

The regulation of type III secretion system of Pseudomonas aeruginosa

Dakang Shen

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UNIVERSITE JOSEPH FOURIER-GRENOBLE 1

SCIENCE & GEOGRAPHIE

THESE

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TITRE DE THESE

Régulation du Système de Sécrétion de Type III de *Pseudomonas aeruginosa*

COMPOSITION DU JURY

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A ma femme, Xia, et à ma fille, Xin-Yu

ABBREVIATIONS

bp	base pair
Da	dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EB	ethidium bromide
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol bis (β-aminoethyl ether) N,N,N’,N’-tetraacetic acid
EMSA	electrophoretic mobility shift assay
HEPES	N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
Max	maximum
mHBS	modified HEPES-buffered saline
MOI	multiplicity of infection
MW	molecular weight
OD	optical density
PBS	phosphate-buffered saline
RLU	relative luciferase units
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-PolyAcrylamide Gel Electrophoresis
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
TEMED	N, N, N’, N’-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
UV	ultraviolet
X-Gal	5-Bromo-4-Chloro-3-Indolyl-b-D-galactopyranoside

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INTRODUCTION

(En anglais)

1. *Pseudomonas aeruginosa* and *P. aeruginosa* infections

P. aeruginosa is a common environmental gram-negative bacillus, which acts as an opportunistic pathogen under several circumstances (Bodey *et al*., 1983). The ubiquitous occurrence of *P. aeruginosa* in the environment (Green *et al*., 1974) is due to several factors, including its abilities to colonize multiple environmental niches and to utilize many environmental compounds as energy sources (Williams and Worsey, 1976).

P. aeruginosa was likely first reported in human infections in 1862 by Luke, who observed rod-shaped particles in blue-green pus of some infections. Similar coloration had been previously observed by Sedillot on surgical dressings, and is now known to be caused by the pigment pyocyanin produced by *P. aeruginosa*. The microorganism was first isolated from infections in 1882 by Gessard, who called it *Bacillus pyocyaneus*. Given the widespread occurrence of *P. aeruginosa* in the environment, it is noteworthy that human disease attributable to it is quite rare in otherwise healthy individuals (Lyczak *et al*., 2000).

Nearly all-clinical cases of *P. aeruginosa* infection can be associated with the compromise of host defense. While many cases of *P. aeruginosa* infection can be attributed to general immunosuppression such as in AIDS patients (Franzetti *et al*., 1992; Kielhofner *et al*., 1992) and in neutropenic patients undergoing chemotherapy (Bendig *et al*., 1987), such scenarios predispose the host to a variety of bacterial and fungal infections, and are not specific to the pathogenesis of *P. aeruginosa*. In this respect, four of the more informative human diseases caused by *P. aeruginosa* are: 1) chronic lung infection in cystic fibrosis patients; 2) burn wound infections; 3) acute ulcerative keratitis in users of extended-wear soft contact lenses; and 4) nosocomial pneumonia.

1.1 *P. aeruginosa* and cystic fibrosis

Cystic fibrois (CF), fatal autosomal recessive disorder, is most prevalent in the Caucasian population with one of every 3,300 live births. CF is one of the most common fatal genetic disorders in the United States, affecting about 30,000 individuals, and a comparable number of people in Europe also have CF (Bye, *et al*., 1994).

Although immense progress has been made in the elucidation of the molecular and cellular pathophysiology of CF since the cloning of the CF gene (Rommens, *et al*., 1989; Riordan *et*

al., 1989; Kerem *et al*., 1989), the median life-span of CF patients at the turn of the century was approximately 40 years of age (Elborn *et al*., 1991) and the great majority of CF patients die from lung infection (Orenstein *et al*., 2002; Boucher, 2004). The most common bacterium to infect the CF lung is *P. aeruginosa*. Lungs of most children with CF become colonized (inhabited long-term) by *P. aeruginosa* before their 10^{th} birthday (www.pseudomonas.com).

CF transmembrane conductance regulator (CFTR) (Rommens, *et al*., 1989; Riordan *et al*., 1989; Kerem *et al*., 1989), gene involved in CF, locates on human chromosome 7. CFTR, normally produced in a number of tissues throughout the body, including the pancreas, sweat glands, vas deferens and intestines, regulates the movement of salt and water in and out of these cells. Mutation of the CFTR gene alters the CFTR protein in people with CF. As a result, one hallmark of CF is the presence of a thick mucus secretion, which clogs the bronchial tubes in the lungs and plugs the exit passages from pancreas and intestines, leading to loss of function of these organs. When CF was first identified as a disease, most affected individuals died within the first few years of life, owing to insufficient absorption of nutrients secondary to pancreatic dysfunction. As treatment for CF patients improved over the years, particularly after the introduction of improved nutritional regiments, patients began living much longer. As a consequence of this increased survival, chronic bacterial lung infection emerged as the primary cause of mortality in CF patients. Although *Staphylococcus aureus* is often cited as an early pathogen for CF patients, no credible study documents direct lung pathology resulting from the presence of *S. aureus* in throat cultures of CF patients. In contrast, it is abundantly clear that *P. aeruginosa* is the most prevalent and problematic pulmonary pathogen in CF patients (Lyczak *et al*., 2000). Once established in the lung of CF patients, *P. aeruginosa* can never be completely eliminated by antibiotics due to the formation of biofilm (Govan and Fyfe, 1978).

In addition to its role in salt transport, CFTR may influence *P. aeruginosa* lung infection directly through its role as an epithelial cell receptor for this microorganism (Pier *et al*., 1996). Because bacterial internalization by airway epithelium constitutes a host defense mechanism, served to minimize the bacterial load in the airway in the normal lung. It was proposed that in the CF lung, epithelium fails to function properly due to the mutation of

CFTR, resulting in abnormally high bacterial carriage, which promotes the establishment of chronic bacterial infection.

To date, 1465 candidate mutations in the CF gene have been identified and deposited in bank of 'CF mutation database' (http://www.genet.sickkids.on.ca/cftr/).

1.2 *P. aeruginosa* and burn wound infections

Bacterial infection following severe thermal injury can be most simplistically attributed to extensive breaches in the skin barrier. The fact that *P. aeruginosa* occurs so commonly in the environment makes it the most common cause of burn wound infections (Mayhall, 2003). Exacerbating this situation, hospitals often harbor multidrug-resistant *P. aeruginosa* that can serve as the source of infection (Hsueh *et al*., 1998). *P. aeruginosa* has been found to contaminate the floors, bed rails, and sinks of hospitals, and has also been cultured from the hands of nurses (Chitkara and Feierabend, 1981). Besides transmission through fomites (contaminant) and vectors, bacterial flora can be carried into a hospital by the patient and can be an important source of infection for the same individual after injury (Phillips *et al*., 1989).

The successful colonization of *P. aeruginosa* at a burn site depends upon not only the concerted impairment of several host immune mechanisms, but also a variety of bacterial virulence factors (Lyczak *et al*., 2000), which will be discussed later.

1.3 *P. aeruginosa* and ulcerative keratitis

Ulcerative keratitis is a rapidly progressing inflammatory response to bacterial infection of the cornea, and has been called the most destructive bacterial disease of the human cornea (Laibson *et al*., 1972). Historically, this infection was usually associated with injury or trauma to the cornea. However, in 1984, cases of ulcerative keratitis were reported among individuals who had suffered no acute injury, but who were users of extended-wear soft contact lenses (Weissman *et al*., 1984; Galentine *et al*., 1984).

In the cornea, the basal epithelial cells expressing the most CFTR and possibly internalizing *P. aeruginosa* are buried beneath of several layers of the superficial epithelial cells, which express undetectable quantities of CFTR protein (Zaidi *et al*., 1999). Corneal injury due to the use of contact lenses permits access of the bacteria to the basal epithelial cells. In such case,

rather than serving to remove *P. aeruginosa*, as occurs in the airway, internalization of *P. aeruginosa* by corneal epithelium creates a reservoir of intracellular bacteria which are capable of replicating within the corneal epithelial cells (Fleiszig *et al*., 1995).

1.4 *P. aeruginosa* and nosocomial pneumonia

Nosocomial pneumonia (NP), a frequent complication in critically ill patients requiring mechanical ventilation, is responsible for a significant in-hospital morbidity and mortality (Kollef, 2004). *P aeruginosa* was the most common Gram-negative bacterial pathogen isolated from the respiratory tract in infected patients with both early-onset NP and late-onset NP, while hospital mortality is significantly greater for patients with early-onset and late-onset NP compared to patients without NP (Ibrahim *et al*., 2000).

2. Virulence factors

It is now recognized that the relatively large genome of *P. aeruginosa* (6.3 Mb) carries an extensive repertoire of genes compatible with the various niches that this organism can occupy, and possesses many potential virulence factors that contribute to its pathogenesis (Stover *et al*., 2000).

2.1 Lipopolysaccharide

Lipopolysaccharide (LPS) molecules are located in the cell wall and thus play an important structural role while mediating interaction with the neighboring environment. The tripartite nature of LPS includes from inner to outer i) a hydrophobic lipid A region, ii) a central core oligosaccharide region and iii) a repeating polysaccharide portion referred to as O antigen or O polysaccharide.

The lipid A region of LPS, composed of a phosphorylated diglucosamine moiety substituted with fatty acids, is thought to be responsible for most of the biological activities of LPS (also referred to as endotoxin) through the induction of the immunomodulating molecules via its interaction with the toll-like receptor 4 signaling complex. (Lynn and Golenbock, 1992; Kopp and Medzhitov, 2003).

The core oligosaccharide region of LPS can be subdivided into an inner and an outer core. The outer core region is believed to be the ligand mediating the association with CF transmembrane regulator protein (CFTR), a receptor for the binding of *P. aeruginosa* to host epithelial surfaces, since strains producing rough LPS (lacking O antigen) and semirough LPS (core-lipid A plus one O-antigen repeat) with a complete core region were ingested more readily than were those expressing wild-type smooth LPS (attachment of the O antigen to core-lipid A) (Pier, *et al*., 1996).

O antigen or O polysaccharide is the most heterogeneous portion of LPS and it confers serum resistance to the organism. *P. aeruginosa* is capable of concomitantly synthesizing two types of LPS referred to as A band and B band. The A-band LPS contains a conserved O polysaccharide region composed of D-rhamnose (homopolymer), while the B-band O-antigen (heteropolymer) structure varies among the 20 O serotypes. It is worth of pointing out that A band is the LPS molecule selectively maintained on the *P. aeruginosa* cell surface during chronic CF lung infections and the presence of anti-A-band antibodies within CF patients correlates with both increased duration of *P. aeruginosa* infections and lower pulmonary function (Rocchetta *et al*., 1999).

Taken together, LPS do play a key role in pathogenesis. In acute infections, a smooth LPS protects the organism from complement-mediated killing and, during chronic lung infections, an altered rough LPS helps the organism evade host defense mechanisms (Goldberg and Pier, 1996).

The regulatory mechanisms governing LPS synthesis and changes in LPS production in *P. aeruginosa* have not been determined.

2.2 Exotoxin A

Exotoxin A (ETA), a 66-kDa protein encoded by *toxA*, is an ADP-ribosylating enzyme (Iglewski and Kabat, 1975; Gray *et al*., 1984). It is generally accepted that ETA is internalized by the cell surface receptor CD91, the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (Kounnas *et al*., 1992) and asserts its cellular toxicity by blocking protein synthesis through ADP ribosylation of translation elongation factor 2 (Perentesis *et al*., 1992). ETA is considered to be the most toxic factor secreted by *P. aeruginosa*.

Maximum production of ETA is usually detected when *P. aeruginosa* is grown in iron-deficient medium (Lory, 1986). The complicated process of ETA production by *P.*

aeruginosa also involves several positive regulatory genes, including *regA*, *ptxR* and *pvdS* (Hamood and Iglewski, 1990; Hamood *et al*., 1996; Hunt *et al*., 2002).

2.3 Alginate

Alginate, a copolymer of mannuronic and guluronic acids (Evans and Linker, 1973), is an exopolysaccharide that makes it very difficult to eradicate *P. aeruginosa* from the lungs of CF patients due to its ability to shield the bacteria from antibiotics and host defenses (Bayer *et al*., 1991; Cabral *et al*., 1987; Learn *et al*., 1987; Hatch and Schiller, 1998; Nivens *et al*., 2001; Pier *et al*., 2001; Lyczak *et al*., 2002). The over-production of alginate in CF patients converts the nonmucoid bacteria to mucoid form, which is associated with a decline of pulmonary function and survival rate (Pedersen *et al*., 1992). Alginate can also act as an adhesin (Doig *et al*., 1987), which facilitates bacterial colonization of the respiratory tract. Only the two bacterial genera *Pseudomonas* (Linker and Jones, 1966) and *Azotobacter* (Pindar and Bucke, 1975) are known to produce alginates.

Most of the genes involved in alginate biosynthesis are clustered in an operon, which comprises 12 genes (*algD*, *alg8*, *alg44*, *algK*, *algE*, *algG*, *algX*, *algL*, *algI*, *algJ*, *algF*, and *algA*) under tight control of the alginate promoter, upstream of *algD* (Chitnis and Ohman, 1993; Schurr *et al*., 1993; Shankar *et al*., 1995).

algC is the only gene involved in alginate synthesis that is not located in the cluster, but it is also involved in lipopolysaccharide synthesis and expressed from its own promoter (Goldberg *et al*., 1993; Zielinski *et al*., 1991).

The alginate switch, including known *algU*, *mucA*, *mucB*, *mucC*, and *mucD*, is responsible for the conversion of *P. aeruginosa* to the alginate-producing forms found predominantly in lungs of CF patients (Deretic *et al*., 1994; Martin *et al*., 1993; Mathee *et al*., 1997; Shankar *et al*., 1995). Regulatory genes *algR*, *algP*, *algQ* and *algB*, are important for transcriptional activation of the alginate biosynthetic genes (Gacesa, 1998; Shankar *et al*., 1995).

Historically, alginate has been considered the major exopolysaccharide of the *P. aeruginosa* biofilm matrix, however, recent chemical and genetic studies have demonstrated that alginate is not involved in the initiation of biofilm formation in *P. aeruginosa* strains PAO1 and PA14 (Wozniak *et al*., 2003; Stapper *et al*., 2004). In fact, exopolysaccharide encoded by *psl*

(polysaccharide synthesis locus) and *pel* (pellicle) genes is implicated in the biofilm formation (Matsukawa and Greenberg, 2004; Jackson *et al*., 2004; Friedman and Kolter, 2004; Vasseur *et al*., 2005).

2.4 Phospholipases

Phospholipases are a heterogeneous group of enzymes that are able to hydrolyse one or more ester linkages in glycerophospholipids and include phospholipase A (PLA), B (PLB), C (PLC) and D (PLD). Phospholipase activity can destabilize host membranes, lyse cells and release lipid second messengers (Salyers and Witt, 1994). *P. aeruginosa* produces several extracellular phospholipases, including i) three PLCs: a haemolytic PLC (PlcH), a non-haemolytic PLC (PlcN) (Ostroff *et al*., 1990) and PLcB (Barker *et al*., 2004); All presently known PLCs of *P. aeruginosa* hydrolyse phosphatidylcholine (PC), which is abundant in lung surfactant (Krieg *et al*., 1988), therefore, PLCs probably has a role in virulence; Anti-PLC antibody is readily detectable in CF patients, and the isolation of strains capable of secreting PLCs is usually associated with a poor clinical status (Granstrom *et al*., 1984); Only PlcB is also active on phosphatidylethanolamine (PE) and required for phospholipid chemotaxis (Barker *et al*., 2004); ii) a PLD (PldA), which plays a role for persistence in a chronic pulmonary infection model (Wilderman *et al*., 2001a); iii) a cytosolic PLA (cPlA2), which plays an important role in the induction of host cell death (Kirschnek and Gulbins, 2006).
Expression of the PlcH is induced under phosphate starvation conditions or in the presence of the osmoprotectants choline and glycine betaine (Sage and Vasil, 1997).

2.5 Rhamnolipids

Rhamnolipids are glycolipidic biosurfactants, which reduce water surface tension and emulsify oil. Rhamnolipids are comprised of mono- and dirhamnose groups linked to 3-hydroxy fatty acids that vary in length, the most common being L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (monorhamnolipid) and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (dirhamnolipid) (Lang and Wullbrandt, 1995; Maier and Soberon-Chavez, 2000; Deziel *et al*., 2003). For the synthesis of monorhamnolipid, the enzyme rhamnosyltransferase 1 (Rt 1) catalyses the rhamnose transfer to β-hydroxydecanoyl-

β-hydroxydecanoate, while rhamnosyltransferase 2 (Rt 2) synthesizes dirhamnolipid from TDP-L-rhamnose and monorhamnolipid (Maier and Soberon-Chavez, 2000). Genes for biosynthesis, regulation and induction of the Rt 1 enzyme are organized in tandem in the *rhlABRI* gene cluster (Ochsner *et al*., 1994a; 1994b; Ochsner and Reiser, 1995). The gene *rhlC* encoding the Rt 2 enzyme has been described (Rahim *et al*., 2001), and is homologous to rhamnosyltransferases involved in lipopolysaccharide biosynthesis. RhlG, a β-ketoacyl reductase encoded by *rhlG*, which contains RhlR binding sites in the promoter region but does not require RhlR for activation, is also involved in biosynthesis of rhamnolipids (Campos-Garcia *et al*., 1998).

Rhamnolipids are found in the sputa of CF patients and can inactivate tracheal cilia of mammalian cells, indicating that they are virulence factors (Hastie *et al*., 1986; Kownatzki *et al*., 1987). Rhamnolipid also play a role in the development of normal biofilm architecture (Davey *et al*., 2003; Lequette and Greenberg, 2005) and might be the actual surfactant involved in swarming motility (Kohler *et al*., 2000; Caiazza *et al*., 2005).

Rhamnolipid production coincides with stationary-phase growth (Hauser and Karnovsky, 1957) and is under RhlR-RhlI quorum-sensing control (Ochsner *et al*., 1994b, 1995; Campos-Garcia *et al*., 1998; Rahim *et al*., 2001).

2.6 Phenazines

Phenazine compounds produced by fluorescent *Pseudomonas* species are biologically active metabolites that function in microbial competitiveness (Mazzola *et al*., 1992) and virulence in human and animal hosts (Mahajan-Miklos *et al*., 1999). *P. aeruginosa* produce a variety of redox-active phenazine compounds, including pyocyanin, phenazine-1-carboxylic acid (PCA), 1-hydroxyphenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) (Budzikiewicz *et al*., 1993).

Genes involved in phenazine biosynthesis in *P. aeruginosa* include two homologous core loci (*phzA1B1C1D1E1F1G1* and *phzA2B2C2D2E2F2G2*) responsible for synthesis of PCA and three additional genes (*phzM*, *phzS*, and *phzH*) encoding unique enzymes involved in the conversion of PCA to pyocyanin and PCN (Mavrodi *et al*., 2001).

From 90 to 95% of *P. aeruginosa* isolates produce pyocyanin (Mavrodi *et al*., 2001), and the presence of high concentrations of pyocyanin in the sputum of CF patients has suggested that this compound plays a role in pulmonary tissue damage observed with chronic lung infections (Wilson *et al*., 1988). This idea is supported by several studies, which demonstrated that pyocyanin contributes in a variety of ways to the pathophysiological effects observed in airways infected by *P. aeruginosa*. (Warren *et al*., 1990; Hussain *et al*., 1997; Vukomanovic *et al*., 1997; Denning *et al*., 1998).

The unusually broad range of biological activity associated with phenazines is thought to be due to their ability to undergo redox cycling in the presence of various reducing agents and molecular oxygen, which leads to the accumulation of toxic superoxide (O_2^-) and hydrogen peroxide (H_2O_2) and eventually to oxidative cell injury or death (Hassan and Fridovich, 1980; Britigan *et al*., 1997).

2.7 Proteases

The major role of proteases in *P. aeruginosa* virulence in various infection models is thought to involve tissue penetration (Cowell *et al*., 2003).

Proteases synthesized by this organism include 2 elastases, LasB encoded by the *lasB* gene (Bever and Iglewski, 1988) and LasA encoded by the *lasA* gene (Olson and Ohman, 1992), alkaline protease (AprA) encoded by the *aprA* gene (Okuda *et al*., 1990), and protease IV encoded by *prpL* gene (Wilderman *et al*., 2001b; Traidej *et al*., 2003).

LasA enhances microbial virulence via modulating host defenses by shedding of syndecan-1, the predominant cell-surface heparan sulphate proteoglycans, which are ubiquitous and abundant receptors/co-receptors of extracellular ligands, including many microbes (Park *et al*., 2001).

LasB decreases the host innate defense by cleaving the N-terminal domain of proteinase-activated receptor 2 and disarms its subsequent activation, which is implicated in the pulmonary innate defense (Dulon *et al*., 2005).

Protease IV contributes to the pathogenicity of *Pseudomonas* keratitis (Engel *et al*., 1997, 1998a; O'Callaghan *et al*., 1996). Purified protease IV (50-200 ng) induced corneal epithelial damage within 3 hours after injection into the corneal stroma and increased the virulence of

protease IV-deficient bacteria (Engel *et al*., 1998b). It appears that a combination of AprA, LasB, LasA and protease IV work in concert to promote tissue invasion (Suter *et al*., 1994; Matsumoto *et al*., 2004).

Proteases production is regulated by quorum-sensing with the first discovery of LasR, regulator of *lasB* in the early 1990s (Gambello and Iglewski *et al*., 1991; Toder *et al*., 1991; Gambello *et al*., 1993).

2.8 Pili

Pili, also known as fimbriae, are thin, hairlike appendages on the surface of many microorganisms, especially gram-negative and gram-positive bacteria. After aspiration or inhalation, the initial step in the establishment of a *P. aeruginosa* infection is the adherence to susceptible host cells via type IV pili (Woods *et al*., 1980). In addition to their role as colonization factors, the type IV pili of many species also play a role in twitching motility (Mattick *et al*., 2002), biofilm formation (O'Toole and Kolter, 1998), natural transformation (Dubnau *et al*., 1999), and bacteriophage infection (Bradley, 1974).

Pilus synthesis and assembly require at least 40 genes, which are located in several unlinked regions on the chromosome (Hobbs *et al*., 1993; Mattick *et al*., 2002). The expression of pili is mainly controlled by a two-component transcriptional regulatory system, PilS and PilR (Hobbs *et al*., 1993) and the alternative sigma factor RpoN (Ishimoto and Lory, 1989). The nature of the environmental signal that triggers the expression of pili is not known. Proteins involved in type IV pili biogenesis share homology with the Xcp secreton, a type II secretion machinery involved in the release of toxins and enzymes in *P. aeruginosa* (Russel, 1998).

2.9 Flagella

Flagella, comprised of the membrane-spanning hook-basal body and an external filament, are complex organelles capable of propelling bacteria through liquids (swimming) and through highly viscous environments or along surfaces (swarming) (McCarter, 2006).

In acute infections, flagella are essential for *P. aeruginosa* pathogenesis. This has been documented in a murine thermal injury model in which motile strains were able to spread rapidly throughout the host and nonmotile variants were restricted to the site of inoculation

and easily cleared (Drake and Montie, 1988). Flagella also play a critical role in the development of biofilms, where they aid in initial surface adhesion as well as biofilm dispersal (O'Toole and Kolter, 1998; Sauer *et al*., 2002). In addition, soluble flagellin is released by motile gram-negative bacteria and acts as a potent inducer of inflammation through interaction with Toll-like receptor 5 (Hayashi *et al*., 2001). Flagellin activates the NF-κB arm of the immune system and provokes the release of proinflammatory mediators such as tumor necrosis factor, interleukin 6, interleukin 8, and nitric oxide by macrophages, monocytes, and epithelial cells (Cobb *et al*., 2004; Honko and Mizel, 2004; Ramos *et al*., 2004).

The biosynthesis and assembly of a functional flagellum are subject to a highly complex and tightly controlled regulatory cascade, which requires coordinate expression of approximately 50 genes encoding structural subunits, regulatory proteins, motor force generators, and chemosensory machinery (Aldridge and Hughes, 2002). In *P. aeruginosa*, flagellar genes are clustered in three distinct regions of the chromosome, and a four-tiered transcriptional regulatory circuit controls flagellum synthesis (Dasgupta *et al*., 2003). The FleQ protein, an NtrC-like transcriptional activator, has been referred to as the master regulator of this pathway, as it belongs to the top tier of the intricate flagellar hierarchy and is required for the expression of all other known flagellar genes with the exception of *fliA* (Arora *et al*., 1997; Jyot *et al*., 2002; Dasgupta *et al*., 2003).

Sequence and functional similarities exist between flagellar basal bodies and the apparatus of the type III secretion system (Blocker *et al*., 2003), another virulence factor that will be discussed later. Flagella probably came first and then the type III secretion system (Saier, 2004).

3. Secretion pathways

Interaction of bacterial pathogens with host cells is particularly characterized by factors that are located on the bacterial surface or are secreted into the extracellular space. Although the secreted bacterial proteins are numerous and diverse and exhibit a wide variety of functions that include proteolysis, haemolysis, cytotoxicity, and protein phosphorylation and dephosphorylation, only a few pathways exist by which bacterial proteins are transported

from the bacterial cytoplasm to the extracellular space by crossing the inner membrane (IM), periplasm, and outer membrane (OM) of the cell envelope. These pathways can be divided into two main groups: (i) *sec*-dependent and (ii) *sec*-independent (Hueck, 1998). All pathways described below, except type IV, have been found in *P. aeruginosa* (Ma *et al*., 2003).

3.1 *Sec*-dependent secretion pathways

Proteins secreted via the *sec*-dependent secretion pathways utilize a common machinery, the *sec* system, the so-called general secretory pathway, which exports proteins with cleavable aminoterminal signal peptide from cytoplasm to periplasm (Economou, 1999). In *E. coli*, the *sec* system mainly comprises a number of inner membrane proteins (SecD-G, SecY, YajC, YidC) (Scotti *et al*., 2000), a cytoplasmic membrane-associated ATPase (SecA), a chaperone (SecB) that binds to presecretory target proteins, and the periplasmic signal peptidase (Hueck, 1998). All of these proteins were found in single copy in *P. aeruginosa* (Ma *et al*., 2003).

Sec-dependent secretion pathways involve two steps including export to the periplasm via the *sec* system by crossing the inner membrane and transport across the outer membrane. The common *sec*-dependent secretion pathways include the type II secretion system, the autotransporter and the chaperone/usher secretion system, which can be differentiated from their mechanisms of secretion across the OM (Kostakioti *et al*., 2005).

3.1.1 Type II secretion system

The type II secretion system is responsible for the extracellular transport of a wide variety of hydrolytic enzymes and toxins, and the production of the type IV pili, which are responsible for adhesion and twitching motility of several bacteria (Sandkvist, 2001).

Substrates of the type II secretion system cross the IM via the *sec* system and fold in the periplasm with the help of the DsbA, which is an enzyme required for disulfide bond formation (Bardwell, *et al*., 1991).

In the case of pullulanase (PulA) secretion (Fig. 1) by *Klebsiella oxytoca*, the best-studied example of type II secretion system, transport across the OM involves 14 proteins, which are encoded by a continuous gene cluster. At least seven of these proteins are located in the cytoplasmic membrane, while PulD and PulS are outer membrane proteins (Hueck, 1998).

PulD is believed to form the translocation channel (Nouwen *et al*., 1999) and is conserved in a variety of gram-negative protein transport systems. PulE, a cytoplasmic membrane-associated ATPase, provides the energy for protein secretion (Possot and Pugsley, 1994).

In *P. aeruginosa*, the type II secretion pathway, also called Xcp, has been shown to be the main pathway for the secretion of many degradative enzymes and toxins, such as elastase, exotoxin A, lipase, phospholipases or alkaline phosphatase (Filloux *et al*., 1990; 1998).

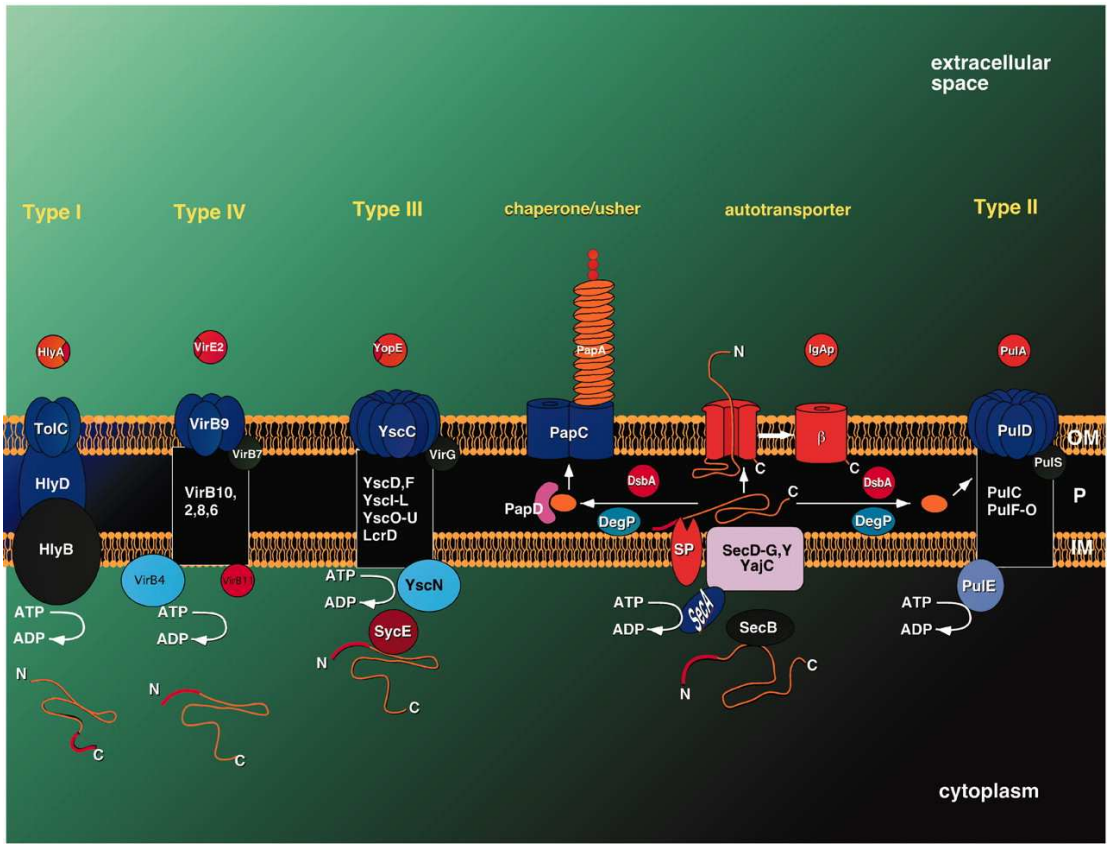


FIG. 1. Schematic presentation of *sec*-dependent and *sec*-independent secretion pathways in gram-negative bacteria. The type I pathway is exemplified by the hemolysin A (HlyA) secretion in pathogenic *Escherichia coli*, the type III pathway by YopE secretion in *Yersinia pestis*, the type IV pathway by the VirE2 protein of *Agrobacterium tumefaciens*, the chaperone/usher pathway by P pilus subunit (PapA) secretion in uropathogenic *E. coli*, the autotransporter pathway by IgA1 protease (IgAp) secretion in *Neisseria gonorrhoeae*, and the type II pathway by pullulanase (PulA) secretion in *Klebsiella oxytoca*. Secreted polypeptides are shown in orange, with their amino- or carboxyl-terminal signal peptides drawn in red. Only proteins secreted by Sec-dependent pathways contain cleavable signal peptides. Proteins secreted by Sec-dependent pathways may fold, fully or partially, in the periplasm with the assistance of periplasmic accessory factors (e.g., DsbA), whereas misfolded secreted proteins in the periplasm are degraded by periplasmic proteases (e.g., DegP). P, periplasm; SP, signal peptide; β, β-domain of autotransporter IgA1 protease; N, amino terminus; C, carboxy terminus. See the text for further details. Reprinted from Kostakioti *et al*., 2005. Except type IV pathway, all pathways shown here have been described in *P. aeruginosa* (Ma *et al*., 2003).

3.1.2 Autotransporter

Autotransporter pathway (Fig. 1) is one of the most widely distributed secretion systems among the gram-negative bacteria (Yen *et al*., 2002; Kostakioti *et al*., 2005). Many autotransporters have been identified, such as immunoglobulin A protease (IgAp) from *Neisseria gonorrhoeae* (Halter *et al*., 1984), vacuolating cytotoxin VacA from *Helicobacter pylori* (Schmitt and Haas, 1994), SepA from *Shigella flexneri* (Benjelloun-Touimi *et al.*, 1995), EspC from enteropathogenic *Escherichia coli* (Stein *et al*., 1996), Hap from *Haemophilus influenzae* (Hendrixson *et al*., 1997), adhesin-involved-in-diffuse-adherence (AIDA-I) autotransporter from *E. coli* (Maurer *et al*., 1999), Hia from *Haemophilus influenzae* (Surana *et al*., 2004).

The main characteristic of this pathway is that autotransporters mediate their own transport, because the information required for transport across the OM resides entirely within the autotransporters themselves. A typical autotransporter contains three domains: an aminoterminal signal sequence for secretion across the IM by the *sec* system, an internal passenger domain (α-domain) and a carboxyterminal domain (β-domain). Following transport across the IM and cleavage of the signal sequence, the β-domain inserts into the OM to form a β-barrel pore, through which the α-domain passes to the cell surface. The α-domain may either remain attached to the bacterial cell or be released to the external milieu by proteolysis (Kostakioti *et al*., 2005).

In *P. aeruginosa*, esterase EstA was identified as an AT (Wilhelm *et al*., 1999) and another two AT homologues were predicted (Ma *et al*., 2003).

3.1.3 Chaperone/usher secretion system

The chaperone/usher pathway is dedicated to the assembly and secretion of pili as well as capsule-like structures. The biogenesis of both structures occurs by similar mechanisms and requires the actions of two secretion components working in concert: a periplasmic chaperone and an outer membrane protein called as ‘usher’ (Kostakioti *et al*., 2005).

Assembly of P and type 1 pili by uropathogenic *E. coli* represents the prototype for the chaperone/usher pathway (Thanassi *et al*, 1998). Following translocation across the IM as unfolded polypeptides via the *sec* system, P pilus subunits (PapA) interact with the

periplasmic chaperone PapD (Fig. 1), which provides a template for their folding in the periplasm and caps interactive surfaces on the subunits, preventing their premature aggregation. Chaperone-subunit complexes are targeted to the P pilus usher, PapC, in the OM, where the chaperone dissociates from the subunits. Dissociation of the chaperone uncaps the interactive surfaces of the subunits, which drives their assembly into pili. In the absence of an usher, chaperone-subunit complexes accumulate in the periplasm but cannot translocate across the OM for assembly into pili on the bacterial surface.

In *P. aeruginosa*, chaperone/usher systems (Cup) are involved in the biofilm formation in early stages (Vallet *et al*., 2001)

3.2 *sec*-independent secretion pathways

Sec-independent pathways tend to allow direct export from the cytoplasm to the extracellular environment in one step, and do not involve periplasmic intermediates and aminoterminal processing of the secreted proteins via the *sec* system. These pathways include the type I secretion system, the type III secretion system and the type IV secretion system, that can be *sec*-dependent but is mostly considered *sec*-independent (Ding *et al*., 2003; Kostakioti *et al*., 2005).

3.2.1 Type I secretion system

Type I secretion system, also called ATP-binding cassette (ABC) protein exporters, is employed by a wild range of gram-negative bacteria for secretion of toxins, proteases and lipases (Binet *et al*., 1997). It exemplified by the secretion system of hemolysin (HlyA) of pathogenic *E. coli* (Koronakis *et al*., 1989). Type I secretion system consists of three polypeptides: an inner membrane transport ATPase (e.g., HlyB), also termed ABC protein, which provides the energy for protein secretion; a membrane fusion protein (e.g., HlyD), which is anchored in the inner membrane and spans the periplasmic space; an integral OM protein (e.g., TolC) that forms a β-barrel with a central hydrophilic pore (Fig. 1). Proteins secreted by the type I secretion system does not contain a cleavable N-terminal signal peptide; instead, they possess a noncleavable C-terminal signal sequence that targets them to the secretion apparatus (Hueck, 1998).

In *P. aeruginosa*, alkaline protease (AprA) is secreted by a type I secretion pathway (Guzzo *et al*., 1991).

3.2.2 Type IV secretion system

Type IV secretion system is characterized by a remarkable functional versatility, as they can transfer both proteins and single-stranded-DNA-protein complexes in a cell-contact-dependent or cell-contact-independent mechanism (Cascales and Christie, 2003; Llosa and O'Callaghan, 2004). Type IV secretion system is generally *sec* independent, with the remarkable exceptions of the *sec*-dependent transport of the *Bordetella pertussis* PT toxin (Cascales and Christie, 2003).

The T-DNA transfer system of *Agrobacterium tumefaciens* has provided the basis of what is known about type IV secretion system (Cascales and Christie, 2003). *A. tumefaciens* transfers at least three macromolecular substrates, including oncogenic T-DNA, single-stranded DNA-binding protein VirE2, and VirF protein, to plant cells during the course of infection (Christie, 2001). Recent studies have proposed that the proteins VirB2, VirB6, VirB8, VirB9, and VirB11 correspond to channel subunits of the type IV secretion apparatus, whereas VirB4, VirB5, VirB7, and VirB10 interact indirectly with the secreted substrate through their direct association with the channel subunits (Cascales and Christie, 2004).

Type IV secretion system is closely related to bacterial conjugation and are thought to have evolved from the conjugation machinery (Cascales and Christie, 2003; Ding *et al*., 2003).

3.2.3 Type III secretion system

The type III secretion system (TTSS) can mediate delivery of bacterial virulence factors directly into host cells, involving protein export not only across the bacterial cell envelope, but also the plasma membrane of the eukaryotic cell. It was first characterized for the Yop proteins of *Yersinia* and has much in common with the flagellar export system (Hueck, 1998).

TTSS, an important virulence factor, serves to secrete and inject proteins effectors of virulence into the cytosol of eukaryotic host cells during infection. The injected protein effectors often resemble eukaryotic factors with signal transduction functions and are capable of interfering with eukaryotic signalling pathways. Redirection of cellular signal transduction

may result in disarmament of host immune responses or in cytoskeletal reorganization, establishing subcellular niches for bacterial colonization and facilitating a highly adapted pathogenic strategy of "stealth and interdiction" of host defense communication lines (Hueck, 1998).

Distinctive features of the TTSS include: (i) the absence of a typical *sec*-dependent cleavable signal sequence in the secreted proteins; (ii) the requirement of specific cytosolic proteins with chaperone-like activity for the secretion of at least some of the effector proteins; and (iii) the requirement of an environmental signal, usually derived from contact with the host cell, for their full activation.

TTSSs are widely used by gram-negative bacteria, including the animal pathogens *Yersinia* spp., *Shigella flexneri*, *Salmonella typhimurium*, enteropathogenic *Escherichia coli* (EPEC), *P. aeruginosa*, and *Chlamydia* spp and the plant pathogens *P. syringae*, *Erwinia* spp., *Ralstonia* (formerly *Pseudomonas*) *solanacearum*, *Xanthomonas campestris*, and *Rhizobium* spp (Hueck, 1998). It is worth of pointing out that *S. typhimurium*, *Yersinia* spp., and *E. coli* have two TTSSs, otherwise, TTSSs are also present in some commensal microorganisms (*Photorhabdus luminscens*, *Sodalis glossinidius*, *Sitophilus zeamais*), symbiotic rhizobia and some nonpathogenic prokaryotes (*P. fluorescens*) (Tampakaki *et al*., 2004).

The prototypical TTSS is exemplified by the plasmid-encoded Yop (*Yersinia* outer proteins) export genes, which is responsible for secretion of Yop virulence determinants *in vitro* (Michiels *et al*., 1991a; 1991b). Genes encoding TTSS, especially those genes encoding the type III secretion apparatus (TTSA), are clustered. In some organisms, these gene clusters are located on plasmids, which are unique to the pathogen and are not found in nonpathogenic relatives (*Yersinia* spp., *S. flexneri*, and *R. solanacearum*). In other pathogens (*S. typhimurium*, EPEC, *P. aeruginosa*, *P. syringae*, *E. Amylovora* and *X. campestris*), the type III secretion gene clusters are located on the chromosomes and often appear to have been acquired during evolution, since related nonpathogenic bacteria lack these pathogenicity islands but share corresponding adjacent sequences (Hueck, 1998).

While genes encoding TTSA are conserved in different gram-negative bacteria and show significant sequence similarities to protein of the flagellar assembly machinery (Kubori, *et al*., 1998), genes encoding secreted protein effectors differ entirely, illustrating how one bacterial

pathogenicity mechanism can give rise to a multitude of diseases that range from bubonic plague in humans to fire blight in fruit trees (Hueck, 1998).

Here we focus on the *P. aeruginosa* TTSS, which is highly homologous to that of *Yersinia* species (Frank, 1997).

4. Type III secretion system of *P. aeruginosa*

4.1 Genetic organization and subcellular function

In *P. aeruginosa*, most TTSS genes are clustered within five continuous operons: *pscNOPQRSTU*, *popNpcr1234DR*, *exsCEBA*, *pcrGVHpopBD* and *exsDpscBCDEFGHIJKL* (Table 1) (Frank, 1997; www.pseudomonas.com). Among these five operons, the operon *exsCEBA* plays a regulatory role (Hovey and Frank, 1995; Dasgupta *et al*., 2004; Rietsch *et al*., 2005; Urbanowski *et al*., 2005), the operon *pcrGVHpopBD* encode type III secretion translocon, and the other three operons encode the TTSA. Genes encoding the TTSS effectors, including *exoS*, *exoT*, *exoU* and *exoY*, are scattered in the chromosome of *P. aeruginosa* among which *exoU* and *exoS* appear to be mutually exclusive in the same strain (Feltman *et al*., 2001).

4.1.1 *pscNOPQRSTU*

Homologs of PscN, protein encoded by *pscN*, are present in all TTSSs described to date and are among the most highly conserved type III secretory proteins (Hueck, 1998). The proteins show similarity to the α- and β-subunits of the F1 component of the bacterial F_0F_1 proton-translocating ATPase (Walker *et al*., 1984) and to the catalytic subunits of eukaryotic as well as archaebacterial ATPases (Woestyn *et al*., 1994).

The subcellular location has not been determined for any of the members of the PscN family, but by homology to the soluble F1 component of F_0F_1 ATPase, it is likely that the PscN ATPases are cytoplasmic proteins. PscN proteins may interact with membrane-bound components of the TTSA to energize secretion or to provide the energy for the assembly of the secretion apparatus, as has been shown for the flagellum biosynthesis homolog FliI (Fan *et al*., 1996).

PscO and PscP, encoded by *pscO* and *pscP*, are homologues to *Yersinia* YscO and YscP,

TABLE 1. Proteins of the TTSS of *P. aeruginosa* and their homologues

Protein	Size[a]	Loc[b]	Yer[c]	Fla[c]	SP[d]	Reference
TTSA						
PscN	440	C?	YscN (89)	FliI	-	Woestyn *et al*., 1994
PscO	158	C?	YscO (57)		-	Bergman *et al*., 1994
PscP	369	Unknown	YscP (41)		-	Bergman *et al*., 1994
PscQ	309	Unknown	YscQ (58)	FliNY	-	Bergman *et al*., 1994
PscR	217	IM?	YscR (90)	FliP	-	Fields *et al*., 1994
PscS	88	Unknown	YscS (88)	FliQ	+	Bergman *et al*., 1994
PscT	262	IM?	YscT (83)	FliR	+	Lewenza *et al*., 2005
PscU	349	IM?	YscU (84)	FlhB	-	Lewenza *et al*., 2005
PopN	288	Secreted	YopN/LcrE (64)		-	Forsberg *et al*., 1991
Pcr1	92	C?	TyeA (69)		-	Forsberg *et al*., 1991
Pcr3	121	Unknown	YscX (64)		-	Yahr *et al*., 1997
Pcr4	109	C?	YscY (60)		-	Yahr *et al*., 1997
PcrD	706	IM	YscV/LcrD (89)	FlhA	-	Plano *et al*., 1991
PcrR	144	Unknown	LcrR (64)		-	Barve and Straley, 1990
PcrG	98	Unknown	LcrG (98)		-	Bergman *et al*., 1991
PcrV	294	Secreted	LcrV (57)		-	Sawa *et al*., 1999
PscC	600	OM	YscC (77)		+	Yahr *et al*., 1996a
PscD	432	C?	YscD (63)	FilG	-	Yahr *et al*., 1996a
PscF	85	Unknown	YscF (81)		-	Quinaud *et al*., 2005
PscH	143	C?	YscH/YopR		-	Yahr *et al*., 1996a
PscI	112	Unknown	YscI (63)		-	Yahr *et al*., 1996a
PscJ	248	Unknown	YscJ (83)	FliF	+	Yahr *et al*., 1996a
PscK	208	C?	YscK (57)		-	Yahr *et al*., 1996a
PscL	214	C?	YscL (75)	FliH	-	Yahr *et al*., 1996a
Translocon						
PopB	390	Secreted	YopB (62)		-	Frithz-Lindsten *et al*., 1998
PopD	295	Secreted	YopD (59)		-	Frithz-Lindsten *et al*., 1998
Regulators						
ExsE	81	Secreted	none		-	Rietsch, *et al*., Urbanowski, *et al*.,2005
ExsB	137	Unknown	VirG (50)		-	Goranson *et al*., 1997
ExsA	278	C	VirF/LcrF (72)		-	Frank *et al*., 1991
ExsD	276	C	none		-	McCaw *et al*., 2002
Effectors						
ExoS	453	Secreted	YopE (45)		-	Yahr *et al*., 1996b
ExoT	457	Secreted	YopE (50)		-	Yahr *et al*., 1996b
ExoY	378	Secreted	none		-	Yahr *et al*., 1998
ExoU	687	Secreted	none		-	Finck-Barbancon *et al*., 1997
Chaperone						
SpcU	137	Unknown	none		-	Finck-Barbancon *et al*., 1998
Orf1	116	Unknown	SycE/YerA (56)		-	Wattiau *et al*., 1993
Pcr2	123	C?	SycN (67)		-	Day and Plano, 1998
PscB	140	C?	YscB (63)		-	Day and Plano, 1998
PcrH	167	Unknown	LcrH/SycD (76)		-	Broms *et al*., 2003; 2006
PscE	67	C	YscE (63)		-	Quinaud *et al*., 2005
PscG	115	C	YscG (69)		-	Quinaud *et al*., 2005
ExsC	145	C?	none		-	Rietsch, *et al*., Urbanowski, *et al*.,2005

a. length of amino acids; b. subcellular localization, C, cytoplasmic, OM, outer membrane, IM, inner membrane; c. Yer, homologue of *Yersinia*; number in parentheses indicate % similarity; Fla, homologue of flagellum; d. signal peptide, predicted by www.pseudomonas.com or by 'SignalP 3.0 Server' from site of http://www.cbs.dtu.dk/services/SignalP/. Different operons or classes are separated by dashed lines.

respectively. These protiens share little or no sequence similarities with other TTSSs genes (Hueck, 1998). Studies showed that YscP acts as a molecular ruler determining the length of needle of TTSA (Journet *et al*., 2003). In combination with YscU, YscP can also regulate the substrate specificity of the TTSS of *Yersinia* (Edqvist *et al*., 2003).

PscQRSTU, encoded by *pscQRSTU*, show remarkably high homology to TTSS genes of other gram-negative bacteria with respect to both individual genes and gene organization (Hueck, 1998). In addition, PscQRSTU show significant sequence similarities to proteins of the flagellar assembly machinery (Table 1) of both gram-negative and gram-positive bacteria. By similarity to the flagellar assembly machinery, all of the respective proteins, including PscN, PscQRSTU, PcrD, PscD, PscJ and PscL (Table 1) are likely to be located in or associated with the inner membrane and may form the cytoplasmic gate (and perhaps a periplasmic extension) of the TTSA. Since the flagellum biosynthesis proteins that are homologous to type III secretion proteins are specifically involved in the flagellum-specific protein export pathway, it is likely that some of the homologous proteins may play similar roles in both systems. For some of the flagellar proteins, their role in assembly of flagellar structural components is known, and several interactions between these proteins have been detected. Therefore, these data may provide hints as to how the respective components of TTSSs might interact (Hueck, 1998).

These findings indicate also that the flagellar assembly machinery and TTSSs evolved from a common ancestor, though little information is available to answer the controversial question, which came first (Saier, 2004)?

4.1.2 *popNpcr1234DR*

Proteins PopN, *Pseudomonas* calcium response (Pcr)1, Pcr2, Pcr3, Pcr4, PcrD and PcrR, encoded by the operon *popNpcr1234DR*, are homologous to *Yersinia* YopN, TyeA, SycN, YscX, YscY, YscV (LcrD) and LcrR respectively (Table 1). *Yersinia* low calcium response (LCR) is characterized at 37°C by either bacterial growth restriction (bacteriostasis) with expression of Yop in low calcium medium or bacterial growth with repression of Yop in medium containing at least 2.5 mM of calcium (Zahorchak and Brubaker, 1982; Brubaker, 2005).

YopN, TyeA and SycN, together with YscB and LcrG, are considered to form a plug of TTSA,

which blocks Yop secretion in the presence of calcium *in vitro* or before contact with a eukaryotic cell *in vivo*, since in same conditions mutational inactivation of any one of the five genes encoding these proteins results in uncontrolled secretion (Forsberg *et al*., 1991; Iriarte *et al*., 1998; Cheng and Schneewind, 2000; Matson and Nilles, 2001; Goss *et al*., 2004; Ferracci *et al*., 2004; 2005).

SycN, together with YscB, functions as a specific chaperone for YopN in Y. *pestis* (Day and Plano, 1998; Jackson *et al*., 1998; Iriarte and Cornelis, 1999).

YscX and YscY are two proteins required for Yop secretion (Iriarte and Cornelis, 1999).

LcrD is essential for the high-level expression and secretion of Yop in low calcium medium (Plano and Straley, 1993).

LcrR is involved in the downregulation of *lcrGVHpopBD* transcription in the presence of calcium and is necessary for *lcrGVHpopBD* expression in the absence of calcium (Barve and Straley, 1990).

4.1.3 *pcrGVHpopBD*

Operon *pcrGVHpopBD*, encodes five proteins, including PcrG, PcrV, PcrH, PopB and PopD, which are homologous to *Yersinia* LcrG, LcrV, LcrH, YopB and YopD, respectively (Frank, 1997).

LcrG/PcrG forms a stable complex with LcrV/PcrV (Skryzpek and Straley, 1993; Nilles *et al*., 1997; DeBord *et al*., 2001; Matson and Nilles, 2001; Nanao *et al*., 2003; Allmond *et al*., 2003). In *Yersinia*, LcrG acts as a negative regulator (plug) that blocks secretion of Yops (Matson and Nilles, 2001).

LcrV/PcrV, located at the tip of the needle of TTSA (Mueller *et al*., 2005), are known to be a protective antigen (Leary *et al*., 1995; Neely *et al*., 2005) and are required for the translocon assembly in eukaryotic cell membranes in conjunction with translocators, YopB-YopD or PopB-PopD (Frithz-Lindsten *et al*., 1998; Sawa *et al*., 1999; Holmstrom *et al*., 2001, Goure *et al*., 2004, 2005).

PcrH, a chaperone, is responsible for the presecretory stabilization and efficient secretion of the PopB and PopD translocators, however, unlike some other translocator-class chaperones (e.g., LcrH of *Yersinia* species, SicA of *Salmonella enterica*, and IpgC of *Shigella* species), PcrH is not involved in the *in vitro* regulation of type III secretion (Broms *et al*., 2003).

4.1.4 *exsCEBA*, see Introduction 6 'regulation of TTSS of *P. aeruginosa*'.

4.1.5 *exsDpscBCDEFGHIJKL*

With few exceptions, the TTSS genes of *P. aeruginosa* and *Yersinia* spp. are highly conserved. One of the exceptions is *exsD*, first gene of the operon *exsDpscB-L*, whose *Yersinia* homologue is the operon *yscA-M*, which lacks of the *exsD* but has additional *yscA* and *yscM*. ExsD, a 32 kDa protein encoded by *exsD*, is unique to *P. aeruginosa* and was identified as the first negative regulator of the *P. aeruginosa* TTSS (McCaw *et al*., 2002). The function of ExsD is described below (see Introduction 6.1.2).

PscB is a protein encoded by *pscB* and is homologous to YscB, which functions as a specific chaperone for YopN in *Y. pestis* (Day and Plano, 1998; Jackson *et al*., 1998).

PscC is encoded by *pscC* and is homologous to YscC, which is the integral outer membrane component of the TTSA of *Yersinia* and belongs to the family of secretins, such as PulD, which is a component of the type II secretion pathway (Fig. 1) (Genin and Boucher, 1994). YscC forms stable ring-like oligomers in the outer membrane, which are thought to function as transport channels of the TTSA (Burghout *et al*., 2004).

PscD, encoded by *pscD*, is considered to be part of the TTSA as its homologue YscD (Plano and Straley, 1995), because a mutation in the *pscD* resulted in a highly attenuated virulence. Based on the observation that a triple-effectors mutant was less attenuated in virulence than the *pscD* mutant, author from the same study concluded that additional *P. aeruginosa* TTSS virulence components remain to be identified (Miyata, *et al*., 2003).

PscE, encoded by *pscE*, and PscG, encoded by *pscG*, are two chaperones for PscF, encoded by *pscF*, which is the main component of the needle of *P. aeruginosa* TTSA (Quinaud, *et al*., 2005). PscE and PscG fulfill roles of preventing premature polymerization of PscF, needle-forming subunit, within the bacterial cytoplasm and maintaining it in a secretion-prone

conformation (Quinaud *et al*., 2005). Needle is essential for secretion and translocation and is the hollow conduit through which effectors travel to reach the target cell (Tamano *et al*., 2000; Jin and He, 2001; Li *et al*., 2002).

PscH is encoded by *pscH* and is homologous to YscH, which is often called as YopR. Though YopR seems not to be implicated in Yop secretion, it is involved in *Yersinia* pathogenesis since the LD50 (lethal dose which causes the death of 50% of test animals) for the mouse of the *yscH* mutant was 10-fold higher than that of the parental strain (Allaoui *et al*., 1995). Lee and Schneewind proposed that together with YopB and YopD, two other proteins that are secreted but not delivered into the cytosol of mammalian cells, YopR may be secreted into the extracellular milieu in order to divert host defenses away from the actual site of infection (Lee and Schneewind, 1999). YopR displays structural similarity with one domain of YopN, a plug of *Yersinia* TTSA (Schubot *et al*., 2005).

PscJ, encoded by *pscJ*, is homologous to YscJ, which is essential for the export of toxins (Michiels *et al*., 1991b; Kim *et al*., 1997, Kang *et al*., 1997; Hauser *et al*., 1998). *pscJ* is one of the few *P. aeruginosa* TTSS genes having an aminoterminal signal peptide (Table 1).

PscL is encoded by *pscL* and is homologous to YscL, which interacts with YscN, the ATPase in *Yersinia* TTSS, and is the regulator of YscN (Blaylock *et al*., 2006). YscL also interacts with YscQ in a complex composed of YscN, YscQ, YscK and YscL (Jackson and Plano, 2000).

4.2 Type III secretion apparatus

TTSA, the secretion apparatus of the TTSS, also called type III secreton or type III injectisome or needle complex, was first identified and biochemically isolated from *S. typhimurium* (Kubori, *et al*., 1998). *Salmonella* TTSA was described as a cylindrically symmetrical object composed of two parts: i) a base, cylinder similar to the flagellar basal body, formed by two pairs of rings (20 and 40 nm in diameter for upper rings and lower rings, respectively) presumed to traverse the inner and outer bacterial membranes and the peptidoglycan; ii) a 7-8-nm-wide and 60-nm-long needle emanating from the base. Rapidly, similar but variant TTSAs of other gram-negative bacteria were visualized under electron microscopy, for instance, the tripartite TTSA of *S. flexneri* having an additional ‘bulb’

between the base and the needle (Blocker *et al*., 1999); the TTSA of EPEC possessing a supplementary sheath-like structure (more than 600 nm) at the tip of the needle (Sekiya *et al*., 2001). Similarity analysis showed that the TTSA is genetically and structurally related to flagellar assembly machinery (Blocker *et al*., 2003) (Fig. 2). It is thought that the TTSA serves as a hollow conduit through which the secreted proteins travel across the two bacterial membranes and the peptidoglycan in one step.

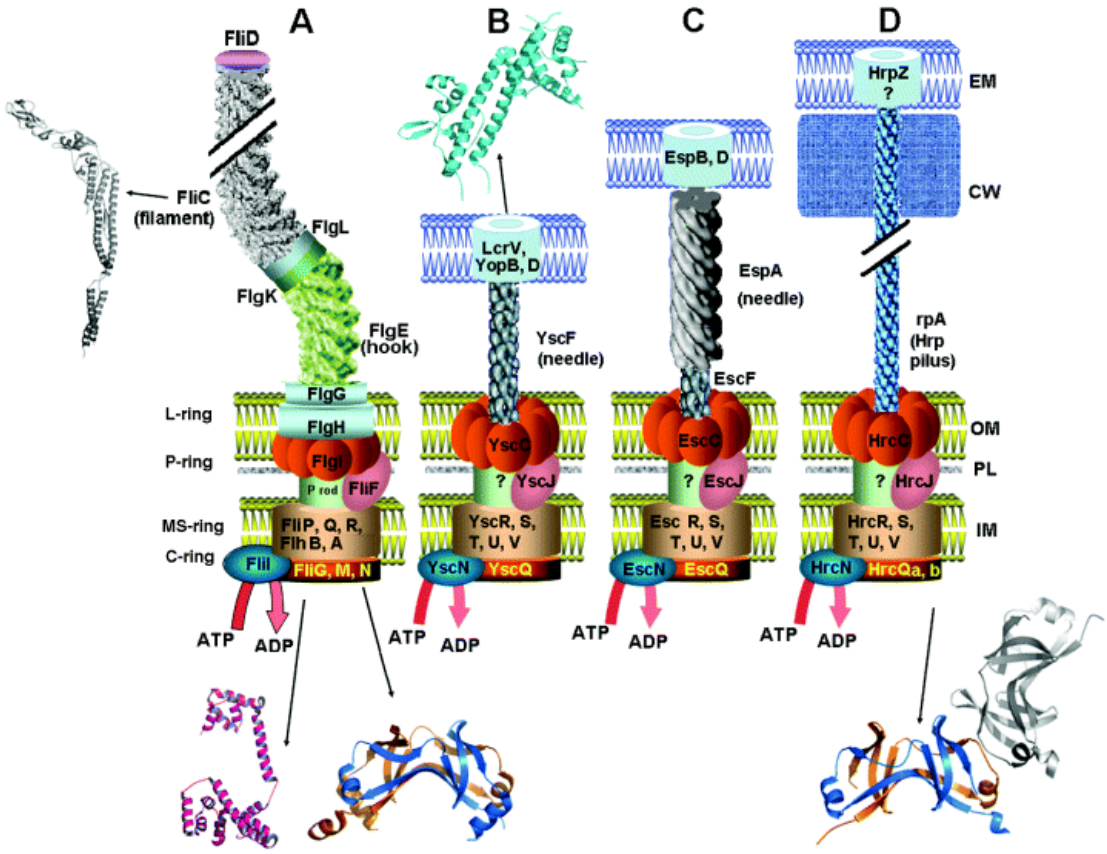


FIG. 2. Schematic representation of the bacterial flagellum (A) and TTSSs in *Yersinia* (B), *E. coli* (C) and *P. syringae* (D). The basal body of flagellum consists of proteins organizing the C-, MS-, P- and L-rings. Only conserved proteins in the TTSSs (and their flagellar homologues) have been drawn and are marked by similar position and colouring. The P rod of flagellum consists of FlgB, FlgC, FlgF and FliE and has no homologous proteins in TTSSs. The question mark in TTSS indicates that a channel structure in the IM has not been identified yet. The major constituent of the needle is YscF in *Yersinia*, EspA in *E. coli*, HrpA in *P. syringae*. Pore-forming proteins are drawn with the same colour in the eukaryotic cell membrane (EM): LcrV, YopB, YopD in *Yersinia*, and EspB, D in *E. coli*. In plant pathogens, putative translocator proteins are HrpZ (*P. syringae*) and HrpF (*X. campestris*). Structurally characterized proteins are shown schematically. Known 3-D structures of FliG, FliN, $HrcQ_B$, LcrV and FliC are shown. OM, outer membrane; PL, peptidoglycan layer; IM, inner membrane of the bacterium; EM, eukaryotic membrane; CW, cell wall of the plant cells. Reprinted from Tampakaki *et al*., 2004.

In *S. typhimurium*, the base and the needle of the TTSA as well as their assembly were visualized under electron cryomicoscopy (Marlovits *et al*., 2004). The base is formed by InvG, PrgJ, PrgH, and PrgK, and the assembly of the base does not require the functional TTSA (Kubori *et al*., 2000, Marlovits *et al*., 2004). These results indicated that the export of the components of the base most likely occurs through the general "*sec*-dependent" secretory pathway. Consistent with this hypothesis, all components of the base structure identified so far exhibit a *sec*-dependent signal sequence (Kubori *et al*., 2000). PrgI, whose assembly requires the base of TTSA, forms the needle. InvJ controls needle length by regulating the assembly of the inner rod (Kubori *et al*., 2000; Marlovits *et al*., 2006).

Salmonella InvG, PrgJ, PrgK, PrgI and InvJ correspond to *P. aeruginosa* PscC, PscI, PscJ, PscF and PscP (Hueck, 1998). PscC and PscJ, possible base of the *P. aeruginosa* TTSA, possess *sec*-dependent signal sequence (Table 1).

PscF has been proved to be the main component of the needle of *P. aeruginosa* TTSA (Quinaud, *et al*., 2005).

4.3 Type III secretion translocon

In addition to the TTSA, direct injection of toxins into the cytoplasm of host cells requires disruption of the cellular membrane at the site of contact by the translocon, a proteinaceous pore, which is presumably formed by proteins that are delivered through the needle of the TTSA (Schoehn *et al*., 2003).

PopB and PopD, 2 translocators secreted by the functional TTSA, are believed to be sufficient to form the *P. aeruginosa* translocon and act in synergy to promote membrane permeabilization (Schoehn *et al*., 2003; Faudry *et al*., 2006). However, some study showed that PcrV is also required for the translocon assembly in eukaryotic cell membranes in conjunction with YopB and YopD (Goure *et al*., 2004; 2005). Thus, the functionality of PcrV in the formation of *P. aeruginosa* translocon is still unclear.

4.4 Type III secretion Effectors

In *P. aeruginosa*, there are four known TTSS secreted effectors, including ExoS, ExoT, ExoY and ExoU. The complement of effectors varies from strain to strain, but, in general, all strains

have *exoT* and about 89% of strains have *exoY*. The presence of *exoS* and that of *exoU* appears to be mutually exclusive (Feltman *et al*., 2001). Strains, such as PA103, a human lung isolate, expressing *exoU* appear to be more acutely cytotoxic and to lyse target cells *in vitro* (Finck-Barbancon *et al*., 1997). The sequenced *P. aeruginosa* strain PAO1 contains *exoS*, *exoT*, and *exoY* but lacks *exoU* (www.pseudomonas.com) (Stover *et al*., 2000). Upon entry into the cytoplasm, effectors disturb the cellular cytoskeleton and initiate inflammatory and apoptotic processes, eventually leading to cell death (Finck-Barbancon *et al*., 1997; Frank, 1997; Yahr *et al*., 1998).

4.4.1 ExoS and ExoT

ExoS and ExoT share76% amino acid identity, and are bifunctional effectors with N-terminal RhoGAP domains and C-terminal ADP-ribosyltransferase (ADPRT) domains. The N termini of ExoS and ExoT also comprise a sequence required for secretion through the TTSA, a chaperone binding region, and a membrane localization domain, which is a hydrophobic region and localizes ExoS and ExoT to intracellular membrane after translocation into host cells through the TTSS (Fig. 3) (Barbieri and Sun, 2004).

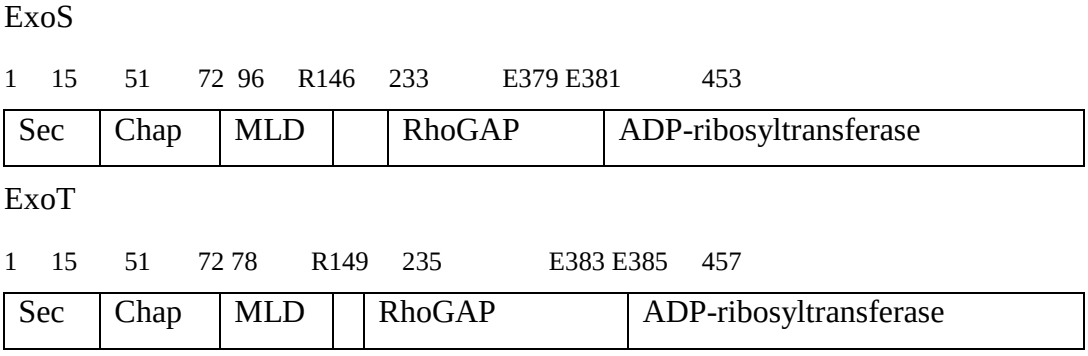


FIG. 3. Functional domains of *P. aeruginosa* ExoS and ExoT. ExoS and ExoT are similar yet distinct. They share 76% amino acid identity and similar functional domain with N-terminal RhoGAP activity and C-terminal ADP-ribosyltransferase activity. The N terminus of ExoS and ExoT are similar, containing sequence required for secretion (Sec), chaperone binding domains (Chap), membrane localization domains (MLD) and RhoGAP domains (RhoGAP). ExoS and ExoT have distinct C-terminal ADP-ribosyltransferase domains. Arginine at position 146 (R146) of ExoS and R149 of ExoT are catalytic residues of the RhoGAP activity. Glutamic acid at position 379 (E379) of ExoS and E383 of ExoT contribute to the transfer of ADP-ribose to target substrates, and E381 of ExoS and E385 of ExoT function as a catalytic residue. Adapted from Barbieri and Sun, 2004.

The GAP domains of either enzyme target small Rho-like GTPases, and can stimulate the reorganization of the actin cytoskeleton by inhibition of Rac and Cdc42, and stimulate actin stress fiber formation by inhibition of Rho (Goehring *et al*., 1999; Pederson *et al*., 1999; Wurtele *et al*., 2001; Krall *et al*., 2000; 2002).

Activation of ADPRT activity needs a soluble eukaryotic protein, FAS (factor activating exoenzyme S) (Coburn *et al*., 1991), which belongs to a highly conserved, widely distributed eukaryotic protein family, collectively designated as 14-3-3 proteins (Fu *et al*., 1993). Though both ExoS and ExoT have ADPRT activity, they ADP-ribosylate different substrates. ExoS has poly-substrate specificity and preferentially targets Ras superfamily proteins that are integral to cell signaling pathways (Iglewski *et al*., 1978; Coburn *et al*., 1989; Coburn and Gill, 1991; Knight *et al*., 1995; McGuffie *et al*., 1998; Radke *et al*., 1999; Fraylick *et al*., 2002a; 2002b; Henriksson *et al*., 2002), whereas ExoT has a more restricted host proteins and specially targets CrkI and CrkII, two host kinases that regulate focal adhesion and phagocytosis (Sun and Barbieri, 2003). ExoS ADPRT activity has effects on cell growth (DNA synthesis), morphology (cell rounding) and cellular adherence (Fraylick *et al*., 2001; Rocha *et al*., 2003), whereas ExoS GAP activity plays an antiphagocytic role (Rocha *et al*., 2003).

4.4.2 ExoY

ExoY, an adenylate cyclase, is homologous to that of *Bordetella pertussis* (CyaA) and *Bacillus anthracis* (EF) (Yahr, *et al*., 1998). Active ExoY results in an elevation of intracellular cAMP and cell-morphology changes. Unlike CyaA and EF, which are secreted by type I and type II secretion pathways respectively, and may exert their effects at sites distal from the site of infection (Gordon *et al*., 1989), ExoY only intoxicates host cells at sites of colonization due to the delivery mechanism of TTSS. An unknown eukaryotic protein, distinct from calmodulin needed for the activity of CyaA and EF, is required for the activation of the adenylate cyclase activity of ExoY (Yahr, *et al*., 1998). Recently, studies showed that ExoY could inhibit *P. aeruginosa* invasion of epithelial cells coinciding with adenylate cyclase-mediated cytoskeleton disruption (Cowell *et al*., 2005). However, ExoY does not seem to be important for infection (Vance *et al*., 2005; Lee *et al*., 2005).

4.4.3 ExoU

ExoU, parallely identified as PepA, is the most cytotoxic (Finck-Barbancon *et al*., 1997; Hauser *et al*., 1998) and possesses lipase activity similar to patatin, human calcium-independent phospholipase A2 (iPLA2) and cytosolic phospholipase A2 (cPLA2) (Sato *et al*., 2003).

ExoU is found in about one-third of clinical isolates, and these ExoU-expressing strains are associated in 90% of cases with severe disease (Hauser *et al*., 2002; Feltman *et al*., 2001). ExoU has been shown to cause fatality in a mouse model of lung infection (Finck-Barbancon *et al*., 1997), and expression of ExoU in *P. aeruginosa* strains lacking it increases virulence in a mouse model of acute pneumonia (Allewelt *et al*., 2000).

Similar to ExoS, ExoT and ExoY (Coburn *et al*., 1991; Fu *et al*., 1993; Yahr *et al*., 1998), ExoU requires an unknown eukaryotic protein cofactor or complex for its phospholipase activity *in vitro* (Sato *et al*., 2005).

4.5 Type III secretion chaperones

Chaperones are proteins that associate transiently with one or several substrates to prevent premature or incorrect intramolecular or intermolecular interactions. In TTSSs, before being secreted, some proteins are stored in the cytoplasm, which is not their final destination, and need to be stabilized, separated from other interaction partners, and maintained in a secretion-competent state with the help of chaperones (Page and Parsot, 2002; Parsot, *et al*., 2003).

Although all type three secretion chaperones do not share sequence similarities, they have common physical characteristics, including i) a low molecular weight (<15 kDa); ii) an acidic pI (<5); iii) a predicted amphipathic a helix near their C-terminal end (Wattiau *et al*., 1994). It is worth pointing out that, genes encoding chaperones usually locate in the vicinity of gene encoding their substrate(s).

Up to date, three classes of type three secretion chaperones have been distinguished based on the function of the substrates: class I chaperones associate with effector(s); class II chaperones associate with translocators; and class III chaperone associate with other components of the TTSS (Page and Parsot, 2002; Parsot, *et al*., 2003; Quinaud *et al*., 2005; Rietsch *et al*., 2005; Urbanowski *et al*., 2005; Yip *et al*., 2005). Recent crystallographic and structural studies have

shed light on the molecular basis of the specific interactions between chaperones and their substrates and the role of these interactions on substrate conformation (Birtalan and Ghosh, 2001; Stebbins and Galan, 2001; Luo *et al*., 2001; Phan *et al*., 2004; Singer *et al*., 2004; van Eerde *et al*., 2004; Buttner *et al*., 2005; Locher *et al*., 2005; Schubot *et al*., 2005; Yip *et al*., 2005 Letzelter *et al*., 2006; Edqvist *et al*., 2006).

Type three secretion chaperones have been suggested to i) act as anti-aggregation and stabilizing factors; ii) be secretion signal and set a secretion hierarchy; iii) function as antifolding factors; iv) participate in the regulation of the TTSS (Page and Parsot, 2002; Parsot, *et al*., 2003; Feldman and Cornelis, 2003).

In *P. aeruginosa*, several chaperones have been described.

4.5.1 Class I chaperones

SpcU

SpcU, specific *P. aeruginosa* chaperone for ExoU, is encoded by *spcU* and is a small 15-kDa acidic protein that possessed a leucine-rich motif associated with the Syc family of cytosolic chaperones for the *Yersinia* Yops. The association of ExoU and SpcU required amino acids 3 to 123 of ExoU. In *P. aeruginosa*, ExoU and SpcU are coordinately expressed as an operon that is controlled at the transcriptional level by ExsA (Finck-Barbancon *et al*., 1998).

Orf1, Pcr2 and YscB

SycE/YerA, SycN and YscB of *Yersinia* spp., homologous to Orf1, Pcr2 and YscB of *P. aeruginosa* respectively (Table 1), are known type three secretion chaperones (Wattiau *et al*., 1993; Day and Plano, 1998) and the structure of SycE has been determined (Birtalan and Ghosh, 2001). However, up to date, no experimental data is available about the function of Orf1, Pcr2 and YscB.

4.5.2 Class II chaperones

PcrH, chaperone of the translocators PopB and PopD, performs a critical function in ensuring the assembly of a functional type III secretion translocon (Broms *et al*., 2003). In the absence of PcrH, *P. aeruginosa* is translocation deficient because of a specific reduction in

presecretory stability and subsequent secretion of PopB and PopD. PcrH exerts its chaperone function by binding directly to PopB and PopD. Recent study showed that tetratricopeptide repeats of PcrH is essential for its activity towards the translocators and subsequently for the translocation of exoenzymes into host cells (Broms *et al*., 2006).

However, unlike the regulatory roles of other class II chaperones, for instance LcrH of *Yersinia* species (Francis *et al*., 2001; Anderson *et al*., 2002), SicA of *Salmonella enterica* (Darwin and Miller, 2001), and IpgC of *Shigella* species (Mavris *et al*., 2002), *in vitro* regulation of *P. aeruginosa* type III secretion does not involve PcrH.

It seems that PcrH is the *bona fide* chaperone, becaue the role of chaperones in the regulation of transcription might not correspond to that of a chaperone that, by definition, does not participate in the final function of its substrate. Thus, it remains to be determined whether or not the class II chaperones act as co-activators or as chaperones of transcriptional activator; that is, whether or not they are associated with activators unpon transcription activation (Parsot, *et al*., 2003).

4.5.3 Class III chaperones

- PscE and PscG, were demonstrated to be chaperones for PscF, which is the main component of the needle of *P. aeruginosa* TTSA (Quinaud, *et al*., 2005). PscE and PscG are absolutely essential for PscF functionality *in vivo* because *P. aeruginosa* PscE and PscG knockout strains both lack PscF and are consequently non-cytotoxic. PscE and PscG trap PscF in a ternary, 1:1:1 complex and fulfill roles of preventing premature polymerization of PscF, needle-forming subunit, within the cytoplasm and maintaining it in a secretion-prone conformation (Quinaud, *et al*., 2005).
- ExsC, has the biochemical characteristics of a type three secretion chaperone, including low molecular mass (16.3 kDa), an acidic isoelectric point (4.6) and a predicted carboxy-terminal amphipathic alpha-helix (Dasgupta *et al*., 2004). Two independent groups showed that ExsC interact with ExsE, a secreted regulator of *P. aeruginosa* TTSS (Rietsch *et al*., 2005; Urbanowski *et al*., 2005). ExsC is required for efficient secretion of ExsE and increase the stability of cytoplasmic ExsE (Urbanowski *et al*., 2005).

4.6 Type III secretion signals

Type three secretion effectors normally produced by one species of bacterium can apparently be secreted by other bacteria with different TTSS, suggesting that all the different TTSSs use a common mechanism for substrate recognition and secretion (Cornelis *et al*., 2003). However, substrates of TTSSs lack a single, defined secretion signal and there is evidence for the existence of three independent secretion signals that direct substrates for secretion through the TTSA (Aldridge and Hughes, 2001).

4.6.1 The 5' mRNA signal

Arguments supporting this hypothesis include, i) frameshift mutations, which completely alter its amino acid profile while largely maintaining its mRNA sequence, introduced into the *yopE* and *yopN* comprising the first 15 codons yield a functional secretion signal (Anderson and Schneewind, 1997). Such tolerance to frameshift mutations of the first 15 codons was observed from other substrates from different organisms though it has limits (Ramamurthi and Schneewind, 2003b; 2005); ii) Codons' synonymous substitutions, which preserve the same amino acid sequence but largely change its mRNA sequence, abrogated the function of *yopQ* minimal secretion signals (Ramamurthi and Schneewind, 2002; 2003a).

To date, we have been unable to discern any commonality in *yop* mRNAs, either at the sequence level or in predicted secondary structures, which could suggest a method of RNA recognition by the secretion machinery.

4.6.2 The N-terminal amino acid signal

Curiously, the same arguments employed in favor of the 5' mRNA signal hypothesis can also be used in support of the amino acid signal hypothesis for type III secretion and include, i) synonymous substitutions at 17 nucleotide positions within codons 2-11 of yopE, resulting in the same amino acid sequence and dramatically changed nucleic acid sequence, produced no secretion defect (Lloyd *et al*., 2001). Similar results have been obtained for the minimal secretion signal of *Salmonella* type III protein InvJ (Russmann *et al*., 2002); ii) frameshift mutations into the first 11 codons of yopE within the context of the full-length *yopE* open reading frame (ORF) results in polypeptides that are poorly secreted (Lloyd *et al*., 2001).

Controversy between mRNA signal and N-terminal amino acid signal seemed to be explainable, as suggested by Ramamurthi and Schneewind (Ramamurthi and Schneewind, 2003b). mRNA signal could be 'bipartite' and composed of a 'minimal secretion signal' (codons 1-10), which does not tolerate frameshift mutation or synonymous substitutions of codons, and a 'suppressor region' (codons 11-15) , which increases the efficiency of secretion and suppresses mutations in the minimal secretion signal. However, proteins with alternating Ser-Ile motif generate amphipathic sequences, which promote type III secretion. These data argued strongly that amino acid sequences at the N-termini of certain Yops constituted a poorly defined, possibly structural motif that functions as an independent secretion signal (Lloyd *et al*., 2002). Computational analysis of 58 known type III substrates revealed that 66% harbor predicted secretion signals (Lloyd *et al*., 2002).

4.6.3 Chaperone signal

Arguments supporting this point of view include, i) in addition to the first secretion signal located in the first 15 amino acids of YopE, a second secretion signal is located downstream between residues 15-100 of YopE and is only recognized by the type III machinery when it is bound to its chaperone, SycE (Cheng *et al*., 1997); ii) chaperone-effector complexes could function as three-dimensional TTSS secretion signals and may endow a temporal secretion hierarchy (Birtalan *et al*., 2002). However, only limited effectors have a chaperone (Feldman and Cornelis, 2003). If chaperones are not essential for recognition of all effectors by the TTSA, why should they be essential for any? It is interesting to note that all known type three secretion translocators, which should be secreted before the effectors, have a respective chaperone. However, no evidence is available for a hierarchized secretion of translocators over effectors, or effectors that have a chaperone over those that do not have any (Parsot *et al*., 2003).

4.6.4 Model for type three secretion signals

Although any one of these signals may be sufficient for secretion, the presence of all three signals (the 5' mRNA signal, the N-terminal amino acid signal and the chaperone signal) may boost the efficiency of protein secretion. Furthermore, the increased dependency on one signal

or another by a substrate may render a particular substrate more or less sensitive to mutagenesis of the RNA or protein sequence (Ramamurthi and Schneewind, 2003b). One hypothesis proposes the presence of three distinct signals that mediate efficient secretion of type III secreted substrates (Karlinsey *et al*., 2000; Aldridge and Hughes, 2001). The first is that some distinct property of *yop* mRNAs mediates the recruitment of translating ribosomes to the TTSA. However, as it has been demonstrated that the mRNA signal must be translated to be functional (Ramamurthi and Schneewind, 2002), the second is that some property of the amino acid sequence allows the nascent polypeptide to engage the secretion apparatus. Finally, the cognate chaperone of the secretion substrate binds to downstream amino acid sequences and prevents the dislocation of the Yop from the secretion channel.

No data about secretion signal in *P. aeruginosa* type III secreted substate is available.

4.7 Posttranslational and/or cotranslational secretion

Corresponding to N-terminal amino acid secretion signal of the TTSS, it has been demonstrated that, some *Shigella* proteins are posttranslationally secreted, meaning these proteins are presynthesized and stored before secretion (Menard *et al*., 1994; Bahrani *et al*., 1997). On the contrary, a 5' mRNA secretion signal favors the cotranslational secretion, meaning that the translation of secreted protein is coupled to its subsequent secretion (Karlinsey *et al*., 2000). Up to date, the only reported case of co-translational secretion is seen in *Shigella* (Page *et al*., 2002).

Unpublished observations in our lab have shown that ExoS/ExoT are presynthesized in the bacterial cytoplasm before secretion.

5. Regulation of TTSSs

5.1 Environment stimuli

Pathogenic bacteria occupy very different infection foci, for instance animal pathogen *Shigella* spp. and *Salmonella* spp. make an intracellular living after successful invasion, whereas *Yersinia* spp., *P. aeruginosa* and EPEC predominantly remain extracellular (Francis *et al*., 2002; Nataro and Kaper, 1998). Therefore, different stimuli could be used to up- or down-regulate the expression of TTSS genes.

5.1.1 Temperature

Most bacteria normally reside in the surrounding environment. However, on contact with an animal host, these bacteria encounter an increase in their growth temperature to 37°C. Temperature increase has long been known to induce TTSS gene expression (Table 2) in *Shigella* spp (Maurelli *et al*., 1984), *Yersinia* spp (Goguen *et al*., 1984; Michiels *et al*., 1990) and EPEC (Rosenshine *et al*., 1996; Umanski *et al*., 2002; Bustamante *et al*., 2001). In *Shigella*, on temperature increase to 37°C, an AraC-like transcriptional activator, VirF, activates VirB which subsequently activates the whole TTSS gene expression (Dorman *et al*., 2001; Beloin *et al*., 2002). As DNA supercoiling is temperature-dependent, only at temperatures below 32°C but not 37°C, H-NS, a nucleiod-associated protein, is able to bind to and repress the promoter activity of VirF (Falconi *et al*., 1998). Similarly, DNA topology has been implicated in the temperature-dependent expression of TTSS genes of *Yersinia* spp., EPEC and enterohemorrhagic *E. coli* (EHEC) (Cornelis *et al*., 1991; Rohde *et al*., 1994; 1999). It is likely that these four pathogens exhibit a comparable physical basis for thermoregulation that probably involves the accessibility for target DNA to H-NS (Francis *et al*., 2002). In addition to temperature, osmolarity, pH and oxygen tension, have been implicated to cause topological changes in local DNA structure. Not surprisingly, these environmental stimuli are all encountered by a pathogen during infection of an animal (Francis *et al*., 2002).

5.1.2 Divalent cations

Divalent cations can function as an extracellular signal to regulate virulence gene expression (Garcia Vescovi *et al*., 1996). In *S. typhimurium*, a two-component system PhoPQ senses the different Mg^{2+} concentrations outside and inside the host cell to inversely regulate two independent TTSSs: pathogenicity island I (SPI-1), which is important in ensuring initial invasion, and SPI-2, which is important for intracellular survival and proliferation (Groisman, 2001; Chamnongpol *et al*., 2003). In *Yersinia* spp. and *P. aeruginosa*, TTSS gene expression *in vitro* is induced in low calcium medium by adding calcium chelator (Goguen *et al*., 1984; Pollack *et al*., 1986; Michiels *et al*., 1990; Yahr and Frank, 1994; Frank, 1997).

TABLE 2. A summary of regulatory characteristics of TTSSs of several animal pathogens

	P. aeruginosa	*Yersinia* spp.	*Shigella* spp.	*Salmonella* spp[b]	EPEC[c]
Environmental stimuli					
Temperature		+	+		+
Divalent cations	Ca^{2+}	Ca^{2+}		Mg^{2+}	Ca^{2+}
Cell contact	+	+	+		+
Serum	+	+	+	+	
Special factors[a]	+	+	+	+	+
Secreted regulator	ExsE	LcrQ (YscM)	OspD1		
AraC-like activator	**ExsA**	**VirF** (**LcrF**)	VirF	HilD, HilC, InvF, RtsA	PerA; GadX
Anti-activator	ExsD, PtrA				
Regulatory chaperone	ExsC	SycH, LcrH, SycN, YscB	IpgC	SicA	
Two-component system	GacS/GacA, RetS, LadS, SadARS	YsrS/YsrR	CpxA/CpxR, OmpR/EnvZ	PhoP/PhoQ, BarA/SirA	EvgS/EvgA
Quorum sensing	RhlI/RhlR			? (AI-2; SdiA)	AI-2; SdiA; QseA
TTSA component		YopN, TyeA, LcrG, YopD			
Others	Vfr, CyaB, RsmA, RpoS, PsrA, TruA, DsbA, HutT, FimL, PtrB, MexCD-OprJ, MexEF-OprN	YmoA, TtsA	**VirB**, IHF, Tgt, MiaA, H-NS, mxiE, Fis,	**HilA**, CsrABC, DsbA, FliZ, FadD, Fis, H-NS, Hha, HU, CpxA, Lon, HilE, PNPase, ppGpp, NpnN	**Ler**, H-NS, IHF, Fis , PerC, BipA, GrlA, GrlR

[a]See text for more details; [b]*Salmonella* spp. pathogenicity island I (SPI-1); [c]enteropathogenic *E. coli*; master transcriptional regulators activating directly the TTSS genes expression are bold and underlined.

5.1.3 Cell contact

The properties of type III-mediated translocation include (i) bacterial and eukaryotic cell contact, (ii) the introduction of translocation proteins into the eukaryotic plasma membrane, and (iii) the formation of a pore or channel through which bacterial effector proteins translocate (Lee, 1997). The *Yersinia* TTSS and effector proteins (Yops) serve as the prototypical model of type III-mediated intoxication (Cornelis and Wolf-Watz, 1997). Bacterial contact with macrophages results in a massive up-regulation of effector *yop* gene expression and subsequent injection of effector Yop substrates through TTSA (Rosqvist *et al*., 1994; Pettersson *et al*., 1996). Elegant studies involving immunoprecipitation,

immunofluorescence, and localization of enzyme activity have shown that the *Yersinia* effectors (YopE, YopH, YopM, and YpkA) are delivered directly into host cells from adherent bacteria (Sory and Cornelis, 1994; Persson *et al*., 1995; Hakansson *et al*., 1996; Boland *et al*., 1996). Host cell contact-dependent activation of the TTSS gene expression is also observed in *Shigella* spp (Menard *et al*., 1994; Watarai *et al*., 1995), EPEC (Rosenshine *et al*., 1996) and *P. aeruginosa* (Vallis *et al*., 1999; Hornef *et al*., 2000).

5.1.4 Serum

Serum is able to activate the TTSS of *Shigella* (Menard *et al*., 1994), *Salmonella* (Zierler and Galan, 1995), *Yersinia* (Lee *et al*., 2001) and *P. aeruginosa* (Vallis *et al*., 1999, Hornef *et al*., 2000).

5.1.5 Other factors

- In *Yersinia* spp., growth in chemically defined media, for example, Dulbecco's minimal Eagle medium, does not lead to type III secretion, even under low-calcium conditions (Lee *et al*., 2001). Two additional signals, **glutamate** and host **serum**, must be provided with the chemically defined media to activate the type III pathway (Lee *et al*., 2001).
- In *Shigella* spp., a group of chemical compounds, including **Congo red**, **Evans blue**, and **direct orange**, are able to induce secretion of Ipa proteins by bacteria suspended in phosphate-buffered saline. In addition, activation of Ipa secretion by Congo red was observed with bacteria harvested throughout the **exponential phase** of growth but not with bacteria in the stationary phase (Bahrani *et al*., 1997). The interaction of bacteria with components of the extracellular matrix, such as **fibronectin**, **laminin** or **collagen type IV**, can stimulate the release of Ipa effector proteins (Watarai *et al*., 1995). **tRNA modification** is also required for the full expression of TTSS genes (Durand *et al*., 2000).
- In *Salemonella* spp., studies showed that, i) **neutral pH**, **low oxygen tension** and **high osmolarity** conditions, favors the maximal expression of TTSS genes (Bajaj *et al*., 1996); ii) rising concentration of **acetate** in the distal ileum (small intestine) provides a signal for TTSS gene expression, where **propionate** and **butyrate**, present in high concentrations in the caecum and colon (large intestine), inhibit the expression of TTSS

genes (Lawhon *et al*., 2002; Gantois *et al*., 2006); iii) **cationic antimicrobial peptides**, a conserved and highly effective component of innate immunity, repress the TTSS genes expression while activate other virulence regulons (Bader, *et al*., 2003); iv) expression of *Salmonella* TTSS genes is repressed in the presence of **bile** (Prouty and Gunn, 2000).

- In EPEC, maximal type III secretion occurs under conditions reminiscent of those in the gastrointestinal tract, including the presence of **sodium bicarbonate**, millimolar concentrations of **calcium** and **$Fe(NO3)_3$** (Kenny *et al*., 1997). The negative regulation of TTSS gene expression was observed in the presence of **ammonium** or in **LB** (Bustamante *et al*., 2001). Otherwise, different **pH** during the passage through the acidic stomach to neutral small intestine determine the need or not of the TTSS gene expression (Shin *et al*., 2001; Francis *et al*., 2002), see part 5.3 'AraC-like transcriptional activator'.

5.2 Secreted regulators

The principle of secreting an inhibitor originates from the flagellar biosynthesis regulation. In this case, expression of the late flagellar biosynthesis genes is repressed by FlgM, an anti-sigma factor that binds to the flagellar biosynthesis-specific sigma factor, sigma 28. Once the basal body and hook of the flagellum have been synthesized, the anti-sigma factor is secreted from the cell by means of the flagellum, freeing sigma 28, which then directs late flagellar gene transcription (Chilcott and Hughes, 2000).

- In *Yersinia* spp., repression of the TTSS gene expression is relieved when LcrQ in *Yersinia pseudotuberculosis*, and two LcrQ homologues, YscM1 and YscM2 in *Yersinia enterocolitic*a, are secreted by the TTSA (Pettersson *et al*., 1996; Stainier *et al*., 1997; Wulff-Strobel *et al*., 2002; Cambronne *et al*., 2000; 2004).
- In *Shigella* spp., the expression of late TTSS effectors, controlled by MxiE and IpgCa, is not initiated until OspD1, anti-activator of MxiE, is secreted by the TTSA (Parsot, *et al*., 2005).
- In *P. aeruginosa*, TTSS gene expression is activated when ExsE, a small inhibitor of TTSS, is secreted through the opened type III secretion channel (Rietsch *et al*., 2005; Urbanowski *et al*., 2005).

The underlying principle in all these systems is the same: a type III secreted negative regulator is at the top of the subsequent regulatory cascade though the details of the regulation appear to differ among the systems.

5.3 AraC-like transcriptional activators

AraC family, is known by virtue of their homology to a 99 amino acid segment of AraC, the first transcriptional activator described. Proteins of AraC family contain two potential α-helix-turn-α-helix DNA binding motifs and regulate diverse bacterial functions, including sugar catabolism, responses to stress, and virulence (Gallegos *et al*., 1997; Martin and Rosner, 2001).

- In *Yersinia*, VirF from *Y. enterocolitica* (Cornelis *et al*., 1987), and LcrF from *Y. pestis* (Yother *et al*., 1986) and *Y. pseudotuberculosis* (Forsberg and Wolf-Watz, 1988), belong to the AraC family. VirF acts as a key transcriptional activator of the expression of TTSS genes, such as *yop* and *yscA-L*, by specially binding to the promoters of these genes (Hueck, 1998). As discussed in part 5.1.1, VirF is only active at 37°C and its expression is modulated by a histone-like protein called *Yersinia* modulator A (YmoA) (Cornelis *et al*., 1991).
- In *S. flexneri*, VirF is an AraC-like positive regulator of the secondary regulatory gene *virB* and the structural gene *icsA*. The product of the *virB* gene in turn activates transcription of the genes coding for the invasion proteins, and for the TTSA which promotes export of the invasion proteins to the bacterial cell surface (Dorman *et al*., 2001).
- In *S. typhimurium*, HilD and HilC, two AraC/XylS family members, activate both indirectly, via HilA, and directly the TTSS gene expression (Schechter *et al*., 1999; Akbar *et al*., 2003). InvF, a third AraC-like regulator encoded by the first gene of the inv-spa gene cluster, is required for the optimal expression of several genes encoding SPI1-secreted proteins (Kaniga *et al*., 1994; Darwin and Miller, 1999; Eichelberg and Galan, 1999). RtsA, a fourth AraC-like regulator, increases expression of the TTSS genes by inducing *hilA* expression (Ellermeier and Slauch, 2003).

- In EPEC, which is a major cause of infantile diarrhea in a number of developing countries and is the prototype of pathogenic bacteria that cause attaching and effacing (A/E) intestinal lesions (Nataro and Kaper, 1998), PerA (Gomez-Duarte and Kaper, 1995; Porter *et al*., 2004) and GadX (Shin *et al*., 2001), two members of the AraC/XylR family of bacterial transcriptional regulators, coordinately regulate the expression of acid tolerance and TTSS genes, because in order to colonize or invade the intestinal epithelium, EPEC must first overcome the hostile acidic environment encountered during passage through the stomach (Francis, *et al*., 2002). In acidic conditions such as in the stomach, GadX (glutamate decarboxylases) activates *gadAB*, genes expressing acid tolerance, but represses PerA (plasmid encoded regulator), activator of Ler (LEE encoded regulator) that is required for the TTSS genes expression (Mellies *et al*., 2006), to ensure survival. After entry into the duodenum, where ingested food contents are deacidified and digested by pancreatic juice, GadX represses *gadAB* and activates PerA to promote a cascade of expression of TTSS genes, which are encoded by a pathogenicity island termed locus of enterocyte effacement (LEE) and are required for the successful colonization of the small intestine.
- In *P. aeruginosa*, AraC-like ExsA is the master activator for TTSS gene expression, see below (6.1.1) for more details.

5.4 Regulatory chaperones

- In *Yersinia* spp., both chaperone LcrH and SycH play a role in the regulation of TTSS genes expression together with their substrate YopD and LcrQ/YscM respectively (Anderson *et al*., 2002; Wulff-Strobel *et al*., 2002; Cambronne *et al*., 2000; 2004).
- In *S. flexneri*, IpgC, chaperone of effectors IpaB and IpaC, binds to MxiE and allows MxiE to bind DNA target sites and activate late TTSS genes when IpgC has been liberated after the secretion of its substrates (Mavris *et al*., 2002; Parsot *et al*., 2005).
- In *S. typhimurium*, the type III secretion chaperone SicA binds and activates InvF, a regulator that is responsible for the transcription of the SPI-1 type III secretion genes in this organism (Darwin and Miller, 2001). This result implied a model whereby SicA is bound to its secretion substrates, SipB and SipC, when *S. typhimurium* are not secreting. When secretion is triggered, SipB and SipC are transported out of the cell, freeing SicA to bind InvF and induce transcription of TTSS genes (Miller, 2002).

- In *P. aeruginosa*, ExsC, chaperone of secreted regulator ExsE, acts as an anti-anti-activator in the regulation of TTSS genes expression, see part 6.1.1 for more details.

5.5 Two-components regulatory system

Two-component system (TCS) is one of the most fundamental and widespread mechanisms of signal perception/transduction in prokaryotes to respond to changes in their surroundings. A canonical TCS consists of a histidine kinase sensor and a cognate response regulator. Signaling through TCS begins with the autophosphorylation of a sensor kinase at a conserved histidine residue, usually in response to an extracellular signal. This phosphate is ultimately the substrate of a phosphotransfer reaction to a conserved aspartate residue in a cognate response regulator domain/protein. Phosphorylation induces a conformational change in the regulatory domain that results in activation of an associated domain that effects the response (Stock *et al*., 2000).

- In *Y. enterocolitica*, YsrS/YsrR (Walker and Miller, 2004), a putative TCS, is required for the NaCl-dependent expression of Ysa TTSS, which is the 2nd *Y. enterocoliticais* TTSS encoded by chromosomal DNA and have only been detected in vitro when cells are cultured at 26°C in a high-NaCl medium (Haller et al., 2000).
- In *S. sonnei*, CpxA/CpxR is involved in regulation of TTSS gene expression through posttranscriptional processing of the regulator VirB, a master regulator of *Shigella* spp. TTSS genes (Mitobe *et al*., 2005). OmpR/EnvZ, another TCS, appears to modulate TTSS genes expression according to environmental conditions (Bernardini *et al*., 1990).
- In *S. typhimurium*, PhoP/PhoQ (Pegues *et al*., 1995), represses the synthesis of *prgHIJK* (PhoP-repressed gene) transcript, which, together with InvG, is required for the assembly of TTSA (Kubori *et al*., 2000, Marlovits *et al*., 2004). In fact, PhoP/PhoQ control *prgHIJK* expression indirectly by regulation of the expression of HilA, which is the master regulator of TTSS expression in *Salmonella* spp. (Bajaj *et al*., 1995; 1996; Eichelberg and Galan, 1999; Lostroh and Lee, 2001). BarA/SirA, another two-component sensor kinase and a response regulator, activates TTSS genes expression via *hilA* and *hilC* (Teplitski *et al*., 2003).

- In EPEC, upon activation of the EvgS/EvgA two-component system, EvgA (the response regulator) activates both *ydeP* and *ydeO* expression and that YdeP and YdeO act conjointly, directly or indirectly repressing the expression of TTSS genes (Nadler *et al*., 2006).
- In *P. aeruginosa*, several TCS play a role in the regulation of TTSS gene expression, see below (6.2.1) for more details.

5.6 TTSA components

- TTSA plug

  In *Yersinia.* spp., YopN, TyeA and SycN, together with YscB and LcrG, are considered to form a plug of TTSA, which blocks Yop secretion in the presence of calcium *in vitro* or before contact with a eukaryotic cell *in vivo*, since in same conditions mutational inactivation of any one of the five genes encoding these proteins results in uncontrolled secretion (Forsberg *et al*., 1991; Iriarte *et al*., 1998; Cheng and Schneewind, 2000; Matson and Nilles, 2001; Goss *et al*., 2004; Ferracci *et al*., 2004; 2005).
- Translocator

  In *Y. enterocolitica*, When YopD is associated with its cognate chaperone LcrH, it represses expression of type III secretion-associated mRNAs (Anderson *et al*., 2002). Secretion of YopD relieves this repression. Accordingly, a *yopD* deletion mutant results in constitutive expression in this system, even in the context of a mutation that inactivates the secretion apparatus (Williams and Straley, 1998).
- In *P. aeruginosa*, a negative regulatory loop has been postulated based on the observation that mutants in components of the secretion apparatus (*pscN*, *pscC* or *pcrD*) fail to accumulate type III-related exoproducts in the cytoplasm (Yahr *et al*., 1996a; Vallis *et al*., 1999; Hornef *et al*., 2000).

In these cases, the alternation of TTSS genes expression is probably due to structural defect of TTSA, so it might not be appropriate to call these TTSA components as regulators.

5.7 Quorum sensing

Quorum sensing (QS), cell-to-cell communication in bacteria, involves producing, releasing,

detecting, and responding to small hormone-like molecules termed autoinducers allowing bacteria to monitor the environment and to alter behavior in a population density-dependent manner in response to changes in the number and/or species present in a community (Waters and Bassler, 2005).

- In *Y. enterocolitica*, production of the Yop proteins in the wild type and in a QS mutant is indistinguishable (Throup *et al*., 1995).
- In *S. flexneri*, maximal expression of TTSS gene and maximal activity of TTSA occur at high cell density. However, autoinducer 2 (AI-2), a QS signaling molecule active in late log phase of *S. flexneri*, does not influence TTSS gene expression (Day and Maurelli, 2001).
- In EPEC, QS controls TTSS genes transcription and protein secretion (Sperandio *et al*., 1999). Such known regulators include AI-2 (Sperandio *et al*., 1999), SdiA (Kanamaru *et al*., 2000), an *E. coli* homologue of QS regulators, and QseA, quorum-sensing *E. coli* regulator A (Sperandio *et al*., 2002). QseA shares homology with several hypothetical *P. aeruginosa* proteins and PtxR, which positively regulates exotoxin A (Hamood *et al*., 1996).
- In *P. aeruginosa*, QS has an important impact on the TTSS expression, see part 6.2.3 for more details.

Taken together, expression of TTSS genes is controlled by multicomponent regulatory networks which integrate a diverse set of environmental cues, probably to restrict the energy-consuming expression of 20 or more proteins to the correct place and time only when they are really needed (Hueck, 1998). Additional regulatory factors implicated in the expression of TTSSs of several animal pathogens are listed in Table 2.

6. Regulation of TTSS of *P. aeruginosa*

The TTSS of *P. aeruginosa* is induced by at least three environmental signals (Yahr and Frank, 1994; Frank, 1997; Vallis *et al*., 1999, Hornef *et al*., 2000): i) *in vivo* contact with eukaryotic host cells; ii) *in vitro* removal of calcium from medium; iii) the presence of serum (due to the existance of type III secretion factors, which were recently identified as albumin and casein (Kim *et al*., 2005). However, a subset of *P. aeruginosa* TTSA component, encoded

by the operon *exsDPscB-L*, and transcriptional activator ExsA, are constitutively expressed, indicating the existence of an expression hierarchy similar to the ordered assembly of the highly related flagellar structure (Wolfgang *et al*., 2003). Such scenario may facilitate the subsequent assembly and localization of additional TTSA components for rapid secretion of presynthesized or cotranslational proteins under TTSS-inducing conditions.

TTSS of *P. aeruginosa* is tightly transcriptionally and posttranscriptionally regulated.

6.1 ExsA-dependent regulation

6.1.1 Regulatory operon *exsCEBA*

- ExsA, a member of the AraC family of transcriptional regulators, exhibits 56% overall amino acid identity to the *Yersinia* VirF/LcrF proteins (Frank, 1997). ExsA functions as a central transcriptional regulator of TTSS because it is required for the expression of all TTSS genes, including itself, by binding to the consensus sequence TXAAAAXA, which is located approximately 51 or 52 bp upstream of the transcriptional start site of four operons (*pscN-U*, *popNpcr1234DR*, *pcrGVHpopBD*, *exsDpscB-L*) encoding the TTSA and translocation machinery, the operon *exsCEBA*, and the effector proteins (Hovey and Frank, 1995). ExsA is constitutively expressed (Wolfgang *et al*., 2003), but little information is available about this control.
- ExsC, a type three secretion chaperone of ExsE, functions as an anti-anti-activator due to the fact that ExsC binds to and sequesters the anti-activator, ExsD, under TTSS-inducing conditions, thus freeing ExsA to activate the expression of TTSS genes (Dasgupta *et al*., 2004).
- ExsE, identified by two independent groups, is a secreted regulator of TTSS in *P. aeruginosa* (Rietsch *et al*., 2005; Urbanowski *et al*., 2005). When the secretion channel is closed, ExsE is complexed with its chaperone, ExsC, in the cytoplasm, and transcription of the TTSS is repressed by sequestration of ExsA by ExsD. On the contrary, when the secretion channel is opened, ExsE is secreted, leaving ExsC free to interact with ExsD and releasing ExsA, thereby allowing liberated ExsA to activate transcription of the TTSS genes (Fig. 4).

- *exsB*, 3rd gene of the operon *exsCEBA*, is not translated. Untranslated *exsB* region mediates either the stability or the translation of *exsA* (Goranson *et al*., 1997).

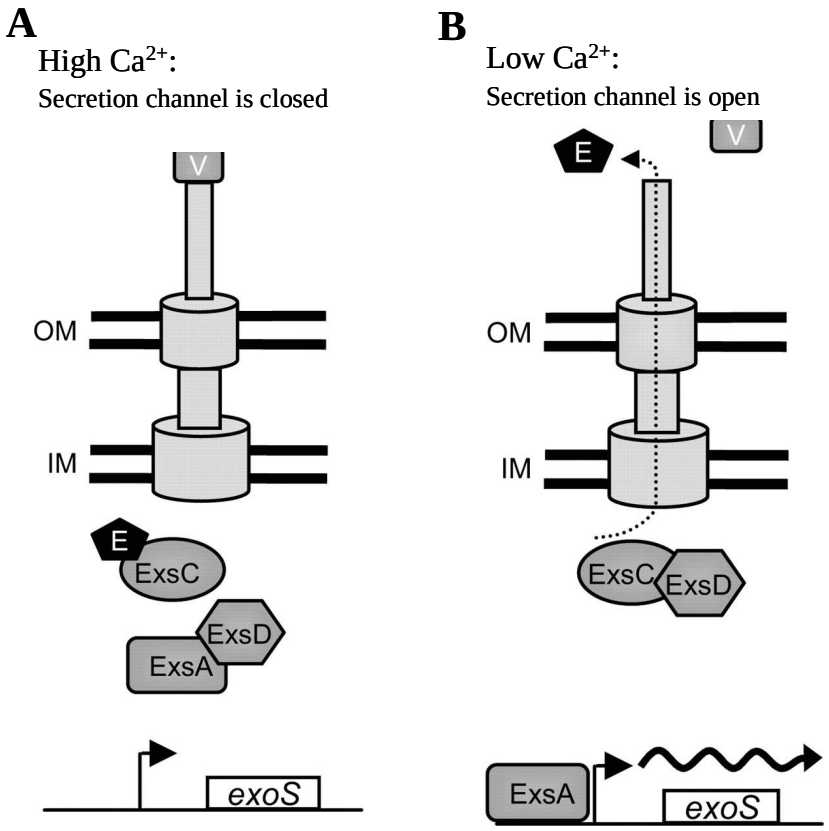


FIG. 4. A model for the coupling of transcription with type III secretory activity in *P. aeruginosa*. All proteins except ExsE (E) and PcrV (V) are indicated by name. The type III secretion apparatus is depicted spanning the inner membrane (IM) and outer membrane (OM). Expression of *exoS* under inducing condition is indicated by a wavy arrow. Reprinted from Rietsch *et al*., 2005.

6.1.2 ExsD

ExsD was identified as the first negative regulator of *P. aeruginosa* TTSS (McCaw *et al*., 2002). Bacterial two-hybrid data indicated that ExsD interacts with the ExsA and functions as an antiactivator to regulate the expression of TTSS genes. ExsD is essential to prevent inappropriate expression of TTSS genes in conditions such as in the absence of the appropriate environmental cue for secretion or in mutants lacking a functional TTSA.

6.1.3 PtrA

PtrA, *Pseudomonas* type III repressor A, can repress TTSS genes expression by inhibition of the ExsA function through a direct binding. The expression of PtrA is highly and specifically

induced by high copper signal, for instance during the infection of mouse burn wound, through a CopR-CopS two-component regulatory system to shut down energy-expensive TTSS (Ha *et al*., 2004).

6.2 ExsA-independent regulation

6.2.1 Two-components regulatory system

- **RetS**, regulator of exopolysaccharide and type III secretion (Goodman *et al*., 2004; Zolfaghar *et al*., 2005), and RtsM, regulator of type III secretion (Laskowski *et al*., 2004), are the same protein identified independently by different groups. RetS, an unusual hybrid two-component signaling protein with a sensor kinase domain followed by two response regulator receiver domains in tandem, is required for expression of TTSS genes and for repression of genes responsible for exopolysaccharide components of the *P. aeruginosa* biofilm matrix. Recent study also showed that, an intact phosphoacceptor site within receiver domain 2, but not receiver domain 1, is absolutely required for RetS activity, and periplasmic domain and phosphorylation of receiver domain 1 may inhibit RetS activation. Furthermore, RetS may serve as a substrate for another sensor kinase (Laskowski and Kazmierczak, 2006).
- **LadS**, another hybrid sensor kinase, and **GacS/GacA**, a canonical TCS, together with RetS, are all involved in reciprocal regulation of TTSS genes expression and exopolysaccharide synthesis (Ventre *et al*., 2006). Signals transduced by these three sensor kinase proteins are hypothesized to ultimately control the levels of a small regulatory RNA, RsmZ, which in turn regulates the reciprocal expression of genes involved in TTSS and biofilm formation by titrating the amount of free, or active RsmA in the cell (Goodman *et al*., 2004; Ventre *et al*., 2006; Burrowes *et al*., 2006). RsmA is a global regulator, which appears to control gene expression at the posttranscriptional level and positively regulates TTSS gene expression (see part 6.2.4) (Heurlier *et al*., 2004; Burrowes *et al*., 2005; Mulcahy *et al*., 2006). Such a system would allow for *P. aeruginosa* to switch between patterns of gene expression that characterize acute infection versus chronic colonization; however, both the signals that are sensed by this system as well as the biochemical relationships between these various regulators remain

uncharacterized. It is possible that RetS and LadS may respond to carbohydrates of host or bacterial origin, because the periplasmic domain of both proteins belongs to a class of bacterial periplasmic sensor modules, 7TMR-DISMED2, which have been identified in a variety of carbohydrates binding proteins (Ventre *et al*., 2006).

- **SadARS**, a three-component regulatory system, is comprised of a putative sensor histidine kinase, SadS, and two response regulators, SadA and SadR. SadARS may either promote or repress TTSS genes expression under different circumstances (Kuchma *et al*., 2005).
- **CopR-CopS**, a canonical TCS, can sense the copper stress during the infection of mouse burn wound and induce the expression of PtrA to shut down energy-expensive TTSS genes expression in such special conditions (Ha *et al*., 2004).

Screening the *P. aeruginosa* genome has led to the identification of 64 response regulators and 63 histidine kinases, as well as 16 atypical kinases (Rodrigue *et al*., 2000). However, the functions of the majority of these regulatory proteins have not been established yet.

6.2.2 Metabolic factors

- Mutants lacking pyruvate dehydrogenase (*aceA* or *aceB*), or a glucose transport regulator (*gltR*) fail to induce the TTSS expression (Dacheux *et al*., 2002, Wolfgang *et al*., 2003).
- In the presence of histidine in the medium, excessive uptake and degradation of histidine due to the overexpression of histidine utilization genes, for instance *hutT*, abolishes the ability to induce *exoS* expression and rends the bacteria non-cytotoxic (Rietsch *et al*., 2004). The cytotoxicity defect can be partially suppressed by an insertion in *cbrA*, gene that encodes the sensor kinase of CbrAB two-component regulatory system involved in sensing and responding to a carbon-nitrogen imbalance.
- A dose-dependent upregulation of TTSS gene expression is seen with increasing NaCl concentrations (Hornef *et al*., 2000). Increasing the concentration of salt in the medium correlates with an increase in the percentage of cells that are able to induce *exoS* expression. It seems that *exoS* expression is in fact controlled by osmolarity because salt could be replaced by sucrose in these experiments. Furthermore, one metabolite controlling the TTSS expression might be derived from acetyl-CoA (Rietsch and

Mekalanos, 2006). Though induction of *exoS* expression by removal of calcium does not work in minimal glucose media, addition of caseamino acids but not just glutamate is able to totally restore its induction (Rietsch and Mekalanos, 2006).

6.2.3 Quorum sensing

QS is crucial in the pathogenesis of *P. aeruginosa* infections and controls virulence factor gene expression in the lungs of CF patients (Juhas *et al*., 2005; Erickson *et al*., 2002). The two intimately linked QS systems in *P. aeruginosa* involve an interaction between a small diffusible molecule, an acylhomoserine lactone (AHL), and a transcriptional activator. The *las* system (Gambello and Iglewski, 1991), consists of the transcriptional activator LasR and the AHL synthase LasI, which directs the synthesis of N-3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL); the *rhl* system (Latifi *et al*., 1995), consists of the transcriptional regulator RhlR and the AHL synthase RhlI, which directs the synthesis of N-butanoyl-homoserine lactone (C4-HSL). The third QS system in *P. aeruginosa* involves a non-AHL-related signal, 2-heptyl-3-hydroxy-4-quinolone, *P. aeruginosa* quinolone signal (PQS) (Pesci *et al*., 1999). PQS adds a further level of complexity to the QS network and provides a link between the *las* and *rhl* systems. QS systems in *P. aeruginosa* are hierarchically arranged with the *las* system being on top of the signalling cascade and have been shown to activate the expression of a wide variety of virulence genes (Juhas *et al*., 2005).

- Expression of TTSS genes, except operon *exsCEBA*, is negatively regulated by RhlI/RhlR, but not LasI/LasR QS system (Hogardt *et al*., 2004; Bleves *et al*., 2005) as well as by the stationary phase sigma factor RpoS (Hogardt *et al*., 2004). Although the mechanism is unknown, it has been proposed that TTSS would not be necessary for bacteria embedded in a biofilm during chronic infection, as most bacterial cells will not have any contact with the host cells.
- Analysis of 5 QS-deficient clinical isolates producing either no or very low level of AHL signals showed that, only one isolate produced normal TTSS effector proteins ExoS and ExoT, two isolates produced ExoT only, and two isolates produced no effector proteins (Schaber *et al*., 2004).

6.2.4 Others

- RsmA, regulator of secondary metabolites, is a small RNA-binding protein and bind to, and affect the translation rate of target mRNAs, a function that is further modulated by one or more, small, untranslated competitive regulatory RNAs (Heeb *et al*., 2006). RsmA plays an important role in the posttranscriptional regulation of a number of virulence-related genes in *P. aeruginosa* (Pessi *et al*., 2001; Heurlier *et al*., 2004; Burrowes *et al*., 2005) and positively regulates TTSS genes expression by controlling other TTSS regulators (Mulcahy *et al*., 2006), because *rsmA* mutant results in decreased expression of five positive regulators, including RetS, ExsA, ExsC, CyaB and Vfr, and one negative regulator, ExsD (Mulcahy *et al*., 2006), and increased production of QS signal C4-HSL (Pessi *et al*., 2001) and multidrug resistance efflux pump MexEF-OprN (Burrowes *et al*., 2006). All these effects, except that on ExsD, make RsmA a positive regulator of the expression of TTSS genes.

  RsmA is highly homologous (85% identity) to *Salmonella* CsrA, which has been shown to be a positive regulator of invasion genes and components of the TTSS (Lawhon *et al*., 2003).
- A membrane-associated adenylcyclase (CyaB), responsible for cAMP synthesis, and a cAMP-binding CRP homologue called Vfr are shown to be required for the expression of TTSS genes (Wolfgang *et al*., 2003; Smith *et al*., 2004; Lory *et al*., 2004). Modulation of intracellular cAMP seems to be an important mechanism controlling TTSS expression.
- *truA*, gene encoding pseudouridinase enzyme, is required for the expression of type III secretory genes. Pseudouridination of tRNAs is proposed to be critical for the translation of TTSS genes or their regulators (Ahn *et al*., 2004).
- *dsbA*, gene encoding a periplasmic thiol/disulphide oxidoreductase affects expression of multiple virulence factors, including TTSS genes. This is probably a non-specific effect resulting from abnormal protein folding caused by the lack of disulphide bonds (Ha *et al*., 2003).
- FimL, homologous to the N-proximal domain of the complex chemosensory protein ChpA, regulates TTSS genes expression by intersecting with Vfr-modulated pathways (Whitchurch *et al*., 2005).

- Overexpression of the MexCD-OprJ and MexEF-OprN, multidrug resistance efflux pumps, is associated with a reduction of TTSS genes expression. Since these pumps can extrude a wide range of compounds belonging to different structural family, it is possible that intracellular signal needed for the activation of TTSS genes expression is extruded (Linares *et al*., 2005).

The regulation of TTSS expression in *P. aeruginosa* is briefly summarized in Fig. 5.

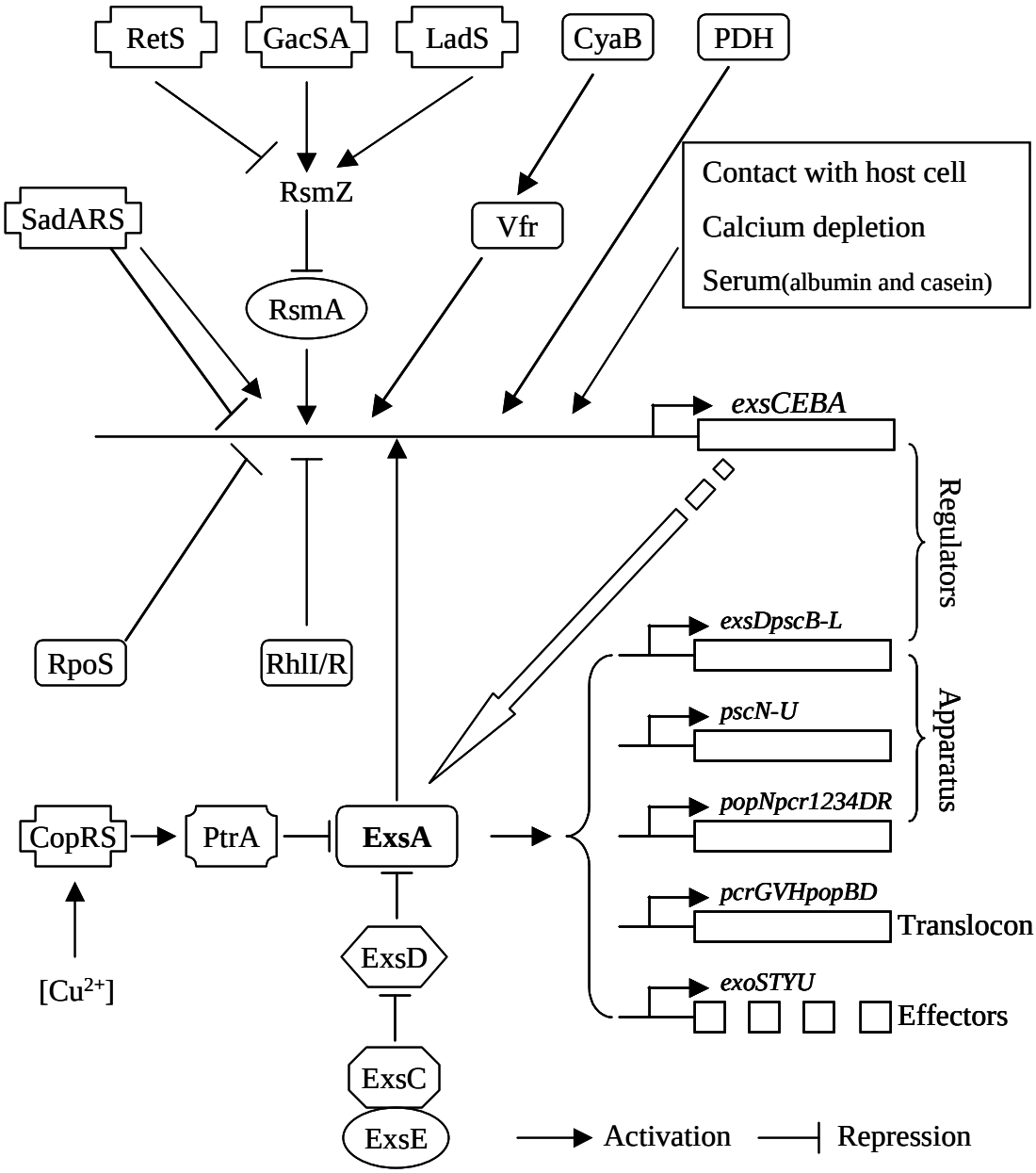


FIG. 5. Regulation of TTSS expression in *P. aeruginosa*. The expression of the operons encoding TTSS regulators, apparatus, translocon and effectors is under the control of the positive transcriptional regulator ExsA. Other main factors implicated in the regulation of TTSS expression are indicated.

INTRODUCTION

(Résumée en français)

1. *P. aeruginosa* et la mucoviscidose

P. aeruginosa, connue depuis longtemps sous le nom de *Bacillus pyocyaneus* ou agent du pus bleu des plaies surinfectée, est un bacille à Gram négatif se développant dans une large gamme d'environnements différents. *P. aeruginosa* est un pathogène opportuniste capable d'infecter un large spectre d'hôtes: plante, animaux et hommes. Chez l'homme, cette bactérie affecte particulièrement les personnes immunodéprimées, les grands brûlés et est la cause principale de morbidité et mortalité des patients atteints de mucoviscidose.

La mucoviscidose, maladie héréditaire autosomique récessive, a pour origine la mutation d'une protéine membranaire appelé cystic fibrosis transmembrane conductance regulator (CFTR) (Rommens, *et al*., 1989; Riordan *et al*., 1989; Kerem *et al*., 1989), qui joue un rôle dans la régulation des échanges ioniques des cellules avec le milieu extérieur. Sur le plan pulmonaire, le disfonctionnement de cette protéine provoque un changement de la composition ionique et une diminution de l'hydratation du mucus bronchique. Ce mucus plus visqueux perturbe le phénomène de clairance ciliaire et constitue un terrain plus propice à la colonisation bactérienne.

Les patients atteints de mucoviscidose développent une infection pulmonaire chronique conduisant à une destruction des tissus pulmonaire par les toxines bactériennes et le relargage du contenu des granules des cellules phagocytaires. Un modèle de pathogénicité suggère deux phases d'infection : l'invasion puis la persistance. Lors de l'invasion initiale du poumon, *P. aeruginosa* exprime une grande quantité de facteurs de virulence neutralisant les défenses immunitaires innées de l'hôte. En suite, durant la seconde phase, une fois la zone colonisée, la bactérie adhèrerait à l'épithélium et adopterait un phénotype mucoïde. Elle se développe alors sous forme de micro-colonies entourées d'un biofilm composé notamment d'un oligosaccharide et l'alginate. Sous cette forme, *P. aeruginosa* est plus résistante face aux défenses de l'hôte et aux antibiotiques. La colonisation de la totalité des poumons se ferait ensuite de proche en proche par essaimage d'individus du biofilm et réactivation du cycle d'invasion-persistance avec expression de facteurs de virulence.

2. Les facteurs de virulence de *P. aeruginosa*

P. aeruginosa possède et utilise un vaste arsenal de facteurs de virulence agissant à différents niveaux au cours de l'infection lui permettant de survivre de nombreux environnements et de coloniser divers types d'hôtes. Le système de sécrétion de type III (SSTT) est un des facteurs de *P. aeruginosa*, il fera l'objet d'un paragraphe séparé.

Le lipopolysaccharide (LPS), couche externe de la membrane, est impliqué dans les interactions directes avec les hôtes ainsi que dans la stimulation de la réponse inflammatoire. Le LPS est composé de trois parties, i) l'antigène-O ou polysaccharide-O, ii) le coeur oligosaccharidique, et iii) la région du lipide A. L'antigène-O, partie la plus distale, est la plus variable et peut même être absent. La présence de l'antigène-O attaché au coeur oligosaccharidique et au lipide A donne le phénotype lisse du LPS, alors que la perte de l'antigène-O aboutit au phénotype rugueux. Selon la présence ou non l'antigène-O, les souches de *P. aeruginosa* sont plus ou moins résistantes à la phagocytose (Pier, *et al*., 1996).

L'exotoxin A, la protéine la plus cytotoxique produite par *P. aeruginosa*, est sécrété sous forme d'une pro-toxine inactive et ensuite internalisée dans la cellule cible par le récepteur à l'α_2-macroglobuline (Kounnas *et al*., 1992). Cette interaction produit le clivage de la pro-toxine. La partie active se fixe et ADP-ribosyle le facteur d'élongation E2 aboutissant à l'inhibition de la synthèse protéique eucaryote et à la mort des cellules cibles (Iglewski and Kabat, 1975; Perentesis *et al*., 1992).

L'alginate, des polymères linéaires de haut poids moléculaire constitués d'acide D-mannuronique et d'acide L-guluronique, est un exopolysaccharide jouant un rôle majeur dans le changement de phénotype de *P. aeruginosa* au cours de l'infection. La surproduction d'alginate rend *P. aeruginosa* mucoïde et lui confère une résistance aux réponses immunitaires de l'hôte ainsi qu'aux antibiotiques (lyczak *et al*., 2002).

Les phospholipases, enzymes extracellulaires, sont capables d'hydrolyser des glycero-phospholipides et ainsi de déstabiliser la membrane cellulaire (Salyers and Witt, 1994). *P. aeruginosa* produit trois phospholipase C, qui peuvent hydrolyser la phosphatidylcholine, très abondante dans le surfactant pulmonaire (Krieg *et al*., 1988 ; Ostroff *et al*., 1990 ; Barker *et al*., 2004). Au moins une phospholipase D et une phospholipase A cytosolique sont aussi produites par *P. aeruginosa* (Wilderman *et al*., 2001a ; Kirschnek and Gulbins, 2006).

Les rhamnolipides, biosurfactants glycolipidiques, peuvent inhiber la fonction ciliaire de l'épithélium respiratoire humain et se trouver dans les crachats des patients atteints de mucoviscidose (Hastie *et al*., 1986; Kownatzki *et al*., 1987). Cependant, le rôle exact des rhamnolipides dans les infections aiguës ou chroniques reste obscur.

Les protéases, qui agissent sur un grand nombre de protéines dont l'élastine, un composant majeur du tissu pulmonaire, sont impliquées dans l'invasion tissulaire (Cowell *et al*., 2003). *P. aeruginosa* peut produire quatre protéases majeures, y compris LasB (Bever and Iglewski, 1988), lasA (Olson JC and Ohman DE, 1992), AprA (Okuda *et al*., 1990) et protéase IV (Wilderman *et al*., 2001b). Il semble que l'ensemble de ces 4 protéases harmonise l'invasion tissulaire (Matsumoto *et al*., 2004).

Les pili, situées sur la membrane externe, sont des structures filamenteuses et sont importants pour l'adhésion aux cellules cibles (Woods *et al*., 1980). Les pili interviennent aussi dans une mobilité de type 'twiching' (déplacement sur des surfaces) et dans le développement du biofilm (O'Toole and Kolter, 1998; Mattick *et al*., 2002).

Le flagelle, organelle de mobilité, est constituée de trois principales parties, le filament, le crochet et le corps basal (Fig. 2). Des travaux sur modèle murin montrent clairement qu'un mutant dépourvu de flagelle est atténué dans sa virulence (Drake and Montie, 1988). Le flagelle est aussi impliqué dans le développement du biofilm (O'Toole and Kolter, 1998 ; Mattick *et al*., 2002).

3. Les différents systèmes de sécrétion bactériens

La sécrétion des facteurs de virulence est réalisée grâce à différents mécanismes qui permettent le transport actif de protéines à travers les membranes bactériennes. On pourrait diviser les différents systèmes de sécrétion en deux groupes selon l'utilisation ou non du système *sec*, qui transporte des protéines contenant une séquence N-terminale clivable du cytoplasme vers l'espace périplasmique (Economou, 1999).

3.1 La sécrétion *sec*-dépendante

Le système de sécrétion de type II, la mieux connue des voies de sécrétion, s'effectue en deux étapes. La première étape consiste en un transport des protéines du cytoplasme vers le

périplasme par le système *sec*. La deuxième étape correspond à l'exportation vers le milieu extérieur à travers la membrane externe par une voie différente de la première. L'exemple le plus étudié est la sécrétion de pullulanase par *Klebsiella oxytoca* (Fig. 1). Chez *P. aeruginosa*, le système de sécrétion de type II, également appelé Xcp, est impliqué dans la sécrétion de nombreuses enzymes et toxines, telles que l'élastase, l'exotoxine A, la lipase, les phospholipases ou la phosphatase alcaline (Filloux *et al*., 1990; 1998).

L'autotransporteur, lui-même contenant non seulement la séquence N-terminale clivable mais aussi l'information pour le passage à travers la membrane externe, peut passer la membrane externe par lui-même (Kostakioti *et al*., 2005) (Fig. 1). Chez *P. aeruginosa*, l'estérase EstA a été identifiée comme autotransporteur (Wilhelm *et al*., 1999) et deux autres homologues ont été proposés (Ma *et al*., 2003).

Le système de sécrétion chaperonne/usher, dédié à l'assemblage et la sécrétion de pili, réalise son rôle en harmonisant la fonction de 2 composants: une chaperonne et une protéine membranaire, 'usher'. L'exemple le plus étudié est l'assemblage les pili de type I par *E. coli* (Fig. 1). Chez *P. aeruginosa*, ce système, appelé Cup, est impliqué dans la formation du biofilm à la phase initiale (Vallet *et al*., 2001).

3.2 La sécrétion *sec*-indépendante

Système de sécrétion de type I, aussi appelé ABC-transporteur, permet le transport de protéines directement du cytoplasme vers le milieu extérieur (Binet *et al*., 1997). Ce système est constitué 3 complexes protéiques: une protéine de transport avec une activité ATPase dans la membrane interne, une protéine formant un pore dans la membrane externe, et une protéine liant les deux premières (Fig. 1). Chez *P. aeruginosa*, la protéase alcaline est sécrétée par le système de sécrétion de type I (Guzzo *et al*., 1991).

Système de sécrétion de type IV, absent chez *P. aeruginosa*, est capable de transporter une grande variété de substrats, y compris protéines, DNA et de complexes nucléoprotéiques. L'exemple le plus étudié est la sécrétion de fragment T du plasmide Ti (T-DNA) d'*Agrobacterium tumefaciens* (Cascales and Christie, 2003) (Fig. 1).

Système de sécrétion de type III, par sa composition et son fonctionnement, est le plus complexe des systèmes de sécrétion bactériens connus et est présent dans de nombreux genres

de bacilles à Gram négatif dont *Yersinia*, *Salmonella*, *Shigella*, les *Escherichia* entéropathogènes, *etc* (Hueck, 1998). La sécrétion de type III est indépendante du système *sec*, cependant son assemblage requiert probablement le système *sec*, car plusieurs composants possèdent des séquences signal similaires à celles trouvées dans le système *sec* (Table 1). L'appareil de sécrétion de type III est composé de plus de vingtaine de protéines et la plupart des protéines de la membrane interne sont homologues aux protéines de l'appareil de biosynthèse du flagelle des bactéries Gram négatif et positif (Table 1). Activé par le contact avec la cellule eucaryote, ce système est dédié à l'injection d'effecteurs directement du cytoplasme de la bactérie dans le cytosol de la cellule cible. Ces effecteurs ont pour effets la mort cellulaire ou le dysfonctionnement de la cellule en détruisant la membrane, en perturbant les cascades de transduction des signaux ou en désorganisant le cytosquelette (Hueck, 1998). Ils permettent aux bactéries pathogènes d'envahir les tissus de l'organisme infecté ou de circonscrire ses défenses. Les SSTTs sont conservés au niveau structural entre les bactéries à Gram négatif et présentent de grandes similitudes avec le corps basal du flagelle (Fig. 2) (Tampakaki *et al*., 2004).

4. Le système de sécrétion de type III de *P. aeruginosa*

4.1 L'organisation génétique

A l'exception des gènes codant les exotoxines, les gènes codant les protéines du SSTT sont regroupés en clusters sur chromosome contrairement au genre *Yersinia* pour lequel le SSTT est situé sur un plasmide. Ils sont organisés en 5 opérons. L'opéron *exsCEBA* code les principaux régulateurs du SSTT, les opéron *pscN-U*, *popNpcr1234DR*, *exsDpscB-L* codent la structure de l'appareil de sécrétion, permettant le passage des effecteurs à travers les membranes bactériennes, et l'opéron *pcrGVHpopBD* code des protéines indiquées dans l'appareil de translocation, permettant l'injection des effecteurs directement dans le cytosol de la cellule cible eucaryote (Table 1) (Frank, 1997).

4.2 Effecteurs

Quatre effecteurs sécrétés par le SSTT sont connus chez *P. aeruginosa* : ExoS, ExoT, ExoY and ExoU. Toutes les souches ne possèdent pas tous les effecteurs (Feltman *et al*., 2001).

ExoS et **ExoT** sont des protéines bifonctionnelles qui partagent 76% d'identité. Elles possèdent une activité GAP spécifique des petites protéines G de la famille Rho ainsi que une activité ADP ribosyltransférase (à l'extrémité C-terminal) sur des substrats différents: respectivement les protéines de la famille Ras et CrkI/II pour ExoS et ExoT (Barbieri and Sun, 2004). L'activité ADP ribosyltransférase n'est active qu'en présence d'une protéine eucaryote, FAS (Factor activating exoenzyme S) (Coburn *et al*., 1991). Ces activités perturbent la transduction des signaux intracellulaires, médiés par Ras par exemple, et conduisent à la mort cellulaire ou au remodelage du cytosquelette d'actine, induisant ainsi une inhibition de la phagocytose ou un changement de morphologie des cellules.

ExoY est une adénylate cyclase qui présente des homologies avec les adénylates cyclases de *Bordetella pertussis* (CyaA) et *Bacillus anthracis* (EF). ExoY induit une augmentation de la concentration en AMP cyclique et son activité nécessite un ou des facteurs eucaryotes inconnus (Yahr, *et al*., 1998). Bien que ExoY inhiberait l'internalisation bactérienne en perturbant le cytosquelette des cellules épithéliales (Cowell *et al*., 2005), il aurait seulement un rôle mineur lors d'une infection pulmonaire aiguë (Vance *et al*., 2005; Lee *et al*., 2005).

ExoU, l'effecteur le plus cytotoxique injecté par le SSTT de *P. aeruginosa*, possède une activité phospholipase de type PLA2 (Sato *et al*., 2003). Il est associé à une lyse rapide des cellules et une forte léthalité en modèle murin (Finck-Barbancon *et al*., 1997). Similaire aux autres effecteurs, il a besoin d'une ou plusieurs protéines eucaryotes inconnues pour son activité phospholipase (Sato *et al*., 2005).

4.3 Chaperonnes

Plusieurs protéines sécrétées par SSTT requièrent la présence de protéines cytosoliques appelées chaperonnes. Les chaperonnes sont caractérisées par une petite taille (< 15 kDa), un pI acide (<5), et en C-terminal une hélice alpha amphiphile capable de fixer leur substrat protéique (Wattiau *et al*., 1994). Les gènes codant ces chaperonnes se trouvent généralement à proximité des gènes codant les protéines qu'elles fixent. Les chaperonnes ont des différents rôles importants, en particulier elles peuvent stabiliser leurs partenaires (Page and Parsot, 2002; Parsot, *et al*., 2003; Feldman and Cornelis, 2003). Les chaperonnes pourraient se diviser en plusieurs groupes selon le type de protéine qu'elles fixent (Page and Parsot, 2002; Parsot, *et al*., 2003). Plusieurs chaperonnes se trouvent chez *P. aeruginosa* (Table 1).

4.4 Signaux de sécrétion

Pour être sécrétés, les effecteurs de SSTT doivent d'abord être reconnus par l'appareil de sécrétion. Trois différents types de signaux ont été proposés, y compris un 5' mRNA, une séquence N-terminale et les chaperonnes (Anderson and Schneewind, 1997; Cheng *et al*., 1997; Lloyd *et al*., 2001; 2002; Birtalan *et al*., 2002; Ramamurthi and Schneewind, 2002; 2003a; 2003b; 2005). L'existence de plusieurs signaux de sécrétion pourraient rendre la sécrétion plus efficace et déterminer la hiérarchie de la sécrétion, par exemple, les protéines nécessaires à la formation de l'appareil de translocation devront être sécrétées avant les effecteurs par l'appareil de sécrétion.

5. La régulation de l'expression du SSTT chez *P. aeruginosa*

Le SSTT de *P. aeruginosa* est activé *in vivo* lors du contact avec la cellule eucaryote, *in vitro* lors de la déplétion calcique dans le milieu de culture et la présence de sérum (Yahr and Frank, 1994; Frank, 1997; Vallis *et al*., 1999, Hornef *et al*., 2000; Kim *et al*., 2005). Pourtant, même en absence des signaux d'activation, l'opéron *exsDpscB-L* (gènes codant l'appareil de sécrétion) et *exsA*, le gène codant l'activateur transcriptionnel, ont une faible expression (Wolfgang *et al*., 2003). En effet, l'ensemble des gènes constituant le SSTT de *P. aeruginosa* est sous le contrôle d'un facteur transcriptionnel, ExsA, qui se trouve dans l'opéron *exsCEBA*. ExsA coordonne l'expression des gènes de l'appareil de sécrétion, de l'appareil de translocation et des effecteurs. Les réseaux de régulation contrôlant l'activation du SSTT agissent principalement sur l'expression ou l'activité d'ExsA, de façon directe ou indirecte. Par conséquent, on peut distinguer deux types de régulation: la régulation ExsA-dépendante et la régulation ExsA-indépendante.

5.1 La régulation ExsA-dépendante

ExsA, homologue de VirF qui contrôle l'expression du SSTT chez *Yersinia*, est un membre de famille des activateurs transcriptionnels de type AraC (Frank, 1997). Il possède une activité de fixation ADN de type 'α-hélice-tour-α-hélice' et se fixe sur les promoteurs des opérons codant les gènes du SSTT au niveau de la séquence consensus TXAAAAXA, à environ 50 paires de base en amont du site d'initiation de la transcription (Hovey and Frank,

1995). ExsA active sa propre synthèse en se fixant sur le promoteur de l'opéron *exsCEBA*, créant ainsi une boucle de rétroaction positive. Une mutation du gène *exsA* conduisant à un défaut d'expression ou une protéine non fonctionnelle, abolit l'expression des gènes du SSTT et aboutit à une souche moins virulente. L'hypothèse d'une bistabilité dans le niveau d'expression de ExsA a été récemment proposée (Filopon *et al*., 2006).

ExsD, codé par le première gène de l'opéron *exsDpscB-L*, est un anti-activateur du SSTT de *P. aeruginosa* (McCaw *et al*., 2002). ExsD est capable de se fixer à ExsA et d'inhiber son activité. Une surexpression d'ExsD induit une inhibition du SSTT et son absence une dérépression de l'opéron *exsCEBA* et par conséquent de l'ensemble des gènes du SSTT.

ExsC, codé par le premier gène de l'opéron *exsCEBA*, est un anti-anti-activateur du SSTT de *P. aeruginosa* par sa capacité à inhiber l'activité de ExsD en libérant ExsA (Dasgupta *et al*., 2004). De plus, ExsC pourrait être la protéine chaperonne d'ExsE (Rietsch *et al*., 2005; Urbanowski *et al*., 2005).

ExsE, positionné au sommet de la cascade de régulation centrée sur l'activité d'ExsA, est un inhibiteur du SSTT de *P. aeruginosa* (Rietsch *et al*., 2005; Urbanowski *et al*., 2005). En effet, les quatre protéines ExsA, ExsD, ExsC et ExsE forment une cascade de régulation sous la dépendance d'ExsA. ExsE, au sommet de cette cascade, couple la transcription des gènes du SSTT avec l'activation de la sécrétion. En absence d'activation, le canal de sécrétion est fermé, ExsE est fixée à ExsC laissant ExsD jouer un rôle d'inhibiteur en se fixant à ExsA. Lors de la présence des signaux d'activation, par exemple la déplétion calcique, ExsE est sécrété, ExsC libéré fixe alors ExsD et permet à ExsA d'induire la transcription des gènes du SSTT (Fig. 4). L'expression des gènes du SSTT doit dépendre d'un équilibre fin entre les concentrations des différents constituants de cette cascade.

PtrA, répresseur du SSTT de *P. aeruginosa*, pourrait inhiber en s'y liant, l'activité d'ExsA et ainsi l'expression des gènes du SSTT. PtrA lui-même est induit par la présence de cuivre en concentration élevée, ainsi que cela est observé lors de l'infection secondaire à une brûlure (Ha *et al*., 2004).

5.2 La régulation ExsA-indépendante

D'autre facteurs, y compris i) les régulateurs à deux composants, par exemple RetS

(Goodman *et al*., 2004; Zolfaghar *et al*., 2005; Laskowski *et al*., 2004), LadS et GacS/GacA (Ventre *et al*., 2006); ii) les facteurs métaboliques, par exemple la pyruvate déhydrogénase (PDH) (Dacheux *et al*., 2002); iii) le quorum sensing, par exemple RhlI/RhlR (Hogardt *et al*., 2004; Bleves *et al*., 2005); iv) les régulateurs globaux, par exemple Vfr (Wolfgang *et al*., 2003); pourraient aussi être impliqués dans la régulation de l'expression des gènes du SSTT chez *P. aeruginosa* (Fig. 5).

6. Objectif du travail

Les objectifs de ce travail sont d'une part de caractériser les protéines impliquées dans la régulation transcriptionnelle de *exsCEBA* et, d'autre part d'identifier des signaux régulateurs de type 'quorum sensing'.

MATERIALS AND METHODS

1. Bacterial media

- Luria-Bertani Medium (LB medium)
  - Tryptone (GibcoBRL®, Paisley, Scotland) 10g
  - Yeast extract (GibcoBRL®) 5g
  - NaCl[a] (VWR International, Fontenay sous Bois, France) 10g

  Add 1 liter of deionized H_2O from Millipore, ultra-pure water system (Millipore SAS, Molsheim, France). Shake until the solutes have dissolved (adjust the pH to 7.0 with 10 N NaOH if necessary). Autoclave at 121°C for 15 minutes. Sterile media can be stored at room temperature.

  [a]If not indicated, the chemical reagents used in this study were either from VWR International or from Sigma®, St. Louis, MO, USA.
- Vogel-Bonner (VB) minimal medium (Vogel and Bonner, 1956)
  - D-(+)-Glucose ($C_6H_{12}O_6$) 4.5g (25mM)
  - Citric acid ($C_6H_8O_7.H_2O$) 2g (9.5mM)
  - Sodium amnonium phosphate ($NaNH_5PO_4.4H_2O$) 3.5g (16.7mM)

  Add 980 ml of tap water (not deionized H_2O). Shake until the solutes have dissolved. Adjust the pH to 7.2 with 10 N NaOH (~ 3ml). Sterilize by filtration through a 0.22 µM filter (Millex® GP, Cork, Ireland).
- LB agar plate
  - Tryptone 10g
  - Yeast extract 5g
  - NaCl 10g
  - Agar (GibcoBRL®) 15g

  Add 1 liter of deionized H_2O. Shake until the solutes have dissolved (adjust the pH to 7.0 with 5 N NaOH, if necessary). Autoclave at 121°C for 15 minutes. When the medium is recovered from the autoclave, swirl it gently to distribute the melted agar evenly throughout the solution. Allow the medium to cool to 50-60°C before adding appropriate antibiotics (Table 3). Plates can then be poured directly from the flask using about 20 ml of medium per 90-mm plate (Dominique Dutscher, Brumath, France). When the medium has hardened completely, invert the plates and store them at 4°C until needed (as fresh as possible, no more than 2 months). The plates should be incubated at 37°C for 1 hour before they are used.
- *Pseudomonas* Isolation Agar (PIA) plate

  Suspend 45g of the PIA (Difco laboratories, Detroit, USA) powder in 1 liter of deionized H_2O supplemented with 20 ml of glycerol. Prepare the PIA plate as described for LB agar plate.

TABLE 3. Antibiotic solutions

	Stock solution[a]	Working concentration		
		Pseudomonas aeruginosa, CHA		*Escherichia coli*
		LB	PIA plate	LB/LB agar plate
Carbenicillin (Cb)	300 mg ml^{-1}	300 µg ml^{-1}	600 µg ml^{-1}	
Gentamicin (Gm)	80 mg ml^{-1}	200 µg ml^{-1}	400 µg ml^{-1}	10/20 µg ml^{-1}
Tetracycline (Tc)[b]	20 mg ml^{-1}	250 µg ml^{-1}	250 µg ml^{-1}	10/20 µg ml^{-1}
Ampicillin (Amp)	100 mg ml^{-1}			100 µg ml^{-1}
Kanamycin (Kana)	50 mg ml^{-1}			50 µg ml^{-1}

[a]Stock solutions of antibiotics dissolved in H_2O (except tetracycline here) should be sterilized by filtration through a 0.22 µM filter. Store antibiotic solutions at -20°C.

[b]Tetracycline should be dissolved in ethanol and don't need be sterilized. Store stock solution in light-tight containers. Magnesium ions are antagonists of tetracycline. Use media without magnesium salts (*e.g.*, LB medium) for selection of bacterial resistant to tetracycline.

2. Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 4.

TABLE 4. Bacterial strains and plasmids used in this study

Strains or plasmids	Description[a]	Source
Strains		
E. coli DH5α	used for plasmid transformation	Stratagene
E. coli BL21(DE3)	used for recombinant protein production	Stratagene
E. coli S17-1	used for mating constructs into *P. aeruginosa*	Simon *et al*.,1983
CHA	mucoid CF isolate	Toussaint *et al*., 1993
Δ*exsA*	*exsA*::Gm mutant of CHA	Dacheux *et al*., 1999
CHA (*pClux*)	*lux* fusion with *exsCEBA* promoter intergrated at the *attB* site of the CHA chromosome	This study
CHA (*pSlux*)	*lux* fusion with *exoS* promoter at the *attB* site of the CHA chromosome	This study
Δ*psrA*	CHA containing an in-frame deletion of amino acids 14-194 of *psrA* ORF	This study
Δ*psrA* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*psrA* chromosome	This study
Δ*psrA* (*pSlux*)	*lux* fusion with *exoS* promoter at the *attB* site of the Δ*psrA* chromosome	This study
Δ*rpoS*	CHA containing an in-frame deletion of amino acids 1-207 of *rpoS* ORF	This study
Δ*rpoS* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*rpoS* chromosome	This study
Δ*psrA* (p*PsrA*) (*pClux*)	Δ*psrA* (*pClux*) complemented by *psrA*	This study
Δ*psrA* (p*PsrA*) (*pSlux*)	Δ*psrA* (*pSlux*) complemented by *psrA*	This study
Δ*psrA* (pDD2) (*pClux*)	Δ*psrA* (*pClux*) containing pDD2 vector	This study
Δ*psrA* (pDD2) (*pSlux*)	Δ*psrA* (*pSlux*) containing pDD2 vector	This study
Δ*lasI*	CHA containing an in-frame deletion of amino acids 1-155 of *lasI* ORF	This study
Δ*lasI* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*lasI* chromosome	This study
Δ*rhlI*	CHA containing an in-frame deletion of amino acids 20-201 of *rhlI* ORF	This study
Δ*rhlI* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*rhlI* chromosome	This study
Δ*lasI rhlI*	CHA containing two in-frame deletions of both *lasI* ORF and *rhlI* ORF	This study
Δ*lasI rhlI* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*lasIrhlI* chromosome	This study
Δ*phnAB*	CHA containing an in-frame deletion of amino acids 1-530 and 1-172 of *phnA* and *phnB* ORF respectively	This study
Δ*phnAB* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*phnAB* chromosome	This study
Δ*trpA*	CHA containing an in-frame deletion of amino acids 36-247 of *trpA* ORF	This study
Δ*trpA* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*trpA* chromosome	This study
Δ*trpA* (*pSlux*)	*lux* fusion with *exoS* promoter at the *attB* site of the Δ*trpA* chromosome	This study
Δ*virA*	CHA containing an in-frame deletion of amino acids 234-677 of *virA* ORF	This study
Δ*virA* (*pClux*)	*lux* fusion with *exsCEBA* promoter at the *attB* site of the Δ*virA* chromosome	This study
Δ*virA* (*pSlux*)	*lux* fusion with *exoS* promoter at the *attB* site of the Δ*virA* chromosome	This study
Plasmids		
pTOPO	Blunt end cloning vector, Ap^r	Invitrogen
pIApC	pUCP20-derived vector containing *exsCEBA* promoter	Dacheux *et al*., 2001
pDD2	pUCP20 derived vector containing *exsA* gene; Ap^r	Dacheux *et al*., 1999
pEX100Tlink	Allelic replacement suicide vector; $Ap^r(Cb^r)$	Quenee *et al*., 2005
pUCGmlox	pUC18-based vector containing the *aacC1* gene flanked by *lox*; Ap^r, Gm^r	Quenee *et al*., 2005
pCM157	Cre recombinase; Tc^r	Marx and Lidstrom, 2002
Mini-CTX-*lux*	mini-CTX-*lux* ; Tc^r	Laskowski *et al*., 2004
Mini-CTX-*exsCp-lux*	mini-CTX-*lux* containing *exsCEBA* promoter ; Tc^r	Laskowski *et al*., 2004
mini-CTX-*exoSp-lux*	mini-CTX-*lux* containing *exoS* promoter ; Tc^r	Laskowski *et al*., 2004
pFLP2	Flp recombinase; $Ap^r(Cb^r)$	Hoang *et al*., 1998
pUCP20	*Escherichia-Pseudomonas* shuttle vector ; $Ap^r(Cb^r)$	West *et al*., 1994
pUC18	For *Pseudomonas* genomic DNA cloning; Ap^r	Yanisch-Perron *et al*., 1985
p*exsA*ind	pUCP20-derived vector containing IPTG-inducilbe *exsA*; $Ap^r(Cb^r)$	Filopon *et al*., 2006
pp*psrA*	pTOPO-derived vector containing *psrA* promoter	This study
pp*fleQ*	pTOPO-derived vector containing *fleQ* promoter	This study
pp*hpA*-Rec	pET15b-derived vector containing *phpA*; Ap^r	This study
p*psrA*-Rec	pET15b-derived vector containing *psrA*; Ap^r	This study
p*vfr*-Rec	pET15b-derived vector containing *vfr*; Ap^r	This study
p*PsrA*	pUCP20-derived vector containing *psrA* ORF as well as its promoter region; $Ap^r(Cb^r)$	This study
pKO-*psrA*	pEX100Tlink-derived vector containing up- and downstream region of *psrA* and Gmlox; Ap^r, Gm^r	This study
pKO-*rpoS*	pEX100Tlink-derived vector containing up- and downstream region of *rpoS* and Gmlox; Ap^r, Gm^r	This study
pKO-*lasI*	pEX100Tlink-derived vector containing up- and downstream regions of *lasI* and *Gmlox*; Ap^r, Gm^r	This study
pKO-*rhlI*	pEX100Tlink-derived vector containing up- and downstream regions of *rhlI* and *Gmlox*; Ap^r, Gm^r	This study
pKO-*phnAB*	pEX100Tlink-derived vector containing up- and downstream regions of *phnAB* and *Gmlox*; Ap^r, Gm^r	This study
pKO-*trpA*	pEX100Tlink-derived vector containing up- and downstream regions of *trpA* and *Gmlox*; Ap^r, Gm^r	This study
pKO-*virA*	pEX100Tlink-derived vector containing up- and downstream regions of *virA* and *Gmlox*; Ap^r, Gm^r	This study

[a] Ap^r, ampicillin resistance ; Gm^r, gentamicin resistance ; Tc^r, tetracycline resistance ; Cb^r, carbenicillin resistance; KO, knock out.

2.1 Strain construction

2.1.1 Strain containing chromosomal transcriptional gene reporter

In order to study precisely the level of transciption of *exsCEBA* and *exoS* promoters, we used the system developped by Laskowsky and colleagues to construct transcriptional gene reporter. In brief, the plasmids mini-CTX-*exsCp-lux* and mini-CTX-*exoSp-lux* were mobilized into *Pseudomonas* strain by mating and vector backbone sequences were excised by Flp recombinase as previously described (Fig. 6) (Hoang *et al*., 1998). Each reporter construct is therefore present as a single, unmarked copy integrated at the chromosomal *attB* site (Laskowski *et al*., 2004).

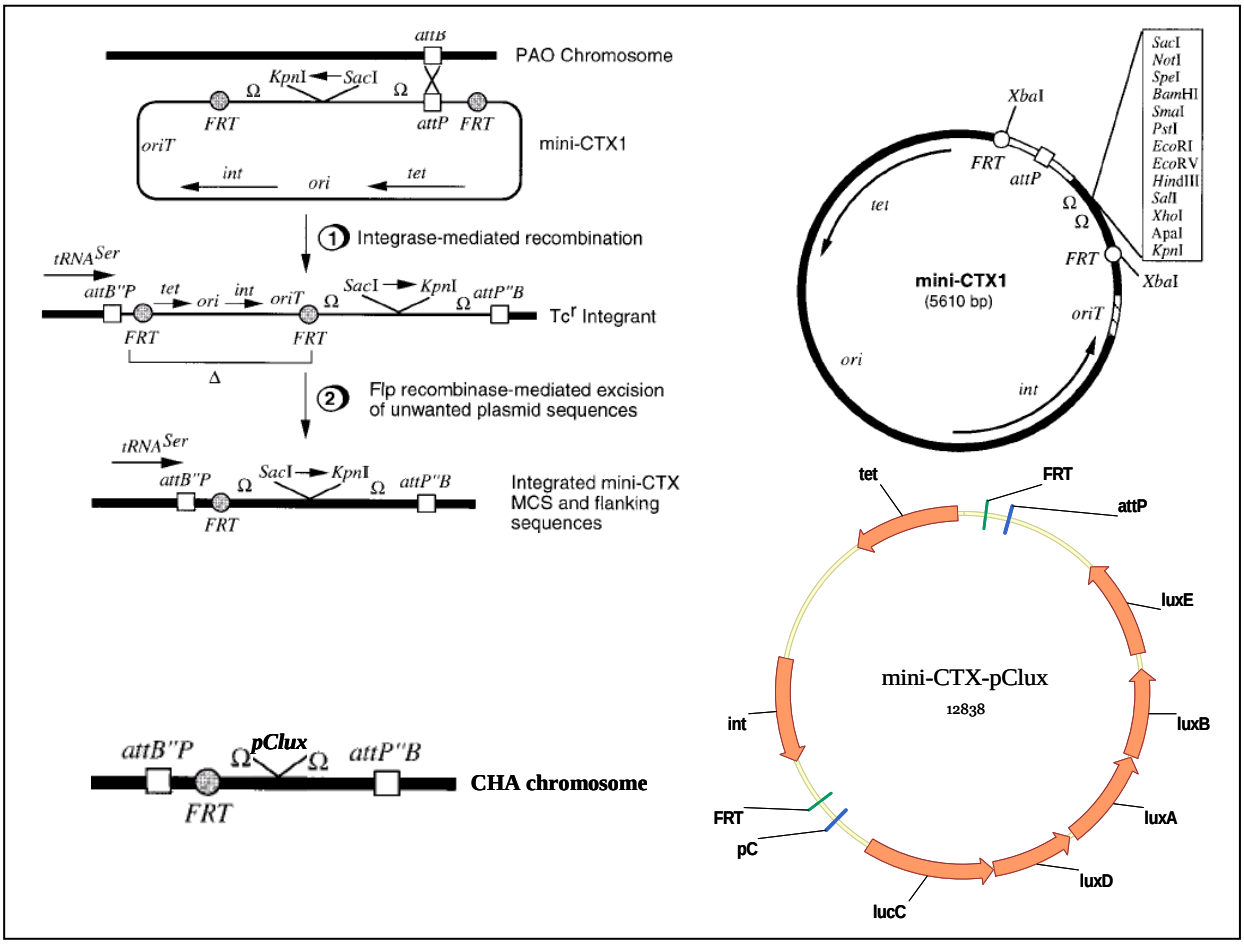


FIG. 6. Construction of transcriptional gene reporter. Adapted from Hoang *et al*., 1998.

MATERIALS

-Bacterial strains

- *P. aeruginosa* wild-type CHA
- *E.coli* S17-1

-Plasmids

- mini-CTX-*exsCp-lux* (a kind gift of Dr. Kazmierczak)
- mini-CTX-*exoSp-lux* (a kind gift of Dr. Kazmierczak)
- pFLP2 (a kind gift of Pr. Schweizer)

Attention, genebank access N° for mini-CTX-*lux* and pFLP2 are AF140579 and 048702, respectively.

-Agar plate

- LB (Tetracycline, 20 µg ml^{-1})
- PIA (Tetracycline, 250 µg ml^{-1})

- PIA (Carbenicillin, 600 µg ml^{-1})
- LB without salt

METHOD

-Preparation of *E.coli* S17-1 containing mini-CTX-*exsCp-lux* or mini-CTX-*exoSp-lux*

1. Transform *E.coli* S17-1 by mini-CTX-*exsCp-lux* and mini-CTX-*exoSp-lux* and select in LB agar plate containing tetracycline (20 µg ml^{-1})

-Mating between *P. aeruginosa* wild-type CHA and *E.coli* S17-1

2. Mix at 1:1 with 50 µl of exponential phase (OD_{600} ≈ 0.5) CHA and *E.coli* S17-1. Deposit the mixture on the surface of LB agar without salt. Incubate the plate overnight in the incubator at 37°C.
3. Transfer the bacteria into a microfuge tube containing 1 ml of LB.
4. Spread 100 µl bacteria (OD_{600} is from 1 to 2) on the PIA containing tetracycline (250 µg ml^{-1}). Incubate the plate overnight in the incubator at 37°C.

-Excision of vector backbone sequences by Flp recombinase

5. Electroporate the mated bacteria with pFLP2. Select on PIA containing carbenicillin (600 µg ml^{-1}). Incubate the plate overnight in the incubator at 37°C.

-Remove pFLP2 plasmid

6. Culture the bacteria (Cb^r) in 2ml of LB without antibiotic overday. Inoculate again in 2 ml of LB overnight. Repeat another overday and overnight.
7. Select the bacteria (Tc^s, Cb^s).
8. Verify the construct by EGTA activation test.

IMPORTANT: To obtain mutant containing pC*lux* and pS*lux*, we begin by mating CHA (pC*lux*) [or CHA (pS*lux*)] with *E. Coli* S17-1 containing plasmid pKO-gene (Fig. 7), though we can also begin by mating mutant with *E. Coli* S17-1 containing gene reporter pC*lux* or pS*lux*.

2.1.2 Mutants

Study of gene function is often carried out by deleting genes in the strains of interest and observing the phenotypic differences between the parental and deleted strains. We used *cre-lox* system developped in our lab combining the sacB-based positive selection to construct mutants (Fig. 7) (Quenee *et al*., 2005). The advantage of *cre-lox* method is that *lox*-flanked antibiotic marker (*aacC*1, for gentamicin resistance) can be removed from the bacterial genome by site-specific recombinase encoded by the *cre* gene.

MATERIALS

-Plasmids

- PKO-*gene* (Table 4)
- pEX100Tlink (Δ*Hind*III) (Quenee *et al*., 2005).
  This vector contains 1) the counterselectable *sacB* marker, 2) an *oriT* for conjugation-mediated plasmid transfer and 3) multiple cloning sites. *SacB* gene allows for positive selection of the segregation of true mutants from the more fequently occuring merodiploids on medium containing 5% sucrose (Levansucrase , encoded by *sacB*, can produce toxic products from sucrose; mutants with simple combination could not survive in the medium containing sucrose due to the presence *sacB*; only mutants with double combination could survive in the 5% sucrose due to the lost of *sacB*, Schweizer and Hoang. 1995).
- pUCGmlox
- pCM157

-Bacteria strains

- CHA
- *E.coli* S17-1

-Agar plate

- LB agar without salt
- PIA containing 5% sucrose
- PIA (Gm_{400})
- PIA (Cb_{600})
- PIA (Tc_{250})

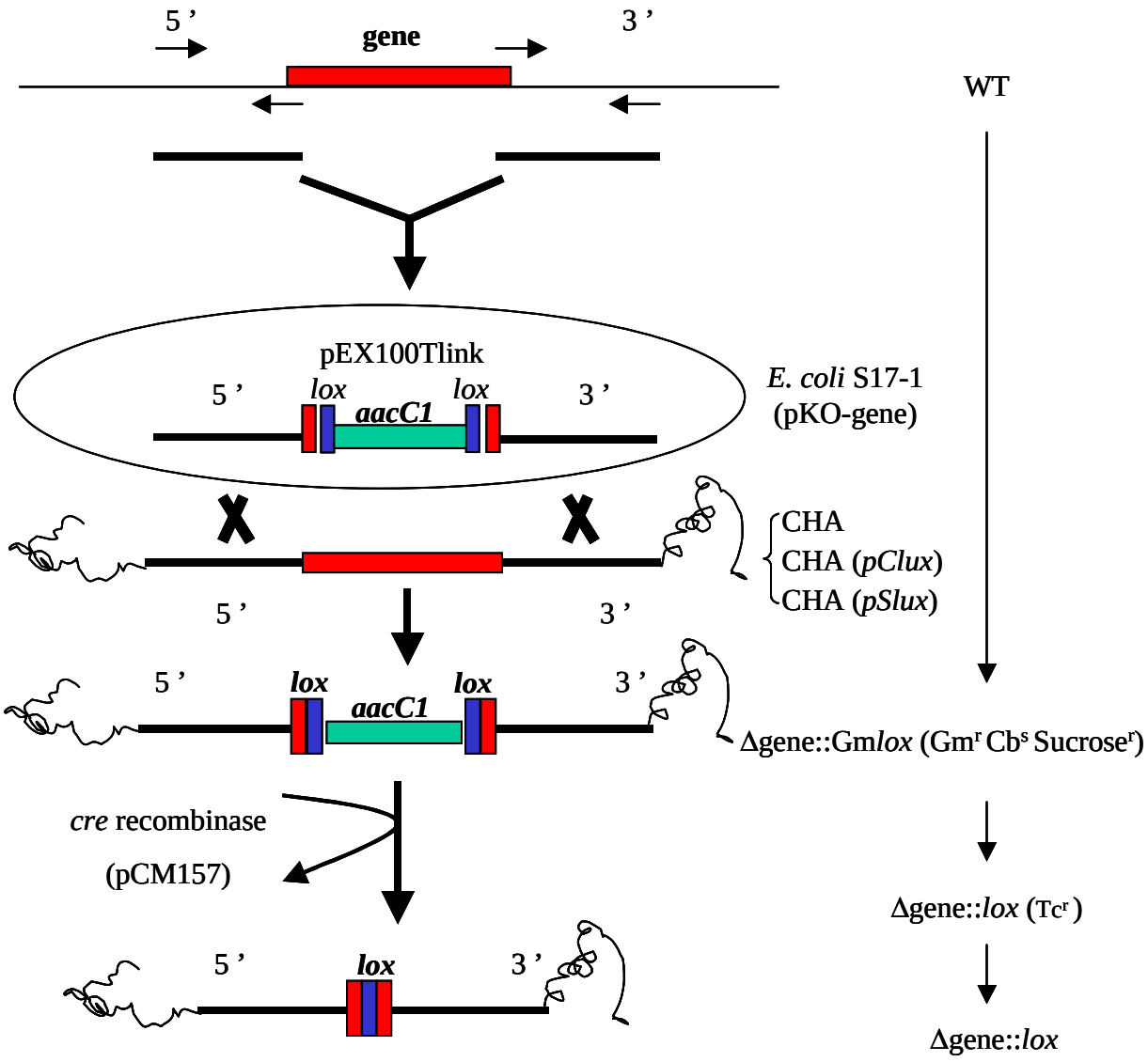


FIG. 7. Procedure for mutant construction. (Also used to construct mutants containig gene reporters).

METHOD

-Preparation of *E.coli* S17-1 containing plasmid pKO-*gene* of interest (Fig. 7).

1. PCR upstream and downstream region (about 0.8-1.5 kb) of gene (to be deleted) by using primer pairs *gene*-U1-*gene*-U2 and *gene*-D1-*gene*-D2, respectively (Table 5).
2. Topo cloning, miniprep, verification by digestion and DNA sequencing.
3. Ligate up-*gene*, down-*gene* into plasmid pEX100Tlink (digested with appropriate enzymes, such as *Eco*R1/*Hind*III, *Hind*III/*BamH*1 and *Eco*R1/*BamH*1 respectively) at 16°C, O.N. Transform *E.coli* DH5α, select on the LA agar plate (Amp_{100}), miniprep to obtain plasmid pEX100Tlink containing up-*gene*-down-*gene*.

TABLE 5. Primer sequences used in this study

Primer	Sequences*	Description
psrA-U1	5′-AGAATTCCGCCGTCGTCATTCGCCGA-3′	Amplification of upstream region flanking *psrA*
psrA-U2	5′-TAAGCTTCCGCATCGAGAATGCGTTCG-3′	
psrA-D1	5′-TAAGCTTGGTGATGCACCTGATGGTGC-3′	Amplification of downstream region flanking *psrA*
psrA-D2	5’-AGGATCCCATCGACAGCTCCCTCAATC-3′	
rpoS-U1	5’-TAGGATCCAGACCCTGAAAGGCGCC-3’	Amplification of upstream region flanking r*poS*
rpoS-U2	5’-AGAAGCTTCGCTGGGTTTTCTGGC -3’	
rpoS-D1	5’-ATAAGCTTCTCGTCCAGCATGATGCC -3’	Amplification of downstream region flanking r*poS*
rpoS-D2	5’-ATGAATTCGAAGACCTGCATCGCCG-3’	
vfr-R1	5’-ACATATGGTAGCTATTACCCACACA-3’	Amplification of recombinant Vfr protein
vfr-R2	5’-AGGATCCTCAGCGGGTGCCGAAGAC-3’	
psrA-R1	5’-ACATATGGCCCAGTCGGAAACCG-3’	Amplification of recombinant PsrA protein
psrA-R2	5′-AGGATCCTCAGGCCTTGGCGGGCGT-3′	
psrA-C1	5′-AGAATTCACTGGTGCGTCATCTGCCGC-3′	Amplification of fragment containing *psrA* ORF as well as its promoter region
psrA-C2	5′-AGGATCCTCAGGCCTTGGCGGGCGT-3′	
p*psrA*-F	5’-AGAATTCGGGGCGTCAGCTTCTGCAT-3’	Amplification of fragment containing *psrA* promoter region
p*psrA*-R	5’-AGAATTCAATGCGTTCGACGGTTTCCG-3’	
p*fleQ*-F	5’-AGAATTCCTACCAGATGTTCGGATAAGT-3’	Amplification of fragment containing v*fr* promoter region
p*fleQ*-R	5’-AGAATTCCGAATGGGTCTCGCTCGACC-3’	
phpA-1	5’-ACATATGGAATTCCTTGTTAAAAGCGT-3’	Verification of Δ*phpA*
phpA-2	5’-AGGATCCGGGTCACTTGGCCCGTTCC-3’	
psrA-1	5’-ATGGCCCAGTCGGAAACCG-3’	Verification of Δ*psrA*
psrA-2	5’-TCAGGCCTTGGCGGGCGT-3’	
rpoS-1	5’-GCGAGTTGGTCATCATCAAAC-3’	Verification of Δr*poS*
rpoS-2	5’-GCATCAAGTGCAAATACACCG-3’	
lasI-U1	5’-ACGGATCCAAGTGGCTATGTCGCCG-3’	Amplification of upstream region flanking *lasI*
lasI-U2	5’-ACAAGCTTCCGGACCCTTGCTAGGC-3’	
lasI-D1	5’-AGAAGCTTGGCCTGGACGTATCGCG-3’	Amplification of downstream region flanking *lasI*
lasI-D2	5’-GGGAATTCTCAGCCTGCCC-3’	
rhlI-U1	5’-TATCTAGACGCGGTGCGCCGCAAGG-3’	Amplification of upstream region flanking *rhlI*
rhlI-U2	5’-ATAAGCTTGCTCGGCGATCATGGCG-3’	
rhlI-D1	5’-ATAAGCTTGCGTCATCGGGCGTTCC-3’	Amplification of downstream region flanking *rhlI*
rhlI-D2	5’-CAGAGCTCTGTCCGGAAATCCTCATGC-3’	
phnAB-U1	5’-ATGGATCCATGTTGAACCACGGTGTCC-3’	Amplification of upstream region flanking *phnAB*
phnAB-U2	5’-ATAAGCTTTCGCCGCAATGGATGTCC-3’	
phnAB-D1	5’-ATAAGCTTGATTCTCACCACCCACGG-3’	Amplification of downstream region flanking *phnAB*
phnAB-D2	5’-ATGAATTCGGGAACTGACGATTGCAG-3’	
trpA-U1	5’-AGAATTCTCAGATCAGCTCGTGGCGG-3’	Amplification of upstream region flanking *trpA*
trpA-U2	5’-TAAGCTTCAGGCGGTCAAGGATGTCCT-3’	
trpA-D1	5’-TAAGCTTCCAGGGAAGACGCGTAGT-3’	Amplification of downstream region flanking *trpA*
trpA-D2	5’-TGGATCCGCACAAGATCAACAACTGC-3’	
virA-U1	5’-AGGATCCCACACCGATGCAGAAGACCG-3’	Amplification of upstream region flanking *virA*
virA-U2	5’-TAAGCTTGTCGTGGAGATAGTCGCGAT-3’	
virA-D1	5’-TAAGCTTAAGGGCGTGGGCAAGGGCAC-3’	Amplification of downstream region flanking *virA*
virA-D2	5’-AGAATTCTGTACCCATGCCTCGACCCT-3’	
lasI-1	5’-GTGGATGCTCAAGAAGGACTAC-3’	Verification of Δ*lasI*
lasI-2	5’-CTGATCGGCAACCTTACCC-3’	
rhlI-1	5’-CGCGGCGCTGGGTCTCATC-3’	Verification of Δ*rhlI*
rhlI-2	5’-GTAACCGCCCTCTTCCGTGCGG-3’	
phnAB-1	5’-GATTCTCTCCCGCCAGGCG-3’	Verification of Δ*phnAB*
phnAB -2	5’-CCATCGACGAGGAACTGAAG-3’	
trpA-1	5’-TCAGCGTGCGTTGCGCACGC-3’	Verification of Δ*trpA*
trpA-2	5’-AAGCATGAGCCGCCTGCAGA-3’	
virA-1	5’-GTCGCGGATATTCTTCGGCG-3’	Verification of Δ*virA*
virA-2	5’-TTGCCGATCTCCGAGTGGAT-3’	
trpA-C1	5’-AGGATCCATGAGCCGCCTGCAGACCCG-3’	Amplification of complete *trpA* ORF for complementation
trpA-C2	5’-TAAGCTTCAGCGTGCGTTGCGCACGC-3’	
aacC1-F	5’-ATGTTACGCAGCAGCAACGA-3’	Amplification of *aacC1* probe for southern blot
aacC1-R	5’-CAAGTTCCCGAGGTAATCGG-3’	

* Restriction enzyme sites are underlined

4. Ligate Gm*lox* (digested from plasmid pUCGmlox by *Hind*III) into *Hind*III digested pEX100Tlink containing up-*gene*-down-*gene* at 16°C, O.N. Transform the ligate into *E.coli* DH5α and select on the LA agar plate (Gm_{10}/Amp_{100}). Miniprep to obtain plasmid pKO*gene* (pEX100Tlink-up-*gene*-Gm*lox*-down-*gene*).
5. Transform pKO*gene* (pEX100Tlink-up-*gene*-Gm*lox*-down-*gene*) into *E.coli* S17-1.

-Mating between CHA/CHA (*pClux*)/CHA (*pSlux*) and *E.coli* S17-1 (pKO-*gene*)

6. Mix at 1:1 of 50 µl exponential phase (OD600 ≈ 0.5) CHA and *E.coli* S17-1 (pKO-gene). Deposit the mixture on the surface of LB agar without salt. Incubate the plate overnight in the incubator at 37°C.
7. Transfer the bacteria into a microfuge tube containing 1 ml of LB.
8. Spread 100 µl bacteria (OD_{600} is from 1 to 2) on the PIA (Gm_{400}). Overnight, 37°C.
9. Inoculate 50 colonies on the PIA containing 5% sucrose. Overnight, 37°C.
   In theory, only mutants with double combination could survive in the presence of 5% sucrose, but in fact all mutants survive after overnight's incubation (with different size of the colonies).
10. Re-inoculate these 50 colonies on the PIA (Gm_{400}) as well as PIA (Cb_{600}). Overnight, 37°C.
11. Select colonies growing on the PIA(Gm_{400}) but not on the PIA (Cb_{600}) for further verification by PCR.

-Excision of Gm*lox* by *Cre* recombinase

12. Electroporate the PCR confirmed colonies (Gm^r/Cb^s) with plasmid pCM157 and select on the PIA (Tc_{250}). Overnight, 37°C.

-Remove pCM157 plasmid

13. Culture the bacteria (Tc^r) in 2ml of LB (without antibiotic) overday. Inoculate again in 2 ml of LB overnight. Repeat another overday and overnight.
14. Select the bacteria (Tc^s, Gm^s).
15. Verify the construct by PCR.

2.1.3 Complementation construct

A complemetation construct has to be constructed to confirm that the phenotype of the mutant is really due to the deletion of the gene but not due to the neighbouring genes that might be affected by a polar effect of this deletion.

MATERIALS

-Primers

- *psrA*-C1
- *psrA*-C2

-plasmid pUCP20

-Materials needed for PCR, agarose gel electrophoresis, Topo cloning, Miniprep, digestion, ligation and transformation.

METHOD

1. Carry out the PCR to produce a 782-bp fragment containing the *psrA* open reading frame (ORF) as well as 80 bp upstream encompassing the promoter region so that *psrA* is under the transcriptional control of its own promoter.
2. Topo cloning, miniprep, verification by digestion and DNA sequencing.
3. Digest and ligate this fragment inot pUCP20 (*EcoR*1-*BamH*1) to generate p*PsrA*.
4. Transform p*PsrA* into strain Δ*psrA* by electroporation.

2.2 Strain maintenance, storage, recovery, culture and quantitation

MATERIALS

-Bacterial strains (Table 4)

-Bacterial media (Materials and Methods 1)

-Special equipments

- Protect Bacterial Preservers (TSC, Lancashire, UK)
- Nunc Cryo Tube, 1.0 ml (NordicCell, Copenhagen, Denmark)

- Toothpick
- Disposable bacterial loop/spreader (VWR International)
- 14 ml of polypropylene round-bottom tube (Becton Dickinson labware, Meylan, France)
- Microbiological safety cabinet class II (Claus Damm, Humleback, Denmark)
- Incubator (Memmert, Schwabach, Germany)
- Bacterial shaker: Novotron platform shaker (TLS, Auckland, New Zealand)
- Spectrophotometer: Uvikon 860 (UVK-LAB, Trappes, France)
- -86°C ULT Freezer (Thermo Forma, Marietta, OH, USA)
- Autoclaver (Slli, Montpellier, France)

METHOD

- *P.aeruginosa* and *E. coli* strains were maintained on PIA plate and LB agar plate respectively with antibiotics as required (Table 3).
- *P. aeruginosa* strains were stored in Protect Bacterial Preservers at -80°C. Briefly, scrape enough *P. aeruginosa* from PIA palte and make a thich suspension in the cryopreservation fluid. Invert intensely the tube and stand it for several minutes. Withdraw as much as fluid as possible and store it at -80 °C.
- *E. coli* strains were stored at -80°C as 40% glycerol stocks in Nunc Cryo Tube. Briefly, add 0.5 ml of sterile 80 % glycerol (sterilized by autoclaving) to 0.5 ml of *E.coli* culture in Nunc Cryo Tube. Mix well and freeze the culture in ethanol-dry ice or in liquid nitrogen. Transfer the tube to -80 °C for long-term storage. 15% glycerol stock is proposed by Molecular Cloning (Sambrook and Russell, 2001).
- To recover the bacteria, scrape the frozen surface of *E. coli* (Nunc Cryo Tube) or pick a frozen bead of *P. aeruginosa* (Protect Bacterial Preservers) with a sterile bacterial spreader, and then immediately streak onto the surface of an LB agar plate or PIA plate containing the appropriate antibiotics. Incubate the plate overnight in the incubator at 37°C.
- Bacterial culture was usually performed in 2 ml of LB with a single colony picked from an agar plate. Grow the bacteria overnight in a shaker at 37°C with agitation of 260 rpm. Wash the overnight bacteria with LB and inoculate at 1:100 for another culture. The volume of culture is best not more than 1 tenth of that of the tube or the flask to assure enough air needed for the growth of the bacteria.
- Bacterial growth level or quantity were estimated by by optical density measuremnt at 600 nm (OD_{600}) using spectrophotometer. We consider that there are 6×10^8 *P. aeruginosa* in 1 ml of LB at an OD_{600} of 1.

2.3 Bacterial transformation

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. DNA do not enter bacteria under their own power, but require assistance traversing the outer and inner cell membranes and in reaching an intracellular site where they can be expressed and replicated. Bacteria which are able to uptake foreign DNA are called ‘competent’ and are obtained either from a commercial source or by treatment with calcium chloride in the early log phase of growth. Transformation are carried either by chemical method, heat-shock method or by physical method, electroporation.

2.3.1 Chemical transformation of *E.coli* DH 5α by heat shock method

MATERIALS

-DNA: ligated mixture,or plasmid, or PCR product, or digested genomic DNA, *et al*.

-Chemically competent DH5α (Invitrogen)
-Thermomixer (Eppendorf, Hamburg, Germany) or a water bath preset to 42°C
-SOC medium (Invitrogen) or LB medium
-LB agar plate containing appropriate antibiotics

METHOD

1. Add 1-3 µl of transforming DNA (up to 25 ng) in 50 µl of competent DH5α. Swirl the tube gently several times to mix their content. Do not mix by pipetting up and down.
   The DNA volume should not exceed 10 % of that of the competent cells.
2. Incubate on ice for 30 minutes.
3. Heat-shock the cells for 45 seconds at 42°C without shaking.
4. Transfer immediately the tube to ice and allow the cells to cool for 1-2 minutes.
5. Add 250 µl prewarmed SOC medium (or LB) and shake the tube at 37°C for 1 hour.
6. Spread 25 µl and 250 µl of transformed cells onto the prewarmed selective plates .
7. Incubate at 37°C overnight.

2.3.2 Transformation of *P. aeruginosa* by electroporation (Enderle and Farwell, 1998)

MATERIALS

-Electroporation cuvettes (BTX®, San Diego, California,USA)
-ECM 399 pulse generator (BTX®)
-Fresh *P. aeruginosa* from PIA (storing the plate at 4°C lowers the transformation efficiency)
-Disposable bacterial loop/spreader (VWR)
-Plasmid (1:10 diluted Miniprep in H_2O or TE, 10 mM Tris-HCl, pH 8.0, 1mM EDTA)
-PIA plate containing appropriate antibiotic
-H_2O (sterile, distilled and deionized)

METHOD

1. Transfer the half loop of fresh *P. aeruginosa* to a 1.5 ml microcentrifuge tube containing 1 ml of H_2O. Suspend the cells by pipetting up and down.
2. Centrifuge at 14 000 rpm for 1 min. Remove the supernatant and resuspend the cells in another 1 ml of H_2O. Vortex to make a homogenos mixture.
3. Repeat step 2 for three times. Suspend the cells in 40 µl of ice H_2O (OD_{600} is about 5, so there would be about 1×10^8 bacteria). Keep on ice.
4. Add 1-3 µl plasmid (10-30 ng) and mix well with the cell. Incubate on ice for 30 minutes.
5. Add the mixture in the cold electroporation cuvette (-20°C for 30 minutes) and pulse the cuvette at 1800V in a ECM 399 pulse generator. Add immediately 1 ml of prewarmed LB and shake at 37°C for 1 hour.
   Cuvettes could be reused by rising in H_2O and ethanol, air-drying and treating with UV light to inactivate any DNA.
6. Spread 50 µl and 500 µl of transformed cells onto the prewarmed PIA plates containing appropriate antibiotic.
   Transformation efficiency is about 1 transformant per 10^6 bacteria.
7. Incubate at 37°C overnight.

2.4 Plasmid manipulation

2.4.1 Miniprep

Many methods have been developed to purify plasmids from bacteria. These methods invariably involve three steps: growth of the bacterial culture, harvesting and lysis of the bacteria and purification of the plasmid DNA. QIAprep Miniprep System provides a fast, simple, and cost-effective plasmid miniprep method for routine molecular biology laboratory applications. QIAprep Spin Miniprep kit using a microcentrifuge is designed for purification of up to 20 μg of high-copy plasmid DNA from 1-5 ml of overnight cultures of *E. coli* in LB medium.

MATERIALS

-1-5 ml of overnight cultures of *E. coli*

-QIAprep® Spin Miniprep kit (Qiagen, Courtaboeuf, France)

-Centrifuge (Eppendorf, Hamburg, Germany)

Relative centrifugal force (RCF)=1.1×10^{-6} $(rpm)^2 r$.

The symbol *r* represents the radial distance (mm) from the center of the rotor to the point for which RCF is required. This is generally equivalent to r_{max} of the rotor. For example, rotor speed (rpm) = 80, 000 rpm and *r* = 20 mm, RCF can be calculated as 142, 080 g usig the equation given above.

METHOD

All procedures were performed according to the instructions of the manual of the kit.

2.4.2 Restriction enzyme digestion

MATERIALS

-Restriction enzymes (Roche, Meylan, France or Invitrogen, Cergy pontoise, France)

-10 × buffer for Restriction enzymes (Roche or Invitrogen)

-Plasmids

-Thermomixer (Eppendorf, Hamburg, Germany) or a water bath preset to 37°C

METHOD

1. In a sterile 0.5 or 1.5 ml microfuge tube, mix:
   - Plasmid 0.1-0.5 μg (1-5 μl)
   - Restriction enzymes (10 U μl^{-1}) 0.5 μl
   - 10 × buffer for restriction enzymes 1 μl
   - H_2O to total volume of 10 μl

   One unit is defined as the amount of enzyme required to hydrolyze 1 μg of λDNA to completion in 60 minutes in a total reaction volume of 50 μl, under optimal assay conditions (salt concentration, pH, temperature). Digestion is generally performed with a 5-fold excess of enzyme whose volume should not be more than 1 tenth of the total volume to avoid side effects, such as star activity.
2. Incubate at 37°C for 2h (mix 1-2 times during the incubation, centrifuge the tube if necessary).
3. Check the digestion by agarose gel electrophoresis (see Materials and Methods 6).

2.4.3 DNA ligation

MATERIALS

-DNA (digested by same restriction enzyme(s) and purified from agarose gel)

- Plasmid
- Insert DNA

-T4 DNA ligase (Invitrogen)

T4 DNA ligase joins fragments of DNA with compatible cohesive termini as well as blunt ended DNA fragments. DNA fragments with cohesive termini can be ligated at 4°C overnight by the addition of 0.01 to 0.1 Weiss units of enzyme per μg DNA. Blunt-ended DNA fragment ligation is usually carried out at 16°C with 1 to 2 Weiss units per μg DNA. DNA ligase catalyzed the formation of a phosphodiester bond between adjacent 3'-OH and 5'-P termini. This phosphodiester bond-forming reaction requires ATP hydrolysis. The 10 × incubation buffer provided in the kit containing ATP. Inorganic pyrophosphate and AMP are released as by-products.

-10 × buffer for T4 DNA ligase (Invitrogen)
-Thermal cycler or a water bath preset to 16°C

METHOD

1. In a sterile 0.5 or 1.5 ml microfuge tube, mix:
   - Plasmid — 20-200 ng (often use 0.1 μl of Miniprep)
   - Insert DNA — 3-fold molar excess (not mass)
   - T4 DNA ligase — 1 μl
   - 10 × buffer for T4 DNA ligase — 1 μl
   - H_2O — to total volume of 10 μl
2. Incubate at 16°C overnight.
3. Carry out a transformatin or conserve it at -20°C .

2.4.4 Transformation

It was performed as described in Materials and Methods 2.3.1 and 2.3.2.

2.4.5 DNA sequencing

It was performered at GENOME express, Meylan, France by delivering 1 μg of plasmid. (generally 10 μl of Miniprep).

2.4.6 Cloning in plasmid vectors

In principle, cloning in plasmid vectors is very straightforward. Closed circular plasmid DNA is cleaved with one or two restriction enzymes and ligated *in vitro* to foreign DNA bearing compatible termini. The products of the ligation reaction are then used to transform an appropriate strain of *E. coli*. The resulting transformed colonies are screened by hybridization, by PCR or by digestion with restriction enzymes to identify those that carry the desired DNA sequences. To generate a high yield of circular recombinants containing a single insert in a predefined orientation, digestion with two restriction enzymes is a good choice which is known as directional cloning.

2.4.6.1 TOPO and PCR-Script cloning

MATERIALS

-PCR cloning kit

- Zero Blunt® TOPO® PCR cloning kit (Invitrogen, San Diego, USA)
  Zero Blunt® TOPO® PCR cloning provides a highly efficient, 5 minutes, one-step cloning strategy for the direct insertin of blunt-end PCR products generated by a proofreading polymerase (i.e. *Pfu*) into a plasmid vector (pCR®-Blunt II-TOPO®).
- PCR-Script® cloning kit (Stratagene, La Jolla, CA, USA)

-PCR product amplified by *Pfu* polymerase
-IPTG (for PCR-Script cloning)

IPTG is a galactose analogue not recognized by β-galactosidase. It is an inducer of the operon in *E. Coli*. IPTG can induce β-galactosidase gene (*lacZ*) expression allowing the selection of Lac+ recombinant bacteria in X-Gal containing medium.

Make a 1M stock solution by dissolving IPTG in deionized H_2O. Sterilize by filtration through a 0.22 μM filter (Millex® GP). Dispense the solution into 1-ml aliquots and store them at -20°C. IPTG is generally used at a 0.5-1mM concentration in LB medium. For LB agar plate, spread 50 μl of 10 mM IPTG and 50 μl of 2% X-gal on the prewarmed plate 30 minutes before spreading transformed *E. coli*.

- X-Gal (for PCR-Script cloning)

X-Gal is a β-galactosidase chromogenic substrate. In the presence of β-galactosidase, X-Gal is hydrolyzed and yields a blue precipitate. X-Gal is used for the identification and selection of β-galactosidase producing bacteria, they produce blue colonies on agar medium.

Make a 2% (20 mg ml^{-1}) stock solution by dissolving X-Gal in dimethylformamide. Use a glass or polypropylene tube. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and should be stored at -20°C. It is not necessary to sterilize X-Gal solutions by filtration. X-Gal is used at a 40 μg ml^{-1} concentration.

-LB agar plate containing

- 50 μg ml^{-1} kanamycin (for Topo cloning)
- 100 μg ml^{-1} ampicillin supplemented with IPTG and X-gal (for PCR-Scrpit cloning)

METHOD

All procedures were performed according to the instructions of the manual of the kit.

2.4.6.2 Expressing cloning

MATERIALS

-DNA

- Plasmid, pET-15b (Novagen, Darmstadt, Germany)
  This vector allows the expression from a T7 promoter of the gene of interest cloned in frame with a sequence for poly His-tag used in immobilized metal affinity chromatography (IMAC) purification prodecure.
- Gene (*phpA*, *psrA*, *vfr*) digested by *Nde*I and *BamH*I

-T4 DNA ligase and 10 × buffer (Invitrogen)

-Chemically competent DH5α (Invitrogen)

-*E. coli* BL21 (DE3) (Novagen)

METHOD

1. Ligate pET-15b and insert DNA.
2. Transform the ligation mixture into DH5α and select on LB agar plate containing Amp_{100}.
3. Miniprep, verify by digestion and DNA sequencing.
4. Transform the pET-15b-gene into *E. coli* BL21 (DE3) as step 2 and select on LB agar containing Amp_{100}. Miniprep, verify by digestion.
5. Small-scale expression of the target protein, see Materials and Methods 16.

2.4.6.3 Genomic DNA cloning

MATERIALS

-DNA

- Plasmid, 7.5 μg pUC18
- 2.5 μg genomic DNA of transposon insertion mutant

-Shrimp alkaline phosphatase (SAP) and 10 × buffer (Invitrogen)

-T4 DNA ligase and 10 × buffer

-Chemically competent DH5α (Invitrogen)
-*E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany)

METHOD

1. Digest plasmid pUC18 and genomic DNA with *Pst*I.
2. Inactivate *Pst*I by heating to 65°C for 15 minutes.
3. Incubate SAP (1 unit/ μg DNA) with restriciton-digested pUC18 at 37°C for 15 to 60 minutes in 10× SAP buffer in a final volume of 30 μl.
   After linearization with a single restriction enzyme, the pUC18 is dephosphorylated to reduce its ability to recircularize during ligation and to increase the proportion of recombinant clones.
4. Inactivate SAP by heating to 65°C for 15 minutes.
5. Use a 10 μl of dephosphorylated pUC18 aliquot (2.5 μg) for ligation with 2.5 μg genomic DNA digested by *Pst*I in a 30 μl volume.
6. Transform the ligation mixture into chemically competent DH5α and select on LB agar plate containing Amp_{100} and Gm_{10}.
7. Miniprep, verify by digestion, southern blot and DNA sequencing.

2.4.6.4 Other cloning

For example, in complementation test, gene was cloned into plasmid pUCP20, a shuttle vector for *E.coli* and *P.aeruginosa*, the procedure is similar as described before, always including digestion, ligation, transformation and verification.

3. Preparation of genomic DNA from *P.aeruginosa*

The standard way to remove proteins from nucleic acid solutions is to extract first with phenol:chloroform and then with chloroform. This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Although phenol denatures proteins efficiently, it does not completely inhibit RNAase activity, and it is a solvent for RNA molecules that contain long tracts of poly(A). Both of these problems can be circumvented by using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1). The subsequent extraction with chloroform:isoamyl alcohol removes any lingering traces of phenol from the nucleic acid preparation.

MATERIALS

-2 ml of overnight cultures of *P.aeruginosa*
-Buffers and solutions

- PBS
  - NaCl 8 g
  - KCl 0.2 g
  - Na_2HPO_4 1.44 g
  - KH_2PO_4 0.24 g

  Add 900ml of deionized H_2O. Shake until the solutes have dissolved. Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving.
- Resuspension buffer
  - 0.15 M NaCl
  - 0.1 M EDTA (pH 8.0)
- 10% SDS

A 10 % SDS (w/v) should be prepared in deionized H_2O and stored at room temperature. SDS is toxic, an irritant, and poses a risk of severe damage to the eyes. Wear appropriate gloves.

- 5M Sodium perchlorate, $NaClO_4$
- Phenol:chloroform:isoamyl alcohol (QBIOgene, Illkirch, France)

- Chloroform:isoamyl alcohol (QBIOgene)
- 100% ethanol (VWR International)
- TE
    - 10 mM Tris-HCl, pH8.0
    - 1 mM EDTA, pH 8.0

-Special equipment

- Vortexer (Bioblock Scientific, Illkirch, France)
- Centrifuge (Eppendorf, Hamburg, Germany)
- Thermomixer (Eppendorf) or a water bath preset to 60°C

METHOD

1. Collect 1.5 ml of overnigh cultures of *P.aeruginosa* in LB and centrifuge for 2 min at 9,000 rpm.
2. Wash pelleted bacteria by adding 1.5 ml of PBS to remove alginate and centrifuge for 1 min at 9,000 rpm.
3. Resuspend pelleted bacteria in 500 µl resuspension buffer and then add 100 µl 10% SDS to lysis the bacteria.
4. Mix gently and incubate at 60°C until the solution becomes clear (about 5 minutes).
5. Add 75 µl 5M $NaClO_4$ and mix well to stop the lysis.
6. Add 675 µl phenol:chloroform:isoamyl alcohol and mix thoroughly by vortexing for 1 min.
7. Centrifuge for 15 min at 14,000 rpm and collect aqueous phase (about 600 µl).
8. Repeat steps 6 through 7 (or just repeat step 7) if there is visible protein contamination in the collected aqueous phases.
9. Add an equal volume of chloroform:isoamyl alcohol and mix well.
10. Centrifuge for 15 min at 14,000 rpm and collect aqueous phase (about 500 µl).
11. Repeat step 10 if there is visible protein contamination.
12. Precipitate DNA by adding 2 volume of cold ethanol (Mix well and see cotton-like pellet).
13. Centrifuge for 15 min at 14,000 rpm at 4°C.
14. Discard the ethanol and wash the pellet by adding 1 ml of 70% ethanol and 30% TE.
15. Centrifuge for 5 min at 14,000 rpm at 4°C.
16. Discard the supernatants and dry the pellet in the air.
17. Dissolve the pellet by adding 50 µl H_2O and store them at -4°C.

4. Quantitation of nucleic acids

Two types of methods are widely used to measure the amount of nucleic acid in a preparation. If the sample is pure (i.e., without significant amounts of contaminants such as proteins, phenol, agarose, or other nucleic acids), spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the bases is simple and accurte.

Sometimes there is not sufficent DNA (<250 ng ml^{-1}) to assay spectrophotometrically, or the DNA may be heavily contaminated with other substances that absorb ultraviolet irradiation and therefore impede accurate analysis. A rapid way to estimate the amout of DNA in such samples is to utilize the ultraviolet-induced fluorescence emitted by EB molecules intercalated into the DNA. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescent yield of the sample with that of DNA molecular weight marker. As little as 1-5 ng of DNA can be detected by this method. This method is used to estimate the amount of DNA digested by restriction enzymes.

MATERIALS

-DNA samples, such as genomic DNA of *P.aeruginosa*.
-Spectrophotometer (Beckman, DU®640, Fullerton, California, USA)

METHOD

Take readings at wavelength of 260 nm and 280 nm. The reading at 260 mm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 μg ml^{-1} for double-stranded DNA, 40 μg ml^{-1} for single-stranded DNA and RNA, and ~ 33 μg ml^{-1} for single-stranded oligonucleotides.
The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure purifications of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0 respectively. If there is contamination with protein or phenol, the OD_{260}/OD_{280} will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

5. Polymerase chain reaction

Polymerase chain reaction (PCR), is a common method of creating copies of specific fragments of DNA. PCR rapidlly amplifies a single DNA molecule into many billions of molecules.

MATERIALS

-Template DNA.

The template can be a fragment of DNA, a preparation of genomic DNA, a plasmid, or any other DNA-containing sample. In the case of mammalian genomic DNA, up to 1.0 μg of DNA is utilized per reaction, an amount that contains ~3×10^5 copied of a single-copy autosomal gene. The typical amounts of yeast, bacterial, and plasmid DNAs used per reaction are 10 ng, 1 ng, and 1 pg, respectively.

-Forward primer and reverse primer.

Each primer should be 20-30 nucleotides in length and have no significant homology with other sequences on either strand of the target genome. G+C content should be between 40 and 60%, with a balanced distribution of all four bases along the length of the primer and a low propensity to form stable secondary structures. Restriction sites can be added to the 5' termini of the primer and these primer-specific restriction sites are transferred to the termini of the target DNA during amplification (It should be sure that there is no primer-specific restriction sites within amplified DNAs). Oligonucleotide primers can be synthesized on an automated DNA synthesizer and can generally be used in standard PCR without further purification. It is recommended that primers be reconstituted at concentrations greater than 10 μM in TE (10 mM Tris-HCl, pH8.0; 1 mM EDTA pH 8.0) and stored at -20°C. We stored all primers at concentration of 100 μM in H_2O at -20°C. Typically 0.1-0.5 μM of each primer is used and higher concentrations of primers favor mispriming, which may lead to nonspecifi amplification. All PCR primers used in this study are listed in Table 5. All primers were designed on the basis of PAO1 genome sequence (*http:www.pseudomonas.com*) and synthesizd by Invitrogen, Cergy pontoise, France.

-Thermostable DNA polymerase.

Taq DNA polymerase is the enzyme of choice for routine amplification of small segments of DNA. However, when greater fidelity is required, other thermostable DNA polymerase may have significant advantages. For example, if the goal is to make faithful copies of a gene, an enzyme with proofreading function is required, whereas if the goal is to clone an amplified product, an enzyme that generates blunt ends may be of advantage, *Pfu* polymerase is the choice in such case. Otherwises, mixtures of two or more DNA polymerases can significantly increase yield and enhence amplification, particularly of longer target DNAs.

- *Taq* DNA polymerase (QBIOgene, Illkirch, France)
- *Pfu* polymerase (Promega, Madison, USA)

-Buffers and solutions.

All thermostable DNA polymerases require free divalent cations, usually Mg^{2+}, for activity. Increasing the concentration of Mg^{2+} to 4.5 mM or 6 mM has been reported to decrease nonspecific priming in some cases and to increase it in others. The optimal concentration of Mg^{2+} must therefore be determined empirically for each combination of primers and template. Optimization can be achieved by comparing the yield obtained from a series of ten PCRs containing concentration of Mg^{2+} ranging from 0.5 mM to 5.0 mM, in 0.5 mM increments. If

possible, preparations of template DNA should not contain significant amounts of chelating agents such as EDTA or negatively charged ions, such as PO_4^{3-}, which can sequester Mg^{2+}. DMSO has been shown to facilitate DNA strand separation (in GC rich difficult secondary structures) because it disrupts base pairing and has been shown to improve PCR efficiency (Sambrook and Russell, 2001). Sometimes 5% DMSO was added in our PCR.

- 10 × amplification buffer (QBIOgene)
- dNTP solution containing all four dNTPs (QBIOgene)

-Additional reagents

- Reagents needed for agarose gel electrophoresis (see Materials and Methods 6)

-Special equipment

- Microfuge tubes (0.25 ml or 0.5 ml)
- Thermal cycler (Mastercycler®, Hamburg, Germany or MJ MiniCycler™, watertown, USA)

  If the thermal cycler is not equipped with a heated lid (for example, MJ MiniCycler™), use either mineral oil or parafin wax to prevent evaporation of liquid from the reaction mixture during PCR. If mineral oil was used to overlay the reaction, remove the oil from the sample by extraction with 150 µl of chloroform for the purpose such as cloning.
- Equipment needed for agarose gel electrophoresis

METHOD

1. In a sterile 0.25 or 0.5 ml microfuge tube, mix in the following order:
   - 10 × amplification buffer 5 µl
   - 5 mM solution of four dNTPs 2 µl
   - 10 µM (pmol μl^{-1}) forward primer 2 µl
   - 10 µM (pmol μl^{-1}) reverse primer 2 µl
   - 5 unit μl^{-1} thermostable DNA polymerase 0.2 µl
   - H_2O 33-38 µl
   - Template DNA 1-5 µl
   - Total volume 50 µl

TABLE 6. Standard reaction conditions for PCR

Mg^{2+}	KCl	dNTPs	Primers	DNA polymerase	Template DNA[a]
1.5 mM	50 mM	200 µM	0.1-1 µM	1-5 units	1 pg to 1 µg

[a]For bacterial colony PCR, pick colony with a steril toothpick and dab the toothpick into a microfuge tube containing 100 µl water. Heat the tube at 95°C for 10 minutes to disrupt the cells. Centrifuge at 6000g for 5 min and use the supernanant as template DNA.

2. Programming PCR

   PCR is an iterative process, consisting of three elements: denaturation of the template by heat, annealing of the oligonucleotide primers to the single-stranded target sequence(s), and extension of the annealed primers by a thermostable polymerase.

   Double-stranded DNA templates denature at a temperature that is determined in part by their G+C content. The higher the proportion of G+C, the higher the temperature required to separate the strands of template DNA. 45 seconds at 94-95°C are recommended for routine amplification of linear DNA templates whose contents of G+C is 55% or less. In the first cycle of PCR, denaturation is sometimes carried out for 5 minutes to increase the probability that long molecules of templae DNA are fully denatured.

   The temperature used for the annealing step (Ta) is critical. If the annealing temperature is too high, the primers anneal poorly to the template and the yield of amplified DNA is very low. If the annealing temperature is too low, nonspecific annealing of primers may occur, resulting in the amplification of unwanted segments of DNA. Annealing is usually carried out 3-5°C lower that the calculating melting temperature at which the oligonucleotide primers dissociate from their templates. The melting temperature (Tm) of hybrids between oligonucleotide primers can be easily estimated using classical equation: Tm (in °C)=2 (A+T) + 4 (G+C), where (A+T) is the sum of the A and T residues in the oligonucleotide and (G+C) is the sum of G and C residues in the oligonucleotide.

Extension of primers is carried out at or near the optimal temperature for DNA synthesis catalyzed by the thermostable polymerase, which in the case of *Taq* DNA polymerase is 72-78°C. Extension is carried out for 1 minute for every 1000 bp of product. For the last cycle of PCR, an extension time that is generally three times longer than in the previous cycles is used to allow completion of all amplified products.

The number of cycles required for amplification depends on the number of copies of template DNA present at the beginning of the reaction and the efficiency of primer extension and amplification. At least 25 cycles are required to achieve acceptabe levels of amplification of single-copy target sequences in mammalian DNA templates.

3. Amplify the nucleic acids using the desired program, an example listed below
    - 94°C 5 min
    - 94°C 45 sec
    - 60°C 45 sec
    - 72°C 1 min (for 1 kb of length of the target DNA)
    - repeat steps 2-4 for another 34 cycles
    - 72°C 7 min
    - 4°C up to overnight

6. Agarose Gel Electrophoresis

Electrophoresis through agarose gel lies near the heart of molecular cloning and is used to separate, identify, and purify DNA fragments. The technique is simple, rapid to perform, and capable of separating fragments of DNA that cannot be separated adequately by other procedures, such as density gradient centrifugation. Furthermore, the location of DNA within the gel can be determined directly by staining with low concentrations of fluorescent intercalating dyes, such as ethidium bromide; bands containing as little as 20 pg of double-stranded DNA can be detected by direct examination of the gel in UV. If necessary, these bands of DNA can be recovered from the gel and used for a variety of purposes.

The two major classes of agaroses are standard agaroses and low-melting temperature agaroses whose melting teperatures are near 95°C and about 65°C respectively. Agarose gels have a great range of separation. DNAs from 50 bp to several megabases in length can be separated on agarose gels of various concentrations and configuraions, see Table 7.

TABLE 7. Range of separation of DNA fragments through different types of agarose

	Size range of DNA fragments separated by agaroses	
Agarose (%)	Standard	Low melting temperature
0.5	700 bp to 25 kb	
0.8	500 bp to 15 kb	800 bp to 10 kb
1.0	250 bp to 12 kb	400 bp to 8 kb
1.2	150 bp to 6 kb	300 bp to 7 kb
1.5	80 bp to 4 kb	200 bp to 4 kb
2.0		100 bp to 3 kb
3.0		50 bp to 1 kb

MATERIALS

-Agarose

- Standard agarose, (SeaKem®GTG® agarose, Rockland, ME, USA)
- Low melting temperature agarose (NuSieve®GTG® agarose, Rockland, ME, USA)

Agarose should be melted in the electrophoresis buffer that provide electrical conductivity and buffering capacity.

-Buffers and solutions

- Electrophoresis buffer, 50 × TAE stock solution, per liter

- Tris base 242 g (2 M)
- Glacial acetic acid 57.1 ml (2 M)
- EDTA (0.5M, pH 8.0) 100 ml (50 mM)

- EDTA (0.5M, pH 8.0)
  Add 186.1g disodium ethylenediaminetetraacetate.2H_2O to 800 ml of deionized H_2O. Stir vigorously and adjust the pH to 8.0 with NaOH (~20g of NaOH pellets, the disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0). Dispense into aliquots and sterilize by autoclaving.
- Ethidium bromide: 10 mg ml^{-1} (20,000 ×) (Sigma).
  EB is a powerful mutagen and is toxic. Avoid breathing the dust. Wear appropriate gloves when working with solutions that contain this dye. Specific handling and disposal procedures are recommended by the laboratory.
- Gel-loading buffer: Blue/Orange 6 × loading dye (Promega, Madison, WI, USA).
  Gel-loading buffers are mixed with the samples before loading into the slots of the gel. These buffers serve three purposes: They increase the density of the sample, ensuring that the DNA sinks evenly into the well; they add color to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels run in 0.5 × TAE at approximately the same rate as linear double-stranded DNA 300 bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double-stranded DNA 4 kb in length.

-Nucleic acids and oligonucleotides

- DNA samples, such as PCR product, digested plasmids.
- Molecular weight marker: 1 kb DNA ladder (100 ng μl^{-1}) (Promega)

-Special equipment

- Electrophoresis apparatus with chamber and comb (Embi Tec, San Diego, CA, USA)
- Gel-sealing tape
- Microwave oven (Bluesky, Meylan, France)
- Electrophoresis power supply device (Apelex, Massy, France)
- UV device (Bioblock Scientific, Illkirch, France)

UV light and/or UV radiation is dangerous and can damage the retina of the eyes. Never look at an unshielded UV light source with naked eyes. View only through a filter or safety glasses that absorb harmful wavelengths. Wear protective appropriate gloves when holding materials under the UV light source.

- Imaging device (Vilber lourmat, Marne-La-Vallee, France)

METHOD

1. Prepare sufficient electrophoresis buffer (0.5 × TAE) to to cast the gel and fill the electrophoresis tank.
2. Prepare a solution of agarose in 0.5 × TAE at an appropriate concentration (usually 1% (w/v)) in an Erlenmeyer flask (the gel should be between 3 mm and 5 mm thick).
3. Heat the slurry in a microwave oven until the agarose dissolves and add EB to a final concentration of 0.5 μg ml^{-1} when the molten gel has cooled (about 55°C).
4. Mix the gel solution thoroughly by gentle swirling and pour the agarose solution into the mold containing an appropriate comb for forming the sample slots in the gel.
5. Allow the gel to set completely (30-45 minutes at room temperature) and mount the gel in the electrophoresis tank.
6. Add electrophoresis buffer (without EB) to cover the gel to a depth of ~ 1 mm.
7. Mix the samples of DNA with 6 × gel-loading buffer.
8. Load the DNA marker and the sample mixture into the slots of the submerged gel.
9. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1-5 V cm^{-1} (measured as the distance between the positive and negative electrodes). Bubbles should be generated at the anode and cathode (due to electrolysis).

10. Run the gel until the bromophenol blue and xylene cyanol EF have migrated an appropriate distance through the gel.
11. Turn off the electric current and remove the leads and lid from the gel tank.
12. Photograph the gel using the imaging device (or just examine the gel by UV device)
13. Imprint and save the image.

7. Recovery of DNA from agarose gel

Many methods and many kits have been developed over the year to recovered DNA from agarose gels. Two such kits were used in our work.

MATERIALS

-Gel Extraction kit

- QIAquick Gel Extraction kit (Qiagen, Courtaboeuf, France)
  QIAquick Gel Extraction kit is designed for extraction of DNA fragments (70 bp to 10 kb) from standard, or low-melting agarose gels in TAE or TBE buffer and DNA cleanup from enzymatic reaction.
- UltraCleanTM15 DNA purification kit (Mo Bio, Solana Beach, USA)
  UltraClean^{TM}15 kit is a 10-15 minute DNA purification kit. DNA can be recovered from TAE and TBE agarose gels, solutions, and enzymatic reactions. The DNA size range that can be purified is 60 bp to 50 kb.

-Agarose gel containing the DNA fragment of interest (at least 100 ng)

-Additional regents

- Isopropanol (needed only for very big or small DNA fragment)

-Special equipment

- Equipment needed for agarose gel electrophoresis
- Scapel
- Thermomixer (Eppendorf, Hamburg, Germany) or a water bath preset to 50°C
- Centrifuge (Eppendorf)
- UV device (Bioblock Scientific, Illkirch, France)

METHOD

All procedures were performed according to the instructions of the manual of the kit.

8. SDS-PAGE of proteins

Almost all analytical electrophoresis of proteins are carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with a reducing agent and heat to dissociate the proteins before they are loaded onto the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bounds almost always proportional to the molecular weigh of the polypeptide and is independent of its sequences, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. At saturaion, ~ 1.4 g of detergent is bound per gram of polypeptide. By using markers of known molecular weight, it is possible ot estimate the molecular weight of the polypeptide chain(s). Modifications to the polypeptide backbone, such as N- or O-linked glycosylaion, however, have a significant impact on the apparent molecular weight. Thus, the apparet molecular weith of glycosylated proteins is not a true reflection of the mass of the polypeptide chain.

In most cases, SDS-PAGE is carried out with a dicontinuous buffer system. The sample and the stacking gel contain Tris-HCl (pH 6.8), the resolving gel contains Tris-HCl (pH8.8), and the electrophoresis buffer contains Tris-glycine (pH 8.3). All components of the system

contain 0.1 % SDS. The SDS-polypeptide complexes in the sample that is applied to the gel are swept along by a moving boundary created when an electric current is passed between the electrodes. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone (or stack) on the surface of the resolving gel. The ability of discontinuous buffer systems to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS-polyacrylamide gels. The SDS-polypeptide complexes move through the resolving gel and are separated according to size by sieving.
The effective range of separation of SDS-polyacrylamide gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross-linking (see Table 8).

TABLE 8. Effective range of separation of SDS-polyacrylamide gels

Acrylamide concentration (%)	Linear range of separation (kDa)
15	10-43
12	12-60
10	20-80
7.5	36-94
5.0	57-212

8.1 SDS-PAGE

MATERIALS

-Acrylamide solution

- 30 % Acrylamide and N,N'-methylene-bis-acrylamide solution, 37.5 :1 (Bio-Rad, Hercules, CA, USA).

Monomers of acrylamide are polymerized into long chains in a reaction initiated by free radicals. In the presence of N,N'-methylene-bis-acrylamide, these chains become cross-linked to form a gel. The porosity of the resulting gel is determined by the length of chains and degree of cross-linking that occurs during the polymerization reaction. Acrylamide (unpolymerized) is a potent neurotoxin and is absorbed through the skin. Wear appropriate gloves. Polyacrylamide is considered to be nontoxic, but it should be handled with care because it might contain small quantities of unpolymerized acrylamide.

- 1.5 M Tris (pH 8.8)
- 1.0 M Tris (pH 6.8)

Dissolve 181.65 g or 121.1 g of Trizma base®, minimum (Sigma) in 800 ml of H2O. Adjust the pH to the desired value by adding concentrated HCl (wear appropriate gloves and perform with great care in a chemical fume hood). Allow the solution to cool to room temperature before making final adjustments of the pH since the pH of Tris solutions is temperature-dependent and decreases ~0.03 pH units for each 1°C increase in temperature. Adjust the volume of the solution to 1 liter with H2O. Dspense into aliquots and sterilize by autoclaving. Store at room temperature. Tris may be harmful.

- 10% SDS

Exclusive use of one brand of SDS is recommended since the pattern of migration of polypeptides may change quite drastically when one manufacturer's SDS is substituted for another's.

- Ammonium persulfate (Bio-Rad)

Ammonium persulfate (AP) provides the free radicls that drive polymerization of acrylamide and bisacrymamide. A small amount of a 10 % (w/v) stock solution should be prepared in deionized H_2O and stored at 4°C. AP decomposes slowly, and fresh solutions should be prepared weekly.

- TEMED (Bio-Rad)

TEMED accelerates the polymerization of acrylamide and bisacrylamide by catalyzing the formation of free radicals from ammonium persulfate. TEMED is extremely destructive to tissues of the mucous membranes and upper respiratory tract, eyes,and skin. Wear appropriate gloves and use in a chemical fume hood

See Table 9 for resolving gel recipes and Table 10 for stacking gel recipes.

TABLE 9. Solutions for resolving gels for SDS-PAGE

Concentration	6 %		8 %		10 %		12 %		15 %	
Components /volume	10ml	30ml	10ml	30ml	10ml	30ml	10ml	30ml	10ml	30ml
H_2O	5.3	15.9	4.6	13.9	4.0	11.9	3.3	9.9	2.3	6.9
30 % crylamide/bis	2.0	6.0	2.7	8.0	3.3	10.0	4.0	12.0	5.0	15.0
1.5 M Tris (pH 8.8)	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5
10 % SDS	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3
10% AP	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3	0.1	0.3
TEMED	0.008	0.024	0.006	0.018	0.004	0.012	0.004	0.012	0.004	0.012

TABLE 10. Solutions for 5 % stacking gels for SDS-PAGE

Components/volume	1 ml	2 ml	5 ml	6 ml
H_2O	0.68	1.4	3.4	4.1
30 % acrylamide/bis	0.17	0.33	0.83	1.0
1.0 M Tris (pH 6.8)	0.13	0.25	0.63	0.75
10 % SDS	0.01	0.02	0.05	0.06
10% AP	0.01	0.02	0.05	0.06
TEMED	0.001	0.002	0.005	0.006

-Protein marker (Amersham Biosciences, Buckinghamshire, UK)

-10 × SDS-PAGE buffer

- 250 mM Tris
- 2.5 M glycine (pH 8.3)
- 1 % (w/v) SDS

-5 × SDS gel-loading buffer

- 250 mM Tris-HCl (pH 6.8)
- 500 mM dithiothreitol
- 10 % (w/v) SDS
- 0.5 % bromophenol blue
- 50 % glycerol

-Protein samples

Samples to be resolved, for example, cell lysates, purified protein.

-Special equipment

- Electrophoresis power supply device (Apelex, Massy, France)
- Vertical electrophoresis apparatus (Amersham Biosciences, San Francisco, CA, USA)
- Thermomixer (Eppendorf, Hamburg, Germany) preset to 99°C
- 20 µl or 50 µl Microliter™ syringe (Hamilton, Bonaduz, Switzerland)

METHOD

1. Assemble the glass plates.
2. Prepare the appropriate volume (5 ml for a minigel, 8 cm × 10 cm × 0.1 cm; and 30 ml for a big gel, 15 cm × 18 cm × 0.15 cm) and concentration (often 12 %) of solution for resolving gel using values given in Table 9.
3. Mix the acrylamide solution and pour it into the gap between the glass plates as soon as the TEMED has been added. Use a Pasteur pipette to carefully overlay the acrylamide solution with H_2O (best containing 0.1 % SDS) in order to prevent oxygen from diffusing into the gel and inhibiting polymerization.

4. Place the gel in a vertical position at room temperature until the polymerization is complete (about 30 minutes). Pour off the overlay and wash the top of the gel with H_2O. Remove any remaining H_2O with the edgy of a paper towel.
5. Prepare the stacking gel (1 ml for a minigel and 5 ml for a big gel) using values given in Table 10 and pour it directly onto the surface of the polymerized resolving gel.
6. Insert quickly a comb into the stacking gel and place the gel in a vertical position at room temperature.
7. Prepare the samples in the SDS gel-loading buffer and heat them, including protein marker, to 100°C for 5 minutes to denature the proteins.
8. Remove the comb carefully after the polymerization is complete (about 30 minutes). Wash the wells immediately with H_2O to remove any unpolymerized acrylamide.
9. Mount the gel in the electrophoresis apparatus and add SDS-PAGE buffer to the top and bottom reservoirs.
10. Load up to 15 µl of each of the samples in a predetermined order into the bottom of the wells using microliter syringe.
11. Apply a voltage of 8V/cm to the stacking gel and then increase the voltage to 15 V/cm to the resolving gel. Run it until the bromophenol blue reaches the bottom of the gel.
12. Mark the orientation of the gel and then the gel can be stained or used to establish an immunoblot as described on the following pages.

8.2 Staining SDS-polyacrylamide gels by Coomassie Brilliant Blue

Unlabeled proteins separated by SDS-PAGE typically are detected by staining, either with Coomassie Brilliant Blue or with silver salts. In a relatively rapid and straight-forward reaction, Coomassie Brilliant Blue binds nonspecifically to proteins but not to the gel, thereby allowing visualization of the proteins as discreet blue bands. Silver staining, although somewhat more difficult to perform, is nearly 100-fold more sensitive than staining with Coomassie Brilliant Blue and is capable of detecting as little as 0.1 ~ 1 ng of polypeptide in a single band. Kits for silver staining are commercially available from Pierce and Bio-Rad.

MATERIALS

-Coomassie Brilliant Blue R 250 (Merck, Darmstadt, Germany). Dissolve 0.25 g of Coomassie Brilliant Blue R250 per 100 ml of methanol:acetic acid: H_20 (5:1:4, v/v/v) solution. Filter the solution through a Whatman No. 1 filter to remove any particulate matter.

-Methanol: glacial acetic acid:H_2O (5:1:4, v/v/v)

-SDS-polyacrylamide gel containg separated proteins (See Materials and Methods 8.1)

METHOD

1. Immerse the gel in the Coomassie Brilliant Blue R 250 solution.
2. Rotate slowly for a minimum of 1 hour at room temperature.
3. Remove the staining solution and save it for future use.
4. Destain the gel by soaking it in the methanol: glacial acetic acid: H_2O (5:1:4, v/v/v) on a slowly rocking platform for 4-8 hours, changing the destaining solution several times (Use methanol: glacial acetic acid: H_2O 3:1:6, v/vv, for a more rapid destainig).
5. Store indefinitely the fixed gel in H_2O containg 20 % glycerol before drying.

8.3 Air drying SDS-polyacrylamide gels

MATERIALS

-Owl Gel-Drying kits (Owl Sepration Systems, Portsmouth, NH, USA)

- Two sets of gel-drying frames

- 50 sheets of precut cellophane

-Methanol (20 %) containing 3% glycerol
-SDS-polyacrylamide gel from Materials and Methods 8.2.

METHOD

1. Soak the fixed gel in 20 % methanol containing 3% glycerol for at least 1 h.
2. Wash the cellophane with H_2O.
3. Place the gel between two sheets of cellophane and remove any bubble if possible.
4. Fix the cellophane on the gel-drying frame and place them in a vertical position at room temperature for several days until the gel is dry.

9. Preparation of labeled DNA probes

Radioactive isotopes, usually ^{32}P and ^{35}S, are used to detect hybridization between labeled probes and target sequences. Although radiolabeled probes can detect minimal quantities of immobilized target DNA (< 1 pg), they suffer from a low stability (always less than 6 weeks). In addition, the exposure to radiation, the storage and disposal of low-level radioactive may be significant.

The most used nonradioactive method was based on the labeling of the nucleic acid probe with biotin. After hybridization, the biotinylated probe was detected via interaction with streptavidin that had been conjugated to a reporter enzyme, usually horse radish peroxidase (HRP). The membrane was then exposed to an enzyme capable of hydrolyzing a colorigenic, fluorogenic, or chemiluminescent substrate. The sensitivity of this systmem derives from the rapid enzymatic conversion of the substrate to a product that is colored or emits visible or UV light at a specific wavelength. The distribution and intensity of the product correspond to the spatial distribution and concentration of target sequences on the membrane.

Digoxigenin (DIG), like biotin, can be chemically coupled to linkers and nucleotides. DIG-labeled hybrids are detected with an anti-DIG Fab fragment conjugated to a reporter enzyme, usually alkaline phosphatase (AP), whose best chromogenic substrates are 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

9.1 Labelig probes with ^{35}S

MATERIALS

-[^{35}S]-dATP (PerkinElmer^{TM}, Boston, MA, USA)
-1 mM dGTP (Roche, Meylan, Rance)
-5 unit μl^{-1} Klenow enzyme (Roche)

DNA polymerase I can be cleaved by mild treatment with subtilisin into two fragments, the larger fragment is known as the Klenow fragment and the smaller fragment is nameless. Klenow fragment, which retains the DNA polymerase and the 3'→5'exonuclease activities of the holoenzyme but lack its powerful 5'→3' exonuclease activity, is used to fill recessed 3' termini of double-stranded DNA.

-10 × SuRE/Cut Buffer H for Restriction enzyme (Roche)
-Linearized DNA: a 280-bp *exsCEBA* promoter (p*C*), 3 μg plasmid pIApC of Miniprep digested by *BamH*1 and purified from agarose gel.
-Thermomixer (Eppendorf, Hamburg, Germany)

METHOD

1. Add the following in a sterile 1.5 ml microfuge tube:
   - 100 ng μl^{-1} linearized DNA 30 μl
   - 1 mM dGTP# 2.5 μl
   - 5U μl^{-1} Klenow enzyme 2 μl

- 10 × SuRE/Cut Buffer H 5 µl
- H_2O 8.5 µl
- [^{35}S]-dATP 2 µl
- Totla volume 50 µl

dGTP were added, *BamH*1 $_{5'}$-G $_{OH3'}$
$_{3'}$-CCTAG $_{P5'}$

2. Mix well.
3. Incubate for 2 hours at 37°C.
4. Stop reaction by incubation for 10 min at 80°C.
5. Decrease slowly the temperature to 25°C and then store the samples at 4°C.
   All procedure was carried out in special room for radioactive manipulation. Always wear appropriate gloves, lab coat and safety goggles. Always monitor thoroughly after using radioisotopes.

9.2 Labeling probes with Biotin

MATERIALS

-Biotin-16-dUTP (Roche, Meylan, Rance)
-1 mM dATP (Roche)
-1 mM dGTP (Roche)
-5 unit µl^{-1} Klenow enzyme (Roche)
-10 × SuRE/Cut Buffer H for Restriction enzyme (Roche)
-Linearized DNA

- A 280-bp *exsCEBA* promoter (p*C*), 3 µg of plasmid pIApC of Miniprep digested by *BamH*1 and purified from agarose gel.
- A 280-bp *psrA* promoter (p*psrA*), 3 µg of plasmid pp*psrA* of Miniprep digested by *Eco*R1 and purified from agarose gel.
- A 280-bp *fleQ* promoter (p*fleQ*), 3 µg of plasmid pp*fleQ* of Miniprep digested by *Eco*R1 and purified from agarose gel.

-Thermomixer (Eppendorf)

METHOD

1. Add the following in a sterile 1.5 ml microfuge tube:
   - 100 ng µl^{-1} linearized DNA 30 µl
   - 1 mM Biotin-16-dUTP 2 µl
   - 1 mM dATP* 2.5 µl
   - 1 mM dGTP# 2.5 µl
   - 5U µl^{-1} Klenow enzyme 2 µl
   - 10 × SuRE/Cut Buffer H 5 µl
   - H_2O 6 or 8.5 µl
   - Totla volume 50 µl

   * Only dATP was added for p*psrA* and p*fleQ*/ *Eco*R1 $_{5'}$-G $_{OH3'}$
   $_{3'}$-CTTAA $_{P5'}$

   # Both dGTP and dATP were added for p*C*/*BamH*1 $_{5'}$-G $_{OH3'}$
   $_{3'}$-CCTAG $_{P5'}$

2. Mix well.
3. Incubate for 2 hours at 37°C.
4. Stop reaction by incubation at 80°C for 10 min.

5. Decrease slowly the temperature to 25°C and then store the samples at -20°C.
6. Test the labeling efficiency using EMSA (see Materials and Methods 15)

9.3 Labeling probes with Digoxigenin

MATERIALS

-10 × PCR DIG labeling Mix (Roche)
- 2 mM dATP
- 2 mM dCTP
- 2 mM dGTP
- 1.9 mM dTTP
- 0.1 mM DIG-11-dUTP

-Thermomixer (Eppendorf)
-Other reagents and special equipment needed for PCR (See Materials and Methods 5)

METHOD

1. PCR (See Materials and Methods 5).
2. Prepare low melting agarose gel (See Materials and Methods 6).
3. Agarose gel electrophoreis (See Materials and Methods 6) and cut the band.
4. Store the band (probe) at -20°C.
   Attention, the PCR product amplified using PCR DIG labeling Mix is bigger than that using normal dNTP mix.

10. Southern hybridization

Southern transfer and hybridization is used to study how genes are organized within genomes by mapping restriction sites in and around segments of genomic DNA for which specific probes are available. Genomic DNA is first digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through a standard agarose gel. The DNA is then denatured *in situ* and transferred from the gel to a solid support (usually a nylon membrane, which is far more durable and has a higher binding capacity than a nitrocellulose membrane). The DNA attached to the membrane is hybridized to a labeled DNA probe (or RNA, or oligonucleotide probe) and bands complementary to the probe are located by an appropriate detection system, for example, by traditional autoradiography or by enzymatic methods. By estimating the size and number of the bands generated after digestion of the genomic DNA with different restriction enzymes, singly or in combination, it is possible to place the target DNA within a context of restriction sites.
In our study, southern hybridization was performed to verify that the mutants were caused by a single transposition event (that is to say there is only one copy of *aacC1* inserted in genomic DNA) and to obtain useful informations for subsequent cloning.

MATERIALS

-Buffers and solutions
- Denaturation solution
  - 1.5 M NaCl
  - 0.5 M NaOH
- Neutralizing buffer
  - 0.5 M Tris (pH7.5)
  - 1.5 M NaCl
- 20 × SSC (neutral transfer buffer)
  - 3M NaCl

    - 300 mM sodium citrate
    - Adjust the pH to 7.0 with NaOH and autoclave.
- Blocker (Roche, Meylan, France)
  Blocking agents prevent ligands from sticking to surfaces. They are used to decrese nonspecific binding of probes in Southern, Northern and Western blotting. If left to their own devices, these probes would bind tightly and nonspecifically to the supporting nitrocellulose or nylon membrane. Without blocking agents, it would be impossible to detect anything but the strongest target macromolecules. Two blocking agents in common use in nucleic acid hybridization are Denhardt's reagent and BLOTTO (Bovine Lacto Transfer Technique Optimizer) (Sambrook and Russell, 2001).
- 10 % (w/v) blocking solution (10 g blocker in 100 ml of detection solution 1)
- Prehybridization/hybridization solution (DIG Easy Hyb, Roche)
    - 5 × SSC
    - 1 % (w/v) blocking solution
    - 0.02 % (w/v) SDS
    - % (w/v) N-lauroylsarcosine (detergent)
- Washing buffer 1: 2 × SSC containing 0.1% SDS
- Washing buffer 2: 0.1 × SSC containing 0.1% SDS
- Detection solution 1
    - 150 mM NaCl
    - 100 mM maleic acid
    - Adjust the pH to 7.5 with NaOH and autoclave.
- Detection solution 2: detection solution 1 containing 1 % (w/v) blocking solution
- Detection solution 3
    - 100 mM Tris
    - 100 mM NaCl
    - 50 mM $MgCl_2$
    - Adjust the pH to 9.5 with HCl and autoclave.
- DIG Nucleic Acid Detection Kit (Roche)
    - Anti-DIG AP-conjugate
    - NBT-BCIP stock solution

-Enzymes and buffers

- *Pst*1 (Invitrogen)

-Nucleic acids and oligonucleotides

- Genomic DNA, ~ 5 µg
- Digoxigenin labeled probe DNA: low-melting temperature agaroses containing 50 µl PCR product of *aacC*1.

-Materials needed for agarose (0.8 %) gel electrophoresis

-Special equipments

- Transparent ruler
- Nylon membranes, positively charged (Roche, Indianapolis, IN, USA)
- Whatman 3MM (Whatman®, Middlesex, UK)
- Model 785 Vacuum blotter unit (Bio-Rad, Hercules, CA, USA)
- Liquid trap
- Cross-linking device: UV Stratalinker® 1800 (Stratagene, La Jolla, CA, USA)
- Water bath, boiling
- Hybridization oven (Bioblock Scientific, Illkirch, France)
- Roller bottles (Schott, Stafford, UK)
- Rotary platform shaker (Stuart Scientific, Watford Herts, UK)

METHOD

-Digestion and electrophoresis of genomic DNA

1. Digest 5 μg genomic DNA with 30 units *Pst1* (generally 5 units per μg DNA) at 37°C overnight in a total volume of 50 μl.
2. Incubate at 80°C for 10 minutes to inactivate the enzyme and wait until the temperature has decreased to 37°C.
3. Add 1 μg RNase to degrade RNA at 37°C for 10 minutes.
   Steps 2 and 3 are not obligatory.
4. Separate the DNA fragments by electrophoresis through an agarose gel (0.8%).
5. Photograph the gel by placing a transparent ruler alongside the gel.
   A complete digestion should give a smear.
6. Place the gel in 0.25 N HCl for 15 minutes with agitation.
7. Remove the 0.25 N HCl and rince the gel twice with water.
   Steps 6 and 7 are recommended by the instruction manuel of Bio-Rad, but we haven't performed these two steps.

-Preparation for vaccum transfer

8. Cut a nylon membrane (cut off a small corner to simplify orientation) and Whatman 3MM according to the size of the gel and wet them with water.
   Use very clean forceps and powder-free gloves, and handle the membrane only at the corners.
9. Setup vaccum blotter. (See instruction manuel of Bio-Rad for more details and informations).

-Vaccum transfer DNA from agarose gels to nylon membrane

10. Start slowly the vaccum source until the gauge reads at 5 inches of mercury.
11. Denature immediately the gel by pouring 15 ml of denaturation solution for 15 minutes.
12. Neutralize the gel by pouring 10 ml of neutralizing buffer for 5 minutes.
13. Repeat step 12.
14. Transfer the gel by pouring 20 × SSC (about 50 ml) for 1 hour. Keep the gel humide.
15. Turn off the vacuum source and soak the membrane in 5 × SSC for 5 minutes.
    Several other methods, such as electrophoretic transfer, can be used to transfer DNA from agarose gels to nylon membrane. Although single-stranded DNA and RNA could be transferred directly, fragments of double-stranded DNA must first be denatured *in situ* as step 11, neutralized as step 12 and then soaked in electrophoresis buffer (0.5 × TAE) before electrophoretic transfer.

-Cross-linking DNA to the membrane by UV irradiation

16. Place the damp membrane on a dry piece of paper and cross-link the DNA to the membrane by UV Stratalinker® 1800 using 'auto cross link' . Cross-link once for each side of the membrane.

-Hybridization of probes to DNA immobilized on nylon membrane

17. Soak the membrane in 5 × SSC for 2 minutes.
18. Roll gently the wetted membrane into a hybridization roller bottle. Add 15 ml of prehybridization solution (at least 0.1 ml per 1 cm^2 of membrane). Close the bottle tightly.
19. Place the hybridization bottle inside a prewarmed hybridization oven at 68°C for 1 h.
    Attention, 42°C for solvents containing 50 % formamide; 65°C for phosphate-SDS solvents.
20. Denature the probe DNA (PCR product of *aacC*1 in low-melting temperature agarose) by heating at 100°C for 5 minutes and chilled rapidly on ice for 2 minutes. Store the probe DNA at 68°C (if not, the agarose will gel).
    Single-stranded DNA or RNA probes need not be denatured.
21. Pour off the prehybridization solution from the hybridization bottle and replace with fresh hybridization solution containing probe.
    Collect both prehybridization and hybridization solutions which could be reused and store them at -20°C.

22. Seal the bottle and replace it in hybridization oven overnight with rolling at 68°C.

-Detection of hybridization

23. Pour off the hybridization buffer and wash the membrane with 25 ml of washing buffer 1 (2 × SSC containing 0.1% SDS, at least 1 ml per 1 cm^2 of membrane) at room temperature for 15 minutes. Repeat another wash. Pour off the wahsing buffer 1.
24. Add 25 ml of washing buffer 2 (0.1 × SSC containing 0.1% SDS, at least 1 ml per 1 cm^2 of membrane) at 68°C for 15 minutes. Repeat another wash.
25. Remove the membrane from the hybridization bottle and place it in a tray with appropriate size (as small as possible to economize the reagent).
26. Soak the membrane in 20 ml of detection buffer 1 (150 mM NaCl, 100 mM maleic acid, pH 7.5) and incubate at room temperature with gentle agitation for 2 minutes. Pour off the detection buffer 1.
27. Add 20 ml of detection buffer 2 (detection buffer 1 containing 1 % (w/v) blocking solution). Incubate at room temperature with gentle agitation for 1 hour. Pour off the detection buffer 2.
28. Add 10 ml of Anti-DIG AP-conjugate solution (1:5000 Anti-DIG AP-conjugate in dection buffer 2). Incubate at room temperature with gentle agitation for 30 minutes. Pour off the solution.
29. Add 20 ml of detection buffer 2 and incubate at room temperature with gentle agitation for 15 minutes. Repeat once again and pour off the solution.
30. Add 20 ml of detection buffer 3 and incubate at room temperature with gentle agitation for 2 minutes. Pour off the solution.
31. Add 10 ml of relevation solution (200 μl NBT-BCIP stock solution in 10 ml of detection buffer 3) and incubate at room temperature with gentle agitation until the bands have appeared (about 30 minutes). Prevent light during the incubation.
32. Remove the membrane and wash it with water for several times.
33. Dry the membrane between two sheets of paper towels.
34. Take a photo and restore the membrane in an envelope.

11. Western blotting

Western blotting is used to identify and measure the size of macromolecular antigens (usually proteins) that react with a specific antibody. The proteins are first separated by electrophoresis through SDS-polyacrylamide gels and then transferred electrophoretically from the gel to a solid support, such as a nitrocellulose, cationic nylon membrane or polyvinylidene difluoride. After the unreacted binding sites of the membrane are blocked to suppress nonspecific adsorption of antibodies, the immobilized proteins are reacted with a specific polyclonal or monoclonal antibody. Antigen-antibody complexes are finally located by radiographic, chromogenic, or chemiluminescent reactions. As little as 1-5 ng of an average-sized protein can be detected by Western blotting.

MATERIALS

-SDS-polyacrylamide gel containg separated proteins (See Materials and Methods 8)

-Ponceau S (Sigma)

Ponceau S may be used to prepare a stain for rapid (5 minutes) reversible detection of protein bands on nitrocellulose membranes. 0.1% (w/v) ponceau S is used in 5 % (v/v) acetic acid.

-Electrophoretic transfer apparatus: semi-dry transfer unit (Amersham Biosciences)

-Whatman 3MM paper (Whatman®, Middlesex, UK)

-Nitrocellulose membrane (Bio-Rad, Hercules, CA, USA)

Nitrocellulose binds proteins chiefly by hydrophobic interaction. The nitrocellulose can be stained with the removable but insensitive stain Ponceau S.

-Semi-dry buffer (pH 8.3)

- Tris base 24 mM
- Glycine 192 mM
- Methanol 20 % (v/v)

-1 × TBS (Tris-buffered saline)

- 10 mM Tris-HCl, pH 8.0
- 150 mM NaCl
- 0.05% Tween 20

-1 × TBST: 1 × TBS containing 0.05% Tween 20
-Blocking solution: 1 × TBST containing 5 % (w/v) nonfat dried milk
-ECL Western blotting detection reagents and analysis system (Amersham Biosciences)
-Autoradiography cassette (Amersham Biosciences)
-X-ray film: Hyperfilm^{TM} ECL^{TM} (Amersham Biosciences)
-Dark room

METHOD

-Transfer proteins from SDS-polyacrylamide gel to nitrocellulose using 'semi-dry' method.

1. Soak gel, nitrocellulose membrane and 6 pieces of Whatman 3MM paper in semi-dry buffer for several minutes.
2. Wet transfer apparatus with H_2O. Place 3 pieces of Whatman 3MM paper on the platform; place nitrocellulose on 3MM paper; place gel on top of nitrocellulose; place another 3 pieces of prewet 3MM paper on top of gel; roll out air bubbles at each step.
3. Switch the transfer apparatus and run it at 1 milliamp/cm^2 (equal to 3-15 V according to the size of the gel) for 60 minutes.

-Confirm transfer effeciency by Ponceau S staining

4. Soak the membrane in 0.1 % (w/v) Ponceau S solution for several minutes (less than 5 minutes) with gentle agitation till the appearance of bands. Wash with H_2O; mark the positions of bands and take a photo if necessary.
5. Wash away the Ponceau S with H_2O or TBS. (The membrane could then be wraped in serawrap and be stored at 4°C or -20°C)

-Western hybridization

6. Put the membrane into 10 ml of blocking solution and incubate at room temperature for 45 min.
7. Pour off the blocking solution and add 10 ml of blocking solution containing 1:5000 anti-His_6 HRP conjugates (=antibody). Incubate for 45 minutes at room temperature with gentle agitation. Collet the solution (containing antibody) which could be reused.
8. Wash the membrane 3 times with TBST for 5 min each time with agitation.
9. Briefly dry the membrane with the edgy of a paper towel and put it on the serawrap.
10. Mix 1 ml of the two ECL detection reagents together and pour off the mixture on the membrane and incubate the membrane for 1 minute.
11. Briefly dry the membrane; wrap the membrane with serawrap and put it into cassette.
12. Expose the membrane in a dark room to ECL hyperfilm from 30 seconds to several minutes.

12. Heparin-Sepharose^{TM} Affinity Chromatography

Ion exchange is a technique that is used to separate closely similar biological molecules from highly complex mixtures on the basis of the charges carried by solute molecules. Since almost all biological molecules are polar and can be charged, ion exchange is widely used and has a very high resolving power. Ion exchangers can base on Sepharose, Sephadex, DEAE-Sephacel, and so on. Polyanionique structure of heparin mimics DNA and binds transcriptional factors.

MATERIALS

-Chromatography system

- HiTrap-heparin column 5 ml and 1 ml (Amersham Biosciences, Buckinghamshire, UK)
- Chromatography accessories
  - Adaptor (Pharmacia Biotech, Sweden)
  - Pump, Pharmcia LKB Pump P-1 (Pharmacia Biotech)
  - Recorder, Pharmcia LKB REC 101 (Pharmacia Biotech)
  - Monitor, Optical unit UV-1 and Control unit UV-1 (Pharmacia Biotech)

-Cell extract from 200 ml of cultures (OD_{600} of 1.5) of mutant Δ*exsA*

-Buffer A

- 25 mM HEPES (pH 7.3)
- 5% Glycerol

-Elution buffer

- NaCl (0.3, 0.42, 0.44, 0.46, 0.48, 0.50, 0.52, 0.54, 0.56, 0.6 M)
- Buffer A

-Addtional equipment

- Cell disruptor (Branson Sonifier®, Danbury, CT, USA)
- Centrifuge (Beckman avanti™ J-25, Ramsey, Minnesota, USA)

METHOD

-Cell extract preparations

1. Inoculate 200 ml of LB in a 1-liter flask with 2 ml of overnight cultures of Δ*exsA* and incubate the cultures with shaking at 37°C until the cultures have reached the OD_{600} of 1.5 (about 3-3.5 hours).
2. Harvest the cells by centrifugation at 5000g for 15 minutes at 4°C.
3. Resuspend the cell pellets in a 50-ml tube of polypropylene with 25 ml of buffer A (generally 1/10 original culture volume).
4. Disrupt the cell by Branson Sonifier using a '2' output, '30' % duty cycle and 'continous' power for 1 minute avoiding any bubble and keeping cold during sonicaion and then put it on ice for 1 minute.
5. Repeat step 4 several times until the solution becomes clear.
6. Centrifuge the solution at 14,000 rpm for 30 minutes at 4°C and filtrate the supernatant (cell lysate) through a 0.22 μM filter.
7. Collect 20 ml of (1.5 μg ml^{-1}) solution in a new tube.
8. Adjust the NaCl concentration to 0.2 M by adding 10 ml of buffer A containing 0.6 M NaCl and store it at 4°C (ready for step 11) or -20°C.

-Equilibration of the HiTrap-heparin column (5 ml)

9. Wash the column with 5 column volumes of sterile H_2O (25 ml).
10. Equilibrate the column with 5 volumes of buffer A containing 0.2 M NaCl.

-Fractination of the lysate

11. Load immediately the cell lysate (30 ml, 1 mg ml^{-1}, from step 8), onto the column and adjust the flow rate at 1 ml min^{-1} and collect the whole flowthrough.
12. Wash the column with at least 10 column volumes of buffer A containing 0.2 M NaCl until the A_{280} of the flowthrough is <0.01 and adjust the flow rate at 2 ml min^{-1}. Collect the fisrt 10 ml of wash soultion.
13. Elute the column with 5 volumes of elution buffer containing 0.3 M NaCl (25 ml) .
14. Collect the first 2-3 volumes of elution solution (about 10-15 ml) according to the A_{280} of the fraction.

15. Repeat steps 13 and 14 using elution buffer containing 0.6 M NaCl.
16. Wash the column and regenerate the column according to the instructions given by Amersham.

-Determine the concentration of each fraction (see Materials and Methods 14).

-Assay each fraction for pC binding activity by EMSA (see Materials and Methods 15).

-Fractionner the preliminarily purified fractions (about 12 ml, eluted from elution buffer containing 0.6 M NaCl) having pC binding activity by a 1 ml HiTrap-heparin column.

Adjust the NaCl concentration of preliminarily purified fractions to 0.3 M with buffer A. Equilibrate the column by the buffer A containing 0.3 M NaCl, wash the column by 10 column volumes of buffer A containing 0.3M NaCl at a flow rate of 0.5 ml min^{-1} and then load the preliminarily purified fractions. Wash the column with at least 10 volumn volumes of buffer A containing 0.3 M NaCl until the A_{280} is <0.01. Elute delicately the column with 10 volumes of elution buffer containing a series of 0.42-0.6 M NaCl at a flow rate of 0.5 ml min^{-1} and monitor the A_{280}. Collect the first 2-3 volumes (about 2-3 ml) of flowthrough (0.1-0.3 mg of each fraction) according to the A_{280} of each fraction. Analyse each fraction for pC binding activity by EMSA.

13. Mass spectrometry analysis

Mass spectrometry (MS) has emerged as an important tool for analyzing and characterizing large biomolecules of varying complexity. The matrix assisted laser desorption/ionization (MALDI) technique (Karas *et al*., 1987), developed in 1987, has increased the upper mass limit for mass spectrometric analysis of biomolecules to over 300,000 Da and has enabled the analysis of large biomolecules by mass spectrometry to become easier and more sensitive. An attractive feature of the time-of-flight (TOF) mass spectrometer is its simple instrumental design. TOF mass spectrometers operate on the principle that when a temporally and spacially well defined group of ions of differing mass/charge (m/z) ratios are subjected to the same applied electric field and allowed to drift in a region of constant electric field, they will traverse this region in a time which depends upon their m/z ratios.
MALDI TOF-MS is now a valuable tool in the biosciences for obtaining both accurate mass determinations and primary sequence information.

MATERIALS

-SDS-polyacrylamide gel stained by Coomassie Brilliant Blue
-Sterile scalpel
-10% alcohol

METHOD

-MALDI sample preparation

1. Depose the gel on the glass plate which has been washed by soap liquid and then by methanol.
2. Cut the bande of interest (as small as possible).
3. Put the band in the eppendorf (biopure, keratin-free) containing 500 µl of 10% alcohol (or 25 mM ammonium bicarbonate, also called ammonium hydrogen carbonate, NH_4HCO_3) and seal the eppendorf with parafilm. Store at 4°C.
   It is important to wear the gloves washed by soap and to avoid any contamination of keratin, such as the hand, the box of gloves.

-MALDI TOF-MS was perfomred by Kuhn L in the laboratory of Protein Chemistry (Director J. Garin), CEA, Grenoble, France.

14. Quantitation of protein

Many methods have been used to determine the concentration of soluble protein. The Bio-Rad Protein Assay, based on the Bradford dye-binding procedure, is a simple and accurate

procedure. It involves the addition of an acidic dye, Coomassie Brilliant Blue G-250 whose absorbance maximum shifts from 465 nm to 595 nm when binding to protein occurs, and subsequent measurement at 595 nm with a spectrophotometer or microplate reader. The color change of dye is in response to various concentrations of protein. Comparison to a standard curve provides a relative measurement of protein concentration.

MATERIALS

-Bio-Rad's protein assay kit (Bio-Rad, Hercules, CA, USA)
-Spectrophotometer:Beckman DU®640 (Beckman coulter, Fullerton, CA, USA)
-Protein sample

METHOD

All procedures were performed according to the instructions of the manual of the kit.

15. Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) has been used extensively for studying DNA-protein interactions. This assay is based on the fact hat DNA-protein complexes migrate slower than unbound DNA in a nondenaturing polyacrylamide or agarose gel, resulting in a 'shift' in migration of the labeled DNA band. To further determine the binding sequence of the protein, DNase I footprint assay should be performed.
The LightShift™ Chemiluminescent EMSA Kit uses a nonisotopic method to detect DNA-protein interactions. Biotin end-labeled DNA containing the binding site of interest is incubated with a nuclear extract, cell lysate or purified factor. This reaction is then subjected to gel electrophoresis on a nondenaturing polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA is detected using the streptavidin-horseradish peroxidase conjugate and LightShift™ chemiluminescent substrate.

MATERIALS

-LightShift™ Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA)
-Biotin-labeled DNA probe (Materials and Methods 9.2)

- *exsCEBA* promoter fragment
- *psrA* promoter fragment
- *fleQ* promoter fragment

-Proteins of interest

- bacterial extract
- recombinant protein, such as rPsrA, rVfr

-Buffers and solutions

- 5 × TBE stock solution
  - Tris base 54 g (0.45 M)
  - Boric acid 27.5 g (0.45 M)
  - EDTA (0.5M, pH 8.0) 20 ml (10 mM)

  A precipitate forms when concentrated stock solution of TBE are stored for long periods of time. To avoid problems, store the 5 × solution in glass bottles at room temperature and discard any batches that develop a precipitate.
- polydIdC (Amersham Biosciences, Buckinghamshire, UK)

  PolydIdC is the non-specific competitor DNA which is used to reduce nonspecific binding.
- 250 mM HEPES (pH 7.3)
- 0.5 M NaCl
- 50 % glycerol
- 5 % nondenaturing polyacrylamide gel solution

- 30 % acrylamide/bis 5 ml
- 5 × TBE 3 ml
- 10 % ammonium persulfate 0.3 ml
- TEMED 30 µl
- H_2O total volume 30 ml

- EMSA electrophoretic buffer: 0.5 × TBE

-Materials needed for polyacrylamide gel electrophoresis

-Additional equipments

- Nylon membranes, positively charged (Roche, Indianapolis, IN, USA)
- Whatman 3MM paper (Whatman®, Middlesex, UK)
- Electrophoretic transfer apparatus: semi-dry transfer unit (Amersham Biosciences, San Francisco, CA, USA)
- Cross-linking device: UV Stratalinker® 1800 (Stratagene, La Jolla, CA, USA)
- Autoradiography cassette (Amersham Biosciences)
- X-ray film: Hyperfilm™ ECL™ (Amersham Biosciences)
- X-ray room

METHOD

-Binding reactions

1. To a sterile 1.5-ml microfuge tube add:
   - 2 µg μl^{-1} polydIdC 1 µl
   - 200 pM biotin-labeled DNA probe 2 µl
   - 250 mM HEPES (pH 7.3) 2 µl
   - 0.5 M NaCl 2 µl
   - 50 % glycerol 2 µl
   - protein of interest 0.01-7.2 µg$
   - H_2O to 20 µl

   MW of rPsrA ≈ PsrA + His-Tag-Thrombin site= 25784*+2012 = 27796 Da

   MW of rVfr ≈ Vfr + His-Tag-Thrombin site= 24225+2012 = 26237 Da

   Suppose 1 µg rPsrA is in the 20 µl reaction volume, the rPsrA concentration is equivalent of 1.7 µM (1/20/27796 M). On the contrary, 1 µM rPsrA in 20 µl reaction volume is equivalent of 0.6 (0.56) µg protein.

   $Various quantity of recombinant proteins was added, however, about 1µg of extract was added for each binding reaction as control; *calculated from www.infobiogen.fr.

   20 µM cAMP was supplemented both in the gel and in the electrophoretic buffer for the binding reaction of rVfr.
2. Mix and incubate the solution at 37°C for 20 to 30 minutes.

-Native polyacrylamide gel electrophoresis

3. Pre-run the gel for 30 to 60 minutes. Apply 120 V for a 15 cm × 18 cm × 0.1 cm gel.
4. Load 20 µl of each example onto the gel and run the gel at 180 V for 2 hours.

   No difference was observed when the electrophoresis was performed at room temperature or at 4° C.

-Electrophoretic transfer binding reactions to positively charged nylon membrane

5. Soak Whatman 3MM paper and nylon membrane in 0.5 × TBE for 1 minute.
6. Sandwich the Whatman 3MM paper (2-3 sheets), nylon membrane, gel and Whatman 3MM paper (2-3 sheets) in the electrophoretic transfer apparatus according to the manufacturer's instructions.
7. Transfer at 1 milliamp per cm^2 of membrane (4-15 V) for 1 hour.

-Cross-linking DNA to the membrane by UV irradiation

8. Place the damp membrane on a dry piece of paper towels and cross-link the DNA to the membrane by UV Stratalinker® 1800 using 'auto cross link' . Cross-link once for each side of the membrane.

Membrane may be stored at -4°C for several days.

-Detection of biotin-labeled DNA

9. Perform according to the instructions but use one third or half volume of reagent recommended by the manufacturer (Pierce).

16. Expression and purification of recombinant protein

16.1 Expression of recombinant protein

Escherichia coli is the system of choice for expression of many heterologous proteins because of the vast fund of knowedge about its genetics, biochemistry and molecular biology; straightforward genetic manipulations; easy and inexpensive culture; high toleration and expression of many foreign proteins. The amount of heterologous proteins produced in *E. coli* will vary depending on the target protein, but it is typically in the range of 1-10 mg of protein per 100 ml of cell culture. However, *E.coli* is not always the host of choice. For example, proteins whose full biological activity requires posttranslational modification (*e.g.*, glycosylation or cleavage at specific sites) may best be expressed in a eukaryotic host.

MATERIALS

-Nucleic acids and oligonucleotides

- Primers *phpA* R1-R2 containing *Nde*I-*Bam*HI respectively (Table 5).
- Primers *psrA* R1-R2 containing *Nde*I-*Bam*HI respectively (Table 5).
- Primers *vfr* R1-R2 containing *Nde*I-*Bam*HI respectively (Table 5).

- Bacterials strains and vectors

- *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany)

E. coli BL21 (DE3) contains a chromosomal copy of the gene for T7 RNA polymerase (T7 gene 1) from bacteriophage DE3, a lambda derivative that also has the immunity region of phage 21 and carries a DNA fragment containing the *lacI* gene and the *lacUV5* promoter. In the absence of inducer, T7 RNA polymease production is inhibited by the *lac* reprssor, encoded by *lacI* gene, which binds to the operator (lacO) and prevents synthesis of T7 gene 1 mRNA. Repression of T7 RNA polymerase is relieved by addition of IPTG, which binds to the repressor and causes a conformational change that lowers the affinity of the repressor for the lacO. T7 RNA polymease then in turn transcribes the target DNA in the plasmid containing bacteriophage T7 promoters. DE3 is inserted into the *int* gene of *E. coli* BL21, preventing DE3 from integrating into or excising from the chromosome without a helper phage. *E. coli* BL21 is deficient in the *lon* protease and lack the *omp*T outer membrane protease that can degrade proteins during purification. *E. coli* BL21 (DE3) is the most widely used host for target gene expression.

- pET-15b (Novagen)

pET-15b vector carries bacteriophage T7 promoters and allows regulated expression (IPTG-inducible) of foreign genes by bacteriophage T7 RNA polymerase recognizing only bacteriophage T7 promoters, which are not present in the *E.coli* chromosomal DNA. Their multiple cloning sites (*Bam*H1, *Xho*1, *Nde*1) allow an inserted coding sequence to be placed under control of the 'natural' promoter for T7 RNA polymerase. Attaching target protein to an N-terminal His-Tag sequence provide a convenient method to 'tag' and purify the target protein by nickel affinity chromatography.

-Media

- LB agar plates containing 100 μg ml^{-1} ampicillin
- LB containing 100 μg ml^{-1} ampicillin

-Additional reagents

- IPTG
- Materials needed for PCR
- Zero Blunt® TOPO® PCR cloning kit

- Materials needed for SDS-PAGE
- Materials needed for Coomassie Brilliant staining

-Special equipment

- Flask of 1 L
- Centrifuge (Beckman avanti^{TM} J-25, Ramsey, Minnesota, USA)

METHOD

-Preparation of *E. coli* BL21 (DE3) containing the recombinant expression vector

1. Amplify *phpA*, *psrA* and *vfr* by PCR and clone them into pCR®II-Blunt-TOPO®
   Sequence the constructs to ensure that no spurious mutations were induced during PCR.
2. Digest *phpA*-Topo, *psrA*-Topo and *vfr*-Topo of plasmid minipreparations as well as pET-15b by *Nde*I-*Bam*HI
3. Ligate *phpA*, *psrA* and *vfr* into pET-15b
4. Transform the ligation mixutre into DH5α, select on LB agar plate containing Amp_{100}, and Miniprep.
5. Transform *E. coli* BL21 (DE3) with pET-15b containing gene of interest.
6. Screen the transformants by restriction enzyme analysis after Miniprep.

-Large-scale expression of the target protein

7. Inoculate 10 ml of LB containing 100 μg ml^{-1} ampicillin in a 100-ml flask with individual colonies of *E. coli* BL21 (DE3) containing gene of interest and incubate the cultures with shaking overnight at 37°C.
8. Inoculate 200 ml of LB containing 100 μg ml^{-1} ampicillin in a 1-liter flask with 2 ml of overnight cultures and incubate the cultures with shaking at 37°C until the culture has reached the mid-log phase of growth (A600 = 0.5- 1.0).
9. Induce expression of recombinant protein by adding 0.5 mM IPTG with shaking but at 30°C for another 3 hours.
10. Harvest the cells by centrifugation at 5000g for 15 minutes at 4°C.
11. Store the cells at -20°C (or at -80°C) before purification.

    An optimization of the induction of target protein expression should be performed in a small volume before large-scale expression, including optimal values of IPTG concentration, incubation temperature and incubation time. Target protein expression could be analysed by SDS-PAGE (resuspend directly each cell pellet in SDS gel-loading buffer) in combination with Coomassie brilliant blue staining and confirmed by Western blot using anti-His_6 antibody.

16. 2 Purification of recombinant protein

A protein carrying an exposed His-6 region will bind to a resin charged with divalent nickel ions. Because of its large binding capacity, as much as 10 mg of recombinant protein may be purified from 1 ml nickel resin. Contaminating proteins can be removed with appropriate washing, and the protein of interest can then be eluted by a soluble competing chelator such as imidazole. Efficient elution is usually achieved with 50-100 mM imidazole at pH 7-8. The lowest effective concentraion should be established using a linear gradient of 10-200 mM imidazole. Because few natural proteins bind with significant affinities to a resin charged with divalent nickel ions, His-6-labeled proteins generated by recombinant techniques can be purified substantially in a single step by metal chelate affinity chromatography.Those natural proteins that do bind to these matrices can almost always be removed in a second chromatography step. Because any chelator can interfere with metal affinity chromatography, buffers used for extraction and chromatography should be free of EDTA or EGTA. The nickel ions resin can be regenerated and reused many times.

MATERIALS

-*E. coli* BL21 (DE3) pellets (from 200 ml of cell cultures) expressing a polyhistidine-tagged protein, PhpA, PsrA or Vfr

-0.22 μM filter (Millex® GP)

-Ni^{2+}-charged chromatography resin: Ni Sepharose™ high performance (Amersham Biosciences).

-Binding buffer (pH7.3)

- 25 mM HEPES (pH 7.3)
- 250 mM NaCl
- 5 mM imidazole

-Imidazole (5 M)

-Elution buffer

- Imidazole (50, 100, 200, 500 mM)
- Binding buffer (pH 7.3)

  In fact, elution buffer of pH 6 is recommended by Molecular Cloning (Sambrook and Russell, 2001).

-Special equipment

- Test-tube rotator (Labinco, Breda, Netherlands)
- Cell disruptor (Branson Sonifier®, Danbury, CT, USA)
- Centricon® YM-10 (Millipore, Bedford, MA, USA)

  Centricon® centrifugal filter units provides efficient concentration of even dilute (ng ml^{-1} to μg ml^{-1}) protein solutions. Uses low binding Ultracel-YM membrance with five cut-offs available: 3,000, 10,000, 30,000, 50,000 and 100,000 nominal molecular weight limit. For instance, Centricon® YM-10 can concentrate proteins having a molecular weigh more than 10 kDa.

METHOD

-Equilibration of the Ni^{2+}-charged chromatography resin

1. Invert gently the bottle of Ni^{2+}-charged chromatography resin to mix the slurry, and transfer 2 ml to a 15-ml tube of polypropylene. Allow the resin to pack under gravity flow.
2. Wash the resin with 25 ml of sterile H_2O.
3. Centrifuge the solution at 1,000 g for 3 minutes at 4°C and discard the supernatant.
4. Equilibrate the solution with 25 ml of binding buffer, centrifuge and discard the supernatant.
5. Repeat step 4 for another time.
6. Equilibrate the solution by rotating gently on test-tube rotator for 15 minutes at 4°C.
7. The resin is now ready for use in step 13.

   Prepacked Ni^{2+}-affinity column (HisTrap™ HP column) is available from Amersham Biosciences, in this case, we directly equilibrate the column and carry out the purification according to the instructions which is similar to that of chromatography. Monitor the A_{280} of each fraction. HisTrap™ HP column could bind at least 40 mg polyhistidine-tagged protein per ml medium.

-Preparation of cell extract

8. Resuspend the *E. coli* BL21 (DE3) cell pellets (Materials and Methods 16.1, step 11) in a 50-ml tube of polypropylene with 25 ml (generally one tenth of original culture volume) of binding buffer (pH7.3) containing 1 mM 4-(2-aminoethyl) benzenesulfonyl-fluoride (AEBSF), and 10 μM pepstatin A, inhibitors of protease.
9. Disrupt the cell by Branson Sonifier using a '2' output, '30' % duty cycle and 'continous' power for 1 minute avoiding any bubble and keeping cold during sonicaion and then put it on ice for 1 minute.
10. Repeat step 9 several times until the solution becomes clear.
11. Centrifuge the solution at 14,000 rpm for 30 minutes at 4°C and collect the supernatant (cell lysate) in a new tube.

12. Filtrate the cell lysate through a 0.22 μM filter and collect it in a new tube.

-Purification of the polyhistidine-tagged protein

13. Add the equilibrated resin in the filtered supernatant and mix them by rotating gently on test-tube rotator for 30 minutes at 4°C.
14. Centrifuge at 1,000 g for 3 minutes at 4°C and collect the supernatant (flowthrough)
15. Wash the bound protein with 25 ml of binding buffer containing 10 mM imidazole and mix them by rotating gently on test-tube rotator for 5 minutes at 4°C.
16. Centrifuge at 1,000 g for 3 minutes at 4°C and collect the supernatant (wash).
17. Elute the bound protein with 5 ml of elution buffer containing 50 mM imidazole and mix them by rotating gently on test-tube rotator for 5 minutes at 4°C.
18. Centrifuge at 1,000 g for 3 minutes at 4°C and collect the supernatant (elution 1, 50 mM imidazole).
19. Repeat steps 17 and 18 using elution buffer containing increasing concentrations of imidazole (100 mM, 200 mM and 500 mM).
20. Assay all the fractions for the presence of the polyhistidine-tagged protein by analyzing 30-μl aliquots by SDS-PAGE in combination with Coomassie brillant blue staining and then Western blot using anti-His_6 antibody.

-Concentration of the polyhistidine-tagged protein (if necessary)

21. Add purified polyhistidine-tagged protein (up to 2 ml) in Centricon® YM-10.
22. Centrifuge at 4,000 g at 4°C (for about 45 minutes) until the volume has greatly decreased (90%)
23. Add 1.5 ml of binding buffer without imidazole and centrifuge to eliminate imidazole.
24. Repeat step 23.
25. Collect concentrated protein containing little imidazole and sotre it at -20°C.

17. Gene reporting analysis: TTSS transcriptional activation

In order to study precisely the level of transcription of *exsCEBA* and *exoS* promoters, reporter strains containing one single copy of transcriptional fusions of *pClux* or *pSlux* were tested in different conditions.

17.1 Transcription in response to calcium depletion

MATERIALS

-Bacteria containing *pClux* or *pSlux*

-0.5M EGTA (pH7.2)

Add 19.02 g of EGTA to 80 ml of H_2O. Mix well by vortexing and adjust the pH to 7.2 with NaOH. Add H_2O to 100 ml. Filtrate through a 0.22 μM filter. EGTA does not dissolve until the pH of the solution is adjusted to ~7.2.

-2M $MgCl_2$

-0.5M $CaCl_2$

-spectrophotoluminometer (Luminoskan Ascent, Labsystems, Finland)

-96 wells plate (Costar®)

METHOD

1. Grow bacteria in 2 ml of LB overnight at 37°C, with aeration and agitation of 260 rpm
2. Wash bacteria 2 times with LB and then inoculate them at 1:100 in 1 ml of LB supplemented with 5mM EGTA/20mM $MgCl_2$ (inducing condition) or LB in the presence of 5mM $CaCl_2$ (non-inducing conditon), for 3-4 hours till an OD_{600} of 1-1.5.
3. Measure relative luciferase units (RLU) of 0.2 ml of culture (1:2, 1:4 and 1:8 dilution by LB) in 96 wells plate using a spectrophotoluminometer. Bacterial quantity was

estimated from OD_{600} (we consider that there is 6×10^8 bacteria in 1ml of LB at OD_{600} of 1). Transcription is then expressed as RLU per 6×10^8 bacteria.

17.2 Transcription in response to human serum

MATERIALS

-Bacteria containing *pClux* or *pSlux*
-Human serum AB
-Modified HEPES-buffered saline (mHBS) (Dacheux *et al*., 1999)

- 15 mM HEPES
- 8 mM glucose
- 4 mM KCl
- 140 mM NaCl
- 0.5 mM $MgCl_2$
- 0.9 mM $CaCl_2$

-spectrophotoluminometer (Luminoskan)
-96-well plate (Costar®)

METHOD

1. Wash O.N. bacteria 2 times with LB and preculture them at 1:100 in 1 ml of LB to OD_{600} of ~1 (about 2 hours).
2. Wash twice with mHBS and plate 5×10^5 bacteria/well in a 96-well plate with or without 10% human serum AB.
3. Read RLU from the entire culture plate immediately after adding bacteriae (t = 0) and at 1 h intervals.

17.3 Transcription in response to cell contact

MATERIALS

-Bacteria containing *pClux* or *pSlux*
-Differentiated PLB-985 cells (Bionda *et al*., 2004)
-mHBS
-spectrophotoluminometer (Luminoskan)
-96-well plate (Costar®)

METHOD

1. Wash PLB-985 2 times with mHBS and plate 10^5 cell/well in a 96-well plate.
2. Add precultured bacteria at a MOI of 5. Control infections were set up under identical conditions in the absence of PLB-985 cells.
3. Read RLU from the entire culture plate immediately after adding bacteriae (t = 0) and at 1 h intervals.

18. Functionality of TTSS

To examine the functionality of TTSS, we analysed the secreted proteins.

MATERIALS

-Bacteria of interest
-LB
-LB containing 5mM EGTA and 20 mM$MgCl_2$
-LB containing 5mM $CaCl_2$

-Acetone
-Trichloroacetic acid (TCA) 50 % solution
Dissolve 110.1 g TCA in 100 ml of deionized H_2O. The resulting solution will contain 50% (w/v) TCA.
-SDS-PAGE associated materials

METHOD

1. Grow bacteria in 2 ml of LB containing appropriate antibiotics at 37°C for overnight.
2. Wash bacteria twice by LB, re-inoculated at 1:100 into inducing or non-inducing LB, and culture at 37°C for 3 h.
3. Collect the supernatant of the bacterial culture (equivalent of 1 ml of OD_{600} 1) by centrifugation 13,000 rpm for 15 min .
4. Add 0.35 ml 50% TCA per ml supernatant (TCA final concentration is 13%) to precipitate proteins at 4°C for 30 min.
5. Collect precipitated proteins by centrifugation 13,000 rpm for 15 min
6. Wash the pellet with cold acetone (-20°C) and centrifuge 5 min; repeat another wash.
7. Dry the pellet in air (about 5 min) and resuspend them in 20 µl 1× SDS-PAGE loading buffer.
8. Perform a 10% SDS-PAGE and stain the gel by Coomassie Brilliant Blue.

19. Resistance to phagocyte killing

Differentiated poly-morphonuclear (PMN) leukocyte-like cells, PLB-985 were used to examine the ability of different strains of *P.aeruginosa* to resist the bacterial killing activity of PMN.

MATERIALS

-PLB-985 cells (GREPI, CHU Grenoble, France, Bionda *et al*., 2004)
PLB-985 are single, round cells and are belong to human acute myeloid leukemia, derivative of HL-60. They are maintained in 90% RPMI 1640 with 10% fetal bovin serum (FBS), 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine at 37°C in a 5% CO_2 atmosphere. Minimum density of subculture is about 0.2×10^6 cells ml^{-1}, doubling time is about 24 hours and maximal density is at about 1.5×10^6 cells ml^{-1} (generally is 1×10^6 cells ml^{-1}). PLB-985 are stored in liquid nitrogen (-196°C) in 70% RPMI 1640, 20% FBS, 10% DMSO at about 5×10^6 cells per ampoule.
-Cell culture incubator (Heraeus, Courtaboeuf, France)
-RPMI 1640 (Invitrogen, San Diego, USA)
-FBS (Invitrogen)
-10, 000 units ml^{-1} penicillin (Invitrogen)
-10, 000 µg ml^{-1} streptomycin (Invitrogen)
-200 mM (100 ×) L-glutamine (Invitrogen)
-N,N-Dimethylformamide (DMF) (Sigma)
-mHBS (See Material and Methods 17.2)

METHOD

1. Expose 5×10^5 PLB-985 cells per ml (20 ml volume) to 0.5% DMF for 5-7 days to obtain differentiated granulocytes whose activity could be estimated as described previously (Bionda *et al*., 2004).
2. Re-inoculate overnight *P. aeruginosa* at 1:100 in LB for 3 hours.
3. Wash bacteria twice with mHBS and adjust them to 2.5×10^8 bac ml^{-1} in mHBS.
4. Wash differentiated PLB-985 twice with mHBS and resuspend 5×10^6 cell in 800 µl mHBS in a culture tube.
5. Add prepared 100 µl of *P. aeruginosa* (2.5×10^7 bac, MOI= 5), 100 µl human serum AB (1 ml total), mix well and then incubate the tube at 37°C wtih gentle agitation.

6. Prepare immediately (time [t]=0) and after different intervals two $1:10^5$ interaction dilutions and plated 50 µl diluted solution onto the prewarmed PIA plates.
7. Count CFU after 16 h of incubation at 37°C and calculate the average value.
   The $1:10^5$ dilution was prepared by adding 50 µl interaction solution in 5 ml of LB ($1:10^2$); then 50 µl in another 5 ml of LB ($1:10^4$) and at the end 100 µl in 900 ul LB (10^5). In theroy, in 50 µl interaction solution (time [t]=0), there should be 50 bacteria ($=0.05\times2.5\times10^7/10^5$).

20. Search of TTSS inhibitor(s) from stationary phase culture supernanant

20.1 Supernatant preparation and its activity on the transcription of *exsCEBA*

MATERIALS

-Strains
- CHA wide type
- Transposon insertion mutants
- CHA (*pClux*)

-Medium
- LB
- VB

-Flask of 500 ml

METHOD

-Supernatant preparation
1. Inoculate overnight bacteria (wash twice with LB) at 1:200 in 100 ml of LB in a flask of 500 ml and grow the bacteria at 37°C with agitation of 260 rpm for 6 hours (OD_{600} should be 3.0-4.0)
2. Centrifuge at 8,000 rpm for 10 min and pour off the supernatant.
3. Wash briefly the pellet with 10 ml of VB, centrifuge and pour off the supernatant.
4. Add 100 ml of VB and culture at 37°C with agitation overnight.
5. Centrifuge at 15,000 rpm for 45 min and collect the supernant which could be stored at -20°C or 4°C.
   Otherwise, we could concentrate the supernatant by lyophilization using Freeze dry system/LYPH LOCK 4.5® (LABCONCO, Kansas, Missouri, USA); then suspend the powder in 10 ml of 70 % alcohol to precipate the salt and protein at -20°C for 1 hour; centrifuge at 15000 rpm at 4°C for 45 minutes; collect the supernatant and then eliminate alcohol by ISS110 Speed VAC® system (Thermo, Waltham, Manchester, USA) and finally resuspend the precipation in 1 ml of H_2O. Such treatment does not influence the activity of supernatant on CHA (*pClux*)/(*pSlux*) (Thesis of Didier FILOPON, December 21, 2005, UJF Grenoble 1).

-Supernatant activity on the transcription of *exsCEBA*
6. Take 1.05 ml of supernatant and add 150 µl mixture as follows:
   - 12 µl precultured CHA (*pClux*) at $OD_{600}\approx1$
   - 120 µl 10 × LB
   - 12 µl/12 µl 0.5 M EGTA/2 M $MgCl_2$*
7. Read RLU immediately after adding bacteriae (t = 0) and at 1 h intervals.

*: replace 12 µl 0.5 M $CaCl_2$ for the negative control.

20.2 Construction and screening transposon insertion mutant library

Transposon insertion mutants were generated by mobilizing the suicidal plasmid pFAC from *E. coli* S17-1 to *P. aeruginosa* CHA as previously decribed (Wong and Mekalanos, 2000; Diaz-Perez *et al.*, 2004).

MATERIALS

-Strains

- Wild-type CHA
- *E.coli* S17-1 (pFAC) (pFAC is a gift from Dr. Campos-Garcia)

-Medium

- LB agar without salt
- PIA Gm400
- LB
- VB

-Digital multichannel pipette 5-50 µl (Titertek®, Titertek, Finland)
-Digital multichannel pipette 50-200 µl (Titertek®)
-96 wells plate

METHOD

-Mating between wild-type CHA and *E.coli* S17-1 (pFAC)

2. Mix at 1:1 of 100 µl exponential phase ($OD_{600} \approx 0.5$) CHA and *E.coli* S17-1. Deposit the mixture on the surface of LB agar without salt. Incubate the plate overnight in the incubator at 37°C.
3. Transfer the bacteria into a microfuge tube containing 1 ml of LB and dilute OD_{600} to 0.02.
4. Spread 100 µl suspension on the PIA Gm400 and incubate the plate overnight in the incubator at 37°C (mating rate is about $1/10^4$, there is about 120 colonies, $0.02\times6\times10^8/10/10^4$).

-Screening mutants whose supernatant do not repress *exoS* promoter activity

5. Inoculate 84 colonies as well as CHA (in 6 different wells, positive control) in 96-well plate containg 100 µl LB/well (conserve 6 empty wells as negative control). Incubate the plate overnight at 37°C with agitation.
6. Transfer 5 µl overnight culture using multichannel pipette into another 96-well plate containing 100 µl LB/well. Incubate the plate 6 hours at 37°C with agitation.
7. Centrifuge the plate at 1200 g at 25°C for 1 hour and pour off the supernatant.
8. Add 100 µl VB/well and incubate the plate overnight at 37°C with agitation.
9. Centrifuge the plate at 1200 g at 25°C for 1 hour and transfer carefully 80 µl supernatant/well using multichannel pipette.
10. Add 20 µl mixture containing
    - 5 µl precultured CHA (*pSlux*) at $OD_{600}\approx1$
    - 10 µl 10 × LB
    - 1 µl/1 µl 0.5 M EGTA/2 M $MgCl_2$*
    - 3 µl H_2O
11. Read RLU immediately after adding bacteriae (t = 0) and at 1 h intervals.

*: replace 1 µl 0.5 M $CaCl_2$ for the negative control.

20.3 Mutant characterization

Mutants whose stationary phase supernanants lost their ability to inhibit *exoS* promoter activity were characterized by genomic DNA cloning into pUC18 and then sequencing, see Material and Methods 2.4.6.3 and 2.4.5. A single transposition event of mutant was verified by southern hybridization, see Material and Methods 10.

21. High Performance Liquid Chromatography

MATERIALS

-HPLC system

- Liquid chromatography: LC-10AD (Shimadzu, Kyoto, Japan)
- UV spectrophotometric detector: SPD-6A (Shimadzu)
- Chromatopac: C-R5A (Shimadzu)
- Pump: Gastorr GT-103 (Laboc)
- C18 cartridge column (Shimadzu)

-Supernatant (filtrated by 0.22 µM filter, see Materials and Methods 20.1)
-Solvent A: 0.1% trifluoroacetic acid (TFA)
-Solvent B: 80% methanol and 20% solvent A

METHOD

Stationary phase culture supernatant from the wild-type CHA and mutant Δ*trpA* were prepared as described in Materials and Methods 20.1 and were filtrated through a 0.22 µM filter. 1 ml of samples were injected into a LC-10AD device, washed with 10 ml of solvent A and eluted with 5 ml of solvent B at a flow rate of 0.5 ml min^{-1}. The linear gradient elution was initially 100% solvent A, programmed to be 100% solvent B over 30 minutes and hold 100% solvent B for 5min. A C18 cartridge column was equilibrated prior and after each injection.

RESULTS

Chapter 1

PsrA is a positive transcriptional regulator of type III secretion system in *Pseudomonas aeruginosa*

Résumé en français

Le système de sécrétion de type III (SSTT) de *Pseudomonas aeruginosa* est induit *in vivo* lors du contact avec la cellule eucaryote et *in vitro* lors de la déplétion calcique dans le milieu de culture. Nous avons observé qu'une protéine précédemment identifiée, PsrA, est nécessaire pour la pleine activation de l'expression des gènes du SSTT chez *P. aeruginosa*. Les analyses de gel retard ont montré que la protéine recombinante PsrA peut se fixer sur la région du promoteur d'*exsCEBA*, qui est l'opéron régulateur du SSTT chez *P. aeruginosa*. Un mutant avec une délétion du gène *psrA* a été construit. En utilisant des fusions transcriptionnelles, nous avons montré que PsrA est nécessaire pour la pleine activation de la transcription d'*exsCEBA* et *exoS*, bien que le mutant Δ*psrA* ait toujours répondu à la déplétion calcique, au sérum, et au contact de cellule hôte. Le mutant Δ*psrA* a montré une diminution marquée de la sécrétion des effecteurs de type III et une faible résistance à la phagocytose par les cellules PLB-985. Le défaut de la transcription des gènes et la sécrétion des effecteurs du SSTT dans le mutant Δ*psrA* a pu être complémenté par l'expression *in trans* de *psrA*. PsrA a été précédemment identifié comme un activateur transcriptionnel de RpoS, un régulateur central pendant la phase stationnaire. Nous avons confirmé que RpoS a un effet négatif sur l'expression des gènes du SSTT. L'ensemble des résultats suggère que PsrA soit un nouveau activateur qui est impliqué directement dans l'expression du SSTT en augmentant le niveau de la transcription d'*exsCEBA*.

En anglais

1. Background

In *P. aeruginosa*, expression of all TTSS genes is coordinately regulated by an AraC-like transcriptional activator, ExsA, which binds to a consensus sequence (TNAAAANA) approximately 50 bp upstream of the transcriptional start site of the TTSS genes (Hovey and Frank, 1995). In recent years, a cascade of proteins has been discovered which may influence posttranscriptionally the activity of ExsA. ExsD, an antiactivator, is a negative regulator that binds ExsA and prevents it from activating TTSS gene transcription (McCaw *et al*., 2002). ExsC has recently been shown to interact with ExsD and is thought to act as an anti-

antiactivator that releases ExsA from inhibition by ExsD (Dasgupta *et al*., 2004). ExsE, a small TTSS secreted protein binding tightly to ExsC and acting as a repressor of the TTSS, has been recently identified (Rietsch *et al*., 2005; Urbanowski *et al*., 2005). ExsE sequesters ExsC when the TTSS channel is closed but is secreted when the channel is activated by a signal such as low calcium concentration, leaving ExsC free to interact with ExsD and releasing ExsA, thereby allowing ExsA to activate TTSS expression. It is interesting that, except for *exsD*, the genes encoding these proteins, *exsA*, *exsC*, and *exsE*, are located in the same operon, *exsCEBA*, but other genes, such as that for PtrA, pseudomonas type III repressor A, may also affect the TTSS through direct interaction with ExsA. PtrA is highly and specifically inducible by a high copper concentration signal and suppresses the TTSS by inhibiting the function of ExsA through direct interaction, although it is not clear whether PtrA blocks the DNA binding ability of ExsA or its ability to interact with the RNA polymerase (Ha *et al*., 2004). Otherwise, defect of TTSS gene expression in mutants, such as deletions of *cyaAB* or *vfr* (Wolfgang *et al*., 2003), *aceAB* (Dacheux *et al*., 2002), and *retS*/*rtsM* (Goodman *et al*., 2004; Laskowski *et al*., 2004; Zolfaghar *et al*., 2005), could be restored to normal level by *in trans* expression of *exsA*, indicating another important transcriptional level of regulation of the TTSS through the activity of promoter of the *exsCEBA* operon (pC). Therefore, it is interesting to study the regulation of *exsCEBA* itself to explore the regulation of the TTSS in *P. aeruginosa*.

Previous work realised in our lab showed that, transcriptional activation of the TTSS is defective in pyruvate dehydrogenase (PDH) mutants of *P. aeruginosa strain* CHA (Dacheux et al., 2002). Work also showed that cell extracts of PDH mutant contain a protein able to bind strongly to the promoter pC. Leucine aminopeptidase, PhpA, one clearly enriched protein in the fraction having the strongest pC binding activity was indentified by Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) (unpublished work realised by Epaulard, *et al*). Furthermore, PhpA was reported to be involved in negative regulation of the expression of *algD*, the first gene of the alginate biosynthetic operon (Woolwine and Wozniak, 1999), and its homologous (56 % amino acid identity) protein in *E. coli*, PepA, is a multifunctional protein, possessing independent aminopeptidase and DNA-binding activities (Charlier *et al*., 2000). The regulation of *algD* transcription in *P. aeruginosa* is related to the aminopeptidase activity of PhpA and the binding of PhpA to the *algD* promoter region has not been studied (Woolwine *et al*., 2001).

2. Objective and strategy

Our objective is to isolate, identify and characterize the role in TTSS regulation of proteins able to act as regulator on the promoter pC. For such purpose, we have used strategies summarized in Fig. 8. Briefly, we prepared extract from mutant Δ*exsA* and fractionnated them by heparin-sepharose affinity chromatography. Using EMSA, we determined each fraction's DNA-binding activity to biotin-labelled promoter pC and collected fractions which have a strong pC binding activity. After separation by SDS-PAGE, unique bands in these fractions were analyzed by MALDI-TOF and 2 co-purified proteins were characterized as Vfr, a known transcriptional activator of TTSS and PsrA, a member of the TetR family of transcriptional regulators. Recombinant proteins PhpA, PsrA and Vfr were produced to determine their binding activity to pC. Mutants construction allowed to further investigate the role of each of these proteins in regulation of TTSS genes expression.

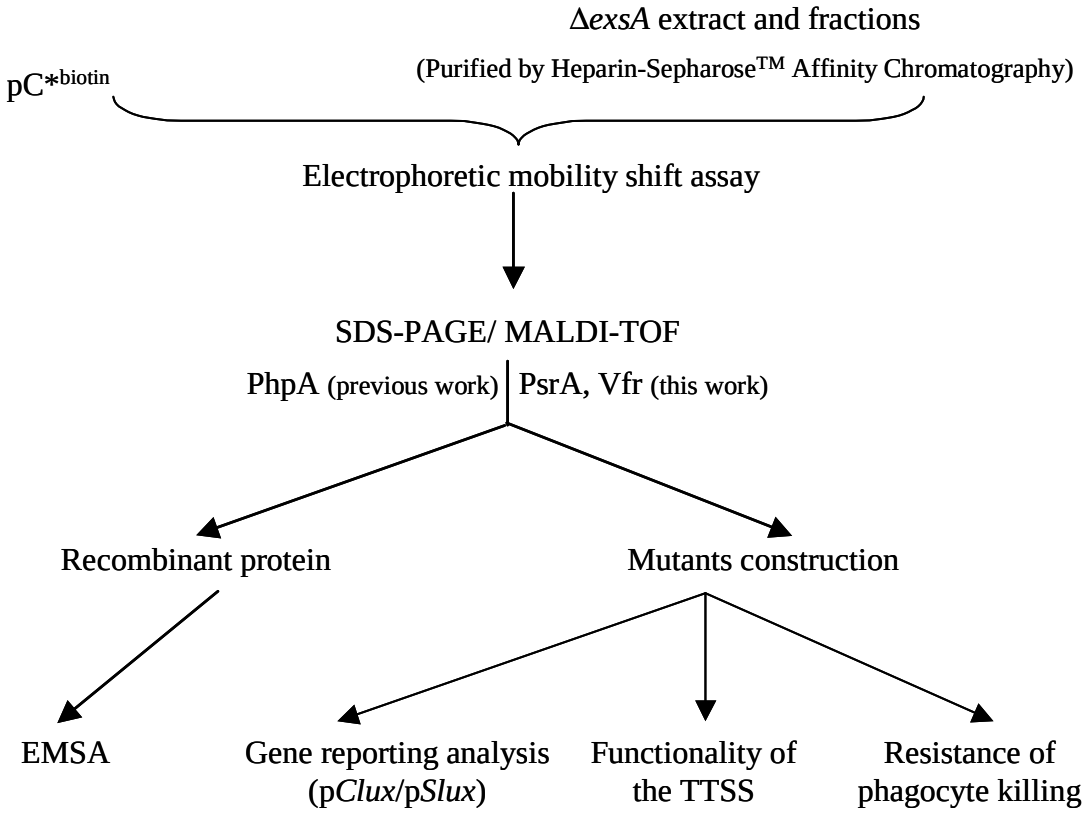


FIG. 8. Strategy in search of unknown regulators acting at promoter of *exsCEBA* operon.

3. Results

3.1 Chromosomal transcriptional gene reporter *pClux* and *pSlux*

In order to study the regulation of *exsCEBA* itself to explore the regulation of TTSS genes expression in *P. aeruginosa*, we have constructed chromosomal transcriptional gene reporter strains containing one single copy of transcriptional fusion of promoter of operon *exsCEBA* with *lux* (*pClux*) and promoter of *exoS* with *lux* (*pSlux*). Construction was realized by mating *E.coli S17* containing plasmid mini-CTX-*exsCp-lux* or mini-CTX-*exoSp-lux* (a kind gift of Dr. Kazmierczak) with our CHA strain. Then plasmid backbone sequences was excised via Flp recombinase as described in Materials and Methods 2.1.1 (Laskowski *et al*., 2004). As seen in Fig. 9, transcription of both *exsCEBA* and *exoS* in the CHA wild-type is quickly activated in TTSS-inducing conditions (calcium depletion by adding EGTA), indicating that chromosomal transcriptional gene reporters *pClux* and *pSlux* provided us a useful tool to study precisely the transcriptional level of *exsCEBA* ane *exoS* promoter.

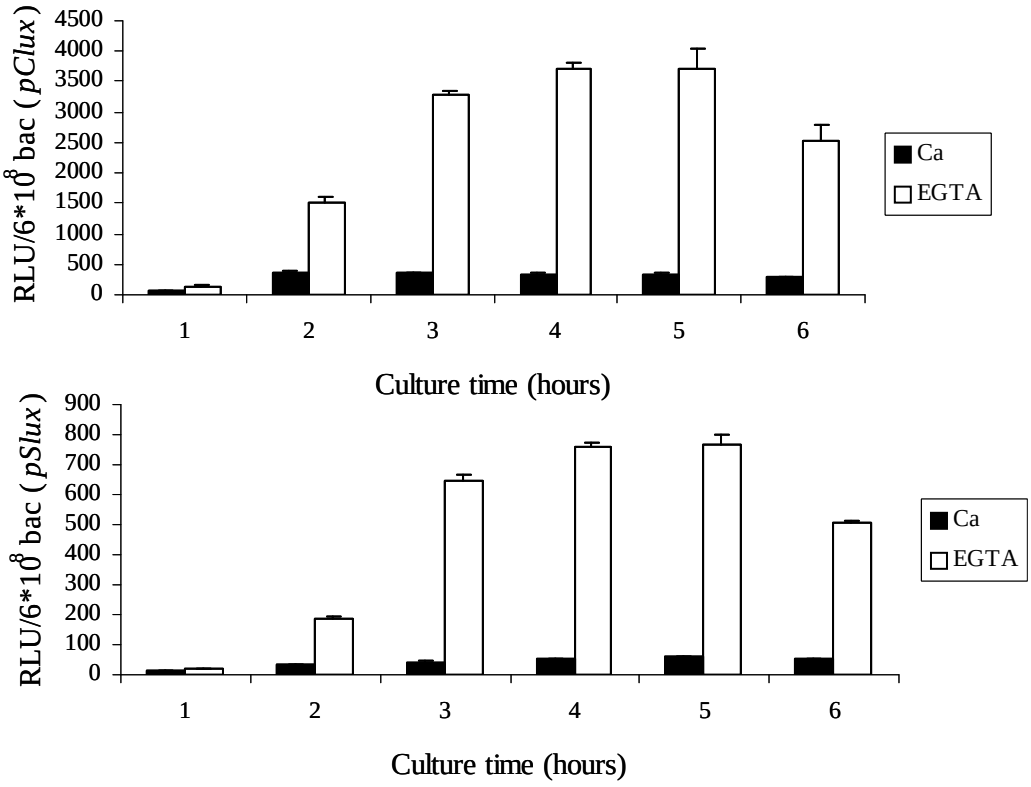


FIG. 9. Analysis of TTSS exprssion by transcriptional gene reporter *pClux* and *pSlux*. The CHA wild-type carrying a single copy of the *pClux* or *pSlux* transcriptional reporter at its chromosome were grown at 37°C from an initial OD_{600} of 0.05 in LB under TTSS-noninducing (black bars, in the presence of 5 mM calcium) or -inducing (white bars, in the presence of 5 mM EGTA and 20 mM $MgCl_2$) conditions. RLU were measured at 1-h intervals over 6h of culture and calculated into normalized bacterial counts (6×10^8 bac). Bars represent the means plus the standard deviations of results for triplicate samples and show the averages of results from three independent experiments. bac, bacteria. Ca, calcium.

It is worth pointing out that maximum expression of both *exsCEBA* and *exoS* happens at the end of four hours culture (Fig. 9) and this phenomenon will be investigated in the second part of work.

3.2 PhpA doesn't play a role in the regulation of TTSS expression

3.2.1 pC binding activity of rPhpA

In order to determine the binding activity of PhpA to promoter pC, we first produced His_6-tagged recombinant protein PhpA (rPhpA) in *E. coli* BL21(DE3). rPhpA was purified by using a nickel affinity column according to the instructions of the manual, analyzed by SDS-PAGE, visualized with Coomassie staining (Fig. 10), and confirmed by Western blotting using anti-His_6 antibody (data not shown).

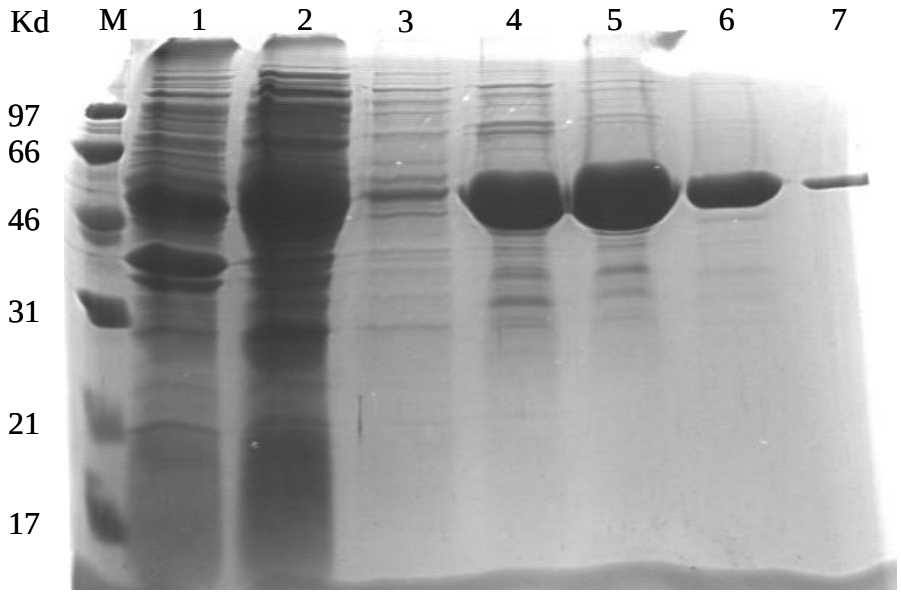


FIG. 10. Expression and purification of recombinant proteins rPhpA. SDS-PAGE analysis of rPhpA, which were expressed from a pET15b vector in *E.coli* BL21 (DE3) and purified using Ni-NTA Agarose with differenct imidazole concentrations in the wash and elution step. Kd, kilodalton; M, protein marker whose sizes are indicated at left side; 1, cell lysate; 2, flow-through; 3: wash solution in the presence of 10 mM imidazole; 4-7: elution containing 50 mM, 100 mm, 200 mM and 500 mM imidazole respectively. PhpA was visualized by a Coomassie Blue-stained 10% SDS-Polyacrylamide gel.

To verify if rPhpA is able to bind the pC, we carried out, as described in Materials and Methods 15, an electrophoretic mobility shift assay, which is usually used to study the interaction between DNA and protein. In this experiment, DNA fragment was a 280-bp pC fragment containing the region of promoter p*CEBA*. It has been shown previously that this pC

fragment fused to *gfp* can be used to monitor transcriptional regulation of the *exsCEBA* operon (Dacheux *et al*., 2001). The pC fragment was obtained from digestion of plasmid pIApC with *BamH*I and then was labeled with biotin-16-dUTP. As shown in Fig. 11, several shifts were appear in the reaction of pC and 4 μg of total extract prepared from strain Δ*exsA*, suggesting beside ExsA, several other proteins could bind to pC. However, using same conditions of EMSA, no shift was observed from the reaction between pC and rPhpA (Fig. 12A) and such phenomenon was also observed using 35^S-labelled pC (Fig. 12B), implying that rPhpA does not bind to pC.

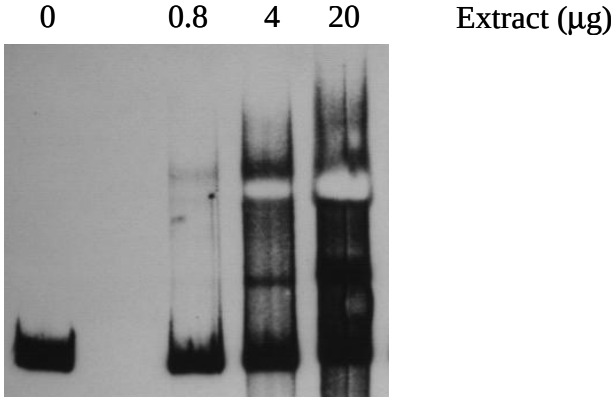


FIG. 11. Analysis of extract's pC binding activity. A 280-bp biotin-labeled pC fragment was incubated with Δ*exsA* extract in the presence of 2 μg of poly(dI-dC) as described in Materials and Methods. The quantity of used extract was indicated on the upside.

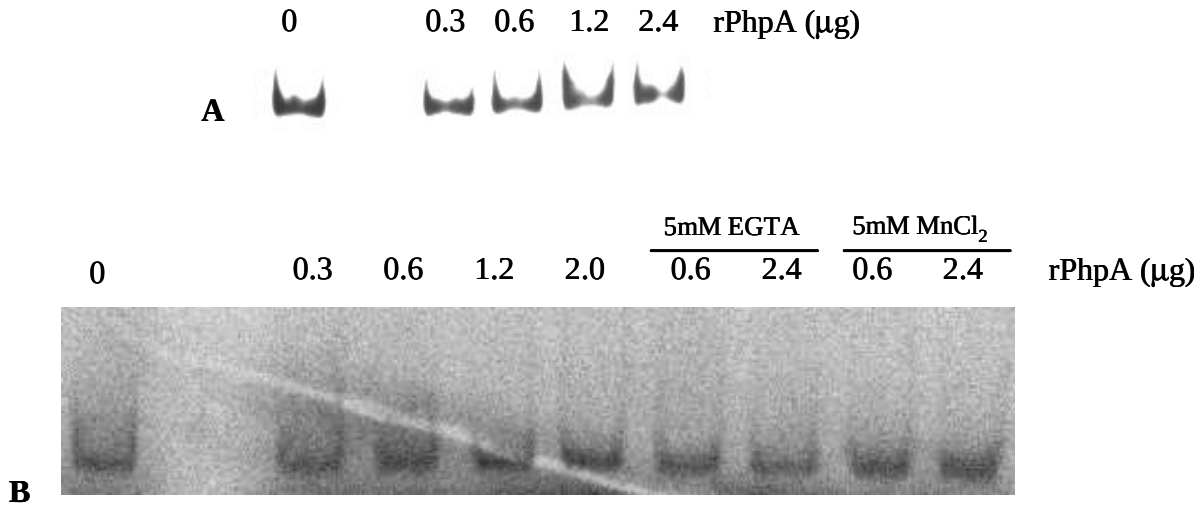


FIG. 12. rPhpA does not bind to the pC region. An EMSA experiment was performed to show the interaction between rPhpA and a 280-bp pC fragment labelled by biotin (A) or by ^{35}S (B). Interaction was carried out in the presence of 2 μg of poly(dI-dC), and purified rPhpA of different quantity was used as indicated in the figure.

3.2.2 Phenotype of mutant Δ*phpA*

To determine the role of PhpA in the regulation of TTSS expression, we first constructed, by allelic exchange, as described in Materials and Methods 2.1.2, an unmarked in-frame deletion mutation of gene *phpA*, yielding the Δ*phpA* strain which was confirmed by PCR. As seen in Fig. 13, with the same primer pair *phpA*-1-*phpA*-2 as listed in Table 5, the size of amplified DNA fragment alters as expected from wild-type to mutant Δ*phpA* due to the insertion and the deletion of the cassette Gm*lox* (*lox*-flanked *aacC1* encoding gentamicin resistance). The amplified DNA fragment became smaller after the excision of cassette Gm*lox*, indicating that the mutant Δ*phpA* has been correctly constructed.

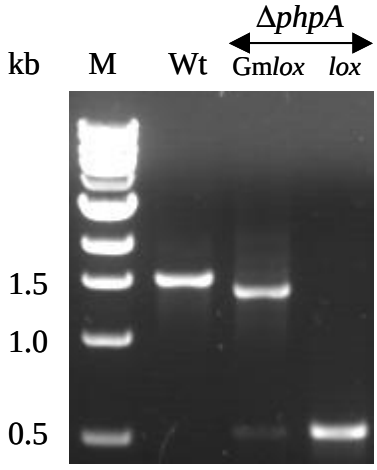


FIG. 13. Characterization of mutant Δ*phpA* by PCR. PCR was performed on genomic DNA prepared from different wild-type CHA and mutant Δ*phpA*. Wt, wild-type CHA; Gmlox, *lox*-flanked *aacC1* encoding gentamicin resistance, is present in the chromosome; *lox*, only *lox* but not *aacC1* is present in the chromosome. M: DNA marker whose sizes are indicated at left side; kb, kilobase.

We then yielded the strain Δ*phpA* containing one single copy of transcriptional fusions of *exsCEBA* with *lux* [Δ*phpA* (*pClux*)], and Δ*phpA* containing one single copy of transcriptional fusions of *exoS* with *lux* [Δ*phpA* (*pSlux*)] by mating *E. coli* S17-1 (pKO-*phpA*) with CHA (*pClux*) and CHA (*pSlux*) as described in Materials and Method 2.1.1. Contrast to wild-type CHA, the deletion of *phpA* does not have an influence on the activation of neither *exsCEBA* nor *exoS* expression in TTSS-inducing conditions (calcium depletion by EGTA) (Fig.14). Taken together, PhpA is not the right protein in the extract of PDH mutant responsible for strong pC binding activity, and PhpA does not play a role in the regulation of TTSS genes expression in *P. aeruginosa*.

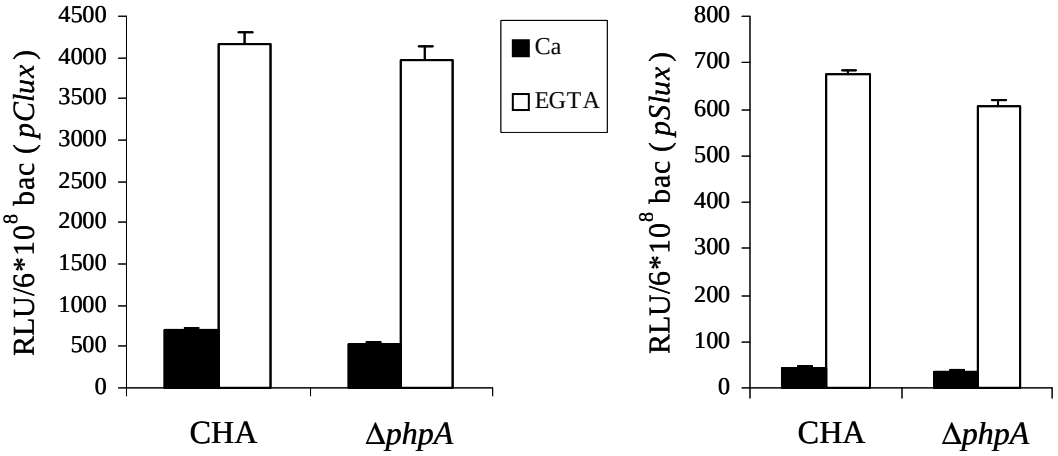


FIG. 14. PhpA has not a regulatory role in the expression of TTSS. The CHA wild-type and Δ*phpA* strains carrying a single copy of the *pClux* (left) or *pSlux* (right) transcriptional reporter at its chromosome were grown at 37°C in LB under TTSS-noninducing (black bars, in the presence of 5 mM calcium) or -inducing (white bars, in the presence of 5mM EGTA and 20 mM $MgCl_2$) conditions. RLU were measured over 4 h of culture and calculated into normalized bacterial counts (6×10^8 bac). Bars represent the means plus the standard deviations of results for triplicate samples and show the averages of results from three independent experiments. bac, bacteria. Ca, calcium.

3. 3 PsrA is an activator of TTSS in *P. aeruginosa*

3.3.1 PsrA was identified from extracts possessing pC binding activity

To identify protein(s) implicated in the regulation of *exsCEBA* expression, we decided to keep identifying protein(s) having an interaction with the DNA fragment containin the region of promoter pC. To exclude the pC binding activity due to the presence of ExsA and the influence of ExsA itself, the strain Δ*exsA* with a deletion of *exsA* (Dacheux *et al*., 1999) was used to produce extracts, which were then fractionnated by heparin-sepharose affinity chromatography through adjusting the concentration of salt. Proteins from the fraction (eluted from 0.44 M NaCl) possessing the strongest pC binding activity and those from the fraction (eluted from 0.46 M NaCl) with no pC binding activity (Fig. 15A) were separated by SDS-10% PAGE and stained with Coomassie blue (Fig. 15B). The bands present in the active fraction and absent in the adjacent nonactive fraction were sliced, and a matrix-assisted laser desorption-time of flight (MALDI-TOF) analysis was performed. Results of the MALDI-TOF analysis were listed in Table 11, where band 1 is the mixture of PsrA and Vfr; band 2 is the mixture of 50S ribosomal protein L14 and putrescine aminotransferase; and band 3 is the mixture of 50S ribosomal protein L14 and 50S ribosomal protein L19. Because Vfr (Wolfgang *et al*., 2003) and PsrA (Kojic and Venturi, 2001; Kojic *et al*., 2002; 2005) are known transcriptional regulators of *P. aeruginosa*, and moreover because Vfr is a suggested

activator of the TTSS by an unknown mechanism, we were interested to know whether Vfr and/or PsrA play a role in TTSS regulation by direct binding to the promoter pC.

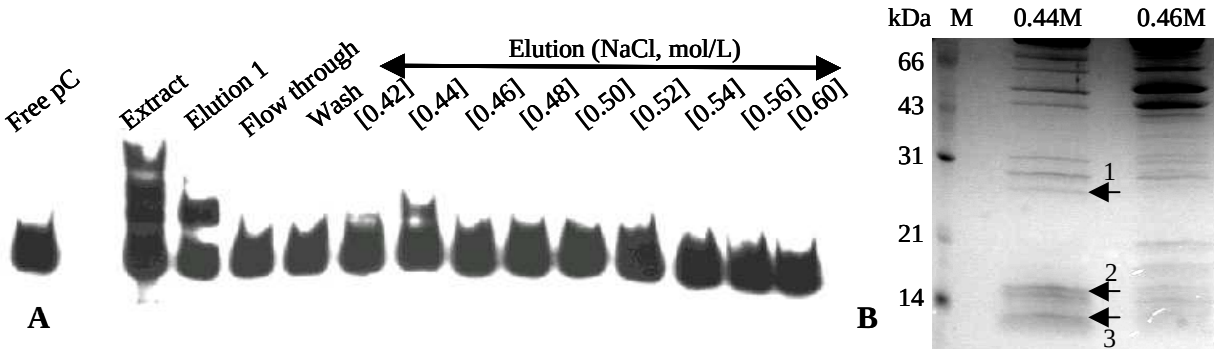


FIG. 15. Isolation of protein(s) possessing pC binding activity. (A) Results of EMSA of Δ*exsA* crude extract or purified fractions on the pC. A 280-bp biotin-labeled pC fragment was incubated with Δ*exsA* extract or a fraction obtained from heparin-Sepharose affinity chromatography in the presence of 2 μg of poly(dI-dC) as described in Materials and Methods. Extract, total extract of Δ*exsA*; elution 1, preliminarily purified extract from 5-ml column; other lanes, fractions collected from second purification with elution at the indicated NaCl concentration. One microgram of protein was added in each lane. (B) SDS-PAGE analysis of fractions of Δ*exsA* extract purified using heparin-sepharose affinity chromatography. The fraction (0.44 M) possessing the pC binding activity and the adjacent fraction (0.46 M) not having any pC binding activity (A) were separated by SDS-10% PAGE and stained with Coomassie blue. The bands present only in the 0.44 M fraction indicated by arrows were sliced, and a MALDI-TOF analysis was performed.

Table 11. Analysis of MALDI-TOF

band	Protein name	Gene name	*PA number
1	PsrA (*Pseudomonas* sigma regulator)	*psrA*	PA3006
	Vfr (virulence factor regulator)	*vfr*	PA0652
2	RplN (50S ribosomal protein L14)	*rplN*	PA4253
	SpuC (putrescine aminotransferase)	*spuC*	PA0299
3	RplN (50S ribosomal protein L14)	*rplN*	PA4253
	RplS (50S ribosomal protein L19)	*rplS*	PA3742

* PA number refers to the *P. aeruginosa* assigned numbers in www.pseudomonas.com.

3.3.2 Recombinant protein PsrA binds to the pC region

To determine if PsrA and/or Vfr could effectively bind to the pC, we produced recombinant protein PsrA (rPsrA) and rVfr in *E. coli* BL21(DE3). Recombinant proteins were purified by using a nickel affinity column chromatography with imidazole gradient elution, analyzed by

SDS-PAGE, visualized with Coomassie staining, and confirmed by Western blotting using anti- His_6 antibody (Fig. 16).

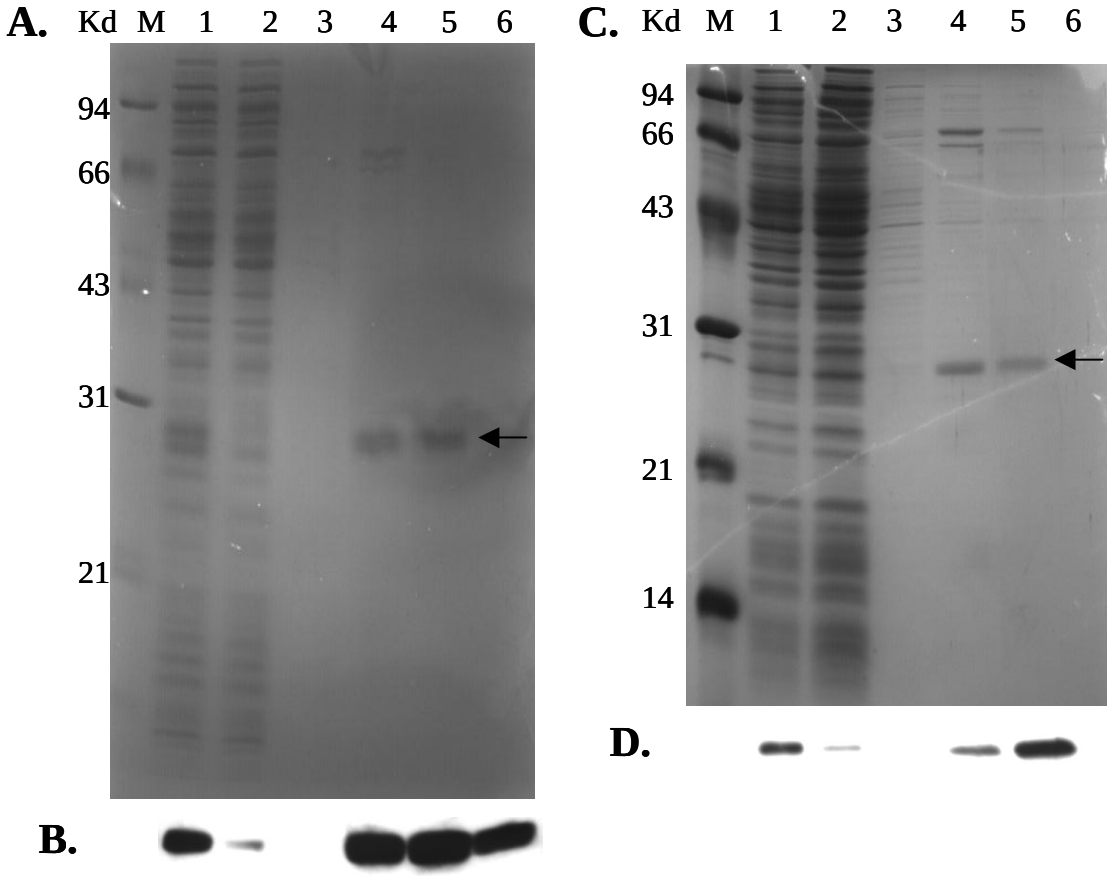


FIG. 16. Expression and purification of recombinant proteins rPsrA and rVfr. SDS-PAGE analysis of rPsrA (A) and rVfr (C), which were expressed from a pET15b vector in *E.coli* BL21 (DE3) and purified using Ni-NTA resin from Invitrogen with different imidazole concentrations in the wash and elution step. Kd, kilodalton; M: protein marker whose sizes are indicated at left side; 1: total cell lysate (5 mM imidazole); 2: flow-through; 3: wash (10 mM imidazole); 4-6: 50 mM, 100 mM and 200 mM imidazole elution respectively. Proteins were visualized by a Coomassie Blue-stained 15% SDS-Polyacrylamide gel. Arrows indicate the position of rPsrA (A) and rVfr (C). Western blot analysis of rPsrA (B) and rVfr by using anti-His_6-HRP antibody, the bands present in (B) and (D) correspond to the bands presented at the level indicated by arrows in (A) and (C) respectively.

To verify if rPsrA and/or rVfr is able to bind the pC, an EMSA was performed. Purified rPsrA and rVfr were tested for their ability to bind the 280-bp DNA pC fragment containing promoter of *exsCEBA* operon. As shown in Fig. 17, the mobility of the pC was retarded in the presence of 1.5 μM rPsrA. It has to be noted that the position at which this retardation occurred is not exactly the same as that due to the preliminarily purified extract from which PsrA was identified. In contrast, no retardation of the pC was observed in the presence of rVfr, even in combination with 20 μM cyclic AMP, a known ligand of Vfr that increase its

capacity to bind DNA. The mixing of rPsrA and rVfr in an equimolar ratio did not modify the retardation profile of rPsrA alone. This suggested that Vfr is not able to bind, at least *in vitro*, to the pC. The binding of rPsrA needed relatively high concentrations of recombinant protein, indicating either a low binding affinity for the pC or poor activity of the recombinant protein. In order to determine the binding specificity of our rPsrA on the pC, we also produced a 280-bp p*psrA*, a known promoter for PsrA (Kojic *et al*., 2002), and a 280-bp p*fleQ*, a proven promoter having binding sequences for Vfr (Dasgupta *et al*., 2002). As shown in Fig. 17, no retardation was observed in the presence of p*fleQ* and rPsrA even with a high concentration of rPsrA. In contrast, retardation was observed with p*fleQ* and rVfr (Fig. 17), indicating (i) that the rVfr protein is correctly folded and active and (ii) that, for the first time, we showed that Vfr is not able to bind the pC. In the same manner, p*psrA* was retarded in the presence of rPsrA at a concentration 10 times lower than that needed for the pC, indicating that rPsrA is active and that, although the binding is specific, the in vitro binding affinity of PsrA for the pC is low.

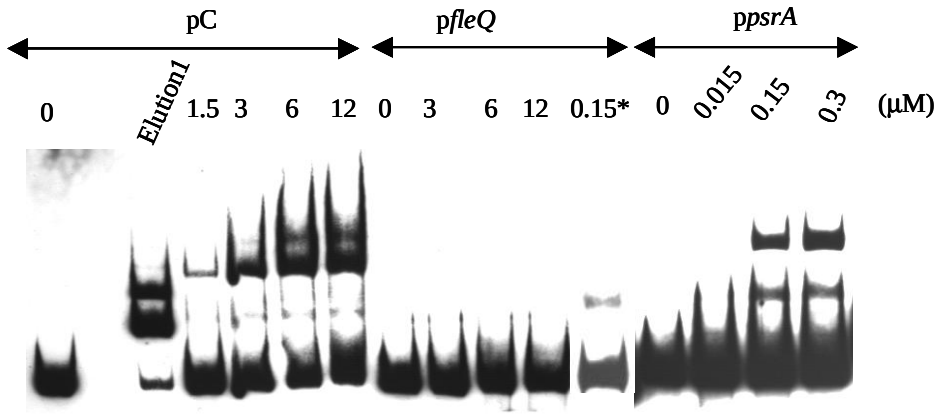


FIG. 17. rPsrA binds to the pC region. An EMSA experiment was performed to show that rPsrA interacts with the pC region. Biotin-labeled DNA fragments containing pC, p*fleQ*, or p*psrA* were incubated in the presence of 2 µg of poly(dI-dC) with purified rPsrA of different concentrations as indicated in the figure. Elution 1, preliminarily purified extract (1 µg) fraction obtained from the first heparin-Sepharose affinity chromatography purification as described in Materials and Methods. *, rVFR was used for this lane to verify its p*fleQ* binding activity.

3.3.3 Mutant Δ*psrA* and Δ*psrA* containing gene reporter *pClux* or *pSlux*

As PsrA is able to bind the pC, we asked whether PsrA was indeed necessary for the transcription of *exsA* and logically the TTSS effectors, such as *exoS*, in response to various known stimuli of the TTSS. Therefore, from our CHA parental strain we constructed, by

allelic exchange, an unmarked in-frame deletion mutation of Δ*psrA* as described in Materials and Methods, yielding the strain Δ*psrA*, and then Δ*psrA* containing one single copy of transcriptional fusions of *exsCEBA* with *lux* [Δ*psrA* (*pClux*)], and Δ*psrA* containing one single copy of transcriptional fusions of *exoS* with *lux* [Δ*psrA* (*pSlux*)], which were confirmed by PCR (Fig. 18).

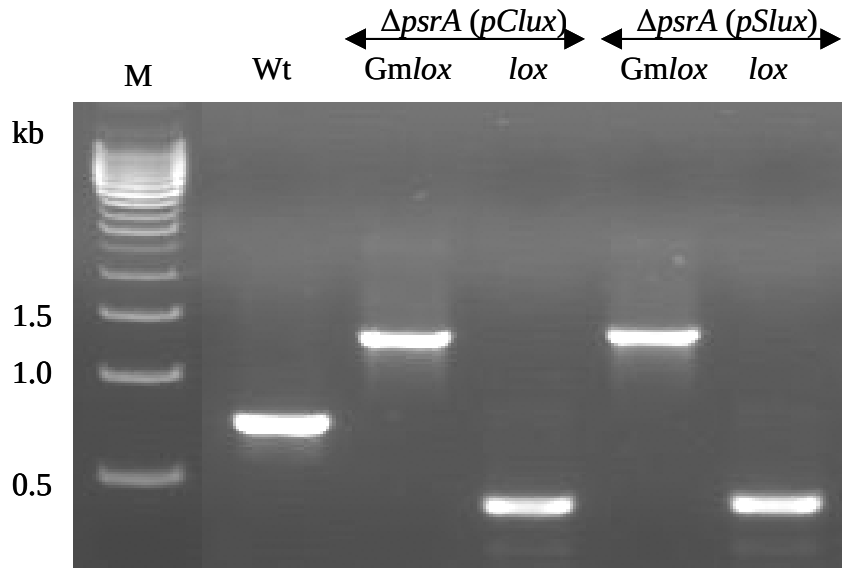


FIG. 18. Mutant analysis by PCR. PCR was performed on genomic DNA prepared from different strains. Wt, wild-type CHA; Gmlox, *lox*-flanked *aacC1* encoding gentamicin resistance, is present in the chromosome; *lox*, only *lox* but not *aacC1* is present in the chromosome. M: DNA marker whose sizes are indicated at left side; kb, kilobase.

3.3.4 PsrA is required for the full transcriptional activation of *exsA* and *exoS*

---PsrA is required for the full activation of *exsA* and *exoS* by calcium depletion

The different strains were cultured from an OD_{600} of 0.05 in TTSS-noninducing conditions (in the presence of calcium) and in TTSS-inducing conditions (in the presence of calcium chelator EGTA). As seen in Fig. 19, the transcriptional level for *exoS* and *exsA* in the CHA wild-type was two- to threefold higher than that in the Δ*psrA* strain over 4 h of growth with EGTA. However, the difference in the fold activation of gene expression between the wild-type strain and the Δ*psrA* strain was quite low (4.7/3.3 and 11.1/10.4). Note that under these culture conditions, no discernible growth difference was observed between the strains. Thus, it seemed that PsrA is implicated in the regulation of the level of expression of *exsA* and hence *exoS* but not in the regulation in response to calcium depletion. As calcium depletion

activation occurs through the release of ExsA from its antiactivator ExsD, our result confirm that PsrA is a positive transcriptional activator of TTSS independent of ExsA.

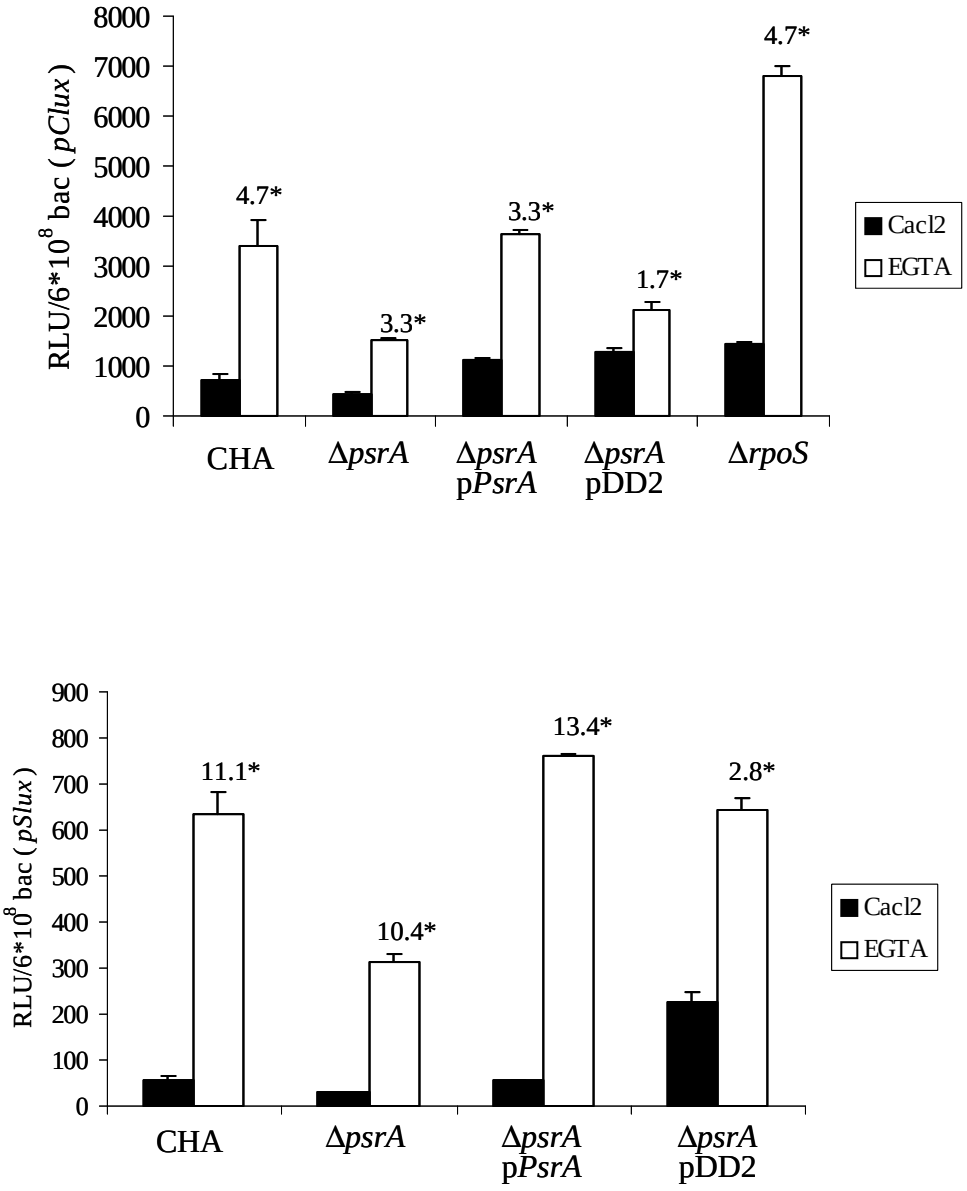


FIG. 19. Full activation of TTSS transcription requires PsrA. The CHA wild-type, Δ*psrA* , Δ*psrA*(p*PsrA*), Δ*psrA*(pDD2), and Δ*rpoS* strains carrying a single copy of the *pClux* (up) or *pSlux* (down) transcriptional reporter at its chromosome were grown at 37°C in LB under TTSS-noninducing (black bars) or -inducing (white bars) conditions. RLU were measured over 4 h of culture and calculated into normalized bacterial counts (6×10^8 bac). Bars represent the means plus the standard deviations of results for triplicate samples and show the averages of results from three independent experiments. *, fold activation of gene expression under inducing conditions compared with that under noninducing conditions. bac, bacteria.

---PsrA is required for the full activation of *exsA* and *exoS* by serum

Since serum is one of the environmental signals that can induce the expression of TTSS in P. aeruginosa (Vallis et al., 1999; Hornef et al., 2000, Kim et al., 2005), we asked what is role of PsrA in such induction. It is interesting that, in the presence of the human serum stimulation for 4 h, the transcription of *exsA* was always about fourfold lower in the Δ*psrA* strain than in the CHA wild-type and the fold activation in psrA was two times lower than that in the wild-type (Fig. 20).

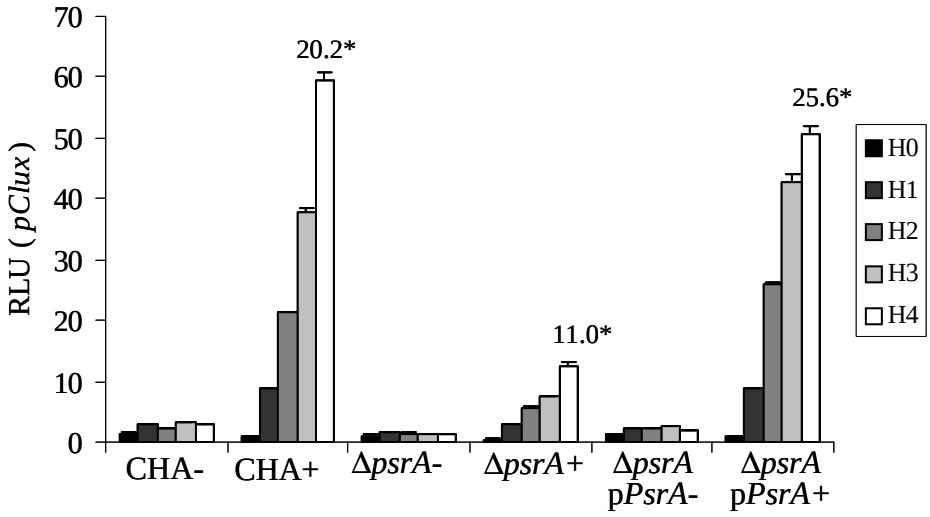


FIG. 20. Activation of TTSS transcription by serum requires PsrA. Cultured bacteria (5×10^5) of the CHA wild-type and strains Δ*psrA* and Δ*psrA* (p*PsrA*) carrying a single copy of the *pClux* transcriptional reporter at its chromosome were incubated with mHBS buffer (–) or 10% human serum AB (+) in a 96-well plate. RLU were measured over 4 h of incubation. RLU readings were taken immediately after addition of serum (t = 0) and at 1-h intervals. Bars represent the means plus the standard deviations of results for triplicate samples and show the averages of results from two independent experiments. *, fold activation of gene expression at hour 4 under inducing conditions compared with that under noninducing conditions. H0 to H4, hours 0 to 4.

Complementation of Δ*psrA in trans* with the plasmid p*PsrA* restored the level of transcription of *exsA* and *exoS* in response to both calcium depletion and serum AB stimuli (Fig. 19 and Fig. 20). Expression *in trans* of *exsA* only partially restored the level of transcription of *exsCEBA* under the calcium depletion condition; however, it completely restored the level of *exoS* expression (Fig. 19). Thus, PsrA appeared to be really required for the full expression of *exsCEBA* and hence the TTSS effectors, such as *exoS*.

---PsrA is required for the full activation of *exsA* upon host cell contact

Besides calcium depletion growth conditions, the expression of the *P. aeruginosa* TTSS and secretion of effector proteins could also be induced after contact with tissue culture cells (Vallis *et al*., 1999; Hornef *et al*., 2000). Therefore, we asked whether PsrA is required for such induction by measuring transcription of the *pClux* reporter construct in wild-type and Δ*psrA* strains following infection of PLB-985 polymorphonuclear leukocyte-like cells. As seen in Fig. 21, *exsCEBA* transcription increased about fourfold at hour 4 in CHA in the presence of PLB-985 cells as did that in the Δ*psrA* strain; on the contrary, the global transcription level of *exsCEBA* in the Δ*psrA* mutant was twofold lower than that in the wild-type strain.

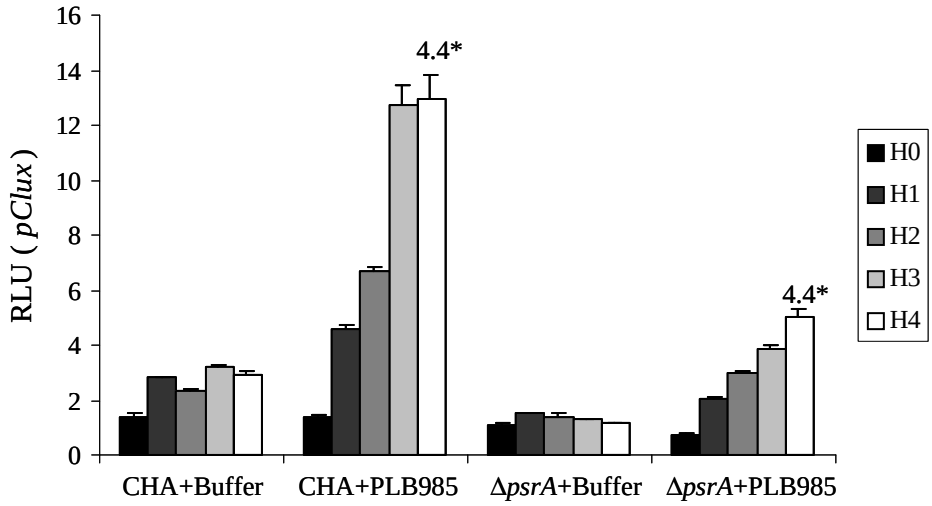


FIG. 21. Transcriptional activation of *exsCEBA* during exposure of bacteria to differentiated PLB-985 cells requires PsrA. RLU produced by 5×10^5 cultured bacteria of CHA and Δ*psrA* strains carrying a single copy of the *pClux* transcriptional reporter at its chromosome were measured over 4 h following bacterial interaction with 10^5 PLB-985 cells or mHBS buffer control in a 96-well plate. RLU readings were taken immediately after the addition of bacteria (t = 0) and at 1-h intervals. Bars represent the means plus the standard deviations of results for triplicate samples and show the averages of results from two independent experiments. *, fold activation of gene expression at hour 4 under inducing conditions compared with that under noninducing conditions. H0 to H4, hours 0 to 4.

3.3.5 Secretion of the type III effectors is decreased in the Δ*psrA* strain

Secretion of TTSS effectors is strongly correlated with *P. aeruginosa* virulence both in animal models and in studies of human disease (Hauser *et* al., 2002). Therefore, we examined the secretion of TTSS effector molecules in wild-type CHA, the Δ*psrA* strain, and the Δ*psrA* strain carrying pPsrA [Δ*psrA* (p*PsrA*)] under TTSS-noninducing or -inducing conditions. As seen in Fig. 22, the Δ*psrA* mutant showed markedly decreased secretion of ExoS, ExoT, and PopB proteins compared to the parental CHA strain. Conversely, complementation of Δ*psrA* restored the strain's ability to secrete ExoS, ExoT, and PopB proteins whose amounts seemed slightly higher than those of proteins from the wild-type CHA strain, confirming the positive regulatory function of PsrA for the TTSS. However, ectopic expression of *psrA* cannot overcome the requirement for a TTSS-inducing signal, such as calcium depletion, as demonstrated by the absence of ExoS, ExoT, and PopB secretion when Δ*psrA*(p*PsrA*) was grown under noninducing conditions, indicating that the presence of EGTA is essential for the secretion (Fig. 22).

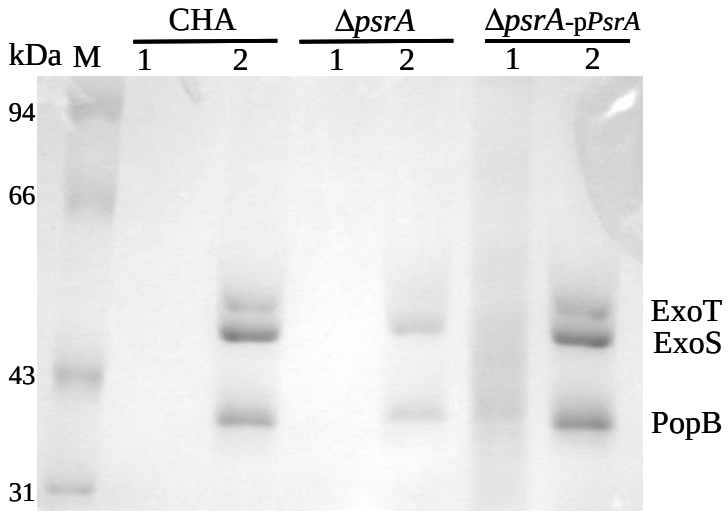


FIG. 22. Secretion of the type III effectors is decreased in the Δ*psrA* strain. Bacteria were grown under TTSS-noninducing (lanes 1) or TTSS-inducing (lanes 2) conditions. Supernatants normalized to bacterial counts (6×10^8 bacteria $lane^{-1}$) were subjected to trichloroacetic acid precipitation. The extracellular proteins were separated by SDS-10% PAGE and stained with Coomassie blue. M, protein marker. Sizes in kilodaltons are indicated at the left.

3.3.6 Strain Δ*psrA* is less resistant to the phagocyte-like PLB-985 cells

To examine the effect of the Δ*psrA* mutation on the ability of *P. aeruginosa* to resist the bacterial killing activity of polymorphonuclear leukocyte-like cells from differentiated PLB-985 cells, CHA, the Δ*psrA* strain, and Δ*exsA*, a TTSS-defective strain, were compared. Fig. 23 shows that the Δ*psrA* strain is less resistant to the phagocyte-like PLB-985 cells than its parental strain, CHA, as the

number of viable bacteria after 80 min of coculture with PLB-985 was two times lower for the Δ*psrA* strain than for wild-type CHA. Nevertheless, the survival of Δ*exsA* in the presence of phagocyte-like PLB-985 cells reveals that, besides the TTSS, other factor(s) may also play a role in the resistance of *P. aeruginosa* to phagocytosis.

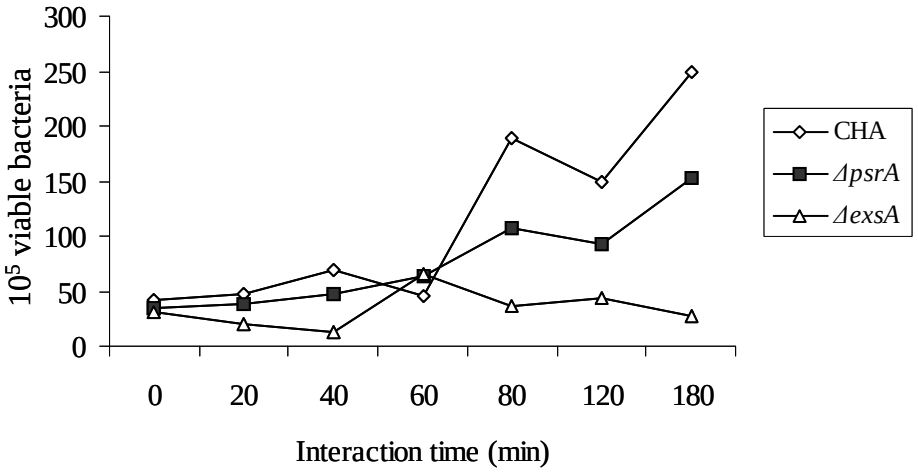


FIG. 23. The Δ*psrA* strain is less resistant to phagocyte-like PLB-985 cells. The numbers of viable bacteria were calculated from colony counts made from a $1:10^5$ dilution over 3 h following bacterial interaction with differentiated PLB-985 cells at different intervals. Points represent the means plus the standard deviations of results for duplicate samples and show the averages of results from two independent experiments.

4. Discussion

The TTSS is known to be tightly controlled by the proteins encoded by the regulatory operon *exsCEBA*, particularly by the central positive regulator ExsA, though many other proteins, such as Vfr, RetS, PtrA, and pyruvate dehydrogenase, are implicated in the regulation of the TTSS (Fig. 24).

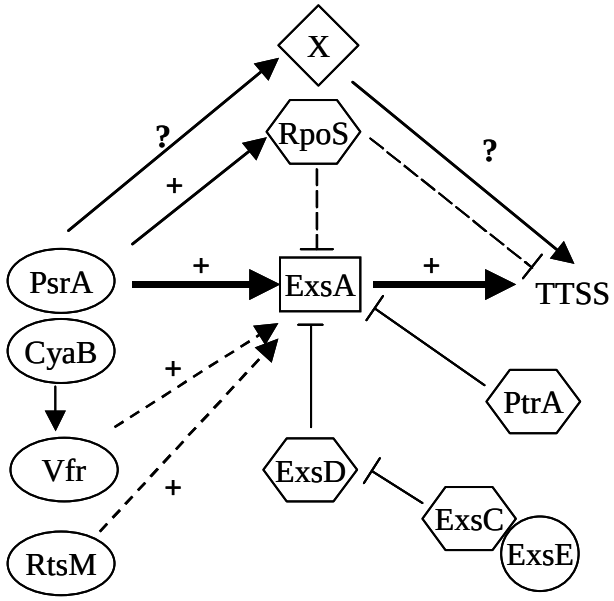


FIG. 24. A model for the regulation of the TTSS. PsrA activates the TTSS through a positive effect (+) on the expression of ExsA and may also regulate another unknown protein(s) (X) which has a role in the TTSS. PsrA also has a positive effect on RpoS, which has a negative effect on the TTSS by an unknown mechanism. Other main molecules involved in the regulation of the TTSS that are mentioned in the text are also indicated.

It is not surprising that *P. aeruginosa* has multiple regulatory networks, because 8% of its genome codes for regulatory genes, indicating that *P. aeruginosa* has dynamic and complicated regulatory mechanisms responding to various environmental signals (Greenberg, 2000). Because of the requirement for a large number of genes, the construction of the type III secretion apparatus is an energy-expensive process for the bacteria, rendering so important the ability to precisely regulate the TTSS in response to the environmental changes.

In this work, we describe a *P. aeruginosa* protein, PsrA, that is required for the full activation of the TTSS regulatory operon *exsCEBA* and hence the expression of effector genes, although the mutant still responds to depletion of calcium, exposure to serum, and contact with tissue culture cells. PsrA was previously described as a regulator involved in positive regulation of RpoS and in negative autoregulation by direct binding to the promoters (Kojic and Venturi, 2001; Kojic *et al*., 2002; 2005). RpoS, a central regulator during stationary phase (Loewen *et*

al., 1998), was identified also as an inhibitor of the TTSS (Hogardt *et al*., 2004) (Fig. 24). The PsrA-dependent induction of the synthesis of RpoS has been shown to take place in the late exponential and stationary phases (Kojic and Venturi, 2001). Here we measured the induction of the TTSS by different stimuli in the first part of the exponential growth phase when no RpoS induction of synthesis could occur. Furthermore, we confirmed the RpoS-dependent inhibition of TTSS synthesis in our strain CHA Δ*rpoS*(*pClux*) (Fig. 19). This indicated that the action of PsrA on TTSS gene transcription is independent of its role in RpoS regulation. A previous study showed that a Δ*psrA* mutant had a 90% reduction in *rpoS* promoter activity (Kojic and Venturi, 2001); considering also the opposite functions of PsrA and RpoS in TTSS regulation, we could speculate that PsrA should have an even more important regulatory ability for the TTSS than that measured here. It seemed that PsrA is implicated rather in the regulation of the basic level of *exsA* and hence *exoS* expression than in the regulation in response to environmental signals, at least calcium depletion and host cell contact, since there was no or only a small difference in fold activation of gene expression under these inducing conditions compared with that under noninducing conditions for the mutant and the wild-type strain (Fig. 19 and Fig. 21). We also always observed that the growth curves of the CHA and Δ*psrA* strains were identical, indicating that the deletion of *psrA* has no metabolic consequences for the CHA strain. Moreover, the complementation *in trans* by a multicopy plasmid harboring the *psrA* gene controlled by its own promoter showed an augmentation of the type III secretion. In addition, we showed that the decrease of *exsCEBA* expression due to *psrA* deletion could not be overcome by expression *in trans* of *exsA*, while *exoS* expression could be restored (Fig. 19). Taken altogether, these results suggest that PsrA is a ExsA-independent positive regulator of *exsCEBA* and hence the whole TTSS genes.

Using the PsrA binding motif (G/CAAAC $N_{2\ to\ 4}$ GTTTG/C, where $N_{2\ to\ 4}$ is any two to four nucleotides) to search against the *P. aeruginosa* genome sequence at http://www.pseudomonas.com revealed that at least 26 genes may be controlled by PsrA (Table 12), indicating that the PsrA is a global regulator. 15 genes were described as target genes of the PsrA regulator (kojic *et al*., 2005) during the process of our submitted paper concerning PsrA. It is reasonable to propose that PsrA could influence the TTSS by activating or repressing genes having a potent regulatory role in TTSS expression (Fig. 24). Identification of such unknown genes as well as the relationship with PsrA will help us to better understand the mechanism by which PsrA affects TTSS expression.

TABLE 12. Possible promoter regions containing PsrA binding sites

PsrA binding site			Region Type	Gene name or N° of PA
CAAAC	AAAC	GTTTG	intergenic	*etfB*-PA2953
CAAAC	AGTG	GTTTC	intergenic	*groES*-PA4387
CAAAC	GCCT	GTTTG	intergenic	PA0505-PA0506
GAAAC	CAGC	GTTTC	intergenic	PA3594-PA3595
CAAAC	CCGC	GTTTC	intergenic	PA4792-PA4793
GAAAC	CG	GTTTC	intergenic	PA4963-parC
CAAAC	ACTT	GTTTG	intergenic	*psrA-lexA* (PA3006-3007)
GAAAC	CG	GTTTC	intergenic	*ptxR-ptxS* (PA2258-2259)
GAAAC	CG	GTTTC	intergenic	*ptxS*-PA2260
GAAAC	GCT	GTTTG	PA_gene	*argJ* (PA4402)
GAAAC	TG	GTTTC	PA_gene	*betB* (PA5373)
CAAAC	CC	GTTTG	PA_gene	*cyoA* (PA1317)
GAAAC	GTAT	GTTTC	PA_gene	*cysC* (PA1393)
CAAAC	GAGC	GTTTC	PA_gene	*ligT* (PA2861)
GAAAC	CGGG	GTTTC	PA_gene	*mmsA* (PA3570)
GAAAC	CC	GTTTC	PA_gene	PA0098
CAAAC	CCG	GTTTC	PA_gene	PA0112
GAAAC	TGAA	GTTTC	PA_gene	PA0805
CAAAC	CGA	GTTTC	PA_gene	PA1736
CAAAC	TCC	GTTTG	PA_gene	PA2674
CAAAC	TTCC	GTTTG	PA_gene	PA3623
GAAAC	GCCC	GTTTC	PA_gene	PA4421
GAAAC	CG	GTTTG	PA_gene	PA4489
GAAAC	CC	GTTTC	PA_gene	*pilK* (PA0412)
CAAAC	GT	GTTTC	PA_gene	*pilT* (PA0395)
GAAAC	CG	GTTTC	PA_gene	*wbpW* (PA5452)
C/GAAAC	N2-4	GTTTG/C		

We do not know the signal(s) to which PsrA responds, but it may play an important role during infection, as Δ*psrA* bacteria are less resistant to phagocyte-like PLB-985 cells. Furthermore, the presence of PsrA homologues in other pathogenic bacteria that employ a TTSS, such as *P. syringae*, raises the possibility that these homologues also play roles in regulating the other TTSS. If this proves to be the case, it will be interesting to ask whether the signal(s) transduced by PsrA and its homologues is also conserved among plant and human pathogens.

Though the rPsrA *in vitro* bound the pC with a relatively low affinity, it is still possible that PsrA may *in vivo* activate the TTSS by directly binding onto it. In fact, pC sequence analysis revealed no presence of a true consensus binding site for PsrA, but some sequences, such as GAAAC at the position -56 from the transcriptional start site, resemble a partial binding site. Considering the complicated regulatory network of the TTSS, it is possible that PsrA may bind the pC *in vivo* at a higher affinity in the presence of another partner(s) or it may also help

another regulator(s) to bind the pC. The copurification of PsrA and Vfr, an known activator of the TTSS (Wolfgang *et al*., 2003), suggests possible interaction of PsrA and Vfr during the process of regulation of the expression of the TTSS. Exploring the interaction of PsrA with another molecule(s) and the pC would enrich understanding of the adaptation of bacteria to living conditions. The extract of the Δ*psrA* strain possessed the same pC binding activity as the one of Δ*exsA* strain (Fig. 25), indicating that the protein(s) having a strong pC binding activity has not been isolated in this work and is possibly the partner of PsrA in TTSS regulation. Isolation and identification of this protein will provide us more information about the regulation of TTSS genes expression via the transcription level of the regulatory operon *exsCEBA*.

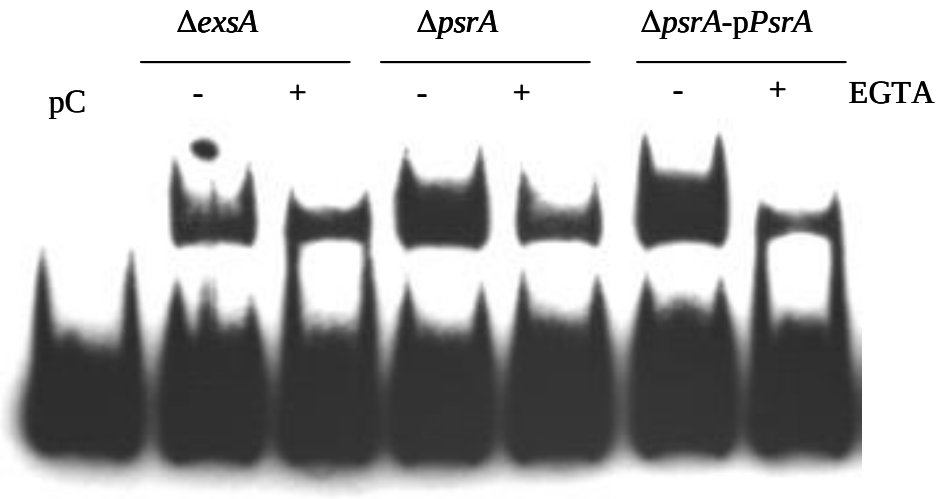


FIG. 25. Extract of Δ*psr* preserve the pC binding activity. Extracts of different strains as indicated were prepared from TTSS non inducing (-) and inducing conditions (+, EGTA). A 280-bp biotin-labeled pC fragment was incubate with 1 μg extract in the presence of 2 μg of poly(dI-dC).

Chapter 2

Study of the inhibition of TTSS genes expression during the stationary phase

Résumé en français

P. aeruginosa est responsable d'infections aiguës à d'infections chroniques. Le système de sécrétion de type III (SSTT) est un facteur de virulence qui semble être très important dans les infections aiguës chez l'humain mais moins important pour maintenir les infections chroniques causées par *P. aeruginosa*. Nous avons observé que l'expression du SSTT est, *in vitro*, dépendante de la densité des bactéries dans le milieux de culture. En outre, le surnageant venant d'une culture en phase stationnaire peut inhiber l'activation du SSTT. Ici nous rapportons l'observation de PARST, un inhibiteur qui est produit dans la phase stationnaire de la culture et réprime l'expression du SSTT *in vitro*. L'analyse des mutants d'insertion de transposon a montré que la production de PARST pourrait dépendre du tryptophane, qui est le précurseur de l'acide d'indole-3-acétic (IAA). IAA-Na et un analogue fonctionnel, potassium acide 1-naphthalacétic (NAA-K) à des concentrations millimolaires sont en effet capable d'inhiber l'expression et la sécrétion du SSTT. Une molécule de *m/z* 224.2, présent uniquement dans le surnageant stationnaire de la souche sauvage CHA mais pas celui du mutant avec la délétion de gène *trpA* a été déterminé par LC-MS. Cette molécule de *m/z* 224.2 a le même poids moléculaire de NAA-K pourrait être l'inhibiteur de l'expression des gènes du SSTT quand les bactéries entrent la phase stationnaire. Ces observations fournissent une autre voie régulatrice dans la transition de l'expression de gènes entre un état avec des cellules à faible densité et celui avec des cellules à forte densité.

En anglais

1. Background

Several reports suggest that functional type III secretion of *P. aeruginosa* is far less common in cystic fibrosis (CF) patient isolates than in isolates from patients with acute infections (Dacheux *et al*., 2000; Roy-Burman *et al*., 2001). Following infection of cystic fibrosis patient airways, *P. aeruginosa* strains evolved to reduce the expression of TTSS (Lee *et al*., 2005) or gradually changed from a type III protein secretion-positive phenotype to a secretion-negative phenotype (Jain *et al*., 2004). This theme seems to be common in bacterial infection, because the expression of other TTSSs were also down-regulated during mammalian cell infection by *Salmonella enterica* serovar Typhimurium, *E. coli* O157 and *Shigella flexneri* (Eriksson *et al*., 2003; Dahan *et al*., 2004, Lucchini *et al*., 2005, Faucher *et al*., 2006). The consistency of these data suggests that the down-regulation of TTSS genes may be a conserved phenomenon that follows successful infection of mammalian cells by these gram-negative pathogens. Presumably, a mechanism should exist for inactivating the TTSS once it has fulfilled its mission.

In enterohemorrhagic and enteropathogenic *E. coli*, study showed that maximum expression of TTSS happens during the transition from the late exponential to the stationary phase (Sperandio *et al*., 1999). In *P. aeruginosa*, we observed that maximum expression of TTSS genes such as *exsCEBA* and *exoS*, happens at 4h of culture (corresponding an OD_{600} of 1.5 to 2.0), and then decreased rapidly with the increase of OD_{600} (Fig. 27, page 131). Furthermore, we observed the existance of unknown inhibitor(s) repressing the expression of TTSS genes when bacteria enter stationary phase in the culture medium. This unknown inhibitor(s) was termed as ‘PARST’ for ‘*Pseudomonas* autorepressor of secretion three’.

2. Objective and strategy

Our aim is to identify gene(s) involved in the production of PARST and finally the nature of PARST. For such purpose, we have used strategy summarized in Fig. 26. Briefly, we first produced a library of about 10^5 mutants using mariner-based transposon *Himar*1::Gm^r from plasmid pFAC (Wong and Mekalanos, 2000; Diaz-Perez *et al*., 2004). Then, we screened about 2,000 mutants for the effect of their stationary phase culture supernatant on the expression of *exoS*. We selected and characterized transposon insertion mutants whose supernatants did lose the ability to inhibit the *exoS* expression. To investigate the nature of PARST, further analysis such as liquid chromatography-mass spectrometry will be performed.

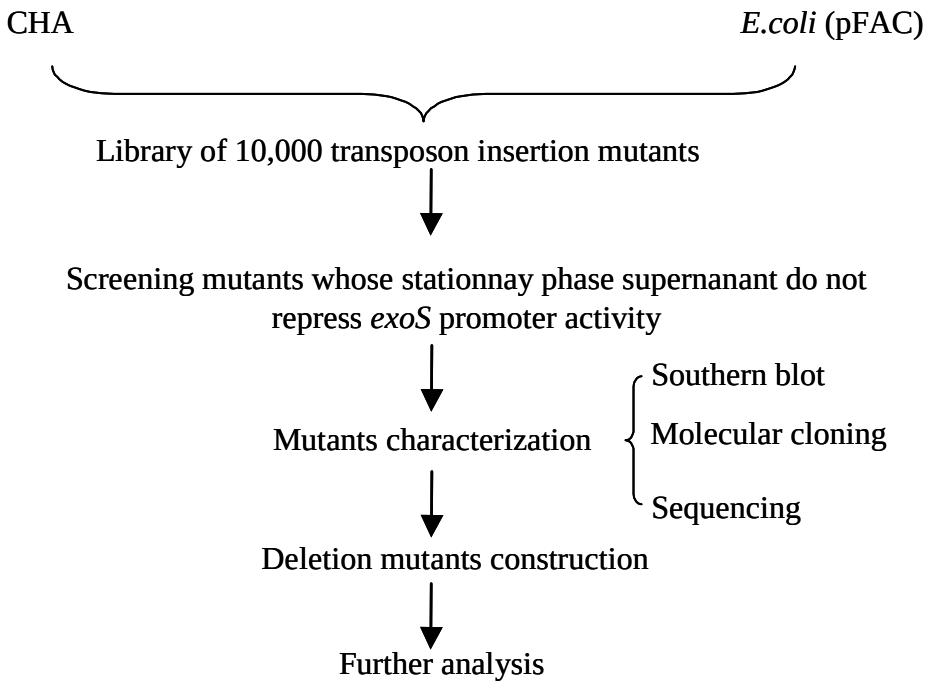


FIG. 26. Strategy in determination of the nature of PARST

3. Results

3.1 TTSS expression is cell density-dependent

Chromosomal transcriptional gene reporters composed of *exsCEBA* protmoter-*lux* fusion (*pClux*), and *exoS* promoter-*lux* fusion (*pSlux*) were used to study precisely the transcriptional level of *exsCEBA* and *exoS* (Shen *et al*., 2006). From the results shown in Fig. 27, it is evident that both *exsCEBA* and *exoS* were expressed in a growth phase-dependent manner when strains were initially cultured from an OD_{600} of 0.05 in LB without or with calcium depletion by EGTA. *ExsCEBA* and *exoS* expression reached their maximums at 4h of culture corresponding an OD_{600} of 1.5 to 2.0 and then decreased rapidly with the increase of OD_{600}. We were not sure why *exsCEBA* and *exoS* expression decline over time but this phenomenon was reminiscent of quorum sensing (QS) regulation, in which a sufficient number of bacteria, the bacterial 'quorum', is needed to induce or repress expression of a group of genes (Fuqua *et al*., 1994). QS having an impact on TTSS expression in *P. aeruginosa* (Schaber *et al*., 2004; Hogardt *et al*., 2004; Bleves *et al*., 2005; Juhas *et al*., 2005) plus report that the maximum expression of TTSS happens during the transition from the late exponential to the stationary phase in enterohemorrhagic and enteropathogenic *E. coli* (Sperandio *et al*., 1999) prompted us to examine the possibility that the reduction of *exsCEBA* and *exoS* expression over time was due to the regulation of QS, which control genes expression at cell density-dependent manner by producing different QS signaling molecules.

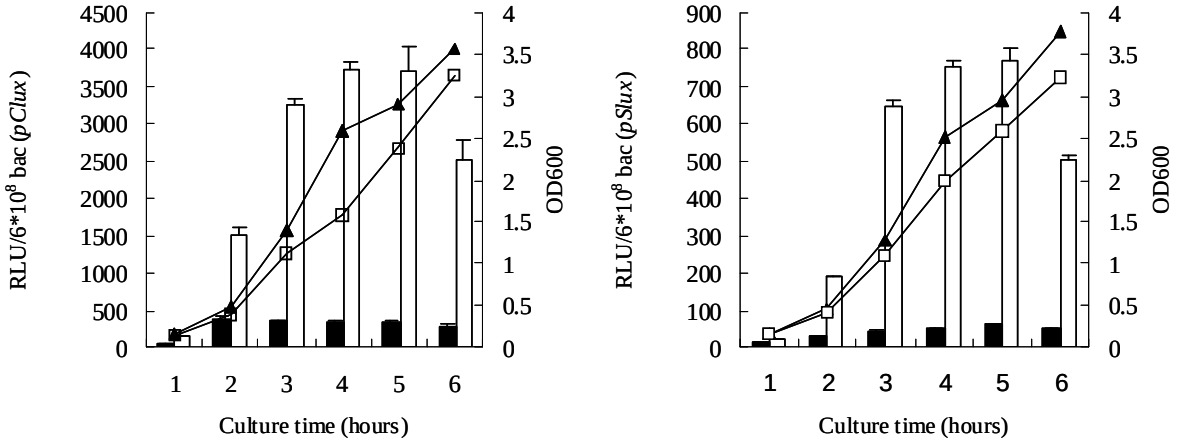


FIG. 27. TTSS expression is cell density-dependent. The CHA wild type carrying a single copy of *pClux* (right) or *pSlux* (left) transcriptional reporter at its chromosome was grown at 37°C in LB under TTSS-noninducing (black bars, in the presence of 5 mM calcium) or inducing conditions (white bars, in the presence of 5mM EGTA and 20 mM $MgCl_2$). RLU were measured over 6h of culture at 1-h intervals and calculated to normalized bacterial counts (6×10^8 bac). Bars represent the mean plus the standard deviation of triplicate samples, and are the average of three independent experiments. Black angles and white squares represent the OD_{600} in noninducing and inducing conditions, respectively. bac, bacteria.

3.2 PARST inhibits *exsCEBA* expression during the stationary phase

To verify our hypothesis mentioned above, we constructed known QS mutants, including Δ*lasI*, Δ*rhlI*, Δ*lasIrhlI* and Δ*phnAB*, and prepared stationary phase culture supernatant as described in Materials and Methods (work realized by Filopon, thesis, UJF grenoble, 2005). Supernatants were then added to the CHA (*pClux*) cultured from an initial OD_{600} of 0.05 in LB under TTSS-inducing conditions in order to evaluate the influence of each supernatant on the *exsCEBA* expression. In contrast to TTSS-noninducing conditions, *exsCEBA* expression was induced about fivefold in inducing conditions (calcium depletion); however, such induction was decreased in the presence of stationary phase culture supernatant from both the wild-type CHA and QS mutants in which the transcriptional level of *exsCEBA* were in the same range and were about twofold lower than that in the absence of supernatant (Fig. 28).

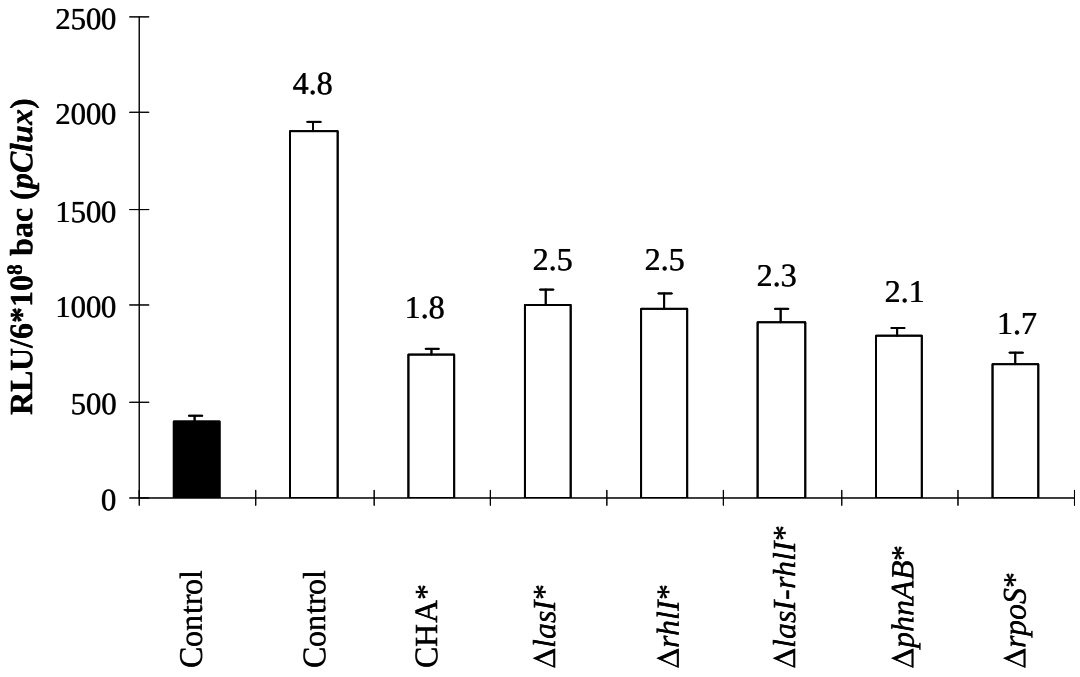


FIG. 28. PARST, but not QS signals, inhibits *exsCEBA* expression during the stationary phase. The wild-type CHA carrying a single copy of *pClux* transcriptional reporter at its chromosome was grown at 37°C in LB under TTSS-inducing conditions (white bars) supplemented with stationary phase culture supernatant from CHA, Δ*lasI*, Δ*rhlI*, Δ*lasIrhlI*, Δ*phnAB and* Δ*rpoS*, indicated as asterisk. RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8 bac). Bars represent the mean plus the standard deviation of triplicate samples, and are the average of three independent experiments. Number indicated above the bar are fold activation of *exsCEBA* expression under inducing conditions compared with that under noninducing conditions (black bar). bac, bacteria. control, no supernatant was added. This work was realized by Filopon, thesis, Université Joseph Fourier-Grenoble 1, 2005.

To totally exclude the role of known QS signal molecules (AHL) in such repression, stationary phase culture supernatant were analyzed by LC-MS. As shown in Table 13, both 3-oxo-C_{12}-HSL and C_4-HSL were produced in the wild-type CHA, on the contrary, respective AHL was absent in the mutants Δ*lasI*, Δ*rhlI* and double mutant Δ*lasIrhlI*. These results indicate that i) there is an inhibitory signal(s) acting on the TTSS expression in the stationary phase culture supernatant, ii) AHL synthase LasI, which directs the synthesis of QS signaling molecule 3-oxo-C_{12}-HSL; AHL synthase RhlI, which directs the synthesis of C_4-HSL and anthranilate synthase PhnAB, which directs the synthesis *Pseudomonas* quinolone signal (PQS) may not be implicated in the production of such TTSS inhibitory signal(s).

Considering the important role of RpoS, which is a known central regulator during the stationary phase (Loewen *et al*., 1998) and recently identified as an inhibitor of the TTSS (Hogardt *et al*., 2004; Shen *et al*., 2006), we prepared the stationary phase culture supernatant from Δ*rpoS* and tested its role on the *exsCEBA* expression. As seen in Fig. 28, deletion of *rpoS* does not change the inhibitory role of supernatant on the *exsCEBA* expression. Taken together, these results indicated that inhibitor(s), different from known QS signals, play(s) a role in repressing TTSS genes expression when bacteria enter stationary phase.

TABLE 13. QS signal analysis by LC-MS

	C4-HSL	3-oxo-C12-HSL
CHA	8399	29.8
Δ*lasI*	4149	0
Δ*rhlI*	0	0
Δ*lasIrhlI*	0	0

(Filopon, thesis, UJF grenoble, 2005)

3.3 Construction and screening transposon insertion mutant library

To identify the gene(s) involved in the production of PARST in the stationary phase, a library of about 10^6 Gm-resistant mutants was generated with the mariner-based transposon *Himar*1::Gm^r from plasmid pFAC (Wong and Mekalanos, 2000; Diaz-Perez *et al*., 2004). About 2,000 mutants were screened for the role of their stationary phase culture supernatants on the expression of *exoS*. Screening was carried out in 96-well plates and optimal procedure described in Materials and Methods 20.2 was determined after different tests. Briefly, stationary phase culture supernatants were prepared in 96-well plate from tansposon insertion mutants as well as CHA wild-type and were tested for the effect on *exoS* expression of CHA (*pSlux*) in TTSS-inducing conditions. Interesting mutantd, whose supernatant seemed to lose

their ability to inhibit the *exoS* expression as exemplified in Fig. 29 (arrowed 'Interesting mutant'), were selected and stored at -20°C for further analysis.

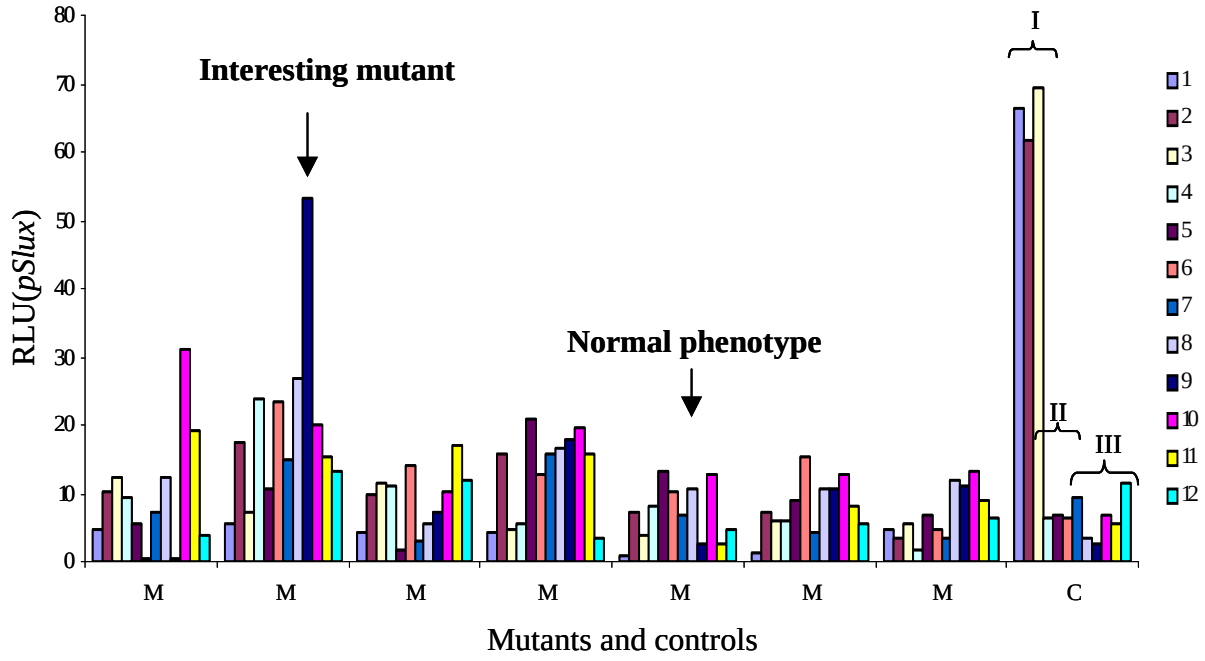


FIG. 29. High throughput screening of transposon insertion mutants that do not inhibit the *exoS* expression. Stationary phase culture supernatants were prepared in 96-well plate from tansposon insertion mutants as well as CHA wild-type and were tested for the effect on *exoS* expression by mesuring *exoS* promter activity in TTSS-inducing condtions (5mM EGTA and 20 mM $MgCl_2$) except for CII. M, mutants; C, controls, I: no PARST (but same volume of VB was added in place of PARST); II, 5 mM $CaCl_2$; III, PARST of wild-type CHA.

From about 2000 screened transposon insertion mutants, twenty mutants were selected in the first round screening and stationary phase culture supernatants of these mutants were prepared in normal culture tube (in replace of 96-well plate). Supernatants were then added to the CHA (*pSlux*) cultured from an initial OD_{600} of 0.05 in LB under TTSS-inducing conditions to evaluate if these mutants could still produce PARST. As seen in Fig. 30, most supernatants of these twenty mutants have partially lost their inhibitory role on the *exoS* expression, particularly three of them, including supernatants from mutants M7, M11 and M14, indicating that these mutants could no longer produce (enough) PARST.

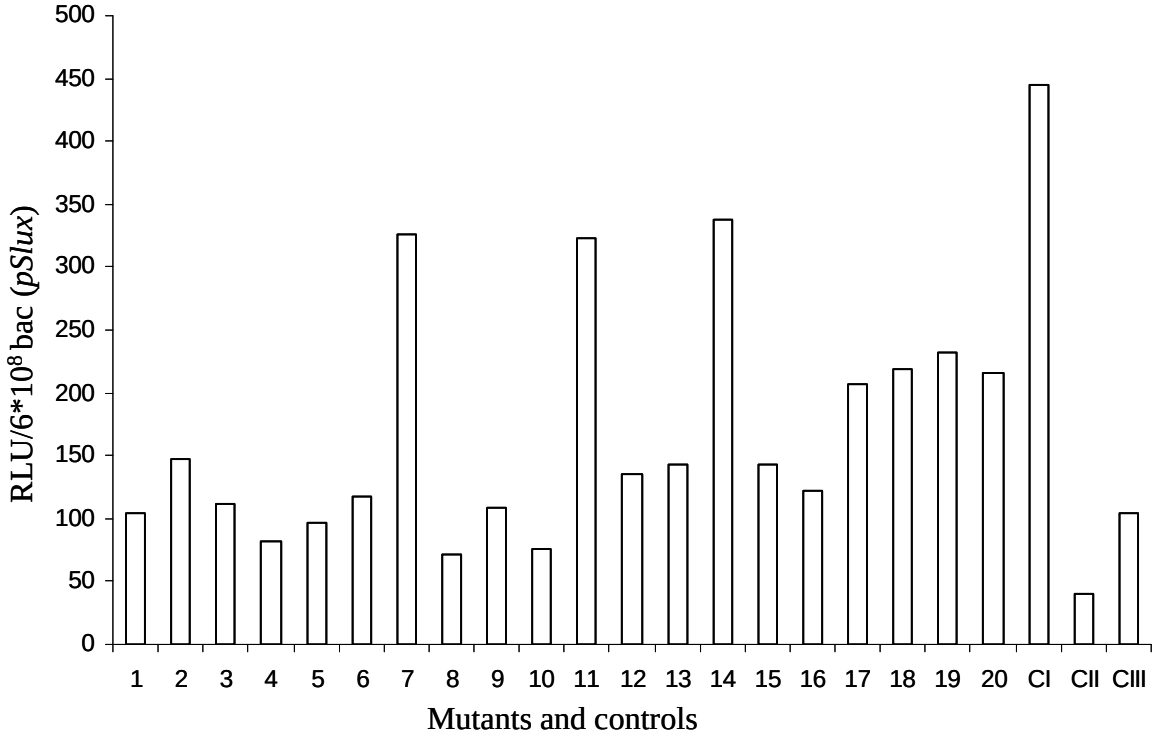


FIG. 30. Second screening of 20 transposon insertion mutants selected from first-round screening. Stationary phase culture supernatant were prepared in normal tube from 20 mutants (numbered from 1 to 20) as well as CHA wild-type (CIII) and were tested for the effect on *exoS* expression by mesuring *exoS* promter activity in TTSS-inducing condtions (5mM EGTA and 20 mM $MgCl_2$) except for CII. CI, control without PARST (but same volume of VB was added in replace of PARST); CII, TTSS-noninducing conditions (5 mM $CaCl_2$); CIII, in the presence of supernatant (PARST) from wild-type CHA.

3.4 Isolation and characterization of mutants not producing PARST

To determine the transposon position in mutant M7, M11 and M14, genomic DNA of these mutants were prepared, digested by *Pst1* and cloned into plasmid pUC18 (Fig. 31).

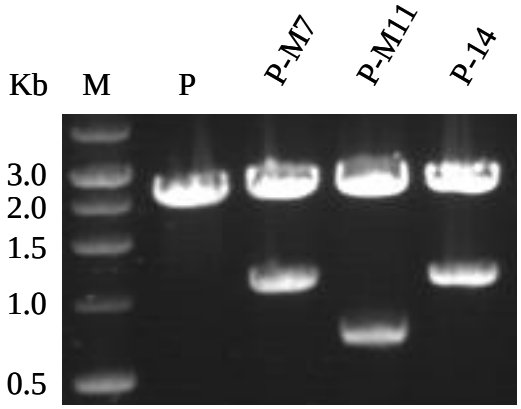


FIG. 31. Molecular cloning of genomic DNA of transposon insertion mutants. Genomic DNA of transposon insertion mutants was digested bt *Pst*1 and ligated into plasmid pUC18 (P). P-M7, P-M11, P-14 mean pUC18 containing transposon Himar1::Gm^r (encoded by *aacC*1) originated from mutant M7, M11 and M14 respectively. M, marker; Kb, kilobase.

The flanking DNA fragments of transposon were then sequenced. Comparison of sequence to PAO1genome sequence (http://www.pseudomonas.com, Stover *et al*., 2000) showed that transposon insertion of M7 and M14 had occurred in the ORF of *trpA* (position of 37371, PA0035), gene encoding tryptophan (Trp) synthase chain, which is implicated in the Trp biosynthesis (Hadero and Crawford, 1986) and *leuC* (position of 3504252, PA3121), gene encoding 3-isopropylmalate dehydratase large subunit, which is implicated in the leucine and isoleucine biosynthesis (Kirino *et al*., 1994), respectively. Unfortunately, we haven't obtain the sequence information about M14 due to unknown reason.

To confirm that all mutants were caused by a single transposition event, southern blot hybridization was performed using digoxigenin-labelled probe *aacC*1 (Fig. 32). As shown in Fig. 33, each mutant has a unique hybridization band, indicating that the mariner-based transposon *Himar*1::Gm^r (encoded by *aacC*1) was indeed inserted in only one site.

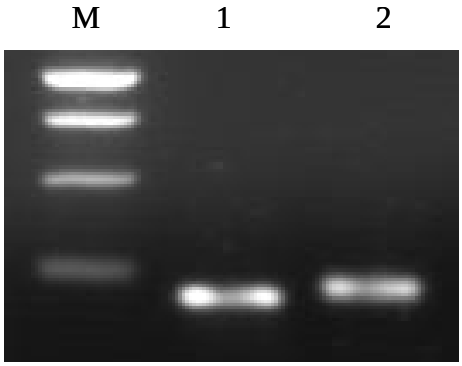


FIG. 32. Preparation of digoxigenin-labelled probe *aacC*1. Digoxigenin-labelled probe *aacC*1 was prepared by PCR using dNTP mixture supplemented with DIG-11-dUTP. Lane 1, PCR with normal dNTP mixture; lane 2, PCR with DIG-11-dUTP-contained dNTP mixture. Attention, fragment amplified from DIG-11-dUTP-contained dNTP is a little bigger (lane2) than that amplified from normal dNTP mixture (lane 1).

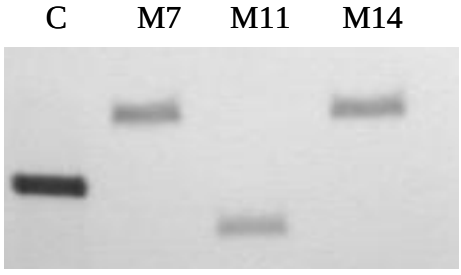


FIG. 33. Characterization of transposon insertion mutants by southern blot. Genomic DNA from transposon insertion mutants (M7, M11 and M14) was digested by *Pst*1, seprated by agarose gel and transfered to nylon membrane. Southern blot was performed using digoxigenin-labelled probe *aacC*1. C, control.

Transposon insertion mutant M7 was selected for further study because M7 affected gene *trpA*, which is implicated in the biosyntheis of tryptophan, a precursor (or itself) of several important signalling molecules in plant, bacteria and human (Fig. 34, Table 14 and Table 15).

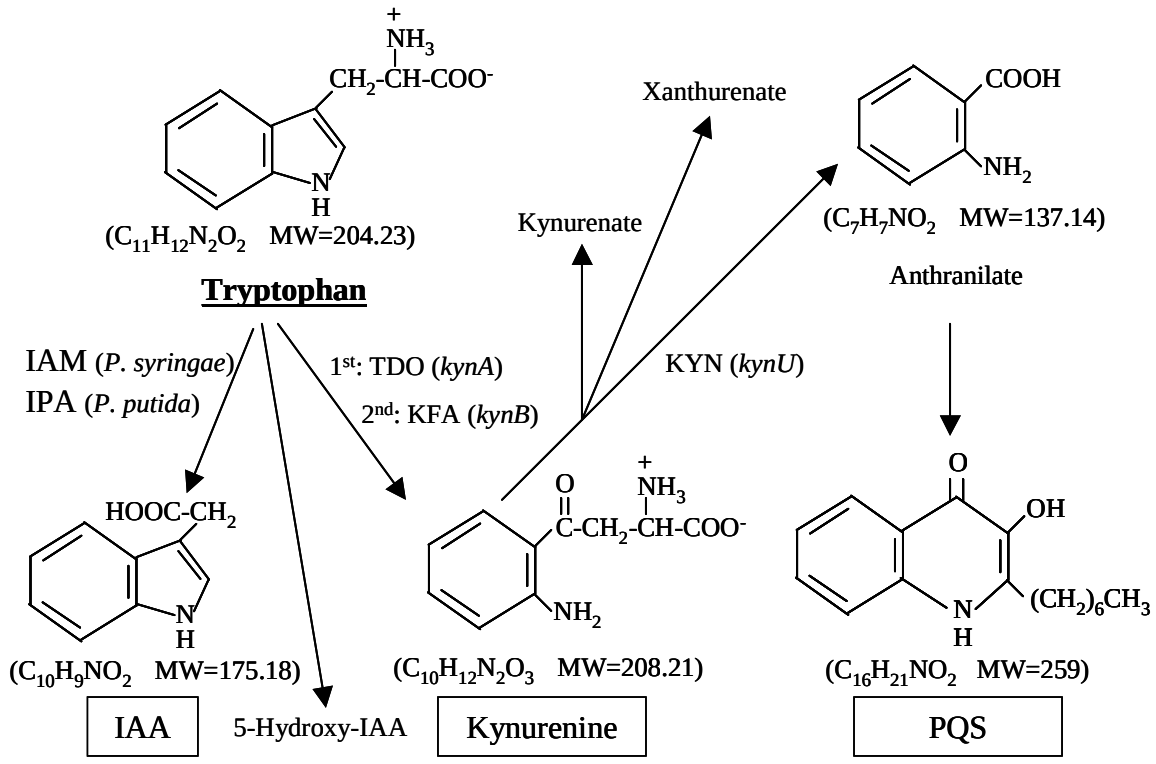


FIG. 34. Tryptophan (Trp) is a precursor of several important signalling molecules in *Pseudomonas* spp. 1) Trp-dependent indole-3-acetic acid (IAA, auxin) biosynthesis. The two most common routes for auxin biosynthesis in bacteria are the indole-3-acetamide (IAM) pathway and indole-3-pyruvic acid (IPA) pathway (Lambrecht *et al*., 2000). In *P. syringae*, Trp monooxygenase (IaaM) converts Trp to IAM, and an IAM hydrolase (IaaH) converts IAM to IAA (Patten and Glick, 1996). The *ipdc*, gene encoding indolepyruvate decarboxylase and the key gene in the IPA pathway for IAA synthesis, was isolated in *P. putida* (Patten and Glick, 2002). 2) Degradation of Trp to kynurenine. In *P. aeruginosa*, tryptophan 2,3-dioxygenase (TDO), encoded by *kynA* (PA2579), and kynurenine formamidase (KFA), encoded by *kynB* (PA 2081), are involved this pathway (Kurnasov *et al*., 2003). 3) *Pseudomonas* quinolone signal (PQS) biosynthesis. Anthranilate is the precursor of PQS (Calfee *et al*; 2001) and all enzymes required in the anthranilate pathway via Trp were characterized in *P. aeruginosa* (Kurnasov *et al*., 2003). KYN, kynureninase, encoded by *kynU* (PA2080).

TABLE 14. *Pseudomonas* spp. possess genes encoding enzymes involved in IAA biosynthesis

	P. syringae (accession N°)	*P. putida* (accession N°)	*P. aeruginosa* [a]PA N°	[b]homology
IAM pathway				
Trp monooxygenase (IaaM)	(AAR06971)		PA4548	$4E^{-04}$
Indole-3-acetamide hydrolase (IaaH)	(P52831)		PA4483	$5E^{-38}$
IPA pathway				
Indolepyruvate decarboxylase (IpdC)		(AAG00523)	PA4977	$5E^{-20}$
			PA4696	$5E^{-20}$
			PA4180	$7E^{-18}$
			PA2035	$1E^{-17}$
			PA2108	$2E^{-17}$

[a]PA number in www.pseudomonas.com; [b]in comparison with proteins of *P.syringae* or *P. putida* in this table.

Table 15. Role of three Trp-derived signalling molecules, IAA, Kynurenine and PQS

Molecules	Functions	References
IAA	1. Phytohormone, plant growth and development (active at nM)	Woodward and Bartel, 2005
	2. A reciprocal signal in bacteria-plant interactions	Lambrecht *et al*., 2000
	3. Inhibit the expression of genes involved in T-DNA transfer (type IV secretion) in *Agrobacterium tumefaciens*	Liu *et al*., 2006
	4. 0.5-1.0 mM IAA causes death of neutrophils	Piers de melo *et al*., 1998
Kynurenine	1. Inhibit T cell proliferation	Hwu *et al*., 2000 ; Frumento *et al*., 2002 ; Terness *et al*., 2002
	2. Inhibit NK cell proliferation (0.5 mM of L-kynurenine)	Frumento *et al*., 2002
	3. Inhibit NK cell activity (0.3-0.6 mM of L-kynurenine)	Della Chiesa *et al*., 2006
PQS	Pseudomonas quorum-sensing signal	Pesci *et al*., 1999

3.5 Tryptophan is implicated in the production of PARST

To determine the role of *trpA* in the production of PARST during the stationary phase, an unmarked in-frame deletion mutation of *trpA* was constructed by allelic exchange as described in Materials and Methods and was confirmed by PCR (Fig. 35).

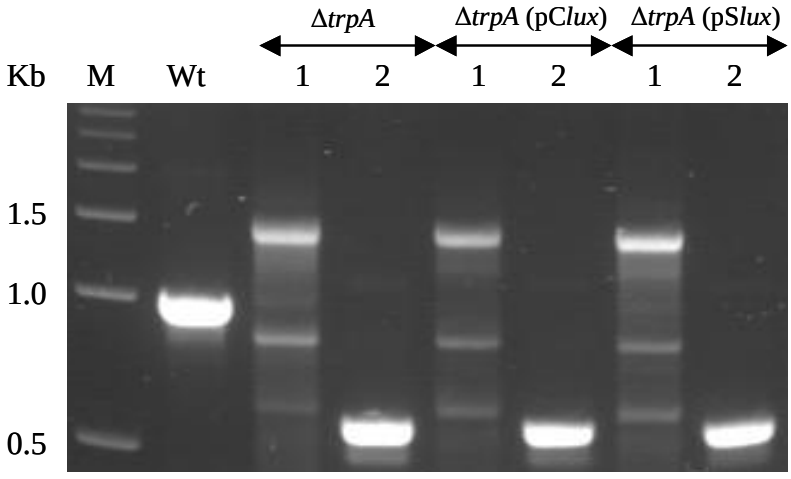


FIG. 35. Mutant analysis by PCR. PCR was performed on genomic DNA prepared from different strains. Wt, wild-type CHA; mutant Δ*trpA* and Δ*trpA* carring a single copy of *pClux*[Δ*trpA* (*pClux*)] or *pSlux*[Δ*trpA* (*pSlux*)]. 1, cassette Gm*lox*, *lox*-flanked *aacC1* encoding gentamicin resistance, is present in the chromosome; 2, only *lox*, but not *aacC1*, is present in the chromosome. M: DNA marker whose sizes are indicated at left side; Kb, kilobase.

Stationary phase culture supernatants were prepared from wild-type CHA and mutant Δ*trpA*, and were added to the CHA (*pClux*) cultured from an initial OD_{600} of 0.05 in LB under TTSS-inducing conditions. In contrast to the supernatant from the wild-type CHA, supernatant from Δ*trpA* lost its ability to inhibit *exsCEBA* expression and gave the same profile as 'control' in which supernatant was replaced by VB (Fig. 36). Complementation of Δ*trpA* by adding 200 μg of Trp ml^{-1} in VB during the preparation of supernatant restored the inhibitory feature of the supernatant from Δ*trpA* on the *exsCEBA* expression, on the contrarily, the addition of Trp during the preparation of supernatant of the wild-type CHA didn't change its inhibitory ability on the *exsCEBA* expression compared to normal prepared supernatant (Fig. 36). These results indicate that, *trpA* may be implicated in the production of PARST during the stationary phase and PARST production seemed to be Trp-dependent.

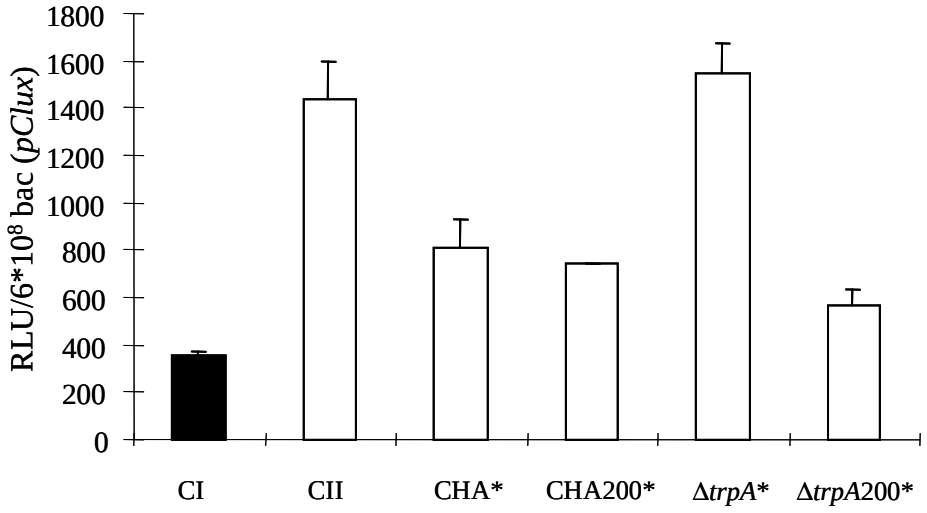


FIG. 36. Trp is implicated in the production of PARST during the stationary phase. The wild-type CHA carrying a single copy of *pClux* transcriptional reporter at its chromosome was grown at 37°C in LB in the presence of stationary phase culture supernatants (indicated as asterisk), which were prepared in VB (CHA and Δ*trpA*) or VB supplemented with 200 μg of Trp ml^{-1} (CHA200 and Δ*trpA*200). RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8 bac). Bars represent the mean plus the standard deviation of triplicate samples, and are the average of three independent experiments. Black bar and white bars represent TTSS-noninducing and inducing conditions, respectively. bac, bacteria. CI, control, TTSS-noninducing conditions (in the presence of 5 mM calcium); CII, control, TTSS-inducing conditions (5 mM EGTA and 20 mM $MgCl_2$).

3.6 Tryptophan itself is not responsible for the TTSS inhibitory activity

Before investigating the role of Trp-derived metabolites in the regulation of TTSS expression, we first we first determined the role of Trp. A serial dilution of L-Trp (Sigma) was added in the CHA (*pClux*) cultured from an initial OD_{600} of 0.05 in LB under TTSS-noninducing and inducing conditions, no difference was observed in culture with or without Trp (Fig. 37), indicating that Trp itself is not responsible for the TTSS inhibitory activity.

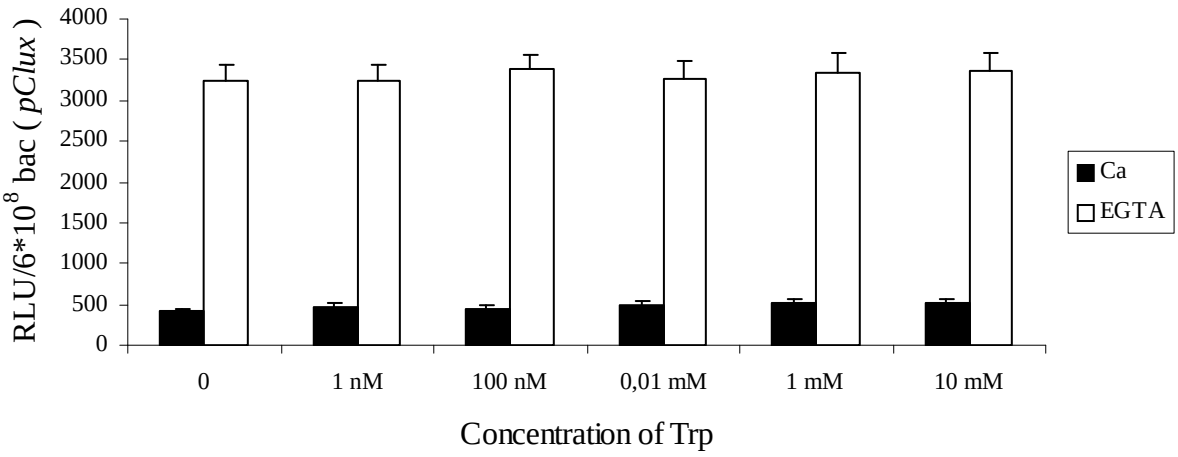


FIG. 37. Trp itself is not responsible for the inhibiton of TTSS expression. The wild-type CHA (*pClux*) were grown at 37°C in LB in the absence or presence of a serial L-Trp (Sigma) under TTSS-noninducing (black bars) or inducing conditions (white bars). RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8 bac). Bars represent the mean plus the standard deviation of triplicate samples, and are the average of three independent experiments. bac, bacteria.

3.7 Kynurenine and PQS are not implicated in the regulation of TTSS expression

Because Trp-derived kynurenine plays an important role in the regulation of T cell-mediated immune response (Table 15), and enzymes involved in the degradation of Trp to kynurenine in *P. aeruginosa* have been characterized (Fig. 34) (Kurnasov *et al*., 2003), we tested the role of kynurenine in the regulaion of TTSS. As seen in Fig. 38, kynurenine does nont seem to be implicated in the regulation of TTSS. Furthermore, PQS signal still exists in Δ*trpA* stationary phase culture supernatant (Filopon personal communication), indicating that PQS is not the PARST.

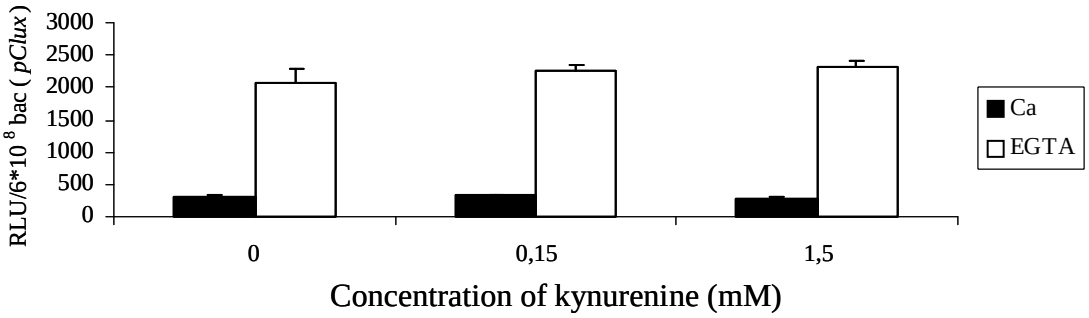


FIG. 38. Kynurenine is not implicated in the regulaion of TTSS expression. The wild-type CHA (*pClux*) were grown at 37°C in LB in the absence or presence of L-kynurenine sulfate salt (Sigma) under TTSS-noninducing (black bars) or inducing conditions (white bars). RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8 bac). Bars represent the mean plus the standard deviation of triplicate samples, and are the average of three independent experiments. bac, bacteria.

3.8 IAA and NAA has a regulatory role in the TTSS expression

3.8.1 IAA and NAA inhibit TTSS expression

Trp-derived auxin was the first discovered plant hormone and was latter determined to be indole-3-acetic acid (IAA) (Cohen *et al*., 2003; Woodward and Bartel, 2005). Because IAA is a reciprocal signalling molecule in bacteria-plant interactions and is critical for the growth and development of plants (Lambrecht *et al*., 2000; Woodward and Bartel, 2005), we examined the role of IAA in the regulation of TTSS expression.

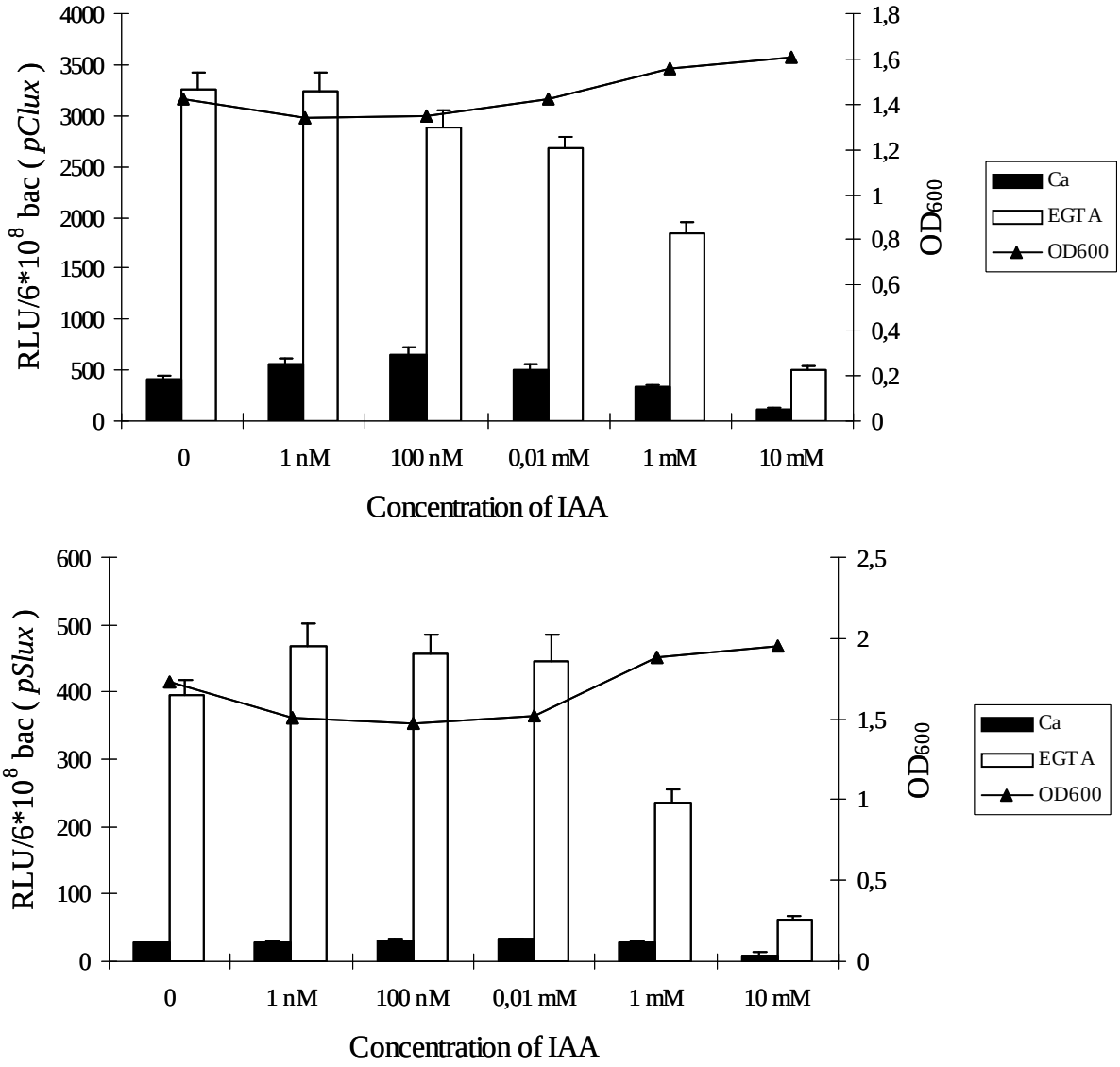


FIG. 39. IAA inhibits TTSS transcription without affecting bacterial growth. The wild-type CHA carrying a single copy of *pClux* or *pSlux* transcriptional reporter at its chromosome were grown at 37°C in LB in the absence or presence of a serial IAA-Na (Sigma) under TTSS-noninducing (black bars) or inducing conditions (white bars). RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8 bac). Bars represent the mean plus the standard deviation of triplicate samples, and are the average of three independent experiments. Black angles represent the OD_{600} under inducing conditions. bac, bacteria.

As shown in Fig. 39, though IAA sodium salt (IAA-Na, Sigma) having no effect on TTSS expression up to concentration of 0.01mM, 1 mM IAA-Na had an evident inhibition on both *exsCEBA* expression and *exoS* expression and 10 mM IAA-Na inhibit totally the TTSS expression with no inhibition of the growth of bacteria.

We also tested the other three commercial IAA derivatives (formula of sodium or potassium salt, Sigma), including indole-3-butyric acid (IBA), 1-Naphthaleneacitic acid (NAA) and (2,4-Dichlorophenoxy) acetic acid (2,4-D) (Woodward and Bartel, 2005), and only NAA-K had a similar inhibitory role to IAA on the TTSS expression (Fig. 40).

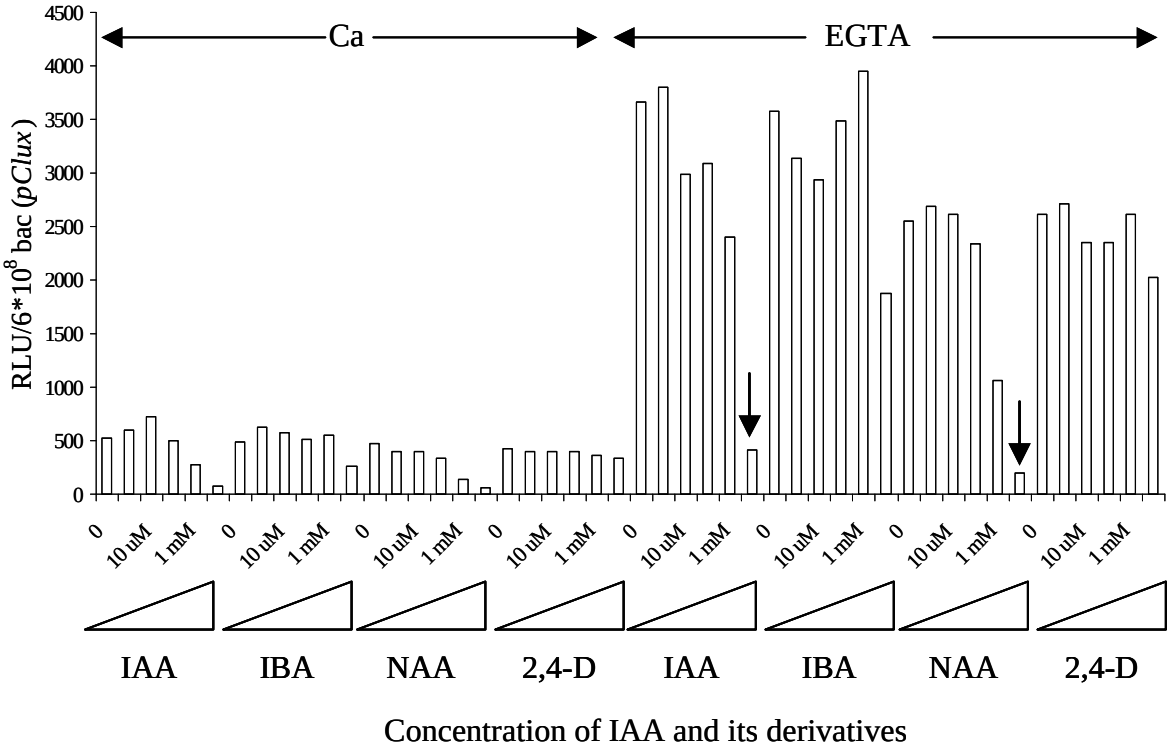


FIG. 40. Both IAA and NAA inhibit TTSS transcription induced by EGTA. The wild-type CHA carrying a single copy of *pClux* transcriptional reporter at its chromosome were grown at 37°C in LB under TTSS-noninducing (in the presence of 5 mM calcium) or inducing conditions (in the presence of 5 mM EGTA and 20 mM $MgCl_2$) in supplemented with or not IAA-Na (Sigma) and derivatives , including IBA-K, NAA-K, 2,4-D-Na (Sigma), of dirrerent concentraions (0, 1 μM, 10 μM, 100 μM, 1 mM and 10 mM). RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8 bac). Ca, calcium.

Besides *in vitro* calcium depletion growth conditions, growth in the presence of serum leads to generalized TTSS expression and secretion (Vallis *et al*., 1999). Therefore, we asked whether IAA could inhibit TTSS expression in the presence of human serum AB stimuli. As seen in Fig. 41, *exsCEBA* transcription was partially inhibited in the presence of 1mM IAA-Na and NAA-K but not IBA-K or 2,4-D-Na. No pH change was observed due to the use of IAA-Na and its derivatives. Considering that IBA is identical to IAA except for two

additional methylene groups in the side chain and the former has no effect on the expression of the TTSS, we conclude that IAA-Na and NAA-K inhibit the expression of TTSS genes specifically.

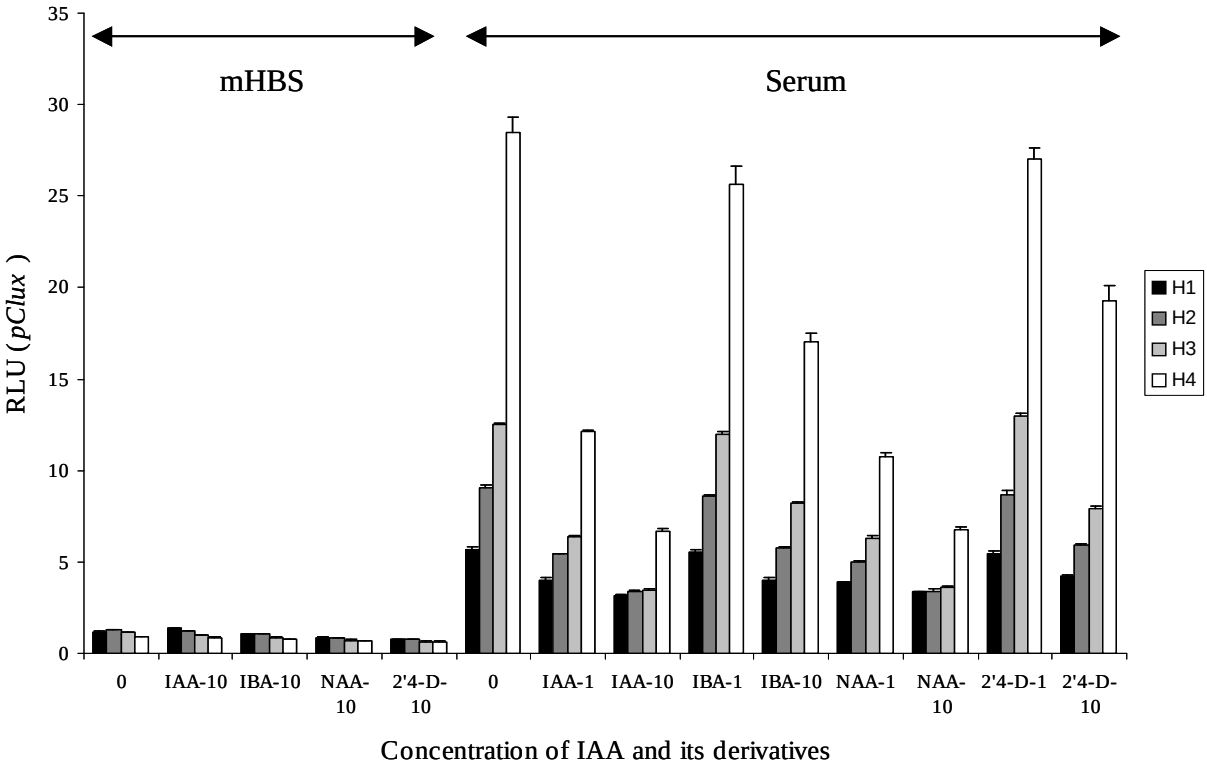


FIG. 41. IAA and NAA inhibit TTSS transcription induced by serum. Cultured bacteria (5×10^5) of the wide-type CHA carrying a single copy of the *pClux* transcriptional reporter at its chromosome were incubated with mHBS buffer or 10% human serum AB in a 96-well plate supplemented with or not 1 mM or 10 mM IAA-Na (Sigma) and its derivatives, including IBA-K, NAA-K, 2,4-D-Na (Sigma). RLU were measured over 4 h of incubation. Bars represent the mean plus the standard deviation of triplicate samples, and are the average of two independent experiments.

3.8.2 IAA inhibits also TTSS secretion

Secretion of TTSS effectors is strongly correlated with *P. aeruginosa* virulence both in animal models and in studies of human disease (Roy-Burman *et al*., 2001, Hauser *et al*., 2002, Smith *et al*., 2004). To examine whether IAA is implicated in the inhibition of TTSS secretion, culture supernatant was analyzed from the wild-type CHA containing plasmid p*exsA*ind, which could express *in trans* exogenous *exsA* by the induction of IPTG. As shown in Fig. 42, only growth of bacteria in the presence of EGTA results in TTSS secretion, however such secretion is prevented in the presence of 5 mM IAA-Na, even with the exogenous expression of ExsA from p*exsA*ind induced by IPTG. In combination with the result of TTSS transcription in the presence of IAA (Fig. 39, 40, 41) and the fact that TTSS transcription is coupled to secretion (Urbanowski *et al*., 2005), we speculated that IAA could block the opening of the translocation pore hence block the release of ExsA and the transcriptional activation of TTSS genes. An equivalent process has been demonstrated for recently identified inhibitory molecules that acts as 'calcium mimetics' on *Yersinia* type III secreion (Nordfelth *et al*., 2005).

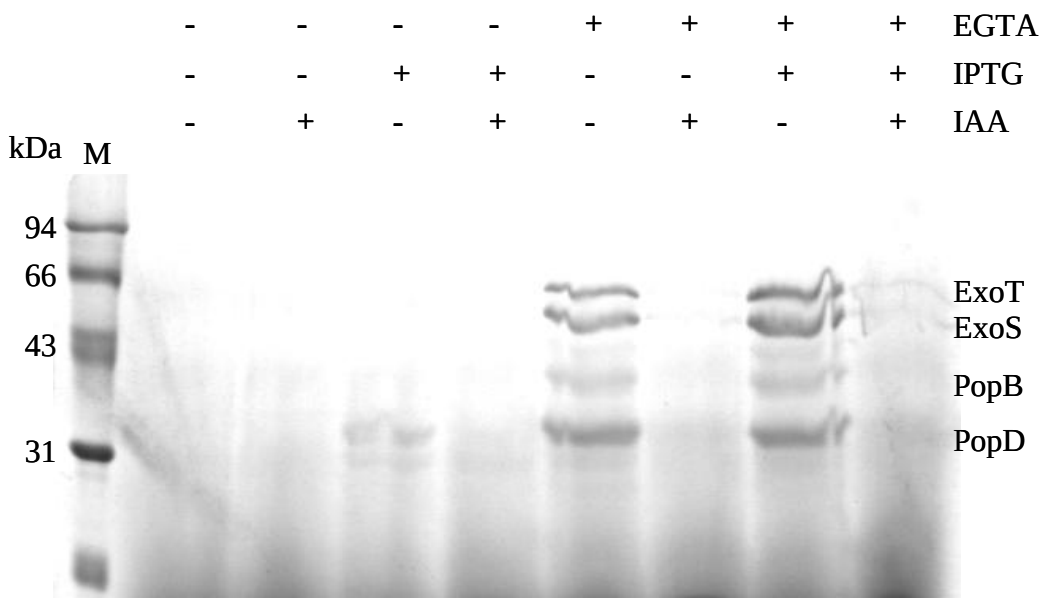


FIG. 42. IAA inhibits TTSS secretion. The wild-type CHA expressing *in trans* ExsA from IPTG-inducible plasmid p*exsA*ind were grown in the absence (-) of presence (+) of 5 mM of IAA-Na under TTSS-noninducing or inducing conditions (+EGTA). Supernatants normalized to bacterial counts (6×10^8 per lane) were subjected to TCA precipitation. The extracellular proteins were separated by 10% SDS-PAGE and stained with Coomassie Blue. M, protein marker, sizes in kilodaltons (kDa) are indicated at the left.

3.8.3 VirA is not involved in the IAA-dependent inhibition of TTSS expression

Since IAA can inhibit the expression of TTSS and might be an inhibitory signal during the stationary phase, we looked for the inhibition of bacterial secretion systems by similar molecules. *Agrobacterium tumefaciens* is a pathogen that cause crown gall tumors in a variety of plants by transferring the oncogenic DNA fragments (T-DNA) into plant cells *via* its type IV secretion system (Introduction 3.2.2). VirA/G, a two component regulatory system, senses the plant signal molecules, typically phenolic compound acetosyringone, and activates the expression of *vir* genes, which are responsible for T-DNA processing and transfer (Brencic and Winans, 2005). It was recently shown that IAA inactivates *vir* genes expression by competing with acetosyringone for interaction with VirA (Liu *et al*., 2006). By searching *Pseudomonas* genome database, we found a protein (PA1243), annotated as a probable sensor/response regulator hybrid, is 49% similar to VirA (www.pseudomonas.com, Stover *et al*., 2000). To investigate if *Pseudomonas* VirA is the receptor of IAA and has a regulatory role in genes expression, such as TTSS expression, we deleted the gene *virA* and yielded the mutant Δ*virA* containing the chromosomal transcriptional gene reporter *pClux* and *pSlux*. As seen in Fig. 43., the deletion of *virA* has no influence on the activation of *exsCEBA* expression as 10 mM NAA still inhibit the activation of *exsCEBA* expression in TTSS-inducing conditions in the absence of VirA sensor. This result indicates that VirA is not involved in the NAA-dependent inhibition of TTSS expression in *P. aeruginosa*.

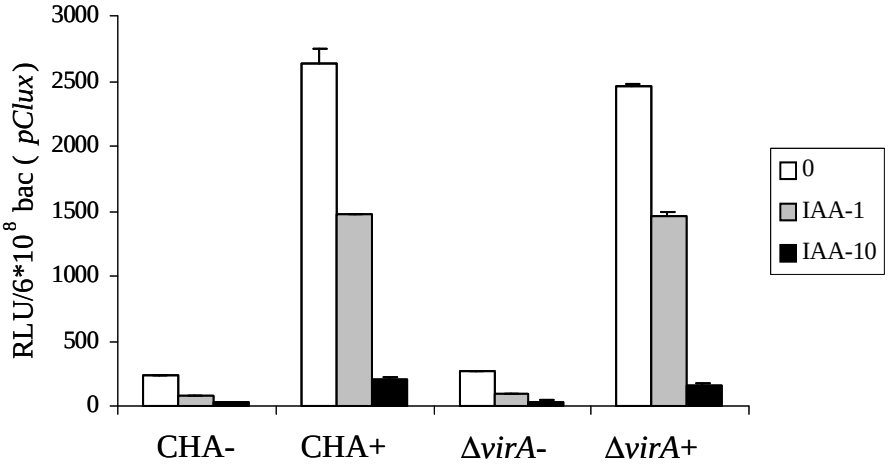


FIG. 43. VirA is not involved in the IAA-dependent inhibition of TTSS expression in *P. aeruginosa*. The wild-type CHA and mutant Δ*virA* carrying a single copy of *pClux* transcriptional reporter at its chromosome were grown at 37°C in LB in the absence or presence of 1mM IAA-Na (IAA-1) or 10 mM IAA-Na (IAA-10) under TTSS-noninducing (-) or inducing conditions (+). RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8 bac). Bars represent the mean plus the standard deviation of triplicate samples, and are the average of three independent experiments. bac, bacteria.

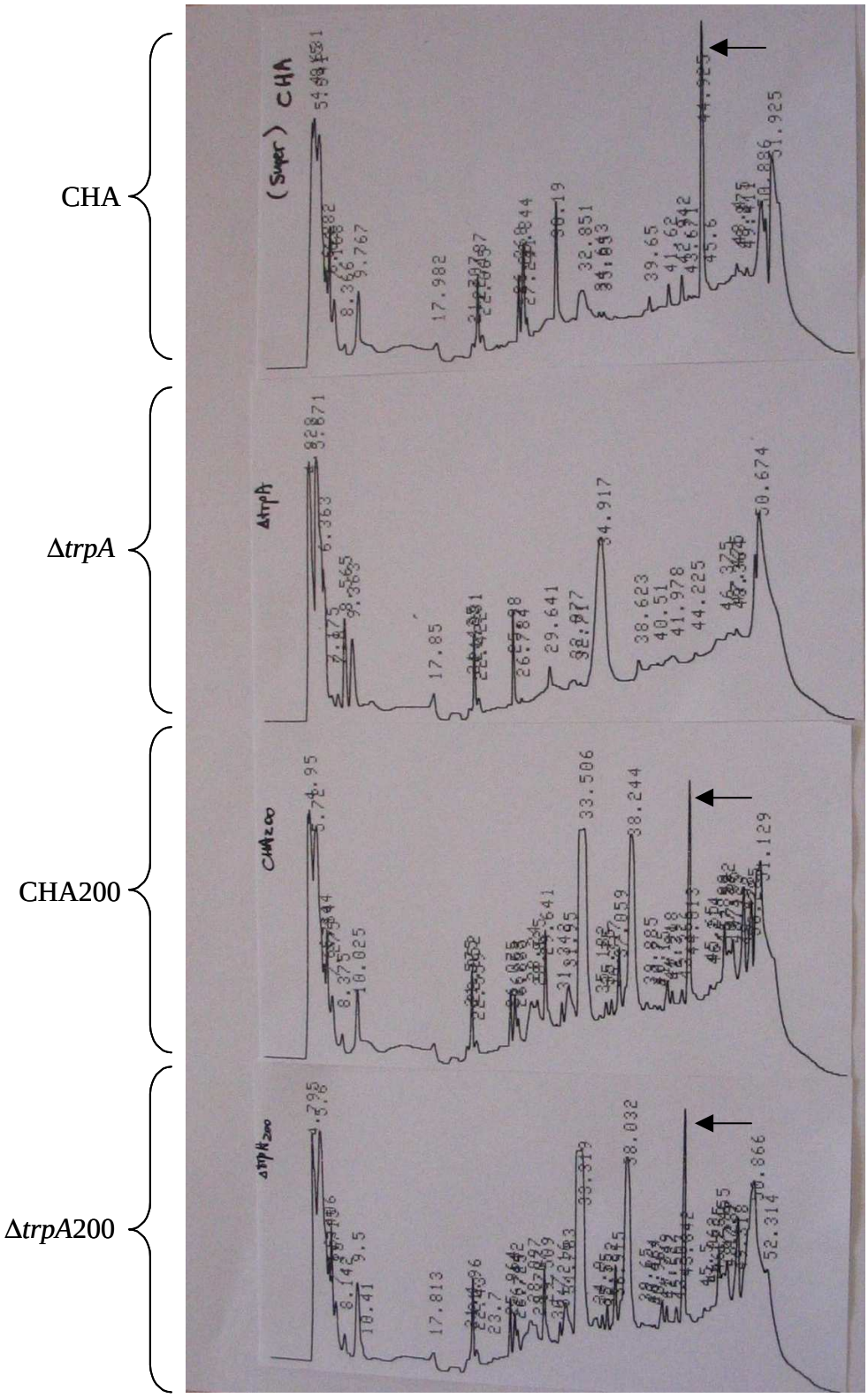


FIG. 44. HPLC analysis of stationary phase culture supernatants. Stationary phase culture supernatants from wild-type CHA and Δ*trpA* were prepared in VB (CHA and Δ*trpA*) or VB supplemented with 200 µg of Trp ml^{-1} (CHA200 and Δ*trpA*200), and HPLC was performed as described in Materials and Methods. Arrow showed the peak, which was only present in the supernatant of the wild-type CHA, absent in the supernatant of Δ*trpA* but reappeared in the supernatant of Δ*trpA* when 200 µg of Trp ml^{-1} was added during the preparation of supernatants.

3.9 Tentative of determination of the nature of PARST

Based on the obtained results before, the production of PARST during the stationary phase depends on Trp, precursor of IAA, which could inhibit both TTSS secretion and expression. We asked here if IAA is indeed in the stationary phase culture supernatant. Using Salkowski's reagent as previously described (Patten and Glick, 2002), we haven't detected IAA in the stationary phase culture supernatant in the wild-type CHA and no difference was observed between the supernatants of different phases (data not shown). It seemed that IAA should not be PARST, however we could not rule out that the special analogs of IAA might exist in the supernatant and regulate TTSS expression *in vivo* at very low concentration, which could not be detected by Salkowski's reagent. To identify the nature of PARST, the wild-type CHA and the Δ*trpA* were collected from stationary phase and then grown overnight in the VB or VB supplemented with 200 μg ml^{-1} Trp. Supernatants were collected by centrifugation, filtrated through a 0.22 μM filter, and then were passed and analysed by HPLC on C18 column as described in Materials and Methods 21. As seen in Fig. 44, peak 44.925 (min of elution, indicated by the arrow) presented in the wild-type CHA was absent from the Δ*trpA* and this same peak reappeared after supplementation by Trp during the culture of strain Δ*trpA*.

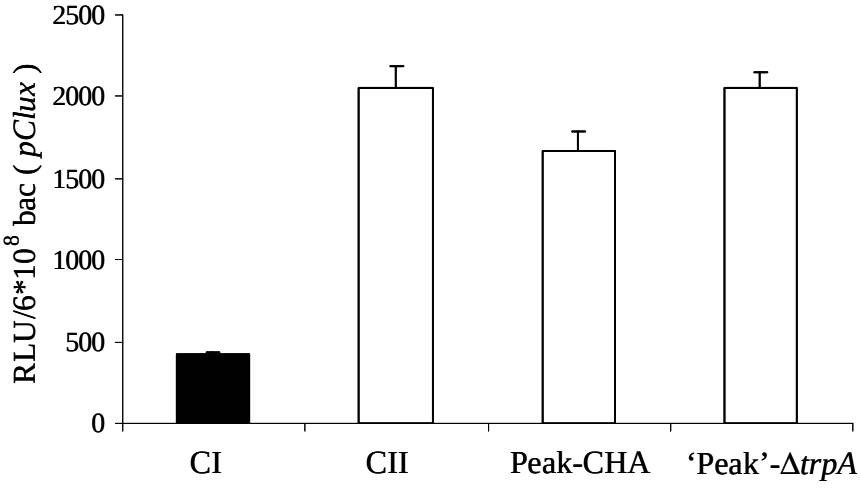


FIG. 45. Peak analysis on the activation of *exsCEBA* expression. During the procedure of HPLC, peak of CHA (Peak-CHA), indicated in Fig. 44, as well as that of trpA ('peak'-Δ*trpA*) appeared at the same time point were collected, evaporated and incubated with CHA carring *pClux* transcriptional report under TTSS-inducing conditions (white bars). RLU were measured over 4h of culture and calculated to normalized bacterial counts (6×10^8). Bar represent the mean plus the standard deviation of 3 reads. CI, control, TTSS-noninducing conditions (black bar); CII, control, TTSS-inducing conditions. Bac, bacteria.

To determine its role on the TTSS expression, peak 44.925 (CHA) was collected, evaporated and resuspended in LB containing CHA (*pClux*) with an initial OD_{600} of 0.05 under TTSS-inducing conditions. A weak inhibition of *exsCEBA* expression (Fig. 45) was observed in the presence of resuspended peak 44.925 (CHA) compared with the presence of the solution collected from Δ*trpA* during HPLC the same time point at which the peak 44.925 (CHA) was collected. We could not exclude the possibility that PARST was not in peak 44.925 (CHA), but even PARST was indeed in the peak, it might have lost a part of its activity during the preparation.

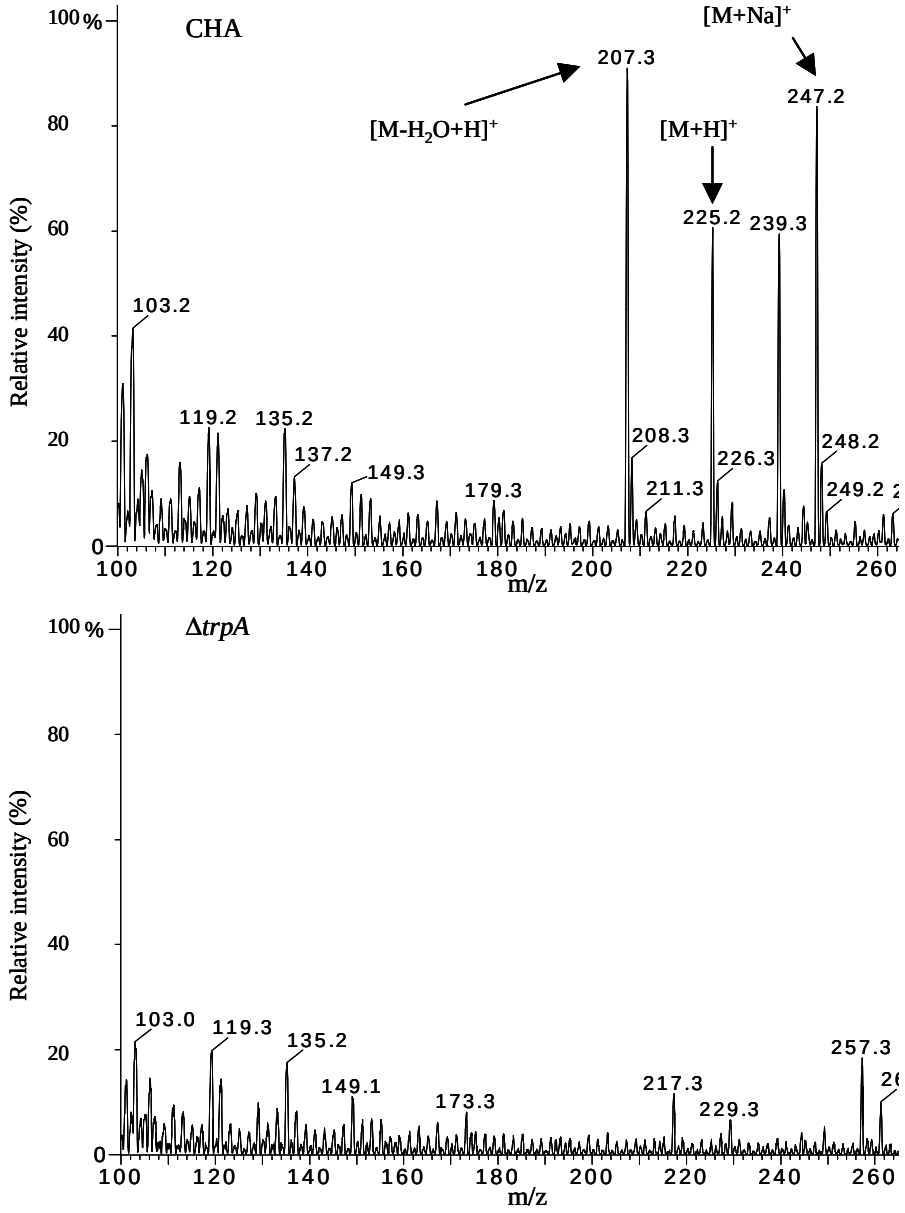


FIG. 46. *m/z* 224 is uniquely present in the stationary phase culture supernatant of CHA. Fractions of the peak 44.925 CHA (as indicated in Fig. 44) and that from Δ*trpA* collected at the same time point during the separation by HPLC were further analyzed by LC-MS as described in Materials and Methods. The extracted ion chromatograms here showed the only detected differences in a scan range from m/z 100 to 1500 between two strains. m/z present only in the wild-type CHA were indicated by arrows.

3.10 PARST might be a molecule of *m/z* 224

Supposing PARST is indeed in the peak 44.925 (CHA) (Fig. 44), we collected this peak as several fractions and that from Δ*trpA* at the same time point during the separation by HPLC. LC-MS analysis showed that 4 molecular ions, including *m/z* 207.3 $[M-H_2O+H]^+$, *m/z* 225.2 $[M+H]^+$, *m/z* 247.2 $[M+Na]^+$ and *m/z* 239 (possibly $[M+CH_2+H]^+$), were uniquely present in the peak of 44.925 (CHA) (Fig. 46).

Therefore, the molecule present in the stationary phase culture supernatant from the wild-type CHA should be *m/z* 224.2 (225.2 minus 1, molecular weight of atom H) and it was reminiscent of NAA-K simply due to the facts that NAA-K has a molecular weigh of 224.3 and can inhibits TTSS expression. So what's the relationship between the *m/z* 224.2 and the NAA-K? Mass spectra analysis of commercial NAA-K exhibited 3 *m/z*, includng *m/z* 187.07, 225.03 and 262.99 corresponding to $[NAA+H]^+$, $[NAA+K]^+$ and $[NAA+2K-H]^+$ respectively. It is possible that *m/z* 207.3, 225.2 and 247.2 in the peak 44.925 (CHA) might correspond to $[NAA-H_2O+K]^+$, $[NAA+K]^+$ and $[NAA+K+Na-H]^+$ respectively. It is normal that no NAA-2K was detected because there is little K but rather Na in the culture medium. Taken together, we speculated that NAA itself or its derivatives could be produced in the stationary phase and inhibits TTSS expression.

4. Discussion

The expression of the TTSS is controlled by multicomponent regulatory networks which integrate a diverse set of environmental cues, probably to restrict the energy-consuming expression of 20 or more proteins to the correct place and time only when they are really needed (Hueck, 1998). Although TTSS is very likely important in acute human infections, it is not necessary for maintaining chronic infection (Dacheux *et al*., 2000; Roy-Burman *et al*., 2001; Hauser *et al*., 2002; Eriksson *et al*., 2003; Jain *et al*., 2004; Dahan *et al*., 2004; Lucchini *et al*., 2005; Lee *et al*., 2005; Faucher *et al*., 2006). *In vitro* culture condition, the expression of the TTSS seems to be cell density-dependent (this study). Presumably, just as a mechanism exists for activating the TTSS genes when they are needed, a mechanism should exist for inactivating the same TTSS genes when they are not required. The data presented here described PARST, an inhibitor of the expression of TTSS, that appears in the stationary phase of culture (high cell density).

Considering that QS is a generic regulatory mechanism controlling in a population density-dependent manner the expression of virulence genes, including TTSS genes (Juhas *et al*., 2005), we ruled out the possibility that the PARST is associated with the QS system (Fig. 28).

The fact that the production of PARST is Trp-dependent (Fig. 36) in combination with the fact that Trp is the precursor of IAA, which has several important features described below, we tested the role of IAA and IAA-like compounds in the regulation of TTSS expression. IAA, the first discovered plant hormone, is critical for plant growth and development and is often potent at nanomolar concentrations in plants (Woodward and Bartel, 2005). Although it is widely accepted that plants use both Trp-dependent and Trp-independent routes to synthesize IAA, none of the pathways is yet defined to the level of knowing each relevant gene, enzyme and intermediate. It is also recognized that many bacteria are able to produce IAA mainly using Trp-dependent pathway (Lambrecht *et al*., 2000) and such pathway is indeed observed in *Pseudomonas* (Patten and Glick, 1996; 2002) (Fig. 34 and Table 14). Recent studies showed that several strains of *P. aeruginosa* isolated from soil (Bano *et al*., 2003; Kumar *et al*., 2005; Ayyadurai *et al*., 2006) could produce IAA and maximum production of IAA (19.1 μg ml^{-1}) by strain *P. aeruginosa* FP10 was observed after 42-h incubation in Dworkin and Foster (DF) salts medium supplemented with 500 μg ml^{-1} of L-tryptophan (Ayyadurai *et al*., 2006).

IAA and one of IAA-like compounds, NAA, could indeed inhibit the expression and the secretion of the TTSS at millimolar concentrations (Fig. 39, 40, 41, 42). However, no IAA was detected from our stationary phase culture supernatant using Salkowski's reagent. Thus, IAA does not seem to be the component of PARST, though genes encoding enzymes needed in the Trp-dependent biosynthesis of IAA do exist in the genome of *P. aeruginosa* PAO1 (Table 14) (www.pseudomonas.com). However, we still have reasons to believe that PARST could be IAA-like compound, because m/z 225.2 (Fig. 46), corresponding to the molecular weight of $[NAA+K]^+$, is only present in the supernatant of the wild-type CHA but not the mutant with the deletion of *trpA* whose product is the precursor in the biosynthesis of IAA. It is possible that undetectable IAA in the culture supernatant might be due to the dilution of IAA, if exist, and the limit of the method we used, in such case, it is possible that IAA or IAA-like compound at very low concentration could be highly active due to its special form. It is worth noting that 1mM of IAA has cytotoxic effect on the cultured neutrophils (Pires de Melo *et al*., 1998). Therefore, it would be interesting if IAA could play a role in the regulation of the transition between two totally different events: inhibiting the expression of the TTSS on the one hand and activating the pathway to kill the neutrophils on the other hand.

Though one study showed that IAA can inhibit the expression of genes required for the transfer of T-DNA (type IV secretion) in *A. tumefaciens* via VirA, a sensor kinase of a two-

component regulatory system (Liu *et al*., 2006), VirA homolog of *P. aeruginosa* is not involved in the IAA-dependent inhibition of the expression of TTSS (Fig. 43).

Recently, one study showed that LadS and RetS, two hybrid sensor kinases of *Pseudomonas*, are implicated in the transition between activation of the TTSS (acute infection) and the biofilm (chronic infection) by sensing an unknown signal (Ventre *et al*., 2006). Therefore, it would be interesting to investigate if IAA or NNA is the signal sensed by LadS/RetS pathway.

We don't know why PARST, probably Trp-derived metabolite, is implicated in the regulation of TTSS, but it is true that metabolic signals/stresses have a profound influence on the TTSS expression. Mutants lacking pyruvate dehydrogenase (*aceA* or *aceB*) (Dacheux *et al*., 2002) or a glucose transport regulator (*gltR*) (Wolfgang *et al*., 2003) fail to induce the TTSS expression under calcium depletion conditions. Similarly, overproduction of MDR efflux pumps (Linares *et al*., 2005) or overexpression of genes involved in histidine transport and metabolism (Rietsch *et al*., 2004) prevents the TTSS expression. A recent study suggests that one metabolite controlling the TTSS expression is derived from acetyl-CoA (Rietsch and Mekalanos, 2006).

It is not surprising that *P. aeruginosa* has multiple regulatory networks, because 8% of its genome codes for regulatory genes, indicating that *P. aeruginos*a has dynamic and complicated regulatory mechanisms responding to various environmental conditions (Greenberg, 2000). In addition to its role in TTSS genes induction, IAA or NAA may serve as a signal to *Pseudomonas* that the living environment is changing, from a low cell density to a high cell density state, and that the bacterium should modify its gene expression to favarite its survival. This possibility could be explored through the use of DNA microarrays or all proteomic analysis under conditions in which the IAA is added or not.

Conclusion and perspectives

En français

P. aeruginosa, comme beaucoup de bactéries Gram-négatives pathogènes de plante ou d'animal, possède un facteur de virulence majeur, le système de sécrétion de type III (SSTT). Il est composé de plus de trente protéines qui s'assemblent pour former un machinerie complexe dont le rôle est de délivrer des effecteurs protéiques directement dans le cytoplasme des cellules cibles (Yahr *et al*., 1995; Frank 1997) où ils agiront pour pervertir différents mécanismes cellulaires de l'hôte, conférant finalement à la bactérie un avantage pour sa survie (Hueck, 1998). La construction, et l'utilisation de ce système de virulence représente un coût énergétique considérable pour la bactérie qui en contre partie a mis en place un contrôle extrêmement précis tant au niveau de l'expression des gènes qu'à celui de l'utilisation du SSTT.

PsrA est un activateur trancriptionnel du SSTT de *P. aeruginosa*

Chez *P. aeruginosa*, l'expression de l'ensemble des gènes du SSTT est contrôlée par ExsA, une protéine qui se fixe sur des séquences spécifiques situées dans les régions régulatrices des différents opérons du SSTT, pour activer leur transcription (Hovey and Frank, 1995). Ceci permet de supposer que la bactérie peut contrôler l'expression du SSTT soit en modifiant l'activité de ExsA soit en contrôlant le niveau d'expression de ExsA lui-même. La première situation a été très bien étudiée et a permis de caractériser les systèmes de contrôle de l'activité de ExsA (McCaw *et al*., 2002; Dasgupta *et al*., 2004; Ha *et al*., 2004; Rietsch *et al*., 2005; Urbanowski *et al*., 2005). Ici nous avons découvert le rôle d'un nouveau facteur de transcription, PsrA, nécessaire pour que la transcription de l'opéron *exsCEBA* qui contient *exsA* et par conséquent celle des autres opérons du SSTT soient maximales.

La recherche des régulateurs impliqués dans le contrôle du niveau d'expression de l'opéron *exsCEBA* est basée sur l'observation faite au laboratoire que des extraits bactériens totaux préparés à partir de culture d'un mutant de délétion de *exsA* contiennent toujours une activité de fixation spécifique à l'ADN du promoteur de l'opéron (pC). Une protéine, PsrA, a pu être identifiée à partir de l'analyse de fractions enrichies en activité de fixation sur pC. La protéine recombinante PsrA (rPsrA) a été préparée pure et elle est effectivement capable de se fixer spécifiquement sur pC. Bien que l'affinité de fixation sur pC soit relativement modeste, cela suggère que PsrA peut être un régulateur de l'opéron *exsCEBA* et donc de l'ensemble des gènes du SSTT. La démonstration du rôle joué par PsrA dans l'expression des gènes du SSTT a été clairement établie par l'étude du phénotype d'un mutant de délétion de *psrA* (Δ*psrA*) obtenu au cours de ce travail. En effet, l'analyse par gène rapporteur a montré que PsrA est nécessaire pour obtenir une activation totale de la transcription de *exsA* dans différentes conditions d'induction comme la déplétion calcique, l'addition de sérum ou le contact cellulaire. La délétion de *psrA* se traduit par une diminution d'un facteur 2 ou 3 du niveau d'expression de *exsA* et peut être complémentée par l'addition, *en trans* du gène *psrA*.

La diminution du niveau d'expression de *exsA* se traduit d'une part par une diminution de

l'expression des gènes du SSTT, en particulier de la toxine ExoS et, d'autre part par une diminution de la sécrétion de ExoS et ExoT. Finalement, le rôle positif de PsrA dans l'expression du SSTT et, par conséquent, dans la virulence de la bactérie a été confirmé par la plus faible résistance à la phagocytose de la souche Δ*psrA*.
Curieusement, les extraits du mutant Δ*psrA* continuent d'avoir cette forte activité de fixation au promoteur de l'opéron *exsCEBA*, indiquant que la protéine PsrA n'est pas la protéine responsable de cette activité. Si on considère que c'est en condition de non activation du SSTT que l'on mesure la plus forte activité de fixation dans les extraits (Figure 25), il est tout à fait légitime d'émettre l'hypothèse que la protéine responsable agirait plutôt comme un inhibiteur de transcription, alors que PsrA agit clairement comme activateur. La présence de ces différentes activités de fixation au promoteur pC, indépendantes de ExsA et de PsrA suggèrent bien que d'autres facteurs de transcription pourraient agir de concert pour donner à la bactérie une grande flexibilité et une grande précision dans le niveau d'expression du SSTT. La caractérisation de ces régulateurs doit se poursuivre afin non seulement d'obtenir plus d'informations sur la capacité des bactéries à contrôler finement le niveau d'expression de leurs gènes, mais aussi pour fournir des cibles potentielles permettant la conception de nouveaux moyens de lutte contre les infections bactériennes.

Le PARST (*Pseudomonas* autorepressor of secretion three) inhibe l'expression du SSTT de *P. aeruginosa* en phase stationnaire

Il avait déjà été observé au laboratoire que l'expression des gènes du SSTT chez *P. aeruginosa* dépend de la densité cellulaire avec un maximum de l'expression des gènes comme *exsCEBA* ou *exoS* vers le milieu de la phase exponentielle correspondant à une DO_{600} de 1.5 à 2 suivit d'une rapide diminution, voire annulation complète de l'expression lorsque que la DO dépasse 2. Ceci indique qu'un mécanisme de type 'quorum sensing' intervient pour inactiver l'expression du SSTT en phase stationnaire. De plus, les surnageants de culture de notre souche ou d'autres souches de *P.aeruginosa* obtenus en fin de phase stationnaire contiennent un signal, très fort, capable de provoquer le blocage de l'expression du SSTT. Nous avons appelé ce signal PARST pour '*Pseudomonas* autorepressor of secretion three'.
Puisque les mécanismes de 'quorum sensing' (QS) sont bien connus chez *P. aeruginosa* pour réguler l'expression de gènes de manière dépendante de la densité cellulaire et que le QS a en effet un impact sur l'expression du SSTT (Sperandio *et al*., 1999; Schaber *et al*., 2004; Hogardt *et al*., 2004; Bleves *et al*., 2005; Juhas *et al*., 2005), nous avons examiné si l'inhibition de l'expression du SSTT constatée à haute densité cellulaire était due ou non aux mécanismes du QS conventionnel. L'activité PARST est maintenue dans les surnageants des différents mutants de délétion des gènes impliqués dans la synthèse des signaux connus du QS. Ceci indique clairement que le PARST est différent des signaux classiques du QS, et que sa synthèse implique des voies nouvelles. C'est la raison pour laquelle nous avons construit une banque de plusieurs milliers de mutants de transposition de notre souche CHA pour identifier les gènes impliqués dans la synthèse du PARST en phase stationnaire.
Le crible de 2000 mutants a révélé que le gène *trpA* codant la sous-unité A de la tryptophane

synthétase ne peut plus produire l'activité PARST dans le surnageant. Un mutant de délétion de *trpA* (Δ*trpA*) a été construit pour préciser le rôle joué par TrpA dans la synthèse du PARST. De manière similaire au mutant d'insertion, aucune activité PARST n'a pu être mesurée dans les surnageants de cultures du mutant de délétion sauf si on ajoute dans le milieu de culture du tryptophane ce qui suggère fortement que le tryptophane est un intermédiaire essentiel dans la synthèse du PARST.

Puisque le tryptophane est le précurseur d'une grande famille de signaux connus chez les plantes et les bactéries, les auxines, nous avons examiné le rôle des représentants principaux de cette famille comme IAA, IBA, NAA et 2,4-D dans l'expression du SSTT chez *P. aeruginosa*. Nous avons observé que l'IAA et aussi le NAA peuvent totalement réprimer l'expression du SSTT et la sécrétion de toxines lorsqu'ils sont ajoutés à la concentration de 5 mM dans les milieux de culture, sans aucune inhibition de la croissance des bactéries. Malheureusement nous n'avons pas pu détecter l'IAA dans les surnageants des cultures de nos souches de *P. aeruginosa*.

Pour déterminer avec précision la nature du signal PARST, nous avons comparer le profil de séparation par HPLC sur colonne C18 des surnageants contenant du PARST issus de la souche CHA à ceux n'en contenant pas issus du mutant Δ*trpA*. Nous avons observé qu'un pic évident était présent dans les surnageant de CHA, absent dans ceux du mutant Δ*trpA* et à nouveaux présents lorsqu'on rajoute du tryptophane dans le milieu de culture du mutant Δ*trpA*. L'analyse par LC-MS de ces surnageants indique qu'une molécule de *m/z* 224.2 est présent dans ce pic issu de CHA. Bien que ceci corresponde au *m/z* du NAA-K, il semble difficile pour l'instant de pouvoir affirmer que le NAA est bien le PARST.

Dans un modèle murin d'infection aiguë à *P. aeruginosa*, le PARST agit positivement pour améliorer la clairance des bactéries, ce qui indique que des inhibiteurs spécifiques du SSTT peuvent être utilisés avec avantage pour lutter contre *P. aeruginosa*. En fait l'expression de gènes de virulence représente désormais de bonnes cibles pour le développement d'antibiotique nouveaux comme cela a été encore récemment démontré chez *Vibrio cholera* où une petite molécule inhibant l'expression de la toxine et des pili s'est révélée capable de protéger les jeunes souris de la colonisation par cette bactérie (Hung *et al*., 2005). Très récemment 2 différentes molécules ont été proposées pour inhiber le SSTT de *Chlamydia trachomatis* afin de lutter contre les fléaux causés par cette bactérie (Muschiol *et al*., 2006; Wolf *et al*., 2006).

Bien que le NAA ne soit probablement pas le PARST, on pourrait examiner son rôle dans l'infection pulmonaire aiguë à *P. aeruginosa*. Si cette molécule a bien un rôle protecteur, il faudra alors identifier par quel récepteur le signal se transmet jusqu'aux systèmes de contrôle de l'expression du SSTT.

En anglais

P. aeruginosa, like many other Gram-negative animal and plant pathogens, possesses a major virulence factor, the type III secretion system (TTSS), where over 30 proteins assemble into a complex machinery designed to deliver bacterial protein (effectors) directly into eukaryotic host cells (Yahr *et al*., 1995; Frank 1997). Injection of the bacterial effectors into the host cells results in various phisiological changes, all of which seem to confer a survival advantage on the bacterial pathogen within the host environment (Hueck, 1998). Because the construction of the type III secretion apparatus is an energy-expensive process due to the requirement for a large number of genes, it is important for the bacteria to tightly regulate the TTSS in response to the environmental changes.

PsrA is a positive transcriptional regulator of type III secretion system in *P. aeruginosa*

In *P. aeruginosa*, expression (transcription) of the entire TTSS genes is controlled by ExsA, a DNA-binding protein acting as a positive transcriptional regulator (Hovey and Frank, 1995), suggesting that *P. aeruginosa* might control the expression of TTSS genes either by influencing the activity of ExsA or by adjusting ExsA's expression level. The first situation has been extensively investigated (McCaw *et al*., 2002; Dasgupta *et al*., 2004; Ha *et al*., 2004; Rietsch *et al*., 2005; Urbanowski *et al*., 2005). Here, we shed light of a new transcriptional activator, PsrA, which is needed for the full transcription of the operon *exsCEBA*, and hence the expression of other TTSS genes.

The research of regulator involved in the regulation of *exsCEBA* expression was based on the observation, that the crude extract from a mutant Δ*exsA* (with a deletion of *exsA* gene) has a strong binding activity to the DNA fragment containin the promoter of the operon *exsCEBA* (pC). One protein, PsrA, was identified from the fraction having the strongest pC binding activity. We further showed that the recombinant protein PsrA (rPsrA) could specifically bind to the pC though the binding affinity is quite low, implying that PsrA might be a regulator of the operon *exsCEBA* and hence interfere the expression of whole TTSS genes. Our hypothese has been justified by analyzing the phenotype of the mutant Δ*psrA* (with a deletion of *psrA* gene). Gene reporter analysis showed that PsrA is required for the full transcription of the *exsCEBA* in different TTSS-inducing conditions, including calcium depletion from the culture medium, contact with eukaryotic host cells and the presence of human serum. The deletion of *psrA* decreased two- to threefold the transcription level of *exsCEBA* in different TTSS-inducing conditions and such defect was restored by *in trans* complementation with *psrA*, confirming that the decrease of the expression level of *exsCEBA* is indeed due to the deletion of *psrA*. Furthermore, the decrease of *exsCEBA* expression could not be totally overcame by the expression of *exsA*, indicating clearly that PsrA controls the transcrption level of *exsCEBA*. As expected, the decrease of *exsCEBA* transcription resulted in a decrease of TTSS

genes expression, such as *exoS*, and of the secretion of type III effectors, for instance ExoS and ExoT. The positive role of PsrA in regulation of the TTSS genes expression was further confirmed by phagocyte killing test, since the strain Δ*psrA* is less resistant to phagocyte-like PLB985 cells.

In fact, we haven't indeed identified the protein from the fraction having the strongest binding activity to pC, since the extract of the strain Δ*psrA* possessed the same pC binding activity as the strain Δ*exsA*. Considering that the pC binding acitvity is more stronger in TTSS-noninducing conditions than in TTSS-inducing condition (Fig. 25), and that the expression of TTSS is induced only in the presence of special enviromental signal, we speculated that the protein to be identifed would more likely to be an inhibitor. Because the extrac of strain Δ*exsA* have different binding activity (Fig. 11 and Fig. 15), it is possible that, beside ExsA and PsrA, other transcriptional regulators might act together on the level of the promoter of the operon *exsCEBA*. In fact, such scenario give *P. aeruginosa* more flexibility in their response to ever changing environmental conditions. The identification of such regulator will provide us more information concerning the regulation of TTSS expression and may provide us targets for new drugs which specially attenuate the bacterial pathogens by directly affecting the function of the main virulence factor TTSS.

PARST inhibits the expression of TTSS of *P. aeruginosa* during the stationary phase

In *P. aeruginosa*, we observed that the expression of TTSS genes is cell density-dependent, more precisely, maximum expression of TTSS genes, such as *exsCEBA* and *exoS*, happens at an OD_{600} of 1.5 to 2.0, and then decreased rapidly with the increase of OD_{600}, indicating that a mechanism exists for inactivation of TTSS in response to the cell density increase. Furthermore, we found an inhibitory signal produced from the stationary phase (high cell density) culture supernatant and termed this unknown inhibitory signal as 'PARST' for '*Pseusomonas* autorepressor of secretion three' in analogy to the other signals of quorum sensing (QS), the 'PAI' for '*Pseudomonas* autoinducer'.

Because QS regulates genes expression at cell density-dependent manner and QS having an impact on the expression of TTSS genes (Sperandio *et al*., 1999; Schaber *et al*., 2004; Hogardt *et al*., 2004; Bleves *et al*., 2005; Juhas *et al*., 2005), we examined whether the repression of TTSS genes expression at high cell-density was due to the regulation of the known QS signals. However, inhibitory actvity still exists in the stationary phase culture supernatant from mutants with the deletion of genes involving the production of known QS signals, indicating that PARST is different from QS signal. A transposon insertion mutant library was constructed to identify potential gene(s) involved in the production of PARST during the stationary phase. After having screened about 2000 insertion mutants, we observed that the mutant with the transposon insertion inside the gene *trpA*, coding the tryptophan (Trp) synthase involved in the biosynthesis of Trp, could no longer produce PARST. A mutant with

the deletion of *trpA* (Δ*trpA*) was constructed to determine the role of *trpA* in the production of PARST. Similiar to its transposon insertion mutant, no evident PARST was produced from Δ*trpA* and such defect could be restored by adding Trp during the preparation of stationary phase culture supernatant, indicating *trpA* might be implicated in the productin of PARST.

Since Trp is the precursor in the biosynthesis of auxin, a plant hormone, we examined the role of different auxins, including IAA, IBA, NAA and 2,4-D, in the regulation of TTSS genes expression. We observed that both IAA and NAA could totally repress the expression and secretion of TTSS genes at a concentration of 10 mM without inhibiting their growth. However, no IAA was detected from the stationary phase culture supernatant.

Oxydative metabolism of Trp also produces kynurenine, but kynurenine was not able to repress the expression of TTSS.

To determine the nature of PARST, we compared the HPLC profile between the supernatant containing PARST, collected from CHA, and the supernatant without PARST, collected from Δ*trpA*. We observed that an evident peak was present in CHA and absent in Δ*trpA*, on the contrary, this peak reappeared in Δ*trpA* when Trp was added during the preparation of supernatant from Δ*trpA*. LC-MS analysis showed that a molecule with a *m/z* 224 was uniquely present in that peak of the supernatant from CHA but not from Δ*trpA*. Though this unknown molecule has the same molecular weight of NAA, one derivate of auxin, they are probably not the same.

In fact, virulence gene expression represents a target for antibiotic discovery, as recently shown for *Vibrio cholerae* where a small molecule inhibitor of both toxin and pili expression, protecting infant mice from colonization (Hung *et al*., 2005). Very recently, 2 different molecules inhibitors of TTSS were identified as potential drug in treatment of *Chlamydia trachomatis*, a Gram-negative bacteria causing sexually transmitted disease and preventable blindness worldwide (Muschiol *et al*., 2006; Wolf *et al*., 2006).

Though NAA is probably not the PARST, we could still examine its possible role in the acute infection of *P. aeruginosa*. If NAA is able to decrease the virulence of *P. aeruginosa* in the acute lung infection, it would be interesting to investigate its receptor on the surface of bacteria.

Nevertheless, work needs to be done to further characterize PARST molecule. The influence of PARST on global gene expression in *P. aeruginosa* should also be studied by proteomic and/or transcriptomic approaches.

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RESUME

Pseudomonas aeruginosa est un pathogène opportuniste responsable d'infections graves chez les personnes immunodéprimées, les grands brûlés et les patients atteints de la mucoviscidose. Cette pathogénicité repose sur de nombreux facteurs de virulence dont le système de sécrétion de type III (SSTT). Activé par le contact avec la cellule eucaryote *in vivo*, le SSTT est dédié à l'injection d'effecteurs protéiques directement de la bactérie dans le cytoplasme de la cellule cible afin de favoriser l'invasion bactérienne.

Nous avons observé une protéine précédemment identifiée, PsrA, nécessaire pour la pleine activation de l'expression du SSTT chez *P. aeruginosa*. Les analyses par retard de migration électrophorétique de fragments du promoteur de l'operon régulateur *exsCEBA* ont montré que la protéine recombinante PsrA pourrait se fixer sur celui-ci. A l'aide de gène rapporteurs, nous avons montré que PsrA est nécessaire pour la pleine activation de la transcription d'*exsCEBA* et *exoS* (gène représentatif pour les effecteurs), bien que le mutant de délétion du gène *psrA* (Δ*psrA*) ait toujours répondu à la déplétion calcique, au sérum, et au contact de cellule hôte. Le mutant Δ*psrA* a montré une diminution marquée de la sécrétion des effecteurs de type III et une faible résistance à la bactéricidie par des cellules de type phagocytaires, PLB-985. Le défaut de la transcription du SSTT ainsi que la sécrétion d'effecteurs dans le mutant Δ*psrA* ont pu être complémentés par l'expression en *trans* de *psrA*. PsrA a été précédemment identifié comme un activateur transcriptionnel de RpoS, un régulateur central pendant la phase stationnaire. Nous avons confirmé que RpoS a un effet négatif sur l'expression des gènes du SSTT dans notre souche. L'ensemble des résultats suggèrent que PsrA est un nouvel activateur qui est impliqué dans l'expression du SSTT en augmentant le niveau de la transcription d'*exsCEBA*.

Dans un second temps, nous nous sommes intéressés au phénomène anciennement observé au laboratoire qui suggère que l'expression du SSTT est dépendant de la densité bactérienne. Nous avons amélioré les outils pour mettre en évidence que le milieu de culture conditionné (surnageant venant de la phase stationnaire) peut inhiber l'activation du SSTT. Nous avons mis en évidence qu'un signal inhibiteur, produit dans la phase stationnaire de la culture, peut réprimer l'expression du SSTT *in vitro*. L'analyse de milliers de mutants de transposition a montré que la production de ce signal dépend du tryptophane, qui est le précurseur de nombreux métabolites dont l'acide d'indole-3-acétic (IAA) et la kynurénine. IAA-Na et un autre membre de cette famille de molécules, le potassium acide 1-naphthalenacétic (NAA-K) aux concentrations millimolaires peuvent en effet inhiber l'expression et la sécrétion du SSTT. La kynurénine n'a pas d'effet. L'identification précise de ce signal nécessite des investigations plus poussées. Ces observations fournissent des arguments en faveur de l'existence d'une autre voie régulatrice dans la transition de l'expression de gènes entre un état avec des cellules à faible densité et celui avec des cellules à forte densité plus connut sous le terme de 'quorum sensing'.

Mots clés: *Pseudomonas aeruginosa*, le système de sécrétion de type III, régulation, PsrA, 'quorum sensing'