Mini-Review

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The role of protein secretion systems in the virulence of the intracellular pathogen *Legionella pneumophila*

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Legionella pneumophila is a Gram-negative facultative intracellular pathogen, which multiplies in protozoa in its natural environment and can cause Legionnaires' disease in man, following infection of alveolar macrophages. In each of the different stages of infection of host cells, virulence proteins need to be delivered to their specific place of action and therefore must cross two barriers: the inner and the outer membrane. To date, several specialized secretion machineries for transport of proteins across the inner and outer membrane have been identified in *L. pneumophila*. Most of these secretion pathways have been shown to affect the virulence of this pathogen. An overview will be given of all the secretion pathways and the proteins transported by these secretion systems identified so far, with special attention paid to those that play a role in the pathogenicity of *L. pneumophila*.

Legionella pneumophila

Legionella pneumophila is a Gram-negative, rod-shaped facultative intracellular pathogen. It is the causative agent of Legionnaires' disease, a very severe form of pneumonia, and can also cause Pontiac fever, a milder, flu-like, self-limiting disease. Legionellae mainly reside in freshwater environments but also in man-made aquatic systems, which are the major sources of infection. In its natural environment *L. pneumophila* multiplies primarily within protozoa, but it can also be associated with biofilms. Protozoa provide nutrients, protection in harsh environmental conditions and also make the bacteria more virulent. Infection takes place by inhalation of contaminated aerosols, followed by replication in human alveolar macrophages (reviewed by Albert-Weissenberger *et al.*, 2007).

After internalization into host cells, *L. pneumophila* resides in a phagosome that does not fuse with the lysosomes of the host cell and then recruits endoplasmic reticulum (ER)-derived vesicles and mitochondria from the host. In this vacuole, the bacteria are converted to a replicative form. When nutrients are depleted, the bacteria enter the transmissive phase and express virulence proteins, resulting in lysis of host cells and the initiation of a new infection round (reviewed by Steinert *et al.*, 2007). In each of these stages of infection, protein secretion is necessary in order to deliver virulence proteins to their specific place of action.

Protein secretion and its role in virulence

To establish a successful pathogen–host interaction, virulence proteins of the pathogen need to be transported

to the bacterial surface, to the extracellular environment or directly into the host cell. This is a challenge for Gram-negative bacteria because secreted proteins must cross the inner and outer membrane. For secretion across the inner membrane, proteins with an N-terminal signal peptide are transported by the Sec or Tat secretion machinery. During translocation the signal peptide is cleaved off by the membrane-bound signal peptidase. For extracellular secretion or direct secretion into host cells, various specialized secretion machineries (named type I–V) exist in Gram-negative bacteria, each responsible for the transport of a specific subset of proteins. These are guided to the correct secretion route based on the presence of different secretion signals. Not all Gram-negative bacteria possess all types of secretion systems (reviewed by Gerlach & Hensel, 2007).

Secretion systems in *L. pneumophila* have been studied extensively because of the essential role of protein secretion in bacterial infection. For transport across the inner membrane the Sec and Tat secretion systems have both been identified in *L. pneumophila*. A lot of attention has been paid to secretion systems for transport across the outer membrane: the type II PilD-dependent Lsp secretion system, the type IVB Icm/Dot secretion pathway and the type IVA Lvh system. In addition, two putative secretion systems have been identified: the type I Lss secretion machinery and a putative type V secretion system identified in *L. pneumophila* strain Paris. In this review we will give an overview of recent knowledge regarding these secretion systems, their protein substrates and their possible role in virulence. An overview of the structural proteins and substrates of the main secretion systems of *L.*

pneumophila is given in Table 1. A schematic representation of the main secretion machineries in Gram-negative bacteria that are also present in *L. pneumophila* is shown in Fig. 1. For details of the different components of these systems the reader is referred to the recent review of Gerlach & Hensel (2007).

Protein transport across the inner membrane

Proteins that need to be transported into or across the inner membrane are generally characterized by an N-terminal signal peptide that is cleaved off by a membrane-bound signal peptidase so that the mature protein is released in the inner membrane or into the periplasm. In *L. pneumophila* Philadelphia two different signal peptidases have been identified: the type I signal peptidase LepB, responsible for cleavage of Sec- and Tat-dependent signal peptides, and the type II signal peptidase LspA, responsible for the processing of signal peptides from lipoproteins. Based on RT-PCR experiments, it was shown that the *lepB* and *lspA* genes are also transcribed when bacteria grow intracellularly in host cells. The functionality of LepB and LspA was demonstrated and LepB was found to be essential for viability, as is the case for other Gram-negative bacteria (Lammertyn *et al.*, 2004; Geukens *et al.*, 2006). It has also been suggested that LspA plays a role in the pathogenicity of *L. pneumophila* because *in silico*-predicted lipoproteins seem to be particularly involved in protein secretion and motility (Geukens *et al.*, 2006).

For transport across the inner membrane, the Sec pathway is generally used. In *Escherichia coli* the main components of this secretion system are a membrane protein complex (the SecYEG translocase) that forms the secretion pore, a cytoplasmic membrane-associated ATPase (SecA) that provides the energy for transport, and a chaperone (SecB). However, also other proteins such as SecD and SecE and YajC can play a role in Sec secretion (reviewed by Gerlach & Hensel, 2007; see Fig. 1). Many Sec translocase subunits are essential for viability and are conserved in

evolution. However, the different *sec* genes are not present in all bacteria and also the essentiality of these genes is not conserved between organisms (van der Sluis & Driessen, 2006). Inspecting the genome of *L. pneumophila* Philadelphia, all *sec* genes except *secG* could be found (Lammertyn & Anné, 2004). The *Legionella* Sec pathway has not been further characterized yet.

In addition to the Sec secretion system, the twin-arginine translocation or Tat secretion pathway can be responsible for transport of proteins across the inner membrane. The Tat system, in contrast to the Sec system, allows transport of folded proteins that have the typical characteristic of bearing two arginine residues in their signal peptide. The exact mechanism of Tat transport is not fully determined yet but a complex of TatB and TatC proteins probably recognizes the Tat substrate, followed by transport through a pore consisting of multiple TatA proteins (reviewed by Lee *et al.*, 2006; see Fig. 1).

In *L. pneumophila* Philadelphia (De Buck *et al.*, 2005) and in *L. pneumophila* strain 130b (Rossier & Cianciotto, 2005) the *tatA*, *tatB* and *tatC* genes were identified. In *L. pneumophila* Philadelphia the *tat* genes are expressed in all growth phases and also when cells grow intracellularly in the host *Acanthamoeba castellanii*. Tat secretion in this strain was shown to play a role in intracellular replication in amoebae and macrophages, which is a first indication of its role in virulence. Furthermore, it is important for stress response, motility and biofilm formation. Several methods were used to identify putative Tat substrates and proteins that are important for the motility and virulence of *L. pneumophila*, such as the virulence protein phospholipase C PlcA, which was shown to be Tat dependent (De Buck *et al.*, 2005). Also an inner-membrane protein, the Rieske iron-sulfur protein PetA, was shown to be a Tat substrate. The Rieske iron-sulfur protein is an essential subunit of mitochondrial and bacterial *bc₁* complexes, which are central redox carriers in respiratory electron-transport chains, and therefore important for growth and viability. It

Table 1. Overview of the main secretion systems in *L. pneumophila*

References can be found in the text.

Secretion system	Structural proteins	Confirmed substrates
Type I Lss system*	LssX, Y, Z, A, B, D	No substrates identified yet
Type II Lsp system	Prepilin peptidase PilD, pseudopilins LspF, G, H, I, J, K, LspD, E, LspC, LspL, LspM	Zinc metalloprotease ProA/Msp, RNase, acid phosphatases, lipases LipA and LipB, phospholipase C PlcA, lysophospholipase A PlaA, phospholipase A PlaB, acyltransferase PlaC, chitinase
Type IVA Lvh system	LvhB2, 3, 4, 5, 6, 7, 8, 9, 10, 11, LvhD4	Plasmid DNA
Type IVB Icm/Dot system	IcmV, W, X, DotA, B, C, D, IcmT, S, R, Q, P, O, N, M, L, K, E, F, C, D, J, B, F, H	Plasmid DNA, RalF, LidA, LepA and LepB, SidC, SidG, SidH, SidJ and DrrA (SidM), SidE paralogues: SdjA, SdeA and SdeC, SidH paralogue: SdhA, VipA, VipD and VipF, VipD paralogues: VpdA and VpdB, YlfA and YlfB, WipA and WipB, LegL3, LegLC4, LegL5, LegL7, LegLC8, LegC5 and LegG2, Ceg10, Ceg23 and Ceg29

*Functionality of this secretion system has not been shown yet.

Fig. 1. Schematic representation of the main secretion systems in Gram-negative bacteria that are also present in *L. pneumophila* (adapted from Gerlach & Hensel, 2007; for detailed information on the different components of the secretion machineries see this reference). OM, outer membrane; IM, inner membrane; CM_{host}, cytoplasmic membrane of host cell; OMP, outer-membrane protein; MPF, membrane fusion protein; ABC, ABC transporter. The symbol represents a β -barrel pore.

was concluded that the Tat pathway is necessary for the correct membrane insertion of this protein (De Buck *et al.*, 2007).

For *L. pneumophila* 130b, the Tat pathway was also shown to play a role in intracellular replication in differentiated U937 cells, but a decrease in intracellular replication in the amoeba *Hartmannella vermiformis* was observed only under iron-limiting conditions. The secretion of the proteins PlcA and PetA was also experimentally confirmed to be Tat dependent (Rossier & Cianciotto, 2005).

Protein transport across the outer membrane

The putative type I Lss secretion system

A type I secretion system can transport proteins in one step across both membranes and consists of three proteins: an inner-membrane ATPase called the ABC (ATP-binding cassette) transporter, a membrane fusion protein spanning the periplasmic space and an outer-membrane protein (reviewed by Gerlach & Hensel, 2007; see Fig. 1).

In *L. pneumophila* a putative type I secretion system named Lss has been identified, based on the identification of a homologue of an ABC transporter. Using Southern blots, the distribution of the *lss* genes was analysed in various *Legionella* strains and it was suggested that a complete *lss* gene cluster is restricted to *L. pneumophila*. Normal expression of the putative *lss* operon was shown by

RT-PCR (Jacobi & Heuner, 2003). A further characterization of this secretion system and its substrates has not been carried out yet and it remains to be determined if this secretion system plays a role in virulence.

The type II PilD-dependent Lsp secretion system

Type II secretion is a two-step process in which transport across the inner membrane is first performed by the Sec or Tat machinery, based on the type of signal peptide of the protein substrates. The proteins then enter the type II machinery, consisting of a multi-subunit complex inserted in the inner membrane and a ring-shaped complex with a central cavity in the outer membrane (reviewed by Gerlach & Hensel, 2007; see Fig. 1).

To date, a type II secretion system in an intracellular pathogen has only been identified in *L. pneumophila* and *Yersinia* sp., which can also have an intracellular phase of survival and growth in macrophages (Cianciotto, 2005). The *L. pneumophila* type II secretion system was first identified based on the presence of PilD, a homologue of the *Pseudomonas* prepilin peptidase that is necessary for the biogenesis of type IV pili and a functional type II secretion system. A *pilD* mutant showed a decrease in replication in amoebae and macrophages. This growth defect was attributed to the absence of type II secretion, because the inactivation of type IV pilus biogenesis has no impact on intracellular growth (Liles *et al.*, 1998). The type II

secretion apparatus itself is encoded by the *lspFGHIJK*, *lspDE*, *lspC*, *lspL* and *lspM* genes (*Legionella* secretion pathway). *lspDE* encode the outer-membrane secretin LspD and the ATPase LspE, *lspF* encodes the inner-membrane protein LspF, *lspGHIJK* encode the pseudo-pilins LspGHIJK, and *lspC*, *lspL* and *lspM* encode conserved components of type II secretion pathways. A decrease in replication in both amoebae and macrophages was observed for single *lspDE*, *lspG*, *lspK* and *lspF* mutants. In addition, the important role of this secretion system in the virulence of *L. pneumophila* was confirmed because type II secretion was shown to play a role in the multiplication and survival of *L. pneumophila* in mammalian lungs (Rossier *et al.*, 2004). Both type IV pili and the type II secretion system also play a role in the colonization and establishment of biofilms by *L. pneumophila* (Lucas *et al.*, 2006).

Several enzymes have been shown to be type II substrates: the major zinc metalloprotease ProA or Msp, an RNase, acid phosphatases, the lipases LipA and LipB, the phospholipase C PlcA, the lysophospholipase A PlaA, the phospholipase A PlaB and the acyltransferase PlaC. None of these type II substrates play a role in intracellular growth in host cells. The proteins ProA, Map acid phosphatase and PlaA were recently also identified in a 2D protein analysis as proteins that were absent or greatly reduced in a type II secretion mutant. Additional differential proteins with a predicted signal peptide were aminopeptidases, an RNase, a chitinase and several proteins with no homology to known proteins. The chitinase was shown to play a role in intracellular replication in the lungs of A/J mice (DebRoy *et al.*, 2006).

Transcription of *pilD* increases at temperatures below 37 °C and thus the type II secretion system promotes growth at lower temperatures (Liles *et al.*, 1998). Information on the regulation of type II secretion is scarce, but recently it was shown that the global regulators LetA and RpoS regulate the secretion of phospholipase A, lysophospholipase A and acyltransferase. Because no other type II substrates or type II-dependent activities were affected, these are probably not direct regulators of the type II machinery (Broich *et al.*, 2006).

The type IVA Lvh and type IVB Icm/Dot secretion systems

The type IV secretion system was first defined based on the *Agrobacterium tumefaciens* T-DNA transfer system (Vir system or type IVA system) and the IncP and IncN bacterial conjugation systems (Tra system or type IVB system). The type IV secretion machinery consists of a channel and a pilus for association with the recipient cell, because the type IV system can also transport proteins into the target cells (reviewed by Gerlach & Hensel, 2007; see Fig. 1).

In *L. pneumophila* a type IVA secretion system named the Lvh (*Legionella vir* homologues) system is present. This secretion system is encoded by 11 *vir* homologues (*lvhB2*, *B3*, *B4*, *B5*, *B7*, *B6*, *B8*, *B9*, *B10*, *B11*, *D4*), situated in a

region with an elevated G + C content, suggesting acquisition from another source (Segal *et al.*, 1999). Interestingly, in *L. pneumophila* strains Philadelphia, Paris and Lens this *lvh* region is encoded in a region that either occurs integrated in the chromosome or can be excised as a plasmid-like element (Steinert *et al.*, 2007). In addition to the *vir* region, five other genes were found in its proximity and named *lvr* genes (*Legionella vir* region). The function of these genes is unknown except for LvrC, which is homologous to the *E. coli* carbon storage regulator CsrA and has been suggested to be a regulator of the expression of the *lvh* genes. However, there is no experimental evidence for this hypothesis (Segal *et al.*, 1999). The Lvh system is important for efficient host cell infection by *L. pneumophila* grown at 30 °C, suggesting a role in environmental conditions (Ridenour *et al.*, 2003). Based on PCR experiments it was shown that *lvh* genes are present in several *L. pneumophila* patient isolates, in contrast to environmental isolates, suggesting a correlation between the presence of these genes and virulence (Samrakandi *et al.*, 2002).

The Icm (intracellular multiplication)/Dot (defective organelle trafficking) system is a type IVB system and is encoded in two pathogenicity regions containing seven genes (*icmV*, *W* and *X* and *dotA*, *B*, *C* and *D*) and 18 *icm* genes (*T*, *S*, *R*, *Q*, *P*, *O*, *N*, *M*, *L*, *K*, *E*, *G*, *C*, *D*, *J*, *B*, *F* and *H*) (reviewed by Segal & Shuman, 1998; Albert-Weissenberger *et al.*, 2007). The specific functions of the Icm/Dot proteins are not known, although some interactions and complexes have been identified. IcmR was identified as a chaperone for IcmQ, which is a pore-forming protein. In addition, the cytosolic components IcmS and IcmW make up a stable complex that facilitates the translocation of substrate proteins. Recently, a subcomplex consisting of five Icm/Dot proteins that bridges the inner and outer membrane was also identified (Vincent *et al.*, 2006b).

Most of the *icm/dot* genes play a role in intracellular replication of *L. pneumophila* in amoebae and macrophages and also in guinea pig infection (Segal & Shuman, 1999; Edelstein *et al.*, 1999). The Icm/Dot system is the secretion system of *L. pneumophila* that is probably the most important for the virulence of this pathogen. The Icm/Dot system plays critical roles in the promotion of phagocytosis, creating a nutrient-rich organelle that escapes the lysosomal degradation pathway, maintaining the integrity of the *L. pneumophila* phagosome, the induction of apoptosis and lysis of the host cells (Molmeret *et al.*, 2007).

To date, type IVB protein substrates have only been identified for the *L. pneumophila* Icm/Dot secretion system (Segal *et al.*, 2005). Some Icm/Dot substrates possess a C-terminal translocation signal for substrate recognition. Several substrates show increased expression in the early stationary growth phase, suggesting an important role in the infectious phase of the growth cycle. Substrates that probably play a role in creating a phagosome that evades the lysosomal degradation pathway are VipA and VipD.

The substrates RalF, LidA and DrrA more likely play a role in the recruitment of ER vesicles. RalF maintains the host ADP ribosylation factor Arf1 in an active state promoting ER recruitment. LidA plays a role in maintaining bacterial cell integrity and possibly also in recruitment of ER vesicles. DrrA is a guanine nucleotide-exchange factor for the host GTPase Rab1 that regulates the vesicular transport processes. Proteins that might be involved in the prevention of early apoptosis are SdhA and SidF. Substrates playing a role in the last step of the infectious cycle are LepA and LepB, which are functionally involved in the release of *Legionella* from protozoa after intracellular multiplication. Several other Icm/Dot substrates, such as the Leg and Ceg proteins, have also been identified, but their precise function is not known. For a list of all known Icm/Dot substrates, the reader is referred to Table 1 and to the recent review of Ninio & Roy (2007). The majority of the Icm/Dot substrates have little or no effect on intracellular growth in host cells, probably because of the presence of several paralogues and functional redundancy (Ninio & Roy, 2007).

Little information is available on the regulation of Icm/Dot secretion. In the promoter region of some *icm* genes a specific binding site was found and the regulatory factors RpoS, RelA and LetA have been shown to exert an effect on the transcription of some *icm* genes. However, no direct binding between consensus motifs and regulators could be shown, so regulation probably takes place in an indirect manner. Upstream of the *icmR* gene a binding site for the response regulator CpxR was identified and direct regulation was demonstrated for the first time (Gal-Mor & Segal, 2003). Regulation of Icm/Dot secretion is the result of altered transcription not only of the *icm/dot* genes, but also of the genes encoding the Icm/Dot substrates. The expression of several Icm/Dot substrate-encoding genes with a conserved regulatory element is controlled by the *L. pneumophila* response regulator PmrA (Zusman *et al.*, 2007).

Regulation of Icm/Dot secretion is also possible at the protein level. The DotL protein has been suggested to play a role in controlling the opening and closing of the secretion pore. Assembly and/or activation of the Icm/Dot secretion complex would probably also be controlled by several other proteins (Vincent *et al.*, 2006a).

Recently, it was suggested that the Lvh system can functionally replace a defective Icm/Dot system under conditions mimicking aquatic and amoeba-encysted niches of *L. pneumophila* (Bandyopadhyay *et al.*, 2007).

In addition to the type IVA Lvh and type IVB Icm/Dot system, a new type IVA and several Tra-like (type IVB) systems have been identified in different *L. pneumophila* strains. The new type IVA secretion system, encoded on a genomic island named Trb-1, was recently identified in the *L. pneumophila* strain Corby as its genome sequence has now become available. On a 65 kb pathogenicity island that is unique to the Philadelphia strain, a type IVB

secretory system, homologous with the Tra proteins of plasmid F of *E. coli*, was identified (Steinert *et al.*, 2007). A type IVB secretion system was also found in *L. pneumophila* strain AA100, but not in the Philadelphia strain. This secretion system consists of homologues of the Tra proteins of the R751 plasmid of *Enterobacter aerogenes* and the RP4 plasmid of *E. coli* (Samrakandi *et al.*, 2002).

A putative type V secretion system

Type V secretion (or the autotransporter secretion system) is a two-step process in which the C-terminal domain of the substrate forms a β -barrel pore in the outer membrane for translocation of the passenger domain, following Sec-dependent transport across the inner membrane based on the signal peptide (reviewed by Gerlach & Hensel, 2007; see Fig. 1). Based on the identification of a gene encoding an autotransporter in *L. pneumophila* strain Paris, the presence of a putative type V secretion system has been proposed (Albert-Weissenberger *et al.*, 2007). The functionality and possible substrates of this secretion system have not yet been characterized.

Conclusion

Now that the genome sequence of several *L. pneumophila* strains (Philadelphia, Paris and Lens) has been completed, knowledge of the secretion systems of this bacterium is increasing very rapidly. Most secretion systems that are present in other bacterial pathogens have also been identified in *L. pneumophila*. Interestingly, *L. pneumophila* is the first and only intracellular pathogen with such an extensively studied type II secretion system. In addition, *L. pneumophila* is almost the only pathogen with a type IVB secretion system and only for *L. pneumophila* type IVB substrates have been identified.

It is remarkable that so many different secretion systems could be observed for different *L. pneumophila* strains. For example, different type IVB secretion systems are present in several *L. pneumophila* strains. A new type IVA system could only be found for *L. pneumophila* strain Corby and a type V secretion system could only be identified in *L. pneumophila* strain Paris. These differences could be explained by the remarkable genome plasticity that has been revealed from comparing the genomes of the sequenced *L. pneumophila* strains. Genomes of other *Legionella* strains are being sequenced at the moment and more information on this subject will become available soon.

In the future, it is to be expected that research will mainly focus on the identification of additional substrates of the known secretion systems, their role in the virulence process of *L. pneumophila* and on the – probably quite complex – regulation of secretion. This information will allow us to better understand the virulence mechanisms of *L. pneumophila*, which is critical on our way towards the development of novel compounds or strategies to better combat this pathogen.

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