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Sortase B, a New Class of Sortase in *Listeria monocytogenes*

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Sortases are transamidases that covalently link proteins to the peptidoglycan of gram-positive bacteria. The genome of the pathogenic bacterium *Listeria monocytogenes* encodes two sortases genes, *srtA* and *srtB*. The *srtA* gene product anchors internalin and some other LPXTG-containing proteins to the listerial surface. Here, we focus on the role of the second sortase, SrtB. Whereas SrtA acts on most of the proteins in the peptidoglycan fraction, SrtB appears to target minor amounts of surface polypeptides. We identified one of the SrtB-anchored proteins as the virulence factor SvpA, a surface-exposed protein which does not contain the LPXTG motif. Therefore, as in *Staphylococcus aureus*, the listerial SrtB represents a second class of sortase in *L. monocytogenes*, involved in the attachment of a subset of proteins to the cell wall, most likely by recognizing an NXZTN sorting motif. The Δ*srtB* mutant strain does not have defects in bacterial entry, growth, or motility in tissue-cultured cells and does not show attenuated virulence in mice. SrtB-mediated anchoring could therefore be required to anchor surface proteins involved in the adaptation of this microorganism to different environmental conditions.

Listeria monocytogenes is a ubiquitous food-borne gram-positive bacterium, responsible for life-threatening infections in humans and animals (17). It is a facultatively intracellular pathogen that is able to enter and multiply in both professional (29) and nonprofessional phagocytes such as epithelial cells (19) or hepatocytes (47). After entry, bacteria rapidly lyse the phagosomal membranes and gain access to the cytosol, where they spread to adjacent cells by an actin-based motility process (for reviews, see references 11, 12, 25, and 46). The interaction of *L. monocytogenes* with host cells is a key event in the pathogenesis of listeriosis, a process that involves a number of surface proteins (see reference 10 for a review). Several distinct mechanisms of cell wall attachment and display of surface proteins have been described in gram-positive bacteria (10, 13, 37). Each mechanism is determined by specific structural motifs in the sequence of the protein. Until very recently, the only surface proteins known to be attached by a covalent linkage to the peptidoglycan (PG) were the LPXTG proteins, exemplified by protein A of *Staphylococcus aureus* and internalin (InlA) of *L. monocytogenes*. In this class, the C-terminal sorting signal is a conserved LPXTG motif that precedes a membrane-spanning hydrophobic domain and a tail that is mostly composed of positively charged residues (31, 44). The LPXTG sorting mechanism was elegantly deciphered in *S. aureus*. Its hydrophobic domains and charged tails retain target staphylococcal proteins in the membrane, allowing recognition and processing of the LPXTG motif by the membrane-anchored sortase, SrtA (33). The anchor structure of cell wall surface proteins in *L. monocytogenes* is also known: a C-terminal threonine is amide linked to the side chain amino group of *m*-diaminopimelic acid within cell wall peptides (14). Thus, in both organisms, the proteins are cleaved between the threonine and the glycine residue of the LPXTG motif and are amide linked to the PG, suggesting that this cell wall sorting mechanism is shared by all gram-positive bacteria.

Analyses of *srtA* deletion mutants demonstrated that SrtA of *L. monocytogenes* acts to process and anchor InlA and at least three other LPXTG proteins and is involved in listerial virulence (5, 20). In *S. aureus*, *srtA* mutants fail to anchor all of the LPXTG surface proteins, causing a reduction in the ability of such sortase mutants to establish animal infections (30). In the gram-positive human commensal organism *Streptococcus gordonii*, inactivation of *srtA* decreases the ability of the bacteria to colonize the oral mucosa in the mouse (6). More recently, genes encoding sortases were identified in *Streptococcus suis* (39), *Streptococcus pyogenes* (3), and the oral pathogens *Streptococcus mutans* (28) and *Streptococcus pneumoniae* (22, 24). Each of these species possess several paralogous copies of *srtA*. For example, *S. suis* possesses as many as five genes encoding sortases or sortase-like proteins (39). In this organism, SrtA is a major sortase that acts to anchor LPXTG proteins to the PG; the roles of the other four putative sortases remain unknown (38).

Bierne et al. recently identified another gene encoding a putative sortase in the genome of *L. monocytogenes*, designated *srtB* (5). Like SrtA, SrtB contains an N-terminal hydrophobic region, which could act as a signal peptide/transmembrane domain, and an essential cysteine residue within the catalytic TLXTC signature sequence, at the C terminus. However SrtB, which is only 23% identical to SrtA, contains different or ad-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Property	Reference
Strains		
BUG 1600	*L. monocytogenes* EGD-e wild-type strain	21
BUG 1777	Isogenic Δ*srtA* mutant	21
BUG 1877	Isogenic Δ*srtB* mutant	This study
BUG 1878	Isogenic Δ*srtA* Δ*srtB* mutant	This study
BUG 1988	Isogenic Δ*srtA* mutant carrying pP1srtB	This study
BUG 1989	Isogenic Δ*srtB* mutant carrying pP1srtB	This study
BUG 1990	Isogenic Δ*srtA* Δ*srtB* mutant carrying pP1srtA	This study
BUG 1991	Isogenic Δ*srtA* Δ*srtB* mutant carrying pP1srtB	This study
BUG 1992	Isogenic Δ*svpA* mutant	This study
Plasmids		
pKSVΔ*srtB*	Thermosensitive plasmid pKSV7 carrying two coligated 500-bp fragments flanking *srtB*	This study
pau1A svpA		This study
pP1*srtA*	Expressing *srtA* under the control of the constitutive promoter pProt	5
pP1*srtB*	Expressing *srtB* under the control of the constitutive promoter pProt	This study

ditional amino acid structures relative to SrtA. SrtB orthologues also exist in the nonpathogenic species *Listeria innocua*, in *S. aureus*, and in a few other gram-positive bacteria whose genomes are available—*S. pyogenes*, *Clostridium difficile*, *Bacillus anthracis*, and *Bacillus halodurans*—suggesting that SrtB belongs to a second specific class of sortases (5, 34).

Very recently, staphylococcal SrtB has been shown to be specifically required to anchor the surface protein IsdC, which is not an LPXTG protein (34). In vitro studies suggest that SrtB recognizes and cleaves an NPQTN peptide motif. Here we studied the role of SrtB in *L. monocytogenes* by constructing Δ*srtB* and Δ*srtA* Δ*srtB* knockout mutants. We show that SrtB has a "sorting" activity distinct from that of SrtA, and we identify SvpA, a virulence factor of *L. monocytogenes* (8), as a target of SrtB. The respective roles of SrtA and SrtB in surface protein anchoring and in the virulence of *L. monocytogenes* are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, mammalian cells, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Plasmids were introduced into *Listeria* strains by electroporation. *Listeria* strains were grown at 37°C in brain-heart infusion (BHI) broth or agar (Difco), supplemented with 8 μg of erythromycin/ml, when carrying plasmids (18). RAW 264.7 mouse macrophages (ATTC TIB-71) were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 2 mM glutamine, 1% nonessential amino acids (Gibco), and 10% fetal calf serum (Sera-Lab) that was decomplemented by heating for 30 min at 56°C. Cells were incubated at 37°C under 10% CO_2.

RNA extraction and reverse transcription-PCR (RT-PCR). Cultures of *L. monocytogenes* (EGD-e) (at an optical density at 600 nm of 0.4) were centrifuged at 4,000 × *g* for 10 min, and the pellet was resuspended in 1 ml of Trizol (Life Technologies) and then transferred to a Bead Beater tube containing 500 μl of 0.1-mm mini glass beads (Polylabo). Bacteria were broken once for 30 s in the Bead Beater at maximum speed. The tubes were centrifuged (13,000 × *g*, 1 min), and the supernatants were transferred to new tubes containing 300 μl of chloroform:isoamyl alcohol. After 10 min of centrifugation at 13,000 × *g*, the aqueous phase was transferred to a tube containing 270 μl of isopropanol. Total RNA was then precipitated overnight at 4°C and washed with 1 ml of 75% ethanol. RNA was resuspended in 60 μl of diethyl pyrocarbonate-treated water. Contaminating DNA was removed by digestion with DNase I according to the manufacturer's instructions (Roche).

RT-PCR experiments were carried out with 1 μg of RNA and 2.5 pmol of specific reverse primers for each amplification. After denaturation at 65°C for 10 min, 12 μl of a mixture containing 2 μl of deoxynucleoside triphosphates (25 mM), 4 μl of 4× buffer, 2 μl of dithiothreitol, 1 μl of RNasin (Promega), and 1.5 μl of Superscript II (Invitrogen) was added. Samples were incubated for 60 min at 42°C, heated at 75°C for 15 min, and chilled on ice. Samples were diluted with 30 μl of H_2O and stored at −20°C. PCR conditions were identical for all reactions. The 50-μl reaction mixtures consisted of 4 μl of the template, 10 pmol of each primer, 1 μl of deoxynucleoside triphosphates (10 mM), 5 μl of 5× buffer, and 0.5 μl of AmpliTaq DNA polymerase from *Thermus aquaticus* (Perkin-Elmer, Branchburg, N.J.) in a Gene Amp system 9600 thermal cycler (Perkin-Elmer).

The following pairs of primers were used to amplify the mRNAs: (i) for *srtB*, primer 1 (5′-TTT GGG GAA AAG TTT GAC CTT-3′) and primer 2 (5′-CTT GAA TAA CCA TAC GTC CTT-3′); (ii) for the cotranscription of *srtB* and *lmo2180*, primer 1 (5′-TTT GGG GAA AAG TTT GAC CTT-3′) and primer 2 (5′-CGC TTT CAA CTA ATC TAG CAC CCT-3′); (iii) for the cotranscription of *lmo2182* and *srtB*, primer 1 (5′-GTG GCG ATT GTT CAT CAA AGT A-3′) and primer 2 (5′-CTT GAA TAA CCA TAC GTC CTT-3′); (iv) for the cotranscription of *lmo2186* and *svpA*, primer 1 (5′-GAA TTC GGG CCT ATG GGT TGA AGG-3′) and primer 2 (5′-GGA TCC GAA AGA GTC ACA GGT GTT G-3′); and (v) for the cotranscription of *svpA* and *lmo2184*, primer 1 (5′-GGA CCA AAA TTG GCA AAA CCG-3′) and primer 2 (5′-TGA ACT AGA CGG AAT TCC AAC GAG-3′).

Chromosomal inactivation and cloning of *srtB*. Two 500-bp fragments flanking *srtB* (*lmo 2181*; GenBank/EMBL accession no. AL591824) (21) were amplified by PCR from EGD-e chromosomal DNA with primers inside and outside the *srtB* locus. Primers for the 5′ fragment were SBS1 [5′-(CGCGGATCC)AATG AACTAATGGTGAAGGAATTG-3′] and SBS2 [5′-(AAAAGGCCT)CCCCA AAAACGATTTTATTTTCAC-3′]; primers for the 3′ fragment were SBS3 [5′-(AAAAGGCCT)TGAGAAAGCACCAAAATATAATAG-3′] and SBS4 [5′-(CCGGAATTC)AATCGATTCGCTTTCAACTAATC-3′]. After restriction of the amplified 5′ and 3′ fragments with BamHI and StuI and with StuI and EcoRI, respectively, the two fragments were coligated into the thermosensitive plasmid pKSV7 (16) digested with BamHI and EcoRI, yielding plasmid pKSVΔ*srtB*. This plasmid was then electroporated into wild-type *L. monocytogenes* (strain EGD-e), as well as into the previously constructed Δ*srtA* mutant (5), to generate a single Δ*srtB* mutant and a double Δ*srtA* Δ*srtB* mutant, respectively. Gene replacement was performed as described previously (16), resulting in a gene containing only the first eight codons and the stop codon of *srtB*. The resulting Δ*srtB* mutants were verified by PCR analysis of chromosomal DNA using couples of internal or flanking primers SB1, SB4, SB10 (5′-GAATTTGC GCAAAATGTGGCG-3′), SB11 (5′-AAATCCGCAAACATGGATCCG-3′), and SB12 (5′-GGAGGAAGTGGCTAAATGTTC-3′). To complement the Δ*srtB* mutation, *srtB* was PCR amplified from EGD-e chromosomal DNA by using primers 5′-(ACGAGCTC)ACGAAAGGAGTTTTAGAGAGT-3′ and 5′-(ACATGCATGC)CTATTATATTTTGGTGCTTTC-3′. The DNA fragment was digested with SacI and SphI and inserted into the SacI and SphI sites in the shuttle plasmid pP1 downstream from the constitutive promoter pProt of the protease gene from *Streptococcus cremoris* (15), yielding plasmid pP1srtB.

Construction of an *svpA* mutant. The mutation in *svpA*, initially constructed in LO28, corresponds to the deletion of a 79-bp internal fragment of *svpA* and insertion of a promoterless *aph3* gene conferring resistence to kanamycin. It was introduced into the chromosome of EGD-e by allelic replacement, using the recombinant integrative plasmid pAUL-A-*svpA*ΩaphA3 described previously (8).

Immunofluorescence analyses. For analysis of SvpA localization at the surfaces of bacteria grown in BHI, overnight cultures of *L. monocytogenes* were grown at 37°C and used immediately (stationary phase) or diluted 1:20 in BHI at 37°C for 3 h (exponential phase). Bacteria were washed twice in phosphate-buffered saline (PBS) and fixed in 3% paraformaldhehyde in PBS for 10 min. They were then labeled with an anti-SvpA polyclonal antibody (8) and an Alexa 488-conjugated secondary antibody (Molecular Probes). To study the distribution of surface proteins strongly bound to PG by using the anti-PG antibody, PBS-washed bacteria were boiled in 4% sodium dodecyl sulfate (SDS) for 30 min, further washed three times with PBS to remove traces of SDS, and processed for immunofluorescence analysis as described above for SvpA. The anti-PG antibody was used in this case at a 1:200 dilution. Preparations were observed with a Zeiss Axiovert 135 microscope. Image acquisition from the Zeiss microscope was carried out with a cooled charge-coupled device camera (Princeton), and the images processed with Metamorph software (Universal Imaging Corporation).

Fractionation experiments and Western blot analysis. Fractions containing highly pure PG were obtained from the different *L. monocytogenes* strains grown

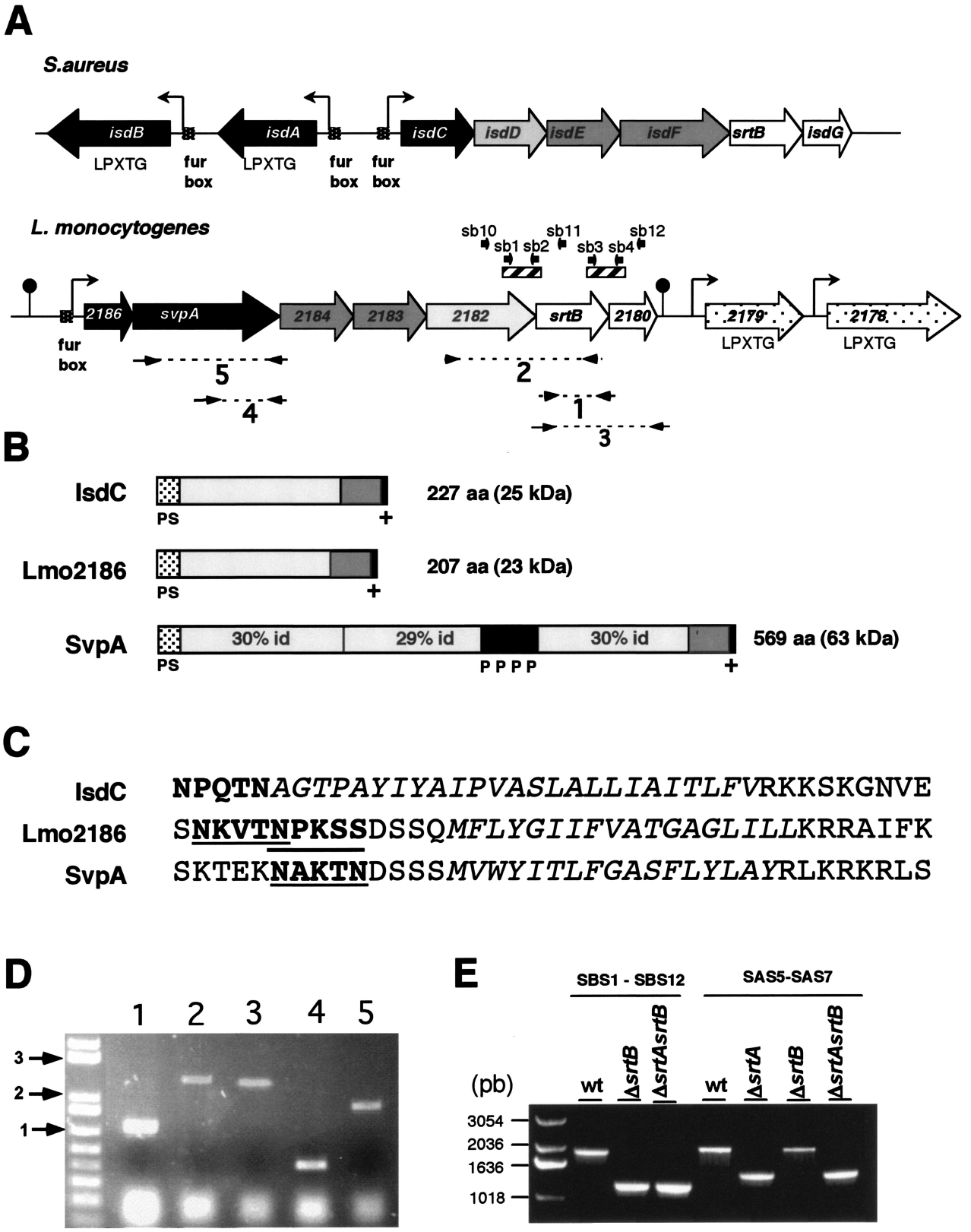

FIG. 1. *srtB* loci in *L. monocytogenes* and *S. aureus*. (A) Genetic organization of the *srtB* locus in *S. aureus* (34) and in *L. monocytogenes* (21). ORFs are designated according to the *L. monocytogenes* genome nomenclature (*lmo*). The *srtB* gene corresponds to *lmo2181*. *lmo2182*, *lmo2183*, and *lmo2184* are homologous to ABC iron transporter-encoding genes; *svpA* (*lmo2185*) and *lmo2186* display homologies with *isdC* in *S. aureus*. *lmo2179* and *lmo2178* encode LPXTG proteins of unknown function that are unrelated to the staphylococcal IsdA and IsdB LPXTG proteins. Bent arrows indicate promoters; circles, putative transcription terminators. Fur boxes are indicated. Solid arrows flanking dotted lines, below the *svpA*

overnight at 37°C in 500 ml of BHI medium, as previously described (5). This "PG fraction" contains proteins that associate with PG, withstanding extensive boiling in 4% SDS. The supernatant fractions were prepared from the same bacterial culture in which the PG fraction was obtained. After the first centrifugation step of the 500-ml culture, 10 ml of the supernatant was filtered with 0.22-μm-pore-size Millipore filters and the proteins were precipitated by a 10% trichloroacetic acid-acetone washing procedure as described previously (23). Equivalent amounts of supernatant fractions (1.5 ml of culture) were loaded for SDS-polyacrylamide gel electrophoresis (PAGE) analysis. The PG fraction was prepared 50-fold concentrated with respect to supernatant fractions. Membrane fractions and cytoplasmic fractions were prepared from bacteria grown overnight in 250 ml of BHI medium. Bacteria were collected by centrifugation (at 10,000 × *g* for 10 min at 4°C), washed in an equal volume of PBS (pH 7.4), and finally resuspended in 5 ml of PBS containing DNase I (30 μg/ml) and an appropriate volume of protease inhibitor cocktail (according to tables provided by Roche). After three freeze-thaw cycles, the bacterial suspension was passed through a French press (1,000 lb/in^2) and centrifuged at low speed (3,000 × *g* for 10 min at 4°C) to remove unbroken cells. The supernatant of this centrifugation was subjected to high-speed centrifugation (at 40,000 × *g* for 30 min at 4°C) to separate the cytosol (supernatant) from the envelope material (pellet). The envelope material was further resuspended in 0.75 ml of PBS containing protease inhibitors. Both the cytosol and membrane fractions were stored at −20°C. Upon addition of an appropriate volume of Laemmli buffer, cytosol and membrane samples corresponding to 1.5 ml of the initial volume culture were used for SDS-PAGE analysis. Proteins were resolved by using the Tris-Tricine buffer system with 10% SDS-polyacrylamide gels (42) or, when specifically indicated, by Tris-glycine SDS (0.1%)-PAGE (7% polyacrylamide) using standard methods (1). Proteins were transferred to Immobilon-P membranes (polyvinylidene difluoride filter, catalog no. IPVH00010; Millipore). Membranes were probed with either monoclonal antibody (MAb) L7.7 (35) to detect InlA, an anti-P60 MAb (a gift from Andreas Bubert, University of Würzburg, Würzburg, Germany), the anti-InlB MAb B4-6 (9), a rabbit polyclonal antiserum (839) to detect proteins present in PG fractions (5), or anti-SvpA polyclonal antibodies (8).

In vitro invasion assays and intracellular multiplication assays. Invasivity tests were performed as described previously (5). Intracellular multiplication assays in RAW 264.7 cells were performed as follows. Cells were infected at a multiplicity of infection of 1:1 and then left 15 min on ice to allow bacterial adherence (time zero). After 45 min of internalization at 37°C, noninvasive bacteria were killed by adding gentamicin at 10 μg/ml. The number of intracellular bacteria was estimated in cell lysates after 45 min (time, 90 min) and at selected intervals (from 90 min to 8 h postinfection).

Animal infections. Animal experiments were performed according to the guidelines of the Institut National de la Santé et de la Recherche Médicale (INSERM) for laboratory animal husbandry. Specific-pathogen-free 6- to 8-week-old female Swiss mice (Janvier, Le Geneset St Isle, France) were used.

(i) Kinetics of bacterial growth in infected organs. Bacteria were grown for 18 h in BHI broth, centrifuged, appropriately diluted in 0.15 M NaCl, and inoculated intravenously (i.v.) (0.5 ml) via the lateral tail vein with a dose of 10^5 bacteria per mouse. Groups of five mice were sacrificed 3 days after infection. Organs (spleens and livers) were aseptically removed and separately homogenized in 0.15 M NaCl. Bacterial counts in organ homogenates were determined on BHI agar plates, as described previously (2).

(ii) LD_{50}. The virulence of each mutant was estimated by the 50% lethal dose (LD_{50}) by using the Probit method (2). Groups of five mice were challenged i.v. with various doses of bacteria, and mortality was monitored over a 14-day period.

RESULTS

The *L. monocytogenes srtB* locus. Bierne et al. previously identified a gene encoding a second putative sortase, *srtB* (*lmo2181*) (5), at a distance of 1,300 kb from *srtA* in the *Listeria* genome (21). Comparison of the *srtB* region of *L. monocytogenes* to the *S. aureus isd* region, which contains the staphylococcal *srtB* gene and which is involved in iron acquisition (34), revealed similarities of genomic organization but not an exact conservation (Fig. 1A). The listerial *srtB* gene is present in a region apparently organized as a seven-gene operon, preceded by a putative promoter and flanked by transcriptional terminators. As in *S. aureus*, this gene cluster encodes a putative iron acquisition system and may be controlled by the iron regulator Fur, because a Fur box exists upstream of the promoter.

The first gene of the operon, *lmo2186*, encodes a protein of 207 amino acids that displays 33% identity with IsdC, the only known target of the staphylococcal sortase SrtB. By contrast, the second gene, *svpA* (*lmo2185*), is not present in the *isd* operon and encodes a 569-amino-acid polypeptide previously identified as a listerial virulence factor (8). SvpA displays three domains that are homologous to regions within Lmo2186 and IsdC, and a proline-rich region possibly involved in protein-protein interactions (Fig. 1B). Both SvpA and Lmo2186 have an N-terminal signal peptide, a C-terminal hydrophobic domain, and a charged tail, resembling sorting signals, albeit without the LPXTG motif. However, an NAKTN motif in SvpA and an NKVTN or NPKSS motif in Lmo2186 may correspond to the NPQTN motif thought to be recognized by SrtB in IsdC (Fig. 1C).

Downstream from the *svp* genes are three genes homologous to components of a putative ferric hydroxamate ABC transporter (see reference 41 for a review). *lmo2184* is predicted to encode a lipoprotein, *lmo2183* is predicted to encode a permease, and *lmo2182* is predicted to encode an ATP-binding protein. The first two share 49 and 38% identity, respectively, with the *isdE* and *isdF* genes in the *isd* locus of *S. aureus*, but the third gene has no homologue in *S. aureus*. The last gene of the locus, *lmo2180*, shares homology with the gene encoding the Gp46 protein of *Listeria* phage 2389 (GenBank/EMBL accession no. NC_003291), a protein of unknown function. The region upstream of this operon does not contain genes homologous to *isdA* and *isdB* in *S. aureus* but instead contains a short open reading frame (ORF) of unknown function and several ORFs homologous either to competence genes, including the negative regulator *mecA*, or to genes involved in amino acid

locus of *L. monocytogenes*, indicate the positions of the primers used in the RT-PCR analysis (see panel D). The Δ*srtB* and Δ*srtA* Δ*srtB* mutants were constructed from *L. monocytogenes* EGD-e by homologous recombination using a thermosensitive vector carrying two short, blunt-end-ligated PCR fragments (hatched rectangles), which were produced with primers SB1–SB2 and SB3–SB4. The in-frame deletion was confirmed by PCR analysis using primers flanking (SB1, SB4, SB10, SB12) (see panel E) or inside (SB11) *srtB*. (B) Schematic representation of IsdC from *S. aureus* and of Lmo2186 and SvpA from *L. monocytogenes*. The N-terminal peptide signal and the C-terminal hydrophobic domain and charged tail are shown. The percentage of identity (id) between the SvpA repeats and Lmo2186 is shown. (C) Sequence alignment of the C-terminal regions of IsdC, Lmo2186, and SvpA. Hydrophobic domains are italicized. The NPQTN cleavage motif in IsdC is boldfaced; putative SrtB cleavage motifs in SvpA and Lmo2186 are underlined. (D) Tris-acetate-EDTA–agarose gel electrophoresis of transcripts amplified by RT-PCR. Numbers (in kilobases) with arrows on the left correspond to sizes on the DNA ladder. Numbers above each lane correspond to those at the bottom of panel A: 1, *srtB*; 2, *lmo2182* and *srtB*; 3, *srtB* and *lmo2180*; 4, *svpA* and *lmo2184*; 5, *lmo2186* and *svpA*. (E) In-frame deletions in mutants were genetically verified by PCR analysis using primers flanking *srtB* (SB1–SB12) or *srtA* (SA5–SA7). DNA fragments were separated on an ethidium bromide-stained agarose gel.

transport and metabolism. Lastly, downstream of the operon are two genes encoding surface proteins with LPXTG motifs, but these display no homology with the LPXTG proteins IsdA and IsdB of *S. aureus* (32).

The organization of the *isd* locus of *S. aureus* is also partially conserved in the gram-positive extremophile *B. halodurans* as well as in *B. anthracis* (34, 36). A closer examination of the locus in *B. halodurans* revealed that the first two genes encode proteins of 221 and 1,071 residues, respectively, that share significant similarity with one another (35.1% identity), as in the *L. monocytogenes srtB* region. Moreover, the large protein shows homology to SvpA (up to 45% identity between residues 365 to 494 of SvpA and residues 385 to 511 encoded by the *B. halodurans orf*). Alignment of the C-terminal domains of the two proteins of *B. halodurans* indicates that the NPKTG motif of the short protein corresponds to an NSKTA motif in the large protein (sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org).

***srtB* is transcribed in bacteria grown in BHI at 37°C.** We analyzed the transcription of the *srtB* region by RT-PCR (Fig. 1D). In the wild-type strain, EGD-e, grown under laboratory conditions (see Materials and Methods), the *srtB* gene is transcribed, suggesting that it encodes a functional protein. The *srtB* gene is cotranscribed with the downstream gene *lmo2180* and with the third gene of the putative ABC transporter (*lmo2182*). Finally, the first two genes of the locus, *lmo2186* and *svpA*, and the first gene of the ABC transporter (*lmo2184*) are all cotranscribed. Taken together, these data suggest that this seven-gene cluster is a single transcriptional unit.

Inactivation of *L. monocytogenes srtB* in wild-type and Δ*srtA* strains. To analyze the function of the *L. monocytogenes* SrtB protein, we inactivated the *srtB* gene in strain EGD-e by introduction of an in-frame deletion, as described previously (5). We also generated the double Δ*srtA* Δ*srtB* mutant. PCR amplifications confirmed the deletion of *srtB* or *srtA* coding sequences in these strains (Fig. 1E and data not shown). The two strains displayed no growth defects in BHI medium, indicating that viability and cell division were not affected under this growth condition. To address a putative modification of the cell wall in the sortase mutants, we also tested their susceptibilities to penicillin and vancomycin, two antibiotics that alter cell wall synthesis, by using disk diffusion tests. We found no differences between the wild-type strain and the Δ*srtA*, Δ*srtB*, or Δ*srtA* Δ*srtB* mutant (data not shown). These results indicate that the sortase mutants have no obvious morphological cell wall alterations.

SrtA has previously been shown to target LPXTG proteins, including the invasion protein InlA (5). We therefore tested whether SrtB had an effect on InlA anchoring. The amounts of InlA in membranes and highly pure PG fractions from the wild-type, Δ*srtA*, Δ*srtB*, and Δ*srtA* Δ*srtB* strains, obtained by a fractionation procedure described previously (5), were determined by Western blotting. This set of samples was also probed with antibodies directed against p60 and InlB, two proteins known to be present in membrane fractions (10). As shown in Fig. 2A, InlA was present in the PG extracts of the wild-type and Δ*srtB* strains, whereas, as expected, it was totally absent in cell walls from Δ*srtA* and Δ*srtA* Δ*srtB* mutants. Membrane proteins did not appear in the PG fraction, indicating that there was no cross-contamination between membrane and PG fractions. This result demonstrates that the listerial SrtB protein is not involved in the anchoring of InlA to PG.

SrtA anchors most of the PG-associated proteins. To determine whether PG-associated proteins distinct from InlA were missorted at the surfaces of Δ*srtB* mutant cells, we used an antiserum raised against proteins present in purified *L. monocytogenes* PG fractions (5) to analyze the surfaces of wild-type, Δ*srtA*, Δ*srtB*, and Δ*srtA* Δ*srtB* cells by immunofluorescence. In all cases, bacteria were boiled in the presence of 4% SDS prior to fixation and antibody labeling (see Materials and Methods). As shown in Fig. 2B, the anti-PG antiserum strongly labeled the wild-type and Δ*srtB* strains, whereas it only weakly labeled the Δ*srtA* mutant. No signal could be detected at the surface of the Δ*srtA* Δ*srtB* double mutant. These results suggested that SrtB anchors only a small subset of surface proteins, whereas SrtA sorts most of the proteins to the PG. These results were confirmed by Western blot analysis of PG and supernatant fractions. Indeed, a small amount of proteins recognized by the anti-PG antiserum was present in PG extracts prepared from the Δ*srtA* mutant, in contrast to the amounts in PG extracts of wild-type and Δ*srtB* strains (Fig. 2C). Interestingly, polypeptides remaining in the PG extracts from the Δ*srtA* mutant, including a major protein of about 60 to 70 kDa, were absent in the Δ*srtA* Δ*srtB* strain extracts, suggesting that they could be targets of SrtB. No immunoreactive species were detected in PG extracts of the double Δ*srtA* Δ*srtB* mutant.

The Δ*srtA* Δ*srtB* strain was complemented for sortase expression with a plasmid expressing either the *srtA* gene (pP1*srtA* [5]) or the *srtB* gene (pP1*srtB* [this study]), placed under the control of a constitutive promoter. The antiserum specific for the listerial PG fraction recognized a pattern of several proteins of diverse apparent molecular weights in the PG fraction from the Δ*srtA* Δ*srtB* strain expressing SrtA, in contrast to the Δ*srtA* Δ*srtB* strain expressing SrtB, in which only a faint smear was detected (Fig. 2C).

Altogether, these results confirmed that SrtA is required for efficient sorting of most of the PG-bound proteins, and they demonstrated that a lack of SrtB affects the association with PG of only a relatively small number of protein species. In addition, the absence of proteins in PG extracts from the double Δ*srtA* Δ*srtB* mutant supports the notion that there is no other sortase activity in *L. monocytogenes*.

Inactivation of *srtB* abolishes association of SvpA with the bacterial surface. Because of the similarity between the region of *srtB* in *L. monocytogenes* and the *isd* locus in *S. aureus*, encoding both SrtB and its target, IsdC (Fig. 1A), it is possible that SvpA and Lmo2186, the first proteins encoded by the region of *srtB*, are substrates of the listerial SrtB. Moreover, SvpA and Lmo2186 have sequence similarities to IsdC, although they do not bear an NPQTN sorting motif. SvpA is located at the bacterial surface of *L. monocytogenes* strain L028 (8). Because a polyclonal anti-SvpA antibody was available, we focused our analysis on this protein. By immunofluorescence, we detected SvpA at the surface of the *L. monocytogenes* EGD-e wild-type strain, both in exponential phase (Fig. 3) and in stationary phase (data not shown). Notably, SvpA was detected either at one pole of the bacterium or laterally, and the intensity of labeling by the anti-SvpA antibody was heterogeneous in the bacterial population. To confirm the anti-SvpA labeling specificities, we generated an allelic *svpA* mutant of

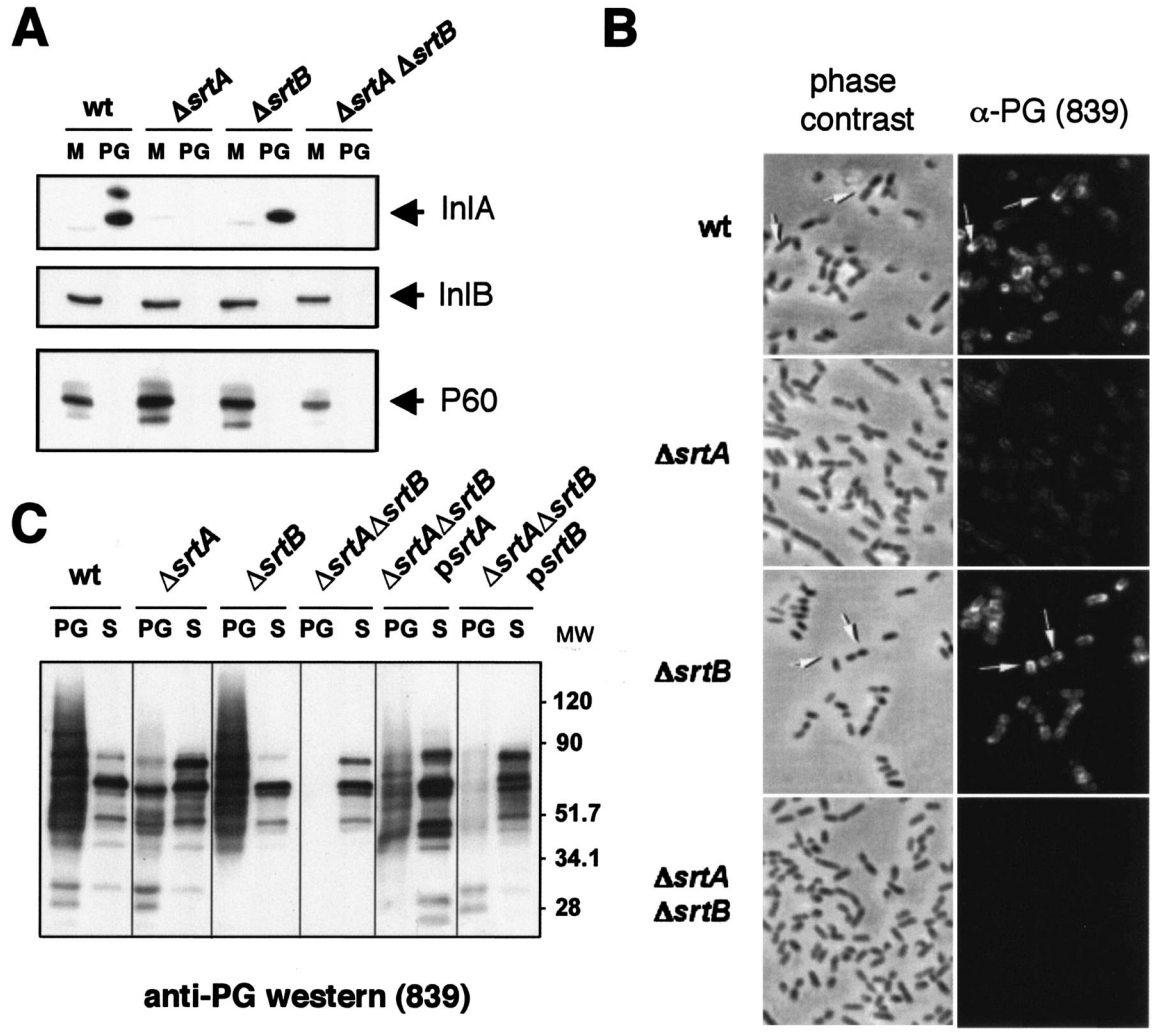

FIG. 2. Roles of SrtA and SrtB in anchoring *Listeria* surface proteins to the PG. (A) Cultures of wild-type (wt), Δ*srtA*, Δ*srtB*, and Δ*srtA* Δ*srtB* EDGe strains were fractionated into membrane (M) and highly pure PG fractions, and proteins were detected by immunoblotting with anti-InlA, -p60, or -InlB antibodies. InlA is missorted in the Δ*srtA* and Δ*srtA* Δ*srtB* mutants. Membrane-associated p60 and InlB proteins do not appear in PG fractions. (B) Immunofluorescence analysis of surface-bound proteins recognized by the polyclonal antiserum 839, which was raised against purified macromolecular *L. monocytogenes* PG. The labeling is mostly polar (arrows). A lack of SrtA notably reduces the amount and number of proteins that associate with PG, whereas a lack of both SrtA and SrtB fully suppresses the detection of these proteins. (C) Immunoblots of highly pure PG and supernatant (S) fractions, prepared from wild type, Δ*srtA*, Δ*srtB*, Δ*srtA* Δ*srtB*, Δ*srtA* Δ*srtB*/pP1*srtA*, and Δ*srtA* Δ*srtB*/pP1*srtB* EDG-e strains and probed with an anti-PG antiserum as previously described (5). The 60- to 70-kDa polypeptide is present in Δ*srtA* and absent in Δ*srtA* Δ*srtB* strains. MW, molecular weight (in thousands).

strain EGD-e (see Materials and Methods). As expected, the antibody did not label the surface of the *svpA* mutant strain. We addressed the role of the sortases SrtA and SrtB in SvpA anchoring. SvpA was no longer detected at the surface of the Δ*srtB* mutant. In contrast, SvpA was present at the surface of the Δ*srtA* mutant as well as at that of the Δ*srtB*/pP1srtB complemented strain, demonstrating that expression of *srtB* in the Δ*srtB* mutant restored surface anchoring (Fig. 3). These results indicate that expression of SrtB is required for the association of SvpA with the bacterial surface.

SrtB anchors SvpA to the cell wall. To investigate whether SvpA is anchored to the PG, we studied the localization of SvpA in cytoplasmic, membrane, PG, and supernatant fractions. A major polypeptide of approximately 66 kDa, a molecular mass compatible with the predicted molecular mass of 63 kDa, was specifically detected in all fractions from the wild-type strain and was absent in the corresponding extracts from the *svpA* mutant (Fig. 4). This result indicates that SvpA is present in the cell wall. However, since the amount of the supernatant fraction loaded in gels was 50-fold diluted compared to that of PG fractions, the amount of SvpA found in PG extracts was small, representing only ~20% of the whole expressed protein. Therefore, the bacterium retains only a small amount of SvpA at the surface, at least for bacteria grown in BHI in vitro. In addition to the 66-kDa protein, immunoreactive species of apparently lower molecular weight were also detected in PG fractions from SrtB-expressing bacteria (Fig. 5A). All of these anti-SvpA immunoreactive species were absent in extracts from the *svpA* mutant, suggesting that they correspond to proteolytic degradation products of SvpA.

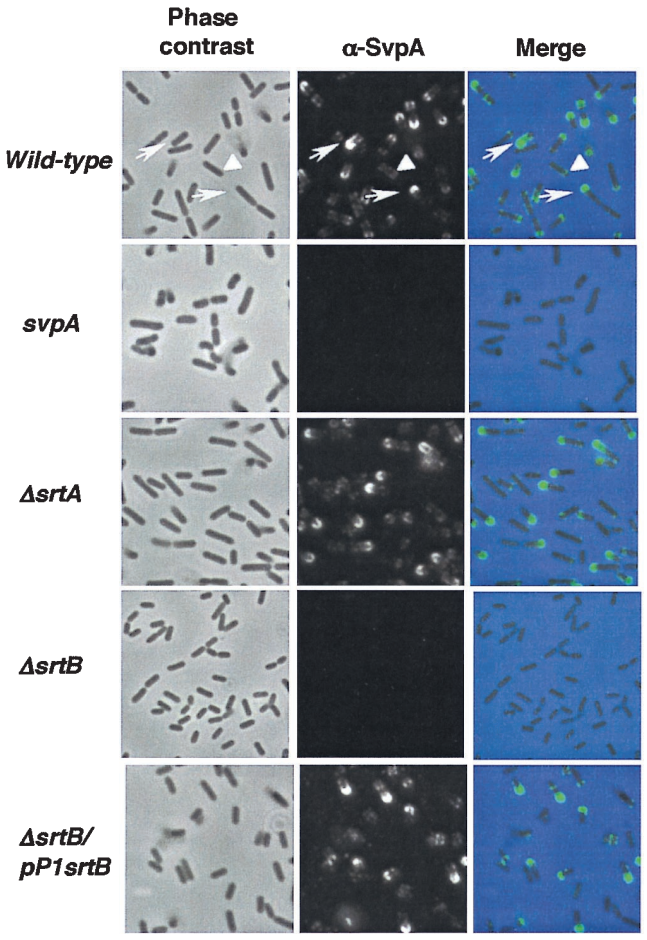

FIG. 3. Display of SvpA on the surfaces of listeriae grown in BHI. The EDGe wild type, Δ*srtA*, Δ*srtB*, and *svpA* strains were analyzed by phase-contrast and immunofluorescence staining with the SvpA-specific polyclonal antibody. The merge image shows the phase contrast in blue and the SvpA labeling in green. SvpA is polarly (arrows) or laterally (arrowheads) localized at cell surfaces in the wild-type bacterial population. Inactivation of *srtB* abolished SvpA surface association, and complementation of the mutation restored it.

We then tested whether the absence of sortases in *L. monocytogenes* affected the localization of SvpA in PG fractions. In agreement with the immunofluorescence experiments, PG extracts from the Δ*srtB* mutant displayed undetectable levels of SvpA, whereas they contained InlA in amounts comparable to those in the wild-type strain (Fig. 5A). Conversely, PG extracts from the Δ*srtA* mutant displayed SvpA but no InlA. PG extracts from the Δ*srtA* Δ*srtB* mutant displayed no detectable levels of either of the two proteins. These results indicate that the presence of SvpA in PG fractions is a SrtB-dependent process.

Complementation of Δ*srtB* and Δ*srtA* Δ*srtB* strains with pP1*srtB* restored SvpA anchoring to the PG. However, amounts of the full-length protein in PG fractions from these complemented mutants were smaller than those in the wild-type or Δ*srtA* strain. A similar effect was observed with the Δ*srtA* strain expressing SrtB. This partial complementation may be due either to an overexpression of SrtB in these strains that would interfere with the normal anchoring process or to instability of SrtB.

SvpA species present in PG and supernatant fractions of strains that have a functional SrtB are probably linked to muropeptide fragments, as a result of muramidase digestion of the cell wall (for PG extracts) or of PG turnover (for supernatant extracts). In the absence of SrtB, the secreted SvpA should not be linked to PG residues. Therefore, one would expect to detect SvpA in the supernatants of Δ*srtB* or Δ*srtA* Δ*srtB* mutants at a molecular weight lower than that detected in supernatants of wild-type and Δ*srtA* strains. To address this hypothesis, we performed SvpA immunoblotting of supernatant fractions, using 7% acrylamide-Tris-glycine gels in order to optimize the separation of protein bands in the 90- to 40-kDa range (see Materials and Methods). Under these conditions, the SvpA forms present in the supernatant fractions of Δ*srtB* and Δ*srtA* Δ*srtB* strains ran with a higher mobility than those detected in all other strains (Fig. 5B, SvpA∗). This result strongly suggests that the SvpA species observed in the supernatant fractions of strains lacking SrtB are not bound to muropeptide molecules.

Overall, these results demonstrate that anchoring of SvpA to the bacterial cell wall is specifically mediated by SrtB.

SrtB-mediated SvpA anchoring is not required for the intracellular infectious process. In order to determine whether inactivation of *srtB* could affect the cellular infectious process of *L. monocytogenes*, we first used the gentamicin survival assay (5) to investigate whether the Δ*srtB* and Δ*srtA* Δ*srtB* mutants displayed any defect in the ability to invade epithelial cells (Caco-2 and Vero) as well as macrophages. Entry of the Δ*srtB* mutant into all cell lines tested was not impaired and was similar to that of the wild-type strain (data not shown). In contrast, entry of the Δ*srtA* Δ*srtB* mutant was significantly decreased only in Caco-2 cells, to the level of entry of Δ*srtA* and Δ*inlA* mutants. Therefore it is evident that inactivation of *srtB* in *L. monocytogenes* has no detectable effect on bacterial entry in the cellular systems tested.

Although inactivation of *svpA* did not affect the entry process, an *svpA* mutant of *L. monocytogenes* strain L028 was reported earlier to be more susceptible than wild-type bacteria to the bactericidal activity of phagosomes in bone marrow macrophages (8). We therefore compared the intracellular replication of isogenic EGD-e *svpA*, Δ*srtB*, Δ*srtA*, and Δ*srtA* Δ*srtB*

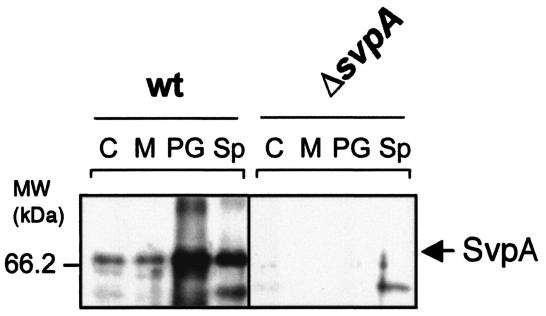

FIG. 4. Localization of SvpA in bacterial compartments. Cytoplasmic (C), membrane (M), PG, and supernatant (Sp) fractions of wild-type (wt) and *svpA* EGD-e strains were analyzed by Western blotting with an anti-SvpA polyclonal antibody. A major polypeptide of ~66 kDa is specifically detected in extracts from the wild-type strain. The Sp fraction is 50-fold diluted with respect to the other fractions.

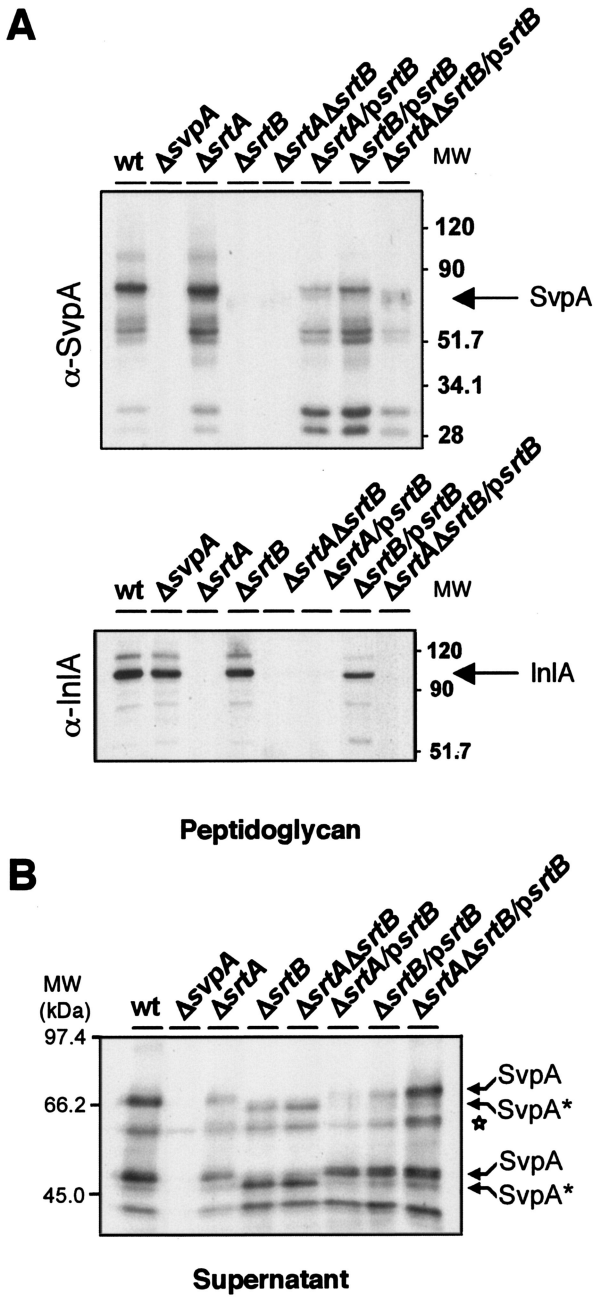

FIG. 5. SrtB is required for SvpA anchoring to PG. (A) PG fractions (as in Fig. 2) of wild-type EDG-e, *svpA*, Δ*srtA*, Δ*srtB*, Δ*srtA* Δ*srtB*, Δ*srtA*/pP1*srtB*, Δ*srtB*/pP1*srtB*, and Δ*srtA* Δ*srtB*/pP1*srtB* strains were analyzed by Western blotting with anti-SvpA (top) or anti-InlA (bottom) antibodies by using the Tris-Tricine electrophoresis system to visualize proteins from high to low molecular weights. Inactivation of *srtB* abolishes the anchoring of SvpA to PG. MW, molecular weight (in thousands). (B) Supernatant fractions of the same strains were analyzed by Western blotting with the anti-SvpA antibody by using the Tris-glycine system to separate proteins in the 90- to 40-kDa range. The SvpA forms present in the samples of Δ*srtB* and Δ*srtA* Δ*srtB* strains (labeled as SvpA*) run with higher mobility than those detected in all other strains (labeled as SvpA), which express SrtB. A 60-kDa band (indicated by the star) that appears in all strains, including the *svpA* mutant, is nonspecific.

mutants to that of wild-type EGD-e in RAW 264.7 macrophages during 8 h of infection (Fig. 6). Wild-type bacteria rapidly multiplied intracellularly, while *svpA* mutant bacteria were partially killed in the first 4 h of infection and surviving bacteria started to multiply thereafter, in agreement with previous observations (8). In contrast, intracellular growth of Δ*srtA*, Δ*srtB*, and Δ*srtA* Δ*srtB* mutants was identical to that of the wild-type strain. Similar results were obtained with epithelial cells (data not shown).

All the sortase mutants were also analyzed by immunofluorescence for actin tail formation in J774 and RAW 264.7 macrophages and in Vero cells. None of them displayed any obvious difference from the wild-type strain in the actin-based motility process (data not shown). These data indicate that inactivation of sortases does not affect the intracytosolic multiplication or motility of *L. monocytogenes*, at least in these cell types.

Effects of *srtB* inactivation on bacterial virulence. Δ*srtA* and *svpA* mutants have been shown to have impaired virulence in the mouse model of infection (5, 20). The role of SrtB in the virulence of *L. monocytogenes* was studied by determining the LD_{50}s of the sortase mutants of EGD-e described in this work, after i.v. inoculation of Swiss mice. The LD_{50} of the Δ*srtB* mutant was $10^{4.5}$ per mouse, e.g., indistinguishable from that of the wild-type strain. In contrast, the LD_{50} of the Δ*srtA* Δ*srtB* double mutant, $10^{5.75}$ per mouse, was ~1.2 log units higher than that of the parental strain (and identical to that of the Δ*srtA* single mutant), reflecting a moderate alteration of bacterial virulence. This result shows that the sortase encoded by *srtB* does not participate in the virulence of *L. monocytogenes*, at least in the mouse model of infection by the i.v. route.

The ability of the Δ*srtB* and Δ*srtA* Δ*srtB* mutants to multiply in the target organs of infected mice was also evaluated. Mice were infected i.v. with 10^5 bacteria per mouse, and bacteria were recovered in the spleens 3 days after inoculation (the time corresponding to the peak of infection). Confirming earlier observations (20), the number of Δ*srtA* mutant bacteria recovered per organ ($10^{7.22 \pm 0.5}$ per mouse) was not significantly lower than the number recorded for the wild-type strain ($10^{7.3 \pm 0.11}$ per mouse), and the counts recorded for the Δ*srtB* mutant were very similar ($10^{7.25 \pm 0.3}$ per mouse). With the Δ*srtA* Δ*srtB* mutant, the bacterial counts in the spleen were slightly lower ($10^{6.8 \pm 0.09}$ per mouse) but not significantly different from those recorded for the two single mutants.

DISCUSSION

L. monocytogenes expresses two sortases that contribute differently to the anchoring of surface proteins. SrtA sorts most of the PG-associated proteins, including internalin and probably all the other LPXTG-containing proteins, while SrtB apparently targets only a few surface proteins, including the SvpA protein, which is not an LPXTG protein. Inactivation of *srtB* does not impair intracellular survival in vitro and does not attenuate bacterial virulence in the mouse, suggesting that the anchorage of SvpA to the cell surface does not play a role in bacterial survival inside cells.

Genes with homology to *srtA* occur in a variety of gram-positive bacteria, usually with more than one sortase-encoding

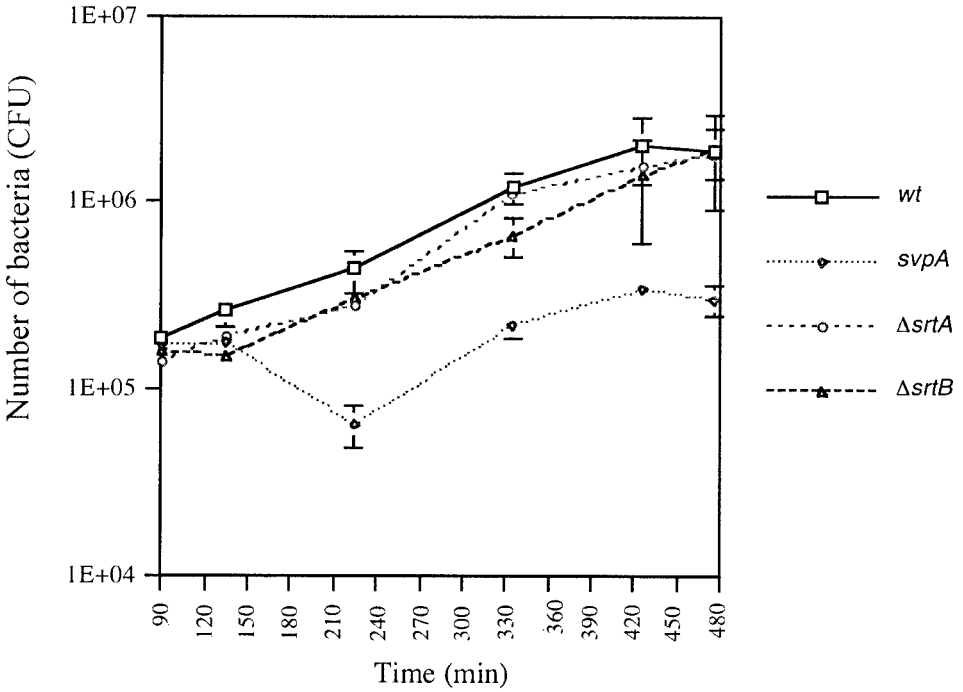

FIG. 6. SrtB-mediated anchoring of SvpA is not required for bacterial growth in macrophages. RAW 264.7 macrophages were exposed to wild-type EGD-e bacteria (squares) or to the Δ*srtA* (circles), Δ*srtB* (triangles), or *svpA* (diamonds) mutant for 15 min at 4°C. After 45 min of internalization at 37°C, gentamicin was added to kill extracellular bacteria, and bacterial survival was monitored for 8 h postinfection. In contrast to that of the *svpA* mutant, the intracellular multiplication of the Δ*srtB* mutant was not affected.

gene homologue in each genome (40). However, genomes may contain several paralogous copies of *srtA*, encoding sortases with the same specificity (e.g., responsible for the attachment of LPXTG proteins), but also *srt* genes expressing sortases with different specificities (e.g., recognizing different target proteins).

Together with the identification of IsdC as a target of SrtB in *S. aureus* (34), our work indicates that class B sortases are responsible for the anchoring of a small subset of cell wall proteins in gram-positive bacteria. We show that the listerial SrtB mediates the sorting of at least one protein, SvpA, which does not bear the classical LPXTG motif. As in *S. aureus*, the gene encoding SrtB and the gene encoding its target, SvpA, are both part of the same locus. Amino acid sequence similarities exist between the products of the first two ORFs of the *svp* operon, SvpA and Lmo2186, and IsdC of the *S. aureus isd* locus. However, only three of the five residues constituting the *S. aureus* probable SrtB recognition motif are conserved in the C-terminal part of SvpA (residues N, T, and N [Fig. 1C]). As mentioned earlier, the genomic organization of the *S. aureus isd* locus is also partially conserved in *B. halodurans* and *B. anthracis*, and in these two organisms, the IsdC homologues contain putative SrtB recognition sequences (NPQTG and NPKTG, respectively). These observations support the hypothesis that the recognition motif of SrtB family members may be less stringent than that recognized by SrtA. This lower stringency may decrease the efficiency of SrtB enzymatic cleavage. The exact mechanism of SrtB-dependent cleavage in *L. monocytogenes* will require further biochemical characterization. Our results suggest that SrtB anchors a small number of proteins. We searched in the *L. monocytogenes* genome for proteins containing an NXXTX sequence upstream of a hydrophobic domain and a charged tail in the C-terminal part. Since only SvpA and Lmo2186 were found, it is tempting to speculate that SrtB may sort only these two proteins.

Immunofluorescence experiments showed that SvpA was detected at the bacterial surface in a SrtB-dependent manner. The anchoring of SvpA to PG was further studied by fractionation experiments and analyses of pure PG preparations of the different mutants constructed. These studies demonstrated that only a fraction of SvpA was anchored by SrtB to the PG. The fact that the extracellular species of SvpA displays a mobility similar to that of the species observed in the PG fraction argues for a release of SvpA from the cell wall as the result of PG turnover, as has been described for internalin (26).

What is the role of SrtB in the environmental adaptation of *L. monocytogenes*? During the course of our study, Schneewind and coworkers demonstrated that the *S. aureus isd* genes were regulated by iron (34) and encoded factors responsible for hemoglobin binding and the passage of heme iron to the cytoplasm (32), thus constituting a potential source of iron for this extracellular pathogen. The *isd* locus of *S. aureus*, initially described by Mazmanian et al. in 2002 (34), was also described in two other papers published the same year, but with different names (*frp* genes in reference 36; *stbA*, *srH*, and *sir* genes in reference 45). The genomic similarities between the region of *srtB* in *L. monocytogenes* and the *S. aureus isd* locus suggest that the *svp-srtB* region might also participate in iron utilization. However, the infectious processes of *S. aureus* and *L. monocytogenes* are distinct (extracellular versus intracellular), imply-

ing different requirements for iron, different sources of iron, and different mechanisms of iron acquisition. Although both pathogens may lyse erythrocytes, and therefore acquire hemoglobin, it is unlikely that this represents a significant source of iron for *L. monocytogenes.* Indeed, the life cycle of *L. monocytogenes* is predominantly intracellular during the whole infectious process in vivo, and bacteremia occurs only transiently, preceding the crossing of the blood-brain barrier (4). Addressing the role of the *svp* locus in iron acquisition may thus reveal unique features that are specific to *Listeria.*

We show here that inactivation of *srtB* does not alter the intracellular survival of *L. monocytogenes* in several cellular models and has no detectable effect on virulence in the mouse model. These results indicate that the SrtB-dependent anchorage of SvpA to the cell wall is not crucial for these processes. The *svpA* mutant of *L. monocytogenes* shows a severe defect in intracellular survival in bone marrow-derived macrophages and has reduced virulence in mice (8). It is possible that the reduced virulence of the *svpA* mutant might be due solely to its restricted growth capacity.

The facts that most SvpA is secreted in the medium and that the surface-bound fraction is not evenly distributed on the surface of the bacterium imply a complex temporal and spatial control of its expression and of its anchoring at the bacterial surface. The surface-anchored SvpA may be functional when the microorganism encounters particular extracellular conditions. It will therefore be interesting to study the expression of *srtB* and of the whole *svp* operon under different growth conditions, especially under iron-controlled conditions.

Several lines of evidence suggest that the regulation of the *svpA* region might be controlled not only by iron availability but also by stress response regulatory genes. Indeed, it was previously shown that expression of SvpA is reduced by MecA and the stress proteins ClpC and ClpP (7, 8). In *Bacillus subtilis*, the adaptor protein MecA, through interaction with ClpC, plays a dual role in the protein quality control network by rescuing or, together with ClpP, by degrading misfolded and/or aggregated proteins (43). It is of interest in this respect that the gene encoding MecA is located in the *svp* operon upstream region. Therefore, deciphering the links between this complex regulatory network, the *srtB* region, and the mechanisms of iron acquisition by *L. monocytogenes* will be an important issue.

In conclusion, SrtB represents a second class of sortase in *L. monocytogenes*, involved in the attachment of SvpA to the cell wall, most likely by recognizing an NXXTN sorting motif. *L. monocytogenes* is the second gram-positive organism, after *S. aureus*, and the first intracellular pathogen shown to express two different types of sorting enzymes, a property that may be shared by other gram-positive bacterial species. Inactivation of *srtB* does not affect listerial virulence in the mouse model, as assessed by LD_{50} or bacterial counts in the spleen after i.v. infection. In *S. aureus*, inactivation of *srtB* has no detectable effect on the establishment of infection, but it does have an effect on long-term persistence (34). At present, the possibility cannot be excluded that during a natural oral infection in humans, an active *srtB* allele might be required to promote full virulence. Future work will therefore be required to address the contributions of the different sortases in listeriosis by using animal models (27) that may be more relevant for human listeriosis than the mouse model. Lastly, *Listeria* exists ubiquitously in several ecological niches, and SrtB maybe required to anchor proteins involved in the adaptation of *Listeria* to particular environmental conditions.

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