The protein secretion systems in *Listeria*: inside out bacterial virulence

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Abstract

Listeria monocytogenes, the etiologic agent of listeriosis, remains a serious public health concern with its frequent occurrence in food coupled with a high mortality rate. The capacity of a bacterium to secrete proteins to or beyond the bacterial cell surface is of crucial importance in the understanding of biofilm formation and bacterial pathogenesis to further develop defensive strategies. Recent findings in protein secretion in *Listeria* together with the availability of complete genome sequences of several pathogenic *L. monocytogenes* strains, as well as nonpathogenic *Listeria innocua* Clip11262, prompted us to summarize the listerial protein secretion systems. Protein secretion would rely essentially on the Sec (Secretion) pathway. The twin-arginine translocation pathway seems encoded in all but one sequenced *Listeria*. In addition, a functional flagella export apparatus, a fimbrilin-protein exporter, some holins and a WXG100 secretion system are encoded in listerial genomes. This critical review brings new insights into the physiology and virulence of *Listeria* species.

Introduction

Bacterial protein secretion has been primarily investigated by researchers studying bacterial infection. In fact, bacterial pathogenicity depends greatly on the ability to secrete virulence factors which are displayed on the bacterial cell surface, secreted into the extracellular milieu or even injected directly into the host cell (Finlay & Falkow, 1997). It also appeared that pathogenic bacteria may be differentiated from their nonpathogenic counterparts by the presence of genes encoding specific virulence determinants, such as adhesins, toxins, enzymes and mediators of motility, often localized in pathogenic islands. Nevertheless, many nonpathogenic organisms also secrete proteins which are relevant to their lifestyles, e.g. environmental saprophytic bacteria may secrete cellulases or other degradative enzymes.

By embracing the notion of biodiversity so important in biology, protein secretion systems have been extensively investigated in a wide range of Gram-negative bacterial species (Fekkes & Driessen, 1999; Thanassi & Hultgren, 2000; Christie, 2001; Sandkvist, 2001; Buttner & Bonas, 2002; Sexton & Vogel, 2002; Blocker *et al*., 2003; Desvaux *et al*., 2003; Delepelaire, 2004; Desvaux *et al*., 2004; Henderson & Desvaux, 2004; Henderson *et al*., 2004; Robinson & Bolhuis, 2004). Following this approach, which was sometimes contested by researchers who instead would have favored an in-depth investigation of the protein secretion in only one model organism, namely *Escherichia coli* (Salmond & Reeves, 1993a; Wandersman, 1993), it appeared that the six major secretory pathways currently recognized (numbered from I to V, plus the chaperone/usher pathway) are not systematically present in a single bacterium and that the extent to which each pathway is used varies from one bacterium to another (Stathopoulos *et al*., 2000; Thanassi & Hultgren, 2000; Christie, 2001; Sandkvist, 2001; Buttner & Bonas, 2002; Thanassi, 2002; Delepelaire, 2004; He *et al*., 2004; Henderson & Desvaux, 2004; Henderson *et al*., 2004; Macnab, 2004). In contrast, consistent information about protein secretion in Gram-positive bacteria is still essentially restricted to *Bacillus subtilis*, which is used as a paradigm (Simonen & Palva, 1993; Tjalsma *et al*., 2000; Van Wely *et al*., 2001; Sharipova, 2002; Tjalsma *et al*., 2004; Preston *et al*., 2005). As the presence of secreted proteins is particularly relevant to bacterial virulence, scattered information can

also be found for some Gram-positive pathogenic bacteria (Van Wely *et al.*, 2001; Pallen *et al.*, 2003; Tjalsma *et al.*, 2004), such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* about the Wss [WXG100 (proteins with WXG motif of ~100 residues) secretion system] (Brodin *et al.*, 2004; Burts *et al.*, 2005; Converse & Cox, 2005), *Clostridium difficile* about the release of TcdA (Toxin of *Clostridium difficile* A) and TcdB via holins (Tan *et al.*, 2001; Voth & Ballard, 2005), or *S. aureus* and Group A *Streptococcus* about the cell surface display of proteins via sortases (Navarre & Schneewind, 1999; Rosch & Caparon, 2004).

The cell envelope of Gram-negative bacteria is composed of two biological membranes, called the cytoplasmic membrane (or inner membrane) and the outer membrane, and therefore the protein secretion system classification based on Types I, II, III, IV and V is restricted to these microorganisms (Salmond & Reeves, 1993b; Henderson *et al.*, 2000). In fact, protein secretion pathways in Gram-negative bacteria are categorized primarily by the outer membrane translocation mechanisms. Whereas in Gram-negative bacteria two biological membranes must be crossed for a protein to be secreted, in Gram-positive bacteria translocation mechanisms through the cytoplasmic membrane can truly permit protein secretion outside the cell. In Gram-positive bacteria, six protein secretion systems are currently recognized:

(1) the Sec pathway (Secretion; TC #3.A.5) (TC#: transport classification number; http://www.tcdb.org; Busch & Saier, 2002);
(2) the Tat pathway (Twin-arginine translocation; TC #2.A.64);
(3) the FEA (Flagella Export Apparatus; TC #3.A.6.1);
(4) the FPE (Fimbrilin-Protein Exporter; TC #3.A.14);
(5) the holins (#1.E);
(6) the Wss (Tjalsma *et al.*, 2000; Pallen *et al.*, 2003; Tjalsma *et al.*, 2004).

To be complete, the MscL family (Large conductance Mechanosensitive ion channel; TC #1.A.22) and the putative Tad (Tight adherence) apparatus should also be added to the list (Ajouz *et al.*, 1998; Kachlany *et al.*, 2000), even though experimental evidence is still awaited. As originally observed in Gram-negative bacteria, recent genomic surveys of the protein secretion in Gram-positive bacteria tend to indicate that the number of secretion systems present and the proteins secreted by each pathway are highly variable from one bacterial species to another (Tjalsma *et al.*, 2000, 2004; Dilks *et al.*, 2003; Yamane *et al.*, 2004; Desvaux *et al.*, 2005).

Listeria monocytogenes is an ubiquitous aeroanaerobic Gram-positive bacterium belonging to the phylum *Firmicutes*, class *Bacilli*, order *Bacillales*, family *Listeriaceae* (Garrity, 2001). This pathogenic bacterium causes severe food-borne disease called listeriosis with an overall 20–30% mortality rate (Roberts & Wiedmann, 2003). In humans, two forms of listeriosis can be discriminated: a mild noninvasive gastrointestinal illness which mainly affects healthy adults, and an invasive disease which manifests itself as septicemia or as neuropathic disease and which occurs most often in adults with underlying immunosuppression (Slutsker & Schuchat, 1999). During the infection cycle, this facultative intracellular parasite can invade and replicate into epithetial cells and macrophages, to further infect liver and spleen (Vazquez-Boland *et al.*, 2001). If the infection is not controlled at this level by an adapted immune response, bacteria can affect the central nervous system and/or foetus in pregnant women. The well established infectious lifecycle of *L. monocytogenes* into epithelial cells and macrophages involves (1) entry and formation of a vacuole, (2) lysis of the vacuole, (3) intracellular replication, (4) actin-based motility, and (5) cell-to-cell spread via formation of a two-membrane vacuole and its lysis (Cossart, 2002). Comparative genomic analyses of *L. monocytogenes* with its counterpart *Listeria innocua*, a closely related nonpathogenic *Listeria* species, revealed that bacterial virulence results from multiple gene acquisition and/or deletion events (Chakraborty *et al.*, 1997; Glaser *et al.*, 2001; Buchrieser *et al.*, 2003; Nelson *et al.*, 2004). *Listeria monocytogenes* is particularly problematic in the food industry as it can grow over a wide range of pH (4.3–9.6), temperatures (1–45 °C), salt concentrations (up to 10%) and water activity (Aw down to 0.93) (Roberts & Wiedmann, 2003). In addition to its ability to survive and multiply under conditions frequently used for food preservation, *L. monocytogenes* also forms biofilms which increases its persistance and resistance within industrial production chain lines (Farber & Peterkin, 1991; Wong, 1998; Chavant *et al.*, 2002, 2004; Kathariou, 2002). Whereas protein secretion is of key importance in both the colonization process and virulence of a microorganism, very little is known about the systems involved in *Listeria* species. The availability of the complete genome sequences of *L. monocytogenes* 1/2a EGDe, 4b F2365, and *L. innocua* Clip11262 (Glaser *et al.*, 2001; Nelson *et al.*, 2004), as well as the unfinished genome sequences of *L. monocytogenes* 1/2a F6854 and 4b H7858 (Nelson *et al.*, 2004) prompted us to summarize the protein secretion systems present by combining bibliographical and genomic analyses to provide new insights into the physiology and virulence of these microorganisms.

Table 1 summarizes the six protein secretion systems found in *Listeria*. Components of a Sec system, a Tat pathway, an FEA, an FPE, the holins, and a Wss, all of which permit protein translocation across the cytoplasmic membrane, could be identified (Fig. 1). As the genomes of *L. monocytogenes* 1/2a F6854 and 4b H7858 are unfinished, some components of the secretion systems described in this review could not be identified; final assembly of these genomic sequences may reveal homologues at a later date. As no definitive conclusion can be drawn from these two unfinished genomes, the information given should only be

Table 1. Components of the predicted protein secretory pathways present in *Listeria* species

				Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
Systems	Components	TC	Predicted cellular location	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
Sec (Secretion system)		#3.A.5																
Transmembrane components	SecY	#3.A.5	Membrane	Lmo2612	16804650	431	LMOf2365_2585	46908784	431	Lin2761	16801822	431	LMOf6854_2732	47097183	431	LMOh7858_2781	47093978	431
	SecE	#3.A.5	Membrane	Lmo0245	16802291	59	LMOf2365_0257	46906478	59	Lin0277	16799354	59	–	–	–	LMOh7858_0271	47094613	59
	SecG	#3.A.5	Membrane	Lmo2451	16804489	77	LMOf2365_2424	46908624	77	Lin2545	16801607	77	–	–	–	–	–	–
	SecDF	#2.A.6.4.1	Membrane	Lmo1527	16803567	754	LMOf2365_1546	46907755	754	Lin1562	16800630	754	LMOf6854_1574	47097024	754	LMOh7858_1628	47094198	754
	YajC	#9.B.18	Membrane	Lmo1529	16803569	109	LMOf2365_1548	46907757	109	Lin1564	16800632	110	LMOf6854_1576	47097026	109	LMOh7858_1630	47094200	754
	YidC	#2.A.9.3.2	Membrane	Lmo1379	16803419	275	LMOf2365_1398	46907607	275	Lin1416	16800484	275	LMOf6854_1421	47095963	275	LMOh7858_1471	47094519	275
				Lmo2854	16804891	287	LMOf2365_2844	46909042	287	Lin2986	16802044	287	LMOf6854_2970	47097239	287	LMOh7858_3117	47093230	287
Cytoplasmic components	FtsY	#3.A.5	Cytoplasm	Lmo1803	16803843	328	LMOf2365_1830	46908034	328	Lin1917	16800983	328	LMOf6854_1862	47097386	328	LMOh7858_1927	47093842	328
	Ffh	#3.A.5	Cytoplasm	Lmo1801	16803841	450	LMOf2365_1828	46908032	450	Lin1915	16800981	450	LMOf6854_1860	47097384	450	LMOh7858_1925	47093840	450
ATPases	SecA		Peripheral	Lmo2510	16804548	837	LMOf2365_2483	46908682	837	Lin2654	16801715	837	LMOf6854_2572	47096103	837	LMOh7858_2659	47093712	837
	SecA2		Peripheral	Lmo0583	16802626	776	LMOf2365_0612	46906828	776	Lin0592	16799667	776	LMOf6854_0624	47096876	776	LMOh7858_0643	47093324	776
Signal peptidases I	SipX		Membrane	Lmo1269	16803309	188	LMOf2365_1287	46907496	188	Lin1308	16800376	188	LMOf6854_1310	47097412	188	LMOh7858_1351	47093917	188
	SipY		Membrane	Lmo1270	16803310	189	LMOf2365_1288	46907497	189	Lin1309	16800377	189	LMOf6854_1311	47097413	189	LMOh7858_1352	47093918	189
	SipZ		Membrane	Lmo1271	16803311	180	LMOf2365_1289	46907498	180	Lin1310	16800378	180	LMOf6854_1312	47097414	180	LMOh7858_1353	47093919	180
Signal peptidases II	Lsp		Membrane	Lmo1844	16803884	154	LMOf2365_1872	46908076	154	Lin1958	16801024	154	LMOf6854_1905	47097694	154	LMOh7858_1969	47094209	154
	LspB		Membrane	Lmo1101	16803141	166	–	–	–	–	–	–	–	–	–	–	–	–
Tat(Twin-arginine translocation)		#2.A.64																
	TatA		Membrane	Lmo0362	16802407	59	–	–	–	Lin0381	16799458	59	LMOf6854_0398	47096693	59	–	–	–
	TatC		Membrane	Lmo0361	16802406	244	–	–	–	Lin0380	16799457	247	LMOf6854_0397	47096692	244	–	–	–
FPE Fimbriae Protein Exporter)	#3.A.14																	
ATPase	ComGA		Membrane	Lmo1347	16803387	340	LMOf2365_1364	46907573	340	Lin1384	16800452	340	LMOf6854_1388	47095930	340	LMOh7858_1434	47093625	340
Membrane protein ComGB			Membrane	Lmo1346	16803386	343	LMOf2365_1363	46907572	343	Lin1383	16800451	343	LMOf6854_1387	47095929	343	LMOh7858_1433	47093624	343
Prepilin peptidase ComC			Membrane	Lmo1550	16803590	236	LMOf2365_1570	46907779	236	Lin1585	16800653	236	LMOf6854_1600	47096714	236	LMOh7858_1654	47093085	236
FEA (Flagella Export Apparatus)		#3.A.6.1																
Transmembrane components	FlhA		Membrane	Lmo0680	16802722	691	LMOf2365_0716	46906931	691	Lin0688	16799763	691	LMOf6854_0726	47095245	691	LMOh7858_0745	47091643	691
	FlhB		Membrane	Lmo0679	16802721	348	LMOf2365_0715	46906930	348	Lin0687	16799762	348	LMOf6854_0725	47095244	348	LMOh7858_0744	47091642	348
	FliR		Membrane	Lmo0678	16802720	253	LMOf2365_0714	46906929	253	Lin0686	16799761	253	LMOf6854_0724	47095243	244	LMOh7858_0743	47091641	253
	FliQ		Membrane	Lmo0677	16802719	90	LMOf2365_0713	46906928	90	Lin0685	16799760	90	LMOf6854_0723	47095242	90	LMOh7858_0742	47091640	90
	FliP		Membrane	Lmo0676	16802718	255	LMOf2365_0712	46906927	255	Lin0684	16799759	255	LMOf6854_0722	47095241	255	LMOh7858_0741	47091639	255
Regulator	FliH		Cytoplasm	Lmo0715	16802757	230	LMOf2365_0751	46906966	230	Lin0723	16799798	230	LMOf6854_0762	47095281	230	LMOh7858_0781	47091678	230
ATPase	FliI		Cytoplasm	Lmo0716	16802758	433	LMOf2365_0752	46906967	433	Lin0724	16799799	433	LMOf6854_0763	47095282	433	LMOh7858_0782	47091679	433
Holins		#1.E																
Holins	TcdE	#1.E.19	Membrane	Lmo0128	16802176	140	LMOf2365_0146	46906368	140	Lin0175	16799252	140	LMOf6854_0141	47095199	140	LMOh7858_0153	47092264	140
	φA118	#1.E.21	Membrane	Lmo2279	16804318	93	–	–	–	Lin0127	16799204	93	LMOf6854_2341	47095981	93	LMOh7858_2415	47092379	93
	φ11	#1.E.11	Membrane	–	–	–	–	–	–	Lin2375	16801438	93	LMOf6854_2656	47096777	93	–	–	–
										Lin1702	16800770	86						
										Lin1295	16800363	87						

Table 1. Continued.

Systems	Components	TC	Predicted cellular location	*Listeria monocytogenes* 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
				Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
Wss (*WXG100* secretion system)		#3.A -																
ATPase	YukAB		Membrane	Lmo0061	16802109	1498	LMOf2365_0072	46906294	1497	Lin0054	16799133	1498	LMOf6854_0072	47095130	1501	LMOh7858_0075	47092188	1498
Transmembrane components	EsaA		Membrane	Lmo0057	16802105	1068	LMOf2365_0068	46906290	1068	Lin0050	16799129	1067	LMOf6854_0068	47095126	1068	LMOh7858_0071	47092184	1068
	EssA		Membrane	Lmo0058	16802106	171	LMOf2365_0069	46906291	171	Lin0051	16799130	170	LMOf6854_0069	47095127	171	LMOh7858_0072	47092185	171
	YukC		Membrane	Lmo0060	16802108	398	LMOf2365_0071	46906293	398	Lin0053	16799132	398	LMOf6854_0071	47095129	398	LMOh7858_0074	47092187	398
Cytoplasmic components	YukD		Cytoplasm	Lmo0059	16802107	83	LMOf2365_0070	46906292	83	Lin0052	16799131	83	LMOf6854_0070	47095128	83	LMOh7858_0073	47092186	83
	EsaC		Cytoplasm	Lmo0062	16802110	131	LMOf2365_0073	46906295	131	Lin0055	16799134	131	–	–	–	–	–	–

*Whole genome shotgun unfinished assembly.

considered as indicative. Consequently, this critical review will mainly focus on completed genome sequence of listerial species, id est *L. monocytogenes* EGDe, 4b F2365, and *L. innocua* Clip11262.

The Sec system (TC #3.A.5.)

Whereas SecA2 (Lenz & Portnoy, 2002; Lenz *et al.*, 2003), signal peptidases (Reglier-Poupet *et al.*, 2003a; Bonnemain *et al.*, 2004; Raynaud & Charbit, 2005) and sortases (Garandeau *et al.*, 2002; Bierne *et al.*, 2004) have been experimentally investigated in *L. monocytogenes*, Sec translocation *per se* has not been experimentally ascertained in *Listeria*. From the prediction of a large number of listerial proteins bearing putative Sec-dependent N-terminal signal peptide (Glaser *et al.*, 2001; Popowska & Markiewicz, 2004a; Trost *et al.*, 2005) and their presence in the extracellular medium or cell envelope as confirmed by proteomic analyses (Calvo *et al.*, 2005; Trost *et al.*, 2005), Sec translocation has been presumed even though direct evidence of a functional listerial Sec system and characterization of its different components is still awaited.

Sec translocase

The heterotrimeric SecYEG complex is the central component of the Sec apparatus; it forms a protein conducting channel through the cytoplasmic membrane (reviewed by Driessen *et al.*, 2001; Veenendaal *et al.*, 2004). The presence, remarkable conservation and essential nature of the Sec translocon have given rise to the notion of a general protein secretion mechanism with *E. coli* as the bacterial paradigm, but have also led to confusing statements (Desvaux *et al.*, 2004). Together with the signal recognition particle (SRP), Ffh/SRP54, and the SRP receptor, FtsY/SRP receptor-α family, SecYEG/Sec61αγβ are ubiquitous in all domains of life (Cao & Saier, 2003), as they are all encoded in *Listeria* ssp. (Table 1, Fig. 2). In *Streptococcus pyogenes*, it was recently shown that protein secretion through the Sec pathway occurs at a single microdomain of the cytoplasmic membrane concentrating the Sec translocons, i.e. the exportal (Rosch & Caparon, 2004, 2005). Although it has been suggested that such a subcellular organization may represent a paradigm in Gram-positive bacteria, it is premature to consider exportal as a general feature of Gram-positive bacteria, considering that it has been reported in only one bacterium, *Streptococcus pyogenes*. Alternatively, in *Bacillus subtilis* rather than an exportal, Sec translocons are organized in clusters following a spiral structure along the longitudinal axis of the cell (Campo *et al.*, 2004). In *E. coli*, the auxiliary complex composed of SecD, SecF and YajC is not essential to Sec-dependent export but

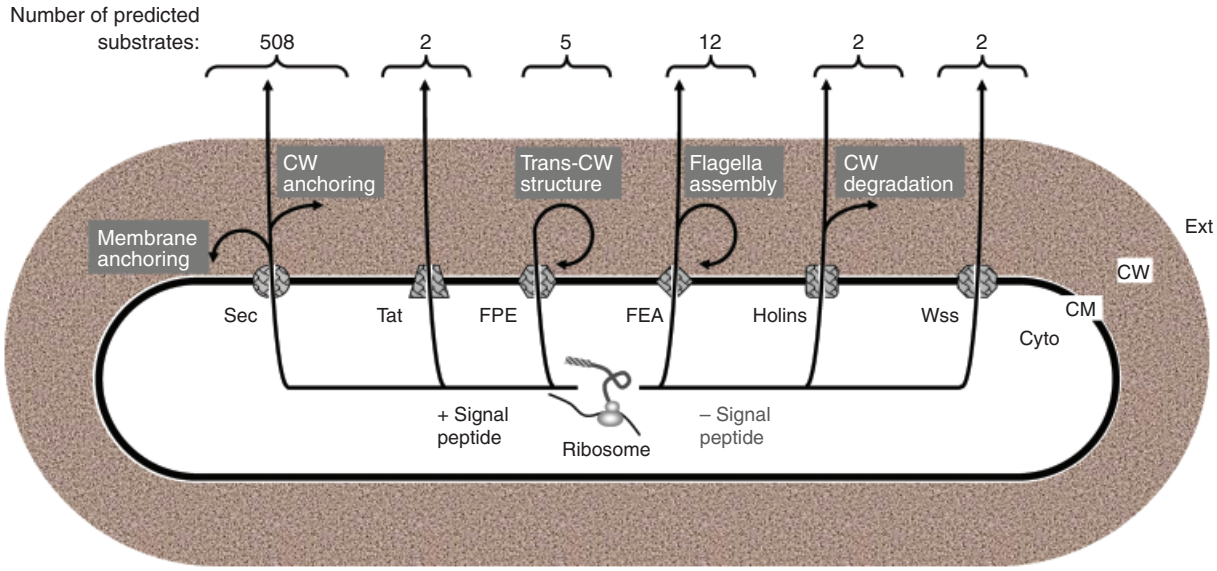


Fig. 1. Schematic representation of protein secretion pathways in *Listeria* ssp. Whereas the protein secretion systems depicted are encoded in all *Listeria* species sequenced so far, except the Tat pathway absent from *L. monocytogenes* 4b F2365, the number of putative substrates is given only for *L. monocytogenes* EGDe. Following bibliographic and bioinformatic analyses, six different protein secretion systems have been found in this microorganism (Table 1). Ribosomally synthetized proteins can be exported to various destinations depending on the presence (+) or absence (−) of an N-terminal signal peptide. Proteins exported by the Sec system can (i) remain anchored in the CM by transmembrane segment or by being covalently attached by their N-terminus to long chain fatty acids of the CM, (ii) be anchored into the CW by loose or covalent interactions, or (iii) be secreted into the extracellular medium or beyond. Some proteins secreted in a SecA2-dependent manner lack a signal peptide. Proteins exported via Tat are most certainly secreted into the extracellular medium. In Gram-positive bacteria, FEP is involved in the formation of *trans*-cell-wall structures but not proper Type 4 pili. FEA is involved in flagella assembly. Proteins exported by holins can be secreted into the extracellular milieu or involved in CW degradation. By analogy to *M. tuberculosis* or *S. aureus*, listerial WXG100 proteins are most probably secreted into the extracellular milieu. Cyto, Cytoplasm; CM, Cytoplasmic Membrane; CW, Cell Wall; Ext, Extracellular milieu; Sec, Secretion apparatus; FPE, Fimbrilin-Protein Exporter; Tat, Twin-arginine translocation; FEA, Flagella Export Apparatus; Wss, WXG100 (proteins with WXG motif of ∼100 amino acyl residues) secretion system.

increases the overall efficiency of the process (de Keyzer *et al*., 2003). As observed in *B. subtilis*, where it is involved in early translocation steps (Bolhuis *et al*., 1998), listerial SecD and SecF are fused into a single protein (Table 1).

Whereas in Gram-negative bacteria, SecB and SRP are considered two distinct protein-targeting pathways converging on the Sec translocon (Valent *et al*., 1998), SecB homologues are not found in Gram-positive bacteria (reviewed by de Cock & Tommassen, 1991; Tjalsma *et al*., 2000; Van Wely *et al*., 2001). In *B. subtilis*, CsaA [Chaperone suppressor of *E. coli* Seca(ts) mutant, protein A] was proposed to be analogous in function to SecB (Muller *et al*., 1992, 2000; Linde *et al*., 2003). However, no homologue to CsaA could be identified in *Listeria* species. In *E. coli*, SecB is now rather considered as only one of a plethora of molecular chaperones available to a newly synthetized protein fated to Sec translocation (reviewed by Randall & Hardy, 2002; Ullers *et al*., 2004). From recent investigations in *E. coli*, it appeared that SRP-independent protein translocation through the Sec translocon might either involve alternative general chaperones such as DnaK, DnaJ, GroEL or GroES, or no chaperone at all (Beha *et al*., 2003). Such chaperones are present in *B. subtilis* but their involvement in protein translocation has not as yet been investigated in any Gram-positive bacteria. It is worth mentioning that in Gram-negative bacteria, SRP is considered to be specific for inner membrane protein translocation and is not involved in the export of secretory proteins into the periplasm (de Gier & Luirink, 2001; Beha *et al*., 2003). As recently reviewed in *B. subtilis* (Sarvas *et al*., 2004), several factors are responsible for folding and quality control of protein exiting from the Sec translocon. In *L. monocytogenes*, a PrsA homologue (Lmo2219) has recently been identified and postulated as essential for cell viability (Milohanic *et al*., 2003). In *B. subtilis*, the chaperone PrsA is considered a putative peptidyl-prolyl *cis/trans* isomerase involved at a late, posttranslocational stage with its expression level constituting a bottleneck for protein secretion (Kontinen & Sarvas, 1993; Sarvas *et al*., 2004).

SecA2

Besides the SecYEG complex, the cytosolic ATPase SecA is also essential to Sec-dependent secretion. Through cycles of ATP binding and hydrolysis, SecA delivers bound precursor

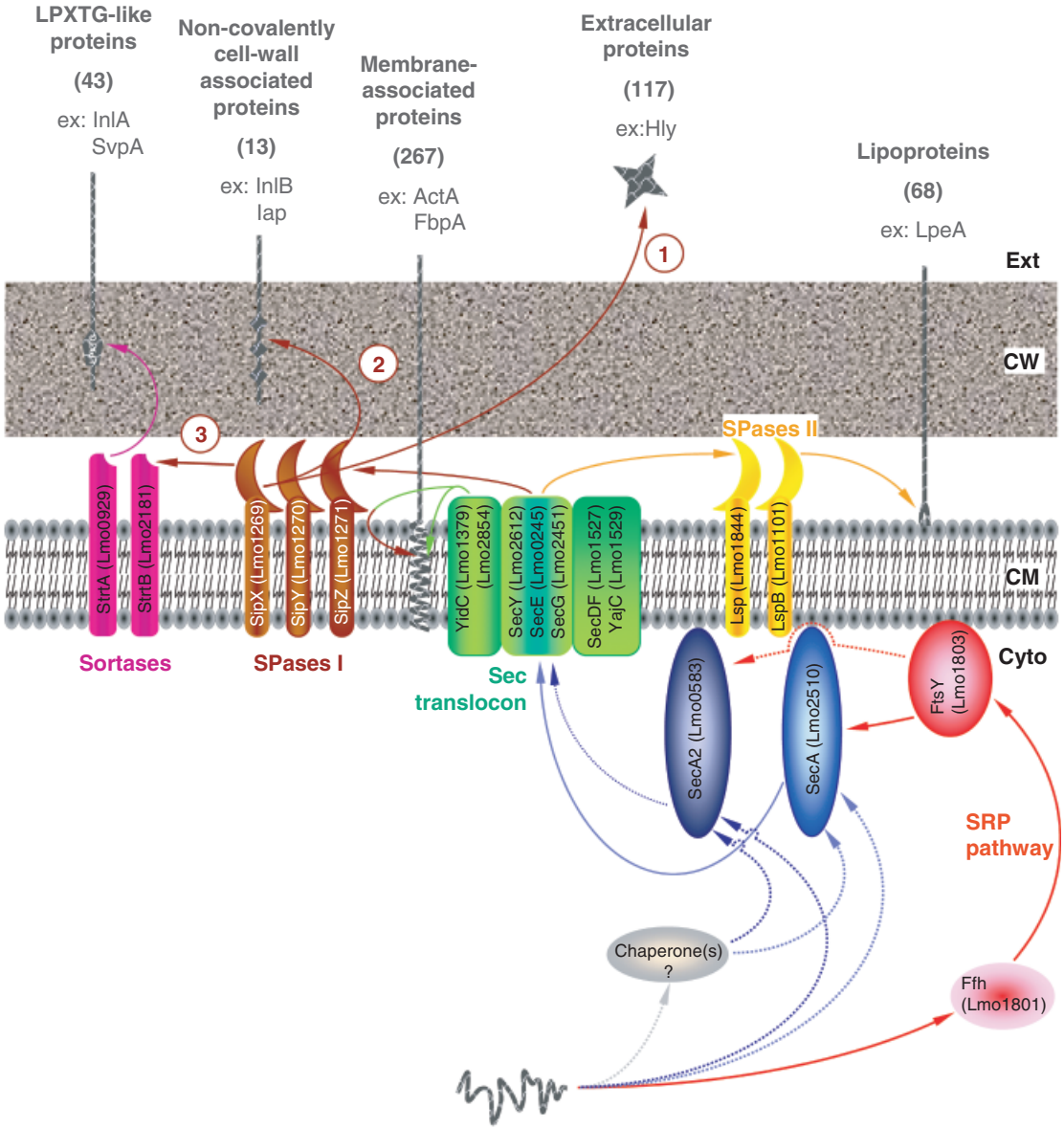


Fig. 2. Schematic overview of the Sec-dependent protein translocation in Gram-positive bacteria on *L. monocytogenes* EGDe. A protein bearing a signal peptide of class 1 or 2 is most commonly targeted via SRP before being translocated through the Sec translocon in a SecA-dependent manner, although SecA2 can also assist SecA-dependent translocation. Alternatively, some proteins with or without a signal peptide can be translocated in a SecA2-dependent manner; the contribution of Sec and/or SRP in this pathway is unknown. It has also been hypothesized that some proteins could be translocated in a SRP-independent manner, without or with unknown alternative chaperones. Once translocated via the Sec apparatus, different scenarios can apply.

(i) After cleavage of their class 2 signal peptide by SPases II, lipoproteins are covalently attached to long-chain fatty acids of the cytoplasmic membrane.

(ii) Proteins bearing an N-terminal signal peptide cleaved by SPases I can be secreted into the extracellular milieu, bind to components of the cell wall via weak interactions, or be substrates to sortases and bind covalently to the cell wall.

(iii) Proteins bearing signal peptide with a stop-transfer sequence are integrated into the CM via YidC and remain anchored by transmembrane domain(s) before being cleaved, or not, by SPases I.

At the top of the schema, the different type of translocated proteins are given with the number of predicted proteins given in brackets as well as key examples. The red arrow represents proteins targeted to the Sec translocon via SRP. The light blue arrow indicates proteins translocated in a SecA-dependent manner. The dark blue arrow indicates SecA2-dependent translocation. The grey arrow indicates proteins targeted to Sec via undetermined molecular chaperone(s). The dashed line indicates a hypothetical pathway, which has not been experimentally demonstrated yet. The green arrow indicates membrane proteins integrated via YidC. The yellow arrow indicates lipoprotein substrates to SPases II. Brown arrows indicate protein substrates to SPases I. The violet arrow indicates protein substrates to sortases. Cyto, Cytoplasm; CM, Cytoplasmic Membrane; CW, Cell Wall; Ext, Extracellular milieu; SPase, Signal Peptidase; SRP, Signal Recognition Particle.

Table 2. Characterized virulence factors substrate of the Sec system in *Listeria* species

	Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
PlcA	Lmo0201	16802247	317	LMOf2365_0212	46906433	317	–	–	–	LMOf6854_0210	47096194	317	LMOh7858_0221	47094336	317
Hly	Lmo0202	16802248	529	LMOf2365_0213	46906434	529	–	–	–	LMOf6854_0211	47096195	529	LMOh7858_0222	47094337	435
ActA	Lmo0204	16802250	639	LMOf2365_0215	46906436	604	–	–	–	LMOf6854_0213	47096197	633	LMOh7858_0224	47093146	630
PlcB	Lmo0205	16802251	289	LMOf2365_0216	46906437	289	–	–	–	LMOf6854_0214	47096198	289	LMOh7858_0225	47093147	289
InlB	Lmo0434	16802478	630	–	–	–	–	–	–	LMOf6854_0470	47096456	630	LMOh7858_0499	47092504	630
Auto	Lmo1076	16803116	572	–	–	–	–	–	–	LMOf6854_1129	47094934	435	–	–	–
SvpA	Lmo2185	16804224	569	LMOf2365_2218	46908419	569	Lin2289	16801353	573	LMOf6854_2249	47095759	569	LMOh7858_2319	47091854	569
Ami	Lmo2558	16804596	917	LMOf2365_2530	46908729	770	Lin2703	16801764	770	LMOf6854_2618	47097101	917	LMOh7858_2709	47093566	748
Sortases substrates															
Vip	Lmo0320	16802365	399	LMOf2365_0338	46906558	422	–	–	–	LMOf6854_0328	47096403	387	LMOh7858_0356	47091457	418
InlA	Lmo0433	16802477	800	LMOf2365_0471	46906689	800	–	–	–	LMOf6854_0469	47096455	800	LMOh7858_0498	47092503	797
InlJ	Lmo2821	16804858	851	LMOf6854_2939	47016058	851	–	–	–	LMOh7858_3085	47093280	916	LMOf2365_2812	46909010	916
SecA2-dependent															
Iap	Lmo0582	16802625	482	LMOf2365_0611	46906827	477	Lin0591	16799666	465	LMOf6854_0623	47096875	480	LMOh7858_0642	47093323	469
FbpA	Lmo1829	16803869	570	LMOf2365_1857	46908061	570	Lin1943	16801009	570	LMOf6854_1889	47096550	570	LMOh7858_1954	47093066	570
Lipoprotein															
LpeA	Lmo1847	16803887	310	LMOf2365_1875	46908079	310	Lin1961	16801027	310	LMOf6854_1908	47097624	310	LMOh7858_1972	47094212	310

*Whole genome shotgun unfinished assembly.

proteins to the Sec translocon leading to the stepwise export of the protein (reviewed by Economou *et al.*, 1995). In *L. monocytogenes*, a novel paralogue of SecA (SecA2) has been identified (Lenz & Portnoy, 2002). The presence of such a paralogue, originally identified in *Mycobacterium smegmatis* (Braunstein *et al.*, 2001), was reported later in *Streptococcus gordonii* (Bensing & Sullam, 2002) and *M. tuberculosis* (Braunstein *et al.*, 2003) but not in Gram-negative bacteria. Unlike *S. gordonii* (Bensing & Sullam, 2002), the duplication of SecA in *Listeria* species is not accompanied by duplication of SecY. Like SecA, SecA2 would couple ATP hydrolysis with Sec-dependent protein translocation across the cytoplasmic membrane (Braunstein *et al.*, 2001; Lenz & Portnoy, 2002); however, the convergence of these two pathways towards Sec translocon has not been investigated in the literature (Fig. 2). Contrary to SecA, SecA2 is not essential for cell viability but would endorse the dual function of assisting SecA in the export of proteins to improve the overall translocation efficiency (Braunstein *et al.*, 2001, 2003), and mediating the secretion of a specific subset of proteins contributing to bacterial virulence, as exemplified with fimbrial adhesins (Chen *et al.*, 2004), platelet-binding protein (Bensing & Sullam, 2002), or superoxide dismutase A (Braunstein *et al.*, 2003). In *L. monocytogenes*, SecA2 is clearly involved in the secretion of the cell-wall hydrolase Iap (Invasion-associated protein) (Lenz & Portnoy, 2002), NamA (N-acetylmuramidase A) (Lenz *et al.*, 2003) and FbpA (Fibronectin-binding protein A) (Dramsi *et al.*, 2004). While Iap, also called p60 (protein of 60 kDa), or CwhA (Cell wall hydrolase A) possesses a putative signal sequence as revealed by bioinformatic analysis using SignalP v3.0 (Signal Peptide prediction; http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al.*, 2004), no signal peptide could be detected in the membrane protein FbpA (Dramsi *et al.*, 2004). Absence of signal peptide was also observed in SodA (Superoxide dismutase A) from *M. tuberculosis* (Braunstein *et al.*, 2003). SecA2 is also encoded in *L. innocua* genome (Table 1), indicating it is not a specific feature of Gram-positive pathogenic bacteria. The contribution of SRP in the SecA2-dependent protein secretion pathway has not been investigated yet.

Signal peptidases

Except for some SecA2-dependent proteins, all proteins targeted to the Sec translocon possess an N-terminal signal peptide. The signal sequence is composed of three domains:
(1) a positively charged amino terminus, called N-domain,
(2) a hydrophobic core region or H-domain,
(3) a consensus signal peptidase (SPase) recognition site, also called C-domain (reviewed by Fekkes & Driessen, 1999).

Sec-dependent translocated proteins can be membrane integrated and/or have their signal peptide removed by the action of a signal peptidase (SPase). Membrane-bound SPases I and II permit the cleavage of class 1 and class 2 signal peptides present in precursor proteins and lipoproteins, respectively (reviewed by Fekkes & Driessen, 1999; Van Wely *et al.*, 2001).

Following cleavage by SPases I, the proteins can either be released into the extracellular medium, bind to cell-wall components by weak interactions, or be covalently anchored to the cell wall via sortases (Fig. 2). In *L. monocytogenes*, three SPases I have been identified and characterized, SipX (Signal peptidase X), SipY and SipZ (Table 1), revealing differential roles in bacterial virulence (Bonnemain *et al.*, 2004; Raynaud & Charbit, 2005). Whereas SipX does not appear to be involved in intracellular multiplication, its absence results in significantly reduced bacterial virulence. SipZ permits secretion of key virulence factors, such as lecithinase phosphatidylcholine-specific phospholipases C (PlcB) and Hly (Haemolysin), i.e. lysteriolysin O, but its absence restricts intracellular bacterial multiplication. Inactivation of *sipY* has no detectable effect (Bonnemain *et al.*, 2004). Despite overlapping substrate specificities between SipX and SipZ, the latter is the major SPase of *L. monocytogenes*, at least with respect to virulence (Bonnemain *et al.*, 2004). It is worth mentioning here that Hly is a member of an expansive family of highly conserved pore-forming toxins, known as cholesterol-dependent cytolysins (TC #1.C.12.) (Alouf, 2000), permitting cytolysin-mediated translocation, in which a bacterial effector protein is translocated into the cytoplasm of an eukaryotic host cell (Madden *et al.*, 2001; Meehl & Caparon, 2004; Desvaux *et al.*, 2006b). In *Listeria*, noncovalent binding of protein to bacterial cell-wall (Desvaux *et al.*, 2006a) involves either (1) modules of around 80 amino acids containing the dipeptide glycine-tryptophan, i.e. modules GW, which interact with lipoteichoic acids, as found in InlB, and represents a total of nine predicted proteins in *L. monocytogenes* EGDe or (2) p60-like proteins interacting via uncharacterized domain(s), i.e. four predicted proteins in *L. monocytogenes* EGDe (Galsworthy *et al.*, 1990; Glaser *et al.*, 2001; Trost *et al.*, 2005; reviewed by Cabanes *et al.*, 2002; Popowska & Markiewicz, 2004a) (Fig. 2).

After cleavage by SPase II, lipoproteins are covalently attached by their N-terminus to long chain fatty acids of the cytoplasmic membrane. In all *Listeria* sequenced so far, the SPase II Lsp (Lipoprotein signal peptidase) could be identified (Table 1). From its characterization in *L. monocytogenes*, it appeared Lsp is involved in maturation of virulence factor LpeA (Lipoprotein promoting entry A) (Reglier-Poupet *et al.*, 2003b), and is of critical importance for efficient phagosomal escape (Bonnemain *et al.*, 2004). In *L. monocytogenes* EGDe, an additional SPase II, LspB, is encoded but remains uncharacterized (Table 1). From bioinformatic analyses, 68 proteins were predicted as lipoproteins in *L. monocytogenes* EGDe (Glaser *et al.*, 2001; Trost *et al.*, 2005).

Sortases

Sortases are responsible for the covalent attachment of proteins to the cell wall of Gram-positive bacteria (reviewed by Navarre & Schneewind, 1999; Ton-That *et al.*, 2004). Protein substrates of this enzyme harbor a conserved C-proximal LPXTG motif, followed by a transmembrane-spanning hydrophobic domain and a hydrophilic charged domain at the C-terminus (Pallen *et al.*, 2001). During secretion through the Sec apparatus, membrane-associated sortase recognizes the LPXTG motif, cleaves it and covalently links the protein to the cell-wall precursor lipid II, which is subsequently incorporated into the cell wall along with the anchored protein (reviewed by Ton-That *et al.*, 2004). It has also been reported that in some Gram-positive bacteria, sortase catalyzes protein polymerization, leading to the ordered assembly of pilus structure on the bacterial cell surface (reviewed by Ton-That & Schneewind, 2004). Variability from the consensus LPXTG motif has been reported (Pallen *et al.*, 2001) and further associated to different sortase subfamilies (reviewed by Comfort & Clubb, 2004). A classification of sortases into four classes designated A, B, C and D has recently been proposed (Dramsi *et al.*, 2005). In *Listeria* species, two sortases are present, SrtA (Sortase A) and SrtB, belonging to the newly defined classes A and B, respectively (Dramsi *et al.*, 2005) (Table 3). In *L. monocytogenes*, SrtA is required for bacterial virulence and permits the cell-wall anchoring of proteins harboring an LPXTG motif, among them the invasion protein InlA (Internalin A) (Bierne *et al.*, 2002; Garandeau *et al.*, 2002). From genomic analyses of *L. monocytogenes* EGDe, 41 proteins were identified with an LPXTG motif; 11 of them are absent from *L. innocua*, including InlA (Glaser *et al.*, 2001; Trost *et al.*, 2005). Whereas SrtA allows the anchoring of most of the proteins into the peptidoglycan, the role of SrtB seems minor (Bierne *et al.*, 2004; Pucciarelli *et al.*, 2005). In *L. monocytogenes* EGDe, SrtB would be involved in the cell-wall anchoring of only two proteins, the virulence factor SvpA (Surface virulence-associated protein A) and Lmo2186 (SvpB) (Bierne *et al.*, 2004) (Table 2). Interestingly, these proteins do not harbor a consensus NPQTN motif or even LPXTG (reviewed by Comfort & Clubb, 2004), but most likely a NXZTN motif, which suggests a lower stringency of the recognition motif of SrtB than SrtA (Borezee *et al.*, 2001; Bierne *et al.*, 2004). Besides SrtA and SrtB, it cannot be excluded that additional enzymes non-ribosomally synthesized and called LPXTGases may also be present and involved in cell-wall anchoring of LPXTG-like proteins in *Listeria* (Lee *et al.*, 2002). In conclusion, a total of 43 sortase-recognized proteins would be encoded in *L. monocytogenes* EGDe (Fig. 2).

Table 3. Sortases in *Listeria* species

	Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
StrA	Lmo0929	16802969	222	LMOf2365_0950	46907163	222	Lin0929	16800000	222	LMOf6854_0976	47096216	222	LMOh7858_0991	47092138	222
StrB	Lmo2181	16804220	246	LMOf2365_2214	46908415	246	Lin2285	16801349	246	LMOf6854_2245	47095755	246	LMOh7858_2315	47091850	246

*Whole genome shotgun unfinished assembly.

YidC homologue

In *E. coli*, the integral membrane protein YidC, which is associated with the SecYEGDF-YajC complex, permits cytoplasmic membrane insertion of polytopic membrane proteins (Scotti *et al.*, 2000). Some authors have suggested the classification of YidC as an alternative inner membrane translocation pathway (Yen *et al.*, 2002a). In fact, it appears the YidC translocation pathway is quite versatile, which does not facilitate a definitive classification, as it is not necessarily SecA-dependent, SecB-dependent or Sec-dependent (Samuelson *et al.*, 2000; Beck *et al.*, 2001; reviewed by Froderberg *et al.*, 2003). In *B. subtilis*, there are two paralogues of YidC, SpoIIIJ and YqjG (reviewed by Tjalsma *et al.*, 2000; Van Wely *et al.*, 2001). Whereas SpoIIIJ is required for sporulation, as gene mutations block sporulation at stage III (Errington *et al.*, 1992), YqjG is dispensable for this developmental process (Murakami *et al.*, 2002). Still, the presence of either SpoIIIJ or YqjG is required for cell viability, indicating that SpoIIIJ and YqjG have different but overlapping functions in *B. subtilis* (Murakami *et al.*, 2002). It was further demonstrated that SpoIIIJ and YqjG are involved in both membrane protein biogenesis and protein secretion (reviewed by Tjalsma *et al.*, 2003; Desvaux *et al.*, 2006a). As in *B. subtilis*, two YidC paralogues are encoded in *Listeria* species (Table 1). In *E. coli*, all inner membrane proteins are SRP- and YidC-dependent (Froderberg *et al.*, 2003). Whereas most integral proteins are not synthesized with a cleavable signal peptide, some integral membrane proteins are (Facey & Kuhn, 2004). These proteins are distinguished from secretory proteins in that they contain additional hydrophobic stop-transfer sequences that anchor the protein in the membrane. ActA is known to remain anchored into the cytoplasmic membrane via a C-terminal hydrophobic tail; however, neither cleavage of its signal peptide nor its integration into the cytoplasmic membrane via Sec and/or YidC has been demonstrated (Kocks *et al.*, 1992; reviewed by Popowska & Markiewicz, 2004a). Similarly, although the cytoplasmic membrane localization of the SecA2-dependent protein FbpA (lacking a signal peptide) has been demonstrated (Dramsi *et al.*, 2004), membrane translocation via Sec and/or YidC has not yet been investigated.

Sec substrates

From a recent *in silico* analysis of the secretome of *L. monocytogenes* EGDe using a battery of web-based bioinformatic tools, 509 coding sequences were predicted as targeted to the Sec translocon (Trost *et al.*, 2005). Among them, 266 coding sequences were predicted as integral and membrane associated proteins, 54 as cell-wall associated proteins, 68 as lipoproteins and 121 as proteins secreted into the extracellular milieu (Trost *et al.*, 2005). The 14 virulence factors

characterized so far as involved in *L. monocytogenes* infection cycle are most likely all translocated via the Sec system (Table 2). For an in-depth understanding of their respective functions in bacterial virulence, which is beyond the scope of this review, we refer the reader to other excellent and updated reviews (reviewed by Dussurget *et al.*, 2004; Krawczyk-Balska & Bielecki, 2004; Popowska & Markiewicz, 2004a). Interestingly, some of the genes encoding these virulence factors are absent from some pathogenic *L. monocytogenes* strains, and some of them are even present in the nonpathogenic species *L. innocua* (Table 2). Compared to each other, the predicted protein size of some orthologues varies slightly. In most cases this is the result of the loss of internal fragment of few base pairs at the genetic level. In *L. monocytogenes* 1/2a F6854 and 4b H7858 the ORF is only partial because located at one end of a contig; as already pointed out, no definitive conclusion can be drawn from these two unfinished genome sequences until completion of genome assembly.

Summing up, a correction must be applied to the number of coding sequences recently predicted as targeted to the Sec translocon in *L. monocytogenes* EGDe (Trost *et al.*, 2005). Instead of 509, 508 coding sequences are most likely targeted to the Sec translocon. As originally reported, there are 68 lipoproteins (Glaser *et al.*, 2001) and 13 noncovalently cell-wall associated proteins, including nine proteins with GW motifs and four p60-like proteins (Glaser *et al.*, 2001; Trost *et al.*, 2005). However, including substrates to StrB, the number of predicted sortase-recognized proteins attains 43 instead of 41 (Glaser *et al.*, 2001; Trost *et al.*, 2005). Including FbpA and the 11 proteins predicted with a hydrophobic anchor, the number of transmembrane proteins reaches 267 instead of 255 (Trost *et al.*, 2005), and removing Type 4 prepilins, ComGC, ComGD, ComGG and ComGE, originally considered to be targeted to the Sec translocon (see 'The fimbrilin protein exporter' below), the number of proteins predicted as secreted into the extracellular medium decreases from 121 to 117 (Trost *et al.*, 2005) (Fig. 2). Recent proteomic analyses of *L. monocytogenes* EGDe subproteome identified (1) 40/68 lipoproteins, including LpeA, (2) 2/9 GW proteins, InlB and Ami, (3) 4/4 p60-like proteins, Iap, p45 (Lmo2505), Nam (N-acetyl muramidase, Lmo2691) and PbpA (Penicillin-binding protein 2A, Lmo1892), (4) 15/43 sortase-recognized proteins, including InlA, SvpA and SvpB, and (5) 54/117 extracellular proteins, including Hly, PlcA and PlcB (Schaumburg *et al.*, 2004; Calvo *et al.*, 2005; Trost *et al.*, 2005).

The Tat system (TC #2.A.64.)

The Tat system denomination comes from the fact that protein precursors translocated through this pathway possess an N-terminal signal peptide harbouring a characteristic and essential twin arginine motif, (S/T)TRRXFLK, which straddles the N-domain and the hydrophobic H-domain (reviewed by Berks *et al.*, 2000). Among prokaryotes, the *E. coli* Tat machinery has been the most investigated over the years (reviewed by Berks *et al.*, 2000, 2003; Müller, 2005; Palmer *et al.*, 2005). The translocation machinery is composed of TatA, TatB, TatC and TatE. TatA, TatB and TatE are homologous, but TatA and TatE, which are more similar to each other than they are to TatB, can partially substitute for each other. TatC is required for interaction of TatA with TatB (Bolhuis *et al.*, 2001). In *E. coli*, TatB and TatC are essential for translocation (Bogsch *et al.*, 1998; Sargent *et al.*, 1999). The generally accepted model proposes a cyclical assembly during Tat translocation (Mori & Cline, 2001; Robinson & Bolhuis, 2004; Berks *et al.*, 2005; Müller, 2005). In the resting state, Tat machinery components are present separately in the cytoplasmic membrane. First, Tat substrate protein precursor binds to the TatBC complex in an energy-independent step, with TatC appearing as the primary site of signal-peptide recognition (Alami *et al.*, 2003). Following this binding step, the TatBC–substrate complex then associates with TatA in a step driven by transmembrane proton electrochemical gradient (Mori & Cline, 2002). This association would persist until completion of protein transport across the membrane driven by proton motive force. Tat signal peptide is subsequently cleaved by signal peptidase and Tat machinery components disassembled. An alternative model, however, has recently been proposed where the membrane integration could precede Tat-dependent translocation and the membrane targeting process may require ATP-dependent N-terminal unfolding-steps energy (Bruser & Sanders, 2003). Still, it is likely that TatB and TatC remain associated throughout the cycle, and TatA most certainly constitutes the protein-conducting channel of the Tat system. TatA complexes form transmembrane ring-shaped structures of variable internal diameters depending on the number of protomers, which thus match the size of the Tat substrate proteins being transported (Gohlke *et al.*, 2005). The cytoplasmic side of the channel is likely closed by a lid that might gate its access. In contrast to the Sec-dependent pathway in which proteins are translocated in an extended conformation, the Tat pathway is considered to transport proteins in a folded state (reviewed by Robinson & Bolhuis, 2001). While translocation of folded protein was originally demonstrated in eukaryotic cells, i.e. plant thylakoid (Roffey & Theg, 1996; Musser & Theg, 2000; Mori & Cline, 2001), in bacteria there is growing evidence that Tat transporter only tolerates folded protein and somehow rejects unfolded ones (Santini *et al.*, 1998; DeLisa *et al.*, 2003; reviewed by Robinson & Bolhuis, 2004; Müller, 2005; Palmer *et al.*, 2005). A recent genomic survey of the prokaryotic protein secretion suggests the Tat pathway instead of the Sec

pathway is used predominantly by certain bacteria and archaea (Dilks *et al*., 2003). In *B. subtilis*, whereas no TatB homologue is present (Dilks *et al*., 2003), two gene clusters containing *tatA* and *tatC* have been reported, as well as a third *tatA* gene found independently elsewhere on the chromosome (Jongbloed *et al*., 2004; reviewed by Van Dijl *et al*., 2002; Yen *et al*., 2002b; Tjalsma *et al*., 2004). The reason for the duplication of *tatC* gene is unknown nor is it known whether some of these TatA proteins could functionally substitute with TatB (Jongbloed *et al*., 2000).

Although components of a Tat pathway were originally identified in *L. innocua* Clip11262 and *L. monocytogenes* EGDe (Dilks *et al*., 2003) as well as in *L. monocytogenes* 1/2a F6854 (Table 1), the functionality of this pathway has not been experimentally investigated. As observed in *B. subtilis*, no TatB homologue could be found. *tatA* and *tatC* genes are systematically clustered together. While *B. subtilis* encodes several paralogues of *tatA* and *tatC*, only one copy of *tatA* and *tatC*, respectively, seems encoded in listerial genomes (Table 1). In *L. monocytogenes* 4b H7858, no components of the Tat pathway could be identified, but, once again, as the assembly of the genome sequence of *L. monocytogenes* 4b H7858 has not been completed, it cannot be excluded that future analyses may reveal their presence. On the other hand this pathway seems absent from *L. monocytogenes* 4b F2365.

Following TATFIND v1.2 search, only two potential substrates to the Tat system could be identified in *L. monocytogenes* EGDe (Dilks *et al*., 2003) (Table 4). A putative β-ketoacyl-acyl carrier protein synthase II is encoded in all sequenced *Listeria* ssp., even *L. monocytogenes* 4b F2365, where the predicted iron-dependent peroxidase is, however, absent. In *L. monocytogenes* 4b H7858, the gene encoding this protein could not be identified either, and the β-ketoacyl-acyl carrier protein synthase II is reported shorter than in other listerial species; however, this latter gene is encoded right at this end of a contig and completion of genome assembly could reveal the presence of both genes at a later date. Compared to some Gram-positive bacteria possessing a Tat pathway, such as *Streptomyces coelicolor*, the number of putative Tat substrates is rather low in *Listeria* (Dilks *et al*., 2003). However, whereas it has long been assumed that Tat signal peptide motif was highly specific and conserved, recent investigations tend to temper such assertions (Robinson & Bolhuis, 2004). Substitution of one or both arginine residues by lysine could still permit targeting and translocation of the protein through the Tat translocon (Ize *et al*., 2002). Some proteins harbouring very distantly related twin arginine motifs, for example the penicillin amidase of *E. coli*, which possesses a signal sequence bearing two arginines separated by an asparagine, can nevertheless be routed towards the Tat pathway (Ignatova *et al*., 2002). Some proteins predicted as Tat substrates

Table 4. Proteins potentially secreted via the Tat system in *Listeria* species

	Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
Iron-dependent peroxidase	Lmo0367	16802412	421	–	–	–	Lin0386	16799463	421	LMOf6854_0403	47096698	421	–	–	–
β-ketoacyl-acyl carrier protein synthase II	Lmo2201	16804240	413	LMOf2365_2234	46908435	413	Lin2304	16801368	413	LMOf6854_2265	47095775	413	LMOh7858_2335	47093726	370

*Whole genome shotgun unfinished assembly.

appeared to be strictly Sec-dependent (Jongbloed *et al.*, 2002). Sec-dependent protein harbouring KK motif can be partially exported by the Tat pathway (Pradel *et al.*, 2003). Thus, the presence of other substrates that could not be identified by bioinformatic approaches is possible and should be further confirmed by experimental work.

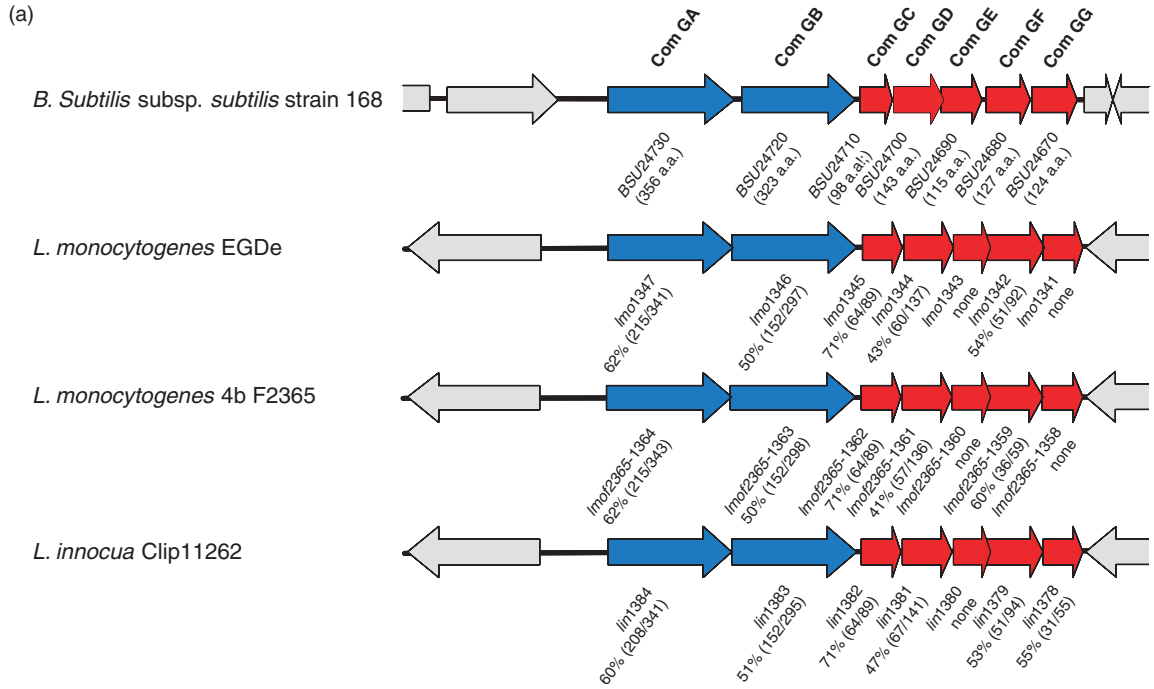
The fimbrilin-protein exporter (FPE; TC #3.A.14.)

The Com (Competence development) pathway, which permits bacterial DNA uptake across the cytoplasmic membrane (reviewed by Dubnau, 1997, 1999; Dubnau & Provvedi, 2000; Chen & Dubnau, 2004), is sometimes mistakenly referred to as the FPE system. Actually, the Com pathway involves both the bacterial competence-related DNA transformation transporter (TC #3.A.11) and the FPE system. The current model in Gram-positive bacteria, essentially based on investigations in *B. subtilis*, suggests that the three proteins necessary for the bacterial competence-related DNA transformation transporter, designated ComEA, ComEC and ComFA, assemble to form a translocation apparatus permitting the transport of DNA across the cytoplasmic membrane, whereas pilin-like proteins are secreted and assembled by the FPE system. Components of the FPE system are encoded by the *comG* locus consisting of seven ORFs (*comGA-GG*) and *comC* located elsewhere on the chromosome. ComGA is an ATPase localized to the cytoplasmic side of the membrane and postulated to act as an energy-transducing protein. ComGB is an integral membrane protein essential for bacterial competence. ComC is a Type 4 prepilin peptidase. The remaining proteins, ComGC, ComGD, ComGE, ComGF and ComGG, exhibit similarities with Type 4 prepilins. In *B. subtilis*, these Type 4 prepilins, exported through the FPE composed of ComC, ComGA and ComGB, do not form a proper Type 4 pilus (Tfp) but a *trans*-cell-wall structure that would function on the outer surface of the cytoplasmic membrane for the binding of DNA, its passage through the cell wall, and its presentation to the DNA translocation machinery (reviewed by Dubnau, 1997, 1999; Dubnau & Provvedi, 2000; Claverys & Martin, 2003; Hamoen *et al.*, 2003). Because proteins encoded by the *comG* operon and *comC* of Gram-positive bacteria resemble proteins found in the Type II secretion system (TTSS), the Tfp assembly apparatus, and Type IV secretion system (TFSS) of Gram-negative bacteria, they have also been collectively called PSTC (Pilus/Secretion/Twitching motility/Competence) (Fussenegger *et al.*, 1997; Dubnau, 1999; Peabody *et al.*, 2003).

The present review constitutes the first report of genes encoding FPE in *Listeria* and, as a consequence, this system has never been experimentally investigated in these bacterial species. In the completed genome sequences for *Listeria*, i.e. *L. monocytogenes* 1/2a EGDe, 4b F2365 and *L. innocua* Clip11262, the *comG* loci present are highly similar in gene sequence and synteny to the *comG* locus from *B. subtilis* (Fig. 3a). Whereas some listerial genes in these loci were annotated as hypothetical, in *L. monocytogenes* 4b F2365 they were systematically and inaccurately annotated as encoding general secretion pathway proteins. As in *B. subtilis*, the gene encoding ComC, a Type 4 prepilin peptidase, does not cluster with *comG* genes but is located elsewhere on the chromosome (Table 1). Whereas significant sequence similarities could be found between proteins encoded by *comGC*, *comGD* and *comGE* from *B. subtilis* and putative proteins encoded by genes at the similar positions in *comG* loci of *Listeria* species (Fig. 3a), alignment using BLAST (Basic Local Alignment Search Tool) 2 sequences (Tatusova & Madden, 1999) could not found sequence similarity between proteins encoded at the *comGF* locus. However, from CDD v2.03 (Conserved Domain Database; http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer *et al.*, 2003) searches, a competence protein ComGF domain was systematically found (COG4940; *E*-values $\leq 1.0 \times 10^{-14}$). Similarly, at the *comGG* locus, no similarity could be found between proteins encoded in *B. subtilis* 168 and *Listeria*, except for Lin1378 from *L. innocua* Clip11262 (Fig. 3a); using Lin1378 as a query, PSI-BLAST [Position-Specific Iterated BLAST; (Altschul *et al.*, 1990, 1997)] searches revealed that listerial proteins encoded at this gene locus were all homologous to ComGG proteins found in other Gram-positive bacterial species (*E*-values $\leq 3.0 \times 10^{-28}$ after four iterations). As in *B. subtilis,* the coding sequences is present downstream of *comGC* overlap (reviewed by Dubnau, 1997); it was suggested that such a gene arrangement would ensure close co-ordination of protein synthesis. In *B. subtilis*, the Type 4 prepilins bear characteristic class 3 N-terminal signal peptides with a consensus (K/R)G(F/Y)BXZE motif present between the n- and h-domains, with cleavage occurring between G and (F/Y) (reviewed by Dubnau, 1997; Tjalsma *et al.*, 2000). In *Listeria* species, although such a consensus is found in ComGC homologues (Fig. 3b), variations around this motif are found in other putative Type 4 prepilins:

(1) glycine residue at position -1 of the cleavage site can be replaced by an alanine residue,
(2) asparagine, serine or proline can replace lysine or arginine residue at -2,
(3) glutamine residue at position $+5$ can be replaced by a phenylalanine.

Some variability in the amino acid present at this latter position was previously reported (reviewed by Tjalsma *et al.*, 2000; Albers & Driessen, 2002). In contrast, phenylalanine at position$+1$ of the cleavage site is highly conserved in all putative listerial Type 4 prepilins as well as leucine residue



(b)

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ComGC_BSU24710_16079527_Bsu          MNEKG FTLVEMLIVLFIISILLLITIPNVTKHNQTIQKKG
ComGD_BSU24700_16079526_Bsu        MNIKLNEEKG FTLLESLLVLSLASILLVAVFTTLPPAYDNTAVRQ
ComGE_BSU24690_16079525_Bsu          MWRENKG FSTIETMSALSLWLFVLLTVVPLWDKLMADEKMAE
ComGG_BSU24670_16079523_Bsu          MYRTRG FIYPAVLFVSALVLLIVNFVAAQYISRCMFEKETK
ComGC_Lmo1345_16803385_Lme         MFKKKIDWRDERG FTLVEMLIVLLVVSVLLLLTIPNIVSQSKSINDKG
ComGD_Lmo1344_16803384_Lme           MKING FTLLEMLLVLTISFTLITLTIFPISSTLSTLREKQ
ComGE_Lmo1343_16803383_Lme           MNKING FSLVESMVSLLLFAMVCSFLLPTAMTIFEKLDHQK
ComGF_Lmo1342_16803382_Lme     MQQINWKKNVSFRSGSPA FTLLETILSITIILSISLLIPLFFQCYNKTIQLSN
ComGG_Lmo1341_16803381_Lme           MKTNA FTLPFTIFIALISILIVTGSASIFKTQIQYEKMIQ
ComGC_LMOf2365-1362_46907571_Lmf   MFKKKIDWRDERG FTLVEMLIVLLVVSVLLLLTIPNIVSQSKSINDKG
ComGD_LMOf2365-1361_46907570_Lmf     MKING FTLLEMLLVLTISFTLITLTIFPISSTLSTLREKQ
ComGE_LMOf2365-1360_46907569_Lmf     MNKING FSLVESMVSLLLFSMVCSFLLPTAMTIFEKLDYQK
ComGF_LMOf2365-1359_46907568_Lmf MQKINRKKNVSFRNGSPA FTLLETILSITIILSISSLIPLFFQCYNKTIQISN
ComGG_LMOf2365-1358_46907567_Lmf     MKTNA FTLPFTIFIALISILIVTGTASIFKTQIQYEKMIQ
ComGC_Lin1382_16800450_Lin         MYKFKIDWKDDRG FTLVEMLIVLLVVSVLLLLTIPNIVTQSKSINNKG
ComGD_Lin1381_16800449_Lin           MKKNA FTLLEMLLVISISLTMITLTIYPIANTITALTEKQ
ComGE_Lin1380_16800448_Lin           MNKVNG FTLVESIVSLLLFSIVCSFLLPISMTIFQKLDQQK
ComGF_Lin1379_16800447_Lin     MQKLIQLKNVHYRKSTSA FTLLETILSITIVLSISSLIPLFFECYHKTIQLSN
ComGG_Lin1378_16800446_Lin           MKNNG FTLPFILFIALITILIVTGSASIFKTQIQYEKQLQ
```

Fig. 3. The *comG* locus in *Listeria*. (a) Comparison of the *comG* loci from completed genome sequence *Listeria* species with *comG* locus of *B. subtilis* ssp. *subtilis* strain 168. Genes of each locus are colored: blue, genes predicted as encoding membrane proteins composing the FPE (Fimbrilin-Protein Exporter); red, genes encoding proteins predicted as Type 4 prepilins. Names of proteins encoded by each gene of the clusters are indicated in bold letters at the top of the schema. Locus tags are indicated under each gene. For *B. subtilis*, the size of the encoded protein is given in number of amino acids. For proteins encoded in *Listeria* species, whenever possible, the percentages of similarity (over *x* matching amino acids out of *y* amino acids: *x*/*y*) are also given in comparison with proteins of *B. subtilis* 168 [results were obtained with BLAST 2 sequences using default parameters except that filter was turned off (Tatusova & Madden, 1999)]. (b) Sequence alignment of predicted signal peptides substrate of the Type 4 prepilin peptidase ComC in completed genome sequence *Listeria* species and in *B. subtilis* ssp. *subtilis* strain 168. The positively charged n-domain is indicated in bold letters and the hydrophobic h-domain in grey letters. The putative recognition sequence by the prepilin peptidase is underlined. Predicted cleavage site is indicated with a gap in the amino acid sequence. Sequence alignment was performed using CLUSTALW (Higgins *et al*., 1994) with minor manual refinement using BIOEDIT v7.0.4.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; Hall, 1999) and shading set up at greater than 65% consensus. Bsu, *B. subtilis* 168; Lme, *L. monocytogenes* 1/2a EGDe; Lmf, *L. monocytognes* 4b F2365; Lin, *L. innocua* Clip11262.

at+3 (Fig. 3b). Compared to *B. subtilis*, the consensus motif found in *Listeria* is slightly different, NGFTLXE. In the end, it seems a complete set of proteins related to FPE system is encoded in *Listeria* species (Tables 1–5) but further experimental investigations would be required to check the functionality of this system.

The flagella export apparatus (FEA; TC #3.A.6.)

The bacterial flagellum can be subdivided into five major parts: the flagella motor/switch, the basal body, the hook/junction proteins, the flagellar filament, and the FEA (reviewed by Macnab, 2003, 2004). In Gram-negative bacteria, the FEA is related to the Type III secretion system together with the Hrp (Hypersensitive response and pathogenicity) pilus export apparatus and the injectisome export apparatus (Young *et al*., 1999; Jin & He, 2001; reviewed by Blocker *et al*., 2003; Pallen *et al*., 2005a, b). Based on studies of Gram-negative bacteria, the FEA is composed of the transmembrane components FlhA, FlhB, FliO, FliP, FliQ and FliR, the chaperone FliJ, the ATPase FliI and its regulator FliH (reviewed by Iino *et al*., 1988; Macnab, 2003, 2004). Except for FlhB, which seems to gate the export pathway, the function of the remaining transmembrane proteins in the transport machinery complex is still unclear (reviewed by Bardy *et al*., 2003; Macnab, 2003, 2004). Proteins secreted through the FEA possess no cleavable signal peptide and conserved signal domains for flagellar protein export have not as yet been clearly recognized (reviewed by Aldridge & Hughes, 2001; Macnab, 2003). Flagellar assembly is regulated at transcriptional, translational and posttranslational levels (reviewed by Aldridge & Hughes, 2002).

Whereas functional flagella are clearly expressed in *Listeria*, FEA components have not been thoroughly investigated. In *L. monocytogenes*, flagellar motility is characterized by a tumbling motion (Galsworthy *et al*., 1990). Flagellar expression is temperature-dependent: fewer flagella are expressed at 37 °C than at 20 °C (Peel *et al*., 1988). It was suggested that motility genes are downregulated at 37 °C by the transcriptional activator of virulence genes PrfA (Positive regulatory factor A) (Michel *et al*., 1998). Additionally, the topoisomerase FlaR (Flagellum R) negatively regulates its own expression as well as flagellin expression (Sanchez-Campillo *et al*., 1995). Recently, it has also been shown that flagellar motility in *L. monocytogenes* EGDe was regulated by MogR (Motility gene Repressor), a transcriptional repressor required for bacterial virulence (Grundling *et al*., 2004), as well as the response regulator DegU (Degradative enzyme U) (Knudsen *et al*., 2004). Thus, it appears that regulation of listerial flagellum assembly and motility is rather complex and not fully understood yet. Besides cell motility and chemotaxis, these flagella also play a crucial role in initial cell attachment to abiotic support as well as in host cell invasion and bacterial virulence (Vatanyoopaisarn *et al*., 2000; Gorski *et al*., 2003; Dons *et al*., 2004; Way *et al*., 2004; Bigot *et al*., 2005). Of the nine FEA components found in Gram-negative bacteria, seven could be identified in *Listeria* (Table 1). Genes encoding FlhA, FlhB, FliP, FliQ, FliR, FliI

Table 5. Putative Type 4 prepilins in *Listeria* species

	Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
ComGC	Lmo1345	16803385	107	LMOf2365_1362	46907571	107	Lin1382	16800450	107	missed	none	107	LMOh7858_1432	47093636	107
ComGD	Lmo1344	16803384	142	LMOf2365_1361	46907570	140	Lin1381	16800449	140	LMOf6854_1386	47095928	142	LMOh7858_1431	47093623	140
ComGE	Lmo1343	16803383	94	LMOf2365_1360	46907569	94	Lin1380	16800448	94	LMOf6854_1385	47095927	94	LMOh7858_1430	47093622	94
ComGF	Lmo1342	16803382	155	LMOf2365_1359	46907568	155	Lin1379	16800447	154	LMOf6854_1384	47095926	155	LMOh7858_1429	47093635	170
ComGG	Lmo1341	16803381	105	LMOf2365_1358	46907567	105	Lin1378	16800446	105	LMOf6854_1383	47095925	105	LMOh7858_1428	47093621	105

*Whole genome shotgun unfinished assembly.

and FliH are present in a 41-gene cluster encoding proteins related to flagellar-motility-chemotaxis (Fig. 4a). In Gram-positive bacteria, organization of flagellar-motility-chemotaxis genes in only one cluster is not always the rule; in *Clostridium acetobutylicum*, for example, these genes are present in two clusters on the chromosome (Nölling *et al*., 2001; Desvaux *et al*., 2005). Following Pfam and CDD searches, neither FliO/FliZ homologue (PF04347; COG3190) nor FliJ homologue (PF02050; COG2882) could be found in listerial species sequenced so far. Absence of genes encoding these proteins had previously been observed in some other bacteria (reviewed by Pallen *et al*., 2005a, b). Based on the flagella model from KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/; Kanehisa *et al*., 2004), a schematic representation of the FEA within the flagellum of *L. monocytogenes* EGDe was attempted (Fig. 4b). By homology with proteins known to be secreted through the FEA in Gram-negative bacteria (Desvaux *et al*., 2006b; reviewed by Aizawa, 2001; Macnab, 2003), 12 proteins, all involved in flagellar morphogenesis, could be identified in *Listeria* ssp. (Table 6). These proteins, lacking a putative signal peptide, are components of the flagellum hook, rod and filament (Fig. 4b). Interestingly, the flagellin FlaA (Flagellin A), which composes the listerial flagellar filament, has been demonstrated to possess a murein-hydrolyzing activity (Popowska & Markiewicz, 2004b).

The holins (TC #1.E.)

Holins are small membrane proteins permitting the cytoplasmic membrane translocation of proteins lacking N-terminal signal sequences (reviewed by Wang *et al*., 2000). Holins originate from phages and are mainly involved in the secretion/activation of enzymes with muralytic activities which hydrolyze the cell-wall polymer as a prelude to cell lysis, a process relevant to bacterial apoptosis (reviewed by Gründling *et al*., 2001; Bayles, 2003). Holins are present as homo-oligomeric complexes that form pores through the cytoplasmic membrane and permit protein translocation concomitant with a passive, energy-independent permabilization event (Haro *et al*., 2003; reviewed by Wang *et al*., 2000). Twenty-one distinct families of holin proteins are currently recognized in TC-DB (Transport Classification DataBase; http://www.tcdb.org/; Busch & Saier, 2002).

The present review constitutes the first report of genes encoding TcdE-like and φ11 holins in *Listeria* and therefore this protein secretion system has never been experimentally investigated in these bacterial species. In all *Listeria* species sequenced so far, we could identify a holin of 140 amino acids long (PF05105; *E*-values $\leq 1.4 \times 10^{-25}$) belonging to the *C. difficile* TcdE family (TC #1.E.19) (Table 1). In *C. difficile, tcdE* is located between the genes *tcdA* and *tcdB*, which encode two large toxins (Tan *et al*., 2001). The holin TcdE then permits the release of TcdA and TcdB into the extracellular medium (Tan *et al*., 2001; Mukherjee *et al*., 2002). In *Listeria*, no genes coding for toxins could be found in proximity to the genes coding for TcdE-like proteins. However, a gene encoding a putative autolysin, an N-acetylmuramoyl-L-alanine amidase (PF01520; *E*-values $\leq 4.2 \times 10^{-23}$) lacking a signal peptide, was systematically present (Table 7). Interestingly, the autolysins Lmo0129 from *L. monocytogenes* EGDe and its orthologue Lin0176 from *L. innocua* (Table 7) were identified in bacterial culture supernatants, suggesting this protein secretion pathway is effectively functional and active in *Listeria* (Trost *et al*., 2005). In *L. monocytogenes*, some autolysins, such as Ami, Iap and Auto, have been demonstrated to contribute to bacterial infection and are even considered as virulence factors (Dussurget *et al*., 2004).

The second family of holin found belongs to the *Listeria* φA118 holin family (PF06946; TC #1.E.21), also called Hol118 (Table 1) (Loessner *et al*., 2000). Interestingly, site-specific integration of A118 prophage occurs into *comK*, a gene encoding an autoregulatory protein specifying the major competence transcription factor (Loessner *et al*., 2000; Lauer *et al*., 2002). Hol118 is a 93 amino acids protein exhibiting three putative transmembrane domains (Loessner *et al*., 2000). The gene encoding Hol118 features a dual start motif giving rise to a second product of 83 amino acids, called Hol118(83) lacking its first transmembrane domain (Vukov *et al*., 2003); such a feature is characteristic of holins and antiholins which are often encoded by the same gene where a dual-start motif yield to two functionally distinct gene products regulating cell autolysis (Bläsi & Young, 1996). While Hol118(83) confers negative control on lytic activity, Hol118 activates autolysis (Vukov *et al*., 2003). When paralelled with CidA/LrgA system (TC #1.E.14) from *S. aureus*, Hol118 can be define as a holin while Hol118(83) as an antiholin (Rice *et al*., 2003). Therefore, φA118 holins would be endolysin exporters involved in programmed cell death, a process that is analogous to apoptosis in eukaryotes (Bayles, 2003). Despite originating from different bacteriophages, Hol118 and Hol500 (93% identity and 99% similarity) belong to the same holin family (Loessner *et al*., 1995). The gene encoding a L-alanoyl-D-glutamate peptidase, i.e. endolysins Ply118 (Phage lysin of φA118) or Ply500, which hydrolyze the cross-linking peptide bridges of peptidoglycan and are therefore responsible for the cell autolysis, systematically clusters with the gene encoding Hol118 homologue (Tables 1 and 7) (Loessner *et al*., 1995; Nelson *et al*., 2004). In sequenced *Listeria* species, neither the genes encoding Ply118 and Ply500 could be found simultaneously nor the gene encoding previously reported endolysin Ply511 (Table 7). Neither φA118 holin, Ply118 nor Ply500 could be identified in *L. monocytogenes* 4b F2365 (Nelson *et al*., 2004).

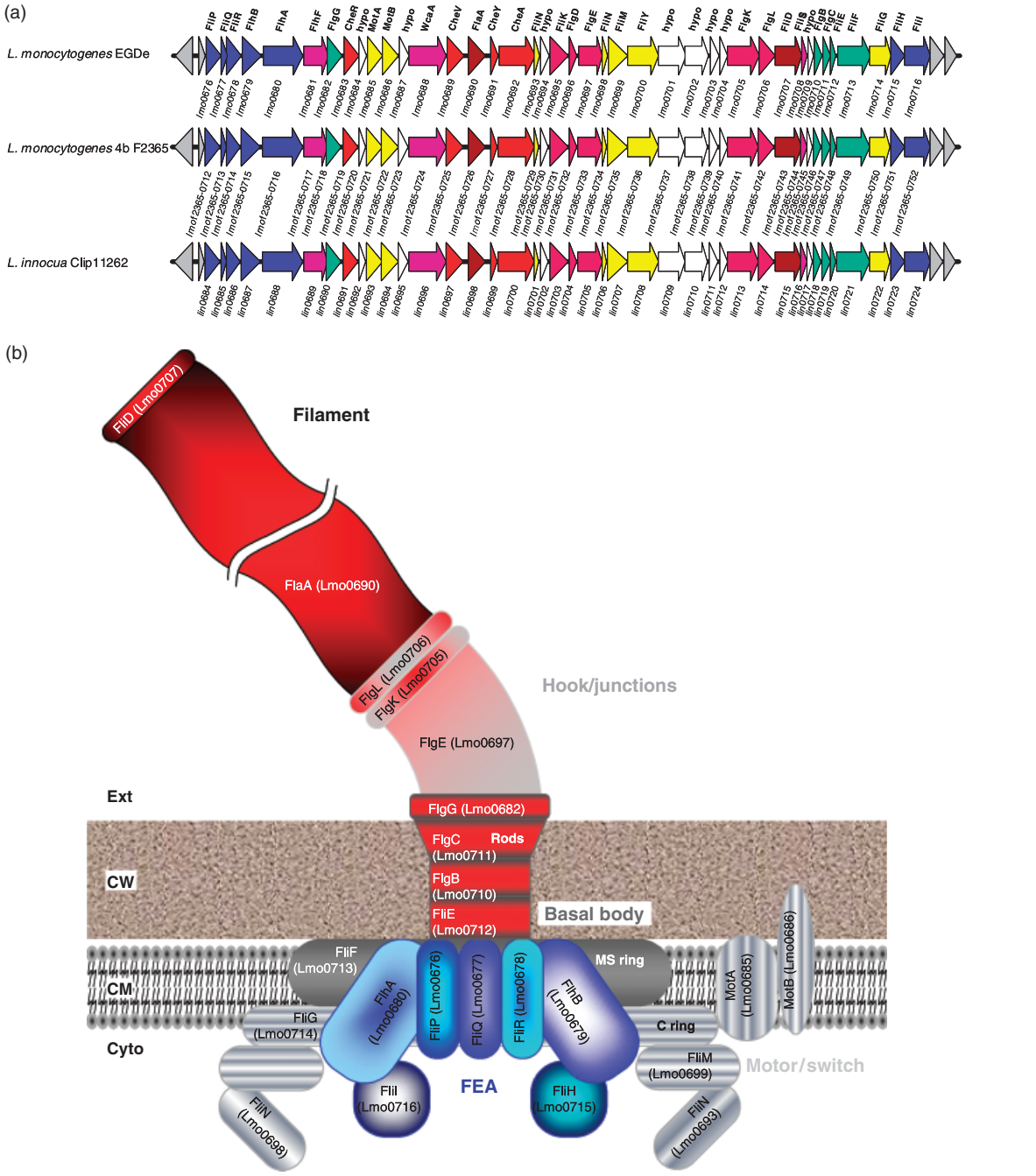

Fig. 4. The flagellum in *Listeria*. (a) The flagellar-motility-chemotaxis gene cluster in completed genome sequence *Listeria* species. Genes related to flagellar-motility-chemotaxis are coloured: blue, genes predicted as encoding components of the FEA; green, genes predicted as encoding components of the basal body; yellow, genes predicted as components of the flagella motor/switch; pink, genes predicted as encoding hook and junction proteins; brown, genes encoding proteins of the flagellar filament; red, genes predicted as encoding proteins related to bacterial chemotaxis; violet, genes predicted as involved global assembly of the flagellum, such as chaperones; white, hypothetical genes encoding proteins of unknown function. Names of proteins encoded by each gene of the clusters are indicated in bold letters at the top of the schema. (b) Schematic representation of a flagellum in *L. monocytogenes* EGDe with a special highlight on the FEA and its substrates. Components of the FEA (Flagellar Export Apparatus) are shown in blue. Proteins secreted via FEA are red. Components of the flagella motor/switch are indicated with white and shaded grey stripes. Components of the basal body are dark grey. Hook and junction proteins are light grey. Proteins of the flagellar filament are black. Cyto, Cytoplasm; CM, Cytoplasmic Membrane; CW, Cell Wall; Ext, Extracellular milieu.

Table 6. Proteins potentially secreted through the FEA in *Listeria* species

	Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
Rod															
FlgB	Lmo0710	16802752	133	LMOf2365_0746	46906961	133	Lin0718	16799793	133	LMOf6854_0757	47095276	133	LMOh7858_0776	47091673	133
FlgC	Lmo0711	16802753	136	LMOf2365_0747	46906962	136	Lin0719	16799794	136	LMOf6854_0758	47095277	136	LMOh7858_0777	47091674	136
FliE	Lmo0712	16802754	98	LMOf2365_0748	46906963	98	Lin0720	16799795	98	LMOf6854_0759	47095278	98	LMOh7858_0778	47091675	98
FlgG	Lmo0682	16802724	259	LMOf2365_0718	46906933	259	Lin0690	16799765	259	LMOf6854_0728	47095247	259	LMOh7858_0747	47091645	259
WcaA	Lmo0688	16802730	637	LMOf2365_0724	46906939	637	Lin0696	16799771	637	LMOf6854_0735	47095254	637	LMOh7858_0753	47091650	637
Hook															
FliK	Lmo0695	16802737	348	LMOf2365_0731	46906946	348	Lin0703	16799778	348	LMOf6854_0742	47095261	348	LMOh7858_0760	47091657	348
FlgD	Lmo0696	16802738	140	LMOf2365_0732	46906947	140	Lin0704	16799779	140	LMOf6854_0743	47095262	140	LMOh7858_0761	47091658	140
FlgE	Lmo0697	16802739	411	LMOf2365_0733	46906948	411	Lin0705	16799780	411	LMOf6854_0744	47095263	411	LMOh7858_0762	47091659	411
FlgK	Lmo0705	16802747	506	LMOf2365_0741	46906956	506	Lin0713	16799788	506	LMOf6854_0752	47095271	506	LMOh7858_0770	47091667	506
FlgL	Lmo0706	16802748	291	LMOf2365_0742	46906957	291	Lin0714	16799789	291	LMOf6854_0753	47095272	291	LMOh7858_0771	47091668	291
Filament															
FlaA	Lmo0690	16802732	287	LMOf2365_0726	46906941	287	Lin0698	16799773	287	LMOf6854_0737	47095256	287	LMOh7858_0755	47091652	287
FliD	Lmo0707	16802749	429	LMOf2365_0743	46906958	429	Lin0715	16799790	429	LMOf6854_0754	47095273	429	LMOh7858_0772	47091669	429

*Whole genome shotgun unfinished assembly.

Table 7. Proteins without putative signal peptide potentially secreted via holins in *Listeria* species

	Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
Autolysin	Lmo0129	16802177	242	LMOf2365_0147	46906369	242	Lin0176	16799253	242	LMOf6854_0142	47095200	246	LMOh7858_0154	47092265	242
Ply118	Lmo2278	16804317	281	–	–	–	–	–	–	LMOf6854_2340	47095980	281	–	–	–
Ply500	–	–	–	–	–	–	Lin0128	16799205	289	–	–	–	LMOh7858_2414	47092378	289
Phagelysin-A	–	–	–	–	–	–	Lin2374	16801437	316	LMOf6854_2655	47096776	235	–	–	–
	–	–	–	–	–	–	Lin2563	16801625	316	–	–	–	–	–	–
Phagelysin-B	–	–	–	–	–	–	Lin1700	16800768	308	–	–	–	–	–	–
AmiC	–	–	–	–	–	–	Lin1296	16800364	277	–	–	–	–	–	–

*Whole genome shotgun unfinished assembly.

The last type of holins belongs to φ11 holin family (TC #1.E.11) and was identified only in *L. monocytogenes* 1/2a F6854 and *L. innocua* (PF04531; *E*-values $\leq 4.8 \times 10^{-7}$). Holins of the φ11 family have been previously found in phage Tuc2009 of *Lactococcus lactis* (Arendt *et al.*, 1994), EJ-1 of *Streptococcus pneumoniae* (Haro *et al.*, 2003) and φ11 of *S. aureus* (Young *et al.*, 1999). EJh, a pneumococcal φ11 holin, is an 85-amino acid-long protein with two transmembrane domains forming transbilayer holes after self-oligomerization as visualized by atomic force microscopy (Haro *et al.*, 2003). EJh is presumably involved in the secretion of EJl, a choline-binding protein and cell-wall amidase (Saiz *et al.*, 2002). In *L. monocytogenes* 1/2a F6854, the gene encoding a EJh homologue clusters with a gene annotated as encoding a phagelysin (LMOf6854_2656), an endolysin that also possesses a D-alanyl-D-alanine carboxypeptidase domain (PF02557; *E*-value = 2.3×10^{-56}) that is usually responsible for vancomycin resistance (Wright *et al.*, 1992) (Tables 1 and 7). In *L. innocua*, three φ11 holins could be identified (Table 1). The gene encoding Lin2375, which is 94% identical and 98% similar to LMOf6854_2656, clusters with a gene originally annotated as hypothetical and encoding a protein, Lin2374, bearing a N-proximal N-acetylmuramoyl-L-alanine amidase domain (PF01510; *E*-value = 4.2×10^{-20}) followed by a D-alanyl-D-alanine carboxypeptidase domain (PF02557; *E*-value = 7.6×10^{-16}). A second copy of the gene encoding Lin2374 is located elsewhere on the chromosome and named *lin*2563 (Table 7). The second gene coding for a φ11 holin, *lin*1702, also clusters with a gene encoding a protein (Lin1700) with a domain organization similar to that of Lin2374. The last gene encoding a φ11 holin in *L. innocua*, Lin1295, clusters with a gene coding for Lin1296, homologous to AmiC (Amidase C; COG0860; *E*-value = 7.0×10^{-18}) from *E. coli*, which is an N-acetylmuramoyl-L-alanine amidase involved in splitting of the murein septum during cell division and bacterial autolysis in the presence of antibiotics (Heidrich *et al.*, 2001).

The WXG100 secretion system (Wss)

Despite the absence of a typical Sec-dependent signal sequence, the small ESAT-6 (Early Secreted Antigen Target of 6 kDa) protein, originally identified in *Mycobacterium tuberculosis*, is secreted into the extracellular milieu (Sørensen *et al.*, 1995). Interestingly, the gene encoding ESAT-6 is located within chromosomic RD1 (Region of Difference 1), which is the only locus specifically deleted in the attenuated vaccine strain *Mycobacterium bovis* BCG (Bacille de Calmette et Guérin) (Calmette *et al.*, 1927; Mahairas *et al.*, 1996; Philipp *et al.*, 1996). Furthermore, the ESAT-6 gene cluster of RD1 appeared to be unique to the phylum *Actinobacteria* and of fundamental importance in mycobacterial virulence (Gey Van Pittius *et al.*, 2001; Guinn *et al.*, 2004). In 2002, however, a new genomic survey revealed the presence of ESAT-6 homologues in both high and low G+C Gram-positive bacteria, within the genera (1) *Corynebacterium, Mycobacterium*, *Streptomyces*, and (2) *Bacillus*, *Clostridium*, *Listeria*, *Staphylococcus*, respectively (Pallen, 2002). Systematically, these proteins are around 100 amino acids long, possess a coil-coil domain and bear a conserved WXG motif; these criteria define a new superfamily of proteins called WXG100, proteins of around 100 residues with WXG motif (Pallen, 2002). This analysis also revealed that genes encoding WXG100 proteins are steadily clustered with YukAB homologues from *B. subtilis* (named according to the rules agreed upon by the international consortium for *B. subtilis* genome sequencing project (Kunst *et al.*, 1995)), which are large membrane-bound ATPases containing two or more FtsK/SpoIIIE domains (FSDs). YukAB was speculated to be part of a novel protein secretion system driving WXG100 protein secretion through the cytoplasmic membrane. Further investigations in *M. tuberculosis* and *M. smegmatis* have identified a total of six to seven proteins involved in the secretion of WXG100, designated Snm (Secretion in mycobacteria) (Stanley *et al.*, 2003; Guinn *et al.*, 2004; Converse & Cox, 2005). A hypothetical model for this secretory apparatus was proposed to be formed of the following:

(1) a YukAB homologue, which can exist either as a single protein or as two interacting proteins both containing ATP-binding sites, called Snm1 and Snm2,
(2) Snm4, a transmembrane protein with 11 α-helices that cross the lipid bilayer,
(3) Snm5, an uncharacterized protein,
(4) an unknown membrane-associated protein with predicted ATP/GTP binding sites, Snm6,
(5) an uncharacterized membrane protein, Snm7,
(6) Snm8, a membrane-anchored serine protease termed mycosin I (Converse & Cox, 2005).

In addition, a cytoplasmic ATP-dependent chaperone of the AAA family seems involved in WXG100 protein secretion in *M. tuberculosis* but not in *M. smegmatis*. More recently, secretion of WXG100 proteins was observed in *S. aureus* and appeared to be required for bacterial pathogenesis (Burts *et al.*, 2005). To date, the WXG100 protein secretion system (Wss) has been experimentally investigated only in *M. tuberculosis*, *M. smegmatis* and *S. aureus*, and this latter investigation therefore constitutes the first report of a functional Wss outside the phylum *Actinobacteria*. The Wss in *S. aureus* seems to be encoded by a locus of eight ORFs, called *ess* (eSAT-6 secretion system). This locus encodes the following:

(1) two WXG100 paralogues, called EsxA (Ess extracellular protein A) and EsxB,
(2) one YukAB homologue,

(3) a polytopic membrane protein with six predicted transmembrane helices, called EsaA (ESAT-6 secretion accessory protein A),
(4) EssA, a protein predicted to possess one transmembrane domain,
(5) another predicted membrane protein homologous to YukC from *B. subtilis*,
(6) a predicted cytoplasmic protein, called EsaC,
(7) another predicted cytoplasmic protein homologous to YukD from *B. subtilis* (Burts *et al.*, 2005).

Because of their predicted membrane location and absolute requirement for WXG100 protein secretion, it was speculated that EssA, YukC and YukAB may form a secretion apparatus. No homologue of Snm4, Snm5, Snm6, Snm7 or Snm8 could be identified in *S. aureus*; this partly explains the original failure to identify a Wss outside the phylum *Actinobacteria* (Gey Van Pittius *et al.*, 2001). Conversely, homologous proteins to EssA, YukC, EsaA and YukD were absent from mycobacteria.

Although genes encoding a Wss was previously reported in *L. monocytogenes* EGDe (Pallen, 2002; Burts *et al.*, 2005), the functionality of this pathway has not been experimentally investigated. Whereas several YukAB paralogues have been reported in several genomes of sequenced bacteria possessing a Wss such as *B. subtilis*, *C. acetobutylicum, Clostridium diphtheriae, M. tuberculosis* or *S. aureus* (Pallen, 2002; Desvaux *et al.*, 2005), in *Listeria* species only one copy of gene encoding YukAB-like protein could be detected in each genome, including the nonpathogenic species *L. innocua* (Table 1). In *L. monocytogenes* 1/2a EGDe, 4b F2365 or *L. innocua* Clip11262, the gene encoding YukAB homologue is present in a cluster with gene sequence and synteny very similar to the *ess* locus described in *S. aureus* Mu50 (Burts *et al.*, 2005) (Fig. 5). Therefore, a putative Wss is encoded in *Listeria* genome. However, no sequence homology could be found between EsaC from *S. aureus* and proteins encoded by genes found in the same position in *Listeria*, i.e. between genes encoding YukAB and WXG100-B homologues (Fig. 5). In addition to being found in the same position in the cluster, these genes encode proteins (i) of the same size, 130–131 amino acid residues, (ii) predicted as soluble and cytoplasmic (PSORTb, Prediction of protein SORTing signals in bacteria; http://www.psort.org/psortb/; Gardy *et al.*, 2005), and (iii) predicted as displaying extended coiled-coil domains with high probabilities (COILS, Coiled-*COILS* prediction; http://www.ch.embnet.org/software/COILS_form.html; Lupas *et al.*, 1991). As in *Mycobacterium* and *S. aureus*, this locus encodes two WXG100 paralogues, WXG100-A [also called Lmesat6 (Way & Wilson, 2005)] and WXG100-B (Tables 1 and 8, Fig. 5). In *M. tuberculosis*, the two WXG100 paralogues, ESAT-6 and CFP-10 (Culture Filtrate Protein 10), play an important role in virulence and are known to interact physically with each other (Pym *et al.*, 2002; Renshaw *et al.*, 2002; Okkels & Andersen, 2004). In *S. aureus*, it also appeared EsxA is absolutely required for synthesis and/or secretion of EsxB, and *vice versa* (Burts *et al.*, 2005). Recent investigation in *L. monocytogenes* suggests that gene locus encoding WXG100-A is not required for bacterial virulence (Way & Wilson, 2005). At this point, however, it is not possible to say whether putative *L. monocytogenes* Wss is functional and/or required for bacterial virulence.

Absent protein secretion systems in *Listeria*

The ABC (ATP-Binding Cassette) transporter superfamily contains both uptake and efflux transport systems for a large variety of substrates – glucides, lipids, proteins, amino acids, peptides, and ions. As indicated by TC-DB, ABCdb (ABC transporter database; http://ir2lcb.cnrs-mrs.fr/ABCdb/; Quentin & Fichant, 2000) and TransportDB (Transport protein DataBase; http://www.membranetransport.org/; Ren *et al.*, 2004) searches, several ABC transporters have been identified in *Listeria*. However, as in all Gram-positive bacteria sequenced so far, none of them is dedicated to protein translocation *per se* but only to the transport of peptides/oligopeptides (Dassa & Bouige, 2001). Peptides translocated by this system generally possess N-terminal signal peptides that are removed by a subunit of the ABC or by specific peptidases (reviewed by Tjalsma *et al.*, 2000).

The Tad export apparatus is a newly characterized secretion system involved in the secretion and assembly of Flp (Fimbrial low-molecular-weight protein) pili (Inoue *et al.*, 1998), which mediate tight adhesion of the bacteria to surfaces (biotic and abiotic) and are essential for colonization as well as pathogenesis (Kachlany *et al.*, 2000; Schreiner *et al.*, 2003). From *in silico* analyses it appears that, in addition to Gram-negative bacteria, the Tad system is also present in some Gram-positive bacteria, essentially within the phylum *Actinobacteria,* class *Actinobacteria*, subclass *Actinobacteridae*, order *Actinomycetales* (*Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *M. bovis* and *Streptomyces coelicolor*) (Planet *et al.*, 2003). More recently, a genomic analysis of the protein secretion systems in *Clostridium acetobutylicum* revealed the presence of a putative Tad system in an additional phylum, *Firmicutes*, class *Clostridia* (Desvaux *et al.*, 2005). It must be stressed that the functionality of this secretion system has not been investigated yet in Gram-positive bacteria. Still, a Tad pathway could not be identified in listerial species, i.e. phylum *Firmicutes*, class *Bacilli*.

Members of the MscL family are also known to permit the release of small proteins such as thioredoxin during osmotic downshift (Ajouz *et al.*, 1998; Pivetti *et al.*, 2003). However, experimental evidence for MscL as a protein secretion pathway in Gram-positive bacteria remains to be demonstrated;

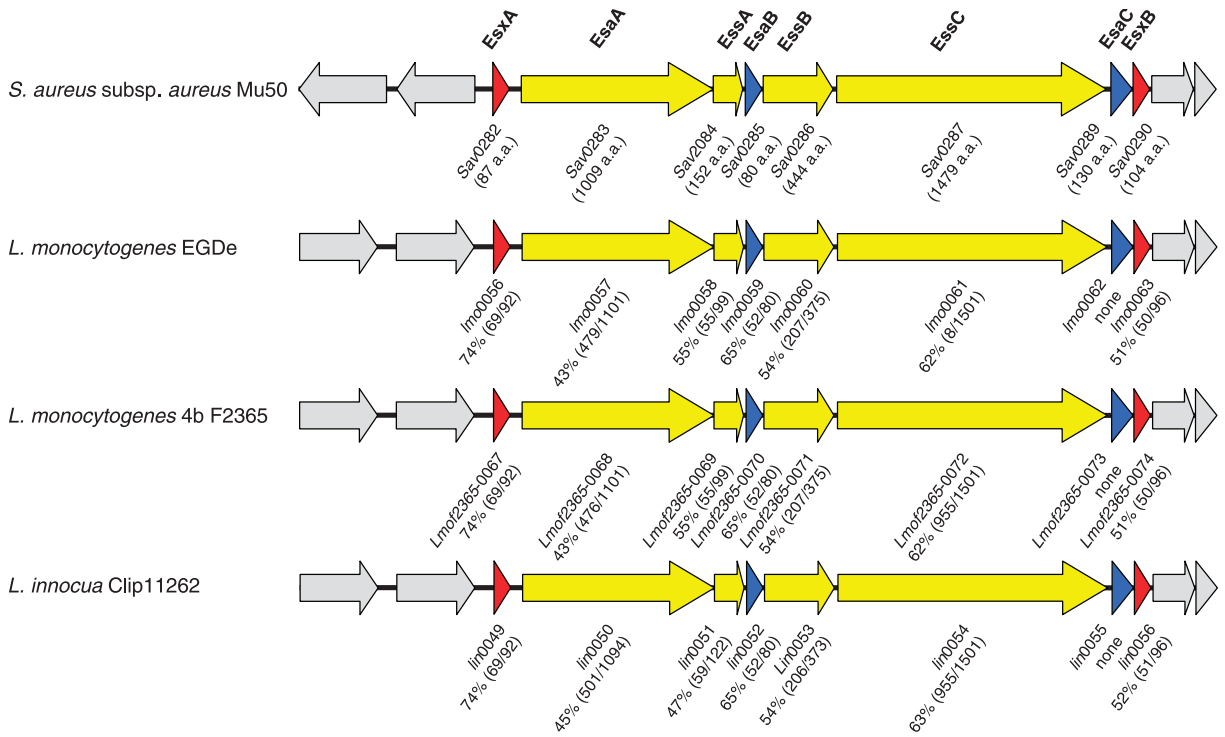

Fig. 5. Comparison of the *wss* loci from completed genome sequence *Listeria* species with the *ess* locus of *S. aureus* ssp. *aureus* Mu50. Genes of each locus are colored: red, genes encoding proteins predicted as secreted in the extracellular medium; blue, genes predicted as encoding soluble cytoplasmic proteins; yellow, genes encoding proteins predicted as transmembrane proteins. Names of proteins encoded by each gene of the clusters are indicated in bold letters at the top of the schema. Locus tags are indicated under each gene. For *S. aureus*, the size of the encoded protein is given in number of amino acids. For proteins encoded in *Listeria* species, whenever possible, the percentages of similarity (over *x* matching amino acids out of *y* amino acids: *x*/*y*) are also given in comparison with proteins of *S. aureus* Mu50 (results were obtained with BLAST 2 sequences using default parameters except that filter was turned off (Tatusova & Madden, 1999).

no homologue of this anecdotal protein secretion system could be identified in *Listeria*.

Conclusion and perspectives

On *L. monocytogenes* EGDe, where 508 proteins were identified as potential substrates of the Sec translocon (Trost *et al.*, 2005), the Sec apparatus appears to be, by far, the primary protein secretion pathway used in *Listeria* (Fig. 1). Among 105 extracellular proteins identified from proteomic analyses of the secretome, only 54 belonged to the 117 extracellular proteins originally predicted with a signal peptide from bioinformatic analyses (Trost *et al.*, 2005). Three of the 14 virulence factors characterized so far in *L. monocytogenes* EGDe were not identified in bacterial culture supernatant, Vip, InlJ and FbpA (Table 2). The remaining 51 proteins identified by proteomic analyses and lacking a signal peptide were hypothesized as secreted via uncharacterized secretion pathways (Trost *et al.*, 2005). This critical review highlights that at least five secretion systems alternative to the Sec-dependent pathway seem to be present for the transport of specific proteins in *Listeria* (Table 1, Fig. 1). Whereas the proteins potentially secreted via Tat, FPE or Wss (Tables 4, 5 and 8) could not be spotted in culture supernatant of *L. monocytogenes* EGDe and *L. innocua*, autolysins Lmo0129 and Lin0176 were identified (Trost *et al.*, 2005). This strongly suggests the protein secretion pathway of holins is active in *Listeria*. Among proteins lacking a signal peptide and present in culture supernatant, several are predicted to be cytosolic (Trost *et al.*, 2005). The presence of such proteins in culture supernatant has also been reported in *B. subtilis* (Antelmann *et al.*, 2001; Tjalsma *et al.*, 2004) and could result from uncharacterized secretion pathways or bacterial cell lysis. Overall, the Sec system, and its substrates, appears to be the best characterized and only experimentally investigated protein secretion pathway in *Listeria*. Much therefore remains to be learned about other protein secretion systems in this genus. Besides protein substrates of the Sec translocon, Wss and holins could potentially be involved in bacterial pathogenesis. Interestingly, like some Sec-dependent virulence factors (Table 2), these two secretion pathways are present in both pathogenic and nonpathogenic *Listeria*. Besides the concept of pathogenicity islands (Hacker & Kaper, 2000; Kolsto *et al.*, 2002;

Table 8. Proteins potentially secreted through the Wss in *Listeria* species

	Listeria monocytogenes 1/2a EGDe			*Listeria monocytogenes* 4b F2365			*Listeria innocua* Clip11262			*Listeria monocytogenes* 1/2a F6854*			*Listeria monocytogenes* 4b H7858*		
	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length	Locus name	GI	Length
WXG100-A	Lmo0056	16802104	97	LMOf2365_0067	46906289	97	Lin0049	16799128	97	LMOf6854_0067	47095125	97	LMOh7858_0070	47092183	97
WXG100-B	Lmo0063	16802111	99	LMOf2365_0074	46879560	99	Lin0056	16799135	99	–	–	–	–	–	–

*Whole genome shotgun unfinished assembly.

Dobrindt *et al*., 2004), this observation fuels the new concept that the frontier between pathogenic and non-pathogenic bacteria is not as neat as originally thought and that so-called virulence factors are probably involved in more general interactions between the microorganism and the host or the environment (Holden *et al*., 2004). Compared to the Sec pathway, few proteins are predicted to be secreted via Tat in *Listeria*. In *B. subtilis*, experimental work revealed a huge discrepancy between prediction of Tat-dependent proteins and proteins truly secreted via Tat (Jongbloed *et al*., 2002; Tjalsma *et al*., 2004). Because of reported variability from the twin arginine motif, experimental work is required to identify proteins passing through this pathway.

In *B. subtilis*, despite being related to the Tfp, the FPE is not involved in the formation of pili *per se* (Chen & Dubnau, 2003, 2004). In Gram-negative bacteria, Tfp are involved in natural bacterial transformation (Fussenegger *et al*., 1997; Koomey, 1998; Averhoff & Friedrich, 2003), and twitching motility, a form of bacterial movement over moist surfaces of crucial importance in bacterial pathogenicity as it permits host colonization and/or the formation of biofilms (Wall & Kaiser, 1999; Mattick, 2002; Nudleman & Kaiser, 2004). Interestingly, it is suggested that Type 4 pili are related to archaeal flagella (Bayley & Jarrell, 1998; Peabody *et al*., 2003). Whereas the presence of pili in Gram-negative bacteria was reported early (Brinton, 1965; Costerton *et al*., 1981; Jones & Isaacson, 1983; Heckels, 1989; Orndorff & Bloch, 1990; Hultgren *et al*., 1991), for a long time their presence in Gram-positive bacteria were considered dubious just because it was against the current 'dogma'. Proving once more such a tenet is foreign to the world of science (Ray, 1991; Johnson, 1999; Bussard, 2005), these structures are now well recognized in these bacteria (Hayashi *et al*., 1985; Fujita *et al*., 1992; Stenfors *et al*., 1997; Rakotoarivonina *et al*., 2002; Ton-That & Schneewind, 2003, 2004; Desvaux *et al*., 2006a). However, in Gram-positive bacterial species the involvement of FPE in the formation of proper Type 4 pili has never been investigated and, except for LPXTG-pilin polymerization via sortase (Ton-That & Schneewind, 2004), no mechanism for pili formation is yet understood. So far, DNA uptake in Gram-positive bacteria has been mostly investigated in *B. subtilis* and *S. pneumoniae* (Dubnau, 1999; Berge *et al*., 2002). Natural competence has also been reported in other Gram-positive bacteria, and bioinformatic analyses have revealed the presence of FPE (Lorenz & Wackernagel, 1994; Peabody *et al*., 2003; Desvaux *et al*., 2005). As Tfp could not be observed on the cell surface of the model organism *B. subtilis*, the FPE had been proposed to allow the assembly of a structure that only traverse the cell wall (Dubnau, 1999; Chen & Dubnau, 2003). In *Listeria* species, however, it is not known whether the FPE (1) is functional, (2) is involved in DNA uptake as in the model

organism *B. subtilis*, (3) permits the secretion of effector proteins as in the related Type II secretion system present in Gram-negative bacteria, and (4), contrary to *B. subtilis*, permits the formation of proper Tfp. So far, *Ruminococcus albus* is the only Gram-positive bacterium in which Tfp have been reported; nevertheless, the secretion apparatus permitting their assembly remains unknown (Pegden *et al.*, 1998; Rakotoarivonina *et al.*, 2002, 2005). Clearly, the state of knowledge on pili formation in Gram-positive bacteria, which to date involves pili formed by protein polymerization via sortase (Ton-That & Schneewind, 2004; Ton-That *et al.*, 2004), Type 4 pili (Rakotoarivonina *et al.*, 2002), or the putative Flp pili (Planet *et al.*, 2003), far behind that in Gram-negative bacteria. Undoubtedly, this field of research requires much deeper investigations. Contrary to pili, flagella are protein cell-surface structures unambiguously present in *Listeria* ssp. and involved in cell motility. As reported in other Gram-negative and Gram-positive bacteria (Borriello, 1998; Tasteyre *et al.*, 2001; Josenhans & Suerbaum, 2002; Ramos *et al.*, 2004; Bendtsen *et al.*, 2005; Desvaux *et al.*, 2006b), flagella also play a key role in bacterial virulence by promoting cell attachment, host invasion and biofilm formation. Although some data are available on regulatory aspects of flagellar expression in *Listeria*, the molecular mechanisms of flagellar assembly have not as yet been thoroughly investigated (Bigot *et al.*, 2005), which is a common trait with Gram-positive bacteria. Contrary to Gram-negative bacteria (Young *et al.*, 1999; Ton-That & Schneewind, 2004), in Gram-positive bacteria the secretion of effector proteins into the extracellular medium via the FEA has only been suggested in *Bacillus thuringensis* (Ghelardi *et al.*, 2002).

From a biotechnological point of view, holins are of particular interest in the dairy industry, especially for product ripening, as bacterial cell autolysis permits the release of intracellular enzymes (de Ruyter *et al.*, 1997). Phage holins have also been used to control pathogenic bacteria all along the food chain (Hudson *et al.*, 2005). Besides the biocontrol of food-borne pathogens, holins have been used for the development of DNA vaccine where self-destruction of intracellular attenuated *Listeria* within the cytosol of macrophages was preprogrammed (Dietrich *et al.*, 1998, 1999). This wide range of promising applications underlines the necessity of investigating the protein secretion via holin at a molecular level. A better understanding of the protein secretion mechanisms involved in this lysis clock could possibly lead to some form of control of *L. monocytogenes* in the course of infection. Interestingly, compared to pathogenic *Listeria* species, *L. innocua* possesses significantly more holins and their associated endolysins. It would be interesting to investigate if and how this could be related to bacterial pathogenicity of *Listeria* species. It is worth noting that in addition to cell-wall hydrolases putatively secreted via the holins, *L. monocytogenes* also secretes murein hydrolases via the Sec pathway, such as Iap, p45, Ami, NamA, or Auto, as well as the flagellin FlaA via the FEA. Besides cell autolysis, murein hydrolases are involved in a myriad of cellular processes including (1) cell growth, (2) cell-wall turnover, (3) peptidoglycan maturation, (4) cell division, (5) formation of cell-surface protein structure, such as flagella and pili, (6) sporulation, (7) chemotaxis, (8) biofilm formation, (9) genetic competence, (10) protein secretion, (11) the lytic action of some antibiotics, and (12) virulence (Koraimann, 2003; Popowska, 2004). Interestingly, from a recent study in the Gram-positive pathogenic bacterium *S. pneumoniae*, it appears that release of the cytoplasmic virulence factor pneumolysin originates from lysis of noncompetent cells and is triggered by competent cells (Guiral *et al.*, 2005). This phenomenon, which involves several cell wall hydrolases, was named allolysis. Allolysis was suggested to stabilize symbiosis with the host, exacerbate infection, contribute to biofilm accretion, and ensure survival of a subpopulation (Gilmore & Haas, 2005; Guiral *et al.*, 2005). It was also hypothesized that some surface-associated pneumococcal proteins usually considered as cytoplasmic, such as enolase or glyceraldehyde-3-phosphate dehydrogenase (Bergmann *et al.*, 2001, 2004), could be released from lysed cells and scavenged by *S. pneumoniae* competent cells (Guiral *et al.*, 2005). These proteins are actually moonlighting outside the cell as they exhibit plasmin(ogen)-binding activity, and thus significantly enhance bacterial virulence (Jeffery, 1999; Bergmann *et al.*, 2003, 2004). Occurrence of allolysis and its involvement in cytoplasmic protein release/cell-surface display, bacterial virulence and/or biofilm formation has not been questioned in other bacteria, including *L. monocytogenes*, and would undoubtedly require further investigations.

So far, a functional Wss has been demonstrated in *Mycobacterium* ssp. and *S. aureus* (Brodin *et al.*, 2004; Burts *et al.*, 2005; Converse & Cox, 2005). Wss plays an important role in bacterial virulence and appears as a key target to develop better vaccine, but the biological function of WXG100 proteins is still unclear (Brodin *et al.*, 2004; Burts *et al.*, 2005). In *Mycobacterium*, WXG100 proteins have been demonstrated to be antigens promoting strong T-cell response, and have been hypothesized to act as cytolysin (Brodin *et al.*, 2004). In *S. aureus*, the role of WXG100 proteins as virulence factors is also unclear, but preliminary analysis suggests Wss may somehow affect the secretion of several other exoproteins (Burts *et al.*, 2005). In *Listeria*, whereas transcription of the gene encoding WXG100-A has been demonstrated (Way & Wilson, 2005), secretion of WXG100 proteins has not yet been reported, and the presence of a functional Wss and its physiological role still remains to be determined. Interestingly, in *Listeria*, the locus *wss* is present in both pathogenic and nonpathogenic

species, and in only one copy. In *Mycobacterium* (Pym *et al*., 2002, 2003; Williams *et al*., 2005), research in this field might lead to the development of an efficient vaccine against listeriosis. However, a recent investigation in *L. monocytogenes* indicates *lmesat6* locus is not related to bacterial pathogenicity (Way & Wilson, 2005). Further investigations on this protein secretion system in *Listeria* are necessary to demonstrate whether this protein secretion system is functional.

Above and beyond a better understanding of listerial virulence – *L. monocytogenes* being used as a paradigm for the study of intracellular pathogen (Chakraborty, 1999; Cossart & Bierne, 2001; Portnoy *et al*., 2002; Cossart & Sansonetti, 2004; Krawczyk-Balska & Bielecki, 2004) to find new strategies or therapies to fight and/or prevent listeriosis – knowledge about protein secretion systems in this pathogenic microorganism could also help in the development of singular biomedical applications. In fact, *L. monocytogenes* has been tested for the development of live-vaccine vector (Paterson & Johnson, 2004; Verch *et al*., 2004), DNA-vaccine vector (Verch *et al*., 2004; Schoen *et al*., 2004, 2005), and cancer vaccine (Brockstedt *et al*., 2004). In the recently proposed concept of inverted pathogenicity, the elaboration of such and other new preventive or therapeutic strategies necessitates the exploitation of microbial toxins (Russmann, 2004); it is, further, legitimate to consider motility, adhesion factors and/or secreted enzymes, which all require the involvement of protein secretion systems at some stage, to hijack pathogen-specific molecular mechanisms.

This critical review provides the first overview of protein secretion systems present in *Listeria* and raises a number of questions: Are homologues of the Sec pathway, such as YidC or SRP, functionally identical between Gram-positive and Gram-negative bacteria? Are chaperones, such as DnaK or GroEL, involved in protein targeting to Sec? Why is there a second SecA paralogue, SecA2, in some Gram-positive bacteria, and not in Gram-negative bacteria? How is SecA2 connected to the Sec apparatus? Is there an exportal in *Listeria*? What are the functions of the predicted extracellularly secreted proteins and cell-surface exposed proteins in *L. monocytogenes*? Are there pili in *Listeria*? Is FEA involved in the secretion of soluble extracellular proteins? Are the Tat, FPE, holins and Wss pathways functional in *Listeria*? What are their protein substrates? What are their physiological role and secretion mechanisms? How is the protein secretion regulated through these different systems? Are there other uncovered protein secretion systems present in *Listeria*? How are the putative secretion systems, as well as the predicted secreted proteins, related to bacterial pathogenicity, bacterial adhesion and/or biofilm formation? Answering such fundamental questions should undoubtedly extend our understanding of Gram-positive bacterial protein secretion outside the model *B. subtilis*. Beyond that, investigations in those directions are an important prerequisite efficiently to develop strategies preventing food contamination, fighting listeriosis or hijacking *L. monocytogenes* for biotechnological exploitation, such as live-vaccine development.

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