Regulation of expression of type I signal peptidases in *Listeria monocytogenes*

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The role of type I signal peptidases (SPases I) is to remove the signal peptides of preproteins exported by the general secretory pathway. The genome of *Listeria monocytogenes* contains a locus encoding three contiguous SPases I (denoted SipX, SipY and SipZ). The authors recently showed that SipX and SipZ perform distinct functions in protein secretion and bacterial pathogenicity. Here, the regulation of *sip* gene expression in broth and in infected eukaryotic cells was studied. The results show that expression of the three *sip* genes is (i) controlled by two distinct promoter regions that respond differently to growth phase and temperature variations, and (ii) influenced by PrfA (the transcriptional activator regulating most of the virulence genes of *L. monocytogenes*) and the stress proteins ClpC and ClpP. It was found that *sip* gene expression was strongly upregulated upon infection of eukaryotic cells when bacteria were still entrapped in the phagosomal compartment. This upregulation is compatible with the need of *L. monocytogenes* to optimize its production of virulence factors in the early stage of the intracellular cycle.

INTRODUCTION

The Gram-positive bacterium *Listeria monocytogenes* is a food-borne pathogen that is widespread in the environment, where it survives hostile conditions, presumably due to the adaptation of various regulatory mechanisms. *L. monocytogenes* is a facultative intracellular bacterial pathogen that causes serious disease in immunocompromised individuals, pregnant women and neonates. Its virulence is due to a capacity to invade and multiply within a wide variety of mammalian cells, including macrophages, hepatocytes, epithelial and endothelial cells (Vazquez-Boland *et al.*, 2001). The molecular mechanisms of this intracellular parasitism have been investigated extensively, and these studies have revealed the crucial role of several secreted proteins, such as listeriolysin O (LLO) and phospholipases, and of surface-exposed proteins, such as internalin A (InlA) and InlB, in the virulence of this pathogen (Cossart, 2004; Dussurget *et al.*, 2004). To date, most of the proteins shown to be directly involved in the virulence of *L. monocytogenes* are either secreted or cell-wall associated.

Bacterial proteins that are exported from the cytoplasm through the general secretory pathway (Sec machinery) are synthesized as precursors with an amino-terminal signal peptide (Pugsley & Possot, 1993). The signal peptide is

required for the targeting of precursor proteins to the cytoplasmic membrane, and for the initiation of their translocation across this membrane. During, or shortly after, the translocation process, most signal peptides are removed by signal peptidases (SPases), which is a prerequisite for the release of secretory proteins from the extracytoplasmic side of the membrane (Paetzel *et al.*, 2000). Thus, SPases play a key role in the transport of protein across membranes in all living organisms (van Wely *et al.*, 2001). In Gram-positive bacteria, the exported proteins are either secreted into the medium, or they remain associated by various means with the bacterial envelope. *In silico* analysis of the genome of *L. monocytogenes* EGD-e revealed that more than 5 % of the coding sequences carry a typical signal peptide cleavable by type I SPases (SPases I) (Cabanes *et al.*, 2002; Glaser *et al.*, 2001).

We have recently reported the identification of a locus containing three contiguous genes encoding three SPases I in the genome of *L. monocytogenes* (Bonnemain *et al.*, 2004). The construction of single and multiple knockout mutants in the *sip* genes showed that SipX and SipZ perform distinct functions in pathogenicity, and that SipZ is the major SPase I of *L. monocytogenes*. Most biological membranes contain one or two SPases I for the removal of signal peptides from the secretory precursor proteins. In this respect, the Gram-positive bacteria *L. monocytogenes* and *Bacillus subtilis* seem to be exceptional, since they possess three and five chromosomally encoded SPases I, respectively (van

Abbreviations: BMM, bone-marrow-derived macrophages; LLO, listeriolysin O; PI-PLC, phosphatidylinositol-specific phospholipase C; SPase, signal peptidase; SPases I, type I signal peptidases.

Roosmalen *et al.*, 2001). To date, the largest number of SPases I has been found in *Bacillus anthracis* and *Bacillus cereus*, which contain six and seven paralogous enzymes, respectively (see van Roosmalen *et al.*, 2004 for a recent review). The unique SPase I of *Escherichia coli*, also known as leader peptidase (Lep), is essential for cell viability, and SPase I limitation results in accumulation of precursors of exported proteins. Similarly, the SPases I SpsB, from *Staphylococcus aureus*, and Sec11p, of the yeast endoplasmic reticulum membrane, are enzymes that are essential for cell viability. In contrast, the SPase I SipS of *B. subtilis* is not essential for cell viability, and mutant *B. subtilis* strains with a disrupted *sipS* gene are still able to process secreted pre-proteins (Bolhuis *et al.*, 1999). This observation seems to indicate that multiple SPases I serve to guarantee a sufficient capacity for protein secretion under various conditions (Tjalsma *et al.*, 1997).

In the present work, we studied the regulation of *L. monocytogenes sip* gene expression in broth and in infected eukaryotic cells. We show that the three *sip* genes are under the control of two distinct promoter regions. Our data reveal that the regulation of *sip* gene expression is temporally controlled, and influenced by complex global regulatory circuits.

METHODS

Bacterial strains, plasmids and growth conditions. We used the reference strain *L. monocytogenes* EGD-e belonging to serovar 1/2a (Glaser *et al.*, 2001), and the strain *L. monocytogenes* L028, which was isolated from a healthy pregnant woman (provided by P. Cossart, Institut Pasteur, Paris, France). *E. coli* strain DH5 α was used for all plasmid constructions prior to their introduction into *L. monocytogenes*. *E. coli* was grown in Luria-Bertani (LB) medium; *L. monocytogenes* was grown in BHI medium or RPMI synthetic medium.

Real-time quantitative PCR. Total RNA isolated from cultures of *L. monocytogenes* grown in two different conditions was converted into DNA with reverse transcriptase, and quantitative PCR using sets of primers from different genes was performed to evaluate the relative levels of expression. Quantification of expression level was performed on EGD-e grown in BHI at 37 °C. We also compared EGD-e growing inside cells [bone-marrow-derived macrophages (BMM) from BALB/c mice and human enterocyte Caco-2 cells] with EGD-e growing in RPMI medium. For RNA preparations, cells were broken in a solution of 1 ml Trizol (Invitrogen) containing mini glass beads using a Bead Beater (Savant). Total RNA was extracted with 300 μ l chloroform/isoamyl alcohol (24:1, v/v), then precipitated with 2-propanol, and resuspended in diethyl-pyrocyanate-treated water. Contaminating DNA was removed by digestion with DNase I.

The absence of contaminating DNA after DNase treatment was confirmed by including a control reaction without reverse transcriptase in the RT-PCR assay (data not shown).

RT-PCR experiments were done with 1 μ g RNA, with 2.5 pmol specific primers for the genes *gyrA* (gyrase, constitutively expressed), and *sipX*, *sipY* and *sipZ*, in a volume of 8 μ l. After denaturation at 65 °C for 10 min, 12 μ l of the mixture containing 2 μ l dNTP (25 mM), 4 μ l 4 $\times$ buffer (provided with the Superscript II), 2 μ l DTT, 1 μ l RNasin

(Promega), and 1.5 μ l Superscript II (Invitrogen), was added. Samples were incubated for 60 min at 42 °C, heated at 75 °C for 15 min, and then chilled on ice. PCR conditions were identical for all reactions. The 25 μ l reactions, performed in sealed tubes, consisted of 12.5 μ l PCR master mix (PE Applied Biosystems) containing Sybr Green, 4 μ l template, and 5 pmol each primer. For real-time quantitative PCR, we used the ABI Prism 7700 sequence detection system with TaqMan Universal PCR master mix (PE Applied Biosystems).

For the infection experiments, three different infections were performed, and each point corresponds to three measurements. The human colon carcinoma cell line Caco-2 (ATCC HTB37) was propagated in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10 % fetal bovine serum at 37 °C with 5 % CO₂. Cells were seeded at approximately 2×10^5 cells ml⁻¹ in tissue culture plates. Monolayers were used 24 h after seeding. Cells were infected with bacteria grown at 37 °C overnight at a multiplicity of 50 bacteria per cell. In experiments involving Caco-2 cells, the induction was measured in infected cells cultivated in RPMI, as compared with *L. monocytogenes* grown in RPMI.

Data analyses. Results were normalized to the amount of gyrase mRNA, which was constant under our growth conditions (Réglier-Poupet *et al.*, 2003). For example, to compare *sipZ* gene expression at pH 7 and 5, for each experiment (carried out three times) we calculated the ratio: (*sipZ* pH 5/*gyrA* pH 5)/(*sipZ* pH 7/*gyrA* pH 7), and then we calculated the means and standard deviations of the three values obtained.

β -Galactosidase assays. We used pTCV-lac, a mobilizable shuttle vector that enables transcriptional fusion to β -galactosidase in a wide range of Gram-positive bacteria, in which it replicates at a low copy number per bacterium (Poyart & Trieu-Cuot, 1997). All oligonucleotides were designed to add *EcoRI* and *BamHI* sites upstream and downstream of the amplified potential promoters (Table 1). The amplified fragments were digested with *EcoRI* and *BamHI*, and cloned into pTCV-lac. Bacteria were grown overnight in BHI broth containing kanamycin. The cultures were then diluted 50-fold in BHI broth supplemented with kanamycin, and cells were collected at different time points during the growth phase. β -Galactosidase was assayed as described by Miller (1972), except that the cells were permeabilized by treatment with 0.5 % toluene and 4.5 % ethanol. The β -galactosidase specific activities, determined in three separate experiments, were expressed as $[10^3 \times (\text{OD}_{420} \text{ of the reaction mixture} - 1.75 \text{ OD}_{550} \text{ of the reaction mixture})] \text{ divided by } [\text{reaction time (min)} \times \text{OD}_{600} \text{ of the quantity of cells used in the assay}]$.

The β -galactosidase assays were performed at least seven times (each time with a new culture). The result given for each experiment is the mean of three measurements.

Gel-mobility-shift DNA-binding assays. *EcoRI/BamHI* DNA fragments, corresponding to the promoter regions of different genes, were generated by PCR, and labelled radioactively by treatment with the Klenow fragment of DNA polymerase I in the presence of a mixture of dGTP, dATP, dTTP (0.08 mM) and [α -³²P]dCTP (40 μ Ci; 1.48 MBq). Binding of protein to DNA was carried out in a 20 μ l reaction mixture containing ³²P-labelled DNA, 1 μ g poly(dI-dC) (Pharmacia), 25 mM sodium phosphate (pH 7), 150 mM NaCl, 0.1 mM EDTA, 2 mM MgSO₄, 1 mM DTT, 10 % (v/v) glycerol. The DNA-binding reaction was initiated by the addition of total crude protein, and incubated at room temperature for 30 min. Samples were then loaded directly onto an SDS-6 % polyacrylamide gel (50 mM Tris, 400 mM glycine, 1.73 mM EDTA) for electrophoresis (10 V cm⁻¹). After overnight electrophoresis, the gels were dried, and scanned with a Molecular Dynamics PhosphorImager. The autoradiographs shown correspond to a 24 h exposure.

Table 1. Strains, plasmids and primers

Strain, plasmid or primer	Characteristics	Reference
<i>L. monocytogenes</i>		
EGD-e	Reference strain, serovar 1/2a	Glaser <i>et al.</i> (2001)
EGD-e P _{sipX}	EGD P _{sipX} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
EGD-e P _{sipZ}	EGD P _{sipZ} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
L028	Wild-type	Laboratory collection
L028 P _{sipX}	L028 P _{sipX} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
L028 P _{sipZ}	L028 P _{sipZ} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ clpC	L028 (<i>clpC</i> ::Tn917)	Nair <i>et al.</i> (2000)
Δ clpC-P _{sipX}	L028 Δ clpC P _{sipX} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ clpC-P _{sipZ}	L028 Δ clpC P _{sipZ} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ clpP	L028 (<i>clpP</i> :: <i>aphA</i> -3'), Km ^R	Gaillot <i>et al.</i> (2000)
Δ clpP-P _{sipX}	L028 Δ clpP P _{sipX} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ clpP-P _{sipZ}	L028 Δ clpP P _{sipZ} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ mecA	L028 (<i>mecA</i> :: <i>aphA</i> -3'), Km ^R	Borezee <i>et al.</i> (2000)
Δ mecA-P _{sipX}	L028 Δ mecA P _{sipX} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ mecA-P _{sipZ}	L028 Δ mecA P _{sipZ} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ prfA	L028 Δ prfA	Sheehan <i>et al.</i> (1996)
Δ prfA-P _{sipX}	L028 Δ prfA P _{sipX} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Δ prfA-P _{sipZ}	L028 Δ prfA P _{sipZ} - <i>lacZ</i> fusion, Km ^R Em ^R	This study
Plasmids		
pTCVlac	Replicative plasmid for constructing transcriptional fusions with <i>lacZ</i> gene carrying <i>erm</i> and <i>aphA</i> -3' genes	Poyart & Trieu-Cuot (1997)
pTCVlac-P _{sipX}	pTCVlac derivative carrying a P _{sipX} - <i>lacZ</i> fusion	This study
pTCVlac-P _{sipY}	pTCVlac derivative carrying a P _{sipY} - <i>lacZ</i> fusion	This study
pTCVlac-P _{sipZ}	pTCVlac derivative carrying a P _{sipZ} - <i>lacZ</i> fusion	This study
Primers		
P _{sipX} forward	5'-CCGGAATTCAGCAGCTCGTGGTGAAGAAGA-3'	
P _{sipX} reverse	5'-CGCGGATCCTTATTTTATCCATCCAAATGCC-3'	
P _{sipY} forward	5'-CCGGAATTCGGCAGACATCCAACTCTGA-3'	
P _{sipY} reverse	5'-CGCGGATCCTAATAATTGATGCGCCCGCT-3'	
P _{sipZ} forward	5'-CCGGAATTCTCGGAGATAATCGCGCGGGA-3'	
P _{sipZ} reverse	5'-CGCGGATCCCGCCCAATCCATGACCATAACC-3'	
Gyr-F (TaqMan)	5'-AAATGCGGACATCATTCTAGACT-3'	
Gyr-R (TaqMan)	5'-TTTAACCCGTCACGAACATCAG-3'	
SipX-F (TaqMan)	5'-CGAAGCGTTTTGACATTATTGTATTT-3'	
SipX-R (TaqMan)	5'-AAGGCCAATGACGCGTTTAA-3'	
SipY-F (TaqMan)	5'-AAGTACTGTTCCAAAAGGGAACTGT-3'	
SipY-R (TaqMan)	5'-AATCCGAAAACACGACTATCTGAACT-3'	
SipZ-F (TaqMan)	5'-GAGCAGCAAAGACAGTCGGAT-3'	
SipZ-R (TaqMan)	5'-TCGGCCAGTAACAAATCGG-3'	

RESULTS

The *sip* genes are under the control of two distinct promoters

The *sip* genes belong to a cluster of five genes (Fig. 1a). Two major transcripts were previously identified by Northern blot analysis (Bonnemain *et al.*, 2004): a large one compatible with a transcript covering the entire locus (*sipX*-*lmo1273*), and a shorter one compatible with a transcription starting upstream of *sipZ* (*sipZ*-*lmo1273*). Here, we decided

to evaluate quantitatively the potential promoter activities of the regions preceding each *sip* gene. For this, we inserted a 200 bp DNA fragment, corresponding to the region immediately upstream of each *sip* coding sequence (denoted P_{sipX}, P_{sipY} and P_{sipZ}), upstream of the reporter *lacZ* gene carried on a Gram-negative/Gram-positive shuttle plasmid (pTCV-lac derivatives, see Methods). The recombinant plasmids were transferred into *L. monocytogenes*, and P_{sip}-dependent β -galactosidase expression was monitored in BHI at different stages of bacterial growth, and in different growth conditions. We used the promoter of the

Fig. 1. (a) Schematic representation of the *sip* locus of *L. monocytogenes*. Putative transcription terminators are indicated (black, strong terminator; grey, weak terminator). Grey arrows indicate the potential promoter fragment upstream of each *sip* gene. (b) Quantification of promoter activity. Expression of β -galactosidase (β -gal) activity for P_{sipX} ($\diamond$), P_{sipY} ($\square$), P_{sipZ} ($\triangle$) and $P_{aphA-3'}$ ($\circ$; positive control) in strain EGD-e growing in LB medium at 37 °C. Bacteria were removed at different time points for measurement of growth (OD₆₀₀) ($\blacktriangle$) and β -galactosidase activity.

aph3' gene as a positive control. Two regions, P_{sipX} and P_{sipZ} , promoted β -galactosidase expression, indicating promoter activity (Fig. 1b). In contrast, no promoter activity was recorded in the fragment upstream of *sipY*, strongly suggesting that *sipY* is transcribed from P_{sipX} (P_{sipY} was used as a negative control in the remaining experiments). In broth, P_{sipX} activity remained relatively constant throughout the entire period of growth, while that of P_{sipZ} reached a peak after 1 h, when cultures were not yet in the exponential phase of growth, and then decreased sharply after 1 h 30 min, which was the beginning of the exponential phase. The maximal β -galactosidase activity recorded with P_{sipZ} was similar to that recorded with P_{sipX} . Such differences in promoter activities have also been observed in *B. subtilis*, where *sipU* and *sipV* are constitutively transcribed, whereas *sipS* and *sipT* are temporally controlled (Tjalsma *et al.*, 1997).

sip promoter activities are influenced by environmental conditions

We monitored P_{sipX} and P_{sipZ} activity at acid and neutral pH values, and at different temperatures. After a preculture at pH 7.2, the bacteria (OD₆₀₀ 0.3) were placed in a new medium at pH 7.2 or 5.5 (bacteria from the initial culture were collected by centrifugation, and resuspended in the same volume of the new medium before inoculation). The β -galactosidase activity was measured at t_0 and 30 min after inoculation. P_{sipX} and P_{sipZ} activities were similar at pH 7.2 and 5.5, indicating that the two major SPases I of *L. monocytogenes* (*sipX* and *sipZ* encoded) are not regulated by acidification (data not shown). We then compared promoter activities at different temperatures (37, 42 and 4 °C, Fig. 2). The activity of P_{sipX} was identical after 30 min at 37 and 42 °C, but increased almost threefold after 30 min growth at 4 °C (Fig. 2a). In contrast, P_{sipZ} activity decreased twofold at 37 °C, was unchanged at 4 °C, and increased weakly at 42 °C (Fig. 2b). We quantified by real-time PCR the expression of *sipX* and *sipZ* at 42 and 4 °C, as compared with 37 °C. The values recorded confirmed the results of

the β -galactosidase assays. Transcription of *sipX* increased at 4 °C, whereas that of *sipZ* increased at 42 °C (data not shown), revealing that, P_{sipX} and P_{sipZ} respond differently to temperature variations.

Pleiotropic regulators modulate the expression of SPase I proteins

Previous studies with *B. subtilis* suggested regulation of SPase I genes by a ClpXP-dependent activator (Pummi *et al.*, 2002). In *L. monocytogenes*, the proteases ClpP and ClpC play an essential role in virulence: ClpP is involved in the rapid adaptative response of the bacterium during the

Fig. 2. Effect of temperature on *sip* expression. (a) P_{sipX} and (b) P_{sipZ} at 4, 37 and 42 °C. Cells were grown in LB medium at 37 °C. At an OD₆₀₀ of 0.3, they were placed at different temperatures. Then β -galactosidase activity was measured at t_0 (black bars) and after 30 min (grey bars). The analysis was carried out three times, and the mean values (± 1 SD) are indicated.

infectious process, and a mutant lacking ClpP is avirulent in the mouse model (Gaillot *et al.*, 2000); ClpC is required for stress tolerance, and contributes to early escape from the phagosomal compartment of macrophages, and *in vivo* survival (Nair *et al.*, 2000).

We tested the role of ClpC and ClpP in the expression of the *sip* locus by introducing plasmid pTCV-lac- P_{sipX} or pTCV-lac- P_{sipZ} into the two knockout mutants $\Delta clpC$ and $\Delta clpP$ of *L. monocytogenes*. Since the mutations had been constructed in strain LO28, we used wild-type EGD-e and LO28 strains as controls. The activities of P_{sipX} and P_{sipZ} were essentially identical in EGD-e and LO28 (approx. 40 β -galactosidase units). The promoter activities of P_{sipX} and P_{sipZ} were three- to fourfold higher in the *clpC* and *clpP* mutants (174 ± 2 and 162 ± 2.6 β -galactosidase units for P_{sipX} in the *clpC* and *clpP* mutants, respectively; 158 ± 0.3 and 167 ± 3 β -galactosidase units for P_{sipZ} in the *clpC* and *clpP* mutants, respectively) compared with the wild-type strains. These results suggest that, as in *B. subtilis*, expression of ClpC and ClpP downregulates transcription of the *sip* genes.

PrfA, the central virulence regulator of *L. monocytogenes*, has been shown to exert a negative control on ClpC expression at the transcriptional level, reflecting the existence of crosstalk between these two systems (Ripio *et al.*, 1998). Moreover, in *B. subtilis*, ClpC controls the expression of competence by forming a complex with MecA to negatively regulate ComK (Bockmann *et al.*, 1996). We therefore tested whether MecA and PrfA could also modulate the expression of the *sip* genes, by monitoring P_{sipX} - and P_{sipZ} -dependent *lacZ* transcription in $\Delta prfA$ and $\Delta mecA$ mutant backgrounds. In these two backgrounds, the activity of both P_{sipX}

and P_{sipZ} was two- to threefold lower than in the wild-type strains (approx. 20 and 10 β -galactosidase units for P_{sipX} in the *mecA* and *prfA* mutants, respectively; approx. 15 and 10 β -galactosidase units for P_{sipZ} in the *mecA* and *prfA* mutants, respectively). Thus, while ClpC and ClpP are involved in the negative regulation of *sip* gene expression, PrfA and MecA are involved in the positive regulation of their expression.

Proteins bind specifically to the promoter regions P_{sipX} and P_{sipZ}

We performed different gel-mobility-shift DNA-binding assays to visualize the binding of proteins to the promoter regions P_{sipX} and P_{sipZ} . First, we used total protein extract from *L. monocytogenes* cells in DNA-binding assays, with 200 bp double-stranded DNA fragments corresponding to P_{sipX} and P_{sipZ} . All the assays were performed in the presence of an excess of non-specific competitor DNA [1 μ g poly(dI-dC)]. Radiolabelled probes corresponding to P_{sipX} and P_{sipZ} were incubated with increasing amounts of total protein extract. The displacement of radiolabelled fragment was proportional to the amount of protein extract added, and was complete at a concentration of 15 μ g total protein (Fig. 3). The same experiment was performed with a radiolabelled probe corresponding to P_{sipY} (a region showing no promoter activity in the previous experiment), and no displacement of radiolabelled fragment among the amount of protein extract was observed (data not shown). Cold competitor chase experiments (as described by Chastanet *et al.*, 2003) were used to demonstrate the specificity of protein binding. The addition of increasing amounts of unlabelled DNA fragments corresponding to P_{sipX} or P_{sipZ} efficiently prevented binding of the radiolabelled probes (Fig. 3a, b). These results indicate that one or several

Fig. 3. Gel-mobility-shift DNA-binding assays. (a) Assays were performed with 0.5 pmol 32 P-labelled P_{sipX} DNA fragment. Lanes: 1, no protein; 2–4, increasing amounts of total protein extract from strain LO28 (2, 5 and 15 μ g in lanes 2, 3 and 4, respectively); 5–8, 15 μ g protein and increasing amounts of unlabelled P_{sipX} DNA fragment (0.1, 0.5, 1 and 2 pmol in lanes 5, 6, 7 and 8, respectively). (b) Assays were performed with 0.5 pmol labelled P_{sipZ} fragment. Lanes: 1, no protein; 2 and 3, increasing amounts of total protein extract from LO28 strain, (5 and 15 μ g in lanes 2 and 3, respectively); 4–6, 15 μ g protein and increasing amounts of unlabelled P_{sipX} DNA fragment (0.1, 0.5 and 2 pmol in lanes 4, 5 and 6, respectively). (c) Assays were performed with 0.5 pmol labelled P_{sipX} fragment. Lanes: 1, no protein; 2–5, 15 μ g total protein extracts from mutant strains $\Delta clpC$, $\Delta clpP$, $\Delta mecA$ and $\Delta prfA$ (lanes 2, 3, 4 and 5, respectively).

proteins bind directly to the promoter regions of *sipX* and *sipZ*.

To determine whether ClpC, ClpP, PrfA and MecA would influence *sip* expression, either by direct binding, or indirectly by modulating the binding of other proteins, to P_{sipX} and P_{sipZ} , the same experiment was performed using total protein extracts from $\Delta prfA$, $\Delta mecA$, $\Delta clpC$ and $\Delta clpP$ isogenic mutant strains. With all the extracts, a similar displacement of the radiolabelled probe was observed (Fig. 3c). Thus, the role of PrfA, MecA, ClpC and ClpP on *sip* gene expression is indirect, and is most likely to involve complex regulatory circuits.

These preliminary observations will need to be followed up with additional experiments to define the actual regulatory mechanisms involved.

Expression of the *sip* genes is upregulated in infected cells

We monitored quantitatively the transcription of each *sip* gene by real-time PCR, upon infection by *L. monocytogenes* of either mouse bone marrow-derived macrophages (BMM) or the human enterocyte Caco-2 cell line (Fig. 4). In both cell types, an increase in the transcription of the three *sip* genes was observed shortly after infection. In mouse BMM (Fig. 4a), a sevenfold induction of *sipZ* was recorded after 30 min of infection; induction levels of *sipX* and *sipY* were similar to each other, and slightly lower (approximately

fivefold induction). In Caco-2 cells (Fig. 4b), significant induction of *sip* gene transcription was also observed after 30 min of infection (approximately fivefold induction of *sipZ*, and three- to fourfold induction of *sipX* and *sipY*). Our electron microscopy observations suggest that, at this early stage of the infection, the majority of bacteria are still localized inside phagosomes (data not shown; Réglier-Poupet *et al.*, 2003). In contrast, after 1 h of infection, about 65 % of the bacteria were surrounded by a meshwork of polymerized actin. After 1 h of infection, the induction of each of the three *sip* genes decreased in the two cell types, but was still about twofold higher than that recorded in broth. These assays suggest that *sip* gene expression might be preferentially upregulated during the intraphagosomal stage of the infectious cycle of *L. monocytogenes*.

DISCUSSION

Regulation of Spase I expression in infected cells

During infection, *L. monocytogenes* modulates its production of virulence factors according to its needs (Bubert *et al.*, 1999). For example, expression of LLO and phosphatidylinositol-specific phospholipase C (PI-PLC) is upregulated in the phagosomal compartment, where these proteins are essential for phagosomal escape; while ActA expression is strongly induced when bacteria have reached the cytosolic compartment of the infected host cell, where the protein is required for actin polymerization and bacterial motility. The upregulation of *sipZ* in the phagosomal compartment is compatible with the necessity to process LLO and PI-PLC efficiently and rapidly in this compartment. In this respect, it is worth recalling that inactivation of *sipZ* impairs the secretion of LLO and the broad-range phospholipase (phosphatidylcholine-specific phospholipase C), restricts intracellular multiplication, and almost abolishes virulence (Bonnemain *et al.*, 2004). The fact that, in broth, acidification of the medium did not affect *sip* expression, suggests that phagosomal acidification is not the signal responsible for the early induction of *sip* expression in cells.

It is tempting to suggest that *L. monocytogenes* has evolved this regulation of SPase I expression as an additional means to control its production of virulence factors. However, it is possible that *L. monocytogenes* simply adapts the synthesis of SPases according to its needs.

PrfA and other factors are involved in the regulation of *sip* gene expression

In agreement with our earlier observations (Bonnemain *et al.*, 2004), we found that the three *sip* genes were regulated by two different promoters, P_{sipX} and P_{sipZ} . Expression of the *sip* genes appeared to be coordinated by complex regulatory signals, including the pleiotropic transcriptional activator PrfA, and stress proteins involved in *L.*

Fig. 4. Induction of *sip* gene expression in mouse BMM and Caco-2 cells. The transcription of *sip* was monitored by real-time quantitative PCR in (a) mouse BMM and (b) Caco-2 cells after 30 min (black bars) and 1 h (grey bars) of infection. The induction ratio is the number of transcripts detected in bacteria in cells during infection divided by the number of transcripts detected in bacteria grown in RPMI medium. Values are expressed relative to the number of *gyrA* transcripts. Errors bars show standard deviations.

monocytogenes pathogenicity. Clp ATPases are believed to form part of post-translation regulatory networks that ensure survival in stress conditions, presumably by acting as molecular chaperones that mediate the repair or scavenging of damaged proteins (Wawrzynow *et al.*, 1996). In *B. subtilis*, Clp ATPases mediate adaptive responses to stress conditions, as well as to many other different processes, including the development of competence, sporulation, exoenzyme synthesis and cell-cycle regulation. Of particular interest, the inactivation of *clp* genes has been shown to upregulate the transcription of three SPase I genes, *sipS*, *sipT* and *sipV*, and of the lipoprotein signal peptidase gene *lsp*, suggesting that Clp proteins control a step in the secretion pathway that is common to both non-lipoproteins and lipoproteins (Pummi *et al.*, 2002).

In *L. monocytogenes*, we found that inactivation of *clpC* or *clpP* increased *sip* gene expression, suggesting that Clp proteins act to downregulate *sip* expression, and we found that inactivation of MecA downregulated *sip* expression, suggesting that MecA could act as both a negative and a positive regulator. We also observed that PrfA influenced *sip* expression, while a large-scale transcriptomic analysis of PrfA-dependent promoters (Milohanic *et al.*, 2003) failed to identify the *sip* genes. Thus, either *sip* gene regulation was below the detection threshold determined by the transcriptomic assay, or, under the studied conditions, the *sip* genes were not regulated.

To date, the interconnection between the PrfA regulon and the regulatory pathways that control stress responses in *L. monocytogenes* is not clearly understood. ClpC expression is negatively controlled at the transcriptional level by the central virulence regulator PrfA. Gel-mobility DNA-binding assays, performed here with total protein extracts devoid of PrfA, ClpC, ClpP or MecA, confirmed the absence of direct action of these proteins on the *sip* promoters, suggesting complex control mechanisms of *sip* gene expression in *L. monocytogenes*.

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