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Inactivation of the *srtA* gene in *Listeria monocytogenes* inhibits anchoring of surface proteins and affects virulence

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Summary

During infection of their hosts, Gram-positive bacteria express surface proteins that serve multiple biological functions. Surface proteins harbouring a C-terminal sorting signal with an LPXTG motif are covalently linked to the cell wall peptidoglycan by a transamidase named sortase. Two genes encoding putative sortases, termed *srtA* and *srtB*, were identified in the genome of the intracellular pathogenic bacterium *Listeria monocytogenes*. Inactivation of *srtA* abolishes anchoring of the invasion protein InlA to the bacterial surface. It also prevents the proper sorting of several other peptidoglycan-associated LPXTG proteins. Three were identified by a mass spectrometry approach. The Δ*srtA* mutant strain is defective in entering epithelial cells, similar to a Δ*inlA* mutant. In contrast to a Δ*inlA* mutant, the Δ*srtA* mutant is impaired for colonization of the liver and spleen after oral inoculation in mice. Thus, *L. monocytogenes srtA* is required for the cell wall anchoring of InlA and, presumably, for the anchoring of other LPXTG-containing proteins that are involved in listerial infections.

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Introduction

The cell wall of Gram-positive bacteria forms a rigid exoskeleton decorated with numerous proteins that have key roles in the interaction of the bacterium with its environment. In the case of pathogens, surface proteins mediate contact with the eukaryotic host by regulating processes such as adhesion, invasion or survival. Surface proteins are attached to the bacterial envelope through interactions between specific domains of the protein and target components of the bacterial surface, such as the membrane, the peptidoglycan or secondary cell wall polymers (reviewed by Navarre and Schneewind, 1999; Cossart and Jonquieres, 2000). One mechanism, referred to as 'sorting', results in the covalent attachment of proteins to the cell wall (Navarre and Schneewind, 1999). These surface proteins contain a C-terminal cell wall sorting signal, consisting of a conserved LPXTG motif followed by a hydrophobic domain of about 20 amino acids and a tail of mostly positively charged residues (Fischetti *et al*., 1990). After synthesis in the bacterial cytoplasm, surface protein precursors are translocated across the membrane by means of an N-terminal secretion signal and are retained from the secretory pathway by their sorting signal. After cleavage between the threonine and the glycine of the LPXTG motif, the carboxyl group of threonine is amide-linked to the amino group of cross-bridges within peptidoglycan precursors (Ton-That *et al*., 1999; 2000). The sorting reaction has been characterized for protein A in *Staphylococcus aureus* (reviewed by Navarre and Schneewind, 1999). The *srtA* gene, encoding sortase, was recently identified through the isolation of *S. aureus* mutants impaired in the ability to anchor protein A to the cell wall (Mazmanian *et al*., 1999). *S. aureus srtA* is required for the C-terminal processing of surface proteins, and recombinant sortase catalyses both cleavage of LPXTG-bearing peptides and the subsequent transpeptidation reaction *in vitro* (Mazmanian *et al*., 1999; 2000; Ton-That *et al*., 1999; 2000). *S. aureus srtA* mutants are unable to anchor surface proteins and are significantly

reduced for virulence in mouse models of infection (Mazmanian *et al*., 2000). It was also shown recently that inactivation of the *srtA* homologue in *Streptococcus gordonii*, a commensal bacterium from oral flora, impairs association of adhesins to the bacterial surface (Bolken *et al*., 2001).

Listeria monocytogenes is a food-borne pathogen responsible for meningoencephalitis and miscarriages, mainly in immunocompromised individuals and pregnant women. This bacterium can cross the intestinal, blood–brain and placental barriers. Pathogenicity is mainly the result of its capacity to survive and replicate in macrophages, to induce its own uptake by non-phagocytic cells and to disseminate from cell to cell by an intracellular motility process (Cossart and Lecuit, 1998). Several bacterial surface proteins are involved in the infectious process, including the invasion proteins InlA (also called internalin) and InlB (reviewed by Cossart and Bierne, 2001). InlA and InlB are attached to the surface by two different mechanisms. InlA is an LPXTG-containing protein that is covalently linked to the peptidoglycan (Lebrun *et al*., 1996; Dhar *et al*., 2000). In contrast, InlB is bound to the bacterial surface through non-covalent interactions with lipoteichoic acid, a membrane-anchored polymer of the cell wall (Jonquières *et al*., 1999). In addition to InlA, *L. monocytogenes* displays 40 other proteins containing an LPXTG motif (Glaser *et al*., 2001). Interestingly, this is the genome containing the largest number of such proteins of any bacterial genomes sequenced so far. To address whether these proteins were anchored at the bacterial surface by a sortase-catalysed mechanism, we looked for the presence of sortase-encoding genes within the *L. monocytogenes* genome and identified two putative *srt* genes, *srtA* and *srtB*. Here, we report the effects of inactivation of *srtA*. *Listeria* mutants lacking *srtA* cannot cleave the InlA sorting signal and are defective in the attachment and display of InlA and several other proteins to the bacterial surface. Furthermore, the *srtA* mutant is defective in bacterial internalization into the human enterocytes Caco-2 and hepatocytes HepG2, but is not defective in cell types for which entry is mediated by the invasion protein InlB or into macrophages. Compared with wild-type *L. monocytogenes*, a Δ*srtA* mutant is affected in colonization of the liver and spleen in a mouse model after oral inoculation. These results provide evidence that SrtA anchors not only InlA but also other LPXTG-containing proteins that play a role in the infectious process.

Results

Identification of srt *genes in* L. monocytogenes

We searched for sequences homologous to the *S. aureus srtA* gene product in the *L. monocytogenes* EGDe strain genome (GenBank/EMBL accession number AL592022; Glaser *et al*., 2001). By BLAST analysis, we found one sequence that is 28% identical to *S. aureus* SrtA and encodes a protein of 222 amino acids with an expected molecular weight of 24.7 kDa. This protein contains, at the N-terminus, a hydrophobic region, which could act as a signal peptide/transmembrane domain and, at the C-terminus, the essential cysteine residue within the catalytic TLXTC signature sequence present in *S. aureus* and *S. gordonii* sortases (Fig. 1). In the *Listeria* genome, *srtA* is flanked by two genes coding for proteins similar to *Bacillus subtilis* open reading frames (ORFs), YhfI, a conserved hypothetical protein, and YxiJ, a 3-methyladenine DNA glycosylase (Fig. 2A). However, this genomic organization is neither conserved in *B. subtilis* (Kunst *et al*., 1997) nor in *S. aureus* (Kuroda *et al*., 2001).

Additional motif searches using the sequence of the catalytic sulphydryl domain of SrtA as a query revealed the existence of a second putative sortase encoding gene, *srtB* (Lmo 2181), at a distance of 1300 kb from *srtA* in the *Listeria* genome. In fact, a recent report demonstrates not only the presence of one, but often multiple sortase homologues and orthologues in all Gram-positive bacterial genomes (Pallen *et al*., 2001). *L. monocytogenes* SrtB is 246 amino acids in length with an expected molecular weight of 28.6 kDa and is 23% identical to SrtA. It contains a putative signal peptide/transmembrane region, the expected TLXTC sequence and two stretches of 13 and 31 amino acids that are not present in SrtA (Fig. 1). Interestingly, SrtA and SrtB homologues were found in the related non-pathogenic species *Listeria innocua* with a very high level of conservation (95% and 91% identity respectively; Glaser *et al*., 2001). BLAST searches on other Gram-positive bacterial genomes revealed the existence of other SrtB-like sequences, particularly in other pathogens such as *S. aureus*, *Bacillus anthracis* (Fig. 1), *Streptococcus pyogenes* and *Clostridium difficile* (data not shown). In *S. aureus* and *B. anthracis*, SrtB sequences were 35% and 44% identical to *L. monocytogenes* SrtB respectively. Consistent with previous observations (Mazmanian and Schneewind, 2002), this result suggests the existence of at least two distinct families of sortases in Gram-positive bacteria.

Inactivation of L. monocytogenes srtA

To analyse the function of the *L. monocytogenes* SrtA protein, we inactivated the *srtA* gene by the introduction of an in frame deletion. This strategy was used to prevent potential polar effects, even though a putative transcriptional terminator is present at the end of *srtA* (Fig. 2A). Deletion mutants (Δ*srtA*) were obtained by allelic exchange in *L. monocytogenes* EGDe, using previously described protocols (Camilli *et al*., 1993; Dramsi *et al*.,

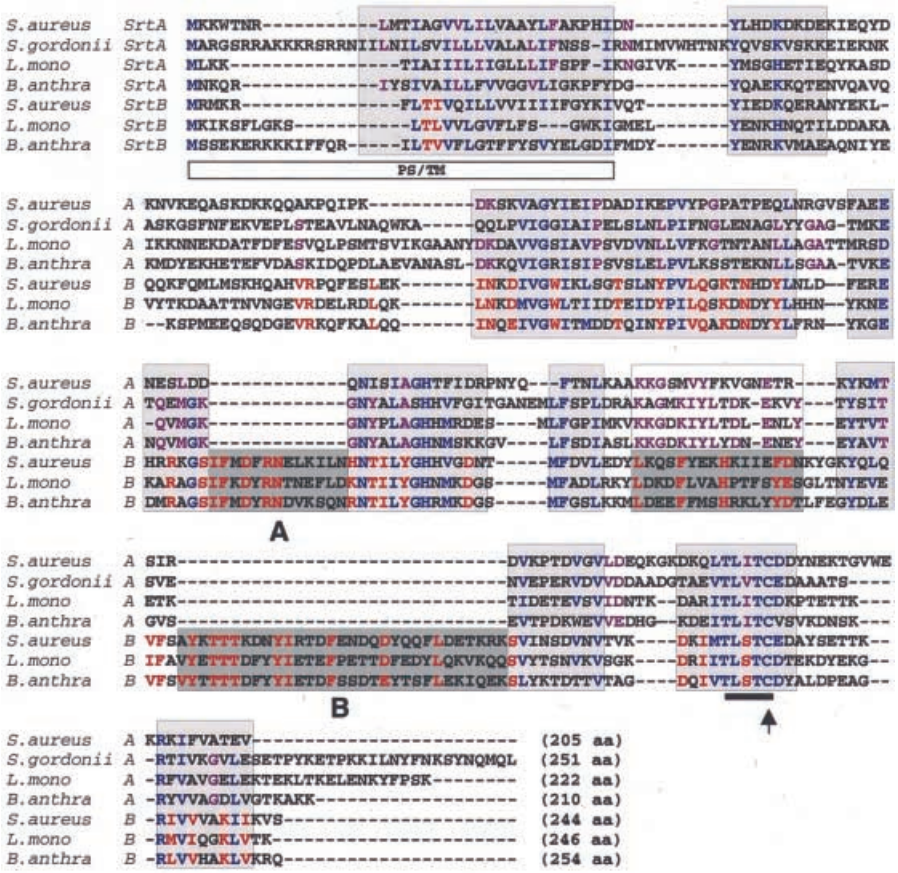

Fig. 1. Multiple sequence alignment of SrtA and SrtB proteins from different species. Species names are indicated (*L. mono*, *L. monocytogenes*; *B. anthra*, *B. anthracis*). The alignment was performed using MATCHBOX (Depiereux and Feytmans, 1992). Dots represent gaps inserted to maximize the alignment. The size of each protein is indicted at the end of each sequence. The boxes indicate regions of conserved structural motifs between all sortase sequences (in light grey), between SrtA sequences (in white) or between SrtB sequences (in dark grey). A and B indicate regions in SrtB that are not present in SrtA. Similar residues present in all sortase types are highlighted in blue; those present only in SrtA or SrtB sequences are in purple or red respectively. The putative signal peptide/transmembrane domain at the N-terminus and the cysteine residue within the catalytic TLXTC signature sequence are shown.

1997). Polymerase chain reaction (PCR) amplification confirmed the deletion of the *srtA* coding sequence in two independent mutant strains (BUG 1777 and BUG 1778; Fig. 2B). The mutants displayed no growth defect in brain–heart infusion (BHI) medium and did not show any morphological alterations, indicating that they were not affected in viability or cell division (data not shown). Both Δ*srtA* mutants behaved similarly in the course of this study; therefore, only results obtained with BUG 1778 are presented below.

We analysed the expression and cellular localization of SrtA within *L. monocytogenes*, including cytosolic, membrane, cell wall and culture medium compartments, by immunoblotting extracts from different fractions of EGDe with specific antibodies raised against purified recombinant SrtA (see *Experimental procedures*). Fractionation was performed as described previously (Jonquières *et al.*, 1999). As for the *S. aureus* SrtA (Mazmanian *et al.*, 2000), sortase was mainly located in the membrane fraction (Fig. 2C). The purity of this fraction was checked by expression of the invasion protein InlB (Jonquières *et al.*, 1999). The mobility of the SrtA protein on SDS–PAGE was estimated at ≈ 30 kDa, slower than the predicted molecular mass of 24.7 kDa. The 30 kDa protein was found in both cytoplasm and membrane fractions, suggesting that the N-terminal signal peptide was not cleaved and may also function as a membrane anchor domain, as already proposed for *S. aureus* SrtA. As expected, the anti-sortase immunoreactive species was absent in the Δ*srtA* strain (Fig. 2D).

In order to complement the mutant, the *srtA* gene from wild-type *L. monocytogenes* was cloned downstream from a constitutive promoter on a multicopy plasmid. Transformation of the mutant Δ*srtA* with plasmid pP1srtA led to an increased expression of sortase compared with the wild-type strain, as shown by immunoblotting bacterial extracts (Fig. 2D). SrtA overexpression resulted in the production of not only native

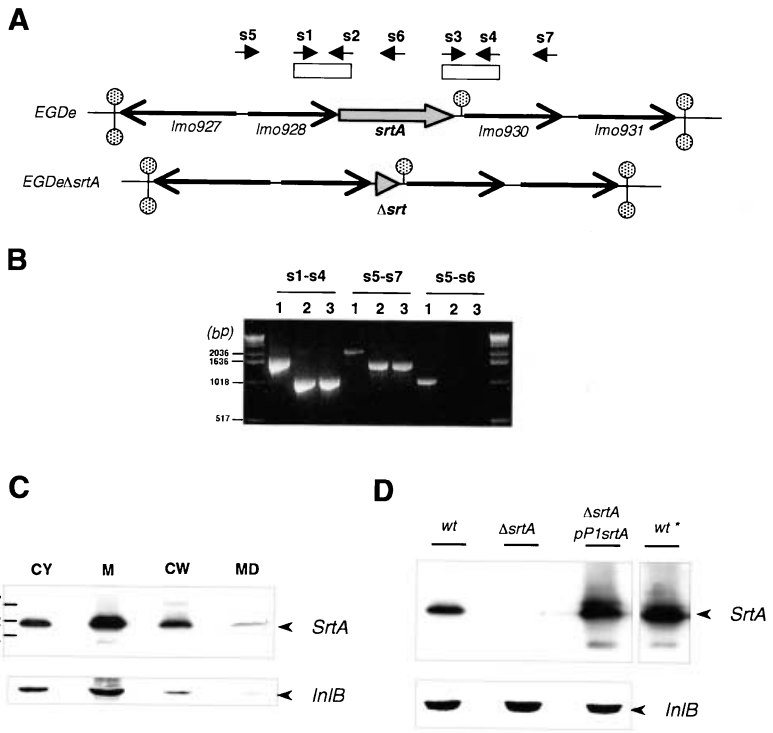

Fig. 2. Chromosomal inactivation of *srtA*.
A. Genetic organization of the *srtA* locus in the EGDe wild type and in the in frame deletion mutant Δ*srtA*. The *srtA* chromosomal region is shown. ORFs are indicated according to the *L. monocytogenes* genome nomenclature (Lmo; Glaser *et al*., 2001). The *srtA* gene (Lmo 0929) is indicated by a grey arrow. Other ORFs, indicated by black arrows, Lmo 0931, Lmo 0930, Lmo 0928 and Lmo 0927, are homologous to YhfJ, YhfI, YxiJ and YfmI in *B. subtilis*, which encode for a lipoate protein ligase A, two proteins of unknown function and a 3-methyladenine DNA glycosylase respectively. Putative transcription terminators are indicated by circles. The Δ*srtA* mutant was constructed from *L. monocytogenes* EGDe by homologous recombination using a temperature-sensitive vector carrying two short PCR fragments blunt end ligated (indicated by open rectangles), which were amplified with primers s1–s2 and s3–s4. The Δ*srtA* mutant carries a truncated *srtA* gene encoding a 7-amino-acid residual peptide, indicated by the arrowhead.
B. The in frame deletion was confirmed by PCR analysis using primers flanking or inside *srtA* (s1–s4, s5–s6 and s5–s6). DNA fragments were separated on ethidium bromide-stained agarose gel.
C. EGDe culture was fractionated into cytosolic (CY), membrane (M), cell wall (CW) and medium (MD) compartments and immunoblotted with anti-SrtA and anti-InlB polyclonal antibodies.
D. Immunoblotting of membrane fractions from wild-type, Δ*srtA* and the complemented strain Δ*srtA*(pP1srtA) with anti-SrtA and anti-InlB antibodies. wt* corresponds to the wild-type lane after overexposure.

30 kDa SrtA but also several additional immunoreactive species. These species can also be found in membrane extracts of wild-type *Listeria* by overexposing immunoreactive signals (Fig. 2D). A faster migrating species of 24 kDa may represent an SrtA degradation product, whereas the slower migrating species could represent sortase molecules that are tethered to surface protein substrates or cell wall fragments, the physiological substrates of the sorting reaction.

Effects of srtA *inactivation in surface anchoring*

To test whether the Δ*srtA* mutant is defective in cell wall anchoring, we first focused on InlA as a representative LPXTG-containing protein, whose sorting is well characterized (Lebrun *et al*., 1996; Dhar *et al*., 2000). The presence of InlA on the bacterial surface was analysed by immunofluorescence and immunogold labelling, using two different InlA-specific monoclonal antibodies (Lebrun *et al*., 1996; Mengaud *et al*., 1996a). Internalin was not detected on the surface of the Δ*srtA* mutant, whereas it was clearly detected on the surface of the *L. monocytogenes* EGDe wild-type strain as well as on the Δ*srtA*(pP1srtA) complemented strain, indicating that expression of *srtA* in the mutant restored surface anchoring (Fig. 3A). Immunogold labelling showed that the average number of InlA-associated gold particles was significantly decreased in the Δ*srtA* strain compared with the wild-type strain (Fig. 3B and C). Immunoblotting demonstrated that the expression of InlA was unaltered in the Δ*srtA* strain (data not shown). We then investigated whether the absence of sortase in *L. monocytogenes* affected the localization of InlA by fractionation experiments of [^{35}S]-methionine pulse-labelled cultures, as described previously (Dhar *et al*., 2000). Wild-type EGDe mainly sorted InlA to the cell wall compartment (Fig. 3D), even though small amounts of InlA were also found in the extracellular medium, as observed previously (Lebrun *et al*., 1996). In contrast, the sortase mutant strain mis-sorted InlA to all cellular compartments. Furthermore, proteolytic degradation fragments of InlA were found within the media and cell wall fractions. These results demonstrate that InlA is no longer properly expressed on the bacterial surface in the Δ*srtA* mutant.

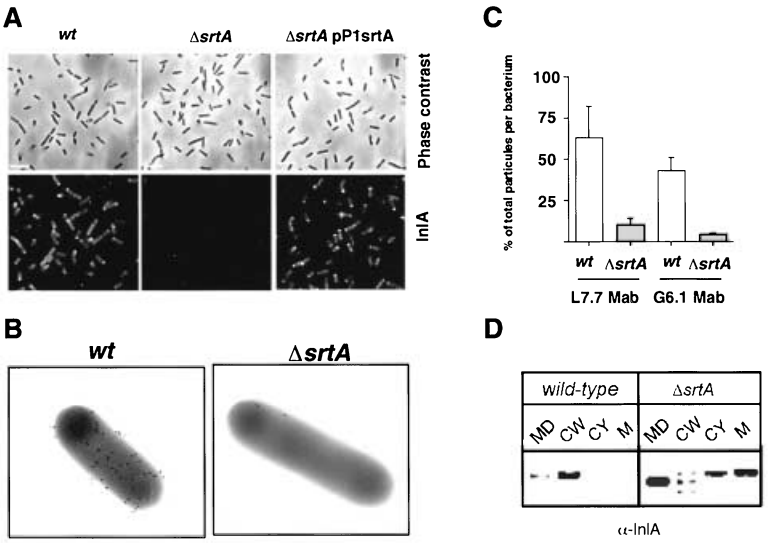

Fig. 3. Display of InlA on the listerial surface.
A. The EGDe wild-type (*wt*), Δ*srtA* and Δ*srtA*(pP1srtA) strains were analysed by phase-contrast (top) and immunofluorescence staining with the InlA-specific mAb L7.7 (bottom). Scale bar = 5 μM.
B. Immunogold labelling of EGDe *wt* and Δ*srtA* with mAb G6.1.
C. Quantification of gold particles on the surface of *wt* and Δ*srtA* with mAbs L7.7 and G6.1 (mean of 7–15 non-dividing bacteria in two independent experiments).
D. Pulse-labelled cultures of wild-type and Δ*srtA* strains were fractionated into medium (MD), cell wall (CW), cytoplasmic (CY) and membrane (M) fractions, and proteins were immunoprecipitated with InlA antibodies. InlA is mis-sorted in the sortase mutant.

To determine whether other peptidoglycan-associated proteins were mis-sorted at the cellular surface of the Δ*srtA* mutant, we prepared an antibody raised against proteins present in a purified *L. monocytogenes* peptidoglycan fraction and used it to analyse highly purified peptidoglycan fractions of the wild-type, Δ*srtA* and Δ*srtA*(pP1srtA) complemented strains. This type of material is purified by mechanical rupture of bacteria and extensive boiling of the cell wall in 4% SDS (see *Experimental procedures*). As a result, only the cell wall proteins that bind strongly to peptidoglycan, such as the set of proteins sorted by the LPXTG motif and covalently attached to peptidoglycan, are those able to withstand the purification procedure. As shown in Fig. 4A, the antiserum specific for the listerial peptidoglycan fraction recognized a pattern of at least 15 proteins of diverse molecular weights in the wild-type strain. Strikingly, these proteins were mostly absent in peptidoglycan extracts from the Δ*srtA* mutant. As expected, the Δ*srtA*(pP1srtA) strain displayed a pattern of peptidoglycan-associated proteins similar to that of the wild type. The amount of InlA anchored to the peptidoglycan was also determined in these fractions (Fig. 4B). The Δ*srtA* mutant had an almost undetectable level of InlA, unlike the wild-type and complemented strain, in which a large amount of InlA was observed anchored to the peptidoglycan. Altogether, these re-sults confirmed that SrtA is required for efficient sorting of InlA to the peptidoglycan, and demonstrate that lack of SrtA causes a notable decrease in the amount and number of other proteins that bind strongly to the peptidoglycan.

To complement the immunochemical analysis and to identify further SrtA-dependent proteins, we chose a 'gel-less' proteome approach. SDS-treated purified cell walls from the wild-type, Δ*srtA* and complemented strains were directly digested with trypsin in order to generate peptides from all proteins that were present in the cell wall fraction. The peptide mixtures were analysed with a quadrupole time-of-flight mass spectrometer (Q-TOF-MS) after electrospray ionization. Peptides were then micro-sequenced, and the corresponding proteins identified. Besides peptides present in all the strains, peptides derived from three proteins were detected only in the wild-type and the Δ*srtA*(pP1srtA) complemented strains. The corresponding proteins were identified as the LPXTG-containing proteins Lmo 0130, Lmo 0880 and Lmo 2714 in the genome sequence (Table 1; Glaser *et al.*, 2001). The corresponding peptide signatures were totally absent in the spectra of the Δ*srtA* mutant. We verified that these peptides were not present in minor amounts in the Δ*srtA* mutant, hidden in the background, by performing tandem mass spectrometry (MS/MS) fragmentation experiments on the masses of these identified peptides (Table 1). No peptides could be found in this way, indicating that the corresponding proteins were not present in cell wall extracts of Δ*srtA*. These results strongly suggest that SrtA targets not only InlA but also at least three other LPXTG-containing proteins to the *L. monocytogenes* cell wall.

Listeria monocytogenes *SrtA recognizes the* S. aureus *protein A sorting signal*

We have shown previously that the sorting signal of staphylococcal protein A (spa) is able to anchor proteins to the *L. monocytogene*s cell wall (Lecuit *et al.*, 1997; Braun *et al.*, 1999). To address the ability of the listerial SrtA to recognize this heterologous signal, we expressed a chimeric protein containing the N-terminal region of InlB fused to the protein A sorting signal (LRR-IR-spa; Braun

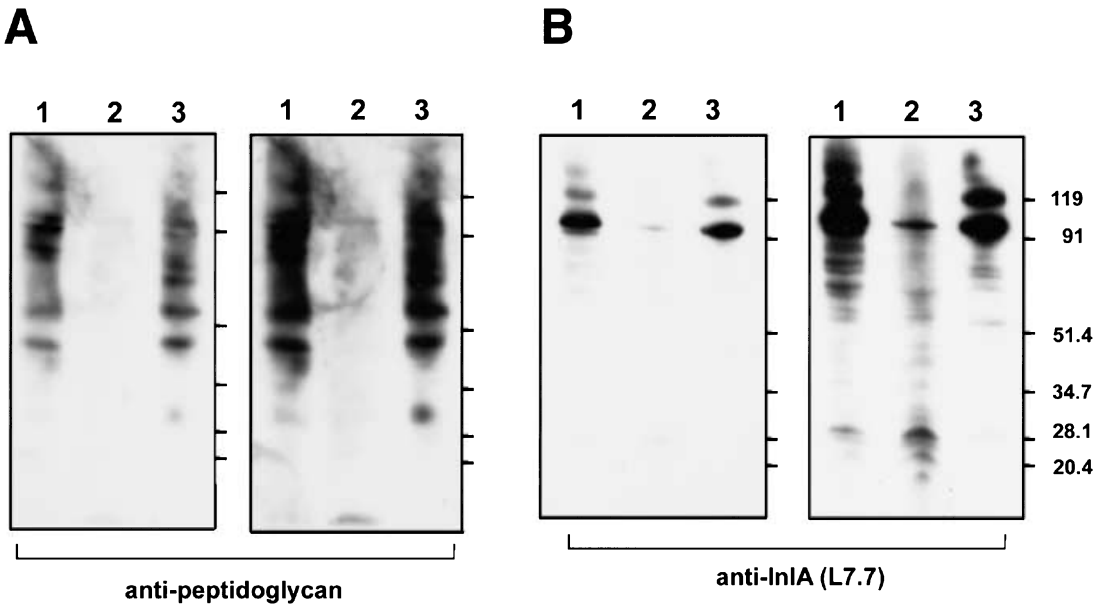

Fig. 4. Role of StrA in anchoring *Listeria* surface proteins to the peptidoglycan. Highly pure peptidoglycan (PG) fractions were prepared from EDGe wild-type (1), Δ*srtA* (2) and Δ*srtA*(pP1strA) (3) strains, as described in *Experimental procedures*. Proteins present in these fractions correspond to those that withstand extensive boiling in 4% SDS, including the proteins sorted by the LPXTG motif.
A. Proteins of the PG fraction recognized by the polyclonal antiserum 839, which was raised to purified macromolecular *L. monocytogenes* peptidoglycan.
B. Levels of InlA protein present in the PG fractions detected with the monoclonal antibody L7.7. Two different exposures of each Western assay are shown. The lack of SrtA notably reduces the amount and number of proteins that associate to peptidoglycan, including InlA.

et al., 1999) in the wild-type strain and in the Δ*srtA* mutant. Protein expression was verified by immunoblotting of cytoplasmic extracts, using InlB affinity-purified antibodies (data not shown). As shown in previous immunofluorescence studies (Braun *et al*., 1997), these antibodies did not allow detection of the native InlB protein on the surface of the wild-type strain (Fig. 5), as InlB is partially buried in the cell wall (Jonquières *et al*., 1999). In contrast, the chimeric LRR-IR-spa protein was efficiently detected at the bacterial surface when expressed into the wild-type strain (Fig. 5). However, the protein A sorting signal was inefficient in targeting the InlB derivative to the cell wall of the Δ*srtA* strain. This result suggests that SrtA is required for the anchoring of any proteins bearing an LPXTG-type C-terminal sorting signal in *L. monocytogenes.*

Effect of srtA *inactivation in the* L. monocytogenes *cellular infectious process* in vitro

We investigated whether the Δ*srtA* mutant displayed a defect in the ability to invade various tissue-cultured non-phagocytic cells, a process requiring InlA and InlB, or for invasion of macrophages, a process that does not require the InlA and InlB invasion proteins. The wild-type strain EGDe, Δ*srtA* as well as Δ*inlA* and Δ*inlB* mutants (Lingnau *et al*., 1995) were examined for their ability to enter into several host cells using the gentamicin survival

Table 1. LPXTG-containing proteins found exclusively in wild-type and Δ*srtA*(pP1srtA) strains.

Accession no.[a]	Size[b] (aa)	Putative function	Masses $[M + 2H]^{2+}$	Peptide sequences
Lmo 0130	784	5′-nucleotidase[c]	574.8	FGPIVEAYPR
			829.9	GTNTVAEGFLPYYVK
			816.4	SVTPNADITAVTEDAK
Lmo 2714	327	Unknown	1095.3	LSTAQTGIQEVTLLGDNEDGK
Lmo 0880	462	Unknown	659.4	ATLSGDNLDAVSR
			805.4	VIATNIQPIALDADR
			676.8	NTTQTVTPYISK

The protein function is based on homology searches with the WU-BLAST algorithm ($<10^{-60}$). The masses of the detected peptides are given in Daltons. The results of the microsequencing experiments of these masses are given by a one-letter code.
a. In GenBank/EMBL accession number AL592022.
b. Number of amino acids in the full-length protein.
c. Highest homology to 5′ nucleotidase from *Streptococcus pyogenes*.

assay (Gaillard *et al*., 1994). We first measured entry into the human epithelial cells Caco-2 and hepatocytes HepG2, which requires InlA and InlB (Dramsi *et al*., 1995; Lingnau *et al*., 1995), and into the monkey epithelial cell line Vero and murine hepatocytes TIB73, which requires only InlB (Dramsi *et al*., 1995; Ireton *et al*., 1996). We also tested entry into J774 and RAW264.7 macrophages. Entry of the Δ*srtA* mutant into Caco-2 cells and HepG2 cells was significantly decreased to the level of the Δ*inlA* mutant (Table 2). In contrast, the Δ*srtA* mutant was internalized into Vero and TIB73 cells as efficiently as wild-type and Δ*inlA* strains, whereas entry of the Δ*inlB* mutant was significantly reduced in those cells, as expected. Furthermore, entry of Δ*srtA* into macrophage cell lines J774 and RAW 264.7 was not impaired and was similar to wild-type or internalin mutant strains. Taken together, these results indicate that inactivation of *srtA* in *L. monocytogenes* caused a similar effect on listerial entry into various cell lines as that observed for the inactivation of *inlA*.

We then determined the effects of complementation on bacterial entry into cells. Unexpectedly, complementation of the Δ*srtA* mutant with the plasmid carrying *srtA* did not restore invasiveness in Caco-2 and HepG2 cells (Table 2). Moreover, overexpression of sortase dramatically reduced InlB-mediated invasion into Vero and TIB73 cells. In contrast, the pathway that leads to *L. monocytogenes* uptake into macrophages was not significantly affected by overexpression of sortase. We verified that bacterial viability or cell division was not affected by overexpression of sortase (data not shown). Taken together, these results suggest that overexpression of SrtA causes a dominant-negative effect on the invasion of *L. monocytogenes* into all the non-phagocytic cells tested, even though it restores the surface localization of InlA.

Finally, to examine whether the Δ*srtA* mutant was affected at any other step in the infection process, we examined its intracellular replication, intracellular motility and cell-to-cell spread in Vero and TIB73 cells. The sortase mutant was identical to the wild-type strain in both intracellular growth during 6 h of infection and in actin tail formation (data not shown), which is required for intra- and intercellular motility. We then analysed the capacity of the Δ*srtA* mutant to form plaques on culture cell monolayers of Vero and TIB73 cells, as well as in mouse L2 fibroblasts (Sun *et al*., 1990). In each case, wild type and Δ*srtA* mutants formed plaques of the same size, indicating that the inactivation of sortase did not alter bacterial cell-to-cell spread.

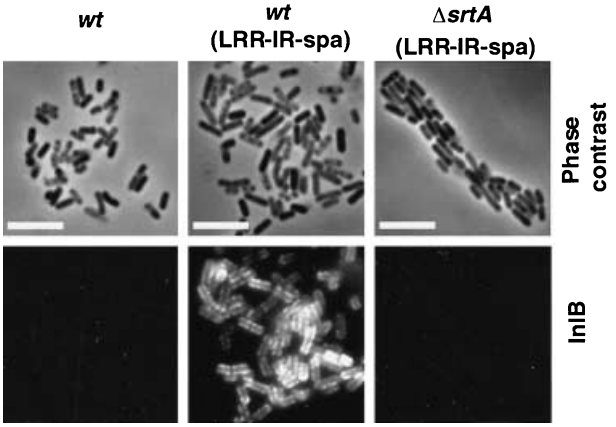

Fig. 5. Display of the fusion protein InlB(LRR-IR)-spa on the listerial surface. The EGDe wild-type strain (*wt*) and the *wt* and Δ*srtA* strains expressing the LRR-IR domain of InlB fused to the protein A sorting signal (LRR-IR-spa) were analysed by phase-contrast (top) and by immunofluorescence staining with InlB affinity-purified antibodies (bottom). Scale bars = 5 μM.

Effects of the srtA *mutation on liver and spleen colonization*

The contribution of *srtA* to *L. monocytogenes* infections was examined using a mouse model of tissue colonization. After oral infection, *L. monocytogenes* can, albeit inefficiently, cross the intestinal barrier and gain access to

Table 2. Comparison of the invasive abilities of *L. monocytogenes* EGDe wild type and derivatives, Δ*srtA*, *inlA*, *inlB* and Δ*srtA*(pP1srtA).

Strains	Relative percentage of entry into cell lines[a]					
	CaCo-2	HepG2	TIB73	Vero	J774	RAW 267.10
Wild type	100	100	100	100	100	100
Δ*srtA*	**3.8 ± 0.8**	**11 ± 3**	97 ± 29	76 ± 5	86 ± 8	102 ± 9
Δ*inlA*	**2.1 ± 1.2**	**15 ± 8**	71 ± 33	87 ± 27	107 ± 6	ND[b]
Δ*inlB*	**1.2 ± 1.1**	**4 ± 2**	**17 ± 8**	**11 ± 6**	87 ± 11	ND[b]
Δ*srtA* (pP1srtA)	**1.7 ± 0.2**	**8 ± 5**	**17.5**[c]	**7 ± 1**	78 ± 25	113 ± 17

a. Cells were infected with the bacterial strains for 1 h, and invasion frequencies were calculated from the number of bacteria that survived incubation in the presence of gentamicin with respect to the total number of inoculated bacteria. The level of entry of the wild-type strain has been artificially reported as 100, and the level of entry of the different mutants is a relative value. Bold letters indicate significant falls in invasion frequencies. Results are the mean ± SD of three to six independent experiments performed in duplicate.
b. Not determined.
c. Result of one experiment performed in duplicate.

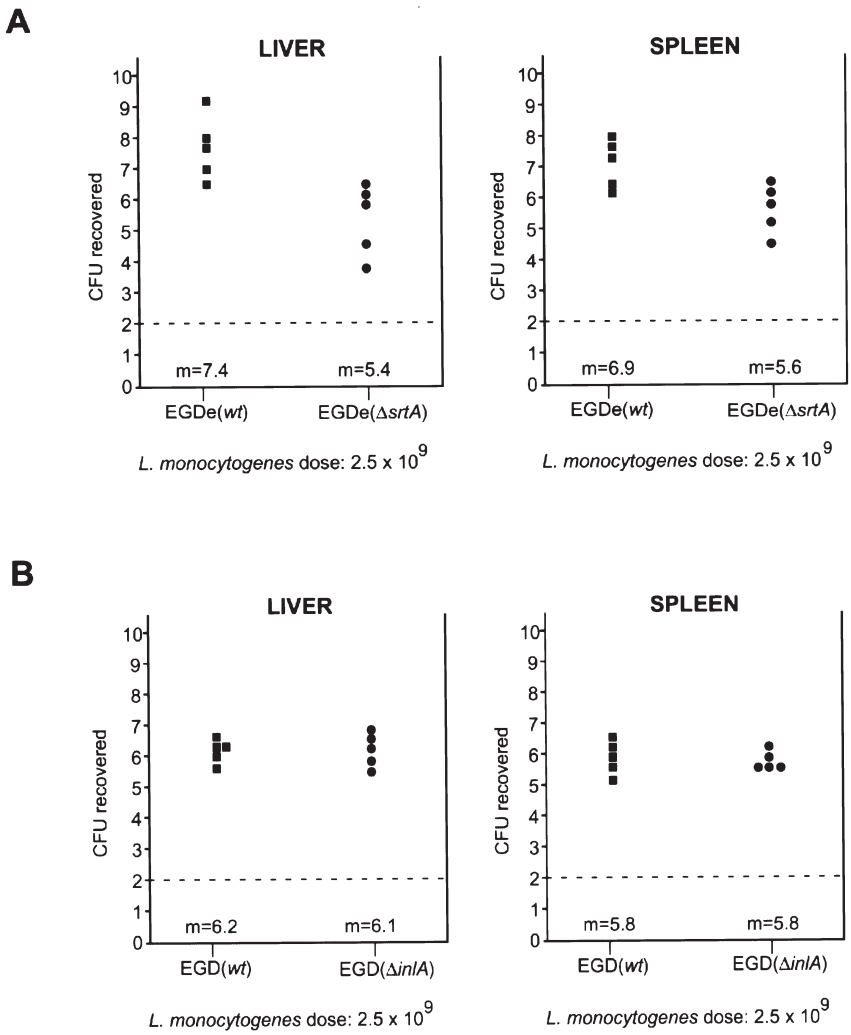

Fig. 6. Determination of colony-forming units (cfu) after oral infection of BALB/c mice. *L. monocytogenes* EGDe and isogenic Δ*srtA* mutants were orally inoculated into five BALB/c mice each, as indicated in *Experimental procedures*. Three days after infection, animals were euthanized, and livers and spleens were homonogenized and plated. Symbols indicate $\log_{10}$ cfu of (A) EGDe (closed squares) and the Δ*srtA* mutant strain (closed circles) and (B) EGD (closed squares) and the Δ*inlA* mutant strain (closed circles). The mean (m) cfu for each strain is shown. The dashed line represents the limit of detection of *L. monocytogenes* in tissues.

deeper tissues, causing abscess formation in various organs. Determination of the level of colonization in livers and spleens of infected mice can be used as a measure of bacterial virulence. BALB/c mice were infected orally with 2.5×10^9 colony-forming units (cfu) of the wild-type EGDe and Δ*srtA* isogenic pair. We also infected mice with a Δ*inlGHE* mutant, which is deleted of three genes encoding LPXTG-containing proteins and is known to be affected in liver and spleen colonization (Raffelsbauer *et al*., 1998). Bacterial counts within infected organs were determined 3 days after infection. As shown in Fig. 6A, the sortase mutant displayed a decrease in bacterial counts of about 2 and 1 $\log_{10}$ units in liver and spleen, respectively, compared with the wild-type strain. The Δ*inlGHE* mutant showed an intermediate level of colonization (data not shown), corroborating previous reports that these internalins may be involved in listerial pathogenesis of mouse infections (Raffelsbauer *et al*., 1998; Schubert *et al*., 2001). In a separate set of experiments, the InlA mutant did not exhibit a reduction in cfus in the same tissues when compared with wild type (Fig. 6B), consistent with our recent observations (Lecuit *et al*., 2001) and with the fact that InlA is unable to interact with murine E-cadherin (Lecuit *et al*., 1999). These results suggest that several LPXTG-containing proteins sorted by SrtA may play a role during murine listeriosis, as the Δ*srtA* mutant has a colonization defect greater than that of the Δ*inlGHE* strain.

Discussion

In this work, we show that the *srtA* gene of *L. monocytogenes* encodes a sortase that is required for cleavage of the InlA sorting signal and targets this invasion protein to the listerial surface. Moreover, we demonstrate that SrtA is required for the proper sorting of several other peptidoglycan-bound proteins. Three of them were identified by a mass spectrometry approach, with the help of the newly determined *L. monocytogenes* sequence (Glaser *et al*., 2001). Together with the previously pub-

lished data in *S. aureus* and *S. gordonii* (Mazmanian *et al*., 2000; Bolken *et al*., 2001), this result confirms the universal function of sortase in anchoring LPXTG-containing surface proteins to the cell wall of Gram-positive bacteria. We also show that the staphylococcal protein A sorting signal, which is capable of anchoring fusion proteins to the listerial surface (Lecuit *et al*., 1997; Braun *et al*., 1999), is a substrate for SrtA of *L. monocytogenes*. In *Listeria*, LPXTG-containing proteins are anchored to the amino group of the *m*-diaminopimelic acid cross-bridge (Dhar *et al*., 2000), whereas in *S. aureus*, they are linked to the pentaglycine cross-bridge (Navarre and Schneewind, 1994). Therefore, listerial and staphylococcal SrtA proteins are conserved in their ability to anchor heterologous sorting signals to peptidoglycan substrates, as expected from previous work (Schneewind *et al*., 1993). However, it is not yet known whether sortases from different bacterial species are able to recognize heterologous cell walls. How are the enzymes able to recognize their substrates? Interactions are likely to occur in the membrane, as sortases are membrane-anchored proteins (Mazmanian *et al*., 2000; this work), and LPXTG proteins and peptidoglycan precursors are transiently retained in the membrane. Future work is needed to characterize more precisely the localization of sortases relative to their substrates in the membrane and to determine hypothetical interactions with other components.

A search for homologies to the catalytic sulphydryl-containing core region of SrtA led to the identification of *srtB,* another gene encoding a putative sortase. SrtB orthologues were also found in the non-pathogenic *L. innocua*, in *S. aureus* as well as in other Gram-positive genomes, such as *S. pyogenes*, *C. difficile* and *B. anthracis*, suggesting the existence of at least two classes of sortases (this work, see also Mazmanian and Schneewind, 2002). These results are in agreement with a recent systematic search for sortase homologues in bacterial genomes, which revealed the existence of sortases in almost all Gram-positive bacteria, usually with more than one sortase per genome (Pallen *et al*., 2001). Our analysis indicates that the second class of sortases encodes two additional motifs compared with the first one. Genes encoding sortase-like proteins are often clustered with genes encoding LPXTG-containing proteins (Pallen *et al*., 2001). This is not the case for listerial *srtA*. In this case, the closest LPXTG-containing protein is located at a distance of 48 kb from *srtA* in the genome (512 kb for *inlA*). In contrast, *srtB* is located in the vicinity of two genes encoding LPXTG-type surface proteins (Lmo 2178 and Lmo 2179). The *L. monocytogenes* genome contains 41 LPXTG-type proteins (Glaser *et al*., 2001). If *srtB* encodes another sortase, does it target some of these proteins? In this work, we show that inactivation of *srtA* fully abrogates the anchoring of InlA and that of at least three other LPXTG-containing proteins (Lmo 0130, Lmo 0880 and Lmo 2714). In addition, nearly all the proteins detected in purified peptidoglycan extracts in the wild-type strain are missing in the Δ*srtA* mutant. Therefore, SrtB probably has a minor role, if any, in the cell wall anchoring of LPXTG proteins in *L. monocytogenes*, as already noticed for *S. aureus* SrtB (Mazmanian *et al*., 2001).

We have analysed the effect of *srtA* inactivation on several steps of the *Listeria* infectious process *in vitro*. A Δ*srtA* mutant is as defective for entry into epithelial cells as a Δ*inlA* mutant, but is not affected in intracellular motility or in cell-to cell spread, at least in the cell types used here. Interestingly, complementation of the Δ*srtA* mutant with *srtA* on a multicopy plasmid, which restored anchoring of InlA, does not restore entry into IniA-permissive cells and, furthermore, inhibits entry into IniB-permissive cells. Overexpression of SrtA in this strain may modify the accessibility or the local concentration of surface proteins that play a role in the interaction of the bacteria with mammalian cells. The nature and role of these LPXTG-containing proteins in the cellular infectious process is unknown.

An attractive feature of sortases is that they may be used as targets for identifying inhibitors that could be of general use in the prevention of Gram-positive bacterial infections. This hypothesis depends on the ability of LPXTG-containing proteins to perform critical functions during infection. In fact, many surface proteins have been identified as virulence factors in several Gram-positive pathogens (Navarre and Schneewind, 1999). Until now, only a few LPXTG-containing proteins of *L. monocytogenes* are believed to be involved in the infectious process. InlA promotes bacterial invasion of epithelial cells by interacting with its host receptor, E-cadherin (Mengaud *et al*., 1996b). Internalin does not interact with the murine E-cadherin, excluding the mouse as a suitable model to test its function *in vivo* (Lecuit *et al*., 1999). In line with this result, our laboratory has recently established that InlA promotes crossing of the intestinal barrier in guinea pigs or in transgenic mice expressing human E-cadherin (Lecuit *et al*., 2001). Other proteins belonging to the internalin multigene family [i.e. proteins containing a signal sequence, leucine-rich repeats (LRR) and a sorting signal (Dramsi *et al*., 1997)] are known to play a role in murine listeriosis. A mutant with a deletion of the *inlGHE* locus and an *inlH* mutant exhibit loss of virulence (Raffelsbauer *et al*., 1998; Schubert *et al*., 2001). However, the role of these proteins is still unknown. Lastly, a mutant in a gene that encodes for a novel internalin-like protein (Lmo 2026) was shown to be affected in multiplication of *L. monocytogenes* in the brain in the mouse model after intravenous infection (Autret

et al., 2001). Here again, the role of this protein remains to be identified.

In this work, we have shown that a Δ*srtA* mutant is reduced in colonization of the liver and spleen after oral infection in mice compared with wild-type *L. monocytogenes*. As InlA has no role in murine infections, this result suggests that SrtA anchors at least one, if not several, other LPXTG-containing protein(s) that are involved in murine listeriosis. This hypothesis is supported by the fact that the Δ*srtA* strain exhibits a more pronounced virulence defect compared with the Δ*inlGHE* mutant. It will be important to identify this (these) protein(s) and also to determine the behaviour of Δ*srtA* and Δ*srtB* mutants in other organs and other animal models. Identification of drugs that inhibit sortase activity may be used as anti-infective therapies to either prevent or cure human infections by *Listeria* or other Gram-positive bacteria.

Experimental procedures

Bacterial strains and plasmids

The *Listeria* strains used in this work were the wild-type *L. monocytogenes* strain EGDe (BUG 1600), whose genome has been sequenced recently (GenBank/EMBL accession number AL591824; Glaser *et al*., 2001), the Δ*inlA* and Δ*inlB* isogenic mutants (Lingnau *et al*., 1995), the Δ*srtA* mutants (BUG 1777 and 1778; this work), the complemented strain Δ*srtA* (pP1srtA) (Bug 1783, this work, i.e. BUG 1778 carrying plasmid pP1srtA) and strain EGDe and Δ*srtA* carrying plasmid pP1B8 [i.e. expressing the LRR-IR domain of InlB fused to protein A sorting signal (Braun *et al*., 1999), BUG 1836 and BUG 1837 respectively; this work]. Note that all these strains are not isogenic with EGD and Δ*inlA* and Δ*inlB* derivatives used previously by our laboratory (Dramsi *et al*., 1995; Braun *et al*., 1997; Braun *et al*., 1999; Lecuit *et al*., 2001). Strains were grown at 37°C in BHI agar (Difco), supplemented with 8 μg ml^{-1} erythromycin when carrying plasmids. The human hepatocarcinoma HepG2 cells, the African green monkey kidney Vero cells, the mouse hepatocytes TIB73 and the mouse macrophages J774 and RAW267.1 were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Sera Laboratory); the human colon carcinoma Caco-2 cells were cultured in MEM + 20% FCS, and the fibroblast cell lines in F12 Ham (Gibco) + 10% FCS. All media were 2 mM glutamine and 1% non-essential amino acids (Gibco). Cells were incubated at 37°C in 10% CO_2.

Sequence analysis

Sequence analyses were performed on the mainframe computer run by the Institut Pasteur Computer Services. Iterative database searches were performed using the BLAST family of programs (Altschul and Koonin, 1998) on genomes of *Listeria* sp. BLAST searches of Gram-positive bacterial genomes were performed on the NCBI Blast web server (http://www.ncbi.nlm.nih.gov/Microb_blast). Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. Multiple alignments were performed using a using local alignment method (MATCHBOX; Depiereux and Feytmans, 1992). Statistical significance of the multiple alignment was evaluated using BLAST-associated *E*-values and estimation of *P*-values by MACAW (Schuler *et al*., 1991).

Chromosomal inactivation and cloning of srtA

Two 500 bp fragments flanking *srtA (*position 966.2 kb on the *L. monocytogenes* EGDe sequence, Lmo 929, GenBank/EMBL accession no. AL591824; Glaser *et al*., 2001) were amplified by PCR from EGDe chromosomal DNA with primers inside and outside the *srtA* locus. Primers for the 5′ fragment were s1 (5′-CGCGGATCCGTGTTGCTTAATTTTATAACC-3′) and s2 (5′-AAAAGGCCTAATTGTTTTCTTTAACATATG-3′); primers for the 3′ fragment were s3 (5′-AAAAGGCCTTAATGAG-GAAAAAAAGACAGC-3′) and s4 (5′-CCGGAATTCGAAAGTTACTTTAAA-CGGCC-3′) (Fig. 2A). After restriction of the amplified 5′ and 3′ fragments with *Bam*HI and *Stu*I, and *Stu*I and *Eco*RI, respectively, the two fragments were coligated into the thermosensitive plasmid pKSV7 (Dramsi *et al*., 1997) digested by *Bam*HI and *Eco*RI, yielding plasmid pKSV7Δ*srtA*. This plasmid was then electroporated into *L. monocytogenes* strain EGDe, and gene replacement was performed as described previously (Camilli *et al*., 1993; Dramsi *et al*., 1997), resulting in a gene containing only the first six codons and the stop codon of *srtA*. Two independent mutants (BUG 1777 and BUG 1778) were verified by PCR analysis of chromosomal DNA using couples of internal or flanking primers s1, s4 and s5 (5′-CGTACATCAAACGAATGAGGG-3′), s6 (5′-ACCTGCTAGCGGATAATTACC-3′) and s7 (5′-TTCTAAATCGCCAACTTCGGG-3′) (Fig. 2A). To complement the Δ*srtA* mutation, *srtA* was PCR amplified from EGDe chromosomal DNA using primers 5′-ACGAGCTCAATAGTTACAAGGAGGAATC-3′ and 5′-ACATGCATGCGAAAGTTACTTTAAACGGCC-3′. The DNA fragment was digested with *Sac*I and *Sph*I and inserted into the *Sac*I and *Sph*I sites in the shuttle plasmid pP1 downstream from the constitutive promoter pProt of the protease gene from *Streptococcus cremoris* (Dramsi *et al*., 1995), leading to plasmid pP1srtA.

Purification of SrtA and generation of polyclonal antibodies

To purify recombinant SrtA from *L. monocytogenes*, *srtA* flanking primers lmsrt-5 (5′-AAGGATCCACCAATGGCCCCGGAAAATT-3′) and lmsrt-3 (5′-AAGGATCCGCTACATGATCTGGATGGTAA-3′) were used to PCR amplify the *srtA* coding sequence deleted in the N-terminal hydrophobic domain. The resulting product and vector were digested with *Bam*HI and ligated into pET16b (Novagen). Protein expression was induced in BL21(DE3) by the addition of 1 mM IPTG, and proteins were purified over Ni-NTA under denaturing conditions (Qiagen). Purity was assessed through Coomassie staining of eluates. Protein (3 mg) was injected into a female, white New Zealand rabbit (Charles River), with boosters given every 3 weeks until sacrifice in week 12. Antiserum was obtained by cardiac puncture according to UCLA Animal Rights Committee protocols.

Microscopy, fractionation and immunoblots

Immunofluorescence and immunogold staining of InlA were performed according to procedures described elsewhere (Lebrun *et al*., 1996) using monoclonal antibodies (mAbs) L7.7 and G6.1 (Mengaud *et al*., 1996a) and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Biosys). Immunogold quantification experiments were performed by counting the InlA-associated gold particles in 7–15 wild-type or Δ*srtA* bacteria, from two independent experiments. Immunofluorescence staining of the chimeric InlB protein LRR-IR-spa was performed as described previously (Braun *et al*., 1997) using affinity-purified InlB polyclonal antibody and FITC-conjugated secondary antibody. For analysis of sortase expression, bacterial proteins were separated into cytoplasm, membrane, cell wall and culture medium, according to a previously described protocol (Jonquières *et al*., 1999). Equivalent amounts of all fractions, representing 100 or 200 μl of bacterial culture, were analysed by immunoblotting. Proteins were boiled in Laemmli sample buffer, resolved on a 14% SDS–PAGE gel and transferred to nitrocellulose membranes. SrtA and InlB were detected using polyclonal antibodies (this work; Braun *et al*., 1997) and horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibodies and the ECL kit (Amersham). For analysis of InlA localization, overnight cultures of *L. monocytogenes* were grown in BHI at 37°C and diluted 1:50 in BHI at 37°C for 3 h, at which time cells were washed and resuspended in minimal media lacking met and cys. After pulse labelling with 5 μl of [^{35}S]-Promix (Amersham), bacterial proteins were separated into cytoplasm, membrane, cell wall and culture medium and immunoprecipitated with anti-InlA specific antibodies, according to a previously described protocol (Dhar *et al*., 2000). Cell wall digestion was performed with 1 mg ml^{-1} endolysin (Ply118; Loessner *et al*., 1996) for 1 h at 37°C. Proteins were separated by SDS–PAGE and visualized by phosphorimager (Molecular Dynamics).

Isolation of peptidoglycan pure fractions

Fractions containing highly pure peptidoglycan were obtained from the different *L. monocytogenes* strains grown overnight at 37°C in 500 ml of BHI medium. Briefly, bacteria were spun down (5000 *g*, 10 min, 4°C) and suspended in 10 ml (final volume) of phosphate-buffered saline (PBS, pH 7.4) containing a cocktail of protease inhibitors (Roche Diagnostics) and 100 μg ml^{-1} DNase. The cells were disrupted by two passages through a French press. Unbroken cells were removed by low-speed centrifugation (5000 *g*, 5 min, 4°C). The supernatant was centrifuged at higher speed (18 000 *g*, 20 min, 4°C) to obtain a pellet containing the envelope material. This material was suspended in 3 ml of PBS (pH 7.4) and slowly mixed with 3 ml of boiling 8% SDS. Boiling at 100°C was carried out for 3 h and then continued overnight at 80°C. Macromolecular peptidoglycan was recovered by high-speed centrifugation (200 000 *g*, 20 min, 30°C). After four washing steps with warm (60°C) distilled water, the peptidoglycan was suspended in 10 mM Tris-HCl, pH 7.6, 0.06% NaCl and treated with α-amylase (100 μg ml^{-1}, 37°C, 90 min; Sigma, ref. A-6380). The sample was subjected to high-speed centrifugation (200 000 *g*, 20 min, 30°C) and the pellet washed once with 'muramidase buffer', 50 mM phosphate buffer, pH 4.9. The pellet (peptidoglycan material) was finally suspended in muramidase buffer and digested with 20 μg ml^{-1} N,O-diacetyl-muramidase (Cellosyl; Hoechst) at 37°C for 18 h. Samples were then incubated at 100°C for 20 min to inactivate the enzyme. Proteins released from macromolecular peptidoglycan were precipitated by simultaneous treatment with acid pH (the pH 4.9 of muramidase buffer) and heat (100°C, 10 min), a step included in the standard procedure to prepare muropeptides for high-performance liquid chromatography (HPLC) analysis (Glauner, 1988). Upon centrifugation at 15 000 *g* for 15 min at 4°C, the pellet containing the precipitated proteins and undigested macromolecular peptidoglycan was suspended in 40 μl of PBS, pH 7.4 buffer. This fraction was named 'peptidoglycan fraction' (PG) and contain proteins that associate to peptidoglycan withstanding extensive boiling in 4% SDS. Upon addition of concentrated Laemmli buffer, the PG fractions were boiled, centrifuged (15 000 *g*, 5 min, 4°C) to remove the undigested peptidoglycan and the supernatant subjected to SDS–PAGE and Western immunoblotting. To detect surface proteins that strongly associate to peptidoglycan, a rabbit polyclonal antiserum (839) was raised against a purified macromolecular (undigested with muramidase) peptidoglycan fraction of the *L. monocytogenes* P14-A strain (Ripio *et al*., 1997).

Proteomic analysis

To generate the cell wall fraction, EGDe wild type, EDGΔ*srtA* and Δ*srtA*(pP1srtA) were grown on 300 ml of BHI at 37°C and 180 r.p.m. The cells were harvested at OD_{600} = 0.8 and centrifuged at 3000 *g* (20 min, 4°C). The cell pellets were washed twice with 1 ml of PBS and once with 1 ml of 1 M Tris-HCl, pH 7.5. After resuspending in 8 ml of 0.1 M Tris-HCl, pH 8, the cells were broken at 1000 p.s.i. in a French press (SLM-Aminco), and 40 μl of 100 mM Pefabloc-SC (Roth Chemicals) was added to prevent proteolysis. The cell disruption by French press was repeated twice; then, 1 μl of benzonase (Merck) was added and incubated for 30 min on ice. Intact cells were removed by centrifugation at 3000 *g* (20 min, 4°C), and the supernatant was centrifuged again at 10 000 *g* (20 min, 4°C) to obtain the cell wall fraction. The pellet was washed with 10 ml of 0.1 M Tris-HCl, pH 8, and twice with 1 ml of 4% SDS, 4% mercaptoethanol, 0.1 M Tris-HCl, pH 8. The solution was treated in a ultrasonicator for 5 min before centrifugation at 10 000 *g* (20 min, 4°C).

For mass spectrometry sample preparation, the cell walls were washed three times with 1 ml of 0.1 M Tris-HCl, pH 8, to remove remaining SDS and mercaptoethanol. The sediment was suspended in 500 μl of trypsin (2 μg ml^{-1}) in 50 mM NH_4HCO_3 (sequencing grade; Promega), incubated at 37°C with constant shaking overnight. The suspension was centrifuged at 10 000 *g*, the supernatant dried down and redissolved in 30 μl of 5% MeOH, 0.5% formic acid. The peptides were concentrated and desalted using C18-ZipTip (Millipore) and eluted in 5 μl of 65% MeOH, 0.5% formic acid.

For electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis and subsequent peptide sequencing 3 μl of ZipTip purified sample was filled into Au/Pd-coated nanospray glass capillaries (Protana). The tip of the capillary was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight mass spectrometry instrument

(Q-TOF II; Micromass) equipped with a nanospray ion source. A capillary voltage between 750 V and 1000 V and a cone voltage of 35 V were applied. Doubly and triply charged peptides were chosen for collision-induced dissociation experiments, and the corresponding parent ions were selectively transmitted from the quadrupole mass analyser into the collision cell. Argon was used as the collision gas, and the kinetic energy was set between 20 eV and 35 eV. The resulting daughter ions were separated by an orthogonal time-of-flight mass analyser. Peptide sequencing and protein identification were carried out with the program PEPTIDE-SEQUENCING within the BIOLYNX software (version 3.4; Micromass) and with the program SONAR (Proteometrics).

In vitro *invasion assays*

Invasivity tests were performed as described previously (Gaillard *et al*., 1994; Mengaud *et al*., 1996b). Briefly, the *Listeria* strains were grown to OD = 0.8–1, washed in PBS and diluted in DMEM such that the multiplicity of infection (MOI) was about 50 bacteria per cell. Bacterial suspensions were added to mammalian cells for 1 h, cells were then washed and non-invasive bacteria were killed by adding 10 $\mu g\,ml^{-1}$ gentamicin for 2 h. After washing, cells were lysed in 0.2% Triton X-100, and the number of viable bacteria released from the cells was assessed by titring on agar plates.

Animal experiments

Five 5- to 6-week-old-female BALB/c mice (Charles River) were inoculated orally with a 100 µl bolus of 2.5×10^9 *Listeria* grown in BHI for 18 h at 37°C, washed once in 0.15 M NaCl and resuspended in 0.15 M NaCl. After 3 days, livers and spleens were removed and homogenized in 0.5% Triton X-100 in water and plated on tryptic soy broth (TSB) supplemented with 50 $\mu g\,ml^{-1}$ nalidixic acid. All mice were treated in accordance with institutional guidelines for the humane treatment of animals (UCLA ARC).

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