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The Sortase SrtA of *Listeria monocytogenes* Is Involved in Processing of Internalin and in Virulence

Caroline Garandeau, Hélène Réglier-Poupet, Iharilalao Dubail, Jean-Luc Beretti, The European *Listeria* Genome Consortium,† Patrick Berche, and Alain Charbit*

INSERM U-411, CHU Necker-Enfants Malades, 75730 Paris Cedex 15, France

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***Listeria monocytogenes* is an intracellular gram-positive human pathogen that invades eucaryotic cells. Among the surface-exposed proteins playing a role in this invasive process, internalin belongs to the family of LPXTG proteins, which are known to be covalently linked to the bacterial cell wall in gram-positive bacteria. Recently, it has been shown in *Staphylococcus aureus* that the covalent anchoring of protein A, a typical LPXTG protein, is due to a cysteine protease, named sortase, required for bacterial virulence. Here, we identified in silico from the genome of *L. monocytogenes* a gene, designated *srtA*, encoding a sortase homologue. The role of this previously unknown sortase was studied by constructing a sortase knockout mutant. Internalin was used as a reporter protein to study the effects of the *srtA* mutation on cell wall anchoring of this LPXTG protein in *L. monocytogenes*. We show that the *srtA* mutant (i) is affected in the display of internalin at the bacterial surface, (ii) is significantly less invasive in vitro, and (iii) is attenuated in its virulence in the mouse. These results demonstrate that *srtA* of *L. monocytogenes* acts as a sortase and plays a role in the pathogenicity.**

Gram-positive bacteria are surrounded by a cell wall envelope containing attached polypeptides and polysaccharides that may interact with host cells and play a role in the virulence of pathogenic species (34). In gram-positive bacteria, several distinct mechanisms of cell wall attachment and display of surface proteins have been recently described (reviewed in reference 5). The only surface proteins known to be covalently linked to the cell wall are the LPXTG proteins, exemplified by protein A of *Staphylococcus aureus* (34). In a pioneer work, Schneewind et al. described a cysteine protease of *S. aureus*, designated sortase, which is responsible for the covalent attachment of protein A, and identified the biochemical processes allowing the anchoring of protein A to the cell wall (27). Very recently, the three-dimensional structure of the sortase of *S. aureus* was determined by nuclear magnetic resonance spectroscopy, thus identifying a catalytic domain responsible for the transpeptidation reaction (17). After synthesis in the bacterial cytoplasm, surface protein precursors are translocated across the membrane and the NH_2-proximal leader peptide is removed by leader peptidase. The COOH-terminal sorting signal is first cleaved by sortase between the threonine and glycine residues of the LPXTG motif. Then, the enzyme covalently links the carboxyl of the threonine to the cell wall peptidoglycan by amide linkage (34, 35, 43, 44, 45). The sorting signal consists of a conserved LPXTG motif followed by a membrane-spanning hydrophobic domain and a tail mostly composed of positively charged residues (18, 28, 42). Notably, it was recently shown that in the gram-positive human pathogen *Listeria monocytogenes*, the surface proteins were also cleaved between the threonine and the glycine residue of the LPXTG motif and amide-linked to the peptidoglycan (7), strongly suggesting that this cell wall sorting mechanism is shared by all gram-positive bacteria.

In *S. aureus*, a mutant lacking the *srtA* gene encoding sortase is defective in the anchoring of surface proteins and accumulated precursor proteins with uncleaved C-terminal sorting signals. As a result, the assembly and display of surface adhesins is abolished and causes a reduction in the ability of sortase mutants to establish animal infections (26). In another gram-positive bacterium, the human commensal *Streptococcus gordonii*, it has been very recently shown that inactivation of *srtA* encoding sortase altered the expression of specific anchored surface proteins containing the canonical LPXTG motif, ultimately decreasing the ability of bacteria to colonize the oral mucosa in the mouse (3). These observations prompted us to search for a gene encoding a sortase homologue in *L. monocytogenes* and to test the effect of the gene disruption on surface protein anchoring and on bacterial virulence.

L. monocytogenes is a ubiquitous food-borne gram-positive bacterium, responsible for life-threatening infections in humans and animals (11). It is a facultative intracellular pathogen able to enter and multiply in both professional (25) and nonprofessional phagocytes such as epithelial cells (12, 13) or hepatocytes (8, 14, 48). The major steps of the intracellular parasitism of *L. monocytogenes* have been deciphered (see references 6 and 47 for reviews). 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

* Corresponding author. Mailing address: Faculté de Médecine Necker, 156, rue de Vaugirard, 75730 Paris Cedex 15, France. Phone: 33 1-40 61 53 76. Fax: 33 1-40 61 55 92. E-mail: charbit@necker.fr.

† The European *Listeria* Genome Consortium is composed of Philippe Glaser, Alexandra Amend, Fernando Baquero-Mochales, Patrick Berche, Helmut Bloecker, Petra Brandt, Carmen Buchrieser, Trinad Chakraborty, Alain Charbit, Elisabeth Couvé, Antoine de Daruvar, Pierre Dehoux, Eugen Domann, Gustavo Dominguez-Bernal, Lionel Durant, Karl-Dieter Entian, Lionel Frangeul, Hafida Fsihi, Francisco Garcia del Portillo, Patricia Garrido, Werner Goebel, Nuria Gomez-Lopez, Torsten Hain, Joerg Hauf, David Jackson, Jurgen Kreft, Frank Kunst, Jorge Mata-Vicente, Eva Ng, Gabriele Nordsiek, Jose Claudio Perez-Diaz, Bettina Remmel, Matthias Rose, Christophe Rusniok, Thomas Schlueter, Jose-Antonio Vazquez-Boland, Hartmut Voss, Jurgen Wehland, and Pascale Cossart.

process. Lysis of the phagosome results mainly from listeriolysin O, a sulfhydryl-activated hemolysin active at acidic pHs. Actin assembly is mediated by the surface protein ActA. The interaction of *L. monocytogenes* with host cells is a key event in the pathogenesis of listeriosis. This process involves a number of surface proteins, including internalin (or InlA), InlB, and ActA. InlA is an 800-amino-acid protein synthesized as a precursor bearing an N-terminal signal peptide and a C-terminal sorting signal with an LPXTG motif (20). It is required for entry into the human enterocyte-like cell line Caco-2 (9, 12) and other cell lines expressing its cellular receptor, the adhesion molecule E-cadherin (21, 31). The in vivo relevance of this molecule for the development of an infectious process has been addressed only very recently (22, 39).

In this work, we identified in silico a gene encoding a sortase-like protein, designated *srtA*, from the recently completed sequence of the genome of *L. monocytogenes* (15). We constructed a knockout mutant of this gene. The phenotypic analysis of the mutant strain revealed that *srtA* of *L. monocytogenes* acts as a sortase involved in processing and anchoring of internalin and therefore is involved in bacterial virulence.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) and Luria-Bertani (Difco Laboratories) broth and agar were used to grow *Listeria* and *Escherichia coli* strains, respectively. We used the reference strain of *L. monocytogenes* EGD belonging to serovar 1/2a, recently sequenced (15). Wild-type bacteria were transformed by electroporation as previously described (37). Strains harboring plasmids were grown in the presence of the following antibiotics: for pCR derivatives, kanamycin (50 μg ml^{-1}); for pAUL-A derivatives, erythromycin (150 μg ml^{-1} [*E. coli*] and 5 μg ml^{-1} [*L. monocytogenes*]).

Genetic manipulations. Total DNA from *Listeria* cells was prepared as described (38). Standard techniques were used for plasmid DNA preparation, fragment isolation, DNA cloning and restriction analysis (41). Restriction enzymes and ligase were purchased from New England Biolabs and used as recommended by the manufacturer. DNA was amplified with *Taq* DNA polymerase (Promega) for 35 cycles of 60 s at 95°C, 60 s at 55°C, and 90 s at 72°C in a Gene Amp System 9600 thermal cycler (Perkin-Elmer, Branchburg, N.J.). Nucleotide sequencing was carried out with *Taq* DiDeoxy terminators and by the DyePrimer Cycling Sequence protocol developed by Applied Biosystems (Perkin-Elmer) with fluorescently labeled dideoxynucleotides and primers, respectively. Fluorescently labeled primers were purchased from Life Technologies, Paisley, Scotland. Labeled extension products were analyzed on an ABI Prism 310 apparatus (Applied Biosystems, Perkin-Elmer). Protein and nucleotide databases were searched using the programs BLASTP and BLASTN (National Center for Biotechnology Information, Los Alamos, N.Mex.), available via the Internet. Protein sequences were aligned by the program CLUSTAL W.

Construction of a *srtA* deletion mutant of *L. monocytogenes*. A 2,042-bp DNA fragment comprising the *srtA* gene (*orf 1592.1*) was amplified by PCR from *L. monocytogenes* (EGDwt) genomic DNA using the following primer pair: primer 1 (5′-GATATTCTGGGTATGCGTCTCGTACATCAAACGAA-3′) and primer 2 (5′-GCAACTTCTGTACTTGCCATATGCACTTTTGTTTTTCC-3′). Oligonucleotides were synthesized by Genset (Paris, France). The AmpliTaq DNA polymerase of *Thermus aquaticus* from Perkin-Elmer was used. The amplified double-stranded DNA fragments were first cloned into the pCR cloning vector using the Invitrogen TA cloning kit (Invitrogen Corporation, San Diego, Calif.). The recombinant plasmids pCR -*rtA* was cut with *Eco*RI and *Kpn*I restriction enzymes (Biolabs NEN, Beverly, Mass.). The fragment comprising the *srtA* gene was purified on Tris-acetate-EDTA–agarose gels and subcloned into the *Eco*RI-*Kpn*I sites of the thermosensitive shuttle vector pAUL-A (24) to give plasmid pAUL -*srtA*. The *srtA* deletion mutant of *L. monocytogenes* (EGDΔ*srtA*) was constructed by deleting a 57-bp internal fragment of *srtA* gene, corresponding to two natural *Nhe*I sites present within the sortase coding sequence, that removed residues 105 to 123 of SrtA. The resulting plasmid pAUL -Δ*srtA* was introduced into EGD by electroporation, and chromosomal integration of the mutated gene was performed by allelic replacement, as described previously (16, 24). The construction was confirmed by PCR sequence analysis of chromosomal DNA from the mutant.

Insertion of a kanamycin resistance cassette into the *Nhe*I site of Δ*srtA*. A promoterless *aphA3* gene, conferring resistance to kanamycin (46), was inserted into the *Nhe*I site of the Δ*srtA* mutant gene carried by plasmid pAUL -Δ*srtA*. The following pair of primers was used to amplify the *aphA3* gene: primer 1 (5′-CTA *GCTAGC*TAGGTGACTAACTAG-3′) and primer 2 (5′-CTA *GCTAGC*TAGC TTGGGTCATTATTCCC-3′) (the *Nhe*I restriction sites are in italics). The resulting plasmid, pAUL -Δ*srtA*::*aphA3*, was introduced into EGD by electroporation, and chromosomal integration of the mutated gene was performed by allelic replacement (see above).

Complementation. For complementation, the entire *srtA* gene and its promoter region (452 bp upstream from the ATG) were introduced in the chromosome of EGDΔ*srtA* by using the integrative plasmid pAT113, a Tn*1545* derivative allowing random insertion in the chromosome of *L. monocytogenes* (references 1 and 23 and references therein). The recombinant plasmid pCR -*srtA* (carrying the *srtA* gene and its promoter region [see above]) was cut with *Eco*RI restriction enzyme (Biolabs NEN). The 1.8-kb *Eco*RI fragment comprising the *srtA* gene was purified on TAE-agarose gels and subcloned into the *Eco*RI site of the integrative plasmid pAT113, to give plasmid pAT113 -*srtA*. Plasmid pAT113-*srtA* was then electroporated into EGDΔ*srtA* and selection on erythromycin (8 μg ml^{-1}). One insertion of the recombinant plasmid that occurred in a region of the chromosome that did not interfere with any known biological activity of *L. monocytogenes* (not shown) was chosen for further analyses.

Reverse transcriptase PCR (RT-PCR). Total RNA was extracted from *L. monocytogenes* (EGD) grown overnight in BHI broth at 37°C. The following pairs of primers were used to amplify the mRNAs: *srtA* primer 1 (5′-AGGAG GAATCATATGTTAAAGAAAACAA-3′) and primer 2 (5′-CCGCTGTCTTT TTTTCCTCATTAT-3′), *orf 1589.1* primer 1 (5′-TCTGAAACGTTTAGGCGT CAAT-3′) and primer 2 (5′-CCGAAGAAGTCACCAAAAATCT-3′), and *orf 1590.1* primer 1 (5′-GGAAGAAGCTGGATTTAAGCTG-3′) and primer 2 (5′-GCCAACTTCGGGTAAATGACTA-3′).

The procedure used was that described in the instructions for the SuperScript One-step RT-PCR System (Life Technologies). Prior to RT-PCR, total RNA samples were incubated for 1 h at 37°C with RNase-free DNase I (Boehringer, Mannheim, Germany) to eliminate any DNA contamination, and DNase was heat inactivated by incubation at 80°C for 15 min.

SDS-PAGE and Western blot analysis. Proteins from culture supernatants and total bacterial extracts were prepared as follows. Fractions of bacterial cultures at different optical densities at 600 nm (OD_{600}) were centrifuged. Supernatants were passed through a 0.22-μm-pore-size filter (Millipore, Bedford, Mass.) and further concentrated with ultrafree Biomax columns with a cutoff of 30 kDa. Samples were finally suspended in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (130 mM Tris-HCl, pH 6.8; 1% SDS; 7% 2-β-mercaptoethanol; 7% sucrose; 0.01% bromophenol blue). The bacterial pellets were suspended in cold water, and bacteria were disrupted using a Fastprep FP120 apparatus (Ozyme; Bio101, Carlsbad, Calif.) with three pulses of 30 s at a speed of 6.5. After a centrifugation of 3 min at 10,000 rpm, lysates were collected and suspended in 1× SDS-PAGE sample buffer. Electrophoresis and Western blotting were carried out as described previously (23) in SDS–8% polyacrylamide minigels (Mini Protean II; Bio-Rad). Loading in each well corresponds to 100 μl of bacterial culture (adjusted to a final OD_{600} of 1). Nitrocellulose sheets were probed either with anti-internalin monoclonal antibody (MAb) L7.7, K18.4, or C20.4 (30) or with polyclonal ActA antibody, obtained from P. Cossart (Pasteur Institute, Paris, France), with an anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody. Antibodies were used at a final dilution of 1:1,000. Antibody binding was revealed by adding 0.05% diaminobenzidine-tetrahydrochloride (Sigma) and 0.03% hydrogen peroxide (Sigma).

Protein sequencing. EGDΔ*srtA* mutant strain was grown in RPMI 1640 synthetic medium containing glucose (3% final concentration) overnight with agitation at 37°C. After centrifugation, cell supernatants were filtered through a 0.22-μm-pore-size Millipore filter and further concentrated with ultrafree columns with a cutoff of 30 kDa (Millipore). Denatured proteins were separated by SDS-PAGE and then transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore). Protein bands were visualized by staining the membrane with amido black. To identify the protein bands corresponding to internalin, a part of the membrane was cut and tested like a classical Western blot with anti-InlA MAb L7.7. For N-terminal sequencing, the two major protein bands detected by the MAb were cut from the polyvinylidene difluoride membrane and sequenced on an Applied Biosystems Procise Sequencer (J. d'Alayer, Laboratoire de Séquençage des Protéines, Institut Pasteur, Paris).

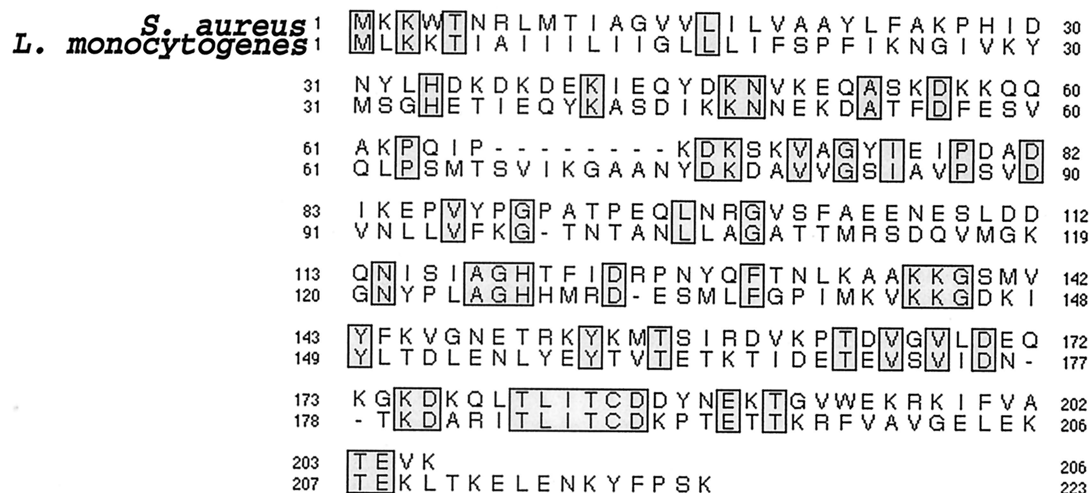

FIG. 1. Alignment of *L. monocytogenes* SrtA with *S. aureus* sortase. Alignment was performed using CLUSTALw. Identical residues are boxed. *L. monocytogenes* SrtA comprises 222 amino acids; the sortase of *S. aureus* comprises 206 amino acids.

Protein solubility in hot SDS. In *L. monocytogenes*, cell wall-anchored internalin is largely insoluble in hot SDS unless the peptidoglycan had been first digested enzymatically. In contrast, the membrane-anchored protein ActA is almost completely extractable in hot SDS without any prior treatment (7). We used this assay to determine the solubility of internalin in hot SDS in the *srtA* mutant. Briefly, *L. monocytogenes* EGD and *srtA* mutant were grown with agitation at 37°C in BHI medium. Two milliliters of each culture (at an OD_{600} of 1) were collected and centrifuged for 20 min at 6,000 rpm at 4°C (in an Eppendorf centrifuge). The bacterial pellets were washed in phosphate-buffered saline and then resuspended in 100 μl of 4% SDS–0.5 M Tris-HCl, pH 8. The suspension was boiled for 10 min and then centrifuged in an Eppendorf centrifuge at 10,000 rpm for 5 min. The supernatant (100 μl) and pellet (resuspended in 100 μl of 4% SDS–0.5 M Tris-HCl, pH 8) fractions were precipitated with 10% ice-cold trichloroacetic acid; the precipitate was washed in 70% acetone and dried. Each sample was finally resuspended in 20 μl of SDS-PAGE sample buffer and denatured by boiling for 10 min prior to loading onto SDS–8% polyacrylamide gels. Ten microliters were loaded per well (corresponding to 1 ml of bacterial culture at an OD_{600} of 1).

Invasion assays. (i) Culture of cell lines. The human colon carcinoma cell line Caco-2 (ATCC HTB37) and the human hepatocellular carcinoma cell line HepG-2 (ATCC HB 8065, kindly provided by S. Dramsi and P. Cossart, Institut Pasteur, Paris) were propagated as previously described (8) in Dulbecco's modified Eagle's medium (25 mM glucose) (Gibco). Cells were seeded at 8×10^4 cells cm^{-2} in 24-well tissue culture plates (Falcon). Monolayers were used 24 h after seeding.

(ii) Invasion assays. The invasion assays were carried out essentially as described previously (33). Briefly, cells were inoculated with bacteria at a multiplicity of infection of approximately 100 bacteria per cell. They were incubated for 1 h to allow the adherent bacteria to enter and were then washed three times with RPMI and overlaid with fresh Dulbecco's modified Eagle's medium containing gentamicin (10 mg $liter^{-1}$) to kill extracellular bacteria. At 2 and 4 h, cells were washed three times and processed for counting of infecting bacteria. For that, cells were lysed by adding cold distilled water. The titer of viable bacteria released from the cells was determined by spreading onto BHI plates. Each experiment was carried out in triplicate and repeated three times.

Virulence in the mouse. Specific-pathogen-free female Swiss mice (Janvier, Le Geneset St. Isle, France), 6 to 8 weeks old, were used. Bacteria were grown for 18 h in BHI broth, centrifuged, washed once, appropriately diluted in 0.15 M NaCl, and inoculated intravenously (i.v.) (0.5 ml) via the lateral tail vein. Groups of five mice were challenged i.v. with various doses of bacteria, and mortality was observed for 10 days. The virulence of strains was estimated by the 50% lethal dose (LD_{50}) using the Probit method and by the bacterial survival in tissues. Bacterial growth was monitored in organs after i.v. inoculation of 10^6 bacteria. Groups of five mice were sacrificed by day 1, 2, 3, 4, and 7. Organs (spleen, liver, and brain) were aseptically removed and separately homogenized in 0.15 M NaCl. Bacterial counts in organ homogenates were determined at various intervals on BHI agar plates, as described previously (1).

RESULTS

Sequence analysis and transcription of the *srtA* gene *of L. monocytogenes*. The Blast search (NCBI Blastp 2 version) of the very recently completed sequence of the genome of *L. monocytogenes* EGD-e serovar 1/2a (15), using the amino acid sequences of SrtA from *S. aureus* (27) and *S. gordonii* (3), led us to identify a single open reading frame (ORF) encoding a sortase-like protein of 222 amino acids (*orf 1592.1*). The protein shares 32% identity over its entire sequence with the sortase from *S. gordonii* and 27% with that of *S. aureus*, with a perfect conservation of the consensus motif around the putative active site cysteine (Fig. 1). When the 222-amino-acid sequence was used to scan the *Listeria* genome database, no additional paralogous sortase with ≥25% overall identity could be found. When only the TLXTC consensus motif was used, a second ORF (*orf 812.1*) encoding another putative sortase-like protein of 246 residues was found. However, in spite of a good conservation of the consensus motif (TLSTC), this ORF showed only a very low overall sequence conservation, with 21 and 16.5% identity with the sortases from *S. aureus* and *S. gordonii*, respectively. Therefore, we decided to focus in the present work on the study of the 222-amino-acid protein encoded by *orf 1592.1*. According to its hydropathic profile, this sortase homologue belongs to the same class of enzymes as *S. aureus* SrtA, which contain an N-terminal segment of hydrophobic amino acids that functions both as a signal sequence and a stop-transfer signal for membrane anchoring (17). Therefore, this gene will be referred to as *srtA* and the corresponding protein will be referred to as SrtA in the rest of this work for simplification.

We then performed the analysis of the region of the *L. monocytogenes* chromosome comprising *srtA*. A canonical AGGAGG Shine-Dalgarno sequence precedes the *srtA* coding sequence (7 bp upstream of the start codon). We identified a potential promoter region immediately upstream of *srtA* (57 bp) that is very similar to the consensus sequence of σ-70-dependent promoters (29), with a TTGCTT −35 region and a TATAAT −10 region spaced by 17 bp. Immediately down-

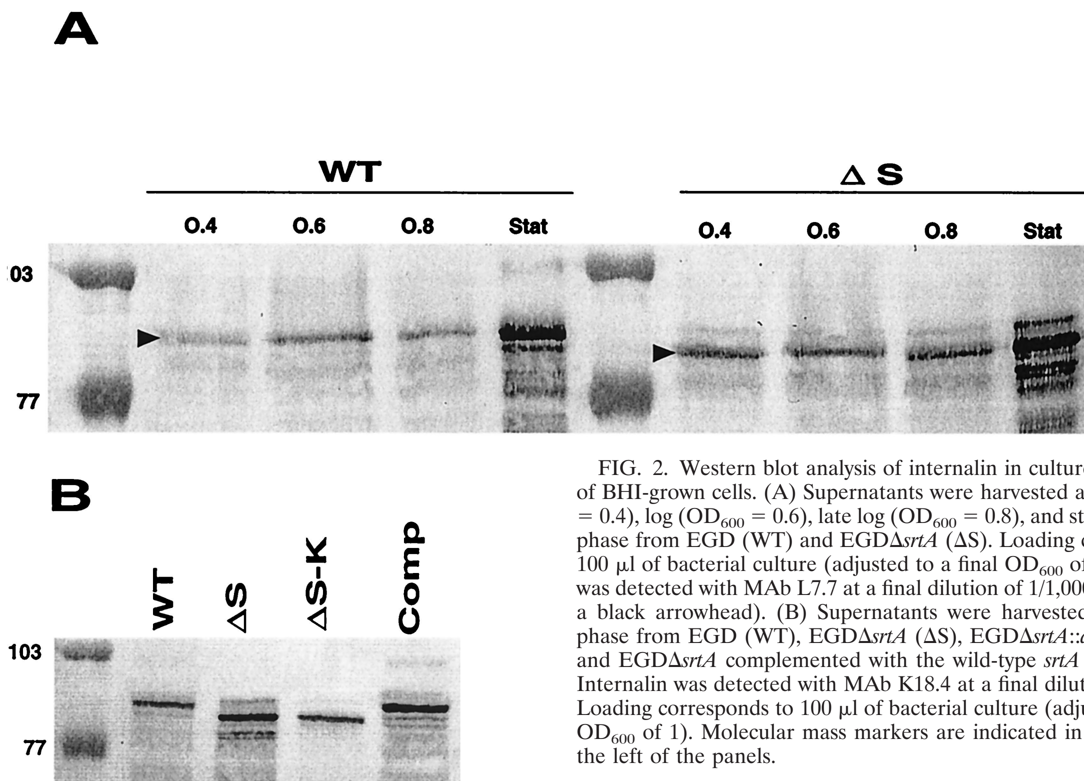

FIG. 2. Western blot analysis of internalin in culture supernatants of BHI-grown cells. (A) Supernatants were harvested at early (OD_{600} = 0.4), log (OD_{600} = 0.6), late log (OD_{600} = 0.8), and stationary (Stat) phase from EGD (WT) and EGDΔ*srtA* (ΔS). Loading corresponds to 100 μl of bacterial culture (adjusted to a final OD_{600} of 1). Internalin was detected with MAb L7.7 at a final dilution of 1/1,000 (indicated by a black arrowhead). (B) Supernatants were harvested at stationary phase from EGD (WT), EGDΔ*srtA* (ΔS), EGDΔ*srtA*::*aphA3* (ΔS-K), and EGDΔ*srtA* complemented with the wild-type *srtA* gene (Comp). Internalin was detected with MAb K18.4 at a final dilution of 1/1,000. Loading corresponds to 100 μl of bacterial culture (adjusted to a final OD_{600} of 1). Molecular mass markers are indicated in kilodaltons to the left of the panels.

stream of *srtA*, a putative transcription terminator is found, with a predicted free energy of the stem-loop structure of −18.2 kcal/mol.

The genes surrounding *srtA* in the chromosome of *L. monocytogenes* encode hypothetical proteins of unknown functions. The two genes upstream of *srtA* encode a putative 3-methyladenine DNA glycosylase (*orf 1593.1*) and a hypothetical protein of unknown function (*orf 1594.1*), sharing 50 and 59% identity with YxlJ and YfnIJ, respectively, two hypothetical proteins of *Bacillus subtilis*. Strikingly, the two genes encoding these putative ORFs are located in two different regions of the *B. subtilis* chromosome, revealing a divergent genetic organization between the two bacterial species. The two genes downstream of *srtA* encode a hypothetical protein of unknown function (*orf 1590.1*) and a putative lipoate ligase (*orf 1589.1*), sharing 47 and 65% identity with YhfI and YhfJ, respectively, two hypothetical proteins encoded by consecutive genes of the *B. subtilis* chromosome. *orf 1589.1* and *orf 1590.1* are followed by a typical rho-independent transcription terminator.

We confirmed by RT-PCR (see Materials and Methods) that the *srtA* gene was transcribed in the wild-type strain grown in BHI medium at 37°C in exponential phase, strongly suggesting that it encodes a functional protein(s). RT-PCR also confirmed that the internal deletion in the *srtA* gene had no polar effect on the transcription of the two downstream genes, *orf 1590.1* and *orf 1589.1* (not shown).

We constructed an *L. monocytogenes srtA* knockout mutant by deletion of an internal fragment of *srtA* gene, encoding residues 105 to 123 of SrtA. The mutation was integrated in the chromosome of *L. monocytogenes* by allelic replacement (EGDΔ*srtA*) (see Materials and Methods). To confirm that the phenotypes observed were due to the Δ*srtA* deletion, we constructed an *srtA*-complemented strain. We also constructed a mutant carrying a kanamycin resistance cassette into the deleted portion of the *srtA* gene (EGDΔ*srtA*::*aphA3*) to check that the deletion Δ*srtA* had completely inactivated SrtA (see Materials and Methods). The properties of these two strains were compared to those of EGDΔ*srtA*.

SrtA acts as a sortase involved in processing and anchoring of internalin in *L. monocytogenes*. The Δ*srtA* mutant did not show any growth defect in BHI medium at 37°C, compared with wild-type EGD (data not shown), indicating that the mutated gene identified is not essential for bacterial multiplication. Internalin was used as a reporter protein to study the sorting defect of the *srtA* mutant, because it is the best-studied LPXTG protein of *L. monocytogenes* involved in the virulence (references 12 and 22 and references therein) against which a series of antibodies are available (30).

Truncated forms of internalin are released in the Δ*srtA* mutant. We found by Western blot analysis, using the anti-internalin MAb L7.7 (Fig. 2A), that substantial amounts of internalin were released in the culture medium during bacterial growth, as previously shown (10). These amounts of internalin released in the culture supernatant of wild-type bacteria were weak in early exponential phase and increased considerably in stationary phase. In the Δ*srtA* mutant, significantly higher amounts of internalin were released as soon as the early exponential phase. At each time point of exponential growth, one major band of internalin was detected by MAb L7.7. Strikingly, this band had a lower molecular weight (MW) in the Δ*srtA* mutant than that in the wild-type strain. Similar results were obtained with the two other anti-internalin MAbs tested, K18.4 and C20.4 (not shown). In stationary phase, mul-

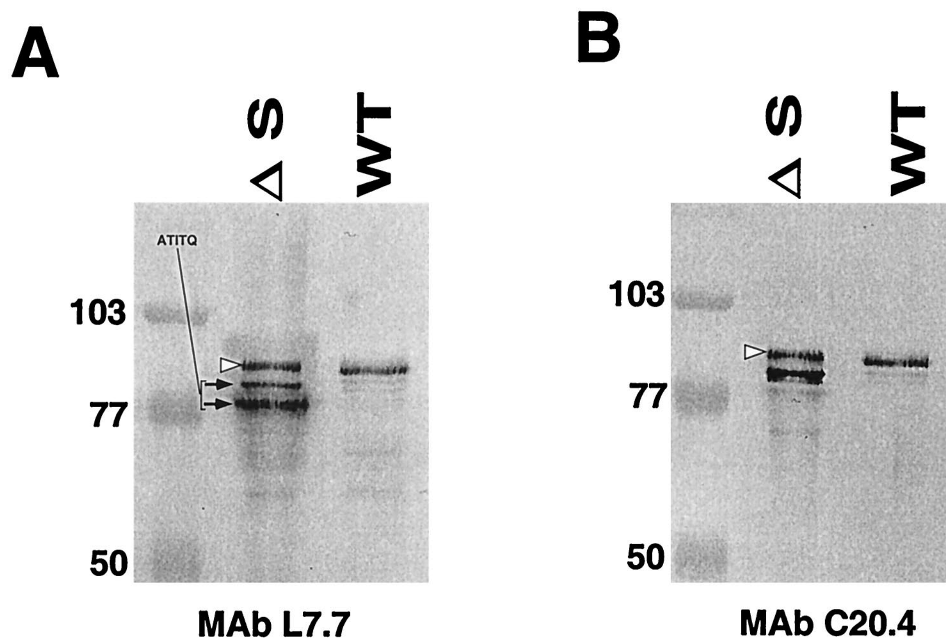

FIG. 3. Western blot analysis of internalin in culture supernatants of RPMI-grown cells. (A) Detection with MAb L7.7. (B) Detection with MAb C20.4. Three major forms of internalin protein were detected by MAb L7.7 in the culture supernatant of the *srtA* mutant. The upper bands detected in the mutant are indicated by open arrowheads. The two forms with a lower apparent molecular mass than that of internalin released by the wild-type strain are indicated by arrows. Both polypeptides have the same N terminus as the mature form of wild-type internalin (ATITQ). Internalin was detected with MAb L7.7 or MAb C20.4 at final dilutions of 1/1,000. Molecular mass markers are indicated in kilodaltons to the left of the panels. ΔS, EGDΔ*srtA*; WT, EGD.

tiple band patterns were detected with both strains. Such patterns have been previously reported and were suspected to reflect partial proteolytic degradation of the C-terminal part of internalin (12, 20, 31). Interestingly, in stationary phase a band with a higher MW was also clearly detected (Fig. 2A). This protein species has an MW compatible with that of a membrane-anchored form of the protein that would be released in the culture supernatant from lysed bacteria, as proposed earlier (20). As shown in Fig. 2B, the MW of the internalin band detected in the supernatant of the complemented strain was identical to that of wild-type EGD (Fig. 2B), confirming that the defect of internalin anchoring and processing observed was due to the Δ*srtA* deletion. The band of internalin detected in the culture supernatant of the mutant strain EGDΔ*srtA*::*aphA3* had the same MW as that detected in EGDΔ*srtA*.

Internalin release was also evaluated in bacteria grown in RPMI synthetic medium. During stationary phase, three major forms of internalin were detected by MAb L7.7 in culture supernatants of the Δ*srtA* mutant, for only one in the wild-type strain (Fig. 3A). As observed in BHI (see above), a band with a slightly higher MW than that of internalin released by the wild-type strain was detected (Fig. 3). We performed the N-terminal protein sequencing of the two lower migrating bands detected by MAb L7.7, showing that both polypeptides had the same N terminus as the mature form of wild-type internalin (ATITQ). This result indicates that, in the Δ*srtA* mutant, an important fraction of internalin is processed by proteolysis at its C terminus. Moreover, MAb C20.4, a MAb directed against the portion of internalin immediately preceding the sorting signal, detected only one of the two truncated forms of internalin (Fig. 3B). These data are compatible with a proteolytic cleavage of internalin close to the natural cleavage site at the LPXTG motif, which would remove the antibody recognition site. The enzyme(s) responsible for this activity remains to be identified.

Internalin is solubilized by SDS treatment in the Δ*srtA* mutant. It is known that only a fraction of internalin is solubilized when wild-type bacteria are treated by SDS, whereas ActA, a protein of *L. monocytogenes* anchored to the bacterial membrane through a hydrophobic tail, is easily solubilized by this treatment (7). When the Δ*srtA* mutant was incubated with hot SDS, we found that membrane-associated internalin was entirely solubilized, with a multiple band pattern, probably reflecting partial degradation (Fig. 4A). Interestingly, the upper protein band detected by MAb L7.7 in the mutant was higher than that detected in wild-type EGD. The size of this upper band and its solubility in SDS are compatible with that of uncleaved internalin that would be anchored to the cytoplasmic membrane through its conserved hydrophobic C-terminal tail. As expected after treatment by SDS, the membrane-anchored protein ActA was almost totally released in supernatants of the wild-type strain and the Δ*srtA* mutant (Fig. 4B).

SrtA is involved in virulence of *L. monocytogenes*. Since internalin is known to be important in the invasion of eucaryotic cells by *L. monocytogenes* (12), we first studied the ability of EGDΔ*srtA* to penetrate into and to replicate within cells in vitro. Using a multiplicity of infection of 100 bacteria/cell, we tested two different types of human cell lines, the enterocyte-like cell line Caco-2 and HepG-2 hepatocytes, previously used as

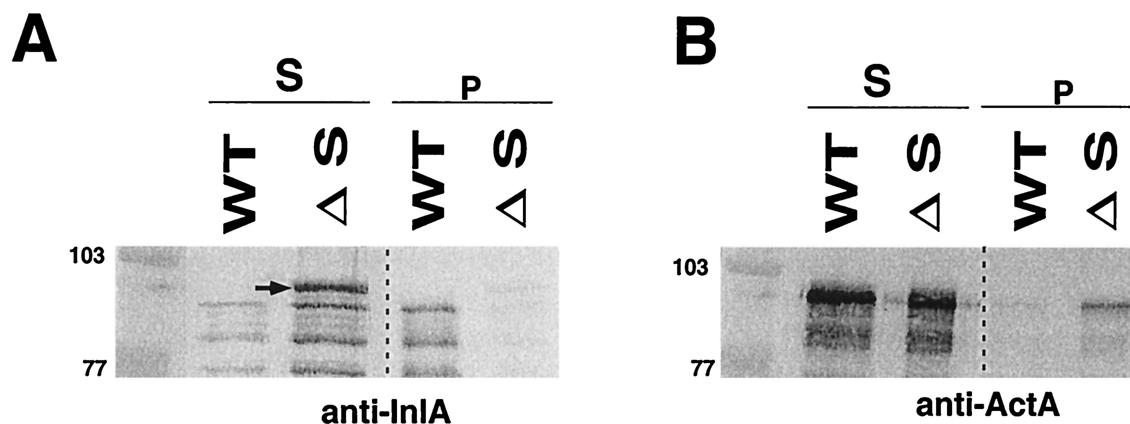

FIG. 4. Western blot analysis of SDS-treated bacteria. In the *srtA* mutant (ΔS), essentially all internalin was solubilized by SDS treatment, while in the wild-type (WT) strain, only a fraction of internalin was solubilized. (A) The uppermost form of internalin released by the mutant strain is indicated by an arrow. Internalin was detected with MAb L7.7 at a final dilution of 1/1,000. (B) In the wild-type strain, as well as in the *srtA* mutant, the membrane-anchored protein ActA is as good as totally released in the supernatant after incubation of the bacteria in hot SDS. ActA was detected with anti-ActA polyclonal serum at a final dilution of 1/1,000. Molecular mass markers are indicated in kilodaltons to the left of the panels. Abbreviations: S, soluble fraction; p, pellet fraction.

model systems to study infection with *L. monocytogenes* (4, 13). In Caco-2 cells, invasion was strongly affected in EGDΔ*srtA* (Fig. 5A). After 2 h of exposure, the intracellular multiplication of mutant bacteria was only 1/20 that of wild-type EGD and ca. 1/10 after 4 h. As shown in Fig. 5A, the phenotype of mutant EGDΔ*srtA*::*aphA3* was almost identical to that of EGDΔ*srtA*, demonstrating that the Δ*srtA* deletion was sufficient to impair SrtA activity. As expected, intracellular multiplication of the complemented mutant was similar to that of wild-type EGD (Fig. 5A), confirming that the phenotype observed was indeed due to the inactivation of *srtA*. In HepG-2 cells (Fig. 5B), the Δ*srtA* mutant showed a less-pronounced growth defect after 2 h of infection (one-sixth that of wild-type EGD). However, after 4 h, this defect corresponded to only a twofold reduction of bacterial counts compared to the wild-type strain.

The role of SrtA in the virulence of *L. monocytogenes* was first studied by determining the LD_{50} after i.v. inoculation of Swiss mice. The LD_{50} of the Δ*srtA* mutant was estimated at $10^{6.4}$ bacteria, almost 2 logs higher than that of the parental strain ($10^{4.5}$ bacteria), indicating that bacterial virulence was attenuated but not completely abolished. The LD_{50} of the mutant EGDΔ*srtA*::*aphA3* was slightly higher, with that of EGDΔ*srtA* at $10^{7.2}$ bacteria. As expected, the complemented strain regained almost complete virulence, with an LD_{50} at $10^{5.3}$.

The ability of the Δ*srtA* mutant to multiply in the target organs of infected mice was then evaluated by injecting mice i.v. with the normally lethal dose of 10^6 bacteria/animal. Earlier studies (2) have shown that upon i.v. inoculation of Swiss mice with 10^6 wild-type EGD organisms, bacterial growth in the spleen and liver reaches a peak at day 3, with up to 10^8 bacteria per organ, ultimately leading to death at day 4 to 5. At this dose, all the mice inoculated with the Δ*srtA* mutant fully recovered from infection. In the spleen, bacterial multiplication of mutant bacteria was almost identical to that of the wild-type strain until day 2, and then bacteria were slowly eliminated, reaching $<10^3$ bacteria per spleen by day 7 (Fig. 6A). The kinetics of survival in the liver showed an increase of bacterial counts up to day 4, reaching ca. 10^7 bacteria, followed by a drop to fewer than 10^3 bacteria per liver by day 7 (Fig. 6B). In the brain, the growth curve of mutant bacteria was similar to that of the wild-type strain until day 3 (Fig. 6C) and paralleled the increased bacteremia. Then, mutant bacteria were completely cleared from the brain by day 7, in contrast to wild-type bacteria (Fig. 6C).

DISCUSSION

We have identified a gene, *srtA*, that encodes a sortase in the genome of *L. monocytogenes* and tested whether inactivation of this gene would have an effect on bacterial virulence. We show that an *srtA* knockout mutant (i) is defective in processing and anchoring of internalin, (ii) has reduced invasiveness in vitro, and (iii) is attenuated in vivo, demonstrating the role of this sortase in the pathogenicity of *L. monocytogenes.*

SrtA of *L. monocytogenes* acts as a sortase. Earlier protein sequence comparison within sortase enzymes from several different gram-positive bacteria (43) revealed a striking conservation of the region surrounding the cysteine 184 (referring to the *S. aureus* sortase numbering), a residue known to be essential for enzyme activity (17, 43). A more recent survey on sortase homologues among predicted proteins from 92 bacterial genomes led to the identification of putative sortase-like proteins in most gram-positive bacterial species (36). Interestingly, in almost every gram-positive bacterium, there was usually more than one putative sortase-like protein. The physiological reasons for this apparent redundancy are at present unknown.

Here, we identified a single *orf* in the genome of *L. monocytogenes* encoding a sortase-like protein. We found that, in a Δ*srtA* mutant, a significant amount of a truncated form of internalin was released in the culture supernatant in early exponential growth. The truncated forms released in stationary phase were shown to correspond to proteolysis at the C terminus of internalin, most likely close to the natural cleavage site at the LPXTG motif. Moreover, solubilization of mem-

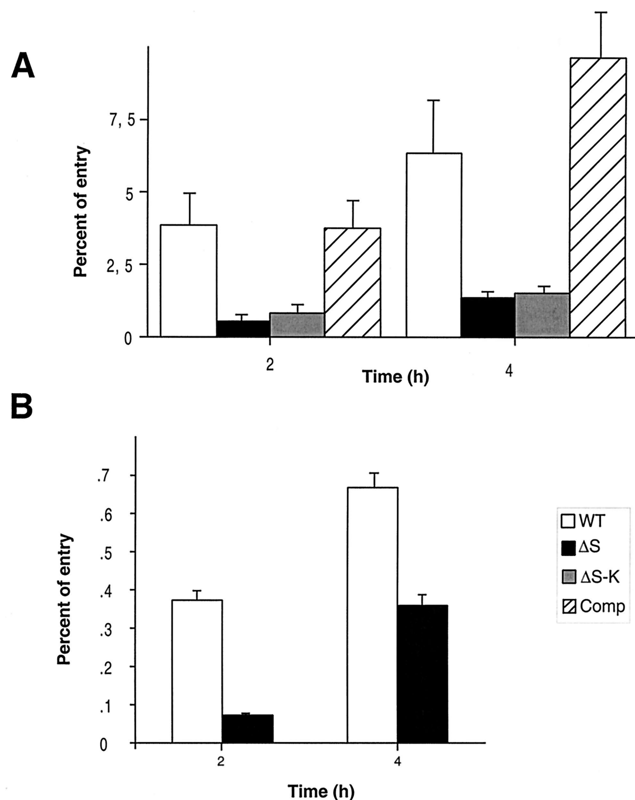

FIG. 5. Invasivity assay of EGD and Δ*srtA* into Caco-2 and HepG-2 cells. Invasiveness was evaluated in two different types of cell lines: Caco-2 cells (A) and HepG-2 cells (B). Cell monolayers were incubated for 1 h at 37°C with approximately 100 bacteria per cell. After washing, the cells were reincubated for 4 h in fresh culture medium containing gentamicin (10 mg liter^{-1}). At 2 and 4 h, the cells were washed again and lysed and viable bacteria were counted on BHI plates. The percentage of entry is the number of bacteria that survived in the presence of gentamicin per number of inoculated bacteria. Values are means of three different experiments. Error bars show standard deviations. Abbreviations: WT, EGD; ΔS, EGDΔ*srtA*; ΔS-K, EGDΔ*srtA*::*aphA3*; Comp, EGDΔ*srtA* complemented with the wild-type *srtA* gene.

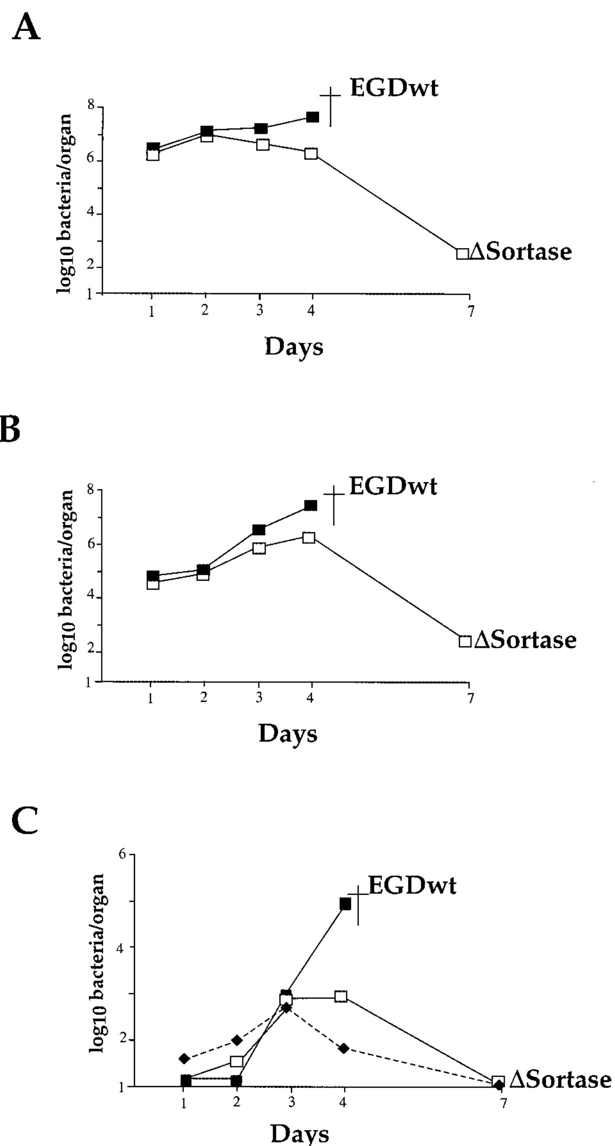

FIG. 6. In vivo survival of the Δ*srtA* mutant. The kinetics of bacterial growth was monitored in organs of mice infected with the *srtA* mutant, compared with EGD as a positive control. Mice were inoculated with 10^6 bacteria. Bacterial survival was monitored in the spleen (A), liver (B), and brain and blood (C) over a 7-day period. The counts in the blood for the *srtA* mutant are represented by dashed lines and black diamonds. Symbols: ■, EGD; □, Δ*srtA*. ■†, death.

brane-anchored proteins by SDS treatment suggested that, in the Δ*srtA* mutant, the form of internalin still associated to the cell wall was anchored to the cytoplasmic membrane through its uncleaved hydrophobic C-terminal tail.

In Caco-2 cells, and to a lesser extent in HepG-2 cells, invasion was diminished in the Δ*srtA* mutant. These results suggest that the inactivation of *srtA* has altered the processing and/or cell wall presentation of one or several proteins that participate in bacterial entry in these two cell types. Remarkably, the invasion defect observed with the *srtA* mutant in Caco-2 cells is significantly less pronounced than that of an *inlA* mutant (ca. 3 logs less than wild-type [references 12 and 31 and unpublished data]). In this respect, it is worth recalling that *L. monocytogenes* strain LO28, which expresses a truncated internalin protein lacking its C-terminal anchor (and thus noncovalently linked to the peptidoglycan) (19), invades Caco-2 cells much less efficiently than EGD (10). In spite of that defect, LO28 is fully virulent in the mouse model (32).

The role of SrtA in virulence was demonstrated in the mouse model. The *srtA* mutant of *L. monocytogenes* was attenuated, but virulence was not abolished. In all the assays performed, complementation of the mutant strain EGDΔ*srtA* with the wild-type *srtA* allele restored wild-type properties, demonstrating that the phenotypes observed were due to the *srtA* mutation.

Functional implications. Several members of the LPXTG protein family have been shown to be involved in the virulence of *L. monocytogenes* (1, 22, 40). Of particular interest, internalin, which is required for entry into human Caco-2 cells in vitro, had as good as no effect on bacterial virulence in the mouse model, even upon administration by the oral route (14, 22).

The work by Cossart and colleagues showed that this was due to a point mutation in the mouse receptor for internalin, E-cadherin (21). The critical role of internalin–E-cadherin interaction in vivo was demonstrated very recently (22), using transgenic mice expressing human E-cadherin. In such mice, *inlA* mutants have a clear defect of virulence after oral infection.

The Δ*srtA* deletion mutant and its derivative carrying a kanamycin resistance cassette (Δ*srtA*::*aphA3*) shared similar in vitro and in vivo properties. However, the fact that EGDΔ*srtA*::*aphA3* was slightly more attenuated (0.8 log) than EGDΔ*srtA* might suggest that the deletion Δ*srtA* did completely abolish SrtA activity. Interestingly, the deleted region did not remove the conserved cysteine comprised within the putative active site of the molecule (17). However, in SrtA from *S. aureus*, the region corresponding to the deleted portion comprises one of the three residues (N98) proposed to constitute the catalytic triad that mediates the transpeptidation reaction, as well as all three acidic residues (E105, E108, and D112) constituting the putative calcium binding site that stimulates catalysis (located near the active site in the tertiary structure) (17). It is thus likely that, in SrtA of *L. monocytogenes*, the deletion of this internal region led to drastic alterations of the structural, and hence functional, organization of the molecule.

One hypothesis to account for the fact that the *srtA* mutant did not completely abolish bacterial virulence could be that additional sortase-like proteins of *L. monocytogenes* participate in the cell wall anchoring of the LPXTG protein involved in bacterial virulence (for example, *orf 812.1*). The presence of multiple sets of genes involved in secretion in the genomes of gram-positive bacteria (see reference 28 for a review) suggests that these organisms might have evolved several distinct pathways for surface protein transport. Another possible explanation could be that the fraction of the LPXTG proteins that remain associated with the cell wall in the *srtA* mutant is sufficient to promote the development of the infectious process. Ionic interactions between noncovalently attached surface proteins and other components of the cell wall (such as teichoic and/or lipoteichoic acids) might account for the conservation of invasiveness and virulence. Since we focused here on the sorting defect of the *srtA* mutant with respect to internalin, further analyses will be required to test the specificity of this sortase on the other LPXTG proteins expressed by *L. monocytogenes*. Finally, it is possible than in human infections by the oral route, a sortase-defective mutant could be more severely impaired in virulence.

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