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Definition of potential targets in the *Pseudomonas aeruginosa* T3SS for a new anti-infectious strategy

Tuan Dung Ngo

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THÈSE

Pour obtenir le grade de

DOCTEUR DE LA COMMUNAUTE UNIVERSITE GRENOBLE ALPES

Spécialité : **Virologie, Microbiologie, Immunologie**

Arrêté ministériel : 25 mai 2016

Présentée par

Tuan-Dung NGO

Thèse dirigée par **Eric FAUDRY**

préparée au sein du **Laboratoire Biologie du Cancer et de
l'Infection**
dans **l'École Doctorale Chimie et Sciences du Vivant**

Définition de cibles potentielles dans le SST3 de *Pseudomonas aeruginosa* pour une nouvelle stratégie anti-infectieuse

Thèse soutenue publiquement le **6 février 2019**,
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CONTENT

INTRODUCTION	8
A. THE CHALLENGE OF MULTI-DRUG RESISTANT BACTERIA FOR HUMAN HEALTH.	9
I. <i>Antibiotic resistances</i>	9
II. <i>Different strategies against infectious diseases</i>	11
1 Phage therapy	11
2 Fecal microbiota transplantation	12
3 Anti-virulence therapy.....	13
B. PSEUDOMONAS AERUGINOSA	15
I. <i>An opportunistic pathogen</i>	15
II. <i>Mechanisms of antibiotic resistances</i>	16
III. <i>Virulence factors as potential targets: the anti-virulence therapy.</i>	18
1 Regulation factors	19
1.1 Description.....	19
1.2 Drug discovery	20
2 Adhesion, biofilm formation and motility.	22
2.1 Description.....	22
2.2 Drug discovery	25
3 Secretion systems.....	27
3.1 Type I secretion system	28
3.2 Type II secretion system	29
3.3 Type V secretion system	33
3.3.1 Type Va Secretion system (auto-transporter).....	33
3.3.2 Type Vb Secretion system (two-partner secretion: TPS).....	34
3.4 Type VI secretion system	35
3.5 Type III secretion system	38
3.5.1 Assembly.....	38
a. Cytoplasmic components.....	42
b. Basal body.....	44
c. Needle complex or injectisome.....	45
d. Translocators	47
3.5.2 Exotoxins.....	51
a. ExoS and ExoT	51
b. ExoY.....	53
c. ExoU	54
d. Besides the four exoenzymes	55
e. A summary of exoproducts secreted by P. aeruginosa secretion systems.	56
3.5.3 T3SS Regulation	62
3.5.4 Role of ATPase for the secretion process.....	65
a. An overview of secretion system ATPase.....	65
b. The T3SS ATPase interacts with exported protein complex.....	70
c. The T3SS ATPase provides energies for the secretion process	72
d. T3SS ATPase contributions to the sorting of exported proteins during the secretion process.....	75
3.5.5 Drug discovery	81
C. SUMMARY AND THESIS OBJECTIVES	89
RESULTS	91
A. POPN ACTS ON PscN TO CONTROL NEEDLE SECRETION BY THE TYPE III SECRETION SYSTEM IN PSEUDOMONAS AERUGINOSA	92
I. <i>Abstract (in French)</i>	92
II. <i>Introduction (in French)</i>	92
III. <i>Result presented in article format</i>	93

IV.	Conclusion (in French)	112
B.	NEW MOLECULES TARGETING A T3SS PROTEIN-PROTEIN INTERFACE EFFICIENTLY AFFECT <i>PSEUDOMONAS AERUGINOSA</i> VIRULENCE	115
I.	Abstract (in French)	115
II.	Introduction (in French)	115
III.	Result presented in article format	116
IV.	Conclusion (in French)	140
C.	TARGETTING OF THE T3SS ATPASE PscN IN <i>PSEUDOMONAS AERUGINOSA</i>	143
I.	Abstract (in French)	143
II.	Introduction (in French)	143
III.	Material and method.	144
1	Bacterial strains, plasmids and primers	144
2	Protein expression and purification.	145
3	ELISA and High-Throughput target-based Screening.	146
4	Cell culture and staining, automated High-Content Screening (HSC) and High-Content Analysis (HCA)	146
5	Analysis of ExoS-bla and PopB-bla secretion.	146
IV.	Results	147
1	Expression of the T3SS ATPase PscN of <i>P. aeruginosa</i>	147
2	PscN interacts with T3SS exported proteins	148
3	Identification of compounds inhibiting the ATPase PscN interaction with the translocator PcrH-PopD by high throughput screening (HTS).	151
4	Characterization of hit compounds ex vivo and in vivo	155
5	Purification of FSS-PscN and target validation of hit compounds.	157
5.1	Purification of FSS-PscN	157
5.2	Target validation	159
V.	Discussion	160
VI.	Conclusion (in French)	163

GENERAL DISCUSSION AND PERSPECTIVE..... 165

A.	THE ATPASE PscN PLAYS AN ESSENTIAL ROLE FOR THE T3SS FUNCTIONING AND BACTERIAL VIRULENCE IN <i>P. AERUGINOSA</i>	166
B.	TWO POTENTIAL TARGETS FOR THE DEVELOPMENT OF ANTI-VIRULENCE STRATEGY AGAINST <i>P. AERUGINOSA</i>	171

ANNEXES..... 174

REFERENCE BIBLIOGRAPHIES..... 190

List of Figure

Figure 1: Time scale of antibiotic deployment and observed antibiotic resistance.....	9
Figure 2: The number on human death caused by AMR in comparison to other causes of death in 2050 ...	10
Figure 3: Classification of bacteria for the development of new antibiotics by WHO	17
Figure 4: Overview of virulence factors of <i>P. aeruginosa</i>	18
Figure 5: Interplay between four quorum sensing circuits in the regulation of <i>P. aeruginosa</i>	19
Figure 6: Interplay of four regulatory pathways (cAMP, c-di-GMP, quorum sensing and two-component system Gac/Rsm) in the regulation of acute/chronic toxicity and biofilm lifestyle of <i>P. aeruginosa</i>	21
Figure 7: Two life styles of <i>P. aeruginosa</i> : Planktonic and Biofilm	22
Figure 8: Flagella assembly (A) and flagellin organization (B)	23
Figure 9: Different secretion systems presented in <i>P. aeruginosa</i>	27
Figure 10: Assembly of Type IV pili and Type II Secretion System	30
Figure 11: T6SS structure	36
Figure 12: Mechanism model of three T6SS in <i>P. aeruginosa</i>	37
Figure 13: Schematic diagrams of the inside-out (a) and outside-in (b) models of the T3SS assembly	40
Figure 14: Components of the Type III Secretion System	42
Figure 15: The 3D reconstruction from cryo-ET of T3SS in <i>Shigella flexneri</i> shows pod-like densities of the export apparatus with six-fold symmetry	43
Figure 16: Cryo-EM structures of the T3SS basal body from <i>Salmonella enterica</i>	44
Figure 17: Proposed ruler (a) and timer (b) models for the mechanism of substrate switching and needle-length control in the needle assembly.....	46
Figure 18: In situ structure analysis of the intact translocator in the membrane of a cell challenged with <i>Salmonella</i>	48
Figure 19: Diagrammatic analysis of translocators of Ysc (<i>Pseudomonas</i> and <i>Yersinia</i>), Ssa-Esc (<i>Salmonella</i> SP2 and <i>E. coli</i>) and Inv-Mxi-Spa (<i>Shigella</i> and <i>Salmonella</i> SP1) systems.....	49
Figure 20: Diagrammatic analysis of <i>P. aeruginosa</i> exotoxins.....	52
Figure 21: An overview of T3SS gene organization (a) and mechanism of T3SS genetic regulation (b).....	62
Figure 22: The Vfr protein binds to the promoter PexsA located upstream of the exsA gene.....	64
Figure 23: Diagrammatic representation of the ATPases of the T2SS, T3SS and T6SS.....	66
Figure 24: Phylogenetic analysis of the PilT-like ATPase family members	67
Figure 25: Structure of the T3SS ATPase.....	69
Figure 26: Cryo-ET of the T3SS basal body and cytoplasmic components in <i>S. flexneri</i> minicells	72
Figure 27: Models of protein export through the T3SS.....	73
Figure 28: Model of T3SS protein export which depends on both ATPase and proton motive force.....	74
Figure 29: Modèle du changement allostérique de conformation de l'ATPase PscN pour réguler la sécrétion de l'aiguille	113
Figure 30: 12(6,4) et 12(4,6) protègent <i>Galleria Mellonella</i> lors d'infection à <i>P. aeruginosa</i>	141
Figure 31: Expression of ATPase PscN in different conditions	147
Figure 32: Purified PscN interacts with T3SS secreted proteins	150
Figure 33: Screening of chemical compounds inhibiting interaction of PscN and PcrH-PopD.....	152
Figure 34: Characterization of 15 hits compounds	156
Figure 35: Four constructions of PscN used in this study	157
Figure 36: Higher purification yield with Flag-Strep-Strep-PscN (FSS-PscN) construct.....	158
Figure 37: Effect of seven compounds on the binding capacity and catalytic activity of PscN.....	159
Figure 38: <i>P. aeruginosa</i> CHA growth in the presence of M6 at indicated concentrations.....	162

List of Table

Table 1: <i>P. aeruginosa</i> resistance mechanisms and current antipseudomonal treatments	17
Table 2: Functions and nomenclature of components of the T3SS and their flagellar homologues	41
Table 3: Summary of secretion systems and secreted proteins in <i>P. aeruginosa</i>.....	61
Table 4: Activities of T3SS ATPase SctN on T3SS proteins.	71
Table 5 Overview of T3SS ATPase expression, purification and biochemical characterization	77
Table 6 Overview of T3SS inhibitors in <i>P. aeruginosa</i>.	85
Table 7: Strains, plasmids and primer	145
Table 8: Hit compounds identified from screening of Prestwick library.....	153

List of Abbreviations

- 1TD:** One-transmembrane domain
- 2TD:** Two-transmembrane domain
- ABC:** ATP-binding cassette
- ADPRT:** ADP-ribosyltransferase
- AMR:** Antimicrobial resistance
- cAMP:** Cyclic adenosine mono-phosphate
- CDI:** Clostridium difficile infection or Contact-depend growth inhibition
- c-di-GMP:** Cyclic diguanylate mono-phosphate
- CF:** Cystic fibrosis
- COPD:** Chronic obstructive pulmonary disease
- C-ring:** Cytoplasmic ring
- Crk:** Cysteine rich receptor-like kinase
- CTD:** C-terminal domain
- ECL:** Enhanced chemiluminescence
- ELISA:** Enzyme-like immunosorbent assay
- EPS:** Exopolysaccharide
- FDA:** Food and Drug Administration
- FMT:** Fecal microbiota transplantation
- FSS-PscN:** Flag-Strep-Strep-PscN
- GAP:** GTPase activating protein
- HSL:** Homo Serine Lactone
- HTRF:** Homogeneous Time Resolved FRET
- IM:** Inner membrane
- IUPAC:** International Union of Pure and Applied Chemistry
- LPS:** Lipopolysaccharide
- mAb:** Monoclonal antibody
- MDR:** Multi-drug resistance
- MLD:** Membrane localization domain
- MST:** Micro Scale Thermophoresis
- NDK:** Nucleoside diphosphate kinase
- NTD:** N-terminal domain

OM: Outer membrane
PC: Phosphatidyl choline
PE: Phosphatidyl ethanolamine
PLA2: Phospholipase A2
PQS: Pseudomonas Quinolone Signal
QS: Quorum sensing
SAR: Structure Activity Relationship
T3FS: Type III Flagellar System
T3SS: Type III Secretion System
T4P: Type IV pili
TMB: Tetramethylbenzidine
TLR: Toll-like receptor
VAP: Ventilator associated pneumonia
WHO: World Health Organization

Chapter I

Introduction

A. The challenge of multi-drug resistant bacteria for human health.

I. Antibiotic resistances

The bacteria are found almost everywhere in our planet: in the air, in the water, in the soil and also in the living organisms. Some bacteria are beneficial for the animal life such as the gut microbiota, forming an equilibrated community of microorganisms in the intestine, defending against pathogens and regulating the immune system. However, the pathogenic bacteria are responsible for many human diseases and the use of antibiotic is essential to combat these bad bugs. The antibiotics are chemical substances from either natural or synthetic source that inhibit the bacteria growth (bacteriostatic) or kill the pathogens (bactericidal). Antibiotic activity was firstly found in 1928 by Alexander Fleming while he was examining a petri dish of *Staphylococcus* contaminated by *Penicillium glaucum* (Fleming, 1929). This was the premise of the discovery of penicillin that was purified and scaled up by Howard Florey and Ernst Boris Chain 12 years later. Its production was next optimized by Norman Heatley, which allowed this antibiotic to be widely use since 1945. The 20th century witnessed a golden time of antibiotics with many drugs discovered and marketed such as Sulfonamide (1932), Streptomycin (1944), Erythromycin (1952), Vancomycin (1956), etc.

Nonetheless, immoderate and inappropriate usage of antibiotics for agriculture and health care as well as the release of these active substances in the environment lead to the development and the diffusion

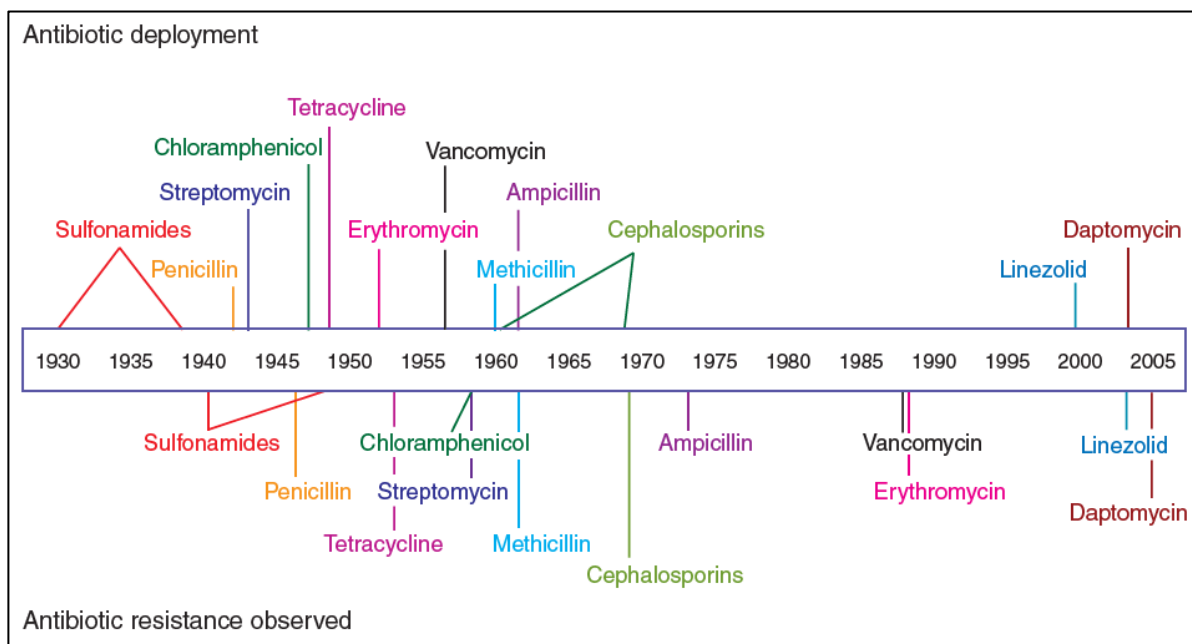


Figure 1: Time scale of antibiotic deployment and observed antibiotic resistance (Clatworthy et al., 2007)

of antimicrobial resistances (AMR) (Palumbi, 2001). Indeed, pathogenic bacteria could evolve to resist the medicines affecting their growth or survival. The natural coexistence of a high number of bacteria from diverse species permits that rare mutations that could occur during selection phase will be widespread among the microorganisms through horizontal gene transfer (Burmeister, 2015; Hawkey and Jones, 2009; Price et al., 2012). This expansion makes antibiotics sooner or later ineffective in therapy against infectious diseases and, throughout the antibiotic era, the AMR becomes a new risk for the human health (Figure 1).

As depicted in Figure 1, more than twenty new classes of antibiotic were deployed from 1935 to 1969 while there were only two new classes that have been marketed since 1969 (Butler and Buss, 2006; Clatworthy et al., 2007), the oxazolidinone Linezolid in 2000 (Zappia et al., 2007) and the lipopeptide Daptomycin in 2003 (Hair and Keam, 2007)). This means that if twenty classes of antibiotic were enough to fight infectious diseases for 50 years (1950-2000), the two new classes may be insufficient for the next 50 years (2000-2050). According to a review on antimicrobial resistance, in 2050 the human deaths due to AMR are expected to reach 10 million per year and this number will exceed the total resulting from

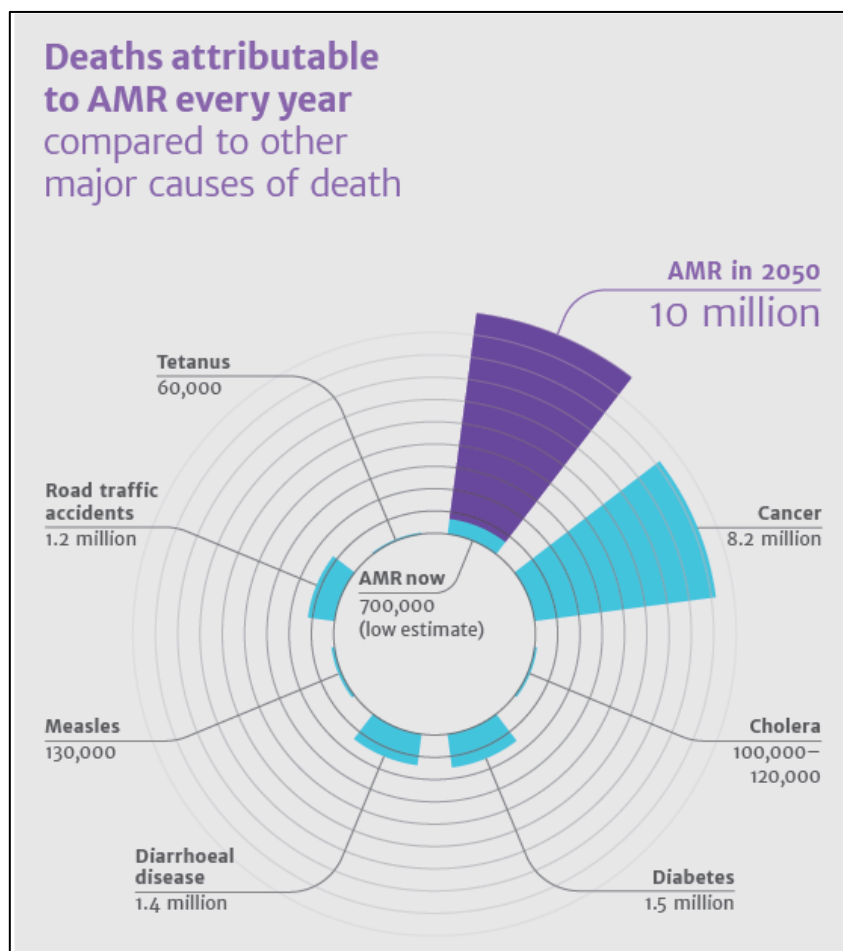


Figure 2: The number on human death caused by AMR in comparison to other causes of death in 2050 (O’neil, 2014).

Cancer and Diabetes, the two other major causes of death in this century (Figure 2) (O'neil, 2014). In this situation, new prophylactic resources against pathogenic bacteria are urgently needed and thus, some new strategies are identified and seem to be very convincing, including phage therapy, fecal microbiota transplantation, and anti-virulence therapy.

II. Different strategies against infectious diseases

1 Phage therapy

The phage therapy has been used to treat bacterial infection in East-Europe for over 80 years (Kropinski, 2006). This approach uses bacteriophages to kill the pathogenic bacteria. Phages are small viruses with a size ranging from 24 to 100 nm in length, which specifically infect and lyse bacteria. Actually, phages are able to inject their DNA into the bacterial cytoplasm and then their genome is replicated and translated by the host enzymes and ribosomes. This leads to a high number of viral copies and their accumulation causes bacterial lysis during the lytic cycle. On the other hand, in the lysogenic cycle, the phage DNA is inserted into the host chromosome and replicated during the bacterial division. Basically, these genes are not expressed until an environmental stimulus, for example a stress induces the lytic viral cycle.

Based on their viral cycles, phages are classified into two types: temperate phages and virulent phages. The temperate phages are able to display both lytic and lysogenic cycles meanwhile the virulent phages are solely lytic. After the lysogenic cycle, the temperate phages switch to the lytic cycle and can transfer DNA of one bacterium to the other by transduction. This transferred DNA could harbor genes encoding for bacterial toxins or antibiotic resistances and there is thus a high risk of creating a new dangerous bacterium. Therefore, the virulent phages are usually exploited for phage therapy to kill bacteria because they only possess the lytic function and are therefore safer.

Although bacteriophages were discovered in 1915 by Frederick William Twort (Twort, 1915) and in 1917 by F.H.d'Herelle (D'Herelle, 2007), they were not widely enough exploited as an antimicrobial strategy in that century because of the development of antibiotic a few years later. However, with the threat of AMR, scientists presently come back to the bacteriophages as a potential manner to treat bacterial infection, leading to more publications and some clinical trials. In 2006, an eight-phages cocktail was able to kill *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* and was approved by the Food and Drug Administration (FDA) for a Phase I clinical trial (ClinicalTrials.gov Identifier: NCT00663091). Actually, these phages were tested in 42 patients with venous leg ulcers. Although no

side effects were observed, the wound healing was the same between the experimental and control groups (Criscuolo et al., 2017; Rhoads et al., 2009).

From 2015, a first randomized, multicentric and open-label Phase I/II clinical trial is currently on going in burn patients who had a sign of infection by either *E. coli* or *P. aeruginosa* (ClinicalTrials.gov Identifier: NCT02116010). This trial uses phage-cocktails from Pherecydes Pharma that are Good Manufacturing Practice (GMP) compliant, containing 12 (*E. coli*) and 13 (*P. aeruginosa*) lytic phages and aims to evaluate their tolerance/safety and their efficacy during treatment. This is an European R&D project funded by the European Commission under the 7th Framework Program. Although it is currently in the recruiting phase, the results of this trial are important as a proof of concept for the advancement of phage therapy and might lead to next clinical studies to assess the feasibility of bacteriophage therapy as the future medicine for bacterial infection treatment.

In addition, other studies assessing the safety and efficacy of phage therapy at a smaller scale were performed such as the T4 phages in young children with diarrhea provoked by enteritoxigenic and/or enteropathogenic *E. coli* (ETEC and/or EPEC) in 2009 (ClinicalTrials.gov Identifier: NCT00937274) (Criscuolo et al., 2017; Sarker et al., 2016) and the French trial of bacteriophage against *Staphylococcus* infection in diabetic foot ulcers patients in 2017 (ClinicalTrials.gov Identifier: NCT02664740). The number of performed clinical trials indicate that phage therapy attracts increasing public and private investments. It is obvious that the bacteriophages offer significant advantages as they only kill the targeted bacteria, do not affect the gut microbiota and limit the cross-resistance among bacteria (Loc-Carrillo and Abedon, 2011). Moreover, the phage displays a good safety as shown in many Phase I clinical trials, and could also evolve rapidly in parallel with the evolution of bacteria. It is a very important argument explaining why these natural enemies of bacteria are believed to be a potential alternative approach for the antibacterial medicines in the AMR era.

2 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is a process in which microorganisms are transplanted from a healthy individual to the intestinal tract of a recipient in order to change the gut microbiota composition, which is known to affect human health. The use of human fecal suspension as therapy was described firstly by Ge Hong in the 4th century in China to treat patients who had food poisoning or severe diarrhea (Zhang et al., 2012). 1200 years later, in the 16th century, Li Shinzhen also showed that fresh, dry or fermented feces called “yellow soup” could effectively treat abdominal diseases including severe diarrhea. In 1958, fecal enemas were described as a cure of pseudomembranous colitis by Ben Eiseman and then,

FMT was introduced in the market and was considered as a medicine (Eiseman et al., 1958; Gupta et al., 2016).

A great achievement of FMT is the efficient treatment of *Clostridium difficile* infection (CDI). An average cure rate of 87-90% was reported with one FMT treatment (Bakken et al., 2011; Burke and Lamont, 2013; Gupta et al., 2016; Kassam et al., 2013; Kelly et al., 2012). Actually, *C. difficile* is a gram positive bacterium causing nosocomial infection. This pathogen is usually found in human colon but is normally inhibited by the healthy gut flora. However, antibiotic therapies can sometimes perturb the balance of intestinal microorganisms and thus, *C. difficile* becomes dominant and causes the CDI. The major symptom of CDI includes watery diarrhea, abdominal pain and pseudomembranous colitis (Bauer et al., 2011). After a treatment by antibiotics such as vancomycin or metronidazole, some pathogens can persist to develop recurrent CDI (rCDI) that is more dangerous and leads to high mortality.

Since 2013, the use of FMT has been allowed by the FDA to treat patients having CDI that does not respond to standard therapies with antibiotics but the regulation of FMT was modified several times from 2013 to 2016 (Hoffmann et al., 2017). The challenge of FMT is that the composition of the microbiota varies among different individuals or within individual at different times. Unlike the probiotics which have low number of bacteria, FMT involves a large number of microorganisms that can totally change the gut environment. Moreover, most of FMT bacteria cannot be cultured *in vitro*. As a result, the control of bacterial species is difficult and a high risk of introducing pathogen exists. However, at least for now, FDA classified FMT as a drug and all the results in the literature support FMT as an approach to treat CDI with high safety and low adverse effect (Kassam et al., 2013; Kelly et al., 2015). To date, FMT is also explored for other intestinal diseases such as inflammatory bowel disease (IBD), gastrointestinal disorders, etc (Gupta et al., 2016; Kelly et al., 2015).

3 Anti-virulence therapy

Pathogenic bacteria possess many virulence factors allowing them to colonize and to infect their hosts. Therefore, anti-virulence therapy refers to strategies employing natural or synthetic small molecules, or antibodies, to inhibit the virulence factors of pathogens. Unlike the phage therapy and the FMT from which administered products are living organisms with active metabolism, the anti-virulence molecules are lifeless compounds like the antibiotics. Hence, they could be adequately controlled with the standard medical and pharmaceutical practices. While antibiotics kill the pathogens or inhibit the bacterial growth, anti-virulent compounds disarm them and bacterial clearance is thus performed by the host immune system. This strategy should elicit less resistance apparition in bacteria, overcoming thus the potential major drawback of classic antibacterial drugs.

In comparison to antibiotics, anti-virulent compounds have both advantages and disadvantages. In addition to the lower evolutionary pressure for resistance development, anti-virulent compounds are specific to the targeted virulence factor and can inactivate this target rapidly. As a result, they have limited impacts on host commensal flora, which is of importance to keep the balance of gut microorganisms. Nevertheless, because of this specificity, many anti-virulence therapies should be available to treat different pathogens or strains with different virulence factors. Finally, the therapeutic effects of anti-virulence compounds may be reduced compared to antibiotics but they can be used as an alternative supplement to increase the efficacy of treatment notably in the context of AMR (Dickey et al., 2017).

With all its advantages, this strategy becomes highly promising and attracts a lot of scientists and companies with a high number of studies and publications. Thank to scientific researches conducted on virulence factor of bacteria over the years, many points and mechanisms are identified as potential targets for anti-virulence development. Here, in the next chapter, the virulence factors as well as the developed anti-virulence compounds will be described for *Pseudomonas aeruginosa*, the principal bacterium that we are working on in the laboratory and during my PhD.

B. *Pseudomonas aeruginosa*

I. An opportunistic pathogen

P. aeruginosa is a non-capsulated and non-sporulating Gram-negative bacterium from the Pseudomonadaceae family. It is aerobic (grows in oxygen-rich medium) but is able to adapt to anaerobic conditions (Vasil, 1986). This bacterium is also called pyocyanic bacillus due to its rod-shaped morphology and its capacity to produce some pigments with a blue-green color (pyocyanin and pyoverdine) (Lau et al., 2004). Basically, *P. aeruginosa* is not very threatening for human but it could be dangerous in some conditions. This pathogen usually attacks patients having immunodeficiency mainly caused by breaches of the epithelium physical barrier as in the case of burns and wounds and, in addition, 40 – 60 % of the mortality due to *P. aeruginosa* are attributed to the ventilator associated pneumonia (VAP) (Alnour and Ahmed-Abakur, 2017). It is also responsible for urinary and other lung infections like chronic obstructive pulmonary disease (COPD) but it is above all the major cause of morbidity and mortality in case of cystic fibrosis (CF). In the CF patients, the mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause a defect in the transfer of chloride ions across the cell membrane, leading to an abnormal production of fluid and mucus mainly in the lung and in the digestive system. The accumulation of mucus creates a favorable environment for the infection by pathogens like *P. aeruginosa* which can escape from the human immune systems (Clunes and Boucher, 2007; Cutting, 2015).

Briefly, *P. aeruginosa* is also famous as an opportunist bacterium that takes advantage of weakness of patients to colonize and to cause nosocomial diseases. In the hospital, this bacterium is widespread in humid environments such as the faucets, siphons, sinks, showers, surfaces of mouth thermometers, nebulizers and humidifiers. The contaminated surfaces, materials and instruments are also a disease vector if the hygiene and the infection controls are not correctly applied (de Abreu et al., 2014; Lanini et al., 2011). With *E. coli* and *S. aureus*, *P. aeruginosa* is one of the major causes of nosocomial infections. It is involved in around 9 – 40% of these infections, with variations according to the geographic location of the hospitals, and the mortality rate can reach up to 61% in vulnerable patients (Bassetti et al., 2008; Gaspar et al., 2013; Ilić and Marković-Denić, 2009; Kakupa et al., 2016; Maschmeyer and Braveny, 2000; McCormack and Barnes, 1983; Obritsch et al., 2005; Smetana et al., 2014).

II. Mechanisms of antibiotic resistances

P. aeruginosa is naturally resistant to many common antibiotics due to a variety of mechanisms such as the expression of the AmpC β -lactamase that degrades antibiotics having β -lactam rings, the synthesis of efflux pumps to reject antibiotics and a low permeability of the outer membrane that prevents the entry of antibiotics into bacteria. *P. aeruginosa* also has the ability to acquire further resistance mechanisms to multiple groups of anti-bacteria agents. Therefore, the infections caused by *P. aeruginosa* are often challenging to treat and may require the use of a combination of antibiotics (Kapoor and Murphy, 2018).

Basically, the four main targets of antibiotics are cell membranes, cell wall synthesis, protein synthesis and nucleic acid synthesis. The typical resistance mechanisms of *P. aeruginosa* and the current antipseudomonal treatments are listed in the Table 1 (Banerjee and Stableforth, 2000; Friedrich, 2017; Gaspar et al., 2013).

Class	Targets in <i>P. aeruginosa</i>	Resistance mechanisms of <i>P. aeruginosa</i>	Antibiotic names – current therapy	Descriptions
Beta-lactams	Bind to and inactivate the penicillin-binding proteins which are responsible for cell wall biosynthesis.	<ul style="list-style-type: none"> • Expression of different β-lactamase enzymes • Efflux pumps 	Piperacillin/tazobactam (penicillin-like) (Lodise et al., 2007)	Ureido-penicillin plus β -lactamase inhibitor
			Ceftazidime, cefepime (cephalosporin-like) or combination Ceftazidime/avibactam (Housman et al., 2014; Stone et al., 2018), Ceftolozane/tazobactam	<ul style="list-style-type: none"> • Less prone to hydrolysis by the most important β-lactamases than Penicillin-like. • Could be combined with a β-lactamase inhibitor
			Meropenem, Doripenem (carbapenem-like) or Aztreonam (monobactam-like)	Broader spectrum and more stable than penicillin-like and cephalosporin-like.
Aminoglycosides	Bind to 30S and 50S ribosomal subunits, thus inhibiting the translation and bacterial protein synthesis	<ul style="list-style-type: none"> • Reduction of membrane permeability • Mex efflux pumps (Poole, 2005) 	Gentamicin	<ul style="list-style-type: none"> • Highly effective but severely toxic • Could be used alone or combined with a β-lactam antibiotic (Scribner et al., 1982)
			Tobramycin	2 to 4 times more active against <i>P. aeruginosa</i> and less toxic than Gentamicin

Quinolones	Bacterial DNA synthesis (topoisomerase II and IV and DNA gyrase) (Bolon, 2009; Grégoire et al., 2010)	<ul style="list-style-type: none"> Point mutation in topoisomerases and DNA gyrase Mex Efflux pumps (Bolon, 2009; Grégoire et al., 2010) 	Ciprofloxacin (fluoroquinolone-like)	Very large spectrum drugs but that leads to rapid resistance development.
Polymyxins	Disruption of bacterial lipopolysaccharide (LPS). Mode of action similar to detergents.	Alteration and regulation of the lipid composition in bacterial membrane (Gutu et al., 2013; Moore et al., 1984)	Polymyxin B or E (colistin) (Zavascki et al., 2007)	Neurotoxic and nephrotoxic. They are only used as the last antibiotic if the others are ineffective.

Table 1: *P. aeruginosa* resistance mechanisms and current antipseudomonal treatments

Although *P. aeruginosa* has many mechanisms of antibiotic resistance, only these drugs are used for the therapy nowadays. As described above, a combination of antipseudomonal medicines is usually necessary for effective treatment but most of the medications are based on old antibiotic classes and new resistances are likely to appear. Indeed, some studies of resistance evolution of *P. aeruginosa* for the drug combinations such as ceftazidime/avibactam are reported in the literature (Sanz-García et al., 2018). Moreover, this pathogen has the ability to acquire multiple resistances and it is called in this case multi-drug resistance (MDR) bacteria. The MDR *P. aeruginosa* account for 12.3% to 36.5% of the total strains from different health care units in different countries (Alnour and Ahmed-Abakur, 2017). These strains

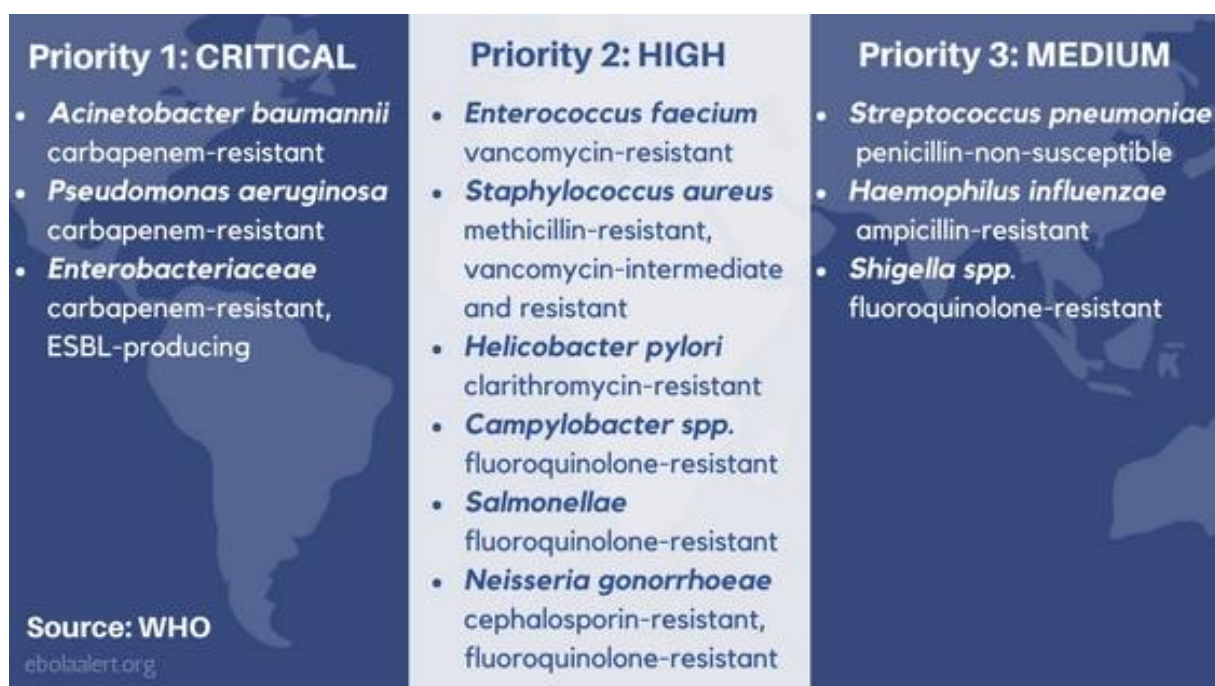


Figure 3: Classification of bacteria for the development of new antibiotics by WHO

are dangerous, hard to treat and urgently deserve a new strategy to control them. The overall mortality rate due to *P. aeruginosa* infection is higher than 20% and this rate increases in case of MDR bacteria (Alnour and Ahmed-Abakur, 2017). For these reasons, with *Acinetobacter baumannii* and *Enterobacteriaceae*, *P. aeruginosa* was classified as a top priority for the development of new antibiotics by the World Health Organization (WHO) in 2017 (Tacconelli et al., 2018).

III. Virulence factors as potential targets: the anti-virulence therapy.

P. aeruginosa possesses many virulence factors allowing its adaptation to different environments and host cells infection. The flagella and Type IV pili are displayed on the surface and involved in the adhesion and motility of bacteria (Bucior et al., 2012). This pathogen synthesizes exopolysaccharides (for example alginate) responsible for the biofilm formation. Otherwise, some molecules called auto-inducer like HomoSerine Lactone (HSL) and Pseudomonas Quinolone Signal (PQS) are secreted by *P. aeruginosa* and play a role in the bacteria communication and regulation via the quorum sensing system. There are also virulence factors secreting bacterial toxins in the extracellular medium such as the Type I, II and V Secretion Systems. Their secreted toxins like elastases, alkaline proteases and phospholipases cause cell

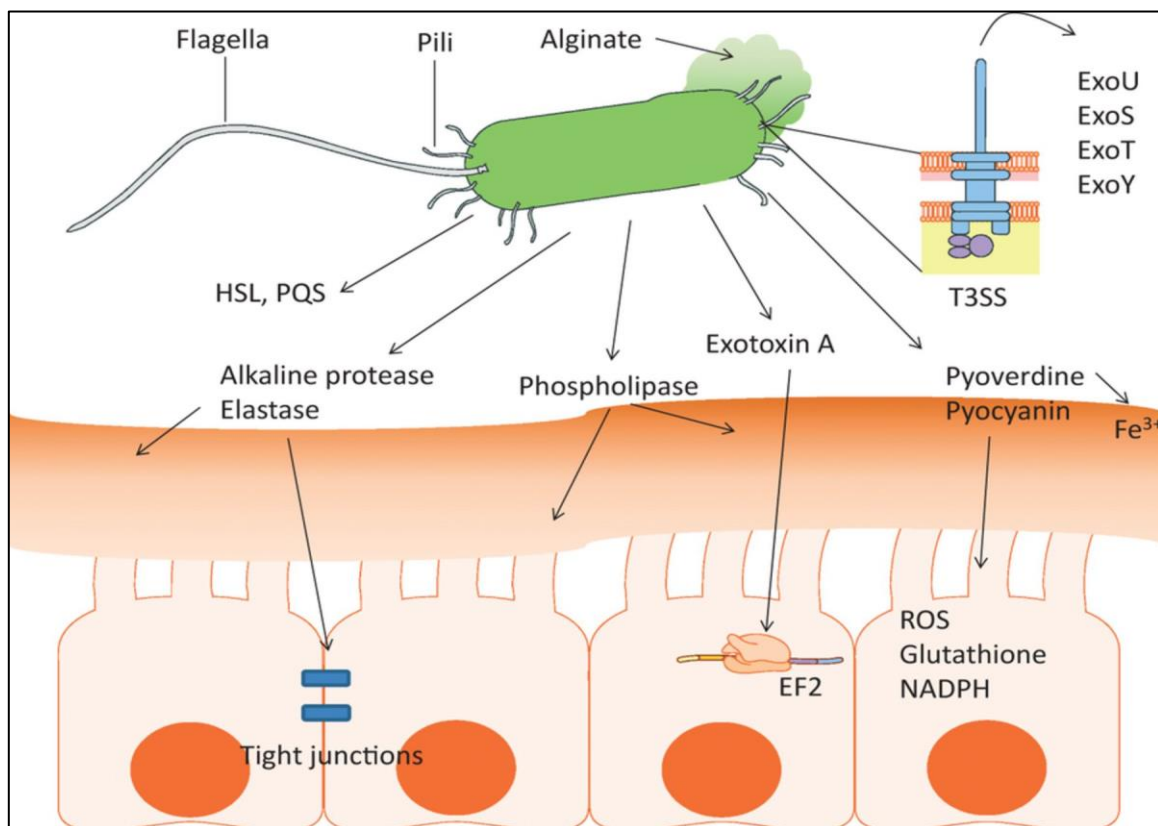


Figure 4: Overview of virulence factors of *P. aeruginosa*. This figure is not exhaustive and does not contain all the virulence factors. The others will be depicted in other figures. (Gellatly and Hancock, 2013).

damages by different mechanisms as depicted in Figure 4 (Gellatly and Hancock, 2013). In addition, the Type III and VI Secretion Systems directly inject exotoxins of *P. aeruginosa* in the cytoplasm of eukaryotic or other competing bacterial (Bleves et al., 2010). These are the main factors responsible for the bacterial invasion and pathogenicity, they are thus the focus of studies made in order to understand their functions and their potential to define new pipelines of anti-virulence drug developments.

1 Regulation factors

1.1 Description

Quorum sensing (QS) is a bacteria communication system enabling them to build a community network and to regulate their metabolism at the population level. With the QS, *P. aeruginosa* communicates and coordinately regulates its pathogenicity to affect host cells or attack other bacteria. Sometimes, a cross-regulation between bacteria species can occur as in the case of the coexistence of *P. aeruginosa* and *S. aureus* in adult cystic fibrosis patients (Fugère et al., 2014). *P. aeruginosa* possesses three main QS circuits including las, rhl and pqs (Lee and Zhang, 2015; Wagner et al., 2016). Both Las QS and Rhl QS use the HSL as auto-inducer for signaling (N-3-oxododecanoyl-L-homoserine lactones and N-butanoyl-L-homoserine for Las and Rhl system respectively) while the Pqs QS synthesizes the PQS and its precursor 2-heptyl-4-hydroxy-quinolone (HHQ). These signaling molecules are produced by the

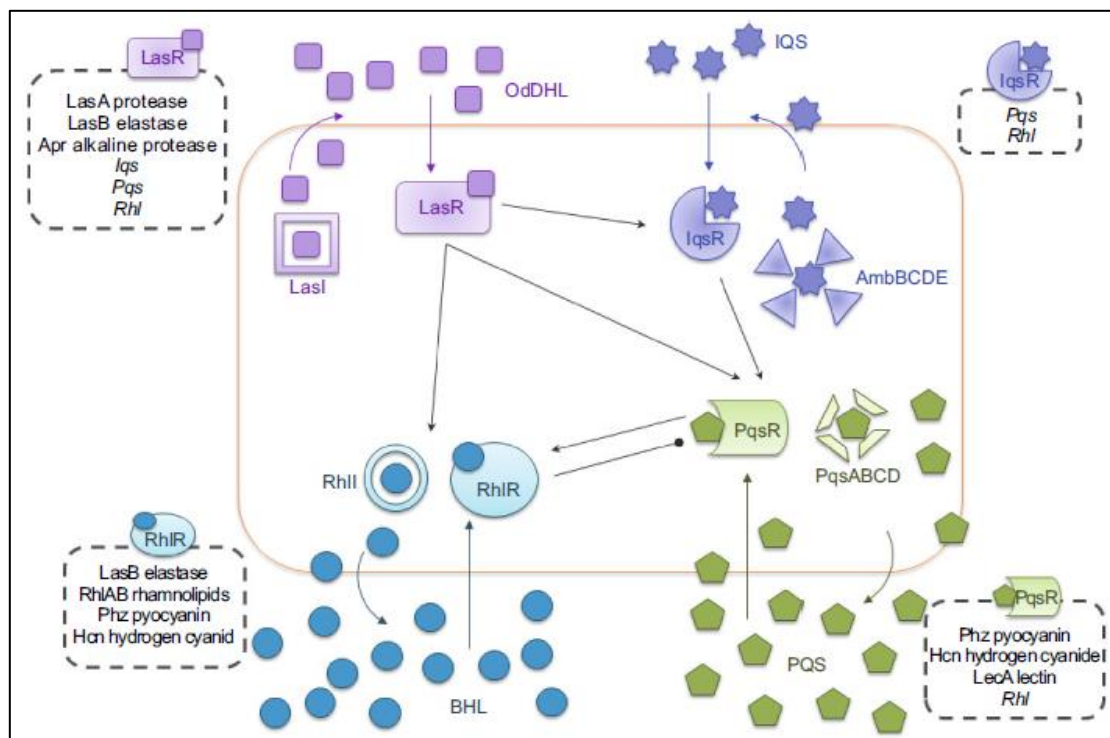


Figure 5: Interplay between four quorum sensing circuits in the regulation of *P. aeruginosa* (Lee and Zhang, 2015).

synthase proteins LasI, RhII and PqsABCD and delivered to the corresponding receptors LasR, RhIR and PqsR for the bacterial regulation. Recently, a fourth QS circuit has been discovered with the 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Integrated Quorum Sensing signal, IQS) as the auto-inducer signaling molecule (Lee et al., 2013). All the four QS systems are interconnected and control most of the pathogenic genes of *P. aeruginosa* as described in Figure 5 (Lee and Zhang, 2015).

A second regulation mechanism of *P. aeruginosa* is the two-component signal transduction system consisting of a sensor and a response regulator. The most famous are the tripartite sensor histidine kinases GacS/RetS/LadS and the response regulator GacA (Bordi et al., 2010; Ventre et al., 2006). Briefly, the three sensors GacS/RetS/LadS are displayed on the bacterial inner membrane and are able to detect unknown signals from outside. These proteins interact with and phosphorylate GacA whose phosphorylated form induces the expression of two small non-coding RNA RsmY and RsmZ. These RNA bind and inhibit the RsmA protein that controls, directly or via the interaction with QS, diverse pathogenic traits of *P. aeruginosa* like the expression of secretion systems and the formation of biofilm (Figure 6), thus controlling the acute/chronic infection switching.

The last main regulation mechanism consists of two second messengers signaling: cyclic diguanylate mono-phosphate (c-di-GMP) and cyclic adenosine mono-phosphate (cAMP). The regulation by cAMP coupled with the Vfr protein will be described in chapter 3.5.3. On the other hand, the c-di-GMP molecule is synthesized by diguanylate cyclases (DGC) and hydrolyzed by phosphodiesterases (PDE) (Jenal and Malone, 2006). Its main function is to stimulate the biofilm formation but concentrations 100-1000 time higher than the physiological levels of this molecule cause an inverse effect (Ishihara et al., 2009). While the QS is involved in the formation of mature biofilm, the c-di-GMP is a factor controlling the switch between planktonic and biofilm lifestyles, meaning that it is responsible for the early stage of biofilm formation (Hengge, 2009; Wagner et al., 2016). Otherwise, this molecule was also shown to have a function on the virulence regulation by inhibiting the secretion systems (Cotter and Stibitz, 2007).

1.2 Drug discovery

The mechanisms and the links between these three main pathways are shown in the Figure 6 (Coggan and Wolfgang, 2012). Due to their important role in the regulation of the pathogenicity of *P. aeruginosa*, they are potential targets for the approach of anti-virulence therapy. Indeed, a number of studies were reported over the years. Two inhibitors and two inducers of Type III Secretion System (T3SS) of *P. aeruginosa* were identified by screening a library of phenolic compounds involved in plant defense signaling. They affected the expression level of the small RNA RsmY and RsmZ which are the key factors of the two-component signal regulation as described above. As a result, the expression of ExoS (an

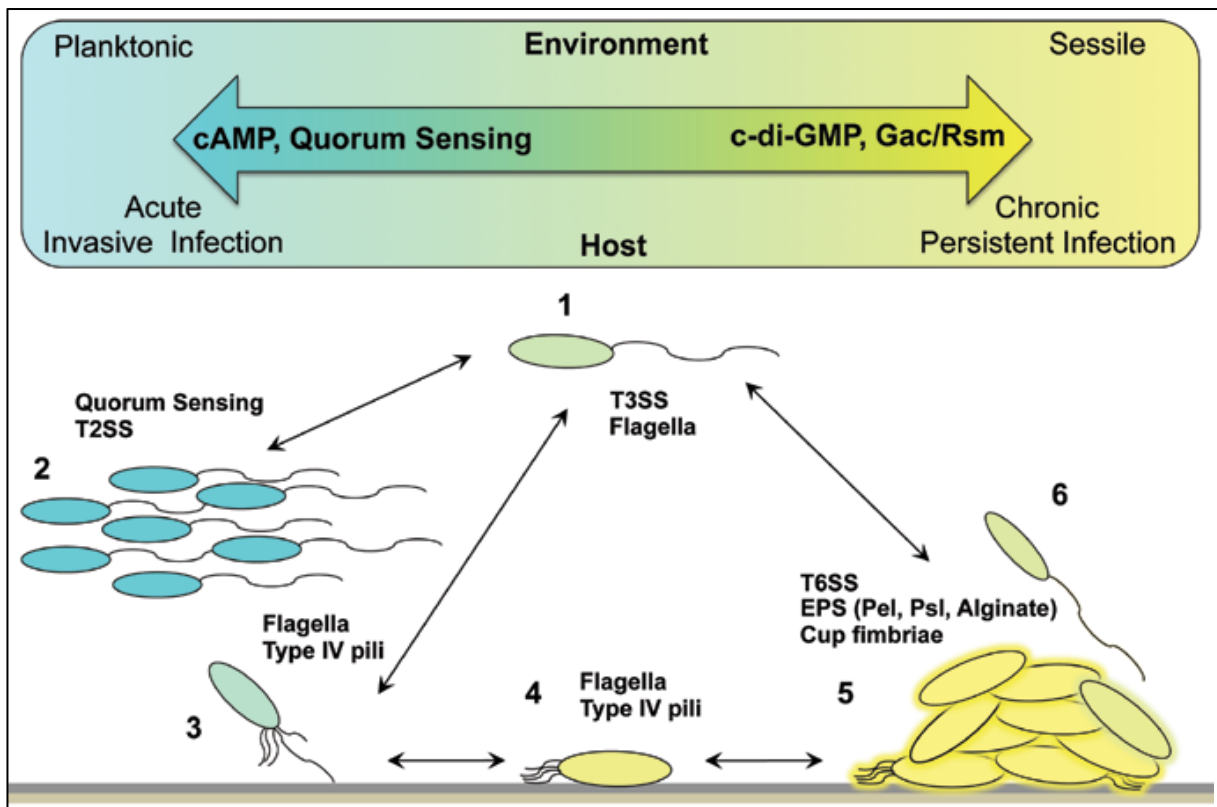


Figure 6: Interplay of four regulatory pathways (cAMP, c-di-GMP, quorum sensing and two-component system Gac/Rsm) in the regulation of acute/chronic toxicity and biofilm lifestyle of *P. aeruginosa* (Coggan and Wolfgang, 2012).

effector of T3SS) was affected and thus, these compounds were suggested to affect the T3SS through the GacSA-RsmYZ-RsmA-ExsA regulatory pathway (Yamazaki et al., 2012).

The three main strategies to inhibit QS are the inactivation of auto-inducer molecules, the blocking of protein synthases or the antagonization of the cognate receptors. Anthranilic acid (AA) analogs were identified as potent inhibitors of PQS signaling. Actually, PQS and its precursor HHQ are synthesized from the primary precursor 4-hydroxy-2-alkyl-quinolines (HAQs), the IUPAC nomenclature name of anthranilic acid. These AA analogs were shown to reduce *P. aeruginosa* PQS levels (Calfee et al., 2001), thus they were proposed to inhibit the protein synthase PqsA. A previous study in *E. coli* indicated that the analogs of AA affected the anthranilate synthase activity (Held and Smith, 1970). In addition, a decrease of the mortality of mice infected by *P. aeruginosa* and a restriction of the pathogens dissemination without perturbing bacterial viability were reported in the presence of these compounds, thus providing a starting point for next development steps and possibly for clinical assays (Lesic et al., 2007). Otherwise, several compounds inhibiting the two other QS circuits, Rhl and Las, were also discovered. For example, meta-bromo-thiol lactone (mBTL) was reported as an inhibitor of the two synthases RhlR and LasR. This compound could block *P. aeruginosa* virulence and the biofilm formation.

In vivo, it was shown to protect *Caenorhabditis elegans* and human epithelial cells from killing by *P. aeruginosa* (O’Loughlin et al., 2013).

As c-di-GMP regulatory molecule is controlled by DGCs and PDEs, the two protein classes are good targets for inhibitors development. Ebselen was identified by high-throughput screening as an inhibitor of *P. aeruginosa* DGC enzyme WspR. This compound and its analog ebselen oxide were shown to reduce diguanylate cyclase activity by covalently modifying cysteine residues, thus affecting the c-di-GMP synthesis. Moreover, these compounds inhibited *in vivo* the biofilm formation and repressed the bacterial swimming motility mediated by the flagella (Lieberman et al., 2014).

2 Adhesion, biofilm formation and motility.

2.1 Description

P. aeruginosa possesses two life styles strictly linked to its environments and its pathogenicity: planktonic and biofilm. The planktonic mode usually occurs when the number of bacteria is low. In this mode, the pathogens are isolated, mobile, and aggressive and cause the acute infection (Furukawa et al., 2006). They are able to attack many kinds of cell and tissues, notably in respiratory and urinary tracts, digestive system and even some innate immune cell like macrophages and neutrophils, causing a rapid spreading in the host and a high mortality in susceptible patients. Indeed, in a healthy person, *P. aeruginosa* could be cleared by the human immune systems when they are in the planktonic form due to some expressed virulence factors like flagellin and lipopolysaccharide (LPS) that trigger the immune response. However, the infection of this pathogens could be severe in patients with immunodeficiency or a weakness of physical barrier like a burn or a wound (Alnour and Ahmed-Abakur, 2017). In these cases, the use of antibiotics is necessary to effectively treat this bacterium because it is generally more susceptible to most, but not all, of antipseudomonal drugs in this growth mode (Spoering and Lewis, 2001).

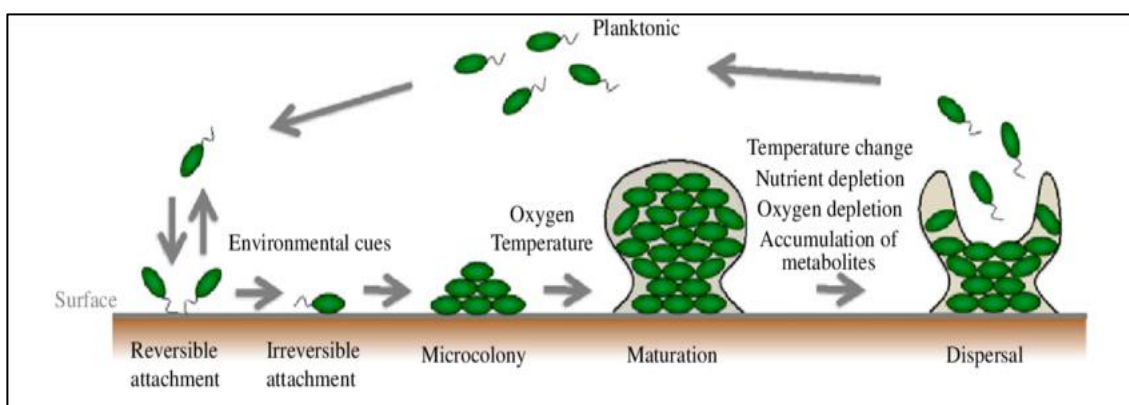


Figure 7: Two life styles of *P. aeruginosa*: Planktonic and Biofilm (Toyofuku et al., 2016).

On the other hand, *P. aeruginosa* is more persistent in the biofilm that appears in some conditions at a high concentration of bacteria. In the biofilm, *P. aeruginosa* lives in a community, sometimes with other bacteria species with efficient network of coordination and regulation (Billings et al., 2013). The biofilm consists of rhamnolipids, extracellular DNA, exopolysaccharides (EPS) and proteins secreted by the bacteria which forms a hydrated bio-polymeric layer and leads to an increase of the bacterial resistance toward the host defense mechanisms and the antibiotic treatments (Flemming and Wingender, 2010). The biofilm formation includes five stages as described in the Figure 7 (Toyofuku et al., 2016). In this mode, many aspects of the bacterial metabolism are reduced because of the lack of nutrients such as carbon source and oxygen. Hence, some antibiotics targeting the bacterial metabolisms like ciprofloxacin (interferes with bacterial replication process) and tetracycline (interferes with bacterial translation process) are less active toward the biofilm (de Beer et al., 1994; Pamp et al., 2008). In addition, the production of some extracellular bacterial appendages needed for acute infection like the flagella, decreases in the biofilm and this lowers the detection by immune cell (Feldman et al., 1998; Gellatly and Hancock, 2013). Therefore, the pathogens are less invasive, inducing a lower cytotoxicity but the infection becomes chronic. This chronic infection of *P. aeruginosa* is usually found and typically described in the pulmonary tissues of cystic fibrosis patients where the presence of the thicker and more viscous host mucus forms a suitable environment for infection development.

One early and important step for either acute infection or the biofilm formation is the adhesion of bacteria on the tissues surface. In *P. aeruginosa*, this step is mediated by some bacteria virulence factors

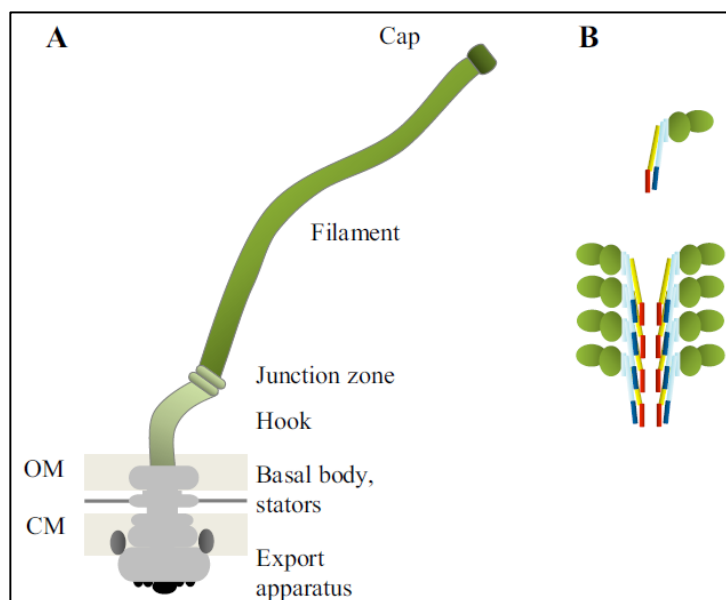


Figure 8: Flagella assembly (A) and flagellin organization (B). The central variable globular domain (green); The conserved N- and C-terminal domains (blue, light blue, yellow, red) include regions binding to TLR5 (light blue, yellow) and those involved in inflammasome formation (blue, red). (Haiko and Westerlund-Wikström, 2013)

displayed on the surface including mainly a single polar flagella and the Type IV pili. The flagella is conventionally known to provide motility function and responsible for the swimming in liquid medium or the swarming on a semi-solid surface (Murray and Kazmierczak, 2006; O'May and Tufenkji, 2011). It is constituted of four parts: a cytoplasm export apparatus, a basal body in the bacterial membrane, a hook connecting the basal body to the filament and a filament made of the main polymeric protein FliC called flagellin (Figure 8A) (Haiko and Westerlund-Wikström, 2013). The role of this protein is important because the *AfliC* mutant is less virulent than the wild-type strain and a decrease of mortality was observed in the infected mouse model of pneumonia (Feldman et al., 1998). Structurally, the monomeric FliC consists of a central variable globular domain shown in green, and the N-, C-terminus conserved regions shown in red, blue, and yellow (Figure 8B). The central domain is exposed outside of the filament, thus it is also proposed to play a critical role in the bacterial adhesion. Indeed, the flagellin FliC and the cap protein FliD which controls the filament polymerization, are essential for the attachment of *P. aeruginosa* in the host because the *AfliC* or *Aflid* mutation affected the binding of some strains to mucin human sputum (Arora et al., 1998; Scharfman et al., 2001). Otherwise, this central domain can activate the innate immune system through its recognition by the host Toll-like receptor 5 (TLR5) (Song and Yoon, 2014). Moreover, the activation of inflammasome is also mediated by the N and C-terminus of FliC, leading to pyroptosis (Halff et al., 2012). Indeed, even the purified form of this flagellin can cause an intense inflammatory response in mouse lung. Therefore, in addition to the traditional motility function, the flagellum turns out to play an important role in the virulence, the immune detection and the adhesion of bacteria. Otherwise, the N and C-terminus of FliC are shown to be involved in the polymerization to form the filament (Samatey et al., 2001; Song and Yoon, 2014).

The second virulence factor involved in the bacterial adhesion as well as the bacterial motility is the Type IV pili (T4P). This is a small appendage which is responsible for the bacterial twitching motility on solid surfaces such as glass or mammalian cells (Mattick, 2002). There are two major types of T4P: Type IVa pili (T4aP) and Type IVb pili (T4bP) and *P. aeruginosa* is the only species in which, both T4aP and T4bP are described in literatures (de Bentzmann et al., 2006; Burrows, 2012; Carter et al., 2010). The difference between T4aP and T4bP is their constituted protein subunits called pilins and the mechanism of pilus assembly. Functionally, the *P. aeruginosa* T4bP is not associated with the bacterial twitching motility but it can be involved in the adherence and biofilm formation (de Bentzmann et al., 2006). In addition, T4bP can transfer bacterial DNA to other *P. aeruginosa* strains by conjugative mechanism (Carter et al., 2010) and was shown to promote bacterial self-association in *Vibrio cholera* and *E. coli* (Anantha et al., 2000; Herrington et al., 1988). Otherwise, the basic functions of the T4aP are the twitching motility and the adhesion to eukaryotic cells. Recently, swarming and walking have also been described as other forms of T4aP-mediated motility (Conrad et al., 2011; Gibiansky et al., 2010; Köhler et al., 2000)

and in collaboration with the flagella, they are involved in the initial colonization of bacteria on host cells to establish the early steps of infection. For the adhesion, T4aP interacts with the glycolipid asialo GM1 located on the mammalian cell surface (Gupta et al., 1994) and it has thus a crucial role in the virulence of *P. aeruginosa*. Indeed, its inactivation was shown to reduce the cytotoxicity (Comolli et al., 1999).

In addition to the flagella and T4P, there are also other virulence factors that are on the bacterial surface and involved in the bacterial attachment and biofilm formation, for example the EPS, the lectins, and the LPS. *P. aeruginosa* produces three EPS: Psl, Pel and alginate, the most abundant, which are the main components of the biofilm and responsible for its stability and architecture (Flemming and Wingender, 2010; Franklin et al., 2011; Wingender et al., 2001). The lectins play a role in the pathogenicity of bacteria because *ΔlecA* and *ΔlecB* mutants decrease the bacterial cytotoxicity and adhesion on eukaryotic cells (Chemani et al., 2009). The lectins recognize host cell targets via their interaction with carbohydrate molecules presented on the host membrane: LecA binds to galactose and its derivatives (Garber et al., 1992; Gilboa-Garber, 1972); LecB binds to fucose, manose and their derivatives (Garber et al., 1987; Gilboa-Garber et al., 1977; Glick and Garber, 1983). Moreover, they are involved in the stability of the EPS matrix through their interplay with the polysaccharides (Diggle et al., 2006; Tielker et al., 2005). LPS is an essential virulence factor whose structure consists of the lipid A inserted in the bacterial outer membrane, a core of oligosaccharide and the most exposed O-antigen responsible for the antigenic specificity of the different strains. A role of LPS in the *P. aeruginosa* adhesion was described in the literature because it binds to the human corneal epithelial protein galectin-3 and the glycolipid asialo GM1 (Gupta et al., 1994, 1997). However, as an important virulence factor, the main effect of LPS is the activation of the innate immune system, thus inducing the inflammation via its interaction with the Toll-like receptor 4 (TLR4) (Pier, 2007).

2.2 Drug discovery

The inhibition of the adhesins responsible for the bacterial adhesion is a potential approach because all of them are not necessary for the bacterial viability but they are essential for the bacterial virulence. One strategy to affect these targets is the inhibition of their production. The synthesis of most adhesins and of the biofilm formation are regulated by three main regulatory pathways including the QS, c-di-GMP and two-component system GacS/GacA as depicted in Figure 6. Some compounds inhibiting these pathways were identified and they were described in the previous chapter.

Another strategy directly targets the adhesins, based on their properties. For example, the lectins LecA and LecB are described to bind to sugar molecules displayed at the eukaryotic cell surface (galactose for LecA and fucose, mannose for LecB). Thus, derivatives of these carbohydrates are developed by

many groups in order to inhibit the binding capacity of the lectins by competitive antagonists. Calixarene-based glycoclusters including galactosides and fucosides were synthesized and were shown to be effective inhibitors of *P. aeruginosa* adhesion. These compounds contain four residues of galactose or fucose, thus they are considered as tetravalent ligands of LecA or LecB. The affinity dissociation constant (K_d) between galactosylated tetravalent glycocluster and LecA is 176 nM, 1000-fold higher than its monovalent parent (150 μM) and around 500-fold higher than the d-galactose or methyl β-d-galactoside (87.5 μM and 70 μM respectively) (Boukerb et al., 2014; Cecioni et al., 2009; Kadam et al., 2011). This means that although the monovalent molecules showed a lower affinity than the basic d-galactose, the increase of valence significantly improves its affinity with LecA. On the other hand, when fucosides were fused to form a tetravalent ligand, it also displayed a nanomolar affinity with LecB (48 nM) but this value is only 6-fold higher than the monovalent molecule or the methyl α-l-fucoside (300 nM and 430 nM) (Boukerb et al., 2014; Sabin et al., 2006). In addition, these two tetravalent glycoclusters were shown to inhibit the biofilm formation and the mouse lung infection at 5 μM (Boukerb et al., 2014). Thus, they represent promising compounds to be tested in clinical assays. In addition to the calixarene-based glycoclusters, other natural and synthetic mannose-based inhibitors of LecB are described in the literature (Hauck et al., 2013; Hofmann et al., 2015; Johansson et al., 2008; Sabin et al., 2006). However, these studies rather focus on their chemical structure and only show effects on the bacterial adhesion or the biofilm formation. Some preclinical assays need to be performed in animals to evaluate their efficacy.

Immunotherapy is an innovative approach to treat severe diseases such as Hepatitis, Ebola virus and different types of cancers. A few groups have reported the use of anti-Flagella (Oishi et al., 1993), anti-LPS and anti-EPS antibodies against *P. aeruginosa* infection. Panobacumab is a fully human monoclonal IgM antibody directed against LPS O-polysaccharide of *P. aeruginosa* serotype O11. This antibody reduced the lung inflammation and enhanced the bacterial clearance from lung in neutropenic mice pneumonia. Moreover, its safety was also reported in humans and it is now tested in a phase II clinical trial (Lu et al., 2011; Secher et al., 2013). Otherwise, a monoclonal antibody (mAb) against Psl (a component of exopolysaccharide) was identified and could inhibit the adherence of *P. aeruginosa* to eukaryotic epithelial cells and reduce the biomass of biofilm. In addition, this antibody could mediate phagocytic activity against mature biofilms and recognize Psl in a chronic infection of pig skin. Recently, the anti-Psl was chemically combined with the anti-PcrV mAb targeting the tip protein of the Type 3 Secretion System, to create a single molecule with bispecific function. This multifunctional mAb (BiS4αPa) offered more protections against *P. aeruginosa* than the mAb parents alone or in combination in a murine pneumonia model and it is ongoing phase I and II clinical trials (DiGiandomenico et al., 2014a; Ray et al., 2017).

3 Secretion systems

Secretion systems are virulence factors allowing the direct secretion of bacterial proteins in the extracellular medium or their injection in bacterial or eukaryotic target cells. Therefore, they play an important role for the bacteria virulence. These machineries are presented in the Figure 9 and classified into two types: the one-step secretion system including the type I, III and VI secretion systems and the two-steps secretion system including the type II and V secretion systems. In two-steps mechanisms, the proteins are first exported into the periplasm by the Sec or Tat systems before they cross the outer membrane thanks to the type II or V secretion systems. Among the secretion systems, the type III secretion system (T3SS) presented in red in Figure 9, is one of the most important virulence factor by which the bacterial exotoxins are injected into mammalian cells in one step and cause rapid and severe cell damages.

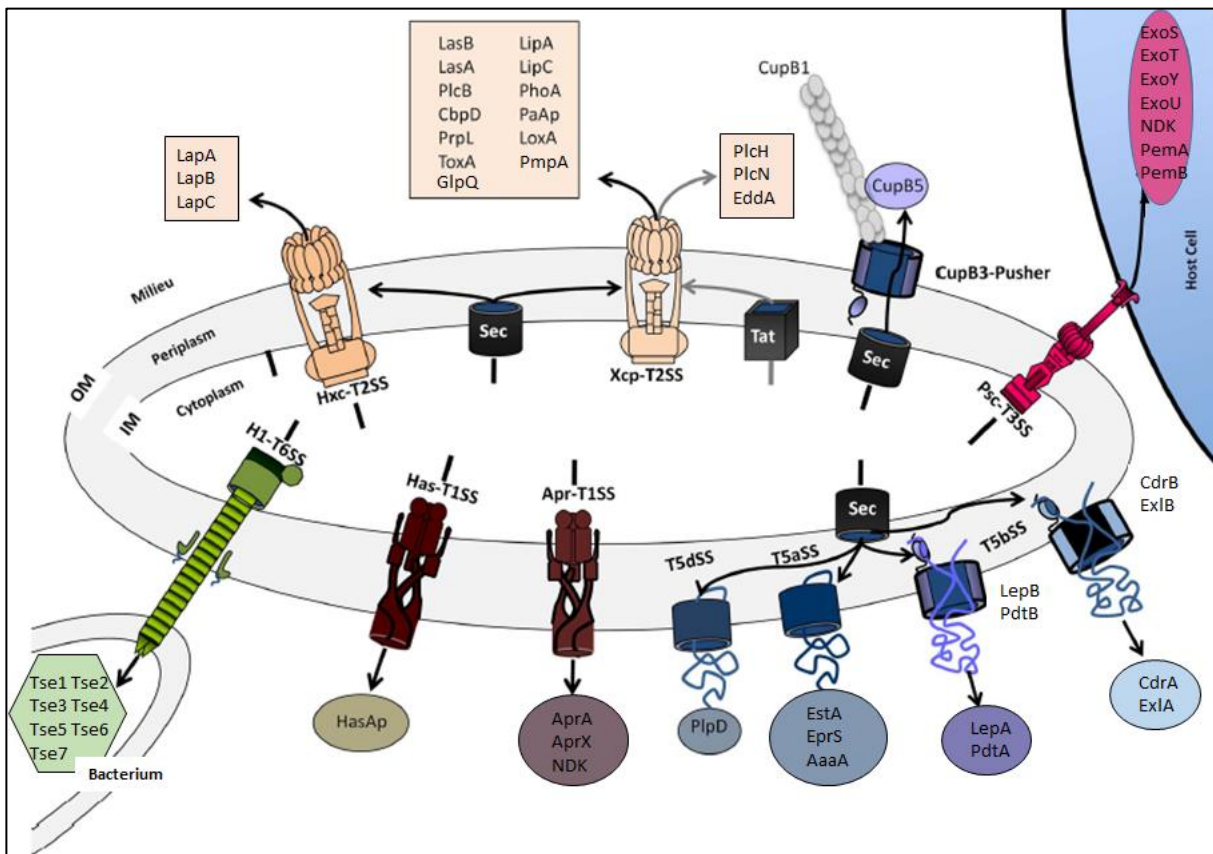


Figure 9: Different secretion systems presented in *P. aeruginosa*. Adapted from (Filloux, 2011)

3.1 Type I secretion system

The type I Secretion System (T1SS) is constituted of three components: an ATP-binding cassette protein (ABC transporter) located in the bacterial cytoplasmic membrane, belonging to a family responsible for the transport of diverse substances by hydrolyzing ATP as an energy source, an outer membrane (OM) protein and an inner membrane (IM) protein that protrudes into the periplasm and connect the ABC transporter to the OM protein. Nowadays, two T1SS are well studied in *P. aeruginosa*: the Apr and Has systems. The components of the Apr system are AprD (ABC transporter), AprF (OM protein) and AprE (IM protein). This system is involved in the secretion of AprX whose function is still unknown and AprA which is an alkaline protease (Guzzo et al., 1991a, 1991b). AprA was reported to block the activation and cause the degradation of human complement proteins. Thus, it affects the phagocytosis (Laarman et al., 2012). As a result, this protease helps *P. aeruginosa* to evade the killing by human neutrophils and is an important factor for the bacterial persistence.

The second T1SS (Has) consists of HasD (ABC transporter), HasF (OM protein) and HasE (IM protein). It secretes the protein HasAp that is known as a heme-binding protein involved in the iron uptake by bacteria (Létoffé et al., 1998). Actually, HasAp can bind to the free heme or heme containing proteins such as hemoglobin in the extracellular medium and then transfers them to the membrane receptor HasR that allows heme molecules internalization into bacteria where they are used as a second iron source, besides the uptake by siderophores (Létoffé et al., 2001; Wandersman and Delepelaire, 2004).

In addition to the two known T1SS effectors, a nucleoside diphosphate kinase (NDK) is proposed to be another toxin also secreted by the T1SS into the extracellular medium. Indeed, like AprA, NDK possesses a conserved motif of 4 amino-acids in the C-terminus required for the protein secretion by the T1SS (Ghigo and Wandersman, 1994; Guzzo et al., 1991a, 1991b). This kinase is present in both cytosolic and membrane associated fraction as well as in a secreted form. Inside bacteria, while the 16kDa cytosolic NDK is almost inactive and does not show specificity to any nucleotides, the membrane NDK (truncated form of 12kDa) uses ATP to phosphorylate GDP into GTP and is required for the alginate EPS synthesis and cellular growth (Sundin et al., 1996). Upon secretion in the extracellular medium, this toxin can be cytotoxic for macrophages by controlling the extracellular ATP level through its kinase activity (Zaborina et al., 1999).

Although the T1SS is well studied, compounds targeting it have not yet been identified. Although many roles are described for this system in the virulence of *P. aeruginosa*, its participation to the bacterial virulence is less crucial than other secretion systems, and consequently it is possible that the research of inhibitor concentrates rather on more promising targets than the T1SS.

3.2 Type II secretion system

The Type II Secretion System (T2SS) secretes proteins that are present in the bacterial periplasm into the extracellular medium (Figure 9). Before that, these proteins are translocated from the bacterial cytoplasm to the periplasm by the general Sec- and Tat-secretion pathways that are the two major systems secreting diverse proteins across the cytoplasmic membrane and are essential for the cellular viability. The secretory pathway (Sec) includes SecA (ATPase) and SecYEG (Translocon) that transport the proteins possessing an N-terminal signal peptide consisting of one (or more) positive charge at the N-terminus, a core of 8-12 hydrophobic residues followed by the cleavage site. Once proteins are targeted to the Sec complexes, they are unfolded and translocated through the pore made of the SecYEG proteins located in the inner membrane, by using the energy provided by the SecA motor. Upon hydrolyzing ATP, this ATPase provides a proton motive force which facilitates the protein translocation and unfolding. The secreted proteins are refolded and released in the bacterial periplasm after cleavage of their signal peptide (Papanikou et al., 2007). In a similar way, an N-terminal signal peptide is necessary for the protein secretion by the Twin Arginine Translocation (Tat) pathway. The positive charge is constituted specifically by a tandem arginine residues and the core of the signal peptide is less hydrophobic than the one of the Sec-pathway. The Tat-system secretes 34 fully folded proteins in *P. aeruginosa* and no protein unfolding process is necessary as in the Sec-export (Ball et al., 2016; Gimenez et al., 2018). In *P. aeruginosa*, there are three types of T2SS including Xcp (Extracellular protein), Hxc (Homologous to Xcp) and Txc (Third Xcp homolog). Most of the effectors of these T2SSs are translocated by the Sec-pathways to the periplasm while only three proteins are demonstrated to be Tat-dependent. These three exotoxins are PlcH and PlcN that are two phospholipase C and EddA that is a phosphodiesterase/alkaline phosphatase D, and they belong to the Xcp T2SS (Figure 9 and Table 3) (Ball et al., 2016; Gimenez et al., 2018; Voulhoux et al., 2001).

The Hxc was described in 2002 as a second T2SS. It secretes the exotoxins LapA and LapB which are alkaline phosphatases and the expression of Hxc/T2SS is regulated by the phosphate condition (Ball et al., 2002). These two effectors are found in the PAO1 strain and, unfortunately, their effect on the bacterial virulence remains unclear. On the other hand, a third exotoxin named LapC is also reported to be secreted by the Hxc/T2SS but this toxin is presented only in PA14-like strains. Despite possessing a high sequence similarity with LapA, this protein does not display the alkaline phosphatase activity. Otherwise, LapC could bind to free phosphate and is proposed to be involved in capturing extracellular phosphate needed for the bacterial survival under phosphate-limiting conditions (Ball et al., 2012).

The Txc was discovered in 2014 in the *P. aeruginosa* strain PA7 that lacks the T3SS. This T2SS secretes the exotoxin CpdE that was shown to be a chitin binding protein. The exact function of this proteins in *P. aeruginosa* pathogenicity is still unknown (Cadoret et al., 2014).

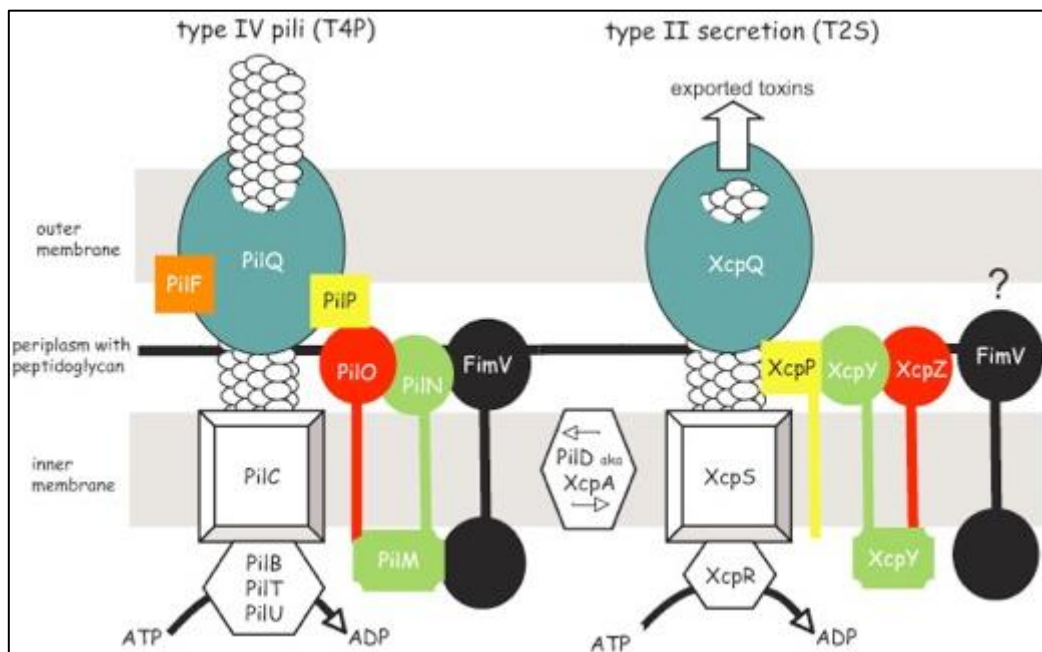


Figure 10: Assembly of Type IV pili and Type II Secretion System (Burrows, 2016)

The Xcp is the classical T2SS constituted by an ATPase (XcpR) facing the bacterial cytoplasm, linked to the inner membrane proteins complex XcpPSYZ of which the XcpP, transiently interacts with the outer membrane secretin XcpQ. This structure is related to the T4P (Figure 10) but one striking difference is the cytoplasmic ATPases (Burrows, 2016). While the T4P displays three distinct ATPases PiiB, PiiT and PiiU, the Xcp/T2SS possesses a single ATPase XcpR which provides energy to push exoproteins from the bacterial cytoplasm through the secretin to the extracellular medium by using ATP as the fuel (Bleves et al., 2010). The secretin XcpQ is a dodecameric protein forming a large ring of 90 Å pore diameter through which, even folded proteins could be transported. To date, there are at least 14 known exoproteins secreted by the Xcp/T2SS as shown in the Table 3. Among them, the most famous are the elastase LasB and the exotoxin A (ToxA) that are targets of potential anti-virulence strategies.

The elastase LasB is a zinc-dependent metalloprotease of 33kDa possessing multiple roles in the infection process. This proteolytic enzyme is present in the periplasm and extracellular fractions. Inside bacteria, LasB plays a role in the biofilm regulation by activating the nucleoside diphosphate kinase (NDK) as described in the chapter of T1SS. Actually, LasB cleaves NDK from 16kDa to its active form of 12kDa (Kamath et al., 1998). Upon secretion outside the bacterium, LasB proteolytic activity degrades the matrix proteins such as elastin (Wretling and Wadström, 1977), collagen (Heck et al., 1986), fibronectin (Beaufort et al., 2011) and VE-cadherin (Golovkine et al., 2014). Hence, it facilitates the cell

damages and bacterial invasion in the acute infection. In addition, LasB is involved in the inactivation of the plasma complement (Hong and Ghebrehiwet, 1992; Schultz and Miller, 1974) as well as in the degradation of many components of the innate and adaptive immune systems (Kon et al., 1999; Kuang et al., 2011; Parmely et al., 1990). Recently, with the alkaline phosphatase AprA effector from T1SS, LasB has been reported to degrade the free flagellin FliC in the medium, thus affecting its recognition by the host immune systems and enhancing the persistence of *P. aeruginosa* (Casilag et al., 2016). Finally, the bacteria swarming motility was also shown to be linked to this elastase (Overhage et al., 2008). As a result, LasB has been considered as a promising target for the anti-virulence development because of its key functions in the pathogenicity of *P. aeruginosa*. N-mercaptoacetyl-Phe-Tyr-amide was identified as a potential inhibitor by incorporating the mercaptoacetyl group to the N-terminus of a dipeptide template obtained from the screening of a dipeptide library ($20^2 = 400$ compounds). This mercaptoacetyl group is known as a metal-chelator that could affect LasB which is a zinc-dependent enzyme. *In vitro*, this compound protected NDK and immunoglobulin G (IgG), which are two substrates of LasB, against the elastase activity. In addition, the biofilm formation was reduced in presence of this compound, as the result of the indirect NDK inhibition (Cathcart et al., 2011). Otherwise, some mercapto-acetamides derivated compounds were identified by an *in silico* screening against LasB. The effect of the “hit” compounds was then tested *in vitro* by evaluating the elastase activity and characterized *in vivo* in the *Caenorhabditis elegans* model. Three “lead” compounds were confirmed to inhibit LasB activity *in vitro* and improved the survival of *C. elegans* against the *P. aeruginosa* infection at a non-toxic concentration (Zhu et al., 2015).

The Exotoxin A (ToxA) is a *P. aeruginosa* extracellular toxic enzyme belonging to the mono-ADP-ribosyltransferase (mADPRT) family present in many bacteria such as *Vibrio cholerae* (cholera toxin), *Corynebacterium diphtheria* (diphtheria toxin), *Bordetella pertussis* (pertussis toxin), *Clostridium botulinum* and *Clostridium lomosum* (C3-like exoenzyme) and *Escherichia coli* (heat-labile enterotoxin). This enzyme transfers an ADP-ribosyl group to targeted proteins by using NAD^+ as a cofactor, thus affecting the protein function in the cells. The mADPRT family is characterized by a low sequence identity, hence each enzyme acts on its own targets but the catalytic domain is structurally conserved. The Exotoxin A possesses three domains including the C-terminal region which is responsible for the enzymatic activity. The N-terminal and central domains have a role in the interaction with eukaryotic receptors and the toxin translocation across the membrane of intracellular compartments, respectively (Allured et al., 1986). This toxin affects the eukaryotic elongation factor-2, which is known as the essential catalytic protein factor of the ribosome, thus slowing the protein synthesis (Foley et al., 1995; Iglewski et al., 1977; Nygård and Nilsson, 1990). As a result, the Exotoxin A is involved in the virulence of *P. aeruginosa* and was reported to induce cell apoptosis (Chang and Kwon, 2007; Du et al., 2010; Jenkins et

al., 2004). Therefore, inhibition of this toxin could be considered as an anti-virulence strategy because it is not required for *P. aeruginosa* growth and viability. A yeast two-hybrid screening was performed to look for peptides that bind to the catalytic domain of ToxA. From 10 million peptide sequences, only three were selected and then were shown to inhibit the ADPRT enzymatic activity *in vitro* (Thompson et al., 2003). Recently, a virtual screening was accomplished to search for compounds inhibiting ToxA by docking them to the active site of this enzyme. The identified “hit” compounds were shown to protect the mammalian C38 cells against the activity of Exotoxin A *in vitro* (Turgeon et al., 2011). All these discoveries could be promising for the development of anti-virulence compounds but they would directly affect the catalytic activity of ToxA which is highly conserved among the ADPRTs. Therefore, these compounds could have a low specificity and potential effects on the human ADPRTs. More studies are necessary to evaluate their efficacy *in vivo*.

In addition to the direct strategy targeting the exotoxins, another approach consists in inhibiting the T2SS assembly. A screening of T2SS inhibition from 73000 compounds of Chembridge and Timtec libraries was performed using an indirect cellular bioluminescence assay of the translation of SecA (ATPase of Sec-pathway). This screening strategy is based on that fact that translocation of SecA is upregulated when T2SS secretion is blocked in *E. coli* (Alksne et al., 2000). Nine of these compounds were shown to inhibit the secretion of the elastase LasB (Sec-mediated pathway) and the phospholipase C PlcH/N (Tat-mediated pathway) but they did not have an impact on the β -lactamase secretion to the periplasm mediated by the Sec-pathway. Therefore, these compounds were suggested to target rather the T2SS than the Sec-system (Moir et al., 2011). Otherwise, thiazolidinone which was identified in a screening for T3SS inhibitors in *S. typhimurium*, showed an effect on the secretion of the elastase LasB of *P. aeruginosa*. In addition, this compound inhibited the twitching motility mediated by T4P but did not affect the flagella-mediated swimming motility. This can be explained by the high similarity between T2SS and T4P as described above in Figure 10. In conclusion, authors suggested that this compound inhibits all the *P. aeruginosa* secretins located in the outer-membrane because this is the only common target of T3SS, T2SS, T4P and not the flagella (Felise et al., 2008).

3.3 Type V secretion system

The Type V Secretion system (T5SS) is the second two-step secretion system and all its known effectors are translocated into the periplasm by the Sec-pathway. In *P. aeruginosa*, there are two T5SS: T5aSS and T5bSS (Figure 9).

3.3.1 Type Va Secretion system (auto-transporter)

T5aSS consists of only one protein harboring an N-terminal signal peptide domain for the Sec-transport, a C-terminal domain that forms a channel with 12 β -barrel strands into the outer-membrane and a central domain (passenger domain) that is transported to the extracellular space after cleavage of the C-terminal structure. Thus, this protein is usually known as auto-transporter. There are three typical auto-transporters in *P. aeruginosa*: EstA (PA5112), EprS (PA3535) and AaaA (PA0238).

The C-terminal domain of EstA is not cleaved. Hence its passenger domain remains bound to the outside of the bacterial membrane and displays an esterase activity responsible for the production of rhamnolipids, a component of bacterial biofilm. In addition, EstA was shown to affect the bacterial motility: swimming, twitching and swarming and this effect is linked to its catalytic activity (Wilhelm et al., 2007).

On the other hand, the passenger domain of EprS is secreted into the extracellular medium by *P. aeruginosa*. This secreted protein possesses a serine protease activity and preferentially cleaves substrates that terminate with arginine or lysine residues. EprS was reported to cleave PARs tethered ligand at a specific site and to induce IL-8 production. As a result, this protein activity induced inflammatory responses in a human bronchiole epithelial cell line EBC-1 (Kida et al., 2013).

Otherwise, the auto-transporter AaaA has been recently reported. This is an arginine-specific aminopeptidase whose passenger and C-terminal domains remain at the bacterial surface. This enzyme is important for the bacterial growth and is involved in the virulence of *P. aeruginosa*. Indeed, its mutant strains were shown to be less virulent and persistent in a chronic mouse wound model (Luckett et al., 2012).

In addition to the EstA, EprS and AaaA, PlpD (a Patatin-like protein lipase) is another auto-transporter protein whose passenger domain is secreted into the extracellular medium by *P. aeruginosa*. However, its C-terminal domain shares a similarity with the transporter protein of a T5bSS (TpsB) with 16 β -barrel strands that will be described below. Thus, this enzyme was proposed to be classified into a new class of auto-transporter: T5SSd (da Mata Madeira et al., 2016; Salacha et al., 2010).

3.3.2 Type Vb Secretion system (two-partner secretion: TPS)

The T5bSS consists of two proteins, one forms a pore in the bacterial outer membrane (TpsB) and the other is secreted (TpsA) into the extracellular medium. Both TpsA and TpsB proteins possess a signal peptide and are translocated across the inner membrane by the Sec-pathway. In the periplasm, TpsB forms a pore with 16 strands β -barrel structure inserted into the outer membrane and a periplasmic domain containing two POTRA (Periplasmic Polypeptide Transport-Associated) motifs responsible for the recognition of TpsA. On the other hand, upon arrival in the periplasm, TpsA partially folds after cleavage of its signal peptide. The N-terminus domain of TpsA contains a conserved sequence called TPS through which, this protein is docked to the POTRA motifs of TpsB. Afterwards it is secreted through the TpsB channel and completely folds. In *P. aeruginosa*, seven putative T5bSSs are found and four of them are characterized: LepA/B, CdrA/B, PdtA/B and ExlA/B.

Like the auto-transporter EprS, the large extracellular protease LepA was shown to digest the tethered ligand region of the human receptors PAR-1, -2 and -4 at a specific site, leading to their activation (Kida et al., 2008). These receptors are considered to play an important role for the immune response and host inflammation. For example, they can activate the transcription factor, nuclear factor-kappa B (NF- κ B) which is essential for the regulation of interleukin 8 (IL-8) expression (Elliott et al., 2001). Indeed, the induction of IL-8 production was reported in the human bronchiole epithelial cell line (EBC-1) in presence of LepA (Kida et al., 2008).

CdrA is a protein involved in the biofilm formation. Its expression is under the control of c-di-GMP, one of the main regulatory pathways as described in the chapter 1.1. This protein promotes the biofilm formation and the bacterial auto-aggregation in liquid medium. By immunoprecipitation, this protein was demonstrated to bind to the exopolysaccharide Psl, thus increasing the biofilm structure stability (Borlee et al., 2010).

The phosphate depletion regulated TPS PdtA/B is expressed in low phosphate condition and has been recently characterized. Unlike the two other T5SS exoproteins, PdtA does not display neither proteolytic activity nor involvement in adhesion and biofilm formation. Its exact function is still unknown but this protein is important for the virulence of *P. aeruginosa*. Actually, a mutant strain Δ pdtA was less aggressive toward the *C. elegans* in comparison to the wild-type strains (Faure et al., 2014).

In addition, another TPS has been recently discovered, called Exolysin A/B (ExlA/B). This TPS is found in *P. aeruginosa* strains that lack the T3SS locus coding for the major virulence factor of classic strains. The reason for the incompatibility of T3SS and exolysin A/B system is still unknown and some strains expressing ExlA/B are hyper-virulent on endothelial cells and in mice in comparison to the archetypal PA7 strain of this outlier cluster (Elsen et al., 2014). Therefore, one may speculate that the

presence of the simpler ExlA/B system is sufficient for the pathogenicity of *P. aeruginosa* and these strains do not need the complex T3SS that basically consumes more energy for its assembly and functioning. More details about T3SS are described in the chapter 3.5. Actually, ExlA is a pore-forming protein causing cell death. In our lab, we demonstrated that this toxin promotes the activation of Caspase 1 that is involved in the maturation of interleukin 1 β (IL-1 β) in macrophages. The matured IL-1 β is released upon cell pyroptosis (Basso et al., 2017). On the other hand, ExlA induced the cleavage of E- and VE-cadherin of epithelial and endothelial cells respectively as the result of the calcium influx through the ExlA pore formed in the host membrane (Reboud et al., 2017).

Although the T5SSs are important for the pathogenicity of *P. aeruginosa* and even one of them displays a cytotoxicity comparable to the T3SS, no inhibitor compounds targeting the T5SS have been reported so far. Basically, the T5SS is the simplest secretion system constituted of one protein (auto-transporter) or two proteins (TPS), hence the only possible strategy is to directly target the toxin or the translocator. However, the most cytotoxic T5SSs (ExlA/B) has been recently discovered and the other seem to be less cytotoxic than the T2SS and T3SS. This can thus explain the lack of study reporting inhibitor to date.

3.4 Type VI secretion system

The type VI secretion (T6SS) is present in many gram negative bacteria and directly delivers toxins in either eukaryotic or prokaryotic cells. This nanomachine possesses a structure that is similar to the needle of the T4 bacteriophages (Figure 11A) and is constituted of 15 – 20 proteins. A homotrimeric protein VgrG and the proteins from the PAAR (Proline-Alanine-Alanine-Arginine) repeat family, form a tail spike (in green) that facilitates the penetration of the T6SS tip into targeted cells. This spike is located at the end point of a tail tube composed by the main protein Hcp that forms a tube with stacked rings of hexamers (in orange in Figure 11A and in red in Figure 11B). The tail tube is surrounded by a cogwheel-like tubule composed by VipA/VipB assembling into a six-start helix and forming a sheath (in gold in Figure 11B) (Chang et al., 2017; Kudryashev et al., 2015). Unlike the bacteriophage in which, all the systems are docked on the baseplate gp25 (in brown) that serves as a platform for the assembly of the tube and the sheath as well as an attachment site of tail fibers (Figure 11A) (Ho et al., 2014; Kostyuchenko et al., 2003), the tube and the sheath of the T6SS are attached to the bacterial membrane through the interaction with TssE/TssK (homologous of baseplate gp25 in phage) and the core complex TssJ/TssL/TssM (Figure 11A) (Ho et al., 2014). This core complex displays a five-fold symmetry and is composed of a base TssL in the inner membrane and the cytoplasm, a tip TssJ in the outer membrane and the arches TssM in the periplasm (Figure 11C) (Durand et al., 2015). The tube and the spike of the T6SS are translocated across the bacterial membrane by using the energy from the sheath contraction

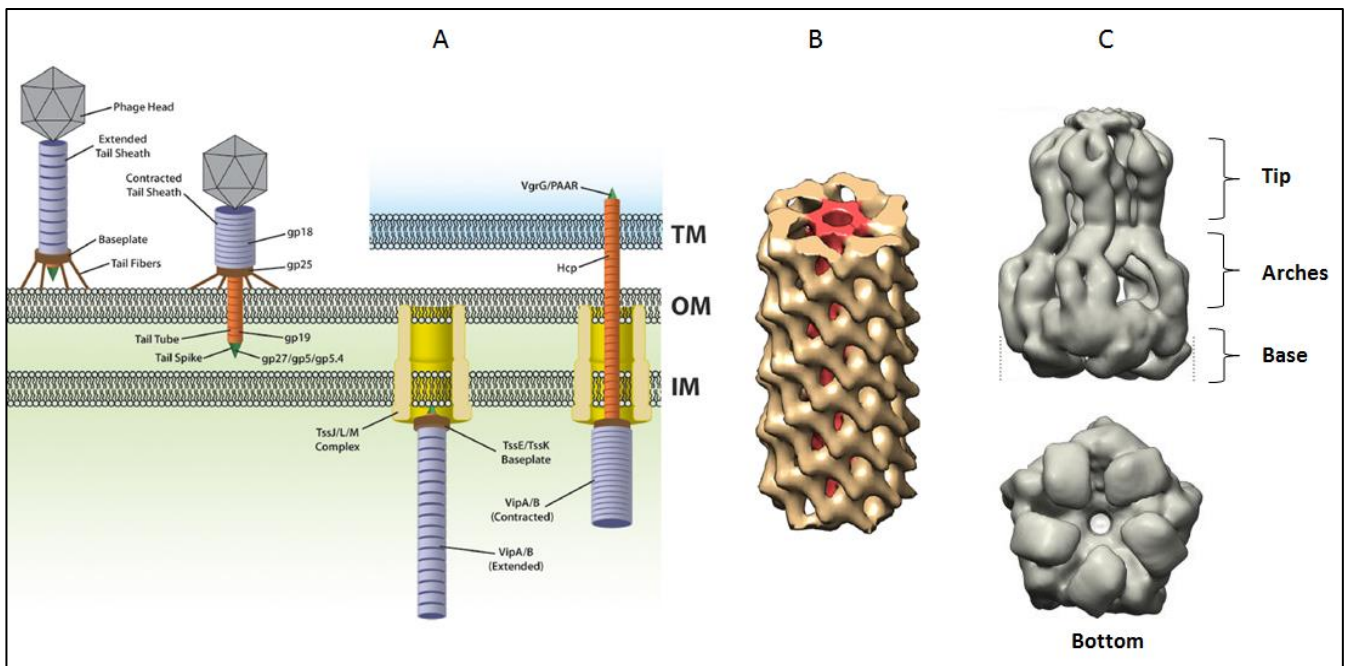


Figure 11: T6SS structure. (A) Assembly of the Type VI Secretion System and its similarity with the T4 bacteriophage. (B) Cryo-ET reconstruction of the T6SS tube (red) and sheath (gold). (C) Negative-stain EM reconstruction of the TssJ/L/M complex. **Adapted from (Ho et al., 2014; Chang et al., 2017; Durand et al., 2015).**

mechanism (Leiman and Shneider, 2012). The contracted sheath is then recognized by the ATPase ClpV that recycles VipA/B, allowing the efficient turnover of the T6SS (Bönemann et al., 2009). In *P. aeruginosa*, the main function of T6SS is to antagonize other rival bacteria, thus gaining growth advantages in some conditions, but it can also target eukaryotic cells. Indeed, while most of the known T6SS effectors target the bacteria, four of them have an effect on eukaryotic cells (Table 3). Besides, some putative T6SS effectors were also pointed out (Sana et al., 2016). In *P. aeruginosa*, the genome contains three gene clusters of T6SS coding for H1-T6SS, H2-T6SS and H3-T6SS. While H1-T6SS secretes toxin targeting only bacteria, the toxins of H2-T6SS and H3-T6SS can affect both prokaryotic and eukaryotic cells.

The H1-T6SS possesses seven characterized toxins Tse1 to 7 (Type 6 secretion exported 1 to 7) (Sana et al., 2016), among them Tse1, Tse2 and Tse3 are the best characterized. Tse1 and Tse3 are secreted into the periplasm of other bacteria where they degrade the peptidoglycan, an essential component of gram negative bacterial cell walls. While Tse1 is an amidase that hydrolyzes the peptide bond connecting two linear sugar chains of the peptidoglycan, Tse3 displays a muramidase activity that cleaves the glycan backbone between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc) residues, two alternative amino sugars of the peptidoglycan polysaccharide chains (Russell et al., 2011). On the other hand, the Tse2 toxin is secreted into the bacterial cytoplasm (Li et al., 2012) and inhibits the cell proliferation, thus providing a growth advantage for *P. aeruginosa* (Hood et al., 2010). The exact

mechanism of Tse2 action is still unknown even if its structure was recently obtained by X-ray crystallography (Robb et al., 2016). Based on its structure, Tse2 was predicted to possess a NAD⁺ dependent phosphotransferase activity. However, no biochemical assay has been performed to confirm this activity.

The phospholipases D (PldA and PldB) are described as toxins secreted by the H2-T6SS and H3-T6SS, respectively. While PldA was shown to have antibacterial activity by degrading the phosphatidylethanolamine (PE) of the bacterial membrane (Russell et al., 2013), the exact mechanism of PldB action is still not clear. Although both proteins that possess a phospholipase D activity were shown to degrade phosphatidylcholine (PC) *in vitro*, their catalytic motifs are distinct and they were classified in different clades. Regarding their effect toward the prokaryotic cells, both PldA and PldB are injected in the periplasm and the cytosolic proteins were shown to produce little effects on bacterial growth. Otherwise, PldA and PldB are involved in the internalization of *P. aeruginosa* into human epithelial cell (Figure 12). Actually, when translocated into eukaryotic cell by the T6SS, they bind to the phosphatidylinositol 3-kinase (PI3K) and protein kinase B/Akt (Akt) (Jiang et al., 2014). Indeed, this PI3K/Akt pathway was shown to be critical for the *P. aeruginosa* internalization (Kierbel et al., 2005). Moreover, the Akt phosphorylation as well as the bacterial internalization were affected in cells infected by *P. aeruginosa* mutants $\Delta pldA$ and $\Delta pldB$ in comparison to wild-type strains (Jiang et al., 2014). Therefore, these T6SS phospholipases could be considered as the exciting target for drug developments.

Nonetheless, to date, no identified compounds have been reported against the T6SS. That could be explained by the relatively recent discovery of bacterial T6SS and by the fact that most of the well

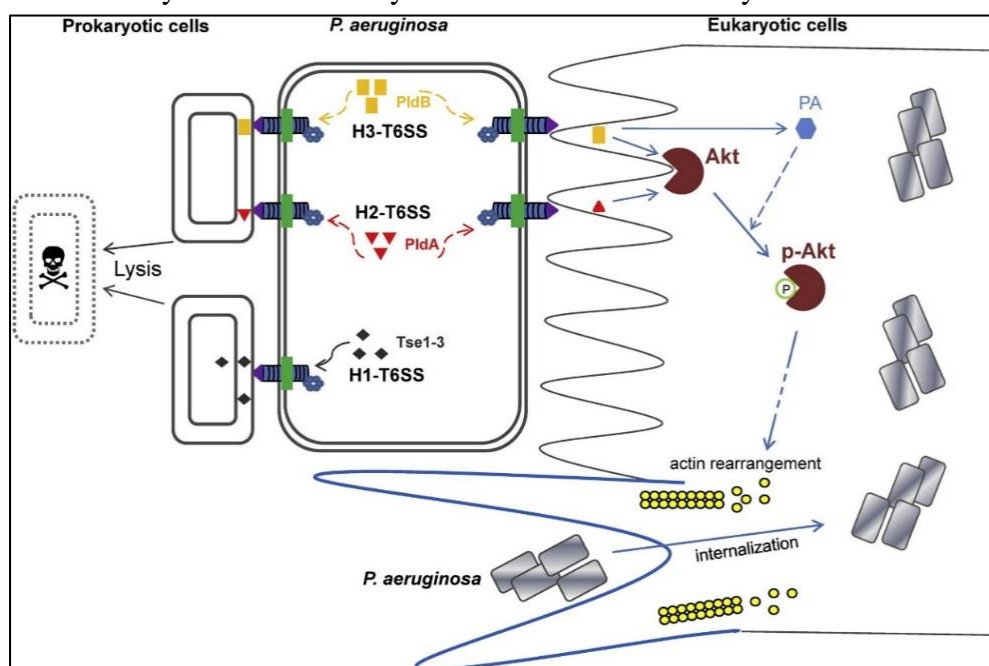


Figure 12: Mechanism model of three T6SS in *P. aeruginosa* versus prokaryotic cell (in the left) and versus eukaryotic cells (in the right). (Jiang et al., 2014)

characterized effectors have effects rather on prokaryotic cells. As a result, the T6SS might not represent an important virulence trait toward human and would not therefore represent a priority for the anti-virulence therapy.

3.5 Type III secretion system

The Type III Secretion System (T3SS) is one of the major virulence factors present in many gram negative bacteria and is responsible for the delivery of effector proteins directly into eukaryotic cells. The N-terminus of the toxins injected by the T3SS harbors the determinant of secretion which is specific to each bacterium. Even if a formal signal sequence is difficult to identify, the T3SS could however be exploited in biotechnologies as a bacterial vector for the protein transfer, for example in vaccination. Actually, an antigen of interest could be fused to a toxin N-terminus and thus be delivered into host cell cytosol through the T3SS (Le Gouëllec et al., 2013). Many companies have developed the bacterial vectorization by using this technique and some promising results in clinical assays were obtained (Le Gouëllec et al., 2012).

In addition, the T3SS can be used as a tool for the production of recombinant protein. Heterologous protein expression is usually performed in *E. coli* strains but it sometimes leads to the production of insoluble proteins in inclusion bodies. Therefore, it is necessary to look for another approach to obtain active protein with high yield of production. For example, in some cases, recombinant proteins are fused to an N-terminal signaling sequence and could be secreted into the bacterial periplasm by the Sec or Tat pathways. In fact, this strategy could be use not only with the Sec or Tat pathways by also with different secretion systems to secrete proteins in the extracellular medium instead of bacterial periplasm (Mergulhão et al., 2005). As a result, no protein extraction is needed and this application was already employed with the T3SS of *P. aeruginosa* (Derouazi et al., 2008).

Nonetheless, for all the applications based on the bacterial T3SS, a good understanding of its function is required. In this chapter, the *P. aeruginosa* T3SS assembly, regulation, exotoxins as well as the ATPase PscN, an essential protein I studied during my PhD will be described. In addition, the anti-virulence compounds targeting this system will also be reviewed.

3.5.1 Assembly

The T3SS is a complex nanomachine constituted of more than 20 proteins that are in monomeric or oligomeric forms and localized in the bacterial cytoplasm, bacterial inner membrane, bacterial periplasm, bacterial outer membrane, extracellular medium, host membrane or host cytoplasm. Based on the structure and the location, the T3SS machinery can be divided into four parts: a cytoplasmic ring (C-ring) and an

export apparatus facing the bacterial cytoplasm, a basal body in the bacterial membrane, a needle complex outside of the bacteria and the translocators in the eukaryotic membrane (Figure 14) (Diepold and Wagner, 2014). The exact mechanism of T3SS assembly is still unclear but two models have been proposed currently and may correspond to different assembly mechanisms in different bacteria: the inside-out and the outside-in models (Figure 13) (Deng et al., 2017).

In the inside-out model (Figure 13a), the inner membrane proteins are the first complex to be formed, followed by the assembly of the export apparatus proteins. Their assembly allows the secretin protein PscC (SctC) to be correctly located in the outer membrane and finally, the needle and translocators are formed. This model seems to match the results obtained for the T3SS of *E. coli* and *Salmonella typhimurium* (Deng et al., 2017; Wagner et al., 2010a). In contrast, the secretin SctC is proposed to be firstly formed in the outside-in model. This outer membrane protein is required for the assembly of SctD, a component of the inner membrane ring. Consequently, after the secretin assembly, the inner membrane complex is assembled, followed by the export apparatus as depicted in the Figure 13b. This outside-in model corresponds to results obtained for the *Yersinia* T3SS (Deng et al., 2017; Diepold et al., 2010). Although the T3SS has a similar structure among gram negative bacteria, their assembly mechanism could be different because of slightly divergent components of each T3SS. Unfortunately, there is no description of the assembly of *P. aeruginosa* T3SS ascertaining to which model it belongs but it is probably the outside-in model because the *P. aeruginosa* T3SS is phylogenetically close to the *Yersinia* one (Cornelis, 2006). Nonetheless, it is evident that the assembly of the T3SS includes two phases: the first one is the assembly of the core machine inside bacteria and the second one is the assembly of needle, tip and translocon outside bacteria. In the next part, the structure as well as the function of each component of T3SS is described based on results obtained in many different bacteria species. Thus, the common name Sct (secretion and cellular translocation) will be used instead of the *P. aeruginosa* name Psc (Pseudomonas secretion component) (Table 2 and Figure 14).

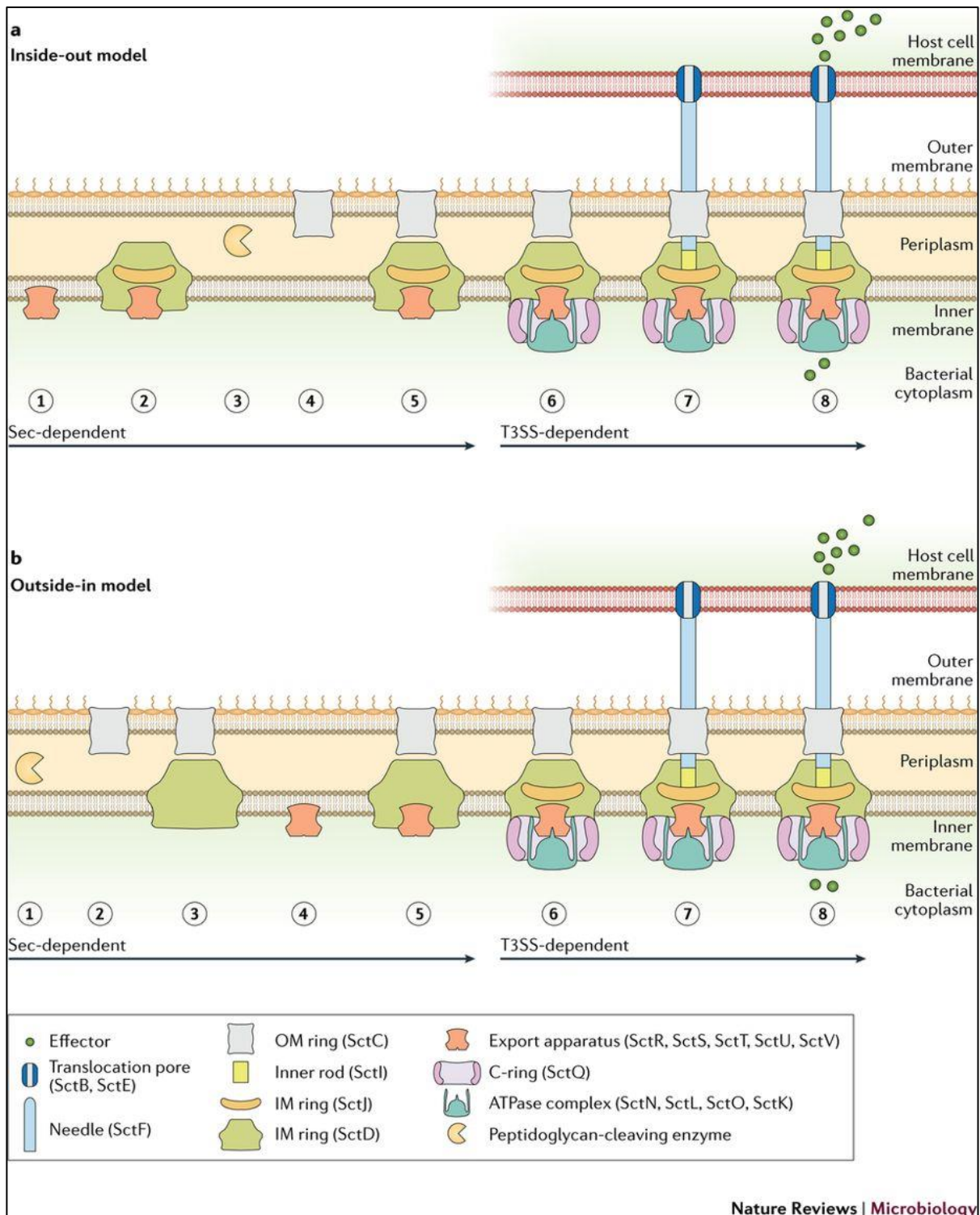


Figure 13: Schematic diagrams of the inside-out (a) and outside-in (b) models of the T3SS assembly (Deng et al., 2017).

Functional name	<i>T3SS family</i>	<i>Ysc</i>		<i>Inv-Mxi-Spa</i>		<i>Ssa-Esc</i>		<i>Hrp-Hrc 1</i>	<i>Hrp-Hrc 2</i>	Flagellar
	Sct name	<i>P. aeruginosa</i>	<i>Yersinia</i>	<i>Shigella</i>	<i>Salmonella SPI</i>	<i>Salmonella SP2</i>	<i>E. coli</i>	<i>P. syringae</i>	<i>R. solanacearum</i>	
Secretin	SctC	PscC	YscC	MxiD	InvG	SsaC	EscC	HrcC	HrcC	–
Outer MS ring protein	SctD	PscD	YscD	MxiG	PrgH	SsaD	EscD	HrpQ	HrpW	FliG
Inner MS ring protein	SctJ	PscJ	YscJ	MxiJ	PrgK	SsaJ	EscJ	HrcJ	HrcJ	FliF
Minor export apparatus protein	SctR	PscR	YscR	Spa24 (SpaP)	SpaP	SsaR	EscR	HrcR	HrcR	FliP
Minor export apparatus protein	SctS	PscS	YscS	Spa9 (SpaQ)	SpaQ	SsaS	EscS	HrcS	HrcS	FliQ
Minor export apparatus protein	SctT	PscT	YscT	Spa29 (SpaR)	SpaR	SsaT	EscT	HrcT	HrcT	FliR
Export apparatus switch protein	SctU	PscU	YscU	Spa40 (SpaS)	SpaS	SsaU	EscU	HrcU	HrcU	FliB
Major export apparatus protein	SctV	PcrD	YscV/L crD	MxiA	InvA	SsaV	EscV	HrcV	HrcV	FliA
Accessory cytosolic protein	SctK	PscK	YscK	MxiK	OrgA	–	–	HrpD	–	–
C ring protein	SctQ	PscQ	YscQ	Spa33 (SpaO)	SpaO	SsaQ	EscQ	HrcQA+B	HrcQ	FliM + FliN
Stator	SctL	PscL	YscL	MxiN	OrgB	SsaK	EscL (Orf5)	HrpE	HrpF	FliH
ATPase	SctN	PscN	YscN	Spa47 (SpaL)	InvC	SsaN	EscN	HrcN	HrcN	FliI
Stalk	SctO	PscO	YscO	Spa13 (SpaM)	InvI	SsaO	Orf15	HrpO	HrpD	FliJ
Needle filament protein (Early substrate)	SctF	PscF	YscF	MxiH	PrgI	SsaG	EscF	HrpA	HrpY	–
Needle chaperones		PscE, PscG	YscE, YscG							
Inner rod protein	SctI	PscI	YscI	MxiI	PrgJ	SsaI	EscI (rOrf8)	HrpB	HrpJ	–
Needle length regulator	SctP	PscP	YscP	Spa32 (SpaN)	InvJ	SsaP	EscP (Orf16)	HrpP	HpaP	FliK
Hydrophilic translocator, needle tip protein (Middle substrate)	SctA	PcrV	LcrV	IpaD	SipD	SseB	–	–	–	–
Hydrophilic translocator partner		PcrG	LcrG			SsaE				
Hydrophobic translocator, pore protein (Middle substrate)	SctE	PopB	YopB	IpaB	SipB	SseC	EspD	HrpK	PopF1, PopF2	–
Hydrophobic translocator, pore protein (Middle substrate)	SctB	PopD	YopD	IpaC	SipC	SseD	EspB	–	–	–
Hydrophobic translocator, chaperone		PcrH	SycB; SycD	IpgC	SicA	SseA	CesAB			
Pilotin		ExsB	YscW	MxiM	InvH	–	–	–	–	–
Gatekeeper	SctW	PopN	YopN/T yeA	MxiC	InvE	SsaL	SepL	HrpJ	HpaA	–
Regulators	ExsA, ExsC, ExsD									

Table 2: Functions and nomenclature of components of the T3SS and their flagellar homologues. Adapted from (Deng et al., 2017)

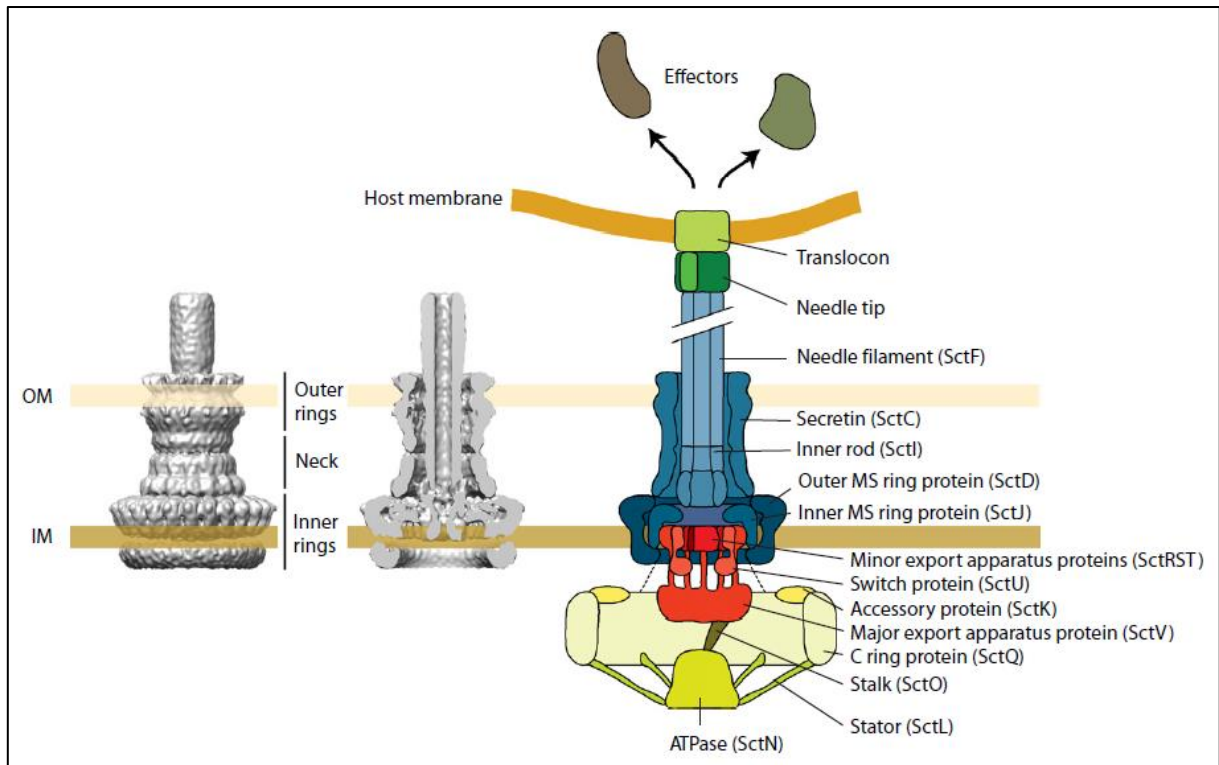


Figure 14: Components of the Type III Secretion System. The left and middle panels represent 3D reconstruction of the T3SS basal body based on Cryo-EM. The right panel represent a drawing of the T3SS with most of its components. (Diepold and Wagner, 2014).

a. Cytoplasmic components

The cytoplasmic components encompass an ATPase complex SctNLOK, a C-ring SctQ and an export apparatus complex SctRSTUV. The ATPase complex is located on the cytoplasmic side and includes the main protein SctN that possesses ATPase activity by which this enzyme provides energy for the T3SS functioning and the secretion process (Akeda and Galan, 2005). The function of SctN will be discussed in more detail in chapter 3.5.4. SctN interacts with SctO (stalk), which anchors SctN to the major protein of the export apparatus SctV, and with SctL (stator) which is its negative regulator (Notti et al., 2015). Actually, SctL decreases the ATPase activity of SctN *in vitro* and the overexpression of SctL affects the T3SS assembly and function *in vivo* (Blaylock et al., 2006; Chatterjee et al., 2013). In addition, the stator plays a role in the connection of the ATPase complex with the C-ring SctQ and possibly with the accessory protein SctK (Diepold and Wagner, 2014; Ibuki et al., 2013; Imada et al., 2016). Structurally, the ATPase complex and the C-ring display a conserved six-fold symmetry which was shown by electron cryotomography and subtomogram averaging (Figure 15) (Makino et al., 2016). The C-ring consists of 22 - 24 subunits of SctQ and is shown to have a dynamic structure. The subunits SctQ in C-ring could be cycled and exchanged with the cytosolic SctQ. It is suggested that SctQ can be involved in

the loading and unloading of substrates during the secretion process (Diepold et al., 2010). In contrast, the function of SctK is unclear.

Regarding the export apparatus complex, one part is protruding in the bacterial cytoplasm and the other part is embedded in the bacterial inner membrane. This complex forms an entry gate for substrates and can be divided into three parts: the major protein SctV, the switch protein SctU and the minor proteins complex SctRST. The SctRST complex has a stoichiometry of 5:1:1 and is described as the first formed complex in the inner membrane during the export apparatus complex assembly (Deng et al., 2017). The switch protein SctU is then recruited to the SctRST complex and is known as an auto-protease (Wagner et al., 2018). SctU is predicted to have a transmembrane domain and a C-terminal domain in the cytoplasm. This C-terminal domain could be cleaved by its auto-protease activity and thus may differentially interact with other T3SS proteins. Therefore, this switch protein was reported to regulate the substrate secretion from early substrate (for example: needle proteins) to middle substrate (translocator proteins) for the T3SS assembly (Deng et al., 2017; Wagner et al., 2018). The last component recruited to the export apparatus complex is the large protein complex SctV. This protein also contains a transmembrane domain and a C-terminal domain that forms a homo-nonameric (nine subunits) ring in the cytoplasm (Abrusci et al., 2013; Deng et al., 2017; Wagner et al., 2018). The exact role of SctV has not been characterized but based on its structure and location, this protein may be an entry point for substrates in the export apparatus.

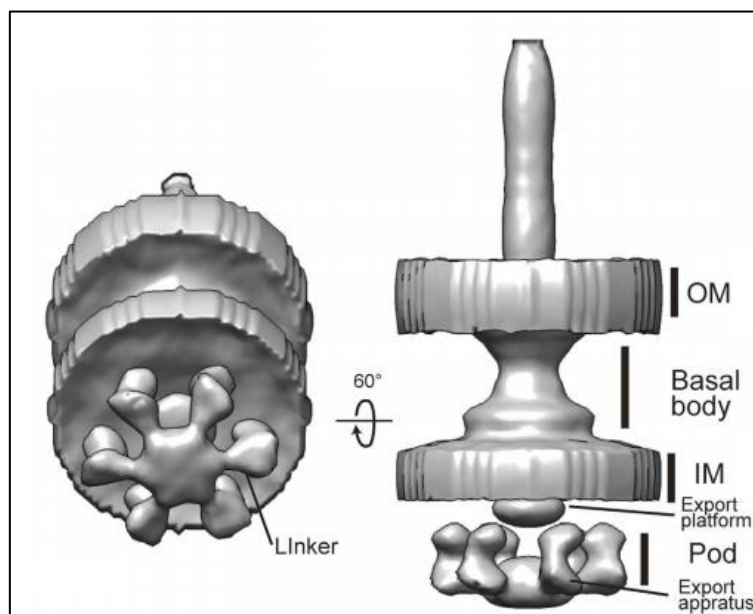


Figure 15: The 3D reconstruction from cryo-ET of T3SS in *Shigella flexneri* shows pod-like densities of the export apparatus with six-fold symmetry (Makino et al., 2016).

b. Basal body

The basal body includes three main proteins: SctD, SctJ and the secretin SctC with a 24:24:15 stoichiometry (Deng et al., 2017). All the three proteins are secreted by the Sec-pathway into the periplasm where they build rings in the bacterial inner and outer membranes. SctD and SctJ form two concentric rings in the inner membrane, with SctD on the outside and SctJ on the inside. Both proteins possess a cytoplasmic domain, a transmembrane domain and a periplasmic domain and they are involved in the connection between the export apparatus and the bacterial inner membrane (Wagner et al., 2018). The secretin SctC contains an N-terminal domain deeply protruding in the periplasm and interacting with the two concentric rings of SctD and SctJ. This protein also has a C-terminal domain that forms a ring in the outer membrane (OM) and a central domain interacting with a pilotin ExsB/YscW/MxiM (in *Pseudomonas*, *Yersinia* and *Shigella* species) that is an outer membrane lipoprotein (Wagner et al., 2018). This pilotin protein is required for the polymerization and the correct insertion of SctC in the OM (Perdu et al., 2015; Rau and Darwin, 2015) but it is not represented in the figure because it was not found in all T3SSs. Upon assembly, the secretin ring forms a periplasmic gate in closed conformation which is stabilized by its N3 domain (Figure 16B) (Worrall et al., 2016). Afterward, the initial rod/needle polymerization within the secretin lumen contacts this N3 domain which in turn unlocks the gate and the final needle assembles with fully open periplasmic gate (Figure 16C) (Hu et al., 2018).

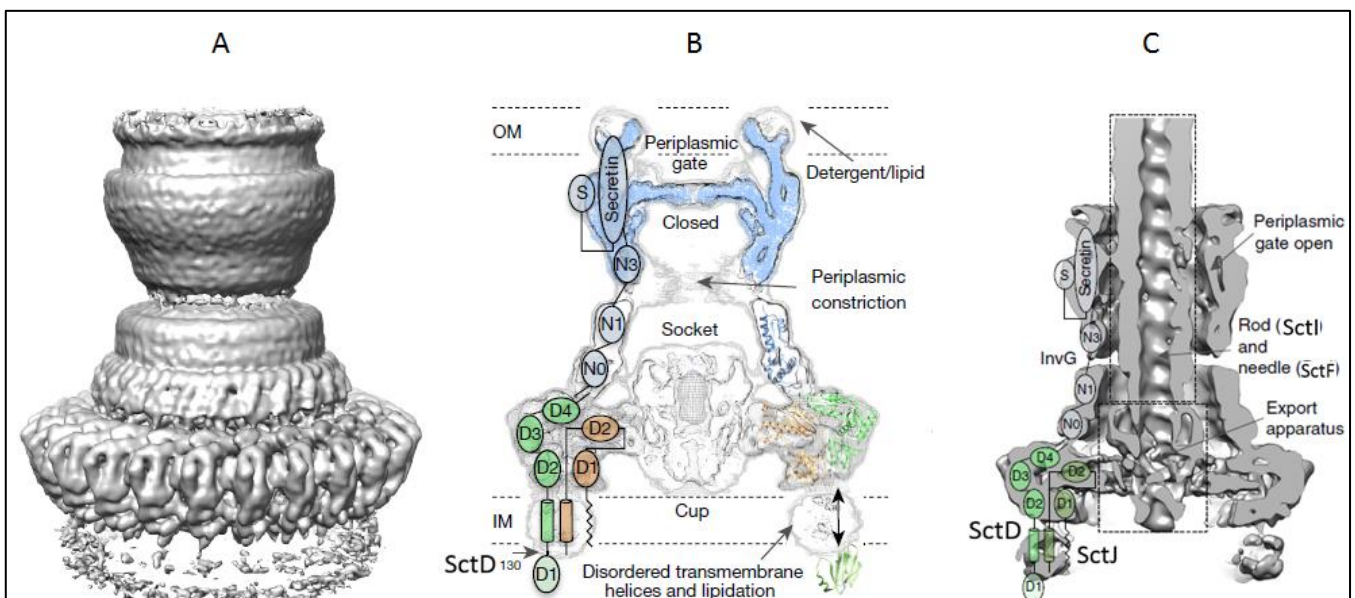


Figure 16: Cryo-EM structures of the T3SS basal body from *Salmonella enterica*. (A) Reconstruction of the T3SS basal body. (B) Central slice view of basal body reconstruction. Closed secretin SctC (light-blue), two concentric inner membrane rings: SctD (light-green) and SctJ (orange). The cytoplasmic domain of SctJ is not displayed in this figure. (C) Central slice view of basal body reconstruction in the presence of the rod/needle filament. **Adapted from (Worrall et al., 2016; Hu et al., 2018)**

c. Needle complex or injectisome

The needle complex is constituted of the inner rod and the needle, respectively made of SctI and SctF subunits that are described as the early substrates of the secretion process. The inner rod SctI is secreted to assemble into a rod inside the T3SS basal body. The structure of SctI appears to be partially unfolded but it is proposed to form a short oligomer of α -helical hairpin (Lefebvre and Galán, 2014; Monlezun et al., 2015). This oligomeric protein is connected to the export apparatus complex by binding to the SctRST protein complex that serves as the platform for the inner rod assembly (Dietsche et al., 2016; Kuhlen et al., 2018; Marlovits et al., 2004). On the other side, SctI is proposed to anchor to the T3SS needle and is important for the needle stability (Marlovits et al., 2006; Monlezun et al., 2015). The binding of SctI to SctF was shown to occur inside bacteria, to depend on residues 83-102 in the C-terminus of SctI and to be essential for the needle assembly (Cao et al., 2017). However, the role in the injectisome assembly of the interaction between the oligomeric form of the needle and the inner rod remains unclear.

On the other hand, the needle SctF subunit is able to self-polymerize and forms a helical filament throughout the bacterial membrane that protrudes in the extracellular medium. The elongation of the needle is controlled by the molecular ruler protein SctP which is also secreted by the T3SS. Actually, in absence of this protein, the needle is abnormally long and no secretion of effectors and translocators is observed (Kubori et al., 2000).

The role of SctP and the mechanism of the needle length control might be different in the different bacteria species and, to date, two models are proposed: the ruler model and the timer model (Galán et al., 2014). In the ruler model, the ruler SctP extends from the cytoplasmic side to the tip of needle complex, thus measuring the length of the needle. Once fully extended, this protein triggers conformational changes on the cytoplasmic side, allowing its secretion which in turn switches the T3SS substrate secretion, thus stopping the secretion of needle subunits and the needle elongation. To support this model, the needle length was demonstrated to correlate with the size of SctP in *Yersinia* (Journet et al., 2003; Wagner et al., 2010b). Moreover, SctP was shown to interact with the switch protein SctU *in vitro* (Ho et al., 2017), whose role is considered to be to switch the secretion from early substrates to middle substrates (Edqvist et al., 2003; Monjarás Feria et al., 2015; Wood et al., 2008).

Otherwise, the timer model is based on the fact that in absence of SctP, the assembly of the inner rod is affected while the needle could nevertheless be formed with an extra-long length in *Salmonella* (Lefebvre and Galán, 2014). This result suggests that the assembly of the needle does not require the inner rod whose assembly would be regulated by SctP. In other words, in the timer model, SctI and SctF are proposed to be secreted and assembled simultaneously and the SctP-dependent completion of the inner rod assembly will terminate the needle growth (Marlovits et al., 2006). Thus, the ruler SctP is proposed

to indirectly regulate the length of needle. This mechanism is supported by the fact that SctI interacts with SctF inside bacteria as described above and that altering their stoichiometry has an impact on the needle length (Marlovits et al., 2006). In fact, extra-long needles are observed when SctF is overexpressed, compared to SctI and it should be noted that this long needle is functional unlike that of the $\Delta sctP$ mutant. As a consequence of the inner rod assembly, proteins at the cytoplasmic side change their conformation, thus inducing a switch, probably through the SctU protein, of substrate secretion, and stopping the needle elongation. This timer model derives from results obtained in *Shigella* and *Salmonella* while the ruler model originates from results obtained in *Yersinia*.

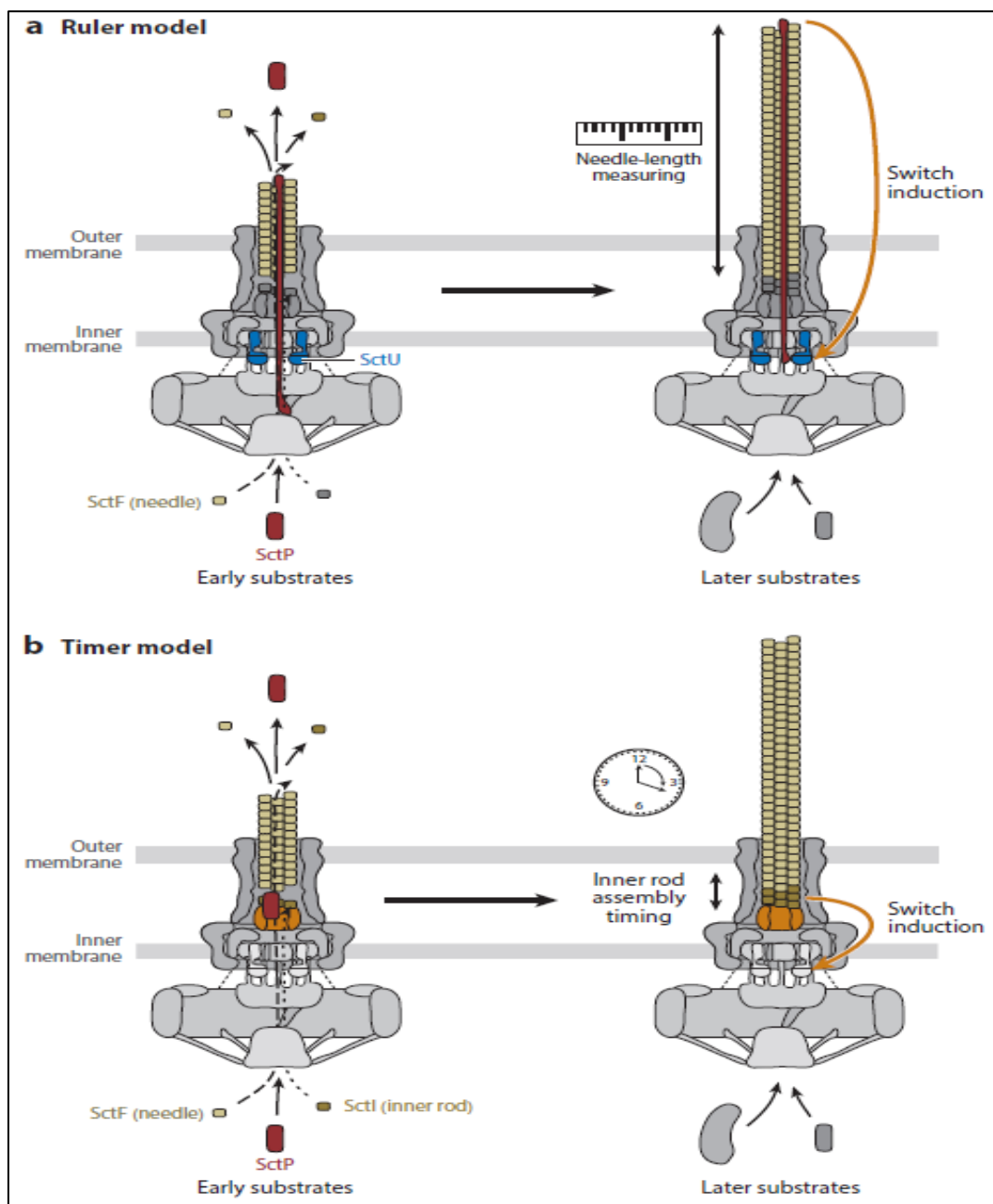


Figure 17: Proposed ruler (a) and timer (b) models for the mechanism of substrate switching and needle-length control in the needle assembly (Galán et al., 2014).

Although two models are proposed, their mechanisms are not conflicting. In fact, one is based on the size of the ruler protein while the other is based on the interplay between needle and inner rod assemblies which are also regulated by this ruler. It is clear that two mechanisms are observed in different bacteria but they can be reconciled by considering the ruler SctP having two distinct roles. Finally, the switching from early substrates to middle substrates is proposed as the main mechanism that stops the needle polymerization for both models. However, this hypothesis seems incompatible with another study showing that a mutant preventing auto-cleavage of SctU is able to control needle length but unable to secrete middle substrates (Monjarás Feria et al., 2015). Thus, the exact mechanism remains unclear and might not depend only on SctU and SctP. Actually, the C-ring and the ATPase complex were described as a sorting platform for the substrate secretion of T3SS (Lara-Tejero et al., 2011). Therefore, another mechanism involved in the control of needle length as well as substrates switching, may still remain to be elucidated.

d. Translocators

The translocators include three proteins located at the extracellular end of the needle complex: SctA, SctB and SctE. In some pathogenic *E. coli* strains, SctA polymerizes in an extended form called filament that is longer than the needle itself but whose function is unknown. In contrast, in most bacteria, this protein forms oligomers (Gébus et al., 2008) and builds the needle tip in complex with SctE (four SctA and one SctE) (Cheung et al., 2015). SctA has a conserved coiled-coil central domain responsible for its interaction with the needle (Lunelli et al., 2011; Rathinavelan et al., 2011). An N-terminal α -helical domain present in the SctA of *Salmonella* and *Shigella* is proposed to prevent the self-oligomerization inside bacteria (Johnson et al., 2007). Meanwhile, SctA of *Pseudomonas* and *Yersinia* (PcrV/LcrV) lack this domain (Chaudhury et al., 2013) but their cognate chaperon (PcrG/LcrG) are reported to have this function in the two species (Allmond et al., 2003; DeBord et al., 2001; Nanao et al., 2003). Located at the extremity of the needle, SctA has a role of sensor of host-cell contact and of assembly platform to assist the correct insertion of the translocation pore (translocon) into the host membrane (Goure et al., 2004; Roehrich et al., 2013). In absence of SctA, both translocons and effectors are secreted into the extracellular medium and no exotoxin is delivered into the host cell cytosol (DeBord et al., 2001; Sawa et al., 1999; Sundin et al., 2004). Besides the structural function, SctA is involved in the regulation of secretion process but this function, which is shown only for PcrV/LcrV of *Pseudomonas* and *Yersinia*, may be linked to their chaperon PcrG/LcrG. In fact, LcrG was shown to be a negative regulator that blocks the secretion of middle and late substrates in *Yersinia*. In contrast, LcrV is described as a positive regulator because it binds to LcrG and thus inhibits LcrG activity (Matson and Nilles, 2001; Nilles et al., 1997). In *Pseudomonas*, the role of PcrV/PcrG is less clear than its homologous protein in *Yersinia*. Nonetheless,

both PcrV and PcrG are suggested to be involved in the negative regulation of expression and secretion of exotoxins (Sundin et al., 2004). Recently, PcrG was shown to interact with distinct components of the T3SS apparatus to control the specificity secretion of effectors and translocators (Lee et al., 2014).

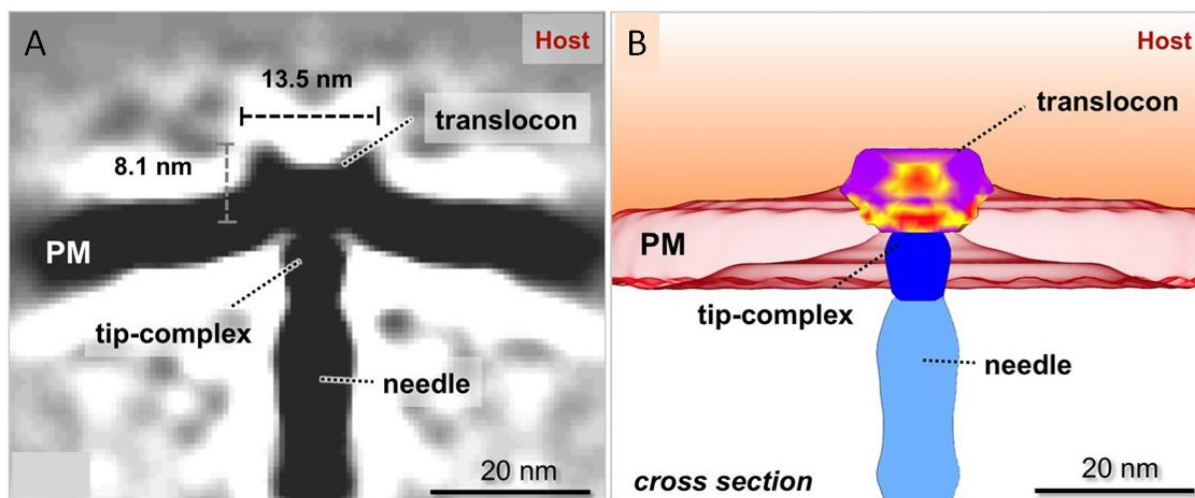


Figure 18: In situ structure analysis of the intact translocator in the membrane of a cell challenged with *Salmonella*. (A) Central section of sub-tomogram averages of the needle, the translocators and the eukaryotic plasma membrane (PM). (B) Surface rendering of the translocon and tip-complex in A. Adapted from (Park et al., 2018)

SctB and SctE are the translocon proteins located in the eukaryotic membrane. Each of these proteins is able to form pores in liposome but can induce vesicle permeabilization in synergy (Faudry et al., 2006) and together, they assemble into a hetero-oligomeric complex which is inserted into host cell membranes with support from tip proteins (Romano et al., 2016). One half of the translocon is embedded in the host membrane while the other half is protruding in the host cytoplasm (Figure 18) (Park et al., 2018). Inside bacteria, SctB and SctE interact with their cognate chaperone responsible for their stability and prevention of self-oligomerization (Discola et al., 2014; Faudry et al., 2007). Unfortunately, unlike the hydrophilic tip complex SctA, the uncovering of the exact structure of the pore is still challenging because the hydrophobic structure makes it unstable *in vitro*, leading to rapid aggregation or degradation. However, SctB and SctE are predicted to harbor one and two transmembrane helices respectively, hence SctB can be called the one-transmembrane domain (1TD) translocator and SctE the two-transmembrane domain (2TD) translocator. In addition, both 1TD and 2TD translocators are predicted to have a coiled-coil structure in the N-terminus and an amphipathic helix in the C-terminus (Figure 19) (Matteï et al., 2011).

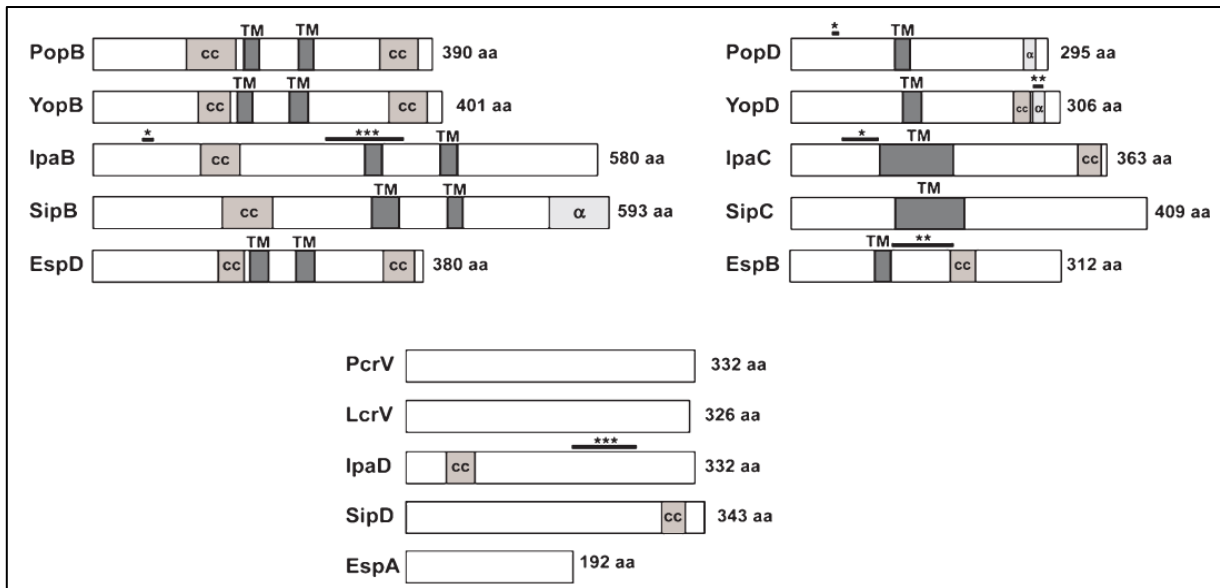


Figure 19: Diagrammatic analysis of translocators of Ysc (*Pseudomonas* and *Yersinia*), Ssa-Esc (*Salmonella* SP2 and *E. coli*) and Inv-Mxi-Spa (*Shigella* and *Salmonella* SP1) systems. TM: Predicted transmembrane region; CC: predicted coiled coil; *: chaperone binding domain; **: predicted interaction domain with the hydrophilic partner SctA; *: predicted interaction domain with the hydrophobic partner; α : predicted amphipathic helix (Mattei et al., 2011).**

The structure of the N-terminus of the 2TD translocator was recently obtained by x-ray crystallography (for IpaB and SipB of *Shigella* and *Salmonella*) and was shown to have a trimeric coiled-coil motif. This coiled-coil motif displayed a similarity with other gram-negative bacteria pore-forming protein such as colicins, thus indicating that this motif has an important role in the assembly of the translocon (Barta et al., 2012). Actually, the N-terminal domain of SctE is essential for its oligomerization (Hume et al., 2003) and its recognition by the chaperone inside bacteria (Discola et al., 2014; Lunelli et al., 2009). Otherwise, two predicted transmembrane helices were identified by evaluating the sequence conservation of SctE among bacteria (Hume et al., 2003; Mattei et al., 2011). The existence of these transmembrane helices was then confirmed by the fact that they are protected from proteolysis by the membrane lipids (Hume et al., 2003). In addition, the insertion of SctE into artificial membranes is very stable and once liposome-associated, this protein cannot be extracted by using additives that usually solubilize the superficially-associated proteins (Discola et al., 2014; Hume et al., 2003). Finally, the C-terminal domain of SctE binds to the T3SS needle tip SctA and is required for the recruitment of the 1TD translocator SctB (Roehrich et al., 2010). This result suggests that SctE is probably secreted before SctB and regulates the secretion of SctB. This hypothesis is supported by the fact that the 2TD translocator could be inserted into membranes in absence of the 1TD translocator. Nonetheless, the pore is not functional and the effector are not translocated in a $\Delta sctB$ mutant (Blocker et al., 1999; Goure et al., 2004).

On the other hand, no structure of the 1TD translocator SctB is available. The N-terminus of SctB is predicted to present a coiled-coil structure and was shown to be involved in the interaction with the

cytoplasmic chaperone (Faudry et al., 2007; Job et al., 2010; Schreiner and Niemann, 2012). SctB contains only one transmembrane helix and unlike the 2TD translocator, the 1TD translocator could be detached from liposome, suggesting that this protein superficially binds to bilayer lipid membrane (Discola et al., 2014). Nonetheless, this protein could not be extracted once associated by wild-type bacteria into the real cell membrane as described in the case of red blood cells (Goure et al., 2004). Like SctE, the C-terminus domain of SctB is reported to interact with SctA. However, it is also involved in the oligomerization of the 1TD translocator meanwhile this function is carried out by the N-terminal domain in the case of the 2TD translocator (Costa et al., 2010; Hume et al., 2003).

Besides the structural function of pore formation in the T3SS assembly and effector funneling, the translocators SctE and SctB are also described as a pore-forming toxin because the purified proteins are able to induce membrane leakage (Faudry et al., 2006; Wager et al., 2013). Actually, the formation of the translocon pore leads to K^+ efflux which in turn cause histone dephosphorylation and disruption of the mitochondria network (Dortet et al., 2018). Consequently, the translocon proteins are suggested to possess a pore-forming activity upon secretion and insertion by the T3SS.

3.5.2 Exotoxins

P. aeruginosa is described to secrete seven effectors into host cell cytosol but only four of them are characterized and are shown to have a severe effect on eukaryotic cells. These four effectors are exoenzyme S (ExoS), exoenzyme T (ExoT), exoenzyme Y (ExoY) and exoenzyme U (ExoU), and they are also known by the name exotoxin. Among them, ExoT and ExoY are present in most *P. aeruginosa* strains while ExoS and ExoU are normally mutually exclusive and very scarcely found within one same bacteria. Therefore, T3SS expressing *P. aeruginosa* can be divided into two groups possessing either ExoS or ExoU.

a. ExoS and ExoT

ExoS and ExoT consist of 453 and 457 residues respectively and share 76% of sequence identity. In the N-terminus, both exotoxins possess a secretion signal (S), a chaperone binding domain (CBD) and a membrane localization domain (MLD) (Figure 20) (Hauser, 2009). The 15 first residues of ExoS and ExoT are responsible for the secretion of these toxin because in absence of these extremity residues, no secreted exotoxins are observed (Yahr et al., 1996a). The CBD of these proteins binds to a common chaperone SpcS (specific Pseudomonas chaperone for ExoS) which is necessary for the optimal secretion of ExoS and ExoT (Shen et al., 2008). The MLD is involved in the targeting of these exotoxins to the plasma membrane of host cell, which is important for their efficient activity. Actually, many substrates of ExoS and ExoT are located at the cell membrane and some mutations in the MLD do not alter the enzymatic activity *in vitro* but abolish *in vivo* effects (Riese and Barbieri, 2002; Zhang et al., 2007).

Functionally, ExoS and ExoT are bifunctional proteins that contain an N-terminal GTPase activating protein (GAP) domain and a C-terminal ADP-ribosyltransferase (ADPRT) domain (Figure 20). The GAP activity of ExoS and ExoT seems to act on the same targets such as Rho, Rac and Cdc42 which are eukaryotic GTPases regulating the organization of the actin cytoskeleton. As a result, once injected into host cells, the GAP activity induces a disruption of cell cytoskeleton that leads to cell rounding (Goehring et al., 1999; Kazmierczak and Engel, 2002; Krall et al., 2000). In addition, the GTPase Rac1 and Cdc42 are required for the induction of phagocytosis and thus, the GAP activity of both proteins was suggested to have an anti-phagocytic function (Lee et al., 2000).

On the other hand, the ADPRT domain of these exoenzymes binds to a common eukaryotic protein acting as a cofactor for their catalytic activity. This protein was first identified as factor associating ExoS (FAS) but is now known as the 14-3-3 protein which is needed for both ExoS and ExoT (Hauser, 2009). Nonetheless, the differences between ExoS and ExoT regard two points. Firstly, the catalytic activity of ExoS is 500 fold higher than the one of ExoT *in vitro* (Liu et al., 1997; Yahr et al., 1996b) and secondly, ExoS and ExoT ADPRT activities are directed toward different targets. In fact, ExoS has a broad substrate

specificity on host proteins and thus this toxin triggers several effects on the host cell such as cell death, cell rounding and DNA synthesis inhibition. The two famous substrate families of ExoS are the GTPases Ras as well as the Ras-like proteins such as RalA, Rabs, and Rac1 (Coburn and Gill, 1991; Fraylick et al., 2002), and the ERM protein family including ezrin, radixin and moesin. The Ras GTPases are shown to be involved in cell proliferation while the ERM proteins participate to the actin processing linked to cell shape, mobility and adhesion (Hauser, 2009; Maresso et al., 2004).

In contrast, ExoT displays activity toward only three substrates Crk1, Crk2 (CT10-regulator of kinase) and phosphoglycerate kinase. Among them, the effect of ExoT on Crk proteins was well characterized (Sun and Barbieri, 2003). Actually the Src Homology 2 and 3 domains (SH2 and SH3) of Crk proteins play an essential role in the integrin-mediated phagocytosis and focal adhesion (Feller, 2001). Thus, the ADPRT activity of ExoT is suggested to be endowed with an anti-phagocytosis function that is independent of that of the GAP domain because they act on distinct substrates. Otherwise, the ADP ribosylation of Crk protein affects the integrin signaling pathway by direct inhibition of Crk binding to focal adhesion protein such as p130cas or paxillin (Deng et al., 2005). Moreover, the ADP ribosylated Crks were cytotoxic, thus inducing the cell death anoikis apoptosis which is a programmed cell death as a consequence of loss of cell adhesion (Wood et al., 2015).

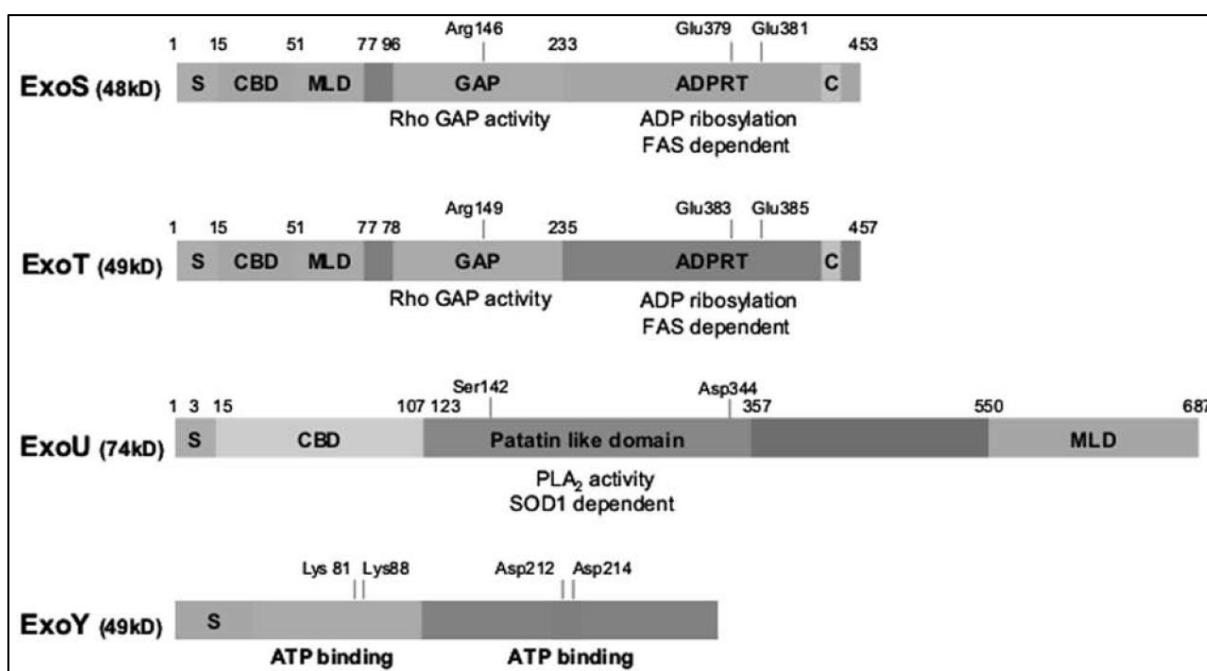


Figure 20: Diagrammatic analysis of *P. aeruginosa* exotoxins. S: Signal peptide; CBD: Chaperone binding domain; MLD: Membrane localization domain; C: C-terminal domain; GAP: GTPase activating protein domain; ADPRT: ADP-ribosyltransferase domain; PLA₂: Phospholipase A₂; SOD1: superoxide dismutase 1. (Galle et al., 2012)

b. ExoY

ExoY is an exotoxin of 387 residues. By analogy with other T3SS substrates, the N-terminus of this toxin is predicted to possess a secretion signal domain and a chaperone binding domain but to date, no chaperone is identified for the exotoxin ExoY (Belyy et al., 2018; Maresso et al., 2006).

The activity of ExoY was initially described as an adenylyl cyclase with two distinct domains that act together for the ATP binding and cyclase activity (Figure 20) (Galle et al., 2012). These catalytic regions shows homology with two adenylate cyclase toxins produced by *Bordetella pertussis* (CyaA) and *Bacillus anthracis* (edema factor EF) (Yahr et al., 1998). The family of bacterial cyclase toxin is characterized by a low activity in the prokaryotic environment to avoid its harmful effect inside bacteria while it becomes active after delivery to the eukaryotic cell through interaction with a host protein acting as a cofactor (Belyy et al., 2018; Yahr et al., 1998). However, unlike CyaA and EF which are activated by eukaryotic calcium-mediator calmodulin, ExoY has been recently shown to bind to filamentous actin (F-actin). Actually, upon association with F-actin, the catalytic activity of ExoY increases 10000 fold *in vitro* and the binding of ExoY to F-actin interfered with the interaction of this filament to other F-actin binding proteins such as Arp2/3, thus inhibiting the F-actin turnover (Belyy et al., 2016). Interestingly, the inhibition of F-actin disassembly resulted from its interaction with ExoY and not from the catalytic activity of this exoenzyme. In fact, no difference on F-actin remodeling was observed upon comparing ExoY harboring a mutation of an essential residue for catalytic activity (K81M) with the wild-type ExoY (Cowell et al., 2005).

On the other hand, the catalytic activity of ExoY is involved in the cyclisation of not only ATP but also other NTP with preference for GMP and UMP (Beckert et al., 2014). This exoenzyme catalyzes the conversion of triphosphate nucleotides into cyclic monophosphate nucleotides (cNMP) interfering with different signaling pathways of host cells (Bähre et al., 2015; Hartwig et al., 2014). Actually, ExoY displays a broad target spectrum depending on eukaryotic cell types and the localization of cNMP in the cell. For example, in epithelial cells, the catalytic activity of ExoY does not contribute to the cytotoxicity but is required for the bleb-niche membrane formation while the epithelial cell rounding is the consequence of actin disruption that results from the binding of this toxin to F-actin (Cowell et al., 2005; Hritonenko et al., 2011). In contrast, in endothelial cells, besides the disruption of F-actin by ExoY binding, the production of cAMP and cGMP activates protein kinase A which in turn phosphorylates the microtubule associated protein Tau. The hyper-phosphorylation of this protein leads to disruption of the microtubule dynamic, resulting in cell rounding (Morrow et al., 2017; Ochoa et al., 2012). In addition, ExoY was recently shown to inhibit the host immune response and this function requires the nucleotidyl cyclase activity (He et al., 2017; Jeon et al., 2017).

c. ExoU

ExoU is the most toxic exoenzyme secreted by *P. aeruginosa* T3SS and possesses a phospholipase A2 (PLA2) activity. Like other exoenzymes, ExoU remains inactive *in vitro* unless an extract from eukaryotic cell is added to the reaction, meaning that the catalytic activity of ExoU requires host cofactors (Sato and Frank, 2004). Indeed, the eukaryotic Cu²⁺, Zn²⁺-superoxide dismutase 1 (SOD1) was identified as the first cofactor of ExoU and this activation did not depend on the enzymatic activity of SOD1 (Sato et al., 2006). Then, the activation mechanism was further determined to be dependent on the ubiquitination of the carboxyl terminal domain of ExoU and SOD1 appeared to be one of the possible ubiquitin donors. In other words, ubiquitylated SOD1 or other ubiquitylated mammalian proteins are necessary for the activation of ExoU (Anderson et al., 2011; Stirling et al., 2006). Later, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) localized in the eukaryotic plasma membrane was reported as a second ExoU cofactor besides ubiquitin and ubiquitinated proteins (Tyson and Hauser, 2013). Actually, the significant conformation change in the C-terminal domain of ExoU in presence of ubiquitin and liposomes combination is different from that occurring in the presence of either ubiquitin or liposome alone. Therefore, the PI(4,5)P2 in the lipid bilayer was suggested to synergistically act with ubiquitin to modify the conformation of the C-terminus and enhance the PLA2 activity of ExoU (Tessmer et al., 2017; Tyson and Hauser, 2013).

Structurally, like other exoenzyme, the 15 first residues at the N-terminus of ExoU are responsible for the secretion signal and are followed by the CBD (Figure 20). The CBD interacts with the identified cytoplasmic chaperone SpcU that is required for the efficient secretion of ExoU (Finck-Barbançon et al., 1998). Otherwise, unlike ExoS and ExoT, the MLD of ExoU is located in the C-terminus and is important for the targeting of ExoU to eukaryotic membrane, where this exoenzyme interacts with host factors for its activation (Rabin et al., 2006). ExoU possesses a patatin-like domain responsible for the PLA2 activity. In fact, the patatin is a major storage protein of potatoes that has PLA2 activity and this protein family is characterized by a serine-aspartate active site dyad (Hirschberg et al., 2001). The structure of ExoU obtained by X-ray crystallography allowed prediction that the Ser142 and Asp344, which form the catalytic PLA dyad, are separated away from each other and are not accessible to the substrate in the non-activated ExoU or when it binds to the chaperone SpcU (Gendrin et al., 2012; Halavaty et al., 2012). In contrast, upon activation by ubiquitin and membrane association, the C-terminal domain of ExoU changes its conformation and the Ser-Asp dyad in the enzymatic center is suggested to move closer to each other, thus displaying full PLA activity (Sawa et al., 2016). This mechanism is supported by analyzing the structure of the human cytosolic phospholipase A2 (homologous to ExoU) obtained by X-ray

crystallography. In the active form, the distance between Serine and Aspartate of the dyad is only 2.9 Å (Dessen et al., 1999), close enough for the PLA activity.

Injection of ExoU by *P. aeruginosa* T3SS causes a rapid cell death in many types of cells including epithelium, endothelium, macrophages and neutrophils (Freitas et al., 2012; Gellatly and Hancock, 2013; McMorran et al., 2003; Rabin and Hauser, 2003). Once injected into the host cell cytosol, ExoU targets membrane-associated phospholipids, thus leading to the plasma membrane disruption and causing cell necrosis. In addition to the direct effect on cell cytotoxicity, ExoU is also involved in the inflammation because a high amount of proinflammatory cytokines is released in the bloodstream as the result of the host cell lysis (Engel and Balachandran, 2009). Therefore, the extensive tissue destruction linked to PLA2 activity of ExoU as well as the response of host inflammation can explain the hyper-virulence of *P. aeruginosa* ExoU positive strains in acute infection.

d. Besides the four exoenzymes

P. aeruginosa T3SS is reported to handle three other effectors: NDK, PemA and PemB. The nucleoside diphosphate kinase (NDK) is secreted by T1SS into extracellular medium as described above. However, this enzyme could also be translocated into eukaryotic cells by the T3SS and this secretion requires a conserved motif in the C-terminus of NDK as for its secretion by the T1SS. This intracellular NDK (iNDK) (in contrast to extracellular NDK secreted by T1SS) is toxic for eukaryotic cell and unlike extracellular NDK, the kinase activity of iNDK is not required for cytotoxicity (Neeld et al., 2014). On the other hand, PemA and PemB (*Pseudomonas* effectors discovered by machine learning) have been recently shown to be injected into host cells by T3SS but they do not display cytotoxic effects. Thus, these effectors were proposed to interfere with the host innate immunity or to regulate specific host pathways for benefits of the bacteria but their exact roles need to be elucidated (Burstein et al., 2015).

e. A summary of exoproducts secreted by *P. aeruginosa* secretion systems.

P. aeruginosa produces and secretes more than 50 effectors through different secretion systems including the one-step secretion systems (Type I, II and V) and the two-steps secretion systems (Type III and Type VI). Considering data from literature, I summarized information related to these proteins including their activity, their mode of action and function (Table 3). They can be classified into four groups based on their targets as well as their catalytic activities. The first one contains non-catalytic effectors whose functions mainly rely on binding to targets, for example: chitin-binding, heme-binding and phosphate-binding. Otherwise, the three other groups are catalytic effectors whose substrates are host proteins (protease, kinase, phosphotransferase), host lipids (phospholipase, lipase) and a host carbohydrate (Muramidase). Most of these effectors secreted by all the secretion systems act on eukaryotic cells, some of those secreted by the T5SS and T6SS act on prokaryotic cells and three effectors secreted by the T1SS and T2SS are involved in optimal growth of bacteria in specific conditions by favoring the uptake of specific substrates.

Regarding the eukaryotic hosts, although the effector proteins have different activities and mechanisms, they usually interfere with the target-cell signaling and help *P. aeruginosa* to lower host immune response, or on the contrary to activate inflammation, to promote the invasion and degradation of host tissues. Regarding the prokaryotic hosts, *P. aeruginosa* inhibits the growth of competing bacteria by injecting into the periplasm the effector toxins that degrade membrane lipids and peptidoglycans or by secreting into the extracellular medium the effectors of the contact-depend growth inhibition (CDI) system. This CDI inhibitory systems require direct cell-to-cell contact between competing bacteria and the extracellular effectors usually bind to host receptors, thus causing several effects on bacterial growth or survival (Ruhe et al., 2013).

Secretion systems		Effectors	Activities	Modes of action and functions	Refs
T1SS	T1SS Apr	AprA	Alkaline protease	Degradation of human complement and escape from phagocytosis	(Guzzo et al., 1991a, 1991b; Laarman et al., 2012)
		AprX	Unknown	Unknown	(Duong et al., 2001)
		Extracellular NDK	Nucleoside diphosphatate kinase	Control of extracellular ATP and induction of macrophage cell death	(Ghigo and Wandersman, 1994; Guzzo et al., 1991b; Sundin et al., 1996; Zaborina et al., 1999)
	T1SS Has	HasAp	Heme-binding	Iron uptake into bacteria	(Létoffé et al., 1998, 2001; Wandersman and Delepelaire, 2004)
T2SS	T2SS Xcp	LasA	Elastase	<ul style="list-style-type: none"> Degradation of elastin and activation of the elastase LasB Degradation of the peptidoglycan of <i>Staphylococcus aureus</i> 	(Braun et al., 1998; Kessler et al., 1993, 1997)
		LasB	Elastase	<ul style="list-style-type: none"> Degradation of diverse substrate of host such as elastin, collagen, fibronectin, VE-cadherin as well as the bacterial flagellin Inactivation of plasma complement and immune system 	(Beaufort et al., 2011; Casilag et al., 2016; Golovkine et al., 2014; Heck et al., 1986; Hong and Ghebrehiwet, 1992; Kamath et al., 1998; Kon et al., 1999; Kuang et al., 2011; Parmely et al., 1990; Schultz and Miller, 1974; Wretlind and Wadström, 1977)
		PlcH	Hemolytic Phospholipase C	Degradation of phosphatidyl- choline (PC) and sphingomyelin (SM) which are common phospholipids in eukaryotic cells, thus leading to cell cytotoxicity and tissues damage.	(Luberto et al., 2003; Stonehouse et al., 2002; Vasil, 2006; Wargo et al., 2011)
		PlcN	Non-hemolytic Phospholipase C	<ul style="list-style-type: none"> Degradation of phosphatidylserine (PS) in eukaryotic cells, less active than PlcH Effect on biofilm formation like PlcB but through an unknown mechanism 	(Lewenza et al., 2017; Ostroff et al., 1990; Vasil, 2006)
		PlcB	Phospholipase C	Degradation of PC in eukaryotic cell and the specific phosphatidylethanolamine (PE) of prokaryotic cells, thus affecting the	(Barker et al., 2004; Lewenza et al., 2017)

			accumulation of biofilm biomass and aggregation.	
		CbpD	Chitin-binding	Unknown but suggested to be involved in adhesion and in mediating colonization of eukaryotic cells surface (Folders et al., 2000)
		ToxA	Mono ADP-ribosyl transferase	Inhibition of protein synthesis by affecting EF-2 at host ribosome, leading to cell death apoptosis (Allured et al., 1986; Chang and Kwon, 2007; Du et al., 2010; Foley et al., 1995; Iglewski et al., 1977; Jenkins et al., 2004; Nygård and Nilsson, 1990)
		PmpA (IMPa)	Metalloprotease	Binding to zinc and displaying protease activity on surface receptors of immune cells such as neutrophil, lymphocyte, monocyte, thus protecting <i>P. aeruginosa</i> against the immune system attacks (Bardoel et al., 2012)
		PrpL	Lysine specific endopeptidase (Protease IV)	Degradation of immunoglobulin G, fibrinogen, plasminogen, complement components and surfactant proteins A, B, D, thus inhibiting host immune response and physical barrier defense. (Engel et al., 1998; Fox et al., 2008; Malloy et al., 2005)
		LipA	Lipase	Control of pyoverdinin production and expression of the sigma factor PvdS through unknown mechanism (Funken et al., 2011)
		LipC	Lipase	Effect on bacterial motility, rhamnolipid production and biofilm formation, thus being suggested to target bacterial lipid membrane (Martínez et al., 1999; Rosenau et al., 2010)
		LoxA	Lipoxygenase	Peroxidation of a broad range of fatty acids and phospholipids in the host membrane, thus leading to many effects such as hemolysis and inflammatory response. (Banthiya et al., 2015, 2016; Deschamps et al., 2016; Vance et al., 2004)
		PhoA	Alkaline phosphatase	Unknown (Filloux et al., 1988)
		PaAP	Amino peptidase	Release of free amino acids or small peptides from protein fragments produced by other proteases, thus providing nutrients that can be uptaken by bacteria (Cahan et al., 2001; Galdino et al., 2017; Kessler and Safrin, 2014)

		GlpQ	Putative glycerophosphoryl diester phosphodiesterase	Hydrolysis of deacylated phospholipids into glycerol 3-phosphate (G3P) with unknown function.	(Voulhoux et al., 2001)
		EddA	Phosphodiesterase and alkaline phosphatase D	Degradation of extracellular DNA as a nutrient source for bacteria growth.	(Ball et al., 2016; Lewenza, 2013)
	T2SS Hxc	LapA	Alkaline phosphatase	Unknown	(Ball et al., 2002)
		LapB	Alkaline phosphatase	Unknown	(Ball et al., 2002)
		LapC	Phosphate binding	Unknown	(Ball et al., 2012)
	T2SS Txc	CbpE	Chitin-binding	Unknown	(Cadoret et al., 2014)
	T3SS		ExoS	GTPase activating and ADP-ribosyltransferase	<ul style="list-style-type: none"> • Subversion of eukaryotic GTPases which are involved in the organization of the actin cytoskeleton. • Ribosylation of diverse eukaryotic proteins causing various effects on cell such as cell proliferation inhibition, cell rounding, cell death, DNA synthesis inhibition.
		ExoT	GTPase activating and ADP-ribosyltransferase	<ul style="list-style-type: none"> • Subversion of eukaryotic GTPases which are involved in the organization of the actin cytoskeleton. • Ribosylation of Crk protein kinase family causing loss of cell adhesion and leading to anoikis apoptosis cell death. 	(Barbieri and Sun, 2004; Kazmierczak and Engel, 2002; Sun and Barbieri, 2003)
		ExoY	Nucleotidyl cyclase	<ul style="list-style-type: none"> • Interaction with F-actin resulting in cell rounding. • Production of cyclic NMP involved in diverse signaling pathway. 	(Yahr et al., 1998)
		ExoU	Phospholipase A2	Rapid degradation of phospholipids in eukaryotic cell membrane, leading to membrane rupture and cell death	(Sato and Frank, 2004)
		PemA	Unknown	Unknown, no cytotoxicity	(Burststein et al., 2015)
		PemB	Unknown	Unknown, no cytotoxicity	(Burststein et al., 2015)
		Intracellular NDK	Nucleoside diphosphatase kinase	Unknown, cytotoxicity	(Neeld et al., 2014)

T5SS	T5SS Auto- transporter	EstA	Esterase	Production of rhamnolipids and effect on bacteria motility	(Wilhelm et al., 2007)	
		EprS	Serine protease	Activation of NF- κ B through protease-activated receptor PAR-1,-2,-4, thus inducing inflammatory response	(Kida et al., 2013)	
		AaaA	Aminopeptidase	Release of free amino terminal arginine from peptide as a source for bacterial growth and virulence	(Luckett et al., 2012)	
		PlpD	Lipase	Degradation of phosphatidylinositols and other phosphatidyl analogs but not phosphatidylethanolamine in bacterial membrane, thus being suggested to target eukaryotic cell membrane.	(da Mata Madeira et al., 2016; Salacha et al., 2010)	
	T5SS Two Partner Secretion	LepA/B	Protease	Digestion of human receptor PAR-1,2,4 and induction of inflammation	(Kida et al., 2008)	
		CdrA/B	Sugar binding	Interaction with the exopolysaccharide Psl and increase of the biofilm structure stability	(Borlee et al., 2010)	
		PdtA/B	Unknown	Unknown but important for bacterial virulence	(Faure et al., 2014)	
		CdiA/B PA0040/PA 0041	Host receptor binding	Contact-depend growth inhibition (CDI) of other bacteria Involved in bacterial adhesion and biofilm formation	(Mercy et al., 2016; Willett et al., 2015)	
		CdiA/B PA2462/PA 2463	Host receptor binding	Contact-depend growth inhibition (CDI) of other bacteria Involved in bacterial adhesion and biofilm formation	(Mercy et al., 2016; Willett et al., 2015)	
		CupB5/Cup B3	Hemagglutinin- like	Formation of fimbriae at bacterial cell surface responsible for the adhesion of bacteria on diverse surfaces.	(Ruer et al., 2008)	
		ExlA/B	Pore forming	Making pore in eukaryotic cell membrane and leading to cell death.	(Basso et al., 2017; Elsen et al., 2014; Reboud et al., 2017)	
	T6SS	H1-T6SS	Tse1	Amidase	Degradation of the peptidoglycans of other bacteria by cleaving the peptide bond.	(Russell et al., 2011)
			Tse2	Putative phosphotransferase	Unknown but involved in the inhibition of growth of other bacteria.	(Hood et al., 2010; Li et al., 2012; Robb et al., 2016)
Tse3			Muramidase	Degradation of the peptidoglycans of other bacterial by cleaving the glycan backbone.	(Russell et al., 2011)	

		Tse4	Pore forming	Formation of pores that disrupt the $\Delta\Psi$ which is one component of the proton motive force, thus inhibiting the bacterial growth when injected in the periplasm.	(LaCourse et al., 2018; Whitney et al., 2014)
		Tse5	Unknown	Unknown mechanism, suggested to be involved in CDI.	(Hachani et al., 2014; Whitney et al., 2014)
		Tse6	Putative mono-ADP-Ribosyltransferase	Degradation of dinucleotides NAD(+) and dinucleotides phosphate NADP(+) of other bacteria, thus displaying an effect.	(Whitney et al., 2015)
		Tse7	Nuclease	Acting on bacterial growth inhibition through ultimate degradation of DNA.	(Hachani et al., 2014; Pissaridou et al., 2018)
	H2-T6SS	PldA	Phospholipase D	<ul style="list-style-type: none"> Degradation of phosphatidyl-ethanolamine in the membrane of other bacteria. Favoring bacteria internalization into eukaryotic cells. 	(Jiang et al., 2014; Russell et al., 2013)
		VgrG2b	Microtubule binding	Interaction with eukaryotic cells γ -Tubulin ring complex (γ -TuRC), thus affecting the organization of cell cytoskeleton and favoring bacteria internalization.	(Sana et al., 2015)
		TseE (Tle4)	Phospholipase A1	<ul style="list-style-type: none"> Degradation of phosphatidyl-ethanolamine in other bacteria membrane. Activation of autophagy through its PGAP1-like domain when translocated into eukaryotic cells. 	(Jiang et al., 2016)
		TseT	TOX-REase-5	Antibacterial polymorphic toxin with unknown mechanism	(Burkinshaw et al., 2018)
	H3-T6SS	PldB	Phospholipase D	<ul style="list-style-type: none"> Inhibition of other bacteria growth through an unknown mechanism Favoring bacteria internalization into eukaryotic cells. 	(Jiang et al., 2014; Russell et al., 2013)
		TseF	Metallophore	Iron uptake into bacteria through interaction with outer membrane vesicles and the PQS system	(Lin et al., 2017)

Table 3: Summary of secretion systems and secreted proteins in *P. aeruginosa*

3.5.3 T3SS Regulation

In *P. aeruginosa*, 43 genes encoding the T3SS machinery, regulators, effectors and chaperones are located in the bacterial chromosome. Most of them are clustered in five different operons except for effectors and effector specific-chaperones (Figure 21). All T3SS genes including those of effectors are under the control of the central regulator ExsA, a protein belonging to the AraC family of transcription factors. Actually, these AraC-family proteins regulate the expression of a variety of genes that are involved in three main functions: carbon mechanism, stress response and pathogenicity (Gallegos et al., 1997). They are characterized by the presence of approximately 100 conserved residues in the C-terminal domain (CTD) that form a helix-turn-helix structure responsible for DNA binding, a variable N-terminal domain (NTD) which is involved in its dimerization and interaction with the ligands, and a

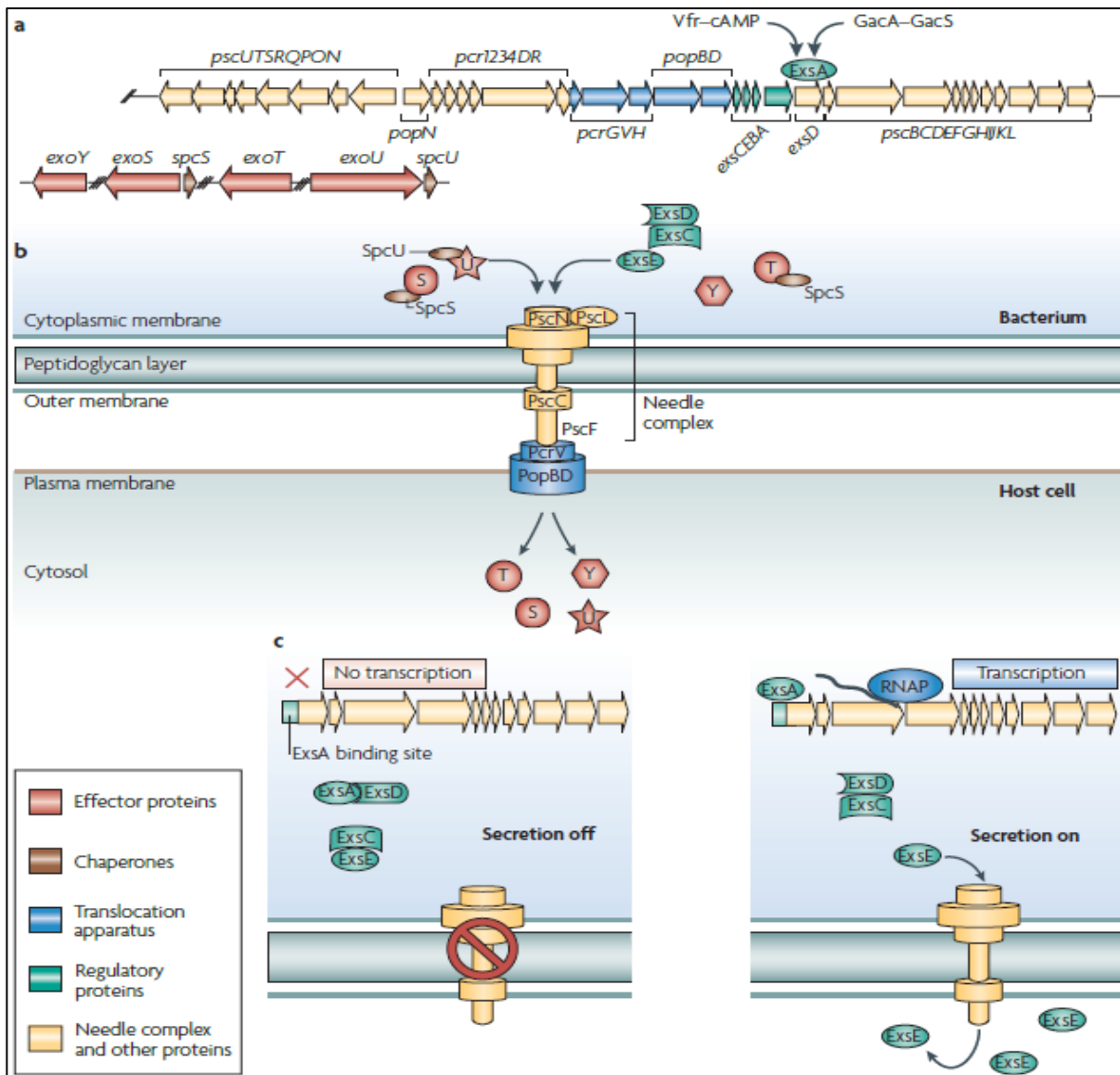


Figure 21: An overview of T3SS gene organization (a) and mechanism of T3SS genetic regulation (b) (Hauser, 2009).

flexible linker that connects the C-terminal and N-terminal parts (Gallegos et al., 1997). In *P. aeruginosa*, the ExsA protein consists of 100 amino acids in CTD, 170 amino acids in NTD and has a crucial role in the transcription activation of the T3SS. The ExsA-CTD binds to T3SS gene promoters while the ExsA-NTD is essential for its dimerization (Marsden et al., 2014) and its interaction with ExsD (Brutinel et al., 2009; Thibault et al., 2009), an anti-activator that inhibits ExsA binding to DNA in non-inducing condition (Brutinel et al., 2010). Besides ExsA and ExsD, two other proteins participate to the regulation cascade connecting secretion and gene synthesis: ExsC which is an anti-anti-activator and ExsE, the negative regulator. ExsC and ExsE are bound together in non-inducing condition. In fact, upon induction by host cell contact or calcium depletion, ExsE is secreted into the extracellular medium by the T3SS, ExsC binds to ExsD which thus releases ExsA in a free form that binds to T3SS promoters and activates gene expression (Figure 21C) (Rietsch et al., 2005). Interestingly, ExsA auto-regulates its own expression as well as that of ExsE and ExsC through the *pexsC* promoter. In addition to ExsA activation through secretion, the T3SS protein expression can be regulated mainly by three global pathways: CyaA/B-cAMP/Vfr, GacSA-RsmY-RsmZ-RsmA and PsrA-RpoS. Moreover, the T3SS gene expression is reported to be regulated by many other pathways whose mechanisms can be directly or indirectly linked to the central regulator ExsA.

The GacSA-RsmYZ-RsmA regulatory pathway is described in the chapter 1.1 as the two components regulator involved in the formation of biofilm and the pathogenicity of *P. aeruginosa*. Actually, the final product of this pathway, RsmA is a positive regulator of *exsA* expression (Mulcahy et al., 2006).

The cyclic AMP (cAMP)-Vfr system (CVS) is a global pathway that regulates the transcription of virulence genes including quorum sensing, Type IV pili, T2SS and T3SS. The CVS pathway includes two adenylate cyclase (CyaA and CyaB), a cAMP phosphodiesterase (CpdA) and the virulence factor regulator (Vfr) transcription factor. Actually, the intracellular cAMP is generated by CyaA and CyaB in response of both high osmolarity (NaCl) and low Ca^{2+} conditions while the cAMP homeostasis is controlled by CpdA (Fuchs et al., 2010a; Wolfgang et al., 2003). The increase of intracellular cAMP activates Vfr which is a DNA binding protein, thus regulating the expression of genes (Fuchs et al., 2010b). Regarding the T3SS, Vfr binds to the promoter *PexsA* that is located immediately upstream of *exsA* gene and controls the expression of ExsA protein (Figure 22) (Marsden et al., 2016a).

PsrA is another transcription factor that positively regulates the transcription of *exsA* by directly binding to the promoter *P_{exsC}* (Kojic et al., 2002; Shen et al., 2012). The activation of PsrA is controlled by its binding to a long-chain fatty acid (LCFA) that inhibits the DNA binding activity of PsrA and thus decreases the expression of the *exsCEBA* operon (Kang et al., 2009). In addition, PsrA binds to the promoter of *rpoS* and positively regulates the expression of RpoS (Kojic et al., 2002) which is a stationary-phase sigma factor of the RNA polymerase. Indeed, RpoS was demonstrated to inhibit the expression of exotoxin ExoS (Hogardt et al., 2004). Therefore, it is suggested that this protein probably affects the central regulator protein ExsA.

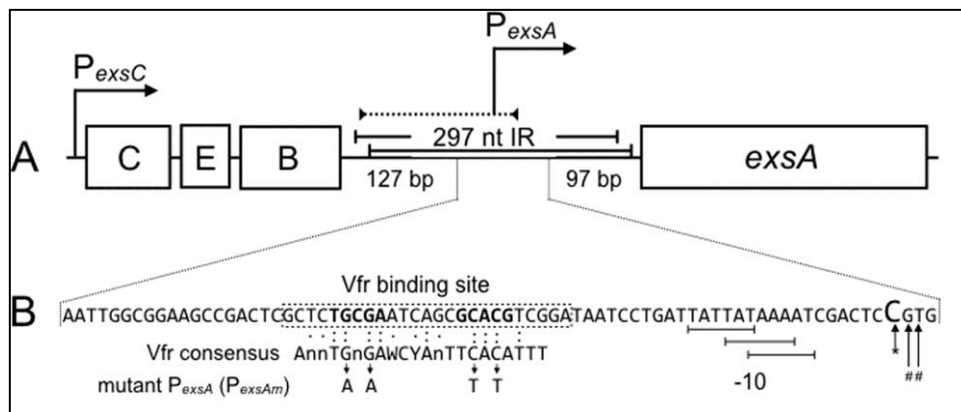


Figure 22: The Vfr protein binds to the promoter *P_{exsA}* located upstream of the *exsA* gene. (A) Organization of *exsC*, *exsE*, *exsB* and *exsA* genes and two promoters *P_{exsC}* and *P_{exsA}*. (B) DNA sequence of the Vfr binding site. (Marsden et al., 2016a)

3.5.4 Role of ATPase for the secretion process

a. An overview of secretion system ATPase

Most of the secretion systems possess an ATPase that provides the energy necessary to secrete their substrates across bacterial membranes. Actually, the T1SS harbors an ATPase from the ABC transporter family which is dimeric and is integrated in the bacterial membrane through its transmembrane domain. This ATPase also possesses a cytoplasmic domain responsible for the nucleotide binding (Filloux, 2011). The ABC transporter was shown to translocate substrates following the ATP switch model. Actually, substrates bind to the pocket that is oriented toward the cytoplasm, in the inward conformation of the transporter. The binding of ATP to the ATPase changes its conformation from inward to outward state, which exposes substrates to the opposite site. In the outward conformation, the transporter has a lower affinity for the substrates which in turn are released to the outside and the ATP hydrolysis reset the conformation of the ATPase (Higgins and Linton, 2004; Wilkens, 2015). However, while this model may be adequate for the small substrates such as drug compounds, the size of substrates secreted by T1SS can be large, from 20kDa (iron scavenger HasA) to 900kDa (LapA, an adhesion protein of *Pseudomonas fluorescens*) (Thomas et al., 2014) and hence, they would not be secreted following the ATP switch model. Although the structure of a T1SS ABC transporter was characterized by X-ray crystallography (Morgan et al., 2017), the mechanism by which the substrate proteins are translocated from the cytoplasm to the extracellular medium by the T1SS, is still unknown (Kanonenberg et al., 2013; Morgan et al., 2017; Thomas et al., 2014).

On the other hand, the ATPases of T2SS, T3SS, Type 3 flagella system (T3FS), T4SS, T4P and T6SS were reported to assemble into a hexameric ring. They were shown to be localized facing the bacterial cytoplasm and to be linked to the bacterial membrane through the interaction with the transmembrane proteins of the secretion systems. Based on the structures and the functions, these enzymes can be classified into three distinct groups: the traffic ATPase of the T2SS, T4SS and T4P, the type III ATPase of the T3SS and T3FS, and the ATPase of the T6SS (Figure 23).

The characterized members of the traffic ATPase family includes the T2SS ATPase GspE (XcpR in *P. aeruginosa*), three ATPase of the T4P PilB, PilT, PilU and an ATPase of the T4SS VirB11. The T4SS also possesses two other ATPases VirD4 and VirB4 binding to VirB11 to form a large ATPase complex that energize substrate export through the T4SS channel (Fronzes et al., 2009). However, the contribution of each ATPase to the secretion process is still unclear. The traffic ATPase family can be distinguished from the other secretion system ATPases by the presence of a conserved C-terminal domain containing an Aspartate box between Walker A and Walker B sites, a Histidine box downstream of Walker B site and of a N-terminal domain that differs among these ATPase (Figure 23) (Planet et al., 2001). The

Asp-box consists of two short aspartate-rich motifs that are suggested to stabilize the nucleotide binding while the His-box contains two histidine residues with unknown function. A tetracysteine (Cys4) motif responsible for metal binding (Camberg and Sandkvist, 2005; Douzi et al., 2012; Possot and Pugsley, 1997) is present in the C-terminal domain (CTD) of GspE, PilB but absent in PilT, PilU and VirB11 (Robien et al., 2003). In addition, GspE and PilB are distinct from the three other ATPases by the presence of 100 to 200 residues stretches in the N-terminal domain (Mancl et al., 2016; Robien et al., 2003). This region called N-terminal domain one (ND1) in the T2SS ATPase GspE, was characterized by X-ray crystallography and was shown to have a helical structure responsible for the interaction with the cytoplasmic domain of the inner membrane protein GspL (XcpY in *P. aeruginosa*) (Lu et al., 2013, 2014). Thus, this region might play a similar role in PilB. In fact, the N-terminal of the inner-membrane protein PilC binds to PilB (Takhar et al., 2013) and is predicted to be located in the central cavity of the hexameric ATPase (McCallum et al., 2017). The second N-terminal domain (ND2), which is present in all traffic ATPase, was shown to possess a α/β sandwich structure (6 anti-parallel β -sheet and 2 or 3 α -helices) in GspE, PilB and PilT with an unknown function (Mancl et al., 2016; Misic et al., 2010; Robien et al., 2003; Satyshur et al., 2007). However, based on the hexameric model, the ND2 seems important for the stability of the protein and both ND2 and CTD could be involved in the oligomerization of the traffic ATPase. In *Xanthomonas campestris*, an additional T2SS ND0 made of the 36 first residues was reported to be necessary for the interaction with GspL like the ND1 (Chen et al., 2005) but this is an exceptional case in the T2SS family. The difference between the two subfamilies GspE/PilB and PilT/PilU (described above and Figure 24) may explain their distinct role in the assembly and the function of T2SS and T4P. Actually, while the ATPase GspE and PilB are involved in the assembly of pseudo-pilins and pilins in T2SS and T4P respectively (Korotkov et al., 2012; Takhar et al., 2013), the ATPase PilT and PilU are required for

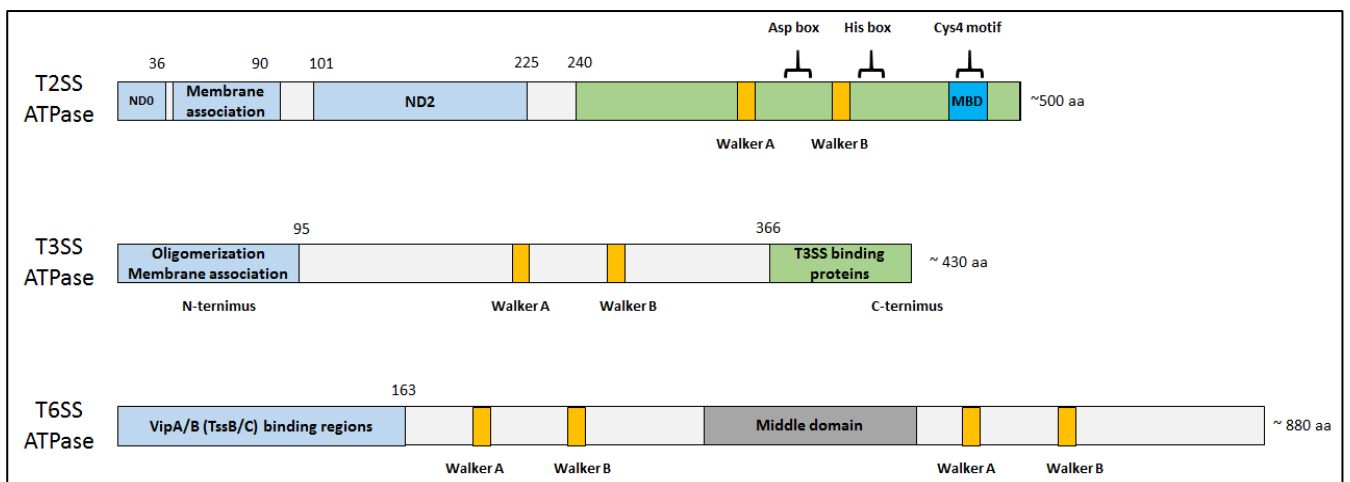


Figure 23: Diagrammatic representation of the ATPases of the T2SS, T3SS and T6SS. ND0: N-terminal domain 0; ND2: N-terminal domain two; MBD: Metal binding domain

the T4P pilins depolymerization (Burrows, 2005; Kurre et al., 2012; Takhar et al., 2013). The functioning of VirB11 is still unclear and is hence not further discussed.

In T2SS, exoproteins and pseudo-pilus proteins are translocated into the periplasm by the Sec and/or Tat secretion pathway (Arts et al., 2007). While the exoproteins bind to the outer-membrane translocator (Douzi et al., 2011; Filloux, 2004; Shevchik et al., 1997), the pseudo-pilins are recruited to the inner membrane protein and are polymerized by probably using energy from the ATPase GspE (McLaughlin et al., 2012; Patrick et al., 2011). The growth of pseudo-pilins pushes the exoproteins throughout the secretin channel like a piston would do (Filloux, 2004; McLaughlin et al., 2012; Nivaskumar and Francetic, 2014). In the same way, the ATPase PilB favors the polymerization of pilins but unlike pseudo-pilins, the pilins extends across the outer membrane and form the T4P outside the bacterium (Craig et al., 2004). Recently, a study showed that upon hydrolyzing ATP, the hexameric PilB changes its conformation which consecutively turns the inner membrane protein PilC in a clockwise rotation with a 60° increment (McCallum et al., 2017). The PilC is then pushed upward in the membrane and would facilitate the extraction of PilA pilin subunits from the membrane and the insertion of PilA into the pilus. On the other hand, the hexameric ATPase PilT works as the antagonist of PilB (Jakovljevic et al., 2008). The C-terminal domain of PilC is predicted to be located in the central pore of the hexameric ATPase PilT (McCallum et al., 2017; Takhar et al., 2013). The conformation change of PilT turns PilC in a counterclockwise rotation, thus pulling PilC toward the cytoplasm and facilitating the pilin depolymerization (McCallum et al., 2017). Otherwise, the ATPase PilU that is present in some bacteria including *P. aeruginosa*, is required for twitching motility (Whitchurch and Mattick, 1994) and is supposed to be involved in the pili retraction (Burrows, 2005) but the exact mechanism is still unknown.

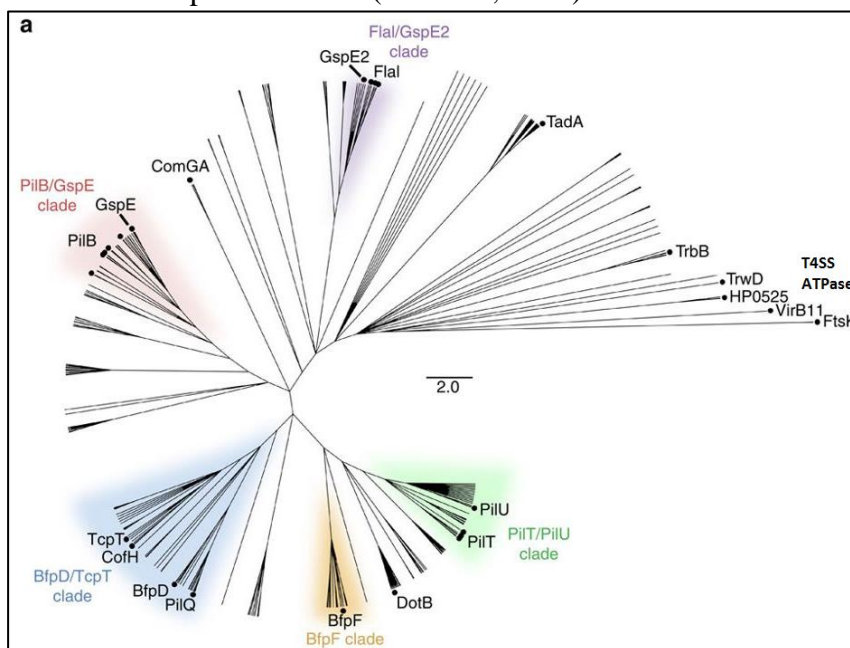


Figure 24: Phylogenetic analysis of the PilT-like ATPase family members (McCallum et al., 2017).

The ATPase of the T6SS, ClpV, has a specific role in the T6SS function that is different from the other secretion system ATPases. Actually, instead of providing energy for substrate translocation, the ClpV recycles the contracted sheath VipA/B, thus contributing to the resetting of the T6SS. ClpV is homologous to Hsp100/ClpB, a heat shock protein in *E. coli* that is involved in the degradation and refolding of misfolded or aggregated proteins (Schlieker et al., 2005; Zietkiewicz et al., 2004). These AAA proteins possess an N-terminal domain with a role of substrate selection and two AAA domains which are separated by a middle domain (Figure 23) (Schlieker et al., 2005). The structure of the N-terminal domain of ClpV was characterized by X-ray crystallography, showing 10 α -helices (α_0 - α_9). The α_1 - α_9 helices of ClpV share a high similarity with Hsp100 while the additional α_0 is only presented in ClpV. This α_0 is oriented perpendicularly to helix α_1 and is responsible for the binding of ClpV to VipA/B (Douzi et al., 2016; Pietrosiuk et al., 2011). Indeed, some mutations in the N-terminal domain of ClpV affected its interaction with the contracted sheath (Basler, 2015; Pietrosiuk et al., 2011).

The type III ATPase is localized in the bacterial cytoplasm and interacts with the membrane-associated proteins of the Type III C-ring and Type III basal body (Figure 14). In this chapter, I summarize data from all the ATPases of T3SSs from different bacteria including their expression method, their purification in full-length or truncated forms, their oligomerization, their available structure as well as their ATP hydrolysis specific activity (Table 5). The structure of SctN shares a high similarity with FliI (ATPase of the flagellar T3SS) and with the α/β -subunit of F1-ATPase which is found in bacterial plasma membrane, mitochondrial inner membrane and chloroplast membrane. SctN possesses two domains corresponding to the ATPase and C-terminal domains of the F1-ATPase while FliI contains all three domains of the F1-ATPase. Unlike FliI whose N-terminal domain was crystallized, showing six stranded β -sheets which are involved in the protein oligomerization, only N-terminus truncated monomers of SctN were characterized by X-ray crystallography. The C-terminus domain contains five α -helices and is considered to interact with the secreted T3SS proteins in the first step of the secretion process. In fact, some single mutations in this domain abolish binding of SctN to effector protein (Akeda and Galán, 2004; Allison et al., 2014; Kato et al., 2015; Zarivach et al., 2007). The central domain displays a high similarity to the ATPase catalytic domain with mixed α/β Rossmann fold containing a parallel nine-stranded β -sheet flanked by three and four α -helices on either sides. The Walker A and B motifs are also located in this domain, responsible for the catalytic activity. A model of the homo-hexameric ring SctN was built *in silico* with the ATP binding pocket interfaces being located between two adjacent subunits of SctN (Burgess et al., 2016a) suggesting that the oligomerization state is essential for the catalytic activity toward ATP and that the ATP binding can also be important for the oligomerization of SctN. Actually, some mutations in this catalytic domain affect the oligomeric form of this type III ATPase, for example InvC G164C and InvC R191H in *Salmonella enterica* (Akeda and Galán, 2004). Conversely, the monomeric

ATPase totally lost or displays much less enzymatic activity in comparison to its oligomeric form. The specific activity of Type III ATPases is different in diverse bacterial species but it is usually cooperative (Table 5). Although atomic structure of the N-terminus domain of SctN is lacking, it was shown to be important for the oligomerization and the membrane association. Actually, the single mutation V51E in InvC, the ATPase of *Samonella enterica*, abolished the ability to associate to the bacteria membrane and different N-terminus truncated form of SctN are not oligomeric. The T3SS ATPase was considered to form hexameric rings or perhaps dodecameric rings by two stacked hexamers (Allison et al., 2014; Müller et al., 2006; Zarivach et al., 2007). However, one publication recently presented a trimeric form of SctN (Burgess et al., 2016b, 2016a).

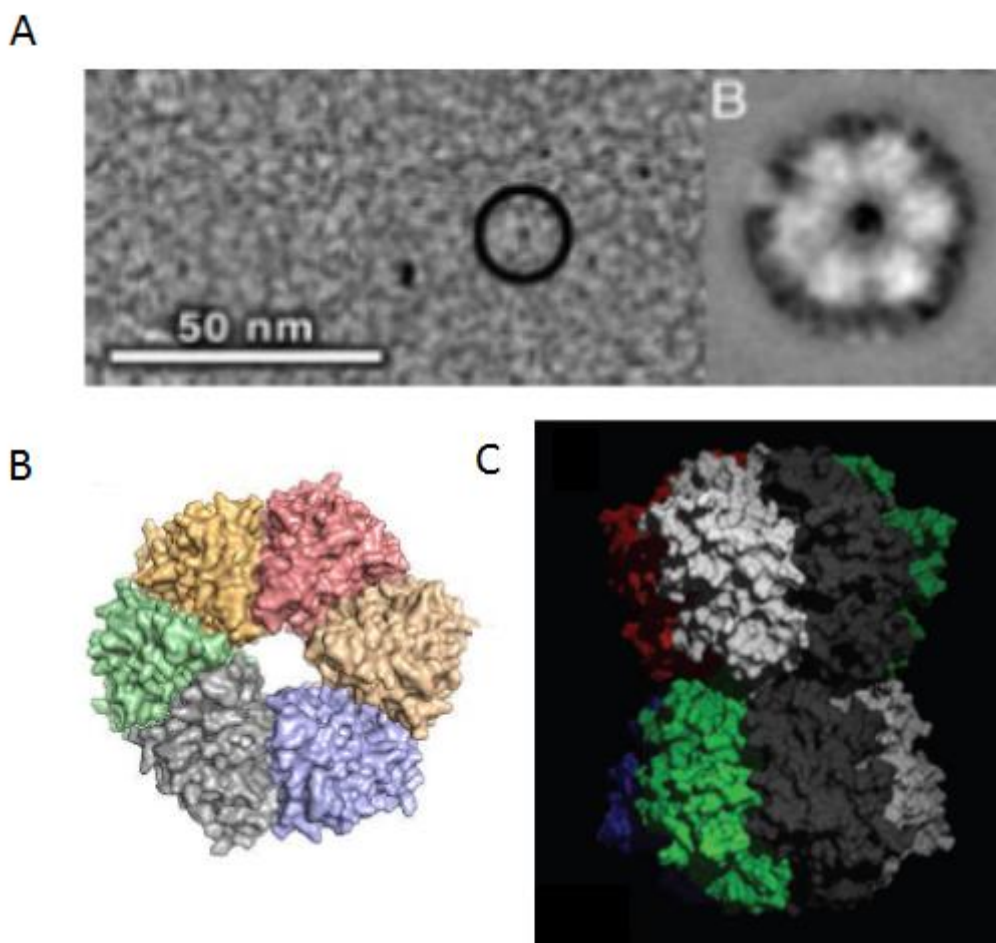


Figure 25: Structure of the T3SS ATPase. (A) Cryo-EM of SctN reveals an hexameric structure. (B) 3D reconstitution of hexameric SctN. (C) Proposed model of dodecameric SctN by two stacked hexamers. Adapted from (Burgess et al., 2016a; Müller et al., 2006).

b. The T3SS ATPase interacts with exported protein complex

The T3SS ATPase SctN is conserved in many gram negative bacteria and is essential for the T3SS function because the deletion mutant $\Delta sctN$ displays no T3SS activity and cannot intoxicate eukaryotic cells. Functionally, T3SS ATPases are considered to interact with the secreted Type III proteins and to deliver them to the secretion channel. Actually, the interaction of SctN with effector proteins, tip/filament proteins, translocon proteins and gate-keeper (described below) proteins was shown *in vitro* (Table 4). However, the inner-rod and needle protein (early substrates of T3SS) interaction with SctN is still speculative. In addition, the preferential interaction of SctN with chaperones, secreted proteins or protein-chaperone complex in the secretion process was not investigated yet. Actually, it is expected that the SctN affinity for secreted T3SS protein alone or in complex with their chaperone is higher than the one for the chaperone alone because the substrate proteins (cargo) are dissociated from their chaperone and subsequently translocated through the secretion channel while the chaperones are released from the complex and stay in the bacterial cytoplasm (Akeda and Galan, 2005; Lorenz and Buttner, 2009; Yoshida et al., 2014). Then, the chaperone proteins are supposed to be degraded or to bind to new cargo proteins for the turnover of the secretion process.

The chaperones of effector were shown to interact with ATPase SctN and by using molecular modelling, these chaperones were docked to the loop of two-helix-finger motif in the C-terminal of SctN (Allison et al., 2014; Zarivach et al., 2007). Confirming this model, a mutation of the conserved residue (V379P) in this region was shown by pull-down assay to abolish the interaction between the ATPase SsaN and the chaperone of effector SrcA in *S. enterica* SPI-2 (Allison et al., 2014). Interestingly, in a screening for mutations of ATPase InvC that affect the substrates secretion in *S. enterica* SPI-1, the same mutation (L376P) inhibiting the secretion of the early substrate InvJ, middle substrates SipB/C and the late substrate effector SctP was identified (Akeda and Galán, 2004). Hence, this two-helix-finger region is pointed out as the binding site of SctN to all the secreted proteins in T3SS but it was not demonstrated that it is the only binding site. It should be noted that to date, no experiment was presented to demonstrate that the mutation in the two-helix-finger region could abolish SctN interaction with translocator, needle and gate-keeper proteins.

	Effector (Late substrates)			Translocator and Tip/Filament (Middle substrates)			Needle complex (Early substrates)	Gate-keeper complex (Regulator)		
	Chaperone	Cargo	Complex	Chaperone	Cargo	Complex		Chaperone	Cargo	Complex
Binding	InvC (Akeda and Galan, 2005) SsaN (Yoshida et al., 2014) EscN (B. B. Finlay, 2003; Zarivach et al., 2007) HcrN	EscN (B. B. Finlay, 2003)	InvC (Akeda and Galan, 2005) EscN (B. B. Finlay, 2003)	SsaN (Yoshida et al., 2014)	Unknown	EscN (Chen et al., 2013)	Unknown	Unknown	Spa47 (Botteaux et al., 2009)	Unknown
Chaperone release	InvC (Akeda and Galan, 2005) HcrN (Lorenz and Buttner, 2009)			SsaN (Yoshida et al., 2014)			Unknown	Unknown		
Substrate unfolding	InvC (Akeda and Galan, 2005)			Unknown			Unknown	Unknown		

Table 4: Activities of T3SS ATPase SctN on T3SS proteins.

c. The T3SS ATPase provides energies for the secretion process

Upon hydrolyzing ATP, this enzyme is supposed to dissociate the chaperone-protein complexes, as shown for effector (Akeda and Galan, 2005; Lorenz and Buttner, 2009) and tip (Yoshida et al., 2014) proteins and to unfold these cargo proteins (only shown for one effector) in order to allow their passage through the narrow T3SS needle (Akeda and Galan, 2005) (Table 4). Nonetheless, the mechanism of pushing an unfolded protein through the secretion channel is not properly understood and remains controversial. In fact, it could be considered that the ATPase SctN provides energy through the hydrolysis of ATP for the protein secretion by funneling the substrates through its central cavity of the hexameric ring for the unfolding. Then, the energy stored in the partially unfolded proteins would contribute to their progression through the T3SS channel (Figure 27A) (Kato et al., 2015). However, some results may not be compatible with this hypothesis. Recently, the high-resolution *in situ* structure of the *Salmonella* T3SS was obtained by cryo-electron tomography (cryo-ET) and sub-tomogram averaging and one can conclude that SctN is connected to the C-ring from the SctN face exposed to the bacterial cytoplasm (Figure 26) (Hu et al., 2015; Makino et al., 2016). In another study on flagella, the FliH-FliI complex (homologous of SctL-SctN) was obtained by X-ray crystallography and demonstrated that FliH (homologous of SctL and thought to connect SctN to the C-ring) interacts with the N-terminal of FliI (Imada et al., 2016). These two findings suggest that the N-terminal part of SctN face the cytoplasm while the C-terminal of SctN, which is probably responsible for substrate protein binding, is oriented toward the entrance of the export

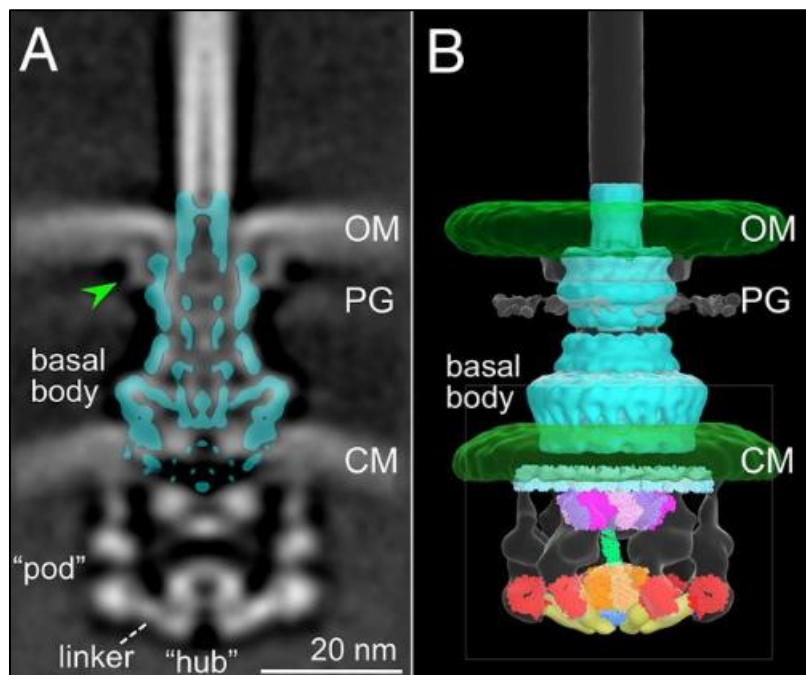


Figure 26: Cryo-ET of the T3SS basal body and cytoplasmic components in *S. flexneri* minicells. (A) Central section (B) Surface rendering, the ATPase is in orange. OM: Outer membrane; PG: Peptidoglycan; CM: Cytoplasmic membrane. (Hu et al., 2015)

apparatus (Hu et al., 2017; Ibuki et al., 2011). Therefore, if the substrate proteins were threaded through the pore of the ATPase, they would be moving in the wrong direction, from the T3SS gate to the bacteria cytoplasm. In addition, the central cavity of SctN may be blocked by SctO because the FliJ (homologous of stalk SctO), which connects the ATPase complex to the inner membrane protein FlhA (homologous of SctV), is reported to possess a structural similarity with the γ subunit of F1-ATPase and is thus supposed to be located in the central pore of Type III ATPase and block this cavity (Ibuki et al., 2011). Hence, this model of substrate secretion through the ATPase pore may be incorrect.

On the other hand, the proton motive force (pmf) was reported as the primary energy source for the protein export in both T3SS and T3FS because dissipating the pmf abolished the flagella assembly and protein secretion by the T3SS (Lee and Rietsch, 2015; Minamino and Namba, 2008; Wilharm et al., 2004). In support to this hypothesis, in the absence of ATPase FliI, the flagellar assembly can proceed if the FliH protein is also removed, although this assembly is not as efficient as in a wild-type strain (Minamino and Namba, 2008). Moreover, the flagella formation could be enhanced by increasing the pmf and flagella substrate levels or removing the inner-membrane proteins FlhA and FlhB (Erhardt et al., 2014; Minamino and Namba, 2008). Furthermore, the catalytic activity of ATPase InvC was shown to be dispensable for the substrate secretion by the T3SS if the pmf is high enough (Erhardt et al., 2014), suggesting that the energy from the pmf is sufficient to maintain the protein export process including the chaperone release, the substrate unfolding and the pushing of unfolded protein through secretion channel (Figure 27B) (Minamino and Namba, 2008; Minamino et al., 2011; Paul et al., 2008). Nonetheless, this model is also

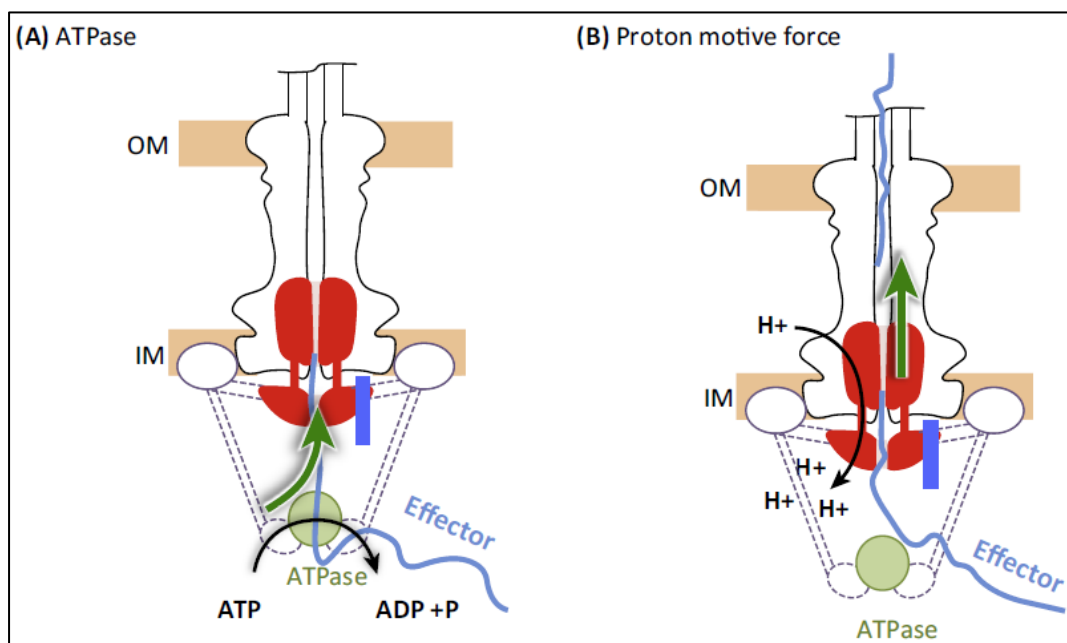


Figure 27: Models of protein export through the T3SS. (A) Secreted substrates are funneled through ATPase cavity and exported by using energies from ATP hydrolysis; (B) Proton motive force (pmf) pulls and exports the secreted proteins by using the flow of proton. OM: outer-membrane, IM: inner-membrane (Lee and Rietsch, 2015).

questionable because of the experiment conditions which are used to modify the pmf. Actually, the addition of chemical compounds inhibiting the pmf as well as gene mutations increasing the pmf, also affects the bacterial physiology such as the ATP level in the cell, which in turn have an incidence on the ATPases activity, or the Sec-dependent export pathway which is strictly dependent on the pmf (Galán, 2008). In addition, no experiment has been reported showing that the pmf could dissociate protein-chaperone complex and unfold substrate proteins as the T3SS ATPase does in a catalytic-dependent manner *in vitro* (Akeda and Galan, 2005). Furthermore, the low secretion of flagellin still observed in the *AfliA*/*AfliH* double mutant could be explained by cross-talk with other unfoldases in bacteria. In fact, the mechanism of substrate recognition of other unfoldases might be similar to the one of a secretion system ATPase and might partially complement its function. Finally, FliH as well as FlhA/B could block the entry to the channel in the absence of the ATPase FliI and hence, their removal could be necessary to observe a low secretion of flagellin (Galán, 2008).

Fundamentally, these two models can be reconciled and both ATP hydrolysis and pmf could be required for the protein export process in different steps. Firstly, secreted proteins in complex with their cognate chaperones are docked to the export gate through the interaction with the Type III ATPase and perhaps others apparatus proteins. Secondly, the Type III ATPase hydrolyzes ATP and provides energies for the chaperone release and the unfolding of the secreted protein. These two activities involve the C-terminal domain of the ATPase which is oriented toward bacterial membrane and the substrate proteins are not funneled through the hexameric ring cavity. Finally, the unfolded proteins are pushed through the secretion channel by using the energy of the pmf. Recently, the structure of SctN in presence of the non-hydrolysable ATP γ S was obtained by X-ray crystallography, showing a conformational change in the luminal loop of the ATPase upon ATP binding (Gao et al., 2018). This loop would be located in the lumen of the ATPase hexameric ring and is considered to interact with the stalk SctO which is located in the central pore of the Type III ATPase as described above. On the other side, SctO binds to the inner-membrane protein SctV and both SctO and SctV are reported to control the pmf for the protein export by T3SS (Lee and Rietsch, 2015; Minamino et al., 2011). Therefore, a model is proposed, connecting the

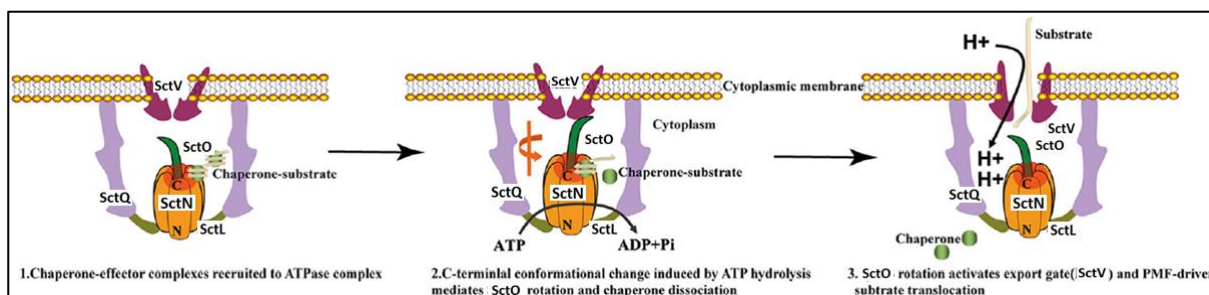


Figure 28: Model of T3SS protein export which depends on both ATPase and proton motive force. Adapted from (Gao et al., 2018).

roles of the ATP hydrolysis and the pmf in the secretion process. Actually, the binding and hydrolysis of ATP would change the conformation of luminal loop of the ATPase which in turn would induce the rotation of SctO as well as the chaperone release and the substrate unfolding. Then, the rotation of SctO would activate the export gate SctV and substrates would thereafter be translocated by the pmf activity (Gao et al., 2018) (Figure 28).

d. T3SS ATPase contributions to the sorting of exported proteins during the secretion process

In addition to the dissociation of the cargo proteins from their chaperones and the energy providing, it might be possible that SctN is involved in the regulation of the secretion process by participating to the sorting of the substrates and, thus allowing them to be secreted hierarchically, a role shown for other cytoplasmic proteins SctO, SctK and SctQ (Lara-Tejero et al., 2011). During T3SS assembly, the needle protein (early substrate) is the first secreted, followed by the translocator (middle substrate) secretion and the effector (late substrate) is secreted and translocated into host cells in the final step of the secretion process. Actually, the ATPase SctN is located at the cytoplasmic extremity of the T3SS. It is thus possible that the ATPase is the first stop of the exported proteins that could be next recognized by other component of the ATPase complex or export apparatus. In addition, the ATPase is suggested to interact with most of the secreted T3SS proteins through the C-terminal domain, hence these proteins might compete to dock to the ATPase if they interact with SctN at the same binding site.

Actually, the sorting of substrates for the secretion is a complex mechanism and it is undoubted that it involves not only the ATPase SctN but also others proteins of the T3SS such as SctO, SctK, SctQ or the proteins which are reported as a regulators such as the needle length regulator SctP, the switch protein SctU and the gate-keeper protein SctW. The SctP and SctU are described in the chapter 3.5.1c and thus they will not be further discussed. The SctW regulates the secretion of effectors and translocators. In fact, an over-secretion of effectors was observed in *ΔsctW* mutant strains of different bacteria while these strains had different phenotypes concerning translocator secretion (Ferracci et al., 2005; Martinez-Argudo and Blocker, 2010; Roehrich et al., 2017; Yang et al., 2007). Actually, in the absence of SctW, a decrease of translocator secretion is observed in *Shigella* (Martinez-Argudo and Blocker, 2010) while it is stimulated in *Yersinia* (Ferracci et al., 2005). In *P. aeruginosa*, the gate-keeper PopN is shown to repress the secretion of the effectors (Yang et al., 2007) but unfortunately, no information concerning the translocators is available. Because the T3SS of *P. aeruginosa* and *Yersinia* are phylogenetically close, it is probable that PopN represses the secretion of both effector and translocator. Interestingly, SctW is shown to interact also with the ATPase SctN and to be secreted by the T3SS like the effectors and

translocators. Besides, the tip protein SctA and its cognate chaperone are described as a negative regulators of the effector secretion (Lee et al., 2014) and they could also bind to the ATPase SctN. Therefore, it would be interesting to study the affinity of SctN with the exported proteins and determine whether SctW and SctA complexes could regulate the secretion process by interacting with the ATPase SctN.

Table 5 Overview of T3SS ATPase expression, purification and biochemical characterization

ATPase	Bacteria	Expression vector and condition	Purification method		Oligomerization form Condition and method			Structure		ATPase specific activity	Refs
			Full-length	Truncated	SEC-MALLS	EM	Model <i>in silico</i>	X-ray crystallography			
FliII ATPase of flagella	<i>Salmonella typhimurium</i>	pGex4T-FliII <i>E. coli</i> BL21 Overnight 25°C	<ul style="list-style-type: none"> ● GST-FliI ● Glutathione agarose ● Thrombin cleaved 	-	Treated with ATP Dimer and a trimer	Treated with AMP-PNP Hexamer	-	-	Cooperativity 0.28 μmol/min/mg	(Claret et al., 2003)	
EscN	<i>Escherichia coli</i> (EPEC)	EscNΔ7; EscN Δ102; EscN Δ102 V393P EscN Δ7 R366D in pET28a <i>E. coli</i> BL21 Overnight 16°C	-	<ul style="list-style-type: none"> ● His-EscN ● Ni-chelating sepharose ● Thrombin cleaved ● Mono Q 	Dimer	-	Hexamer model based on monomer EscNΔ102 V393P	EscNΔ102 V393P 8mg/ml	-	(Zariva et al., 2007)	
		pET19b-EscN <i>E. coli</i> BL21 pLysS 30°C for 4h	<ul style="list-style-type: none"> ● His-EscN ● Nickel column ● Dialyze overnight against buffer containing DTT 	-	Only SEC Treated with ATP/ADP or AMP-PNP Monomer Dodecamer	-	-	-	Cooperativity 0.41 μmol/min/mg	(Andrade et al., 2007)	
		pET28a His-EscN <i>E. coli</i> BL21 30°C for 3h	<ul style="list-style-type: none"> ● His EscN ● Nickel column with Urea 8M 	-	-	-	-	-	-	(B. B. Finlay, 2003)	

Table 5: Overview of T3SS ATPase expression, purification and biochemical characterization (continued)

ATPase	Bacteria	Expression vector and condition	Purification method		Oligomerization form Condition and method			Structure		ATPase specific activity	Refs
			Full-length	Truncated and mutated proteins	SEC-MALLS	EM	Model <i>in silico</i>	X-ray crystallography			
EscN (continued)	<i>Escherichia coli</i> (EPEC) (continued)	pET16b EscN and EscN Δ97 <i>E. coli</i> BL21 37°C for 3h	•His EscN	•His EscN Δ97	Only SEC	-	-	-	-	(Chen et al., 2013)	
			•Nickel column	•Nickel column	Hexameric EscN Monomeric EscN Δ97	-	-	-	-		
InvC	<i>Salmonella enterica</i> SPI-1	pQE60 InvC (InvC-6His)	•Ni-nitrilotriacetic acid column	G164C; K165E; R223H; R191H; R189G	Only SEC	-	-	-	-	(Akeda and Galan, 2005; Akeda and Galán, 2004)	
			•Normal denaturing condition with 8M of Urea		Monomer and Hexamer	-	-	-	-		
SsaN	<i>Salmonella enterica</i> SPI-2	pET26 InvC (10His-InvC-3FLAG) <i>E. coli</i> BL21	•Ni-nitrilotriacetic acid (NTA) agarose	Y385A;G383A, E384A, G388A, E306A, E308A, E309A, E310A, D312A	-	-	Hexamer model	-	2μmol/ min/mg	(Kato et al., 2015)	
			-	•SsaN Δ89 His •Nickel chelating resin •MonoQ	Only SEC Monomer and Dimer	-	Hexamer model	SsaN Δ89-His	-		(Allison et al., 2014; Cooper et al., 2010)

Table 5: Overview of T3SS ATPase expression, purification and biochemical characterization (continued)

ATPase	Bacteria	Expression vector and condition	Purification method		Oligomerization form Condition and method			Structure	ATPase specific activity	Refs
			Full-length	Truncated	SEC-MALLS	EM	Model <i>in silico</i>			
SsaN (continued)	<i>Salmonella enterica</i> SPI-2	SsaN-Myc-6His <i>E. coli</i> Top10	• Ni-NTA agarose (Qiagen)	-	-	-	-	-	Cooperative 0.35µmol/min/mg	(Yoshida et al., 2014)
YscN	<i>Yersinia enterocolitica</i>	pBB7 (pET derivative) YscN <i>E. coli</i> BL21 30°C for 10h	• His-YscN		Only SEC				Cooperative 10mmol/min/mg	(Blaylock et al., 2006)
			• Ni-NTA column		Treated with AMP-PNP					
			• Denaturing condition with 6M Urea	-						
			• YscN-His	Ni-NTA resin column denaturing condition	Only SEC				2.95µmol/min/mg	(Chatterjee et al., 2013)
			• Ni-NTA column	YsaNA(1-5), YsaNA(1-20), YsaNA(411-430), YsaNA(426-430), YsaNA(21-410)	Monomer, Dodecamer					
			• Normal and denaturing condition		Confirmed by cross-linking and DLS					

Table 5: Overview of T3SS ATPase expression, purification and biochemical characterization (continued)

ATPase	Bacteria	Expression vector and condition	Purification method		Oligomerization form Condition and method			Structure	ATPase specific activity	Refs
			Full-length	Truncated	SEC-MALLS	EM	Model <i>in silico</i>			
Spa47	<i>Shigella flexneri</i>	pTYB21 (chitin-tag) CBD-Spa47 <i>E. coli</i> Tuner 17°C for 20h	<ul style="list-style-type: none"> ● CBD-Spc47 ● Chitin affinity column ● Q Sepharose FF anion ● Buffer exchange colupn 	Spa47 Δ 1-79 K165A; E188A, R350A Same protocol as	Only SEC Monomer, Dimer, Trimer Confirmed by analytical ultra-centrifugation	-	Hexamer model	Spa47 Δ1-79	1.48μmol/min/mg (Calculated from data in publication)	(Burgess et al., 2016b, 2016a)
HrcN	<i>Pseudomonas syringae</i>	pET16b 10His-HrcN <i>E. coli</i> BL21 22°C for 3h	<ul style="list-style-type: none"> ● 10His-HrcN ● Ni2-nitrilotriacetic acid Fast Flow resin 	-	Only SEC Monomer, hexamer, dodecamer or aggregated protein Confirmed by rate zonal centrifugation	Double hexamer ring	-	-	Cooperative 43mmol/min/mg	(Müller et al., 2006; Pozidis et al., 2003)
HrcN	<i>Xanthomonas campestris</i>	pDSK604-HrcN <i>E. coli</i> BL21 37°C	<ul style="list-style-type: none"> ● Strep-HrcN ● Streptactin sepharose 	-	No SEC-MALLS data but oligomerization was shown by co-purification of Strep-HrcN and HrcN-Myc	-	-	-	0.4μmol/min/mg	(Lorenz and Buttner, 2009)

3.5.5 Drug discovery

The T3SS is one of the most important virulence factors of *P. aeruginosa*, thus this machinery is considered as the most promising target for the development of anti-virulence therapies. Three main strategies to inhibit the T3SS are reported. The first one is to directly neutralize the exotoxins of *P. aeruginosa* that are secreted proteins but that act inside the eukaryotic cells and may not be accessible in the extracellular medium if they travel inside the T3SS needle. The second one is to block the T3SS assembly by targeting one of the essential proteins of this system and the last one is to affect the expression of T3SS by interfering with its regulation. These two last strategies seem to be more difficult because anti-virulence compounds need to pass through the two bacterial membranes. However, most of the drug discovery use a phenotypical screening to look for some chemical compounds that inhibit the T3SS functioning and have a minimal effect on bacterial growth. While the identification of inhibitor is the essential step, the determination of their targets is still an important challenge. In this chapter, I summarize all the anti-virulence compounds targeting the T3SS of *P. aeruginosa* including their structures, their targets, their mechanisms, the employed screening methods and their efficacies *in vitro* and *in vivo* (Table 6).

Among them, four chemical compounds in different clusters were shown to inhibit the enzymatic activity of T3SS exoenzymes, two for ExoS and two for ExoU. Nonetheless, the activity of these compounds was mainly shown *in vitro* and *in vivo* results are lacking. Moreover, targeting only one exotoxin seems not to be broad enough to inhibit the T3SS of diverse *P. aeruginosa* strains and there might be a high conservation of the catalytic activity between bacterial and human proteins, thus leading to a high risk of side effects by poor specificity.

A derivative of hydroxyquinolone – INP1855 was recently discovered as affecting the ATPase activity of the T3SS ATPase in *Y. pestis* and *P. aeruginosa*. This compound protected cells *ex vivo* and mice *in vivo* against *P. aeruginosa* infection. Thus, it becomes a promising compound that could be next submitted to clinical trials. However, as INP1855 is predicted to target the ATPase activity, its specificity could be questioned as for the compounds inhibiting exoenzymes. In fact, it might be possible that INP1855 also inhibits the activity of human ATPases.

The phenoxyacetamide family was identified and developed by Microbiotix pharmaceutical company as the best chemical compounds inhibiting the T3SS functioning in *P. aeruginosa* with an IC₅₀ of 0.8 μ M for MBX1641, the best analog, regarding the inhibition of the exotoxin ExoS secretion. This compound showed a protective effect on cells *ex vivo* and on mice *in vivo*. Therefore, like INP1855, MBX1641 is very promising and could be tested in clinical trials. By looking for inhibitor-resistant mutants, the needle protein PscF was identified as the apparent target of MBX1641 because three single mutations in *pscF*

were necessary and sufficient to promote resistance to a variety of phenoxyacetamide analogs. By structural modeling, each of the three mutated residues were localized in different PscF subunits and they were very close each to one other. Thus, this compound was predicted to bind to the polymerized PscF and to inhibit subunit-subunit interaction. Nonetheless, an *in vitro* experiment confirming the MBX1641 interaction with the needle protein is still lacking.

Another compound being the focus of intense studies is Thiazolidinone. This molecule was firstly identified and studied in *S. typhimurium*. Then, it also showed a protective effect in other bacteria such as *P. aeruginosa* and *P. syringae*. Actually, Thiazolidinone inhibited the effector secretion by the T3SS and protected macrophage cells from infection by *S. typhimurium*, decreased the twitching motility and the LasB secretion by T2SS in *P. aeruginosa*, and protected the leaves of Tobacco plants from infection by *P. syringae*. The broad effect spectrum of this compound is a challenge to exactly determine its target. Therefore, Thiazolidinone was predicted to affect the secretin proteins in the outer membrane of bacteria because this is the only protein common to the T2SS, T3SS and T4P. However, like MBX1641, an *in vitro* experiment is needed for confirmation.

To inhibit the regulation of the T3SS in *P. aeruginosa*, three compound clusters were identified (Table 6) but only one of them, N-hydroxybenzimidazole, is promising. In fact, the two other compounds display a broad activity spectrum and are suggested to have multi-targets including the T3SS. Moreover, these compounds mainly show their effects *in vitro* or on the secretion of T3SS effectors and information on their *in vivo* efficacy are lacking. On the other hand, N-hydroxybenzimidazole was identified by an *in vitro* screening looking for molecules that inhibit the binding to DNA of the regulator protein LcrF (homologous of ExsA) in *Y. pseudotuberculosis*. This compound was also shown to be effective for ExsA in *P. aeruginosa*. The use of a target-based screening for N-hydroxybenzimidazole identification reduces the possibility of low specificity, thus limiting the possible side effects. In addition, this compound was shown to inhibit the bacterial burden in the lung and increase the survival of mice *in vivo*, thus it becomes a promising compound that should be further tested in clinical trials.

Besides the strategy using small chemical compounds to target the T3SS, some antibodies against the tip protein PcrV (homologous of the V-antigen LcrV in *Y. pestis*) have been developed. Actually, the anti-PcrV antibody blocks the function of PcrV, thus affecting the insertion of the translocators into the eukaryotic cell membrane (Goure et al., 2005). In 1999, rabbit anti-PcrV polyclonal antibodies were demonstrated for the first time to enhance macrophage survival and to reduce bacterial burden in mice. Interestingly, this protection was not observed when using anti-PopD or anti-ExoU antibodies, meaning that only PcrV is accessible for antibody-mediated neutralization (Sawa et al., 1999). Moreover, the protective activity of this antibody did not require the Fc fragment because the Fab fragment was sufficient to inhibit *P. aeruginosa* T3SS (Shime et al., 2001). Three years later, a monoclonal antibody (mAb)

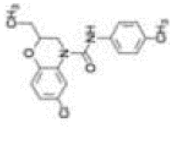
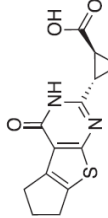
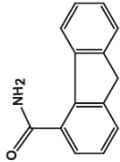
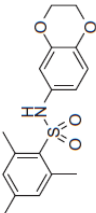
toward PcrV was generated: mAb166 which also showed a protective capacity against *P. aeruginosa* in mice but its efficacy was somehow lower than the rabbit polyclonal anti-PcrV. Authors suggested that the polyclonal antibodies bind to multiple sites on PcrV and block its function more efficiently. Thus, several monoclonal antibodies would be needed to be equally effective. In addition, like for the polyclonal anti-PcrV, the Fab fragment of mAb166 displayed the same level of protection as the whole IgG, indicating that the protective activity is not dependent on the Fc fragment (Frank et al., 2002). Latter, this anti-PcrV monoclonal antibody was shown to protect rats against *P. aeruginosa* in an acute lung infection model as well as to reduce the lung inflammation caused by chronic *P. aeruginosa* infection in a murine model (Faure et al., 2003; Imamura et al., 2007).

Based on the Fab of mAb166, an engineered humanized anti-PcrV was generated in 2009, Fab1A8 that binds to PcrV with a two-fold higher affinity than mAb166. Moreover, this humanized antibody possesses a higher potency in the improvement of mice survival in an acute pulmonary infection model, thus being qualified for clinical uses (Baer et al., 2009). This Fab1A8 was then developed by Kalabios Pharmaceuticals with a PEGylating process which is known to extend serum half-life and to protect against inactivation in lung (Chapman, 2002), thus generating a PEGylated humanized Fab fragment of anti-PcrV mAb, KB001. From 2010 to 2014, Kalabios was in partnership with Sanofi Pasteur to develop the KB001 program. This mAb was tested in phase I/II clinical trials for ventilator-associated pneumonia (VAP) and for chronic pneumonia in cystic fibrosis (CF). While KB001 was demonstrated to be safe, tolerated with a favorable pharmacokinetic profile and promoted a reduction of *P. aeruginosa* pneumonia incidence in ventilated patients colonized by this bacterium (NCT00691587) (François et al., 2012; Sawa et al., 2014), the phase II clinical trial (NCT00638365 and NCT01695343) in people with cystic fibrosis failed (Anantharajah et al., 2016a; Dickey et al., 2017; Milla et al., 2014). This KB001 program was then abandoned in 2014 and both companies Kalabios and Sanofi did not indicate the reasons. It might be possible that the T3SS is low expressed in chronic infections and thus, CF patients are not the appropriate population for the clinical assays (Anantharajah et al., 2016a).

On the other hand, another monoclonal antibody against PcrV was developed by MedImmune. The purified recombinant PcrV was used to immunize VelocImmune mice which express human antibody variable regions, thus generating humanized antibodies in Fab regions. The variable heavy (VH) and variable light (VL) chains of the most potent monoclonal antibody obtained, V2L2, were then fused to human IgG1 Fc and C-kappa-coding region to convert it to a fully humanized antibody V2L2MD. In comparison to the Fab of mAb166, the V2L2MD displays 10 to 20 fold higher affinity to PcrV and more efficacy in the protection from bacteria cytotoxicity *ex vivo* as well as in the protection of murine models (Warrener et al., 2014). This V2L2MD mAb was then combined with an anti-Psl mAb to generate a bispecific mAb (BiS4aPa) as described in the chapter 2.2. This multifunctional mAb, MEDI3902 is

ongoing a phase I and II clinical trials (NCT02255760 and NCT02696902) (Dickey et al., 2017; DiGiandomenico et al., 2014b; Le et al., 2018; Ray et al., 2017).

Table 6 Overview of T3SS inhibitors in *P. aeruginosa*.

Name	Structure	Library and Screening method	Target	Mechanism	Bacterial spectrum	MIC	IC50 (best analogue)	In vivo – Preclinical	Refs
Exosin 5138		<ul style="list-style-type: none"> • 56,000 compounds from LOPAC, SPECTRUM and ChemDiv libraries • Growth restoration of <i>S. cerevisiae</i> (Yeast) that contains yeast vector expressing ExoS 	ExoS	Inhibition of ADPRT activity	<i>P. aeruginosa</i>	Unknown	12µM Decrease of ADPRT activity <i>in vitro</i>	Unknown	(Arnold et al., 2008)
ME0805		<ul style="list-style-type: none"> • Diverse libraries: compounds with MW up to 300Da from PARP inhibitors • Enzymatic assay based on fluorescence energy transfer 	ExoS	Inhibition of ADPRT activity	<i>P. aeruginosa</i>	Unknown	1.3µM Inhibition of ADPRT activity of secreted ExoS	Unknown	(Pinto et al., 2016; Saleeb et al., 2018)
Pseudolipasin A		<ul style="list-style-type: none"> • 50,000 compounds in ChemBridge library • Secretion assay of ExoU fused to β-lactamase reporter + Nitrocefine • Injection assay in CHO cells of ExoU-Bla using CCF4-AM substrates for fluorescence detection 	ExoU	Inhibition of phospholipase activity	<i>P. aeruginosa</i>	Unknown	7µM: PLA2 activity 1-7.5µM : protection of CHO cell (LDH assay)	Unknown	(Lee et al., 2007)
Arylsulfonamides derived		<ul style="list-style-type: none"> • About 1000 compounds in-house chemical library • Growth restoration of Yeast as in the case of ExoS, this time, the vector contains ExoU 	ExoU	Unknown	<i>P. aeruginosa</i>	5µM Restore yeast growth by 60%	Unknown	Unknown	(Kim et al., 2014)

T3SS Exotoxins

Table 6: Overview of T3SS inhibitors in *P. aeruginosa* (continued).

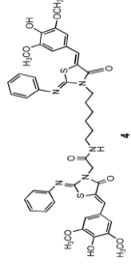
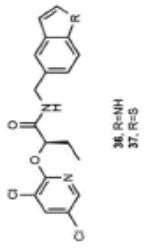
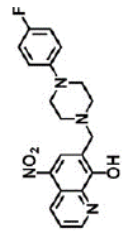
Name	Structure	Library and Screening method	Target	Mechanism	Bacterial spectrum	MIC	IC50 (best analogue)	In vivo – Preclinical	Refs
T3SS Secretin		<ul style="list-style-type: none"> 92000 compounds from nature and synthetic sources (NSRB) Effector secretion assay using reporter of phospholipase activity using PED6 substrate 	PscC and secretin of T2SS, T4P	Unknown	<i>P. aeruginosa</i> <i>S. typhimurium</i>	Unknown	5µM Secretion assay (protein rapporteur as screening method)	Unknown Only <i>in vivo</i> experiment on tobacco plant by <i>P. syringae</i>	(Felise et al., 2008; Kline et al., 2009)
Phenoxyacetamide - MBX1641		<ul style="list-style-type: none"> 80,000 compounds of Cambridge and Tintec libraries Luminescence assay by using ExoT fused to luxCDABE reporter gene 	PscF subunit	Effect on interaction of needle subunits thus inhibition of needle assembly	<i>P. aeruginosa</i>	Unknown	0.8µM Secretion assay (protein rapporteur as screening method)	Abscess formation in mouse infected by <i>P. aeruginosa</i>	(Aiello et al., 2010; Berube et al., 2017; Bowlin et al., 2014; Williams et al., 2015)
Derivate of Hydroxyquinoline - INP1855		<ul style="list-style-type: none"> 17,500 synthetic small organic molecules at Umeå Small Molecule Screening Facility Inhibit transcription of Yop promoter with luxAB reporter gene 	PscN YscN	Inhibition of ATPase activity	<i>P. aeruginosa</i> <i>Y. pestis</i>	60µM Lactate Dehydrogenase (LDH) assay	Unknown	Survival assay on murine acute lung infection	(Anantharajah et al., 2016b; Enquist et al., 2012)

Table 6: Overview of T3SS inhibitors in *P. aeruginosa* (continued).

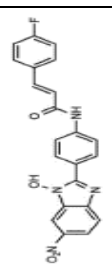
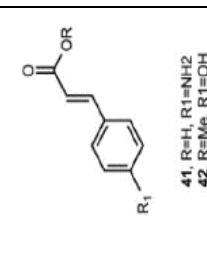
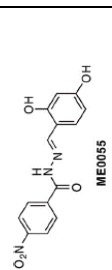
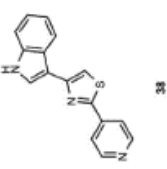
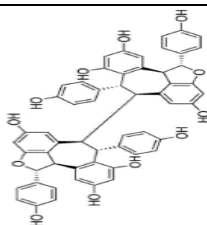
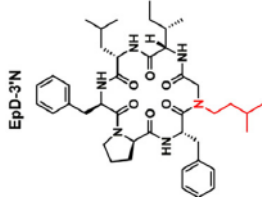
Name	Structure	Library and Screening method	Target	Mechanism	Bacterial spectrum	MIC	IC50 (best analogue)	In vivo – Preclinical	Refs
N-hydroxybenzimidazole		<ul style="list-style-type: none"> 2000 compounds from CambridgeSoft Corp., Cambridge, MA/Restore DNA binding assay using chemiluminescence 	Many AraC protein family including ExxA and LcrF	Inhibition DNA binding activity	<i>P. aeruginosa</i> <i>Y. pseudotuberculosis</i>	Unknown	3.9µM for LcrF and 8µM for ExxA (DNA binding assay – EMSA)	Inhibition of bacterial burden in murine lung Increase in mice survival	(Bowser et al., 2007; Garrity-Ryan et al., 2010; Kim et al., 2009; Marsden et al., 2016b)
derivate of Plant phenolic compound (p-coumaric acid)	 41, R=H, R1=NH2 42, R=Me, R1=OH	<ul style="list-style-type: none"> Inhouse synthesis, derivate of salicylic acid Secretion of ExoS-GFP measured by flow cytometry (FACS) 	GacSA-RmsYZ-RmsA-ExxA	Strong induction of expression of RmsY and RmsZ	<i>P. aeruginosa</i>	250µM Secretion assay – Western blot	Unknown	Unknown	(Yamazaki et al., 2012)
Salicyliden acylhydrazide alone or in complex with Galium III		<ul style="list-style-type: none"> 9400 unique substances (ChemBridge DiverSet F) Inhibit transcription of Yop promoter with luxAB reporter gene 	WtbA (NAD(P)H quinone-oxidoreductase), Tpx (thiol peroxidase), FolX (dihydroneopterin-tri-P-epimerase)	<ul style="list-style-type: none"> Effect on T3SS regulation and also flagella Direct effect on T3SS basal apparatus and affect assembly of needle (shown by electron microscopy) T3SS expression and deprivate iron available in cell 	<i>P. aeruginosa</i> <i>Y. pseudotuberculosis</i> <i>E. coli</i> 0157 <i>S. flexneri</i> <i>C. trachomatis</i> <i>sevovar D</i>	20µM (>90% of inhibition of Secretion Tir effector -WB) and 20µM (100% survival of macrophage J774 infected by <i>S. flexneri</i>)	Unknown	Unknown	(Bailey et al., 2007; Dahlgren et al., 2007, 2010; Kauppi et al., 2003; Nordfeith et al., 2005; Rzhepishevskaya et al., 2014; Slepkin et al., 2007; Tree et al., 2009; Veenendaal et al., 2009; Wang et

Table 6: Overview of T3SS inhibitors in *P. aeruginosa* (continued).

Name	Structure	Library and Screening method	Target	Mechanism	Bacterial spectrum	MIC	IC50 (best analogue)	In vivo	Refs
C15,19,20,22,24,38 Aromatic compounds		<ul style="list-style-type: none"> • 100000 compounds from ChemDiv 2, 3, 4, Maybridge 3, 4 and Biomol • Injection assay in Hep-2 cells of YopE fused to E-TEM-bla proteins using CCF2-AM substrates for fluorescence detection 	Unknown	Reduction of the translocation of effector	<i>P. aeruginosa</i> <i>Y. pseudotuberc ulosis</i>	60µM <i>P. aeruginosa</i> infection model of Hep-2 cells	Unknown	Unknown	(Harm on et al., 2010)
(-)- Hopeaphenol Resveratrol tetramer		<ul style="list-style-type: none"> • Leaf extracted derived from two Papua New Guinean rainforest plants: <i>Anisoptera thurifera</i> and <i>A. polyandra</i> • Luminescence assay by using YopE fused to luxCDABE reporter gene • Phosphatase activity assay of secreted YopH 	Unknown Suggested to be located on bacteria surface	Reduction of the translocation of effector	<i>P. aeruginosa</i> <i>Y. pseudotuberc ulosis</i>	Unknown	Secretion assay – Western blot 3.3-6.6 µM in <i>Yersinia</i> 50-100µM in <i>Pseudomonas</i>	Unknown	(Zetterström et al., 2013)
Membrane permeable cyclic Peptomers		<ul style="list-style-type: none"> • 20 synthetic peptides and peptomers based on natural product phepropeptin D • Inhibition of NF-κB activation in HEK293 cell line by using fusion of NF-κB -GFP 	Unknown	Reduction of the translocation of effector	<i>P. aeruginosa</i> <i>Y. pseudotuberc ulosis</i>	Unknown	Secretion assay – Coomassie 15µM in both <i>Yersinia</i> and <i>Pseudomonas</i>	Unknown	(Lam et al., 2017)
No determined									

C. Summary and thesis objectives

Currently, the antibiotic resistance is a threat for the human health and new prophylactic resources against infection diseases are urgently needed. According to a review on antimicrobial resistance (AMR), the human deaths due to AMR are expected to reach about 10 million per year in 2050 (O'neil, 2014). The difficulty to treat infectious diseases can be dramatic in case of infections by multi-drug resistance (MDR) pathogenic bacteria such as *P. aeruginosa* (Alnour and Ahmed-Abakur, 2017). This bacterium is naturally resistant to many common antibiotics and has the ability to acquire further resistance mechanisms to multiple groups of anti-bacteria agents (Kapoor and Murphy, 2018). Consequently, it is classified on the top of the priority list for the development of new antibiotics by the World Health Organization in 2017 (Tacconelli et al., 2018).

P. aeruginosa possesses two life-styles, planktonic and biofilm, allowing it to adapt to different environments, notably in the human body and on implanted devices. It is therefore a major cause of several chronic and acute infections (Furukawa et al., 2006). Furthermore, this bacterium harbors many virulence factors to invade organisms and to manipulate or kill host cells. For example: the flagella and Type IV pili for bacterial adhesion and motility, exopolysaccharides for biofilm formation, quorum sensing for bacterial communication and secretion systems for intoxication of eukaryotic cells (Figure 4). Most of these factors are crucial for the virulence of *P. aeruginosa* but not required for the bacterial growth and viability. Hence, the anti-virulence strategy aims at inhibiting only virulence factors of bacteria, thus eliciting lower evolutionary pressure for the resistance apparition than the classic antibiotics.

Once in planktonic life-styles, *P. aeruginosa* displays the type III secretion system (T3SS), its major virulence factor allowing the direct injection of four exotoxins from the bacterial cytoplasm into host cells. This nanomachine is constituted of more than 20 proteins and could be divided into four parts: cytoplasmic components including a cytoplasmic ring and an export apparatus, a basal body in the bacterial membrane, a needle complex and translocators in the eukaryotic cell membrane (Figure 14). In the absence of the T3SS, *P. aeruginosa* normally grows but is much less virulent than the wild-type strain. Therefore, inhibition of this system is a potential strategy in the context of AMR.

As a consequence, the first goal of my thesis was to study the functioning of the T3SS in *P. aeruginosa* to better characterize potential anti-virulence targets. An essential protein of this system is the ATPase PscN, considered to interact with the secreted Type III proteins and to deliver them to the secretion channel (Table 4). Upon hydrolyzing ATP, the ATPases from this family were shown to dissociate chaperone-protein complexes and to unfold secreted proteins in order to allow their passage through the narrow T3SS needle (Akeda and Galan, 2005; Yoshida et al., 2014). Therefore, my objective was to characterize activities of the ATPase PscN and give insights into its participation to the assembly

and functioning of the T3SS in *P. aeruginosa*. Furthermore, the result of this study could allow to look for and to develop chemical compounds inhibiting activities of PscN, thus impairing the T3SS and the virulence of this bacterium.

Secondly, I took the opportunity to characterize compounds formerly identified by a screening as inhibitors of the interaction between PscE and PscG, the two cognate chaperones present in the bacterial cytoplasm in complex with the needle protein PscF. This screening was previously performed by the former post-doc Sophie Plé, who also pointed out that the interaction of PscE, PscF and PscG inside the bacteria is important for their stability and that inhibition of their interaction leads to the degradation of these proteins, thus inhibiting the T3SS needle assembly. Actually, single or double point mutations introduced within the binding sites of PscE-PscG lead to a decrease or abolition of *P. aeruginosa* virulence (Ple et al., 2010). A series of chemical compounds was identified, inhibiting *in vitro* the interaction between the two proteins and the analogs of the two best hit compounds were synthesized in collaboration with Yung-Sing Wong (DPM Lab, Grenoble). Hence, my second objective was to develop different assays and to characterize effects of these analog compounds including their toxicity and their efficacy *ex vivo* on cells and *in vivo* on bacteria or on animal models. The approaches developed in this work could also be utilized to characterize compounds inhibiting the activity of PscN as described above.

Chapter II

Results

In order to comply with the rule of the EDCSV-UGA doctoral school, each part of this chapter includes an abstract, an introduction and a conclusion in French.

A. PopN acts on PscN to control needle secretion by the Type III Secretion System in *Pseudomonas aeruginosa*

I. Abstract (in French)

Contexte : Le Système de Sécrétion de Type III (SST3) est un des facteurs de virulence les plus importants de *P. aeruginosa*. Il est constitué par plusieurs protéines dont l'ATPase PscN qui joue un rôle indispensable pour son assemblage et son fonctionnement. Cette enzyme est capable de se lier et dissocier des complexes chaperonnes-substrats du SST3 avant leur sécrétion. Ainsi, elle pourrait être impliquée dans la hiérarchie de sécrétion de ces protéines par un mécanisme qui reste à déterminer.

Résultats: Un vecteur d'expression codant pour PscN fusionné avec un Flag-tag et deux Strep-tag nous a permis de purifier cette protéine par chromatographie d'affinité. *In vitro*, l'interaction de PscN avec des composants du SST3 a été étudiée grâce à un test ELISA et confirmé par HTRF (Homogeneous Time Resolved FRET) et MST (MicroScale Thermophoresis). En particulier, cette dernière méthode a permis d'estimer les constantes de dissociation (Kd) qui représentent l'affinité entre une protéine et son ligand. Les résultats montrent que PscN a plus d'affinité pour des complexes chaperonne/protéine sécrétée que pour des chaperonnes toutes seules, ce qui est cohérent avec le fait que les chaperonnes sont libérées dans le cytoplasme des bactéries après la dissociation du substrat sécrété. Par ailleurs, PscN a la capacité de se lier avec les complexes de l'effecteur ExoU-SpcU, des translocateurs PcrH-PopB/D, de l'aiguille PscE-PscF-PscG et du « gate-keeper » PopN-Pcr1-Pcr2-PscB (PopN complexe). Parmi ces complexes, ExoU-SpcU a le plus de affinité avec PscN alors que PscE-PscF-PscG a un très grand Kd et probablement n'interagit pas avec l'ATPase à des concentrations physiologiques dans les conditions testées. Toutefois, en présence du complexe « gate-keeper » PopN, l'affinité entre le complexe de l'aiguille et l'ATPase augmente considérablement. Ce résultat suggère que le complexe PopN agit sur PscN pour contrôler la reconnaissance de PscE-PscF-PscG à la porte de sécrétion du SST3 et ainsi la sécrétion de la protéine de l'aiguille PscF.

II. Introduction (in French)

Le système de sécrétion de type III (SST3) est présent chez plusieurs bactéries pathogènes à gram-négatif et responsable de l'injection direct d'exotoxines bactériennes dans des cellules hôte (Hauser, 2009). Une protéine indispensable de ce système est l'ATPase SctN car en son absence, aucune protéine du SST3 n'est sécrétée et les bactéries ne peuvent pas intoxiquer les cellules eucaryotes.

Fonctionnellement, il est décrit dans la littérature que cet enzyme est capable d'interagir avec des protéines secrétées du SST3 y compris des effecteurs, des translocateurs et le « gate-keeper ». En plus, elle assure la dissociation des complexes chaperonne-substrat avant la sécrétion de ces substrats protéiques (Table 4).

La sécrétion des protéines du SST3 doit être régulée de sorte que les « early substrate » (par exemple: protéine de l'aiguille) soit secrétés en premier. Ensuite, les « middle substrate » (protéines du translocon et la pointe de l'aiguille) sont secrétés pour former un pore dans la membrane de la cellule eucaryote et, enfin, les « late substrate » (des exotoxines) sont injectés dans les cellules hôte. La régulation de la sécrétion est un processus compliqué qui nécessite une interaction entre différentes protéines régulatrices comme la « ruler » SctP (Ho et al., 2017; Journet et al., 2003), la « switch » SctU (Monjarás Feria et al., 2015; Wood et al., 2008), le « gate-keeper » SctW (Roehrich et al., 2017; Yang et al., 2007), le « tip » SctV (Lee et al., 2014) et trois protéines cytoplasmiques SctO, SctK and SctQ (Lara-Tejero et al., 2011). En outre, il faut noter que l'ATPase SctN est capable d'interagir avec les protéines secrétées du SST3 et elle serait ainsi probablement le premier partenaire de ces protéines car cet enzyme se trouve à l'extrémité cytoplasmique du SST3 (Figure 26). Par conséquent, il est possible que SctN soit impliquée dans la hiérarchie de sécrétion.

Chez *P. aeruginosa*, l'ATPase PscN présente une forte similarité de séquence avec d'autres SctN (Zarivach et al., 2007). Cet enzyme jouerait donc un rôle essentiel pour l'assemblage et le fonctionnement du SST3 dans *P. aeruginosa*. Ainsi, nous avons purifié cette protéine et l'avons caractérisée biochimiquement pour mettre en évidence ses interactions avec des protéines secrétées du SST3. Ensuite, l'affinité de PscN avec ses protéines partenaires a été évaluée, ce qui nous permet de comprendre mieux son rôle dans le fonctionnement du SST3 et de présenter l'hypothèse d'un mécanisme par lequel l'ATPase participerait à la régulation de la sécrétion des protéines du SST3.

III. Result presented in article format

The experiments included in this paper were mainly performed by the PhD student Tuan-Dung NGO with the help of

- Caroline PERDU for the creation of the *ΔpscN* mutant strain and preparation of mass spectrometry samples.
- Bakhos JNEID for setting up the interaction assay based on ELISA
- Michel RAGNO for the assistance with protein purification

The illustrations and first version of this article were prepared by the PhD student Tuan-Dung NGO and the text was then edited by other authors.

The gate-keeper PopN complex acts on the ATPase PscN to regulate the T3SS secretion switch from early to middle substrates in *Pseudomonas aeruginosa*

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❖ Abstract

Pseudomonas aeruginosa is an opportunistic bacterium whose main virulence factor is the Type III Secretion System. The PscN ATPase of this machinery is considered to be localized at the bacterial cytoplasmic extremity of the apparatus and to participate to the recognition, chaperone complex dissociation and unfolding of exported T3SS proteins. A protein-protein interaction ELISA assay revealed the interaction of PscN with a wide range of exported T3SS proteins including needle, translocator, gate-keeper and effector. These interactions were further confirmed by Microscale Thermophoresis that also indicates a preferential interaction of PscN with secreted proteins or protein-chaperone complex rather than with chaperones alone, agreeing with the release of the chaperones in the bacterial cytoplasm after the dissociation from their exported proteins. Moreover, we disclose a new role of the gate-keeper complex and the ATPase in the regulation of early substrates recognition by the T3SS. This finding shed a new light on the mechanism of secretion switch from early to middle substrates.

❖ Introduction

The Type III Secretion System (T3SS) is present in many gram negative bacteria, being responsible for the delivery of effector proteins directly into eukaryotic cells. This nanomachine is constituted of more than 20 proteins mainly assembled in oligomers, which are located in the bacterial cytoplasm, bacterial inner membrane, bacterial periplasm, and bacterial outer membrane. An extracellular protrusion, the needle, connects the bacterial apparatus to the host membrane and cytoplasm. The conserved ATPase SctN is essential for the T3SS function and bacteria deleted of the corresponding gene *sctN* display hampered T3SS secretion and cannot intoxicate eukaryotic cells. This enzyme is localized at the bacterial cytoplasmic extension of the T3SS and indirectly interacts with the membrane-associated proteins of the Type III C-ring SctQ and the basal body.

The T3SS ATPase was first reported to belong to the AAA+ superfamily whose members are characterized by the formation of a hexameric ring and whose protein substrate is unfolded and translocated through the pore of this ring upon ATP hydrolyzing (Akedo and Galan, 2005; Sauer and Baker, 2011). Then, the atomic structure of this enzyme was determined, revealing a structural homology to the F1-ATPase rather than the AAA+ ATPase (Erhardt et al., 2010; Gao et al., 2018). This enzyme is thus suggested to assemble into a hexameric ring but the central cavity is blocked by the stalk SctO whose role and structure are similar to the γ subunit of F1-ATPase (Gao et al., 2018). Indeed, the T3SS ATPase was shown to form hexameric rings or perhaps dodecamer made of two stacked hexamers (Akedo and Galán, 2004; Andrade et al., 2007; Chatterjee et al., 2013; Claret et al., 2003; Müller et al., 2006). However, Burgess et al. recently reported a trimeric form of SctN in *Shigella flexneri* (Burgess et al., 2016a, 2016b).

The structure of the N-terminus truncated monomer of SctN was characterized by X-ray crystallography, showing two main domains (Allison et al., 2014; Burgess et al., 2016b; Zarivach et al., 2007). The C-terminus domain contains five α -helices and is considered to interact with the secreted T3SS proteins in the first step of the secretion process. Indeed, some single mutations in this domain abolish the binding of SctN to effector protein (Allison et al., 2014). The central domain displays a high similarity to the ATPase catalytic domain with mixed α/β Rossmann fold containing a parallel nine-stranded β -sheet flanked by three and four α -helices on either sides. The Walker A and B motifs, which are responsible for the catalytic activity, are also found in this domain. Based on the similarity with the α/β subunit of F1-ATPase, a model of the homo-hexameric ring SctN was built *in silico* with the ATP binding pocket interfaces located between two adjacent subunits of SctN (Allison et al., 2014; Burgess et al., 2016b). It was thus suggested that the oligomerization state is essential for the catalytic activity toward ATP. Despite the absence of available structure of the full-length protein, the N-terminal domain of SctN was shown to be important for the oligomerization and the membrane association. Actually, the single mutation V51E in the *Salmonella enterica* InvC ATPase impaired the ability to associate with the bacteria membrane.

Functionally, T3SS ATPases are proposed to be involved in the secretion process by acting on three different steps. Firstly, it participates in the recognition of the exported proteins at the entrance of the export apparatus. Secondly, upon hydrolyzing ATP, this enzyme was shown to provide energy for the dissociation of chaperone-protein complexes, as shown for effector (Akeda and Galan, 2005; Lorenz and Buttner, 2009) and filamentous-tip proteins (Yoshida et al., 2014). Finally, the ATP hydrolysis energy could be used to unfold secreted proteins in order to allow their passage through the narrow T3SS needle (Akeda and Galan, 2005). The interaction of SctN with effector proteins and their chaperones (Akeda and Galan, 2005; Akeda and Galán, 2004; Allison et al., 2014; B. B. Finlay, 2003; Cooper et al., 2010; Lorenz and Buttner, 2009; Yoshida et al., 2014; Zarivach et al., 2007), a tip-filamentous protein (Chen et al., 2013) and a translocon chaperone protein (Yoshida et al., 2014) was shown *in vitro*. Based on structural modelling, the chaperone of an effector was shown to dock to the loop of the two-helix-finger motif in the C-terminal of SctN (Allison et al., 2014). Thus, this region was pointed out as an important site for the recognition of secretion substrate by SctN. However, whether the exact binding site on SctN is identical for all the exported proteins remains to be investigated.

In addition to these roles, it might be possible that SctN is involved in the regulation of the secretion process by

participating to the substrates sorting, thus allowing them to be secreted hierarchically. To assemble the T3SS, the needle protein (early substrate) is first secreted, followed by the translocator (middle substrate) secretion and the effector (late substrate) is then secreted and translocated into host cells at the final stage of the secretion process. Nonetheless, the sorting of substrates for the secretion is a complex mechanism and undoubtedly requires an interplay between the known, and maybe unknown, regulator proteins such as SctP that controls the length of T3SS needle (Ho et al., 2017; Journet et al., 2003; Wagner et al., 2010), SctU that controls the switch from early to middle substrates (Edqvist et al., 2003; Monjarás Feria et al., 2015; Shen et al., 2012; Wood et al., 2008), SctW that controls the switch from middle to late substrates (Ferracci et al., 2005; Martinez-Argudo and Blocker, 2010; Roehrich et al., 2017; Yang et al., 2007), the putative negative effector regulator SctV in complex with its cognate chaperone in *Pseudomonas aeruginosa* and *Yersinia pestis* (Lee et al., 2014; Matson and Nilles, 2001; Nilles et al., 1997; Sundin et al., 2004) and other cytoplasmic proteins that were proposed to be the substrate sorting platform SctO, SctK and SctQ (Lara-Tejero et al., 2011). Indeed, the global collaboration between these proteins and possibly the ATPase SctN to control the secretion process is still unclear.

In *P. aeruginosa*, the T3SS ATPase is named PscN. In this study, the full-length PscN was fused to tandem tags which promoted its high expression in a modified *P. aeruginosa* strain and allowed its purification. With an ELISA-based assay, we confirmed the interaction of PscN with a variety of secreted T3SS proteins and revealed for the first time the interaction with the needle complex (PscE-PscF-PscG). The dissociation constants of PscN and its partners were further determined by microscale thermophoresis (MST), showing a higher affinity between PscN and the protein complexes than with the chaperones alone. Finally, examining the affinity of PscN alone or bound to the gate-keeper complex PopN-Pcr1-Pcr2-PscB, revealed that this complex can modulate partners recognition by PscN, suggesting a new mechanism by which PscN participates in the regulation of the secretion process.

❖ Results

The ATPase PscN interacts *in vivo* with a wide range of T3SS proteins. The ATPase SctN is anchored to the T3SS apparatus through interactions with the stalk SctO and the stator SctL. To look for soluble partners of this protein in *P. aeruginosa*, the full-length PscN was fused to a Strep-tag and expressed in the *P. aeruginosa* CHA strain harboring a chromosomal deletion of the *pscN* gene. The Strep-PscN was then pulled-down on Streptactin beads and the corresponding fractions were next analyzed by mass spectrometry. Among

the identified proteins, several T3SS proteins were co-purified with PscN, including the exoenzymes S, T, Y, the translocator PopB, PopD and their cognate chaperone PcrH, the stator PscL, the chaperone PscB of the gate-keeper complex and two proteins involved in T3SS transcriptional regulation: ExsD and ExsA (Table 1). Most of these partner proteins were shown to be secreted by the T3SS (Belyy et al., 2018; Goure et al., 2004; Maresso et al., 2006; Shen et al., 2008; Yahr et al., 1996; Yang et al., 2007) being consistent with a role of the ATPase PscN in the recognition of exported protein as the first step of the secretion process.

Overexpression of PscN fused to a triple tag in a modified *P. aeruginosa* strain allows high purification yield of the full-length protein. Based on sequence similarity, PscN is predicted to be the ATPase of the *P. aeruginosa* T3SS but it has never been characterized biochemically due to the difficulty to purify it. After several attempts with a construct harboring a StrepTag at PscN N-terminus, a fusion protein with one Flag and one Twin-Strep tag fused to the N-terminus of *pscN* (called FSS-PscN) was expressed in *E. coli* BL21 and purified using Streptactin Sepharose (GE) beads. Strikingly, FSS-PscN showed a higher yield of purification than the Strep-PscN (Figure 1A). This could be due to the fact that the Twin-Strep tag increases the binding to the affinity beads in comparison to a single Strep-tag. Moreover, the introduction of the triple tag lowers the pI of this enzyme from 7 to 6.2, possibly making it more soluble in the working buffer. Nonetheless, a high amount of one contaminant protein, identified as GroEL by mass spectrometry (data not shown), was present above the band corresponding to PscN. The presence of GroEL suggested that the FSS-PscN folding is challenging in *E. coli*. We therefore tested the expression of FSS-PscN in *P. aeruginosa* ADD1976, which is an engineered *P. aeruginosa* strain adapted to the pET system (Table 2). Remarkably, a higher amount of purified protein and a lower contamination were observed in comparison to the expression in *E. coli* (figure 1A).

To ascertain whether the long tag (50 residues) could affect the conformation and function of the protein, we compared the cytotoxicity of the wild-type strain to the one of a deletion mutant strain Δ *pscN* complemented with *fss-pscN* cloned into the vector pIApG under the control of a T3SS promoter. For this purpose, the cytotoxicity of these strains was tested by incubating them with the macrophage J774 cell-line and the lactate dehydrogenase (LDH) release was measured to monitor cell death. Indeed, the complementation with FSS-PscN could restore the virulence of *P. aeruginosa* mutant strain Δ *pscN* (Figure 1B), thus indicating that the introduction of one Flag and two Strep tags is not deleterious for the activity of PscN.

Furthermore, FSS-PscN was incubated with ATP at 37°C and the released phosphate was measured using a

colorimetric malachite green assay to test whether the full-length purified enzyme is catalytically active *in vitro*. The ATPase PscN displayed a specific activity of 0.1 μ mol/min/mg which was comparable to other T3SS ATPases including FliI, EscN, InvC, SsaN and HcrN (Andrade et al., 2007; Claret et al., 2003; Kato et al., 2015; Lorenz and Buttner, 2009; Yoshida et al., 2014). This result confirmed that the full-length purified FSS-PscN was fully active. Moreover, the catalytic activity of PscN requires the presence of Mg^{2+} or Zn^{2+} and was optimal at pH 9 (Figure S1).

Confirmation of PscN interaction with effector, translocator, needle and gate-keeper proteins and complexes by ELISA and HTRF. It has been shown that the T3SS SctN interacts with exported proteins such as effector, translocator and gate-keeper proteins (Akeda and Galan, 2005; Akeda and Galán, 2004; Allison et al., 2014; B. B. Finlay, 2003; Botteaux et al., 2009; Cooper et al., 2010; Lorenz and Buttner, 2009; Zarivach et al., 2007). To explore the binding spectrum of the ATPase PscN from *P. aeruginosa*, an ELISA-based assay was performed with purified FSS-PscN and purified partner proteins including effector, translocator, needle and gate-keeper proteins (the cargos) and their chaperones in complex or alone. The partner proteins fused to a 6His-tag at the N-terminus were purified on a Nickel affinity column, followed by size exclusion chromatography (SEC). These potential partner proteins were then coated on an ELISA plate and Bovine Serum Albumin (BSA) and PBS were used as negative controls. Afterward, FSSN was incubated at four different concentrations and its binding was detected by anti-PscN and secondary HRP-conjugated antibodies. The signal intensity was much higher with the tested proteins in comparison to negative controls and was dependent on the concentration of PscN (Figure 2). These results gave evidences that PscN interacts with the tested proteins, thus confirming the role of the ATPase PscN in the recognition of exported proteins for the first step of the secretion process.

To examine whether recombinant PscN could interact in solution with secreted proteins bound to their cognate chaperones, an HTRF assay was employed with four protein complexes including ExoU-SpcU (effector complex), PcrH-PopD (translocator complex), PscE-PscF-PscG (needle complex) and PopN-Pcr1-Pcr2-PscB (gate-keeper complex). For this assay, we took advantage of the presence of a Flag-tag on PscN and a His-tag on its partners, enabling their labelling with antibodies directed toward these tags. Two pairs of donor and acceptor antibodies were used: anti-Flag-M2-Eu cryptate and anti-His-d2 or anti-Flag-M2-d2 and anti-His-Tb. However, only interaction of PscN with the translocator and gate-keeper complexes could be detected (Figure S2), thus leading to three hypothesis: i) the effector

and needle complexes do not interact in solution with the ATPase PscN; ii) the His-tags of effector and needle complexes are not accessible upon binding to PscN and iii) the binding site of the effector and needle complexes on PscN is different from that of the translocator and the gate-keeper complexes. Consequently, signal could only be detected with those complexes that are closed enough to the fluorophore-tagged N-terminus of PscN.

PscN binds to several classes of T3SS complexes and has a higher affinity for the effector complex. Another assay detecting protein binding in solution was required to investigate whether the absence of HTRF signal was artefactual. Therefore, microscale thermophoresis (MST) was employed to confirm the binding in solution and to determine the affinity between the ATPase PscN and the exported proteins in *P. aeruginosa*. In these experiments, PscN was incubated with different concentrations of protein ligands and a modification of its thermophoresis signal indicated a binding. A titration curve for each ligand, which represents the bound fraction depending on ligand concentrations, was obtained and the determination of the dissociation constants (K_d) was based on data from three independent replicate experiments. As shown by the binding curves, PscN and the effector complex ExoU-SpcU displayed a high affinity ($K_d = 45$ nM). In contrast, the measured affinity was lower with the translocator complex PcrH-PopB/D ($K_d = 4.7$ μ M) and the gate-keeper complex PopN-Pcr1-Pcr2-PscB ($K_d = 0.8$ μ M). The K_d of PscN and the needle complex (PscE-PscF-PscG) could not be determined due to a much lower affinity (predicted $K_d > 150$ μ M) (Figure 3A and Table S2), suggesting that the ATPase PscN probably did not interact with needle proteins in these conditions.

PscN preferentially interacts with the protein complexes than with chaperones alone. The SctN ATPase was shown to dissociate the cargo-chaperone complex by using energy from ATP hydrolysis in *S. typhimurium* (Akeda and Galan, 2005; Yoshida et al., 2014) and *Xanthomonas* (Lorenz and Buttner, 2009). It is likely that the cargo protein is subsequently secreted through the secretion channel while the chaperone is proposed to be released in the bacterial cytoplasm. To verify this hypothesis, we examined the interaction preference of PscN for the complex, cargo or chaperone proteins. Through titration experiment using MST, the K_d s of the ATPase PscN and the effector ExoU or the cargo-chaperone complex ExoU-SpcU were measured to be 31 nM, 45 nM respectively. In contrast, the K_d of PscN and the chaperone alone was, 1000 times higher, 41 μ M (Table S2 and Figure 3B). In similar experiments with the translocator chaperone PcrH bound or not to its cargos PopB and PopD, the K_d s of the ATPase and the complex PcrH-

PopB, 4.9 μ M, or PcrH-PopD, 4.8 μ M, were 6 fold-lower than the one of PscN and the chaperone PcrH alone ($K_d = 31$ μ M) (Figure 3C). Unfortunately, it is not possible to assess the K_d of PscN and PopB or PopD alone because these proteins are not stable *in vitro* without their cognate chaperone PcrH (Faudry et al., 2007; Wager et al., 2013). Nevertheless, taken together these results indicate that PscN would rather bind to cargo or complex proteins than to the chaperone protein alone. This confirms the hypothesis that the chaperones are released in the bacterial cytoplasm after the complex dissociation.

The gate keeper complex acts on PscN to regulate the binding of the needle complex protein to the ATPase. It has been shown that the gate-keeper protein SctW is involved in the secretion switch between translocator and effector proteins. A Δ sctW mutant strain was demonstrated to over-secrete the effectors in *P. aeruginosa*, *Shigella* and *Yersinia*. In contrast, the effect of this deletion mutation on the translocator secretion is controversial (Ferracci et al., 2005; Roehrich et al., 2017; Yang et al., 2007). Interestingly, this protein SctW, which is secreted by the T3SS, was shown to interact with the ATPase SctN in *Shigella* and the authors suggested that the gate-keeper inhibited the effector secretion by blocking its binding sites on the ATPase (Botteaux et al., 2009). As described in this work, ELISA, HTRF and MST assays showed that PopN (SctW in *P. aeruginosa*) complex interacts with the ATPase PscN. Therefore, to examine whether PopN complex could play a role in the substrate sorting through binding to the ATPase, K_d s were measured between the effector, translocator or needle complexes and PscN bound or not to the gate-keeper complex. To this purpose, PscN was previously incubated with the PopN complex at a concentration of 800 nM equal to the K_d , yielding therefore half of the PscN population bound to the PopN complex. Unfortunately, it was not possible to use higher PopN complex concentration in this experiment because of the yield of its purification. Since only PscN is monitored by MST, a change in the apparent K_d would indicate an effect of the bound gate-keeper complex in the recognition capacities of the ATPase. While PscN alone almost does not bind to the needle complex (K_d estimated > 45 μ M), the apparent K_d of PscN in presence of the gate-keeper was higher for PscE-PscF-PscG than for PcrH-PopB, 5.6 μ M and 12.3 μ M respectively (Table S2 and Figure 4). This result showed that binding of the gate-keeper to PscN dramatically modified its relative affinities for the translocator complex PcrH-PopB and the needle complex PscE-PscF-PscG, thus promoting the loading of the needle to PscN instead of the translocator. On the other hand, PopN complex had no effect on the binding of ExoU-SpcU to the ATPase PscN (Figure 4), indicating that SctW does not block effector secretion through a direct competition for SctN

binding, on the contrary to a suggested hypothesis (Botteaux et al., 2009).

However, it would be possible that a direct interaction between the PscF complex and the PopN complex bound to PscN would be responsible for the apparent increase of the PscN affinity for the needle complex protein. To rule out this possibility, SEC analysis of the individual complexes and a mixture of the PscF and PopN complexes were performed. The superposition of the chromatograms clearly shows that there was no interaction between the needle and gate-keeper complexes while this assay was performed at protein concentration close to the highest concentration used in MST (Figure S3). Therefore, this work disclosed a new role of the gate-keeper and the ATPase in the control of substrate hierarchical secretion in *P. aeruginosa*.

❖ Discussion

The ATPase SctN is essential for the assembly and function of the T3SS. It was first described to interact with exported proteins in *Salmonella*, *E. coli*, *Shigella* and *Xanthomonas* species (Akedo and Galan, 2005; Akedo and Galán, 2004; Allison et al., 2014; B. B. Finlay, 2003; Botteaux et al., 2009; Cooper et al., 2010; Lorenz and Buttner, 2009; Yoshida et al., 2014; Zarivach et al., 2007). However, an extensive comparison of the interactions between the ATPase and the secreted proteins remained to be performed to get insight in its function.

To date, only structures of the N-terminus truncated monomers of SctN were obtained by X-ray crystallography (Allison et al., 2014; Burgess et al., 2016b; Zarivach et al., 2007), suggesting that the N-terminal domain of SctN is flexible and/or partially unfolded. Some groups were able to express and purify the His-tagged recombinant full-length protein from *E. coli* (Akedo and Galan, 2005; Andrade et al., 2007; Chatterjee et al., 2013; Yoshida et al., 2014). Unfortunately, we were not able to obtain the ATPase PscN (SctN name in *P. aeruginosa*) in the same production condition despite many attempts. This indicates that although the ATPase SctN shares 40-50% of sequence identity among the gram-negative pathogenic bacteria (Zarivach et al., 2007), the T3SS ATPase of *P. aeruginosa* is more difficult to obtain than the one of other bacteria. Therefore, in order to get the purified protein, we fused PscN to a triple tag and achieved efficient production and purification from an engineered *P. aeruginosa* strain (Arora et al., 1997; Brunschwig and Darzins, 1992). Consequently, we could obtain recombinant full-length PscN in sufficient quantity for biochemical analysis. Furthermore, this purified PscN is catalytically active and the relatively long tag at the N-terminus is not deleterious for its activity *in vivo*.

By using a protein-protein interaction ELISA assay, we demonstrated that the ATPase PscN interacts with the chaperones, cargos and complexes of effector, translocator, needle and gate-keeper proteins. Of importance, the interaction of the ATPase with the needle complex is described for the first time, thus complementing the overview of T3SS ATPase binding capacity. This result was next confirmed by MST assay and the affinity between PscN and the four complexes was determined, showing that PscN has higher affinity for effector proteins, less for translocator and gate-keeper proteins and does not bind to needle protein in the tested concentration range. Furthermore, PscN preferentially interacts with complexes than with the chaperone proteins. Thus, this confirms that the chaperones are released in the bacterial cytoplasm after the dissociation from cargo proteins.

Using structural modeling *in silico*, chaperones of effector were docked to the loop of a two-helix-finger motif in the C-terminal of SctN (Allison et al., 2014; Zarivach et al., 2007) and the mutation of a conserved residue (V379P) in this region abolished the interaction of the ATPase SsaN and the effector chaperone SrcA in *S. enterica* SPI-2 *in vitro* (Allison et al., 2014). Interestingly, the corresponding mutation (L376P) was also identified from the screening of loss-of-function mutations of the ATPase InvC in *S. enterica* SPI-1 and affects the secretion of the early substrate InvJ, middle substrates SipB/C and the effector SctP (Akedo and Galán, 2004). Hence, this two-helix-finger region was pointed out as the binding site of SctN to exported proteins in T3SS and these proteins might compete to dock to this site on the ATPase. In this case, it would be tempting to speculate that different affinities would dictate the order of proteins secretion. Nonetheless, the affinity of PscN for translocator ($K_d = 4900$ nM) and effector ($K_d = 45$ nM) seems contradictory to the hierarchical secretion of these substrates because the translocator are secreted prior to the effector. Actually, an interpretation solely based on differences in affinities would be too simplistic. First, because the concentrations of the different PscN partners in the bacteria are not known and second because these partners are continually expressed after T3SS activation and a significant decrease of their concentrations during T3SS secretion is speculative. On the other hand, PscN bound to either ExoU-SpcU or PcrH-PopB had almost the same affinity for each substrate as the free-PscN (Figure S4). Therefore, ExoU-SpcU and PcrH-PopB seem not to compete each other to dock to the ATPase PscN.

The different binding site of substrates on the ATPase could be supported by the fact that no inhibition effect on the binding of PscN to ExoU-SpcU, PcrH-PopB and PscE-PscF-PscG was observed in the presence the gate-keeper PopN complex. In contrast, binding of PopN complex to PscN promoted an increase in affinity between PscN and PscE-

PscF-PscG. We therefore propose a model of allosteric conformational change of PscN that regulates its recognition of the needle protein complex (Figure 5). This allosteric regulation of SctN would participate to the mechanisms of the T3SS secretion switch from early to middle substrate, which also involves SctP and SctU but that are still globally unclear.

Actually, the T3SS needle length is controlled by the ruler SctP (Ho et al., 2017; Journet et al., 2003; Wagner et al., 2010) while the auto-cleavage of SctU allows the secretion of translocators and effectors (Frost et al., 2012; Sorg et al., 2007; Wood et al., 2008). However, the stop of further needle elongation and the switching of substrate secretion might be operated by two separated mechanisms because some mutations preventing auto-cleavage of SctU abolished secretion of middle and late substrates but did not affect the control of early substrate secretion and the needle length (Monjarás Feria et al., 2015; Wood et al., 2008). The termination of the needle elongation might therefore require another mechanism, independent from SctU. Indeed, focused on exported substrates recognition by the T3SS before secretion, our findings give new insights on the regulation of needle assembly. We propose a new function of the gate-keeper SctW complex, which would act on the interaction of SctN with the needle SctF complex. We suppose that SctN binds to SctW complex in the resting state before the beginning of the T3SS secretion process (Figure 5). This binding would allow the needle complex recognition by the T3SS through the interaction with the ATPase. When the T3SS needle reaches the expected length, a signal is transmitted probably from the ruler SctP to proteins present in the cytoplasmic export gate including the ATPase bound to the gate-keeper complex. This signal could then stimulate the release of the SctW complex from SctN, which in turn would decrease its affinity for the needle complex and prevent the recognition of this complex by the T3SS. The release of the gate-keeper complex from the ATPase is supported by the fact that in *P. aeruginosa*, the gate-keeper proteins is also shown to be tethered to the inner-membrane protein PcrD (SctV name in *P. aeruginosa*) and to assemble into a complex with PcrG (the chaperone of the tip protein PcrV in *P. aeruginosa*) where they have a second function, the regulation of the next secretion process switch from middle to late substrates (Lee et al., 2014), along with the sorting platform (Lara-Tejero et al., 2011).

Localized at the cytoplasmic extremity of the apparatus, the T3SS ATPase may be the first platform that screens and loads exported substrates to the T3SS. The loaded proteins would be next secreted in a timing that integrates signals from regulators such as the ruler SctP, the switch SctU and/or the gate-keeper SctW. Of importance, our findings reveal a new role of the gate-keeper complex in the control of needle subunit secretion, thus complementing an overview for the

mechanisms by which the secretion switches from early to middle substrates.

❖ Materials and methods

Bacterial strains and plasmids. The *E. coli* and *P. aeruginosa* strains and plasmids used in this study are listed in the Table 2. The *P. aeruginosa* deletion strain $\Delta pscN$ was constructed using the method of Slice Overlapping Extension PCR (SOE-PCR). The Strep-tagged PscN construction was generated by PCR using primers NdeI-Strep-PscN and PscN-HindIII (Table S1) and the amplified fragments were cloned into the pIApG plasmid. To generate Flag-Strep-Strep-PscN construct, a DNA fragment containing NcoI-Flag-Strep-Strep-NterminusPscN-NotI synthesized by Invitrogen, was cloned into the NcoI/NotI sites of pIApG-Strep-PscN. Both constructions pIApG-Strep-PscN and pIApG-FSS-PscN were transformed into *P. aeruginosa* CHA $\Delta pscN$. Otherwise, the genes encoding Strep-PscN and FSS-PscN were cloned from pIApG to pET15b or pET15bVP vectors using NcoI/BamHI sites. The pET15b-Strep-PscN, pET15b-FSS-PscN were next transformed into *E. coli* BL21 (DE3) while the pET15bVP-FSS-PscN was transformed into *P. aeruginosa* PAO1 ADD1976 (Arora et al., 1997; Brunschwig and Darzins, 1992).

LDH release assay. Macrophages from the J774 cell line were grown overnight in a 96 well plate at a density of 50000 cells per well. *P. aeruginosa* CHA WT, $\Delta pscN$, and $\Delta pscN$ /pIApG-FSS-PscN were grown at 37°C in LB medium to OD 1 and added to the J77 cells at a multiplicity of infection (MOI) of 10. Cell death was monitored at 2h, 3h and 4h post-infection using a cytotoxicity detection kit (lactate dehydrogenase (LDH); Roche). The assay was performed in triplicate.

Protein expression and purification. For PscN-partners pull-down experiments, *P. aeruginosa* CHA harboring pIApG-Strep-PscN was grown at 37°C in LB medium containing 20 mM of MgCl₂ and 5 mM of EGTA to OD 1. Harvested cells were resuspended in buffer containing 100 mM Tris pH8, 150 mM NaCl and 1 mM EDTA. Cells were lysed by sonication and 1% triton X100 was added to solubilize membrane fraction for 14h at 4°C. The non-soluble membranes were eliminated by ultracentrifugation at 125000g for 1h30 at 4°C and PscN in soluble fraction was purified using Streptrap HP column on an Akta purifier system. The purified Strep-PscN fractions were eluted in buffer containing 2.5 mM desthiobiotin and next analyzed by mass spectrometry.

E. coli BL21(DE3) harboring Strep-PscN and FSS-PscN and PAO1 ADD1976 harboring FSS-PscN were grown at 37°C in 500ml of LB medium with appropriate antibiotic and

were induced with 1mM IPTG at OD 1 for 2h at 28°C. Cells were harvested and resuspended in 20 ml of binding buffer containing 50 mM Tris pH9, 50 mM Arginine, 50 mM Glutamate, 150 mM NaCl, 10% Glycerol, 5 mM TCEP and 1% Triton X100. Cell suspension was lysed by sonication and centrifuged at 200000g for 30 minutes. Soluble proteins were next incubated with Streptactin sepharose beads for 15h at 4°C and consecutively washed with binding buffer containing 1% and 0.1% of triton X100. Proteins were then eluted with 2.5 mM desthiobiotin.

E. coli BL21 (DE3) harboring 6His tagged to ExoU-SpcU, ExoU, SpcU, PcrH-PopB, PcrH-PopD, PcrH, PcrV, PscEFG, PscE-PscG, PopN complex were grown at 37°C in 500 ml of LB medium with appropriate antibiotic and were induced with 1 mM IPTG at OD 0.6 for 3h. Harvested cells were resuspended in 20 ml of IMAC buffer (25 mM Tris pH8, 500 mM NaCl) containing 10 mM Imidazole. Cell suspension was lysed by sonication and centrifuged at 200,000 g for 30 minutes. Soluble proteins were purified using Histrap HP column on an Akta purifier system. The proteins were eluted by the IMAC buffer supplemented with 200 mM Imidazole and subsequently injected onto a Supedex 200 Increase size exclusion column pre-equilibrated with a buffer containing 50 mM Tris pH8 and 150 mM NaCl.

Protein quantification and ATPase activity. The fractions of all the purified protein were quantified on Agilent Bio-analyzer chips. Samples were prepared according to Agilent protein 80 kit and analyzed on a 2100 Bio-analyzer device (Agilent technologies). The ATPase activity of FSS-PscN was determined by measuring the release of total phosphate at 37°C for 30 minutes which is detected by the malachite green method (Casabona et al., 2013).

ELISA. 50µl of each tested proteins ExoU-SpcU, ExoU, SpcU, PcrH-PopB, PcrH-PopD, PcrH, PcrV, PscEFG, PscE-PscG, PopN complex diluted in PBS at 0.1µM, were coated on ELISA 96 well plates for 15 hours at 4°C. The BSA diluted in PBS at 0.1 µM and PBS buffer were used as negative controls. The wells were then washed three times with 200 µl of PBS/0.1% Tween 20 (PBST) and incubated with 200 µl of PBS containing BSA 4% for 4h. The solution was next removed and plates were washed three times. Afterward, 50 µl of purified FSS-PscN diluted in PBST containing BSA 4% (PBST.BSA 4%) at 12.5 nM, 25 nM, 50 nM and 100 nM, were incubated for 1h at room temperature (RT°). The wells were subsequently washed three times with 200µl of PBST and 50 µl of anti-PscN antibody (1/2000 in PBST.BSA 4%) were incubated for 1h at RT°. Three additional washes with 200 µl of PBST were performed before 50 µl of anti-rabbit-HRP antibody (1/40000 in PBST.BSA 4%) were added for 1h at RT°. The wells were then washed with PBST (three time) and ELISA revealed

using the Pierce ECL Western Blotting Substrate (ThermoFisher: 32106). The chemi-luminescence was measured using a FluoStar BMG reader.

HTRF. Purified FSS-PscN, ExoU-SpcU, PcrH-PopD, PscE-PscF-PscG and PopN complexes were diluted in PBS/0.1% Tween 20 containing BSA 0.05% (PBST.BSA 0.05%) at indicated concentrations. The fluorophore-conjugated antibodies Flag-d2, His-Tb, His-d2 were diluted in PBST.BSA 0.05% while Flag-Eu³⁺ was diluted in PBST.BSA 0.05% containing 400 mM KF. The concentration of fluorophore-conjugated antibodies was used following the recommendation from the manufacturer (Cisbio). Experiments were performed in 384 well plates with a total volume of 20 µl per well. Reagents were sequentially added: 5 µl PscN, 5 µl ligand protein and 5 µl anti-Flag-Eu³⁺/anti-His-d2 or anti-His-Tb/anti-Flag-d2. After 6 hours of incubation at room temperature, the fluorescence was measured on an Infinite M1000 Tecan reader.

Microscale thermophoresis (MST). Purified FSS-PscN fractions were buffer-exchanged to a buffer containing 50 µM HEPES pH 8, 150 mM NaCl, 10% glycerol and 0.1% triton X100 by using a desalting column PD Spin Trap G25 (GE healthcare: 28-9180-04). This FSS-PscN was then labeled with the Monolith Protein Labeling Kit RED-NHS (MO-L001) following the manufacturer instruction (Nanotemper) and diluted in the purification binding buffer (see above) containing 0.1% triton X100 and without TCEP. For MST measurements, labeled FSS-PscN was diluted at 100 nM in the buffer containing 50 mM Tris pH 6.7, 50 mM NaCl, 5 mM DTT and 0.1% Pluronic F127. The ligand proteins were prepared at indicated concentration in SEC buffer containing 50 µM Tris pH 8 and 150 mM NaCl. PscN and ligand were mixed in 1:1 v/v ratio, resulting in a final buffer which contains 50 mM Tris pH7.1, 100 mM NaCl, 2.5 mM DTT and 0.05% Pluronic F127. The thermophoresis signal was then measured on a Monolith NT.115 device (Nanotemper) with premium capillaries (MO-K025). Data from three independent replicates were used to estimate the Kd using MO.Affinity Analysis software provided by the manufacturer Nanotemper.

For competition assay, the same experiments were performed except that FSS-PscN was diluted at 100 µM in the buffer described above containing the partner protein at the indicated concentrations.

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❖ Author Contributions

TD.N. and E.F. designed experiments, TD.N., M.R., C.P., B.J., and E.F. performed experiments, TD.N., A.D., I.A., A.R. and E.F. analyzed and discussed the data, A.R. provided reagents. TD.N. and E.F. wrote the manuscript. All authors contributed to and edited the manuscript.

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Table 1: T3SS proteins detected by mass spectrometry after pull-down of Strep-PscN.

Description	Masse	Protein abundance (%)
ATP synthase in type III secretion system	48173	21,8
Exoenzyme S	48273	0,4
Type III export protein PscL	23982	1,2
Translocator protein PopB	39962	0,4
Putative uncharacterized protein exsD	31680	0,5
Type III secretory apparatus protein PcrD	77168	0,1
Exoenzyme T	48485	0,2
Regulatory protein PcrH	18615	0,6
Exoenzyme S synthesis regulatory protein exsA	31957	0,2
Adenylate cyclase ExoY	45500	0,1
Type III export apparatus protein pscB	15415	0,3
Translocator outer membrane protein PopD	31332	0,1

Table 2: Strains and plasmids used in this work.

Strain	Description	Source or reference
CHA <i>ΔpscN</i>	CHA <i>pscN</i> deletion mutant	This study
CHA <i>ΔpscN</i> /pIApG-Strep-PscN	<i>ΔpscN</i> carrying Strep-PscN in pIApG	This study
CHA <i>ΔpscN</i> /pIApG-Flag-Strep-Strep-PscN	<i>ΔpscN</i> carrying Flag-Strep-Strep-PscN in pIApG	This study
PAO1 ADD1976/pET15bVP-Flag-Strep-Strep-PscN	Flag-Strep-Strep-PscN expressed in modified PAO1 strain and in modified pET15b (N-terminal FSS)	This study (Arora et al., 1997; Brunschwig and Darzins, 1992)
BL21 (DE3)/pET15b-Flag-Strep-Strep-PscN	Flag-Strep-Strep-PscN expressed in BL21 and in pET15b (N-terminal FSS)	This study
BL21 (DE3)/pET15b-Strep-PscN	His-Strep-PscN expressed in BL21 and in pET15b (N-terminal His Strep)	This study
BL21 (DE3)/pET15b-PscE-PscF-PscG	PscE-PscF-PscG complex expressed in BL21 and in pET15b (N-terminal His)	(Quinaud et al., 2005)
BL21 (DE3)/pET15b-PscE-PscG	PscE- PscG complex expressed in BL21 and in pET15b (N-terminal His)	(Quinaud et al., 2005)
BL21 (DE3)/pET15b-ExoU	ExoU expressed in BL21 and in pET15b (N-terminal His)	(Gendrin et al., 2012)
BL21 (DE3)/pET15b-SpcU	SpcU expressed in BL21 and in pET15b (N-terminal His)	(Gendrin et al., 2012)
BL21 (DE3)/pET Duet-ExoU-SpcU	ExoU-SpcU complex expressed in BL21 and in pET15b (N-terminal His)	(Gendrin et al., 2012)
BL21 (DE3)/pET15b-PcrV	PcrV expressed in BL21 and in pET15b (N-terminal His)	(Goure et al., 2004)
BL21 (DE3)/pET15b-PcrH	PcrH expressed in BL21 and in pET15b (N-terminal His)	(Schoehn et al., 2003)
BL21 (DE3)/pET30b-PcrH-PopB	PcrH-PopB complex expressed in BL21 and in pET15b (N-terminal His)	(Schoehn et al., 2003)
BL21 (DE3)/pET30b-PcrH-PopD	PcrH-PopD complex expressed in BL21 and in pET15b (N-terminal His)	(Schoehn et al., 2003)
BL21 codon plus "RP"/pET28bTEV- His-PopN-Pcr1-Pcr2-PscB	PopN complex expressed in BL21 and in pET28b (N-terminal His)	Arne Rietsch, USA (This study)

Figure 1: Fusion of a triple tag to PscN allows high purification yield upon expression in a modified *P. aeruginosa* strain and does not impair its activity. (A) Strep-PscN and FSS-PscN were expressed in *E. coli* BL21 and/or *P. aeruginosa* PAO1 ADD1976 and purified using Streptactin sepharose beads. Elution fractions from each condition were analyzed by Coomassie Blue. (B) Macrophages from the J774 cell line were incubated with *P. aeruginosa* CHA WT, $\Delta pscN$ or complemented strains at MOI of 10. Cell death was monitored at 2h, 3h and 4h post-infection through lactate dehydrogenase release quantification.

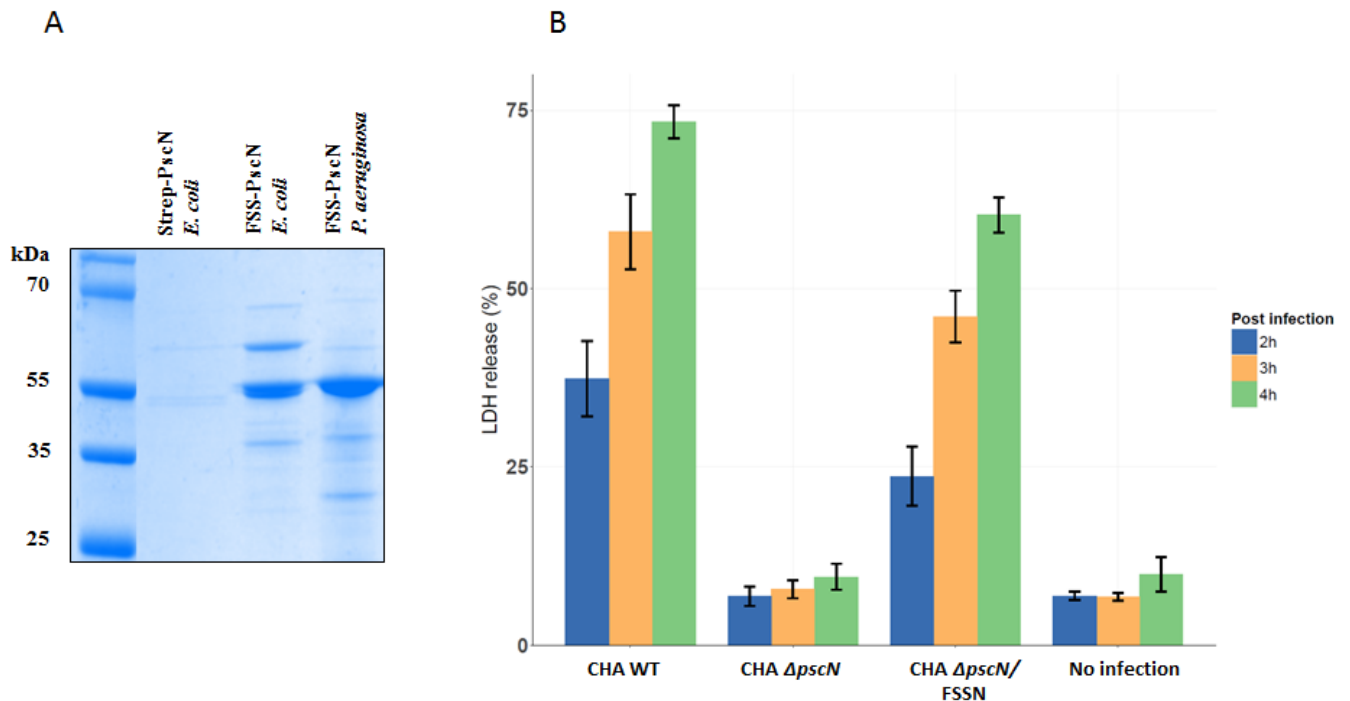


Figure 2: The ATPase PscN binds to T3SS soluble partners and their chaperones. The T3SS exported proteins, their chaperones alone or in complex were purified on Nickel column and 0.1 μ M of each protein was next used to assess their interaction with the purified FSS-PscN at 12.5 nM, 25nM, 50 nM and 100 nM using an ELISA assay. The BSA at 0.1 μ M and PBS were used as negative controls.

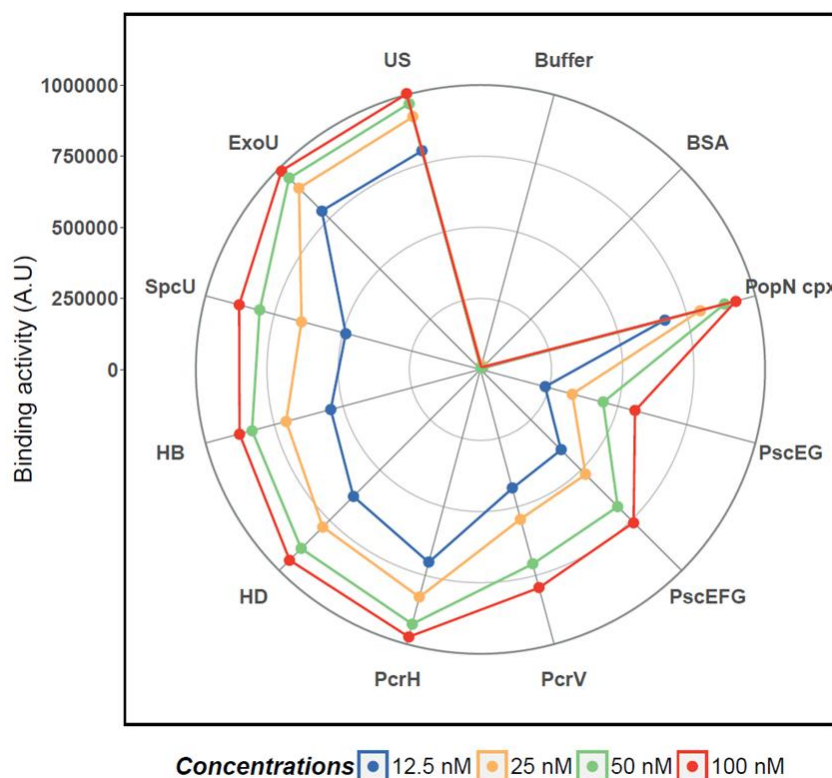


Figure 3: Microscale Thermophoresis allows the measurement of affinities between PscN and its T3SS soluble partners. FSS-PscN was labeled with MST dye and 100 nM of labeled protein were incubated with different concentrations of ligands. A titration fitted curve for each ligand, which represents the bound fraction depending on ligand concentrations, and K_d was obtained (A) Affinity between PscN and four complexes of effector, translocator, needle or gate-keeper. (B) Affinity between PscN and chaperone, effector or chaperone-effector complex. (C) Affinity between PscN and chaperone or chaperone-translocator complexes.

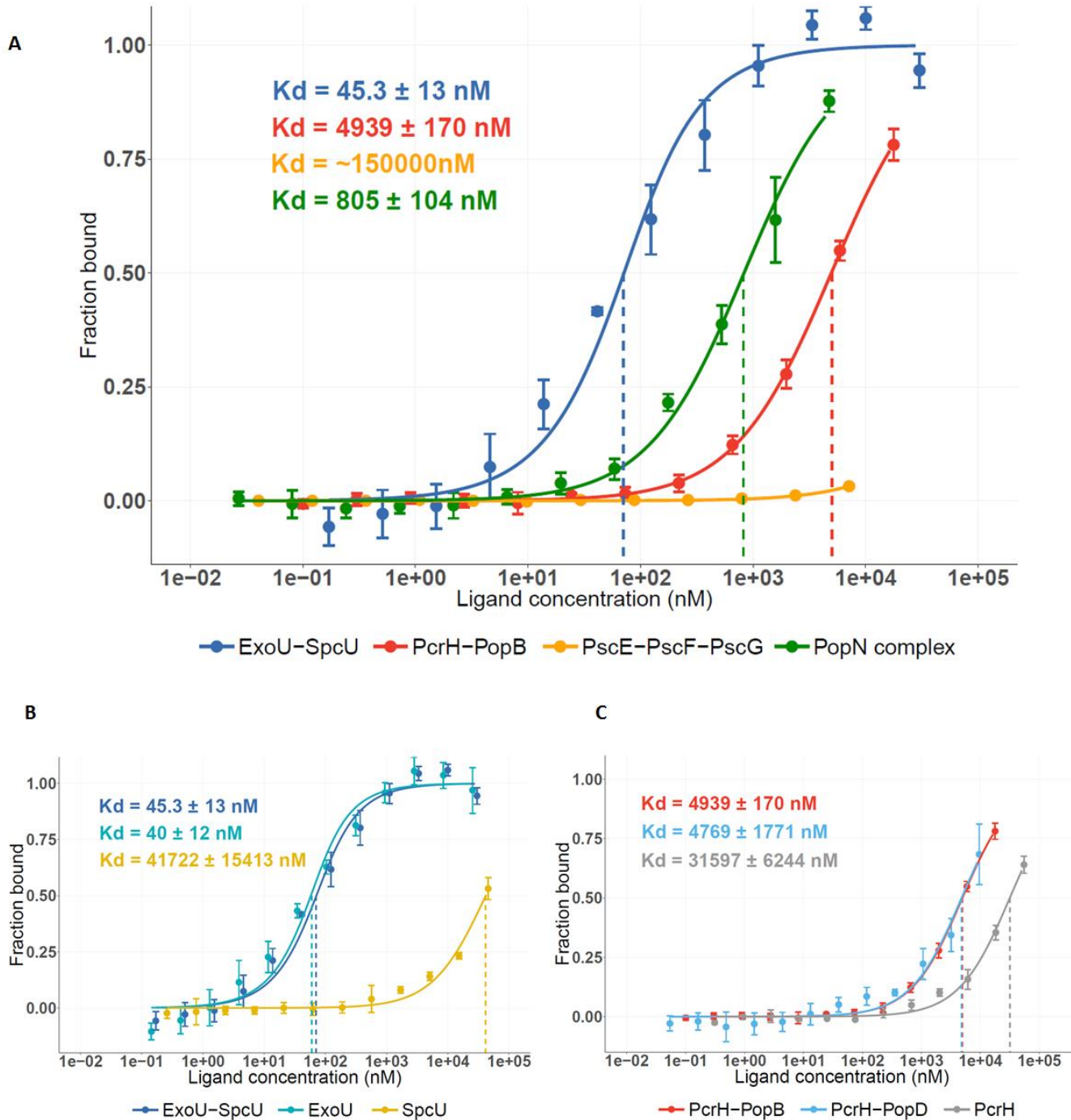


Figure 4: PopN complex acts on PscN to regulate the secretion process. FSS-PscN was labeled with MST dye and diluted at 100 nM in buffer containing or not PopN complex at 800nM. The mixtures were next incubated with different concentrations of three complexes: ExoU-SpcU, PcrH-PopB and PscE-PscF-PscG and a titration fitted curve of each condition was obtained. A significant shift of apparent K_d of PscN and PscE-PscF-PscG in presence of PopN complex indicates that the gate-keeper plays a role in the loading of needle complex on the ATPase.

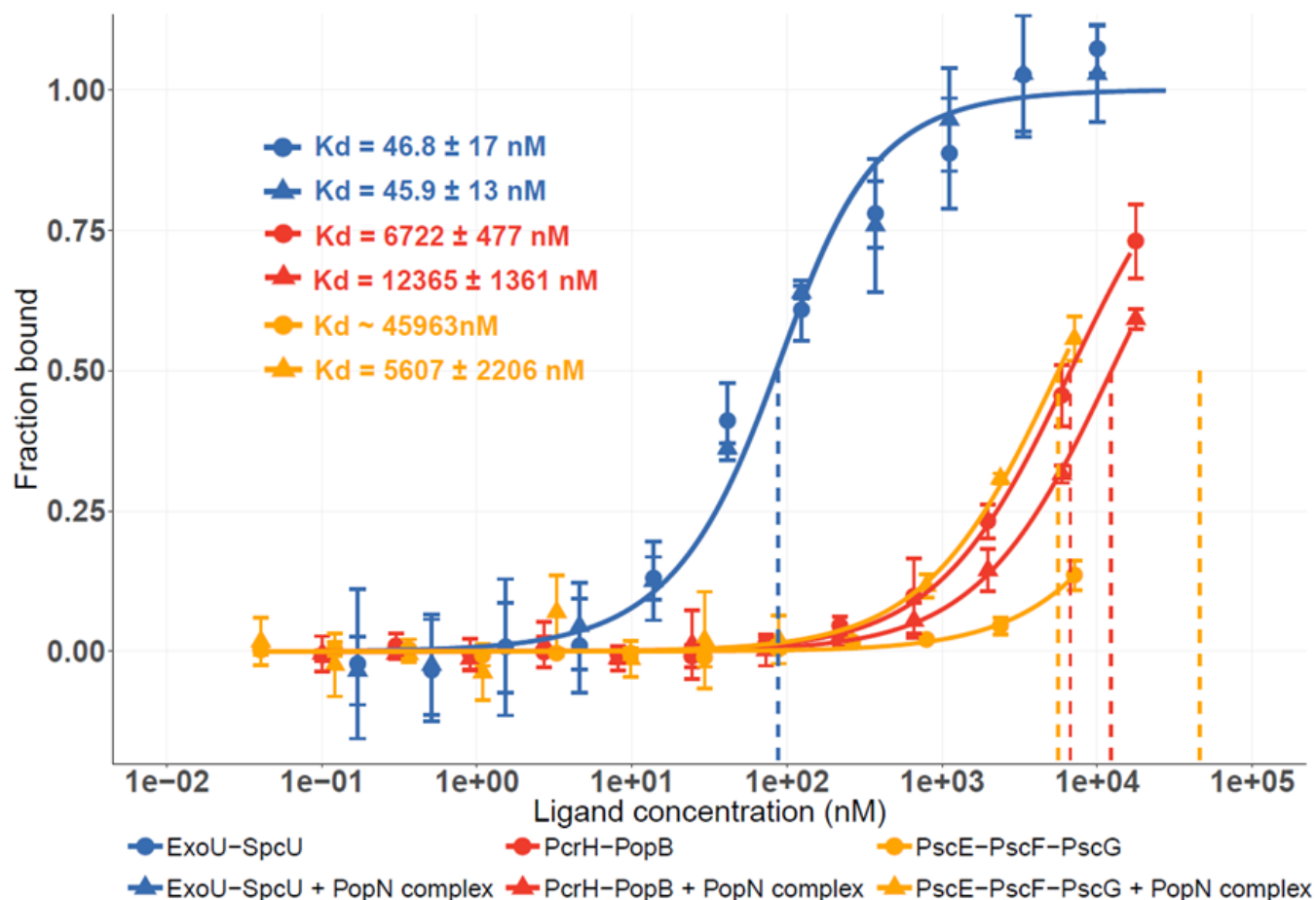


Figure 5: Model of allosteric conformation change of the ATPase PscN controlling the needle protein export. (1) In the presence of PopN complex, PscN is in the “needle binding on” state which facilitates its interaction with the needle complex and has less affinity for the translocator complex. The needle subunit is therefore secreted to form the T3SS needle. The switch protein PscU prevents the secretion of translocators and effectors (2) PopN is detached from PscN, leading to the “needle binding off” state which in turn does not interact with needle complex and has more affinity for the translocator complex. The needle subunit could not be recruited to the export gate, thus stopping the needle elongation. PscU auto-cleavage allows the secretion of translocators. PopN complex: PopN-Pcr1(1)-Pcr2(2)-PscB(B); Needle complex: PscE-PscF-PscG; Translocator complex: PcrH-PopB/D; Effector complex: ExoU-SpcU

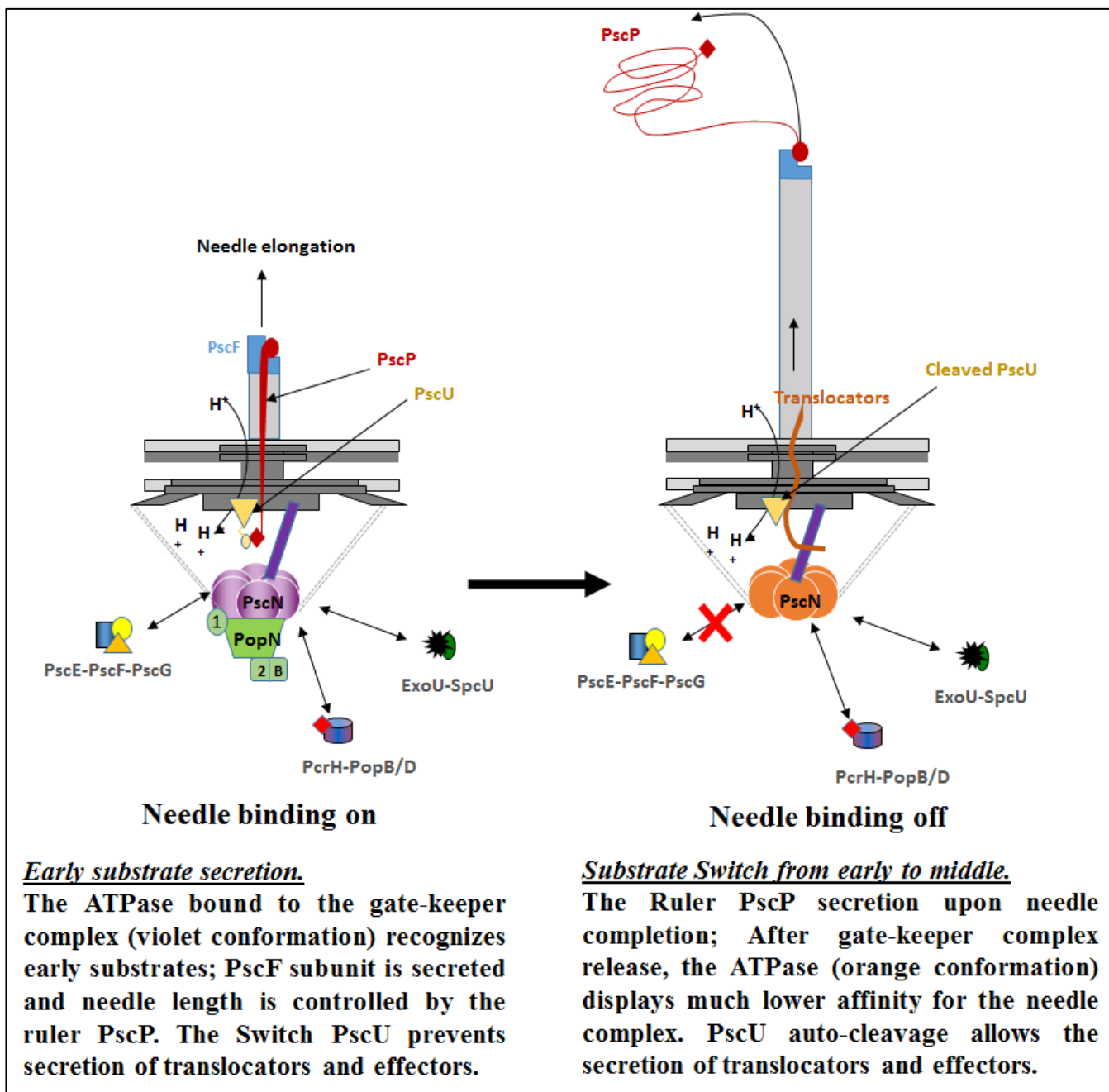


Table S1: Primers and DNA fragment used in this work.

Name	Sequence 5'-3'
Delta_pscN_1	CCG GGC GCC TCG AGC TTC TGC TG
Delta_pscN_2	ATG GCG CTG ATC CAG CGC CTG GTG CTG CTG
Delta_pscN_3	TGG ATC AGC GCC ATG CGG CC
Delta_pscN_4	CCG GGA GCG GAG CCG TAT CCA C
NdeI Strep _PscN	AAC <u>CAT ATG GCC AGC TGG AGC CAC CCG CAG TTC GAG AAG CCG GGC</u> ATG CCC GCG CCT CTC TCT CCT C
PscN_Hind	TGG <u>AAG CTT</u> TCA TGC CGA GAG GCT CCG CAA CTG CGC G
NcoI FSS _NterPscN_Not	<u>CCATGGGCTCGAGCTCCGACTACAAGGACGACGACAAG</u> TTCGAGCTCCGGCAGCGC GAGCGCGTGGAGCCACCCGAGTTCGAGAAGGGCGGCGGCAGCGGCGGCAGCGG CGGCAGCGCGTGGAGCCACCCGAGTTCGAGAAGATCGAGGGAAGGCATATGCCCCGC CCTCTCTCTCTCATCGTCCGGATGCGCCACGCCATCGAAGGCTGCCGCGGATCCAGA TCCGCGGGCGGGTACCCAGGTCACCGGAACCCTGCTCAAGGCCGTGGTCCCCGGCGTGC GCATCGGCGAACTCTGCCAGTTGCGCAATCCCGACCAGAGCCTGGCGCTGCTCGCCGAGG TCATCGGCTTCCAGCAGCACCAGGCGTGTCAACCCGCTCGGCGAGATGCTCGGGGTTT CCTCCAACACCGAAGTCAGCCCTACCGGCGGCATGCATCGCGTGGCGGTTCGGAGAGCACC TGCTCGGGCAGGTGCTCGACGGTCTCGGCCGCCCTTCGACGGCAGCCCGCCGGCCGAGC CGGCGGCCTGGTATCCGGTCTACCGGGATGCCCGCAACCGATGAGCCGGCGCCTGATAG <u>AGCGGCCCGCT</u>

Table S2: Affinity of PscN for T3SS secreted proteins. The Kds (nM) between exported cargos, their chaperones alone or in complex and the ATPase PscN were measured by MST. ND: non-determined. Orange: affinity decrease; Green: affinity increase

Protein			Kd (nM)	Kd (nM)	Kd (nM)	
			Optimal condition	In the absence of PopN complex	In the presence of PopN complex	
Effector	Complex	ExoU-SpcU	45,4 ± 13,2	46,8 ± 17	45,9 ± 13	
	Cargo	ExoU	32 ± 12,2	ND	ND	
	Chaperone	SpcU	41722 ± 15413	ND	ND	
Translocator	Translocon	Complex	PcrH-PopB	4939 ± 170	6722 ± 477	12365 ± 1361
		Complex	PcrH-PopD	4769 ± 1772	ND	ND
		Cargo	PopB/PopD	ND	ND	ND
		Chaperone	PcrH	31597 ± 6245	ND	ND
	Tip	Complex	PcrV-PcrG	ND	ND	ND
		Cargo	PcrV	2648 ± 220	4205 ± 623	13104 ± 2475
		Chaperone	PcrG	ND	ND	ND
Needle	Complex	PscE-PscF-PscG	>150000	>45000	5607 ± 2206	
	Cargo	PscF	ND	ND	ND	
	Chaperone	PscE-PscG	>150000	ND	ND	
Gate-keeper	Complex	PopN-Pcr1-Pcr2-PscB	806 ± 105	1174 ± 40	ND	
	Cargo	PopN-Pcr1	ND	ND	ND	
	Chaperone	Pcr2-PscB	ND	ND	ND	

Figure S1. The ATPase activity of PscN. Purified FSS-PscN was incubated with 5 mM ATP and 5 mM MgCl₂ at indicated pH (A) or with 5 mM ATP and 5 mM of indicated divalent cations at pH 9 (B) for 30 minutes. The released phosphate was measured using the malachite green method.

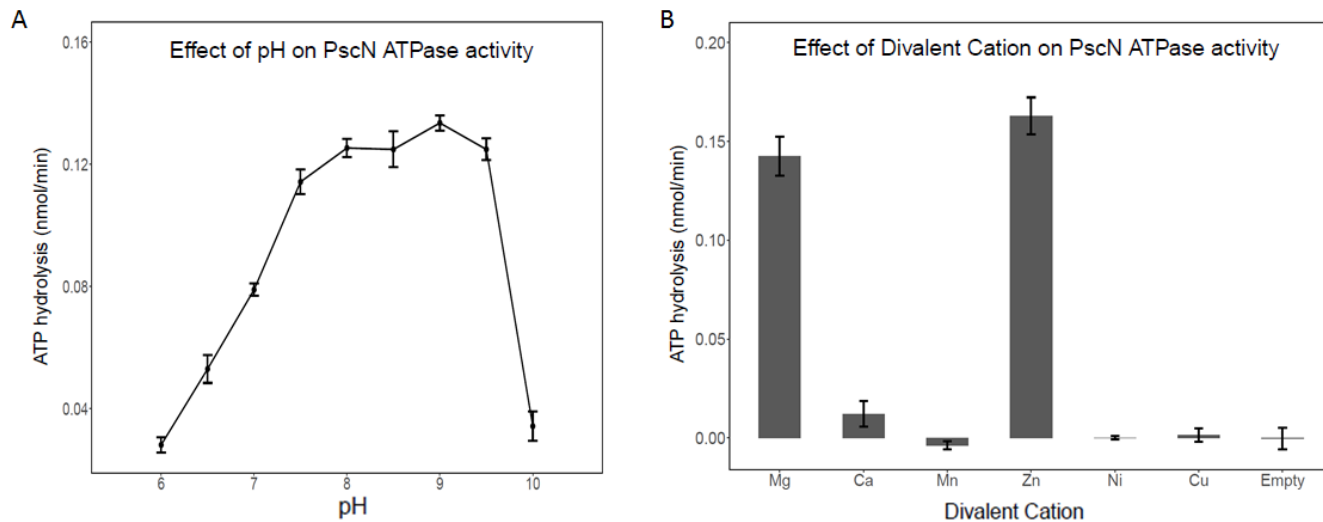


Figure S2: The binding of the ATPase PscN to exported protein complexes, detected by HTRF assay. Effector (ExoU-SpcU), translocator (PcrH-PopD), needle (PscE-PscF-PscG) or gate-keeper (PopN cpx) complexes were mixed with PscN at indicated concentrations in the presence of two pairs of fluorophore-tagged antibodies anti-Flag-Eu³⁺ and anti-His-d2 (A) or anti-Flag-d2 and anti-His-Tb (B). The binding activity was detected by measuring time-resolved fluorescence transfer.

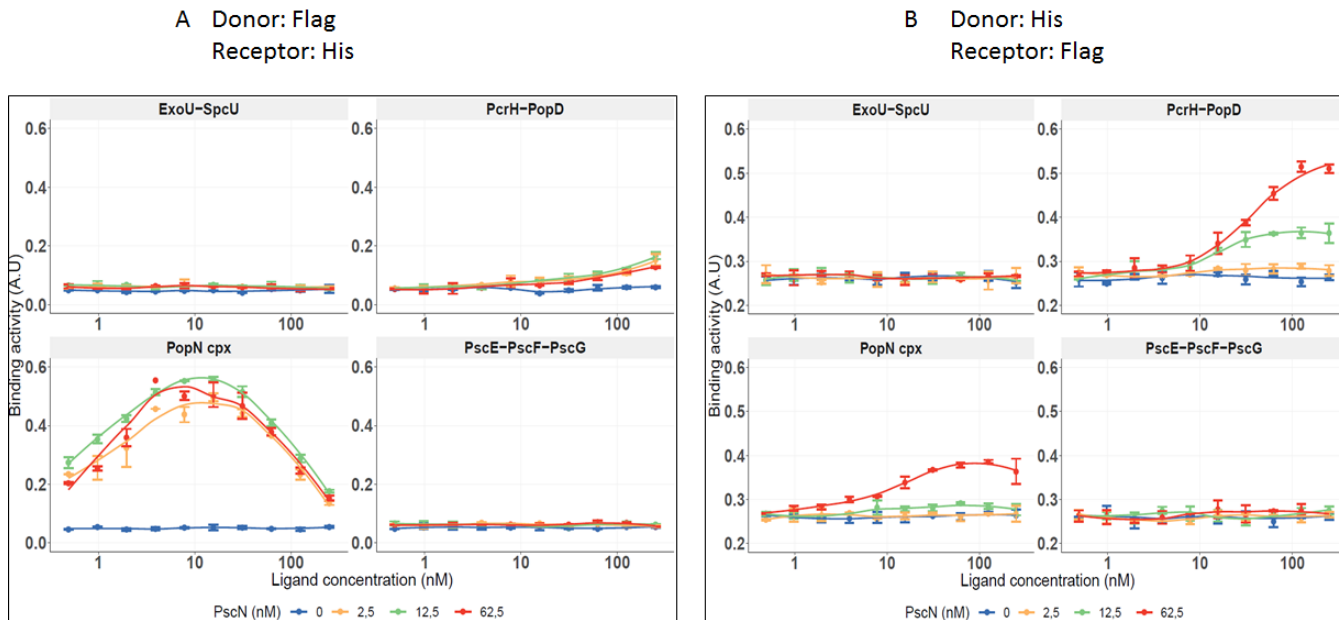


Figure S3: Size exclusion chromatography analysis of PopN complex and PscF complex. PscF complex at 5 μM , PopN complex at 1 μM or a mixture of the two complexes were injected onto a Superdex 200 increase column. The two peaks obtained in the mixture are similar to each peak form the two complexes alone, indicating an absence of interaction between PscF and PopN complexes.

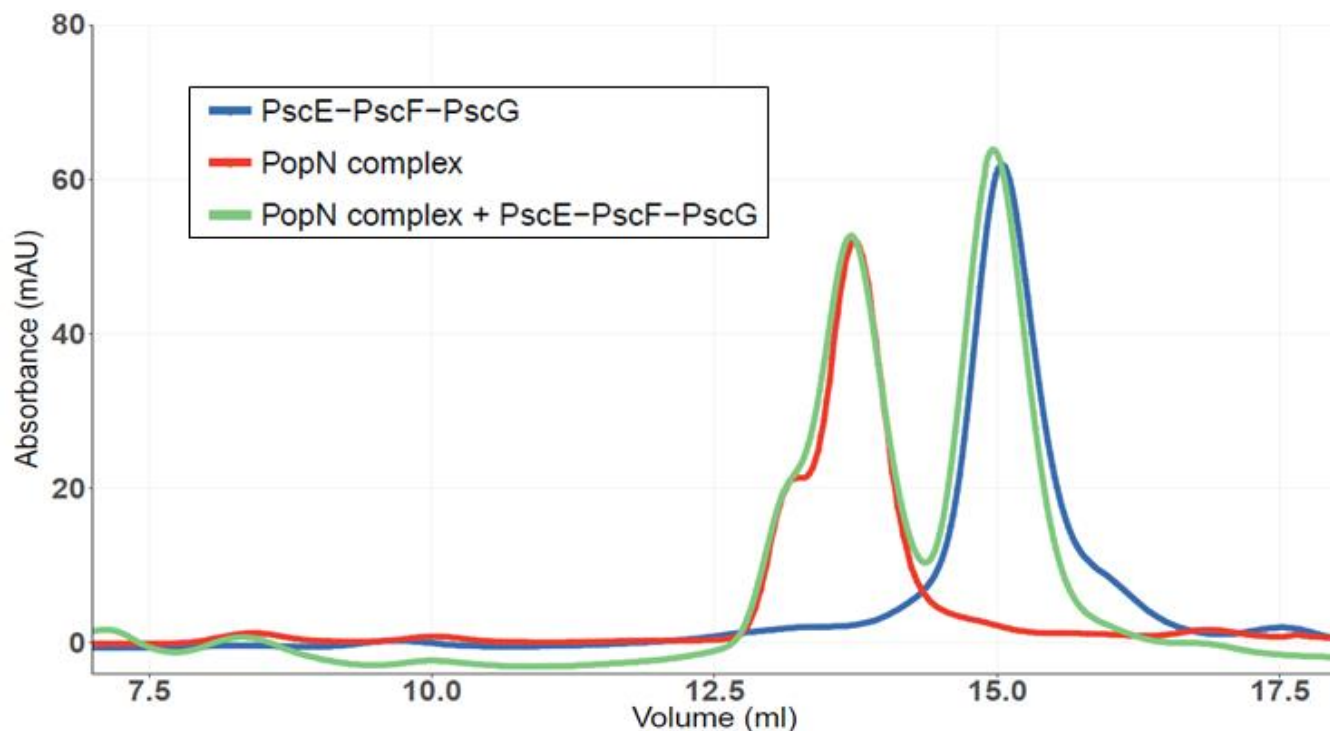
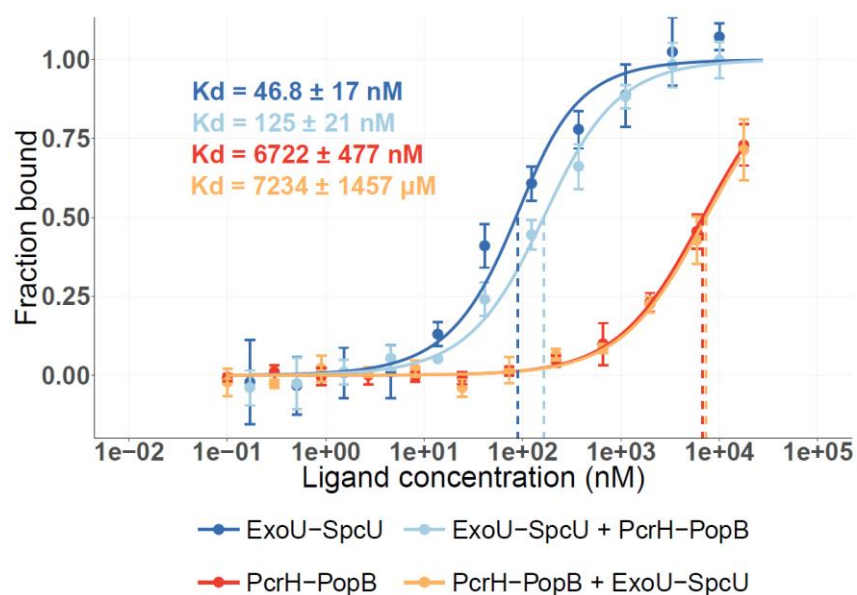


Figure S4: Competition between secreted proteins for the interaction with PscN. The presence of PcrH-PopB slightly decreases the affinity between ExoU-SpcU and PscN while the effector complex does not affect the affinity between the ATPase and the translocator complex.



IV. Conclusion (in French)

En utilisant trois techniques (ELISA, HTRF et MST), nous avons démontré l'interaction de l'ATPase PscN avec les protéines sécrétées du SST3 y compris des effecteurs, des translocateurs, l'aiguille et le « gate-keeper ». De plus, par MST, l'affinité de PscN avec différentes protéines partenaires a été mesurée et les résultats montrent que PscN a plus d'affinité avec l'effecteur ExoU-SpcU, moins d'affinité avec les translocateurs PcrH-PopB/D et le complexe « gate-keeper » PopN et n'interagit pas probablement avec le complexe de l'aiguille PscE-PscF-PscG. Par ailleurs, PscN se lie préférentiellement aux complexes protéines-chaperonne par rapport aux chaperonnes tous seules, ce qui confirme l'hypothèse de la libération des chaperonnes après la dissociation des protéines sécrétées.

Il a été attribué au « gate-keeper » un rôle de régulateur qui contrôle la sécrétion des translocateurs et des effecteurs du SST3 (Roehrich et al., 2017). Chez *P. aeruginosa*, nous avons révélé un autre rôle de ce complexe PopN dans la régulation de sécrétion de l'aiguille via l'interaction avec l'ATPase PscN. En effet, en présence du « gate-keeper », l'affinité de PscN pour le complexe de l'aiguille augmente, ce qui favorise sa reconnaissance par le SST3 et permet sa livraison à la machinerie de sécrétion. Il faut noter que les deux complexes se lient à PscN mais sans entrer en compétition. Donc, il est possible que le complexe PopN régule de manière allostérique PscN en modifiant sa conformation pour modifier sa reconnaissance des substrats (Figure 29). En fait, lors de la liaison au complexe PopN, PscN serait dans une conformation « needle binding on » qui facilite son interaction avec le complexe de l'aiguille et diminue l'affinité avec le complexe de translocateur. La sous-unité PscF est donc sécrétée pour former l'aiguille du SST3. L'élongation de l'aiguille est contrôlé par le « ruler » PscP (Journet et al., 2003) qui à son tour pourrait transmettre un signal au « switch » PscU (Edqvist et al., 2003; Wood et al., 2008). Ce « switch » est connu comme une protéine dont l'auto-clivage du domaine C-terminal est essentiel pour la sécrétion de translocateurs et effecteurs (Frost et al., 2012; Monjarás Feria et al., 2015; Sorg et al., 2007). Pourtant, il a récemment été montré que cet auto-clivage est indépendant de la formation de l'aiguille (Monjarás Feria et al., 2015) et donc, il ne pourrait pas être induit par le « ruler » même si les deux protéines semblent interagir directement *in vitro* (Ho et al., 2017). Cette contradiction laisse penser qu'il existerait un mécanisme reliant la régulation de l'élongation de l'aiguille et la sécrétion de translocateur qui ne dépendrait pas seulement des protéines « ruler » et « switch ».

Par ailleurs, le recrutement du complexe PopN à la protéine de membrane interne PcrD est indispensable pour réguler la sécrétion des translocateurs et des effecteurs (Dewoody et al., 2013; Lee et al., 2014). Donc, il devrait exister un mécanisme qui provoque le détachement du complexe PopN de l'ATPase PscN et son transfert ensuite à la PcrD (une protéine du SST3 dans la membrane interne). Cette hypothèse est appuyée par le fait qu'en absence du complexe PopN (« needle binding off »), l'affinité de PscN pour le complexe de l'aiguille est diminuée fortement tandis que celle pour le complexe de translocateur est augmentée. Ce changement de conformation de l'ATPase aboutirait à la fin de l'élongation de l'aiguille et au début de la sécrétion des translocateurs. Pour conclure, grâce à cette étude, nous proposons un mécanisme par lequel, le « gate-keeper » et l'ATPase participent au passage de la sécrétion de « early substrate » à « middle substrate ».

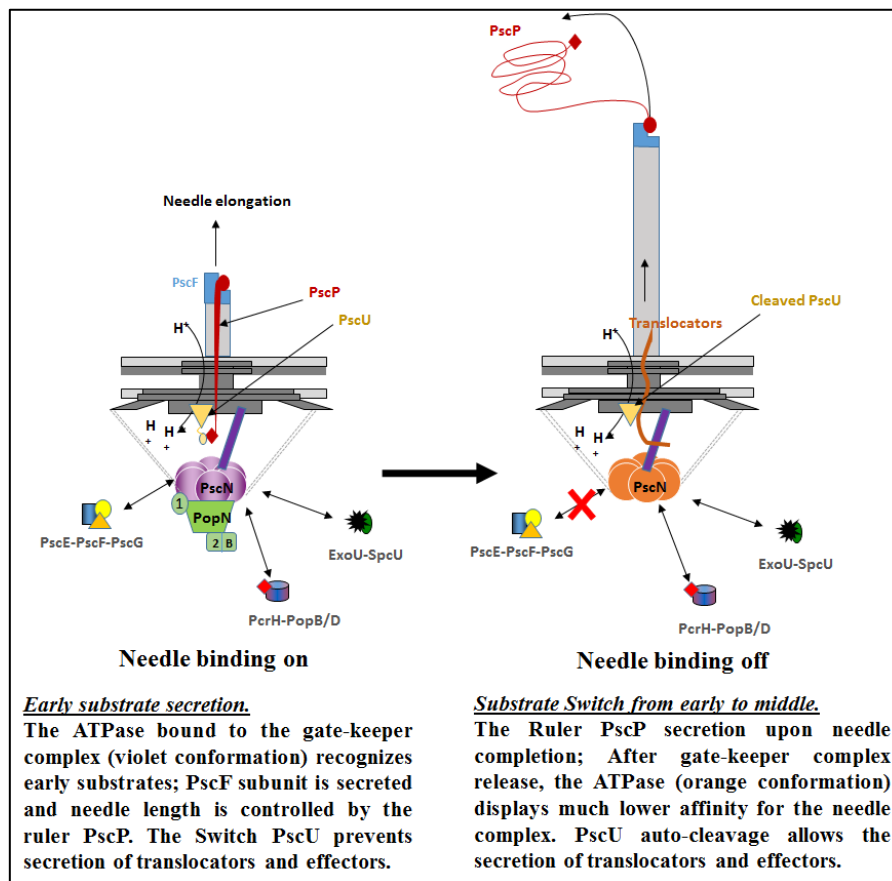


Figure 29: Modèle du changement allostérique de conformation de l'ATPase PscN pour réguler la sécrétion de l'aiguille. (1) Lors de la liaison au complexe PopN, PscN serait dans une conformation « needle binding on » qui facilite son interaction avec le complexe de l'aiguille. La sous-unité PscF est donc sécrétée pour former l'aiguille du SST3. PscU empêche la sécrétion des translocateurs et effecteurs. (2) En absence du complexe PopN (« needle binding off »), l'affinité de PscN pour le complexe de l'aiguille est diminuée fortement. La sous-unité PscF n'est plus livrée à la machinerie de sécrétion. L'auto-clivage de PscU permet la sécrétion des translocateurs et effecteurs.

B. New molecules targeting a T3SS protein-protein interface efficiently affect *Pseudomonas aeruginosa* virulence

I. Abstract (in French)

Contexte. *P. aeruginosa* est une des bactéries pathogènes les plus dangereuses, responsable de maladies nosocomiales et le System de Sécrétion de Type III (SST3) est son facteur de virulence majeur. L'aiguille de ce système est formée de la protéine PscF, qui a la capacité d'auto-polymériser. Dans le cytoplasme de la bactérie, cette protéine forme une complexe avec ses deux chaperonnes PscE and PscG qui sont nécessaires pour sa stabilité et pour empêcher sa polymérisation prématurée avant sa sécrétion (Ple et al., 2010). Par conséquent, la perturbation de l'interaction entre PscE et PscG est une cible potentielle pour la recherche d'anti-virulents.

Résultats. PscE et PscG fusionnées avec des His-tag ont été purifiées et utilisées pour un criblage à haut débit basé sur un test ELISA. Les touches identifiées ont été optimisées pour obtenir 21 molécules réparties en trois classes structurales. Ces analogues ont ensuite été caractérisés *ex vivo* par imagerie cellulaire à haut débit et *in vivo* sur la bactérie. Les résultats indiquent que deux molécules sont prometteuses, capables d'inhiber les dommages cellulaires induits par le SST3 de différentes souches de *P. aeruginosa*. Aux concentrations actives, ces deux composés ne sont pas toxiques pour des cellules eucaryotes et ont un effet minimal sur la croissance, mobilité, et métabolisme des bactéries. De plus, une diminution de la sécrétion des exotoxines ExoS et ExoT et du translocateur PopD est observée *in vivo* en présence des deux molécules, démontrant qu'ils inhibent le fonctionnement du SST3. Enfin, ces composés montrent un effet de protection contre l'infection de *P. aeruginosa* dans le modèle *in vivo* d'insecte *Galleria mellonella*.

II. Introduction (in French)

La résistance aux antibiotiques est une menace pour la santé publique car selon une revue sur la résistance antimicrobienne (RAM), le nombre de décès dus à la RAM en 2050 est estimé à environ 10 millions, un nombre qui dépassera celui du Cancer et du Diabète réunis (O'neil, 2014). Cela met en évidence les difficultés de la thérapie actuelle à traiter efficacement les maladies infectieuses. De plus, ce problème est encore plus accentué dans des cas des infections causées par des bactéries multi-résistantes dont *P. aeruginosa* (Alnour and Ahmed-Abakur, 2017). Ce pathogène est capables de former un biofilm et de s'adapter à différents environnements. En plus d'infections aiguës, il est responsable d'infections chroniques, et est la cause principale de morbidité et mortalité chez les patients atteints de mucoviscidose (Alnour

and Ahmed-Abakur, 2017; Cutting, 2015; Murray et al., 2007). Cette bactérie est reconnue comme un des premiers agents responsables de maladies nosocomiales pour lesquelles le Système de Sécrétion de Type III (SST3) est son facteur de virulence majeur (Obritsch et al., 2005). C'est pourquoi, l'inhibition de ce système est une stratégie potentielle pour lutter contre la virulence de cette bactérie dans un contexte où la résistance aux antibiotique rend nécessaire la découverte d'alternatives thérapeutiques.

L'aiguille du SST3 est formée de la protéine PscF, qui a la capacité d'auto-polymériser (Quinaud et al., 2007). Dans le cytoplasme de la bactérie, cette protéine forme une complexe avec ses deux chaperonnes PscE et PscG qui sont nécessaires pour sa stabilité et pour empêcher sa polymérisation à l'intérieur de la bactérie. Il a été montré que des mutations ponctuelles dans l'interface entre PscE et PscG diminuent ou abolissent la cytotoxicité de *P. aeruginosa* (Ple et al., 2010). Par conséquent, notre objectif est d'identifier et développer des composés chimique qui inhibent l'interaction entre PscE et PscG, afin de déstabiliser le complexe PscE-PscF-PscG et d'atténuer l'activité du SST3.

III. Result presented in article format

The experiments included in this paper were mainly performed by the PhD student Tuan-Dung NGO with the help of

- Caroline BARETTE for screening and drug robotic handling.
- Sophie PLE for setting up the ELISA, screening and chemical compound development
- Yung-Sing WONG for the chemical compound development

The illustrations (except Figure 1, schemes and tables) and first version of this article (except the introduction) were prepared by the PhD student Tuan-Dung NGO and the text was then edited by other authors.

New molecules targeting a T3SS protein-protein interface efficiently affect *Pseudomonas aeruginosa* virulence

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❖ Abstract

Pseudomonas aeruginosa is an opportunistic pathogen which is naturally resistant to many common antibiotics and it is acquiring new resistance traits at an alarming pace. Targeting the bacterial virulence factors by an anti-virulence strategy therefore represents a promising alternative approach besides antibiotic therapy. The type III Secretion System (T3SS) of *P. aeruginosa* is one of its main virulence factor and disrupting interactions between components of this machinery efficiently lowers the bacteria virulence. Using a high-throughput ELISA target-based screening of two chemical libraries, we identified several compounds inhibiting the interaction between PscE and PscG, the two cognate chaperones of the needle subunit PscF of *P. aeruginosa* T3SS. The best compounds from each library were selected to build new and larger hybrid analogues whose efficacy and toxicity were evaluated using High-content screening and phenotypic analysis. Among these optimized molecules, two leads had a minimal effect on bacterial fitness, were almost nontoxic for eukaryotic cell and specifically inhibited the T3SS and the bacterial virulence *ex vivo* on cells and *in vivo* in *Galleria mellonella*. Our work further demonstrated that the structural combination makes the compounds less toxic than their parent precursors while gaining efficacy.

❖ Introduction

The World Health Organization recently published a list of antibiotic-resistant pathogenic bacteria for which the search for new treatments is a high priority (Tacconelli et al., 2018). This list includes the gram-negative bacterium *Pseudomonas aeruginosa*. This pathogen is responsible for severe chronic infections, especially in people with cystic fibrosis (Murray et al., 2007). Due to its multiple forms of resistance to antibiotics, its capacity to form biofilms and to adapt to different environments, it easily colonizes hospital settings. It represents a serious public health problem in terms of nosocomial infection and is the first cause of mortality in ventilator associated pneumonia and burn infections (Kalanuria et al., 2014; Patel et al., 2012).

Faced with the adaptation of this bacterium to antibacterial treatments, it is urgent to develop other therapeutic approaches that do not rely solely on

bactericidal or bacteriostatic activities (Burrows, 2018; Wagner et al., 2016). An emerging alternative approach is based on the inhibition of bacterial virulence to reduce its pathogenic character during infection, without affecting its integrity so as to reduce the selection of resistance to treatments (Dickey et al., 2017). In this perspective small molecules have been shown effective in targeting pathogen-specific virulence factor (Johnson and Abramovitch, 2017).

P. aeruginosa pathogenicity strongly relies on its type III secretion system (T3SS), a conserved virulence trait among gram-negative bacteria (Deng et al., 2017). This complex protein machinery allows the direct injection of toxins into the eukaryotic target cells and can be divided into three main structural parts: the basal body inserted in the two bacterial membranes and prolonged by a cytoplasmic protrusion; the needle that connect the bacterium to the target cell and the translocon, a

translocation pore formed in the host cytoplasmic membrane and linked to the needle by the tip protein (Figure 1A).

In the bacterial cytoplasm, the premature polymerization of the needle subunit PscF is prevented by its association to two small co-chaperones, PscE and PscG (Quinaud et al., 2005). The crystallographic structure of the trimeric complex (Quinaud et al., 2007) revealed that the PscF amphipathic helix involved in the needle polymerization mainly interacts with the concave surface of PscG (Figure 1B). Nevertheless, the stabilization of the complex through a large interaction surface between PscG and PscE (Figure 1C) was shown to be essential for the T3SS functioning as residues mutations in the interface disrupt complex formation and abolished T3SS-mediated cytotoxicity (Ple et al., 2010).

Use of therapeutic antibodies that target the translocon via the recognition of the PcrV tip protein demonstrated the effectiveness of anti-T3SS approach in murine infection models (Warrener et al., 2014). Furthermore, chemical inhibitors have been identified to inhibit bacterial virulence mainly through high throughput phenotypic screening campaigns. However, the exact mechanism of action of these molecules remains to be corroborated to T3SS inhibition (Anantharajah et al., 2016; Charro and Mota, 2015; Duncan et al., 2012; Gu et al., 2015; Kline et al., 2012; Tsou et al., 2013).

Herein, we describe our strategy to identify bioactive small molecules able to inhibit T3SS assembly. In this context, we have developed an *in vitro* ELISA screening test based on the interaction between PscG and PscE, which is essential for PscF stabilization. Protein-protein interaction inhibition with small molecules is an unconventional target class where large surface interaction must be inhibited while meeting the challenge of specificity (Modell et al., 2016; Scott et al., 2016). The interaction surface between the chaperones PscG-PscE is wide (estimated at 1321 Å², figure 1C) and is one of the most challenging interfaces to inhibit (Smith and Gestwicki, 2012). Our approach therefore consisted in linking two different clusters identified by ELISA screening to make a larger hybrid molecule capable of filling this surface while evolving toward ligands of higher affinity. After selection of the best molecule thanks to cellular assays involving multiparametric and orthogonal tests, we identified two closely related compounds that demonstrated *in vivo* antivirulence activity in the *Galleria mellonella* infection model.

❖ Result and Discussion

A screen for inhibitors of the PscE-PscG interaction identified hit molecules from different chemical families. In order to identify compounds capable of inhibiting the protein-protein interaction between PscG and PscE, an ELISA test was developed using the recombinant proteins individually produced and purified (Figure 2, A and B). PscG was first immobilized onto an ELISA plate and the screened molecules were then incubated followed by a PscE incubation. The presence of PscE bound to PscG was then detected using mouse anti-PscE antibodies and secondary antibodies. Two chemical libraries were screened, one from the Prestwick collection (1280 compounds) that contains only approved drugs with known safety and bioavailability and the other from a set of natural product-like compounds (120 compounds) coming from the laboratory's work. Primary screening was performed at a compound concentration of 50 µM. By setting the threshold at 40% inhibition, fourteen compounds were identified in the Prestwick collection and one in the laboratory collection (Table S1). Among Prestwick's best hits, clioquinol (Scheme 1) was selected for further work because hydroxyquinoline scaffold has already been identified as effective in inhibiting the secretion system in *Pseudomonas aeruginosa*, although the exact inhibition mechanism has not yet been identified (Anantharajah et al., 2016, 2017). In addition, it is easy to incorporate an alkyne function into this molecule allowing subsequent coupling by click reaction. In the in-house library, the identified structure **1** belongs to the 3-alkyl pyridine pyridinium alkaloid (3-APPA) family (Volk and Köck, 2004) which can be easily diversified in its structure, especially in length and size (Gil et al.; Kaiser et al., 1998).

The combination of hit molecules is beneficial to target the large interaction surface. To increase the affinity of our two hits, the idea was to combine them together to make a larger hybrid to better fill the area to be inhibited. An approach based on a Split/Mix/Click sequence has been implemented (Scheme 2). To join the two parts, the alkyne group was set on clioquinol (Scheme 1) (Cironi et al., 2004) and the azide group on the 3-APPA derivatives (Scheme 2). Eighteen 3-APPA **11(n,n')** were synthesized (Gil et al.; Kaiser et al., 1998), then coupled with **3** according to a tandem deprotection/click conversion (Fletcher et al., 2008) to give eighteen hybrid 3-APPA/cliоquinol **12(n,n')**. This set of new products **2, 3, 4, 11(n,n')** and

12(n,n') was evaluated on the ELISA test (Table S2). For the clioquinol derivatives **2**, **3** and **4**, the presence of an acetate does not disrupt activity. Several 3-APPA **11(n,n')** showed good ability to inhibit PscG/PscE interaction close to or even superior to clioquinol. Of importance, the hybrid molecules **12(n,n')** include the major part of the highly active compounds (Figure 2C). This suggests that combination of hit molecules is beneficial to target the large interaction surface between PscG/PscE. Among the synthesized molecules, twenty new analogues of clioquinol, **11(n,n')** and **12(n,n')** derivatives were chosen for the subsequent characterization steps

Characterization of the cellular effects of 21 molecules allowed the selection of promising candidates. To evaluate the toxicity and efficacy of hit compounds on cellular models, we assessed their effects on the growth of *P. aeruginosa* PA103 strain, their toxicity for A549 human epithelial pulmonary cell and the protection from ExoU-mediated *P. aeruginosa* cytotoxicity using the PA103 strain. All the twenty molecules, along with the clioquinol, as reference (Table S2), were tested at three concentrations: 3 μ M, 10 μ M and 30 μ M in triplicates and the data were represented in radar plots in order to get a synthetic view of the results (Figure 3). The bacterial growth was monitored with measurements of the optical density of the cultures at 600 nm (OD₆₀₀) in order to reject compounds with potent antibiotic activity. In this assay, only one third of the compounds exhibited bacterial growth inhibition less than 25% even at the higher concentration.

On the opposite, the detection of Propidium Iodide (PI) incorporation into eukaryotic A549 cells incubated with the molecules indicated that the compounds were globally not toxic for the mammalian cells. Furthermore, their protective effect against the *P. aeruginosa* virulence was tested with the PA103 strain whose T3SS injects the ExoU effector that induces rapid eukaryotic cell death revealed by PI labeling. In this third orthogonal assay, **4** (Scheme 1) was not active whereas the other compounds displayed varying ranges of inhibition of the bacteria-mediated cytotoxicity. Of importance, their protective effect was often correlated with inhibition of the bacteria growth, which is not the desired activity pattern.

The antibiotic-like compounds were thus rejected but based on these first results, seven compounds **11(6,6)**, **12(10,10)**, **12(4,6)**, **12(6,4)**, **12(8,12)**, **2** and clioquinol were selected for further characterization and comparison of chemical families in another set of assays: two compounds of the clioquinol cluster,

one compound of 3-APPA cluster and four combination compounds. These seven compounds exhibited minimal effect on bacterial growth and cell toxicity and inhibited the virulence of the PA103 strain.

Further characterization of 7 molecules pointed out two lead candidates. Further characterization was conducted on three orthogonal phenotypic assays employing the *P. aeruginosa* CHA strain that injects the ExoS effector and not ExoU. Indeed, *P. aeruginosa* strains inject ExoU or ExoS and the majority of the clinical strains are ExoS⁺ which induces cytoskeleton disruption through hijacking of cell signaling (Hauser, 2009). Thus, the inhibition of ExoS secretion was quantified, as well as the protection of A549 cells from damages caused by the CHA strain, measured by two automated microscopy approaches. Similarly to the previous characterization, the seven compounds were tested at three concentrations and in triplicate and the results were represented in radar plots (Figure 4). First, a secretion assay of the effector ExoS was employed in which the β -lactamase fused to the C-terminal ExoS was quantified in the extracellular media and all the tested compounds were able to inhibit the secretion of ExoS.

For the cell protection assays, in one experiment cells membranes were labelled and cell shrinkage was quantified by measuring cell confluence decrease. In the second experiment, the recently described CLIQ-BID (bright nuclei) method was employed because it can reveal cellular damages caused by the bacteria as well as toxicity or cell stress caused by the compounds on their own (Wallez et al., 2018). Indeed, while the seven compounds protected cells from the cell retraction induced by toxin to different extents, five of them did not exhibit protection properties in the cell stress assay. It is possible that these molecules are slightly toxic for the eukaryotic cells, inducing a cell stress that prevent detection of a possible anti-virulent activity. Indeed, one advantage of the CLIQ-BID method is to sensitively detect compounds toxicity, allowing to reject molecules whose toxic effects could dominate the protective effect (Wallez et al., 2018). On the other hand, the **12(6,4)** and **12(4,6)** compounds were able to protect A549 cells from ExoS-mediated CHA damages detected by both cell shrinkage and cell stress assay and to inhibit the T3SS-mediated ExoS-bla secretion. Thus, **12(6,4)** and **12(4,6)** were selected as potential “lead” compounds. Interestingly, both **12(6,4)** and **12(4,6)** belong to the combination cluster and their structures are very close, since the length of the linker between the pyridine and the pyridinium is the same.

Long-term toxicity study on HepG2 cells. Toxicity monitoring toward A549 cells revealed that the combination of the chemical clusters somehow makes the compounds less toxic than their parent precursors, while maintaining their protective properties (Figure 3). In this assay, the toxicity of **12(4,6)** and **12(6,4)** was evaluated with the A549 cells death assay, detecting PI incorporation during 5 h. This incubation is usually used in comparable studies (Anantharajah et al., 2017) and no toxicity could be observed at concentrations up to 100 μ M. However, to get information on longer term toxicity using a common toxicity model, these lead compounds were tested on the hepatic cells line HepG2 for 48 h. After cell incubation with the compounds, dead cells were enumerated by fluorescence microscopy using PI incorporation as a viability marker (Figure 5A). A significant increase of HepG2 mortality could only be detected at 50 and 100 μ M for both **12(4,6)** and **12(6,4)**. In addition, the mitochondrial activity was measured with the standard MTT assay after 48 h of incubation with the molecules (Figure 5B). Again, a significant toxicity was observed at the highest concentrations, at 50 and 100 μ M for **12(4,6)** and 100 μ M for **12(6,4)**. In conclusion, while **12(4,6)** displays a somehow higher toxicity than the **12(6,4)**, both compounds are non-toxic at concentrations that protect cells from T3SS-mediated effects.

12(6,4) and 12(4,6) specifically inhibits T3SS secretion. In order to assess the specificity of the T3SS inhibition by the lead compounds, direct secretion experiments on bacteria were performed at three concentrations 5 μ M, 12.5 μ M and 30 μ M. The secretion of three T3SS-exported proteins ExoS, ExoT and PopD of the *P. aeruginosa* CHA strain was monitored by Western Blot (Figure 6A). A strong inhibition of ExoS and ExoT secretion could be observed at 12.5 and 30 μ M, with a threshold effect, while the inhibition of PopD secretion was more correlated to the concentration. Quantification of the western-blot analysis was made on three independent experiments (Figure 6B), confirming the dose-dependent inhibition. IC₅₀ could be estimated around 10 μ M for the secretion of the effectors ExoS and ExoT and 20 μ M for the secretion of translocator PopD. Despite a trend indicating that **12(6,4)** is a more potent inhibitor than **12(4,6)**, this is not statistically significant.

In order to ascertain that the reduced T3SS secretion was not indirectly due to a decrease of bacteria fitness, the bacterial growth was monitored for 24h in the presence of the lead compounds (Figure 6C). The OD₆₀₀ kinetics revealed almost no effect of the compounds, except at the end of the

stationary phase where a faint decrease was observed for unexplained reason. Furthermore, the intracellular ATP levels in the exponential phase was quantified after growth (Figure 6D), displaying no difference in the presence or absence of the compounds. Furthermore, no inhibition of *Escherichia coli* and *Yersinia enterocolitica* growth was observed (Supplemental Figure S1 A) while the T3SS architecture and functioning are relatively conserved among gram-negative pathogens. Nonetheless, **12(6,4)** and **12(4,6)** were not able to inhibit the cell shrinkage caused by *Y. enterocolitica* whose T3SS is phylogenetically close to the one of *P. aeruginosa* (Supplemental Figure S1 B). Consequently, **12(6,4)** and **12(4,6)** were confirmed as specific anti-virulence agents that do not affect bacterial viability.

The flagella machinery architecture is similar to the T3SS and its functioning is also dependent on ATP hydrolysis and the proton-motive force gradient. Therefore, to confirm the specificity of the lead compounds toward the T3SS, we assessed the flagella-dependent swimming motility of *P. aeruginosa* in the presence of the compounds on low-density agar plates and compared to a mutant devoid of functional flagella (Figure 7A and C). No inhibition could be detected, indicating that **12(4,6)** and **12(6,4)** did not affect the flagellar-specific T3SS.

Besides, the Type 4 pilus (T4P) is also a multi-protein appendage that spans the inner and outer membranes as the T3SS and the flagella. The twitching motility which is T4P-dependent was thus observed in the presence of the compounds or in a deletion mutant of a gene essential for T4P function (Figure 7B and C). Similarly to the flagellum, the compounds did not inhibit the T4P. This is of particular importance because the T3SS-mediated intoxication is dependent on the T4P (Golovkine et al., 2016) and the observed cell protection could have resulted from T4P inhibition. Taken together, these findings confirmed that the two lead compounds **12(6,4)** and **12(4,6)** are specific inhibitors of the T3SS.

Protection of animal model from bacterial infection. Different animal models are available to assess *P. aeruginosa* virulence and results obtained in mice and insects (*G. mellonella*) are correlated (Jander et al., 2000). Moreover, *G. mellonella* is a good model to evaluate the efficacy of antimicrobial agents (Tsai et al., 2016). Therefore a dose of 50 wild-type bacteria was injected into the larvae and 30 min later the compounds, or DMSO, were subsequently injected. As a control, larvae were infected with a *P. aeruginosa* deletion mutant of the gene *pscE*, essential for the T3SS, and then injected

with DMSO. The survival of the insects was monitored for 20 h (Figure 8). The effect of T3SS absence is clearly observed with longer time of survival upon infection with the *ΔpscE* mutant. Strikingly, injection with either **12(6,4)** or **12(4,6)** could significantly improve the survival of larvae, thus indicating that these inhibitors have an *in vivo* protective efficacy.

❖ Conclusions

In this study, we designed a high throughput target-based screening to identify inhibitors targeting the interaction of two cognate chaperones PscE and PscG of the T3SS needle subunit protein PscF. The clioquinol and 3-APPA scaffolds were selected as the basis for building new, larger hybrid analogues. As a result, their combination generally resulted in more inhibition efficiency in the protein-protein interaction ELISA assay. Based on this *in vitro* evaluation, 21 compounds from the three clusters (clioquinol, 3-APPA and hybrid 3-APPA/clioquinol) were next characterized by several biochemical and cellular assays, revealing two lead compounds **12(6,4)** and **12(4,6)**. These compounds have minimal effects on bacterial growth and displayed protective effects on A549 eukaryotic cell line infected by two different *P. aeruginosa* strains.

12(6,4) and **12(4,6)** were further demonstrated to protect *G. mellonella* against *P. aeruginosa* infection, as shown by a survival experiment *in vivo*. Therefore, this new family of antivirulence compounds, which targets a key protein-protein interaction in the formation of the *P. aeruginosa* T3SS, would help to propose a new solution to treat bacterial infections that should reduce the development of resistance in this harmful pathogen.

❖ Experimental section

Protein purification and High-Throughput target-based Screening ELISA assay. 6His-PscE and 6His-PscG were expressed in *E. coli* BL21 (DE3) Star using pET plasmids (Quinaud et al., 2005). *E. coli* strains were grown in 1L of LB broth and induced at OD_{600nm} of 0.9-1 for 3 h at 37°C. Cells containing 6His-PscE were harvested and resuspended in IMAC buffer (25 mM Tris pH 8, 500 mM NaCl and 2% glycerol). Cell suspension were lysed by French press or sonication and centrifuged at 200,000 g for 30 min. Soluble 6His-PscE was purified with Ni-NTA sepharose beads and eluted by three steps of IMAC containing 50 mM, 100 mM and 500 mM of imidazole. For purification of 6His-

PscG, harvested cells were resuspended in IMAC containing 6M urea. After centrifugation, solubilized 6His-PscG was purified on a HistrapHP column and protein refolding was performed column by decreasing the urea concentration from 6 M to 0 M in IMAC buffer. 6His-PscG was then eluted by three steps of IMAC containing 100 mM, 250 mM and 500 mM of imidazole. Both proteins were dialyzed against Tris/NaCl buffer (25 mM Tris, 250 mM NaCl, pH8).

For screening, 50 μL of PscG protein at 1 μM (for the screening of Prestwick and in-house library compounds) or 2.2 μM (for the screening of synthesized analog compounds) were coated on ELISA NUNC 96 well plates for 8h at 4°C. The wells were then washed three times with 200 μL of PBS/0.1% Tween 20 (PBST) and incubated with 200 μL of milk 5% in PBST overnight at 4°C. The solution was next removed by dripping and a mix of 40 μL of buffer (25 mM Tris pH 8, 250 mM NaCl), 50 μL of PscE at 25 μM (screening of library compounds) or 3 μM (screening of analog compounds) and 10 μL of inhibitors diluted in DMSO was added on plates for 4 h of incubation at room temperature (RT°). The wells were subsequently washed three times with 200 μL of PBST and incubated with 50 μL of anti-PscE antibody (1/2,000 in PBST) for 2h at RT°. Three additional washes with 200 μL of PBST were performed before incubation with 50 μL of anti-mouse-HRP antibody (1/5000 in PBST) for 2 h. The wells were then washed with PBST (three times) and revealed with the TMB kit. The absorbance was read at 450 nm.

Chemical synthesis of clioquinol, 3-APS and hybrid 3-APS/clioquinol analogs.

To be written by Yung-Sing WONG.

Bacterial Strains and Growth Conditions.

P.aeruginosa cystic fibrosis clinical strains CHA, its *ΔpscE* and *ΔpscF* mutant (Ple et al., 2010), and the strains expressing ExoS-Bla fusion protein CHA *ΔexoS/ExoS-Bla* and CHA *ΔpscF/ExoS-Bla* (Verove et al., 2012) have been previously described. Other strains were *P.aeruginosa* PA103, *Yersinia enterocolitica* MRS40, its *ΔHOPEMN* mutant and *Escherichia coli* DH5a. All the bacteria were grown in LB media (Luria Bertani-Difco) at 37°C except *Y.enterocolitica* strains which were grown at 28°C. For infection, overnight cultures were diluted in fresh media to OD of 0.1 and culture were grown for 2.5 to 3h to reach OD of 1. Bacteria were then incubated with eukaryotic cells at a multiplicity of infection (MOI) of 10.

Compound dilution and preparation. All the compounds were diluted in DMSO at 20mM (stock compounds). They were then serially diluted in DMSO and distributed in 96 well plates (ABgene: 0796) (1.5 μ L per well) at different concentrations with a robotic system. These plates were used for compounds dilution in culture media (DMSO final concentration of 0.5%) to perform the High-Content Screening (HSC).

Cell culture and staining, automated High-Content Screening (HSC) and High-Content Analysis (HCA)

Cell death: A549 cell were cultured overnight with 5000 cells in 80 μ L/well in Black μ clear 384-well plates (Greiner) in RPMI supplemented with 10% FBS at 37°C in a humidified incubator and with an atmosphere of 5% CO₂. For screening, medium was replaced with 60 μ L of fresh medium containing 0.25 μ M Syto24 (Life Technologies: S7559), 0.5 μ g/mL Propidium Iodide (PI) (Sigma Aldrich: P4170), *P.aeruginosa* PA103 at MOI of 10 and in presence of compounds diluted at three concentrations (30 μ M, 10 μ M, 3 μ M). The kinetic acquisitions were performed using the automated microscope Incucyte® Zoom System (Essen Bioscience). One image per well were acquired every 45min for 5h with the 4X objective. The RED channel was used for PI staining detection, the GREEN channel was used for Syto24 staining and the PHASE image channel was used to monitor cell morphology. All the system were maintained at 37°C and 5% CO₂ in a humidified incubator. The image analysis were performed by using the Basic Analyzer of Incucyte Zoom software. Top-Hat background subtraction was applied and edge split was set to -24 to separate closely-space objects. The Red Object Count (1/Well) in RED channel was obtained for the dead cell count (PI incorporation) by applying a threshold of 2.4 Requested Conversation Unit (RCU) and a radius of 100 μ m. The Green Object Count (1/Well) in GREEN channel was obtained for the total cell count at the starting time point by applying a threshold of 12 (RCU) and a radius of 30 μ m. Data were used to calculate the percentage of PI positive cells at each time point.

Cell shrinkage: A549 cell were cultured overnight at 12500 cells in 100 μ L/well in Black μ clear 96-well plates (Greiner) in RPMI supplemented with 10% of FBS. Two hours before infection, cells were washed two times with 100 μ L of PBS to remove cellular debris before incubation with 100 μ L of fresh medium containing Dii dye (Life Technologies: D282) (10 μ g/mL) for 1 h. Cells were then washed two times with 100 μ L PBS and one time with 100 μ L of RPMI containing 10% FBS before infection.

Cells were incubated with *P.aeruginosa* CHA or the *ApseE* mutant (MOI = 10) in presence of compounds diluted at three concentrations (30 μ M, 10 μ M, 3 μ M). Microscopy and imaging were performed using the same protocol as described above, except that the objective was set to 10X, the acquisition time of RED channel was used for Dii staining detection and the image acquisition was performed every 15 min for 4 h. For analysis, the Red Object Confluence (Percent) in RED channel was measured to quantify the cell area by applying a threshold of 0.3 and a radius of 50 μ m. Data were used to calculate the percentage of cell shrinkage at each time point in comparison to the total cells area at the starting point.

For *Yersinia enterocolitica* infection experiment, Hela cells and Cells tracker green (Ctg) (Life Technologies: C7025) dye were used. The same protocol as described above was used for CTg staining detection.

Bright nuclei: A549 cell were cultured overnight at 12500 cells/100 μ L/well in Black μ clear 96-well plates (Greiner) in RPMI supplemented with 10% of FBS at 37°C. Two hours before infection, medium was replaced with 100 μ L of fresh RPMI containing 10% FBS and Hoechst Vital (Sigma Aldrich: B1155) (1 μ g/mL) to label cell nuclei. After one hour of incubation, cells were washed two times with 100 μ L of medium and then incubated with *P.aeruginosa* CHA or the *ApseE* mutant (MOI = 10) in presence of compounds. The image acquisitions were performed using the automated microscope ArrayScan VTI (Thermo Scientific) as previously described (Wallez et al., 2018). In brief, four images per well were acquired with the 5X objective every 15 min for 4 h. Analysis were performed by measuring the nucleus fluorescence intensity using HCS Studio v6.5.0 software and the bright nuclei reflecting stressed cells were discriminated by applying a threshold of 1200 fluorescence arbitrary units (a.u).

Analysis of ExoS-bla secretion. *P.aeruginosa* CHA *LexoS/ExoS-Bla* and CHA *ApseF/ExoS-Bla* were grown overnight in LB containing Carbenicillin (300 μ g/mL). Culture were then diluted in LB containing EGTA (5 mM) and MgCl₂ (20 mM), and distributed (90 μ L per well) in a 96 well plate (REF 353072) in presence of compounds diluted at three concentrations (30 μ M, 10 μ M, 3 μ M). The bacterial final OD was 0.001 and plates were put into an incubator at 37°C with shaking of 300rpm. After 3 h of culture, 9 μ L of Nitrocefin (Calbiochem: 484400) pre-diluted in PBS (250 μ g/mL) were added and the OD 490_{nm} was measured after 15 min of incubation at 37°C to detect β -lactamase activity. To control the absence of direct effect of compounds on β -lactamase activity, bacteria were cultured in 3 mL of

LB containing EGTA and MgCl₂ at OD of 0.1 for 3 h at 300 rpm and 37°C. 500 µL of bacteria culture were then centrifuge at 11000 g for 5 min and the supernatant containing ExoS-Bla was diluted 50 times in LB Mg²⁺ EGTA. The diluted supernatant was distributed in a 96 well plate (REF 353072) containing compounds and the β-lactamase activity was detected by adding Nitrocefin as described above. Absorbances were measured on a SAFAS Microplate Readers MP96.

Molecule toxicity. HepG2 cell were seeded the day before the experiment at 10000 cells in 100µL/well in Black µclear 96-well plates (Greiner) in DMEM supplemented with 10% of FBS at 37°C in a humidified incubator with an atmosphere of 5% CO₂. On the day of experiment, medium was replaced with 80 µL of fresh medium containing **12(6,4)** and **12(4,6)** compounds at eight concentrations (from 0 to 100 µM as described in Figure 5). After 2 days of incubation, PI was added to numerate dead cells by Incucyte® microscopy as described above. The medium was then removed and the cell proliferation was assessed with the MTT Kit following the manufacturer instruction (Sigma Aldrich: Tox1-1KT). Statistical analysis were performed using R (version 3.4.4) for the comparison of multiple groups by one-way ANOVA. The Dunnett post hoc test was applied for comparison to the control group of DMSO.

Bacterial motility. *P. aeruginosa* CHA swimming motility was performed by inoculating bacteria on top of LB agar made with 10 g/l tryptone, 0.3% agarose and 5 g/L NaCl. Petri dishes were incubated at 37°C for 6 h and the diameters of the swimming rings were measured for three independent biological replicates.

P. aeruginosa CHA twitching motility was performed by inoculating bacteria at the interface between the plastic petri dish and LB agar made with 10 g/L tryptone, 5 g/L yeast extract, 1% agarose and 10 g/L NaCl. Petri dishes were incubated at 37°C for 32 h and agar was removed. The zone of twitching was then revealed by Coomassie blue staining and the twitching ring diameters were measured for three independent biological replicates.

Bacterial intracellular ATP measurement. *P. aeruginosa* CHA overnight cultures were grown in 3 mL of LB in presence or absence of **12(6,4)** and **12(4,6)** until DO₆₀₀ of 1. Bacterial cultures were centrifuged and bacterial pellets were resuspended in Tris-EDTA solution. The bacterial suspensions were then heated at 100°C for 5 min and the ATP content was dosed using the Kit luminescence following the manufacturer instruction (Invitrogen: A22066).

Statistical analysis were performed using R (version 3.4.4) for the comparison of multiple groups by one-way ANOVA.

Western blot analysis of ExoS, ExoT and PopD secretion. *P. aeruginosa* CHA was grown overnight in LB containing and culture were then diluted in 3 mL of fresh LB containing EGTA (5 mM), MgCl₂ (20 mM) and **12(6,4)** or **12(4,6)** at three concentration (30 µM, 12.5 µM, 5 µM). Bacteria were incubated at 37°C under shaking for 2.5 h and 1 mL of bacterial culture was centrifuged at 11000 rpm for 3 min. Supernatant and pellet fractions were then analyzed by western blot with anti-Exoenzyme S and anti-PopD antibodies.

Galleria mellonella infection. Calibrated larvae (reference #101040) were purchased from Sudestappats (Queige, France) and used on the day of receipt. Overnight cultures of *P. aeruginosa* CHA WT and *ApscE* were grown in LB until OD₆₀₀ of 1. Bacterial culture were then centrifuged and the pellet were washed three time with sterile PBS. Afterward, the bacterial pellet were diluted in PBS to obtain a calculated dose of 5 bacteria/µL. The larvae were injected with 10 µL of bacteria solution (50 bacteria) near the penultimate, or last pro-legs. Another group was injected with 10 µL PBS only as a control. One hour after bacteria injection, **12(6,4)** or **12(4,6)** were injected at a final concentration of 12.5 µM in the infected larvae. The larvae masses slightly varied around 250 mg and were used to calculate treatment doses (250 mg equal to 250 mL). The infected larvae were then placed in a 100 mm Petri dish lined with paper towel (10 larvae per dish) and incubated at 37°C for 20 h. After 12 to 13h, infected larvae were monitored every 30 min to enumerate deaths. Data from four independent experiments using groups of 10 larvae were pooled to obtain n = 40. The survival data were plotted using Kapan-Meier estimation. The statistical comparison was performed using the log-rank test and false discovery rate (FDR) correction was applied.

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❖ Author contribution

TD.N., S.P., I.A., YS.W., and E.F. designed experiments, TD.N., S.P., C.B., YS.W., and E.F. performed experiments, TD.N., S.P., YS.W., I.A., and E.F., analyzed and discussed the data and TD.N., YS.W., and E.F. wrote the manuscript. All authors contributed to and edited the manuscript.

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❖ Figures

Figure 1: The co-chaperones PscE and PscG in the context of the Type Three Secretion System. (A) Before secretion, the PscF protein is dissociated from its co-chaperones PscE and PscG and then auto-assembles to form a needle-like structure. Afterwards, PcrV forms the needle tip and the translocon inserts into the eukaryotic plasma membrane to allow the injection of the toxins. (B) Crystallographic structure of the PscE-PscF-PscG complex (PDB: 2UWJ). PscE is represented in blue, PscF in orange and PscG in green. (C) Interaction surface between PscE (Ribbon and lateral chains) and PscG (solid surface).

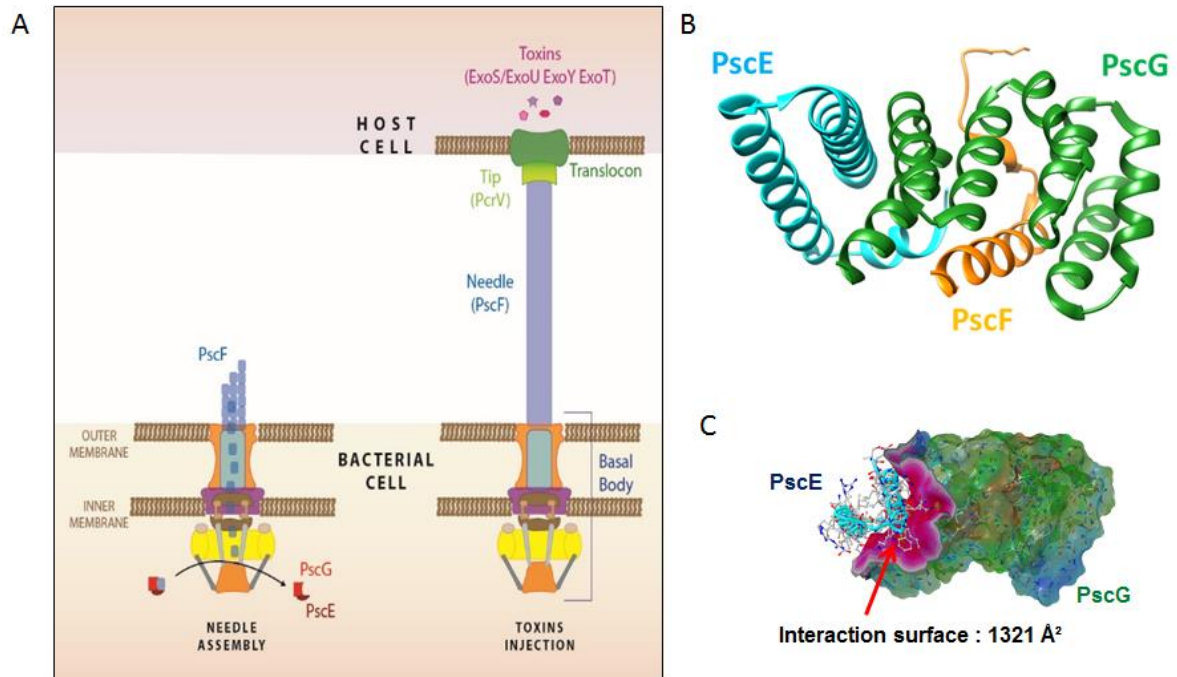


Figure 2: Screening of chemical compounds inhibiting PscE-PscG interaction. (A) PscE and PscG were purified using Nickel chromatography and Wash and Elution fractions were analyzed by SDS-PAGE followed by Coomassie staining. (B) Target-based screening ELISA assay. (C) Inhibition of PscE-PscG interaction revealed by the ELISA assay for clioquinol or analogs (red triangle), 3-APPA or analogs (blue diamond) and hybrid 3-APPA/clioquinol (green circle) at 50 μ M.

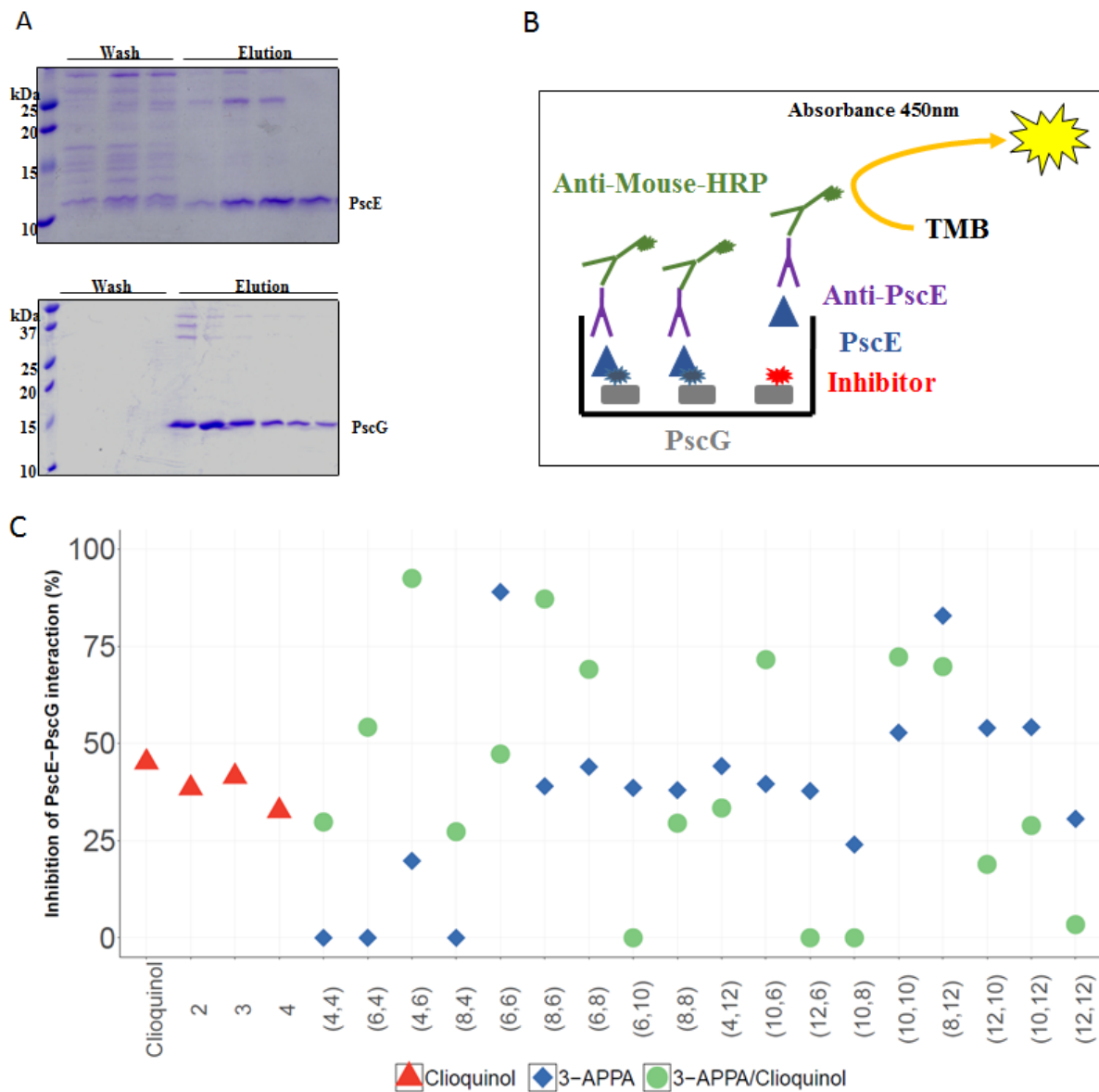


Figure 3: Characterization of 21 selected compounds. The effect of 21 selected compounds on bacterial growth, eukaryotic cell toxicity and bacterial virulence was assessed in triplicates at 3, 20 and 30 μM . All experimental points are represented on radar plots (coordinate polar). The three axis correspond to the three assays: bacterial growth inhibition monitored by OD_{600} , cell toxicity and infection inhibitions detected by PI incorporation. The four circles correspond to 25, 50, 75 and 100% of inhibition.

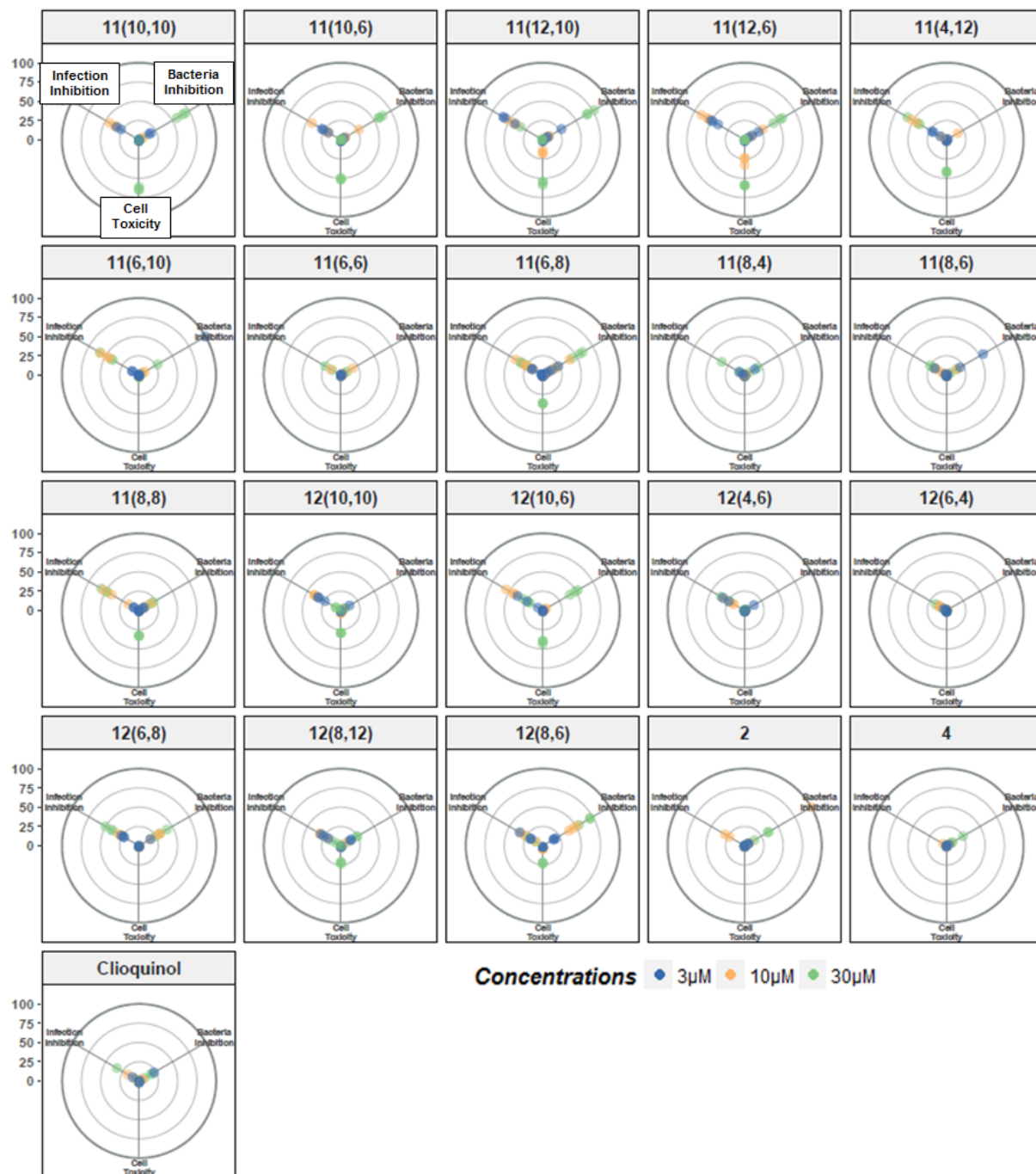


Figure 4: Characterization of selected compounds. The effect of seven selected compounds was assessed in triplicates at 3, 20 and 30 μM by three orthogonal assays: the secretion of ExoS-bla detected by beta-lactamase activity quantification, the cell stress monitored by bright nuclei detection and cell shrinkage quantified by cell area measurement. All experimental points are represented on radar plot (coordinate polar) in percentage of inhibition. Three axis correspond to 3 independent assays: ExoS secretion, Cell stress and cell shrinkage. The four circles correspond to 25, 50, 75 and 100% of inhibition.

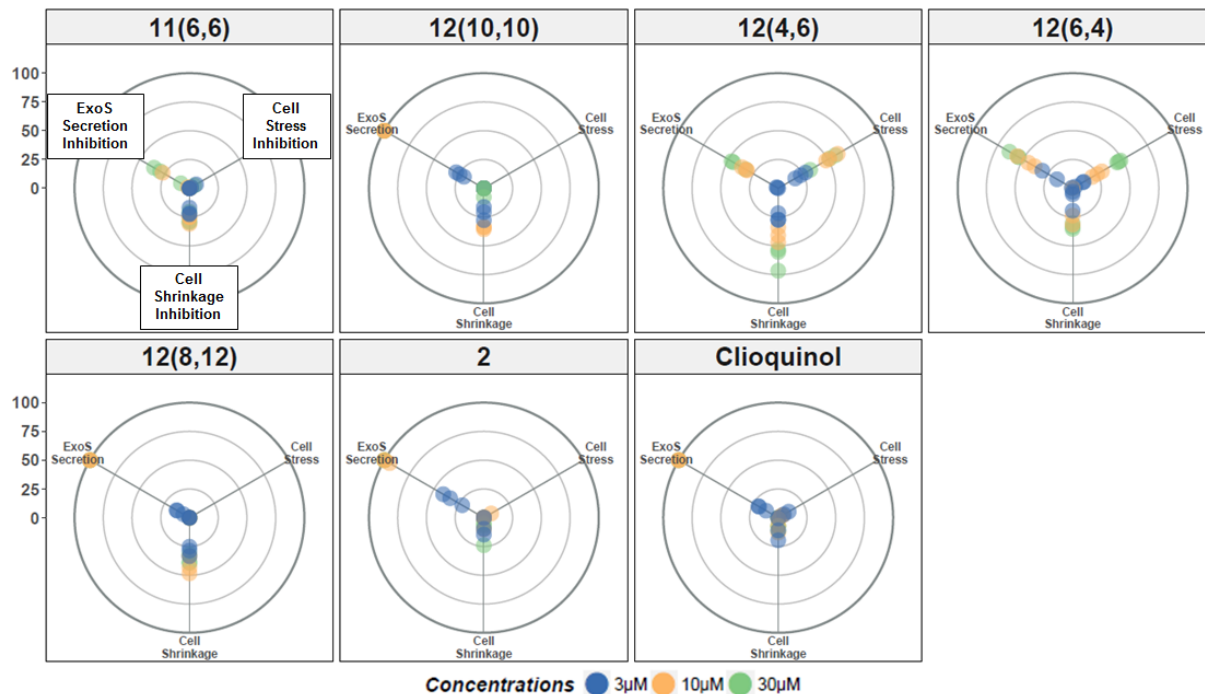


Figure 5: Toxicity of 12(6,4) and 12(4,6) towards eukaryotic cells. (A) Dead cells were enumerated through Propidium Iodide staining of HepG2 cells after 48 h of incubation with **12(6,4)** or **12(4,6)** at the indicated concentrations. (B) Cell proliferation was measured by the MTT assay on HepG2 cells after 48 h of incubation with **12(6,4)** or **12(4,6)** at the indicated concentrations. NS: non-significant; *: $p < 0.05$; **: $p < 0.001$.

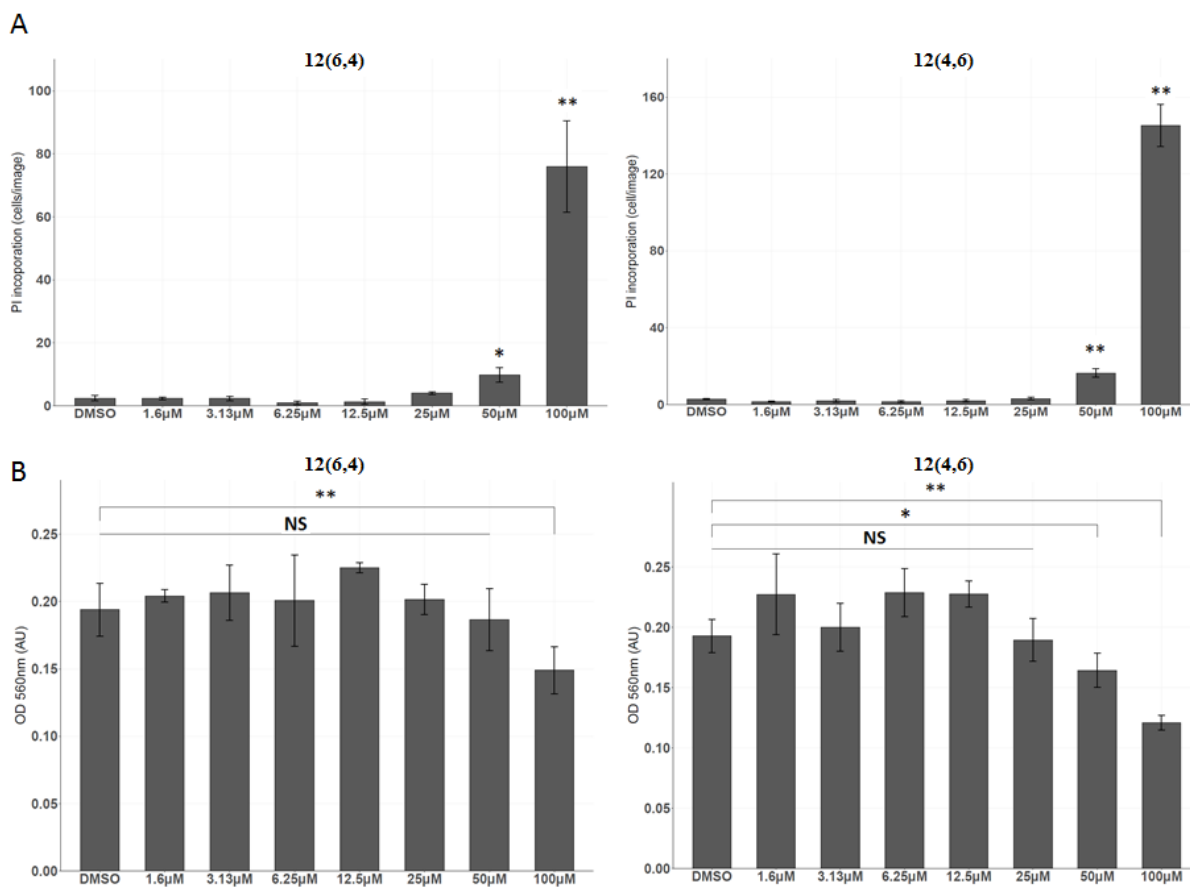


Figure 6: 12(6,4) and 12(4,6) inhibit *P. aeruginosa* T3SS and do not affect bacterial fitness. (A) *P. aeruginosa* CHA was grown in the presence of 12(6,4) or 12(4,6) at 5, 12.5, 30 μM or DMSO. The secretion of two effectors ExoS, ExoT and one translocator PopD was detected by western blot. (B) Western-Blot quantification of three independent experiments allowed the determination of IC_{50} of the compounds for the secretion of the three proteins. (C) The growth of *P. aeruginosa* CHA in LB medium containing DMSO, 12(6,4) or 12(4,6) at 12.5 μM was monitored for 24 h by measuring the Optical Density at 600 nm. (D) The concentration of intracellular ATP of *P. aeruginosa* CHA was determined in the exponential phase growth in the presence of DMSO, 12(6,4) or 12(4,6). No significant difference was found.

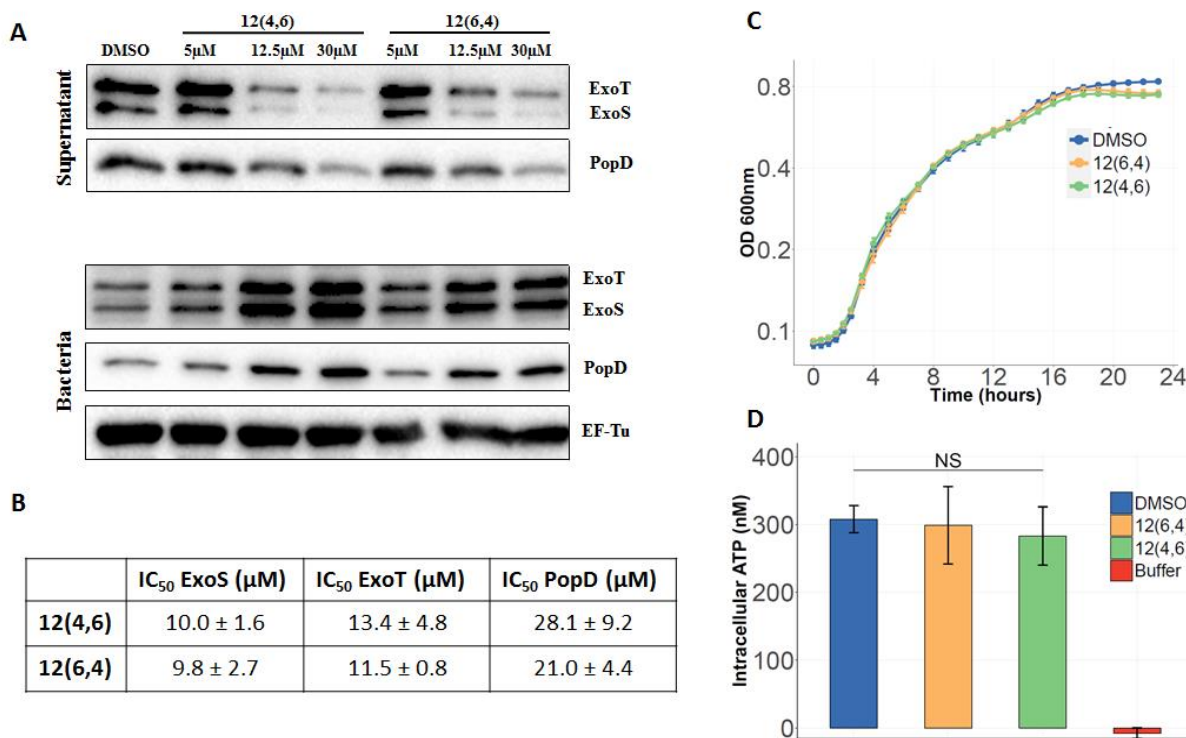
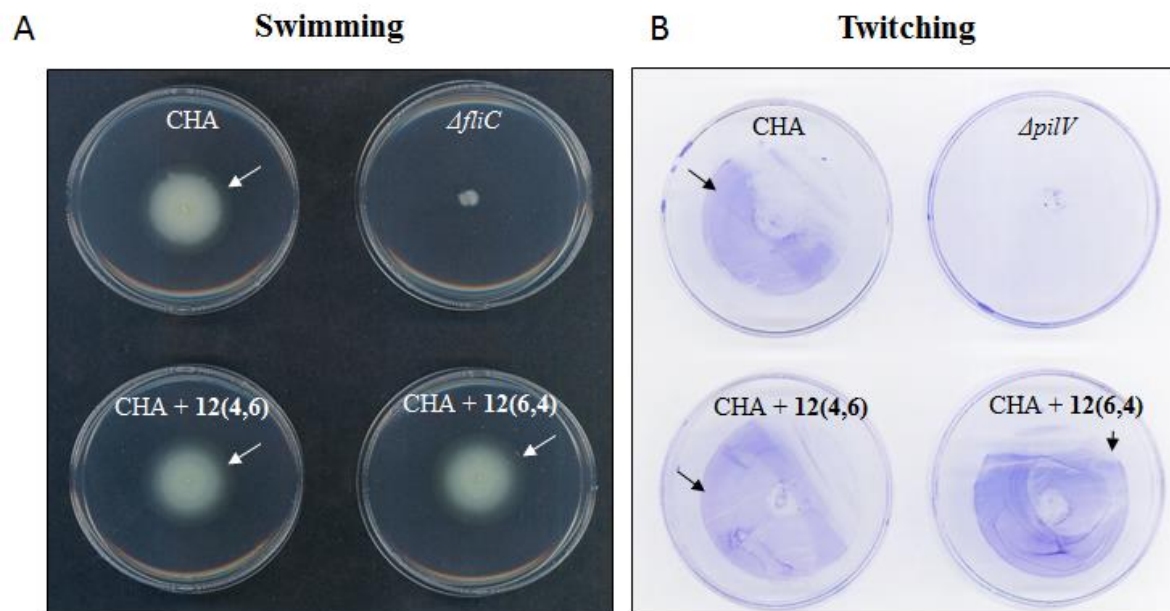


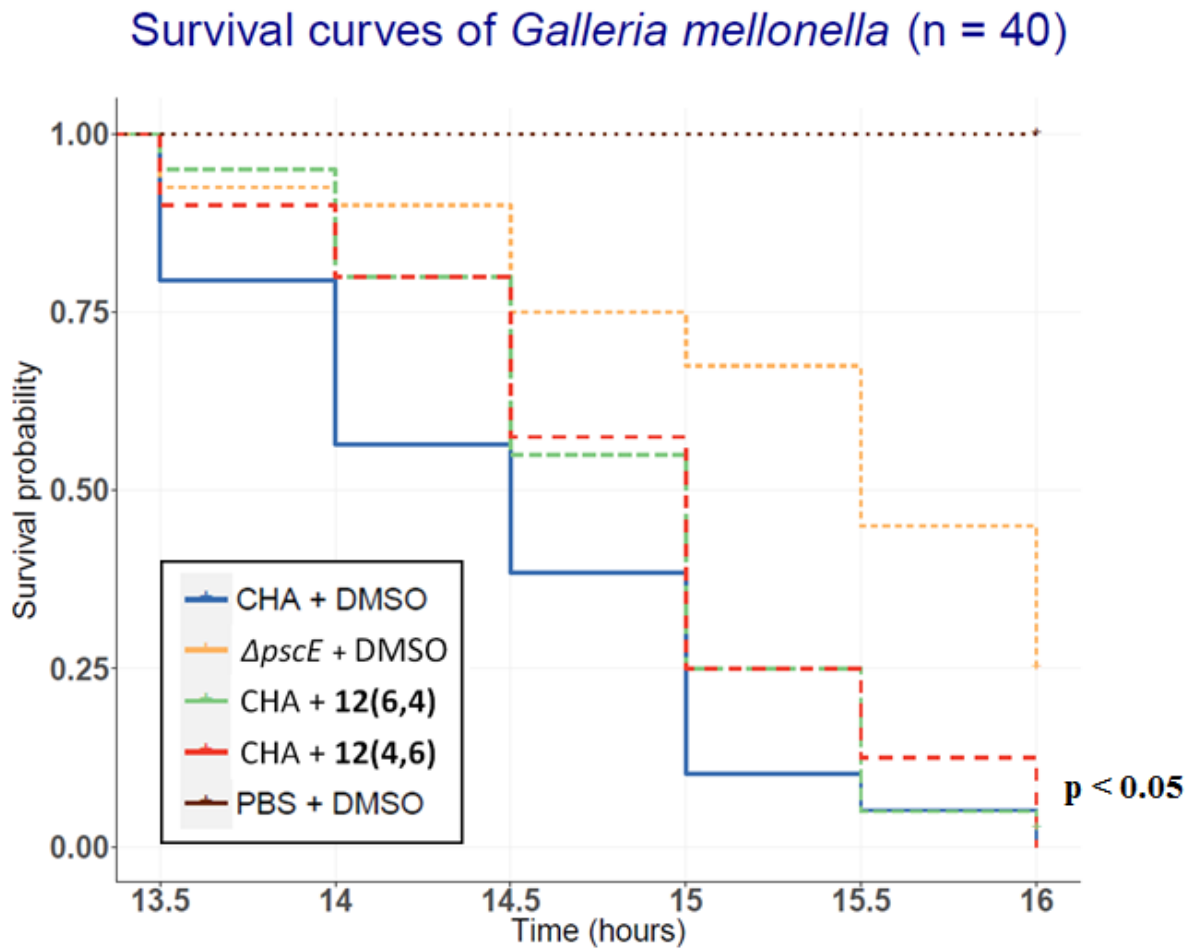
Figure 7: 12(6,4) and 12(4,6) do not affect flagella nor T4 pili of *P. aeruginosa*. Two different types of bacteria motility was assessed on agar plates: the swimming and twitching motilities that are respectively dependent on flagella and T4 pili. (A) Swimming assay of *P. aeruginosa* CHA strain in the presence of **12(6,4)** or **12(4,6)** at 12.5 μ M or DMSO. The strain Δ *fliC* devoid of functional flagella was used as a negative control. (B) Twitching assay of *P. aeruginosa* CHA strain in the presence of **12(6,4)** or **12(4,6)** at 12.5 μ M or DMSO. The strain Δ *pilV* devoid of functional pili was used as a negative control. (C) The diameters of the motility rings were measured for three independent replicates. The table presents the means and standard deviations for the two motility assays. The only statistically significant differences are observed for the mutant controls.



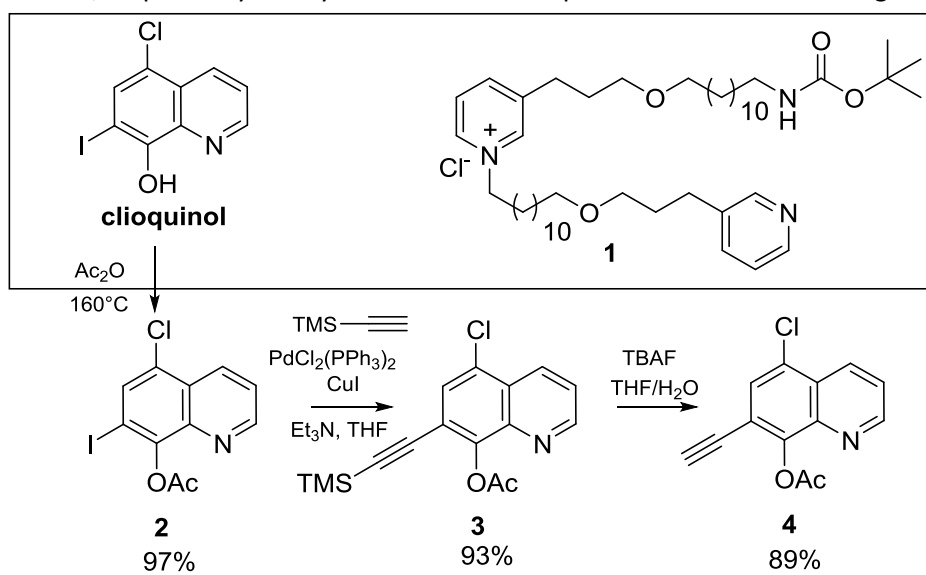
C

Bacterial strains and condition	Swimming Diameter (mm)	Twitching Diameter (mm)
CHA WT	22.2 \pm 0.3	33 \pm 2
CHA Δ <i>fliC</i> or Δ <i>pilV</i>	3.5 \pm 0.5	2 \pm 2
CHA WT + 12(4,6) at 12.5 μ M	21.7 \pm 0.3	34.5 \pm 0.6
CHA WT + 12(6,4) at 12.5 μ M	21.3 \pm 0.6	35.3 \pm 1

Figure 8: 12(6,4) and 12(4,6) protect *Galleria mellonella* from *P. aeruginosa* infection. Insect larvae (n=40) were infected with a dose of 50 bacteria and were injected 30 min later with **12(6,4)**, **12(4,6)** at 12.5 μ M, or DMSO. The strain $\Delta pscE$ was used as a negative control. The survival of the insects was monitored for 20 h and survival curves were represented with a Kapan-Meier plot. A significant difference ($p < 0.05$) between the larvae that received DMSO and the compounds indicate that **12(6,4)** and **12(4,6)** protect from *P. aeruginosa* infection.



Scheme 1. Clioquinol and 3-APPA **1** as best hits from Prestwick and in-house natural product-like chemical libraries, respectively and synthesis of new clioquinol derivatives according to Cironi *et al.*



Scheme 2. Split/mix/click synthetic route to obtain 3-APPA **11(n,n')** and hybride 3-APPA/clioquinol **12(n,n')**

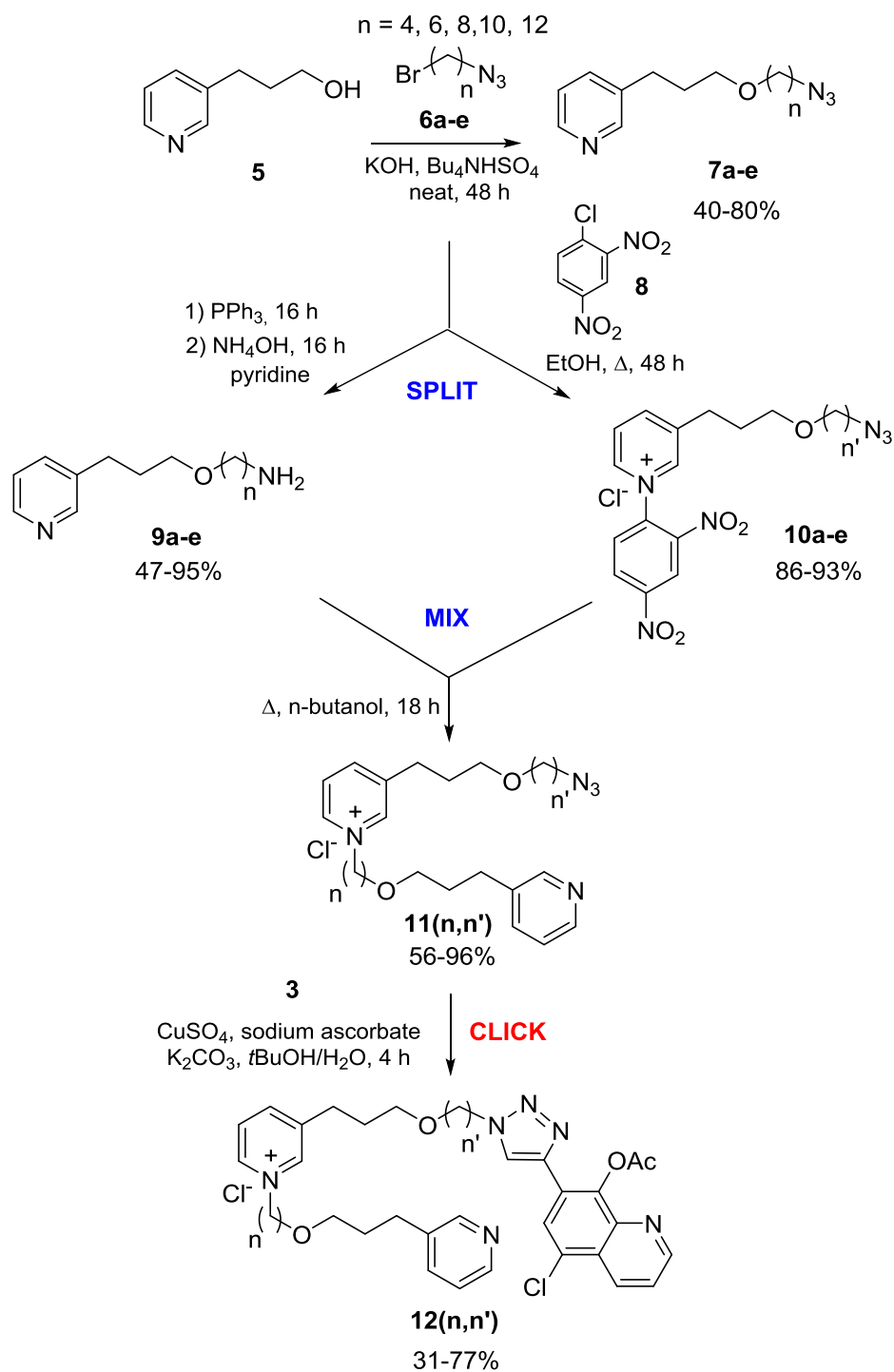
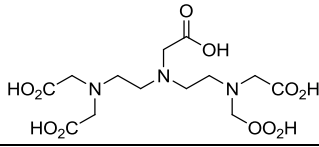
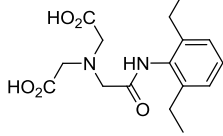
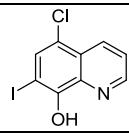
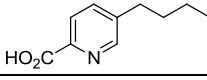
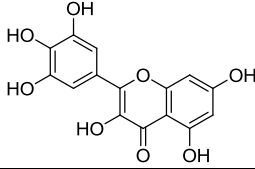
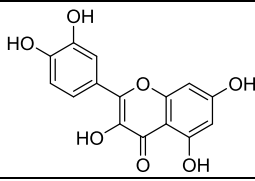
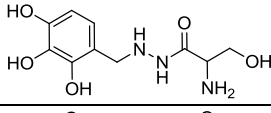
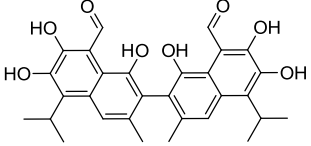
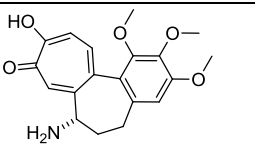
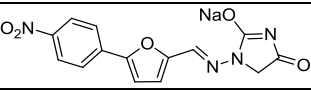
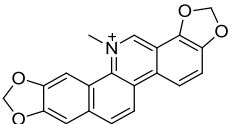
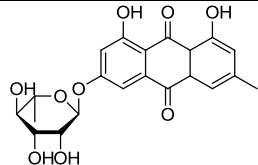
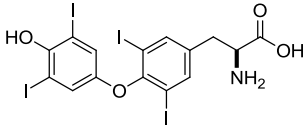
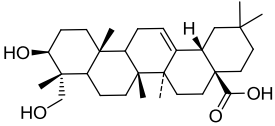
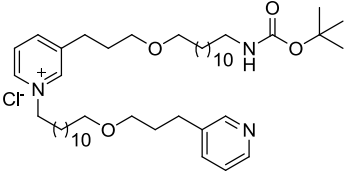


Table S1: Selected compounds from the primary screening of PscE-PscG interaction

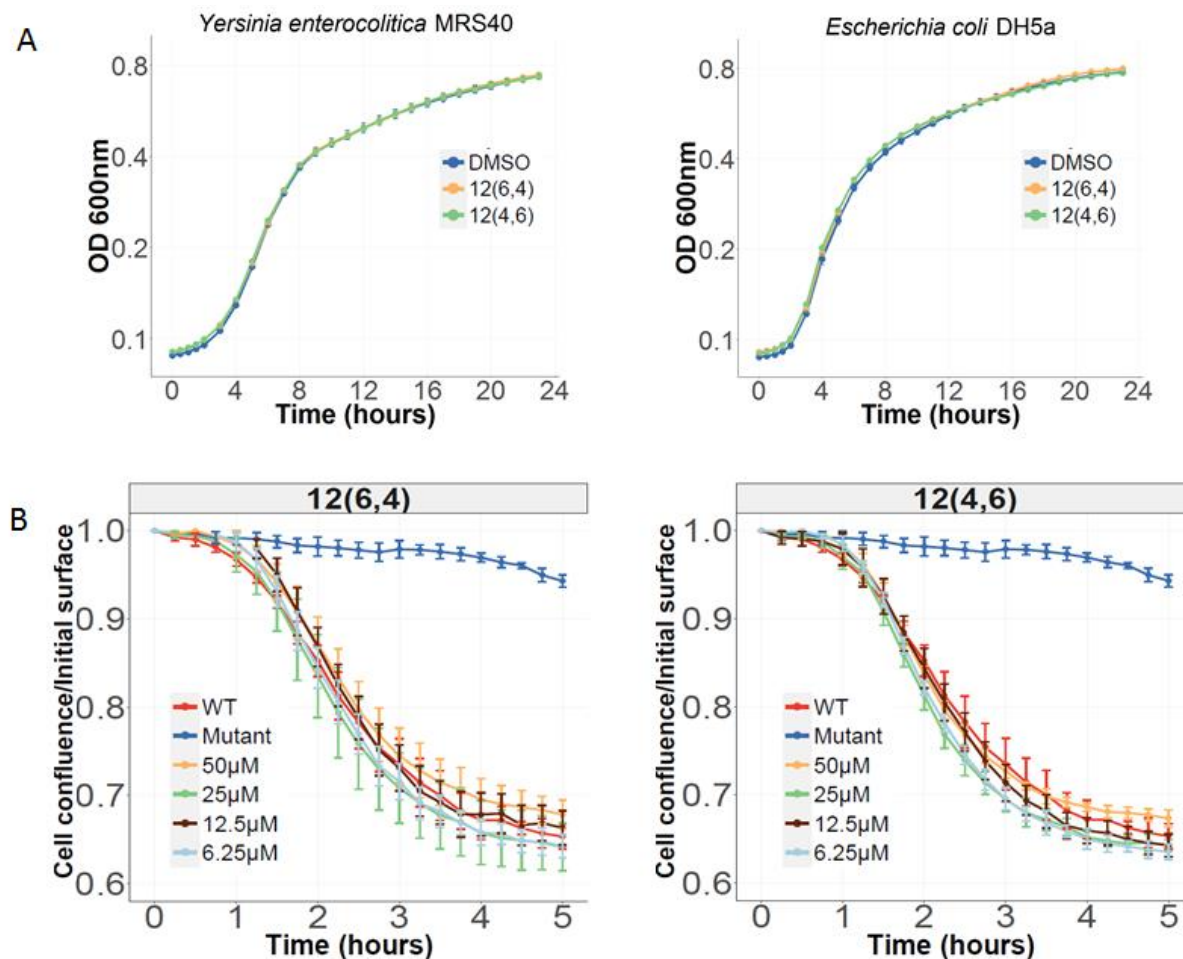
Cluster	Molecules	Name	Known properties	% Inhibition at 50 μ M
Prestwick hits from 1280 compounds				
Siderophore		Pentetic acid		94%
		Etifenin		85.5%
Pyridine-like		Clioquinol	Antifungal Antiprotozoal	93.7%
		Fusaric acid		79.4%
Phenolic compounds		Myricetin	Anti-oxidant	80.5%
		Quercetin	Anti-oxidant	36.5%
		Benserazide	Decarboxylase inhibitor	86.3%
		Gossypol		73.9%
Micellous		Deacetylcolchicine	Tubulin inhibitor	71.8%
		Dantrolene	Muscle relaxant	59.3%
		Sanguinarine	Antiseptic	58.7%

		Frangulin A		54%
		Thyroxine	Hormone	48%
		Hederagenin		46.6%
Natural product-like hits from 120 compounds				
3-Alkyl pyridine/pyridinium alkaloid (3-APPA)				56%

TableS2: Inhibition of the PscE-PscG interaction in the ELISA test for the synthetic analogs. The compounds selected for the characterization *ex vivo* and *in vivo* are highlighted in grey.

Cluster	Compound	% inhibition at 50 μ M
Clioquinol analogs		
Reference	Clioquinol	75%
Clioquinol-like	2	64%
	3	54.3%
	4	69%
3-APPA analogs 11(n,n') and combination 3-APPA/clioquinol 12(n,n')		
Reference	Clioquinol	45.2%
3-APPA 11(n,n')	11(4,4)	No inhibition
	11(6,4)	No inhibition
	11(4,6)	19.8%
	11(8,4)	No inhibition
	11(6,6)	89%
	11(8,6)	39%
	11(6,8)	44%
	11(6,10)	38.6%
	11(8,8)	38%
	11(4,12)	44.2%
	11(10,6)	39.6%
	11(12,6)	37.8%
	11(10,8)	24%
	11(10,10)	52.8%
	11(8,12)	82.9%
	11(12,10)	54%
	11(10,12)	30.6%
11(12,12)	54.2%	
3-APPA/clioquinol 12(n,n')	12(4,4)	29.8%
	12(6,4)	54.2%
	12(4,6)	92.5%
	12(8,4)	27.3%
	12(6,6)	47.3%
	12(8,6)	87.2%
	12(6,8)	69.1%
	12(6,10)	No inhibition
	12(8,8)	29.5%
	12(4,12)	33.4%
	12(10,6)	71.6%
	12(12,6)	No inhibition
	12(10,8)	No inhibition
	12(10,10)	72.3%
	12(8,12)	69.8%
	12(12,10)	18.9%
	12(10,12)	28.9%
12(12,12)	3.4%	

Figure S1: Effect of W550 and W887 on other gram negative bacteria. (A) **12(6,4)** and **12(4,6)** do not affect the growth of *Yersinia enterocolitica* and *Escherichia coli* at 12.5 μ M. (B) **12(6,4)** and **12(4,6)** do not display protective effect in Hela cells against *Y. enterocolitica*. Hela cells were incubated with *Y. enterocolitica* for 5 h in presence of **12(6,4)** and **12(4,6)** at four concentrations. Images were acquired every 15 min by using Incucyte® automated microscopy and cell shrinkage was assessed by measuring the cell confluence at each time point and normalized by cells area at the starting point.



IV. Conclusion (in French)

Des études sur la pathogénicité bactérienne ont souligné l'importance du SST3 pour la virulence de *P. aeruginosa*. Ainsi, ce facteur de virulence a attiré beaucoup d'investissements pour développer des antimicrobiens inhibant la sécrétion de toxines. Un tour d'horizon des inhibiteurs du SST3 chez *P. aeruginosa* montre que plusieurs composés ont été développés, ciblant potentiellement le régulateur transcriptionnel ExsA (Bowser et al., 2007; Kim et al., 2009), l'ATPase PscN (Anantharajah et al., 2016b; Enquist et al., 2012), la sécrétine PscC (Felise et al., 2008; Kline et al., 2009), l'aiguille PscF (Aiello et al., 2010; Williams et al., 2015), le translocateur PcrV et les exotoxines ExoS et ExoU (Arnoldo et al., 2008; Kim et al., 2014; Lee et al., 2007; Pinto et al., 2016). La plupart de ces composés ont été initialement identifiés par un criblage phénotypique, ce qui rend difficile la détermination de leur cible moléculaire. C'est pourquoi, dans cette étude, nous présentons une stratégie d'utilisation d'un criblage ciblé de composés chimiques pour identifier des inhibiteurs du SST3 de *P. aeruginosa*. Cette stratégie nous permet de réduire les risques d'inhibition non-spécifique et de limiter les effets indésirables.

Ainsi, nous avons identifié des molécules inhibant l'interaction PscE-PscG qui sont les deux chaperonnes de la protéine PscF formant l'aiguille du SST3 en utilisant un criblage basé sur un test ELISA. Ces molécules ont été optimisées structurellement pour créer des analogues qui ont été ensuite caractérisées sur des modèles cellulaires *ex vivo* et sur les bactéries *in vivo*, désignant deux composés prometteurs **12(6,4)** et **12(4,6)**. Ces molécules sont en fait obtenues en fusionnant chimiquement deux « clusters » qui proviennent des criblages de deux chimiothèques différentes, ce qui résulte en un effet synergique *in vitro* et *in vivo*. De plus, **12(6,4)** et **12(4,6)** ont pu améliorer la survie de *G. mellonella* après une infection à *P. aeruginosa* (Figure 30) alors qu'elles n'ont presque pas d'effet sur la croissance, mobilité et métabolisme de bactéries. Donc, ces molécules sont confirmées comme des anti-virulents et non des antibiotiques. Par ailleurs, **12(6,4)** et **12(4,6)** ne sont pas capables d'inhiber les dommages cellulaires causés par d'autres bactéries comme il est montré pour *Yersinia enterocolitica* bien que son SST3 soit phylogénétiquement proche de celui de *P. aeruginosa* (Cornelis, 2006). Ce résultat indique un spectre bactérien étroit et un signe de spécificité pour *P. aeruginosa*.

Pour conclure, ce travail inclut plusieurs étapes allant de la découverte de touches jusqu'au test préclinique dans un modèle d'insecte (Hit to Lead). Nous avons défini une nouvelle cible et mis en évidence l'efficacité de deux composés chimiques pour une stratégie d'anti-virulence contre le SST3 de *P. aeruginosa*. La prochaine étape de ce projet est de tester l'efficacité des deux composés « lead » dans des modèles animaux plus complexes comme le poisson zèbre et/ou la souris.

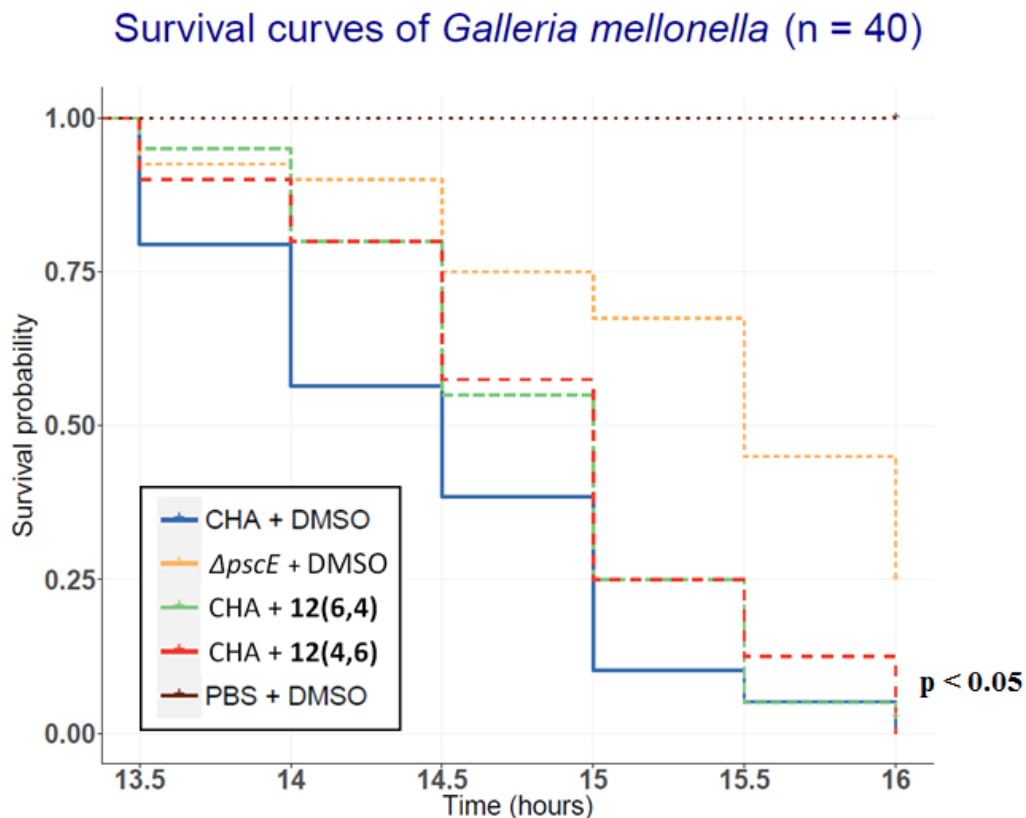


Figure 30: 12(6,4) et 12(4,6) protègent *Galleria Mellonella* lors d'infection à *P. aeruginosa*. Des larves d'insecte ont été infectées avec une dose de 50 bactéries et injectées 30 minutes après avec **12(6,4)** et **12(4,6)** à la concentration de 12.5 μ M ou du DMSO. La souche $\Delta pscE$ est utilisée comme control négatif. La survie des larves est surveillée pendant 20 h et les courbes de survie sont représentées par un graphique de Kaplan-Meier. Une différence significative (p -value < 0.05) entre des larves traitées par le DMSO et celles traitées par un de deux composés, montre que ces composés les protègent lors d'infection à *P. aeruginosa*.

C. Targetting of the T3SS ATPase PscN in *Pseudomonas aeruginosa*

I. Abstract (in French)

Contexte. L'ATPase PscN est une protéine importante du système de sécrétion de type III (SST3) chez *P. aeruginosa*. Par conséquent, cet enzyme est une cible potentielle pour la recherche d'anti-virulent dans un cadre général de résistance accrue aux antibiotiques qui rend nécessaire la découverte d'alternative thérapeutique.

Résultats. Cette enzyme est très difficile à exprimer sous forme soluble et à purifier sans doute pour des questions de repliement. C'est pourquoi, des optimisations de l'expression de cette protéine ont été effectuées chez *E. coli* et PscN soluble fusionné à une étiquette Strep a été purifiée partiellement sur colonne StrepTrap HP. La capacité d'interaction de PscN avec ses partenaires protéiques du SST3 a été montrée par un test ELISA, ce qui permît ensuite un criblage à haut débit pour identifier des inhibiteurs qui bloquent l'interaction de PscN avec le complexe translocateur/chaperonne PcrH-PopD. Ensuite, des touches identifiées ont été caractérisées en utilisant des approches de microscopie automatisée et des tests colorimétriques. Sept molécules ont montré une efficacité sur une modèle cellulaire *ex vivo* et sur la bactérie *in vivo*. Par ailleurs, l'optimisation de la purification de PscN a été poursuivie afin d'obtenir la protéine pure pour des caractérisations biochimiques *in vitro*. Le meilleur rendement de purification a été obtenu en utilisant la construction de Flag-Strep-Strep-PscN (FSS-PscN) exprimé dans une souche d'expression de *P. aeruginosa*. La FSS-PscN purifié a été ensuite utilisé pour valider la spécificité des molécules identifiées envers l'interaction de PscN avec PcrH-PopD. Finalement, une seule molécule a été retenue mais possède une action de détergent dont il faudra tenir compte pour de futurs développements.

II. Introduction (in French)

Pseudomonas aeruginosa est une pathogène opportuniste qui cause des maladies nosocomiales et infecte les patients atteints de mucoviscidose. Le système de sécrétion de type III (SST3) est un de ses facteurs de virulence le plus important et permet d'injecter quatre exotoxines directement de la bactérie dans la cellule cible (Hauser, 2009). Une protéine très importante de ce système est l'ATPase qui est présente et conservée dans plusieurs bactéries

gram négative. Il est considéré que cet enzyme interagit avec les protéines sécrétées du SST3 et les livre au canal de sécrétion (Akeda and Galan, 2005; Allison et al., 2014; Yoshida et al., 2014; Zarivach et al., 2007). Dans un deuxième temps, cet enzyme fournit l'énergie pour la dissociation des complexe protéine-chaperonne et finalement, elle déplie la protéine sécrétée pour son passage à travers l'aiguille du SST3 (Akeda and Galan, 2005; Yoshida et al., 2014).

P. aeruginosa résiste naturellement à plusieurs antibiotiques, ce qui cause un problème majeur pour la thérapie contre ce pathogène. Récemment, l'organisation mondiale de la santé (OMS) a classé cette bactérie comme prioritaire pour le développement de nouveaux antibiotiques (Tacconelli et al., 2018). Chez *P. aeruginosa*, l'ATPase PscN est essentielle pour sa virulence car le mutant de délétion *ΔpscN* n'est pas cytotoxique (chapitre II-A). Donc, cette protéine pourrait être une cible potentielle pour la recherche d'anti-virulents contre ce pathogène. Par conséquent, notre objectif est tout d'abord d'étudier et caractériser l'ATPase PscN afin de comprendre son rôle pour la virulence de *P. aeruginosa*. Par ailleurs, nous cherchons des petites molécules chimiques inhibant l'interaction de PscN avec le complexe de translocator PcrH-PopD. En effet, l'interaction de PscN avec des partenaires est plus spécifique que son activité enzymatique et des inhibiteurs de l'activité catalytique pourraient présenter une activité croisée contre des ATPase humaines.

III. Material and method.

1 Bacterial strains, plasmids and primers

E. coli strains, plasmids and primers used in this study are listed in the Table 7. *P. aeruginosa* PAO1 ADD1976/pET15bVP-FSS-PscN, BL21/pET15b-FSS-PscN, BL21/pET15b-Strep-PscN and *E. coli* strains used for overexpression of T3SS secreted proteins were described in the chapter II-A. His- and Strep-tagged PscN constructions were generated by PCR using primers NdeI-Strep-PscN and PscN-HindIII or NdeI-PscN and PscN-Strep-HindIII. The amplified fragments were cloned into NdeI/HindIII sites of pET15b vector in which, the His-tag was already present upstream of the NdeI site. The pET15b-His-Strep-PscN and pET15b-His-PscN-Strep were next transformed into *E. coli* BL21 (DE3).

Table 7: Strains, plasmids and primer

Strains and plasmids		
Strain	Description	Source or reference
BL21 (DE3)/pET15b- His-Strep-PscN	His-Strep-PscN expressed in BL21 and in pET15b (N-terminal His Strep)	This study
BL21 (DE3)/pET15b- His-PscN-Strep	His-PscN-Strep expressed in BL21 and in pET15b (N-terminal His and C-terminal Strep)	This study
Primers used		
Name	Sequence 5'-3'	Use
<u>NdeI</u> <u>Strep</u> PscN	AAC <u>CAT ATG GCC AGC TGG AGC CAC CCG CAG TTC</u> <u>GAG AAG CCG GGC</u> ATG CCC GCG CCT CTC TCT CCT C	Add NdeI site and Strep-tag at N-terminal of <i>pscN</i>
PscN <u>Hind</u>	TGG <u>AAG CTT</u> TCA TGC CGA GAG GCT CCG CAA CTG CGC G	Add HindIII site at C-terminal of <i>pscN</i>
<u>NdeI</u> PscN	AAC <u>CAT ATG</u> CCC GCG CCT CTC TCT CCT C	Add NdeI site at N-terminal of <i>pscN</i>
PscN <u>Strep</u> <u>Hind</u>	TGG <u>AAG CTT</u> TCA <u>GGC CTT CTC GAA CTG CGG GTG</u> <u>GCT CCA GCT CGG GCC</u> TGC GCA GAG GCT CCG CAA CTG CGC G	Add Strep-tag and HindIII site at C-terminal of <i>pscN</i>

2 Protein expression and purification.

For the ELISA assay and screening, *E. coli* BL21 (DE3) harboring Strep-PscN were grown at 37°C for 4 hours. Harvested cells were resuspended in 20ml of binding buffer containing 100mM Tris pH8, 150mM NaCl and 1mM EDTA. Cell suspension was lysed with the microfluidizer apparatus and centrifuged at 200,000g for 30 minutes. Soluble proteins were purified on StrepTrap HP column and eluted using the binding buffer containing 2.5mM desthiobiotin.

For comparison of purification yield between Strep-PscN (*E. coli*), FSS-PscN (*E. coli*) and FSS-PscN (*P. aeruginosa*), these proteins were expressed and purified in the same condition. See chapter II-A for further details.

Most of the secreted proteins were purified as previously described (chapter II-A). For PopB purification, the purified His-PcrH-PopB was dialyzed in buffer containing 25mM Tris pH8, 500mM NaCl, 10mM Imidazole and 6M guanidine. The dialyzed proteins were loaded onto HisTrap HP column and purified PopB was present in the flow-through fraction while His-PcrH was retained on the column.

3 ELISA and High-Throughput target-based Screening.

The ELISA assay was performed as described before (chapter II-A) except that TMB substrate (absorbance reading) or ECL-western blot substrate were used (luminescence reading).

For screening, 50 μ l of the PcrH-PopD complex diluted in PBS at 0.1 μ M was coated on ELISA plate white (Greiner 655 074) overnight. The wells were then washed three times with 200 μ l of PBS/0.1% Tween 20 (PBST) and incubated with 150 μ l of PBS containing BSA 4% for 4 hours at room temperature (RT $^{\circ}$). The solution was next removed by dripping. Afterward, a bacterial extract that contains Strep-PscN and inhibitors were diluted in PBST containing 4% BSA (PBST.BSA 4%). While the final concentration of inhibitor was 10 μ M, bacterial extract was used at dilutions from 10 to 20 times to get an optimal signal/noise ratio according to preliminary experiments. Then, the mixed solution was added on plates and incubated for 1 h at RT $^{\circ}$. The wells were subsequently washed three times with 200 μ l of PBST and 50 μ l of anti-PscN antibody (1/1000 in PBST.BSA 4%) were incubated for 1 h at RT $^{\circ}$. Three additional washes with 200 μ l of PBST were performed before 50 μ l of anti-rabbit-HRP antibody (1/40000 in PBST.BSA 4%) were added for 1 h. The wells were then washed with PBST (three times) and revealed with ECL substrate and luminescence reading.

4 Cell culture and staining, automated High-Content Screening (HSC) and High-Content Analysis (HCA)

The cell death, cell shrinkage and cell stress (bright nuclei) assays were performed using two microscopy approaches as previously described (chapter II-B). In this study, *P. aeruginosa* PAO1 strain was used for cell shrinkage and cell stress assays instead of CHA strains and the compounds were diluted at four concentrations: 100 μ M, 30 μ M, 10 μ M and 3 μ M.

5 Analysis of ExoS-bla and PopB-bla secretion.

The ExoS-bla secretion was monitored as previously described (chapter II-B). In this study, compounds were diluted at four concentrations: 100 μ M, 30 μ M, 10 μ M and 3 μ M.

To monitor PopB-bla secretion, *P. aeruginosa* CHA Δ *popBpopD*/PopB-Bla and CHA Δ *pscF*/PopB-Bla were used. The same protocol as for ExoS-bla was employed except that bacterial culture started at OD of 0.025 before it was put into an incubator at 37 $^{\circ}$ C for 3 hours.

IV. Results

1 Expression of the T3SS ATPase PscN of *P. aeruginosa*

Many assays that were previously performed in the laboratory showed that low amounts of the ATPase PscN were produced in *P. aeruginosa*, when expressed under the control of native promoters, while this protein was found in the inclusion body upon overexpression in *E. coli*. Therefore, it was necessary to optimize the expression condition of PscN. The Strep-PscN (SN) construction, which was created as described in the chapter II-A, was used for the optimization of protein expression. In addition, the *pscN* gene fused to a Strep-tag in the N-terminus or C-terminus was directly cloned into pET15b vector, generating two constructs: His-Strep-PscN (HSN) and His-PscN-Strep (HNS) (the 6His-tag was already present in the pET15b vector) which were designed to purify PscN using tandem tag affinity purification (Figure 35). The two expression vectors were then transformed into *E. coli* BL21 (DE3). Because of the aforementioned inclusion body problem of PscN overexpressed in *E. coli*, we decided to grow the bacterial culture at low temperature 18°C for 20h. As frequently observed, SN and HSN

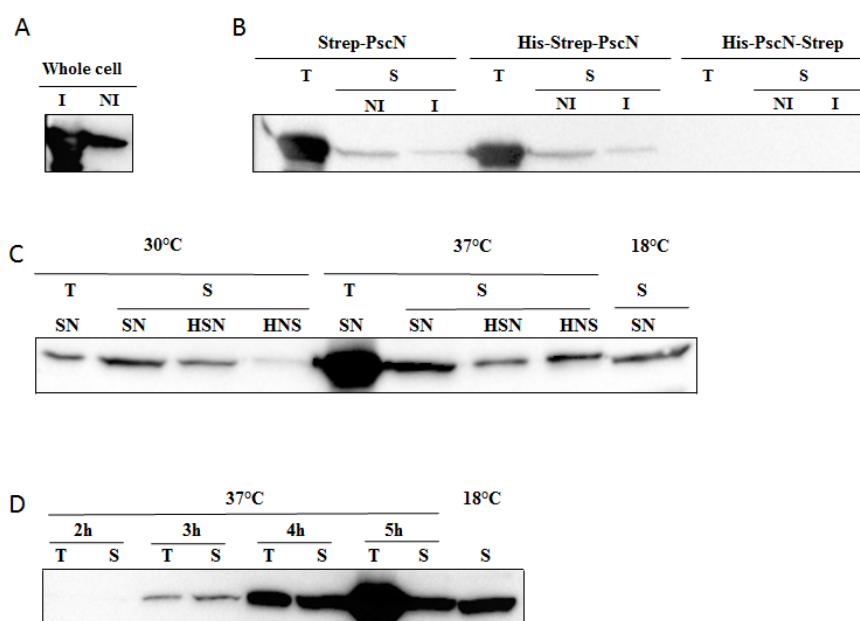


Figure 31: Expression of ATPase PscN in different conditions. (A) Strep-PscN expressed in inducing and non-inducing condition at 18°C. (B) Expression and solubility of SN, HSN and HNS in inducing and non-inducing condition at 18°C. (C) Solubility of SN, HSN and HNS at 30°C and 37°C in non-inducing condition. (D) Expression and solubility of Strep-PscN in non-inducing condition at 37°C after 2h,3h,4h and 5h.

T: Total fraction (whole cell lysate); S: soluble fraction (bacterial extract clarified by ultracentrifugation); NI: no induction; I: induction; SN: Strep-PscN; HSN: His-Strep-PscN; HNS: His-PscN-Strep.

were also expressed in non-inducing condition although their production was lower (Figure 31A). However, more soluble protein was detected in absence of IPTG induction (Figure 31B), confirming that PscN forms insoluble aggregate (inclusion bodies) when a large amount of proteins is synthesized. Furthermore, no PscN production was revealed with HNS construction at 18°C (Figure 31B).

To improve the production of soluble protein and to lower the culture time, protein expression was tested at higher temperatures 30°C or 37°C and, of importance, in the non-inducing condition for 5h. In comparison to the culture at 18°C, the same amount of soluble SN and HSN was observed at 30°C and more soluble SN was produced at 37°C. Otherwise, the soluble HNS was detected at 30°C and higher yield was obtained at 37°C (Figure 31C). These findings indicated that soluble PscN was more expressed at 37°C in the non-inducing condition. Moreover, the fusion of a Strep-tag at the C-terminus could somehow affect the conformation and/or folding of PscN, resulting in a low production at 18°C and 30°C. Finally, the introduction of 6His upstream Strep-PscN did not improve the yield of soluble protein. The optimization of Strep-PscN production was further optimized by modifying the total culture time. Actually, the maximum of soluble proteins was obtained after 4 hours and the proteins accumulated afterward in the inclusion body during longer duration of growth (Figure 31D).

2 PscN interacts with T3SS exported proteins

The soluble fraction of Strep-PscN expressed in *E. coli* BL21 (DE3) for 4h at 37°C in non-inducing condition was purified using StrepTrap HP column. The result showed that PscN was partially purified and there were two other contaminant protein bands present above and below that of PscN which was predicted to be 50 kDa (Figure 32A). Otherwise, the same amount of PscN was revealed in bacterial extract and flow-through fractions, suggesting that Strep-PscN poorly bound to StrepTrap HP column (Figure 32B). This suggests that the Strep-tag fused to the N-terminus of PscN is not totally accessible. As a result, only a small amount of PscN was found in the elution fraction, at a concentration equal to that in the clarified bacterial extract. Of note, the purification attempts of the other constructs, using HisTrap or StrepTrap columns, yielded the same poor protein binding, revealing a poor affinity tag accessibility.

The T3SS ATPase is considered to interact with exported proteins as the first step of the secretion process. In the chapter II-A, we mentioned that T3SS partner proteins of Strep-PscN

were identified by mass spectroscopy. To confirm the binding capacity of this protein *in vitro*, we firstly assessed the interaction of Strep-PscN with a translocator complex PcrH-PopD using an ELISA assay (Figure 32C). The result showed that PscN, present in both elution fraction and bacterial extract, was able to interact with PcrH-PopD yielding the same signal intensity (Figure 32D). Afterward, the elution fraction was utilized to evaluate the interaction of PscN with other proteins secreted or non-secreted by T3SS. We found that PscN could bind to translocator,

needle or an exotoxin in complex with their cognate chaperone (Figure 32E). For translocators, a high signal intensity with proteins responsible for the pore formation and their chaperone (PcrH, PcrH-PopB, PcrH-PopD) was observed while the signal was lower in the case of tip protein and its partner (PcrV, PcrG, PcrV-PcrG).

For needle proteins, the use of a truncated monomeric form of PscF or the polymerized form resulted in a lower signal intensity in contrast to the chaperone PscE-PscG or the needle

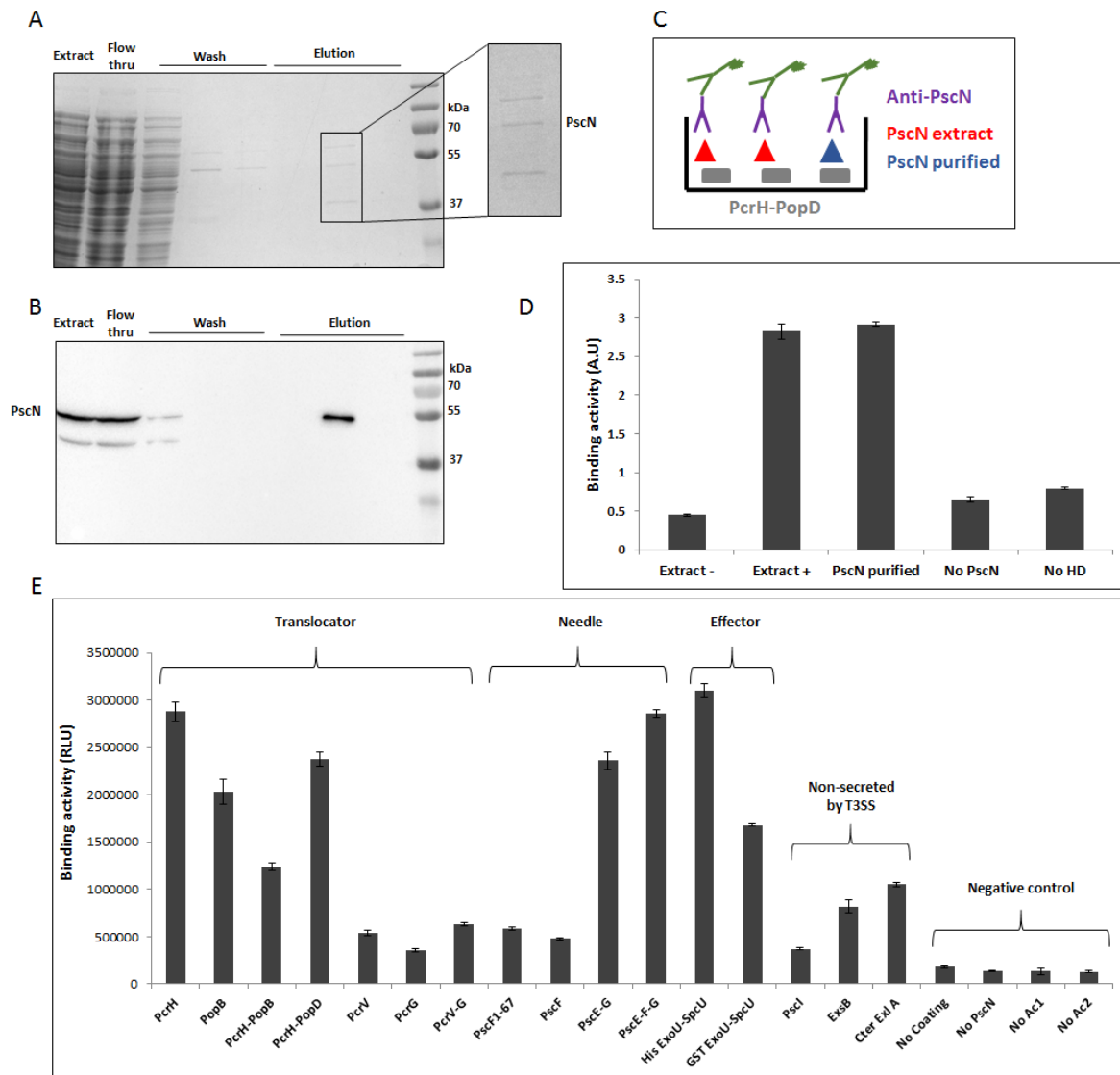


Figure 32: Purified PscN interacts with T3SS secreted proteins. (A) Strep-PscN was purified using StrepTrap HP column and fractions were analyzed by SDS-PAGE and coomassie staining. **Extract:** bacterial extract clarified by ultracentrifugation, **Flowthru:** no binding to the affinity column. (B) Western blot of fractions described in figure A. (C) ELISA assay principle. (D) Binding activity of purified Strep-PscN or PscN presented in bacterial extract to PcrH-PopD revealed by ELISA. (E) Binding activity of purified Strep-PscN to indicated proteins revealed by ELISA.

complex PscE-PscF-PscG. These results suggested that the chaperone PscE-PscG was responsible for the recognition of needle complex protein to the ATPase PscN.

Effector ExoU-SpcU fused to either GST-tag or 6His-tag in N-terminus was used to test the interaction with the ATPase PscN. ELISA result showed that both GST-ExoU-SpcU and His-ExoU-SpcU were able to bind to PscN and the his-tagged protein displayed higher signal intensity (Figure 32E). The lower signal intensity with GST-ExoU-SpcU could be explained by the presence of the large GST protein (26kDa) which could decrease the accessibility of PscN binding site on ExoU-SpcU.

For the non-secreted protein or ExlA (a T3SS protein), the binding signal was low but still higher than the negative controls that miss one of the ELISA partner. It might be possible that PscN interacts with these proteins with a low affinity. However, it should be noted that we used a partially purified PscN and contaminants could partially participate to non-specific ELISA signal.

3 Identification of compounds inhibiting the ATPase PscN interaction with the translocator PcrH-PopD by high throughput screening (HTS).

The ATPase PscN plays an important role in the virulence of *P. aeruginosa* because the $\Delta pscN$ deletion mutant is not cytotoxic *ex vivo*. Hence, this protein represents a potential target for anti-virulence strategy against *P. aeruginosa* infection. Actually, inhibitors of the T3SS ATPase YscN in *Yersinia pestis* were identified and these compounds affected the catalytic activity of this enzyme (Swietnicki et al., 2011). In other studies, in *P. aeruginosa*, derivatives of hydroxyquinolone – INP1855 and INP1750 are reported as promising compounds which could protect cells *ex vivo* and mice *in vivo* against *P. aeruginosa* infection (Anantharajah et al., 2016b, 2017). However, these inhibitor may inhibit the ATPase activity of PscN and raise concerns about their specificity because they also affect the flagellum function. Furthermore, targeting the catalytic activity might elicit cross activity toward bacterial and human nucleosidases. For these reasons, our strategy is to inhibit the interaction capacity of PscN with its T3SS partners because this is a specific activity of the PscN ATPase.

As described above, PscN in both bacterial extract and purified fraction were able to interact with the translocator protein PcrH-PopD, as revealed by ELISA assay. Thus, we designed a screening based on this ELISA assay to look for chemical compounds inhibiting interaction of PscN with PcrH-PopD (anti-PscN compounds) (Figure 33A). Instead of purified PscN, the bacterial extract was used for screening because it was available in larger volume and contained the same concentration of PscN as well as displayed a similar signal intensity of interaction with PcrH-PopD as the purification fraction (Figure 32B and D). We performed the screening with 1280 compounds from the Prestwick library and found out 15 hits compounds when setting a threshold of 50% of inhibition. These hits were next confirmed by a secondary screening (Figure 33B) and the percentage of inhibition varied from 10% to 65% (Table 8).

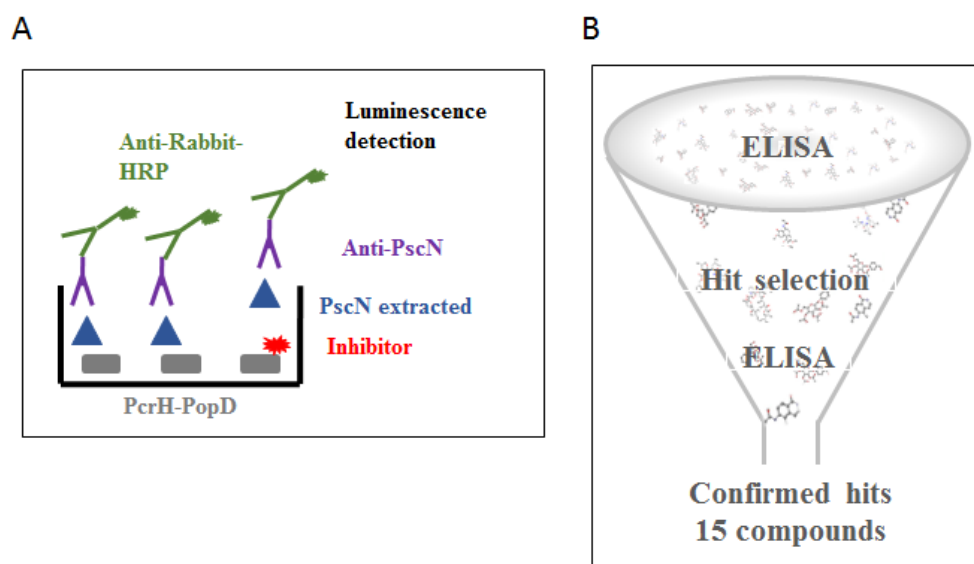
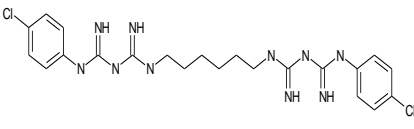
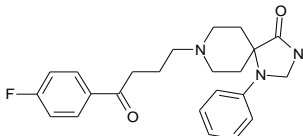
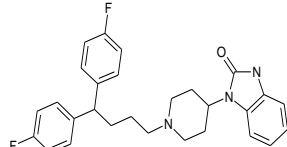
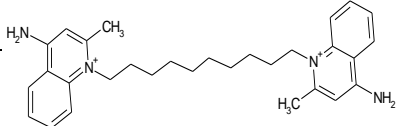
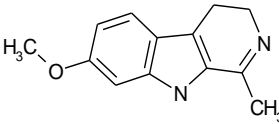
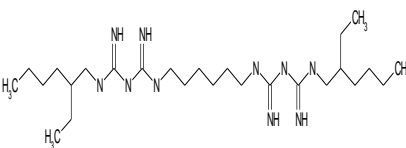
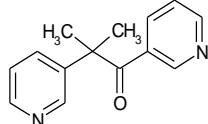
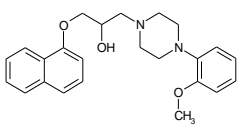
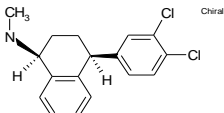
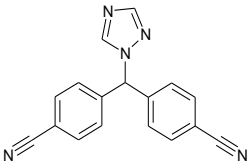
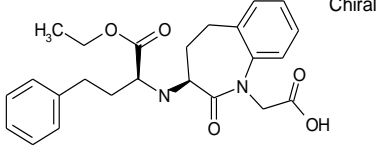
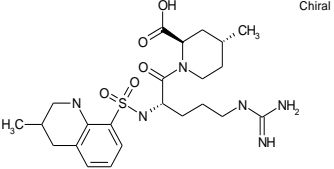
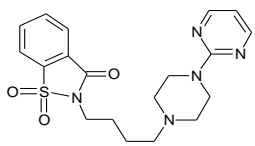
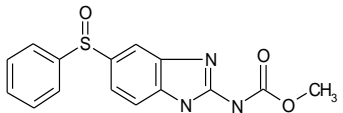
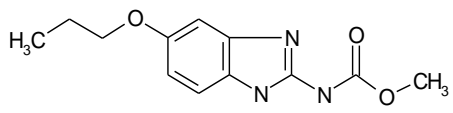


Figure 33: Screening of chemical compounds inhibiting interaction of PscN and PcrH-PopD.

(A) Screening mechanism based on ELISA assay. (B) Screening process

Table 8: Hit compounds identified from screening of Prestwick library. Compounds selected from characterization *in vivo* and *ex vivo* are highlighted in orange.

Molecules	Name	% Inhibition	Structure	Action mechanism
M1 or PW143	Chlorhexidine	38.67%		Detergent
M2 or PW288	Spiperone	25.91%		D2 antagonist
M3 or PW308	Pimozide	36.37%		Dopamine antagonist
M4 or PW388	Dequalinium dichloride	55.80%		Blocker of Ca ²⁺ activated K ⁺ channel Detergent
M5 or PW610	Harmaline hydrochloride dihydrate	19.87%		Monoamine oxidase inhibitor
M6 or PW777	Alexidine dihydrochloride	64.35%		Detergent
M7 or PW904	Metyrapone	17.80%		steroid 11?-hydroxylase inhibitor
M8 or PW975	Naftopidil dihydrochloride	30.41%		Adrenergic receptor antagonist
M9 or PW1014	Sertraline	30.54%		5-HT uptake inhibitor

M10 or PW1025	Letrozole	19.83%		Aromatase inhibitor
M11 or PW1129	Benazepril HCl	15.34%		Non-sulfhydryl angiotensin- converting enzyme (ACE) inhibitor
M12 or PW1228	Argatroban	9.99%		Direct thrombin inhibitor
M13 or PW1292	Ipsapirone	11.64%		Partial 5-HT1A agonist
M14 or PW1459	Oxfendazol	19.43%		Unknown
M15 or PW1460	Oxibendazol	17.63%		Unknown

4 Characterization of hit compounds *ex vivo* and *in vivo*

To evaluate the efficacy of these hit compounds on bacteria *in vivo*, we assessed their effects on the secretion of two proteins secreted by the T3SS: the effector ExoS and the translocator PopB. The secretion assay used a β -lactamase reporter protein fused to the C-terminal of ExoS or PopB. On the other hand, the efficacy of the compounds was figured out on cellular model by testing the protection from ExoU positive *P. aeruginosa* using the PA103 strain and ExoS positive *P. aeruginosa* using the PAO1 strain. Cell death resulting from ExoU intoxication was revealed by PI labeling and cell shrinkage resulting from ExoS intoxication was measured using a membrane labeling. The cell death and cell shrinkage inhibition were monitored using automated microscopy approaches. All the 15 compounds were tested at four concentrations: 3 μ M, 10 μ M, 30 μ M and 100 μ M in triplicates and the data were represented in radar plots in order to get a synthetic view of the results (Figure 34).

Actually, we wanted to select compounds inhibiting cell shrinkage, cell death and PopB-bla secretion or all the four traits because the inhibition target is the interaction between PscN and PcrH-PopD. This inhibition is expected to block the secretion of translocator PopD, thus inhibiting the T3SS assembly and functioning and bacterial cytotoxicity. As PopB and PopD are two translocators of T3SS, it is possible that they bind to PscN in the same manner and compounds that affect the secretion of PopD could also inhibit that of PopB. In contrast, the binding site on the ATPase PscN of effector and translocator proteins is probably different as described in the chapter II-A. Consequently, it is hard to predict whether ExoS is secreted when PopD binding to PscN is disrupted. Thus, this criterion is less important for compound selection. Indeed, seven compounds M1, M3, M5, M6, M8, M9 and M13 presented the expected characteristics (Figure 34).

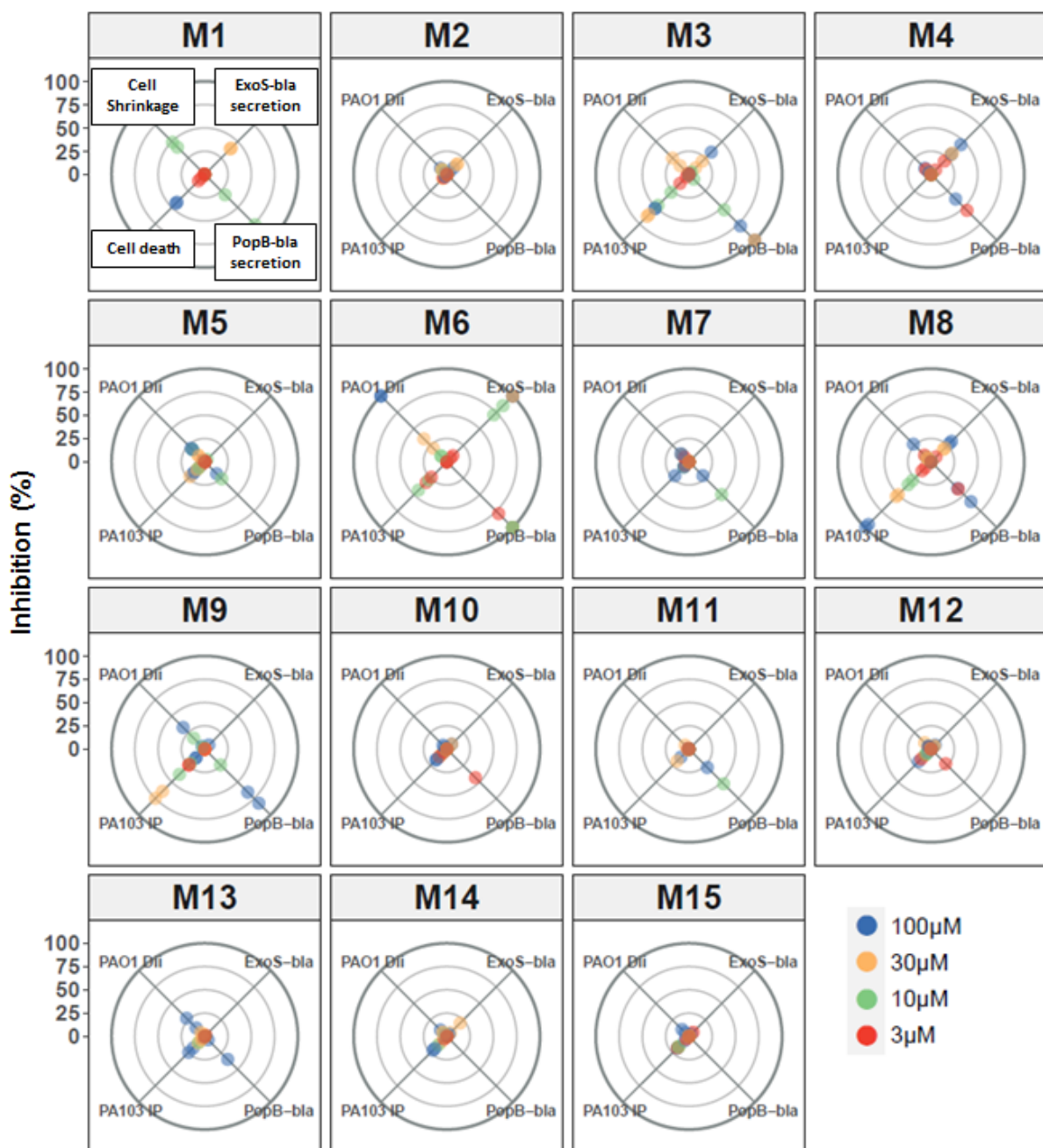


Figure 34: Characterization of 15 hits compounds. 15 compounds were characterized by four orthogonal assays including the secretion of ExoS-bla and PopB-bla detected by beta-lactamase activity, the cell death measured by counting PI positive cells and cell shrinkage assessed by monitoring the cell area. The results are shown by radar plot (coordinate polar) in percentage of inhibition. The four axis correspond to 4 independent assays: ExoS secretion, PopB secretion, and cell death and shrinkage. The four circles correspond to 25, 50, 75 and 100%.

5 Purification of FSS-PscN and target validation of hit compounds.

5.1 Purification of FSS-PscN

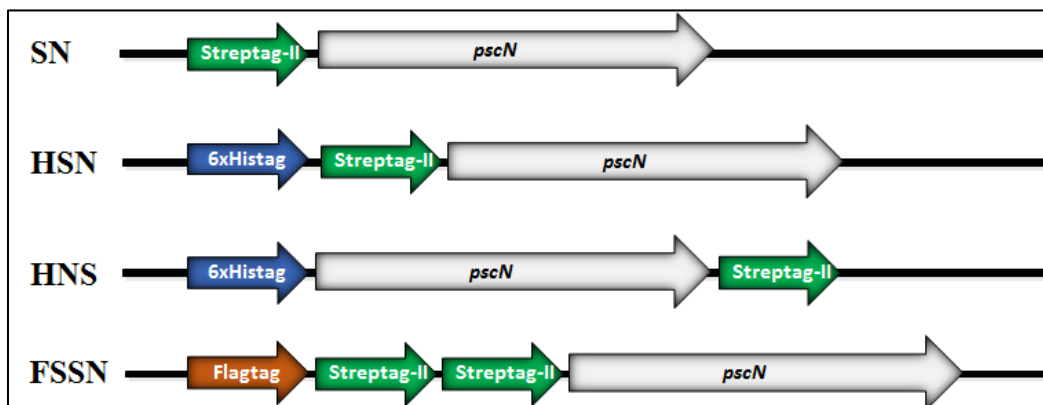


Figure 35: Four constructions of PscN used in this study

As described above, this screening was performed using the bacterial extract that contains Strep-PscN. This raises a major concern related to the target specificity because of non-specific bindings in ELISA assays. Hence, pure PscN is required for validation of the target of the identified compounds and it was thus necessary to optimize the PscN purification. We therefore designed a construct of PscN in which one Flag-tag and two Strep-tag were fused to the N-terminal of *pscN* (Figure 35) as described in the chapter II-A. The twin-Strep-tag is expected to increase affinity with the StrepTrap column matrix to circumvent the observed low binding to the purification column of Strep-PscN. On the other hand, the Flag-tag, which possesses many negative aspartate residues, decreases the pI of the protein, thus making it potentially more soluble in working buffer. Moreover, it permits purifying PscN using tandem affinity purification strategy. This construct was firstly cloned into pET15b vector (without the 6His), transformed into *E. coli* BL21 (DE3) for expression and purified with a StrepTrap HP column. The result showed that FSS-PscN was more soluble and the soluble FSS-PscN almost totally bound to the StrepTrap HP affinity column (Figure 36A). Moreover, the amount of purified FSS-PscN was much higher than that of purified Strep-PscN, thus indicating that the Flag-twin-Strep at the PscN N-terminal could significantly increase the purification yield (Figure 36B).

In order to optimize the production and solubilization of FSS-PscN, we decided to move to *P. aeruginosa* as an expression strain in the hope that the translation and folding machineries of this bacterium could better cope with this challenging protein production. A coupled T7 system, which allows the overexpression of genes in *P. aeruginosa* including a PAO1 strain with T7 polymerase gene inserted in its chromosome and a modified pET15b vector that can

replicates in both *P. aeruginosa* and *E. coli*, was used (Arora et al., 1997; Brunschwig and Darzins, 1992). FSS-PscN purified from *P. aeruginosa* was obtained in higher amount and purity in comparison to that from *E. coli* (described in chapter II-A or Figure 36B). Moreover, this purified FSS-PscN is active (ATPase specific activity estimated 0.1 $\mu\text{mol}/\text{mg}/\text{min}$) and its binding capacity was confirmed by three different assays: ELISA, HTRF and MST (chapter II-A).

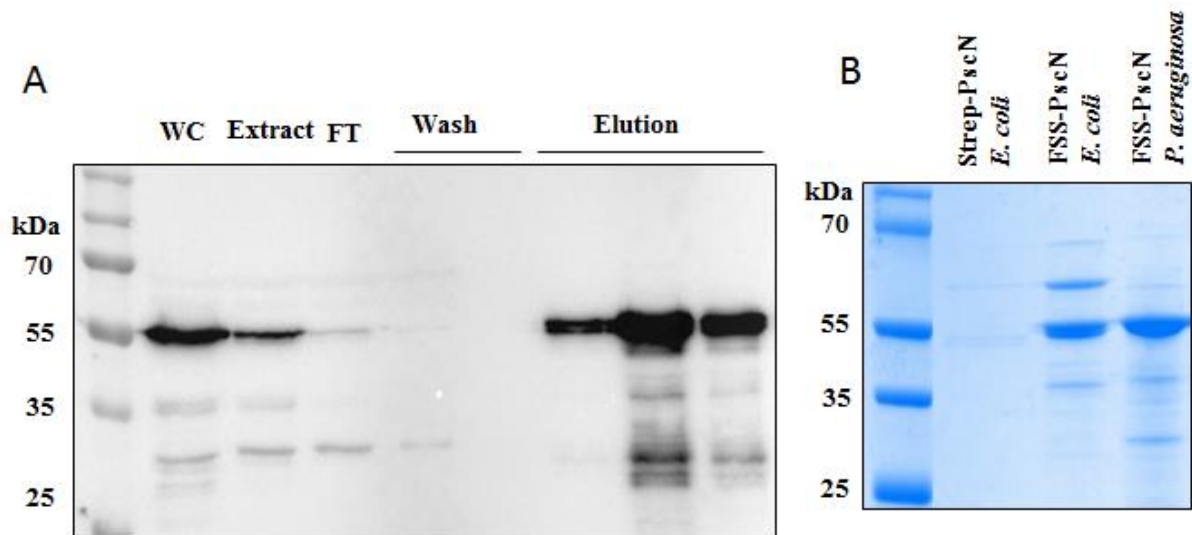


Figure 36: Higher purification yield with Flag-Strep-Strep-PscN (FSS-PscN) construct. (A) FSS-PscN was produced in *E. coli* BL21 (DE3) and purified by StrepTrap HP column. Indicated fractions were analyzed by western blot using anti-PscN antibody. (B) Elution fraction of purified FSS-PscN expressed in *P. aeruginosa* PAO1 ADD1976 in comparison to that from *E. coli* BL21 (DE3) or purified Strep-PscN. **WC**: Whole cell lysate, **Extract**: bacterial extract clarified by ultracentrifugation, **FT**: Flow-through (no binding to the affinity column)

5.2 Target validation

To ascertain the target specificity of hit compounds, ELISA assays were performed to evaluate interaction of PcrH-PopD with FSS-PscN purified from *P. aeruginosa* in the presence of the seven best selected molecules at indicated concentrations (Figure 37, yellow lines). The result showed that M3, M5, M8, M9 and M13 did not have an effect on the binding capacity of PscN. Consequently, their *ex vivo* and *in vivo* T3SS and virulence inhibition should originate from an unknown mechanism which does not involve the binding of the ATPase PscN to its partners, for example by targeting another T3SS component or other virulence factors. On the

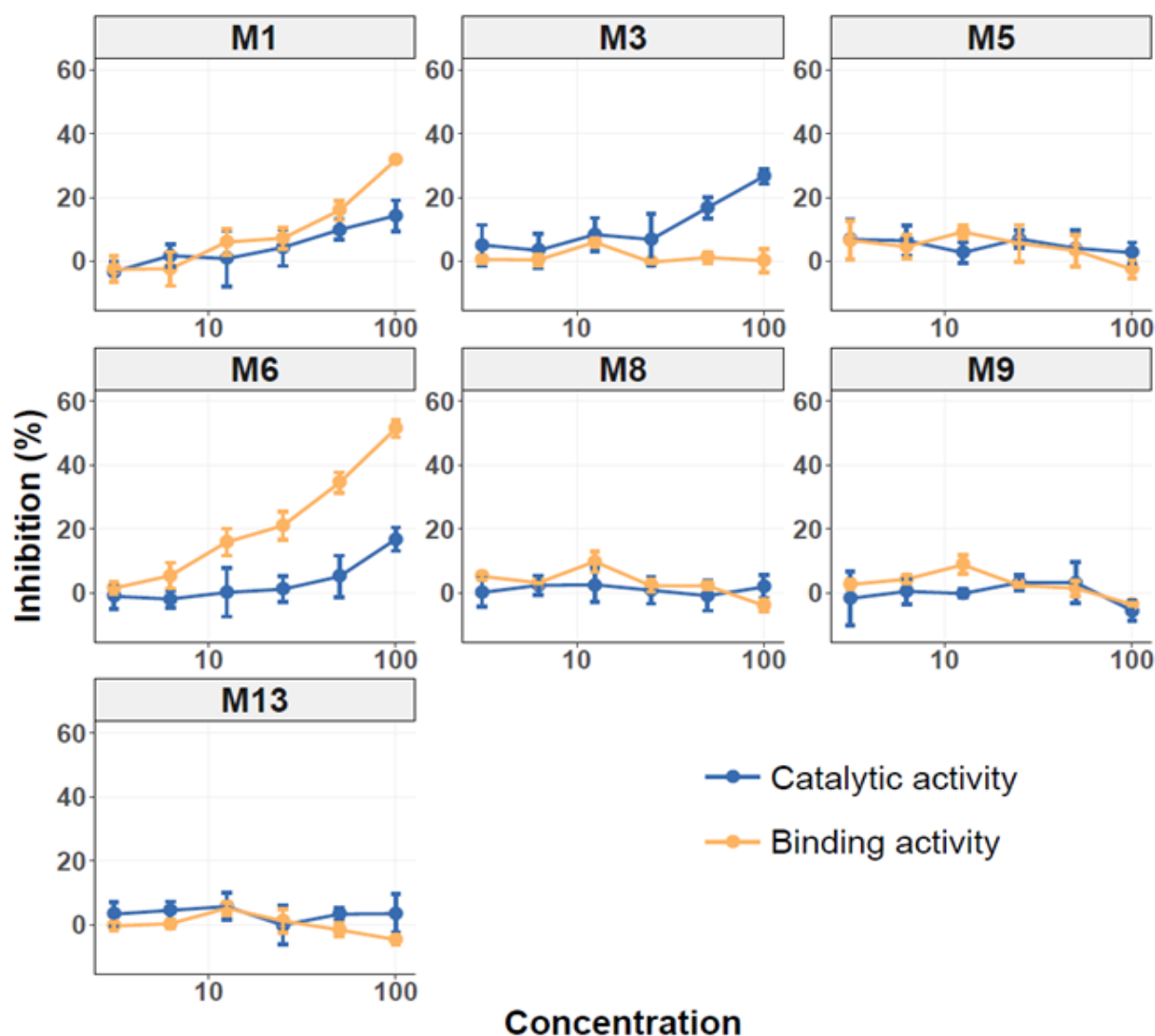


Figure 37: Effect of seven compounds on the binding capacity and catalytic activity of PscN. The purified FSS-PscN was incubated with seven compounds at indicated concentrations and was utilized to determine the ATPase activity using Malachite Green revelation and binding to PcrH-PopD using ELISA assay.

other hand, M1 and M6 were able to inhibit the interaction of PscN with PcrH-PopD, thus confirming that they target this protein-protein interaction.

Otherwise, to assess the direct effect of these compounds on the structure and folding of PscN, we incubated these compounds with PscN and measured the ATPase activity as the readout (Figure 37, blue lines). The result indicated that the M1 and M6 slightly inhibited PscN catalytic activity at high concentrations (100 and 50 μ M for M6 and 100, 50 and 25 μ M for M1). Thus, it is possible that at these concentration, the two compounds denature PscN, resulting in the inhibition of both binding capacity and catalytic activity of this enzyme.

V. Discussion

The ATPase PscN is an essential protein for the T3SS function in *P. aeruginosa*. However, it had never been characterized biochemically *in vitro* due to the challenge of its production and purification. We therefore created some constructs of this protein using a strep-tag in N-terminal or C-terminal of the protein to facilitate its purification. Although PscN was not highly produced in soluble form and was mainly found in inclusion body when expressed in *E. coli*, we could finally obtain this enzyme after many optimization steps of its production and partially purify it using the Strep-PscN construct. The PscN partial purification and the use of antibodies represented an opportunity to avoid non-specific activity of other proteins presented in bacterial extract. Using ELISA, this was the first time that PscN was shown to interact with the T3SS secreted proteins, albeit it was also reported for T3SS ATPases of other bacteria like *S. enterica* SPI-1 (Akeda and Galan, 2005), *S. enterica* SPI-2 (Allison et al., 2014; Yoshida et al., 2014), *E. coli* (Zarivach et al., 2007), *S. flexneri* (Botteaux et al., 2009), *Xanthomonas campestris* (Lorenz and Buttner, 2009). This binding spectrum reflects the involvement of T3SS ATPases in the first step of the secretion process.

P. aeruginosa is an opportunistic pathogen which is classified at the top of the priority list for the development of antibacterial compounds by WHO (Tacconelli et al., 2018). We therefore looked for chemical molecules inhibiting the binding capacity of the ATPase PscN because it is specific for *P. aeruginosa* and has an important role for T3SS functioning as described above. During the setting up of a screening protocol, we performed an experiment showing that the partially purified PscN displays a binding signal to PcrH-PopD as high as that obtained with the bacterial extract while the volume of bacterial extract solution was much

higher. Thus, bacterial extract containing PscN was used for the screening with ELISA assay. 15 chemical compounds were identified and they belong to the Prestwick library whose compounds are drugs approved by the FDA.

Taking advantage from high content screening approaches used for the molecules inhibiting the interaction of PscE-PscG (anti-PscE-PscG compounds) as described in the chapter II-B, we characterized the anti-PscN hit compounds *in vivo* on bacteria and *ex vivo* on eukaryotic cells. The results showed seven promising compounds selected from 15 identified hits. Some of them were further tested by the CLIQ-BID method which can reveal cellular damages caused by the bacteria as well as toxicity or cell stress caused by the compounds on their own (Wallez et al., 2018). Surprisingly, although the compounds come from the Prestwick library, most of them caused cell stress detected by our method at the low concentration of 10 μ M (result not shown). Moreover, some molecules were toxic for eukaryotic cells at higher concentration, resulting in the cell death detected by PI incorporation. For this reason, the inhibition of cell death mediated by the ExoU exotoxin could not be detected for these compounds at high concentration as depicted in Figure 34 (For example: M6 inhibited the cell death caused by *P. aeruginosa* PA103 at 3 μ M and 10 μ M but it was toxic for eukaryotic cell at 30 μ M and 100 μ M). Consequently, the toxicity of Prestwick compounds could be called into question. This could be explained by the fact that some compounds are only used on human skin and not for oral or internal administration. Thus, a higher toxicity threshold could be tolerated. Otherwise, it might be possible that compounds that are toxic at a cellular level could even be used *in vivo* because animals or human could physiologically cope with the balance between efficacy and toxicity of drugs. Hence, the toxicity criterion at the cellular level may only be a supplemental information which should not be used to reject one chemical cluster.

As described in the chapter II-A, we designed a new construct with one Flag-tag and two Strep-tag fused to the N-terminus of PscN. This construct allowed us to obtain pure active PscN with high yield of purification upon expression in *P. aeruginosa*. By using the purified FSS-PscN, we validated the target specificity of anti-PscN hit compounds to rule out the possible false-positive identification from screening experiment employing the bacterial extract. Strikingly, only two (M1 and M6) of the seven best compounds were able to inhibit the interaction between PscN and PcrH-PopD and the M6 was more potent. Furthermore, the relation of these two molecules to the detergent family raises the possibility that they could unfold PscN and that the effect on binding inhibition is a result of the protein denaturation.

Indeed, our results showed a decrease of ATPase activity of PscN in the presence of the two compounds at 25, 50 and 100 μM for M1 and 50 and 100 μM for M6. However, M6 still deserves further investigation because it inhibits the interaction of PscN and PcrH-PopD at concentrations that does not affect ATP hydrolysis by this enzyme.

The Alexidine dihydrochloride (M6) is described by Prestwick as displaying an antibacterial activity. Thus, the identification of this compound is not consistent with our initial objective of development of anti-virulence drugs. Regarding *P. aeruginosa*, the M6 inhibited the growth of CHA strains at concentration above 10 μM (Figure 38). Therefore, we cannot conclude that the *in vivo* and *ex vivo* effects of M6 are the result of either or both of its antibiotic and anti-virulent properties. However, for therapeutic application, a compound with both activities can even be better. The next steps of this project are to perform some structure-activity relationship (SAR) studies of the hit compound M6 to lower its toxicity toward eukaryotic and possibly prokaryotic cells and to increase its efficacy. Afterward, new analogs could be characterized with approaches that we employed for the anti-PscE-PscG compounds (chapter II-B).

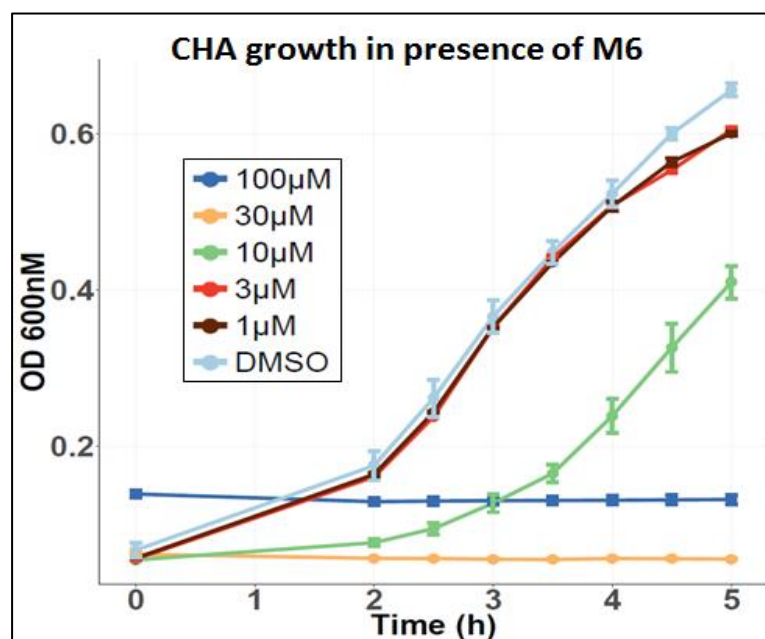


Figure 38: *P. aeruginosa* CHA growth in the presence of M6 at indicated concentrations.

VI. Conclusion (in French)

Concernant l'expression et la purification de l'ATPase PscN, bien que cette protéine soit peu exprimée sous forme soluble et se trouve principalement dans les corps d'inclusion chez *E. coli*, les différentes optimisations de production de cette enzyme nous ont aidé à augmenter la quantité de protéine soluble et à la purifier ensuite partiellement sur la colonne d'affinité StrepTrap HP. De plus, pour la première fois, la capacité d'interaction de PscN avec des protéines sécrétées par le SST3 a été montrée en utilisant un test ELISA. En fait, cette activité a été décrite dans la littérature chez d'autre bactérie comme *S. enterica* SPI-1 (Akeda and Galan, 2005), *S. enterica* SPI-2 (Allison et al., 2014; Yoshida et al., 2014), *E. coli* (Zarivach et al., 2007), *S. flexneri* (Botteaux et al., 2009), *Xanthomonas campestris* (Lorenz and Buttner, 2009). Cela confirme l'activité de l'ATPase du SST3 reflétant la première étape du processus de sécrétion et cette activité de liaison peut être une cible potentielle pour le développement d'anti-virulents.

Par conséquent, en se basant sur un test ELISA, nous avons cherché des molécules inhibitrices de l'interaction entre PscN et le complexe PcrH-PopD et identifié 15 molécules actives après des criblages primaires et secondaires. En utilisant des approches de « high content screening » (HCS) développées dans notre laboratoire, ces 15 molécules ont été caractérisées *in vivo* sur la bactérie and *ex vivo* sur un modèle cellulaire. Les résultats ont montré que sept « touches » étaient assez prometteuses car elles inhibaient la sécrétion de la toxine bactérienne ExoS et du translocateur PopB. De plus, ces molécules ont pu protéger des cellules eucaryotes contre les dommages causés par deux souches de *P. aeruginosa* qui secrètent ExoS ou ExoU.

En outre, comme indiqué dans le chapitre II-A, la nouvelle construction FSS-PscN nous a permis d'obtenir PscN pure et active. Cette FSS-PscN purifiée a été utilisée pour revalider le fait que les sept meilleurs molécules ciblent effectivement l'interaction entre PscN et PcrH-PopD car nous avons utilisé un extrait bactérien contenant PscN pour réaliser le criblage, ce qui peut être source de faux-positifs. Parmi les sept molécules, cinq molécules n'ont pas confirmé de capacité d'inhibition de l'interaction entre FSS-PscN et PcrH-PopD. Les inhibitions observées pendant la caractérisation *in vivo* et *ex vivo* ont donc pu provenir d'un autre mécanisme qui n'implique pas la capacité de liaison de PscN, par exemple l'inhibition d'un autre composant du SST3 ou d'autres facteurs de virulence. En revanche, les deux molécules qui sont actives, appartiennent au groupe structural des détergents, ce qui oblige à se

demander si elles ont un effet direct de dénaturation de PscN. En effet, en utilisant un test d'activité de l'ATPase, une seule molécule est capable d'inhiber l'interaction entre PscN et PcrH-PopD à des concentrations où elle n'affecte pas l'activité catalytique de PscN.

Cette molécule, Alexidine dihydrochloride, est décrite par le fournisseur Prestwick comme un composé qui possède une activité antibactérienne. Nous avons montré que ce composé inhibe la croissance de *P. aeruginosa* CHA à des concentrations supérieures à 10 μM (Figure 38). C'est pourquoi, nous n'avons pas pu conclure que l'inhibition observée pendant les caractérisations *in vivo* et *ex vivo* pour cette molécule est le résultat de l'activité antibiotique ou anti-virulente ou les deux. Cependant, pour des applications thérapeutiques, cela peut être un avantage de disposer d'une molécule qui possède les deux activités. Il est ensuite prévu de réaliser une étude Structure Activity Relationship (SAR) afin d'optimiser sa structure en diminuant sa toxicité et augmentant sa capacité à inhiber les interactions protéiques de PscN. Une fois que les analogues seront obtenus, nous planifions des tests pour caractériser leur efficacité comme nous l'avons fait pour les molécules qui inhibent l'interaction de PscE-PscG (chapitre II-B).

Chapter III

General discussion and perspective

P. aeruginosa is well-known opportunistic pathogen which is classified in the top list of germs causing nosocomial diseases. This bacterium is naturally resistant to many common antibiotics and has the capacity to acquire multiple resistances. Hence, this reveals a major problem for therapy to efficiently treat infection by *P. aeruginosa*. In 2017, WHO classified this bacterium as a critical priority for the development of new antibiotics. However, antibiotics resistance was rapidly observed after their deployment over years (Clatworthy et al., 2007). It is therefore necessary to define alternative approaches such as the anti-virulence strategy which could supplement classic antibiotics in order to increase the efficacy of treatment and decrease pressure for resistance development. Consequently, studies of virulence factors are an essential step to understand virulence mechanisms and to identify potential therapeutic targets. In the laboratory, we are working on *P. aeruginosa* T3SS which is its major virulence factor. Thus, blocking of this machinery could be a promising strategy to fight against this pathogen.

A. The ATPase PscN plays an essential role for the T3SS functioning and bacterial virulence in *P. aeruginosa*

The T3SS is described to deliver effector protein directly from the bacterial cytosol into host cells. This machinery is constituted of more than twenty proteins, some of them are essential for the T3SS functioning, including the ATPase. This enzyme displays many functions in the T3SS assembly: the recognition of exported proteins, the dissociation of cargo-chaperone complexes and the unfolding of secreted cargo proteins (Akedo and Galan, 2005; Yoshida et al., 2014). In our laboratory, the ATPase PscN of *P. aeruginosa* T3SS was previously studied by former trainees and a PhD student, revealing its crucial role because *P. aeruginosa* is not able to intoxicate eukaryotic cells in its absence (chapter II-A). Besides, due to low expression and solubility, the biochemical characterization of this protein is very challenging while it is fundamental to get insight in its functioning. Therefore, different steps to optimize the expression and the purification of PscN were undertaken.

Firstly, three PscN constructions were created using a Strep-tag fused to the N-terminal or C-terminal of PscN and a His-tag, or not, upstream to the fusion proteins, generating Strep-PscN, His-Strep-PscN and His-PscN-Strep. Unfortunately, we were not able to obtain a high yield of purification after many attempts of optimization and the main problem remained the low binding of the protein on affinity purification columns. This obstacle led us to generate a fourth PscN construction with one Flag-tag and two Strep-tag fused to the N-terminal of PscN.

The purification was further improved when FSS-PscN was overproduced in *P. aeruginosa* expression strain thanks to its regulation and folding machineries (Arora et al., 1997; Brunschwig and Darzins, 1992). The FSS-PscN was readily purified using Streptactin sepharose beads and in buffer containing Triton X100 and TCEP. The detergent allows us to improve the purity of purified FSS-PscN while the reducing agent cleaves the inter-molecular disulfide bonds, thus preventing the protein aggregation. Unfortunately, Triton absorbs light in UV and interferes with many biochemical and biophysical techniques but we are not able to substitute it by another detergent. All these factors allowed us, in the first time, to obtain high quantities of pure and active PscN which could be next subjected to biochemical characterization.

The binding of PscN to T3SS secreted proteins was first evaluated using an ELISA assay. This revealed an interaction of PscN with all the tested proteins including the effector, translocator, needle and gate-keeper proteins. These findings are consistent with that obtained for the T3SS ATPases from other gram-negative pathogenic bacteria (Table 4). Nonetheless, we described for the first time the interaction of the ATPase with the needle complex proteins, thus complementing the overview of T3SS ATPase capacities. These interactions of PscN were next validated for translocator and gate-keeper complexes using HTRF assay while no interaction was observed for effector and needle complexes in the same experiment condition. However, we cannot definitively conclude on this experiment because there could be an issue of accessibility of fused-tag on each protein for the fluorophore-conjugated antibodies recognition during interaction. In fact, the close dependence of HTRF signal on the distance between the donor and the acceptor is a major drawback of this technique. Therefore, MST assay, which is the third approach, was employed to confirm the binding in solution as well as to measure the dissociation constants (K_d) between PscN and each T3SS ligand proteins. While PscN interacts with effector, translocator and gate-keeper complexes, this enzyme does not bind to needle protein in the tested conditions. Thus, the interaction with needle protein which was found using the ELISA assay, is called into question because the ELISA assay is known to sometime display a high background and non-specific binding. However, if the needle complex could not bind to the ATPase, it might exist another mechanism to deliver this complex to the T3SS export gate and to dissociate the subunit PscF from its chaperones PscE-PscG if we take into consideration that the complex PscE-PscF-PscG is very stable *in vitro* (Ple et al., 2010).

Furthermore, the MST results also show that PscN and effector complex have a higher affinity ($K_d = 45$ nM) than PscN and the translocator complex ($K_d = 4900$ nM). Although the concentration of each protein complex inside the bacteria, which is still unknown, is also important for their recognition by the ATPase, a 100 times lower PscN affinity for the translocator than for the effector is apparently contradictory with the hierarchical secretion because the translocator should be secreted before the effector. Consequently, in addition to the recognition by the ATPase, the substrate secretion could be regulated by other mechanisms than bipartite interactions. Actually, the gate-keeper protein is characterized as a regulator controlling the secretion switch from middle substrate (translocator) to late substrate (effector) (Roehrich et al., 2017; Yang et al., 2007). Interestingly, we show also that PopN-Pcr1-Pcr2-PscB (gate-keeper complex in *P. aeruginosa*) interacts with the ATPase PscN with a K_d of 800 nM. We therefore wondered if this secretion control could be due to a modulation of the interaction of the two substrates with the T3SS ATPase. Actually, in the presence of the PopN complex, the affinity between PscN and ExoU-SpcU (effector) was not modified and the one between PscN and PcrH-PopB (translocator) only slightly decreased. These results indicate that the repression of effector secretion by PopN complex could occur by an unknown mechanism which does not involve its interaction with the ATPase. In contrast, we found out that the affinity between PscN and PscE-PscF-PscG (needle complex) strongly increased when PscN is bound to the PopN complex. This finding reveals a new function of gate-keeper proteins and allowed us to elucidate the mechanism of needle complex recognition by the T3SS (chapter II-A).

To date, the secretion switch from early to middle substrates in the secretion process still remains controversial. Actually, considering data gathered from literature, this substrate secretion switch involves two known proteins: the ruler PscP and the switch PscU. The ruler PscP controls the needle length (Ho et al., 2017; Journet et al., 2003) while the auto-cleavage of the C-terminal of the switch protein PscU is essential for the secretion of translocator and effector proteins (Frost et al., 2012; Sorg et al., 2007; Wood et al., 2008). Thus, it might exist a mechanism of signal transmission from PscP to PscU upon needle completion, leading to the cleavage of this switch protein. (Monjarás Feria et al., 2015; Wood et al., 2008). This hypothesis is supported by the fact that the C-terminal of the ruler protein was shown to interact with the cytoplasmic C-terminal domain of the switch protein *in vitro* (Ho et al., 2017; Wagner et al., 2018). Consequently, the start of translocator secretion was initially proposed as the main

mechanism of the stop of further needle elongation and the two proteins PscP and PscU were presented to be sufficient to control the secretion switch from early to middle substrates. However, a recent identified mutations that block auto-proteolysis of SpaS (homologous of PscU in *Salmonella enterica* SPI-1) abolished the secretion of middle substrates and late substrates while the secretion of early substrate and the needle length were unaffected (Monjarás Feria et al., 2015). Authors also indicated that the cleavage of SpaS C-terminus occurred upon folding of this proteins and before the end of needle formation. In other words, the mechanisms of the stop of needle elongation and of substrate specificity switching caused by auto-cleavage of SpaS would be completely independent. As a consequence, another mechanism would be required to prevent the further secretion of early substrates when middle substrates are secreted.

In this context, our findings on the new function of the T3SS ATPase and the gate-keeper complex could complement the understanding of this switching process. In fact, before secretion, the exported substrates need to be recognized by the T3SS. According to the cryo-electron topography of the T3SS, the ATPase is localized at the cytoplasmic extremity of the apparatus (Figure 26). This enzyme could thus be considered as the first stop of the exported proteins and should play an important role in the recognition of these proteins by the T3SS. We therefore propose that in the initial stage, the ATPase PscN bound to the gate-keeper PopN complex recognizes the needle complex PscE-PscF-PscG. The needle subunit PscF could be next dissociated from its chaperone PscE-PscG using the energy from ATP hydrolysis by PscN (Akedo and Galan, 2005; Yoshida et al., 2014) and secreted using the proton motive force (pmf) (Lee and Rietsch, 2015; Wilharm et al., 2004) for the needle elongation. The length of needle is controlled by the ruler protein PscP and the effectors and translocators secretion is repressed by the switch protein PscU. When the needle reaches expected length, the secretion of PscP could transmit a signal not only to the switch protein PscU but also to other proteins in cytoplasmic export gate including the ATPase bound to the gate-keeper complex. This signal transmission would subsequently stimulate the release of the PopN complex from PscN. Upon gate-keeper complex release, the ATPase changes its conformation in an allosteric mode, resulting in a very low affinity with the needle complex and blocking its recognition by the T3SS and the secretion of PscF. On the other hand, the switch PscU auto-cleavage, which depends or not on the needle completion, would allow the secretion of translocators.

Although our hypothesis could describe the secretion switch from early to middle substrates in the T3SS secretion process, we give insights on the hierarchical secretion of translocators (middle substrates) and effectors (late substrates) by considering only their affinity with the ATPase PscN. However, besides binding to the ATPase, the secretion of the exported proteins might be also regulated by complex machineries such as the sorting platform, which is localized between the ATPase and the secretin (Lara-Tejero et al., 2011).

In addition to the gate-keeper complex, other regulatory proteins might also modulates substrate recognition by the ATPase. Indeed, the ruler protein is also secreted by the T3SS (Agrain et al., 2005; Payne and Straley, 1999) and probably interacts with the ATPase (Riordan et al., 2008). In addition, the tip protein PcrV in complex with its chaperone PcrG is described as a negative regulator of effector protein secretion (Lee et al., 2014; Sundin et al., 2004). PcrV is secreted by the T3SS (Goure et al., 2004) and the complex PcrV-PcrG should probably bind to the ATPase PscN. Hence, we plan to purify the tip complex PcrV-PcrG and the ruler PscP and to evaluate their effect on the affinity between exported proteins and the T3SS ATPase PscN. These experiments will be performed in the next few month. Thereafter, the possible binding and influence of cytoplasmic domains of the structural components of the basal body will also be assessed.

Besides, we have another project related to the structure of the ATPase PscN. To date, only N-terminal truncated monomeric T3SS ATPases were characterized using X-ray crystallography (Allison et al., 2014; Burgess et al., 2016a; Zarivach et al., 2007). Thus, it would be very interesting to obtain the crystal structure of the full-length PscN in the monomeric and/or oligomeric forms. Our work revealed that the effector ExoU-SpcU and PscN has a high affinity. We will therefore attempt to obtain crystals of the ATPase in complex with ExoU-SpcU. Of importance, the structure of the complex ExoU-SpcU was solved by the team of our collaborator Andrea Dessen (Gendrin et al., 2012). The interaction with effector proteins could stabilize a conformation of the T3SS ATPase and could drive the crystallization of the full-length PscN.

B. Two potential targets for the development of anti-virulence strategy against *P. aeruginosa*

As mentioned above, the study of virulence factors is a fundamental step to identify new potential targets for therapy. In the laboratory, we developed two pipelines for anti-virulence strategy against *P. aeruginosa* T3SS based on our understanding of this machinery. The first one concerns the essential ATPase PscN whose binding to T3SS secreted proteins is being characterized as described in the chapter II-A. The second target is the interaction between the needle proteins PscE, PscG and PscF. These proteins were previously studied by the former post-doc Sophie Plé, revealing that their interaction is important for their stability inside bacteria and that inhibition of their interaction leads to the degradation of these proteins, thus inhibiting the T3SS needle assembly (Ple et al., 2010). Therefore, based on an ELISA assay, we performed screenings with the help of the CMBA platform (CEA-Grenoble screening facility) to look for chemical inhibitors blocking the two promising targets: the ATPase and the needle complex.

Actually, in such a target-based screening method two essential steps need to be performed. The first one is the *in vitro* screening on the defined targets as described above and the second one is the demonstration of the effects *in vivo* of the identified hits. In comparison to another strategy of phenotypic screening, this target-based screening may possess some advantages and disadvantages. Basically, the preparation of sufficient target quantity for the screening is a challenge because some targets (protein or DNA) may sometime be difficult to produce (Payne et al., 2007). However, many information are usually available on the targets and are useful to design experiments. Regarding the hit's specificity, there is no evidence to conclude which strategy is better but hits from target-based strategy often have narrow spectrum and, because the target is defined, the confirmation of *in vivo* effect is usually straightforward. On the other hand, the phenotypic screening is based on an *in vivo* approach looking for compounds that inhibit a phenotype related to a virulent factor. Hence, hits from this strategy already have *in vivo* effect but the determination of their molecular target is a critical challenge (Payne et al., 2007). Nonetheless, thanks to our understanding on the T3SS ATPase and the T3SS needle complex in *P. aeruginosa*, the target-based screening approach perfectly matches our objective and is feasible in the laboratory. However, it should also be reconsidered that both targets are located inside bacteria, representing thus a challenge because the *in vitro* selected drugs will have to pass through the bacterial membranes in order to have *in vivo* effects

(Benedetto Tiz et al., 2018). Therefore, many experiments are required to confirm their efficacy on cellular models as well as *in vivo* on bacteria and on animal models.

Regarding PscE and PscG, Sophie performed screenings to look for chemical inhibitors blocking the interaction between the two chaperones and identified hits from two different libraries. Among these compounds, the best hits (Clioquinol from Prestwick library and 3-APPA from DPM library), which present high percentage of inhibition *in vitro*, were selected for the structure activity relationships (SAR) study. Many analogs of each hit compound as well as of hybrid Clioquinol/3-APPA were created and we selected 21 of them for *ex vivo* and *in vivo* characterizations. Two compounds were afterward shown to be promising because they almost did not affect bacterial fitness, were not toxic for eukaryotic cells at working concentration and inhibited the bacterial virulence *ex vivo* on cells and *in vivo* in an insect model (*Galleria mellonella*). Moreover, the two compounds are closely related and belong to the structure hybrid cluster. In this study, we provide a rare example of use of target-based screening for the identification of inhibitor of bacterial virulence. Furthermore, the combination of hit molecules from two different family could somehow decrease their toxicity and gain efficacy in comparison to their parents.

On the other hand, we showed that PscN interacts with the effector ExoU-SpcU, translocator PcrH-PopB/PopD, needle PscE-PscF-PscG and gate-keeper PopN complexes (chapter II-A). However, the project to look for compounds inhibiting the binding capacity of PscN started early before the interaction of PscN with PscE-PscF-PscG and PopN complex was revealed by MST assay. Otherwise, the secretion of ExoU exotoxin is specific for *P. aeruginosa* PA14-like strains and targeting only one exotoxin seems not to offer a broad enough activity spectrum. For these reasons, the interaction of PscN with the translocator complex was the target of the inhibitor second screening project. Furthermore, PcrH-PopD complex was selected because more signal intensity was observed in the ELISA assay (Figure 32E).

However, the purification of PscN was not optimum at that moment and the ELISA-based screening was not performed with the pure protein. Indeed, the binding signal to PcrH-PopD of partially purified PscN was almost the same as the one of the bacterial extract containing PscN (Figure 32D) while the quantity of the partially purified protein was insufficient for the whole screening. Consequently, based on ELISA assay, the screening was performed using purified PcrH-PopD and the bacterial extract containing PscN although there was a risk of non-specific binding and inhibition despite the detection with anti-PscN antibodies. 15 hit compounds from

the Prestwick library were identified and characterized *ex vivo* on a cellular model and *in vivo* on bacteria. Seven compounds were selected and further used to revalidate the target specificity on ELISA assay using the purified FSS-PscN which was obtained long time after. Only two compounds: Chlorhexidine and Alexidine dihydrochloride, which belong to the structural family of detergent, showed ability to inhibit PscN binding to its partner. Unfortunately, we cannot completely rule out the possibility of direct unfolding effect on the ATPase for Chlorhexidine whereas it is less probable for Alexidine dihydrochloride (Figure 37). Therefore, only Alexidine dihydrochloride was retained for further studies. This work reveals the disadvantage of using ELISA assay for the screening when protein extracts are used. In our case, we identified a series of hit compounds and most of them were not confirmed. The selected compound is known as an antibacterial and thus does not fulfill the criteria of our initial objective for the development of anti-virulent compounds. However, because it inhibits the interaction of PscN with PcrH-PopD at concentrations that does not affect the catalytic activity, it might be possible that this compound possess two different properties: antibiotic and anti-virulent.

In conclusion, we presently have two different series of T3SS chemical inhibitors targeting either the ATPase PscN or the needle complex PscE-PscF-PscG in *P. aeruginosa*. Although they were identified from two different projects, the techniques used for the molecular identification and characterization are almost the same. In other words, we established a process pipeline for the development of new anti-virulence compounds from the discovery to the preclinical research on an insect model. Most of the methodologies are high-throughput and large-scale using 96 or 384 wells plates, and are combined with statistical and automated computational analyses of the results. In further steps, animal models such as zebrafishes and mice will be used to test the efficacy of the two lead compounds inhibiting interaction of PscE and PscG. Otherwise, the scaffold of anti-PscN hit compound will be used for the chemical development and SAR study. Afterward, the analogs compounds will be characterized taking advantage from the developed techniques used for the project on the needle co-chaperones.

Chapter IV

Annexes

Annexe 1: Poster communication in the conference VLM (Vaincre La Mucoviscidose) in Paris, Feb-2016.

Titre et auteurs

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

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Objectifs

Définition de cibles potentielles dans l'ATPase PscN pour une nouvelle stratégie anti-infectieuse.

Méthodes

- Test d'interaction de l'ATPase PscN avec des autres protéines du System de Sécrétion de Type III de *P. aeruginosa* (ELISA, Co-purification, TR-FRET, BiFc, Biacore).
- Test de dissociation des protéines du SST3 avec leur chaperonne (Co-purification).

Résultats

- Démonstration d'activité d'interaction de l'ATPase PscN partielle purifiée avec ces partenaires
- Identification certaines protéines sécrétées qui se lient avec l'ATPase PscN par ELISA

Conclusions

Bien que l'ATPase PscN soit peu exprimée sous forme soluble et soit très difficile à purifier, les différentes optimisations de cette enzyme ont été mise en place et nous ont aidés à obtenir une protéine un peu propre avec la quantité acceptable pour réaliser des tests biochimiques. De plus, pour la première fois, l'activité de PscN purifié a été démontrée grâce au test d'ELISA pour la capacité de se lier avec les autres protéines sécrétées du SST3 de *P. aeruginosa*.

Eventuellement, pour identifier les molécules inhibitrices de cet enzyme dans le but de la thérapeutique, il faut optimiser la production afin d'avoir une grande quantité de cette protéine purifiée pour le criblage. Dans notre organisme, nous avons réalisé des criblages qui basent sur la technique d'ELISA pour chercher des molécules bloquant l'interaction de cet enzyme avec un partenaire sécrété du SST3. Des résultats intéressants ont été obtenus.

Remerciements

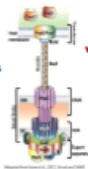
Ce travail est supporté par les associations de Vaincre la Mucoviscidose et de Grégory LEMARCHAL.

INHIBITION OF PSEUDOMONAS AERUGINOSA TYPE III SECRETION SYSTEM

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 Biosciences and Biotechnology Institute of Grenoble – CEA Grenoble
 BCI Lab – Team Bacterial Pathogenesis and Cellular Responses

BACKGROUND

- *Pseudomonas aeruginosa* is a gram-negative pathogenic bacteria responsible for nosocomial infection.
- Associated with deterioration of the cystic fibrosis patients health status.
- Type III Secretion System (T3SS) is a major virulence factor present in many gram-negative bacteria.
- ATPase enzyme (PscN) required for the T3SS activity.

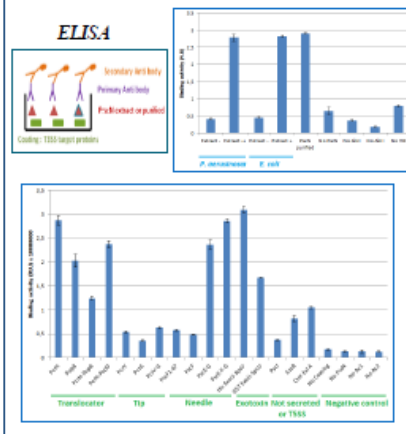


OBJECTIVES

1. To understand the role of the ATPase PscN, a key factor of the T3SS assembly and functioning.
 - > Binding to other T3SS secreted proteins.
 - > Dissociation of these proteins from their chaperons.
 - > Unfolding of these proteins before secretion.
2. To screen for anti-virulent drugs inhibit PscN binding activity.

RESULTS

BINDING ACTIVITY



SCREENING

Anti-PscN
Purified PscN
Inhibitor

PerI-PopD

Collaboration with CMB Platform of CEA

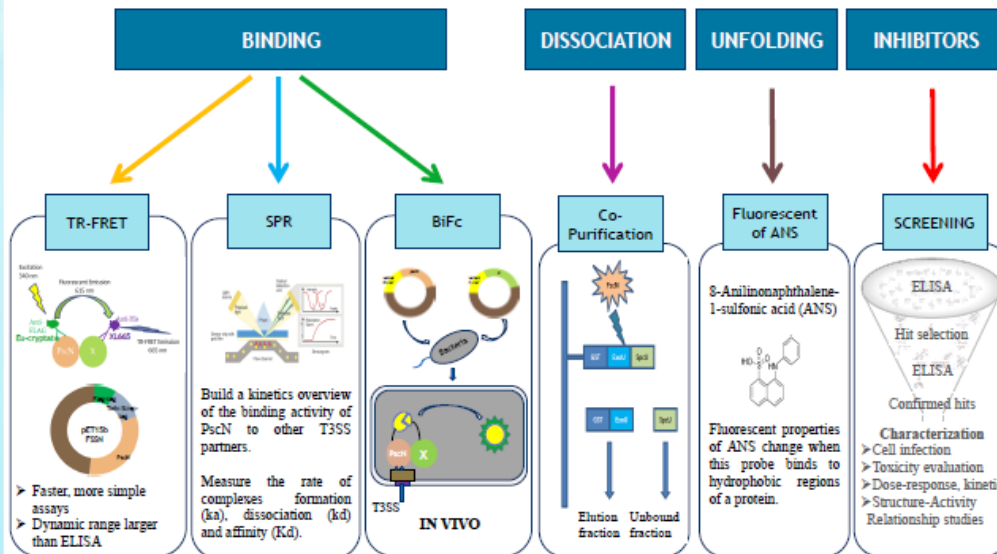
- > Deletion mutant *DpscN* lost its T3SS cytotoxicity.
- > Anti virulence factor should elicit less resistance than antibiotic.
- > Interaction of PscN with its partners is a more specific target than its catalytic activity.

- Many ELISA parameters were optimized.
- Full automation with robotic system.
- Good robustness : Z' factor = 0.7.

$$Z\text{-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

SCREENING

FUTURE STRATEGIES



CONCLUSIONS

- > PscN was purified by affinity chromatography (Srep-tactin column).
- > Binding activity of the purified protein was demonstrated.
- > Binding to many T3SS secreted proteins like the translocators, the needle complex and an exotoxin.
- > Interaction inhibitors will be looked for shortly.

This work is being supported by the Vaincre la Mucoviscidose Association and the Gregory LEMARCHAL Association



Annexe 2: Poster communication in the meeting of Type III Secretion System in Tübingen, Germany, April – 2016.

Abstract title

Type III Secretion System ATPase *Pseudomonas aeruginosa*

Authors: Tuan Dung NGO, Caroline PERDU, Bakhos JNEID, Ina ATTREE and Eric FAUDRY
BioSciences and Biotechnologies Institute of Grenoble, 17 avenue des Martyrs, 38054 Grenoble France

Abstract

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen responsible of nosocomial infections. The Type III secretion system is one of its main virulence factors, allowing the direct injection of four exotoxins into the targeted eukaryotic cells. One important protein of this system is the conserved ATPase, named PscN in *P. aeruginosa*. As expected from the literature, this enzyme was found to be essential for the function of the T3SS because the deletion mutant $\Delta pscN$ lost its ability to secrete and inject toxins. Complementary vectors, containing the wild type gene *pscN* or fusion gene with 6-His-tag or genes mutated in the putative active site, allowed to demonstrate that: i) the presence of a 6-His-tag at the N-terminus of the protein is not deleterious for its activity; ii) its activity can be abolished by single mutations and iii) these mutations appear to be dominant negative. Furthermore, we attempted to purify PscN from *P. aeruginosa* and *E. coli* and for the first time, the binding of PscN on ExoU-SpcU (one exotoxin) and PcrH-PopD (chaperone-translocator complex) was shown by ELISA. Further experiments will be undertaken to determine whether PscN can dissociate these protein complexes and unfold the secreted proteins in order to allow their passage through the narrow T3SS needle.

Type III Secretion System ATPase of *Pseudomonas aeruginosa*

Tuan Dung NGO, Caroline PERDU, Bakhos JNEID, Ina ATTREE and Eric FAUDRY

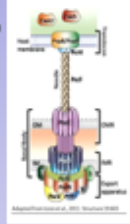
Biosciences and Biotechnology Institute of Grenoble – CEA Grenoble

BCI Lab – Team Bacterial Pathogenesis and Cellular Responses



Background

- Pseudomonas aeruginosa* is a gram-negative pathogenic bacteria responsible for nosocomial infection.
- Type III Secretion System (T3SS) is a major virulence factor present in many gram-negative bacteria.
- ATPase enzyme is required for the T3SS activity.



Objectives

- To understand the role of the ATPase *PscN* in T3SS activity
- Creation of mutation in *pscN* gene
- Study the *PscN* role with complementing vector containing *pscN* wild-type or mutated
- To express and purify the ATPase *PscN*
- To test the binding activity of *PscN* to some partners of the T3SS, before secretion.

Conclusions

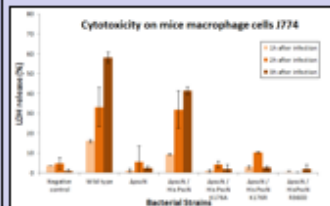
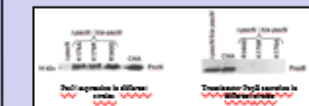
- The deletion mutant *ΔpscN* strain lost its ability to secrete and inject toxin.
- Complementing vector containing the fusion gene with *exHistag* or *StrepTag* can restore T3SS activity.
- Some mutations inside *pscN* gene were created and appeared to be dominant negative.
- Recombinant *PscN* was purified by *HisTrap* or *StrepTactin* columns but its yield is low.
- Binding activity of *PscN* to some T3SS partners was demonstrated by ELISA.

Outlook

- To attempt to express *PscN* in *E. coli*
- To optimize the purification of *PscN* by changing some buffer parameters such as pH, salt concentration, additive.
- To build an overview of *PscN* binding activity to many T3SS proteins.
- To assess the *PscN* ability for the dissociation of protein-chaperone complex and the unfolding of secreted protein in order to pass through the narrow T3SS needle.

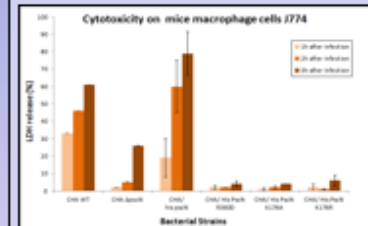
Results

T3SS activity in mutated strains



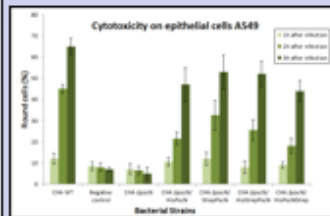
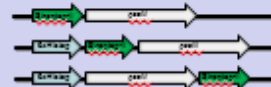
Mutated *pscN* strains are not able to secrete toxin and lost their cytotoxicity.

Dominant negative effect

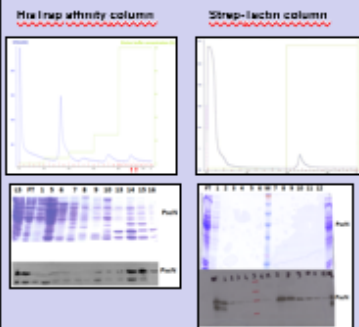


Mutated *pscN* in complementing vector abrogate wild-type cytotoxicity.

New Constructions for *PscN* study



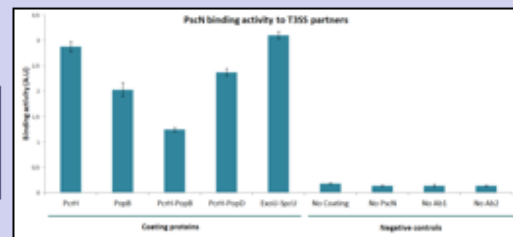
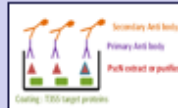
Purification of *PscN*



- A lot of contaminating proteins
- PscN* only detected by Western blot.

Binding activity

ELISA



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Annexe 3: Scientific communication of the project on the characterization and inhibition of the ATPase PscN.

- ✓ *Oral and poster communications in the conference EYIM (European Young Investigators Meeting) on Cystic Fibrosis in Paris, Feb-2017.*
- ✓ *Poster communication in the conference VLM (Vaincre La Mucoviscidose) in Paris, Feb-2017.*

Summary

Abstract title

Characterization and Inhibition of *Pseudomonas aeruginosa* Type III Secretion System ATPase

Authors: Tuan Dung NGO¹, Juliane BROCK^{1,2}, Caroline BARETTE¹, Ina ATTREE¹ and Eric FAUDRY¹

¹ BioSciences and Biotechnologies Institute of Grenoble

² Institut de Biologie Structurale, Grenoble

Background

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that severely affects cystic fibrosis patients. The Type III Secretion System (T3SS) is one of its main virulence factors, allowing the direct injection of four exotoxins into the targeted eukaryotic cells. One important protein of this system is the conserved ATPase, named PscN in *P. aeruginosa*, considered to interact with the secreted Type III proteins and to deliver them to the secretion channel. By hydrolyzing ATP, this enzyme is supposed to dissociate chaperone-protein complexes and to unfold secreted proteins in order to allow their passage through the narrow T3SS needle.

Aims

Our work aims to purify the ATPase PscN and to demonstrate the interaction of PscN with other secreted proteins, including the translocon proteins (PcrH-PopB/D, PcrG-PcrV), the needle complex (PscE-PscF-PscG) and exotoxins (ExoU-SpcU, ExoS/T-SpcS). Furthermore, we will look for chemical compounds inhibiting the binding capacity of ATPase PscN, thus impairing T3SS functioning.

Methods

We constructed an expression vector with tandem tags fused to the N-terminus of *pscN*, which allow us to purify PscN using affinity columns. *In vitro*, the interaction of PscN with other complexes was studied by Pull-down and ELISA assay. The screening was performed using High-Throughput Screening (HTS) of small compounds from Prestwick chemical library at the CMBA platform of CEA-Grenoble, based on ELISA assay. The hits were confirmed and characterized *in vitro* by ELISA and *ex vivo* by High Content Analysis (HCA) of cells infection.

Results

PscN was purified and displayed an ATPase activity similar to other known T3SS ATPases. By ELISA, we also demonstrated the interaction of PscN with secreted protein-chaperone complexes or the chaperones alone but not with the secreted proteins alone.

Besides, we were searching for compounds impairing the interaction between PscN and the PcrH-PopD complex. A first screening identified 15 active molecules, seven of these were selected and confirmed in a second screening, showing over 20% of inhibition at 10 μ M concentration *in vitro*. The *ex vivo* results show promising inhibition patterns, that could be the base for a future protection of the eukaryotic cell against bacterial infection.

Conclusions

The ATPase PscN has an essential role for the T3SS function in *P. aeruginosa*. *In vitro* characterization of purified PscN showed its interaction with the secreted proteins (the first step of the secretion process). Furthermore, we identified some chemical molecules, inhibiting the interaction of PscN with the translocon protein (PcrH-PopD), and characterized them *in vitro* and *ex vivo*. The structure-activity relative (SAR) studies of these molecules will be done soon.

Acknowledgements

This work is supported by the Vaincre la Mucoviscidose and the Grégory LEMARCHAL associations. We thank Emmanuelle SOLEILHAC for her help with HCA and Michel RAGNO for excellent technical assistance.

Summary (in French)

Titre et auteurs

Caractérisation et inhibition de l'ATPase du Système de Sécrétion de Type III dans *Pseudomonas aeruginosa*

Auteurs: Tuan Dung NGO¹, Juliane BROCK^{1,2}, Caroline BARETTE¹, Ina ATTREE¹ and Eric FAUDRY¹

¹ BioSciences and Biotechnologies Institute of Grenoble

² Institut de Biologie Structurale, Grenoble

Contextes

Pseudomonas aeruginosa est un dangereux pathogène opportuniste qui cause des maladies nosocomiales et infecte les patients atteignant la mucoviscidose. Le système de sécrétion de type III (SST3) est un des plus importants facteurs de virulence qui permet d'injecter directement quatre exotoxines dans les cellules hôtes par une seule étape. Une protéine très importante de ce système est l'ATPase PscN qui est considéré d'interagir avec des protéines sécrétées du SST3 and de les délivrer au canal de sécrétion. Après, cet enzyme fournit d'énergies pour la dissociation de complexe protéine-chaperonne et finalement, elle déplie la protéine sécrété pour sa passage à travers l'aiguille du SST3.

Objectifs

Notre objectif est de purifier l'ATPase PscN et de démontrer l'interaction de PscN avec des autres protéines secrétées du SST3. Par ailleurs, nous cherchons des petites molécules chimiques qui inhibent l'activité d'interaction de PscN, ce qui empêche le fonctionnement du SST3.

Méthodes

Nous avons créé un vecteur d'expression contenant pscN fusionné avec des « tandem-tags » qui nous permettent de purifier cette protéine par la chromatographie d'affinité. *In vitro*, l'interaction de PscN avec ses partenaires de type III est étudié par le test d'ELISA. Le criblage des molécules est réalisé en utilisant le criblage à haute débit (High Throughput Screening (HTS)) pour les molécules de la banque de Prestwick (Prestwick chemical library) en collaboration avec la plateforme CMBA du CEA. Des touches (hits) ont été identifiées et caractérisées *in vitro* par le test d'ELISA et *ex vivo* par l'infection cellulaire en utilisant "High Content Analysis (HCA)".

Résultats

PscN est purifié et dispose une activité ATPase comparable avec celles des autres ATPase connues de Systèmes de Sécrétion de Type III. De plus, la protéine PscN a été caractérisée par plusieurs techniques de biophysique et de biochimie et les résultats montrent que la PscN purifiée est monomérique et n'est pas fortement repliée. Nous avons montré aussi par un test d'ELISA que PscN a la capacité d'interagir avec des protéines secrétées par le SST3.

Par ailleurs, nous cherchons des molécules inhibitrices de l'interaction entre PscN et la complexe PcrH-PopD et le criblage primaire nous a identifié 15 molécules actives. Après le criblage secondaire, nous avons confirmé 7 molécules avec le taux d'inhibition supérieur à 20% à 10 μ M. *Ex vivo*, des résultats préliminaires indiquent des hits assez prometteurs. Nous prévoyons une étude Structure Activity Relationship (SAR) afin d'optimiser nos molécules.

Conclusions

L'ATPase PscN joue un rôle essentiel pour le fonctionnement du SST3 de *P. aeruginosa*. *In vitro*, PscN a été caractérisé et a montré son interaction avec les protéines secrétées (la première étape de la sécrétion). De plus, nous avons identifié 7 molécules chimiques qui inhibent l'interaction de PscN avec la protéine du translocateur (PcrH-PopD), et leur avons caractérisé *in vitro* et *ex vivo*. Nous prévoyons une étude Structure Activity Relationship (SAR) afin d'optimiser nos molécules.

Remerciements

Ce travail est supporté par les associations de Vaincre la Mucoviscidose et de Grégory LEMARCHAL. Grand merci à Emmanuelle SOLEILHAC pour l'utilisation de HCA et à Michel RAGNO pour des assistances techniques.

INHIBITION OF *PSEUDOMONAS AERUGINOSA* TYPE III SECRETION SYSTEM

Tuan Dung NGO¹, Juliane BROCK¹, Caroline BARETTE², Ina ATTREE¹ and Eric FAUDRY¹

BioSciences and BioTechnology Institute of Grenoble – CEA Grenoble

¹BCI Lab – Team Bacterial Pathogenesis and Cellular Responses and ²BGE Lab – Team Genomics and Chemogenomics

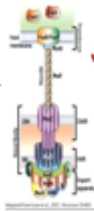
BACKGROUND

Pseudomonas aeruginosa is a gram-negative pathogenic bacteria responsible for nosocomial infection.

Associated with deterioration of the cystic fibrosis patients health status.

Type III Secretion System is one of the major virulence factors present in many gram-negative bacteria.

The ATPase PscN is required for the T3SS activity.

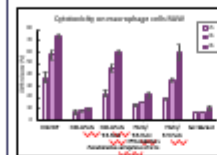


OBJECTIVES

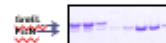
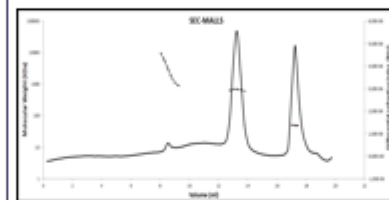
- To purify the ATPase PscN and to demonstrate its activities in the T3SS assembly and functioning.
 - Binding to the T3SS secreted proteins.
 - Dissociation of these proteins from their chaperons.
 - Unfolding of these proteins before secretion.
- To screen for anti-virulent drugs inhibiting the PscN binding activity.

RESULTS

ATPase purification

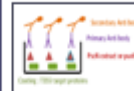


- Tandem-tag fused to the N-terminus of *pscN* (T-T-PscN) is not deleterious for its activity.
- PscN is purified using Affinity column and Size Exclusion Chromatography (SEC) column.
- SEC-MALLS showed pure monomeric PscN



Activity

ELISA



ATPase activity of monomeric PscN : 0.06 $\mu\text{mol}/\text{min}/\text{mg}$
 ⇒ Comparable to other type III ATPase

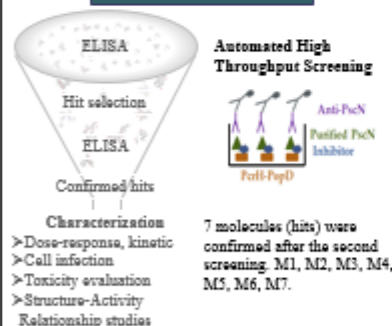
ELISA binding assay
 ⇒ PscN binds to T3SS secreted proteins

⇒ Monomeric PscN is active.



SCREENING AND CHARACTERIZATION

SCREENING



Characterization in vitro

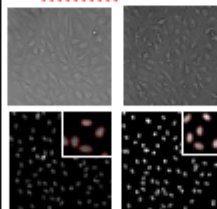
- 7 Molecules
- 5 concentrations: 100 μM , 30 μM , 10 μM , 3 μM and 1 μM
- ELISA assays
- PscN interaction with translocon, needle complexes and exotoxin

10 μM	PerH-PopB	PerH-PopD	PerH	PscD-PscF-PscO	ExoU-SpC1
M1	46.60%	43.20%	15.50%	12.30%	11.20%
M2	16.90%	16.70%	6.60%	3.90%	0.00%
M3	23.90%	17.30%	8.50%	0.00%	0.00%
M4	39.60%	38.20%	17.20%	5.60%	0.00%
M5	48.00%	47.20%	33.20%	44.50%	33.90%
M6	14.40%	28.60%	15.60%	0.00%	0.00%
M7	16.20%	23.20%	8.20%	0.00%	0.00%

Most molecules specifically inhibit the interaction of PscN with the translocon. M1 and M5 show less specificity.

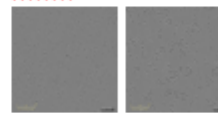
Characterization ex vivo

Bright nuclei using Arravscan™ HCA



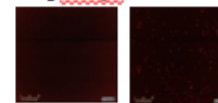
- Cells are labeled to visualize the nuclei
- Nuclei are brighter when cells are stressed

Retracted cell surface using Incucyte® LCA



- Cell shape modification reflects cytotoxicity.
- Injected ExoS induces cell retraction.

Propidium iodide (PI) entry using Incucyte® LCA



ExoU induction of cell death is revealed by an entry of PI inside the cells.

% inhibition (2.5h p.i.)	CHA WT		PAO2F WT		CHA ΔExoS - ExoU	
	Bright nuclei	Retracted cell surface	10 μM	3 μM	10 μM	3 μM
M1	0%	0%	20%	38%	0%	22%
M2	0%	0%	43%	34%	26%	37%
M3	0%	0%	40%	35%	72%	26%
M4	72%	53%	58%	57%	0%	0%
M5	0%	0%	0%	67%	0%	0%
M6	0%	0%	24%	17%	63%	4%
M7	0%	0%	67%	52%	26%	20%

All molecules, except M5, are promising compounds

CONCLUSIONS

- PscN was purified by affinity and size exclusion chromatography.
- Monomeric PscN is active and shows binding activity to many T3SS secreted proteins like the translocators, the needle complex and an exotoxin.
- 7 hits were identified and 6 molecules are still promising inhibitors.
- The structure-activity relative (SAR) will be performed shortly.

This work is being supported by the **Vaincre la Mucoviscidose Association** and the **Grégoire LEMARCHAL Association**



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Annexe 4: Scientific communication of the project on the development of anti-virulent compounds inhibiting PscE-PscG interaction.

- ✓ *Oral communication in the conference G-RREMI (groupe Régional de Recherche en Microbiologie des Interactions) in Lyon, Dec-2017.*
- ✓ *Poster communication in the conference VLM (Vaincre La Mucovisidose) in Paris, Feb-2018.*
- ✓ *Oral communication in the conference SFM (Société Française de Microbiologie) in Paris, Oct-2018.*

Summary

Abstract title

PscE-PscG interaction: a novel target for anti-virulence strategies in *Pseudomonas aeruginosa* Type III Secretion System

Authors: Tuan-Dung Ngo¹, Sophie Plé^{1*}, Caroline Barette¹, Emmanuelle Soleilhac¹, Ina Attrée¹, Yung-Sing WONG² and Eric Faudry¹

¹ BioSciences and Biotechnologies Institute of Grenoble, France

² Département de Pharmacochimie Moléculaire de Grenoble, France

*Current adress: PX'Therapeutics SAS, Grenoble, France

Background

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes nosocomial diseases and infects cystic fibrosis patients. The Type III Secretion System (T3SS) is one of the most important virulence factors, allowing the direct injection of four exotoxins into the targeted eukaryotic cells. PscF is the protein forming the secretion needle and has the ability to self-polymerize. In the bacteria cytoplasm, it stays in complex with two cognate chaperones PscE and PscG that are necessary for its stability and to prevent PscF polymerization inside bacteria. The *P.aeruginosa* mutant $\Delta pscE$ and $\Delta pscG$ are not cytotoxic for eukaryotic cells due to the absence of T3SS activity. Moreover, point mutations in the interface of PscE-PscG impair the T3SS needle formation and decrease or abolish the bacterial cytotoxicity.

Aims

Our work aims to identify chemical compounds that interfere with the PscE-PscG interaction, thus impairing the needle formation and the T3SS functioning. This would represent a new strategy to develop an anti-virulence compound that would elicit less resistance than classical antibiotics.

Methods

Two expression vectors were constructed with His-tag fused to the N-terminus of *pscE* and *pscG* that allow us to purify the recombinant proteins. The screening was performed using

High-Throughput Screening (HTS) of small chemical compounds from the Prestwick and a homemade chemical libraries at the CMBA platform of CEA-Grenoble, based on an ELISA assay. The hits were selected and 24 molecules were created after some chemical structure modifications. These optimized compounds were characterized *ex vivo* by High Content Analysis (HCA) of live cell infection kinetics and *in vivo* on the bacteria.

Results

The Hits molecules identified from the screening were optimized to obtain 24 compounds that are classified into five classes based on their molecule structure. The 24 optimized compounds are highly soluble and have a high percentage of inhibition *in vitro* (from 38% to 92%) at 50 μ M.

The *ex vivo* characterization identified two promising compounds that inhibit the cell infection caused by T3SS positive *P.aeruginosa* strains, are non-toxic for eukaryotic cells and have a minimal effect on bacterial growth. Moreover, a decrease of secretion of the exotoxin ExoS and the translocator PopD was observed in the presence of the two compounds indicating that they inhibit the T3SS functioning.

Conclusions

Based on the screening of chemical compounds, some chemical molecules inhibiting the interaction of PscE-PscG, the two cognate chaperones of needle protein PscF, were identified. These compounds were chemically modified and the optimized compounds were characterized, showing two promising compounds (Leads) with *in vitro*, *ex vivo* and *in vivo* inhibition properties. Some experiments on insect or animal models will be performed soon for these two compounds, to strengthen the proof of our anti-virulence strategy.

Acknowledgements

This work is supported by the Vaincre la Muscovisidose and the Grégory LEMARCHAL associations.

Summary (in French)

Titre et auteurs

Interaction de PscE-PscG: une nouvelle cible pour stratégies de l'anti-virulence dans *Pseudomonas aeruginosa*

Auteurs: Tuan-Dung Ngo¹, Sophie Plé^{1*}, Caroline Barette¹, Emmanuelle Soleilhac¹, Ina Attrée¹, Yung-Sing WONG² and Eric Faudry¹

¹ BioSciences and Biotechnologies Institute of Grenoble, France

² Département de Pharmacochimie Moléculaire de Grenoble, France

*Current adress: PX'Therapeutics SAS, Grenoble, France

Contextes

Pseudomonas aeruginosa est un dangereux pathogène opportuniste qui cause des maladies nosocomiales et infecte les patients atteignant la mucoviscidose. Le système de sécrétion de type III (SST3) est un des plus importants facteurs de virulence qui permet d'injecter directement quatre exotoxines dans les cellules hôtes par une seule étape. Ces exotoxines sont traversées par une aiguille dont la protéine principale est PscF qui a la capacité d'auto-polymériser. Dans le cytoplasme de bactérie, cette protéine forme un complexe avec ses deux chaperonnes PscE et PscG qui sont nécessaires pour sa stabilité et la prévention de sa polymérisation à l'intérieur de bactérie.

Objectifs

Notre travail a pour objectifs d'identifier et développer des composés chimiques qui interfèrent l'interface d'interaction de PscE-PscG, ce qui puisse déstabiliser le complexe PscE-PscF-PscG et inhiber l'activité de SST3. En effet, des mutations ponctuelles dans l'interface de PscE-PscG ont été montrées pour diminuer ou arrêter la cytotoxicité de *Pseudomonas aeruginosa*.

Méthodes

Deux vecteurs d'expression pour exprimer PscE et PscG avec le His-tag ont été construits pour la purification de protéine recombinante. Basé sur un test d'ELISA, le criblage à haute débit (high-throughput screening, HTS) d'inhibiteur a été réalisé au Platform CMBA de CEA avec des composés de la banque de Prestwick et une « home-made » banque. Des touches ont été sélectionnées et 23 analogues sont créés après quelques étapes de modification structurale. Ces molécules sont combinées et optimisées et ensuite caractérisées *ex vivo* par l'imagerie cellulaire à haut débit (high-content screening, HCS) de cinétique d'infection des cellules vivantes et *in vivo* sur la bactérie.

Résultats

Des touches identifiées par le criblage sont optimisées pour obtenir 23 molécules qui sont classées à trois classes basées sur leur structure. Ces optimisées composés sont très solubles et ont un grand pourcentage d'inhibition *in vitro* (de 38% à 92%) à 50 μ M.

Des résultats *ex vivo* nous donnent deux composés prometteuses qui inhibent l'infection causées par des souches de *P.aeruginosa* dépendent du SST3. Ces molécules ne sont pas toxiques pour des cellules eucaryotes et ont un effet minimal sur la croissance bactérienne. De plus, une diminution de sécrétion d'exotoxine ExoS et de translocateur PopD est observée *in vivo* en présence de ces deux composés, ce qui montre qu'ils inhibent le fonctionnement de SST3.

Conclusion et discussion

Basé sur le criblage de composés chimiques, nous avons identifié des molécules inhibant l'interaction de PscE-PscG qui sont les deux chaperonnes de protéine de l'aiguille PscF. Ces molécules sont optimisées structuralement et ensuite caractérisées *ex vivo* dont le résultat nous

données deux prometteuses composés (Leads). Nous visons à des essais sur des modèles de l'insecte et l'animal afin de renforcer la preuve de notre stratégie d'anti virulence dans le contexte où la résistance aux antibiotiques est un problème majeur de la thérapeutique actuelle.

Remerciements

Ce travail est supporté par les associations de Vaincre la Mucoviscidose et de Grégory LEMARCHAL.

Interaction entre PscE-PscG : une nouvelle cible pour la stratégie d'anti-virulence contre *Pseudomonas aeruginosa*

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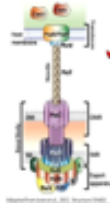
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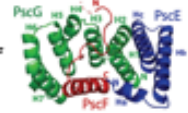
Contexte

- Pseudomonas aeruginosa* est une bactérie pathogène opportuniste qui cause des maladies nosocomiales et infecte les patients atteints de mucoviscidose.
- Le système de sécrétion de type III (SST3) est un des facteurs de virulence les plus importants présents dans plusieurs bactéries gram-négatives.
- L'aiguille du SST3 est principalement formée de la protéine PscF qui forme un complexe avec ses deux chaperonnes PscE and PscG dans le cytoplasme de la bactérie. Ces chaperonnes sont nécessaires pour la stabilité de PscF et pour empêcher la polymérisation de PscF à l'intérieur de la bactérie.
- Des mutations ponctuelles dans l'interface avec PscE et PscG altèrent ou abolissent la cytomotilité de *P. aeruginosa*.



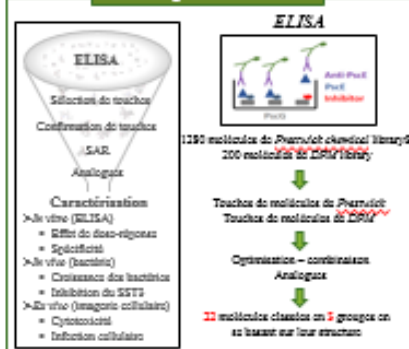
Objectifs

- Identifier et développer des composés chimiques qui
 - Perturbent l'interaction entre PscE et PscG.
 - Désassemblent le complexe PscE-PscF-PscG à l'intérieur de la bactérie.
 - Inhibent l'activité de SST3.



Criblage à haut débit

Criblage automatisé



Molécules identifiées et optimisées

Molécules de Prestwick et analogues			Molécules du DPM et analogues			Combinaisons		
Classe	Structure	Activité (%)	Classe	Structure	Activité (%)	Classe	Structure	Activité (%)
Alcaloïdes		100%	Alcaloïdes		100%	Alcaloïdes		100%
Alcaloïdes		100%	Alcaloïdes		100%	Alcaloïdes		100%
Alcaloïdes		100%	Alcaloïdes		100%	Alcaloïdes		100%
Alcaloïdes		100%	Alcaloïdes		100%	Alcaloïdes		100%

Caractérisation ex vivo et in vivo

Deux molécules prometteuses identifiées par imagerie cellulaire à haut débit

1. Conditions optimales : Plate, Réactifs, 1 seul
2. Imagerie : Microscope automatisé, caméra
3. Analyse : Logiciel automatisé

1.0 cellule/cellule (10⁵ cells/well)
2. Stabilité aux AMS
3. Stabilité de l'illumination

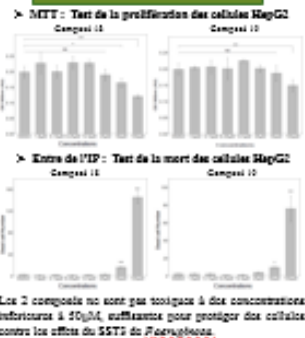
➤ **Noyaux brillants (Stress cellulaire)**

➤ **Surface de cellule rétractée**

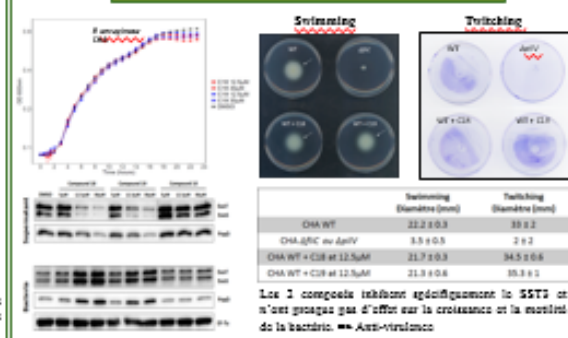
➤ **Entrée de l'iodure de propidium (IP)**

- Les cellules sont marquées pour visualiser le noyau.
- Leur noyau est brillant quand les cellules sont stressées par plusieurs facteurs comme l'infection ou les molécules.
- Les cellules sont marquées pour visualiser la membrane.
- Leur rétraction est induite par l'oxotocaine BtoU, une glycohalogène qui dégrade la membrane cellulaire.
- L'entrée de IP est le résultat de la mort cellulaire induite par l'oxotocaine BtoU, une glycohalogène qui dégrade la membrane cellulaire.

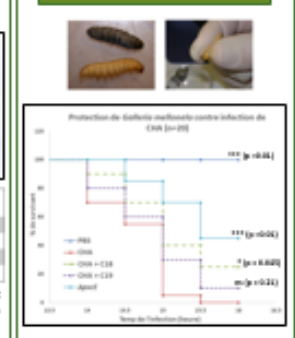
Toxicité



Inhibition spécifique du SST3



Protection in vivo



CONCLUSIONS

Basé sur le criblage automatisé de complexes chimiques, nous avons identifié des molécules inhibant l'interaction de PscE-PscG qui sont les deux chaperonnes de la protéine PscF formant l'aiguille du SST3. Ces molécules ont été optimisées structurellement et caractérisées sur des modèles eucaryotes et sur les bactéries, débouchant sur deux composés prometteurs. Un de ces composés améliore considérablement la survie des larves *Galleria mellonella* infectées par *P. aeruginosa* CRA alors que l'autre semble avoir le même effet mais avec moins d'efficacité.



Annexe 5: Poster communication in the meeting of bacterial secretion systems in Baeza, Spain, November – 2018.

Abstract title

Interaction of *Pseudomonas aeruginosa* T3SS ATPase with secreted proteins and chaperones

Authors: Tuan-Dung NGO, Caroline PERDU, Michel RAGNO, Bakhos JNEID, Ina ATTREE and Eric FAUDRY

Bacterial Pathogenesis and Cellular Responses – CEA, 17 av des Martyrs, Grenoble, France

Abstract

The T3SS ATPase is named PscN in *P. aeruginosa* and SctN in the unified T3SS nomenclature. Its role in the energization of the secretion and the selection of the proteins to be secreted is not clear. On the other hand, it was shown to bind to complexes made of secreted proteins and their chaperones, to dissociate these complexes and unfold the secreted proteins. However, its binding to all the classes of secreted proteins was not demonstrated and it is not known whether it contributes to the establishment of a hierarchical secretion of the different T3SS components. The objective of this work is to get a more exhaustive view of the panel of proteins PscN is able to interact with and to characterize these interactions at the molecular level. Purification of the ATPase was optimized, based on its overexpression in *P. aeruginosa* and the fusion with a triple tag on its N-terminus. Taking advantage of the availability of many recombinant proteins belonging to different classes of secreted proteins and chaperones, the putative interactions were screened using an ELISA-derived assay. Then, Microscale Thermophoresis allowed the determination of the affinity constants, revealing strong differences between the protein classes. Determinants of proteins recognition are now under investigation.

Interaction of *Pseudomonas aeruginosa* T3SS ATPase with secreted proteins and chaperones

Tuan Dung NGO, Michel RAGNO, Caroline PERDU, Bakhos JNEID, Ina ATTREE and Eric FAUDRY

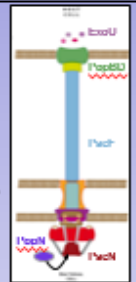
Biosciences and Biotechnology Institute of Grenoble – CEA Grenoble

BCI Lab – Team Bacterial Pathogenesis and Cellular Responses



Background

- Pseudomonas aeruginosa* is responsible for nosocomial infections and is a severe threat for Cystic Fibrosis patients.
- Type III Secretion System (T3SS) is a major virulence factor of *P. aeruginosa*.
- The T3SS ATPase (SciN/PscN) binds to and dissociates chaperone/cargo protein complexes.
- Role in substrate sorting and secretion hierarchy?



Objectives

- To understand the possible contribution of the T3SS ATPase to the sorting of the secreted proteins and to the secretion hierarchy:
- Optimization of PscN expression and purification.
- Screening of the potential partners of PscN.
- Determination of dissociation constants (K_d).
- To explore the formation of ternary complexes:
- Modulation of affinities.
- Competition for the same binding site.

Conclusions

- The introduction of a triple polar tag fused to the N-terminus of PscN dramatically increased the protein yield without affecting its function.
- PscN binds to the chaperones, the secreted cargo proteins and the complexes with different affinities.
- For a given protein complex, PscN displays a lower affinity for the chaperone in agreement with its recycling in the cytoplasm after its dissociation from the cargo.
- The "Gate keeper" PopN complex promotes the loading of the needle complex on PscN.

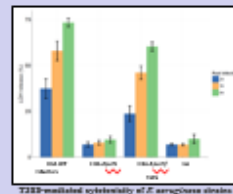
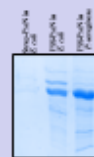
Outlook

- Investigate the possible modulation by other regulators, like ParV and ParG.
- Explore the interaction of PscN with other T3SS components: domains of the C-ring (sorting platform), ExsE (transcription regulation cascade), PscL (negative regulator of ATP hydrolysis)...
- Determine if the partners bind to the same sites on PscN.
- Examine the consequences, in terms of affinities, of point-mutations described *in vivo*.

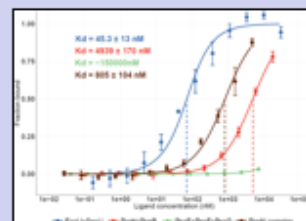
Results

Expression and purification

Fusion of PscN to a Flag-Strap-Strap (FSS) tag allows high purification yield upon expression in a modified *P. aeruginosa* strain, without affecting T3SS function

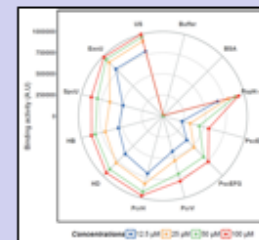


Different affinities for different complexes



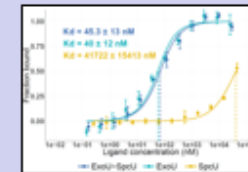
Binding affinities measured by Microscale Thermophoresis reveals large differences between different classes of protein complexes

PscN binds to a wide range of T3SS proteins



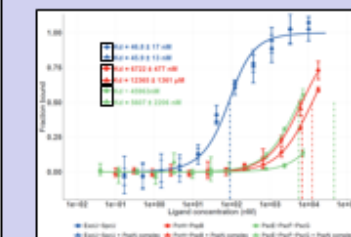
Binding of PscN to chaperones, cargo and protein complexes, detected by ELISA.

Lower affinity for the chaperones

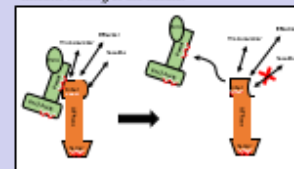


The lower affinity for the chaperones is consistent with the recycling of the chaperones in the cytoplasm after their dissociation from the cargo.

Binding of the "Gate keeper complex" on PscN increases its affinity for the needle complex



Allosteric regulation drastically modifies the recognition of the needle complex by PscN. Release of the "Gate keeper" PopN complex, inhibits loading of the needle.



Chapter V

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Characterization and inhibition of *Pseudomonas aeruginosa* Type III Secretion System

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes nosocomial diseases and infects cystic fibrosis patients. The Type III Secretion System (T3SS) is one of its most important virulence factors, allowing the direct injection of four exotoxins into the target eukaryotic cells. An important protein of T3SS is the conserved ATPase, named PscN that is involved in the assembly and functioning of this system. In this work, we demonstrated the interaction of PscN with T3SS secreted cargo proteins and with their chaperons in complex or alone using ELISA, HTRF and MST assays. Of important, MST (Microscale Thermophoresis) allowed us to determine the dissociation constants (Kd) of these proteins and PscN, showing the interaction preference of this enzyme for the cargo or complex proteins rather than for the corresponding chaperons alone. This confirms the hypothesis that the chaperons are released in the bacterial cytoplasm after the complex dissociation. Otherwise, we assess the Kd between the effector, translocator or needle complexes and PscN bound or not to the gate-keeper complex which is described as a regulator of substrate sorting for the secretion. The results showed that the binding of the gate-keeper to PscN dramatically increases its relative affinity for the needle complex, thus revealing a new role of the gate-keeper in the loading of the needle complex to the ATPase for the control of substrate hierarchical secretion in *P. aeruginosa*.

In parallel, committed to the anti-virulence strategy, we take the opportunity to characterize the *ex vivo* and *in vivo* effects of compounds identified by a previous *in vitro* screening to inhibit the interaction of PscE and PscG. These proteins are the two cognate chaperons of the T3SS needle protein PscF in the bacterial cytoplasm. This interaction had been shown to be a valid anti-virulence target because single or double point mutations introduced within the binding site between PscE and PscG lead to a decrease of *P. aeruginosa* virulence. This work points out two best leads which belong to the structural hybrid cluster combining hits from two different chemical libraries. The two compounds inhibit the cell damages caused by T3SS positive *P. aeruginosa* strains, are non-toxic for eukaryotic cell and have minimal effect on bacterial fitness. They were also shown to be specific for T3SS and could protect *Galleria mellonella* against *P. aeruginosa* infection.

Key words: *Pseudomonas aeruginosa*, Type III Secretion System, ATPase, Anti-virulence

Caractérisation et inhibition du Système de Sécrétion de Type III de *Pseudomonas aeruginosa*

Pseudomonas aeruginosa est un pathogène opportuniste gram-négatif qui cause des maladies nosocomiales et infecte les patients atteints de la mucoviscidose. Le Système de Sécrétion de Type III (SST3) est un de ses facteurs de virulence les plus importants et permet d'injecter directement quatre exotoxines dans les cellules hôtes. Une protéine très importante du SST3 est l'ATPase PscN qui est impliquée dans l'assemblage et le fonctionnement de ce système. Dans ce travail, nous mettons en évidence l'interaction de PscN avec des protéines sécrétées du SST3, avec leurs chaperonnes seul ou en complexes, en utilisant trois techniques de ELISA, HTRF and MST. En particulier, la MST (Microscale Thermophoresis) nous a permis de déterminer les constantes de dissociation (Kd) entre ces protéines et PscN. Les résultats montrent que l'ATPase interagit préférentiellement avec les protéines sécrétées ou les complexes plutôt que les chaperonnes, ce qui confirme le fait que celles-ci sont libérées dans le cytoplasme bactérien après la dissociation des complexes. Par ailleurs, en évaluant le Kd entre des complexes d'effecteur, de translocateur ou d'aiguille et l'ATPase liée ou non au complexe « Gate-keeper », qui est connu comme un régulateur de sécrétion de protéines sécrétées par SST3, nous montrons que l'affinité de PscN avec le complexe de l'aiguille est considérablement augmentée en présence de ce régulateur. Ainsi, un nouveau rôle est assigné au complexe « Gate-keeper » dans la régulation de la sécrétion hiérarchique du SST3, en contrôlant le chargement du complexe de l'aiguille sur l'ATPase.

En parallèle, nous avons ainsi l'opportunité de caractériser les effets *ex vivo* et *in vivo* de molécules identifiées dans un criblage antérieur *in vitro* en raison de leur capacité à inhiber l'interaction entre PscE et PscG. Ces deux protéines sont les deux chaperonnes cytoplasmiques de PscF, la protéine de l'aiguille du SST3. Cette interaction est une cible intéressante pour une stratégie anti-virulence car des simples ou doubles mutations ponctuelles dans l'interface d'interaction de PscE-PscG diminuent la virulence de *P. aeruginosa*. Ce travail met en avant les deux meilleures molécules « leads » dont la structure est très proche et qui appartiennent à un cluster hybride combinant les hits de deux bibliothèques de molécules. Ces deux composés inhibent les dommages cellulaires causés par des souches de *P. aeruginosa* possédant le SST3, ne sont pas toxiques pour des cellules eucaryotes et ont un effet minimal sur la croissance, la mobilité et le métabolisme de la bactérie. De plus, ces « leads » peuvent améliorer considérablement la survie de *Galleria mellonella* lors d'infection par *P. aeruginosa*.

Mots clés: *Pseudomonas aeruginosa*, Système de Sécrétion de Type III, ATPase, Anti-virulence