Protein secretion in *Legionella pneumophila* and its relation to virulence

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Abstract

Protein secretion is a universal process of fundamental importance for various aspects of cell physiology including the infection of a host organism by a bacterial pathogen. Many Gram-negative pathogens export virulence-associated proteins across one or two cell membranes to their place of action using a wide plethora of secretory pathways with the objective of infecting the host. For *Legionella pneumophila*, a facultative intracellular, human pathogen which is ubiquitously found in natural and artificial aquatic environments, two major secretory pathways known to be involved in virulence have been described. These are the PilD-dependent Lsp type II secretion pathway and the type IV secretion system encoded by the *dot/icm* genes. In addition, a second type IV system, with high sequence similarity to the *Agrobacterium tumefaciens* VirB system for conjugal transfer of oncogenic DNA, is present. Albeit dispensable for intracellular growth, this type IV system is important for efficient host cell infection at lower temperatures. Further more, evidence exists for the presence of at least one type I secretion system in *L. pneumophila* as well as for the presence of a twin arginine dependent translocation (Tat) pathway. This is a recently detected, signal peptide-dependent, secretion pathway complementary to the well-known Sec-dependent pathway for protein transport across the cytoplasmic membrane.

Keywords: *Legionella pneumophila*; Protein secretion; Virulence

1. Introduction

In order to establish a successful infection, bacterial pathogens have to subvert cellular processes of their hosts allowing efficient colonization and evasion of the host immune response. This pathogen–host interaction is especially based on factors that are located on the bacterial surface or are secreted into the extracellular environment. In this respect, protein secretion systems play a crucial role by delivering these effector molecules to their place of action. Protein secretion in bacteria is a remarkably complex biological process that involves substrate recognition followed by delivery of the substrate across the membrane barrier. As much as 30% of the genome codes for proteins that associate with, integrate into or are translocated across membranes [1]. In Gram-negative bacteria, proteins have to cross both the cytoplasmic membrane and outer membrane during their journey across the bacterial cell envelope in order to reach the extracellular environment. To achieve export of virulence factors such as pili, degradative enzymes, adhesins and toxins, bacteria have evolved numerous complex multimeric transporter systems. For a general overview of the different protein secretion systems in Gram-negative pathogens we refer to the comprehensive reviews of Lee and Schneewind [2] and Yen et al. [3]. Some of these pathways transport proteins across the bacterial cell envelope in one single step (e.g., type I or ABC protein mediated transporter and the
contact-dependent type III transporter). Others (type II, type IV and the autotransporters) comprise two steps with a periplasmic intermediate. However, not all of the secretory pathways are present in one organism. The scope of this minireview is to give a survey of the different secretion systems present in the human pathogen Legionella pneumophila. Attention will be given in particular to pathways with a demonstrated functionality. The role of these pathways in the virulence process or cell physiology and their possible substrates will be discussed briefly. In addition, the putative presence of other secretion systems based on homology analysis will be discussed in view of its potential importance with respect to the infection capacity.

2. Legionella pneumophila

L. pneumophila is the causative agent of Legionnaires’ disease in man. It is a Gram-negative facultative intracellular parasite of fresh water amoebae, monocytes and alveolar macrophages. L. pneumophila is an inhabitant of natural and man-made fresh water environments, where it is either present in a biofilm-associated state or where it can reside and proliferate as an intracellular pathogen of various protozoa. Industrial settings and the use of technical devices such as air conditioning systems, whirlpools and spas, showers, dental unit water lines and other medical devices, vegetable misters and fountains together with a larger population of elderly and immuno-compromised persons have led to clearly increased infection rates. L. pneumophila has nowadays become an important cause of both community acquired and nosocomial pneumonias. Susceptible individuals acquire the disease upon inhalation of contaminated aerosols. When L. pneumophila cells reach the lungs, the bacteria are phagocytosed by the alveolar macrophages and replicate to high numbers within a specialised vacuole capable of evading the endosomal degradation pathway. Ultimately host cell death and lysis as well as the extracellular action of bacterial degradative enzymes result in damage of the lung tissue.

For L. pneumophila two major secretory systems known to be involved in pathogenesis have been described. These are the Lsp type II pathway and the type IV secretion system encoded by the dot/icm genes. In addition, a second type IV pathway exists with a high sequence similarity to the Agrobacterium tumefaciens VirB system. Furthermore, evidence exists for the presence of a type I secretion apparatus. These pathways are described below. It is noted here that by sequencing the genome of the endemic L. pneumophila strain Paris, evidence was found for the presence of a type V auto-transporter secretion. The system is absent in the L. pneumophila Philadelphia strain demonstrating that the presence of a particular secretion system can be strain specific [4]. Knowledge of protein transport machineries for transport across the cytoplasmic membrane in L. pneumophila is also accumulating.

3. Protein transport across the cytoplasmic membrane

Most secretory proteins are produced as preproteins with a typical tripartite aminiterminal signal peptide, necessary for targeting the preprotein to the secretion apparatus, which is cleaved off after translocation across the cytoplasmic membrane. For signal peptidase-dependent protein transport across the cytoplasmic membrane two different pathways have been described. While for Sec-dependent transport substrate proteins are translocated in an unfolded state [5], the Tat pathway catalyses the movement of folded protein substrates across the inner membrane [6].

Analysis of the developing L. pneumophila database shows the presence of all sec genes known so far except secG (unpublished data).

In addition, we identified and isolated the tat genes (tatA, tatB and tatC) of L. pneumophila. TatA and tatB are cotranscribed, while tatC is situated elsewhere on the chromosome. Reverse transcription PCR experiments showed that the different tat genes are transcribed under standard culturing conditions as well as upon intracellular growth in Acanthamoeba castellanii [7].

Tat-dependent precursors typically carry two arginine residues in a consensus motif at the boundary of the positively charged N-region and the weakly hydrophobic region of their signal peptide. A preliminary screening for putative L. pneumophila Tat substrates resulted in a list of 20 different proteins. Current investigations aim at determining the exact role of the L. pneumophila Tat translocase and its substrates with respect to virulence as, in general, it is becoming increasingly clear that the Tat system is involved in the virulence of various human and plant pathogens such as A. tumefaciens, Rhizobium leguminosarum and Pseudomonas aeruginosa.

Both for Sec- and Tat-dependent translocation, following transport across the cytoplasmic membrane, the typical signal peptide is removed by the action of a membrane-bound endopeptidase, called the signal peptidase. The mature protein is then released at the other side of the membrane. Recently, we isolated and characterised the type I signal peptidase (lepB) gene of L. pneumophila [8]. The lepB gene is also transcribed when the cells grow intracellularly. As for most Gram-negative bacteria, the L. pneumophila lepB gene seems to be unique and its function was found to be essential for viability, as proved by specific inhibitor studies. These observations could make the L. pneumophila LepB an interesting new target for antibacterial therapy.
4. Protein transport across the outer membrane

4.1. Type II secretion

Type II secretion seems to be typically associated with organisms that colonize surfaces. In most cases these organisms do not invade cells. *L. pneumophila* is the only intracellular pathogen found to date with a known type II secretion system. Secretion via the type II secretion pathway, often called the general secretory pathway (GSP), occurs in two membrane translocation steps that can be separated both genetically and biochemically [9]. Following Sec- or Tat-dependent translocation across the inner membrane, protein translocation across the outer membrane occurs in a subsequent step via the main terminal branch (MTB) of the GSP by means of the type II secretion apparatus or the secreton [10]. Two groups described the existence of type II protein secretion in *L. pneumophila* based either on the discovery of the prepilin peptidase (PilD) encoded by the *pilBCD* locus [11] or on the presence of the *lsp* genes encoding conserved components of type II machineries in other bacteria. Besides being involved in adhesive type IV pilus biogenesis and assembly, PilD promotes type II secretion by processing pseudopilins which assemble into a functional type II secretion apparatus. The analysis of a PilD knock out mutant has shown that PilD is necessary for intracellular growth in both amoebae and macrophages. Since inactivation of type IV pilus biogenesis has no severe impact on intracellular growth, the growth defect of a PilD mutant was completely attributed to the defect in type II secretion. *L. pneumophila* has analogs for most of the genes that have been implicated in type II secretion of other bacteria [13]. These are encoded on 5 regions scattered over the chromosome in contrast to most other bacteria where the type II genes are clustered in one or two regions. So far, only one type II secretion system was found in *L. pneumophila* by database analysis while for *P. aeruginosa* two complete and a third incomplete MTB system were found. *L. pneumophila* is the first intracellular pathogen containing a type II system for secretion of

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<th>Secretion type</th>
<th>Structural proteins</th>
<th>Substrates</th>
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<tr>
<td>Type I&lt;sup&gt;b&lt;/sup&gt;</td>
<td>LssB</td>
<td>ABC transporter transmembrane consensus, ATPase domain</td>
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<td></td>
<td>LssD in locus</td>
<td><em>E. coli</em> HlyD homolog</td>
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<td>Type II</td>
<td>PilD</td>
<td>Prepilin peptidase</td>
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<td>LspD</td>
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<td>LspF</td>
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<td>LspG,H,I,J,K</td>
<td>Pseudopilins</td>
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<td>LspL,M,C</td>
<td>Conserved components promoting secretion</td>
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<td>LepB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Type I signal peptidase</td>
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<td>TatA,B,C&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>Translocase for twin arginine dependent precursors</td>
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<tr>
<td>Type IVa</td>
<td>Lvh B2,3,4,5,6, 7,8,9,10,11,D4</td>
<td>Specific functions mostly unknown</td>
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<tr>
<td>Type IVb</td>
<td>24 Dot/Icm proteins in different gene loci</td>
<td>Specific functions mostly unknown</td>
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<sup>a</sup> The reader is referred to the text for references.

<sup>b</sup> Putative, functionality has not been proven yet.

<sup>c</sup> Secretion components responsible for translocation across the inner membrane are included here, although they can also play a role in type IV secretion pathways.

<sup>d</sup> Being a substrate has still to be proven.
degradative enzymes. The PilD dependent type II secretion machinery was shown to be involved in the secretion of various enzymes as outlined in Table 1 [12,14–18]. Nevertheless, with the exception of a mild growth defect in case of inactivation of the metalloprotease, none of these secreted activities were found to be essential for intracellular growth indicating that the absence of other yet unidentified type II substrates or functional redundancy of type II substrates are responsible for the growth defect observed for the Lsp and PilD mutants. The role of type II secretion in virulence was confirmed as recent animal studies revealed that type II secretion is involved in colonizing the mammalian lung [19]. Interestingly, the L. pneumophila type II secretion system was found to facilitate growth at lower temperatures [20].

4.2. Type IV secretion

Type IV protein secretion pathways have long been recognised as systems responsible for the conjugal transfer of plasmids. More recently it became clear that these systems are actually macromolecular transporters that carry nucleic acids, nucleic acid-associated proteins or proteins, not only across the bacterial cell envelope but in several also across the plasma membrane of the host cell, a feature shared by type III secretion systems. As generally accepted for type II secretion, type IV dependent proteins are assumed to be secreted in a two steps process starting with a Sec-dependent translocation across the cytoplasmic membrane resulting in a periplasmic intermediate. Whether Tat-dependent transport can also be the first step of a type IV secretion pathway, remains to be seen.

Type IV substrates can either be directly exported into the host cell or can be excreted in the extracellular environment. Type IV secretion systems have been divided into two subclasses [21]. Type IVA systems contain extensive homology to the virB operon encoded system of A. tumefaciens which delivers oncogenic nucleo-protein particles to plant cells. A protein conduit is formed between the pathogen and the host cell that ensures the efficient transfer of proteins or DNA. The L. pneumophila type IVA system contains 11 lvh (Legionella VirB homologs) genes homologous to genes of other type IV systems, only missing the virB1 homolog, and are arranged in a similar manner. The Lvh system was found to be dispensable for intracellular growth in the two hosts, HL-60-derived macrophages and A. castellanii [22], but was found to play an important role in host cell infection by L. pneumophila grown at 30 °C [23]. In addition, it functions as a conjugational DNA transfer system [22].

Probably the most important secretion system of L. pneumophila with respect to virulence is the type IVB secretion pathway encoded by the dot/icm genes. In contrast to the large number of type IVA systems only a limited number of type IVB systems are currently known [24]. The Dot/Icm apparatus is a macromolecular complex that comprises a large number of membrane proteins and several proteins predicted to have a cytoplasmic location. The Dot/Icm proteins are encoded by two separate pathogenicity regions on the chromosome. The first region carries icmXWV and dotABCD while the second region contains 17 genes, icmTSRQPONMLKEGCDJBF. Primer extension analyses indicated that these genes are probably organised in nine transcriptional units [25]. The system shares significant sequence similarity with the plasmid encoded conjugation system of IncI plasmids R64 and ColIb-P9. Most of the Dot/Icm proteins are predicted to reside in the bacterial membrane suggesting that they are structural components of a membrane bound transport apparatus. Except for a few proteins, the specific functions of most of the Icm proteins are not known. However, some interactions between Icm proteins could be determined. IcmR was suggested to act as a chaperone of IcmQ [26]. DotB, being a part of the type IV system, has extensive similarity to the PilT ATPase family of proteins found in bacterial type II systems [27]. A putative secretion system closely related to the L. pneumophila Dot/Icm system is now found in the intracellular pathogen Coxiella burnetii [28].

The complex role of the Dot/Icm system in cellular processes including virulence was recently reviewed by Molmeret et al. [29] and will not be discussed in detail here. Briefly, the Dot/Icm based type IV secretion system is absolutely required for the infection process of L. pneumophila. This system is not required for intracellular growth as such but plays a crucial role in creating a nutrient rich organelle which is no longer capable of entering the default lysosomal degradation pathway and supports intracellular multiplication. As this pathway is additionally involved in promoting the uptake of the bacterial cells by phagocytosis [30], in inducing caspase-3-dependent apoptosis of the host cells [31], in pore formation-mediated lysis of the host cell, and in subsequent egress of the bacteria from the host cells [32], it can certainly be considered as a key virulence system of L. pneumophila. This is the first example of the role of a type IV secretion system of a bacterial pathogen in the induction of apoptosis [33]. As for the Lvh system, the Dot/Icm system can mediate conjugal DNA transfer.

Although type IV secretion systems have been found in many pathogens, the number of identified type IV substrates is surprisingly small [34]. For the L. pneumophila Dot/Icm system much effort is underway to identify the proteins secreted by this system. Failure to efficiently identify effector molecules is presumed to be the consequence of their functional redundancy. This implies that inactivation of a single effector has no or
only a slight phenotypic effect as its function is taken over by another substrate molecule. DotA, a polytopic predicted inner membrane protein, surprisingly was found to be secreted into the culture medium in a Dot/Icm dependent manner [35]. It is hypothesized that the DotA protein may form a kind of channel in the host cell membrane. The secretion of two other substrates RaIF and LidA by *L. pneumophila* and their export across the membrane of *L. pneumophila*-containing phagosomes was shown to be Dot/Icm dependent as well [36,37]. While the exact biochemical function of LidA remains to be elucidated, its importance in maintaining bacterial cell integrity in the presence of the Dot/Icm complex could be demonstrated. RaIF is a guanine nucleotide exchange factor for the ARF (ADP ribosylation factor) family of proteins. By exchange of GDP for GTP, RaIF maintains ARF in an active membrane bound state in this way promoting ER recruitment by the phagosome. Both proteins were shown to be involved in the recruitment of vesicles during the biogenesis of the replicative vacuole but are not absolutely required for *L. pneumophila* intracellular growth. Therefore, additional Dot/Icm secreted proteins are believed to exist. Very recently, Luo and Isberg [38] identified a large number of additional substrates of the Icm/Dot system (Sid proteins) based on the observation that proteins that are targeted to mammalian host cells can also be transferred interbacterially. Database analysis showed no significant orthologs in other bacteria although many paralogs were found to be present in the *L. pneumophila* database. SidC secretion and translocation across the phagosomal membrane was confirmed to be Dot/Icm-dependent. Mutants affected in LidA, RaIF and the Sid proteins still show considerable growth confirming the possible functional substitution by other effector molecules and thus the functional redundancy of the substrates.

Even though all the *icm* and *dot* genes are part of one system required for intracellular growth and even though their promoters are probably recognised by the vegetative σ factor, it seems that they are subjected to a different regulation mediated by several regulatory factors [25]. As for example extracellular LidA could only be observed upon contact of *L. pneumophila* with macrophages and no secreted LidA could be detected in culture medium, it was supposed that the activity of the type IV secretion system is regulated. Some regulatory proteins such as RpoS, RelA and LetA have been shown to be involved in the regulation of *dot/icm* genes in an indirect manner [39]. In addition, regulation of the *icmR* gene encoding a putative chaperone of IcmQ, was found to be directly regulated by the CpxR response regulator which is additionally involved in the expression of two other *icm* genes [40]. Initial analysis of upstream DNA regions of the *dot/icm* genes showed the presence of 12 regulatory elements involved in controlling the expression of eight *dot/icm* genes and operons. It is clear that only the first steps were taken on the way to the unravelling of the complex cascade mechanism of *dot/icm* regulation.

5. One-step transport across both membranes

5.1. Type I secretion

Like type III secretion systems, type I secretion machineries, also called ABC protein mediated exporters, transport proteins such as toxins or proteases in a single step across both bacterial membranes directly from the cytosol into the external environment without the involvement of a periplasmic intermediate. Proteins secreted in this way carry a carboxyterminal secretion signal [41]. Recently, Jacobi and Heuner [42] described the first putative type I secretion system of *L. pneumophila*. The system is encoded by the *lssXYZABD* locus, which seems to be restricted to *L. pneumophila* strains. LssB and LssD show sequence similarity with type I secretion systems of *Vibrio cholerae* and *Salmonella* typhi. Homologs of ToIC, the outer membrane component of the E. coli I secretion system, are also present in the genome of *L. pneumophila*. Although, in various type I secretion systems, the target protein encoding gene is often located upstream of the gene coding for the ABC transporter, no possible substrate could be identified. As secretion of pore forming toxins is often type I dependent, the recent cloning of the *L. pneumophila* *rtxA* gene is of interest. RtxA was found to play a role in entry of and replication in protozoa and human macrophages [43,44]. Downstream of the *lss* locus, a signalling protein encoding gene, named *lssE*, has been identified but its exact role remains to be elucidated [42].

6. Conclusion

Knowledge of *L. pneumophila* secretion systems, their substrates and importance with respect to the virulence process has rapidly expanded during the last five years using a combination of biochemistry and molecular biology. The identified pathways seem to act basically similarly as occurring in other Gram-negative bacteria, but with different substrates and variations in the composing proteins of the secretion machineries. Therefore, much of the molecular details of the key components of the secretory apparatuses in *L. pneumophila* remain to be uncovered in order to understand the importance of protein secretion across both membranes and the assignment of specific virulence factors in infectivity and pathogenicity. As pathogenic bacteria must assemble and secrete virulence factors in order to interact with host tissues and cause disease, a better, in-depth knowledge
of the different protein secretion systems would allow the development of new strategies for interfering with the pathogenicity process. As for other bacteria the presence of various secretion pathways including some essential key enzymes such as the signal peptidase in case of type II secretion, opens up the possibility of designing novel drugs and compounds able to inhibit or interfere with given secretory mechanisms. Thus, the identification of many additional factors and regulatory processes remains a huge challenge for the future.

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References


