Identification in *Listeria monocytogenes* of MecA, a Homologue of the *Bacillus subtilis* Competence Regulatory Protein

ELISE BOREZEE,[1] TAREK MSADEK,[2] LIONEL DURANT,[1] AND PATRICK BERCHE[1]*

*INSERM U411, Faculté de Médecine Necker, 75730 Paris Cedex 15,[1] and Unité de Biochimie Microbienne
(URA 1300, CNRS), Institut Pasteur, Paris,[2] France*

We identified in *Listeria monocytogenes* a gene encoding a protein homologous to MecA, a regulatory protein acting with ClpC and ComK in the competence pathway of *Bacillus subtilis*. In *L. monocytogenes*, MecA is involved, along with ClpC and ClpP, in the downregulation of a 64-kDa secreted protein. In *B. subtilis*, the MecA protein of *L. monocytogenes* behaves as a regulatory protein, controlling the transcription of *comK* and *comG*. Complete or disrupted ComK homologues were also found in *L. monocytogenes*. However, we failed to detect competence in various strains of *L. monocytogenes*, including those with intact ComK. Our results suggest that the functions of MecA in the saprophytes *L. monocytogenes* and *B. subtilis* have presumably diverged in response to their respective ecological niches.

The gram-positive bacterium *Listeria monocytogenes* is a food-borne pathogen widely spread in the environment, where it survives hostile conditions, presumably due to a rapid stress response. Among the stress proteins characterized for *L. monocytogenes*, the Clp ATPases are members of the HSP-100–Clp family belonging to a highly conserved class of universal molecular chaperones involved in the stress resistance and virulence of this pathogen (4, 14, 16). A homologue of ClpC (formerly designated MecB) in *Bacillus subtilis* acts both as a general stress protein and as a regulatory factor controlling the expression of competence (8). In the competence pathway, ClpC forms a complex with MecA to negatively regulate ComK (19), a transcriptional activator controlling the late competence genes required for the binding, processing, and internalization of transforming DNA (2). In the absence of ComS, MecA recruits ComK to the ClpC-ClpP proteolytic complex. When ComS is present, MecA is degraded by this complex (18, 19). The identification of ClpC (11, 12) and ClpP (3) in *L. monocytogenes* suggested that these proteins might have functions similar to those of their *B. subtilis* homologues. In this work, we searched in *L. monocytogenes* for MecA and other homologues of the *B. subtilis* competence pathway.

The *mecA* locus in *L. monocytogenes*. A 3-kb fragment was cloned and sequenced from an *XbaI*-digested genomic library constructed in pUC19 from *L. monocytogenes* (strain LO28) and screened with a 584-bp intragenic *B. subtilis mecA* probe by colony hybridization at low stringency (50°C). We found an open reading frame (ORF) (GenBank accession no. AF103794) encoding a putative protein of 217 amino acids and showing 49% and 42% identities with MecA of *B. subtilis* and *Bacillus firmus*, respectively, and 33% identity with YpbH of *B. subtilis*, a MecA homologue of unknown function. Another ORF, located upstream, encodes a putative protein of 144 amino acids and 82% identical to YjbD of *B. subtilis*, of unknown function (Fig. 1A). Alignment of the MecA sequences of *B. subtilis* and *L. monocytogenes* revealed two conserved domains, at the N terminus (up to residue 78) and the C terminus (after residue 125), with 74 and 45% identities, re-

spectively, and separated by a variable spacer region (data not shown). In *B. subtilis*, the N-terminal domain binds ComK and ComS and the C-terminal domain is involved in binding ClpC (10). MecA orthologues have also been identified in The Institute for Genomic Research genome database for several species of gram-positive bacteria, including *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Streptococcus mutans*, and *Staphylococcus aureus* (10). Using specific primers for *mecA* and *yjbD*, we showed by PCR that these genes are highly conserved and adjacent in strains of *L. monocytogenes* (EGD-E, ATCC 19115, ATCC 19111, CNL880203, CHUT861141, CNL895793, CNL895795, INRA119, and INRA85), *Listeria ivanovi* (ATCC 19119 and SLCC2379), *Listeria innocua* (ATCC 33090, CHUT861158, and INRA86), *Listeria seeligeri* (CHUT860478, CHUT861166, and CHUT861167), and *Listeria welshimeri* (CHUT860477) (data not shown). These strains were previously described (20).

Transcriptional analysis of the *mecA* locus. Northern blot analysis of total RNA from strain LO28 grown in brain heart infusion (BHI) broth at 37 and 42°C was performed with intragenic probes for *mecA* and *yjbD*. These two genes are strongly expressed during the exponential growth phase at 37°C (Fig. 1B, lanes 1 and 6) and more weakly expressed in the stationary phase (lanes 2 and 7). Transcription of these genes was not induced at an elevated temperature in the exponential phase (Fig. 1B, lanes 3 and 8) or the stationary phase (lanes 4 and 9), in contrast to that of *clpC* (12). We found two *mecA* transcripts of ~0.8 and ~1.5 kb and three *yjbD* transcripts of ~0.5, ~0.6, and ~1.5 kb. The detection of a 1.5-kb transcript by both probes strongly suggests that *mecA* and *yjbD* are co-transcribed as an operon. This notion was confirmed by the results of a transcriptional analysis of a *mecA*::*aphA-3* mutant of LO28 (described below) showing an 0.8-kb increase in the size of the larger transcript (2.3 kb), corresponding to the insertion of the kanamycin resistance cassette (*aphA-3*) into *mecA* (Fig. 1B, lanes 5 and 10). The 0.8-kb *mecA* transcript is also increased in size by the *aphA-3* insertion into *mecA* (Fig. 1B, lane 10), yielding a 1.5-kb transcript.

Construction and phenotypic analysis of a *mecA* mutant. A *mecA* mutant (*mecA*::*aphA-3′*) was constructed from LO28 by deletion of a 225-bp internal fragment (nucleotides 199 to 423) and insertion of a promoterless *aphA-3′* gene into the *mecA* gene using a previously described procedure (3). Using the

* Corresponding author. Mailing address: INSERM U411, Faculté de Médecine Necker, 156 Rue de Vaugirard, 75730 Paris Cedex 15, France. Phone: (33) 1 40 61 53 71. Fax: (33) 1 40 61 55 92. E-mail: berche@necker.fr.
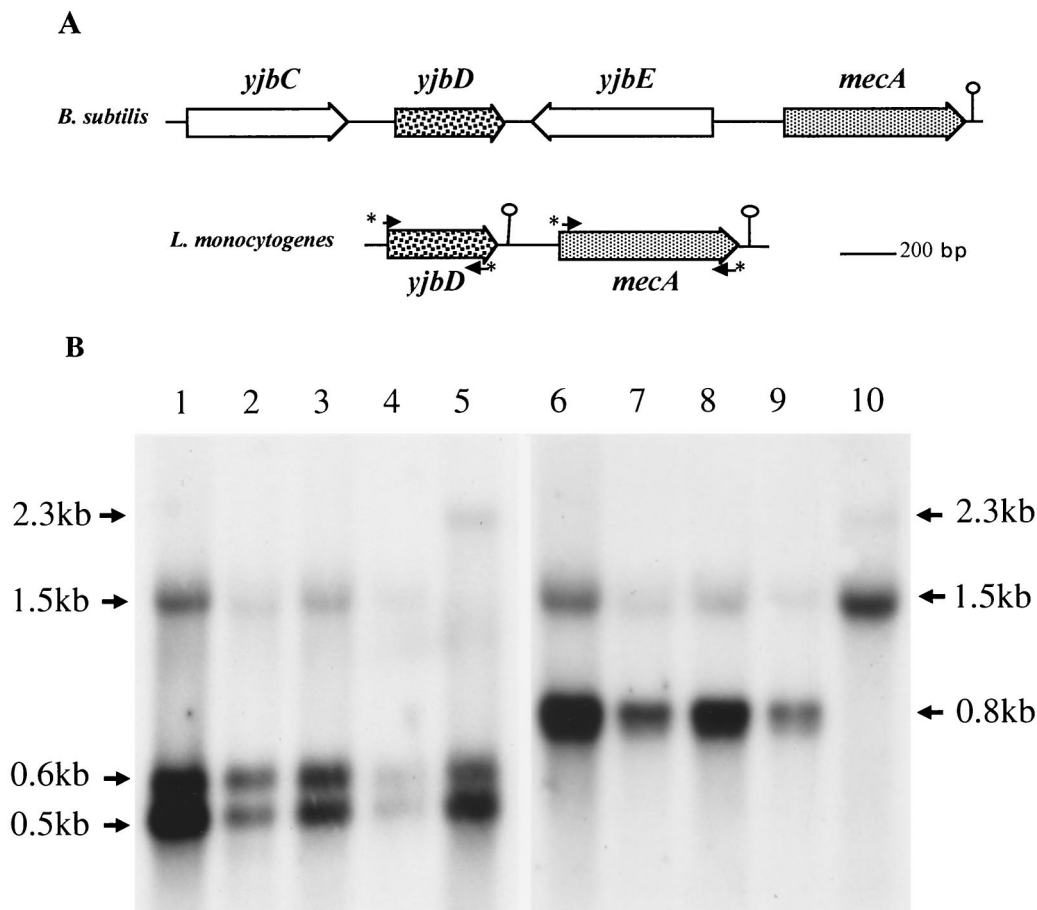
A

B

FIG. 1. (A) MecA regions in *B. subtilis* and *L. monocytogenes* LO28. Small arrows with asterisks indicate the positions of the primers used for PCR amplification of the *mecA* and *yjbD* regions. (B) Northern blot analysis of total RNA extracted from strain LO28 grown in BHI broth at 37 and 42°C. RNA samples were separated and hybridized with an intragenic *yjbD* probe (403 bp) (lanes 1 to 5) or *mecA* probe (584 bp) (lanes 6 to 10). These probes were obtained by PCR from the chromosomal DNA of LO28: *yjbD*, primers 5′-CCCGATAAGGAGTGTGAATG-3′ and 5′-GCGCTTCACGTAGTTGATACG-3′; *mecA*, primers 5′-CCCTTCATTGTCAATGA C-3′ and 5′-ACTAACGGCATTGTCAATG-3′. Lanes: 1 and 6, 37°C, exponential phase; 2 and 7, LO28, 37°C, stationary phase; lanes 3 and 8, LO28, 42°C, exponential phase; 4 and 9, LO28, 42°C, stationary phase; 5 and 10, *mecA* mutant, 37°C, exponential phase. The locations and sizes of mRNAs are indicated by arrows.

same strategy, we repeatedly failed to obtain a *yjbD* mutant of this strain; such a mutation might be lethal for the bacterium. Then, the *mecA* mutant was complemented using plasmid pAT18 (17) harboring *mecA* and its promoter region (1,123 bp). Controls included mutant and wild-type LO28 transformed with pAT18. There was no difference between the *mecA* mutant and the wild-type bacterium with regard to morphology during the exponential and stationary growth phases at 4, 30, 37, and 42°C; motility at 22°C; hemolysis on blood agar plates; and metabolic profiles on API strips (Biomerieux, Marcy l'Etoile, France). However, the exponential growth of the *mecA* mutant in BHI broth at 37°C was much slower than that of the wild-type bacterium. As measured by optical density, the generation time was almost twice that of the wild-type strain (1 h versus 0.5 h) but ultimately reached a similar value at the end of the exponential growth phase (Fig. 2). No difference in bacterial growth was observed between LO28 and the mutant transformed or not transformed by pAT18 alone (data not shown), indicating that the multicopy plasmid itself does not restrict bacterial growth. Transformation of the *mecA* mutant with pAT18-*mecA* partially restored bacterial growth at 37°C. A similar growth curve was found for LO28 transformed with pAT18-*mecA*, suggesting that large amounts of MecA might be responsible for this apparent incomplete restoration

of growth (Fig. 2). The same growth curves were obtained by measuring CFU, showing a good correspondence between viability and optical density (data not shown). So, the absence of MecA in *L. monocytogenes* did not result in a loss-of-viability phenotype, in contrast to the situation for *B. subtilis* (5). We also constructed, with the same strategy (3), a double *mecA clpC* mutant from a previously described *clpC* mutant of LO28 (11); the double mutant displayed a phenotype similar to that of the *clpC* mutant at 42°C. (data not shown).

MecA, ClpC, and ClpP of *L. monocytogenes* downregulate a 64-kDa secreted protein. As MecA of *B. subtilis* is a negative regulatory protein, we compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis the total and secreted protein profiles of *mecA* mutant and wild-type bacteria by using a procedure previously described (13). We found overexpression of a 64-kDa protein (p64) in the culture supernatant of the *mecA* mutant compared to LO28 (Fig. 3, lanes 5 and 2, respectively). An internal fragment of digested p64 was separated on DEAE-C$_{18}$ and sequenced as VISEPAVTTPVTLSD (J. D'Allayer, Institut Pasteur, Paris, France). According to the *Listeria* genome database (strain EGD-E), this fragment corresponds to an ORF encoding a putative protein of 569 amino acids (63.4 kDa). This ORF was amplified by PCR and sequenced in LO28 (GenBank accession no. AF282221), and the
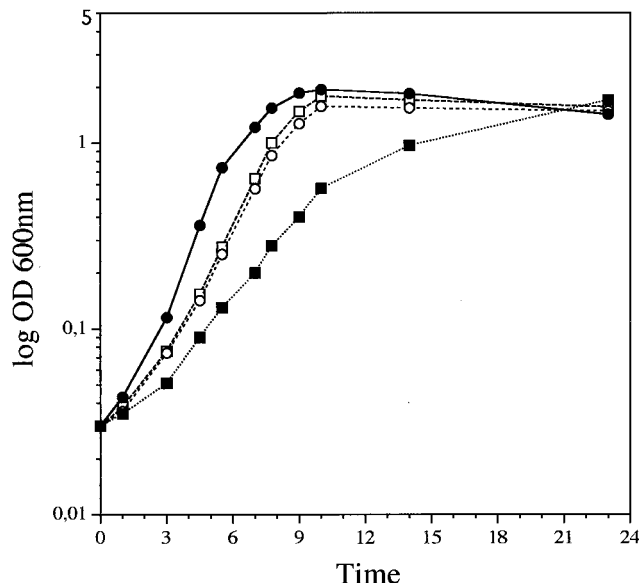
FIG. 2. Growth of *L. monocytogenes* LO28 *mecA* mutant at 37°C and complementation. Symbols: ●, LO28/pAT18; ○, LO28/pAT18-*mecA*; ■, LO28 *mecA* mutant/pAT18; □, LO28 *mecA* mutant/pAT18-*mecA*. Bacteria were grown in BHI medium, and the optical density (OD) at 600 nm was measured at various intervals.

deduced protein showed 100% identity with p64 from strain EGD-E. The p64 protein contains a signal sequence and two repeated domains with significant identity (~32%) to pXO1-88, a putative protein of unknown function encoded by virulence plasmid pXO1 of *Bacillus anthracis*. The expression of p64 was further examined with *clpC* and *clpP* mutants (3, 11) to determine whether MecA, ClpC, and ClpP had a common role in the regulation of this protein, as for *B. subtilis* ComK. The amount of p64 was also increased in these mutants (Fig. 3, lanes 6 and 7) but not in two other mutants (*prfA* and *oppA*) from strain LO28, used as controls (Fig. 3, lanes 3 and 4). These results suggest that MecA might be a regulatory protein, acting with ClpC and ClpP to downregulate a 64-kDa secreted protein.

L. monocytogenes MecA inhibits comK and comG transcription in B. subtilis. We studied the function of *L. monocytogenes* MecA in *B. subtilis* by using two *B. subtilis* strains, QB4673 and QB4842, in which transcriptional *lacZ* fusions with the promoter region from *B. subtilis comK* or *comG* were integrated as single copies at the *amyE* locus (8). Into these two strains, we introduced as a single copy in the *thrC* locus the *mecA* gene of *L. monocytogenes* LO28 under the control of the xylose-inducible promoter. We then disrupted *mecA* of *B. subtilis* by allelic replacement and integration of an *aphA-3* cassette, yielding strains QB8066 and QB8067 (for the methodology used, see reference 9). When bacteria were grown at 37°C in Luria-Bertani broth without xylose (*mecA* null mutant background), transcription of *comK* and *comG* was strongly induced (reaching in about 3 h ~600 to 700 U mg of protein^{-1}; in contrast, transcription was strongly downregulated by the addition of xylose (10-fold decrease) (data not shown). These results indicate that *L. monocytogenes* MecA is functional in the competence regulatory cascade of *B. subtilis*.

Identification of comK in L. monocytogenes. The previous results led us to search for *comK* in *L. monocytogenes*. From the *Listeria* genome database (strain EGD-E), we found in EGD-E a *comK*-like truncated gene cleaved into two parts and

separated by a 42-kb region containing several ORFs encoding phage-related products. Some were very similar to ORFs from the recently sequenced bacteriophage A118 of *L. monocytogenes*, which was also found to be integrated in a *comK*-like gene (6). The *comK*-like truncated gene from strain LO28 was sequenced (GenBank accession no. AF191727) and was almost identical to that in EGD-E. Upstream from '*comK*, the last gene of the phage was *int*, encoding a protein of 472 amino acids and 95% identical to the putative integrase of bacteriophage A118. The inactivation of *comK* might therefore explain the previous failure to demonstrate competence in LO28 (12). So, we screened by Southern blotting with intragenic probes for '*comK* and *int* the 18 strains of *Listeria* used here. *comK* was present in all strains tested, whereas *int* was present in only 5 of 10 *L. monocytogenes* strains, including EGD-E, and was absent from the other *Listeria* strains, except for 1 of 3 strains of *L. innocua*. *comK* from three integrase-negative strains was sequenced. The ComK proteins of *L. monocytogenes* strains ATCC 19115 and CNL895793 (GenBank accession no. AF191725 and AF191724, respectively) were almost identical proteins of 202 amino acids (99% identity), sharing 32% identity with ComK of *B. subtilis*. ComK of *L. seeligeri* strain CHUT860478 (GenBank accession no. AF191726) was 199 amino acids long and 33% identical to ComK of *B. subtilis*.

Competence tests in L. monocytogenes. Two *L. monocytogenes* strains with complete *comK* and their isogenic *mecA* deletion mutants, constructed as described above, were tested for competence in a two-step nutrient shiftdown procedure previously described (7), except for the composition of the competence minimal medium; this medium was adapted to *Listeria* by the addition (per liter) of L-leucine (100 mg), L-isoleucine (100 mg), L-valine (100 mg), L-methionine (100 mg), L-arginine (100 mg), L-cysteine (100 mg), L-histidine (100 mg), riboflavin (4 mg), biotin (4 mg), thiamine (1 mg), and thioctic acid (0.01 mg). Plasmid pMK4 was added to cultures in exponential or stationary growth phase at a concentration of 1 or 10 μg ml^{-1}, and transformants were selected on BHI agar supplemented with chloramphenicol at 10 μg ml^{-1}. *B. subtilis* strain 168, used as a control, was efficiently transformed with pMK4, even in the competence medium adapted for *Listeria*, but we repeatedly failed to transform *Listeria*, suggesting that this microorganism is not competent under the conditions tested. It remains possible that *Listeria* requires unusual conditions for competence. However, it is important to recall that high-level natural transformation of *B. subtilis* could be demonstrated only for a few strains isolated following extensive UV and X-ray mutagenesis (1, 15). The highly transformable strain
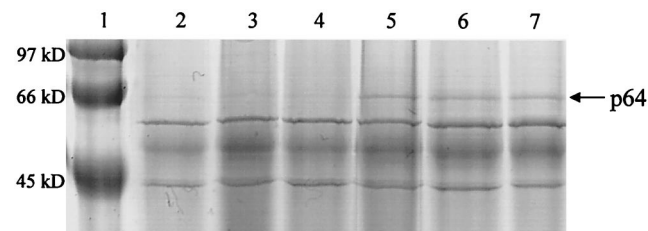

FIG. 3. A secreted 64-kDa protein accumulates in the culture supernatant of *L. monocytogenes* in the absence of MecA, ClpC, or ClpP. Bacteria were cultured in BHI medium until mid-log growth phase, and supernatants were collected and filtered. Supernatant protein extracts were prepared by trichloroacetic acid precipitation as previously described (13). Extracts with equal protein concentrations were applied to a sodium dodecyl sulfate–10% polyacrylamide gel and subjected to electrophoresis. Lane 1, molecular size marker; lane 2, LO28; lane 3, LO28 *oppA* mutant (unpublished data); lane 4, LO28 *prfA* mutant (12); lane 5, LO28 *mecA* mutant; lane 6, LO28 *clpC* mutant; lane 7, LO28 *clpP* mutant.

168 was then chosen for most studies. Thus, the situation for *L. monocytogenes* might be reminiscent of that for *B. subtilis*, with a cryptic DNA uptake apparatus presumably allowing only a very low level of natural transformation in its natural habitat.

From the *Listeria* genome sequence database, we found homologues for most of the early competence proteins (AbrB, CodY, DegU, SpoOKA, SpoOKB, SpoOKC, SpoOKD, and SpoOKE) and late competence proteins (ComC, ComEA, ComEB, ComEC, ComER, ComFA, ComFC, ComGA, ComGB, ComGC, ComGD, and ComGF) of *B. subtilis*, which form the surface structure capable of internalizing DNA. However, several homologues for early and late competence proteins involved in the competence of *B. subtilis* are absent in *L. monocytogenes*, including ComQ, ComS, ComX, ComFB, ComGE, and ComGG. The alternative is that these proteins may be involved in other, still-undefined functions in *L. monocytogenes*, presumably related to the transduction of environment signals. Our results suggest that the saprophytic species *L. monocytogenes* and *B. subtilis* have developed and adapted similar and complex regulatory cascades in response to specific environmental signals encountered in their respective ecological niches.

REFERENCES

1. **Burkholder, P. R., and N. H. Giles, Jr.** 1947. Induced biochemical mutations in *B. subtilis*. Am. J. Bot. **34:**345–348.
2. **Dubnau, D.** 1997. Binding and transport of transforming DNA by *Bacillus subtilis*: the role of type-IV pilin-like proteins—a review. Gene **192:**191–198.
3. **Gaillot, O., E. Pellegrini, S. Bregenholt, S. Nair, and P. Berche.** 2000. The ClpP serine protease is essential for the intracellular parasitism and virulence of *Listeria monocytogenes*. Mol. Microbiol. **35:**1286–1294.
4. **Gottesman, S., and M. R. Maurizi.** 1992. Regulation by proteolysis: energy-dependent proteases and their targets. Microbiol. Rev. **56:**592–621.
5. **Hahn, J., J. Bylund, M. Haines, M. Higgins, and D. Dubnau.** 1995. Inactivation of *mecA* prevents recovery from the competent state and interferes with cell division and the partitioning of nucleoids in *Bacillus subtilis*. Mol. Microbiol. **18:**755–767.
6. **Loessner, M. J., R. B. Inman, P. Lauer, and R. Calendar.** 2000. Complete nucleotide sequence, molecular analysis and genome structure of bacteriophage A118 of *Listeria monocytogenes*: implications for phage evolution. Mol. Microbiol. **35:**324–340.
7. **Msadek, T., V. Dartois, F. Kunst, M. L. Herbaud, F. Denizot, and G. Rapoport.** 1998. ClpC of *Bacillus subtilis* is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation. Mol. Microbiol. **27:**899–914.
8. **Msadek, T., F. Kunst, and G. Rapoport.** 1994. MecB of *Bacillus subtilis*, a member of the ClpC ATPase family, is a pleiotropic regulator controlling competence gene expression and growth at high temperature. Proc. Natl. Acad. Sci. USA **91:**5788–5792.
9. **Nair, S., I. Derre, T. Msadek, O. Gaillot, and P. Berche.** 2000. CtsR controls class III heat shock gene expression in the human pathogen *Listeria monocytogenes*. Mol. Microbiol. **35:**800–811.
10. **Persuh, M., K. Turgay, I. Mandic-Mulec, and D. Dubnau.** 1999. The N- and C-terminal domains of MecA recognize different partners in the competence molecular switch. Mol. Microbiol. **33:**886–894.
11. **Rouquette, C., C. de Chastellier, S. Nair, and P. Berche.** 1998. The ClpC ATPase of *Listeria monocytogenes* is a general stress protein required for virulence and promoting early bacterial escape from the phagosome of macrophages. Mol. Microbiol. **27:**1235–1245.
12. **Rouquette, C., M. T. Ripio, E. Pellegrini, J. M. Bolla, R. I. Tascon, J. A. Vazquez-Boland, and P. Berche.** 1996. Identification of a ClpC ATPase required for stress tolerance and in vivo survival of *Listeria monocytogenes*. Mol. Microbiol. **21:**977–987.
13. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
14. **Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist.** 1996. HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. **21:**289–296.
15. **Spizizen, J.** 1958. Transformation of biochemically deficient strains of *B. subtilis* by deoxyribonuclease. Proc. Natl. Acad. Sci. USA **44:**1072–1078.
16. **Squires, C., and C. L. Squires.** 1992. The Clp proteins: proteolysis regulators or molecular chaperones? J. Bacteriol. **174:**1081–1085.
17. **Trieu-Cuot, P., C. Carlier, C. Poyart-Salmeron, and P. Courvalin.** 1991. Shuttle vectors containing a multiple cloning site and a *lacZ* alpha gene for conjugal transfer DNA from *Escherichia coli* to gram-positive bacteria. Gene **102:**99–104.
18. **Turgay, K., J. Hahn, J. Burghoorn, and D. Dubnau.** 1998. Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. EMBO J. **17:**6730–6738.
19. **Turgay, K., L. W. Hamoen, G. Venema, and D. Dubnau.** 1997. Biochemical characterization of a molecular switch involving the heat shock protein ClpC, which controls the activity of ComK, the competence transcription factor of *Bacillus subtilis*. Genes Dev. **11:**119–128.
20. **Van Langendonck, N., E. Bottreau, S. Bailly, M. Tabouret, J. Marly, P. Pardon, and P. Velge.** 1998. Tissue culture assays using Caco-2 cell line differentiate virulent from non-virulent *Listeria monocytogenes* strains. J. Appl. Microbiol. **85:**337–346.