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Mécanismes moléculaires impliqués dans la régulation post-traductionnelle du système de sécrétion du type VI chez *Pseudomonas aeruginosa*

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A mi mamá,

Que me crió para que no le tuviera miedo a nada.

A mi papá,

De quien he heredado casi todo lo que odio/amo de mi carácter.

Córdoba te espera para pescar, Mar del Plata para correr.

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Summary

Figure List

Table List

Principal abbreviations

ABC transporter: ATP-binding complex transporter

CDI: contact-dependent inhibition of growth

CF: cystic fibrosis

C-ter: C terminus

DDM: n-Dodecyl β-D-maltoside

EAEC: enteroaggregative *Escherichia coli*

GFP: green fluorescent protein

GI: genomic island

HPLC: high pressure liquid chromatography

IDS: identification of self

IF: immunofluorescence

IM: inner membrane

IPTG: Isopropyl β-D-1-thiogalactopyranoside

kDa: kilo Daltons

LPS: lipopolysaccharide

MS: mass spectrometry

N-ter: N terminus

Nano-LC: nano liquid chromatography

OD_{600}: Optical density at 600nm

OM: outer membrane

OMP: outer membrane protein

QS: quorum sensing

SDS: sodium docecyl sulphate

SDS-PAGE: SDS polyacrylamide gel eletrophoresis

Sec: general secretion machinery

T1SS-T6SS: type I secretion system to type VI secretion system

Tat: twin arginine translocation

TPP: threonine phosphorylation pathway

Foreword

Pseudomonas aeruginosa is a human opportunistic pathogen that can cause acute and chronic infections. Despite numerous efforts, the mechanisms of host-pathogen interaction and pathogen survival during infections are still to be elucidated.

Amongst a number of different macromolecular assemblies, *P. aeruginosa* possesses an active type VI secretion system. The type VI secretion system HSI-I (H1-T6SS) is negatively regulated by the global regulator RetS, as shown by transcriptomic analyses. H1-T6SS activity is also controlled at a posttranslational level by a kinase/phospatase pair, PpkA and PppA, respectively, that modulate the phosphorylation level of Fha1. Fha1 phosphorylation is needed for the secretion of the so-called "effectors" of the H1-T6SS.

The **principal objective** of this work was to decorticate the functioning of this novel secretion system in *P. aeruginosa*, in particular to study the role of four novel players, TagT, S, R and Q, in its posttranslational activation.

We showed in the laboratory that *tagT* and *tags* encode a novel ABC-transporter with ATPase activity, indispensable for Hcp1 secretion in *P. aeruginosa*. I sought to further biochemically and functionally characterize TagT, S, R and Q. The first part of the Results describes the principal studies concerning these proteins and includes a publication in Environmental Microbiology and the additional results obtained after this work was published.

The second axis of the results involves the characterization of the cell envelope of *P. aeruginosa*. In an effort to characterize the *tagTS* knockout phenotype, we carried out shotgun mass spectrometry analyses of the inner and outer membranes of *P. aeruginosa.* These experiments lead us to the discovery of TagR association with the outer membrane, as long as the identification of hundreds of envelope-associated proteins. The second Chapter of the Results section of this manuscript describes our vision of P. aeruginosa envelope, making an emphasis in the architecture of the H1-T6SS.

Finally, in the last part of the Results section, I present the results obtained concerning MagD (PA4489), the macroglobulin-like protein of *P. aeruginosa*. The inner membrane sub-proteome included several proteins encoded in a six-gene operon PA4487-PA4491, listed as "conserved hypothetical" proteins. These findings include PA4489, the macroglobulin-like protein of *P. aeruginosa*. We showed that these proteins form an inner membrane bound complex important for *P. aeruginosa* virulence towards a *Drosophila* model.

Introduction

Chapter I. *Pseudomonas aeruginosa*: an opportunistic pathogen

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacillus, capable of colonizing a diversity of niches that include moist environments such as the soil and plant-matter, and that can infect a variety of hosts, including animals and humans. *P. aeruginosa* was first described in 1882 by Carle Gessard when he noticed that wounds from soldiers turned to a blue-green colour. Since then, this bacterium has been extensively studied and has even been declared as one of the six "top-priority dangerous, drug resistant microbes" by the Infectious Diseases Society of America (Talbot *et al.*, 2006, Boucher *et al.*, 2009).

The "pyocyanin bacillum", as it is called in the medical jargon, is a facultative aerobe capable of metabolizing up to fifty different carbon sources when developing in anaerobiosis, and it can use nitrate as electron acceptor when in an anaerobic environment (Vasil, 1986). This mesophilic bacterium has an optimal growth between 30 and 37°C, but it can grow in the range of temperatures from 4°C to 42°C. *P. aeruginosa* is highly motile, being able to swim, twitch and swarm. This opportunistic pathogen is a versatile bacterium, which can adapt to the changes in its surrounding environment and persist in a broad range of conditions. Importantly, its multiresistance to antibiotics in addition to the features mentioned above make *P. aeruginosa* an important subject of study in the pursue to its eradication.

1.01 GENOME

The complete genome sequence of the laboratory strain PAO1 was determined in 2000 by Stover and colleagues (Stover *et al.*, 2000). Its genome of 6.3 million base pairs includes 5570 predicted open reading frames (ORFs), making it the largest bacterial genome to be sequenced in its totality at that time (Figure 1). Since then, other clinical *P. aeruginosa* isolates have been sequenced, such as PA14, PA7, LESB58, PA2192 and C3719 (Lee *et al.*,

2006, Mathee *et al.*, 2008, Roy *et al.*, 2010, Winstanley *et al.*, 2009). Their genome sizes vary between 5.5 and 7 Mbp and the major part of the genome is found in all strains studied so far.

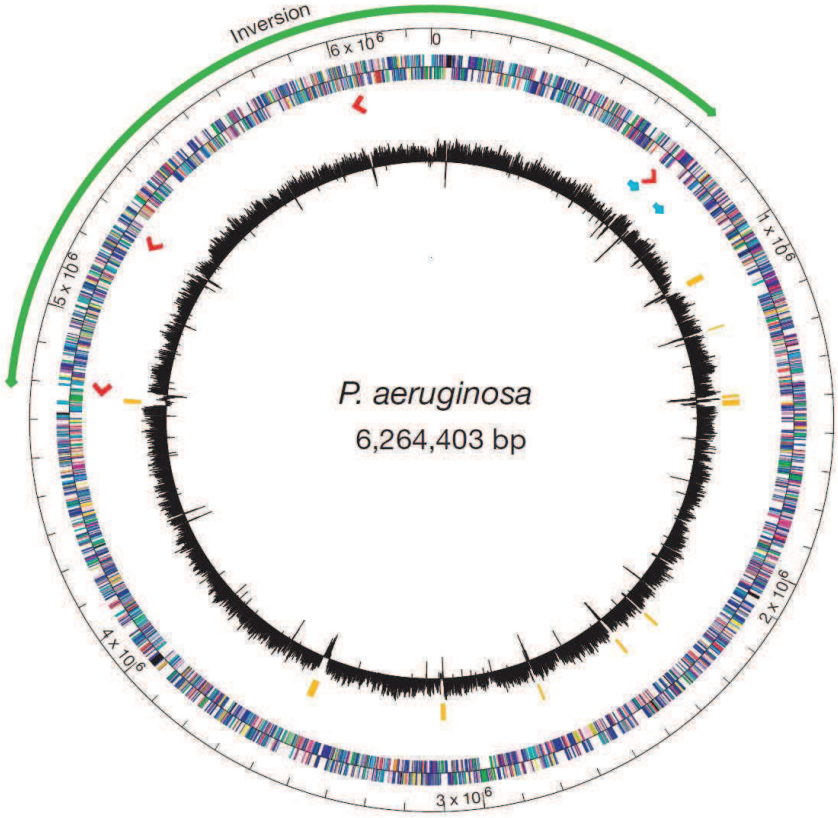

Figure 1 The genome of *P. aeruginosa* PAO1 strain, taken from Stover *et al.* (2000). The green aarow indicates the inverted region that resulted from a homologous recombination event between *rrnA* and *rrnB*.

A comparative microarray-based study revealed that 90% of the genome (referred to as the core genome) of *P. aeruginosa* is conserved between different strains, either clinical or environmental isolates. Interestingly, the non-conserved genes, referred to as the accessory genome, are not randomly distributed throughout the genome but arranged in discrete clusters called Regions of Genome Plasticity (RGPs)(Mathee *et al.*, 2008). The accessory genome was acquired recently by horizontal genetic transfer, and can be recognized by its particular G+C content and/or codon usage. *P. aeruginosa* genome is frequently described as having a mosaic structure, since the core genome is interrupted by the inserted blocks of the accessory "foreign" genome (Mathee *et al.*, 2008, Spencer *et al.*, 2003). The accessory genome endows remarkable plasticity, since it can be acquired from other bacteria, phages

or even other taxa (genomic islands, GI). It is thought that the acquisition of new genetic material is due to environmental pressure. GIs generally carry genes that encode for virulence factors, toxins and proteins related to antibiotic resistance, thus promoting *P. aeruginosa* adaptability to many environments and its persistence within various host species. One example is the GI PAPI-1, that carries ORFs that appear to promote pathogenicity, for example a putative T3SS effector and a chaperone usher pathway gene cluster (He *et al.*, 2004, Qiu *et al.*, 2006).

The sequencing of PAO1 strain genome revealed that 8.4% of the open reading frames (ORFs) are involved in regulation, and a disproportionate large number of genes predicted to encode for OM proteins that could be involved in adhesion, motility, antibiotic efflux, virulence factor export, and environmental sensing by two component systems. Furthermore, a large number of transporters and enzymes involved in nutrient uptake and metabolism were also identified (Lee *et al.*, 2006, Mathee *et al.*, 2008, Roy *et al.*, 2010, Winstanley *et al.*, 2009, Stover *et al.*, 2000).

Recent studies revealed that *P. aeruginosa* genomes also codes for intergenic small RNAs (sRNAs) that are involved in posttranscriptional control of gene regulation (Livny *et al.*, 2006), usually by binding to messenger RNAs and affecting their stability (Brencic & Lory, 2009). High-throughput cDNA sequencing (RNA-seq) has revealed over 500 sRNAs encoded in *P. aeruginosa* genome, most of them with no orthologous bacterial sequences in other bacteria (Gomez-Lozano *et al.*, 2012, Wurtzel *et al.*, 2012).

1.02 LIFESTYLE

P. aeruginosa can have two different lifestyles in the environment, either mobile (also called planktonic) or biofilms (that are aggregates or surface-associated polymicrobial communities).

A transcriptome study on *P. aeruginosa* PA14 strain using RNA sequencing evidenced that biofilms have distinct patterns of gene expression during development, implying that there are different actors implicated at specific biofilm formation stages. Also, distinct patterns of

gene expression were determined in planktonic cultures in stationary phase of growth and biofilms cultures, suggesting that different molecular mechanisms orchestrate the conversion between planktonic and biofilm communities (Dotsch *et al.*, 2012).

In biofilms, the microorganisms themselves account for less that 10% of the dry mass, whereas the matrix accounts for over 90%. The matrix is the extracellular material in which the microbial cells are embedded, a compact gel of polysaccharides produced by the biofilm-forming cells themselves. Proteins, DNA and lipids can also be part of the matrix. The roles of the different substances present in the matrix are diverse and include adhesion (allowing the initial steps in the colonization of the surface), aggregation of bacterial cells, enzymatic activity (the enzymes secreted by the cells can degrade a variety of compounds such as biopolymers), protection against desiccation and of hosts immune defense, amongst others (Flemming & Wingender, 2010).

P. aeruginosa produces at least three exopolysaccharides that are required for biofilm formation: alginate, Psl (for Polysaccharide Synthesis Locus) and Pel (for PELicule). Alginate is the major exopolysaccharide in mucoid strains, which are the predominant isolates from chronically CF infected patients. Pel and Psl are critical for biofilm formation on abiotic surfaces.

The matrix of *P. aeruginosa* biofilms contains extracellular DNA and proteins (Mulcahy *et al.*, 2011, de Bentzmann *et al.*, 2012). A recent study using a proteomics approach has discovered that approximately 30% of the matrix-associated proteins were OMPs, some of which were already identified as present in OMVs. Proteins derived from cell debris and secreted proteins (such as exoenzymes LasA and LasB) were found in *P. aeruginosa* matrix (Toyofuku *et al.*, 2012). *P. aeruginosa* can also form free exopolysaccharides biofilms, where release of DNA to the extracellular milieu appears as a key process (de Bentzmann *et al.*, 2012, Allesen-Holm *et al.*, 2006). Of note, these biofilms presented hypersensitivity to antibiotics, probable due to their unusual matrix (de Bentzmann *et al.*, 2012).

It has been proposed that different matrix materials play distinct roles during bacterial biofilm formation. Conserning exopolisaccharydes in *P. aeruginosa* biofilm formation, Psl and Pel are involved in formation of microcolony structures and in subpopulation interactions, since mutants in Pel or Psl are unable to develop the characteristic mushroom-

like structures observed by laser scanning confocal microscopy (Figure 2). A model for *P. aeruginosa* PAO1 strain biofilm formation has been suggested by Molin and coworkers (Figure 2, Yang *et al.*, 2011b). The different steps (1 to 4) include:

1 – Individual planktonic cells attach to the surface, also called substratum, by means of the pilus, the flagellum and the extracellular DNA (eDNA in Figure 2)

2 – Irreversible attachment: bacteria form microcolonies. Pel and Psl polysaccharides are required for this step, as well as rhamnolipids.

3 – Subpopulations interact with each other, early stage of biofilm architecture. This step is also called maturation (Stoodley *et al.*, 2002).

4 – Mature biofilms formed by macrocolonies. The mushroom-like shape is characteristic of *P. aeruginosa* mature biofilms.

The last step is biofilm dispersion, where motile bacteria “escape” from inside the mushroom when this opens and can colonize another site. Two reports evidenced this “regulated” life-cycle by showing different phenotypes during biofilm development (Davies & Geesey, 1995, Sauer *et al.*, 2002).

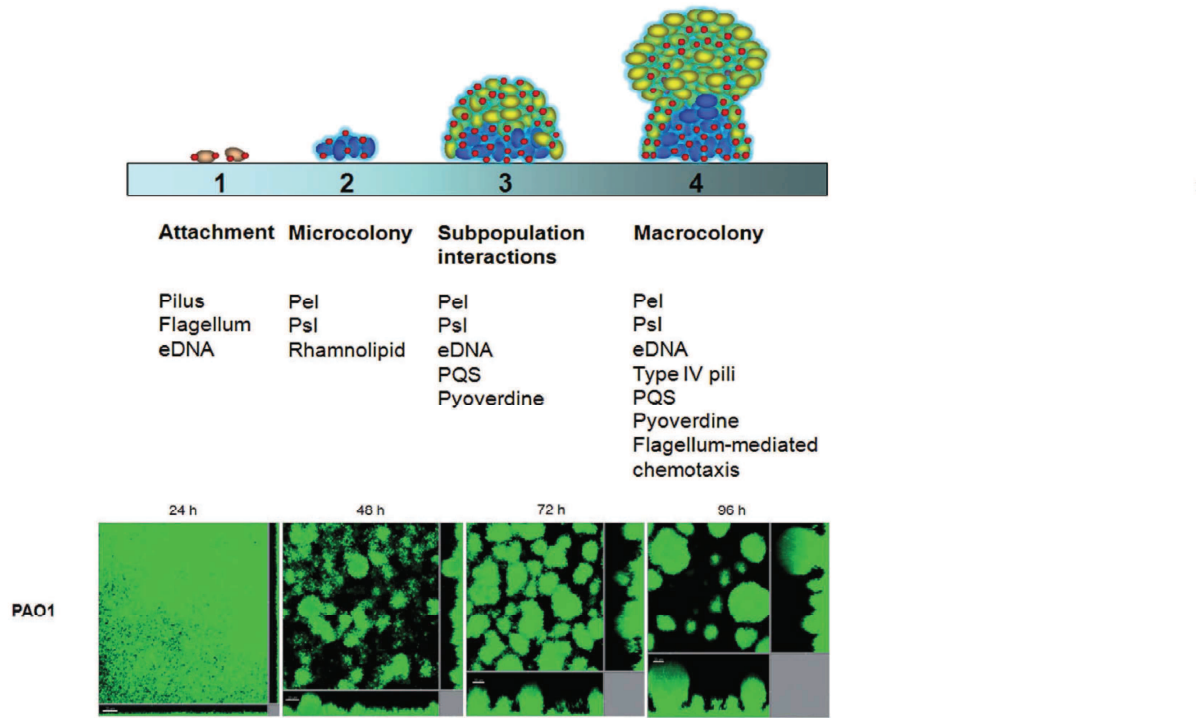

Figure 2 A) Biofilm development by *P. aeruginosa* PAO1. Red spheres represent eDNA, blue and yellow cycles represent non-motile and motile cells respectively. Psl and Pel exopolysaccharides are represented in light blue. For each step (1-4), the principal actors are listed. B) PAO1 biofilm formation. Bacteria were grown on a flow-cell chamber and images were taken with a confocal microscope every 24h. The bar represents 20µm. Modified from Yang *et al.* (2011b).

1.03 *P. AERUGINOSA* INFECTIONS

P. aeruginosa is able to infect insects, plants, and animals, including humans. Depending on the virulence factors that it expresses and the immunitary response of the host, it can cause either an acute or a chronic infection. Human diseases caused by *P. aeruginosa* include bacteremia in severe burn victims, chronic lung infection in cystic fibrosis (CF) patients, and acute ulcerative keratitis in patients that have a prolongated use of contact lenses (Lyczak *et al.*, 2000). Acute infections are characteristic in immune-compromised patients, for instance patients that had surgery, cancer and HIV (Lyczak *et al.*, 2000). This type of infection occurs rapidly and is aggressive, causing inflammation and in the most extreme cases, septic shock. Planktonic bacteria are associated with acute infection, because they produce “aggressive”

virulence factors that allow the injury of the host (there is induction of target cell death). Following infection, *P. aeruginosa* is able to colonize the lungs, the kidney and the urinary tract. If this happens, the results might be fatal.

Chronic infections are related to biofilm-forming *P. aeruginosa*. Biofilms are more resistant to antibiotics and phagocytosis, and have non-motile bacteria that lose the ability to produce some of the virulence factors needed for an acute infection. As a consequence, *P. aeruginosa* is less toxic and persists in the host. During chronic infections, a slow but progressive destruction of the tissue can be observed.

Chronic infections by *P. aeruginosa* are responsible for fatality in many CF patients. CF is an autosomal recessive genetic disorder caused by mutations in the CF transmembrane conductance regulator (CFTR), a channel that regulates ion passage through the epithelium (Quinton, 1983) and that affects mostly the lungs. The unbalanced ion composition results in an altered mucus layer in the respiratory organ, which will be dehydrated and thicker than a wild-type mucus (Lyczak *et al.*, 2002, Hauser *et al.*, 2011). In consequence, there is no normal clearance of the respiratory tract, generating a favourable environment for chronic microbial colonization and survival. Even though numerous species can be found in the CF airways, *P. aeruginosa* is usually the predominant pathogen present in about 80% of adult patients (Figure 3A).

P. aeruginosa has an enormous genetic and metabolic flexibility that allows it to adapt to the milieu and persist within the airways of CF patients. Moreover, the genotypes and phenotypes of the strains present in late stages of the disease differ substantially from those that initially colonize the lungs. It has been suggested that the mutations that appear in these strains are due to selection, since they were advantageous for life within the host (Smith *et al.*, 2006, Rau *et al.*, 2010). Almost all the strains that were studied by Smith *et al* (Smith *et al.*, 2006) had loss-of-function mutations in genes the code for factors used by bacteria to invade and injure the host, such as *exsA*, the T3SS master regulator and *lasR* a QS transcriptional regulator (Figure 3B).

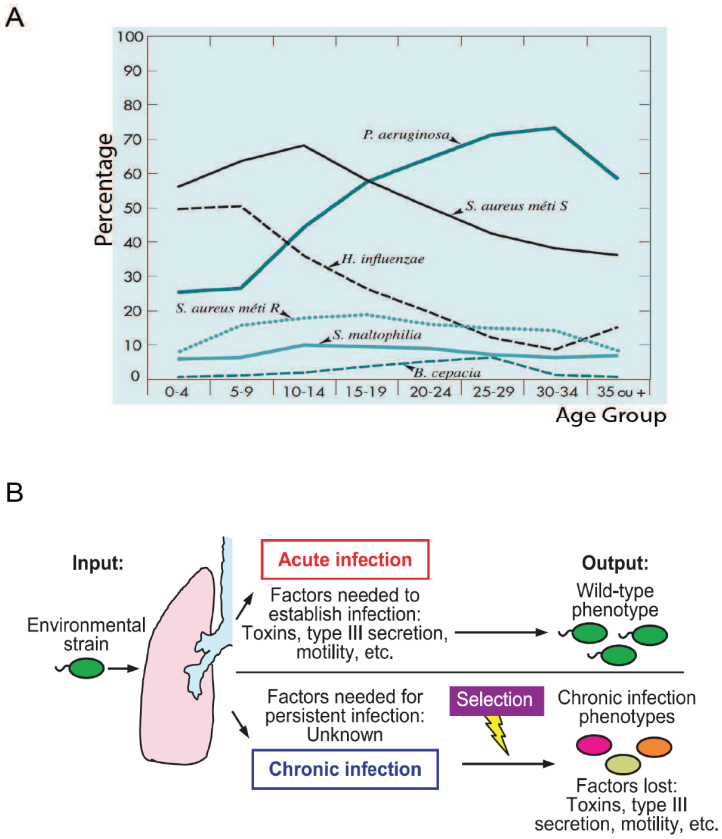

Figure 3 *P. aeruginosa* and CF. A) Bacterial colonization percentage versus patient age group of the five bacterial species considered important from a clinical point of view. Source: Observatoire Nationale de la Mucovisidose, France. B) *P. aeruginosa* factors needed for acute/chronic infection and the evolution of the input strain. Taken from Nguyen & Singh (2006).

1.04 REGULATION OF VIRULENCE FACTORS IN *P. AERUGINOSA*

RetS and LadS are global regulators that respond to an unknown signal and reciprocally regulate the expression of virulence factors (Goodman *et al.*, 2004, Ventre *et al.*, 2006). RetS and LadS are hybrid sensor kinases that converge on sRNAs RsmZ and RsmY by modulating the activity of GacS, responsible for the phosphorylation of GacA (Figure 4). Phosphorylated GacA activates the transcription of RsmZ and RsmY, that have an impact on the amount of free RsmA, a RNA-binding protein that acts as a posttranslational regulator. RsmA promotes the translation of T3SS, T2SS, type VI pili, *lipA* genes, associated with acute infection and a planktonic way of life. Inversely, RsmA downregulates the *psl*, *pel*, HSI-I locus and other

virulence factor genes associated with chronic infections and biofilm formation (Sonnleitner & Haas, 2011). Hence, a *retS* knockout mutant has very low levels of RsmZ and RsmY, resulting in a chronic-like virulence phenotype in *P. aeruginosa*. In addition to RetS and LadS, the Hpt phosphorelay system specifically regulates *rsmY* expression, leading to the expression of genes associated with swarming motility and biofilm formation (Bordi *et al.*, 2010).

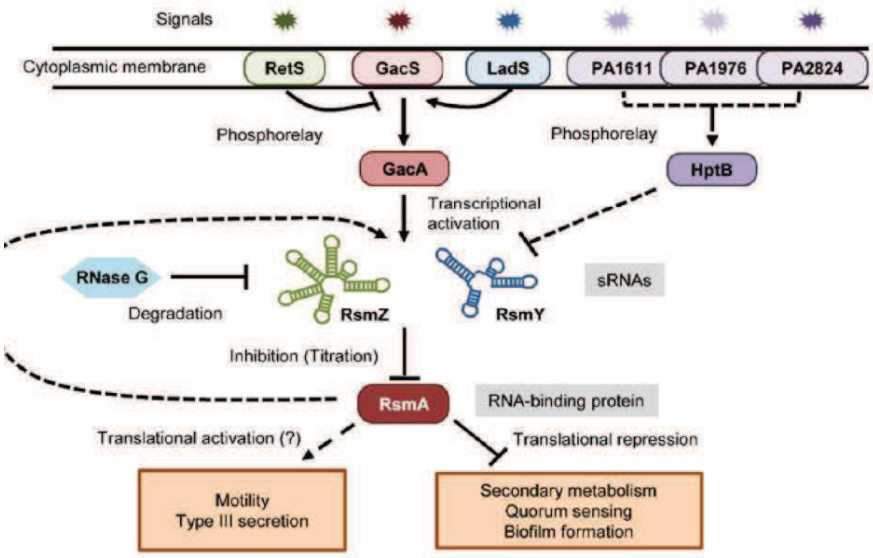

Figure 4 Model of Gac/Rsm system in *P. aeruginosa*. Taken from Sonnleitner & Haas (2011).

cAMP and di-cGMP are secondary metabolites whose intracellular levels impact the regulation of virulence factors. cAMP acts as an allosteric activator Vfr (for virulence factor regulator), a transcriptional regulator that controls the expression of numerous genes (Suh *et al.*, 2002, Wolfgang *et al.*, 2003). In particular, Vfr upregulates the expression of virulence factors important for acute *P. aeruginosa* infections, such as T3SS and T2SS genes, secreted toxins and QS systems Las and Rhl, that themselves control hundreds of genes (including many virulence factors, Croda-Garcia *et al.*, 2011). di-cGMP modulates motility and biofilm formation in an inverted manner.

P. aeruginosa possesses three QS systems, namely *las*, *rhl* and *pqs*, that shape gene expression pattern. Each system consists of genes involved in autoinducer synthesis, a

diffusible molecule that is used to monitor cell population density, and a regulator, a transcription factor (Figure 5). LasI and RhlI produce signals of the family of the acyl-homoserine lactones (acyl-HSL) and in the *pqs* system the products of *pqsABCD* and *pqsH* are involved in the synthesis of the autoinducer 2-heptyl-3-hydroxy-4-quinolone (PQS). Importantly, each autoinducer only binds to its cognate regulator protein.

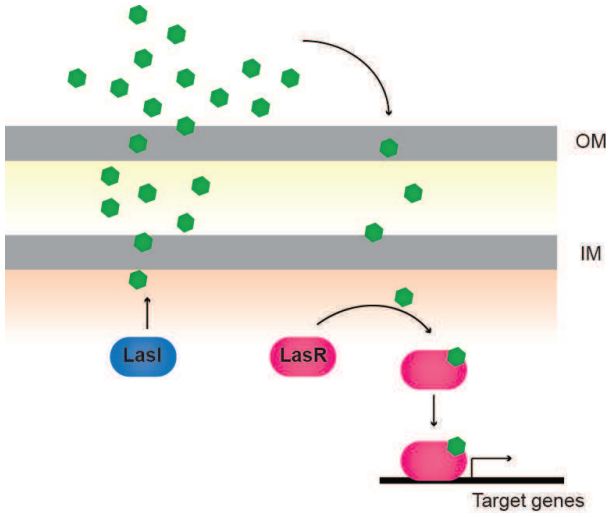

Figure 5 QS *las* system. LasI is a synthase enzyme that will be in charge of synthesizing the autoinducer (acyl-HSL, green hexagon). The autinducer can diffuse through the membrane and will accumulate in the milieu. Once inside the bacterium, it will bind to LasR, a transcriptional factor. The LasR-acyl-HSL complex will activate specifically the transcription of target genes.

The QS systems of *P. aeruginosa* are organized in a hierarchical fashion, forming a complex network (Dekimpe & Deziel, 2009). The Las system positively regulates *rhl* and *pqs* systems. These two coordinately control the transcription of more than a hundred genes, including genes involved in virulence factor production, biofilm maturation, and motility phenotypes (Deziel *et al.*, 2005). The Las system is responsible for the activation of genes such as *lasB*, *aprA* and *exoA*, and together with the *rhl* system they regulate at least three hundred genes (Schuster *et al.*, 2003).

The GacA/GacS regulatory system affects QS posttranscriptionally, since RsmA represses the synthesis of acyl-HSL signals in the absence of RsmZ. RsmA also regulates the production of several quorum-controlled virulence factors, repressing some (hydrogen cyanide and pyocyanin), while inducing others (lipase and rhamnolipids)(Heurlier *et al.*, 2004).

Loss-of-function mutations in the anti-sigma factor MucA are common in CF isolates (Pulcrano *et al.*, 2012). In *P. aeruginosa mucA* knockouts the alternative sigma factor AlgU is liberated and active. AlgU upregulates of the expression of the alginate biosynthetic operon genes and of AlgR, that inhibits *vfr* expression. Hence, CF strains frequently become mucoid and non motile (Pulcrano *et al.*, 2012, Tart *et al.*, 2005, Jones *et al.*, 2010).

Importantly, di-cGMP intracellular levels are controlled partially by RetS, evidencing that there is a continuous crosstalk between the systems, that form a complex regulatory circuit. This regulatory network provides to *P. aeruginosa* the ability to respond to a wide range of stimuli in a finely tuned and progressive fashion.

1.05 VIRULENCE FACTORS OF *P. AERUGINOSA*

A virulence factor is a molecule that is produced –but not necessarily secreted- by a bacterium and which allows the colonization, persistence, multiplication and/or entry of the bacterium into the host.

P. aeruginosa produces a vast repertoire of virulence factors, sometimes encoded by GIs, such as PAPI-1. These include secreted factors such as exotoxin A and elastases A and B, surface factors including LPS and lectins, and toxins that are injected directly into the host like the T3SS toxins (reviewed in Kipnis *et al.*, 2006).

(a) SURFACE VIRULENCE FACTORS

(i) FLAGELLUM AND TYPE IV PILI

P. aeruginosa possesses a polar flagellum of 15-20 µm long that provides it with the ability to swim when in a liquid environment and to swarm (with the help of type IV pili) when in a semi-solid one. Flagella are considered as virulence factors because flagellin (FliC) is able to bind to Toll-receptors TLR5 and TLR2, inducing an inflammatory response (Feldman *et al.*, 1998, Kipnis *et al.*, 2006).

Type IV pili are also polar surface proteic appendices, but are smaller in size (they have ~6nm diameter and are only several microns long, Paranchych *et al.*, 1986, Burrows, 2012). Pili are involved in twitching, spreading of bacteria along a solid surface. Just like flagella, pili are involved in bacterial adhesion to epithelial cells, because they bind to glycolipids asialo-GM1 and GM2 (Tang *et al.*, 1995, de Bentzmann *et al.*, 1996).

(ii) FIMBRIAE

Fimbriae, also called Cup for Chaperon Usher Pathway, have a role in *P. aeruginosa* adhesion to biotic and abiotic surfaces. They are proteic appendages formed by a pilin that have in their distal tip an adhesin, capable of interacting with its receptor in host cells (Vallet *et al.*, 2001). They can be present in numerous copies in the genome. PAO1 strain possesses four Cup systems, CupA, B, C and E whereas PA14 strain has an additional Cup system, CupE, encoded in PAPI-1 (He *et al.*, 2004).

(iii) RHAMNOLIPIDS

Rhamnolipids are glycolipidic surface-active molecules produced by *P. aeruginosa*. They are biosurfactants that can act as detergents towards eukaryotic cells membranes, disrupting them. It has even been shown that rhamnolipid-secreting *P. aeruginosa* are able to disrupt epithelial tight-junctions (Zulianello *et al.*, 2006). Moreover, these compounds can inhibit phagocytosis (Jensen *et al.*, 2007). Rhamnolipids also have a role in correct biofilm formation and in the maintenance of its architecture (Davey *et al.*, 2003, Boles *et al.*, 2005).

(iv) LECTINS

Lectins are proteins that bind specifically to carbohydrate structural epitopes. *P. aeruginosa* produces two lectins, LecA and LecB, that recognize and bind to galactose and fucose, respectively (Glick & Garber, 1983). LecB is localized to the OM and has a role in biofilm formation (Tielker *et al.*, 2005). LecA has been identified in the extracellular milieu of a culture (Plotkowski *et al.*, 1994) and in the extracellular matrix of biofilm (Diggle *et al.*, 2006). LecA has been shown to influence biofilm architecture in *P. aeruginosa* most surely at

biofilm maturation step, since a *lecA* mutant is able to adhere to different abiotic substrates (Diggle *et al.*, 2006). Finally, LecA and LecB facilitate bacterial adhesion to epithelial cells, since bacteria depleted of lectins were found to be less virulent (Chemani *et al.*, 2009).

(v) LIPOPOLYSACHARIDES

The outer leaflet of the OM of Gram-negative bacteria is composed of LPS. These amphipatic molecules are considered as virulence factors in *P. aeruginosa* for several reasons. First, they extend several tenths of nm out of the cell, protecting the bacterium from approaching agents. In addition, LPS can bind to several eukaryotic receptors, such as TLR4/CD14 and asialo-GM1 (Hajjar *et al.*, 2002, Gupta *et al.*, 1994). LPS are also capable of binding CFTR, which affects the clearance of the bacteria from the lung (Pier *et al.*, 1997).

LPS are mostly constituted of three parts: i) lipid A, ii) the core, and iii) the O-antigen. The latter, also called O-polysaccahride, is the outermost part of LPS. It is a polymer that varies in its repetitive units (mostly monosaccharides, but also non-carbohydrate substituents) and the way in which these units are linked between each other. Hence, O-antigens are very variable between species and also amongst bacterial strains. The bacterial serotype is defined by the chemical structure of this component of LPS. The O-antigen and the lipid A can vary over time, e.g. during a life-time colonization of a CF patient. These changes can favour the evasion of the recognition by the host, further implying a role of LPS in *P. aeruginosa* virulence (Smith *et al.*, 2006).

(b) SECRETED VIRULENCE FACTORS

P. aeruginosa also produces virulence factors that are secreted to the extracellular milieu by means of specific secretion systems. Figure 6 depicts the principal effects the secreted toxins have on the host epithelial cells.

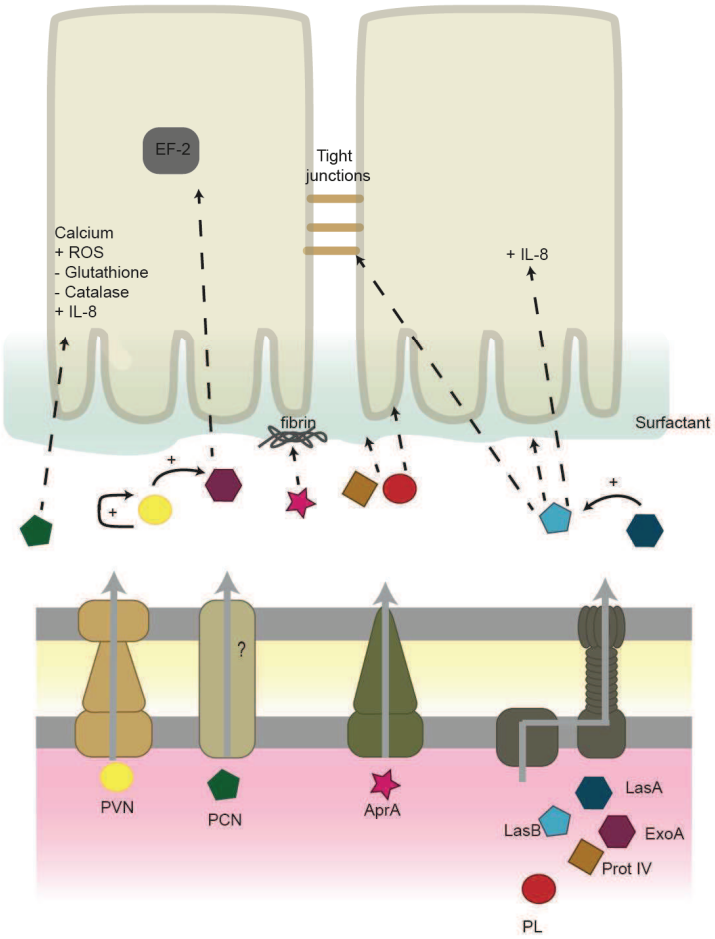

Figure 6 Principal secreted factors of *P. aeruginosa* and their most relevant effects on the host. PVN: pyoverdine, PCN: pyocyanin, PL: phopholipases C, Prot IV: proteinase IV, ROS: reactive oxygen species, EF-2: elongation factor-2. The "+" symbol indicates enchanced activity. Grey arrows indicated transport. The T3SS exotoxins have not been included and are described further on.

The secreted virulence factor that is considered as the most toxic is the exotoxin A (ExoA), an ADP-ribosyl transferase secreted by the T2SS. ExoA inhibits the elongation factor-2 (EF-2), leading to cell death at a comparable level with the diphtheria toxin (Kipnis *et al.*, 2006, Pavlovskis *et al.*, 1978, Miyazaki *et al.*, 1995).

The alkaline protease AprA is a zinc-dependent metalloprotease that is secreted by its own T1SS. It is a fibrin lysing protease that can degrade the C1q and C3 components of the

complement, as well as cytokines (Hong & Ghebrehiwet, 1992, Parmely *et al.*, 1990). Its complete mechanism of action remains unknown.

Another protease secreted by *P. aeruginosa* is protease IV, that has been studied mostly for its role in keratitis. Protease IV is able to cleave host proteins including fibrinogen, plasminogen, immunoglobulin G, C3 and C1q (Engel *et al.*, 1998). This protease can also degrade surfactant proteins A, B and D, suggesting that it could have a role in acute lung injury associated with *P. aeruginosa* (Malloy *et al.*, 2005).

LasA is one of the most abundant proteases produced by *P. aeruginosa*. It is a zinc metalloprotease that is secreted by the Xcp T2 machinery and cleaves peptide bonds subsequent to Gly-Gly pairs in proteins and peptides preferentially (Kessler *et al.*, 1997).

LasA has a variety of attributed roles. For example, LasA has staphylolitic activity (it is able to cleave the peptidoglycan pentaglycine interpeptides), thus it is important as a defense strategy against *Staphylococci* competitors. It can even have a detrimental effect on *Staphylococci* growth when added into the culture (Kessler *et al.*, 1992). Also, LasA is directly involved in virulence by enhancing syndecan-1 shedding at the epithelial cells surface both *in vitro* and *in vivo*; most likely by activation of the host cell's shedding machinery (Park *et al.*, 2000, Park *et al.*, 2001).

Finally, elastase B (LasB) is the second zinc metalloprotease that is secreted by the T2SS, capable of disrupting the tight junctions of epithelial cells thanks to the degradation of elastin and collagen (Azghani *et al.*, 1993, Azghani, 1996). It is known as a pro-inflammatory factor since it can induce an inflammatory response after 4h infection in an animal model (measured by the IL-8 concentration and the neutrophil recruitment, (Kon *et al.*, 1999). Also, it can alter lung permeability (Peterson *et al.*, 1992) and modify innate immune response by degrading surfactant proteins A and D (Mariencheck *et al.*, 2003).

P. aeruginosa produces and secretes at least three phospholipases C: PlcN, PlcH and PlcB (Barker *et al.*, 2004, Ostroff *et al.*, 1990). As it name indicates, these enzymes are able to cleave phospholipids, their target being eukaryotic membrane phospholipids, in particular phosphatidylcholine (Kipnis *et al.*, 2006).

Pyoverdine is a yellow-green and fluorescent iron-chelating siderophore that recovers iron from the environment and is able to internalize it into the cell's cytoplasm. It is essential for *P. aeruginosa* virulence towards mice, since pyoverdine-depleted mutants are unable to kill their hosts (Meyer *et al.*, 1996, Takase *et al.*, 2000). In addition, pyoverdine regulates the production of ExoA, an endoprotease and itself, further implying a strong role in *P. aeruginosa* virulence (Lamont *et al.*, 2002).

Pyocyanin is a green-blue pigment that is essential for *P. aeruginosa* virulence in animal models (Lau *et al.*, 2004a, Lau *et al.*, 2004b, Mahajan-Miklos *et al.*, 1999). It is a redox-active phenazine compound able to kill mammalian and bacterial cells *in vitro* and *in vivo* through the generation of reactive oxygen intermediates (ROS), since it modulates the glutathione redox cycle and inactivates the catalase activity (O'Malley *et al.*, 2004, O'Malley *et al.*, 2003, Muller, 2002). It has also been demonstrated that it can modulate the amount of calcium as Ca^{2+} in the target cells cytoplasm, which has a negative effect in cell signalling (Denning *et al.*, 1998).

Finally, there are four exotoxins that *P. aeruginosa* can secrete via the T3SS. These will be described in the next section.

(c) Secretion Systems

As described in the previous section, many of the virulence factors produced by *P. aeruginosa* are secreted to the extracellular milieu or injected directly into the host. In order to be able to export these molecules out of the cell, *P. aeruginosa* assembles envelope machineries called secretion systems that span both membranes.

Some substrates traverse the cell envelope in one step. This is the case of the T1SS, the T3SS and the T6SS. Other molecules are secreted by a two–step mechanism. They are first translocated into the periplasm by the Sec or Tat system and then they are taken over by the specific OM complex which allows them to get out of the cell. The T2SS and the T5SS are examples of two-step mechanisms of export present in *P. aeruginosa*.

(i) T1SS

T1SSs consist of three indispensible membrane proteins: an IM ATP-binding complex (ABC) transporter, an IM adaptor protein or MFP (for membrane-fusion protein) and a substrate-specific OMP of the TolC family. The OMP and the ABC transporter are linked through the MFP, so that secretion is carried out in one step, bypassing the periplasm (Delepelaire, 2004). T1-secreted proteins are recognized in the cytoplasm due to their C-ter secretion sequence, that interacts with the ABC transporter. This signal sequence is absolutely required for secretion via the T1SS, but its role is not completely understood (Delepelaire, 2004). Once the T1 substrate interacts with the IM complex, there is assembly of the membrane-spanning complex and secretion can occur.

P. aeruginosa possesses four T1SS, but only two of them have been characterized (Ma *et al.*, 2003, Filloux, 2011). Their known substrates are: alkaline phosphatase AprA, lipase LipA, HasA and AprX (PA1245). AprA, LipA and AprX are secreted by the same T1SS, the Apr system. HasA is secreted by the HasDEF system, that has been shown to be very similar to the T1SS involved in iron uptake in *Serratia marcescens* (Ma *et al.*, 2003, Filloux, 2011).

(ii) T2SS

T2SSs are broadly conserved amongst Gram-negative bacteria. The periplasmic intermediates need to be folded correctly in the periplasmic space in order to be recognized by the secretion machinery (Filloux, 2011). The export of the exoproteins requires the assembly of the pseudopilus, that pumps them through the OM channel, formed by oligomerized secretins (Filloux *et al.*, 1998, Douzi *et al.*, 2009, Douzi *et al.*, 2011).

P. aeruginosa PAO1 has two complete T2SS. The Xcp (for extracellular protein) system, that is expressed constitutively, and the Hcx system, expressed only in phophate limitation conditions (Ball *et al.*, 2002, Voulhoux *et al.*, 2001). Interestingly, a third putative T2SS is present in PA7 (Roy *et al.*, 2010). The Xcp machinery has several known substrates, including LasA and LasB, LipA and phospholipases C (for a complete updated list see (Filloux, 2011). In

contrast, there is only one known protein exported by the Hcx system, the alkaline phosphatase LapA (Ball *et al.*, 2002, Filloux, 2011).

(iii) T3SS

T3SSs are complex membrane-spanning proteic complexes that are used by a wide range of bacteria in order to inject toxins directly from the bacterium's cytoplasm into the cell's cytosol. In the case of *P. aeruginosa*, the T3SS machinery is associated with acute infections and is activated upon contact with eukaryotic cells.

This multi-protein structure is composed of at least twenty proteins, that form a membrane-spanning channel that is called the basal body, an extracellular needle and three proteins at the distal tip of the needle that form the translocon. The translocon inserts into the host's membrane and forms a pore (Mattei *et al.*, 2011).

Four substrates required for toxin injection are exported through the T3SS of *P. aeruginosa* once the translocon is in place: ExoS, T, Y and U. ExoS and ExoT have a GTPase activating protein (GAP) domain and an ADP-ribosyl transferase domain (ADPRT). ExoY has an adenylate cyclase activity and finally, ExoU is the most potent cytotoxin, that has a phospholipase activity (Engel & Balachandran, 2009). Interestingly, most *P. aeruginosa* strains only have three T3-toxins at a time encoded in their genome. Indeed, ExoS and ExoU are mutually exclusive and only co-occur rarely in the same genome (Berthelot *et al.*, 2005, Kulasekara *et al.*, 2006).

(iv) T5SS

The T5SSs are the most simple of all the secretion systems, because the information needed for the transport is all contained in either one or two polypeptides (Filloux, 2011).

In the case of the T5aSS or autotransporter system (named as such since there are no need for additional proteins), the primary sequence of the secreted proteins is conserved. The N-ter carries the signal sequence for translocation via the Sec general mechanism. The C-ter, also referred to as the transporter domain, on the contrary, forms a β-barrel structure that will be inserted in the OM. In between these two domains, there is a passenger domain, the

functional domain that has been proposed to be exported out of the bacterium through the β-barrel structure (Filloux, 2011). Once in the bacterial surface, the passenger domains can be modified and stay associated to the β-barrel through non-covalent interactions or it can be processed and released to the extracellular milieu (Henderson *et al.*, 2004).

There are three T5aSS in *P. aeruginosa*. Only one of them remains to be characterized, PA0328, predicted to have metallopeptidase activity(Ma *et al.*, 2003, Filloux, 2011). EstA T5aSS has been functionally characterized as carrying lipolytic activity (Wilhelm *et al.*, 1999, Wilhelm *et al.*, 2007). EprS is a serine protease that activates host inflammatory responses (Kida *et al.*, 2013).

There is another pathway of the T5SS, called the T5bSS or the two-partner secretion pathway. In this case, the passenger domain (also called exoprotein) and the β-barrel domain are translated as separate peptides, TpsA and TpsB family members respectively (Henderson *et al.*, 2004). Both proteins are translocated to the periplasm by the Sec machinery. Then, once TpsB is inserted in the OM, it recognizes a TCS sequence in TpsA and the transport begins.

P. aeruginosa PAO1 carries at least five putative T5bSSs. Of these, only two have been characterized: LepA/LepB (LepA bears proteolytic activity) and CdrA/CdrB (CdrA has c-diGMP activity and promotes biofilm formation). Finally, there is a TspA family protein that has no TspB partner, CupB5 (Ma *et al.*, 2003, Filloux, 2011).

(v) T6SS

The T6SS is the main object of study of this work and will be described in Chapter III.

Chapter II. Contact-dependent Bacterial-Bacterial Interactions

Bacteria rarely exist as mono-culture communities. They form complex mixed - sometimes highly organized - communities, such as biofilms, where individuals are in close contact. Contact-dependent interactions are widespread amongst bacteria and serve different purposes. They have been implicated both in cooperation and in competition between bacteria, as well as in cell-cell signalling and cell-cell exchange of molecules. The establishment of contact-dependent interactions has been shown to coordinate a bacterial population in a spatio-temporal manner, resulting in modulation of a coordinated and adapted response to a stimulus and/or an environmental condition, which leads to survival of a community in complex niches.

There is a growing list of contact-dependent processes amongst bacterial cells. These phenomena can be divided into cargo-delivering (for example T6SS and nanotubes) and cell-cell signalling and communication (such as IDS, CDI, C-signal transmission and alignment of FrzCD). This chapter focuses on the cell-cell contact mechanisms between bacteria and their implication on bacterial behaviour.

2.01 CONTACT-DEPENDENT INHIBITION

Contact–dependent inhibition of growth (CDI) systems are widely distributed throughout α-, β- and γ-proteobacteria, including uropathogenic *E. coli*, *Burkholderia pseudomallei* and possibly *Yersinia pestis* (Aoki *et al.*, 2010). The CDI mechanism is able to control bacterial metabolism and growth in a reversible fashion (Aoki *et al.*, 2009) and thus contribute to the competitive fitness and the coordination of growth of Gram-negative bacteria occupying the same ecological niche (Hayes *et al.*, 2010, Hayes & Low, 2009).

CDI was first described in 2005 by Aoki and colleagues, when they noticed that bacterial growth is regulated by direct cell-cell contact in *E. coli* isolate EC93 (Aoki *et al.*, 2005). For this to happen, the "inhibitor" cells had to be in logarithmic phase of growth and protein synthesis had to occur, whereas the growth phase of the target cells was of no importance, since target cells were inhibited at any growth phase. During this study, the authors also discovered that the supernatants of culture had no effect on the target's growth, implying that this phenomenon was not due to a secreted factor. The fact that growth inhibition is contact-dependent was assessed by separating the cells (inhibitor from target) by means of a membrane. In this case, growth inhibition was not observed.

This phenomenon is due to CdiA and CdiB, two proteins that share homology with two-partner secretion proteins. Bacteria do not inhibit their own growth because they also produce CdiI, which is encoded with *cdiA* and *cdiB* in the same operon and confers them with "immunity" (Aoki *et al.*, 2005). Importantly, CdiI appears to protect the bacterium only from its cognate CDI system, by binding to the polymorphic C-ter region of its cognate CdiA *in vitro* and *in vivo*. This interaction neutralizes the toxic enzymatic activity of CdiA (Aoki *et al.*, 2010, Nikolakakis *et al.*, 2012, Morse *et al.*, 2012). CdiB is a predicted OM β-barrel protein needed for the assembly and transport of CdiA on the cell surface (Choi & Bernstein, 2010, Mazar & Cotter, 2007). CdiA of *E. coli* EC93 isolate is a toxin with tRNase activity dependent on the biosynthetic enzyme CysK, its cofactor located in the cytoplasm of target cells. It has been demonstrated that these two proteins form a stable complex *in vitro*, which is needed for the activation of the enzymatic activity of CdiA both *in vitro* and *in vivo* (Diner *et al.*, 2012).

It was shown later on that BamA and AcrB mutants were more sensible to CDI than wild-type cells (Aoki *et al.*, 2008). Interestingly, the complex that BamA forms with BamB-E and SurA does not play a role in CDI, since mutants in these proteins do not suppress CDI. In this same study it was also demonstrated that BamA is the receptor for CDI, since when bacteria were cultured in the presence of anti-BamA antibodies, the binding between CDI^+ cells and the targets was blocked, thus blocking CDI phenomenon. AcrB is an IM component of a multidrug efflux pump. AcrB was proposed as the antiporter used as CdiA port of entry to the bacterium's cytoplasm. In the current model, CdiA binds to BamA and then is internalized by AcrB (Figure 7).

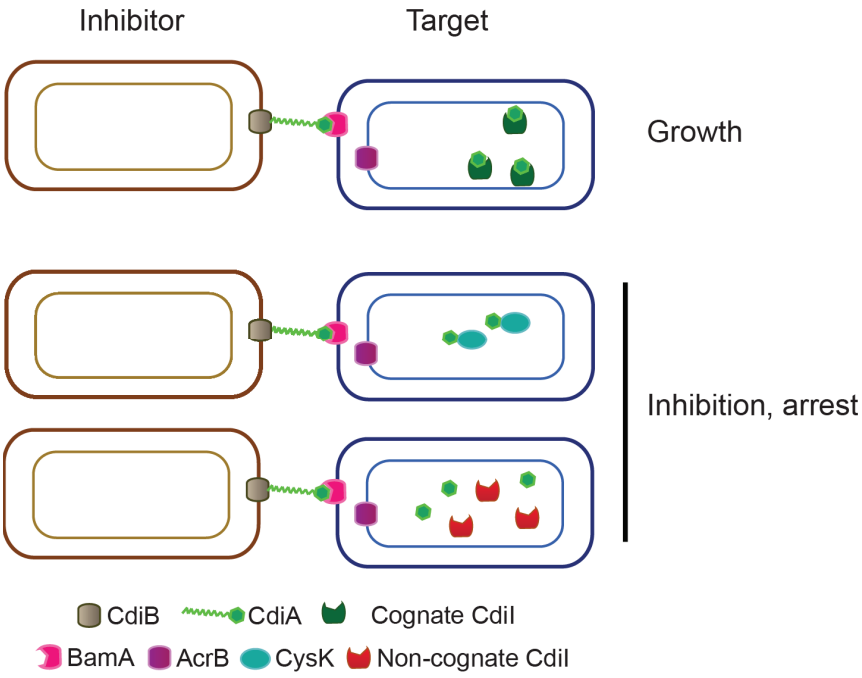

Figure 7 Proposed CDI mechanism in *E. coli*. Three different states that may take place when an inhibitor bacterium encounters a target. CdiA likely forms an extended filament on the cell surface and CdiB is important for its translocation across the OM. The interaction of the cell surface protein CdiA with target cells triggers growth inhibition. The molecular targets of characterized CDI systems include membranes, DNA and RNA (Aoki *et al.*, 2010, Aoki *et al.*, 2009), which is consistent with the distribution of toxin/immunity pairs in diverse bacteria (Aoki *et al.*, 2010, Poole *et al.*, 2011) and with CDI-inhibited cells being metabolically downregulated: significant reductions in respiration, proton-motive force and ATP levels have been reported (Aoki *et al.*, 2009). CdiA forms a complex with CysK when in the target cytoplasm (Diner *et al.*, 2012).

As mentioned above, CDI systems are widespread amongst Gram-negative bacteria. It has been shown that *E. coli*, *B. thailandensis*, *B. pseudomallei* and *Dickeya dadantii* 3937 have functional CDI systems, reminiscent of *E. coli* EC93 strain (Aoki *et al.*, 2008, Aoki *et al.*, 2005, Nikolakakis *et al.*, 2012, Anderson *et al.*, 2012). Moreover, *Biberstania trehalosi* and *Pasteruella multocida*, both Gram-negative bacteria responsible for severe bronchopneumonia in big-horn sheep, are able to inhibit the growth of *Mannheimia haemolytica*, the major cause of this disease, in a contact-dependent fashion (Dassanayake *et al.*, 2010, Bavananthasivam *et al.*, 2012). Finally, it was shown that *P. fluorescens* EPS62e can inhibit the growth of *Erwinia amylovora*, a plant pathogen (Cabrefiga *et al.*, 2007). Of note, the CDI mechanisms in these last examples have not been elucidated yet.

A study on *Burkholderia* species showed that they carry a distinct class of CDI system, which differs from the one found in *E. coli* EC93 isolate both in gene order and content. Of note, these loci were generally located in genomic islands. In particular, *B. thailandensis* expresses

its CDI system stochastically and requires it for aggregation of a solid surface and biofilm formation. Remarkably, in contrast to *E. coli* EC93 isolate CDI, *B. thailandensis* system only mediates inter-bacterial competition on a solid surface, revealing a new role for CDI systems (Anderson *et al.*, 2012).

Interestingly, there are some reports that show CDI phenomena when bacteria are in stationary phase of growth (SCDI, Lemonnier M., 2008, Murat *et al.*, 2008). Another example is calf-isolated *E. coli* strains that can inhibit the growth of *E. coli* K-12 strains lacking *cdiBAI* (Sawant *et al.*, 2011).

Why inhibit the growth of itself/other bacteria? CDI might be a wide spread mechanism used to control the overall growth of a bacterial (mixed or not) population: for example, it could limit the number of bacteria within a tissue and allow the population to be masked and therefore attenuate the host inflammatory responses. Also, it could be used to compete out bacteria in the same ecological niche, if nutritional resources are scarce. Interestingly, *E. coli* EC93 isolate was the predominant *E. coli* strain from fecal pellets of a commercial rat colony. In this strain, the *cdi* locus is constitutively expressed, explaining maybe why it was the predominant strain and a gained competitive advantage.

2.02 ALLOLYSIS

Allolysis, also called fratricide, is the phenomenon in which siblings kill each other. Allolysis is similar to autolysis, but not identical, since it is induced by other cells of the same (Guiral *et al.*, 2005) or closely related (Kreth *et al.*, 2005) species as a response to stress.

The best characterized allolysis is that occuring in *Streptococcus pneumoniae* (Figure 8). *S. pneumoniae* can exist as a mixed population when, as a consequence of environmental cues, cells differentiate either into competent or into non competent cells (reviewed in (Claverys & Havarstein, 2007). All cells produce lytic factors, such as LytA (an N-acetylmuramoyl-L-alanine amidase), LytC (a lysozyme), CbpD (for *C*holine *B*inding *P*rotein, also a cell wall hydrolase) but only competent cells synthesize CibAB (Garcia *et al.*, 1986, Garcia *et al.*, 1999, Kausmally *et al.*, 2005, Guiral *et al.*, 2005, Eldholm *et al.*, 2010). CibAB is thought to be the

trigger of fratricide, because *cibAB* are predicted to code for a two-peptide bacteriocin that could sensibilize the membrane of the non competent, sensitive cells (Guiral *et al.*, 2005). It has also been proposed that CbpD might be the trigger of allolysis, since it is absolutely needed for allolysis to occur (Eldholm *et al.*, 2010). Importantly, cell-cell contact is needed so that CibAB can access the membrane. Upon cell contact, incompetent, and thus defenseless cells, are lysed by their competent, killer sisters.

Allolysis can also occur between relatives, and it is thus called sobrinicide. It has been described that *S. pneumoniae* can attack *Streptococcus oralis* and *Streptococcus mitis*, both related bacteria that inhabit the same environment, in a CbpD-dependent manner (Johnsborg *et al.*, 2008). CbpD is sufficient to kill and lyse target cells during allolysis, however, its activity is strongly enhanced when LytA and LytC are present (Eldholm *et al.*, 2009). Moreover, killing is more efficient when the two autolysins are provided by the target cell.

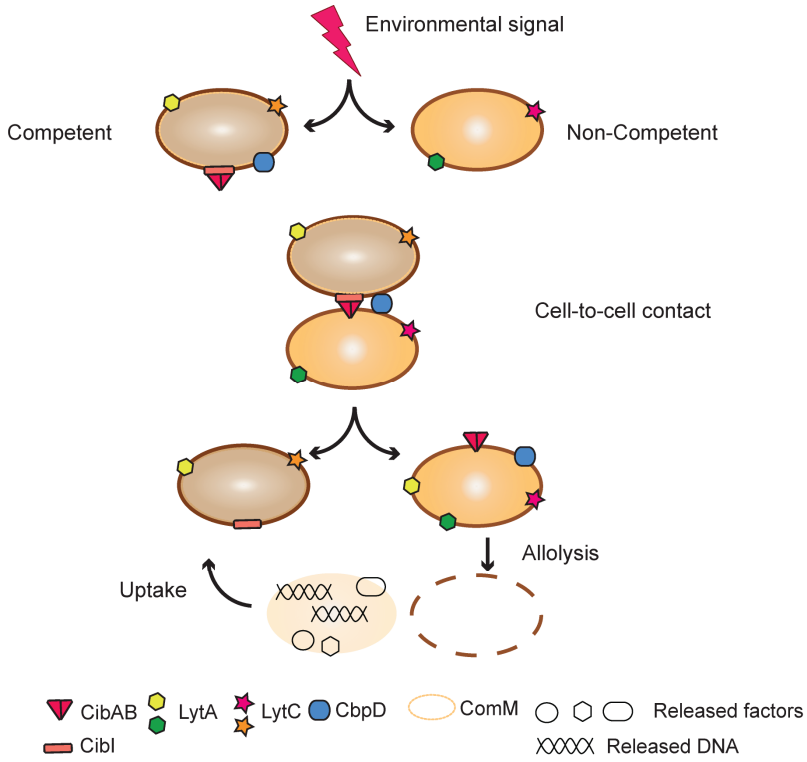

Figure 8 Fratricide in *S. pneumoniae*. Adapted from Claverys & Havarstein (2007). Only competent cells can accumulate CibABI and CbpD on the surface. Killing cells activate the hydrolases of the non killing siblings upon cell-cell contact. CibI and ComM confer competent cells with immunity. It has been proposed that LytC directly or indirectly interacts with CbpD, which activates LytC. In a second time, CbpD triggers the lytic activity of LytA, that cannot act on living cells but only in lysed cells (Eldholm *et al.*, 2009).

Why do competent cells only attack non competent ones? Competent cells synthesize protecting factors that confer immunity, and thus prevent their own lysis. These factors are two predicted membrane-anchored proteins, CibC (Guiral *et al.*, 2005) and ComM (Havarstein *et al.*, 2006). The mechanisms underlying the protection remain unknown.

As a result of cell lysis, there is a release of cell material to the extracellular milieu. It has been proposed that allolysis can be used by competent cells to uptake the DNA of their siblings, favouring genetic exchange. Moreover, killing cells can profit from the nutrient release. Finally, Claverys and colleagues suggested that allolysis could be a way in which bacteria enhance their virulence by the simultaneous liberation of virulence factors produced by the different pneumococcal strains (Guiral *et al.*, 2005). Fratricide in *Enterococcus faecalis* is responsible for governing extracellular DNA release and biofilm development (Thomas *et al.*, 2009) and is regulated by proteases release. Allolysis has not been reported in Gram-negative bacteria, even though spatio-temporal cell death was documented within *P. aeruginosa* biofilms (Webb *et al.*, 2003). It appears that cell death is induced by the release of a bacteriophage that exists as a prophage by part of the population. Cell lysis results in the release of cellular constituents, including DNA, important component of the biofilm matrix (Webb *et al.*, 2003, van Schaik *et al.*, 2005).

Of note, another allolysis process, referred to as cannibalism and first described in *B. subtilis*, will not be presented in this section, since cell-cell contact is not necessary (Gonzalez-Pastor *et al.*, 2003). This is also the case of toxin-antitoxin systems, that some authors consider as part of allolysis processes.

2.03 IDENTIFICATION OF SELF

Proteus mirabilis is a swarming Gram-negative bacterium that forms different colony boundaries when different strains growing on agar encounter each other and do not merge. These boundaries are called the “Dienes line” (Dienes, 1947). Close cell proximity is required for the Dienes line formation, since swarming *P. mirabilis* cells cannot form the Dienes line when separated by a membrane (Budding *et al.*, 2009). Moreover, when a colony of *P.*

mirabilis was challenged with the extracellular content of a swarm of another *P. mirabilis* strain, the Dienes line was not formed, further supporting the idea that factors involved in this phenomenon are not diffusible (Senior, 1977). However, the presence of molecule(s) left behind in the slime trails deposited by cell moving at colony edges (identified because they are visible traits) cannot be ruled out (Gibbs & Greenberg, 2011).

How is it possible that *P. mirabilis* recognizes itself from others? It has recently been described that *P. mirabilis* cells can discriminate themselves thanks to the products of the *idsABCDE* operon (*ids* for *Id*entification of *S*elf, (Gibbs *et al.*, 2011, Gibbs *et al.*, 2008). No biochemical studies have been pursued up to date, hence little is known about the *idsABCDE* products. However, it as been established that IdsA and IdsB share homologies with Hcp and VgrG, respectively. Hcp and VgrG are essential components of the T6SS (see Chapter III). IdsC, IdsD and IdsE, on the contrary, share no homology with known proteins, and they are thought to be involved in strain-specific recognition. Finally, *idsF* codes for a conserved hypothetical protein in bacteria (Gibbs *et al.*, 2008). Greenberg and colleagues have screened for *P. mirabilis* mutants that would form boundaries with their parents. Using swarming assays and assessing boundary formation, they were able to propose a model of self identification, in which i) *idsB*, *idsC*, *idsF* code for proteins needed in self versus non-self recognition and can be switched amongst strains, ii) *idsD* and *idsE* encode for molecular identifiers for self identity and are strain specific, iii) *idsA* is not essential for boundary formation but is encoded in the same operon as the rest of the *ids* genes.

Interestingly, *P. aeruginosa* presents a comparable process to IDS in *P. mirabilis*. This has become clear when, in an effort to develop an easy-to-perform and affordable test to distinguish between different *P. aeruginosa* strains, the authors decided to adapt the Dienes mutual inhibition test that is used for *P. mirabilis*. In this test, two strains are challenged against each other and the formation of the Dienes line is assessed. If the strains are closely related, there will be no evident formation of the Dienes line (Pfaller *et al.*, 2000). On the contrary, if the strains are genetically dissimilar, there will be boundary formation at the intersection of the swarming colonies on an agar plate. 15 isolates of *P. aeruginosa* were tested and the results were in accordance with the ribotyping studies (Munson *et al.*, 2002). The genetic basis of this process is *P. aeruginosa* is still to be explored and the T6SS influence on this phenomenon is still to be assessed.

2.04 PROTEIN AND LIPID TRANSFER

Myxococcus xantus is a Gram-negative bacterium that inhabits soil environments. It possesses two polar engines, called the A – for adventurous – and the S – for social –, that allow it to glide in the soil surface (Hodgkin & Kaiser, 1979). The A engine mechanism of action is less known, but the S engine is known to resemble a type IV pili that needs Tgl, an OM lipoprotein, for its assembly (Kaiser, 1979, Nudleman *et al.*, 2006). In an effort to elucidate the gliding mechanism of *M. xantus*, (Hodgkin & Kaiser, 1977) isolated non-motile mutants and discovered that they could recover their motility when put in contact with wild-type cells. It is now known that this is due to the shuttle of OM lipoproteins, OM lipids and OM-anchored proteins between adjacent *M. xantus* cells (Nudleman *et al.*, 2005, Wei *et al.*, 2011, Pathak *et al.*, 2012). Importantly, cytoplasmic and periplasmic proteins have never been reported as transferred between cells, nor have been IM lipoproteins (Wei *et al.*, 2011).

Interestingly, protein transfer amongst *M. xantus* is kin-dependent, because at least two proteins, TraA and TraB (both predicted to be localized to the OM) are necessary for transfer both in donor and recipient cells. Also, this suggests that transfer might be bi-directional, which makes this process different from known secretion and conjugative systems already described (Pathak *et al.*, 2012). Protein exchange assays using fluorescent protein mCherry revealed that fluorescence intensity was not the same in every bacterium, since bright donors could be depicted next to dim recipiens. This implies that protein transfer might be regulated, as if this process were constitutive, all bacteria (this is, donors and recipients) would appear equally fluorescent (Wei *et al.*, 2011). A working model of protein transfer is depicted in Figure 9.

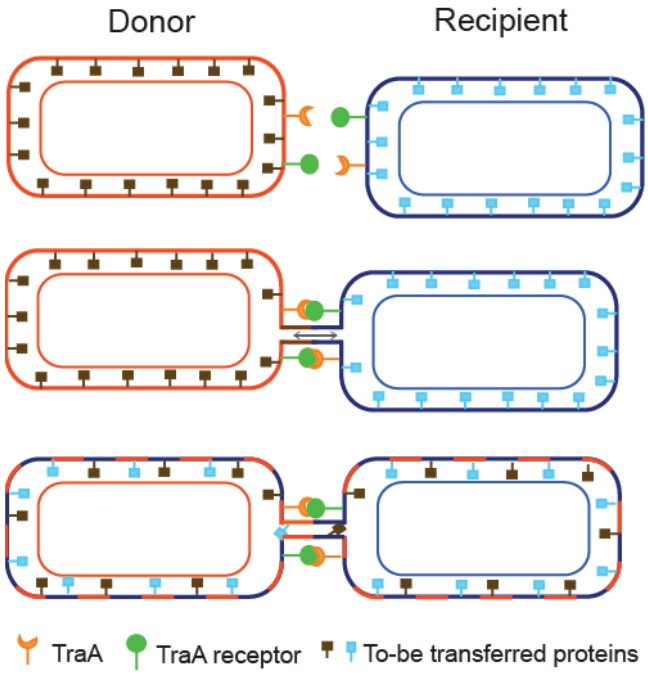

Figure 9 Working model of protein transfer in *M. xantus*. Bacteria are able to recognize each other by means of TraA. When there is cell- cell contact, OM of donor and recipient cells are fused, thus transfer of proteins can occur. Importantly, OM lipids are also exchanged (represented as dashed OM in both bacteria). TraA is thought to be the sensor protein because it possesses a domain for lectine recognition. TraB, a predicted OM-associated protein, is not designed here, but it has been shown as necessary for transfer (Pathak *et al.*, 2012). How bacteria separate once the shuttle is finished is still unknown.

Why do bacteria exchange lipids and OMPs? It has been proposed that this process can result in a gain of fitness, since a non-motile bacterium can become motile, maybe due to the recovery of Tgl OM lipoprotein, essential for S-driven motility. Moreover, it might work as spatial cues to co-ordinate cell behaviours and share resources in a community –which will reduce population heterogeneity -, thus it will have a role in signalling as well.

Interestingly, *M. xantus* is not the only bacterium where membrane fusion and protein exchange has been reported. *Borrelia* spp. have been shown to be able to fuse their OM, even though there is no evidence of material exchange between sisters yet (Kudryashev *et al.*, 2011). Also, bridging between neighbouring cells (formation of nanotubes, see further) involves membrane fusion (Dubey & Ben-Yehuda, 2011).

2.05 C-SIGNAL TRANSMISSION AND ALIGNMENT OF FRZCD

As mentioned above, *M. xantus* is a Gram-negative bacterium, able to glide and swarm when growing on a solid surface. This bacterium has a complex life cell cycle. In the presence of nutrients, the so-called vegetative cells are motile, and they grow and divide. Upon starvation, cells at a high density and on a solid surface trigger a developmental program that culminates in non-motile, aggregated cells, that form spore-filled fruiting bodies (for a review Kaiser, 2004). The morphogenetic changes are spatially and temporally coordinated by the cell-cell contact dependent on the C-signalling program.

The C-signal is the product of *csgA.* CsgA can exist in two surface-exposed forms (Shimkets & Rafiee, 1990), a 25kDa peptide (p25) and a 17kDa peptide corresponding to the C-ter of p25 (p17). Both are localized to the OM of *M. xanthus*, but only p17 is considered as the C-signal (Kim & Kaiser, 1990b, Kim & Kaiser, 1990c, Lobedanz & Sogaard-Andersen, 2003). p25 cleavage is dependent on PopC, a protease that is accumulated in the cytoplasm of vegetative cells (Rolbetzki *et al.*, 2008). PopC forms a cytoplasmic complex with PopD, a soluble protein that shares features with T3SS effector chaperones (Konovalova *et al.*, 2012). Upon starvation, RelA induces degradation of PopD, releasing PopC and allowing its secretion to the extracellular milieu. PopC then cleaves p25, causing p17 accumulation. This suggests that RelA acts as the sensor of starvation. A model of C-signalling is shown in Figure 10.

Of note, it has been demonstrated that PopC is slowly secreted but rapidly degraded once in the extracellular milieu of starving cells. Hence, it has been proposed that PopC acts *in cis* and ensures the slow accumulation of p17 to the surface of the cell.

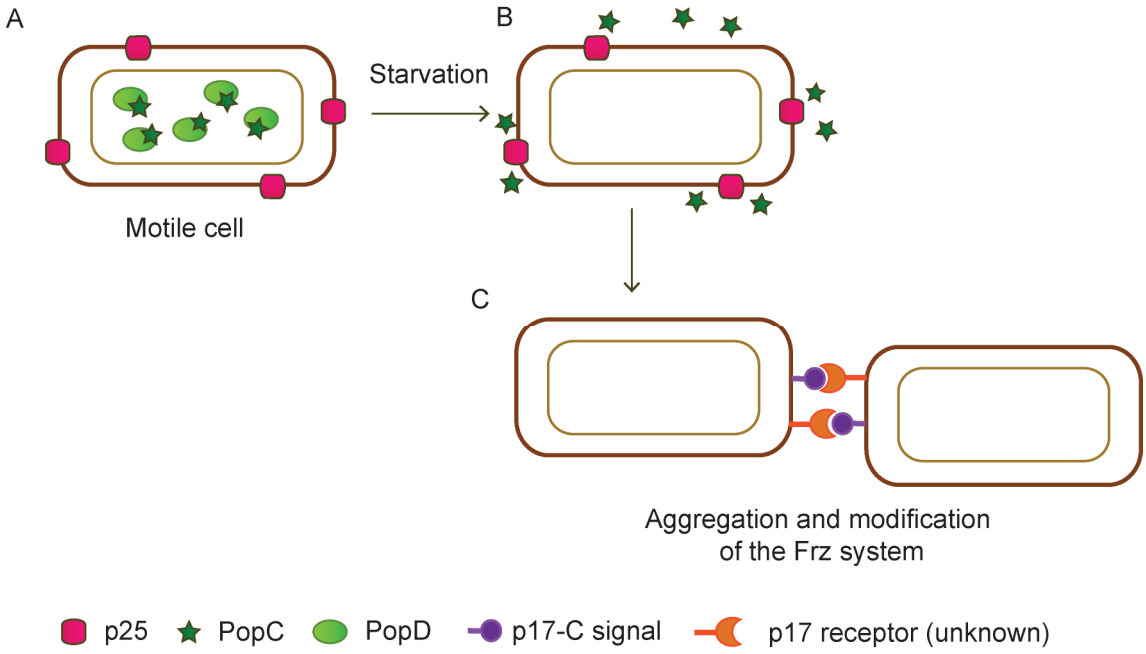

Figure 10 Model of C-signalling in *M. xanthus*. Bacteria are represented as rectangles for simplification. A) PopB-C form a complex in the cytoplasm and p25 is present in the OM of the bacterium. B) Upon starvation, PopD is degraded and PopC is exported outside the cell in a RelA-dependent manner. C) p25 is cleaved probably *in cis* by PopC, generating p17, also referred to as the C signal. Now, recognition of other starving cells is possible, thanks to the p17 receptor, still unknown.

As mentioned above, p17 is anchored to the OM and in consequence not freely diffusible. This further supports the model of contact-dependent C-signal transmission between *M. xanthus* cells. How do *M. xanthus* cells recognize each other? This question remains to be answered, since the p17 receptor has not been identified yet.

C-signal transmission depends upon cell motility and alignment, since transmission is only possible when a pair of cells makes end-to-end contact with each other, meaning that they are both motile (Kim & Kaiser, 1990a, Kim & Kaiser, 1990b, Kroos *et al.*, 1988). Interestingly, when cells are forced to align in an end-to-end pattern motility requirement is bypassed, suggesting that the C-signal transmission event is geometrically constrained to the cell ends, further supporting the cell-cell contact necessity for the signalling. C-signalling changes specific motility parameters that promote aggregation and formation of fruiting bodies (Jelsbak & Sogaard-Andersen, 2002, Jelsbak & Sogaard-Andersen, 2003). Moreover, this phenomenon induces chemical modification of the so called “frizzy” (Frz) transduction system, that also affects motility (Sogaard-Andersen & Kaiser, 1996).

The Frz system is homologue to the *E. coli* chemosensory system called Che (Bustamante *et al.*, 2004), and it regulates and coordinates the A- and S-motility systems of the bacterium, more precisely reversal frequency of the cells (for a review see Zusman *et al.*, 2007). FrzCD has been described as a cytoplasmic protein (Bustamante *et al.*, 2004) and how this protein senses the extracellular stimuli is still an open question. In order to further characterize FrzCD, (Mauriello *et al.*, 2009) have used immunofluorescence (IF) assays and time-lapse video microscopy to monitor its localisation in living cells. IF assays revealed that FrzCD forms helical filaments that span the cell length and that possibly co-localizes with MreB, an actin homologue. Furthermore, this novel localisation pattern was not dependent of the others actors of the Frz pathway (FrzA, B and E), another unusual feature for a chemoreceptor. FrzCD highly dynamic clusters were dependent on the ability of cells to reverse their direction and on the side-to-side contacts between *M. xanthus* cells. This last observation suggests that alignments might be somehow involved in the timing of cell reversals.

2.06 TYPE IV SECRETION SYSTEM

Type IV secretion systems (T4SS) are multiproteic complexes that can translocate DNA and proteins across the bacterial envelope. They are considered as key mechanisms in horizontal gene transfer in bacteria, since they promote the widespread transfer of antibiotic resistance, metabolic functions and virulence determinants. Also, T4SSs can deliver toxic effectors directly into eukaryotic cells (Backert & Meyer, 2006). In order to translocate substrates, cell-cell contact is not always a requisite: *Neisseria gonorrhoeae* can secrete DNA to the extracellular milieu and *Helicobacter pylori* can uptake DNA from the extracellular milieu (for reviews Cascales & Christie, 2003, Alvarez-Martinez & Christie, 2009). In this section, only the T4SSs that mediate bacterial cell-cell contact will be described.

T4SSs are cell-envelope-spanning multiproteic structures that are composed of three different sub-structures: i) an IM complex, that identifies the T4 substrates and allows their entrance to the channel, ii) a secretion channel, iii) an external pilus that contains proteins

that might act as adhesins and that mediate cell contacts (Alvarez-Martinez & Christie, 2009, Hayes *et al.*, 2010).

Cell-cell contact between mating cells is a crucial step in conjugation and DNA transfer. This contact is mediated by the F-pilus of *E. coli*. The F-pilus is the pilus formed by the T4SS that is encoded by the F plasmid, and is a homopolymeric tube-like structure extruding from the inside of the cell. By means of time-lapse video microscopy, Babic and colleagues (Babic *et al.*, 2008) were able to show that DNA transfer occurs through the F pilus *in vivo*.

It has been suggested that the F-pilus could serve to bring cells into contact (observed by electron microscopy by (Achtman *et al.*, 1978). More precisely, the tip of the pilus would be involved in cell-cell recognition, since conjugation takes place only when the tip of F-pilus makes contact with recipient cells. The F-pilus undergoes cycles of extension and retraction, which allows the cell to sense the eventual presence of a recipient cell in a liquid milieu (Jacobson, 1972, Sowa *et al.*, 1983, Clarke *et al.*, 2008). In particular, retraction of the F pilus might bring the cells together once the extended pilus established contact with a recipient cell (Clarke *et al.*, 2008). Then, stabilized mating pairs form mating junctions, and DNA can be transferred (Durrenberger *et al.*, 1991). Importantly, the F-pilus is long (2 to 20 µm) and is very flexible, reason why extension/retraction cycles are possible. However, other conjugative pili such as the T-pili of *Agrobacterium tumefaciens* are not as resistant, and the mechanisms by which they mediate DNA transfer are still unknown. Interestingly, there have been studies that show that non-pilus forming mutants can still efficiently transfer DNA, indicating that the assembly of the conjugative pili might not be required for this process and that a bifurcation within the assembly can exist (Christie *et al.*, 2005).

How does this complex mediate cell-cell contact? A lot of effort has been made in the identification of specific receptors for conjugative pili. It cannot be ruled out that only physical, rather than biochemical, properties mediate recognition. The promiscuous nature of adhesion, important for biofilm formation, probably underlies broad-host range conjugative DNA transfer. Conjugative pili have affinity for LPS, even though LPS-deficient *E. coli* mutants can still act as recipients during conjugation (Anthony *et al.*, 1994, Perez-Mendoza & de la Cruz, 2009). Also, Gram-positive conjugative pili can bind lipoteichoic acids (Clewell, 2007).

Pili are composed of a pilin (VirB2 in *A. tumefaciens*) subunit and a minor pilin attached to the tip of the pilus (VirB5 in *A. tumefaciens*, (Aly & Baron, 2007). The minor pilin has been suggested to function as an adhesin (Schmidt-Eisenlohr *et al.*, 1999), which supports the finding of the minor pilin being situated at the distal extremity of the pilus, and the pilus being involved in recipient cell contact-establishment and recognition.

TraC, the VirB5 homologue encoded by the conjugative plasmid pKM101, has seen its structure resolved in 2003 (Yeo *et al.*, 2003). This study showed that VirB5 is implicated in protein-protein interactions, and discovered that mutated variants of VirB5 that can still be incorporated in the pilus have a dramatic effect on conjugative transfer. Interestingly, both decrease and increase of conjugative transfer have been observed. It has then been proposed by the authors that VirB5 plays a role in bacterial phage attachment. How VirB5 mediates cell recognition remains unanswered.

2.07 NANOTUBES

Recently, direct cell-cell bridges independent of the T4SS and T6SS have been discovered. These so-called nanotubes were first observed in *Bacillus subtilis* grown on a solid surface, by means of high-resolution scanning electron microscopy (Dubey & Ben-Yehuda, 2011). They appear as membrane protrusions when being formed. Nanotubes can connect adjacent cells and have various sizes, and can even be located in different parts of the cell. Moreover, it has been shown by immuno-electron microscopy that cytoplasmic molecules can be transferred through these inter-bacterial bridges, indicating an active transport. The molecular mechanism by which this phenomenon occurs is still unknown, even though it seems that the speed of transfer is inversely proportional to the size of the molecule, based on the two examples studied, GFP and calcein (Dubey & Ben-Yehuda, 2011). Molecules that can be transferred through these tubular extensions include small cytoplasmic proteins, non-conjugative plasmids (the transfer of plasmids did not need any intrinsic plasmid elements) and small molecules. Importantly, there is only transfer of non-heritable traits, and it has been suggested that both cells can act as donor and as recipient (Figure 11).

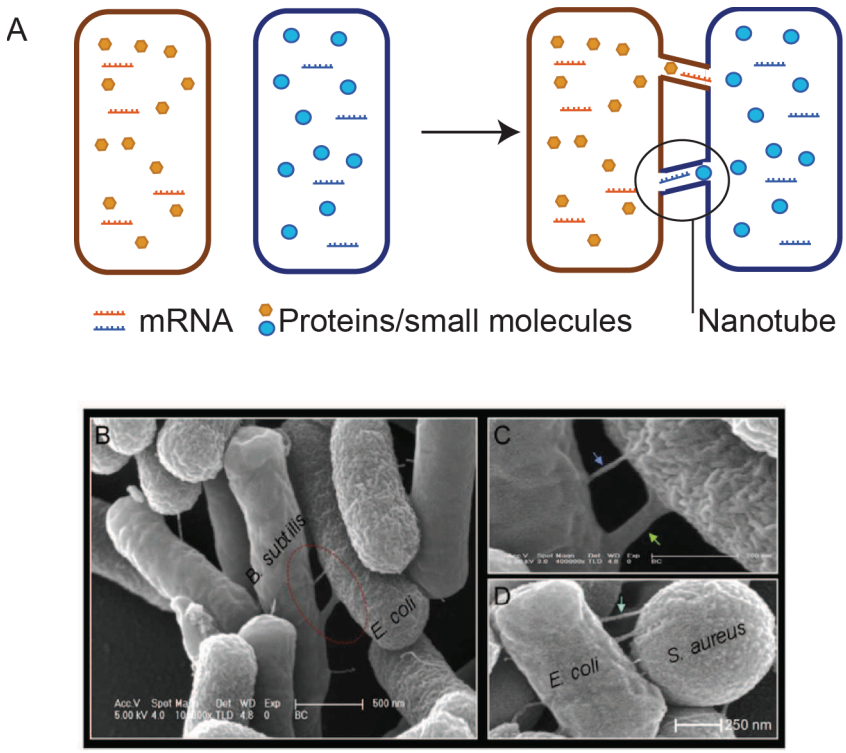

Figure 11 Nanotubes in bacteria, adapted from Dubey & Ben-Yehuda (2011). A) Representation of a model for nanotube-dependent transfer between adjacent cells. For simplicity, cells are represented as rectangles. B) Nanotubes can be observed by high-resolution scanning electron microscopy when cells are grown on a solid surface. In this case, nanotubes between Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*) are shown. C) Close-up of the zone delimited in red in B). D) Nanotubes formed between adjacent *E. coli* and *S. aureus*. Arrows indicate nanotubes. B, C and D) were reproduced from Dubey & Ben-Yehuda (2011).

Nanotube-bridging of cells is not *B. subtilis* exclusive. In fact, this phenomenon has been observed between Gram-positive and Gram-negative bacteria (Figure 11), suggesting that it is ubiquitous (Dubey & Ben-Yehuda, 2011). Generally, tube length ranged up to 1µm, whereas width ranged approximately from 30 to 100nm. Smaller tubes tended to be clustered connecting nearby cells intimately, apprearing to “stich” one cell to another.

Why are nanotubes synthesized? Which is the triggering signal for nanotube synthesis? What is their structure? How does the bridging between cells occur? It is known up-to-date that nanotubes are SDS-sensible, and based on transmission electron miscroscopy (TEM) observations, Dubey and Ben-Yehuda suggested that these inter-bacterial bridges between neighbouring cells are multilayered structures that contain cell wall, membrane and cytoplasmic material. Even though there is no knowledge concerning the regulation and

triggering of nanotube synthesis (Dubey & Ben-Yehuda, 2011), it is to be noted that they share similarity with OMVs, which frequently emerge from the cell wall of Gram-positive and Gram-negative bacteria. For instance, biogenesis of OMVs in Gram-negative bacteria is not random blebbing of the OM but in fact occurs at specific cell surface points (Hoekstra *et al.*, 1976) and OMVs can adhere to and fuse with cell wall of other bacteria. Dongre and collaborators (Dongre *et al.*, 2011) discuss that the initiation of nanotubes might require a similar framework to that of OMVs, protruding at specific sites on the cell at specific time-points and as a consequence of curvature-inducing molecules in the periplasm.

2.08 TYPE VI SECRETION SYSTEM

The type VI secretion system (T6SS) is a contact-dependent cargo-delivery system that has been discovered recently. The T6SS is the central object of study of this Thesis and will be described later in Chapter III.

2.09 OTHER EXAMPLES OF CONTACT-DEPENDENT INTERACTIONS

The examples of contact-dependent interactions among bacteria described above are not the only ones that have been studied. There are other processes that depend on the direct contact between bacteria in order to establish cooperation or competition relationships.

Nanowires are extracellular pili-like structures that have been identified in several metal-reducing bacteria. These structures are conductive and can transfer electrons from donor cells to acceptors (such as Mn(VI) and Fe(III)) without the need of shuttle molecules, El-Naggar *et al.*, 2008, Gorby *et al.*, 2006, Reguera *et al.*, 2005). The first verification of electrical transport through these filamentous appendages was done recently by means of homemade electrodes (El-Naggar *et al.*, 2010). Interestingly, *Geobacter sulfurreducens,* a nanowire-producing bacterium, can form biofilms when grown on a solid surface, such as an

anode. It has been demonstrated that electron transfer can happen from the cells that are far away from the surface, implying that nanowires traverse the multicellular structure (Reguera *et al.*, 2006) and that bacteria do not need to be in contact with the anode in order to contribute to current production (as is the case case in microbial fuel cells).

During symbiosis, two or more species establish a persistent long-term relationship through intimate interactions. This is the case of green sulfur bacteria and *Betaproteobacteria*. These associations can be highly structured, and are called consortia. In consortia, bacteria depend metabolically on each other, and it has been shown that some species cannot occur in pure cultures (Overmann & Schubert, 2002). In order to elucidate the basis for the close cell-cell interaction in phototrophic consortia, Wanner and colleagues (Wanner *et al.*, 2008) used SEM, TEM and 3D-image reconstruction. They discovered that there are subcellular structures exclusively present in symbiotic bacteria: 150-nm-long hair-like filaments that interconnect neighbouring cells. These appendages seem to be formed of OM and are thought to enlarge the surface area and facilitate the putative transfer of molecules between partners. However, free diffusion of molecules through these channels using an approach similar to those described for nanotubes (Dubey & Ben-Yehuda, 2011) has not been confirmed yet (Muller & Overmann, 2011).

Coaggregation is a widespread phenomenon between bacteria that has mainly been studied in multi-species biofilms of the oral cavity, but its role in complex niches is not completely understood (for reviews Kolenbrander, 2000, Rickard *et al.*, 2003a). It has been suggested that coaggregation can protect cells from stress and shear (Rickard *et al.*, 2003a, Rickard *et al.*, 2003b). Also, mutualistic relationships can be established, as is the case of *S. oralis* and *Actinomyces naeslundii* (Palmer *et al.*, 2001). Interestingly, coaggregation seems to be a specific process between bacteria, since the surface-exposed coaggregation adhesins and their cognate receptors expressed by bacteria have different features. Moreover, a sequence of colonization has been proposed for dental plaque, further implying that there is a temporal coordination in plaque formation (Kolenbrander, 2000). Notably, *S. oralis* and *S. gordonii* form part of the primary colonizers of the tooth surface and coaggregate with each other, but not with late colonizers such as *Treponema* spp or *H. pylori*, that coaggregate with a bridge organism, capable of interacting with primary and late colonizers. In consequence, if the bridging organism is not present, late colonizers will not be able to form part of the

dental plaque (Kolenbrander, 2000). Coaggregation adhesins have been identified on the surface of cells, either anchored to the cell wall of Gram-positive bacteria forming fibrillar structures either associated with external appendages. The receptors of these adhesins are less well studied and little is known about their structure and localisation (Kmet *et al.*, 1995, McNab *et al.*, 1999, McNab *et al.*, 1996, Egland *et al.*, 2001).

Examples in which coaggregation between bacteria does not involve biofilm formation can be cited. Thermophilic *Pelotomaculum thermopropionicum* has been shown to aggregate with *Methanothermobacter thermautotrophicus* when grown on a syntrophic coculture, possibly by means of flagellum-like structures (Shimoyama *et al.*, 2009). In these conditions, H_2 flux exists between bacteria (Ishii *et al.*, 2006, Ishii *et al.*, 2005). It has even been shown that there is an effect of the bacterial distance on the flux of electron carriers: when bacteria form aggregates in wheat reactors, the flux is a hundred times bigger than when bacteria are in suspension (de Bok *et al.*, 2004).

BspA is a secreted and cell-surface associated protein indispensible for the coaggregation between periodontal *Tannerella forsythia* and other bacteria when forming synergistic biofilms in mixed cultures (Ikegami *et al.*, 2004, Sharma *et al.*, 2005). Moreover, it has been demonstrated that the transcription of *bspA* gene is downregulated when *T. forsythia* forms biofilms, and the effect is more drastic when the bacterium forms mixed biofilms with other bacteria of the oral cavity. The regulation of *bspA* gene is the result of cell-cell interactions and more precisely, of contact-dependent cell-cell interactions (Inagaki *et al.*, 2005). The regulation of genes by contact-dependent interactions between bacteria has also been shown in *Porphyromonas gingivalis* (Xie *et al.*, 2000).

2.10 Implications of Contact-Dependent Interactions Among Bacteria

The objective of this chapter was to introduce the reader to different ways by which bacteria can communicate with each other, in an intra- or inter- species fashion and in a cell-cell contact-dependent manner. As described above, the interactions between bacteria are not

always beneficial, since cooperation can meet competition. One thing is clear: bacterial populations employ contact-dependent phenomena in order to coordinate their multi-cellular, community behaviours in a tempo-spatial manner. For example, aggregation upon starvation in *M. xanthus* has no sense if bacteria are not close enough to each other. Moreover, bacteria need to answer the question: is my immediate neighbourneighbour my kin or not? Contact-dependent signalling (IDS) seems an efficient approach to do this, since it allows *P. mirabilis* to recognize partners.

Contact-dependent interactions confer bacteria with the ability to transduce positional, spatial information, something that is not achieved during QS signalling, that relies on diffusible factors. Insight on local cell densities amongst a population is gained, in particular, the presence of competitors. Moreover, there are different type of signals that can trigger contact-dependent mechanisms, such as stress in the case of allolysis, starvation for C-signalling and protein-protein interactions for conjugation and possibly also for protein transfer in *M. xanthus*. Bacteria have developed a remarkable variety of inter- and intra-species contact-dependent communication systems, including the T6SS, that has proven to be a straightforward immediate transfer of information that can cross the inherent species barrier.

Chapter III. The Type VI Secretion System

The T6SS is exclusively found in Gram-negative bacteria and is widespread amongst them. It is an envelope-embeded complex that Gram-negative bacteria use to inject toxins and/or effectors directly into eukaryotic and/or prokaryotic cells in a contact-dependent manner (Figure 12). T6SS are mosaic structures, composed of elements reminiscent of a membrane-anchoring complex of T4SS and bacteriophage-like tail tube and sheath structures that share homology with the T4 phage tail.

The main purpose of a T6SS is killing of a target cell, but it can also be implicated in signalization. In consequence, the T6SS constitutes an original multiproteic membrane-spanning complex that allows Gram-negative bacteria to communicate with other cells in many contexts and with diverse outputs.

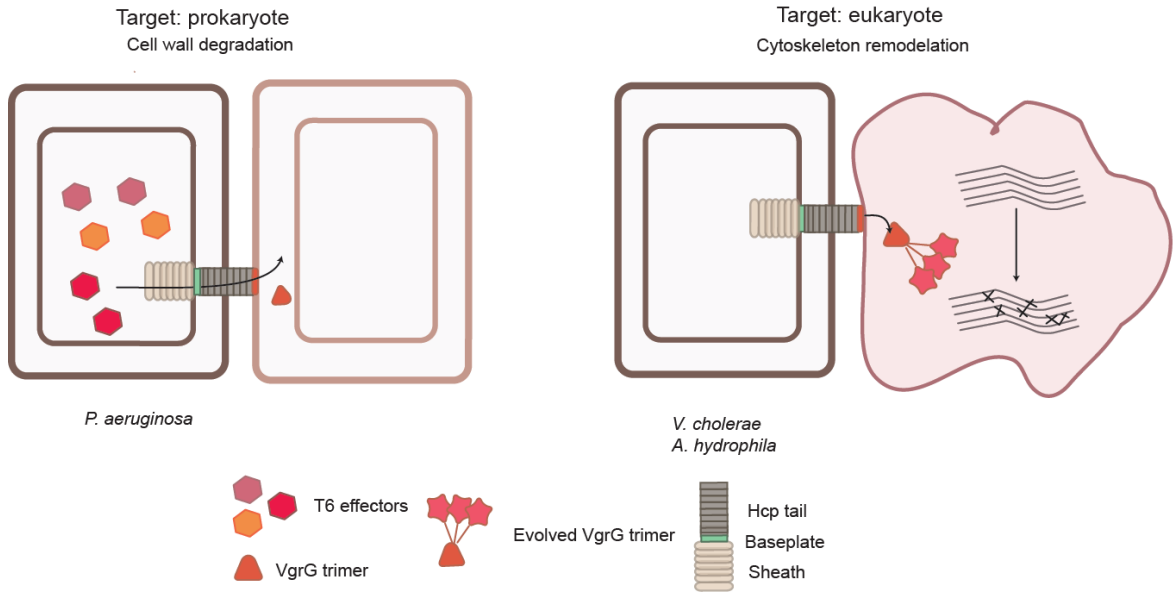

Figure 12 T6SS can target both prokaryotic (left) and eukaryotic (right) cells. Most evolved VgrG trimers act upon injection into eukaryotic cell cytosol by remodeling the actin cytoskeleton.

3.01 DISTRIBUTION IN THE MICROBIAL WORLD

The first *in silico* analyses were undertaken in 2003 (Das & Chaudhuri, 2003), where *icmF* was identified as a protein involved in *V. cholerae* virulence. Also, *icmF* gene homologues were identified in many bacteria, sometimes in multiple copies. Moreover, the authors identified a conserved gene cluster surrounding *icmF*.

It is now known that T6SS are widespread amongst Gram-negative bacteria. (Boyer *et al.*, 2009) undertook the first large-scale genome screening in order to elucidate the phylogenetic distribution, gene content, organization and evolution of the T6SSs. In all, more than 500 genomes were screened, which included all the complete genome sequences at that time (September 2007). Using as bait sixteen conserved T6-genes, they were able to identify 176 loci from 92 different bacteria containing at least five bait genes. These results were in accordance with another study that determined putative T6SS-encoding gene clusters in over one fourth of sequenced Gram-negative bacteria (Bingle *et al.*, 2008). Interestingly, T6SSs are largely confined to proteobacteria (and in particular gamma proteobacteria), even though they also occur in planctomycetes and acidobacteria (Persson *et al.*, 2009, Bingle *et al.*, 2008, Boyer *et al.*, 2009, Shrivastava & Mande, 2008).

The presence of multiple copies of almost complete T6SS loci was found in one third of the genomes harbouring such a system (Boyer *et al.*, 2009). It has been proposed that different loci in one genome have distinct evolutionary history, which means that they have been likely acquired by horizontal transfer and not by duplication (Bingle *et al.*, 2008, Das & Chaudhuri, 2003). T6SS gene loci that can be grouped into five different phylogenetic clusters (Barret *et al.*, 2011, Boyer *et al.*, 2009), further implying that they have not arisen by duplications. Moreover, a recent study has demonstrated that TssB1 and TssC1 from *P. aeruginosa* (that form the tail sheath, described further on in this chapter) encoded by a given cluster interact in a specific manner. TssB1-TssC1 do not cross-interact with TssB2 and TssC2 from another T6-gene cluster to form hetero-complexes, further suggesting that there is no redundancy between multiple T6SSs within one organism (Lossi *et al.*, 2013). Indeed, out of the *Pseudomonas* spp. that have been sequenced, all but one encode at least one T6SS gene cluster (Barret *et al.*, 2011).

As mentioned above, there are putative T6SS-encoding loci all along the gamma proteobacteria. Even though at first this secretion system was thought to have a role only in pathogenesis, this point of view has changed, since the list of T6SS-encoding microbes includes symbionts, pathobionts, non pathogenic bacteria, commensals, mutualists along with pathogens (Jani & Cotter, 2010, Boyer *et al.*, 2009).

3.02 TYPE VI SECRETION SYSTEM: WHAT FOR?

There is a growing list of T6SS-associated phenotypes, summarized in Table 1. Interestingly, several classes of T6SS can be distinguished. There are T6SS that are associated with virulence towards a mammal host, or are necessary for plant and fish invasion and infection. Many T6SS have been characterized as having anti-bacterial activity, either intra- or inter-species, where the T6SS is decisive in the outcome of the bacterium when challenged with a prey. This can be of high importance in the establishment and persistence of a given pathogen during an infection within the host.

Moreover, there have been T6SS proposed to be involved in "antivirulence". In these cases, the secretion system would have a role in the regulation of cell density within the host, being a key factor in disease progression and microbe persistence by limiting colonization and regulating cell density independently of host cells. Other phenotypes that have been associated to T6SS are stress sensing, biofilm formation and persistence in mixed biofilms. These show that T6SS might be involved in diverse social behaviours and as mediators in inter-bacterial interactions.

As already mentioned, one bacterial genome may encode more than one T6SS, and it has been proposed that there is predominance of T6SSs persistence in environmentally adapted bacteria. For example, even though *Burkholderia* spp are closely related, not all of them encode the same amount and type of T6SS. *B. pseudomallei* is a highly virulent pathogen that can be found in many different environments and that possesses six evolutionaty distinct T6SS. *B. mallei* is a close-related bacterium, less virulent and an obligate host-associated bacterium, that encodes five T6SSs. Of these, three appear degraded and have

mutations in core genes that might inactivate the machineries. Since *B. mallei* derived from *B. pseudomallei*, it is possible that some of the T6SS have becomed dispensable in the transition from a free-living to a strict host-associated pathogen. In conclusion, the T6SS that each species bears might be related to their environment and way of life (Schwarz *et al.*, 2010a).

T6SS can inject effectors directly into their targets. There are two classes of effectors that have been identified up-to-date. On one hand, evolved VgrG proteins, that have catalytic domains with detrimental activities for the recipient, such as actin remodelling and peptidoglycan disruption (see below) (Hachani *et al.*, 2011, Brooks *et al.*, 2013, Durand *et al.*, 2012, Ma & Mekalanos, 2010).

On the other hand, there are classical toxins that are not generally part of T6SS-encoding gene clusters and are not necessary for Hcp and VgrG export. On the contrary, they rely on the T6 machinery to be injected directly into the recipient. These effectors are toxin-antitoxin pairs in which the toxic component has lytic activity, such as peptidoglycan degrading activity, and the antitoxin pair confers immunity. This class of effectors has been characterized in *P. aeruginosa* (Hood *et al.*, 2010, LeRoux *et al.*, 2012, Russell *et al.*, 2011), *B. thailandensis, P. fluorescens, S. typhimurium* (Russell *et al.*, 2012) and *S. marcescens* (English *et al.*, 2012). Immunity to effectors is specific.

T6SS effectors have been found to be part of a phylogenetically disperse superfamily that can be divided into four divergent families composed of peptidoglycan amidase enzymes (Figure 13A)(Russell *et al.*, 2012). Family assignment depends on conserved motifs surrounding predicted catalytic sites. By testing cross-complementation of immunity proteins between families, Russell 2012 demonstrated that effector families that are identical in catalytic activity are not redundant in function. Interestingly, the effectors studied in *S. marcescens* (members of family four) are also non redundant (English *et al.*, 2012). This might explain why there is such diversity in effector sequence even though some have overlapping cell wall amidase catalytic activity (Figure 13B). The authors argue that it might be linked to immunity protein recognition, as it has been demonstrated for members of family four in *S. marcescens* (English *et al.*, 2012). This is also the case in other contact-dependent interaction systems, such as CDI (Chapter II).

V. cholerae harbours at least three effector-immunity pairs that differ from the predicted superfamily predicted by (Russell *et al.*, 2012). Indeed, none of the effectors identified using a transposon mutagenesis and deep sequencing (Tn-seq) approach was included as a putative T6-associated effector in (Russell *et al.*, 2012). Interestingly, two of the *V. cholerae* effectors are both necessary for antibacterial and antieukaryotic activity in this bacterium, most likely by acting downstream of the evolved cross-linking acting VgrG1. TseL has putative lipase activity, suggesting that it may target membrane-associated lipids. VasX associated to phospholipids in vitro (Miyata *et al.*, 2011). These two proteins are large (>70kDa) compared to other effectors described (>50kDa). TseL interacs with VgrG3 and its secretion depends on this interaction. Thus, TseL is likely secreted in a complex that includes VgrG3 and this might be true for other T6SS "big" effectors (>50kDa and thus unable to fit into Hcp tubes) that would bind to VgrG orthologs, bypassing the Hcp tight tube.

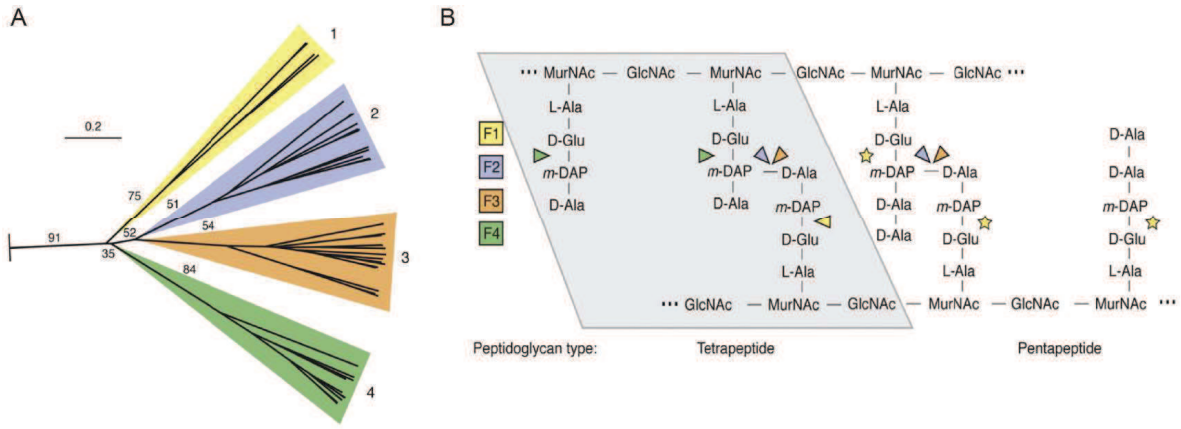

Figure 13 A) Phylogenetic tree of T6S effectors based on alignment of catalytic motifs. Scale bar indicates evolutionary distance in amino acid substitution per site. B) Cleavage sites of effector families 1-4 (F1-4), as indicated with arrows. Stars indicate the putative cleavage sites of Tse1 of *P. aeruginosa*. GlcNAc: *N*-acetylglucosamine; MurAc: *N*-acetylmuramic acid. Adapted from Russell *et al.* (2012).

What would be the benefit for a bacterium to encode different T6SS? As it will be described further on, the T6SS encoded in a same genome are regulated in different ways, implying that they respond to different stimuli. Different T6SS may have specialized functions associated to these stimuli. For example, many bacteria that encode several T6SS have multiple hosts and are able to survive in diverse environmental conditions. This is the case of

P. aeruginosa and *B. pseudomallei*, that employ different T6SS to target prokaryotic and eukaryotic cells (Table 1).

Microorganism	T6-associated Phenotypes	Reference
	Virulence towards eukaryotes	
A. hydrophila SSU	Virulence in mouse model of infection. Translocation of Hcp into eukaryotic cells induces apoptosis through caspase 3 activation.	(Suarez *et al.*, 2008)
Agrobacterium tumefaciens	Efficiency of tumor generation in plant host.	(Wu *et al.*, 2008)
Bordetella bronchiseptica strain RB50	Immunomodulation and pathogenesis, both *in vitro* (macrophages) and *in vivo* (mice model).	(Weyrich *et al.*, 2012)
B. cenocepacia	Deregulation of Rho family GTPases in macrophages, disruption of actin cytoskeleton.	(Rosales-Reyes *et al.*, 2012)
B. mallei, T6SS-1	Virulence in hamster model. Intracellular growth in macrophages and actin-based motility.	(Burtnick *et al.*, 2010, Schell *et al.*, 2007)
B. mallei, T6SS-5	Virulence in hamster model, intra-macrophage survival.	(Shalom *et al.*, 2007)
B. pseudomallei, T6SS-1	*In vitro* cytotoxicity towards RAW 264.7 macrophages. Ability to grow intra-cellularly.	(Burtnick *et al.*, 2011)
B. thailandensis, T6SS-5	Defence against innate immune responses of the host in a murine melioidosis model.	(Schwarz *et al.*, 2010b)
Campylobacter jejuni	Survival in a bile salt, deoxycholic acid, host cell adherence and invasion *in vitro*, colonisation of animal model.	(Lertpiriyapong *et al.*, 2012)
Edwardsiella tarda	Virulence towards fish. Internalization and intracellular proliferation, persistence in immune-related organs (in fish).	(Zheng & Leung, 2007) (Wang *et al.*, 2009, Rao *et al.*, 2004)
E. coli K1 strain RS218	Contribution in pathogenesis.	(Zhou *et al.*, 2012)
Avian pathogenic *E. coli*	Adherence and invasion of epithelial cells, intra-macrophage survival, expression of type 1 fimbriae.	(de Pace *et al.*, 2011, de Pace *et al.*, 2010)
***P. aeruginosa*, HSI-II locus**	**Modulation of the interactions with epithelial cells, internalization into epithelial cells.**	**(Sana *et al.*, 2012)**
***P. aeruginosa*, HSI-I locus**	**Attenuated virulence in mouse model when this locus is interrupted by a transposon. Fitness during colonization.**	**(Potvin *et al.*, 2003)**
P. syringae pv. tomato DC3000	Competition advantage against yeasts.	(Haapalainen *et al.*, 2012)
Salmonella enterica serovar Typhi	Cytotoxicity towards human epithelial cells, systemic infection in mice.	(Wang *et al.*, 2011, Mulder *et al.*, 2012)

V. cholerae strain V52	Virulence towards amoeba *Dictyostelium discoideum* infection model. *In vivo* actin cross-linking in infant mice that produces inflammatory diarrhea and facilitates replication of *V. cholerae* within the intestine, irreversible cytoskeleton disruption.	(Pukatzki *et al.*, 2006, Pukatzki *et al.*, 2007, Ma & Mekalanos, 2010, Ma *et al.*, 2009a)
	Targeting of prokaryotes	
B. thailandensis, T6SS-1	Bacterial competition and persistence in the environment.	(Schwarz *et al.*, 2010b)
Citrobacter rodentium, CTS1	Growth advantage in anti-bacterial competition experiments with *E. coli*.	(Gueguen & Cascales, 2013)
***P. aeruginosa*, HSI-I locus**	**Bacteriolytic effector delivery to other Gram-negative bacteria, anti-bacterial properties.**	**(Hood *et al.*, 2010, Russell *et al.*, 2011)**
P. syringae pv. tomato DC3000	Bacterial fitness in inter-microbial competition against enterobacteria.	(Haapalainen *et al.*, 2012)
Serratia marcescens strain Db10	Anti-bacterial killing activity.	(Murdoch *et al.*, 2011)
Vibrio cholerae strain V52	Anti-bacterial properties, intra- and inter-species.	(MacIntyre *et al.*, 2010, Unterweger *et al.*, 2012)
	Antivirulence	
Helicobacter hepaticus	Limitation of colonization and intestinal inflammation in a mouse model. This promotes a balanced relationship with the host.	(Chow & Mazmanian, 2010)
Pectobacterium atrosepticum	T6-deficient mutants are more effective in maceration of the host plant.	(Mattinen *et al.*, 2008)
Rhizobium leguminosarum	Repression of formation of functional nodules on pea roots and ability to fix nitrogen.	(Bladergroen *et al.*, 2003)
	Other	
B. thailandensis, T6SS-1	Persistence in mixed biofilms (assessed as flow-cell biofilm formation).	(Schwarz *et al.*, 2010b)
Enteroaggregative *E. coli*, *sci-1*	Biofilm formation.	(Aschtgen *et al.*, 2008)
Avian pathogenic *E. coli*	Biofilm formation and motility.	(de Pace *et al.*, 2011)
***P. aeruginosa*, HSI-I locus**	**Enhanced antibiotic resistance linked to biofilm formation.**	**(Zhang *et al.*, 2011a)**
Vibrio anguillarum	Regulation of quorum sensing and stress response.	(Weber *et al.*, 2009)
Yersinia pestis	No effect on flea infection model. No attenuation in murine bubonic and inhalation plague models of infection. Effect on intracellular survival in macrophages.	(Robinson *et al.*, 2009)

Table 1 Summary of the T6-associated phenotypes reported up-to-date.

3.03 Architecture of the Type VI Secretion Apparatus

T6SS are encoded by large gene clusters of more than 20 kb and of up to 25 genes (Boyer *et al.*, 2009, Filloux *et al.*, 2008, Bingle *et al.*, 2008). Among them, thirteen genes have been identified as conserved throughout all T6SS clusters, both using a wide bioinformatic analysis and systematic mutagenesis analysis in *E. tarda* and *V. cholerae* V52 (Boyer *et al.*, 2009, Zheng *et al.*, 2011, Zheng & Leung, 2007). These genes code for the conserved core components of the T6SS and have been named *tss*, for type six secretion genes. The complete list of conserved genes is given in Table 2.

Interestingly, and further supporting the idea of specialized T6SS with distinct functions, there are other genes associated to T6SS gene clusters that are not conserved and are called accessory components, the *tag* genes (for type six associated genes). *tag* genes code for proteins with diverse functions, indispensible for the T6 assembly, function and/or regulation (these components will be described further on).

COG number	Gene name
	P. aeruginosa
3522	*tssK*
3519	*tssL*
3455	*tssF*
3516	*tssB*
3520	*tssG*
3517	*tssC*
3157	*hcp*
3518	*tssE*
3523	*tssM*
3501	*vgrG*
0542	*clpV*
3515	*tssA*
3521	*tssJ*

Table 2 List of conserved T6SS-genes and their features. COG: Clusters of Orthologous Groups of proteins.

The T6S machinery is composed of two major sub-structures, one sharing homology with bacteriophage T4 tail and needle-like structure, and the membrane-spanning peptidoglycan-bound complex that forms the anchoring complex, resembling the T4SS machinery.

(a) Bacteriophage T4 Tail and Needle-like Structure

T6SS are characterized by the presence of two conserved proteins: Hcp (for hemolysin coregulated protein) and VgrG (for valine-glycine repeat protein G). T6SS functionality is frequently assessed by the ability of bacteria to produce and/or secrete these two "so-called" effectors, although these components are now considered as being part of the export machinery. It was shortly after the T6SS were first described that structural studies revealed the striking similarities between several T6SS components, including Hcp and VgrG, and proteins of bacteriophages. T6 bacteriophage-like subunits are core components of the nanomachinery (Figure 14).

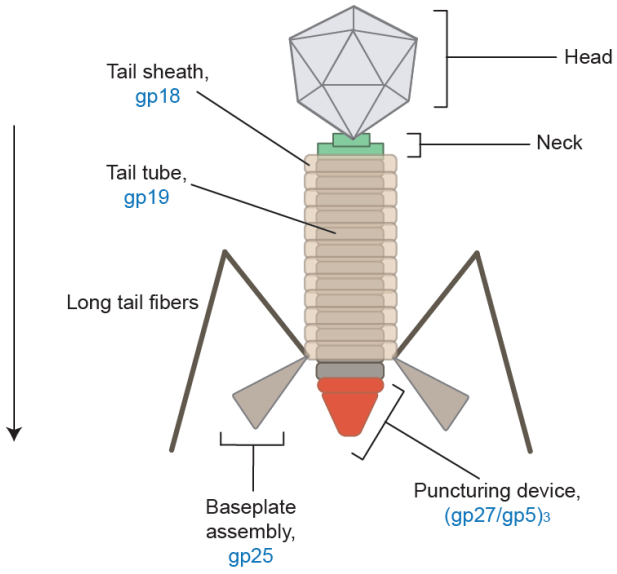

Figure 14 Schematic representation of bacteriophage T4. Subunits that will be described in this section have been written in blue. The arrow indicates the sense in which DNA will be injected.

(i) The Tail Tube

Hcp is small protein (~ 17kDa) secreted by all T6SS. Hcp from *P. aeruginosa* and in *E.* tarda forms a hexameric rings with an internal diameter of approximately 40Å, rings being stacked in a head-to-tail or in a head-to-head fashion (Figure 15, Mougous *et al.*, 2006, Jobichen *et al.*, 2010). Hcp is able to form structured nonhelical nanotube-like assemblies *in vitro*, which can be stabilized by the introduction of disulfide bonds (Ballister *et al.*, 2008). Hcp shares homology with the protein that forms the tail tube of phage T4, gp19 (Pell *et al.*, 2009)(Figure 14 and Figure 15B, Leiman *et al.*, 2009), further supporting the idea that Hcp may form a tubular structure that could traverse the cell envelope for substrate secretion.

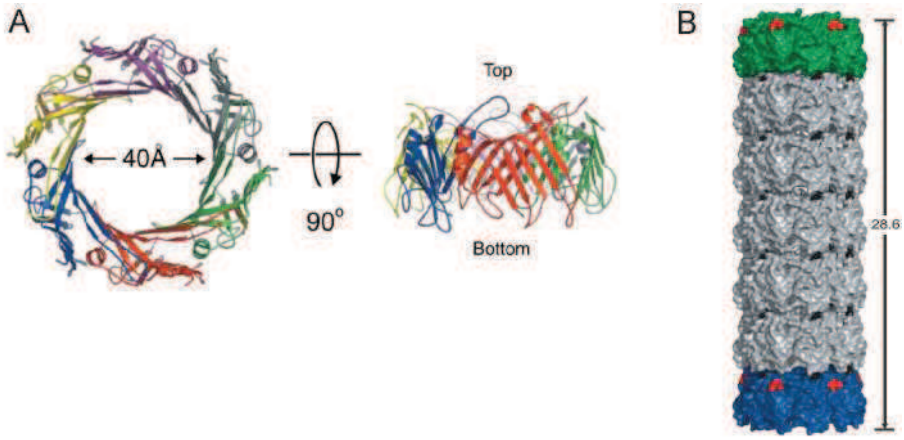

Figure 15 Hcp structural studies. A) Top view of Hcp hexamer from *P. aeruginosa*. Individual subunits are represented in different colours. Taken from Mougous *et al.* (2006). B) Model of Hcp nanotube. Source: Ballister *et al.* (2008).

(ii) The Cell-Puncturing Complex

VgrG protein family is also found in the extracellular milieu. Some bacterial genomes, such as *P. aeruginosa*, encode up to ten different VgrGs (Pukatzki *et al.*, 2007, Boyer *et al.*, 2009, Hachani *et al.*, 2011).

Based on structural analysis of VgrGs and its similarity to the T4 puncturing device, it has been proposed that they are localized in the distal tip of the Hcp tube and act as a membrane-puncturing device. There are several lines of evidence that support this notion. First, the crystal structure of the N-ter domain of the VgrG encoded by *E. coli* CFT073 showed that VgrG architecture is comparable to the gp5-gp27 complex, despite their low

sequence identity (Figure 16, Leiman *et al.*, 2009). This complex forms trimers, necessary for the cell puncturing activity. Accordingly, VgrGs can also multimerize, as is the case in *P. aeruginosa* (Hachani *et al.*, 2011). Secondly, Hcp secretion is VgrG secretion-dependent (Pukatzki *et al.*, 2007, Zheng & Leung, 2007, Hachani *et al.*, 2011). Whether this deficiency is due to an incomplete assembly of the bacteriophage-like needle or to the inability of the bacterium to sense a triggering signal is still an open question.

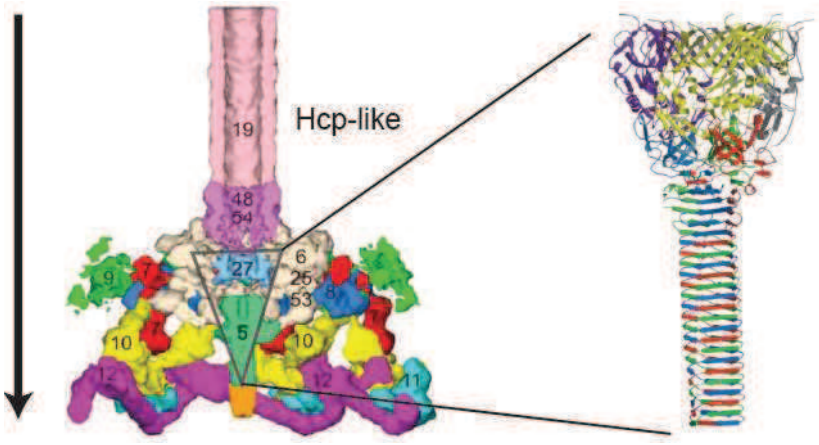

Figure 16 VgrG protein trimers share structural homology with the puncturing device formed by gp5-gp27. The arrow indicates the trajectory of the substrates. Adapted from Leiman *et al.* (2009), Leiman *et al.* (2004).

VgrGs are thought to carry out different tasks within the T6SS. Evolved VgrGs are proteins that carry an "effector" domain, which can function as pathogenic factors. Up-to-date, only three of these evolved VgrGs have been studied from a functional point of view, *V. cholerae* VgrG1 and VgrG3, and *A. hydrophila* VgrG. VgrG1 from *V. cholerae* and VgrG protein from *A. hydrophila* can remodel the host cell actin cytoskeleton (Figure 12, Ma *et al.*, 2009a, Suarez *et al.*, 2010). Interestingly, *A. hydrophila* T6SS is known to target exclusively eukaryotic cells, but *V. cholerae* employs its T6SS to target also other Gram-negative bacteria. The role of its VgrG3 has been recently shown as having peptidoglycan-degrading activity that destroys the cell wall of Gram-negative bacteria (Brooks *et al.*, 2013). In consequence, the evolved domain of VgrG3 has an impact in interbacterial competition, which might influence *V. cholerae* outcome when encountering other Gram-negative bacteria in the environment and/or in the human gastrointestinal gut (Brooks *et al.*, 2013). Whether different VgrG

proteins within a given microorganism are systematically recruited to the T6 machinery or whether there are external cues that regulate specific VgrG recruitment is not known.

How do evolved VgrGs function in the case of eukaryotic-cell targeting? Based on the functional and structural data, it can be speculated that the C-ter domain of the protein functions as the sheath and punctures the cell. Once in the cytosol, the active N-ter domain can modify the host cytoskeleton. Supporting this hypothesis, it has been demonstrated that VgrG1 of *V. cholerae* has a functional actin cross-linking domain (ACD) (Durand *et al.*, 2012). The two domains are connected by a peptide predicted to be naturally disordered that could be the target of host proteases once the protein is in the cytosol. Indeed, the C-ter domain is most likely the one that first enters the cell, since it is located downstream of the gp5-like helical structure (this structure is known to be the puncturing domain of bacteriophages, Rossmann *et al.*, 2004).

(iii) The Tail Sheath

The viral sheath consists of more than a hundred oligomerized gp18 subunits arranged as rings that form a hollow tube, able to accommodate the viral tail (Leiman *et al.*, 2004). TssB and TssC subunits have been reported as forming a hollow tubular cogwheel protein complex of several hundreds of angstroms long in *V. cholerae* and in *P. aeruginosa*. The Internal diameter of the TssB/TssC complex is of approximately 10nm, big enough to accommodate the Hcp tube, estimated to have an external diameter of 9nm (Figure 17A, (Bonemann *et al.*, 2009, Basler *et al.*, 2012, Lossi *et al.*, 2013). TssB/TssC do not share sequence similarity with gp18, the sheath-forming subunit. However, by modelization of a cross section of TssB/TssC tubules it was shown that they shared structural organization (Figure 17B, Cascales & Cambillau, 2012). Hcp co-fractionates with TssB and TssC homologues in *F. novicida*, further supporting the hypothesis that TssB and TssC form a tube-like structure able to engulf the Hcp filament (Figure 17, de Bruin *et al.*, 2011).

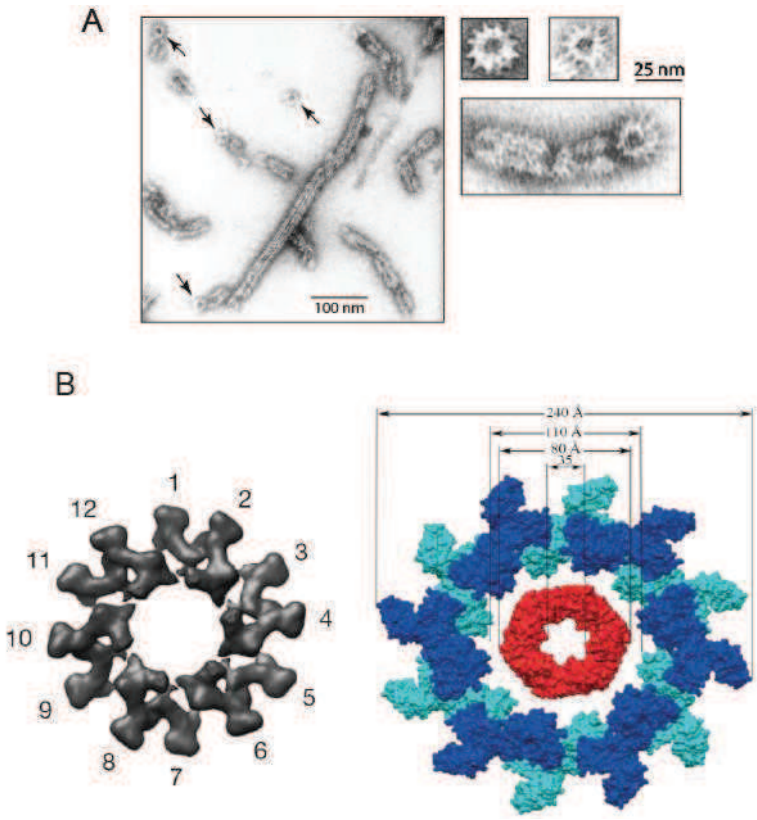

Figure 17 TssB-TssC complex. A) TssB and TssC homologuess in *V. cholerae* (VipA and VipB, respectively) form tubular structures *in vitro*, as shown by electron microscopy analysis. Taken from Bonemann *et al.* (2009). B) The hollow sheath-like TssB/TssC (left panel and blue and light blue in the right panel) structures could accommodate the Hcp tail (red). Adapted from Cascales & Cambillau (2012), Basler *et al.* (2012).

Direct interaction between TssB and TssC has been demonstrated in several bacterial species, including *V. cholerae*, *B. cenocepacia* and *P. aeruginosa* (Bonemann *et al.*, 2009, Aubert *et al.*, 2010, Lossi *et al.*, 2013). Both subunits have been shown to localize in the cytoplasm and in the OM (Bonemann *et al.*, 2009, Aubert *et al.*, 2010). Cytoplasmic structures assemble from the IM and in a perpendicular fashion to the cell envelope (Basler *et al.*, 2012). It has been proposed that the formation of the TssB/TssC complex might be TssC-driven, since the TssB homologue in *V. cholerae* (VipA) does not form tubules *in vitro* in the absence of its partner, and in *P. aeruginosa* the C-ter of TssC is required for tubule formation as well. Furthermore, TssC homologue (VipB) is needed for the interaction of the complex with ClpV in *V. cholerae*, a conserved T6-ATPase (Bonemann *et al.*, 2009, Lossi *et al.*, 2013).

In an effort to elucidate which proteins associate with TssB/TssC sheaths in *V. cholerae*, mass spectrometry analysis for protein identification was carried out on purified TssB/TssC structures. Four T6 proteins were identified: ClpV, VCA0109, VCA0111 and VCA0114. ClpV had already been shown as directly interacting with TssB (Bonemann *et al.*, 2009, Pietrosiuk *et al.*, 2011). VCA0109 is gp25 homologue, a protein that probably forms part of the baseplate and that is localized in the cytoplasm of *P. aeruginosa* (see below). Finally, VCA0111 and VCA0114 (TssG and TssK, respectively, see Table 2) are conserved T6SS components but have not been studied yet.

(iv) The Baseplate Assembly

TssE (VCA0109 in *V. cholerae*) shares approximately 40% sequence similarity with gp25 of T4 bacteriophage (Leiman *et al.*, 2009, Lossi *et al.*, 2011), a highly conserved structural component of the baseplate of phages with contractile tails (Kostyuchenko *et al.*, 2003, Yap *et al.*, 2010). Moreover, TssE shows also remarkable similarity with gp25 secondary and tertiary structures as revealed by prediction tools (Lossi *et al.*, 2011). *P. aeruginosa* TssE is localized in the cytoplasm and has no lysozyme activity, unlike T4 gp25 protein family (Lossi *et al.*, 2011).

T4 bacteriophage baseplate, a macromolecular complex composed of at least 130 proteins of 14 different families, is required for initiation of tail tube and sheath assembly. It is assembled around the "hub", a central three-fold-symmetric cylindrical structure (Rossmann *et al.*, 2004). In T4, gp25 forms a wedge-shaped complex with gp6 and gp53 that is localized around the centre of the baseplate and has a key role in the connection of its central and peripheral parts (Kostyuchenko *et al.*, 2003). It is conceivable to propose that TssE might form part of the baseplate of the T6SS, that would assemble in the inner leaflet of the IM of the bacterium prior to secretion, and that the baseplate would be the linker structure between the tail and spike complex and the membrane-anchored complex.

Several questions remain unanswered. Does TssE form part of a larger IM baseplate-like structure? Does TssE interact with TssM and/or TssL in the inner leaflet of the IM? How is TssE recruited, if so, to the IM machinery complex?

Interestingly, it has been observed by electron microscopy that the T6S machinery presents a bell-shaped platform that connects the sheath to the IM (Basler *et al.*, 2012). TssB/TssC elongated tubes assembly is TssE-dependent in *V. cholerae* and they can be co-purified. Gp25 interacts with gp5 -gp27 trimer in T4 bacteriophages, suggesting that TssE may have a similar role in the T6SS (Basler & Mekalanos, 2012, Basler *et al.*, 2012, Kapitein *et al.*, 2013).

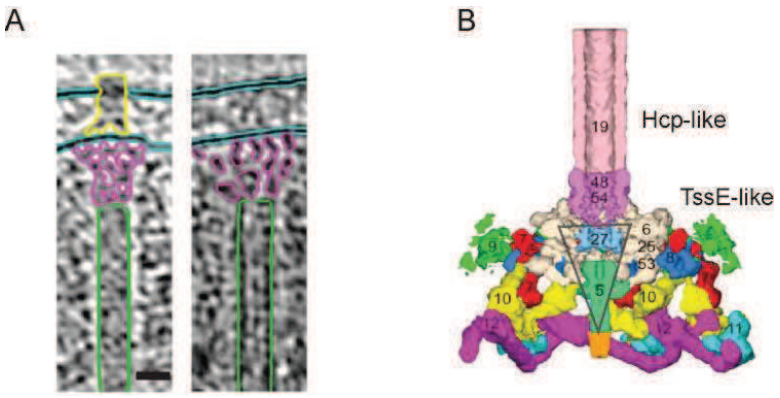

Figure 18 T6 baseplate-like structure. A) Bell-shaped baseplate is shown in yellow and violet, in an extended (left panel) and in a contracted (right panel) structure, as shown by electron cryotomographic imaging of intact cells. Taken from Basler *et al.* (2012). Scale bar represents 20nm. B) Reconstruction of the extended T4 baseplate and tube complex. The grey triangle depicts the homologues of VgrG trimers. Gp25 is localized in a complex with gp6 and gp53, herein white. Adapted from Rossmann *et al.* (2004).

(b) MEMBRANE-ASSOCIATED COMPONENTS

The T6S machinery is anchored to the envelope of Gram-negative bacteria by several membrane-associated proteins that are bound to peptidoglycan. This complex has been isolated by Cascales and co-workers (Aschtgen *et al.*, 2010a), and is composed at least by TssJ, TssM and TssL (Figure 19). All these proteins are core components of the T6SS (Table 2), indispensible for the secretion of Hcp and VgrG.

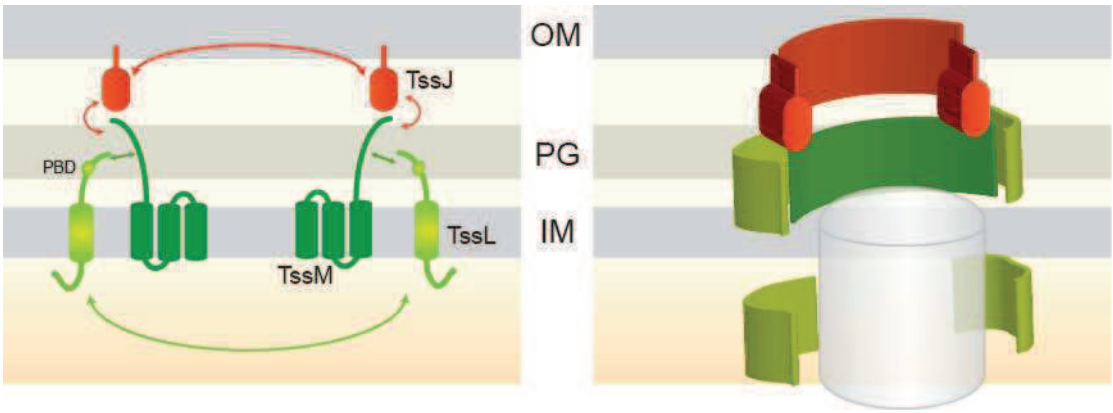

Figure 19 Membrane-spanning complex of the T6SS. *Left panel:* 2D representation of the three proteins that from the complex. As TssM (dark green) interacts both with OM TssJ (red) and IM TssL (light green), it virtually spans the cell envelope. TssL bears a PBD (peptidoglycan-binding) domain, thus anchoring the complex to the cell wall. *Right panel:* 3D representation. Autoassociation of TssJ and TssL might form a ring shaped core that could hold the bacteriophage-like assembly (grey cylinder). OM: outer membrane; PG: peptidoglycan; IM: inner membrane.

TssJ is an OM lipoprotein able to homodimerize (Aschtgen *et al.*, 2008, Rao *et al.*, 2011). Its structure has been reported for EAEC and *S. marcescens* (Felisberto-Rodrigues *et al.*, 2011, Rao *et al.*, 2011). It consists of a particular arrangement of parallel β-sheets connected by an intermediate loop with low level of sequence identity that is required for interaction with TssM in EAEC (Felisberto-Rodrigues *et al.*, 2011).

TssL is an IcmH-like integral IM protein (Ma *et al.*, 2009b, Aschtgen *et al.*, 2012). IcmH, or DotU, is needed for the correct functioning of T4bSS in *Legionella pneumophila*, since knockout mutants are impeded in intracellular growth in *Acanthamoeba castellanii* (Zusman *et al.*, 2004). The C-ter of TssL homologue in EAEC is oriented towards the periplasm (Aschtgen *et al.*, 2012). Its N-ter folds as a hook-like structure composed of two three-helix bundles and is necessary for TssL autoassociation (Durand *et al.*, 2012a).

TssL proteins can bear a peptidoglycan-binding domain (PBG), thought to stabilize the apparatus in the cell envelope by anchoring it to the cell wall. In the case of EAEC, TssL does not have a PBD, but it interacts with TagL, another integral IM able to bind peptidoglycan *in vivo* and *in vitro* (Aschtgen *et al.*, 2010a, Aschtgen *et al.*, 2010b). TagL is an accessory T6SS protein, that co-occurs with TssL proteins without a PDB domain (Aschtgen *et al.*, 2010b). In the particular case of *P. aeruginosa*, TssL possesses a PF00691 domain present in OmpA family of proteins, known to specifically interact with peptidoglycan.

TssM is an integral IM protein that has three trans-membrane domains in EAEC and a large periplasmic domain (744 residues in EAEC, Felisberto-Rodrigues *et al.*, 2011). It is conserved in all T6SS and may function as a nucleotide-binding (NTP-binding) protein. For example, in *A. tumefaciens* TssM has ATP-binding and hydrolysis activity, the latter being crucial for Hcp recruitment and interaction with TssM periplasmic domain (Ma *et al.*, 2009b, Ma *et al.*, 2012). On the other hand, TssM of *E. tarda* does not require its NTP-binding domain for Hcp secretion (Zheng & Leung, 2007). Moreover, some TssM do not possess NTP-binding domains. These findings argue that TssM-associated ATPase activity is not widespread through T6SS and that it might be related to specific needs of each bacterium.

TssM, in addition of interacting with TssJ, is able to form a complex with the C-ter periplasmic domain of TssL (Zheng & Leung, 2007, Ma *et al.*, 2009b, Felisberto-Rodrigues *et al.*, 2011). TssJ and TssL are both able to homodimerize, in the OM and in the IM, respectively. Yeast two-hybrid assays suggested that TssM is oligomeric. It is then plausible to propose that these three proteins form a membrane-spanning complex, that is bound to peptidoglycan and has a ring shape-like structure. Of note, ring shape-like structures seem to be a common feature of different membrane-spanning machineries, such as T3SS and T4SS (Schraidt & Marlovits, 2011, Fronzes *et al.*, 2009).

Does the membrane-spanning complex function as a channel and support for the syringe-like complex? It has been suggested that the TssM-L-J complex can accommodate the phage-like injection machinery. The interaction of TssM via its periplasmic domain both with Hcp and TssB supports this model, where the membrane-spanning complex would stabilize the needle-like complex.

3.04 MECHANISM OF SECRETION/INJECTION, A DYNAMIC STORY

As discussed in the previous sections, bioinformatic, structural and biochemical analyses suggest that T6SSs are contractile injection systems reminiscent of tailed phages, that employ a syringe-like macromolecuar nanomachine to puncture host cells membrane. As depicted in Figure 20, a mechanism of injection can be proposed. In first instance, there is assembly of the apparatus (Figure 20, steps 1 and 2). The assembly process is not completely understood, even though it has been shown that Hcp can form tubules *in vitro* (Ballister *et al.*, 2008) as well as TssB/TssC (Bonemann *et al.*, 2009, Lossi *et al.*, 2013). Some T6SS conserved components are indispensible for TssB/TssC sheath formation in *V. cholerae* and *P. aeruginosa*, such as TssE, Hcp and TssM (Lossi *et al.*, 2013, Basler *et al.*, 2012, Kapitein *et al.*, 2013). Probably upon an environmental signal, contraction of TssB/TssC tubules occurs, which is thought to expel the Hcp tail and the VgrG spike (Figure 20, step 3). After Hcp and VgrG expelling from the donor cell, the latter is thought to puncture the recipient membrane and detach from the Hcp nanotube so that toxins can pass through the conduit. This statement is supported by the fact that extended TssB/TssC structures exhibited central density as observed by electron microscopy, whereas contracted ones appeared hollow (Basler *et al.*, 2012).

In bacteriophages, host recognition occurs through a reversible interaction of the conserved tip of the long fibers with the OMP OmpC or with LPS (Yu & Mizushima, 1982). This triggers conformational changes in the short fibers, that extend and bind to the outer core of LPS in an inrreversible fashion. This binding is followed by contraction of the outer tail sheath, penetration of the bacterial membrane by the hollow inner tail tube (Leiman *et al.*, 2009, Leiman *et al.*, 2004). Interestingly, structural sutides revealed that the aromatic and positive residues on the surface of the fiber tips are most likely receptor-binding determinants with the receptor in the bacterial surface (Bartual *et al.*, 2010). Up-to-date, homologues of these fiber tip components have not been found associated to T6SS (Figure 20).

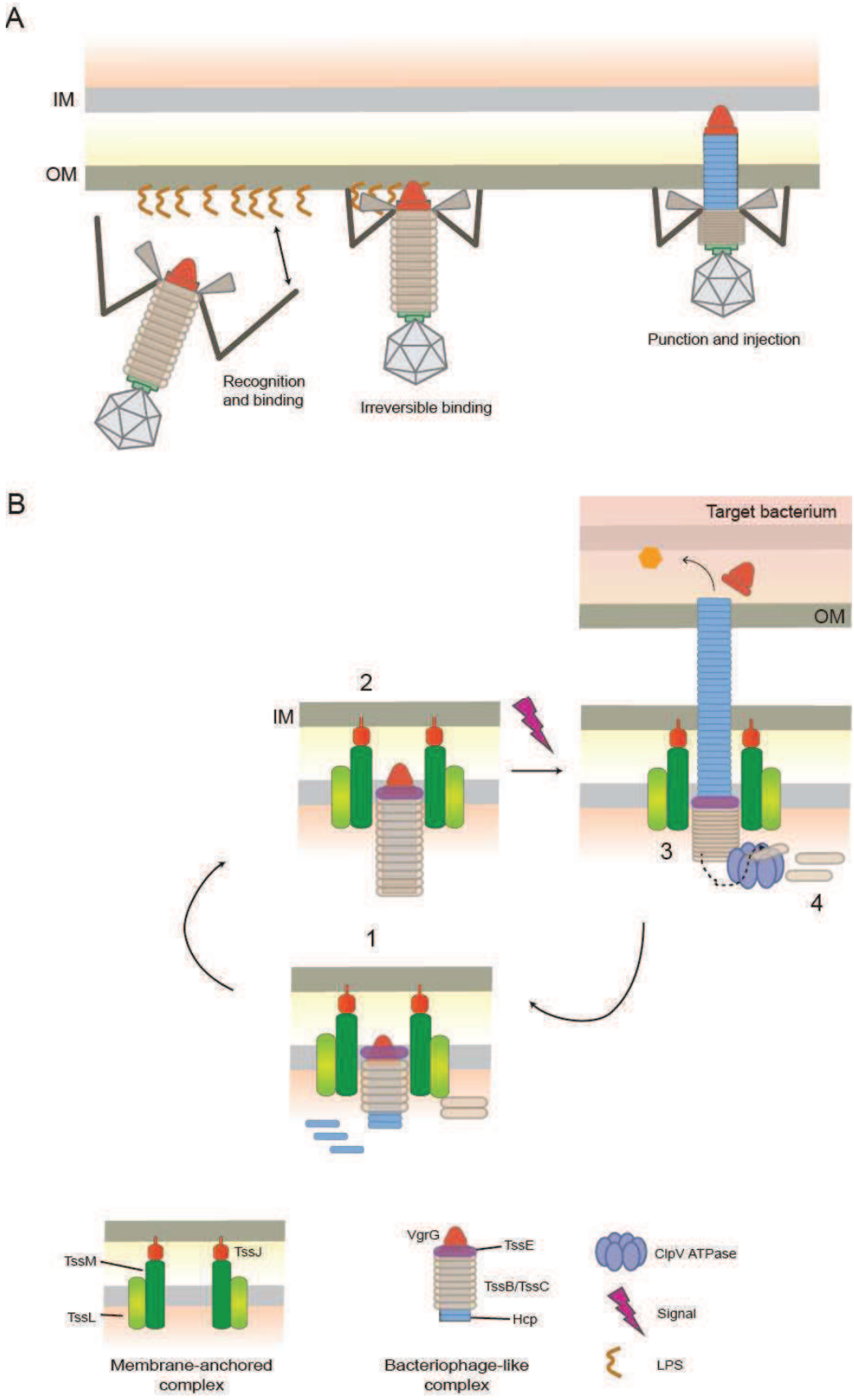

Figure 20 Comparison of the injection mechanism by T4 bacteriophages (A) and T6SS (B). See the text for details.

In the case of bacteriophages, the syringe-like complex is used only once to inject DNA into the host. On the contrary, T6SS can be reused (Figure 20, step 4). It has been suggested that ClpV, a conserved Hsp100/Clp family of AAA+ of the T6S, is responsible for the recycling of the TssB/TssC tubules. ClpV is able to disassemble and remodel the tubules *in vitro* and interacts directly with TssC in a specific manner (Bonemann *et al.*, 2009, Kapitein *et al.*, 2013, Pietrosiuk *et al.*, 2011). Moreover, it has been shown that ClpV homologue in *V. cholerae* binds only to contracted TssB/TssC sheaths *in vivo* (Kapitein *et al.*, 2013, Basler *et al.*, 2012), further supporting the idea that it is involved specifically in turnover of the apparatus upon contraction and not in its assembly. Finally, it has also been demonstrated that ClpV prevents the formation of non-productive TssB/TssC tubules formed spontaneously in the cell. This would assure the presence of a pool of non-assembled TssB and TssC units, needed for T6S assembly upon cue sensing (Kapitein *et al.*, 2013).

T6SS are highly dynamic structures that follow assembly/disassembly contact-dependent cycles. These cycles have been monitored by time-lapse fluorescence microscopy in several microorganisms (Basler & Mekalanos, 2012, LeRoux *et al.*, 2012, Kapitein *et al.*, 2013, Brunet *et al.*, 2012). For example, *P. aeruginosa* cells can respond to T6SS activity in a neighbouring sister cell with an increase in their own T6SS dynamics. This phenomenon has been baptized bacterial duelling (Figure 21, Basler & Mekalanos, 2012), and has also been documented for EAEC cells. Indeed, the propagation of T6SS activities was shown to increase over time, spreading through the bacterial lawn (LeRoux *et al.*, 2012, Brunet *et al.*, 2012). This rapid propagation of T6SS activities could contribute to coordination in a spatial and temporal fashion of a bacterial community to eliminate competing bacteria. Also, it could have an impact in rapid propagation of a signal within cooperative microorganisms.

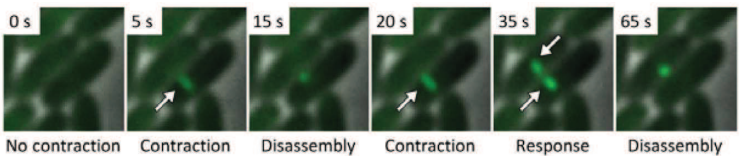

Figure 21 T6S duelling between *P. aeruginosa* sister cells. TssB fused to GFP was monitored by time-lapse fluorescence microscopy. Reproduced from Basler & Mekalanos (2012).

Finally, two reports describe inter-bacterial intoxication via T6SS in a quantitative manner *in vivo* (Brunet *et al.*, 2012, LeRoux *et al.*, 2012). By monitoring prey lysis over time, they demonstrated that cell-cell contact is needed for T6SS-mediated intoxication but it is not the cue for T6SS activation. Moreover, the T6SS is not an extended extracellular appendage, since immediate cell-contact (less than 200nm) was needed for prey lysis (LeRoux *et al.*, 2012). The authors proposed that T6SS-associated attacks may act as a cue to the donor that the susceptible cell is in its vicinity, conceivable given the broad distribution of T6SS among Gram-negative bacteria.

3.05 REGULATION OF TYPE VI SECRETION SYSTEMS

Gram-negative bacteria possessing T6SS can inhabit diverse niches, such as soil, water and specific host tissues. T6SS is not always associated to pathogenic bacteria, but also to symbionts, pathobionts, etc. It has been shown that there are at least 13 conserved genes encoded in T6 loci, but that there can be many more associated genes not conserved throughout all T6SS. Indeed, it has been suggested that these accessory genes contribute to adaptation of the T6SS to specific needs of each individual bacterium (Schwarz *et al.*, 2010a).

Following the same line of thought, it is not surprising that different T6SS gene clusters of a bacterium can be under distinct regulatory mechanisms. This might indicate that bacteria respond to dissimilar stimuli by activating/repressing one or the other T6SS gene cluster and sometimes by modulating different regulatory systems, as in the case of *P. aeruginosa*.

As described in Table 3 and in Figure 22, there are different levels of regulation: transcriptional (including QS and two component systems), posttranscriptional, and posttranslational regulatory cascades. Transcriptional regulation appears to be the most common, both activation and repression have been documented. Finally, there is no evident universal regulation pathway of T6SSs, maybe due to the diversity of bacteria that have a T6SS and their different ecological characteristics (Leung *et al.*, 2011, Bernard *et al.*, 2011, Silverman *et al.*, 2012).

Microorganism	Input and/or type of regulation	Reference
	Iron	
E. tarda	Activation by iron limitation. Repression by the Fur protein by direct binding to the *evp* cluster promotor	(Chakraborty *et al.*, 2011, Wang *et al.*, 2009)
EAEC	Fur repression. DNA methylation activation	(Brunet *et al.*, 2011)
***P. aeruginosa*, HSI-II**	**Negative regulation by iron**	**(Sana *et al.*, 2012)**
	Two component systems and global regulators	
B. pseudomallei, T6SS-1	Upregulation by VirAG system when inside of host cells	(Chen *et al.*, 2011)
B. cenocepacia	Upregulation by QS and negative regulation by sensor kinase hybrid AtsR	(Chambers *et al.*, 2006, O'Grady *et al.*, 2009, Aubert *et al.*, 2008)
EAEC	Regulation by global regulator AggR	(Dudley *et al.*, 2006)
V. cholerae	Negative regulation by TsrA global regulator	(Zheng *et al.*, 2011)
E. tarda	Upregulation by EsrAB TCS	(Wang *et al.*, 2009)
S. enterica serovar Typhi	Upregulation of TCS PmrAB and RcsB. Posttranscriptionally regulated by Hfq	(Wang *et al.*, 2011)
P. aeruginosa, HSI-I	RetS/LadS and Gac/Rsm pathways	(Mougous *et al.*, 2006, Brencic *et al.*, 2009, Brencic & Lory, 2009)
P. fluorescens	GacS/GacA controlled activation	(Hassan *et al.*, 2010)
P. syringae	RetS/LadS and Gac/Rsm pathways	(Records & Gross, 2010)
	Alternative sigma factor	
A. hydrophila	Regulation by sigma-54 factor (σ^{54})-dependent transcriptional regulator VasH	(Suarez *et al.*, 2008, Bernard *et al.*, 2011)
V. cholerae	VasH is an activator of *vas* cluster transcription (it *activates* σ^{54}). The *hcp* operons are regulated by RpoN.	(Pukatzki *et al.*, 2006, Kitaoka *et al.*, 2011, Bernard *et al.*, 2011, Dong & Mekalanos, 2012)
Pectobacterium atrosepticum	σ^{54} – dependent activation of transcription	(Bernard *et al.*, 2011)
Marinomonas spp.	σ^{54} – dependent activation of transcription	(Bernard *et al.*, 2011)
Vibrio alginolyticus	RpoN and VasH homologue-dependent activation of transcription	(Sheng *et al.*, 2012)
	Quorum sensing	
***P. aeruginosa*, HSI-II**	**Activation by Las and Rhl QS systems. Reciprocal regulation with HSI-I locus**	**(Lesic *et al.*, 2009)**
***P. aeruginosa*, HSI-III**	**Activation by Las and Rhl QS systems. Reciprocal regulation with HSI-I locus**	**(Lesic *et al.*, 2009)**
V. cholerae	LuxO regulator-dependent downregulation	(Zheng *et al.*, 2010, Ishikawa *et al.*, 2009)

Microorganism	Input and/or type of regulation	Reference
B. cenocepacia	Positive regulation by QS and negative regulation by sensor kinase hybrid AtsR	(Chambers *et al.*, 2006, O'Grady *et al.*, 2009)
Vibrio alginolyticus	Positive regulation by LuxO and negative regulation by LuxR	(Sheng *et al.*, 2012)
Y. pseudotuberculosis	QS-dependent activation	(Zhang *et al.*, 2011b)
P. aeruginosa, HSI-I	LasR-dependent repression	(Lesic *et al.*, 2009)
A. hydrophila	QS-mediated repression	(Khajanchi *et al.*, 2009)
P. atrosepticum	Upregulation by QS	(Liu *et al.*, 2008)
	Environmental conditions	
R. leguminusarum	Temperature-dependent activation	(Bladergroen *et al.*, 2003)
Y. pseudotuberculosis	Temperature- and growth phase-dependent T6 activity	(Zhang *et al.*, 2011b)
A. tumefaciens	pH-mediated regulation of transcription	(Wu *et al.*, 2012)
Y. pestis	Low temperature-dependent induction	(Robinson *et al.*, 2009)
V. cholerae	Activation under high-osmolarity and low temperature conditions	(Ishikawa *et al.*, 2012)
	Other	
P. aeruginosa, HSI-I	Surface association acts as a cue for posttranscriptional activation	(Silverman *et al.*, 2011)
B. pseudomallei, T6SS-5	Induction in macrophages	(Shalom *et al.*, 2007)
P. atrosepticum	Induction in potato tuber extracts	(Mattinen *et al.*, 2008)
P. aeruginosa, HSI-I	Posttranslational activation by a kinase/phosphatase pair	(Mougous *et al.*, 2007, Hsu *et al.*, 2009)

Table 3 Recapitulative of T6SS regulation in different Gram-negative bacteria. TCS: two component system.

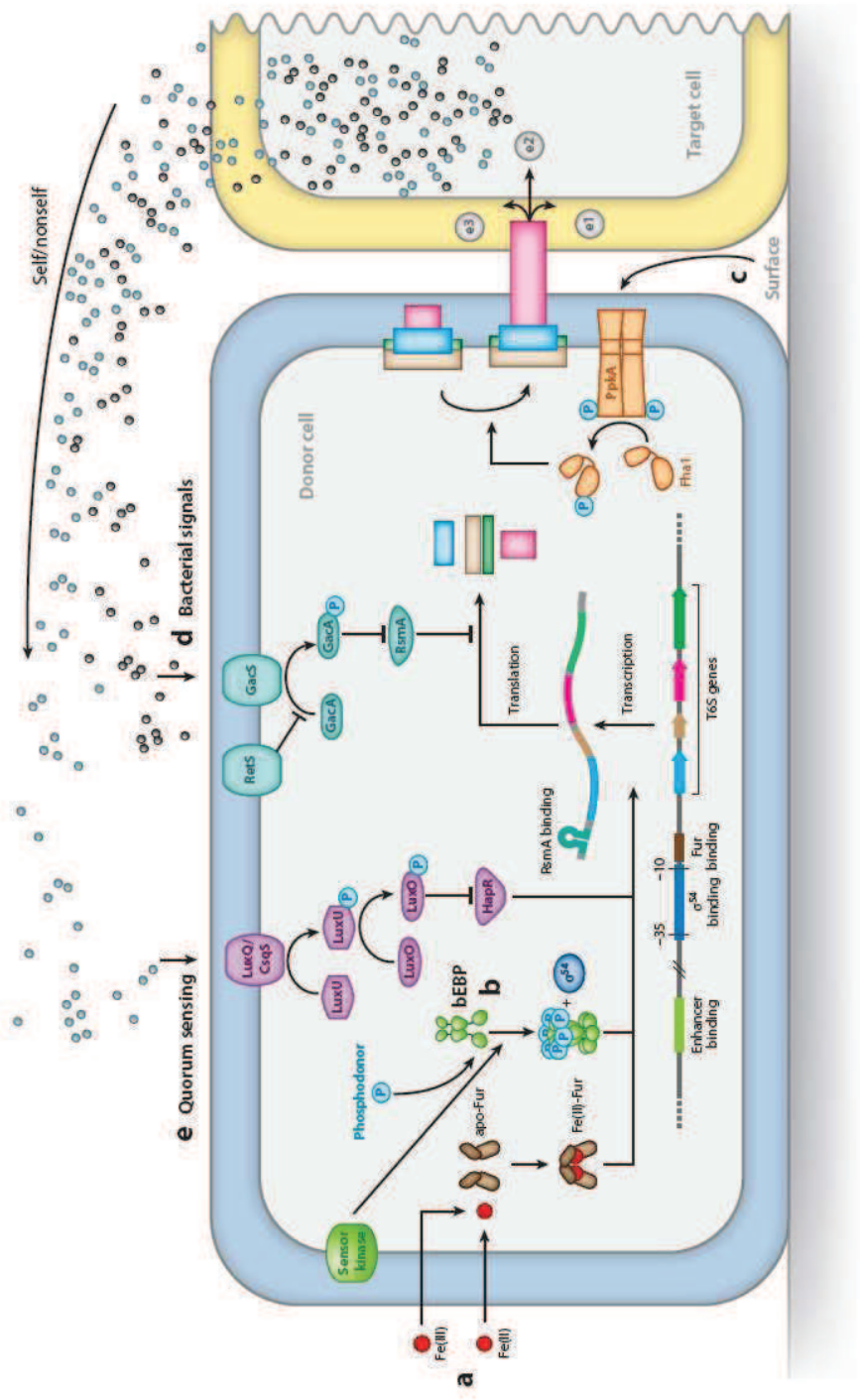

Figure 22 Schematic representation of diverse regulatory systems that modulate T6S expression and activation. A) Fur transcriptional regulation. B) Alternative sigma-dependent transcriptional activation. C) Surface association posttranslational activation. D) Self/non-self bacteria-derived signals regulation at a posttranscriptional level. E) Self/non-self bacteria-derived signals regulation at a transcriptional level. Reproduced from Silverman *et al.* (2012).

EXPERIMENTAL PROCEDURES

Most of the methods used in this study have been described in (Casabona *et al.*, 2013). The experimental procedures presented herein include those that have not been previously described.

Chapter IV. Molecular Biology Techniques

4.01 GENETIC CONSTRUCTIONS

Genes of interest were polymerase chain reaction (PCR)-amplified using PAO1 genomic DNA as a template, cloned into PCR-Blunt II-TOPO (Invitrogen) and sequenced (Beckman Coulter Genomics) before cloning into final destination vectors. The list of oligonucleotides with their appropriate restriction sites and of the plasmids used in this study is given Table 4 and Table 5, respectively.

Table 4 Oligonucleotides used in this study.

Oligo	Sequence (5' → 3')	Characteristics
JP1	AGGATCCCATGATCCTGCGCCTGCAC	PCR TagT for pETDuet-1
JP2	TAAGCTTTCAGCCAACTTCCTCGAG	PCR TagT for pETDuet-1
JP4	TAAGCTTTCAAACATCGCGTAATCCC	PCR TagS for pETDuet-1
WalkerA-antisens	GAGCAGGGTGCTGGCGCCGCAGCCGCT	Mutagenesis Walker A in TagT
WalkerA-sens	AGCGGCTGCGGCGCCAGCACCCTGCTC	Mutagenesis Walker A in TagT
Ch-Xbas	TCTAGAATGGTGAGCAAGGGCGAG	PCR mCherry for pJN105
Ch-Sacas	GAGCTCTCACTTGTACAGCTCGTCC	PCR mCherry for pJN105
70RBSEco	GAATTCAAACCAGGAGTGAGATGC	TagQ for fusion with mCherry
70longXba	TCTAGAGGCCTTGGCGCAGCTCGG	TagQ for fusion with mCherry
TssJ-F-EcoRI	GGGAATTCAGGAGAAACAACGTCAATGAG	TssJ for fusion with mCherry

Oligo	Sequence (5' → 3')	Characteristics
TssJ-R-XbaI	CCTCTAGAGGGCGCGGGACGCGCG	TssJ for fusion with mCherry
QnolipoNde	CCATATGGCCAGCAGCGGCGTCGGCTCG	PCR TagQ for pET15b
QnolipoBam	CGGATCCTCAGGCCTTGGCGCAGCTC	PCR TagQ for pET15b
F-TagR-NcoI	CCATGGGGATGTTTGAGAAAGCCATTCTTCC	PCR TagR for pET52b
R-TagR-SacI	GAGCTCACGCCCGCTGGACGCGAG	PCR TagR for pET52b
70-deltaCys s	GTGCTGCTCAGCGGCGCCAGCAGCGGCGTC	Mutagenesis Cys30 in TagQ
70-deltaCys as	GACGCCGCTGCTGGCGCCGCTGAGCAGCAC	Mutagenesis Cys30 in TagQ
Q TGA s	GCTGCGCCAAGGCCTGATCTAGAATGGTGAG	Mutagenesis TGA in TagQwt/ΔC-mCherry
Q TGA as	CTCACCATTCTAAGTCAGGCCTTGGCGCAGC	Mutagenesis TGA in TagQwt/ΔC-mCherry
Δ70-F1	CCCGGGCGCAGCGCCCTCGATG	*tagQ* deletion
Δ70-R1	TTCCTTCGGCACCTTGGGGCCGACGCCGCTGCTGGCG	*tagQ* deletion
Δ70-F2	CCCAAGGTGCCGAAGGAAGCG	*tagQ* deletion
Δ70-R2	CCCGGGGCGCAGATGGCGTTGTTC	*tagQ* deletion
TagRF1	GGATCCGCCATTCTTCCGCTCGC	*tagR* deletion
TagRR1	TGTATCAGGTTTCCGGCGCCTCGTACTTGCCGATGAAG	*tagR* deletion
TagRF2	CGCCGGAAACCTGATACAGTC	*tagR* deletion
TagRR2	GAATTCGCCCGCTGGACGCGAG	*tagR* deletion
TagR-52b-fw	GGCCATGGAAGGCGACTCGCCGGACAATC	PCR *tagR* to clone in pET52b for overproduction
TagR-52b-rv	GGGTCGACACGCCCGCTGGACGCGAGC	PCR *tagR* to clone in pET52b for overproduction

Table 5 Plasmids used in this study. Their antibiotic resistance and other characteristics are described. Ap^R: ampicillin resistance, Kn^R: kanamycin resistance, Gm^R: gentamycin resistance, Tc^R: tetracycline resistance.

Plasmid	Characteristics	Reference
	Protein production in E. coli	
pETDuet- 1	Ap^R, vector designed for the co-expression of two genes (bicistronic contruction), possibility of hexa-histidine tagging	Novagen
pETDuet-6his*tagT*	Ap^R, *BamH*I-*Hind*III insertion of *tagT*. TagT carries a hexa-histidine tag	(Casabona *et al.*, 2013)
pETDuet-6his*tagTS*	Ap^R, *BamH*I-*Hind*III insertion of *tagTS*. Only TagT carries a hexa-histidine tag	(Casabona *et al.*, 2013)

Plasmid	Characteristics	Reference
pETDuet-6his*tagT*$^{K/A}$*S*	*tagT* carries a single mutation in the WalkerA domain	(Casabona *et al.*, 2013)
pET52b	Ap^R, plasmid used for overexpression of proteins. Possibility to add a N-ter Strep tag or a C-ter 10 histidine tag	Novagen
pET52b-*hcp*	Ap^R, plasmid used for overexpression of Hcp1 carrying a N-ter Strep tag	(Casabona *et al.*, 2013)
pET52b-*tagR*$_{10His}$	Ap^R, plasmid used for overexpression of TagR carrying a C-ter deca-histidine tag	This study
pET30b	Km^R, plasmid used for overexpression of proteins, possibility of N-ter histidine or S tag	Novagen
peT30b-*tagQnl*	Km^R, plasmid used for overexpression of TagQnl, a version of TagQ deprived of signal peptide and lipobox. TagQnl carries no tag	This study
pET15b	Ap^R, plasmid used for overexpression of proteins, possibility of N-ter hexa-histidine tag	Novagen
pET15b-*tagQnl*	Ap^R, plasmid used for overexpression of TagQnl, a version of TagQ deprived of signal peptide and lipobox. TagQnl carries a hexa-hisitdine tag in its N-ter	(Casabona *et al.*, 2013)
	mCherry fusions	
pJN105	Gm^R, broad host range vector that carries the p*BAD* promoter from *E. coli*. Arabinose-inducible.	(Newman & Fuqua, 1999)
pJN-*mCherry*	Gm^R, pJN105 carrying *Xba*I-*Sac*I insertion of *mCherry*	(Casabona *et al.*, 2013)
pJN-*tagQ-mCherry*	Gm^R, pJN-*mCherry* carrying a *EcoR*I-*Xba*I insertion of *tagQ*.	(Casabona *et al.*, 2013)
pJN-*tagQ*ΔCys-*mCherry*	Gm^R, site-directed mutagenesis of pJN-*tagQ-mCherry* has been performed in order to delete Cys30.	(Casabona *et al.*, 2013)
pJN-*tssJ 1-mCherry*	Gm^R, pJN-*mCherry* carrying a *EcoR*I-*Xba*I insertion of *tssJ1*.	(Casabona *et al.*, 2013)
	Complementation	
pJN-*tagQ*-$_{TGA}$-*mCherry*	Gm^R, pJN-*tagQ-mCherry* carrying the TGA stop codon of *tagQ*	(Casabona *et al.*, 2013)
pJN-*tagQ*ΔCys-$_{TGA}$-*mCherry*	Gm^R, pJN-*tagQ*ΔCys-*mCherry* carrying the TGA stop codon of *tagQ*	(Casabona *et al.*, 2013)
	Fluorescent P. aeruginosa	
mini-CTX1	Tc^R, integration plasmid that carries an oriT for conjugation-mediated plasmid transfer	(Hoang *et al.*, 2000)
pIApX2	Ap^R, carries the *gfp* gene under the control of pX2, a strong constitutive promotor of *P. aeruginosa*.	(Thibault *et al.*, 2009)
mini-CTX-pX2-*gfp*	Tc^R, *EcoR*I-*Hind*III insertion of pX2-*gfp* from pIApX2	(Casabona *et al.*, 2013)
	Inter-bacterial competition assays	
pBluescript	Ap^R, carries *lacZ*, allowing blue/white colony screen	Lab collection

To carry out pull down experiments, 6his-*tagTS* from pETDuet-*tagTS* were cloned *Xba*I-*Hind*III into pIApX2 (Thibault *et al.*, 2009) to give pIApX2-6his-*tagTS.*

4.01 CONSTRUCTION OF VECTORS FOR THE CO-OVERPRODUCTION OF TAGQ AND TAGR IN *E. COLI*

In order to be able to co-produce TagQ and TagR in *E. coli*, *tagR* and *tagQ* were cloned without their respective signal peptides into different expression vectors (with different antibiotic resistances), and only *tagR* was fused to a deca-histidine tag.

tagQ was excised from pET15b-*tagQnl* using *Nde*I-*Bam*HI and then cloned into pET30b, generating pET30b-*tagQnl*.

tagR was PCR-amplified using TagR-52b-fw and TagR-52b-rv and cloned into routine cloning vector PCR-Blunt II-TOPO. As *Nco*I-*Sal*I digestion generated 3 fragments of approximately the same size, *tagR* was sub-cloned using *EcoR*I into pUC18, which allowed a white/blue colony screen in the presence of IPTG and X-Gal. Then, *tagR* was cloned into its final destination vector pET52b using *Nco*I-*Sal*I sites.

4.02 PLASMID INCORPORATION IN *P. AERUGINOSA*

All replicative plasmids were introduced in *P. aeruginosa* strains by transformation (Chuanchuen *et al.*, 2002). Constructs used to generate in-frame deletions of *tag* genes or a *tse1-VSV-G* chromosomal fusion were previously reported (Silverman *et al.*, 2011).

Chapter V. Microbiology techniques

5.01 Strains and Growth Conditions

P. aeruginosa PAO1 strain used in this study was obtained from Ph.D. J. Mougous (University of Washington) and it is the reference strain that has been sequenced (Stover *et al.*, 2000).

P. aeruginosa strains were regularly grown in Luria-Bertani (LB, DIFCO®) medium at 37°C and 300 r.p.m. supplemented with carbenicillin 100-250 µg mL^{-1}, gentamycin 200 µg mL^{-1} and tetracycline 200 µg mL^{-1} when needed. *E. coli* strains were grown in LB medium at 37°C and 300 r.p.m. supplemented with ampicillin 100 µg mL^{-1}, gentamycin 50 µg mL^{-1}, kanamycin 25 µg mL^{-1}, tetracycline 10 µg mL^{-1} as required.

The solid medium PIA (Pseudomonas Isolation Agar, DIFCO®) was used to grow selectively *P. aeruginosa*.

The strains used in this study are listed in Table 6.

Table 6 Summary of the strains used in this study.

Strain	Characteristics	Reference
	P. aeruginosa	
PAO1	Reference wild-type strain	(Stover *et al.*, 2000)
PAO1Δ*retS*	PAO1 reference strain with *retS* deletion	(Mougous *et al.*, 2006)
PAO1Δ*retS*Δ*tagTS*	PAO1 reference strain with *retS* and *tagTS* deletion	(Casabona *et al.*, 2013)
PAO1Δ*retS*Δ*tagR*	PAO1 reference strain with *retS* and *tagR* deletion	(Casabona *et al.*, 2013)
PAO1Δ*tagQ*	PAO1 reference strain with *tagQ* deletion	(Silverman *et al.*, 2011)
PAO1Δ*tagR*	PAO1 reference strain with *tagR* deletion	(Silverman *et al.*, 2011)
PAO1Δ*tagS*	PAO1 reference strain with *tagS* deletion	(Silverman *et al.*, 2011)
PAO1Δ*tagT*	PAO1 reference strain with *tagT* deletion	(Silverman *et al.*, 2011)
PAO1Δ*ppkA*	PAO1 reference strain with *ppkA* deletion	(Mougous *et al.*, 2007)
PAO1Δ*pppA*	PAO1 reference strain with *pppA* deletion	(Mougous *et al.*, 2007)
PAO1Δ*tssM1*	PAO1 reference strain with *tssM1* deletion	(Mougous *et al.*, 2006)

PAO1Δ*clpV1*	PAO1 reference strain with *clpV1* deletion	(Mougous *et al.*, 2006)
	E. coli	
TOP10	Chemically-treated competent cells, used for rutine cloning	Invitrogen
XL21blue	Chemically-treated competent cells that allow white/blue screening, used for transformation of site-directed mutagenesis plasmids	Stratagene
DH5α	Strain used for interbacterial competition assays	Lab collection
BL 21	Used for protein production	Invitrogen
BL21(DE3)Star	Used for protein production, contain T7 RNA polymerase	Invitrogen
SHuffle T7	Protein production, can form disulfide bonds in the cytoplasm	Invitrogen

5.02 INTERBACTERIAL COMPETITION ASSAYS

E. coli DH5α was transformed with pBluescript and plated on LB plates supplemented with 100 µg mL^{-1} ampicillin and 40µg mL^{-1} X-gal. Blue colonies were selected and every culture used in competition assays was started from an isolated blue colony.

In *P. aeruginosa* versus *E. coli* DH5α-pBluescript competition assays, a volume of an O/N culture equivalent to OD_{600}=1 was centrifuged 1 min at 13000 r.p.m. and resuspended in 100 µL of fresh LB. Then, bacteria were mixed at a 1:1 ratio and spotted (20µl) on a dry LB plate. Once the drops were absorbed, the competitions assays were let 5h at 37°C. After incubation, competitions were recovered and resuspended in 1 mL LB. Serial dilutions were performed and spotted (20 µL) on LB plates supplemented with 40-80 µg mL^{-1} X-gal. After O/N incubation at 37°C, this method allowed white/blue colony screen.

Of note, *E. coli* DH5α-pBluescript were systematically spotted on LB plates supplemented with ampicillin in order to control that pBluescript was stable during the competition assay. The same control was done if *P. aeruginosa* donor strains harboured a plasmid.

Chapter VI. Biochemistry Techniques

6.01 Inner and Outer Membrane Separation by Discontinuous Sucrose Gradient

Inner and outer membranes of *P. aeruginosa* cells were separated by a discontinuous sucrose gradient as described in Viarre *et al.* (2009), with modifications. Briefly, 500 mL cultures of *P. aeruginosa* at OD_{600} of 1 were harvested by centrifugation. In the case of PAO1Δ*tagQ* + *tagQ*ΔCys, bacteria were grown with appropriate additives and induced by 0.01% arabinose at OD_{600} 0.5. Pellets were resuspended in 25 mL of 10 mM Tris-HCl, 20% sucrose, 10 mg mL^{-1} DNase, 10 mg mL^{-1} RNase, pH 7.4, and were disrupted by using a Microfluidizer at 15,000 psi. Unbroken cells were removed by 15 min centrifugation at 6,000 x *g*. Total membrane fraction was obtained by ultracentrifugation at 100,000 x *g* and resuspended in 500 µL of 20% sucrose containing PIC. The total membrane fraction was then applied at the top of a discontinuous sucrose gradient composed of 1.5 mL layers of 60%, 55%, 50%, 45%, 40%, 35% and 30% of sucrose in 10 mM Tris-HCl, 5 mM EDTA, pH 7.4 (from bottom to top). Sucrose gradients were centrifuged at 90,000 x *g* at 4°C for 36-72 h, and 500 µL fractions were collected from the top. Figure 23A depicts a classical separation. All fractions were then characterized by SDS-PAGE, Western Blot analysis and NADH oxidase activity. Antibodies against T2SS protein XcpY (Michel *et al.*, 1998), kindly gifted by R. Voulhoux (CNRS, Marseille, France), were used as an inner membrane marker and porins visualized directly on Coomassie blue-stained SDS-PAGE or OprF detection with specific antibodies as markers of the outer membrane. NADH oxidase activity was determined as described elsewhere by measuring the NADH consumption at 340 nm of 50 µL of each fraction (Aubert *et al.*, 2010). Representative IM and OM fractions used in this study were analyzed for total protein concentration using the BCA assay (Uptima®).

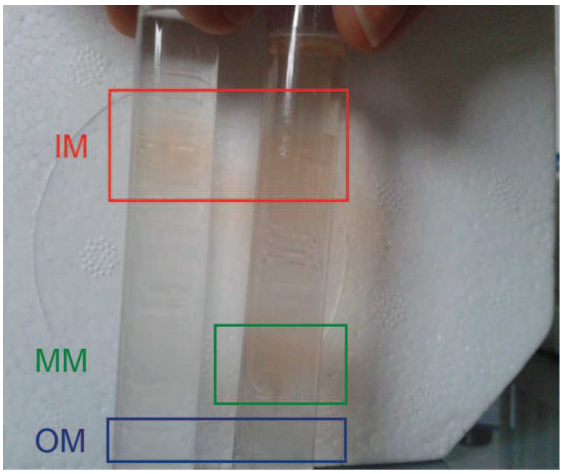

Figure 23 Fractionation of *P. aeruginosa* by discontinuous sucrose gradient. Left: Good, typical separation. Right: "Bad separation". IM: inner membrane ring. OM: outer membrane ring. MM: mixed (IM+OM) membrane ring.

This fractionation technique was also used to generate samples for a high-throughput analysis of the IM of PAO1 using a proteomics approach. In order to obtain a better separation of the IM/OM, the layers in the discontinuous sucrose gradient were modified, so that the IM and OM rings would have more spacing between them. The gradient was then composed of more layers and of different sucrose percentages, as follows (from bottom to top, the volume is indicated): 55% (1.4ml), 50% (1.5ml), 45% (1.5mL), 42.5% (1.3ml), 40% (1.5ml), 37.5% (1.3ml), 35% (1.5ml) and 30% (1.0ml). A photo of these gradients is presented in Figure 23.

The shotgun proteomics approach used for the study of the IM/OM of *P. aeruginosa* allowed us to identify several proteins that form part of the H1-T6SS (see below, Results). For example, TssB1 was identified preferentially in the OM, which was surprising since TssB homologue of *V. cholera* has been shown as being cytoplasmic and localized to the IM (Basler *et al.*, 2012). We thus decided to study more in detail the localisation of TssB1. In order to discard the possibility that TssB was found in the OM fraction due to the formation of high-molecular weight TssB1-TssC1 tubular structures (Bonemann *et al.*, 2010, Bonemann *et al.*, 2009, Basler *et al.*, 2012), discontinuous sucrose gradients were performed, but with the particularity that the formation mixed IM/OM vesicles was favoured. In this case, we expect that only proteins anchored and/or associated to the membranes will be present in the mixed vesicles, since these vesicles will migrate differently in the gradient from the IM

and the OM (Figure 23). Thus, if TssB1-TssC1 tubular structures are really associated to the membrane, the proteins will be present in the mixed vesicles fraction. On the contrary, if TssB1-TssC1 complex happens to migrate equally to the OM, the proteins will not be present in the mixed vesicles fraction.

Briefly, O/N cultures were diluted up to OD600 0.05 and grown at 37°C and 225 r.p.m for 16h. Then, cells were harvested and broken by sonication cycles in an ice-water bath. Finally, the separation was performed as described above, and it will be referred to as a "Bad separation" (Figure 23).

6.02 PROTEINASE K ACCESSIBILITY

The equivalent to $2x10^9$ bacteria was harvested from an O/N culture by centrifugation at 7000 r.p.m. and washed twice with 20mM Tris-HCl, pH 8.0, 100mM NaCl. A total of 100µg of Proteinase K was added when indicated. Samples were incubated for 30min at 37°C and the reaction was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 5mM followed by centrifugation. Bacteria were resuspended in loading buffer and analyzed by SDS-PAGE and immunoblotting.

In order to check the enzyme's activity in the conditions used for the assay, purified TagQ –generated to raise specific antibodies- was added before the incorporation of the Proteinase K. In this case, after the centrifugation step to stop the reaction, supernatants were also recovered and analyzed for TagQ presence/absence.

6.03 IMMUNOFLUORESCENCE ASSAYS

For anti-TagQ staining, bacteria were harvested at mid-log phase by centrifugation and fixed with 4% paraformaldehyde for 2h on a slide coated with fibronectin and permeabilized by 0.5% triton X-100 for 5 min at room temperature. Then, fixed bacteria were washed three

times with PBS before a 30 min blocking step with 2% BSA in PBS. Anti-TagQ was used at a 1/500 dilution and the incubation lasted for 1 h. As secondary antibody, anti-mouse-FITC or anti-mouse-Cyt were used, as indicated. Secondary antibodies were also diluted 1/500 and incubation lasted for 1 h (this step was done in the dark).

In order to label the cells without a permeabilization step, an IF was carried out in liquid. GFP-expressing bacteria were harvested at mid-log phase by centrifugation and washed three times with PBS. After a 30min blocking step (with 2% BSA), cells were washed three times and incubated for 1 h with anti-TagQ 1/500 with agitation. The same steps as described above were repeated for secondary antibody incubation. Stained cells were observed with a Leica TCS-SP2 operating system (Manheim, Germany).

6.04 CO-IMMUNOPRECIPITATION ASSAYS

Co-immunoprecipitation assays were carried out using a Dyabeads® kit (Invitrogen). Briefly, O/N cultures of PAO1DtagQ + tagQDC were diluted to OD_{600} of 0.15 and cultured with shaking up to mid-log phase of growth. At this point, approximately $5*10^9$ cells were harvested by centrifugation and lysed by sonication in lysis buffer (50mM Tris-HCl pH 7.4, 100mM NaCl, 5mM EDTA). Unbroken cells were eliminated by a centrifugation step at 4°C and 6000 x g. After that, soluble proteins were incubated with previously α-TagQ antibody-loaded Dynabeads® for 1h in a wheel. When cross-linking was carried out, it was done as recommended by the manufacturer. After incubation, three washes were carried out using the Washing buffer of the commercial kit. Elution was done using the Elution buffer as recommended by the manufacturer. Samples were analyzed by western blot.

6.05 PULL-DOWN ASSAYS

O/N cultures of PAO1Δ*retS*Δ*tagTS* or PAO1Δ*retS*Δ*tagTS* + 6*hisTagTS* were diluted to OD_{600} of 0.1 and incubated with shaking up to mid-log phase of growth. At this point, approximately

$9*10^9$ bacteria were harvested by centrifugation and broken by sonication in lysis buffer (50mM Na_2PO_4, 100mM NaCl, 10mM imidazole) supplemented or not with 0.5% of n-Dodecyl β-D-maltoside (DDM). Unbroken cells were eliminated by a centrifugation step. Then, soluble proteins were put in contact with magnetic Ni^{2+} beads (Millipore®) previously equilibrated with lysis buffer. Incubation was set up for 1h in a wheel at room temperature. After incubation, beads were washd three times with wash buffer (50mM Na_2PO_4, 100mM NaCl, 20mM imidazole) and eluted with elution buffer (50mM Na_2PO_4, 100mM NaCl, 300mM imidazole). Samples were analyzed by SDS-PAGE and immunoblotting.

6.06 CO-PRODUCTION OF TAGR AND TAGQ

Indicated *E. coli* strains were grown O/N and diluted to OD600 of 0.05. Cells were grown up to OD_{600} ~ 0.4-0.6 and induced by addition of 1mM IPTG. Induction was carried out at 37°C, 300r.p.m. and for 4hs or at 16°C, 120r.p.m. and for 16h. After that, cells were harvested by centrifugation and broken by sonication in lysis buffer (50mM Na_2PO_4, 300mM NaCl). Unbroken cells were eliminated by a centrifugation step and soluble proteins were obtained by ultracentrifugation at 42000 r.p.m. for 30min. Soluble proteins were then incubated with magnetic Ni^{2+} beads (Millipore ®) for 1h at 4°C and in a wheel. Wash and elution steps were carried out as in Section 3.14. Samples were analyzed by SDS-APGE and immunobloting.

6.07 IMMUNOBLOTTING

The conditions used for western blotting during this study are described in Table 7. Commercial antibodies were used as recommended by the manufacturers. SDS-PAGE were transferred to nitrocellulose membranes (0.22µm pore size, Whatman®) using the semi dry blot apparatus Novex mini cell system (Invitrogen). The blots were developed by ECL Detection Kit (Amersham) and Millipore HRP Substrate.

Table 7 SDS-PAGE and Western blotting details of the proteins studied.

Protein	Size (kDa)	SDS-PAGE	Antibody source	WB Dilution	Secondary antibody (HRP-coupled)
TagQ	31.7*	15	(Casabona *et al.*, 2013)	10000	Mouse
TagR	62	12	(Casabona *et al.*, 2013)	1000	Mouse
RpoA	36.6*	15	NeoClone	5000	Mouse
DsbA	23.4*	15	Romé Voulhoux, CNRS Marseille	2000	Rabbit
OprF	37.6*	15	(von Specht *et al.*, 1995)	1000	Mouse
XcpY	41.3*	15	Romé Voulhoux, CNRS Marseille, (Michel *et al.*, 1998)	2500	Rabbit
TssJ1-mCherry	43.9**	12	Clontech (anti-mCherry)	1000	Mouse
ClpV1-gfp	126		Sigma-Aldrich (anti-GFP)	1000	Mouse
TssB1-gfp	46	15	Sigma-Aldrich (anti-GFP)	1000	Mouse
Hcp1	17.4*	15	(Casabona *et al.*, 2012)	5000	Guinea Pig
Tse1-VSV-G	16.4*	15	Sigma-Aldrich (anti-VSV-G)	5000	Rabbit
Fha1-VSV-G	53.3*	7.5	Sigma-Aldrich (anti-VSV-G)	5000	Rabbit
Tse1	16.4*	15	(Casabona *et al.*, 2013)	2000	Rabbit
TagRHis10	62	12	(Casabona *et al.*, 2013)(anti-TagR) ; Sigma-Aldrich (anti-HisHRP)	1000 (anti-TagR) 5000 (anti-HisHRP)	Mouse (anti-TagR)
TssB1	18.8*	15	Alain Filloux, Imperial College of London (Hachani *et al.*, 2011)	1000	Rabbit
VgrGa	72.0*	8	Alain Filloux, Imperial College of London (Hachani *et al.*, 2011)	1000	Rabbit

* Predicted, (Winsor *et al.*, 2011) ; **Software-predicted (VectorNTI, Invitrogen).

RESULTS AND DISCUSSION

Chapter VII. H1-T6SS of P. aeruginosa

7.01 INTRODUCTION

The *P. aeruginosa* genome encodes three potential T6SS in Hcp secretion islands I to III (HSI-I to III). As described in Chapter III, in *P. aeruginosa* the expression of T6S genes as well as the T6S effectors secretion is finely regulated. The T6SS encoded by HSI-I locus (hereafter H1-T6SS) is the most extensively studied and is the subject of this study.

Previous studies showed that LasR orchestrates the transcriptional regulation of the three HSI loci of *P. aeruginosa* (Lesic *et al.*, 2009). LasR downregulates the transcription of HSI-I locus, implying that under environmental conditions that stimulate high QS activity there will be repression of HSI-I gene transcription. The opposite effect is observed for HSI-II and HSI-III loci, in accordance with them being implicated in *P. aeruginosa*-mediated pathogenesis towards eukaryotic cells (Lesic *et al.*, 2009, Sana *et al.*, 2012).

HSI-I gene products are also under posttranscriptional regulation. In this case, the RNA-binding protein RsmA is able to bind to the 5' leader sequence of two target HSI-I mRNAs and trigger their degradation, thus unabling the transcription (Goodman *et al.*, 2004, Brencic & Lory, 2009). RsmA is part of the Gac/Rsm regulatory pathway, which modulates the co-ordinated expression of virulence genes in *P. aeruginosa* (Goodman *et al.*, 2004, Burrowes *et al.*, 2006). The Gac/Rsm pathway itself is co-ordinately regulated by two-component sensor kinases RetS and LadS (Goodman *et al.*, 2004, Ventre *et al.*, 2006).

Finally, H1-T6SS activity depends on at least five additional proteins. Four proteins, PpkA, PppA, TagR and Fha1, form part of the so called threonine phosphorylation pathway (TPP) (Hsu *et al.*, 2009), and TagF, a negative regulator acting independently of the TPP (Silverman *et al.*, 2011).

When the studies presented herein were started, a model for TPP had been just proposed. This pathway depends on a transmembrane serine-threonine kinase, PpkA, which dimerizes

and autophosphorylates to then phosphorylate Fha1, a protein that contains a Forkhead-associated domain. Once phosphorylated, Fha1 can recruit ClpV1 to the IM, triggering the effectors export. TagR, a periplasmic protein that acts upstream of PpkA, is essential for the activation of the system. It has been proposed that TagR could be a co-receptor of the signal that activates H1-T6SS secretion. The activity of PpkA is antagonized by its cognate phosphatase, PppA, which de-phosphorylates Fha1. In conclusion, the modulation of the phosphorylation status of Fha1 determines the triggering of H1-T6SS activation (Hood *et al.*, 2010, Hsu *et al.*, 2009, Mougous *et al.*, 2007). Fha1 is phosphorylated at Thr362, thus the name of TPP.

Independent of the TPP, another pathway has been described recently (Silverman *et al.*, 2011). In this case, Fha1 is negatively regulated by TagF. The environmental cues that govern the two pathways are different, since surface growth induces TPP activation, but not the TagF-dependent pathway (Silverman *et al.*, 2011).

The two posttranslational pathways described here are summarized in Figure 24.

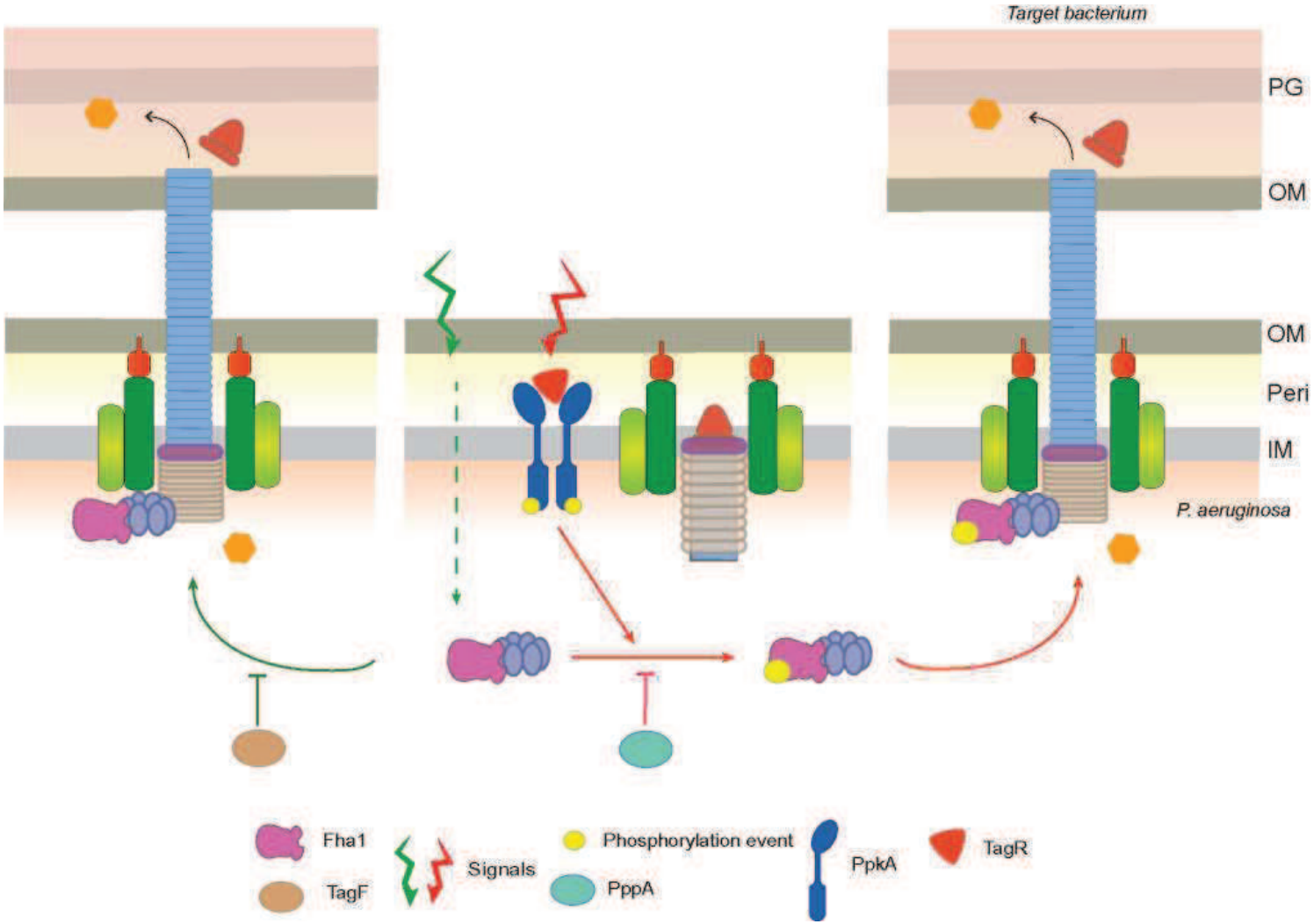

Figure 24 Schematic representation of the two posttranslational pathways that modulate H1-T6SS activity in *P. aeruginosa*. Red pathway: TPP. The environmental cue is associated with surface growth, that triggers dependent of TagR, PpkA dimerization and auto-phosphorylation. Green pathway: independent of TPP. The signal that derepresses TagF is unknown. Proteins not depicted in the legend have already been described in Figure 20 (Chapter III).

Interestingly, the genes coding for posttranslational regulatory actors are found in the same operon, with several uncharacterized non conserved *tag* genes, *tagT*, *tagS* and *tagQ* (Figure 25).

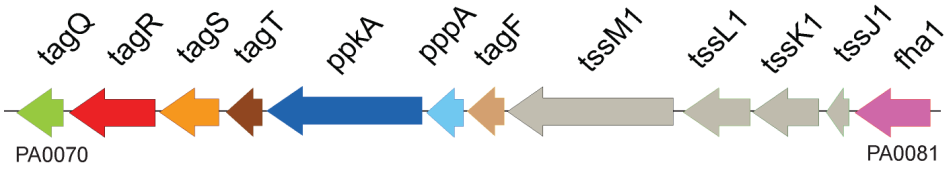

Figure 25 HSI-I operon that harbours the genes coding for the posttranslational regulatory proteins. Colour code is in accord with Figure 24. Conserved T6SS genes are depicted in grey.

This part of the manuscript describes the results I have obtained in an effort to characterize TagT, TagS and TagQ. First, a recent publication giving insights into biochemical properties of these three proteins is presented, where it is demonstrated that TagT, TagS and TagQ are indeed additional players in the posttranslational activation of H1-T6SS (Casabona *et al.*, 2013). During this study, several questions remained unanswered. For example, a direct interaction between TagQ and TagR in the OM was suggested, but not experimentally demonstrated. Also the orientation of TagQ was still unkown. Finally, the question whether Tag proteins are necessary for intra-bacterial species T6-targeting was not addressed. The additional results presented here were aimed to gain insights into these unresolved questions.

7.02 Results and Discussion

(a) Publication in Environmental Microbiology

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An ABC transporter and an outer membrane lipoprotein participate in posttranslational activation of type VI secretion in *Pseudomonas aeruginosa*

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Summary

Pseudomonas aeruginosa **is capable of injecting protein toxins into other bacterial cells through one of its three type VI secretion systems (T6SSs). The activity of this T6SS is tightly regulated on the posttranslational level by phosphorylation-dependent and -independent pathways. The phosphorylation-dependent pathway consists of a Threonine kinase/phosphatase pair (PpkA/PppA) that acts on a forkhead domain-containing protein, Fha1, and a periplasmic protein, TagR, that positively regulates PpkA. In the present work, we biochemically and functionally characterize three additional proteins of the phosphorylation-dependent regulatory cascade that controls T6S activation: TagT, TagS and TagQ. We show that similar to TagR, these proteins act upstream of the PpkA/PppA checkpoint and influence phosphorylation of Fha1 and, apparatus assembly and effector export. Localization studies demonstrate that TagQ is an outer membrane lipoprotein and TagR is associated with the outer membrane. Consistent with their homology to lipoprotein outer membrane localization (Lol) components, TagT and TagS form a stable inner membrane complex with ATPase activity. However, we find that outer membrane association of T6SS lipoproteins TagQ and TssJ1, and TagR, is unaltered in a Δ*tagTS* background. Notably, we found that TagQ is indispensible for anchoring of TagR to the outer membrane fraction. As T6S-dependent fitness of *P. aeruginosa* requires TagT, S, R and Q, we conclude that these proteins likely participate in a transmembrane signalling pathway that promotes H1-T6SS activity under optimal environmental conditions.**

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Introduction

Bacteria cope with their environment through an arsenal of secreted macromolecular products that are transported across the bacterial envelope by protein complexes called secretion systems. Gram-negative bacteria possess six secretion machineries, each of divergent composition and function (Desvaux *et al.*, 2009; Bleves *et al.*, 2010). *Pseudomonas aeruginosa*, an opportunistic human pathogen associated with a variety of acute and chronic diseases, possesses five of these, including the types III and VI secretion systems (T3SS and T6SSs) (Bleves *et al.*, 2010). A defining feature of these two protein export machines is that they allow for the export of cargo proteins directly into eukaryotic and/or prokaryotic cells (Cornelis, 2010; Schwarz *et al.*, 2010). While the composition and mechanism of the T3SS has been studied in great detail (Galan and Wolf-Watz, 2006; Mattei *et al.*, 2010), the existence of T6SS has been described only recently and their function and composition are still largely unexplored.

T6SSs are encoded by genes organized in operons within genetic islands. In some instances, multiple T6SS are present in a given bacterial genome (Cascales, 2008; Filloux *et al.*, 2008; Boyer *et al.*, 2009). Bioinformatics studies revealed that T6SSs are composed of 13 highly conserved core components and a set of additional proteins termed Tags (type six secretion-associated genes) (Shalom *et al.*, 2007; Boyer *et al.*, 2009). Two core components are similar to IcmF and DotU of type IV secretion

system (T4SS) (Fronzes *et al*., 2009; Ma *et al*., 2009), and one protein, ClpV, belongs to a family of AAA+ ATPases (Bonemann *et al*., 2009). A hallmark of all T6SS is the presence of two conserved proteins, Hcp (haemolysin co-regulated protein) and VgrG (valine-glycine repeat protein G). These proteins share sequence and structural homology with tail tube and spike proteins of bacteriophage, respectively (Mougous *et al*., 2006; Pukatzki *et al*., 2007; 2009; Leiman *et al*., 2009), and are proposed to form an injection device. Some specialized VgrG proteins possess C-terminal domains that contain eukaryotic cell effector activities. An example is VgrG-1 of *Vibrio cholerae*, which can cross-link host actin (Pukatzki *et al*., 2007).

Hcp Secretion Islands I–III (HSI-I to III) of *P. aeruginosa* encode three potential T6SSs. The HSI-I-encoded T6SS (H1-T6SS) has been shown to be active in chronic *P. aeruginosa* infections, as sputum of chronically infected cystic fibrosis patients contains Hcp1 and the serum of these patients shows the presence of Hcp1-specific antibodies (Mougous *et al*., 2006). Furthermore, a mutation in HSI-I operons affected the survival of *P. aeruginosa* in a rat model of chronic respiratory infection (Potvin *et al*., 2003). The H1-T6SS specifically exports at least three proteins, Tse1, Tse2 and Tse3 (type VI secretion exported 1–3). These proteins are important for fitness in interbacterial competition assays (Hood *et al*., 2010). While the target of Tse2 is not known, Tse1 and Tse3 are toxins targeting peptidoglycan of adjacent bacteria (Russell *et al*., 2011).

As with other secretion systems of *P. aeruginosa*, the H1-T6SS is finely regulated at several levels. Goodman *et al*. have shown that HSI-I operons are posttranscriptionally regulated by the Gac/Rsm pathway. Two sensor kinase/response regulator hybrid proteins, RetS and LadS, reciprocally regulate the H1-T6SS through this pathway (Goodman *et al*., 2004; Moscoso *et al*., 2011). A second level of regulation is exerted directly on H1-T6SS activity and depends on a set of accessory genes within HSI-I (Fig. S1). Among the proteins encoded by these genes are a trans-membrane threonine protein kinase, PpkA, a PP2C-type phosphatase, PppA, and a periplasmic protein, TagR, which promotes dimerization-induced activity of the kinase (Mougous *et al*., 2007; Hsu *et al*., 2009). The cytoplasmic target of PpkA is a Forkhead Associated domain (FHA)-harbouring protein, Fha1. Fha1 is in a complex with the ClpV1 ATPase, and upon phosphorylation of Fha1, effector export is triggered (Mougous *et al*., 2007; Hsu *et al*., 2009). This regulatory pathway will be referred to hereafter as the threonine phosphorylation pathway (TPP; Silverman *et al*., 2011). Reminiscent of T3SS activity, the intoxication of target cells by T6S effectors requires close cell–cell contact (Pettersson *et al*., 1996; Hayes *et al*., 2011). Interestingly, the TPP is stimulated when *P. aeruginosa* is grown on a surface, as observed by increased levels of phosphorylated Fha1 and Hcp1 secretion (Silverman *et al*., 2011). These findings suggest that the TPP responds to specific physiological stimuli. In addition to this pathway, TagF, a protein encoded upstream of *pppA*, has been reported to function as a negative posttranslational regulator of the H1-T6SS that acts independently of the TPP (Silverman *et al*., 2011).

Three uncharacterized genes, *tagT*, *tagS* and *tagQ*, neighbour genes involved in the TPP. In the current study, we found that the proteins encoded by these genes act upstream of PpkA in the TPP and are required for efficient protein transport through the T6S machinery. We demonstrated that in a heterologous host, TagT and TagS form a membrane-bound complex with ATPase activity, features characteristic of bacterial ABC transporters. We also investigated the localization of TagQ and showed that its outer membrane localization requires a conserved cysteine within the lipo-box sequence. TagQ, but not TagTS, is required for association of the kinase activator, TagR, with the outer membrane fraction. These findings, together with *in silico* analysis of available genomes, illustrate the complexity and novelty of trans-membrane signalling that lead to tuning of H1-T6SS activity.

Results

TagT, TagS and TagQ participate in posttranslational regulation of the H1-T6SS

TagT, TagS and TagQ are non-conserved T6SS components (Boyer *et al*., 2009) encoded within the HSI-I operon that contains each of the known posttranslational regulators of the system (*tagR*, *ppkA*, *pppA* and *tagF*) (Mougous *et al*., 2007; Hsu *et al*., 2009; Silverman *et al*., 2011). Furthermore, conserved synteny of *tag* genes with *ppkA* and *pppA* in *Pseudomonas* species, *Pseudomonas fluorescens*, *Pseudomonas mendocina* and *Pseudomonas brassicacearum* (http://www.pseudomonas.com/), suggests their functional relationship (Fig. S1).

Our previous work on the H1-T6SS has shown that the basal activation of the system in wild-type cells is exceedingly low. Indeed, under planktonic conditions, the quantity of secreted Hcp1 is below standard detection levels. However, using more sensitive detection methods, we found that basal Hcp1 secretion levels can be distinguished from background levels observed in an H1-T6SS-inactive strain (Δ*ppkA*) (Fig. 1A). In this study, we utilize this basal level of Hcp1 secretion as a means to investigate genes involved in H1-T6SS activation. To evaluate the contribution of TagT, TagS and TagQ to T6S activity, Hcp1 secretion levels were assayed in strains with *tagT*, *tagS* or *tagQ* deletions. Secreted Hcp1 levels of these strains were compared with strains that abrogate Hcp1 secretion (Δ*ppkA* and Δ*tagR*). As shown in Fig. 1A,

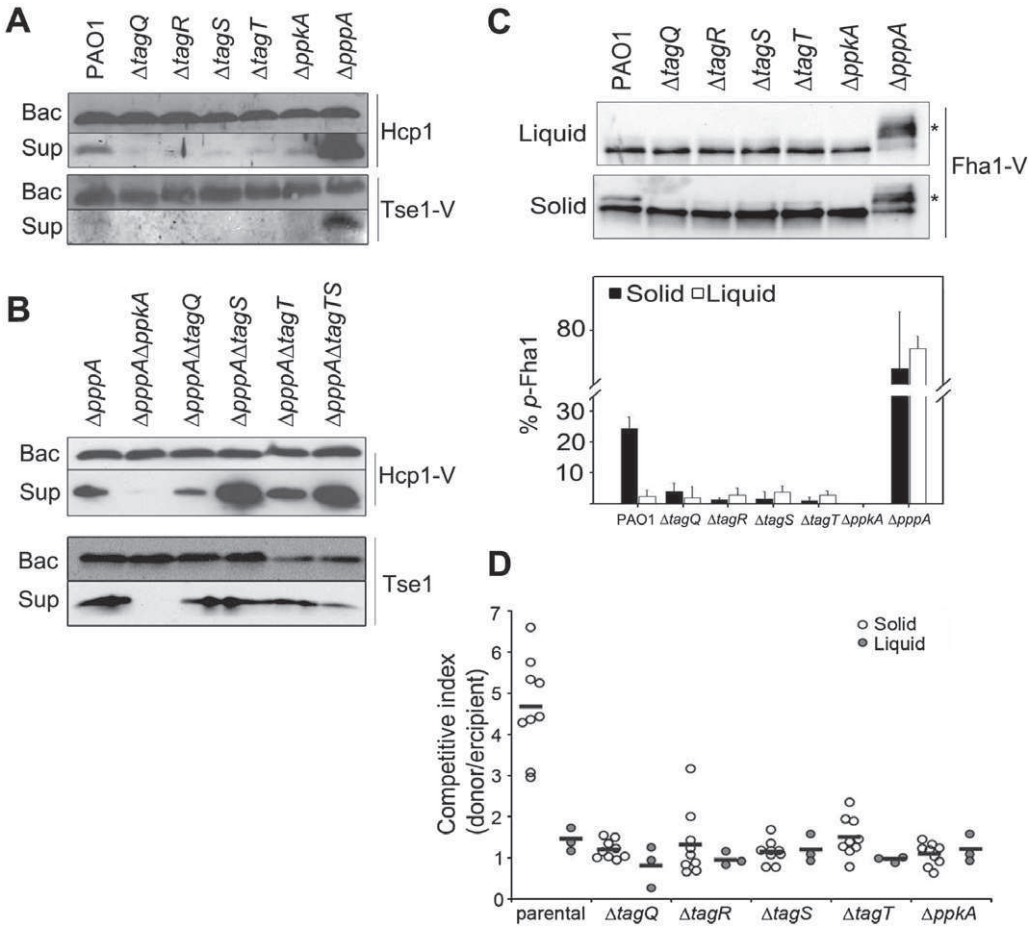

Fig. 1. TagT, TagS and TagQ are required for activation of the H1-T6SS.
A. Analysis of Hcp1 (structural component) and Tse1 (effector) export from strains containing in-frame deletions of *tag* genes.
B. Cellular and secreted Hcp1 and Tse1 from strains lacking Δ*pppA* and indicated *tag* gene.
C. Western blot analysis of Fha1 from indicated strains grown either in liquid or on solid medium. The band corresponding to phosphorylated Fha1 (*p*-Fha1) is indicated by an asterisk. Lower panel corresponds to the quantification of *p*-Fha1. Experiments were performed in triplicate (black = solid grown, white = liquid grown). Bac: bacteria, sup: supernatant.
D. *P. aeruginosa* requires *tagT*, *S*, *R* and *Q* for an H1-T6SS-dependent fitness advantage against competing bacteria. The competitive index is plotted for competitions between each indicated donor strain and a H1-T6SS-susceptible recipient strain of *P. aeruginosa* (PAO1 Δ*tse2* Δ*tsi2*) (Hood *et al*., 2010) (white = solid grown, gray = liquid grown).

individual isogenic PAO1 mutants (Δ*tagT*, Δ*tagS* and Δ*tagQ*) were impaired in Hcp1 export.

The H1-T6SS exports three low-molecular weight effectors encoded by genes outside of the HSI-I locus, Tse1, Tse2 and Tse3 (Hood *et al*., 2010). To determine the effect of *tagT*, *tagS* and *tagQ* genes on Tse export, we analysed secretion levels of Tse1 in the mutant strains. To detect Tse1, a chromosomal fusion of vesicular stomatitis virus G encoding sequence (VSV-G) to *tse1* (Tse1–V) was used (Hood *et al*., 2010). As in the case of Hcp1, the quantity of secreted Tse1–V was decreased in strains lacking *tagT*, *tagS* and *tagQ*. To gain information regarding the functional hierarchy of the Tag proteins relative to the TPP, we examined Hcp1 and Tse1 export in mutants prepared in the Δ*pppA* background. Strains lacking both the *tag* genes and *pppA* did not display a decrease in Hcp1 secretion levels (Fig. 1B), suggesting that these proteins represent regulatory accessory components that act upstream of the kinase/phosphatase checkpoint. Interestingly, the deletion of *tagS* in strains lacking *pppA* reproducibly resulted in higher levels of exported Hcp1, but not Tse1, relative to *pppA* and the other *tag* genes. Possible explanations for this finding are discussed below.

Phosphorylation of Fha1 requires PpkA and is promoted by growing bacteria on solid medium (Silverman *et al*., 2011). To determine if TagT, TagS and TagQ affect phosphorylation of Fha1, we assayed phosphorylated Fha1 (*p*-Fha1) levels in *tag* deletion strains. A chromosomal *fha1-VSV-G* fusion was used and *p*-Fha1 levels were detected by electrophoretic mobility shift (Fig. 1C). Consistent with previous studies, we observed an increase in *p*-Fha1 levels in the wild-type background

when grown on solid versus liquid media. Interestingly, individual *tag* deletion strains abrogated surface growth-dependent *p*-Fha1 levels. As expected, under liquid growth conditions, only low levels of *p*-Fha1 were detected in all strains containing PppA. Taken together with the secretion phenotypes observed, these results suggest that TagT, TagS and TagQ act upstream of PpkA in the TPP.

tagT, S *and* Q *are required for T6S-dependent fitness*

Previous studies have shown that the TPP activates the H1-T6SS during surface growth and therefore is required for H1-T6S-dependent fitness against competing bacteria (Silverman *et al*., 2011). We hypothesized that if TagT, S and Q act upstream of PpkA in the TPP, these proteins should contribute to H1-T6SS-dependent fitness. Using growth competition assays, we assessed the fitness of donor strains containing in-frame deletion of *tagT*, *S* or *Q* relative to a Tse2-sensitive recipient strain (Δ*tse2* Δ*tsi2*) (Fig. 1D). This experiment confirmed that *tagT*, *S* or *Q* are required for H1-T6SS-dependent fitness. As an additional control, we included a donor strain lacking TagR, a protein previously demonstrated to act upstream of PpkA in the TPP. This strain also displayed a loss of H1-T6SS-dependent fitness. Together with their involvement in promoting surface-dependent Fha1 phosphorylation, these findings support a critical role for TagT, S and Q in TPP activation during surface growth.

TagT and TagS form a membrane-bound complex with ATPase activity

TagT and TagS share sequence signatures with bacterial ABC transporters (Davidson *et al*., 2008). The *tagT* gene encodes a protein of 26 kDa with Walker domains (Walker A and Walker B) and other conserved features of ATPases associated with ABC transporters as shown in Fig. 2A. The *tagS* gene encodes a protein of 42 kDa, predicted to be an integral membrane protein with four hydrophobic trans-membrane helices (TMH) and a long periplasmic segment of 233 amino acids between TMH1 and TMH2. BLAST analysis showed high homology of TagS and TagT with membrane components of the lipoprotein outer membrane localization (Lol) complex (Narita and Tokuda, 2006), sharing 56.0%/38.5% and 40.0% similarity with LolE/LolC and LolD of *P. aeruginosa*, respectively (Tanaka *et al*., 2007) (Fig. 2A). Based on sequence homology, the predicted TagT protein belongs to a family of MecA/FtsE/SalX ATPases of bacterial ABC transporters (see also *Discussion*).

To investigate the mechanism by which TagT, S and Q regulate the H1-T6SS through the TPP, we purified the proteins and conducted biochemical analyses. Co-production of TagT and TagS resulted in formation of a stable protein complex associated with *Escherichia coli* membranes that could be solubilized by a detergent and purified to homogeneity by affinity chromatography (Fig. 2B). As mentioned previously, TagT harbours all conserved signatures of classical ATPases. In order to test whether the TagTS complex is capable of ATP hydrolysis, the complex was incubated in the presence of ATP and magnesium and the formation of inorganic phosphate (Pi) was quantified by a malachite green method (Van Veldhoven and Mannaerts, 1987). Notably, the TagTS complex displayed significant ATPase activity varying, in three independent purifications, between 70 and 100 nmolPi min^{-1} mg^{-1}. The specific activities of the TagTS complex was consistent with activities of several bacterial ABC transporters reported to date (Ravaud *et al*., 2006; Ward *et al*., 2007; Torres *et al*., 2009). Moreover, the ATPase activity of the complex was sensitive to orthovanadate (Fig. 2C), a small organic molecule that impedes ATP hydrolysis by interfering with binding of ATP to the Walker A motif (Pezza *et al*., 2002). Finally, to confirm that ATP hydrolysis was due to the TagTS complex, we replaced a conserved amino acid within the Walker A motif of TagT (K/A: Lys 44 to Ala) and purified the complex (Fig. 2B). The K/A mutation abolished ATPase activity of the TagTS complex (Fig. 2C). In conclusion, these results clearly show that the TagTS complex possesses ATPase activity that is dependent on the conserved Walker A motif within the TagT ATPase.

TagQ encodes an outer membrane lipoprotein

The last gene of the operon, *tagQ*, encodes a 31.7 kDa protein with a stretch of hydrophobic and uncharged amino acids at the N-terminus. This sequence is characteristic of a signal peptide and conserved lipo-box with an invariable Cysteine, a sequence recognized by signal peptidase II (Babu *et al*., 2006) (Fig. 3A). In order to study the localization of TagQ in *P. aeruginosa*, we created a fusion protein between TagQ and the red fluorescent protein mCherry, and examined its localization by confocal microscopy using green fluorescent protein (GFP)-expressing *P. aeruginosa* strains (GFP TagQ-mCherry). TagQ-mCherry was readily detected and localized around the periphery of bacterial cells (Fig. 3A). To gain information on the precise localization of TagQ, *P. aeruginosa* cells were treated with lysozyme to create spheroplasts, bacterial cells lacking the peptidoglycan layer. *P. aeruginosa* spheroplasts, while maintaining an intact internal membrane, appear to have a crescent-shaped external membrane as it begins to dissociate from the rest of the spheroplast (Lewenza *et al*., 2008). When *P. aeruginosa* GFP TagQ-mCherry was treated to obtain spheroplasts, the majority of cells retained mCherry fluorescence in the

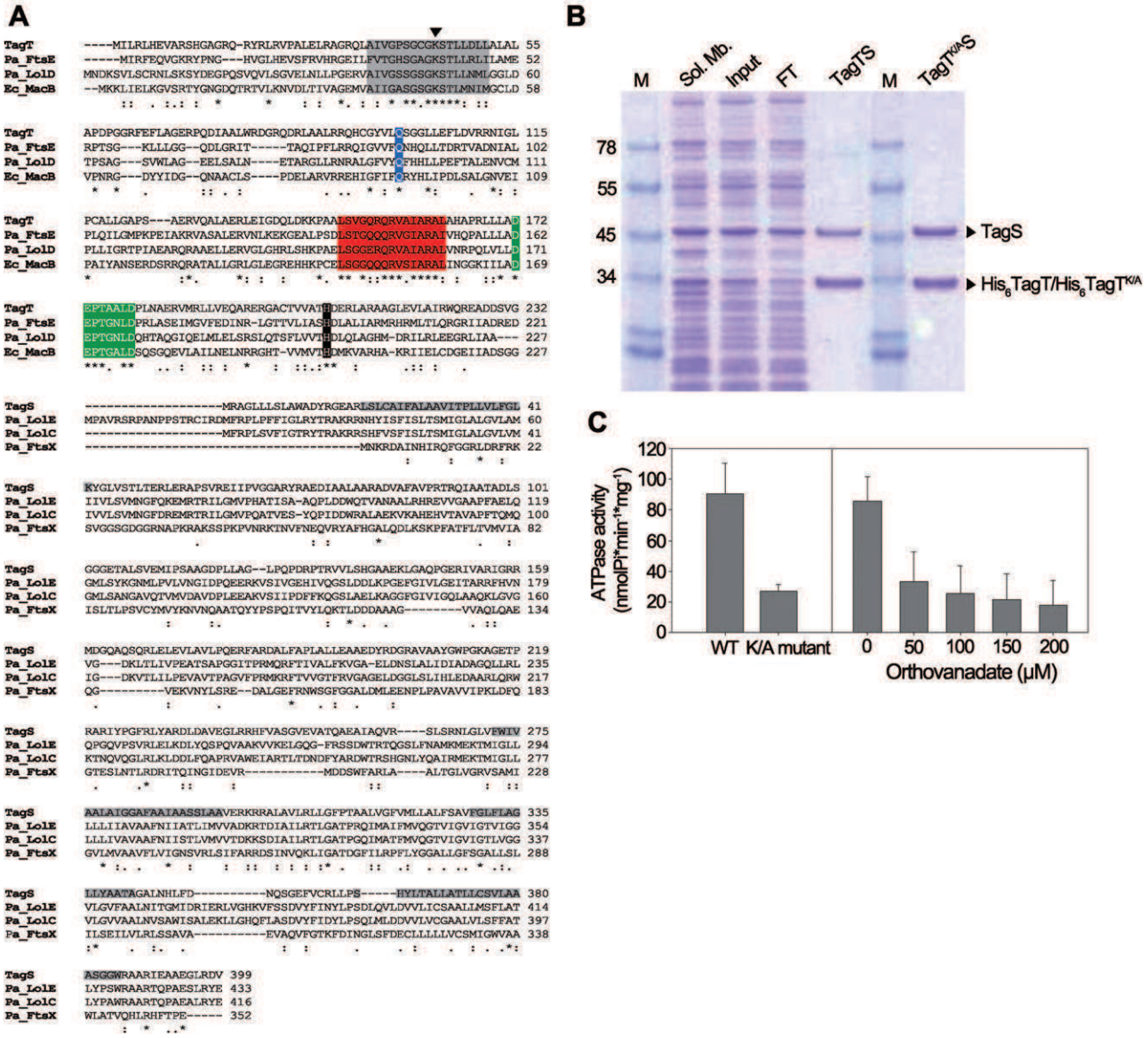

Fig. 2. TagT and TagS form a membrane-bound complex harbouring ATPase activity.
A. Sequence alignment of TagT and TagS with ABC transporters of the same family in *P. aeruginosa* (Pa) and *E. coli* (Ec). Conserved features of TagT are highlighted in colours: Walker A domain (grey), 'Q' motif (blue), ABC transporter signature (red), Walker B motif (green) and 'H' signature (black). The arrow indicates the conserved Lys residue mutated in the TagT$^{K/A}$S mutant. TagS TMHs are shown in grey background.
B. SDS-PAGE analysis of different fractions obtained during His$_6$-TagTS expression, solubilization and purification. M: molecular weight marker (kDa); Sol. mb: detergent-solubilized membranes; FT: flow through; TagTS: wild-type complex eluted with 200 mM imidazole; TagT$^{K/A}$S: Walker A mutant eluted with 200 mM imidazole.
C. Fractions collected in 200 mM imidazole elution step were analysed for ATPase activity by malachite green assay. The ATPase activity of the wild-type complex was inhibited by orthovanadate in a dose-dependent manner.

crescent-shaped labelling pattern particularly visible under xyz scan (Fig. 3A), strongly suggesting the association of TagQ with the outer bacterial membrane. Lipid modifications occur at a conserved cysteine within a lipobox sequence of lipoproteins and promote its association with membranes. To test the requirement for Cys30 in TagQ-mCherry localization, we created a mutant fusion protein, TagQΔCys-mCherry, and checked its localization (Fig. 3A). *P. aeruginosa* GFP TagQΔCys-mCherry cultures systematically showed a mixed population, with the majority of cells harbouring mCherry at the periphery and some cells displaying overlapping cytoplasmic GFP and mCherry. This result suggests that the absence of Cys30 affects the efficient transport of the protein across the inner membrane. The majority of spheroplasts obtained from TagQΔCys-mCherry *P. aeruginosa* lost outer mem-

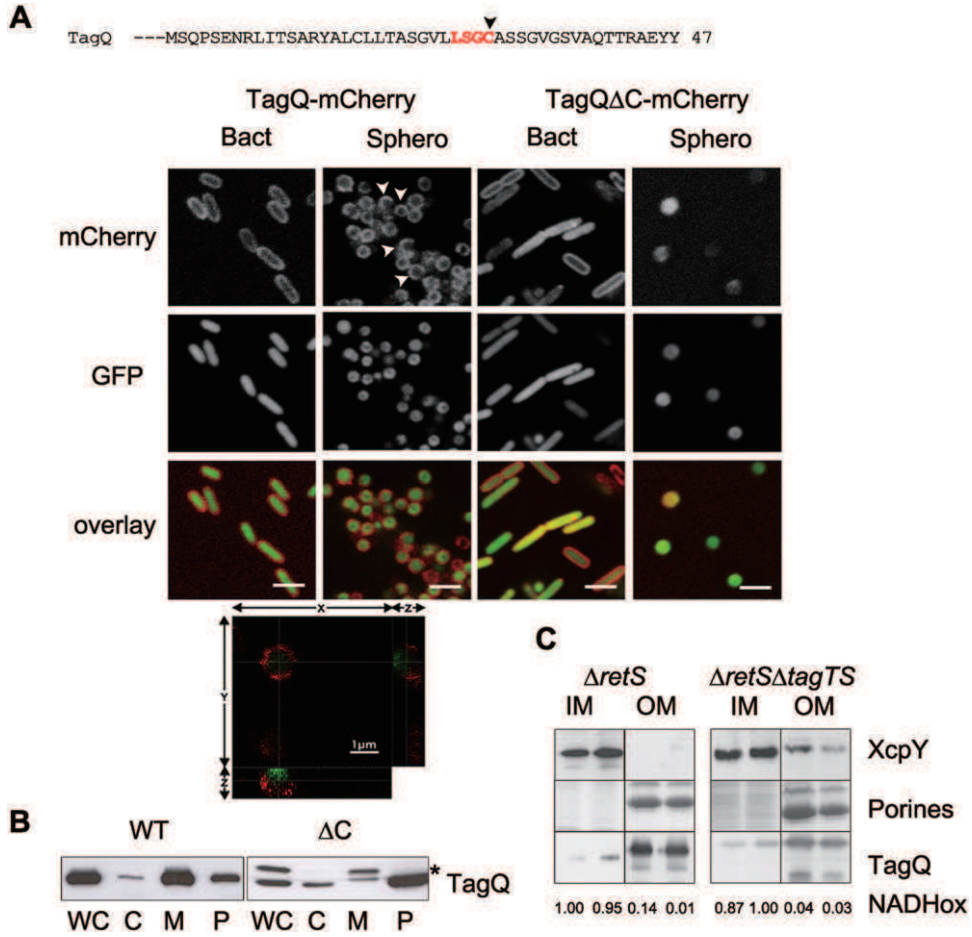

Fig. 3. TagQ is an outer membrane lipoprotein that localizes independent of TagTS.
A. The N-terminal sequence and lipo-box (in red) of TagQ is shown. The mutated cysteine is indicated by an arrow. Confocal microscopy images of PAO1Δ*retS*-GFP producing TagQ-mCherry or TagQΔCys-mCherry. TagQ-mCherry is localized at the bacterial periphery. White arrows on spheroplast images indicate the crescent-shape labelling of the OM. The bar represents 2 µm. Bact: bacteria; Sphero: spheroplasts. The confocal image in XYZ confirms the presence of the protein in the OM.
B. Subcellular fractionation of PAO1 Δ*tagQ* complemented with a plasmid encoding TagQ (WT) or TagQΔCys (ΔC). Whole cells (WC), cytosol (C), membranes (M) and periplasm (P) were analysed by Western blot using specific antibodies against TagQ. ΔCys mutation results in partial processing of the protein (indicated with an asterisk) and its accumulation in the periplasm.
C. Discontinuous sucrose gradient separation of inner and outer membranes in PAO1Δ*retS* and PAO1 Δ*retS*Δ*tagTS*. Fractions were characterized by NADH oxidase (NADHox) activity, SDS-PAGE and Western blotting using anti-TagQ antibodies. NADH oxidase activity is represented relative to the fraction with the highest level activity (noted as 1.0). XcpY was used as an IM marker and porines visualized in Coomassie blue stained gels as an OM marker. The representative IM and OM fractions are indicated. The analysis of the whole gradient is shown in Fig. S2.

brane labelling, strongly suggesting that in most cases the protein had lost its lipid anchor and was released by lysozyme treatment (Fig. 3A). These observations were immuno-quantified by fractionation of membranes and periplasm of Δ*tagQ* strains ectopically expressing wild-type *tagQ* or *tagQΔCys* (Fig. 3B). The deletion of Cys30 resulted in partial processing of the protein, as two anti-TagQ-specific polypeptides were visualized in total bacterial extracts. In addition, while the majority of wild-type TagQ was found to be associated with membrane fractions, the majority of the mutated protein was recovered in periplasmic fractions, in concordance with confocal microscopy observations.

The Lol-like TagTS complex is dispensable for OM localization of TagQ and TssJ1

As the TagTS complex shows significant homology with lipoprotein recycling systems of *E. coli* and *P. aeruginosa*, we first hypothesized that this complex is involved in the transport of specific lipoproteins of the H1-T6SS. To compare membrane distribution of TagQ in different strains, we set up membrane fractionation experiments on discontinuous sucrose gradients coupled to immunodetection. These experiments were performed using *P. aeruginosa* Δ*retS* background to obtain higher expression of the whole HSI-I locus. The quality of separation

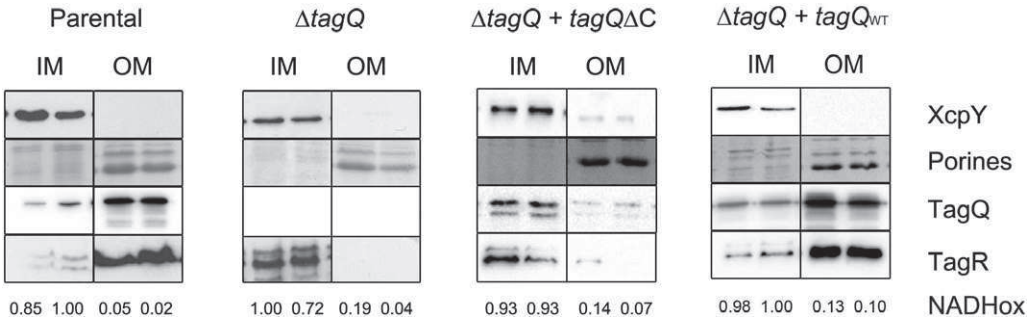

Fig. 4. Outer membrane localized TagQ is required for outer membrane anchoring of TagR. Membrane separations and analysis were performed as described in Fig. 3C. Strains used were: PAO1 (parental), PAO1Δ*tagQ* and PAO1Δ*tagQ* expressing either TagQΔCys or $TagQ_{WT}$. Only the representative IM and OM fractions are shown. TagR and TagQ were detected by specific antibodies.

between inner (IM) and outer membranes (OM) was systematically determined by measuring the activity of NADH oxidase and by Coomassie blue staining of SDS-PAGE gels of each recovered fraction. As shown in Fig. 3C, in accordance with microscopy experiments, the majority of TagQ was found in OM fractions. In order to determine whether the Lol-like ABC transporter TagTS was involved in localization of TagQ, we fractionated membranes of Δ*retS*Δ*tagTS* in an identical manner and detected no significant difference of TagQ distribution between the two strains (Figs 3C and S2 for complete gradients). The second lipoprotein of the H1-T6SS is TssJ1 (PA0080). TssJ1 shares 50% similarity with OM lipoprotein SciN involved in assembly of the Sci-1 T6SS of enteroaggregative *E. coli* (Aschtgen *et al*., 2008). To ascertain whether TssJ1 is targeted to the OM in *P. aeruginosa*, we constructed a fusion between TssJ1 and mCherry, and examined its localization by confocal microscopy (not shown) and by fractionation on discontinues sucrose gradients. Similar to our findings with TagQ, TssJ1-mCherry associated with the OM and its localization was not significantly altered in the *tagTS* mutant (Fig. S3). Together these results show that the ABC transporter TagTS, despite its strong homology to the Lol system, does not participate in membrane targeting of two H1-T6SS-specific lipoproteins.

TagQ is required for TagR association with the OM

Preliminary nanoLC/LC mass spectrometry data performed on inner and outer membrane fractions (M.G. Casabona and Y. Couté, unpublished) indicated the presence of TagR, a positive regulator of the TPP, in OM fractions. This result was intriguing, as TagR is predicted to be a soluble protein and was shown to fractionate with the periplasm, wherefrom it promotes dimerization and activation of PpkA (Hsu *et al*., 2009). To further address the localization of TagR, we raised anti-TagR antibodies and analysed fractionated membranes from wild-type PAO1, confirming that at least one portion of TagR associated with outer membranes (Fig. 4). This association was significant, as a soluble periplasmic protein, DsbA, was not found in any of the membrane fractions (not shown). Whereas TagR OM localization was not altered in a *tagTS* mutant (Fig. S4), the absence of *tagQ* clearly influenced the distribution of TagR between inner and outer membrane fractions (Fig. 4). In accordance, a strain expressing *tagQΔCys in trans*, resulted in TagR mislocalization, demonstrating that OM-anchored TagQ is essential for OM localization of TagR. Of note, TagR was found dispensable for OM localization of the TagQ lipoprotein (Fig. S5).

In silico *genome wide analysis of TagTSR-like systems*

Participation of the TagTS complex in trans-membrane signalling involving a periplasmic protein TagR and an inner membrane-bound Ser/Thr kinase prompted us to perform *in silico* analysis of all available complete bacterial genomes to search for homologues of these proteins. Both TagT and TagS attributed COGs (COG1136-COG4591) are frequently adjacent to other ABC transporter-specific COGs involved in peptide and drug transport, specifically COG0845 and COG0577 which are membrane components of the AcrA and SalY family of multidrug efflux pumps and antimicrobial peptides transport systems, respectively. We found also two strong associations between COG1136-COG4591 tandem and regulatory partners. In 90 *Firmicutes*, ABC transporters are encoded adjacent to homologues of the OmpR family two-component regulatory system (COG0642 and COG0745). In addition, in 100 *Enterobacteriaceae*, ABC transporters are encoded upstream of NagC (COG1940), a transcriptional regulator involved in sugar transport. This observation suggests a more general functional relationship between the TagTS family of ABC transporters with trans-membrane signalling and regulation. Finally, we examined the local organization of chromosomal regions

encoding the ATPase component (TagT, COG1136), the predicted permease component (TagS, COG4591) and COG1262 (TagR) in all available microbial genomes. TagQ was excluded from this screen as it lacks an attributable COG number. As shown by a maximum likelihood tree (Fig. 5), *tagT*, *S* and *R* are predominately found within T6SS-encoding loci in *Pseudomonas (P. aeruginosa, Pseudomonas fulva*, *P. fluorescens*, *P. brassicacearum)*. We found that in *Rhodobacter sphaeroides*, an α-proteobacterium that possess a T6SS locus with similar gene content of HSI-I, *tagT*, *S* and *R* are neighbouring genes coding for PpkA and PppA homologues. However, *tagT*, *S* and *R* are not exclusively found in bacterial genomes encoding T6SS, suggesting that these components may play a role in different cellular processes.

Discussion

Our current study identified TagT, TagS and TagQ as new components of a posttranslational regulatory pathway that modulates the activity of the H1-T6SS of *P. aeruginosa.* We found that these components participate in a phosphorelay system that is stimulated by surface growth conditions. Phosphorelay systems are classical ways for bacteria to regulate adaptive cellular responses induced by external stimuli; however, to our knowledge, this is the first example of an ABC transporter complex, TagTS, participating in trans-membrane signalling that involves a Ser/Thr kinase-dependent phosphorylation pathway.

Some parallels can be made with a large family of 'co-sensor' ABC transporters that interact with membrane-integrated histidine kinases (HK) (Tetsch *et al*., 2008; Tetsch and Jung, 2009). These proteins clearly play a regulatory role in adaptive responses to certain environmental stimuli (Tetsch *et al*., 2008). This is well illustrated by the ABC transporter PstSCAB of *E. coli* that is linked to sensing inorganic phosphate in phosphate-limiting conditions. It has been proposed that the membrane components of this ABC transporter transmit the signal towards the membrane-integrated HK, PhoR, which signals to a transcriptional regulator (response regulator, RR) (Makino *et al*., 1989). A role for related systems in resistance against antimicrobial peptides was recently proposed (Coumes-Florens *et al*., 2011; Dintner *et al*., 2011). For example, it has been experimentally demonstrated in *Staphylococcus aureus* that the detection of, and response to, antibiotic peptide bacitracin requires the interplay between two ABC transporters, BraD/BraE and VraD/VraE, and the HK/RR system BraS/BraR (Hiron *et al*., 2011). Interestingly, our *in silico* analysis showed that genes encoding TagTS-like proteins (COG1136-COG4591) frequently co-occur with genes encoding proteins that participate in two component-regulatory systems. In addition, our *in silico* analysis highlights the association of TagTS-like proteins with NagC, a transcriptional regulator involved in the response to *N*-acetylglucosamine and peptidoglycan in *E. coli* and *P. aeruginosa* (Pennetier *et al*., 2008; Korgaonkar and Whiteley, 2011), further implying their participation in signal recognition and transmission. It is worth noting that in at least one non-*Pseudomonas* T6SS, *Vibrio anguillarum*, an inner membrane polypeptide belonging to a major facilitator superfamily of transporters is required for regulating Hcp export and two additional periplasmic proteins contribute to this signalling (Weber *et al*., 2009).

The TagTS complex could be involved in export or import of a small molecule required for activating the H1-T6SS, or it may play a structural role by stabilizing other Tag proteins. Future experiments aim to determine whether TagT ATPase activity and a long periplasmic loop present in TagS are required for its role in signalling. The apparent conflicting effects on Hcp1 export between Δ*pppA* Δ*tagS* versus Δ*pppA* Δ*tagTS* (Fig. 1) suggest a possible second role for the integral inner membrane domain of TagS, independent of its ATPase partner, TagT. Interestingly, a long periplasmic loop present in TagS-related proteins, LolC and BraB, mediates the detection of cognate substrates, lipoproteins and bacitracin respectively, and it was proposed, for BraB, to interact through the inner membrane with the HK partner.

Outer membrane-localized TagQ is a top candidate for signal detection. The region from amino acids 68 to 114 of TagQ is annotated as PF05433, a family of proteins that include several *Rickettsia* genus specific 17 kDa surface antigens, annotated also as a conserved trans-membrane alpha-helical region containing glycine zipper motifs (http://pfam.sanger.ac.uk). There is rising evidence that OM lipoproteins play crucial roles in trans-membrane signalling in bacteria. A well-characterized example is the Rcs phosphorelay in *E. coli*, which reflects envelope stress response activated by peptidoglycan stress and antibiotics. In this complex system, RcsF, an OM lipoprotein, is proposed to transmit the signal to the IM-located HK sensor and allow further signal transduction from the cell envelope to the cytoplasm (Farris *et al*., 2010; Leverrier *et al*., 2011).

What may be the link between IM TagTS, OM TagQ and the IM PpkA-kinase? The finding that some portion of TagR is also associated with the OM indicates that TagTS, TagR and TagQ may represent a unique complex participating in trans-membrane signalling. Taking into account the sequence predictions for TagR, its role in signal transduction, and our current findings, we hypothesize that TagR associates with membranes through interactions with other OM proteins, such as TagQ. The distribution of TagR between inner and outer membranes was clearly

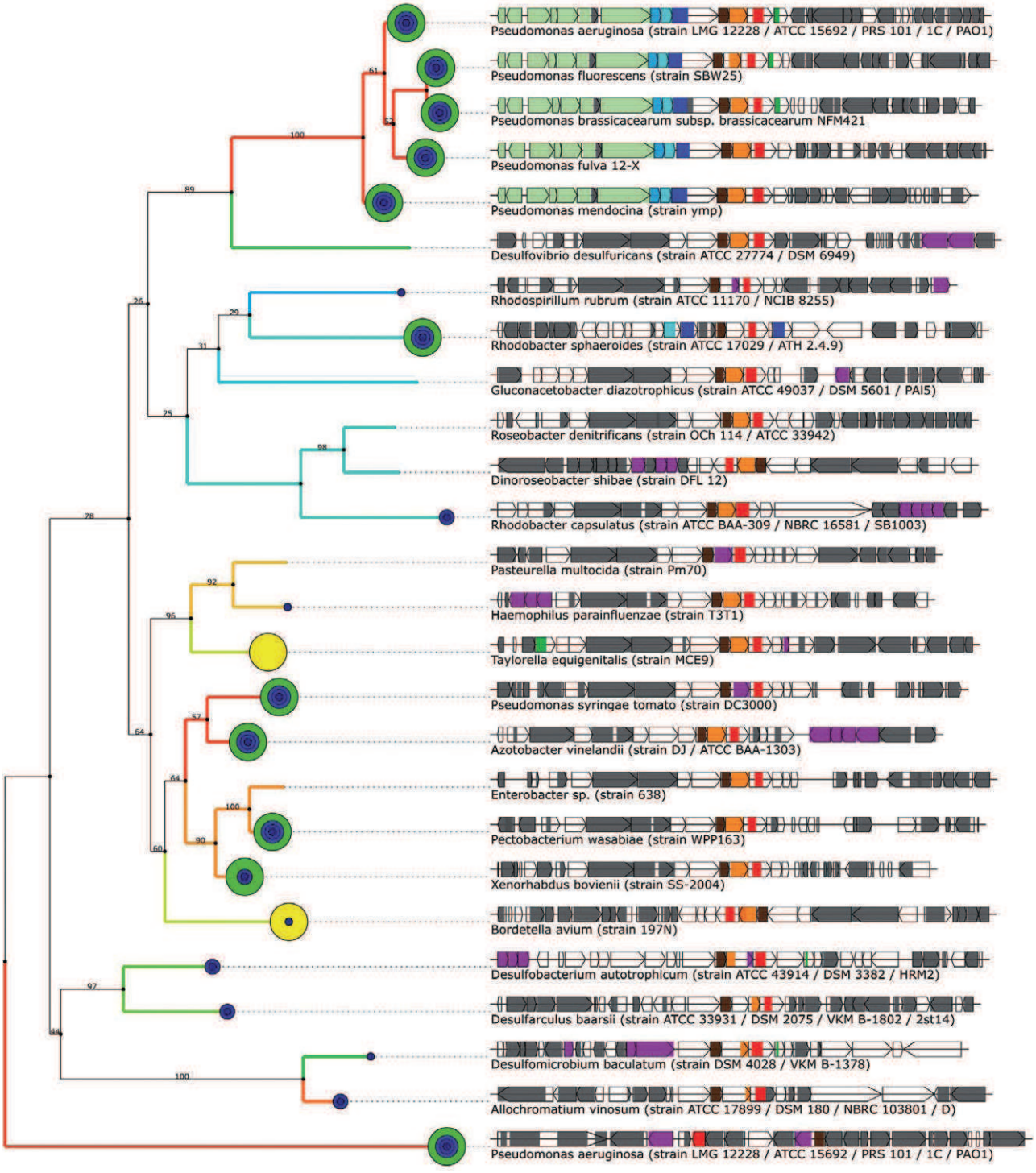

Fig. 5. *In silico* analysis of *tagTSR*-like genes. Maximum likelihood tree of TagR and TagR-like proteins (matching COG1262) with genomic context of the associated genes, together with the periplasmic PvdO protein of *P. aeruginosa* involved in pyoverdine maturation (Yeterian *et al.*, 2010) (and its genomic context) used as outgroup. Green and yellow circles indicate genomes with complete or incomplete T6SS respectively. Inside of circles, blue points indicate the presence of *pppA*/*ppkA*/*fha1*. Coloured boxes represent hits on the COG database; colours are according to Fig. S1, except for purple boxes representing hits on ABC transporter related COGs.

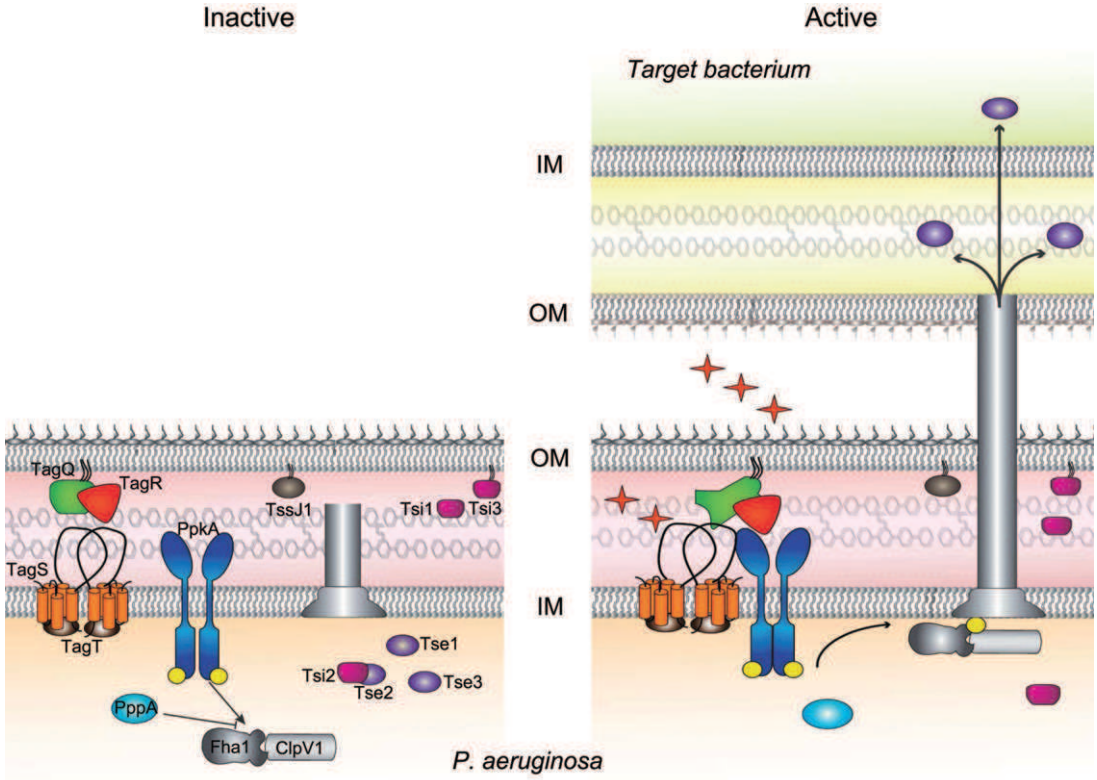

Fig. 6. Model for trans-membrane signalling leading to H1-T6SS activation. The H1-T6S machinery is represented as a single tunnel. Tse1–3 are T6S exported effectors. Molecular players biochemically characterized in this study are: TagQ (OM lipoprotein, *Pseudomonas* specific), TagS and TagT (ABC transporter), TagR (OM associated protein) and TssJ1 (conserved OM lipoprotein). The Ser/Thr kinase, PpkA, and the phosphatase, PppA, as well as the phosphorylation target protein Fha1, are represented. A putative signal is designated by red stars. Proteins are represented using the same colour code as the ORFs in Fig. S1, and yellow spheres indicate phosphorylation. IM and OM of *P. aeruginosa* and the target bacterium are shown.

affected by the absence of TagQ, leaving open the possibility that TagQ may correctly position TagR to interact with the kinase.

Finally, what is the nature of the signal(s) that activate the H1-T6SS? Silverman *et al*. discovered that surface growth of *P. aeruginosa* induces the TPP (Silverman *et al*., 2011), and, here, we show that *tagQ*, *tagR*, *tagS* and *tagT* are important in surface-induced phosphorylation of Fha1. Moreover, using growth competition assays we showed that the fitness of Tag mutants was impaired. All together these results imply that the predicted ABC transporter, TagTS, and the OM lipoprotein TagQ are essential players in intrabacterial communication through H1-T6SS activity. The rapid detection of neighbouring cells may thus be essential to turning on the injection device.

Although we have no evidence yet demonstrating direct interactions between the four Tag proteins (except for TagT and TagS) and the kinase, PpkA, we propose a model (Fig. 6) in which TagTS and TagQ participate in detection and transmission of an environmental signal to PpkA, by modulating either localization or conformation of the kinase activator TagR, leading to rapid response of «attacking» bacteria in highly competitive multi-species niches, such as infecting tissue. Our future work will aim at discovering the signal nature and deciphering the connections between Tag proteins and their link with other components of trans-membrane signalling in T6SS.

Experimental procedures

Genetic constructions

Genes of interest were PCR amplified using PAO1 genomic DNA as a template, cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced before cloning into final destination vectors. The list of oligonucleotides with appropriate restriction sites is given in Table S1. For overexpression, *tagT* and *tagTS* were amplified and cloned into pETDuet-1. In the pETDuet-*tagTS* bicistronic vector, only *tagT* is fused to a histidine tag encoding sequence. For Hcp1 overexpression, the gene was cloned into pET52b giving Hcp1-Strep fusion. The *tagQ* gene was amplified so that it lacks the sequence encoding the first 29 amino acids and the predicted lipo-box (TagQΔ2-30). It was cloned into pET15b by fusing *tagQ* to a sequence encoding hexa-histidine tag on N-terminus. For TagR overproduction, the *tagR* gene was amplified and

cloned into pET52b resulting into TagR-His10 protein. All fusions to *mCherry* were constructed using pJN105-derived plasmids, which contain an arabinose inducible promoter. The gene encoding mCherry was amplified and cloned into *Xba*I and *Sac*I sites of pJN105 (Newman and Fuqua, 1999) giving pJN-*mCherry*. The DNA sequence containing the ribosome binding site of *tagQ* and the whole gene was cloned upstream of *mCherry*-encoding gene using *Eco*RI and *Xba*I sites, giving pJN-*tagQ-mCherry*. *tssJ1*-encoding sequence was amplified and cloned in the same manner to give pJN-*tssJ1-mCherry*. Site-directed mutagenesis (QuikChangeII Site-Directed Mutagenesis kit, Stratagene) was employed to generate $TagT^{K/A}S$ mutant and TagQΔCys, using pETDuet-*tagTS* and pJN-*tagQ-mCherry* as templates respectively. The TGA stop codon was introduced between TagQ and mCherry encoding sequence by site directed mutagenesis using pJN-*tagQ-mCherry* and pJN-*tagQ*ΔCys-mCherry. To constitutively express GFP, pX2-*gfp* (Thibault *et al*., 2009) was transferred in mini-CTX1 (Hoang *et al*., 2000) and inserted into the chromosome of *P. aeruginosa* strains as described. All replicative plasmids were introduced in *P. aeruginosa* strains by transformation. Constructs used to generate in-frame deletions of *tag* genes or a *tse1-VSV-G* chromosomal fusion were previously reported (Silverman *et al*., 2011).

Pseudomonas aeruginosa strains were grown in Luria-Bertani (LB) medium at 37°C supplemented with carbenicillin 100–250 μg ml^{-1}, gentamycin 200 μg ml^{-1} and tetracycline 200 μg ml^{-1} when needed. *E. coli* strains were grown in LB medium at 37°C supplemented with ampicillin 100 μg ml^{-1}, gentamycin 50 μg ml^{-1}, kanamycin 25 μg ml^{-1}, tetracycline 10 μg ml^{-1} as required.

Fha1 phosphorylation assays

Cellular samples of Fha1 from liquid and solid grown cultures were prepared and analysed as previously described (Mougous *et al*., 2006; Hsu *et al*., 2009). Western blots were developed using chemiluminescent substrate (SuperSignal West Pico Substrate, Thermo Scientific) and imaged with a FluorChemQ (ProteinSimple). Densitometry was performed as previously described (Silverman *et al*., 2011) using AlphaView®Q software (ProteinSimple). The percentage of phosphorylated Fha1 was determined by measure band intensity of phosphorylated and total Fha1 from three independent experiments. The values were normalized to Δ*ppkA*, which was set at 0% *p*-Fha1.

Interbacterial growth competition assays and quantification

Growth competition assays were performed as previously described (Silverman *et al*., 2011). Each donor and recipient strain contained constitutively expressing yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP) respectively. pUCP18-mini-Tn7 containing *yfp* or *cfp* (inserted at the neutral phage attachment site, *attB*) was used to construct these strains (Lambertsen *et al*., 2004). The plasmids were introduced into *P. aeruginosa* via four-parental mating conjugation or electroporation (Choi and Schweizer, 2006). Vector backbones were not removed. To observe a H1-T6SS-dependent fitness advantage, Tse2 and Tsi2 were overexpressed in the donor strain. Donor strains harboured pPSV18::PA2702-PA2703 for constitutive expression of Tse2 and Tsi2, and recipient strains harboured the empty vector, pPSV18 (Rietsch *et al*., 2004). Overnight cultures were mixed at a 1:1 ratio to a total density of approximately 1.0×10^8 cfu ml^{-1} in 1 ml of LB medium. Competitions were grown on 0.2 μM polycarbonate membranes on LB agar for 18 h at 37°C, or in 2 ml of LB with shaking. Cells were re-suspended in LB medium and spotted onto 1.0% agarose PBS pads and imaged as described (Silverman *et al*., 2011). YFP and CFP filters were used to image the two cell populations. Assays were performed in triplicate. Three fields containing 100 to 200 cells were imaged for each competition. To determine the competitive index (the number of YFP positive cells to CFP positive cells), NIS-Elements computer-assisted morphometry was used to count YFP and CFP cells.

TagTS expression and protein purification

Escherichia coli BL21(DE3)Star cells harbouring pETDuet-*tagTS* were grown in LB medium at 37°C and 200 r.p.m. At OD_{600} of 0.7, the expression of *tagT* and *tagS* was induced with 0.5 mM IPTG for 4 h. Cells were recovered by centrifugation and lysed in buffer containing 25 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole, pH 8.0 and cocktail of protease inhibitors (PIC, Roche). The lysis was achieved by passage of cells through a Microfluidizer (M-100P, Microfluidics, USA) at constant pressure of 10 000 psi. After centrifugation at 200 000 *g* for 1 h, membranes were recovered in solubilization buffer containing 25 mM Tris-HCl, 500 mM NaCl, 2% n-dodecyl-β-D-maltopyranoside (DDM), 15% glycerol, pH 8.0 and PIC. The solubilization was performed in a glass beaker at 4°C for 1 h 30 min by agitation. The obtained suspension was further centrifuged at 200 000 *g* for 1 h. Solubilized material was loaded at 0.5 ml min^{-1} on HisTrapHP Ni^{2+}-column previously equilibrated in solubilization buffer. The washes and elution was performed on AktaPurifier FPLC apparatus (GE Healthcare) with buffer A (25 mM Tris-HCl, 500 mM NaCl, 0.05% DDM, 15% glycerol, pH 8.0) and buffer B (25 mM Tris-HCl, 500 mM NaCl, 0.05% DDM, 15% glycerol, 200 mM imidazole, pH 8.0). The washes were performed with 30 and 80 mM imidazole obtained by mixing buffers A and B, and elution was performed using buffer B. Fractions eluted from the column were analysed on SDS-PAGE and the gel was stained by Coomassie Brillant Blue R-250.

ATPase activity and orthovanadate assay

ATPase activity was quantified by measuring inorganic phosphate Pi by a malachite green method (Van Veldhoven and Mannaerts, 1987). The reaction mixture (100 μl) containing 0.5 mM ATP, 1 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.5, and 1 μg of purified TagTS complex was incubated for 30 min at 37°C. When indicated, the orthovanadate was added to protein samples at indicated concentrations before the reaction. The reaction was stopped by adding 800 μl of malachite green solution. Malachite green solution was prepared 30 min in advance by mixing 3 vols of 0.045% malachite

green with 1 vol. of 4.2% ammonium molybdate in 4 M HCl and 1/50 of volume of Triton X-100. Greenish colour resulting from precipitation of Pi was measured at 640 nm.

Secretion assays

Overnight cultures were diluted at OD_{600} of 0.02 and incubated at 37°C with shaking up to mid-log phase, in the presence of antibiotics when needed. At this point, 250 µl of bacteria were harvested by centrifugation at 7000 *g* and re-suspended in 100 µl of loading buffer and stored at –20°C until use. Extracellular proteins were precipitated by a TCA-sarkosyl method (0.5% final volume of sarkosyl and 7.5% final volume of TCA) (Chevallet *et al*., 2007) after a double centrifugation of 1250 µl of culture and 1 h incubation on ice. Pellets were re-suspended in a final volume of 25 µl of loading buffer. Samples were then analysed by SDS-PAGE and immunoblotting.

Proteomic analyses by mass spectrometry (MS)

Protein bands were manually excised from the gels and washed. Proteins were *in-gel* digested with trypsin (Promega, sequencing grade) and peptides extracted from gel slices (Shevchenko *et al*., 1996). The dried extracted peptides were re-suspended in 5% acetonitrile and 0.1% trifluoroacetic acid and analysed by online nanoLC-MS/MS (Ultimate 3000, Dionex and LTQ-Orbitrap XL, Thermo Fischer Scientific). The nanoLC method consisted in a 15 min gradient ranging from 5% to 40% acetronitrile in 0.1% formic acid at a flow rate of 300 nl min^{-1}. Peptides were sampled on a 300 µm × 5 mm PepMap C18 precolumn and separated on a 75 µm × 150 mm RP column (PepMap C18, Dionex). MS and MS/MS data were acquired using Xcalibur (Thermo Fischer Scientific) and processed automatically using Mascot Daemon software (version 2.3, Matrix Science). Searches against the PAO1 database, SwissProt-Trembl_decoy (*E. coli* taxonomy) and contaminants databases (534637 sequences) were performed using an in-house version of Mascot 2.3. ESI-TRAP was chosen as the instrument, trypsin/P as the enzyme and two missed cleavages was allowed. Precursor and fragment mass error tolerances were set respectively at 10 ppm and 0.6 Da. Peptide modifications allowed during the search were: Carbamidomethyl (C, fixed), Deamidated (NQ, variable), Oxidation (M, variable) and Acetyl (Protein N-term, variable). The IRMa soft (Dupierris *et al*., 2009) was used to filter the results by query homology threshold $P < 0.01$ and a minimum of two peptides per protein.

Spheroplast preparation and confocal microscopy

Overnight cultures of *P. aeruginosa*-GFP strains producing TagQ-mCherry, TagQΔC-mCherry or TssJ1-mCherry fusion proteins were diluted at OD_{600} of 0.15 in 3 ml of LB. Cultures were incubated until mid-log phase of growth in the presence of antibiotics and then induced for 1.5 h by 0.25% arabinose. At this point, cells were harvested and spheroplasts were created (Imperi *et al*., 2009). Briefly, 1 ml of culture was centrifuged at 6000 *g* for 5 min. The pellet was re-suspended in TSE buffer (0.1 M Tris-acetate, 16% saccharose, 5 mM EDTA, pH 8.2) and lysozyme was added at a final concentration of 50 µg $µl^{-1}$. Spheroplasts were incubated on ice and centrifuged at 1000 *g* for 5 min after the addition of $MgSO_4$ to a final concentration of 0.1 M. Finally, spheroplasts were re-suspended in TSM buffer (0.05 M Tris-acetate, 8% sucrose, 10 mM $MgSO_4$, pH 8.2). 1 ml culture of arabinose-induced bacteria was harvested by centrifugation at 7000 *g* for 5 min and re-suspended in 100 µl of LB.

Specimens were analysed by confocal laser scanning microscopy, using a Leica TCS-SP2 operating system (Manheim, Germany). GFP and mCherry fluorescences were excited and collected sequentially (400 Hz line by line) by using 488 nm for GFP and 543 nm for mCherry excitation. Fluorescence emissions were collected from 500 to 537 nm for GFP and from 557 to 625 nm for mCherry.

Fractionation of P. aeruginosa

Cultures of *P. aeruginosa* grown for 16 h were diluted to an OD_{600} of 0.15 in 30 ml of LB cultures with antibiotics when needed. Cultures were incubated with agitation until OD_{600} of 0.85 and at this point, 100 µl of cells were harvested by centrifugation as a total bacteria fraction. The rest of the cultures was centrifuged at 6000 *g* for 10 min. Pellets were washed with 10 ml of TMP buffer (10 mM Tris-HCl, 200 mM $MgCl_2$, PIC) and re-centrifuged at 6000 *g* for 10 min. Pellets were re-suspended in 1 ml of TMP and incubated for 30 min at 300 r.p.m. and 23°C in the presence of 0.5 mg ml^{-1} lysozyme. Next, bacteria were centrifuged at 8000 *g* for 15 min at 4°C, obtaining the periplasm fraction (supernatant) and spheroplasts (pellet). At this point, the proteins present in the periplasm fraction were precipitated (as described in *Secretion assays*). Spheroplasts were then re-suspended in 1 ml of TMP, recentrifuged and the pellet, recovered in 1 ml of TM buffer (30 mM Tris-HCl, 10 mM $MgCl_2$, pH 8.0), was disrupted by sonication. Unbroken spheroplasts were eliminated by a low speed centrifugation and the supernatant was ultracentrifugated for 30 min at 100 000 *g* with a TLA120 rotor at 4°C to obtain the cytosolic fraction (supernatant) and the total membrane fraction (pellet). All fractions were re-suspended in loading buffer and heated at 100°C for 10 min before SDS-PAGE and immunoblotting analysis.

Inner and outer membrane separation

Inner and outer membranes of *P. aeruginosa* cells were separated by a discontinuous sucrose gradient as described (Viarre *et al*., 2009). Briefly, 500 ml cultures of *P. aeruginosa* at OD_{600} of 1 were harvested by centrifugation. In the case of PAO1Δ*tagQ* + *tagQ*ΔCys, bacteria were grown with appropriate additives and induced by 0.01% arabinose at OD_{600} 0.5. Pellets were re-suspended in 25 ml of 10 mM Tris-HCl, 20% sucrose, 10 mg ml^{-1} DNase, 10 mg ml^{-1} RNase, pH 7.4, and were disrupted by using a Microfluidizer at 15 000 psi. Unbroken cells were removed by 15 min centrifugation at 6000 *g*. Total membrane fraction was obtained by ultracentrifugation at 100 000 *g* and re-suspended in 500 µl of 20% sucrose containing PIC. The total membrane fraction was then applied at the top of a discontinuous sucrose gradient

composed of 1.5 ml layers of 60%, 55%, 50%, 45%, 40%, 35% and 30% of sucrose in 10 mM Tris-HCl, 5 mM EDTA, pH 7.4 (from bottom to top). Sucrose gradients were centrifuged at 90 000 *g* at 4°C for 36–72 h, and 500 µl of fractions were collected from the top. All fractions were then characterized by SDS-PAGE, Western Blot analysis and NADH oxidase activity. Antibodies against T2SS protein XcpY (Michel *et al*., 1998), kindly gifted by R. Voulhoux (CNRS, Marseille, France), were used as an inner membrane marker and porines visualized directly on Coomassie blue-stained SDS-PAGE as markers of the outer membrane. NADH oxidase activity was determined as described elsewhere by measuring the NADH consumption at 340 nm of 50 µl of each fraction (Aubert *et al*., 2010).

Hcp1, TagQ, TagR and Tse1 expression for antibody production

The pET52b-*hcp1*, pET15b-*tagQ*Δ2-30 and pET15b-*tagR* expression vectors were introduced into *E. coli* BL21(DE3)Star. Expression was induced at OD_{600} of 0.7 by 0.5 mM IPTG and lasted for 4 h. Bacteria were lysed by the Microfluidizer at 10 000 psi and the proteins were purified on appropriate affinity columns using AktaPurifier (GE Healthcare). TagR-His_{10} was obtained by solubilization of inclusion bodies using 6 M guanidine. Antibodies were raised in guinea pig for Hcp1 (Eurogentec, Belgium) and in mouse for TagQ and TagR (Agro-Bio, France).

The anti-Tse1 polyclonal rabbit antibody was raised against purified Tse1 (GenScript).

Immunoblotting

For Western blotting, antibodies were used at dilutions: anti-Hcp1 1:5000, anti-XcpY 1:1000, anti-TagQ 1:20000, anti-TagR 1:1000 and anti-Tse1 1:2000. Commercial antibodies anti-VSV-G (Sigma Aldrich) and anti-mCherry (Clontech) were used as recommended by the manufacturers. Secondary antibodies were anti-rabbit (Zymed), HRP-coupled anti-guinea pig (Invitrogen) and HRP-coupled anti-mouse (Sigma), all used at 1:5000 dilution. Western blots were developed by ECL Detection Kit (Amersham) or Millipore HRP Substrate.

In silico *bacterial genome scanning for* tagTSR

Annotated genomes were downloaded (September 2011) from the Genome Reviews ftp site (ftp://ftp.ebi.ac.uk/pub/databases/genome_reviews/) (Sterk *et al*., 2006) and http://www.pseudomonas.com/ for *Pseudomonas* genomes unavailable in Genome Reviews (Winsor *et al*., 2011). Predicted protein sequences for all genomes were aligned with *rpsblast* (Altschul *et al*., 1997) against the COG section of the CDD database (September 2011) (Marchler-Bauer *et al*., 2009). COG hits were considered positive if their alignment covered at least 30% of the COG PSSM and had an *E*-value $\leq 10^{-6}$. TagR and *tagR*-like sequences were aligned using *muscle* (Edgar, 2004). Based on this alignment, a maximum likelihood tree with 100 bootstrap replicates was computed using PhyML (Guindon *et al*., 2010).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Organization of the *P. aeruginosa* (PA) HSI-I operon containing tag genes and comparison with *P. mendocina* ymp (Pmen), *P. fluorescens* (PFL) and *P.* ***brassicacearum*** **(PSEBR)**. Orthologues are represented in the same colour. Gene and COG numbers are indicated.

Figure S2. TagQ localization to the OM is not influenced by TagTS. Complete analysis of discontinuous sucrose gradients of PAO1Δ*retS* and PAO1Δ*retS*Δ*tagTS* are shown. NADH oxidase activity and XcpY are used as IM markers, and porines are used as OM markers. TagQ was detected using specific antibodies. Note that the NADH oxidase activity is represented relative to the fraction of highest activity. IM and OM are indicated.

Figure S3. The TagTS complex does not influence TssJ1-mCherry localization to the outer membrane. Discontinuous sucrose gradient separation of TssJ1-mCherry in PAO1Δ*retS* and PAO1Δ*retS*Δ*tagTS* strains. NADH oxidase activity and controls were as described in Fig. S2. Fusion protein TssJ1-mCherry was detected by anti-mCherry antibodies.

Figure S4. TagTS does not influence TagR localization to the OM. Discontinuous sucrose gradient separations shown in Fig. S2 were further analysed using anti-TagR antibodies, showing that TagR is localized to the OM both in PAO1Δ*retS* and PAO1Δ*retS*Δ*tagTS*.

Figure S5. TagR does not play a role in the OM localization of TagQ.

A. Confocal microscopy analysis of PAO1Δ*retS*Δ*tagR* expressing GFP and harbouring TagQ-mCherry. White arrows show the OM of *P. aeruginosa* that remains partially attached to spheroplasts after lysozyme treatment (see text for details). Bact: bacteria, Sphero: spheroplasts. The bar represents 1 μm.

B. Discontinuous sucrose gradient separation of the IM and OM of PAO1Δ*retS*Δ*tagR*. All fractions were analysed as described in Fig. S2.

Table S1. List of oligonucleotides used for genetic constructions.

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Supplementary Figure Legend

Figure S1. Organization of the *P. aeruginosa* (PA) HSI-I operon containing *tag* genes and comparison with *P. mendocina* ymp (Pmen), *P. fluorescens* (PFL) and *P. brassicacearum* (PSEBR). Orthologues are represented in the same colour. Gene and COG numbers are indicated.

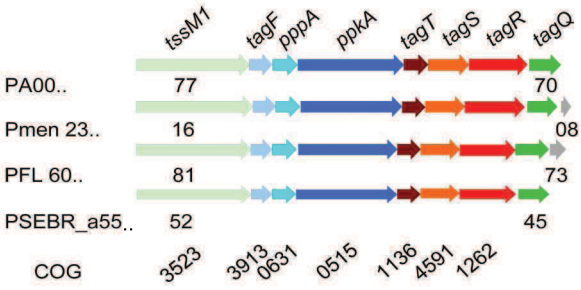
Figure S2. TagQ localization to the OM is not influenced by TagT-S. Complete analysis of discontinuous sucrose gradients of PAO1Δ*retS* and PAO1Δ*retS*Δ*tagTS* are shown. NADH oxidase activity and XcpY are used as IM markers, and porines are used as OM markers. TagQ was detected using specific antibodies. Note that the NADH oxidase activity is represented relative to the fraction of highest activity. IM and OM are indicated.

Figure S3. The TagTS complex does not influence TssJ1-mCherry localization to the outer membrane. Discontinuous sucrose gradient separation of TssJ1-mCherry in PAO1Δ*retS* and PAO1Δ*retS*Δ*tagTS* strains. NADH oxidase activity and controls were as described in Figure S3. Fusion protein TssJ1-mCherry was detected by anti-mCherry antibodies.

Figure S4. TagT-S does not influence TagR localization to the OM. Discontinuous sucrose gradient separations shown in Figure S2 were further analyzed using anti-TagR antibodies, showing that TagR is localized to the OM both in PAO1Δ*retS* and PAO1Δ*retS*Δ*tagTS*.

Figure S5. TagR does not play a role in the OM localization of TagQ. A. Confocal miscroscopy analysis of PAO1Δ*retS*Δ*tagR* expressing GFP and harbouring TagQ-mCherry. White arrows show the OM of *P. aeruginosa* that remains partially attached to spheroplasts after lysozyme treatment (see text for details). Bact: bacteria, Sphero:

spheroplasts. The bar represents 1 μm. **B.** Discontinuous sucrose gradient separation of the IM and OM of PAO1Δ*retS*Δ*tagR*. All fractions were analysed as described in Figure S2.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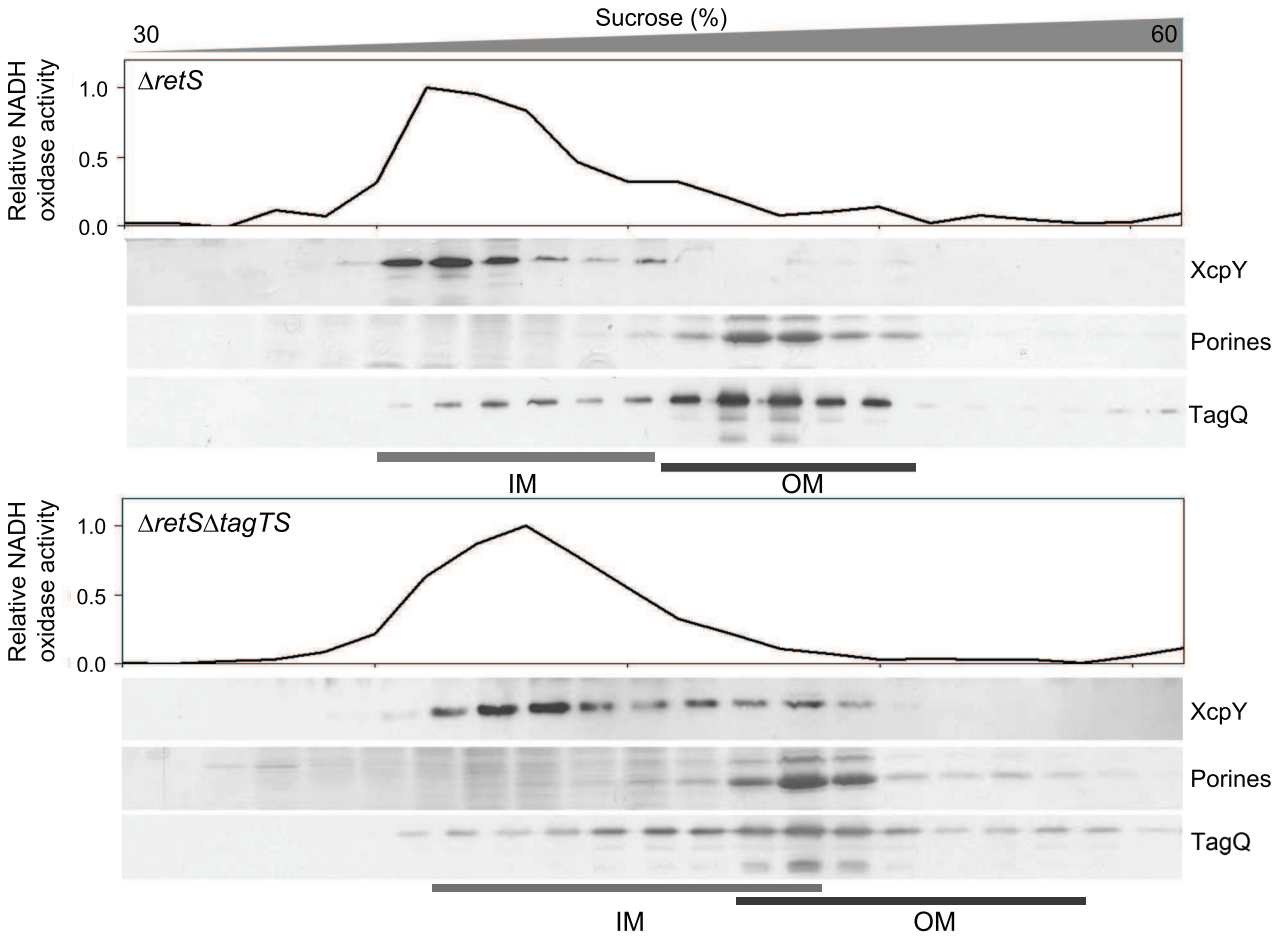
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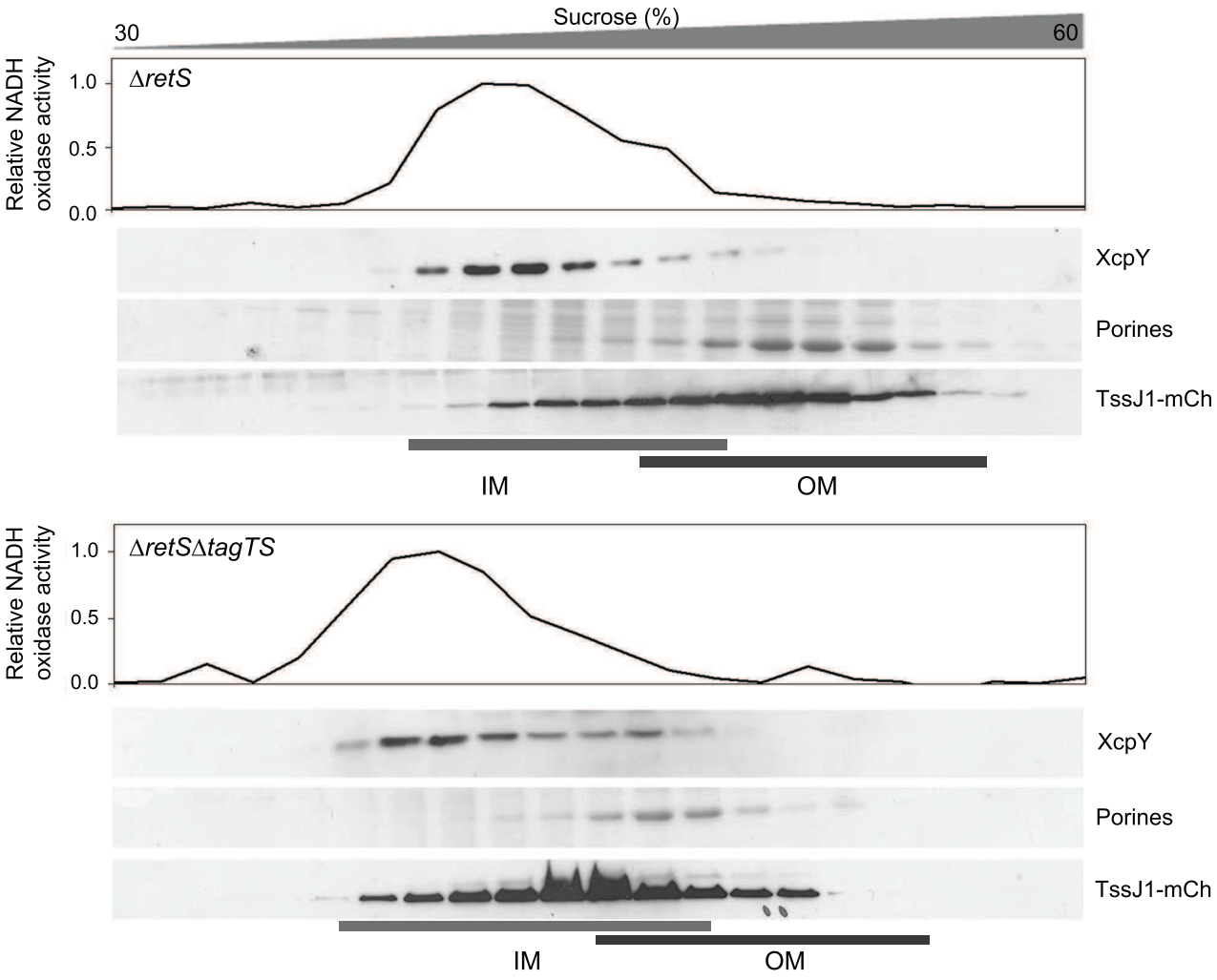
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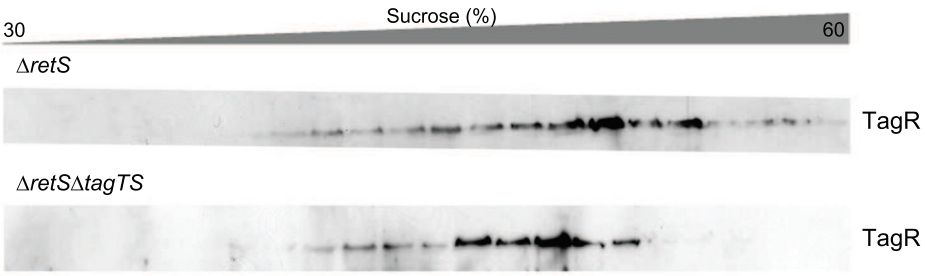
Sucrose (%)
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ΔretSΔtagTS
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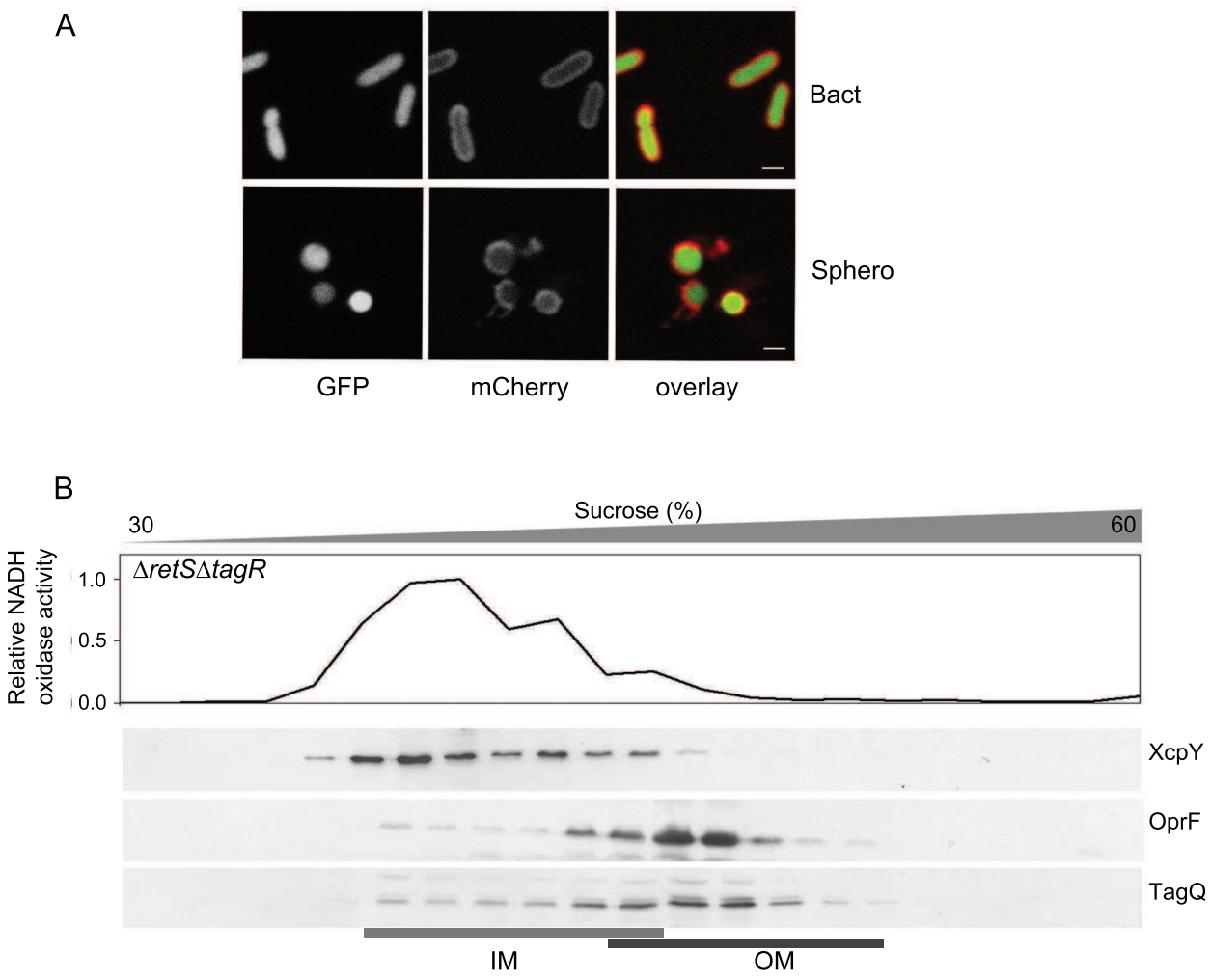
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(b) SEARCH FOR INTERACTIONS BETWEEN TAG PROTEINS

Our *in silico* approach showed that *tagT*, *S* and *R* co-occur both in T6SS-encoding and non-encoding bacteria. Moreover, our findings also suggested that TagS could have a TagT-independent role, since Δ*pppA*Δ*tagS* and Δ*pppA*Δ*tagST* knockout mutants do not share the same Hcp1 secretion profile. Finally, TagS has a predicted periplasmic loop, similar to the one in charge of substrate recognition in LolC and BraB. All these data prompted me to investigate whether TagS could interact with OM TagR and/or TagQ. In order to do this, I took advantage of the bicistronic construction that was used to purify the 6HisTagTS membrane-bound complex from *E. coli* and constructed a *P. aeruginosa* expression plasmid carrying *6his-tagTS*. The vector was introduced into PAO1Δ*retS*Δ*tagTS* so as to have an overexpression of HSI-I locus (Δ*retS*) and no competition with the endogenous proteins (Δ*tagTS*).

As expected, two polypeptides were recovered using magnetic beads coupled to Ni^{2+} (not shown), including 6HisTagT (detected using α-His antibody, Figure 26), indicating that the ABC transporter complex could be purified using this technique. On the contrary, TagQ and TagR were not detected in the elution fraction, suggesting that they do not interact with the TagTS complex under the conditions tested (Figure 26). As a control, the same experiment was carried out with the non-transformed strain, and there was no histidine-tagged protein recovered (data not shown).

The assay was carried out either in presence or absence of a mild non-ionic detergent, n-Dodecyl β-D-maltoside (DDM). The latter condition did not allow the recovery of 6HisTagT, probably because membrane solubilization is critical for integral membrane protein complex recovery.

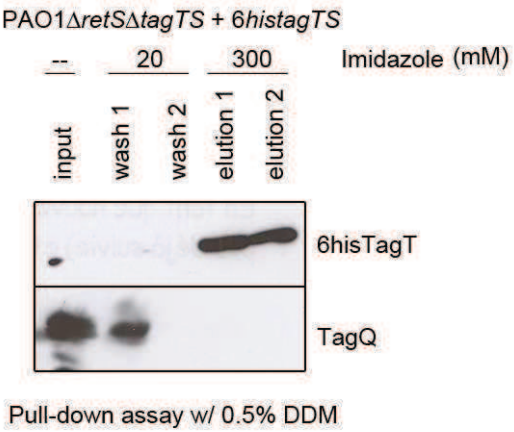

Figure 26 Pull down assay using magnetic Ni^{2+} beads. Western blots anti-his and anti-TagQ are shown. Only the condition with detergent is presented.

TagR is not associated to the OM in the absence of TagQ (Figure 4 in Casabona *et al.*, 2013). I then investigated whether TagS influences TagR localisation. For that, discontinuous sucrose gradients were carried out in a Δ*tagQ*Δ*tagS* background. In this case, TagR was localized to the IM, as it co-fractionated with XcpY, the IM marker (Figure 27). This approach confirmed that TagS does not influence the association of the periplasmic protein to the IM in the absence of TagQ.

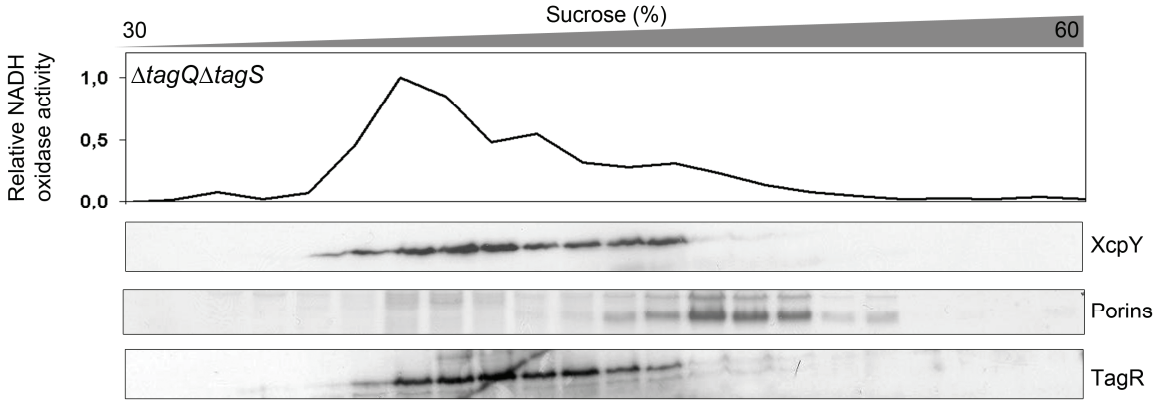

Figure 27 Discontinuous sucrose gradients of PAO1Δ*tag*QΔ*tagS*. XcpY and the NADH oxidase activity are used as IM markers. Porins are used as OM markers. The NADH oxidase activity is represented relative to the highest activity, noted as 1,0.

TagR associates to the OM in a TagQ-dependent fashion, implying that they might interact in this compartment (Figures 4 and 7 in Casabona *et al.*, 2013). To address this question, α-TagQ immunoprecipitations (IP) were carried out in a PAO1Δ*tagQ* + *tagQ*ΔCys background, where *tagQ*ΔCys is under the control of an arabinose-inducible promotor. TagQΔCys is not anchored to the membrane because the conserved Cys residue that is lipidated has been deleted. Importantly, the protein is still translocated into the periplasm (Figure 3 in (Casabona *et al.*, 2013). In this case, the use of detergents can be avoided, and there is less risk of disrupting potential protein-protein interactions.

As shown in Figure 28A, TagQ was immunoprecipitated using α-TagQ antibodies. On the contrary, TagR was detected in the input, but not in the wash and elution fractions. The band observed in the elution fractions corresponded to the heavy chains of the α-TagQ antibodies, eluted with the interacting proteins. This became evident when the experiment was carried out by cross-linking the immunoprecipitant antibodies to the magnetic beads, since the band was no longer detected (Figure 28B). The same experiments were carried out using α-TagR antibodies, but they were not immunoprecipitant in the conditions tested (not shown).

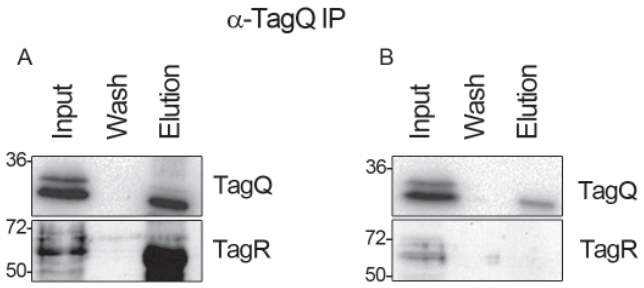

Figure 28 α-TagQ IP. A) α-TagQ antibodies were not cross-linked to the magnetic beads. B) Cross-linking was performed using BS^3. Molecular weight markers (in kDa) are shown.

To further investigate the possible interaction between TagQ and TagR, I co-produced TagR and TagQ in the cytoplasm (Sec N-ter signals were deleted) of *E. coli*, where only TagR was carrying a histidine tag. Both genes were cloned into different plasmids, but were under the control of the same IPTG-inducible promotor.

TagR had already been overproduced in *E. coli* for antibody production, and formed highly insoluble aggregates localized in inclusion bodies. This is why 10HisTagR solubility was tested upon overexpression. Two different *E. coli* strains were used: BL21(DE3)Star, commonly used for protein overexpression; and SHuffle T7®, a strain capable of forming disulfide bonds in its cytoplasm. The latter was chosen because both proteins of interest are periplasmic and have Cys. Moreover, TagQ possesses at least one disulfide bond (personal communication with Viviana Job, IBS, Grenoble). As shown in Figure 29A, it was possible to overexpress 10HisTagR in both strains at 16 and 37°C, but soluble 10HisTagR was produced only in SHuffle T7®. On the contrary, TagQ was soluble in all the conditions tested (data not shown). In consequence, further assays were carried out in SHuffle T7® strain and induction was carried out at 16°C.

Once the expression conditions were set up, I tested whether 10HisTagR could be purified using magnetic beads coupled to Ni^{2+} (Millipore©). As shown in Figure 29B, 10HisTagR can be recovered by this technique when overproduced in SHuffle T7®. Importantly, the input of this experiment is only the soluble fraction of 10HisTagR.

When co-produced with TagQ, 10HisTagR formed high molecular complexes, indicating that the protein might have formed aggregates. TagQ, on the other hand, is produced as a soluble molecule (Figure 29C). We conclude that this approach was not adapted due to the different properties of the proteins. Since TagQ is more soluble than TagR, there is less apparent 10HisTagR relative to soluble TagQ.

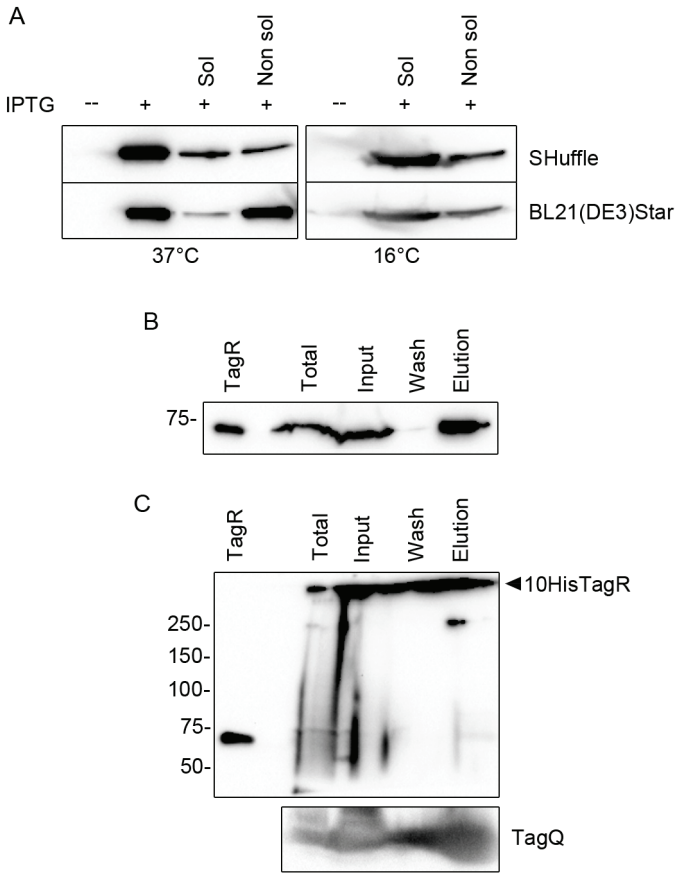

Figure 29 Co-production of TagQ and 10HisTagR in *E. coli*. A) Production of 10HisTagR under different conditions. Sol: soluble fraction; Non sol: insoluble fraction. B) 10HisTagR recovery using magnetic Ni^{2+} beads. C) Co-production and purification of 10HisTagR and TagQ. Molecular weight markers (in kDa) are shown.

A plausible reason why TagR-TagQ interaction could not be detected *in vitro* is that TagQ might be localized to the outer leaflet of the OM. To address this question, IF assays and proteinase K accessibility experiments were carried out. First, I tested whether the α-TagQ antibodies could be employed to detect TagQ in IF classical experiments. As shown in Figure 30A and B, upon membrane permeabilization of PAO1 and PAO1 + *tagQmCherry* respectively, TagQ is localized to the periphery of bacteria, as already reported (Casabona *et al.*, 2013). Next, in order to assess whether TagQ is localized to the inner or outer leaflet of the OM, IF were carried out in liquid without the permeabilization step, as described in Experimental Procedures. Bacteria used in these experiments constitutively expressed GFP in the cytoplasm, so that integrity of the membranes could be evaluated (green fluorescent bacteria were considered to have intact membranes). Only bacteria that were no longer

green appeared red-labelled, suggesting that the α-TagQ antibody could enter the cell and bind to TagQ. This indicates that TagQ faces the periplasmic space (Figure 30C).

In order to confirm these results, a biochemical approach in which bacteria were incubated in the absence/presence of proteinase K was set up. If TagQ is facing the extracellular milieu, it should be degraded by proteinase K. As expected, TagQ was only degraded when a strong anionic detergent such as SDS was added, meaning that TagQ is protected by OM integrity (Figure 30D). As a control, purified TagQ was added to the reaction mix, so as to prove that if accessible, it could be a proteinase K target. These results prove that TagQ is facing the periplasm in *P. aeruginosa* PAO1.

Our results and the model of H1-T6SS posttranslational activation that we have proposed suggest that TagR might act as a shuttle between the IM and the OM. In this case, TagQ would be its partner in the OM. Which protein, if there is one, could be the IM partner? As described above, TagTS were ruled out by discontinuous sucrose gradients and pull down assays.

It has been shown that TagR is necessary for the activation of PpkA and it has been proposed that they interact directly (Hsu *et al.*, 2009), even though this has never been demonstrated. In order to address this question, the localisation of TagR in a Δ*tagQ*Δ*ppkA* mutant was studied. If TagR is anchored to the IM through PpkA kinase in the absence of TagQ, one would expect the loss of TagR in the IM after a discontinuous sucrose gradient separation.

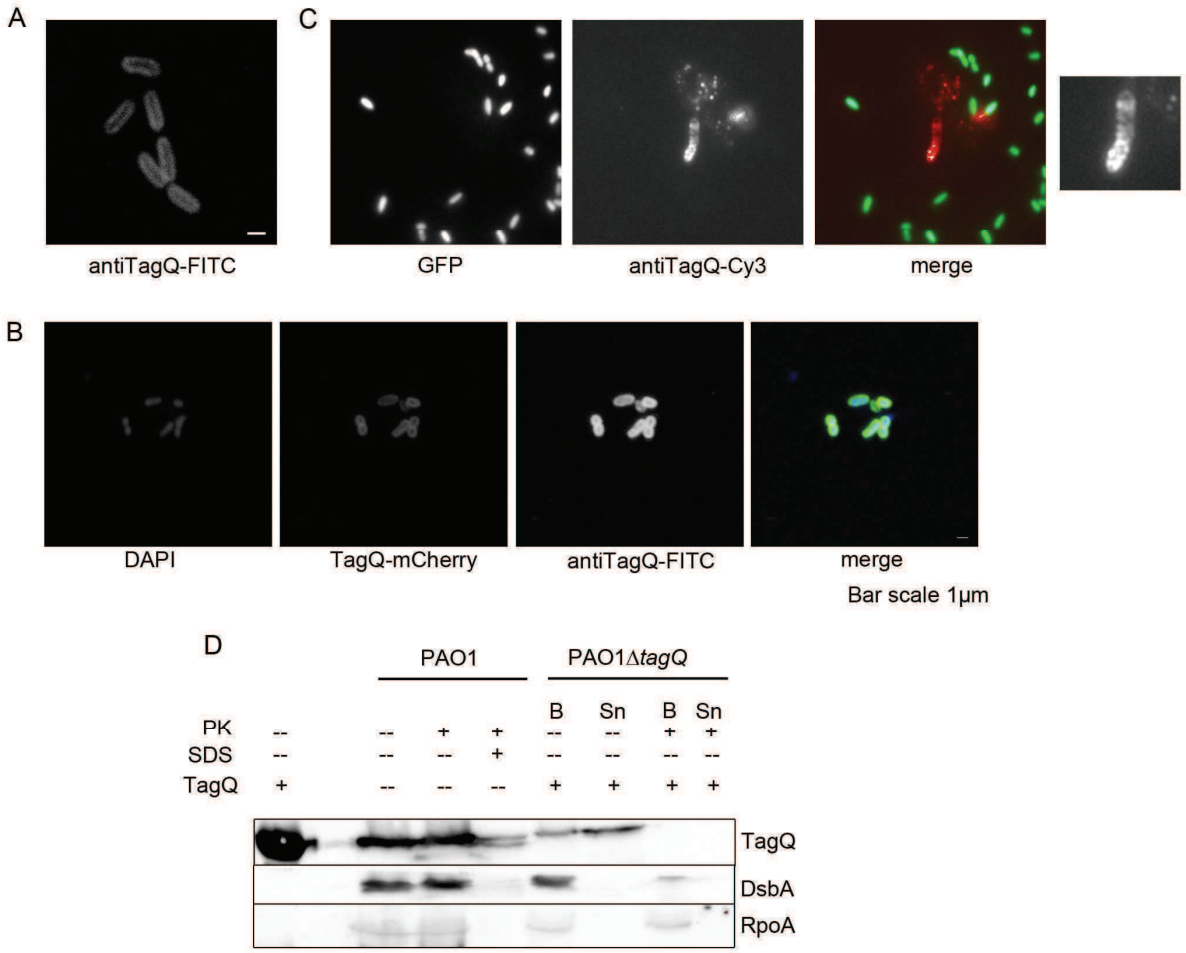

Figure 30 TagQ faces the periplasmic space. A) IF assay in permeabilized *P. aeruginosa* PAO1 background. B) Same IF assay as in A but using PAO1 + *tagQmcherry*. C) IF in liquid milieu using PAO1-gfp. D) Proteinase K (PK) accessibility assay. Purified TagQ was added to the reaction mix of PAO1Δ*tagQ* as a control. DsbA and RpoA are periplasmic and cytoplasmic controls, respectively. B: bacteria, Sn: supernatant of the reaction mix. For IF experiments, α-TagQ was used as primary antibody and α-mouseFITC was used as secondary antibody.

This was not the case, since TagR co-fractionated with porins, the OM marker, in a Δ*tagQ*Δ*ppkA* mutant (Figure 31). The low amount of TagR detected in the fractions compared to previous assays prompted me to analyze the different fractions from discontinuous sucrose gradients in order to quantify the amount of TagR present in each of them.

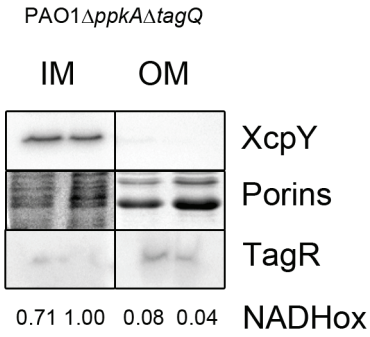

Figure 31 Discontinuous sucrose gradient separation of PAO1DppkADtagQ. XcpY and NADH oxidase activity (NADHox) are IM markers. Porins are OM markers.

To quantify TagR in the different IM/OM samples, I chose in an arbitrary fashion one sample and referred all TagR quantities as detected by western blot to it. The selected value was the average of the two IM fractions of PAO1 (Figure 32). Relative amounts of TagR between samples were then normalized to the total protein concentration of each sample (calculated using BCA assay). These preliminary results suggest that there are comparable amounts of TagR in the IM of the parental strain and the mutant strains tested. Also, they imply that, contrary to what is expected, TagR is in the same relative proportions in both membranes in a Δ*ppkA*Δ*tagQ* mutant. One may argue that this association of TagR with the membrane is due to the anchoring complex formed by TssM-L-J. This is not the case, since TagR is also localized to the OM in a Δ*tssM1* mutant (Figure 33).

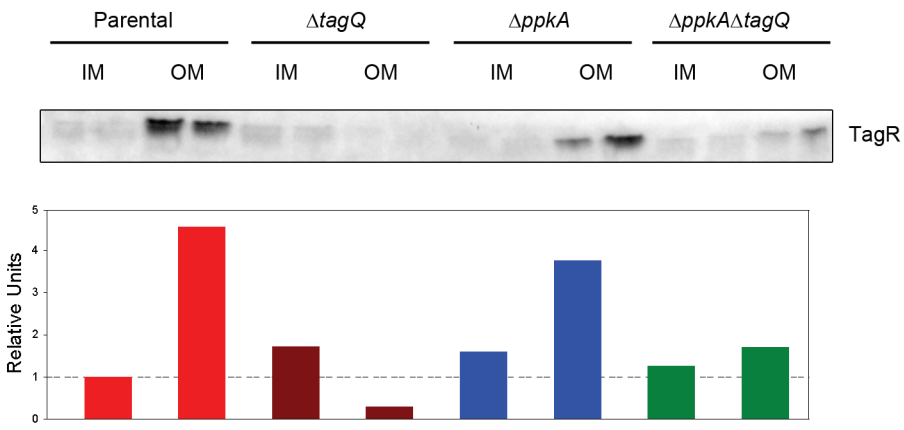

Figure 32 Relative quantification of TagR in the IM and OM of different *P. aeruginosa* T6-mutant strains. The parental strain is PAO1. Different bar colours are shown for each strain studied.

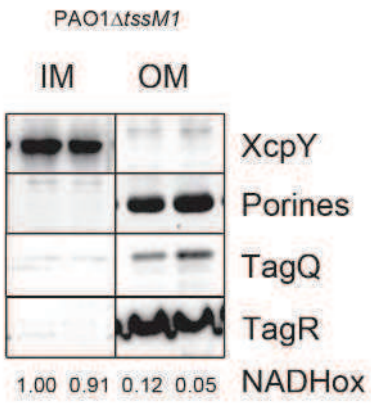

Figure 33 TagR is localized to the OM in PAO1Δ*tssM1*. Controls are the same as Figure 31.

(c) TagT, S, R and Q are Indispensable in Inter-species Fitness of *P. aeruginosa*

As depicted in Figure 24, separate inputs modulate H1-T6SS activation in *P. aeruginosa* PAO1. Our data demonstrated that TagT, S, R, and Q are necessary for Fha1 phosphorylation and intra-bacterial fitness. However, no evidence of importance in inter-bacterial competitions was shown. We set up competition assays between *P. aeruginosa* as a competitor and *E. coli* K12 as prey and we tested whether TagT, S, R and Q were necessary for H1-T6SS-mediated inter-bacterial competition assays. It is plausible to propose that the environmental cues that trigger the H1-T6SS can be different when a bacterium faces itself or another, as is the case of *P. mirabilis*, that needs T6 homologue proteins for self identification (Gibbs *et al.*, 2008). As shown in Figure 34, TagT, S, R and Q are indispensable for PAO1 T6-mediated fitness against other Gram-negative bacteria, since *E. coli* K12 is able to develop after being challenged with different PAO1Δ*tag* mutants. This suggests that surface-induced phosphorylation of Fha1 is necessary for inter-bacterial competition and argues that there are separate inputs that contribute to the activation of H1-T6SS (Figure 24 and (Silverman *et al.*, 2011).

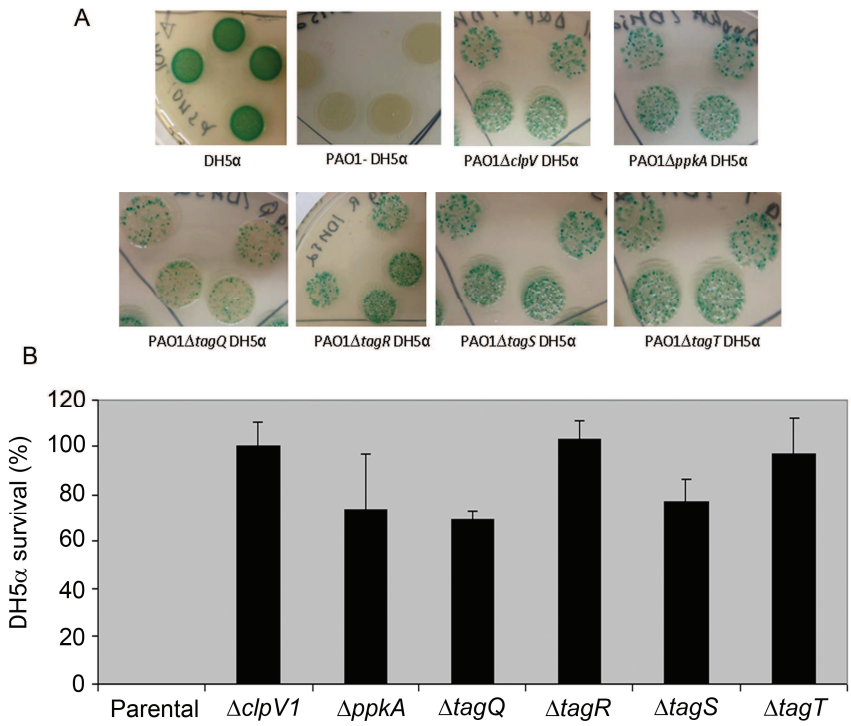

Figure 34 Tag proteins are needed for T6-related fitness in *P. aeruginosa* against *E. coli*. A and B) Competition assays between different PAO1 strains (donor) and *E. coli* p*Bluescript* (recipient).

(d) DISCUSSION

Recently, data obtained by time-lapse fluorescence microscopy at a single-cell level, supporting the hypothesis that TagTSRQ are involved in sensing an exogenous T6SS attack have been published (Basler *et al.*, 2013). As shown in Figure 35, *pppA* and *tagT* mutants are unable to respond to the T6SS-mediated attack of a neighbouring cell by "counter-attacking" it (action known as "duelling", Chapter III). Importantly, the loss of duelling activity does not produce a deficiency in the assembly and firing (contraction of the sheath) of the H1-T6SS. Also, TagT, PpkA and PppA are necessary for H1-T6-mediated killing of *V. cholerae* and *Acetinobacter baylyi* (Basler *et al.*, 2013). These results suggest that *P. aeruginosa* detects T6SS-associated attack signals even if they have no lethal consequence. Similar to our conclusions, these authors proposed that TagTSRQ-PpkA/PppA-Fha1 may form a regulatory module involved in direct sensing of envelope perturbations such as OM breach, peptidoglycan disruption/modification and/or IM perforation upon exogenous T6SS attack.

Envelope perturbations will then be the point where the new T6SS will be assembled and "fired" (Figure 36).

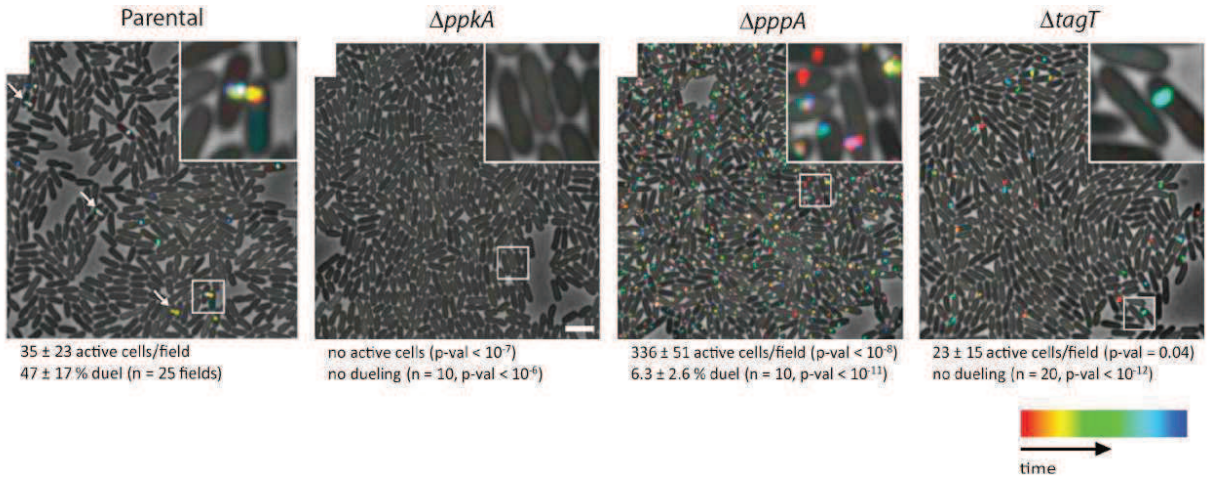

Figure 35 T6SS duelling depends on PpkA, PpkA and TagT. ClpV1-GFP localisation was followed for 3min and temporally colour coded. The bar represents 3µm. n indicates the amount of fields analyzed; p value is compared to the parental strain. Adapted from Basler *et al.* (2013).

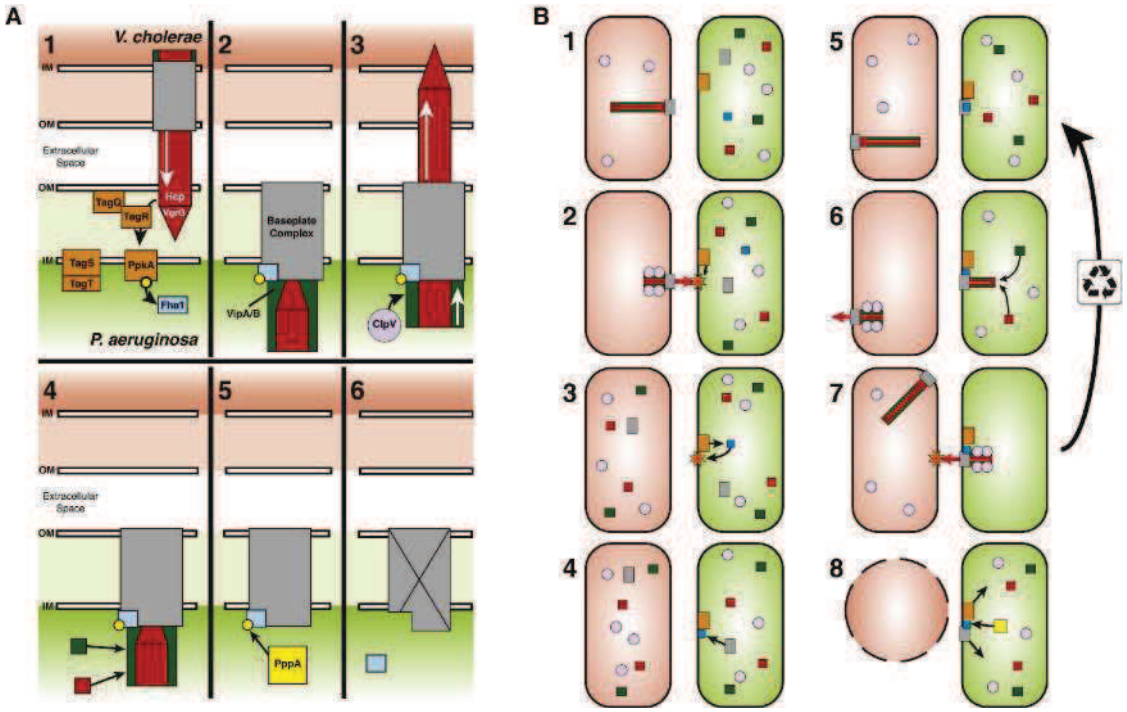

Figure 36 Model for TagTSRQ-mediated aiming. A) 1/T6SS-mediated attack fom V. cholerae is sense by TagTSRQ-PpkA-Fha1 module, resulting in Fha1 phosphorylation. 2/Assembly of the baseplate complex by *P. aeruginosa* at the place of the attack. 3/Firing and reclycling. 4-6/The baseplate and tail/pike/sheath can be reused. B) Dynamics of V. cholerae and *P. aeruginosa* T6SS-mediated interactions. 1/ *V. cholerae* (pink) will assembly and fire its T6SS spontaneously and eventually hit *P. aeruginosa* (green). 2-7/ *P. aeruginosa* senses

the assault and assemblies and fires its H1-T6SS. 8/ *V. cholerae* cell dies. Reproduced from Basler *et al.* (2013).

In this study only the TagT-associated phenotype was studied. It would be interesting to assess the phosphorylation state of Fha1 under the conditions of the assays presented in Basler *et al.* (2013) and Figure 34, as well as the dynamics of the duelling and firing of the H1-T6SS in *tagS*, *Q* and *R* knockout mutants.

The results presented herein show that TagR association to the OM is TagQ-dependent but anchoring complex-independent. Moreover, preliminary data showed that the combination of PpkA and TagQ absence disrupts the wild-type association of TagR to the IM/OM. In order to decorticate the TagTSRQ-PpkA/PppA-Fha1 regulatory module, it would be interesting to construct a version of TagQ that anchors to the IM and monitor TagR localisation and H1-T6-activation in these conditions. OM lipoproteins of P. aeruginosa can be targeted to the IM by substitution of the "+3" and "+4" residues (Narita & Tokuda, 2007). These experiments would be important to gain insight into whether TagQ-TagR could be the co-sensors of the T6-activating signal.

There is evidence that OM lipoproteins can be the direct sensors of signalling pathways, as is the case of RcsF of the Rcs pathway, responsible for phosphorelay triggering (Majdalani & Gottesman, 2005). What could be the role of TagTS ABC transporter upon envelope modification? ABC transporters have already been implicated in the transport and/or flipping of lipids and/or LPS in Gram-negative bacteria (Davidson *et al.*, 2008). Others, such as Pglk of *C. jejuni*, are required for glycan assembly (Kelly *et al.*, 2006).

TagTS possesses four predicted trans-membrane domains, such as LolCDE and FtsEX, which might indicate that it is not a transporter, that usually have six to eight trans-membrane domains (Davidson *et al.*, 2008). Maybe TagTS is involved in the facilitation of the delivery of a molecule/protein, as does the LolCDE system. Interestingly, it has been shown that in the case of MsbA, in charge of the translocation of LPS from the IM to the OM in *E. coli* (Doerrler *et al.*, 2004), ATP binding increases the accessibility of its periplasmic regions to H_2O. This is consistent with the idea of ATP triggering an alternation in the exposure of a chamber from the interior to the exterior side or leaflet of the membrane (Dong *et al.*, 2005). We have

shown that TagTS can bind and hydrolyse ATP, one can imagine that the ATP binding can trigger a conformational change important for envelope disruption recognition.

Other possibility is that TagTS modulates the activity of another protein by means of ATP hydrolysis, in a similar way as FtsEX of *E. coli* (Yang *et al.*, 2011a). FtsEX regulates the catalytic activity of a cell wall hydrolase, EnvC, at the Z-ring at the division site by directly recruiting it, thanks to ATP binding driven conformational changes (Yang *et al.*, 2011a).

There is an ABC transport system, the MlaE-MlaF-MlaB complex, that prevents phospholipid accumulation in the outer leaflet of the OM of *E. coli* (Malinverni & Silhavy, 2009). This ABC transporter forms part of the Mla pathway, that includes both an OM lipoprotein and a periplasmic protein. Maintenance of lipid asymmetry may happen by retrograde trafficking of phospholipids from the OM to the IM (Malinverni & Silhavy, 2009).

TagTS could recognize and/or interact with phospholipids, LPS and maybe molecules issued from cell envelope modification upon OM breaching by an endogenous T6SS attack. It can be proposed that TagTS can interact with the effectors from other T6SS. Studies at the single-molecule level would be of high importance to decipher the exact role of this ABC transporter in the H1-T6SS activation of *P. aeruginosa*.

We have shown that a Walker A mutant of the TagTS complex ($TagT^{K/A}TagS$) conserves 30% of its original ATPase activity. Intriguingly, when cloned under a *P. aeruginosa* strong constitutive promoter, *tagT*$^{K/A}$*tagS* are able to complement Hcp1 secretion (not shown). In order to check whether the ATPase activity is crucial for H1-T6SS activity in *P. aeruginosa*, it would be interesting to construct a double mutant by disrupting also the Walker B domain of $TagT^{K/A}$ to completely abrogate the catalytic activity and clone it under its cognate promoter. Then, Hcp1 secretion and the level of Fha1 phsophorylation could be measured to assess function and activation of the H1-T6SS, respectively.

To gain insight into whether TagTS is able to sense cell envelope disruptions/modifications, it can be tested whether it interacts with phospholipids and/or peptigoglycan. If disruptions in the cell envelope are cues sensed by TagTS, one could expect that the ABC transporter would recognize them. Also, a VgrG mutant that cannot breach the OM could be constructed

in order to check for H1-T6SS activation under exogenous T6SS attack that does not leads to OM breaching.

Is cell envelope disruption the real cue for H1-T6SS activation? In order to answer to this question, it is plausible to propose to expose bacteria to a low power pulsed laser radiation (Nandakumar *et al.*, 2006) and check for H1-T6SS activation. This has been already done for eukaryotic cells to study the effects of local membrane disruption (Minamikawa *et al.*, 2011, Rau *et al.*, 2006).

In order to go further, it would be interesting to construct a *tagQ-mCherry* fusion under the control of *tagQ* cognate promoter and in *P. aeruginosa* chromosome (by allelic replacement) and follow the dynamics under different culture conditions, to study whether there is formation of punctuate foci under specific conditions. The same could be done concerning TagR, using another fluorescent protein in order to be able to study both TagQ and TagR dynamics. This approach has been previously used to study interactions and dynamics of other T6SS-components (Mougous *et al.*, 2006, Basler & Mekalanos, 2012, Kapitein *et al.*, 2013). Novel techniques can be used, such as TIRF (total internal reflection fluorescence) microscopy which allows single-molecule tracking and is specially adapted for the visualization of single-molecules near a surface (Trache & Meininger, 2008). This technique has already been employed in *P. aeruginosa* to study the localisation of proteins involved in pyoverdine biosynthesis (Guillon *et al.*, 2012).

Chapter VIII. Characterization of *P. aeruginosa* envelope: a proteomics approach

Biological membranes of Gram-negative bacteria are far from being static compartments. Indeed, their protein and phospholipid contents vary from one strain to the other and in the same bacterium are conditioned by environmental conditions. Membrane structural and functional characteristics strongly depend on their protein repertoires, which consists of integral as well as peripherally associated proteins. These proteins are involved in critical physiological processes, including nutrient uptake, adhesion, energy production, environmental sensing and antibiotic resistance.

P. aeruginosa is a versatile bacterium, able to rapidly adapt to changing environments. The capacity to dynamically modify the protein content of its cell envelope plays an essential role in the adaptability of the bacterium. As a first step to gain insight into the multiple functions fulfilled by the different compartments of cell envelope, the establishment of their protein repertoires has been undertaken. However, even though exhaustive proteomics studies of the periplasm, OM and OMVs have been carried out, the protein content of IM was still elusive.

Our previous work on the TagTSRQ regulatory module demonstrated the power of shotgun proteomics as a tool for the profiling of IM and OM, since it allowed the discovery of TagQ-dependent TagR association of the OM.

In the first part of this Chapter, I will present my work on characterization of the *P. aeruginosa* IM sub-proteome, as a manuscript submitted to Proteomics. Secondly, to go further in the description of cell envelope compartments, we tempted to establish a quantitative comparison of IM and OM proteomes using label-free quantitative proteomics in collaboration with the Proteomic facilities of the Institute (EDyP, BGE, iRTSV, Dr. Yoahn Couté and Dr. Yves Vandenbrouk).

8.01 CHARACTERIZATION OF THE PROTEIN CONTENT OF THE IM OF PAO1

(a) INTRODUCTION

Mass spectrometry (MS)-based proteomics has become a powerful method for large-scale identification and quantification of proteins in a variety of biological samples from prokaryotic to eukaryotic organisms (Cox & Mann, 2011).

One of the universally used proteomic methods is shotgun (or discovery) proteomics. This technique allows global measurement of protein content, giving a wide insight into particular phenotypes. Shotgun proteomics is the method of choice for a without *a priori* characterization of the protein repertoires of complex samples, since no prior knowledge is required. Shotgun proteomics is a typical discovery technology that can be used for the generation of protein inventories (Domon & Aebersold, 2010).

Many cellular compartments have been characterized using shotgun proteomics (organellar proteomics). This method can complement traditional microscopy-based approaches in cell-biology (Cox & Mann, 2011). Indeed, when combined, these techniques can provide data on subcellular localisation for the detected proteins in a time-resolved manner (Andersen *et al.*, 2005).

In a classical shotgun experiment, proteins are digested with trypsin. The generated peptides are then fractionated on a reverse-phase column using an acetonitrile gradient by liquid chromatography working at nanoflow (nanoLC) and injected online in a mass spectrometer. This instrument switch along time into 2 modes: MS to monitor the exact masses of the peptides eluting from the nanoLC system and MS/MS to fragment and obtain sequence information for these peptides. The spectra acquired for a particular sample by the mass spectrometer are then submitted to various probabilistic algorithms in order to match experimental and theoretical spectra and identify peptides and proteins from a database (Cox & Mann, 2011, Domon & Aebersold, 2010 and Figure 37). The proteome coverage for a

particular sample will depend on its complexity, on the dynamic range of its protein content and on the limit of detection of the mass spectrometer used.

Recent developments have brought proteomics from acquisition of qualitative data to generation of quantitative information. Two main families of quantitative approaches co-exist: one using differential labeling of the samples to be compared and the other without labeling (label-free). With the development of dedicated softwares, label-free quantitative proteomics becomes more and more popular. Two ways of quantitative information inference from label-free analyses can be used: spectral counting and extracted ion chromatogram (Neilson *et al.*, 2011). The first one compares the numbers of MS/MS spectra that identified a protein whereas the other one relies on the abundances measured by the mass spectrometer in MS mode for each identified peptide and protein.

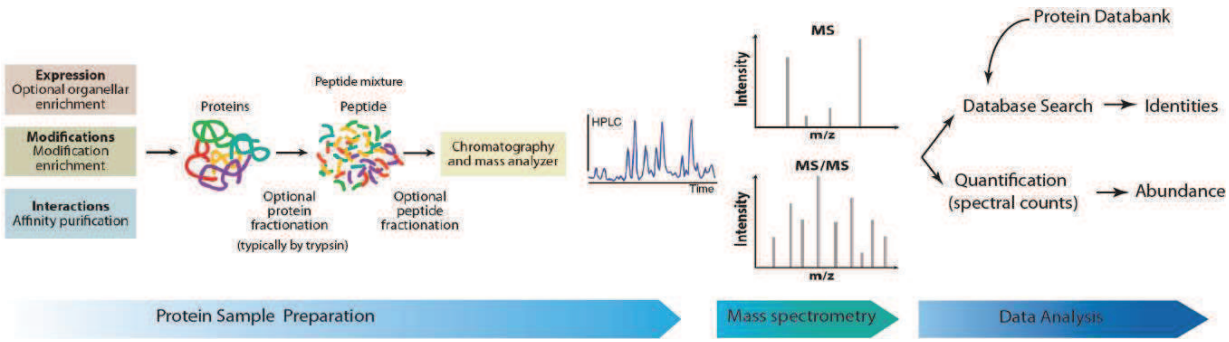

Figure 37 Outline of a generic shotgun proteomics workflow. Adapted from Cox & Mann (2011).

(b) MANUSCRIPT SUBMITTED TO PROTEOMICS

DataSET Brief

Proteomic characterization of *Pseudomonas aeruginosa* PAO1 inner membrane

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Keywords: Inner membrane / Pseudomonas aeruginosa / Discontinuous sucrose gradient / Bacterial proteomics / MS-MS analysis

Total number of words: 2'489

Abstract

Pseudomonas aeruginosa is a gram-negative bacterium that can inhabit a wide variety of environments and infect different hosts. Human infections by this microbe are nowadays perceived as a major health problem due notably to its multi-resistance. The present dataset provides the first description of *P. aeruginosa* inner membrane proteome. To achieve this, we combined efficient separation of membranes from PAO1 reference strain using discontinuous sucrose gradient centrifugation and mass spectrometry-based proteomic analysis. A core list of 991 non-redundant proteins was established and analyzed in terms of trans-membrane domains, signal peptide and lipobox sequence prediction. Furthermore, functional insights into membrane-spanning and membrane-associated protein complexes have been explored.

Pseudomonas aeruginosa is a ubiquitous opportunistic human pathogen capable of causing both acute and chronic life-threatening infections. Due to multi-drug resistances, hospital-acquired *P. aeruginosa* infections are becoming an increasing world-wide health problem. This led to the classification of *P. aeruginosa* among "ESCAPE" micro-organisms for which there is an urgent need for novel antimicrobial molecules [1]. Bacterial envelope and associated macromolecular structures are evidently among the best targets for developments of new generation drugs.

The cell envelope of Gram-negative bacteria is a multilayered structure composed of the inner membrane (IM), the periplasm and the outer membrane (OM). Some of the functions of this structure include: (i) protecting bacteria from hostile and changing environments; (ii) acting as a barrier allowing the selective passage of nutrients and waste products; (iii) providing a surface for different reactions to occur (i.g. oxidation/reduction of substrates). Surprisingly, while the periplasm and the OM proteomes of *P. aeruginosa* have been previously explored using proteomic approaches [2, 3, 4, 5], the IM protein content has never been characterized using such strategies. IM is composed of a phospholipid bilayer that was previously shown to harbour molecular actors of major importance for cell biogenesis and function, such as multi-component transporters, protein-sensors that are part of signalling and regulatory systems and protein export machineries involved in virulence [6]. Therefore, we believe that uncovering the IM proteome of *P. aeruginosa* will be of special interest for the study of its lifestyle and virulence.

In order to study the assembly of membrane multimolecular machineries of *P. aeruginosa*, we have set up separation of bacterial IM and OM using differential centrifugation, adapted from [7]. Briefly, 500 ml culture of *P. aeruginosa* PAO1 strain was grown up to mid-log phase at 37°C with shaking. Bacteria were harvested by centrifugation and lysed at 15'000 psi using a Microfluidizer in buffer A (25 ml of 10 mM Tris-HCl, 20% sucrose, 10 mg ml^{-1} DNase, 10 mg ml^{-1} RNase, pH 7.4). Non disrupted cells were eliminated by a centrifugation step at 6'000 x g. Total membrane fraction was obtained by ultracentrifugation at 100'000 x g for 1h and resuspended in 500 µl of 20% sucrose. As shown in Fig. 1A, this membrane fraction was largely devoid of cytosolic and periplasmic markers, respectively RpoA and DsbA, whereas it was clearly enriched in XcpY and OprF membrane proteins. In order to separate IM and OM fractions, total membranes were loaded on top of a discontinuous sucrose gradient

composed of eight layers of sucrose (from bottom to top, volume): 55% (1.4ml), 50% (1.5ml), 45% (1.5mL), 42.5% (1.3ml), 40% (1.5ml), 37.5% (1.3ml), 35% (1.5ml) and 30% (1.0ml). Ultracentrifugation at 90'000 x g for 72 h was then carried out. Finally, 500 µl fractions were collected from the top and analyzed for NADH oxidase activity [8] (Fig. 1B) and protein pattern using SDS-PAGE (Fig. 1C). The efficiency of IM and OM separation was proven to be successful since NADH oxidase activity (IM marker) was restricted to fractions in the first third of the gradient while porins (OM markers) were restricted to fractions in the last third of the same gradient. In order to evaluate the purity of IM fractions, we analysed by immunoblotting the distribution of XcpY (IM marker, [9]) and OprF (OM marker, [10]) in the 2 central fractions from first and last parts of the gradient (Fig. 1B). As shown in Fig. 1A, XcpY was enriched in IM fractions and nearly undetectable in OM fractions, while the opposite was observed for OprF, showing the efficient separation of the two membrane systems.

Three independent biological replicates of IM samples (combination of the 2 central fractions positive for NADH oxidase activity) were prepared, their protein content extracted in Laemmli buffer and heated at 95°C during 5 min [11]. Proteins were then stacked in the top of a SDS-PAGE gel (NuPAGE 4-12%, Invitrogen) and stained with Coomassie blue. Gel slices (1 per biological replicate) were excised and proteins in-gel digested by trypsin as described in [12]. Resulting peptides were analyzed by online nanoLC-MS/MS (Ultimate 3000 and LTQ-Orbitrap Velos pro, Thermo Fischer Scientific). Two analytical replicates were acquired per biological replicate. Raw data were processed using Mascot Distiller v. 2.3.2, and database search was carried out with Mascot 2.4 against the PAO1-UW strain protein sequence databank (February 1, 2012 release from www.pseudomonas.com, 5'572 entries). More details about experimental procedures are available in Supporting Information. Identification results were filtered using the IRMa software [13] (version 1.30.4): conservation of rank 1 peptides, peptide identification FDR < 1% as calculated by employing a reverse database strategy, and minimum of 1 specific peptide per identified protein group (PG). Mass spectrometry identifications were then compiled and structured within dedicated databases, grouped and compared using a homemade software (hEIDI, manuscript in preparation). For each biological replicate, the spectral count values of identified PGs (number of spectra assigned per PG in the sample) were calculated. Finally, results were

filtered again in order to conserve only PGs that exhibited a minimum of 2 specific spectral counts in every biological replicates. This allowed sorting out a list of 991 PGs that we considered as *P. aeruginosa* PAO1 strain IM reference proteome (Supplementary Table). Interestingly, all of these PGs except two were composed of a single protein and were therefore non-ambiguous in terms of protein identity.

We investigated the knowledge about these 991 proteins, notably by taking advantage of the expert-curated Pseudomonas Genome Database [14] (www.pseudomonas.com). About one half of them possessed a described function (protein name confidence classes 1 and 2) whereas the function of the other half was still elusive (protein name confidence classes 3 and 4) (Table 1). In parallel, information about number of trans-membrane domains (TMDs, [15]), presence of signal peptide [16] and lipobox sequence [17] were gained (Supplementary Table). As shown in Table 1, 47% of identified proteins from class 1 and 2 harbour at least one predicted TMD. Notably integral membrane proteins with up to 35 predicted TMDs were found. Twenty percents of the identified proteins exhibit a signal peptide probably allowing their export across the inner membrane and 62 are predicted lipoproteins according to the recent list of *P. aeruginosa* proteins harbouring the lipobox sequences [17]. Proteins from our IM repertoire were then categorised according to their reported subcellular localisation in the Pseudomonas Genome Database. For this, we restricted the analysis to localisation demonstrated for proteins in *P. aeruginosa* or for counterparts in other organisms (subcellular localisation confidence levels 1 and 2). Proteins with no demonstrated localisation represented about half of the repertoire (Fig. 2A, left pie chart). Interestingly, the majority of these proteins are predicted to associate with IM (subcellular localisation confidence levels 3 and 4, Fig. 2A, right pie chart). For proteins with already described localisation, three main groups emerged: IM (10.4% of identified proteins), outer membrane vesicles (OMVs, 15.7%) and multiple localisations (14.2%). The important proportion of proteins annotated as component of OMV was not surprising as these vesicles were reported to be composed of cell envelope, including IM [18]. Most of the proteins described as exhibiting multiple localisations were partly found either in the IM and/or as being associated with OMVs. Altogether, these results strongly suggest that the present dataset mainly contain *bona-fide* IM proteins and/or proteins that cross IM during

export. It also allows proposing a residence cellular compartment for numerous proteins with until now unattributed localisation.

Proteins with the highest confidence rating (protein name confidence classes 1 and 2) were grouped into PseudoCAP functional categories retrieved from the Pseudomonas Genome Database. From this subset of proteins (52.5% of identified proteins), a large spectrum of function was represented, highlighting the complexity of *P. aeruginosa* IM. A main part of these proteins were involved in secretion and transport (19%), as well as biosynthesis and metabolism (33%). Since about half of the identified proteins were not predicted to contain TMDs, we decided to look more in detail at membrane-associated and envelope-spanning machineries, for which the existence of protein-protein interactions could be supposed. Our strategy was to explore proteins encoded within same operons, which likely indicates their involvement in same cellular process and/or forming macromolecular complexes. For each PseudoCAP functional category highlighted by our analysis, one or two representative operons were chosen (Fig. 2C). As examples, proteins of operons encoding part of type II (Xcp) and type VI secretion (Hcp-secretion island 1) machineries were readily detected (7 components of T2SS and 7 of T6SS), while only a part of them harboured predicted TMDs. Same results were observed for operons encoding type 4 pili (*pil*), the cytochrome oxidase (*cco*) and NADH dehydrogenase (*nuo*) complexes, proteins involved in chemotaxis (*pct*), transporters such as Bra and Fts complexes involved in cell division and cell wall biogenesis. These results suggested that an important part of the IM proteome of *P. aeruginosa* is probably maintained through protein-protein interactions. Further proteomic studies of IM fractions submitted to stringent washings should allow the differential analysis of membrane-embedded and IM-associated proteins.

In this work, we delivered the first IM sub-proteome of a *P. aeruginosa* strain. This represents an important step that paves the way for a better understanding of the regulation of molecular machineries contained within *P. aeruginosa* membranes. Furthermore, this dataset provides a knowledge platform that could stimulate novel drug developments.

Acknowledgments

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The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [19] with the dataset identifier PXD000107 and DOI 10.6019/PXD000107.

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Figure Legends

Table 1. Characterization of the IM reference proteome. The 991 identified proteins were classified according to the protein name confidence class they belong to in the Pseudomonas Genome Database. The number of proteins in which presence of TMDs, signal peptide and lipobox were found is indicated.

Figure 1. Analyses of cellular fractions obtained during membranes preparation from *P. aeruginosa* PAO1 strain. A) Western blot analyses of total bacteria (Bac), total membranes (MB_T), IM and OM fractions using antibodies directed against RpoA (cytoplasmic marker), DsbA (periplasmic marker), XcpY (IM marker) and OprF (OM marker). B) Analysis of membrane sub-fractions for NADH oxidase (NADHox) activity (IM marker). Activity is represented relative to the fraction with the highest level activity (noted as 1.0). C) Analysis of membrane sub-fractions by SDS-PAGE and protein staining by Coomassie blue. Positions of porins (OM markers) are indicated. The 2 fractions of IM and OM tested in Fig. 1A are shown.

Figure 2. Mining of the IM reference proteome. A) Pie graphs representing the diverse localisation distributions of the 991 identified proteins (left pie chart). For each group, the proteins of unknown localisation were analyzed and classified corresponding to their predicted location (right pie charts). B) Classification into functional classes based on PseudoCAP (Pseudomonas Genome Database). The percentage of proteins present in each category is indicated. C) Representative operons of the different categories described in B). Genes whose products have been identified in this study are marked with an asterisk.

Table 1.

	Number of Proteins	Number of proteins with predicted TMD (%)	Number of proteins with predicted signal peptide (%)	Number of proteins with predicted lipobox (%)
Total	991	465 (46.9%)	198 (20.0%)	62 (6.3%)
Classes 1+2	520	195 (37.5%)	69 (13.3%)	23 (4.4%)
Classes 3+4	471	270 (57.3%)	129 (27.4%)	39 (8.3%)

Figure 1.

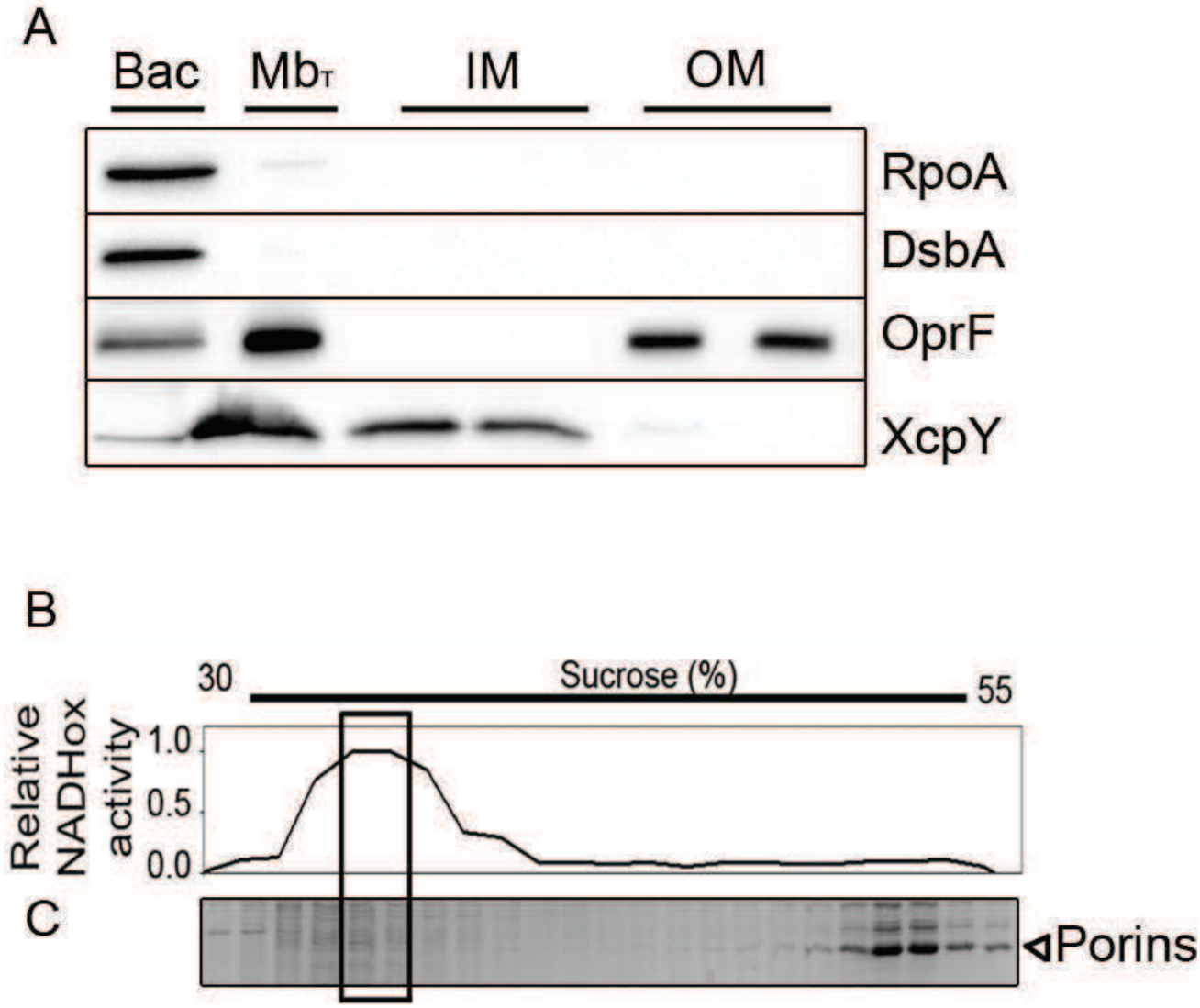
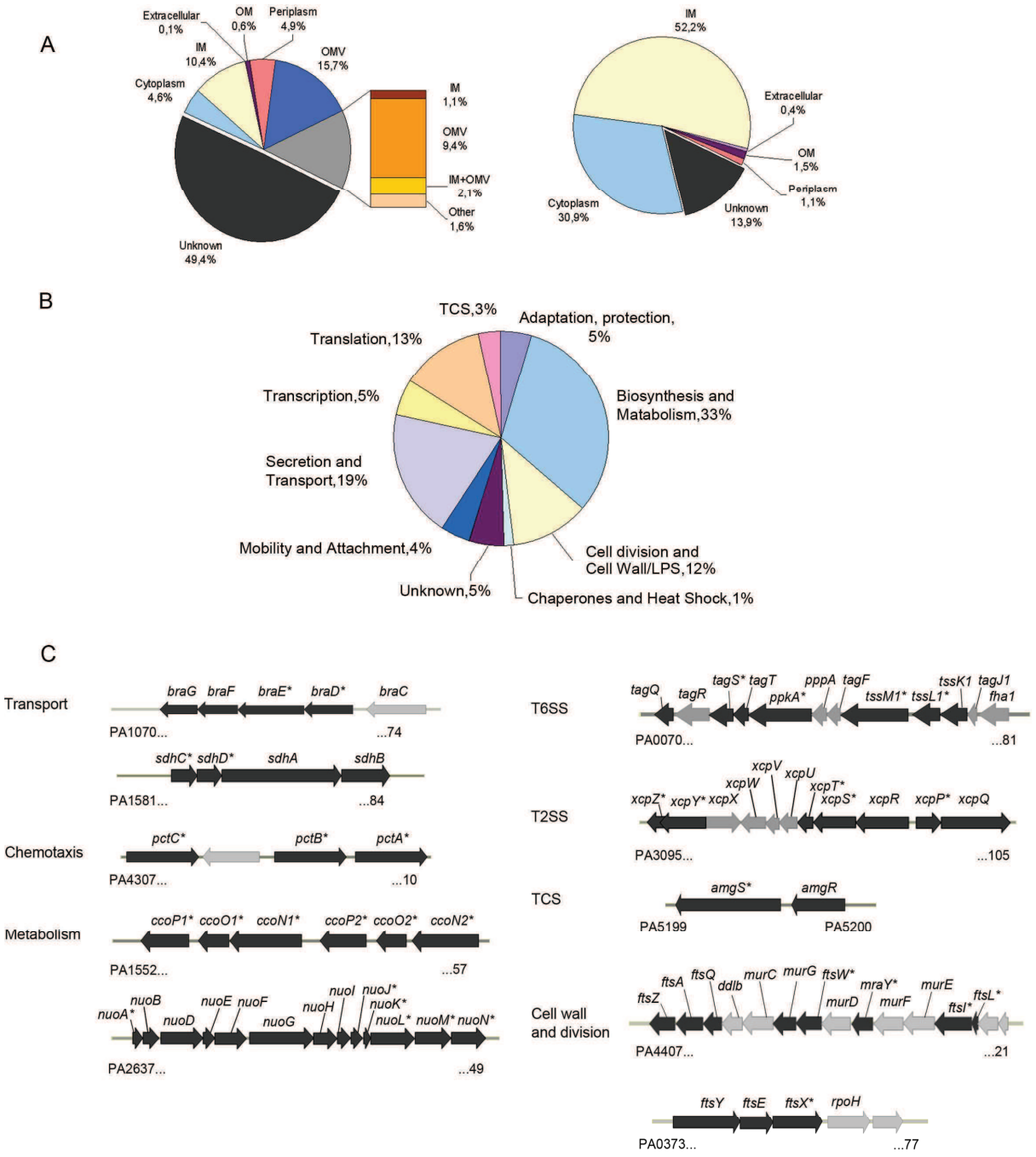

A
Bac
MbT
IM
OM
RpoA
DsbA
OprF
XcpY
B
30
Sucrose (%)
55
Relative NADHox activity
1.0
0.5
0.0
C
Porins

Figure 2.


A
Extracellular 0,1%
OM 0,6%
Periplasm 4,9%
IM 10,4%
OMV 15,7%
Cytoplasm 4,6%
Unknown 49,4%
IM 1,1%
OMV 9,4%
IM+OMV 2,1%
Other 1,6%
IM 52,2%
Extracellular 0,4%
OM 1,5%
Periplasm 1,1%
Cytoplasm 30,9%
Unknown 13,9%
B
TCS,3%
Adaptation, protection, 5%
Translation,13%
Transcription,5%
Biosynthesis and Matabolism,33%
Secretion and Transport,19%
Mobility and Attachment,4%
Cell division and Cell Wall/LPS,12%
Unknown,5%
Chaperones and Heat Shock,1%
C
Transport
braG braF braE* braD* braC
PA1070... ...74
sdhC* sdhD* sdhA sdhB
PA1581... ...84
Chemotaxis
pctC* pctB* pctA*
PA4307... ...10
Metabolism
ccoP1* ccoO1* ccoN1* ccoP2* ccoO2* ccoN2*
PA1552... ...57
nuoA* nuoB nuoD nuoE nuoF nuoG nuoH nuoI nuoJ* nuoK* nuoL* nuoM* nuoN*
PA2637... ...49
T6SS
tagQ tagR tagS* tagT ppkA* pppA tagF tssM1* tssL1* tssK1 tagJ1 fha1
PA0070... ...81
T2SS
xcpZ* xcpY* xcpX xcpW xcpV xcpU xcpT* xcpS* xcpR xcpP* xcpQ
PA3095... ...105
TCS
amgS* amgR
PA5199 PA5200
Cell wall and division
ftsZ ftsA ftsQ ddlb murC murG ftsW* murD mraY* murF murE ftsI* ftsL*
PA4407... ...21
ftsY ftsE ftsX* rpoH
PA0373... ...77

(c) DISCUSSION

After our work was submitted, a report describing the cytoplasmic peripheral IM sub-proteome of *E. coli* BL21(DE3) strain using an extensive biochemical approach combined with shotgun proteomics was published (Papanastasiou *et al.*, 2012). The authors set up sequential washes of inverted IMVs (periplasmic proteins cannot be accessed since they are in the lumen of the vesicles) using different chemical agents, such as KCl and DDM. The resulting washed IMVs were submitted to surface trypsinolysis, and the soluble peptides were analyzed by shotgun proteomics. This approach allowed the authors to position 342 proteins at the periphery of the inner leaflet of the IM of *E. coli* according to kind of association. Protein-protein interactions within several IM complexes were also examined experimentally by differential centrifugation of chemically treated IM vesicles coupled to and size-exclusion chromatography follwed by MS analysis.

In an attempt to generate a global view of part of the IM sub-proteome of *P. aeruginosa*, I took advantage of the "bird's eye" generated by Papanastasiou *et al* (2012), and compared the proteins presented in their investigation with the proteins identified in our study. As depicted in Figure 39, I classified proteins into three categories. The first category consists of *E. coli* proteins that do not present evident homologues in *P. aeruginosa* (as annotated in UniProtKB, the Universal Protein Resource (UniProt) Knowlegdebase, http://www.uniprot.org/). Secondly, those that share homology in both microorganisms were divided into two sub-categories: i) proteins that were not identified in our study, and ii) proteins identified in both sub-proteomes. Of note, even if the small and large ribosomal subunits appear as identified in both proteomes, the proteins that form part of them have not been compared one by one. There are at least 160 proteins that have been identified in both sutdies (not taking into account ribosomal subunits).

In the case of *E. coli* BL21(DE3), its peripherome includes at least mannose and glucose-specific phosphotransferase permeases (PTS permeases, bird eye in Papanastasiou *et al.*, 2012). A wide genomic analysis of bacterial genomes revealed that *E. coli* strains can have between 17 to 26 functional PTS systems, where pathogenic strains possess extra PTS systems compared to the non pathogenic *E. coli* K12 (Barabote & Saier, 2005). Some gamma-

proteobacteria have been reported as having few PTS permeases, as is the case of *Pseudomonads*. Indeed, *P. aeruginosa* possesses only two PTS permeases, comprising one fructose and one N-acetylglucosamine-specific (Barabote & Saier, 2005). In our work, we have identified both FruA (PA3560) and NagE (PA3761), both known components of the fructose and the N-acetylglucosamine PTS transporters, respectively.

Other group of proteins identified in both sub-proteomes are proteases and chaperones involved in heat-shock response, such as DnaJ and DnaK. Since many heat-shock proteins are highly conserved among species (Rosen & Ron, 2002), it is possible to infer their localisation using studies in other bacteria. Importantly, under the conditions of our assay, heat-shock protein production is not up-regulated, which might explain why not all the proteins involved in this process were detected.

Papanastasiou *et al* (2012) have set up experiments allowing enrichment of samples with proteins that interacted in different ways with the inner leaflet of the IM by means of adapted biochemical treatments. This approach and the fact that many of the actors involved in molecule transport/metabolism are tightly regulated under specific environmental cues might explain why many of the transporters found in *E. coli* were not identified in *P. aeruginosa* (for example, glutamine, histidine and arginine transporters).

Previously reported sub-proteomes of *P. aeruginosa* illustrate the dynamics of the protein composition profile of the bacterium under different environmental conditions (Imperi *et al.*, 2009, Toyofuku *et al.*, 2012, Hare *et al.*, 2012, Hare *et al.*, 2011, Nouwens *et al.*, 2000), reflecting that *P. aeruginosa* envelope is a highly dynamic compartment. The proteomic approach presented herein is a methodology that provides insights of a genotype at a phenotypic level in a spatio-temporal manner. Taking into account that the microorganisms used in both studies are different, it is not surprising that the sub-proteomes compared herein are different as well. The purity of our samples suggests that the proteins that are present in the IM sub-proteome and are cytoplasmic of periplasmic are interacting with IM proteins and/or form part of membrane-spanning complexes (Figure 1 of DataSet Brief). This is comforted by the important number of proteins overlapping between our study and peripherome of *E. coli* (Figure 39).

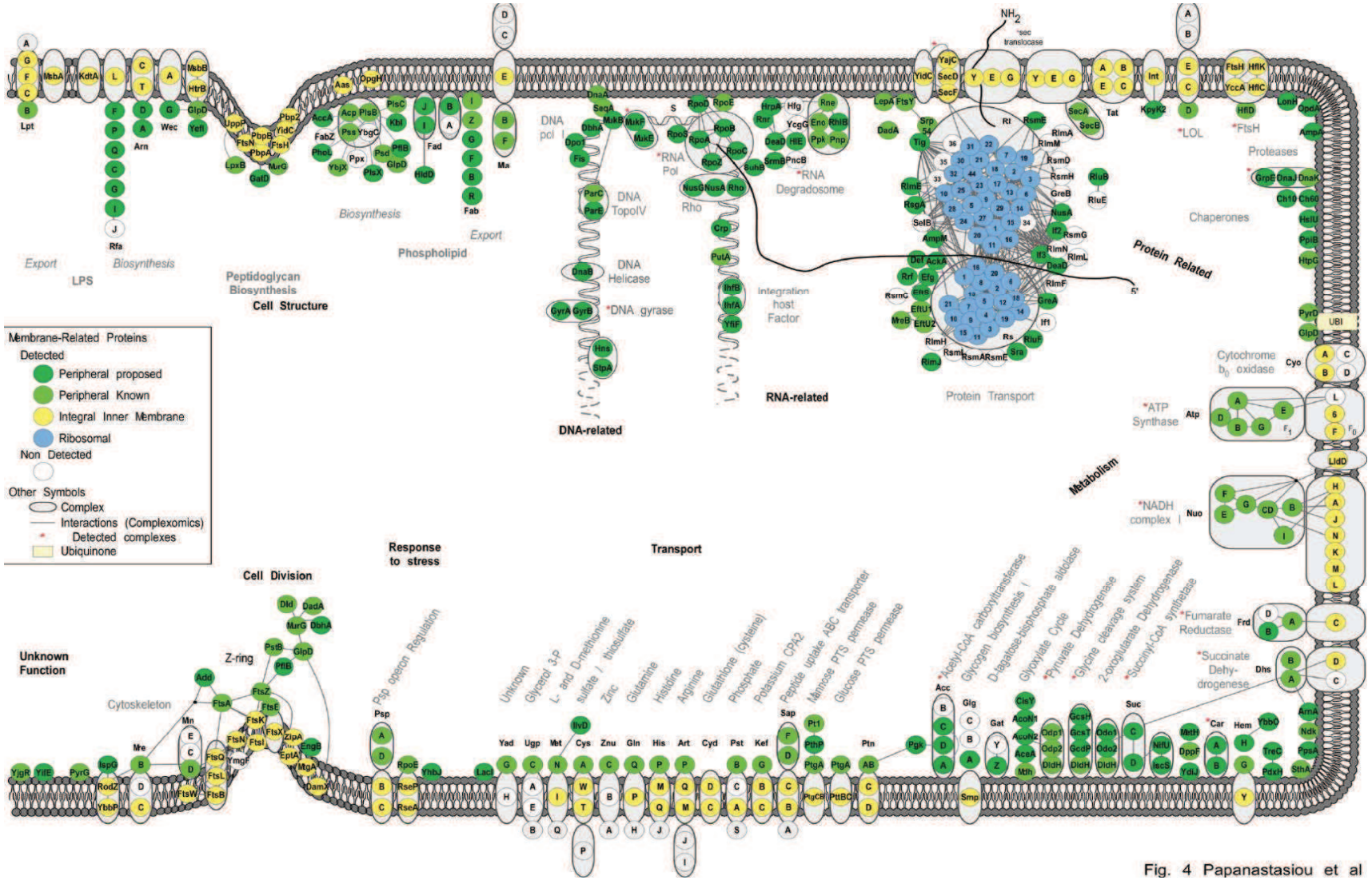


Figure 38 Bird's-eye view of the E. coli peripherome, taken from Papanastasiou *et al.* (2012).

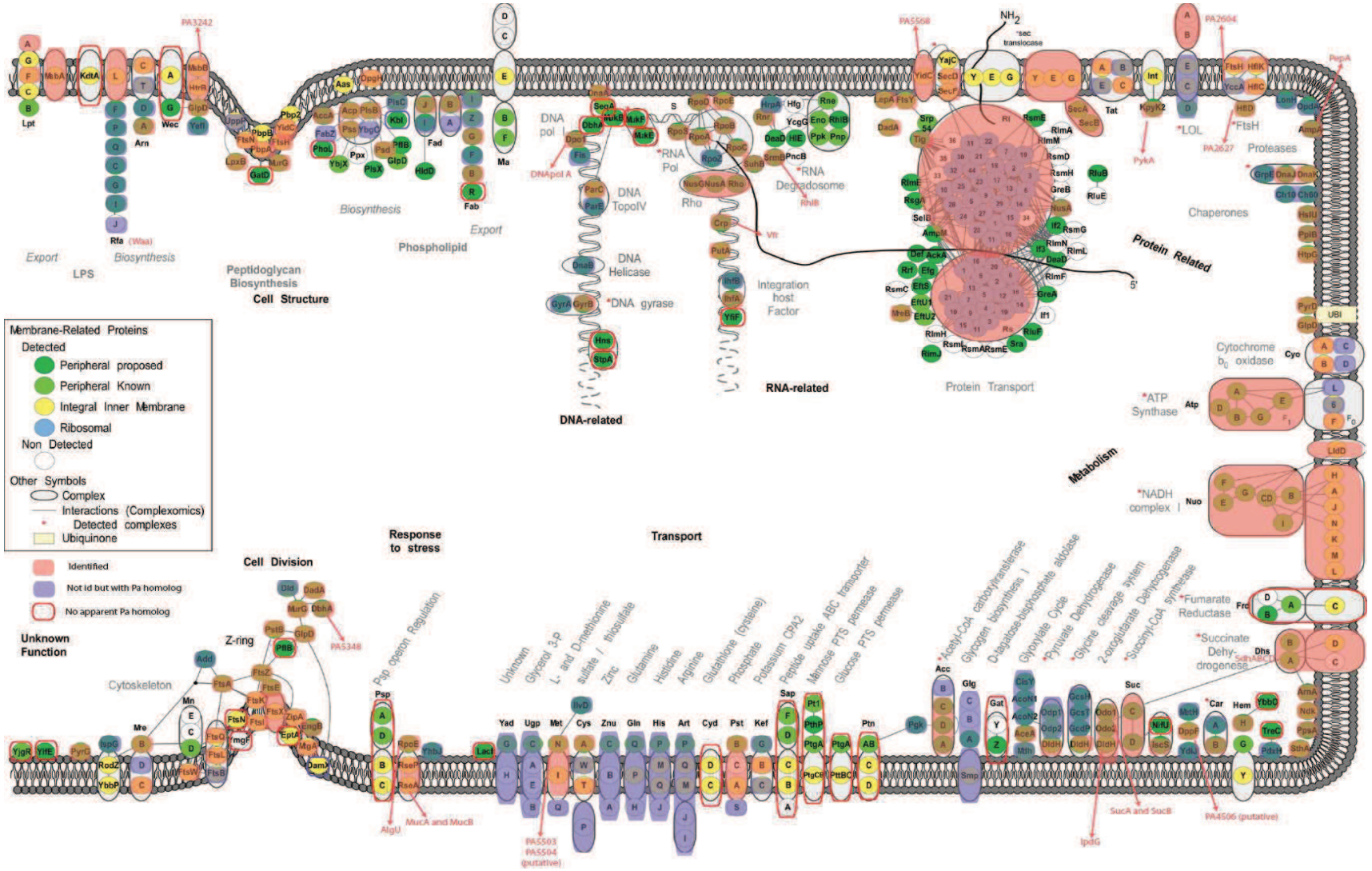


Figure 39 Comparison of the *E. coli* peripherome (Figure 38) and the results obtained in this study. Three protein categories have been identified and added to the figure legend. See the text for details.

8.02 CHARACTERIZATION OF THE IM VERSUS THE OM OF PAO1

(a) APPROACH

The OM of several *P. aeruginosa* strains has been previously characterized by means of mass spectrometry methods concerning their protein content (Nouwens *et al.*, 2000, Nouwens *et al.*, 2003, Peng *et al.*, 2005). The approaches used in these studies include two-dimensional (2-D)-gel electrohporesis coupled to shotgun proteomics. Strikingly, so far no comparison of the IM versus the OM protein repertoires has been undertaken in a quantitative manner. While datasets providing general descriptions of organelles are most useful, differential localisation of proteins within membrane compartments is crucial for the understanding of their functioning and regulation.

In order to compare the IM versus the OM protein components, discontinuous sucrose gradient separation was carried out coupled to shotgun proteomics and bioinformatic analyses. This approach was set up to decipher global assembly of molecular machineries in the *P. aeruginosa* envelope, notably the H1-T6SS, but can be applied to other envelope complexes, as shown for the macroglobuling-like complex (Robert-Genthon *et al*, to be submitted). All methods used have been described in the submitted manuscript, Casabona *et al*, Proteomics. The experimental workflow is depicted in Figure 40.

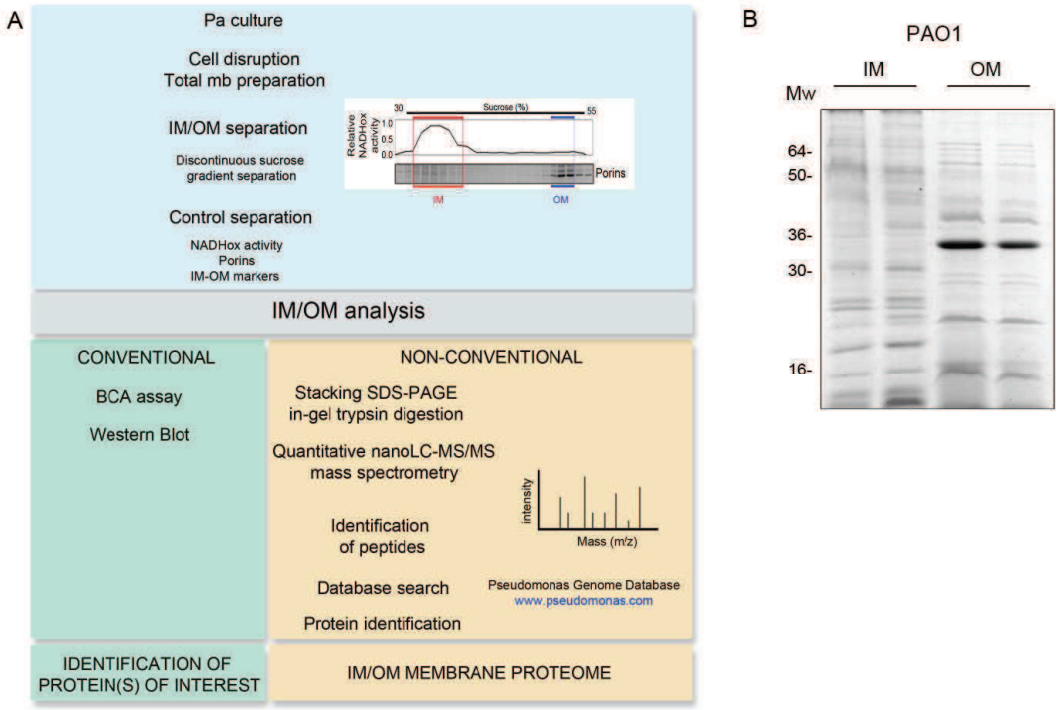


Figure 40 A) Experimental workflow of the IM and OM separation and analysis/characterization of *P. aeruginosa.* B) Total protein content of PAO1 IM and OM discontinuous sucrose gradient fractions pooled for MS-MS analysis. Mw: molecular weight, in kDa.

(b) RESULTS

As for the IM sub-proteome determination, two samples corresponding to the IM or the OM were pooled. As shown in Figure 40B, their protein content was different. Using this approach, a core list of 1707 non-redundant proteins could be established (the complete list can be obtained on request from Ina ATTREE, ina.attree-delic@cea.fr, and will be published in the future). Almost 70% of the proteins identified could be attributed a resident compartment, 65% of the proteins being localized preferentially in the IM and 35% in the OM. 30% of the proteins could not be assigned a localisation, probably because they are distributed between both the IM and the OM, or because the variations in relative amounts between biological replicates do not allow discrimination with the statistical test used (t-test).

We have identified 573 proteins (33.6%) listed as "hypothetical proteins" or "conserved hypothetical proteins". These proteins can now be classified as "proteins of unknown function" because we have confirmed by MS analysis that they are produced in *P. aeruginosa*. Thus, the existence of these proteins is no longer hypothetical.

In order to gain insight in the functions associated with the IM and the OM, I further classified "Class 1 and 2" proteins according to their PseudoCAP category. All categories are represented in both compartments (not shown). The most striking feature is the percentage of proteins (45%) involved in biosynthesis and metabolism in the IM. Proteins involved in energy production such as cytochromes and ATP synthases are localized in the IM (Stenberg *et al.*, 2005), whereas only few enzymes are known to be present in the OM, as is the case of a phospholipase (called PldA, Snijder *et al.*, 1999), a protease (OmpT, Vandeputte-Rutten *et al.*, 2001) and a modifying-LPS enzyme (PagP, Hwang *et al.*, 2002) in *E. coli*.

The IM is semipermeable, since certain small molecules without charges, such as O_2, CO_2 and H_2O, can diffuse freely. Molecules that cannot diffuse, such as proteins, are transported through the IM thanks to special multiproteic complexes that include ABC transporters, importers, exporters and antiporters. Fifty-four proteins classified as being part of ABC transporters were identified as residents of the IM, including phosphate, methionine and polyamine transporters, as well as putative ATP binding components and permeases (Table 8). Importantly, this was not the case in the OM, since only three proteins were annotated as "putative ABC transporter components" and a fourth protein identified was BraC, known to be the periplasmic component of the Bra transporter (Hoshino *et al.*, 1992).

PA Number	Gene Name	Produc Name
	Inner Membrane	
PA0281	*cysW*	Sulfate transport protein CysW
PA0302	*spuF*	Polyamine transport protein PotG
PA0303	*spuG*	Polyamine transport protein PotH
PA0304	*spuH*	Polyamine transport protein PotI
PA0888	*aotJ*	Arginine/ornithine binding protein AotJ
PA0889	*aotQ*	Arginine/ornithine transport protein AotQ
PA0890	*aotL*	Arginine/ornithine transport protein AotM
PA0892	*aotP*	Arginine/ornithine transport protein AotP
PA1070	*braG*	Branched-chain amino acid transport protein BraG
PA1071	*braF*	Branched-chain amino acid transport protein BraF

PA Number	Gene Name	Produc Name
PA1073	*braD*	Branched-chain amino acid transport protein BraD
PA1339	NA	Amino acid ABC transporter ATP binding protein
PA1340	NA	Amino acid ABC transporter membrane protein
PA1341	NA	Amino acid ABC transporter membrane protein
PA1342	NA	Probable binding protein component of ABC transporter
PA1475	*ccmA*	Heme exporter protein CcmA
PA1476	*ccmB*	Heme exporter protein CcmB
PA1807	NA	Probable ATP-binding component of ABC transporter
PA1808	NA	Probable permease of ABC transporter
PA1809	NA	Probable permease of ABC transporter
PA1861	*modC*	Molybdenum transport protein ModC
PA2811	NA	Probable permease of ABC-2 transporter
PA2812	NA	Probable ATP-binding component of ABC transporter
PA2986	NA	Conserved hypothetical protein
PA2988	NA	Conserved hypothetical protein
PA3314	NA	Probable ATP-binding component of ABC transporter
PA3315	NA	Probable permease of ABC transporter
PA3400	NA	Pypothetical protein
PA3607	*potA*	Polyamine transport protein PotA
PA3608	*potB*	Polyamine transport protein PotB
PA3672	NA	Probable ATP-binding component of ABC transporter
PA3827	NA	Conserved hypothetical protein
PA3828	NA	Conserved hypothetical protein
PA3891	NA	Probable ATP-binding component of ABC transporter
PA4461	NA	Probable ATP-binding component of ABC transporter
PA4503	NA	Probable permease of ABC transporter
PA4504	NA	Probable permease of ABC transporter
PA4506	NA	Probable ATP-binding component of ABC transporter
PA4688	*hitB*	Iron (III)-transport system permease HitB
PA4706	NA	Probable ATP-binding component of ABC transporter
PA4707	NA	Probable permease of ABC transporter
PA4997	*msbA*	Transport protein MsbA
PA5074	NA	Probable ATP-binding component of ABC transporter
PA5076	NA	Probable ATP-binding component of ABC transporter
PA5094	NA	Probable ATP-binding component of ABC transporter
PA5217	NA	Probable binding protein component of ABC iron transporter
PA5317	NA	Probable binding protein component of ABC dipeptide transporter
PA5366	*pstB*	ATP-binding component of ABC phosphate transporter
PA5367	*pstA*	Membrane protein component of ABC phosphate transporter
PA5368	*pstC*	Membrane protein component of ABC phosphate transporter
PA5376	NA	Probable ATP-binding component of ABC transporter
PA5500	*znuC*	Zinc transport protein ZnuC
PA5503	NA	Probable ATP-binding component of ABC transporter
PA5504	NA	D-methionine ABC transporter membrane protein

PA Number	Gene Name	Produc Name
	Outer Membrane	
PA1074	*braC*	Branched-chain amino acid transport protein BraC
PA4223	NA	Probable ATP-binding component of ABC transporter
PA5095	NA	Probable permease of ABC transporter
PA5230	NA	Probable permease of ABC transporter

Table 8 ABC transporters identified as IM or OM residents in PAO1.

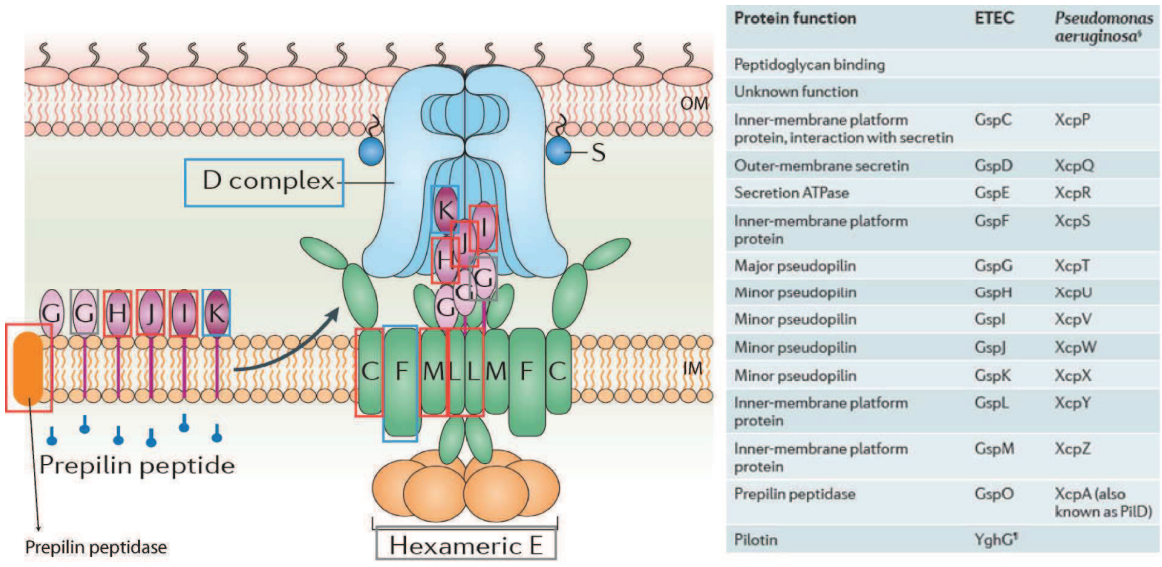
Porins are pore-forming proteins that are required to facilitate diffusion in nutrient-limited environments. *P. aeruginosa* can uptake different nutrients thanks to a variety of porins, which are exclusively localized in the OM. Accordingly, in our study proteins classified as porins or putative porins were only found as OM residents (Table 9). OprF is the major non-specific porin of *P. aeruginosa* (Hancock *et al.*, 1979) and it allows the free diffusion of polysaccharides of 2000-3000 daltons. Other porins present in *P. aeruginosa* belong to the OprD family and have different specificities for substrates. For example, OprB allows the penetration of solutes of <300 daltons, OprD facilitates the diffusion of amino acids and peptides, and OprO is a pyrophosphate-selective channel (Nikaido, 2003). Porins are also relevant in antibiotic resistance. For example, OprD is specific for imipenem diffusion. When there is OprD alteration, as is the case of many *P. aeruginosa* clinical isolates, imipemen is excluded from the bacterium and as a consequence, the microorganism becomes resistant to the antibiotic (Rodriguez-Martinez *et al.*, 2009, Li *et al.*, 2012). The variety of porins in *P. aeruginosa* OM reflects its ability to develop in a wide range of environments.

PA Number	Gene Name	Product name
PA0162	*opdC*	Histidine porin OpdC
PA4501	*opdD*	Glycine-glutamate dipeptide porin OpdP
PA0755	*opdH*	Cis-aconitate porin OpdH
PA2113	*opdO*	Pyroglutamate porin OpdO
PA3186	*oprB*	Glucose/carbohydrate outer membrane porin OprB precursor
PA3790	*oprC*	Putative copper transport outer membrane porin OprC precursor
PA0958	*oprD*	Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor
PA0291	*oprE*	Anaerobically-induced outer membrane porin OprE precursor
PA1777	*oprF*	Major porin and structural outer membrane porin OprF precursor
PA4067	*oprG*	Outer membrane protein OprG precursor
PA1178	*oprH*	PhoP/Q and low Mg2+ inducible outer membrane protein H1 precursor
PA0427	*oprM*	Major intrinsic multiple antibiotic resistance efflux outer membrane

PA Number	Gene Name	Product name
		protein OprM precursor
PA2495	*oprN*	Multidrug efflux outer membrane protein OprN precursor
PA3280	*oprO*	Pyrophosphate-specific outer membrane porin OprO precursor
PA2760	*oprQ*	OprQ
		Putative
PA4208	*opmD*	Probable outer membrane protein precursor
PA2391	*opmQ*	Probable outer membrane protein precursor

Table 9 Complete list of the porins identified in our study.

Bacterial membranes are spanned by molecular machineries such as the flagellum and one-step secretion systems (T2SS, T3SS and T6SS in the case of *P. aeruginosa*). These macromolecular complexes are formed both by integral and peripheral membrane proteins that interact between each other. As shown in Figure 41, several components of the Xcp T2SS could be identified and out of eleven proteins identified, only one, XcpS, did not correspond to its known localisation, since it was identified as an OM resident. XcpX, found in the OM, is thought to localise to the extremity of the psudoilus and to interact with the OM complex Korotkov *et al.* (2012).



Protein function	ETEC	*Pseudomonas aeruginosa*[a]
Peptidoglycan binding		
Unknown function		
Inner-membrane platform protein, interaction with secretin	GspC	XcpP
Outer-membrane secretin	GspD	XcpQ
Secretion ATPase	GspE	XcpR
Inner-membrane platform protein	GspF	XcpS
Major pseudopilin	GspG	XcpT
Minor pseudopilin	GspH	XcpU
Minor pseudopilin	GspI	XcpV
Minor pseudopilin	GspJ	XcpW
Minor pseudopilin	GspK	XcpX
Inner-membrane platform protein	GspL	XcpY
Inner-membrane platform protein	GspM	XcpZ
Prepilin peptidase	GspO	XcpA (also known as PilD)
Pilotin	YghG[b]	

Figure 41 Localisation of the Xcp T2SS components. Red: IM, blue: OM, grey: indistinguishable. Adapted from Korotkov *et al.* (2012).

Our approach has allowed us to detect numerous players of the H1-T6SS machinery (Table 10). PAO1 reference strain was used for the comparison of the IM versus the OM, because preliminary data showed that this strain has a better yield of IM/OM separation than a *retS* mutant (not shown). We have not attributed localisation to proteins that have not been identified in all biological triplicates (Table 10, NA). However, some proteins, such as TssE and TssF, have been found once or twice and in a *retS* background, probably due to the HSI-I locus overexpression, and their localisation needs to be confirmed. As described in Chapter III, the localisation of some of these T6SS proteins has already been described in several microorganisms, but not in *P. aeruginosa*.

PA Number	Protein	Identified	Expected Localisation	Localisation**
PA0070	TagQ	Yes	OM	OM
PA0071	TagR	Yes	Periplasmic	OM
PA0072	TagS	Yes	IM	IM
PA0073	TagT	Yes	IM	IM
PA0074	PpkA	Yes	IM	IM
PA0075	PppA	Yes*	Cytoplasmic	NA
PA0076	TagF	No	Cytoplasmic	NA
PA0077	TssM1	Yes	IM	IM
PA0078	TssL1	Yes	IM	IM
PA0079	TssK1	Yes	Cytoplasmic	ND
PA0080	TssJ1	Yes	OM	OM
PA0081	Fha1	Yes*	Cytoplasmic	NA
PA0082	TssA1	Yes*	Cytoplasmic	NA
PA0083	TssB1	Yes	Cytoplasmic	OM
PA0084	TssC1	Yes	Cytoplasmic	OM
PA0085	Hcp1	Yes	Cytoplasmic/Secreted	IM
PA0086	TagJ1	Yes*	Cytoplasmic	NA
PA0087	TssE1	Yes*	Cytoplasmic	NA
PA0088	TssF1	Yes*	Cytoplasmic	NA
PA0089	TssG1	No	Cytoplasmic	NA
PA0090	ClpV1	Yes	Cytoplasmic	OM
PA0091	VgrG1	Yes*	Cytoplasmic/Secreted	NA
PA1844	Tse1	Yes*	Cytoplasmic/Secreted	NA
PA1845	Tsi1	No	Periplasmic	NA
PA2702	Tse2	Yes*	Cytoplasmic/Secreted	NA
PA2703	Tsi2	No	Cyotplasmic	NA
PA3484	Tse3	Yes	Cytoplasmic/Secreted	ND
PA3485	Yes	Yes	OM	OM

Table 10 H1-T6SS proteins. *Not in every sample. **In our studies. NA: not applicable, ND: not distinguishable.

As expected, the proteins that form the anchoring complex, TssJ-L-M, were found in the IM in accord to what has already been published in other microorganisms.

Strikingly, TssB1 and TssC1 were found associated with the OM. Some authors claim that this might be due to the size of TssB/TssC tubules (de Bruin *et al.*, 2011). TssB/TssC tubules could be large enough to be pelleted by ultracentrifugation and might therefore be an artefact in discontinuous sucrose gradient separation. Of note, the sheath homologues in *V. cholerae* were obtained after an ultracentrifugation step after cell lysis (Basler *et al.*, 2012). In order to test whether this is true, I carried out "good" and "bad" discontinuous sucrose gradient separations in parallel (as detailed in Experimental procedures). In this experiment, mixed vesicles (formed of both IM and OM) are created prior to differential centrifugation ("bad" separation). If TssB1 of *P. aeruginosa* co-fractionates with the OM as an artefact, it will be detected in the same percentage of sucrose fractions as in a "good" separation. On the contrary, if TssB1 is associated to the OM, it will co-fractionate with the mixed vesicles, that will migrate in the sucrose gradient between the IM- and the OM-rings (see Experimental Procedures, Figure 23). As shown in Figure 42, TssB1 associated with mixed IM/OM vesicles after performing a "bad separation", indicating that TssB1, and probably TssC1, are associated to the OM in the conditions tested. TssC1 homologue in *B. cenocepacia* has been reported as tightly associated with the OM (Aubert *et al.*, 2010).

Basler *et al.* (2012) have imaged by electron microscopy the sheaths from *V. cholerae*, that they obtained after lysozyme treatment coupled to differential centrifugation. They were only able to purify contracted sheaths, possibly because of spontaneous sheath contraction during cellular disruption and purification. This might be the case in our studies too, indicating that maybe upon contraction TssB/TssC homologues are in contact with the OM of the bacterium. Importantly, TssB1 also localized to the OM both in a Δ*tssM1* and in a Δ*clpV1* mutant, further indicating that the association with the OM might be due to contracted versions of the sheaths (Figure 43).

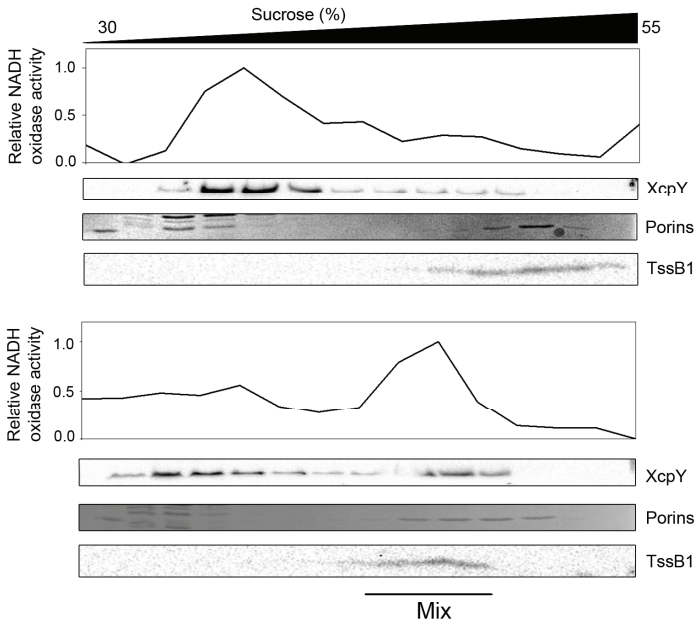


Figure 42 Good (upper panel) versus bad (lower panel) discontinuous sucrose gradient separation. NADH oxidase activity and XcpY are used as IM markers. Porins, detected by SDS-PAGE and Coomasie blue staining, were used as OM markers.

ClpV1 homologues have been shown to interact with TssB-like proteins (Bonemann *et al.*, 2009, Kapitein *et al.*, 2013, Pietrosiuk *et al.*, 2011), in particular with contracted sheaths (Kapitein *et al.*, 2013). In our studies, ClpV1 systematically co-fractionated with the OM. If contracted TssB1/TssC1 tubules are associated with the OM in *P. aeruginosa*, this would explain ClpV1 localisation in our studies (Table 10).

Hcp1 was found to co-fractionate with the IM in every strain sutdied (Table 10, Figure 43 and also in Δ*retS*, not shown). Also, preliminary data suggested that VgrG1 and Tse1 are also associated with the IM (Figure 43 and not data shown). This would indicate that the effectors of the H1-T6SS are docked in the IM, so that when the machinery is assembled, bacteria can recruit and fire the effectors as fast as possible.

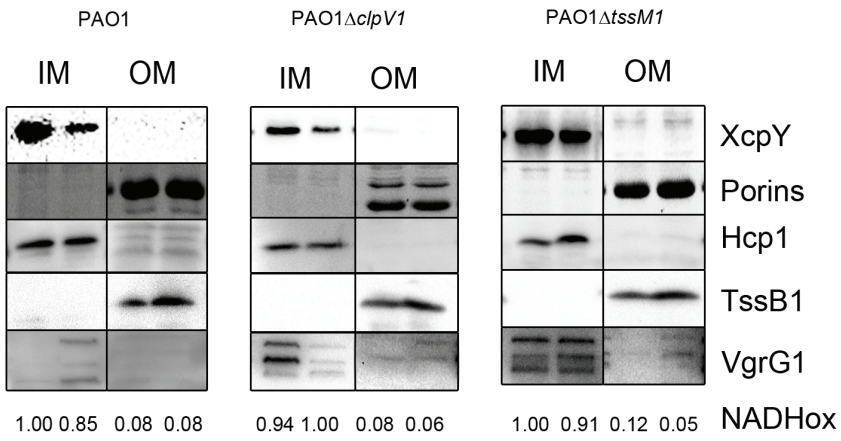


Figure 43 IM and OM separation by discontinuous sucrose fractionation. XcpY and NADH oxidase activity (NADHox) were used as IM markers. Porins, detected by SDS-PAGE and Coomasie blue staining, were used as OM markers.

(c) DISCUSSION

The fractionation method used in these studies coupled to shotgun proteomics proved to be an efficient strategy for the study of macromolecular assemblies of both the IM and the OM of *P. aeruginosa*, accounting for integral and peripheral membrane proteins. Proteins annotated as periplasmic or cytoplasmic might interact with membrane components and should not be considered as contaminants, since they can form part of membrane-bound machineries.

We have been able to identify integral membrane proteins with up to twenty-five trans-membrane domains (PA1054, listed as "hypothetical protein"). Identification of integral IM proteins with numerous trans-membrane domains is considered to be a challenge, since they are usually expressed at low levels in the cell and they do not have charged residues in the membrane-spanning domains, what make their identification a difficult task (Barrera & Robinson, 2011).

When the IM and the OM samples are clustered, it becomes evident that OM samples differ amongst them (EX in Figure 44), which might explain why some proteins could not be attributed a residence compartment. This could also be due to proteins with multiple

localizations of a protein within the cell. To this regard, it is possible that more than three biological replicates are needed for statistical discrimination of less abundant proteins.

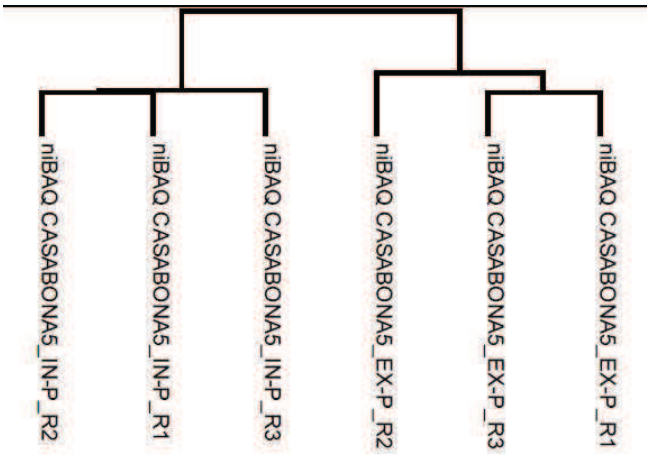


Figure 44 IM and OM samples clustering of *P. aeruginosa* PAO1 strain. IN: inner membrane. EX: outer membrane. R1-3: replicates 1 to 3. Samples EX-R1 and EX-R3 are more similar between them than EX-R2. On the contrary, IM samples are all similar.

Our approach was adapted for quantitative label-free analysis and allowed us to compare the IM and the OM of *P. aeruginosa*. In particular, this comprehensive analysis and the results obtained for the H1-T6SS mutants (Figure 43) has allowed me to propose a model of how the H1-T6SS is arranged to span the cell envelope in *P. aeruginosa* (Figure 45), in which the T6SS effectors (Hcp and VgrG) and the toxins are docked in the IM and the contracted sheaths are associated with the OM. The deletion of *tssM1* does not influence the association of the effectors to the IM, suggesting that the anchoring complex is not involved in this association or that the effectors interact with TssL1, the other IM component of the anchoring complex.

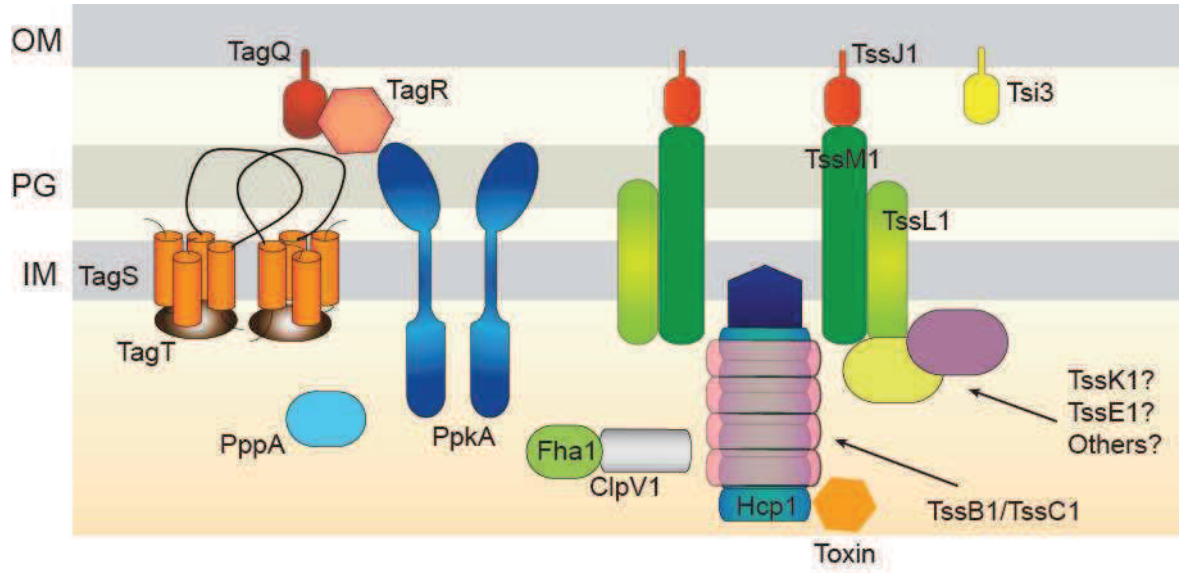


Figure 45 Global vision of the H1-T6SS machinery of *P. aeruginosa*, based on studies of protein homologues and our studies concerning TagTSRQ and TssJ1. Hcp1 hexameric rings, toxins and VgrG associate with the IM. TssK1 is a predicted cytoplasmic protein that was identified in our studies but could not be attributed a resident compartment. Proteins that have never been identified are not represented.

To my knowledge, this is the first study revealing insights into the global membrane arrangement of the H1-T6SS of *P. aeruginosa*. Our future studies will to decipher the assembly of other H1-T6SS components, such as TssK1 and TssE1, that can be detected by shotgun proteomics. In order to do this, their localisation will be studied using Selected Reaction Monitoring (SRM) applied to proteomics in wild-type and H1-T6SS mutants (PAO1 as reference strain, Δ*retS*, Δ*tssM1*, Δ*clpV1*, Δ*ppkA* and Δ*pppA*). In contrast to conventional shotgun proteomic studies, SRM measurements are quantitative analyses strictly targeting a predetermined set of peptides for which prerequisite information such as the MS response is necessary (Lange *et al.*, 2008). In our case, this previous information has already been collected in the shotgun proteomic assays. We have chosen two representative peptides per protein, for XcpY and XcpX (IM markers), OprF (OM marker), TssL1, TssK1, PpkA, ClpV1, TssB1, TssC1, Fha1, TagQ and TssJ1. These experiments are ongoing (in collaboration with the Proteomics facility of our institute, Dr Couté and Dr Adrait).

Ultimately, the novel findings will be verified using a cellular approach. Proteins of interest will be fused to fluorescent proteins to carry out at single-cell level analysis, as it has been proposed for TagTSQR regulatory module components. Also, spheroplast observation by confocal microscopy can be used to decipher localisations in *P. aeruginosa*, as I have already

done for TssJ1-mCherry and TagQ-mCherry. To this purpose, the constructions for TssK1, TssE1, Hcp1, TssB1 and ClpV1 are under way.

Chapter IX. Identification and Characterization of a Novel Macroglobulin-like complex in *P. aeruginosa* Envelope

9.01 INTRODUCTION

As described in the previous Chapter, our proteomics approach to study the envelope of *P. aeruginosa* allowed the identification of a big number of proteins of unknown function. Amongst these, PA4489, a protein in which we we were in particulary interested in our laboratory.

Analysis of the genetic environment of PA4489 (hereafter *magD*) revealed that *magD* is encoded in a putative six gene operon, that we named *magABCDEF* (PA4492-4489). Previous studies showed that the mRNA of *magA* (PA4492) is a direct target of RsmA, and therefore is co-regulated with HSI-I (Brencic & Lory, 2009). Moreover, transposon insertion mutants in *magB*, *magD* and *magF* were found to have attenuated virulence towards a *Drosophila melanogaster* host model (Potvin *et al.*, 2003). Since bioinformatic analyses showed that *magD* codes for a human α2-macroglobulin (α_2M) homologue, we sought to biochemically, functionally and structurally characterize the α_2M-like protein of *P. aeruginosa* PAO1 as a possible player in its virulence.

α2-macroglobulins are broad-spectrum protease inhibitors that play essential roles in the innate immune system of eukaryotic species. Hence, they are evolutionarily conserved and even though they can be divided into different protein families, sequence analysis showed that α_2Ms derived from a common ancestor (Rehman *et al.*, 2012). Sequencing of bacterial genomes during the past years revealed the presence of homologue α_2M-like genes, including PA4489 of *P. aeruginosa* PAO1.

MagD has been found in OM vesicles (Choi *et al.*, 2011) and is exported to the periplasm using a PhoA reporter fusion (Lewenza *et al.*, 2005). I have participated in the determination of its localisation by carrying out fluorescence microscopy experiments. The first 64 residues of MagD were fused to mCherry ($MagD_{1-64}$-mCherry) and the red labelling was monitored in GFP-expressing bacteria and spheroplasts. Moreover, since shotgun proteomic analysis of the IM and the OM of *P. aeruginosa* revealed that MagD was associated preferentially with the IM, I carried out discontinuous sucrose gradients in order to confirm these results.

We have also shown in this study that MagD can be present in two forms in *P. aeruginosa*. The cleaved form, of 100kDa, is *P. aeruginosa*-specific and is dependent of the presence of the other proteins encoded by the *mag* operon.

Immunoprecipitation assays coupled to shotgun proteomics allowed us to identify MagD partners in the envelope. Moreover, MagD is crucial in the stability of the proteins that form this membrane-associated complex, MagA, MagB and MagF, but not MagC, apparently a non interactant.

9.02 MANUSCRIPT AS SUBMITTED TO JOURNAL OF BIOLOGICAL CHEMISTRY

Running title: Pa Macroglobulin

Unique biochemical and structural features of *Pseudomonas aeruginosa* α2-Macroglobulin homolog

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SUMMARY

Human pathogens frequently use protein mimicry to manipulate the host cells thus promoting their survival. Here we show that opportunistic pathogen *Pseudomonas aeruginosa* synthesizes a structural homolog of the human α2-macroglobulin protein, a large spectrum protease inhibitor and important player of innate immunity. SAXS analysis demonstrated that the *P. aeruginosa* protein MagD (PA4489) adopts the structure of the human protein and changes the conformation upon the binding of the human neutrophil elastase. MagD synthesis is under control of a general virulence regulatory pathway implying inner membrane sensor RetS and the RNA-binding protein RsmA and it undergoes cleavage from a 167kDa to a 100kDa form in all clinical isolates tested. Fractionation and immunoprecipitation experiments showed that MagD is translocated to the bacterial periplasm and resides within the inner membrane in a complex with three other molecular partners MagA, MagB and MagF, all of them encoded by the same six-gene genetic element. Inactivation of the whole 10kb operon on the PA01 genome resulted in mislocalization of uncleaved, *in trans*-provided, MagD and in its rapid degradation. In conclusion, in this work we identified in pathogenic bacteria a human macroglobulin homolog that may play a role in host-pathogen interaction, probably by binding host proteases and/or antimicrobial peptides.

Introduction

The α2-macroglobulin (A2M) is a highly conserved large spectrum protease inhibitor present in plasma that plays essential roles in innate immunity in humans and other metazoans. The main function of human A2M is to entrap a target proteinase, which may be of endo- or exogenous origins, and to eliminate it from the blood circulation using the specific cell surface receptor and endocytosis. A2M is a glycosylated protein formed of four 1451 amino-acids subunits and several conserved domains (Figure 1, Sottrup-Jensen *et al.*, 1989, Armstrong & Quigley, 1999). The target protease interacts first with the "bait region" of A2M which occurs simultaneously with a proteolytic cleavage of the A2M, and is followed by the formation of a covalent bond between the two molecules through a thioester formed in the conserved cysteine-glutamine region (CXEQ region) (Arakawa *et al.*, 1989, Delain *et al.*, 1992, Qazi *et al.*, 2000). This "venus flytrap" mechanism (Meyer *et al.*, 2012) involves important conformational changes of the protein and also conducts to exposure of the "receptor binding domain" required for binding of the complex to cell surface receptor identified as Low density lipoprotein Receptor-related Protein LRP (Sottrup-Jensen *et al.*, 1986, Sottrup-Jensen et al., 1989, Doan & Gettins, 2007). The binding of the A2M to LRP results in clearance of A2M and its cargos through the endocytic degradation pathways.

A2M belongs to a family of proteins including the C3 complement molecule, sharing macroglobulin domains (MG) and a thioester domain (TED) characterized by the CXEQ sequence. Although the C3 molecule is composed of two peptide chains, its activation pathway includes proteolytic cleavage and conformational changes similar to the one observed for A2Ms, observations recently highlighted by the determination of the A2M crystal structure and its comparison with high-resolution structure of C3 (Janssen *et al.*, 2006, Marrero *et al.*, 2012).

Availability of hundreds of bacterial genomes for bioinformatic analysis allowed the identification of A2M homologs in different bacterial clades including proteobacteria (Budd *et al.*, 2004). Notably, based on uneven phylogenetic distribution, the authors suggested the acquisition of the MG genes directly from the metazoan hosts as a colonization and/or defence factors. Predicted bacterial macroglobulin proteins (bMGs) could be classified in two subfamilies according to conserved protein domains and genetic environment of bMG-encoding genes. In majority of bacteria, the MG-encoding gene neighbours the gene encoding a penicillin-binding protein (PBP) 1C involved in cell wall biogenesis. This first class of bMG harbors in common a conserved signal peptide and overlapping lipobox sequence with the Cys residue at the N-terminal region of the protein,

suggesting their export into bacterial periplasm and membrane anchoring. Furthermore, this class of proteins possesses a conserved bait region and the CLEQ motif required for thioester formation (Budd et al., 2004 and Figure 1). The second class of bMGs is encoded within six-gene operons, all encoding proteins of unknown function. In this case, the predicted MG homolog harbors the signal peptide but no Cys residues throughout the whole sequence, which suggests its distinct mechanism in localization and substrate capture. A study concerning first class of bMGs performed on *Escherichia coli* A2M (ECAM) showed that indeed the protein is capable of binding human neutrophil elastase and undergoes proteolytic cleavage (Doan & Gettins, 2008). Furthermore, we recently showed by Small Angle X-ray Scattering (SAXS) analysis and electron microscopy that ECAM folds into an elongated molecule reminiscent of C3 complement molecule and undergoes important conformational changes upon protease binding (Neves *et al.*, 2012, Stover *et al.*, 2000).

P. aeruginosa is a Gram-negative opportunistic pathogen for humans provoking acute and chronic infections. Due to its resistance to majority of nowadays available antibiotics, *P. aeruginosa* is considered as one of the most preoccupying infectious agents frequently associated with nosocomial infections. *P. aeruginosa* is capable of growing either as planktonic, mobile bacteria or residing in sessile, biofilm communities (Coggan & Wolfgang, 2012, Mikkelsen *et al.*, 2011, Goodman *et al.*, 2004). These two ways of life are linked to differential expression of number of virulence genes, some of which being under control of membrane associated sensors RetS, LadS and GacS, among others (Goodman et al., 2004, Ventre *et al.*, 2006).

Although the genome sequence of *P. aeruginosa* strain PA01 has been available since 2001 (Stover et al., 2000), still more than 30% of genes are annotated as of unknown function. In this work we biochemically and structurally characterized the PA4489 gene product and showed that it encodes a bacterial A2M of 167 kDa which can be cleaved into a 100 kDa form. The structural analysis of the recombinant protein demonstrated the global fold reminiscent to MG-like molecules A2M and C3. *P. aeruginosa* A2M lacks the lipobox sequence and a second conserved Cys within the TED domain (Figure 1A), however it is capable of binding the neutrophil elastase *in vitro*. The protein is expressed in majority of *P. aeruginosa* strains and is regulated by the RetS-GacS pathway and the RNA-binding protein RsmA. The PA4489, renamed MagD, is a periplasmic, membrane attached protein forming a complex with at least three proteins encoded in the same six-gene operon. These results suggest that the macroglobulin-like protein in pathogenic bacteria may mimic its human homolog by trapping different substrates noxious for bacterial life.

Materials and Methods

Bacterial strains and growth conditions

P. aeruginosa and *E. coli* were grown at 37°C with agitation in LB broth supplemented with antibiotics when needed. Antibiotics used were ampicillin (50 μg/ml), tetracycline (10 μg/ml), gentamycin (25 μg/ml) and kanamycin (25 μg/ml) for *E. coli* and carbenicilline (300 μg/ml), tetracycline (100 μg/ml) and gentamycin (200 μg/ml) for *P. aeruginosa*. Transformed *P. aeruginosa* strains were cultured on Pseudomonas Isolation Agar (Difco) plates. For analysis, over-night cultures were diluted to optical densities at 600nm (OD_{600}) of 0.1 and cultivated further until OD_{600} of 1.0 – 1.5. Bacterial cells were harvested by centrifugation and immediately treated for further experiments or frozen at -20°C. *P. aeruginosa* strains were obtained from different laboratories as indicated in Supplementary Table 1.

Genetic constructions

P. aeruginosa strains deleted for *magD* sequences were constructed as follows. The *magD* sequence corresponding to codons 37 to TGA was obtained by PCR and cloned into *Sma*I-digested pEX100T vector (Schweizer, 1992). Then, the internal sequence was deleted by *Pst*I digestion and religation of the vector. The genetic construct used to delete the whole *magABCDEF* operon was obtained by splicing by overlap extension (SOE) PCR. The mutants were created by homologous recombination using pRK2013 as a helper plasmid (Konyecsni & Deretic, 1988). Mutants were created in PA01 and PA01Δ*retS* backgrounds

for *magD* deletion, and only in PA01 for the whole operon deletion (Δoperon). The fusion between the first 64 amino acids of MagD and mCherry protein was obtained by PCR amplification and cloning of the obtained fragment into pJN-mCherry (Casabona *et al.*, 2013). For overexpression in *E. coli*, the *magD* gene lacking first 111 nucleotides was PCR amplified, sequenced and cloned as *Eco*RI-*Hin*dIII fragment into pETDuet-1 vector (Novagen). Overexpressed protein lacks first 37 amino-acids and harbors hexa-histidine sequence at N-terminus. *magA*, *B*, *C*, *E* and *F* genes were synthesised using *E. coli* codon usage and cloned into pUC57 by GeneScript (http://www.genscript.com). The sequences harbor *Nde*I and *Bam*HI restriction sites at 5' and 3' ends, respectively, and are made so to encode proteins without signal peptide. For *magB*, the sequence encoding putative transmembrane helix was also excluded. All genes were cloned into pET15b for overexpression. For complementation, *magD* gene was synthetized by Proteogenix (http://www.proteogenix.fr/) and cloned into mini-CTX derivative pSW196 (Baynham *et al.*, 2006) harboring arabinose-inducible promoter *pBAD* to drive *magD* expression. The *rsmA* gene was PCR amplified and cloned, after sequencing, into pIApX2 vector. As a control, pIApX2-GFP (Thibault *et al.*, 2009) was introduced in the same *P. aeruginosa* strain and cultivated under same conditions. The list of primers used for amplifications are given in Supplementary Table 1.

Protein expression and antibody production

The plasmids were introduced into *E. coli* BL21(DE3) Star (Invitrogen) and protein production was induced by IPTG in 500 ml of LB during 3h at 37°C. The His_6-$MagD_{37\text{-coli}}$ protein was obtained as soluble, while other Mag proteins were obtained in inclusion bodies. Until now we have not been able to obtain His_6-MagC protein. Proteins were purified by Ni-affinity chromatography using standard protocols (Novagen) and Akta purifier (GE Healthcare). Rabbit polyclonal serum raised against His_6-$MagD_{37\text{-coli}}$ was obtained from Covalab (http://www.covalab.com). Antibodies against other Mag proteins were obtained by immunization of mice by AgroBio (http://www.agro-bio.com).

Bacteria and spheroplast preparation for $MagD_{1\text{-}64}$mCherry analysis

GFP-expressing bacteria and spheroplasts were prepared as described (Casabona *et al.*, 2013). Briefly, overnight cultures were diluted to OD_{600} 0.15 and incubated up to mid-log phase at 37°C with shaking. Induction was carried out by the addition of 0.25% arabinose for 1.5h. At this point, 1mL of cells were harvested and spheroplasts were created as described (Casabona *et al.*, 2013, Imperi *et al.*, 2009). In order to visualize intact bacteria, 1mL of culture was harvested by a rapid centrifugation step, and resuspended in 100µL fresh LB. Visualization was carried out with a Zeiss Axiovert operating system.

Fractionation of P. *aeruginosa*

Fractionation of bacterial cells was performed using exponential grown cultures (OD_{600} =1). The pellet equivalent to 2.10^9 bacteria was resuspended in 1ml buffer A (10mM Tris-HCl, 200mM $MgCl_2$, pH8) in presence of protease inhibitor cocktail (Complete, Roche), 0.5mg/ml lysozyme, and incubated for 30 min at 4°C with gentle agitation. The periplasmic fraction was recovered after centrifugation at 8000 g, 15min, 4°C. After one wash, the pellet, resuspended in 10mM Tris-HCl, 10mM $MgCl_2$, pH8, was disrupted by sonication. Unbroken bacteria were eliminated by centrifugation 8000g, 15min. Then, the supernatant was ultracentrifuged at 200000 g for 45min at 4°C (rotor TLA120 Beckman) to obtain the cytosolic fraction (supernatant) and the total membrane fraction. *E. coli* RNA polymerase (RpoA) and disulphide oxidoreductase (DsbA) were used as internal markers for cytosolic and periplasmic fraction, respectively. All fractions were resuspended in 4x SDS-PAGE loading buffer, incubated 5min at 100°C before SDS-PAGE or western blotting analysis.

Inner and outer Membrane separation

Inner and outer membranes of PAO1Δ*retS* were obtained as described previously with minor modifications (Casabona et al., 2013). Briefly, overnight cultures of PAO1Δ*retS* were diluted to $0D_{600}$ 0.15 and cultured to mid-log phase of growth. At this point, cells were harvested by centrifugation and resuspended in 25ml buffer A (10 mM Tris-HCl, 20% sucrose, 10 mg/ml DNase, 10 mg /ml RNase, pH 7.4). Cells were broken using a Microfluidizer at 15000 psi and total membrane fraction was

obtained by a centrifugation step at 100000*g* for 1h. This sample was resuspended in 500µL of 20% sucrose and loaded on top of a discontinuous sucrose gradient composed of eight layers of sucrose (from bottom to top, volume): 55% (1.4ml), 50% (1.5ml), 45% (1.5mL), 42.5% (1.3ml), 40% (1.5ml), 37.5% (1.3ml), 35% (1.5ml) and 30% (1.0ml). Centrifugation at 90000 g was carried for 72h and 500µL fractions were collected from the top. Fractions were characterized by SDS-PAGE, immunoblotting, and NADH oxidase activity. Porins were used as outer membrane markers. NADH oxidase activity and XcpY were used as inner membrane markers (Aubert *et al.*, 2010, Michel *et al.*, 1998).

Immunoblotting analysis

Western blotting analyses were done on Hybond LFP-PVDF transfer membrane (GEHealthcare) after electrotransfert in Laemmli buffer containing 20% ethanol. The membranes were blocked with 5% non-fat dry milk before incubation with primary antibodies overnight at 4°C. Dilutions of polyclonal antibodies were anti-MagD 1:40000, anti-MagA, -MagB, -MagE and -MagF 1:1000, anti-RpoA (Neoclone) 1:10000, anti-DsbA 1:10000 (obtained from R. Voulhoux, CNRS, Marseille), anti-XcpY 1:2000 (Michel *et al.*, 1998), anti-TagQ 1: 10.000 (Casabona *et al*, 2013). The secondary HRP-conjugated antibodies against rabbit or mouse were obtained from Sigma and used with a dilution of 1:50000. Detection was performed with Luminata Western HRP substrate Kit (Millipore).

Immunoprecipitation

Total extracts from PAO1Δ*retS* or PAO1Δ*retSΔmagD* cultures (equivalent to 30 OD_{600} units) resuspended in buffer A (10mM Tris-HCl pH8, 20% sucrose) and protease inhibitor cocktail (Complete, Roche) were obtained using Microfluidizer. Unbroken bacteria were eliminated by centrifugation at 8000g for 10 min prior to use. Protein A-magnetic beads (Immunoprecipitation Kit Dynabeads Protein A, Invitrogen) were incubated with anti-MagD immunopurified antibodies for 30mn at room temperature in Antibodies Binding Buffer–Invitrogen Kit. The covalent cross-link was realised using 5mM BS3 (Bis-sulfosuccinimidyl-suberate). After washes with Wash Buffer-Invitrogen Kit, the anti-MagD coupled beads were added to total extracts of PAO1Δ*retS* or PAO1Δ*retSΔmagD* and the incubation was carried out for 2h at room temperature in buffer A containing 100mM NaCl. The magnetic beads were washed three times and the elution was performed for 10 min at 70°C with reagents provided by the manufacturer.

Mass spectrometry

Three independent biological replicates of IP samples were prepared, their protein content extracted in Laemmli buffer and heated at 95°C during 5 min. Proteins were then stacked in the top of a SDS-PAGE gel (NuPAGE 4-12%, Invitrogen) and stained with Coomassie blue. Gel slices (1 per biological replicate) were excised and proteins in-gel digested by trypsin. Resulting peptides were analyzed by online nanoLC-MS/MS (Ultimate 3000 and LTQ-Orbitrap Velos pro, Thermo Fischer Scientific). Two analytical replicates were acquired per biological replicate. Raw data were processed using Mascot Distiller v. 2.3.2, and database search was carried out with Mascot 2.4 against the PAO1-UW strain protein sequence databank (February 1, 2012 release from www.pseudomonas.com, 5'572 entries). Identification results were filtered using the IRMa software (version 1.30.4): conservation of rank 1 peptides, peptide identification FDR < 1% as calculated by employing a reverse database strategy, and minimum of 1 specific peptide per identified protein group (PG). Mass spectrometry identifications were then compiled and structured within dedicated databases, grouped and compared using a homemade software (hEIDI, manuscript in preparation). For each biological replicate, the spectral count values of identified PGs (number of spectra assigned per PG in the sample) were calculated.

Results

Expression and regulation of an α2-Macroglobulin-like protein in *P. aeruginosa*

Bioinformatic analysis indicated that the *P. aeruginosa PA4489* gene encodes a homolog of the human α2-Macroglobulin (A2M) (www.pseudomonas.com,Winsor *et al.*, 2011). The predicted protein of 167kDa shares 20% identity and 53% similarity with *E. coli*

macroglobulin-like protein (ECAM, Doan & Gettins, 2008) and 45% similarity with human A2M (accession No P01023). It harbors a conserved N-terminal signal peptide that targets proteins across the bacterial inner membrane. In comparison to ECAM, *P. aeruginosa* macroglobulin-like protein does not possess a Cys residue within the signal peptide, conserved in bacterial lipoproteins and is thus predicted as a soluble periplasmic protein. In addition, *P. aeruginosa* protein lacks a second conserved Cys present in the A2M active site (CXEQ) forming the thioester bond involved in covalent binding of its substrates (Figure 1A). In contrast to ECAM, whose gene is adjacent to penicillin-binding protein (PBP) gene *pbpC*, the genetic environment of *PA4489* predicts an operon with five additional open reading frames that we named *magABCDEF* (PA4492-4487), the A2M-like protein (PA4489) being encoded by *magD* (**FIGURE 1B**). The specific antibodies raised against the recombinant MagD protein detected two distinct polypeptides in crude extracts of *P. aeruginosa* strains, one corresponding to the predicted size of the native MagD protein of approx. 167kDa (without the predicted signal peptide) and a second one of 100kDa; no labelling was observed in a PAO1 strain deleted for the *magD* sequence demonstrating that the two polypeptides are MagD-specific (**FIGURE 2A**). Recent studies showed that the the leader sequence of *PA4492* mRNA is a direct target of RsmA, an RNA-binding protein belonging to the virulence regulatory cascade including two membrane sensors RetS and LadS and the two-component system GacS/GacA (Brencic & Lory, 2009, Goodman et al., 2004, Ventre et al., 2006). In order to explore whether the synthesis of MagD is influenced by RetS and RsmA, we performed immunoblotting analysis of two laboratory strains PA01 and PAK deficient for the RetS sensor and of PA01 overexpressing RsmA. Indeed, the deletion of *retS* resulted in increase of MagD quantities detected in whole cell lysates, whereas overexpression of RsmA resulted in complete turn-off of the MagD synthesis (**FIGURE 2A**). MagD was readily detected in several *P. aeruginosa* strains, mostly clinical isolates obtained from different laboratories with some variations in synthesis levels, except for mucoid clinical isolate CHA where the two MagD-specific peptides were barely detected (**FIGURE 2B**). This absence of MagD expression in CHA is due to the perturbation of the same RetS-RsmA regulatory pathway (Sall et al., submitted). Interestingly, in some strains, the 167kDa polypeptide was more abundant whereas in other strains was the one of 100kDa, suggesting that the specific cleavage of the protein may be somehow regulated. Notably, the recombinant MagD protein lacking the first 37 amino acids obtained from *E. coli* ($MagD_{37\text{-coli}}$) was never cleaved, further indicating specific *P. aeruginosa* cleavage (see latter).

MagD shares structural similarities with human Macroglobulin, C3 and ECAM

Strong domain homology of MagD with human A2M protein prompted us to undertake the structural studies of purified $MagD_{37\text{-coli}}$ by SAXS. Experiments were performed with native protein, methylamine-reacted and after incubation with elastase. All samples were purified after reaction by gel filtration. The data are presented with the form log I(s) versus s (nm^{-1}), where I is the measured intensity and s is the scattering angle. The intensity curves for native MagD and methylamine-treated (red and blue, respectively, Fig. 3A) show strikingly similar shape and nearly superpose on the low angle region, indicating that no major conformational change happens upon incubation with methylamine. Differently, *E. coli* macroglobulin and C3 alter their structure when the thioesther bond present on CxEQ motif is disrupted by amines (Neves et al., 2012, Gros *et al.*, 2008). The absence of structure alteration upon methylamine reaction in MagD might be explained by the lack of CxEQ motif. On the contrary, however, the curve representing MagD after elastase reaction (green, Fig. 3A) shows that the side maximum shifts to higher angles after protease incubation and suggests that MagD structure seems to undergo a conformational change. However, the structure change might not be as drastic as in ECAM or C3 (Neves et al., 2012, Gros *et al.*, 2008) since is shows a discrete shift. Moreover the parameters radius of gyration (Rg) and Dmax calculated from the curves were almost the same for native (Rg 4.57, Dmax 18.3 nm) and for methylamine-reacted (Rg 4.61 nm, Dmax 17.7 nm), respectively. However, elastase-treated parameters were different (Rg 5.20 nm and

Dmax 20.3 nm). The parameters corroborate the fact that MagD conformation might change upon incubation with a protease but not with methylamine.

Using the scattering data collected on ID14-3, we initially calculated models of native ECAM using GASBOR (Svergun *et al.*, 2001) with default options. After ten independent models were generated, they were averaged by DAMAVER. Subsequently, a refined averaged model was calculated using GASBOR by employing a fixed core input file calculated by DAMSTART. The envelope of the elastase-reacted form of MagD (Fig. 3A and B) indicates a discrete conformational modification, generating a slightly larger structure. Notably, the native conformation is remarkably similar to *E. coli* native α2-macroglobulin SAXS structure (Neves et al., 2012).

MagD is a periplasmic protein associated preferentially with inner membranes

To explore the localization of MagD in *P. aeruginosa*, we first constructed the fusion protein between first 64 amino acids encompassing the signal peptide of MagD and the fluorescent mCherry protein. The $MagD_{1-64}$-mCherry-encoding fusion was introduced into *P. aeruginosa* PA01 strain and the localization of the protein was examined by microscopy both in whole bacteria and in spheroplasts expressing cytoplasmic GFP, as described recently (Casabona *et al.*, 2013). As presented in **FIGURE 4A**, the $MagD_{1-64}$-mCherry fusion localized to the bacterial periphery without any co-localization with cytoplasmic GFP (upper panel). The mCherry labelling was absent from spheroplast preparations, strongly suggesting that the fusion protein was lost during preparation, consequence of being addressed to the periplasmic space. To complete these observations, *P. aeruginosa* lysates were fractionated in cytoplasmic, membrane and periplasmic fractions and analysed by immunoblotting. Interestingly, by this approach, the majority of the native MagD was found associated with bacterial membranes (**FIGURE 4B, right panel**). This was confirmed by high content mass spectrometry analysis of *P. aeruginosa* inner and outer membranes where MagD peptides were systematically found in inner membrane preparation (Casabona *et al.*, submitted). Indeed, western blot analysis performed on membranes separated by centrifugation on sucrose gradients confirmed the presence of MagD in inner membrane preparations with some minor protein fraction found associated with outer membranes (**FIGURE 4B**). This finding was intriguing as MagD does not possess any hydrophobic regions nor predicted lipobox sequences that would attach the protein to the lipid moieties. We thus postulated that the protein could be associated to the membranes by interacting with other partners.

Protein partners of MagD include three proteins of the *mag* operon

Following the hypothesis that MagD associates with *P. aeruginosa* inner membranes through its protein partners, we searched first in mass spectrometry proteomic data for proteins of the *mag* operon that were membrane-associated. Notably, in addition to MagD, peptides of two proteins encoded by the operon, MagB (PA4491) and MagF (PA4487), were readily found in membrane preparations (Casabona *et al.*, submitted). In order to identify direct MagD partners, we set up immunoprecipitation (IP) experiments coupled with nanoLC-MS/MS analysis using whole bacterial lysates. Anti-MagD IPs were done in parallel with extracts of PAO1Δ*retS* (parental) and PAO1Δ*retS*Δ*magD* (Δ*magD*). Different steps of IP were checked by SDS-PAGE, revealed by silver staining (**FIGURE 5A**) and western blotting using anti-MagD antibodies. As shown on **FIGURE 5B**, anti-MagD antibodies immunoprecipated both MagD polypeptides (167 and 100 kDa) and additional proteins visible by silver staining that were absent from control experiments. Mass spectrometry analysis of the total IP eluate uncovered a number of potentially interesting partners including MagA (PA4492), MagB (PA4491) and MagF (PA4487) encoded within the operon same operon as MagD, which were further investigated (**Table 1 and Supplementary Table 2**). Proteins encoded by the *mag* operon display several interesting features (**Table 2**). First, all of them except MagB possess signal peptides and are predicted to be addressed to the periplasm. Second, MagB harbors one predicted

hydrophobic helix (amino acids 26 to 48). Third, the first and the last gene of the operon encode proteins, MagA and MagF that share 105 out of 269 identical amino acids (not shown). All are annotated in Pseudomonas genome database (www.pseudomonas.com) as being of "unknown function". We overexpressed and raised the antibodies against several Mag proteins and used them to confirm IP-MS/MS data and explore their localization. Indeed, MagA, MagB and MagF were specifically co-eluted with MagD, while no protein was eluted in washing fractions (**FIGURE 5B**). Interestingly, while these three MagD partners were readily detected in crude extracts of parental strain, no signal was obtained from the strain lacking MagD, suggesting the co-stabilization between the proteins of the presumable macroglobulin complex.

Mag complex formation is required for macroglobulin stability, cleavage and membrane anchoring

To investigate the localization and stability of MagA, MagB and MagF in parental strain PA01Δ*retS* and its MagD-deficient isogenic mutant, we performed immunoblotting analysis of different cellular fractions. The Δ*retS* strain was used here in order to enhance the detection of Mag proteins. Notably, the three proteins that were identified as MagD partners by IP/MS analysis were found preferentially associated with bacterial membranes, while they were absent from the MagD-deficient strain **(FIGURE 6A**). Importantly, the product of the fifth gene of the operon, MagE (PA4488), was not affected by the *magD* deletion, and was found preferentially in cytoplasmic fractions further supporting the idea that genetic construction had no polar effect on downstream genes. To ascertain this conclusion, we provided MagD in *trans* from the chromosomally inserted plasmid pSW196 (Baynham *et al.*, 2006) and induced its expression by arabinose. Clearly, MagD synthesis restored the presence of other three Mag proteins in the Δ*retS*Δ*magD*/pSW196-*magD* strain (**FIGURE 6B**). Here, we noticed that the dosage of MagD versus other Mag proteins encoded by the chromosome was important as high concentrations of arabinose used for induction resulted in degradation of the protein (not shown and see later). Therefore, four proteins, including the *P. aeruginosa* macroglobulin MagD, co-stabilize each other within the *P. aeruginosa* envelope by creating a multimolecular complex.

In order to investigate the role of proteins encoded by the *mag* operon regarding MagD *per se*, we deleted the whole operon of 10kb on the PA01 chromosome (the deletion could have never been obtained in the Δ*retS* background) and then provided *magD in trans*, inducing its expression by arabinose. In Δoperon genetic background (the Δop strain) we observed an evident, rapid degradation of the *in trans* provided MagD protein. Interestingly however, when the MagD protein could be detected, it appeared only as non-cleaved 167kDa form (**FIGURE 6C, right panel**). Furthermore, this non-cleaved form was found preferentially in bacterial periplasm and was absent from bacterial membranes. Thus, the Mag proteins encoded by the operon are essential for correct localization of MagD by anchoring it to the inner membrane and for its proteolytic cleavage into a 100 kDa form.

Discussion

In this work, we characterized a novel protein complex that resides within *P. aeruginosa* envelope which structurally mimics MG molecules of innate immunity, A2M and the C3 complement. The bioinformatics analysis indicated that the PA4489 gene encoded by the operon harboring six genes codes for a 167 kDa protein with several MG-domains in its N-ter half (www.pseudomonas.com, Winsor et al., 2011, Budd et al., 2004). *PA4489* gene product, MagD, is homologous to *E. coli* A2M-like protein ECAM, for which it was shown that it binds *in vitro* the human neutrophil elastase and undergoes the cleavage similar to the human A2M (Neves et al., 2012, Doan & Gettins, 2007, Harpel *et al.*, 1979). Contrary to ECAM, MagD of *P. aeruginosa* lacks the two cysteine residues essential for its localization and function. The first cysteine, belonging to the lipobox region, is thought to anchor the protein to the membrane. The second cysteine, within the CLEQ motif, conserved between *E. coli* and human A2M, participates in formation of the thioester required for covalent binding of substrate proteases. Despite these differences,

recombinant MagD is capable of elastase binding and changes its conformation upon the reaction. The structural resemblance between MagD and human C3 molecule (Doan & Gettins, 2007, Marrero et al., 2012, Neves et al., 2012) revealed by SAXS is striking and may indicate similar way of action. Moreover, the C3 complement molecule undergoes the proteolytic cleavage upon activation (Janssen et al., 2006, Dodds & Law, 1998). Interestingly, MagD appears to be cleaved from 167kDa to a 100kDa form in all clinical isolates tested. Unfortunately, up to date, we were unable to determine the site of the cleavage. Furthermore, it seems that the cleaved fragment of the protein is rapidly degraded, as only 100kDa polypeptide could be detected both by immunodetection and mass spectrometry analysis.

Despite the absence of the conserved Cys in the lipobox region and no predicted trans-membrane domain, MagD associates with bacterial IM through interactions with another protein which we identified as being probably MagB. MagB is encoded by the same operon, fractionates to the IM and harbors one predicted trans-membrane helix. We found, by IP experiments followed by shotgun proteomics identification, that in addition to the MagD-MagB complex, MagA and MagF participate in the complex formation. This was corroborated by the fact that both proteins MagA and MagF were found in IM fractions and moreover, in the absence of MagD, MagA, MagB and MagF were degraded. On contrary, MagE, encoded by the fifth gene of the operon, was equally well expressed and stable in wild-type and *magD*-deleted strains. To our surprise, MagE, although possessing the signal peptide which should allow its transfer across the inner membrane, was found only in bacterial cytoplasm. We cannot exclude that the predicted signal peptide is not functional or that a specific external signal is required for protein translocation. It is possible that cytoplasmic MagE interacts with the short Nter domain of MagB and links the Mag complex with other cytoplasmic components. Finally, one additional protein is encoded by the *mag* operon, MagC, which was never identified by mass spectrometry analysis of neither IP eluate nor IM and OM fractions (not shown). MagC was also absent from recent analysis of periplasmic compartment (Imperi et al., 2009). This could suggest that MagC is also cytoplasmic but its participation in complex formation needs to be examined in the future. The global structure of MagD by SAXS was obtained in absence of its partners indicating that C3-like fold does not require complex formation. The MagD partners could rather participate in proper localization of the complex and its processing/activation. Indeed, when MagD is found in bacteria without its partners (the Δop strain), it is still translocated into the periplasmic space, but it does not anchor to the membrane, it is not correctly processed and it rapidly degrades.

It was shown previously that the leader sequence of the *PA4492* (*magA*) mRNA is a direct target of the RNA-binding protein, RsmA (Brencic & Lory, 2009). This suggests that the *mag* operon is part of a regulon comprising several operons, all under control of the same regulatory cascade. Indeed, RsmA binds and down regulates the expression of a battery of genes all involved in formation and/or maintenance of the *P. aeruginosa* biofilm, including *psl* and *pel* operons necessary for exopolysaccharide synthesis (Brencic & Lory, 2009). RsmA activity is counter balanced by two small regulatory RNAs, RsmY and RsmZ, whose expression is under control of three inner membrane sensors, LadS, RetS and GacS (Ventre et al., 2006, Goodman et al., 2004, Brencic & Lory, 2009). The fact that MagD synthesis is up-regulated in the conditions that favor biofilm formation, e.g during chronic infections, is intriguing. Indeed, during chronic infections and growth in biofilms, *P. aeruginosa* down regulates the majority of so-called aggressive virulence factors, such as the Type III Secretion System (T3SS) machinery that injects toxins within the host cell (Hauser, 2009). T3SS toxins are major players in defence against host immune system, notably phagocytosis by macrophages and neutrophils (Hauser, 2009, Dacheux *et al.*, 2000, Dacheux *et al.*, 1999). The absence of the active T3SS in biofilm conditions renders the bacterium more fragile to external deleterious molecules, such as proteases and antimicrobial peptides. In these conditions, the overexpression of the protease inhibitor may be essential for bacterial survival. Indeed, binding of the elastase by the recombinant protein suggests that MagD may play a function similar to the human A2M by

trapping external proteases and inactivating them. Effectively, there are several examples where the pathogenic bacteria evolved the resistance mechanisms against metazoan immune molecules. For example, many bacterial species, including *P. aeruginosa*, harbor the periplasmic serine protease inhibitor, ecotin, which protects bacteria against the neutrophil attack (Eggers *et al.*, 2004). Another example is a conserved bacterial protein, Ivy, capable of binding and inhibition of the host lysozyme (Abergel *et al.*, 2007, Monchois *et al.*, 2001). Macroglobulin-like molecules are encoded in diverse bacterial species; however only few possess the complex genetic environment similar to *P. aeruginosa* (Budd et al., 2004). In majority of bacteria, the macroglobulin-encoding gene is located in the vicinity of PBP1C (Budd et al., 2004), an enzyme involved in peptidoglycan synthesis (Mattei *et al.*, 2010), suggesting that bacterial MGs may play a protective role, notably during bacterial division and cell wall synthesis.

In addition to three Mag proteins, the IP-MS/MS experiment allowed the identification of few additional putative partners, notably TssM1, recovered with 19 spectral counts specifically from the parental strain. Intriguing, TssM1 is a conserved inner membrane protein of the Type VI Secretion System (H1-T6SS) machinery which plays a crucial role in bacteria-bacteria interactions (Russell *et al.*, 2011, Schwarz *et al.*, 2010, Basler & Mekalanos, 2012). *P. aeruginosa* H1-T6SS-encoding genes, situated in the HSI-I cluster (Hcp Secretion Island-I), are co-regulated with the *mag* operon by the same regulatory cascade, RsmA targeting several H1-T6SS mRNAs (Brencic & Lory, 2009). Interestingly, T6SSs inject proteins within the periplasm of the neighboring species attacking directly the peptidoglycan chain (Russell et al., 2011, English *et al.*, 2012). We cannot exclude that the macroglobulin complex identified in this work plays a protective role against a bacterial aggressor.

First evidences of possible role of *mag* genes in bacterial virulence came from work of (Potvin *et al.*, 2003). Using high-throughput screening of *P. aeruginosa* mutants attenuated in a chronic rat infection model, these authors identified three mutants with independent transposon insertions in *PA4491* (*magB*), *PA4489* (*magD*) and *PA4488* (*magE*) genes (Potvin *et al.*, 2003). Further phenotypic characterization of these mutants showed that they are affected either in motility (swarming) or attenuated in alternative model host *Drosophila melanogaster*. Deciphering exact molecular mechanisms of MagABDF function will be a challenge of future studies.

In conclusion, identification of the macroglobulin-like complex in the bacterial envelope suggests a novel way of defence the pathogen has develop in order to survive. Targeting this complex may be a way to cripple this pathogen during antibiotic therapy.

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Figure Legends

Figure 1. *P. aeruginosa* PA4489 shares conserved domains with A2M. A/ Representation of human A2M protein with its conserved domains compared with *E. coli* protein (ECAM, YfhM) and product of the *P. aeruginosa* PA4489 gene, renamed MagD. Conserved Cys residues are indicated within the lipobox sequence and CLEQ motif, forming the thioester. Note the absence of both Cys residues in MagD. SS, signal peptide; MG, macroglobuline. RBD, receptor-binding domain. **B/** Genetic organization of the PA4489 gene within the operon of six genes, all predicted as encoding proteins of unknown function (www.pseudomonas.com). The operon consisting of *PA4492-PA4487* was named *mag* operon and genes *magA* to *magF*, with *PA4489* encoding *P. aeruginosa* A2M homolog.

Figure 2. Synthesis of MagD (PA4489) in *P. aeruginosa* is under control of RetS and RsmA. A/ Immunoblot analysis of crude *P. aeruginosa* extracts revealed by specific antibodies rose against recombinant His_6-$MagD_{37\text{-coli}}$ protein (line 7). *P. aeruginosa* strains were grown until mid-exponential phase and cells were recovered directly for analysis. Two MagD-specific peptides were identified: a first form corresponding to entire protein of 167kDa probably lacking predicted signal peptide of 26 amino acids and a second, cleaved form corresponding to a protein of approximately 100kDa. Note the increase and decrease of MagD synthesis in PA01Δ*retS* and PA01+RsmA, respectively. **B/** Detection of MagD polypeptides in various *P. aeruginosa* isolates, including cystic fibrosis isolates TB, CHA, SCV, and Liverpool epidemic strain (LES400). All the strains, except CHA, express high levels of MagD with different ratio between 167 and 100kDa forms.

Figure 3. Small-angle X-ray scattering (SAXS) results for native, methylamine-treated, and elastase-reacted MagD. A/ The radially averaged scattered X-ray intensity was plotted as a function of the momentum transfer *s*. Scattering patterns for MagD in native form (red), after reaction with methylamine (blue) and elastase (green) were recorded in 8.2, 0.3, 0.6 mg/mL, respectively. Inset, detail of differences in curve shape. **B**/ Distance distributions *p*(r) of native, methylamine-reacted and elastase-reacted of MagD. All curves were normalized. **C/ and D/ *Ab initio* models of MagD generated by SAXS.** Each model results from averaging 10 individual models calculated by the program GASBOR using: native (red) ECAM, elastase-treated ECAM (green). GASBOR was used in "user" mode, following default options, except for the total number of residues, which corresponded to entire MagD (1516 residues). The envelopes are based on the *p*(r) functions shown in A/ and B/, and the GNOM files generated were used as input for GASBOR. The models are drawn to scale.

Figure 4. MagD is translocated to the periplasm but appears attached to the inner membrane. A/ $MagD_{1\text{-}64}$-mCherry fusion was expressed from arabinose-inducible promoter carried within pJN105. The plasmid was introduced into different *P. aeruginosa* strains expressing cytoplasmic GFP. Spheroplasts obtained by lysozyme treatment and bacteria, both embedded in 1% agarose were observed by fluorescent microscopy using appropriate filters. Note the peripheral labelling in bacteria and absence of labelling in spheroplasts. **B/** Fractionation and immunoblot analyses of MagD. Whole bacterial cells (B) were fractionated into cytoplasm (C), total membranes (M) and periplasm (P), and immunoblotted with anti-MagD antibodies. RpoA and DsbA were used as cytoplasmic and periplasmic markers, respectively. Total membranes were further separated by centrifugation on sucrose gradients. Two inner (IM) and two outer membrane (OM) fractions were analysed by

measuring NADH oxidase activity, and by immunoblotting with anti-XcpY as an IM marker. OM is characterized by porins (36kDa) visualized by Coomassie staining. MagD is preferentially present in IM.

Figure 5. Immunoprecipitation coupled with LC-MS/MS analysis identifies potential MagD partners, including three proteins encoded by the *mag* operon. A/ Silver stained SDS-polyacrylamide gel showing steps of IP experiments. Immunopurified anti-MagD antibodies were used with parental (PA01Δ*retS*) and Δ*magD* strain. Twenty microliters of input (I), washing fraction (W) and eluate (E) were loaded on the gel. **B/** Immunoanalysis of input (I), washing (W) and eluate (E) by anti-MagD antibodies showing the presence of MagD only in eluate of parental strain. Following LC-MS/MS analysis (Table 1), the same fractions were analysed for presence of three proteins encoded within the operon, namely MagA, MagB and MagF.

Figure 6. Membrane localized MagA-MagB-MagD-MagF complex is required for macroglobuline localization and stability. A/ PA01Δ*retS* (parental) and PA01Δ*retS*Δ*magD* (Δ*magD*) bacterial cells (B) were fractionated into cytoplasm (C), total membranes (M) and periplasm (P) and revealed by antibodies directed against MagA, MagB, MagF and MagD, as control. Three Mag proteins co-fractionate with MagD in parental strain, and are absent in Δ*magD* mutant. **B/** Total extracts of PA01Δ*retS* (parental), PA01Δ*retS*Δ*magD* (Δ*magD*), PA01Δ*retS*Δ*magD* in which MagD was provided *in trans* by pSW196 (Δ*magD*+*magD*) or controlled strain (Δ*magD*+*ctrl*) were examined for presence of Mag proteins by western blotting. Note that the expression of *magD in trans* hinders the degradation of MagA, MagB and MagF. **C/** PA01 and its isogenic mutant in which the whole *mag* operon was deleted (Δop), in presence or absence of *magD* provided *in trans*, were analyzed for the two MagD forms. In the separated panel, membrane and periplasmic fractions were prepared and analyzed. DsbA was used either as loading control, or marker for the periplasm. Anti-TagQ was used as marker for total membranes. In the Δop strain, MagD is absent from membranes and present only as MagD 167kDa form in the periplasm.

Figure 7. Schematic representation of *P. aeruginosa* macroglobulin complex Mag localized to the bacterial envelope. MagD is reminiscent of C3 and A2M molecules by its structure. It is translocated across the inner membrane to the periplasm and resides associated with the inner membrane probably through interactions with MagB, harboring one transmembrane domain on its C-terminus. MagA and MagF, encoded by the same operon, are also partners of membrane-associated Mag complex.

Tables

Table 1. Identification of MagD partners as revealed by immunoprecipitation coupled to mass spectrometry analyses.

Locus Tag*	Gene name	Product name	Mean SC PA01Δ*retS*	Mean SC PA01Δ*retS* Δ*magD*	Enrichment
PA4491	*magB*	MagB	86.5	2.5	34.6
PA3068	*gdhB*	NAD-dependent glutamate dehydrogenase	30	1	30
PA4487	*magF*	MagF	34.5	1.5	23
PA2462		Possible hemagglutinin (DUF637)	63	4	15.8
PA4492	*magA*	MagA	19	0	WT only
PA0077	*tssM1*	TssM1	19	0	WT only
PA5304	*dadA*	D-amino acid dehydrogenase, small subunit	18	0	WT only

Proteins immunoprecipitated from parental and Δ*magD* strain using anti-MagD antibodies were analysed using shotgun proteomics. Proteins identified with at least 10 spectral counts (SC) in two biological replicates and either only in parental or enriched at least 10 times in parental versus Δ*magD* are listed. Proteins encoded by the same operon as MagD are highlighted. *From www.pseudomonas.com. The whole list of identified proteins is provided in Supplementary data.

Table 2. Characteristics of MagA-F proteins.

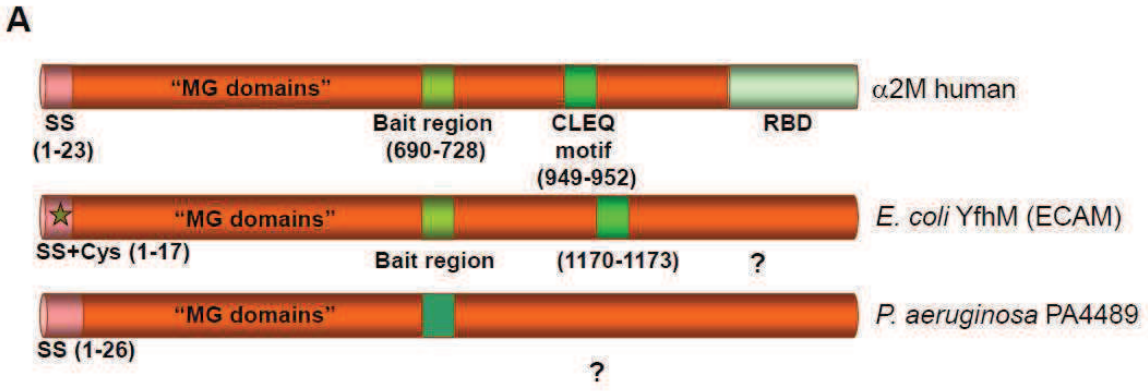
PA No*	Protein name	COG	pI/Mw (Da)	TMH**	Signal peptide***
PA4492	MagA	COG4676	5.78/29297		GLPA(25)LAE
PA4491	MagB	COG4685	5.12/64215	aa 26-48	
PA4490	MagC	COG3234	9.90/24001		GVARG(21)EPA
PA4489	MagD (A2M)	COG2373	5.48/167429		AAVQA(26)EDT
PA4488	MagE	COG5445	8.82/61710		LLLRA(20)AEA
PA4487	MagF	COG4676	5.12/28145		LVAWA(19)DNPV

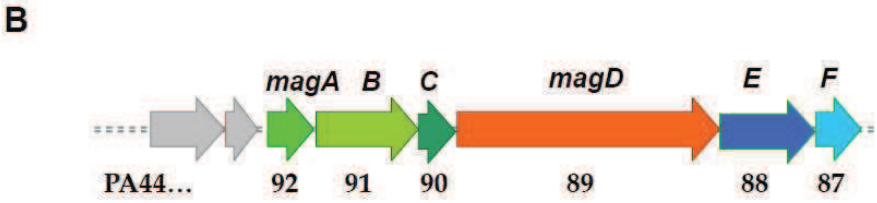
* Pseudomonas genome database http://www.pseudomonas.com/

** TMHMM http://www.cbs.dtu.dk/services/TMHMM/

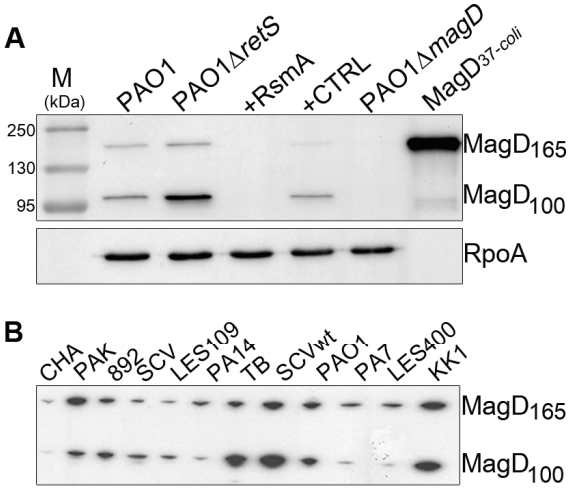
*** SignalP http://www.cbs.dtu.dk/services/SignalP/

COG: conserved ortholog group

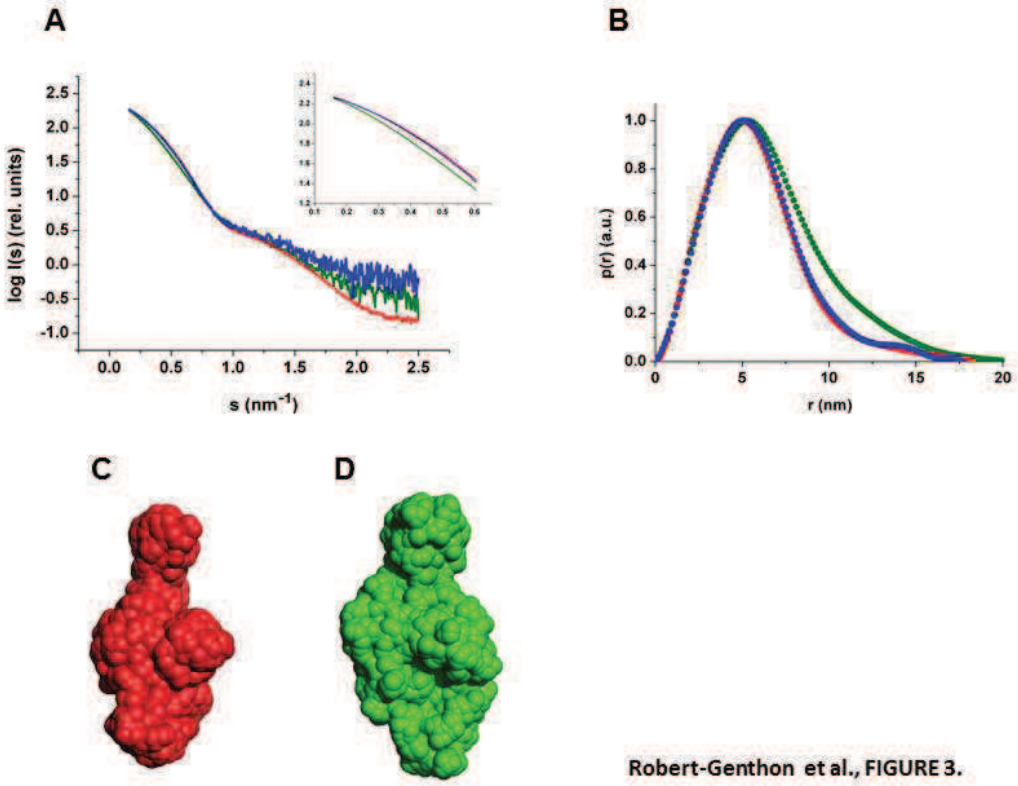




Robert-Genthon et al., FIGURE 1.



Robert-Genthon et al., FIGURE 2.



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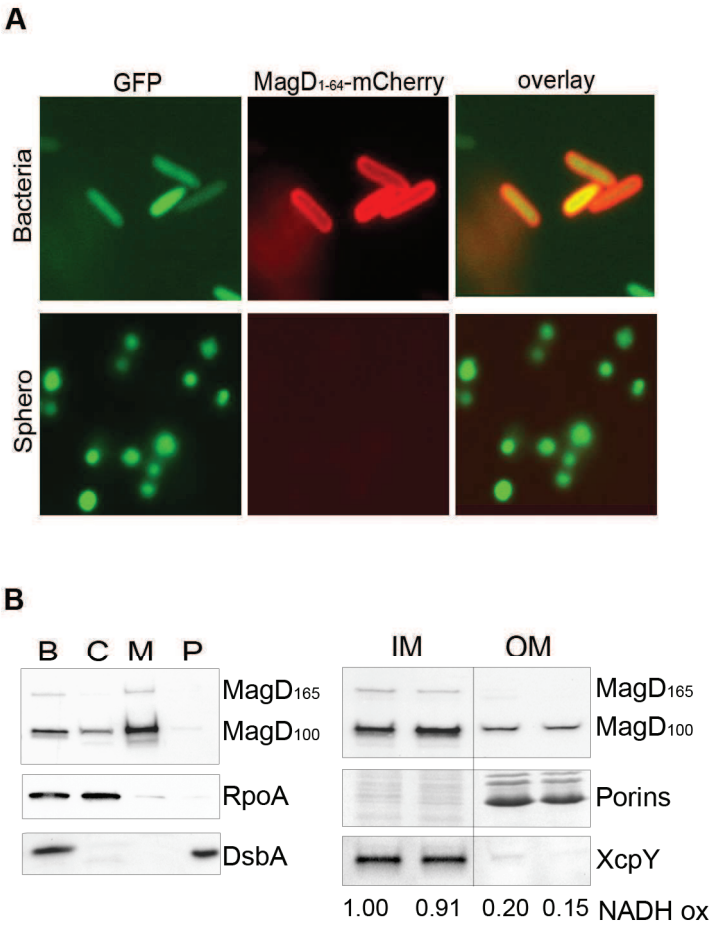
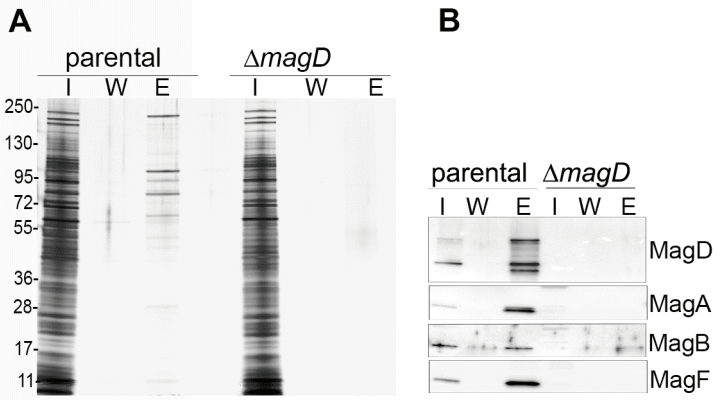


Robert-Genthon et al., FIGURE 4.



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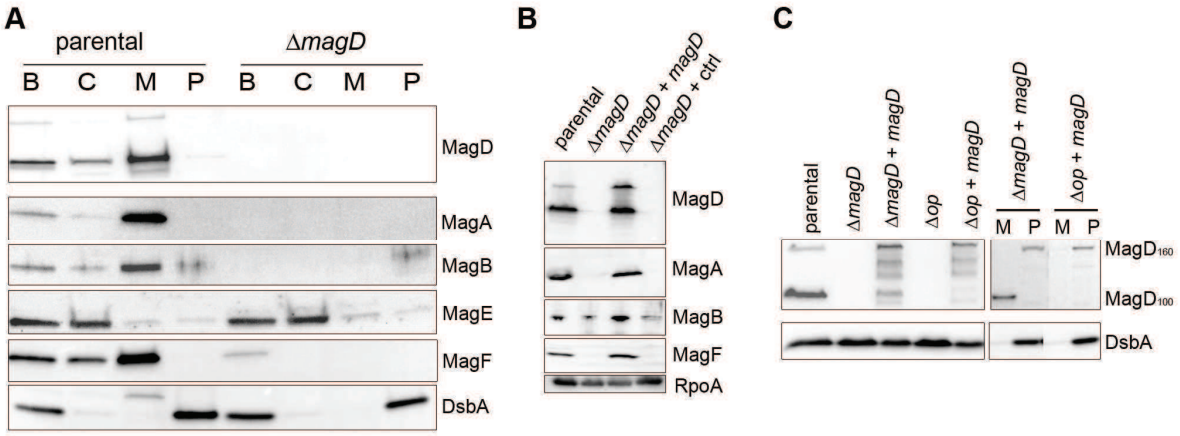
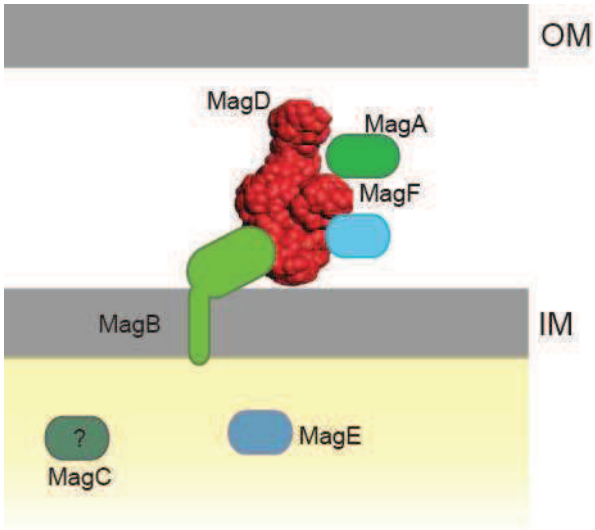


Robert-Genthon et al. FIGURE 6.



Robert-Ghenton et al. FIGURE 7.

Supplementary Table 1. *P. aeruginosa* strains and oligonucleotides used in this study.

Strain	**Characteristics**	**Origin/Ref**
PA01	Wound isolate, sequenced laboratory strain	J. Mougous, U. Washington
PA01Δ*retS*	RetS-deficient PA01 mutant	J. Mougous, U. Washington
PAO1Δ*magD*	MagD-deficient PA01 mutant	This study
PA01Δ*retS*Δ*magD*	RetS-, MagD-deficient PA01 mutant	This study
PAO1Δ*operon*	*mag* operon-deficient PA01 mutant	This study
PAK	Clinical isolate, widely used laboratory strain	S. de Bentzmann, CNRS, Marseille
892 TBCF121838)	CF isolate	B. Tümmler, Hannover Medical School (Salunkhe *et al.*, 2005)
SCV	CF isolate	I. Steinmetz, Hannover Medical School (von Gotz *et al.*, 2004)
LES109	CF epidemic strain	C. Winstanley, U. Liverpool
PA14	Virulent clinical isolate	A. Filloux, Imperial College (Rahme *et al.*, 1995)
TB (TB10839)	CF isolate	B. Tümmler, Hannover Medical School (Salunkhe et al., 2005)
SCVwt	Isogenic strain of SCV	I. Steinmetz, Hannover Medical School (von Gotz et al., 2004)
PA7	Wound isolate	PH Roy, Université Laval, Québec (Roy *et al.*, 2010)
LES400	CF epidemic strain	C. Winstanley, U. Liverpool (Salunkhe et al., 2005)
KK1	CF isolate	S. de Bentzmann, CNRS, Marseille (Bastonero *et al.*, 2009)
Primer name	**Sequence (5'…)**	**Characteristics**
MacroOE s	tacgaattcgatggccggcgaatccttcttcctg	Amplification of *magD* for overproduction
MacroOE as	tacaagctttcactcgaccttgaccttggccagc	Amplification of *magD* for overproduction
MagDrbsEco	gaattccctctctcaatgaatcgcg	Construction of *magD* -mCherry fusion
MagDXba	tctagagggcgcttcgagacgg	Construction of *magD* -mCherry fusion
MagD test1	atccgcggcgcaacaacgtg	Screening for *magD* deletion mutants

MagD test2	agcagttggctggcggtctg	Screening for *magD* deletion mutants
Mut Mag-F1	cccgggaaaacccgatgtcggtggat	Construction of Δoperon by SOE PCR
Mut Mag-R1	tctgcggcgtcggcgtggcaccgaagg ctcggctagtg	Construction of Δoperon by SOE PCR
Mut Mag-F2	ccacgccgacgccgcagaag	Construction of Δoperon by SOE PCR
Mut Mag-R2	cccgggccgtgcggcgcaggaagcg	Construction of Δoperon by SOE PCR

Supplementary Table 1. *P. aeruginosa* strains and oligonucleotides used in this study.

Supplementary References

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CONCLUSIONS AND PERSPECTIVES

P. aeruginosa is an important opportunistic human pathogen that can cause acute and chronic infections, notably in CF patients. This bacterium wide variety of virulence factors, including the H1-T6SS, active during chronic infections. In this work, we have characterized TagT, TagS, TagR and TagQ as additional players of the posttranslational activation of the H1-T6SS. We have shown that TagT and TagS form a membrane-bound ABC transporter with ATPase activity. TagQ is an OM lipoprotein that faces the periplasm. TagR is a periplasmic protein that associates to the OM in a TagQ-dependent manner. We propose that TagTSRQ form a novel signalling module in charge of sensing exogenous T6SS attacks in *P. aeruginosa*, most probably by sensing cell envelope disruption or interacting with exogenous T6SS effectors. The sensing by TagTSRQ module promotes local Fha1 phosphorylation that leads to T6S machinery assembly and firing.

In this study we also deliver the first comprehensive analysis of the protein repertoire of the IM of *P. aeruginosa*, by means of differential centrifugation coupled to shotgun mass spectrometry. A list of 991 non redundant proteins was obtained and analyzed. Moreover, a quantitative label-free approach was used in order to compare the IM and the OM sub-proteomes of *P. aeruginosa* and we obtained a list of more than 1700 non redundant proteins that could be attributed a residence compartment, 33.6% amongst them previously listed as “hypothetical proteins” or “conserved hypothetical proteins”.

This approach allowed the identification of numerous proteins that are associated to the membranes through protein-protein interactions, indicating that the cell envelope proteome is a complex network maintained by interactions. The full spectrum of biological activities was represented in both sub-proteomes, denoting the dynamism of these compartments. In particular, it allowed proposing a global view of the membrane assembly of the H1-T6SS of *P. aeruginosa*. To our knowledge, this is the first work reporting the localization of H1-T6SS components in *P. aeruginosa*, in particular the OM association of TssB1-TssC1. In our hands, after a “bad separation” of the IM and the OM, TssB1 co-fractionated with mixed vesicles, indicating that TssB1 – and probably TssC1- are tightly associated with the OM in the conditions tested.

Finally, we identified a novel IM-anchored complex co-regulated with H1-T6SS. This complex is composed of MagA-MagB-MagD-MagF, MagD being the homologue of a human A2macroglobulin of *P. aeruginosa*. In this work, we have shown in collaboration with Dr Dessen laboratory at IBS (Grenoble, France), that MagD shares structural features with human macroglobulin and the C3 complement. Despite the absence of lipobox and trans-membrane domain(s), MagD was found to associate with the IM of *P. aeruginosa* in our shotgun proteomics analyses. This might be possible due to the multiple interactions with MagB, MagA and MagF, all found to immunoprecipitate with MagD. Since previous studies have shown that several Mag proteins are necessary for infection in *Drosophila* and murine models, we propose that these proteins might form a complex involved in "innate immunity" of *P. aeruginosa* and thus it may have a role in host-pathogen interaction.

Overall, this work presents evidence of a novel signalling module of *P. aeruginosa*, in charge probably of T6SS exogenous attack sensing. Also, high-throughput mass spectrometry analyses of the IM and the OM of *P. aeruginosa* as a tool to study protein localization and envelope machineries identification are provided. In particular, insights into the macroglobulin-like membrane-bound complex are given.

ANNEXES

Institut de Recherche en Technologies et Sciences du Vivant (iRTSV)

CEA-Grenoble, France. January 2013

The scientific newsletter of our institute is a bimestral electronique publication aiming to pass along the new discoveries and technologies from each laboratory. The article presented herein was published in the February 2013 Newsletter. It describes the findings obtained in the article that we published in Environmental Microbiology and some preliminary results of inter-bacterial competition assays.

L E T T R E S C I E N T I F I Q U E

Guerre civile bactérienne

Les microbes vivent dans presque tous les environnements, formant des communautés poly-microbiennes appelées biofilms. Pendant la colonisation et l'infection des tissus, les agents pathogènes et commensaux cohabitent, interagissent, communiquent et combattent pour les éléments nutritifs. Dans la plupart des cas, un contact direct de cellule à cellule est nécessaire pour ce phénomène.

L'équipe *Pathogenèse Bactérienne et Réponses Cellulaires* du laboratoire Biologie du Cancer et de l'Infection cherche à élucider les mécanismes moléculaires de la pathogénicité bactérienne et à décrypter les réponses cellulaires de l'hôte à l'infection. Une nouvelle nano-machinerie moléculaire capable de pourvoir « la bonne santé » de bactéries en leur permettant de mener une guerre intra- et inter- espèces a été récemment identifiée dans l'enveloppe bactérienne (*Figure 1*). Cette structure ressemble au bactériophage ; appelée système de sécrétion de type VI (SST6), elle se présente sous la forme d'un appendice retrouvé à la surface de la bactérie et composé de plus de 15 protéines qui agissent en tant que dispositifs d'injection de toxines ou d'autres effecteurs. Dans une étude bio-informatique précédente [1], SST6 avait été caractérisé comme étant un complexe de 13 protéines retrouvées à la fois chez les bactéries pathogènes et les non pathogènes. De plus, cette étude avait mis en évidence que certaines bactéries, tel le pathogène opportuniste humain *Pseudomonas aeruginosa*, présentaient un certain nombre de gènes accessoires spécifiques localisés dans le même *opéron* que celui qui code pour des protéines connues comme étant impliquées dans la régulation de l'activation de SST6.

Une nouvelle étude a été réalisée en collaboration avec le groupe du Dr Joseph Mougous de l'Université de Washington dans le but de caractériser les produits de ces gènes accessoires, souvent associés aux besoins spécifiques de la bactérie, afin de mieux comprendre le rôle de SST6 dans les interactions intra- et inter- bactériennes. Dans ce but, les chercheurs ont construit des mutants de *P. aeruginosa* délétés des gènes accessoires *tagS*, *tagT*, *tagR* et *tagQ* (*Figure 2*). Les systèmes de sécrétion dans ces bactéries mutantes se sont avérés être correctement assemblés mais incapables de sécréter leur toxines, suggérant que ces protéines ont un rôle dans la régulation de l'activation du SST6. Avec l'aide de la plate-forme protéomique de l'iRTSV, il a été montré que TagQ est une lipoprotéine qui est localisée sur la membrane externe de *P. aeruginosa* et qui est indispensable pour l'ancrage de TagR, un acteur clef dans l'activation du SST6, sur cette même membrane. Enfin des approches biochimiques ont permis de montrer que TagT et TagS forment un nouveau *transporteur ABC* intra-membranaire, lui aussi indispensable à l'activation du SST6.

Ces études ont permis de proposer un modèle rendant compte du fait que les protéines considérées comme accessoires pour le système de sécrétion SST6 participent très probablement à une voie de signalisation trans-membranaire qui favorise la mise au point du dispositif d'injection dans des conditions environnementales appropriées.

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[1] **Boyer F, Fichant G, Berthod J, Vandenbrouck Y and Attree I.** Dissecting the bacterial type VI secretion system by a genome wide *in silico* analysis: What can be learned from available microbial genomic resources? *BMC Genomics*, 2009, 10: 104

[2] **Casabona MG, Silverman JM, Sall KM, Boyer F, Coute Y, Poirel J, Grunwald D, Mougous JD, Elsen S and Attree I.** An ABC transporter and an outer membrane lipoprotein participate in posttranslational activation of type VI secretion in *Pseudomonas aeruginosa*. *Environmental Microbiology*, 2013, 15(2): 471-486

Un opéron est un groupement de gènes qui sont transcrits ensemble en ARN messager.
Les transporteurs ABC forment un vaste ensemble de protéines transmembranaires dont le rôle est le transport unidirectionnel de part et d'autre de la membrane cytoplasmique de diverses substances (ions, stérols, macromolécules...) en utilisant l'énergie fournie par l'hydrolyse de l'ATP.

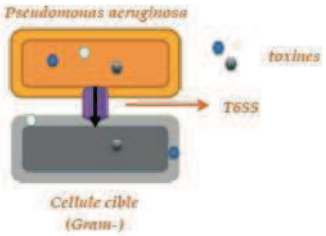

Figure 1. L'injection des toxines via SST6 est dépendante d'un contact entre deux cellules.

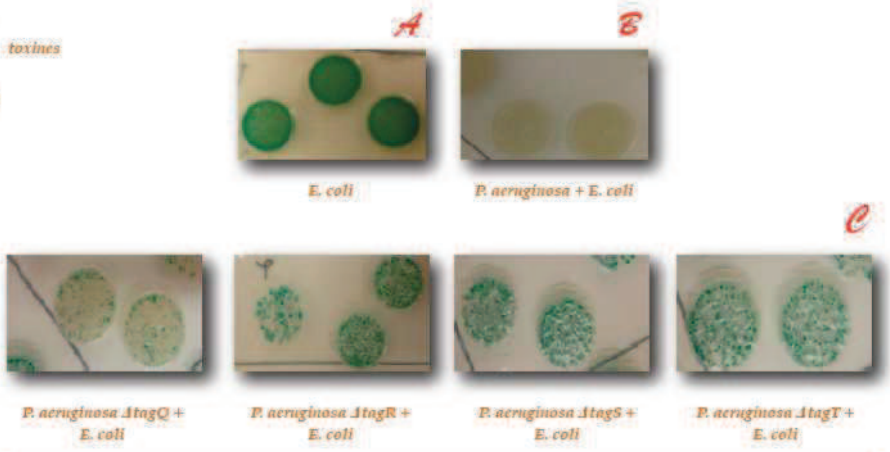

Figure 2. Tests de compétition entre P. aeruginosa et Escherichia coli, une autre bactérie à Gram négatif.
A - Mono-culture de Escherichia coli (colonies colorées).
B - Test de compétition entre E. coli et une souche sauvage de P. aeruginosa. Toutes les colonies de E. coli sont tuées.
C - Dans ces tests de compétition, les quatre mutants de délétion de P. aeruginosa ΔtagQ, ΔtagR, ΔtagS et ΔtagT, sont mis en culture en présence de E. coli. Leur capacité à injecter une toxine dépendante du SST6 est évaluée au travers de ce test visuel.

12th French CF Young Investigator Meeting

Paris, France. 4th March 2011

Poster presentation

The role of a novel ABC transporter in the T6SS of *P. aeruginosa*

Casabona M.G. [1], Sall K. [1], Giraud C.[2], Ragno M. [1], Elsen S. [1], de Bentzmann S.[2] and Attrée I.[1]

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Objective: *Pseudomonas aeruginosa* is an opportunistic pathogen, resistant to many antibiotics that can cause severe infections and death in cystic fibrosis patients. The main objective of this work is to study the assembly and function of one of the three type six secretion systems (T6SS-HSI I) of *P. aeruginosa*. This protein export nanomachine is known to be involved in the chronic stage of infections.

Methods: A combination of genetic and biochemical approaches were used in this study. *P. aeruginosa* mutants were generated in order to study different phenotypes as a consequence of an incomplete secretion machine. Confocal microscopy was used to study protein localisation and flow cell systems were used to study biofilm formation.

Results: TagS and TagT are two proteins encoded in the same operon as different proteins that are involved in the post-translational regulation of the T6SS-HSI I. We have purified the complex TagST, after heterologous expression, from *E. coli* membranes, and shown that it possesses an ATPase activity. We have demonstrated that this ABC-transporter is necessary for the correct functioning of the T6SS, since in deletion mutants the export of the effector protein Hcp is deeply affected. We have also been able to prove that, despite of high homology with a general lipoprotein transport system (the Lol system), TagST ABC transporter is not implicated in the outer membrane localisation of the two lipoproteins encoded in the T6SS locus, TagQ and TssJ. In the search for other phenotypes, we have tested the biofilm formation of different mutants. We have demonstrated, using flow cell system, that a deletion mutant of the ABC transporter is not able to form the usual biofilm "mushrooms".

Discussion and Conclusion: The results obtained in this study suggest that the novel ABC-transporter associated with T6SS is implicated in the biofilm formation by *P. aeruginosa.*

The full understanding of its functioning may contribute in the future to combat *P. aeruginosa* linked infections.

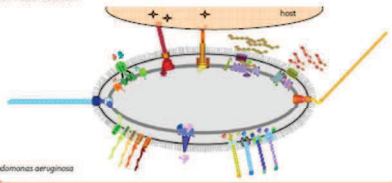
Ce travail fait l'objet d'un soutien par l'Association Vaincre la Mucoviscidose

The role of a novel ABC transporter in the T6SS of *P. aeruginosa*

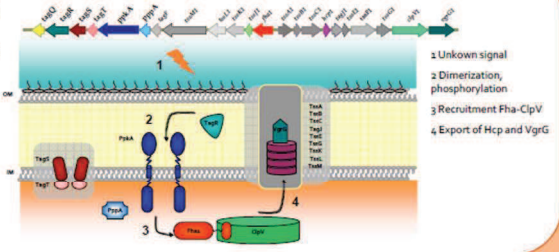
Casabona M.G. [1], **Sall K.** [1], **Giraud C.**[2], **Ragno M.**[1], **Elsen S.** [1], **de Bentzmann S.**[2] **and Attrée**[1] [1]UMR 1036 INSERM, Biology of Cancer and Infection; CNRS, ERL 5261, Bacterial Pathogenesis and Cellular Responses, iRTSV, CEA/Gre, France [2]Laboratoire d'Ingénierie des Systèmes Macromoléculaires, CNRS – Aix Marseille Université, 31 Chemin Joseph Aiguier, 13402 Marseille, France.

Introduction

- The Type 6 Secretion System (T6SS) can inject toxins directly into eukaryotes and prokaryotes. It was first described by J. Mougous (Mougous et al, Science, 2006)
- There are three T6SS in *P. aeruginosa*: T6SS-1 is involved in chronic infection in cystic fibrosis and biofilm formation



How does it work?



T6SS of Pa and Cystic Fibrosis

- Hcp is is present in the sputum of CF patients infected with *P. aeruginosa* **(Figure 1)**
- Hcp is regulated at two levels:

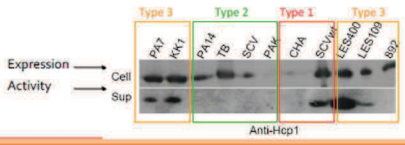


Figure 1 Hcp production and secretion in different clinic isolates.

TagST are necessary for Hcp secretion

- The double deletion mutant PAO1Δ*retS*Δ*tagST* presents a diminished capacity in Hcp export in comparison to PAO1Δ*retS*, similarly to Δ*retS*Δ*tagR* mutant (Hsu *et al* 2009). This deficiency is restored when a replicating plasmid carrying ***tagS*** and ***tagT*** is incorporated (Figure 2).
- Hcp export was lower when the complementation was done with a *tagStagT**, a construction carrying a point mutation in the key residue for the ATPase's TagT *in vitro* activity.

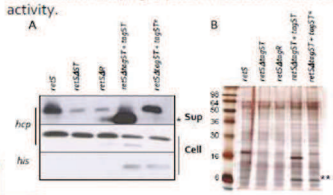


Figure 2 TagS and TagT are necessary for Hcp secretion. A) Immunoblotting αHcp. For Hcp secretion tests, cells are harvested at a OD_{600} = 0,95. The equivalent to 1,5x10⁸ bacterial cells is loaded on gel and the equivalent to 750µL of supernatants is precipitated. The αHis blot was done as control of the expression of 6hisTagT in complemented strains. B) Silver stained SDS-PAGE gel used as a control of secretion profiles corresponding to the parental strain and the complemented mutants.

TagST are necessary for biofilm fomation

- The formation of biofilms is crucial the chronic infection by Pa. Biofilms have higher antibiotic and stress resistance.
- As shown in Figure 3A, PAO1Δ*retS* and PAO1Δ*retS*Δ*tagST* 3D mushroom-like structures after 1 day incubation, even though the deletion mutant presents less. Only the parental strain presents the 3D structures in the 4th day.
- The TagST complex has a critical role in the biofilm persistence in PAO1 in a ΔretS background, even though its growth is not affected in the conditions (Figure 3B)

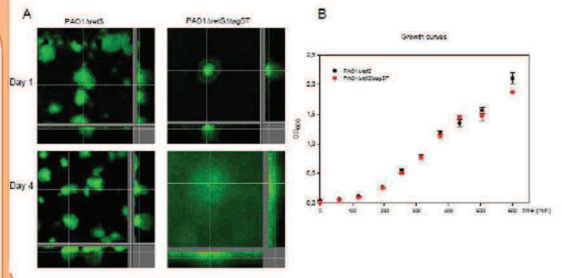


Figure 3 Biofilm formation in PAO1Δ*retS* and PAO1Δ*retS*Δ*tagST* mutants under dynamic conditions A) Biofilms were grown in flow cell chambers with minimal medium M63 supplemented with 0,2% glucose and 0,5% CAS amino acids. B) Growth control curves in the same milieu used for the flow cell chamber experiments.

TagST do not influence TagQ's localization

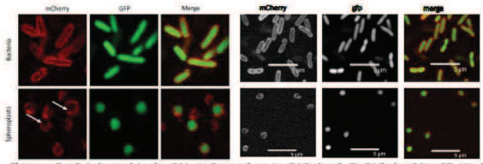


Figure 4 TagQ is located in the OM. A) Parental strain, PAO1ΔretS. B) PAO1ΔretSΔtagST. We have analyzed its localization by fusing TagQ to the red fluorescent protein mCherry under an inducible promoter and introducing it in PAO1ΔretS-GFP strains. Using confocal microscopy, we have observed that TagQ-mCherry has a peripherical location in *P. aeruginosa*'s bacterial cells. Next, spheroplasts were created. P. aeruginosa's spheroplasts have the particularity that the OM stays partially attached in the form of a "croissant" after lyzozyme treatment

- TagST is an ABC-transporter with unknown substrate. It presents high homology with the system in charge of addressing lipoproteins to the outer membrane (OM).
- TagQ is addressed to the OM in PAO1Δ*retS* (Figure 4A)
- TagQ is also addressed to the OM in PAO1Δ*retS*Δ*tagST* (Figure 4B)

Conclusions and perspectives

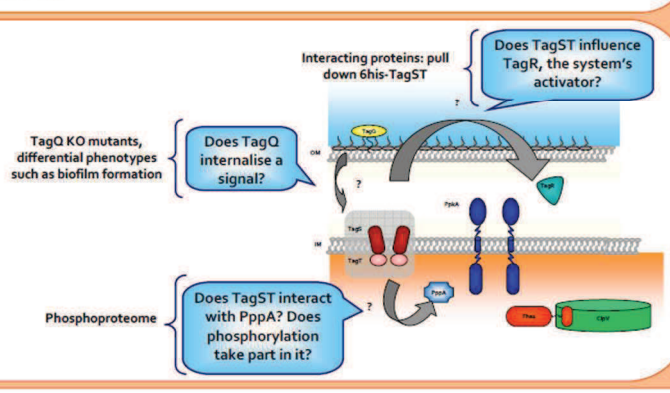
✓TagST is necessary for Hcp secretion

✓ TagST are necessary for biofilm formation in a Δ*retS* background (hyper active T6SS)

✓TagST do not influence TagQ's localization, despite its homology to the Lol System, in charge of addressing OM lipoproteins to the OM

Is the TagST complex involved in posttranslational regulation?

The full understanding of its functioning may contribute in the future to combat *P. aeruginosa* linked infections.



References Mougous *et al.* 2006 Hsu *et al.* 2009

6th European CF Young Investigator Meeting

Paris, France. 24 – 27 April 2012

Poster and Oral Presentation

The role of an OM lipoprotein in the H1-T6SS of *Pseudomonas aeruginosa*

Casabona M.G. [1]**, Silverman J.M.** [2]**,** Sall K. [1], Couté Y. [3], Grunwald D. [1], Mougous J.D. [2], Elsen S. [1] and Attrée I.[1]

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Background: *P. aeruginosa* is able to inject bacteriolytic toxins directly into other bacteria through one of its three type six secretion systems (T6SS), the H1-T6SS. This export machine is of high importance in complex niches such as the lungs, as it can serve as a defense mechanism for *P. aeruginosa* against competing bacteria.

Aim: The aim of this study is the characterization of TagQ, a T6-*Pseudomonas* specific protein that is encoded in the same operon as PpkA1, PppA1 and Fha1, actors of posttranslational regulation of H1-T6SS.

Methods: Deletion mutants were generated to study their impact on H1-T6SS function. Competition assays were used to analyze the fitness of *P. aeruginosa*. Confocal miscroscopy and discontinuous sucrose gradients were used to study the localisation of TagQ. The inner and outer membranes were further analyzed by discovery nanoLC/LC-mass spectrometry.

Results: We show that TagQ is indispensable for the function and activation of H1-T6SS by acting upstream of the PpkA-PppA phosphorylation checkpoint. We demonstrate that TagQ is a lipoprotein addressed to the OM. We discovered that periplasmic TagR, the PpkA kinase activator, is localized to the OM and that this association relies on the presence of TagQ.

Conclusions: The results presented herein identify and characterize TagQ as a novel player in the posttranslational regulation of *P. aeruginosa* H1-T6SS. The full understanding of its functioning may contribute in the future to combat *P. aeruginosa*-linked infections.

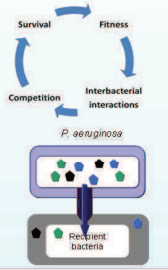
Acknowledgements: MGC is supported by a VLM doctoral fellowship.

The role of an OM lipoprotein in the T6SS of *Pseudomonas aeruginosa*

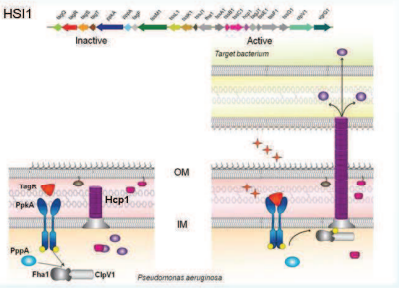
Casabona M.G.[1], Silverman J.M.[3], Sall K.[1], Couté Y.[2], Grunwald D.[1], Mougous J.D.[3], Elsen S.[1], Attrée I.[1]

[1]UMR 1036 INSERM, Biology of Cancer and Infection; CNRS, ERL 5261, Bacterial Pathogenesis and Cellular Responses, iRTSV, CEA/Grenoble, France [2]INSERM, U1035, Laboratoire Biologie à Grande Echelle, F-38054 Grenoble, France [3]Department of Microbiology, University of Washington, Seattle, WA 98195

Introduction



- The Type 6 Secretion System (T6SS) is present in a large number of Gram(-) bacteria [1].
- *P. aeruginosa* harbours 3 different T6SS-encoding loci (HSI 1-3). The HSI1 T6SS is expressed in chronic infections of CF patients: Hcp1 is present in the sputum of these patients [1,2].
- The HSI1 T6SS injects bacteriolytic enzymes directly into other Gram(-) bacteria [3,4].
- The posttranslational activation of HSI1 T6SS involves a Ser-Thr kinase, PpkA, capable of dimerization and autophosphorylation. PppA is a protein phosphatase that downregulates the system. Periplasmic TagR is also indispensible for the HSI1 T6SS activation [5,6].



1/ HSI1 T6SS in *P. aeruginosa* Isolates

- Hcp1 is present in the sputum of CF patients infected with *P. aeruginosa*
- Hcp1 and T6SS are regulated at two levels:

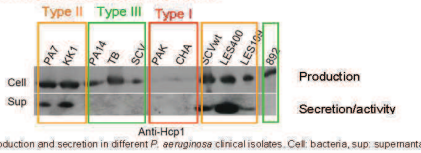


Fig1. Hcp1 production and secretion in different *P. aeruginosa* clinical isolates. Cell: bacteria, sup: supernatant.

2/ The Tag Proteins are Indispensible for T6SS Activity

- The Tag proteins (*t*ype *s*ix *a*ssociated *g*enes) are non conserved in all T6SS.
- Deletion mutants have been created in order to study their role in secretion and T6SS function

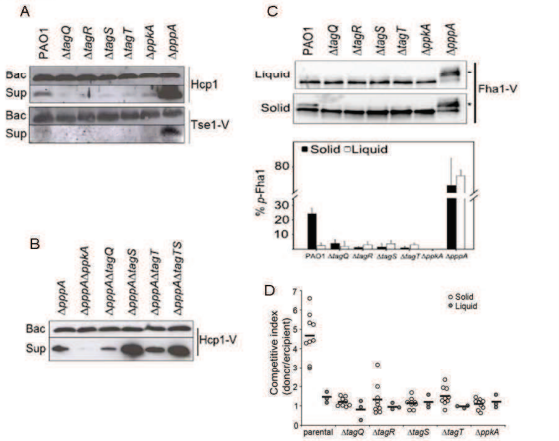


Fig2. A) Hcp1 and Tse1 production and secretion in Δ*tag* strains grown on liquid medium. B) Cellular and secreted Hcp1 from strains lacking Δ*pppA* and indicated *tag* gene. C) Fha1 phosphorylation (*p*-Fha1, marked with an asterisk) in solid and liquid medium. The percentage of *p*-Fha1 is also shown (lower panel). D) *P. aeruginosa* requires the *tag* genes for HSI1 T6SS-dependent fitness. The competitive index is plotted for competitions between each indicated donor strain and a HSI1 T6SS-susceptible recipient strain of *P. aeruginosa*. Bac: bacteria, sup: supernatant.

3/ TagQ is an Outer Membrane lipoprotein

- TagQ is predicted to be an OM lipoprotein
- TagQ is a *Pseudomonas* specific T6SS protein

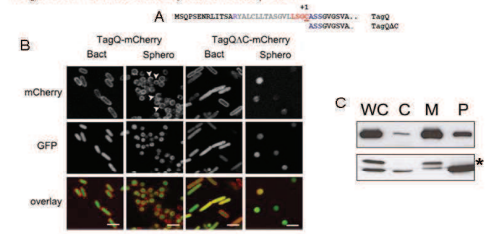


Fig3. A) The lipobox of TagQ is shown in red. The underlined C is the conserved C that has been deleted in TagQΔC. B) TagQ is an OM lipoprotein. GFP-expressing *P. aeruginosa* bacteria and spheroplasts expressing TagQ-mCherry or TagQΔC-mCherry were analyzed using laser scanning confocal microscopy. Arrows indicate the OM. Bact: bacteria, Sphero: spheroplasts. The bar represents 2 µm. C) Subcellular separation and immunoquantification of TagQ in PAO1Δ*tagQ* expressing either TagQ (WT) or TagQΔC (ΔC). The asterisk indicates the partial clivage of TagQΔC. WC: whole cells, C: cytosol, M: membrane fraction, P: periplasm.

4/ TagR's Association to the OM is TagQ-dependent

- TagR has been shown to be localized to the periplasm [6].
- TagR has no predicted trans-membrane domains.
- A fraction of TagR is associated to the OM

Fig4. Subcellular separation and immunoquantification of TagR in PAO1 (A) and PAO1Δ*tagQ* (B). WC: whole cells, C: cytosol, M: membrane fraction, P: periplasm.

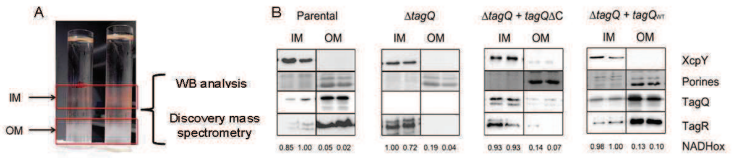


Fig5. A) Representative discontinuous sucrose gradient samples. The IM (red ring) and the OM (white lower ring) are shown. Fractions were collected from the top and analyzed by WB and discovery mass spectrometry. B) Representative IM and OM fractions. NADH oxidase activity (NADHox) and XcpY were used as IM markers. Porines were used as OM markers. Parental strain: PAO1.

Conclusions and Revised Model of HSI1 T6SS Activation

The genes of the *tag* operon are indispensible for the functioning and posttranslational regulation of the HSI1 T6SS of *P. aeruginosa*.

TagQ is an OM lipoprotein.

TagR, the kinase activator, is localized to the OM.

TagR localization to the OM depends on the correct localization of TagQ to the OM.

Do TagRSTQ form a complex? Do they interact? Pull-down experiments
Bacterial two-hybrid systems

What is the signal that triggers the trans-membrane signaling?

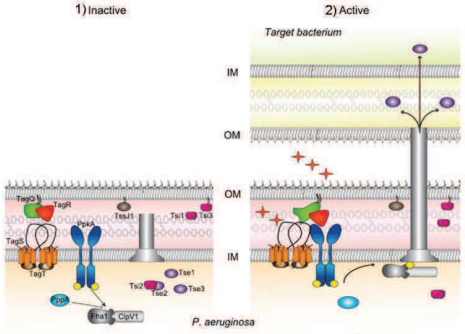


Fig6. Revised model of HSI1 T6SS: During the inactive state (1), TagR and TagQ might interact in the OM, and thus TagR is not accesible to PpkA and no phosphorelay is possible. Upon an unknown signal, during the active state (2), TagQ suffers a conformational change making TagR accesible. Then, PpkA dimerizes and autophosphorylates, triggering the cargo proteins secretion. The proteins are represented using the same colour code as in Fig1 and the T6SS machinery is represented as a single tunnel.

References

[1] Boyer *et al.*, 2009.
[2] Mougous *et al.*, 2006.
[3] Hood *et al.*, 2010.
[4] Russel *et al.*, 2011.
[5] Mougous *et al.*, 2007.
[6] Hsu et al., 2009.

112th General Meeting – American Society for Microbiology

San Francisco, United States of America.16-19th June 2012

Poster Presentation

Trans-envelope signaling leading to H1-T6SS activation requires an OM lipoprotein and an ABC transporter

Casabona M.G. [1], Silverman J.M. [2], Sall K. [1], Couté Y. [3], Grunwald D. [1], Mougous J.D. [2], Elsen S. [1] and Attrée I.[1]

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Background: *Pseudomonas aeruginosa* injects bacteriolytic toxins directly into other bacteria through one of its three type six secretion systems (T6SS), the H1-T6SS, which increases its fitness. This export machine is posttranslationally regulated by a eukaryotic-like phosphorylation pathway, which includes a kinase-phosphatase pair.

Aim: The aim of this study was to characterize TagS, TagT, TagR and TagQ, T6-Pseudomonas specific proteins that are encoded in the same operon as Ppka1, Pppa1 and Fha1, actors of posttranslational regulation of H1-T6SS.

Materials: Deletion mutants in T6S-Pseudomonas specific genes were generated and the function of the H1-T6SS was assayed in vitro and in growth fitness assays. Biochemical approaches and confocal microscopy were used to characterize TagS-TagT complex and determine the localisation of TagQ. Finally, a discovery-based assay using nanoLC/LC-mass spectrometry on inner and outer membranes was employed to decipher the connections between Tag proteins and actors of the posttranslational pathway.

Results: TagT, TagS, TagR and TagQ were found indispensable for activation of the H1-T6SS, acting upstream of the PpkA-PppA phosphorylation checkpoint. TagT-TagS has the signatures of bacterial ABC-transporters and forms an IM bound complex endowed with ATPase activity. We discovered that TagR, the PpkA kinase activator, is localized to the OM and that this anchor relies on the presence of TagQ, an OM lipoprotein.

Conclusions: The results presented herein identify and characterize TagT, TagS and TagQ as novel players in the posttranslational regulation of the H1-T6SS in *P. aeruginosa*. Taking into account our bioinformatic analysis we propose that the Tag proteins mentioned above represent a novel module participating in the detection and/or trans-membrane signaling through the bacterial envelope. The full understanding of the functioning of the H1-T6SS may contribute in the future to combat *P. aeruginosa*-linked infections.

Trans-envelope signaling leading to H1-T6SS activation requires an outer membrane lipoprotein and an ABC transporter

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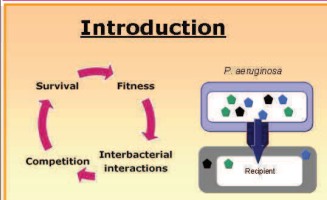
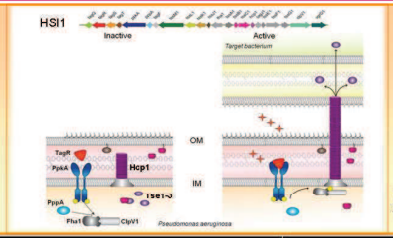
Introduction





- The Type VI Secretion System (T6SS) is present in a large number of Gram(-) bacteria [1].
- *P. aeruginosa* harbours 3 different T6SS-encoding loci (HSI 1-3). The H1-T6SS is expressed in cystic fibrosis patients chronically infected by *P. aeruginosa*: Hcp1 is present in the sputum of these patients [1,2].
- The H1-T6SS injects bacteriolytic effectors (Tse1-3) directly into other Gram(-) bacteria [3,4].
- The posttranslational activation of H1-T6SS involves a Ser-Thr kinase, PpkA, capable of dimerization and autophosphorylation. PpkA is a protein phosphatase that downregulates the system. Periplasmic TagR is also indispensible for the H1-T6SS activation (Fig1) [5,6].

Fig1. Posttranslational activation of H1 T6SS of *P. aeruginosa*. The putative signal is represented with red stars. Inner membrane (IM) and outer membrane (OM) of *P. aeruginosa* and the target bacterium are shown. Tse1-3 are T6S exported effectors.

TagQ is an Outer Membrane lipoprotein

TagQ is a *Pseudomonas* specific T6SS protein.

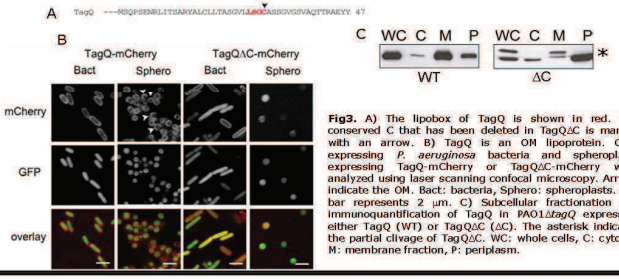


Fig3. A) The lipobox of TagQ is shown in red. The conserved C that has been deleted in TagQΔC is marked with an arrow. B) TagQ is an OM lipoprotein. GFP-expressing *P. aeruginosa* bacteria and spheroplasts expressing TagQ-mCherry or TagQΔC-mCherry were analyzed using laser scanning confocal microscopy. Arrows indicate the OM. Bact: bacteria, Sphero: spheroplasts. The bar represents 2 µm. C) Subcellular fractionation and immunoquantification of TagQ in PAO1Δ*tagQ* expressing either TagQ (WT) or TagQΔC (ΔC). The asterisk indicates the partial clivage of TagQΔC. WC: whole cells, C: cytosol, M: membrane fraction, P: periplasm.

TagT-TagS form an ABC transporter with ATPase activity

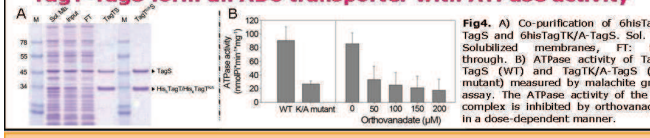


Fig4. A) Co-purification of 6hisTagT-TagS and 6hisTagTK/A-TagS. Sol. Mb. Solubilized membranes, FT: flow through. B) ATPase activity of TagT-TagS (WT) and TagTK/A-TagS (K/A mutant) measured by malachite green assay. The ATPase activity of the WT complex is inhibited by orthovanadate in a dose-dependent manner.

The Tag Proteins are Indispensible for T6SS Activity

The Tag proteins (*t*ype six *a*ssociated *g*enes) are non conserved in all T6SS. Deletion mutants have been created in order to study their role in secretion and T6SS function.

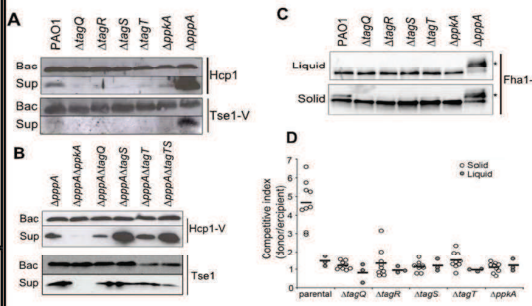


Fig2. Hcp1 and Tse1 production and secretion in Δ*tag* (A) or Δ*pppA*Δ*tag* strains (B) grown on liquid medium. C) Fha1 phosphorylation (marked with an asterisk) in solid and liquid medium. D) *P. aeruginosa* requires the *tag* genes for H1-T6SS-dependent fitness. The competitive index is plotted for competitions between each indicated donor strain and a H1-T6SS-susceptible recipient strain of *P. aeruginosa*. Bac: bacteria, sup: supernatant.

TagR's Association to the OM is TagQ-dependent

TagR is a periplasmic protein [6].
TagR has no predicted transmembrane domains.

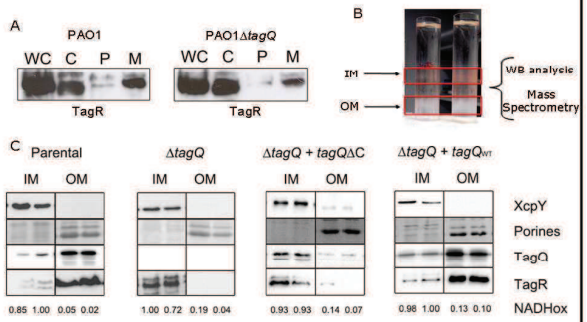


Fig5. A) Subcellular fractionation and immunoquantification of TagR in PAO1 and PAO1Δ*tagQ*. WC: whole cells, C: cytosol, M: membrane fraction, P: periplasm. B) Representative discontinuous sucrose gradient samples. The IM (red, upper ring) and the OM (white, lower ring) are shown. Fractions were collected from the top and analyzed by WB and discovery mass spectrometry. C) Representative IM and OM fracions. NADH oxidase activity (NADHox) and XcpY were used as IM markers. Porines were used as OM markers. Parental strain: PAO1.

Conclusions and Revised Model of H1-T6SS Activation

The genes of the *tag* operon are indispensible for the functioning and posttranslational regulation of the H1-T6SS of *P. aeruginosa* and act upstream of the PpkA-PppA phosphorylation checkpoint.

TagQ is an OM lipoprotein.

TagR association to the OM depends on the correct localization of TagQ to the OM.

Do TagRSTQ form a complex? Do they interact? Pull-down experiments
Bacterial two-hybrid

What is the signal that triggers the trans-membrane signaling?

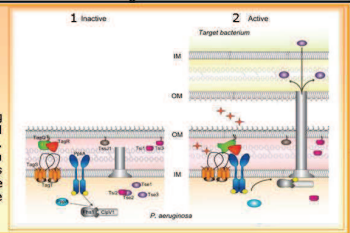


Fig6. Revised model of H1-T6SS posttranslational activation. During the inactive state (1), TagR and TagQ might interact in the OM, and thus TagR is not accesible to PpkA and no phosphorelay is possible. Upon an unknown signal, during the active state (2), TagQ suffers a conformational change making TagR accesible. Then, PpkA dimerizes and autophosphorylates, triggering the cargo proteins secretion. The proteins are represented using the same color code as in Fig1 and the H1-T6SS machinery is represented as a single tunnel [7].

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[3] Hood *et al.*, 2010
[4] Russel *et al.*, 2011
[5] Mougous *et al.*, 2007
[6] Hsu *et al.*, 2009
[7] Casabona, Silverman *et al.*, *in press* 2012

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Mécanismes moléculaires involucrées dans la régulation post-traductionnelle du système de sécrétion du type VI chez *Pseudomonas aeruginosa*

La bactérie à Gram-négatif *Pseudomonas aeruginosa* est un pathogène humain opportuniste qui peut causer des infections chroniques pouvant conduire à la mort des patients, et plus particulièrement ceux atteints de la mucoviscidose. Il a été montré qu'un de ses trois systèmes de sécrétion de type VI (SST6) est actif durant les infections chroniques, le SST6-H1. *P. aeruginosa* est capable d'injecter des toxines de type bactériolytique directement dans le périplasme des autres bactéries à Gram-négatif grâce au SST6-H1, ce qui laisse penser que cette nanomachine pourrait être capitale dans la compétitivité de *P. aeruginosa* dans les niches polymicrobiennes, comme par exemple un poumon infecté. Cette nanomachine insérée dans l'enveloppe bactérienne est régulée au niveau post-traductionnel par une voie de phosphorylation ressemblant à celles des eucaryotes. Cette voie est constituée par une kinase, PpkA, et une phosphatase, PppA, qui modulent ensemble le niveau de phosphorylation de la protéine Fha1. Nous avons démontré que quatre protéines spécifiques de *Pseudomonas* appelées TagT, TagS, TagR et TagQ, agissent en amont du couple PpkA/PppA, et sont indispensables pour l'activation du SST6-H1. De plus, elles sont aussi nécessaires lors de compétitions entre *P. aeruginosa* et d'autres bactéries. Nous avons montré que TagR, connue comme étant une protéine périplasmique, est en fait associée à la membrane externe et cette localisation dépend de TagQ, une lipoprotéine ancrée dans le feuillet interne de la membrane externe. TagT et TagS forment un transporteur de type ABC qui a une activité d'ATPase.
L'association de TagR à la membrane externe a été mise en évidence par des études de protéomique à haut débit qui avaient pour but la caractérisation des membranes externe et interne de *P. aeruginosa*. Grâce à l'analyse des résultats, unmodèle de l'assemblage du SST6-H1 au sein de l'enveloppe a pu être proposé. Ce travail a permis l'identification de plus de 1700 protéines, parmi elles un complexe multi-protéique incluant MagD, une protéine homologue à la macroglobuline humaine. Les résultats obtenus lors de la caractérisation de ce complexe sont aussi présentés dans ce manuscrit.
Mots-clés : **Pseudomonas aeruginosa*, SST6, régulation post-traductionnelle, protéomique à haut débit, sous-protéome***

Molecular mechanisms involved in the post-translational regulation of type VI secretion system in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a human opportunistic pathogen that can cause severe infections and death in chronically infected cystic fibrosis (CF) patients. It has been shown that one of its three Type VI Secretion Systems (T6SS), the H1-T6SS, is active during chronic infections in CF patients. *P. aeruginosa* injects bacteriolytic toxins directly into other Gram-negative bacteria by means of its H1-T6SS, which could be of high importance in its outcome in complex niches such as an infected lung. This trans-envelope nanomachine is posttranslationally regulated by a eukaryotic-like phosphorylation pathway, which includes a kinase-phosphatase pair, PpkA and PppA, respectively. In this work, TagT, TagS, TagR and TagQ, *Pseudomonas* specific T6SS proteins that are encoded in the same operon as Ppka, PppA and Fha1, were analysed functionally and biochemically. We found that these four proteins are indispensable for the activation of H1-T6SS, by acting upstream of the phosphorylation checkpoint. Moreover, they were also needed for intra- and inter-species fitness mediated by H1-T6SS. We discovered that TagR, a periplasmic protein, associates with the outer membrane (OM) of *P. aeruginosa* in a TagQ-dependent manner. TagQ is an OM lipoprotein that faces the periplasm. TagT and TagS form a membrane-bound complex, an ABC transporter, with ATPase activity.
TagR association with the OM was discovered by shotgun mass spectrometry analyses of the OM and the inner membrane (IM) of *P. aeruginosa*. In this work, the IM and OM sub-proteomes of *P. aeruginosa* are also presented, with highlights on T6SS global assembly. Moreover, these two sub-proteomes allowed the identification of a novel envelope-associated complex with macroglobulin-like protein, MagD. The studies concerning this protein and its partners in *P. aeruginosa* are also presented in this manuscript.
Keywords: **Pseudomonas aeruginosa*, T6SS, posttranslational regulation, shotgun proteomics, IM-OM sub-proteomes***