SvpA, a novel surface virulence-associated protein required for intracellular survival of *Listeria monocytogenes*

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A previously unknown protein, designated SvpA (surface virulence-associated protein) and implicated in the virulence of the intracellular pathogen *Listeria monocytogenes*, was identified. This 64 kDa protein, encoded by *svpA*, is both secreted in culture supernatants and surface-exposed, as shown by immunogold labelling of whole bacteria with an anti-SvpA antibody. Analysis of the peptide sequence revealed that SvpA contains a leader peptide, a predicted C-terminal transmembrane region and a positively charged tail resembling that of the surface protein ActA, suggesting that SvpA might partially reassociate with the bacterial surface by its C-terminal membrane anchor. An allelic mutant was constructed by disrupting *svpA* in the wild-type strain LO28. The virulence of this mutant was strongly attenuated in the mouse, with a 2 log decrease in the LD₅₀ and restricted bacterial growth in organs as compared to the wild-type strain. This reduced virulence was not related either to a loss of adherence or to a lower expression of known virulence factors, which remained unaffected in the *svpA* mutant. It was caused by a restriction of intracellular growth of mutant bacteria. By following the intracellular behaviour of bacteria within bone-marrow-derived macrophages by confocal and electron microscopy studies, it was found that most *svpA* mutant bacteria remained confined within phagosomes, in contrast to wild-type bacteria which rapidly escaped to the cytoplasm. The regulation of *svpA* was independent of PrfA, the transcriptional activator of virulence genes in *L. monocytogenes*. In fact, SvpA was down-regulated by MecA, ClpC and ClpP, which are highly homologous to proteins of *Bacillus subtilis* forming a regulatory complex controlling the competence state of this saprophyte. The results indicate that: (i) SvpA is a novel factor involved in the virulence of *L. monocytogenes*, promoting bacterial escape from phagosomes of macrophages; (ii) SvpA is, at least partially, associated with the surface of bacteria; and (iii) SvpA is PrfA-independent and controlled by a MecA-dependent regulatory network.

Keywords: microbial pathogenicity, surface protein, bacterial competence

INTRODUCTION

Listeria monocytogenes is a ubiquitous Gram-positive, facultative intracellular bacterium responsible for severe food-borne infections in humans and many animals (Gray & Killinger, 1966). The virulence of this pathogen

is due to its capacity to invade and multiply within host cells, including macrophages, hepatocytes, epithelial and endothelial cells. Early after internalization, bacteria disrupt the phagosomal membrane and access the cytoplasm where they polymerize actin and spread from cell to cell (Gaillard *et al.*, 1987; Tilney & Portnoy, 1989). Each step of the intracellular parasitism is dependent upon the production of virulence factors, including internalin (InlA), listeriolysin O (LLO), phospholipases and ActA, which are controlled by the

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pleiotropic transcriptional activator PrfA (Cossart & Lecuit, 1998).

Like other intracellular pathogens, *L. monocytogenes* is exposed to hostile conditions during intracellular survival, including starvation, low pH, chemical and enzymic stresses and elevated temperature. *L. monocytogenes* has evolved a complex adaptive network to maintain cell viability under stress and ensure persistence and growth both in the environment and occasionally in host tissues. In this network, stress proteins such as Clp (caseinolytic proteins) play a crucial role. Clp possess an ATPase activity and belong to the 100 kDa heat-shock protein (HSP100) Clp family of highly conserved and universal molecular chaperones (Schirmer *et al.*, 1996). They are involved in the folding and assembly of proteins, and in the regulation of ATP-dependent proteolysis, ultimately promoting stress-induced resolubilization of aggregates (Gottesman & Maurizi, 1992; Schirmer *et al.*, 1996; Squires & Squires, 1992). In *Escherichia coli*, ClpP alone degrades peptides less than 7 aa long (Woo *et al.*, 1989). When associated with ClpA or ClpX, ClpP expresses protease activity against specific substrates determined by the ATPase subunit (Gottesman *et al.*, 1998; Larsen & Finley, 1997; Wang *et al.*, 1997). In *L. monocytogenes*, we previously found that two Clp, ClpC and ClpE, play an important role in stress tolerance and *in vivo* intracellular survival (Nair *et al.*, 1999; Rouquette *et al.*, 1996, 1998). We also identified a stress-induced serine protease ClpP, required for growth under stress conditions and playing a crucial role in intracellular survival of *L. monocytogenes* (Gaillot *et al.*, 2000). ClpP modulates the production of LLO under stress conditions (Gaillot *et al.*, 2000).

Clp also play regulatory functions in *Bacillus subtilis*, a genetically related soil Gram-positive bacterium. The *B. subtilis* ClpC protein, which is highly homologous to ClpC of *L. monocytogenes*, negatively controls bacterial competence, a physiological state allowing bacteria to efficiently internalize exogenous DNA. Indeed, ClpC of *B. subtilis* forms a ternary complex with MecA and ComK, leading to the sequestration and degradation of ComK, the key transcriptional activator of competence (Turgay *et al.*, 1998). Recently, we identified a MecA homologue in *L. monocytogenes*. This protein mimics the regulatory function of *B. subtilis* MecA, repressing transcription of *comK*, and subsequently *comG*, when introduced in *B. subtilis* (Borezée *et al.*, 2000a). Although we failed to demonstrate any competence in *L. monocytogenes*, many homologues of late competence gene products and competence regulatory proteins of *B. subtilis* are present in this pathogen (Borezée *et al.*, 2000a). Interestingly, we found that a 64 kDa secreted protein (p64) of *L. monocytogenes* accumulates in the supernatant of *mecA*, *clpC* and *clpP* mutants. These results suggested that MecA of *L. monocytogenes* might belong to a signal transduction network involved in the regulatory processes of this pathogen (Borezée *et al.*, 2000a).

In this work, we studied the role of this secreted protein

by constructing an allelic mutant of the gene encoding this protein. We found that the 64 kDa protein is required for intracellular survival and virulence of *L. monocytogenes*. This protein is a novel PrfA-independent factor implicated in the virulence of *L. monocytogenes*. It was designated SvpA for surface virulence-associated protein, encoded by the gene *svpA*.

METHODS

Bacterial strains, growth conditions and transformation. We used *L. monocytogenes* reference strain LO28 and *E. coli* DH5. All strains were routinely grown in Brain Heart Infusion (BHI) medium. Antibiotics were used at the following concentrations: ampicillin, 100 µg ml⁻¹ for *E. coli*; erythromycin (Em), 8 µg ml⁻¹ for *L. monocytogenes* and 200 µg ml⁻¹ for *E. coli*; kanamycin (Km), 50 µg ml⁻¹; colistin, 10 µg ml⁻¹; nalidixic acid, 50 µg ml⁻¹. Constructs were introduced into *Listeria* strains by conjugation or electroporation as described previously (Poyart *et al.*, 1993). Bacterial growth and phenotype analysis of strains were performed as described by Rouquette *et al.* (1996). The metabolic profiles were determined on an API strip (50 substrates; BioMérieux).

DNA manipulations, RNA extraction and reverse transcription (RT) PCR. Chromosomal DNA, plasmid extraction, electrophoresis, restriction enzyme analysis, hybridizations and amplification by PCR were performed according to standard protocols (Sambrook *et al.*, 1989). DNA sequencing was performed with the ABI-Prism 310 Sequencer (Perkin Elmer). Total RNA was extracted as described by Celli & Trieu-Cuot (1998) from *L. monocytogenes* cultures grown until mid-exponential phase in BHI broth at 37 °C and subjected to RT-PCR, as described previously (Borezée *et al.*, 2000b). The following primers were used to amplify *svpA* and *LiM00807.1* from chromosomal DNA or total RNA of LO28 and the *svpA* mutant strains: *svpa* (5'-CGGGATCCAAGG-GGGATTATAATGAAGAAATTATGG-3') and *svpb* 5'-TCTAGATAACCCCGACCTAACTCGC-3'); 807a (5'-GG-GGCCAAATTTGATCGTA-3') and 807b (5'-GTGCTTC-TGGCGCATTC-3'), respectively.

Construction of an *svpA*-deleted mutant. An *svpA* mutant (LO28 *svpA*Δ*aphA3*) was constructed by deletion of a 79 bp internal fragment of *svpA* (nt 559–638) and insertion of a promoterless *aphA-3* gene conferring resistance to Km (Menard *et al.*, 1993) by double recombination. The deletion/replacement mutant of *svpA* was constructed by inserting a 964 bp *EcoRI*–*Bam*HI LO28 DNA fragment (–397 to +557), an 855 bp *Bam*HI *E. faecalis* DNA fragment carrying *aphA-3* and a 1119 bp *Bam*HI–*Xba*I LO28 DNA fragment (+634 to +1746), between the *EcoRI* and *Xba*I sites of the thermosensitive shuttle vector pAUL-A (Chakraborty *et al.*, 1992) to give plasmid pAUL-*svpA*Δ*aphA3*. Positions are given relative to the translation initiation codon of *svpA*. These three DNA fragments were generated by PCR using the following primers: mut1 (5'-GAATTCGGGCCTATGGGT-TGAAGGGAACGC-3') and mut2 (5'-GGATCCGAAAGA-GTCACAGGTGTTG-3'); km1 (5'-CGGGATCCCCGACTA-ACTAGGAGGAATA-3') and km2 (5'-CGGGATCCCCGG-GTCATTATTCCCTCC-3'); mut3 (5'-GGATCCTGCTTG-GTTGAAAGTG-3') and *svpb*. pAUL-*svpA*Δ*aphA3* was introduced into LO28 by electroporation and transformants were selected for Em resistance at 30 °C. We used a gene replacement procedure described by Chakraborty *et al.* (1992) to obtain an isogenic mutant carrying the disrupted *svpA* gene on the chromosome. The genotype of the mutant was confirmed by PCR sequencing and Southern blotting.

Fig. 1. Schematic representation of the SvpA protein of *L. monocytogenes*. The SvpA peptide sequence contains a signal peptide at the N terminus (N), a large region with a central proline-rich domain and a C-terminal region (C) composed of a transmembrane helix and a positively charged tail. The amino-acid sequence of the C-terminal region is indicated below and its hydrophobic domain is underlined. The amino acid sequence of the proline-rich domain is also indicated; proline residues are in bold.

Overproduction, purification of His₆-SvpA and His₆-MecA, and antibody production. Plasmids pET-14b and pET-20b(+) were used for protein overexpression and purification (Novagen). The *L. monocytogenes* *svpA* and *mecA* genes were amplified by PCR using primers: *svp1* (5'-CATATGAAGA-AATTATGGAAAAAGGCTTAGTAGC-3') and *svp2* (5'-GGATCCTTAACCTCAATCTTTTACGTTTAAATCG-3'), *mec1* (5'-CCATGGAAATTGAACGAATTAATGAGG-3') and *mec2* (5'-GGATCCGAGAAGTGTTTCTAATTTGCTTTAATG-3'), respectively. The *svpA* and *mecA* PCR products were then cloned into the *NdeI/BamHI* sites of plasmid pET-14b and the *NcoI/BamHI* sites of pET20b(+), to generate pET*svpA* and pET*mecA*, respectively. These plasmids were used to transform *E. coli* strain BL21DE3 in which the T7 RNA polymerase gene is under the control of the inducible *lacUV5* promoter. The recombinant strains were grown in LB medium at 37 °C to mid-exponential phase (OD₆₀₀ = 0.7). IPTG (1 mM) was then added and incubation continued for 2 h. The cells were centrifuged, resuspended in 1/50 of the culture volume of PBS, disrupted by sonication and cell debris removed by centrifugation. *E. coli* crude extracts were loaded on a 1 ml Poly-His Protein Purification Resin column (Roche) previously equilibrated with PBS and the His₆-tagged proteins were eluted with an imidazole gradient (10–500 mM). The eluted fractions were subjected to SDS-PAGE as described by Laemmli (1970). Protein concentrations were determined using the Bio-Rad protein assay (Bradford, 1976). Molecular size references markers were obtained from Life Technologies. The His₆-tagged proteins were used for custom antibody production in rabbits (Centre de Production Animale, Olivet, France).

Western blot analysis. Cultures of LO28 and mutant strains in the exponential growth phase were pelleted and supernatants collected and filtered. Supernatant protein extracts were prepared by TCA precipitation as described by Sambrook *et al.* (1989) and further concentrated with ultrafree columns (Millipore). The bacterial pellet was resuspended in 1/20 of the culture volume of Tris (10 mM)/EDTA (1 mM) and sonicated as described by Rouquette *et al.* (1998). Bacterial debris was removed by centrifugation and the resulting supernatant consisted of the cytoplasmic proteins. Supernatant and cytoplasmic protein extracts were analysed by Western blotting as described by Geoffroy *et al.* (1991). The membranes were incubated with rabbit polyclonal antibodies for His₆-SvpA and His₆-MecA, or with mouse mAbs directed against purified LLO, ActA, InlA, InlB, PC-PLC (obtained

from P. Cossart, Institut Pasteur, Paris, France). Anti-rabbit or anti-mouse immunoglobulin-peroxidase conjugates were used for immunodetection (Sigma). Enzymic activity was revealed by the addition of diaminobenzidine tetrahydrochloride (Sigma) supplemented with hydrogen peroxide (0.1 %).

Infection of macrophages. Macrophages were infected at a bacterium/macrophage ratio of 1:1 and 15:1 for growth curves and microscopic studies, respectively. Bone-marrow-derived macrophages from C57/BL6 mice were cultured and infected as previously described (Gaillot *et al.*, 2000). After 15 min of bacterial adherence on ice, macrophages were exposed for 15 min at 37 °C (time 0). The number of intracellular bacteria was estimated in cell lysates at selected intervals (from 0 to 8 h post-infection). Double fluorescence labelling of F-actin and bacteria was performed as described by Kocks *et al.* (1992) using phalloidin coupled to Oregon Green 488 (Molecular Probes) and a rabbit anti-*Listeria* serum (J. Rocourt, Institut Pasteur, Paris, France) revealed with an anti-IgG antibody coupled to Alexa 546 (Molecular Probes). Images were scanned on a Zeiss LSM 510 confocal microscope.

Culture of Caco-2 cells and adhesion assays. The human colon carcinoma Caco-2 cell line was propagated as described by Gaillard & Finlay (1996). All incubations were carried out in a 10% CO₂ atmosphere at 37 °C. Cells were seeded at 10⁵ cells cm⁻² onto 12 mm diameter glass coverslips in 24-well plates. Monolayers were used 24 h after seeding. Bacteria were grown for 16 h in BHI broth, pelleted, washed once and diluted appropriately in DMEM. Cells were inoculated with bacteria at a ratio of 100 bacteria per cell and incubated for 1 h. They were subsequently washed, fixed and processed for immunolabelling as described by Milohanic *et al.* (2000). Coverslips were mounted on slides and examined by fluorescence microscopy. Each assay was carried out in triplicate and repeated twice. Adherent bacteria were counted by examining 50 cells in randomly selected microscope fields.

Processing for electron microscopy. Macrophages were infected at a bacterium/macrophage ratio of 15:1, fixed for 1 h at room temperature and processed as described previously (de Chastellier & Berche, 1994). The percentage of intraphagosomal or intracytoplasmic bacteria was determined at selected intervals post-infection (0, 4, 6 h) for 50–100 different cell profiles (about 100 bacteria were examined per time point). For immunogold labelling, bacteria were grown overnight in BHI broth and processed as described previously

Fig. 2. Growth of *L. monocytogenes* LO28 wild-type (●) or *svpA* mutant (■) in BHI broth at 37 °C. Wild-type or *svpA* bacteria were grown at 37 °C and OD₆₀₀ was measured at various intervals.

(Gaillard *et al.*, 1991). The grids were incubated for 1 h with rabbit anti-*Listeria* or rabbit anti-SvpA antibodies and further incubated with goat anti-rabbit IgG conjugated to 10 nm gold particles.

Mouse virulence assay. Six- to eight-week-old female Swiss mice (Janvier) were inoculated intravenously (i.v.) with various doses of bacteria (Gaillard *et al.*, 1996). Mortality was followed over a 14-d period on groups of five mice. The LD₅₀ was determined by the probit method. Bacterial growth was

followed in organs (spleen and liver) of mice infected i.v. with 8×10^5 bacteria as described by Nair *et al.* (1999).

RESULTS

The *svpA* gene encodes a secreted protein

We previously identified a secreted p64 protein of 569 aa (63.4 kDa) of unknown function (Borezée *et al.*, 2000a), encoded by a gene of 1707 nt designated *svpA* (GenBank accession no. AF282221). The genetic organization of the *svpA* region of LO28 is similar to that of strain EGD-E recently sequenced by the *Listeria* Genome Consortium. *svpA* corresponds to ORF LiM00806.1 of EGD-E and is located upstream of ORF LiM00807.1, separated by a 114 bp non-coding region. There is neither an obvious promoter nor any *prfA* boxes detectable in the upstream region of *svpA*. A transcriptional analysis by Northern blotting failed to detect *svpA* transcripts in the parental strain LO28 and in the *mecA* mutant, presumably due to unstable transcripts. However, we detected in LO28 specific *svpA* transcripts by RT-PCR using the primers svpa and svpb (data not shown).

As previously reported (Borezée *et al.*, 2000a), the SvpA protein is identical to the product of ORF LiM00806.1 of strain EGD-E from the *Listeria* genome project. SvpA shares several repeated homologies with an ORF of 82 aa encoded by the virulence plasmid pXO1 from *Bacillus anthracis* (~33% identity). Analysis of the peptide sequence of SvpA revealed three distinct regions: a peptide leader sequence with a predicted signal peptidase cleavage site between residues 28 and 29, a large region containing a proline-rich domain (residues 316 to 348), forming a predicted strong secondary structure and a stretch of 19 hydrophobic amino acids, presumed to

Fig. 3. Immunogold labelling of wild-type strain (a and c) and *svpA* mutant strain (b and d), using an anti-*Listeria* serum (a and b) or an anti-SvpA serum (c and d). In these representative pictures SvpA was detected on the surface of wild-type bacteria, but *svpA* mutant bacteria were not significantly labelled.

Fig. 4. Growth of *L. monocytogenes* LO28 wild-type (●) or *svpA* mutant (■) in the spleen (a) or the liver (b) of mice inoculated i.v. with $10^{5.6}$ bacteria.

form a transmembrane helix at the C-terminal extremity (residues 545–563) of the protein (Fig. 1). The hydrophobic region is followed by a tail of six residues, most of which are positively charged. The presence of a predicted peptide signal associated with a cleavage site is in accordance with the fact that SvpA is secreted in the culture supernatant. Nevertheless, it is possible that SvpA might reassociate to the bacterial surface by its predicted C-terminal transmembrane region.

Construction and phenotypic analysis of a deleted *svpA* mutant

An allelic mutant was constructed by deletion of an internal fragment of *svpA* and insertion of an *aphA-3* cassette by double-crossover into the chromosomal DNA of strain LO28, using a two-step procedure described by Chakraborty *et al.* (1992). The mutant was

verified by Southern blotting and PCR sequencing of the *svpA* region. A transcriptional analysis was performed in wild-type and *svpA* mutant strains by RT-PCR using specific primers of *svpA* and of the downstream ORF LiM00807.1 mentioned above. In this mutant, the *svpA* transcript was larger in size due to the *aphA-3* cassette insertion. The ORF LiM00807.1 transcript was present in both strains (data not shown), indicating that there is no polar effect due to the *aphA-3* gene insertion in the mutant.

As compared with the parental strain LO28, the phenotypic analysis of the *svpA* mutant did not reveal any difference with respect to microscopic morphology (Gram staining), aspect of colonies, motility at 22 °C, the profiles of 50 metabolic characters using API-CH50, or the haemolytic activity on horse-blood agar plates (data not shown). Ultra-thin sections of bacteria were also examined by electron microscopy, which detected neither morphological difference nor any alteration in the bacterial cell wall (data not shown). Growth of the *svpA* mutant in BHI broth was moderately delayed at 37 °C, reaching a lower bacterial density at the stationary phase as compared to the wild-type strain (Fig. 2).

SvpA is a surface-exposed protein

Although SvpA is detected in culture supernatants, its predicted structure indicates a C-terminal sequence consisting of a 19 aa hydrophobic domain and a positively charged tail. This situation is reminiscent of that of other *L. monocytogenes* proteins, i.e. InlA (Gaillard *et al.*, 1991) or ActA (Domann *et al.*, 1992; Kocks *et al.*, 1992; Lebrun *et al.*, 1996), which are secreted and reassociated with the bacterial surface. We therefore tested the hypothesis that SvpA could be partially exposed on the bacterial surface. Wild-type and *svpA* mutant bacteria were immunolabelled using anti-SvpA serum. Whole bacteria were stained by the immunogold method and examined by electron microscopy. As a control, bacteria were also labelled with an anti-*Listeria* serum, revealing a strong labelling surrounding wild-type and *svpA* mutant bacteria (Fig. 3a, b). With the anti-SvpA serum, wild-type bacteria were efficiently stained (about 102 gold particles per bacterium) (Fig. 3c), whereas a background labelling was seen with the *svpA* mutant (about 25 gold particles per bacterium) as illustrated in Fig. 3(d). We conclude that the secreted SvpA might at least partially associate to the bacterial surface.

As the SvpA protein is present at the surface of *L. monocytogenes*, it might be implicated in the binding to non-professional phagocytic host cells. We tested the role of SvpA in the binding of *L. monocytogenes* to Caco-2 cells, using a method described by Milohanic *et al.* (2000). We did not find any difference in the capacity to adhere to Caco-2 cells between the wild-type (32 ± 11 bacteria per cell islet) and *svpA* mutant strains (36 ± 12 bacteria per cell islet), suggesting that SvpA itself does not play a role in adhesion.

(a)

(b)

(c)

Fig. 5. (a) Western blot analysis of culture supernatant extracts of wild-type and *svpA* mutant *L. monocytogenes* revealed with anti-InlA, anti-InlB, anti-LLO, anti-ActA and anti-PC-PLC antibodies. The expression of the known virulence factors LLO, ActA, PC-PLC, InlA and InlB is not altered in the *svpA* mutant. (b) Western blot analysis of culture supernatant extracts of wild-type, *svpA*, *mecA*, *clpC*, *clpP* and *prfA* mutant *L. monocytogenes* using anti-SvpA antibodies. The expression of SvpA is controlled by MecA, ClpC and ClpP but does not depend on PrfA. (c) Western blot analysis of cytoplasmic extracts of wild-type, *mecA*, *svpA*, *clpC* and *clpP* mutant *L. monocytogenes* revealed with anti-MecA antibodies. The MecA protein of *L. monocytogenes* accumulates in the *clpC* and *clpP* mutants.

SvpA is required for the virulence of *L. monocytogenes*

We studied the role of the secreted SvpA protein in the virulence of *L. monocytogenes*. The LD₅₀ of LO28 and an *svpA* mutant were determined by inoculating (i.v.) Swiss mice with increasing doses of bacteria. The LD₅₀ value of the *svpA* mutant was 10^{7.2} bacteria per mouse, indicating a 2 log decrease compared to the wild-type strain (10⁵). Bacterial survival in organs was monitored over a period of 3 d by following the number of viable bacteria in the spleen and the liver of mice infected i.v. with 10^{5.6} bacteria (Fig. 4). In contrast to the wild-type strain, which rapidly grew in organs until the death of mice, mutant bacteria were progressively eliminated in the liver and the spleen over the 3-d period. The growth of the *svpA* mutant was severely restricted in the spleen and the liver, with a 3–4 log difference compared to the wild-type strain by day 3 post-infection (Fig. 4). We examined the expression of the main virulence factors in supernatants of wild-type and mutant bacteria (InlA, InlB, LLO, ActA, PC-PLC). They were not altered in the *svpA* mutant, as shown by Western blotting analysis with specific anti-sera (Fig. 5a). These results show the

role of SvpA as a novel secreted factor required for virulence of *L. monocytogenes*.

The expression of SvpA is controlled by MecA, ClpC and ClpP, and is PrfA-independent

Using SDS-PAGE analysis, we previously found that SvpA (formerly termed p64) was overexpressed in the supernatants of the *mecA*, *clpP* and *clpC* mutants (Borezée *et al.*, 2000a). This finding was confirmed by Western blotting analysis using a rabbit anti-SvpA serum raised against purified His-tagged SvpA. SvpA was not detected in the supernatant of the *svpA* mutant, as opposed to the wild-type strain, thus confirming that the expression of SvpA is abrogated in the mutant (Fig. 5b). SvpA is overexpressed in the *mecA*, *clpP* and *clpC* mutants (Fig. 5b). In contrast, the expression of SvpA is similar in LO28 and in a *prfA* mutant of LO28, showing that PrfA is not necessary to induce expression of *svpA* (Fig. 5b). We also purified His₆-MecA with the His-tag system and prepared a rabbit anti-MecA serum. The expression of MecA was similar in cytoplasmic extracts of LO28 and of the *svpA* mutant (Fig. 5c), showing that SvpA does not regulate MecA. On the other hand, MecA

Fig. 6. Growth of *L. monocytogenes* LO28 wild-type (●) or *svpA* mutant (▲) in macrophages. Bone-marrow-derived macrophages from C57/BL6 mice were exposed for 15 min (time 0) to bacteria (1 bacterium per cell) and bacterial survival was followed for 8 h post-infection.

accumulated in the *clpC* and *clpP* mutants (Fig. 5c). This result is consistent with the apparent control of the *B. subtilis* MecA level by the ClpC/ClpP protease complex (Msadek *et al.*, 1998; Turgay *et al.*, 1998).

One might expect that the overexpression of SvpA could exacerbate the level of virulence of *L. monocytogenes*. So, we compared the level of virulence of LO28 with that of the *mecA* mutant. No significant difference was found, as determined by LD₅₀ and the growth curves in the spleen and liver over a 3 d period as described above (data not shown). Conversely, we also tested the virulence of LO28 transformed with a multicopy plasmid harbouring *mecA*, which down-regulates the expression of SvpA. The virulence was attenuated in this strain as shown by the LD₅₀ value estimated at 10⁷ per mouse as compared to 10⁵ for the wild-type strain. Altogether, these results indicate that: (i) SvpA is down-regulated by MecA, ClpC and ClpP; (ii) SvpA is not controlled by the transcriptional activator PrfA controlling virulence genes; and (iii) the level of MecA is also controlled by ClpC and ClpP.

SvpA is required for intracellular growth in macrophages

The behaviour of the *svpA* mutant and LO28 were studied in bone-marrow-derived macrophages from C57/BL6 mice. Macrophages were exposed to a bacterium/cell ratio of 1:1. As shown in Fig. 6, a similar

amount of bacteria associated with macrophages was found at the onset of infection, suggesting that SvpA is not involved in the uptake by macrophages. After a latent phase of 2 h, wild-type bacteria rapidly multiplied inside macrophages, inducing cellular lysis 6 h post-infection. In contrast, *svpA* mutant bacteria were partially killed in the first 2 h post-infection (decrease of 0.5 log), suggesting that the *svpA* mutant might be more susceptible to the bactericidal activity of phagosomes than wild-type bacteria. Surviving bacteria grew slowly until 6 h post-infection. Then, the number of viable bacteria remained stationary, without significant lysis of macrophages. These results indicate that SvpA plays an important role in the intracellular survival of *L. monocytogenes*.

SvpA facilitates the bacterial escape from phagosomes of macrophages

The intracellular fate of wild-type and *svpA* mutant bacteria was then studied by confocal microscopy in bone-marrow-derived macrophages (bacterium/cell 15:1). Infected cells were examined at various intervals (0, 4 and 8 h) after double staining with an anti-*Listeria* antibody and with β -phalloidin to visualize F-actin. As found above, the early bacterial uptake (time 0) was similar for the wild-type and mutant bacteria (Fig. 7a and d). At 4 and 8 h post-infection, most wild-type bacteria were associated with polymerized actin, either surrounded by sheaths of actin, or, more often, forming comets tails (Fig. 7b and c). In contrast, at 4 h post-infection, most mutant bacteria were apparently confined within phagosomes, with few bacteria associated with polymerized actin and comets (Fig. 7e). At 8 h post-infection, the total number of intracellular and intracytoplasmic mutant bacteria associated with actin or comets slowly increased, but remained much lower than those seen with wild-type bacteria (Fig. 7c and f).

We performed a quantitative electron microscopic study on macrophages infected by LO28 or the *svpA* mutant under the conditions described above (see Methods). Intracytoplasmic wild-type bacteria reached 69 and 92 % of the total number of intracellular bacteria 4 and 6 h post-infection, respectively. In contrast, only 22 and 41 % of *svpA* mutant bacteria were visible inside the cytoplasm 4 and 6 h post-infection. Typical aspects of intracellular wild-type and mutant bacteria 4 and 6 h post-infection are illustrated in Fig. 8(a–f). As expected, many wild-type bacteria escaped from the phagosomes at 4 h post-infection (Fig. 8a) and most bacteria were located inside the cytoplasm and surrounded with polymerized actin after 6 h (Fig. 8c, e). In contrast, a majority of *svpA* mutant bacteria remained confined in phagosomes where they were progressively destroyed (Fig. 8b, d, f). Rare bacteria surrounded by polymerized actin (Fig. 8b, d) and actin comets (Fig. 8d) were seen for the mutant 4 and 6 h post-infection, respectively. These results indicate that SvpA promotes the phagosomal escape of intracellular bacteria and the subsequent access to the cytoplasm of macrophages.

Fig. 7. Confocal microscopy of bone-marrow-derived mouse macrophages infected (15 bacteria per cell) with LO28 (a–c) or *svpA* mutant (d–f). Macrophages were observed at 0 (a, d), 4 (b, e) and 8 h (c, f) post-infection. F-actin was stained with phalloidin (green). Bacteria were labelled with an anti-*Listeria* serum (red). The majority of *svpA* mutant bacteria are not associated with actin and the intracellular growth is restricted.

DISCUSSION

In this study we identified a novel secreted protein, SvpA, involved in the virulence of *L. monocytogenes*. The role of this protein was studied by constructing an *svpA*-deleted mutant. The *in vitro* phenotypic analysis of this mutant revealed that it closely resembles the wild-type strain, except that its growth was impaired at 37 °C (Fig. 2). The most important finding is that the virulence of a deleted *svpA* mutant is strongly attenuated with a 2 log drop of the LD₅₀ value ($10^{7.2}$ versus 10^5). This loss of virulence was due to the restriction of bacterial growth in the spleen and liver of infected mice receiving a lethal challenge. We showed that SvpA plays an important role in the intracellular survival of *L. monocytogenes*. Mutant bacteria remained confined in phagosomes of bone-marrow macrophages, in contrast to wild-type bacteria, which rapidly escaped from phagosomes. This longer exposure to bactericidal activity of phagosomes in macrophage might explain that mutant bacteria were partially killed in the first 2 h post-infection, as compared to wild-type bacteria (Fig. 6). As a result, the intracellular growth was reduced in the mutant, with few bacteria escaping the phagosomes and multiplying in the cytoplasm of macrophages, without significant cellular lysis 6 h post-infection (Fig. 6). By confocal microscopy (Fig. 7) and quantitative electron micro-

scopy, we confirmed that SvpA facilitates the bacterial escape from phagosomes since only 41% of mutants were seen in the cytoplasm 6 h post-infection, compared to 92% for wild-type bacteria. Many mutant bacteria remained confined in phagosomes (Fig. 8). These results indicate that SvpA promotes intracellular survival by facilitating the phagosomal escape of bacteria in macrophages.

Although the 64 kDa SvpA is released in the culture supernatant (Fig. 5), this protein is also associated and exposed to the bacterial surface, as demonstrated by immunogold labelling (Fig. 3). However, SvpA is apparently not involved in bacterial adhesion to bone-marrow macrophages and Caco-2 cells, since an *svpA* mutant was efficiently internalized by macrophages and adhered normally to Caco-2 cells. The SvpA protein might be anchored to the bacterial membrane through its C-terminal hydrophobic region. Indeed, analysis of the peptide sequence revealed that SvpA possesses a peptide leader sequence, a large region containing a proline-rich domain and a predicted transmembrane segment followed by a short, positively charged tail at the C terminus (Fig. 1). This structure has similarity with some domains of ActA and InlA. Interestingly, these proteins are secreted and reassociated with the surface by their C-terminal domain. The hydrophobic

Fig. 8. Distribution of *L. monocytogenes* within macrophages. Bone-marrow-derived mouse macrophages were infected with the wild-type (a, c, e) or *svpA* mutant (b, d, f) strains and processed for electron microscopy at selected times post-infection (a–f). Ultra-thin sections of macrophages 4 (a, b) and 6 h (c–f) post-infection. Arrows indicate intracytoplasmic bacteria. About 70 and 90 % of wild-type bacteria are free in the cytoplasm 4 (a) and 6 h (c, e) post-infection, respectively. Cytoplasmic bacteria are surrounded by actin sheets and many of them by a double membrane, a result typical of cell-to-cell spreading in adjacent cells. In contrast, the majority of *svpA* mutant bacteria are enclosed within phagosomes (b, d, f). The small fraction of mutant bacteria accessing the cytoplasm could polymerize actin, as shown by an actin sheet (b, d) and a typical actin comet (d).

region of InlA is preceded by the consensus motif LPXTG, required for the covalent anchoring of the protein to the cell wall (Lebrun *et al.*, 1996). Nevertheless, surface proteins from Gram-positive bacteria exist which are not covalently anchored to the cell wall.

This is the case for ActA, a virulence factor involved in actin assembly and intracellular movement, which possesses a hydrophobic domain of 22 residues followed by 4 charged residues, without the LPXTG motif (Kocks *et al.*, 1992). This surface protein is assumed to be

anchored to the cytoplasmic membrane via its C-terminal transmembrane helix (Chakraborty, 1999). Similarly, SvpA could be tethered to bacteria without an LPXTG motif. Another interesting feature is that SvpA also possesses a proline-rich region of 32 aa. ActA also contains a central domain with four proline-rich repeats involved in the direct binding to cytoskeletal components of infected cells (Chakraborty, 1999; Niebuhr *et al.*, 1997). It is known that these proline-rich motifs are often involved in protein-protein interactions (Kay *et al.*, 2000). Although the significance of the proline-rich domain of SvpA is unknown, it is predicted to form a secondary structure that might interact with host-cell components during the intracellular parasitism of *L. monocytogenes*. Since SvpA presumably acts inside phagosomes, one could speculate that SvpA might interact with the components of the phagosomal membrane to promote bacterial escape from this cellular compartment. However, the function of SvpA remains unknown.

By studying the regulation of SvpA, we found that it is not controlled by the transcriptional activator controlling virulence genes, PrfA: a *prfA* mutant produced the same amount of SvpA in culture supernatants as that of wild-type bacteria (Fig. 5b). On the other hand, SvpA was down-regulated by MecA, ClpC and ClpP. Using an antiserum raised against purified SvpA, we found by Western blotting analysis that SvpA accumulates in the culture supernatants of *mecA*, *clpC* and *clpP* mutants, as compared to the wild-type bacteria (Fig. 5b). SvpA might be part of a MecA-dependent regulatory network proposed previously (Borezée *et al.*, 2000a). We previously found by heterologous complementation in *B. subtilis* that MecA of *L. monocytogenes* mimics the regulatory function of the *B. subtilis* MecA protein, presumably forming a complex with ClpC and ClpP to sequester and degrade ComK, a transcriptional activator of the late competence genes in *B. subtilis* (Turgay *et al.*, 1997, 1998). The regulation of SvpA by MecA might be indirect and the expression of SvpA could be regulated by an unknown factor repressed by the MecA/ClpC/ClpP complex. We also found in the present study that *L. monocytogenes* MecA accumulates in the cytoplasm of the *clpC* and *clpP* mutants (Fig. 5c), suggesting that MecA might also be controlled by the ClpC/ClpP protease complex in this pathogen. In *B. subtilis*, MecA acts as a linker protein targeting ComK for proteolysis and is regulated by the same proteolytic complex (Msadek *et al.*, 1998; Turgay *et al.*, 1998). Although no competence could be detected in *L. monocytogenes* (Borezée *et al.*, 2000a; Rouquette *et al.*, 1998), homologues for many competence factors of *B. subtilis* exist in *L. monocytogenes*. The MecA-dependent network of this pathogen might play an important role in bacterial physiology, including the down-regulation of SvpA, a factor implicated in virulence.

In conclusion, our results suggest that SvpA is a novel PrfA-independent factor required for intracellular survival and belongs to a regulatory network involving MecA, ClpC and ClpP. SvpA facilitates bacterial escape

from phagosomes. SvpA might protect intraphagosomal bacteria from bacterial killing, and/or act synergistically with other virulence factors (listeriolysin O, phospholipases) or even directly to promote bacterial escape from phagosomes.

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