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Sandra Anne Angèle Renier

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Déterminants protéiques de la voie de sécrétion Sec impliqués dans la formation de biofilm chez *Listeria monocytogenes*

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Liste des abréviations

ABC : ATP-binding cassette

Abs : absorbance

AFSSA : Agence Française de Sécurité Sanitaire des Aliments

AI-2 : auto-inducteur 2

Ami : amidase

ANSES : Agence Nationale de SEcurité Sanitaire

Auto: autolytic protein

Bap : biofilm associated protein

CAMP : Christie-Atkins-Munch-Peterson test

CM: cytoplasmic membrane

CS: cell surface

CW: cell wall

CWBD : cell-wall binding domain

CwhA : cell wall hydrolase A

DGAL : Direction générale de l'alimentation

DGCCRF : Direction générale de la consommation, de la concurrence et de la fraude

DGS : Direction générale de la santé

DO : densité optique

ECM : matrice extracellulaire

EM: extracellular milieu

EPS : exopolysaccharide

FbpA : fibronectin-binding protein A

FEA flagellum export apparatus

FPE fimbrillin-protein exporter

GW: Glycine Tryptophane

Hly: Hémolysine

Hpt : Hexose phosphate transporter

Iap : invasion associated protein

IMP: integral membrane protein

InlA : internaline A

InlB : internaline B

InVS : Institut de Veille Sanitaire

Lap : Listeria adhesion protein

LB : lysogeny broth

LC : liquid chromatography

Lgt : lipoprotein diacylglyceryl transferase

LLO : listeriolysine O

Lnt : lipoprotein aminoacyl transferase

LpeA : lipoprotein promoting entry

Lsp : lipoprotein signal peptidase

LysM : lysin motif

MCDB : molecular, cellular and developmental biology

MS : mass spectrometry

msIMP: multi-spanning IMP

MurA : muramidase A

NC: non-classical secretion

PGBD : peptidoglycan-binding domain

pH : potential d'hydrogène

Plc : phospholipases C

PlcA : phosphatidylinositol phospholipase C

PlcB : phosphatidylcholine phospholipase C

QS : quorum sensing

SCL: subcellular localization

SCWPs : Secondary cell wall polymers

Sec : secretion

SLHD : S-layer homology domain

SP: signal peptide

SPase : signal peptidase

ssIMP: single-spanning IMP

SvpA (surface virulence-associated protein)

Tat : twin-arginine translocation

TMD: domaine transmembranaire

Unc-SP : uncleaved SP

Wss : WXG100 secretion system

wt : wild type

WXG100 : protein of about 100 amino acid with a conserved WXG motif

INTRODUCTION

Listeria monocytogenes est une bactérie pathogène à Gram positif impliquée dans une infection d'origine alimentaire, la listériose. Celle-ci évolue généralement sous forme de cas sporadiques mais avec parfois des cas groupés voire des épidémies. Bien que son incidence soit moins importante que celle de *Salmonella* et *Campylobacter*, qui sont les agents infectieux les plus souvent en cause dans les toxi-infections alimentaires, *L. monocytogenes* reste néanmoins très importante en raison d'un taux de mortalité élevé, qui varie de 20% à 35% des cas (Fratamico, Bhunia et al. 2005; Riemann and Cliver 2006). Après une décroissance très significative du nombre de cas de listériose entre les années 1986 et 2001 (d'un facteur 3), due à l'instauration d'importantes mesures de lutte notamment dans les industries agroalimentaires, l'incidence de la listériose s'est stabilisée entre 2002 et 2005 autour de 0,35 cas/100 000 habitants. Cependant, une augmentation de cette incidence a été constatée ces dernières années en France (0,45 à 0,50 cas/100 000 habitants) comme dans d'autres pays européens, sans que son origine soit précisément connue (Allerberger and Wagner ; AFSSA 2009). La listériose affecte principalement des populations considérées comme à risque, les personnes immunodéprimées, les femmes enceintes, les nouveau-nés et les personnes âgées (Farber and Peterkin 1991). Une infection par *L. monocytogenes* peut se manifester par (i) des formes non-invasives, généralement peu graves, telles que gastro-entérites, angines, infections cutanées, ou (ii) des formes invasives qui peuvent être graves, telles que les bactériémies ou des infections du système nerveux central (Vazquez-Boland, Kuhn et al. 2001). En France, la récente réémergence de la listériose est principalement imputable à des formes bactériémiques chez des personnes âgées de plus de 60 ans, en particulier celles présentant un terrain à risque (pathologies associées) (AFSSA 2009). Le cycle infectieux de la listériose fait intervenir de nombreux facteurs de virulence et un mécanisme complexe d'infection; il a été très largement étudié et est maintenant clairement défini. En quelques mots, les étapes majeures du cycle infectieux de *L. monocytogenes* font intervenir les protéines pariétales InlA (internaline A) et InlB, qui sont impliquées dans l'adhésion de la bactérie à la surface de la cellule eucaryote et son entrée dans l'hôte par la phagocytose. La libération de la listeriolysine O (LLO) et des phospholipases C (PlcA et PlcB) permet aux bactéries de sortir de la vacuole phagocytaire, tandis que la protéine membranaire ActA, en induisant la polymérisation des monomères d'actine de la cellule hôte, contribue à la motilité bactérienne intracellulaire et à sa propagation de cellules en cellules.

Bien que *L. monocytogenes* soit connue comme un pathogène opportuniste, c'est avant tout une bactérie ubiquiste présente dans des environnements variés comme les sols, les eaux et sur les végétaux. De ce fait, elle peut se retrouver et persister dans les ateliers de production

et de transformation des aliments. Sa persistance est en grande partie due à sa capacité à se développer dans une large gamme de température (de -0,4 à 45 °C), de pH (de 4,3 à 9,6), de sel (jusqu'à 10%) mais également à une faible activité en eau (jusqu'à une Aw de 0.93). De plus, *L. monocytogenes* est capable d'adhérer à diverses surfaces telles que le verre, le métal (acier inoxydable), le caoutchouc ou encore le plastique (polystyrène) et de se développer par la suite en biofilm (Frank and Koffi 1990; Blackman and Frank 1996; Norwood and Gilmour 1999; Di Bonaventura, Piccolomini et al. 2008). La formation de biofilms contribue fortement à la résistance accrue des bactéries à des conditions environnementales difficiles (dessiccation, déplétion en nutriments, etc) et à des traitements technologiques (cycles de nettoyage-désinfection) ou pharmacologiques (antibiotiques) (Folsom and Frank 2006; Tessema, Moretro et al. 2009). Ces propriétés permettent à *L. monocytogenes* de persister dans les chaînes de production pendant de longues périodes, ce qui pose de sérieux problèmes en terme de sécurité alimentaire puisque les biofilms sont des sources importantes de (re)contamination des aliments (Moretro and Langsrud 2004; Gounadaki, Skandamis et al. 2008; Koutsoumanis, Pavlis et al. 2010). L'élimination des biofilms contenant des pathogènes tel que *L. monocytogenes* est donc primordiale dans les ateliers de production et transformation des produits alimentaires afin d'assurer leur sécurité microbiologique.

SYNTHESE BIBLIOGRAPHIQUE

- CHAPITRE 1-
LISTERIA MONOCYTOGENES

1. Historique

C'est en 1926 que le Dr Everitt George Dunne Murray décrit pour la première fois la bactérie aujourd'hui appelée *Listeria monocytogenes.* Celle-ci était isolée du foie de lapins malades et de cochons d'Inde, et nommée *Bacterium monocytogenes* en raison d'une forte quantité de monocytes présents dans la circulation sanguine des sujets infectés (Murray 1926). Un an plus tard, J.H. Harvey Pirie isolait ce même microorganisme à partir du foie de gerbille et la nommait *Listerella hepatholytica* en hommage à Lord Joseph Lister, un pionnier dans le domaine de l'antiseptie dans la chirurgie opératoire (Pirie 1927). Cependant, ce nom de « *Listerella »* n'a pas été retenu puisqu'il avait déjà été donné à un groupe de moisissures. De même, ne possédant pas les caractéristiques du genre *Bacterium,* celui proposé par Murray était écarté et c'est ainsi que le nom définitif de *Listeria monocytogenes* lui a été attribué en 1940 (Pirie 1940). Depuis son isolement, de nombreuses épidémies de listériose ont été répertoriées chez divers animaux à travers le monde (Gray and Killinger 1966). Chez l'homme, le premier cas de listériose n'a été confirmé qu'en 1929 par A. Nyfeldt (Nyfeldt 1929). Malgré de nombreux rapports relatant des cas de méningites, d'encéphalites associées à cette bactérie, aussi bien chez l'homme que chez les animaux, la communauté scientifique n'a commencé à s'intéresser à cette dernière qu'à partir de la moitié des années 50. Et ce n'est qu'en 1981, suite à une épidémie de listériose associée à la consommation de nourriture contaminée (Schlech, Lavigne et al. 1983), que *L. monocytogenes* a été l'objet d'une surveillance accrue avec, dans les années 1990, l'instauration de mesures de prévention dans la filière agroalimentaire.

2. Caractéristiques microbiologiques et biochimiques du genre *Listeria*

Le genre *Listeria* appartient au phylum des *Firmicutes*, à la classe des *Bacilli*, à l'ordre des *Bacilliales* et à la famille des *Listeriacae*. Ce genre comporte actuellement neuf espèces :

Listeria monocytogenes, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. seeligeri* *L. welshimeri*, *L. rocourtiae et L. weihenstephanensis* (Rocourt and Buchrieser 2007; Graves, Helsel et al. 2010; Leclercq, Clermont et al. 2010; Orsi, den Bakker et al. 2011; Lang Halter, Neuhaus et al. 2012).

Parmi ces espèces, seulement deux sont pathogènes : (i) *L. monocytogenes*, reconnue comme étant l'agent responsable d'infections chez l'homme et les ruminants, et (ii) *L. ivanovii*, particulièrement impliquée dans les cas d'avortements des ruminants et est, dans des cas très rares, capable aussi d'infecter l'homme (Guillet, Join-Lambert et al. 2010).

Les différentes espèces du genre *Listeria* possèdent des caractères biochimiques communs. Ce sont toutes des bactéries aérobies/anaérobies facultatives, à catalase positive et oxydase négative. Elles sont capables de fermenter de nombreux glucides : glucose (sans production de gaz), fructose, mannose, amygdaline, salicine, cellobiose, maltose, tréhalose et arabitol. Elles hydrolysent l'esculine et réduisent le lait tournesolé. En revanche, elles ne produisent pas d'indole, et ne possèdent pas d'activité uréase et gélatinase (Larpent 2000; Graves, Helsel et al. 2010; Leclercq, Clermont et al. 2010; Lang Halter, Neuhaus et al. 2012).

La différentiation des espèces du genre *Listeria* est principalement fondée sur les caractères mentionnés dans le tableau 1 :

Tableau 1 : Caractères biochimiques de différenciation des espèces du genre *Listeria.* (Rocourt, Jacquet et al. 2000; Graves, Helsel et al. 2010; Leclercq, Clermont et al. 2010; Lang Halter, Neuhaus et al. 2012)

	Hémolyse	CAMP test *S. aureus*[a]	CAMP test *R. equi*[b]	D-Xylose[c]	L-rhamnose[c]	α méthyl D-mannoside[c]	Ribose[c]	Mannitol[c]
L. monocytogenes	+	+	-	-	+	+	-	-
L. ivanovii (subsp ivanovii)	+	-	-	+	-	-	+	-
L. ivanovii (subsp londoniensis)	+	-	+	+	-	Variable	-	-
L. innocua	-	-	-	-	Variable	+	-	-
L. welshimeri	-	-	-	+	Variable	+	-	-
L. seeligeri	+	+	-	+	-	-	-	-
L. grayi	-	-	-	-	-	Non défini	-	+
L. rocourtiae	-	-	-	+	+	+	+	+
L. weihenstephanensis	-	-	-	+	+	-	+	+
L. marthii	-	Non défini	Non défini	-	-	Non défini	Non défini	-

[a] synergistic β-hemolysis with *Staphylococcus aureus;* [b] synergistic β-hemolysis with *Rhodococcus equi;* [c] production d'acides à partir de (+: positif; -: négatif)

L. monocytogenes est un petit bacille à Gram positif, non capsulé, non sporulé, formant des bâtonnets aux bouts arrondis mesurant de 1 à 2 µm de long sur 0,4-0,5 µm de diamètre. Cette bactérie est capable de se déplacer lorsqu'elle est cultivée à une température comprise entre 20 et 25°C grâce à la présence de flagelles péritriches. A température infectieuse (37°C), elle est immobile ou très faiblement mobile. *L. monocytogenes* forme des colonies lisses, transparentes, aux bords réguliers, β-hémolytiques sur gélose au sang. Cependant, dans certaines conditions de stress (acide, salin…) (Bereksi, Gavini et al. 2002; Hazeleger, Dalvoorde et al. 2006; Giotis, Blair et al. 2007) ou en mode de croissance sessile (Monk, Cook et al. 2004), les cellules peuvent s'allonger jusqu'à atteindre une longueur de 55 µm (Bereksi, Gavini et al. 2002) et former des colonies dites rugueuses. *L. monocytogenes* peut aussi se retrouver sous une troisième forme, appelée « L-form », qui correspond à des bactéries dépourvues de peptidoglycanes et de paroi cellulaire. Par ailleurs, un récent rapport décrit l'existence d'une génération de souches de *L. monocytogenes* présentant une morphologie de type L-form qui est stable et non réversible (Dell'Era, Buchrieser et al. 2009).

Pour différencier les souches de *L. monocytogenes*, différentes méthodes peuvent être mises en œuvre. La plus ancienne et la plus utilisée de nos jours est le sérotypage. Cette méthode est basée sur la détection de 14 antigènes somatiques (O) et 4 antigènes flagellaires (H). L'utilisation de différents antisérums entraîne ou non l'agglutination des souches testées. Treize sérovars ont ainsi pu être définis (Seeliger and Höhne 1979) chez *L. monocytogenes* : 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e et 7. Au total, 17 sérovars ont été différenciés dans le genre *Listeria* (Tableau 2). Au vu de la distribution des sérovars, aucune corrélation n'a pu être établie entre les espèces et les sérovars, à l'exception du sérovar 5 qui ne correspond qu'à l'espèce *ivanovii* (Tableau 2).

Tableau 2 : Distribution des sérovars parmi quelques espèces de *Listeria* (Jones and Seeliger 1991)

Espèce	Sérovars
L. monocytogenes	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e et 7
L. innocua	3, 6a, 6b, 4ab
L. ivanovii	5
L. seeligeri	1/2a, 1/2b, 1/2c, 4b, 4c, 4d et 6
L. welshimeri	1/2a, 4c, 6a, 6b

Avec le développement de méthodes plus fiables basées sur des techniques moléculaires, comme l'électrophorèse en champ pulsé, le ribotypage (Cheng, Siletzky et al. 2008) ou encore l'analyse du polymorphisme électrophorétique des enzymes (ou « Multilocus Enzyme Electrophoresis »), l'espèce *L. monocytogenes* a d'abord été subdivisée en 2 linéages, le Linéage I (appelé également Division II) et le Linéage II (appelé également Division I), puis en un troisième linéage, Linéage III (ou Division III), qui a été défini sur la base de la variation de gènes de virulence. Les différents sérovars de *L. monocytogenes* semblent être associés à des linéages spécifiques : le Linéage I contient les sérovars 1/2b, 3b, 4b, 4d et 4e ; le Linéage II contient les sérovars 1/2a, 1/2c, 3a et 3c, et le Linéage III contient les sérovars 4a et 4c. Cependant, la corrélation entre les sérovars et les linéages ne doit pas être considérée comme stricte et absolue puisque, par exemple, certains sérovars sont sous-représentés ou encore absents (4ab ou 7). De plus, certains sérovars sont représentés dans différents linéages comme le 4b (Linéage I et II) (Liu, Lawrence et al. 2006). Enfin, plus récemment, la méthode MLST (MultiLocus Sequence Typing) a conduit à une autre distribution des souches de *L. monocytogenes* qui consiste en 23 complexes monoclonaux et 22 singletons (Ragon, Wirth et al. 2008).

3. Environnement

L. monocytogenes est une bactérie ubiquiste, tellurique, très largement répandue dans les environnements naturels (sols, eaux, végétaux) ainsi que sur les surfaces abiotiques des ateliers de production et de transformation des aliments (Freitag, Port et al. 2009; Piveteau, Depret et al. 2011). Cette bactérie peut donc passer d'un mode de vie saprophyte à un mode de vie parasitaire lorsqu'elle est ingérée par un animal ou par l'Homme. Certains animaux, dont l'Homme, peuvent héberger *L. monocytogenes* sans développer de maladies (Olier, Pierre et al. 2003; Olier, Rousseaux et al. 2004). Ces animaux sont alors qualifiés de porteurs sains et sont susceptibles de disséminer les bactéries dans l'environnement par le biais des déjections. Les ruminants joueraient un rôle important dans le maintien de *L. monocytogenes* dans l'environnement via un cycle d'enrichissement fécal/oral (Fenlon 1999). De plus, la bactérie est capable d'adhérer sur des surfaces et de former des biofilms, ce qui est particulièrement problématique dans les filières agroalimentaires. En effet, les aliments mis en contact avec *L. monocytogenes* peuvent être conservés pendant de longues périodes à basse température, ce qui n'empêche pas son développement étant donné son caractère psychrotrophe ; elle peut ainsi contaminer le futur consommateur. Malgré l'utilisation de

divers agents désinfectants, certaines souches de *L. monocytogenes* persistent et posent ainsi un réel problème aux industriels dont l'image peut être ternie suite à des cas d'épidémies de listériose liés à leurs produits. Dans ce cas, aux conséquences sanitaires parfois graves peuvent venir s'ajouter des conséquences économiques qui peuvent être dramatiques pour l'entreprise.

4. La listériose

4.1 Pathologie chez l'animal

La listériose se déclare généralement sous forme sporadique chez certains animaux (oiseaux, ruminants, rongeurs) suite à une contamination par l'intermédiaire d'environnements souillés et en particulier par l'ingestion de végétaux contaminés par des porteurs. *L. ivanovii* peut être pathogène pour les petits ruminants comme les ovins (Chand and Sadana 1999) alors que *L. innocua* n'est généralement pas considéré comme pathogène, bien qu'elle ait été isolée chez des animaux présentant des signes cliniques (Walker, Morgan et al. 1994). Entre 1998 et 1999, 428 cas de listériose ont été recensés en France dont 259 cas chez les bovins et 108 chez les ovins (Vaissaire 2000). Les principales formes de listériose se caractérisent par des atteintes nerveuses, des entérites, des septicémies et des formes génitales à l'origine d'avortements. Chez les bovins, les avortements sont les conséquences les plus fréquentes alors que chez les ovins, les formes méningo-encéphaliques sont plus courantes. Des cas de listériose ont ainsi été rapportés chez de nombreuses espèces animales telles que volailles, oiseaux, chevaux, chiens, chevreuils, lapins, lièvres et sangliers (Vaissaire 2000).

4.2 Pathologie chez l'Homme

Chez l'Homme, la listériose est une maladie dont les manifestions cliniques sont variées, souvent peu spécifiques et différentes selon le terrain de la personne atteinte. Il existe différentes formes de listériose : (i) la forme non invasive qui se traduit par des gastro-entérites, des infections cutanées ou des angines, dont le diagnostic reste exceptionnel, et (ii) la forme invasive qui est beaucoup plus grave. Cette dernière forme touche préférentiellement les sujets dont le système immunitaire est perturbé soit naturellement comme les femmes enceintes, les nouveau-nés et les personnes âgées soit en raison d'une maladie ou de son traitement (SIDA, cancer, diabètes…).

Chez la femme enceinte, la listériose se traduit par un épisode fébrile, le plus souvent spontanément résolutif en quelques jours. La contamination du fœtus (listériose fœto-maternelle) par *L. monocytogenes* s'effectue par voie hématogène transplacentaire, pouvant entraîner la mort *in utero*, un avortement ou un accouchement prématuré selon le stade de la grossesse. L'enfant peut également être contaminé lors de l'accouchement, on parle alors de listériose néonatale. L'infection du nouveau-né se révèle très rapidement et se présente généralement sous forme de septicémie, prenant le plus souvent l'allure d'une détresse respiratoire.

Chez l'adulte, la forme de listériose invasive la plus fréquente est la forme bactériémique fébrile isolée, rencontrée principalement chez les immunodéprimés. Les secondes formes invasives les plus fréquentes sont les infections du système nerveux central, se traduisant le plus souvent par une méningite aigüe. Les formes neuro-méningées regroupent également les méningo-encéphalites, les rhomboencéphalites et les abcès cérébraux. Chez les personnes de moins de 60 ans n'appartenant pas aux groupes à risque, la forme prédominante (plus de 90% des cas) est la forme méningée. Cependant, chez ces sujets, le pronostic vital est plutôt bon avec une létalité inférieure à 5%. Chez les personnes immuno-déprimées, le pronostic est beaucoup plus péjoratif avec 30% de séquelles suite à des rhomboencéphalites et entre 25 et 35% de décès suite à des abcès cérébraux (Beaufort, Goulet et al. 2009). Dans 99% des cas, la contamination menant au développement de la listériose est d'origine alimentaire (Orsi, den Bakker et al. 2011). Dans la catégorie des toxi-infections alimentaires, la listériose a été la deuxième cause de mortalité en France juste après la salmonellose (Vaillant, de Valk et al. 2005), mais également aux Etats-Unis (Mead, Slutsker et al. 1999). Malgré les efforts des industriels de la filière alimentaire et la mise en place d'un système de surveillance efficace en France, qui a contribué à faire baisser globalement le nombre de cas de listériose ces dernières années, le risque sanitaire reste néanmoins élevé.

5. Epidémiologie de la listériose

En France, la listériose est une maladie à déclaration obligatoire depuis 1999 et fait l'objet d'une surveillance continue. Le Centre National de Référence (CNR) des *Listeria* réalise le typage des souches d'origine humaine (Goulet, Jacquet et al. 2006), tandis que le Laboratoire National de Référence (LNR) des *Listeria* s'attache aux souches d'origine alimentaire.

Dans le cadre de la déclaration obligatoire, chaque cas de listériose doit être déclaré à la DASS par l'intermédiaire d'un formulaire standardisé où sont recueillies des informations tels que la forme clinique de la maladie, le site (ou les sites) de prélèvement de la bactérie, l'existence de pathologies associées… Ces informations permettent de suivre l'évolution dans le temps de l'incidence des cas et des caractéristiques de cette infection. De plus, les investigations épidémiologiques ainsi que les expertises des souches auprès du CNR des *Listeria*, ont également pour objectif d'identifier les produits alimentaires susceptibles de présenter un risque pour la santé publique. Lorsque des cas groupés de listériose sont repérés par le CNR, ceux-ci sont signalés à l'Institut de Veille Sanitaire (InVS) pour investigation. En cas de suspicion sur un aliment, la cellule « *Listeria* » constituée de représentants de la Direction générale de la santé (DGS), de l'InVS, de la Direction générale de l'alimentation (DGAL), de la direction générale de la consommation, de la concurrence et de la fraude (DGCCRF), de l'ANSES et du CNR, décide des interventions complémentaires à réaliser, et ceci afin d'éviter un épisode épidémique.

Avant l'instauration de mesures de lutte vis-à-vis de *L. monocytogenes* dans la filière agroalimentaire, les études réalisées dans les années 1990 estimaient le nombre de cas de listériose en France à plus de 1000 par an. Au moment de la mise en place de la déclaration obligatoire en 1999, l'incidence de listériose était de 0.45 cas/100 000 habitants contre 1.5 cas/100 000 habitants en 1986 soit 3 fois moins importante (Goulet, Espaze et al. 1989; Goulet, de Valk et al. 2001). Depuis l'incidence des cas de listériose a continué de baisser jusqu'à se stabiliser entre 2002 et 2005 autour de 0.35 cas/100 000 habitants. A partir de 2006, un renversement de tendance a été observé avec une augmentation notable de l'incidence allant jusqu'à 0.51 cas/100 000 habitants en 2009 (Figure 1).

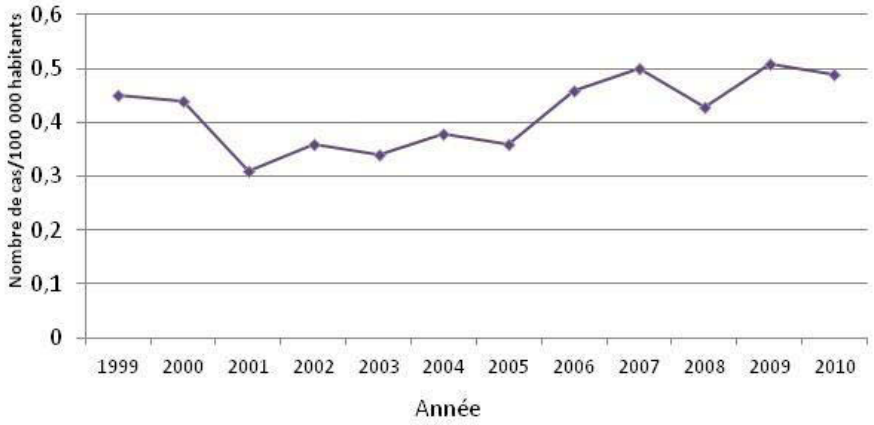

Figure 1 : Incidence de la listériose (nombre de cas /100 000 habitants) entre 1999 et 2010 (Source InVS)

Alors que la forme materno-néonatale de listériose est en constante diminution depuis 1999, l'évolution de l'incidence de la listériose est expliquée par une forte augmentation du nombre de cas de la forme bactériémique (Figure 2), le nombre de cas de forme neuroméningée restant relativement stable au cours du temps.

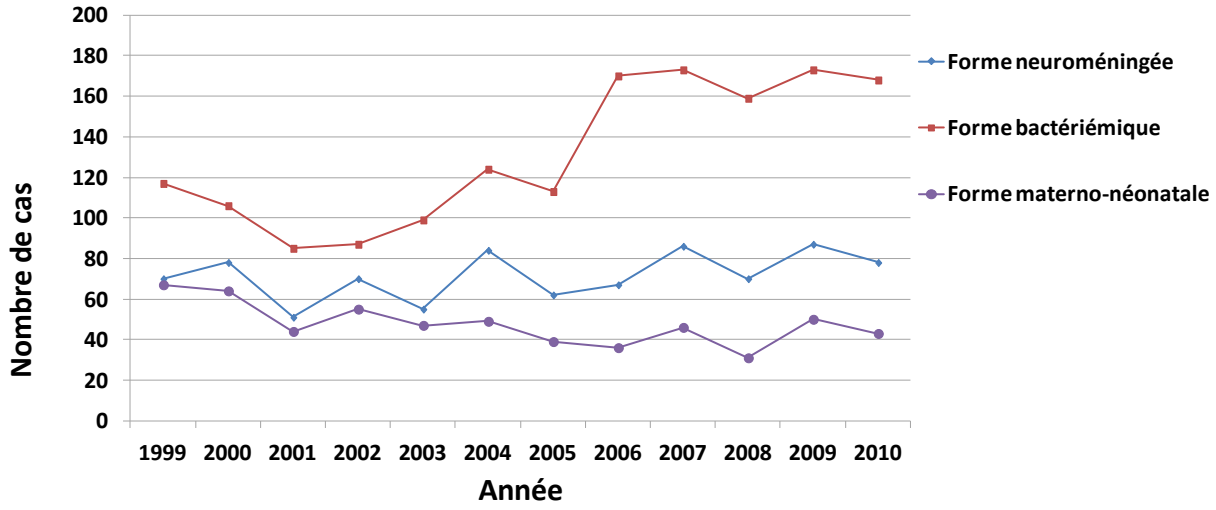

Figure 2 : Nombre de cas des différentes formes de listériose entre 1999 et 2010 (Source InVS)

Cette augmentation de l'incidence de listériose de type bactériémique a entraîné une augmentation du nombre de décès non-materno-néonatal. En effet, au cours temps, l'évolution de ces deux paramètres suivent la même tendance (Figures 2 et 3). Le pourcentage de décès, quant à lui, reste stable autour de 22% pour les non-materno-néonatales et 30% pour toutes les formes invasives (Beaufort, Goulet et al. 2009).

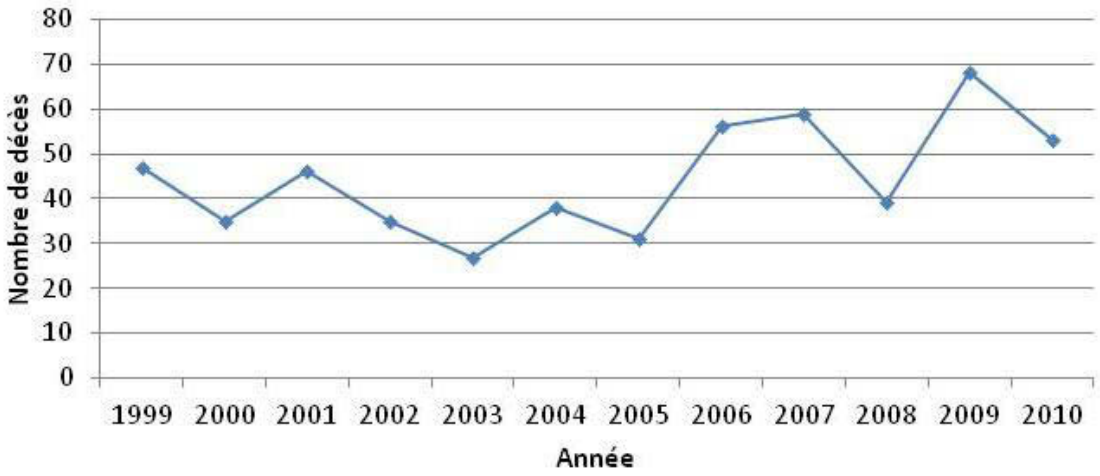

Figure 3 : Nombre de décès par an liés à une listériose non materno-néonatale (Source InVS)

Cette augmentation est marquée chez les sujets âgés de plus de 60 ans (+51%) et tout particulièrement chez les sujets dont l'âge est ≥ 75 ans (+59%) (Figure 4). C'est ce que révèle

un rapport sur l'évolution de l'incidence des listérioses non materno-néonatales par classe d'âge entre les périodes de 2001-2005 et 2006-2007 (Beaufort, Goulet et al. 2009).

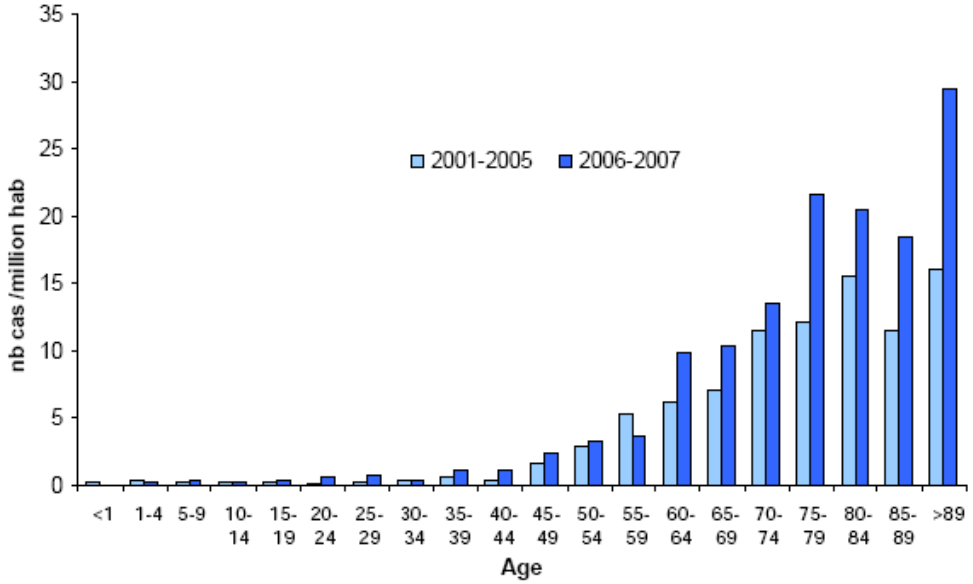

Figure 4 : Évolution de l'incidence par classe d'âge des listérioses non materno-néonatales entre 2001-2005 et 2006-2007 (Goulet, Hedberg et al. 2008)

La listériose évolue sous forme de cas sporadiques, auxquelles viennent s'ajouter des cas groupés voire des épidémies. Les aliments incriminés lors des cas de listérioses depuis 2005 sont listés dans le Tableau 3 ; on trouve principalement des produits laitiers et carnés, et plus rarement des produits de la mer ou des végétaux.

Les principales souches à l'origine de cas de listériose humaine appartiennent aux sérovars 4b, 1/2a et 1/2b, avec une prédominance du sérovar 4b depuis 1987 à l'exception d'une période de 1992 à 1995. Récemment, de nombreux pays ont montré une inversion des sérovars à l'origine des infections humaines (du sérovar 4b aux sérovars 1/2a et 1/2b). Cette inversion accompagne la montée des formes bactériémiques/septicémiques par rapport aux formes neuroméningées (Swaminathan and Gerner-Smidt 2007). Cependant, cette tendance n'a pas été observée en France.

Tableau 3 : Epidémies survenues depuis 1995 dans le monde et produits impliqués (liste non exhaustive)

Pays	Produits impliqués	Années	Références
Canada	Viandes prêtes à consommer	2008	(InVS)
USA	Lait (Pasteurisé)	2007	(Anonyme 2008)
Royaume-Uni	Sandwichs	2007	(Little, Barrett et al. 2008)
Suisse	Fromage à pâte molle (Pasteurisé)	2005	(Bille, Blanc et al. 2006)
Royaume-Uni	Sandwichs	2004	(Gillespie, McLauchlin et al. 2006)
Etats-Unis	Fromage de type mexicain	2003	(Swaminathan and Gerner-Smidt 2007)
Royaume-Uni	Beurre	2003	(Gillespie, McLauchlin et al. 2006)
France	Mortadelle	2003	(InVS)
Royaume-Uni	Sandwichs	2003	(Gillespie, McLauchlin et al. 2006)
Canada	Fromage (Cru)	2002	(Swaminathan and Gerner-Smidt 2007)
USA	Viande de dinde prête à consommer	2002	(Food and Drug Administration 2003)
France	Saucisse à tartiner	2002	(InVS)
Japon	Fromage à pâte molle et à croûte lavée	2001	(Makino, Kawamoto et al. 2005)
Suède	Fromage à pâte molle	2001	(Lundén, Tolvanen et al. 2004)
USA	Viande de dinde prête à consommer	2000	(Food and Drug Administration 2003; McLauchlin, Mitchell et al. 2004)
France	Époisses (Cru)	1999	(Goulet, de Valk et al. 2001)
USA	Charcuterie	1999	(Food and Drug Administration 2003)
Royaume-Uni	Sandwichs	1999	(Gillespie, McLauchlin et al. 2006)
France	Livarot, Pont-l'Evêque (Cru)	1997	(De Buyser, Dufour et al. 2001; Lundén, Tolvanen et al. 2004)
Italie	Salade de maïs (et de thon)	1997	(Gianfranceschi, Gattuso et al. 2007)
Canada	Crabe	1996	(McLauchlin, Mitchell et al. 2004)
France	Brie de Meaux (Cru)	1995	(Goulet, Jacquet et al. 1995)

6. Pathogénicité

L'infection par *L. monocytogenes* est réalisée après ingestion de nourriture contaminée. Les bactéries atteignent la barrière intestinale puis la traverse pour rejoindre la circulation sanguine. Elles vont par la suite pouvoir se multiplier au niveau du foie et de la rate, atteindre le système nerveux central ou encore traverser la barrière placentaire chez les femmes enceintes (Figure 5).

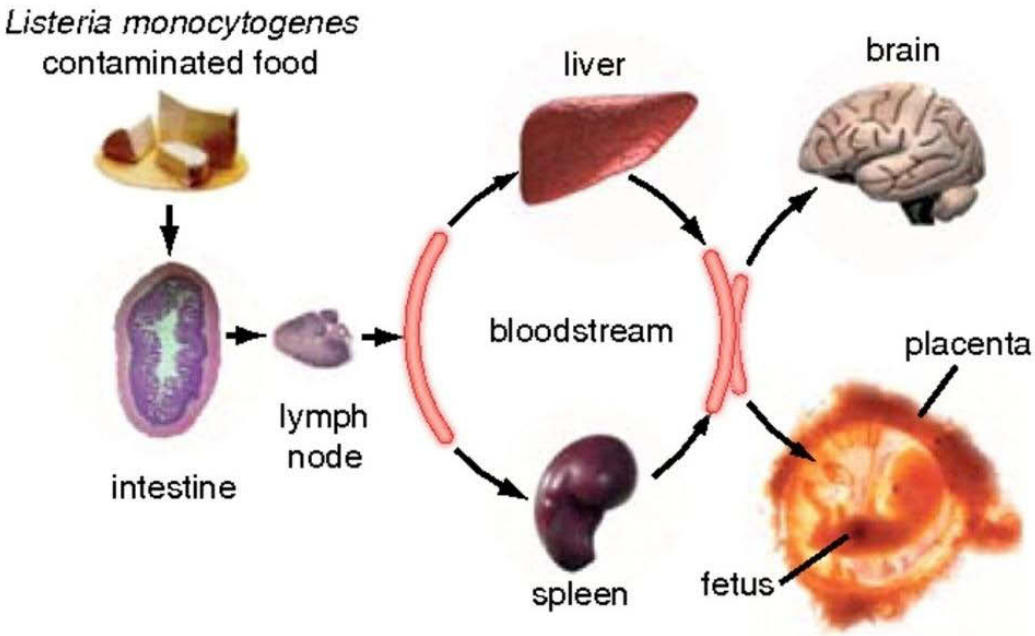

Figure 5 : Mode d'infection par *L. monocytogenes* (Cossart 2011)

L. monocytogenes est donc capable de pénétrer dans différents types de cellules eucaryotes : (i) les cellules phagocytaires comme les macrophages qu'elle parvient à neutraliser ainsi que (ii) les non-phagocytaires comme les cellules épithéliales, hépatocytes, fibroblastes, cellules de l'endothélium et du système nerveux (Vazquez-Boland, Kuhn et al. 2001; Barbuddhe and Chakraborty 2009). Après avoir pénétré les cellules non-phagocytaires, *L. monocytogenes* suit un cycle infectieux intracellulaire qui se décline en plusieurs étapes : (i) adhésion et internalisation de la bactérie dans la cellule hôte, (ii) lyse de la vacuole d'internalisation, (iii) multiplication dans la cellule hôte, (iv), mouvements intracellulaires et (v) passage dans les cellules adjacentes qui s'accompagne d'une nouvelle internalisation avec une double membrane, suivie de (vi) la lyse de cette double membrane (Figure 6).

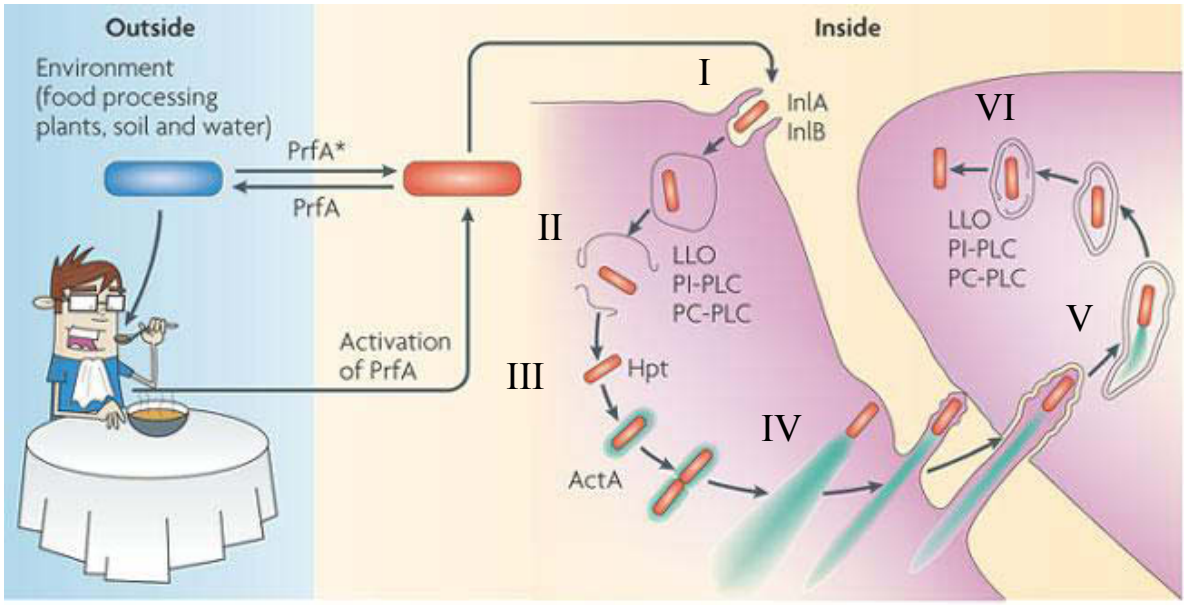

Figure 6 : De la vie saprophytique au pathogène intracellulaire : les différentes étapes du cycle d'infection de *L. monocytogenes* (Freitag, Port et al. 2009)

Les mécanismes moléculaires impliqués dans les différentes étapes d'infection depuis la pénétration cellulaire sont relativement bien connus et font intervenir un certain nombre de facteurs de virulence dont la plupart sont régulés par le facteur de transcription PrfA.

6.1 Adhésion et pénétration cellulaire

6.1.1 Les internalines

L'efficacité de pénétration varie selon les lignées cellulaires ou le type de cellules utilisées et n'atteint jamais le taux d'entrée observé chez les macrophages. L'entrée dans les cellules a été étudié dans le détail depuis la découverte des protéines appelées internalines, InlA et InlB, (Lecuit, Ohayon et al. 1997; Braun, Ohayon et al. 1998) et leur récepteurs respectifs, l'E-cadhérine (E-cad) et Met. Ces protéines possèdent un domaine dans leur partie N-terminale constitué de régions riches en leucine (LRR : Leucin Rich Region) composées de 22 acides aminés répétés en tandem (Schubert, Gobel et al. 2001). Ces domaines LRR intègrent des unités de reconnaissance pour les interactions protéine-protéine ainsi que pour l'activation de protéines aussi bien procaryotes qu'eucaryotes. Ces protéines sont toutes deux liées à la paroi cellulaire mais de façon différente puisque InlA possède un motif LPXTG permettant une fixation covalente aux peptidoglycanes de la paroi alors que InlB est liée aux acides lipotéichoïques de la paroi par des interactions électrostatiques faisant intervenir les modules GW.

La protéine InlA permet l'entrée de la bactérie dans les cellules épithéliales après s'être fixée à son récepteur spécifique, la E-cadhérine. Leur interaction entraîne une cascade de réactions et une réorganisation du cytosquelette menant à son internalisation. InlA permettrait également le franchissement de la barrière placentaire (Lecuit, Nelson et al. 2004). Cependant, son rôle reste encore à démontrer. En effet, un facteur supplémentaire interviendrait dans l'infection placentaire puisqu'il a été montré que la transmission materno-fœtale s'effectue chez des souris qui n'ont pas le récepteur d'InlA et chez des cochons d'Inde infectés avec un mutant dépourvu du gène *inlA* (Bakardjiev, Stacy et al. 2004). Etant donné que les cellules épithéliales qui sont au contact du liquide céphalorachidien expriment également la E-cadhérine, il est supposé que InlA joue un rôle dans le franchissement de la barrière hémato-encéphalique (Seveau, Pizarro-Cerda et al. 2007).

La protéine InlB, quant à elle, est impliquée dans l'invasion d'une plus grande variété de cellules. Plusieurs récepteurs pour InlB ont été identifiés : gClqR, le récepteur tyrosine kinase Met (c-Met) et les glycosaminoglycanes (GAGs) (Braun, Ghebrehiwet et al. 2000; Shen, Naujokas et al. 2000). InlB interagit avec les GAGs par l'intermédiaire de modules GW. Les récepteurs GAG facilitent le détachement des protéines InlB de la surface bactérienne qui vont pouvoir se regrouper pour activer fortement c-Met par la suite et induire l'entrée de la bactérie (Banerjee, Copp et al. 2004). L'activation de c-Met par InlB est espèce dépendante. En effet, chez la souris, InlB n'est pas impliqué dans le franchissement de la paroi intestinale, mais il est important pour la colonisation du foie et de la rate (Disson, Grayo et al. 2008). Chez le lapin et le cochon d'Inde, la virulence n'est pas atténuée lorsque ces derniers sont infectés par le mutant Δ*inlB* (Khelef, Lecuit et al. 2006). Enfin, des études récentes ont montré que InlB jouait un rôle important dans le franchissement de la barrière placentaire, en collaboration avec InlA (Disson, Grayo et al. 2008).

D'autres membres de la famille des internalines sont impliqués dans l'adhésion aux cellules eucaryotes. C'est notamment le cas de InlF et InlJ. En effet, InlF permet d'augmenter l'adhésion et l'entrée de *L. monocytogenes* dans les cellules hôtes suite à l'inactivation de la voie RhoA/ROCK (Kirchner and Higgins 2008). InlF est donc un exemple de facteur de virulence dont l'action n'est significative que sous certaines conditions et chez des espèces spécifiques. InlJ est une protéine uniquement exprimée à la surface cellulaire dans le sang et le foie des animaux infectés (Sabet, Toledo-Arana et al. 2008). Lorsque le gène *inlJ* est délété, la virulence de *L. monocytogenes* est fortement réduite suite à une infection par voie intraveineuse et intragastrique (Sabet, Lecuit et al. 2005).

6.1.2 Lap et LapB

Lap (Listeria adhesion protein), initialement appelée p104, est une protéine d'adhésion de 104 kDa, présente chez toutes les espèces du genre *Listeria* excepté chez *L. grayi*. La fonction primaire de cette protéine multitâche (« moonlighting ») est une activité alcool acétaldéhyde déshydrogénase lorsqu'elle est présente dans le cytoplasme ; elle interagit avec le récepteur Hsp60 des cellules hôtes permettant l'adhésion de la bactérie aux cellules intestinales lorsqu'elle est présente à la surface cellulaire (Pandiripally, Westbrook et al. 1999; Wampler, Kim et al. 2004; Burkholder and Bhunia 2010; Jagadeesan, Koo et al. 2010). La réduction de la virulence chez une souris, suite à l'administration par voie orale d'une souche dépourvue du gène *lap,* confirme l'importance de Lap en tant que facteur de virulence (Burkholder, Kim et al. 2009).

LapB, qui n'a rien à voir avec Lap, est une protéine de 184 kDa à motif LPXTG, dont le gène est absent chez les espèces non pathogènes du genre *Listeria.* L'expression du gène *lapB* est régulée positivement par le facteur PrfA et augmente fortement au niveau de la rate de souris infectées. LapB est nécessaire à l'adhésion et à l'entrée de *L. monocytogenes* dans les cellules épithéliales ainsi que pour la virulence de cette dernière suite à une infection par voie orale ou par voie intraveineuse. La fonction d'adhésine est située au niveau de la partie N-terminale de la protéine, qui interagit probablement avec un récepteur de la cellule hôte (Reis, Sousa et al. 2010).

6.1.3 FbpA

FbpA (fibronectin-binding protein A) est une protéine de 65 kDa exposée à la surface bactérienne et localisée à la membrane malgré l'absence de peptide signal et de domaines transmembranaires, tout comme ses homologues du genre *Streptococcus* (Dramsi, Bourdichon et al. 2004). FbpA est capable de fixer la fibronectine humaine immobilisée et augmente l'adhésion de *L. monocytogenes* aux cellules épithéliales lorsque de la fibronectine exogène est présente. De plus, l'expression de FbpA affecte le niveau en protéine de deux facteurs de virulences majeurs : InlB et la listériolysine O (LLO) décrite plus loin. Non seulement FbpA est capable de fixer la fibronectine mais elle jouerait le rôle de chaperonne permettant de stabiliser et/ou d'assurer la sécrétion de LLO et InlB (Dramsi, Bourdichon et al. 2004).

6.1.4 Ami

Ami est une protéine de 99 kDa présentant une activité N-acétylmuramoyl-L-alanine amidase (McLaughlan and Foster 1998). Elle est liée aux acides lipotéichoïques de la paroi bactérienne par des modules GW qui peuvent également permettre l'adhésion aux cellules épithéliales humaines (Milohanic, Jonquieres et al. 2001; Milohanic, Jonquieres et al. 2004). Ami améliore la colonisation des hépatocytes de souris et notamment en interagissant avec les glycosaminoglycanes (Asano, Kakizaki et al. 2012). Son absence entraîne une réduction de la virulence chez les souris (Asano, Sashinami et al. 2011).

6.1.5 CwhA

CwhA (cell wall hydrolase A) est une protéine de 60 kDa également appelée p60 ou encore Iap (invasion associated protein). CwhA est une hydrolase pariétale impliquée dans la division cellulaire chez *L. monocytogenes*. Son absence entraîne la formation de courts filaments cellulaires pendant la phase exponentielle de croissance qui retrouvent une taille normale en phase stationnaire (Pilgrim, Kolb-Maurer et al. 2003). De plus, le mutant Δ*cwhA* montre une capacité inférieure à celle de la souche sauvage à pénétrer dans les cellules hôtes telles que les fibroblastes ou les cellules épithéliales (Kuhn and Goebel 1989; Ruhland, Hellwig et al. 1993). Le mutant peine également à se déplacer au sein de la cellule hôte du fait de son incapacité à former la queue d'actine lui permettant de passer d'une cellule à une autre (Pilgrim, Kolb-Maurer et al. 2003). En effet, l'absence de CwhA conduit à une mauvaise localisation de la protéine ActA qui se situe tout autour de la cellule au lieu d'être regroupée au niveau d'un pôle. La réduction de virulence observée chez le mutant Δ*cwhA* serait principalement due à cette absence de motilité et de propagation plutôt qu'à la faible réduction d'invasion cellulaire, ce qui a conduit à proposer le nom de CwhA plutôt que Iap pour cette protéine (Pilgrim, Kolb-Maurer et al. 2003).

6.1.6 LpeA

LpeA (lipoprotein promoting entry) est une lipoprotéine de 35 kDa qui est nécessaire à l'entrée de *L. monocytogenes* au niveau des hépatocytes et des cellules épithéliales de l'intestin. De façon étrange, le mutant dépourvu du gène *lpeA* survit beaucoup mieux dans les macrophages que la souche sauvage ce qui a pour conséquence d'exacerber faiblement la virulence de *L. monocytogenes* chez la souris (Réglier-Poupet, Pellegrini et al. 2003).

6.1.7 Auto

Auto est une protéine autolytique de 64 kDa possédant un domaine N-acétylglucosamidase qui est activé par un clivage protéolytique et dont l'activité est optimale à pH acide (Bublitz, Polle et al. 2009). Tout comme Ami et InlB, Auto est associée à la paroi cellulaire par des modules GW (Cabanes, Dussurget et al. 2004) et impliquée dans l'entrée de *L. monocytogenes* dans différentes lignées de cellules épithéliales. C'est un facteur de virulence important puisque son absence entraîne une forte réduction de cellules viables suite à une injection par voie veineuse chez la souris ou par voie orale chez le cochon d'Inde (Cabanes, Dussurget et al. 2004). Il a donc été proposer de définir Auto comme un facteur de virulence nécessaire au contrôle de l'architecture de la surface cellulaire de *L. monocytogenes* lors de son exposition à une cellule hôte (Camejo, Carvalho et al. 2011).

6.2 Lyse de la vacuole d'internalisation

Suite à son entrée dans une cellule hôte, *L. monocytogenes* est temporairement enfermée dans une vacuole phagocytaire avant de s'échapper dans le cytoplasme. LLO est le principal déterminant responsable de la libération de *L. monocytogenes* des vacuoles primaires mais également secondaires (Portnoy, Jacks et al. 1988; Gedde, Higgins et al. 2000). LLO, aussi appelée Hly (hémolysine) , est une protéine de 58,7 kDa sécrétée dans le milieu extracellulaire faisant partie de la famille des toxines cytolysine cholestérol-dépendant (Gilbert 2010). Les mutants dépourvus du gène codant LLO *(hly)* ont des difficultés à atteindre le cytoplasme et sont moins virulents (Kathariou, Metz et al. 1987; Portnoy, Jacks et al. 1988; Cossart, Vicente et al. 1989). L'activité de LLO est optimale au pH acide présent au sein de la vacuole phagocytaire. Puis cette activité diminue à pH neutre dans le cytoplasme, évitant d'engendrer trop de dégâts dans la cellule (Beauregard, Lee et al. 1997). LLO se fixe à la membrane sous forme de monomères qui s'oligomérisent en complexe formant un pore. La destruction de la membrane vacuolaire est améliorée par deux phospholipases, une phosphatidylinositol phospholipase C de 36 kDa (PlcA) et une phosphatidylcholine phospholipase C de 33 kDa (PlcB) (Camilli, Goldfine et al. 1991; Geoffroy, Raveneau et al. 1991; Leimeisterwachter, Domann et al. 1991; Mengaud, Braunbreton et al. 1991; Goldfine and Knob 1992). Les deux enzymes coopèrent avec la LLO dans la lyse des membranes vacuolaires primaires et secondaires. PlcB est exprimée comme une proenzyme dont l'activation est permise à pH acide par la métalloprotéase Mpl (Camilli, Goldfine et al. 1991;

Raveneau, Geoffroy et al. 1992; Marquis and Hager 2000). La protéine chaperonne PrsA2 (32,7 kDa) joue également un rôle important dans cette étape du cycle infectieux puisqu'elle permet l'activation et la stabilisation de LLO et PlcB (Alonzo, Port et al. 2009). Son absence entraîne une réduction de la virulence et de la propagation de cellules en cellules chez le modèle murin.

La protéine SvpA (surface virulence-associated protein) est une protéine pariétale de 63,4 kDa, également retrouvée dans le milieu extracellulaire, dont l'absence réduit significativement la virulence chez les souris (Borezée, Pellegrini et al. 2001). En effet, cette réduction est due à l'incapacité de *L. monocytogenes* de s'échapper des phagosomes des macrophages.

6.3 Survie et multiplication intracellulaire

L. monocytogenes est remarquablement bien adaptée à survivre dans les macrophages et autres cellules. Une fois qu'elle s'est échappée de la vacuole, elle peut se multiplier dans le cytosol en utilisant le glucose-1-phosphate comme source d'énergie. Ce processus métabolique dépend de la synthèse de Hpt, un transporteur d'hexose phosphate qui est nécessaire à la prolifération de la bactérie dans les organes de souris (Goetz, Bubert et al. 2001; Chico-Calero, Suarez et al. 2002).

Certaines protéines jouent également des rôles importants dans la survie intracellulaire comme LplA1, une lipoate ligase, ou PycA, une pyruvate carboxylase, dont l'absence entraîne un arrêt de multiplication cellulaire (O'Riordan, Moors et al. 2003; Keeney, Stuckey et al. 2007). D'autres sont nécessaires à une multiplication optimale comme Fri (ferritine), RelA ((p)ppGpp synthétase), PrsA2, et OppA (transporteur d'oligopeptides) (Borezée, Pellegrini et al. 2000; Taylor, Beresford et al. 2002; Dussurget, Dumas et al. 2005; Chatterjee, Hossain et al. 2006).

6.4 Mobilité intracellulaire et propagation de cellules en cellules

La protéine de surface ActA (70,3 kDa) est un des principaux facteurs de virulence chez *L. monocytogenes*. Elle possède dans sa partie C-terminale un domaine transmembranaire lui permettant de rester encrée à la membrane cytoplasmique (Domann, Wehland et al. 1992; Vazquezboland, Kocks et al. 1992; Garcia-del Portillo, Calvo et al. 2011). ActA est responsable de la polymérisation des filaments d'actines à un pôle de la cellule bactérienne, conduisant à la formation d'une queue d'actines permettant à la bactérie de se déplacer dans le

cytoplasme mais également d'être propulsée d'une cellule à une autre (Kocks, Marchand et al. 1995; Pizarro-Cerda and Cossart 2006).

6.5 Régulation des facteurs de virulence

PrfA contrôle l'expression des principaux facteurs de virulence de chaque étape du cycle d'infection de *L. monocytogenes* (Chakraborty, Leimeisterwachter et al. 1992). Le cœur du régulon PrfA est composé de 10 gènes codant pour des facteurs de virulence (Figure 7).

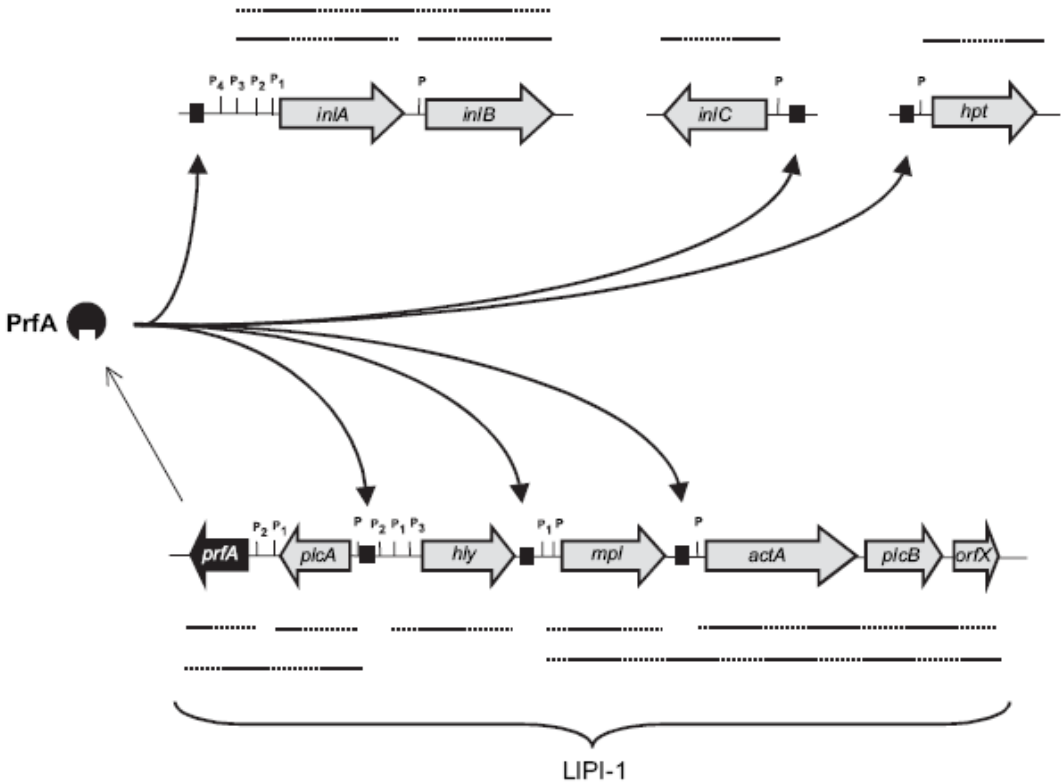

Figure 7 : Le régulon PrfA (Scortti, Monzo et al. 2007). Les boîtes PrfA sont représentées par des carrés noirs. Les promoteurs connus et les transcripts sont représentés par la lettre P et les lignes en pointillés, respectivement.

Le régulon PrfA comprend l'îlot de pathogénicité LIPI-1 qui code LLO, ActA, PlcA, PlcB, MpI et PrfA, ainsi que trois loci supplémentaires : l'opéron *inlAB* et les gènes monocistroniques *inlC* et *hpt*. De plus, différentes études ont identifié près de 160 gènes dont l'expression est régulée directement ou indirectement par PrfA (Ripio, Vazquez-Boland et al. 1998; Milohanic, Glaser et al. 2003; Cabanes, Sousa et al. 2005). PrfA se présente sous la forme d'homodimères qui activent la transcription de gènes en se fixant sur une séquence spécifique du promoteur appelée boîte PrfA (Scortti, Monzo et al. 2007). Il a été montré qu'un

mutant dépourvu de PrfA était incapable de croître dans une cellule hôte et était 100 000 fois moins virulent que la souche sauvage chez une souris (Freitag, Rong et al. 1993).

Une étude récente a révélé que le facteur σ^B était impliqué dans la régulation d'une grande quantité de gènes (≈ 200). Ces gènes sont prédits comme jouant des rôles dans la résistance aux stress, le métabolisme des hydrates de carbone, le transport de solutés et la virulence (Hain, Hossain et al. 2008). Certains gènes régulés par PrfA le sont également par σ^B, comme *inlA* et *inlB*. Il a été suggéré que σ^B permettrait de moduler l'expression de certains facteurs de virulence afin d'éviter une trop forte expression de ces derniers qui pourrait causer de trop gros dégâts à la cellule hôte (Ollinger, Bowen et al. 2009)

Les mécanismes de virulence chez *L. monocytogenes* sont très bien documentés. Des informations sont disponibles aussi bien sur les protéines de surfaces qui sont responsables des interactions avec les cellules hôtes, que sur les protéines de régulation permettant de passer d'une forme de vie saprophyte à une forme intracellulaire parasite. Dans sa forme saprophyte, *L. monocytogenes* est capable d'adhérer à des surfaces biotiques ou abiotiques, et de former des biofilms. Cependant, les mécanismes moléculaires mis en jeu ne sont pas encore complétement élucidés.

- CHAPITRE 2-
LA FORMATION DE BIOFILM CHEZ *L. MONOCYTOGENES*

1. Qu'est-ce qu'un biofilm ?

Un biofilm est définit comme le développement sessile d'une communauté de micro-organismes adhérente à une surface et généralement englobée dans une matrice d'exopolymères (Costerton, Lewandowski et al. 1995). La formation d'un biofilm comprend plusieurs étapes (Figure 8). La première consiste en l'attachement initial de la cellule à une surface, suivi d'une adhésion irréversible. Puis, des microcolonies se forment et se développent en un biofilm mature. Des cellules du biofilm peuvent ensuite se libérer et redevenir planctoniques pour aller coloniser d'autres surfaces (O'Toole, Kaplan et al. 2000; Hall-Stoodley and Stoodley 2002).

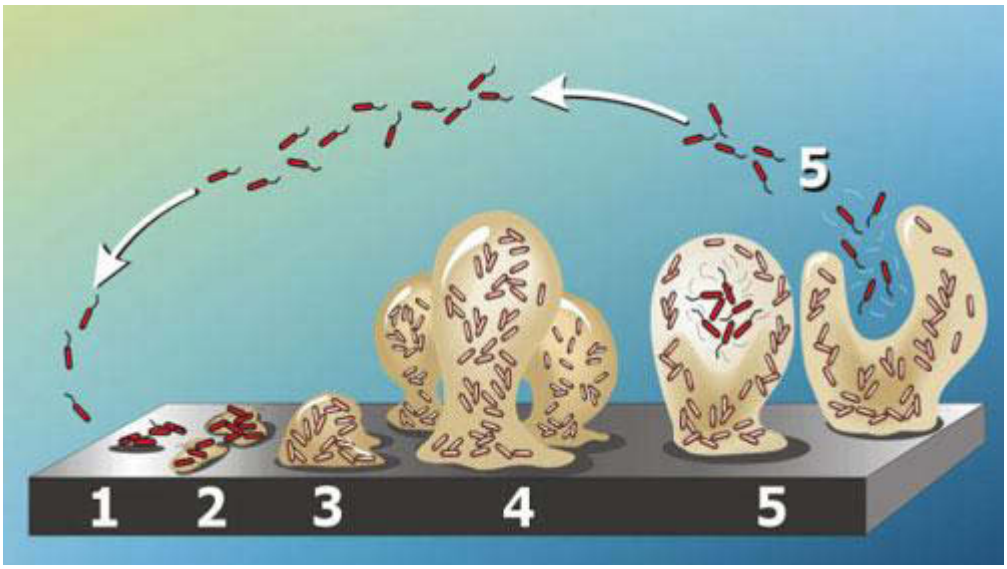

Figure 8 : Cycle de formation d'un biofilm : (1) attachement initial des cellules, (2) adhésion irréversible, (3) formation de microcolonies, (4) développement d'un biofilm mature, (5) dispersion des cellules planctoniques.

Les biofilms sont retrouvés dans divers environnements, qu'ils soient naturels, médicaux ou industriels. Ils sont capables de coloniser aussi bien les surfaces biotiques

qu'abiotiques, dans des environnements favorables à la vie comme dans les environnements les plus inhospitaliers (O'Toole, Kaplan et al. 2000).

Les végétaux représentent un habitat favorable aux communautés bactériennes. Des biofilms ont été fréquemment détectés au niveau de racines de diverses plantes (Davey and O'toole 2000). Ces communautés ont un rôle plutôt positif dans l'environnement puisqu'elles participent à la production et à la dégradation de la matière organique, de certains polluants environnementaux et au recyclage de certains éléments, comme par exemple le carbone, l'azote ou encore le soufre (Davey et O'Toole, 2000). Cependant, certains biofilms peuvent être également source de maladies chez les végétaux (Vojnov, do Amaral et al. 2010).

Dans les environnements médicaux, le développement de biofilms est très problématique puisque ces derniers sont responsables de plus de 60% de cas d'infections nosocomiales (Lewis 2001). En effet, les biofilms peuvent se former sur la quasi-totalité du matériel moderne comme les cathéters, les valves cardiaques, les prothèses, les stérilets, les seringues, les sutures, les lentilles de contacts… (Costerton, Cheng et al. 1987; Davey and O'toole 2000). Il est d'ailleurs reconnu que la majorité des infections chroniques était associée à des biofilms (Costerton, Veeh et al. 2007).

Dans les milieux industriels, les biofilms posent de gros problèmes d'hygiène avec des risques pour la santé humaine, mais ils peuvent également avoir des conséquences économiques importantes. Leur développement dans les ateliers de production et de transformation des aliments peut constituer une source de contamination bactérienne persistante qui peut mener à la détérioration de l'aliment et/ou à la transmission de maladies. Certains biofilms sont également responsables de phénomènes de biocorrosion sur des surfaces métalliques telles que la fonte, les aciers au carbone, des alliages de cuivre, l'aluminium ainsi que des aciers inoxydables. La présence du biofilm induit, à la surface du métal, des modifications physico-chimiques impliquées dans les processus de corrosion qui se manifestent sous forme de piqûres ou de cavernes. Les installations industrielles susceptibles d'être endommagées par la biocorrosion sont diverses : systèmes de transport des eaux brutes et de servitude, échangeurs de chaleur, circuits anti-incendie, déminéralisateurs et condenseurs.

2. Avantages écologiques du biofilm

2.1 Protection contre l'environnement

La plupart des espèces bactériennes formant des biofilm produisent une matrice d'exopolymères qui enrobe les cellules. Cette matrice est composée d'un mélange de composants, tels que des exopolysaccharides (EPS), des protéines, des acides nucléiques et d'autres substances (Sutherland 2001). Parmi ces composants, les EPS ont été particulièrement étudiés et certains avantages leur ont été attribués.

La matrice d'EPS d'un biofilm peut procurer un environnement favorable au développement de certaines bactéries. En effet, les bactéries qui sécrètent des EPS sont capables de former des agrégats qui peuvent constituer un squelette dans lequel d'autres organismes ne formant pas de biofilm peuvent être englobés. De cette façon, certaines bactéries anaérobies strictes peuvent ainsi vivre dans un microenvironnement où le niveau d'oxygène demeure très faible.

De plus, la matrice d'EPS peut empêcher l'accès de certains agents antimicrobiens au biofilm, en agissant comme un échangeur d'ions qui réduit la diffusion des composés du milieu environnant vers le biofilm (Gilbert, Das et al. 1997). Cette caractéristique dépend de la nature de l'agent et des bactéries productrices de biofilm (Hoiby, Bjarnsholt et al. 2010). Cet effet semble être très prononcé avec les antibiotiques hydrophiles et chargés positivement tels que les aminoglycosides (Nickel, Ruseska et al. 1985; Nichols, Dorrington et al. 1988; Nichols, Evans et al. 1989; Subramanian, Shanmugam et al. 2012). Il a également été montré que les EPS permettaient d'emprisonner des métaux, des cations ainsi que des toxines (Flemming 1993; Joshi and Juwarkar 2009; Ruas-Madiedo, Medrano et al. 2010). De plus, non seulement les biofilms sont capables de concentrer les métaux, mais ils jouent également un rôle important dans leur transfert au sein des écosystèmes (Farag, Woodward et al. 1998).

Enfin, il a été montré que la présence d'EPS fournissait une protection contre divers stress environnementaux, tels que les radiations UV, les changements de pH, les chocs osmotiques et la dessiccation (Flemming 1993).

2.2 Disponibilité en nutriments et coopération métabolique

Il a été montré qu'au sein de certains biofilms, les microcolonies sont séparées par des canaux aqueux. Ces canaux forment un réseau de circulation permettant, d'une part, d'acheminer l'oxygène et les nutriments dans les régions enfouies du biofilm, et, d'autre part,

d'évacuer les déchets (Costerton, Lewandowski et al. 1995). Les caractéristiques métaboliques des bactéries au sein d'un biofilm sont différentes de celles de ces mêmes bactéries en condition planctonique. L'architecture du biofilm permet la mise en place d'une coopération métabolique entre les membres de la communauté bactérienne. Par exemple, pour la dégradation de la matière organique en méthane et en dioxyde de carbone en condition anaérobie, l'association d'au moins trois catégories de microorganismes est nécessaire. Tout d'abord, les bactéries fermentatives initient le catabolisme en produisant des alcools et des acides qui seront utilisés par la suite par les bactéries acétogènes. Au final, les bactéries méthanogènes obtiennent l'énergie nécessaire pour convertir l'acétate, le dioxyde de carbone et l'hydrogène en méthane (Davey and O'toole 2000). Un biofilm apparaît donc comme un environnement idéal pour la mise en place d'interactions syntrophiques.

2.3 Brassage génétique

Le transfert horizontal de gènes joue un rôle important dans l'évolution des communautés bactériennes. En effet, c'est un moyen rapide pour les bactéries d'acquérir de nouveaux caractères, et notamment des capacités d'adaptation et de résistance à un environnement hostile. Or, les biofilms permettraient d'augmenter le brassage génétique entre les diverses espèces, en facilitant le transfert horizontal de gènes (Madsen, Burmolle et al. 2012). En effet, il a été montré que les phénomènes de conjugaison étaient plus fréquents au sein d'un biofilm qu'en condition de croissance planctonique (Hausner and Wuertz 1999; Sorensen, Bailey et al. 2005). Il en est de même pour les phénomènes de transformation qui permettent non seulement le transfert de petits fragments d'ADN mais également de plasmides initialement considérés comme non mobiles (Hendrickx, Hausner et al. 2003; Maeda, Ito et al. 2006). De plus, il a été montré que certains plasmides possédaient des gènes codant pour des facteurs impliqués dans la formation de biofilm. C'est notamment le cas de plasmides contenant des gènes codant des fimbriae chez *E. coli* isolés de souches capables d'adhérer à des surfaces abiotiques (Norman, Hansen et al. 2008). Il existerait donc des interconnections importantes entre la formation de biofilm et le transfert horizontal de gènes via les éléments génétiques mobiles, qui s'autoréguleraient de façon positive (Madsen, Burmolle et al. 2012).

L. monocytogenes est considérée comme un faible producteur de biofilm comparé à d'autres genres bactériens comme *Pseudomonas aeruginosa* ou *Staphylococcus aureus.* De plus, contrairement à ces dernières, la présence de matrice extracellulaire de nature

polysaccharidique n'a jamais été mise en évidence chez *L. monocytogenes*. Cependant, selon les souches et les conditions de cultures, différentes architectures de biofilms ont pu être mises en évidence.

3. L'architecture des biofilms chez *L. monocytogenes*

L'architecture des biofilms de *L. monocytogenes* a été étudiée par différentes techniques comme la microscopie électronique à balayage (Kalmokoff, Austin et al. 2001; Chavant, Martinie et al. 2002; Borucki, Peppin et al. 2003), la microscopie à épifluorescence (Lundén, Miettinen et al. 2000; Kalmokoff, Austin et al. 2001; Carpentier and Chassaing 2004; Monk, Cook et al. 2004; Pan, Breidt et al. 2006) et la microscopie confocale à balayage laser (LSMC) (Chae and Schraft 2000; Rieu, Briandet et al. 2008). Plusieurs tentatives de modélisation de formation de biofilm ont été réalisées (Kreft and Wimpenny 2001; Zameer, Gopal et al. 2010).

L. monocytogenes est capable de coloniser aussi bien les surfaces hydrophiles comme l'acier inoxydable, que les surfaces hydrophobes telles que le polytetrafluoroéthylène (PTFE) (Figure 9) (Chavant, Martinie et al. 2002; Renier, Hebraud et al. 2011).

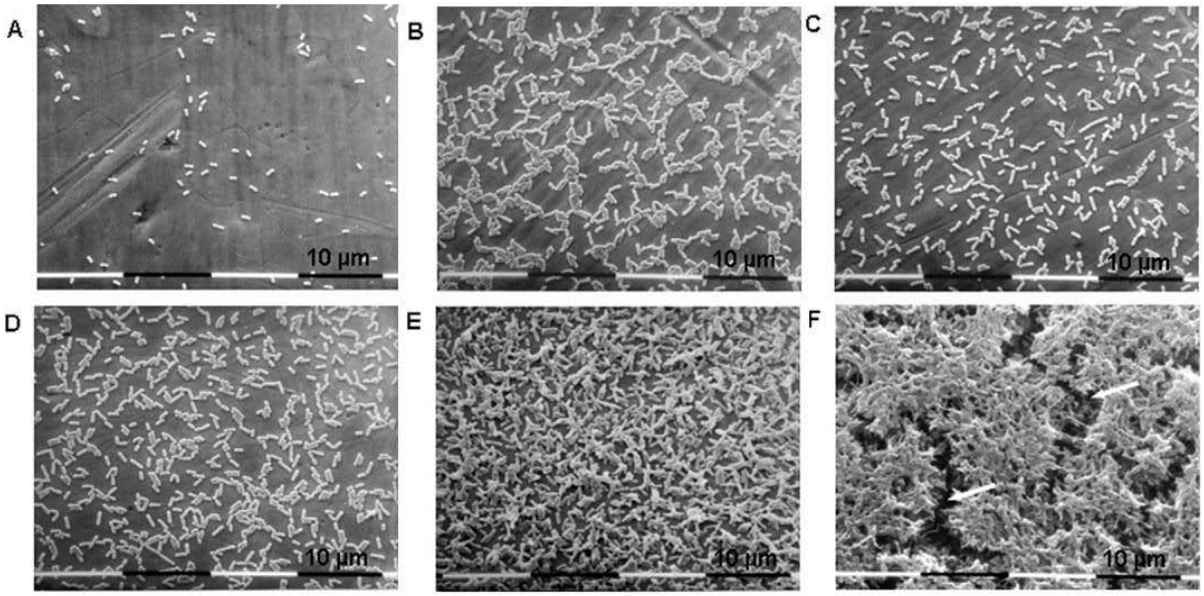

Figure 9 : Formation de biofilm au cours du temps chez *L. monocytogenes* EGDe (Renier, Hebraud et al. 2011). Biofilm sur coupon d'acier après A) 10 secondes, B) 5 heures, C, 8 heures, D) 24 heures, E) 5 jours, F) 7 jours d'incubation à 20°C. Les flèches blanches indiquent des canaux putatifs.

En condition statique, certaines souches produisent des biofilms à structure tridimensionnelle en forme de champignon parsemés de canaux et de pores, alors que d'autres ne forment que des agrégats cellulaires épars ou des tapis monocouches bactériens (Chae and Schraft 2000; Kalmokoff, Austin et al. 2001; Chavant, Martinie et al. 2002; Borucki, Peppin et al. 2003). Une structure particulière de biofilm en forme de rayon de miel a également été observée chez certaines souches colonisant de l'acier inoxydable ou des articles en plastique (Figure 10) (Marsh, Luo et al. 2003). En condition dynamique (flow-cell) une nouvelle structure tridimensionnelle a été caractérisée par LSCM, consistant en des microcolonies en forme de pelotes entourées d'un réseau de chaînes « tricotées » (Figure 10) (Rieu, Briandet et al. 2008).

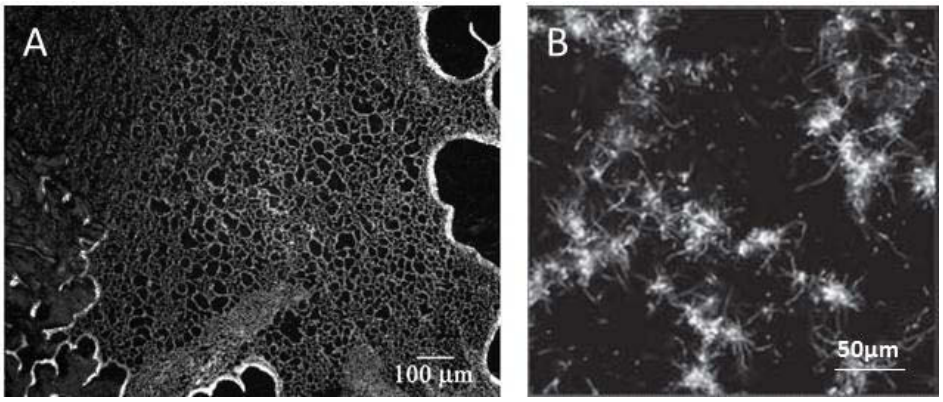

Figure 10 : Structures de biofilm particulières. A) en nid d'abeilles (Marsh, Luo et al. 2003) et B) en forme de pelotes entourées d'un réseau de chaînes tricotées (Rieu, Briandet et al. 2008)

4. Phylogénie et formation de biofilm

Différentes études ont montré que les souches de *L. monocytogenes* présentaient des différences significatives quant à leur capacité à adhérer à des surfaces abiotiques (Norwood and Gilmour 1999; Lundén, Miettinen et al. 2000). Une hypothèse a alors été formulée selon laquelle cette capacité d'adhésion et de formation de biofilms serait conservée chez les souches appartenant à un même linéage phylogénétique.

L'étude d'un grand nombre d'isolats a suggéré que les sérovars présentaient des différences significatives pour leur capacité à adhérer à l'acier inoxydable et en particulier les souches appartenant au sérovar 1/2c qui ont montré le plus haut niveau d'attachement (Norwood and Gilmour 1999; Lundén, Miettinen et al. 2000). De plus, cette forte capacité

d'adhésion est pour la plupart des cas corrélée à des souches dites persistantes, c'est-à-dire qui demeurent toujours présentes malgré les traitements désinfectants.

Plusieurs travaux ont également montré une corrélation entre la phylogénie et la capacité de *L. monocytogenes* à former des biofilms. Cependant, les conclusions énoncées par deux équipes divergent (Djordjevic, Wiedmann et al. 2002; Borucki, Peppin et al. 2003). En effet, alors que Djordjevic *et al*. (2002) ont montré que certaines souches appartenant au Linéage I (sérovars 1/2b et 4b) formaient des biofilm plus importants que les souches appartenant au Linéage II (sérovars 1/2a et 1/2c), l'inverse était démontré un an plus tard par (Borucki, Peppin et al. 2003). Il n'existerait pas de corrélation non plus entre la persistance d'une souche dans un atelier et sa capacité à former un biofilm (Nilsson, Ross et al. 2011).

Au vu de ces résultats, aucune conclusion ne peut être établie concernant la potentielle corrélation entre les Linéages et la capacité à former un biofilm. Alors que la forte capacité d'adhésion semble être corrélée à la persistance de la bactérie, ce n'est pas le cas pour la capacité à former un biofilm. Ceci n'est finalement pas surprenant puisque *L. monocytogenes* est généralement isolée à partir de biofilms contenant plusieurs genres bactériens. Il a d'ailleurs été montré que des faibles producteurs de biofilm, par exemple la souche 4b, pouvaient former d'importants biofilms en présence d'un fort producteur de biofilm comme la souche SK1387 1/2a. Il a alors été suggéré que la matrice extracellulaire produite par cette dernière souche adhérait à la surface et améliorait la croissance sessile de la souche 4b, lui conférant une meilleure protection contre les facteurs de stress environnementaux (Pan, Breidt et al. 2009).

5. Effet de l'environnement sur la formation de biofilm chez *L. monocytogenes*

Le passage d'une forme planctonique à une forme sessile nécessite de profonds changements physiologiques, qui se produisent par la régulation de l'expression de gènes en réponse à divers signaux environnementaux. L'importance des conditions environnementales, telles que la nature des surfaces, le milieu nutritif et la température de croissance, dans la formation de biofilm a été mise en évidence (Moltz and Martin 2005). Une étude récente a également montré que l'architecture d'un biofilm pouvait varier selon les conditions de cultures, qu'elles soient statique ou dynamique (Rieu, Briandet et al. 2008).

Les facteurs biotiques peuvent également jouer un rôle dans la formation de biofilm et ils seraient de la plus grande importance concernant la persistance de certaines souches, notamment dans les ateliers de transformation des aliments (Zottola and Sasahara 1994). Les biofilms déjà établis peuvent avoir un impact positif ou négatif sur la formation de biofilm par *L. monocytogenes* (Carpentier and Chassaing 2004). L'inhibition du développement de *L. monocytogenes* peut être due à une compétition vis à vis des nutriments (Besse, Beaufort et al. 2008; Guillier, Stahl et al. 2008) ou à la sécrétion d'agents antimicrobiens comme les bactériocines produites par *Lactococcus lactis* par exemple (Leriche, Chassaing et al. 1999; Liu, O'Conner et al. 2008). En revanche, la présence de micro-organismes comme *Staphylococcus capitis* ou *S. aureus,* dans certaines conditions, améliore la formation de biofilm par *L. monocytogenes* (Carpentier and Chassaing 2004; Rieu, Lemaitre et al. 2008). Il a d'ailleurs été montré que l'ajout de surnageant de *S. capitis* ou de *S. aureus* est suffisant pour stimuler le développement de biofilm chez *L. monocytogenes*. Cet effet positif est par ailleurs supprimé lorsque ce surnageant est traité par la protéinase K, mais pas après ultrafiltration (3 kDa cut-off), ce qui suggère qu'une ou des molécules peptidiques présentes dans le surnageant de *S. aureus* pourraient être responsables de l'amélioration de la formation de biofilm (Rieu, Lemaitre et al. 2008). *Pseudomonas fragi* a également été démontré comme nécessaire à la mise en place de biofilm par *L. monocytogenes* (Sasahara and Zottola 1993). Afin de déterminer les propriétés des biofilms qui influencent la fixation initiale de *L. monocytogenes*, un ensemble de biofilms de *L. lactis* avec des architectures, des porosités, des types de matrices et des propriétés de surface de cellules individuelles différentes ont été créés (Habimana, Meyrand et al. 2009). Cette étude suggère que la structure poreuse des biofilms résidents améliore l'adhérence de *L. monocytogenes*, alors que les exopolysaccharides produits par les biofilms résidents empêchent son adhésion.

Au cours de l'infection du tractus gastro-intestinal, *L. monocytogenes* se trouve dans un environnement particulier avec des conditions sous-optimales, notamment exposée aux sels biliaires. Néanmoins, *L. monocytogenes* est capable de survivre, coloniser et pénétrer dans les cellules épithéliales. La bile a d'ailleurs été récemment montrée comme améliorant la fixation initiale sur des surfaces plastiques et la formation de biofilm. Ainsi, lors de l'infection, l'exposition à la bile pourrait améliorer la formation de biofilms chez *L. monocytogenes*, et par conséquent pourrait contribuer à sa survie en facilitant la colonisation du tractus gastro-intestinal (Begley, Kerr et al. 2009).

6. Déterminants moléculaires impliqués dans la formation de biofilm

6.1 Le flagelle

L. monocytogenes possède quatre à six flagelles péritriches par cellule (Figure 11), composés de milliers de monomères de flagellines codées par le gène *flaA*.

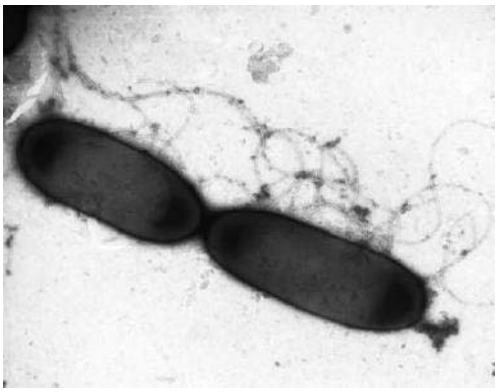

Figure 11 : *L. monocytogenes* avec ses flagelles péritriches (Mauder, Williams et al. 2008)

La biosynthèse du flagelle est régulée par la température (Peel, Donachie et al. 1988). En effet, l'expression de *flaA* est arrêtée à des températures supérieures à 30°C, auxquelles les souches de *L. monocytogenes* ne sont plus mobiles. Le contrôle de la biosynthèse des flagelles est plutôt complexe car elle implique au moins cinq facteurs de régulation, (i) FlaR (flagellin regulator) (Sanchez-Campillo, Dramsi et al. 1995), PrfA (positif regulatory factor A) (Michel, Mengaud et al. 1998), DegU (dégradation enzymes regulator) (Knudsen, Olsen et al. 2004), MogR (motility gene repressor) (Gründling, Burrack et al. 2004) et GmaR (glycotransferase and motility antirepressor) (Shen and Higgins 2006). Tout comme PrfA (Scortti, Monzo et al. 2007), la plupart de ces régulateurs contrôlent également l'expression de facteurs de virulence. Jusqu'à présent, FlaA est la première et unique protéine de surface dont la glycosylation ait été rapportée chez *L. monocytogenes*.

Il a été montré chez plusieurs genres bactériens que les flagelles jouaient un rôle très important dans les premières étapes de la formation du biofilm (O'Toole, Kaplan et al. 2000). Chez *L. monocytogenes*, les premières études réalisées sur les flagelles, en tant que structures potentiellement impliquées dans la formation de biofilms, ont montré que leur absence

affectait l'adhésion initiale. Cependant, aucune différence quantitative n'était constatée au bout de 24 heures de formation de biofilm (Vatanyoopaisarn, Nazli et al. 2000).

Des recherches complémentaires ont confirmé l'implication des flagelles dans les premiers stades d'adhésion, mais pas en tant qu'adhésine (Lemon, Higgins et al. 2007). Un mutant possédant des flagelles dépourvus d'O-glycosylation forme aussi bien des biofilms que la souche sauvage. Par contre, la quantité de biofilm adhérée est largement réduite chez un mutant possédant un flagelle paralysé.

De nombreuses études ultérieures ont confirmé l'importance de la motilité dans les premières étapes de la formation de biofilm (Tresse, Lebret et al. 2006; Gueriri, Cyncynatus et al. 2008; Tresse, Lebret et al. 2009). Toutefois, Todhanakasem et Young (2008) ont démontré que l'absence de flagelle ou de motilité avait un effet plus complexe sur le développement du biofilm. Ces derniers ont notamment montré chez les mutants dépourvus de flagelles ou dont le flagelle était paralysé, la formation d'un « hyper biofilm » (HB) en condition de culture continue (Todhanakasem and Young 2008). Ce phénotype HB n'apparaît pas en condition statique, probablement en raison des différences dues à de nombreux changements survenant dans le milieu de culture lorsque les cellules ont atteint la capacité maximale de croissance, comme les changements de pH ou de disponibilité en nutriments. Ainsi, bien que le flagelle permette l'amélioration de l'adhésion initiale chez *L. monocytogenes* par la mobilité qu'il procure à la fois en condition statique que dynamique, ce flagelle n'est pas nécessaire à l'adhésion, ni à la formation de biofilm.

6.2 Les déterminants de la communication cellulaire

Au cours du développement du biofilm et de sa maturation, des mécanismes cellulaires complexes sont mis en place. Ces mécanismes nécessitent une régulation coordonnée de l'expression de gènes mettant en jeu des systèmes de communication entre cellules (Dunny and Leonard 1997; Hardman, Stewart et al. 1998; Waters and Bassler 2005; von Bodman, Willey et al. 2008). Parmi ces systèmes de signalisation cellule-cellule, le quorum sensing (QS) a été le plus étudié. Il fait spécifiquement référence à un comportement multicellulaire aboutissant à la régulation de divers processus physiologiques, qui est seulement induit lorsque les cellules ont atteint un certain seuil de densité. Ce mécanisme est fondé sur la production et la libération de molécules de signalisation nommées auto-inducteurs. La détection d'auto-inducteurs à partir d'un certain seuil de concentration conduit à une réponse bactérienne. Il existe deux archétypes de systèmes de QS chez *L. monocytogenes* : le système

LuxS/auto-inducteur 2 (AI-2) présent chez les bactéries à Gram négatif et à Gram positif, et le système de signalisation *agr* (accessory gene regulator) caractéristique des bactéries à Gram positif (Dunny and Leonard 1997; Miller and Bassler 2001; Waters and Bassler 2005).

Listeria monocytogenes est capable de produire une molécule similaire à AI-2 par l'intermédiaire de la protéine LuxS (Challan Belval, Gal et al. 2006). Selon les genres bactériens, la délétion du gène *luxS* affecte la formation de biofilm de façon différente (Blehert, Palmer et al. 2003; Cole, Harwood et al. 2004; Wen and Burne 2004). Chez *L. monocytogenes*, la souche mutante *luxS* forme un biofilm plus dense que la souche sauvage (Challan Belval, Gal et al. 2006; Sela, Frank et al. 2006). Cependant, l'ajout d'AI-2 de synthèse dans le surnageant du mutant *luxS* ne restaure pas le phénotype de la souche sauvage. En revanche, la S-ribosyle homocystéine (SRH), précurseur de l'AI-2 (Figure 12), s'accumule dans le surnageant du mutant (Challan Belval, Gal et al. 2006). Ces résultats montrent que SRH est capable de modifier le nombre de cellules adhérées, mais pas l'AI-2, et que le gène *luxS* participe à la répression de la formation de biofilms en convertissant la SRH en AI-2.

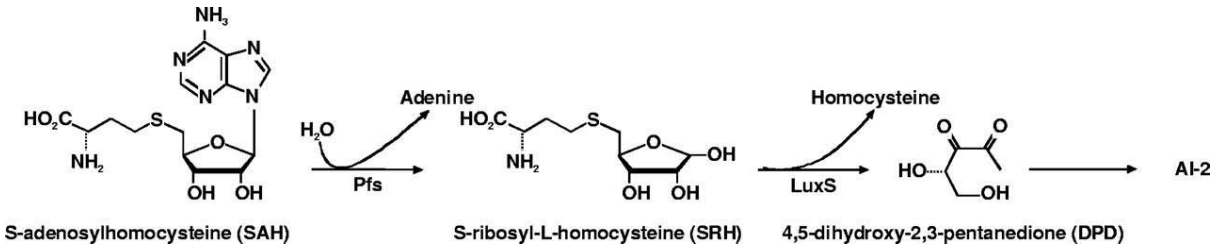

Figure 12 : Voie de synthèse de l'AI-2 (Challan Belval, Gal et al. 2006)

De plus, le précurseur du SRH, la S-adénosyl homocystéine (SAH) qui est un composé toxique, s'accumule également dans le surnageant du mutant *luxS*. L'ajout de SAH dans le surnageant de la souche sauvage n'augmente pas sa capacité d'adhésion. De ce point de vue, le rôle physiologique de l'AI-2 ne peut être limité qu'à la détoxification de SAH chez *L. monocytogenes*.

Le système *agr* a d'abord été décrit chez *S. aureus*, puis a été identifié et étudié chez *L. monocytogenes*. Chez *S. aureus*, le locus *agr* est composé de quatre gènes (*agrB*, *agrD*, *agrC* et *agrA*) organisés en opéron (Autret, Raynaud et al. 2003). AgrD est le peptide précurseur de l'AIP (peptide autoinducteur) qui est maturé par AgrB, une endopeptidase membranaire. AgrC correspond à une histidine kinase qui est activée par l'AIP tandis qu'AgrA est le régulateur transcriptionnel qui induit la transcription de deux transcripts divergents RNA II et RNA III. Ce dernier régule positivement l'expression de plusieurs gènes

codant des facteurs de virulence et négativement l'expression de plusieurs gènes codant des facteurs d'adhésion. Le locus *agr* de *L. monocytogenes* comprend les quatre gènes dans le même arrangement que chez *S. aureus*, *agrBDCA* (Figure 13). Cependant, la structure de l'AIP n'est pas connue à ce jour et il n'existe pas d'équivalent au transcrit RNAIII de *S. aureus* en amont de l'opéron *agr* chez *L. monocytogenes*.

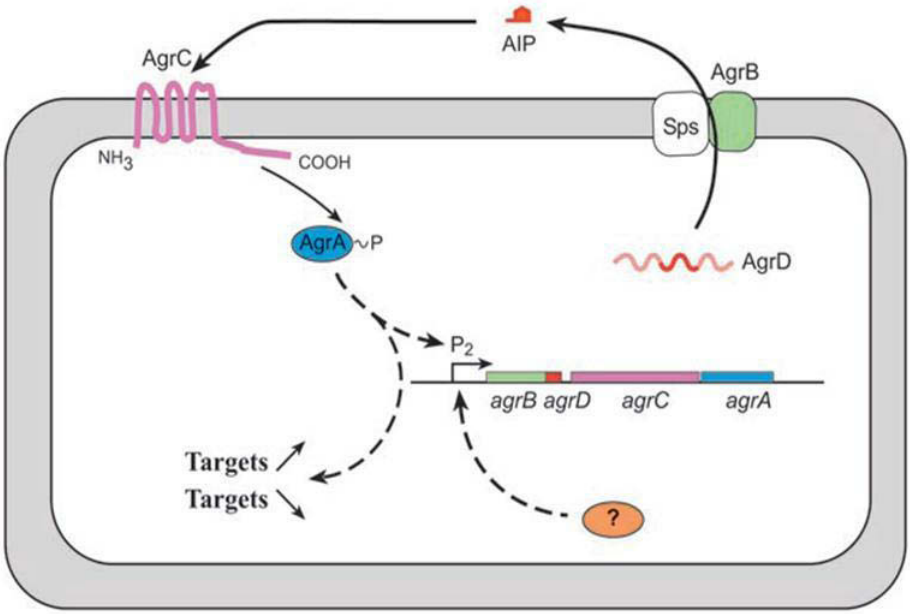

Figure 13 : Le système *agr* chez *L. monocytogenes* (Garmyn, Gal et al. 2009). AgrD est maturé par AgrB pour former AIP. Ce dernier va activer AgrC qui va phosphoryler le régulateur transcriptionnel AgrA.

Il a été récemment montré que le système *agr* jouait un rôle important dans la formation de biofilm chez *L. monocytogenes* (Rieu, Weidmann et al. 2007; Riedel, Monk et al. 2009). Les premières étapes de formation de biofilm sont notamment affectées chez des souches mutantes Δ*agrA* et Δ*agrD* dans les 24 premières heures d'incubation, mais pas par la suite (Rieu, Weidmann et al. 2007). L'expression des gènes *agr* augmente progressivement au cours de la formation de biofilm en condition dynamique et cette expression est maximale à la périphérie des structures en pelotes formées par les cellules (Rieu, Briandet et al. 2008). De plus, lors de l'utilisation d'un autre milieu de culture (BHI dilué au 1/10 par rapport à du TBS), la formation de biofilm chez le mutant *agrD* est plus affectée que ce qui avait précédemment été montré (Riedel, Monk et al. 2009). Le phénotype sauvage est cependant rétabli chez le mutant Δ*agrD* après l'ajout du surnageant de la souche sauvage (*wt*) cultivé dans du BHI ou en mélangeant les souches avec un rapport cellulaire de 90:10 (Δ*agrD*:*wt*) (Riedel, Monk et al. 2009).

Une intéressante conséquence de la suppression du gène *agrD* est la diminution de l'internaline A (InlA) dans la paroi cellulaire (Riedel, Monk et al. 2009), indiquant que l'expression des gènes de virulence est également régulée par le système *agr*. Par ailleurs, les souches de *L. monocytogenes* exprimant la protéine InlA sous sa forme tronquée améliorent considérablement leur capacité à former un biofilm comparées aux souches exprimant la forme complète (Franciosa, Maugliani et al. 2009). Comme il a été montré pour d'autres systèmes (Davies, Parsek et al. 1998; Hall-Stoodley and Stoodley 2002; Sauer, Camper et al. 2002), les régulations mises en place au cours du développement sessile sont complexes et sans doute transitoires. Ces systèmes de signalisation sont très certainement impliqués dans des régulations physiologiques avec des conséquences indirectes sur la formation de biofilm. Cependant, le rôle du système *agr* sur le métabolisme de *L. monocytogenes* n'est pas connu à ce jour. Il ne semble pas impliqué dans la virulence, ni dans la motilité, ni dans la croissance planctonique à différentes températures ou dans différentes conditions de stress (NaCl, éthanol, H_2O_2) (Autret, Raynaud et al. 2003; Williams, Bauer et al. 2005). De plus, il n'a jamais été montré que ce système était un mécanisme permettant l'évaluation de la densité cellulaire, dans le but de coordonner le comportement de l'ensemble de la population chez *L. monocytogenes* (Garmyn, Gal et al. 2009) ; en d'autres termes, la mise en évidence de la présence d'un système de QS reste encore à démontrer chez *L. monocytogenes*.

6.3 La matrice extracellulaire

La matrice extracellulaire, qui englobe les cellules dans un biofilm, est un mélange complexe d'exopolysaccharides, d'ADN extracellulaire (ADNe), de protéines, et d'autres polymères extracellulaires (polyglutamate, acides téchoïques, etc), permettant la stabilisation et la protection des biofilms (Sutherland 2001). La présence, les proportions respectives et la contribution de ces différents composants sont très variables d'une espèce bactérienne à une autre et peuvent même être souche-dépendante. Alors que les exopolysaccharides sont décrits comme les principaux composants de la matrice extracellulaire dans de nombreux biofilms bactériens (Whitfield 1988; Vu, Chen et al. 2009), leur présence systématique et leur contribution dans la formation de biofilm chez *L. monocytogenes* restent quelque peu controversés. Une matrice extracellulaire épaisse et collante a été observée chez certaines bactéries modèles formant des biofilms, comme par exemple chez *Staphylococcus epidermidis* (Götz 2002), mais ce n'est pas le cas de *L. monocytogenes*. La présence de fibres reliant les cellules individuelles entre elles chez *L. monocytogenes* a été

observée en microscopie électronique (Borucki, Peppin et al. 2003; Marsh, Luo et al. 2003; Hefford, D'Aoust et al. 2005; Zameer, Gopal et al. 2010). Il a tout d'abord été envisagé que ces structures résultaient de la déshydratation de l'échantillon, suite à son traitement pour les observations microscopiques. La déshydratation aurait conduit à un rétrécissement de la matrice d'exopolymères, laissant en évidence uniquement les structures fibrillaires. En parallèle, la coloration au rouge de ruthénium a révélé la présence d'hydrates de carbone extracellulaires sur les contours des cellules de *L. monocytogenes* (Borucki, Peppin et al. 2003; Zameer, Gopal et al. 2010). Cependant, ces fibrilles n'ont jamais été identifiées comme des exopolysaccharides. De plus, le rouge de ruthénium peut également colorer les hydrates de carbone présents sur la surface cellulaire autre que les exopolysaccharides, tels que les composés glycosylés (peptidoglycanes, acides téichoïques ou protéines). Jusqu'à présent, les analyses génomiques réalisées chez *L. monocytogenes* n'ont pas révélé la présence de voie de biosynthèse d'exopolysaccharides tels que l'alginate présent chez *Pseudomonas aeruginosa* ou le poly-N-acétylglucosamine présent chez *Staphylococcus aureus* (Harmsen, Lappann et al. 2010). Ces mêmes fibrilles ont également été qualifiées de structures similaires aux fimbriae (Folio 2003). Des investigations plus poussées seraient nécessaires pour confirmer l'origine de ces fibrilles chez *L. monocytogenes*.

Comme chez de nombreux genres bactériens, y compris *Staphylococcus* (Qin, Ou et al. 2007; Rice, Mann et al. 2007; Izano, Amarante et al. 2008), *Pseudomonas* (Whitchurch, Tolker-Nielsen et al. 2002; Allesen-Holm, Barken et al. 2006) et *Bacillus* (Vilain, Pretorius et al. 2009), il a été récemment montré chez *L. monocytogenes* que d'ADN extracellulaire (ADNe) jouait un rôle important dans les premières phases de formation de biofilm (Harmsen, Lappann et al. 2010). En effet, l'ajout de DNaseI réduit significativement la fixation cellulaire, affectant la formation ultérieure du biofilm, alors que l'ajout de RNases et de protéinases ne produit aucun effet. Cependant, le rôle exact de l'ADNe dans la formation de biofilm reste encore flou. Ainsi, dans une culture sans ADNe, l'ajout d'ADN génomique de *L. monocytogenes*, de fragments d'ADN de faible poids moléculaire ou d'ADN de sperme de saumon ne restore pas la capacité à adhérer. Par contre, l'ajout combiné, dans une culture dépourvue d'ADNe, d'ADN de haut poids moléculaire et de surnageant de culture traité enzymatiquement avec la DNase I et la protéinase K restore l'adhésion des cellules. Ces résultats suggérent l'implication dans l'adhésion initiale d'un ou plusieurs composés de nature non protéique présents dans le surnageant de culture. Le composé identifié est un peptidoglycane et plus particulièrement, le N-acétyl-glucosamine (NAG). Le NAG, en présence d'ADN de haut poids moléculaire, pourrait former un polymère de nature similaire

au poly-NAG (PNAG) de *Staphylococcus* ou *E. coli*, qui permettrait l'adhésion et la formation de biofilm même si d'autres composants de la matrice sont absent dans le milieu (Harmsen, Lappann et al. 2010). Cependant, d'autres investigations sont nécessaires pour définir, à un niveau moléculaire, les interactions existantes entre ces différents composants et leur rôle exact dans l'adhérence et la formation de biofilms chez *L. monocytogenes*.

Des études ont également montré que des protéines de surface et des protéines extracellulaires jouaient un rôle essentiel dans l'adhésion initiale de la bactérie à une surface (Smoot and Pierson 1998; Longhi, Scoarughi et al. 2008). Ainsi, (Smoot and Pierson 1998) ont montré que l'adhésion de *L. monocytogenes* à l'acier inoxydable et au caoutchouc synthétique était réduite de 99% lorsque de la trypsine était ajoutée au milieu. De plus, *L. monocytogenes* ne parvient pas à former un biofilm suite à un traitement avec de la serratiopeptidase, une métalloprotéase extracellulaire produite par *Serratia marcescens* (Longhi, Scoarughi et al. 2008). Des décrochements de biofilm ont également été mis en évidence suite à un traitement avec l'endopeptidase K (Franciosa, Maugliani et al. 2009).

6.4 Protéines associées au biofilm (Bap)

Les protéines Bap (biofilm-associated protein) appartiennent à une famille de protéines de surface impliquées dans la formation de biofilm (Lasa and Penades 2006). La protéine Bap a été identifiée pour la première fois chez une souche de *S. aureus* isolée de mammites (Cucarella, Solano et al. 2001). Elle a été retrouvée ensuite chez divers genres bactériens comme *Enterococcus faecalis* (Toledo-Arana et al. 2001), mais aussi chez des bactéries à Gram négatif, telles que *Pseudomonas fluorescens* (Hina et al. 2003 et *Salmonella enterica* sv. *typhimurium* (Latasa et al. 2005). Toutes les protéines identifiées en tant que Bap partagent des caractéristiques structurales communes. Ce sont des protéines de surface de poids moléculaires élevés avec un domaine central contenant des séquences répétées successives d'acides aminés et un motif LPXTG d'ancrage à la paroi cellulaire dans la région C-terminale. Des expériences *in vitro* ont montré que, chez *S. aureus*, Bap n'était pas seulement impliquée dans l'étape d'adhésion initiale mais aussi, avec le polysaccharide PIA/PNAG, dans l'agrégation des cellules entre elles et la maturation du biofilm. Les protéines Bap jouent également un rôle important dans les processus d'infection bactérienne et peuvent parfois être présentes dans les éléments mobiles (Lasa and Penades 2006).

Récemment, une analyse *in silico* réalisée sur le génome de *L. monocytogenes* a révélé un cadre ouvert de lecture codant une protéine similaire à Bap (Lmo0435) (Jordan, Perni et al.

2008). Cette protéine a été désignée BapL car elle présente une structure similaire à celles des protéines Bap et est impliquée dans la fixation des cellules à des surfaces abiotiques. Chez *L. monocytogenes* 10403s, le mutant *lmo0435* montre une réduction significative du niveau d'adhésion par rapport à son parent isogénique. Cependant, contrairement à d'autres protéines de type Bap, BapL ne participe pas à la virulence de la bactérie (Jordan, Perni et al. 2008). Bien que BapL semble jouer un rôle important dans l'adhésion, seuls quatre isolats de *L. monocytogenes* sur 17 testés possèdent le gène codant cette protéine. De plus, certaines souches ne possédant pas BapL adhérent nettement mieux que d'autres qui la possèdent. BapL peut donc contribuer à la fixation de certaines souches de *L. monocytogenes*, mais son rôle dans le développement du biofilm n'a pas été clairement établi. Une réduction de l'adhésion n'empêche pas la formation ultérieure de biofilm chez le mutant n'exprimant pas BapL. Ces résultats vont à l'encontre de ce qui avait été montré précédemment chez les autres genres bactériens, dont l'aptitude à former un biofilm était fortement affectée suite à la suppression de Bap (Lasa and Penades 2006). Par conséquent, des analyses complémentaires s'avèrent nécessaires afin de déterminer plus précisément la (ou les) fonction(s) de BapL chez *L. monocytogenes* et si son appartenance à la famille des protéines Bap est bien justifiée au niveau fonctionnel.

6.5 Autres déterminants moléculaires

Suite au criblage d'une banque de transposons de *L. monocytogenes*, deux mutants d'insertion au niveau des gènes *relA* et *hpt* ont présenté une capacité à former un biofilm inférieure à celle de la souche sauvage (Taylor, Beresford et al. 2002). Le gène *relA* code une guanosine pentaphosphate synthétase qui catalyse la formation de l'alarmone (p) ppGpp, tandis que le gène *hpt* code une 6-oxopurine phosphoribosyltransférase qui convertit la base purique (guanine) en guanosine monophosphate (GMP). L'étude de ces deux mutants a montré qu'ils possédaient une capacité d'adhésion similaire à celle de la souche sauvage. Par contre, les cellules sont incapables de se développer en biofilm par la suite. Ceci suggère que les deux gènes sont essentiels à un mode de croissance sessile de *L. monocytogenes*. Il a également été constaté que ces deux mutants étaient incapables de synthétiser (p) ppGpp en réponse à une privation de nutriments (Taylor, Beresford et al. 2002). Le niveau d'expression du gène de *relA* augmente après l'adhésion initiale, montrant la mise en place d'une réponse stringente suite à cette adhésion. Ces résultats suggèrent fortement que la capacité de mettre

en place une réponse stringente et des adaptations physiologiques suite à une carence en nutriments est essentielle pour la croissance cellulaire en mode sessile.

Le diguanylate cyclique (c-di-GMP), un autre dérivé de la guanine, joue un rôle crucial en tant que messager secondaire, en particulier pour la transition entre la forme planctonique mobile et la forme sédentaire (biofilm), chez de nombreux genres bactériens (Lasa 2006; Hengge 2009). Ce système de transduction de signal implique de nombreuses diguanylate cyclases ainsi que la phosphodiestérase, c'est-à-dire des protéines présentant des motifs GGDEF et EAL / HD-GYP respectivement. Selon la base de données Pfam, le génome de *L. monocytogenes* code 4 protéines présentant un domaine GGDEF (PF00990: Lmo1912, Lmo2174, Lmo0531 et Lmo1911) et trois protéines présentant un domaine EAL (PF00563: Lmo1914, Lmo0111 et Lmo0131). Chez *Listeria*, cependant, la voie de signalisation du c-di-GMP n'a jamais été démontrée. Une molécule signal secondaire supplémentaire, le di-adénosine monophosphate cyclique (c-di-AMP), a été récemment montrée chez *L. monocytogenes* comme permettant le déclanchement d'une voie cytosolique de l'immunité innée au cours d'une infection (Woodward, Iavarone et al. 2010).Le c-di-AMP n'a pas été beaucoup étudié chez les bactéries, mais il est supposé qu'il interviendrait dans la régulation de la sporulation bactérienne (Bejerano-Sagie, Oppenheimer-Shaanan et al. 2006). *L. monocytogenes* ne sporulant pas (Seeliger and Jones 1986), il serait intéressant d'étudier l'effet du c-di-AMP dans la physiologie de la bactérie, y compris dans la formation de biofilm.

Dans des conditions de culture continue, les biofilms de *L. monocytogenes* forment des pelotes entourées de réseaux cellulaires. Le facteur de réponse SOS, YneA, est spécifiquement activé en condition de culture continue, tout comme RecA qui est nécessaire à son activation (van der Veen and Abee 2010). La suppression des gènes *yneA* et *recA* conduit à un biofilm sensiblement réduit dans ces mêmes conditions, alors qu'aucune différence significative n'a été observée dans des conditions statiques de formation de biofilm. Contrairement à la souche sauvage qui forme un biofilm avec des structures particulières, c'est à dire des pelotes entourées d'un réseau de chaînes tricoté (Rieu, Briandet et al. 2008), les mutants *yneA* et *recA* présentent quelques plaques de cellules adhérées après 24 h, qui se développent en très petites microcolonies après 48 h. Les facteurs de réponse SOS, YneA et RecA, ne sont pas nécessaires à l'adhésion initiale de la bactérie mais sont essentiels pour le développement ultérieur de biofilms.

Récemment, il a été montré que le régulateur de virulence PrfA était impliqué dans la formation de biofilm (Lemon, Freitag et al. 2010). Tout comme les deux protéines

précédemment citées, PrfA ne serait pas nécessaire à l'adhésion initiale de *L. monocytogenes*, du moins à 20°C. Sachant que les régulations réalisées par PrfA sont dépendantes de la température, il est dommage que ces mêmes tests n'aient pas été effectués à des températures plus élevées comme 30 ou 37°C. Comme PrfA régule positivement à la fois des gènes de virulence et des gènes impliqués dans la formation de biofilm, ce régulateur pourrait jouer un rôle global dans la transition du mode de vie saprophyte au mode de vie parasite (Lemon, Freitag et al. 2010).

Suite à une croissance sessile prolongée sur des coupons d'acier inoxydable en bioréacteur, le passage d'un morphotype lisse à morphotypes dits rugueux a été montré au cours de la formation de biofilm chez *L. monocytogenes* (Monk, Cook et al. 2004). Les bactéries présentant ce morphotype rugueux apparaissent spontanément lors de la formation de biofilm et forment des chaînes cellulaires qui permettraient d'améliorer la colonisation de surfaces (Monk, Cook et al. 2004). Ce morphotype apparaît également chez *L. monocytogenes* lorsqu'elle est cultivée en condition de stress salin, acide ou alcalin ainsi qu'à de fortes températures (Brzin 1975; Bereksi, Gavini et al. 2002; Geng, Kim et al. 2003; Hazeleger, Dalvoorde et al. 2006; Giotis, Blair et al. 2007). La délétion du gène *secA2* codant une ATPase du système Sec (Voir Chapitre 3- La voie de sécrétion Sec- 2.3. SecA2) conduit également à la formation de longs filaments cellulaires, de même que la mutation simultanée des deux hydrolases pariétales MurA et p60, sécrétées de façon SecA2-dépendante (Machata, Hain et al. 2005). Ces informations ont permis d'émettre l'hypothèse selon laquelle SecA2 serait impliquée dans la transition d'un morphotype lisse à un morphotype rugueux dans l'environnement. Alors que le mutant Δ*secA2* ainsi que les mutants Δ*p60* et *ΔmurA* ont été largement étudiés pour leur implication dans la virulence chez *L. monocytogenes*, aucune étude n'a été réalisée concernant leur capacité à coloniser un milieu. Il serait donc intéressant de regarder l'effet du morphotype rugueux sur la capacité de *L. monocytogenes* à former un biofilm mais également de déterminer l'implication des protéines sécrétées de façon SecA2-dépendante.

6.6 Conclusion

Un faible nombre de déterminants moléculaires impliqués dans la formation de biofilm est connu actuellement chez *L. monocytogenes*. De plus, pour certains d'entre eux comme BapL, le flagelle ou l'ADN extracellulaire, des investigations supplémentaires seraient nécessaires pour préciser leur rôle potentiel. D'autres protéines sembleraient avoir un rôle indirect dans la

formation de biofilm, comme les protéines associées au QS ou les facteurs de réponse SOS. Concernant la recherche de déterminants moléculaires situés à l'interface cellule-surface, il serait judicieux de s'intéresser aux protéines sécrétées et plus particulièrement au MSCRAMM (microbial surface components recognizing adhesive matrix molecules) qui sont connus pour être impliqués dans la colonisation des surfaces biotiques et abiotiques comme dans la virulence (Desvaux, Dumas et al. 2006; Desvaux and Hebraud 2006; Desvaux and Hébraud 2008). Pour cela, il est important de connaître le sécrétome de *L. monocytogenes*, c'est-à-dire l'ensemble des protéines sécrétées ainsi que les systèmes de sécrétion présents. La suite de l'exposé traitera en particulier du système Sec (sécrétion) qui constitue la principale voie de sécrétion chez *L. monocytogenes*.

- CHAPITRE 3- LA VOIE DE SECRETION SEC

1. Les Systèmes de sécrétion chez *L. monocytogenes*

Chez les bactéries à Gram positif, différents mécanismes permettent aux protéines d'être sécrétées, c'est-à-dire de traverser la membrane plasmique de la bactérie. Sept systèmes de sécrétion ont été décrits : les systèmes (i) Sec (sécrétion) et (ii) Tat (twin-arginine translocation) qui permettent également la translocation de protéines à travers la membrane cytoplasmique des bactéries à Gram négatif, les systèmes (iii) FEA (flagella export apparatus),(iv) FPE (fibrillin-protein exporter), (vi) WSS (WXG100 secretion system), (v) les holins et les transporteurs ABC (ATP-binding cassette) (Desvaux, Khan et al. 2005; Desvaux, Hebraud et al. 2006). Ces différents systèmes de sécrétion ont été prédits chez *L. monocytogenes,* suite à une analyse génomique, de même que leurs substrats (Figure 14) (Desvaux and Hebraud 2006; Desvaux, Dumas et al. 2010)

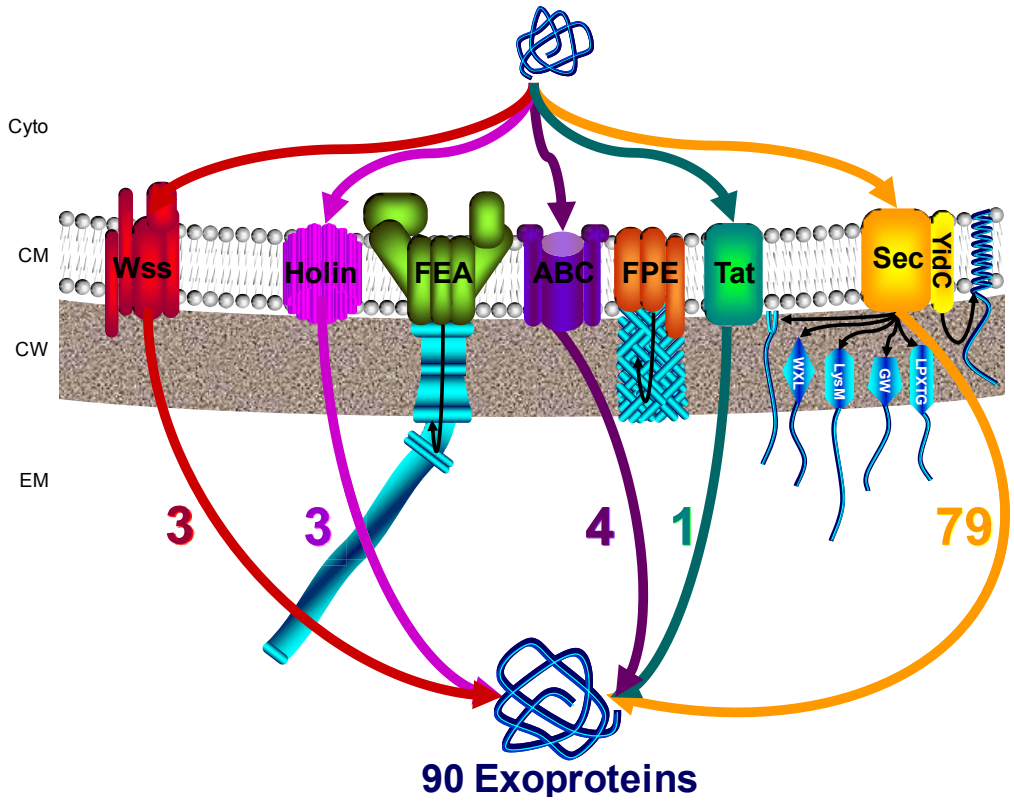

Figure 14 : Les systèmes de sécrétion identifiés chez *L. monocytogenes* (Desvaux, Dumas et al. 2010)

Les protéines passant par les systèmes Sec, Tat, FPE et ABC possèdent un peptide signal caractéristique, nécessaire à leur translocation. Pour le système Sec, le peptide signal donne également des informations sur la localisation finale de la protéine.

L'étude de l'exoprotéome, c'est-à-dire des protéines sécrétées dans le milieu extracellulaire, a révélé expérimentalement une proportion importante de protéines ne possédant pas de peptides signaux et prédites comme cytoplasmiques. Le système permettant la sécrétion de ces protéines est à l'heure actuelle inconnu et l'on parle alors de voie de sécrétion non-classique (NC) (Bendtsen, Kiemer et al. 2005). La détection de ces protéines dans le milieu extracellulaire a été de nombreuses fois attribuée à une lyse cellulaire lors de manipulations expérimentales. Cependant, certaines de ces protéines ont été retrouvées dans le milieu extracellulaire par plusieurs groupes de recherche lors de différentes approches expérimentales et chez différents genres bactériens, appuyant l'hypothèse selon laquelle elles pourraient être sécrétées par des cellules intactes. Certaines protéines qui présentent une fonction cytoplasmique bien caractérisée se sont révélées comme ayant un rôle physiologique différent dans le milieu extracellulaire. C'est notamment le cas de la glycéraldéhyde 3-phosphate déshydrogénase (GAPDH) ou de la protéine DnaK qui, dans le milieu extracellulaire, sont capables de fixer le plasminogène humain (Lenz, Mohammadi et al. 2003; Schaumburg, Diekmann et al. 2004). Ces protéines ayant une double fonction sont qualifiées de protéines multitâches (« moonlighting »).

2. Le système Sec : la translocase

Compte tenu du nombre de protéines potentiellement adressé à ce système, la voie Sec est considérée comme la principale voie de sécrétion chez *L. monocytogenes* (Desvaux and Hebraud 2006). Les différents constituants de cette voie ont été prédits chez plusieurs souches de *L. monocytogenes* et sont résumés dans le tableau 4.

Le système de translocation des protéines, appelé la translocase, est constitué du translocon SecYEG formant un canal à travers la membrane plasmique, et de l'ATPase SecA, (Du Plessis, Nouwen et al. 2011).

2.1 Le translocon

Le canal formé par les protéines SecYEG permet de transloquer les substrats à travers la membrane plasmique. SecY est une protéine de 48 kDa, très hydrophobe, formant des hélices

α qui traversent plusieurs fois la membrane plasmique. C'est le constituant le plus important du translocon, il est essentiel à la viabilité cellulaire et à la translocation. SecE est une protéine plus petite, de 14 kDa, mais toute aussi importante que SecY. Inversement, SecG n'est pas essentiel mais permettrait d'améliorer la translocation des pré-protéines, particulièrement à des basses températures (Nishiyama, Hanada et al. 1994) et lorsque les forces protomotrices sont réduites (Hanada, Nishiyama et al. 1996).

Tableau 4 : Composants prédits de la voie de sécrétion Sec chez deux souches de *L. monocytogenes* (Desvaux and Hebraud 2006)

Composants	TC	Localisation cellulaire prédite	*Listeria monocytogenes* 1/2a EGDe			*Listeria monocytogenes* 4b F2365		
			Nom du Locus	GI	Longueur	Nom du Locus	GI	Longueur
Système Sec	#3.A.5							
Composants transmembranaires								
SecY	#3.A.5	Membrane	Lmo2612	16804650	431	LMOf2365_2585	46908784	431
SecE	#3.A.5	Membrane	Lmo0245	16802291	59	LMOf2365_0257	46906478	59
SecG	#3.A.5	Membrane	Lmo2451	16804489	77	LMOf2365_2424	46908624	77
SecDF	#2.A.6.4.1	Membrane	Lmo1527	16803567	754	LMOf2365_1546	46907755	754
YajC	#9.B.18	Membrane	Lmo1529	16803569	109	LMOf2365_1548	46907757	109
YidC	#2.A.9.3.2	Membrane	Lmo1379	16803419	275	LMOf2365_1398	46907607	275
			Lmo2854	16804891	287	LMOf2365_2844	46909042	287
Composants cytoplasmiques								
FtsY	#3.A.5	Cytoplasme	Lmo1803	16803843	328	LMOf2365_1830	46908034	328
Ffh	#3.A.5	Cytoplasme	Lmo1801	16803841	450	LMOf2365_1828	46908032	450
ATPases								
SecA		Periphérique	Lmo2510	16804548	837	LMOf2365_2483	46908682	837
SecA2		Periphérique	Lmo0583	16802626	776	LMOf2365_0612	46906828	776
Signal peptidases I								
SipX		Membrane	Lmo1269	16803309	188	LMOf2365_1287	46907496	188
SipY		Membrane	Lmo1270	16803310	189	LMOf2365_1288	46907497	189
SipZ		Membrane	Lmo1271	16803311	180	LMOf2365_1289	46907498	180
Signal peptidases II								
LspA		Membrane	Lmo1844	16803884	154	LMOf2365_1872	46908076	154
LspB		Membrane	Lmo1101	16803141	166	–	–	–

TC (transport classification), GI (Gene identifier)

SecDF et YajC forment un complexe qui interagit avec le canal SecYEG de façon transitoire afin de stimuler la translocation (Duong and Wickner 1997; de Keyzer, van der Does et al. 2003). Bien que SecD et SecF ne soient pas essentielles, leur inactivation chez *E. coli* résulte en une importante réduction de la sécrétion et du taux de croissance cellulaire (Pogliano and Beckwith 1994). Chez *L. monocytogenes* tout comme chez *B. subtilis,* SecD et

SecF sont fusionnées en une seule et même protéine impliquée dans les premières étapes de translocation des pré-protéines (Bolhuis, Broekhuizen et al. 1998).

Chez *Streptococcus pyogenes*, il a été montré que la sécrétion de protéines par le système Sec était réalisée au niveau d'un seul domaine relativement réduit de la membrane cytoplasmique, appelé « exportal », où sont concentrés les translocons Sec (Rosch and Caparon 2004; Rosch and Caparon 2005). Cette organisation cellulaire n'a pu être considérée comme un modèle chez les bactéries à Gram positif puisque la même année, une deuxième organisation a été mise en évidence. En effet, chez *B. subtilis*, les translocons Sec sont organisés en amas qui suivent une structure spiralée le long de l'axe longitudinal de la cellule (Campo, Tjalsma et al. 2004).

2.2 L'ATPase SecA

Tout comme le complexe SecYEG, SecA est une protéine essentielle à la sécrétion Sec-dépendante. L'énergie fournie par les cycles répétés de fixation et d'hydrolyse de l'ATP permet la libération des protéines initialement fixées à SecA et leur translocation à travers le canal SecYEG. C'est une protéine cytoplasmique qui se situe principalement au niveau de la membrane plasmique. En effet, elle interagit particulièrement avec la principale boucle cytoplasmique de SecY (Matsumoto, Yoshihisa et al. 1997; Karamanou, Bariami et al. 2008) ainsi qu'avec les têtes hydrophiles des phospholipides membranaires, mais avec une moindre affinité (Lill, Dowhan et al. 1990). La structure tridimensionnelle de SecA a été caractérisée chez différents organismes comme *E. coli*, *B. subtilis* ou *Mycobacterium tuberculosis*. Dans la plupart des cas, SecA se présente sous forme de dimères antiparallèles à l'exception de *Thermus thermophilus,* chez qui les dimères sont parallèles (Du Plessis, Nouwen et al. 2011).

2.3 SecA2

Il y a quelques années, une deuxième ATPase homologue à SecA a été découverte chez de nombreuses bactéries à Gram positif comme *Streptococcus gordonii*, *Mycobacterium tuberculosis*, *M. smegmatis*, *M. tuberculosis, Clostridium difficile*, *Staphylococcus aureus* et *Listeria monocytogenes* (Rigel and Braunstein 2008; Siboo, Chaffin et al. 2008). Contrairement à la protéine SecA, SecA2 n'est pas essentielle à la viabilité cellulaire. De plus, celle-ci est plus petite que SecA en raison d'une troncation dans sa partie C-terminale responsable de l'interaction avec la protéine chaperone SecB et les phospholipides présents chez les bactéries à Gram négatif (Breukink, Nouwen et al. 1995).

Néanmoins, toutes les protéines SecA2 possèdent, comme SecA, le domaine moteur de l'ATPase, qui inclut (i) les deux sites de fixation de l'ATP, NBF1 et NBF2, (ii) le domaine IRA1 (régulateur intracellulaire de l'hydrolyse de l'ATP) et (iii) le domaine de fixation de la pré-protéine HWD (Karamanou, Vrontou et al. 1999). L'inactivation de SecA2 entraîne un défaut de septation aboutissant à la formation de longs filaments cellulaires et au développement de colonies dites « rugueuses », contrairement aux colonies classiques formées par *L. monocytogenes* qui sont qualifiées de « lisses » (Figure 15).

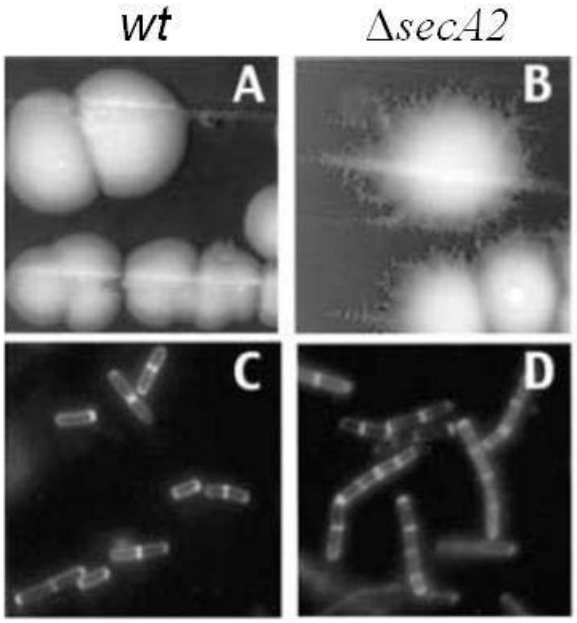

Figure 15 : Colonies formées par la souche sauvage (A) et le mutant Δ*secA2* (B) chez *L. monocytogenes* et photographies de cellules respectives (C et D) (Lenz and Portnoy 2002)

Plusieurs études ont montré que le défaut de septation était en grosse partie dû à une réduction de la sécrétion de deux hydrolases pariétales, CwhA et MurA (muramidase A) également nommé NamA (N-acetylmuramidase A) (Lenz and Portnoy 2002; Lenz, Mohammadi et al. 2003). En effet, l'inactivation simultanée des gènes codant ces hydrolases entraîne l'apparition du phénotype rugueux (Machata, Hain et al. 2005). Une analyse comparative, effectuée par électrophorèse monodimensionnelle, a permis d'identifier sept protéines sécrétées de façon SecA2-dépendante dans le milieu extracellulaire, parmi lesquelles 4 possèdent un peptide signal, dont CwhA et MurA, et trois autres n'en possèdent pas (Lenz, Mohammadi et al. 2003). Certaines de ces mêmes protéines ont également été identifiées en moindre quantité chez le mutant Δ*secA2* lors de l'analyse comparative des protéines de surface. Des études indépendantes menées ultérieurement ont permis d'identifier deux autres protéines ne possédant pas de peptides signaux mais étant sécrétées de façon

SecA2-dépendante, à savoir la superoxyde dismutase (MnSOD) (Archambaud, Nahori et al. 2006) ainsi que la protéine Lap (listerial adhesion protein) dont la fonction cytoplasmique initialement connue est d'être une alcool acétaldéhyde déshydrogénase (Burkholder, Kim et al. 2009; Jagadeesan, Koo et al. 2010). L'ensemble des protéines sécrétées de façon SecA2 dépendante est résumé dans le tableau 5.

Tableau 5 : Ensemble des protéines sécrétées de façon SecA2-dépendente chez *L. monocytogenes* (Lenz, Mohammadi et al. 2003; Archambaud, Nahori et al. 2006; Burkholder, Kim et al. 2009)

Description	Localisation*
Protéines avec peptide signal	
PBP 2B	SN, CS
N-acetylmuramidase (NamA)	SN, CS
Transporteur de phéromone(OppA)	SN, CS
CwhA	SN
Maltose maltodextrine ABC transporteur	CS
Lipoprotéine antigénique (TcsA)	CS
Lipoprotéine conservée	CS
Protéines sans peptide signal	
Sous unité β' de la ARN polymerase	SN, CS
Sous unité β de la ARN polymerase	SN, CS
Sous unité E2 de la pyruvate dehydrogenase	CS
DnaK	CS
GroEL	SN, CS
EF-Tu	CS
Enolase	CS
Phosphomannose isomerase	CS
Protéine ribosomal L19	CS
Protéine ribosomal S9	CS
MnSOD	SN
LAP	SN

*Fraction cellulaire où ont été identifiées les protéines : SN, surnageant) ; CS, surface cellulaire.

SecA2 jouerait donc un rôle important dans la sécrétion des protéines ne possédant pas de peptides signaux, initialement décrites comme des protéines cytoplasmiques sécrétées par des voies non-classiques. Récemment, une nouvelle protéine nommée DivIVA, a été décrite chez *L. monocytogenes* comme influençant l'activité de la voie de sécrétion SecA2 (Halbedel, Hahn et al. 2012). En effet, la délétion du gène *divIVA* entraîne la formation de longs filaments cellulaires comme Δ*secA2*. La protéine DivIVA est localisée au niveau des pôles cellulaires et a pour rôle, entre autres, de recruter les protéines p60 et MurA afin qu'elles puissent être sécrétées par la voie SecA2-dépendante. Les deux mutants Δ*secA2* et Δ*divIVA*

présentant un même phénotype et étant affectés de façon similaire pour leur capacité d'infection, une possible collaboration entre ces deux protéines a donc été suggérés.

Le mode d'action de SecA2 dans la sécrétion de protéines n'a pas encore été mis en évidence. Chez *M. smegmatis,* il a été supposé que SecA2 assisterait SecA et que les protéines SecA2-dépendantes emprunteraient le translocon SecYEG pour traverser la membrane cytoplasmique (Braunstein, Brown et al. 2001). En effet, contrairement à d'autres genres bactériens exprimant la protéine SecA2, *M. smegmatis*, tout comme *L. monocytogenes*, ne possèdent pas le gène codant une protéine homologue à SecY, nommée SecY2 (Rigel and Braunstein 2008). Chez *Streptococcus gordinii*, un domaine AST (accessory sec transport) a été identifié à environ 20 acides aminés de la partie N-terminale des protéines matures sécrétées de façon SecA2 dépendantes (Bensing and Sullam 2010). Ce domaine n'est pas retrouvé chez aucune des protéines sécrétées par SecA2 chez *L. monocytogenes*.

2.4 Les protéines associées au système de sécrétion Sec

Afin de diriger les protéines vers la translocase, deux voies existent : (i) la translocation co-traductionnelle faisant intervenir une particule de reconnaissance de signal (SRP), nommée Ffh/SRP54, ainsi que le récepteur du SRP faisant partie de la famille des récepteurs α FtsY/SRP, et (ii) la translocation post-traductionnelle utilisant la protéine chaperone SecB.

Concernant la voie SRP, la particule SRP va reconnaître et fixer le peptide signal de la chaîne polypeptidique naissante ou un futur segment transmembranaire afin de bloquer la traduction entreprise par les ribosomes. Le complexe formé est alors reconnu par le récepteur FtsY. Après des cycles de fixation et d'hydrolyse de 4 ATP, la particule SRP se sépare du complexe et la chaîne polypeptidique naissante est transférée au translocon (Valent, Scotti et al. 1998; de Leeuw, Kaat et al. 2000). La traduction peut ainsi suivre son cours pendant la translocation à travers la membrane cytoplasmique, permettant la mise en place des protéines membranaires intrinsèques (IMPs). En effet, chez les bactéries, le système SRP est principalement impliqué dans l'adressage des IMPs (de Gier and Luirink 2001). SRP et FtsY sont des protéines essentielles pour la croissance bactérienne, ce qui souligne l'importance des IMPs en surface (Luirink, High et al. 1992). Cependant, une étude récente menée chez *E. coli,* suggère que la voie SRP permettrait également d'adresser au translocon des chaînes polypeptidiques naissantes de protéines cytoplasmiques (Bornemann, Jockel et al. 2008), ce qui pourrait expliquer la présence de ces protéines dans le milieu extracellulaire.

SecB à une double fonction de chaperone moléculaire en empêchant le repliement de la protéine synthétisée dans le cytoplasme puis en l'adressant à la translocase (Bechtluft, Nouwen et al. 2010). Contrairement à la voie SRP qui est ubiquitaire dans le monde vivant (Cao and Saier 2003), la voie SecB n'est pas retrouvée chez les bactéries Gram positif (Decock and Tommassen 1991; Tjalsma, Bolhuis et al. 2000; van Wely, Swaving et al. 2001). Chez *B. subtilis*, la protéine chaperone CsaA jouerait un rôle similaire à SecB dans l'export de protéines (Linde, Volkmer-Engert et al. 2003). Cependant, aucune protéine homologue à CsaA n'a été identifiée chez les différentes espèces de *Listeria*.

2.5 YidC

YidC est une protéine membranaire associée au translocon SecYEG, qui joue un rôle dans l'insertion des protéines au niveau de la membrane (Du Plessis, Nouwen et al. 2011). YidC interagit provisoirement avec le translocon pendant l'insertion des IMPs et cette interaction implique également SecD et SecF par la formation d'un complexe hétérotétramérique YidC-SecDF-YajD (Nouwen and Driessen 2002). Cependant, il a été montré chez *E. coli* que YidC était également capable à elle seule de fonctionner comme un insertase (Samuelson, Chen et al. 2000; Price and Driessen 2008; Price and Driessen 2010), suggérant sa possible implication dans une voie alternative de translocation à travers la membrane qui serait Sec-indépendante. Chez *B. subtilis*, il existe deux paralogues de YidC, nommés YqjG et SpoIIIJ, ou plus récemment OxaA1 et OxaA2 respectivement (Tjalsma, Bolhuis et al. 2000; van Wely, Swaving et al. 2001; Saller, Fusetti et al. 2009). Ils ont tout d'abord été montrés comme nécessaires à la sporulation (Errington, Appleby et al. 1992). La double inactivation des gènes *spoIIIJ* et *yqjG* est létale pour la bactérie alors qu'aucune différence de croissance n'est observée chez les simples mutants par rapport à la souche sauvage. Ces résultats suggèrent que SpoIIIJ and YqjG ont des fonctions communes mais également des fonctions qui leur sont propres. Par la suite, il a été montré que les deux protéines jouaient un rôle dans la biogénèse des protéines membranaires mais aussi dans la sécrétion (Tjalsma, Bron et al. 2003; Desvaux, Dumas et al. 2006). Tout comme *B. subtilis*, *L. monocytogenes* possède ces deux paralogues de YidC (Tableau 4) (Desvaux and Hebraud 2006).

3. Les Protéines transloquées par le système Sec

Une fois les pré-protéines transloquées à travers de la membrane cytoplasmique par le système Sec, celles-ci peuvent être maturées par les différents embranchement de la voie Sec selon leur localisation finale (Figure 16) permettant ainsi: (i) l'intégration des protéines membranaire, (ii) la maturation des lipoprotéines, (iii) l'ancrage covalent des protéines LPXTG à la paroi, (iv) l'association non-covalente à la paroi de protéines possédant des CWBDs (cell wall binding domains), et (v) le relarguage dans le milieu extracellulaire d'exoprotéines.

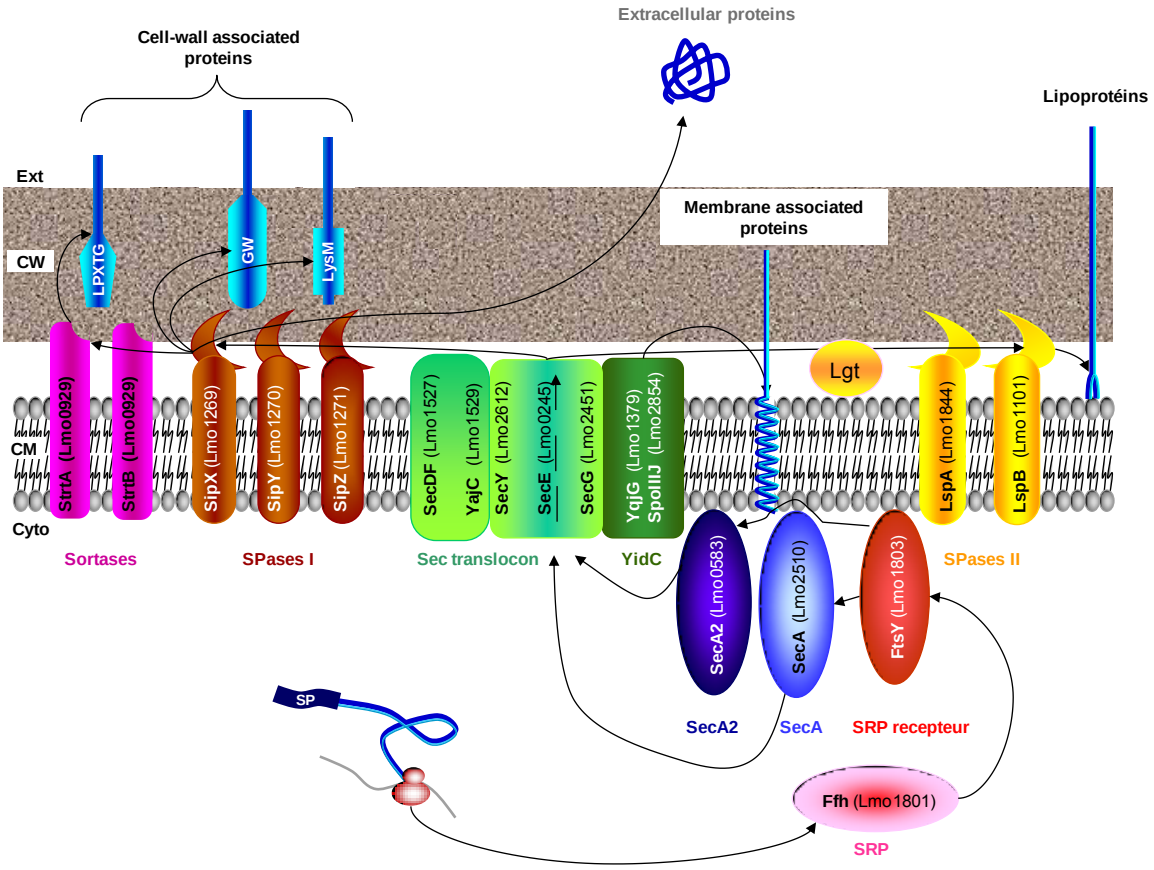

Figure 16 : Schéma général de la voie de sécrétion Sec (Desvaux and Hebraud 2006)

Les pré-protéines peuvent être maturées par les peptidases signal de type I (SPases I). Dans ce cas, les protéines sont soit : (i) relarguées dans le milieu extracellulaire, (ii) ancrées à la membrane plasmique par un ou plusieurs domaines transmembranaires pour les IMPs, (iii) liées à la paroi cellulaire de façon non-covalente ou de façon covalente pour les protéines LPXTG qui sont reconnues par les sortases. Les pré-protéines maturées par les peptidases

signal de type II (SPases II) sont ensuite liées de façon covalente aux parties lipidiques de la membrane plasmique et correspondent donc aux lipoprotéines.

3.1 Les peptides signaux

Les peptides signaux (SP) possèdent au moins trois fonctions. La première est de permettre la reconnaissance de la pré-protéine par le récepteur du système de sécrétion. Ce dernier la transmet par la suite à la translocase qui permet son transfert à travers la membrane cytoplasmique (Hartl, Lecker et al. 1990; Dalbey, Chen et al. 2000). La deuxième est de servir de déterminant topologique de la pré-protéine. En effet, les peptides signaux initient la translocation de la partie C-terminal hydrophile de la pré-protéine alors que la partie N-terminale du SP reste située du côté interne de la membrane plasmique (Dalbey 1990; Andersson, Bakker et al. 1992). Enfin, la troisième fonction du SP est d'empêcher le repliement des chaînes polypeptidiques naissantes, ce qui évite l'activation d'enzymes potentielles au sein de la cellule et maintient la compétence des pré-protéines à être transloquées sous forme dépliée (Liu, Topping et al. 1989).

Les SP sont composés de trois domaines distincts : (i) le domaine N dans la partie N-terminale du peptide signal qui est chargé positivement et possède au moins un résidu arginine ou lysine, (ii) le domaine H, formé d'une suite de 10 à 15 résidus hydrophobes qui semblent adopter une conformation en hélice alpha dans la membrane plasmique (Nielsen, Engelbrecht et al. 1997) et (iii) le domaine C qui possède le site de clivage pour les SPases (Fekkes and Driessen 1999). Chez *B. subtilis*, quatre classes de SP ont été distinguées sur la base du site de reconnaissance de la SPase (Figure 17).

La première classe de SP correspond au peptide signal dit « classique », présent sur les pré-protéines maturées par les SPases I, qui reconnaissent la séquence « Ala-X-Ala ». Un sous-groupe de cette classe contient un motif RR (« twin-arginine »), qui est caractéristique des protéines sécrétées par le système de sécrétion Tat. La deuxième catégorie de SP est présente sur les pré-lipoprotéines qui sont maturées par les SPase II. La différence majeure entre ce SP et le précédant est la présence d'un domaine conservé nommé la boîte lipo (« lipobox »). La troisième classe de SP correspond à celle des pré-pilines qui sont maturées par une SPase spécifique, ComC (Chung and Dubnau 1995). Le site de reconnaissance de cette dernière SPase est, contrairement aux autres SP, localisé entre le domaine N et le domaine H, laissant ce dernier domaine attaché à la pré-piline mature après le clivage (Pugsley 1993; Chung and Dubnau 1995; Chung and Dubnau 1998). Enfin, la quatrième

classe de SP est retrouvée chez les bactéricines et phéromones exportées par les ABC transporteurs (Tjalsma, Bolhuis et al. 2000). Ces différents types de peptides signaux sont retrouvés chez *L. monocytogenes* (Desvaux and Hebraud 2006; Desvaux, Dumas et al. 2010).

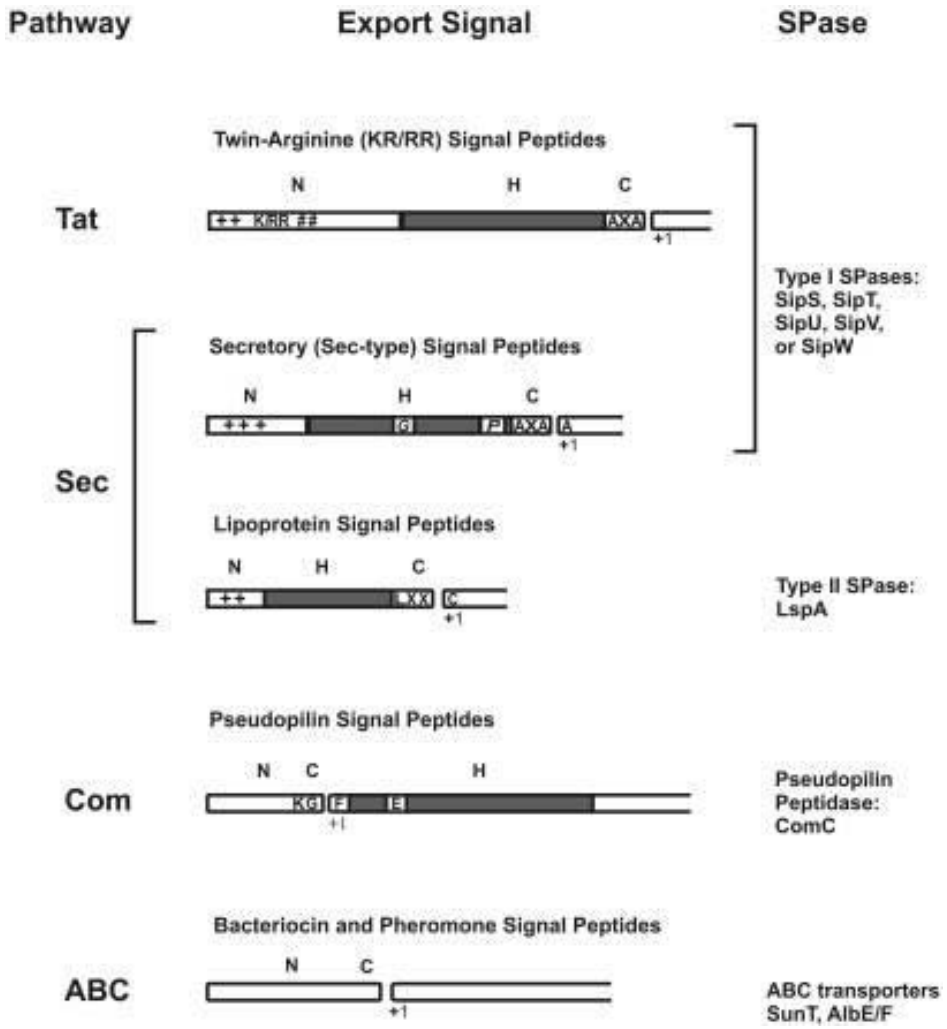

Figure 17 : Classification des peptides signaux chez *B. subtilis* (Tjalsma, Antelmann et al. 2004)

3.2 Les peptidases signal de type I

Chez les bactéries à Gram positif, la présence de plusieurs SPases I pour une même espèce bactérienne est communément observée. Le plus grand nombre de SPase I a été montré chez *B. cereus* qui contient sept gènes codant des paralogues de ces protéines. Chez *L. monocytogenes*, trois paralogues ont été identifiés au sein d'un locus et caractérisés par rapport à leur implication dans la virulence. Ces SPases sont nommées SipX, SipY et SipZ ; elles sont très proches de SipS et SipT, les principales SPases de *B. subtilis* avec 38 à 44% d'identité (Bonnemain, Raynaud et al. 2004; Raynaud and Charbit 2005). L'inactivation de

SipX n'affecte pas la multiplication intracellulaire de *L. monocytogenes* mais réduit significativement sa virulence d'un facteur 100 environ. SipZ permet la sécrétion de certains facteurs de virulence comme la phosphatidylcholine phospholipases C (PlcB) ou la listériolysine O (LLO). De plus, son absence réduit également la capacité de la bactérie à se multiplier dans les macrophages, les cellules épithéliales et les hépatocytes. A l'inverse des deux autres SPases, l'inactivation de *sipY* n'a aucun effet détectable. Bien que SipX et SipZ interviennent dans des aspects différents de la pathogénicité bactérienne, SipZ semble être la principale SPase chez *L. monocytogenes* (Bonnemain, Raynaud et al. 2004). Comme il a été mentionné précédemment, les SPases I sont responsables de la maturation de différentes catégories de protéines : (i) les exoprotéines, (ii) les protéines membranaires et (iii) les protéines pariétales.

3.2.1 Les exoprotéines

Chez *L. monocytogenes*, une analyse du sécrétome, c'est-à-dire de l'ensemble des protéines sécrétées ainsi que des éléments constituant les systèmes de sécrétion, a permis de prédire 90 exoprotéines dont 79 sont sécrétées par la voie Sec, 1 par la voie Tat, 3 par les holins, 3 par le système Wss et enfin 4 bactériocines par les ABC transporteurs (Figure 14) (Desvaux, Dumas et al. 2010). Parmi le 79 exoprotéines sécrétées par la voie Sec, des fonctions potentielles ont pu être attribuées pour 49 d'entre elles, suite à des recherches de motifs conservés ainsi que des BLAST. Parmi ces exoprotéines sont retrouvées les facteurs de virulence comme la LLO, PlcA, PlcB et InlC. Des enzymes de dégradation intervenant dans le catabolisme des hydrates de carbone sont également prédites comme sécrétées dans le milieu extracellulaire, telles que les chitinase A et B. Ces protéines sont impliquées dans le catabolisme des chitines, les deuxièmes polymères d'hydrate de carbone les plus importants après la cellulose, qui représentent une source majeur de carbone et d'azote pour les bactéries (Gooday 1990 ; Guedon, Payot et al. 2000). L'activité chitinolytique est certainement mise en place chez *L. monocytogenes* lorsque cette dernière vit en condition saprophytique dans les sols. Diverses exoprotéases et exopeptidases ont été identifiées chez *L. monocytogenes* ainsi que diverses protéines ne possédant pas de domaine de liaison à la paroi cellulaire mais étant impliquées dans la biogénèse, dégradation ou maturation de la paroi cellulaire comme la protéine p45.

Dans les faits, plusieurs études de l'exoprotéome ont montrées que des protéines appartenant à d'autres catégories étaient retrouvées systématiquement dans le milieu

extracellulaire (Trost, Wehmhoner et al. 2005; Desvaux, Dumas et al. 2010) comme par exemple les protéines pariétales CwhA et MurA, les protéines cytoplasmiques DnaK, GroEL, GroES, la protéine membranaire ActA ainsi qu'un grand nombre de lipoprotéines.

3.2.2 Les protéines intégrales membranaires

Il existe différentes catégories de protéines intégrales membranaires (IMP) selon la présence d'un ou plusieurs domaines transmembranaires (TMD) et de son orientation (Figure 18) (Desvaux and Hébraud 2008).

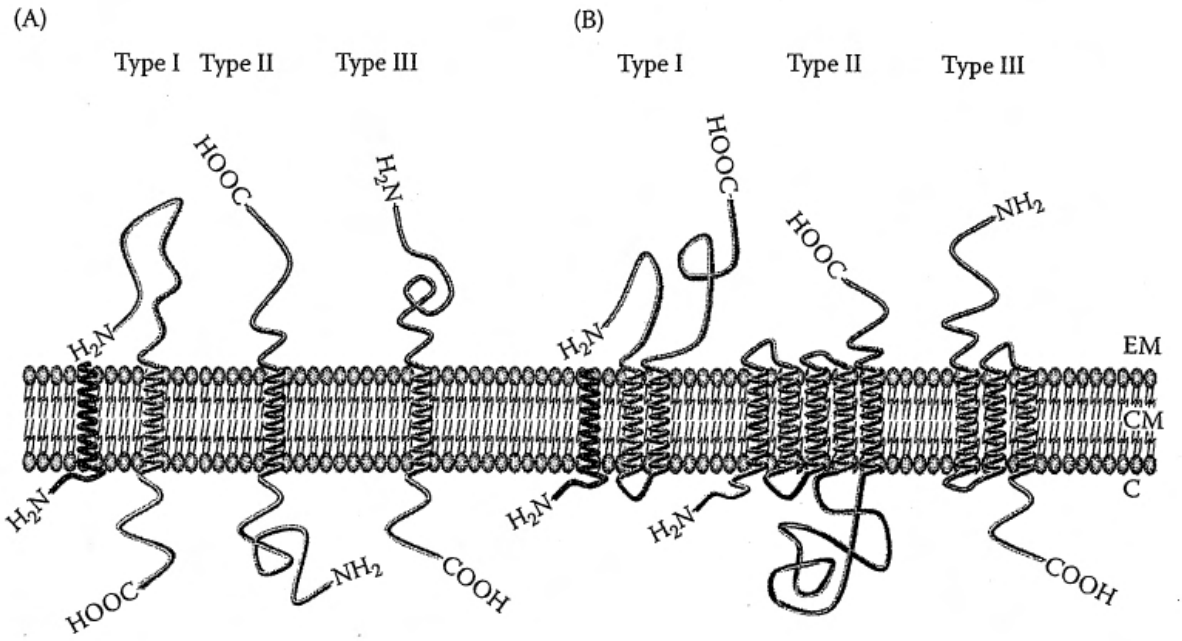

Figure 18 : Classification et topologie des IMPs de la membrane cytoplasmique (Desvaux and Hébraud 2008).

Les IMPs sont tout d'abord divisées en deux groupes : (A) les IMPs présentant un seul TMD (ss-IMP pour « single spanning IMP») et (B) les IMPs présentant au moins deux TMDs (ms-IMP pour « multi- spanning IMP ») (Goder and Spiess 2001). Puis, au sein de ces deux groupes, trois types d'IMPs peuvent être différenciés en fonction de leur premier TMD (situé vers la région N-terminal). En effet, les IMPs de type I (IMP I) correspondent aux IMPs possèdant un peptide signal clivable et dont la partie N-terminale du premier TMD se situe à l'extérieur de la cellule et sa partie C-terminale à l'intérieur (N_{out}-C_{in}). A l'inverse, pour les IMPs de type II (IMP II), le premier TMD possède la partie N-terminale à l'intérieur et C-terminale à l'extérieur de la cellule (N_{in}-C_{ou}). Dans ce cas, l'unique TMD des ss-IMPs et le

premier TMD des ms-IMPs correspondent à un signal d'ancrage, c'est-à-dire à un peptide signal non clivé. Le premier TMD des IMPs de type III (IMP III) à quant à lui une topologie N_{out}-C_{in} comme les IMPs I, mais les IMP III sont dépourvues de peptide signal (Desvaux and Hébraud 2008). Chez la souche *L. monocytogenes* EGD, 1204 IMPs ont été prédites (Desvaux and Hebraud 2009), parmi lesquelles figure le facteur de virulence ActA qui est une ss-IMP I.

3.2.3 Les protéines pariétales

3.2.3.1 Les protéines pariétales covalentes et sortases

Chez *L. monocytogenes* comme chez les autres bactéries à Gram positif, le seul mécanisme permettant d'attacher de façon covalente les protéines à la paroi cellulaire fait intervenir des protéines appelées sortases (Navarre and Schneewind 1999). Les substrats des sortases possèdent un motif particulier LPXTG (où X= n'importe quel acide aminé), qui est suivi par un domaine transmembranaire hydrophobe et un domaine chargé positivement en position C-terminale. Pendant la sécrétion à travers le translocon SecYEG, les sortases reconnaissent le motif LPXTG et le clivent entre la thréonine (T) et la glycine (G). Un intermédiaire acyl-enzyme est alors formé entre une cystéine du site actif de la sortase et le groupe carboxylique de la thréonine de la nouvelle partie C-terminale de la protéine. Le groupe anime du pentaglycine (Gly_5) du lipide II (base du peptidoglycane) clive cet intermédiaire au niveau de la liaison thioester et forme une liaison amide entre la thréonine et le lipide II. Des réactions de transglycosylation et de transpeptidation permettent l'incorporation du groupement obtenu dans la paroi cellulaire (Schneewind and Missiakas 2012) (Figure 19).

Une certaine variabilité dans la séquence consensus du domaine LPXTG a été montrée (Pallen, Lam et al. 2001) puis associée à des sous-familles de sortases (Comfort and Clubb 2004). Celles-ci ont été classées en 4 catégories nommées A, B, C et D (Dramsi, Trieu-Cuot et al. 2005). Chez les différentes espèces de *Listeria*, deux sortases ont été identifiées appartenant à la classe A (SrtA) et B (SrtB) (Dramsi, Trieu-Cuot et al. 2005). Chez *L. monocytogenes*, SrtA joue un rôle dans la virulence bactérienne. En effet, elle permet l'ancrage des protéines à LPXTG à la paroi cellulaire, dont le facteur de virulence InlA (Bierne and Cossart 2002; Pucciarelli, Calvo et al. 2005). Des analyses génomiques ont permis d'identifier 43 protéines présentant un motif LPXTG, parmi celles-ci 11 sont absentes chez *L. innocua* dont InlA (Trost, Wehmhoner et al. 2005; Desvaux and Hebraud 2006). Alors que la protéine SrtA est impliquée dans l'ancrage de la majorité des protéines à la paroi

cellulaire, la protéine SrtB semble jouer un rôle mineur (Bierne and Cossart 2002; Pucciarelli, Calvo et al. 2005).

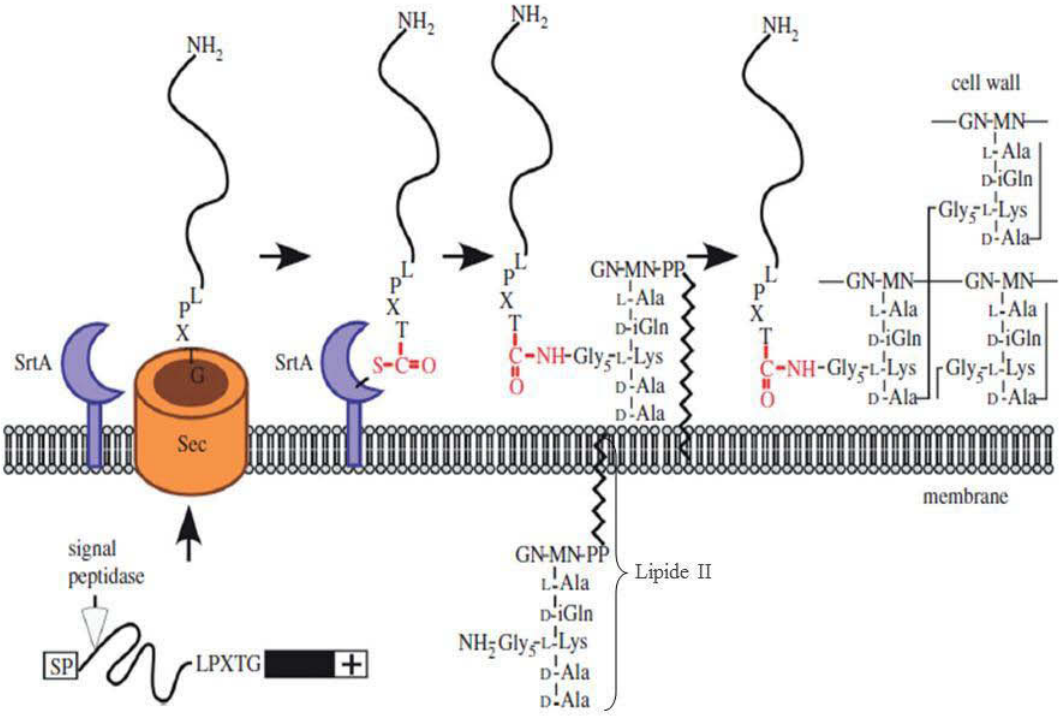

Figure 19 : La voie de maturation des protéines à motif LPXTG chez les bactéries à Gram positif (Schneewind and Missiakas 2012). MN : Acide N-acétylmuramique ; GN : N-acétylglucosamine.

SrtB possèderait uniquement deux substrats, les facteurs de virulence SvpA (protéine de surface associée à la virulence A) et SvpB (Bierne and Cossart 2002). Ces deux protéines ne possèdent pas de séquence consensus LPXTG ni NPQTN, mais une séquence NXZTN, ce qui suggère une plus faible spécificité de reconnaissance du motif pour SrtB que pour SrtA (Borezée, Pellegrini et al. 2001; Bierne, Garandeau et al. 2004).

Chez de nombreux genres bactériens, des protéines à LPXTG sont impliquées dans l'adhésion et la formation de biofilm (Shimoji, Ogawa et al. 2003; O'Neill, Pozzi et al. 2008; Hendrickx, Willems et al. 2009; Velez, Petrova et al. 2010). Chez *L. monocytogenes*, aucune protéine à domaine LPXTG n'a été caractérisée pour une éventuelle implication dans la formation de biofilm.

3.2.3.2 Les protéines non covalentes

Certaines protéines maturées par les SPases I peuvent être liées à la paroi cellulaire par des liaisons non-covalentes. Ces liaisons s'effectuent par la présence de domaines particuliers permettant l'attachement à la paroi (CWBD). Parmi les domaines jusqu'à présent caractérisés et référencés dans les banque de données de motif conservé comme InterPro (IPR) (Mulder, Apweiler et al. 2007) ou Superfamily (SSF) (Wilson, Madera et al. 2007), on compte les domaines LysM (Lysine motif), GW (Glycine Tryptophane), WXL (Tryptophane X Leucine), CWBD1 (cell wall binding domain of Type 1), CWBD2, SLHD (S-layer homology domain), PGBD1 (peptidoglycan-binding domain of Type 1), PGBD2, PGBD3 et PGBD4 (Desvaux, Dumas et al. 2006; Desvaux and Hébraud 2008).

3.2.3.2.1 LysM (IPR002482)

Le domaine LysM (lysin motif) tient son nom de sa présence chez de nombreuses lysines bactérienne. Ce domaine forme des liaisons directement avec le peptidoglycane. Cette liaison est entravée par des polymères secondaires de la paroi cellulaire (SCWPs) tels que les acides lipotéichoïques (Steen, Buist et al. 2003). Le domaine LysM, composé de 40 résidus d'acides aminés et de 1 et 6 copies en tandem, est généralement localisé dans la partie N ou C-terminale de la protéine. Ce domaine est retrouvé chez plus de 1500 protéines, provenant aussi bien de cellules procaryotes qu'eucaryotes. Les protéines possédant ce domaine peuvent jouer un rôle dans la dégradation de la paroi cellulaire mais peuvent également avoir des fonctions de peptidase, chitinase, estérase, réductase, nucléase, ou avoir des rôles d'antigènes ou de fixation de protéines comme l'albumine, l'élastine ou l'immunoglobuline. Parmi les protéines possédant ce motif sont retrouvées Iap et MurA chez *L. monocytogenes*.

3.2.3.2.2 GW (SSF82057)

Le module GW correspond à un motif de 80 acides aminés contenant des dipeptides Gly-Trp. Ce motif permettant d'établir des liaisons non-covalentes entre la protéine et la paroi cellulaire a été découvert suite à l'étude de l'InlB chez *L. monocytogenes* (Jonquieres, Bierne et al. 1999). Ces modules sont trouvés en général en multicopies, comme c'est le cas pour l'InlB ou l'amidase (Ami) avec 3 et 8 modules GW, respectivement. Plus le nombre de modules est important et plus la fixation à la paroi cellulaire est forte. Les modules GW interagissent avec les acides lipotéichoïques permettant leurs maintiens au sein de la paroi. Cependant, la présence d'un seul module GW ne permet pas à une protéine d'être retenue au niveau de la paroi. Celle-ci sera alors relarguée dans le milieu extracellulaire (Braun, Dramsi

et al. 1997; Jonquieres, Bierne et al. 1999; DeDent, Bae et al. 2008). En plus du genre *Listeria*, ces modules ont été identifiés chez une autolysine de surface de plusieurs espèces de *Staphylococcus* (Cabanes, Dehoux et al. 2002). Ces autolysines sont souvent caractérisées pour intervenir dans l'adhésion aux surfaces biotiques, comme Aas (autolysin/adhesin of *Staphylococcus saprophyticus* (Hell, Meyer et al. 1998), ou abiotiques et dans la formation de biofilm (Hirschhausen et al. 2012 PLoS One 7:e40353).

3.2.3.2.3 WXL

Suite à une analyse génomique réalisée chez *Enterococcus faecalis*, un nouveau motif de liaison à la paroi cellulaire a été découvert et appelé le domaine WXL. (Brinster, Furlan et al. 2007). Ce domaine conservé est caractérisé par un premier motif WXL suivi par un deuxième motif YXXX[LIV]TWXLXXXP. Ces deux motifs sont éloignés l'un de l'autre par un certain nombre de résidus pouvant aller de 66 à 247 acides aminés. Le domaine WXL a été identifié dans les séquences codantes des génomes de nombreuses bactéries à Gram positif, y compris *L. monocytogenes* (Brinster, Furlan et al. 2007). Il a été montré chez *E. faecalis*, que la présence du motif WXL permettait de maintenir les protéines au niveau de la surface cellulaire, ce qui n'est pas le cas lorsque le domaine est délété. De plus, le domaine WXL permet de lier directement la protéine au peptidoglycane sans l'intervention d'autres protéines ou de carbohydrates. Chez certaines bactéries à Gram positif, les protéines possédant ce domaine présentent des fonctions particulières. En effet, certaines protéines à domaine WXL sont notamment impliquées dans la dégradation et l'utilisation d'oligo- ou poly-saccharides d'origines végétales (Siezen, Boekhorst et al. 2006), alors que d'autres permettraient des interactions entre différentes espèces bactériennes (Brinster, Furlan et al. 2007). Chez *L. monocytogenes*, la fonction des 4 protéines possédant un domaine WXL n'a pas été déterminée et leur présence à la surface cellulaire n'est pas encore démontrée.

3.2.3.2.4 CWBD1 (IPR018337)

Le CWBD1, précédemment appelé CBD (cholin-binding domain), est un domaine de liaison spécifique aux cholines, des acides téichoïques et lipotéichoïques (Fernandez-Tornero, Lopez et al. 2001). Ce domaine est constitué de plusieurs séquences répétées de 20 acides aminés et est caractérisés par la présence de résidus aromatiques conservés. Le nombre de copies peut aller de 1 à plus de 50 chez *Clostridium acetobutylicum* (Desvaux, Khan et al. 2005), mais, dans la majorité des cas, ce nombre se situe autour de 5 ou 10 copies. CWBD1 est absent chez *L. monocytogenes* et se trouve essentiellement chez les bactéries à Gram positif de l'ordre des *Clostridiales* et *Lactobacillales* (Desvaux, Dumas et al. 2006).

3.2.3.2.5 CWBD2 (IPR007253)

Le domaine CWBD2 est constitué d'environ 100 acides aminés retrouvés en multiples copies en tandem, entre 1 et 3, et généralement localisés dans les régions N et C-terminales. Les éléments de la paroi cellulaire interagissant avec CWBD2 sont inconnus. Ce domaine, présent chez les membres de la famille des *Bacillaceae* et des *Clostridiaceae*, n'est pas retrouvé chez *L. monocytogenes*.

3.2.3.2.6 SLHD (IPR001119)

Le domaines SLHD a été découvert suite à l'étude de glycoprotéines qui forment la couche S observée chez certains organismes procaryotes (Sára and Sleytr 2000). Les protéines de la couche S se fixent très fortement à la paroi cellulaire par des interactions non-covalentes. Chez les bactéries Gram positif, le SLHD interagit avec les SCWPs ou avec le peptidoglycane (S'ara 2001). Cependant, la spécificité et l'affinité de ces interactions ne sont pas encore expliquées. Cette couche S est absente chez *L. monocytogenes.*

3.2.3.2.7 PGBD1 (IPR002477)

PGBD1 correspond à un domaine de 70 acides aminés présent en position N ou C-terminale. Ce domaine est généralement présent en une seule copie mais parfois peut atteindre 9 copies dans certaines protéines. Chez les bactéries à Gram positif, comme les classes des *Clostridia*, des *Bacilli* et des *Actinobacteria*, les protéines possédant ce domaine sont impliquées dans la dégradation de la paroi cellulaire. Les preuves de l'implication de ce domaine dans la fixation de protéines au niveau de la paroi sont toujours en attente.

3.2.3.2.8 PGBD2 (IPR014927)

PGBD2 est un domaine d'une soixantaine d'acides aminés impliqué dans l'attachement au peptidoglycane mais absent des protéines de *L. monocytogenes*. Il est essentiellement retrouvé chez des bactéries des ordres *Rubrobacterales*, *Actinomycetales*, *Bacillales*, *Thermoanaerobacteriales* et *Clostridiales*.

3.2.3.2.9 PGBD3 (IPR018537)

Ce domaine d'environ 70 acides aminés permettant l'attachement au peptidoglycane n'est pas retrouvé chez *L. monocytogenes* et n'a été identifié que chez quelques bactéries des phyla *Actinobacteria* ou *Firmicutes*, essentiellement des genres *Rothia* et *Acetohalobium*.

3.2.3.2.10 PGBD4 (IPR018537)

PGBD4 correspond à un domaine d'environ 110 acides amines dont la structure tridimensionnelle a été résolue et forme un repliement α/β constitué de 4 hélices α et 9 feuillet β (Biarrotte-Sorin, Hugonnet et al. 2006). Ce domaine généralement trouvé en duplicat permet de lier deux chaînes de peptidoglycane. Absent des protéines de *L. monocytogenes*, il est esentiellement présent chez des bacéries du phylum *Actinobacteria* ou *Firmicutes*, classes *Clostridia*, *Lactobacillales* et *Bacillales*.

3.3 Les peptidases signal de type II

3.3.1 La voie de maturation des lipoprotéines

Les pré-pro-lipoprotéines possèdent un peptide signal spécifique contenant une lipobox qui est reconnue par les SPases II. Après la translocation de ces pré-pro-lipoprotéines à travers le translocon SecYEG, la maturation en lipoprotéine fonctionnelle s'effectue en trois étapes (Figure 20).

Tout d'abord, un groupement diacylglycéryl est transféré au groupement sulphydryl de la cystéine en position N-terminale. Cette étape est réalisée par une diacylglycéryl transférase nommée Lgt. Puis la SPase II clive le peptide signal au niveau de la lipobox (Tjalsma, Zanen et al. 1999). La modification apportée par Lgt est nécessaire à l'activité de la SPase II chez *E. coli* et d'autres bactéries (Hussain, Ichihara et al. 1980; Tokunaga, Loranger et al. 1982), ce qui n'est pas le cas chez *L. monocytogenes* (Baumgartner, Karst et al. 2007). Enfin, la cystéine de la partie N-terminale est acylée à un acide gras par une lipoprotéine aminoacyl transférase nommée Lnt. Les groupements diacylglycéryl et acyl dérivent des phospholipides de la membrane plasmique permettant de fixer les lipoprotéines à la membrane. Chez les bactéries à Gram positif à faible GC%, aucune protéine orthologue de Lnt n'a été identifiée et la maturation des lipoprotéines s'achève suite au clivage du peptide signal par la SPase II.

L. monocytogenes possède 2 SPases II, LspA (lipoprotein signal peptidase A) et LspB. LspA est impliquée dans la maturation du facteur de virulence LpeA (lipoprotein promoting entry A) mais n'est pas indispensable à la survie de la bactérie (Réglier-Poupet, Frehel et al. 2003) ; LspB n'a encore jamais été caractérisée. Chez *L. monocytogenes*, 68 protéines ont été prédites comme étant des lipoprotéines (Glaser, Frangeul et al. 2001; Trost, Wehmhoner et al. 2005). Bien que la localisation primaire des lipoprotéines soit en surface de la cellule, de

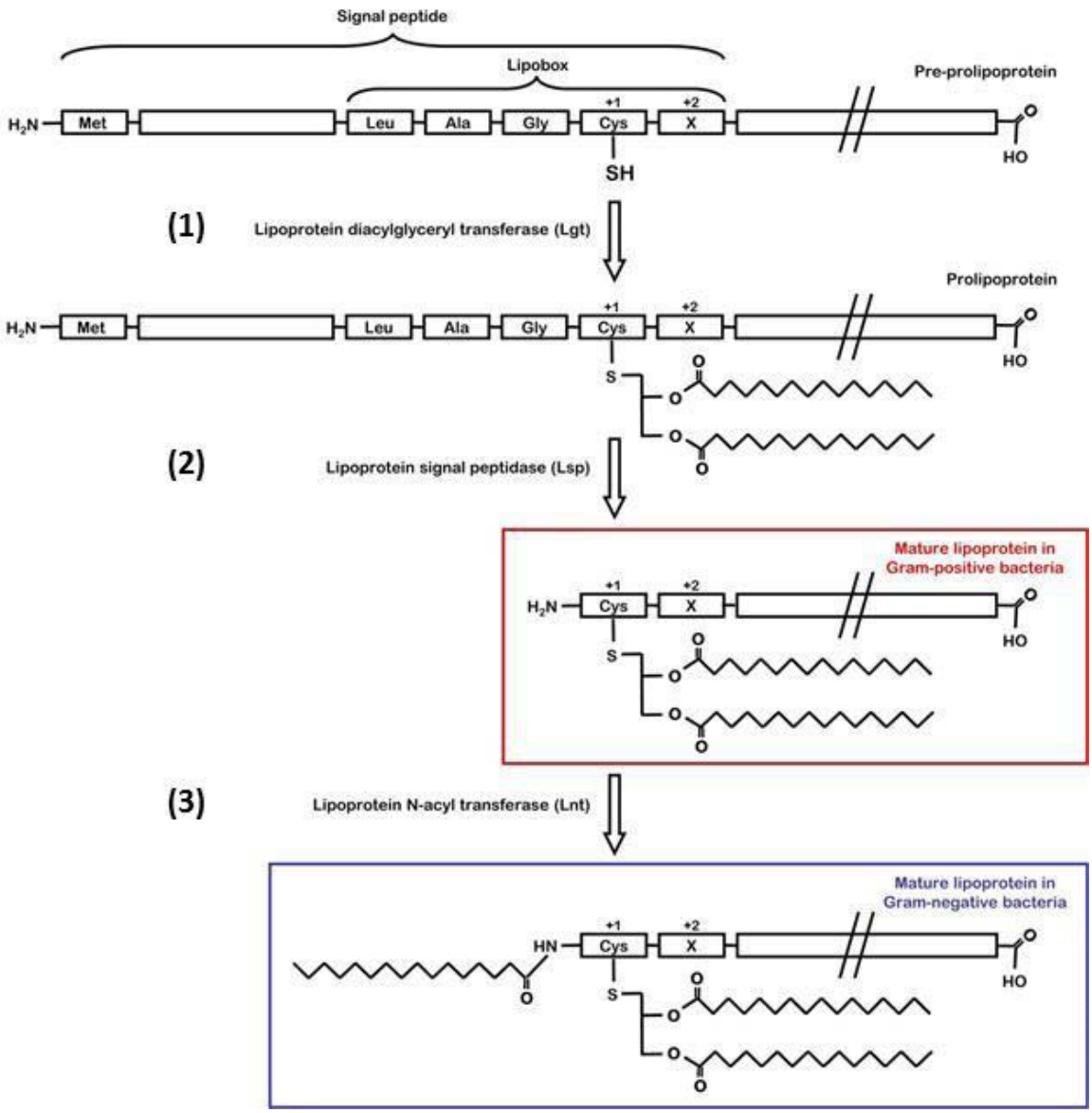

Figure 20 : Voie de maturation des lipoprotéines (Kovacs-Simon, Titball et al. 2011)

nombreuses lipoprotéines sont retrouvées dans le milieu extracellulaire (Desvaux, Dumas et al. 2010). Ces lipoprotéines possèdent une Glycine en position C+2, c'est-à-dire deux acides aminés après le site de clivage des SPase II. Il a été montré que cette position est très importante pour le relargage des lipoprotéines dans le milieu extracellulaire chez les bactéries à Gram positif (Antelmann, Tjalsma et al. 2001; Tjalsma and van Dijl 2005; Sibbald, Ziebandt et al. 2006).

3.3.2 Les lipoprotéines

Les lipoprotéines peuvent intervenir dans divers processus physiologiques (Sutcliffe and Russell 1995). Elles peuvent soit (i) être responsables de la résistance à un antibiotique, comme la pénicillinase chez *Bacillus licheniformis* (Lai, Sarvas et al. 1981; Nielsen, Caulfield

et al. 1981; Smith, Tai et al. 1981), (ii) faire partie de systèmes de transports, comme les ABC transporteurs, (iii) jouer le rôle d'adhésines (Jenkinson 1994), ou encore être impliquées (iv) dans les systèmes de signalisation (Tam and Saier 1993), (v) dans la sporulation (Errington, Appleby et al. 1992), (vi) dans les phénomènes de conjugaison (Tanimoto, An et al. 1993) ou (vii) dans la virulence (Baumgartner, Karst et al. 2007).

Chez *L. monocytogenes*, l'analyse de l'exoprotéome de la souche sauvage et du mutant Δ*lgt* a permis d'identifier 26 lipoprotéines (Baumgartner, Karst et al. 2007). En effet, en absence de Lgt lors de leur maturation par les SPases II, les lipoprotéines sont relarguées dans le milieu extracellulaire puisqu'elles ne sont plus fixées au groupement diacylglycéryl de la membrane cytoplasmique. Parmi ces 26 lipoprotéines ont été identifiées des lipoprotéines impliquées dans le transport ou dans l'agrégation cellulaire en réponse à des phéromones, un antigène stimulant les cellules T (Lmo1388), une lipoprotéine jouant potentiellement le rôle de chaperone (Lmo2219), deux lipoprotéines jouant un rôle dans la virulence, LpeA et OppA (Borezée, Pellegrini et al. 2000; Réglier-Poupet, Pellegrini et al. 2003), ainsi que diverses lipoprotéines de fonction inconnue. L'implication des lipoprotéines dans la virulence de *L. monocytogenes* a été mise en évidence de façon indirecte par l'étude du mutant Δ*lspA*, qui est incapable de s'échapper du phagosome suite à son entrée dans un macrophage (Réglier-Poupet, Pellegrini et al. 2003). Cependant, l'implication des lipoprotéines dans la formation des biofilms n'a jamais été étudiée.

RESULTATS

1. Contexte et objectifs

Listeria monocytogenes est une bactérie pathogène à Gram-positif impliquée dans de nombreuses toxi-infections alimentaires. En effet, elle est l'agent étiologique de la listériose, une maladie rare qui touche principalement les personnes immunodéprimées telles que les femmes enceintes, les nouveaux nés, les personnes âgées ou atteintes de graves maladies (SIDA, cancer, diabète…). Bien que la fréquence des cas de listériose ait diminuée grâce aux efforts des industriels de la filière agroalimentaire, le risque sanitaire reste élevé avec une incidence de 5,1 cas/million d'habitants en France pour 2006 et un taux de mortalité de 20 à 30%. La persistance de *L. monocytogenes* dans les chaînes de production agroalimentaires, est en partie due à sa forte capacité à se développer dans une large gamme de température, de pH et de salinité. De plus, *L. monocytogenes* est capable de former des biofilms sur diverses surfaces, lui permettant de résister aux traitements technologiques et aux stress environnementaux.

Plusieurs études ont montrées que les protéines de surface jouaient un rôle dans le développement de biofilm chez *L. monocytogenes* (Smoot and Pierson 1998; Longhi, Scoarughi et al. 2008; Franciosa, Maugliani et al. 2009). Mis à part la protéine BapL (Jordan, Perni et al. 2008) et le rôle controversé des flagelles (Vatanyoopaisarn, Nazli et al. 2000; Tresse, Lebret et al. 2009), qui interviendraient plutôt dans la phase d'adhésion initiale, les évènements et les déterminants moléculaires inhérents au mode de croissance en biofilm ne sont pas identifiés. La sécrétion des protéines, qui permet la translocation de protéines au travers de la membrane cytoplasmique, constitue un évènement clé dans la présence d'effecteurs à l'interface entre la cellule bactérienne et son environnement. Différents systèmes de sécrétion permettent le transport de protéines au travers de la membrane cytoplasmique chez les bactéries à Gram positif. Parmi ces systèmes, la voie Sec semble empruntée par la majorité des protéines secrétées chez *L. monocytogenes* (Desvaux and Hebraud 2006; Desvaux, Dumas et al. 2010). Selon leur nature et leur destination finale, les protéines vont emprunter différentes embranchements pour être soit sécrétées dans le milieu extracellulaire, intégrées à la membrane cytoplasmique, liées de façon covalente à la membrane cytoplasmique, liées de façon covalente ou non à la paroi cellulaire, ou enfin libérée dans le milieu extracellulaire (Fig. 16).

L'objectif du travail réalisé dans le cadre de ma thèse était de caractériser les déterminants protéiques impliqués dans l'adhésion et la formation de biofilm chez

L. monocytogenes, plus particulièrement au niveau des protéines de surface et des systèmes de sécrétion associés, par une approche de biologie intégrative. Pour cela, une étude génomique rationnelle modélisant la sécrétion des protéines de *L. monocytogenes* a tout d'abord été réalisée afin d'identifier les protéines du sécrétome et de prédire leur localisation finale au niveau de la membrane, de la paroi ou dans le milieu extracellulaire. Les résultats de cette analyse génomique ont permis de générer des hypothèses *in silico* et d'identifier les voies de sécrétion et des produits de gènes cibles potentiellement impliqués dans la formation de biofilm. Ainsi, une attention particulière a été portée sur deux aspects de la voie de sécrétion Sec : la voie alternative de sécrétion SecA2 et la voie de maturation des lipoprotéines. La stratégie adoptée dans les deux cas est une approche ciblée visant à inactiver d'une part des gènes codant des protéines impliquées dans ces voies de sécrétion et d'autre part des gènes codant les substrats de ces voies, afin de déterminer leur implication dans l'adhésion et la formation de biofilm. La caractérisation phénotypique des mutants obtenus a été effectuée (i) par la mise en œuvre de différentes techniques d'évaluation de la formation du biofilm à des stades précoces et matures et (ii) par la visualisation de la morphologie des cellules et de l'architecture des biofilms par des techniques de microscopie en condition de croissance dynamique et/ou statique. Les approches ciblées sur les deux mécanismes de sécrétion de la voie Sec (SecA2 et voie de maturation des lipoprotéines) ont été complétées par des approches globales impliquant la protéomique visant à identifier expérimentalement la ou les protéines sécrétées par ces voies et potentiellement impliquées dans la formation de biofilm.

Il est à noter que le financement de ce travail de thèse a été assuré par le programme de l'Union Européenne ProSafeBeef 2007-2012 (7ème PCRDT). Nos objectifs s'inscrivaient dans le "WorkPackage 1.2.: Persistence and virulence of key microbial pathogens in the beef chain" avec, plus précisément pour notre équipe de recherche, la caractérisation de la persistance de *L. monocytogenes* sous forme de biofilms dans les environnements de fabrication et de transformation des produits de la filière bovine. Dans le cadre de ce WP, nous avons collaboré avec des partenaires irlandais (Dr Geraldine Duffy, TEAGASC, Dublin), norvégiens (Dr Even Heir, Nofima Food, Ås) et grecs (Prof. George Nychas, Agricultural University of Athen, Athènes). En dehors de ce programme, nous avons également noué des collaborations avec le Danemark (Prof Susanne Knøchel, Univerity of Copenhagen), la Pologne (Dr Magdalena Popowwska, University of Warsaw) et une autre équipe irlandaise (Prof. Colin Hill, University College of Cork).

2. Analyse génomique des protéines sécrétées chez *Listeria monocytogenes*

Les cellules vivantes interagissent avec leur environnement par l'intermédiaire de protéines, qui peuvent être localisées dans les enveloppes cellulaires (paroi, membrane) ou encore sécrétées dans le milieu extracellulaire. La prédiction de la localisation subcellulaire de ces protéines permet de fournir des pistes sur leur fonction et d'appuyer les données expérimentales en protéomique. Afin d'identifier l'ensemble des protéines sécrétées chez *L. monocytogenes*, une analyse génomique rationnelle modélisant la sécrétion des protéines a été développée. Cette approche originale prend en compte les différents systèmes de sécrétion identifiés chez *L. monocytogenes* ainsi que les différents motifs signaux impliqués dans la localisation finale de ces protéines afin de prédire leur présence dans le cytoplasme, la membrane, la paroi ou bien le milieu extracellulaire.

ARTICLE n°1

Sandra RENIER, Pierre MICHEAU, Régine TALON, Michel HÉBRAUD and Mickaël DESVAUX.

Final subcellular location of extracytoplasmic proteins in monoderm bacteria: Generic and rational secretomics-based method for genomic and proteomic analyses of secreted proteins

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Subcellular Localization of Extracytoplasmic Proteins in Monoderm Bacteria: Rational Secretomics-Based Strategy for Genomic and Proteomic Analyses

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Abstract

Genome-scale prediction of subcellular localization (SCL) is not only useful for inferring protein function but also for supporting proteomic data. In line with the secretome concept, a rational and original analytical strategy mimicking the secretion steps that determine ultimate SCL was developed for Gram-positive (monoderm) bacteria. Based on the biology of protein secretion, a flowchart and decision trees were designed considering (i) membrane targeting, (ii) protein secretion systems, (iii) membrane retention, and (iv) cell-wall retention by domains or post-translocational modifications, as well as (v) incorporation to cell-surface supramolecular structures. Using *Listeria monocytogenes* as a case study, results were compared with known data set from SCL predictors and experimental proteomics. While in good agreement with experimental extracytoplasmic fractions, the secretomics-based method outperforms other genomic analyses, which were simply not intended to be as inclusive. Compared to all other localization predictors, this method does not only supply a static snapshot of protein SCL but also offers the full picture of the secretion process dynamics: (i) the protein routing is detailed, (ii) the number of distinct SCL and protein categories is comprehensive, (iii) the description of protein type and topology is provided, (iv) the SCL is unambiguously differentiated from the protein category, and (v) the multiple SCL and protein category are fully considered. In that sense, the secretomics-based method is much more than a SCL predictor. Besides a major step forward in genomics and proteomics of protein secretion, the secretomics-based method appears as a strategy of choice to generate *in silico* hypotheses for experimental testing.

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Introduction

All living cells interface with their surrounding through proteins that are located in the cell envelope, displayed on the cell surface or released into the extracellular milieu, and even beyond when injected into a host cell. Such proteins are translocated in the first instance through biological membranes by protein-conducting channels, *i.e.* translocons. In bacteria, several secretion systems enabling protein transport from the inside to the outside of the cell have been characterised. Distinction must be made between bacteria possessing (i) one biological membrane (the cytoplasmic membrane), the so-called monodermata, and (ii) two biological membranes (the inner membrane and outer membrane), the so-called didermata [1,2,3]. Until recently and in contrast to diderm bacteria [4,5,6,7,8], comprehensive knowledge on protein secretion systems in monoderm species was restricted to the non-pathogenic *Bacillus subtilis* species [9,10,11] or scattered among different species for specific systems [12,13,14,15].

Because of the absence of an outer membrane, the numerical classification of protein secretion systems does not apply to monodermata and export across the cytoplasmic membrane actually corresponds to a secretion event [16]. As in didermata, the Sec (secretion) and Tat (twin-arginine translocation) machineries are found in the cytoplasmic membrane but additional secretion systems can be present in monoderms, *i.e.* the FPE (fimbrilin-protein exporter), ABC (ATP-binding cassette) transporters, FEA (flagellum export apparatus), holins (hole-forming) and Wss (WXG100 secretion system) [17,18]. As thoroughly explain by several specialists in the field of bacterial protein secretion [2,19,20,21,22,23,24], we will abstain to use the "T7SS" terminology to describe the Wss in monoderms, which is actually ascribed to the chaperone-usher pathway in diderm-LPS and at best only apply to diderm-mycolate, which is restricted to bacteria of the genus *Mycobacterium*.

As in any living cell, extracytoplasmic proteins cover a vast variety of functions, including nutrient uptake, chemosensing, motility, adhesion or cell envelope biogenesis. Moreover, their subcellular localization (SCL) and biological functions provide distinguishing clues regarding the physiology, lifestyle, position and interactions of the bacterial cell in an ecological niche and more generally in an ecosystem, as revealed for example by extracellular degradative enzymes in saprophytes or cell-surface virulence factors in pathogens. The final SCL of a protein results from a series of molecular mechanisms, involving post-translational and/or post-translocational modifications. The secretome concept is very useful for considering these different steps as it

includes both the proteins secreted across the cytoplasmic membrane to the membrane cell wall interface, the cell wall or the extracellular environment, and importantly, the secretory machineries themselves [10,25,26]. As such and contrary to what it sometimes misconceptualized by some authors, the secretome is not a proteome *per se*, let alone the subset of extracellular proteins, that is actually the exoproteome [1]. In bacteria with a Gram-positive cell-envelope architecture, the proteins actively transported *via* these secretion systems, the so-called secreted proteins, can have radically different final destinations and be either (i) anchored to the cytoplasmic membrane, (ii) associated with the cell wall, (iii) released into the extracellular milieu, or even (iv) injected into a host cell [1]. Description of SCL now follows the Gene Ontology (GO) recommendations for describing "Cellular component", one of the three structured controlled vocabularies [27].

Because experimental investigation of the membrane and cell wall proteomes is hindered by technical limitation of protein extraction from their subcellular fractions, genomic prediction of SCL has been the subject of intense research effort. Numerous localization predictors have been developed for predicting the final destination of proteins. These bioinformatic tools can be divided into (i) specialized prediction tools, essentially based on the identification of signal peptides or retention sequences to the membrane or cell wall, *e.g.* SignalP [28], LipoP [29], TMHMM [30] or CW-PRED [31], and (ii) global prediction tools indicating the protein final SCL, *e.g.* PSORTb [32], LocTree [33], CELLO [34] or Gpos-mPLoc [35]. Such ensemble classifiers based on support vector machine (SVM) or neural network (NN) have been constructed on algorithms with a rationale somehow disconnected from the biology of the system investigated. Each of these tools having its own prediction limits, though, an alternative and powerful strategy consists in combining predictions [36]. For Gram-positive bacteria, different pipelines have been developed to predict final location of protein, *e.g.* Augur [37], LocateP [38] or SurfG+ [39], but none of them is comprehensive. A momentous limitation is that, by essence, their workflows are not evolutive but established once and for all and cannot be willingly adjusted in light of new findings in the field. Consequently, new specialized prediction tools cannot be swiftly implemented since any modifications remain at the discretion of their designers. In addition, results of some of these tools are frozen in databases and interrogations cannot be readily performed on demand or on newly available bacterial genomes.

In order to provide a much more comprehensive, reliable, flexible and adjustable prediction of protein SCL in monoderm bacteria, we aimed at developing a strategy for analysing genomic and proteomic data for secreted proteins. The originality of the method resides in the use of an extensive number of readily available bioinformatic tools organized in a workflow and decision trees mimicking very closely the molecular steps encountered by a protein in the course of secretion. Compared to all available predictions tools to date, this methodology embraces the secretome concept as it does not only consider the presence of various domains that can target or retain the secreted proteins within the cell-envelope of monoderm bacterium but most importantly considers the presence/absence of protein secretion pathways. Taking into account (i) it potentially expresses a prodigious amount of extracellular proteins and cell-surface proteins, which are membrane or cell-wall attached [4,40,41], (ii) it is a pathogenic Gram-positive bacterium contrary to the paradigm *B. subtilis*, and (iii) numerous experimental proteomic approaches have been dedicated to its extracytoproteome, including the surface and extracellular proteomes [42,43,44,45,46,47], it prompts us to focus on the extracytoproteome of *Listeria monocytogenes* as a case study.

Results

Design of the secretomics-based method for genomic analysis of secreted proteins in monoderm bacteria

The rationale for analyzing the final SCL of secreted proteins is based on the biology of protein secretion, *i.e.* the secretome. This concept provides an integrated and global view by considering protein routing, transport systems, post-translocational (translational) modifications and subcellular location [10,25,26]. Based on the secretome in monoderm bacteria (**Figure 1**), the steps considered are (i) the targeting of protein to the membrane by an N-terminal signal peptide, (ii) the protein secretion systems present, *i.e.* Sec, Tat, ABC, FPE, FEA, holin and/or Wss, (iii) the membrane retention of secreted proteins by domains or post-translational modifications, (iv) the cell-wall retention of secreted proteins by domains or post-translational modifications, and (v) the incorporation to cell-surface supramolecular structures. A summary of the different abbreviations in use in relation to protein secretion, especially protein categories and subcellular localizations, is provided in **Table 1.**

As one of the cornerstones of the secretomics-based method, the type of signal peptide (SP) is defined (**Figure 2**). Besides, the search in parallel of all known protein secretion pathways in monoderm bacteria constitutes the keystone of this analytical strategy. Secretion pathways not only focus on translocases of the secretion systems but include the associated post-translocation maturation pathways, namely the respective signal peptidases, the lipoprotein maturation pathway, and the covalent cell-wall anchoring sortase pathway (**Table 2**). It is important to stress that the absence of a SP cannot rule out protein secretion by alternative systems as detailed below. Presence of the different types of SP is associated with the respective secretion systems, whereas substrates to alternative secretion systems lacking SP are subjected to similarity search (**Figure 3**). Together with pseudopilus and flagellum secreted *via* FPE and FEA respectively, other surface supramolecular structures are considered, namely S-layer, pilus and/or cellulosome. For integral membrane protein (IMP) and besides the presence of transmembrane domain (TMD), the presence of uncleaved N-terminal SP, which could serve as a signal anchor, is utterly considered. Secreted proteins, which are neither predicted as retained to the membrane, cell-wall nor subunits of surface appendages, are predicted as exoproteins. Once the different protein categories are defined, SCL can be predicted (**Figure 3**). Cytoproteins potentially secreted by non-classical pathway (NC) are also predicted as localized in the extracellular milieu. Position +2 of the cleavage site (C+2) is checked as an indicator that the lipoprotein can be potentially released into the extracellular milieu [26,48]. The number of GW modules is carefully considered for protein SCL in the extracellular milieu rather than in the cell wall. Besides discriminating the protein category from the SCL to avoid any confusing statement or misleading interpretation, the secretomics-based method provides a detailed description of protein routing and permits considering multiple SCL for a given protein.

SCL prediction of secreted proteins in *L. monocytogenes* following the secretomics-based method

Refining the analysis of genome-encoded proteins exhibiting a SP in *L. monocytogenes* EGD-e and consolidating the results with the prediction of uncleaved SP, it appears that 723 proteins exhibit a SP, *i.e.* 1 protein with a Tat-SP, 3 an ABC-SP and 5 a FPE-SP as well as 224 with a SP of Type I (SP I), 74 with a SP II and 416 with an uncleaved SP (Unc-SP) (Table S1). The protein secretion pathways (**Table 2**) are associated with the identification of their

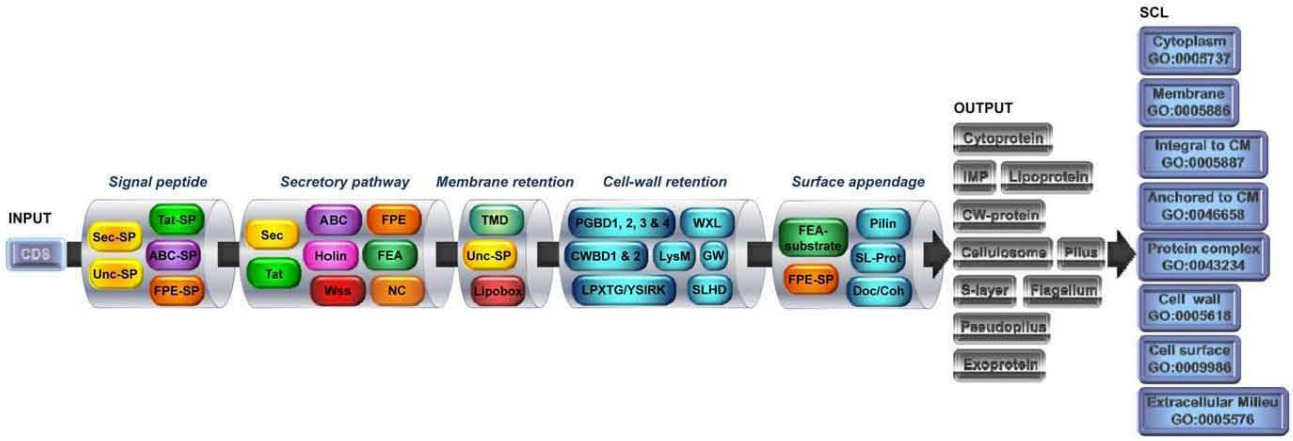

Figure 1. Synoptic view of the secretomics-based method for monoderm bacterium. Based on the biology of protein secretion, the coding sequences (CDS) are sequentially analysed in a workflow for (i) signal peptide (SP), (ii) secretory pathway, (iii) membrane retention, (iv) cell-wall retention, and (v) surface appendage. For each step, a combination of different tools allows defining different databases, as indicated in the detailed flowchart (**Figure 2**). From there, the resulting databases are analysed as depicted in the detailed decision trees (**Figure 3**). In the end, proteins are discriminated into different categories and different SCL are predicted. Sec-SP: Sec-dependent SP; Unc-SP: uncleaved SP; TMD: transmembrane domain; PGBD: peptidoglycan-binding domain; CWBD: cell-wall binding domain; SLHD: S-layer homology domain; SL-Prot: S-layer protein; Doc/Coh: dockerin/cohesin domain; IMP: integral membrane protein; GO: gene ontology.
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respective protein substrates. Altogether and following majority vote approach, 741 proteins appear secreted, including 714 proteins targeted to Sec, 1 to Tat, 4 to ABC (including a leaderless bacteriocin), 3 to holin, 3 to Wss, 5 to FPE and 11 to FEA (Table S1). In addition, 162 IMPs lack a SP but could nonetheless be targeted and translocated within the membrane *via* YidC. Moreover, 108 proteins primarily predicted as cytoproteins, were further predicted as putatively secreted by NC. Altogether, proteins secreted *via* these different pathways are predicted as either located to the cytoplasmic membrane (CM; GO:0031226), cell wall (CW; GO:0009275), cell surface (CS; GO:0009986) and/or extracellular milieu (EM; GO:0005576).

Table 1. Summary of the abbreviations in use in relation to protein secretion.

Abbreviation	Full name
Protein categories	
IMP	integral membrane protein
ssIMP I/II/III	single-spanning IMP of type I/II/III
msIMP	multi-spanning IMP
CW-protein	parietal protein
Subcellular localization (SCL)	
CM	cytoplasmic membrane
CW	cell wall
CS	cell surface
EM	extracellular milieu
Other	
SP I/II	signal peptide of type I/II
Unc-SP	uncleaved SP
TMD	α-helical transmembrane domain
CWBD1/2	cell-wall binding domain of type 1/2
PGBD1/2/3/4	peptidoglycan-binding domain of type 1/2/3/4
SLHD	S-layer homology domain
Sec	secretion
Tat	twin-arginine translocation
FEA	flagellum export apparatus
FPE	fimbrillin-protein exporter
ABC	ATP-binding cassette
Wss	WXG100 secretion system
NC	non-classical secretion

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Location intrinsic to the cytoplasmic membrane (GO:0031226). This location splits into two further classes, i.e. either integral to the cytoplasmic membrane (GO:0005887) or anchored to the cytoplasmic membrane (GO:0046658).

Among the 686 predicted IMPs (GO:0005887) as revealed by majority-vote scheme, 524 bear an N-terminal SP including 100 with a SP I, 8 with a SP II and 416 with an Unc-SP (Table S2). From the most recent review [40], the number of protein with a C-terminal "hydrophobic tail" was estimated at only 10, no estimate have ever been provided for N-terminal "hydrophobic tail" proteins [49]. It is also worth noting that the view that only single-spanning IMP (ssIMP) with N-terminal or C-terminal TMD can be considered as surface exposed is simplistic. On the one hand, it is not only the TMD position but rather its orientation that should be considered. On the other hand, even a short strand of amino acid interacting with the external side, such as a loop in multi-spanning IMP (msIMP) can have significant biological function. Based on topogenic elements defined by TMD orientation, where Type I and Type II modules have N_{out} C_{in} and N_{in} C_{out} orientations respectively [50], ssIMPs are further classified into (i) Type I where the ssIMP remains membrane-integrated by a Type I module following cleavage of an N-terminal SP, (ii) Type II where the ssIMP remains membrane-integrated by a Type I module, such an Unc-SP for instance, and (iii) Type III where the ssIMP remains membrane-integrated by a Type I module but is deprivated of an N-terminal SP [51,52,53]. Following the standard nomenclature and among the 157 ssIMP, 20 are of Type I (ssIMP I), *i.e.* exhibiting a cleavable SP, 120 are ssIMP II (including 87 exhibiting an Unc-SP), and 17 ssIMP III (Table S2). Among the

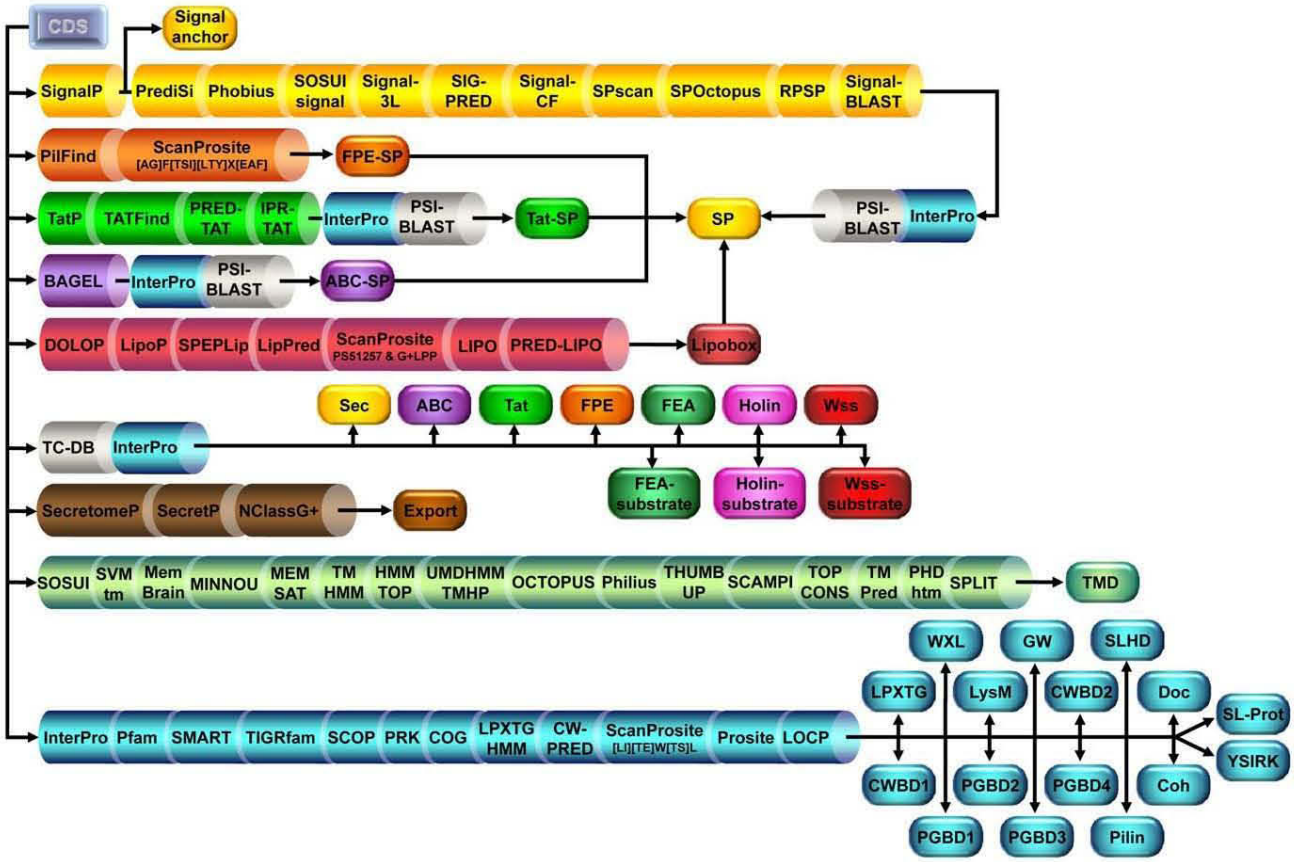

Figure 2. Comprehensive flowchart of the secretomics-based method in a monoderm bacterium. The analysis considered the (i) signal peptide (SP), (ii) type of SP (Signal anchor, FPE-SP, Tat-SP, ABC-S and, lipobox), (iv) protein secretion systems, (v) exported proteins lacking a SP (Export, FEA-, Holin, and Wss-substrates), (iv) transmembrane domain (TMD), and (iv) relevant conserved domains (LPXTG, WXL, LysM, CWBD1,...*etc*). Details of the prediction tools used for the analysis and definition of the databases are provided inthe Materials & Methods section.
doi:10.1371/journal.pone.0042982.g002

529 ms IMP (msIMP), 88 exhibit a cleavable Sec-SP and 323 an Unc-SP (Table S2). From literature survey, the number of IMP in *L. monocytogenes* was estimated at 267 [4], proteins with a C-terminal "hydrophobic tail" was estimated at only 10 [40] and no estimate had ever been provided for N-terminal "hydrophobic tail" proteins [49]. Besides the fact that the terminology in usage for IMPs in *L. monocytogenes* is quite inadequate, the estimate is here considerably changed and qualitatively improved.

Among the 74 lipoproteins (GO:0046658) predicted through majority voting (Table S3), 41 exhibited a glycine residue at position +2 of the cleavage site, conferring them a double possible location in the CM and the EM. Besides, 8 lipoproteins are also predicted as IMP (2 ssIMP I and 6 msIMP); 5 of them (Lmo0269, Lmo0641, Lmo0821, Lmo2687 and Lmo2793) are predicted as lipoproteins for the first time. While one-third of them have unknown function, even after PSI-BLAST search, the majority of them would be substrate-binding protein from ABC transport system. The combination of comprehensive prediction tools allowed here correcting the previous prediction of 68 lipoproteins [40].

Location at the cell wall (GO:0009275). Parietal proteins (CW-protein) covalently anchored to the cell-wall bear a C-terminal LPXTG domain. Of note, the LPXTG-domain refers to the original regular expression of the motif now surpassed by HMM profiles encompassing the diversity of the cell-wall covalent-anchoring domain, *i.e.* the LPXTG-protein family [54]. In *L. monocytogenes*, the 43 LPXGT-proteins previously mentioned have been here predicted [40,49], including two LPXTG-proteins substrate to SrtB, a lipoprotein (Lmo1136) and a LXPTG protein bearing LysM domains (Lmo880) (Table S4). No YSIRK motif (IPR005877) could be identified within Sec-dependent signal peptides of these proteins; when present, this motif is systematically associated with a LPXTG domain for efficient protein secretion [55] and/or specific final localization within the bacterial cell wall [56].

Besides proteins covalently linked to the cell wall, several CW-proteins are predicted attached by alternative means (Table S4). Contrary to the most recent review [40], only 5 instead of 9 GW-proteins are estimated as CW-proteins. Indeed, the 4 other proteins only exhibit one GW module, which is insufficient for binding to lipoteichoic acids in a noncovalent manner, and are consequently considered here as located extracellularly (Table S6) [57,58,59]. In addition, 4 WXL-proteins and one PGBD1-protein (Lmo1851) have been also identified. Previously, the PGBD1-protein had been announced as a IMP because of the presence of a TMD [40]; however, the only TMD predicted is located in the cleavable N-terminal signal peptide of Type I.

Location at cell surface (GO:0009986). According to GO, this term is intended to annotate gene products that are attached to the CM (intrinsic or loosely bound, GO:0031226 and GO:0031232, respectively) or to the CW (integral or loosely bound, GO:0009275 and GO:0010339, respectively), and thus not

Table 2. Protein secretion pathways in *L. monocytogenes* EGD-e as revealed by the secretomics-based method.

Secretion pathway[a]	Protein ID	Annotation[b]	Similarity search[c]
Sec			
Translocase	Lmo2612	Sec translocon, subunit SecY	TC#3.A.5, COG0201, IPR002208, PIRSF004557, TIGR00967, SSF103491, PF00345
	Lmo0245	Sec translocon, subunit SecE	TC#3.A.5, COG0690, IPR001901, IPR005807, TIGR00964, PF00584
	Lmo2451	Sec translocon, subunit SecG	TC#3.A.5, COG1314, IPR004692, TIGR00810, PF03840
	Lmo1527	Sec transcolon, bifunctional subunit SecDF	TC#2.A.6.4, TC#3.A.5, IPR03335, PF07549, PF02356
	Lmo1529	Sec transcolon, subunit YajC	TC#9.B.18, COG1862, TIGR00739, PF02699
	Lmo2510	Sec translocase, ATPase, SecA	TC#3.A.5, COG0653, IPR000185, TIGR00963
	Lmo0583	Sec translocase, ATPase, SecA2	TC#3.A.5.10, COG0653
	Lmo1803	Signal recognition particle (SRP) receptor subunit, FtsY	TC#3.A.5, COG0552, IPR004390, IPR000897, TIGR00064, SSF47364
	Lmo1801	Signal recognition particle (SRP), Ffh	TC#3.A.5, COG0541, IPR004780, TIGR00959, SSF47446
Insertase	Lmo1379	YidC insertase, OxaA1 (YqjG)	TC#2.A.9, COG0706
	Lmo2854	YidC insertase, OxaA2 (SpoIIIJ)	TC#2.A.9, COG0706
SPase	Lmo1269	Signal peptidase of Type I, SipX	COG0681, IPR000223, TIGR02227, SSF51306
	Lmo1270	Signal peptidase of Type I, SipY	COG0681, IPR000223, TIGR02227, SSF51306
	Lmo1271	Signal peptidase of Type I, SipZ	COG0681, IPR000223, TIGR02227, SSF51306
	Lmo1844	Signal peptidase of Type II, lipoprotein signal peptidase, LspA	COG0597, IPR001872, TIGR00077, PF01252
	Lmo1101	Signal peptidase of Type II, lipoprotein signal peptidase, LspB	COG0597, IPR001872, TIGR00077, PF01252
CM anchoring	Lmo2482	Prolipoprotein diacylglyceryltransferase, Lgt	COG0682, IPR001640, TIGR00544, PF01790
CW anchoring	Lmo0929	Sortase A, SrtA	COG3764, IPR005754, TIGR01076, SSF63817, PF04203
	Lmo2181	Sortase B, SrtB	COG4509, IPR009835, IPR015986, PIRSF030150, TIGR03064, SSF63817, PF07170
Tat			
	Lmo0362	Twin-arginine translocase protein A, TatA	TC#2.A.64, COG1826, IPR003369, IPR006312, TIGR01411, PF02416
	Lmo0361	Twin-arginine translocation protein, TatC	TC#2.A.64, COG0805, IPR002033, TIGR00945, PF00902
ABC			
	Lmo0607	ABC-type bacteriocin exporter, peptidase domain, ATP-binding/permease protein	TC#3.A.1, COG2274
	Lmo0608	ABC-type bacteriocin exporter, peptidase domain, ATP-binding/permease protein	TC#3.A.1, COG2274
	Lmo2580	ABC-type antimicrobial peptide transport system, ATPase component	TC#3.A.1, COG1136
	Lmo2581	ABC-type antimicrobial peptide transport system, permease component	TC#3.A.1, COG0577
	Lmo2751	ABC-type bacteriocin exporter, peptidase domain, ATP-binding/permease protein	TC#3.A.1, COG2274
	Lmo2752	ABC-type bacteriocin exporter, peptidase domain, ATP-binding/permease protein	TC#3.A.1, COG2274
	Lmo0107	ABC-type bacteriocin exporter, peptidase domain, ATP-binding/permease protein	TC#3.A.1, COG2274
	Lmo0108	ABC-type bacteriocin exporter, peptidase domain, ATP-binding/permease protein	TC#3.A.1, COG2274
FPE			
	Lmo1347	Fimbrilin-protein exporter, ATPase component, ComGA	TC#3.A.14
	Lmo1346	Fimbrilin-protein exporter, membrane component, ComGB	TC#3.A.14
	Lmo1550	Type 4 prepilin peptidase, ComC	COG1989, IPR000045, PF01478
FEA			
	Lmo0680	Flagellar export apparatus, membrane subunit FlhA	TC#3.A.6, COG1298, IPR001712, PF00771

Table 2. Cont.

Secretion pathway[a]	Protein ID	Annotation[b]	Similarity search[c]
	Lmo0679	Flagellar export apparatus, membrane subunit FlhB	TC#3.A.6, COG1377, IPR006135, PF01312
	Lmo0678	Flagellar export apparatus, membrane subunit FliR	TC#3.A.6, COG1684, IPR002010, PF01311
	Lmo0677	Flagellar export apparatus, membrane subunit FliQ	TC#3.A.6, COG1987, IPR002191, PF01313
	Lmo0676	Flagellar export apparatus, membrane subunit FliP	TC#3.A.6, IPR018035, PF02108
	Lmo0715	Flagellar export apparatus, peripheral subunit FliH	TC#3.A.6, COG1338, IPR005838, PF00814
	Lmo0716	Flagellar export apparatus, ATPase subunit FliI	TC#3.A.6, COG1157, IPR005714, TIGR01026
Holin			
	Lmo0128	Holin TcdE-like	TC#1.E, COG4824, IPR006480, TIGR01593, PF05105
	Lmo2279	Holin phage A118	TC#1.E, IPR009708, PF06946
Wss			
	Lmo0061	WXG100 secretion system, ATPase component, YukAB (EssC)	TC#9.A.44, IPR023839, TIGR03928
	Lmo0057	WXG100 secretion system, membrane component, EsaA	TC#9.A.44, IPR023838, TIGR03929
	Lmo0058	WXG100 secretion system, membrane component, EssA	TC#9.A.44, IPR018920, PF10661, TIGR03927
	Lmo0060	WXG100 secretion system, membrane component, YukC (EssB)	TC#9.A.44, IPR018778, PF10140, TIGR03926
	Lmo0059	WXG100 secretion system, peripheral component, YukD (EsaB)	TC#9.A.44, IPR14921, PIRSF037793, PF08817
	Lmo0062	WXG100 secretion system, peripheral component, EsaC	TC#9.A.44

[a]Protein secretion systems: Sec (Secretion), Tat (Twin-arginine translocation), ABC (ATP-binding cassette), holin (hole forming) and Wss (WXG100 secretion system) pathways.
[b]Some annotations were corrected respective to the similarity search performed as described in the Material & Methods section. More extensive and detailed annotations are available in Table S1.
[c]Similarity search were based on interrogations of dedicated databases as described in the Material & Methods section.
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only covers IMP, lipoprotein and CW-protein categories but also includes protein complexes (GO:0043234).

Concerning secreted proteins belonging to supramolecular cell-surface appendages (GO:0043234 AND 0009986), neither S-layer proteins, cellulosome components nor prepilins with LPXTG domains could be identified in *L. monocytogenes*. Nonetheless, 11 flagellar components are predicted secreted and assembled by the FEA (Table S5). In addition, 5 genes encoding for pseudo-prepilin exported by the FPE are identified. Surprisingly, these supramolecular structures had rarely been reported as part of the surface proteins in *L. monocytogenes* [40,60].

Location into the extracellular milieu (GO:0005576). 80 exoproteins were predicted as localized extracellularly, including 69 proteins transported *via* Sec, 1 protein secreted *via* Tat, 4 bacteriocins translocated *via* ABC transporters, 3 proteins exported *via* holins and 3 proteins secreted *via* Wss (Table S6). While three proteins were predicted with a Tat-SP in the first place (Table S1), only the Dyp (dye decolorising peroxydase)-type peroxydase Lmo0367 was finally predicted as a Tat substrate (Table S6). Among the five predicted bacteriocins, four of them would be secreted *via* ABC transporters, *i.e.* Lmo0335, Lmo0615, Lmo2574, and Lmo2753, which is leaderless. As a lactococcin 972 homolog Lmo2776 exhibits an N-terminal SP is most certainly exported in Sec-dependent manner.

Besides, some lipoproteins (Table S3) and primarily cytoplasmic proteins were also predicted with extracellular localization following NC (Table S1). For example, it can be noted that some proteins lacking a SP can be secreted in a route involving the alternative cytosolic ATPase SecA2, a paralogue of SecA, that most certainly converge to the Sec translocon [4,61].

Comparison with SCL predictors applied to the extracytoproteome of *L. monocytogenes*

With 8 different predictable SCL in agreement with GO terms in Gram-positive bacteria, *i.e.* EM (GO:0005876), CS (GO:0009986), cell-surface protein complex (GO:0043234 AND 0009986), CW (GO:0009275), intrinsic to the CM (GO:0031226), integral to the CM (GO:0005887), anchored to the CM (GO:0046658), and cytoplasm (GO:0005737), other predictors never reach such a level of discrimination (Tables 3 and S7). Usually, 4 SCLs are considered as in CELLO [34], PSORTb [62] and Gpos-mPLoc [35] with the cytoplasm, membrane, cell wall and extracellular milieu. SubLoc [63] or LocTree [33] only differentiate cytoplasmic from extracellular location, and Augur the surface from the extracellular milieu [37]. Of note, results of some of these tools are not strictly in accordance with the GO and suffer of several misconceptions regarding the field of protein secretion [1], *e.g.* (i) SurfG+ [39] and LocateP [38] misleadingly refer to "secreted" for EM location, (ii) they mixes up SCLs with protein categories using descriptors such as LPXTG, multi-transmembrane or N-terminally anchored, which are further not in agreement with standart nomenclature for IMP for instance, (iii) SurfG+ considers "potentially surface exposed (PSE)" needs a certain amino acid length to cross the cell wall, whereas first, this is protein folding that would rather matter, and second, there is no need for a protein to poke out the confine of the cell wall to interact with their environment [60]. In addition and contrary to SubLoc, LocTree, Augur, PSORTb, SurfG+ or LocateP, the secretomics-based method allows attributing multiple SCL for a single protein.

A major and original advance from the secretomics-based analysis resides in the clear and systematic differentiation of the protein category from the protein SCL. To objectively evaluate

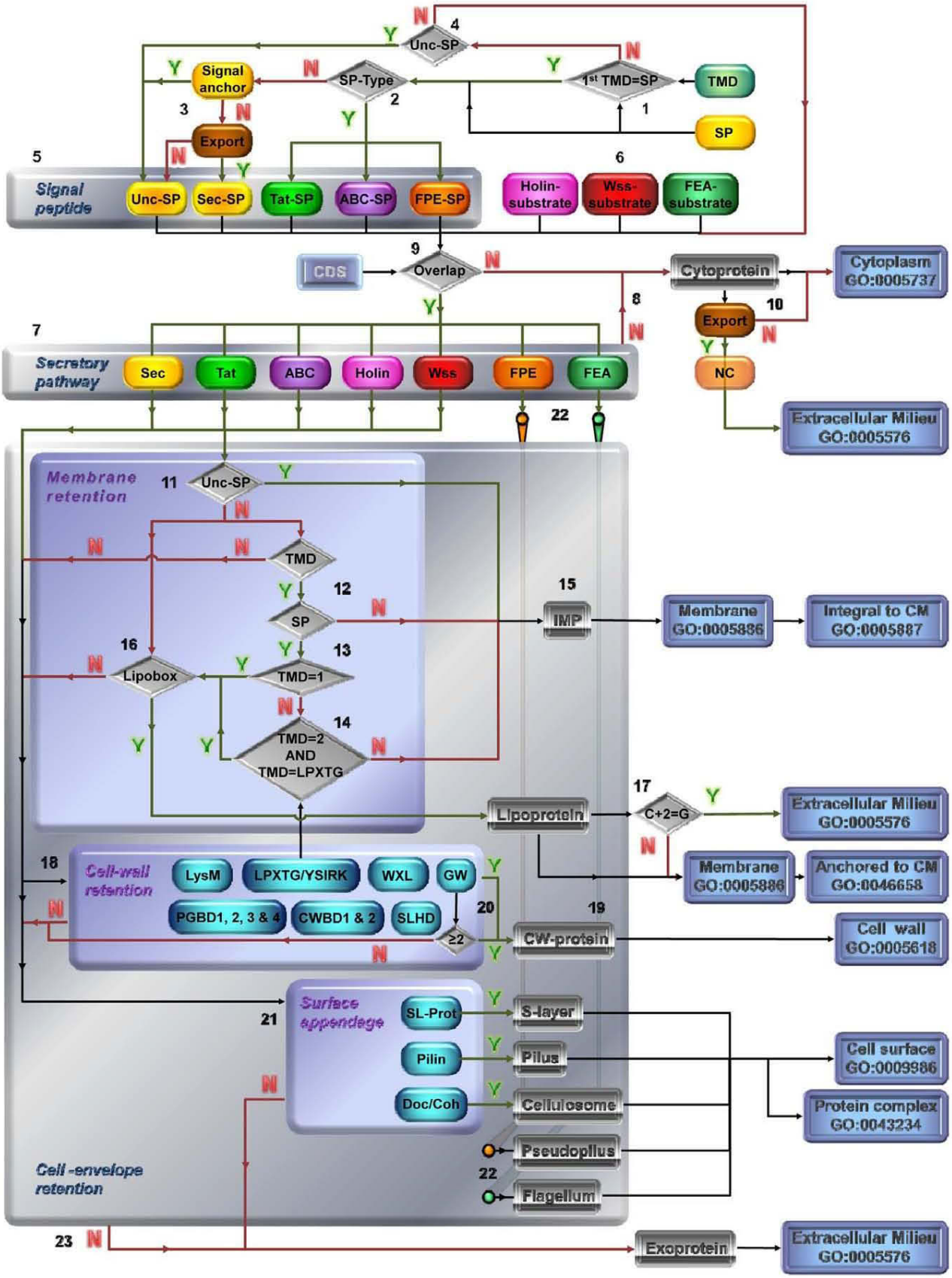
Unc-SP
Signal anchor
SP-Type
1st TMD=SP
TMD
SP
Export
Signal peptide
Unc-SP
Sec-SP
Tat-SP
ABC-SP
FPE-SP
Holin-substrate
Wss-substrate
FEA-substrate
CDS
Overlap
Cytoprotein
Cytoplasm GO:0005737
Export
NC
Extracellular Milieu GO:0005576
Secretory pathway
Sec
Tat
ABC
Holin
Wss
FPE
FEA
Membrane retention
Unc-SP
TMD
SP
TMD=1
Lipobox
TMD=2 AND TMD=LPXTG
IMP
Membrane GO:0005886
Integral to CM GO:0005887
Lipoprotein
C+2=G
Extracellular Milieu GO:0005576
Membrane GO:0005886
Anchored to CM GO:0046658
Cell-wall retention
LysM
LPXTG/YSIRK
WXL
GW
PGBD1, 2, 3 & 4
CWBD1 & 2
SLHD
≥2
CW-protein
Cell wall GO:0005618
Surface appendage
SL-Prot
Pilin
Doc/Coh
S-layer
Pilus
Cellulosome
Pseudopilus
Flagellum
Cell surface GO:0009986
Protein complex GO:0043234
Cell -envelope retention
Exoprotein
Extracellular Milieu GO:0005576

Figure 3. Detailed decision trees for prediction of protein category and SCL of secreted proteins. (1) Proteins exhibiting N-terminal SP are extracted from TMD database (Y). (2) The different types of SP (Tat-SP, ABC-SP and FPE-SP) are extracted (Y). (3) Absence of a signal anchor (N) and export (Y) define Sec-dependent SP (Sec-SP). (1) Proteins with TMD but no predicted SP (N), (4) are checked for uncleaved SP (Unc-SP), *i.e.* TMD of at least 7 amino acid within the first 100 N-terminal residues and with N_{in}-C_{out} topology (Type II signal) (Y). (3) Unc-SP also comprises proteins with signal anchor (Y) and SP categorised as non-exported (N). (5) From the types of SP are clearly defined. Together with (4) proteins with TMD but no SP (N) and (6) protein substrates of holins, Wss and FEA, (7) the presence of the respective protein secretion systems is checked (Y). (8) When the respective protein secretion system is absent (N) or (9) proteins are not predicted as secreted (N), proteins are considered as cytoproteins and located in the CP. (10) Cytoproteins predicted as exported by NC (Y) are further considered as located extracellularly. (7) Secreted proteins and their respective secretion system are defined from there. (11) Translocated proteins with Unc-SP (Y) are IMPs. (11) Translocated proteins without Unc-SP (N), and (12) with TMD (Y) but no SP (N) are IMPs. (13) Remaining translocated proteins with a cleavable SP (Y) and a single predicted TMD (TMD = 1) (Y) cannot be IMP, and are checked for (16) the presence of a lipobox. (13) Remaining translocated proteins with more than one TMD (N) are checked for (14) the absence of overlap (N) with SP region and LPXTG domain respectively (TMD = 2 AND TMD = LPXTG) to be IMP, otherwise (Y) are checked for (16) the presence of a lipobox. (15) From TMD topology prediction (**Figure 2**), IMPs are further subcategorised and considered as integral to CM. (16) Presence of a lipobox (Y) define lipoproteins anchored to the CM. (17) The presence of glycine residue at position C+2 (C+2 = G) (Y) indicates potential release into the EM [26,48]. (18) Presence of cell-wall retention domains define (19) parietal proteins (CW-protein) that are further subcategorised (**Figure 2**) and considered as located at the CW. (20) Proteins with less tha 2 GW modules is not defined as CW-protein located at the CW [57,58]. (21) Proteins part of S-layer, pilus and cellulosome, as well as (22) pseudopilus and flagellum are defined. (23) Secreted proteins with none of the cell-envelope retention are as exoproteins located in the EM. N: No, Y: Yes.
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the performance to predict the protein SCL, the secretomics-based method and other SCL predictors were tested against a dataset of 337 distinct *L. monocytogenes* proteins, which actual location is defined and referenced (Table S7). SubLoc and LocTree exhibit the lowest overall performance as indicated by MCC values lower than 0.5 (**Table 3**). While they both show quite similar percentages of overall and single accuracy for SCL prediction, they exhibit an imbalance in their specificity with regard to the sensivity for protein prediction into the cytoplasm. With quite similar percentages of overall and single accuracy for SCL prediction, CELLO and Gpos-mPloc have MCC values just above 0.5. They both present an imbalance in their sensivity with regard to the specificity for SCL prediction to the EM and CW, especially CELLO, which appears inappropriate for EM and CW prediction as its sensitivity is null in the latter case.

With correct overall performance, Augur and PSORTb also present an imbalance in their sensivity/specificity for SCL prediction to the EM (**Table 3**). While the Augur database identifies the so-called "cell anchor" responsible for cell surface location [37], it suffers from numerous inaccuracies. The presence of LRR and NLPC/P60 domains are considered as cell anchors, which are not. Other well-known cell-wall binding domains, namely PGBD, CWBD, SLHD or WXL domain, are not taken into account. The absence of SP when a "cell anchor" is present does not underdetermine the surface prediction. From there, considering a "single secretome analysis" is performed, when just identifying all secreted proteins with SP and without "cell anchor", is misleading. In the end, the proportion of genome encoded *L. monocytogenes* proteins, which SCL prediction is in agreement with the secretomics-based method is quite low, *i.e.* 67% (Table S7); with a number of Sec-secreted exoproteins estimated at 112, only 49% are in agreement with our present investigation as the remaining proteins exhibit cell-envelope retention domains. Conscious of the ambiguities in the Gram nomenclature especially in the field of protein secretion [1,2], PSORTb proposes a confusing description to differentiate the analysis for bacteria with Gram positive or negative staining and those "positive with outer membrane" and "negative without outer membrane". In line with a phylum level perspective on bacterial cell envelope architecture, however, the terminology of monoderm and diderm bacteria, which can even be discriminated between diderm-LPS and diderm-mycolate bacteria, is much more appropriate especially in the field of secretomics [1,2,64].

Among tested SCL predictors, SurfG+ and LocateP have the highest overall performances (**Table 3**). At a genome scale level, the agreement in SCL predictions of *L. monocytogenes* proteins between these tools and the secretomics-based approach is quite high, *i.e.* 95% and 92% respectively (Table S7). Back to the performance evaluation metrics, however, LocateP present an imbalance in its sensivity with regard to the specificity for SCL prediction to the EM, as well as to the CW for SurfG+ (**Table 3**). While the attempt by LocateP to differentiate the IMPs between multi-transmembrane, C-terminally and N-terminally anchored proteins with or without cleavable SP can be acknowledged, it also results in misprediction to the cytoplasm for several ssIMP II or III where the TMD is not located in the N-terminal or C-terminal region and then do not stand within the classification above (Table S7). Similarly in SurfG+ the differentiation of IMP topology into Loop-out, Nterm-out or Cterm-out tails does not compile with the standard nomenclature for IMP. Moreover, LocateP only considers LPXTG motif for SCL to the cell wall; as a result several proteins predicted as IMPs or exoproteins in *L. monocytogenes* EGD-e are in fact CW-proteins with LysM, GW, PGBD or WXL domains. Finally, the possibility for a single protein to belong to different protein categories is not fully considered by either of these two tools.

Compared to all other SCL predictors, the secretomics-based methods clearly outperform them both considering single and overall performances with accuracy reaching or close to 100% and MCC reaching or close to 1 for all of the 8 SCLs considered by the approach (**Table 3**). The secretomics-based method is the sole to take into consideration protein cell-surface protein complexes (GO:0043234 AND 0009986), *i.e.* S-layer (GO:0030115), cellulosome (GO:0043263), flagellum (GO:0019861), pseudo-pilus and pilus (GO:0009289). Actually, PSORTb considers flagellar components but it has not been especially designed to identify substrates to the FEA [62]; as a result, it could only find 3 out of the 11 components secreted by FEA in *L. monocytogenes* EGD-e and misleadingly predict them as located into the extracellular milieu (Table S7). Protein substrates of FPE or FEA are systematically mispredicted by other SCL predictors, most often as located in the cytoplasm or release in the extracellular milieu.

As the keystone of the secretomics-based method, none of the SCL predictors consider the secretion pathways, including protein routing, transport mechanisms and post-translocational modifications. In fact, LocateP was the best attempt to mimic secretion process but it is restricted to only 3 protein secretion systems, Sec, Tat and ABC protein exporter, plus NC secretion. Another major difference is that the pipeline misses some treads and does not follow the sequential steps of the biology of protein secretion, *e.g.* TMD prediction is considered before SP, processing of Type 4 prepilin is ruled out, or non-covalent anchoring of proteins to the cell wall is overlooked.

Table 3. Performance evaluation metrics of the secretomics-based methods compared to other SCL predictors.

Tool	Actual location	GO[a]	Performance[b]				
				MCC	Accuracy	Sensitivity	Specificity
Secretomics-based method			Single				
	Extracellular milieu	GO:0005876		0.914	97.8	89.8	99.3
	Cell surface	GO:0009986		0.965	98.2	97.0	100.0
	Cell surface protein complex	GO:0043234 AND 0009986		1.000	100.0	100.0	100.0
	Cell wall	GO:0009275		1.000	100.0	100.0	100.0
	Intrinsic to the CM	GO:0031226		1.000	100.0	100.0	100.0
	Anchored to the CM	GO:0046658		1.000	100.0	100.0	100.0
	Integral to the CM	GO:0005887		1.000	100.0	100.0	100.0
	Cytoplasm	GO:0005737		1.000	100.0	100.0	100.0
			Overall	0.988	99.5	98.5	99.9
SubLoc			Single				
	Extracellular milieu	GO:0005876		0.267	70.0	65.3	70.8
	Cell surface	GO:0009986		n/a	n/a	n/a	n/a
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		n/a	n/a	n/a	n/a
	Intrinsic to the CM	GO:0031226		n/a	n/a	n/a	n/a
	Anchored to the CM	GO:0046658		n/a	n/a	n/a	n/a
	Integral to the CM	GO:0005887		n/a	n/a	n/a	n/a
	Cytoplasm	GO:0005737		0.420	63.5	94.0	48.3
			Overall	0.396	66.7	85.5	60.8
LocTree			Single				
	Extracellular milieu	GO:0005876		0.298	68.5	73.5	67.7
	Cell surface	GO:0009986		n/a	n/a	n/a	n/a
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		n/a	n/a	n/a	n/a
	Intrinsic to the CM	GO:0031226		n/a	n/a	n/a	n/a
	Anchored to the CM	GO:0046658		n/a	n/a	n/a	n/a
	Integral to the CM	GO:0005887		n/a	n/a	n/a	n/a
	Cytoplasm	GO:0005737		0.556	71.5	100.0	57.0
			Overall	0.471	70.0	92.0	63.0
CELLO			Single				
	Extracellular milieu	GO:0005876		0.030	73.8	20.4	82.8
	Cell surface	GO:0009986		n/a	n/a	n/a	n/a
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		n/a	91.2	0.0	100.0
	Intrinsic to the CM	GO:0031226		0.572	78.8	77.7	79.7
	Anchored to the CM	GO:0046658		n/a	n/a	n/a	n/a
	Integral to the CM	GO:0005887		n/a	n/a	n/a	n/a
	Cytoplasm	GO:0005737		0.749	86.8	97.4	81.2
			Overall	0.553	82.6	69.5	87.1
Gpos-mPloc			Single				
	Extracellular milieu	GO:0005876		0.248	73.5	55.1	76.6
	Cell surface	GO:0009986		n/a	n/a	n/a	n/a
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		0.230	89.3	23.3	95.8
	Intrinsic to the CM	GO:0031226		0.627	81.8	73.6	88.0
	Anchored to the CM	GO:0046658		n/a	n/a	n/a	n/a
	Integral to the CM	GO:0005887		n/a	n/a	n/a	n/a

Table 3. Cont.

Tool	Actual location	GO[a]	Performance[b]				
				MCC	Accuracy	Sensitivity	Specificity
	Cytoplasm	GO:0005737		0.745	87.7	91.5	85.9
			Overall	0.571	83.1	72.7	86.6
Augur			Single				
	Extracellular milieu	GO:0005876		0.310	85.0	32.7	93.8
	Cell surface	GO:0009986		0.691	83.5	76.4	93.6
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		n/a	n/a	n/a	n/a
	Intrinsic to the CM	GO:0031226		n/a	n/a	n/a	n/a
	Anchored to the CM	GO:0046658		n/a	n/a	n/a	n/a
	Integral to the CM	GO:0005887		n/a	n/a	n/a	n/a
	Cytoplasm	GO:0005737		n/a	n/a	n/a	n/a
			Overall	0.654	84.3	67.7	93.8
PSORTb			Single				
	Extracellular milieu	GO:0005876		0.280	86.9	20.4	97.4
	Cell surface	GO:0009986		n/a	n/a	n/a	n/a
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		0.730	95.6	76.7	97.4
	Intrinsic to the CM	GO:0031226		0.680	84.1	72.3	93.2
	Anchored to the CM	GO:0046658		n/a	n/a	n/a	n/a
	Integral to the CM	GO:0005887		n/a	n/a	n/a	n/a
	Cytoplasm	GO:0005737		0.831	92.4	88.9	94.2
			Overall	0.714	89.7	70.9	95.9
SurfG+			Single				
	Extracellular milieu	GO:0005876		0.378	87.5	34.7	95.8
	Cell surface	GO:0009986		0.470	70.4	54.8	90.4
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		0.888	98.3	86.7	99.4
	Intrinsic to the CM	GO:0031226		0.803	90.3	90.5	90.1
	Anchored to the CM	GO:0046658		0.802	95.7	91.9	96.2
	Integral to the CM	GO:0005887		0.828	92.4	86.8	95.2
	Cytoplasm	GO:0005737		0.819	90.6	100.0	85.9
			Overall	0.730	89.3	77.2	94.0
LocateP			Single				
	Extracellular milieu	GO:0005876		0.392	88.6	26.5	98.4
	Cell surface	GO:0009986		n/a	n/a	n/a	n/a
	Cell surface protein complex	GO:0043234 AND 0009986		n/a	n/a	n/a	n/a
	Cell wall	GO:0009275		0.866	97.9	76.7	100.0
	Intrinsic to the CM	GO:0031226		0.789	89.2	94.6	85.2
	Anchored to the CM	GO:0046658		0.825	96.0	97.3	95.9
	Integral to the CM	GO:0005887		0.807	91.0	93.0	90.0
	Cytoplasm	GO:0005737		0.814	90.3	100.0	85.5
			Overall	0.790	92.1	87.9	93.4

[a]Subcellular location follow the GO (Gene Ontology) for cellular component.
[b]Performance was evaluated for single and overall SCL predictions for each tools. MCC (Matthews Correlation Coefficient) and other statistical metrics were calculated as described in the Materials & Methods section. Sensitivity, specificity and accuracy are expressed in %. Detailed performance evaluation metrics are provided in Table S7.
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Comparison with experimental proteomic analyses of the extracytoproteome of *L. monocytogenes*

Globally, the SCL from the secretomics-based approach is in good agreement with experimental data as revealed from the analyses of different extracytoplasmic fractions in *L. monocytogenes* EGD-e (Table S8). Information on protein trafficking has been experimentally confirmed for only a few proteins and concerns the (i) secretion of proteins dependent on the SPases I (SipX, SipY or SipZ) or Spase II LspA, (ii) SecA2-dependent protein secretion, (iii) protein anchoring to the CM by the prolipoprotein diacylglyceryltransferase Lgt, (iv) non-covalent anchoring to cell wall *via* GW repeats, or (v) covalent anchoring of proteins to the cell wall *via* sortases, *i.e.* SrtA and SrtB. Protein secretion system in used was demonstrated for none of them.

Concerning exoproteins, 26 were experimentally identified, mostly in culture supernatant but also in multi-fractions for some of them, namely membrane, cell wall and/or cell surface exposed fractions (Table S8). SP cleavage site was confirmed for only 3 of them, the involvement of SPase I SipZ for 2 of them, and the SecA2-dependent secretion for one of them. Most exoproteins experimentally identified are predicted secreted *via* Sec and two of them would be substrates of holin or ABC exporter, respectively.

Among CW-proteins, 35 were experimentally identified, essentially in the cell wall but some of them were also found in the membrane and/or supernatant fractions (Table S8). While the presence in the supernatant of CW-proteins covalently attached to the cell wall can be more surprising than those linked by weak interactions, it must considered that LPXTG-protein SCL is strictly associated with cell-wall biogenesis. Cell wall anchoring *via* sortases, SrtA or SrtB, was confirmed for 15 LPXTG-proteins but SP cleavage site as well as the involvement of SPase I SipZ and/or SipX was confirmed for only 2 CW-proteins, and the SecA2-dependent secretion for one of them.

With 117 IMP experimentally identified and categorized into ssIMP I, ssIMP II, ssIMP III and msIMP (Table S8), 36 lipoproteins essentially originate from membrane fraction (GO:005624). Again, some of them were also found in the cell wall, supernatant and/or cell surface exposed fractions. For IMP, the involvement of SPase I was confirmed for only one of them as well as SecA2-dependent translocation. Interestingly, most lipoproteins identified in the supernatant exhibited a G at C+2. SecA2-dependent secretion was demonstrated for 4 of them and the involvement of SPase II LspA for only 2 of them. SP cleavage site was confirmed for none. Anchoring to the CM by Lgt was actually confirmed for 26 lipoproteins.

With 192 representatives, the cytoproteins represent the largest category of experimentally identified proteins in extracytoplamic fractions of *L. monocytogenes* (Table S8). While only 6 of them could be predicted as secreted *via* NC in the first place, SecA2-dependent translocation was demonstrated for 12 of them. This category stresses the importance to discriminate the GO terms for SCL from the GO terms for the different cell fractions. For example, the presence of ribosomal proteins in membrane fractions is certainly related to SRP (Signal Recognition Particule)-dependent pathway, where ribosomes interact closely with the Sec translocon in the course of co-translational translocation. The SCL of such proteins would then be extrinsic to the CM (GO:0019897) and more precisely on the internal side (GO:0031234), still they would be identified in the membrane fraction (GO:0005624) and even more precisely peripheral to membrane of membrane fraction (GO:0000300) depending on the experimental protocol applied. Besides being secreted in a SecA2-dependent manner, the primary glycolytic enzyme enolase was demonstrated to moonlight when located extracytoplasmically since, together with DnaK, EF-TU and GAPDH, it binds to human plasminogen [44].

Discussion

Compared with other SCL predictors for Gram-positive bacteria available to date, the secretomics-based method clearly outperforms them in term of single and overall performances. Accordingly, (i) protein routing is described and detailed in terms of secretion system, post-translocational modification, cell-envelope retention and/or incorporation to cell-surface supramolecular structures, (ii) the number of distinct SCL, as well as the different protein categories is the most comprehensive for monoderm bacterial cell, (iii) detailed description of protein type and topology is provided, (iv) it unambiguously differentiates the SCL from the protein category, and (v) the possibility for a single protein to belong to different protein categories as well as to have multiple SCL is fully considered. In that sense, the secretomics-based method is much more than a SCL predictor. In *L. monocytogenes*, this approach conveyed a highly significant quantity of new information, *e.g.* i) by considering for the first time ABC transporters in protein secretion, ii) presenting the first ever comprehensive estimate of IMPs with careful concern about their topology (msIMP, ssIMP I, II, or III) and double affiliation (to lipoprotein for instance), iii) reporting cell-surface supramolecular structures (flagellum and pseudo-pilus), iv) correcting the prediction of the number of lipoproteins or v) of non-covalently cell-wall anchored proteins by including PGBD1 and excluding proteins exhibiting only one GW domain. While protein attachment to the lipoteichoic acids of the cell wall is clearly modulated by the number of GW modules, i.e. complete release with one GW module and complete retention with 8 GW modules [57,58], the proportion of retention/release from the bacterial cell for proteins with an in-between number of GW modules remain to be established. As a boundary, this combinatorial approach is based on the biology of protein secretion, which means it relies on the veracity of the knowledge in the field at a given time. This is also the strength of the secretomics-based method as revealed by its performance and its ability to be readily adjusted in light of new findings in the field.

As the keystone of the method, the protein secretion pathways in Gram-positive bacteria are fully taken into consideration and provided for each secreted proteins. Such an in-depth integration of the secretome concept for genomic analysis had never been achieved before. LocateP is the only SCL predictor attempting to reconsider protein secretion systems in monoderm bacteria but it misses some points, *e.g.* non-covalent cell-wall anchoring of secreted proteins, cell-surface supramolecular structures, or alternative secretion systems to Sec, Tat and ABC pathways [38]. The recently developed CoBaltDB, which combines at once prediction outputs from bioinformatic tools related to protein SCL is a valuable tool to get data prior to the secretomics-based analysis following the decision tree here described for monoderm bacteria [65]. While the basic limit of these tools resides in their lack of flexibility as new predictions tools, new basic findings in the field of protein secretion or genomes are made available, the secretomics-based approach can on the contrary be readily implemented within an Excel data table (Table S1). Nonetheless, we are currently working to make also available in the near future an online tool to ease and fasten the use of the secretomics-based method. Though, as for all pipelines combining results of individual prediction tools and developed to date, the secretomics-based method is based on majority-voting scheme for predictions; for example, LocateP applies unique, consensus and/

or unanimously scheme at different stages of the decision trees [38]. Taking lipoprotein prediction tools for instance, they display different overall performances but also distinct precision and recall values [66], which should even be further considered in their single performances for the cleavage site prediction for instance. An awaited development in the field is the attempt to benchmark each individual tool for the subsequent weightings of each prediction.

Besides genomic analysis, this rational strategy is also useful for categorizing, analyzing, and supporting proteomic data. As an example, the prediction of a lipoprotein even by only one tool can be of high relevance if it backups some experimental proteomic data. An interesting aspect is the possibility to generate a database containing mature protein sequences, where any cleavable parts of pre(pro)proteins are removed [43]. The root of proteomics is indeed the protein identification based on expected peptide sizes deduced from *in silico* trypsinization of amino acid sequence. Confrontation of experimental data with the mature database can then allow experimental determination of the cleavage site in SP or sorting signal [43].

The secretomics-based method allows rigorous interpretation of experimental data by clearly differentiating the predicted SCL GO terms from the experimental cell fraction GO terms. While GO terms are available for proteins identified in membrane fractions (GO:0005624, 0000299, 0000300), they are still awaited for cell wall, surface exposed and supernatant fractions. This further pinpointed some limitations for SCL prediction. Despite very recent improvement in SignalP for example [67], straight prediction in bacteria of uncleaved SP serving as a signal anchor are still unavailable and thus requires intricate analyses (**Figure 3**). Due to limited experimental data on the IWZ (inner wall zone) [68], for which no GO term is as yet available, prediction of IWZ protein is not reliable. No bioinformatic tools have been developed to predict secreted peripheral proteins (GO:0019897, 0010339). Similarly, no SCL prediction into the cytosol of a host cell for substrates to cytolysin-meditated translocation could be performed.

By considering all molecular mechanisms known to date, the present secretomics-based analysis constitutes the ultimate step in mimicking the protein secretion steps for prediction of the final SCL. Contrary to all other SCL predictors, it does not only supply a static snapshot of protein SCL but also offers the full picture of the secretion process dynamics. Such insight provides strong basis to generate a wealth of *in silico* hypotheses that further fuel experimental work, *e.g.* demonstrating the secretion of a protein *via* a specific secretion system, its post-translational modification by specific sorting pathway, or the biological significance of peripheral proteins. Hopefully, this analytical strategy should be inspirational for the development of rational secretomics-based approaches dedicated to diderm-LPS (Gram-negative) bacteria. As a leitmotif in the field of bacterial protein secretion, the reason for the presence of primarily cytoproteins in the supernatant is an important issue. Besides autolysis, several alternative hypotheses can be formulated (i) allolysis, (ii) uncovered secretion systems, (iii) piggybacking, or (iv) leakage [69]. As exemplified with *L. monocytogenes*, the secretomics-based method provides the most updated and comprehensive genomic analysis of the extracytoproteome in a monoderm bacterium and should promote further experimental testing based on generated *in silico* hypotheses.

Materials and Methods

Bioinformatic analyses were performed from web-based servers or under Linux environment with Sun Grid Engine (SGE) server hosted at INRA MIGALE Bioinformatics Platform from (INRA, Jouy-en-Josas, France) with a Intel Quad Core W3520 2.67 GHz. The cluster is organized around 4 computing groups, (i) AMD Quad Core 8354 2.8 GHz, (ii) Intel Quad Core E5520 2.27 GHz, (iii) Intel Quad Core 5340 2.33 GHz, and (iv) Intel Dual Core 5140 2.33 GHz. The complete genome, coding sequences (CDS) and annotation files for *L. monocytogenes* EGD-e were downloaded from GenBank (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/Listeria_monocytogenes/).

Searches against various databases were performed using different tools, namely RPS-BLAST v2.2.19 (Reverse Position-Specific BLAST) [70], HMMER v2.3.2 for hidden Markov models (HMM) [71], InterProScan v4.3 [72], or ScanProsite v1.0 [73]. Interrogated databases included InterPro (IPR) v32.0 [74], Pfam (PF) v24.0 [75], SMART (SM) v6.1 [76], TIGRfam (TIGR) v10.1 [77], SuperFamily (SSF) SCOP v1.73 [78,79], PIRSF v2.74 [80], PRK v3.0 [81], COG v1.0 [82] and Prosite (PS) v20.7 [83]. Position-specific iterated BLAST (PSI-BLAST v2.2.25) [70] searches were executed against UniProtKB v2011_07 [84] until convergence with unlimited number of database sequences for matrix building.

Identification of N-terminal signal peptide

N-terminal signal peptide (SP) were predicted combining results from (i) SignalP v2.0 and v3.0 using both neural network (NN) and hidden Markov model (HMM) [85] with truncation set either at 35, 70, 140 or disabled and predictions with best scores and propensity were considered, (ii) PrediSi v1.0 [86], (iii) Phobius v1.0 [87], (iv) SOSUIsignal v1.0 [88], (v) Signal-3L v1.0 [89], (vi) SIG-PRED v1.0, (vii) Signal-CF v1.0 [90], (vii) SPScan v1.0, an implementation of von Heijne's weight matrix approach with McGeoch criteria where prompted parameters and optional parameter -ADJustscores were used and predictions with best scores and propensity were considered [91,92], (viii) SPOctopus v1.0 [93], (ix) RPSP v1.0 [94], and (x) Signal-BLAST v1.0 [95]. Tat SP prediction was performed from (i) TatP v1.0 [96], (ii) TATFIND v1.4 [97], (iii) PRED-TAT v1.0 [98], and (iv) InterPro (IPR006311, IPR019546, PS51318, PF10518, TIGR01409). Whenever possible, all these previously cited tools were trained on prokaryotes, bacteria or Gram-positive bacteria. Pseudopilin-like SP were searched from PilFind v1.0 and with ScanProsite syntax [73] for consensus motif [AG]-F-[TSI]-[LTY]-x-[EAF] located between the N- and H-domains of the SP [4]. SP for protein substrates of ABC transporters were identified using BAGEL v1.0 [99]. Uncleaved signal peptides, *i.e.* N-terminal signal anchors, were predicted from SignalP using HMM trained on eukaryotes [85]. Identification of proteins with no signal peptide and secreted *via* alternative systems was based on genomic proximity with the genes encoding the respective secretion pathway as described below, coupled to similarity searches, namely substrates to holin and Wss (IPR018921, IPR010310, TIGR03930, PF10663, PF06013). Prediction of non-classical secreted proteins, *i.e.* lacking a signal peptide and translocated *via* unknown secretion system, was performed from (i) SecretomeP v2.0 [100], (2) SecretP v2.0 [101] and (3) NClassG+ v1.0 [102] trained on Gram-positive bacteria.

Identification of protein secretion systems

Protein secretion systems were identification performing BLAST search against TC-DB v2011_07 [103], namely Sec (TC#3.A.5), Tat (TC#2.A.64), ABC (TC#3.A.1), FPE (TC#3.A.14), FEA (TC#3.A.6.2), holins (TC#1.E), and Wss (TC#9.A.44) pathways. To discriminate ABC transporters involved in protein secretion, results were compiled with BAGEL [99]. Results were refined by similarity searches, also to identify

signal peptidases of Type I (SPase I, IPR000223, COG0681, PRK10861, TIGR02227, SSF51306), SPaseII (COG0597, PRK00376, PRK01574, IPR001872, TIGR00077, PF01252) and prepilin peptidase (COG1989, IPR000045, PF01478), the lipoprotein maturation pathway for some Sec- or Tat substrates, namely in addition to SPase II, the prolipoprotein diacylglyceryltransferase Lgt (COG0682, PRK00052, PRK12437, PRK13108, IPR001640, TIGR00544, PF01790) and the apolipoprotein N-acyltransferase (Lnt) (COG0815, IPR004563, TIGR00546) and the covalent cell-wall anchoring sortase (COG3764, IPR005754, IPR009835, IPR015986, IPR23365, IPR022445, TIGR01076, TIGR03064, TIGR03784, SSF63817, PF04203, PF07170, PIRSF029877, PIRSF030150) pathway for some Sec-substrates.

Identification of transmembrane hydrophobic domains and lipoproteins

Transmembrane hydrophobic α-helice domain (TMD) were predicted combining (i) TMHMM v2.0 [30], (ii) SVMtm v1.0 [104] (iii) THUMBUP v1.0 [105], (iv) SOSUI v1.10 [106], (v) HMMTOP v2.0 [107], (vi) PHDhtm v8.94_69 [108], (vii) UMDHMMTMHP v1.0 [105], (vii) MEMSAT v3.0 [109], (ix) MemBrain v1.0 [110], (x) OCTOPUS v1.0 [111], (xi) MINNOU v1.0 [112], (xii) Philius v1.0 [113], (xiii) SCAMPI v1.0 [114], (xiv) TMPred v1.0 [115], (xv) SPLIT v4.0 [116], and (xvi) TOPCONS v1.0 [117].

For the identification of lipobox, sequences were submitted to (i) DOLOP v2.0 [118], (ii) LipoP v1.0 [29], (iii) SPEPLip v1.0 [119], (iv) LipPred v1.0 [120], (v) ScanProsite for a scan with both PS51257 profile and G+LPP v2.0 pattern [121], (vi) LIPO [122], and (vii) PRED-LIPO [123].

Identification of cell-wall attachment domains

Besides similarity search from InterPro (IPR001899, IPR017502, IPR019931, IPR017503, IPR019948, TIGR01167, TIGR03063, TIGR03065, PF00746, PS50847), LPXTG domain were specifically identified by LPXTG-HMM profile [54] and CW-PRED v2.0 [31]. The concomitant presence of YSIRK domain (IPR005877, PF04650, TIGR01168) within Sec-SP was also checked [55,56]. WXL domain were found scanning for the motif [LI]-[TE]-W-[TS]-L with ScanProsite where C-terminal location of the motif was also taken into account [124]. Similarity searches were performed for other cell-wall binding motifs, namely (i) LysM (IPR018392, IPR002482, PF01476, SM00257, SSF54106), (ii) GW (SSF82057), (iii) choline-binding domain, also called cell-wall binding domain of type 1 (CWBD1; IPR018337, PF01473, PS51170, SSF69360), (iv) CWBD2 (IPR007253, PF04122), (v) peptidoglycan-binding domain of type 1 (PGBD1; IPR002477, PF01471, SSF47090), (vi) PGBD2 (IPR014927, PF08823), (vii) PGBD3 (IPR018537, PF09374, SSF53955), PGBD4 (IPR022029, PF12229, SSF143985), and S-layer homology domain (SLHD; IPR001119, PF00395, PS51272).

Identification of cell-surface supramolecular structure

Besides the identification of pseudo-pilus components based on the identification of proteins with pseudopilin-like signal peptides as described above, pilus in Gram-positive bacteria was identified from LOCP [125]. Identification of proteins substrates to FEA followed similarity search (IPR001029, IPR00149, IPR010809, IPR003481, IPR013384, IPR002371, IPR020013, IPR005648, IPR021136, IPR001444, IPR006300, IPR006299, IPR001624, COG1345, COG1334, COG1344, COG1256, COG1749, COG1843, COG4786, COG4787, COG1815, COG1558, COG1677, SSF64518, SSF117143), as well as proteins substrates to Wss (IPR018921, IPR010310, TIGR03930, PF10663, PF06013). Identification of cellulosome components was based on dockerin/cohesin domains (IPR002102, IPR018452, IPR016134, IPR002105, IPR018242, IPR009034, IPR002883) and for S-layer on SLHD (IPR001119, COG1361) coupled to PSI-BLAST search.

The secretomics-based method

The method is truly based on the biology of protein secretion in monoderm bacterium (**Figure 1**). The different types of N-terminal SP targeting a protein to the membrane are discriminated into SP specific for Sec, Tat, ABC and FPE, as well as uncleaved SP (Unc-SP). The protein secretion systems considered are Sec, Tat, ABC, holins, Wss, FPE, FEA, and non-classical secretion (NC). Both transmembrane domain (TMD), including uncleaved SP, or post-translational modifications, *i.e.* lipobox, are considered for membrane retention. Domains (PGBD, CWBD, WXL, LysM, SLHD, GW) or post-translational modification (LPXTG) are considered for cell-wall retention. Besides flagellum and pseudo-pilus secreted *via* the FPE and FEA, S-layer, pilus and cellulosome are also considered as cell-surface supramolecular structures.

The coding sequences (CDS) are screened simultaneously following a flowchart (**Figure 2**), which provides results from the prediction tools described above and organizes to define the different databases as exemplified in Table S1, namely (i) signal peptide (SP), (ii) type of SP, (iv) protein secretion systems, (v) export (proteins lacking a SP), (iv) transmembrane domain (TMD), and (iv) relevant conserved motifs. Results of different tools for similar prediction as combined into a majority vote approach, *i.e.* SP, FPE-SP, Tat-SP, ABC-SP, Lipobox, Export and TMD. Using specific tools, proteins exhibiting SP specific for Tat, FPE and ABC transporters are defined as well as proteins with signal anchor, *i.e.* uncleaved SP, or lipobox in SP of Type II (SP II); combined with the 14 distinct predictions resulting from 11 tools, proteins exhibiting a N-terminal SP are delineated after checking the compatibility with functional annotation verified by similarity search with InterProScan and/or PSI-BLAST. Besides secretion systems, substrates of FEA, holin and Wss are identified following BLAST search against TC-DB. Exported proteins are identified using specific tools for NC. Prediction of proteins with TMD combined 16 tools, 12 of them provide further information on protein topology. Topological information allows to categorised proteins into msIMP, ssIMP I, ssIMP II or ssIMP III. Proteins with a lipobox in SP II are extracted from 8 distinct predictions. Finally, identification of conserved motifs for cell wall anchoring or surface appendages results from interrogation of 10 distinct databases. Parietal proteins are discriminated between LPXTG-, WXL-, SLHD-, LysM-, GW-, CWBD1/2- and/or PGBD1/2/3/4-protein.

From there and as a key step of the the secretomics-based method, the resulting databases are analysed as depicted in the detailed decision trees (**Figure 3**). As a result of the decision trees, proteins are discriminated into 10 distinct primary categories; IMPs can be further discriminated into multi-spanning IMP (msIMP), single-spanning IMP of Type I (ssIMP I), II (ssIMP II) or III (ssIMP III), whereas parietal protein (CW-protein) can be dicrimnated into 12 subtypes (LPXTG-protein, LysM protein, CWBD1-protein,...*etc*). In agreement with GO terms, the secretomics-based method provides 4 primary SCL, CP (GO:0005737), CM (GO:0005886), CW (GO:0009275) and EM (GO:0005576); protein complex (GO:0043234), cell surface (GO:0009986), integral (GO:0005887) and anchored to CM (GO:0046658) SCL are further discriminated.

Subcellular localization predictors

Predictions for SCL were performed from pipelines, *i.e.* Augur v2010_07 [37], LocateP v1.0 [38] and SurfG+ [39], as well as support vector machines (SVMs), *i.e.* SubLoc v1.0 [63] and LocTree v1.0 [33] trained on prokaryotes where only extracellular and cytoplasmic SCL are considered, whereas CELLO v2.5 [34], PSORTb v3.0.2 [62] and Gpos-mPLoc v1.0 [35] trained on Gram-positive bacteria considered extracellular, cell wall, membrane and cytoplasmic subcellular compartments as well as multiple localization sites.

Performance evaluation metrics

Sensitivity, specificity, accuracy and MCC (Matthews Correlation Coefficient) of the SCL methods were calculated from the four basic statistics, *i.e.* true positives (TP), false negatives (FN), false positives (FP) and true negatives (TN) [126]. Sensitivity was calculated as TP/(TP+FN), specificity as TN/(TN+FP), accuracy as (TP+TN)/(TP+FN+FP+TN), and MCC as $[(TP\times TN)-(FN\times FP)]/\sqrt{[(TP+FN)\times(TP+FP)\times(TN+FP)\times(TN+FN)]}$. Sensitivity, specificity and accuracy were expressed as percentage, whereas MMC values can vary between −1 and 1. Taking the extracellular milieu (EM) as an example of SCL prediction, (i) TP corresponds to instances both predicted and actually located in the EM, (ii) FN to instances actually located in the EM but not predicted in the EM, (iii) FP to instances predicted in the EM but actually not located in the EM, and (iv) TN to instances both predicted and actually not located in the EM. Besides the overall performance of a given tool, single performances were evaluated for each of the different SCL considered. A dataset was constructed from *L. monocytogenes* proteins, which location was experimentally confirmed and/or acknowledged from literature survey (Table S7). It consisted of 337 distinct proteins localized in the (i) extracellular milieu (EM; 49 proteins), (ii) cell surface (CS; 199 proteins), (iii) protein complex (PC; 16 proteins), (iv) cell wall (CW; 30 proteins), (v) intrinsic to the cytoplasmic membrane (CM; 148 proteins), (vi) anchored to the CM (aCM; 37 proteins), (vii) integral to the CM (iCM; 114 proteins), and/or (viii) cytoplasm (CP; 117 proteins) (Table S7).

Supporting Information

Table S1 Complete results from secretomics-based genomic analysis allowing prediction of protein types and SCL in *L. monocytogenes* EGD-e.
(XLSX)

Table S2 Summarised information about protein categories, secretion pathways and GO terms for IMPs, lipoproteins, cell-wall proteins, subunits of supramolecular cell-surface appendages and exoproteins, respectively, as predicted by the secretomics-based method in *L. monocytogenes* EGD-e.
(PDF)

Table S3 Summarised information about protein categories, secretion pathways and GO terms for IMPs, lipoproteins, cell-wall proteins, subunits of supramolecular cell-surface appendages and exoproteins, respectively, as predicted by the secretomics-based method in *L. monocytogenes* EGD-e.
(PDF)

Table S4 Summarised information about protein categories, secretion pathways and GO terms for IMPs, lipoproteins, cell-wall proteins, subunits of supramolecular cell-surface appendages and exoproteins, respectively, as predicted by the secretomics-based method in *L. monocytogenes* EGD-e.
(PDF)

Table S5 Summarised information about protein categories, secretion pathways and GO terms for IMPs, lipoproteins, cell-wall proteins, subunits of supramolecular cell-surface appendages and exoproteins, respectively, as predicted by the secretomics-based method in *L. monocytogenes* EGD-e.
(PDF)

Table S6 Summarised information about protein categories, secretion pathways and GO terms for IMPs, lipoproteins, cell-wall proteins, subunits of supramolecular cell-surface appendages and exoproteins, respectively, as predicted by the secretomics-based method in *L. monocytogenes* EGD-e.
(PDF)

Table S7 Detailed performance evaluation metrics and comparison of prediction results in *L. monocytogenes* EGD-e between secretomics-based method and available SCL predictors.
(XLSX)

Table S8 Secretomic analysis of extracytoplasmic proteins experimentally identified in *L. monocytogenes* EGD-e.
(PDF)

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Author Contributions

Conceived and designed the experiments: SR MD. Performed the experiments: SR PM MD. Analyzed the data: SR RT MH MD. Contributed reagents/materials/analysis tools: SR PM MD. Wrote the paper: SR RT MH MD.

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Table 2S: **Integral membrane proteins (IMPs) in *L. monocytogenes* EGD-e as revealed by the secretomics-based method.**

Protein ID	Annotation[a]	Protein Type[b]	Secretion pathway	GO[c]
Single-spanning integral membrane proteins				
Lmo0058	WXG100 protein secretion system, membrane component, EssA	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0082	Protein of unknown function	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0204	Actin-assembly inducing protein, ActA	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0528	Protein of unknown function, COG5298	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0530	Protein of unknown function	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0552	Protein of unknown function, DUF916 domain, CscA-like	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0586	Protein of unknown function, DUF916 domain, CscA-like	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0701	Protein of unknown function	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1021	Signal transduction histidine kinase, LiaS	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1205	Cobalt transport protein, CbiN	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1378	Signal transduction histidine kinase, LisK	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1461	Protein of unknown function	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1610	Protein of unknown function	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2061	Protein of unknown function with GLE1 domain, COG4549	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2410	Membrane protein of unknown function	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2451	Sec translocon, subunit SecG	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2534	F_0F_1 ATP synthase, subunit C	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2706	Protein of unknown function	ssIMP I	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0821	Lipoprotein of unknown function	ssIMP I-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo2793	Lipoprotein of unknown function	ssIMP I-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo0060	WXG100 protein secretion system, membrane component, YukC (EssB)	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0066	Protein of unknown function, COG5444	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0292	Trypsin-like serine protease with C-terminal PDZ/DHR/GLGF domain	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0383	Methylmalonate-semialdehyde dehydrogenase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0502	Sugar phosphate isomerase involved in capsule formation	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0702	Protein of unknown function	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0763	Phosphohydrolases	ssIMP II	YidC	0031226, 0005887, 0009986

Lmo0890	Sulphate transporter/antisigma-factor antagonist STAS	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0974	D-alanine--D-alanyl carrier protein ligase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1075	Teichoic acids export protein ATP-binding subunit	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1097	Protein of unknown function	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1106	ATPase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1194	Cobalamin (vitamin B12) biosynthesis CbiD	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1198	Cobalamin (vitamin B12) biosynthesis CbiG	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1374	Catalytic domain of dehydrogenase complex,biotin/lipoyl attachment	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1395	DNA-binding λ repressor-like, COG1426	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1414	Thiolase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1433	Pyruvate/oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1552	Valyl-tRNA synthetase, class Ia	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1663	Asparagine synthase, glutamine-hydrolyzing	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1820	Serine/threonine protein kinase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo1927	3-dehydroquinate synthase, AroB	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo2202	β-ketoacyl-acyl carrier protein synthase III, FabH	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo2390	Thioredoxin reductase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo2757	DNA helicase, ATP-dependent, RecQ type	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo2769	ABC-type multidrug transport system	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo2833	Maltose phosphorylase/glycosyl hydrolase/vacuolar acid trehalase	ssIMP II	YidC	0031226, 0005887, 0009986
Lmo0039	Carbamate kinase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0052	Signal transduction phosphoesterase YybT type with GDEF and DHH domains	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0089	F_0F_1-type ATP synthase, δ subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0104	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0186	Protein of unknown function with 3D and G5 domains	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0193	Macrolide transporter subunit MacA	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0217	Septum formation initiator	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0245	Sec translocon, subunit SecE	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0289	Signal transduction YycH protein	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0290	Protein of unknown function, COG4853	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0299	Phosphotransferase system cellobiose-specific component IIB	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0353	Acyl-CoA N-acyltransferase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo0354	Acyl-coenzyme A synthetase/AMP-(fatty) acid ligase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0362	Twin-arginine translocase protein A, TatA	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0376	Transcriptional regulator	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0392	Protein of unknown function with DUF1432, COG4864	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0393	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0399	Phosphotransferase system, fructose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0427	Phosphotransferase system, fructose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0441	Cell division protein FtsI/Penicillin-binding protein 2, transpeptidase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0442	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0443	Transcriptional regulator	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0465	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0477	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0478	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0479	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0481	Streptococcal 67 kDa myosin-cross-reactive antigen like	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0592	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0595	O-acetylhomoserine/O-acetylserine sulfhydrylase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0628	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0661	Carboxymuconolactone decarboxylase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0675	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0686	Flagellar motor protein MotB	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0746	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0810	Spermidine/putrescine ABC transporter, substrate-binding protein	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0914	Phosphotransferase system, lactose/cellobiose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0926	Transcriptional regulator	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0929	Sortase A, SrtA	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0951	Protein of unknown function with α/β-hydrolase fold and DUF1801, COG4814	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0962	Listeria epitope, LemA	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0971	D-alanine esterification of (lipo-)teichoic acid protein, DltD	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1026	Transcriptional regulator	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1049	Thiamine/molybdopterin biosynthesis ThiF/MoeB-like protein	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1095	Phosphotransferase system, lactose/cellobiose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo1103	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1128	Lysophospholipase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1215	Muramidase flagellum-specific with a single GW domain, FlgJ-type	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1269	Signal peptidase of Type I, SipX	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1270	Signal peptidase of Type I, SipY	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1271	Signal peptidase of Type I, SipZ	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1380	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1399	HD superfamily hydrolase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1484	DNA uptake competence protein, ComEA	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1495	Protein of unknown function with DUF1510 domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1499	Aminodeoxychorismate lyase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1511	Lysophospholipase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1529	Sec transcolon, subunit YajC	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1547	Cell shape-determining protein, MreC	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1715	S-adenosyl-L-methionine-dependent methyltransferase type 11	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1720	Phosphotransferase system, lactose/cellobiose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1813	Iron-sulphur-dependent L-serine dehydratase beta subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1861	Protein of unknown function, COG4698	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1892	Membrane carboxypeptidase (penicillin-binding protein), PbpA	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1898	Protein of unknown function, COG5353	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1924	Prephenate dehydrogenase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2034	Cell division septal protein, FtsQ	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2036	UDP-N-acetylmuramoylalanine-D-glutamate ligase, MurD	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2040	Cell division protein, FtsL -like	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2051	Protein of unknown function with PDZ domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2056	Ca^{2+} chelating serine protease with SCP/PR1 domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2089	Esterase/lipase	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2181	Sortase B, SrtB	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2229	Carboxypeptidase (penicillin-binding protein)	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2257	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2258	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2280	Protein of unknown function, COG1422	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo2373	Phosphotransferase system, lactose/cellobiose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2420	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2442	Protein of unknown function, DUF218 domain, COG1434	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2485	Stress-responsive transcriptional regulator	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2486	Protein of unknown function, UCP012569 type, COG3595	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2518	Transcriptional regulator	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2680	K+ transporting ATPase, C subunit, KdpC	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2683	Phosphotransferase system, lactose/cellobiose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2710	Protein of unknown function	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2762	Phosphotransferase system, lactose/cellobiose-specific IIB subunit	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2805	Protein of unknown function with DUF1310 domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2806	Protein of unknown function with DUF1310 domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2807	Protein of unknown function with DUF1310 domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2808	Protein of unknown function with DUF1310 domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2809	Protein of unknown function with DUF1310 domain	ssIMP II, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1303	Cell division suppressor protein, YneA, with LysM domain	ssIMP II-LysM-protein, Unc-SP	Sec, YidC	0031226, 0005887, 0009275, 0009986
Lmo1941	Protein of unknown function, YpbE-like, LysM and ATPase domains	ssIMP II-LysM-protein, Unc-SP	Sec, YidC	0031226, 0005887, 0009275, 0009986
Lmo0069	Protein of unknown function	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo0504	Protein of unknown function	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo0545	Transcriptional activator	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo0825	Hydroxymethylglutaryl-CoA reductase	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1088	Glycosyl/glycerophosphate transferases involved in teichoic acid biosynthesis, TagB	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1238	Ribonuclease PH	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1306	Protein of unknown function, COG3763	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1351	Rhodanese-related sulfurtransferase	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1412	Topology modulation protein	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1538	Glycerol kinase	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1594	Negative regulator of septation ring formation	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo1885	Xanthine phosphoribosyltransferase	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo2302	Protein of unknown function	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo2325	Protein of unknown function	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo2533	F_0F_1 ATP synthase, subunit B	ssIMP III	YidC	0031226, 0005887, 0009986

Lmo2664	Threonine dehydrogenase, Zn-dependent dehydrogenase	ssIMP III	YidC	0031226, 0005887, 0009986
Lmo2836	Threonine dehydrogenase, Zn-dependent dehydrogenase	ssIMP III	YidC	0031226, 0005887, 0009986
Multi-spanning integral membrane proteins				
Lmo0014	Cytochrome AA3-600 quinol oxidase, subunit I	msIMP	YidC	0031226, 0005887, 0009986
Lmo0024	Phosphotransferase system, mannose/fructose/sorbose/N-acetylgalactosamine IID	msIMP	YidC	0031226, 0005887, 0009986
Lmo0027	Phosphotransferase system, β-glucoside-specific IIABC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo0048	Post-translational modification protein of the autoinducing quorum-sensing peptide	msIMP	YidC	0031226, 0005887, 0009986
Lmo0061	WXG100 protein secretion system, ATPase component, YukAB (EssC)	msIMP	YidC	0031226, 0005887, 0009986
Lmo0098	Phosphotransferase system, mannose/fructose/sorbose family IID component	msIMP	YidC	0031226, 0005887, 0009986
Lmo0121	Phage-related protein	msIMP	YidC	0031226, 0005887, 0009986
Lmo0155	ABC-type Mn2+/Zn2+ transport system, permease component	msIMP	YidC	0031226, 0005887, 0009986
Lmo0176	Sugar uptake permease	msIMP	YidC	0031226, 0005887, 0009986
Lmo0321	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo0332	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo0349	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo0358	Phosphotransferase system, fructose IIC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo0405	Phosphate transporter	msIMP	YidC	0031226, 0005887, 0009986
Lmo0424	Sugar transport	msIMP	YidC	0031226, 0005887, 0009986
Lmo0459	M trans-acting positive regulator	msIMP	YidC	0031226, 0005887, 0009986
Lmo0508	Phosphotransferase system, galactitol-specific IIC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo0518	Protein of unknown function with DUF1304, COG3759	msIMP	YidC	0031226, 0005887, 0009986
Lmo0529	Glycosyltransferases, probably involved in cell wall biogenesis	msIMP	YidC	0031226, 0005887, 0009986
Lmo0543	Phosphotransferase system sorbitol-specific component IIBC	msIMP	YidC	0031226, 0005887, 0009986
Lmo0559	Mg2+ and Co2+ transporters	msIMP	YidC	0031226, 0005887, 0009986
Lmo0577	Protein of unknown function, COG3610	msIMP	YidC	0031226, 0005887, 0009986
Lmo0578	Threonine/serine exporter family, ThrE	msIMP	YidC	0031226, 0005887, 0009986
Lmo0589	Protein of unknown function, COG4852	msIMP	YidC	0031226, 0005887, 0009986
Lmo0604	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo0618	Protein kinase-like	msIMP	YidC	0031226, 0005887, 0009986
Lmo0647	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo0648	Mg2+ and Co2+ transporters	msIMP	YidC	0031226, 0005887, 0009986
Lmo0722	Pyruvate oxidase	msIMP	YidC	0031226, 0005887, 0009986

Lmo0733	Lambda repressor-like, DNA-binding	msIMP	YidC	0031226, 0005887, 0009986
Lmo0738	β-glucoside-specific PTS system components IIABC	msIMP	YidC	0031226, 0005887, 0009986
Lmo0744	ABC-type antimicrobial peptide transport system, ATPase component	msIMP	YidC	0031226, 0005887, 0009986
Lmo0779	Protein of unknown function with DUF986 domain, COG4811	msIMP	YidC	0031226, 0005887, 0009986
Lmo0781	Phosphotransferase system, mannose/fructose/sorbose family IID component	msIMP	YidC	0031226, 0005887, 0009986
Lmo0793	Protein of unknown function with DUF554 domain, COG1811	msIMP	YidC	0031226, 0005887, 0009986
Lmo0803	Na+/H+ antiporter	msIMP	YidC	0031226, 0005887, 0009986
Lmo0826	Na+/phosphate symporter	msIMP	YidC	0031226, 0005887, 0009986
Lmo0867	Protein of unknown function with DUF988 domain, COG4708	msIMP	YidC	0031226, 0005887, 0009986
Lmo0910	Protein of unknown function, UCP032908-like, COG4194	msIMP	YidC	0031226, 0005887, 0009986
Lmo0933	Undecaprenyl phosphate 4-deoxy-4-formamido-L-arabinose transferase	msIMP	YidC	0031226, 0005887, 0009986
Lmo0992	Tellurium resistance membrane protein, TerC	msIMP	YidC	0031226, 0005887, 0009986
Lmo0993	Cation transporter	msIMP	YidC	0031226, 0005887, 0009986
Lmo1013	Mechanosensitive ion channel MscS	msIMP	YidC	0031226, 0005887, 0009986
Lmo1015	ABC-type proline/glycine betaine transport system, permease component, GbuB	msIMP	YidC	0031226, 0005887, 0009986
Lmo1035	Phosphotransferase system, beta-glucoside-specific IIABC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo1064	Mg2+ and Co2+ transporters, CorA-like	msIMP	YidC	0031226, 0005887, 0009986
Lmo1167	Aquaporin, glycerol uptake facilitator, GlpF	msIMP	YidC	0031226, 0005887, 0009986
Lmo1214	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo1230	Colicin V production protein	msIMP	YidC	0031226, 0005887, 0009986
Lmo1255	Phosphotransferase system, trehalose-specific IIBC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo1261	Protein of unknown function with GYF domain	msIMP	YidC	0031226, 0005887, 0009986
Lmo1284	Protein of unknown function with DUF205 domain, COG0344	msIMP	YidC	0031226, 0005887, 0009986
Lmo1318	Peptidase M50, putative membrane-associated zinc metallopeptidase	msIMP	YidC	0031226, 0005887, 0009986
Lmo1337	Peptidase S54, rhomboid	msIMP	YidC	0031226, 0005887, 0009986
Lmo1346	Fimbrilin-protein exporter, membrane component, ComGB	msIMP	YidC	0031226, 0005887, 0009986
Lmo1353	Protein of unknown function with DUF1385 domain, COG3872	msIMP	YidC	0031226, 0005887, 0009986
Lmo1422	ABC-type proline/glycine betaine system, oxgall and bile exclusion system, BilEB	msIMP	YidC	0031226, 0005887, 0009986
Lmo1425	ABC-type proline/glycine betaine transport systems, permease component, OpuCD	msIMP	YidC	0031226, 0005887, 0009986
Lmo1427	ABC-type proline/glycine betaine transport systems, permease component, OpuCB	msIMP	YidC	0031226, 0005887, 0009986
Lmo1443	Branched-chain amino acid transporter	msIMP	YidC	0031226, 0005887, 0009986
Lmo1446	ABC-type Mn2+/Zn2+ transport systems, permease component, ZurM	msIMP	YidC	0031226, 0005887, 0009986

Lmo1482	Competence protein ComEC/Rec2	msIMP	YidC	0031226, 0005887, 0009986
Lmo1500	Selenite transport protein, SNARE-associated protein	msIMP	YidC	0031226, 0005887, 0009986
Lmo1516	Ammonium permease	msIMP	YidC	0031226, 0005887, 0009986
Lmo1539	Major intrinsic protein	msIMP	YidC	0031226, 0005887, 0009986
Lmo1650	Protein of unknown function with DUF1453 domain, COG4846	msIMP	YidC	0031226, 0005887, 0009986
Lmo1665	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo1740	ABC-type amino acid transport system, permease component	msIMP	YidC	0031226, 0005887, 0009986
Lmo1776	Protein of unknown function, COG4843	msIMP	YidC	0031226, 0005887, 0009986
Lmo1812	L-serine dehydratase, alpha subunit	msIMP	YidC	0031226, 0005887, 0009986
Lmo1844	Signal peptidase of Type II, lipoprotein signal peptidase, LspA	msIMP	YidC	0031226, 0005887, 0009986
Lmo1853	Heavy metal translocating P-type ATPase	msIMP	YidC	0031226, 0005887, 0009986
Lmo1911	Protein with GGDEF domain	msIMP	YidC	0031226, 0005887, 0009986
Lmo1912	Protein with GGDEF domain	msIMP	YidC	0031226, 0005887, 0009986
Lmo1919	Zinc metallopeptidase	msIMP	YidC	0031226, 0005887, 0009986
Lmo1971	Sugar-specific PTS, UlaA	msIMP	YidC	0031226, 0005887, 0009986
Lmo1999	Glucosamine 6-phosphate synthetase, amidotransferase and phosphosugar isomerase	msIMP	YidC	0031226, 0005887, 0009986
Lmo2000	Phosphotransferase system, mannose/fructose/sorbose family IID component	msIMP	YidC	0031226, 0005887, 0009986
Lmo2029	Protein of unknown function, YGGT-like, COG0762	msIMP	YidC	0031226, 0005887, 0009986
Lmo2037	UDP-N- phosphotransferase, MraY	msIMP	YidC	0031226, 0005887, 0009986
Lmo2075	O-sialoglycoprotein endopeptidase	msIMP	YidC	0031226, 0005887, 0009986
Lmo2081	Camphor resistance CrcB protein	msIMP	YidC	0031226, 0005887, 0009986
Lmo2096	Phosphotransferase system, galactitol-specific IIC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo2105	Fe2+ transport system protein B	msIMP	YidC	0031226, 0005887, 0009986
Lmo2120	Protein of unknown function with DUF147 domain, CHP00159-like, COG1624	msIMP	YidC	0031226, 0005887, 0009986
Lmo2170	2-nitropropane dioxygenase, NPD	msIMP	YidC	0031226, 0005887, 0009986
Lmo2174	Protein with GGDEF domain	msIMP	YidC	0031226, 0005887, 0009986
Lmo2221	Protein of unknown function, P-loop nucleoside triphosphate hydrolases, COG4717	msIMP	YidC	0031226, 0005887, 0009986
Lmo2224	Protein of unknown function with DUF445 domain, UCP032178-like, COG4399	msIMP	YidC	0031226, 0005887, 0009986
Lmo2287	Tape measure domain bacteriophage A118	msIMP	YidC	0031226, 0005887, 0009986
Lmo2335	2-O-a-mannosyl-D-glycerate specific PTS transporten components IIABC	msIMP	YidC	0031226, 0005887, 0009986
Lmo2347	ABC-type amino acid transport system, permease component	msIMP	YidC	0031226, 0005887, 0009986
Lmo2367	Phosphoglucose isomerase	msIMP	YidC	0031226, 0005887, 0009986

Lmo2378	Na(+)/H(+) antiporter subunit A, MnhA	msIMP	YidC	0031226, 0005887, 0009986
Lmo2380	Multisubunit Na+/H+ antiporter, MnhB subunit	msIMP	YidC	0031226, 0005887, 0009986
Lmo2381	Monovalent cation/H+ antiporter subuni D	msIMP	YidC	0031226, 0005887, 0009986
Lmo2383	Monovalent cation/H+ antiporter subunit F	msIMP	YidC	0031226, 0005887, 0009986
Lmo2482	Prolipoprotein diacylglyceryltransferase, Lgt	msIMP	YidC	0031226, 0005887, 0009986
Lmo2490	Protein of unknown function, COG4897	msIMP	YidC	0031226, 0005887, 0009986
Lmo2502	PDZ/DHR/GLGF	msIMP	YidC	0031226, 0005887, 0009986
Lmo2519	Glycosyl transferase, family 4	msIMP	YidC	0031226, 0005887, 0009986
Lmo2526	UDP-N-acetylglucosamine enolpyruvyl transferase	msIMP	YidC	0031226, 0005887, 0009986
Lmo2550	Undecaprenyl phosphate 4-deoxy-4-formamido-L-arabinose transferase	msIMP	YidC	0031226, 0005887, 0009986
Lmo2602	MgtC/SapB transporter	msIMP	YidC	0031226, 0005887, 0009986
Lmo2638	NADH dehydrogenase, FAD-containing subunit	msIMP	YidC	0031226, 0005887, 0009986
Lmo2641	Terpenoid synthase	msIMP	YidC	0031226, 0005887, 0009986
Lmo2649	Sugar-specific PTS, UlaA	msIMP	YidC	0031226, 0005887, 0009986
Lmo2665	Phosphotransferase system, galactitol-specific IIC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo2669	Protein of unknown function with DUF1113 domain, COG4905	msIMP	YidC	0031226, 0005887, 0009986
Lmo2679	Osmosensitive K+ channel histidine kinase	msIMP	YidC	0031226, 0005887, 0009986
Lmo2682	K+ transporting ATPase, A subunit, KdpA	msIMP	YidC	0031226, 0005887, 0009986
Lmo2717	Cytochrome bd-type quinol oxidase, subunit 2, CydB	msIMP	YidC	0031226, 0005887, 0009986
Lmo2718	Cytochrome bd-type quinol oxidase, subunit 1, CydA	msIMP	YidC	0031226, 0005887, 0009986
Lmo2733	2-O-a-mannosyl-D-glycerate specific PTS transporten components IIABC	msIMP	YidC	0031226, 0005887, 0009986
Lmo2767	Protein of unknown function, UCP033111-like, COG4858	msIMP	YidC	0031226, 0005887, 0009986
Lmo2772	Phosphotransferase system, beta-glucoside-specific IIABC component	msIMP	YidC	0031226, 0005887, 0009986
Lmo2778	Protein of unknown function	msIMP	YidC	0031226, 0005887, 0009986
Lmo2787	Phosphotransferase system, β-glucoside-specific IIABC component, BvrB	msIMP	YidC	0031226, 0005887, 0009986
Lmo0003	Na+-driven multi antimicrobial extrusion protein MatE	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0008	Cardiolipin synthetase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0015	Cytochrome AA3-600 quinol oxidase, subunit III	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0016	Cytochrome AA3-600 quinol oxidase, subunit IV, QoxD	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0023	Phosphotransferase system, mannose/fructose/sorbose/N-acetylgalactosamine IIC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0034	Phosphotransferase system, lactose/cellobiose IIC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0042	Selenite transport protein, SNARE-associated protein, DedA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo0050	Signal transduction protein with a C-terminal ATPase domain	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0070	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0073	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0074	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0088	F0F1-type ATP synthase, subunit c	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0094	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0097	Phosphotransferase system, mannose/fructose/sorbose family IIC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0107	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0108	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0128	Holin TcdE-like	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0137	ABC-type dipeptide/oligopeptide/nickel transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0180	ABC-type sugar transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0195	ABC-type macrolide transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0215	Polysaccharide biosynthesis protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0220	Peptidase M41, FtsH	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0253	Peptidase M56, BlaR1	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0283	ABC-type metal ion transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0288	Signal transduction histidine kinase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0296	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0298	Phosphotransferase system cellobiose-specific component IIC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0350	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0361	Twin-arginine translocase protein C, TatC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0373	Phosphotransferase system cellobiose-specific component IIC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0388	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0389	Low temperature requirement protein A, LtrA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0400	Phosphotransferase system, fructose-specific IIC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0403	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0417	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0419	Protein of unknown function with DUF1211 and , COG3548	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0421	Cell division membrane protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0428	Phosphotransferase system, fructose-specific IIC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0450	Protein of unknown function with DUF1275 and , COG3619	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo0452	Transglutaminase-like	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0453	Protein of unknown function with DUF58 domain, COG1721	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0456	Purine-cytosine permease and related proteins	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0475	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0495	Protein of unknown function with DUF6, COG0697	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0519	Drug resistance transporter EmrB/QacA subfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0523	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0524	Sulfate permease, transporter MFS superfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0527	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0531	Protein of unknown function and GGDEF domain	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0544	Phosphotransferase system, enzyme II sorbitol-specific factor	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0555	Amino acid/peptide transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0573	Xanthine/uracil/vitamin C permease	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0584	Autoinducer-2 exporter family, AI-2E (PerM)	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0593	Formate/nitrite family of transporters	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0596	Acid-resistance membrane protein, COG3247	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0600	Protein of unknown function with DUF1700 domain and , COG3752	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0603	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0605	Na+-driven multidrug efflux pump	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0607	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0608	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0616	Glycerophosphoryl diester phosphodiesterase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0621	Permease, COG0730	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0622	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0639	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0642	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0644	Membrane-bound phosphoglycerol transferase, alkaline phosphatase superfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0645	Amino acid/polyamine transporter I	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0653	Protein of unknown function with DUF975 domain, COG5523	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0656	Protein of unknown function with DUF420 domain, COG2322	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0668	ABC-type polysaccharide/polyol phosphate export system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0672	Protein of unknown function with DUF805 domain and , COG3152	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo0677	Flagellar export apparatus, membrane subunit FliQ	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0679	Flagellar export apparatus, membrane subunit FlhB	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0680	Flagellar export apparatus, membrane subunit FlhA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0685	Flagellar motor protein MotA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0713	Flagellar MS-ring protein, FliF	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0723	Chemotaxis methyl-accepting receptor, signalling	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0731	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0743	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0748	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0750	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0757	ABC-type transport system, multi-copper enzyme maturation, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0766	ABC-type sugar transport systems, permease components	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0771	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0782	Phosphotransferase system, sorbose-specific IIC subunit	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0787	D-alanine/D-serine/glycine permease	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0795	Chloramphenicol-sensitive protein, RarD	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0798	lysine transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0804	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0805	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0808	ABC-type spermidine/putrescine transport system, permease component I	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0809	ABC-type spermidine/putrescine transport system, permease component II	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0818	Cation transport ATPase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0836	Phosphate-starvation-inducible E	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0838	Sugar phosphate antiporter, UhpT	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0841	Cation transport ATPase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0843	Protein of unknown function with DUF1294 domain, COG3326	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0853	Small multidrug resistance protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0854	Small multidrug resistance protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0861	ABC-type sugar transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0876	Phosphotransferase system cellobiose-specific component IIC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0882	Protein of unknown function with DUF304 domain and , COG3402	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0883	Protein of unknown function , UCP026631-like, COG3428	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo0901	Phosphotransferase system, cellobiose-specific IIC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0908	Autoinducer-2 Exporter family, AI-2E (PerM)	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0912	Formate/nitrite transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0915	Phosphotransferase system cellobiose-specific component IIC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0920	Protein of unknown function with DUF318 domain and , COG0701	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0925	ABC-type multidrug transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0927	Membrane-bound phosphoglycerol transferase, alkaline phosphatase superfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0932	Selenite transport protein, SNARE-associated protein, YdjX	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0937	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0947	Cyanate permease	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0949	Protein of unknown function, UPF0118-like, COG0628	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0952	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0954	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0963	Heat shock protein HtpX	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0980	ABC-type polysaccharide/polyol phosphate export system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0985	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0987	Multidrug/hemolysin exporter, CylA/B	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0990	Na+-driven multidrug efflux pump	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0994	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0995	Fucose 4-O-acetylase and related acetyltransferases	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0998	Abortive infection protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0999	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1020	Cell wall-active antibiotics response protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1024	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1025	Protein of unknown function, COG1289	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1037	Protein of unknown function with DUF817 domain, COG3739	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1056	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1061	Signal transduction histidine kinase, BaeS	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1062	ABC-type transport system involved in lipoprotein release, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1071	Cell cycle protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1074	ABC-type polysaccharide/polyol, permease component, COG1682	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1079	Protein of unknown function, COG4485	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo1100	Heavy metal translocating P-type ATPase, CadA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1101	Signal peptidase of Type II, LspB	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1105	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1107	Transfer Clostridial Plasmid Conjugative System, Tcp-Conj protein family	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1109	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1112	Cell divisionFtsK/SpoIIIE	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1132	ABC-type transport system cysteine/glutathione, ATP-binding component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1148	Cobalamin synthase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1186	Ethanolamine utilisation protein, EutH	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1192	Cobalamin biosynthesis protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1206	Cobalt ABC transporter CbiQ, permease subunit, CbiQ	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1224	ABC-type antimicrobial peptide transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1250	Major facilitator superfamily MFS-1	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1291	Acyltransferase family 3	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1352	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1385	Protein of unknown function with DUF161 domain and , COG1284	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1386	DNA segregation ATPase FtsK/SpoIIIE and related proteins	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1390	ABC-type uncharacterized transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1396	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1409	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1410	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1417	Permease of the major facilitator superfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1419	Autoinducer-2 exporter family, AI-2E (PerM)	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1424	Mn2+ and Fe2+ transporters of the NRAMP family, MntH-like	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1429	Thiamine transporter YuaJ	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1432	Cytochrome c oxidase, subunit I	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1440	Protein of unknown function with DUF1189 domain	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1442	AzlC-like	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1464	Diacylglycerol kinase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1466	Membrane-associated HD superfamily hydrolase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1506	Macrolide exporter family, MacB	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1508	Signal transduction histidine kinase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo1526	Protein of unknown function, COG5416	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1546	Cell shape-determining protein, MreD	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1550	Type 4 prepilin peptidase, ComC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1569	Protein affecting phage T7 exclusion by the F plasmid, FxsA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1584	Protein with RDD domain	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1617	Drug resistance transporter EmrB/QacA subfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1624	Polysaccharide biosynthesis protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1625	Polysaccharide biosynthesis protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1626	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1637	ABC-type transport system, multi-copper enzyme maturation, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1640	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1651	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1652	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1655	VanZ like protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1672	O-succinylbenzoic acid--CoA ligase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1677	1,4-dihydroxy-2-naphthoate octaprenyltransferase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1682	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1695	Lysyl-tRNA synthetase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1697	Co/Zn/Cd cation transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1706	Ribonuclease BN-related	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1712	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1723	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1732	ABC-type sugar transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1741	Signal transduction histidine kinase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1746	ABC-type transport system, involved in lipoprotein release, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1748	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1761	Na+-dependent transporters of the SNF family	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1762	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1839	Xanthine/uracil permease, PyrP	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1845	Hypoxanthine/guanosine uptake transporter, PbuG	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1846	Multidrug efflux protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1848	ABC-type iron (Fe2+)/zinc (Zn2+)/copper (Cu2+) transprt system	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo1864	HylII	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1869	Aromatic acid exporter family, ArAE	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1870	Selenite transport protein, SNARE-associated protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1884	Xanthine permease	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1909	Protein of unknown function with DUF161 domain, COG1284	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1920	Protein of unknown function with DUF1405 domain, COG4347	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1947	Signal transduction histidine kinase, ResE	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1957	ABC Fe3+-siderophore transport system, permease component, FhuG	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1963	ABC-type transport system, multi-copper enzyme maturation, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1966	5-bromo-4-chloroindolyl phosphate hydrolysis protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo1980	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2008	ABC-type sugar transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2011	Signal transduction protein with a C-terminal ATPase domain	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2012	Protein of unknown function with DUF624 domain and , COG5578	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2043	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2045	Protein of unknown function , UCP030092-like	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2057	Protohaem IX farnesyltransferase, CtaB	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2059	Voltage-gated potassium channel	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2063	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2064	Large-conductance mechanosensitive channel, MscL	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2065	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2066	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2071	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2082	Camphor resistance CrcB protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2087	Multidrug efflux pump, VmrA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2115	Bacitracin exporter, BceAB (BarAB, YtsCD)	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2116	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2123	ABC-type maltose transport systems, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2124	ABC-type sugar transport systems, permease components	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2127	Abortive infection protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2129	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2130	Amino acid transporters	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo2145	Niacin/nicotinamide transporter, NiaY	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2147	Sulfate exporter family, efflux pump, YeiH	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2148	Protein of unknown function with DUF1361 domain and , COG4330	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2150	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2169	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2177	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2194	ABC-type dipeptide/oligopeptide/nickel transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2195	ABC-type dipeptide/oligopeptide/nickel transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2197	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2204	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2207	Protein interacting with FtsH	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2214	ABC-type transporter, EcsB	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2218	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2226	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2228	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2231	Cation efflux protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2232	Hemolysin, CBS domains	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2237	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2238	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2239	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2254	Xanthine/uracil/vitamin C permease	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2265	Protein of unknown function with DUF1516 domain	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2330	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2348	ABC-type amino acid transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2353	NhaP-type Na+/H+ and K+/H+ antiporters	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2355	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2357	Acid-resistance membrane protein, COG3247	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2371	ABC-type transport system, involved in lipoprotein release, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2377	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2382	Monovalent cation/H+ antiporter subunit E	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2384	Monovalent cation/H+ antiporter subunitGF	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2387	Chloride channel, core	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo2399	Hemolysins and related proteins containing CBS domains	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2404	4-toluene sulfonate uptake permease family	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2405	Protein of unknown function with DUF1634 domain and , COG4272	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2409	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2418	ABC-type metal ion transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2421	Signal transduction histidine kinase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2423	Cation efflux protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2427	Rod shape-determining protein RodA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2428	Rod shape-determining protein RodA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2430	Transport system permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2435	Protein of unknown function, COG5658	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2463	Drug exporters of the RND superfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2466	Protein of unknown function , COG4269	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2469	Amino acid/polyamine transporter I	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2484	Protein of unknown function , COG1950	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2492	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2497	Phosphate transport system permease protein 2	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2498	Phosphate ABC transporter, permease protein PstC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2500	Signal transduction histidine kinase, PhoR	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2503	Cardiolipin synthetase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2506	Cell division protein, FtsX	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2508	Protein of unknown function with DUF161 domain and , COG1284	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2527	Protein of unknown function with unlceaved signal peptide, YwzB-like, COG4836	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2535	F0F1 ATP synthase, subunit A	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2536	F0F1 ATP synthase, subunit I	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2549	Wall teichoic acid glycosylation protein, GtcA	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2553	Protein of unknown function , CHP00374-like, COG0392	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2563	Peptidase M50	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2567	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2570	Peptide-antibiotic killer factor immunity protein, SdpI family, SdpC (YvaZ)	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2575	Cation efflux protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2581	ABC-type antimicrobial peptide transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986

Lmo2582	Signal transduction histidine kinase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2588	Drug resistance transporter EmrB/QacA subfamily	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2599	Cobalt transport protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2634	Cobalt transport protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2635	1,4-dihydroxy-2-naphthoate octaprenyltransferase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2681	K+ transporting ATPase, B subunit, KdpB	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2684	Phosphotransferase system, lactose/cellobiose IIC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2689	Cation transport ATPase	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2708	Phosphotransferase system, lactose/cellobiose IIC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2715	ABC transporter, cysteine exporter family, permease/ATP-binding protein, CydD	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2716	ABC transporter, cysteine exporter family, permease/ATP-binding protein, CydC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2725	Na+-driven multidrug efflux pump	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2738	Hemolysins and related proteins containing CBS domains	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2741	Drug efflux system protein, MdtG	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2751	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2752	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2763	Phosphotransferase system cellobiose-specific component IIC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2768	Protein of unknown function	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2775	Bacteriocin-associated integral membrane protein	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2777	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2783	Phosphotransferase system cellobiose-specific component IIC	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2799	Phosphotransferase system, mannitol-specific IIBC component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2816	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2818	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2826	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2837	ABC-type sugar transport system, permease component	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2838	ABC-type sugar transport systems, permease components	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo2850	MFS general substrate transporter	msIMP, Unc-SP	Sec, YidC	0031226, 0005887, 0009986
Lmo0029	Protein of unknown function	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0037	Amino acid/polyamine transporter I	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0057	WXG100 protein secretion system, membrane component, EsaA	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0136	ABC-type dipeptide/oligopeptide/nickel transport system, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986

Lmo0169	Sugar uptake permease	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0179	ABC-type sugar transport system, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0234	Protein with PIN domain superfamily, COG4956	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0365	High-affinity Fe2+/Pb2+ permease	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0404	Protein of unknown function	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0414	Divalent heavy-metal cations transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0444	Phage infection protein, YhgE, C-terminal	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0448	Amino acid/polyamine transporter I	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0449	Protein of unknown function	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0576	Protein of unknown function with DUF1085 domain	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0591	Protein of unknown function with DUF1295 domain, COG3752	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0626	Protein of unknown function	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0632	Phosphotransferase system, fructose-specific IIC component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0650	Phage infection protein, YhgE	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0666	Protein of unknown function with DUF423 domain, COG2363	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0676	Flagellar export apparatus, membrane subunit FliP	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0678	Flagellar export apparatus, membrane subunit FliR	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0767	ABC-type sugar transport system, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0831	Malonate transporter, MdcF	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0839	MFS general substrate transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0847	ABC-type amino acid transport system, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0860	ABC-type sugar transport systems, permease components	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0872	Arabinose efflux permease	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0897	Sulphate anion transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0921	Protein of unknown function with DUF1980 domain, COG3689	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0959	Glycosyl transferase, family 4	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0973	D-alanine esterification of lipoteichoic acid and wall teichoic acid protein, DltB	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0981	MFS general substrate transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0991	Tellurium resistance membrane protein, TerC	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1004	Protein of unknown function with DUF218 domain, COG1434	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1040	ABC-type molybdate transport system, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1131	ABC-type transport system cysteine/glutathione, ATP-binding component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986

Lmo1190	Dimethylbenzimidazole porter, CblT	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1204	Cobalamin (vitamin B12) biosynthesis CbiM	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1210	Orotate transporter, OroP	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1211	Orotate transporter, OroP	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1226	Drug exporters of the RND superfamily	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1252	Protein of unknown function with DUF161 domain, COG1284	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1300	Arsenical pump membrane protein	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1316	Phosphatidate cytidylyltransferase, CdsA	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1391	Deoxyribonucleoside permease, RnsD	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1416	Glycopeptide antibiotics resistance protein	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1527	Sec transcolon, bifunctional subunit SecDF	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1568	Protein of unknown function with DUF441 domain, COG2707	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1623	Phosphatidic acid phosphatase type 2/haloperoxidase	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1686	Protein of unknown function with DUF939 domain, COG4129	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1690	Protein of unknown function with DUF457 domain, COG1988	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1696	Glycopeptide antibiotics resistance protein	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1731	ABC-type sugar transport systems, permease components	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1945	Riboflavin uptake transporter, YpaA	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo1958	ABC Fe3+-siderophore transport system, permease component, FhuB	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2001	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific IIC	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2009	ABC-type polysaccharide transport system, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2017	Phosphatidic acid phosphatase type 2/haloperoxidase	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2058	Cytochrome oxidase assembly protein, CtaA	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2062	Copper resistance D	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2070	Abortive infection protein	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2092	BCCT transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2122	Maltodextrose utilization protein MalA	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2135	Phosphotransferase system, fructose-specific IIC component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2140	ABC-type Na+ efflux pump, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2171	MFS general substrate transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2183	Transport system permease protein	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2249	Phosphate transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986

Lmo2250	ABC arginine/histidine transport system, permease component	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2255	Protein of unknown function	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2264	Protein of unknown function, GlnB-like, COG1284	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2279	Holin phage A118	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2360	Phage infection protein, YhgE	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2362	Glutamate:g-aminobutyrate antiporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2379	Multisubunit Na+/H+ antiporter, MnhB subunit	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2386	Acid phosphatase/vanadium-dependent haloperoxidase	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2443	Protein of unknown function	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2612	Sec translocon, subunit SecY	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2640	Heptaprenyl diphosphate synthase	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2688	Cell cycle protein	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2745	ABC-type bacteriocin/lantibiotic exporters, ATP-binding/permease protein	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo2845	MFS general substrate transporter	msIMP, SP	Sec, YidC, SPase I	0031226, 0005887, 0009986
Lmo0013	AA3-600 quinol oxidase subunit II, QoxA	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo0269	ABC-type dipeptide/oligopeptide/nickel transport systems, permease component	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo0641	Heavy metal translocating P-type ATPase	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo1379	YidC insertase, OxaA-like protein, OxaA1 (YqjG)	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo2687	Cell division protein, FtsW	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo2854	YidC insertase, OxaA-like protein, OxaA2 (SpoIIIJ)	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986

[a]Some annotations were corrected respective to the homology search performed as described in the Material & Methods section. More extensive and detailed annotations are available in Table 1S.

[b]Single-spanning IMP (ss-IMP) are categorized into (i) Type I, *i.e.* IMP exhibiting a cleavable SP in addition to another TMD positioned upstream, (i) Type II, *i.e.* IMP exhibiting a signal anchor, which is a TMD with N_{in}-C_{out} orientation (Type I module), that could be an uncleaved SP (Unc-SP), (iii) Type III, *i.e.* IMP exhibiting an reverse signal-anchor, which is a TMD with N_{out}-C_{in} orientation (Type II module).

[c]Subcellular location follow the GO (Gene Ontology) for cellular component. Besides location to intrinsic to the cytoplasmic membrane (GO:0031226), IMPs are more precisely integral to cytoplasmic membrane (GO:0005887) and consequently at cell surface (GO:0009986).

Table 3S: **The 74 lipoproteins in *L. monocytogenes* EGD-e as revealed by the secretomics-based method.**

Protein ID	Annotation[a]	Protein category	Secretion pathway[b]	GO[c]
Lmo0047	Peptidase M4, PepSY	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo0135	ABC-type dipeptide transport system, substrate-binding protein family 5 component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0152	ABC-type oligopeptide transport system, substrate-binding protein family 5 component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0153	ABC-type metal ion transport system, substrate-binding protein component, surface adhesin	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0181	ABC-type sugar transport system, substrate-binding protein family 1 component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0207	Lipoprotein of unknown function with DUF1307 domain, YehR-like, COG4808	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0255	Lipoprotein of unknown function with DUF1307 domain, YehR-like, COG4808	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0285	ABC-type metal ion transport system, substrate-binding protein component, surface antigen	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0303	Protein of unknown function, lysin rich	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0324	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo0355	Fumarate reductase flavoprotein subunit	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0366	Iron/lead transport substrate-binding protein, YcdO-like, PbrT-like	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo0460	Adhesin with RGD motif, LRR (Leucine-rich repeat) and DUF285 domain, RNI-like	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0510	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo0517	Phosphoglycerate mutase	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0541	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0617	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0768	ABC-type sugar transport system, substrate-binding protein family 1 component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0791	Protein of unknown function, YcdA-like spore lipoprotein	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0859	ABC-type trehalose/maltose transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo0945	Metallo-β-lactamase family protein, DNA-binding ComEC/Rec2-like protein	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo0953	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1016	ABC-type proline/glycine betaine transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1041	ABC-type molybdate transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1068	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1073	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1265	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986

Lmo1340	Protein of unknown function, YqgU-like	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1388	CD4+ T-cell stimulating antigen, TcsA, substrate-binding protein ABC transport system	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1426	ABC-type betaine/choline transport system, osmoprotectant, OpuCC	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1444	Peptidylprolyl isomerase, foldase, PrsA1	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1649	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1653	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1671	ABC-type metal ion transport system, substrate-binding protein component, surface adhesin	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1730	ABC-type sugar transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1738	ABC-type amino acid transport/signal transduction, surface adhesin	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1757	Sex pheromone cAM373 biosynthesis, CamS	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1800	Receptor-linked protein tyrosine/serine phosphatase	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1847	ABC transport, metal-binding protein, surface adhesin, lipoprotein promoting entry, LpeA	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1903	Thioredoxin, Bacteriocin transport accessory protein	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo1959	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2007	ABC-type sugar transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2023	L-aspartate oxidase, NadB	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2079	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2080	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2125	ABC-type maltose transporter, substrate-binding protein family 1 component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2184	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2196	ABC-type oligopeptide transport system, substrate-binding protein family 5 component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2219	Peptidylprolyl isomerase, foldase, PrsA2	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2331	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2349	ABC-type amino acid transport/signal transduction, surface adhesin	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2416	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2417	ABC-type metal ion transport system, substrate-binding protein component, surface antigen	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2431	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2446	Glycoside hydrolase, family 31	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2499	ABC-type phosphate transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2569	ABC-type oligopeptide transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2578	α/β hydrolase with a lipobox motif	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986

Lmo2594	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2595	Protein of unknown function	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2636	Thiamine biosynthesis lipoprotein ApbE-like	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2637	Major membrane immunogen, electron transport complex RnfABCDGE type	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo2642	Phosphoesterase	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2812	D-alanyl-D-alanine carboxypeptidase	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986
Lmo2839	ABC-type sugar transport system, substrate-binding protein component	Lipoprotein	Sec, Lgt, SPase II	0031226, 0046658, 0009986,0005576
Lmo1136	Protein of unknown function with LRR (Leucine-rich repeat), internalin-like	LPXTG-Lipoprotein	Sec, Lgt, SPase II,SrtA	0031226, 0046658, 0009275, 0009986
Lmo0013	AA3-600 quinol oxidase subunit II, QoxA	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986,0005576
Lmo0269	ABC-type dipeptide/oligopeptide/nickel transport systems, permease component	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo0641	Heavy metal translocating P-type ATPase	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo0821	Protein of unknown function	ssIMP I-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo1379	YidC insertase, OxaA-like protein, OxaA1 (YqjG)	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo2687	Bacterial cell division protein, FtsW	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo2793	Protein of unknown function	ssIMP I-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986
Lmo2854	YidC insertase, OxaA-like protein, OxaA2 (SpoIIIJ)	msIMP-Lipoprotein	Sec, YidC, Lgt, SPase II	0031226, 0005887, 0046658, 0009986,0005576

[a]Some annotations were corrected respective to the similarity search performed as described in the Material & Methods section. More extensive and detailed annotations are available in Table 1S.

[b]Following secretion *via* the Sec translocon, protein exhibiting a signal peptide of Type II (SP II) are taken in charge by the lipoprotein maturation pathway consisting of the prolipoprotein diacylglyceryltransferase (Lgt) and signal peptidases of Type II (SPase II) in *L. monocytogenes* EGD-e (Table 1).

[c]Subcellular location follow the GO (Gene Ontology) for cellular component. Besides location to intrinsic to the cytoplasmic membrane (GO:0031226), lipoproteins are more precisely anchored to cytoplasmic membrane (GO:0046658) and consequently at cell surface (GO:0009986). In some cases, the release of lipoproteins in the extracellular milieu (GO:0005876) can be expected by the presence of a glycine residue at position +2 of the SP cleavage site.

Table 4S: **The 58 secreted proteins anchored to the cell wall in *L. monocytogenes* EGD-e as revealed by the secretomics-based method.**

Protein ID	Annotation[a]	Protein category	Secretion pathway[b]	GO[c]
Covalent anchoring				
Lmo0130	Bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase, CpdB	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0159	Collagen-binding protein	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0160	Collagen-binding protein	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0171	Protein of unknown function with LRR (Leucine-rich repeat), PKD, Ig-like, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0175	Protein of unknown function with PKD, DUF1085 motif	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0262	Internalin G, InlG, with LRR (Leucine-rich repeat)	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0263	Internalin H, InlH, with LRR (Leucine-rich repeat)	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0264	Internalin E, InlE, with LRR (Leucine-rich repeat)	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0320	Virulence protein with LPXTG motif, Vip, recognition of Gp96 receptor	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0327	Protein of unknown function with LRR (Leucine-rich repeat), DUF1085, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0331	Protein of unknown function with LRR (Leucine-rich repeat), PKD motif, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0333	Internalin I, InlI, with LRR (Leucine-rich repeat), PKD motif	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0409	Internalin F, InlF, with LRR (Leucine-rich repeat)	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0433	Internalin A, InlA, with LRR (Leucine-rich repeat)	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0435	Biofilm-associated protein, BapL	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0463	Protein of unknown function	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0514	Protein of unknown function with LRR (Leucine-rich repeat), PKD motif, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0550	Protein of unknown function with LPXTG motif, CscD-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0610	Protein of unknown function with LRR (Leucine-rich repeat), PKD motif, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0627	Collagen-binding protein	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0725	Protein of unknown function	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0732	Protein of unknown function with LRR, DUF1085, bacterial Ig-like, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0801	Protein of unknown function with LRR (Leucine-rich repeat), PKD motif, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0835	Protein of unknown function	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0842	Invasin/intimin cell-adhesion protein with bacterial Ig-like domain	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo1115	Collagen-binding protein	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986

Lmo1289	Protein of unknown function with LRR (Leucine-rich repeat), PKD motif, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo1290	Internalin K, InlK, Recruitment of the Major Vault Protein, with PKD motif	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo1413	Protein of unknown function with DUF1085 domain	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo1666	Bacterial adhesin with hyalin, cadherin domains, PKD repeats and RGD motif	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo1799	Protein of unkonwn function with Ig fold, transmembrane, ATPase	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2026	Protein of unknown function with LRR, DUF1085, bacterial Ig-like, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2085	Collagen-binding protein	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2178	Collagen-binding protein	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2179	Protein of unknown function with DUF1085 domain, Cna-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2185	Surface virulence-associated protein, SvpA (P64), NEAr transporter	LPXTG-protein	Sec, SPase I, SrtB	0009275, 0009986
Lmo2186	Surface virulence-associated protein, SvpB, NEAr transporter	LPXTG-protein	Sec, SPase I, SrtB	0009275, 0009986
Lmo2396	Protein of unknown function with LRR (Leucine-rich repeat), DUF1085, internalin-like	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2576	Collagen-binding protein	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2714	Protein of unknown function	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo2821	Internalin J, InlJ, with LRR (Leucine-rich repeat)	LPXTG-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo0880	Collagen-binding protein with peptidoglycan-binding LysMs	LPXTG-LysM-protein	Sec, SPase I, SrtA	0009275, 0009986
Lmo1136	Protein of unknown function with LRR (Leucine-rich repeat), internalin-like	LPXTG-Lipoprotein	Sec, Lgt, SPase II, SrtA	0031226, 0046658, 0009275, 0009986
Non-covalent anchoring				
Lmo0582	Cell-wall hydrolase, protein of 60 KDa (P60), invasion associated protein, Iap	LysM-protein	Sec, SPase I	0009275, 0009986
Lmo2522	Protein of unknown function	LysM-protein	Sec, SPase I	0009275, 0009986
Lmo2691	Autolysin, N-acetylmuramoyl-L-alanine amidase, MurA	LysM-protein	Sec, SPase I	0009275, 0009986
Lmo1303	Cell division suppressor protein, YneA	ssIMP II-LysM-protein	Sec, YidC	0031226, 0005887, 0009275, 0009986
Lmo1941	Protein of unknown function, YpbE-like, ATPase	ssIMP II-LysM-protein	Sec, YidC	0031226, 0005887, 0009275, 0009986
Lmo0549	Protein of unknown function with LRR (Leucine-rich repeat), CscB-like	WXL-protein	Sec, SPase I	0009275, 0009986
Lmo0551	Protein of unknown function, CscB-like	WXL-protein	Sec, SPase I	0009275, 0009986
Lmo0585	Protein of unknown function, CscB-like	WXL-protein	Sec, SPase I	0009275, 0009986
Lmo0587	Protein of unknown function, CscB-like	WXL-protein	Sec, SPase I	0009275, 0009986
Lmo0434	Internalin B, InlB, with LRR (Leucine-rich repeat)	GW-protein	Sec, SPase I	0009275, 0009986
Lmo1076	N-acetylmuramoyl-L-alanine amidase, Auto	GW-protein	Sec, SPase I	0009275, 0009986
Lmo2203	N-acetylmuramoyl-L-alanine amidase	GW-protein	Sec, SPase I	0009275, 0009986
Lmo2558	N-acetylmuramoyl-L-alanine amidase family 2, autolysin, Ami	GW-protein	Sec, SPase I	0009275, 0009986

Lmo2591	N-acetylmuramoyl-L-alanine amidase	GW-protein	Sec, SPase I	0009275, 0009986
Lmo1851	Peptidase S41	PGBD1-protein	Sec, SPase I	0009275, 0009986

[a]Some annotations were corrected respective to the similarity search performed as described in the Material & Methods section. More extensive and detailed annotations are available in Table 1S.

[b]Following secretion *via* the Sec translocon, N-terminal signal peptide of Type I (SP I) is cleaved by signal peptidases of Type I (SPase I) whereas C-terminal LPXTG domain is cleaved and covalently linked to peptidoglycan precursor by sortase A (SrtA) or SrtB in *L. monocytogenes* EGD-e (Table 1).

[c]Subcellular location follow the GO (Gene Ontology) for cellular component. LPXTG-, LysM-, WXL-, GW- and PGBD1-proteins are anchored to the cell wall (GO:0009275) and consequently at cell surface (GO:0009986).

Table 5S: **The 16 secreted proteins part of supramolecular cell-surface appendage in *L. monocytogenes* EGD-e as revealed by the secretomics-based method.**

Protein ID	Annotation[a]	Protein Type	Secretion pathway[b]	GO[c]
Pseudo-pilus				
Lmo1341	Type 4 pseudo-prepilin, ComGG	FPE-substrate	FPE, ComC	0043234, 0009986
Lmo1342	Type 4 pseudo-prepilin, ComGF	FPE-substrate	FPE, ComC	0043234, 0009986
Lmo1343	Type 4 pseudo-prepilin, ComGE	FPE-substrate	FPE, ComC	0043234, 0009986
Lmo1344	Type 4 pseudo-prepilin, ComGD	FPE-substrate	FPE, ComC	0043234, 0009986
Lmo1345	Type 4 pseudo-prepilin, ComGC	FPE-substrate	FPE, ComC	0043234, 0009986
Flagellum				
Lmo0682	Flagellar basal body rod protein, FlgG	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0690	Flagellin, FlaA	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0695	Flagellar hook protein, FliK	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0696	Flagellar hook capping protein, FlgD	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0697	Flagellar hook protein, FlgE	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0705	Flagellar hook-associated, FlgK	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0706	Flagellar hook-associated, FlgL	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0707	Flagellar capping protein, FliD	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0710	Flagellar basal body rod protein, FlgB	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0711	Flagellar basal body rod protein, FlgC	FEA-substrate	FEA	0043234, 0009986, 00019861
Lmo0712	Flagellar hook-basal body protein, FliE	FEA-substrate	FEA	0043234, 0009986, 00019861

[a]Some annotations were corrected respective to the similarity search performed as described in the Material & Methods section. More extensive and detailed annotations are available in Table 1S.

[b]Pseudo-pilus are secreted and assembled by FPE after cleavage of the signal peptide of class 3 by signal peptidase ComC. Flagellum components are secreted and assembled by FEA (Table 1).

[c]Subcellular location follow the GO (Gene Ontology) for cellular component. Pseudo-pilus and flagellum (GO:00019861) are supramolecular protein complexes (GO:0043234) exposed at cell surface (GO:0009986).

Table 6S: **The 80 secreted exoproteins in *L. monocytogenes* EGD-e as revealed by the secretomics-based method.**

Protein ID	Annotation[a]	Secretion pathway[b]	GO[c]
Lmo0017	5'-nucleotidase/2',3'-cyclic phosphodiesterase, UDP-sugar hydrolase	Sec, SPase I	0005576
Lmo0019	Cell wall hydrolase with SH3 domain	Sec, SPase I	0005576
Lmo0086	Protein of unknown function with fibronectin type III-like fold	Sec, SPase I	0005576
Lmo0087	Protein of unknown function	Sec, SPase I	0005576
Lmo0105	Chitinase, ChiB	Sec, SPase I	0005576
Lmo0201	Phosphatidylinositol phospholipase C, PlcA	Sec, SPase I	0005576
Lmo0202	Listeriolysin O (Thiol-activated cytolysin) (LLO) Hly (HlyA) (LisA)	Sec, SPase I	0005576
Lmo0203	Zinc metalloproteinase, Mpl (PrtA)	Sec, SPase I	0005576
Lmo0205	Phosphatidylcholine phospholipase C, PlcB (prtC)	Sec, SPase I	0005576
Lmo0206	Protein of unknown function	Sec, SPase I	0005576
Lmo0275	Metallo-β-lactamase	Sec, SPase I	0005576
Lmo0394	NLP/P60-type cell wall hydrolase	Sec, SPase I	0005576
Lmo0408	Protein of unknown function with DUF1312 domain, COG5341	Sec, SPase I	0005576
Lmo0412	Potein of unknown function	Sec, SPase I	0005576
Lmo0415	Peptidoglycan GlcNAc deacetylase	Sec, SPase I	0005576
Lmo0438	Nuclear targeted protein A, IntA	Sec, SPase I	0005576
Lmo0461	Protein of unknown function	Sec, SPase I	0005576
Lmo0462	Protein of unknown function	Sec, SPase I	0005576
Lmo0516	Poly-β-glutamate biosynthesis enzyme	Sec, SPase I	0005576
Lmo0540	β-lactamase-type transpeptidase	Sec, SPase I	0005576
Lmo0601	Protein of unknown function with WD-40 repeat, COG3595	Sec, SPase I	0005576
Lmo0638	Protein of unknown function	Sec, SPase I	0005576
Lmo0671	Protein of unknown function	Sec, SPase I	0005576
Lmo0724	Protein of unknown function UCP032442 type, COG4990	Sec, SPase I	0005576
Lmo0745	Protein of unknown function	Sec, SPase I	0005576
Lmo0755	SGNH hydrolase-type esterase	Sec, SPase I	0005576
Lmo0778	Protein of unknown function	Sec, SPase I	0005576

Lmo0849	Amidase	Sec, SPase I	0005576
Lmo0881	Protein of unknown function	Sec, SPase I	0005576
Lmo0950	Protein of unknown function, α/β hydrolase fold, DUF1801 domain, COG4814	Sec, SPase I	0005576
Lmo1104	NLP/P60-type cell wall hydrolase	Sec, SPase I	0005576
Lmo1216	Muramidase flagellum-specific with a single GW domain, FlgJ-type	Sec, SPase I	0005576
Lmo1264	Metalloprotease with zincin-like fold	Sec, SPase I	0005576
Lmo1333	Aminodeoxychorismate lyase	Sec, SPase I	0005576
Lmo1334	Protein of unknown function	Sec, SPase I	0005576
Lmo1438	Cell division protein FtsI/Penicillin-binding protein 2, transpeptidase	Sec, SPase I	0005576
Lmo1518	Protein of unknown function	Sec, SPase I	0005576
Lmo1521	N-acetylmuramoyl-L-alanine amidase with a single GW domain, YrvJ-type	Sec, SPase I	0005576
Lmo1585	Peptidase S49	Sec, SPase I	0005576
Lmo1601	Protein of unknown function with TMP (tape measure protein) domain, COG4980	Sec, SPase I	0005576
Lmo1602	Protein of unknown function with DUF948, COG4768	Sec, SPase I	0005576
Lmo1654	Metalloprotease with zincin-like fold	Sec, SPase I	0005576
Lmo1656	Protein of unknown function	Sec, SPase I	0005576
Lmo1752	Protein of unknown function	Sec, SPase I	0005576
Lmo1786	Internalin C, InlC, with LRR (Leucine-rich repeat)	Sec, SPase I	0005576
Lmo1855	Peptidase M15B and M15C, D,D-carboxypeptidase VanY/endolysins	Sec, SPase I	0005576
Lmo1862	SGNH hydrolase-type esterase	Sec, SPase I	0005576
Lmo1883	Chitinase, ChiA	Sec, SPase I	0005576
Lmo1913	Six-hairpin glycosidase-like	Sec, SPase I	0005576
Lmo2027	Protein of unknown function with leucine-rich repeat (LRR) protein, COG4886, internalin-like	Sec, SPase I	0005576
Lmo2039	Cell division protein FtsI/Penicillin-binding protein 2, transpeptidase	Sec, SPase I	0005576
Lmo2074	α/β hydrolase, pepditase S9/S15	Sec, SPase I	0005576
Lmo2093	Sec-translocated protein of unknown function	Sec, SPase I	0005576
Lmo2106	Metallo-dependent phosphatase	Sec, SPase I	0005576
Lmo2119	Protein of unknown function YbbR-like	Sec, SPase I	0005576
Lmo2156	Protein of unknown function YxeA-like with conserved DUF1093 domain	Sec, SPase I	0005576
Lmo2217	Protein of unknown function, COG4980	Sec, SPase I	0005576
Lmo2439	Protein of unknown function YxeA-like, COG5294	Sec, SPase I	0005576

Lmo2444	Glycosyl hydrolase family 31 with galactose-binding like domains	Sec, SPase I	0005576
Lmo2467	Chitin binding protein	Sec, SPase I	0005576
Lmo2470	Protein of unknown function with leucine-rich repeat (LRR) protein, COG4886, internalin-like	Sec, SPase I	0005576
Lmo2504	Peptidase M23	Sec, SPase I	0005576
Lmo2505	Peptidoglycan lytic protein P45 (protein of 45 kDa), Spl (secreted protein with lytic property)	Sec, SPase I	0005576
Lmo2568	Protein of unknown function YxeA-like, COG5294	Sec, SPase I	0005576
Lmo2639	Protein of unknown function with DUF1312 domain, COG5341	Sec, SPase I	0005576
Lmo2686	Protein of unknown function	Sec, SPase I	0005576
Lmo2713	YkuD family protein (former ErfK/YbiS/YcfS/YnhG family protein)	Sec, SPase I	0005576
Lmo2754	Peptidase S11, D-alanyl-D-alanine carboxypeptidase A	Sec, SPase I	0005576
Lmo2776	Bacteriocin, lactococcin 972	Sec, SPase I	0005576
Lmo0367	Dyp-type peroxidase	Tat, SPase I	0005576
Lmo0335	Bacteriocin, class II microcin	ABC	0005576
Lmo0615	Bacteriocin	ABC	0005576
Lmo2574	Bacteriocin	ABC	0005576
Lmo2753	Bacteriocin leaderless	ABC	0005576
Lmo0129	Cell wall hydrolase, autolysin	Holin	0005576
Lmo2278	Endolysin Ply118	Holin	0005576
Lmo2284	Polygalacturonase	Holin	0005576
Lmo0056	WXG100-A, Lmesat6	Wss	0005576
Lmo0062	WXG100 protein secretion system, peripheral component, EsaC	Wss	0005576
Lmo0063	WXG100-B	Wss	0005576

[a]Some annotations were corrected respective to the similarity search performed as described in the Material & Methods section. More extensive and detailed annotations are available in Table 1S.

[b]Exoprotein with a SP is cleaved by signal peptidase of Type I (SPase I) when secreted *via* Sec or Tat or concomitantly to secretion *via* ABC transporter (Table 1).

[c]Subcellular location follow the GO (Gene Ontology) for cellular component. Exoprotein as released into the extracellular milieu (GO:0005576).

Table 8S: **Secretomic analysis of extracytoplasmic proteins experimentally identified in *L. monocytogenes* EGD-e.**

Protein ID	Annotation[a]	Secretomic analysis				Experimental analysis			References
		Protein type[b]	SCL[c]	Secretion pathway[d]	SP[e]	Fraction[f]	Secretion pathway[g]	C+1[h]	
Exoprotein									
Lmo0201	Phosphatidylinositol phospholipase C, PlcA		EC	Sec;SPase I	I (30)	mb/sn		30	[1,2,3]
Lmo0202	Listeriolysin O (LLO), Hly		EC	Sec;SPase I	I (25)	mb/cw/sn	SipZ	25	[1,2,3,4,5,6]
Lmo0203	Zinc metalloproteinase, Mpl (PrtA)		EC	Sec;SPase I	I (25)	sn			[1,2]
Lmo0205	Phosphatidylcholine phospholipase C, PlcB (PrtC)		EC	Sec;SPase I	I (26)	mb/sn	SipZ		[1,2,3,4]
Lmo0412	Sec-translocated protein of unknown function		EC	Sec;SPase I	I (59)	sn			[1]
Lmo0540	β-lactamase-type transpeptidase		EC	Sec;SPase I	I (47)	sn			[1]
Lmo0601	Protein of unknown function with WD-40 repeat, COG3595		EC	Sec;SPase I	I (29)	mb			[3]
Lmo0950	Protein of unknown function with DUF1801 domain, COG4814		EC	Sec;SPase I	I (33)	mb/se/sn			[2,3,7]
Lmo1216	Muramidase flagellum-specific with a single GW domain, FlgJ-type		EC	Sec;SPase I	I (21)	sn			[2]
Lmo1333	Aminodeoxychorismate lyase protein family		EC	Sec;SPase I	I (33)	mb/sn			[1,2,3]
Lmo1438	Cell division protein FtsI/Penicillin-binding protein 2, transpeptidase		EC	Sec;SPase I	I (51)	sn			[1,2]
Lmo1521	N-acetylmuramoyl-L-alanine amidase with a single GW domain, YrvJ-type		EC	Sec;SPase I	I (30)	sn			[1,2]
Lmo1601	Protein of unknown function of unknown function, COG4980		EC	Sec;SPase I	I (26)	mb			[3]
Lmo1602	Protein of unknown function with DUF948, COG4768		EC	Sec;SPase I	I (21)	mb			[3]
Lmo1752	Protein of unknown function of unknown function		EC	Sec;SPase I	I (34)	sn			[1,2]
Lmo1786	Internalin C, InlC, with LRR		EC	Sec;SPase I	I (34)	sn			[1,2]
Lmo1862	SGNH hydrolase-type esterase		EC	Sec;SPase I	I (26)	mb			[3]
Lmo1883	Chitinase, ChiA		EC	Sec;SPase I	I (30)	sn			[1,2]
Lmo2039	Cell division protein FtsI/Penicillin-binding protein 2, transpeptidase		EC	Sec;SPase I	I (49)	se/sn	SecA2		[1,8]
Lmo2119	Protein of unknown function, YbbR-like		EC	Sec;SPase I	I (29)	mb			[3]
Lmo2156	Protein of unknown function YxeA-like with DUF1093 domain		EC	Sec;SPase I	I (31)	sn			[1,2]
Lmo2504	Peptidase M23		EC	Sec;SPase I	I (26)	sn			[1,2]
Lmo2505	Peptidoglycan lytic protein P45 (protein of 45 kDa), Spl		EC	Sec;SPase I	I (28)	mb/cw/se/sn		28	[1,2,3,6,7,9,10]
Lmo2754	Peptidase S11, D-alanyl-D-alanine carboxypeptidase A		EC	Sec;SPase I	I (32)	sn			[2]
Lmo0129	Cell wall hydrolase, autolysin		EC	Holin	-	sn			[2]
Lmo2574	Bacteriocin		EC	ABC	ABC (21)	mb			[3]
CW-protein									
Lmo0130	Bifunctional phosphodiesterase/nucleotidase, CpdB	LPXTG	CW/CS	Sec;SPase I-SrtA	I (35)	cw/sn	SrtA	35	[1,6,9,10]
Lmo0160	Collagen-binding protein with LPXTG domain	LPXTG	CW/CS	Sec;SPase I-SrtA	I (37)	cw	SrtA		[6,9,10]

Lmo0262	Internalin G, InlG, with LRR and LPXTG domain	LPXTG	CW/CS	Sec;SPase I-SrtA	I (28)	cw	SrtA		[6,9,10]
Lmo0263	Internalin H, InlH, with LRR and LPXTG domain	LPXTG	CW/CS	Sec;SPase I-SrtA	I (31)	cw/se/sn	SrtA		[2,6,9,10,11]
Lmo0320	Virulence protein, Vip, recognition of Gp96 receptor	LPXTG	CW/CS	Sec;SPase I-SrtA	I (32)	se			[12]
Lmo0327	Protein of unknown function, DUF1085, internalin-like	LPXTG	CW/CS	Sec;SPase I-SrtA	I (27)	cw	SrtA		[6,9,10]
Lmo0331	Protein of unknown function with LRR, PKD, internalin-like	LPXTG	CW/CS	Sec;SPase I-SrtA	I (28)	cw			[6]
Lmo0333	Internalin I, InlI, with LRR, PKD motif	LPXTG	CW/CS	Sec;SPase I-SrtA	I (29)	cw			[6]
Lmo0433	Internalin A, InlA, with LRR	LPXTG	CW/CS	Sec;SPase I-SrtA	I (36)	cw/se/sn	SipZX,SrtA		[2,4,6,9,10,13,14]
Lmo0435	Biofilm-associated protein, BapL	LPXTG	CW/CS	Sec;SPase I-SrtA	I (30)	cw			[6]
Lmo0514	Protein of unknown function with LRR, PKD motif, internalin	LPXTG	CW/CS	Sec;SPase I-SrtA	I (27)	cw			[6]
Lmo0610	Protein of unknown function with LRR, PKD motif, internalin	LPXTG	CW/CS	Sec;SPase I-SrtA	I (29)	cw	SrtA		[6,9,10]
Lmo0842	Invasin/intimin cell-adhesion protein with Ig-like motif	LPXTG	CW/CS	Sec;SPase I-SrtA	I (34)	cw	SrtA		[6,9,10]
Lmo0880	Collagen-binding protein	LPXTG-LysM	CW/CS	Sec;SPase I-SrtA	I (26)	cw/sn	SrtA		[2,6,9,10]
Lmo1290	Internalin K, InlK, Recruitment of the Major Vault Protein	LPXTG	CW/CS	Sec;SPase I-SrtA	I (31)	se			[15]
Lmo1413	Protein of unknown function with DUF1085 motif	LPXTG	CW/CS	Sec;SPase I-SrtA	I (30)	cw	SrtA		[6,9,10]
Lmo1666	Bacterial adhesin, hyaline/cadherin domain, RGD motif	LPXTG	CW/CS	Sec;SPase I-SrtA	I (27)	cw/sn	SrtA		[2] [6,9,10]
Lmo2085	Collagen-binding protein	LPXTG	CW/CS	Sec;SPase I-SrtA	I (28)	cw	SrtA		[6,9,10]
Lmo2178	Collagen-binding protein	LPXTG	CW/CS	Sec;SPase I-SrtA	I (30)	cw			[6]
Lmo2185	Surface virulence-associated protein, SvpA, NEAr transporter	LPXTG	CW/CS	Sec;SPase I-SrtB	I (29)	cw/se/sn	SrtB		[2,6,9,10,16,17,18]
Lmo2186	Surface virulence-associated protein, SvpB, NEAr transporter	LPXTG	CW/CS	Sec;SPase I-SrtB	I (28)	cw/sn	SrtB		[2,6,9,10]
Lmo2714	Protein of unknown function	LPXTG	CW/CS	Sec;SPase I-SrtA	I (27)	cw/sn	SrtA		[1,2,6,9,10]
Lmo2821	Internalin J, InlJ, with LRR (Leucine-rich repeat)	LPXTG	CW/CS	Sec;SPase I-SrtA	I (26)	se			[19]
Lmo0585	Sec-translocated protein of unknown function, CscB-like	WXL	CW/CS	Sec;SPase I	I (27)	sn			[1]
Lmo1851	Peptidase S41	PGBD1	CW/CS	Sec;SPase I	I (57)	mb/sn			[2,3]
Lmo0434	Internalin B, InlB, with LRR (Leucine-rich repeat)	GW	CW/CS	Sec;SPase I	I (36)	se/sn	SipZ		[2,4,7,20]
Lmo2203	N-acetylmuramoyl-L-alanine amidas	GW	CW/CS	Sec;SPase I	I (36)	sn			[1]
Lmo2558	N-acetylmuramoyl-L-alanine amidase family 2, autolysin, Ami	GW	CW/CS	Sec;SPase I	I (31)	se/sn	GW		[2,7,21]
Lmo1076	N-acetylmuramoyl-L-alanine amidase, Auto	GW	CW/CS	Sec;SPase I	I (27)	se/sn			[2,22]
Lmo2591	N-acetylmuramoyl-L-alanine amidase	GW	CW/CS	Sec;SPase I	I (27)	sn			[1,2]
Lmo0582	Cell-wall hydrolase, invasion associated protein, Iap (P60)	LysM	CW/CS	Sec;SPase I	I (26)	mb/cw/se/sn	SecA2	26	[1,2,3,6,7,8,9,10,23,24]
Lmo2522	Protein of unknown function	LysM	CW/CS	Sec;SPase I	I (25)	sn			[1,2]
Lmo2691	Autolysin, N-acetylmuramoyl-L-alanine amidase, MurA	LysM	CW/CS	Sec;SPase I	I (53)	cw/sn			[1,2,6,7,9,10]
Lmo1303	Cell division suppressor protein, YneA	ssIMP II-LysM	MB/CW/CS	Sec;YidC	SA	sn			[2]
Lmo1941	Protein of unknown function, YpbE-like	ssIMP II-LysM	MB/CW/CS	Sec;YidC	SA	mb/cw			[3,6]
IMP									
Lmo0204	Actin-assembly inducing protein, ActA	ssIMP I	MB/CS	Sec;SPase I	I (30)	mb/cw/sn	SipZX		[1,2,3,4,6]
Lmo0289	Signal transduction YycH protein	ssIMP II	MB/CS	Sec;YidC	SA	mb			[3]
Lmo0971	D-alanine esterification of teichoic acid, DltD	ssIMP II	MB/CS	Sec;YidC	SA	mb			[3]

Lmo1499	Aminodeoxychorismate lyase	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1547	Cell shape-determining protein, MreC	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo2410	Protein of unknown function	ssIMP I	MB/CS	Sec;SPase I	I (34)	sn	[2]
Lmo0186	Protein of unknown function with 3D and G5 domains	ssIMP II	MB/CS	Sec;YidC	SA	sn	[1,2]
Lmo0217	Septum formation initiator	ssIMP II	MB/CS	Sec;YidC	SA	sn	[2]
Lmo0292	Serine protease with C-terminal PDZ/DHR/GLGF domain	ssIMP II	MB/CS	YidC	-	mb/se/sn	[2,3,7]
Lmo0290	Protein of unknown function, COG4853	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0392	Protein of unknown function with DUF1432, COG4864	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0441	Cell division protein FtsI/Penicillin-binding protein 2	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0443	Transcriptional regulator	ssIMP II	MB/CS	Sec;YidC	SA	mb/se/sn	[1,2,3,7]
Lmo0929	Sortase A, SrtA	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0962	Listeria epitope, LemA	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1026	Transcriptional regulator	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1075	Teichoic acids export protein ATP-binding subunit	ssIMP II	MB/CS	YidC	-	mb	[3]
Lmo1395	DNA-binding λ repressor-like, COG1426	ssIMP II	MB/CS	YidC	-	mb/sn	[2,3]
Lmo1399	HD superfamily hydrolase	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1495	Protein of unknown function with DUF1510 domain	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1511	Lysophospholipase	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1529	Sec transcolon, subunit YajC	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1663	Asparagine synthase, glutamine-hydrolyzing	ssIMP II	MB/CS	YidC	-	mb	[3]
Lmo1715	S-adenosyl-L-methionine-dependent methyltransferase type 11	ssIMP II	MB/CS	Sec;YidC	SA	sn	[2]
Lmo1820	Serine/threonine protein kinase	ssIMP II	MB/CS	YidC	-	mb	[3]
Lmo1861	Protein of unknown function, COG4698	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1892	Carboxypeptidase, PbpA, Glycosyl transferase family 51	ssIMP II	MB/CS	Sec;YidC	SA	mb/cw	[3,9,10]
Lmo1898	Protein of unknown function, COG5353	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1927	3-dehydroquinate synthase, AroB	ssIMP II	MB/CS	YidC	-	mb	[3]
Lmo2036	UDP-N-acetylmuramoylalanine-D-glutamate ligase, MurD	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo2056	Ca^{2+} chelating serine protease with SCP/PR1 domain	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo2089	Esterase/lipase	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo2229	Carboxypeptidase (penicillin-binding protein)	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo2486	Protein of unknown function, UCP012569 type, COG3595	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo2518	Transcriptional regulator	ssIMP II	MB/CS	Sec;YidC	SA	mb/sn	[1,2,3]
Lmo2710	Protein of unknown function	ssIMP II	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1351	Rhodanese-related sulfurtransferase	ssIMP III	MB/CS	YidC	-	mb	[3]
Lmo1594	Negative regulator of septation ring formation	ssIMP III	MB/CS	YidC	-	mb	[3]
Lmo2533	F_0F_1 ATP synthase, subunit B	ssIMP III	MB/CS	YidC	-	mb	[3]
Lmo0008	Cardiolipin synthetase	msIMP	MB/CS	Sec;YidC	SA	mb	[3]

Lmo0014	Cytochrome AA3-600 quinol oxidase, subunit I	msIMP	MB/CS	YidC	-	mb	[3]
Lmo0015	Cytochrome AA3-600 quinol oxidase, subunit III	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0098	Phosphotransferase system, mannose/fructose family IID	msIMP	MB/CS	YidC	-	mb	[3]
Lmo0136	ABC-type dipeptide/oligopeptide/nickel transporter, permease	msIMP	MB/CS	Sec;SPase I	I (33)	mb	[3]
Lmo0220	Peptidase M41, FtsH	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0234	Protein of unknown function, PIN domain, COG4956	msIMP	MB/CS	Sec;SPase I	I (65)	mb	[3]
Lmo0288	Signal transduction histidine kinase	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0296	Protein of unknown function	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0524	Sulfate permease, transporter MFS superfamily	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0531	Protein of unknown function with GGDEF domain	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0584	Permease	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0607	ABC bacteriocin exporter, peptidase, ATP-binding/permease	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0608	ABC bacteriocin exporters, peptidase, ATP-binding/permease	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0626	Protein of unknown function	msIMP	MB/CS	Sec;SPase I	I (32)	mb	[3]
Lmo0644	Phosphoglycerol transferase, alkaline phosphatase superfamily	msIMP	MB/CS	Sec;YidC	SA	sn	[1,2]
Lmo0650	Phage infection protein, YhgE	msIMP	MB/CS	Sec;SPase I	I (37)	mb	[3]
Lmo0653	Protein of unknown function with DUF975 domain, COG5523	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0680	Flagellar export apparatus, membrane subunit FlhA	msIMP	MB/CS	Sec;SPase I	I (42)	sn	[2]
Lmo0781	Phosphotransferase system, mannose family IID	msIMP	MB/CS	YidC	-	mb	[3]
Lmo0782	Phosphotransferase system, sorbose-specific IIC subunit	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0841	Cation transport ATPase	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo0847	ABC-type amino acid transport system, permease	msIMP	MB/CS	Sec;SPase I	I (31)	mb	[3]
Lmo0927	Phosphoglycerol transferase, alkaline phosphatase superfamily	msIMP	MB/CS	Sec;YidC	SA	mb/sn	[1,2,3]
Lmo0933	Phosphate 4-deoxy-4-formamido-L-arabinose transferase	msIMP	MB/CS	YidC	-	mb	[3]
Lmo0952	Protein of unknown function with uncleaved signal peptide	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1015	ABC-type betaine transport system, permease, GbuB	msIMP	MB/CS	YidC	-	mb	[3]
Lmo1061	Signal transduction histidine kinase, BaeS	msIMP	MB/CS	Sec;YidC	SA	sn	[2]
Lmo1064	Mg^{2+} and Co^{2+} transporters, CorA-like	msIMP	MB/CS	YidC	-	mb	[3]
Lmo1261	Protein of unknown function with GYF domain	msIMP	MB/CS	YidC	-	mb	[3]
Lmo1291	Acyltransferase family 3	msIMP	MB/CS	Sec;YidC	SA	se/sn	[2,7]
Lmo1422	ABC-type betaine uptake system, bile exclusion system, BilEB	msIMP	MB/CS	YidC	-	mb	[3]
Lmo1424	Ion transporters of the NRAMP family, MntH-like	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1427	ABC-type betaine transport systems, permease, OpuCB	msIMP	MB/CS	YidC	-	mb	[3]
Lmo1440	Protein of unknown function with DUF1189 domain	msIMP	MB/CS	Sec;YidC	SA	mb	[3]
Lmo1516	Ammonium permease	msIMP	MB/CS	YidC	-	mb	[3]
Lmo1527	Sec transcolon, bifunctional subunit SecDF	msIMP	MB/CS	Sec;SPase I	I (24)	mb	[3]
Lmo1626	Protein of unknown function	msIMP	MB/CS	Sec;YidC	SA	mb	[3]

Lmo1637	ABC transporter, copper enzyme maturation, permease	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo1695	Lysyl-tRNA synthetase	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo1740	ABC-type amino acid transport system, permease	msIMP	MB/CS	YidC	-	mb		[3]
Lmo1741	Signal transduction histidine kinase	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo1839	Xanthine/uracil permease, PyrP	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo1853	Heavy metal translocating P-type ATPase	msIMP	MB/CS	YidC	-	mb		[3]
Lmo1884	Xanthine permease	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo1919	Zinc metallopeptidase	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2017	Phosphatidic acid phosphatase type 2/haloperoxidase	msIMP	MB/CS	Sec;SPase I	I (29)	mb		[3]
Lmo2029	Protein of unknown function, YGGT-like, COG0762	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2059	Voltage-gated potassium channel	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2064	Large-conductance mechanosensitive channel, MscL	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2120	Protein of unknown function, COG1624	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2140	ABC-type Na^+ efflux pump, permease component	msIMP	MB/CS	Sec;SPase I	I (45)	mb		[3]
Lmo2194	ABC dipeptide/oligopeptide/nickel transporter, permease	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2195	ABC dipeptide/oligopeptide/nickel transporter, permease	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2221	Protein of unknown function, COG4717	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2232	Hemolysin, CBS domains	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2254	Xanthine/uracil/vitamin C permease	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2287	Tape measure domain bacteriophage A118	msIMP	MB/CS	YidC	-	sn		[2]
Lmo2335	2-O-mannosyl-D-glycerate specific PTS transporter	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2360	Phage infection protein, YhgE	msIMP	MB/CS	Sec;SPase I	I (33)	mb		[3]
Lmo2367	Phosphoglucose isomerase	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2371	ABC transporter, lipoprotein release, permease	msIMP	MB/CS	Sec;SPase I	I (41)	mb		[3]
Lmo2418	ABC-type metal ion transport system, permease	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2469	Amino acid/polyamine transporter I	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2503	Cardiolipin synthetase	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2526	UDP-N-acetylglucosamine enolpyruvyl transferase	msIMP	MB/CS	YidC	-	se/sn	SecA2	[8]
Lmo2535	F_0F_1 ATP synthase, subunit A	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2550	Phosphate 4-deoxy-4-formamido-L-arabinose transferase	msIMP	MB/CS	YidC	-	mb/sn		[2,3]
Lmo2612	Sec translocon, subunit SecY	msIMP	MB/CS	Sec;SPase I	I (42)	mb		[3]
Lmo2638	NADH dehydrogenase, FAD-containing subunit	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2681	K^+ transporting ATPase, B subunit, KdpB	msIMP	MB/CS	Sec;YidC	SA	sn		[2]
Lmo2715	ABC cysteine exporter, permease/ATP-binding protein, CydD	msIMP	MB/CS	Sec;YidC	SA	mb		[3]
Lmo2718	Cytochrome bd-type quinol oxidase, subunit 1, CydA	msIMP	MB/CS	YidC	-	mb		[3]
Lmo2745	ABC bacteriocin exporter, peptidase, ATP-binding/permease	msIMP	MB/CS	Sec;SPase I	I (47)	mb		[3]
Lmo2751	ABC bacteriocin exporter, peptidase, ATP-binding/permease	msIMP	MB/CS	Sec;YidC	SA	mb		[3]

Lmo0013	AA3-600 quinol oxidase subunit II, QoxA	msIMP-Lipo	MB/CS/EC	Sec;YidC-Lgt-SPase II;C+2=G	II (23)	mb/se/sn		[1,2,3,7]
Lmo0641	Heavy metal translocating P-type ATPase	msIMP-Lipo	MB/CS	Sec;YidC-Lgt-SPase II	II (25)	mb		[3]
Lmo2854	YidC insertase, OxaA-like protein, OxaA2 (SpoIIIJ)	msIMP-Lipo	MB/CS/EC	Sec;YidC-Lgt-SPase II;C+2=G	II (27)	mb		[3]
Lipoprotein								
Lmo0047	Peptidase M4, PepSY		MB/CS	Sec;Lgt-SPase II	II (21)	mb/se/*	Lgt	[3,7,25]
Lmo0135	ABC-type dipeptide transport system, substrate-binding protein family 5		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (20)	mb/se/sn/*	Lgt	[1,2,3,7,25]
Lmo0152	ABC-type oligopeptide transport system, substrate-binding protein family 5		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (22)	mb/sn/*	Lgt	[2,3,25]
Lmo0153	ABC-type metal ion transport system, substrate-binding protein, surface adhesin		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	mb/sn		[2,3]
Lmo0181	ABC-type sugar transport system, substrate-binding protein family 1		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	*	Lgt	[25]
Lmo0285	ABC-type metal ion transport system, substrate-binding protein, surface antigen		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (23)	mb/se		[3,7]
Lmo0355	Fumarate reductase flavoprotein subunit		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (22)	mb		[3]
Lmo0541	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (23)	mb/*	Lgt	[3,25]
Lmo0791	Lipoprotein of unknown function, YcdA-like spore lipoprotein		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	mb/se/*	Lgt	[3,7,25]
Lmo0945	Metallo-β-lactamase family protein, DNA-binding ComEC/Rec2-like protein		MB/CS	Sec;Lgt-SPase II	II (19)	*	Lgt	[25]
Lmo1016	ABC-type proline/glycine betaine transport system, substrate-binding protein		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	mb		[3]
Lmo1041	ABC-type molybdate transport system, substrate-binding protein		MB/CS	Sec;Lgt-SPase II	II (19)	mb/se		[3,7]
Lmo1068	Lipoprotein of unknown function		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (20)	mb/se/sn/*	Lgt	[1,2,3,7,25]
Lmo1073	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (19)	se/*	Lgt	[7,25]
Lmo1388	CD4+ T-cell stimulating antigen, TcsA, ABC transporter, binding protein		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (23)	mb/se/sn/*	SecA2,Lgt	[1,2,3,7,8,25]
Lmo1426	ABC-type choline transport system, osmoprotectant, binding protein, OpuCC		MB/CS	Sec;Lgt-SPase II	II (23)	mb/se		[3,7]
Lmo1671	ABC-type metal ion transport system, substrate-binding protein		MB/CS	Sec;Lgt-SPase II	II (22)	mb/se/sn/*	Lgt	[2,3,7,25]
Lmo1730	ABC-type sugar transport system, substrate-binding protein component		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (22)	se		[7]
Lmo1738	ABC-type signal transduction system, substrate-binding protein family 3		MB/CS	Sec;Lgt-SPase II	II (20)	mb/se/sn/*	Lgt	[2,3,7,25]
Lmo1757	Sex pheromone cAM373 biosynthesis, CamS		MB/CS	Sec;Lgt-SPase II	II (18)	mb/se/*	Lgt	[3,7,25]
Lmo1800	Receptor-linked protein tyrosine/serine phosphatase		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (22)	*	Lgt	[25]
Lmo1847	ABC-type metal transport system, binding protein, surface adhesin, LpeA		MB/CS	Sec;Lgt-SPase II	II (19)	mb/se/sn/*	Lgt	[2,3,7,25,26]
Lmo1959	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	mb		[3]
Lmo2079	Lipoprotein of unknown function		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	sn/*	Lgt	[2,25]
Lmo2125	ABC-type maltose transporter, substrate-binding protein family 1		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (23)	se	SecA2	[8]
Lmo2196	ABC-type oligopeptide transport system, substrate-binding protein family 5		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (23)	mb/se/sn/*	SecA2,Lgt	[1,2,3,8,25]
Lmo2219	Peptidylprolyl isomerase, foldase, PrsA2		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	mb/se/*	LspA,Lgt	[3,7,25,27]
Lmo2331	Lipoprotein of unknown function		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (22)	mb/se	Lgt	[3,7,25]
Lmo2349	ABC-type signal transduction system, substrate-binding protein family 3		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (21)	mb/sn		[2,3]
Lmo2416	Lipoprotein of unknown function		MB/CS	Sec;Lgt-SPase II	II (21)	sn/*	Lgt	[2,25]
Lmo2417	ABC-type metal ion transport system, substrate-binding protein, surface antigen		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (20)	mb/se/*	Lgt	[3,7,25]
Lmo2431	ABC-type Fe^{3+}-hydroxamate transport system, substrate-binding protein		MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (20)	mb/*	Lgt	[3,25]
Lmo2569	ABC-type oligopeptide transport system, substrate-binding protein		MB/CS	Sec;Lgt-SPase II	II (23)	mb/*	Lgt	[3,25]

Lmo2595	Lipoprotein of unknown function	MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (22)	*	Lgt	[25]
Lmo2636	Thiamine biosynthesis lipoprotein ApbE-like	MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (20)	mb/se/*	Lgt	[3,7,25]
Lmo2637	Immunogen protein, NADH-(ubi-)quinone oxireductase subunit	MB/CS/EC	Sec;Lgt-SPase II;C+2=G	II (23)	mb/se/sn/*	SecA2,Lgt	[2,3,7,8,25]
Cytoprotein							
Lmo0796	Lipid/polyisoprenoid-binding, YceI-like	CP/EC	NC	-	sn		[1,2]
Lmo0900	Protein of unknown function with conserved DUF1801 domain, COG5646	CP/EC	NC	-	sn		[2]
Lmo1439	Manganese-superoxide dismutase, MnSOD	CP/EC	NC	-	se/sn	SecA2	[1,2,7,28]
Lmo2297	Phage minor structural GP20	CP/EC	NC	-	sn		[2]
Lmo2487	Protein of unknown functionYvlB-like, COG3595	CP/EC	NC	-	mb		[3]
Lmo2705	Polyketide cyclase/dehydrase	CP/EC	NC	-	mb		[3]
Lmo0020	DNA-binding transcriptional regulator, FrlR	CP	-	-	mb		[3]
Lmo0044	30S ribosomal protein S6, RpsF	CP	-	-	sn		[2]
Lmo0096	Phosphotransferase system, sorbose subfamily IIB	CP	-	-	mb		[3]
Lmo0118	Antigen A, LmaA	CP	-	-	sn		[2]
Lmo0119	Protein of unknown function	CP	-	-	sn		[2]
Lmo0154	ABC-type Mn/Zn transport system, ATPase	CP	-	-	mb		[3]
Lmo0194	ABC-type macrolide transport system, ATPase	CP	-	-	mb		[3]
Lmo0196	Stage V sporulation protein G	CP	-	-	sn		[1]
Lmo0197	Sporulation stage V, protein G	CP	-	-	mb/se		[3,7]
Lmo0199	Phosphoribosyl pyrophosphokinase	CP	-	-	mb		[3]
Lmo0211	50S ribosomal protein L25	CP	-	-	sn		[1]
Lmo0214	Transcription-repair coupling factor (superfamily II helicase)	CP	-	-	se		[7]
Lmo0223	Cysteine synthase A, CysA	CP	-	-	mb/se/sn		[1,2,3,7]
Lmo0229	Transcriptional repressor of class III stress genes	CP	-	-	mb		[3]
Lmo0237	Glutamyl-tRNA synthetase, class Ic	CP	-	-	mb		[3]
Lmo0248	50S ribosomal protein L11, RplK	CP	-	-	mb		[3]
Lmo0249	50S ribosomal protein L1	CP	-	-	mb		[3]
Lmo0251	50S ribosomal protein L7/L12, RplL	CP	-	-	mb/sn		[2,3]
Lmo0254	Protein of unknown function	CP	-	-	mb		[3]
Lmo0258	DNA-directed RNA polymerase, subunit β	CP	-	-	se/sn	SecA2	[8]
Lmo0259	DNA-directed RNA polymerase, subunit β-prime	CP	-	-	se/sn	SecA2	[8]
Lmo0284	ABC-type metal ion transport system, ATPase	CP	-	-	mb		[3]
Lmo0395	Acyl-CoA N-acyltransferase, GCN5-related, Nat	CP	-	-	sn		[2]
Lmo0407	Protein of unknown function with DUF1149 domain, COG4835	CP	-	-	mb		[3]
Lmo0454	AAA-type ATPase chaperone, MoxR	CP	-	-	mb		[3]
Lmo0526	DNA-binding protein, MerR-like	CP	-	-	sn		[2]
Lmo0539	Tagatose-1,6-bisphosphate aldolase	CP	-	-	sn		[2]

Lmo0560	Glutamate dehydrogenase/leucine dehydrogenase	CP	-	-	mb/sn		[2,3]
Lmo0583	Sec translocase, ATPase, SecA2	CP	-	-	mb		[3]
Lmo0667	ABC-type multidrug transport system, ATPase	CP	-	-	mb		[3]
Lmo0739	β-glucosidase/6-phospho-β-glucosidase/β-galactosidase	CP	-	-	se		[7]
Lmo0756	ABC-type multidrug transport system, ATPase	CP	-	-	mb		[3]
Lmo0774	Sphingosine kinase	CP	-	-	mb		[3]
Lmo0785	Transcriptional antiterminator	CP	-	-	mb		[3]
Lmo0807	ABC-type spermidine/putrescine transport system, ATPase	CP	-	-	mb		[3]
Lmo0848	ABC-type polar amino acid transport system, ATPase	CP	-	-	mb		[3]
Lmo0851	Protein of unknown function	CP	-	-	mb		[3]
Lmo0866	Superfamily II DNA and RNA helicase	CP	-	-	mb		[3]
Lmo0943	DNA-binding protein, ferritin-like, Dps type	CP	-	-	mb/sn		[2,3]
Lmo1002	Phosphotransferase system, phosphocarrier HPr protein	CP	-	-	sn		[2]
Lmo1003	Phosphoenolpyruvate-protein kinase, PTS system EI	CP	-	-	mb		[3]
Lmo1014	ABC-type proline/glycine betaine transport system, ATPase	CP	-	-	mb		[3]
Lmo1023	K^+ transport systems, NAD-binding	CP	-	-	mb		[3]
Lmo1053	Pyruvate/2-oxoglutarate dehydrogenase complex, β subunit, PdhB	CP	-	-	mb/se		[3,7]
Lmo1054	Dihydrolipoamide acyltransferase (E2)	CP	-	-	mb/se	SecA2	[3,7,8]
Lmo1055	Dihydrolipoamide dehydrogenase	CP	-	-	mb/se/sn		[1,3,7]
Lmo1059	Disulfide isomerase with thioredoxin-like fold	CP	-	-	se		[7]
Lmo1067	GTP-binding protein TypA	CP	-	-	mb		[3]
Lmo1072	Pyruvate carboxylase	CP	-	-	mb		[3]
Lmo1077	CDP-glycerol glycerophosphotransferase	CP	-	-	mb		[3]
Lmo1080	Nucleotide-diphospho-sugar transferase, glycosyl transferase family 2	CP	-	-	mb/sn		[2,3]
Lmo1084	dTDP-4-dehydrorhamnose reductase	CP	-	-	mb		[3]
Lmo1087	Threonine dehydrogenase, Zn-dependent dehydrogenase family	CP	-	-	mb		[3]
Lmo1090	Glycosyltransferase involved in cell wall biogenesis	CP	-	-	mb		[3]
Lmo1091	Glycosyl transferase, family 2	CP	-	-	mb		[3]
Lmo1125	Protein of unknown function	CP	-	-	sn		[2]
Lmo1182	Propanediol utilization protein, PduL	CP	-	-	sn		[2]
Lmo1221	Phenylalanyl-tRNA synthetase, class IIc, α subunit	CP	-	-	mb		[3]
Lmo1222	Phenylalanyl-tRNA synthetase, class IIc, β subunit	CP	-	-	mb		[3]
Lmo1267	Trigger factor, FKBP-type peptidyl-prolyl cis-trans isomerase	CP	-	-	mb		[3]
Lmo1279	ATP-dependent protease, HslVU (ClpYQ), ATPase subunit	CP	-	-	mb		[3]
Lmo1299	Glutamine synthetase type I	CP	-	-	mb		[3]
Lmo1305	Transketolase, Tkt	CP	-	-	sn		[2]
Lmo1314	Ribosome recycling factor	CP	-	-	se		[7]

Lmo1357	Acetyl-CoA carboxylase, biotin carboxylase	CP	-	-	mb		[3]
Lmo1360	Tetrahydrofolate dehydrogenase/cyclohydrolase	CP	-	-	mb		[3]
Lmo1364	Cold shock protein, CspA type	CP	-	-	sn		[1]
Lmo1389	ABC-type sugar transport system, ATPase component	CP	-	-	mb		[3]
Lmo1398	Recombinase A, RecA	CP	-	-	mb		[3]
Lmo1421	ABC-type betaine uptake system, bile exclusion system, BilEA	CP	-	-	mb		[3]
Lmo1428	ABC-type proline/glycine betaine transport system, ATPase	CP	-	-	mb		[3]
Lmo1435	Dihydrodipicolinate synthase subfamily	CP	-	-	mb		[3]
Lmo1473	DnaK class I heat-shock chaperone protein	CP	-	-	mb/se/sn	SecA2	[1,2,3,7,8]
Lmo1475	Negative transcriptional regulator of class I heat shock protein	CP	-	-	mb		[3]
Lmo1517	Nitrogen regulatory protein PII	CP	-	-	mb		[3]
Lmo1519	Aspartyl-tRNA synthetase	CP	-	-	mb		[3]
Lmo1544	Septum site-determining protein, MinD	CP	-	-	mb		[3]
Lmo1548	Cell shape determining protein MreB/Mrl	CP	-	-	mb		[3]
Lmo1570	Pyruvate kinase	CP	-	-	mb		[3]
Lmo1571	6-phosphofructokinase, Pfk	CP	-	-	mb/sn		[2,3]
Lmo1572	Acetyl-CoA carboxylase, α subunit	CP	-	-	mb		[3]
Lmo1580	Universal stress protein, UspA, Adenine nucleotide α hydrolases-like	CP	-	-	mb/sn		[2,3]
Lmo1600	Phospho-2-dehydro-3-deoxyheptonate aldolase, subtype 2	CP	-	-	mb		[3]
Lmo1606	DNA segregation ATPase FtsK/SpoIIIE	CP	-	-	mb		[3]
Lmo1611	Peptidase M42	CP	-	-	mb		[3]
Lmo1634	Bifunctional aldehyde/alcohol dehydrogenase, listerial adhesion protein, Lap	CP	-	-	se	SecA2	[7,29]
Lmo1636	ABC-type multidrug transport system, ATPase component	CP	-	-	mb		[3]
Lmo1641	Aconitase/iron regulatory protein 2	CP	-	-	mb		[3]
Lmo1647	Phospholipid/glycerol acyltransferase	CP	-	-	mb		[3]
Lmo1657	Translation elongation factor Ts	CP	-	-	mb/se/sn		[2,3,7]
Lmo1658	30S ribosomal protein S2	CP	-	-	mb		[3]
Lmo1713	Cell shape determining protein MreB/Mrl	CP	-	-	mb		[3]
Lmo1737	Glycerol dehydrogenase	CP	-	-	mb		[3]
Lmo1739	ABC-type polar amino acid transport system, ATPase component	CP	-	-	mb		[3]
Lmo1744	Nucleoside-diphosphate-sugar epimerases	CP	-	-	mb		[3]
Lmo1755	Glutamyl-tRNA amidotransferase subunit A, GatA	CP	-	-	mb		[3]
Lmo1764	Phosphoribosylglycinamide synthetase	CP	-	-	mb		[3]
Lmo1765	Phosphoribosylaminoimidazolecarboxamide formyltransferase, PurH	CP	-	-	mb/sn		[2,3]
Lmo1769	Phosphoribosylformylglycinamidine synthase II	CP	-	-	mb		[3]
Lmo1773	Adenylosuccinate lyase, PurB	CP	-	-	mb/sn		[2,3]
Lmo1774	Phosphoribosylaminoimidazole carboxylase, ATPase subunit	CP	-	-	mb		[3]

Lmo1775	1-(5-Phosphoribosyl)-5-amino-4-imidazole-carboxylate carboxylase, PurE	CP	-	-	sn		[2]
Lmo1783	50S ribosomal protein L20	CP	-	-	mb		[3]
Lmo1809	Phospholipid biosynthesis protein, PlsX type	CP	-	-	mb		[3]
Lmo1829	Fibronectin-binding protein A, FbpA, with DUF814	CP	-	-	mb	SecA2	[30]
Lmo1835	Carbamoyl phosphate synthase, large subunit, glutamine-dependent	CP	-	-	mb		[3]
Lmo1849	ABC-type Mn/Zn transport system, ATPase component	CP	-	-	mb		[3]
Lmo1867	Phosphoenolpyruvate synthase/pyruvate phosphate dikinase	CP	-	-	sn		[2]
Lmo1875	ABC-type sugar transport system, ATPase component	CP	-	-	mb		[3]
Lmo1877	Formyltetrahydrofolate synthetase	CP	-	-	mb		[3]
Lmo1934	Histone-like bacterial DNA-binding protein	CP	-	-	sn		[2]
Lmo1964	ABC-type multidrug transport system, ATPase component	CP	-	-	mb		[3]
Lmo1967	Toxic anion resistance protein, TelA type	CP	-	-	mb/se		[3,7]
Lmo2016	Cold-shock protein, DNA-binding	CP	-	-	sn		[2]
Lmo2020	Cell division initiation protein, DivIVA	CP	-	-	mb		[3]
Lmo2021	Small molecule binding protein with 3H domain	CP	-	-	se		[7]
Lmo2030	Protein of unknown function with DUF552 domain, COG1799	CP	-	-	mb		[3]
Lmo2032	Cell division protein, FtsZ	CP	-	-	mb		[3]
Lmo2033	Cell division protein, FtsA	CP	-	-	mb		[3]
Lmo2035	N-acetylglucosaminyltransferase, MurG	CP	-	-	mb		[3]
Lmo2068	Chaperonin GroEL	CP	-	-	mb/se/sn	SecA2	[1,2,3,7,8]
Lmo2069	Chaperonin GroES	CP	-	-	sn		[1,2]
Lmo2103	Phosphotransacetylase, Pta	CP	-	-	sn		[2]
Lmo2110	Mannose-6-phosphate isomerase	CP	-	-	se	SecA2	[8]
Lmo2114	ABC-type antimicrobial peptide transport system, ATPase	CP	-	-	mb		[3]
Lmo2118	Phosphoglucosamine mutase	CP	-	-	mb		[3]
Lmo2139	ABC-type multidrug transport system, ATPase	CP	-	-	mb		[3]
Lmo2154	Ribonucleotide-diphosphate reductase, β subunit	CP	-	-	mb		[3]
Lmo2192	ABC-type oligopeptide transport system, ATPase	CP	-	-	mb		[3]
Lmo2193	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase	CP	-	-	mb		[3]
Lmo2206	Chaperonin ClpB	CP	-	-	mb		[3]
Lmo2215	ABC-type multidrug transport system, ATPase	CP	-	-	mb		[3]
Lmo2248	Phosphate transport regulator, distant homolog of PhoU	CP	-	-	mb		[3]
Lmo2296	Bacteriophage coat protein	CP	-	-	sn		[2]
Lmo2323	Protein of unknown function	CP	-	-	mb		[3]
Lmo2332	Resolvase	CP	-	-	mb		[3]
Lmo2372	ABC-type antimicrobial peptide transport system, ATPase	CP	-	-	mb		[3]
Lmo2389	NADH dehydrogenase, FAD-containing subunit	CP	-	-	mb		[3]

Lmo2391	Nucleoside-diphosphate-sugar epimerase	CP	-	-	mb		[3]
Lmo2411	ABC-type transport system involved in Fe-S cluster assembly, permease, SufB	CP	-	-	mb		[3]
Lmo2414	ABC-type transport system involved in Fe-S cluster assembly, permease	CP	-	-	mb		[3]
Lmo2415	SUF system FeS cluster assembly, SufC, ATPase	CP	-	-	mb/se		[3,7]
Lmo2419	ABC-type metal ion transport system, ATPase	CP	-	-	mb		[3]
Lmo2425	Glycine cleavage H-protein, GcvH	CP	-	-	se/sn		[2,7]
Lmo2455	Enolase	CP	-	-	mb/se/sn	SecA2	[1,2,3,7,8]
Lmo2456	Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	CP	-	-	mb/sn		[1,3,7]
Lmo2458	Phosphoglycerate kinase	CP	-	-	se/sn		[1,2,7]
Lmo2459	Glyceraldehyde-3-phosphate dehydrogenase	CP	-	-	mb/se/sn		[1,2,3,7]
Lmo2468	Peptidase S14, ClpP	CP	-	-	mb		[3]
Lmo2507	Cell division ATP-binding protein, FtsE	CP	-	-	mb		[3]
Lmo2510	Sec translocase, ATPase, SecA	CP	-	-	mb		[3]
Lmo2511	Ribosomal protein S30Ae/σ54 modulation protein	CP	-	-	se/sn		[1,7]
Lmo2525	Cell shape determining protein MreB/Mrl	CP	-	-	mb		[3]
Lmo2528	F_0F_1 ATP synthase, subunit ε	CP	-	-	mb		[3]
Lmo2529	F_0F_1 ATP synthase, subunit β	CP	-	-	mb		[3]
Lmo2530	F_0F_1 ATP synthase, subunit γ	CP	-	-	mb		[3]
Lmo2531	F_0F_1 ATP synthase, subunit α	CP	-	-	mb		[3]
Lmo2532	F_0F_1 ATP synthase, subunit δ	CP	-	-	mb		[3]
Lmo2539	Glycine/serine hydroxymethyltransferase, GlyA	CP	-	-	mb/sn		[2,3]
Lmo2554	Glycosyl transferase, family 1	CP	-	-	mb		[3]
Lmo2556	Fructose/tagatose/ketose bisphosphate aldolase class II, FbaA	CP	-	-	se		[7]
Lmo2557	Sphingosine kinase	CP	-	-	mb		[3]
Lmo2580	ABC-type peptide transport system, ATPase	CP	-	-	mb		[3]
Lmo2596	30S ribosomal protein S9	CP	-	-	mb/se	SecA2	[3,8]
Lmo2597	50S ribosomal protein L13, RplM	CP	-	-	mb		[3]
Lmo2600	ABC-type cobalt transport system, ATPase	CP	-	-	mb		[3]
Lmo2601	ABC-type cobalt transport system, ATPase	CP	-	-	mb		[3]
Lmo2605	50S ribosomal protein L17	CP	-	-	mb		[3]
Lmo2613	50S ribosomal protein L15	CP	-	-	mb		[3]
Lmo2615	30S ribosomal protein S5	CP	-	-	mb		[3]
Lmo2618	30S ribosomal protein S8	CP	-	-	mb		[3]
Lmo2620	50S ribosomal protein L5	CP	-	-	mb		[3]
Lmo2626	30S ribosomal protein S3	CP	-	-	mb		[3]
Lmo2627	50S ribosomal protein L22	CP	-	-	mb		[3]
Lmo2629	50S ribosomal protein L2, RplB	CP	-	-	mb		[3]

Lmo2631	50S ribosomal protein L4	CP	-	-	mb/sn		[2,3]
Lmo2632	50S ribosomal protein L3	CP	-	-	mb		[3]
Lmo2653	Elongation factor EF-Tu, TufA	CP	-	-	mb/se/sn	SecA2	[2,3,7,8]
Lmo2654	Translation elongation factor EFG/EF2	CP	-	-	mb/se		[3,7]
Lmo2756	DNA topoisomerase III, TopB	CP	-	-	mb		[3]
Lmo2758	Inositol-5-monophosphate dehydrogenase	CP	-	-	mb		[3]
Lmo2781	Glycoside hydrolase	CP	-	-	mb		[3]
Lmo2785	Catalase, Kat	CP	-	-	mb		[3]
Lmo2792	λ repressor-like, DNA-binding domain	CP	-	-	mb		[3]
Lmo2795	DNA-binding transcriptional regulator with SIS domain	CP	-	-	ec		[2]
Lmo2853	RNA-binding protein	CP	-	-	mb		[3]

[a]Some annotations were corrected respective to the homology search performed as described in the Methods section. More extensive and detailed annotations are available in Table 1S.

[b]Besides exoproteins, parietal proteins (CW-protein) could be categorized between those covalently anchored to the cell wall via a LPXTG domain (LPXTG) and those attached via non-covalent interaction, namely with GW repeat, LysM, WXL or PGBD1 domains. Considering integral membrane protein (IMP), multi-spanning IMP (msIMP) are discriminated from single-spanning IMP (ssIMP), which are further categorized into (i) Type I (ssIMP I), *i.e.* IMP exhibiting a cleavable SP in addition to another TMD positioned upstream, (i) Type II (ssIMP II), *i.e.* IMP exhibiting a signal anchor, which is a TMD with N_{in}-C_{out} orientation (Type I module), that could be an uncleaved SP (Unc), (iii) Type III (ssIMP III), *i.e.* IMP exhibiting an reverse signal-anchor, which is a TMD with N_{out}-C_{in} orientation (Type II module). Proteins exhibiting a lipobox were predicted as lipoproteins (Lipo) anchored to cytoplasmic membrane.

[c]Subcellular location (SCL) follows the GO (Gene Ontology) for cellular component: cytoplasm (CP; GO:0005737), intrinsic to membrane (MB; GO:0031226), cell wall (CW; GO:0009275), cell surface (CS; GO:0009986) and extracellular milieu (EC; GO:0005576).

[d]The predicted routes follow by the secreted proteins are detailed in terms of (i) translocation system, namely Sec translocon, ABC exporter or holin, as well as membrane integration via YidC or secretion via non-classical way (NC), (ii) post-translocational modification, namely cleavage by signal peptidase of Type I (SPase I) or II (SPase II), protein acylation by the prolipoprotein diacylglyceryltransferase Lgt, covalent anchoring to cell wall by sortase A (SrtA) or B (SrtB), (iii) presence of a glycine residue at position +2 from the cleavage site (C+2=G).

[e]The signal peptide (SP) are differentiated between SP of Type I (I), Type II (II), signal anchor (SA), *i.e.* uncleaved signal peptide, or SP targeting protein to ABC exporter. The position +1 from the cleavage site (C+1) is given in bracket.

[f]Whenever possible, fraction of bacterial cell where the protein was experimentally identified follows the GO (Gene Ontology) for cellular component: membrane fraction (mb; GO:005624), cell wall fraction (cw), surface exposed fraction (se) and supernatant (sn). Asterisk indicates protein identified in supernatant of an isogenic mutant for the Lgt maturation pathway resulting in the release of lipoprotein into the extracellular milieu [25].

[g]Experimental information on the secretion route is provided for SecA2-dependent protein secretion, SP cleavage by signal peptidase of Type I (SPase I) SipX and/or SipZ, or signal peptidase of Type II (SPase II) LspA, covalent protein anchoring to cell wall by sortase A (SrtA) or B (SrtB), non-covalent attchment to cell wall *via* GW repeats, or protein anchoring to the cytoplasmic membrane by the prolipoprotein diacylglyceryltransferase Lgt.

[h]Experimental identification of cleavable SP is provided with the position +1 from the cleavage site (C+1).

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3. Implication de la voie SecA2 dans la formation de biofilm chez *Listeria monocytogenes*

Les protéines sécrétées par le système Sec sont transloquées à travers la membrane cytoplasmique par l'intermédiaire d'une translocase. Cette translocase est composée du translocon SecYEG-SecDF-YajD et d'une ATPase SecA qui fournit l'énergie nécessaire à la translocation. Contrairement à SecA, son paralogue nommé SecA2 n'est pas essentiel à la viabilité cellulaire et formerait, en association avec le translocon Sec, une voie de sécrétion alternative. L'inactivation de la voie SecA2 conduit à la formation de longs filaments cellulaires et de colonies qualifiées de rugueuses. Ce morphotype est en grande partie dû à la réduction de la sécrétion de deux hydrolases pariétales, CwhA et MurA, impliquées dans la division cellulaire. De plus, la délétion simultanée des gènes codant ces deux hydrolases restaure le phénotype rugueux. L'apparition spontanée d'un tel morphotype chez *L. monocytogenes*, et notamment lors de la formation d'un biofilm, nous a conduits à nous interroger sur le rôle de la voie SecA2 et des deux principales protéines sécrétées de façon SecA2-dépendante, CwhA et MurA, dans la formation de biofilm.

Afin de répondre à cette question, les mutants de délétion Δ*secA2*, Δ*murA*, Δ*cwhA* et Δ*murA*Δ*cwhA* ont été construits et testés pour leur aptitude à former un biofilm par comparaison avec la souche sauvage. Diverses techniques ont été mises en œuvre afin d'étudier les différentes phases de formation de biofilm : (i) le Biofilm Ring Test pour les phases précoces ainsi que (ii) la coloration au cristal violet pour les phases tardives de formation de biofilm. L'adhésion initiale des différentes souches a également été testée en condition statique et dynamique, par coloration au cristal violet et par des observations en microscopie, respectivement. L'architecture des biofilms a été caractérisée en microscopie confocale, également en condition statique et dynamique.

Le morphotype rugueux ayant été isolé au sein de biofilms chez *L. monocytogenes*, nous nous sommes interrogés sur les conséquences de l'apparition de longs filaments cellulaires au sein d'un biofilm. Des essais de formation de biofilm ont par conséquent été effectués en co-cultures.

Dans cette partie du travail, l'implication de la voie SecA2 dans la formation de biofilm a été plus particulièrement étudiée, mais également celle des deux hydrolases pariétales responsables du phénotype rugueux. Néanmoins, la voie SecA2 ne se limite pas à la sécrétion de ces deux protéines. En effet, une première étude, réalisée par électrophorèse

monodimensionnelle, a permis d'identifier un faible nombre des protéines sécrétées de façon SecA2-dépendante (Lenz 2003). Les auteurs ont caractérisé 7 protéines dans le milieu extracellulaire, parmi lesquelles 4 possèdent un peptide signal (SP), dont CwhA et MurA, mais pas les 3 autres. Comme à l'heure actuelle, il est impossible de prédire les protéines sécrétées par la voie SecA2, la recherche de ces protéines SecA2 dépendante a été appronfondie en réalisant une analyse comparative par électrophorèse bidimensionnelle de l'exoprotéome de la souche sauvage et du mutant *ΔsecA2* cultivés à 20°C et à 37°C.

ARTICLE n°2

Sandra RENIER, Nelly CACCIA, Julien DESCHAMPS, Julie SZLAVIK, Susan JOYCE, Magdalena POPOWSKA, Colin HILL, Susanne KNØCHEL, Romain BRIANDET, Michel HÉBRAUD et Mickaël DESVAUX.

The SecA2 Protein Export Pathway Impacts Biofilm Formation in *Listeria monocytogenes*

Pour publication dans *Environmental Microbiology*

The SecA2 protein export pathway impacts biofilm formation in *Listeria monocytogenes*

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Running title: SecA2 impacts biofilm formation in *L. monocytogenes*

Keywords: *Listeria monocytogenes*, Biofilm formation, Bacterial adhesion, SecA2 translocase, protein secretion system, Cell-wall hydrolase.

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ABSTRACT

Listeria monocytogenes presents a dichotomic lifestyle as an ubiquitous saprophytic species and an opportunistic foodborne pathogen. Besides its capacity to grow in a wide range of environmental stressful conditions, *L. monocytogenes* has the ability to adhere and colonize surfaces. Morphotype variation to long-chain cells forming rough colonies has been reported from different isolates, including biofilms, and associated with *secA2*. This SecA paralogue is not essential and would form an alternative translocase associated to the Sec pathway. The morphotype modification is mainly attributed to a lower amount of two SecA2-dependent secreted cell-wall hydrolases, CwhA and MurA. Investigating for the first time the involvement of the SecA2 protein export pathway in biofilm formation, it first appeared that its inactivation leads to the formation of thick and rough aerial biofilm but with lower surface coverage. Compared to the wild type strain, this morphotype enhances the listerial biofilm formation in environmental conditions. Simultaneous deletion of *murA* and *cwhA* appears to be sufficient to induce the Δ*secA2* biofilm morphotype. Regulation of *L. monocytogenes* morphotype by *secA2* and its consequences on bacterial physiology, especially surface colonization are further discussed.

INTRODUCTION

Listeria monocytogenes is an opportunistic pathogen Gram-positive bacterium involved in several foodborne disease outbreaks. Infection mainly occurs after ingestion of contaminated food (Gahan and Collins, 1991) and affects predominantly pregnant women, neonates, the elderly and immunocompromised patients (Farber and Peterkin 1991). In the food industry, *L. monocytogenes* represents a major problem as a source of contamination of raw and processed foods. Besides its ability to grow in a wide range of pH (4.3 to 9.6), temperature (1 to 45°C), salt concentration (up to 10% NaCl) and water activity (up to Aw of 0.93), *L. monocytogenes* adheres and colonizes working surfaces, which contributes to its strong resistance to technological treatments and environmental stresses (Carpentier and Cerf 2011). While different definitions can be found in the literature (Dunne 2002), a biofilm can be broadly defined as the sessile development of microbial cells. The cells adhering to each other and/or to surface or interface are generally surrounded by a matrix of extracellular polymers (Kolter 2005). In *L. monocytogenes*, such an exopolysaccharide matrix has never been confirmed (Nilsson, Ross et al. 2011; Renier, Hebraud et al. 2011) but, in certain growth conditions, extracellular DNA could play a role in adhesion and early stages of biofilm formation (Harmsen, Lappann et al. 2010). Actually, cell-surface proteins would rather be the major adhesion factors concurring to biofilm formation (Smoot and Pierson 1998; Longhi, Scoarughi et al. 2008; Franciosa, Maugliani et al. 2009) especially the flagella and some other proteins, which identity remains elusive (Renier, Hebraud et al. 2011).

In any case, cell-surface displayed proteins in monoderm bacteria need to be first translocated across the cytoplasmic membrane (Desvaux, Dumas et al. 2006; Desvaux, Hebraud et al. 2009). From the most recent proteogenomic analyses and with 714 proteins exhibiting an N-terminal signal peptide (Renier, Micheau et al. 2012), the Sec (Secretion) pathway appears as the main route for protein secretion in *L. monocytogenes* (Desvaux and Hebraud 2006). The Sec translocase, composed of the transmembranar SecYEG translocon and the peripheral SecA ATPase, is essential for the passage of preproteins through the cytoplasmic membrane (Du Plessis, Nouwen et al. 2011). Like in some other Gram-positive pathogenic bacteria, a paralogue of SecA, named SecA2, has been uncovered in *L. monocytogenes*. Upon deletion of the *secA2* gene, cell morphotype is changing from discrete cells forming smooth colonies in *L. monocytogenes* wild type (*wt*) to long-chain cells forming rough colonies (Lenz and Portnoy 2002). Similar morphotypes has been isolated from clinical patients, food samples and biofilms (Rowan, Candlish et al. 2000; Monk, Cook et al. 2004), and reversible conversion has been observed upon acid-, temperature- and salt-

induced stress environment (Brzin 1975; Bereksi, Gavini et al. 2002; Geng, Kim et al. 2003; Hazeleger, Dalvoorde et al. 2006; Giotis, Blair et al. 2007). In *L. monocytogenes* Δ*secA2* mutant, the phenotype modification is mainly attributed to a lower amount of two extracellular cell-wall hydrolases, namely CwhA (cell-wall hydrolase A), also called Iap (invasion associated protein) or p60 (protein of 60 kDa), and MurA (muramidase A), also called NamA (N-acetylmuramidase A) (Lenz, Mohammadi et al. 2003; Machata, Hain et al. 2005). Simultaneous deletion of *cwhA* and *murA* leads to rough morphotype (Machata, Hain et al. 2005). Recently, a study showed that strains lacking the *divIVA* gene also form rough colonies (Halbedel, Hahn et al. 2012). Actually, DivIVA is involved in the recruitment of CwhA and MurA to the cell poles prior to export in a SecA2-depedent manner.

While rough colony variants and/or related mutants have been extensively studied with regards to their virulence level, a few investigations were dedicated to their biofilm formation ability. Contradictorily, rough colony isolates variants were shown to give enhanced biofilm formation (Monk, Cook et al. 2004), whereas deletion of *divIVA* led to defective sessile development (Halbedel, Hahn et al. 2012). Despite the important role of SecA2 and the two associated cell-wall hydrolases CwhA and MurA in the conversion to the rough colony morphotype, their contribution in biofilm formation has never been questioned as yet. This prompted us to investigate the involvement of the SecA2 protein export pathway at different steps of biofilm formation and under different environmental conditions relevant to the physiology of *L. monocytogenes*, especially the growth temperature.

RESULTS

The deletion of the secA2 gene enhances the colonization of surfaces at 20°C but leads to the formation of a fragile biofilm

The involvement of the SecA2 pathway in *L. monocytogenes* biofilm formation was investigated at temperatures relevant to its pathogenic and saprophytic lifestyles, i.e. 37°C, the temperature encountered in the course of a human infection, and 20°C, a standard ambient temperature. Early and later stages of sessile development were considered and evaluated by the BioFilm Ring Test (BRT) (Chavant, Gaillard-Martinie et al. 2007) and the crystal violet methods (Borucki, Peppin et al. 2003), respectively.

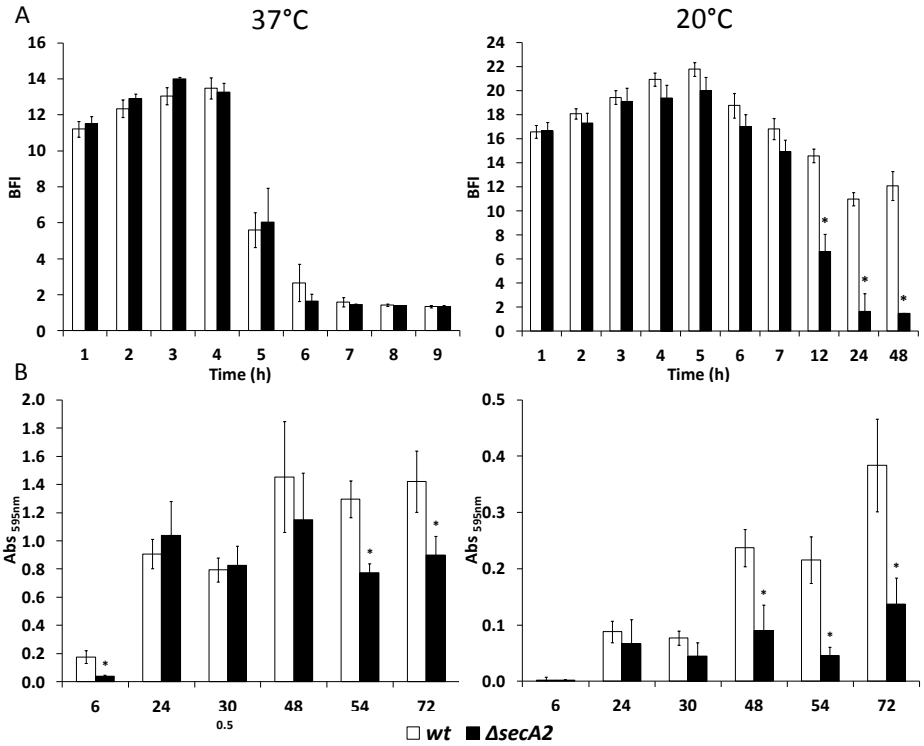

Figure 1: Biofilm formation of *L. monocytogenes* EGD-e *wt* and the isogenic mutant Δ*secA2* at 37°C and 20°C. (A) Biofilm formation at early stages of sessile development assayed with the BRT. (B) Biofilm formation at late stages of sessile development assayed with the crystal violet method. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

At 37°C, deletion of the *secA2* gene had no impact in the early stages of biofilm formation as both the *wt* and the Δ*secA2* mutant strains blocked the microbeads (BioFilm Index [BFI] <2) from and after 6 hours of incubation (Fig. 1A). While no significant difference could be either observed up to 48 hours sessile growth (Fig. 1B), the amount of sessile biomass for the Δ*secA2* strain was significantly reduced from and after 54 h. When the

experiments were performed at 20°C, the results considerably changed since the microbeads remained unblocked until 48 h incubation with *L. monocytogenes wt* (Fig. 1A). Following sessile development with the crystal violet method and compared to 37°C, biofilm formation ability was impaired for the *wt* strain (Fig. 1B). Regarding the Δ*secA2* mutant, microbeads were partially (12 h) and then completely blocked but only from and after 24 h of incubation at 20°C (Fig. 1A). While the Δ*secA2* mutant blocked the microbeads earlier than *L. monocytogenes wt* at 20°C, the sessile biomass of both strains were approximately the same during the first 30 h and even appeared significantly lower with the Δ*secA2* mutant from and after 48 h of biofilm development (Fig. 1B). This would suggest the bacterial cells of the Δ*secA2* mutant are less adherent than *L. monocytogenes wt*; while bacteria blocked the microbeads in BRT, cells are detached during the washing steps of the crystal violet method. Bacterial adhesion assays performed at both temperatures in static (Fig. 2A) and dynamic (Fig. 2B) conditions showed a significantly reduced adhesion at 37°C and an almost absence of adhesion at 20°C for the isogenic *L. monocytogenes* Δ*secA2* mutant compared to the *wt*. Therefore, it seems the deletion of the *secA2* gene enhances the surface colonization but leads to the formation of a fragile, poorly cohesive biofilm at 20°C as the sessile biomass is more easily detached.

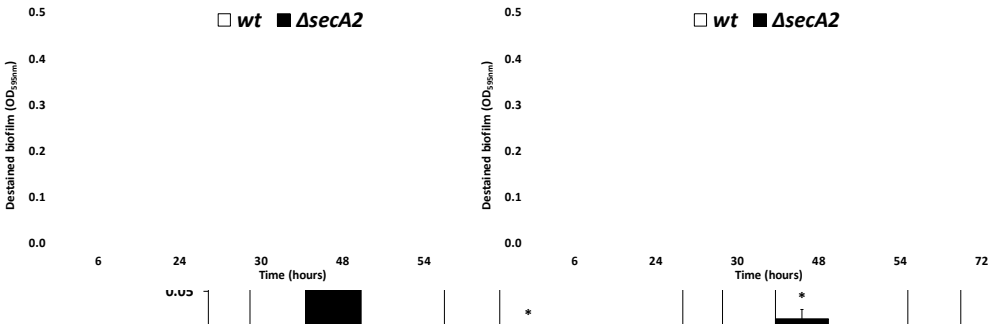

Figure 2: Bacterial adhesion of *L. monocytogenes* EGD-e *wt* and the isogenic mutant Δ*secA2* at 37°C and 20°C. (A) Initial adhesion assay based on crystal violet staining. (B) Bacterial adhesion assayed under liquid flow. Statistical significance of the results is indicated by an asterisk ($P < 0.05$). IAR: initial adherent rate.

Simultaneous deletion of murA and cwhA genes exacerbates the ΔsecA2 biofilm phenotype

In order to investigate the involvement of the two major proteins secreted by the SecA2 pathway in biofilm formation and bacterial adhesion, the Δ*murA,* Δ*cwhA* and Δ*murA*Δ*cwhA* mutants were compared to the *wt*.

Deletion of the *murA* or *cwhA* gene were both reported to lead to the formation of chain cells but at a much lesser extent than cells of the *L. monocytogenes* Δ*secA2* mutant (Machata,

Hain et al. 2005). Though, their behavior towards biofilm formation and bacterial adhesion differed significantly (Fig. 3 and 4). Indeed, at 37°C the Δ*murA* mutant blocked the microbeads in the BRT later, whereas the Δ*cwhA* mutant blocked them earlier than the *wt* strain (Fig. 3A).

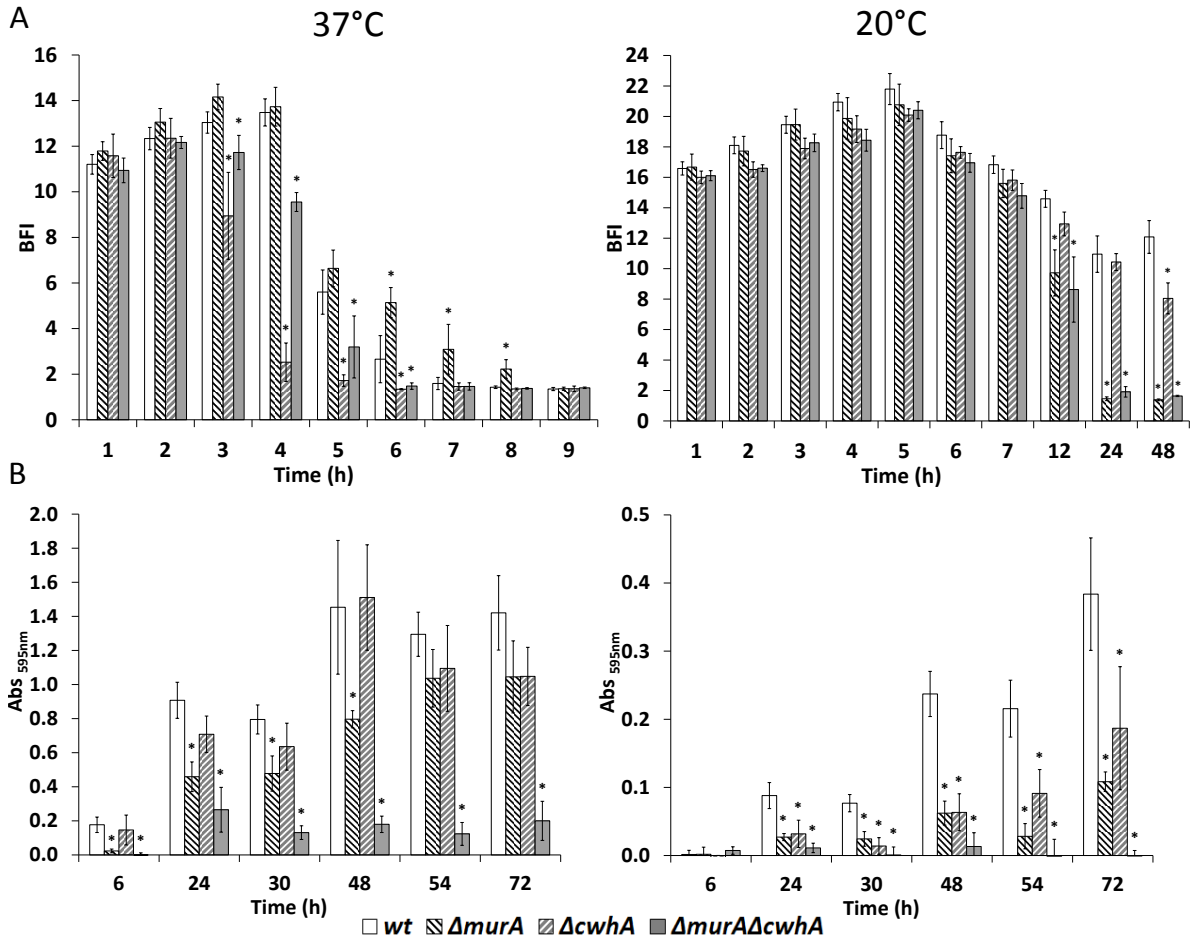

Figure 3: Biofilm formation of *L. monocytogenes* EGD-e *wt* and the isogenic mutants Δ*murA,* Δ*cwhA* and Δ*murA*Δ*cwhA* at 37°C and 20°C. (A) Biofilm formation at early stages of sessile development assayed with the BRT. (B) Biofilm formation at late stages of sessile development assayed with the crystal violet method. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

As follow by the crystal violet method, the sessile biomass was significantly reduced until 48 h in the course of biofilm formation for the isogenic *L. monocytogenes* Δ*murA* mutant but no difference was observed for Δ*cwhA* mutant compared to *L. monocytogenes wt* at 37°C (Fig. 3B). Regarding bacterial adhesion, no difference under static or dynamic conditions could be observed for the Δ*murA* mutant, whereas it was significantly decreased upon deletion of *cwhA* at 37°C (Fig. 4).

With the BRT at 20°C, the Δ*murA* mutant behaved as the Δ*secA2* strain by completely blocking the microbeads from and after 24 h incubation, whereas deletion of *cwhA* did not impact the early stages of sessile development as the microbeads remained unblocked like

L. monocytogenes wt until 48 h (Fig. 3A). Whatever the deletion of *murA* or *cwhA*, the sessile biomass was significantly reduced during the entire kinetics of biofilm formation compared to the *wt* strain (Fig. 3B). This was associated with a severely decreased bacterial adhesion in both static and dynamic conditions for the Δ*murA* mutant (Fig. 4). Despite the initial adhesion was similar to the *wt* strain, the rate of initial attachment (IAR) was nevertheless significantly reduced under a liquid flow with the Δ*cwhA* mutant.

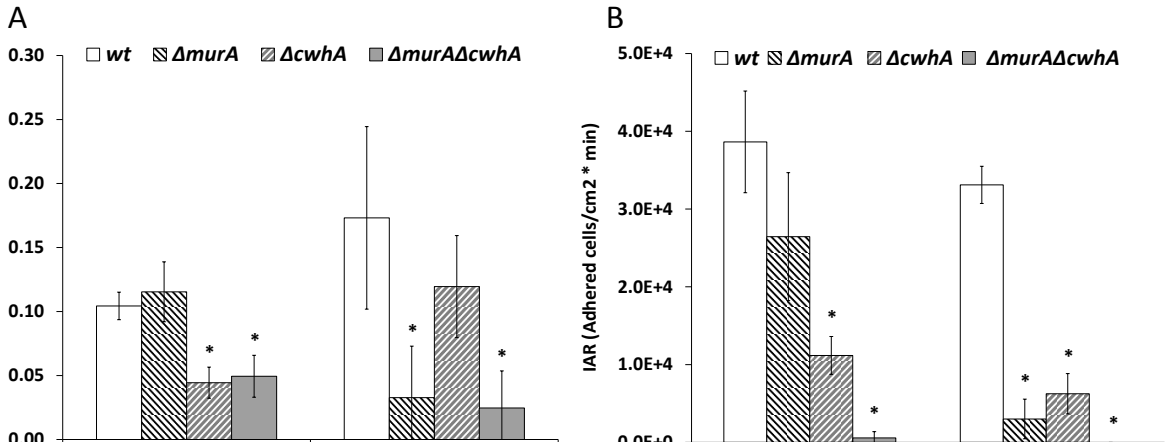

Figure 4: Bacterial adhesion of *L. monocytogenes* EGD-e *wt* and the isogenic mutants Δ*murA,* Δ*cwhA* and Δ*murA*Δ*cwhA* at 37°C and 20°C. (A) Initial adhesion assay based on crystal violet staining. (B) Bacterial adhesion assayed under liquid flow. Statistical significance of the results is indicated by an asterisk ($P < 0.05$). IAR: initial adherent rate.

Simultaneous deletion of *murA* and *cwhA* was previously shown to lead to the formation of filamentous cells and rough colonies similar to the Δ*secA2* mutant (Machata, Hain et al. 2005). From the BRT, microbeads are blocked earlier than the *wt* strains both at 37 and 20°C (Fig. 3A). The sessile biomass of the Δ*murA*Δ*cwhA* strain, however, was severely reduced at 37°C and almost absent at 20°C (Fig. 3B). Actually, initial bacterial adhesion at 37 and 20°C was once again affected by the deletion of both genes as attachment was much lower and almost undetectable under a liquid flow when compared the *wt* strain (Fig. 4). The simultaneous deletion of *murA* and *cwhA* genes provokes a more pronounced effect on *L. monocytogenes* initial adhesion and biofilm formation as previously observed with the Δ*secA2* mutant (Fig. 1 and 2).

Deletion of the secA2 gene, or simultaneous deletion of the murA and cwhA genes, changes dramatically the architecture of L. monocytogenes biofilm

The biofilm spatial organization of the *L. monocytogenes wt* and mutants were compared under static condition using confocal laser scanning microscope (CLSM). After

24 h of sessile growth at 37°C, the *wt* strain formed a biofilm that covered almost entirely and homogeneously the surface with an average thickness of 30 µm (Fig. 5A and C).

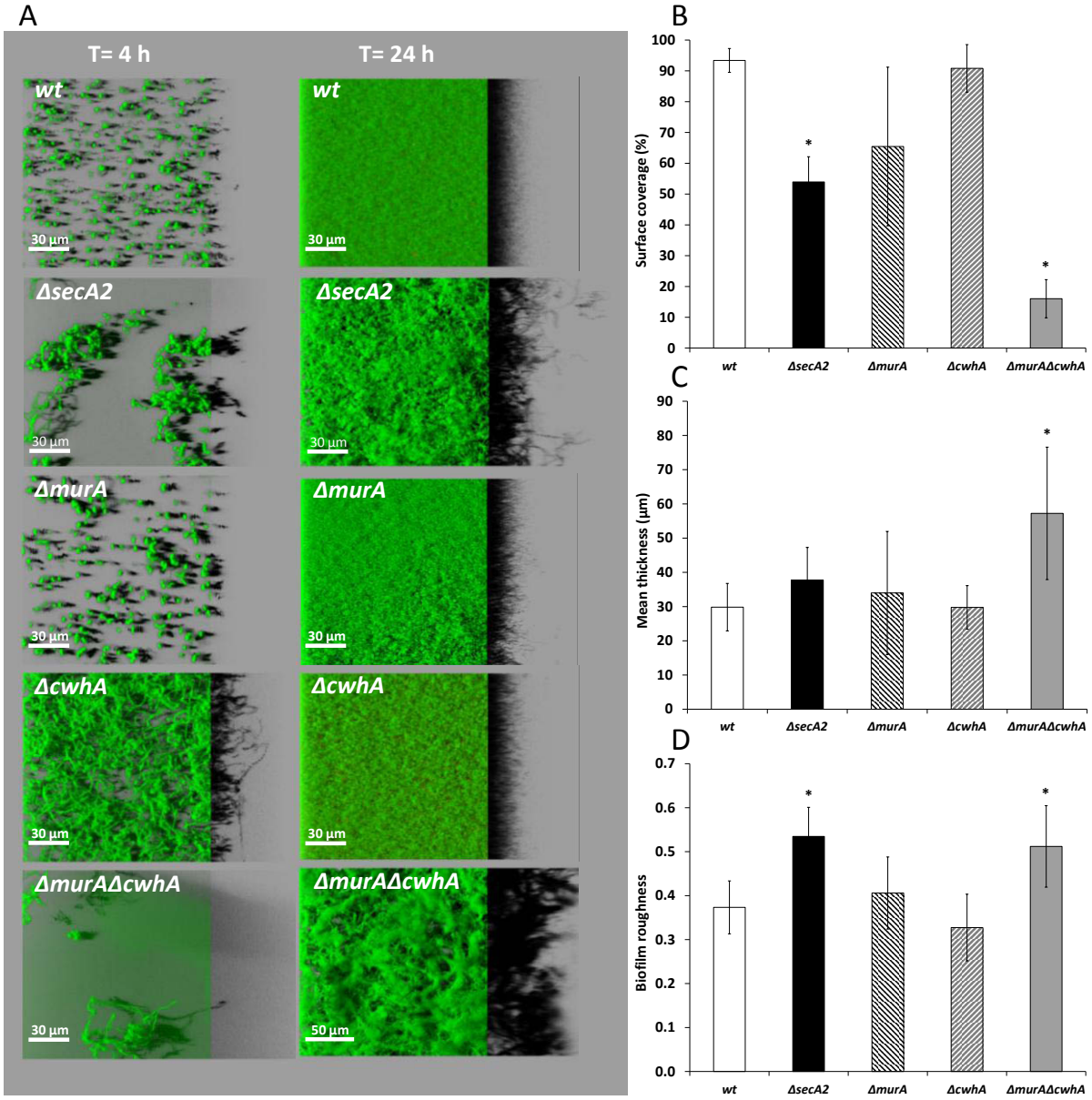

Figure 5: Biofilm formation of *L. monocytogenes wt* and its Δ*secA2*, Δ*murA*, Δ*cwhA* and Δ*murA*Δ*cwhA* mutants bearing pNF8 under static condition at 37°C. (A) CLSM images. The side view projections were acquired after 4 h and 24 h of biofilms development. (B) Surface coverage, (C) thickness and (D) roughness were obtained on biofilms of the *wt* and mutant strains after 24 h of growth and resulted from image analyses as described in the Experimental Procedures. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

On the contrary, the Δ*secA2* mutant formed a porous biofilm with aerial structures (Fig. 5A), which surface coverage is heterogeneous and frankly reduced (Fig. 5B). While no significant difference could be evidenced between the biofilm thickness of the *wt* and Δ*secA2* strains, the biofilm roughness for the Δ*secA2* mutant is significantly higher than the one for *L. monocytogenes wt* (Fig. 5D). The single deletion of *murA* or *cwhA* gene did not affect the

architecture of *L. monocytogenes* biofilm (Fig. 5). Of notice, the surface coverage at 4 h of sessile development was significantly higher (35 %) for Δ*cwhA* mutants when compared to *L. monocytogenes wt* (3 %) (Fig. 5A), which correlated with previous observations using the BRT (Fig. 3A). Image analyses of CLSM observations confirmed previous data about the exacerbated effect of the simultaneous deletion of *murA* and *cwhA* over Δ*secA2* biofilm phenotype, which led to the formation of a biofilm with even more aerial structures (Fig. 5A). Compared to *L. monocytogenes wt*, (i) surface coverage was significantly reduced and lesser than 20% (Fig. 5B), (ii) the average thickness was higher and could reach up to 75 µm (Fig. 5C), as well as (iii) the biofilm roughness (Fig. 5D). At 37°C in dynamic conditions using flow cells, a similar trend could be observed from CLSM image analyses when comparing the biofilms of the Δ*secA2* or Δ*murA*Δ*cwhA* mutant strains with *L. monocytogenes wt* (Fig. 6A). The surface coverage was significantly reduced and even more for the double mutant (Fig. 6B). Biofilms were significantly rougher and thicker for Δ*murA*Δ*cwhA* whereas the thickness did not significantly differ for Δ*secA2*. Altogether, these results confirm previous observations at early and later stage of biofilm development as well as bacterial adhesion in static and dynamic conditions.

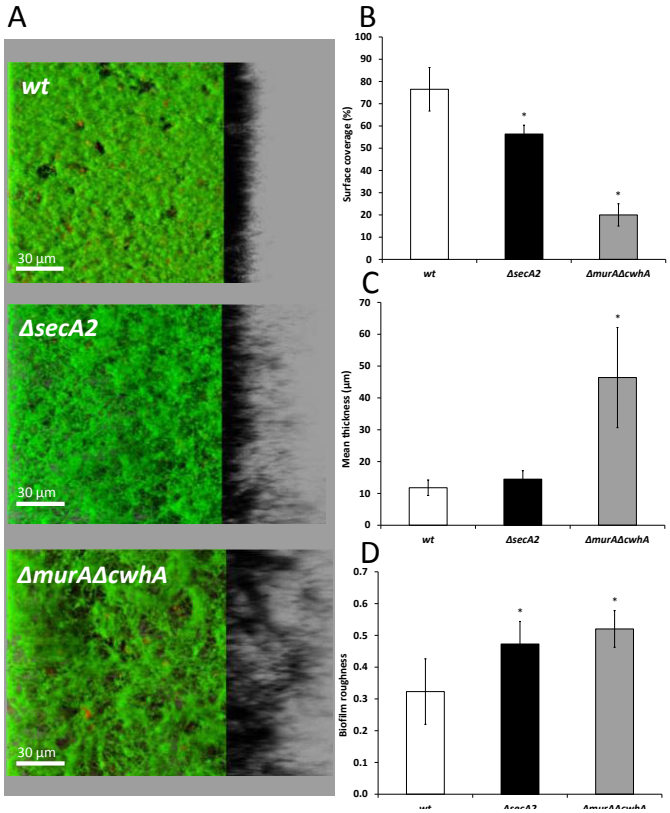

Figure 6: Biofilm formation of *L. monocytogenes wt* and its Δ*secA2* and Δ*murA*Δ*cwhA* mutants bearing pNF8, after 24 h growth at 37°C under flowing condition. (A) CLSM images. (B) Surface coverage, (C) thickness and (D) roughness resulted from image analyses as described in the Experimental Procedures. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

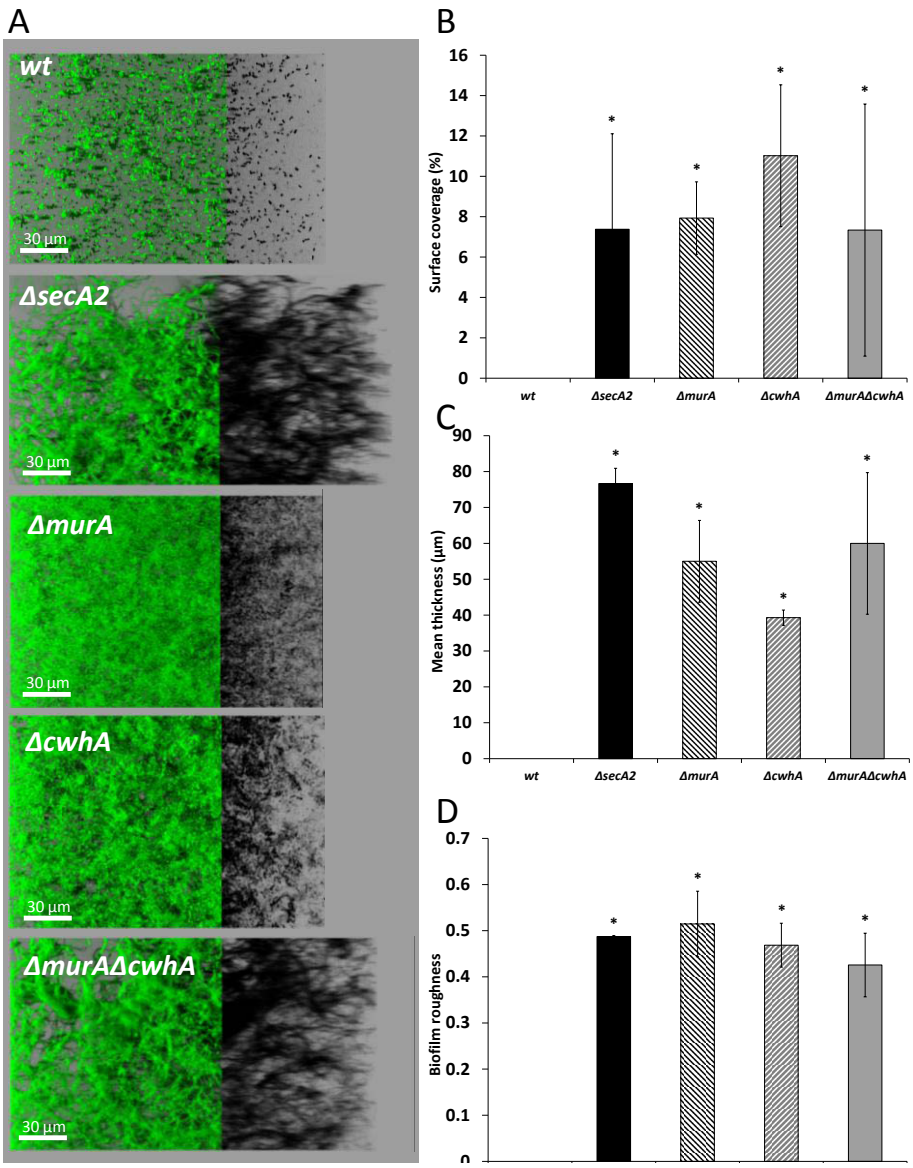

Figure 7: Biofilm formation of *L. monocytogenes wt*, Δ*secA2*, Δ*murA*, Δ*cwhA* and Δ*murA*Δ*cwhA* mutants bearing pNF8 under static condition at 20°C. (A) CLSM images. The side view projections were acquired after 4 h and 24 h of biofilm development. (B) Surface coverage, (C) thickness resulted from image analyses as described in the Experimental Procedures.

After 24 hours of incubation at 20°C, CLSM observation in static conditions revealed that the *wt* did not form biofilm, instead the cells were highly motile (Fig. 7A). While connected to the surface by a reduced proportion of cells as surface coverage did not exceed 10 % for all the mutants (Fig. 7B), interestingly enough, the *L. monocytogenes* Δ*secA2* and Δ*murA*Δ*cwhA* mutants formed an aerial biofilm even thicker in average than the ones observed at 37°C, i.e. 77 µm and 60 µm respectively (Fig. 7C). Under dynamic conditions, *L. monocytogenes wt* did not form a biofilm after 48 h of flow at 20°C contrary to Δ*secA2* and Δ*murA*Δ*cwhA* mutants (Fig. 8A). Biofilm formation of the mutant strains was irregular as it only appeared in some

area of the flow cell (Fig. 8B). While the roughness was similar, the mean thickness was significantly higher than the ones observed at 37°C (Fig. 8C and 8D).

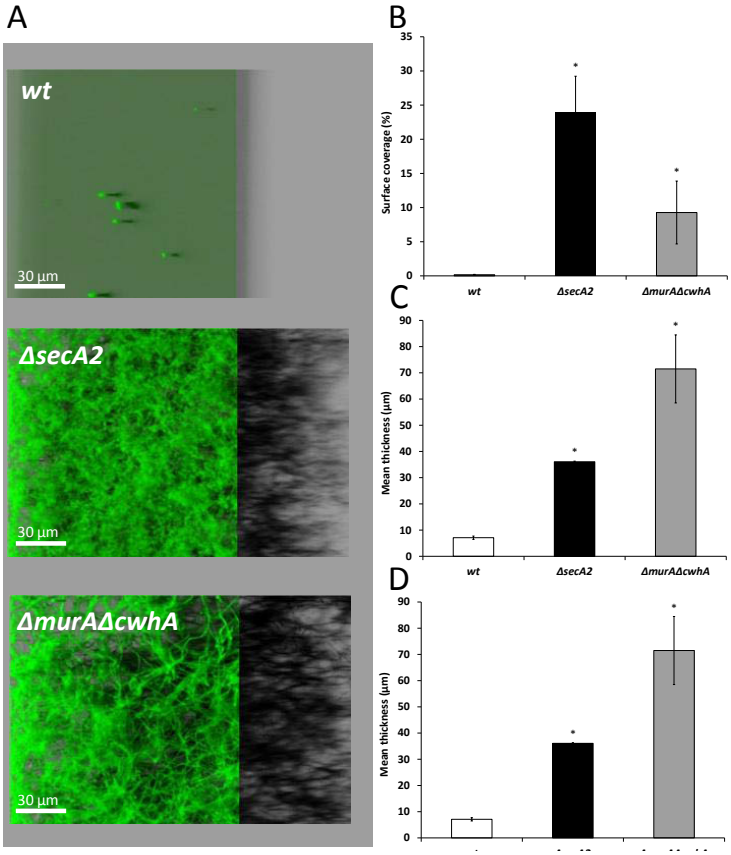

Figure 8: Biofilm formation of *L. monocytogenes wt* and its Δ*secA2* and Δ*murA*Δ*cwhA* mutants bearing pNF8 after 48 h growth at 20°C under flowing condition at 20°C. (A) CLSM images, (B) Surface coverage, (C) thickness and (D) roughness resulted from image analyses as described in the Experimental Procedures. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

Mixed cultures lead to the formation of an aerial biofilm

Considering the rough morphotype was previously reported and isolated from *L. monocytogenes* biofilms (Monk, Cook et al. 2004), its contribution to sessile development was questioned by setting co-culture experiments of *L. monocytogenes wt* with Δ*secA2* or Δ*murA*Δ*cwhA* mutants respectively. While the amount of sessile biomass changed significantly over 48 h of incubation at 37°C, no difference could be observed at 20°C (Fig. 9). At 37°C, CLSM observations revealed that mixed biofilms displayed an aerial structure with uniform repartition of the *wt* and mutant strains (Fig. 10 and 5). On the contrary, the co-culture biofilms at 20°C were exclusively formed by the mutant cells, i.e. either Δ*secA2* or Δ*murA*Δ*cwhA* strain, and only few scattered red cells. This shows the inability of *L. monocytogenes wt* to establish themselves within the biofilm most certainly as result of the motility (Fig. 7). Whatever the temperature, the morphotype of those co-culture

biofilm resemble the one observed in single culture of Δ*secA2* or Δ*murA*Δ*cwhA* strain, that is the formation of aerial architecture (Fig. 5 and 7).

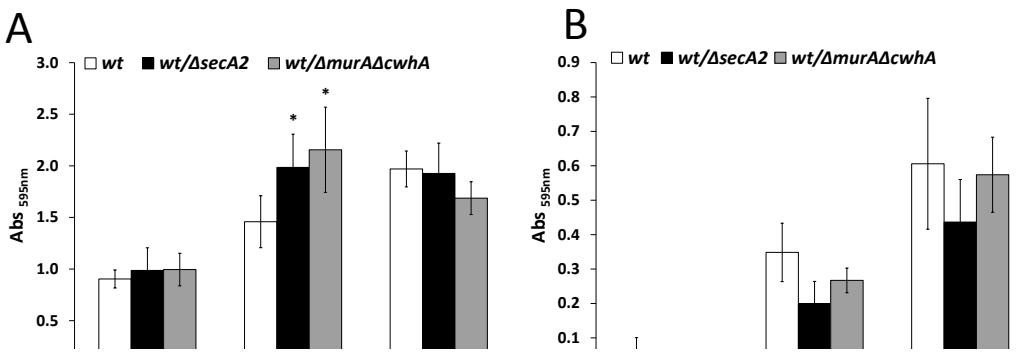

Figure 9: Biofilm formation of *L. monocytogenes* EGD-e *wt* and mixed cultures of *wt*/Δ*secA2* and *wt*/Δ*murA*Δ*cwhA* at late stages of sessile development assayed with the crystal violet method at 37°C (A) and 20°C (B). Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

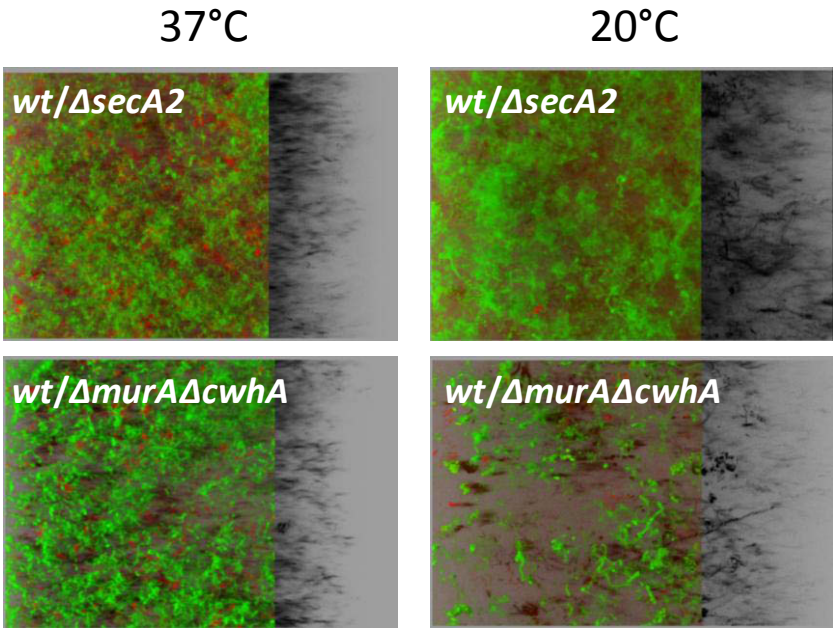

Figure 10: LCMS images of mixed cultures of *wt*/Δ*secA2* and *wt*/Δ*murA*Δ*cwhA* after 24 h growth at 37°C and 20°C. *L. monocytogenes wt* was colored in red. ΔsecA2 and Δ*murA*Δ*cwhA* mutant strains were colored in green.

DISCUSSION

Considering its importance in listerial physiology in term of genetic expression, especially virulence factors (Toledo-Arana, Dussurget et al. 2009; de las Heras, Cain et al. 2011) but also biofilm formation (Chavant, Martinie et al. 2002; Moltz and Martin 2005; Lemon, Freitag et al. 2010) *L. monocytogenes* was here investigated at temperatures relevant to its ecological niche oscillating between saprophytism and human pathogen (Gray, Freitag et al. 2006; Freitag, Port et al. 2009). From the present investigation, the temperature appeared to have a great influence on biofilm formation of rough-colony forming *L. monocytogenes*, namely deficient in the SecA2 pathway or in its two main substrates, i.e. the cell-wall hydrolases CwhA and MurA.

At 37°C and compared to the *wt* strain, biofilms of *L. monocytogenes* Δ*secA2* or Δ*murA*Δ*cwhA* were not as covering and actually detached more easily due to their lower adherence ability. When settled, however, the biofilms in static or dynamic conditions were rougher, and in the case of *L. monocytogenes* Δ*murA*Δ*cwhA*, much thicker. At 20°C, *L. monocytogenes* Δ*secA2* or Δ*murA*Δ*cwhA* formed biofilms in both static and dynamic conditions contrary to the *wt* strain. These biofilms were as thick and rough as the biofilms observed at 37°C but they detached easily and were not as covering. When *L. monocytogenes wt* is co-cultured with *L. monocytogenes* Δ*secA2* or Δ*murA*Δ*cwhA* strain at 37°C, they both participate to biofilm formation, which result in an denser and aerial architecture. When co-cultured with *L. monocytogenes wt* at 20°C, *L. monocytogenes* Δ*secA2* or Δ*murA*Δ*cwhA* still form a biofilm with aerial architecture but the *wt* strain still do not contribute to biofilm formation.

The deletion of both cell-wall hydrolase genes, *murA* and *cwhA*, known to be secreted by the SecA2 pathway is sufficient to restore the rough morphotype and biofilm morphotype. However, Δ*murA*Δ*cwhA* biofilm appeared more fragile than the Δ*secA2* mutant as the adhered biomass is frankly lower after biofilm assay in static condition (Fig. 2B). This may be due to the absence of both hydrolase in the double mutant. Actually, in the Δ*secA2* mutant, the secretion of MurA seems to be totally abolished whereas CwhA secretion is only reduced (Lenz and Portnoy 2002). This dramatically reduction of adhered biomass has been also reported in the listerial isogenic *divIVA* mutant, in which the MurA and CwhA secretion was reduced, leading to the formation of filamentous cells (Halbedel, Hahn et al. 2012). At 20°C, single deletion of *murA* or *cwhA* led to the formation of elongated cells and biofilms. The ability to form biofilm at this temperature would be mostly dependent on the cell morphology

rather than in the absence of MurA or CwhA *per se*. Considering that filamentous cells spontaneously appeared during biofilm formation, we wanted to evaluate its impact by performing mixed-culture biofilms.

While alga-like streamers projecting from stainless steel surface had been previously described upon development of spontaneous rough colony isolate (Monk, Cook et al. 2004), our investigation report a new kind of biofilm architecture in *L. monocytogenes*. Whatever the temperature, the main feature of the *L. monocytogenes* Δ*secA2* or Δ*murA*Δ*cwhA* biofilms was the formation of aerial and fluffy structures, which heterogeneously covered the substrate leading to the formation of a rougher and thicker biofilm than the *wt* biofilm. This highlights the high polymorphism of *L. monocytogenes* biofilm architectures ranging from monolayer, honey-comb, three-dimensional mushroom-shaped and most recently a network of knitted chains (Chae and Schraft 2000; Chavant, Martinie et al. 2002; Borucki, Peppin et al. 2003; Rieu, Briandet et al. 2008). This latter phenotype was shown to be dependent on RecA and YneA (van der Veen and Abee 2010), activated by the SOS response, leading to the elongation of cells and allowing a better stress resistance (van der Veen, van Schalkwijk et al. 2010). From there, it is tempting to hypothesize about a connection between the SOS response in *L. monocytogenes* and the SecA2 pathway, which could have implication in adaption to its external environment and biofilm formation

The combinatory approach here performed (i.e. bacterial adhesion in static and dynamic conditions, early and late stages of sessile development using BRT and the crystal violet method, CLSM in static and dynamic conditions) proved necessary and powerful to comprehend the biofilm formation in *L. monocytogenes* and prevented hasty conclusions. For instance at 20°C, the absence of surface colonization (as revealed by the BRT but contradicted by the crystal violet method) was actually confirmed by CLSM observations. In this condition, *L. monocytogenes wt* do not grow in a sessile mode; only a few single bacterial cells adhered, certainly by flagellum-mediated attachment (Lemon, Freitag et al. 2010), and the remaining bacteria were motile and swam in the liquid medium (Fig. supplementary material video).

SecA2 has been previously shown to be essential for increased listerial virulence (Lenz, Mohammadi et al. 2003; Halbedel, Hahn et al. 2012). In this study, we demonstrated that the inactivation of the SecA2 pathway allowing rough morphotype provides an advantage in listerial surface colonization in environmental conditions. Regulation by *secA2* could be involved in the conversion from rough morphotype in environmental conditions to virulent smooth morphotype in condition of infection. The enhancement of biofilm formation ability

by the *L. monocytogenes* rough colony morphotype should be further considered as potential risk factor for contamination of industrial production chain line and food products. Considering that environmental biofilms are generally multispecies rather than monospecies, morphotype conversion could have consequences on *L. monocytogenes* implantation and interaction with other bacterial species in biofilm and would request much further in depth investigations.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. Routinely, cells of *L. monocytogenes* were cultivated in BHI (brain-heart infusion) broth or BHI agar plates at 20°C or 37°C. If necessary, X-Gal (100 µg ml^{-1}) was added or antibiotics at the following concentrations: erythromycin (5 µg ml^{-1}), kanamycin (50 µg ml^{-1}). For all cloning procedures, *Escherichia coli* TOP 10 was used as the standard plasmid host (Sambrook and Russell 2001).

Construction of in-frame ΔsecA2, ΔmurA and ΔmurAΔcwhA L. monocytogenes mutants and gene complementation

The genes encoding SecA2 (lmo0583) and MurA (lmo2691) were deleted by allelic exchange using pMAD vector as described previously (Arnaud, Chastanet et al. 2004). From *L. monocytogenes* EGD-e genomic DNA purified with Wizard® Genomic DNA Purification Kit (Promega), upstream and downstream DNA fragments flanking the gene of interest were amplified by high-fidelity PCR using TaKaRa LA Taq DNA polymerase with two couples of primers, i.e. Fw1/Rv2 and Fw3/Rv4 respectively (Table 2). The two PCR products then served as a matrix for the SOE-PCR using Fw1/Rv4 primers. Following standard molecular cloning technique (Sambrook and Russell 2001), the resulting amplicon was cloned into pMAD following DNA restriction digestion with NcoI and MluI, ligation, transformation into *E. coli* TOP10 (Invitrogen) and selection on LB (lysogeny broth) agar with ampicillin (100 µg ml^{-1}). After purification from *E. coli* using Nucleospin Plasmid QuickPure (Macherey-Nagel), the resulting plasmid pMAD-Δ*secA2* and pMAD-Δ*murA* were electroporated into *L. monocytogenes* EGD-e and also in the in-frame Δ*cwhA* mutant for pMAD-*ΔmurA* (Monk, Gahan et al. 2008) with the selection performed on BHI agar containing erythromycin. As previously described (Arnaud, Chastanet et al. 2004), blue-white screening was applied to select gene knockout events. The isogenic mutants were identified by colony PCR with outFw/outRv primers using GoTaq DNA polymerase (Promega) and were further confirmed by DNA sequencing (GATC-Biotech) on both strands using primers Fw1 and Rv4, respectively.

For gene complementation, the entire CDS (coding sequence) was amplified from genomic DNA by PCR using TaKaRa LA Taq DNA polymerase and the primers

SecA2BspHFw/SecA2PstRv and MurANcoFw/MurAPstRv, respectively. The amplicon was cloned into pIMK2 (Monk *et al*., 2008) following DNA digest with NcoI/PstI restriction enzymes, ligation, electroporation into *E. coli* TOP10 and selection on LB agar with kanamycin. After plasmid purification, the resulting pIMK2-*secA2* and PIMK2-*murA* were electroporated into *L. monocytogenes* EGD-e Δ*secA2* and *L. monocytogenes* EGD-e Δ*murA*, respectively. Site-specific integration of the plasmid was confirmed following plating on by kanamycin BHI agar and colony PCR with primers SecA2BspHFw/ SecA2PstRv and MurANcoFw/MurAPstRv respectively.

Biofilm formation assay at early stages of sessile development

The assay was conducted using the BioFilm Ring Test® (BRT) (Chavant, Gaillard-Martinie et al. 2007) following BioFilm Control (BFC, France) supplier recommendations from overnight cultures *L. monocytogenes* EGD-e *wt* or mutant strains adjusted at $OD_{600nm} = 0.01$ (approximately 10^5 CFU ml^{-1}) in sterile BHI medium. Briefly, a suspension of paramagnetic microbeads (Ton5: 2.8 µm in diameter) was added at 10 µl ml^{-1} final concentration, homogenized by vortexing prior to 200 µl loading into 96-wells BFC Polystyrene Microtiter plates or 8-wells BFC Polystyrene Strips and static incubation at 20°C or 37°C. Control wells were filled with sterile BHI and Ton5. For reading at the different time points, wells of microtiter plates were first covered with 100 µl of BFC Contrast Liquid prior to scanning before and after one-minute magnetization using a BFC Magnetic Rack. Results were expressed as Biofilm Formation Index (BFI) (Chavant, Gaillard-Martinie et al. 2007; Macé, Seyer et al. 2008). Basically, in the course of bacterial sessile development, BFI decreases and a BFI ≤ 2 indicates a full immobilization of the paramagnetic microbeads. At least 5 independent experiments with at least two repeats each were performed for each strain and incubation time.

Biofilm formation assay at late stages of sessile development.

The assay is based on the crystal violet method (Borucki, Peppin et al. 2003). Briefly, overnight cultures of *L. monocytogenes* strains were adjusted at 0.01 (OD_{600nm}) in sterile BHI medium and 200 µl loaded into the wells of a 96-wells polystyrene microtiter plate prior to static incubation at 20 or 37°C. At different time points, the supernatant was removed from the wells, which were washed with TS (tryptone salt) and fixed with absolute ethanol (20 min). After emptying and air-drying the wells, 200 µl of an aqueous solution of crystal

violet (0.1%) was added and left for 10 min. After washing with water, the bound dye was solubilized with 200 µl of an aqueous solution of acetic acid (33 %). Contents of each well (150 µl) were transferred to a clean microtiter plate and absorbance was finally measured using a microtiter plate reader set to 595 nm. At least 5 independent experiments with at least two repeats each were performed for each strain and incubation time.

Initial adhesion

This assay is similar to the biofilm formation assay but with following changes. Briefly, overnight cultures of *L. monocytogenes* strains were adjusted at 1.5 (OD_{600nm}) in sterile BHI medium and loaded into the wells of a 96-wells polystyrene microtiter plate prior to static incubation at 20 or 37°C. After one hour, the supernatant was removed from the wells, which were washed with TS and directly stained with an aqueous solution of crystal violet (0.1%). After washing, the bound dye was solubilized in acetic acid (33 %), then transfert to a clean microtiter plate where the absorbance was finally measured. At least 5 independent experiments with at least two repeats each were performed for each strain.

Adhesion assay under liquid flow

Initial adhesion under liquid flow was assayed using a standard protocol which was recently described (Szlavik, Paiva et al. 2012). However, instead of microscope slides, we used plastic coverslips (Agar Scientific, dimension 22 mm×22 mm) which are made from clear unbreakable polystyrene. Briefly, cells were diluted in citric acid-Na_2HPO_4 buffer (pH 6.6) to a final volume of 50 ml and OD_{600}=0.100±0.005 (cell density of 10^8 CFU/ml). The tested bacterial solution was connected to a peristaltic pump (Spectec, Perimax 16/1) with a pumping velocity of 0.76 giving a wall shear stress of 0.0505. The chamber was mounted on an inverted microscope (Zeiss, Axiovert 25) with an attached camera (QImaging, MicroPublisher 3.3). After activation of the pumps, consecutive pictures were taken in three separate vistas every 5 min for 30 min and the adhered cells were enumerated. The median was selected for each time point and the initial adhesion rate (IAR) was calculated using linear regression from the medians. All adhesion tests were performed at least in duplicates.

Biofilm growth conditions for CLMS

(i) Static biofilm experiments.

Overnight cultures of *L. monocytogenes* strains carrying the pNF8 plasmid expressing the GFP, were adjusted at 0.01 (OD_{600nm}) in sterile BHI medium. 200 µl of these cultures were pipetted into the wells of 96 wells polystyrene microtiter plate with a µclear base (Greiner Bio-one, France) which enables high resolution fluorescence imaging. Then, the plates were incubated at 20 or 37°C. After 2 hours, the medium was removed, and 200 µl of fresh BHI was added. Biofilm development was evaluated by microscopic observations after 24 h of incubation. Three independent experiments were performed for each strain.

(ii) Flow-cell biofilm experiments

Biofilms were performed in flow-cells (DTU Systems Biology, Denmark) with individual channel dimensions of 1 by 4 by 40 mm. Flow chambers were inoculated with overnight cultures of the EGDe(pNF8), Δ*secA2*(pNF8) and Δ*murA*Δ*cwhA*(pNF8) strains adjusted at 0.01 (OD_{600nm}) in fresh BHI medium. After inoculation (2 ml), the medium flow was stopped for 1 h to allow bacterial adhesion, and thereafter the medium was pumped through the flow cells at 4 ml/h by using a peristaltic pump (model 205S; Watson Marlow, Falmouth, England, United Kingdom). Two independent experiments with two replicates each were made.

(iii) Co-cultured assays

Static and flow-cell biofilm experiments were also performed in co-cultures. Overnight cultures of *L. monocytogenes* EGDe and Δ*murA*(pNF8) or *L. monocytogenes* EGDe and Δ*cwhA*(pNF8) were mixed in equal quantity and then, adjusted at 0.01 (OD_{600nm}) before being added in wells or inoculated in the flow-cell chamber. Then, sessile cells were stained with the red nucleic acid stain SYTO61 (0.01%) (Molecular Probes, Invitrogen) before microscopic observation.

CLSM and image processing.

Horizontal plane images of the biofilms were acquired using a Leica SP2 AOBS CLSM (Leica Microsystems, France) at the MIMA2 microscopy platform

(http://www6.jouy.inra.fr/mima2_eng/). When necessary the CLSM allowed simultaneous monitoring of GFP and SYTO61 dyes. The excitation wavelength used for GFP was 488 nm, and emitted fluorescence was recorded within the range of 500 to 550 nm. The red fluorescent nucleic acid stain SYTO61 was excited at 633 nm, and the emitted fluorescence was collected in the range of 650 to 700 nm. Images were collected through a 63x Leica oil immersion objective (numerical aperture, 1.4).

3D projections were performed with IMARIS software (Bitplane, Zürich, Switzerland). The biofilm structural parameters (thickness, roughness and substratum coverage) were evaluated using the PHLIP Matlab program developed by J. Xavier (http://phlip.sourceforge.net/phlip-ml). For each experiment, at least 3 microscopic fields were analyzed. Considering the heterogeneity of the Δ*secA2* and Δ*murA*Δ*cwhA*, only images containing a biofilm were considered for the analysis.

Statistical analysis

In order to test the significance of the differences observed in each assay between the *wt* and the different mutants, a pair Student's *t*-test was performed. Differences were considered significant at $P < 0.05$.

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SUPPLEMENTARY MATERIALS

Oligonucleotide sequences used in this study are provided in Table 1S. Growth curves for the *wt* and mutant strains performed at 20°C and 37°C are provided in Figure 1S.

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TABLE

Table 1. Strains and plasmids used in this study.

Name	Relevant characteristics	Source/reference
Plasmids		
pMAD	Amp^R, Em^R, *bgaB*	Arnaud *et al*. (2004)
pIMK2	Site-specific listerial integrative vector, p_{help}, Kan^R	Monk *et al*. (2008)
pMAD-Δ*secA2*	Amp^R, Em^R, *bgaB, ΔsecA2*	This work
pMAD-Δ*murA*	Amp^R, Em^R, *bgaB, ΔmurA*	This work
pIMK2*secA2*	Site-specific listerial integrative vector, p_{help}-*secA2*, Kan^R	This work
pIMK2*murA*	Site-specific listerial integrative vector, p_{help}-*murA*, Kan^R	This work
pNF8	Em^R, Mob+(IncP), oriR pAMβ1, oriR pUC; Pdlt-*gfpmut1*	Fortinea *et al*. (2000)
L. monocytogenes strains		
EGD-e	&	Glaser *et al*. (2001)
Δ*secA2*	Δ*secA2* (lmo0583)	This work
Δ*murA*	Δ*murA* (lmo2691)	This work
Δ*cwhA*	Δ*cwhA* (lmo0582)	Monk et al. (2008)
Δ*murA*Δ*cwhA*	Δ*murA*Δ*cwhA*	This work
Δ*secA2*:: pIMK2*secA2*	Δ*secA2*:: pIMK2*secA2* with SecA2 overexpressed from the phelp promoter integrated at $tRNA^{Arg}$	This work
Δ*murA*:: pIMK2*murA*	Δ*murA*:: pIMK2*murA* with MurA overexpressed from the phelp promoter integrated at $tRNA^{Arg}$	This work
Δ*cwhA*:: pIMK2*cwhA*	Δ*cwhA*:: pIMK2cwhA with CwhA overexpressed from the phelp promoter integrated at $tRNA^{Arg}$	Monk et al. (2008)
Δ*murA*Δ*cwhA*		This work
EGDe pNF8		This work
Δ*secA2* pNF8		This work
Δ*murA* pNF8		This work
Δ*cwhA*pNF8		This work
Δ*murA*Δ*cwhA* pNF8		This work

SUPPLEMENTARY MATERIALS

Table 1S. Oligonucleotides used in this study

Name	Oligonucleotide sequence (5'-3')[a]	Restriction site
DSecA2NcoFw1	TTATCCATGGCCAGCTGTAGCCGCGATAGTT	NcoI
DSecA2Rv2	TTTTTCGATCATCATAATTCTGTCTCAT	
DSecA2Fw3	ATGAGACAGAATTATGATGATCGAAAAATGAACTTGATCCAGACGGCTTAAT	
DSecA2MluRv4	TAATACGCGTACCTCGCTTCCACTTATGTTTCTCTA	MluI
SecA2outFw	TCAGCAGCAGCAACCTCATTATTTAC	
SecA2outRv	CCCCGGGAATGATTTGAACAAG	
SecA2BspHFw	TATATCATGAGACAGAATTATGATGATCGAAAAATAG	BsphI
SecA2PstRv	ATATCTGCAGTTGGAAAAAATCAGACGCAAGTTG	PstI
DMurA-NcoFw1	TTTATACCATGGATTCCATATCTTTGCTGCCCATTT	NcoI
DMurARv2	TGTAAGGCTTCAAGGATTCGTTCT	
DMurAFw3	AGAACGAATCCTTGAAGCCTTACAGTGAATGTAAAAAGCCTACCTCGTG	
DMurA-MluRv4	TTTATAACGCGTCACGTTTATCATCGGGACAGAAGA	MluI
MurAoutFw	TAGTTTTTTCATTTTCCTTGCCTCC	
MurAoutRv	AAGTAAGAAATATCGTGCTCGGAAT	
MurA-NcoFw	TGAGTTGCCATGGCAAAAACGAGAAAAGAACGAATCCT-	NcoI
MurA-PstRv	TGAGTTGCTGCAGTCACTTAATTGTTAATTTCTGACCAACATGAAT	PstI

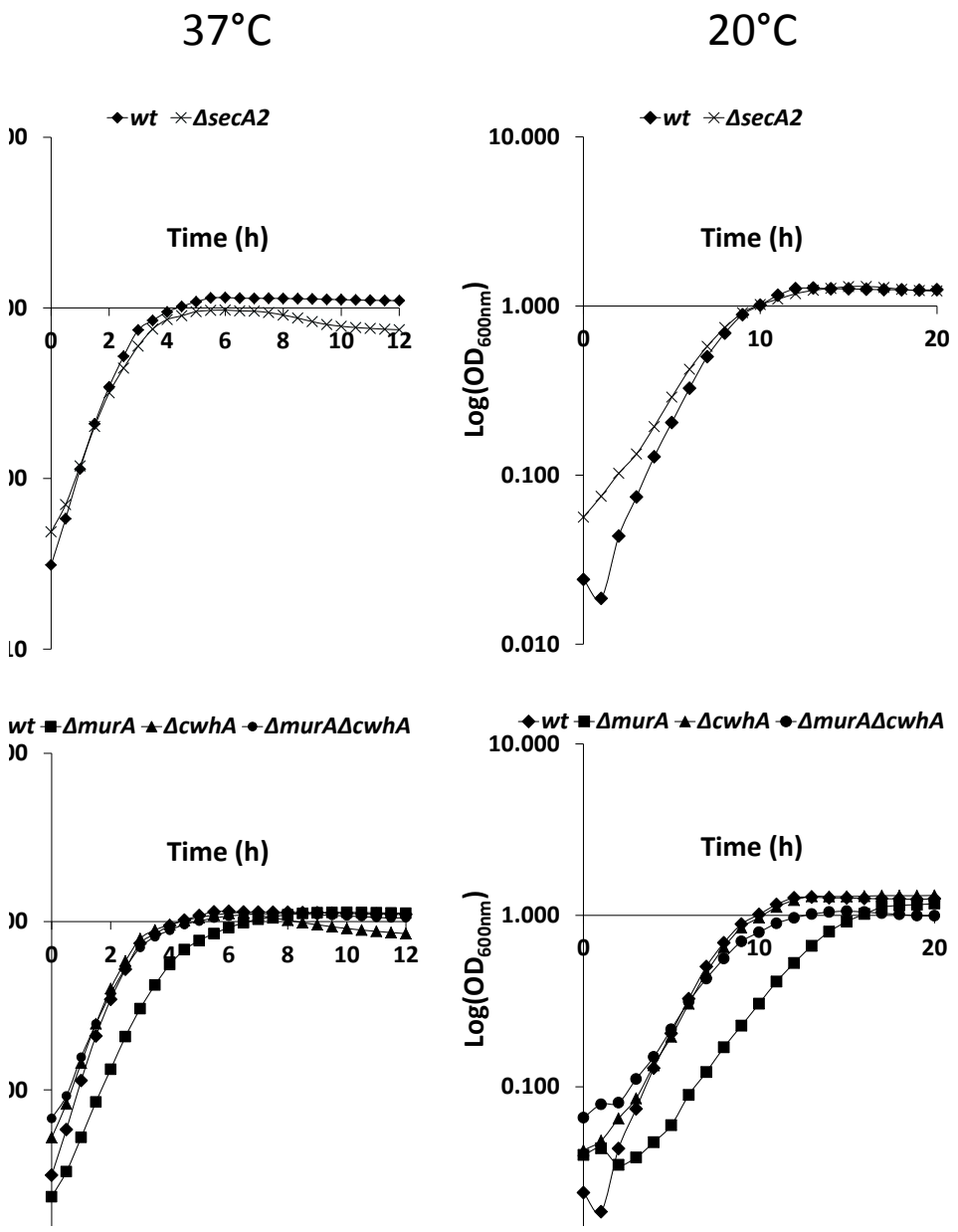

Figure 1S: Growth curves for the *wt* and mutant strains performed at 20°C and 37°C.

ARTICLE n°3

Sandra RENIER, Christophe CHAMBON, Didier VIALA, Caroline CHAGNOT, Michel HÉBRAUD and Mickaël DESVAUX.

Exoproteomic analysis of the SecA2-dependent secretion in *Listeria monocytogenes* EGD-e

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Exoproteomic analysis of the SecA2-dependent secretion in *Listeria monocytogenes* EGD-e

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ABSTRACT

As part of the Sec translocase, the accessory ATPase SecA2 is present in some pathogenic Gram-positive bacteria. In *Listeria monocytogenes*, deletion of *secA2* results in filamentous cells that forms rough colonies and have lower virulence. However, only a few proteins have been identified that are secreted by this pathway. This investigation aims to provide the first exoproteomic analysis of the SecA2-dependent secretion in *L. monocytogenes* EGD-e. By using media and temperatures relevant to bacterial physiology, we demonstrated that the rough colony and elongated bacterial cell morphotypes are highly dependent on growth conditions. Subsequently, comparative exoproteomic analyses of the Δ*secA2* versus wt strains were performed in chemically defined medium at 20 °C and 37 °C. Analyzing the proteomic data following the secretomics-based method, part of the proteins appeared routed towards the Sec pathway and exhibited an N-terminal signal peptide. For another significant part, they were primarily cytoplasmic proteins, thus lacking signal peptide and with no predictable secretion pathway. In total, 13 proteins were newly identified as secreted via SecA2, which were essentially associated with cell-wall metabolism, adhesion and/or biofilm formation. From this comparative exoproteomic analysis, new insights into the *L. monocytogenes* physiology are discussed in relation to its saprophytic and pathogenic lifestyle.

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1. Introduction

Listeria monocytogenes is a Gram-positive bacterium, which can cause severe food-borne infections in humans and animals [1,2]. This opportunistic pathogen can survive in with very diverse environments including soil, water, vegetation or abiotic surfaces in food processing plants, the gastro-intestinal tracts and the cytosol of eukaryotic cells in the course of an infection [3,4]. Temperature plays a key role in the regulation of gene expression in *L. monocytogenes*, especially virulence factors, and consequently in the switch from a saprophytic to a pathogenic bacterial cell [5–8]. Accordingly, *L. monocytogenes* secretes a broad range of proteins involved in various important processes like infection [9], cell-wall metabolism [10] or surface colonization [11].

In *L. monocytogenes*, the transport of proteins across the cytoplasmic membrane, i.e. corresponding to secretion and also called export in monoderm bacteria, can be performed by seven systems [12–14], namely the (i) Sec (secretion), (ii) Tat (twin-arginine translocation), (iii) FEA (flagella export apparatus), (iv) FPE (fimbrilin–protein exporter), (v) holins, (vi) ABC (ATP-binding cassette) transporters and (vii) Wss (WXG100 protein secretion system). With 714 proteins predicted to

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possess an N-terminal signal peptide (SP) [15], the Sec pathway is considered to be the main route for protein secretion in *L. monocytogenes*. The essential Sec translocase is composed of the heteromeric SecYEG translocon, which forms a protein-conducting channel through the cytoplasmic membrane, and the peripheral SecA ATPase. The energy provided by repeated cycles of ATP hydrolysis, allows the translocation of the preprotein in an unfolded state through the SecYEG channel [16].

A few years ago, a paralogue to SecA, named SecA2, was identified in several pathogenic Gram-positive bacteria, namely *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Streptococcus gordonii*, *Clostridium difficile*, *Staphylococcus aureus*, *Bacillus anthracis* and *L. monocytogenes* [17–19]. Unlike the SecA ATPase, SecA2 exhibits a smaller size because of a truncation in the C-terminal region and is generally not essential for cell viability [17,20]. In *S. gordonii* and *Streptococcus parasanguinis*, an accessory Sec translocase consists of the SecA2 ATPase and a second translocon formed by SecY2 along with other transmembrane proteins Asp1, Asp2, and Asp3 [21]. This SecA2/SecY2Asp1-3 translocase has been extensively studied and is involved in the export of GspB [22] and Fap1 [23], which are two large serine-rich (SRR) glycoproteins. The preprotein GspB exhibits a 90 amino acid long SP and an adjacent AST (the accessory Sec transport) domain of 24 residues [24,25]. *S. aureus* also possesses a locus encoding an accessory Sec translocase (SecA2/SecY2Asp1-3), which is required for the specific export of the SRR glycoprotein SraP [18]. However, in *Mycobacteria* and *L. monocytogenes*, this locus is absent [17]. As no SecY2 or Asp proteins have been identified, it is most likely that SecA2 interacts with the Sec translocon [13]. SecA2 may assist the SecA/Sec translocase and improve the secretion efficiency of some dedicated proteins [26,27]. In *M. segmentis*, two lipoproteins have been shown to be exported via the SecA2-only system [28], as well as the superoxide dismutase (Sod) [29]. In *L. monocytogenes*, Sod is also secreted in a SecA2-dependent manner [30]. Since its absence leads to a reduced virulence [31], SecA2 may play an important role in the physiopathology of *L. monocytogenes*.

Indeed, bacterial cells lacking SecA2 present a typical morphology consisting in long chain cell forming rough colony. The cell division defect is mainly due to a lower amount of two cell-wall hydrolases, named CwhA (cell-wall hydrolase A), formerly called p60 (protein of 60 kDa) or Iap (Invasion associated protein), and MurA (muramidase A), also called NamA (N-acetylmuramidase A), which are secreted in a SecA2-dependent manner [31–33]. Following comparative analysis by one-dimensional gel electrophoresis (1-DE), it appeared SecA2 was required for the normal secretion of 7 proteins to the extracellular milieu; 25 bands were resolved from the culture supernatant of the *L. monocytogenes* parent strain [31]. While 4 of these proteins exhibited an N-terminal SP (including CwhA and MurA), the remaining proteins lack one. In addition to the Sod [30], LAP (*Listeria* adhesion protein), which is primarily a cytoplasmic alcohol acetaldehyde dehydrogenase, was also identified as a protein lacking a SP but which was secreted extracellularly in a SecA2-dependent manner during anaerobiosis [34,35]. Thus, the SecA2-pathway also facilitates the secretion of proteins devoid of an N-terminal signal peptide, which cannot be predicted by proteogenomic analysis [15]. Altogether, this prompted us to investigate the SecA2-dependent pathway in *L. monocytogenes* by exoproteomic analysis.

2. Material and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes EGD-e *wt* (wild type) [36] and the isogenic in-frame *secA2* deletion mutant were cultured in a chemically defined medium, i.e. MCDB202 (CryoBioSystem). Nutrient broth medium was prepared according to supplier instructions at a final glucose concentration of 5 g L^{-1} and pH adjusted at 7.3. From −80 °C stock culture (previously grown in MCDB202), bacterial strains were plated on MCDB202 agar (15 g L^{-1}) and incubated 24–48 h at 20 or 37 °C. A preculture was set up from one isolated bacterial colony and grown in MCDB202 broth at the relevant temperature in an orbital shaker (170 rpm). From an inoculum in the exponential growth phase, a 250-mL culture was set up, adjusted to 0.01 (OD_{600nm}) and incubated as described. For proteomic experiments, sampling was performed in late exponential growth phase (OD_{600nm} = 0.5, 12 h at 37 °C and 16 h at 20 °C).

2.2. Microscopic observation

Microscopic observations were performed with an inverted contrast phase microscope (Olympus LH50A). For microscopic images of bacterial colonies, the strains were grown on MCDB202 agar plates at 20 and 37 °C for 24 h and resultant colonies were observed at 150× original magnification. For visualization of bacterial cells, cultures grown in MCDB broth, at 20 and 37 °C, were sampled during the exponential phase and fixed onto glass slides for microscopic analysis at 600× original magnification.

2.3. Construction of in-frame ΔsecA2 L. monocytogenes mutant and gene complementation

The gene encoding SecA2 (*lmo0583*) was deleted by allelic exchange using pMAD [37]. Fragments of DNA flanking the *secA2* gene were amplified from *L. monocytogenes* EGD-e genomic DNA by PCR using high-fidelity TaKaRa LA Taq DNA polymerase with two primers, i.e. DSecA2NcoIFw1/DSecA2Rv2 (TTATCCATG GCCAGCTGTAGCCGCGATAGTT/TTTTTCGATCATCATAATTCTG TCTCAT), and DSecA2Fw3/DSecA2MluRv4 (ATGAGACAGAATTA TGATGATCGAAAAATGAACTTGATCCAGACGGCTTAAT/TAATA CGCGTACCTCGCTTCCACTTATGTTTCTCTA). The two PCR products then served as a matrix for the SOE-PCR using DSecA2NcoIFw1/DSecA2MluRv4. Following standard molecular cloning technique [38], the resulting amplicon was cloned into pMAD following DNA restriction digestion with NcoI and MluI, ligation, transformation into *Escherichia coli* TOP10 (Invitrogen) and selection on LB (lysogeny broth) agar with ampicillin (100 μg mL^{-1}). After purification from *E. coli*, the resulting plasmid pMAD-Δ*secA2* was electroporated into *L. monocytogenes* EGD-e [39] with the selection performed on BHI (brain-heart infusion) agar containing erythromycin (5 μg mL^{-1}). As previously described [37], blue-white screening was applied to select gene knockout events. The isogenic mutant *L. monocytogenes* EGD-e Δ*secA2* was identified by colony PCR using GoTaq DNA polymerase (Promega) and SecA2outFw/SecA2outRv (TCAGCAGCAGCAACCTCATTATTTAC/CCCCGGGAATGATTT GAACAAG) and was further confirmed by DNA sequencing

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(GATC-Biotech) on both strands using primers DSecA2NcoIFw1 and DSecA2MluRv4, respectively.

For gene complementation, the entire *secA2* CDS (coding sequence) was amplified from genomic DNA by PCR using TaKaRa LA Taq DNA polymerase and the primers SecA2BspHFw1/SecA2PstRv2 (TATATCATGAGACAGAATTATGATGATCGAAAAATAG/ATATCTGCAGTTGGAAAAAATCAGACGCAAGTTG). The amplicon was cloned into pIMK2 [40] following DNA digest with NcoI/PstI restriction enzymes, ligation, electroporation into *E. coli* TOP10 and selection on LB agar with kanamycin (50 μg mL^{-1}). After plasmid purification, the resulting pIMK2-*secA2* was electroporated into *L. monocytogenes* EGD-e Δ*secA2*. Site-specific integration of the plasmid was confirmed following plating on kanamycin BHI agar and colony PCR using GoTaq DNA polymerase with primers SecA2BspHFw1/SecA2PstRv2.

2.4. *Precipitation of extracellular proteins*

The supernatants were recovered after culture filtrations (0.2 μm), and 0.2 mM phenylmethylsulfonyl fluoride was added to inhibit protease activity. Supernatants were concentrated in a final volume of about 5 mL using a Vivacell-100 centrifugal concentrator (5 kDa cut-off; Sartorius Stedim). To evaluate potential cell lysis, specific enzyme activity of aminopeptidase C was assayed as previously described [12,41]. Sodium deoxycholate (0.2 mg mL^{-1}) was added to the solution, and the solution was incubated for 30 min on ice. Sodium deoxycholate supports protein precipitation, which was carried out by the addition of 10% (wt/vol) trichloroacetic acid and incubation overnight at 4 °C. After centrifugation (12,000 rpm, 30 min, 4 °C), the precipitate was washed with ice-cold acetone and solubilized in IEF buffer as previously described [42]. Three protein extractions from 3 independent cultures were carried out and at least two 2-DE gels per sample were run.

2.5. *Two-dimensional gel electrophoresis (2-DE)*

For IEF, precast immobilized pH gradient (IPG) strips with a pH 3–10 nonlinear gradient or pH 4–7 linear gradient were passively rehydrated for 17.5 h in a reswelling tray with 400 μL of IEF buffer containing 0.3% (vol/vol) ampholytes for pH 3–10 or 0.15% (vol/vol) ampholytes for pH 4–7 and 0.15% (vol/vol) ampholytes for pH 5–7, 2 mM TBP, and 50 μg of proteins. The proteins were first subjected to IEF for a total of 66,450 Vh (7 h at 50 V, 2 h at 200 V, linear gradient to 1000 V in 2 h, 1 h at 1000 V, linear gradient to 8000 V in 5 h, and 8000 V till the end). The strips were equilibrated twice for 15 min in an equilibration solution (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% [wt/vol] sodium dodecyl sulfate [SDS], 30% [vol/vol] glycerol) containing 2 mM TBP (tributylphosphine) for the first step and 2.5% (wt/vol) iodoacetamide and traces of bromophenol blue for the second step. The second-dimension electrophoresis (SDS-polyacrylamide gel electrophoresis) was carried out with 12.5% acrylamide gel in a Multicell Protean II XL system (Bio-Rad). The gels were silver stained as previously described [12,43].

2.6. *2-DE gel image and statistical analyses*

At least 5 reproducible gels from 3 independent cultures for each bacterial strain were selected and included for image analysis. The amount of proteins loaded on each gel allowed protein spots to be within the linear range of silver staining (0–60 ng/spot) as

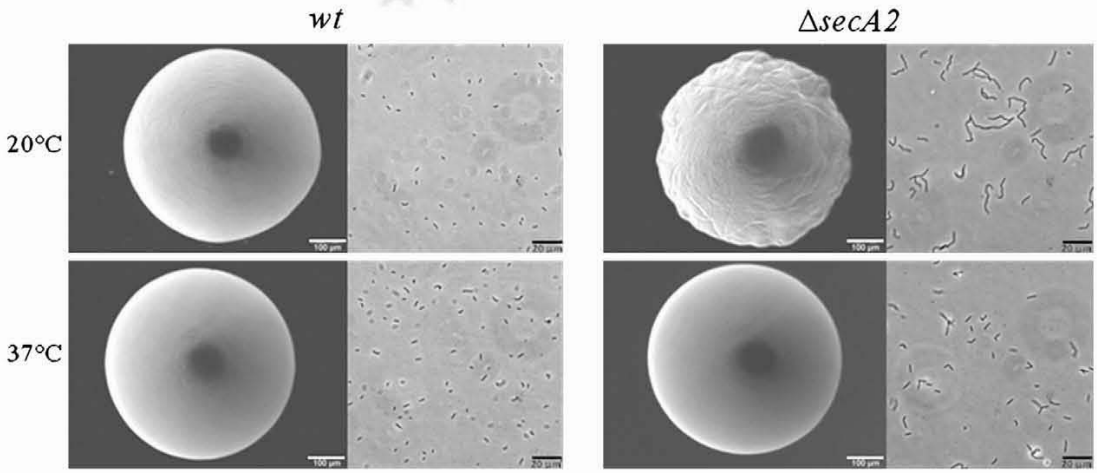

Fig. 1 – Microscopic analysis of *L. monocytogenes* EGD-e *wt* and Δ*secA2* morphologies in chemically defined medium. (On the left side) Morphology of colonies of both strains grown in MCDB202 agar plates was compared by microscopic observations (150× original magnification) at 20 °C and 37 °C. The wild type (*wt*) shows a smooth outline at 37 °C and 20 °C as well as the Δ*secA2* mutant at 37 °C. Δ*secA2* only exhibits irregular edges at 20 °C. Bars, 100 μm. (On the right side) Cells were grown in MCDB202 at 20 °C and 37 °C. Pictures of individual cells of wild type and Δ*secA2* mutant were taken in exponential phase (600× original magnifications). The Δ*secA2* mutant exhibits long filaments at 20 °C but not at 37 °C, according to the morphology of respective colonies. At 37 °C, the morphology of Δ*secA2* cells seems to be intermediated between the long filaments of Δ*secA2* at 20 °C and the small individual cells of the wild type. Bars, 20 μm.

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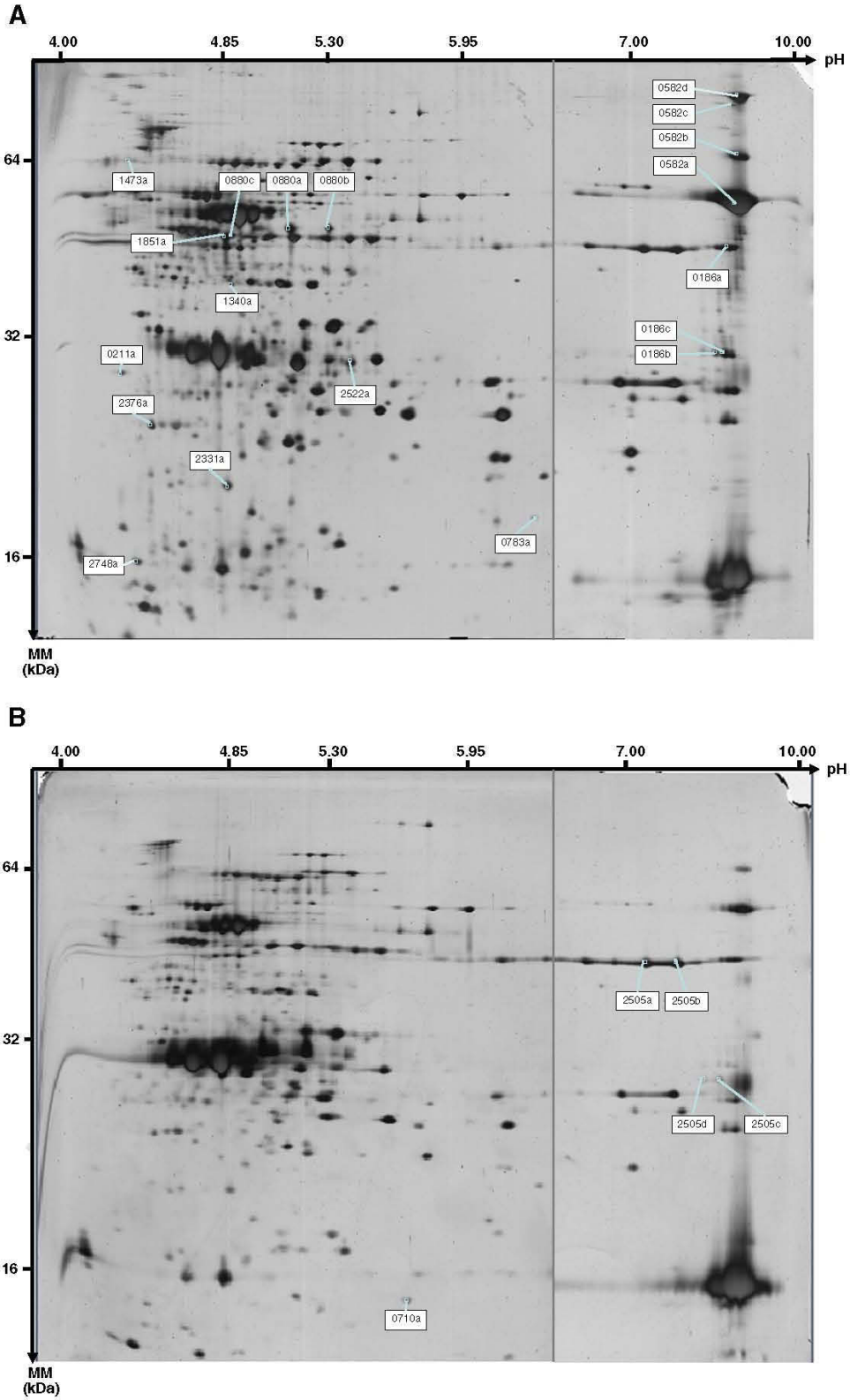

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previously described [44–46]. Silver stained 2-DE gels were scanned by a GS-800 imaging densitometer (Bio-Rad).

Statistical image analysis was performed using Progenesis Samespots software, version 4.1 (Nonlinear Dynamics). For each independent analysis according to the temperature and the pH gradient, gels were aligned on a reference gel (*wt*). After background subtraction, spot autodetection and quantification were performed by the software. Each spot was assigned a relative value corresponding to a spot volume. To compare the Δ*secA2* mutant to the *wt*, an experimental design was set up with two conditions, i.e. *wt* and Δ*secA2*, where corresponding gels were added. Differential spot intensity was considered significant at $p<0.01$ using ANOVA (analysis of variance) procedure.

2.7. *Identification of proteins by mass spectrometry*

Semipreparative 2-DE gels containing 500 μg of proteins were run for protein spot identification as previously described [12]. Protein spots were stained with colloidal Coomassie blue, and spots of interest were excised in the gel and subjected to the following treatments. First, the spots were washed in 25 mM ammonium bicarbonate – 5% acetonitrile for 30 min and twice in 25 mM ammonium bicarbonate – 50% acetonitrile for 30 min each. The spots were then dehydrated with 100% acetonitrile. The dried gels were reswelled in 25 mM ammonium bicarbonate containing 20 ng μL^{-1} trypsin. Digestion was performed at 37 °C for at least 5 h. The resulting peptides were extracted with 100% acetonitrile. After 15 min at 37 °C, each sample was mixed with saturated cyano-4-hydroxycinnamic acid onto the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) target. Using MALDI-TOF mass spectrometry (MS) (Voyager DE-Pro, Applied BioSystems) and Voyager software for data collection and analysis, positive-ion MALDI mass spectra were recorded in the reflectron mode. Identifications by nano-liquid chromatography (LC) coupled to electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) (LTQVelos, Thermo Scientific) were performed when identification from MALDI-TOF MS failed. Monoisotopic peptide masses were assigned and used for database searches with Mascot v2.2.0. Interrogations were performed against a home database containing distinct entries corresponding to the predicted mature proteins in *L. monocytogenes* EGD-e [12], i.e. DBMature-LmoEGDe, provided as supplementary material. The following parameters were considered for the searches: a maximum ion mass tolerance of 25 or 50 ppm, possible modification of cysteines by carbamidomethylation, as well as partial oxidation of methionine.

2.8. *Bioinformatic analyses*

As previously described, bioinformatic analyses were performed from the computational resources provided by INRA MIGALE Bioinformatics Platform (INRA, Jouy-en-Josas, France) [12]. The secretomics-based method was applied for proteogenomic analyses of protein secretion and protein subcellular location (SCL) in *L. monocytogenes* as previously and thoroughly explained [15].

Sequence alignments were performed using ClustalW [47] from BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Similarity searches were performed from InterProScan v4.3 [48] against InterPro (IPR) v32.0 [49].

3. Results

3.1. *Rough colony morphotype of L. monocytogenes Δ*secA2* is dependent on growth medium and temperature*

Considering a chemically defined medium is more appropriate to investigate the physiology of a microorganism rather than undefined complex media such as LB or BHI [50], the colony morphology of *L. monocytogenes* was investigated in MCDB202. Two different temperatures known to induce radically different gene expression profiles in *L. monocytogenes* were used, i.e. 37 °C (a temperature encountered in the course of an infection in a human host) and 20 °C (a standard ambient temperature). Following plating on MCDB202 agar plates, rough colonies were observed with *L. monocytogenes* Δ*secA2* grown at 20 °C but, surprisingly enough, not at 37 °C (Fig. 1). The rough colony morphotype was observed both at 37 °C and 20 °C using an undefined complex medium. As previously reported, microscopic observations further revealed this colony morphotype was associated with the formation of elongated bacterial cells (Fig. 1). While not as elongated as at 20 °C, *L. monocytogenes* Δ*secA2* cells were still longer than the *wt* at 37 °C. The mutant strain was complement with pIMK2-*secA2*, which integrates into the listerial chromosome and expresses the *secA2* gene from the constitutive *Phelp* promoter. The introduction of this *secA2* expression construct successfully rescued the smooth colony and bacillus cell phenotypes as observed with the *wt* strain (Fig. 1S), demonstrating the modifications of colony and bacterial-cell morphologies were the result of the *secA2* mutation. Besides confirming the role of SecA2, this morphotype appeared to depend on growth conditions. In order to gain further insight in the physiology of protein secretion of *L. monocytogenes*, the SecA2-dependent secretion pathway was investigated in chemically defined medium MCDB202 at 37 °C and 20 °C.

3.2. *Exoproteomic analysis of the SecA2-dependent secretion in L. monocytogenes*

To investigate the extracellular proteome, proteins isolated from the culture supernatant were separated by 2-DE gel. In order to improve the resolution in the acidic pH range as expected from our previous proteomic data [12,51], proteins were migrated in two different pH ranges in the first dimension, i.e. IEF with a nonlinear pH 3–10 or with a linear pH 4–7 (Figs. 2 and 3). Differential exoproteomic analyses of *L. monocytogenes* Δ*secA2* versus the *wt* were performed at 20 °C and 37 °C. Following

Fig. 2 – 2-DE gels of the exoproteome at 20 °C of *L. monocytogenes* EGD-e *wt* (A) and Δ*secA2* (B). Proteins were run in the first dimension by IEF in non-linear pH 3–10 or in linear pH 4–7 IPG strips, and in SDS-PAGE 12.5% acrylamide gel in the second dimension. As materialized by the vertical gray line, these figures are actually photomontages combining 2-DE gels in pH 3–10 (right side) and in pH 4–7 ranges (left side). Original 2-DE gels are provided as supplementary material (Fig. 2S).

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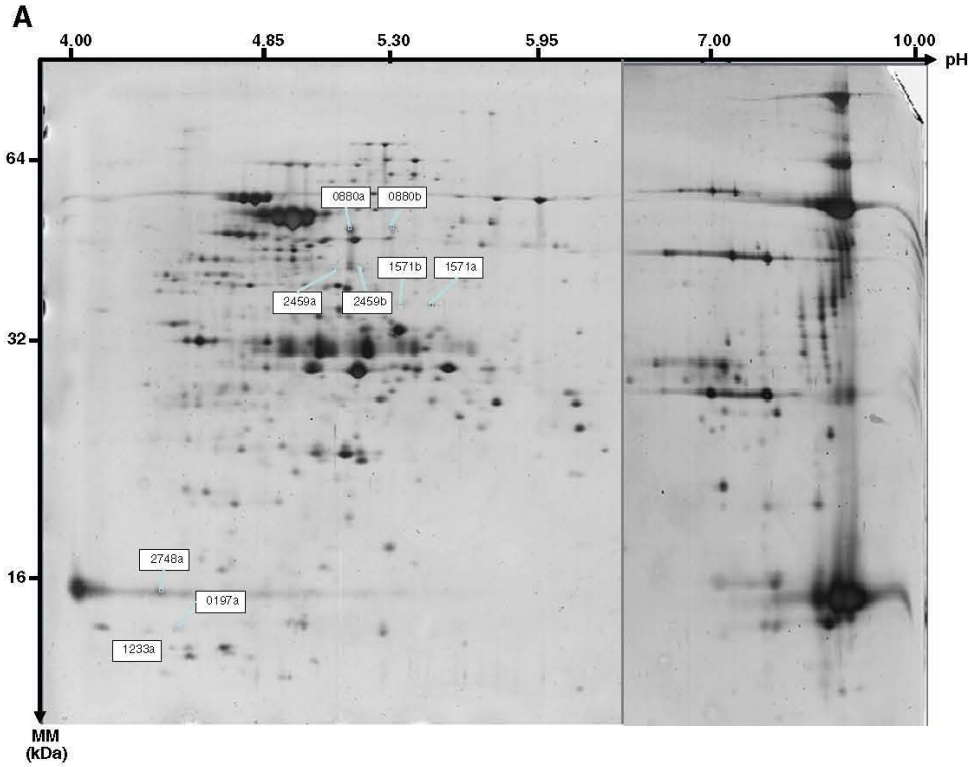

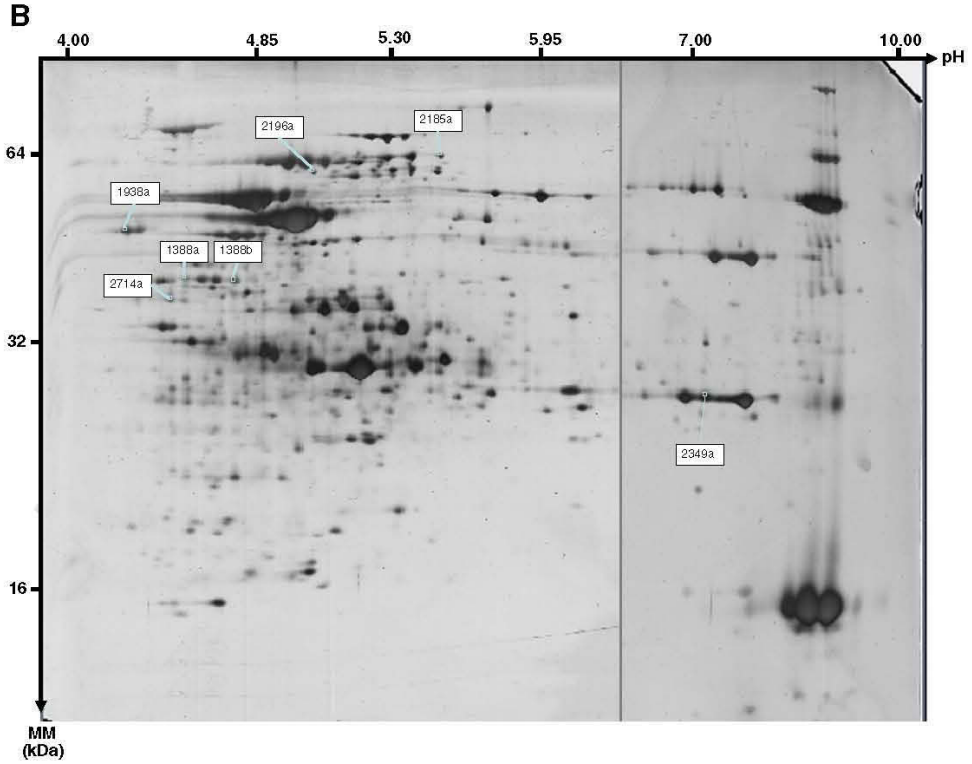

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statistical image analysis, only spots reproducibly found from one gel to another for the same strain and in statistically significant different amounts between the *L. monocytogenes wt* and Δ*secA2* were included. Following peptide mass fingerprinting (PMF) identification, a few protein spots were not considered further as they could either simply not be identified or correspond only to truncated protein forms. In the end, a total of 24 distinct proteins, which were present in different amount, i.e. secreted differentially, at 20 and/or 37° were identified in the culture supernatant.

3.2.1. *Comparative proteomic analysis of L. monocytogenes Δ*secA2* versus the wt at 20 °C*

Statistical image analyses revealed that 24 protein spots displayed significant different protein spot levels between the extracellular fraction of *L. monocytogenes* Δ*secA2* and the *wt* strain at 20 °C (Fig. 2). Following identification by MALDI-TOF MS, or LC–ESI-MS/MS, 14 distinct proteins were identified as present in different amount (Table 1). Of those, 12 were down-secreted and 2 proteins were found in a higher amount in the extracellular fraction of the Δ*secA2* mutant when compared to the *wt* strain.

Several cell-wall hydrolases exhibiting N-terminal SP were identified as differentially exported to the extracellular milieu of *L. monocytogenes* Δ*secA2* and *wt* (Table 1 and Fig. 2). Following a previously described strategy based on the use of a mature genomic database for PMF [12,15], the cleavage site (C+1) was experimentally validated from which the presence and size of these SP were deduced (Table 1S). As it could be expected from the morphology of listerial cells, secretion of CwhA was significantly reduced in the Δ*secA2* mutant at 20 °C (Table 1 and Fig. 2). However, under these conditions no differential secretion of MurA to the extracellular milieu could be observed between Δ*secA2* and *wt* strain. In contrast, a homologue of ClpP (caseinolytic protease; Lmo1851; SSF52096: E-value=3.9×10^{-76}), which is known to specifically degrade some cell-wall hydrolases such as MurA [52], was down-secreted in the mutant strain (Table 1 and Fig. 2). A muralytic transglycosylase (Lmo0186), belonging to the SpsB (stationary phase survival subfamily B) family involved in cell-wall morphogenesis [53], was also, and for the first time, shown to be significantly down-secreted in a Δ*secA2* background. Interestingly, SpsB was originally predicted to be a Type II integral membrane protein with an uncleaved SP serving as a signal anchor [15]. Indeed, a transmembrane domain (TMD) of about 20 residues was identified within the first 35 amino acids of the N-terminal region by all TMD prediction tools. While no cleavable SP was predicted, the h-domain possesses a glycine towards the end, a feature known to facilitate the cleavage by the Type I signal peptidase (SPase I) [54]. A closer look, further indicated that 4 out of 14 tools predicted a cleavage site (C+1) at position 30, 37, 42 or 49, this latter prediction being here experimentally confirmed (Table 1S) [15]. Surprisingly, the flagellar basal body rod FlgB (Lmo0710) was also found up-secreted in the supernatant of the Δ*secA2* mutant. Contrary to other proteins identified, this protein is normally secreted and assembled by the flagellar export apparatus (FEA) [13]. Considering cell and cell wall morphology are modified as a result of differential secretion of some cell-wall hydrolases [32,55], variation in the presence of flagella components in the supernatant is most certainly ectopic.

Remarkably, Lmo0880 is absent from the supernatant of Δ*secA2* mutant and thus exhibits the greatest quantitative difference compared to the *wt* (Table 1 and Fig. 2). This collagen-binding protein (IPR008456: E-value=7.3×10^{-45}) is reported here for the first time to be secreted in a SecA2-dependent manner. While its SP could be experimentally confirmed following the secretomics-based method [15] (Tables 1 and 1S), this is not the case for two lipoproteins of unknown function (Lmo1340 and Lmo2331). Despite refining prediction results to improve the original annotation, no function could be inferred from similarity searches for those proteins (Table 1S).

While a set of cytoplasmic listerial protein lacking SP is predicted to be secreted by non-classical secretion (NC) [15], none of the primarily cytoplasmic proteins were identified here, except for Lmo2748, which is predicted by NClassG+ (Tables 1 and 1S). As previously reported the molecular chaperone DnaK and ribosomal proteins were under-secreted in the supernatant of Δ*secA2* strain [31].

3.2.2. *Comparative proteomic analysis of L. monocytogenes Δ*secA2* versus the wt at 37 °C*

Proteomic analysis performed from the supernatants of the *wt* and Δ*secA2* strains at 37 °C revealed that 20 protein spots displayed significant different protein spot levels (Fig. 3) and finally corresponded to 13 distinct proteins (Table 2).

As expected from the absence of morphological variation and contrary to 20 °C, no differences in the export of cell-wall hydrolases could be identified (Table 2 and Fig. 3); MurA and CwhA were present in similar amounts in the extracellular milieu of the *wt* and Δ*secA2* strains.

Four (TcsA, SvpA, OppA and Lmo2714) of the five proteins observed in higher quantity in the supernatant of the Δ*secA2* mutant were previously reported as implicated in the virulence of *L. monocytogenes* (Table 2 and Fig. 3). As observed at 20 °C, the collagen-binding protein Lmo0880 was absent from the supernatant of the Δ*secA2* mutant (Table 2 and Fig. 3) and is therefore predicted to be secreted in a SecA2-dependent manner. Except for the secreted protein of unknown function Lmo2714, no SP could be experimentally confirmed for the remaining predicted Sec-secreted proteins. This is most certainly due to peptides between the putative SP cleavage site and the trypsinization site only 1 to 4 amino-acid residues long and thus too short to be detected (Table 1S). Surprisingly, 3 of them were substrate-binding lipoproteins up-secreted in the Δ*secA2* mutant exhibiting a glycine at position +2 of the putative SP cleavage site. Such a feature is encountered in lipoproteins released into the

Fig. 3 – 2-DE gels of the exoproteome at 37 °C of *L. monocytogenes* EGD-e *wt* (A) and Δ*secA2* (B). Proteins were run in the first dimension by IEF in non-linear pH 3–10 or in linear pH 4–7 IPG strips, and in SDS-PAGE 12.5% acrylamide gel in the second dimension. As materialized by the vertical gray line, these figures are actually photomontages combining 2-DE gels in pH 3–10 (right side) and in pH 4–7 ranges (left side). Original 2-DE gels are provided as supplementary material (Fig. 3S).

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Unknown function												
Lmo1340	Lipoprotein of unknown function	1340a	−(2.7)	1639	5.1	37.4	4.9	44	14	59	Sec	24
Lmo2331	Lipoprotein of unknown function	2331a	−(1.9)	689	4.9	22.4	4.9	22	5	32	Sec	24
Primarily cytoplasmic												
Lmo0211	50S ribosomal protein L25	0211a	−(2.5)	88	4.4	22.6	4.5	35	9	52		no
Lmo0783	Phosphotransferase system, sorbose subfamily IIB component	0783a	−(4.1)	55	5.9	15.3	6.1	19	5	43		no
Lmo1473	DnaK class I heat-shock chaperone protein	1473a	−(5)	711	4.7	64.4	4.5	64	10	25		no
Lmo2376	Peptidyl-prolyl cis-trans isomerase, cyclophilin-type	2376a	−(1.6)	63	4.6	21.4	4.6	29	8	47		no
Lmo2748	Pyridoxamine 5′-phosphate oxidase	2748a	−(4.8)	202	4.7	15.7	4.5	15	2	20	NC	no

[a] Compared to original GenBank record, some annotations were corrected following bioinformatic analysis by similarity searches as described in the Materials and methods and detailed in Table 1S provided as supplementary material.
[b] The first four digits used as spot identifier (ID) corresponds to the identification number for proteins in *L. monocytogenes* EGD-e, for example the spot "0186a" corresponds to protein Lmo0186.
[c] Difference in protein spot intensity in the Δ*secA2* mutant compared to the wild type (*wt*), where −(x) means x-fold lower and +(x) means x-fold higher in Δ*secA2*.
[d] Mascot score was obtained against the databank DB-Mature-LmoEGDe v2.0 provided as supplementary material, where score greater than 50 are significant ($p<0.05$). Mascot scores of proteins identified by nanoLC–MS/MS are underlined.
[e] Theoretical average p*I* and molecular mass (MM) are given for the predicted mature exoprotein, that is, devoid of signal peptide.
[f] Expressed as percentage.
[g] Protein secretion pathways as predicted by the secretomics-based method [15]. Sec: Sec translocase; FEA: flagella export apparatus; NC: non-classical secretion.
[h] Size of the cleavable N-terminal signal peptide (SP) predicted by the secretomics-based method [15] or experimentally validated as indicated by the asterisk. Detailed analysis is provided as supplementary material in Table 1S. no: no predicted SP.

PROOF

supernatant of Gram-positive bacteria [56,57]. Once again, the differential presence in the supernatant of primarily cell envelope proteins is most certainly ectopic and results from abnormal cell morphology [32,55].

The remaining proteins present in lower amounts in the extracellular milieu of Δ*secA2* compared to *L. monocytogenes wt* were predicted to be localized to the cytoplasm (Table 2 and Fig. 3). Except for the pyridoxamine 5′-phosphate oxidase (Lmo2748; IPR011576: E-value = 5.1×10^{-18}), these other cytoplasmic proteins were not differentially exported at 20 °C (Figs. 1 and 2). While none of them were predicted to be secreted by NC secretion, some of them have already been reported to be moonlighting proteins, e.g. the glyceraldehyde-3-phosphate dehydrogenase (Lmo2459) [41].

4. Discussions

Secreted proteins are the main active determinants promoting the direct interactions between the bacterial cell and its surrounding environment. As such, knowledge of protein secretion is of the utmost importance in understanding the physiology of a pathogenic bacterial species. While some SecA2-dependent secreted proteins were shown to contribute to *L. monocytogenes* virulence [30,31,34], the identification of proteins secreted by this pathway had never been performed by proteomics before. In this context, this investigation provides the first exoproteomic analysis of the SecA2-dependent secretion in *L. monocytogenes* EGD-e. In total, 13 proteins have been newly identified as secreted in a SecA2-dependent manner.

L. monocytogenes Δ*secA2* was previously reported to form rough colony and long cell filaments when grown in undefined complex media, such as LB or BHI [31–33]. Using chemically defined medium and two different temperatures relevant to the lifestyle of this microorganism, these phenotypes here appeared to be highly dependent on growth medium and temperature. Such a relationship between changes in colony morphology and the type of nutrient medium was also observed for *M. smegmatis* [26]. In *L. monocytogenes*, the formation of long bacterial cell is associated with a lower amount of cell-wall hydrolases [31,32,58]. From the present investigation, *L. monocytogenes* Δ*secA2* exhibits a rough colony phenotype at 20 °C, correlated with a significantly reduced secretion of CwhA, whereas at 37 °C, the amount of CwhA and MurA in the supernatant was not significantly altered between the mutant and *wt* strains, which both formed smooth colonies. In this study, additional cell-wall hydrolases appeared down-secreted in a Δ*secA2* background at 20 °C, namely the muralytic transglycosylase SpsB (Lmo0186) and the murein transglycosylase MltD (Lmo2522; PRK10783: E-value = 8.0×10^{-11}).

The SecA2 pathway is often presented as contributing to bacterial virulence [29,31]. In addition to the previously uncovered exoproteins Sod and Lap [30,34] or surface protein FbpA [59], which are not cell-wall hydrolases [60], several virulence factors appeared to be secreted in the SecA2-depedent manner, i.e. TcsA [61], SvpA [62], OppA [63] and Lmo2714 [64]. While the *L. monocytogenes* Δ*secA2* mutant displays invasion and cell-to-cell spread defects [58], this is mostly a result of down-secretion of the cell-wall hydrolase CwhA, which was originally, but misleadingly, considered to be a virulence factor for invasion and formerly named Iap [55]. Indeed, this phenotype is an indirect consequence of the loss of bacterial septum formation, due to a lower amount of CwhA, which results in mislocation of some key cell-surface virulence factors, namely the internalin InlA promoting host cell invasion and ActA involved in actin polymerization for cell-to-cell spreading [55]. In other words, SecA2-dependent cell-wall hydrolases can interfere and have collateral effect on the correct final location of some surface proteins, which can be released into the extracellular milieu. This could also explain the higher abundance for flagellar component FlgB and the cell-wall hydrolase SpI (Lmo2505) in the extracellular milieu of *L. monocytogenes* Δ*secA2* but not for the ribosomal protein Lmo1938. Considering the mechanism of cell-wall covalent anchoring by sortases [65], an imbalance between the rates of LPXTG protein secretion and cell-wall biogenesis (coinciding with cell division) can result in misincorporation to the peptidoglycan of the proteins Lmo2185 and Lmo2714, and thus their release into the extracellular milieu. Interestingly, lipoproteins OppA (Lmo2196) and TscA (Lmo1388, formerly called Csa) were previously found in lower amounts on the cell surface in *L. monocytogenes* Δ*secA2* [33]. Together with Lmo2349, their ectopic location and higher amount in the extracellular milieu could result from the propensity of lipoproteins exhibiting a G at position C+2 to be released, following natural shaving by cell-surface proteases [56,57,66].

No conserved domain could be identified following protein sequence alignment of the SP or the mature part of the SecA2-dependent secreted proteins, such as AST in *Streptococcus* [24,25]. Besides preproteins, several proteins down-secreted in culture supernatant of *L. monocytogenes* Δ*secA2* were devoid of N-terminal SP. The absence of aminopeptidase C in the supernatants clearly ruled out extensive cell lysis in late exponential phase. Also, these proteins primarily predicted to be located in the cytoplasm but experimentally established as part of the exoproteome and present in statistically significant different amounts between the *L. monocytogenes wt* and mutant strains are secreted by the SecA2 pathway. Among them, the export of DnaK and ribosomal proteins was previously reported to be SecA2-dependent [31]. From our investigation, the prediction of NC secretion for Lmo2748 no longer applies and it becomes part of the protein set secreted via SecA2. While several proteins lacking a SP have been newly identified in this study, two proteins previously reported to be secreted extracellularly in a SecA2-dependent manner could not be identified, i.e. Sod [30] and LAP [34]. This could result from the use of different experimental conditions, namely the growth medium, redox potential and/or temperature. Of note, additional proteins were previously reported to show a dependence on SecA2 but were found in the cell surface fraction not in the supernatant [31]. In some cases, the SecA2-dependent secretion of primarily cytoplasmic proteins was further associated with moonlighting activity as previously demonstrated for GAPDH and DnaK in *L. monocytogenes* [41]. When localized extracytoplasmically, these proteins can bind human plasminogen [31,41]. While the moonlighting activities of these other proteins remain to be determined, the mechanism by which SecA2 allows the secretion of such proteins is also unresolved.

Besides moonlighting GAPDH and DnaK, other SecA2-dependent secreted proteins would be involved in bacterial adhesion. The collagen-binding protein Cbp (Lmo0880) showed a strong dependence upon SecA2 as it could not be detected

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Table 2 – Exoproteins identified by MALDI-TOF MS or LC–ESI-MS/MS present in different amount in Δ*secA2* compared to *wt* at 37 °C. Q3

Protein ID	Annotation [a]	Spot ID [b]	Δ*secA2* vs. *wt* [c]	Mascot score [d]	Theoretical [e]		Experimental		Queries matches	Sequence coverage [f]	Secretion pathway [g]	SP [h]
					pI	MM	pI	MM				
Adhesion												
Lmo0880	Collagen-binding protein, Cbp	0880a	−(9.3)	55	5.6	47.0	5.1	46	7	20	Sec	25*
		0880b	−(6.4)	51	5.6	47.0	5.3	47	4	17		
Unknown function												
Lmo2714	Cell-wall protein of unknown function	2714a	+(1.9)	4692	5.0	31.9	4.6	37	15	63	Sec	26*
Membrane transport												
Lmo1388	CD4+ T-cell stimulating antigen, TcsA	1388a	+(1.8)	4813	5.1	35.7	4.6	39	25	81	Sec	27
	Substrate-binding protein ABC transport system	1388b	+(1.6)	18,710	5.1	35.7	4.7	38	34	88		
Lmo2185	NEAr transporter, SvpA	2185a	+(3.3)	98	5.8	60.3	5.5	65	13	34	Sec	28
Lmo2196	ABC-type oligopeptide transport system, OppA	2196a	+(6.5)	4791	5.3	59.4	4.9	61	28	52	Sec	27
Lmo2349	ABC-type amino acid transport system	2349a	+(1.6)	6626	8.0	26.5	7	24	15	77	Sec	29
Primarily cytoplasmic												
Lmo0197	Sporulation stage V, protein G	0197a	−(4.4)	52	4.6	11.4	4.6	12	3	38		no
Lmo1233	Thioredoxin	1233a	−(6)	231	4.3	11.6	4.4	12	5	59		no
Lmo1571	6-Phosphofructokinase	1571a	−(6.2)	147	5.7	34.4	5.4	36	17	48		no
		1571b	−(3.7)	2616	5.7	34.4	5.3	36	15	51		no
Lmo1938	30S ribosomal protein S1	1938a	+(5)	3688	4.6	41.4	4.4	47	21	66		no
Lmo2459	Glyceraldehyde-3-phosphate dehydrogenase, GAPDH	2459a	−(6.9)	2514	5.4	36.3	5.0	39	15	61		no
		2459b	−(6.7)	1179	5.4	36.3	5.2	39	8	29		no
Lmo2748	Pyridoxamine 5′-phosphate oxidase	2748a	−(5.4)	202	4.7	15.7	4.4	15	2	20	NC	no

[a] Compared to original GenBank record, some annotations were corrected following bioinformatic analysis by similarity searches as described in the Materials and Methods and detailed in Table 1S provided as supplementary material.
[b] The first four digits used as spot identifier (ID) corresponds to the identification number for proteins in *L. monocytogenes* EGD-e, for example the spot "0582a" corresponds to protein Lmo0582.
[c] Difference in protein spot intensity in the Δ*secA2* mutant compared to the wild type (*wt*), where −(x) means x-fold lower and +(x) means x-fold higher in Δ*secA2*.
[d] Mascot score was obtained against the databank DB-Mature-LmoEGDe v2.0 provided as supplementary material, where score greater than 50 are significant ($p<0.05$). Mascot scores of proteins identified by nanoLC–MS/MS are underlined.
[e] Theoretical average pI and molecular mass (MM) are given for the predicted mature exoprotein, that is, devoid of signal peptide.
[f] Expressed as percentage.
[g] Protein secretion pathways as predicted by the secretomics-based method [15]. Sec: Sec translocase; FEA: flagella export apparatus; NC: non-classical secretion.
[h] Size of the cleavable N-terminal signal peptide (SP) predicted by the secretomics-based method [15] or experimentally validated as indicated by the asterisk. Detailed analysis is provided as supplementary material in Table 1S. no: no predicted SP.

in the supernatant of *L. monocytogenes* ΔsecA2. This is quite remarkable since SecA2-dependent proteins reported so far in pathogenic Gram-positive bacteria show a marked decrease in secretion but not complete abolition of export, e.g. CwhA in *L. monocytogenes* [15,31,33]. According to the most recent model of the SecA2-only system, SecA2 ATPase activity is required to assist SecYEG mediated export of a specific subset of secreted proteins, which features are not understood as yet [67]. From most recent genomic analysis [15,68], the *L. monocytogenes* genome encodes 7 additional collagen-binding proteins (Lmo0159, Lmo0160, Lmo0627, Lmo1115, Lmo2085, Lmo2178 and Lmo2576), but to date none of them have been experimentally characterized as yet. Interestingly, SpsB (Lmo0186) exhibits a G5 domain (IPR011098: E-value=3.7×10^{-28}) described as binding N-acetylglucosamine and is involved in adhesion and biofilm formation [69]. Actually, the involvement of the SecA2 pathway in *L. monocytogenes* adhesion and biofilm formation was suggested [40] but this aspect would clearly require much further investigations.

Of interest, TcsA the CD4$^+$ T-cell stimulating antigen and OppA the oligopeptide-binding protein transport system were previously observed to be more secreted in a PrfA activated mutant [70]. The SecA2 pathway was demonstrated to be required for long-term protective immunity and in the priming of memory CD8$^+$ T cells [71,72]. Therefore, it is tempting to speculate that a relationship exists between the regulation of virulence factor expression by PrfA, the immune response, and the SecA2 pathway. Clearly, this deserves further in-depth investigations. Considering the physiopathology of *L. monocytogenes*, a primary event is the crossing of the intestinal barrier. The intestinal mucus consists of two distinct layers, i) an outer, loosely attached layer densely colonized with bacteria, and ii) an inner, compact layer devoid of bacteria [73–75]. While *L. monocytogenes* binds to the major component of intestinal mucus MUC2, it cannot to the cell surface mucin MUC1 [76]. The possible involvement of the SecA2 pathway at this infection step requires further investigations.

From the present study, it appears the influence of SecA2 on listerial physiology is dependent on environmental conditions, an aspect that was so far overlooked. This highlights that much has to be gained from investigating *L. monocytogenes* in different experimental conditions in order to understand its physiology as a saprophyte in the environment or as a pathogen in the course of an infection. This first global protein analysis of the SecA2-depedent secretion provided new insights on the interaction of *L. monocytogenes* with its surroundings. Further investigations should focus on its protein surfaceome to complete this secretomic analysis and to provide complementary information on SecA2-dependent secreted determinants potentially involved in *L. monocytogenes* cell-wall metabolism, virulence, adhesion and/or biofilm formation [11,77].

Pictures of colony and cell of complemented *L. monocytogenes* ΔsecA2::pIMK2-secA2 in MCDB202 agar and BHI agar at 20 and 37 °C are provided in Fig. 1S. Original 2-DE gels with IEF in non-linear pH 3–10 or in linear pH 4–7 IPG strips for the exoproteome of *L. monocytogenes* EGD-e *wt* and ΔsecA2 at 37 °C are provided in Fig. 2S. Original 2-DE gels with IEF in non-linear pH 3–10 or in linear pH 4–7 IPG strips for the exoproteome of *L. monocytogenes* EGD-e *wt* and ΔsecA2 at 20 °C are provided in Fig. 3S. Detailed results of the bioinformatic analysis as described in the Materials and Methods are provided in Table 1S. Database in Mascot format containing the set of pre-proteins and different versions of the mature proteins potentially expressed in *L. monocytogenes* EGD-e, which was here used in the course of proteomic analysis for protein-spot identification, is provided as DB-MD-Pre-Mature-LmoEGDe v2.0.

These supplementary materials are available free of charge via the Internet at http://pubs.acs.org/. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.11.027.

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Figure 1S. Microscopic analysis of *L. monocytogenes* EGDe Δ*secA2*::pIMK2-*secA2* morphologies in chemically defined medium and in undefined complex medium. (On the left side) Morphology of colonies of Δ*secA2*::pIMK2-*secA2* grown in MCDB202 and BHI agar plates (150× original magnification) at 20 and 37°C. The smooth morphotype was restored. Bars, 100 µm. (On the right side) Cells were grown in MCDB202 or BHI at 20°C and 37°C. Pictures of individual cells Δ*secA2*::pIMK2-*secA2* were taken in exponential phase (600× original magnifications). Bars, 20 µm.

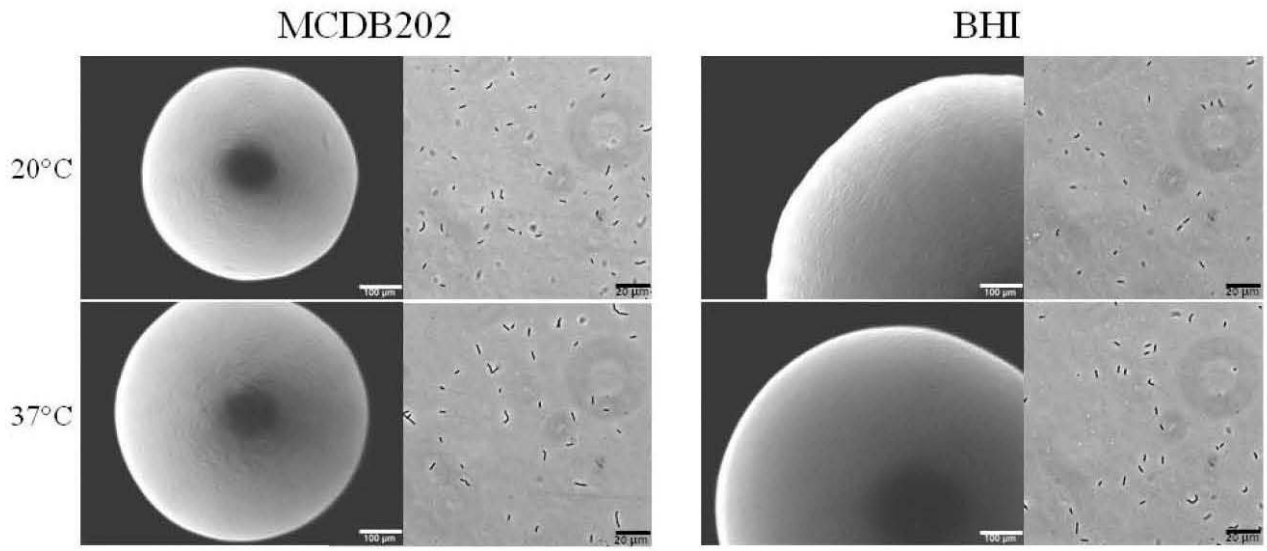
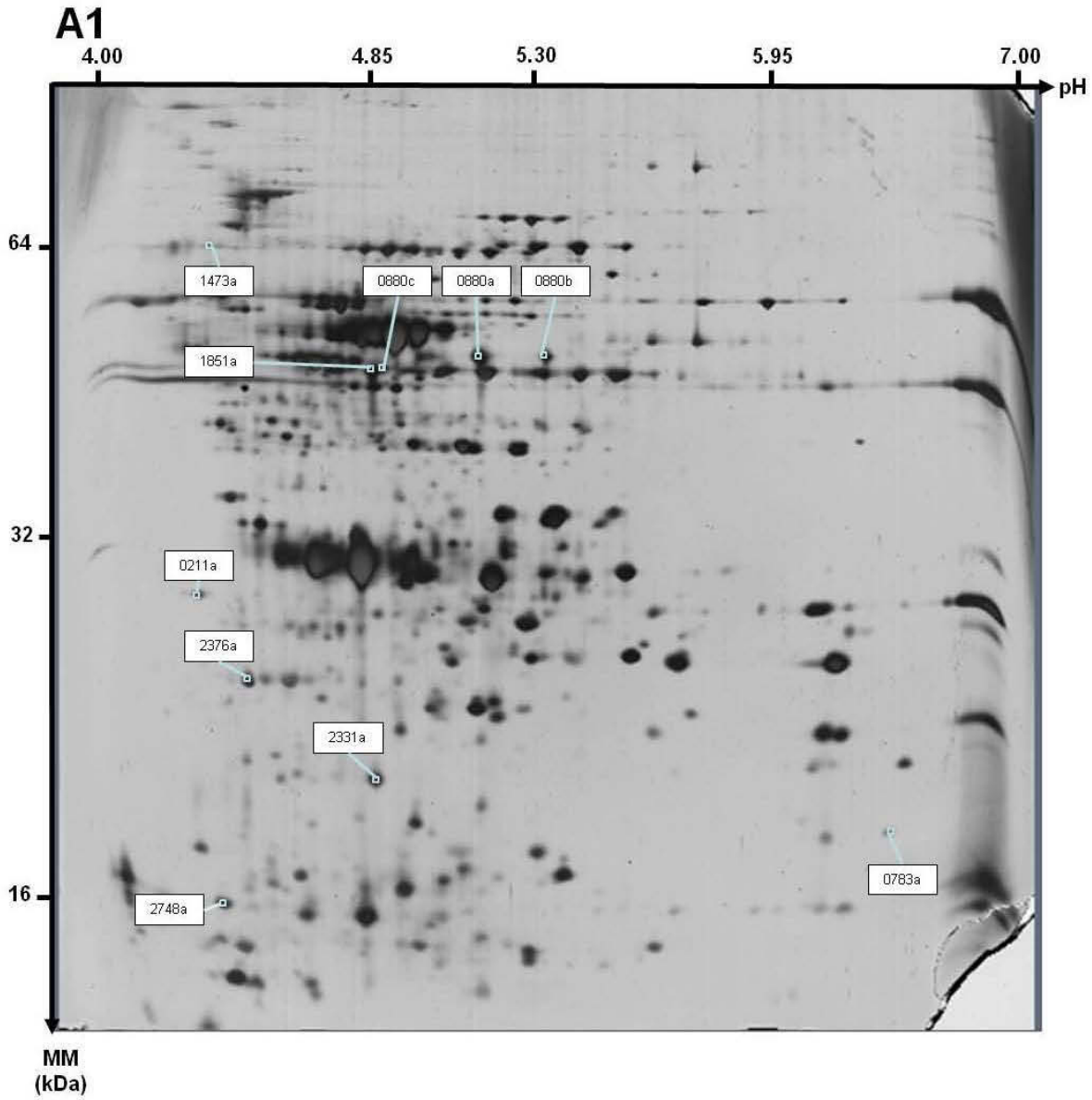
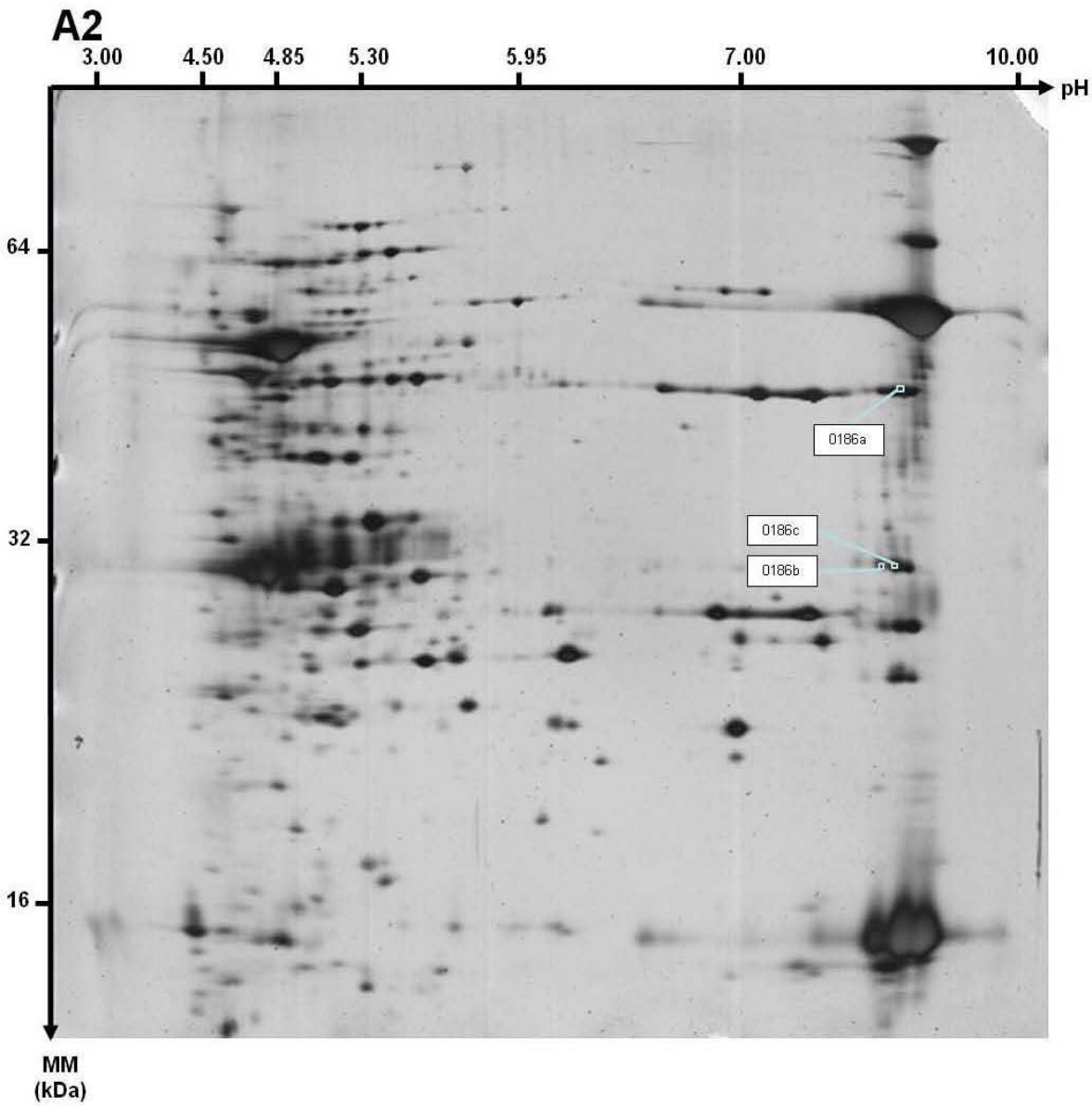


Figure 3S: 2-DE gel of the exoproteome at 20°C of *L. monocytogenes* EGD-e wt (A) and Δ*secA2* (B). Proteins were run in the first dimension by IEF in linear pH 4-7 (1) or in non-linear pH 3-10 IPG strips (2), and in SDS-PAGE 12.5% acrylamide gel in the second dimension.

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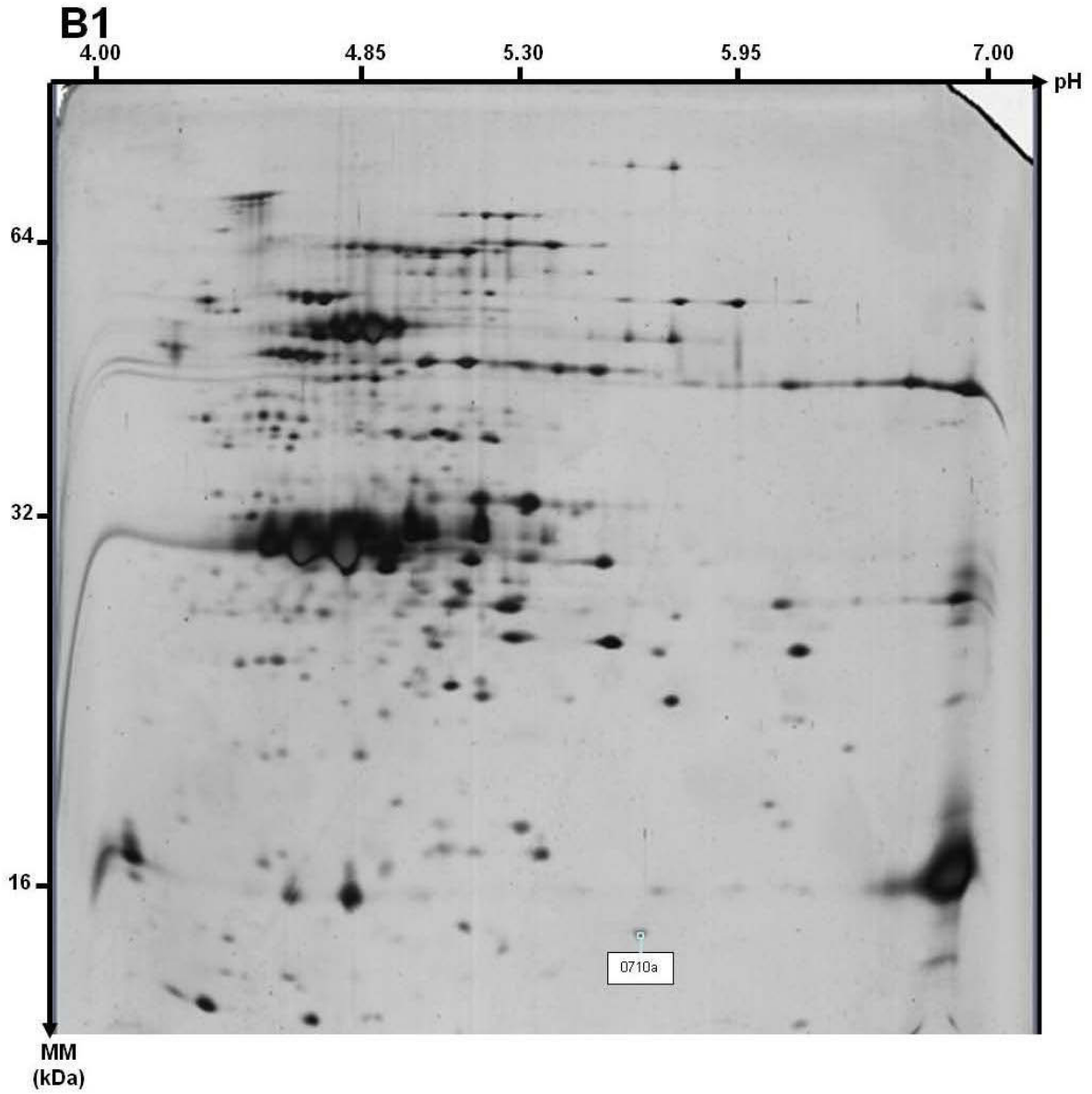
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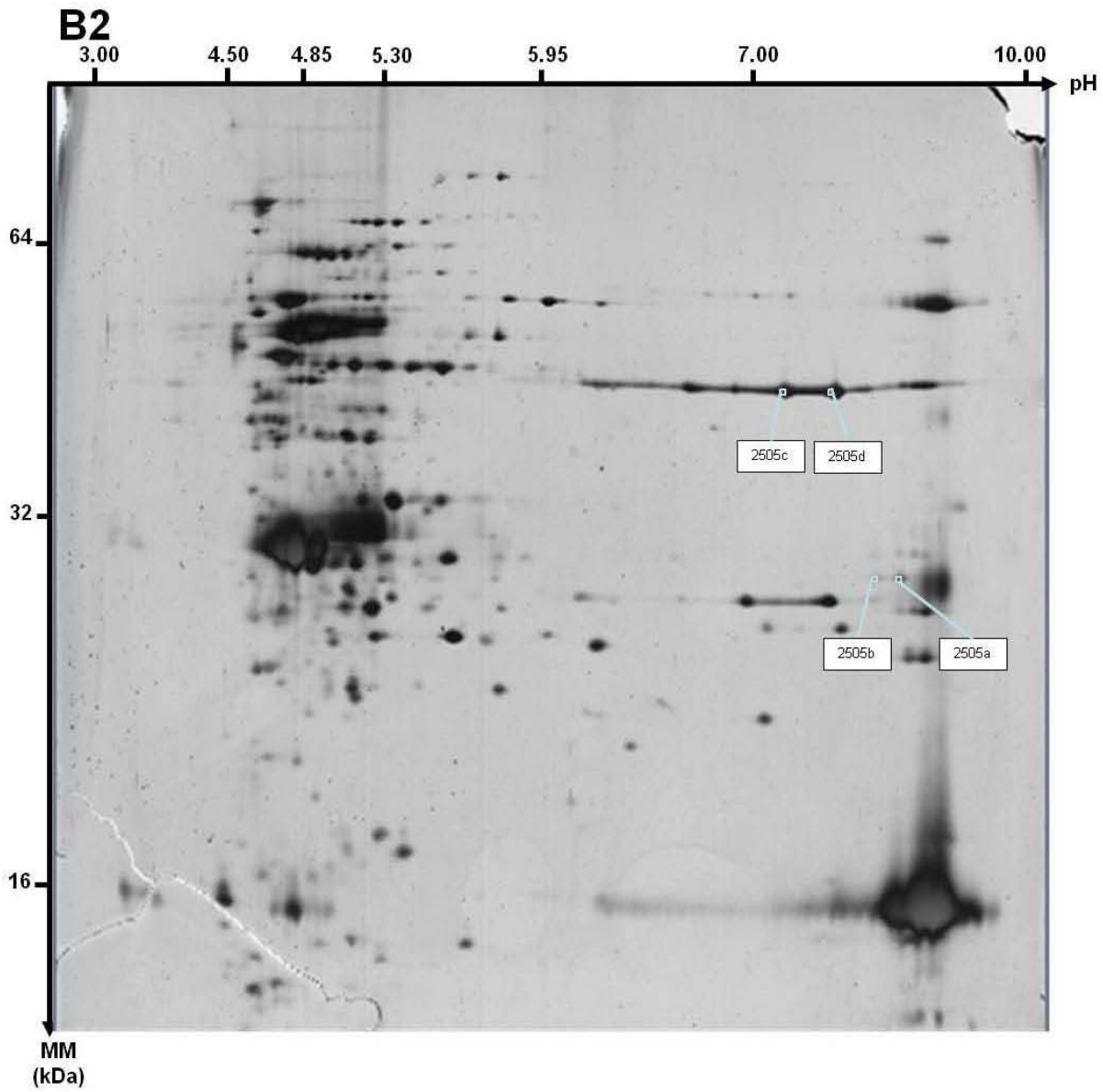
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2505d
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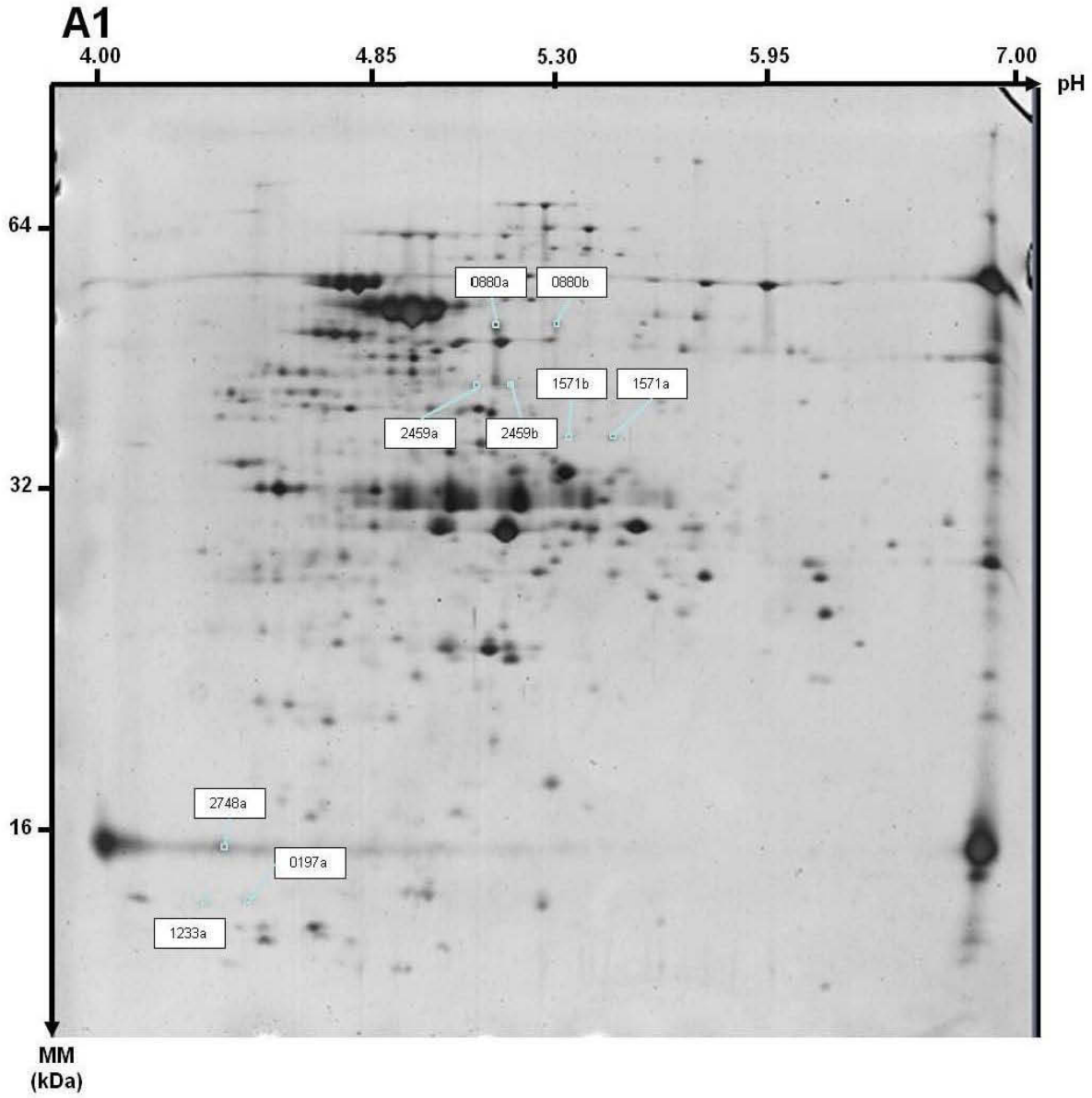
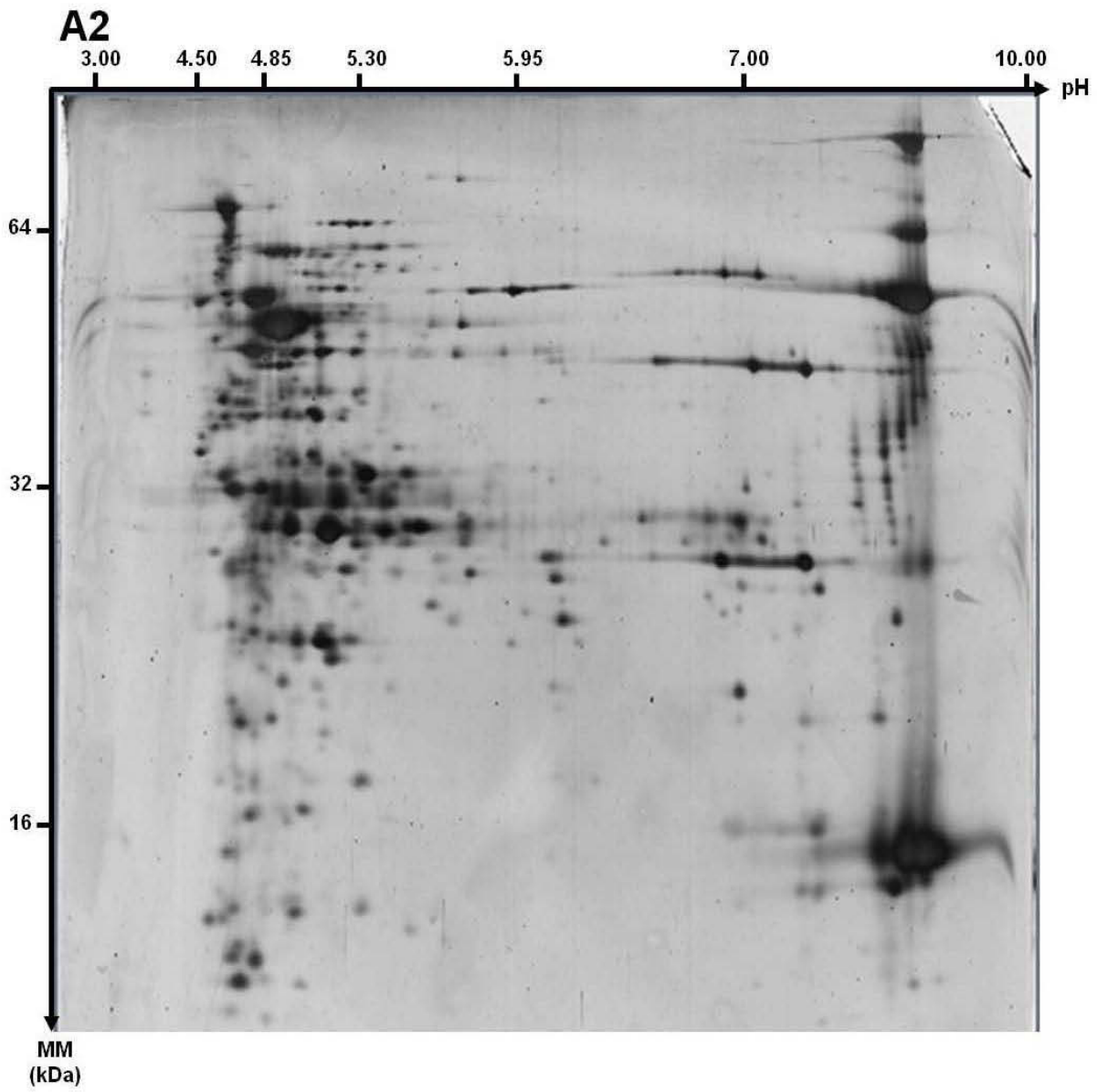
Figure 4S. 2-DE gel of the exoproteome at 37°C of *L. monocytogenes* EGD-e *wt* (A) and Δ*secA2* (B). Proteins were run in the first dimension by IEF in linear pH 4-7 (1) or in non-linear pH 3-10 IPG strips (2), and in SDS-PAGE 12.5% acrylamide gel in the second dimension.

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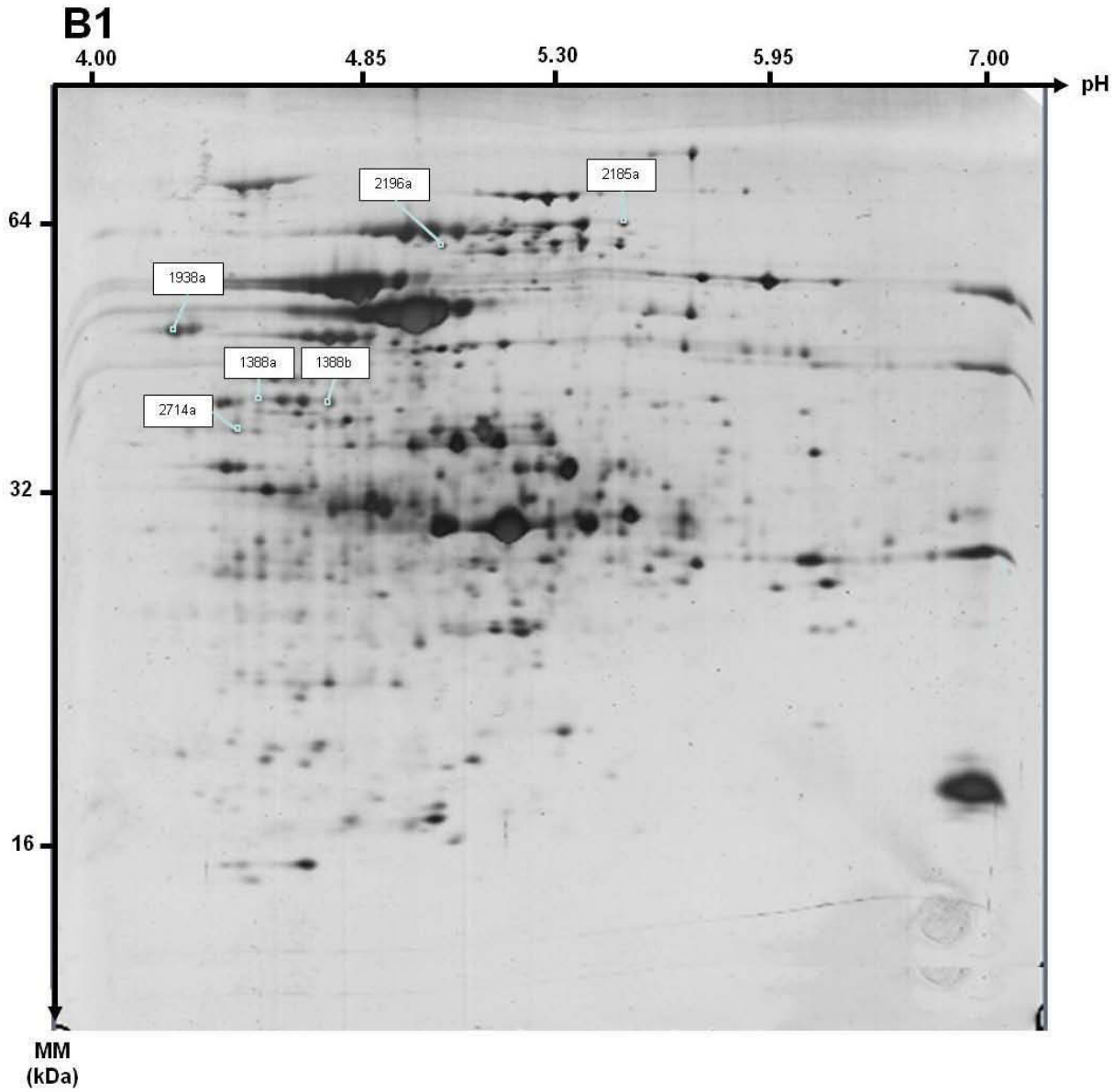
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1388b
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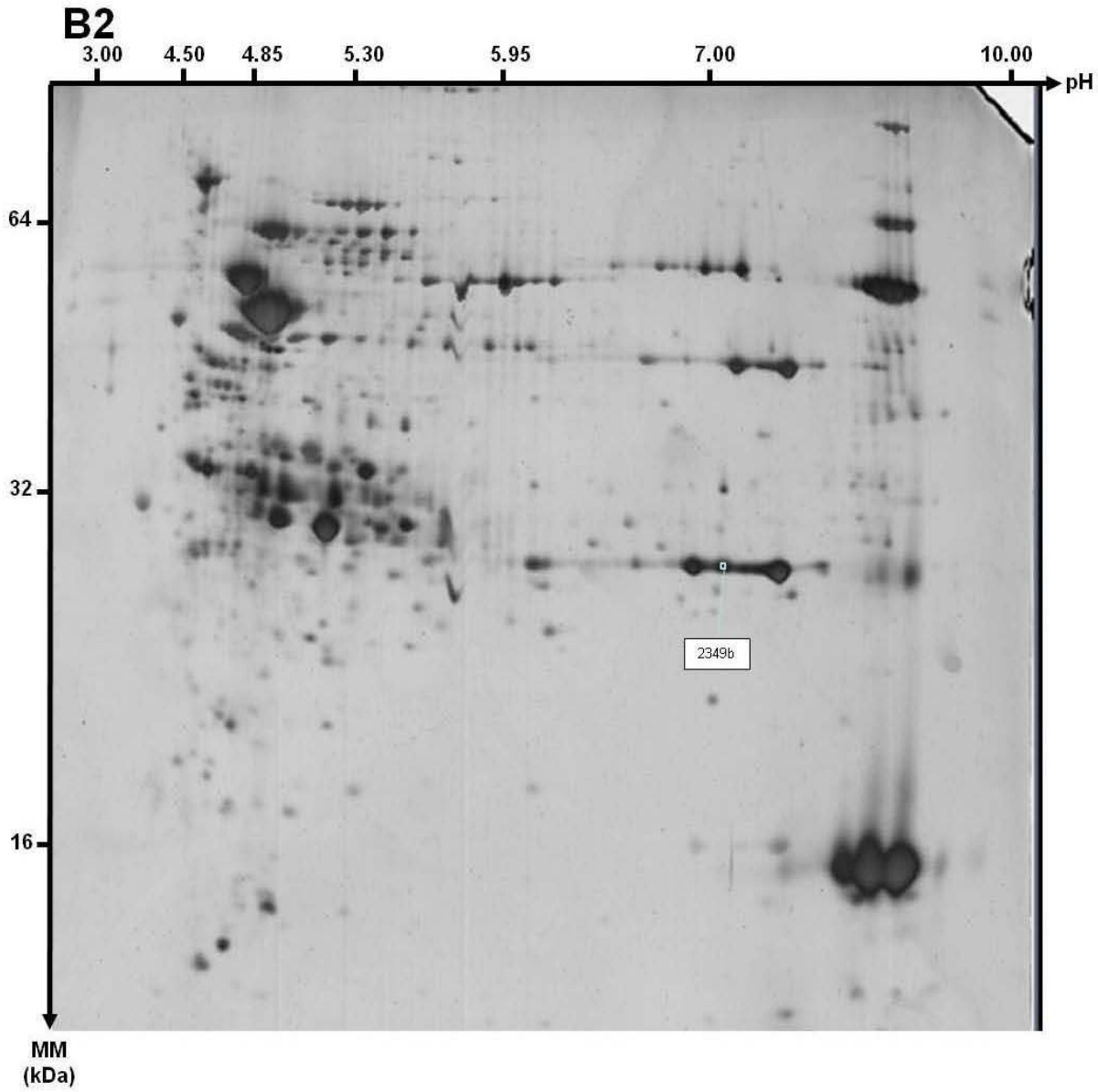
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4. La voie de maturation des lipoprotéines

Les lipoprotéines appartiennent à une catégorie de protéines de surface liées de façon covalente à la membrane cellulaire. Chez les bactéries à Gram positif, elles peuvent intervenir dans différents processus physiologiques comme la sporulation, la conjugaison, la résistance à des antibiotiques, le transport de substrats, la virulence ou encore l'adhésion (Sutcliffe and Russell 1995). Chez *L. monocytogenes*, les lipoprotéines constituent le plus important groupe de protéines de surfaces après les protéines membranaires (Renier, Micheau et al. 2012). L'inactivation de la voie de maturation des lipoprotéines est une stratégie permettant d'éliminer toutes les lipoprotéines normalement présentes à la membrane cytoplasmique et donc pertinent pour étudier l'implication des lipoprotéines dans la formation de biofilm. Pour cela, un mutant de délétion du gène *lgt* codant une diacylglycéryl transférase a été construit. Cette protéine permet de lier les lipoprotéines à la membrane cellulaire avant leur maturation par les peptidases signal de type II (SPases II). Par conséquent, la délétion du gène *lgt* génère théoriquement un mutant totalement dépourvu de lipoprotéines en surface cellulaire. L'étude des mutants des gènes codant les SPases II, LspA et LspB, permet d'obtenir des informations sur la voie préférentiellement empruntée par la ou les lipoprotéines impliquées dans la formation de biofilm et souligne l'importance de la maturation dans l'activité des lipoprotéines. En effet, les lipoprotéines non maturées restent ancrées à la membrane par leur SP sous forme de prolipoprotéine. En revanche, la délétion simultanée de *lgt* et du gène codant l'une des deux SPases II, permet le relargage d'une partie des lipoprotéines qui sont maturées par la SPase II restante.

Les mutants cités ci-dessus ont été testés pour leur capacité à former un biofilm en comparaison avec la souche sauvage. Les phases précoces et tardives de formation de biofilm ont été évaluées par les techniques du Biofilm Ring Test et de coloration au cristal violet, respectivement. Des informations sur l'architecture des biofilms ont été fournies par des observations en microscopie confocale. Enfin, l'expression des gènes codant les lipoprotéines a été suivie au cours de la formation de biofilm et a permis de se focaliser sur l'étude de trois d'entre elles. Par ailleurs, les SPases II n'ayant jamais été caractérisées, les exoprotéomes des doubles mutants Δ*lgt*Δ*LspA* et Δ*lgt*Δ*LspB* ont été comparés à celui de la souche sauvage et du mutant Δ*lgt* afin d'identifier les lipoprotéines maturées spécifiquement ou préférentiellement par LspA et LspB.

ARTICLE n°4

Sandra RENIER, Nelly CACCIA, Christophe CHAMBON, Didier VIALA, Julien DESCHAMPS, Romain BRIANDET, Alain CHARBIT, Michel HÉBRAUD and Mickaël DESVAUX

Involvement of lipoproteins in *Listeria monocytogenes* biofilm formation: Exoproteomic analysis of the lipoprotein maturation pathway

Pour publication dans *PLoS One*.

Involvement of lipoproteins in *L. monocytogenes* biofilm formation: Exoproteomic analysis of the lipoprotein maturation pathway

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Running title: biofilm formation in *L. monocytogenes*

Keywords:*Listeria monocytogenes*, biofilm formation, bacterial adhesion, lipoproteins, signal peptidases.

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ABSTRACT

The opportunistic foodborne pathogen *Listeria monocytogenes* is the etiological agent of listeriosis, a rare but serious infection. This ubiquitous species can switch from saprophyte to pathogen but also formed biofilm, which increase its resistance and persistence. Among cell surface proteins, lipoproteins constitute an important group of cell envelope proteins in *L. monocytogenes*. These proteins synthesized in the form of preprolipoproteins are translocated across the cytoplasmic membrane by the Sec system before being matured by the diacylglyceryl transferase (Lgt) followed by their signal peptide cleavage by a signal peptidase of Type II (SPase II). Two paralogues of SPase II are encoded in the genome of *L. monocytogenes* EGD-e, i.e. LspA and LspB. To investigate the importance of the lipoproteins in biofilm formation in *L. monocytogenes*, mutants lacking *lgt*, *lspA* and *lspB* genes were constructed. Their characterization reveals that the absence of lipoproteins on the cell surface is critical for biofilm formation and that LspA is required for optimal biofilm formation in *L. monocytogenes*. Genetic expression of lipoproteins during biofilm formation allowed the identification of LraI family lipoproteins potentially involved in biofilm formation and espacially LpeA which deletion restored Δ*lgt* phenotype. To decipher the specificity of LspA and LspB in lipoprotein maturation pathway, the lipoproteome of *L. monocytogenes* was investigated following off-gel and label-free quantitative proteomic approach. From this investigation, LspA appears to be the major SPase II involved in lipoprotein maturation.

INTRODUCTION

Listeria monocytogenes is an ubiquitous Gram-positive bacteria and the etiological agent of a rare but serious disease, namely listeriosis. Infection can manifest in two different ways: (i) a non-invasive form, which mainly results in gastroenteritis, and (ii) an invasive form, which is more severe (meningitis, encephalitis, abortion) and touched preferentially individuals whose immune system is somehow compromised, namely pregnant women, neonates, the elderly and patients with some serious diseases such as cancer or AIDS (Allerberger and Wagner 2010). As stunning feature, *L. monocytogenes* can be found in the environment as a saprophyte in the soil and make a transition into an intracellular pathogen following food consumption by a host (Gray, Freitag et al. 2006; Ivanek, Grohn et al. 2006; Freitag, Port et al. 2009). This opportunistic foodborne pathogen has been isolated from an extensive range of food products, including vegetables, milk, cheeses, fish and meat products as well as various ready-to-eat products (Little, Barrett et al. 2008; Mataragas, Stergiou et al. 2008; Cordano and Jacquet 2009; Kushwaha and Muriana 2009; Ells and Truelstrup Hansen 2010). *L. monocytogenes* is particularly problematic in the food industry since it can survive and multiply under conditions frequently used for food preservation (Gandhi and Chikindas 2007). Moreover, *L. monocytogenes* adheres to food contact surfaces and can further establish a biofilm, which protects bacteria from desiccation, antimicrobials and sanitizing agents. While cell surface proteins appear to be the major adherence factors in *L. monocytogenes* (Smoot and Pierson 1998; Longhi, Scoarughi et al. 2008; Franciosa, Maugliani et al. 2009), few of them has been identified as associated with biofilm formation (Renier, Hebraud et al. 2011).

Surface proteins play essential roles in the interactions of bacteria with their surroundings. In Gram-positive bacteria and following the gene ontology terms (GO) for subcellular localization, cell surface proteins (GO:0009986) can be (i) covalently or non-covalently linked to the cell wall (GO:0009275), or (ii) intrinsic to the cytoplasmic membrane (CM) (GO:0031226), i.e. either integral (GO:0005887) or anchored to the CM (GO:0046658). This last category constitutes an important group of surface proteins in *L. monocytogenes*, since most recent genomic analysis following a secretomics-based method estimated at 74 the number of lipoproteins in this species (Renier, Micheau et al. 2012). Lipoproteins can exhibit in broad range of important physiological functions like antibiotic resistance, solute transport, signaling, sporulation, conjugation, adhesion and bacterial virulence (Sutcliffe and Russell 1995). Lipoproteins are synthetized in the form of preprolipoproteins possessing a N-terminal

signal peptide (SP) of Type II bearing a consensus sequence named the lipobox (commonly Leu_{-3}-Ala_{-2}-Gly_{-1}-Cys_{+1}) (Kovacs-Simon, Titball et al. 2011). After the translocation through the Sec system, the maturation of the pre-prolipoproteins emerging on the external side of the CM is initiated by the diacylglyceryl transferase (Lgt), which recognizes the lipobox and transfers a diacylglyceryl moiety from a phosphatidylglycerol to the sulfhydryl group of the very conserved Cys_{+1} of the lipobox. Then, the SP II of the prolipoprotein is cleaved off by a signal peptidase of Type II (SPase II). In Gram-negative and high-GC Gram-positive bacteria, the N-terminal cysteine is further modified by addition of an amide-linked fatty acid by apolipoprotein aminoacyl transferase (Lnt). While no orthologue of this enzyme is encoded in the genomes of low-GC Gram-positive bacteria, the presence of triacylated lipoproteins in *Staphylococcus aureus* and *Acholeplasma laidlawii* was recently reported suggesting that an analogue to Lnt would be present but remains to be uncovered (Kurokawa, Ryu et al. 2012). In *L. monocytogenes*, though, only diacylated lipoproteins have been identified.

While early genomic analysis indicated that the lipoprotein maturation pathway in *L. monocytgenes* was constituted of Lgt (Lmo2482) and a SPase II (Lmo1844), called LspA (lipoprotein signal peptidase A) (Glaser, Frangeul et al. 2001), most recent secretomic analyses revealed the presence of an overlooked SPase II paralogue, named LspB (Lmo1101), (Desvaux and Hebraud 2006; Renier, Micheau et al. 2012). Recent investigations showed that the deletion of the *lgt* gene in *L. monocytogenes* led to a bacterial surface totally devoid of lipoproteins (Baumgartner, Karst et al. 2007). In the absence of their diacylglyceryl moiety, these proteins were released from the CM into the extracellular milieu upon cleavage of their N-terminal SP by SPase II. Indeed, unlike in *Escherichia coli* (Tokunaga, Tokunaga et al. 1982), the modification of the conserved cysteine is not necessary to the activation of the SPase (Baumgartner, Karst et al. 2007). Besides lipoproteomic analysis based on the exoproteome, the inactivation of *lgt* and *lsp* further allowed to stress the importance of lipoproteins in bacterial virulence (Réglier-Poupet, Frehel et al. 2003; Baumgartner, Karst et al. 2007; Machata, Tchatalbachev et al. 2008). Altogether, this prompted us to investigate the importance of the lipoproteins in biofilm formation in *L. monocytogenes* but also to decipher the specificity of LpsA and LpsB in *L. monocytogenes* lipoprotein maturation pathway.

RESULTS

The absence of lipoproteins on the cell surface is critical for biofilm formation in L. monocytogenes

In order to determine the potential involvement of lipoproteins in *L. monocytogenes* biofilm formation, an isogenic *lgt* deletion mutant was constructed and compared to the wild type (*wt*) strain. Different stages of biofilm formation were considered, i.e. the early and later stages of sessile development, which were followed by the BioFilm Ring Test (BRT) (Chavant, Gaillard-Martinie et al. 2007) and the crystal violet methods (Borucki, Peppin et al. 2003) respectively. Experimentations were performed at two temperatures relevant to the listerial physiology as a saprophyte and pathogen, i.e. 20°C and 37°C, respectively. The growth rate of *L. monocytogenes* was not affected by the deletion of *lgt* in any of these temperatures (Fig. 1S).

Whatever the temperature, the deletion of the *lgt* gene had no impact on the early stages of *L. monocytogenes* biofilm formation. Indeed, at 20°C neither the *wt* nor Δ*lgt* strains could block the microbeads within 24 h of incubation (BioFilm Index [BFI] > 2), whereas at 37°C both strains blocked the microbeads (BioFilm Index [BFI] < 2) from and after 6 h of incubation (Fig. 1A). Following later stages of biofilm formation with the crystal violet method, however, the sessile biomass was significantly lower in the Δ*lgt* strain from and after 48 h and 24 h of incubation at 20°C and 37°C, respectively (Fig. 1B). Complementation of the *lgt* gene in the mutant strain restored *wt* biofilm phenotype (Fig. 2S). At 20°C and compared to the *L. monocytogenes* EGD-e *wt,* initial bacterial adhesion of the Δ*lgt* mutant is affected and could explain the reduced sessile biomass previously observed (Fig. 1B and 1C). At 37°C, however, this decrease was not associated with a lower initial bacterial adhesion, which was similar between *wt* and Δ*lgt* strains (Fig. 1C). Altogether, it appeared that a *L. monocytogenes* strain devoid of lipoproteins on its cell surface is impaired in adhesion and biofilm development abilities at 20°C and 37°C respectively.

Maturation of lipoproteins by LspA is required for optimal biofilm formation at 37°C

While the *lspA* gene was previously shown to encode a SPase II required for lipoproteins maturation (Réglier-Poupet, Frehel et al. 2003), *lspB* had never been characterized as yet (Desvaux, 2006). In order to investigate the involvement of lipoprotein

maturation in biofilm formation, the isogenic *lspA* and *lspB* deletion mutants were constructed and tested for their ability to form a biofilm. In the first instance, transcriptional analysis by RT-PCR revealed that *lspB* was indeed transcribed in both planktonic and sessile growth conditions (Fig. 3S).

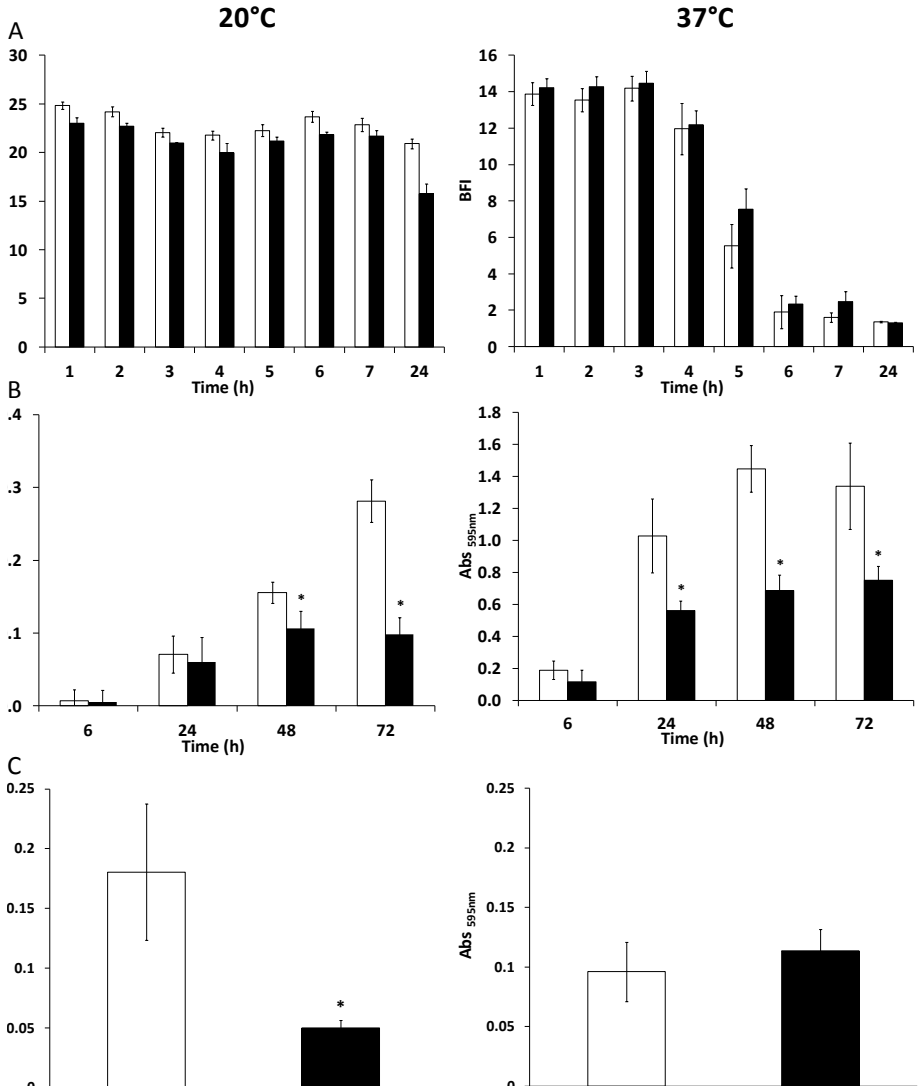

Figure 1: Biofilm formation and bacterial adhesion of *L. monocytogenes* EGD-e *wt* and the isogenic mutant Δ*lgt* at 37°C and 20°C. (A) Biofilm formation at early stages of sessile development assayed with the BRT. (B) Biofilm formation at late stages of sessile development and (C) initial adhesion assay assayed with the crystal violet method. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

As for the Δ*lgt* mutant, deletion of *lspA* or *lpsB* did not affect the early stages of biofilm formation neither at 20°C nor at 37°C (Fig. 2A). As follow by the crystal violet method, however, the sessile biomass was modified upon deletion of *lspA* at both 20°C and 37°C. LspA thus appeared to be required for efficient biofilm formation, whereas LspB is only required at 20°C (Fig. 2B). Regarding bacterial adhesion, no differences could be observed

upon deletion of *lspA* but it was significantly reduced in the Δ*lspB* mutant at 20°C (Fig. 2C), meaning LspB would process lipoprotein(s) involved in initial adhesion at 20°C. Complementation of Δ*lspA* confirmed that LspA is required for biofilm formation at 37°C (Fig. 4S). However, the complementation of Δ*lspB* did not restore the initial adhesion observed in the *wt* à 20°C (Fig. 5S).

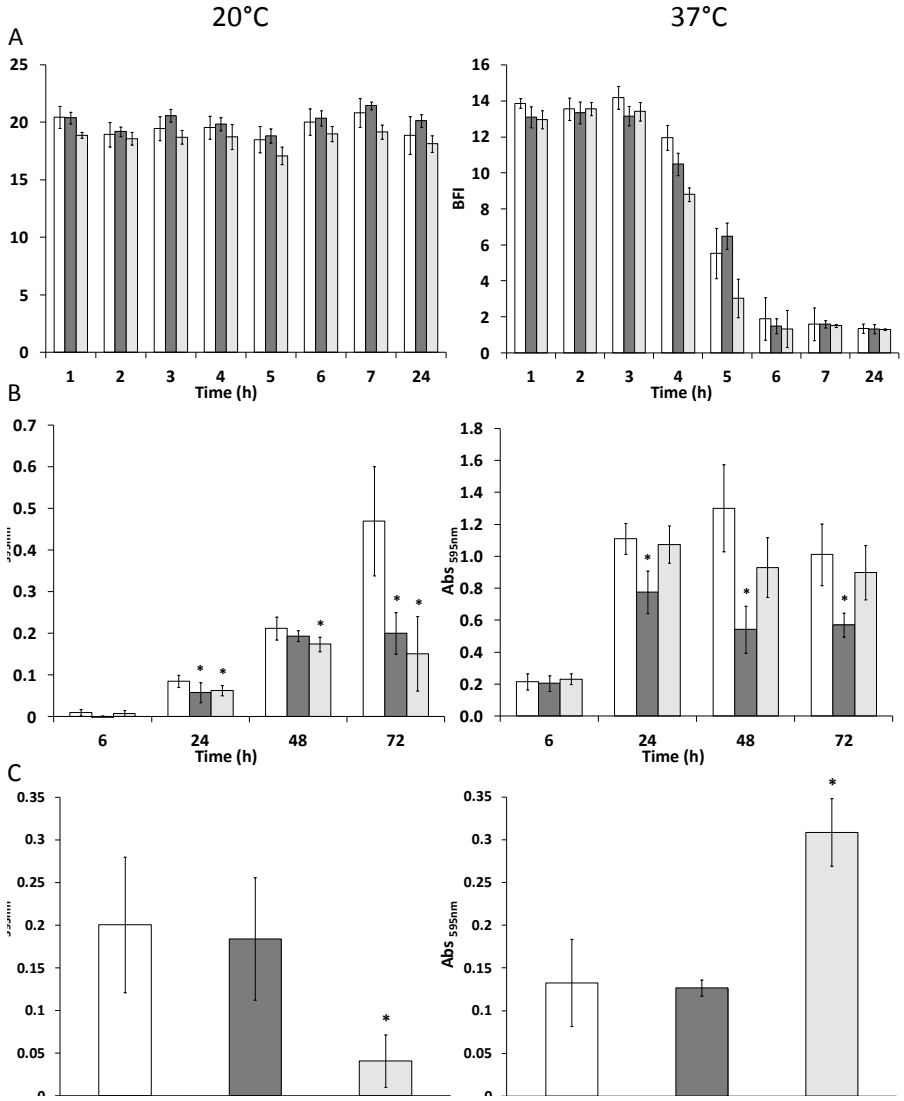

Figure 2: Biofilm formation of *L. monocytogenes* EGD-e *wt* and the isogenic mutants Δ*lspA* and Δ*lspB* at 37°C and 20°C. (A) Biofilm formation at early stages of sessile development assayed with the BRT. (B) Biofilm formation at late stages of sessile development and (C) initial adhesion assay (see Experimental procedures) assayed with the crystal violet method. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

To confirm the involvement of the SPase II in biofilm formation, the double mutant Δ*lgt*Δ*lspA* and Δ*lgt*Δ*lspB* were constructed. In the case of the Δ*lgt*Δ*lspA* mutant for instance, lipoproteins matured by LspA remain anchored in the cytoplasmic membrane by their SP and are not post-translationally modified by Lgt but lipoproteins processed by LspB are released

into the supernatant. Firstly, the growth rates of double deletion mutants were not affected compared to *L. monocytogenes wt* (Fig. 5S). As expected at early stage of biofilm formation, neither the *wt* nor the double mutant strains could block the microbeads within 24 h of incubation at 20°C (Fig. 3A). However, from and after 48 h of biofilm formation, lower sessile biomass associated with decreased initial adhesion was observed for both double mutants (Fig. 3B and 3C). These results confirmed the presence of lipoprotein(s) processed by LspB was required for efficient initial bacterial adhesion at 20°C. While at 37°C the Δ*lgt*Δ*lspA* mutant tended to block the microbeads one hour earlier compared to the *wt* (Fig. 3A), the sessile biomass was nonetheless significantly lower in the both double mutants, with a more pronounced decreased with the Δ*lgt*Δ*lspB* mutant (Fig. 3B).

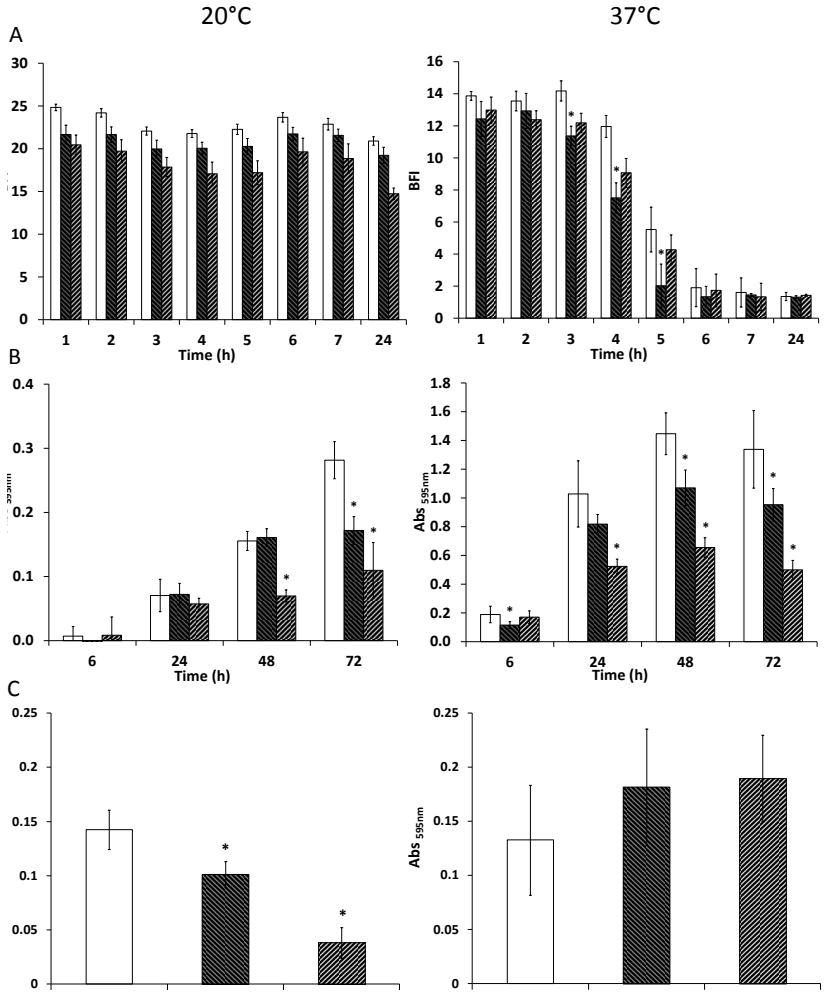

Figure 3: Biofilm formation of *L. monocytogenes* EGD-e *wt* and the isogenic mutants Δ*lgt*Δ*lspA* and Δ*lgt*Δ*lspB* at 37°C and 20°C. (A) Biofilm formation at early stages of sessile development assayed with the BRT. (B) Biofilm formation at late stages of sessile development and (C) initial adhesion assay (see Experimental procedures) assayed with the crystal violet method. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

This result confirmed lipoprotein(s) important for biofilm formation are more specifically matured by LspA rather than LspB at 37°C. Indeed, in the Δ*lgt*Δ*lspB* mutant, the cell surface is totally devoid of lipoproteins processed by LspA, whereas in the Δ*lgt*Δ*lspA* LspA-dependent lipoproteins are not subjected to post-translocational modification by Lgt but remain anchored to the cytoplasmic membrane by their SP.

To get further insight in the biofilm architecture of the *L. monocytogenes wt* and mutant strains, microscopic observations were made under static condition using confocal laser scanning microscope (CLSM). After 24 hours of incubation at 20°C, strains were only able to adhere to the surface that confirms the results previously obtained with the BRT. Indeed, microscopic observation revealed motile cells swimming in the media with only a few individual adhering cells. The increase of sessile biomass over time observed in the *wt* by the crystal violet method (Fig. 1B) would mostly result from bacterial adhesion (Fig. 1C) rather than sessile development *per se*.

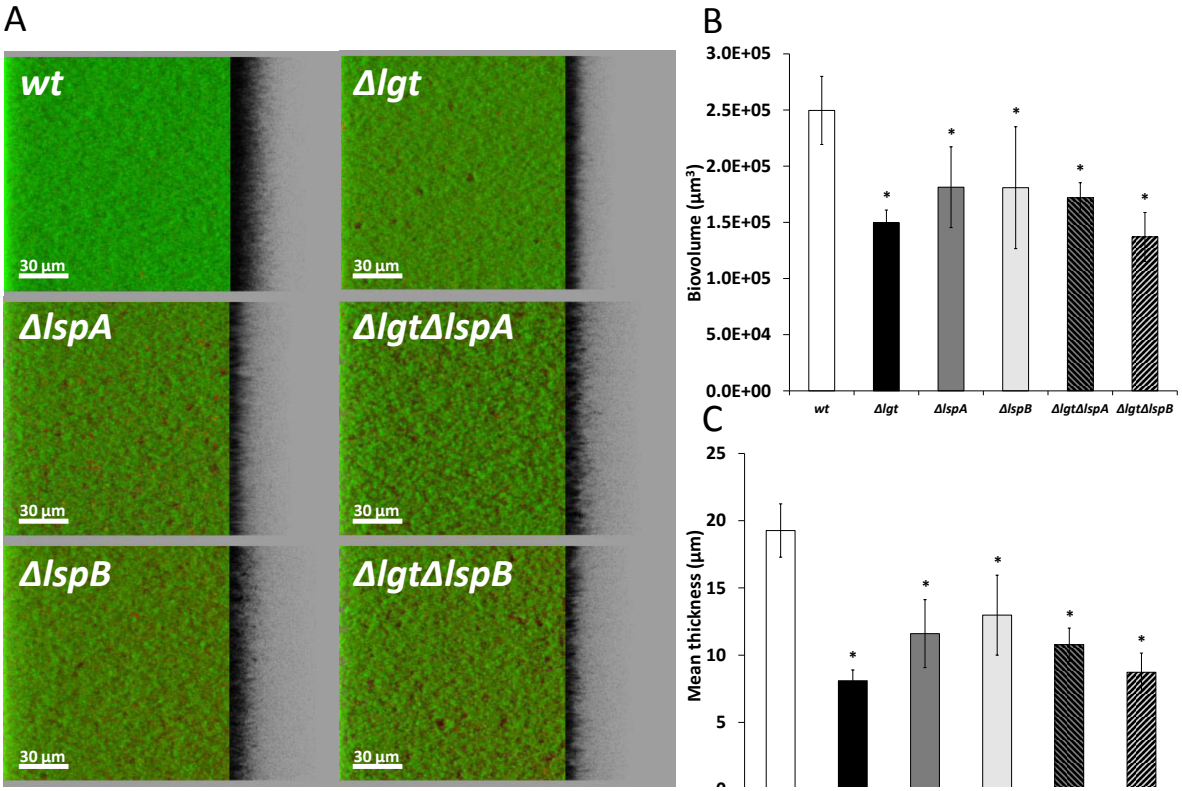

Figure 4: Biofilm formation of *L. monocytogenes wt* and its mutants bearing pNF8 under static condition at 37°C. (A) CLSM images. The side view projections were acquired after 4 h and 24 h of biofilm development. (B), biovolume and (C) thickness were obtained on biofilms of the *wt* and mutant strains after 24 h of growth and resulted from image analyses as described in the Experimental Procedures. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

At 37°C and after 24 h sessile growth, all strains formed a biofilm that covered almost entirely and homogeneously the surface (Fig. 4A). In agreement with data obtained with the

crystal violet method, however, total biovolumes were significantly lower for all mutants when compared to *L. monocytogenes* EGD-e *wt* (Fig. 4B). In addition, mean thickness of mutants was significantly lower than the *wt* strain (Fig. 4C). Considered together with (i) the sessile biomass of the Δ*lgt*Δ*lspB* strain that did not significantly increase over 72 h of biofilm development (Fig. 3B), (ii) a similar initial bacterial adhesion between the *wt* and Δ*lgt*Δ*lspB* (Fig. 3C), and (iii) restoration of the *wt* biofilm formation phenotype in the complemented Δ*lspA* strain (Fig. 4S), these data provide a body of evidences for the importance LspA-dependent lipoprotein(s) in *L. monocytogenes* biofilm formation at 37°C.

Identification of LraI family lipoproteins potentially involved in biofilm formation

In order to identify some individual lipoprotein(s) potentially involved in *L. monocytogenes* biofilm formation at 37°C, genetic expression was monitored for all the genes predicted to encode a lipoprotein by at least one of the 8 prediction tools applied in the secretomics-based method (Renier, Micheau et al. 2012). While the number of lipoproteins in *L. monocytogenes* EGD-e was estimated at 74 upon majority voting, 90 distinct genes were predicted as encoding a putative lipoprotein at least once. From there, gene transcripts were measured by RT-PCR at different time points of *L. monocytogenes* biofilm formation (Table 2).

Globally, the relative gene expression decreased for a majority of the lipoproteins genes all along biofilm formation (Table 2). Only two genes, which transcripts could not be detected at time 0, were over-expressed from and after 6 h and 12 h of sessile development, namely *lmo1671* and *lmo0153* respectively. Unexpectedly, these two genes originally annotated as hypothetical proteins in GenBank are actually homologous to adhesins belonging to LraI (lipoprotein receptor antigen I) family (COG0803: *E*-value=3.0×10^{-79} for Lmo0153 and *E*-value=7.0×10^{-70} for Lmo1671) together with Lmo1847 (COG0803: *E*-value=6.0×10^{-79}). Contrary to *lmo0153* and *lmo1671*, *lmo1847*, which encodes the protein LpeA (lipoprotein promoting entry A) (Réglier-Poupet, Pellegrini et al. 2003), was strongly expressed all along the biofilm formation (Table 2). This prompted us to investigate the possible involvement of these 3 lipoproteins in *L. monocytogenes* biofilm formation by functional genetic analysis.

LpeA is required for efficient biofilm formation

While the deletion of *lmo0153* could not be achieved, the isogenic *L. monocytogenes* deletion mutants for *lpeA* and *lmo1671* were constructed and further tested for their biofilm

formation ability at 37°C. As expected from previous result, the deletion of *lpeA* or *lmo1671* did not affect the early stages of biofilm formation as monitored with the BRT (Fig. 7S). As follow by the crystal violet method, the sessile development of the Δ*lmo1671* mutant did not differ from the *wt* (Fig. 5). The sessile biomass of the Δ*lpeA* mutant, however, was significantly decreased and similar to what is observed with of the Δ*lgt* mutant (Fig. 5). Complementation of *lpeA* in the Δ*lpeA* mutant strain partially restored *wt* biofilm phenotype (Fig. 8S). Considering that the bacterial growth and the initial bacterial adhesion were not affected in the Δ*lpeA* strain (Fig. 6S), LpeA was demonstrated to be an important determinant for efficient biofilm formation in *L. monocytogenes*.

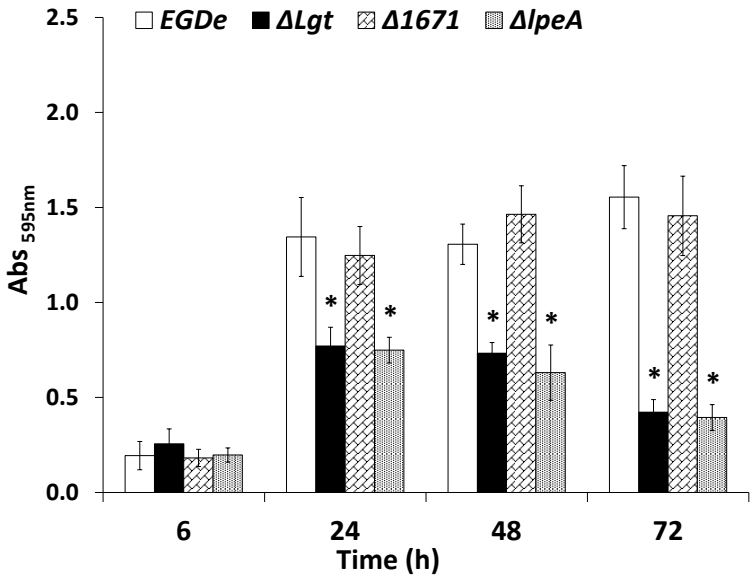

Figure 5: Biofilm formation of *L. monocytogenes* EGD-e *wt* and the isogenic mutants Δ*lmo1671* and Δ*lpeA* at 37°C. Biofilm formation at late stages of sessile development was assayed with the crystal violet method. Statistical significance of the results is indicated by an asterisk ($P < 0.05$).

Analysis of the specificity of LspA and LspB in L. monocytogenes lipoprotein maturation

In order to determine the substrate specificity for the two SPases II, namely LspA and LspB, the lipoproteome of *L. monocytogenes wt* and some isogenic mutants for the lipoprotein maturation pathway was investigated. Actually, this exoproteomic analysis was based on the differential release into the extracellular milieu of the lipoproteins upon deletion of *lgt* versus Δ*lgt*Δ*lspA* or Δ*lgt*Δ*lspB*. This subproteome was investigated following off-gel and label-free quantitive proteomic approach by LC-MS/MS.

In the first instance, comparing the supernatants of the *Δlgt* and *wt* strains, 27 out of the 74 lipoproteins predicted by majority vote in *L. monocytogenes* (Renier, Micheau et al. 2012) were here experimentally identified (Table 3). While 6 of them were present in similar amount in the extracellular milieu of both the *L. monocytogenes wt* and the isogenic mutant,

15 lipoproteins were totally absent from the *wt* supernatant and 5 lipoproteins were found in a lower amount (Table 3). Surprisingly, the lipoprotein QoxA, was found in a higher amount in the *wt* compared to Δ*lgt* mutant. Together with 10 out of the 11 other lipoproteins present in the extracellular milieu of *L. monocytogenes wt* (Table 3), it exhibited a glycine residue at position +2 of their SP II cleavage site (C+2) as predicted by the secretomics-based method (Renier, Micheau et al. 2012), which is considered as major determinant in lipoprotein release into the supernatant of Gram-positive bacteria (Antelmann, Tjalsma et al. 2001; Tjalsma and van Dijl 2005).

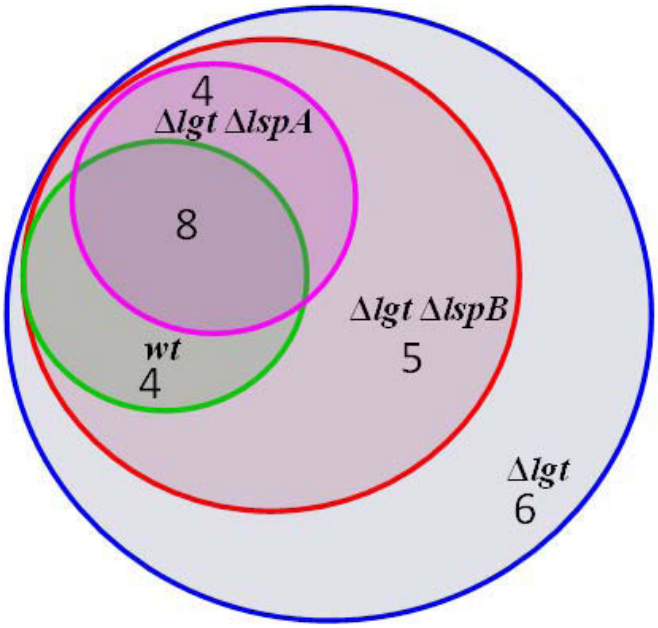

Figure 6: Repartition of lipoproteins identified in the *wt* and mutants supernatants. A total of 27 lipoproteins were identified in the supernatant of Δ*lgt*, including 6 which were exclusively found in Δ*lgt*. Among these 27 lipoproteins, 21 were found in the supernatant of Δ*lgt*Δ*lspB*, including 5 that were absent from the *wt* and Δ*lgt*Δ*lspA* supernatants. Finally, among lipoproteins identified in Δ*lgt*Δ*lspA,* 12 lipoproteins were found in the supernatant of Δ*lgt*Δ*lspA* and the *wt*, including 8 lipoproteins common to all strains.

Comparing the exoproteome of *L. monocytogenes* Δ*lgt*Δ*lspA* with the *Δlgt* strain, 18 lipoproteins appeared present in different amounts including 15 that were absent from the supernatant of the *Δlgt* mutant and 3 lipoproteins were found in a lower amount (Table 3). These lipoproteins then appeared specifically matured by LspA but not exclusively for 3 of them, namely Lmo0152 and the two virulence associated lipoproteins TcsA and OppA. Comparing the supernatant of Δ*lgt*Δ*lspB* with *Δlgt,* 6 lipoproteins were actually absent indicating they were specifically matured by LspB (Table 3). However, these same lipoproteins were also specifically matured by LspA suggesting both SPases II are required for their maturation. On one hand, the 9 lipoproteins absent from the supernatant of

L. monocytogenes Δ*lgt*Δ*lspA* but present in the extracellular milieu of the Δ*lgt*Δ*lspB* strain would be specifically and exclusively matured by LspA. On the other hand, no lipoprotein were absent from supernatant of *L. monocytogenes* Δ*lgt*Δ*lspB* and present in the extracellular milieu of the Δ*lgt*Δ*lspA*. In addition, 9 lipoproteins were found in similar amount between Δ*lgt*Δ*lspA* and *Δlgt* but also between Δ*lgt*Δ*lspB* and *Δlgt* (Fig. 6). Among them, 5 lipoproteins were absent or found in a lower amount from the supernatant of *L. monocytogenes wt*, indicating they could be equally matured either by LpsA or LpsB. Three other lipoproteins were found in similar amount between the *wt* and *Δlgt* strains indicating neither LspA nor LspB appeared specific for maturation of these putative lipoproteins.

DISCUSSION

In this study, the involvement of a large group of cell-envelope proteins in *L. monocytogenes*, namely lipoproteins, was for the first time investigated in relation to biofilm formation. By inactivating a key mechanism for cell-surface display of lipoproteins in Gram-positive bacteria, namely the diacylglyceryl transferase Lgt, none proteins were acylated and were consequently all released from the cytoplasmic membrane upon cleavage by SPase II (Baumgartner, Karst et al. 2007). This strategy had previously allowed demonstrating the importance of lipoproteins in *L. monocytogenes* virulence (Réglier-Poupet, Frehel et al. 2003). From the present investigation, *L. monocytogenes* Δ*lgt* devoid of lipoproteins on its cell surface was significantly impaired in its surface adhesion and colonization abilities, which definitively established the involvement of lipoproteins in *L. monocytogenes* sessile development. It further appeared the SPase II LspA over LspB was required for optimal biofilm formation. Following genetic expression and functional genetic analyses originally based on *in silico* hypothesis generated from the secretomics-based method (Renier, Micheau et al. 2012), an adhesin of the LraI family was demonstrated as a key lipoprotein involved in *L. monocytogenes* biofilm formation, namely LpeA. Together with OppA, this lipoprotein was previously shown to be matured by LspA and to participate in *L. monocytogenes* virulence (Réglier-poutet 2001).

LraI (lipoprotein receptor antigen I) family proteins play a dual role in metal ion transport and adhesion (Elsner, Kreikemeyer et al. 2002; Papp-Wallace and Maguire 2006). Adherence properties of LraI range from (i) binding to extracellular matrix (ECM) components, especially laminin with LmB or Lsp from *S. agalactiae* and *S. pyogenes* respectively or fibrin with FimA from *S. parasanguis* (Viscount, Munro et al. 1997; Spellerberg, Rozdzinski et al. 1999; Elsner, Kreikemeyer et al. 2002), (ii) eukaryotic cells, e.g. PsaA from *S. pneumonia* to pneumocytes (Berry and Paton 1996), and (iii) to other bacterial cells, e.g. ScaA in coaggregation of *S. gordonii* with *Actinomyces naeslundii* (Andersen, Ganeshkumar et al. 1993). While LpeA was not involved in adhesion to eukaryotic cells, it was previously demonstrated as required for *L. monocytogenes* virulence, especially internalization into host cells as Lsp, which is required for internalization of *S. pyogenes* (Elsner, Kreikemeyer et al. 2002; Réglier-Poupet, Pellegrini et al. 2003). Two additional paralogues of the LraI family were here uncovered for the first time in *L. monocytogenes*, namely Lmo0153 and Lmo1671, and their functional characterization in

adhesion to ECM components, eukaryotic and/or bacterial cells would also require further investigations.

From the investigation of the lipoproteome, it appeared some lipoproteins were specifically and even exclusively matured by LspA. Proteins specifically matured by LspB, though, also required LpsA for complete post-translocational modification. Some other lipoproteins could have their SP II equally cleaved by LspA or LspB. While no conserved domain could be identified following protein sequence alignment within SP to explain the specific and/or exclusive lipoprotein maturation by LspA or LspB, further in-depth investigations of structure-function relationships are clearly required. Of note, the presence of lipoproteins in the supernatant of *L. monocytogenes* EGD-e was already and previously reported from several exoproteomic investigations (Trost, Wehmhoner et al. 2005; Baumgartner, Karst et al. 2007; Desvaux, Dumas et al. 2010). While glycine at position C+2 is considered of major importance in lipoprotein release into the extracellular milieu, its function as a signal for lipoprotein exclusion from the cytoplasmic membrane remains to be demonstrated. Indeed, their ectopic location in the extracellular milieu could result from natural shaving by specific cell-surface proteases (Antelmann, Tjalsma et al. 2001; Tjalsma and van Dijl 2005; Westers, Westers et al. 2008). Interestingly, the virulence associated lipoproteins OppA and TscA were previously found in lower amount on the cell surface in *L. monocytogenes* Δ*secA2*, suggesting a relationship between the lipoprotein maturation and SecA2-depedent secretion pathways that would require further investigations (Lenz and Portnoy 2002).

While the involvement of lipoproteins in *L. monocytogenes* biofilm formation was here uncovered for the first time, their contribution to surface colonization in different environmental conditions depending on the nature of the support (stainless steel, glass,…etc), temperature, pH or redox would require further studies. Considering the *L. monocytogenes* fitness, the influence of environmental conditions on listerial physiology should not be overlooked. The unexpected involvement of member of LraI family protein in *L. monocytogenes* biofilm formation is particularly exciting and questioned their possible contribution to colonization of biotic surface not only in the course of an infection but also during contamination of food, especially animal products (Chagnot, Listrat et al. 2012). Indeed, none of the three paralogues of the LraI family have been characterized as yet in *L. monocytogenes* with regards to adhesion to laminin. This working hypothesis sounds like the promise of exciting new development in the field of listerial adhesion/colonization of ECM components.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. Routinely, cells of *L. monocytogenes* were cultivated in BHI (brain-heart infusion) broth or BHI agar plates at 20°C or 37°C. If necessary, X-Gal (100 µg ml^{-1}) and antibiotics were added in the following concentrations: erythromycin (5 µg ml^{-1}), kanamycin (50 µg ml^{-1}). For all cloning procedures *Escherichia coli* TOP 10 was used as the standard plasmid host (Sambrook and Russell 2001). For proteomic experiments, the wild type (*wt*) and the double mutants Δ*lgt*Δ*lspA* and Δ*lgt*Δ*lspB*, were cultured in a chemically defined medium, i.e. MCDB202 (CryoBioSystem). Nutrient broth medium was prepared according to supplier instructions at a final glucose concentration of 5 g L^{-1} and pH adjusted at 7.3. From -80°C stock culture (previously grown in MCDB202), bacterial strains were plated on MCDB202 agar (15 g L^{-1}) and incubated 24-48 h at 20 or 37°C. A preculture was set up from one isolated bacterial colony and grown in MCDB202 broth at the relevant temperature in an orbital shaker (170 rpm). A 250-ml culture was adjusted at 0.01 (OD_{600nm}) from an inoculum in the exponential growth phase and incubated as described above prior to sampling in late exponential growth phase (OD_{600nm} = 0.3) after 8 h of incubation.

Construction of L. monocytogenes mutants and gene complementation

The genes encoding Lgt (lmo2482), LspB (lmo1101), LpeA (lmo1847) and Lmo1671 (lmo1671) were deleted by allelic exchange using pMAD vector as described previously (Arnaud, Chastanet et al. 2004). From *L. monocytogenes* EGD-e genomic DNA purified with Wizard® Genomic DNA Purification Kit (Promega), upstream and downstream DNA fragments flanking the gene of interest were amplified by high-fidelity PCR using TaKaRa LA Taq DNA polymerase with two couples of primers, i.e. Fw1/Rv2 and Fw3/Rv4 respectively (Table 2). The two PCR products then served as a matrix for the SOE-PCR using Fw1/Rv4 primers. Following standard molecular cloning technique (Sambrook and Russell 2001), the resulting amplicon was cloned into pMAD following DNA restriction digestion with NcoI and MluI, ligation, transformation into *E. coli* TOP10 (Invitrogen) and selection on LB (lysogeny broth) agar with ampicillin (100 µg ml^{-1}). After purification from *E. coli* using Nucleospin Plasmid QuickPure (Macherey-Nagel), the resulting plasmid pMAD-Δ*lgt*, pMAD-Δ*LspB,* pMAD-*ΔlpeA* and pMAD-*Δlmo1647* were electroporated into

L. monocytogenes EGD-e for single mutant construction and pMAD-Δ*lgt* was also electroporated in Δ*lspA* and Δ*lspB* for the construction of the double mutants Δ*lgt*Δ*lspA* and Δ*lgt*Δ*lspB*, respectively. The selection was performed on BHI agar containing erythromycin. As previously described (Arnaud, Chastanet et al. 2004), blue-white screening was applied to select gene knockout events. The isogenic mutants were identified by colony PCR with outFw/outRv primers using GoTaq DNA polymerase (Promega) and were further confirmed by DNA sequencing (GATC-Biotech) on both strands using primers Fw1 and Rv4, respectively.

For gene complementation, the entire CDS (coding sequence) was amplified from genomic DNA by PCR using TaKaRa LA Taq DNA polymerase and the primers Fw5/tRv6. The amplicon was cloned into pIMK2 (Monk *et al*., 2008) following DNA digest with NcoI/PstI restriction enzymes, ligation, electroporation into *E. coli* TOP10 and selection on LB agar with kanamycin. After plasmid purification, the resulting plasmids were electroporated into respective *L. monocytogenes* EGD-e mutants. Site-specific integration of the plasmid was confirmed following plating on by kanamycin BHI agar and colony PCR with primers Fw5/Fw6.

Biofilm formation assay at early stages of sessile development

The assay was conducted using the BioFilm Ring Test® (BRT) (Chavant, Gaillard-Martinie et al. 2007) following BioFilm Control (BFC, France) supplier recommendations, from overnight cultures *L. monocytogenes* EGD-e *wt* or mutant strains adjusted at $OD_{600nm} = 0.01$ (approximately 10^5 CFU ml^{-1}) in sterile BHI medium. Briefly, a suspension of paramagnetic microbeads (Ton5: 2.8 µm in diameter) was added at 10 µl ml^{-1} final concentration, homogenized by vortexing prior to 200 µl loading into 96-wells BFC Polystyrene Microtiter plates or 8-wells BFC Polystyrene Strips and static incubation at 20°C or 37°C. Control wells were filled with sterile BHI and Ton5. For reading at the different time points, wells of microtiter plates were first covered with 100 µl of BFC Contrast Liquid prior to scanning before and after one-minute magnetization using a BFC Magnetic Rack. Results were expressed as Biofilm Formation Index (BFI) (Chavant, Gaillard-Martinie et al. 2007; Macé, Seyer et al. 2008). Basically, in the course of bacterial sessile development, BFI decreases and a BFI ≤ 2 indicates a full immobilization of the paramagnetic microbeads. At least 5 independent experiments with at least two repeats each were performed for each strain and incubation time.

Biofilm formation assay at late stages of sessile development

The assay is based on the crystal violet method (Borucki, Peppin et al. 2003). Briefly, overnight cultures of *L. monocytogenes* strains were adjusted at 0.01 (OD_{600nm}) in sterile BHI medium and 200 µl loaded into the wells of a 96-wells polystyrene microtiter plate prior to static incubation at 20 or 37°C. At different time points, the supernatant was removed from the wells, which were washed with TS (tryptone salt) and fixed with absolute ethanol (20 min). After emptying and air-drying the wells, 200 µl of an aqueous solution of crystal violet (0.1%) was added and left for 10 min. After washing with water, the bound dye was solubilized with 200 µl of an aqueous solution of acetic acid (33 %). Contents of each well (150 µl) were transferred to a clean microtiter plate and absorbance was finally measured using a microtiter plate reader set to 595 nm. At least 5 independent experiments with at least two repeats each were performed for each strain and incubation time.

Initial adhesion

This assay is similar to the biofilm formation assay but with the following changes. Briefly, overnight cultures of *L. monocytogenes* strains were adjusted at 1.5 (OD_{600nm}) in sterile BHI medium and loaded into the wells of a 96-wells polystyrene microtiter plate prior to static incubation at 20 or 37°C. After one hour, the supernatant was removed from the wells, which were washed with TS and directly stained with an aqueous solution of crystal violet (0.1%). After washing, the bound dye was solubilized in acetic acid (33 %), then transfer to a clean microtiter plate where the absorbance was finally measured. At least 5 independent experiments with at least two repeats each were performed for each strain.

Biofilm growth conditions for CLSM images

Overnight cultures of *L. monocytogenes* strains carrying the pNF8 plasmid expressing the GFP, were adjusted at 0.01 (OD_{600nm}) in sterile BHI medium. 200 µl of these cultures were loaded into the wells of 96 wells polystyrene microtiter plate with a µclear® base (Greiner Bio-one, France) which enables high resolution fluorescence imaging. Then, the plates were incubated at 20 or 37°C. After 2 hours, the medium was removed, and 200 µl of fresh BHI was added. Biofilm development was evaluated by microscopic observations after 24 h of incubation. Three independent experiments were performed for each strain.

CLSM and image processing

Horizontal plane images of the biofilms were acquired using a Leica SP2 AOBS CLSM (Leica Microsystems, France) at the MIMA2 microscopy platform (http://www6.jouy.inra.fr/mima2_eng/). The excitation wavelength used for GFP was 488 nm, and emitted fluorescence was recorded within the range of 500 to 550 nm. Images were collected through a 63x Leica oil immersion objective (numerical aperture, 1.4).

3D projections were performed with IMARIS software (Bitplane, Zürich, Switzerland). The biofilm structural parameters (thickness and biovolume) were evaluated using the PHLIP Matlab program developed by J. Xavier (http://phlip.sourceforge.net/phlip-ml). For each experiment, at least 3 microscopic fields were analyzed.

Statistical analysis

In order to test the significance of the differences observed in each assay between the *wt* and the different mutants, a pair Student's *t*-test was performed. Differences were considered significant at $P < 0.05$.

RNA extraction and cDNA preparation

Overnight cultures of *L. monocytogenes* EGD-e *wt* were adjusted at 0.01 (OD_{600nm}) in plastic Petri dishes containing 25 ml of sterile BHI medium prior to incubation at 37°C. At different time points, the supernatant was removed from the Petri dishes and washed twice with TS (tryptone salt). Biofilm cells were resuspended in 10 ml of fresh BHI by scraping the bottom of the Petri dishes. Cells were harvested by centrifugation (6000 rpm, 5 min), resuspended in 500 µl of TE (10 mM Tris, 1 mM EDTA, pH 8) and were transferred in a tube containing 600 mg of zirconium beads (0.1 mm), 50 µl of acid phenol (pH 4) and 3.5 µl of β-mercaptoethanol. Then, cells were disrupted in a FastPrep®-24 (MP Biomedicals) for two rounds of 60 s at 6.5 m s^{-1} separated by 5 min of incubation in ice. After addition of chloroform (200 µl), tubes were mixed by inversion and centrifuged for 20 min at 13000 rpm, 4°C. The aqueous phase was recovered and purified with NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's recommendation. Nucleic acid concentrations were estimated by measuring the absorbance at 260 nm using a NanoDrop 1000. Before reverse transcription (RT), 10 µg of total RNA were treated with 2 U of Turbo DNase (Ambion) as

described by the manufacturer. The absence of chromosomal DNA contamination was checked by PCR. cDNA were then synthesized by using the Superscript Reverse Transcriptase (Invitrogen) as recommended.

RT-PCR

RT-PCR were performed with cDNA synthesized with total RNA extracted from five different biofilm incubation time as described above. Specific primers of proteins predicted at least once as lipoproteins (Renier, Micheau et al. 2012) and the housekeeping gene gyrB were designed (Table 1S).

Precipitation of extracellular proteins

The supernatants were recovered after culture filtrations (0.2 µm), and 0.2 mM phenylmethylsulfonyl fluoride was added to inhibit protease activity. Na deoxycholate (0.2 mg mL^{-1}) was added to the solution, and the solution was incubated for 30 min on ice. Na deoxycholate supports protein precipitation, which was carried out by the addition of 10 % (wt/vol) trichloroacetic acid and incubation overnight at 4°C. After centrifugation (12000 rpm, 30 min, 4°C), the precipitate was washed with ice-cold acetone and solubilized in a buffer compatible with mass spectrometry consisting of 8 M urea and 2 M thiourea. Protein concentrations were evaluated by the Bradford protein assay. Three protein extractions from 3 independent cultures were carried out.

Samples preparation before LC-MS/MS

As protein concentrations of mutant extracts including lipoproteins were higher than the one of the *wt*, the same volumes of protein samples (corresponding to 5 µg for the *wt*) were concentrated in the stacking gel of a SDS-PAGE according to Laemmli (1970). The run was stopped before the sample reached the resolving gel (15 min, 25 mA/gel) and samples were fixed in the stacking gels with a solution consisting of 10% acetic acid and 25% isopropanol. After 1 h of coloration in a colloidal Coomassie blue solution (10% acetic acid, 50% methanol, 0.25% Coomassie Blue Brillant R250) following by 20 min of destaining in a solution of 10 % acetic acid and 15 % methanol, bands were excised and subjected to the following treatments. First, the bands were washed in 25 mM ammonium bicarbonate–5 % acetonitrile (ACN) for 30 min and twice in 25 mM ammonium bicarbonate–50 % ACN for 30 min each. The spots were then dehydrated with 100 % ACN. The dried gels were reswelled in

25 mM ammonium bicarbonate containing 200 ng of trypsin, for the *wt* and the Δ*lgt*Δ*lspA* mutant and 400 ng of trypsin for the Δ*lgt* and Δ*lgt*Δ*lspB* mutants. Digestion was performed overnight at 37°C. The resulting peptides were extracted with 100 % (ACN), before evaporation and addition of 20 µl of 2 % ACN and 0.05 % trifluoroacetic acid.

Identification of proteins

The identifications was performed by nano-liquid chromatography (LC) coupled to electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) (LTQVelos, Thermo Scientific). Monoisotopic peptide masses were assigned and used for database searches with Mascot v2.2.0. Interrogations were performed against a database containing distinct protein entries corresponding to the mature set of *L. monocytogenes* EGD-e (desvaux Dumas 2010). The following parameters were considered for the searches: a maximum ion mass tolerance of 25 or 50 ppm, possible modification of cysteines by carbamidomethylation, as well as partial oxidation of methionine.

Relative quantification

The relative amount of proteins was estimated using Progenesis LC-MS software (Nonlinear dynamics). The Δ*lgt* mutant was compared to the *wt*, whereas Δ*lgt*Δ*lspA* and Δ*lgt*Δ*lspB* were compared with Δ*lgt* in order to evaluate the relative participation of each SPase in lipoprotein maturation. Differential protein expression was considered significant at $p < 0.01$ using ANOVA (Analysis of Variance) procedure.

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TABLES

Table 1. Strains and plasmids used in this study.

Name	Relevant characteristics	Source/reference
Plasmids		
pMAD	Amp^R, Em^R, *bgaB*	Arnaud *et al*. (2004)
pIMK2	Site-specific listerial integrative vector, p_{help}, Kan^R	Monk *et al*. (2008)
pMAD-Δ*lgt*	Amp^R, Em^R, *bgaB, Δlgt*	This work
pMAD-Δ*lspB*	Amp^R, Em^R, *bgaB, ΔlspB*	This work
pMAD-Δ*lpeA*	Amp^R, Em^R, *bgaB, ΔlpeA*	This work
pMAD-Δ*1671*	Amp^R, Em^R, *bgaB, Δ1671*	This work
pIMK2*lgt*	Site-specific listerial integrative vector, P_{help}-*lgt*, Kan^R	This work
pIMK2*lspA*	Site-specific listerial integrative vector, P_{help}-*lspA*, Kan^R	This work
pIMK2*lspB*	Site-specific listerial integrative vector, P_{help}-*lspB*, Kan^R	This work
pIMK2*lpeA*	Site-specific listerial integrative vector, P_{help}-*lpeA*, Kan^R	This work
pNF8	Em^R, Mob+(IncP), oriR pAMβ1, oriR pUC; Pdlt-*gfpmut1*	Fortinea *et al*. (2000)
L. monocytogenes strains		
EGD-e	Wild type, serovar 1/2a strain	Glaser *et al*. (2001)
Δ*lgt*	Δ*lgt* (*lmo2482*)	This work
Δ*lspA*	Δ*lspA* (*lmo1844*)	Réglier et al. (2003)
Δ*lspB*	Δ*lspB* (*lmo1101*)	This work
Δ*lgt*Δ*lspA*	Δ*lgt*Δ*lspA*	This work
Δ*lgt*Δ*lspB*	Δ*lgt*Δ*lspB*	This work
Δ*lpeA*	Δ*lpeA* (*lmo1847*)	This work
Δ*1671*	Δ*1671* (*lmo1671*)	This work
Δ*lgt*:: pIMK2*lgt*	Δ*lgt* strain expressing constitutively Lgt	This work
Δ*lspA*:: pIMK2*lspA*	Δ*lspA* strain expressing constitutively LspA	This work
Δ*lspB*:: pIMK2*lspB*	Δ*lspB* strain expressing constitutively LspB	This work
Δ*lpeA*:: pIMK2*lpeA*	Δ*lpeA* strain expressing constitutively LpeA	This work
EGD-e(pNF8)	wt strain expressing constitutively fluorescent protein GFPmut1	This work
Δ*lgt(*pNF8)	Δ*lgt* strain expressing constitutively fluorescent protein GFPmut1	This work
Δ*lspA*(pNF8)	Δ*lspA* strain expressing constitutively fluorescent protein GFPmut1	This work
Δ*lspB*(pNF8)	Δ*lspB* strain expressing constitutively fluorescent protein GFPmut1	This work
Δ*lgt*Δ*lspA(*pNF8)	Δ*lgt*Δ*lspA* strain expressing constitutively fluorescent protein GFPmut1	This work
Δ*lgt*Δ*lspB(*pNF8)	Δ*lgt*Δ*lspB* strain expressing constitutively fluorescent protein GFPmut1	This work

Table 2: Relative Gene expression of the 90 predicted lipoprotein genes in the course *L. monocytogenes* biofilm formation. (-) no expressed, (+) weakly expressed, (++) highly expressed and (+++) strongly expressed. The maintenance gene *gyrB* was used as a reference for relative genetic expression (Meinersmann, Phillips et al. 2004). Grey shading indicates genes encoding proteins of the LraI family.

	Time (h)				
Gene locus	0	6	12	24	48
gyrB	++	++	++	++	++
lmo0013	+++	+++	++	+++	++
lmo0023	-	-	-	-	-
lmo0047	++	++	+	+++	++
lmo0135	+++	++	++	+++	+++
lmo0152	++	++	++	+++	+++
lmo0153	-	-	+	+	++
lmo0181	++	+	-	-	-
lmo0204	++	+	+	+	+
lmo0207	+	-	-	-	+
lmo0255	+	-	-	-	-
lmo0269	+	+	+	-	+
lmo0285	+	++	+	+	+
lmo0303	++	++	+	+	+
lmo0324	-	-	-	-	-
lmo0355	++	++	++	++	++
lmo0366	+	-	-	-	-
lmo0460	-	-	-	-	-
lmo0510	+	+	-	-	-
lmo0517	-	-	-	-	-
lmo0541	++		-	-	-
lmo0617	+	+	+	+	+
lmo0641	+	++	+	+	++
lmo0724	+	+	-	-	-
lmo0768	-	-	-	-	-
lmo0791	+++	++	++	++	++
lmo0821	+	+	+	+	-
lmo0859		-	-	-	-
lmo0903	+++	+++	+++	+++	+++
lmo0945	++	++	+	+	+
lmo0953	+	-	-	-	-
lmo1016	+	+++	+++	+++	+
lmo1041	++	++	-	+	-
lmo1068	+++	+++	++	+++	++
lmo1073	+	+++	+	+	-
lmo1136	+	+	+	+	+
lmo1146	+	+	-	-	-
lmo1265	-	-	-	-	-

lmo1274	+	+	-	-	-
lmo1340	+++	+	+	+	+
lmo1379	+++	+++	+++	+++	++
lmo1384	++	++	+	+	+
lmo1388	+++	+++	+++	+++	+++
lmo1426	+++	++	+	+	-
lmo1444	-	+	-	-	-
lmo1508	+++	++	+	+	+
lmo1649	+++	++	++	++	++
lmo1653	-	+	-	-	-
lmo1671	-	+	+	+	++
lmo1730	++	-	-	-	-
lmo1738	++	++	+	++	+
lmo1757	++	++	++	+	+
lmo1786	+	+	-	-	-
lmo1800	-	-	-	-	-
lmo1847	+++	+++	+++	+++	+++
lmo1903	++	+++	+	+	+
lmo1959	+	-	-	-	-
lmo2007	++	+	-	-	-
lmo2023	-	-	-	-	-
lmo2056	++	+++	++	++	++
lmo2079	+	+	-	+	+
lmo2080	+++	++	++	++	++
lmo2125	+	-	-	-	-
lmo2156	+++	++	+++	+++	+++
lmo2172	-	-	-	-	+
lmo2184	+++	-	-	-	+
lmo2196	+++	+++	+++	+++	+++
lmo2219	+++	+++	+++	+++	+++
lmo2313	+	++	+	+	++
lmo2331	+++	+++	++	++	++
lmo2349	+	-	-	++	++
lmo2382	+++	++	+	++	+
lmo2416	+++	+++	++	++	+++
lmo2417	+	+	+	+	+
lmo2420	+	+	+	+	+
lmo2431	+	+	+	+	+
lmo2446	-	-	-	-	-
lmo2499	-	-	-	-	-
lmo2545	++	++	++	++	+
lmo2569	++	++	++	++	+
lmo2578	++	+	+	+	+
lmo2594	+	+	+	+	+
lmo2595	+	+	-	+	+
lmo2636	++	++	+	+	+

lmo2637	++	++	+	+	+
lmo2642	+	++	+	+	+
lmo2687	+	+	-	-	-
lmo2793	-	-	-	-	-
lmo2812	-	-	-	-	-
lmo2839	-	-	-	-	-
lmo2854	+++	+++	+++	++	++

Table 3: Comparison of the lipoprotein amounts between the *wt*, Δ*lgt*Δ*lspA* or Δ*lgt*Δ*lspB* respective to Δ*lgt*

Protein	Function	*wt* vs Δ*lgt*	Δ*lgt*Δ*lspA* vs Δ*lgt*	Δ*lgt*Δ*lspB* vs Δ*lgt*	C + 2[a]
Lmo0013	AA3-600 quinol oxidase subunit II, QoxA	+2.54	=	=	G
Lmo0047	Peptidase M4, PepSY	-	-	-	S
Lmo0135	ABC-type dipeptide transport system, substrate-binding protein family 5 component	-4.94	=	=	G
Lmo0152	ABC-type oligopeptide transport system, substrate-binding protein family 5 component	-2.53	-3.31	=	G
Lmo0153	ABC-type metal ion transport system, substrate-binding protein component, surface adhesin, LraI family	-	-	-	G
Lmo0181	ABC-type sugar transport system, substrate-binding protein family 1 component	-	-	=	G
Lmo0541	ABC-type Fe3+-hydroxamate transport system, substrate-binding protein component	-	-	=	G
Lmo0791	Lipoprotein of unknown function, YcdA-like spore lipoprotein	=	-	=	G
Lmo1016	ABC-type proline/glycine betaine transport system, substrate-binding protein component	-	-	=	G
Lmo1068	Lipoprotein of unknown function	=	=	=	G
Lmo1073	ABC-type Fe3+-hydroxamate transport system, substrate-binding protein component	-	-	-	G
Lmo1388	CD4+ T-cell stimulating antigen, TcsA, substrate-binding protein ABC transport system	-2.72	-4.4	=	G
Lmo1671	ABC-type metal ion transport system, substrate-binding protein component, surface adhesin, LraI family	-	-	-	S
Lmo1738	ABC-type amino acid transport/signal transduction system, substrate-binding protein family 3 component, surface adhesin	-	-	=	S
Lmo1757	Sex pheromone cAM373 biosynthesis, CamS	-	-	-	A
Lmo1847	ABC-type metal ion transport system, substrate-binding protein component, surface adhesin, LraI family, lipoprotein promoting entry, LpeA	-	=	=	S
Lmo2079	Lipoprotein of unknown function	-1.28	-	=	G
Lmo2184	ABC-type Fe3+-hydroxamate transport system, substrate-binding protein component	-	-	-	G
Lmo2196	ABC-type oligopeptide transport system, substrate-binding protein family 5 component	-5.7	-5.8	=	G
Lmo2219	Peptidylprolyl isomerase, foldase, PrsA2	-	=	=	G
Lmo2331	Lipoprotein of unknown function	=	-	=	G
Lmo2349	ABC-type amino acid transport/signal transduction system, substrate-binding protein family 3 component, surface adhesin	=	=	=	G
Lmo2416	Lipoprotein of unknown function	=	=	=	S
Lmo2417	ABC-type metal ion transport system, substrate-binding protein component, surface antigen	-	=	=	G
Lmo2431	ABC-type Fe3+-hydroxamate transport system, substrate-binding protein component	-	-	=	G
Lmo2569	ABC-type oligopeptide transport system, substrate-binding protein component	-	=	=	Q
Lmo2637	Major membrane immunogen lipoprotein with FMN-binding domains, electron transport complex RnfABCDGE type subunit, Na(+)-translocating NADH-(ubi-)quinone oxireductase subunit	=	-	=	G

[a]C+2: Position +2 respective to the cleavage site

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SUPPLEMENTARY MATERIALS

Table 1S. Oligonucleotides used in this study for the mutant construction and complementation

Name	Oligonucleotide sequence (5'-3')[a]	Restriction site
DlgtNcoFw1	TTTATACCATGGCACCTTAAGACCTCGCATAATCGT	NcoI
DlgtRv2	TTGAAAGTAGGGGAATTATGGGTATCGTCGTTTGAAAATGAATCCGCCA	
DlgtFw3	TACCCATAATTCCCCTACTTTCAA	
DlgtMluRv4	TTTATAACGCGTCAGGAAGCAGTGGTGTTGGTAAAAG	MluI
LgtoutFw	TTCTAACATGAAATCTGGCTGG	
LgtoutRv	CAAATGTGTACGAAGCGGACTC	
LgtNcoFw	TTAACCATGGGTAATGGTGTTCAGCCAC	NcoI
LgtPstRv	ATATCTGCAGTTTCCCAGTCATAATTTCTTCCTTTC	PstI
LspABsphFw	TGAGTTGTCATGA**A**TTATTATCTAATCACCTTAGCAG	NcoI
LspAPstRv	TGAGTTGCTGCAGTTATTTAATTCCTTTCGTTTTGCGGT	PstI
DLpsBNcoFw1	TTTATACCATGGCTTGAATAATCTTTTTCATCAGTG	NcoI
DLspBRv2	AAGGCTACATTTTTTTGTAACTCTTATTTTTCATATCAACTCACTT	
DLspBFw3	AGTTACAAAAAAATGTAGCCTT	
DLspBMluRv4	TTTATAACGCGTAATTGAACAAACAGCAAAAGC	MluI
LspBoutFw	TAAGTGATTCCATTGTAAAGTC	
LspBoutRv	ATTGTAACAGTGAAATACCTTG	
LspBBsphFw	TGAGTTGTC**ATG**AAAAATAAGACAATTATATCAAAG	BsphI
LspBPstRv	ATATCTGCAGTCTTCTACTTGAAAAAAGGCTACA	PstI
DLpeANcoFw1	TTTATACCATGGAATCCGCAAATAGCGTCTGC	NcoI
DLpeARv2	GGCTAAACCATCATGGATTTTTTCATTCGATAACCTCCCT	
DLpeAFw3	AGTTACAAAAAAATGTAGCCTT	
DLpeAMluRv4	TTTATAACGCGTCCTTTGCGAAAAGTAGTACG	MluI
LpeABoutFw	AGAGATCATCGGCATTGGC	
LpeAoutRv	ATTGGTGCAGCTACATTCGG	
LpeABsphFw	TGAGTTGTCATGAAAAAAATAATAGTTGTATCCCTTTTCG	BsphI
LpeAPstRv	TGAGTTGCTGCAGTTATTTGGCTAAACCATCATGGA	PstI

Table 2S. Oligonucleotides used in this study for RT-PCR

	Foward primers		Reverse primers	
Gene	Name	Oligonucleotide sequence (5'-3')	Name	Oligonucleotide sequence (5'-3')
lmo0006	GyrBFw	AGACACTAAATACAGCATTCAACGATA	GyrBRw	TCAACATTTCAAAAGTTTCATCGGCATCAA
lmo1101	LspBFw	TAGGTGCTACCCTTACAGAC	LspBR	ATCTGCCCTTCTAAAATGCTCC
lmo0013	Lmo0013Fw	GGTTGTGGTGACTTGACGGT	Lmo0013Rv	CGTGCATATCTGGCTCGTAG
lmo0023	Lmo0023Fw	AACAGGAACGCTTGCTGGAA	Lmo0023Rv	AAGTCAGGAATCGACGCACC
lmo0047	Lmo0047Fw	GCAACAGTAGGGATGACGGT	Lmo0047Rv	GCGTTAATCGCGTCTTCATAGC
lmo0135	Lmo0135Fw	TTCTGGCGCTAGTTCAGACA	Lmo0135Rv	ACGGTCAGAAGCGTAGTTCG
lmo0152	Lmo0152Fw	CTGGGGTTGCTGACGGAA	Lmo0152Rv	CTTTGAAGAGAGCGGAGTACC
lmo0153	Lmo0153Fw	ATGCGGTGCTAGCAACGATA	Lmo0153Rv	ATCCTTTGCGCTTGGTTCGT
lmo0181	Lmo0181Fw	TGGCAGTAGTAGTGGCGAT	Lmo0181Rv	GAGCATCTCCACCTGCGA
lmo0204	Lmo0204Fw	GCGTGCGATGATGGTGGTT	Lmo0204Rv	CCTCGCTTGGTTGCTCTTCT
lmo0207	Lmo0207Fw	TGCTTCGTTAGGTGTGGCTT	Lmo0207Rv	ACCATTTGAAGACATACCAGGGA
lmo0255	Lmo0255Fw	GTCGCTTCCCAATACTCTGC	Lmo0255Rv	AGTTCCACCAAGTAAGGAAGC
lmo0269	Lmo0269Fw	ACCGAGAGCGCAAATGTGAC	Lmo0269Rv	AGCTGTCCGGTCGTTACTAC
lmo0285	Lmo0285Fw	GCAGCCGCAGCTATCAATAC	Lmo0285Rv	CGTCTTTGTTGGCACTACGAAC
lmo0303	Lmo0303Fw	GTGGGTTGTGGTAAAGAGGT	Lmo0303Rv	TCCCTCTGCATCAATGTCCA
lmo0324	Lmo0324Fw	GCCTTGTGTTTATGAAGAGGCA	Lmo0324Rv	TTCGTCGCCGCCTTGTTC
lmo0355	Lmo0355Fw	GGTGGTCACGGAACAAACGA	Lmo0355Rv	TTTCGCTCATTCCGCCTGTT
lmo0366	Lmo0366Fw	AGCGGTGTTGGTTGTTGGAT	Lmo0366Rv	TTTGACCGCGTTTGCTTGCT
lmo0460	Lmo0460Fw	GAGCAGCCTATTGCCGATTC	Lmo0460Rv	CCAAGCGGTTGGTTTACTACT
lmo0510	Lmo0510Fw	ACGATAGGCAAGGATGGTAGT	Lmo0510Rv	ACAGCGACATATTTACCATCGCT
lmo0517	Lmo0517Fw	CTGGCCTGCTGAAGACTATG	Lmo0517Rv	CCGATGCTTAGTCCGTGAGA
lmo0541	Lmo0541Fw	CGCTTGTGGCAGTTCTTCAG	Lmo0541Rv	CGCTTACGTTTTGAAGTGCGACA
lmo0617	Lmo0617Fw	TGCATGTGGTGGGACAGAAG	Lmo0617Rv	TGCGATGGCATTGTTACCTTCA
lmo0641	Lmo0641Fw	CGTTAGCTGCGGCAATAACAC	Lmo0641Rv	GAAAATGCTGCCGCTGCTTC
lmo0724	Lmo0724Fw	CAAATGGATGCTCCGACGCT	Lmo0724Rv	CGACGAACGCCTTATCTGGA
lmo0768	Lmo0768Fw	TGTTATGTGGGGCTCTTGGT	Lmo0768Rv	TATCAGGGAGTTCGCCACC
lmo0791	Lmo0791Fw	TGCAGATGCAGTATCAGAAGCT	Lmo0791Rv	CATGCCGCCCATAAAGAACG
lmo0821	Lmo0821Fw	CTTGCTGGATGTGCCGATGT	Lmo0821Rv	AGGCGAAAATCCCTGCTACA
lmo0859	Lmo0859Fw	GCTGGGTCATTACTCACTGC	Lmo0859Rv	AGCTTTCGCCATTTCATCCCA
lmo0903	Lmo0903Fw	GGCGGTTGCTGCTTGTAG	Lmo0903Rv	ACACATGCACAGCACTTTCTTGA
lmo0945	Lmo0945Fw	TCCAAGCAGAAGATGGCACA	Lmo0945Rv	ACCAATATGGTCTGCGTGCG
lmo0953	Lmo0953Fw	GGAGCTGTCGCTGGAGTT	Lmo0953Rv	TGAAGAGATGAAACGCCACCT
lmo1016	Lmo1016Fw	CAACCCTTGCTCCTTATGACG	Lmo1016Rv	TGTCATTGCCGCAGTGGAAC
lmo1041	Lmo1041Fw	GAGGATACGCCTGAGAAAGTC	Lmo1041Rv	GTTCGCGAATTTGTCCAGAACC
lmo1068	Lmo1068Fw	TAAATCTGTCGGCGGGAGTT	Lmo1068Rv	AGCCCTCTAGCAGTTTTACC
lmo1073	Lmo1073Fw	TGCACTAGGACTTGGTGATGA	Lmo1073Rv	CAAGCATCGATTCGTGTCCAAGA
lmo1136	Lmo1136Fw	AGAAGTCATCCAGATGCCAAGA	Lmo1136Rv	ATAGGATTGGCGCTAACATTCAG
lmo1146	Lmo1146Fw	ATGTGCCTGGTGGTTGTGTT	Lmo1146Rv	AAGTCAAACGAGCCGTCACC
lmo1265	Lmo1265Fw	GGAAAAGGGAGAAGAGTATACGC	Lmo1265Rv	TTGCTTGCCCGCCATCTA
lmo1274	Lmo1274Fw	GGTTTACGAAGCACCACCGA	Lmo1274Rv	TTCCTATGGCCATTCCGCTA
lmo1340	Lmo1340Fw	ATGCTGCTCGAGAGTATGATGA	Lmo1340Rv	CTCTTTTGCTCCGCTGCCA
lmo1379	Lmo1379Fw	CACCGGATATGGTGCTTGC	Lmo1379Rv	GGGGCTGTGAAGGAAACGA
lmo1384	Lmo1384Fw	GGCCGAATTTTGGTGGCTAC	Lmo1384Rv	ATATCAGCGAAGCGAGCGTC
lmo1388	Lmo1388Fw	TCCTAGGTGCTTGTGGTTCG	Lmo1388Rv	GGTCATCAACGCCACCAGTA
lmo1426	Lmo1426Fw	GCTGCTCTTTACCAGGACTT	Lmo1426Rv	GTTGATCCGAGGTTGTTGACGA

lmo1444	Lmo1444Fw	ACTACTGCTCGCTGGTTGTG	Lmo1444Rv	GTTGTTGCACGACCTCATTTCC
lmo1508	Lmo1508Fw	GATTGGGGTGCTTGTCTCC	Lmo1508Rv	GCATCGTTGCATCTTGTGGC
lmo1649	Lmo1649Fw	GCACTAGGATTGTCGCTTGC	Lmo1649Rv	GATCTGCGCCAACTGCTC
lmo1653	Lmo1653Fw	GGGAAAGTAACGCAAGAAACTGG	Lmo1653Rv	TCTTCCCGAATTCCACCTTGT
lmo1671	Lmo1671Fw	TTGCTCAGACGAGAGCGACA	Lmo1671Rv	CCAGGCGGATAAATGCTGTG
lmo1730	Lmo1730Fw	ATTAATGGCGTGTGGTGGGT	Lmo1730Rv	CGAGTTCTTTTAGGGACTTGGC
lmo1738	Lmo1738Fw	AGAGGACCAGTGGAGTAGAA	Lmo1738Rv	CGTCCAGTCAATCGGTGTGAAT
lmo1757	Lmo1757Fw	TCGGCTTAACGCTTGTGCTT	Lmo1757Rv	TGTCATGATGCCCGTTTCCG
lmo1786	Lmo1786Fw	AAGACCCAGATGGAAGATGGAT	Lmo1786Rv	TATAGCCTCAGTCTCCCCAA
lmo1800	Lmo1800Fw	AAGTAACAGGAGCAGGGGTA	Lmo1800Rv	TGGCTTCCTGGTTTGAGTGT
lmo1847	Lmo1847Fw	ATTGTACCTGTTGGCGTGGA	Lmo1847Rv	ACCAACCGTTCCCTGTTTCTA
lmo1903	Lmo1903Fw	GTAGGGGCATGTAGCGAT	Lmo1903Rv	CGCCCAACGTAAACAAAGCC
lmo1959	Lmo1959Fw	GCTGGAAGTGAGCAGGTC	Lmo1959Rv	CCCCACAGGCTTCATTCCA
lmo2007	Lmo2007Fw	CGGAAGCGATGAGAAGGCA	Lmo2007Rv	TGCATCCGTTTACCGTAGTCG
lmo2023	Lmo2023Fw	ATCGTTGGGAGCGGCATTTC	Lmo2023Rv	TTTGACACTGCCGCTGCC
lmo2056	Lmo2056Fw	ACAGCCGAAGATACAGCGAC	Lmo2056Rv	CACCGGGTCACCAAACTCTT
lmo2079	Lmo2079Fw	ACTGGTGGCATGTGGTCTAA	Lmo2079Rv	TTTGCCTCAGGTTGGTCTGT
lmo2080	Lmo2080Fw	GCAGAAGTGCCTGCTTTAGTC	Lmo2080Rv	CGGCTGTATTGCTACTCACTG
lmo2125	Lmo2125Fw	GTTTCCGTTGACGCAGGCT	Lmo2125Rv	CTGGAGCAGTTCCAGCAG
lmo2156	Lmo2156Fw	TAGCCGCAGTTCTTCTAACTGT	Lmo2156Rv	CGGGTTTCCCTGCGTCTT
lmo2172	Lmo2172Fw	AGGATGGCGATACTGTTGCT	Lmo2172Rv	CCCGACGATCACCAAGCG
lmo2184	Lmo2184Fw	TTAGTTGGCTGCGGAAAGGA	Lmo2184Rv	ACTAGACGGAATTCCAACGAGG
lmo2196	Lmo2196Fw	ATGGTATTCCTGCCATTGCC	Lmo2196Rv	TGCAGTTACAGGGTCTCCG
lmo2219	Lmo2219Fw	ACGGAGATCAATTCTCTGCAGTT	Lmo2219Rv	GTGTCAGTATTGGCTTCGGTAG
lmo2313	Lmo2313Fw	AGGTGTGAGCGGTTGTGG	Lmo2313Rv	TCTCCCGCAAATGAATCTGTGT
lmo2331	Lmo2331Fw	CGGTGGAAAAGTCCCTGTCA	Lmo2331Rv	GGTTGTACAGTGAAGACGCC
lmo2349	Lmo2349Fw	CGGTTGGAACAGGAACGCA	Lmo2349Rv	AGCACCAAGGCTAACGAGTA
lmo2382	Lmo2382Fw	ACGGTGCATGTAAGTCGGAT	Lmo2382Rv	ATGCGAGCATCGTCACTTCC
lmo2416	Lmo2416Fw	ATTCCTCCTCTTCCTACAGCG	Lmo2416Rv	AGATGCTATCAGAAGCGTCGTA
lmo2417	Lmo2417Fw	GGCGTGAAACCACAAGATGC	Lmo2417Rv	TGCTACAACGTCGCCTTCTTC
lmo2420	Lmo2420Fw	GCTTGTATTACGGTTATTGCGGT	Lmo2420Rv	CTGACTAGCCATTTGAGAGGC
lmo2431	Lmo2431Fw	TATGCACTTGGTGGAACGGT	Lmo2431Rv	ATCAGCACCAAGAGAGGCAA
lmo2446	Lmo2446Fw	CCAGAAGGCGAATGGGTTGA	Lmo2446Rv	ACCAAGTTGGTATCCATCTGTCA
lmo2499	Lmo2499Fw	GCATGTGGAAGTGACAGCAG	Lmo2499Rv	TGGAAGCTGCTTCAACGAGT
lmo2545	Lmo2545Fw	CTTGGAAACTGGGTCGTAGG	Lmo2545Rv	TGAACGGAAGCGTATCAGGC
lmo2569	Lmo2569Fw	AGCATGTCAGTCTGGCGATA	Lmo2569Rv	CTATTGTCTGCGAGCGTTGGA
lmo2578	Lmo2578Fw	GCGCGTTATCTCTCAGTGCT	Lmo2578Rv	TGTATCACCACCGCTTCCAT
lmo2594	Lmo2594Fw	GCCGCAATACGCTTTGACATC	Lmo2594Rv	GGTCTATTTGGGCTGGTGAA
lmo2595	Lmo2595Fw	ACTTACCGTTGACGACCCTA	Lmo2595Rv	AGTAAATCTCCCGCTGTCATCT
lmo2636	Lmo2636Fw	GCGCTTGTTGTATCAGCGTG	Lmo2636Rv	CAACCGTGCCCATTAGAAAGTC
lmo2637	Lmo2637Fw	GTTGCGGTAGCAGTGACGA	Lmo2637Rv	ACCTTTCCAGCCTTTGTCGT
lmo2642	Lmo2642Fw	GCTTGTAGTTCCGCAAGTGG	Lmo2642Rv	TCCCGTTGTCGGTTAGTGATG
lmo2687	Lmo2687Fw	TGCGCAGCAGACAAATCAGT	Lmo2687Rv	ACCTAGGAGCGTAATGACCA
lmo2793	Lmo2793Fw	TCCTTATGCCCGAGCAGT	Lmo2793Rv	GCCAAATGCGATGAGATATGCC
lmo2812	Lmo2812Fw	AGAACGTGGTCCGTCAGAGA	Lmo2812Rv	AAGTCCGCTAGCACTGACAA
lmo2839	Lmo2839Fw	TGTCACACTTGCTCGCTCTG	Lmo2839Rv	AGTTTGTTGCGCCTTCGTAGT
lmo2854	Lmo2854Fw	TGCTTTCGGGTTGTGGCTAT	Lmo2854Rv	CCGACTGCATAACTTCCACC

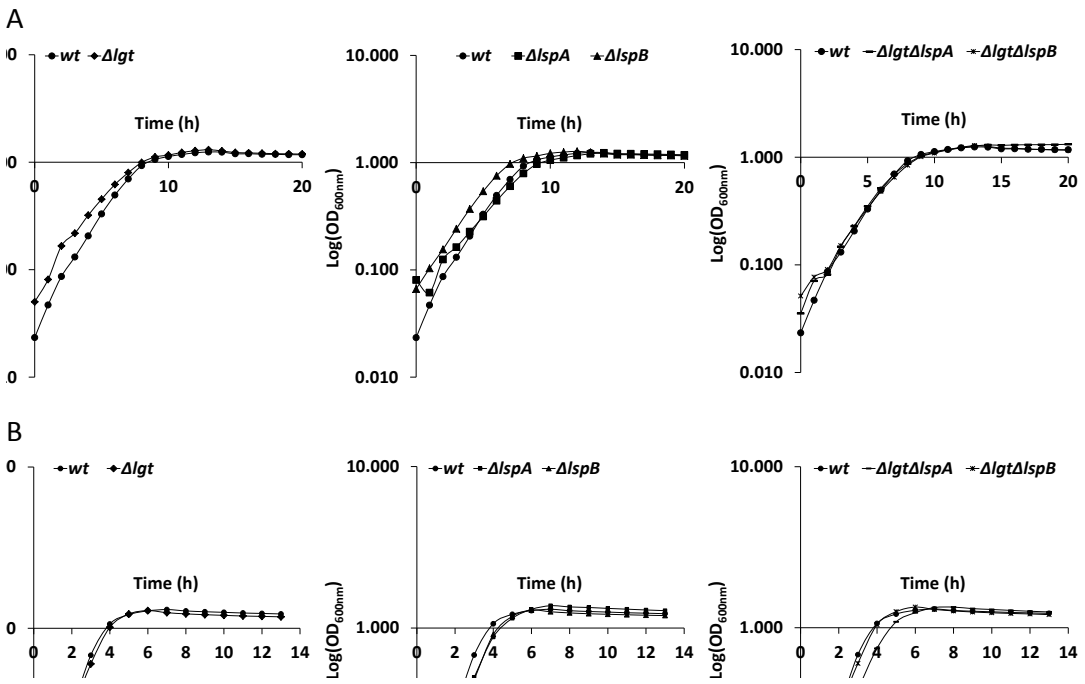

Figure 1S: Growth curves for the *L. monocytogenes wt, Δlgt, ΔlspA, ΔlspB, ΔlgtΔlspA* and *ΔlgtΔlspB* strains in BHI medium at 20°C (A) and 37°C (B).

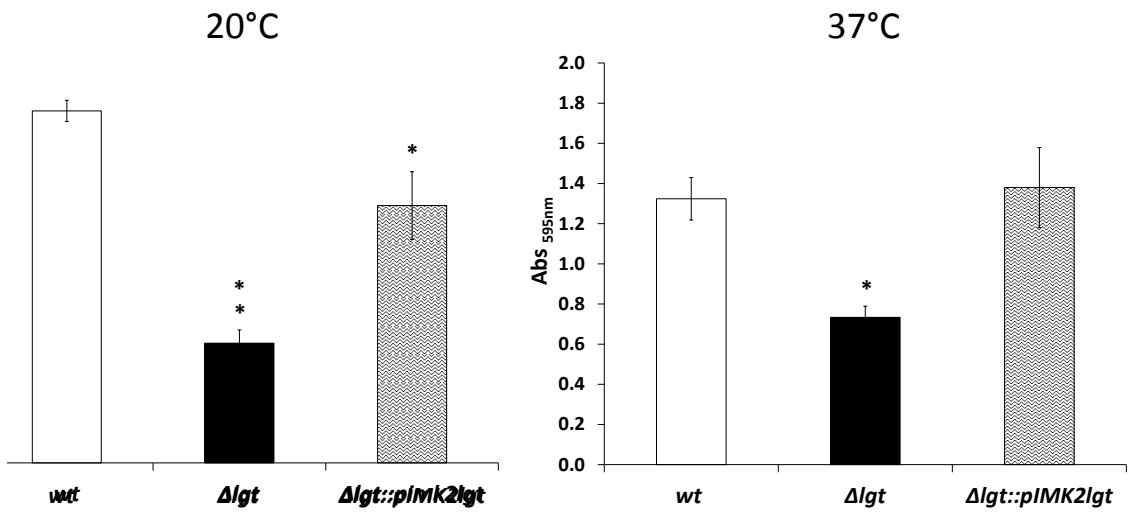

Figure 2S: Comparison of sessile biomass formed by *L. monocytogenes* EGD-e *wt,* Δ*lgt* and the complemented strain Δ*lgt*::pIMK2*lgt* after 72 h and 48 h of biofilm formation at 20°C and 37°C, respectively.

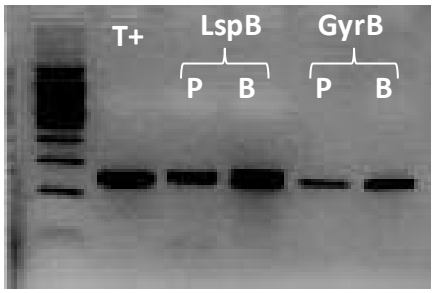

Figure 3S: RT-PCR were performed with cDNA synthesized with total RNA extracted from planktonic cells (P) and sessile cells (B) grown during 24 h in BHI at 37°C.

T+ : LspB amplification on genomic DNA.

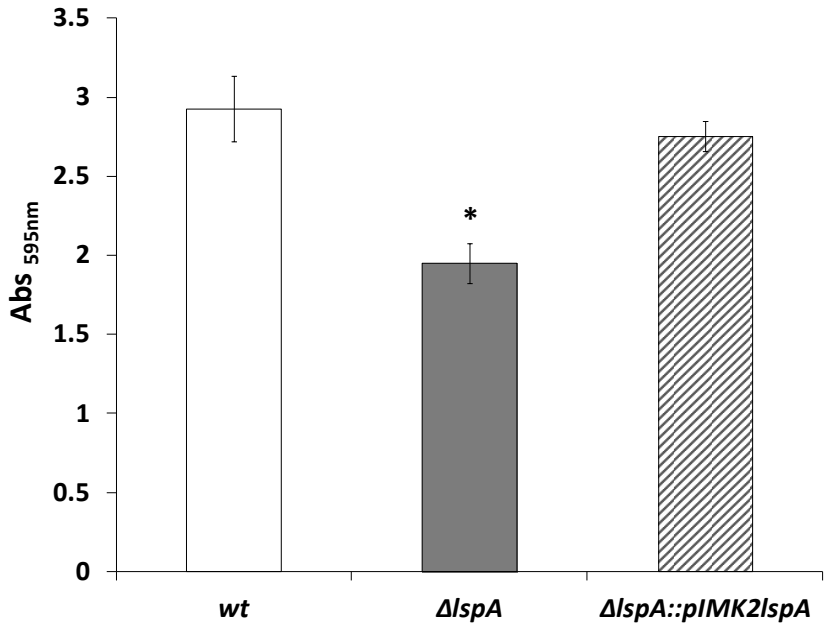

Figure 4S: Comparison of sessile biomass formed by *L. monocytogenes* EGD-e *wt,* Δ*lspA* and the complemented strain Δ*lspA*::pIMK2*lspA* after 48 h of biofilm formation at 37°C.

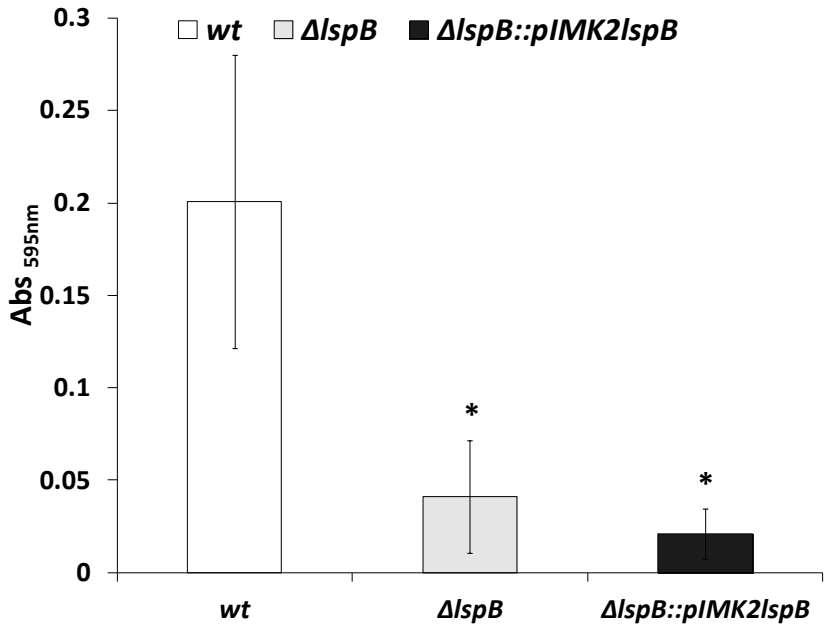

Figure 5S: Initial adhesion of *L. monocytogenes wt*, Δ*lspB* and the complemented strain Δ*lspB*::*pIMK2lspB*

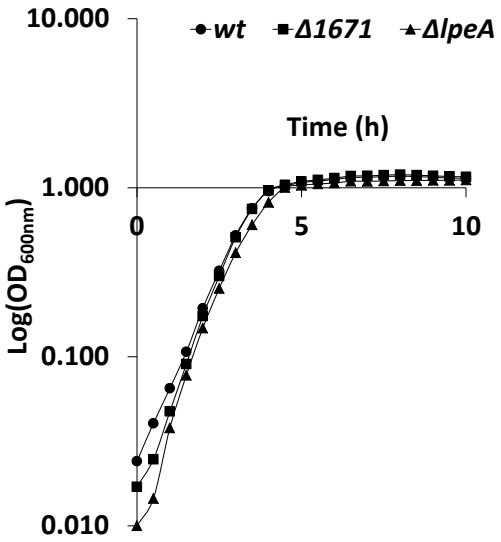

Figure 6S: Growth curves for the *L. monocytogenes wt,* Δ*1647* and *ΔlpeA* strains in BHI medium at 37°C.

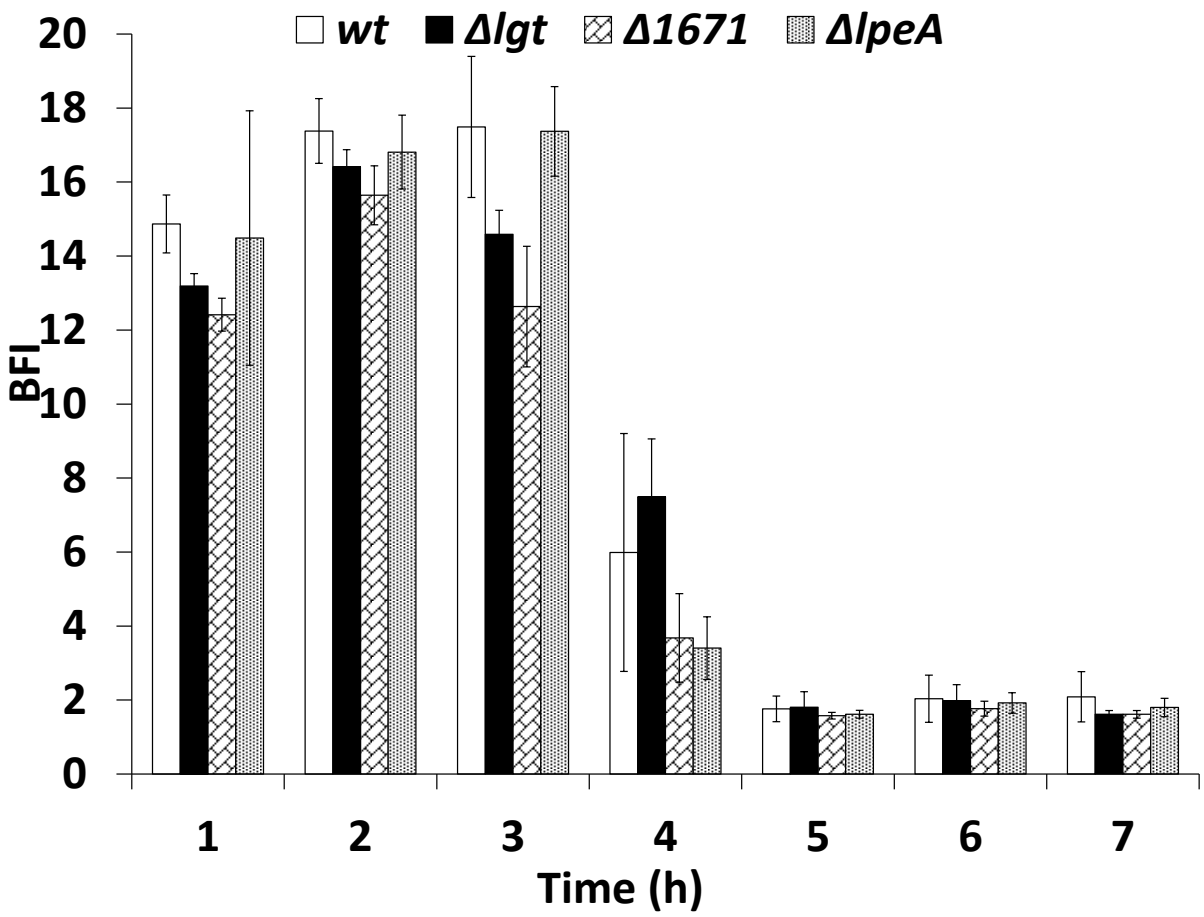

Figure 7S: Biofilm formation by *L. monocytogenes* EGD-e *wt* and the isogenic mutants Δ*1671* and Δ*lpeA* at 37°C. Early stages of sessile development were assayed with the BRT.

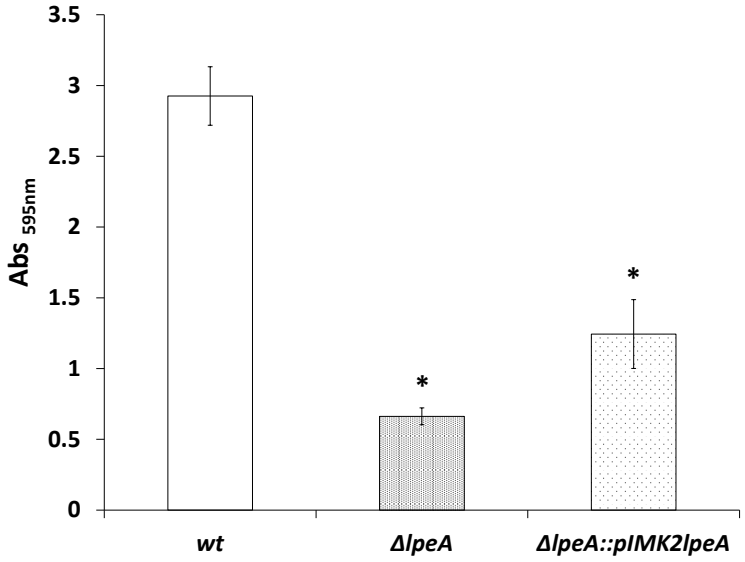

Figure 8S: Comparison of sessile biomass formed by *L. monocytogenes* EGD-e *wt,* Δ*lpeA* and the complemented Δ*lpeA*::pIMK2*lpeA* strains after 48 h of biofilm formation at 37°C.

DISCUSSION

Listeria monocytogenes est une bactérie pathogène et l'agent étiologique de la listériose. Elle est capable d'adhérer et de coloniser des surfaces, notamment dans les ateliers de transformation et conditionnement des produits alimentaires, ce qui augmente le risque de contamination des aliments et, par voie de conséquence, celui des individus qui les consomment. Contrairement à un grand nombre d'espèces bactériennes, *L. monocytogenes* ne sécrète pas ou très peu d'EPS qui sont généralement les principaux constituants de la matrice extracellulaire des biofilms (Flemming 1993; Sutherland 2001). Plusieurs études ont par ailleurs démontré que les protéines de surfaces pouvaient être impliquées dans la formation de biofilm. En effet, les protéines de surfaces permettent aux cellules bactériennes d'interagir avec le milieu environnant. La translocation de ces protéines à travers la membrane cytoplasmique est réalisée par les systèmes de sécrétion. Ainsi, afin de mieux cibler les protéines de surface potentiellement impliquées dans la formation de biofilm ainsi que les systèmes de sécrétion associés à ces protéines, une méthode de prédiction de la localisation subcellulaire, basée sur la biologie de la sécrétion des protéines et l'analyse du sécrétome (« sécrétomique »), a été développée chez les bactéries à Gram positif . Cette méthode s'est révélée plus performante que les autres prédicteurs de localisation subcellulaire connus, car elle prend en compte le trajet emprunté par les protéines en termes de système de sécrétion, les modifications postérieures à la translocation, les motifs de rétention au niveau des enveloppes cellulaires et de l'incorporation dans les structures supramoléculaires. Elle donne également des informations sur la catégorie de la protéine ainsi que sa topologie. De plus, la possibilité d'appartenir à plusieurs catégories de protéines et de posséder différentes localisations subcellulaires est prise en considération. En prenant comme modèle d'étude *L. monocytogenes*, nous avons pu améliorer les prédictions de protéines sécrétées chez cet organisme. Par exemple, 74 lipoprotéines ont été prédites par cette analyse alors que ce nombre n'était que de 68 auparavant. Cependant, certaines voies de sécrétion ne peuvent pas être prédites par cette méthode, c'est particulièrement le cas des protéines sécrétées par la voie SecA2.

La voie SecA2 est une voie alternative faisant intervenir l'ATPase SecA2, une protéine homologue à la protéine essentielle du système Sec, SecA. La délétion de ce gène entraîne la formation de longs filaments cellulaires et le développement de colonies qualifiées de rugueuses. Les longs filaments cellulaires sont dus à un défaut de septation qui résulte principalement de la réduction de la sécrétion de deux hydrolases pariétales sécrétées de façon SecA2 dépendante, CwhA et MurA. En effet, la délétion simultanée des deux gènes restaure le phénotype rugueux. L'implication d'un tel phénotype dans la formation de biofilm a été

brièvement étudiée et a conduit à des conclusions contradictoires. Monk et al. (2004) suggèrent que l'apparition d'un phénotype rugueux améliore la formation de biofilm alors que Halbedel et al (2012) observent le contraire par l'étude d'un mutant dépourvu du gène *divIVA*. La protéine DivIVA est une chaperonne qui permet de recruter les protéines CwhA et MurA pour leur sécrétion au niveau des pôles de la cellule (Halbedel, Hahn et al. 2012). Afin d'y voir plus clair, l'étude du mutant Δ*secA2* ainsi que du double mutant Δ*murA*Δ*cwhA* a été initiée sur leur capacité à former des biofilms. Une nouvelle architecture de biofilm a pu être mise en évidence expliquant les résultats précédemment exposés. En effet, ces mutants forment un biofilm hétérogène ne recouvrant qu'une faible partie des surfaces de colonisation, avec des structures aériennes comme des filaments qui s'enroulent et s'entremêlent. Cette architecture poreuse et aérienne avec relativement peu de cellules adhérées a pour conséquence que le biofilm est plus fragile et se décroche facilement. Le phénotype de biofilm observé est exacerbé chez le double mutant Δ*murA*Δ*cwhA* en raison de l'absence totale d'expression de ces protéines, alors que chez le mutant Δ*secA2*, leur sécrétion est grandement réduite mais pas totalement absente (Lenz, Mohammadi et al. 2003). Le défaut de septation observé chez ces mutants entraîne une mauvaise localisation des autres protéines de surface comme cela a été montré pour ActA et InlA (Pilgrim, Kolb-Maurer et al. 2003). Les hydrolases CwhA et MurA n'auraient pas un rôle d'adhésine, mais leur absence aurait des effets collatéraux entrainant une mauvaise localisation de protéines directement impliquées dans l'adhésion.

Par ailleurs, les mutants Δ*secA2* et Δ*murA*Δ*cwhA* forment des biofilms aussi bien à 20°C qu'à 37°C, ce qui n'est pas le cas pour la souche sauvage. En effet, le Biofilm Ring Test (BRT) a montré qu'à 20°C, la souche sauvage de *L. monocytogenes* n'était pas capable de bloquer les microbilles contrairement aux autres mutants, à l'exception de Δ*cwhA*. Les observations en microscopie confocale ont confirmé que seules quelques cellules étaient fixées à la surface pour la souche sauvage. Sur une surface hydrophobe telle que le polystyrène, la capacité d'adhésion puis de formation de biofilm de *L. monocytogenes* est très faible contrairement à une surface hydrophile telle que l'acier inoxydable (Chavant, Martinie et al. 2002; Rieu, Briandet et al. 2008). Tandis que l'expression du flagelle chez *L. monocytogenes* est dépendante de la température, avec des cellules bactériennes mobiles à 20°C mais pas à 37°C (Peel, Donachie et al. 1988), les flagelles participent à l'adhésion initiale qui se trouve plus importante à 20°C qu'à 37°C (Tresse, Lebret et al. 2009). Aussi, le défaut de mobilité des mutants Δ*secA*2 et Δ*murA*Δ*cwhA* (résultant du défaut de septation) entraîne une réduction de l'adhésion initiale à 20°C mais favorise le développement sous

forme sessile. Ces résultats soulignent l'importance de la température et de la nature de la surface utilisée pour la formation de biofilm chez *L. monocytogenes*. Ils montrent aussi la complémentarité des tests d'adhésion BRT et cristal violet, le premier permettant de révéler la phase précoce d'adhésion des cellules au support, le second permettant de colorer la biomasse d'un biofilm mature indépendamment de son architecture. Ces résultats confortent ainsi la nécessité de mettre en œuvre différentes approches (BRT, cristal violet, microscopie) et de tester différents paramètres (température, milieu de culture, nature du support, conditions statiques ou dynamiques,...*etc*) pour caractériser la capacité d'adhésion et de formation de biofilm par les bactéries.

En condition de stress environnementaux (températures, acidité et salinité extrêmes), *L. monocytogenes* peut former de façon réversible des longs filaments (Bereksi, Gavini et al. 2002; Geng, Kim et al. 2003; Giotis, Blair et al. 2007). En condition de culture continue, le défaut de septation survient avec la formation de biofilm sous forme de pelotes entourées d'un réseau de cellules (Rieu, Briandet et al. 2008). Ce phénotype est dépendent de RecA et YneA qui sont activées lors de la réponse SOS (van der Veen, van Schalkwijk et al. 2010). On peut donc émettre l'hypothèse qu'en condition de stress, les déterminants moléculaires impliqués dans la réponse SOS interagissent avec SecA2 en inactivant la protéine ou en réprimant l'expression du gène, permettant ainsi à la bactérie de se développer de façon sessile et d'être plus résistante aux stress. De plus, la délétion du gène *secA2* entraîne une réduction de la virulence chez *L. monocytogenes* (Lenz, Mohammadi et al. 2003). On peut donc penser que l'expression de *secA2* ou son activation est régulée en fonction de l'environnement de la bactérie avec la présence d'un morphotype qui favoriserait le développement de *L. monocytogenes* en condition infectieuse (37°C) et un morphotype rugueux qui favoriserait sa persistance en condition saprophyte (20°C). Il serait par ailleurs intéressant d'étudier les interactions de ce morphotype avec d'autres espèces qui sont connues pour former des biofilms dans les mêmes environnements que *L. monocytogenes*.

Etant donné l'importance de cette voie de sécrétion aussi bien pour la virulence que pour la formation de biofilms, l'étude globale des protéines sécrétées de manière SecA2 dépendante s'est avéré nécessaire. Pour cela, les exoprotéomes de la souche sauvage et du mutant Δ*secA2* aux deux températures citées précédemment ont été comparé. Il a pu être constaté que le mutant Δ*secA2* formait des colonies rugueuses à 20°C mais pas à 37°C. Ces conditions présentaient une opportunité d'étudier l'exoprotéome chez le mutant Δ*secA2* qui ne possède pas de défauts de septation et de constater qu'à 37°C, les protéines CwhA et MurA étaient effectivement retrouvées dans les mêmes proportions dans les surnageants du mutant

et de la souche sauvage. Cette analyse a permis d'identifier 13 nouvelles protéines SecA2 dépendante dont une partie ne possède pas de peptides signaux. Cette voie alternative pourrait, en effet, jouer un rôle dans la sécrétion des protéines ne possédant pas de peptides signaux, prédites comme sécrétées par des voies non-classiques, et qui sont retrouvées dans le milieu extracellulaire de façon récurrente. C'est notamment le cas de certaines protéines multitâches (« moonlighting ») qui possèdent une fonction supplémentaire en dehors de celle définie dans le cytoplasme. Parmi ces protéines identifiées, la GAPDH et DnaK jouent un rôle dans l'adhésion au plasminogène (Schaumburg, Diekmann et al. 2004). Les autres protéines identifiées sont essentiellement associées au métabolisme de la paroi cellulaire (SpsB, SpI et MltD), à l'adhésion au collagène (Cbp) ou à la formation de biofilm (SpsB). Ces résultats fournissent de nouvelles pistes de recherches pour la compréhension de la physiologie de *L. monocytogenes* au cours de la colonisation de surfaces abiotiques mais également biotique. Cependant, pour compléter la recherche des protéines sécrétées de façon SecA2-dépendante, l'étude devrait être étendue aux subprotéomes des fractions membranaires et pariétales, ce qui pourrait être envisagé par des techniques (i) classiques de fractionnement cellulaire (Planchon, Desvaux et al. 2009), (ii) de « shaving » visant à raser les protéines de la surface cellulaire suite à l'action de protéases (Tjalsma and van Dijl 2005; Westers, Westers et al. 2008), ou encore (iii) de marquage spécifique des protéines de surface afin de les isoler ultérieurement par chromatographie d'affinité (Hempel, Pane-Farre et al. 2010). Ces protéomes peuvent ensuite être étudiés soit par des approches classiques en électrophorèse bidimensionnelles couplés à de la MS soit par des approches hors gel de type LC-MS/MS.

Outre les protéines membranaires intégrales (IMP), les lipoprotéines constituent le groupe le plus important de protéines de surface chez *L. monocytogenes* mais dont l'implication dans la formation de biofilm bactérien n'avait pas encore été envisagée dans la littérature scientifique. Pour cela, le mutant Δ*lgt* dont les lipoprotéines sont relarguées dans le milieu extracellulaire a été étudié. Selon la température, il est apparu que les lipoprotéines pouvaient être impliquées à différents stades de la formation du biofilm. En effet, à 20°C, les lipoprotéines ne semblent jouer un rôle que dans la phase d'adhésion initiale car le mutant tout comme la souche sauvage ne sont pas capables de se développer de façon sessile. En revanche, à 37°C les lipoprotéines joueraient un rôle dans le développement tardif du biofilm. En effet, l'adhésion initiale n'est pas affectée, alors que l'épaisseur moyenne et le biovolume du biofilm de Δ*lgt* sont fortement réduits. A 37°C, la maturation des lipoprotéines par LspA est essentielle au bon développement du biofilm. L'analyse de l'expression des gènes codant les lipoprotéines au cours de la formation du biofilm ainsi que l'analyse génomique basée sur

l'étude du sécrétome, ont permis de cibler 3 lipoprotéines potentiellement impliquées dans la formation de biofilm. La construction des mutants et leur étude vis-à-vis de leur capacité à former un biofilm a permis de déterminer une lipoprotéine impliquée dans les étapes tardives de formation de biofilm, c'est-à-dire LpeA. Comme les deux autres lipoprotéines Lmo0153 et Lmo1671, cette protéine fait partie de la famille LraI dont les membres possèdent notamment des fonctions d'adhésion. Chez *L. monocytogenes*, LpeA est impliquée dans l'invasion cellulaire mais non dans l'adhésion aux cellules eucaryotes (Réglier-Poupet, Pellegrini et al. 2003). Tandis que l'implication de LpeA a pu être clairement démontrée, sa fonction exacte dans la formation du biofilm reste à être déterminer. En effet, certains membres de la famille LraI pouvant adhérer à la laminine, une protéine fibreuse de la matrice extracellulaire (ECM), LpeA pourrait permettre (i) l'adhésion aussi bien à des protéines de l'ECM ou à des polymères synthétiques tels que polystyrène (Shimoji, Ogawa et al. 2003), (ii) l'autoaggrégation des cellules bactériennes pour la formation de structures tridimensionnelles, (iii) également l'adhésion à des cellules eucaryotes, ou encore (iv) des phénomènes de co-aggrégation entraînant l'adhésion à d'autres genres bactériens et participant à la formation de biofilms multi-espèces. Par ailleurs, l'implication vis-à-vis de ces différents phénomènes d'adhésion des deux autres paralogues de la famille LraI encodés dans le génome de *L. monocytogenes* nécessiterait des études ultérieures. Quoi qu'il en soit, la participation de *L. monocytogenes* à des biofilms multi-espèces et les mécanismes d'interaction moléculaire sont des aspects qui n'ont pas encore suscité d'importantes études dans la littérature scientifique (Leriche, Chassaing et al. 1999; Bremer, Monk et al. 2001; Guillier, Stahl et al. 2008; Habimana, Meyrand et al. 2009; Lourenco, Machado et al. 2011) et qui pourtant pourrait se révéler un aspect particulièrement pertinent et essentiel dans la persistance de cette espèce aussi bien dans les environnements agro-alimentaires que naturels.

Afin de déterminer l'éventuelle spécificité d'action des deux Spases II sur la maturation des lipoprotéines, des doubles mutants Δ*lgt*Δ*lspA* et Δ*lgt*Δ*lspB* ont également été construit. Les exoprotéomes des deux doubles mutants ont ensuite été comparés à celui de la souche sauvage et du mutant Δ*lgt*. Les exoprotéomes analysés à partir de cultures à 37°C ont montré que la protéine LspA était responsable de la maturation de la quasi-totalité des lipoprotéines. Certaines lipoprotéines n'ont été retrouvées que dans le surnageant du simple mutant indiquant que la présence des deux SPases II était indispensable à la maturation de certaines lipoprotéines. Au contraire, d'autres lipoprotéines sont retrouvées dans tous les surnageants en quantités équivalentes soulevant la question de leur vraie nature. Compte tenu de l'influence

de la température sur la physiologie de *L. monocytogenes*, il serait également pertinent d'étudier la spécificité de maturation des lipoprotéines par les SPase II à 20°C.

Lors de ces trois ans de thèse la voie de sécrétion Sec chez *L. monocytogenes* a été caractérisée d'une part par rapport à la sécrétion de protéines extracellulaires SecA2 dépendante et d'autre part sur la maturation des lipoprotéines. Parallèlement, ces deux embranchements de la voie de sécrétion Sec ont été étudiés pour leur implication dans la formation de biofilm. De ce point de vue, d'autres embranchements de la voie de sécrétion Sec restent encore à explorer notamment la maturation des protéines pariétales ancrées de manière covalentes (protéines LPXTG maturés par la sortase) ou non-covalentes (LysM, GW, WXL et PGBD) ainsi que les IMPs (intégrée par OxaA1/OxaA2) (Fig. 16). Ces pré(pro)protéines sont maturées par des SPases de type I, au nombre de 3 chez *L. monocytogenes* (SipX, SipY et SipZ), qui ont déjà fait l'objet de travaux mais seulement pour leur implication dans la virulence (Bonnemain, Raynaud et al. 2004). Concernant les protéines LPXTG, elles constituent après les lipoprotéines le groupe de protéines de surface le plus abondant chez *L. monocytogenes*. Le décryptage de cette embranchement est rendue complexe car il nécessiterait de combiner des mutants pour les deux sortases SrtA et SrtB avec des mutants pour les trois SPases I puis de réaliser une analyse protéomique comparative du subprotéome pariétale particulièrement difficile à résoudre (Schaumburg, Diekmann et al. 2004). Néanmoins, l'étude des protéines LPXTG est particulièrement pertinente puisque certaines d'entre elles sont impliquées dans différents processus d'adhésion/colonisation chez d'autres espèces bactériennes comme *S. gordonii* (Davies, Svensater et al. 2009) ou *Lactobacillus rhamnosus* (von Ossowski, Satokari et al. 2011).

Outre le système Sec et le FEA (Flagella Export Apparatus) impliqué dans la formation des flagelles, *L. monocytogenes* possède 5 autres systèmes de sécrétion qui n'ont pas encore été caractérisés expérimentalement, c'est-à-dire les systèmes Tat, Wss, holins, ABC transporteurs et le FPE (Fimbrilin-Protein Exporter). Comme l'a révélé la méthode basée sur la sécrétomique, les protéines potentiellement sécrétées par ces derniers systèmes de sécrétion ne semblent pas posséder de fonctions particulièrement impliquées dans la formation de biofilm, à l'exception du système FPE. En effet, la présence de fimbriae chez *L. monocytogenes* n'a jamais été mise en évidence, mais a seulement été suggérée à plusieurs reprises dans la littérature par des observations microcopiques (Borucki, Peppin et al. 2003; Zameer, Gopal et al. 2010; Renier, Hebraud et al. 2011). Or, tout comme les flagelles, le rôle des pili dans l'adhésion, la co-agrégation (Telford, Barocchi et al. 2006) et la formation de biofilm a été décrit chez certaines bactéries à Gram-positif comme *Streptococcus pyogenes*

(Manetti, Zingaretti et al. 2007) ou *Enterococcus faecalis* (Nallapareddy, Singh et al. 2006). La formation potentielle de pili par ce système de sécrétion pourrait constituer une nouvelle piste de recherche à explorer.

CONCLUSIONS

Les travaux de recherche réalisés pendant cette thèse avait pour but de rechercher des déterminants protéiques impliqués dans la formation de biofilm chez *L. monocytogenes*. Ainsi, l'analyse génomique du sécrétome a permis d'améliorer les prédictions de localisation et de fonction des protéines mais surtout d'identifer des gènes cibles codant pour des déterminants protéiques de la voie Sec potentiellement impliqués dans le développement sessile. Ces prédictions ont fourni de nouvelles informations sur les mécanismes moléculaires liès à la sécrétion des protéines chez cette espèce grâce à une approche générique applicable à d'autres bactéries à Gram positif et permettant de générer de nombreuses hypothèses *in silico* non seulement par rapport à la colonisation mais aussi à divers autres processus physiologiques.

L'étude de la voie SecA2-dépendante a permis de souligner l'importance de cette voie et du morphotype rugueux dans la formation de biofilm, en particulier à des températures où *L. monocytogenes* est mobile. L'intégration de plusieurs techniques visant à étudier les différentes étapes de formation de biofilm (BRT, cristal violet, CLSM), et la réalisation des expériences dans différentes conditions (cultures en conditions statiques et dynamiques, à différentes températures), soulignent l'importance de multiplier les tests et les conditions de cultures afin d'obtenir des résultats cohérents et de ne pas émettre de conclusions trop hâtives.

La comparaison des exoprotéomes de la souche sauvage et du mutant Δ*secA2* a permis d'augmenter le nombre initialement connu de protéines sécrétées de façon SecA2-dépendante. Cette analyse ayant été réalisée à 20°C et 37°C, les protéines identifiées constituent de futures cibles pour la compréhension de la physiologie de *L. monocytogenes* au cours de la colonisation de surfaces abiotiques et biotiques, mais également au cours d'une infection. Si les mécanismes de virulence et de formation de biofilm sur les surfaces présentes dans les ateliers de transformation des aliments ont été largement étudiés, aucune étude n'a été réalisée sur la colonisation des produits alimentaires par *L. monocytogenes*.

Enfin, l'étude de la voie de maturation des lipoprotéines a permis de mettre en évidence l'importance des lipoprotéines dans les phases tardives de formation de biofilm et notamment de la protéine LpeA qui est également connue pour être impliquée dans la virulence. L'étude de la spécificité des Spas II a révélé l'importance majeure de LspA dans la maturation des lipoprotéines et la faible participation de LspB dans les conditions testées.

Outre la régulation de la voie SecA2 ou l'analyse fonctionnelle des lipoprotéines chez *L. monocytogenes*, ce travail ouvre à présent des perspectives sur de nombreuses autres directions de recherche. D'une part sur les relations entre les autres embranchements de la voie Sec (sortase, SPases I, YidC) et la formation de biofilm, mais aussi les systèmes de

sécrétion alternatifs (Tat, ABC, holin, FEA, FPE). Au-delà des processus de colonisation, l'implication de ces derniers systèmes (à l'exception du FEA) dans la physiologie de *L. monocytogenes* reste à être déterminée, ne serait ce qu'au niveau de leur implication dans la virulence. Plus générallement, tous ces systèmes de sécrétion et les protéines sécrétées qui leurs sont associées, c'est-à-dire le sécrétome, revêtent une grande importance dans toutes les interactions des cellules bactériennes à leur environnement, aussi bien dans le sol, le tube digestif d'animaux ou de l'Homme, les aliments ou des chaînes de fabrication agro-industrielle. Tandis que dans un écosystème, différents types d'interactions microbiennes peuvent s'établir depuis le mutualisme à la pathogenie, par rapport à la cellule bactérienne , il faut distinguer (i) la perception de molécules ou de stimili physiques, (ii) les relations avec d'autres entités biologiques tels que des cellules eucaryotes, *Archaea*, bactériennes (de la même ou d'espèces différentes) ou bactériophages, et (iii) les contacts de surfaces/intefaces (biotiques ou abiotiques) déclenchant la formation de biofilm. Par ailleurs, depuis l'Environnement en passant par l'aliment puis l'Homme, la physiologie de *L. monocytogenes* va étre différemment régulée aux niveaux de la synthèse (transcriptionnel, post-transcriptionnel, traductionnel, post-traductionnel) et de l'activité protéique, ce qui peut notamment avoir des conséquences sur son infectiosité. La compréhension de ces processus complexes à un niveau global et intégré nécessitera encore de nombreuses années d'étude. L'apport, au moins partiel, de ces connaisances fondamentales semble néamoins un pré-requis indipensable au développement rationnel de stratégies/pratiques innovantes pour limiter et/ou prévenir la contamination de produits alimentaires par *L. monocytogenes* et donc les toxi-infections alimentaires.

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ANNEXE

environmental microbiology

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Minireview

Molecular biology of surface colonization by *Listeria monocytogenes*: an additional facet of an opportunistic Gram-positive foodborne pathogen

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Summary

The opportunistic and facultative intracellular pathogenic bacterium *Listeria monocytogenes* causes a rare but severe foodborne disease called listeriosis, the outcome of which can be fatal. The infection cycle and key virulence factors are now well characterized in this species. Nonetheless, this knowledge has not prevented the re-emergence of listeriosis, as recently reported in several European countries. *Listeria monocytogenes* is particularly problematic in the food industry since it can survive and multiply under conditions frequently used for food preservation. Moreover, this foodborne pathogen also forms biofilms, which increase its persistence and resistance in industrial production lines, leading to contamination of food products. Significant differences have been reported regarding the ability of different isolates to form biofilms, but no clear correlation can be established with serovars or lineages. The architecture of listerial biofilms varies greatly from one strain to another as it ranges from bacterial monolayers to the most recently described network of knitted chains. While the role of polysaccharides as part of the extracellular matrix contributing to listerial biofilm formation remains elusive, the importance of eDNA has been demonstrated. The involvement of flagella in biofilm formation has also been pointed out, but their exact role in the process remains to be clarified because of conflicting results. Two cell–cell communication systems LuxS and Agr have been shown to take part in the regulation of biofilm formation. Several additional molecular determinants have been identified by functional genetic analyses, such as the (p)ppGpp synthetase RelA and more recently BapL. Future directions and questions about the molecular mechanisms of biofilm formation in *L. monocytogenes* are further discussed, such as correlation between clonal complexes as revealed by MLST and biofilm formation, the swarming over swimming regulation hypothesis regarding the role of the flagella, and the involvement of microbial surface components recognizing adhesive matrix molecules in the colonization of abiotic and biotic surfaces.

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Introduction

Listeria monocytogenes is a Gram-positive pathogen involved in numerous foodborne disease outbreaks. Although its involvement in food poisoning lags behind that of *Salmonella* and *Campylobacter*, which remain the prominent threats to food safety worldwide, *L. monocytogenes* is singular because of its high mortality rate, which ranges from 20% to 35% (Fratamico *et al.*, 2005; Riemann and Cliver, 2006). Without any obvious single reason, but rather a combination of several factors, cases of listeriosis have increased in several European countries in recent years (Allerberger and Wagner, 2010). Listeriosis mainly affects high-risk groups, including immunocompromised patients, pregnant women, newborns and the elderly (Farber and Peterkin, 1991). *Listeria monocytogenes* infection can manifest as (i) a mild non-invasive gastrointestinal illness which can be misdiagnosed in healthy adults, or (ii) an invasive disease which manifests as septicemia or neuropathic disease (Vazquez-Boland *et al.*, 2001). In France, the recent re-emergence of listeriosis is mainly attributable to bacteremia in people over 60 years of age, particularly those with a predisposition, i.e. co-morbidities (ANSES, 2009). The infectious cycle, key virulence factors and pathogenesis mechanisms of *L. monocytogenes* have been extensively investigated and are now clearly defined (Cossart,

2002; Dussurget *et al.*, 2004; Hamon *et al.*, 2006). Briefly, the major steps of intracellular parasitism involve the cell wall proteins InlA (internalin A) and InlB for adhesion to the surface of the eukaryote cell and entry into the host cell via phagocytosis. Released listeriolysin O (LLO) and phospholipases C (PlcA and PlcB) then enable bacteria to escape from the phagocytic vacuole, whereas membrane-anchored ActA (actin assembly) is responsible for actin-based motility allowing for cell-to-cell spread.

Although at first glance *L. monocytogenes* is generally regarded as a pathogen, it should primarily be considered as a saprophytic bacterium well adapted for survival in the environment (Fenlon, 1999). It is able to contaminate and thrive in the food-processing environment thanks to its ability to grow in a wide range of temperatures (–0.4 to 45°C), pH (4.3 to 9.6) and salt concentrations (up to 10% NaCl) as well as at low water activity (*Aw* down to 0.90) (Seeliger and Jones, 1986; ICMSF, 1996). Moreover, *L. monocytogenes* adheres to food contact surfaces, such as glassware, metal (stainless steel), rubber and plasticware (polystyrene) where it can further establish a biofilm (Frank and Koffi, 1990; Blackman and Frank, 1996; Norwood and Gilmour, 1999; Beresford *et al.*, 2001; Chae *et al.*, 2006; Di Bonaventura *et al.*, 2008). Biofilm-coated surfaces are particularly difficult to decontaminate, since biofilms protect the embedded bacteria from desiccation, antimicrobials and sanitizing agents (Folsom and Frank, 2006; Tessema *et al.*, 2009). This ability potentially allows the persistence of *L. monocytogenes* for long periods of time in the processing environment and is therefore a food safety hazard since biofilms are an important source of contamination when food products come into contact with them (Moretro and Langsrud, 2004; Gounadaki *et al.*, 2008; Poimenidou *et al.*, 2009; Koutsoumanis *et al.*, 2010). *Listeria monocytogenes* has been isolated from an extensive range of food products, including vegetables, milk, soft and farmhouse cheeses, fish and meat products as well as various ready-to-eat products (Ells and Truelstrup Hansen, 2006; 2010; Mataragas *et al.*, 2008; Panagou and Nychas, 2008; Cordano and Jacquet, 2009; Kushwaha and Muriana, 2009; O'Brien *et al.*, 2009; Pintado *et al.*, 2009; Takahashi *et al.*, 2009; Little *et al.*, 2010). The elimination of *L. monocytogenes* biofilms in processing plants appears critical for improving food safety. This review summarizes current knowledge on the molecular mechanisms responsible for the process whereby *L. monocytogenes* colonizes food products and food-processing environments. A better understanding of the molecular determinants responsible for its ability to colonize biotic (i.e. of biological origin) and abiotic supports is a prerequisite for the development of new strategies that could limit and even prevent contamination as well as subsequent food infections.

Listerial phylogeny and biofilm formation

Listeria monocytogenes exhibits a high level of heterogeneity from one strain to another, and several techniques have been developed over the years to discriminate isolates (Chen and Knabel, 2008). Among phenotypic methods, serotyping has been the most widely used and can differentiate four serogroups and 13 distinct serovars, named 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7. This classification is based on serological reactions with variations of the 15 somatic (O-factor subtyped from I to XV) and 5 flagellar (H-factor subtyped from A to E) antigens with specific antisera (Seeliger and Hohne, 1979; Seeliger and Jones, 1986). While *L. monocytogenes* is generally regarded as a pathogen, the level of virulence is highly variable from one *L. monocytogenes* strain to another, as a significant proportion of isolates is hypovirulent and even apathogenic (Roche *et al.*, 2001; 2003). The degree of virulence of *L. monocytogenes* strains could correlate with some serovars, as the majority of human listeriosis cases are caused by three serovars, i.e. 1/2a, 1/2b and 4b (Schuchat *et al.*, 1991). A genoserotyping technique based on multiplex PCR has been developed to facilitate and speed up discrimination of the isolates (Doumith *et al.*, 2004). Five genoserotypes (or PCR groups) are distinguished: genoserotype IIa (serovars 1/2a and 3a), genoserotype IIb (serovars 1/2b, 3b and 7), genoserotype IIc (serovars 1/2c and 3c), genoserotype IVb (serovars 4b, 4d and 4e) and genoserotype L (containing other serovars).

With the development of more reliable methods based on molecular tools, this bacterial species was separated into three major divisions, i.e. Genomic Division I (also designated Lineage II), Genomic Division II (also designated Lineage I) and Genomic Division III (or Lineage III) (Cheng *et al.*, 2008). Based on the screening of *L. monocytogenes* strain libraries, the different serovars appeared to be associated with specific lineages: (i) Lineage I contains serovars 1/2b, 3b, 4b, 4d and 4e; (ii) Lineage II contains serovars 1/2a, 1/2c, 3a and 3c; and finally, (iii) Lineage III contains serovars 4a and 4c. The distribution between serovars and lineages (and probably genoserotypes) should not be considered as strict and absolute as, for example, some serovars were under-represented or not represented at all (e.g. 4ab or 7) or were distributed between different lineages, e.g. 4b found in both lineages I and III (Liu *et al.*, 2006). More recently, an additional step in genotypic methods was reached using MLST (multilocus sequence typing) where the *L. monocytogenes* species emerged as distributed into 23 clonal complexes and 22 singletons (Ragon *et al.*, 2008).

Several studies have noted that *L. monocytogenes* strains show significant differences in their ability to

adhere to food-processing surfaces (Norwood and Gilmour, 1999; Lunden *et al.*, 2000). The hypothesis that the ability to adhere and then to form biofilms might be conserved within phylogenetic lineages was therefore formulated. The study of a large number of isolates suggested that serovars presented significant differences in their ability to adhere to stainless steel; strains within serovar 1/2c especially showed the highest levels of attachment (Norwood and Gilmour, 1999; Lunden *et al.*, 2000). Moreover, this high capacity for adherence was associated with strain persistence. Subsequent investigations found a correlation between phylogeny and the ability to form biofilms, but their conclusions differed (Djordjevic *et al.*, 2002; Borucki *et al.*, 2003). Djordjevic and colleagues (2002) observed that strains associated with Lineage I (serovars 1/2b and 4b) produced more biofilm than did strains from Lineage II (serovars 1/2a and 1/2c), whereas Borucki and colleagues (2003) reported exactly the opposite. These investigations also disagree regarding the relationships between persistence and the ability to form biofilms. However, neither of them observed a direct correlation between biofilm formation and serovars. Conflicting conclusions might result from differences in sample sizes and strains used in the analyses, but not in methodology; eight common strains were tested in biofilm formation following their respective protocols and the results of the two methods were not statistically different (Borucki *et al.*, 2003). In a recent report, however, a correlation between serovars and the ability to form biofilms was noted, but involved only 1/2a and 4b *L. monocytogenes* strains (Pan *et al.*, 2009).

In view of these results, no conclusion about the putative correlation between the ability to form biofilms and lineage can be established. While a high capacity for adhesion seems to be correlated with persistence, the same cannot be said for the capacity to form biofilms. This is not that surprising considering *L. monocytogenes* most likely exists as a member of a complex bacterial community in the natural environment or in food-processing plants. *Listeria monocytogenes* could take advantage of other bacterial biofilms, which could explain the persistence of low biofilm producers when co-cultured with *Pseudomonas fragi*, for example (Sasahara and Zottola, 1993). It has also been shown that low biofilm producers, i.e. *L. monocytogenes* 4b strains, can form higher-density biofilm in the presence of a high biofilm producer, e.g. *L. monocytogenes* SK1387 1/2a strain, in mixed-culture biofilm. It has been suggested that the extracellular matrix produced by *L. monocytogenes* SK1387 strain sticks to the surface and improves sessile growth of serovar 4b *L. monocytogenes* strains, conferring greater protection against environmental stresses (Pan *et al.*, 2009). These data highlight the need to take into account the interaction of *L. monocytogenes* with other bacterial species in biofilm communities in order to determine how they influence each other. For example, in co-culture with *Staphylococcus aureus*, the sessile population of *L. monocytogenes* increased significantly (Rieu *et al.*, 2008a); conversely, some isolates of *Enterococcus durans*, *Lactobacillus plantarum* and *Lactococcus lactis* ssp. *lactis* were found to greatly inhibit *L. monocytogenes* biofilm formation (Zhao *et al.*, 2004).

Biofilm architecture

A biofilm can be broadly defined as a community of microorganisms adhering to a surface (Costerton *et al.*, 1995). Bacterial cells in a biofilm are generally surrounded by a self-produced extracellular matrix. Biofilm formation can be divided into several key steps in which the adhesion of planktonic bacteria is followed by their subsequent proliferation to form microcolonies (Fig. 1). The next step is the maturation of the biofilm in a three-dimensional structure, and finally some bacteria are released from the biofilm and dispersed, enabling cells to colonize other surfaces (O'Toole *et al.*, 2000; Hall-Stoodley and Stoodley, 2002).

The architecture of *L. monocytogenes* biofilm has been investigated by several techniques as scanning electron microscopy (Kalmokoff *et al.*, 2001; Chavant *et al.*, 2002; Borucki *et al.*, 2003), epifluorescence microscopy (Lunden *et al.*, 2000; Kalmokoff *et al.*, 2001; Carpentier and Chassaing, 2004; Monk *et al.*, 2004; Pan *et al.*, 2006) and laser-scanning confocal microscopy (LSCM) (Chae and Schraft, 2000; Rieu *et al.*, 2008b). Several attempts have also been made to model *L. monocytogenes* biofilm formation (Kreft and Wimpenny, 2001; Zameer *et al.*, 2010). Pioneering work on the structure of *L. monocytogenes* biofilm using scanning electron microscopy and epifluorescence microscopy revealed the ability of this bacterial species to colonize hydrophilic (stainless steel) and hydrophobic (polytetrafluoroethylene [PTFE]) surfaces (Chavant *et al.*, 2002). In static culture conditions, some strains of *L. monocytogenes* produced three-dimensional mushroom-shaped biofilms with well-distributed channels and pores, whereas other ones produced only sparse aggregates of cells or a bacterial monolayer (Chae and Schraft, 2000; Kalmokoff *et al.*, 2001; Chavant *et al.*, 2002; Borucki *et al.*, 2003). In similar conditions using either stainless steel or plasticware surfaces, a honeycomb-like biofilm structure has also been reported for some other strains (Marsh *et al.*, 2003). Using LSCM, a novel three-dimensional structure has been characterized in *L. monocytogenes* EGD-e under dynamic conditions (flow-cell), where ball-shaped microcolonies are surrounded by a network of knitted chains (Rieu *et al.*, 2008b). Following prolonged sessile growth

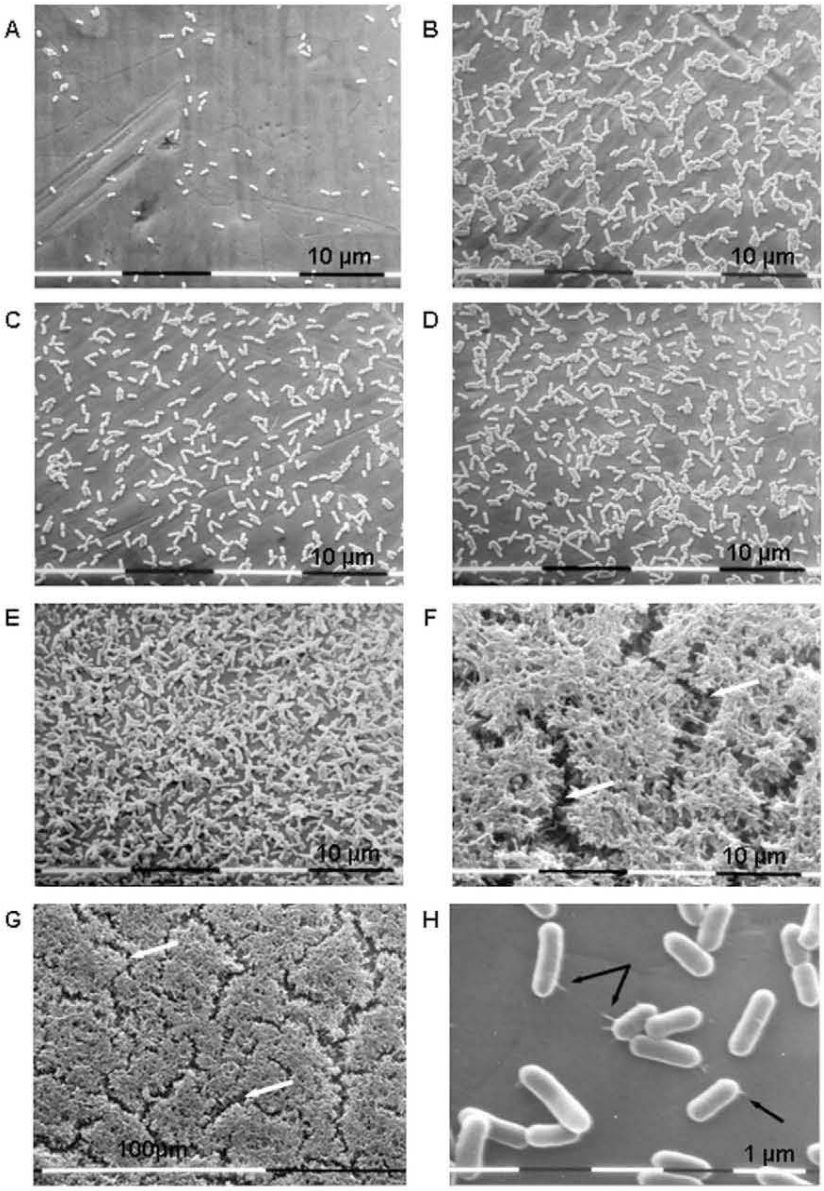

Fig. 1. Scanning electron microscopy observations of *L. monocytogenes* biofilm formation. *Listeria monocytogenes* was grown at 20°C in BHI on stainless steel chips after 10 s (A), 5 h (B), 8 h (C), 24 h (D), 5 days (E) and 7 days (F and G) of adhesion. White arrows indicate the putative water channel (F and G). Detail of 30-minute sessile cells with fimbriae-like structures as indicated by black arrows (H).

on stainless steel coupons in a bioreactor, morphotypic conversion from the common smooth colony morphology to a succession of rough colony variants has been reported to occur in the course of *L. monocytogenes* biofilm formation (Monk *et al.*, 2004). Bacterial cell variants exhibiting rough colony morphology appeared spontaneously in the biofilm and formed chain cell structures allowing increased surface colonization (Monk *et al.*, 2004). In the end, it appears that the biofilm structure of *L. monocytogenes* depends on a multitude of parameters, namely the strain, the type of surface as well as other environmental conditions, such as pH, medium and temperature.

Effect of environmental factors on biofilm formation

The switch from the planktonic to sessile state requires profound physiological changes, which occur after the regulation of gene expression in response to different environmental signals. The importance of environmental conditions, such as the nature of the surfaces, the growth temperature and medium, on biofilm formation of various *L. monocytogenes* strains has been highlighted (Moltz and Martin, 2005). The most recent investigation of the structural dynamics of *L. monocytogenes* biofilm formation using LSCM revealed different biofilm architectures when grown in static versus dynamic conditions (Rieu

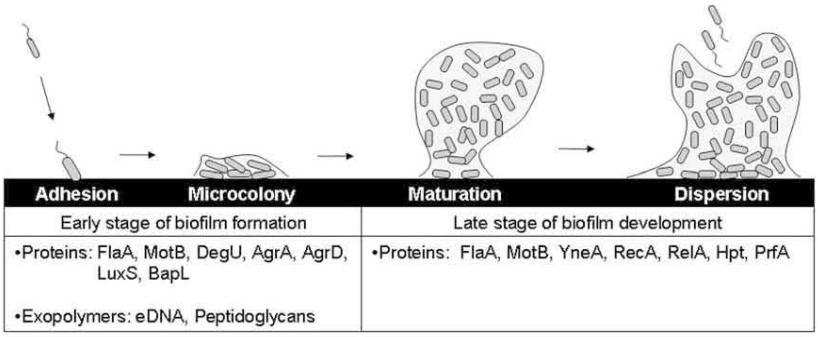

Fig. 2. Schematic representation of molecular determinants as yet uncovered and involved in early and late stages of biofilm formation in *L. monocytogenes*. Detailed descriptions of the roles of the different molecular determinants are provided in the text.

et al., 2008b). This investigation pointed out the importance of culture conditions in biofilm formation and the need for complementary approaches.

In food-processing environments, biotic factors also play a role in biofilm development (Zottola and Sasahara, 1994). Resident biofilm could have negative or positive effects on biofilm formation by *L. monocytogenes* (Carpentier and Chassaing, 2004). The inhibition of *L. monocytogenes* development could occur by competition for nutrients (Gnanou Besse *et al.*, 2008; Guillier *et al.*, 2008) or by the secretion of antimicrobial agents like bacteriocins produced by *Lactococcus lactis*, for example (Leriche *et al.*, 1999; Liu *et al.*, 2008). In contrast, the presence of microorganisms like *Staphylococcus capitis* or *S. aureus* could improve *L. monocytogenes* biofilm formation (Carpentier and Chassaing, 2004; Rieu *et al.*, 2008a). Addition of cell-free supernatant from either *S. capitis* or *S. aureus* biofilm was sufficient to stimulate biofilm development of *L. monocytogenes*. Interestingly, this positive effect was abolished when supernatant was treated with proteinase K, but not after ultrafiltration (3 kDa cut-off), suggesting that peptide molecules within the supernatant of *S. aureus* could be involved in the improvement of biofilm formation (Rieu *et al.*, 2008a). *Pseudomonas fragi* has also been shown to be necessary for the establishment of *L. monocytogenes* biofilm (Sasahara and Zottola, 1993). In order to determine the biofilm properties that influence the initial fixation of *L. monocytogenes*, a set of *L. lactis* model resident biofilms with different architectures, porosities, types of matrices and individual cell surface properties has been created (Habimana *et al.*, 2009). This study suggests that the porous structure of resident biofilms improves the adhesion of *L. monocytogenes*, whereas exopolysaccharides produced by resident biofilms prevent its adhesion.

During infection of the gastrointestinal tract, *L. monocytogenes* is in a particular environment with suboptimal conditions, including exposure to bile. Nevertheless, *L. monocytogenes* is able to survive, colonize and enter epithelial cells. Bile has recently been shown to improve the initial attachment to plastic surfaces and biofilm formation of *L. monocytogenes* cells. So, during infection, the exposure to bile may enhance biofilm formation of *L. monocytogenes*, and consequently may contribute to its survival and facilitate colonization of the gastrointestinal tract (Begley *et al.*, 2009).

Molecular determinants of biofilm formation

Common molecular determinants are seen in biofilm formation by Gram-positive bacteria, namely exopolysaccharides, Bap (biofilm-associated protein) and bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) (Götz, 2002; Lasa, 2006). However, some of these determinants clearly differ or have not yet been considered for biofilm development in *L. monocytogenes*. Molecular determinants characterized to date at the different stages of the biofilm formation process in *L. monocytogenes* are shown in Fig. 2. They include essentially motility factors, cell transduction signal systems, exopolymers, BapL as well as some other molecular determinants, such as second messengers.

Flagella

Flagella are very important for biofilm formation in several bacterial species (O'Toole *et al.*, 2000). In *L. monocytogenes*, the flagellum is composed of thousands of flagellin monomers encoded by the *flaA* gene. *Listeria monocytogenes* has four to six peritrichous flagella per cell, the biosynthesis of which is regulated by temperature (Peel *et al.*, 1988). The transcription of *flaA* is stopped at temperatures above 30°C, where *L. monocytogenes* strains are not motile. Control of flagella biosynthesis is rather complex as it involves at least five regulators, namely FlaR (flagellin regulator) (Sanchez-Campillo *et al.*, 1995), PrfA (positive regulatory factor A) (Michel *et al.*, 1998), DegU (degradation enzymes regulator) (Knudsen *et al.*, 2004), MogR (motility gene repressor) (Gründling *et al.*, 2004) and GmaR (glycosyltransferase and motility antirepressor) (Shen *et al.*, 2006). Like PrfA (Scortti *et al.*, 2007), most of these regulators also control expression of

virulence factors required for full virulence of *L. monocytogenes*. Interestingly, GmaR is bifunctional as it also glycosylates FlaA with β-O-linked N-acetylglucosamine (Schirm *et al.*, 2004). So far, FlaA is the first and only surface protein reported to be glycosylated in *L. monocytogenes*.

The first studies of the involvement of flagella as a putative adhesive structure in biofilm formation showed that the absence of flagella affected the initial stage of adhesion, but did not influence final levels of attachment achieved over longer periods of time (Vatanyoopaisarn *et al.*, 2000). Following the generation of unflagellated as well as nonmotile *L. monocytogenes* mutant strains, it was demonstrated that flagellated but non-motile bacterial cells do not adhere to or invade human epithelial cells more efficiently than nonflagellated listerial cells (O'Neil and Marquis, 2006). Rather than acting as adhesins, the flagella as mediators of motility enhance the adhesion of *L. monocytogenes* to targeted host cells. In the colonization of the intestines, motile bacteria appear to outcompete nonmotile bacteria, suggesting that flagellum-mediated motility enhances infectivity soon after bacterial ingestion (O'Neil and Marquis, 2006). However, it should be stressed that in this study the rapid transfer of *L. monocytogenes* from a temperature enabling flagella expression to 37°C for *in vitro* invasion assay would allow the presence of motile flagella in a significant proportion of the bacterial population. Moreover, the *L. monocytogenes* 10403S strain used in this study is a peculiar model as it exhibits deregulation of *flaA* expression at 37°C (Gründling *et al.*, 2004). Altogether, results and conclusions drawn from this investigation might not be generalized to other *L. monocytogenes* strains. Using the same *L. monocytogenes* strain, though, further investigations using abiotic surfaces confirmed the involvement of flagella in the early stages of attachment, but not as surface adhesins (Lemon *et al.*, 2007). A motile mutant of *L. monocytogenes* with an altered flagellar surface formed biofilm as well as the wild-type. In addition, comparison of adhesion between flagellum-minus cells and paralysed-flagellum cells after centrifugation revealed a significant difference, suggesting that paralysed-flagella interfere slightly with contact between putative surface adhesin(s) and abiotic surfaces. However, comparison of biofilm formation in wild-type bacteria and both non-motile mutants showed a biofilm-defective phenotype, suggesting that flagellum-mediated motility plays a predominant role in biofilm formation in *L. monocytogenes* (Lemon *et al.*, 2007). Swarming ability, which is a specialized form of movement that enables flagellated bacteria to coordinately move atop solid surfaces, is distinct from the simple swimming ability conferred by flagella, which is an individual and non-cooperative movement (Henrichsen, 1972). Swarming over swimming regulation might further explain the importance of flagella as motile determinants rather than adhesins in biofilm formation (Desvaux and Hébraud, 2009).

Many later studies have confirmed the importance of motility in the first stages of biofilm formation (Tresse *et al.*, 2006; 2009; Gueriri *et al.*, 2008), except for one which demonstrated that defects in flagella or motility have a more complex effect on biofilm development. Using flow cells, mutant strains lacking flagella (*ΔflaA* and *ΔflgL*) or affected for motility (*ΔmotB*) were impaired in initial bacterial attachment but subsequently displayed a hyper-biofilm phenotype (HB) (Todhanakasem and Young, 2008). This HB phenotype was not previously found under static conditions using microtitre plate assays, probably because of differences due to the numerous changes that occur when bacteria reach a high density under static conditions, as changes in pH and/or nutrient availability. So, although flagellum-mediated motility improves initial attachment under both static and dynamic conditions, it is not necessary for attachment and biofilm formation and interferes with biofilm development under dynamic conditions.

Cell–cell communication

During biofilm development and maturation, complex cellular mechanisms are involved requiring coordinated regulation of gene expression by cell–cell communication (Dunny and Leonard, 1997; Hardman *et al.*, 1998; Waters and Bassler, 2005; von Bodman *et al.*, 2008). Among cell–cell signalling systems, quorum sensing (QS) has been the most investigated and specifically refers to cell density-linked coordinated gene expression in populations that experience threshold signal concentrations to induce a synchronized population response. In other terms, QS is just an example of multicellular behaviour in prokaryotes leading to regulation of diverse physiological processes, which is only induced when bacteria are at high cell population densities. This mechanism is based on the production and release of signal molecules named auto-inducers. The subsequent detection of auto-inducers from a certain threshold concentration leads to bacterial responses. There are two archetypal QS systems in *L. monocytogenes*: the auto-inducer 2 (AI-2) LuxS system found in both Gram-negative and Gram-positive bacteria, and the peptide-mediated QS signalling system Agr characteristic of Gram-positive bacteria (Dunny and Leonard, 1997; Miller and Bassler, 2001; Waters and Bassler, 2005).

Listeria monocytogenes is able to produce AI-2-like molecules via the LuxS orthologue (Challan Belval *et al.*, 2006). Depending on the bacterium, the *luxS* mutation affects biofilm formation differently (Blehert *et al.*, 2003; Cole *et al.*, 2004; Wen and Burne, 2004). In *L. monocy-*

togenes, the *luxS* mutant strain forms denser biofilm than the parental strain (Challan Belval *et al.*, 2006; Sela *et al.*, 2006). However, the addition of synthetic AI-2 to the *luxS* mutant supernatant does not restore the phenotype of the parental strain. S-ribosyl homocysteine (SRH), the precursor of AI-2, may explain the denser biofilm phenotype, and was able to increase the number of attached cells. Furthermore, SRH was found in larger quantities in *luxS* mutant supernatant than in parental strain supernatant (Challan Belval *et al.*, 2006). These results suggest that the *luxS* gene participates in repression of biofilm formation, probably by converting SRH to AI-2. It would be interesting to elucidate the mechanisms by which SRH allows an increase of attached cells and the precise role of *luxS* in attachment and biofilm formation in *L. monocytogenes*, by comparing genetic expression between sessile and planktonic cells, for example. Besides SRH, a toxic precursor, S-adenosyl homocysteine (SAH), also accumulated in culture supernatant of *luxS* mutant (Challan Belval *et al.*, 2006). As AI-2 though, addition of SAH could not increase the number of attached cells. From this point of view, the physiological role of AI-2 may be limited to the detoxification of SAH in *L. monocytogenes*.

The Agr system is a peptide-mediated QS system initially described in *S. aureus* and present in *L. monocytogenes*. The *agr* locus is composed of four genes (*agrB*, *agrD*, *agrC* and *agrA*) organized as an operon (Autret *et al.*, 2003). These genes encode the histidine kinase AgrC and the response regulator AgrA of a two-component system, as well as a precursor peptide AgrD, which is matured into an autoinducing peptide by AgrB. Whereas the *agr* system was known to be involved in the production of virulence factors, it has recently been shown to play an important role in biofilm formation in *L. monocytogenes* (Rieu *et al.*, 2007; Riedel *et al.*, 2009). It has been shown that the adhesion and the first stage of biofilm formation are affected in *agrA* and *agrD* mutant strains, within the first 24 h of incubation, but not afterwards (Rieu *et al.*, 2007). Interestingly, *agr* gene expression increased progressively over the incubation period in flowing conditions and its activity was maximal in cells outside ball-shaped microcolonies (Rieu *et al.*, 2008b). Using another culture medium (10-fold diluted BHI versus TSB), biofilm formation of a mutant Δ*agrD* was affected more than originally shown (Riedel *et al.*, 2009). The wild-type biofilm phenotype could be restored by adding supernatant of *L. monocytogenes* EGD-e grown in full-strength BHI to the Δ*agrD* strain or by mixing Δ*agrD* cells with wild cells in a cell ratio of 90:10 respectively (Riedel *et al.*, 2009). Considering that the *agr* system probably controls the expression of genes involved in different physiological processes, it will be interesting to identify *agr*-dependent mechanisms allowing biofilm formation. An interesting consequence of *agrD* deletion is the decrease of internalin A (InlA) in the cell wall compared with both wild-type and the complement strain (Riedel *et al.*, 2009), indicating that the expression of virulence genes is also regulated by the *agr* system. *Listeria monocytogenes* strains expressing truncated InlA exhibited significantly enhanced biofilm-forming ability compared with those expressing full-length InlA (Franciosa *et al.*, 2009). As observed in other systems (Davies *et al.*, 1998; Hall-Stoodley and Stoodley, 2002; Sauer *et al.*, 2002), developmental regulations involved during biofilm formation appear complex and probably transitory. As a general trend, these signalling systems are most certainly involved in physiological regulation beyond biofilm formation itself. Moreover, there is no demonstration that the *agr* system is a mechanism to assess cell density in order to coordinate behaviour of the whole population in *L. monocytogenes* (Garmyn *et al.*, 2009); in other words, evidence of a QS system is still awaited in this species.

Extracellular matrix

The extracellular matrix, which encompasses communities of cells in biofilm, is a complex mixture of exopolysaccharides, DNA, proteins, and other extracellular polymeric substances (polyglutamate, teichoic acids, etc.) that plays a structure-stabilizing and protective role in biofilm (Sutherland, 2001). The presence, respective proportions and contribution of these different compounds are highly variable from one bacterial species to another and can even be strain-dependent. While exopolysaccharides have been described as the main component of the extracellular matrix of numerous bacterial biofilms (Whitfield, 1988; Guedon *et al.*, 2000; Desvaux and Petitdemange, 2001; Desvaux *et al.*, 2001a,b; Desvaux, 2005; 2006; Vu *et al.*, 2009; Flemming and Wingender, 2010), their systematic presence and contribution in *L. monocytogenes* biofilm remain somewhat contentious. It is true that a thick and gummy extracellular polymeric substance as observed in some model biofilm-forming bacteria, e.g. *Staphylococcus epidermidis* (Götz, 2002), is not present in *L. monocytogenes*. The presence of fibres binding individual *L. monocytogenes* cells to another and to the surface has been reported on the basis of electron microscopy observations (Borucki *et al.*, 2003; Marsh *et al.*, 2003; Hefford *et al.*, 2005; Zameer *et al.*, 2010). It has been speculated though that these structures result from the complete dehydration used in processing samples for electron microscopy, leading to massive shrinkage of the polymeric matrix, leaving only thin fibrillar structures. In parallel, ruthenium red staining has revealed the presence of extracellular carbohydrate in the close surroundings of the *L. monocytogenes* cells, which is consistent with the presence of exopolysaccharides

(Borucki *et al.*, 2003; Zameer *et al.*, 2010). Nevertheless, because microscopically observed loose meshes of fibrils have not been directly identified as exopolysaccharides and considering that ruthenium red may also bind carbohydrates on the bacterial cell surface that are unrelated to exopolysaccharides, e.g. glycosylated compounds such as peptidoglycan, teichoic acids or proteins, these data are not conclusive. So far, genomic analyses in *L. monocytogenes* have not revealed the presence of biosynthetic pathways for exopolysaccharides like alginate in *Pseudomonas* or poly-*N*-acetylglucosamine in *Staphylococcus* (Harmsen *et al.*, 2010). An alternative interpretation of these fibrils, which requires further investigation, resides in the report of putative fimbriae-like structures on the *L. monocytogenes* cell surface (Folio, 2003) (Fig. 1).

As for several bacteria species, including *Staphylococcus* (Qin *et al.*, 2007; Rice *et al.*, 2007; Izano *et al.*, 2008), *Pseudomonas* (Whitchurch *et al.*, 2002; Allesen-Holm *et al.*, 2006) and *Bacillus* (Vilain *et al.*, 2009), it has been recently shown that biofilm matrix of *L. monocytogenes* contains extracellular DNA (eDNA) that plays an important role in initial adhesion and the early stage of biofilm formation (Harmsen *et al.*, 2010). The addition of DNaseI significantly reduces cellular attachment resulting in reduced biofilm formation. In contrast, enzymatic removal of both RNA and proteins does not alter the adhesion capacity. While the presence of exogenous DNA alone has no effect, cell attachment can be restored by the additional supply of culture supernatant enzymatically treated with DNaseI and proteinase K. When combined with eDNA, components other than proteins would then permit bacterial adhesion (Harmsen *et al.*, 2010). The identification of the peptidoglycan, and more specifically *N*-acetyl glucosamine (NAG), as the causative agent also raises contradictions. It has been shown that the addition of peptidoglycan alone to DNaseI-treated bacteria appears sufficient to restore initial attachment, but eDNA has been described as having a major role in initial attachment. However, the growth medium used was different from one set of experiments to another, i.e. the complex medium BHI and the chemically defined medium HTM respectively. Nonetheless, the length of eDNA is quite important for attachment of *L. monocytogenes* cells since cell adhesion is abolished when low-molecular-weight (LMW) DNA is added to the peptidoglycan. Thus, LMW DNA may act as an inhibitor of components involved in adhesion. Further investigations are required to define at a molecular level the interactions of these components (eDNA, LMW DNA, peptidoglycan, NAG) and their roles in adhesion and biofilm formation in *L. monocytogenes*.

While the importance of eDNA in the early stages of biofilm formation has been demonstrated in *L. monocytogenes*, other studies have shown that surface/extracellular proteins play an essential role at least in the initial attachment to a surface (Smoot and Pierson, 1998; Longhi *et al.*, 2008). It has been shown that the adhesion of *L. monocytogenes* to stainless steel and synthetic rubber is reduced by 99% when trypsin is added to the medium (Smoot and Pierson, 1998). Moreover, *L. monocytogenes* fails to produce biofilm following treatment with serratiopeptidase, an extracellular metalloprotease produced by *Serratia marcescens* (Longhi *et al.*, 2008), while *L. monocytogenes* biofilm detachment occurs following treatment with endopeptidase K (Franciosa *et al.*, 2009).

Biofilm-associated protein (Bap)

Bap (biofilm-associated protein) belongs to a family of surface proteins involved in biofilm formation (Lasa and Penadés, 2006). Bap was first identified in a *S. aureus* mastitis isolate and then found in diverse bacterial species like *Enterococcus faecalis*, but also Gram-negative bacteria, e.g. *Pseudomonas fluorescens* and *Salmonella enterica* sv. *typhimurium* (Cucarella *et al.*, 2001; Toledo-Arana *et al.*, 2001; Hinsa *et al.*, 2003; Latasa *et al.*, 2005). All Bap-related proteins have common structural features. They are surface proteins of high molecular weight that contain a core domain of tandem repeats and confer upon bacteria the capacity to form a biofilm. They play a relevant role in bacteria infectious processes and can occasionally be contained in mobile elements (Lasa and Penadés, 2006).

Recently, an *in silico* analysis of the genome of *L. monocytogenes* revealed an open reading frame (Lmo0435) for a protein similar to Bap (Jordan *et al.*, 2008). This protein was designated BapL because it presents Bap-like structural features and is required for cell attachment to abiotic surfaces. The *lmo0435* mutant of *L. monocytogenes* 10 403 s shows a significant reduction in attachment level compared with its isogenic parent. In contrast with other Bap-related proteins, BapL is not required for virulence (Jordan *et al.*, 2008). Although BapL seems to play an important role in adhesion, only four out 17 *L. monocytogenes* isolates tested possessed the gene encoding this protein. Furthermore, some *bapL*-negative strains adhered significantly better than *bapL*-positive strains such as *L. monocytogenes* 10 403 s and EGD-e, while other strains were strongly impaired in their attachment ability. BapL can contribute to the attachment of some *L. monocytogenes* strains, but its role in biofilm development has not been clearly established. A reduced level of attachment does not prevent the *bapL* mutant from forming a biofilm. This is in marked contrast to all other bacterial species encoding Bap, where deletion of the *bap*-related gene systematically led to defective biofilms (Lasa and Penadés, 2006). Consequently, the capacity of the *lmo0435* mutant to form biofilm should be tested in different conditions to confirm whether or not that BapL is

really functional and actually belongs to the Bap family. Moreover, other proteins with putative Bap structural features should be sought throughout the *L. monocytogenes* genome available to date.

Other molecular determinants

By screening a transposon bank of *L. monocytogenes*, two mutants disrupted in the *relA* and *hpt* genes show impaired biofilm formation (Taylor *et al.*, 2002). The *relA* gene encodes a guanosine pentaphosphate synthetase catalysing the formation of the alarmone (p)ppGpp, whereas the *hpt* gene encodes a 6-oxopurine phosphoribosyltransferase that converts the purine base (guanine) into the corresponding nucleotide, i.e. guanosine monophosphate (GMP). Both mutants have been shown to adhere to microtitre plates to a degree comparable to that of the wild-type during the first hour of incubation. However, subsequently the attached bacteria were apparently unable to grow. This suggests that both genes are essential for *L. monocytogenes* growth after attachment to an inert surface. Interestingly, *relA* and *hpt* mutants were unable to synthesize (p)ppGpp in response to nutritional starvation (Taylor *et al.*, 2002). Moreover, the level of transcription of the gene *relA* increased after initial attachment, proving that a stringent response is established after attachment. These results strongly suggest that the ability to establish a stringent response and undergo physiological adaptations to nutrient deprivation is essential for the subsequent growth of the adhered bacteria. It would be interesting to determine the role of (p)ppGpp during biofilm formation and understand its regulation in *L. monocytogenes*.

The cyclic diguanylate (c-di-GMP) is another guanine derivative, which plays a crucial role as second messenger, especially for transition between motile planktonic and sedentary biofilm-associated modes of growth in a wide variety of bacteria (Lasa, 2006; Hengge, 2009). This signal transduction system involves multiple diguanylate cyclase and phosphodiesterase enzymes, i.e. proteins exhibiting GGDEF and EAL/HD-GYP motifs respectively. According to the Pfam database, the genome of *L. monocytogenes* EGD-e encodes four proteins exhibiting a GGDEF domain (PF00990: Lmo1912, Lmo2174, Lmo0531 and Lmo1911) and three proteins with an EAL domain (PF00563: Lmo1914, Lmo0111 and Lmo0131). In *Listeria*, though, the c-di-GMP signalling network in biofilm formation has not been deciphered nor has its role even been questioned. An additional secondary signal molecule, i.e. cyclic diadenosine monophosphate (c-di-AMP), has recently been shown in *L. monocytogenes* to trigger a cytosolic pathway of innate immunity in the course of infection (Woodward *et al.*, 2010). c-di-AMP has scarcely been investigated in bacteria, but it is thought to regulate bacterial sporulation (Bejerano-Sagie *et al.*, 2006). While sporulation does not occur in *L. monocytogenes* (Seeliger and Jones, 1986; Payot *et al.*, 1999; Desvaux and Petitdemange, 2002), the possible role of c-di-AMP in listerial physiology, including biofilm formation, merits further investigation.

Under continuous flow conditions, *L. monocytogenes* forms biofilm composed of a network of knitted chains (Rieu *et al.*, 2008b). And yet the SOS response factor YneA is specifically activated during continuous flow biofilm formation, with RecA required for its activation (van der Veen and Abee, 2010). The deletion of the *yneA* and *recA* genes leads to a significantly reduced biofilm under flowing conditions, whereas no significant differences were observed under static biofilm conditions. Compared with the wild-type strain which formed a biofilm composed of a complex structure of elongated cells forming a network of knitted chains, *yneA* and *recA* mutants presented some patches of adhered cells after 24 h, which developed to very small microcolonies after 48 h. Elongated cells were not observed for these mutants. The SOS response factors YneA and RecA were not required for initial attachment but were essential for this type of biofilm development.

Recently, the virulence regulator PrfA has been shown to be involved in the regulation of biofilm development (Lemon *et al.*, 2010). The *prfA* mutant presents a defective biofilm compared with the wild-type. However, the mutant and wild-type *L. monocytogenes* showed similar adhesion to glass at 20°C, suggesting that biofilm defects occurred after initial surface adhesion. Considering regulation by PrfA is temperature-dependent, adhesion tests were unfortunately not performed at higher temperatures, especially 30°C and 37°C. As PrfA positively regulates both virulence genes and genes involved in biofilm formation, it may play a global role in modulating *L. monocytogenes* lifestyle (Lemon *et al.*, 2010).

Conclusion and perspectives

Further investigations are clearly necessary to decipher the molecular mechanisms specific to biofilm formation in *L. monocytogenes* (Table 1). Thanks to recent advances in phylogenetic clustering of *L. monocytogenes* isolates, especially MLST, correlations with biofilm-forming capacity as well as biofilm architecture can be formulated, and require further investigation. Future studies to establish more precisely the role of flagella in biofilm formation should test the swarming over swimming regulation hypothesis. As recently demonstrated in *Caulobacter crescentus* where eDNA masks the adhesive properties of newly synthesized holdfast to enable the escape of motile flagellated cells from the biofilm (Berne *et al.*, 2010; Kirkpatrick and Viollier, 2010), differential release of DNA

Table 1. Key questions for future research on molecular mechanisms of biofilm formation in *L. monocytogenes*.

Question	Rationale
Is there a relationship between clonal complexes and the ability to form biofilm?	At the moment, MLST analysis of *L. monocytogenes* is the most representative of the biodiversity of the species. Investigating the correlations between these clonal complexes and biofilm formation is more pertinent than considering the serovars or lineages.
What are the different natures and contributions of the potential exopolysaccharides and other exopolymers?	While the presence of exopolysaccharides is suggested, their secretion must be ascertained following biochemical identification and deciphering of the biosynthetic pathway(s). The contribution of exopolysaccharides to biofilm formation with respect to other alternative exopolymers such as eDNA should also be investigated.
What are the different roles for the flagella in biofilm formation?	Contradictory results emerge from different investigations on the role of the flagella in biofilm formation. While environmental conditions such as growth media or temperatures may account for the contradictory results, the swarming over swimming regulation hypothesis should be tested, as should the interaction with eDNA and other putative exopolymers.
What is the contribution of the secretome to biofilm development?	The secretion systems and secreted proteins, which can remain anchored to the cell envelope or are released into the extracellular milieu, could be involved at different stages of biofilm formation. This involves the study of MSCRAMM, which mediate colonization of both abiotic and biotic surfaces, including food products.
How is the signalling and regulation network engaged in the course of sessile growth?	Signal transduction seems to occur at least via the LuxS and Agr systems. Together with the involvement of second messengers (p)ppGpp, c-di-GMP and possibly c-di-AMP, much remains to be learned about the regulation network associated with biofilm development.

depending on culture conditions might explain the contradictory results regarding the role of the flagella in biofilm development in *L. monocytogenes*. Besides exopolymers produced by some bacteria exhibiting anti-adhesion and biofilm inhibition properties (Valle *et al.*, 2006), the use of synthetic or natural compounds that could outcompete QS and then prevent biofilm formation (Rice *et al.*, 1999; 2005; de Nys *et al.*, 2006; Chorianopoulos *et al.*, 2010) has not yet been investigated in *L. monocytogenes*.

As functional determinants interfacing the cell with its surroundings (Desvaux *et al.*, 2003; 2005a,b; 2006a; 2009a,b; Henderson *et al.*, 2004), secreted proteins certainly deserve more careful attention, especially MSCRAMM (microbial surface components recognizing adhesive matrix molecules), which can both take part in colonization of abiotic and biotic surface and in bacterial virulence (Desvaux and Hébraud, 2006; 2008; Desvaux *et al.*, 2006b). Protein secretion and its consequences are under active investigation in our laboratory. While the infection cycle of *L. monocytogenes* does not leave room for surface colonization (Tilney and Portnoy, 1989; Sleator *et al.*, 2009), the presence of several genes coding for MSCRAMM suggests that interactions might occur at some stage in the lifetime of this bacterial species. In other words, interactions with biotic surfaces could occur not only in the course of infection but also in the environment. When considering a foodborne pathogen, its involvement in the colonization of food products and food-processing environments is a legitimate and exciting hypothesis, which has so far been overlooked, but is currently being tested by our group. Several MSCRAMM proteins putatively involved in adhesion to fibronectin, mucin and collagen have been reported in *L. monocytogenes* (Bierne and Cossart, 2007). Few have been functionally and experimentally characterized, but internalins InlB, InlC and InlJ have been shown to bind human mucin MUC2 (Lindén *et al.*, 2008). FpbA (fibronectin binding protein A) is a surface protein associated with the membrane by an undetermined mechanism (Dramsi *et al.*, 2004). Nonetheless, FpbA has been confirmed to bind human fibronectin, contributing to eukaryotic cell adhesion and participating in bacterial virulence. The example of other investigated fibronectin-binding proteins suggests that MSCRAMM proteins may also be involved in adhesion and colonization of abiotic surfaces such as polystyrene (Shimoji *et al.*, 2003; O'Neill *et al.*, 2008).

Apart from proteins directly involved in bacterial adhesion to and colonization of both biotic and abiotic surfaces, characterization of gene determinants responsible for the regulation of biofilm formation remains a key challenge in *L. monocytogenes*. Considering the multifactorial nature of biofilm formation, obtaining a clear and unequivocal altered phenotype is often hampered when mutating a single gene encoding a structural protein. However, such a phenotype could most certainly be observed by mutating genes encoding signal transducers or transcriptional regulators controlling the expression of several genes encoding proteins physically engaged in cell attachment or the colonization process. In the context of microbial food safety, the influence of environmental conditions on biofilm formation and the regulation network engaged in the course of sessile growth are of crucial

importance for the development of practices and policies to limit and even prevent the contamination of food-processing plants and ultimately of food products. Multidisciplinary strategies, such as genomics, transcriptomics, proteomics, functional genetics and state-of-the-art microscopic techniques, are undoubtedly required to identify, target and characterize these gene products (Tremoulet *et al.*, 2002; Planchon *et al.*, 2007; 2009; Dumas *et al.*, 2008; 2009a,b; Stewart and Franklin, 2008; Wood, 2009; Desvaux *et al.*, 2010). The implementation of these complementary and readily available approaches promises major findings ahead in the field of molecular biology of surface colonization by *L. monocytogenes*. In the fight against listeriosis and following the adage 'mieux vaut prévenir que guérir' or 'an ounce of prevention is worth a pound of cure', research with the long-term aim of prevention complements investigations of listerial virulence whose shorter-term purpose is curative with the development of new treatments for infected people.

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RESUME

Listeria monocytogenes est une bactérie pathogène impliquée dans la toxi-infection alimentaire à l'origine de la listeriose, une maladie peu fréquente mais avec un taux de mortalité de 25 % chez l'homme. Cette bactérie est capable de former un biofilm lui permettant de mieux résister aux stress environnementaux ainsi qu'aux traitements de décontamination. Une nouvelle stratégie d'analyse génomique a été développée et a permis de cibler des systèmes de sécrétion et des protéines potentiellement impliqués dans la formation de biofilm. L'inactivation de la voie SecA2 entraîne la formation d'un biofilm aérien et par conséquent fragile. Ce morphotype est capable de croître de façon sessile à 20°C sur du polystyrène alors que ce n'est pas le cas pour la souche sauvage. De nouvelles protéines sécrétées de façon SecA2 dépendante ont été identifiées par l'étude de l'exoprotéome du mutant Δ*secA2* en comparaison avec celui de la souche sauvage. Le rôle des lipoprotéines dans la formation de biofilm ainsi que leur maturation par les peptidases signal de type II, LspA et LspB, a également été abordé. La combinaison d'une analyse de l'expression des gènes codant les lipoprotéines au cours de la formation de biofilm avec l'analyse génomique basé sur le sécrétome a permis de cibler trois lipoprotéines, dont LpeA qui serait impliquée dans les phases tardives de formation de biofilm. Enfin, l'importance majeure de LspA dans la maturation des lipoprotéines, a été mise en évidence par l'étude de l'exoprotéome des doubles mutant Δ*lgt*Δ*lspA* et Δ*lgt*Δ*lspB* en comparaison avec celui de Δ*lgt*.

Mots-clés : *Listeria monocytogenes* ; biofilm ; système de sécrétion ; SecA2 ; MurA ; CwhA ; lipoprotéines ; peptidases signal ; exoprotéome.

Protein determinants of the Sec secretion pathway involved in *Listeria monocytogenes* biofilm formation

ABSTRACT

Listeria monocytogenes is a foodborne pathogenic bacteria responsible for listeriosis, a rare but high mortality rate disease in humans (25 %). This bacterium can form biofilm allowing a better resistance to environmental stresses as well as decontamination treatments. A new strategy for genomic analysis was developed and allowed to target secretion systems and proteins potentially involved in biofilm formation. Inactivation of the SecA2 pathway leads to the formation of an aerial and fragile biofilm. This morphotype is able to grow in a sessile mode at 20 °C on polystyrene whereas this is not the case for the wild type strain. New proteins secreted in a SecA2 manner were identified by comparing the Δ*secA2* exoproteome to the one of the wild type. The role of lipoproteins in biofilm formation and their maturation by the signal peptidase II, LpsA and LspB, was also tackled. Combining expression analysis of genes encoding lipoproteins during biofilm formation with genomic analysis based on the secretome allowed targeting three lipoproteins, including LpeA, which appeared to be involved in the later stages of biofilm formation. Finally, the importance of LspA in the maturation of lipoproteins, was highlighted by comparing of the double mutant Δ*lgt*Δ*lspA* and Δ*lgt*Δ*lspB* exoproteomes to the one of Δ*lgt*.

Keywords : *Listeria monocytogenes* ; biofilm ; secretion system ; SecA2 ; MurA ; CwhA ; lipoproteins ; signal peptidases ; exoproteome.

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