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clpB, a Novel Member of the *Listeria monocytogenes* CtsR Regulon, Is Involved in Virulence but Not in General Stress Tolerance

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Clp-HSP100 ATPases are a widespread family of ubiquitous proteins that occur in both prokaryotes and eukaryotes and play important roles in the folding of newly synthesized proteins and refolding of aggregated proteins. They have also been shown to participate in the virulence of several pathogens, including *Listeria monocytogenes*. Here, we describe a member of the Clp-HSP100 family of *L. monocytogenes* that harbors all the characteristics of the ClpB subclass, which is absent in the closely related gram-positive model organism, *Bacillus subtilis*. Transcriptional analysis of *clpB* revealed a heat shock-inducible σ^A-type promoter. Potential binding sites for the CtsR regulator of stress response were identified in the promoter region. In vivo and in vitro approaches were used to show that expression of *clpB* is repressed by CtsR, a finding indicating that *clpB* is a novel member of the *L. monocytogenes* CtsR regulon. We showed that ClpB is involved in the pathogenicity of *L. monocytogenes* since the Δ*clpB* mutant is significantly affected by virulence in a murine model of infection; we also demonstrate that this effect is apparently not due to a defect in general stress resistance. Indeed, ClpB is not involved in tolerance to heat, salt, detergent, puromycin, or cold stress, even though its synthesis is inducible by heat shock. However, ClpB was shown to play a role in induced thermotolerance, allowing increased resistance of *L. monocytogenes* to lethal temperatures. This work gives the first example of a *clpB* gene directly controlled by CtsR and describes the first role for a ClpB protein in induced thermotolerance and virulence in a gram-positive organism.

Listeria monocytogenes is a gram-positive pathogen implicated in food-borne infections and is responsible for meningitis, septicemia, and gastroenteritis—diseases with a high degree of mortality for immunocompromised hosts. During the past few years, this bacterium has been extensively studied, and it has become a model for intracellular growth (51) because of its abilities to escape from the phagosome, grow in the cytosol, and efficiently invade neighboring cells.

Several virulence proteins that are required for the key steps of the infectious process have been identified to date. InlA and InlB are required for entrance of *L. monocytogenes* into epithelial cells; listeriolysin O is required for escape from the phagosome; ActA is required for actin polymerization, cell-cell mobility, and invasion; and PlcB is required for lysis of the two-membrane vacuole (7). All virulence genes identified so far are under the positive control of a single regulator, PrfA. Due to the secondary structure of its mRNA, this activator, which acts as a thermosensor (28), is present only at the host temperature.

In addition to these major virulence factors are many proteins that are involved in pathogenicity of *Listeria*. These proteins, known as stress proteins, are important because they allow persistence and rapid adaptation during the infectious process. The accumulating body of data regarding several pathogens indicates that acid or oxidative stress proteins and (more recently) heat shock proteins (HSPs) and chaperones play an important role in virulence. Indeed, synthesis of the two major *Staphylococcus aureus* chaperones, DnaK and GroESL, was shown to be induced during infection of human epithelial cells (52); in *L. monocytogenes*, expression of the *groESL* operon is induced during intracellular infection, while DnaK is required for efficient phagocytosis with macrophages (15, 22). Another class of stress proteins, the Clp family, has also been shown to play a major role in the virulence of several pathogens: ClpP was shown to control expression of the attachment invasion locus (*ail*) of *Yersinia enterocolitica*, whereas in *Salmonella enterica* serovar Typhimurium, inactivation of *clpP* prevents growth and survival within macrophages (24, 69). Systematic genome-wide approaches such as signature-tagged mutagenesis revealed the roles of several *clp* genes, including *clpE*, *clpC*, and *clpL* of *Streptococcus pneumoniae* (23, 33, 48), as well as *clpX* of *S. aureus* (38).

Clp proteins are ubiquitous among prokaryotes and eukaryotes, and they function both as proteases and chaperones (19). Bacterial genomes are endowed with different sets of *clp* paralogs encoding Clp-HSP100 ATPase subunits, belonging to groups A, B, C, D, E, or L, and that are distinguished by their N-terminal domains and the central spacer regions between the two ATP-binding sites. *clpP*, which encodes the proteolytic subunit of the Clp ATP-dependent protease, is usually present as a single copy, but up to five copies per genome can coexist, as shown in *Streptomyces lividans* and *Streptomyces coelicolor* (8, 65, 66). The ClpP proteolytic subunit requires association with an ATPase subunit in order to be active, giving rise to a multimeric complex presenting structural and functional analogies with the eukaryotic proteasome (50). The ATPase subunits can also act in the absence of ClpP, forming a smaller complex with chaperone activity. It is interesting that ClpB of

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
L. monocytogenes		
LO28	Virulent *L. monocytogenes* reference strain	67
LM2000	Δ*clpB*	This study
LM2001	Δ*ctsR*::*aphA3*	This study
B. subtilis		
168	*trpC2*	Laboratory stock
QB4991	*trpC2* Δ*ctsR amyE*::(*'lacZ aphA3*)	10
QB8059	*trpC2 amyE*::(*clpB'-bgaB cat*) Δ*ctsR*	pDL73/74→QB4991
QB8060	*trpC2 amyE*::(*clpB'-bgaB cat*) Δ*ctsR thrC*::(*pxylctsRLmospc*)	pxyl59/60→QB8059
Plasmids		
pMAD	pE194 derivative with a thermosensitive origin of replication for deletion replacement of genes in gram-positive bacteria	M. Arnaud and M. Débarbouillé, unpublished
pMAD Δ*clpB*	pMAD derivative, for deletion replacement of the *L. monocytogenes clpB* gene	This study
pMAD Δ*ctsR*	pMAD derivative, for deletion replacement of the *L. monocytogenes ctsR* gene	This study
pDL	Integrative plasmid for constructing transcriptional fusions with the *B. stearothermophilus bgaB* gene	70
pDL73/74	pDL derivative carrying a *clpB'-bgaB* fusion	This study
pXT	Plasmid allowing integration at the *thrC* locus and expression from the P*xylA* xylose-inducible promoter	9
pxyl59/60	pXT derivative carrying the *ctsR* coding sequence of *L. monocytogenes*	42

Escherichia coli does not interact with the proteolytic subunit and is exclusively considered a chaperone (68). However, although some ClpB proteins have been characterized for both eukaryotes and bacteria, no phenotypes have been described as yet for low-G+C gram-positive bacteria.

In *L. monocytogenes*, three *clp* genes have been shown to play a role in virulence. ClpC is required for intracellular growth and in vivo survival in host tissues by promoting early escape from the phagosomal compartment (54, 55) and is also necessary for cell adhesion and invasion (44). The ClpE ATPase plays a role in *L. monocytogenes* virulence also (43), and an *L. monocytogenes clpP* mutant presents a defect in intracellular replication (16).

A Δ*clpB* mutant of *Y. enterocolitica*, a major gastrointestinal pathogen, presents a decrease in invasin and flagellin expression, characteristics that are encoded by the two virulence genes *inv* and *fleB* (2). For *S. enterica* serovar Typhimurium, the *clpB* mutant was discovered during a systematic search for mutants deficient in colonization of the chicken alimentary tract and was shown to be attenuated for virulence in 1-day-old chicks (64). Finally, a Δ*clpB* mutant of *Francisella novicida* was isolated during a screen for genes required for in vitro growth in thioglycolate-elicited mouse peritoneal macrophages (21).

Analysis of the complete genome of *L. monocytogenes* EGDe (18) reveals several uncharacterized genes encoding proteins belonging to the Clp family, two of which are preceded by potential binding sites for the CtsR regulator of stress response (10). Here we have characterized the *clpB* gene of *L. monocytogenes*. Using both in vivo and in vitro approaches, regulation of *clpB* was studied, showing a direct control by CtsR. This repression was demonstrated to be thermosensitive. A deletion mutant was constructed, and functional analysis revealed a role for ClpB in terms of the virulence of *L. monocytogenes*. We also show that, although ClpB has no obvious role in terms of stress tolerance, it is required for induced thermotolerance of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and transformation. Bacterial strains used in this work are listed in Table 1. *E. coli* K12 strain TG1 [Δ(*lac proAB*) *supE thi hsd*Δ5 (F′ *traD36 proAB lacI*^{q} *lacZ* ΔM15)] (17) was used for cloning experiments.

E. coli was grown in Luria-Bertani (LB) medium. Electroporation procedures were used for transformation with selection on LB plates supplemented with ampicillin (100 μg/ml), erythromycin (200 μg/ml), or kanamycin (25 μg/ml). *L. monocytogenes* LO28 was routinely grown in brain heart infusion (BHI) complex medium. Constructs were introduced into LO28 strains by electroporation. The following antibiotics were used at the indicated concentrations: erythromycin (8 μg/ml), kanamycin (50 μg/ml), and spectinomycin (60 μg/ml). *Bacillus subtilis* was grown in LB medium and transformed as previously described by using plasmid DNA (40). Transformants were selected on SP plates supplemented with chloramphenicol (5 μg/ml) or spectinomycin (100 μg/ml).

β-Galactosidase activity was estimated on plates by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) hydrolysis. β-Galactosidase-specific activities were determined as previously described (39–41) and were expressed as Miller units per milligram of protein.

Basal stress resistance experiments were performed as follows. Overnight cultures were diluted 100-fold in BHI medium and grown at 37°C with vigorous shaking until the optical density at 600 nm (OD_{600}) reached 0.3. Exponentially growing cultures were then divided into two parts, and one of which was subjected to one of the following stress conditions: 2% NaCl (wt/vol); 0.01% sodium dodecyl sulfate (SDS) (wt/vol); 15, 30, or 60 μg of puromycin per ml (values indicate final concentrations); or growth at 42, 44, 48, or 55°C. Growth was then monitored for an additional 3 h. For induced thermoresistance, overnight cultures were diluted 100-fold in BHI medium and placed at 37°C with vigorous shaking until the OD_{600} reached 0.3. Prior to heat treatment at 60°C, cultures were divided into two parts, one of which was maintained at 37°C, while the other half was preincubated at the nonlethal temperature of 48°C for 20 min. Both cultures were then incubated at 60°C, and aliquots were quickly transferred to ice, diluted in ice-cold BHI, and immediately plated on BHI; CFU were then counted. The induced thermoresistance experiment was repeated four times, yielding the same results.

DNA manipulations. Chromosomal DNA preparation, plasmid isolation, restriction enzyme analysis, and amplification by PCR were performed according to standard protocols (57). DNA sequences were determined by the dideoxy chain termination method (59) using modified T7 DNA polymerase (63) (Pharmacia). DNA concentrations were calculated by measuring UV spectroscopy at 260 nm.

Mutant and plasmid constructions. All oligonucleotide positions are given relative to the translation initiation codon. The plasmids used in this study are

listed in Table 1. Plasmid pDL (71) was used for constructing transcriptional fusions with the *Bacillus stearothermophilus bgaB* gene, which encodes a thermostable β-galactosidase (25), with subsequent integration at the *B. subtilis amyE* locus. A *clpB′-bgaB* transcriptional fusion was constructed using a 189-bp *Eco*RI/*Bam*HI DNA fragment corresponding to the *clpB* upstream region, which was generated by PCR by using oligonucleotides ID73 (−193) (5′-GAAGAATTCA TGTTCTTACTCCGCC-3′) and ID74 (−5) (5′-GGAGGATCCTTATAAAAG ATAAGTC-3′). This fragment was cloned between the *Eco*RI and *Bam*HI sites of plasmid pDL to give plasmid pDL73/74. Linearization of this plasmid at the unique *Pst*I site and transformation of the *B. subtilis* QB4991 strain with selection for chloramphenicol resistance yielded strain QB8059, in which the *clpB′-bgaB* fusion was integrated as a single copy at the *amyE* locus. The linearized pxyl59/60 plasmid (42) was then introduced in these strains by transformation and selection for spectinomycin to give strain QB8060, in which the *L. monocytogenes ctsR* gene is placed under the control of the P*xylA* xylose-inducible promoter and integrated as a single copy at the *thrC* locus.

A markerless Δ*clpB* deletion mutant of *L. monocytogenes* was constructed using plasmid pMADΔ*clpB*. The mutant was constructed by first using PCR to generate two DNA fragments of 829 and 761 bp, using oligonucleotide pairs AC189 (5′-AATGGATCCCACATCCGAGCGAGTAAACAC-3′) and AC190 (5′-TAAGTCGACTCATTCGTCCTCCTTATAAAA-3′) and AC192 (5′-CACGTCGACTGAAAGGGAAAACTTTGGTTG-3′) and AC193 (5′-TATC CATGGAATATTTATTTACTGGTTTTA-3′), corresponding, respectively, to the chromosomal DNA regions that are directly upstream and downstream from the *clpB* gene. These fragments were cloned in pMAD, a pRN5101 derivative carrying a thermosensitive origin of replication (M. Arnaud and M. Débarbouillé, unpublished data), and the resulting pMADΔ*clpB* plasmid was electroporated in the LO28 strain with selection for erythromycin. Integration and excision of pMADΔ*clpB* was performed as previously described (4) but with a nonpermissive temperature growth of 40°C, thus yielding strain LM2000 (Δ*clpB*), in which the entire *clpB* coding sequence was removed. PCR amplifications were performed in order to confirm the gene deletion.

The *ctsR* deletion mutant was obtained by transforming the LO28 strain by plasmid pMADΔ*ctsR*. For this purpose, DNA fragments of 1,044 and 1,031 base pairs corresponding to the upstream and downstream chromosomal DNA regions from *ctsR* were amplified by using oligonucleotide pairs AC212 (5′-GGCG GATCCCTCCTAAAGAGTAACGGAGGC-3′) and AC213 (5′-ACTGAATTC CAATACTTGTTTCAAATAAGC-3′) and AC214 (5′-TGAGAATTCGGATTT TAGAGGCGATGTTAG-3′) and AC215 (5′-TATCCATGGTCTTTATCAAAA GCATAAC-3′), respectively. The *aphA3* kanamycin resistance gene, deprived of its transcription initiation and termination signals, was then cloned at the *Eco*RI site between the two fragments just described. The resulting pMADΔctsR plasmid was introduced into strain L028, and the integration-excision procedure was performed as was described for the *clpB* deletion, thus yielding strain LM2001.

Mouse virulence assay. Six- to 8-week-old pathogen-free Swiss female mice (Janvier, Le Genset St. Isle, France), were used in this study. Groups of five mice were injected intravenously with doses of *L. monocytogenes* LO28 and Δ*clpB* mutant ranging between 5×10^5 and 5×10^8 bacteria. Mortality was observed over a 14-day period. The 50% lethal dose was determined by the Probit method. Mice were killed by cervical dislocation in accordance with the policies of the Animal Welfare Committee of the Faculté Necker (Paris, France).

Gel mobility shift DNA-binding assays. A 189-base-pair *Eco*RI/*Bam*HI DNA fragment corresponding to the promoter region of *clpB* was generated by PCR using oligonucleotides ID73 and ID74. Radiolabeling, DNA-binding, and gel shift experiments were performed as previously described (10).

DNase I footprinting. A 229-base-pair DNA fragment used for DNase I footprinting was prepared by PCR using *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) and oligonucleotides ID75 (−147) (5′-AAATTCAGAAGATCTG CCAACC-3′) and ID76 (+82) (5′-CTTATGTTCTGATGCAATAGC-3′). Labeling and DNase I treatment were performed as previously described (10).

RNA extraction and primer extension. *L. monocytogenes* strains were grown in BHI medium at 37°C with aeration until the OD_{600} reached 0.5; half of the culture was then shifted to 42°C, and incubation was undertaken for another 10 min. Cells were pelleted and frozen immediately, and RNA extraction and primer extension were then performed as previously described (5) using radiolabeled oligonucleotide AC209 (+22) (5′-GTGTAAATTTTTGTAAATCCAT TC-3′). Radioactive gels were exposed to storage phosphor screens and scanned with a Molecular Dynamics Storm 860 optical scanner. Quantitation of primer extension products was performed using the ImageQuant 5.1 software package (Molecular Dynamics).

RESULTS

Genome sequence analysis reveals ClpB ATPase in *L. monocytogenes*. Analysis of the complete genome of *L. monocytogenes* EGDe reveals a protein with a deduced 63% amino acid sequence identity with ClpB of *Lactococcus lactis* and 52% with that of *E. coli* (Fig. 1). The ATG initiation codon of *clpB* is preceded by a classical ribosome binding site (RBS), tAAG GAGG, at a suitable distance, and this sequence encodes a predicted protein of 866 amino acid residues with a calculated molecular mass of 97.5 kDa. We note that a GTG codon is located 151 codons downstream from the ATG codon and is also preceded by a typical RBS, (AgAGGAGG), at an appropriate distance of 7 bp. This potential internal translation initiation site suggests the existence, as shown for *E. coli*, of a smaller form of ClpB, with 716 amino acid residues and a theoretical molecular mass of 80.6 kDa (47, 62).

Analysis of the amino acid sequence of the protein revealed two typical Clp signature motifs (60, 61). Indeed, two ATP-binding sites are present, one with a single Walker A and two Walker B motifs and the other presenting only one of each Walker motif, a finding that is characteristic of HSP100 proteins (Fig. 1). There are also two repeated Clp amino-terminal domain motifs (ClpN), which are typical of ClpA and ClpB proteins (3) but are also present in most ClpC proteins.

This Clp ATPase also presents a long central domain separating the two ATP-binding sites, approximately 130 amino acids in length, which is characteristic of ClpB proteins. This domain contains a predicted coiled-coil motif (37; http://smart.embl-heidelberg.de), which may be involved in multimerization. Analysis of the carboxy-terminal domain, between the second ATP-binding site and the PDZ-like sensor and substrate discrimination domain, revealed the absence of the IGF loop required for interaction with the ClpP proteolytic subunit (30). This suggests that, as in *E. coli*, ClpB function in *L. monocytogenes* is restricted to chaperone activity without any interaction with ClpP.

***clpB* is a novel member of the *L. monocytogenes* CtsR regulon.** Three *clp* genes of *L. monocytogenes* have been shown to be controlled by the CtsR repressor of stress response genes (42), and many *clpB* genes are known to be heat shock-induced genes. To investigate a potential mechanism of transcriptional regulation of *clpB*, we analyzed the sequence of the promoter region, thereby revealing the presence of a potential binding site for CtsR (GGTCAAA AAA GGTCAgA) (see Fig. 3B), suggesting that ClpB may be a novel member of the *L. monocytogenes* CtsR regulon.

We used *B. subtilis* as a heterologous host to test whether CtsR plays a role in controlling *clpB* expression. For this purpose, a transcriptional fusion was constructed between the *L. monocytogenes clpB* promoter region and the *bgaB* gene of *B. stearothermophilus*, which encodes a thermostable β-galactosidase (25) (see Materials and Methods). The fusion was integrated as a single copy at the *amyE* locus of *B. subtilis* strain QB4991, in which the endogenous *ctsR* gene is deleted (10). The *L. monocytogenes ctsR* gene was then integrated as a single copy at the *thrC* locus, under the control of the P*xylA* xylose-inducible promoter, by using plasmid pxyl59/60 (42), thus leading to strain QB8060.

Strain QB8060 was grown at 37°C in the presence or absence

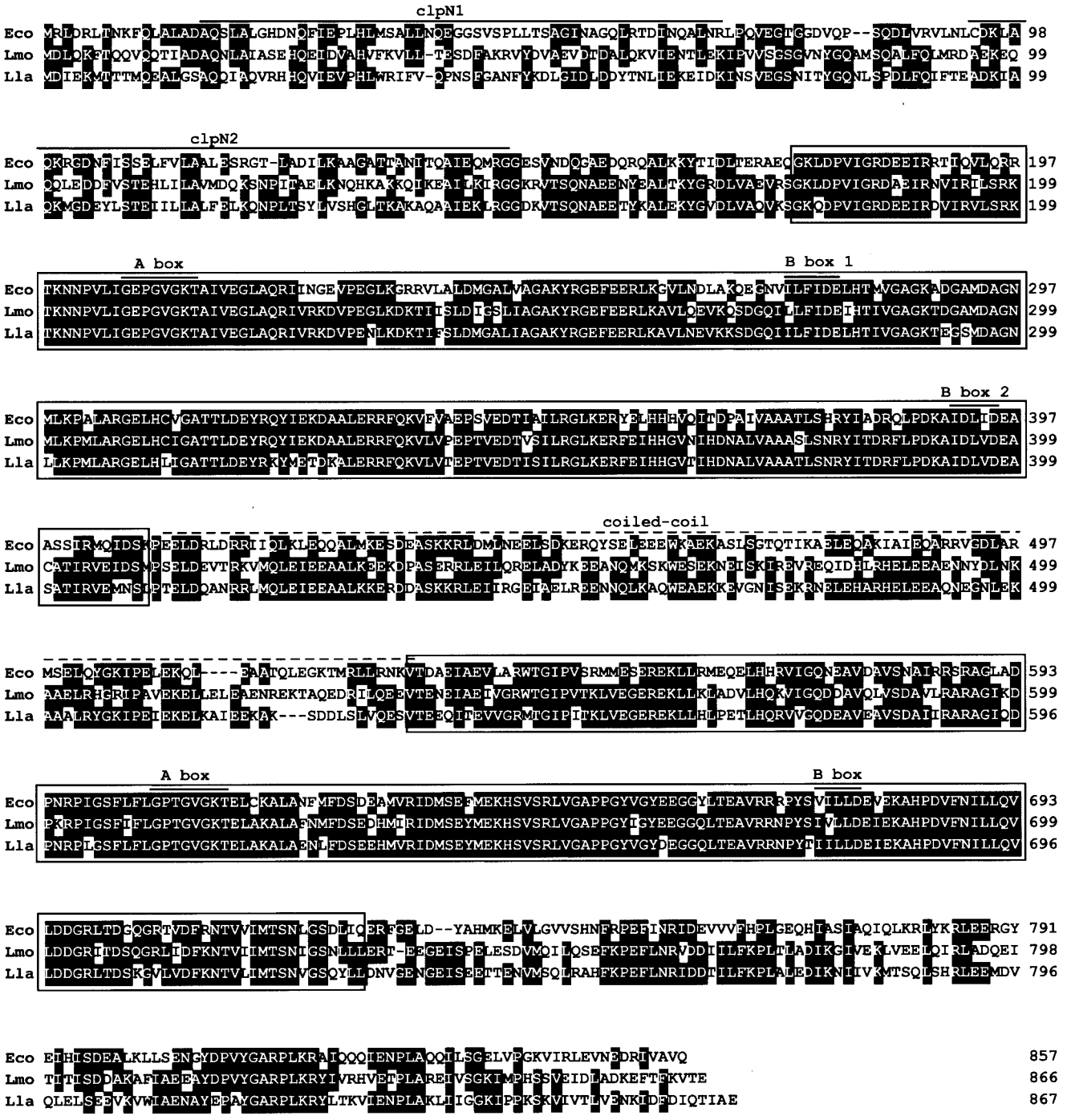

FIG. 1. Alignment of the ClpB amino acid sequence of *L. monocytogenes* with those of *E. coli* and *L. lactis*. Numbers indicate positions in the amino acid sequence. Identical residues are shaded. The conserved nucleotide-binding regions are boxed. Conserved Walker motifs (A box and B box) and predicted ClpN and coiled-coil motifs are overlined.

of xylose, and β-galactosidase activities were assayed. As shown in Fig. 2, *clpB′-bgaB* was expressed up to approximately 550 Miller units · mg^{-1} of protein at 37°C in the absence of the repressor (without xylose). This expression was repressed 15-fold (35 Miller units · mg^{-1} of protein) in the presence of CtsR of *L. monocytogenes* (with xylose). However, when the culture was shifted to high-temperature conditions (48°C) instead of 37°C, expression was induced up to 47-fold (1,650 Miller units · mg^{-1} of protein) in the presence of CtsR (with xylose; data not shown). These results demonstrate that *clpB* of *L. monocytogenes* is under negative regulation by CtsR and that this repression is thermosensitive.

***clpB* is expressed from a σ^A-dependent heat-inducible promoter.** In order to demonstrate the thermoinducibility of *clpB* and a role for CtsR in its regulation, an analysis of *clpB* transcription in *L. monocytogenes* was performed by using primer

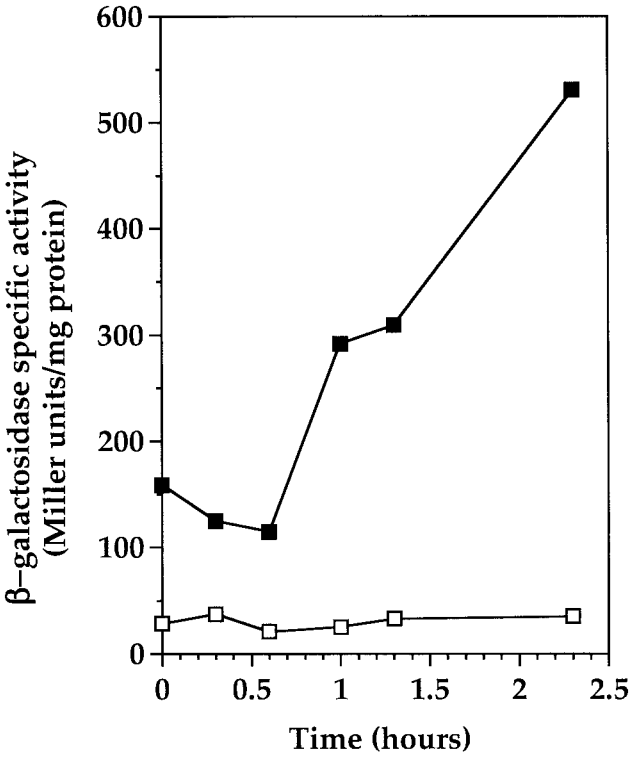

FIG. 2. *clpB* is negatively regulated by CtsR in the heterologous host *B. subtilis*. Levels of expression of *clpB'-bgaB* (strain QB8060, *clpB'-bgaB* Δ*ctsR pxylctsRLmo*) in LB medium at 37°C in the presence (□) or absence (■) of xylose are shown. Symbols indicate β-galactosidase activities expressed as Miller units/mg of protein as a function of time.

extension experiments. First, the transcription initiation site was determined by using RNA from *L. monocytogenes* cells grown in BHI at 37°C and harvested in mid-exponential phase (see Materials and Methods). This procedure revealed a single transcriptional start site 45 bp upstream from the ATG start codon of *clpB* (Fig. 3A and B). Consensus −10 and −35 sequences recognized by the $E\sigma^A$ RNA polymerase holoenzyme were identified upstream from the transcriptional start site, suggesting a σ^A-dependent promoter (Fig. 3B). A comparative transcriptional analysis of RNA expression at 37 and 42°C was performed by primer extension. As shown in Fig. 3A, *clpB* was expressed at a low basal level during growth in BHI at 37°C (Fig. 3A, lane 1), and transcription was increased fourfold when the culture was shifted to 42°C (Fig. 3A, lane 3), a finding which is consistent with a thermosensitive transcriptional regulation.

Repression of *clpB* by CtsR was examined in vivo in *L. monocytogenes*. The LM2001 Δ*ctsR* mutant strain was constructed by deleting the entire *ctsR* coding sequence and replacing it with the *aphA3* kanamycin resistance gene. This resistance cassette was deprived of its transcription initiation and termination signals in order to rule out any polar effects on expression of the downstream genes. Expression of *clpB* at 37°C in the wild-type (L028) (Fig. 3A, lane 1) and Δ*ctsR* (LM2001) (Fig. 3A, lane 2) strains was followed by primer extension analysis, which revealed increased transcription of *clpB* (6.5-fold) in the absence of CtsR. It is interesting that *clpB* derepression at 42°C is only partial since expression levels are higher in the *ctsR* deletion mutant at 37°C (Fig. 3A, lane 2) than in the wild-type strain at 42°C (Fig. 3A, lane 3), thus suggesting a limited inactivation of CtsR at this temperature. In conclusion, the in vivo evidence indicates that *clpB* expression is repressed by CtsR and is heat shock inducible.

CtsR binds specifically to the *clpB* promoter region. An in vitro approach was used to demonstrate a direct interaction between CtsR and the *clpB* promoter region. Histidine-tagged CtsR of *L. monocytogenes*, presenting a carboxy-terminal extension of six histidine residues, was overproduced and purified by using a Ni-nitrilotriacetic acid agarose column (42). This recombinant protein was used in gel mobility shift DNA-binding assays with a 189-bp radiolabeled PCR-generated DNA fragment corresponding to the *clpB* promoter region. This DNA fragment, extending from positions −193 to −5 relative to the translation initiation codon, was incubated with increasing amounts of purified CtsR in the presence of nonspecific competitor DNA [poly-(dI-dC)]. As shown in Fig. 4A, CtsR bound specifically to the radiolabeled fragment, leading to progressive displacement of the probe to the single higher-molecular-weight protein/DNA complex. Although an incomplete displacement was observed even at the highest CtsR concentrations, the single DNA/protein complex suggests the presence of only one CtsR-binding site in this promoter. These results demonstrate that CtsR of *L. monocytogenes* represses *clpB* expression by binding directly to the promoter region.

DNase I footprinting assays were performed for *L. monocytogenes* DNA fragments corresponding to the *clpB* promoter region in order to precisely determine the location of the CtsR-binding site. When the nontemplate strand of *clpB* DNA was end labeled, CtsR protected a region extending from positions −42 to −20 (Fig. 4B and D). When the template strand

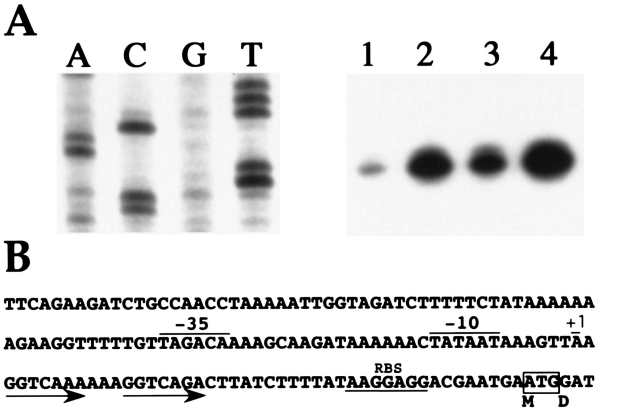

FIG. 3. (A) Primer extension analysis of *clpB* expression at 37°C (lanes 1 and 2) or following a 10-min heat shock at 42°C (lanes 3 and 4). Total RNA (20 μg) extracted from *L. monocytogenes* L028 (lanes 1 and 3) and LM2001 (Δ*ctsR*) (lanes 2 and 4), was used as a template for reverse transcriptase. The corresponding DNA sequence is shown on the left. (B) Nucleotide sequence of the *L. monocytogenes* L028 *clpB* promoter region. Potential −35 and −10 promoter sequences are overlined; the transcriptional start site is indicated by +1; the CtsR direct-repeat operator sequence is indicated by arrows; the potential RBS sequence is underlined; the translational start site is boxed, and the deduced amino acid sequence is indicated below the nucleotide sequence.

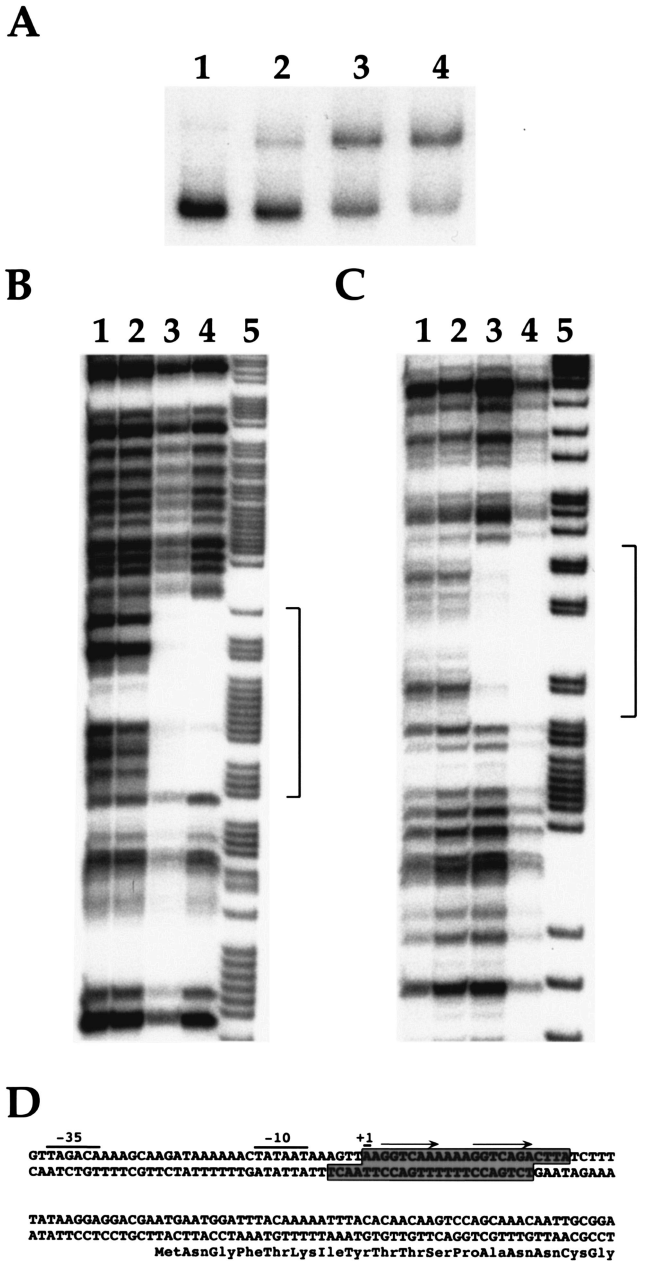

FIG. 4. (A) CtsR binds specifically to the *clpB* promoter region. DNA-binding reactions were performed with radiolabeled DNA fragments (10,000 cpm) corresponding to the *clpB* promoter region. Lane 1, no protein; lane 2, 7 ng; lane 3, 70 ng; lane 4, 700 ng. (B and C) DNase I footprinting analysis of CtsR binding to the *clpB* promoter region. Each lane contains 50,000 cpm of radiolabeled DNA fragment corresponding to the nontemplate strand (panel B) or the template strand (panel C) of the *L. monocytogenes clpB* promoter region. Fragments were incubated with increasing amounts of purified CtsR. Lane 1, no protein; lane 2, 35 ng; lane 3, 350 ng; lane 4, 3,500 ng; lane 5, Maxam and Gilbert reactions of the corresponding DNA fragment. Brackets indicate regions protected by CtsR. (D) Nucleotide sequence of the *clpB* promoter region. The DNase I protected area is boxed, and arrows indicate the CtsR direct-repeat recognition sequence. Positions are numbered relative to the translational initiation codon.

was end labeled, the protected region extended from positions −46 to −24 (Fig. 4C and D). All positions given are relative to the respective translational start site.

A single region within the *clpB* promoter is protected from DNase I cleavage, a finding which is in agreement with the single protein/DNA complex observed in the gel mobility shift DNA-binding assay (Fig. 4A). This protected region overlaps the transcriptional start site of *clpB* and contains the predicted CtsR direct-repeat recognition sequence (GGTCAAA AAA GGTCAGA) (Fig. 4D). These results indicate that CtsR negatively regulates *clpB* expression by directly binding to its operator sequence in the promoter region.

In conclusion, using both in vitro and in vivo approaches, we have shown that *L. monocytogenes clpB* is a heat shock gene that is under the negative regulation of CtsR, extending the *L. monocytogenes* CtsR regulon.

ClpB is involved in virulence of *L. monocytogenes*. Since ClpP, ClpC, and ClpE of *L. monocytogenes* have been shown to play a role in virulence (16, 43, 54), we therefore examined the virulence of an *L. monocytogenes* Δ*clpB* mutant in a murine model.

For this purpose, we constructed the LM2000 mutant strain of *L. monocytogenes*, in which the entire coding sequence of *clpB* was deleted (see Materials and Methods). Virulence of the Δ*clpB* strain was assayed by intravenous inoculation as described in Materials and Methods and was compared to that of the wild-type L028 strain. The 50% lethal dose of the Δ*clpB* mutant was $5.4 \times 10^{6.3}$ bacteria, whereas that of L028 was $5.4 \times 10^{4.2}$ bacteria. The Δ*clpB* mutant thus displays a significant decrease in virulence (100-fold).

We monitored the survival of mice for 12 days after an inoculation of 5.4×10^5 bacteria. Mice infected with the wild-type strain began to die after 5 days, and all were dead after the 10th day, whereas all animals infected by strain LM2000 (Δ*clpB*) were still alive after 12 days (Fig. 5). These results clearly show that ClpB plays a significant role in the pathogenicity of *L. monocytogenes*.

In order to determine whether ClpB of *Listeria monocytogenes* affects expression of virulence genes, primer extension experiments were carried out for the wild-type and for the Δ*clpB* and Δ*ctsR* mutants in order to compare expression of the *L. monocytogenes hly* gene, which encodes listeriolysin O, a major virulence determinant. Expression was identical for all three strains (data not shown), a finding which indicated that the major virulence PrfA regulon is not controlled by ClpB or CtsR and that the role for ClpB in virulence is most likely due to its chaperone activity rather than to a regulatory role in virulence gene expression.

ClpB is not required for general stress response but is necessary for heat shock-induced thermotolerance. Since several Clp proteins are involved in virulence and because many are also essential for resistance to various stress conditions, one might argue that Clp protein effects on pathogenicity may be indirect consequences of generally lowered cell fitness, thus leading to increased sensitivity to stress when invading the host.

A functional analysis of ClpB was undertaken, during which survival of the Δ*clpB* mutant was examined under different stress conditions. The LM2000 (Δ*clpB*) mutant strain had no obvious phenotype, since the mutant cells showed no morpho-

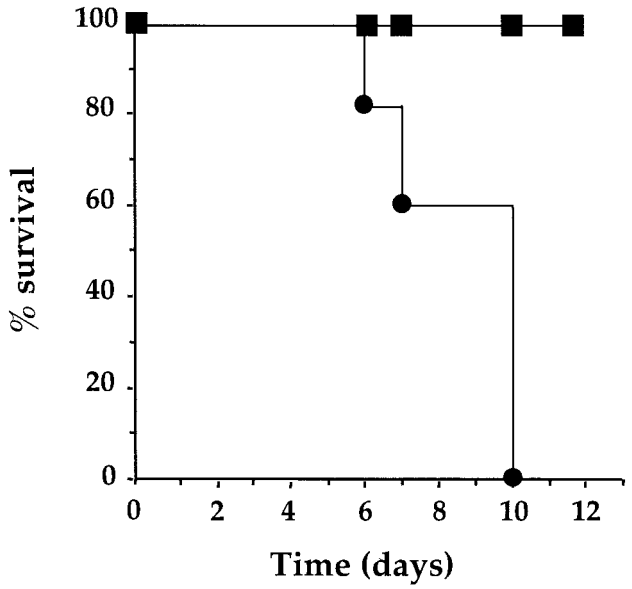

FIG. 5. ClpB is involved in virulence of *L. monocytogenes*. Survival curves for Swiss mice after intravenous inoculation with 5×10^5 bacteria of the wild-type L028 (●) or the mutant strain Δ*clpB* (■) are shown.

logical defects and because the growth curve in BHI at 37°C was identical to that of the LO28 reference strain (data not shown).

The stress resistance of the Δ*clpB* strain was evaluated under various conditions, such as heat stress, treatment with puromycin, the presence of salt, and SDS-induced stress, all of which are known to require the activity of other Clp proteins. Wild-type and mutant strains were grown in BHI medium until an OD_{600} of 0.3 was reached, cultures were divided into two parts, and one part was subjected to stress conditions (see Materials and Methods). The results presented in Fig. 6 summarize data obtained for typical growth curves for each stress condition. As shown in Fig. 6, growth of the wild-type and mutant strains was affected when the temperature was equal to or greater than 42°C, when the concentration of puromycin was greater than 30 μg/ml, or in the presence of 0.01% SDS. However, no difference was observed between the Δ*clpB* and the L028 reference strain, since the two strains grew equally well under all conditions tested. In conclusion, *L. monocytogenes* ClpB is not required for general stress adaptation, a finding that is contrary to the situation for gram-negative bacteria such as *E. coli* (29), *Brucella suis* (12), or *Helicobacter pylori* (1).

A recent study reports the induction of *L. monocytogenes clpB* during growth at low temperature (36), a condition which seems to induce the activity of most of the general stress proteins; for the cyanobacterium *Synechococcus* sp., ClpB was shown to be involved in cold adaptation (49). Growth at low temperature is an important part of the *L. monocytogenes* life cycle and is one which favors food contamination and outbreaks of food-borne disease. The role of ClpB in adaptation of *L. monocytogenes* to cold stress was tested. An overnight culture grown at room temperature was diluted and placed at 5°C for 4 days or was first grown to the mid-exponential phase at 37°C before shifting the culture to a temperature of 5°C. In both cases, the growth rate of the Δ*clpB* mutant at 5°C was the same as that of the parental strain (data not shown), suggesting that ClpB of *L. monocytogenes* is not involved with adaptation to cold stress.

It was previously shown that *L. monocytogenes* has a higher survival rate to lethal temperatures following previous exposure to a sublethal temperature (46, 56). This phenomenon is known as induced thermotolerance, a characteristic which stands in contrast to basal thermotolerance and has been described as occurring in many bacteria. ClpB was shown to be required for induced thermotolerance in the cyanobacterium *Synechococcus* sp. (13) and in the eukaryote *Saccharomyces*

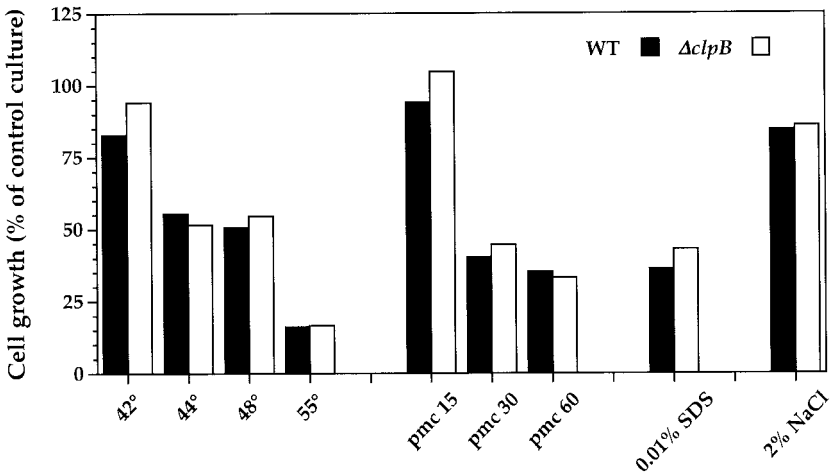

FIG. 6. ClpB is not required for stress resistance. The L028 wild-type (WT) strain and the Δ*clpB* mutant strain were grown exponentially at 37°C with aeration in BHI medium until the OD_{600} reached 0.3. The culture was divided into two parts, one of which was subjected to various stresses. Stresses assayed were temperature shifts to 42, 44, 48, or 55°C; addition of puromycin to a final concentration of 15 (pmc15), 30 (pmc30), or 60 (pmc60) μg/ml; 0.01% SDS; or 2% NaCl. Values represent the percentage of cell growth with respect to the control culture performed in the absence of stress (grown in BHI at 37°C) 2 h after the stress was applied. In all cases, there was no significant difference in the growth curves between the wild-type and the mutant strains.

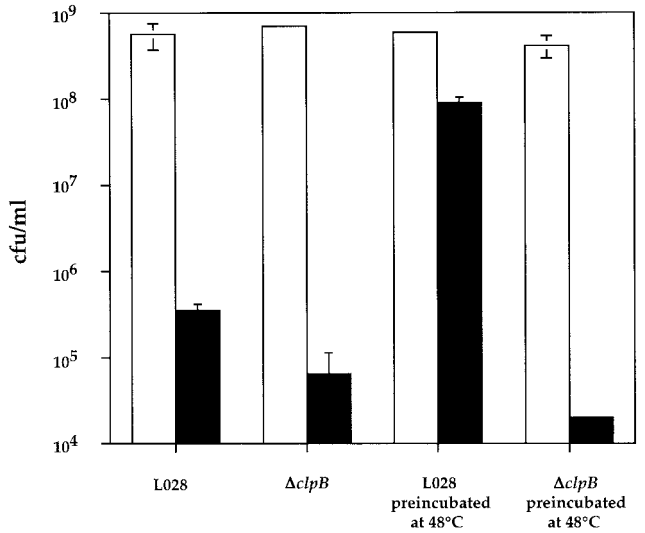

FIG. 7. ClpB is required for induced thermotolerance. Cultures of wild-type L028 and Δ*clpB* mutant strains were grown exponentially until the OD_{600} reached 0.3. Half of the culture was preincubated for 20 min at the nonlethal temperature of 48°C, while the other half was maintained at 37°C. After preincubation, both cultures were incubated at 60°C, and cell survival was evaluated by plating diluted aliquots. White bars indicate CFU values before incubation at 60°C; black bars indicate CFU values after 5 min of stress.

cerevisiae (58). In order to test the involvement of ClpB in induced thermotolerance of *L. monocytogenes*, we incubated both wild-type L028 and Δ*clpB* mutant strains in liquid BHI medium at 37°C until an OD_{600} of 0.3 was reached. The cultures were divided into two parts; one half was maintained at 37°C, and the other was preincubated at 48°C for 20 min. Both cultures were then subjected to heat treatment at 60°C. As shown in Fig. 7, preincubated wild-type cells presented an increased resistance to lethal heat shock, since after 5 min of incubation at 60°C, the survival rate was approximately 100-fold higher than that for untreated cells. In contrast, no induced thermotolerance could be observed for the Δ*clpB* strain, which remained as sensitive to lethal temperatures as were the untreated cells. Consequently, contrary to the situation for wild-type L028, a preincubation at 48°C did not protect Δ*clpB* cells, thus revealing a role for ClpB in induced thermotolerance.

DISCUSSION

Clp-HSP100 proteins make up a ubiquitous family of ATPases that act both as chaperones and as ATPase subunits for the Clp ATP-dependent protease. Most of them are induced by stress and are implicated in stress tolerance. Moreover, Clp proteins are involved in crucial steps of the infectious process for many gram-positive and gram-negative bacteria as well as in lower eukaryotes. In *L. monocytogenes*, ClpC, ClpP, and ClpE are involved in virulence and are required for thermotolerance and resistance to salt stress (16, 43, 44, 54). Their expression is thermoinducible and is under the negative control of CtsR, a repressor that binds to a heptad operator sequence in the promoter region (42).

Analysis of the complete sequence of *L. monocytogenes* EGDe (18) revealed several new *clp* genes. Just as for its closest relative, *B. subtilis*, we noted the presence of genes encoding orthologs to ATPase subunits ClpY (65.5% identity) and ClpX (81% identity) and to the proteolytic subunit ClpQ (78.5% identity). Surprisingly, and contrary to the situation for *B. subtilis*, there are two additional *clp* genes, both of which are preceded by potential operator sites for the CtsR repressor in their promoter regions. One of the encoded proteins shared 40% amino acid sequence identity with the ClpP proteolytic subunit of *L. monocytogenes* and is now referred to as ClpP2.

The second new *clp* gene revealed during our analysis has no ortholog in the low-G+C gram-positive model bacterium *B. subtilis* but shares strong similarities with *clpB* of *L. lactis*. We performed a systematic search for ClpB homologs to determine the extent of its distribution among low-G+C gram-positive bacterial genomes. Unlike the situation for *B. subtilis*, ClpB orthologs are found in all staphylococci, clostridia, and enterococci, as well as in *L. lactis*, *Listeria innocua*, and most bacilli. Streptococci seem to be the only group without this homolog, despite the presence in *S. mutans* of a ClpB-like protein presenting the characteristically long spacer region between both ATP-binding sites (34). However, the very divergent sequence places this paralog far from all the other known eubacterial *clpB* genes, suggesting a recent acquisition by way of horizontal transfer. Consequently, ClpB ATPases are well represented among low-G+C gram-positive bacteria.

Examination of the *clpB* promoter sequence revealed a typical σ^A promoter and a potential CtsR-binding site. In this work, we showed that CtsR represses the expression of *clpB* in *L. monocytogenes*. We showed in vitro that CtsR binds directly to the *clpB* promoter region. The gel mobility shift experiments reveal a single protein/DNA complex. This was confirmed by DNase I footprints in which only one protected region was observed, a finding which corresponded to the predicted CtsR box and overlapping the transcriptional start site.

A detailed DNA motif analysis of the complete genome of *L. monocytogenes* allowed us to determine that there appear to be only five members of the CtsR regulon: the *ctsR-clpC* operon, *clpP*, *clpE*, *clpB*, and potentially *clpP2*. Interestingly, although the *dnaK* operon is also preceded by a canonical CtsR operator, it does not seem to be controlled by this regulator, although the repressor can bind to this sequence in vitro (4).

Stress induction of *clp* genes is generally correlated with a role in stress resistance. Data from *E. coli* suggest that, unlike the other Clp ATPases, ClpB does not associate with the ClpP proteolytic subunit and has no effect on protease activity (68); consequently, ClpB acts exclusively as a chaperone.

In agreement with this activity, ClpB has been shown to have three closely related functions in several gram-negative bacteria and in eukaryotes: (i) resistance to high-temperature stress in *H. pylori*, *B. suis*, and *E. coli* (1, 12, 29, 62); (ii) cold acclimatization in *Synechococcus* sp.; and (iii) induced thermotolerance to lethal stress in the cyanobacterium *Synechococcus* sp. (13) and in the eukaryote *S. cerevisiae* (35, 58). HSP101, a member of the Clp/HSP100 family that is present in plants, has also been shown to be implicated in induced thermotolerance in both *Arabidopsis thaliana* (53) and maize (45). However, until now, only two *clpB* genes have been described for gram-positive bacteria, those of *Streptomyces albus* G (20) and *L. lactis* (27), and no obvious phenotype was associated with the

respective mutants. Indeed, the Δ*clpB* mutant of *L. lactis* was still resistant to temperature, salt, and puromycin stresses (27), and no thermosensitivity was observed for the *S. albus* mutant (C. Grandvalet, personal communication), even though both genes were shown to be thermoinducible. Here, we demonstrate that ClpB is required for induced thermotolerance of *L. monocytogenes*, which allows for better survival of lethal conditions when cells have been exposed to a nonlethal stress.

ClpB is required for induced thermotolerance of *L. monocytogenes*; this fact may contribute to the persistence of this bacterium and the health hazard it constitutes. Indeed, this bacterium has the ability to grow in a wide range of temperatures, even during the refrigeration process or after high-temperature short-time pasteurization and is considered one of the most thermotolerant bacteria among non-spore-forming foodborne pathogens (11, 14). It is clear that the ability of *L. monocytogenes* to grow at high temperatures is an important problem for food processing, and our results suggest that ClpB may be partially responsible for this adaptation faculty.

A deletion of *clpB* is associated with a reduction in virulence of several eukaryotes and gram-negative bacteria, such as *Leishmania major* (26), *Leishmania donovani* (6, 31, 32), *S. enterica* serovar Typhimurium (64), *Y. enterocolitica* (2), and *F. novicida* (21). However, the exact function of ClpB—and more generally, that of Clp homologs—in virulence is still unclear because their targets have not yet been discovered. Clp proteins, because of their central role in protein folding, are important factors for efficient growth and cell fitness. ClpP proteolytic subunits, for example, have pleiotropic roles, and their deletion, even in optimum conditions, greatly affects growth (5, 40). In most cases, Clp proteins involved in virulence are also required for stress survival, and since infection is one of the most stressful conditions encountered by bacteria, one can argue that effects observed in a *clp* deletion mutant are due to a deficiency in cell fitness. We have shown here that ClpB is not required for general stress survival of *L. monocytogenes*, with the exception of induced thermotolerance at 60°C. Thus, it is tempting to speculate that the significant reduction in *L. monocytogenes* virulence of the Δ*clpB* mutant might not be due to a reduction in survival ability or in adaptation to stressful conditions but rather to a specific alteration in a key process for pathogenic development, where ClpB probably acts as a chaperone. This speculation is supported by the fact that expression of the *hly* gene, a major virulence determinant belonging to the PrfA regulon, is not modified in the Δ*clpB* or Δ*ctsR* mutants.

In conclusion, our results demonstrate a role for ClpB in induced thermotolerance and present the first evidence for a role for ClpB in virulence of *L. monocytogenes*; our work constitutes the first description of phenotypes for a *clpB* gene in gram-positive bacteria.

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