lmo1273, a novel gene involved in *Listeria monocytogenes* virulence

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Listeria monocytogenes is a foodborne pathogen able to infect humans and many other mammalian species, leading to serious, often fatal disease. We have previously identified a five-gene locus in the genome of *L. monocytogenes* EGD-e which comprised three contiguous genes encoding paralogous type I signal peptidases. In the present study, we focused on the two distal genes of the locus (*lmo1272* and *lmo1273*), encoding proteins sharing significant similarities with the YlqF and RnhB proteins, respectively, of *Bacillus subtilis*. *lmo1273* could complement an *Escherichia coli* *rnhA-rnhB* thermosensitive growth phenotype, suggesting that it encodes a functional RNase H. Strikingly, inactivation of *lmo1273* provoked a strong attenuation of virulence in the mouse model, and kinetic studies in infected mice revealed that multiplication of the *lmo1273* mutant in target organs was significantly impaired. However, the mutation did not impair *L. monocytogenes* intracellular multiplication or cell-to-cell spread in cell culture models. Transcriptional profiles obtained with an *lmo1273*-overexpressing strain were compared to those of the wild-type strain, using microarray analyses. The data obtained suggest a pleiotropic regulatory role of *lmo1273* and possible links with amino acid uptake.

Received 15 July 2008
Revised 24 October 2008
Accepted 8 November 2008

INTRODUCTION

The Gram-positive bacterium *Listeria monocytogenes* is a foodborne pathogen that is widespread in the environment. This facultative intracellular pathogen is responsible for sporadic severe infections in humans and numerous animal species (Farber & Peterkin, 1991; Vazquez-Boland *et al.*, 2001). *L. monocytogenes* is capable of invading most host cells, and the molecular mechanisms of its intracellular parasitism have been investigated extensively (Hamon *et al.*, 2006). We have previously identified a five-gene locus, designated *sip* (*lmo1269–lmo1273*), in the genome of *L. monocytogenes* EGD-e, encoding three paralogous type I signal peptidases (SPases

I) (Bonnemain *et al.*, 2004) involved in bacterial virulence. We showed that this locus was under the control of two distinct promoters, one preceding *lmo1269* (designated *sipX*) and a second preceding *lmo1271* (designated *sipZ*) (Raynaud & Charbit, 2005). The two distal genes of the locus (*lmo1272* and *lmo1273*), which are co-transcribed with the SPase I genes (Raynaud & Charbit, 2005), encode proteins sharing significant similarities with the YlqF and RnhB proteins, respectively, of *Bacillus subtilis*. In the latter organism, RnhB is involved in RNA–DNA heteroduplex degradation (Itaya *et al.*, 1999; Ohtani *et al.*, 1999a) and YlqF participates in ribosome biogenesis (Matsuo *et al.*, 2006; Uicker *et al.*, 2007).

Currently, at least 15 RNases are known in *Escherichia coli* and at least 10 in *B. subtilis* (Condon, 2003). Among these RNases, RNase H (Crouch, 1990; Katayanagi *et al.*, 1990) has been defined as an enzyme that specifically degrades the ribonucleotide moiety in RNA–DNA hybrid molecules (Ohtani *et al.*, 1999a, b). RNases H are classified into two major families, type I and type II, which are evolutionarily unrelated. Bacterial RNases HI (encoded by *rnhA*), eukaryotic RNases H1 and retroviral RNases H are members of the type I RNase H family. Bacterial RNases

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Abbreviations: BMM, bone-marrow-derived macrophages; i.v., intravenous(ly); SPase I, type I signal peptidase.

The microarray data associated with this paper have been deposited in the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>) under the accession number E-MEXP-1162.

A supplementary table of primers and three supplementary figures are available with the online version of this paper.

HII and RNases HIII (encoded by *rnhB* and *rnhC*, respectively), archaeal RNases HII and eukaryotic RNases H2 are members of the type II RNase H family (Zhang *et al.*, 1997). The role of RNase H in bacterial cell growth has recently been reassessed in *B. subtilis* (Fukushima *et al.*, 2007). This study revealed that *B. subtilis* possesses four RNase H encoding genes (*rnhB*, *rnhC*, *ypdQ* and *ypeP*). Although RNase H had been previously reported to be essential in *B. subtilis*, the four genes could be simultaneously deleted, demonstrating that RNase H activity is dispensable for the bacterium. However, the growth rates of mutants with two or more *rnh* genes inactivated were low, indicating that this activity is required for normal cell growth and may thus participate in DNA replication.

The genome of *L. monocytogenes* EGD-e encodes one putative RNase HI (*lmo1880*), one putative RNase HII (*lmo1273*) and one putative RNase HIII (*lmo1228*). The aim of the present work was to address the role of *lmo1273* in virulence. The data presented show that *lmo1273* controls the expression of multiple genes and is involved in the *in vivo* survival of *L. monocytogenes*.

METHODS

Bacterial strains, plasmids and growth conditions. We used the reference strain of *L. monocytogenes* EGD-e, which belongs to serovar 1/2a (Glaser *et al.*, 2001). *E. coli* strain DH5 α was used for all plasmid constructions prior to their introduction into *L. monocytogenes*. *E. coli* was grown in LB; *L. monocytogenes* was grown in BHI rich medium. Plasmids pUC19 (Vieira & Messing, 1985) or LITMUS28 (New England Biolabs) were used to clone all PCR-amplified sequences from EGD-e and to construct the required deletions. Deletions were then transferred to the shuttle vector pAUL-A (thermosensitive in *Listeria*: Lingnau *et al.*, 1995) for subsequent recombination into the chromosome. Strains harbouring plasmids were grown in the presence of the following antibiotics: for pUC19, pTrc99A and LITMUS28 derivatives, ticarcillin 100 $\mu\text{g ml}^{-1}$; for pAUL-A derivatives, erythromycin 150 $\mu\text{g ml}^{-1}$ (*E. coli*) and 5 $\mu\text{g ml}^{-1}$ (*L. monocytogenes*) and kanamycin 50 $\mu\text{g ml}^{-1}$; for pPL2 derivatives, chloramphenicol, 5 $\mu\text{g ml}^{-1}$. PCR amplifications were performed using *Taq* polymerase (Finnzymes), according to the manufacturer's recommendations. Restriction enzymes and ligase were purchased from New England BioLabs. Primers were purchased from Eurogentec.

Construction of Δ *lmo1273* chromosomal deletion mutant. We replaced the coding sequence (nucleotides 5–780) of the *lmo1273* gene by the promoterless *aphA3* cassette conferring kanamycin resistance. A 760 bp *EcoRI*/*Bam*HI-digested double-stranded DNA fragment (A) upstream of *lmo1273* and a 790 bp *Bam*HI–*Sal*I DNA fragment (B) downstream of *lmo1273* were amplified by PCR using *L. monocytogenes* EGD-e genomic DNA with primers 1-2 and 3-4 respectively (see Supplementary Table S1, available with the online version of this paper, for primer sequences). After digestion, fragments A and B were successively cloned into pUC19 vector between the *EcoRI* and the *Sal*I site. The pUC19-AB plasmid was digested with *Bam*HI, and the 800 bp *aphA3* cassette flanked by two *Bam*HI restriction sites was inserted into pUC19-AB. The 2.4 kb *EcoRI*–*Sal*I A-*aphA3*-B fragment was then cloned into the thermosensitive shuttle vector pAUL-A. Plasmid pAUL-A-*aphA3*-B was then electroporated into *L. monocytogenes* EGD-e (Park & Stewart, 1990) and the transformants were selected for Em^R at 30 °C.

The *L. monocytogenes* Δ *lmo1273* chromosomal mutant was obtained by allelic replacement, using the classical two-step procedure described previously (Lingnau *et al.*, 1995). First, chromosomal integration of the recombinant plasmid was achieved by growing the bacteria at the non-permissive temperature of 37 °C. Then, the excision event eliminating the plasmid carrying the wild-type *lmo1273* copy was obtained after repeated passages at replication-permissive temperature (30 °C). This procedure led to a Δ *lmo1273* chromosomal mutant of EGD-e expressing kanamycin resistance. The resulting EGD- Δ *lmo1273* mutant was verified by sequencing analysis of chromosomal DNA using pairs of internal or flanking primers.

Construction of a Δ *lmo1272* chromosomal deletion mutant. We attempted to construct a Δ *lmo1272* mutant by the same procedure in wild-type EGD-e. Two DNA fragments flanking the *lmo1272* gene (see primers in Supplementary Table S1) were ligated and cloned into the thermosensitive plasmid pAUL-A, yielding pAUL-A-*lmo1272*. This plasmid was then introduced into EGD-e. Chromosomal integration of the recombinant plasmid was obtained (using the procedure described above for construction of the *lmo1273* mutant). The second crossover event, leading to excision of the plasmid, was screened by growing the recombinant bacteria at a permissive temperature for up to 100 generations in the absence of erythromycin. All the clones recovered were either still Em^R (i.e. had not lost the integrated plasmid) or were Em^S but had lost the plasmid carrying the mutated *lmo1272* region, reconstituting a wild-type chromosomal region. Thus, the second crossover event occurred only when the wild-type *lmo1272* allele was kept on the bacterial chromosome, strongly suggesting that the deletion of this gene was not tolerated.

Generation of an internal *lmo1273* deletion (*lmo1273-ID* strain). We also attempted to construct a Δ *lmo1272*-*lmo1273* double mutant. For this, a 600 bp *EcoRI*–*Sma*I double-stranded DNA fragment comprising an internal portion of *lmo1272* (A) and a 900 bp *Sma*I–*Sal*I double-stranded DNA fragment comprising the distal portion of *lmo1273* and downstream region (B) were amplified by PCR from *L. monocytogenes* (EGD-e) genomic DNA, using primers listed in Supplementary Table S1. After digestion, fragments A (*EcoRI*–*Sma*I) and B (*Sma*I–*Sal*I) were ligated and cloned into the *EcoRI*–*Sal*I sites of pUC19. The vector was digested with *Sma*I and the 900 bp *aph3'* cassette (conferring Km resistance) flanked by two *Sma*I restriction sites was inserted into pUC19-AB. To permit allele exchange in *L. monocytogenes* the *EcoRI*–*Sal*I fragments containing the Km cassette were subcloned into the corresponding restriction sites of the thermosensitive shuttle vector pAUL-A, to give plasmid pAUL-A : A-*aph3'*-B. The *L. monocytogenes* chromosomal mutant was constructed by allelic replacement of the A-*aph3'*-B fragment by the classical two-step procedure routinely used in our laboratory. The construction was checked by PCR sequence analysis. Unexpectedly, the resulting construct possessed an intact *lmo1272* gene and carried only an internal deletion of 224 bases within *lmo1273* (fusing out of frame the first 433 bp and the last 125 bp of the gene).

Construction of the Δ *lmo0847* chromosomal deletion mutant. We generated a deletion of the *lmo0847* sequence by allelic replacement using the pMAD thermosensitive shuttle vector (Arnaud *et al.*, 2004). Briefly, upstream and downstream sequences of the gene were amplified by PCR using primers 51-52 and 53-54 (see Supplementary Table S1) and cloned in pMAD between the *EcoRI* and the *Bam*HI unique site. The resulting plasmid pMAD- Δ *lmo0847* was electroporated in EGD-e. Integration of the plasmid into the chromosome was obtained after 48 h of culture at restrictive temperature (42 °C). Then, the excision event eliminating the plasmid carrying the wild-type *lmo0847* copy was obtained after repeated passages at replication-permissive temperature (30 °C). The resulting EGD- Δ *lmo0847* mutant was verified by sequence analysis of chromosomal DNA using pairs of flanking primers.

Electron microscopy. Bacteria grown to the exponential phase (OD₆₀₀ 0.6) were processed for observation by electron microscopy. Fixation of the bacteria and embedding in Epon resin was done as described by Frehel & Leduc (1987). Thin sections were stained with uranyl acetate and lead citrate before observation with a JEOL 100 CX II transmission electron microscope.

Complementation studies

Functional complementation in *E. coli*. The coding sequence of the *lmo1273* gene of *L. monocytogenes* EGD-e was amplified by PCR with primers 17 and 18. The PCR product was digested by *Nco*I and *Bam*HI and cloned into the pTrc99A vector (Pharmacia Biotech; GenBank accession number M22744), which allows IPTG-inducible expression of the insert via the hybrid *trp/lac* promoter. pTrc99A-*lmo1273* was then electroporated into the temperature-sensitive *E. coli* strain MIC2067 (kindly provided by M. Itaya). The temperature-sensitive growth of this strain resulted from the *rnhA* and *rnhB* mutations (Itaya *et al.*, 1999). We tested, at various IPTG levels, the ability of strains MIC2067 and MIC2067/pTrc99A-*lmo1273* to form colonies on plates incubated at 37 °C or 42 °C, compared to the number of colony-forming units (c.f.u.) obtained at 30 °C.

Complementation of the *lmo1273* deletion using the integrational vector pPL2. The *lmo1273* coding sequence (including the ribosome-binding site) was amplified from EGD-e genomic DNA by PCR using primers 22 and 24. To ensure efficient transcription, *lmo1273* was cloned downstream of the P_{*sipX*} promoter (Raynaud & Charbit, 2005), the main promoter of the *sip* locus. The P_{*sipX*} region was amplified with primers 20 and 21. Digested P_{*sipX*} and *lmo1273* PCR products were subcloned together in the LITMUS28 cloning vector. Digestion of the resulting plasmid with *Sac*I and *Kpn*I produced a 1 kb insert, which was ligated into the *Sac*I/*Kpn*I-restricted vector pPL2 (Lauer *et al.*, 2002). The resulting pPL2-P_{*sipX*}-*lmo1273* vector was electroporated into *L. monocytogenes* Δ*lmo1273* and a transformant was selected on BHI chloramphenicol plates. Integration of the resulting vector in the tRNA^{Arg} region was confirmed by PCR as described by Lauer *et al.* (2002).

Expression of *lmo1273* under P_{*sipX*} promoter control in pAT28 (pP_{*X*}-*lmo1273*) in EGD-e. A strain overexpressing *lmo1273* was constructed by placing the *lmo1273* gene under the control of the P_{*sipX*} promoter in the multicopy plasmid pAT28. Briefly, P_{*sipX*} was amplified using primers 19 and 21, and *lmo1273* was amplified using primers 22 and 23. The two DNA fragments obtained were cloned together in pUC19. The *Eco*RI/*Sph*I-digested product was then cloned into pAT28 and the resulting vector introduced in EGD-e by electroporation. The final construction was checked by sequencing and *lmo1273* expression measured by quantitative RT-PCR.

Transcriptomic analyses: RNA isolation, labelling with Cy dyes, hybridization and microarray analysis. Bacteria were grown in BHI to OD₆₀₀ 1.0 (late exponential phase) and RNA was stabilized using the RNeasy Protect Bacteria reagent (Qiagen) according to the manufacturer's recommendations. For each strain, RNA was extracted from three independent biological samples. Briefly, bacterial total RNA was isolated, reverse transcribed and hybridized to a whole-genome microarray, and microarray data were analysed as described previously (Chatterjee *et al.*, 2006). Hybridizations were performed with cDNAs from (i) EGD-e versus Δ*lmo1273* and (ii) EGD-e versus the overexpressing strain (EGD-e/pP_{*X*}-*lmo1273*). After hybridizations, microarray slides were imaged with a Generation III array scanner (GE Healthcare) and the fluorescent signal intensities from each spot on the microarray slide were quantified using ImaGene 7.5 software (BioDiscovery). The cut-off for significantly differentially expressed genes was set with a *q*-value of ≤5% and a fold change ≥2. The microarray data associated with this paper have

been deposited in the ArrayExpress database (<http://www.ebi.ac.uk/arrayexpress>) under the accession number E-MEXP-1162.

Quantitative PCR analysis. Real-time RT-PCR was conducted on total RNA samples isolated independently from the transcriptome analysis experiment (three independent biological samples). Total RNA was extracted as described above, with the only modification that RNA-protected bacteria were lysed by mechanical disruption in a Fastprep apparatus instead of by chemical disruption using lysozyme, mutanolysin and proteinase K. Forward and reverse primer were purchased from Eurogentec and are described in Supplementary Table S1. cDNA was obtained using the Superscript II kit from Invitrogen according to the manufacturer's recommendation, with 250 ng total RNA. Quantitative real-time PCR was performed on an Applied Biosystems 7700 sequence detection thermocycler for 40 cycles with 5 μl of 1/10 cDNA dilution, 10 μl of Power SYBR green PCR master mix 2 × (Applied Biosystems) and 5 pmol of each primer. For each pair of primers, a water control and a control without reverse transcriptase for each RNA sample (to ensure absence of genomic DNA contamination) were included. The quantity of cDNA for each gene was normalized to the quantity of *gyrA* cDNA. PCR products were run on a 2% agarose electrophoresis gel to check specificity of the amplification.

Intracellular multiplication

Cell cultures. Mouse macrophage cell line J774 (ATCC TIB67) and human colon carcinoma cell line Caco-2 (ATCC HTB37) were propagated as previously described (Dramsé *et al.*, 1995) in DMEM containing 10% fetal bovine serum. Cells were seeded at a concentration of 2 × 10⁵ cells per well in 12-well tissue culture plates (Falcon). Monolayers were used 24 h after seeding. Bone-marrow-derived macrophages (BMM) from a BALB/c mouse were obtained and cultured as described previously (de Chastellier & Berche, 1994).

Kinetics of infection. The invasion assays were carried out essentially as described previously (Bigot *et al.*, 2006). Briefly, cell monolayers were incubated for 30 min (BMM and J774) or 1 h (Caco-2) at 37 °C with the bacterial suspensions in Dulbecco's modified Eagle medium (multiplicities of infection 0.1 for BMM, 1 for J774, and 100 for Caco-2) to allow the bacteria to enter. After washing (time zero of the kinetic analysis), the cells were incubated for several hours in fresh culture medium containing gentamicin (50 μg ml⁻¹) to kill extracellular bacteria. At several time points, cells were washed three times in RPMI and processed for counting of infecting bacteria. Cells were lysed with distilled water. The titre of viable bacteria released from infected cells was determined by spreading dilutions of the lysate onto plates of BHI medium. The assay was performed in triplicate for each strain and time point in an experiment. Each experiment was repeated at least twice.

Plaque formation assays. These assays were performed essentially as described by Chatterjee *et al.* (2006). L929 murine fibroblast cells were grown to monolayers in 6-well tissue culture plates in RPMI 1640 supplemented with 5% fetal calf serum (FCS). The cells were infected with 8 μl of a 10⁻⁴ dilution of a washed bacterial culture with an OD₆₀₀ of 1.0 for 2 h at 37 °C in a humidified incubator and subsequently incubated for 30 min in the presence of 50 μg gentamicin ml⁻¹. The agarose overlay contained 1% agarose in minimal essential medium, 5% FCS, glutamax (PAA Laboratories), nonessential amino acids (Gibco), and 10 μg gentamicin ml⁻¹. Experiments were carried out in duplicate. After 3 days incubation at 37 °C, wells were stained with neutral red and examined for plaque formation.

Determination of virulence in the mouse model.

Infection of mice. Specific-pathogen-free 6- to 8-week-old female Swiss mice (Janvier) were used. Bacteria were grown for 18 h in BHI

broth, centrifuged, appropriately diluted in 0.15 M NaCl, and inoculated (0.5 ml) intravenously (i.v.) into mice via the lateral tail veins. Groups of five mice were challenged i.v. with various doses of bacteria, and mortality was monitored for 10 days. The virulence of the mutant was estimated by using the 50% lethal dose (LD₅₀) with the Probit method (Finney, 1971). For strains carrying pAT28 or a derivative plasmid, mice were pre-treated with spectinomycin (1 mg per mouse per day) in order to overcome *in vivo* instability of the recombinant plasmid.

Kinetics studies. Mice were inoculated i.v. in the lateral tail veins. At days 1, 3, 4, 5 and 6, groups of five mice were sacrificed, and their spleens, livers and brains were aseptically removed and separately homogenized in 0.15 M NaCl. Bacterial numbers in organ homogenates were determined at various intervals on BHI plates. Five hundred microlitres of bacterial suspension containing 10^{6.1} bacteria were injected per mouse. Animal experiments were performed according to the INSERM guidelines for husbandry of laboratory animals.

RESULTS

lmo1273 encodes an RNase H homologue

The *sip* locus of *L. monocytogenes* comprises five genes (*lmo1269*–*lmo1273*). The three first genes of the locus (*lmo1269*–*lmo1271*) encode paralogous copies of SPases I, and were hence designated *sipX*, *sipY* and *sipZ*. The role of SPases I is to remove the signal peptides of preproteins exported by the general secretory pathway. Whereas *SipX* and *SipZ* perform distinct functions in pathogenicity, *SipZ* is the major SPase I of *L. monocytogenes* (Bonnemain *et al.*, 2004).

The fourth gene of the locus, *lmo1272*, encodes a protein of 287 amino acids homologous to *B. subtilis* YlqF (58% amino acid identity). It has been experimentally demonstrated that *B. subtilis* *ylqF* encodes a GTP-binding protein that participates in the late step of 50S ribosomal subunit assembly (Matsuo *et al.*, 2006). Moreover, *ylqF* has been shown to be an essential gene in *B. subtilis* (Uicker *et al.*, 2007). GTPases control a wide variety of biological processes, including protein synthesis and cell cycle control. Most bacteria generally encode several highly conserved GTP-binding proteins of unknown function (at least 11 in *B. subtilis*), many of which are essential for growth (Morimoto *et al.*, 2002). The last gene of the locus, *lmo1273*, encodes a putative RNase HII of 261 amino acids, sharing 51% amino acid identity with the *rnhB*-encoded RNase HII of *B. subtilis*.

We have shown previously that the expression of the *sip* locus is regulated by two different promoters, *P_{sipX}* and *P_{sipZ}*, producing a large transcript covering the entire locus (*sipX*–*lmo1273*) and a shorter one compatible with a transcription starting upstream of *sipZ* (*sipZ*–*lmo1273*) (Bonnemain *et al.*, 2004; Raynaud & Charbit, 2005). Notably, the genomic organization and DNA sequence of the locus are highly conserved in the other sequenced *Listeria* genomes, including in *L. innocua* and in the recently sequenced genome of *L. welshimeri* (Hain *et al.*, 2006). A computer-assisted analysis of all the complete

bacterial genomes available at the Center for Genomic Research (TIGR, <http://www.tigr.org/tigr-scripts/CMR2/CMRGenomes.spl>), searching for the presence of a putative *rnhB* gene in the immediate vicinity of a *sip* and/or *ylqF* gene, revealed that several genomes (including those of strains of *Bacillus anthracis*, *Clostridium tetani* and *Lactobacillus sakei*) had a genetic organization similar to that of *L. monocytogenes* (Supplementary Fig. S1). In a number of other genomes, and in particular in all the mycobacterial genomes available, the genes *sip* and *rnhB* are contiguous and in the same orientation. Finally, as in *B. subtilis* and in *Streptococcus* species, the gene *rnhB* can be preceded by a *ylqF* orthologue, but these genes are not located next to any *sip* gene.

Functional complementation in *E. coli*

Several thermosensitive mutants of *E. coli* that form colonies at 30 °C but not at 42 °C as a result of *rnhA* and *rnhB* double mutation have been previously described (Itaya *et al.*, 1999). The thermosensitive phenotype can be relieved by delivering plasmids that carry a functional RNase H gene. This system, which provides a simple and convenient way to probe the functionality of an RNase H-encoding gene, has been successfully used to identify *rnhB* and *rnhC* genes of *B. subtilis*. Here, we used the *E. coli* strain MIC2067 (an *rnhA*–*rnhB* double mutant strain, kindly provided by M. Itaya) and transformed it with plasmid pTrc99A–*lmo1273*, carrying the *lmo1273* gene under IPTG-inducible promoter control. Complementation of the thermosensitive phenotype was tested at various IPTG levels, by evaluating the capacity of the recombinant *E. coli* to form colonies on plates incubated at 37 °C or 42 °C. As shown in Fig. 1, while MIC2067 was severely impaired in its ability to form colonies at 37 °C and 42 °C; normal growth was restored in the complemented strain upon IPTG induction (25 µM IPTG) at these two temperatures (i.e. the number of c.f.u. recorded at these two temperatures was similar to that at 30 °C).

Construction and analysis of Δ *lmo1273* chromosomal mutants of EGD-e

All our attempts to inactivate *lmo1272* by allelic exchange were unsuccessful (see Methods for details), suggesting that *lmo1272* is an essential gene in *L. monocytogenes*, as the homologous *ylqF* gene is in *B. subtilis* (Uicker *et al.*, 2007). In contrast, inactivation of *lmo1273* was tolerated and we successfully generated two chromosomal Δ *lmo1273* deletion mutants of EGD-e: a deletion removing the entire coding sequence (designated Δ *lmo1273*) and an internal deletion of 224 bases (designated *lmo1273-ID*).

We first evaluated the growth of the two *lmo1273* mutants in BHI medium, at various temperatures (from 4 °C to 42 °C). The growth rate of the two mutants was identical and only slightly reduced as compared to that of wild-type EGD-e (Supplementary Fig. S2a). We examined the wild-

Fig. 1. Functional complementation assay. *E. coli* MIC2067 is a temperature-sensitive strain resulting from the inactivation of the *rnhA* and *rnhB* genes (Itaya *et al.*, 1999). MIC2067 was transformed with a plasmid carrying *lmo1273* under the control of an IPTG-inducible promoter (pTrc99A-*lmo1273*). The capacity of the recombinant strain to form colonies on plates incubated at the non-permissive temperatures of 37 °C or 42 °C is shown here using plates with 25 μ M IPTG and appropriate antibiotics.

type strain and the *lmo1273-ID* mutant by electron microscopy in stationary phase at 37 °C. In thin sections both wild-type and mutant bacteria showed a typical morphology of dividing bacteria, with formation of septum, a normal cell wall thickness, and a normal bacterial size ($\sim 2 \mu$ m) (Supplementary Fig. S2b). Thus, inactivation of *lmo1273* did not create any major defect in cell wall biogenesis or cell division capacity.

Preliminary proteomic analyses (1D-gel analyses; not shown) did not reveal any visible difference in the protein pattern of the *lmo1273* mutants as compared to wild-type EGD-e. Moreover, Western blot analyses of culture supernatants, using either anti-LLO or anti-ActA polyclonal antibodies, did not reveal any detectable defect in protein production in the Δ *lmo1273* mutant as compared to EGD-e (data not shown).

Effect of the Δ *lmo1273* deletion on intracellular multiplication and cell-to-cell spread

The ability of the mutant strains to penetrate into and replicate within cells was studied in non-professional phagocytic mammalian epithelial cells (Caco-2). There was no significant difference in the number of viable bacteria between the mutant and the wild-type (although the number of mutant bacteria was slightly lower) at any time in the growth curve (Fig. 2a). We also monitored the intracellular multiplication of the Δ *lmo1273* mutant in bone-marrow-derived macrophages (BMM) and in the murine macrophage-like cell line J774. The mutant did not show any growth defect in either cell type (Fig. 2b, c), indicating that the *lmo1273* gene product is not critical for intracellular survival of *L. monocytogenes*.

We finally examined plaque-formation ability, a measure of intracellular growth and cell-to-cell spread of these bacteria. As shown in Fig. 2(d), the *lmo1273* mutant did not show any visible defect in plaque formation, in terms of either numbers or size of plaques, compared to the wild-type strain.

Role of *lmo1273* in bacterial virulence

We first studied the role of the *lmo1273* gene in bacterial virulence by determining the LD₅₀ of the mutant strains after i.v. inoculation of Swiss mice (Table 1). The LD₅₀ of the Δ *lmo1273* mutant was estimated at $10^{6.5}$. A similar value was recorded with the internal deletion mutant (*lmo1273-ID*, $10^{6.6}$). These values are 100-fold higher than that of the wild-type strain EGD-e ($10^{4.5}$), reflecting a substantial attenuation of virulence, suggesting that *lmo1273* might play a role in virulence. When *lmo1273* was reintroduced under the control of the *P*_{sipX} promoter in the chromosome of the Δ *lmo1273* mutant (strain Δ *lmo1273*/pPL2-*P*_{sipX}*lmo1273*), the virulence of the strain was partially restored (LD₅₀ $10^{5.5}$).

We next monitored the kinetics of bacterial survival in the spleens, livers and brains of mice infected with a dose of $10^{6.1}$ bacteria per mouse. At this dose, the mice infected with EGD-e or the complemented strain Δ *lmo1273*/pPL2-*P*_{sipX}*lmo1273* died within the first 4 days following infection. In contrast, all the mice infected with the mutant strain showed reduced symptom of illness and none of them died. As shown in Fig. 3, the Δ *lmo1273* mutant showed a drastic reduction of multiplication in all the target organs tested. In the spleen, at day 3, a 10-fold reduction in bacterial counts was recorded as compared with EGD-e; and at day 6, 95 % of the bacteria were eliminated from the spleen. In the liver, at day 3 a 150-fold reduction in bacterial counts was recorded; and at day 6, bacteria had been eliminated from nearly all the mice. Moreover, the brains of mice infected with the Δ *lmo1273* mutant strain were never highly infected, while the count in the brain reached 10^5 – 10^6 c.f.u. with the wild-type or the complemented strain.

Taken together, these data demonstrate that the Δ *lmo1273* mutant is severely impaired in *in vivo* survival.

Overexpression of *lmo1273* in wild-type EGD-e

Transformation of EGD-e with a multicopy plasmid carrying *lmo1273* preceded by the promoter of *sipX* (EGD-e/p*P*_{sipX}*lmo1273*) led to the overproduction of *Lmo1273*. As shown in Table 3, the amount of *lmo1273* transcripts produced by this strain is about eightfold higher than that in EGD-e (see below). As observed for the Δ *lmo1273* strain, the overproducing strain had a slight growth defect in BHI at 37 °C compared to a control strain carrying the pAT28 plasmid (Fig. 4a). EGD-e/p*P*_{sipX}*lmo1273* appeared to be attenuated *in vivo*, as determined by LD₅₀ analysis in the mouse model (Table 1) and by monitoring survival in infected target organs (Fig. 4b). The LD₅₀ value

Fig. 2. Intracellular survival. (a, b, c) Kinetics of intracellular multiplication of enterocytic Caco-2 cells (a), J774 macrophage-like cells (b) and bone-marrow-derived macrophages (BMM) from BALB/c mice (c). Invasion and intracellular multiplication was monitored over a 9 h period in the presence of gentamicin ($50 \mu\text{g ml}^{-1}$ final concentration). Data shown represent the means $\pm$ SD of one of at least two independent experiments performed in triplicate with similar results. (d) Cell-to-cell spreading assay. L929 murine fibroblast cells were infected with *L. monocytogenes* EGD-e (left) or its isogenic $\Delta lmo1273$ mutant (right) for 2 h at 37°C in a humidified incubator and subsequently incubated for 30 min in the presence of $10 \mu\text{g ml}^{-1}$ gentamicin. The plates were then agarose overlaid and were observed for plaques after an additional incubation at 37°C 3 days post-infection.

of $10^{6.2}$ recorded with EGD-e/pP_X*lmo1273* should be compared to that of $10^{5.4}$ recorded with EGD-e carrying pAT28 without insert, thus reflecting a 6.3-fold reduction of virulence. A ~ 100 -fold decrease in the number of bacteria recovered in the spleens, livers and brains of infected animals collected 5 days after infection was observed with EGD-e/pP_X*lmo1273* as compared to EGD-e/pAT28. These results indicated that overproduction of *lmo1273* impaired virulence of *L. monocytogenes*.

Table 1. Lethal dose 50% (determined in Swiss mice 10 days after i.v. inoculation)

Strain	LD ₅₀
EGD-e	$10^{4.5}$
EGD-e $\Delta lmo1273$	$10^{6.5}$
EGD-e <i>lmo1273-ID</i>	$10^{6.6}$
EGD-e $\Delta lmo1273$ /pPL2-P _X <i>lmo1273</i>	$10^{5.5}$
EGD-e/pAT28	$10^{5.4}$
EGD-e/pP _X <i>lmo1273</i>	$10^{6.2}$
EGD-e $\Delta lmo0847$	$10^{5.2}$

Transcriptomic analyses

Since *lmo1273* is co-transcribed with *sipZ* (*lmo1271*) in *L. monocytogenes* (Bonnemain *et al.*, 2004), we first investigated the potential involvement of this putative RNase HII in regulation of *sip* genes by real-time PCR, using *sip* primers (see Supplementary Table S1). The levels of *sip* transcripts were similar in the $\Delta lmo1273$ mutant and in the wild-type strain during exponential growth in BHI medium (data not shown). We then evaluated the impact of either the deletion or the overexpression of *lmo1273* on the transcriptome of EGD-e, using microarray technology. Transcriptomic analyses were carried out to compare (i) wild-type EGD-e with its isogenic $\Delta lmo1273$ mutant; and (ii) wild-type EGD-e with the *lmo1273*-overproducing strain (EGD-e/pP_X*lmo1273*). No significant difference was apparent from the comparison of the wild-type versus the $\Delta lmo1273$ mutant. However, analysis of the expression profile of the overexpressing strain (EGD-e/pP_X*lmo1273*) revealed that 88 genes were differentially transcribed compared to the wild-type strain. Among these genes, 17 were downregulated (see Table 2) and 71 were upregulated (see Table 3). The deletion or the overexpression of *lmo1273* had no impact on the expression of any of the other four genes of the *sip* locus, including *lmo1272*.

Fig. 3. Kinetics of *in vivo* multiplication. The growth of EGD-e (◆), its isogenic $\Delta lmo1273$ mutant (○) and the complemented strain ($\Delta lmo1273/pPL2-Px/lmo1273$; ◇) were monitored over a 6 day period in target organs of mice infected i.v. with a dose of $10^{6.1}$ bacteria per mouse. (a) Spleen; (b) liver; (c) brain at day 3 after i.v. inoculation. Values and error bars represent the means and standard deviation of the number (in $\log_{10}$) of bacteria per organ (five mice per point). † indicates that the majority of mice infected with the wild-type or the complemented strain died before day 4. Thus only three mice infected with EGD-e and two mice infected with $\Delta lmo1273/pPL2-Px/lmo1273$ could be sacrificed at day 4.

Among the 17 genes that were downregulated when *lmo1273* was overexpressed, 7 genes encode transporters, mainly amino acid or oligopeptide transporters (5/7).

Among the genes upregulated when *lmo1273* is over-expressed, we found genes encoding mainly proteins of unknown functions (33/71) and genes associated with

Table 2. Genes downregulated in the context of *lmo1273* overexpression

Functional category/gene†	Description‡	Ratio§	IC
Transport			
<i>lmo0135</i>	Oligopeptide ABC transporter binding protein	-2.0	M +
<i>lmo0152</i>	Oligopeptide ABC transporter binding protein	-2.8	M +
<i>lmo0847</i>	Glutamine ABC transporter, binding and transport protein	-12.4	
<i>lmo0848</i>	Amino acid ABC transporter, ATP binding protein	-5.8	
<i>lmo0798</i>	Lysine-specific permease	-2.4	
<i>lmo0920</i>	Putative ABC transporter permease protein	-4.1	
<i>lmo2254</i> ^(B)	Xanthine-uracil permease	-2.1	
Metabolism			
<i>pyrAa (lmo1836)</i>	Highly similar to carbamoyl-phosphate synthetase	-2.3	
<i>lmo1885</i>	Similar to xanthine phosphoribosyltransferase	-2.0	
<i>lmo1969*</i>	2-Dehydro-3-deoxyphosphogluconate aldolase	-3.9	M + C +
Other functions			
<i>lmo0960</i>	Similar to peptidase		
<i>glyQ (lmo1459)</i>	Similar to glycyl-tRNA synthetase alpha chain	-2.3	M -
Unknown			
<i>lmo0903</i>		-2.2	M +
<i>lmo0921</i>	Putative membrane protein	-4.1	
<i>lmo1210</i>		-3.8	
<i>lmo1865</i>		-2.2	M +
<i>lmo2409*</i>		-2.8	C -

†Genes labelled with an asterisk (*) have no orthologue in the non-pathogenic species *L. innocua*. Genes labelled with ^(B) have been previously identified as sigma B-downregulated genes (Hain *et al.*, 2006).

‡Description given by the Listlist web server <http://genolist.pasteur.fr/Listlist/index.html>.

§Ratio of wild-type EGD-e to EGD-e/p_{Px}*lmo1273*. The minus sign indicates that the genes are repressed when *lmo1273* is overexpressed.

||IC: genes which have been previously reported as regulated during intracellular infection of macrophages (M) or Caco-2 cells (C). +, Intracellularly upregulated genes; -, intracellularly downregulated genes.

Fig. 4. Overexpression of *lmo1273* in wild-type EGD-e. The gene *lmo1273*, expressed under the control of the *sipX* promoter (P_X) and carried on pAT28 (pP_Xlmo1273), was introduced into EGD-e by electroporation. EGD-e transformed with pAT28 alone was used as a control. (a) Bacterial growth in BHI broth; (b) bacterial counts in the target organs at day 5 after i.v. inoculation of 10^{5.4} bacteria per mouse. Values and error bars represent the means and standard deviation of the number (in log₁₀) of bacteria per organ (five mice per point). * indicates a *P*-value < 0.05 (Student's *t*-test).

metabolism (16/71) or transport functions (7/71). Five genes encode proteins predicted to be associated with peptidoglycan (PG), three of which have previously been experimentally identified as being anchored to the PG by their LPXTG motif by the sortase A (Pucciarelli *et al.*, 2005). This differential expression of surface proteins (transport proteins or PG-associated proteins) prompted us to analyse adhesion to non-phagocytic cells. However, no significant difference in adhesion to Caco-2 cells was observed for the overproducing strain compared to the wild-type (data not shown). Four putative transcription regulators are upregulated, two of which have no orthologue in the non-pathogen *L. innocua*. Another four genes encode proteins related to stress. Finally, 30 of the 71 genes are preceded by a sigma B promoter as identified by Kazmierczak *et al.* (2003), suggesting that they might also be regulated in response to stress conditions.

Interestingly, more than 50 % of the genes identified by this transcriptomic analysis (49/88 identified) were also found to be regulated during infection of macrophages (Chatterjee *et al.*, 2006) or Caco-2 cells (Joseph *et al.*, 2006). It is also worth noting that 10/88 genes have no orthologue in the non-pathogenic species *L. innocua* (Glaser *et al.*, 2001).

To verify the microarray transcription data, we conducted real-time RT-PCR analysis for 10 % of the differentially regulated genes. The fold induction of *lmo1273* determined by the two techniques was the same. Qualitative correlation between the two experiments was observed ($r=0.86$; see Supplementary Fig. S3) despite some quantitative variations between the results of the hybridization and the real-time RT-PCR, as previously reported (Joseph *et al.*, 2006; Stintzi, 2003).

Inactivation of *lmo0847* impairs virulence

Among the 17 downregulated genes identified by the transcriptomic analysis of the *lmo1273*-overexpressing

strain, two genes *lmo0847* and *lmo0848*, possibly belonging to the same operon, were the most severely downregulated (-12.4 and -5.8 , respectively; Table 2). These two genes, which could be direct targets of *Lmo1273*, encode a putative glutamine transporter of the ABC family (Glaser *et al.*, 2001). Of interest, ArpJ, a paralogue of *Lmo0847*, has been previously shown to be important intracellularly (Klarsfeld *et al.*, 1994). In order to determine if the downregulation of this transporter could account for, at least in part, the phenotype of the *lmo1273* mutants, we constructed a deletion mutant of the gene *lmo0847* (see Methods). The LD₅₀ of this mutant was 10^{5.2} in Swiss mice by the i.v. route. The level of attenuation of this mutant (about fivefold, as compared to wild-type EGD-e) is of the same order as that of the *lmo1273*-overexpressing strain EGD-e/pP_Xlmo1273 (about fivefold as compared to the control strain EGD-e/pAT28). Thus, the downregulation of *lmo0847*–*lmo0848* expression could account by itself for the decrease of virulence in the *lmo1273*-overexpressing strain.

DISCUSSION

We addressed here the role of the *lmo1273* gene in *L. monocytogenes* virulence. For this, we constructed two chromosomal deletion mutants as well as an overexpressing strain. We monitored the intracellular survival properties of the recombinant *L. monocytogenes* strains, in cell culture assays and *in vivo*. We also performed a whole-genome transcriptomic analysis of a strain overproducing *Lmo1273* to identify *Lmo1273*-regulated genes.

lmo1273, a novel gene involved in *L. monocytogenes* virulence

Inactivation of *lmo1273* had no detectable impact on intracellular survival of *L. monocytogenes* in any of the cell culture assays that we performed. However, it provoked a

Table 3. Genes upregulated in the context of *lmo1273* overexpression

Functional category/gene [†]	Description [‡]	Ratio [§]	IC
Metabolism			
<i>lmo0539</i> ^(B)	Tagatose-1,6-bisphosphate aldolase	2.0	
<i>lmo0669</i> ^(B)	Similar to oxidoreductase, short chain dehydrogenase motif	2.6	
<i>lmo0722</i> ^(B)	Similar to pyruvate oxidase	2.2	
<i>lmo1045</i>	Similar to molybdopterin converting factor (subunit 1)	2.6	
<i>lmo1048</i>	Similar to molybdenum cofactor biosynthesis protein B	2.8	C+
<i>glpD</i> (<i>lmo1293</i>)	Similar to glycerol-phosphate dehydrogenase	2.9	M+
<i>lmo1375</i> ^(B)	Similar to aminotripeptidase	4.0	M+
<i>lmo1538</i>	Similar to glycerol kinase	2.4	M+ C+
<i>lmo1789</i>	Weakly similar to NAD(P)H oxidoreductase chain B	3.2	
<i>lmo1830</i> ^(B)	Short chain dehydrogenase	2.8	M+
<i>lmo2230</i> ^(B)	Putative arsenate reductase	2.3	M+
<i>lmo2434</i>	Highly similar to glutamate decarboxylases	4.9	C+
<i>lmo2571</i> ^(B)	Similar to nicotinamidase	3.4	M+
<i>lmo2572</i> ^(B)	Similar to dihydrofolate reductase chain A	3.4	M+
<i>lmo2695</i> ^(B)	Dihydroxyacetone kinase (Dak1)	2.7	M+ C+
<i>lmo2696</i> ^(B)	Dihydroxyacetone kinase (Dak2)	2.7	M+
Transport			
<i>lmo0648</i>	Magnesium transporter, member of the CorA family	3.2	C-
<i>lmo1018</i>	Similar to <i>E. coli</i> copper homeostasis CutC	2.3	
<i>lmo1516</i>	Similar to ammonium transporter NrgA	2.5	M- C+
<i>lmo1539</i>	Similar to glycerol uptake facilitator	2.6	M+
<i>lmo1637</i> ^(B)	Similar to membrane protein, putative ABC transporter permease	2.1	M+
<i>lmo2581</i>	ABC transporter permease protein	2.9	
<i>lmo2741</i>	Major facilitator family transporter (multidrug efflux)	2.1	
Cell-wall associated protein			
<i>lmo0610</i> ^(B)	Protein with LPXTG motif, putatively similar to internalin [#]	6.0	M+
<i>lmo0732</i>	Putative peptidoglycan-bound protein, LPXTG motif	2.8	
<i>lmo0880</i> ^(B)	Protein with LPXTG motif [#]	2.3	M+
<i>lmo2085</i> ^{*(B)}	Protein with LPXTG motif [#]	4.0	
<i>lmo2522</i>	Similar to hypothetical cell wall binding protein from <i>B. subtilis</i>	2.1	
Transcription regulator			
<i>lmo0445</i> ^{*(B)}	Similar to transcription regulator	2.9	M+
<i>lmo0602</i> ^(B)	Weakly similar to transcription regulator	2.9	M+
<i>lmo0649</i>	Similar to transcription regulator, GntR family	2.6	C-
<i>lmo2672</i> ^{*(B)}	Weakly similar to transcription regulator, AraC family	3.5	M+
Stress protein			
<i>lmo0292</i>	Similar to heat-shock protease HtrA	2.2	M+
<i>lmo0515</i> ^(B)	Universal stress family protein	4.8	M+
<i>lmo2673</i> ^(B)	Universal stress protein family	3.0	M+
<i>lmo2748</i> ^(B)	Similar to <i>B. subtilis</i> stress protein YdaG, general stress protein 26	3.5	M+
Other functions			
<i>lmo1273</i>	Putative RNase H	8.6	
<i>lmo2511</i> ^(B)	Similar to ribosomal subunit interface protein	2.0	
Unknown			
<i>lmo0069</i> [*]		3.4	
<i>lmo0083</i> [*]		2.1	
<i>lmo0133</i>	Similar to <i>E. coli</i> YjdI	3.2	
<i>lmo0134</i> ^(B)	Similar to <i>E. coli</i> YjdJ	3.1	
<i>lmo0170</i> ^(B)		2.0	
<i>lmo0274</i>		2.6	
<i>lmo0321</i>		3.8	M+
<i>lmo0406</i>	Similar to <i>B. subtilis</i> YyaH	2.2	C-
<i>lmo0466</i> [*]		2.2	
<i>lmo0589</i>		2.7	
<i>lmo0642</i>		3.0	
<i>lmo0654</i>		2.3	C+

Table 3. cont.

Functional category/gene†	Description‡	Ratio§	IC
<i>lmo0670</i> ^(B)	Similar to <i>B. subtilis</i> YwnB	3.0	
<i>lmo0794</i>		2.5	M + C +
<i>lmo0911</i> ^(B)		2.2	M +
<i>lmo0937</i>		2.1	
<i>lmo0953</i> ^(B)	Similar to <i>B. subtilis</i> YwmG	2.7	M +
<i>lmo0994</i> ^(B)		2.8	M +
<i>lmo1124</i>		3.3	
<i>lmo1140</i> ^(B)		4.0	M + C +
<i>lmo1241</i> ^(B)		4.9	M +
<i>lmo1518</i>		2.4	M +
<i>lmo1526</i> ^(B)		2.9	M +
<i>lmo1694</i> ^(B)		2.3	M +
<i>lmo1704</i>		2.1	
<i>sepA</i> (<i>lmo2157</i>) [*] ^(B)		2.4	C +
<i>lmo2158</i> ^(B)	Similar to <i>B. subtilis</i> YhfK	2.6	M +
<i>lmo2387</i> [*] ^(B)		3.1	
<i>lmo2391</i> ^(B)		2.7	M +
<i>lmo2570</i> ^(B)		3.3	M +
<i>lmo2603</i> ^(B)		2.4	
<i>lmo2697</i> ^(B)		2.6	M +
<i>lmo2740</i>		2.1	

† Genes labelled with an asterisk (*) have no orthologue in the non-pathogenic species *L. innocua*. Genes labelled ^(B) have been previously identified as sigma B-upregulated genes (Hain *et al.*, 2006).

‡ Description given by the Listlist web server <http://genolist.pasteur.fr/Listlist/index.html>. Proteins labelled # have been experimentally identified as anchored to the peptidoglycan by the sortase SrtA (Pucciarelli *et al.*, 2005).

§ Ratio of EGD-e/pP_X*lmo1273* to wild-type EGD-e.

|| IC: genes which have been previously reported as regulated during intracellular infection of macrophages (M) or Caco-2 cells (C). +, Intracellularly upregulated genes; –, intracellularly downregulated genes.

severe attenuation of virulence in the mouse model. Moreover, overexpression of *lmo1273* (in a wild-type EGD-e genetic background) also led to an attenuation of virulence, implying that not only *lmo1273* expression is required to promote an efficient infection but also an appropriate level of expression. Most of the *L. monocytogenes* attenuated mutants described so far exhibited various growth defects in cell culture models (impaired adhesion, phagosomal escape, intracytosolic multiplication, movement or cell-to-cell spread...) associated – or not – with *in vivo* attenuation. The non-canonical virulence phenotype of the *lmo1273* mutants has however also been reported (Engelbrecht *et al.*, 1998; Sabet *et al.*, 2008). Of particular interest, the absence of *in vitro* defects in an *in vivo*-attenuated *inlJ* mutant of *L. monocytogenes* was recently explained by showing that *InlJ* (a surface protein of the internalin family) was not produced by bacteria grown *in vitro* (including in infected cells) but was specifically expressed at the bacterial surface *in vivo* (Sabet *et al.*, 2008). In the case of *lmo1273*, the situation is probably more complex, since overexpression of *lmo1273* modulates the expression of many other genes. Inactivation of *lmo1273* may alter the bacterial fitness in infected organs

and/or influence bacterial recognition by the host immune system.

Previous studies have shown that the three bacterial RNases H are required for the removal of RNA moieties of RNA–DNA hybrids such as primers from Okazaki fragments during lagging-strand DNA synthesis, and may thus be directly involved in DNA replication (Crouch, 1990; Qiu *et al.*, 1999). However, only RNase HII is capable of removing single RNA from DNA–RNA/DNA–DNA hybrids, such as those produced when RNA is misincorporated into DNA (Haruki *et al.*, 2002; Rydberg & Game, 2002). A recent extensive phylogenetic analysis of 353 prokaryotic genomes revealed that the RNase H group has evolved in such a way that genes encoding RNase HI and RNase HIII are inherited in a mutually exclusive manner, which may be attributed to the functional redundancy of the two enzymes. Of note, it has been shown, in yeast, that RNase HII is involved in both DNA replication and DNA repair (Arudchandran *et al.*, 2000; Rydberg & Game, 2002). Moreover, in *Haemophilus influenzae*, *rnhA* mutants lacking RNase HI activity show an approximately threefold increase in the mutation rates of tetranucleotide repeats

(Bayliss *et al.*, 2005), thus affecting phase variation, a key process in the pathogenicity of this organism.

Thus, in *L. monocytogenes* *rnhB* might also be involved in DNA replication and/or DNA repair, which remains to be experimentally determined.

***Lmo1273*, a novel pleiotropic regulator of *L. monocytogenes*?**

In order to get further insight into the mechanisms by which *Lmo1273* could influence bacterial virulence, we performed whole-genome microarray analyses. These analyses revealed significant differences between EGD-e and an *Lmo1273*-overproducing strain (EGD-e/*plmo1273*). In contrast, no major difference could be detected between EGD-e and the Δ *lmo1273* mutant, suggesting that, under the conditions used in our assays, the level of expression of *lmo1273* in EGD-e is probably low.

Seventeen downregulated genes were recorded when *lmo1273* was overexpressed. Notably, approximately one-third (5/17) of the genes encode amino acid or oligopeptide transporters. A significantly larger number of genes appeared to be upregulated when *lmo1273* was overexpressed. These genes encompassed a variety of predicted functions, and included genes associated with metabolism (16/71) or transport functions (7/71). Of interest, among the four genes encoding proteins bearing an LPxTG motif and predicted to be associated with peptidoglycan (PG), three of them have been experimentally shown to be PG-anchored.

Strikingly, 39 genes out of the 71 upregulated are sigma B-dependent genes (Hain *et al.*, 2008), supporting the notion of a link between *lmo1273*-dependent regulation and stress response in *L. monocytogenes* (Hain *et al.*, 2008). Thirty of these 39 genes are preceded by a sigma B consensus box and could be directly regulated by sigma B. Of note, *lmo1272* is also preceded by a putative sigma B promoter sequence. However, the transcriptomic analysis of the sigma B regulon of *L. monocytogenes* (Hain *et al.*, 2008) reveals that none of the genes of the *sip* locus is under the control of sigma B.

Interestingly, almost half of the genes (7/17) that were repressed upon overproduction of *lmo1273* belonged to amino acid and/or oligopeptide transport systems. This observation prompted us to evaluate the virulence of a mutant in one of these transporters. We chose *lmo0847*, the most severely downregulated gene (see Table 2). We found that inactivation of *lmo0847*, encoding a putative glutamine binding protein of a dedicated ABC transporter, led to a modest but significant reduction of virulence. It is thus reasonable to assume that the strong attenuation of virulence of the *lmo1273* mutant is due to a sum of effects, and in particular on genes involved in nutritional adaptation in its animal host.

Future work will be required to determine the precise molecular mechanisms by which the *lmo1273* gene product

functions and controls gene expression. We obtained preliminary indications that *lmo1273* might encode a genuine RNase H. At this stage, it cannot be excluded that RNase H activity might also provide a means for *L. monocytogenes* to regulate expression of a series of genes whose expression is under the control of antisense RNAs. The fact that *lmo1272* and *lmo1273* are expressed from the same transcript may also reflect a link between RNase H activities and ribosomal biogenesis.

Finally, the fact that inactivation of *rnhB* led to the production of viable bacteria strongly suggests that, as observed in other bacterial species possessing more than one RNase H, the lack of RNase HII can be compensated at least in part by the other RNase H encoded by the *L. monocytogenes* genome.

ACKNOWLEDGEMENTS

This work was supported by CNRS, INSERM and Université Paris V. Work carried out in Giessen was supported by EuroPathoGenomics Network, and the DFG through SFB535 Project B14 (T. H. and T. C.).

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Edited by: H. Ingmer