



Type 1 secretion system in *Legionella pneumophila* : substrate localization and role during the infectious cycle

Hussein Kanaan

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Hussein KANAAN

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Système de sécrétion de type 1 chez *Legionella pneumophila* : localisation de son substrat et rôle lors du cycle d'infection

**Type 1 secretion system in *Legionella pneumophila*: substrate localization and
role during the infectious cycle**

Directeur de thèse : Christophe GILBERT

Co-directeur de thèse : Ali CHOKR

Devant le jury composé de :

Dr. Sylvie ELSÉN

Pr. Guillermo MARTINEZ-DE-TAJADA

Pr. Jean-Marc BERJEAUD

Pr. Patricia DOUBLET

Dr. Christophe GILBERT

Pr. Ali CHOKR

CEA-Grenoble

Universidad de Navarra

Université de Poitiers

Université Lyon 1

Université Lyon 1

Université Libanaise

Rapporteure

Rapporteur

Rapporteur

Examinatrice

Directeur de thèse

Co-directeur de thèse

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LIST OF ABBREVIATIONS

- **ABC:** ATP binding cassette
- **ADP:** Adonesine diphosphate
- **ArF:** ADP ribosylation factor
- **ATP:** Adonesine triphosphate
- **AYE:** ACES-buffered yeast extract
- **BAP:** Biofilm associated proteins
- **BCYEA:** Buffered charcoal yeast extract agar
- **BSA:** Bovine serum albumin
- **BTLCp:** bacterial transglutaminase-like cysteine proteinase
- **CDC:** Center for disease control and prevention
- **c-di-GMP:** Bis-(3'-5')-cyclic dimeric guanosine monophosphate
- **CDM:** Chemically defined liquid medium
- **CFU:** Colony forming unit
- **CLD:** C39-like domain
- **COP:** Coat protein
- **CPD:** Cysteine protease domain
- **DC:** District of Columbia
- **DFA:** Direct fluorescent antibody
- **DGC:** Diguanylate cyclases
- **DNA:** Deoxyribonucleic acid
- **Dot:** Defect in organelle trafficking
- **DUF:** Domain of unknown function
- **ECDC:** European center for disease prevention and control
- **ECT:** Electron cryotomography
- **EHEC:** Enterohemorrhagic *Escherichia coli*
- **ELDSNet:** European Legionnaires' disease surveillance network
- **EPEC:** Enteropathogenic *Escherichia coli*
- **EPS:** Exopolysaccharide
- **ER:** Endoplasmic reticulum
- **EWGLI:** European working group for *Legionella* infections
- **GCAT:** Glycerophospholipid cholesterol acyltransferase
- **Gsp:** General secretion pathway
- **GTP:** Guanosine triphosphate
- **HGT:** Horizontal gene transfer
- **Icm:** Intracellular multiplication
- **IFA:** Indirect fluorescent antibody
- **IM:** Inner membrane
- **kb:** Kilobases
- **kDa:** KiloDaltons
- **LAMP:** Lysosomal associated membrane glycoproteins
- **Lcl:** *Legionella* collagen-like
- **LCV:** *Legionella* containing vacuole
- **Lgt:** *Legionella* glucosyltransferase
- **Lp1:** *Legionella pneumophila* serogroup 1
- **LPS:** Lipopolysaccharides
- **Lsp:** *Legionella* secretion pathway
- **Lss:** *Legionella* secretion system
- **Lvh:** *Legionella* VirB homolog
- **MAPK:** Mitogen-activated protein kinase

- **MARTX:** Multifunctional-autoprocessing RTX
- **MFP:** Membrane fusion protein
- **MIP:** Macrophage infectivity potentiator
- **MOMP:** Major outer membrane protein
- **mRNA:** Messenger ribonucleic acid
- **NBD:** Nucleotide binding domain
- **NFκB:** Nuclear factor-κB
- **NO:** Nitric oxide
- **OM:** Outer membrane
- **OMP:** Outer membrane protein
- **OMV:** Outer membrane vesicles
- **PAGE:** Polyacrylamide gel electrophoresis
- **PAL:** Peptidoglycan-associated lipoprotein
- **PAP:** Phosphatidic acid phosphatase
- **PAS:** Per-Arnt-Sim domain
- **PCR:** Polymerase chain reaction
- **PDE:** Phosphodiesterase
- **PI:** Phosphoinositides
- **PI4P:** Phosphoinositol 4-phosphate
- **PlaA:** Phospholipase A
- **PP:** Periplasm
- **ppt:** Pyrimidine phosphoribosyl transferase
- **RNA:** Ribonucleic acid
- **RND:** Resistance Nodulation Division
- **ROS:** Reactive oxygen species
- **RTX:** Repeats in toxin
- **SCV:** *Salmonella* containing vacuole
- **SDS:** Sodium dodecyl sulfate
- **SPI:** *Salmonella* pathogenicity island
- **Spp.:** Species
- **SRP:** Signal recognition particle
- **T1SS:** Type 1 secretion system
- **T2SS:** Type 2 secretion system
- **T3SS:** Type 3 secretion system
- **T4CP:** Type 4 coupling protein
- **T4SS:** Type 4 secretion system
- **T5SS:** Type 5 secretion system
- **T6SS:** Type 6 secretion system
- **T7SS:** Type 7 secretion system
- **T9SS:** Type 9 secretion system
- **Tat:** Twin arginine translocation
- **TEM:** Transfer Electron micrograph
- **TMD:** Transmembrane domain
- **TPS:** Two-partner secretion
- **UPEC:** Uropathogenic *Escherichia coli*
- **vWA:** von Willebrand factor type A
- **WHO:** World health organization

RESUME

Legionella pneumophila est responsable d'une forme de pneumonie, la legionellose ou de maladie du légionnaire. Entre 2012 et 2015, les cas annuels ont grimpé de 5848 à 7069 en Europe, la France, l'Allemagne, l'Italie et l'Espagne correspondant à 69% du total. De façon inquiétante, la mortalité était de 8,2% faisant de cette maladie un réel enjeu de santé publique. Un facteur de virulence produit par cette bactérie est la protéine RtxA (~700 kDa) de la famille des protéines RTX (Repeats in ToXin) sécrétée via un système de sécrétion de type 1.

Dans ce travail, *in vitro*, la protéase périplasmique LapG clive la partie N-terminale de RtxA au sein d'un motif di-alanine (position 108-109). La construction de mutants déficients dans l'expression de LapG et LapD a révélé une localisation de RtxA sous le contrôle de ces deux protéines, mécanisme semblable au modèle LapA décrit chez *P. fluorescens*. Un mutant $\Delta lapG$ maintient RtxA à la surface de cellules, à l'opposé d'un mutant $\Delta lapD$. Nous avons identifié des systèmes homologues T1SS/LapDG dans de nombreuses espèces *Legionella* ainsi que d'autres gammaproteobactéries.

Concernant la virulence de *L. pneumophila*, les mutants déficients pour le T1SS (IssBD/tolC) étaient plus altérés dans leur virulence que des mutants du système LapDG. Nous avons également montré, grâce à des expériences de compétition, que *L. pneumophila* semble cibler les cellules hôtes via la protéine RtxA. L'utilisation d'anticorps spécifiques anti-RtxA nous a permis de détecter RtxA à la surface des cellules hôtes, mais aussi de réduire de la virulence de *L. pneumophila*, suggérant un rôle important de RtxA lors du processus d'infection, bien que non limitant.

Mots clés : *Legionella pneumophila* ; virulence ; système de sécrétion de type 1 ; Protéine RTX.

ABSTRACT

Legionella pneumophila is the causative agent of a form of pneumonia called legionellosis or Legionnaires' disease. Between 2012 and 2015, the reported European cases of legionellosis increased from 5,848 to 7,069 cases per year where France, Germany, Italy and Spain accounted for 69% of the reported cases. Worryingly, the case fatality of incidents was 8.2% making this disease a considerable health concern. One virulence factor produced by this bacterium is a large protein (~700 kDa) belonging to the RTX (Repeats in ToXin) family called RtxA secreted by the type 1 secretion system.

The hereby work reveals that, *in vitro*, LapG periplasmic protease cleaves RtxA N-terminus in the middle of a di-alanine motif (a.a. 108-109). We also show using *lapG* and *lapD* mutant strains, that RtxA release is controlled by these two proteins similar to *Pseudomonas fluorescences* LapA. We observed that a strain lacking LapG protease maintains RtxA on the cell surface, while a strain lacking LapD does not exhibit cell surface RtxA. Interestingly, we identified the presence of homologous potential T1SS/LapDG systems in many *Legionella* species and other Gammaproteobacteria.

Regarding *L. pneumophila* virulence, our work showed that mutants for *L. pneumophila* T1SS (*lssBD/tolC*) were more disruptive to its virulence than *lapG/lapD* mutants. We also hypothesize, by challenging infection, that *L. pneumophila* might be actively targeting its host via RtxA. Additionally, by observing *rtxA* mutants as well as detecting RtxA on host surface briefly after inoculation and attenuating virulence by using anti RtxA antibodies, we assume an important but not limiting role for this protein in the infection process.

Key words: *Legionella pneumophila*; virulence; type 1 secretion system; RTX protein.

INTRODUCTION

A. Legionnaires' disease

By definition, legionellosis or Legionnaires' disease is a form of severe pneumonia that was first identified in the early 1977 (Campese *et al.*, 2015, Cunha *et al.*, 2016). The microorganism behind this outbreak was unknown at the time and was hence named *Legionella pneumophila* (Cunha *et al.*, 2016). Today, the original strain isolated during this outbreak is known as *Legionella pneumophila* subsp. *pneumophila* strain Philadelphia 1.

1. Brief history

The first reported incident of this disease where the causative agent was identified took place in Philadelphia, U.S.A where a major pneumonia outbreak occurred after the annual meeting of the American Legion in 1976 which coined the term "Legionnaires' disease". This meeting was held at the Bellevue-Stratford hotel shown in Figure 1 (Fraser, 2005, Cianciotto, 2009, Cunha *et al.*, 2016). However, incidents of pneumonia had occurred prior to the previously mentioned outbreak of 1976, some of which were the outbreak of 78 cases in 1957 in Austin, Minnesota and that of 81 cases at the St. Elizabeth's hospital in Washington, DC in 1965. Another widely known but also unsolved outbreak occurred during the year 1968 in a health department building in Pontiac, Michigan. In this outbreak, the majority of visitors developed symptoms of an influenza like illness, but interestingly, it occurred only among those present when the air conditioning evaporative condenser was operating. Subsequent investigations failed to reveal any responsible agent(s). On the other hand, exposure of guinea pigs to water aerosols from the same evaporative condenser caused development of pneumonia, while the same filtered aerosols failed to produce the same result. Nevertheless, attempts to culture an agent from this water failed as well (Fraser, 2005).



Figure 1: The Bellevue-Stratford hotel, Philadelphia, U.S.A (Boucher, 1976).

Regarding the 1976 outbreak, there were 182 affected patients of which 29 or 16% had died (Fraser, 2005, Cunha *et al.*, 2016). During the onset of the outbreak, swine flu was thought to be the cause but

was ruled out later alongside the suspicion of easily grown bacteria. Therefore, the difficulties in uncovering the causative agent contributed to the spread of the disease where regular culturing methods did not reveal the true culprit. Further testing was performed by Joseph McDade, a rickettsia specialist, where he observed clusters of organisms in livers of guinea pigs inoculated with Legionnaires' disease material. He proceeded to isolate these organisms as he would for rickettsia and prepared reagents for direct and indirect fluorescent antibody tests (DFA/IFA). Subsequent DFA and IFA testing on specimens from the Philadelphia outbreak revealed a bacterium that they hence named *Legionella pneumophila*. This discovery also helped solve the earlier outbreaks mentioned above (Fraser, 2005).

2. Clinical symptoms/manifestations

Legionnaires' disease is not always easy to diagnose due to its non-specific symptoms. Generally, the incubation period ranges between 2 to 14 days and the general symptoms include headaches, myalgia, asthenia and anorexia, in addition to digestive disorders such as diarrhea, nausea and vomiting (Sabria & Yu, 2002, Campese *et al.*, 2015, Cunha *et al.*, 2016). Fever at 39-40°C is almost always present, followed by chills, cough, dyspnea and possible neurological abnormalities, all symptoms similar to a pneumococcal pneumonia (Fields *et al.*, 2002, Cunha *et al.*, 2016).

Legionellosis manifestations mainly affects susceptible patients due to age, previous conditions or immunosuppression. Recovery can be slow and patients are susceptible to relapse, especially those suffering from immunosuppression where also the mortality rate will be higher than immuno-competent individuals (Cunha *et al.*, 2016). Chronic lung disease, smoking and age exceeding 50 years are common risk factors for Legionnaires' disease. Receipt of an organ transplant also constitutes a major risk factor for acquiring this disease (Sabria & Yu, 2002, Cunha *et al.*, 2016).

Pontiac fever is generally a less severe form of Legionnaires' disease with similar symptoms, but a real characterization of Pontiac fever is yet to be determined since its pathogenesis is still obscure (Cunha *et al.*, 2016).

3. Reservoir and transmission

Legionella withstands a maximum temperature of 50°C for a few hours but generally cannot multiply at temperatures below 20°C (Cunha *et al.*, 2016). However, *Legionella* can survive in temperatures as low as 4°C (Paszko-Kolva *et al.*, 1993). Although some *Legionella* spp. (*pneumophila* and *longbeachae*) are indirect human pathogens, they are ubiquitous in aquatic environments, water distribution systems and even in soil where they survive as intracellular parasites of amoebae and protozoa, their natural hosts as represented in Figure 2 (Cunha *et al.*, 2016). Moreover, the control of biofilms containing

Legionella is an important measure against their eradication since they prove harder to eliminate once established in biofilms (Campese *et al.*, 2015, Cunha *et al.*, 2016).

Regarding the transmission of *L. pneumophila*, the main mode is through inhalation of infected aerosols. The major mechanisms behind this are man-made systems such as water-cooling towers, artificial reservoirs, domestic plumbing equipment, thermal spas and other industrial equipment corresponding to the majority of community acquired legionellosis (Campese *et al.*, 2015, Cunha *et al.*, 2016). Environmental factors related to weather conditions such as rain fall, humidity and temperature were also associated with the incidence of Legionnaires' disease (Campese *et al.*, 2015). Furthermore, potting soil can be a medium of transmission, especially for a species called *Legionella longbeachae* where not washing hands after gardening can lead to the uptake of the organisms and subsequently contracting the disease. Instances of this mode of transmission are more frequent in Australia and New Zealand (Campese *et al.*, 2015, Cunha *et al.*, 2016, Kenagy *et al.*, 2017).

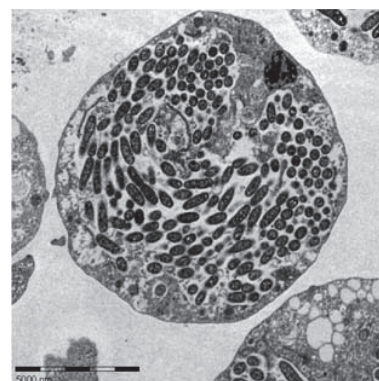


Figure 2: *Hartmanella vermiformis* amoeba infected with *Legionella pneumophila* (Holland/Ozel).

Hospital acquired legionellosis have also been recorded frequently, it is linked to the presence of *Legionella* in the water supply where aspiration and use of aerosol generating devices within hospitals are common modes of transmission (Sabria & Yu, 2002, Cunha *et al.*, 2016). Disturbingly, a study on the water supplies of 20 hospitals in Catalonia (northeast Spain) revealed the presence of *L. pneumophila* in 17 (85%) of these hospitals which can possibly be the case in other hospitals (Sabria *et al.*, 2001). This can happen despite the appropriate maintenance of water distribution systems in hospitals since it helps in controlling *Legionella* growth but has little effect on colonization (Sabria & Yu, 2002).

4. Pathogenesis

In this part, the intracellular life cycle of *Legionella* will not be addressed as it will be discussed in detail later on. However, it is important to emphasize that *L. pneumophila* is an opportunistic and accidental pathogen of humans. It replicates in the human eukaryotic cell in a manner similar to that in amoebae even though human cells are not its main host (Campese *et al.*, 2015, Cunha *et al.*, 2016).

The *Legionella* genus comprises around 60 species and 70 serogroups where 30 species are documented to be pathogenic for humans (Campese *et al.*, 2015). *L. pneumophila* serogroup 1 (Lp1) is the most virulent species and a major cause of human disease, virulence can even vary within the different strains of the same species. *Legionella* virulence factors are diverse, some are involved in the early stages of the infection cycle and more precisely in adhesion and entry into the host. These include the flagellum,

pili and bacterial surface proteins. *L. pneumophila*, *L. longbeachae*, *L. anisa* and several others can survive as intracellular pathogens by avoiding phagosome-lysosome fusion after their internalization and this brings us to the most crucial virulence factor of *Legionella* which is the Type IV-B Dot/Icm (Defective for organelle trafficking/Intra cellular multiplication) secretion system (T4SS). This secretion system can transport approximately 300 effector proteins from the internalized bacterium to the host cytoplasm, these effectors disrupt and hijack many host processes such as the lysosomal fusion mentioned earlier and consequently create a specialized niche for replication in the phagosome called the *Legionella* containing vacuole (LCV). These effectors can also modulate the host anti-apoptotic pathway and disrupt the phagosomal and host cell membranes to escape into the extracellular environment. Other virulence factors include cytotoxins (Lgt: a family of glucosyltransferases), heat shock proteins, lipopolysaccharides (LPS), phospholipases, metalloproteases, β -lactamases and other virulence factors (Campese *et al.*, 2015, Cunha *et al.*, 2016).

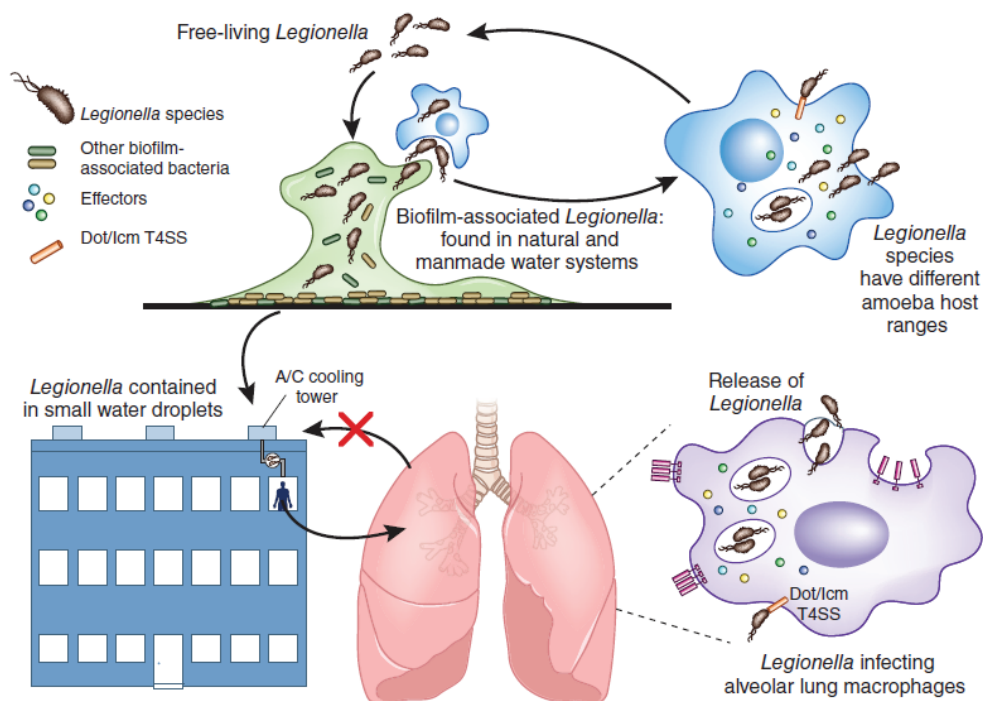


Figure 3: The *Legionella* life cycle Most *Legionella* species are able to persist in their natural hosts, amoebae or other water protozoa and are also capable of surviving in multispecies biofilms. *Legionella* uses effector proteins secreted by its T4SS to infect and survive within amoebae or human alveolar macrophages. Spread of *Legionella* is facilitated by man-made system such as cooling towers where they are dispersed inside contaminated water droplets. From (Comas, 2016).

As displayed in Figure 3 above, *L. pneumophila* can be considered as an accidental human pathogen since its delivery to humans is almost entirely dependent on man-made systems (Abdelhady & Garduno, 2013, Comas, 2016). It was also shown that *L. pneumophila* cells that emerge from macrophages are less able to initiate infections and also less antibiotic resistant compared to *Legionella* cells emerging

from amoebae which supports the previous statement that *L. pneumophila* is an accidental human pathogen (Abdelhady & Garduno, 2013). The authors also proposed that this fact could explain the non-communicable state of legionellosis. However, a recent publication includes evidence of a first person-to-person transmission and this must be taken into consideration in the future regarding modes of transmission of *L. pneumophila* (Borges *et al.*, 2016).

B. *Legionella pneumophila*

Legionella spp. are Gram-negative bacilli ranging in size between 2 to 20 μm (Figure 5), they are generally described as fastidious which can be ironic since they inhabit very hostile environments such as water plumbing systems and artificial reservoirs, but it is meant in the sense that under laboratory conditions, this bacterium is dependent on specific growth requirement factors not strictly required by other bacteria such as L-cysteine and iron (Winn, 1996).

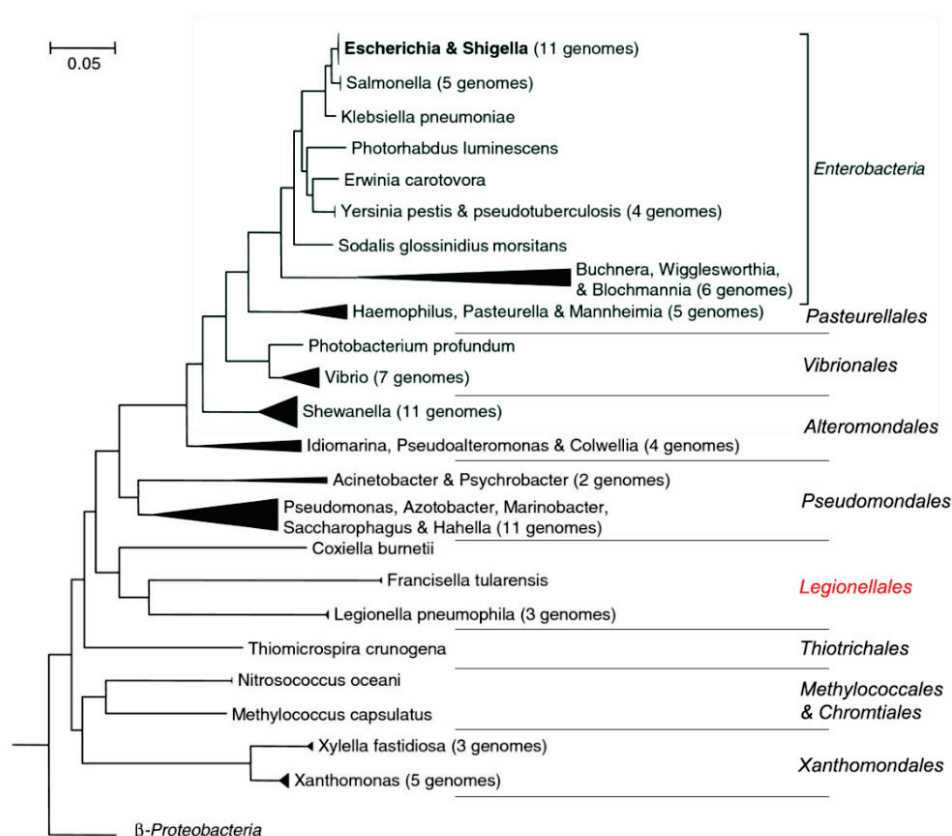


Figure 4: Phylogeny of the γ -Proteobacteria This phylogenetic tree was generated from concatenated alignments of highly conserved proteins. The β -Proteobacteria is a sister group of the γ -Proteobacteria. The scale bar signifies 5% amino acid divergence. Adapted from (Price *et al.*, 2008).

L. pneumophila belongs to the *Legionellales* order of the γ -Proteobacteria class as shown in Figure 4. *L. pneumophila* is also phylogenetically related to *Coxiella burnetti*, they are both intracellular pathogens that use similar virulence mechanisms to manipulate their hosts and can cause lung infections in humans (Sauer *et al.*, 2005, Qiu & Luo, 2017).



Figure 5: Transmission electron micrograph of *L. pneumophila* (Science, 2016)

Legionella can grow only on specific culture media such as the buffered charcoal yeast extract agar (BCYEA) and in ACES-buffered yeast extract (AYE) in addition to chemically defined liquid medium (CDM) since it allows for the simple control of nutrients concentration (Chatfield & Cianciotto, 2013). It can be detected after 3 to 5 days of incubation; young colonies are 0.5-1 mm in diameter. In case a bacterium is suspected to be *Legionella* it must be Gram stained and plated onto two different media in the presence and absence of L-cysteine to prove its dependency on this amino acid (Cunha *et al.*, 2016). They are obligate aerobes where they derive their energy from the metabolism of amino acids and not carbohydrates. *Legionellae* developed several methods to acquire iron from their host cells or *in vitro* media which is important for their survival (Murray *et al.*, 2016).

When in replicative phase, *L. pneumophila* are avirulent, sodium resistant and non-flagellated. However, transmissive phase bacteria are virulent, flagellated and highly motile (Molofsky & Swanson, 2004). The switch from replicative to transmissive phase is initiated by amino acid and/or fatty acid starvation at the end of the infectious cycle allowing the transmission to new hosts, this happens due to the depletion of host resources (Byrne & Swanson, 1998).

The *Legionella* intracellular life cycle in protozoa or monocytes is relatively similar and consists of several stages starting with entry, intracellular survival and replication and finally lysing the host and exiting to the extracellular environment. Figure 6 illustrates these steps.

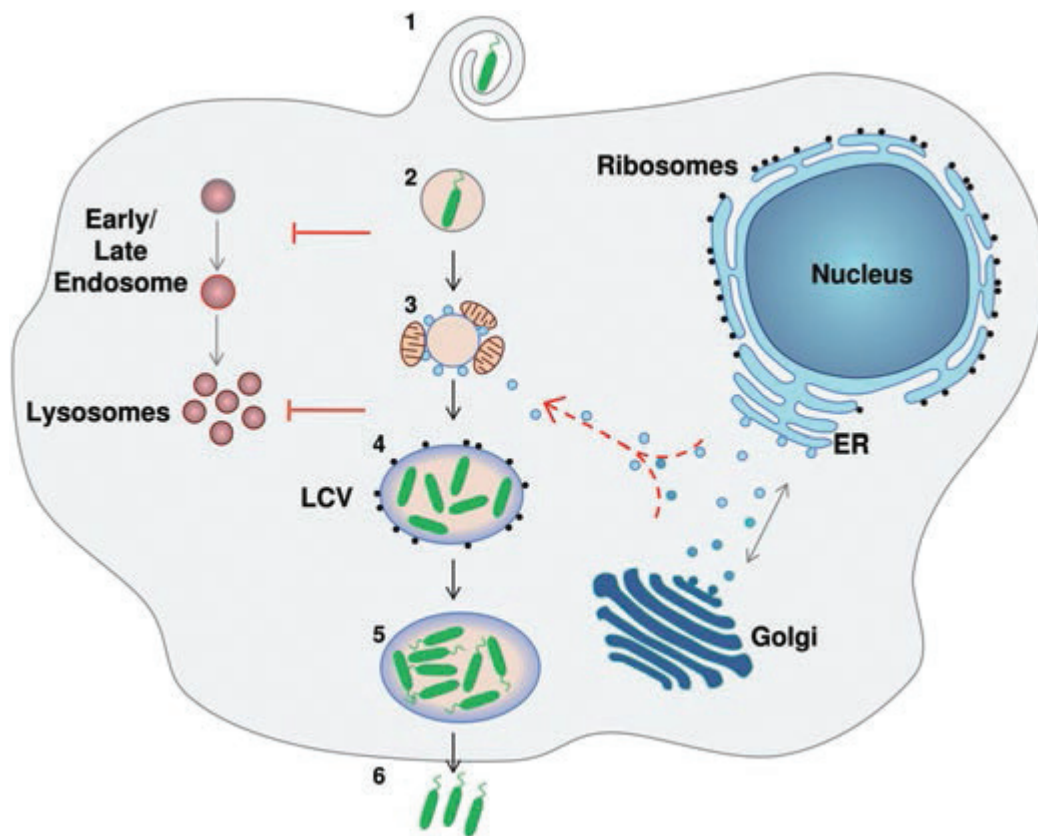


Figure 6: Intracellular lifecycle of *L. pneumophila* (1) through coiling phagocytosis, the host cell is able to internalize *L. pneumophila*. (2) After uptake, the bacterium will be situated in a LCV that will evade fusion with endosomes and later delivery to lysosomes. (3) during the first hour after uptake, the LCV will recruit mitochondria and disrupt the secretory pathway by also recruiting endoplasmic reticulum (ER) derived vesicles to the LCV instead of the Golgi apparatus. (4) the mature LCV will recruit ribosomes that will facilitate the replication of its bacteria. (5) in this vacuole, the bacteria will undergo several rounds of replication and ultimately become virulent and flagellated. (6) The LCV will burst and that will be followed by lysis of the host and release of the bacteria to repeat the cycle again in neighbouring cells. Adapted from (Franco *et al.*, 2009).

1. Entry into the host cell

As mentioned earlier, the natural hosts for *L. pneumophila* are free-living protozoa and more specifically amoebae with the human alveolar macrophages being an accidental host for this microorganism. It has also been found that *L. pneumophila* can enter and survive within different mammalian cell types (epithelial cells and fibroblasts) (Samrakandi *et al.*, 2002). This process comprises a series of steps that will be discussed below.

a) Finding an appropriate host

Establishing close proximity between *Legionella* and its host is the first step of entry and the infectious cycle, that is achieved through motility and with a possible implication of chemotaxis by the bacteria and/or its host cells. Evidence for chemo-attractants has not been established so far but *L. pneumophila* motility has been addressed on many occasions.

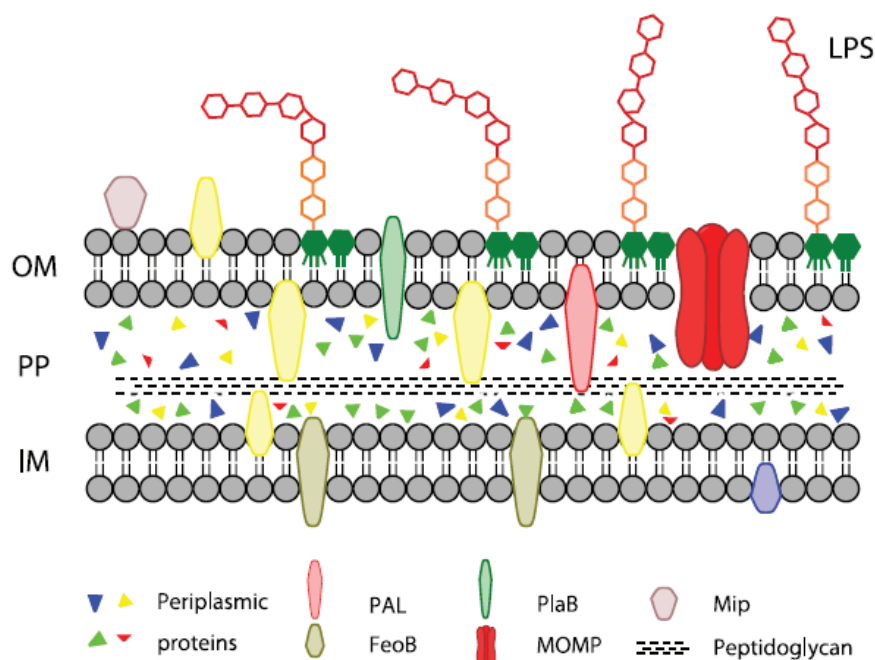


Figure 7: Representation of the *L. pneumophila* cell envelope IM, inner membrane; PP, periplasm; OM, outer membrane; LPS, lipopolysaccharides; PAL, peptidoglycan-associated lipoprotein; FeoB, iron transporter; PlaB, phospholipase A/lysophospholipase A; MOMP, major outer membrane protein; MIP, macrophage infectivity potentiator. From (Shevchuk *et al.*, 2011).

Regarding motility, the *L. pneumophila* flagellum is 14-20 nm in diameter, primarily single, subpolar and gently curved. The expression of flagella is affected by the growth phase, nutrients, temperature and viscosity. The latter can be correlated to the fact that *L. pneumophila* has to find its host in the mucous of mammalian lungs. Moreover, the observation of flagella during infection in human lungs hints at their possible role in pathogenesis (Samrakandi *et al.*, 2002). A study on *L. pneumophila* with mutated *flaA* gene showed a reduced ability to infect amoebae and macrophages (Dietrich *et al.*, 2001). On the contrary, another study found that flagella may not be as crucial for intracellular replication. A mutation in the *L. pneumophila* *fliI* gene that affects flagella secretion, does not reduce the ability to replicate intracellularly in macrophages (Merriam *et al.*, 1997). However, this study does not consider the potential implication of flagella in entry into the host. Importance of motility can be observed in aquatic habitats where host cells are less abundant.

b) Attachment to host cells

Regarding this matter, it is not easy to distinguish the adherence of *Legionella* to its host apart from phagocytosis events. Several methods have been applied to try to separate these two events but many problems with these techniques prevented a clear result from being obtained (Samrakandi *et al.*, 2002).

Among many factors affecting adherence in *L. pneumophila*, the first identified protein with such role is the major outer membrane protein (MOMP) shown in Figure 7 above (Krinos *et al.*, 1999, Samrakandi *et al.*, 2002). It is also the most abundant outer membrane protein with a subunit size of approximately 29 to 30 kDa. MOMP is present in all *Legionella* species (Samrakandi *et al.*, 2002). It has been shown to play a role in attachment to host cells (Samrakandi *et al.*, 2002, Shevchuk *et al.*, 2011), where the use of anti-MOMP antibodies can reduce or even abolish adherence and virulence (Krinos *et al.*, 1999, Samrakandi *et al.*, 2002) which hints at the adhesive role of this protein. It is also worthy to note that adhesins can be classified as afimbrial such as the case with MOMP and fimbrial such as pili. *L. pneumophila* has several genes related to pili production, one of which is the *pilE* gene. Although *pilE* mutants exhibit wild-type intracellular replication, adherence to the host is greatly reduced (Samrakandi *et al.*, 2002). In addition to the previous, a protein called Legionella collagen-like (Lcl) protein was shown to contribute to adherence and invasion of host cells via its repeat units (Vandersmissen *et al.*, 2010).

c) Entry into the host

L. pneumophila has the ability to infect several cell types and exhibits more than one mechanism of entry which makes it difficult to characterize whether each host requires a specific mode of entry (Samrakandi *et al.*, 2002). Therefore, there is yet a lot to be discovered regarding internalization by eukaryotic cells, more specifically whether this entry is related to targeted pathogen virulence versus being a host-directed response such as the uptake of *L. pneumophila* by amoebae as a food source or via phagocytosis by monocytes following an immune response (Samrakandi *et al.*, 2002, Newton *et al.*, 2010).

Concerning entry into phagocytes, studies have reported two main mechanisms, either by conventional phagocytosis or an atypical coiling phagocytosis displayed in Figure 6 (step 1). The prevalence of these mechanisms in entry is also up to debate since some research indicates that conventional phagocytosis is most common while others support coiling phagocytosis. Macropinocytosis has also been observed for bone marrow derived macrophages (Samrakandi *et al.*, 2002, Newton *et al.*, 2010).

Regarding interaction with monocytes, several studies confirmed that complement receptors were playing a role in entry of *L. pneumophila*, these interactions being most efficient when *L. pneumophila*

is also interacting with Fc receptors. Probably the MOMP mentioned earlier will interact to complement while Fc interactions are mediated through anti-LPS antibodies (Samrakandi *et al.*, 2002). However, opsonin independent entry has been observed in macrophages and that can be correlated to the fact that complement levels in the lungs are generally low and that *L. pneumophila* can also invade non-phagocytic cells such as epithelial cells that do not express high levels of complement or Fc receptors. This supports the hypothesis of the virulence directed invasion of *L. pneumophila* (Samrakandi *et al.*, 2002, Newton *et al.*, 2010).

To expand briefly on non-opsonic uptake, several bacterial factors implicated in host invasion were characterized: EnhC, LpnE, RtxA, LvhB2 and HtpB. EnhC for example, is a periplasmic protein that maintains the integrity of the cell wall and therefore probably contributes indirectly to invasion. HtpB also known as HSP60 (heat shock protein 60) is a surface located chaperonin, it was demonstrated to have a role in entry as well as early LCV development by associating with mitochondria following invasion (Samrakandi *et al.*, 2002, Newton *et al.*, 2010, Zhan *et al.*, 2015). RtxA will be addressed in detail in coming chapters, briefly it is a large protein that belongs to Repeats in Toxin family and it harbors several repeats and domains that may have a role mainly in adherence to host membranes (D'Auria *et al.*, 2008). Deletions of *enhC* and *rtxA* genes reduced entry of *L. pneumophila* into epithelial cells and monocytes by 50%. Furthermore, RTX proteins showed an ability to bind $\beta 2$ integrins which hints at entry being a dual mechanism between bacterium and host (Samrakandi *et al.*, 2002). Another study on *L. pneumophila* RtxA also hints at a role in adhesion and entry in amoebae as well as a possible role in intracellular survival and trafficking (Cirillo *et al.*, 2002). Moreover, disrupting the secretory apparatus of RtxA in *L. pneumophila* leads to attenuated virulence of these mutants (Fuche *et al.*, 2015).

2. Intracellular survival

As previously mentioned, when microorganisms are engulfed by phagocytes, they are eliminated after being delivered to the lysosomal system, where bacteria unspecialized for intracellular life are digested in the phagolysosome which comprises an acidic environment that harbors various activated hydrolytic enzymes. Whereas in the case of *L. pneumophila*, lysosomal fusion is evaded, and recruitment of various host components takes place to the LCV.

The success of *L. pneumophila* as an intracellular pathogen and its ability to avoid host defense mechanisms is almost entirely dependent upon the Dot/Icm Type IV secretion system which spans both bacterial membranes as well as the phagosomal membranes to inject effector proteins directly into the host cytoplasm. It is essential for the virulence of *L. pneumophila* (Xu & Luo, 2013). Development of sensitive protein translocation assays as well as various genetic and bioinformatic methods uncovered a very large number of effector proteins that are transported by the Dot/Icm system (Luo & Isberg, 2004,

Zusman *et al.*, 2008, Heidtman *et al.*, 2009, Huang *et al.*, 2011, Zhu *et al.*, 2011, Xu & Luo, 2013). So far there are around 275~300 effectors where most are still hypothetical proteins with no obvious homology to proteins of known function, however some do form distinct families with homologous members. It is also interesting that a deletion of a single effector gene rarely impairs intracellular growth, hinting at a probable functional redundancy among these proteins (Xu & Luo, 2013).

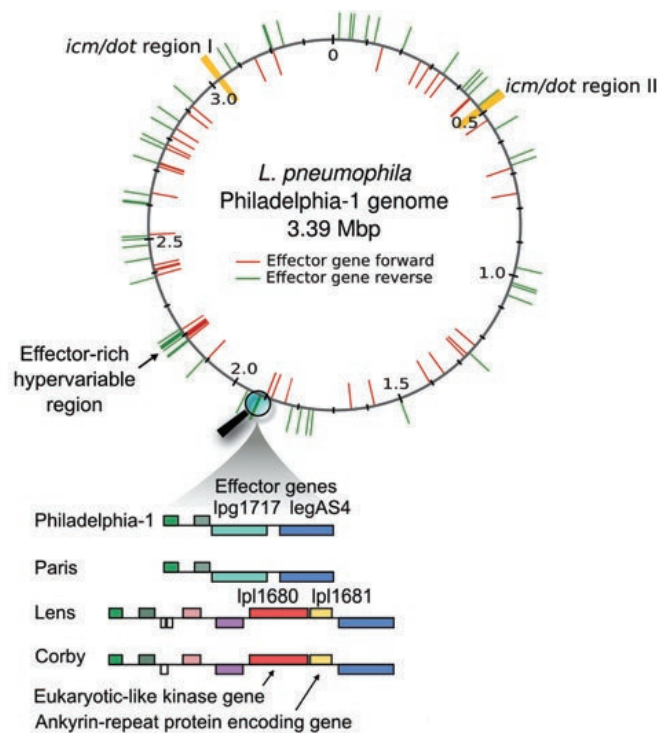


Figure 8: Representation of the *L. pneumophila* Philadelphia-1 effectors location Dot/Icm effector genes are distributed throughout the genome on the chromosome with some effector rich regions. The closeup shows a variable effector containing region of the indicated *L. pneumophila* strains. The effector gene *lpg1717* is present in Philadelphia-1 and Paris but absent in Lens and Corby. There it is replaced with two eukaryotic like protein encoding genes (*lpl1680* and *lpl1681*). Their encoded proteins were found to be Dot/Icm substrates. Adapted from (Franco *et al.*, 2009).

Figure 8 Above shows that the effector genes of *L. pneumophila* are widely distributed throughout the genome with no apparent genetic organization. However, there is a region that is enriched in effector genes. The 25 *dot/icm* genes are located on two loci indicated in Figure 8, these genes are strictly conserved in the *L. pneumophila* strains mentioned above. Closely related genes were also identified in the intracellular pathogens *Coxiella burnetii* and *Rickettsiella gyrali* (Franco *et al.*, 2009).

There are various ways in which these effectors manipulate host processes, including the following examples.

a) Interference with the endocytic pathway

By default, invading microorganisms or any foreign particles are eliminated from professional phagocytes by being engulfed into phagosomes. This phagosome eventually matures into a digestive vacuole via the endocytic pathway. The progression of interaction of this phagosome with the endosomal network leads to the acidification and degradation of its contents (Newton *et al.*, 2010, Xu & Luo, 2013). The phagolysosome is characterized by its low pH, presence of hydrolytic enzymes, reactive oxygen species (ROS) and bactericidal peptides. This acidification is essential for the proper function of the components of the phagolysosome, this is controlled mainly by the vacuolar-ATPase (v-ATPase) machinery which is a proton pump driven by ATP (Adenosine triphosphate) hydrolysis (Xu & Luo, 2013).

Under normal circumstances, the phagosome acquires early endosome markers such as Rab5 which belongs to a family of small GTPases (Guanosine triphosphate) specialized in vesicle trafficking. This is followed by the recruitment of Rab7 and lysosomal associated membrane glycoproteins (LAMPs) that constitute the late endosomal markers, which eventually promotes phagolysosomal fusion. However, in the case of *L. pneumophila*, this fusion is delayed allowing the bacteria to persist within this phagosome for extended periods of time (Hubber & Roy, 2010, Newton *et al.*, 2010, Xu & Luo, 2013). Endocytic markers mentioned earlier such as Rab5, Rab7 and LAMP-1 are absent from LCV surface until 18 to 24 hours after formation. Oppositely, Dot/Icm *Legionella* mutant ($\Delta dotA$) vacuole acquires these host proteins within minutes of uptake. Therefore, the evasion mechanism implicated in LCV formation is concerted by the T4SS effectors (Hubber & Roy, 2010, Newton *et al.*, 2010). In consequence, *L. pneumophila* is able to maintain a neutral pH in the phagosome for at least 6 hours whereas vacuoles with non-pathogenic bacteria become acidified within 15 minutes of their formation (Xu & Luo, 2013).

Several Dot/Icm effectors that target the endocytic pathway were identified. VipA, VipD and VipF are able to interfere with lysosomal protein trafficking. Despite the fact that *L. pneumophila* avoids fusion of its vacuole with the lysosome, it was found that the v-ATPase is indeed present on the membrane of the LCV. This suggests that *L. pneumophila* can antagonize the v-ATPase activity. SidK which is a substrate of the Dot/Icm is able to bind VatA, the catalytic subunit of the v-ATPase leading to arrest of proton translocation (Xu & Luo, 2013). Studies also implicate the LPS vesicles targeted to the LCV membrane in delaying the fusion of endosomal vacuoles with lysosomes thereby enhancing the ability to avoid lysosomal fusion (Hubber & Roy, 2010, Xu & Luo, 2013). In addition to the previous, *L. pneumophila* also relies on recruiting host cell Rab1 to delay acidification of the LCV where knockdown of Rab1 is associated with greater acidification and accumulation of the late endosomal marker LAMP-1 (Misch, 2016).

b) Hijacking the secretory pathway

Although the previous step is crucial for intracellular survival, *L. pneumophila* also relies in its intracellular cycle on creating an organelle permissive for bacterial replication, the LCV, where remodeling of this vacuole by the host secretory pathway is required for the progression of the bacterial life cycle (Hubber & Roy, 2010).

Briefly, in eukaryotic cells, the secretory pathway is defined as the transport of ER (endoplasmic reticulum) synthesized proteins to the Golgi complex and then downstream to cellular destinations or the extracellular environment. In general, trafficking is established through several types of coated vesicles such as the COPII responsible for transporting newly synthesized peptides from the ER to the Golgi apparatus. At the *trans*-Golgi network, the proteins will be sorted to their final destinations via clathrin coated vesicles; retrograde transport towards the ER is mediated by COPI vesicles (Xu & Luo, 2013). *L. pneumophila* is able to intercept and hijack vesicle trafficking between the ER and the Golgi apparatus, this serves to facilitate the conversion of the LCV plasma membrane into a membrane with ER characteristics (Hubber & Roy, 2010, Xu & Luo, 2013). This is corroborated by studies showing that the inhibition of ER-Golgi trafficking will consistently block the development of the LCV (Kagan & Roy, 2002). It is also notable that these cell biological modifications of the LCV require a functional Dot/Icm system (Kagan *et al.*, 2004).

L. pneumophila manipulates this pathway by recruiting specific regulators of vesicle trafficking such as members of the Arf (ADP-ribosylation factor), Rab and Sar families of small GTPases in addition to controlling GTP cycling (Newton *et al.*, 2010, Xu & Luo, 2013). For example, Arf1 and Rab1 will be recruited to and activated on the LCV as shown in Figure 9. Arf1 regulates COPI-coated retrograde trafficking, it is recruited and enriched on the LCV membrane in a Dot/Icm dependent manner through the bacterial effector protein RalF. Rab1 promotes the fusion of ER-derived vesicles with Golgi compartments, similar to Arf1 it is recruited to the LCV in a Dot/Icm dependent manner; the Dot/Icm effector responsible for Rab1 recruitment is SidM/DrrA. Rab1 recruitment contributes to the fusion of ER-derived vesicles to the LCVs (Hubber & Roy, 2010, Newton *et al.*, 2010, Xu & Luo, 2013).

c) Modulation of host ubiquitin pathways

Ubiquitination is a post-translational modification that has several functions, it regulates the activity, half-life and localization of various proteins. It is a eukaryotic exclusive process involved in many cellular mechanisms such as proteasomal degradation, signaling cascade and DNA repair (Kerscher *et al.*, 2006). Due to the importance of this process as well its implication in the host's immune system, pathogens developed the ability to hijack this process to facilitate colonization, mainly by effectors that mimic the host ubiquitin ligase (E3 ligase). In *L. pneumophila* this can be demonstrated by enrichment

of ubiquitinated proteins on its phagosome seen in Figure 9. One effector active in this domain is LubX that shows clear mimicry to host E3 ligase (Hubber & Roy, 2010, Xu & Luo, 2013).

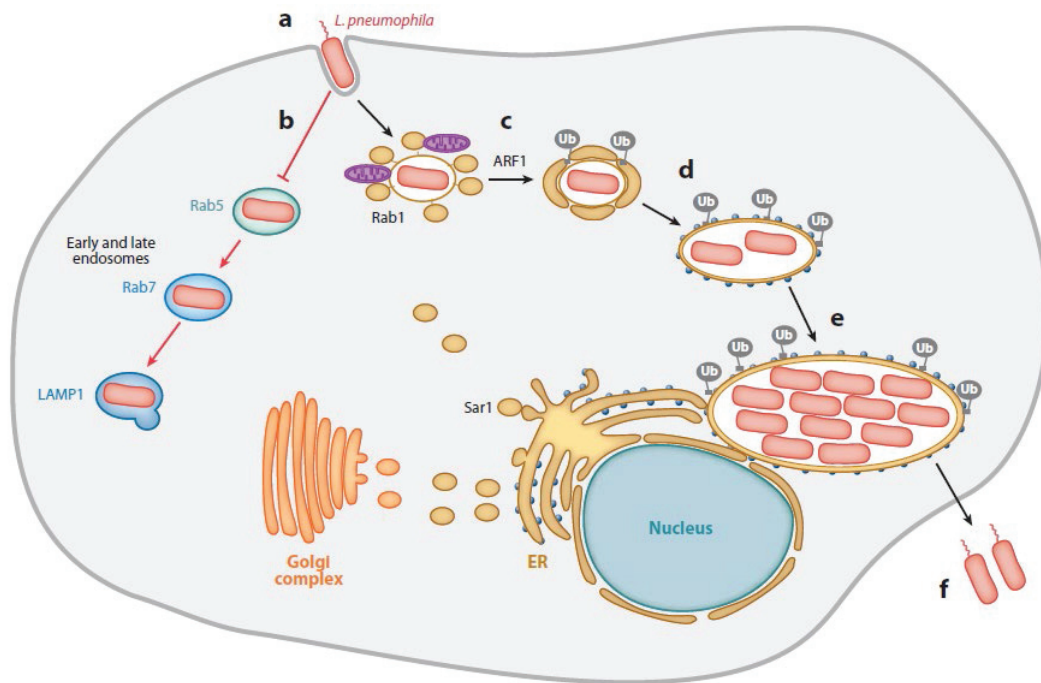


Figure 9: *L. pneumophila* modulation of host cell trafficking (a) First step is uptake of *L. pneumophila* by its eukaryotic host. (b) The normal endocytic pathway is blocked demonstrated here by red inhibition line and arrows. (c) The *L. pneumophila* containing vacuole will hijack host factors Rab1 and Sar1 to facilitate fusion of ER-derived vesicles with the LCV. (d) The remodeling continues as host Arf1 is implicated in fusion of ER membranes and proteins into the lumen of the LCV. Ubiquitinated (Ub) proteins localize to the LCV 1 hour after infection to drive further LCV development. (e) LCV will resemble the rough endoplasmic reticulum and provide a niche for extensive bacterial replication. (f) Finally, the *Legionellae* will be released allowing further infection cycles. Adapted from (Hubber & Roy, 2010).

d) Interference with host cell death pathways

Programmed cell death or apoptosis is a prevalent mechanism in multicellular organisms and is crucial for various cellular events. One mechanism of interest that utilizes apoptosis is the defense against infection, where elimination of the replication niche constituted by the host cell itself via apoptosis will hinder the bacterial infection process. Therefore, many intracellular pathogens have developed mechanisms to suppress the apoptotic process in order to further progress their intracellular growth (Xu & Luo, 2013).

In an interesting contradiction, challenge of macrophages with *L. pneumophila* was found to activate caspase-3, the executioner caspase (Newton *et al.*, 2010, Xu & Luo, 2013). Caspase-3 usually promotes

apoptosis through catalyzing the cleavage of key cell survival and maintenance proteins (Seervi & Xue, 2015). Despite activating caspase-3, *L. pneumophila* inhibits apoptosis in macrophages until the later stages of infection. It might be that *L. pneumophila* benefits from several other functions of caspase-3, such as inhibiting phagolysosomal fusion and other involvements in vesicle trafficking (Newton *et al.*, 2010, Xu & Luo, 2013).

The *L. pneumophila* Dot/Icm transport system is also implicated here through delivery of effectors with anti-apoptotic properties. For example, SidF inhibits apoptosis by direct interactions with pro-apoptotic members of the Bcl2 family. Inhibition of apoptosis is also correlated to the induction of expression of host genes that promote cell survival, such induction is achieved by activation of NFκB (nuclear factor κB) which is the major the regulator of cell survival and immune response (Newton *et al.*, 2010, Xu & Luo, 2013).

e) Exploitation of host lipid metabolism

It is well established that lipids play essential roles in many cellular processes such as signaling, vesicle trafficking and organelle definition. Of particular interest are a group of small lipids called phosphoinositides (PIs), they are critical in various cellular processes such as defining intracellular organelle identity, cell signaling, proliferation and membrane trafficking. An important function of these PIs that *L. pneumophila* takes advantage of is defining different cellular compartments, for example, phosphoinositol 4-phosphate (PI4P) is abundant in the Golgi apparatus (Hubber & Roy, 2010, Seervi & Xue, 2015). *L. pneumophila* Dot/Icm effectors SidC, SdcA and SiDm/DrrA are capable of binding PI4P. Moreover, these proteins exist in abundant concentration on the LCV surface suggesting an enrichment of PI4P on its membrane. This may allow the LCV membrane to mimic the *cis*-Golgi compartment, facilitating the fusion with ER-derived vesicles (Hubber & Roy, 2010, Xu & Luo, 2013).

3. Origin of *L. pneumophila* eukaryotic effectors

After discussing the importance of *L. pneumophila* effectors in its intracellular life cycle and the ability of these proteins to interact and mimic eukaryotic host proteins, it is natural to investigate their origin and how *L. pneumophila* was able to acquire and evolve this large arsenal of effectors.

Considering the fact that *L. pneumophila* is mainly a protozoal intracellular pathogen, the co-evolution of *L. pneumophila* with fresh water amoebae is reflected in its genome sequence (Gomez-Valero *et al.*, 2011a, Gomez-Valero *et al.*, 2011b, Best & Abu Kwaik, 2018). The analysis of *L. pneumophila* genomes revealed a very large number of eukaryotic-like proteins and proteins containing motifs mainly found in eukaryotes (Gomez-Valero *et al.*, 2011b). Many of these genes are virulence factors used by *L.*

pneumophila to subvert host functions and allow intracellular replication (Gomez-Valero *et al.*, 2011a, Gomez-Valero *et al.*, 2011b).

Acquisition of these genes may have taken place either via horizontal gene transfer (HGT) from host cells or from other bacteria or may have evolved by convergent evolution (Gomez-Valero *et al.*, 2011a, Best & Abu Kwaik, 2018). HGT within the genus *Legionella* and within the species *L. pneumophila* has also been reported, both instances are portrayed in Figure 10. Moreover, *L. pneumophila* has all the necessary features for incorporating foreign DNA, since they are naturally competent and possess an intact recombination machinery (Stone & Kwaik, 1999). Their genomes are very dynamic therefore HGT and recombination events would play an important role in their evolution (Gomez-Valero *et al.*, 2011b, Buchrieser & Charpentier, 2013, Khodr *et al.*, 2016). An example of this is the *L. pneumophila* gene *ralF* which was the first gene described to have been acquired from eukaryotes by HGT, since its product RalF carries a eukaryotic Sec7 domain (Gomez-Valero *et al.*, 2011a).

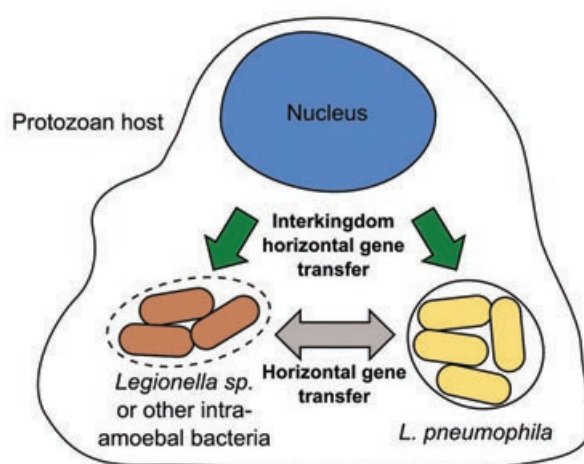


Figure 10: Scenario of acquisition of eukaryotic-like genes by *L. pneumophila* Acquisition of eukaryotic like genes can occur between *L. pneumophila* and its host during an infection event or even among *L. pneumophila* strains or any other intracellular pathogen of amoebae. Adapted from (Franco *et al.*, 2009).

In addition to the previous, the majority of effector genes with eukaryotic-like domains contain G+C biases when compared to other *L. pneumophila* genes which supports the theory of that acquisition occurred via HGT (de Felipe *et al.*, 2005). This is true since GC-biased gene conversion, a process impacted mainly through recombination events, is more prominent in eukaryotes than in bacteria (Lassalle *et al.*, 2015). Acquisition of eukaryotic genes is especially interesting since around 85% of eukaryotic genes have introns. Although that percentage is lower in protozoa, it may not represent a barrier for the acquisition of a coding sequence carrying a eukaryotic domain. For the acquired sequence to become an effector it has to be recognized by the T4SS through a C-terminal signal (Nagai *et al.*,

2005). It is interesting to note that since some acquired coding sequences may still be undergoing evolution, not all eukaryotic-like genes in *L. pneumophila* would become effectors (Franco *et al.*, 2009).

C. Bacterial secretion systems

Secretion is an essential process for all cell types, for this purpose, bacteria have evolved various methods to secrete a wide range of substrates including small molecules, proteins and DNA. These substrates have important roles in the interaction of the bacterium with its environment in addition to several physiological processes such as adhesion, adaptation and survival. In case of pathogenic bacteria, secretion systems constitute a crucial factor for pathogenesis and inter-bacterial competition; more specifically in *L. pneumophila*, the secreted proteins are used to manipulate the host and establish a replicative niche (XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016).

In order to achieve successful substrate transport, the bacterium will have to translocate substrates across its membrane(s) and in some cases across a third membrane, the host cell membrane. The Gram-negative bacterial envelope is composed of two membrane layers and a periplasmic space in between, while for Gram-positive bacteria, the envelope consists of a cytoplasmic membrane followed by a thick peptidoglycan layer. Therefore, bacteria evolved complex machineries to facilitate protein transport (XU & LIU, 2014). Depending on the secretion system, the substrates have three fates: remain associated to the outer membrane (OM), or released into the extracellular space, or injected into a target cell (Gerlach & Hensel, 2007).

In Gram-negative bacteria, transport machinery can either span both the inner membrane (IM) and the OM, or the OM only. These constitute the main groups of Gram-negative bacterial transport systems which are subdivided into seven secretion systems. There are five double membrane spanning systems that have been identified to date and classified as, the type I secretion system (T1SS), T2SS, T3SS, T4SS, T6SS. The T5SS only spans the OM. Finally, Mycobacteria which possess a Gram-negative like cell envelope, encode a T7SS that is mostly restricted to these bacteria and has not been observed elsewhere (Costa *et al.*, 2015). Since 2009 a new secretion system discovered in *Porphyrromonas gingivalis* has been proposed as type IX (Sato *et al.*, 2010, Sato *et al.*, 2013). A T9SS has no significant homologies with known secretion systems is involved in gliding motility and has also been identified in some Gram-negative bacteria (Kita *et al.*, 2016). The substrates of these systems comprise usually at their N or C-terminal a signal sequence that allows for the identification of the substrate by its transporter. Table 1 shows the various types of Gram-negative secretion systems.

Table 1: Classes of protein secretion systems

(Green & Mecsas, 2016)

| Secretion apparatus | Signal sequence | Steps in secretion | Folded substrates | Number of membranes | Gram stain |
|---------------------|-----------------|--------------------|-------------------|---------------------|--------------|
| T1SS | C-terminus | 1-2 | No | 2 | Gram (-) |
| T2SS | N- terminus | 2 | Yes | 1 | Gram (-) |
| T3SS | N- terminus | 1-2 | No | 2-3 | Gram (-) |
| T4SS | C- terminus | 1 | No | 2-3 | Gram (-) |
| T5SS | N- terminus | 2 | No | 1 | Gram (-) |
| T6SS | Unknown | 1 | Unknown | 2-3 | Gram (-) |
| T7SS | C- terminus | 1-2 | Yes | 1-3 | Mycobacteria |
| T9SS | C-terminus | 2 | Yes | 1 | Gram (-) |
| Sec | N- terminus | 1 | No | 1 | Gram (+/-) |
| Tat | N- terminus | 1 | Yes | 1 | Gram (+/-) |

The secretion of substrates across the bacterial envelope involves either a one-step or two-step secretion mechanism. In the case of double membrane spanning systems, with the exception of T2SS, all secretion systems (T1SS, T3SS, T4SS, T6SS) use a one-step transport mechanism, this means that substrates are transported directly from the bacterial cytoplasm into the extracellular space or into a target cell cytosol. On the other hand, T2SS uses a two-step mechanism for transport where substrates are first translocated into the periplasmic space by IM-spanning transporters and are subsequently transferred to the OM or the extracellular medium by a dedicated OM-spanning secretion system. Most secretion systems transport unfolded or partially folded substrates, with the exception of T2SS and possibly T6SS that can secrete folded or partially folded proteins (Costa *et al.*, 2015).

Another family of transporters that exists in but not exclusive to Gram-negative bacteria are the general secretion (Sec) and twin arginine translocation (Tat) which are most commonly used to transport proteins across the cytoplasmic membrane (Natale *et al.*, 2008). They are highly conserved and have been identified in all domains of life (bacteria, archaea and eukarya) (Papanikou *et al.*, 2007). Most proteins transported by these systems usually stay inside the cell, either in the periplasm or attached to the inner membrane. However, in Gram-negative bacteria substrates of these systems can be transported to the outside of the cell with the help of another secretion system, *i.e.* T2SS or T5SS (Green & Mecsas, 2016).

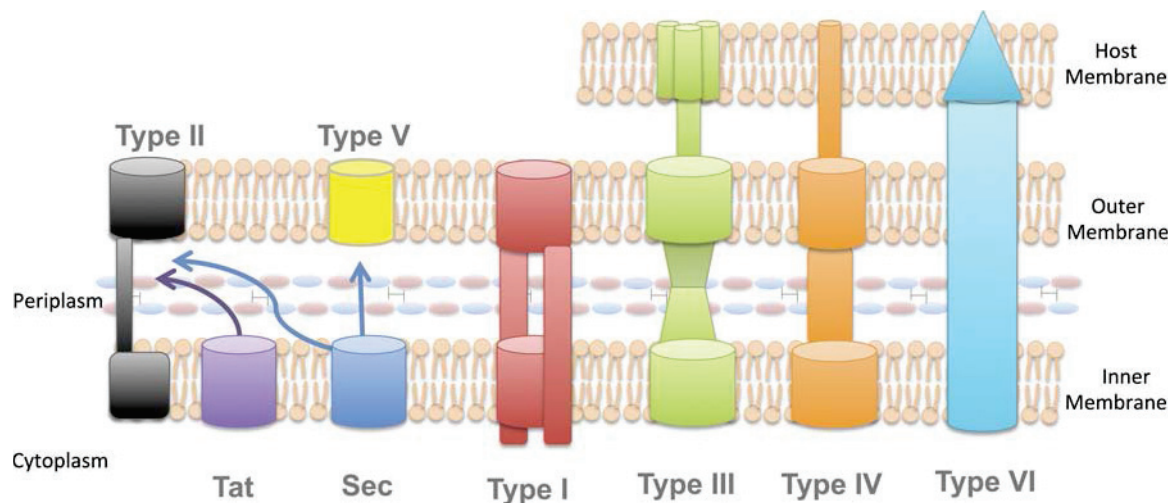


Figure 11: Secretion systems in Gram-negative bacteria several types of transporters are used by Gram-negative bacteria to transport proteins across one, two, or three membranes. Some substrates are transported in two-steps by the Sec and Tat mechanisms, where these proteins cross the IM via the Sec or Tat secretory pathways then through T2SS and T5SS if these proteins were destined for transport across the OM. One step transport across both bacterial membranes in Gram-negative bacteria includes the T1SS, T3SS, T4SS, T6SS. These transporters contain a periplasm spanning channel for secretion of proteins from cytoplasm directly to the outside of the cell. Additionally, the T3SS, T4SS, T6SS are capable of transporting a substrate across an additional host cell membrane to deliver specific proteins to the cytosol of their target cells (Green & Meccas, 2016)

Before addressing the different mechanisms of these Gram-negative bacterial secretion systems individually, it is important to know which of these systems are functional in *L. pneumophila*. A study on 74 whole genome sequences from 19 species of *Legionella* confirmed the presence of type II and type IVB secretion systems in all *Legionella* strains tested, while type IVA was randomly distributed among different species (Qin *et al.*, 2017). The type 1 secretion system was found to be restricted to *L. pneumophila* (Qin *et al.*, 2017) and indeed functional (Fuche *et al.*, 2015, Qin *et al.*, 2017). However, carefully looking at genomes in many *Legionella* species reveals the presence of T1SS main components and this point has to be clarified. The type VI secretion system was also detected but only in three non-*L. pneumophila* strains (Qin *et al.*, 2017). The different secretion systems in Gram-negative bacteria will be detailed below with a special focus on those present in *L. pneumophila*.

The table below shows the components and substrates of *L. pneumophila* secretion systems.

Table 2: Examples of secretion system components and substrates in *Legionella* (Lammertyn & Anne, 2004)

| Secretion system type | Structural proteins | | Substrates |
|-----------------------|---------------------------------------|--|--|
| Type I | | | RtxA, <i>L. pneumophila</i> virulence factor (Adhesin/toxin) ^a |
| | LssB | ABC transporter with ATPase domain | |
| | LssD | MFP, <i>E. coli</i> HlyD homolog | |
| | LssXYZABDE | Unconfirmed function | |
| Type II | | | PhospholipaseA/C, Zn metalloprotease, Lipases, RNase, pNPPC hydrolase, Lysophospholipase |
| | PilD | Prepilin peptidase | |
| | LspD | Outer membrane secretin | |
| | LspE | ATPase | |
| | LspF | Inner membrane protein | |
| | LspG,H,I,J,K | Pseudopilins | |
| | LspL,M,C | Conserved secretion components | 20 putative Tat-dependant substrates |
| | LspO | | |
| | LepB6 | Type I signal peptidase | |
| | TatA,B,C | Translocase for Tat dependant precursors | |
| Type IVA | | | Plasmid DNA |
| | Lvh B2-11, D4 | Unconfirmed functions | |
| Type IV B | | | Plasmid DNA, DotA, Various effector proteins (RaIF, LidA, Sid molecules...) |
| | 25 Dot/Icm proteins in different loci | Unconfirmed functions | |

^a (Fuche *et al.*, 2015)

1. The Sec secretion pathway

Substrates of the Sec pathway are primarily unfolded proteins. This system comprises three components: a protein targeting component, a motor protein, and the membrane integrated channel all together forming the SecYEG translocase (Papanikou *et al.*, 2007). Proteins translocated by this pathway have many roles where some of them promote virulence of bacterial pathogens, for example *Vibrio cholerae* and *Klebsiella pneumoniae* (Korotkov *et al.*, 2012). Export by this system is dependent on a hydrophobic signal sequence at the N-terminus of the protein, it is usually 20 amino acids long. Proteins that are destined for secretion to the periplasm or the extracellular environment will contain a SecB signal sequence, while those meant to remain in the inner membrane contain a signal recognition particle (SRP) (Green & Meccas, 2016).

a) SecB Pathway

As mentioned earlier, in Gram-negative bacteria, proteins using the Sec pathway whose final destination is the periplasm or the outside of the cell contain a removable signal sequence recognized by the SecB proteins. It functions as a chaperone that binds proteins and prevents their folding (Randall & Hardy, 2002). As shown in Figure 12, SecB will deliver its substrate to SecA which will guide both proteins to the SecYEG channel and at the same time serve as the ATPase that provides energy for substrate translocation (Hartl *et al.*, 1990).

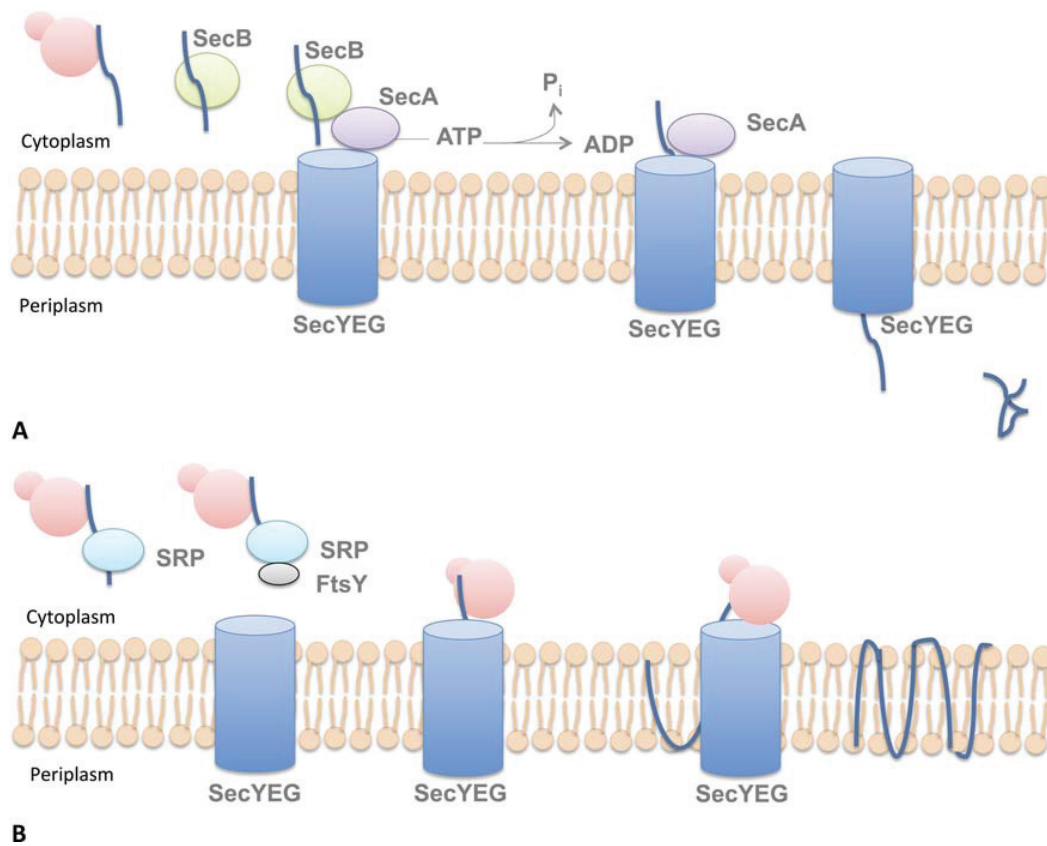


Figure 12: Scheme of export via the SecB and SRP pathways The Sec pathway transports unfolded substrates that can be released into the periplasm or become embedded in the IM. **(A)** Proteins destined for periplasm or extracellular release contain a removable SecB signal. SecB binds substrates and prevents them from folding while delivering them to SecA. SecA guides both proteins the SecYEG channel and also serves as the ATPase to provide energy for translocation. **(B)** Also utilizing the Sec pathway are proteins destined to remain in the IM. However, these proteins contain a signal recognized by the SRP. The SRP binds proteins as they emerge from the ribosome and recruits a docking protein FtsY. FtsY delivers the ribosome-protein complex to the SecYEG channel where it is translocated across the cytoplasmic membrane. During secretion, the transmembrane domain of the substrate is able to escape through the side of the channel and into the IM where it remains attached. Adapted from (Green & Mecsas, 2016).

It is important to note that the SecB signal sequence will be cleaved by the signal peptidase I prior to transport through the channel, where the protein will be folded upon arriving to the periplasm (Mogensen & Otzen, 2005). Most of the proteins delivered by the SecB system remain in the periplasm, some will ultimately become extracellular. These proteins will be transported across the outer membrane with the help of T2SS and T5SS (Green & Mecsas, 2016).

b) SRP pathway

Proteins that are meant to remain in the inner membrane can also be transported by the Sec system through the SRP pathway. Since transmembrane proteins contain hydrophobic domains, they are usually unstable in the cytoplasm. For this reason, SRP pathway secretion utilizes a co-translational method of export that couples the translation of the protein by the ribosome with secretion through the SecYEG channel. This pathway relies on an SRP particle which contains a small 4.5S RNA bound to a protein called Ffh (Luirink & Sinning, 2004). As seen in Figure 12, SRP will bind the transmembrane domains of the protein as they emerge from the ribosome (Sijbrandi *et al.*, 2003). Consequently, the SRP will bind a docking protein FtsY which will deliver the ribosome-protein complex to the SecYEG channel. Translation of the protein will drive its secretion through the channel (Green & Mecsas, 2016).

2. The Tat secretion pathway

In contrast to the previous system, this pathway primarily secretes folded proteins. It is important since certain proteins undergo post-translational modifications in the cytoplasm which can initiate their folding (Berks *et al.*, 2005). The Tat secretion pathway consists of two or three subunits: TatA, TatB, TatC (in Gram-positive bacteria) (Pop *et al.*, 2002). As the name implies, the Tat signal sequence contains a pair of twin arginines in the motif S-R-R at the N-terminus of the folded protein (Green & Mecsas, 2016). However, the Tat signal sequences are usually longer than their Sec counterparts, these sequences are removed after transport by specific peptidases such as the leader peptidase and thylakoidal peptidase (Lee *et al.*, 2006). In contrast to Gram-positive bacteria where Tat system substrates are released extracellularly, in Gram-negative bacteria the Tat secreted proteins can either remain in the periplasm or be transported across the OM by the T2SS. The Tat pathway is important for survival of both pathogenic and non-pathogenic bacteria. Some examples of pathogenic bacteria that require a functional Tat pathway for full virulence are *Pseudomonas aeruginosa* and *Escherichia coli* (Green & Mecsas, 2016). The figure below illustrates this system.

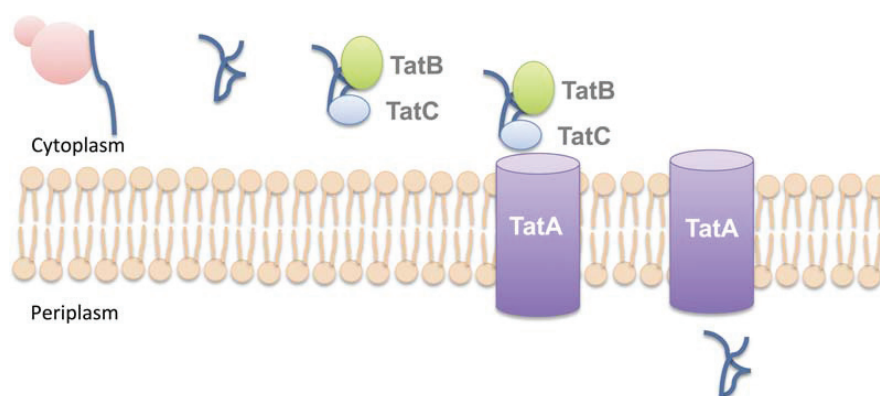


Figure 13: Secretion via the Tat pathway Folded proteins are translocated across the cytoplasmic membrane via the Tat pathway. In Gram-negative bacteria, TatB and TatC recognize a specific Tat signal on substrates. TatB and TatC then recruit TatA to the cytoplasmic membrane where it forms a channel. Folded proteins are translocated across this channel into the periplasm. Adapted from (Green & Mecsas, 2016).

3. The type I secretion system

T1SSs mediate the secretion of a large variety of protein substrates from the cytoplasm of Gram-negative bacteria to the extracellular medium in a single step across both bacterial membranes (Delepelaire, 2004, Lecher *et al.*, 2012, Kanonenberg *et al.*, 2013, Thomas *et al.*, 2014, XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016). The transported substrates include toxins, lipases, heme-binding and S-layer proteins, in addition to adhesins and proteins with RTX motifs (Lecher *et al.*, 2012, Green & Mecsas, 2016). They range in size from small proteins such as the 20 kDa iron scavenger HasA, the 110 kDa HlyA hemolysin, up to a molecular weight of 900 kDa for LapA, a large adhesion protein from *Pseudomonas* spp. (Thomas *et al.*, 2014).

In general, T1SSs consist of three indispensable components where two of these three reside in the IM. The complex is composed of an ATP-binding cassette transporter (ABC), a membrane fusion protein (MFP) and a pore forming outer membrane protein (OMP) (Kanonenberg *et al.*, 2013, XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016). Some bacteria may have several T1SSs, where each is dedicated to transporting one or few unfolded substrates (Delepelaire, 2004). In general, T1SSs are Sec independent and usually but not always, contain an uncleaved C-terminal signal sequence that is recognized by the T1SS (XU & LIU, 2014, Green & Mecsas, 2016). The motif of recognition is glycine rich and usually consists of the following consensus, Gly-Gly-X-Gly-X-Asp that binds calcium ions (Delepelaire, 2004, Costa *et al.*, 2015). However, in hemophore protein HasA of *Serratia marcescens*, secretion is not entirely dependent on its C-terminal sequence as it contains multiple regions that maximize secretion efficiency (Masi & Wandersman, 2010).

The inner membrane component or ABC transporter plays several critical functions, it provides energy for substrate translocation via ATP hydrolysis, it interacts with the MFP and it also participates in substrate recognition (Kanonenberg *et al.*, 2013). The basic structure of an ABC transporter consists of

four modules, two transmembrane domains (TMD) and two nucleotide binding domains (NBD) (Davidson *et al.*, 2008). However, ABC transporters of the T1SS only comprise one TMD and one NBD which are encoded by a single gene, the functional unit on the other hand is a dimer of this protein. The transmembrane domain helices form the translocation pathway of the substrate across the IM (Hollenstein *et al.*, 2007). While the NBDs are responsible for supplying energy via nucleotide binding and hydrolysis and the coordination of a cofactor (Mg^{2+}) (Oswald *et al.*, 2006). The TMDs of T1SSs exhibit sequence variability which is probably due to their implication in recognition and binding of different substrates, while NBDs show high sequence homology among T1SSs since they function solely as power supplies (Kanonenberg *et al.*, 2013). The MFP of the T1SS is also implicated in substrate recognition through its cytoplasmic N-terminal domain (Balakrishnan *et al.*, 2001). Regarding the OMP of this system, it forms a pore in the outer membrane through which the substrate will pass in an unfolded state. Interestingly, T1SSs usually use the multipurpose protein TolC as their OMP (Delepelaire, 2004). This protein is also involved in exporting molecules and other compounds and is recruited to the system after substrate recognition (Balakrishnan *et al.*, 2001). In addition to the previous, T1SSs are related to the resistance-nodulation-division (RND) family of multidrug efflux pumps. RND pumps mostly secrete antibacterial compounds out of the cell. RND pumps and T1SSs share a similar structural organization, both utilize TolC as the outer membrane protein and both consist of a MFP (AcrA-like in the case of RND pumps). However, the inner membrane component of RND pumps (AcrB-like) does not belong to the ABC family (Green & Meccas, 2016). In contrast to T1SSs that use ATP as an energy source to transport their substrates, RND pumps rely on a proton gradient to translocate the substrate (Eicher *et al.*, 2014).

Table 3: Examples of T1SS substrates (Thomas *et al.*, 2014)

| Organism | Substrate | Function | Amino acids | T1SS components |
|-----------------------|-----------|-----------------|-------------|-----------------|
| <i>P. aeruginosa</i> | HasA | Heme-binding | 219 | HasD/HasE/HasF |
| <i>E. coli</i> | HlyA | RTX toxin | 1024 | HlyB/HlyD/TolC |
| <i>P. aeruginosa</i> | AprA | Protease | 479 | AprD/AprE/AprF |
| <i>S. marcescens</i> | LipA | Lipase | 613 | LipB/LipC/LipD |
| <i>P. fluorescens</i> | LapA | Adhesin | 888 | LapB/LapC/LapE |
| <i>S. marcescens</i> | SlaA | S-layer protein | 259 | LipB/LipC/LipD |

In Table 3 above we can see the diversity in function and size of T1SS substrates signifying the importance and versatility of this secretion system in Gram-negative bacteria.

The first studied T1SS was in uropathogenic *E. coli* (UPEC) for the secretion of an RTX toxin known as hemolysin A (HlyA). The hemolysin system is composed of three components. HlyB which is the ABC transporter, the membrane fusion protein is HlyD and the OMP is TolC. These components

representing the general structure of a T1SS transporter are displayed in Figure 14. The T1SS is also encoded by other Gram-negative bacteria such as *V. cholerae*, *P. aeruginosa* as well as *L. pneumophila* for the transport of RTX toxins, proteases and other virulence factors. The *L. pneumophila* T1SS is called the *Legionella* secretion system (Lss) and will be discussed further on (XU & LIU, 2014).

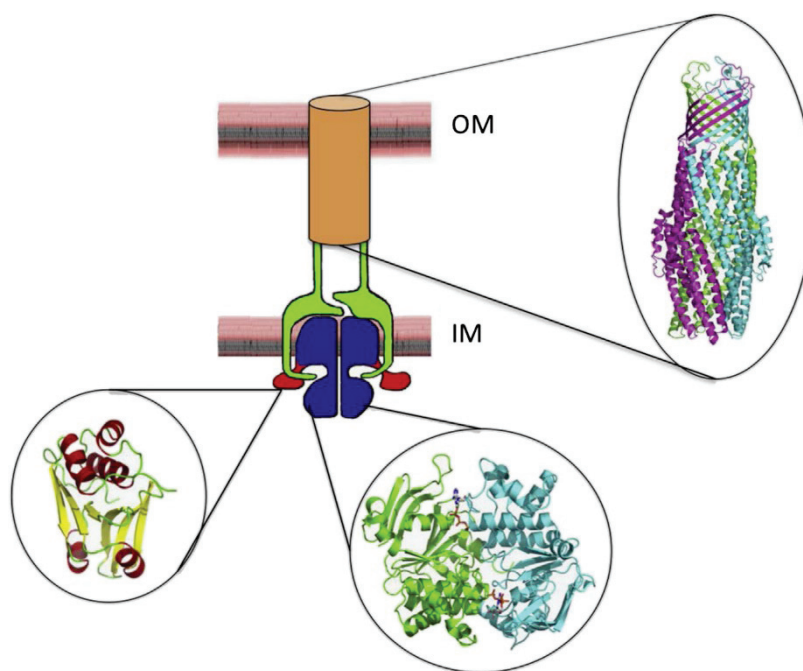


Figure 14: General schematic representation of a T1SS involved in RTX protein secretion The ABC transporter is illustrated in blue with the C39 like domains (CLD) highlighted in red, the MFP in green and the OMP in orange. The structures shown are those of the *E. coli* hemolysin A (HlyA) transporter. The ABC transporter in this case is HlyB with the structures of its NBD and CLD represented in circles. The OMP is TolC, a water filled channel in the OM with its structure also shown above. From (Kanonenberg *et al.*, 2013).

T1SSs do not exist in permanently associated static complexes, they rather assemble upon substrate recognition. More specifically, the ABC transporter and the MFP always form a complex, but the entire system only assembles after interaction of the substrate with the ABC and/or MFP (Kanonenberg *et al.*, 2013). This is illustrated in Figure 15 below.

T1SSs substrates usually comprise a C-terminal secretion signal as mentioned earlier in Table 1. This is evident in the figure below in case of RTX proteins where secretion only happens after translation has finished (Delepelaire, 2004, Masi & Wandersman, 2010). In the bacterial cytoplasm, RTX proteins remain in an unfolded state. This happens since RTX proteins share the presence of characteristic carboxy-proximal tandem repetitions of the Ca^{2+} binding RTX nonapeptides of a consensual sequence GGxGxDxxx. Calcium binding to these repeats allows the proper folding and acquisition of the protein's biological activity. This cannot happen inside the cytoplasm where calcium concentrations are usually low ($< 100 \text{ nM}$) (Linhartova *et al.*, 2010, Bumba *et al.*, 2016). It was also proposed that this folding

aspect of RTX proteins once its C-terminus reaches the extracellular milieu serves as a translocation ratchet that pulls this protein through the T1SS ducts (Bumba *et al.*, 2016).

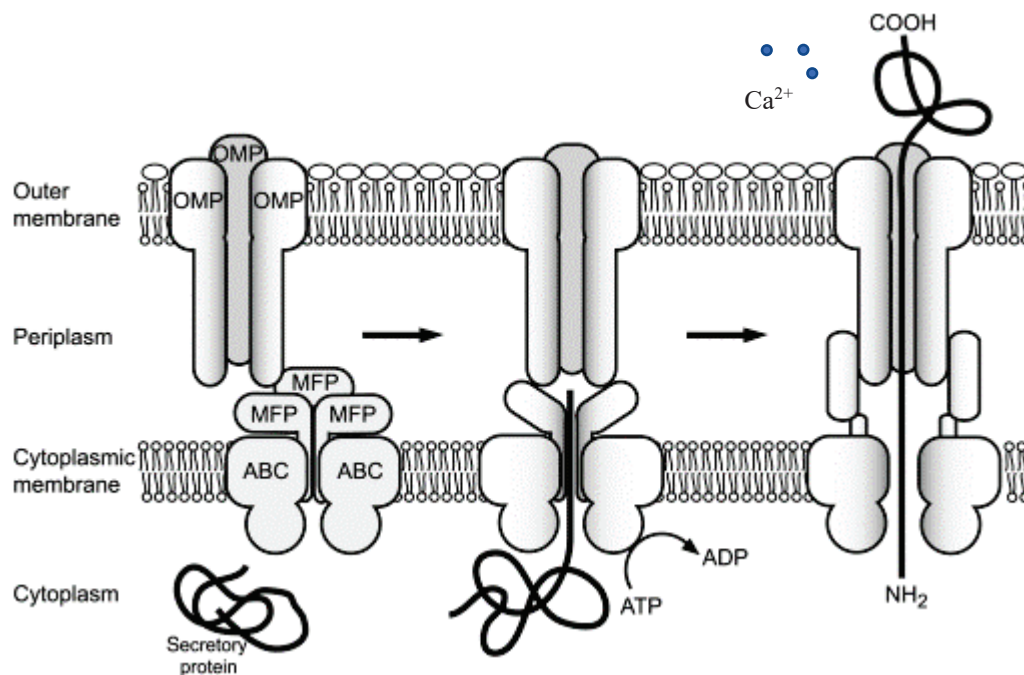


Figure 15: General structure of a T1SS secreting a substrate After the complete translation of the RTX substrate, the C-terminal signal sequence will interact with the ABC/MFP complex which will trigger the assembly of the complete secretory apparatus by contacting the trimeric OMP. A channel spanning both membranes will be formed in which the RTX protein will be exported in a single step to the extracellular medium. Higher concentrations of Ca^{2+} in the extracellular medium will allow the folding of the RTX protein by binding to the RTX repeats. This will lead to the activation of the protein. Adapted from (Linhartova *et al.*, 2010).

a) T1SS ABC transporters

Coming back to the ABC transporters, by default, they contain four canonical domains, two TMDs and two NBDs. However, many also contain additional features at their cytosolic N-termini. This allows for the classification of ABC transporters into 3 different groups:

(1) C39-containing ABC transporters

Proteins destined for secretion are targeted to their transporters by a specific signal peptide, in this case an N-terminal signal sequence that is cleaved during transport, it is called the leader peptide (Kanonenberg *et al.*, 2013). These small proteins are called bacteriocins or microcins and are secreted by Gram-negative bacteria. However, this is usually a feature of Gram-positive bacteria since the secretion of small antimicrobial peptides is not common in Gram-negative bacteria (Duquesne *et al.*,

2007b, Duquesne *et al.*, 2007a). The ABC transporters responsible for the secretion of such compounds contain an additional N-terminal domain that exhibits Ca^{2+} dependent proteolytic activity (Wu & Tai, 2004). This domain resembles a C39 peptidase which is a member of the papain superfamily. They cleave polypeptides C-terminal to a canonical double glycine motif (GG). After interaction of the leader peptide with the C39 domain, this will lead to cleavage at the C-terminal site of the GG motif allowing the mature protein to be secreted to the extracellular space via its specific TISS (Havarstein *et al.*, 1995). C39 domains also play a role in the translocation activity of these ABC transporters. As for the leader sequence, in addition to contributing to the stability of the mRNA and recognition by this specific TISS, its interaction with the C39 domain will likely keep the substrate in an unfolded state until secretion occurs (Kanonenberg *et al.*, 2013).

(2) CLD-containing ABC transporters

Another family of ABC transporters contains an N-terminal domain that strongly resembles a C39 domain but is lacking any proteolytic activity due to the absence of the essential catalytic cysteine (Lecher *et al.*, 2012). Therefore, these domains are called C39 like domains (CLD) and their substrates do not contain an N-terminal leader peptide. CLD is essential for the secretion of RTX proteins, for example the hemolysin HlyA mentioned earlier where the CLD interacts with the C-terminal of HlyA containing the three RTX repeats. However, the secretion signal in the last 60 amino acids was not required, it is only necessary to target the substrate to the transport machinery (Kanonenberg *et al.*, 2013). It is important to note that all ABC transporters dedicated to the transport of RTX proteins contain a CLD. Since RTX proteins are usually very large, some can be greater than 1000 kDa in size, it is important that they remain unfolded and without aggregation in the cytoplasm. A dedicated chaperone molecule has never been shown to play this role, however the interaction of the N-terminal domain of the substrate with the CLD suggests that it also plays a role in preventing the protein's aggregation or degradation in the cytoplasm. It seems that the CLD has evolved from the C39 domain even though they greatly differ (Lecher *et al.*, 2012).

(3) ABC transporters without appendix

Some TISS ABC transporters are composed only of the general domains described for these transporters. Therefore, these transporters do not contain any additional N-terminal domains, their substrates may contain RTX repeats but no leader N-terminal peptide (Delepelaire, 2004). An example is the secretion system of HasA in *Serratia marcesens*, it does not belong to the RTX family but contains nonetheless a C-terminal secretion signal. Moreover, it has been shown that numerous regions in HasA polypeptide interact with the ABC transporter (Masi & Wandersman, 2010). The other difference

regarding this system is the requirement of the chaperone SecB to prevent the folding of this protein, which is a state incompatible with secretion (Kanonenberg *et al.*, 2013).

b) T1SS MFP/adaptor protein

This protein consists of a short N-terminal fragment in the cytoplasm, a unique transmembrane fragment and a large periplasmic part. Mutations in the C-terminal and as well as the central region of HlyD, the MFP of the Hly system, are deleterious for substrate secretion (Delepelaire, 2004). This protein is also probably responsible for the mechanical force exerted on the OMP (TolC) leading to the opening of the channel (Zgurskaya & Nikaido, 1999). As mentioned before, the substrate will trigger the assembly of the secretion apparatus via an initial interaction with the ABC protein. Regarding the MFP, its role in this process is not always the same. In the Has and Prt systems, the MFP does not interact by itself with the substrate, but this seems to be the case in the Hly system. This interaction is mediated by the cytoplasmic N-terminus of HlyD which is 59 amino acids long and is longer than those in the other systems mentioned earlier. Another notable difference is the presence of a stable complex between the MFP and the ABC transporter in case of Hly system, as opposed to that in Has/Prt systems (Delepelaire, 2004).

c) OMP

All T1SSs in Gram-negative bacteria include an OMP, TolC is the best characterized (Delepelaire, 2004, Ferhat *et al.*, 2009). In the *E. coli* Hly system, TolC is 55 kDa and also serves as an outer membrane component for several processes such as type 1 secretion and drug export via RND type systems which means it is connected to both systems (Andersen, 2003, Gerlach & Hensel, 2007). In *L. pneumophila*, a TolC homolog (64 kDa) was demonstrated to play a role in multidrug resistance and an essential role in its virulence in addition to stress resistance (Ferhat *et al.*, 2009). TolC is a trimeric protein, anchored in the OM by beta strands, and comprises a large periplasmic part formed almost uniquely of alpha helices, it is almost closed at the periplasmic end and wide open at the OM surface (Delepelaire, 2004).

d) Lss, the *L. pneumophila* T1SS

The reason for including information about the *E. coli* hemolysin system is because it represents a model T1SS which is the best characterized so far. In addition to that, many similarities exist between this system and the *L. pneumophila* Lss system.

In *E. coli*, the operon consists of *hlyC*, *hlyA*, and the exporter genes *hlyB* and *hlyD*. As mentioned earlier, HlyA is the RTX pore forming toxin, HlyC is an acyl transferase that acylates two internal lysines in HlyA in order to activate its hemolytic activity. HlyB is the ABC protein and HlyD is the MFP. TolC, the outer membrane protein is not encoded within the Hly operon (Thomas *et al.*, 2014). After a putative RTX toxin was identified in *L. pneumophila* (Cirillo *et al.*, 2000), this triggered the identification of a six gene locus in *L. pneumophila* that constitutes a T1SS but no substrate was identified at that time (Jacobi & Heuner, 2003). However, it was later established that *L. pneumophila* RtxA was indeed a substrate of the LssBD/TolC secretion system (Fuche *et al.*, 2015). Comparison of the *lss* operon of *L. pneumophila* with *hly* operon from *E. coli* is displayed in the figure below.

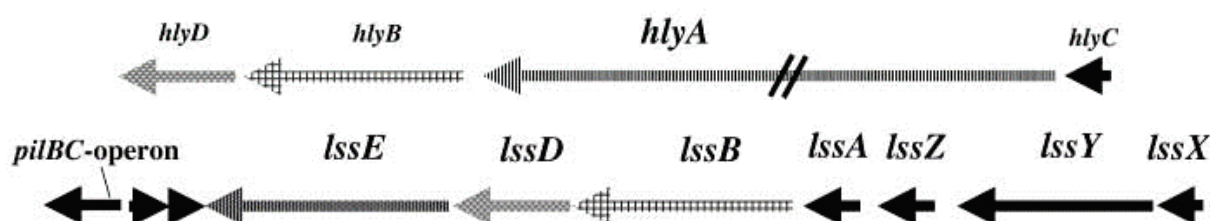


Figure 16: Comparison of the *lss* locus of *L. pneumophila* Philadelphia and T1SS of *E. coli* The *lss* *L. pneumophila* operon (bottom) consists of the displayed genes *lssXYZABDE*. ORFs with similar function are shaded accordingly. The *E. coli* ABC transporter and MFP genes *hlyB* and *hlyD* are similar to *L. pneumophila* *lssB* and *lssD* respectively. In both cases the *tolC* gene is not encoded within the T1SS operon, but contrastingly, the Hly operon from *E. coli* includes the substrate it transports (HlyA) where this is not the case in *L. pneumophila*. The *L. pneumophila* genes *lssXYZAE* do not appear to share homology with *E. coli* genes. Adapted from (Jacobi & Heuner, 2003).

As displayed in Figure 16, the *lss* locus is located next to a *pilBC* locus encoding necessary genes for type IV secretion. The first gene of the operon, *lssX*, encodes a putative protein with similarity to LipB protein of various bacteria, hence it may encode a putative lipolate biosynthesis protein. Downstream of *lssX*, we find *lssY* coding the LssY protein that contains various transmembrane regions and a PAP2 (phosphatidic acid phosphatase) super family domain usually found in some phosphatases. *lssA* and *lssZ* genes encode two small proteins with no significant similarity to any proteins. Further downstream, *lssB* encodes a 718-amino acid protein that contains an ABC transporter transmembrane sequence and an ATPase domain. *L. pneumophila* *lssD* gene was found to encode a protein of 378 amino acids, this protein comprises a signal peptide in the first 26 amino acids and a HlyD-family secretion proteins signature. The LssB/LssD proteins share significant identities with T1SS proteins of *V. cholerae* (45%/48.6%) and *E. coli* (31%/29%) in addition to other bacteria. The final protein encoded by the operon is LssE which may be implicated in signal transduction. It is similar to the sensory box GGDEF family of several bacteria (Jacobi & Heuner, 2003). The putative protein functions of the different

proteins encoded by this operon and the identities of these genes between the Philadelphia and Corby strains are displayed Figure 17 below.

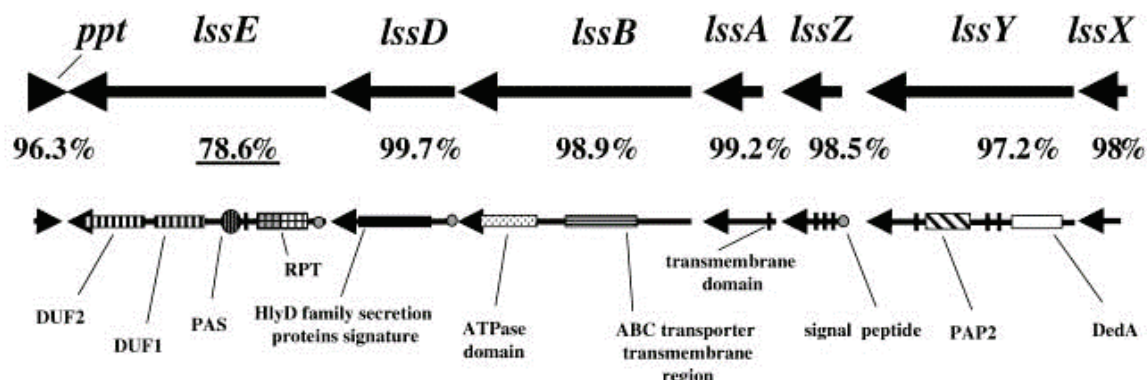


Figure 17: Putative protein domains of the genes encoded in the *lss* locus the first line of arrows represents the genes constituting the *lss* secretion system in *L. pneumophila* Philadelphia strain. The second line shows the putative proteins and motifs. The percentages indicate the identity between the genes of the *lss* operon in Philadelphia and Corby strains of *L. pneumophila*. High identity is observed among these genes indicating this system is conserved. Abbreviations: DedA, DedA-related protein family; PAP2, type 2 phosphatidic acid phosphatase family; RPT, internal repeats; PAS, Per-Arnt-Sim domain found in signaling proteins; DUF1, unknown function domain with GGDEF motif; DUF2, domain of unknown function; ppt, pyrimidine phosphoribosyl transferase. Adapted from (Jacobi & Heuner, 2003).

Although T1SSs are described to transport substrates in a single step from the cytoplasm to extracellular medium, sub-groups belonging to this class including that of *L. pneumophila* have been recently shown to use a two-step mechanism with a periplasmic intermediate (Smith *et al.*, 2018a). This will be discussed in the coming parts after presenting all the details of this system.

4. The type II secretion system

The T2SS (also called Gsp or general secretion pathway and Lsp in case of *Legionella*) is conserved in many Gram-negative bacteria. It transports folded proteins from the periplasm into the extracellular environment. The channel for this system is present in the outer membrane only so the first step of delivering the substrate to the periplasm is achieved via the Sec or Tat secretion pathways which are present in *L. pneumophila* (Cianciotto, 2005, XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016). Therefore, T2SS substrates must have a Sec or Tat N-terminal cleavable signal for secretion. Since the T2SS secretes folded substrates, any proteins translocated by the Sec system must be folded in the periplasm prior to export via the T2SS (Green & Mecsas, 2016). So far, *L. pneumophila* is the only known intracellular pathogen with a functional T2SS (Lammertyn & Anne, 2004, Qin *et al.*, 2017).

Moreover, a study confirmed the presence of T2SS genes in all *L. pneumophila* strains and at the same time in other *Legionella* species (Qin *et al.*, 2017).

Bacteria use the T2SS to transport a variety of substrates including degradation enzymes such as lipases and phosphatases in addition to some bacterial toxins (XU & LIU, 2014). The table below summarizes some examples of T2SS substrates with focus on *L. pneumophila*. This secretion system is often correlated with bacterial pathogens for several reasons, genes encoding the core components are present in many pathogens as well. Also, the nature of this system's substrates which are generally degradative enzymes suggests that it promotes damage to host cells (Cianciotto, 2005). In some cases, type II exoenzymes have been shown to contribute to virulence, as is the case of *P. aeruginosa* exotoxin A (Sandkvist, 2001). And finally, T2SS genes mutation can attenuate virulence in relevant models of disease. For example, a mutation in *L. pneumophila* T2SS LspF protein abolishes the ability for survival in lungs of infected mice. Therefore, in *L. pneumophila*, T2SS facilitates pathogenesis by promoting bacterial growth in intracellular niches (Rossier *et al.*, 2004). On the other hand, T2SS also promotes growth of bacteria in environmental niches. These bacteria can be considered metabolically diverse and often reside in "extreme" environments. This can be observed through the T2SS functions in environmental non-pathogens such as the manganese oxidation in *Pseudomonas putida* and lipase secretion by *Pseudomonas alcaligenes* (Cianciotto, 2005). Interestingly, this can also be the case in *L. pneumophila* where it was shown that T2SS might promote its persistence in aquatic habitats by facilitating intracellular growth in fresh water amoeba as well as extracellular growth at low temperatures (12-25°C) (Rossier *et al.*, 2004, Soderberg *et al.*, 2004).

Table 4: Examples of T2SS substrates and functions in different bacteria (Cianciotto, 2009)

| Organism | Protein/Function | Other phenotypes |
|------------------------------------|--|--|
| <i>E. coli</i> (EHEC) ^a | StcE metalloprotease | Adherence to epithelial cells |
| <i>P. aeruginosa</i> | Alkaline phosphatase, chitin-binding protein, exotoxin A, lipases (LipA, C) | Cytotoxicity for CHO ^b cells (only in absence of T3SS) |
| <i>V. Cholerae</i> | Cholera toxin, endochitinase, lipase, neuraminidase | Outer membrane assembly, rugose polysaccharide production |
| <i>L. pneumophila</i> | ProA (MspA) – zinc metalloprotease (promotes amoebal infection) PlcA – phospholipase C PlaA - lysophospholipase A ChiA - chitinase (promotes lung infection) SrnA - type 2 ribonuclease (promotes amoebal infection) LapA - leucine, phenylalanine and tyrosine aminopeptidase LipA – mono- and triacylglycerol lipase | Virulence in murine pneumonia model, intracellular infection of macrophages and amoeba, extracellular growth at low temperatures |

^a EHEC: enterohemorrhagic *E. coli*

^b CHO: Chinese hamster ovary

Regarding the structure of a T2SS, it is composed mainly of 4 parts and at least 12 core proteins. There is an OM complex, a periplasmic pseudopilus, an IM platform and a cytoplasmic ATPase (Cianciotto, 2014, XU & LIU, 2014, Costa *et al.*, 2015). More specifically, the cytosolic ATPase is the E protein; three inner membrane proteins that create a platform for the ATPase are proteins F, L and M; protein C is a Linker protein that connects the IM platform to the OM secretin; multiple major and minor pseudopilins form a pilus-like structure that spans the periplasm and the corresponding proteins are T2S G, H, I, J, K. Finally, the outer membrane secretin forms the secretion pore, T2S D. Protein O is an inner membrane peptidase that clips pseudopilins before their integration with the apparatus (Cianciotto, 2014). These structure of a T2SS is illustrated in Figure 18.

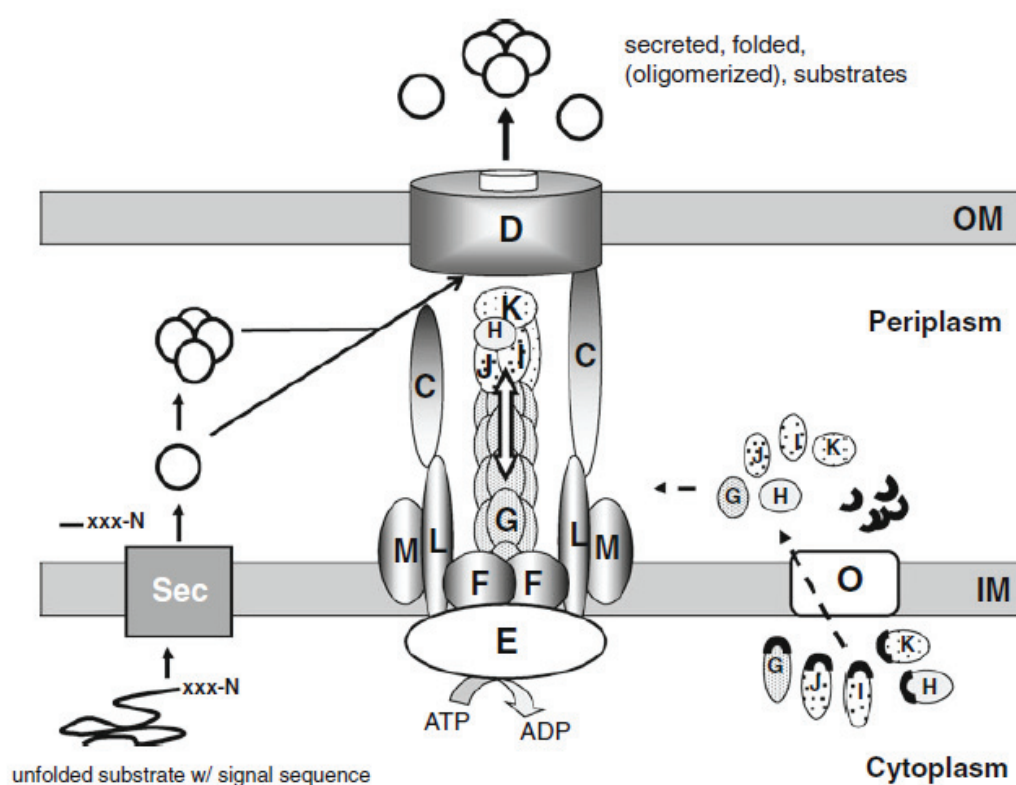
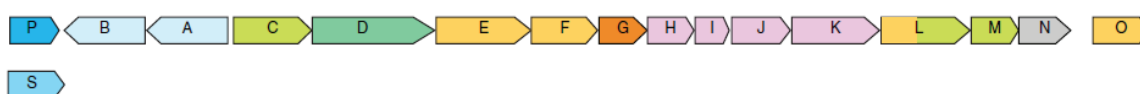


Figure 18: Structural model of a T2SS in Gram-negative bacteria T2SS components are indicated by single letter designations C-M. A Sec system substrate is recognized via its N-terminal signal sequence and is transported to the periplasm where sometimes oligomerization occurs. Within the periplasm, substrates are recognized by the secretion apparatus. ATP hydrolysis by the inner membrane ATPase, a pilus like structure composed of major (G) and minor (H, I, J, K) pseudopilins will push the substrate through the OM secretin in a piston like manner. The pre-pilin peptidase (O) cleaves and N-methylates pseudopilins prior to their integration into the T2SS apparatus. Substrates that are folded within the cytoplasm will cross the IM via the Tat pathway; it is not displayed in this figure. From (Cianciotto, 2014).

The mechanism of T2SS secretion is interesting since it pushes the substrates outside mechanically rather than forming a channel for translocation. Concerning the inner membrane platform proteins, only T2S C, L, M have one transmembrane segment while protein F has multiple segments. T2S C binds to

the periplasmic domains of T2S D, thereby connecting the IM platform to the OM complex. The T2S E, which is the cytoplasmic ATPase, is a ring shaped hexamer that is thought to be recruited to the IM platform through its interaction with T2S L and T2S F (Costa *et al.*, 2015). T2SS comprises a pseudopilus that in contrast to a fully formed pilus which extends to the extracellular space, remains within the periplasm. The pseudopilus is composed of one major (G) and four minor subunits (H, I, J, K). The pseudopilins are inserted into the IM by the SecYEG translocon and later cleaved by the peptidase T2S O (Korotkov *et al.*, 2012). The minor pseudopilins assemble at the tip of the T2S G helical filament to complete the formation of the pseudopilus (Cisneros *et al.*, 2012). After the T2SS substrates are transported to the periplasm via the Sec or Tat pathways, it has been suggested that ATP hydrolysis is used to power the assembly of the periplasmic pseudopilus and to push substrates through the OM channel via the secretin by extension of the pseudopilus in a piston like manner (Nivaskumar *et al.*, 2014). It is worthy to note that the T2SS pseudopilus is evolutionarily connected to the Type IV pilus as well as archaeal pili and flagella (Nivaskumar & Francetic, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016).

A) Type II secretion system of *Aeromonas hydrophyla*



B) Competence Type IV pili *Streptococcus pneumoniae*



C) Type IV a pili of *Pseudomonas aeruginosa*

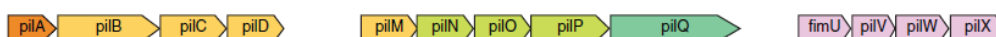


Figure 19: Genetic organization and composition of T2SS (A); in comparison with type IV pilus assembly systems in Gram-positive (B); and in Gram-negative bacteria (C). components are indicated by single letter designations C-S. Common color code is used for genes belonging to the corresponding modules. Components of the same module are depicted with common colors. The module gene encoding the major pseudopilin subunit (G) is shown in orange, minor pseudopilin subunits (HIJK) in pink. The gene encoding the connecting module in Gram-negative bacteria (C) is in light green and the secretin (D) in dark green. From (Nivaskumar & Francetic, 2014).

As seen in Figure 19, the genetic organization of the T2SS from the Gram-negative *Aeromonas hydrophyla* shares a considerable amount of similarity with type IV pilus in Gram-negative and even Gram-positive bacteria. Core components such as the type 2 pseudopilus subunits (dark orange and pink) can be detected in all these systems.

In *L. pneumophila*, the first evidence for the existence of a T2SS was the discovery of *PilD*, the gene encoding the pseudopilin peptidase (T2S O) (Liles *et al.*, 1998). Consequently, genome analysis of *L. pneumophila* Philadelphia-1 revealed the locus *lspFGHIJK* encoding the respective proteins. Further studies on strain 130b revealed genes encoding T2S D and E (*lspDE*), T2S C (*lspC*), T2S L and M (*lspLM*) where Mutational analysis of *lspDE* confirmed their role in secretion (Cianciotto, 2014). Moreover, genome sequencing of several *L. pneumophila* strains including Alcoy, Paris, Lens, Corby and Philadelphia-1 showed that *L. pneumophila* contains a full set of T2SS genes (D'Auria *et al.*, 2010) and possibly other strains do too (Costa *et al.*, 2012). T2SS mutants of *L. pneumophila* did not display growth anomalies when grown in bacteriological media (AYE broth or BCYE agar) at 37°C. Thus, T2SS is not required for extracellular growth under laboratory conditions (Cianciotto, 2014).

a) T2SS substrates and phenotypes in *L. pneumophila*

As mentioned briefly in Table 4, various substrates rely on the T2SS for translocation in *L. pneumophila* where most of these substrates are associated to virulence. These include various acid phosphatases, phospholipases C, lysophospholipase A, glycerophospholipid cholesterol acyltransferase (GCAT) and many more (Cianciotto, 2014). Mutations in the structural genes encoding T2SS substrates do not completely abolish their corresponding activities, this suggests that *L. pneumophila* has more than one secreted phosphatase, lipase, phospholipase C, etc. (Cianciotto, 2014). Also, among the identified substrates there are ProA (metalloprotease), Map (phosphatase), PlaA (phospholipase A) (DebRoy *et al.*, 2006) and SrnA (ribonuclease) (Rossier *et al.*, 2009).

Regarding observed phenotypes, *L. pneumophila* exhibits surface translocation when grown on media containing 0.5-1% agar (Stewart *et al.*, 2009). Interestingly, *L. pneumophila* mutants lacking flagella and/or type IV pili still behave as the wild-type, indicating that the observed behavior is not swarming or twitching motility. This translocation is regarded as sliding motility since a translucent film composed of a lipid containing surfactant is visible on the agar in front of the spreading *legionellae* (Stewart *et al.*, 2011). *L. pneumophila* *lsp* mutants are defective for surface translocation and surfactant expression and so are TolC mutants indicating the probable joint effort of these two protein systems to achieve this phenotype (Stewart *et al.*, 2009, Stewart *et al.*, 2011).

b) T2SS and extracellular survival in *L. pneumophila*

It has been suggested that *L. pneumophila* T2SS plays a role in extracellular replication. *L. pneumophila* strain 130b mutants replicate normally between 30-37°C but are defective for growth in media at temperatures between 12 and 25°C (Soderberg *et al.*, 2004). *lsp* mutants also exhibited reduced survival

in tap water incubated between 4 and 25°C, these trials were conducted to mimic aquatic habitats (Soderberg *et al.*, 2008). However, T2SS mutants can grow better when plated next to wild-type or wild-type culture supernatants, suggesting the presence of secreted factor(s) that promote growth at low temperatures (Soderberg *et al.*, 2004, Soderberg *et al.*, 2008). It is interesting that when wild-type *L. pneumophila* is grown at “low temperatures”, new proteins appear in the supernatants. Another study also indicates that some T2SS substrate genes are over-expressed when *L. pneumophila* are grown at 20°C in a biofilm (Cianciotto, 2014). This information implicates the T2SS in planktonic persistence of *L. pneumophila* and in consequence as a factor in disease transmission.

c) T2SS and intracellular infection of amoebae

T2SS mutants are impaired for the intracellular infection of freshwater amoebae such as *Acanthamoeba castellanii* and *Hartmanella vermiformis*. The wild-type infection potential is restored when a copy of the T2SS gene is reintroduced, confirming its role in infection (Cianciotto, 2014). This phenotype is not related to the entry potential as *lsp* mutants are not defective for entry (Soderberg *et al.*, 2008). Mutational analysis on two T2SS substrates, ProA and SrnA, shows that the observed defect is greater than the corresponding single mutants, indicating that the role of T2SS in amoebal infection is a result of the combined effects of several secreted proteins (Rossier *et al.*, 2009). SrnA is a ribonuclease that might be degrading host RNA in order to obtain nutritional nucleotides and phosphate or simply to alter host function. This information demonstrates that the reduced ability of T2SS *lsp* mutants to infect amoeba hosts is due to the loss of secreted effectors (Cianciotto, 2014).

d) T2SS and models of lung infection

Mutants of *L. pneumophila* T2SS are impaired in the murine model of Legionnaire’s disease (McCoy-Simandle *et al.*, 2011). In contrary to wild-type *L. pneumophila* strain 130b that increases tenfold in the lungs of A/J mice, *lspF* mutants exhibit no CFU (colony forming unit) increase and are cleared rapidly (Rossier *et al.*, 2004). Therefore, T2SS is a key player in *L. pneumophila* virulence. Specifically, it has been suggested that T2SS substrate ChiA chitinase is required for survival in the lungs. Mutants lacking ChiA are impaired around fourfold when tested *in vivo* (DebRoy *et al.*, 2006). Interestingly, *chiA* mutants can grow normally in macrophages *in vitro* and the reduced survival was apparent in the later stages of infection. This hints that ChiA helps in the persistence of *L. pneumophila* rather than initial replication. However, since mammals do not have chitin, it maybe that the degradation of a chitin like factor in the lungs helps in *L. pneumophila* survival or this enzyme might have a dual function and another substrate (Cianciotto, 2014). The T2SS substrate ProA is also suggested to aid in lung infection of *L. pneumophila* by promoting the destruction of lung tissue (DebRoy *et al.*, 2006).

e) **Role of the T2SS in intracellular infection of macrophages**

L. pneumophila T2SS mutants display a reduced ability to infect U937 cells, a human macrophage-like cell line. In these cells, T2SS mutants exhibit tenfold reduced recovery 48 hours post inoculation. This reduced infection potential can be complemented by the re-introduction of the *lsp* genes (Rossier *et al.*, 2004). The *lsp* mutants are also impaired for infection of human peripheral blood monocytes and THP-1 cells, also a human macrophage like cell line. Microscopic observation of infected U937 cells reveal the greatly reduced numbers of intracellular mutant bacteria compared to the wild-type even at 16 hours post infection (Cianciotto, 2014). Regarding the exact substrates responsible for this phenotype, many T2SS substrate mutants were tested but all were able to grow normally. This data indicates that the T2SS is implicated in *L. pneumophila* growth within macrophages, but the identity of the key effector(s) remains unknown (Cianciotto, 2014).

f) **Role of the T2SS in intracellular infection of epithelial cells**

Alveolar epithelial cells were identified as a potential niche for *L. pneumophila* growth during lung infection (Newton *et al.*, 2006). *lspF* and *lspDE* mutants displayed an inability to grow in epithelial monolayers at 1 to 3 days post infection but are present in cells at t_0 even after treating the monolayers with gentamycin to eradicate any residual extracellular bacteria, indicating that the entry mechanism into epithelial cells is not impaired by these mutations (Cianciotto, 2014). These phenotypes were restored after complementation with the corresponding deleted gene confirming the role of T2SS (McCoy-Simandle *et al.*, 2011). However, the specific T2SS substrates responsible for this phenotype are unknown but likely act through facilitating intracellular growth as already mentioned in macrophages (Cianciotto, 2014).

5. **The Type III secretion system**

Unlike the T2SS discussed previously which is prevalent in all classes of bacteria, T3SSs are found exclusively in Gram-negative bacteria that live in close association with their eukaryotic hosts, *i.e.* bacterial pathogens and symbionts, for example, *Salmonella*, *Shigella*, *Yersinia*, *Pseudomonas* and *E. coli* (XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016). Despite this diversity, the T3SS apparatus and secretion mechanisms are very well conserved (Buttner, 2012). This secretion system is embedded in both bacterial membranes and has been described as an injectisome because of its needle-like structure. Because of this unique structure, T3SSs are able to translocate substrates across a eukaryotic host membrane in addition to both bacterial membranes in a single step thereby transferring substrates to the plasma membrane or into the cytoplasm of a target eukaryotic cell (XU & LIU, 2014,

Costa *et al.*, 2015, Green & Mecsas, 2016, Deng *et al.*, 2017). The T3SS substrates are called effector proteins, they modulate or subvert specific host cell functions hence promoting bacterial invasion and colonization; these processes include manipulating host immune responses, cytoskeletal dynamics, vesicle transport and signal transduction pathways (Deng *et al.*, 2017). The secretion signal for these substrates is not cleaved and located at the N-terminus (XU & LIU, 2014, Green & Mecsas, 2016). The signals are often encoded within the first 100 residues of the protein and ensure that the secretion proceeds in a coordinated and hierarchical manner (Costa *et al.*, 2015). Many of these effectors require chaperones to guide them to the T3SS base, where they are secreted in an ATP-dependent and unfolded state (XU & LIU, 2014, Green & Mecsas, 2016). T3SS chaperones specifically engage with the substrates' secretion signals, ensuring stabilization of the protein and guiding it to the recognition site of the secretion complex (Costa *et al.*, 2015). Some substrates of the T3SS are listed in Table 5.

Table 5: Examples of T3SSs and substrates in different bacterial pathogens (Coburn *et al.*, 2007)

| Organism | Structure/Translocator | Effectors | Associated Disease ^a |
|--|--|--------------------------------|---|
| <i>Yersinia species</i> (<i>Y. pestis</i> , <i>Y. enterocolitica</i> , <i>Y. pseudotuberculosis</i>) | Ysc injectisome, YopB, YopD, LcrV | YopH, E, T, O and YpkA, P/J, M | Plague, enterocolitis, mesenteric lymphadenitis |
| EPEC/EHEC ^b | EspA/B/D (translocators) | Tir, Map, Cif, Orf3 | Intestinal inflammation, and bloody diarrhea, possibility of renal failure and septic shock |
| <i>Shigella species</i> (<i>S. dysenteriae</i> , <i>S. flexneri</i> , <i>S. boydii</i>) | Mxi/Spa (apparatus), IpaB/C (translocators), IpgC, (chaperone) | IpaA/B, VirA, IpaH | Bacillary dysentery (shigellosis) |
| <i>P. aeruginosa</i> | PopB and PopD (translocators), PcrV, SpcU (chaperone) | ExoS, ExoT, ExoU, ExoY | Pneumonia (nosocomial and occasionally community acquired), airway infection in cystic fibrosis, urinary tract infection, various clinical infections |

^a Only human diseases are mentioned in the table

^b EPEC: enteropathogenic *E. coli*

The T3SS has a core of nine proteins that are highly conserved among known systems. Eight of these proteins are shared with the flagellar apparatus found in many bacteria and are evolutionary related to the flagellin (XU & LIU, 2014, Green & Mecsas, 2016, Deng *et al.*, 2017). The secretion and cellular translocation (Sct) prefix was suggested as a unified nomenclature for conserved components of the T3SS (Hueck, 1998). In addition to the core nine proteins, T3SSs have an additional 10 to 20 proteins that play important roles in the proper function of this system. The structural components are typically located in a few operons, which can be found either in pathogenicity islands in the bacterial chromosome

or on plasmids (Green & Mecsas, 2016, Deng *et al.*, 2017). Several studies suggest that these gene clusters were probably acquired by horizontal gene transfer. Therefore, bacteria that are evolutionary distinct may have closely related systems and vice versa (Buttner, 2012, Green & Mecsas, 2016).

As mentioned earlier, T3SSs descended from flagella which are bacterial motility organelles that comprise a membrane-embedded basal body, attached through an extracellular hook, to a 10-20 μm long filament. The extracellular flagellar components are assembled through a T3SS (Macnab, 2003).

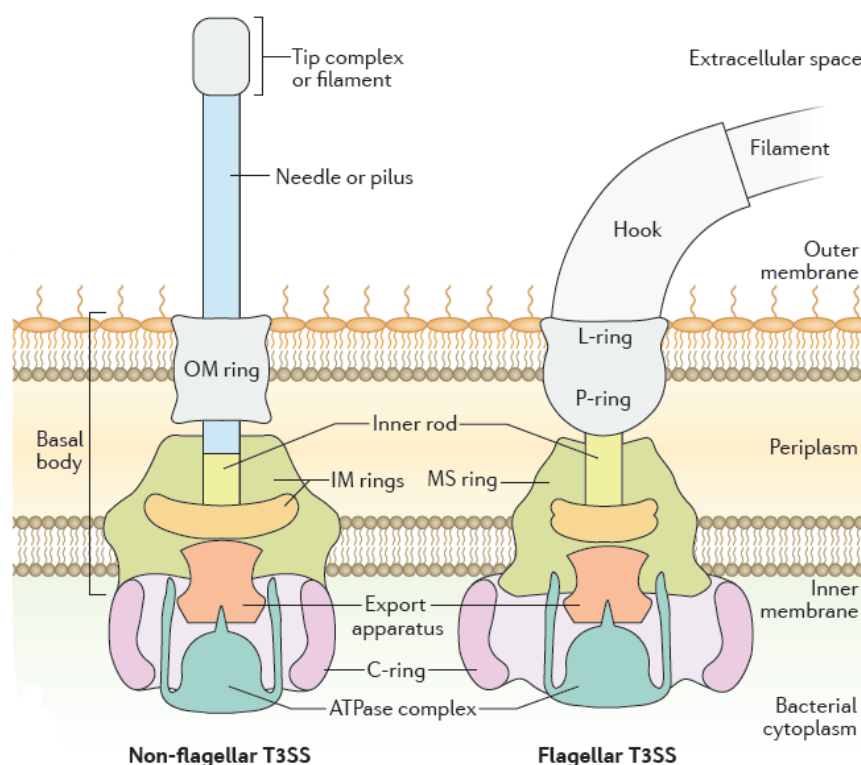


Figure 20: Structural representation of secretory and flagellar T3SSs IM ring: inner membrane ring, L-ring: lipopolysaccharide ring, MS ring: membrane/supermembrane ring, OM ring: outer membrane ring, P-ring: peptidoglycan ring. From (Deng *et al.*, 2017).

Flagellar and non-flagellar T3SSs (NF-T3SSs) are similar in structure as shown in Figure 20. Of the thirteen proteins that are shared among almost all NF-T3SSs, nine share sequence homology with flagellar components (Abby & Rocha, 2012). Phylogenetic studies of the core T3SS proteins revealed that the flagellar T3SS evolved first to transport extracellular components of the flagellum. NF-T3SSs then diverged from flagellar T3SSs initially losing flagellum-specific proteins but gaining the IM ring and inner rod components (Abby & Rocha, 2012). Thus, it changed from a motility organelle to a specialized secretion machine.

Concerning its structure, the T3SS is mainly composed of three components, a base complex or basal body, the needle component and the translocon. The base complex contains cytoplasmic components and spans the inner and outer membranes, forming a socket-like structure consisting of several rings with a central rod. Protruding from this socket and rod-like structure is a filament called the needle, it extends through the secretin and into the extracellular space (Green & Mecsas, 2016). The needle has a hollow core where the lumen of the tube is ~ 25 Å in diameter, thus only fully unfolded substrates can be transported through the narrow secretion channel (Costa *et al.*, 2015). Finally, the tip complex, which is on the outer end of the needle, is crucial for sensing contact with host cells and regulating secretion of effectors. It is also necessary for inserting the translocon into host cell membranes (Picking *et al.*, 2005). It is useful to note that translocons are assembled after contact with host cells to form the required pore for effector delivery (Holmstrom *et al.*, 2001). The structure of a T3SS complex is depicted in Figure 21.

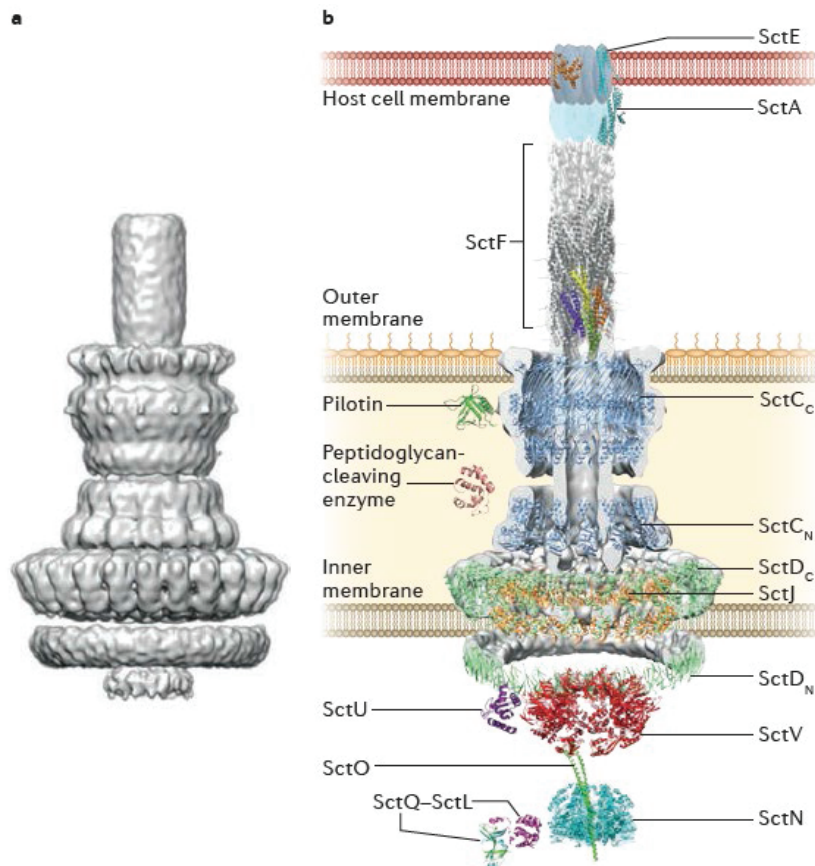


Figure 21: Structural overview and the components of a T3SS (a) The overall architecture of the basal body, needle and inner rod in *Salmonella enterica*, Salmonella pathogenicity island 1 (SPI-1) type III secretion system as determined by cryo-electron microscopy. (b) Starting from the bottom with the ATPase complex of the NF-T3SS, composed of SctN (ATPase), SctL, SctO. SctQ is a cytoplasmic ring. The export apparatus consists of SctU and SctV in addition to other components (not shown). Coming to the basal body, it consists of SctD and SctJ (inner membrane rings), SctC is the secretin or outer membrane ring and finally the needle, SctF. The needle tip is composed of SctA and translocation pore is SctE. Only the core components are shown in this figure as T3SSs can contain additional proteins. Distinct ring structures belonging to the same module are labeled as subscripts. Adapted from (Deng *et al.*, 2017).

Briefly regarding the effects of T3SS and its substrates. *Salmonella* spp. for example have two T3SSs encoded by two pathogenicity islands, SPI-1 and SPI-2. The SPI-1 is activated and required upon initial contact where it is used to invade the host, establish the *Salmonella*-containing vacuole (SCV), and inhibit host cell apoptosis. It translocates effector proteins targeting host pathways such as cytoskeleton rearrangement, ubiquitination, modulation of Rho GTPase signaling (XU & LIU, 2014, Deng *et al.*, 2017). On the other hand, The SPI-2 T3SS is only induced after the bacterium is internalized, it is used to manipulate SCV trafficking and maturation to promote survival and replication (van der Heijden & Finlay, 2012). *Chlamydia* spp. also requires the T3SS to establish an intravacuolar niche for replication and dissemination (Beekman & Vanrompay, 2010). On the other hand, *Shigella* spp. uses a T3SS to facilitate the escape from the *Shigella*-containing vacuole, the cytoplasmic survival and the ability to modulate host immunity (Raymond *et al.*, 2013). In a different approach, enteropathogenic *E. coli* and

enterohemorrhagic *E. coli* require the T3SS to attach to the intestinal epithelium of the host and subsequently inducing cytoskeletal rearrangements and interfering with the integrity of the epithelial barrier. They also use the T3SS to interrupt nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling to avoid host immune activation (Santos & Finlay, 2015).

Regarding *L. pneumophila* and the T3SS, a study found flagellum-encoding genes in 14 out of 21 *L. pneumophila* strains tested. However, no non-flagellar T3SSs were found (Qin *et al.*, 2017).

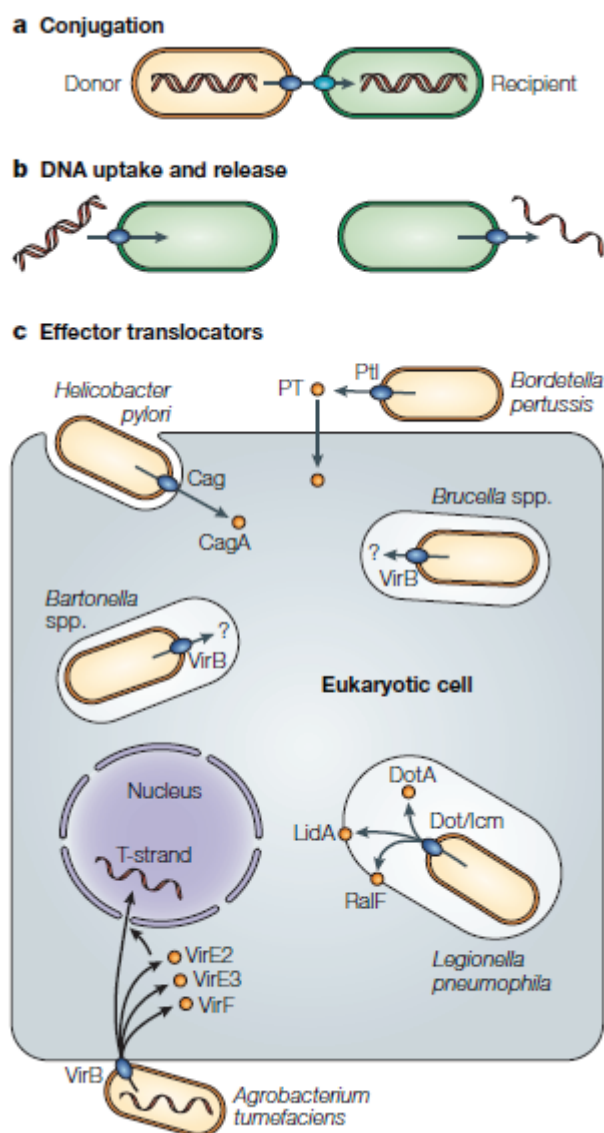
6. The Type IV secretion system

The T4SS is particularly interesting due to several facts. First is the presence of this system mainly in Gram-negative bacteria as well as Gram-positive bacteria and some *archaea* (Wallden *et al.*, 2010, Costa *et al.*, 2015, Ghosal *et al.*, 2017); Second, its impressive diversity on a functional level as well as the nature of its substrates (DNA and proteins) (XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016). That is represented by the collective capacity of T4SSs to perform multiple functions (shown in Figure 22) including (i) bacterial conjugation by recognizing and translocating single stranded DNA substrates to recipients; (ii) bacterial pathogenesis, as it delivers effector proteins to eukaryotic target cells; (iii) DNA exchange with the extracellular milieu; (iv) contribution to biofilm development and (v) delivery of killing toxin to bacterial neighbors (Grohmann *et al.*, 2018). Therefore, T4SSs can be classified into 3 functional categories. Conjugative T4SSs mediate transfer of DNA between bacterial cells which plays a role in genome plasticity and diversity. Also, it often provides a selective advantage for the cell such as antibiotic resistance (Waksman & Orlova, 2014). A second type mediates protein translocation where substrates range in size from small effector proteins to large protein complexes, it is mostly found in pathogenic bacteria (Wallden *et al.*, 2010). And a third group that mediates DNA uptake (transformation) and release from the extracellular milieu such as in *Helicobacter pylori* (Wallden *et al.*, 2010, Waksman & Orlova, 2014). In addition to the previous, the T4SS is able to transport substrates across both the inner and outer membranes, and like the T3SS, the T4SS can span an additional host membrane allowing the direct translocation of substrates in to the cytoplasm of the recipient cell (XU & LIU, 2014, Green & Mecsas, 2016).

Despite their diversity in substrates and functions, T4SSs are evolutionarily linked to the bacterial conjugation apparatus (Backert & Meyer, 2006, Green & Mecsas, 2016). They are also implicated in the pathogenesis of various bacteria through their different modes of action. In *L. pneumophila*, a T4SS is indispensable for intracellular survival in host cells (Lammertyn & Anne, 2004).

Figure 22: Main functions of the T4SS

The three main subfamilies of T4SS are displayed here. **(a)** Conjugation machinery delivers DNA to recipient bacteria and other cell types by cell-cell contact. **(b)** DNA uptake and release systems exchange DNA with the extracellular environment without the need for direct cell contact with target cells. **(c)** Finally, effector translocators deliver DNA or protein substrates to eukaryotic cells during infection. This process can vary remarkably among the bacterial pathogens shown. For example, *Agrobacterium tumefaciens* uses its T4SS to translocate oncogenic T-DNA (transfer DNA) and accessory proteins into plant cells. Whereas *L. pneumophila* translocates around 300 effector proteins into its host cytoplasm during infection in order to modulate host processes to its advantage. Similarly, *H. pylori* can transport effectors via its T4SS such as CagA that eventually causes gastritis or peptic ulcer. *Bordetella pertussis* PT toxin (pertussis toxin) causes whooping cough when it colonizes the respiratory tract. *Brucella* and *Bartonella* spp. use a VirB system during intracellular infection and cause Brucellosis (Malta fever) and cat-scratch disease respectively. From (Cascales & Christie, 2003).



Depending on genetic and structural organization, T4SS transporters can be divided into two major classes, type IVA such as the VirB/D4 in *A. tumefaciens*, and type IVB such as the Dot/Icm system in *L. pneumophila* (Backert & Meyer, 2006). *L. pneumophila* also possesses a type IVA system called *Lvh* (*Legionella* VirB homologs) where *lvh* genes were found in 40 *L. pneumophila* and seven non-*L. pneumophila* strains. The order and sequence of T4SS *lvh* genes are highly conserved among these strains (Qin *et al.*, 2017). However, the best characterized T4SS is the one of *A. tumefaciens*. It consists of 11 VirB proteins (VirB1 to VirB11) and one VirD4 protein (Waksman & Orlova, 2014, XU & LIU, 2014). On the other hand, the *Legionella* Dot/Icm T4SS is more complex than most T4SSs as it consists of ~27 components rather than 12 (Ghosal *et al.*, 2017). It is encoded by two separate pathogenicity regions on the chromosome, the first region carries *icmXWV* and *DotABCD*. The second region contains 17 genes *icmTRSQPONMLKEGCDJBF* (Lammertyn & Anne, 2004, Qin *et al.*, 2017).

A similarity between T4ASS and T4BSS occurs in the C-terminus of DotG that matches part of the VirB10 sequence. The operons' organization is represented in Figure 23.

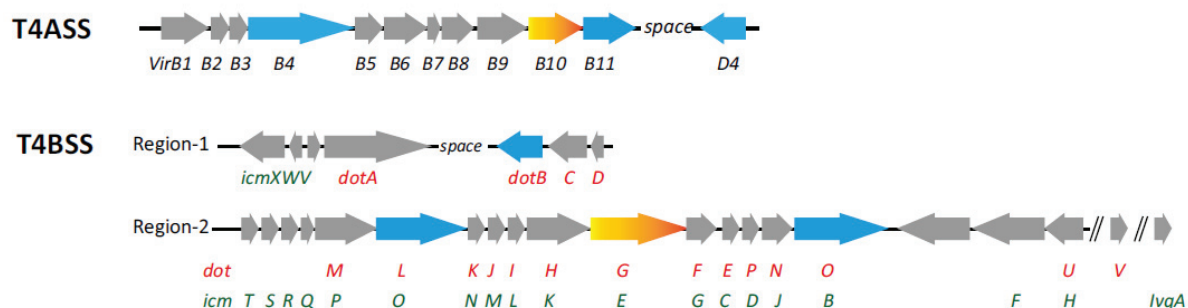


Figure 23: Genetic organization of the type IV A&B secretion systems Operons of the type IVA (VirB/D4 from *A. tumefaciens*) and IVB (Dot/Icm from *L. pneumophila*) systems are schematically represented above. Genes in blue are ATPases. VirB10 and DotG colored in orange show sequence similarity, with both proteins having a conserved TrbI domain in their C-terminus. From (Ghosal *et al.*, 2017).

Effectors translocated by the T4SS are typically recognized through a C-terminal secretion signal (Christie *et al.*, 2014). Studies also suggest that like the T3SS, some T4SS substrates interact with chaperones for proper delivery to the secretion apparatus (Nagai *et al.*, 2005, Kubori & Nagai, 2016). Table 6 contains examples of T4SS and substrates, their distribution in different organisms and their pathogenicity.

Table 6: Examples of T4SSs and substrates in various bacterial pathogens (Backert & Meyer, 2006)

| | Organism | T4SS Structure | Effectors | Associated disease |
|------------------------|------------------------|----------------|--|---|
| Extracellular pathogen | <i>A. Tumefaciens</i> | VirB/D4 (pTi) | T-DNA, VirD2, VirD5, VirE2, VirE3... | Crown gall tumors (plant cells) |
| | <i>H. pylori</i> | cagPAI | CagA, peptidoglycan | Gastritis, ulcer, cancer (human gastric epithelial cells, phagocytes) |
| Intracellular pathogen | <i>L. pneumophila</i> | Dot/Icm | RalF, LidA, Lepb, SidA-SidH, VipA, VipD... | Legionnaires' disease (human alveolar macrophages) |
| | <i>Bartonella spp.</i> | VirB/D4 | BepA-BepG | Intraerythrocytic bacteremia (mammalian erythrocytes) |

a) Type IVA secretion system

As mentioned earlier, the best characterized T4ASS is that of *A. tumefaciens*. Components of this secretion system are present in multiple copies (Waksman & Orlova, 2014, Green & Mecsas, 2016)

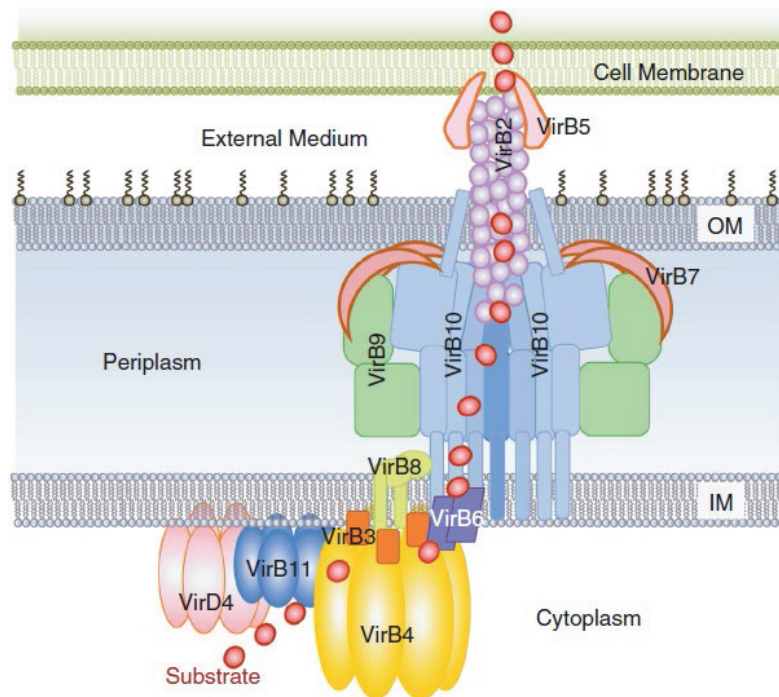


Figure 24: Structural organization of the type IVA secretion system The T4ASS comprises 3 ATPases that energize substrate translocation, VirB4, VirB11 and VirD4 (it is also a coupling protein). These proteins in addition to VirB3, the bitopic VirB8 and polytopic VirB6 constitute the cytoplasmic IM portion of the complex. VirB7, VirB9 and VirB10 compose the periplasmic part of the secretion system. Whereas VirB2 and VirB5 form the outer part of the secretion system. Red dots are the substrates being translocated within the secretion apparatus. From (Waksman & Orlova, 2014).

The 12 components of a T4ASS (VirB1 to VirB11 and VirD4) are organized in three major subcomplexes (Figure 24). The cytoplasmic inner membrane complex consists of three ATPases, VirB4, VirB11 and VirD4 in addition to VirB3, VirB6 and parts of VirB8 and VirB10 (Waksman & Orlova, 2014). VirD4, also known as the type IV coupling protein (T4CP), is a hexameric ATPase that physically couples early DNA and protein substrate processing reactions to the translocation machinery (Christie *et al.*, 2014). The second subcomplex is the core complex and it is composed of three proteins: VirB7, VirB9 and VirB10 (Figure 24). It functions as the scaffolding for other T4SS components, it also forms a channel that spans both bacterial membranes and participates actively in substrate transfer through the T4SS (Waksman & Orlova, 2014). The third subcomplex consists of the VirB2 and VirB5 proteins that make the extracellular pilus which is crucial for direct contact with the recipient cell and may act as a conduit for substrate delivery (Waksman & Orlova, 2014). One final component that is not displayed in

the above figure is VirB1, a periplasmic lytic transglycosylase that degrades the peptidoglycan layer and is required for pilus biogenesis (Costa *et al.*, 2015). T4ASSs in other bacteria may adopt a different nomenclature with few probable deviations in the compositions of these subcomplexes, but they are likely conserved in the overall organization.

Regarding *L. pneumophila* Lvh T4ASS, it contains 11 *lvh* genes homologous to genes of other T4ASSs only missing a VirB1 homolog (Lammertyn & Anne, 2004). This system was not linked to bacterial infection potential under normal conditions. However, *lvh* mutants exhibit a significant defect in entry and intracellular replication when the bacteria are pre-treated at low temperature (Lammertyn & Anne, 2004, XU & LIU, 2014). It also functions as a conjugational DNA transfer system (Lammertyn & Anne, 2004).

b) Type IVB secretion system

In this part we will focus on the *L. pneumophila* T4BSS. The Dot/Icm secretion system is a class of T4SSs that is related to conjugation systems of the IncI conjugative plasmids. However, it is much more complex than the typical T4ASS (Kubori & Nagai, 2016, Ghosal *et al.*, 2017). Therefore, T4SSs closely related to the *Legionella* Dot/Icm are classified as T4BSSs to distinguish them from the conventional T4ASSs related to the VirB system (Kubori & Nagai, 2016). Early studies on the *L. pneumophila* Dot/Icm T4SS revealed that it efficiently transfers DNA (Voth *et al.*, 2012). Later on, the *L. pneumophila* protein RalF was found to be translocated into host cells in a *dot/icm*-dependent manner (Nagai *et al.*, 2002). Therefore, RalF was the first effector protein to be identified in *L. pneumophila*. So far, it has been shown that ~300 substrates are transported by *L. pneumophila* into the host cells cytosol via the Dot/Icm system (Zhu *et al.*, 2011). This T4BSS is essential to *Legionella* virulence as *dot/icm* deficient mutant are unable to create proper LCV and to proliferate within amoebae or macrophages (Brand *et al.*, 1994).

Regarding substrate recognition, early studies showed that a C-terminal region in RalF was essential for its transport via the Dot/Icm T4BSS into host cells where removal of this signal abolished its translocation (Nagai *et al.*, 2005). Apart from a few exceptions, it is now well established that T4SS substrates have a C-terminal secretion signal (Kubori & Nagai, 2016). This task may also be facilitated by two cytosolic proteins, IcmS and IcmW which probably act as chaperones to direct secretion of multiple effectors (Voth *et al.*, 2012, Kubori & Nagai, 2016). Moreover, the binding of IcmS and IcmW to effector proteins promotes a conformational change exposing their C-terminal secretion signal (Voth *et al.*, 2012).

Concerning the structure of this system, the translocation pore consists of DotC, DotD, DotF, DotG and DotH (Figure 25). These proteins are analogous to the VirB6-VirB10 complex in T4ASSs. Also, as

mentioned earlier, DotG and VirB10 share a considerable sequence similarity where its cytosolic portion may play a role in substrate recognition (Voth *et al.*, 2012, Kubori & Nagai, 2016). DotL is distantly related to type IV coupling proteins and was suggested to function as a T4CP through binding and mediating substrate transfer to the translocation machinery along with DotM and DotN (Kubori & Nagai, 2016).

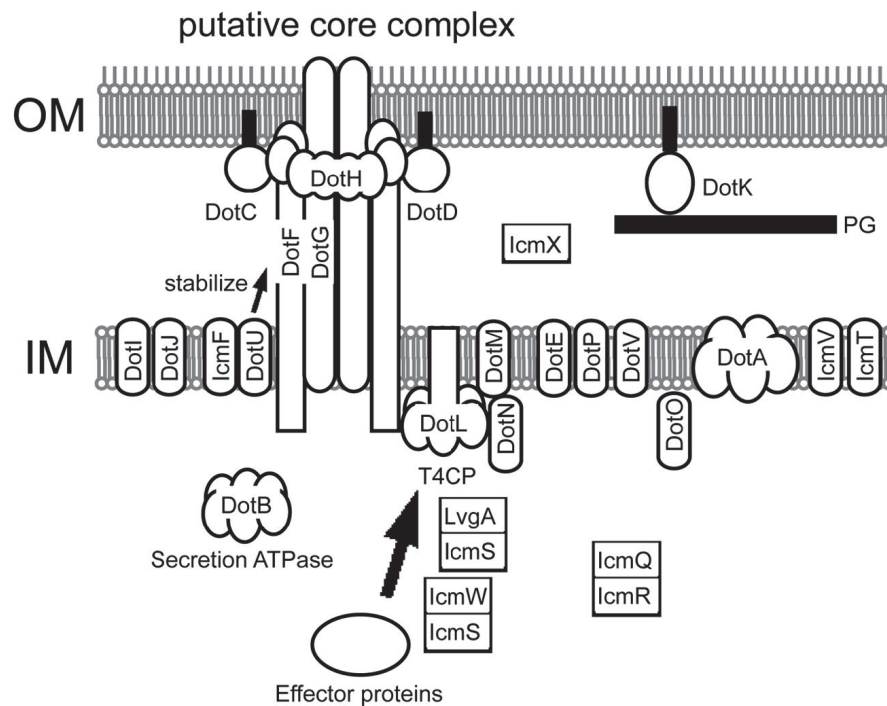


Figure 25: Structure of a *L. pneumophila* type IVB secretion system The Secretion system core complex is formed by DotC, DotD, DotG, DotH. Assembly of the system probably begins when outer membrane proteins, DotC and DotD recruit the periplasmic DotH forming a DotC-D-H complex. The carboxy-terminal region of DotG participates in the outer membrane complex resulting in a structure spanning both membranes. DotF then participates in this complex forming the complete channel for translocation. DotU and IcmF are inner membrane proteins involved in stabilization of the secretion complex. Several Dot/Icm proteins depicted above are still of unknown function. PG: peptidoglycan. From (Nagai & Kubori, 2011).

DotA is a mysterious yet critical component of the T4BSS, it is required for Dot/Icm activity and defective *dotA* mutants are incapable of intracellular replication (Nagai & Kubori, 2011). It is well conserved in T4BSSs and it is somehow secreted to the extracellular medium in culture grown *L. pneumophila* in a Dot/Icm-dependent fashion (Nagai & Kubori, 2011, Voth *et al.*, 2012). DotK is a lipoprotein that probably tethers the secretion complex to peptidoglycan (Figure 25) (Voth *et al.*, 2012). IcmQ and IcmR partner to form pores in membranes (Coers *et al.*, 2000).

Recently, electron cryotomography (ECT) has been applied to visualize the *in situ* structure of the Dot/Icm system. As shown in Figure 26, they appear as cone shaped particles near the cell poles almost adopting a “Wi-Fi” symbol conformation (Ghosal *et al.*, 2017). Distinct densities were resolved in the

subtomogram average, a hat like structure with α and β densities near the outer membrane; a stem, a stalk and γ densities in the periplasmic region. Weaker densities called wings extend from the inner membrane to the periplasm, and finally rod-like densities in the inner membrane towards the cytoplasm (Ghosal *et al.*, 2017).

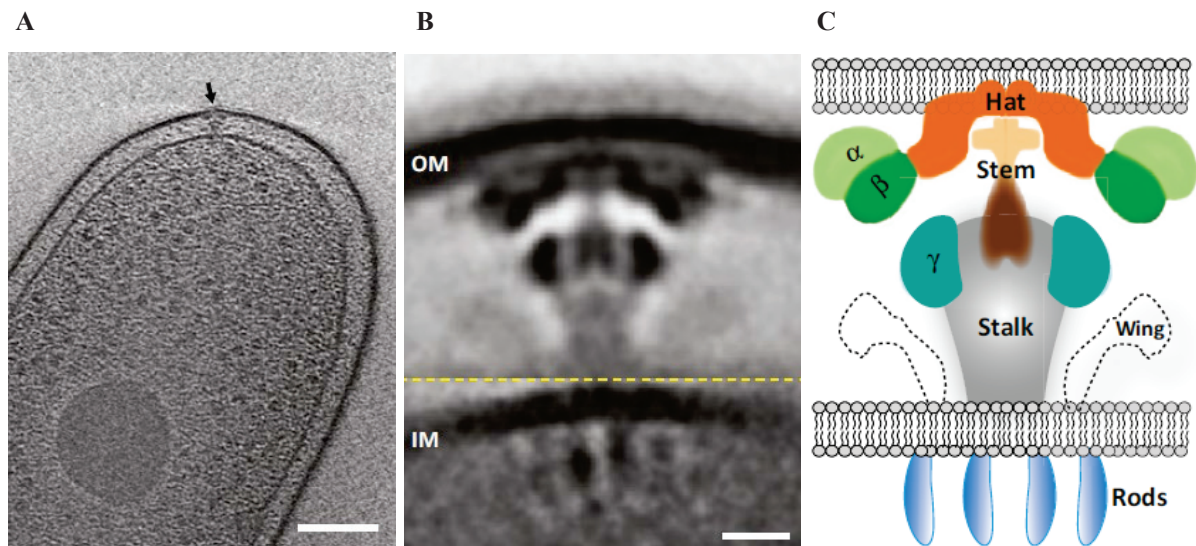


Figure 26: High resolution imagery of *L. pneumophila* Dot/Icm system (A) electron cryotomography of *L. pneumophila* cell slices, black arrow points to Dot/Icm particles. Scale bar 100 nm. (B) subtomogram average of wild-type Dot/Icm particles generated using 386 particles. Scale bar 10nm. (C) a schematic representation of the subtomogram average labeling the most prominent densities. Adapted from (Ghosal *et al.*, 2017)

7. The type V secretion system

The type V secretion is a unique system also known as the autotransporter system, this is due to the fusion of the substrate and its secretion pore in a single polypeptide. This means that a single polypeptide can drive its own secretion through the outer membrane (XU & LIU, 2014, Costa *et al.*, 2015, Fan *et al.*, 2016, Green & Mecsas, 2016). This system is found in various Gram-negative bacteria including environmental and pathogenic species (van Ulsen *et al.*, 2014). Its substrates are mainly virulence proteins including adhesins, proteases and toxins which are important for bacterial survival and virulence (XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016). These substrates are translocated in a two-step process similar to that of T2SS, they possess an N-terminal Sec signal allowing the translocation across the inner membrane via the Sec system. This signal sequence is cleaved upon passage to the periplasm (van Ulsen *et al.*, 2014). Some examples of these substrates are shown in Table 7.

Table 7: Examples of T3SS substrates and functions (van Ulsen *et al.*, 2014, Green & Meccas, 2016)

| Organism | Protein substrate | Function |
|--------------------------------|---------------------------|--|
| <i>Neisseria gonorrhoeae</i> | Immunoglobulin A protease | Cleavage of host antibodies |
| <i>Shigella flexneri</i> | IcsA | Adhesion & actin based intracellular motility |
| | SigA | Cytopathic effect on host cells by cleaving intracellular targets |
| <i>Yersinia enterocolitica</i> | YadA | Promotes translocation of T3SS substrates into hosts & mediates resistance to host complement system |
| <i>Helicobacter pylori</i> | VacA | Pore formation & activation of apoptosis |
| <i>E. coli</i> | TibA | Adhesin/invasion, bacterial aggregation & biofilm formation |

Another type of substrates using the T5SS usually utilizes partner proteins to form OM pores. This allows to divide T5SS into two major subtypes depicted in Figure 27, the autotransporters (type Va) and the two-partner secretion systems (type Vb). However, T5SS also includes 3 additional subtypes Vc, Vd and Ve (van Ulsen *et al.*, 2014).

Concerning autotransporter secretion, they are present in all Gram-negative bacterial genera (van Ulsen *et al.*, 2014). The transported proteins consist of several domains, a translocator domain at the C-terminus that is responsible for the formation of the outer membrane channel, a linker domain and a passenger domain that constitutes the functional part of the autotransporter protein. And sometimes, a protease domain is present to cleave the passenger domain after it passes through the channel (Leyton *et al.*, 2012). After transporting the unfolded autotransporter protein through the IM via the Sec system, the Sec signal will be cleaved off and the translocator domain of the matured protein will assemble in the outer membrane forming a 12-stranded β -barrel, usually with the assistance of accessory factors (Henderson & Nataro, 2001, van Ulsen *et al.*, 2014, Green & Meccas, 2016). After that, the passenger domain will transverse this channel reaching the extracellular milieu. Once it reaches the cell surface, most autotransporter passenger proteins are proteolytically cleaved and can remain attached to the cell surface via non-covalent interactions or be released into the extracellular milieu (Leyton *et al.*, 2012, van Ulsen *et al.*, 2014, Green & Meccas, 2016).

Regarding the two-partner secretion (TPS), it is important to note that the majority of T5SS substrates are secreted via the autotransporter mechanism. However, a few depend on several polypeptides for transport outside the cells (Green & Meccas, 2016). As the name implies, the first partner of this system is the secreted protein generically named TpsA and the other partner carries the β -barrel domain for pore formation named TpsB (van Ulsen *et al.*, 2014). Both partners contain an N-terminal Sec signal for transport through the IM. Once in the periplasm, TpsB inserts into the outer membrane as a 16-stranded β -barrel. As for the secreted protein TpsA, after cleavage of its signal peptide, it targets its N-terminal Tps domain to the TpsB allowing the recognition and secretion across the outer membrane. After that,

the TpsA protein may be released or remains attached to the cell surface (Henderson *et al.*, 2004, van Ulsen *et al.*, 2014). The TPS system is mainly responsible for transporting large virulence proteins such as the filamentous hemagglutinin of *Bordetella pertussis* and the large adhesins HWM1 and HWM2 of *Haemophilus influenzae* (Lambert-Buisine *et al.*, 1998, McCann & St Geme, 2014).

The figure below depicts the basic structure of the two subtypes a and b discussed earlier in addition to type Vc.

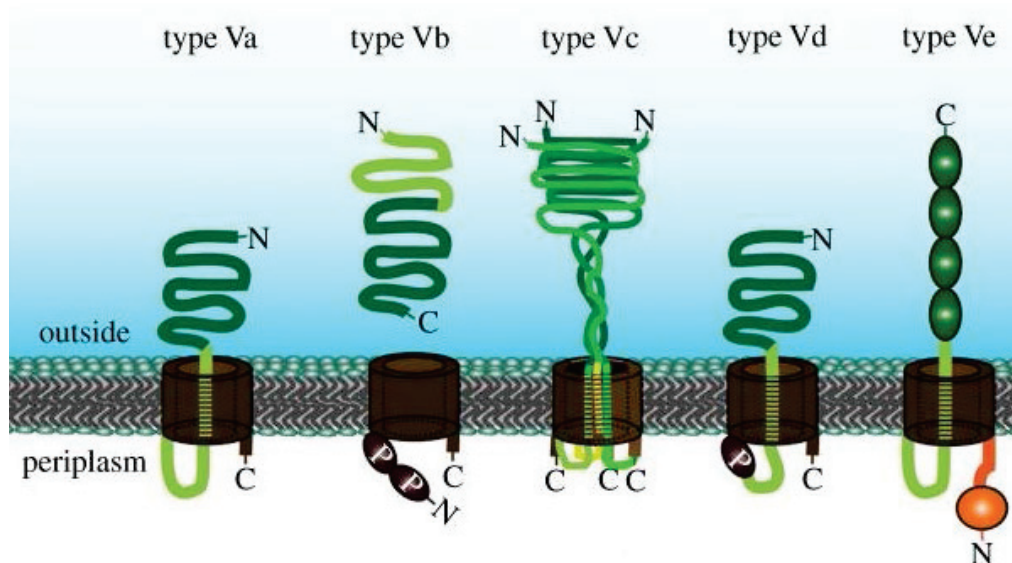


Figure 27: Schematic representation of the Type V secretion systems This system usually comprises four functional domains (signal, passenger [dark green], linker [light green] and β -barrel [brown]). Substrates use the Sec system for passage through the IM, the signal sequence is cleaved then the β -barrel inserts into the OM forming a pore. After that, the passenger domain will cross through the β -barrel and into the extracellular medium. Type Vc are similar to type Va autotransporters but are usually trimeric (Leo *et al.*, 2012).

A subcategory of the T5SS is the chaperone usher pathway. It involves proteins secreted with the help of two other proteins, the usher proteins that forms the β -barrel channel in the OM and a periplasmic protein which facilitates folding of the substrate prior to delivery to the channel (Waksman & Hultgren, 2009). This pathway is used to assemble and secrete multi-subunit appendages called pili or fimbriae, thus contributing to pathogenicity and biofilm formation (Costa *et al.*, 2015).

In conclusion, the Va subtype corresponds to monomeric autotransporters (Gawarzewski *et al.*, 2014), Vb to two-partner systems (ur Rahman *et al.*, 2014), Vc to trimeric autotransporters (Lyskowski *et al.*, 2011), Vd to patatin-like autotransporters (Salacha *et al.*, 2010) and Ve to the inverse autotransporters (intimin-invasin protein family) (Leo *et al.*, 2015). However, recently, few authors proposed to modify this nomenclature and to rename some type V secretion systems as type VII (Desvaux *et al.*, 2009) As

the type VII secretion system nomenclature is still referring to a specific secretion system identified in *Mycobacterium* species with no common feature of the type 1 pilus secretion system (Groschel *et al.*, 2016). On the other hand, this new nomenclature does not seem to be adopted by all authors. A clarification will be necessary in the future to avoid any confusion between the T7SS of *Mycobacteria* and T7SS in Gram-negative bacteria.

Concerning *L. pneumophila*, a unique potential autotransporter protein (lpp0779) corresponding to a Type Va secretion system has been first described in *Legionella pneumophila* Paris genome (Cazalet *et al.*, 2004). It contains an N-terminal leader peptide for secretion across inner membrane and a C-terminal domain forming a pore in the outer membrane. The autotransporter passenger domain composed of hemagglutinin repeats can pass to the cell surface via this pore and presents high homologies with *E. coli* autotransporters. AIDA-I and Ag43, two proteins that are implicated in virulence mediating adherence to mammalian cell and also involved in cell-cell aggregation (Cazalet *et al.*, 2004). Astonishingly, the presence of this autotransporter seems to be restricted to a really small panel of strains in *L. pneumophila* species such as Paris or Leg01/20 and in one other *Legionella* strain: sp. 39-23. However, the role of this T5aSS in *Legionella* virulence still has to be demonstrated.

8. The type VI secretion system

The T6SS is one of the most recently identified transport system in Gram-negative bacteria, it was functionally defined in 2006 (XU & LIU, 2014, Costa *et al.*, 2015, Green & Mecsas, 2016). It is a cell envelope spanning machine responsible for translocating effector proteins into both eukaryotic and prokaryotic cells cytoplasm where it plays an important role in pathogenesis and bacterial competition (Ho *et al.*, 2014).

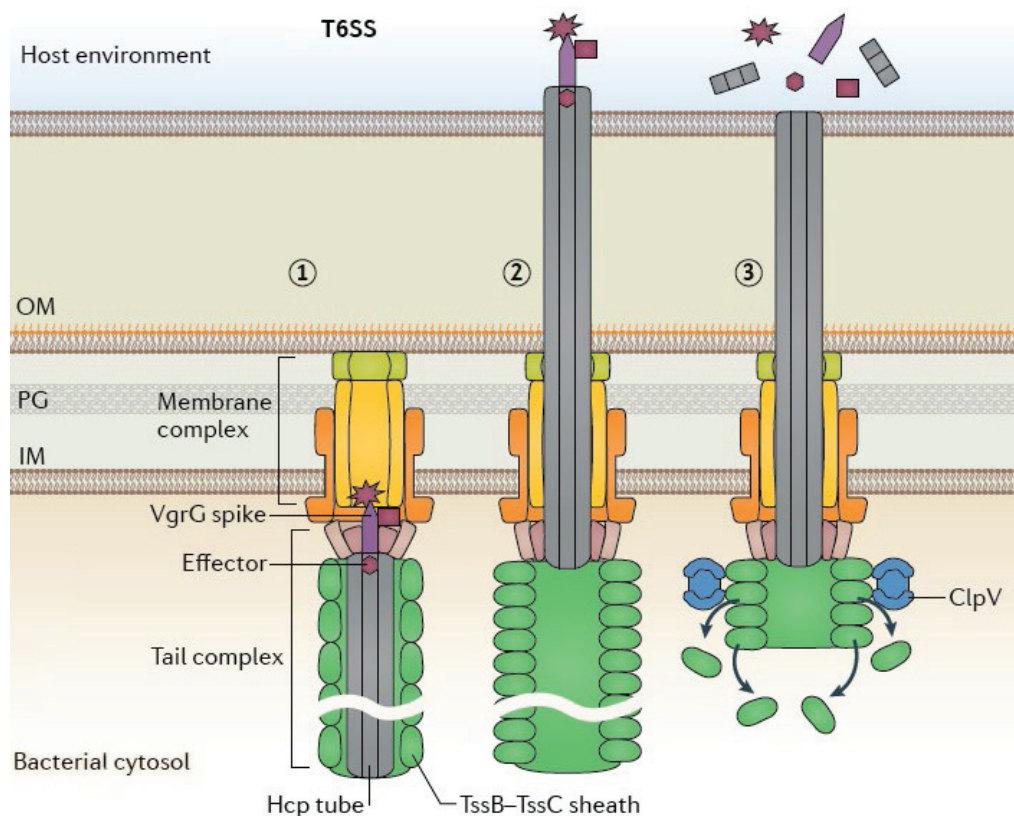


Figure 28: Mode of action of a type VI secretion system **Step 1.** The T6SS tail complex assembles onto the membrane complex. Through the hemolysin co-regulated protein, effectors are recruited to the spike-tube complex via the extension domains of VgrG. **Step 2.** Following the stimulation via an unknown extracellular signal, the TssB-TssC sheath contracts leading to the ejection of the spike-tube complex across a target membrane, thereby delivering effector proteins into the cell. **Step 3.** ClpV ATPase disassembles the contracted TssB-TssC sheath, allowing a new T6SS to be reassembled using the released subunits. Adapted from (Costa *et al.*, 2015).

This system was initially discovered in *V. cholerae* and *P. aeruginosa*. However, database search suggests that it is widespread among almost 100 bacterial species (XU & LIU, 2014). These systems are well conserved among Gram-negative bacterial species where nearly a quarter of sequenced genomes contain genes for T6SS components (Russell *et al.*, 2011). In principle, this system injects protein substrates from the bacterial cytoplasm into recipient eukaryotic or other bacterial cells in a single step and in a contact dependent manner. The translocated effectors function in both bacterial pathogenesis and competition (XU & LIU, 2014, Green & Mecsas, 2016).

Regarding structure, the T6SS is very large with up to 21 proteins encoded within a contiguous cluster. 13 of these proteins are considered as core components and appear to be conserved in all T6SSs where they play a structural role in the secretion apparatus (XU & LIU, 2014, Green & Mecsas, 2016). The machinery consists of two main complexes, a membrane complex that comprises IM proteins which are homologous to components of the T4SS, and a tail complex that contains components that are evolutionarily related to contractile bacteriophage tails (Costa *et al.*, 2015). It was hypothesized that

T6SSs may have arisen from inverted phage tails that eject proteins outside of the bacterial cell (Green & Mecsas, 2016). The proposed mechanism of substrate delivery is that during secretion, the energy generated by sheath contraction propels protein substrates through the inner tube, then a tip formed by VgrG punctures the target membrane leading to substrate delivery (shown in Figure 28). It is useful to note that several studies implicate VgrG and Hcp in virulence and pathogenicity of several bacteria confirming these proteins as effectors as well (Zhou *et al.*, 2012, Wang *et al.*, 2018). A study showed that the C-terminal domain of *V. cholerae* VgrG-1 is able to cause actin cross-linking in amoebae and mammalian cells (XU & LIU, 2014).

T6SS effectors can have various functions, many of them are directed against the bacterial cell wall and membrane supporting the role in bacterial competition (Green & Mecsas, 2016). Many of these effectors are encoded next to a gene that provides immunity to the effector, thereby preventing self-intoxication (Russell *et al.*, 2014). Antibacterial substrates can be divided into two groups, the T6SS amidase effector (Tae) proteins and the T6SS glycoside hydrolase effector (Tge) proteins, they function to degrade the peptidoglycan component of the bacterial cell wall (XU & LIU, 2014). A third group is also responsible for degrading the lipid component, they are called the T6SS lipase effector (Tle) proteins (Russell *et al.*, 2013).

In *Legionella*, a study by Qin *et al* in 2017 identified the presence of *icmF* gene in 3 strains, in *Legionella* it is a T4SS gene but also it is one of the T6SS in *E. coli*. Another 3 *Legionella* strains harbored a gene cluster identified as T6SS.

9. The type VII secretion system

First instances of the type VII secretion system were identified in mycobacteria. These bacteria are generally characterized by the presence of fatty acids called mycolic acids in their cell envelope which form the main constituent of a second hydrophobic layer surrounding the cytoplasmic membrane. This outer membrane serves as a protective layer against desiccation, antimicrobial compounds and mechanical stress (Houben *et al.*, 2014). Therefore, the cell envelope of these bacteria is diderm and protein secretion is as problematic as for Gram-negative bacteria (Ates *et al.*, 2016).

The first identified secreted proteins of *Mycobacterium tuberculosis* were EsxA and EsxB, their surrounding genes were found to play a role in their secretion and this region is now known as the *esx-1* locus and contains 20 genes. It is worthy to note that ESX-1 is a major virulence factor for *M. tuberculosis* (Houben *et al.*, 2014, Ates *et al.*, 2016). Interestingly, a role of ESX-1 in a special conjugation process in *M. smegmatis* has been identified (Houben *et al.*, 2014). These ESX systems are required for secretion over the diderm mycobacterial cell envelope and were named type VII secretion systems (Ates *et al.*, 2016). As mentioned earlier, type VII secretion system are not exclusive for

pathogenic mycobacteria; they can also be found in non-pathogenic mycobacteria and other bacteria notably in several *firmicutes* species such as *Bacillus* and *Staphylococcus* (Ates *et al.*, 2016, Bottai *et al.*, 2017).

Mechanism of transport via T7SSs is not fully elucidated yet, for example it is proposed that secretion comprises a single step but no outer membrane components have been identified (Houben *et al.*, 2014, Ates *et al.*, 2016, Bottai *et al.*, 2017). Concerning substrates of this system, they do not contain classical signal sequences and therefore do not depend on Sec or Tat for secretion. However, they do contain a C-terminal secretion motif (Houben *et al.*, 2014). A model of transport by T7SS is depicted in Figure 29.

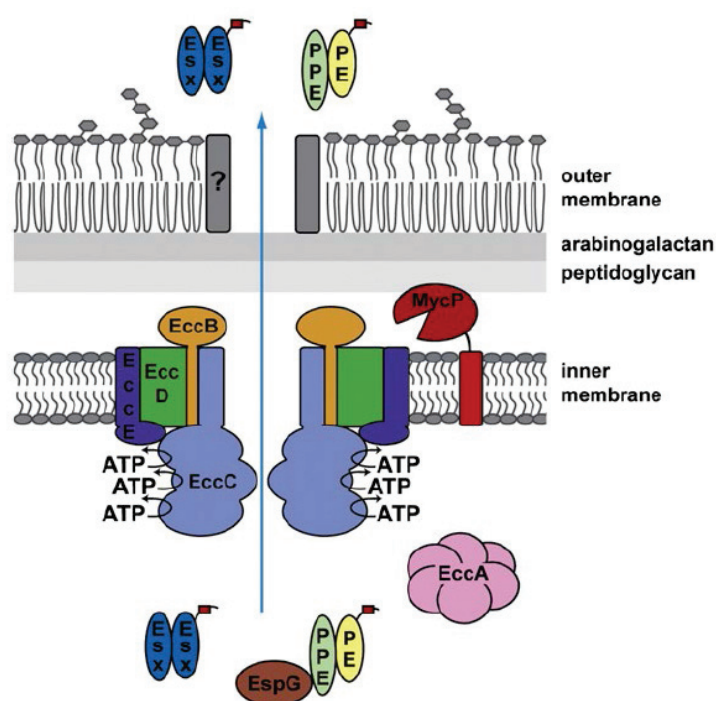


Figure 29: Model of T7SS in mycobacteria Both Esx and PE/PPE are exported as dimers by the secretion machinery where they are recognised via a C-terminal signal motif. EspG (brown) and EccA (pink) act as cytosolic chaperones for PE/PPE dimers. Energy for transport is provided through ATP hydrolysis by EccC (light blue) ATPase. Mycosin (MycP [red]) is not part of the core complex but essential for successful secretion. The inner membrane complex consists of EccB, EccC, EccD and EccE. In this model, T7S is a two-step process where the channel in the outer membrane refers to a hypothetical pore. (Houben *et al.*, 2014)

10. The type IX secretion system

The recently discovered T9SS (Por secretion system [PorSS] or Perigote) is the protein export pathway of bacteria of the Gram-negative Fibrobacteres-Chlorobi-Bacteroidetes superphylum. T9SS can play two roles, the first is providing a means of movement or gliding motility (Lasica *et al.*, 2017). This is evident in Bacteroidetes where they exhibit a unique and rapid gliding motility in which cell surface adhesins move on helical tracks (Shrivastava *et al.*, 2016), this mechanism is tightly linked to the T9SS through adhesin export (McBride & Nakane, 2015). The second role concerning T9SSs is pathogenicity, this can be seen in *Porphyromonas gingivalis*, a human oral pathogen that is the major causative agent of periodontitis, where T9SS translocates proteins especially virulence factors to the outer membrane (Lasica *et al.*, 2017, Lauber *et al.*, 2018).

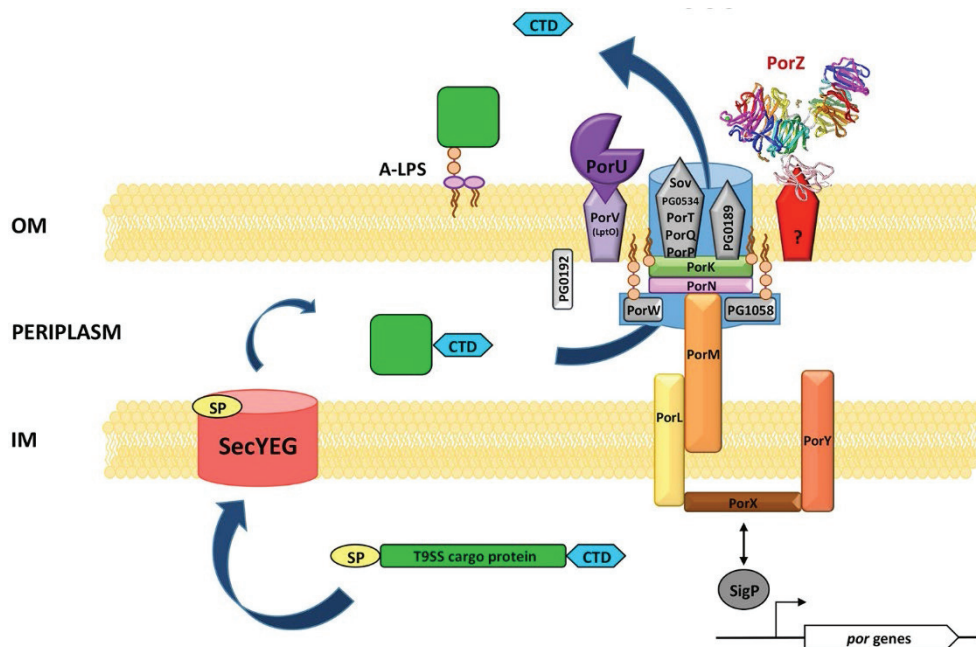


Figure 30: Hypothetical model of the *Porphyromonas gingivalis* T9SS the OM translocon components are not yet fully characterized, it is shown as a background blue shape containing known components. As shown, the T9SS substrate carries two sorting signals: a classic N-terminal signal peptide (SP) directing the protein to the SecYEG translocon and a conserved C-terminal domain (CTD) recognized by the T9SS. Substrates are generally folded in the periplasm, then CTD directs proteins for further translocation across the OM. Finally, this CTD is cleaved off by PorU sortase and the secreted protein anchored to the cell surface by attachment of A-LPS to its C-terminal end. Two-component system PorX/ProY and sigma factor SigP have regulatory effect on *por* genes but exact mechanisms need to be clarified. From (Lasica *et al.*, 2017).

Regarding T9SS substrates, they are large multi-domain proteins that fold in the periplasm before being exported through an outer membrane translocon. Their transport is directed by a C-terminal domain that guides substrate export (Lauber *et al.*, 2018). This transport is a two-step process where the cargo proteins are first guided by a classical signal peptide to the Sec machinery in the inner membrane. This

signal sequence is cleaved upon translocation to the OM by a protease with sortase-like activity and an anionic LPS is attached to the newly formed C-terminus. Finally, this transported protein could be secreted to the extracellular milieu or attached to the bacterial surface (Lasica *et al.*, 2017). The function of the T9SS is regulated by a two-component system but the specific environmental signal(s) that trigger it has not been identified (Lasica *et al.*, 2017). A schematic representation of transport by T9SS is shown above.

D. RtxA – a *L. pneumophila* virulence associated protein

The RTX (repeats in toxin) group of proteins is a large and growing family of proteins with diverse biological functions, these toxins are important virulence factors secreted by various Gram-negative bacteria (Linhartova *et al.*, 2010, Chenal *et al.*, 2015). Traditionally, they have been categorized in two groups, hemolysins, which affect a wide variety of cells and leukotoxins that are more cell type specific (Lally *et al.*, 1999, Satchell, 2011). RTX toxins falling in these groups are able to form pores in the membranes of their eukaryotic target cells and this directly contributes to their cytotoxic activities (Chenal *et al.*, 2015). However, as this protein family has been expanding, several other classes have been identified such as the multifunctional-autoprocessing RTX (MARTX) toxins originally identified in *Vibrio* species (Satchell, 2011), as well as RTX proteins with adhering capacities that play a role in biofilm formation and hence named biofilm associated proteins (BAPs) such as the *P. fluorescens* LapA (El-Kirat-Chatel *et al.*, 2014).

A characteristic feature of RTX proteins group is the presence of several glycine and aspartate-rich nonapeptide repeats with a consensus sequence GGXGXDXUX (G: glycine, D: aspartate, U: any large hydrophobic amino acid, X: any amino acid) (Lally *et al.*, 1999, Linhartova *et al.*, 2010, Satchell, 2011, Kanonenberg *et al.*, 2013, Chenal *et al.*, 2015). This RTX motif constitutes a calcium binding sequence (Kanonenberg *et al.*, 2013, Chenal *et al.*, 2015, Bumba *et al.*, 2016). Some RTX proteins have even been shown to bind calcium in solution (Ostolaza *et al.*, 1995). Another feature is that all RTX proteins are secreted via a dedicated type I secretion system that is similar in most cases except for MARTX secretion which is achieved via an atypical four-component T1SS rather than the traditional three-components (Linhartova *et al.*, 2010, Chenal *et al.*, 2015). Also, in most cases, the T1SS genes are in the same genetic locus as the RTX substrates as displayed in Figure 31. The secretion signal for these proteins is noncleavable and located at the C-terminus of the polypeptide downstream to the RTX motifs (Kanonenberg *et al.*, 2013, Chenal *et al.*, 2015). Regarding the biological functions of these proteins, various functions have been observed such as pore-forming toxins, metalloproteases, lipases, iron-regulating proteins, nodulation-related proteins, proteins implicated in S-layer formation, bacterial adherence/motility or host-receptor interaction (Chenal *et al.*, 2015). It was hypothesized that the family

of RTX containing bacteria may have originated from *Pasteurellaceae* and spread to different species via several horizontal gene transfer events (Chenal *et al.*, 2015).

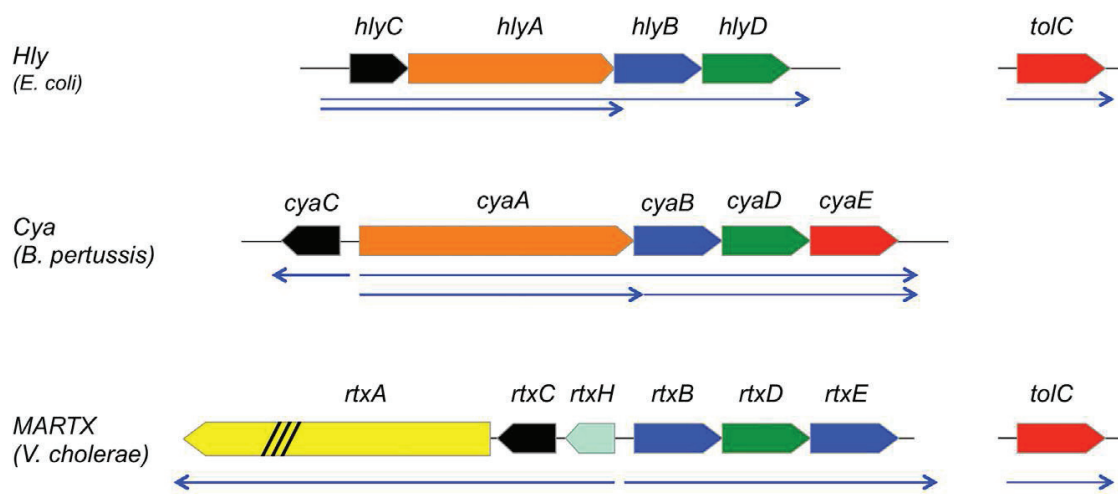


Figure 31: Genetic organization of various *rtx* loci Arrows represent the different coding regions of RTX protein operons. *rtxA* genes coding for RTX proteins are in orange and yellow. *rtxC* genes coding for acyltransferases required for activation of cytolytic function of some RTX hemolysins are in black. *rtxB* coding for the IM ABC transporter are in blue. *rtxD* genes coding for the MFPs are in green and genes encoding the OMP of the T1SS are in red. The arrows indicate different transcripts. In these examples, the RTX protein is in close proximity to its corresponding secretion system. Adapted from (Chenal *et al.*, 2015).

RTX proteins share many common features, one is being large in general, mostly greater than 50 kDa in size and frequently very large up to 1000 kDa (Kanonenberg *et al.*, 2013). They are usually acidic and the RTX repeats can vary in number from 6 to more than 50 (Linhartova *et al.*, 2010, Chenal *et al.*, 2015). Moreover, RTX proteins display calcium dependent activities which in case of cytolytic RTX toxins results from the ability to form short-lived cation selective pores in lipid membranes (Chenal *et al.*, 2015). In fact, calcium binding is required for functional activation of these proteins, calcium binds to the RTX motifs and triggers their folding into a parallel β -roll structure that is required for maturation of this toxin as shown in Figure 32 (Chenal *et al.*, 2015). Interestingly, since calcium ion concentration is low in the cytoplasm (300-500 nM) compared to concentration in the extracellular space which is in the mM range, this constitutes an efficient mechanism to prevent folding of these proteins inside the cytoplasm and promoting folding after secretion via the T1SS (Kanonenberg *et al.*, 2013).

In addition to calcium requirement for proper folding and export (ratchet mechanism) and maturation, RTX toxins are generally synthesized as inactive proteins that require post-translational activation prior to their export. This is carried out by RtxC which acylates one or two key lysine residues (K564 and K690 in HlyA) in the middle region of RTX polypeptide (Lally *et al.*, 1999, Chenal *et al.*, 2015). This acylation is a key factor in attachment of the RTX toxin to the cell membrane of target cells (Lally *et al.*, 1999).

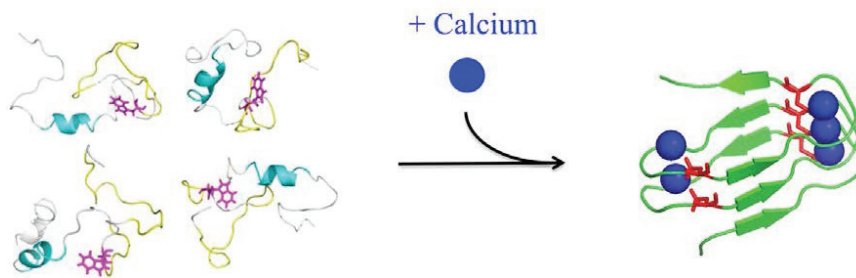


Figure 32: Structure of an RTX domain before and after calcium-induced folding The RTX domain of the alkaline protease from *P. aeruginosa* (residues 326-377). In absence of calcium (left), RTX motifs are disordered and adopt a pre-molten globule conformation. Binding calcium ions will trigger compaction, dehydration and folding of the motifs into stable β -roll conformation. From (Chenal *et al.*, 2015).

Regarding the structure of RTX proteins, they generally comprise four distinct regions: (i) a N-terminal hydrophobic region that is 200-300 residues long and contains several hydrophobic and amphiphilic α -helical structures, these structures interact and insert into the target cell membrane (Valeva *et al.*, 2008). (ii) a central region harboring one or two lysine residues that are fatty-acylated by the RtxC acyltransferase (Stanley *et al.*, 1994). (iii) an RTX domain that comprises a variable number of calcium-binding nonapeptide repeats which are essential for RtxA to recognize target cells (Chenal *et al.*, 2015). (iv) a C-terminal secretion signal (50-60 amino acids) that is recognized by components of the T1SS machinery (Gray *et al.*, 1986, Jarchau *et al.*, 1994). Some of these features are displayed in Figure 33.

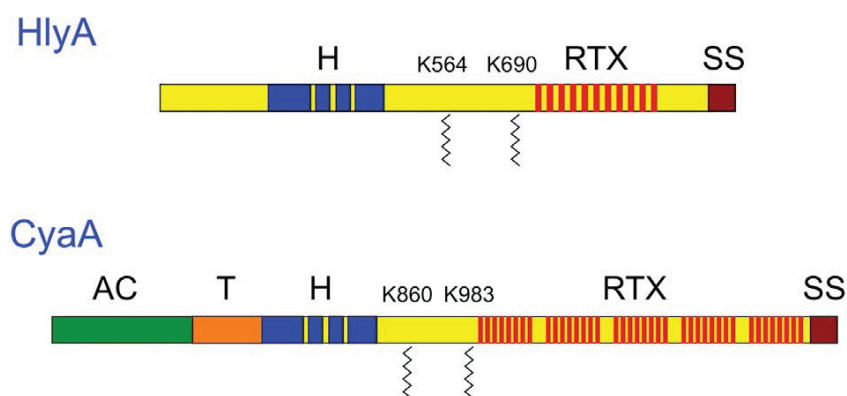


Figure 33: Schematic organization of HlyA and CyaA RTX toxins Hemolysin A (Hly A) from pathogenic *E. coli* and Adenylate cyclase toxin (CyaA) from the *Bordetella* species are displayed above. The different domains of these proteins are indicated by the following symbols. H: hydrophobic domains; RTX: RTX motifs; SS: secretion signal; AC: adenylate cyclase (CyaA catalytic domain) and T: CyaA translocation domain. The acylated lysine residues are also noted above. From (Chenal *et al.*, 2015)

Concerning the attachment of RTX toxins to host membrane, it is thought to comprise at least two phases: a passive adsorption phase onto the target cell surface and a membrane insertion phase that results in an irreversible conformational change (Lally *et al.*, 1999). The acylated residues play an important role in this attachment as well other factors such as electrostatic attraction and protein-protein interactions, these acyl groups could be the initial site of interaction between RTX toxins and their target cells. Moreover, the RTX repeat domain does not appear to be involved in cell lysis but is indeed implicated in toxin-target cell interactions (Lally *et al.*, 1999). As for RTX toxin induced cell death, studies showed that LtxA from *Actinobacillus actinomycetemcomitans* when present in high concentrations can induce very rapid cell death similar to necrosis, while at lower concentrations, target cells exhibit morphological and biochemical changes associated with apoptosis (Lally *et al.*, 1999). This can be attributed to the size and ion permeability of pores formed in these different cases. The reason behind this might be the oligomerization of RTX molecules leading to fusion of transmembrane pores and consequently rapid cell death. While at low concentrations, individual pores will only lead to injury of cells and progressive slow cell death (Lally *et al.*, 1999).

1. HlyA-A model RTX hemolysin

The most well defined RTX toxin is the α -hemolysin HlyA, it is produced by a variety of uropathogenic *E. coli* (UPEC) strains, and occasionally by enterohemorrhagic or enteropathogenic (EHEC/EPEC) *E. coli* (Chenal *et al.*, 2015). The operon responsible for secretion of this protein is *hlyCABD*, it is usually encoded in pathogenicity islands (Bielaszewska *et al.*, 2014, Chenal *et al.*, 2015). HlyA polypeptide consists of 1024 amino acids (110 kDa) and is also subject to posttranslational modification by fatty acylation of two lysine residues, Lys-564 and Lys-90 (Chenal *et al.*, 2015). This central region containing the acylated amino acids also contributes to cell selectivity (Pellett & Welch, 1996). Its N-terminal domain is thought to interact with and insert into target cells membranes in order to create cation selective pores that will trigger cell death (Hyland *et al.*, 2001). Its RTX-containing region stretches between residues 724 and 852, it contains 11 to 13 nonapeptide repeats that are responsible for calcium binding which triggers conformational changes that are crucial for target cell recognition (Chenal *et al.*, 2015). And as most RTX proteins, the last 60 residues on the C-terminus harbor a secretion signal recognized the HlyB/D/TolC machinery (Chenal *et al.*, 2015).

HlyA can attach to a variety of mammalian cells particularly epithelial cells, lymphocytes and leukocytes (Chenal *et al.*, 2015). As mentioned earlier, it binds and inserts into cell membranes thereby voiding its barrier function causing leakage of ions, amino acids and nucleotides, and upon extensive pore formation can cause cell lysis (Chenal *et al.*, 2015). This happens at high concentrations of HlyA and is likely to be the result of direct association of the toxin with the cell membrane in a receptor independent manner (Chenal *et al.*, 2015). *In vitro* experiments revealed that HlyA can bind to and form

cation selective pores in planar lipid membranes consisting solely of phospholipids indicating that a receptor is not required (Bakas *et al.*, 1996). However, glycophorin, a membrane protein in erythrocytes was shown to act as a receptor for HlyA (Cortajarena *et al.*, 2001). In addition to the previous, the $\beta 2$ integrin LFA-1 (lymphocyte function associated antigen, also known as *CD11a/CD18*) was shown to serve as a cell surface receptor for HlyA on polymorphonuclear neutrophils (PMNs) (Lally *et al.*, 1997). HlyA can also affect non-immune cells such as in a renal epithelial line and endothelial cells (Chenal *et al.*, 2015).

The EHEC hemolysin (EhxA) can exist in two biological forms shown in Figure 34, either as free hemolysin protein or as hemolysin associated with outer membrane vesicles (OMVs) that are released by EHEC during growth (Balsalobre *et al.*, 2006, Bielaszewska *et al.*, 2014). When associated with OMVs, the hemolysin is further stabilized thus prolonging its hemolytic activity when compared to the free toxin. Moreover, these two forms also have different specificities since the free toxin lyses human microvascular endothelial cells by pore formation, whereas OMV associated toxin does not lyse these cell types but after its internalization it targets mitochondria and triggers caspase-9 mediated apoptosis (Bielaszewska *et al.*, 2014). This toxin is expressed during infection and its transcription is significantly upregulated upon contact of EHEC-HlyA producing bacteria with human intestinal epithelial cells (Brockmeyer *et al.*, 2011). Moreover, the transcription level of EHEC-*hlyA* gene is significantly higher in highly pathogenic strains compared to less pathogenic EHEC O157:H7 strains (Abu-Ali *et al.*, 2010). This proves the importance of this toxin in pathogenesis.

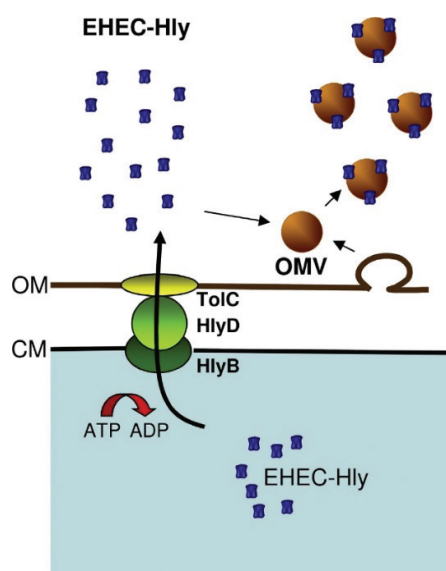


Figure 34: Secretion mechanism of free and vesicle associated EHEC hemolysin EHEC hemolysin Hly is secreted via a tripartite T1SS formed by HlyB, HlyD and TolC. The high affinity of Hly to outer membrane vesicles released from the bacterial cells, most free Hly toxin get rapidly associated with OMVs. OM: outer membrane, CM: cytoplasmic membrane. From (Bielaszewska *et al.*, 2014).

2. MARTX and other large RTX adhesins

The largest and most diverse category of RTX proteins are those that function as loosely attached adhesins. They play a role in interbacterial interactions during biofilm formation or bacterium-host interaction during infection and their encoding genes are usually the largest in the genome (Satchell, 2011). Biofilm studies in the recent years have revealed the involvement of large repetitive RTX proteins called biofilm associated proteins (BAPs) (Satchell, 2011).

Two well characterized examples are LapA and LapF in *Pseudomonas* species. For example, during biofilm formation, LapA is required for irreversible attachment to surfaces (Hinsa *et al.*, 2003). While LapF is not required for surface attachment, it is necessary for cell to cell interactions during later stages leading to proper architecture of the biofilm (Martinez-Gil *et al.*, 2010). These proteins are described briefly below.

a) LapA

A well characterize member of this family is LapA which is required for biofilm formation in both *Pseudomonas fluorescens* and *Pseudomonas putida* (Hinsa *et al.*, 2003, El-Kirat-Chatel *et al.*, 2014). LapA follows the general rules for RTX proteins as it is secreted by a T1SS, it can also be recovered from supernatants after vortexing of bacterial cultures which indicates its peripheral attachment to the bacteria (Hinsa *et al.*, 2003). LapA of *P. putida* is very large and composed of around 8682 residues, it consists of four domains (shown in Figure 35): (i) a short 277 amino acid N-terminal domain; (ii) and consists of 9 nearly perfect 100 a.a. repeats; (iii), it has 29 imperfect repeats of 218 to 225 a.a. sequence; and finally a C-terminal domain with 13 RTX repeats (Satchell, 2011).

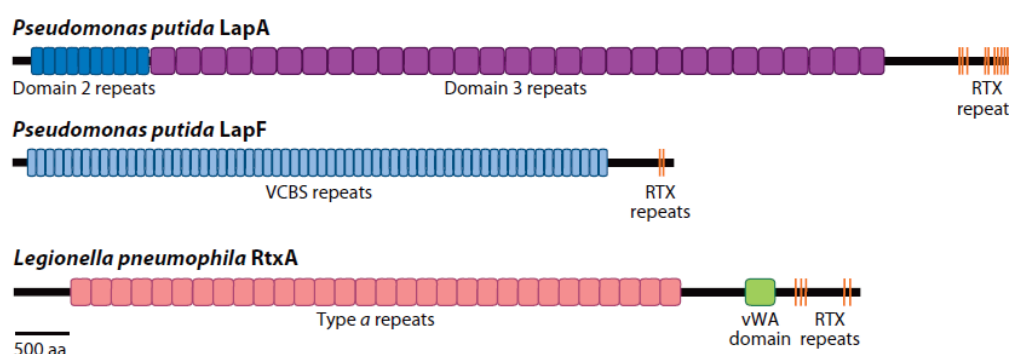


Figure 35: Schematic representation of large RTX proteins Orange lines represent the nonapeptide RTX repeats, other repeat domains are shown as colored squares with different colors representing different repeat sequences. The *L. pneumophila* (Paris strain illustrated above) RtxA contains the traditional components of an RTX protein in addition to a Von Willebrand factor type A domain (vWA) at the C-terminal region upstream of the RTX repeats. Adapted from (Satchell, 2011).

b) LapF

Regarding this RTX protein, its encoding gene is the second largest in *P. putida* and is also required for biofilm formation. It is smaller than LapA with approximately 6310 amino acids (Figure 35). Interestingly, the locus encoding LapF contains genes for type I secretion probably indicating it is secreted by its own T1SS rather than sharing one with LapA (Satchell, 2011). Its N-terminal domain is composed of 152 residues followed by 64 imperfect repeats of 83 to 91 amino acids which are not shared with LapA, and finally a C-terminus with only two copies of RTX repeats (Martinez-Gil *et al.*, 2010).

c) MARTX proteins

Multifunctional auto-processing RTX proteins are among the newly recognized RTX protein families, they were initially classified with the hemolysins subfamily of RTX proteins (Lin *et al.*, 1999). However, they were later separated as a distinct subfamily of toxins due to various features that distinguish them from pore forming RTX toxins (Satchell, 2007). There are similarities between this group and the previously described adhesins due to the frequent large size of proteins and presence of C-terminal RTX repeats, but these two subfamilies are indeed considered distinct (Satchell, 2011). The first MARTX toxin was identified in *Vibrio cholerae* where it was shown to contribute to the virulence of the bacterial host (Lin *et al.*, 1999).

Concerning the genetic locus encoding these proteins, the MARTX gene cluster contains two divergent operons: (i) *rtxH/rtxC/rtxA*; RtxH is a conserved hypothetical protein of unknown function, RtxC is the putative acyltransferase and RtxA is the toxin itself (Satchell, 2011); some studies suggest that RtxC is not necessary for MARTX function *in vitro* but contributes to toxicity *in vivo* (Satchell, 2011); (ii) the divergently transcribed *rtxBDE* operon encodes three proteins for a type 1 secretion system that works in conjunction with a TolC protein to export the toxin (Chenal *et al.*, 2015). After export, the toxin can be found in supernatant fluids either free or associated to OMVs and the outer membrane (Boardman *et al.*, 2007). The repeat regions of MARTX proteins cover as much as half of the peptide sequence and can be found at both N and C termini. Moreover, these repeats are well conserved between the toxins produced by different species (Satchell, 2007).

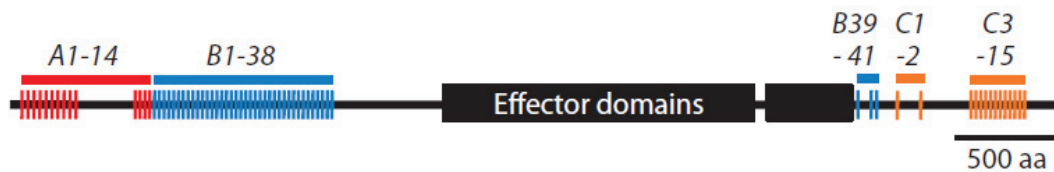


Figure 36: Repeat structure of *Vibrio cholerae* MARTX protein The line schematic shows repeat locations indicated as vertical lines. The MARTX proteins comprise a central effector domain in addition to the three types of repeats, N-terminal A and B repeat region. The B region also has 3 repeats on the C-terminal side. The 15 RTX repeats are located also at the C-terminus. Adapted from (Satchell, 2011).

As shown in Figure 36, there are 3 classes of repeats: 15 non-classical RTX repeats at the C-terminus in addition to 18 to 20 amino acid glycine rich A and B repeats at the N-terminus (Satchell, 2011). Because the RTX repeats of MARTX toxins are distinct from classical RTX repeats, they have been named C repeats in agreement with their C terminal location (Satchell, 2007). The repeat region function has not been properly elucidated but might be involved in forming pore-like structures in target membranes (Satchell, 2011).

A conserved feature of MARTX toxins is a cysteine protease domain (CPD) (Figure 37) required for auto-processing (Sheahan *et al.*, 2007). Auto-processing refers to the enzymatic release of effector domains from the main peptide sequence. The CPD is activated via the binding of inositol phosphate signal molecules, preferentially inositol hexakisphosphate (InsP₆) (Prochazkova *et al.*, 2009). The inducing role of InsP₆ of MARTX autoproteolysis is a critical factor for the function of this protein. The exact mechanism of this action is unknown but the CPD and the effector domains must reach the cytosol to allow the processing to take place since InsP₆ is a molecule found in the cytosol of eukaryotic cells (Irvine & Schell, 2001).

Regarding the effector domains, as a result of CPD auto-processing, they are released from the large toxin where they are free in the eukaryotic cytosol to induce cytotoxic and cytopathic effects (Satchell, 2011). As much as 10 domains can be recognized in MARTX toxins where only 2 have been characterized for function, these two include the actin cross linking domain and the Rho GTPase-inactivation domain. On a related note, it has been proposed that novel MARTX toxins can arise by recombination with *rtxA* genes acquired via horizontal gene transfer (Satchell, 2011). As for their involvement in virulence, a specific MARTX variant in *V. cholerae* has been shown to help induce cell rounding and loss of tight junction integrity when added to epithelial cells due to actin cross-linking and Rho GTPase-inactivating activities (Fullner & Mekalanos, 2000). Moreover, *V. cholerae* mutants that express MARTX but not other cytotoxins successfully disable macrophages and prevent bacterial engulfment suggesting that the immune system is the target of *V. cholerae* MARTX toxin (Ma *et al.*, 2009). These proteins contribute as well to biofilm formation in the environment (Satchell, 2011). In addition to hemolysins, adhesins and MARTX discussed above, the RTX family also comprises

proteases, lipases, S-layer proteins, nodulation proteins and probably many others (Linhartova *et al.*, 2010). This hints at the large diversity of this family members and that there is yet a lot to be discovered concerning this family.

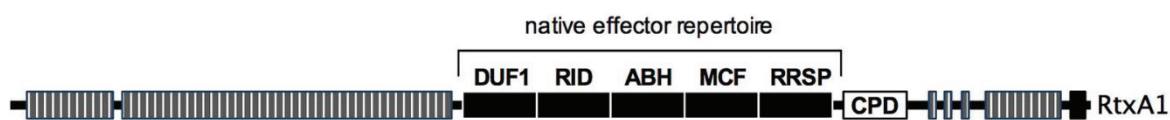


Figure 37: Schematic representation of *Vibrio vulnificus* MARTX protein Toxins produced by *V. vulnificus* MARTX protein RtxA1 are shown above in addition to other domains. DUF1: domain of unknown function in the first position; RID: Rho inactivation domain; ABH: Alpha/Beta hydrolase domain. MCF: Makes caterpillars floppy-like domain; RRSP: Ras/Rap1 Specific Protease domain; CPD: cysteine protease domain; striped boxes represent repeat regions. Adapted from (Gavin *et al.*, 2017).

3. *L. pneumophila* RtxA

Information regarding the *L. pneumophila* RtxA is still limited compared to other well described RTX toxins. However, many similarities are shared with the general features of the RTX family such as the C-terminal secretion signal, RTX repeats motif and the central repeat region (Figure 35) (D'Auria *et al.*, 2008). Regarding the secretory pathway, it has already been established through previous work in our team that RtxA is indeed transported via a T1SS LssB/LssD/TolC (Fuche *et al.*, 2015). As for the protein itself, RtxA has been previously detected in *L. pneumophila* strains (Table 8) (Cirillo *et al.*, 2002, D'Auria *et al.*, 2008). RtxA in *L. pneumophila* is a very large protein (approximately 7000 amino acids) with repeated structures belonging to at least 3 protein family domains (D'Auria *et al.*, 2008). The ability of *L. pneumophila* to invade and replicate within macrophages and amoebae has been directly linked to RtxA, where it plays an important role in the pathogenicity through adherence to cell membranes (Cirillo *et al.*, 2001, D'Auria *et al.*, 2008, Satchell, 2011). However, other studies show that *L. pneumophila* also encodes a protein called *Legionella* collagen-like (Lcl) protein which plays a role in adhesion and biofilm formation (Abu Khweek & Amer, 2018). Interestingly, this protein has a C-terminal consensus motif of outer membrane proteins and a large region of collagen like repeats where the number of repeat units has an influence on its adhesion characteristics (Vandersmissen *et al.*, 2010). This suggests that adhesion/invasion capabilities of *L. pneumophila* is probably a result of the actions of several factors.

Table 8: *L. pneumophila rtxA* structure highlights (D'Auria *et al.*, 2008)

| Strain | Locus position | Length (aa)* | Repeats* |
|-------------------------|------------------------|--------------|---|
| Paris (CR628336) | lpp0699 | 6764** | 30 (type <i>a</i>) |
| Lens (CR628337) | Lpl0681 | 7910 | 26 (type <i>b1</i>), 9 (type <i>b2</i>) |
| Corby (CP000675) | Lpc2649 | 6289 | 4 (type <i>c1</i>), 21 (type <i>c2</i>) |
| Alcoy (EU054322) | Lpa00614 | 4669 | 16 (type <i>c</i>) |
| Philadelphia (AE017354) | Lpg0644-spacer-lpg0645 | 1487/865/681 | 6 (type <i>d</i>) |

Strain name with accession number is represented in parenthesis, followed by GenBank locus tag; length of amino acids; number and type of repeats. *length of polypeptide and number of repeats estimated according to sequences published in the relative Genome Project. **Recent sequencing using PacBio technique revealed true number of residues as 6764 rather than previously estimated 7679.

Concerning structural organization, studies revealed a high diversity of the *rtxA* gene compared to those from the flanking regions which are highly conserved in sequence and order, this hints that *rtx* undergoes a particular intragenic evolution (D'Auria *et al.*, 2008, Satchell, 2011). More precisely, the genes located at 5' of *rtx* are strictly conserved, both in sequence and order, whereas this synteny and nucleotide similarity of those at the 3' end do not follow this pattern (D'Auria *et al.*, 2008). As for the gene structure, a study on five *L. pneumophila* strains (Paris, Lens, Corby, Alcoy, Philadelphia) revealed that the N terminus region of RtxA protein is highly conserved, and this region is followed by a variable number of tandem repeats (Table 8). These repeats contain domains involved in host-membrane interaction with a wide variability either in copy number or even nucleotide composition (D'Auria *et al.*, 2008).

RtxA protein is clearly divided into two regions, the N-terminal, involved in adhesion, and the C-terminal region which is conserved in the different strains, is also involved in adhesion and possibly pore formation in host membranes (D'Auria *et al.*, 2008, Satchell, 2011). Between those termini, exists a variable repeat region with different kinds of adhesion domains. In the Paris strain type *a* repeats, similarity was found with the "Thrombospondin type 3 repeat" of human endothelial cells (D'Auria *et al.*, 2008). This domain was shown to bind fibrinogen, fibronectin, laminin and type V collagen (Kvansakul *et al.*, 2004). Going towards the C-terminal part, additional domains can be detected such as von Willebrand factor type 1 (vWA) and several blocks of tandem repeats identified as HemolysinCabind domains (hemolysin calcium binding) (D'Auria *et al.*, 2008). The vWA domain is involved in adhesion processes, thus RtxA may contact another vWA protein on the macrophage or amoeba cell, facilitating its adhesion and entry thereby impacting virulence (D'Auria *et al.*, 2008, Satchell, 2011). The HemolysinCabind domain is related to adhesion and cytotoxicity probably by pore formation (D'Auria *et al.*, 2008). These domains form blocks of tandem repeats which are different in the studied strains (light pink in Figure 38): 3+2 in Paris, 1+3+2 in Lens, 3+3+2 in Corby, Alcoy and Philadelphia where these motifs comprise the RTX repeats of RtxA. As the name implies, ability to bind

calcium by these repeats as other RTX proteins indicate the presence of a conformational change upon binding calcium ions (D'Auria *et al.*, 2008). The *rtx* gene structure of the different *L. pneumophila* strains is displayed in the figure below.

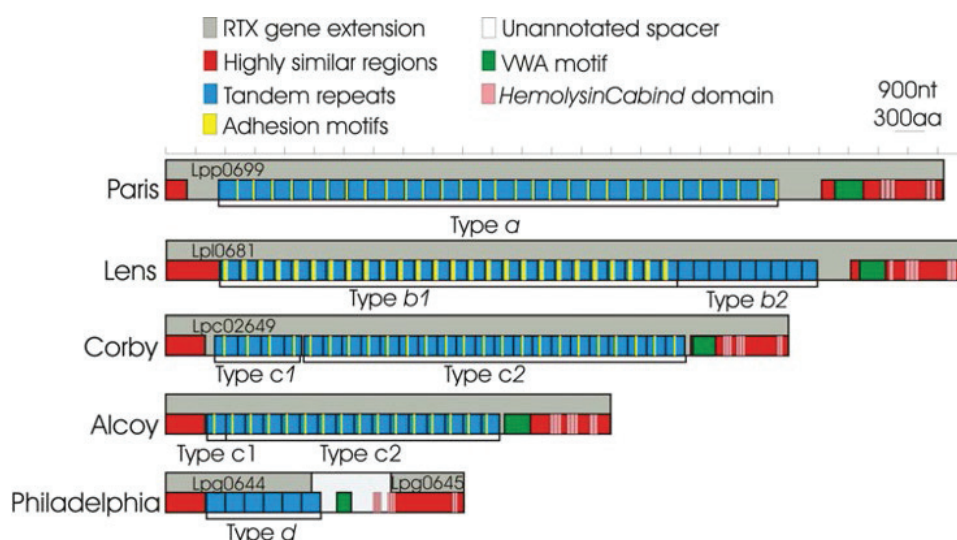


Figure 38: Comparison of *rtxA* gene organization in five *L. pneumophila* strains The structure of *rtxA* genes in the studies strain are displayed above. Different domains are represented using different colors, a conservation of the N and C termini is noticed across the analyzed genes. Adapted from (D'Auria *et al.*, 2008).

Regarding the variety of the repeats region seen in Figure 38, it seems to be acquired by the two mechanisms involved in concerted evolution, intragenic gene conversion and/or unequal crossing over (D'Auria *et al.*, 2008).

The involvement of RtxA in *L. pneumophila* virulence has been addressed in several publications. The inactivation of *rtxA* gene lead to a measurable loss of virulence in mice, where complementation of the deleted gene lead to a restoration of the lost virulence (Cirillo *et al.*, 2001). One of the major observations was that adherence to monocytic and epithelial cells was reduced almost 50% in *rtxA* mutants compared to wild-type *L. pneumophila* (Cirillo *et al.*, 2001). This was also confirmed to be the case in amoebae (Cirillo *et al.*, 2002). It was proposed that RtxA might use the β_2 integrin as a possible receptor on mammalian cells, however in *Acanthamoeba* so far there are no identified receptors homologous to integrins in contrast to *Hartmannella* and *Entamoeba* (Cirillo *et al.*, 2001, Cirillo *et al.*, 2002). RtxA has also been implicated in intracellular survival though with not enough evidence, for instance inhibition of lysosomal fusion. This might suggest that RtxA is a modular protein that can assume several functions (Cirillo *et al.*, 2002). Interestingly, as much as RtxA is important in *L. pneumophila* virulence, it is not absolutely required for the pathogenesis as $\Delta rtxA$ mutants were able to invade and replicate within host cells albeit to a lesser extent than wild-type *L. pneumophila* (Cirillo *et al.*, 2001, Cirillo *et al.*, 2002). Moreover, disrupting the RtxA secretion system (LssB and LssD defective mutants) resulted in a delay

of *L. pneumophila* entry into amoebae compared to wild-type in the first few hours post exposure (Fuche *et al.*, 2015).

E. Release regulation of RTX adhesins & its relation to secretion

As mentioned previously, the RTX family is a diverse and continually expanding group of proteins. A certain subgroup of this family is the RTX adhesins or biofilm associated proteins where the *P. fluorescens* pf0-1 LapA protein is perhaps the most well studied member. Biofilm formation is a mechanism of adaptation to changing environmental conditions used by pathogenic and environmental bacteria (Chatterjee *et al.*, 2012). In order to form biofilms, bacterial cells must undergo a transition from planktonic to a sessile mode of life by committing to stable surface attachment. This transition, irreversible attachment, is the first committed step in biofilm formation and is followed by cell-cell interactions (Boyd *et al.*, 2014, El-Kirat-Chatel *et al.*, 2014). This transition is fostered by diverse mechanisms but triggered originally by environmental cues that favor biofilm formation (Boyd *et al.*, 2014). In this state, microbial cells are surrounded in a self-secreted extracellular matrix composed mainly of exopolysaccharides (EPS), proteins and nucleic acids (Hall-Stoodley *et al.*, 2004). Pathogenic bacteria that are able to form biofilms have been associated with numerous persistent and nosocomial infections in humans, an example is the biofilm formation by *P. aeruginosa* in cystic fibrosis patients' lungs (Chatterjee *et al.*, 2014). Another example is biofilm formation on various medical implants such as catheters and artificial hips leading to severe medical complications (Percival *et al.*, 2015). A very important feature of bacteria in biofilms is the ability to withstand antibiotic treatment where many clinically relevant antibiotics are ineffective in the treatment of biofilm associated bacterial infections (Mah & O'Toole, 2001).

1. Release of RTX adhesins

The *P. fluorescens* adhesin LapA is smaller than the *P. putida* LapA described previously but still a large RTX protein (5,218 amino acids ~520kDa). It consists of the traditional RTX repeats (six repeats), a C-terminal secretion signal and a characteristic amino acid repeat region (37 repeats) (Boyd *et al.*, 2014). It also comprises a von Willebrand factor type A domain (El-Kirat-Chatel *et al.*, 2014). LapA in *P. fluorescens* mediates adhesion and biofilm formation on every abiotic surface tested to date, including hydrophobic and hydrophilic surfaces (Boyd *et al.*, 2014, El-Kirat-Chatel *et al.*, 2014). Expanding briefly on the decision to transition from a planktonic to a biofilm lifestyle, it is orchestrated by the ubiquitous bacterial second messenger, bis-(3'-5')-cyclic dimeric GMP (c-di-GMP), a dinucleotide responsible for modulating different aspects of bacterial physiology (Hengge, 2009, Boyd *et al.*, 2014, Chatterjee *et al.*, 2014). c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases

(DGCs) containing a GGDEF domain and hydrolyzed by phosphodiesterases (PDEs) with either EAL or HD-GYP domain (Chatterjee *et al.*, 2014). In *P. fluorescens* and probably other bacteria, the availability of nutrients and more precisely inorganic phosphate (P_i), controls the production of c-di-GMP inside cells (Chatterjee *et al.*, 2014). Briefly, under conditions of low P_i concentrations, the periplasmic cysteine protease LapG cleaves LapA from the cell surface thereby releasing the adhesin into the supernatant and preventing attachment or biofilm formation (Newell *et al.*, 2011). An inner membrane c-di-GMP effector protein LapD regulates LapG's activity in a c-di-GMP dependent manner. LapD from *P. fluorescens* contains both catalytically inactive GGDEF and EAL domains, with the latter being the exclusive c-di-GMP binding module. Binding of c-di-GMP causes a conformational change in LapD, and through an inside-out signaling mechanism, LapD binds LapG preventing LapG dependent cleavage of LapA from the cell surface and thereby promoting attachment (Chatterjee *et al.*, 2012, Boyd *et al.*, 2014, El-Kirat-Chatel *et al.*, 2014). More specifically, c-di-GMP bound LapD interacts with the periplasmic protease LapG through its Per-Arnt-Sim (PAS) like domain, preventing cleavage of cell surface adhesin LapA (Chatterjee *et al.*, 2014). LapG will specifically target the N-terminus of LapA resulting in cleavage and release of the bulk of the protein from cell surface which subsequently leads to destabilizing cell attachment (Boyd *et al.*, 2014, Chatterjee *et al.*, 2014). These events are represented in Figure 39. The LapG cleavage site at the N-terminus of LapA was also found to be conserved in other RTX adhesins as shown in Figure 40. Mutant *P. fluorescens* strains with deleted *lapG* gene accumulate the LapA adhesin on the cell surface leading to a hyper adherent biofilm (El-Kirat-Chatel *et al.*, 2014). Research have shown that the LapD-LapG interaction can be targeted. Therefore, in bacteria using these mechanisms, it presents a way for controlling biofilm formation (Chatterjee *et al.*, 2014). LapD and LapG orthologs are present in a variety of bacteria, including several pathogens such as *L. pneumophila*, which will be addressed in the coming part (Chatterjee *et al.*, 2012, Chatterjee *et al.*, 2014). Therefore, understanding these systems is of great interest and is a quickly developing field in the past few years.

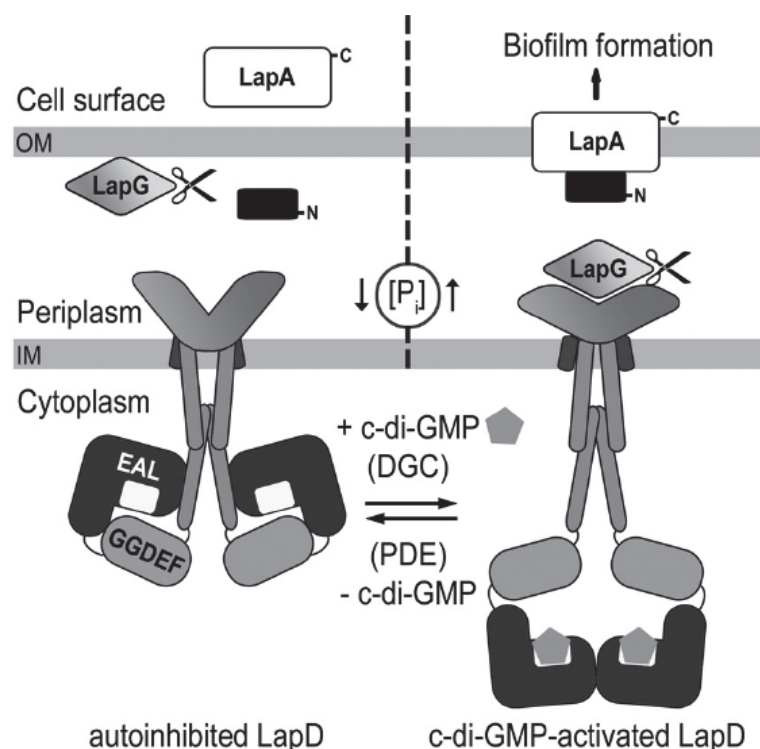


Figure 39: The LapA/LapDG system in *P. fluorescens* A model of LapD mediated biofilm regulation in *P. fluorescens* through controlling the release of cell surface adhesin LapA. (Right) in case of abundance of nutrients in the environment depicted by an increase in phosphate P_i, multiple DGCs will be activated and contribute to a high intra-cellular c-di-GMP levels. LapD through its EAL domain will bind cytosolic c-di-GMP causing a conformational change that will sequester the periplasmic protease LapG and stopping it from cleaving/releasing LapA which leads to promotion of biofilm formation. (Left) limiting phosphate increases the expression of a PDE which in turn lowers intracellular c-di-GMP levels, this will maintain LapD in its autoinhibited state and LapG will be free to cleave the N-terminus of LapA releasing it from cell surface and causing a depression in *P. fluorescens* biofilms. Adapted from (Chatterjee *et al.*, 2012).

Concerning the periplasmic protease LapG, it is a calcium dependent cysteine protease (Boyd *et al.*, 2012, Chatterjee *et al.*, 2012). Calcium binding to residues D134 and E136 which are near the critical C135 active site residue, are required for LapG activity in *P. fluorescens* both *in vivo* and *in vitro* (Boyd *et al.*, 2012). Moreover, mutations in the calcium binding residues (D134 and E136) abolished the ability of LapG proteins to interact with LapD indicating that calcium binding is necessary for LapG to adopt a conformation that allows interaction with the protein that regulates its activity (Boyd *et al.*, 2012).



Figure 40: Sequence alignment of the LapG cleavage site of several putative adhesins Several adhesins were identified by their close proximity to genes encoding LapG homologs. The LapA cleavage site is indicated by asterisks (two possible sites identified) and putative alternative cleavage sites are indicated by arrows. The conserved residues are in grey or black. The predicted helical domain is underlined. The protein sequences belong to: LapA, *P. fluorescens* Pf0-1; PFL_0133, *P. fluorescens* Pf-5; PP_0168, *P. putida* KT2440; PSEEN0141, *Pseudomonas entomophila* L-48; ECA3266, *Pectobacterium atrosepticum*SCRI1043; DP0516, *Desulfotalea psychrophila* LSv54; Rfer_3766, *Rhodospirillum rubrum* DSM 15236; Patl_2658, *Pseudomonas aeruginosa* T6c; Sden_0384, *Shewanella denitrificans* OS217; Bpro_0306, *Polaromonas* sp. strain JS666. Adapted from (Boyd *et al.*, 2014).

As mentioned previously, LapD protein has GGDEF and EAL domains that lack enzymatic activity, though still able to bind c-di-GMP via the EAL domain (Chatterjee *et al.*, 2014). And since bacteria contain large c-di-GMP signaling networks made of DGCs and PDEs that can direct cellular activities sensitive to c-di-GMP levels, this raises questions regarding the control and specificity of this system and how these systems avoid undesired crosstalk (Dahlstrom *et al.*, 2016). So, for these networks to have order, a mechanism likely exists that allows DGCs to specifically signal their targets and it has been suggested this is done via physical interactions (Dahlstrom *et al.*, 2015). A DGC in *P. fluorescens* called GcbC was found to interact physically with LapD, where a LapD mutant unable to bind c-di-GMP can still interact with GcbC (Dahlstrom *et al.*, 2015). More specifically, a specific region in GcbC named the $\alpha 5^{\text{GGDEF}}$ helix is recognized by the $\alpha 2^{\text{EAL}}$ helix on LapD. This $\alpha 5^{\text{GGDEF}}$ helix is conserved in other species and can be used to predict interactions (Dahlstrom *et al.*, 2015). This GcbC-LapD interaction results in increased LapA localization to the cell surface (Giacalone *et al.*, 2018). Interestingly, GcbC shows little potential to synthesize c-di-GMP *in vitro*. However, when LapD is present, GcbC activity is significantly enhanced which indicates that engaging with LapD receptor stimulates its activity (Giacalone *et al.*, 2018).

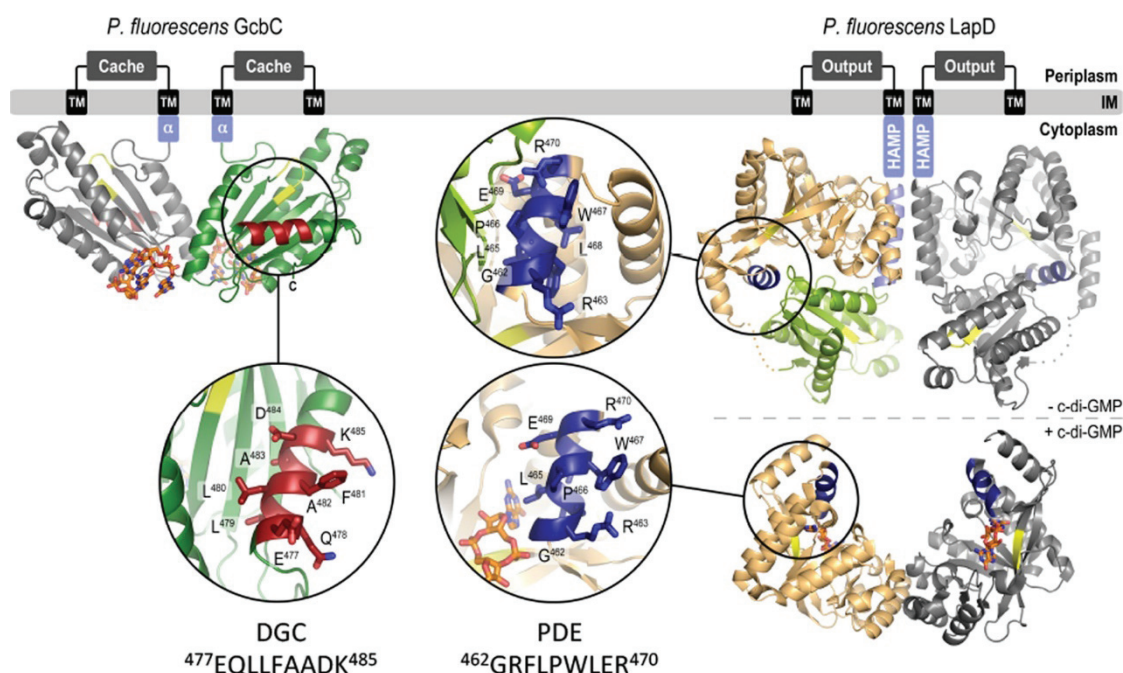


Figure 41: Model of LapD-GcbC interaction On the left is GcbC bound to c-di-GMP in inactive dimer. The magnification shows the $\alpha 5^{\text{GGDEF}}$ helix (residues 477 to 485) in red. On the right and above is LapD in its unbound and below in the c-di-GMP bound active state. The magnification shows the $\alpha 2^{\text{EAL}}$ helix of LapD in dark blue. This helix is exposed in the c-di-GMP bound state. From (Dahlstrom *et al.*, 2015).

While DGCs synthesize c-di-GMP, many DGCs also contain an inhibitory site (I-site) that binds c-di-GMP to stop excess production of this molecule, thereby controlling the amount of c-di-GMP available to bind target proteins (Dahlstrom *et al.*, 2016). It was shown that the I-site of GcbC is essential for interaction with LapD, where signaling between the DGC (GcbC) and its target LapD, is a combined function of I-site protein-protein dependent interaction and the level of c-di-GMP (Dahlstrom *et al.*, 2016). This autoinhibitory site found on many DGCs, locks the enzyme in an inactive state when bound to c-di-GMP to prevent further production of this molecule (Dahlstrom *et al.*, 2016). It is important to note that LapD in *P. fluorescens* seems also to interact with a dozen different DGCs, so this process is not restricted to GcbC alone (Giacalone *et al.*, 2018).

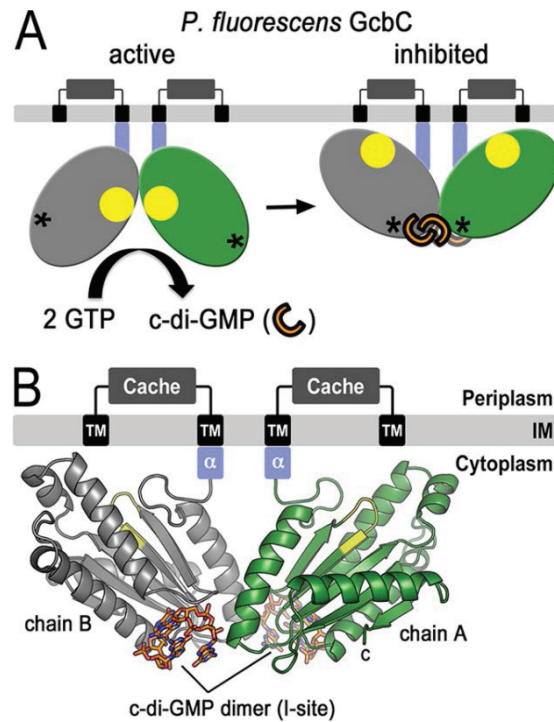


Figure 42: Architecture of the *P. fluorescens* GcbC I-site (A) a model for the negative feedback inhibition of GcbC by c-di-GMP. The yellow circles and the asterisks represent the active and inhibitory sites respectively, on the GGDEF domain. (B) the crystal structure shows of GcbC shows the dimer of the GGDEF domains binding two molecules of c-di-GMP at the canonical I-site. Adapted from (Dahlstrom *et al.*, 2016).

Disruption of this I-site leads to deregulation of c-di-GMP production, and mutations in this region also lead to decreased biofilm formation. However, certain targeted I-site mutations result in elevated levels of biofilm formation (Dahlstrom *et al.*, 2016). Therefore, manipulation of this system can turn its components on or off depending on the nature of the alterations.

2. Secretion of certain cell-surface regulated RTX proteins

We mentioned previously that RTX proteins are secreted via T1SSs in a single step process directly from the cytoplasm to the extracellular medium. While that is true, a recent study shows that it's not entirely accurate for all T1SSs. A distinct subgroup of T1SSs that are linked with bacterial transglutaminase-like cysteine proteinase (BTLCP) uses a two-step secretion mechanism (Smith *et al.*, 2018a). This model was based on the LapA of *P. fluorescens* Pf0-1 that uses an N-terminal retention module that anchors the adhesin at the cell surface in an intermediate state, while threaded through the outer membrane TolC-like protein LapE. This secretion intermediate is then cleaved by the BTLCP family LapG protein to release LapA (Smith *et al.*, 2018a).

The rationale behind this thinking that lead to the previously mentioned findings is that LapD-LapG interaction based environmental conditions, controls the localization of LapA adhesin and subsequently biofilm formation ability. Therefore, LapA has to be tethered to the cell surface via a certain mechanism prior to its potential release. Interestingly, LapG and LapD homologs are found together in the genomes of more than 1300 bacterial species over 120 genera in the proteobacteria (Smith *et al.*, 2018b) which suggests that this localization strategy could be common among this group of organisms. This can also be said about *L. pneumophila* where it comprises this system and the BTLCP-linked adhesin RtxA that is associated with enhanced virulence (Cirillo *et al.*, 2000).

In support of the previous assumption, mutational analysis of *P. fluorescens* LapA showed that a mutant lacking D31-95A residues is unable to associate with the cell surface and is secreted directly into the supernatant, this mutant is defective for biofilm formation. Thus, this region of LapA N-terminus was termed the “retention module” (Smith *et al.*, 2018a). Consistent with this idea that LapA adhesin is anchored in a TolC-like component of the T1SS, secretion competition experiments showed that cell-surface associated LapA blocks secretion of a secretion peptide containing only LapA’s secretion signal (Smith *et al.*, 2018a). This suggests that LapA occupies a component of its transport machinery when attached to cell surface. Regarding this component, studies on the retention module of a LapA like adhesin (MpIBP from *Marinomonas primoryensis*) revealed that it adopts a structure that is too large to pass through TolC (Guo *et al.*, 2017). Concerning LapA, It was found that its retention module contains well folded N-terminal region that prevents extracellular secretion and a poorly folded C-terminal region that could thread through the OM LapE pore (Smith *et al.*, 2018a). Additional evidence towards LapE arise from the *Shewanella* spp. AggA which is a TolC-like protein that transports the RTX adhesin BpfA (Wu *et al.*, 2013). Bioinformatic studies suggest that AggA belongs to a subclass of TolC-like proteins involved with secreting large T1SS adhesins (Theunissen *et al.*, 2009). This group includes LapE of *P. fluorescens* Pf0-1 and other LapE like proteins linked with LapD and LapG proteins, This suggests that the outer membrane component of T1SS adhesins transporters is distinct from the general TolC, RND and other outer membrane proteins (Smith *et al.*, 2018a).

Figure 43 shows key differences between the classic RTX secretion mechanism and the one theorized to occur with BTLCP-linked RTX proteins.

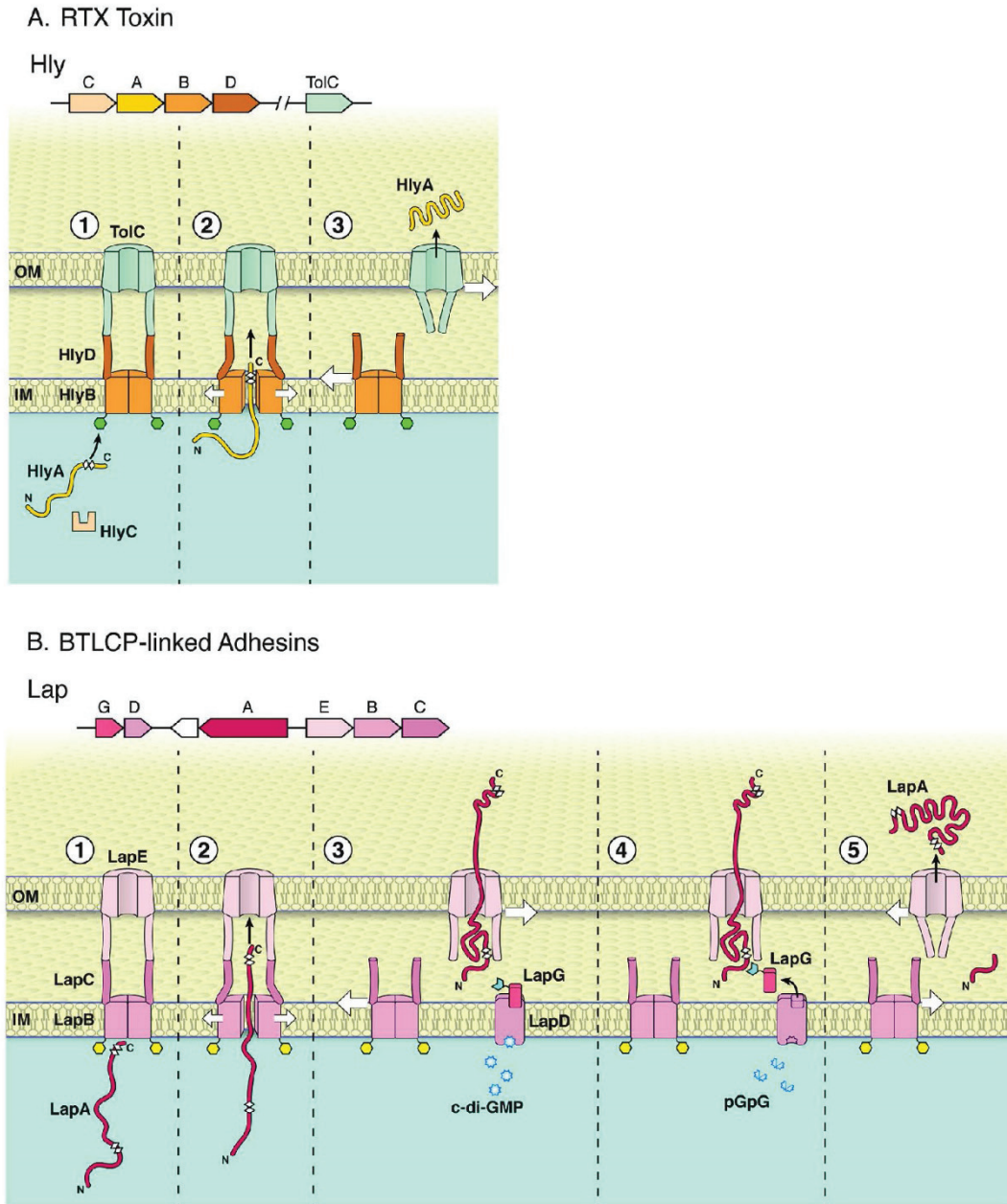


Figure 43: Overview of classic and BTLCP-linked RTX secretion A T1SS is composed of an ABC transporter, MFP and an OM protein. **(A)** secretion of the RTX toxin HlyA, Top is the operon containing HlyC (acyltransferase that activates HlyA), HlyA (RTX toxin), HlyB (ABC transporter), HlyD (MFP) and the distantly located TolC OM pore in *E. coli*. First, the glycine rich motif of HlyA will bind HlyB then TolC will be recruited and the secretion through the channel will take place. Finally, HlyBD-TolC complex will dissociate following the secretion of HlyA into the extracellular environment. **(B)** Secretion of the BTLCP-linked RTX adhesin in *P. fluorescens* pf0-1, top is the operon containing LapG (periplasmic BTLCP that cleaves LapA), LapD (c-di-GMP sensory protein that is able to sequester LapG), LapA (BTLCP-linked RTX adhesins), LapE (TolC like OM pore), LapB (ABC transporter), LapC (MFP). LapA secretion commences via interaction with CLD of Lap (yellow hexagon). The secretion takes place in a C-terminal to N-terminal direction. Secretion of LapA halts during translocation where it is threaded through LapE but its N-terminal cleavable retention domain remains in the periplasm and multiple adhesive repeats exposed at the cell surface. LapA is fixed at the cell surface as a biofilm promoting secretion intermediate. When c-di-GMP levels are reduced, LapG is released from LapD where it cleaves LapA at a dialanine site in the retention module. This cleaved LapA is released from the cell surface and the now free LapE can perform the cycle again. Adapted from (Smith *et al.*, 2018a).

3. LapD/G system and *L. pneumophila*

Unlike *Pseudomonas*, *L. pneumophila* is a facultative intracellular bacterium and is also able to grow in biofilms, although it will most likely colonize existing biofilms rather than producing its own (Declerck, 2010). Therefore, a mechanism for cleaving surface adhesin RtxA in *L. pneumophila* can probably regulate both infection potential and biofilm formation due to implication of RtxA in host attachment as discussed earlier.

Regarding this matter, research has found a similar system to that in *P. fluorescens* to exist also in *L. pneumophila*. It was first uncovered using bioinformatic tools that an orthologous system indeed exists in *L. pneumophila*, and that its genome encodes 21 predicted proteins with GGDEF and/or EAL domains where some of these were shown to impact intracellular growth, motility and biofilm formation (Chatterjee *et al.*, 2012). Studies on *L. pneumophila* Philadelphia 1 indicated the existence of genes similar those encoding the LapD/LapG system of *P. fluorescens*, shown in Figure 44.

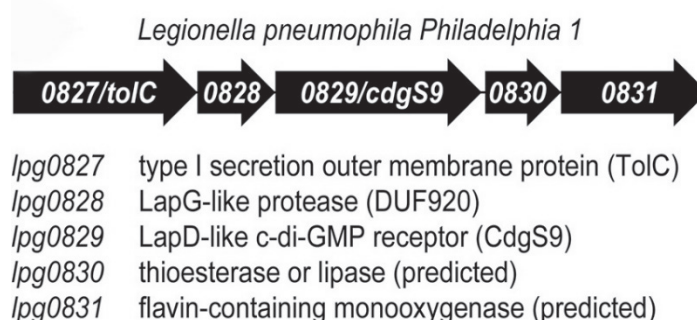


Figure 44: Genetic map of the LapD/LapG containing operon in *L. pneumophila* The LapD ortholog in *L. pneumophila* is called CdgS9 or lpg0829. The operon also includes the LapG ortholog and a predicted TISS component, a TolC-like outer membrane component. Adapted from (Chatterjee *et al.*, 2012).

The interaction between LapG and LapD was first confirmed. It was shown that *P. fluorescens* LapG was able to interact with the periplasmic output domain of LapD from both *P. fluorescens* and *L. pneumophila* (CdgS9). This was also observed between the LapD and LapG from *L. pneumophila*. However, binding of *L. pneumophila* LapG to the output domain of *P. fluorescens* LapD was detectable but weaker. This indicates that the mode of binding is specific and conserved across distantly related bacterial species (Chatterjee *et al.*, 2012).

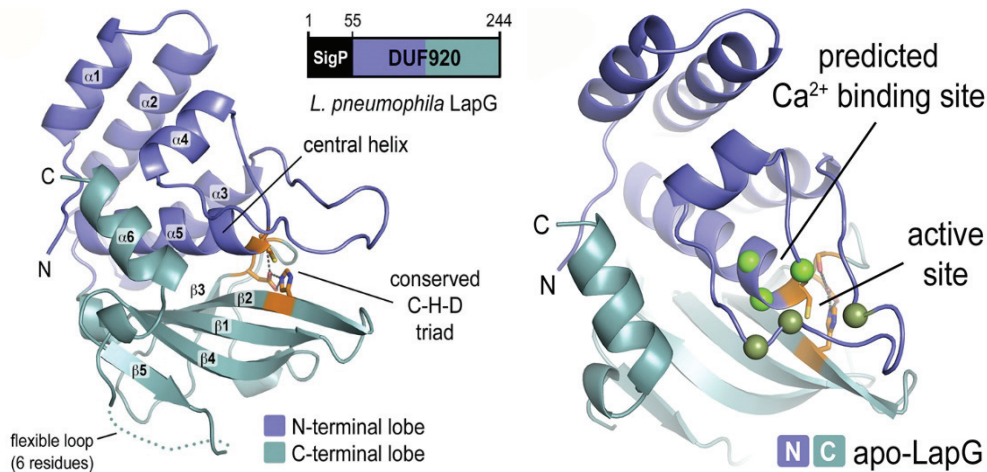


Figure 45: Crystal structure of *L. pneumophila* LapG (Left) crystal structure of LapG excluding the signal peptide (residues 1-55) is shown, LapG belongs to the domain of unknown function 920 (DUF920) family of proteins. It also has a cysteine-histidine-aspartate (C-H-D) catalytic triad at the active site. (Right) Position of putative calcium binding sites in LapG. Adapted from (Chatterjee *et al.*, 2012).

LapG in *L. pneumophila* was also found to contain calcium binding sites thereby proving dependence on calcium ion binding for proper function (Figure 45). It was hypothesized that calcium binding to LapG aids in catalysis by providing increased access to the substrate or possibly by altering enzyme structure or dynamics. Calcium binding may also place LapG in close proximity to its substrate, LapA (Chatterjee *et al.*, 2012).

The *L. pneumophila* LapG catalytic activity was confirmed using the *P. fluorescens* LapA as a model substrate. It was found that *L. pneumophila* LapG successfully processed the N terminus of LapA albeit less efficiently than *P. fluorescens* LapG (Chatterjee *et al.*, 2012).

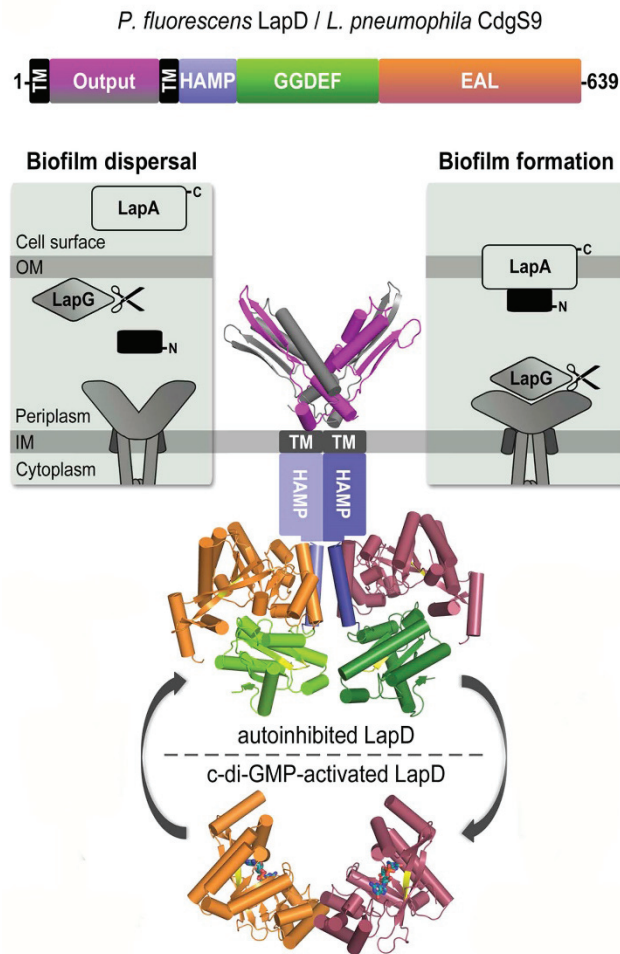


Figure 46: The LapD signaling system The primary structure and domain organization of LapD is shown above. LapD in *P. fluorescens* and its ortholog CdgS9 in *L. pneumophila* share a similar structure, the cytoplasmic GGDEF and EAL domains. It is followed by a HAMP domain which is a crucial signal relay module not only in bacteria but also eukaryotes. The conformational changes in the GGDEF-EAL domains are sensed by the HAMP domain which propagates the signal to the output domain that in turn changes its conformation resulting in change in affinity to LapG. The cartoon summarizes the process of LapD-mediated biofilm regulation in *P. fluorescens* which was discussed earlier. Adapted from (Chatterjee *et al.*, 2014).

As mentioned earlier, the *L. pneumophila* LapD ortholog exists and was called CdgS9. As seen in Figure 46, the PAS-like periplasmic domain (output domain) of LapD that engages LapG is flanked by two putative transmembrane helices, the second of which connects to an intracellular, juxtamembrane HAMP domain followed by the aforementioned GGDEF-EAL domain module (Chatterjee *et al.*, 2014). Gene deletion of *cdgS9* didn't result in an obvious phenotype, yet overexpression of CdgS9 has an effect on intracellular growth which suggests functional relevance of this protein (Chatterjee *et al.*, 2014).

As we mentioned earlier regarding c-di-GMP signaling in *L. pneumophila*, depending on the strain, the genome contains between 22 and 24 genes encoding GGDEF/EAL proteins. It's worthy to note that *L. pneumophila* harbors a particularly high rate of potential c-di-GMP metabolizing enzymes (Pecastaings

et al., 2016). Both virulence and biofilm formation in *L. pneumophila* have been linked to c-di-GMP signaling, it was shown that overproduction of most GGDEF/EAL proteins impacts the ability to successfully replicate in amoebae or macrophages (Allombert *et al.*, 2014). On the other hand, the DGC Lpg1057 is involved in *L. pneumophila* biofilm since overproduction of this c-di-GMP metabolizing enzyme leads to a hyper biofilm phenotype. However, little is known about *L. pneumophila* biofilm formation (Carlson *et al.*, 2010). More recent studies show that five GGDEF/EAL encoding genes in *L. pneumophila* Lens strain (*lpl0075*, *lpl0329*, *lpl1176*, *lpl1054* and *lpl1559*) whose deletion led to unusual biofilm formation (Pecastaings *et al.*, 2016). Interestingly, it was also established that intracellular concentration of c-di-GMP is not clearly affected by deletion of genes encoding DGCs involved in biofilm formation or surface attachment (Abel *et al.*, 2013, Pecastaings *et al.*, 2016). In addition to the previous, *L. pneumophila* also seems to escape the general rules for biofilm formation generally dictated by c-di-GMP and nitric oxide (NO) (Pecastaings *et al.*, 2016). This suggests the importance of c-di-GMP signaling networks in the lifestyles of *L. pneumophila*, however, much of the mechanisms surrounding these networks are still unknown.

PROJECT AIMS

Legionella pneumophila is a continuing health concern that causes disease worldwide. Older data from the European working group for *Legionella* infections (EWGLI) show that European cases of Legionellosis increased steadily from 1161 cases in 1994 to 4546 cases in 2004. These numbers include nosocomial, travel associated, and community acquired cases which were consequently increasing. More recently, under monitoring of the European Legionnaires' disease surveillance network (ELDSNet), there were 30532 reported cases between 2011 and 2015 where France, Germany, Italy and Spain accounted for 69% of these cases. For this reason and the involved fatality risk associated with this disease, major organizations such the Center for Disease Control and Prevention (CDC), the World Health organization (WHO) and the European Center for Disease Prevention and Control (ECDC) continuously try to raise awareness regarding this disease in an effort to reduce its incidence. On the research front, studying this bacterium will help understand its virulence in depth which consequently serves in reduction of this disease's impact.

My work in this PhD project aims to explore several aspects of the *L. pneumophila* RtxA, a virulence associated protein, mainly the mechanisms of its regulation on the cell surface in confirmation of the similarities between this system and that of *P. fluorescens*. In addition to previous, we attempt to find partners of the RtxA release system that are responsible for its regulation, this is particularly important in light of recent evidence that RtxA secretion may occur in a two-step manner. We also investigate the effect of deletion mutations of RtxA and components of its secretion and release systems on *L. pneumophila* virulence towards amoebae. Therefore, in order to test all of the above, we also had to develop a clean and efficient genetic manipulation tool in *L. pneumophila* which will be presented in the coming part.

I. Protocol: Scar-free genome editing in *Legionella pneumophila*

Genetic manipulation is a key weapon in the arsenal of any researcher, more specifically biologists. However, certain risks always accompany such manipulation including the alteration of untargeted loci that may produce erroneous results or even be detrimental to the subject being manipulated. Also, some procedures are time consuming, have low success rate and even stressful for the organism.

Here we describe a protocol that relies on the natural transformability of *L. pneumophila* where we use a linear DNA fragment consisting of a selection/counter selection cassette for homologous recombination with a targeted region of the *L. pneumophila* chromosome followed by another recombination to remove the first cassette after proper selection. This two-step method ensures an accurate and efficient way to generate scar-free deletions, insertions, single nucleotide mutations and various transcriptional/translational fusions in the *L. pneumophila* chromosome.

The main advantage of this method is that there is no exogenous “unwanted” DNA left in the genome of the final mutated clone. This protocol has been successfully used to construct all the mutant strains described in the following Ph.D. work.

This new genetic manipulation method has been published in a book chapter of *Legionella*; Methods and Protocols in Methods in Molecular Biology series (vol. 1921)



Chapter 6

Scar-Free Genome Editing in *Legionella pneumophila*

Nathalie Bailo, Hussein Kanaan, Elisabeth Kay, Xavier Charpentier, Patricia Doublet, and Christophe Gilbert

Abstract

Studying bacterial physiology and pathogenesis often requires isolation of targeted mutants. From the early days of bacterial genetics, many genetic tools have been developed to achieve this goal in a lot of bacteria *species*, and a major key is to be able to manipulate the targeted genome region with a minimum impact on the rest of the genome. Here, we described a two-step protocol relevant in *Legionella pneumophila*. This efficient two-step protocol uses the natural transformability of *L. pneumophila* and linear DNA fragments as substrates for recombination without the necessity of intermediate hosts to amplify targeted DNA. Based on a suicide cassette strategy, this genetic toolbox enables to generate clean scar-free deletions, single-nucleotide mutation, transcriptional or translational fusions, as well as insertion at any chosen place in *L. pneumophila* chromosome, therefore enabling multiple mutations with no need of multiple selection markers.

Key words *Legionella pneumophila*, Natural transformation, Genome editing, Recombination, Suicide cassette

1 Introduction

In recent years, many genetic protocols have been designed to create deletions, insertions, or mutations in bacterial chromosome. However, most of these published techniques need to antecedently alter the bacterial genetic content, for example, by introducing a plasmid expressing a recombinase [1, 2]. Furthermore, even called “markerless” strategies often leave a few nucleotide scars on the bacterial chromosome (i.e., “FRT scar” in case of λ Red recombinase). One of the most advanced methods to avoid any scar on the genome was described in *Bacillus subtilis* using an *Escherichia coli* toxin-encoding gene, *mazF*, as a counterselectable marker [3]. In this method described by Zhang et al. intermediate manipulations required the cloning of target sequence in *E. coli* plasmids, and the final step is obtained through a single-crossover event between two directed repeat sequences introduced in the first step-modified

chromosome. One disadvantage of this method is the time-consuming cloning step in *E. coli* which is also associated with potential issues related to the cloning of heterologous sequence (toxicity, mutagenesis).

Another exciting field of bacterial genome modification is the use of CRISPR-Cas systems based on endogenous or heterologous CRISPR-Cas machinery [4, 5]. However, in this latter case, pre-modification of the bacterial host is needed to introduce the CRISPR-Cas genes into its chromosome or to provide them on a replicative plasmid that must be cured at the end of the process to remove all foreign DNA from the mutated bacteria (counterselected or naturally lost). Both native homologous and λ Red recombinase-helped recombination have been successfully used in many Gram-negative and Gram-positive bacterial genera such as *Bacillus*, *Clostridium*, *Escherichia*, *Lactobacillus*, *Streptococcus*, *Streptomyces*, and *Mycobacterium* [6–10]. An interesting technology using polymer-derivatized CRISPR nanocomplex has also been described to target methicillin-resistant *Staphylococcus aureus* strains, but this protocol seemed more suitable for medical application rather than as a routine protocol in bacterial genetic application [11].

The purpose of our work was to set up an efficient and easy-to-perform scar-free genome editing method in *Legionella pneumophila* with minimal host manipulation. CRISPR-Cas systems are present in some *L. pneumophila* strains but are not widely spread, thus making it not suitable for a universal use [12]. Therefore, taking into account the possibility to use the natural transformability of *Legionella pneumophila* [13, 14], we designed a genetic toolbox to enable the synthesis of linear DNA fragments as substrates for recombination without the necessity of intermediates host to amplify targeted DNA, based on Zhang et al.'s work (*mazF*-based method; [3]). This efficient two-step protocol enables to generate clean scar-free deletions [15] and single-nucleotide mutation, transcriptional or translational fusions, as well as insertion at any chosen place in *L. pneumophila* chromosome (unpublished data; see **Note 1**), therefore allowing multiple mutations with no need of multiple selection markers.

The general principle is to create a “first-step” mutant harboring a resistance/suicide cassette in place of the targeted chromosomal DNA region and, in a “second step,” to replace the resistance/suicide cassette by the appropriate wanted DNA fragment using inducible counterselection. To achieve that goal, our strategy was to use *E. coli mazF* gene which encodes the toxin part of a toxin-antitoxin module (*mazEF*) involved in cell death, for example, during stressful conditions [16]. Indeed, MazF is a ribonuclease active on mRNAs, resulting in cellular growth arrest if not inhibited by its cognate antitoxin MazE [17]. It is important to note that MazF/MazE system is not present in *L. pneumophila*. In

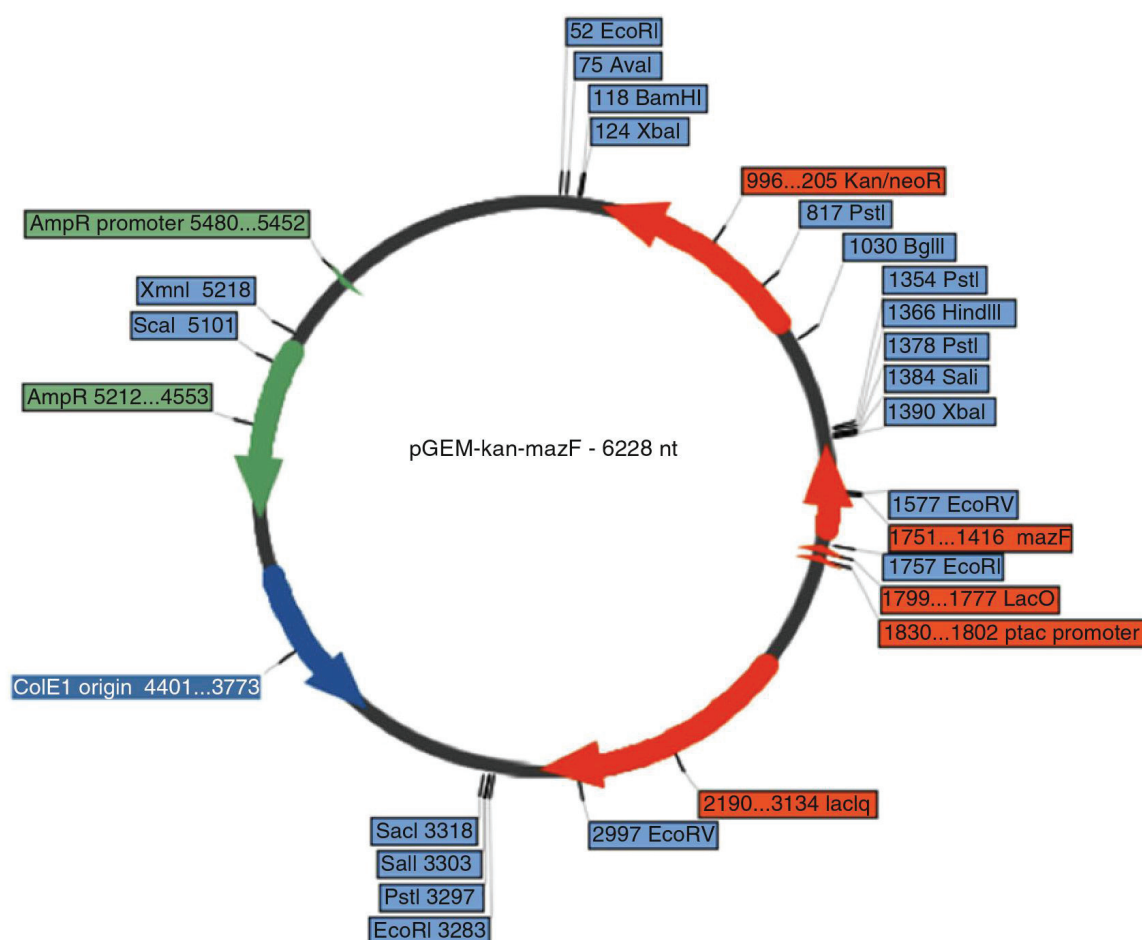


Fig. 1 Plasmid map of pGEM-*kan-mazF*. The pGEM-*kan-mazF* plasmid is derived from pGEM-T vector (PROMEGA). All the features corresponding to MazFCass are shown in orange. A few classical restriction sites are also mentioned

order to control the “suicide” role of *mazF* during the procedure, its expression is dependent on the classical *Ptac/lacI* inducible system. Therefore, during the first step of the protocol, there is no synthesis of MazF toxin, thus enabling growth of the recombinant mutant in presence of the antibiotic corresponding to the resistance gene carried by the insertion cassette. The “suicide” induction is done during the second step of the protocol using IPTG as inducer of *Ptac/lacI* system, leading to death of the non-recombinant second-step clones.

All the constructs to create the resistance/suicide cassette (MazFCass) have been done in *E. coli* strain DH5 α , and the final plasmid pGEM-*kan-mazF* (Fig. 1) is maintained in that strain which is used to produce the plasmid DNA for purification (Table 1). This cassette displays three main features: (1) a selectable marker usable in *L. pneumophila*, kanamycin resistance or gentamycin resistance; (2) a counterselectable marker usable in

Table 1
List of bacterial strains and plasmids

| Strain | Genotype | Source |
|---------------------------------|---|-----------|
| <i>Escherichia coli</i> strains | | |
| DH5 α | <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK-mK+)</i> | [18] |
| | DH5 α /pGEM-kan-mazF | This work |
| <i>Legionella pneumophila</i> | | |
| Strain Paris | Clinical wild-type isolate | [19] |

L. pneumophila, *E. coli* mazF toxin; and (3) an endogenous-inducible mechanism, *lacI* repressor/*Ptac* promoter (inducible by IPTG) (Fig. 2a). A joint PCR technique is used to obtain the mutated DNA fragment (Fig. 2b, c) which is the substrate of natural transformation (Fig. 2d). Among all the transformed clones obtained, one Kan^R IPTG^S is stored as the “first-step mutant” and confirmed by PCR. This “first-step mutant” will be the basis of the succeeding constructions. In the second step of this technique, the DNA fragment may correspond to a deletion of the targeted region or to any new DNA inserted between the two flanking regions (upstream fragment U; downstream fragment D) (Fig. 2e). This DNA fragment is then used to transform “first-step mutant” cells in order to be integrated in the recipient genome by homologous recombination. To select the second-step recombinants, the *mazF* expression is induced by IPTG, promoting death of non-recombinant clones and selecting recombinant clones thus insensitive to IPTG induction due to the loss of the resistance/suicide cassette. The final clones are selected as Kan^S IPTG^R clones and are confirmed by PCR before storage (Fig. 2f).

2 Materials

2.1 DNA Manipulation Components

1. Template plasmid pGEM-kan-mazF as the source for the resistance/suicide cassette by PCR.
2. Set of 2 primers to amplify the 3236 bp resistance/suicide cassette from plasmid pGEM-kan-mazF:
Forward primer MazFCass-F:
5'-CGACTCACTATAGGGCGAATTGGGCCGCTTTCCAGTCGGGAAACCTG-3'.
Reverse primer MazFCass-R:
5'-CATATGCCACCGACCCGAGCAAACCCGAAGAAGTTGTCCATATTGGCCAC-3'

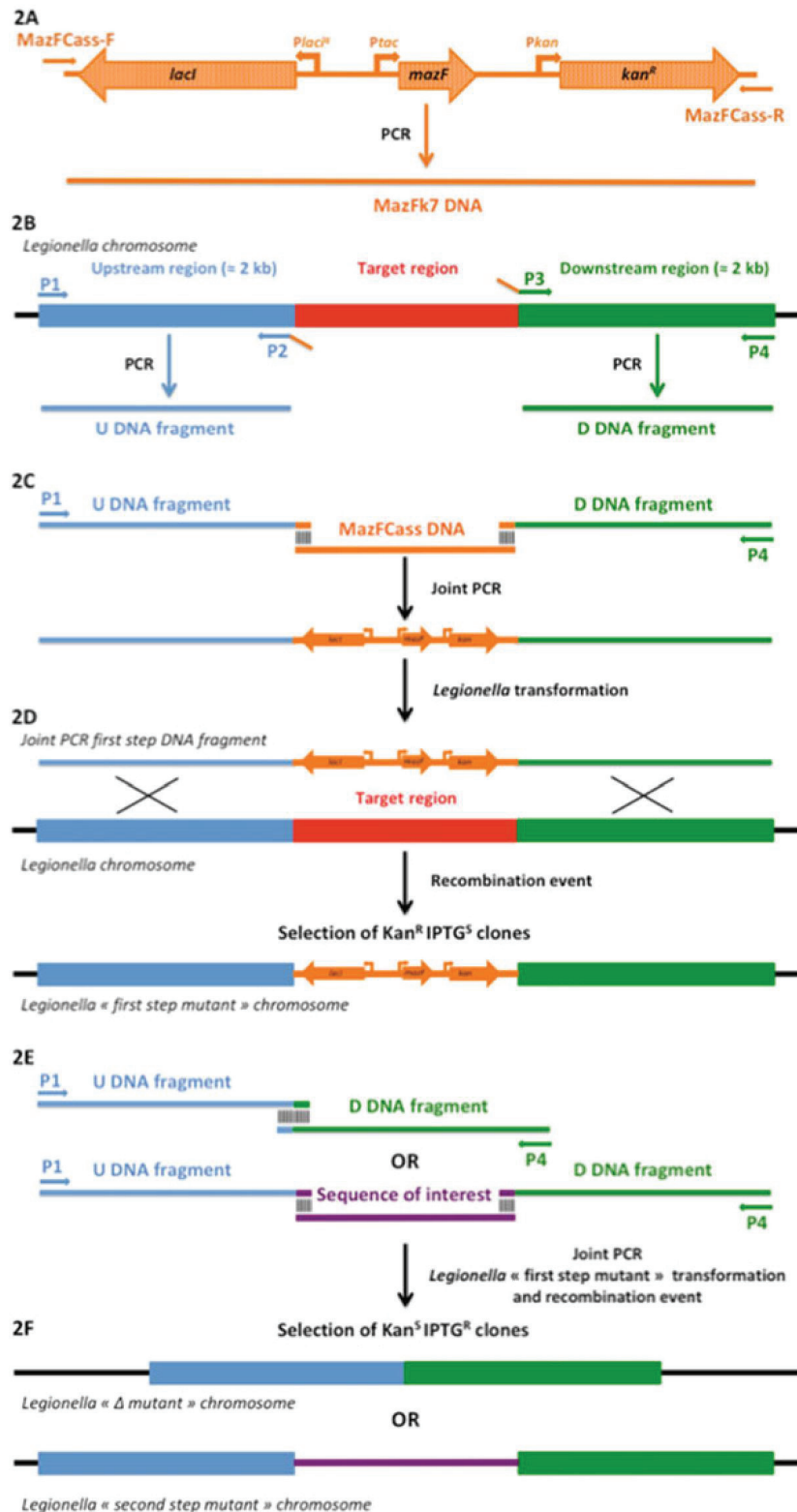


Fig. 2 Procedure to construct deletion or insertion mutants by natural transformation. (a) PCR of Kan^R/suicide cassette constructed on an *E. coli* plasmid. (b) PCR of flanked DNA region of the targeted region (P2 and P3 primers contain extensions homologous to MazFk7-S and MazFk7-R, respectively). (c) Joint PCR to produce the substrate DNA for natural transformation. (d) Genetic recombination and selection of “first-step mutant.” (e) Joint PCR design of second-step DNA substrates. (f) Genetic recombination and selection of final mutants

3. Template wild-type *Legionella pneumophila* chromosomal DNA sample.
4. A set of primers to amplify 1–2 kb flanking regions of the sequence to modify (Fig. 2; see **Note 2**). Typically, as shown on Fig. 2, P1 and P4 primers are used for all the constructs (first-step mutant and second-step mutants). P2 and P3 primers are designed suicide cassette/suicide cassette, meaning designed to do the first joint PCR: reverse P2 primer carries a 5' extension for hybridization with MazFCass-F primer (5'-GGCCCAATTCGCCCTATAGTGAGTCG-3') and forward P3 primer carries a 5' extension for hybridization with MazFCass-R primer (5'-GGGTTTGCTCGGGTCGGTGGCATATG-3'). Other primers necessary for the second joint PCR step are designed (and named P5, P6, P7, P8, and so on) to perform the desired construct (i.e., deletion, insertion, single point mutation, or fusion; Fig. 2e) following the same approach (see **Note 3**).
5. Thermocycler with suitable consumables (e.g., PCR 0.2 mL tube) and reagents: buffer, dNTPs, high-fidelity polymerase.
6. Ultrapure water (distilled or deionized water).
7. Agarose gel electrophoresis equipment and reagents.
8. Reagents to purify PCR products from PCR reaction or from agarose gel (e.g., Qiagen PCR Purification Kit and Gel Extraction Kit).
9. Microcentrifuge and 1.5 mL tubes.

2.2 Natural Transformation Components

1. Parent *Legionella pneumophila* strain (e.g., strain Paris).
2. Charcoal-yeast extract plates (CYE plates): AGAR 15 g/L, activated charcoal 2 g/L, yeast extract 10 g/L, ACES 10 g/L, L-cysteine 0.4 g/L, and iron pyrophosphate 0.25 g/L. And pH is adjusted to 6.9 with potassium hydroxide solution.
3. ACES-buffered yeast extract broth (AYE) sterilized by filtration on 0.2 µm membrane. The composition of AYE is as follows: yeast extract 12 g/L, ACES 10 g/L, L-cysteine 0.4 g/L, and iron pyrophosphate 0.3 g/L. And pH is adjusted to 6.9 with potassium hydroxide solution.
4. 10–16 mL sterile tubes suitable for culture in incubator shaker.
5. 37 °C incubator shaker.
6. Centrifuge.

2.3 Recombinant Isolation Components

1. CYE plates supplemented with appropriate antibiotic (Kan 15 µg/mL) or with IPTG (0.5 mM) when needed.
2. Microcentrifuge and 1.5 mL tubes.

3. Thermocycler with suitable consumables (e.g., PCR 0.2 mL tube) and reagents: buffer, dNTPs, and economical screening polymerase.
4. Ultrapure water (distilled or deionized water).
5. Agarose gel electrophoresis equipment and reagents.

3 Methods

3.1 Prepare Substrate DNA Fragments for Recombination Steps

1. Using high-fidelity DNA polymerase and standard conditions, perform the amplification of DNA fragments needed for all double-joint PCR protocols:
 - (a) Resistance/suicide cassette using plasmid pGEM-*kan-mazF* (100 ng) as template and MazFCass-F/MazFCass-R primers (20 pmoles of each).
 - (b) Upstream DNA fragment for first-step mutant using wild-type *L. pneumophila* chromosomal DNA sample (100 ng) as template and P1/P2 primers (20 pmoles of each).
 - (c) Downstream DNA fragment for first-step mutant using wild-type *L. pneumophila* chromosomal DNA sample (100 ng) as template and P3/P4 primers (20 pmoles of each).
 - (d) Upstream DNA fragment for second-step mutant using wild-type *L. pneumophila* chromosomal DNA sample as template (100 ng) and P1/P5 primers (e.g., P5 designed to delete the target gene) or P1/P6 primers (e.g., P6 designed to introduce a point mutation in the target gene) or P1/Px depending of the desired construct (20 pmoles of each primer).
 - (e) Downstream DNA fragment for second-step mutant using wild-type *L. pneumophila* chromosomal DNA sample as template (100 ng) and P7/P4 primers (e.g., P7 designed to delete the target gene) or P8/P4 primers (e.g., P8 designed to introduce a point mutation in the target gene) or Py/P4 depending on the desired construct (20 pmoles of each primer).
2. Analyze PCR products on agarose gel, and if in agreement with the expected ones, purify each fragment (a–e) by extraction from agarose gel (*see Note 4*).
3. Using high-fidelity DNA polymerase and standard conditions, perform the amplification of double-joint PCR DNA fragments:
 - (f) First-step joint DNA fragment using a + b + c DNA fragments obtained above in equimolar concentrations with a

total quantity around 100 ng as template and P1/P4 primers (20 pmoles of each) (*see Note 5*).

- (g) Second-step joint DNA fragment using d + e DNA fragments obtained above in equimolar concentrations with a total quantity around 100 ng as template and P1/P4 primers (20 pmoles of each) (*see Note 5*).
- 4. Analyze aliquots of PCR products on agarose gel, and if in agreement with the expected ones, purify each fragment (f and g) from PCR reactions. Purification by extraction from agarose gel is also suitable.
- 5. Keep purified DNA fragments f and g at -20°C till needed.

3.2 First-Step Mutant Isolation

1. Streak *L. pneumophila* wild-type strain (e.g., *L. pneumophila* strain Paris) on CYE plate, and incubate for 72 h at 37°C .
2. With a sterile loop, scrap off *Legionella* culture from CYE plate, and resuspend in 2 mL of AYE in a 10–16 mL sterile tube.
3. Measure OD at 600 nm of the *Legionella* suspension.
4. From the *Legionella* suspension, inoculate three tubes of 5 mL AYE with 3 different initial OD (600 nm): 0.0125, 0.025, and 0.05.
5. Incubate for 12 to 18 h at 37°C with shaking.
6. Measure the OD (600 nm) of the three cultures, and choose the culture tube with OD between 1.1 and 1.5, corresponding to the competence state of *L. pneumophila*.
7. Centrifuge the culture tube with right OD for 5 min. at $4000 \times g$ at room temperature.
8. Remove 3 mL of supernatant, and gently resuspend the bacteria cell pellet in 2 mL of supernatant left.
9. Add purified DNA fragment f (volume corresponding to 300 ng to 1 μg of DNA) in the 2 mL *Legionella* suspension, and gently homogenize (e.g., using a vortex at low speed).
10. Incubate 8–24 h at 30°C without shaking (*see Note 6*).
11. Plate on 5 CYE plates (400 μL on each plate) containing 15 $\mu\text{g}/\text{mL}$ of kanamycin.
12. Incubate the plates for 2–5 days at 37°C (*see Note 7*). After this period, CYE plates can be stored at room temperature for 1 week.
13. Pick up 30–50 Kan^R isolated colonies, and for each one, make 2 patches on 2 plates, CYE + kanamycin (15 $\mu\text{g}/\text{mL}$) and CYE + IPTG (0.5 mM), to identify the right clones that must be Kan^R IPTG^S (*see Note 8*).
14. Incubate 24–48 h at 37°C .

15. Re-isolate the correct mutant clones (Kan^R IPTG^S) on CYE + Kan (15 µg/mL) plates, and incubate at 37 °C for 24 h for further analysis.

3.3 First-Step Mutant Analysis

1. From CYE + Kan (15 µg/mL) plates, scrap off one colony (do it for ten correct mutant clones), and resuspend in 50 µL of ultrapure water in a 1.5 mL tube.
2. Heat at 95 °C for 15 min.
3. Centrifuge at $13,000 \times g$ for 5 min, and transfer 5 µL of the supernatant in a PCR microtube.
4. Perform amplification by PCR with economical DNA polymerase and standard conditions using the 5 µL supernatant as template and P1/P4 primers (20 pmoles of each primer).
5. Analyze aliquots of PCR products on agarose gel to confirm the insertion of resistance/suicide cassette (MazFCass) in mutant chromosomes (*see* **Note 9**).
6. Select one or two clones to store as first-step mutant.

3.4 Second-Step Mutant Isolation

1. Streak *L. pneumophila* first-step mutant on CYE + Kan (15 µg/mL) plate, and incubate for 72 h at 37 °C.
2. With a sterile loop, scrap off *Legionella* mutant culture from CYE + Kan (15 µg/mL) plate, and resuspend in 2 mL of AYE in a 10–16 mL sterile tube.
3. Measure OD at 600 nm of the *Legionella* suspension.
4. From the *Legionella* suspension, inoculate three tubes of 5 mL AYE with 3 different initial OD (600 nm): 0.0125, 0.025, and 0.05.
5. Incubate for 12–18 h at 37 °C with shaking.
6. Measure the OD (600 nm) of the three cultures, and choose the culture tube with OD between 1.1 and 1.5, corresponding to competence state of *L. pneumophila*.
7. Centrifuge the culture tube with right OD for 5 min. at $4000 \times g$ at room temperature.
8. Remove 3 mL of supernatant, and gently resuspend the bacteria cell pellet in 2 mL of supernatant left.
9. Add purified DNA fragment g (volume corresponding to 300 ng to 1 µg of DNA) in the 2 mL *Legionella* suspension, and gently homogenize (e.g., using a vortex at low speed).
10. Incubate 8–24 h at 30 °C without shaking (*see* **Note 6**).
11. Prepare 10^{-1} , 10^{-2} , and 10^{-3} dilutions of the previous incubated suspension in AYE (*see* **Note 10**).
12. Plate 100 µL of each dilution on CYE plates containing 0.5 mM IPTG.

13. Incubate the plates for 2–5 days at 37 °C (*see Note 8*). After this period, CYE plates can be stored at room temperature for 1 week.
14. Pick up 30–50 IPTG^R isolated colonies, and for each one, make two patches on two plates, CYE + kanamycin (15 µg/mL) and CYE + IPTG (0.5 mM), to identify the right clones that must be Kan^S IPTG^R (*see Note 11*).
15. Incubate 24–48 h at 37 °C.
16. Re-isolate the correct mutant clones (Kan^S IPTG^R) on CYE + IPTG (0.5 mM) plates, and incubate at 37 °C for 24 h for further analysis.

3.5 Second-Step Mutant Analysis

1. From CYE + IPTG (0.5 mM) plates, scrap off one colony (do it for ten correct mutant clones), and resuspend in 50 µL of ultrapure water in a 1.5 mL tube.
2. Heat at 95 °C for 15 min.
3. Centrifuge at 13,000 × *g* for 5 min, and transfer 5 µL of the supernatant in a PCR microtube.
4. Perform amplification by PCR with economical DNA polymerase and standard conditions using the 5 µL supernatant as template and P1/P4 primers (20 pmoles of each primer).
5. Analyze aliquots of PCR products on agarose gel to confirm the construct (i.e., deletion, insertion, and so on) in mutant chromosomes (*see Note 9*).
6. Select one or two clones to store as clean mutant (second-step mutant).

4 Notes

1. Using this protocol, it is possible to design primers targeting one desired chromosomal region as an insertion site for new genes (e.g., reporter gene). In that case, attention must be paid in the primer design to restore the intact native flanking DNA region of insertion during the second step of the procedure.
2. Even if we successfully obtained mutants with 500 bp flanking region to promote homologous recombination, the efficacy of the protocol was clearly enhanced using at least 1 kb DNA homologous flanking region. Moreover, in our hands, 2 kb DNA flanking region was successfully used to manipulate *L. pneumophila* chromosome regardless of the target site.
3. Classically, primers 5' extensions for hybridization during the second joint PCR procedure are designed to have 20–30 nucleotides able to hybridize, which is sufficient.

4. In major cases, purification of initial PCR fragments by extraction from agarose gel is preferable to avoid any contamination with the primers used during the PCR. Indeed, primers used are often of 40–60 bp, an oligonucleotide length not always eliminated using PCR Purification Kit.
5. In few cases, joint PCR may be tricky to perform and may need optimization of the amount of each target DNA fragment. Thus, instead of equimolar concentrations of each DNA fragment, increasing the amount of MazFCass DNA fragment is often a successful solution.
6. Alternatively, transformation protocol from **steps 4 to 10** can be achieved as follows:
 - (a) The *Legionella* suspension is used to inoculate 2 mL AYE at a starting OD (600 nm) of 0.05.
 - (b) Add purified DNA fragment (volume corresponding to 300 ng to 1 µg of DNA) in the 2 mL *Legionella* suspension, and gently homogenize (e.g., using a vortex at low speed).
 - (c) Incubate 24 h at 30 °C with shaking to reach the stationary phase, and go on with **step 11** of the protocol.
7. Generally, small colonies appeared within 2 days, but 4 days is often the best incubation time to get colonies easy to pick up for further analysis. It is important to note that colonies may be of a large variety of size, and it is worth to pick up different ones to make the patches.
8. This analysis step is crucial as few mutant clones may present a Kan^R IPTG^R phenotype. One hypothesis of this phenotype appearance is the possible leak in the inducible *lac* expression system used, therefore selecting mutation in *mazF* gene or *Ptac* in recombinant bacteria. In our hands, the proportion of phenotype was as low as 0% but up to 60% depending of the targeted DNA region. Obviously, to be able to go on the second step of the protocol, the first-step mutant has to be Kan^R IPTG^S.
9. If the length of PCR products is questionable, for example, if the expected size of mutant DNA fragment is closed to the wild-type DNA fragment size, PCR products can be purified using PCR Purification Kit to perform restriction analysis.
10. The second step of the procedure is clearly more efficient, and dilutions of natural transformation sample are necessary to limit the number of colonies on CYE + IPTG plates. Plating 10⁻¹, 10⁻², and 10⁻³ dilutions allows to get at least 1 plate with adequate number of colonies, between 30 and 300, to go on further analysis.

11. Once again, this analysis step is crucial as few mutant clones may present Kan^R IPTG^R phenotype, certainly corresponding to the appearance of mutation in *mazF* gene or *Ptac* promoter instead of homologous recombination with g DNA fragment during the natural transformation step. In our hands, mutants spontaneously resistant to IPTG appear with a frequency of 10⁻⁶. Thus, this event is relatively rare and if all clones display a Kan^R IPTG^R phenotype, it is the consequence of bad first-step mutant selection. Therefore, the protocol must be restarted to isolate a clear Kan^R IPTG^S mutant.

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II. Article manuscript: The *Legionella pneumophila* LapD/LapG system is directly involved in localization of the virulence associated protein RtxA on the cell surface.

Legionella pneumophila is an intracellular pathogenic organism, one of its virulence factors is a cell surface protein belonging to the RTX family known as RtxA. Similar to some bacteria expressing RTX adhesins such as *Pseudomonas fluorescens* LapA, *L. pneumophila* contains genes (*lapG/lapD*) that are homologous to those existent in *P. fluorescens* and responsible for regulating LapA presence on the cell surface.

Our aim was to verify that *L. pneumophila* RtxA is indeed a substrate of such regulation system. In fact, previous work in our laboratory has shown that RtxA is the substrate of *L. pneumophila* type 1 secretion system but no evidence of a link between T1SS and LapD/LapG system was described. By consequence, our intent was also to verify whether the interaction of RtxA with these proteins results in regulating its presence on the cell surface. We also used bioinformatics tools to study the presence of RtxA type 1 secretion system components (*lssB/lssD*) across *legionella* species as well as for *lapG/lapD*. Finally, we investigated the possibility of interaction of *L. pneumophila* LapD with other diguanylate cyclases that might function in conjunction with LapD to regulate RtxA location similar to other bacterial models, using a bacterial two-hybrid assay.

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Title: The *Legionella pneumophila* LapD/LapG system is directly involved in localization of the virulence associated protein RtxA on the cell surface.

Hussein Kanaan,^{a,b,c} Claire Andrea,^a Ali Chokr,^{b,c} Annelise Chapalain,^a Patricia Doublet,^a Christophe Gilbert,^{a,#}

^aInternational Center for Infectiology Research (CIRI), INSERM U1111, ENS Lyon, CNRS UMR5308, Université Lyon 1, Université de Lyon, Lyon, France.

^bLaboratory of Microbiology, Department of Biology, Faculty of Sciences I, Lebanese University, Hadat Campus, Beirut, Lebanon.

^cPlatform of Research and Analysis in Environmental Sciences (PRASE), Doctoral School of Sciences and Technologies, Lebanese University, Hadat Campus, Beirut, Lebanon.

Running title: RtxA cleavage and release from *Legionella*

#Correspondence to: Christophe Gilbert, CIRI U1111 INSERM, Bât. Lwoff, 10 rue Raphael Dubois, 69622 Villeurbanne Cedex, France. Tel: (33) 4 72 43 13 66, Fax: (33) 4 72 43 26 86.
christophe.gilbert.bio@univ-lyon1.fr

Keywords: *L. pneumophila*, *P. fluorescens*, RtxA, LapD, LapG, Type 1 secretion system, virulence.

A. Abstract

Legionella pneumophila is the causative agent of a form of pneumonia called legionellosis or Legionnaires' disease. This bacterium produces an extremely large protein belonging to the RTX (Repeats in ToXin) family called RtxA which is often correlated to its virulence. We previously reported that RtxA is transported by a dedicated type 1 secretion system (T1SS) to the cell surface. However, it was not clear whether this protein is released directly to the extracellular milieu or kept on the cell surface pending release after certain environmental cues, which is the case in closely related models such as LapA in *Pseudomonas fluorescens*. The hereby work reveals that, *in vitro*, the LapG periplasmic protease cleaves RtxA N-terminus in the middle of a di-alanine motif (position 108-109). Interestingly, we identified the presence of homologous potential T1SS/LapDG systems in many *Legionella* species and other Gammaproteobacteria.

We also show, using *lapG* and *lapD* mutant strains and immunofluorescence microscopy, that RtxA release is under the control of these two proteins. We observed that a strain lacking LapG protease maintains RtxA on the cell surface, while a strain lacking LapD does not exhibit cell surface RtxA because of its continuous cleavage and release. These findings demonstrate that the presence of this large virulence protein on the cell surface is regulated in *L. pneumophila*, similar to the *P. fluorescens* LapA model and this probably occurs via an intermediary state during protein secretion as recently described for LapA in *P. fluorescens*. However, cyclic-di-GMP fine regulation and proteins partners remain unknown.

B. Importance

Several Gram-negative bacteria utilize RTX proteins as a tool for survival through biofilm formation, virulence and many others. Some bacteria producing such proteins do not secrete them directly to the extracellular medium, in fact they are maintained on the cell surface to perform specific functions after which they are released based on distinct signals. Here we show that RtxA, a *Legionella pneumophila* surface RTX protein implicated in virulence, is subject to release from cell surface following cleavage between two alanine bases (*in vitro* characterization of cutting site) by a periplasmic protease named LapG.

C. Introduction

The *Legionella* genus comprises around 60 species including 70 serogroups. 30 of these species are pathogenic to humans, especially *Legionella pneumophila* serogroup 1 (Lp1) that is responsible for more than 90% of clinical cases (1-3). *L. pneumophila* is a Gram-negative intracellular bacillus and is the causative agent of a severe form of pneumonia called Legionnaires' disease which accounts for 2-9% of cases of community acquired pneumonia (1, 2, 4). These bacteria inhabit freshwater environments as a parasite of protozoa especially amoebae which are considered as their natural hosts (1). However, humans are considered to be an accidental host to *L. pneumophila*. In fact, legionellosis is almost exclusively a result of man-made environments such as water-cooling towers and air conditioning systems where bacteria use the dispersed aerosols to infect human alveolar macrophages (1, 5). Nosocomial instances of legionellosis have also been recorded frequently due to presence of *Legionella* in hospitals' water supply despite appropriate maintenance of water distribution systems (6). *L. pneumophila* is also regarded as an opportunistic pathogen since it mainly affects susceptible patients due to age, previous conditions and immunosuppression (2, 3). Moreover, the mortality rate of Legionnaires' disease ranges from 7-25% despite appropriate antibiotic treatment which renders this disease a public health concern (7).

In the case of pathogenic bacteria, secretion systems constitute a crucial factor for pathogenesis and interbacterial competition mainly through damaging and/or manipulating the host, evading the immune system and establishing a replicative niche in case of intracellular pathogens such as *L. pneumophila* (8-10). Seven bacterial secretion systems have been identified to date and they fall into two main categories: those which direct their substrates to the extracellular medium such as the type 1 secretion system (T1SS), T2SS, T5SS and T7SS; and those that inject their substrates into the host cytoplasm by spanning several membranes, such as the T3SS, T4SS and T6SS (8, 11). Regarding *Legionella*, a recent study reports the presence of T1SS, T2SS, T4SS and the T6SS which was previously unknown (12). The T2SS and T4SS have been directly correlated to the virulence of *L. pneumophila* and especially the T4SS that

enables this bacterium to assume the intracellular lifestyle (13). Briefly, the Dot/Icm T4BSS is responsible for transporting approximately 300 effector proteins from the internalized bacterium to the host cytoplasm, these effectors disrupt and hijack many host processes such as the lysosomal fusion and consequently create a specialized niche for replication in the phagosome called the *Legionella* containing vacuole (LCV) (14). Regarding the T1SS, we previously reported that it is responsible in *L. pneumophila* for the secretion of a large RTX protein called RtxA (15). Typically, T1SSs consist of three components: an inner membrane ABC transporter that relies on ATP for substrate transport, a periplasmic membrane fusion protein and an outer membrane protein; in *L. pneumophila* these are named LssB, LssD and TolC respectively (15). These proteins form a channel spanning both bacterial membranes for substrate secretion. Usually, this secretion system transports its substrate in a single step to the extracellular medium. However, recent studies suggest that some RTX transporting T1SSs mediate transport via a two-step process that involves a periplasmic intermediate (16).

RTX proteins have been implicated in the virulence of various bacteria, the most studied model is the pore forming *Escherichia coli* hemolysin A (HlyA) (17, 18). The biofilm associated protein of *P. fluorescens* LapA is also well studied example (19). The *L. pneumophila* RtxA shares the general features of RTX proteins such as a C-terminal secretion signal, an RTX repeats motif (GGXGXDX) and a central repeat region (20). It has been frequently linked to *L. pneumophila* virulence where *rtxA* mutants exhibited a measurable loss of virulence and reduction in adhesion capabilities to monocytic and epithelial cells (21). RtxA has also been implicated in intracellular survival such as inhibition of lysosomal fusion though with not enough evidence (22). In the closely related adhesin LapA from *P. fluorescens*, studies report that biofilm formation in this bacterium is dependent on the presence of LapA on the cell surface and that specific environmental signals orchestrate this process (23). Briefly, the inner membrane effector protein LapD (member of GGDEF/EAL domain protein) in conjunction with a diguanylate cyclase (24) senses changes in intracellular cyclic-di-GMP levels and regulates accordingly the activity of a periplasmic protease LapG by sequestering or releasing it in the periplasm (25). This protease, when free, can cleave the N-terminus of LapA leading to its release and biofilm dispersal and vice versa (26). In *L. pneumophila*, functional LapG and LapD homologs were identified, but their role with T1SS was not studied (27, 28).

To date, it is not known whether *L. pneumophila* RtxA is regulated on the cell surface in a manner similar to other closely related adhesins. In this paper, we provide evidence that RtxA^{NH2} is cleaved by LapG *in vitro*. Interestingly, it seems that the T1SS and LapD/LapG coevolved in *L. pneumophila* species and harbored specific characteristics compared to the equivalent systems present in other *Legionella* species. Moreover, we proved that this cleavage ultimately results in the release of RtxA from the cell surface suggesting that *L. pneumophila* can modulate its virulence associated protein according to specific conditions. Finally, we attempted to investigate the possible interaction between the RtxA secretion system components and the LapD/LapG system.

D. Results

1. *In vitro* cleavage of RtxA by LapG

Previous studies report that some RTX family proteins are susceptible to cleavage by periplasmic proteases in a controlled regulatory process (23, 26). And since homologs of both substrate and enzyme were identified in *L. pneumophila* (27, 29), we hypothesized that the periplasmic protease LapG can indeed cleave RtxA. For this purpose, a COOH-His-tagged fragment of RtxA^{NH2} (492 amino acids ~ 54 kDa) was cloned in an appropriate plasmid and overproduced in *E. coli* as well as a NH₂-His-tagged LapG protein minus its secretion signal (188 amino acids ~ 22 kDa). Both cloning and purification were conducted independently.

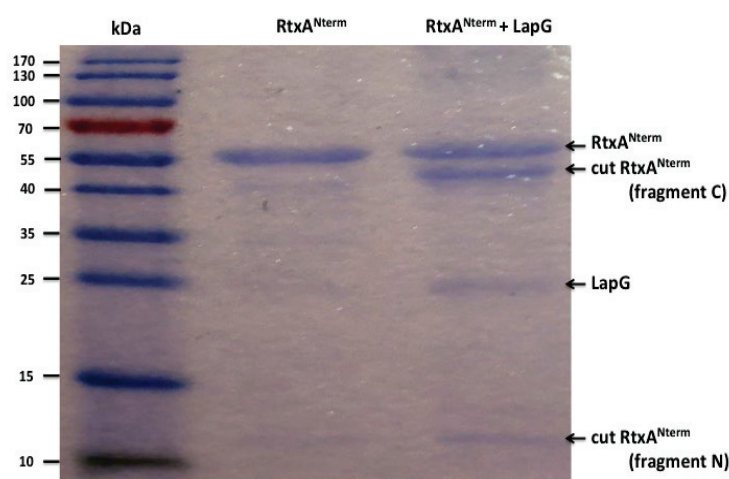


Figure 1. Gel electrophoresis of RtxA^{NH2} incubated with LapG protease

Purified RtxA^{NH2} (shown in the second lane ~54 kDa) was incubated with LapG (~23 kDa) in a suitable buffer containing 40 mM MgCl₂ and 80 mM CaCl₂ for 2 hours at 37 °C. The third lane represents the outcome of the incubation showing two protein bands at 42 and 12 kDa corresponding to the COOH and NH₂ fragments of the cleaved RtxA^{NH2} in addition to residual uncleaved amount of the latter protein and LapG. Proteins were run on a 12% polyacrylamide gel, PageRuler™ prestained protein ladder is used.

Following co-incubation of the two purified proteins in a suitable buffer for 2 hours, reaction mixture components were visualized using SDS-PAGE (**Figure 1**). In the control lane corresponding to RtxA^{NH2} incubated alone in the assay buffer, no degradation was observed during the 2 hours incubation. On the contrary, the lane containing the co-incubated proteins show 2 protein bands (42 and 12 kDa) in addition to LapG and RtxA^{NH2} which indicates that an amount of RtxA^{NH2} was cleaved *in vitro*. Furthermore, the sum of the size of the 2 RtxA^{NH2} post-cleavage fragments is in agreement with the given size of our

recombinant protein (54 kDa). These fragments also coincided with previous reports in *P. fluorescens* stating that the most probable cleavage site is around 108th or 109th residue (**Figure 2**) (23).

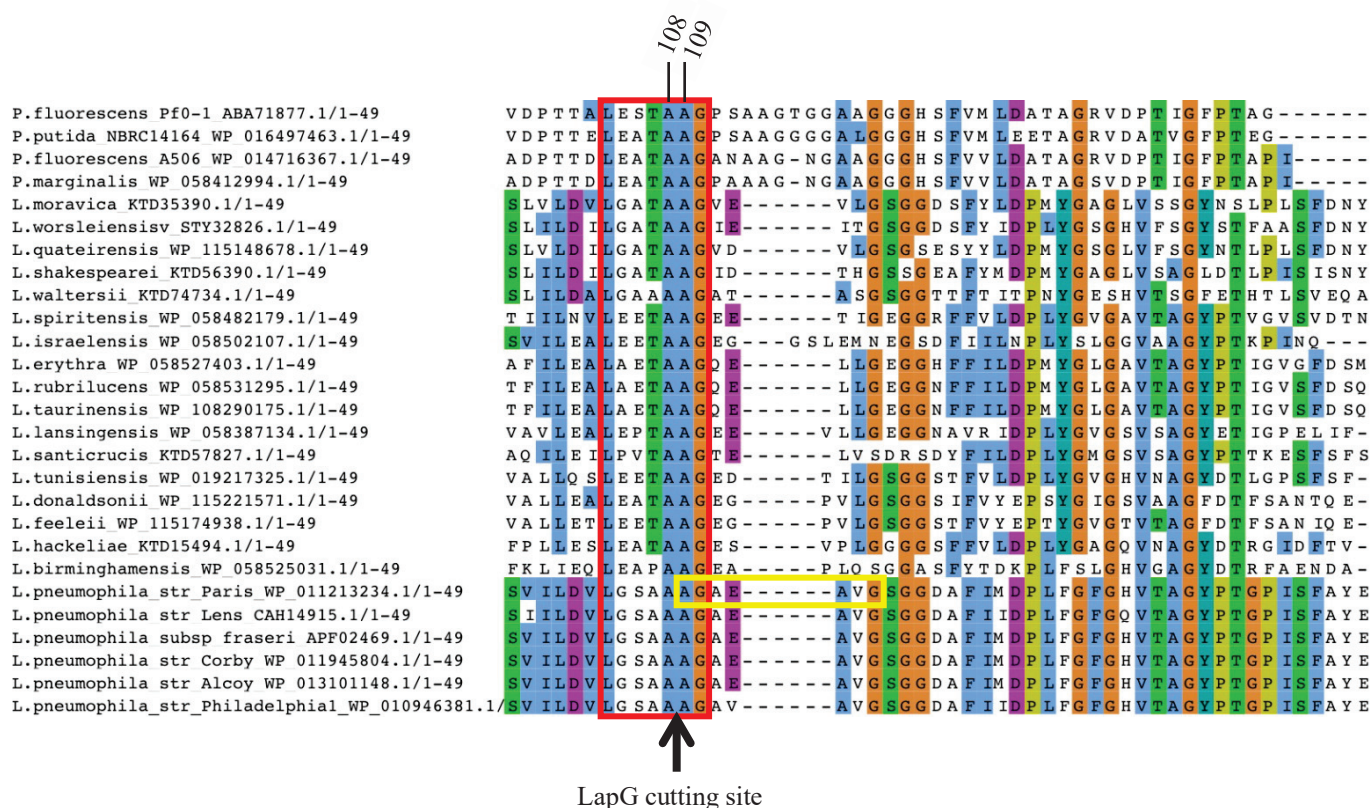


Figure 2. Primary sequence alignment of LapG cleavage site region of various potential RtxA proteins within *Legionella* species with *Pseudomonas* LapA proteins

Cleavage site between two alanine residues is indicated by an arrow. The yellow box surrounded sequence is the outcome of Edman degradation analysis of the cleaved RtxA C-fragment, cleavage occurring between residues 108 to 109. This position is well conserved in most of RtxA and LapA proteins with few variations (from 106 to 112 for the first alanine).

Therefore, to further pinpoint the exact *in vitro* cleavage site, the C-fragment (42 kDa) of the cleaved RtxA^{NH2} was sequenced by Edman degradation and the identity of its 7 N-terminal amino acids was found to be AGAEAVG, indicating that the cleavage occurs immediately between residues 108 and 109. This result experimentally confirmed the putative cleavage site proposed for other RTX proteins, especially in *P. fluorescens* (23) (**Figure 2**). Interestingly, using protein BLAST search, RtxA N-terminus homologues were identified among many *Legionella* species and alignment of LapG cutting site region revealed few differences. Especially an A in position 107 (before the double A recognized cutting site) that is present in all *L. pneumophila* RtxA proteins in substitution of a T present in most of cutting sites among LapA family proteins (**Figure 2**). Therefore, *L. pneumophila* RtxAs seem to define a well conserved clade in *Legionella* group which are in any case close relatives compared to *Pseudomonas* LapA.

2. Phylogeny of T1SS and LapD/LapG system among *Legionella* species

Recently, published work claimed that T1SS is restricted to *L. pneumophila* species among the genus *Legionella* (12, 16), an observation not in agreement with RtxA homologues search in all *Legionella* species as reported in the previous paragraph. In fact, the authors searched for the entire *lss* operon in *Legionella*, but the whole genetic organization does not seem to be conserved among the genus, which may have led to an erroneous conclusion. Moreover, it is interesting to note that few potential C39 family peptidases have been identified in different *Legionella* species as mentioned on MEROPS, the peptidase database (30). This family of transporters is involved in bacteriocin secretion and is closely relative to T1SS transporters family. Thus, to elucidate this point more precisely, we performed protein BLAST searches on *Legionella* genomes already published looking for classical C39 exporters (ABC-type bacteriocin/lantibiotic exporters) and C39-like T1SS exporters (HlyB and LapB/LssB families). The results were consistent with the presence of both types of transporters in *Legionella* species and alignments were performed with representative strains to build a phylogenetic tree (**Figure 3**). In case of ABC-type bacteriocin/lantibiotic exporters, only *Escherichia coli* and *Legionella* homologues are reported in the tree whereas few other bacterial species are included in the C39-like exporters family. Interestingly, recently published genomes of 3 *L. pneumophila* species (D-5265 reported on the tree, D-4040 and D7787) revealed the presence of C39 and C39-like exporters co-existing on the chromosome. It is the first identification of a bacteriocin exporter in *L. pneumophila* and it seems closer to *E. coli* colicin system than to many other putative bacteriocin exporters in *Legionella* group. Up to date, the bacteriocin substrates have not been characterized in *Legionella* genus, neither the bacterial species sensitive to these bacteriocins. Concerning the C39-like T1SS exporters involved in “adhesin-like” export, highly conserved LssB protein is recovered in *L. pneumophila* species inside a clade formed by *Legionella* LssB homologues (**Figure 3**). A similar tree was obtained comparing LssD/LapD and HlyD families’ proteins, the periplasmic associated component of T1SS (data not shown). In addition, the comparison of LapG and LapD proteins, components of RtxA/LapA cutting system was performed to construct phylogenetic trees. Searching of LapG homologues in the bacterial species identified previously did not permit to identify close proteins in *Burkholderia* genus, but the phylogeny observed with all the other species was similar to the LssB/LapB phylogeny (**Figure 4**). A similar tree was obtained using LapD homologous proteins (data not shown).

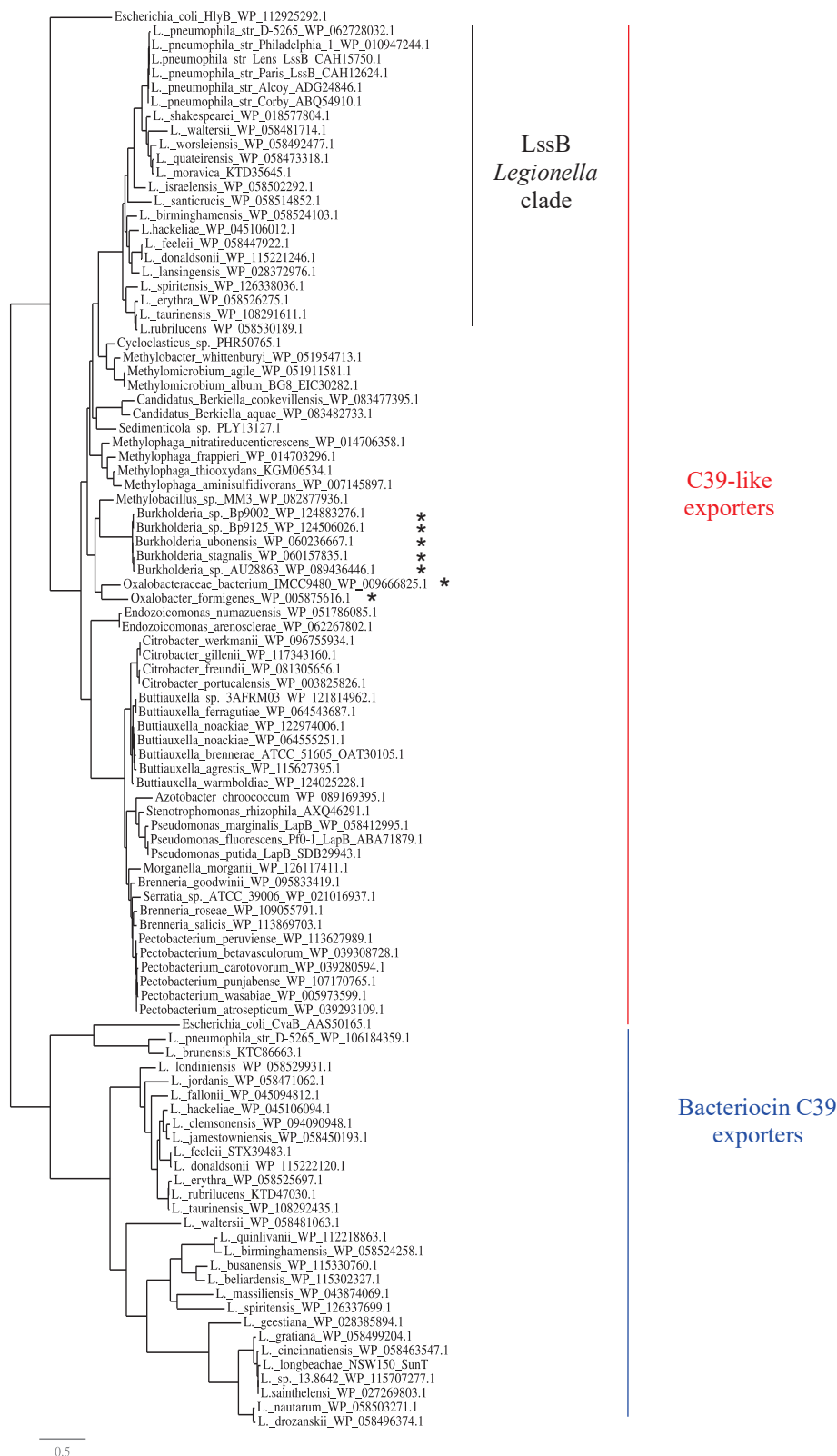


Figure 3. C39 and C39-like exporters phylogenetic tree inferred using maximum-likelihood (PhyML 3.0 software)

The proteins reported in the tree were chosen for their high similarities with *Pseudomonas* LapB or *E. coli* CvaB proteins. Only one representative protein is conserved in each bacterial species except in *Legionella pneumophila*. All bacterial species are gammaproteobacteria except 7 pointed with an asterisk which are in betaproteobacteria class. The branch length is proportional to the number of substitutions per site (scale at the bottom). L: *Legionella*.

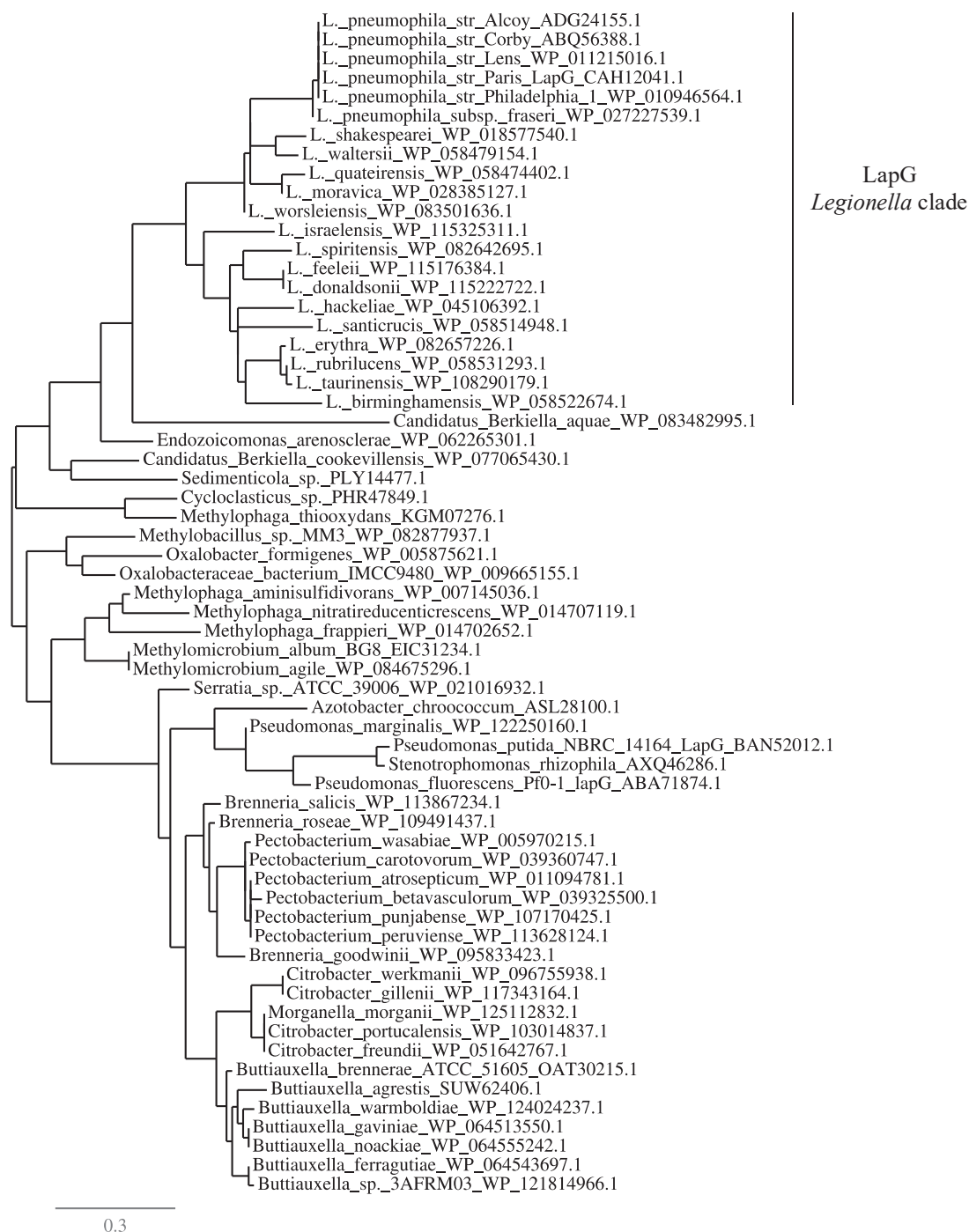


Figure 4. LapG family proteins phylogenetic tree inferred using maximum-likelihood (PhyML 3.0 software)
The proteins reported in the tree were chosen for their high similarities with *Pseudomonas* LapG. Only one representative protein is conserved in each bacterial species except in *Legionella pneumophila*. All bacterial species are gammaproteobacteria except 2 pointed with an asterisk which are in betaproteobacteria class. The branch length is proportional to the number of substitutions per site (scale at the bottom). L: *Legionella*.

3. RtxA release from cell surface is controlled by LapD/LapG system

RTX proteins are transported to the extracellular space by T1SSs and that is the case of *L. pneumophila* RtxA as we proved in a previous publication (15). However, the possible fates of the protein after its passage through the secretion machinery were not clear. Following evidence from *P. fluorescens* (26) and building on our previous results, we also hypothesized that LapG cleavage of RtxA results in its release from the cell surface.

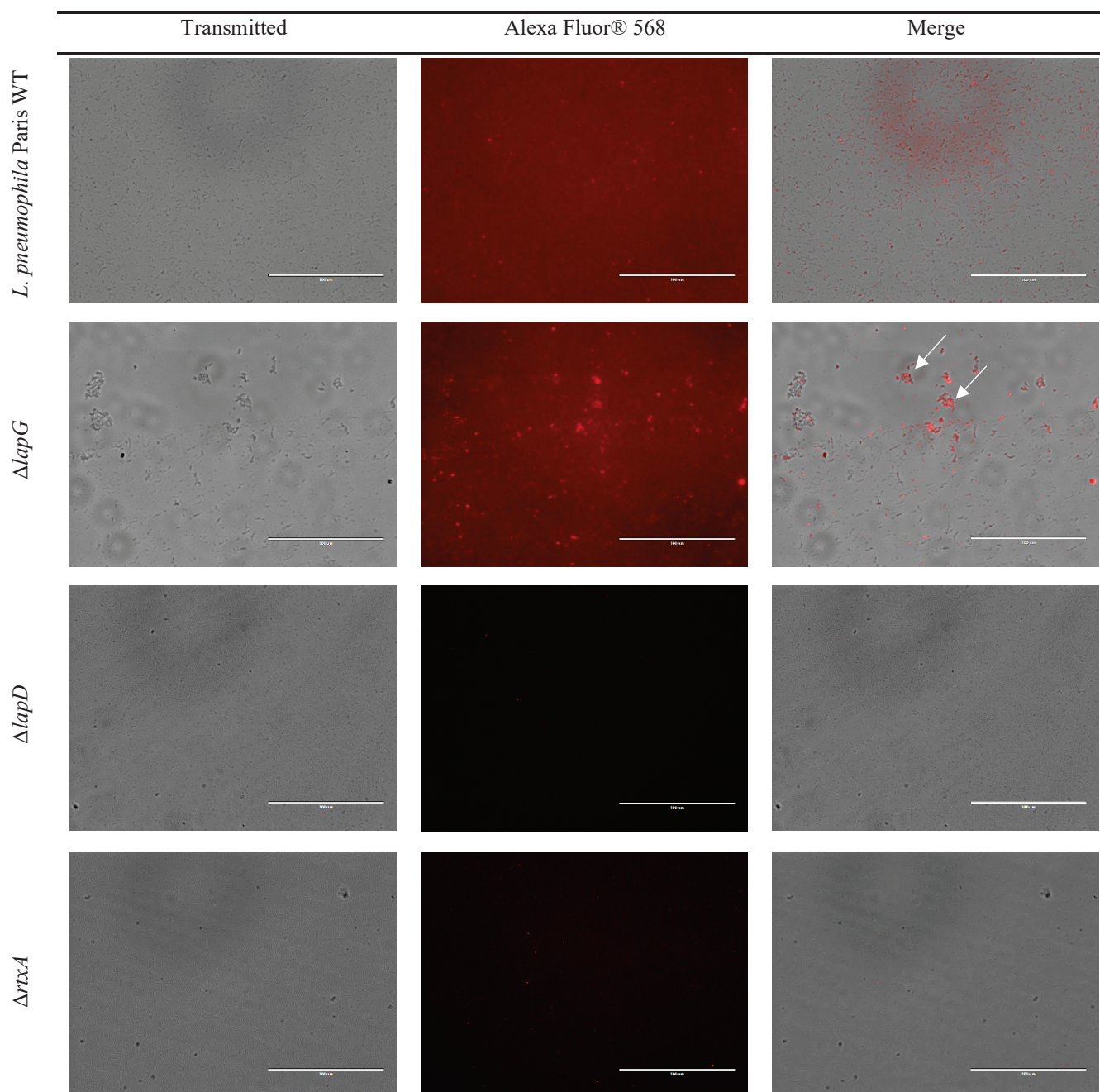


Figure 5. Immunofluorescence microscopy of four *L. pneumophila* strains using anti RtxA^{COOH} antibodies
L. pneumophila Paris wild-type, $\Delta lapG$ mutant, $\Delta lapD$ mutant and $\Delta rtxA$ mutant were used in an indirect immunofluorescence assay using antibodies targeting the C-terminus of RtxA protein. The secondary antibody was Alexa Fluor® 568 goat anti-rabbit (Invitrogen Inc. USA). Scale bar shown is 100 μ m. Aggregated bacteria are pointed out with a white arrow.

For this purpose, specific rabbit polyclonal antibodies against the C terminal region of RtxA were produced. Clean *L. pneumophila* $\Delta lapG$ and $\Delta lapD$ deletion mutants were designed and constructed to disrupt the hypothetical RtxA regulatory machinery in addition to $\Delta rtxA$ deletion strain for use as a negative control in immunofluorescence microscopy. Briefly, *L. pneumophila* were grown for 3 days then fixed on microscope slides for immunoblotting and visualization. The images shown in **figure 5** correspond to blotting assays using anti-RtxA^{COOH} antibodies at 0.374 $\mu\text{g ml}^{-1}$ followed by secondary anti-IgG antibodies conjugated to an Alexa Fluor® 568 fluorescent element. Transmissive phase and fluorescence images were taken then merged.

As expected, no fluorescence at all was seen in the case of $\Delta rtxA$ mutant strain as it is defective for RtxA production when comparing with the wild-type (WT, **Figure 5**). Therefore, our negative control is efficient and no cross-reaction of our primary antibodies with other *Legionella* proteins is detected. As for the WT strain, almost all bacteria were marked with red fluorescent dots, our experimental growth conditions seemed to maintain a significant amount of *L. pneumophila* RtxA on cell surface. Coming to $\Delta lapD$, the absence of fluorescence allowed us to conclude that no RtxA was present at the surface of the cells. This result is in agreement with the proposed role of LapD. Indeed, LapD controls the activity of the periplasmic protease LapG by physically sequestering it close to the outer-face of the inner membrane and preventing RtxA N-terminus periplasmic cleavage. Therefore, the absence of LapD in $\Delta lapD$ mutant strain may result in continuously free LapG in the periplasm, that is able to cleave and release RtxA from the cell surface. Finally, the strain lacking *lapG* exhibits lots of red dots at the surface of the cells corresponding to RtxA protein embedded into the outer membrane (**Figure 5**). Moreover, it is worth noting that some phenotypic characteristics such as aggregation which may be due to saturation of the cell surface with RtxA protein which in turn may cause clumping of the cells. Therefore, all these results confirmed that, LapD/LapG system controls the RtxA localization (embedded or released) in *L. pneumophila* by blocking or promoting its N-terminus cleavage in the periplasmic space.

4. Assessment of interaction between T1SS, LapD/LapG and other diguanylate cyclases

In order to further investigate which components of the Lss T1SS interact with LapG or LapD to allow cleavage of RtxA after its transport through the secretion channel, a bacterial two hybrid system was designed. Our hypothesis was corroborated by recent studies on *P. fluorescens* that prove the presence of an intermediary state for RtxA during secretion where cleavage can occur (16). Protein interactions were estimated by the presence/intensity of the blue color of transformants on LB-X-Gal plates.

The upper portion of **Table 1** shows the results obtained with our different combinations. Positive and negative controls were taken into account when estimating the results. As expected, we observed a

strong interaction between LssB and LssD components of the T1SS. We then detected a possible faint interaction between LapD and LssE (lpp1475), a protein belonging to the *lss* operon. LssE is an inner membrane protein belonging to the GGDEF/EAL domain proteins family but the GGDEF domain is weakly conserved (SGDQF) and no diguanylate cyclase activity was detected in our hands (Anne Vianney, personal communication). Therefore, LssE may be involved in LapD deactivation by degrading c-di-GMP bound to LapD. Indeed, LapD proteins in *L. pneumophila* or *P. fluorescens*, though containing denaturated GGDEF/EAL domains, lack diguanylate cyclase activity and are unable to synthesize c-di-GMP, neither to degrade it (no diguanylate phosphodiesterase activity) (31). Finally, we detected interaction between LssE and LssZ, another protein of the T1SS operon. LssZ is an inner membrane protein of unknown function (**Table 1**).

Table 1. Bacterial two-hybrid screening for partners among LapD and *lss* operon encoded proteins (top), between LapD and GGDEF/EAL domains proteins (bottom)

| | LssX | LssY | LssZ | LssA | LssB | LssD | LssE |
|------|------|------|------|------|------|------|------|
| LssY | Ø | | | | | | |
| LssZ | Ø | Ø | | | | | |
| LssA | Ø | Ø | Ø | | | | |
| LssB | Ø | Ø | Ø | Ø | | | |
| LssD | Ø | Ø | Ø | Ø | +++ | | |
| LssE | Ø | Ø | ++ | Ø | Ø | Ø | |
| LapD | Ø | Ø | Ø | Ø | Ø | Ø | + |

| | lpp0299 | lpp0440 | lpp0809 | lpp0942 | lpp1311 | lpp2355 |
|------|---------|---------|---------|---------|---------|---------|
| LapD | Ø | Ø | Ø | Ø | Ø | Ø |

To pursue further the identification of the diguanylate cyclase protein responsible for LapD activation by providing c-di-GMP, 6 possible *L. pneumophila* GGDEF domain proteins were chosen based on a BLAST against *P. fluorescens* protein GcbC that was shown to activate LapD (24). The lower portion of **Table 1** shows the outcome of our two-hybrid investigation between LapD and the 6 candidate diguanylate cyclases, but no interaction was observed for these combinations under our experimental conditions. However, c-di-GMP networks are considered to be complex and may require stringent conditions to operate properly.

E. Discussion

RTX proteins are produced by a variety of Gram-negative bacteria and with diverse functions where some are cytotoxins, hemolysins, proteases, lipases and adhesion associated proteins (32). Moreover, such proteins from many bacteria are directly implicated in human diseases, namely the Hemolysin A from enterohemorrhagic *E. coli* (17, 18); or indirectly which is the case with *L. pneumophila* RtxA (21). Therefore, understanding the mechanisms that govern their secretion and possible regulation on the cell surface is crucial to target and combat their activities and role in pathogenesis.

P. fluorescens is a well-studied model in this regard, and its RTX protein LapA localization was shown to undergo precise regulation that in turn controls biofilm formation as this protein is involved in adhesion (23). Homologs to the proteins controlling LapA cell surface localization, LapG and LapD, were discovered in *L. pneumophila* (27, 28), but were not studied for their role with their *Legionella* potential substrate, RtxA. Therefore, in this work we demonstrated that *L. pneumophila* periplasmic protease LapG effectively cleaves RtxA in its N terminal region, *in-vitro*. Moreover, by sequencing the first 7 amino acids of the cleaved fragment we were able to determine for the first time that the exact cleavage site was between amino acids 108 and 109, even if we can't exclude other possible alternative cutting sites *in vivo*. Previous research regarding the *L. pneumophila* LapG reveals it is capable of cleaving the *P. fluorescens* LapA^{Nterm} albeit less efficiently than *P. fluorescens* native LapG protein (27). In this study we proved that *L. pneumophila* RtxA is indeed a substrate for LapG and the few changes in amino acids surrounding the cutting site of LapA compared to RtxA may explain the difference of cleaving efficacy observed by Chatterjee *et al.* (27). Moreover, looking for T1SS and LapD/LapG proteins among bacterial translated genomes, we clearly identified homologous proteins for both systems in all *Legionella* species, and on a wider scale, in many gammaproteobacteria species, which seems to rule out the previous observation by Qin *et al.* (12). Thus, the presence of a T1SS can't be clearly associated with higher virulence of *L. pneumophila* strains towards macrophages as questioned by Smith *et al.* (16); and may be involved during the first steps of all eukaryotic cells' infection, *i.e.* Protozoa, pneumocytes and macrophages. Despite this fact, the RtxA proteins harbored highly variable regions between strains and that may have an impact on their role towards host cells, therefore modifying their range. The presence of large repeat regions complicates the identification of entire *rtxA* genes in *Legionella* genomes using classical high-throughput Illumina sequencing, but the development of Nanopore-based sequencing (Oxford Nanopore Technologies) may help in the future to better characterize the RtxA proteins family which may contribute to understand the differential role of these huge proteins among strains. It is worth noting that few *Legionella* species such as *L. longbeachae* do not possess any Lss T1SS but harbor a bacteriocin C39 exporter, though not characterized yet. Looking at the phylogeny of the C39 and C39-like exporters, the assumption of independent acquisition event of these two transporters types in *Legionella* species can be made, a hypothesis reinforced by the fact that both systems can be present in the same strain. As *L. longbeachae* is a well-known legionellosis agent,

the absence of Lss T1SS suggests that the first infection steps to enter amoeba or macrophages may involve different mechanisms compared to *L. pneumophila*.

Building on our previous results, we assessed the effect of RtxA cleavage on its localization on the cell surface. Using *L. pneumophila* mutants and antibodies specific to the RtxA^{COOH}, we were able to determine that in $\Delta lapD$ mutant strains, no RtxA was detected on the cell surface. Given our previous findings that LapG cleaves RtxA, this suggests that LapD exerts certain control regulating LapG activity where in the absence of this protein, LapG was free to continuously cleave RtxA and release it from the cell surface. Moreover, in the $\Delta lapG$ mutant strain, detected fluorescence signifies the presence of RtxA on the cell surface and this permanent localization was associated with cell aggregation when compared to WT and other mutant strains. This can be attributed to the saturation of *L. pneumophila* cell surface with RtxA, knowing that its C-terminal region possesses a von Willebrand type A domain involved in adhesion as well as several adhesion motifs in its central repeat region (20). Therefore, we can safely conclude that LapD has regulatory potential over LapG protease activity, and that RtxA^{NH2} cleavage results in its release from the cell surface.

Recent studies show that although T1SSs are known to transport substrates in a single step to the extracellular medium, a distinct sub-group of T1SS machinery linked with bacterial transglutaminase-like cysteine proteinase (BTLCP) uses a two-step secretion mechanism (16). This intermediary step during secretion presents a plausible opportunity for the cleavage process to take place. This study also suggests that an outer membrane protein other than the classical TolC may be involved in stabilization of an RTX adhesin on the cell surface via its N-terminal fragment. Therefore, we assessed via bacterial two-hybrid assays, the possible interactions between proteins of the T1SS operon and between those proteins and LapD. Under our experimental conditions we were only able to detect interactions between LssB/LssD, LssE/LssZ and LssE/LapD. The first interaction is quite expected since these proteins are adjacent components of the T1SS (ABC transporter and the membrane fusion protein). LssE, a predicted member of GGDEF/EAL domains protein family, but potentially only displaying diguanylate phosphodiesterase activity, therefore able to degrade c-di-GMP which may induce the LapD return to an inactive state. Up to date, no role can be proposed for LssZ and further studies must clarify its involvement in RtxA release. Moreover, LapD activation according to recent research occurs via physical interaction with another protein able to synthesize c-di-GMP (33). Thus, few other diguanylate cyclases candidates mentioned in **Table 1** were included in the two-hybrid experiment to assess their possible interaction with LapD but none appeared to interact under our conditions.

So far, the studied RTX surface proteins that undergo a regulated release process, are biofilm associated proteins. Taken together, our findings show for the first time that an RTX protein in *L. pneumophila* which is linked to its virulence is also susceptible to similar regulation. This raises question whether this is a way for *Legionella* to modulate its virulence potential. Despite the previous statement, we also

detected cellular aggregation in mutant strains unable to release RtxA which might hint at role in biofilm formation as well, but mono-species *Legionella* biofilms are weak compared to multi-species biofilms including *Legionella*. Furthermore, *Legionella* biofilm formation has also been recently reported as T4SS-dependent (34), meaning many factors are involved. We also conclude that LapD is a central module in this regulation process, similar to its homolog in *P. fluorescens*. Further research is needed to clarify the specifics of RtxA cleavage, and to unravel possible partners of LapD that allow such process to be tightly controlled in conjunction with the infection cycle of *Legionella pneumophila*. The dynamic and transitory state of such proteins' interaction will require *in vivo* experimental techniques to enable the characterization of cascade events involved in RtxA embedding in outer membrane and further release from the cell surface.

F. Materials and methods

Strains and growth conditions

Strains used in this study are listed in **table S1**. Regarding *L. pneumophila*, the Paris strain was used in the hereby presented work, it was grown on buffered charcoal yeast extract (BCYE) agar or in liquid AYE (ACES buffered yeast extract) medium. Cultures were grown at 30°C or 37°C depending on the experiment. Isopropyl β -D-1-thiogalactopyranoside (IPTG; 1 mM), Kanamycin (15 $\mu\text{g ml}^{-1}$) or chloramphenicol (5 $\mu\text{g ml}^{-1}$) were added when appropriate. *E. coli* strains were grown in lysogeny broth with rotation (LB) or agar at 37°C unless mentioned otherwise. Appropriate antibiotics were added when necessary according to the following concentrations; Kanamycin (50 $\mu\text{g ml}^{-1}$) and/or Ampicillin (100 $\mu\text{g ml}^{-1}$).

Plasmid construction

DNA constructs used in this study were made by using *E. coli* BL21 or DH5- α as the host. Schematic representations of the key plasmids are shown in **figure S2** in supplemental material. *L. pneumophila* Paris strain genomic DNA was used as template for PCR production of desired inserts. The recombinant plasmids were verified by PCR and enzymatic digestion prior to electroporation into the *E. coli* strains.

Gene deletion in *L. pneumophila*

Gene-specific deletions in *L. pneumophila* were carried out using the homologous recombination method (35). Mutant strains were derived from *L. pneumophila* wild-type (WT) Paris strain. Briefly,

this two-step process relies on the natural competence of *L. pneumophila* and results in clean scar-free mutants. In our work, the 2kb regions flanking the gene to be deleted were amplified by PCR. In the first step, a resistance/suicide inducible cassette which is in this case kanamycin/MazF, is inserted between the flanking regions by double joint PCR. This constructed cassette given to *L. pneumophila* competent cells will replace the gene of interest and will allow for primary selection on kanamycin supplemented media. Successful colonies are Kan resistant and IPTG sensitive. The cassette integration was confirmed by PCR. In the second step, the gene flanking regions were joined by PCR and used for natural transformation of the previously obtained “first step mutants”. The aim is to replace the Kan/MazF cassette which will produce clean gene deletions. Colonies corresponding to deleted mutants are Kan^s IPTG^r and deleted DNA regions were also confirmed by PCR.

***In vitro* LapG cleavage analysis**

RtxA^{NH2} (nucleotides 1-1490 from *rtxA* lpp0699) was cloned into a pET-30 plasmid upstream a 6xHis Tag using NdeI/SalI restriction sites to insert the DNA fragment (**Figure S2**). The constructed plasmid allowed for production of N-terminal fragment of 505 amino acids including 6 histidine with a molecular mass of 53.2 kDa. In brief, the constructed plasmid was transformed into *E. coli* strain BL21 by electroporation. Recombinant protein was produced by a 2-hour induction with 1 mM IPTG of an exponential culture that reached an OD_{600nm} of 0.5. Cells were collected and broken using a French pressure cell (20,000 Psi). The recombinant protein was purified using Talon® metal affinity resin (Takara Bio USA Inc). Similarly, *L. pneumophila* LapG was produced in *E. coli* BL21 by cloning nucleotides 169-735 (lpp0890) using BamHI/PstI restriction sites to insert the DNA fragment in a pQE-30 plasmid downstream a 6xHis tag (**Figure S2**). The recombinant protein (22.5 kDa) was purified as described earlier. To assess the protease activity of *L. pneumophila* LapG on RtxA, the purified proteins were co-incubated for 3 hours at 37°C in the presence of 40 mM CaCl₂ and 80 mM MgCl₂. The hydrolyzed fragments were observed using SDS-PAGE, and the first 7 amino acids of the cleaved RtxA^{NH2} (C fragment) were sequenced by Edman degradation.

Production of RtxA C-terminus polyclonal antibodies

The desired *rtxA* DNA fragment encoding the C-terminus protein part was selected to be expressed and purified to obtain a protein sample for antibody production. Briefly, RtxA^{COOH} (nucleotides 19,482-20,309 from *rtxA* lpp0699) was cloned (using BamHI/EcoRI restriction sites; **Figure S3**) into a pGEX-6P-3 plasmid downstream a glutathione S-transferase (GST) tag followed by an HRV 3C site for cleavage by a PreScission protease. The constructed plasmid allowed for production of C-terminus fragment of 278 amino acids. This plasmid was then transformed into XL1-Blue *E. coli* strain.

Recombinant protein was produced using an overnight culture followed by a 2-hour induction with 1 mM IPTG. Cells were collected and broken using a French pressure cell (20,000 Psi). The recombinant protein was purified using GST affinity resin (Protino® Glutathione Agarose 4B; Macherey-Nagel, Germany) for GST tagged proteins. The purity and size of the expressed protein was assessed by SDS-PAGE and quantified using a modified Bradford assay (Roti®-Quant; Carl Roth, Germany). Purified polyclonal antibodies were produced commercially (Covalab, France) by immunization of rabbits with purified RtxA C-terminus, then passing the antisera through columns charged with our recombinant proteins followed by elution. Specificities of the antibodies were confirmed by western blots of purified protein and whole cell lysates.

Immunofluorescence microscopy

To visualize RtxA proteins on the surface of *L. pneumophila*, we modified a procedure based on a previous protocol (36). The first step was preparing glass coverslips by washing them in Ethanol/HCl solution (1M) for 1 hour then coating with poly-L-lysine (Sigma P8920). The following steps were carried out at room temperature (RT). *Legionella* cells were normalized to O.D_{600nm} 1.0 and 300 µl of this suspension were spread on the previously prepared cover slips, then left to adhere for 30 minutes. The excess suspension was then gently aspirated and replaced with 3.7% formaldehyde and left 30 minutes to fix the cells. After rinsing with phosphate-buffered saline (PBS pH 7.0), non-specific sites were blocked by 3% bovine serum albumin (BSA) solution for 30 minutes. After blocking, the slides were washed again with PBS and 100 µl (1:10000 or 0.374 µg ml⁻¹) of primary antibody (rabbit anti-RtxA^{COOH}) were added on the cover slips followed by incubation for 1 hour. After several washes with PBS, 50 µl of fluorescence conjugated secondary antibody (Alexa Fluor® 568 goat anti-rabbit, Invitrogen Inc. USA) were incubated with the cover slips for 1 hour in the dark. The slips were then washed twice and mounted on glass slides with 20 µl mounting medium (Mowiol®+DAPCO) (Sigma-Aldrich, USA) then cells were visualized using an epifluorescence microscope (Thermo Fisher EVOS FL, USA).

Bacterial Two-Hybrid assay

The following experiment was carried out according to the protocol of Bacterial Adenylate Cyclase Two-Hybrid (BACTH) system by Euromedex, France. Standard molecular biology techniques were applied to insert the desired *L. pneumophila* genes into the “bait” and “prey” vectors, pKT25/pKNT25 and pUT18/pUT18C. The constructed plasmids were produced in *E. coli* DH5-Alpha strain, purified then confirmed by enzymatic digestion. The proteins of *L. pneumophila* chosen for investigation are shown in **table 1** in the results section. Since most chosen proteins are theorized inner membrane

proteins, bioinformatics prediction tools (37, 38) were applied to predict their orientation. The desired genes were then fused N or C-terminally to the T18 or T25 fragments of the previously mentioned plasmids in a way that maintains these fragments in the cytoplasm after expression. Suitable combinations of the constructs were co-transformed into the reporter strain *E. coli* BTH101 (*cya-99*) on LB-X-Gal (40 µg ml⁻¹) medium containing 25µg ml⁻¹ kanamycin and 50 µg ml⁻¹ ampicillin in addition to 1 mM IPTG to increase β-galactosidase expression. Positive controls were GCN4 leucine zipper motifs cloned into pKT25 and pUT18C. Negative controls comprised co-transforming a plasmid carrying the gene of interest along with a suitable empty vector with no insert to eliminate the possibility of cross reactions. The co-transformants were streaked onto the LB-X-Gal medium and incubated for 1 day at 30°C followed by 2 days at room temperature. Transformants with successful protein interaction were blue, otherwise they remained white.

Sequences searches and alignments and Phylogenetic studies.

Homologous proteins sequences were searched on NCBI website (<https://www.ncbi.nlm.nih.gov/>) using Blastp software. Proteins of interest (one per species identified) were downloaded as Fasta files to perform further analysis. Alignment of RtxA cutting site regions was done using Jalview software (version 2.10.5; (39)). Phylogenetic trees were inferred using maximum-likelihood with PhyML 3.0 software online pipeline ([http://phylogeny.lirmm.fr](http://phylogeny.lirmm.fr;); (40)).

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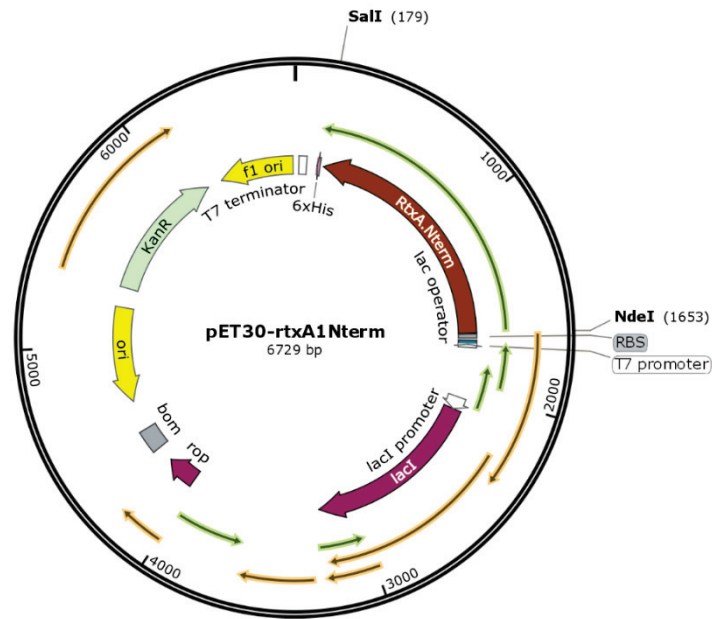
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I. Supplemental material

| Table S1. Bacterial strains used in this study | | |
|--|--|-------------------------------------|
| Strain | Genotype | Reference |
| | | |
| | <i>Escherichia coli</i> | |
| XL1-Blue | <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIqZΔM15 Tn10</i> (Tetr)] | Stratagene |
| DH5-Alpha | F ⁻ Φ 80 <i>lacZΔM15 Δ(lacZYA-argF) U169 recA1</i> <i>endA1 hsdR17 (rK⁻, mK⁺) phoA supE44 λ⁻ thi-1 gyrA96</i> <i>relA1</i> | Invitrogen |
| BL21 | F ⁻ <i>ompT hsdS_B(r_B⁻, m_B⁻) gal dcm</i> | Stratagene |
| B121/pREP4- <i>groESL</i> | F ⁻ <i>ompT hsdS_B(r_B⁻, m_B⁻) gal dcm</i> carrying the plasmid pREP4- <i>groESL</i> (coding for LacI, GroES et GroEL) | Stratagene |
| BTH101 | F ⁻ , <i>cya-99, araD139, galE15, galK16, rpsL1 (Str^r), hsdR2,</i> <i>mcrA1, mcrB1</i> | Euromedex |
| | <i>Legionella pneumophila</i> | |
| Paris-Wild type | <i>L. pneumophila</i> serogroup 1, Paris strain | (Cazalet <i>et al.</i> , 2004) * |
| Paris $\Delta lapG$ | $\Delta lpp0890$ | This study |
| Paris $\Delta lapD$ | $\Delta lpp0891$ | This study |
| Paris $\Delta rtxA$ | $\Delta lpp0699$ | This study |

*Cazalet C, Rusniok C, Bruggemann H, Zidane N, Magnier A, Ma L, Tichit M, Jarraud S, Bouchier C, Vandenesch F, Kunst F, Etienne J, Glaser P, Buchrieser C. 2004. Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. Nat Genet 36:1165-73.

A



B

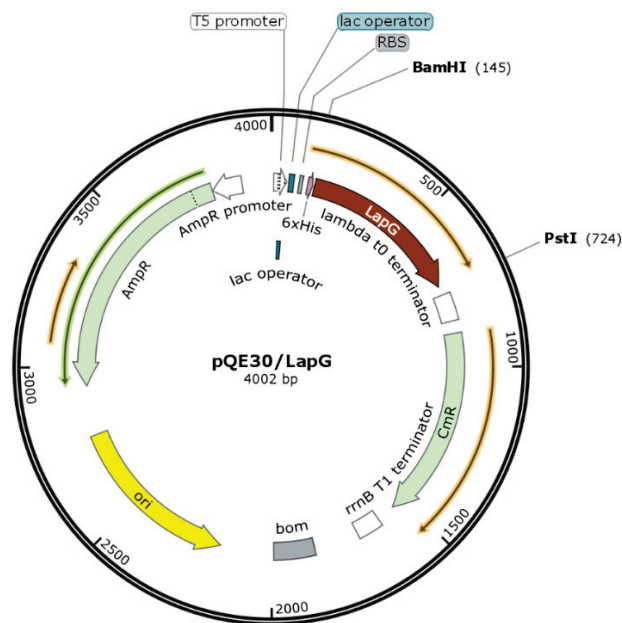


Figure S2. Plasmids constructions used for RtxA N-terminus and LapG production in *E. coli*

Standard molecular biology techniques were used to construct the above derived plasmids (from pET30(a) and pQE30) for overproduction and purification of his-tagged proteins. The polypeptides intended for overproduction were fused in frame with his-tag for easy purification, upstream in case of RtxA N-terminus (A) or downstream in case of LapG (B). pET30(a) and pQE30 vectors enable the control of fused protein expression using an inducible promoter system.

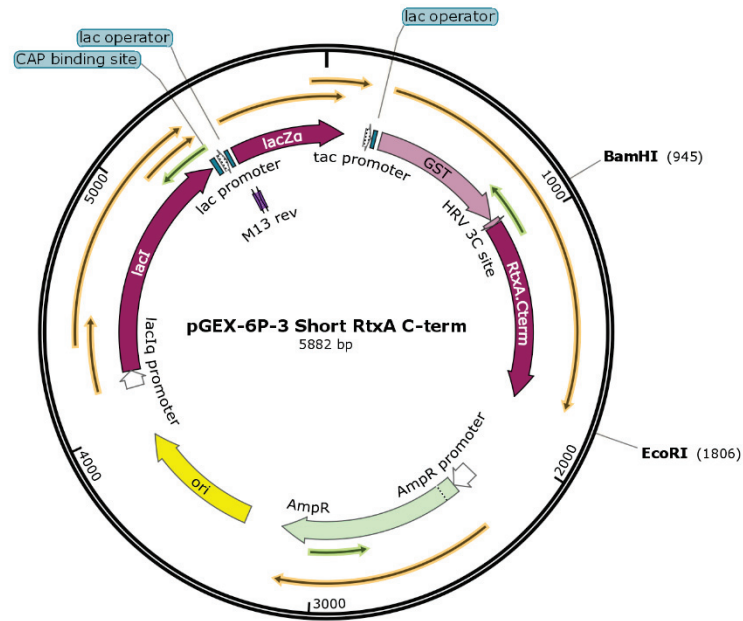


Figure S3. Plasmid construction used for RtxA C-terminus production in *E. coli*

Standard molecular biology techniques were used to construct the above derived plasmid from pGEX-6P-3 for overproduction and purification of a GST-tagged protein. The polypeptides intended for overproduction was fused in frame with GST (downstream) for easy purification. pGEX-P-3 vector enable the control of fused protein expression using an inducible promoter system.

J. Unpublished additional experiment 1: Investigation of interactions between T1SS components, RtxA and LapG via pull-down assays

Based on the findings in our work that RtxA in *L. pneumophila* can undergo cleavage by a periplasmic protease LapG resulting in its release from the cell surface, and even prior to recent findings in *Pseudomonas fluorescens* that point to a subtype of T1SS linked to bacterial transglutaminase-like cysteine proteinase (BTLCP) that can retain the N-terminus of LapA in the outer membrane secretion channel (TolC complex) tethering the entire protein pending cleavage. We tried via protein pull-down assays to establish interactions between RtxA in *L. pneumophila* and components of the T1SS channel as well as between the latter and LapG, the protease involved in RtxA cleavage. The goal was to identify interactions which serve to prove that indeed RtxA is kept in the secretion channel (TolC) and that LapG must somehow gain access to its N-terminal region for cleavage.

For the purpose of this experiment we cloned and overproduced specific regions of the desired proteins in order to avoid transmembrane region. We predicted these transmembrane regions using bioinformatics protein sequence analysis tools (TMPred, TMHMM). Protein parts chosen for interaction assessment are as follows (polypeptides chosen from LssB and LssD represent their possible periplasmic regions):

- LapG (excluding its secretion signal)
- RtxA N-terminus (a.a. 1 – 492)
- LssB (a.a. 1 – 171)
- LssB (a.a. 438 – 718)
- LssD (a.a. 34 – 378)

lapG (lpp0890) and RtxA^{NH2} (lpp0699) were destined for 6xHis tagging and were then cloned into pQE30 and pET-30a(+) plasmids respectively. *lssB* (lpp1473) and *lssD* (lpp1474) were cloned downstream a glutathione S-transferase (GST) tag region in pGEX-6P-3 plasmid. The obtained constructs were transformed into XL-1 Blue *E. coli* strain for overproduction.

Using classical native purification protocols for histidine and GST tagged proteins, we successfully produced and purified the desired proteins, which were then verified for their size using SDS-PAGE. Regarding pull-downs, bait proteins were kept attached to their respective resin and incubated for 1 to 2 hours at ambient temperature with the purified prey protein (in some cases we used several prey proteins in a single pull-down). Regular protein purifications were then conducted, and the contents of washing and elution solutions were analyzed by SDS-PAGE.

The table below represents the different combinations we assessed in order detect potential protein-protein interactions.

Table 9: Pull-down assessment between components of T1SS, RtxA and LapG in *L. pneumophila*

| | Bait protein | Prey protein(s) | Pull-down type |
|---------------|---------------------|---|----------------|
| Experiment 1 | LapG | LssD (34-378) | 6xHis |
| Experiment 1' | LssD (34-378) | LapG | GST |
| Experiment 2 | LapG | LssB (438-718) | 6xHis |
| Experiment 2' | LssB (438-718) | LapG | GST |
| Experiment 3 | LapG | LssB (1-171) | 6xHis |
| Experiment 3' | LssB (1-171) | LapG | GST |
| Experiment 4 | LapG | LssB (1-171) LssB (438-718) LssD (34-378) | 6xHis |
| Experiment 5 | RtxA ^{NH2} | LssD (34-378) | 6xHis |
| Experiment 5' | LssD (34-378) | RtxA ^{NH2} | GST |
| Experiment 6 | RtxA ^{NH2} | LssB (1-171) LssB (438-718) LssD (34-378) | 6xHis |

Unfortunately, under our experimental conditions we were never able detect an interaction between the tested proteins. A possible reason to this result is that we are trying to assess complex interactions that require stringent conditions not easily reproducible *in vitro*. However, data from (Smith *et al.*, 2018b) suggest that the N-terminal region of LapA in *P. fluorescens* is anchored to the membrane via the TolC outer membrane component of the T1SS (LapE in *P. fluorescens*). This keeps the large adhesion protein anchored in the membrane pending signals that release and activate the periplasmic cysteine proteinase LapG, consequently cleaving and releasing RtxA from the cell surface. Unfortunately, technical difficulties did not allow us to test the periplasmic part of TolC component in the pull-down assay, but future work will focus on that. Following the similarity patterns between these models, one can presume that RtxA in *L. pneumophila* behaves in a similar manner, but that has yet to be elucidated as *in vitro* assays did not provide any clue of interaction between the LapD/LapG system and RtxA/T1SS. Experimental *in vivo* protocols may be needed to clarify this link.

III. Article manuscript: New insights into the role of RtxA and the Type I secretion system in *Legionella pneumophila* virulence

Implication of *rtxA* in *L. pneumophila* virulence has been previously described. The most probable mechanism of action of RtxA is through its involvement in adhesion and entry into the host. In light of our previous work describing a dedicated Type 1 secretion system (LssB/LssD/TolC) responsible for RtxA translocation to the cell surface, and the discovery of functional RtxA location regulation mechanism (LapG/LapD) led us to investigate how manipulation of these systems would affect *L. pneumophila* virulence.

We used microscopy to study differences in *L. pneumophila* virulence among the mutants of the systems mentioned above. We also wanted to assess if *L. pneumophila* is able to reach and target host cells via competition experiments. Then we verified the implication of RtxA in early infection stages using immunofluorescence and antibodies specific to this protein and we studied the effect of “inoculating” an infection medium with Anti RtxA antibodies to assess whether it affects *L. pneumophila* virulence. The use of antibodies against *L. pneumophila* RtxA in co-immunoprecipitation assays from amoebae and macrophage infected samples allowed us to identify potential host proteins interacting with RtxA at the early steps of infection.

This manuscript is still in preparation and additional experiments are going on to reinforce the final version of this paper prior to submission. Especially confocal microscopy that will help to better localize RtxA protein and/or potential host protein partners on cells surface during the entry/phagocytosis process. Another point will be to solve a problem we faced with complemented strains and work is going on to use a new strategy to achieve this goal as the one used routinely in the laboratory is not suitable in the case of $\Delta lapD$ complementation. This problem is exposed in an extra paragraph after the manuscript (Additional experiment 2).

Title: New insights into the role of RtxA and the Type I secretion system in *Legionella pneumophila* virulence

Hussein Kanaan,^{a,b,c} Nathalie Bailo,^a Ali Chokr,^{b,c} Johann Guillemot,^a Patricia Doublet,^a Christophe Gilbert,^{a,#}

^aInternational Center for Infectiology Research (CIRI), INSERM U1111, ENS Lyon, CNRS UMR5308, Claude Bernard University - Lyon 1, Lyon, France.

^bLaboratory of Microbiology, Department of Biology, Faculty of Sciences I, Lebanese University, Hadat Campus, Beirut, Lebanon.

^cPlatform of Research and Analysis in Environmental Sciences (PRASE), Doctoral School of Sciences and Technologies, Lebanese University, Hadat Campus, Beirut, Lebanon.

Running title: RtxA is involved in *L. pneumophila* virulence

#Correspondence to: Christophe Gilbert, CIRI U1111 INSERM, Bât. Lwoff, 10 rue Raphael Dubois, 69622 Villeurbanne Cedex, France. Tel: (33) 4 72 43 13 66, Fax: (33) 4 72 43 26 86. christophe.gilbert.bio@univ-lyon1.fr

Keywords: *Legionella pneumophila*, *Acanthamoeba castellanii*, U937 macrophage, virulence, RtxA, Type 1 secretion system, LapD, LapG

A. Abstract

L. pneumophila is a Gram-negative bacterium responsible for a form of pneumonia called legionellosis or Legionnaire's disease. One of the virulence enhancing factors possessed by this organism is a very large cell surface protein belonging to the RTX (Repeats in ToXin) family called RtxA. This protein has often been correlated to the initial entry steps of *L. pneumophila* to its host. In a previous study we reported that RtxA is transported to the cell surface by a dedicated type 1 secretion system (T1SS). We also proved that *L. pneumophila* RtxA similar to other RTX proteins such as *pseudomonas fluorescens* LapA, is regulated on the cell surface and can be released under specific environmental conditions. In the current work, we investigated the *L. pneumophila* virulence via clean deletion mutants. It was apparent that mutants for *L. pneumophila* T1SS (*lssBD/tolC*) were more disruptive to its virulence than deletion mutants of the LapG/LapD system responsible for release of RtxA. We also hypothesize based on competition experiments that *L. pneumophila* might target its host rather than be consumed by phagocytic cells.

Regarding RtxA implication in virulence, we were able using specific anti-RtxA antibodies to detect this protein on amoebae surface only 20 minutes following exposure to *L. pneumophila*. In addition to the previous, using *in vitro* antibody protection experiments using anti C-terminus RtxA antibodies we were able to reduce the virulence of *L. pneumophila*.

B. Importance

RTX proteins have been involved in pathogenesis mainly as hemolysins. In *L. pneumophila*, RtxA has been linked to early virulence stages mainly through adhesion or host recognition and probably through other mechanisms later on. Here we study the impact of manipulation of RtxA secretion and release systems on *L. pneumophila* virulence, as well as *in vitro* protection against infection using Anti-RtxA antibodies.

C. Introduction

Infectious diseases caused by pathogenic microorganisms are among the leading causes of death worldwide (1). Many of these pathogens have evolved a wide array of approaches to colonize and invade human organs, despite the efforts of various host defense mechanisms (2). Such virulence properties of pathogenic bacteria might include the bacterial capsule, cell wall, toxins and adhesins (1). Among these bacteria is the intracellular pathogen *Legionella pneumophila*. This bacterium is a Gram-negative bacillus and it is the causative agent of a severe form of pneumonia called Legionnaires' disease which represents 2-9% of cases of community acquired pneumonia (3-5). The *Legionella* genus comprises approximately 60 species including 70 serogroups; thirty of these species are pathogenic to humans where *L. pneumophila* serogroup 1 (Lp1) is responsible for more than 90% of clinical cases (3, 4, 6). *Legionella* exists in freshwater environments as a parasite of protozoa such as amoebae that are considered to be their natural hosts (3). However, man-made systems such as water-cooling towers and air conditioning systems provided means for these bacteria to reach and infect human alveolar macrophages via the dispersed aerosols (3, 7). *L. pneumophila* primarily affects susceptible patients due to age, preexisting conditions or immunosuppression and are hence considered opportunistic pathogens (4, 6). In spite of the previous statement, the mortality rate of Legionnaires' disease ranges from 7-25% even with appropriate antibiotic treatment which renders this disease a public health concern (8).

Secreted proteins such as toxins and various effector proteins are common aggressive bacterial virulence factors that serve for host colonization. In addition to the previous, adhesins which are utilized by pathogenic bacteria, are also known to play important roles in infection (9). In general, these virulence factors are required to establish a successful infection, where invading pathogens have to subvert host cellular processes to allow for efficient colonization and evasion of host immune response (10). Therefore, bacterial secretion systems are important factors in pathogenesis as they are responsible for transporting such proteins. This is particularly true in the case of intracellular pathogens, where such protein transport systems are required by invading organisms to manipulate/evade host processes and more specifically in the case of *L. pneumophila*, to establish a replicative niche inside its host (11-13).

In general, bacterial secretion systems usually secrete substrates to the extracellular medium or inject them into host cytoplasm; to date, seven secretion systems have been described (11, 14). In *Legionella*, a recent genomic analysis reports the presence of type 1 secretion system (T1SS), T2SS, T4SS and the T6SS in a few isolates (15). The Dot/Icm (Defect in Organelle Trafficking/IntraCellular Multiplication) T4BSS is heavily implicated in the pathogenesis of *L. pneumophila* where it transports around 300 effector proteins directly from the internalized bacterium to the host cytoplasm. These proteins hijack many host processes subsequently allowing extensive replication of *Legionella* in the phagosome that is called the *Legionella* containing vacuole (LCV) (16, 17).

Regarding T1SSs, an early study reports the presence of a putative type 1 secretion system in many *L. pneumophila* strains. However, in most T1SSs, the target protein encoding gene is located upstream of the secretion system transporter protein, but none were identified in *Legionella* (18). In our previous work, we were able to verify that a *L. pneumophila* T1SS is indeed present and functional and its substrate is an RTX (Repeats in ToXin) protein called RtxA (19). The T1SS in *L. pneumophila* is composed of three components: LssB, an inner membrane ABC transporter that relies on ATP for substrate transport. LssD, a periplasmic membrane fusion protein and TolC, an outer membrane protein (18, 19). These proteins form a channel spanning both bacterial membranes through which the substrate is secreted to the extracellular medium. However, recent studies suggest that some RTX transporting T1SSs mediate transport via a two-step process that involves an outer membrane embedded intermediate (20).

Various RTX proteins have been implicated in the virulence of many bacteria, they usually function as pore forming toxins to lyse or damage target cells (1). The most studied model is the pore forming toxin of uropathogenic and enterohemorrhagic *Escherichia coli* hemolysin A (HlyA) (21, 22). Among many other classes of RTX proteins, the *Pseudomonas fluorescens* biofilm associated adhesin LapA is also well described in literature (23). Structurally, The *L. pneumophila* RtxA shares the general features of RTX proteins such as a C-terminal secretion signal, an RTX repeats motif (GGXGXDX) and a central repeat region (24). In *L. pneumophila*, RtxA has been linked to pore forming activity though it depends on host cell type, and it probably performs less efficiently than specialized pore forming RTX toxins such as HlyA (19, 25). It has also been implicated in adherence to host cells as this protein harbors a von Willebrand motif in its C-terminal region and several adhesion motifs in the central region (24, 26). For these reasons, RtxA has been frequently linked to *L. pneumophila* virulence and more specifically in the initial entry step. RtxA was shown to affect adherence to monocytes, epithelial cells and amoebae (25, 26). In addition to the previous, *rtxA* mutants exhibited a measurable loss of virulence towards monocytic and epithelial cells (25). RtxA has also been implicated in intracellular survival such as inhibition of lysosomal fusion though with not enough evidence (26).

Studies on the biofilm associated RTX protein LapA in *P. fluorescens* revealed that it is kept or released by cleavage from the cell surface in response to certain environmental factors that favor or block biofilm formation (27). Briefly, the inner membrane effector protein LapD and in conjunction with a diguanylate cyclase (DGC) senses changes in intracellular cyclic-di-GMP levels and regulates accordingly the activity of a periplasmic protease LapG by sequestering or releasing it in the periplasm (28). This protease, when free, can cleave the N-terminus of LapA leading to its release and biofilm dispersal. On the other hand, when this protease is sequestered, LapA is kept on the surface promoting biofilm formation (29). In addition to homologs of these proteins being discovered in *L. pneumophila* (30, 31), recent findings in our lab point that *L. pneumophila* RtxA adopts a similar regulation mechanism. Therefore, RtxA presence on the cell surface can be modulated following specific signals.

In this study, we try to further establish using various mutants, the role of RtxA and its secretion system in *L. pneumophila* virulence. We also investigate the possible consequences of blocking RtxA using specific antibodies on *L. pneumophila* virulence.

D. Results

1. RtxA plays a role in early infection steps of *A. castellanii*

The *rtxA* gene has been previously correlated to *Legionella* virulence, mainly adherence and entry into various host cells (25, 26). In the current work, we used clean deletion mutants of *rtxA*, its T1SS components and the LapD/LapG system responsible for releasing RtxA from the cell surface. A mutant strain, $\Delta dotA$, defective for dot/icm T4SS and hence intracellular replication was used as a negative control. As a control, all constructed mutants were tested for growth in AYE medium and no difference in growth capacity/fitness compared to wild-type strain was noticed (data not shown).

In this experiment, we followed the progress of infection for the different strains using light microscopy. It is worth to mention that all infection experiments made during this work followed a special protocol; *L. pneumophila* cells were added to host cells (amoebae or macrophages) without any centrifugation, hence avoiding forced contact and adhesion to host cells. Severity of the infection was based on amoebae mortality as well as morphology since they assume a round shape when infected (32), compared to the elongated morphology with possible pseudopods. In **figure 1**, it is clear that three days post infection, the strain lacking *rtxA* had a similar impact on amoeba as the strain $\Delta dotA$ that is incapable of intracellular replication. However, after five days the amoeba cells infected with the *rtxA* mutant strain are clearly under stress albeit less severely than the WT strain. And after seven days, many WT infected amoebae died and the $\Delta rtxA$ infected amoeba cells are all stressed at the difference of cells infected with $\Delta dotA$ strains. This suggests that RtxA is important for the initiation of infection. However, this protein

is not a limiting factor in *L. pneumophila* virulence as the infection can proceed without it but less efficiently. We also noticed that its absence causes a delay of entry into amoebae.

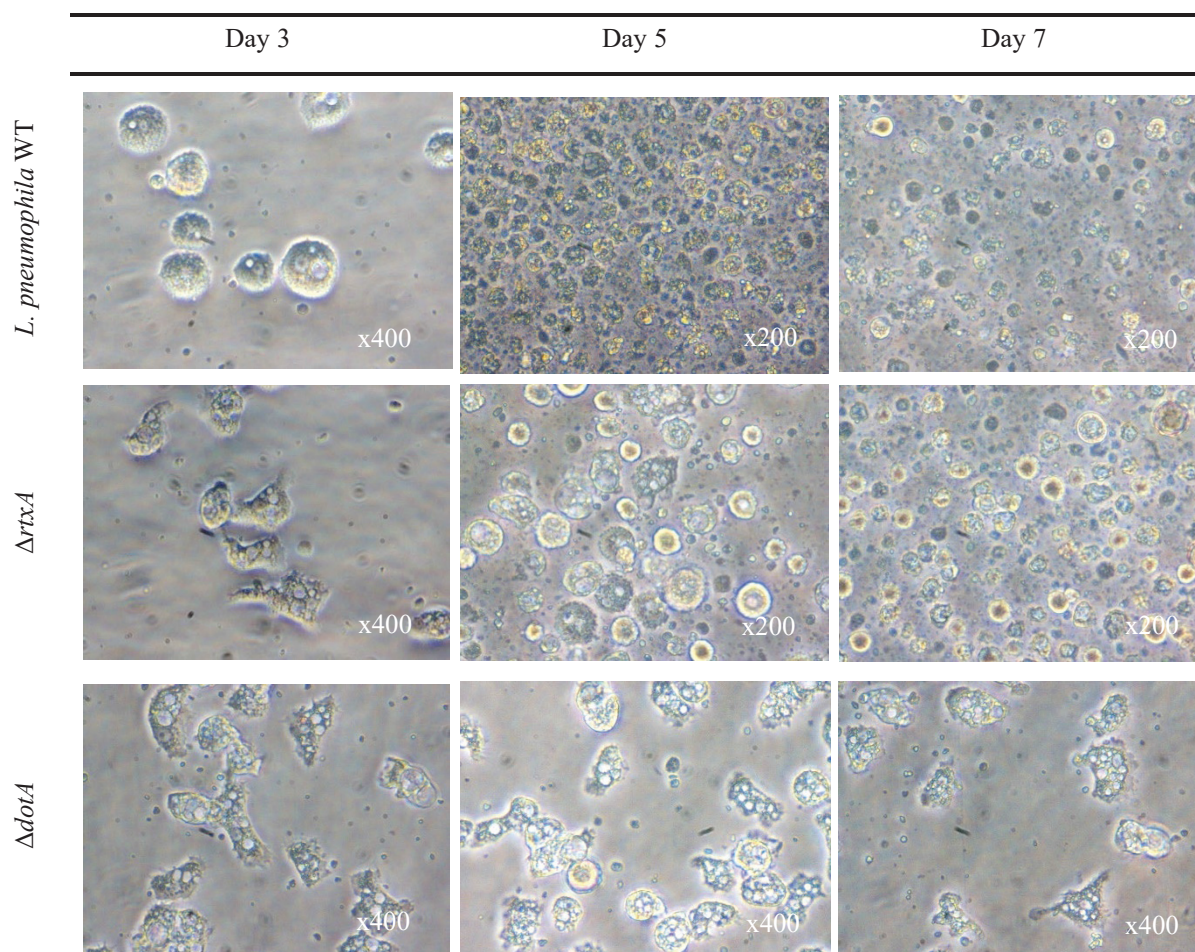


Figure 1. Impact of *L. pneumophila* RtxA on the severity of *Acanthamoeba castellanii* infection

Three *L. pneumophila* strains (WT, $\Delta rtxA$ and $\Delta dotA$) were used for infection of *A. castellanii* at MOI 0.1 in a medium lacking growth requirements for both bacteria and its host. Light microscopy images were captured at 3 time points post infection. Round morphology of *A. castellanii* corresponds to stressed infected cells. An infection with a strain deficient in the type IV secretion system ($\Delta dotA$) which is incapable of intracellular replication was used as a control.

In the next part we investigated mutant strains with deleted components of the T1SS responsible for transporting RtxA to the cell surface and the LapD/LapG proteins involved in localization. **Figure 2** images were captured three days after infection at MOI 1. Two patterns can be observed regarding the severity of *A. castellanii* infection. Mutant strains for the T1SS, $\Delta lssBD$ and $\Delta tolC$ display lower virulence toward *A. castellanii* than mutant strains for *lapD* and *lapG* as the amoebae were more stressed in the latter case. Interestingly, $\Delta lapD$ infected cells were more similarly stressed as the WT infected cells whereas $\Delta lapG$ infected amoebae seemed to have a slight delay of infection. We can say that

disrupting the T1SS will prevent the secretion of RtxA and the effects of these deletions can be seen in the lower virulence of these strains when compared to WT. However, it seems that manipulating the presence of RtxA on the cell surface ($\Delta lapG$) versus being constantly released ($\Delta lapD$) didn't drastically attenuate the virulence of *L. pneumophila* towards *A. castellanii* even if RtxA release seemed to be more favorable. Complemented strains for our mutants were also constructed, e.g., *L. pneumophila* $\Delta lapD$ /pXDC50-*lapD* and $\Delta lapG$ /pXDC50-*lapG*. However, their growth phenotypes were altered, especially in the case of $\Delta lapD$ /pXDC50-*lapD* strain (data not shown). Therefore, no infection experiment could be performed with these complemented strains up to this date.

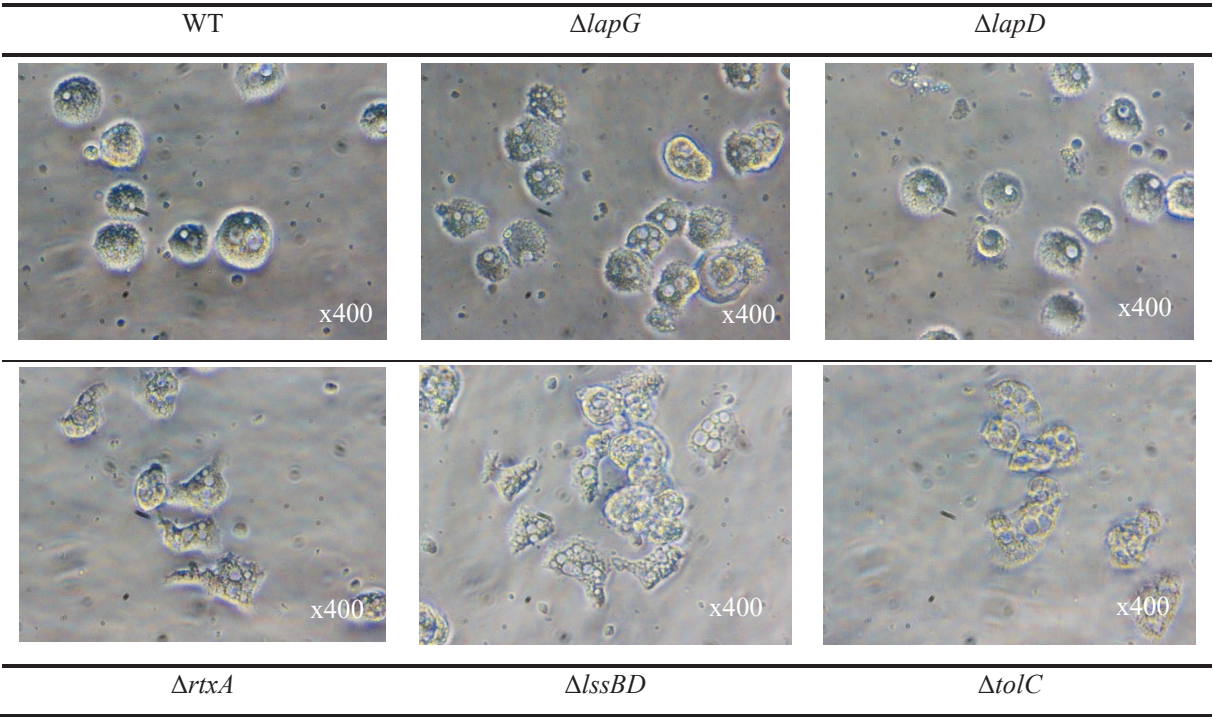


Figure 2. Importance of RtxA secretion and release systems in *L. pneumophila* virulence
A. castellanii were infected with *L. pneumophila* strains (WT, $\Delta lapG$, $\Delta lapD$, $\Delta lssBD$, $\Delta tolC$ and $\Delta rtxA$) at MOI 1 in a growth inhibitory medium. Light microscopy images were taken 3 days post infection. Round morphology of *A. castellanii* corresponds to stressed infected cells.

In conclusion, transporting RtxA by the T1SS to the cell surface appears to be the crucial factor in *L. pneumophila* virulence. We can also hypothesize that the presence of RtxA in the infection medium whether on the cell or released may enhance the virulence of *L. pneumophila*.

2. Amoebae selective feeding does not alleviate *L. pneumophila* virulence

Phagotrophic protists including amoeba can be very selective consumers that recognize prey organisms (33). Also, there might exist certain characteristics of prey cells that potentially influence selective

feeding by protists (34). Therefore, we attempted to investigate the effects of presence of alternative prey cells to the amoebae in the infection medium on the infection potential of *L. pneumophila*. We carried out an *A. castellanii* infection with *L. pneumophila* WT, $\Delta dotA$ and $\Delta rtxA$ at MOI 1 in the presence of 100 fold excess cells of *E. coli* MG1655 in the infection medium. **Figure 3** shows that after three days, *A. castellanii* cells infected with WT *Legionella* were significantly stressed much like a regular infection with no *E. coli*, as shown in **figure 1**. Moreover, as expected, the *rtxA* mutants displayed lower virulence than WT after three and seven days compared to WT.

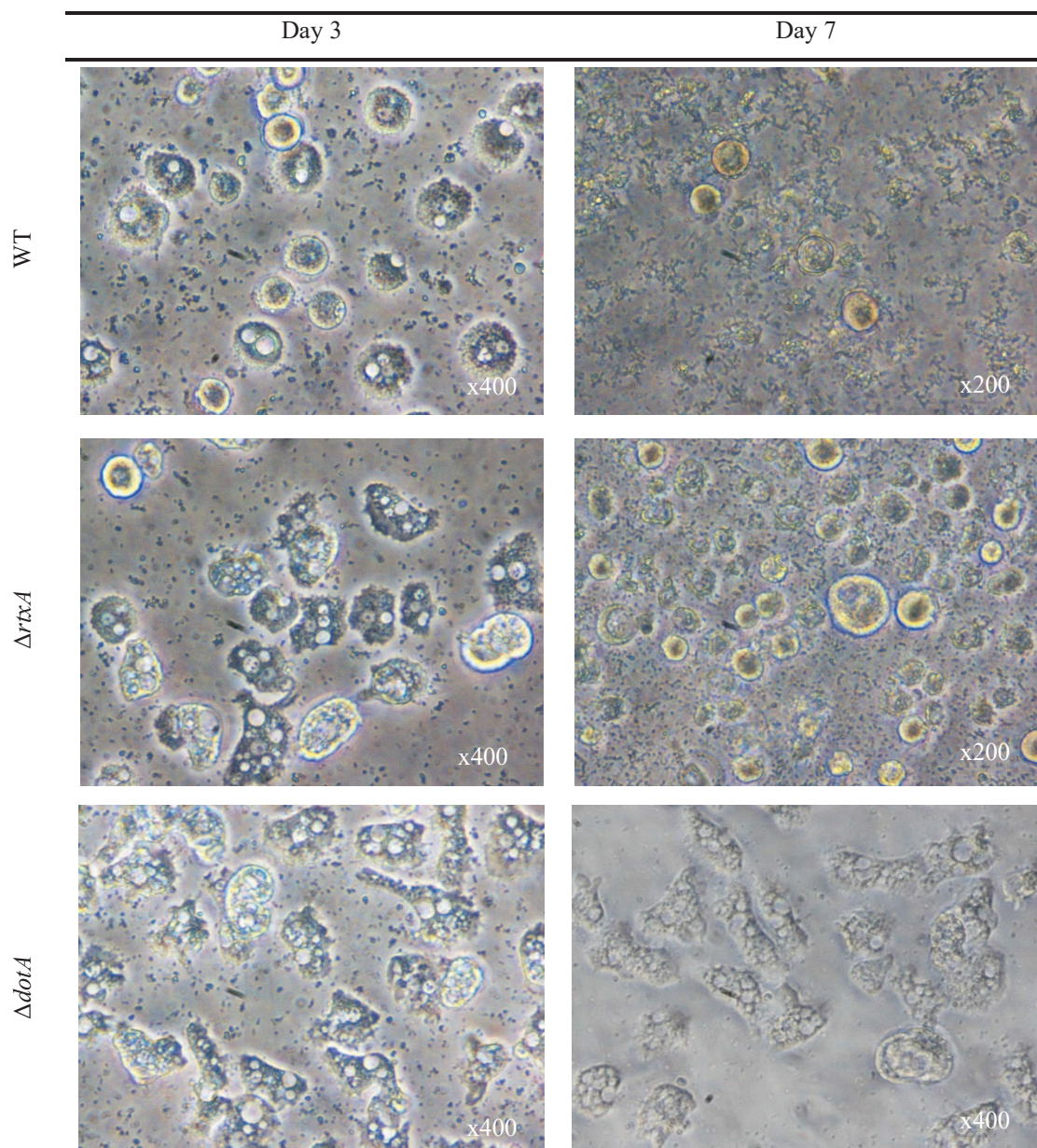


Figure 3. *L. pneumophila* may target *A. castellanii* during infection

Infection of *A. castellanii* cells with different *L. pneumophila* strains (WT, $\Delta rtxA$, $\Delta dotA$) at MOI 1 in a growth inhibitory medium in the presence of 100-fold *E. coli* cells (MG1655) as a source of nutrition for amoebae. Observations were made after 3 and 7 days. Round morphology of *A. castellanii* corresponds to stressed infected cells.

This simple experiment allows us to hypothesize that despite the presence of a preferred alternative food source for the amoebae in the infection medium, *L. pneumophila* virulence and infection still progressed in a manner similar to a classic infection with no *E. coli* cells as food. Therefore, keeping in mind that we did not force any contact between amoebae and *L. pneumophila* or *E. coli*, we can assume that *L. pneumophila* actively targets its host cell rather than simply being engulfed and consumed by phagocytic cells. However, the role of RtxA in this process has to be elucidated since the mutants were less virulent toward *A. castellanii* cells (compared to WT), but no evidence of a delay compared to infection without *E. coli* in excess has been noticed.

3. RtxA is detected on the surface of host cells early during infection

Firstly, to assess the efficiency of *L. pneumophila* infection towards human cells, we used U937 monocytes as hosts for $\Delta lapG$ *L. pneumophila*, and we allowed the infection to proceed for only 15 minutes at MOI 10 to minimize entry and study early implication of RtxA in infection. Using fluorescent bacteria (**figure 4A**), we showed that *L. pneumophila* is heavily detected on the surface of host cells as early as 15 minutes post inoculation. As already seen during amoeba infections, *L. pneumophila* seemed to adhere to U937 macrophages immediately after exposure.

Therefore, we performed new infections in amoebae and macrophages for 20 minutes where we used anti RtxA^{COOH} antibodies in immunofluorescence experiments to visualize RtxA. The infection was performed at MOI 10 and exposure time was limited to 20 minutes. Immunofluorescence imagery in **figure 4B** showed that we can detect RtxA on both hosts' surface as soon as 20 minutes post inoculation. This coincides with previous research implicating RtxA in adherence to host cells and it raises questions of its involvement in *L. pneumophila* recognition towards its host cells.

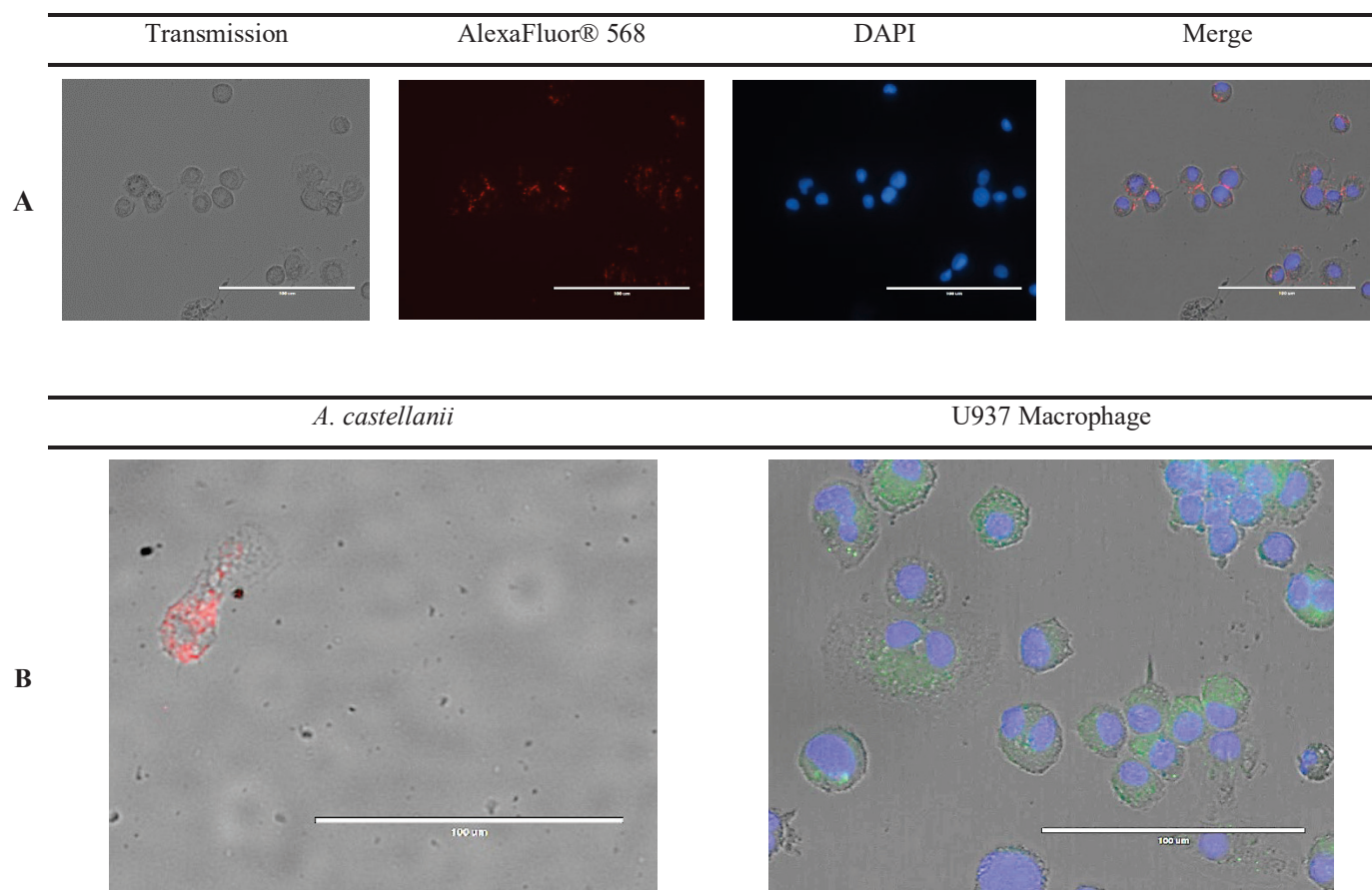


Figure 4. *L. pneumophila* is detected on its host early during infection

(A) U937 macrophages infected with $\Delta lapG$ *L. pneumophila* Paris mutant strain expressing mCherry after 15 minutes of inoculation. (B) (Left) *A. castellanii* were infected with 3 days old $\Delta lapG$ *L. pneumophila* Paris strain, Anti-RtxA^{COOH} antibodies linked to a red fluorescence element (Alexa Fluor® 568 goat anti-rabbit), fluorescence is detected on the amoebae surface 20 minutes post infection. (Right) U937 macrophages where infected in a similar manner but using a green fluorescence element (Alexa Fluor® 488 goat anti-rabbit). Appropriate controls with no primary antibody were taken into account. Scale bar: 100 μ m.

4. Protection against *L. pneumophila* infection of *A. castellanii*

Protection against infection of various bacteria by immunization of the host with parts of their virulent protein(s) has been documented before. More specifically, a C-terminal region of *Vibrio vulnificus* multifunctional auto-processing RTX protein (MARTX) called RtxA1 implicated in virulence was used to immunize mice and resulted in significant protection against lethal challenge with *V. vulnificus* (35). We took an *in vitro* approach using purified *L. pneumophila* anti-RtxA^{COOH} antibodies and *A. castellanii* as host cells in an attempt to assess its effects on the function of RtxA in adherence/entry.

Since our antibodies were preserved in glycerol that might affect *L. pneumophila* virulence, appropriate controls were taken. **Figure 5** shows that glycerol on its own is able to reduce the infection potential of *L. pneumophila* by approximately 36% even at 1% glycerol concentration. However, the curve

representing an infection in presence of $37.4 \mu\text{g ml}^{-1}$ Anti-RtxA^{COOH} (containing an equivalent of 1% glycerol) was able to further decrease the peak fluorescence and consequently infection efficiency by approximately 62%. Taking into consideration that significance analysis confirmed the significance of our results at $P < 0.0001$, this suggests that Anti RtxA antibodies are able to hinder the infection process by binding and possibly disrupting RtxA activity.

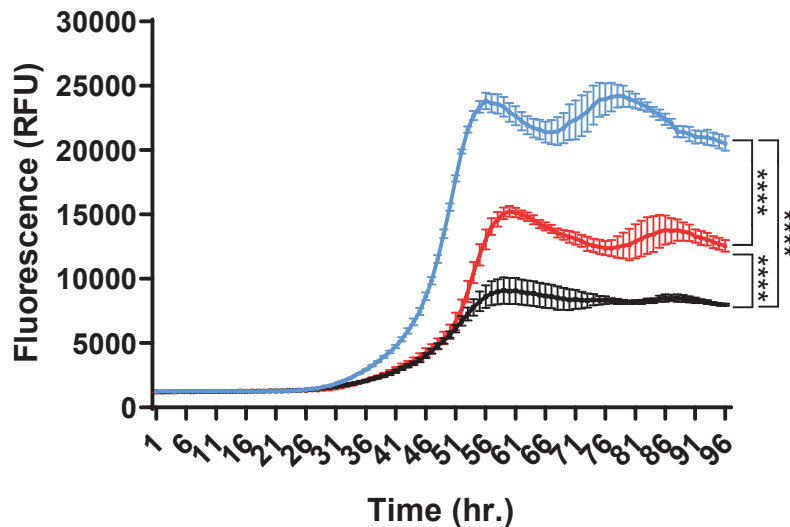


Figure 5. Anti-RtxA^{COOH} antibodies attenuate *L. pneumophila* infection of *A. castellanii*

L. pneumophila Paris wild-type (WT) was used to infect *A. castellanii* cells at MOI 1. The curves represent variation in mCherry fluorescence versus time reflecting the number of bacteria. Results are displayed as mean fluorescence (3 replicates) \pm standard error of the mean. —: WT-pXDC50; —: WT-pXDC50 + 1% Glycerol; —: WT-pXDC50 + Anti-RtxA^{COOH}. **** $P < 0.0001$ indicates that the means of our results are significantly different among each other.

5. Co-immunoprecipitation of RtxA protein targets

Antibodies directed towards either the N-terminus (anti-RtxA^{NH2}) or the C-terminus (anti-RtxA^{COOH}) of RtxA were coupled to a resin and used for co-immunoprecipitation (coIP) assays on infected *Acanthamoeba castellanii* cells or macrophages, or non-infected cells for controls. Non-infected *A. castellanii* or macrophages cell lysates did not bind to the anti-RtxA^{COOH} coupled resin, nor to the anti-RtxA^{NH2} coupled resin (data not shown).

A. castellanii cells were then infected with one-day old *L. pneumophila* str Paris plate culture and the infection was stopped after 30 minutes by fixation and cross-linking of the infected cells. CoIP assays with the anti-RtxA^{NH2} resin did not reveal any prospective partners but a few protein bands were detected in the elution fraction when passing infected *A. castellanii* lysates through an anti-RtxA^{COOH} column.

The same experiments were conducted on macrophages, infected for 30 minutes (**Figure 6**). Three proteins with a molecular mass of around 170 kDa, 60-65 kDa and 45 kDa, respectively, were revealed when passing the lysates through the antibody-coupled columns. Interestingly, the proteins appeared in the elution fractions of both the anti-RtxA^{COOH} and the anti- RtxA^{NH2} coIP assays.

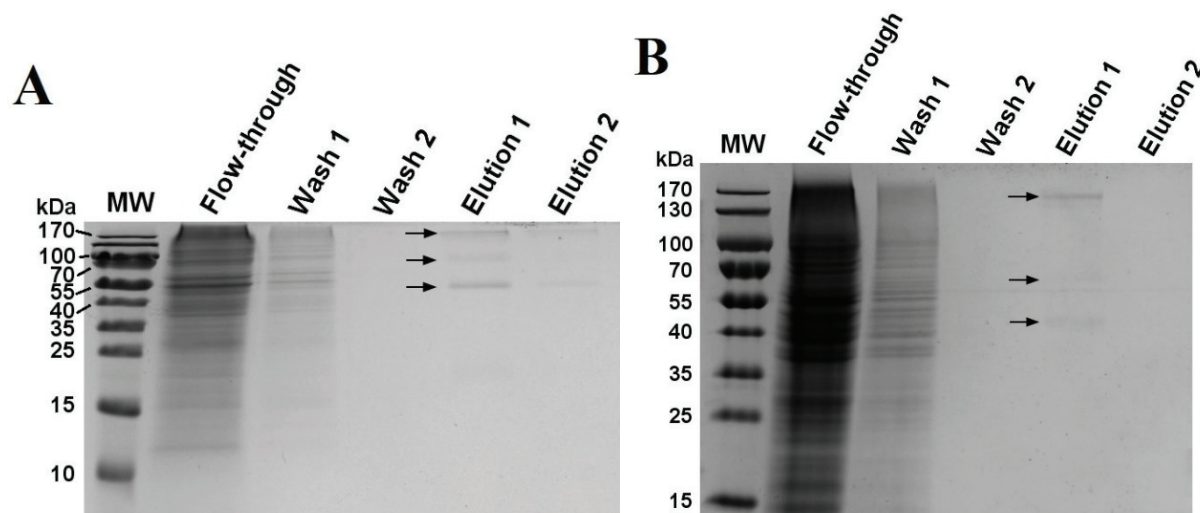


Figure 6. Co-immunoprecipitation (coIP) assays of macrophage lysates with anti-RtxA-antibodies
 Samples were analysed by SDS-PAGE (A) CoIP of infected macrophage lysates with an anti-RtxA^{COOH} antibody (30 min. infection). (B) CoIP of infected macrophage lysates with an anti-RtxA^{NH2} antibody (30 min. infection). Gels were stained with Coomassie blue. In both cases, the arrows point out the three proteins bands clearly identified in elution fractions that were not present in control CoIP using uninfected macrophages.

The three promising elution fractions, one from *A. castellanii* infection and two from macrophages infections were sent for identification by mass spectrometry (MS). Identified peptides were compared against human, amoeba and *L. pneumophila* proteomes. Analyzing the MS data, a protein disulfide-isomerase (PDI), P4HB, appeared to be specific for both macrophages CoIP elution fractions with a good score and in case of *A. castellanii* CoIP sample, a peroxiredoxin 2 was significantly detected.

E. Discussion

Various virulence factors are utilized by bacteria to colonize their host. Membrane associated virulence factors are particularly important for adhesion and possibly evasion of the host cell (36). One such class of membrane associated virulence factors include some RTX proteins produced by a variety of Gram-negative bacteria. RTX proteins have diverse functions and range from being cytotoxins, hemolysins, proteases, lipases, and adhesion associated proteins (37). In *L. pneumophila*, we previously reported that

RtxA complies with same guidelines and is secreted by a LssBD/TolC T1SS (19) and that its localization on the cell surface is regulated by the combined actions of LapG and LapD proteins.

Our work proved that deletion of *rtxA* definitely affects *L. pneumophila* virulence as the infection of amoebae was less severe in a Δ *rtxA* genetic background compared to the wild-type. Moreover, disrupting RtxA secretion mechanism, namely the ABC transporter/membrane fusion protein pair LssBD and the outer membrane protein TolC, resulted in reduced virulence while deletion of *lapG* and *lapD* did not result in clear virulence reduction in our conditions. Therefore, this suggests that the presence of RtxA during the infection process whether be it released or on cell surface, impacts the entry capacity of *L. pneumophila* into its host cells. Furthermore, in a simple competition experiment with *L. pneumophila* in the presence of 100-fold more *E. coli*. Virulence towards *A. castellanii* was still similar to an infection with no *E. coli*. This leads us to believe that *L. pneumophila* do not necessarily act as prey to phagocytic cells, but on the contrary, they can actively target and infect host cells or to promote phagocytosis.

Using anti-RtxA antibodies specific to the C-terminal region in immunofluorescence imagery. We revealed fluorescent spots on host cells surface signifying the presence of RtxA on the surface of these cells briefly after exposure (20 minutes in our assays). This allows us to implicate RtxA in the initial entry steps of *L. pneumophila* probably through adherence since RtxA harbors various adhesion motifs in C terminal as well as central repeat region (24). Moreover, addition of anti-RtxA C-terminal antibodies can affect infection potential of *L. pneumophila*. In the presence of 37.4 $\mu\text{g ml}^{-1}$ of antibodies, there was approximately a 62% reduction in virulence but glycerol accounts for around 36% of this effect as seen in the glycerol control.

Therefore, we focused on coIP assays that successfully provided prospective targets of RtxA through SDS-PAGE analysis. MS identification of the proteins contained in the eluted fractions revealed the protein disulfide isomerase (PDI) P4HB as a potential partner of RtxA in macrophages. P4HB was present in both macrophage samples with good coverage and with peptides different from the ones usually found in cases of non-specific binding to agarose (38). PDI is a family of multifunctional enzymes involved in redoxprocesses of disulfide bonds. P4HB catalyzes the formation and isomerization of disulfide bonds through redox properties and participates in protein folding (39). PDIs are mostly localized in the endoplasmic reticulum but P4HB can also be found on the cell surface (40) where it has mostly a reductase function. Interestingly, although PDIs have not yet been studied in relation to legionellosis, the importance of these proteins has been described in different host-pathogen relations. Extracellular PDI participates in membrane fusion of HIV and Th2 cells by reducing thiol bonds of viral proteins (39). Cell surface PDI is required for host cell adhesion and entry of several *Chlamydia* species (41). *Chlamydia* is a genus of gram-negative intracellular pathogens that infect a range of eukaryotic cells and requires a structurally intact PDI at the surface of the host cell for adherence. The reduction activity of PDI is necessary for entry. The authors do not specify the involved

PDI in their experiments, but one of their previous articles contains a sequence of the protein (42) and protein Blast search on this sequence revealed 95% of identity with P4HB suggesting participation of a protein close to the one we identified through coIP.

Furthermore, there is evidence that PDI regulates the activity and clustering of integrins (43). and several RTX toxins (HlyA, LtxA) have been described to interact with $\beta 2$ integrins. Integrins are cell surface receptors which mediate multiple processes, including adhesion to other cells and the extracellular matrix (44). Thus, RtxA may stimulate adhesion of *L. pneumophila* strains to the host cell surface through the action of PDI on these receptors.

An enzyme with a predicted disulfide redox function, peroxiredoxin 2, was present in the coIP amoeba lysate. This enzyme contains a putative thioredoxin domain, similar to the ones found in PDI (45). The subcellular location of this protein *A. casellanii* is not known and its function is not experimentally proven, but previous work on *Amoeba proteus* mentioned the role of a peroxiredoxin 2 in response to oxidative stress and phagocytosis, however reported to be in the cytoplasm (46).

Taken altogether, our data point out the potential active targeting mechanism set up by *L. pneumophila* to facilitate its entry into hosts cells in a RtxA-dependent manner. The interaction between PH4B and RtxA needs to be confirmed through different experiments. However, in the scientific context, PDI looks promising as a partner or recruited protein for RtxA and as a host enzyme involved with *L. pneumophila* entry. Considering that an amoeba protein with thioredoxin functions might also interact with RtxA, further work will focus on the relationship of RtxA and *L. pneumophila* with disulfide reductase proteins. For example, looking at *L. pneumophila* host cell entry in the presence of reductase inhibitors (bacitracin, PACMA 31, Loc14) may help to precise the mechanism, but preliminary results showed that the bactericidal activity of bacitracin against *Legionella* does not permit its use in an infection assay. *In cellulo* immunolabeling and colocalization techniques at initial steps of infection using anti-RtxA antibodies and anti-PH4B proteins may also help to visualize these proteins interaction.

F. Materials and methods

Strains and growth conditions

Regarding *L. pneumophila*, the Paris strain was used in the hereby presented work, it was grown on buffered charcoal yeast extract (BCYE) agar or in liquid AYE medium. Cultures were grown at 30°C or 37°C depending on the experimental procedure. Kanamycin (15 $\mu\text{g ml}^{-1}$) or chloramphenicol (5 $\mu\text{g ml}^{-1}$) were added when appropriate. *E. coli* strains were grown in lysogeny broth (LB) with rotation or on agar at 37°C unless mentioned otherwise. Appropriate antibiotics were added when necessary according to the following concentrations; Kanamycin (50 $\mu\text{g ml}^{-1}$) and/or Ampicillin (100 $\mu\text{g ml}^{-1}$).

Axenic *Acanthamoeba castellanii* cells were grown on proteose-yeast extract-glucose (PYG) medium at 30°C and split once a week, appropriate antibiotics were supplemented. Human U937 cells were maintained at 37°C and 5% CO₂ in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS). Differentiation into macrophages was initiated by adding Phorbol 12-myristate 13-acetate (PMA) at a final concentration of 100 ng ml⁻¹.

Plasmid construction

DNA constructions used in this study were made by using *E. coli* BL21 or DH5- α as the host. *L. pneumophila* Paris strain genomic DNA was used as template for PCR production of the desired inserts. The recombinant plasmids were verified by PCR and enzymatic digestion prior to electroporation into the *E. coli* strains. Plasmids pXDC50 (47) and pXDC116 were obtained from Xavier Charpentier. The appropriate plasmids were inserted into *L. pneumophila* by electroporation (2400 V, 400 Ω , 25 μ F).

Gene deletion in *L. pneumophila*

Gene-specific deletions in *L. pneumophila* were carried out using the homologous recombination method (48). Mutant strains were derived from *L. pneumophila* wild-type (WT) Paris strain. Briefly, this two-step process relies on the natural competence of *L. pneumophila* and results in clean scar-free mutants. In our work, the 2kb regions flanking the gene to be deleted were amplified by PCR. In the first step, a resistance/suicide inducible cassette which is in this case kanamycin/MazF, is inserted between the flanking regions by double joint PCR. This constructed cassette given to *L. pneumophila* competent cells will replace the gene of interest and will allow for primary selection on kanamycin supplemented media. Successful colonies are Kan resistant and IPTG sensitive. The cassette integration was confirmed by PCR. In the second step, the gene flanking regions were joined by PCR and used for natural transformation of the previously obtained “first step mutants”. The aim is to replace the Kan/MazF cassette which will produce clean gene deletions. Colonies corresponding to deleted mutants are Kan^s IPTG^r and deleted DNA regions were also confirmed by PCR.

Production of RtxA N- and C-terminus polyclonal antibodies

The desired fragments were designed, expressed and purified to obtain a protein sample for antibody production. Briefly, the RtxA^{NH2} (nucleotides 14-1490 from *rtxA* lpp0699) was cloned into a pET-30 plasmid upstream a 6xHis Tag; the RtxA^{COOH} (nucleotides 19,482-20,309 from *rtxA* lpp0699) was cloned into a pGEX-6P-3 plasmid downstream a glutathione S-transferase (GST) tag followed by an HRV 3C site for cleavage by a PreScission protease. The constructed plasmids allowed for production

of N and C-termini fragments of 492 and 278 amino acids respectively. In brief, the constructed plasmids were transformed to BL21 and XL1-Blue *E. coli* strains respectively. Recombinant proteins were produced using an overnight culture followed by a 2-hour induction with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG). Cells were collected and broken using a French pressure cell (20000 Psi). The recombinant proteins were purified using Talon® metal affinity resin (Takara Bio) for his-tagged proteins, and GST affinity resin (Protino® Glutathione Agarose 4B; Macherey-Nagel, Germany) for GST tagged proteins. The purity and size of the expressed proteins were assessed by SDS-PAGE and quantified using a modified Bradford assay (Roti®-Quant; Carl Roth, Germany). Purified polyclonal antibodies were produced commercially (Covalab, France) by immunization of rabbits with purified RtxA N-terminus or C-terminus, then passing the antisera through columns charged with our recombinant proteins followed by elution. Specificities of the antibodies were confirmed by western blots of whole cell lysates.

Intracellular growth of *L. pneumophila*

A. castellanii cells were washed then seeded into a 24-well tissue culture treated microplate (Greiner CELLSTAR®, Germany) at 1×10^5 cells/well and left to adhere for 2 hours at 30°C. In order to inhibit any extracellular growth of *L. pneumophila*, the infection medium used in this experiment is a modified proteose-yeast extract medium lacking peptone, yeast extract and glucose required by *Legionella*. Amoebae were infected at a multiplicity of infection (MOI) of 0.1 with bacterial suspensions made by dilution of late-stationary phase cultures of *L. pneumophila* strains. It is important to note that many protocols require centrifugation of the microplates after inoculation with bacteria to force contact between *L. pneumophila* and its host; we skipped this step to challenge the *Legionella* infection process. Plates were then incubated at 30°C and microscopic observation performed daily to follow the progress of infection. To further challenge the infection process, we repeated the same procedure described above at MOI 1 but with supplementing the infection medium with 1×10^7 *E. coli* MG-1655/well (i.e. 100x *Legionella*) as a source of nutrients for *A. castellanii*.

Protection against infection using Anti-RtxA antibodies

A. castellanii cells were washed then seeded into a 96 well microplate (Greiner CELLSTAR®, Germany) at 1×10^5 and left to adhere for 2 hours at 30°C. Amoebae were infected at a MOI of 1 with bacterial suspensions made by dilution of late-stationary phase cultures of *L. pneumophila* strains. The infection medium used (proteose-yeast extract) is bacteriostatic for *Legionella*, only allowing intracellular growth. The *Legionella* strains used in this experiment were transformed with a pXDC50 plasmid expressing mCherry red fluorescent protein. The infection medium in both cases was supplied

with chloramphenicol (5 $\mu\text{g ml}^{-1}$) and IPTG (1 mM). Before inoculation, we added different concentrations of purified anti-RtxA^{COOH} to the bacterial suspension. Appropriate controls were performed including a *Legionella* anti-LegK4 antibody as well as various concentrations of glycerol since it was used to preserve our antibodies. To measure fluorescence, the plates were incubated for 3 days in a Tecan Infinite® F200 pro at 30°C.

Fluorescent microscopy of *L. pneumophila* infection of phagocytic cells

U937 Cells were seeded and then infected with ΔlapG *L. pneumophila* as previously described, however the infection was allowed to proceed for 15 minutes only. The infection was stopped and several washes PBS to remove non adherent cells. Then we added 250 μl of 4% paraformaldehyde (PFA) solution and left 30 it minutes to fix the cells. Finally, we washed again and added 250 μl PBS and the cells were visualized using an epifluorescence microscope (EVOS® FL; Thermo Fisher, USA).

Immunofluorescence microscopy of *L. pneumophila* infection

Infection experiments were carried out mainly in 96 well plates (Greiner CELLSTAR®, Germany). Regarding U937 cells, 1×10^5 monocytes were seeded in each well and left to differentiate into mature macrophages for 2~3 days. Macrophages were infected at a MOI of 10 with bacterial suspensions made by dilution of late-stationary phase cultures of *L. pneumophila* strains; the infection medium used was RPMI-1640+10% FBS. The plate was then left for 20 minutes at 37°C. As for *A. castellanii*, 1×10^5 cells/well were seeded and left overnight at 30°C, *L. pneumophila* were prepared as described before, but the infection medium in this case is the modified bacteriostatic proteose-yeast extract medium. After inoculation, the plate was left for 20 minutes at 30°C for the infection to proceed. The following steps were common for both cell types and were carried out at room temperature. The infection was stopped then we added 250 μl of 4% paraformaldehyde (PFA) solution and left 30 it minutes to fix the cells. After washing with phosphate-buffered saline (PBS pH 7.0), we added 250 μl PBS+0.1% glycine to reduce background fluorescence. Non-specific sites were blocked by PBS+3% bovine serum albumin (BSA) solution for 1 hour. The wells were washed again and 100 μl (1:10000 or 0.374 $\mu\text{g ml}^{-1}$) of primary antibody (rabbit anti-RtxA^{COOH}) were added and left to incubate for 1 hour. After several washes with PBS, 50 μl of red fluorescence conjugated secondary antibody (Alexa Fluor® [568 for *A. castellanii* and 488 for U937 cells] goat anti-rabbit antibodies; Invitrogen Inc. USA) were added and the plates were left in the dark for 1 hour. Finally, we washed the wells twice with PBS and the cells were visualized using an epifluorescence microscope (EVOS® FL; Thermo Fisher, USA).

Cross-linking of infected cells

A. castellanii cultures were grown in flasks for three days before infection. The cells were counted and then pelleted through centrifugation at 720 g for 10 min at 20°C. The pellet was suspended in PY medium for a final concentration of 2×10^7 cells/mL. *L. pneumophila* str Paris was added to the solution at MOI 10. Infection was carried out at 30°C during 30 min or 1 h.

Infection of macrophages was carried out in the Petri dish used for their differentiation from monocytes. *L. pneumophila* str Paris solution was added to the plate at MOI 10 and the plate was then kept at 37°C for 30 min or 1 h at CO₂ of 5%.

Infected cells were centrifuged for 10 minutes at 25°C at 720 g for amoebae and 300 g for macrophages. The pelleted cells were cross-linked with a 1% formaldehyde-stabilized solution and quenching was done with a solution of glycine 2,5 M. Cells were washed with PBS 1X pH 7.4, pelleted and then suspended in 600 µL of IP Lysis/Wash Buffer (Pierce® Kit) for lysis with a Fast-Prep beader (MP Biochemicals).

Co-immunoprecipitation assays

Cell extracts from lysed infected cells were clarified using a Pierce® Crosslink Immunoprecipitation Kit column (ThermoFisher). Antibodies targeting the N-terminus or C-terminus of RtxA were coupled to the A/G protein resin from the Pierce® Kit and preserved in a solution containing 0.02% azide. Co-immunoprecipitation assays were performed after incubating the clarified cell lysates with one of both antibodies coupled to the resin. All was done according to the manufacturer's instructions.

Statistical analysis

Protection against *L. pneumophila* infection using anti-RtxA antibodies were performed in triplicate and the results were displayed as mean values \pm standard errors of the mean. Differences in protection efficiencies were evaluated by ordinary two-way ANOVA for analysis of variance followed by Tukey's multiple comparisons test of significance for means. These analyses were performed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. P values less than 0.05 were considered statistically significant.

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I. Unpublished additional Experiment 2: Follow-up of *L. pneumophila* infection of *A. castellanii*

In order to assess the effects of disruptions of the LapD/LapG system on *L. pneumophila* virulence towards amoebae, we produced deletion mutants of their respective genes and these mutants alongside the wild-type were transformed with pXDC50 plasmid with an inducible promoter upstream mCherry. We also constructed pXDC50 plasmids containing the deleted gene to study the complementation of deletion as well as overexpression (if the plasmid was transformed into wild-type *L. pneumophila*). These complementation experiments are essential to validate the phenotypes observed when using the deleted strains as complemented strain must restore the WT phenotype if no other gene is affected.

Briefly, amoebae were seeded in a 96 well tissue culture treated microplate at 1×10^5 cells/well. Bacterial suspensions of the different *L. pneumophila* mutants were prepared in a bacteriostatic medium containing IPTG (1 mM) for plasmid induction. Then 200 μ L of each suspension was inoculated to infect amoebae at MOI 1. Plates were then incubated for 96 hours at 30°C and fluorescence was measured every hour. The same number of bacteria (1×10^5 CFU/well) grown in AYE rich *Legionella* medium were also inoculated in separate wells to verify the fitness of bacteria regardless of their virulence.

In Figure 47, the graphs (mean of triplicate experiment \pm standard error of the mean) show the progress of infection via measured mCherry fluorescence and bacterial growth via the absorbance OD_{595nm}. It is worthy to mention first that $\Delta dotA$ mutants lack a component of the T4SS required for intracellular growth and that can be seen via its normal growth in AYE but failure in infection (Figure #H). This phenotype is our negative control of *L. pneumophila* infection capacity. Interestingly, the wild-type strain overexpressing LapG protein that should continuously cleave RtxA, showed higher intracellular growth than the wild-type (~3000 vs. ~2300 RFU) and the infection started without any delay compared to the WT strain (Figure #A and #C). Concerning the strain lacking *lapG* keeping RtxA on the surface (Figure #F), or the complemented strain (Figure #G), no noticeable intracellular growth phenotype can be seen compared to the WT strain. However, in this experiment, the results mainly pointed out the replication capacity of *Legionella* in amoebae, not the initial steps of infection.

In case of LapD overexpression in *L. pneumophila*, our results clearly showed that overexpressing LapD drastically affected *Legionella* cells for growth in liquid medium (Figure #B) with a lesser extent in the $\Delta lapD$ genetic background (Figure #E). In the last case, we can notice the delay of growth start and a low capacity of replication inside amoebae. Other growth assays confirmed the weak fitness of LapD overexpressing strains of *Legionella* in liquid medium, but also on CYE plates with mainly a delay of growth. This is probably due to the fact that this protein can bind c-di-GMP via its GGDEF/EAL domain which will disrupt various host processes that rely on this second messenger and will in consequence

affect the general fitness of this bacterium. However, $\Delta lapD$ mutants which should always cleave and release RtxA had similar infection potential to that of wild-type (Figure #D).

Taking in account all these results, it was clearly apparent that our strategy to complement mutant strains by over expressing LapD using a plasmid can't be used and a new strategy has to be set up to achieve that goal. One better solution may be the complementation with one single copy of *lapD* with its own promoter reinserted into the *L. pneumophila* Paris chromosome. Our choice is to reinsert this gene in another chromosomal location and all the generation of mutations process is now carried out on $\Delta lapD$ and $\Delta lapG$ mutants to construct these new complemented strains and to test their capacity of infection.

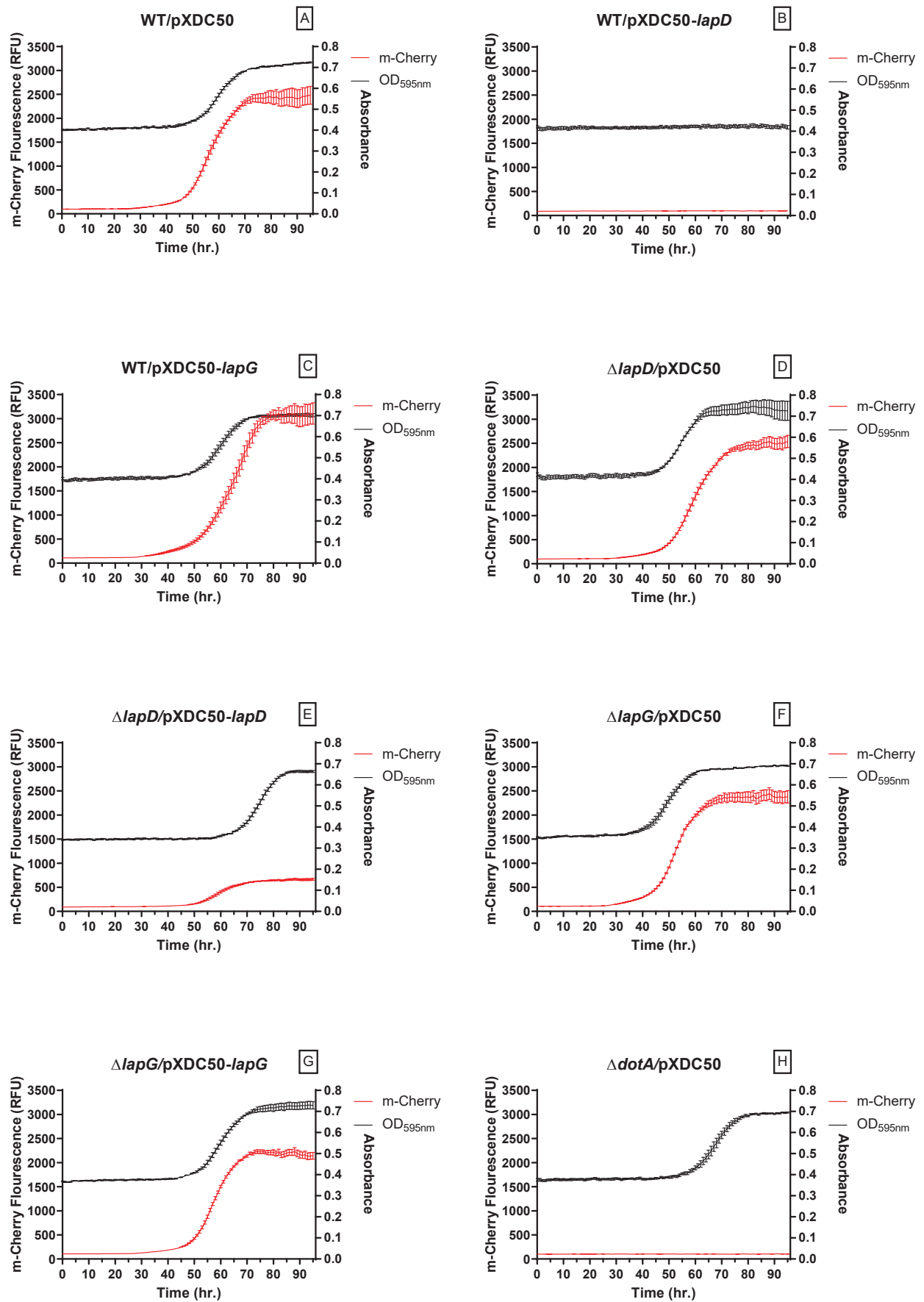


Figure 47: Infection of *A. castellanii* by $\Delta lapG$ *L. pneumophila* at MOI 1

L. pneumophila WT, mutants, and complementation strain were used to infect *A. castellanii* at MOI 1 for 4 days at 30°C in a multimode plate reader (TECAN infinite F200 pro) mCherry fluorescence represent *L. pneumophila* virulence in amoebae while OD_{595nm} represents its growth in AYE medium.

IV. Conclusions and perspectives

Our data in this work elucidate new and important aspects concerning *L. pneumophila* virulence, type 1 secretion machinery and the relation between both. For instance, we confirmed that the *L. pneumophila* surface adhesin, RtxA, is susceptible to cleavage by a periplasmic protease LapG that is in turn moderated by an inner membrane protein with a periplasmic domain, LapD. We also proved that this cleavage results in RtxA release from the cell surface. This kind of regulation of a cell surface RTX adhesin has been recorded previously for several bacteria such as *Pseudomonas fluorescens* with its LapA (Boyd *et al.*, 2012) and *Shewanella oneidensis* BpfA (Zhou *et al.*, 2015). It is also worthy to note that *Pseudomonas aeruginosa* secretes a cell surface adhesin, CdrA, that is involved in biofilm formation and regulated by LapD/LapG homologs despite not being an RTX protein, in fact it is secreted by a T5SS (Rybtke *et al.*, 2015). Interestingly, these bacteria regulate their surface adhesins in order to control biofilm formation.

Regarding *L. pneumophila*, several publications report its capacity to be included in biofilms in their natural habitat, meaning complex biofilms comprising other bacterial species and protozoans. However, its potential to form monospecies biofilms seems to be limited. One team succeeded in visualizing this biofilm formation by *L. pneumophila* with confocal laser scanning microscopy, setting up a suitable medium to promote it (Pecastaings *et al.*, 2010). Previous work in the laboratory in collaboration with this team was done to assess the possible role of RtxA in biofilm formation. The *lssBD* deletion mutant of strain *L. pneumophila* Lens was compared to the WT strain as shown in Figure 48. The experiment was conducted twice with the same observation, which was in fact difficult to interpret. Apart from the WT strain, no clear fluorescent spots (bacterial cells) could be seen with the T1SS defective strain and the fluorescence was diffuse. One hypothesis is that the fluorescence may correspond to cellular debris and SYTO 9, labelling released DNA inside these debris. This could be in agreement with a defective capacity of $\Delta lssBD$ mutant strain to form biofilms in such condition, therefore inducing the attached bacterial cells to die (Pécastaings, personal communication). Based on the medium identified to promote biofilm formation, different experiments were conducted during my thesis to assay biofilm formation with *L. pneumophila* Paris WT and derivatives mutants ($\Delta rtxA$, $\Delta lssBD$, $\Delta lapD$, $\Delta lapG$) as it would have been an easy phenotypic test and would have confirmed the role of RtxA in this process. Unfortunately, monospecies biofilms were too weak to enable reproducible experiments and to compare phenotypic characteristics of the biofilms formed. It is however interesting to note that the $\Delta lapG$ strain displayed an “aggregated” phenotype as seen using anti-RtxA antibodies with immunofluorescence microscopy (figure 5 of the first manuscript), that may be the consequence of RtxA remaining embedded in the outer membrane. Such a role will be in agreement with the classical postulate that biofilm formation is associated with high level of c-di-GMP, therefore keeping the adhesin LapA on the cell surface of *Pseudomonas fluorescens*. New experiments will be conducted using $\Delta lapD$ and $\Delta lapG$ mutants to

clarify the role of RtxA in biofilm formation, but working with complex biofilms might also be necessary to achieve that goal. Other clues of this biofilm aspect will be discussed below.

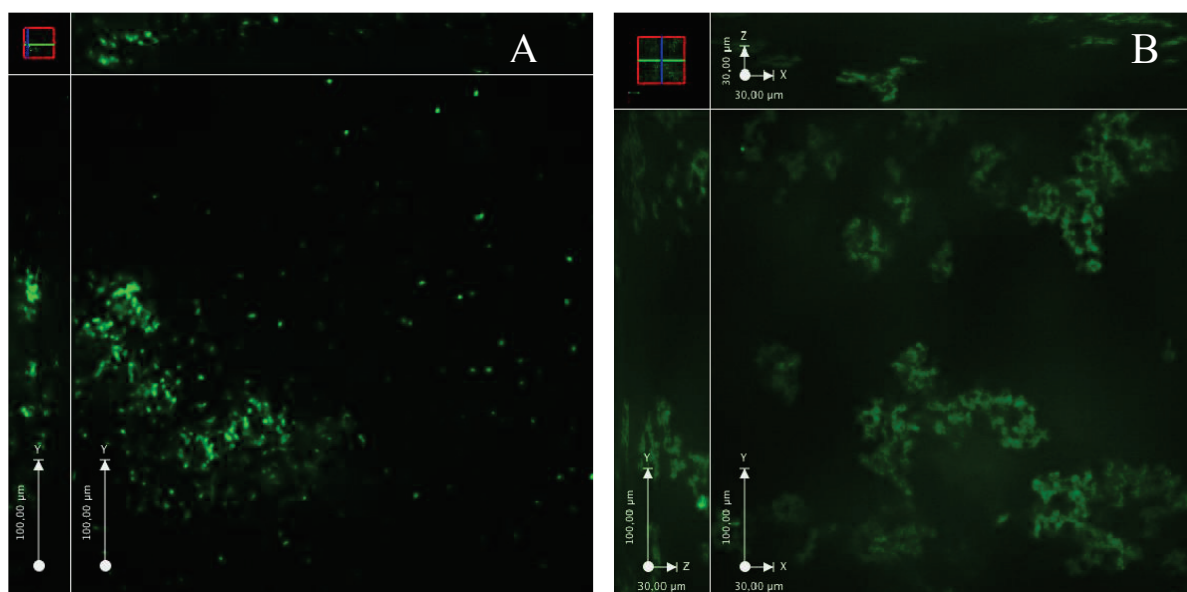


Figure 48: Observation of SYTO 9 stained *L. pneumophila* cells in the biofilm by confocal laser scanning microscopy

(A) *L. pneumophila* Lens WT strain, (B) *L. pneumophila* $\Delta lssBD$ strain. SYTO 9 is a cell-permeant nucleic acid stain that shows significant fluorescent shift when bound to nucleic acids.

Consequently, the *L. pneumophila* RtxA has not been clearly linked to biofilm formation in this bacterium, but it has been frequently associated to virulence and initial attachment to host cells (Cirillo *et al.*, 2001). Therefore, proving that such a large RTX adhesin is detected during the earlier steps of host attachment and undergoes potentially selective cleavage and release, creates a class of adhesins that are not mainly related to biofilm formation but are regulated in a highly similar manner. This opens questions on whether *L. pneumophila* can regulate its virulence potential via RtxA under specific conditions. This requires future work to study RtxA expression as well as release during the various stages of *Legionella* lifecycle especially the transmissive versus the replicative phase. An attempt to achieve that was done by constructing chromosomal translational fusions in *L. pneumophila* Paris targeting *lssD* and *lapD* genes using GFP (Green Fluorescent Protein) as a reporter (data not shown). This choice of translational fusion proteins was taken because it may have answered two questions: detecting the synthesis during *Legionella* growth stages and localize the fused protein in bacterial cells. However, neither epifluorescence microscopy nor cytofluorometry allowed us to detect a fluorescent signal, whatever the physiological state of *L. pneumophila*. Astonishingly, previous work in the laboratory (using RTqPCR) pointed out the transcriptional increase of *lssB* during the stationary phase (Mourad Ferhat's thesis). Different hypotheses can be proposed, one is that the level of transcription is too low to provide enough GFP fused protein to be detected. A second one is that the fused proteins do

not generate an active GFP. In order to test it, new experiments will be conducted to detect the GFP-fused proteins by western blot using anti-GFP antibodies. A third hypothesis is that translational regulation may modulate the expression of *lssB* or *lapD*, therefore the mRNA levels observed in our previous work do not reflect the level of proteins produced. New experiments are programmed to elucidate these points; especially new genetic constructs will be produced to generate transcriptional fusions in *L. pneumophila* Paris. In fact, generating transcriptional fusions with *lssBD* operon, *lapGD* operon and *rtxA* may solve two problems: GFP will be functional and post-transcriptional regulation may be avoided by *gfp* harboring its own RBS (if there is no polar effect of this potential regulation). All these data will be essential to better understand the dynamic of T1SS, RtxA and LapDG during the bacterial cycle and will help to define more precisely the sampling time to study this system.

Although *rtxA* is not confirmed in non-*pneumophila* species, and that its sequence is not strictly conserved among *L. pneumophila* strains (Cazalet *et al.*, 2008) especially in terms of its repeat regions (D'Auria *et al.*, 2008), our BLAST searches revealed some interesting findings regarding this matter. First, we discovered the presence of RtxA N-terminus homologs in various *Legionella* species. In fact, the whole gene is not clearly annotated in all these genomes as its internal repeats are often a source of sequencing uncertainty. Therefore, looking at the partially conserved N-terminus region was a better way to point out this potential presence. Moreover, the LssB and LssD components of the T1SS responsible for RtxA transport was also detected in translated genomes of non-*L. pneumophila* species, a statement not in agreement with published work restricting the presence of T1SS to *L. pneumophila* species (Qin *et al.*, 2017, Smith *et al.*, 2018a). In addition to the previous, LapD and LapG, responsible for RtxA localization were also found in these *Legionella* species. This indicates that high sequence variability and low conservation of the genetic organization (especially in case of *lss* operon) among the *Legionella* genus, could have led previously to restricting RtxA and the T1SS to *L. pneumophila* species, which contradicts with our current findings. Based on our work, it seems that the global system including RtxA, T1SS and LapG/LapD is present in most of *Legionella* species, except *L. longbeachae* and may participate in common physiological functions such as biofilm formation or virulence towards the natural hosts (*i.e.* amoebae).

In the previously mentioned models (*i.e.* *Pseudomonas*) and in *L. pneumophila*, the RTX location/regulation is controlled by LapD, an inner membrane protein with a periplasmic domain and LapG, an entirely periplasmic protein. On the other hand, RtxA is transported by a dedicated T1SS whose hallmark is one step secretion from the cytoplasm through a periplasmic channel to the extracellular medium. These facts conflict as LapG needs to access the N-terminal region of the RTX protein in the T1SS channel for cleavage to occur. To answer this raised question, we used pull-down assays and bacterial two-hybrid assays to investigate possible protein-protein interactions, especially between the N-terminal region of RtxA and components of the T1SS that prove RtxA is retained in the channel pending cleavage, as well as potential interaction between LapG and the T1SS. Unfortunately,

in our conditions we only detected evident LssB and LssD interactions in *E. coli*. However, recent findings by (Smith *et al.*, 2018b) confirm our hypothesis where they state that in *P. fluorescens*, the LapA adhesin is anchored and consequently blocked via its N-terminal region in the LapE duct, equivalent to TolC canal in *L. pneumophila*. Moreover, a new class of T1SS was suggested in a recent study also by (Smith *et al.*, 2018a), this sub-group is linked to bacterial transglutaminase-like cysteine proteinase (BTLCP) and involves a two-step secretion mechanism, a process that requires a periplasmic intermediate of the substrate; here the RTX protein. This data offers more insight into RTX secretion in general and especially in BTLCP systems such as in *L. pneumophila*. Additional work will be needed in *Legionella* to establish the link between RtxA N-terminal region and the TolC or other components of the T1SS, or even interactions with LapG/LapD system. Moreover, as the interactions between the proteins involved in these mechanisms may be transient, the best results may be obtained using an *in vivo* method that enable us to study the mechanism at different time points of bacterial lifecycle. Recent work using proximity-dependent biotinylation (APEX2-dependent biotinylation) succeeded in deciphering the role of one T6SS component in the dynamic of this mechanism in *Escherichia coli* (Santin *et al.*, 2018). Moreover, other authors pointed out the efficacy of this labeling in the periplasmic compartment (Ganapathy *et al.*, 2018), which makes this tool perfectly adapted to study the T1SS/RtxA/lapGD machinery in *Legionella*. Briefly, APEX2 is an engineered variant of the ascorbate peroxidase that oxidizes phenol derivatives to phenoxyl radicals in the presence of hydrogen peroxide. Hence, APEX2 converts biotin-phenol (substrate provided during the experiment) to short-lived, small-distance diffusive biotin-phenoxyl radicals, therefore ending with biotinylation of proteins that share the same space with the bait protein (APEX2-fused protein), approximately within a 20-nm radius (Rhee *et al.*, 2013). The proximity-biotinylated proteins can then be enriched using streptavidin binding and identified by mass spectrometry. Thanks to the genetic manipulation tool developed during this work, we will be able to construct *L. pneumophila* strains expressing the APEX2-fused bait proteins to identify their potential partners *in vivo*, keeping the genetic background and the general mechanisms regulation as close as possible to the wild-type context. Work is now going on targeting LapG, LapD, LssB and LssD as baits.

Despite LapD being the central regulator for the cleavage/release system, current data in both *L. pneumophila* and *P. fluorescens* points to the inability of this protein to synthesize/degrade c-di-GMP, the second messenger regulating this system. In fact LapD only possesses denaturated GGDEF/EAL domains that lack diguanylate cyclase activity and are unable to synthesize c-di-GMP, neither to degrade it (no diguanylate phosphodiesterase activity) (Newell *et al.*, 2009, Chatterjee *et al.*, 2012). Therefore, LapD must rely on partner protein(s) that can activate/inhibit this system by producing/degrading c-di-GMP bound by LapD. Recently, a physical interaction between GcbC (diguanylate cyclase) and LapD in *P. fluorescens* was described and was shown to be necessary for the LapD activation (Dahlstrom *et al.*, 2015). Furthermore, the regulation network involving c-di-GMP and consequently GGDEF/EAL

proteins appeared to be highly complex and other works identified nine potential GGDEF/EAL proteins interacting with LapD (Giacalone *et al.*, 2018). What complicates this issue even further is that *L. pneumophila* contains more than 20 predicted proteins with GGDEF or EAL domains (up to 27 in strain Paris). Among the 7 candidates we chose, some by analogy to *Pseudomonas* proteins; our bacterial two-hybrid assays revealed only a moderate interaction between LapD and LssE, an inner membrane protein belonging to the *lss* T1SS operon. This protein harbors a weakly conserved (SGDQF) GGDEF/EAL domain with no apparent diguanylate cyclase activity. Therefore, additional work is already under way to test new candidates for possible interactions using the bacterial two-hybrid assay, especially GGDEF/EAL proteins pointed out for their role in biofilm formation in *L. pneumophila* from previous collaborative work in the laboratory (Pecastaings *et al.*, 2016). However, once again, the new APEX2-fused bait proteins strategy that is in setup phase in the laboratory may be a suitable tool to detect such transient interactions *in vivo* and we will consider it in the future work. Figure 49 below is to help imagine the structure of this T1SS and its relation with cleavage machinery and the substrate.

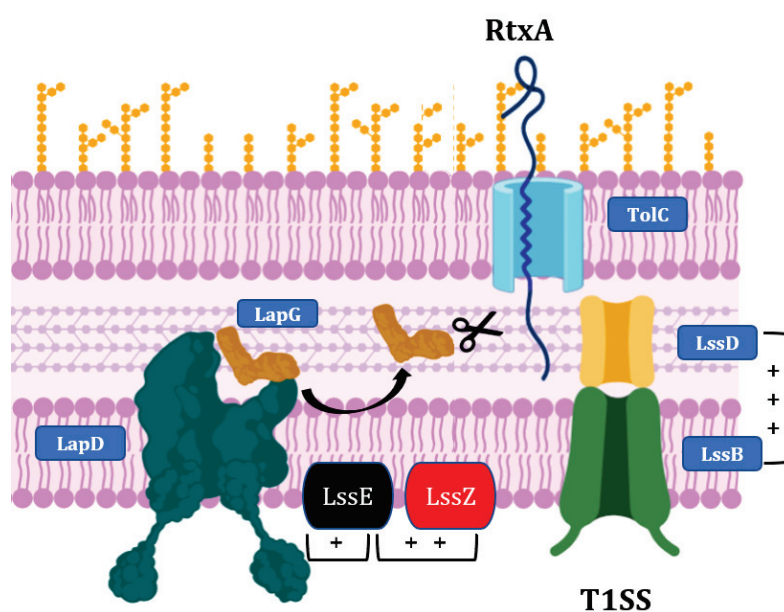


Figure 49: Model of interaction of T1SS, LapD/LapG, RtxA and partners The T1SS in *L. pneumophila* is theorized to retain the substrate in the TolC component of the secretion channel until LapG protease is released by LapD and cleaves the N-terminal domain of RtxA releasing it from the cell surface. The (+) signals represent strength of interaction between indicated components in a bacterial two-hybrid assay conducted in *E. coli*. LssB and LssD served as a control where we detected strong interaction. Slight interaction was detected between LapD and LssE (protein with degenerate GGDEF/EAL) domain, and a moderate interaction between LssE and LssZ (unknown function).

Regarding *L. pneumophila* virulence, we confirmed via constructed mutants, previous reports implicating RtxA in the infectious cycle. But in light of our findings, we also investigated the importance of RtxA location in *L. pneumophila* virulence. First, we proved that *L. pneumophila* lacking *rtxA* exhibits

considerable delay in entry/infection of amoeba cells as well as mutants lacking T1SS components LssB and LssD. However, we noticed via *lapD/lapG* mutants that RtxA localization did not seem to be highly impactful on *L. pneumophila* infection potential in our conditions. This means that RtxA secretion is exclusive to this secretion system and that under our experimental conditions, the ability to secrete RtxA is more prominent during the infectious cycle than its location. Therefore, data from our experiments corroborate and add to previous findings implicating RtxA in virulence (Cirillo *et al.*, 2001, Cirillo *et al.*, 2002). Moreover, our preliminary data regarding virulence attenuation using Anti-RtxA^{COOH} antibodies further implicate RtxA in *L. pneumophila* virulence towards amoebae. Host immunization experiments such as those described by (Lee *et al.*, 2014) can provide additional information about these effects *in vivo* and whether this immunization can contribute to a more efficient immune response. In conclusion, we can say that RtxA indeed participates in virulence but certainly not as a strictly limiting factor as infections can still proceed albeit with a noticeable delay.

Adding to the previous, we would like to mention again that in all our infection experiments, we did not force any contact between *L. pneumophila* and its host and that the MOI we used ranged between 0.1 (which is considerably low) and 10. Despite the previous, and occasionally limiting exposure time of *L. pneumophila* to its host for as low as 15 to 20 minutes, we could detect *L. pneumophila* and RtxA on the surface of amoebae and macrophages. This means that this bacterium is definitely potent and capable of targeting its host and that is even more evident in the *E. coli* competition experiments where *Legionella* infection proceeded normally despite the presence of an excess alternative food source for amoebae. On this regard, it is interesting to note that recent data from (Konig *et al.*, 2019) revealed that chlamydial endosymbionts of *Acanthamoeba* spp. provide the host with protection against different strains of *L. pneumophila*, but it seems that this protection process occurred during the replication step inside amoebae as *Legionella* containing vacuoles were seen indicating that the initial entry was not affected. Additional experiments will be needed to more accurately detect RtxA (confocal microscopy) during the infectious cycle, and to study the virulence of *L. pneumophila* and the constructed mutants towards various cell types. Interestingly, thanks to the anti-RtxA antibodies produced during this work, co-immunoprecipitation enabled to identify 2 potential host target proteins with reductase activities, a protein disulfide isomerase (PDI) from U937 macrophages and the peroxiredoxin 2 from *Acanthamoeba*. It is worth to note that cell surface PDI are required for host cell adhesion and entry of several Chlamydia species (Abromaitis & Stephens, 2009) and that peroxiredoxin 2 has been reported to participate to phagocytosis process in amoeba (Park *et al.*, 2005). Future work on RtxA role in virulence will focus on these candidates.

The last point to mention in this work is the difficulties encountered working on this complex machinery in *Legionella pneumophila*. For example, our attempts to clone the long *rtxA* gene (around 20 kb) are not yet successful, even obtaining the full DNA fragment of this gene by PCR (including many repeats) was difficult. New strategies will have to be set up to achieve that goal and to be able to validate the

$\Delta rtxA$ phenotype. For other reasons, the classical plasmidic complementation method in use in the laboratory appeared to be deleterious when applied to *lapD* gene, probably by unbalancing the c-di-GMP into the bacteria and consequently affecting growth process. To bypass this problem, we must consider complementation without overexpression of LapD, for example by reintroducing the *lapD* gene into *L. pneumophila* chromosome under its own promoter. Concerning the anti-RtxA antibodies, even the RtxA^{COOH} fragment proved difficult to obtain where different plasmids and *E. coli* strains were used to achieve proper production of this polypeptide.

In conclusion, *L. pneumophila* is a potent intracellular organism that relies on a plethora of virulence factors to be labeled as a successful pathogen. The virulence factor, RtxA, which seems to be the sole T1SS substrate in *Legionella*, participates in its pathogenesis where it plays an important role in efficacy of initial entry into the host cell, but may also be implicated in other *Legionella* processes such as intracellular replication and biofilm formation. This protein's presence on the cell surface is regulated by complex processes that are likely influenced by environmental conditions. Interestingly, the two well established locations, embedded in outer membrane or release in the extracellular medium seem to be tightly regulated by the LapG/LapD complex that may be closely linked to biofilm formation capacity as already pointed out for other bacterial species, but is not easily associated with virulence yet. Understanding the role of T1SS and its cognate substrate RtxA may help in the future to better control the biofilm formation and/or the capacity of *Legionella* species to infect natural host in water habitats, therefore their opportunity to multiply within the environment, and consequently to better control the risk sources of human infection.

V. Technical sheets: Strains, Plasmids, Primers and Protocols

A. Strains used in this study

| Strain | Genotype | Reference |
|---------------------------|---|-------------|
| <i>Escherichia coli</i> | | |
| XL1-Blue | <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacIqZΔM15 Tn10</i> (Tetr)] | Stratagene |
| DH5-Alpha | F ⁻ <i>Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK⁻, mK⁺) phoA supE44 λ⁻ thi-1 gyrA96 relA</i> | Invitrogen |
| BL21 | F ⁻ <i>ompT hsdS_B(r_B⁻, m_B⁻) gal dcm</i> | |
| BL21/pREP4- <i>groESL</i> | F ⁻ <i>ompT hsdS_B(r_B⁻, m_B⁻) gal dcm</i> carrying the plasmid pREP4- <i>groESL</i> (coding for LacI, GroES et GroEL) | Stratagene |
| MG1655 | F ⁻ , <i>lambda⁻</i> , <i>rph-1</i> | ATCC® 47076 |
| DHM1 | F ⁻ , <i>cya-854, recA1, endA1, gyrA96 (Nal^r), thi1, hsdR17, spoT1, rfbD1, glnV44(AS)</i> | Euromedex |
| BTH101 | F ⁻ , <i>cya-99, araD139, galE15, galK16, rpsL1 (Str^r), hsdR2, mcrA1, mcrB1</i> | Euromedex |

Legionella pneumophila

| | | |
|---------------------|---|--------------------------------|
| Paris | <i>L. pneumophila</i> serogroup 1, Paris strain | (Cazalet <i>et al.</i> , 2004) |
| Paris <i>ΔdotA</i> | <i>Δlpp2740</i> | This study |
| Paris <i>ΔtolC</i> | <i>Δlpp0889</i> | This study |
| Paris <i>ΔlapG</i> | <i>Δlpp0890</i> | This study |
| Paris <i>ΔlapD</i> | <i>Δlpp0891</i> | This study |
| Paris <i>ΔlssBD</i> | <i>Δlpp1473, Δlpp1474</i> | This study |
| Paris <i>ΔrtxA</i> | <i>Δlpp0699</i> | This study |

| Eukaryotic cells | Phenotype | Reference |
|---------------------------------|------------------------------------|------------------|
| <i>Acanthamoeba castellanii</i> | Environmental isolate | |
| U937 Macrophages | Human monocyte cell line | ATCC® CRL-1593.2 |
| A549 Pneumocytes | Human type II pneumocyte cell line | ATCC® CCL-185 |

B. Plasmids used in this study

Base plasmids

| Plasmid | Characteristics | Reference |
|------------|---|--------------------|
| pXDC50 | Utilizable plasmid in <i>Legionella</i> , mCherry expression (Ptac promoter), <i>lacIq</i> , CmR | Xavier Charpentier |
| pXDC116 | Utilizable plasmid in <i>Legionella</i> , GFP expression (Ptac promoter), <i>lacIq</i> , CmR, KanR | Xavier Charpentier |
| pET-30a(+) | Bacterial expression plasmid, T7 promoter, 6xHis tag – N&C ^{term} , KanR | |
| pQE30 | Bacterial expression plasmid, T5 promoter, 6xHis tag – N ^{term} , AmpR | |
| pGEX-6p-3 | Bacterial expression plasmid, Ptac promoter, GST tag – N ^{term} , AmpR, PreScission cleavage site | |
| pKT25 | Derivative of the pSU40 low copy number plasmid. It allows fusion of inserts with T25 fragment (first 224 amino acids of CyaA), lac promoter, KanR, in frame fusions at the C-term of T25 polypeptide | Euromedex |
| pKNT25 | Derivative of the pSU40 low copy number plasmid. It allows fusion of inserts with T25 fragment (first 224 amino acids of CyaA), lac promoter, KanR, in frame fusions at the N-term of T25 polypeptide | Euromedex |
| pUT18 | Derivative of the pUC19 high copy number plasmid, it allows fusion of inserts with T18 fragment (amino acids 225 to 399 of CyaA), lac promoter, AmpR, in frame fusions to the N-term end of T18 polypeptide | Euromedex |
| pUT18C | Derivative of the pUC19 high copy number plasmid, it allows fusion of inserts with T18 fragment (amino acids 225 to 399 of CyaA), lac promoter, AmpR, in frame fusions to the C-term end of T18 polypeptide | Euromedex |

Plasmids for mCherry expression and deletion complementation in *L. pneumophila*

| Plasmid | Characteristics | Reference |
|-------------|--|--------------------|
| pXDC50 | Utilizable plasmid in <i>Legionella</i> , mCherry expression (Ptac promoter), <i>lacIq</i> , CmR | Xavier Charpentier |
| pXDC50-LapG | <i>lapG</i> gene of Paris strain (<i>lpp0890</i>) cloned by XbaI/SphI in pXDC50 (under Ptac control); <i>lacIq</i> , CmR | This study |
| pXDC50-LapD | <i>lapD</i> gene of Paris strain (<i>lpp0891</i>) cloned by XbaI/SphI in pXDC50 (under Ptac control); <i>lacIq</i> , CmR | This study |
| pXDC50-TolC | <i>tolC</i> gene of Paris strain (<i>lpp0889</i>) cloned by XbaI/SphI in pXDC50 (under Ptac control); <i>lacIq</i> , CmR | This study |

Plasmids for overproduction of RtxA and LapG in *E. coli*

| Plasmid | Characteristics | Reference |
|------------------------------------|--|------------|
| pET30a-RtxA ^{NH2} | N-term of RtxA (492 a.a.) belonging to gene <i>lpp0699</i> cloned by NdeI/SalI in pET30 (T7 promoter); KanR | This study |
| pQE30-LapG | <i>lapG</i> gene of Paris strain (<i>lpp0890</i> without signal sequence) (192 a.a.) cloned by BamHI/PstI in pQE30 (T5 promoter), KanR | This study |
| pQE30-long RtxA ^{COOH} | C-term of RtxA (a.a. 5831-6764) belonging to gene <i>lpp0699</i> cloned by BamHI/PstI downstream a 6His tag in pQE30 (T5 promoter), AmpR | This study |
| pQE30-short RtxA ^{COOH} | C-term of RtxA (a.a. 6490-6764) belonging to gene <i>lpp0699</i> cloned by BamHI/PstI downstream a 6His tag in pQE30 (T5 promoter), AmpR | This study |
| pGEX6p3-long RtxA ^{COOH} | C-term of RtxA (a.a. 5831-6764) belonging to gene <i>lpp0699</i> cloned by BamHI/SalI downstream a GST tag in pGEX6p3 (Ptac promoter), AmpR | This study |
| pGEX6p3-short RtxA ^{COOH} | C-term of RtxA (a.a. 6490-6764) belonging to gene <i>lpp0699</i> cloned by EcoNI/EcoRI downstream a GST tag in pGEX6p3 (Ptac promoter), AmpR | This study |

Plasmids for pull-down assays

| Plasmid | Characteristics | Reference |
|---------------------|---|------------|
| pGEX6p3-LssB1.171 | N-term of LssB (a.a. 1-171) belonging to gene <i>lpp1473</i> cloned by EcoRI/SalI downstream a GST tag in pGEX6p3 (Ptac promoter), AmpR | This study |
| pGEX6p3-LssB437.718 | C-term of LssB (a.a. 438-718) belonging to gene <i>lpp1473</i> cloned by EcoRI/SalI downstream a GST tag in pGEX6p3 (Ptac promoter), AmpR | This study |
| pGEX6p3-LssD34.378 | LssD (a.a. 34-378) belonging to gene <i>lpp1474</i> cloned by EcoRI/SalI downstream a GST tag in pGEX6p3 (Ptac promoter), AmpR | This study |
| pQE30-LapD25.125 | N-term of LapD (a.a. 25-125) belonging to gene <i>lpp0891</i> cloned by BamHI/PstI downstream a 6His tag in pQE30 (T5 promoter), KanR | This study |
| pQE30-LapD175.639 | C-term of LapD (a.a. 175-639) belonging to gene <i>lpp0891</i> cloned by BamHI/PstI downstream a 6His tag in pQE30 (T5 promoter), KanR | This study |
| pGEX6p3-LapD25.125 | N-term of LapD (a.a. 25-125) belonging to gene <i>lpp0891</i> cloned by EcoRI/SalI downstream a GST tag in pGEX6p3 (Ptac promoter), AmpR | This study |

Plasmids for bacterial two hybrid assays

| Plasmid | Characteristics | Reference |
|-------------|---|------------|
| pKNT25-LapD | <i>lapD</i> gene of Paris strain (<i>lpp0891</i>) cloned by SphI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pKT25-LssA | <i>lssA</i> gene of Paris strain (<i>lpp1472</i>) cloned by XbaI/KpnI in pKT25 (lac promoter) downstream the T25 polypeptide, KanR | This study |
| pUT18C-LssA | <i>lssA</i> gene of Paris strain (<i>lpp1472</i>) cloned by XbaI/KpnI in pUT18C (lac promoter) downstream the T18 polypeptide, AmpR | This study |
| pKT25-LssB | <i>lssB</i> gene of Paris strain (<i>lpp1473</i>) cloned by XbaI/KpnI in pKT25 (lac promoter) downstream the T25 polypeptide, KanR | This study |
| pUT18C-LssB | <i>lssB</i> gene of Paris strain (<i>lpp1473</i>) cloned by XbaI/KpnI in pUT18C (lac promoter) downstream the T18 polypeptide, AmpR | This study |
| pUT18C-LssD | <i>lssD</i> gene of Paris strain (<i>lpp1474</i>) cloned by XbaI/KpnI in pUT18C (lac promoter) downstream the T18 polypeptide, AmpR | This study |
| pKNT25-LssE | <i>lssE</i> gene of Paris strain (<i>lpp1475</i>) cloned by SphI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-LssE | <i>lssE</i> gene of Paris strain (<i>lpp1475</i>) cloned by SphI/KpnI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |
| pKT25-LssX | <i>lssX</i> gene of Paris strain (<i>lpp1469</i>) cloned by XbaI/KpnI in pKT25 (lac promoter) downstream the T25 polypeptide, KanR | This study |
| pUT18C-LssX | <i>lssX</i> gene of Paris strain (<i>lpp1469</i>) cloned by XbaI/KpnI in pUT18C (lac promoter) downstream the T18 polypeptide, AmpR | This study |
| pKNT25-LssY | <i>lssY</i> gene of Paris strain (<i>lpp1470</i>) cloned by SphI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-LssY | <i>lssY</i> gene of Paris strain (<i>lpp1470</i>) cloned by SphI/KpnI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |
| pKNT25-LssZ | <i>lssZ</i> gene of Paris strain (<i>lpp1471</i>) cloned by SphI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-LssZ | <i>lssZ</i> gene of Paris strain (<i>lpp1471</i>) cloned by SphI/KpnI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |

Plasmids for bacterial two hybrid assays

| Plasmid | Characteristics | Reference |
|----------------|--|------------|
| pKNT25-lpp0299 | Paris strain gene (<i>lpp1471</i>) cloned by XbaI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-lpp0299 | Paris strain gene (<i>lpp1471</i>) cloned by XbaI/KpnI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |
| pKNT25-lpp0440 | Paris strain gene (<i>lpp0440</i>) cloned by XbaI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-lpp0440 | Paris strain gene (<i>lpp0440</i>) cloned by XbaI/KpnI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |
| pKT25-lpp0809 | Paris strain gene (<i>lpp0809</i>) cloned by XbaI/KpnI in pKT25 (lac promoter) downstream the T25 polypeptide, KanR | This study |
| pUT18C-lpp0809 | Paris strain gene (<i>lpp0809</i>) cloned by XbaI/KpnI in pUT18C (lac promoter) downstream the T18 polypeptide, AmpR | This study |
| pKNT25-lpp0942 | Paris strain gene (<i>lpp0942</i>) cloned by XbaI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-lpp0942 | Paris strain gene (<i>lpp0942</i>) cloned by XbaI/KpnI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |
| pKNT25-lpp1311 | Paris strain gene (<i>lpp1311</i>) cloned by PstI/SmaI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-lpp1311 | Paris strain gene (<i>lpp1311</i>) cloned by PstI/SmaI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |
| pKNT25-lpp2355 | Paris strain gene (<i>lpp2355</i>) cloned by XbaI/KpnI in pKNT25 (lac promoter) upstream the T25 polypeptide, KanR | This study |
| pUT18-lpp2355 | Paris strain gene (<i>lpp2355</i>) cloned by XbaI/KpnI in pUT18 (lac promoter) upstream the T18 polypeptide, AmpR | This study |

C. Primers used in this study

Protein Pull-down primers

| Name | Sequence 5'-3' | Site | Cloned gene |
|---------------------|--|-------|---------------------|
| LssD34-378pGEX6P3S | ATGCGAATTCTGATGAAGTAACTACAGGACAAGGG | EcoR1 | <i>lssD</i> lpp1474 |
| LssD34-378pGEX6P3R | GTTTTTAGTCGACAA TAACGCTCTCTAAG TGCTG | Sal1 | |
| LssB438-718pGEX6P3S | GCCCCGAATTCTCAAGTATCCACTC | EcoR1 | <i>lssB</i> lpp1473 |
| LssB438-718pGEX6P3R | GAGTTTTTGTCGACGACTATTCCTCACC | Sal1 | |
| LssB0-171pGEX6P3S | TCAGAATTCTACTCCGAGAAGACAATCATGGTC | EcoR1 | <i>lssB</i> lpp1473 |
| LssB0-171pGEX6P3R | AGGGTCGACAGCTATTCGGAATACAATGGC | Sal1 | |
| LapD25-125pQE30S | CGGAACTTATGGATCCACTATGAATAATGCGCG | BamH1 | <i>lapD</i> lpp0891 |
| LapD25-125pQE30R | CTCCTGCTTCTGCAGACTAATCCATAATTAAGGATG | Pst1 | |
| LapD175-639pQE30S | AAGAGCTCTTGCAACCTTTAAAACGAGTCACAG | Sac1 | <i>lapD</i> lpp0891 |
| LapD175-639pQE30R | CTGTTTTGTCGACCGTTATTTTATTAATTCTACAGAAG | Sal1 | |
| LapD25-125pGEX6P3S | CGGAACTTAGAATTCCACTATGAATAATGCG | EcoR1 | <i>lapD</i> lpp0891 |
| LapD25-125pGEX6P3R | CTGGTCTCGACCACTAATCCATAATTAAGG | Sal1 | |

Bacterial Two-Hybrid System primers (T1SS operon)

| Name | Sequence 5'-3' | Site | Cloned gene |
|----------|---|------|---------------------|
| DHlssAS | ATTTCTAGATATGTGTGTAATTTACCAGTATAGTGCGTCTATGAAAC | Xba1 | <i>lssA</i> lpp1472 |
| DHlssAR | CCAATGGTACCATTAAGCAGTAAATTTAGTCTTTTAAAGGGTTTG | Kpn1 | |
| DHlssYSS | GACGCATGCTAAATAATAGGAAATTATGAATTTGTTAGCAGATTATGTG | Sph1 | <i>lssY</i> lpp1470 |
| DHlssYR | GTCAGTGGTTGCACATAATCTGCTAACAAATTCATAGGTACCTAT | Kpn1 | |
| DHlssBS | TAATCTAGAGAATACTCCGAGAAGACAATCATGGTCTC | Xba1 | <i>lssB</i> lpp1473 |
| DHlssBR | TTCGGTACCTATTCCTCACC GTTACCCAG | Kpn1 | |
| DHlapDS | AATAGCATGCGTGATAGACCATGACATTAAC TAAGAAAATGG | Sph1 | <i>lapD</i> lpp0891 |
| DHlapDR | TCATCTGGTACCTTTATTAATTCTACAGAAGATAAAAAGCGTCC | Kpn1 | |
| DHlssES | AGAGCATGCCTGACAGGGATGTTATATTATGCC | Sph1 | <i>lssE</i> lpp1475 |
| DHlssER | GATCATGAGGCAAATAAATAGGTACCTGTAAAAGTTGCGTA | Kpn1 | |
| DHlssDS | GAATCTAGATATGAAAAAACTCACAGCTCAAAAACCTTTACCC | Xba1 | <i>lssD</i> lpp1474 |
| DHlssDR | GTTTTTACACGGTACCTAACGCTCTCTAAGTGCT | Kpn1 | |
| DHlssXS | TGTCTAGAGATGAAGGAGTTCACTGCAACCCGAGA | Xba1 | <i>lssX</i> lpp1469 |
| DHlssXR | GTCAGTGGTTGCACATAATCTGCTAACAAATTCATAGGTACCTAT | Kpn1 | |
| DHlssZS | ATTGCATGCATAAGGGATTGAGTCAATGCATATCTTGGC | Sph1 | <i>lssZ</i> lpp1471 |
| DHlssZR | TTGGGTACCGAACTCCTTTTTTGTGCAATAAGAGCATTTG | Kpn1 | |

Bacterial Two-Hybrid System primers (GGDEF candidates)

| Name | Sequence 5'- 3' | Site | Cloned gene |
|--------------|--|------|--------------|
| DH.lpp0809.S | GCTCTAGATCAAGAATTAAAGAAAAAAATTGTTAACACAATTAGC | XbaI | lpp0809 |
| DH.lpp0809.R | TAGGTACCATATTCCACTATAATATTTCTACCCTGTTCTTTCC | KpnI | |
| DH.lpp0942.S | GCTCTAGACAATTAATGGACATTGGATAAGATGACTTCC | XbaI | lpp0942 |
| DH.lpp0942.R | GGGGTACCAGAATGTCATCTTCTGAAAGTAGCTTTTTTACTACAATTTGG | KpnI | |
| DH.lpp1311.S | AACTGCAGAACATACCCTTTTACTAACTACTTGTATGAT | PstI | lpp1311 |
| DH.lpp1311.R | TCCCCCGGGAGAAACAACAATCATATGTTTATTAATAAATTTGAATGGCG | SmaI | |
| DH.lpp0299.S | GCATCTAGAGCTTATGACGGGATATTGATGATATGAAATC | XbaI | lpp0299 |
| DH.lpp0299.R | ACACGGTACCGAGGATGATATTTCCGAGAATAAAATTACCTTATT | KpnI | |
| DH.yddV.S | GCTCTAGATGAATAATTATGCATCAAAAGAAGCGGTCTTC | XbaI | yddV lpp0440 |
| DH.yddV.R | GGGGTACCTTGGCTCTCAATTCTTGCGAGCCTAACGATTTAG | KpnI | |
| DH.lpp2355.S | GCTCTAGAGTATTTAAGGCCAAATCATGGTTATCCCAG | XbaI | lpp2355 |
| DH.lpp2355.R | GGGGTACCCAATAGTCAGCGATTGGATGGATACTCGT | KpnI | |

L. pneumophila gene deletion primers

| Name | Sequence 5'- 3' | Deleted gene |
|------------------|---|-------------------|
| CassMazF-F | CGACTCACTATAGGGCGAATTGGGCCGCTTTCCAGTCGGGAAACCTG | Kan/MazF cassette |
| CassMazF-R | CATATGCCACCGACCCGAGCAAACCCGAAGAAGTTGTCCATATTGGCCAC | |
| lapGdel1 | TGACTCACGGTCAGTATTGGC | lapG lpp0890 |
| lapGdel2J1ter | GGCCCAATTGCGCCCTATAGTGAGTCGGAGCCAATTCCTTTAGCCTTTC | |
| lapGdel3J1ter | GGGTTTGCTCGGGTCGGTGGCATATGAAAAAGGTGATAGACCATGACATTAAC | |
| lapGdel2J2bis | GTTAATGTCATGGTCTATCACCTTTTTATAACACTAAGCCTTCTTGGGC | |
| lapGdel3J2bis | GCCCAAGAAGGCTTAGTGTTATAAAAAGGTGATAGACCATGACATTAAC | |
| lapGdel4 | AAAAACGTATTTGCAGTTTGCC | |
| lpp0891del1 | CGTTTGTATGCTTACTCCATCG | lapD lpp0891 |
| lpp0891del2J1bis | GGCCCAATTGCGCCCTATAGTGAGTCGATGGTCTATCACCTTTTTTATTCC | |
| lpp0891del3J1bis | GGGTTTGCTCGGGTCGGTGGCATATGTAACGAGATGAAAAACAGATTATTC | |
| lpp0891del2J2 | GAATAATCTGTTTTTCATCTCGTTAATGGTCTATCACCTTTTTTATTCC | |
| lpp0891del3J2 | GGAATAAAAAAGGTGATAGACCATTAACGAGATGAAAAACAGATTATTC | |
| lpp0891del4 | TCAGGTCGTTTCAAAGTCCC | |
| lpp0889del1 | TCCTCTCAGTGGTGCTGTG | tolC lpp0889 |
| lpp0889del2J1 | GGCCCAATTGCGCCCTATAGTGAGTCGTCCTCATTCCTTATTATTGTTATTT | |
| lpp0889del3J1 | GGGTTTGCTCGGGTCGGTGGCATATGATGTTGAAGGAGTCCTGGTTAC | |
| lpp0889del2J2 | GTAACCAGGACTCCTTCAACATTCCTCATTCCTTATTATTGTTATTTG | |
| lpp0889del3J2 | CAAAATAACAATAATAAGGAATGAGGAATGTTGAAGGAGTCCTGGTTAC | |
| lpp0889del4 | ACCGTCTGTAACCTGCCTGC | |
| rtxA1del1 | AGTATTTGGGGTTCTTTCTGG | |

| | | |
|-------------|--|--|
| rtxA1del2J1 | GGCCCAATTCGCCCTATAGTGAGTCGGTCTGTCTCCTCAAAATATACTATTA | <i>rtxA</i> lpp0699 |
| rtxA1del2J2 | ATTACCAATGTAATGAGTTCATCGTTCTGTCTCCTCAAAATATACTATTATT | |
| rtxA1del3J1 | GGGTTTGCTCGGGTCGGTGGCATATGGATGAACTCATTACATTGGTGAAT | |
| rtxA1del3J2 | GATGAACTCATTACATTGGTGAAT | |
| rtxA1del4 | CAAAATCAACATCCTGCCC | <i>lssB</i> , <i>lssD</i> lpp1473, lpp1474 |
| lssBDdel1 | CATATGCCCTATCACAAGGAG | |
| lssBDdel2J1 | GGCCCAATTCGCCCTATAGTGAGTCGATGATTTTTATGATTATTCTTTATA | |
| lssBDdel2J2 | TTTCCAAAATGTTTTTACACACTAAATGATTTTTATGATTATTCTTTATAT | |
| lssBDdel3J1 | GGGTTTGCTCGGGTCGGTGGCATATGTTAGTGTGTAAAAACATTTTGAAA | |
| lssBDdel3J2 | TTAGTGTGTAAAAACATTTTGAAA | |
| lssBDdel4 | AATTAATCCTTTTCCAGGGTG | |

L. pneumophila gene complementation in pXDC50

| Name | Sequence 5'-3' | Site | Amplified gene |
|-----------------|--------------------------------------|------|---------------------------|
| lpp0889pxdcSens | AGCATCTAGAATAAGGAATGAGGAATGAGAAAGG | XbaI | <i>TolC</i> lpp0889 |
| lpp0889pxdcRev | AGCAGCATGCGCTATAACACTAAGCCTTCTTGGG | SphI | |
| lapGpxdcSens | AAAGTCTAGAGGAATTGGCTCATGTTG | XbaI | <i>lapG</i> lpp0890 |
| lapGpxdcRev | TCATGGGCATGCACCTTTTTTATTCCATACG | SphI | |
| lpp0891xdcSens | TGAAACGTCTAGAATAAAAAAGGTGATAG | XbaI | <i>lapD</i> lpp0891 |
| lpp0891pxdcrev | ATGCCTTGAGCATGCTCTGTTTTTCATC | SphI | |
| SlssBD | CCTTTAAGTCGACTAAATTTACTGCTTAATATAAAG | SalI | <i>lssB</i> , <i>lssD</i> |
| RlssBD | ATGCATGCTACCGTAACAACCTTCATCAACG | SphI | lpp1473, lpp1474 |

Overexpression of *L. pneumophila* proteins

| Name | Sequence 5' - 3' | Site | Amplified gene |
|-----------------------|--------------------------------------|-------|----------------|
| lapGsensPQE30bis | TTGCGGATCCCCTTTAATCAGTGTGGAAAAAATTC | BamH1 | lapG lpp0890 |
| lapGrevPQE30 | CAATCTGCAGTTTTATTCCATACGTTTCATTAGAGC | PstI | |
| N-terminus RtxA | | | |
| RtxAsensPet30 | ACAGCATATGTTAGCTGAATCTGTTATCGG | NdeI | rtxA lpp0699 |
| RtxArevPet30 | GTCAAGTCGACCGCTTCATCATAG | SalI | |
| C-terminus RtxA | | | |
| rtxA21136S | ACAGGATCCCGTGTCTATGGAAATGGAAGCC | BamH1 | rtxA lpp0699 |
| rtxA23113S | ACAGGATCCGATATTACGGATGAACAACTCAACTCC | BamH1 | |
| rtxA24001RPstIpQE30 | ACACTGCAGCGAGAAAAATGTCGCGAGG | PstI | |
| rtxA24001RSalIpGEX6P3 | ACGCGTCGACGCAGCGAGAAAAATGTCGCGAGG | SalI | |

D. Protocols

1. Growth media/requirements for bacterial and eukaryotic cells

For both *Escherichia coli* and *Legionella pneumophila*, we estimated the concentration of bacterial suspensions based on the optical density at 600 nm (OD_{600nm}). We considered OD_{600nm} of 1 to be equivalent to 10⁹ CFU/mL for *L. pneumophila* and OD_{600nm} of 0.4 to be equivalent to 3x10⁸ CFU/mL for *E. coli*. Counting eukaryotic cells was done using a hemocytometer.

a) Legionella pneumophila

We used ACES (*N*-(2-acetamido)-2-aminoethanesulfonic acid)-buffered yeast extract (AYE) for *L. pneumophila* growth in liquid medium. It is composed of yeast extract (12 g/L), ACES (10 g/L), L-cysteine (0.5 g/L) and iron (III) pyrophosphate (0.3 g/L). The pH of the mixture was then adjusted to 6.9 using potassium hydroxide (KOH 10N). The medium is then filtered using a 0.22 µm filtration unit (Rapid-Flow™, Nalgene).

Regarding solid media, we used an agar medium based on BCYE (buffered charcoal yeast extract). It is composed of *Legionella* agar base (15 g/L) with activated charcoal (2 g/L), yeast extract (10 g/L). This medium was autoclaved for 20 minutes at 120°C. It was then supplemented with ACES (10 g/L), iron pyrophosphate (0.25 g/L), cysteine (0.275 g/L), this solution was prepared and autoclaved separately for 20 minutes at 120°C.

These media were supplied when necessary with the appropriate antibiotics (Sigma); kanamycin (10 µg/mL) or chloramphenicol (5 µg/mL). IPTG (Isopropyl β-D-1-thiogalactopyranoside, Roth) was added when necessary at 1 mM for both solid and liquid media.

L. pneumophila was grown at 37°C with agitation unless mentioned otherwise.

b) Escherichia coli

As liquid growth medium, we used the lysogeny broth (LB) for all cultures. Purchased from Roth, it is composed of tryptone (10 g/L), yeast extract (5 g/L) and sodium chloride (5 g/L). the pH was then adjusted at 7 and the medium autoclaved for 20 minutes at 120°C. For solid media, we added a 12 g/L sterile agar solution in 1:1 ratio to the LB broth. The obtained medium was poured in petri plates and cooled for later use.

These media were supplemented with the following antibiotics when necessary: ampicillin (100 µg/mL), chloramphenicol (20 µg/mL), Kanamycin (50 µg/mL), tetracycline (15 µg/mL). IPTG was added at 1 mM final concentration for liquid and solid media. For bacterial two-hybrid assays, we added the chromogenic substrate for β-galactosidase, X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, Roth) at a final concentration of 40 µg/mL.

Unless mentioned otherwise, *E. coli* were cultured at 37°C with agitation.

c) Eukaryotic cells

Acanthamoeba castellanii were cultured in PYG medium (Peptone, Yeast extract, Glucose). This medium consists of peptone (20 g/L), yeast extract (1 g/L), MgSO₄ (15 mM), CaCl₂ (40 mM), Sodium citrate (3.4 mM), Fe(NH₄)₂(SO₄)₂ (50 µM), Na₂HPO₄ (2.5 mM), KH₂PO₄ and supplemented with glucose (0.1 M). Infection experiments were performed in a bacteriostatic variant of the PYG (PY special) medium that lacks peptone and yeast extract. *A. Castellanii* cultures and infections were achieved at 30°C.

For U937 monocytes, they were cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS). Differentiation to macrophages was performed over 3 days before utilization by adding phorbol 12-myristate 13-acetate (PMA 100 ng/mL). The cells were cultured at 37°C with 5% CO₂. In certain cases, such as prolonged infection in a multimode plate reader, we used CO₂ independent medium (Gibco).

2. Molecular biology techniques

a) DNA manipulation

(1) Chromosomal DNA extraction

This protocol was utilized for *E. coli* as well as *L. pneumophila*, starting from a culture obtained on solid or overnight liquid medium, we prepared a suspension in 800 µL sterile water. We centrifuged the tube for 5 minutes at 10,000xg, the pellet was suspended in 400 µL sterile water. The cells were lysed by adding 20 µL of 10% SDS and 200 µL of proteinase K solution (20 mg/mL). The mixture was then incubated with agitation for 1 hour at 37°C. Eight hundred microliters of a phenol/chloroform/isoamyl alcohol (25/24/1) solution were added and the mixture homogenized by gentle inversion of the tube for 15 to 20 minutes then it was centrifuged for 5 minutes at 14,000xg. The aqueous phase is aspirated and subjected to a second extraction with chloroform/isoamyl alcohol (24/1) mixture. Two volumes of cold ethanol (-20°C) and 1/10 volumes of solution III (5M potassium acetate, 11.5% glacial acetic acid) were

then added to the collected aqueous phase to precipitate the DNA. The chromosomal DNA is then visible and can be recovered with a platinum wire, it is then dried near a flame of a Bunsen burner and resuspended in 50 μ L ultrapure water.

(2) Plasmid DNA extraction

DNA extraction kits were used for this purpose as well as a house method based on silica affinity. The following method represents a miniprep protocol, the same procedure is used for a maxiprep, but all amounts were multiplied by 10.

An overnight bacterial culture in rich medium containing suitable antibiotic(s) for plasmid selection was prepared, of which 1 mL were transferred to an Eppendorf tube and centrifuged for 10 minutes at 10,000xg. The obtained pellet is then suspended in 100 μ L of solution I (Tris HCl 25 mM, EDTA Na₂ 10 mM, glucose 50 mM) adjusted to pH 8 and supplemented with 2 mg/mL lysozyme and 100 μ g/mL RNase A, this mixture was incubated for 5 minutes at ambient temperature. Cells are lysed by adding 200 μ L of solution II (NaOH 0.2 mM, 1% SDS), the tubes are then inverted gently to homogenize the mixture. To precipitate the DNA, we added 150 μ L of solution III (5M potassium acetate, 11.5% glacial acetic acid). The tubes are then centrifuged for 10 minutes at 14,000xg. The supernatants are transferred to new tubes and 200 μ L of silica solution is added. The tubes are then left on a rotator for 20 minutes at ambient temperature to bind DNA to the silica beads. The tubes are centrifuged for 2 minutes at 14,000xg, the supernatant discarded, and the pellet washed with 500 μ L ethanol wash (52 % ethanol, 4 M NaCl, 1 M Tris HCl, 0.2 M EDTA), this was repeated twice and then tubes centrifuged again and supernatants discarded. Then the tubes were transferred to a dry bath and incubated at 55 °C for 5 minutes with open caps. We then added 50 μ L of elution buffer (Tris 10 mM), and the pellet was resuspended by heavily vortexing the tubes. Then they were returned for another incubation at 55°C for 5 minutes and then centrifuged, the final supernatant was transferred to a new tube and DNA concentration measured using a NanoDrop™ ND-2000.

b) DNA amplification by Polymerase Chain Reaction (PCR)

Most of the amplifications were performed using PrimeSTAR® 2X PCR mix (Takara Bio), the mix contains PrimeSTAR HS high fidelity DNA polymerase. We typically used the following reaction mixture:

- DNA template 2 μ L
- Primers (10 μ M) 2 μ L each
- PrimeSTAR mix 25 μ L
- Ultrapure water 21 μ L

The PCR reaction is automated and comprises 5 steps:

- Initial denaturation for 30 seconds at 98°C (increased to 2 minutes if PCR on colony to lyse cells)
- Denaturation for 10 seconds at 98°C
- Hybridization or annealing of the primers for 30 seconds at a specified temperature T_m depending on the used primers. It is calculated according to the following equation; T_m (°C): $4[G+C] + 2[A+T]$, where G, C, A and T corresponds to the number of each nucleotide in both primers.
- Elongation at 72°C, for a defined time depending on the DNA polymerase and the size of the amplicon (5 to 10 seconds/kb for PrimeSTAR®)
- Final elongation for 10 minutes at 72°C

The denaturation, annealing and elongation steps (steps 2 through 4) are repeated 30 to 35 times, all the primers used are listed in the technical sheet.

c) Verification and purification of PCR products

Following each PCR, we verified the size and purity of the obtained DNA fragments by electrophoretic analysis on agarose gels. We prepared a gel containing 0.5 to 1.2% of agarose; in order to visualize DNA under ultraviolet light, we added ethidium bromide at a final concentration of 1 µg/mL. If the DNA band in question was destined for purification, we replaced ethidium bromide with SYBR Safe (Invitrogen) for visualization under blue light (to avoid DNA damage caused by ultraviolet radiation). The DNA migration is performed at 100 to 120V/gel for 15 to 30 minutes. In case of DNA purification, the desired DNA band was excised and purified using a QiaQuick Gel extraction kit (Qiagen) following the manufacturer's instructions.

After verification, the desired DNA fragments can be directly purified from remnants of the PCR reaction using columns containing a silica membrane, we used QiaQuick PCR purification kit (Qiagen) for this purpose.

d) Cleavage by restriction enzymes

The following reaction mixture was typically used:

- DNA to digest 0,5 to 5 µg
- Endonuclease 1U per µg of DNA
- Reaction buffer 2 µL
- Ultrapure water up to 20 µL

The enzymes were used according to the manufacturer's instructions (Fermentas or New England Biolabs). The most common procedure is incubation of the mixture for 2 hours at 37°C, then analysis on agarose gel. Finally, the reaction product must be purified to remove residual endonucleases (see purification of PCR product above).

e) Ligation of DNA fragments

DNA fragments were ligated with vector DNA (plasmid) using T4 or T7 DNA ligase (NEB). This reaction was performed at room temperature between 15 to 120 minutes depending on ligation type. The following mixtures were used:

- Insert + vector in a molar ratio 3:1
- DNA Ligase 1U
- Buffer 10X for T4 or 2X for T7 ligase

The reaction mixture is generally 20 µL or adjusted to 20 µL using ultrapure water

3. Bacterial transformation

In this study we used electroporation to transform both *E. coli* and *L. pneumophila* with desired plasmid constructs or heat shock for plasmid DNA/ligation mixtures in *E. coli*. However, we used natural transformation for chromosomal genetic manipulation in *L. pneumophila* via linear DNA fragments as mentioned in our published book chapter.

a) *E. coli* transformation

(1) Preparation of competent cells

From an overnight culture of the desired strain for example DH5-Alpha, we inoculate 200 mL of LB (with appropriate antibiotics if necessary). The cultures are placed at 37°C under agitation and we

monitored the bacterial growth by measuring the optical density OD_{600nm} until it was between 0.4 and 0.6. The flasks were then put on ice and then the cells collected by centrifugation at 8000xg for 15 minutes at 4°C. The pellets were then washed with 50 mL cold sterile water and centrifuged (this step is performed twice). Finally, the pellets were resuspended in an equal volume of cold 20% glycerol solution, 100 µL aliquots were prepared and conserved at -80°C.

(2) Electroporation of competent cells

Previously prepared aliquots were thawed gently on ice. Plasmid DNA to be transformed or DNA ligation mixture (10 to 100 ng) was mixed with the thawed cells, this was then transferred to a cold electroporation cuvette (Eurogentec, 2 mm gap). This cuvette was subjected to an electric field (2500 V, 10 µF, 600 Ω) in an Eppendorf® electroporator 2510. Directly after this step, 900 µL of LB medium is added to the cells and the tubes incubated at 37°C for 1 hour for the cells to recover and express the antibiotic resistance genes. Using sterile glass beads, 100 µL of this culture is spread on LB agarose medium (with appropriate antibiotic) for selection of the introduced plasmid. The plates are then incubated for 16 to 24 hours at 37°C.

(3) Heat shock of competent cells

Similar to electroporation, competent cells aliquots are thawed on ice and mixed with DNA to be transformed. The cells were then left on ice for 30 minutes where DNA will adhere to cell walls, they are then placed at 37°C for 2 minutes (cells will absorb the DNA), the cells are returned on ice for 5 minutes. Then 900 µL of LB were added and cells placed at 37°C for 1 hour for the cells to recover. Using sterile glass beads, 100 µL of this culture is spread on LB agarose medium (with appropriate antibiotic) for selection of the introduced plasmid. The plates are then incubated for 16 to 24 hours at 37°C.

b) *L. pneumophila* transformation

(1) Preparation of competent cells

From bacterial stock, fresh *L. pneumophila* cultures are prepared by inoculating BCYE agar and incubating the plates at 37°C for 3 days. A large number of bacteria is collected using a 10 µL inoculation loop and then resuspended in 1 mL cold sterile water. After centrifugation at 6000xg for 10 minutes at 4°C, the cells were washed with 1 mL of cold sterile water (This step was repeated 3 times). The cells were finally suspended in an appropriate volume of 10% sterile glycerol solution. 100 µL aliquots are finally prepared and conserved at -80°C until usage.

(2) Electroporation of competent cells

An aliquot of cells is thawed on ice, then mixed with the desired amount of plasmid DNA. This mixture is then transferred to cold electroporation cuvette (2 mm) and subjected to an electric field (2400 V, 25 μ F, 400 Ω) in a Gene Pulser® II (Bio-Rad). Immediately after the electric shock, we add 900 μ L AYE medium and place the tubes at 30°C for 1 to 2 hours. 200 μ L of this culture is then spread (using disposable cells spreaders) on BCYE agar supplemented with the appropriate antibiotics. The plates are then incubated at 30°C for 3 days.

(3) Natural transformation

From freshly prepared *L. pneumophila* on BCYE agar plates incubated at 37°C for 3 days, we inoculate 2 mL of AYE medium using a sterile loop. Then from this *Legionella* suspension, we prepare three tubes of 5 mL AYE at OD_{600nm} 0.0125, 0.025 and 0.05. We incubate these tubes at 37°C with shaking for 12 to 18 hours. Following bacterial growth, we measure the OD_{600nm} and choose the culture tube with optical density between 1.1 and 1.5. This tube is centrifuged at 4000xg at room temperature, 3 mL of the supernatant is removed, and the cells resuspended in the remaining volume. The purified DNA fragment is then added (300 ng to 1 μ g) and homogenized with the cells. The culture is then incubated for 8 to 24 hours at 30°C without shaking. 400 μ L of this culture is then spread on BCYE agar plates containing the appropriate antibiotics and incubated for 2 to 5 days at 37°C.

4. Biochemistry techniques

a) Protein overproduction in *E. coli*

An overnight culture of the bacterial strain expressing the desired recombinant protein is used to inoculate 50 mL LB broth supplemented with appropriate antibiotics at an OD_{600nm} of 0.05. when the OD_{600nm} reaches 0.5, we transfer 1 mL of this culture to an Eppendorf tube and leave it on ice, this will be the uninduced protein sample for SDS-PAGE analysis. Recombinant protein expression is then induced by adding IPTG at a final concentration of 1 mM. The culture is then incubated at 37°C for 2 to 3 hours with agitation. A second 1 mL sample is then transferred to a microcentrifuge tube that will constitute the induced protein sample. The remainder of the culture is then centrifuged at 10,000xg for 15 minutes, the supernatants discarded, and the pellet washed with sterile water. A final centrifugation is then performed, and the pellets stored at -20°C pending protein purification.

b) Protein purification

- I. For 6xHistidine tagged proteins in native conditions, cell pellets were thawed on ice for 15 minutes and then resuspended in 5 mL cold lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole). The cells are then lysed by passing through a French pressure cell at 20,000 psi. The obtained lysate is centrifuged at 10,000xg for 30 minutes at 4°C, the recombinant protein is usually present in the supernatant.

We used 50 to 100 µL of Talon® metal affinity resin (Takara Bio Inc USA) to bind 6xHis tagged proteins, they were washed twice using the lysis buffer and added to the previously obtained supernatants. The tube was then left on a rotator for 30 minutes at room temperature for the binding to take place. The obtained mixture is then transferred to a purification column (by gravity) and the flow through was collected. Then we washed the loaded beads three times with 2 mL washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole) and the fractions were preserved separately. For the elution, we used 200 µL elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole), also this was repeated three times and the fraction kept separately. Finally, we suspended the beads in 200 µL elution buffer and all the collected fractions were analyzed by SDS-PAGE.

- II. Under denaturing conditions for 6xHis tagged proteins, we used Ni-NTA Agarose (Qiagen). First, we thawed the cell pellet for 15 minutes on ice and re-suspended it in buffer B (100 mM NaH₂PO₄, 10 mM Tris.Cl, 8 M Urea, pH 8) at 5 mL/gram of wet weight. Then we rotate the suspension for 1 hour at room temperature to completely lyse the cells. Centrifuge lysate at 10,000xg for 20-30 min at room temperature to pellet the cellular debris. During this step, the agarose has to be prepared for binding following this procedure:

- a. Transfer 1 mL of beads to a 2mL microcentrifuge tube
- b. Centrifuge at 1000xg for 1 minute
- c. Wash with 800 µL buffer B
- d. Centrifuge again and discard supernatant
- e. Wash again

We then added 1 mL of 50% Ni-NTA slurry to 4-5 mL of lysate and rotate for 1 hour at room temperature. Load lysate-resin mixture carefully into an empty column (Ni-NTA Superflow, Qiagen). Collect the flow through. Wash twice with 4 mL buffer C (100 mM NaH₂PO₄, 10 mM Tris.Cl, 8 M Urea, pH 6.3). Elute the recombinant protein four times with 0.5 mL buffer D (100 mM NaH₂PO₄, 10 mM Tris.Cl, 8 M Urea, pH 5.9) then another four times with 0.5 mL buffer E (100 mM NaH₂PO₄, 10 mM Tris.Cl, 8 M Urea, pH 4.5). the collected samples are then analyzed by SDS-PAGE.

III. For GST tagged proteins, the pellets were thawed on ice for 15 minutes and then resuspended in 5 mL cold lysis buffer (PBS 1mM PMSF [phenylmethylsulfonyl fluoride], pH 7.4). The cells are then lysed by passage through a French pressure cell at 20,000 psi. The obtained lysate is centrifuged at 10,000xg for 30 minutes at 4°C, the recombinant protein is also usually present in the supernatant.

We used 50 to 100 µL of GST affinity resin (Protino® Glutathione Agarose 4B; Macherey-Nagel, Germany) to bind GST tagged proteins, they were washed twice using the lysis buffer and added to the previously obtained supernatants. The tube was then left on a rotator for 30 minutes at room temperature for the binding to take place. The obtained mixture is then transferred to a purification column (by gravity) and the flow through was collected. Then we washed the loaded beads three times with 2 mL washing buffer (PBS 1mM PMSF) and the fractions were preserved separately. For the elution, we used 200 µL elution buffer (Tris 50 mM [tris(hydroxymethyl)aminomethane], Glutathione 10mM), also this was repeated three times and the fraction kept separately. Finally, we suspended the beads in 200 µL elution buffer and all the collected fractions were analyzed by SDS-PAGE.

c) GST and 6xHis tag pulldowns

Both pulldowns were carried out in a similar manner. The bait proteins either GST or 6xHis tagged, were kept attached to their respective beads and not eluted. On the other hand, an approximately equal amount of the prey protein(s) was added to the loaded bait beads and incubated with gentle rotation at room temperature for 1 hour. Subsequently, we carried out a regular protein purification depending on the nature of the tag as mentioned above. The obtained fractions (flow through, washing, elution) were analyzed by SDS-PAGE to assess protein interaction.

d) *In vitro* LapG cleavage of RtxA N-terminus

RtxA^{NH2} (nucleotides 1-1490 from *rtxA* lpp0699) was cloned into a pET-30 plasmid upstream a 6xHis Tag using NdeI/SalI restriction sites to insert the DNA fragment. The constructed plasmid allowed for production of N-terminal fragment of 505 amino acids including 6 histidine with a molecular mass of 53.2 kDa. In brief, the constructed plasmid was transformed into *E. coli* strain BL21 by electroporation. Recombinant protein was produced by a 2-hour induction with 1 mM IPTG of an exponential culture that reached an OD_{600nm} of 0.5. Cells were collected and broken using a French pressure cell (20,000 Psi). The recombinant protein was purified using Talon® metal affinity resin (Takara Bio USA Inc). Similarly, *L. pneumophila* LapG was produced in *E. coli* BL21 by cloning nucleotides 169-735 (lpp0890) using BamHI/PstI restriction sites to insert the DNA fragment in a pQE-30 plasmid

downstream a 6xHis tag. The recombinant protein (22.5 kDa) was purified as described earlier. To assess the protease activity of *L. pneumophila* LapG on RtxA, the purified proteins were co-incubated for 3 hours at 37°C in the presence of 40 mM CaCl₂ and 80 mM MgCl₂. The hydrolyzed fragments were observed using SDS-PAGE. We then sequenced the first 7 amino acids of the cleaved RtxA^{NH₂} (C fragment) by Edman degradation.

e) Protein analysis by SDS-PAGE and western blot

(1) Polyacrylamide gel electrophoresis (SDS-PAGE)

This procedure is used for protein analysis by migration in a denaturing polyacrylamide gel driven by an electric field. Prior to loading, protein samples are mixed 1:1 with a loading buffer (2x) composed of:

- Tris-HCl pH 6.8
- Glycerol 20%
- SDS 4%
- β-Mercaptoethanol 0.1M
- Bromophenol blue 0.005%

The mixture of samples and cracking buffer is heated for 10 minutes at 100°C, then loaded on a polyacrylamide gel containing SDS, as described by Laemmli (1970). After migration, the proteins are stained using Coomassie blue.

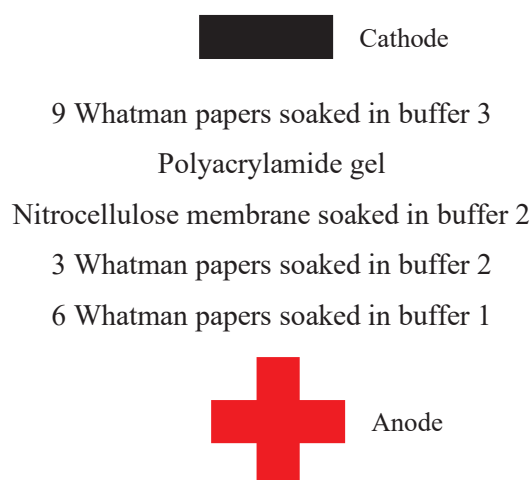
(2) SDS-PAGE for *L. pneumophila* whole cell extracts

Regarding gel electrophoresis with *L. pneumophila* cell lysate, we prepare a bacterial suspension in 1 mL ultrapure water from *Legionella* plate cultures. Calculate using OD_{600nm} the volume of suspension needed to pellet 5x10⁸ cells. The centrifugation is carried out at 10,000xg for 5 minutes, supernatant is discarded. The pellet is resuspended in 50 µL ultrapure water then the cells are lysed by heating for 15 minutes at 95°C. Centrifuge again at 10,000xg for 5 minutes and transfer the supernatants to a new tube, the lysates are then mixed with an equal volume of cracking buffer (2x), heat the sample for 10 minutes at 100°C then proceed with SDS-PAGE.

(3) Immuno-revelation by western blot

In this procedure, proteins migrated by SDS-PAGE are transferred to a nitrocellulose membrane under the effect of an electric current. This is carried out via a semi-dry transfer of polypeptides, for this purpose we use 3 buffers; buffer 1 (Tris HCl 300 mM, methanol 20%), buffer 2 (Tris HCl 25 mM, methanol 20%) and buffer 3 (Tris HCl 25 mM, EACA 40 mM, methanol 20%).

We used Whatman® 3MM chromatography paper to “sandwich” the polyacrylamide gel and nitrocellulose membrane according to the following layout.



The transfer takes place from cathode to anode under constant electric current; 0.8 mA/cm² of gel for 60 to 90 minutes, or 0.2 mA/cm² overnight. To verify the transfer, we can optionally stain the membrane with Ponceau red dye then rinse with de-ionized water.

After the transfer is complete, nonspecific binding sites are blocked by incubating the nitrocellulose membrane with 15 mL TBS 3% BSA (Tris HCl 50 mM, NaCl 150 mM, 3% Bovine serum albumin (Albumin Fraction V, Roth)). The membrane + blocking solution is subjected to gentle agitation for 1 hour at room temperature. After removing the blocking solution, the membrane is incubated in TBS 0.1% Tween 20 (TBST) 1 % BSA in the presence of primary antibodies, it is also agitated for 1 hour at room temperature or overnight at 4°C. The membrane is then washed 3 times with 15 mL TBST and then incubated with TBS 0.1% Tween 20 (TBST) 1 % BSA in the presence of secondary antibodies. After that, we wash the membrane twice with TBST and a final wash with TBS. Revelation is performed using luminol based SuperSignal™ West Pico chemiluminescent substrate kit (Thermo).

5. Protein interaction via bacterial two-hybrid system

For this purpose, we used the Euromedex BACTH (Bacterial Adenylate Cyclase-based Two-Hybrid) system kit. This represents a fast approach to detect protein-protein interaction *in vivo* (*E. coli*). Briefly, this system is based on interaction-mediated reconstitution of adenylate cyclase activity in *E. coli*. The desired proteins are fused to T18 and T25 fragments which constitute the catalytic domain of adenylate cyclase (*cyaA*). Heterodimerization of these hybrid proteins restores *cyaA* activity leading to production

of cAMP which consequently binds to the catabolite activator protein (CAP), this complex regulates gene transcription in *E. coli*.

In *E. coli*, the expression of *lacZ* gene encoding β -galactosidase is positively controlled by cAMP/CAP. Hence, bacteria expressing interacting hybrid proteins will form blue colonies on LB rich medium in the presence of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside 40 μ g/mL).

Standard molecular biology techniques described above were used to insert the desired *L. pneumophila* genes into the “bait” and “prey” vectors, pKT25/pKNT25 and pUT18/pUT18C. The constructed plasmids were produced in *E. coli* DH5-Alpha strain, purified then confirmed by enzymatic digestion. Since most chosen proteins are theorized inner membrane proteins, bioinformatics prediction tools (TMpred, TMHM) were used to predict their orientation. The desired genes were then fused N or C-terminally to the T18 or T25 fragments of the previously mentioned plasmids in a way that maintains these fragments in the cytoplasm after expression. Suitable combinations of the constructs were co-transformed into the reporter strain *E. coli* BTH101 (*cya-99*) then plated on LB-X-Gal medium containing 25 μ g/mL kanamycin and 50 μ g/mL ampicillin in addition to 1 mM IPTG to increase β -galactosidase expression. Positive controls were GCN4 leucine zipper motifs cloned into pKT25 and pUT18C. Negative controls comprised co-transforming a plasmid carrying the gene of interest along with a suitable empty vector with no insert to eliminate the possibility of cross reactions. The co-transformants were streaked onto the LB-X-Gal medium and incubated for 1 day at 30°C followed by 2 days at room temperature. Transformants with successful protein interaction were blue, otherwise they remained white.

6. Co-immunoprecipitation assays

a) Cross-linking of infected cells

A. castellanii cultures were grown in flasks for three days before infection. The cells were counted and then pelleted through centrifugation at 720xg for 10 min at 20°C. The pellet was suspended in PY medium for a final concentration of 2×10^7 cells/mL. *L. pneumophila* str Paris was added to the solution at MOI 10. Infection was carried out at 30°C during 30 min or 1 h.

Infection of macrophages was carried out in the Petri dish used for their differentiation from monocytes. *L. pneumophila* str Paris solution was added to the plate at MOI 10 and the plate was then kept at 37°C for 30 min or 1 h at CO₂ of 5 %.

Infected cells were centrifuged for 10 minutes at 25°C at 720xg for amoebae and 300xg for macrophages. The pelleted cells were cross-linked with a 1% formaldehyde-stabilized solution and

quenching was done with a solution of glycine 2,5 M. Cells were washed with PBS 1X pH 7.4, pelleted and then suspended in 600 µL of IP Lysis/Wash Buffer (Pierce® Kit) for lysis with a Fast-Prep beader (MP Biochemicals).

b) Co-IP assays

Cell extracts from lysed infected cells were clarified using a Pierce® Crosslink Immunoprecipitation Kit column (ThermoFisher). Antibodies targeting the N-terminus or C-terminus of RtxA were coupled to the A/G protein resin from the Pierce® Kit and preserved in a solution containing 0.02% azide. Co-immunoprecipitation assays were performed after incubating the clarified cell lysates with one of both antibodies coupled to the resin. All was done according to the manufacturer's instructions.

7. Genome editing in *Legionella pneumophila*

The principle behind this procedure is to construct by PCR a linear DNA fragment consisting of a selection cassette for homologous recombination with a targeted region of the *L. pneumophila* chromosome, this is followed by another recombination using a second construct to remove the first cassette after proper selection.

A detailed description for producing clean *L. pneumophila* mutants using natural transformation is previously detailed in our published book chapter.

8. Phenotypic studies of *Legionella pneumophila*

a) In vitro growth of *L. pneumophila*

From *L. pneumophila* cultivated on solid media for 3 days at 37°C, we prepare bacterial suspensions in AYE broth at 1×10^5 CFU/mL. 100 µL of these suspensions are transferred in triplicate to a 96 well plate (black, flat and clear bottom; Greiner Bio-One). The plates were then incubated at 30° or 37°C in a multi-mode plate reader (Tecan Infinite F200 pro) and the OD_{595nm} is measured every hour for at least 3 days. Also, the fluorescence of *L. pneumophila* harbouring pXDC50 can be measured by supplementing the suspensions with IPTG to induce mCherry expression.

b) Amoeba plate test

The following protocol is based on the original method described by (Albers *et al.*, 2005). *Acanthamoeba castellanii* are detached from the flask by incubation on ice for 15 minutes followed by

gentle tapping. They are then counted by a hemocytometer, centrifuged for 10 minutes at 4°C and 4000xg and the pellet washed with 10 mL PY medium to remove traces of antibiotics. Then they are centrifuged again, and the pellet resuspended in a volume of PY medium to reach 3×10^6 Cells/mL. 3.5 mL of this suspension is then spread on BCYE agar (12cm x 12cm square plate) and spread using a cell spreader. The plate is left for 2 hours for the suspension to dry and create a carpet of amoebae. During this time, we prepared bacterial suspensions of *L. pneumophila* at OD_{600nm} 1, these suspensions are used to create serial 10-fold dilutions until 10^{-8} . 3 µL of each suspension is then spotted on the amoebae carpet (avoid touching the agar), after drying at room temperature for 20 minutes, the plates are incubated at 30°C for 3 to 5 days until bacterial colonies are observed.

c) Microscopic observation of *L. pneumophila* infection of Amoebae

As described earlier, *A. castellanii* cells, were washed then seeded into a 24-well tissue culture treated microplate (Greiner CELLSTAR®, Germany) at 1×10^5 cells/well and left to adhere for 2 hours at 30°C. The infection medium used here is a special PY medium described above (PY special) to prevent all extracellular growth of *L. pneumophila*. The bacteria were cultured for 3 days at 37°C on BCYE agar containing appropriate antibiotics. 24 hours prior to infecting the amoebae, *L. pneumophila* are transferred to new BCYE plates containing IPTG to induce mCherry expression (pXDC50 plasmid). Bacterial suspensions are prepared at a final concentration necessary to infect amoebae at a multiplicity of infection (MOI) of 0.1. After inoculation, it is important to note that we did not centrifuge the plate as many protocols recommend, we skipped this step to challenge the *Legionella* infection process. Plates were then incubated at 30°C and microscopic observation performed daily to follow the progress of infection. To further challenge the infection process, we repeated the same procedure described above at MOI 1 but with supplementing the infection medium with 1×10^7 *E. coli* MG-1655/well (i.e. 100x *Legionella*) as a source of nutrients for *A. castellanii*.

d) Infection by *L. pneumophila* in a multimode plate reader (fluorescence)

Regarding *A. castellanii*, cells were washed then seeded in a 96-well tissue culture treated microplate (Greiner) at 1×10^5 cells/well and left to adhere for 2 hours at 30°C. The medium used for infection is the bacteriostatic PY special described earlier. 24 hours prior to infecting the amoebae, *L. pneumophila* are transferred to BCYE plates containing IPTG to induce mCherry expression. By measuring the OD_{600nm}, we prepared bacterial suspensions in PY special (plus chloramphenicol and IPTG) at a final concentration 5×10^5 CFU/mL. 200 µL of these suspensions are used to inoculate the wells in order to infect the amoebae at MOI 1. The plates were then incubated at 30° (Amoebae) or 37°C (U937 cells) in

a multi-mode plate reader (Tecan Infinite F200 pro) and fluorescence corresponding to bacterial growth (mCherry; excitation: 560 nm, emission: 635 nm) measured every 60 minutes for 3 to 4 days.

For U937 monocytes, 1×10^5 cells were seeded in each well and left to differentiate into mature macrophages for 2~3 days. Same MOI was used as above but the infection medium in this case was a CO₂ independent medium (GIBCO®) supplied with L-glutamine (2 mM).

Protection against infection using Anti-RtxA antibodies: to test the efficiency of Anti-RtxA^{COOH} antibodies in hindering the virulence of *L. pneumophila*. The procedure is the same as described above. However, we incubated different concentrations of purified anti-RtxA^{COOH} with the bacterial suspensions for 1 hour prior to inoculation.

e) Immunofluorescence microscopy of *L. pneumophila* RtxA

The purpose of this experiment is to localize RtxA C-terminus, whether if it's on the cell surface or released. For this experiment we modified a procedure based on a previous protocol (Buddelmeijer *et al.*, 1998). The first step was preparing glass coverslips by washing them in Ethanol/HCl solution (1M) for 1 hour then coating with poly-L-lysine (Sigma P8920). The following steps were carried out at room temperature (RT). *Legionella* cells were normalized to OD_{600nm} 1.0 and 300 µl of this suspension were spread on the previously prepared cover slips, then left to adhere for 30 minutes. The excess suspension was then gently aspirated and replaced with 3.7% formaldehyde and left 30 minutes to fix the cells. After rinsing with phosphate-buffered saline (PBS pH 7.0), non-specific sites were blocked by 3% bovine serum albumin (BSA) solution (PBS 3% BSA) for 30 minutes. After blocking, the slides were washed again with PBS and 100 µl (1:10000 or 0.374 µg/mL) of primary antibody (rabbit anti-RtxA^{COOH}) were added to the cover slips followed by incubation for 1 hour. After several washes with PBS, 50 µl of fluorescence conjugated secondary antibody (Alexa Fluor® 568 goat anti-rabbit, Invitrogen Inc. USA) were incubated with the cover slips for 1 hour in the dark. The slips were then washed twice and mounted on glass slides with 20 µl mounting medium (Mowiol®+DAPCO) (Sigma-Aldrich, USA) then cells were visualized using an epifluorescence microscope (Thermo Fisher EVOS FL, USA).

f) Immunofluorescence infection microscopy of *L. pneumophila*

Infection experiments were carried out mainly in 96 well plates (Greiner CELLSTAR®, Germany). Regarding U937 cells, 1×10^5 monocytes were seeded in each well and left to differentiate into mature macrophages for 2~3 days. Macrophages were infected at a MOI of 10 with bacterial suspensions made by dilution of late-stationary phase cultures of *L. pneumophila* strains; the infection medium used was

RPMI-1640 + 10% FBS. The plate was then left for 20 minutes at 37°C. As for *A. castellanii*, 1x10⁵ cells/well were seeded and left overnight at 30°C, *L. pneumophila* were prepared as described before. Also, the infection medium in this case is the modified bacteriostatic proteose-yeast extract medium (PY special). After inoculation, the plate was left for 20 minutes at 30°C for amoebae and 37°C for U937 cells in order for the infection to proceed. The following steps were common for both cell types and were carried out at room temperature. The infection was stopped then we added 250 µl of 4% paraformaldehyde (PFA) solution and left it 30 minutes to fix the cells. After washing with phosphate-buffered saline (PBS pH 7.0), we added 250 µl PBS + 0.1% glycine to reduce background fluorescence. Non-specific sites were blocked by PBS + 3% bovine serum albumin (BSA) solution for 1 hour. The wells were washed again and 100 µl (1:10000 or 0.374 µg/mL) of primary antibody (rabbit anti-RtxA^{COOH}) were added and left to incubate for 1 hour. After several washes with PBS, 50 µl of red or green fluorescence conjugated secondary antibody (Alexa Fluor® [594 for *A. castellanii* and 488 for U937 cells] goat anti-rabbit antibodies; Invitrogen Inc. USA) were added and the plates were left in the dark for 1 hour. Finally, we washed the wells twice with PBS and the cells were visualized using an epifluorescence microscope (EVOS® FL; Thermo Fisher, USA).

9. Sequences searches and alignments and Phylogenetic studies

Most *L. pneumophila* DNA and protein sequences were obtained from the Legiolist server (<http://genolist.pasteur.fr/LegioList/>). *rtxA* sequence was obtained by PacBio next generation sequencing.

Homologous proteins sequences were searched on NCBI website (<https://www.ncbi.nlm.nih.gov/>) using Blastp software. Proteins of interest (one per species identified) were downloaded as Fasta files to perform further analysis. Alignment of RtxA cutting site regions was done using Jalview software (version 2.10.5; (Waterhouse *et al.*, 2009)). Phylogenetic trees were inferred using maximum-likelihood with PhyML 3.0 software online pipeline (<http://phylogeny.lirmm.fr/>; (Dereeper *et al.*, 2008)).

10. Statistical analysis

Protection against *L. pneumophila* infection using anti-RtxA antibodies were performed in triplicate and the results were displayed as mean values ± standard errors of the mean. Differences in protection efficiencies were evaluated by ordinary two-way ANOVA for analysis of variance followed by Tukey's multiple comparisons test of significance for means. These analyses were performed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. P value less than 0.05 were considered statistically significant.

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Type 1 secretion system in *Legionella pneumophila*: substrate localization and role during the infectious cycle

Legionella pneumophila is the causative agent of a form of pneumonia called legionellosis or Legionnaires' disease. Between 2012 and 2015, the reported European cases of legionellosis increased from 5,848 to 7,069 cases per year where France, Germany, Italy and Spain accounted for 69% of the reported cases. Worryingly, the case fatality of incidents was 8.2% making this disease a considerable health concern. One virulence factor produced by this bacterium is a large protein (~700 kDa) belonging to the RTX (Repeats in ToXin) family called RtxA secreted by the type 1 secretion system.

The hereby work reveals that, *in vitro*, LapG periplasmic protease cleaves RtxA N-terminus in the middle of a di-alanine motif (a.a. 108-109). We also show using *lapG* and *lapD* mutant strains, that RtxA release is controlled by these two proteins similar to *Pseudomonas fluorescens* LapA. We observed that a strain lacking LapG protease maintains RtxA on the cell surface, while a strain lacking LapD does not exhibit cell surface RtxA. Interestingly, we identified the presence of homologous potential T1SS/LapDG systems in many *Legionella* species and other Gammaproteobacteria.

Regarding *L. pneumophila* virulence, our work showed that mutants for *L. pneumophila* T1SS (*lssBD/tolC*) were more disruptive to its virulence than *lapG/lapD* mutants. We also hypothesize, by challenging infection, that *L. pneumophila* might be actively targeting its host via RtxA. Additionally, by observing *rtxA* mutants as well as detecting RtxA on host surface briefly after inoculation and attenuating virulence by using anti RtxA antibodies, we assume an important but not limiting role for this protein in the infection process.

Key words: *Legionella pneumophila*; virulence; type 1 secretion system; RTX protein.

Système de sécrétion de type 1 chez *Legionella pneumophila* : localisation de son substrat et rôle lors du cycle infectieux

Legionella pneumophila est responsable d'une forme de pneumonie, la légionellose ou de maladie du légionnaire. Entre 2012 et 2015, les cas annuels ont grimpé de 5848 à 7069 en Europe, la France, l'Allemagne, l'Italie et l'Espagne correspondant à 69% du total. De façon inquiétante, la mortalité était de 8,2% faisant de cette maladie un réel enjeu de santé publique. Un facteur de virulence produit par cette bactérie est la protéine RtxA (~700 kDa) de la famille des protéines RTX (Repeats in ToXin) sécrétée via un système de sécrétion de type 1.

Dans ce travail, *in vitro*, la protéase périplasmique LapG clive la partie N-terminale de RtxA au sein d'un motif di-alanine (position 108-109). La construction de mutants déficients dans l'expression de LapG et LapD a révélé une localisation de RtxA sous le contrôle de ces deux protéines, mécanisme semblable au modèle LapA décrit chez *P. fluorescens*. Un mutant $\Delta lapG$ maintient RtxA à la surface de cellules, à l'opposé d'un mutant $\Delta lapD$. Nous avons identifié des systèmes homologues T1SS/LapDG dans de nombreuses espèces *Legionella* ainsi que d'autres gammaproteobactéries.

Concernant la virulence de *L. pneumophila*, les mutants déficients pour le T1SS (*lssBD/tolC*) étaient plus altérés dans leur virulence que des mutants du système LapDG. Nous avons également montré, grâce à des expériences de compétition, que *L. pneumophila* semble cibler les cellules hôtes via la protéine RtxA. L'utilisation d'anticorps spécifiques anti-RtxA nous a permis de détecter RtxA à la surface des cellules hôtes, mais aussi de réduire de la virulence de *L. pneumophila*, suggérant un rôle important de RtxA lors du processus d'infection, bien que non limitant.

Mots clés : *Legionella pneumophila* ; virulence ; système de sécrétion de type 1 ; Protéine RTX.