Bacterial secreted proteins: secretory mechanisms and role in pathogenesis

Mickaël Desvaux, Michel Hébraud

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Listeria monocytogenes

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Mickaël Desvaux and Michel Hébraud

Abstract

As a monoderm prokaryote, protein secretion systems in *Listeria monocytogenes* are distinct from those encounter in diderm bacteria, still they remain the gates for expressing protein functions outside the intracellular bacterial cell compartment. Despite the fact that protein secretion is a key factor in virulence of a pathogen, fewer studies have been dedicated to pathogenic Gram-positive bacteria compared to Gram-negative bacteria and *L. monocytogenes* is no exception. Among the six protein secretion systems identified in *L. monocytogenes*, only proteins putatively translocated via the Sec pathway are indisputably involved in bacterial virulence. The 16 secreted virulence effectors characterized to date are either (i) associated with the cytoplasmic membrane, i.e. as integral membrane proteins or lipoproteins, (ii) associated with the cell wall, i.e. covalently in a sortase-dependent manner or via cell-wall binding domains, or (iii) released in the extracellular milieu. Identification of several candidates as putative secreted virulence factors as well as the availability in the near future of a large amount of *Listeria* genomic data from different sequencing projects promise a very exciting time in the field of listerial protein secretion and should provide further insights into how *L. monocytogenes* interacts with its biotic or abiotic surroundings.

Introduction

The genus *Listeria* is hitherto circumscribed to only six species namely *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii* and *L. grayi* (Vaneechoutte *et al.*, 1998); it is worth noting that the previously distinct species *L. murrayi* is now assigned to the single species *L. grayi* (Rocourt *et al.*, 1992). *L. ivanovii* is comprised of two subspecies, i.e. *ivanovii* and *londiniensis*, whereas the subspecies of *L. grayi* are subsp. *grayi* and subsp. *murrayi*. These Low G+C% Gram-positive bacteria belong to phylum BXIII *Firmicutes*, class III *Bacilli*, order I *Bacillales*, family IV *Listeriaceae* (Garrity, 2001). While it shares the same family with the closely related genus II *Brochothrix*, taxonomic analyses further revealed that the genus *Listeria* occupies an intermediary position in class III *Bacilli* between genus *Bacillus* (order I *Bacillales*, family I *Bacillaceae*) and genus *Lactobacillus* (order II *Lactobacillales*, family I *Lactobacillaceae*) (Jones, 1988). Phylogenetic analyses also suggest that the different *Listeria* species would have diverged only very recently (Collins *et al.*, 1991; Sallen *et al.*, 1996). *L. grayi* represents the deepest branch within the genus and the remaining species split into (i) a first lineage composed of *L. monocytogenes* and *L. innocua* and (ii) a second lineage composed of *L. welshimeri*, *L. ivanovii* and *L. seeligeri* (Schmid *et al.*, 2005). *L. monocytogenes* is the etiologic agent of listeriosis in human and animals, whereas *L. ivanovii* is almost only associated with infections in animals (Vazquez-Boland *et al.*, 2001b). The remaining *Listeria* species are apparently apathogenic, which would have resulted in the course of evolution from two independent deletion events of the virulence gene cluster as represented by *L. innocua* and *L. welshimeri*, respectively (Schmid *et al.*, 2005). Listerial strains were early serotyped according to variation of 15 somatic O (subtyped from I to XV) and five flagellar H (subtyped

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from A to E) antigens derived from methods employed on Gram-negative pathogen *Salmonella* (Seeliger and Hohne, 1979; Seeliger and Jones, 1986). Though, due to differences in cell envelope structure between Gram-negative and Gram-positive bacteria, O antigens could not be of lipopolysaccharidic nature in *Listeria* and serological identification could only result from cross-reactivity of antisera. Indeed, it appeared later on that the somatic component of the serotypic designation in *Listeria* resides primarily in teichoic acids, structurally and antigenically as a result of glycosidic substitutions of the ribitol phosphate units (Fiedler *et al.*, 1983; Uchikawa *et al.*, 1986). Concerning H antigens, it is worth noting that variation of flagella expression in different growth conditions can result in discrepant serological results (Peel *et al.*, 1988a; Peel *et al.*, 1988b). Altogether, 17 distinct serotypes (or serovars) have been established in *Listeria* spp. including 13 for *L. monocytogenes*, i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Molecular subtyping analyses further divided *L. monocytogenes* into three major phylogenetic divisions, with prevalent distribution of serotypes (i) 1/2b, 3b, 3c, 4b, 4d and 4e in lineage I, (ii) 1/2a, 1/2c, and 3a in lineage II, and (iii) 4a, 4b and 4c in lineage III (Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997; Nadon *et al.*, 2001; Ward *et al.*, 2004; Chen *et al.*, 2007). Importantly, while good correlation (though not absolute) between these three monophyletic lineages and most common serotypes could be ascertained, serotype 4b could be found in both lineages I and III, indicating this serotype could not be used for lineage attribution since such *L. monocytogenes* do not represent a distinct evolutionary group (Ward *et al.*, 2004). Determining irrefutably the serotype of some *Listeria* isolates can be very challenging as phenotypic characteristics may vary in function of environmental conditions. Therefore, these serological methods have now been largely overtaken by molecular methods in order to clearly identify and differentiate listerial strains (Liu, 2006), nonetheless serotyping information is still widely used in everyday practice.

Following an epidemic in animal care houses in Cambridge (UK), *Listeria monocytogenes* was discovered in 1924 by Murray *et al.* (1926); however, the final name of this bacterial species was coined by Pirie in 1940 and named after Lord Lister, a British surgeon, and the fact that an increase in the number of circulating monocytes, i.e. a monocytosis, was found in infected rabbits and guinea pigs (Gray and Killinger, 1966; Farber and Peterkin, 1991). The first case of human listeriosis dates back to 1929 in Denmark (Nyfelt, 1929). From an epidemiological point a view, human listeriosis must be considered as a rare disease as indicated by a low incidence with only 2–10 reported cases per million populations per year (Schuchat *et al.*, 1991; Ramaswamy *et al.*, 2007). In France and the USA, however, listeriosis is one of most frequent food-borne causes of death since it ranks only second after salmonellosis with a high mortality rate ranging between 20% and 30% (Mead *et al.*, 1999; Vaillant *et al.*, 2005). While overall case fatality rate is not as high in other European countries, listeriosis incidence has significantly increased between 2001 and 2005 in Germany as it increased from 0.26 per 100 000 inhabitants, i.e. 217 cases, to 0.62 per 100 000 inhabitants, i.e. 519 cases (Koch & Stark, 2006). Listeriosis essentially breaks out in weakened patients namely immunodepressed individuals, pregnant women, neonates and elderly (Vazquez-Boland *et al.*, 2001b). In healthy individuals, listerial infection can manifest as a more or less severe gastroenteritis (Swaminathan and Gerner-Smidt, 2007). Nonetheless, human listeriosis is a serious food-borne illness caused essentially by consumption of contaminated processed food such as soft cheeses, diary products, smoked fish, processed meats and delicatessen (Farber and Peterkin, 1991; Vazquez-Boland *et al.*, 2001b). The presence of *L. monocytogenes* in foodstuffs mainly results from its ability to survive and multiply under conditions frequently used for food preservation, i.e. low pH, low temperature, low *Aw* and high salt concentrations (Roberts and Wiedmann, 2003). In addition, *L. monocytogenes* also forms biofilms, which increases its persistence and resistance within industrial production chain lines (Chavant *et al.*, 2002, 2004) and might facilitate infection (Roberts and Wiedmann, 2003). It is worth stressing that *L. monocytogenes*, like other *Listeria* spp., is a chemoorganoheterotroph, ubiquitous in the environment where it lives as a saprophyte. The level of virulence is highly variable from

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one bacterial strain to another and there is no direct correlation between clinical isolates and those originating from the food chain. Indeed, listerial strains of serovar 4b are responsible of most serious cases of human listeriosis, i.e. essentially in outbreaks of invasive listeriosis (Farber and Peterkin, 1991; Goulet *et al.*, 2006; Swaminathan and Gerner-Smidt, 2007), but *L. monocytogenes* 4b are rarely isolated from food or from industrial environments (Aarnisalo *et al.*, 2003; Lukinmaa *et al.*, 2004; Thevenot *et al.*, 2005). Out of 13 serotypes only the three, 1/2a, 1/2b and 4b, account for more than 95% of human listeriosis cases (Gellin and Broome, 1989). *L. monocytogenes* is considered as a model of opportunistic intracellular pathogen with an infection cycle now well defined (Cossart and Mengaud, 1989; Cossart, 2002; Hamon *et al.*, 2006). This facultative intracellular pathogen is not only able to multiply in macrophages but in a wide variety of eukaryotic cells such as nonprofessional phagocytes, fibroblasts, hepatocytes, enterocytes, epithelial, endothelial or nerve cells (Vazquez-Boland *et al.*, 2001b). The first step of host cell invasion involves internalization of *L. monocytogenes* with concomitant formation of a vacuole. At this stage, the vacuole is lysed releasing the bacterium into the host cell cytosol where it then starts to replicate. By polymerizing host cell actin to form a tail, *L. monocytogenes* is then able to propel itself inside the cytosol and spread to neighbouring cells. Invasion of another eukaryotic cell involves formation of a double-membrane protrusion, which must be lysed to initiate a new infection cycle. At each stage of the cell infection, different listerial molecular determinants are implicated, in particular secreted virulence factors.

Relationships between protein secretion systems and virulence in *Listeria monocytogenes*: an overview

The ability of a bacterium to infect an host relies essentially on secretion of virulence factors (Finlay and Falkow, 1997; Lee and Schneewind, 2001). Early, proposal of research projects dealing with protein secretion of diverse bacteria was controversial in the scientific communities since some stubbornly favoured in-depth investigations in only one model bacterium, i.e. *Escherichia coli* (Salmond and Reeves, 1993; Wandersman, 1993). In the following years, though, investigating protein secretion systems in a wide range of Gram-negative bacterial pathogens finally proved to be wise and judicious as it has led to the discovery of at least six major protein secretion systems, which otherwise would not have been discovered. Indeed, such investigations indicated that these secretory pathways are never all systematically present in a single bacterium and that the number and function of secreted proteins varies hugely from one bacterium to another. In Gram-negative bacteria, these functionally independent systems with respect to outer membrane translocation mechanisms have been numbered from type I to type VI secretion system (T1SS to T6SS) (Blight *et al.*, 1994; Koster *et al.*, 2000; Thanassi and Hultgren, 2000; Stathopoulos *et al.*, 2000; Desvaux *et al.*, 2004; Henderson *et al.*, 2004; Kostakioti *et al.*, 2005; Economou *et al.*, 2006). In contrast to Gram-negative bacteria, research on protein secretion in Gram-positive bacteria has remained focused on a single and apathogenic microorganism used as a paradigm, i.e. *Bacillus subtilis* (Simonen and Palva, 1993; Tjalsma *et al.*, 2000; Van Wely *et al.*, 2001; Sharipova, 2002; Tjalsma *et al.*, 2004; Yamane *et al.*, 2004; Kostakioti *et al.*, 2005). Only scattered information can be found among some Gram-positive pathogenic bacteria namely *Bacillus cereus*, *Mycobacterium tuberculosis*, Group A *Streptococcus*, *Staphylococcus aureus* and *Clostridium difficile* (Tjalsma *et al.*, 2004; Desvaux and Hébraud, 2006). Since the cell envelope of Gram-positive bacteria is composed of only one biological membrane instead of the two found in Gram-negative bacteria, protein secretion only relies on translocation systems present in the cytoplasmic membrane and thus nomenclature in use is different from Gram-negative bacteria. Altogether, six major protein secretion systems are described in Gram-positive bacteria, namely (i) Sec (secretion), (ii) Tat (twin-arginine translocation), (iii) FEA (flagellar export apparatus), (iv) FPE (fimbrillin-protein exporter), (v) holins (hole-forming), and (vi) Wss (WXG100 secretion system). Following a rational approach for genomic analysis of Gram-positive protein secretion systems (Desvaux *et al.*, 2005), each

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of these six systems could be identified in *L. monocytogenes*, providing new insight in bacterial virulence (Desvaux and Hébraud, 2006) (Fig. 14.1).

Predicted and characterized protein secretion systems in *Listeria monocytogenes*

Few studies have been devoted to protein secretion systems per se in *Listeria*. Their identification essentially results from bioinformatic analyses (Desvaux and Hébraud, 2006). A critical and general issue in the research field of protein secretion is the actual proof of protein transport across membranes through a pore forming a translocon, i.e. a protein-conducting channel. Once such evidences have been generated in a given organism, e.g. for the Sec translocon in eukaryotic cell (Simon and Blobel, 1991), it is rarely demonstrated and verified again in other organisms even though scientific rigorousness would justify it. *Listeria* is no exception since protein translocation across each of the six systems identified following in silico analyses has not been as yet ascertained by experimental investigations.

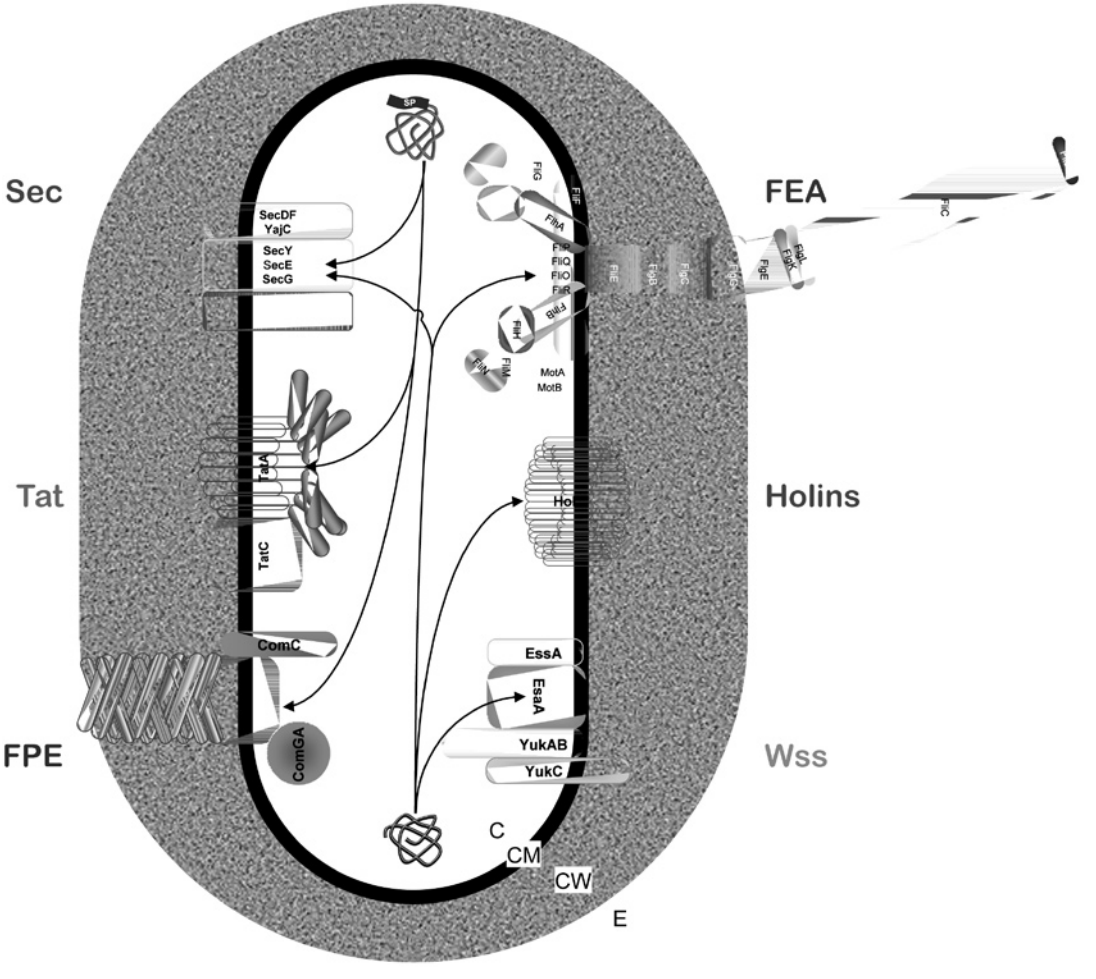

Figure 14.1 Protein secretion systems present in *Listeria monocytogenes*. Proteins bearing a N-terminal SP (at the top of the picture) can be translocated across the CM either in a Sec, Tat or FPE dependent manner. Proteins which do not exhibit a SP can be translocated via the FEA, Holins or Wss; nonetheless, some of the proteins lacking a N-terminal SP can use the Sec pathway. SP, signal peptide. C, cytoplasm; CM, cytoplasmic membrane; CW, cell wall; extracellular milieu; Sec, secretion; Tat, twin-arginine translocation; FPE, fimbrilin-protein exporter; FEA,: flagella export apparatus; Wss, WXG100 (proteins with WXG motif of ~100 amino acyl residues) secretion system.

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Sec, Tat and FPE allow secretion of proteins bearing an N-terminal signal peptide, whereas FEA, Holins and Wss are involved in translocation of proteins lacking a signal peptide (Fig. 14.1). Protein targeting to these latter systems is not clearly elucidated. Signal peptides allow targeting to cytoplasmic membrane translocators. In the course of translocation, they are generally cleaved off by signal peptidases. N-terminal signal peptides are composed of N-, H- and C-domains, i.e. an N-terminal hydrophilic domain, followed by an hydrophobic domain and a cleavage site. In the Sec pathway, three signal peptidases of type I (SPases I) have been uncovered, i.e. SipX (signal peptidase), SipY and SipZ (Bonnemain *et al.*, 2004; Raynaud and Charbit, 2005). Absence of SipY has no detectable effect on virulence, but SipX and SipZ have overlapping substrate specificities and are involved in secretion of key virulence factors (Bonnemain *et al.*, 2004). While FPE uses its own signal peptidase, i.e. ComC (competence), where cleavage occurs at the cytoplasmic side of the membrane between N- and H-domains (Dubnau, 1997), Tat substrates are cleaved off by SPase I (Sargent *et al.*, 2006). In *Listeria*, FPE substrates bear a conserved cleavage site with a motif [NPRS][GA]▼F[TS]L[VLP][EF] (Desvaux and Hébraud, 2006). Both Sec and Tat substrates are cleaved in the C-domain, but the latter is differentiate by the presence of a conserved twin-arginine motif straddling the N- and H-domains. Some Sec substrates exhibit a lipobox present in the C-domain (Tjalsma *et al.*, 2000). Signal peptides of such lipoproteins are cleaved off by another type of signal peptidase, i.e. SPase II. Two SPases II are encoded in *L. monocytogenes* EGDe, i.e. LspA (lipoprotein signal peptidase) and LspB (Desvaux and Hébraud, 2006), but only one, LspA, has been functionally characterized (Reglier-Poupet *et al.*, 2003a; Bonnemain *et al.*, 2004). In Gram-positive bacteria, some Sec substrates possess a YSIRK motif localized at the beginning of the H-domain that is required for efficient protein secretion (Bae and Schneewind, 2003). Such a motif is systematically associated with the LPXTG motif involved in covalent protein anchoring to cell wall. Finally, other Sec substrates exhibit uncleavable N-terminal signal peptides allowing protein anchoring to the cytoplasmic membrane.

Protein secretion with N-terminal signal peptide: Sec (secretion), Tat (twin-arginine translocation) and FPE (fimbrillin-protein exporter) pathways

All listerial virulence factors characterized so far are most certainly transported via the Sec system. Rather than the archaic terminology of general secretory pathway (GSP), the term Sec pathway is now clearly preferred as it is much less ambiguous and not as confusing (Desvaux *et al.*, 2004). Compared to the subset of proteins translocated via the five alternative secretion systems, Sec is by far the major pathway involved in protein secretion in *L. monocytogenes* (Desvaux and Hébraud, 2006). Translocation across the SecYEG-SecDF-YajC protein conducting channel is energized by the cytosolic ATPase SecA. From investigations in *E. coli*, proteins are known to be secreted via the Sec translocon in stepwise fashion where about 25 amino acid residues are translocated through each cycle of ATP binding/hydrolysis (Driessen *et al.*, 2001). As has also been observed in some other Gram-positive bacteria, especially pathogens, a SecA paralogue called SecA2 is present in *L. monocytogenes* (Lenz and Portnoy, 2002). Convergence of SecA and SecA2 routes into the Sec transcolon remains to be verified. On one hand, SecA2-dependent secretion is a promoter of bacterial pathogenesis in *L. monocytogenes* (Lenz *et al.*, 2003). On the other hand, SecA2 is also encoded in apathogenic *L. innocua* (Desvaux and Hébraud, 2006). Interestingly, some proteins secreted via SecA2 do not exhibit a recognizable signal peptide (Braunstein *et al.*, 2003; Dramsi *et al.*, 2004; Archambaud *et al.*, 2006). Two paralogues of YidC are present in *L. monocytogenes*, i.e. SpoIIIJ and YqjG (Desvaux and Hébraud, 2006). From investigations in *E. coli*, YidC is essential and required for integration of all membrane proteins (Froderberg *et al.*, 2003). The YidC pathway appears to be versatile as it can involve the Sec translocon or operate independently of it. Contrary to the situation in *B. subtilis* (Errington *et al.*, 1992; Tjalsma *et al.*, 2000; Van Wely *et al.*, 2001; Murakami *et al.*, 2002), YidC homologues have not been investigated in *Listeria* and thus their role in bacterial virulence remains to be elucidated.

Only 1 Tat substrate homologous to an iron-dependent peroxidase could be predicted in *L. monocytogenes* EGDe genome but its actual

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secretion has not been ascertained (Desvaux and Hébraud, 2006; Dilks *et al*., 2003). It can be further noticed that despite a high level of similarity (99%) with predicted Tat substrate Lin2304 from *L. innocua*, the orthologue Lmo2201, a putative β-ketoacyl-acyl carrier protein synthase II, is not predicted as a Tat substrate (Dilks *et al*., 2003). As in all Gram-positive bacteria, the Tat translocon in *Listeria* seems composed of TatA and TatC only (Dilks *et al*., 2003); though, TatB is an essential component in *E. coli* (Sargent *et al*., 2006). Contrary to the situation in *B. subtilis* (Tjalsma *et al*., 2000), only one copy of the genes encoding TatA and TatC are found in the same locus in *L. monocytogenes* genomes (Desvaux and Hébraud, 2006). The functionality and role of Tat pathway in listerial pathogenesis remain to be determined.

FPE is involved in translocation and assembly of a particular subset of secreted proteins, i.e. type 4 prepilins, involved in bacterial competence (Dubnau, 1997). As in *B. subtilis* (Chen *et al*., 2006), the five type 4 prepilins encoded in *L. monocytogenes* might be assembled into the cell wall to form a trans-wall structure rather than a true pilus (Desvaux and Hébraud, 2006). In *B. subtilis*, this macromolecular protein structure was named competence pseudopilus. Still, in listeria its presence remains to be experimentally established as well as functionality and role of FPE in pathogenesis if any. Notably, competence development, and thus DNA uptake by this process, has so far never been reported in *Listeria*.

Protein secretion without N-terminal signal peptide: FEA (flagella export apparatus), holin (hole-forming) and Wss (WXG100 secretion system) pathways

FEA is essentially involved in translocation and assembly of flagellar components (Macnab, 2003). In Gram-negative bacteria, FEA also allows secretion of virulence factors released freely in the extracellular milieu (Young *et al*., 1999); among Gram-positive bacteria, secretion of extracellular virulence factors via FEA has so far been reported only in *B. thuringensis* and *B. cereus*, namely haemolysin and phosphatidylcholine phospholipase C (Ghelardi *et al*., 2002; Ghelardi *et al*., 2007). Flagella are primarily involved in motility of *L. monocytogenes*, which is characterized by a tumbling motion (Galsworthy *et al*., 1990), but flagella expression is essentially regulated as a function of growth temperature (Peel *et al*., 1988b). Indeed, as *L. monocytogenes* can synthesize up to six peritrichous flagella at 20°C, few are expressed at 37°C, the temperature of infected host. It is worth noting though that strain to strain variation has also been reported. As a general feature of bacteria, listerial flagellin is recognized by the Toll-like receptor 5 in mammalian cells and thus activates innate immune system (Hayashi *et al*., 2001). Though, flagellin FlaA is not essential for *L. monocytogenes* pathogenesis (Way *et al*., 2004) it increases the efficiency of epithelial cell invasion (Dons *et al*., 2004; Bigot *et al*., 2005). Flagella do not enhance the adhesion of *L. monocytogenes* to targeted host cells but they do function as invasion factors (O'Neil and Marquis, 2006). Swarming ability, which is a specialized form of movement that enables flagellated bacteria to coordinately move atop solid surfaces, is distinct from the simple swimming ability conferred by flagella, which is an individual and non-cooperative movement (Henrichsen, 1972). Swarming would further explain the importance of flagella as motile determinants rather than adhesins in biofilm formation (Vatanyoopaisarn *et al*., 2000; Lemon *et al*., 2007). Control of flagella biosynthesis is rather complex as it involves at least five regulators, namely FlaR (flagellin regulator) (Sanchez-Campillo *et al*., 1995), PrfA (positive regulatory factor A) (Michel *et al*., 1998), DegU (degradation enzymes regulator) (Knudsen *et al*., 2004), MogR (motility gene repressor) (Grundling *et al*., 2004) and GmaR (glycosyltransferase and motility anti-repressor) (Shen *et al*., 2006). As PrfA and, in addition to regulating flagellar genes (Scortti *et al*., 2007), most of these regulators also control expression of virulence factors required for full virulence of *L. monocytogenes*. Interestingly, FlaA is glycosylated with β-O-linked *N*-acetylglucosamine via GmaR, which is bifunctional (Schirm *et al*., 2004; Shen *et al*., 2006). While flagella glycosylation is not essential for cell motility (Lemon *et al*., 2007), its importance in bacterial virulence remains to be investigated.

Holins are xenologues presumed to be of phagic origin (Fitch, 2000). They form homo-

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oligomeric pore complex in the cytoplasmic membrane enabling passive but specific protein translocation (Ziedaite *et al.*, 2005). Proteins secreted and activated by this system are essentially enzymes dedicated to cell wall degradation leading to cell autolysis (Wang *et al.*, 2000; Gründling *et al.*, 2001; Bayles, 2003). In *L. monocytogenes* EGDe, a prophage of the A118 family, coding notably for holin Hol118, is inserted in the gene *comK* (Loessner *et al.*, 2000; Desvaux and Hébraud, 2006). Some holins, however, like LmoO128 belonging to TcdE (toxin of *Clostridium difficile*) family, do not seem to be part of a cluster encoding for a lysogenic phage and thus would be suggestive of an ancient horizontal gene transfer in *Listeria*. The report of the putative autolysin Lmo0129 in bacterial supernatants (Trost *et al.*, 2005) provides the first clue that the holin pathway might be functional in *Listeria* (Desvaux and Hébraud, 2006). In *Clostridium difficile*, TcdE plays a key role in bacterial virulence since it is involved in secretion of two large bacterial toxins, i.e. TcdA and TcdB (Tan *et al.*, 2001; Mukherjee *et al.*, 2002). In apathogenic *L. innocua*, the number of encoded holins is higher than in pathogenic *L. monocytogenes*, which is correlated with a higher number of lysogenic phage (Glaser *et al.*, 2001; Desvaux and Hébraud, 2006). The functionality and involvement of each holin and their putative substrates in pathogenesis wait to be experimentally clarified in *Listeria*.

As indicated by its name, the Wss is dedicated to secretion of WXG100 protein, i.e. protein of about 100 amino acid residues, with a coil-coil domain and bearing a conserved WXG motif in their central region (Pallen, 2002a). The function of WXG100 proteins is currently unknown (Brodin *et al.*, 2004) but Wss is of crucial importance in bacterial pathogenesis in *Mycobacterium tuberculosis* (Converse and Cox, 2005) as well as *Staphylococcus aureus* (Burts *et al.*, 2005). In mycobacteria, this system is the focus of numerous research investigations aiming at developing new vaccines (Pym *et al.*, 2003; Williams *et al.*, 2005; Maue *et al.*, 2007). Indeed, part of this system is located in RD1 (region of difference 1) which is the only locus specifically deleted from vaccine strain *M. bovis* BCG (bacille Calmette–Guérin) (Philipp *et al.*, 1996; Pym *et al.*, 2002). Contrary to the situation in other bacteria where it has been identified, only one *wss* gene cluster was uncovered in *L. monocytogenes* (Desvaux and Hébraud, 2006). Comparing these gene clusters between *L. monocytogenes* and *S. aureus*, synteny appears very conserved and genes highly similar (Burts *et al.*, 2005). So far, functionality of listerial Wss has not been investigated. Though, in the sense it is required for the virulence of pathogens previously mentioned, it was surprising to find out that the WXG100 protein in *L. monocytogenes* is not essential for bacterial virulence (Way and Wilson, 2005). Importantly, a nearly identical *wss* cluster is also found in genome of apathogenic *L. innocua* (Desvaux and Hébraud, 2006).

Other protein secretion systems in *Listeria monocytogenes*?

None of ABC (ATP-Binding Cassette) transporters encoded in genomes of sequenced *L. monocytogenes* seems to be dedicated to secretion of proteins per se but, as a general feature of Gram-positive bacteria, to transport of short amino acid chains devoid of tertiary structure, i.e. peptides/oligopeptides (Desvaux and Hébraud, 2006). Genomic analyses failed to identify an MscL (large conductance mechanosensitive ion channel) homologue in *L. monocytogenes* (Desvaux and Hébraud, 2006). In Gram-negative bacteria, members of this protein family are involved in the release of small proteins upon osmotic shock but no evidence of a similar function in Gram-positive bacteria is as yet available (Ajouz *et al.*, 1998). Similarly, no Tad (Tight adherence) system seems to be encoded in listerial genome. In Gram-negative bacteria, this system related to T2SS allows secretion and assembly of Flp (fimbrial low-molecular-weight protein) pili (Kachlany *et al.*, 2001; Tomich *et al.*, 2007). Genomic analyses allowed the identification of this protein secretion system in several Gram-positive bacteria namely *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *M. bovis*, *Streptomyces coelicolor* as well as *Clostridium acetobutylicum* (Planet *et al.*, 2003; Desvaux *et al.*, 2005), though its expression in Gram-positive bacteria remains to be established. It should be stressed that from the apparent absence of some genes in a given bacterial genome it cannot be excluded that functional analogues or distant

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homologues are actually present; confirmation of true absence requires deeper experimental investigation.

Gram-negative bacteria release outer membrane vesicles (OMVs) enabling the delivery of proteins (Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006). As a general trend, OMVs would allow interactions and material exchanges between prokaryotic and eukaryotic cells in their environment. A trait which is rarely highlighted is that naturally occurring cytoplasmic membrane vesicles (CMVs) have been reported in diverse Gram-positive bacteria (Dorward and Garon, 1990; Mayer and Gottschalk, 2003; Klieve *et al.*, 2005). The composition and role of CMVs, however, have not been thoroughly investigated. Because the structure and biogenesis of bacterial cytoplasmic membranes and outer membranes are different, the mechanisms of formation of CMVs in Gram-positive bacteria and OMVs in Gram-negative bacteria is almost certainly distinct (Zhou *et al.*, 1998; Mayer and Gottschalk, 2003). Such membrane vesicles could constitute another type of protein secretion system or more exactly of bacterial excretion system as CMV or OMV proteins are either present into the vesicle lumen or the membrane but are not truly released into the extracellular milieu. Nonetheless, should the vesicle fuse with that of a target host cell, the proteins will truly be released into the cytosol of that cell. In Gram-negative bacteria, OMVs are produced by both pathogenic and apathogenic bacteria but are considered as potent virulence factors and as participating in biofilm formation (Kuehn and Kesty, 2005; Schooling and Beveridge, 2006). Presence of naturally occurring CMVs have been reported in closely related *Bacillus* spp. (Dorward and Garon, 1990), but their presence in *Listeria* spp. remains unknown. However, CMVs were early observed in *L. monocytogenes* following cell envelope fractionation procedures (Ghosh and Carroll, 1968).

In *Streptococcus pneumoniae*, secretion of the key virulence factor pneumolysin, which is devoid of a N-terminal signal peptide, was for some time puzzling. It recently appeared that pneumolysin is not translocated across the cytoplasmic membrane but actually released extracellularly following autolysis (Guiral *et al.*, 2005). More precisely, cell lysis occurred only in non-competent cells and was triggered by competent cells of the same species. This specific autolytic phenomenon was named allolysis, in opposition to heterolysis, a term reserved to describe the lysis of other species. Allolysis involves regulation of competence development and cell wall degradation mechanisms. It is then tempting to speculate that it can be somehow related to FPE and holins pathways. Selective lysis of siblings seems to be a complex and tightly controlled process used by different bacterial species where it contributes to pathogenesis by coordinating the release of intracellular virulence factors (Gilmore and Haas, 2005;Guiral *et al.*, 2005). Moreover, it could explain moonlighting of primarily cytoplasmic proteins on the pneumococcal cell surface such as glyceraldehyde 3-phosphate dehydrogenase or enolase, which then exhibits plasmin(ogen)-binding activity (Bergmann *et al.*, 2001; Bergmann *et al.*, 2004). The presence of such proteins with similar moonlighting functions have been reported in *L. monocytogenes* (Schaumburg *et al.*, 2004), although the occurrence of allolysis has not yet been addressed experimentally in *Listeria*.

Taking the example of the novel T6SS originally discovered in *Vibrio cholerae* in 2006 (Pukatzki *et al.*, 2006) and subsequently considered as a key virulence determinant in *Escherichia coli* (Dudley *et al.*, 2006), *Pseudomonas aeruginosa* (Mougous *et al.*, 2006) and *Burkholderia mallei* (Schell *et al.*, 2007), and encoded in several other pathogenic Gram-negative bacteria (Folkesson *et al.*, 2002; Moore *et al.*, 2002; Bladergroen *et al.*, 2003; Das and Chaudhuri, 2003), it cannot be ruled out that other protein secretion systems involved in bacterial pathogenesis will be uncovered in the near future in Gram-positive bacteria, including *L. monocytogenes*.

Secreted virulence factors in *Listeria monocytogenes*

In Gram-positive bacteria and, contrary what is sometimes wrongly assumed, the final location of a protein translocated across the cytoplasmic membrane by a protein secretion system is not inexorably the extracellular milieu. Indeed, such a secreted protein, i.e. a protein transported by a secretion system, can either (i) anchor to the

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cytoplasmic membrane, (ii) associate with the cell wall, (iii) be released into the extracellular milieu or (iv) be released beyond this, e.g. in the cytosol of an infected eukaryotic cell. Describing location of cellular components according Gene Ontology (GO) Consortium (Harris *et al.*, 2004), membrane-associated proteins are localized to the cytoplasmic membrane, i.e. GO:0005886 (Fig. 14.2). However, different subclasses of membrane-associated proteins can be distinguished. Proteins with a covalently attached moiety embedded in the cytoplasmic membrane correspond to GO:0031226, *i.e.* 'intrinsic to plasma membrane'. This class of membrane-related location can be further subdivided into GO:0005887 and GO:0046658 referring respectively to (i) 'integral to plasma membrane' with integral membrane proteins (IMPs) where one or more parts of the amino acid sequence span the cytoplasmic membrane, and (ii) 'anchored to plasma membrane' with lipoproteins. The second membrane-related location to be distinguished is GO:0019897, i.e. 'extrinsic to plasma membrane' which could only refer to proteins present on the external side of the plasma membrane, i.e. GO:0031232, in the case of secreted proteins. GO:0005618 and GO:0005887 correspond to cell wall and extracellular milieu respectively. In *L. monocytogenes*, most secreted virulence factors responsible for key steps of intracellular parasitism are encoded in the Listeria pathogenicity island 1 (LIPI-1), i.e. *hly*, *plcA*, *actA* and *plcB*, whose transcription is regulated by PrfA (positive regulatory factor A) (Vazquez-Boland *et al.*, 2001a). The core PrfA regulon also includes secreted virulence factors encoded by *inlA* and *inlB* in a second cluster (Scortti *et al.*, 2007) and other virulence gene such as *inlC* (Engelbrecht *et al.*, 1996) or *vip* (Cabanes *et al.*, 2005). Other proteins whose expression is PrfA independent are also involved in bacterial virulence (Table 14.1).

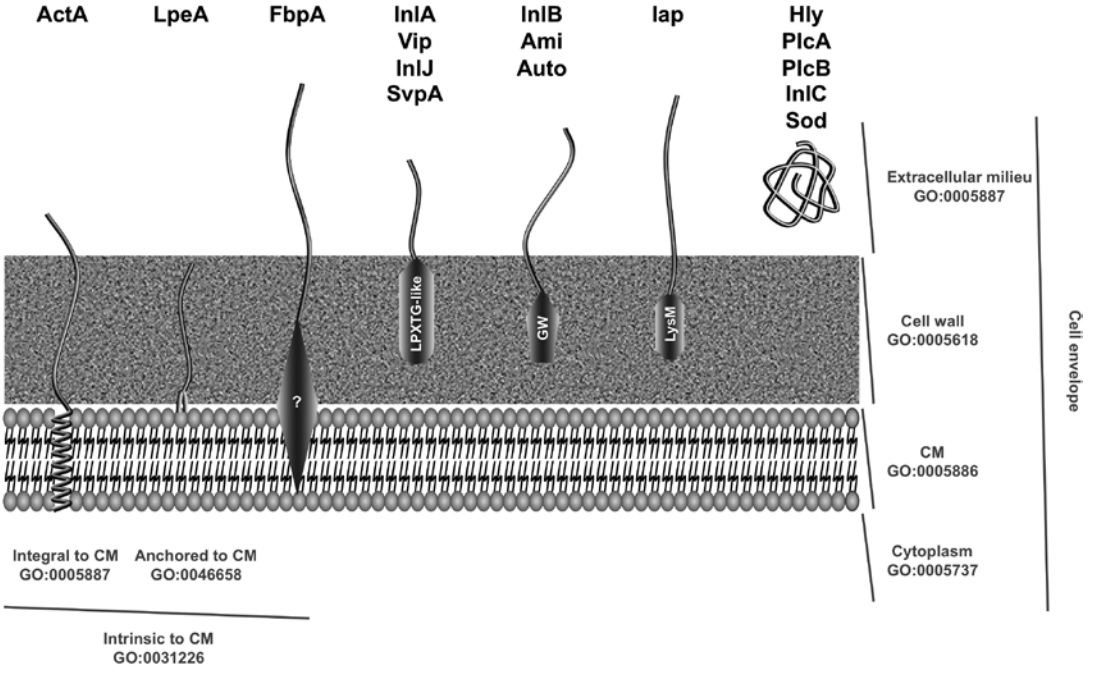

Figure 14.2 Subcellular localization of the 16 secreted virulence factors characterized to date in *L. monocytogenes*. Once translocated via the Sec translocon, proteins can (i) anchor to CM (GO:0031226) by transmembrane domain(s), i.e. integral membrane proteins (GO:0005887), or be lipoproteins (GO:0046658); (ii) associate with the cell wall (GO:0005618) covalently, i.e. proteins with LPXTG-like motifs, or by non-covalent interactions via various cell-wall binding domains; or (iii) be released into the extracellular medium (GO:0005887). CM: Cytoplasmic membrane; GO Gene Ontology.

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Table 14.1 Secreted virulence factors encoded in complete genome sequenced *Listeria*

Subcellular localization	Protein category[a]	Protein name	Locus name[b]	GI[c]	Length[d]	Note	Protein architecture[e]
Membrane associated	IMP	ActA	Lmo0204	16409569	639	PrfA-regulated transcription	SP1[1–29]_ActA(5x)[30–639]
			LmoF2365_0215	46879700	604		SP1[1–29]_ActA(5x)[30–604]
			–	–	–		
			–	–	–		
	Lipoprotein	LpeA	Lmo1847	16411301	310	LspA-dependent membrane anchoring	SP2[1–18]_SBPbac9[19–309]
			LmoF2365_1875	46881349	310	PrfA-regulated transcription	SP2[1–18]_SBPbac9[19–309]
			Lin1961	16414462	310		SP2[1–18]_SBPbac9[19–309]
			Lwe1866	116742160	310		SP2[1–18]_SBPbac9[19–309]
	Unknown	FbpA	Lmo1829	16411283	570	SecA2-dependent secretion	FbpA[1–447]_DUF814[454–539]
			LmoF2365_1857	46881331	570		FbpA[1–447]_DUF814[454–539]
			Lin1943	16414444	570		FbpA[1–447]_DUF814[454–539]
			Lwe1848	116742142	570		FbpA[1–447]_DUF814[454–539]
Cell-wall associated	LPXTG protein	InlA	Lmo0433	16409810	800	StrA-dependent cell-wall anchoring	SP1[1–35]_LRR1(13x)[99–404]_LRRa[439–496]_BLr(3x)[525–707]_LPXTG[759–797]
			LmoF2365_0471	46879954	800	PrfA-regulated transcription	SP1[1–35]_LRR1(13x)[99–404]_LRRa[439–496]_BLr(3x)[525–707]_LPXTG[759–797]
			–	–	–		
			–	–	–		
		Vip	Lmo0320	16409684	399	StrA-dependent cell wall anchoring	SP1[1–31]_LPXTG[370–399]
			LmoF2365_0338	46879823	422	PrfA-regulated transcription	SP1[1–31]_LPXTG[394–422]
			–	–	–		
			–	–	–		
		InlJ	Lmo2821	16412321	851	StrA-dependent cell wall anchoring	SP1[1–25]_LRR1(10x)[94–392]_MucBP(3x)[506–779]_LPXTG[813–851]

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		LmoF2365_2812	46882283	916		SP1[1–25]_LRR1(10x)[94–392]_MucBP(5x)[506–849]_LPXTG[878–916]
		–	–	-		
		–	–	-		
	SvpA	Lmo2185	16411655	569	StrB-dependent cell wall anchoring	SP1[1–28]_NEAT(3x)[30–482]_LPXTG[536–569]
		LmoF2365_2218	46881690	569	Fur-regulated transcription	SP1[1–28]_NEAT(3x)[30–482]_LPXTG[536–569]
		Lin2289	16414801	573		SP1[1–28]_NEAT(3x)[30–483]_LPXTG[540–573]
		Lwe2202	116742496	574		SP1[1–28]_NEAT(3x)[30–484]_LPXTG[541–574]
GW protein	InlB	Lmo0434	16409811	630	PrfA-regulated transcription	SP1[1–35]_[LRR1(6x)[99–229]_LRRa[264–321]_BLr[350–391]_GW(3x)[392–629]
		–	–	–		
		–	–	–		
		–	–	–		
	Ami	Lmo2558	16412046	917		SP1[1–30]_Ami2[108–259]_GW(8x)[273–915]
		LmoF2365_2530	46882001	770		SP1[1–30]_Ami2[108–259]_GW(6x)[276–769]
		Lin2703	16415239	770		SP1[1–30]_Ami2[108–259]_GW(6x)[276–769]
		Lwe2508	116742802	604		SP1[1–30]_Ami2[107–258]_GW(4x)[275–603]
	Auto	Lmo1076	16410478	572		SP1[1–26]_GluAmi[97–239]_GW(4x)[245–571]
		–	–	–		
		–	–	–		
		–	–	–		
LysM protein	Iap	Lmo0582	16409958	482	SecA2-dependent secretion	SP1[1–25]_LysM[28–70]_SH3.3[86–139]_LysM[201–243]_NLPC/P60[379–481]

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Table 14.1 *continued*

Subcellular localization	Protein category[a]	Protein name	Locus name[b]	GI[c]	Length[d]	Note	Protein architecture[e]
			LmoF2365_0611	46880093	477		SP1[1–27]_LysM[30–72]_SH3.3[87–140]_LysM[202–244]_NLPC/P60[374–476]
			Lin0591	16413031	465		SP1[1–25]_LysM[28–70]_SH3.3[85–138]_LysM[199–241]_NLPC/P60[362–464]
			Lwe0549	116740847	524		SP1[1–27]_LysM[30–72]_SH3.3[86–139]_LysM(2x)[198–358]_NLPC/P60[421–523]
Extracellular		Hly	Lmo0202	16409567	529	SipZ-dependent secretion	SP1[1–24]_ThioCyto[57–524]
			LmoF2365_0213	46879698	529	PrfA-regulated transcription	SP1[1–24]_ThioCyto[57–524]
			–	–	–		
			–	–	–		
		PlcA	Lmo0201	16409566	317	PrfA-regulated transcription	SP1[1–29]_PIPLCXc[61–200]
			LmoF2365_0212	46879697	317		SP1[1–29]_PIPLCXc[61–200]
			–	–	–		
			–	–	–		
		PlcB	Lmo0205	16409570	289	SipZ-dependent secretion	SP1[1–25]_ZnPLPC[26–289]
			LmoF2365_0216	46879701	280	PrfA-regulated transcription	SP1[1–18]_ZnPLPC[19–280]
			–	–	–		
			–	–	–		
		InlC	Lmo1786	16411240	296	PrfA-regulated transcription	SP1[1–33]_LRR1(4x)[97–204]_LRRa[239–296]
			LmoF2365_1812	46881287	297		SP1[1–34]_LRR1(4x)[98–205]_LRRa[240–297]

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	–	–	–		
	–	–	–		
Sod	Lmo1439	16410868	202	SecA2-dependent secretion	SodFeN[2–90]_SodFeC[95–198]
	LmoF2365_1458	46880935	202		SodFeN[2–90]_SodFeC[95–198]
	Lin1478	16413951	202		SodFeN[2–90]_SodFeC[95–198]
	Lwe1456	116741750	202		SodFeN[2–90]_SodFeC[95–198]

[a]Proteins are here categorised as a function of mechanism involved in cell-envelope anchoring.

[b]Lmo: *L. monocytogenes* 1/2a EGDe; LmoF2365: *L. monocytogenes* 4b F2365; Lin: *L. innocua* 6a Clip11262; Lwe: *L. welshimeri* 6b SLCC5334.

[c]GI: GenInfo Identifier in GenBank database.

[d]Length of primary protein sequence expressed as number of amino acid residues.

[e]Protein domains were predicted from Pfam (PF), LipoP (LP), SignalP (SP) and Superfamily (SSF). ActA: actin assembly protein (PF05058); DUF814: domain of unknown function (PF05670); FbpA: fibronectin binding protein A (PF05833); GluAmi: glucosaminidase (PF01832); GW: GW domain (SSF82057); LBr: *Listeria-Bacteroides* repeat domain (PF09479); LPXTG: LPXTG domain (PF00746); LRR1: leucine rich repeat (PF00560); LRRa: LRR adjacent (PF08191); LysM: lysin motif (PF01476); NEAT: near transporter domain (PF05031); NLPC/P60: cell-wall peptidase of p60 protein family (PF00877); PIPLCXc: phosphatidylinositol-specific phospholipase C × domain (PF00388); SH3.3: Src homology domain (PF08239); SodFeN: iron/manganese superoxide dismutases N-terminal domain (PF00081); SodFeC: iron/manganese superoxide dismutases C-terminal domain (PF02777); SP1: signal peptide of class 1 (SP); SP2: signal peptide of class 2 (LP); SBPbac9: periplasmic solute binding protein (PF01297); ThioCyto: thiol-activated cytolysin (PF01289); ZnPLPC: zinc dependent phospholipase C (PF00882). Numbers in brackets indicate the numb er of repeats. Numbers in square brackets indicate positions along the amino acid sequence.

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Membrane-associated virulence factors

Among the different subclasses of membrane-associated proteins, virulence factors characterized in *L. monocytogenes* are found in proteins intrinsic to the plasma membrane including both proteins integral to the plasma membrane and proteins anchored to the plasma membrane. However, only three virulence factors have been investigated to date, i.e. one IMP, one lipoprotein and a protein anchored to the membrane by an unknown mechanism (Table 14.1).

Membrane-integrated virulence factors

In *L. monocytogenes* EGDe, 11 proteins are predicted with an hydrophobic domain located at the carboxyl terminus (Glaser *et al.*, 2001). In *Listeria*, IMPs with domains exposed on the bacterial cell surface are generally considered to be restricted to those proteins with such hydrophobic C-terminal tails (Cabanes *et al.*, 2002). IMPs can be primarily split into (i) single membrane-spanning domain, i.e. with only one α-helical transmembrane domain (TMD), and (ii) multi-membrane spanning domain proteins, i.e. with at least two TMDs (Goder and Spiess, 2001). Two TMD topologies can be discriminated, N_{out}–C_{in}, corresponding to type I, or C_{out}–N_{in},corresponding to type II. These topogenic elements are actually uncleavable signal peptides present along the amino acid sequence. IMPs are categorised according to the topology of the most N-terminal TMD (von Heijne, 2006). Depending on IMP topology, some loops located between two TMDs or regions present at sequence extremities can be cell surface exposed. As in *E. coli*, all IMPs are most certainly translocated and integrated into the cytoplasmic membrane in a YidC-dependent manner (Froderberg *et al.*, 2003). In *L. monocytogenes* EGDe, 1204 IMPs have been predicted compared to 733 in *L. monocytogenes* F2365 (Nelson *et al.*, 2004). Among IMPs, ActA (actin assembly) is the only virulence factor with an hydrophobic tail uncovered so far. ActA is a type I IMP as the preprotein exhibits a cleavable N-terminal signal peptide and the mature protein possesses a single C-terminal TMD with a N_{out}–C_{in} topology.

ActA promotes actin nucleation once *L. monocytogenes* is present in the cytosol of an infected host cell (Domann *et al.*, 1992; Kocks *et al.*, 1992). Recruitment and asymmetric polymerization of host actin by ActA propels *L. monocytogenes* through the host cytoplasm by forming a comet tail (Tilney and Portnoy, 1989; Dabiri *et al.*, 1990). Polar distribution of ActA on the surface of *L. monocytogenes* is necessary for efficient actin-based motility (Smith *et al.*, 1995; Rafelski and Theriot, 2005). Polarization of ActA was proposed as a direct consequence of the differential cell wall growth rates along the bacterium and would also depend on the relative rates of protein secretion, protein degradation and bacterial growth (Rafelski and Theriot, 2006). ActA is a central virulence factor as it allows spreading directly from one host cell to another (Hamon *et al.*, 2006). This mode of propagation through tissues limits contact of *L. monocytogenes* with extracellular milieu and thus phagocytes, antibodies, bactericides or antimicrobial drugs. The N-terminal region of ActA is essential to actin-based motility and shows homology with the C-terminal region of WASP (Wiskott–Aldrich syndrome protein) ((May *et al.*, 1999; Mayer and Gottschalk, 2003). This region is a nucleation-promoting factor (NPF) activating the host complex formed by Arp2 (actin-related protein 2) and Arp3, which closely resemble the structure of monomeric actin and serve as initiation sites for biosynthesis of new actin filaments. Besides providing intracellular movement to the bacterial cell, ActA is also necessary for entry into the eukaryotic cell (Alvarez-Dominguez *et al.*, 1997; Suarez *et al.*, 2001). The N-terminal region of ActA exhibits heparan sulphate (HS)-binding domains. Following specific interaction of ActA with heparan sulphate proteoglycan (HSPG) receptors present on the surface of epithelial cells, *L. monocytogenes* would attach and enter the infected host cell.

Lipoprotein virulence factors

Following translocation through the Sec translocon, lipoproteins are tethered to the cytoplasmic membrane by a non-polypeptidic covalently attached hydrophobic anchor (Tjalsma *et al.*, 2004). Interestingly, in *E. coli*, YidC is involved in translocation of some lipoproteins (Froderberg *et al.*, 2004), and some lipoproteins are integrated to the membrane by TMDs and

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may consequently be considered as IMPs (Van Bloois *et al.*, 2006). In Gram-positive bacteria, attachment of lipoproteins to the outer surface of the membrane requires two post-translational modifications involving Lgt (prolipoprotein diacylglyceryl transferase) and Lsp (Tjalsma *et al.*, 1999). In *L. monocytogenes*, lipidation by Lgt, where a N-acyl diglyceride moiety from a membrane glycerophospholipid is transferred to the thiol group of the lipobox cysteine, is not a prerequisite for cleavage of the N-terminal signal peptide by Lsp (Baumgärtner *et al.*, 2007). According to the most recent evaluation based on a new hidden Markov model (HMM), the number of lipoproteins in *L. monocytogenes* EGDe and F2365 is estimated to 62 and 56 respectively (Baumgärtner *et al.*, 2007). Among all these lipoproteins, the only secreted virulence factor characterized so far is LpeA (lipoprotein promoting entry).

As indicated by its name, LpeA is involved in bacterial entry into eukaryotic cells and constitutes the first lipoprotein reported to promote cell invasion by an intracellular pathogen (Reglier-Poupet *et al.*, 2003b). Despite homology with PsaA (pneumococcal surface adhesin A) from *Streptococcus pneumoniae*, a lipoprotein belonging to the LraI (lipoprotein receptor antigen I) family (Sampson *et al.*, 1994), LpeA is not implicated in bacterial cell adherence. Members of the LraI family play a dual role in adhesion and transport as they are known as the binding protein components of ABC (ATP-binding cassette) transporters specific for metal ions, essentially Zn or Mn (Claverys, 2001). In *L. monocytogenes*, the gene encoding LpeA is transcribed under the control of PrfA (Scortti *et al.*, 2007), and is localized in an operon resembling those of the ABC transporter family, although the role of LpeA in any kind of transport remains to be established (Reglier-Poupet *et al.*, 2003b). Nonetheless, LpeA is clearly a cell surface exposed invasin favouring the entry of *L. monocytogenes* into non-professional phagocytes and is required to enable the bacterium to escape from the phagosomal compartment.

Other membrane-associated virulence factors

FbpA (fibronectin binding protein A) is described as bacterial cell surface exposed protein in *L. monocytogenes* (Dramsi *et al.*, 2004). It is clearly sublocalized to the cytoplasmic and membrane fractions, and consequently must be considered as a protein intrinsic to cytoplasmic membrane. Mechanisms involved in anchoring this protein to the membrane are completely unknown as FbpA lacks conserved domains involved in cell-envelope attachment. It must be stressed, however, that failure to identify a lipobox or TMD following bioinformatic analyses does not mean such regions are absent as they might be present but non-predicted. Unravelling the underlying mechanism permitting membrane association of FbpA should require systematic experimental analysis of the structure–function relationships in order to reveal region(s) involved in membrane anchoring. Like Iap, FbpA does not display a N-terminal signal peptide and takes the SecA2 route for secretion (Dramsi *et al.*, 2004). As indicated by its name, FbpA binds human fibronectin and it was demonstrated to contribute to eukaryotic cell adherence (Dramsi *et al.*, 2004). In addition, it could function as a molecular chaperone as it interacts and post-transcriptionnally modulates the expression levels of the virulence factors listeryolysin O and InlB. Inactivation of *fbpa* resulted in virulence attenuation of the mutant and low liver colonization in mice inoculated intravenously, confirming the role of FbpA in listerial pathogenesis (Dramsi *et al.*, 2004).

Cell-wall associated virulence factors

With nine proteins characterized, this class of a subcellular localized virulence factors has been the most investigated in *L. monocytogenes* (Table 14.1). Among cell-wall associated proteins, virulence factors characterized in *L. monocytogenes* include proteins covalently attached, i.e. LPXTG proteins, and non-covalently attached to cell wall, i.e. GW and LysM proteins.

Virulence factors covalently attached to the cell wall

Once translocated via Sec, some listerial proteins bearing a C-terminal sorting signal, consisting of a pattern varying around LPXTG followed by an hydrophobic region and a positively charged tail, are recognized by membrane sortase (Navarre and Schneewind, 1999). Among Gram-positive bacteria, *L. monocytogenes* encodes the highest

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number of LPXTG proteins (Pallen *et al.*, 2001). Transpeptidase sortase cleaves LPXTG at the T-G bond and further catalyses the formation of an amide link between T and lipid II precursor, resulting in protein incorporation into the cell wall in the course of peptidoglycan biogenesis (Ton-That *et al.*, 2004). This is the only mechanism as yet discovered allowing covalent anchoring of a protein to the bacterial cell wall. Two sortases with different specificities, i.e. SrtA (sortase of class A) and SrtB, have been identified in *L. monocytogenes* (Bierne *et al.*, 2002, 2004).

Out of 43 LPXTG-like proteins encoded in *L. monocytogenes* EGDe (Boekhorst *et al.*, 2005), 41 are presumably recognized by SrtA including three characterized virulence factors (Glaser *et al.*, 2001). Consequently, inactivation of SrtA affects virulence of *L. monocytogenes* (Bierne *et al.*, 2004). The first SrtA substrate identified as a key virulence factor was a member of the internalin family, i.e. InlA (internalin A) (Gaillard *et al.*, 1991). In Gram-positive bacteria, *L. monocytogenes* encodes the highest number of internalins (Bierne *et al.*, 2007). Twenty-five members of the internalin family are encoded in *L. monocytogenes* EGDe, although only InlA and InlB have been demonstrated to be involved in internalization. So, rather than a function associated with bacterial entry into eukaryotic host cells, internalins are basically defined as proteins containing leucine-rich repeats (LRR), which pinpoints that their name is somehow misleading (Bierne *et al.*, 2007). As all internalins, InlA possess a N-terminal signal peptide of class 1 and is thus is predicted to be translocated via Sec. While InlA bears a C-terminal LPXTG motif, this is not a general feature of internalins. InlA possess two LRR regions separated by an immunoglobulin-like domain, named inter-repeat (IR), which are necessary and sufficient for *L. monocytogenes* entry into human epithelial cells (Lecuit *et al.*, 1997). Like ActA, InlA is polarly localized (Lebrun *et al.*, 1996). Interestingly, some truncated forms of InlA are encoded in some naturally occurring *L. monocytogenes* strains, more often in strains isolated from food products rather than clinical isolates (Jonquieres *et al.*, 1998; Jacquet *et al.*, 2004). InlA binds E-cadherin on the surface of the eukaryotic host cell inducing *L. monocytogenes* internalization (Mengaud *et al.*, 1996). Despite pronounced structural similarity (Schubert *et al.*, 2002), InlA recognizes E-cadherin from human, guinea pig and rabbit but fails to bind the murine E-cadherin (Lecuit *et al.*, 1999). This species-specific interaction results from variation of a single amino acid in murine E-cadherin. E-cadherin is a transmembrane glycoprotein involved in cell–cell adhesion via formation of adherent junctions. Upon InlA binding, local cytoskeletal rearrangements in the host cell, involving intracellular α- and β-catenin complex (Yen *et al.*, 2002), ARHGAP10 (Rho GTPase-activating protein 10) (Sousa *et al.*, 2005), as well as unconventional myosin VIIA and its ligand vezatin (Kussel-Andermann *et al.*, 2000; Sousa *et al.*, 2004), result in *L. monocytogenes* uptake in a process not fully understood (Hamon *et al.*, 2006).

Another LPXTG internalin involved in listerial virulence was uncovered later on, i.e. InlJ (Sabet *et al.*, 2005). LRR in InlJ differs from prototypical LRR as it contains a conserved cysteine, and thus defines a new subfamily of LRRs. In contrast to InlA, InlJ contains MucBP (mucin-binding protein) domains. InlJ is neither involved in *Listeria*-induced phagocytosis, intracellular replication nor intercellular spreading. While deletion of *inlJ* results in significantly attenuated virulence in orally acquired listeriosis, its exact role in pathogenesis remains to be elucidated (Sabet *et al.*, 2005).

The SrtA-dependent protein Vip (virulence protein) is required for entry into some mammalian cells (Cabanes *et al.*, 2005). This cell surface protein, positively regulated by PrfA the master regulator of *L. monocytogenes* virulence genes, binds the endoplasmic reticulum resident chaperone Gp96 (glycoprotein of 96 kDa) from the infected host cell. Interaction with this Vip cellular receptor, expressed at the cell surface, is critical for *L. monocytogenes* entry and invasion of eukaryotic host cells. Vip appeared clearly as a key virulence factor as revealed by bacterial counts in organs from mice orally inoculated with wild-type (wt) and Δ*vip L. monocytogenes* strains (Cabanes *et al.*, 2005).

Out of 48 LPXTG-like proteins encoded in *L. monocytogenes* F2365 (Boekhorst *et al.*, 2005), only three are presumably substrates of SrtB, as opposed two as definitively ascertained in *L.*

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monocytogenes EGDe (Pucciarelli *et al.*, 2005). As substrates of SrtB, these proteins possess a C-terminal NPXTX-like motif diverging from the usual LPXTG pattern (Comfort and Clubb, 2004). Only one of them has been clearly identified as a virulence factor in *L. monocytogenes*, i.e. SvpA (surface virulence-associated protein A) (Borezee *et al.*, 2001). SvpA is required for intracellular growth in macrophages and facilitates bacterial escape from phagosomes. Its expression is not regulated by PrfA. However, the operon encoding SvpA and SrtB is under the control of Fur (ferric uptake regulation), a global transcriptional repressor responsive to iron availability (Newton *et al.*, 2005). Moreover, SvpA exhibits three NEAT (near transporter) domains, which are essentially found in proteins from pathogenic bacteria and appear to be associated with iron transport in Gram-positive bacteria, possibly as receptors of siderophore-ferric iron complexes (Andrade *et al.*, 2002). Actually, all LPXTG-like protein substrates of SrtB appear to be involved in iron metabolism (Andrade *et al.*, 2002; Comfort and Clubb, 2004). Acquisition of iron is recognized as a key step in the development of any pathogen in its host (Ratledge and Dover, 2000). Nevertheless, utilization of haemin, haemoglobin or ferrichrome could not be demonstrated for SvpA and thus, its clear implication in Fe^{3+} uptake in relation to listerial virulence requires further investigations.

Virulence factors non-covalently attached to the cell wall

Among proteins non-covalently attached to the cell wall, GW proteins are the most represented in *L. monocytogenes* with nine in strain EGDe and seven in F2365 (Glaser *et al.*, 2001; Billion *et al.*, 2006). A GW module is about 80 amino acids long with a highly conserved glycine-tryptophan dipeptide and is often found in multicopy, which increases the strength of attachment to the cell wall accordingly (Jonquieres *et al.*, 1999; Cabanes *et al.*, 2002). Indeed, GW modules interact with lipoteichoic acid of the cell wall, which result in anchoring and cell surface exposure of protein. Only three GW proteins are currently characterized as virulence factors in *L. monocytogenes*.

Besides LIPI-1, InlB is encoded with InlA in a second gene cluster necessary for invasion and intracellular replication of *L. monocytogenes* where it forms an operon of only two genes, i.e. *inlAB* (Gaillard *et al.*, 1991). Like InlA, InlB is a surface protein, whose expression is regulated by PrfA, but it constitutes a second subfamily of internalins as it is anchored to the cell wall via three GW modules (Bierne *et al.*, 2007). InlB is a key virulence factor required for *L. monocytogenes* internalization into many types of eukaryotic cells. Indeed, InlB interacts via LRR or GW domains with three host cell ligands, (i) hepatocyte growth factor receptor (HGF-R), a tyrosine kinase receptor (Shen *et al.*, 2000), (ii) gC1q-R (globular part of the complement component 1, Q subcomponent receptor), an acidic multiligand-binding glycoprotein (Braun *et al.*, 2000), and (iii) proteoglycans, namely glycosaminoglycans (GACs) such as heparan sulphates (Jonquieres *et al.*, 2001). It also suggested that interactions with GACs and gC1q-R facilitates and/or enhances binding of InlB with HGF-R. Clearly, InlB interacts with HGF-R at a different site than the natural ligand hepatocyte growth factor (HGF) (Niemann *et al.*, 2007; Veiga and Cossart, 2007). Binding to these ligands induce signal transduction and specific activation of complex cellular response pathways, involving for example phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) or Arp2/3 complex (Bierne and Cossart, 2002; Dussurget *et al.*, 2004; Hamon *et al.*, 2006). Ultimately, signalling via these pathways leads to cytoskeletal rearrangements and *L. monocytogenes* internalization following clathrin-mediated endocytosis (Veiga and Cossart, 2005; Niemann *et al.*, 2007).

The cell-wall binding domain of Ami (amidase) exhibits the highest number of GW modules among *L. monocytogenes* EGDe proteins, i.e. 8 (Cabanes *et al.*, 2002). Ami is an autolysin also implicated in bacterial adhesion to the eukaryotic host cell (McLaughlan and Foster, 1998; Milohanic *et al.*, 2001). The adhesion capacity of Ami to target mammalian cells resides only in its cell-wall binding domain (Milohanic *et al.*, 2001). However, component(s) recognized by Ami on the surface of eukaryotic cells remain to be determined. The role of Ami in virulence was demonstrated by intravenous inoculation of mice with inactivated *ami* mutant

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of *L. monocytogenes*, which resulted in a reduced mortality and capacity of bacteria to colonize the liver. Polymorphism was observed among *ami* from different *L. monocytogenes* isolates (Jacquet *et al.*, 2002). In the epidemic *L. monocytogenes* 4b CHUT82337, Ami exhibits only 6 GW modules and is less able to bind human eukaryotic cells than Ami from *L. monocytogenes* 1/2a EGD. Phylogenic analysis further revealed that difference in sequences of the Ami GW domains in various serovars of *L. monocytogenes* correlate with the somatic antigens.

A second autolysin, Auto, with four GW modules is also involved in listerial virulence (Cabanes *et al.*, 2004). Indeed, an *aut* deletion mutant inoculated intravenously in mice or orally in guinea pigs results in attenuated virulence. Auto is necessary but not sufficient for *L. monocytogenes* entry into eukaryotic cells. Unlike Ami, Auto is not involved in adhesion but is required for invasion of *L. monocytogenes* into non-phagocytic eukaryotic cells. Auto was suggested as being implicated both in the early step of intestinal barrier crossing and at later stages of the infectious process. However, *aut* is not systematically present in all *L. monocytogenes* isolates (Desvaux and Hébraud, 2006).

The LysM (lysin motif) is another type of cell-wall anchoring motif about 40 amino acid long folded as three α-helices and binding directly to peptidoglycan (Steen *et al.*, 2003). Six LysM proteins, which bear between one and four copies of LysM, are encoded in each *L. monocytogenes* EGDe and F2365 (Desvaux and Hébraud, unpublished data). Only one LysM protein, Iap (invasion associated protein), is currently recognized as a virulence factor in *L. monocytogenes* (Kuhn and Goebel, 1989). It is worth stressing that in the literature, Iap is also called p60 (protein of 60 kDa) or CwhA (cell wall hydrolase A) (Pilgrim *et al.*, 2003). Contrary to early assumptions (Wuenscher *et al.*, 1993), Iap is not an essential gene product of *L. monocytogenes*. In the first instance, deletion of *iap* leads to abnormal *L. monocytogenes* cell division; as such a mutant is impaired in its ability to form a septum, which results in longer filamentous bacterial cells (Pilgrim *et al.*, 2003). Consequently, ActA and InlA, which normally have a polar localization (Kocks and Cossart, 1993), are unevenly distributed (Pilgrim *et al.*, 2003). Considering that ActA distribution depends on relative rates of cell wall growth, protein secretion, protein degradation and bacterial growth (Rafelski and Theriot, 2006) and that bacterial shape and ActA localization affect initiation of actin-based motility (Rafelski and Theriot, 2005), it is not that surprising that the Δ*iap* mutant exhibits a deficiency in actin polymerization, intracellular movement and intercellular spreading (Pilgrim *et al.*, 2003). Since mutation in *iap* interferes with the correct surface distribution and function of several virulence factors, attenuation of invasiveness and virulence with this *L. monocytogenes* mutant is most certainly an indirect consequence, which challenges the assumption that Iap is a listerial virulence factor (Pilgrim *et al.*, 2003). In another investigation, though, an in-frame deletion of *iap* did not cause chaining of bacterial cells or other growth defects (Lenz *et al.*, 2003). SecA2-dependent secretion of Iap is important for infection of host tissue as it contributed to *L. monocytogenes* persistence in livers of infected mice (Lenz and Portnoy, 2002; Lenz *et al.*, 2003). Surprisingly, peptidoglycan digestion by Iap is required for *L. monocytogenes* virulence but this function does not contribute to the release of other SecA2-dependent or independent virulence factors. It was hypothesized that release of peptidoglycan fragments, e.g. muramyl peptides, could modulate the regulation of the host inflammatory responses (Lenz *et al.*, 2003). However, in another report, and in agreement with earlier studies (Kuhn and Goebel, 1989; Pilgrim *et al.*, 2003), deletion of *iap* was once again reported to result in formation of cell chains and thus its involvement in cell division was confirmed (Machata *et al.*, 2005). Thus, mutation in *iap* seems to definitely result in significant differences to bacterial cell morphology and growth rate. In order to investigate the role of Iap independently from all listerial virulence genes, an investigation using *B. subtilis* cells expressing listerial haemolysin Hly alone or in combination with Iap revealed that Iap significantly increased adherence and invasion of eukaryotic host cells when compared with *B. subtilis* haemolytic strain (Wisniewski *et al.*, 2006). Iap seems to play a role in listeriolysin-mediated haemolytic activity. This study further confirmed the role of Iap as murine

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hydrolase involved in cell division. Nonetheless, establishing the exact mechanisms directly responsible for listerial virulence by Iap (if any) clearly requires further investigations.

Extracellular virulence factors

According the most recent estimation in *L. monocytogenes* EGDe, 117 proteins are predicted as being secreted via Sec because of the presence of an N-terminal signal peptide of class 1 and released into the extracellular milieu as they do not exhibit an additional region involved in cell-envelope attachment (Trost *et al.*, 2005; Desvaux and Hébraud, 2006). Four of them are currently characterized as key virulence factors. While the number of secreted proteins lacking a N-terminal signal peptide of class 1 has not been thoroughly estimated in *L. monocytogenes* (Bendtsen *et al.*, 2005), one of them was identified as a listerial virulence factor (Table 14.1).

Listeriolysin O (LLO), encoded by *hly* (haemolysin), is a key determinant in intracellular life step of the *L. monocytogenes* infection (Dussurget *et al.*, 2004; Kayal and Charbit, 2006; Schnupf and Portnoy, 2007). This protein secreted extracellularly, presumably via Sec, and is matured in a SipZ-dependent manner (Bonnemain *et al.*, 2004). LLO is required to escape from vacuoles formed after internalization or cell-to-cell spreading, i.e. primary and secondary phagosomes (Cossart *et al.*, 1989; Dramsi and Cossart, 2002). A highly significant attenuation of *L. monocytogenes* virulence is observed in a murine model of listeriosis in the absence of active LLO (Gaillard *et al.*, 1986; Kathariou *et al.*, 1987; Portnoy *et al.*, 1988; Kathariou *et al.*, 1990). LLO is a member of the cholesterol-dependent cytolysin (CDC) family, which is responsible for membrane lysis of virtually any mammalian cell (Bhakdi *et al.*, 1998; Alouf, 2000; Billington *et al.*, 2000; Alouf, 2003; Palmer, 2004). By sensing the acidic pH of the phagosome, LLO is subjected to conformational changes inducing its activation, i.e. oligomerization of monomers resulting in pore formation in the host plasmic membrane (Jones and Portnoy, 1994; Schuerch *et al.*, 2005). In addition, CDCs permit permeation of macromolecules of up to 100 kDa into the host cell cytoplasm (Walev *et al.*, 2001), and have been recently described as generally involved in cytolysin-mediated translocation (CMT) (Madden *et al.*, 2001; Meehl and Caparon, 2004; Tweeten and Caparon, 2005). In this model, CDCs form large oligomeric pores in cholesterol-containing membranes of target eukaryotic cells (Tilley *et al.*, 2005), which then permit the translocation of effector molecules (Tweeten and Caparon, 2005). Misleadingly, CMT was described as a T3SS, even though only FEA is phylogenetically related to this secretion system and this terminology is restricted to Gram-negative bacteria (Desvaux *et al.*, 2006b). Besides LLO, the most widely known members of the CDC family playing a role in virulence of pathogenic Gram-positive bacteria are (i) streptolysin O from *Streptococcus pyogenes*, the causative agent of numerous suppurative infections (Bisno and Stevens, 1996), (ii) perfringolysin O from *Clostridium perfringens*, the etiological agent of gas gangrene (Rood, 1998), and (iii) pneumolysin from *S. pneumoniae*, a causative agent of pneumonia and meningitis (Hirst *et al.*, 2004). Unlike other bacteria, *L. monocytogenes* is the only pathogen to secrete this type of cytolysin inside a host cell. In the host cell cytosol, LLO is degraded by the N-end rule pathway, which regulates *L. monocytogenes* virulence as it reduces the toxicity level of this CDC during infection (Kayal and Charbit, 2006; Schnupf *et al.*, 2007). Translocation of proteins synthesized by *L. monocytogenes* through LLO in a CMT manner has never been thoroughly investigated, though LLO-dependent translocation of PlcA (phospholipase C protein A) has been suggested (Schnupf and Portnoy, 2007). Surprisingly, LLO activates different signalling pathways in the host cell that elicit different cellular responses (Dussurget *et al.*, 2004; Schnupf and Portnoy, 2007), e.g. eukaryotic cell apoptosis, nitric oxide formation, interleukin secretion, expression of cell adhesins, induction of inflammatory cytokines, mucin exocytosis or production of interferons. CDCs, including LLO, are partly involved in such cellular responses by acting as antagonists of Toll-like receptors (Park *et al.*, 2004; Srivastava *et al.*, 2005). While membrane damage due to pore formation by LLO could also be an indirect cause of cell signalling (Tang *et al.*, 1994), CMT of factors involved in activation of these response pathways cannot be ruled out. It

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is known that pores form by LLO are permeable to calcium cations, an important second messenger, where oscillation of Ca^{2+} concentrations modulates cellular responses (Repp *et al.*, 2002).

After invasion, *L. monocytogenes* gains access to the host cell cytoplasm by escaping from membrane-bound phagosomes following the combined action of LLO and two extracellularly secreted phospholipases C, i.e. PlcA and PlcB (Goldfine and Wadsworth, 2002; Krawczyk-Balska and Bielecki, 2004; Dussurget *et al.*, 2004). While *plcA* (phospholipase C protein A) encodes a phosphatidylinositol phospholipase C (PI-PLC), *plcB* encodes a phosphatidylcholine phospholipase C (PC-PLC). The combined action of these two phospholipases C allows the efficient hydrolysis of a broad-range of mammalian phospholipids resulting in degradation of vacuolar membranes. As with Hly, SipZ is involved in maturation of PlcB (Bonnemain *et al.*, 2004). The secreted and PrfA-regulated metalloproteinase Mpl is involved in maturation of PlcB and is thus necessary for full virulence expression of *L. monocytogenes* (Domann *et al.*, 1991; Mengaud *et al.*, 1991) but it cannot be considered as a virulence factor stricto sensu.

Unlike the three other internalins characterized to date as being implicated in listerial virulence, i.e. InlA, InlB and InlJ, the internalin InlC is not associated with the cell wall but is released into the extracellular milieu following translocation, presumably through the Sec translocon (Engelbrecht *et al.*, 1996). Consequently, InlC belongs the third and last subfamily of internalins, which are smaller, i.e. about 35 kDa, lack domains involved in covalent or non-covalent attachment to the cell wall, and are thus predicted to be extracellular (Bierne *et al.*, 2007). *inlC* is monocistronic and PrfA-regulated (Engelbrecht *et al.*, 1996). InlC is likely to play a role in a late stage of *L. monocytogenes* infection rather than internalization of *L. monocytogenes*. Intracellular replication of *L. monocytogenes* Δ*inlC* in infected eukaryotic cells appeared comparable with that of wt strain. Although function of InlC in pathogenesis remains unclear(Bergmann *et al.*, 2002; Chatterjee *et al.*, 2006; Joseph *et al.*, 2006), its involvement was demonstrated as significant virulence attenuation was observed in an intravenous mouse model using a deletion mutant.

Besides Iap and FbpA, a primarily cytoplasmic manganese superoxide dismutase (MnSod) is secreted in a SecA2-dependent manner in its most active non-phosphorylated form (Archambaud *et al.*, 2006). Dephosphorylation of MnSod requires Stp (serine-threonine phosphatase), which is involved in regulation of *L. monocytogenes* virulence (Archambaud *et al.*, 2005). Once in the extracellular milieu or in infected cells, MnSod becomes phosphorylated and its antioxidant potential appears as a critical factor for *L. monocytogenes* pathogenesis. Indeed, a Δ*sod* mutant was subject to increased bacterial death within macrophages and dramatic virulence attenuation in intravenously infected mice compared with its wild-type parent (Archambaud *et al.*, 2006).

Other secreted virulence factors in *Listeria monocytogenes*?

As in other Gram-positive bacteria (Bendtsen *et al.*, 2005; Scott and Barnett, 2006), some proteins lacking a N-terminal signal peptide and not predicted to be secreted via FEA, holins or Wss are reported to be exposed on the bacterial cell surface or present in the extracellular milieu, and could thus be secreted by uncharacterized secretion pathway(s) in *L. monocytogenes* (Schaumburg *et al.*, 2004; Trost *et al.*, 2005; Desvaux and Hébraud, 2006). Alternatively, it is possible that some of these proteins could be released extracellularly following allolysis (Guiral *et al.*, 2005). Among proteins present in the *L. monocytogenes* cell wall, the primarily cytoplasmic proteins enolase, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), DnaK (desoxyribonucleic acid replication factor K) and EF-Tu (elongation factor – transfer fraction unstable component) were demonstrated to be strong human plasminogen binders (Schaumburg *et al.*, 2004). While molecular mechanisms involved in protein binding to the bacterial cell wall remain to be determined, these proteins would moonlight on the bacterial cell surface and might play a role in *L. monocytogenes* invasion mechanisms. The 104-kDa protein Lap (*Listeria* adhesion protein) also lacks a N-terminal signal peptide and is localized on the bacterial surface as well as in the cytoplasm (Jaradat *et al.*, 2003). Indeed, Lap was further identified as an alcohol acetal-

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dehyde dehydrogenase encoded by *lmo1634* and exhibiting an iron-containing alcohol dehydrogenase domain at the N-terminus and an aldehyde dehydrogenase domain at the carboxyl end (Kim *et al.*, 2006). Lap mediates adhesion of *L. monocytogenes* to intestinal cells (Pandiripally *et al.*, 1999; Young *et al.*, 1999). Invasiveness appeared to be proportional to variable adhesion levels in different types of infected host cell (Jaradat *et al.*, 2003). The surface-expressed mammalian heat shock protein Hsp60 was identified as a receptor for Lap (Wampler *et al.*, 2004). While Lap possibly plays an important role during the intestinal phase of infection, especially with intestinal cells originating from the lower part of small intestine and from the upper part of large intestine (Jaradat *et al.*, 2003), to date evidence of this protein as a virulence factor has not been provided.

Using signature-tagged transposon mutagenesis to uncover new genes involved in virulence, 10 distinct loci were identified in *L. monocytogenes* (Autret *et al.*, 2001). Among them, three proteins predicted to be localized in the cell envelope appeared to be related to virulence, i.e. a LPXTG internalin Lmo2026, an integral membrane protein YtgP (named according to the European *Bacillus subtilis* genome sequencing project; Kunst *et al.*, 1995), and a membrane-anchored protein PtbX (penicillin-binding protein X). While attenuation of these mutants is unlikely to be due to a defect in cell internalization or multiplication in the spleen and liver, invasion of the brain was more limited, suggesting a defect in the efficiency of crossing the blood–brain barrier. Transposon insertion in *lmo2026* caused a strong inhibition of intracellular replication. However, further investigations, namely complementation experiments, are necessary to confirm the involvement of these proteins in virulence and to determine their precise role in pathogenicity.

The role of the LPXTG internalin InlH in listerial virulence has not as yet been clearly demonstrated. However, it was observed that deletion mutants in *L. monocytogenes* were affected in their ability to colonize spleen and liver cells (Young *et al.*, 1999; Schubert *et al.*, 2001). Nonetheless, InlH is not involved in bacterial invasion, intracellular multiplication or intercellular spreading. The gene encoding InlH would result from a recombination event between *inlC2* and *inlD*, where InlC2 diverges from InlH only by one amino acid in the IR domain and 12 residues in the second LRR region (Dramsi *et al.*, 1997; Young *et al.*, 1999). However, inactivation of *inlC2* had no effect on colonization ability of *L. monocytogenes*.

In Gram-positive bacteria, 6 different domains involved in non-covalent cell wall attachment of proteins have been uncovered (Desvaux *et al.*, 2006a), though proteins bearing an S-layer homology domain (SLHD), cell wall binding domain of type 1 (CWBD1) or CWBD2 could not be identified in *L. monocytogenes* (Bierne and Cossart, 2007). Besides GW and LysM, a third kind of cell wall binding motif present in some *L. monocytogenes* proteins has recently been uncovered, i.e. WXL (Brinster *et al.*, 2007). As in most low G+C% Gram-positive bacteria where they were reported, the four listerial WXL proteins are encoded in two *csc* (cell surface complex) clusters and are speculated to form cell surface protein complexes involved in degradation and utilization of plant oligo- and/or polysaccharides (Siezen *et al.*, 2006). One of them is predicted to be a member of the internalin family (Lmo0549). Though, none of these proteins have been experimentally investigated in *L. monocytogenes*, and thus their expression, function, subcellular localization and physiological role, including contribution to pathogenicity, remain to be elucidated.

Following genomic analyses, the first listerial protein exhibiting ChW (clostridial hydrophobic domain with conserved tryptophan residue) domains was identified as encoded in the genome of *L. monocytogenes* F2365, i.e. LmoF2365_1900 (Desvaux and Hébraud, unpublished data). ChW domains are speculated to be involved in protein anchoring to the cell wall, where they could form cell surface protein complexes implicated in degradation of plant cell wall polymer (Nölling *et al.*, 2001). Once again, experimental investigations are awaited to ascertain the function of this putative listerial serine protease and its potential physiological role, including in bacterial virulence.

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Conclusion

Besides complete genome sequences of *L. monocytogenes* 1/2a EGDe and 4b F2365 (Glaser *et al.*, 2001; Nelson *et al.*, 2004), the incomplete genomes of *L. monocytogenes* 1/2a F6854 and *L. monocytogenes* 4b H7858 are publicly available (Nelson *et al.*, 2004). Additionally, an *L. monocytogenes* strain of serotype 4a is currently being sequenced as well as another *L. monocytogenes* strain from a project run by the Hubei Entry/Exit Inspection and Quarantine Bureau (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi; http://www.genomesonline.org) (Hain *et al.*, 2006b). Moreover, a huge genome sequencing project run by the Broad Institute of Massachusetts Institute of Technology (MIT) and Harvard on 20 different *L. monocytogenes* strains is under way (http://www.genomesonline.org; http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Out of the six listerial species and besides *L. monocytogenes*, two complete listerial genome sequences are published, namely *L. innocua* CLIP 11262 serotype 6a (Glaser *et al.*, 2001) and *L. welshimeri* SLCC 5334 serotype 6b (Hain *et al.*, 2006b). Genome sequences of the remaining three *Listeria* species should soon become accessible, namely from bacterial strains *L. grayi* CLIP 12515, *L. seeligeri* 1/2b SLCC 3954 and *L. ivanovii* PAM 55 serotype 5 (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi; http://www.genomesonline.org)(Hain *et al.*, 2006a). Once available, comparison of all these genome sequences should provide new insights in virulence of *L. monocytogenes* and serve as a basis for generation of in silico-informed hypothesis before fuelling experimental research (Pallen, 2002b). Genomic predictions of protein motifs associated with cell wall anchoring or cell adhesion allowed the identification of putative virulence factors (Bierne and Cossart, 2007). Pathogenic bacteria are considered to be differentiated from their non-pathogenic counterparts by the presence of genes encoding specific virulence determinants; secreted proteins often play a key role, e.g. in adhesion, motility or toxicity (Finlay and Falkow, 1997). Definition of a protein determinant as a virulence factor is essentially based on the molecular Koch's postulates first formulated by Stanley Falkow (Falkow, 1988): (i) the phenotype should be associated with pathogenic members of a genus or species and the gene in question should be present in all pathogenic members and absent from non-pathogenic members of the genus or species; (ii) inactivation of the specific gene should lead to loss in virulence; and (iii) gene complementation should lead to restoration of pathogenicity. However, it appeared that strict application of these postulates could lead to hazy conclusion as a virulence gene could be defined as such regardless of its function in the complex process of pathogenicity (Wassenaar and Gaastra, 2001). While it could be argued that molecular Koch's postulates is just a first base towards defining more precisely the role of such determinants in bacterial virulence (Falkow, 2004), an unambiguous definition of what is a virulence factor is quite useful in the post-genomic era considering the ever-growing number of coding sequences (CDS) available and the need for clear annotation in databases. Besides, in science as in philosophy, defining a term or a concept is an essential prerequisite to develop any coherent, logical and rational thinking. For example, in *L. monocytogenes*, should a protein such as the metalloproteinase Mpl that contributes to maturation of PlcB be really considered as a virulence factor? Should proteins required for full virulence of *L. monocytogenes* but also expressed in non-pathogenic listeria such as *L. innocua* really be regarded as virulence factors? How can moonlighting proteins having a clear distinct function at a different subcellular localization, such as enolase, which is also supposedly involved in virulence, be annotated? Although a coherent classification of virulence factors has already been proposed (Wassenaar and Gaastra, 2001), it has so far not been applied thoroughly to proposed virulence determinants in pathogenic bacteria and *L. monocytogenes* is no exception. Ultimately, considering the distinction between pathogenic and non-pathogenic bacteria is not as neat as originally thought (Ochman and Moran, 2001; Dobrindt *et al.*, 2004), defining a protein as a virulence factor can be even more difficult to establish (Holden *et al.*, 2004). Indeed, some so-called virulence factors are involved in more general interactions with the host or the environment as they are also present in commensal, symbiotic or environmental bacteria. From this point of view, *L. monocytogenes* is a perfect

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example of such a switch from environmental to pathogenic bacterial strains; even so the molecular mechanisms involved are as yet not fully understood (Gray *et al*., 2006). While answers to the underlying question of what really makes a pathogen remains open, virulence potential of *L. monocytogenes* can be beneficially used in order to develop therapies to fight listeriosis and even other diseases such as cancer (Brockstedt *et al*., 2004; Paterson and Johnson, 2004; Schoen *et al*., 2004; Starks *et al*., 2004; Verch *et al*., 2004; Schoen *et al*., 2005). In this new concept of inverted pathogenicity or patho-biotechnology (Russmann, 2004; Sleator and Hill, 2006; Sleator and Hill, 2007), the pathogen-specific molecular mechanisms of *L. monocytogenes* can be hijacked on purpose. To achieve this, in depth knowledge of effector molecules and protein secretion systems present in *L. monocytogenes* are part of the prerequisites for developing efficient new strategies.

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