JOURNAL OF BACTERIOLOGY, May 1994, p. 3040–3048
0021-9193/94/$04.00+0

Vol. 176, No. 10

Plasmid-Borne Cadmium Resistance Genes in *Listeria monocytogenes* Are Similar to *cadA* and *cadC* of *Staphylococcus aureus* and Are Induced by Cadmium

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Received 22 November 1993/Accepted 2 March 1994

pLm74 is the smallest known plasmid in *Listeria monocytogenes*. It confers resistance to the toxic divalent cation cadmium. It contains a 3.1-kb *Eco*RI fragment which hybridizes with the *cadAC* genes of plasmid pI258 of *Staphylococcus aureus*. When introduced into cadmium-sensitive *L. monocytogenes* or *Bacillus subtilis* strains, this fragment conferred cadmium resistance. The DNA sequence of the 3.1-kb *Eco*RI fragment contains two open reading frames, *cadA* and *cadC*. The deduced amino acid sequences are similar to those of the *cad* operon of plasmid pI258 of *S. aureus*, known to prevent accumulation of Cd^{2+} in the bacteria by an ATPase efflux mechanism. The cadmium resistance determinant of *L. monocytogenes* does not confer zinc resistance, in contrast to the *cadAC* determinant of *S. aureus*, suggesting that the two resistance mechanisms are slightly different. Slot blot DNA-RNA hybridization analysis showed cadmium-inducible synthesis of *L. monocytogenes cadAC* RNA.

Listeria monocytogenes is a gram-positive bacterial pathogen responsible for opportunistic infections in humans and animals. Over the last decade, it has been implicated in a number of outbreaks and several sporadic episodes of listeriosis that have been traced to contaminated food (9). It is ubiquitous and found in the environment (9), probably because of its ability to grow in extreme conditions, including low temperatures and high concentrations of NaCl. Recently, it was shown that 35.8% of *L. monocytogenes* strains are able to survive in the presence of Cd^{2+} (26). In *L. monocytogenes*, cadmium resistance is more frequently plasmid borne than chromosomal: of cadmium-resistant strains, 12.8% are plasmid free (26).

Cadmium is a heavy metal, and its cation is toxic to microbial and other life forms. It enters bacteria via transport systems for essential divalent cations. The toxic effect of Cd^{2+} is believed to result from the inhibition of respiration caused by binding of the ions to sulfhydryl groups on essential proteins (54). However, some bacteria contain cadmium resistance determinants and are thus less susceptible to its toxic effect.

Six genetic determinants in bacteria are known to express cadmium resistance. Most prevent the accumulation of Cd^{2+} by active cation efflux; others sequester Cd^{2+} to small binding proteins, analogous to metallothioneins. Three cadmium resistance mechanisms are known in *Staphylococcus aureus*: two of them (*cadAC* and *cadB*) are plasmid borne and confer resistance to Cd^{2+} and Zn^{2+}, and the third is chromosomal and confers resistance to Cd^{2+} only (50, 58).

The *cadAC* determinant mediates a cadmium and zinc efflux mechanism and has been cloned and sequenced (37). *cadAC* comprises two genes, which encode the CadC and CadA polypeptides. CadC is a low-molecular-weight soluble protein, and CadA is a P-type ATPase, which allows efflux of cadmium. CadC is necessary for full resistance, but its role in efflux is not clear (61). DNA sequences similar to the *cadA* and *cadC* genes have been identified in the chromosome of the alkaliphilic *Bacillus firmus* OF4; the *cadC* gene from *B. firmus* partially complements sodium sensitivity in an *nhaA* mutant of *Escherichia coli* (19). *B. firmus* OF4 can resist cadmium concentrations as high as those of *S. aureus* harboring the entire *cadAC* determinant (61). However, there is no evidence that the chromosomal *cadA* and *cadC* genes of *B. firmus* confer cadmium resistance.

The *cadB* determinant (8, 36) probably acts via a cation sequestration mechanism (40) and contains two genes, *cadX* and *cadB* (7). The product of *cadB* is a 23.3-kDa protein unrelated to any proteins presently in the data banks. CadX is a small polypeptide with weak similarity to CadC.

The chromosomal determinant of *S. aureus* is a cadmium efflux system allowing resistance to lower concentrations of cadmium than the plasmid-borne *cadAC* determinant (58). The two cadmium efflux mechanisms of *S. aureus* were previously shown to be encoded by different genes on the basis of lack of hybridization of the *cadAC* determinant with total cellular DNA from chromosomally cadmium-resistant *S. aureus* strains (58). However, recent DNA sequence analysis shows that the genes are similar (reported in reference 49).

Cadmium resistance in the gram-negative bacterium *Alcaligenes eutrophus* is effected by a plasmid-borne efflux system, the *czc* system (35). It confers resistance to cobalt, zinc, and cadmium. The CzcABC proteins form a complex, which is predicted to actively transport the cations out of the bacterial cell (34). CzcD and CzcR are involved in regulation of *czc* expression (33). The *czc* genes do not show homology to any of the *S. aureus* genes.

In *Pseudomonas putida*, the plasmid-borne cadmium resistance determinant probably encodes a cadmium efflux system (16). However, no sequence data are available.

The last type of cadmium resistance involves low-molecular-weight, cysteine-rich proteins related to eucaryotic metallothioneins that sequester Cd^{2+}. These metalloproteins have

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TABLE 1. Bacterial strains and plasmid

Strain or plasmid	Relevant characteristics[a]	Plasmid	Reference or source
E. coli			
MC1061	F⁻ *araD139* Δ(*ara-leu*)*7696* Δ*lacY74 galU galK hsr hsm strA*		5
TG1	K-12, Δ(*lac-pro*) *supE thi hsdD5* F′ *traD36 proAB*⁺ *lacI*ᵠ *lacZ*ΔM15		Gibson (Medical Research Council, Cambridge, United Kingdom)
B. subtilis 168	*trpC2*		Laboratory stock
L. monocytogenes			
LO28	Clinical isolate of serovar 1/2c		Hospital Ramon y Cajal Collection (55)
Lm24	Serotype 1, Cd^s	pLm24	26
Lm40	Serotype 1, Cd^r	pLm40	26
Lm74	Serotype 1, Cd^r	pLm74	26
Lm101	Serotype 1, Cd^r	pLm101	26
Lm106	Serotype 4, Cd^r	pLm106	26
Lm162	Serotype 4, Cd^s	pLm162	26
Plasmid pKPY11	3.0-kb *Xba*I fragment containing entire *cadAC* operon of pI258 cloned into pT7-5		61

[a] Cd^s, cadmium sensitive; Cd^r, cadmium resistant.

been found in *Synechococcus* sp., *P. putida*, and *Thiobacillus thiooxidans* (15, 39, 44).

The aim of the present study was to determine the genetic and molecular basis of plasmid-borne cadmium resistance in *L. monocytogenes*. In this article, we present the characterization and complete nucleotide sequence of the cadmium resistance determinants of *L. monocytogenes* and provide direct evidence that cadmium resistance is induced by Cd^{2+}.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Table 1 lists the bacterial strains used. *E. coli* strains were grown on LB medium, *L. monocytogenes* strains were grown on brain heart infusion (BHI) broth or agar (Difco Laboratories, Detroit, Mich.), and *B. subtilis* strains were grown in BHI supplemented with 1% glucose. Ampicillin was added to a concentration of 100 μg/ml in agar and 25 μg/ml in liquid medium; chloramphenicol was added to 5 μg/ml as appropriate. All antibiotics, $3CdSO_4 \cdot 8H_2O$, and $ZnSO_4 \cdot 7H_2O$ were purchased from Sigma Chemical Co., St. Louis, Mo. Plasmid pUC18 (59) was used to clone DNA fragments in *E. coli*.

DNA techniques. Standard recombinant DNA techniques (45) were used for cleavage of DNA with restriction endonucleases (Boehringer, Mannheim, Germany, and Appligene, Illkirch, France), dephosphorylation of DNA ends with heat-killed phosphatase (Epicentre Technologies, Madison, Wis.), and ligation of DNA fragments with T4 DNA ligase (Amersham, Les Ulis, France). *L. monocytogenes* plasmids (pLm24, pLm40, pLm74, pLm101, pLm106, and pLm162; see Table 1) were isolated as described in reference 2 except that 5 mg of lysozyme (Sigma) per ml was added to the lysis solution. For Southern blot hybridization, DNA was cleaved with *Eco*RI and electrophoresed overnight at 20 V in 0.8% agarose. The DNA was transferred to a Hybond N membrane (Amersham) by the method of Southern (52). A *cadAC*-specific probe was prepared as follows. The 3.0-kb *Xba*I fragment of pKPY11, containing the entire *cadAC* resistance determinant of *S. aureus* (61), was isolated from a 1% agarose gel with the GeneClean kit (Bio 101, Inc., La Jolla, Calif.). The DNA was labeled with [^{32}P]dCTP with a random primer labeling kit

TABLE 2. Cadmium resistance of *L. monocytogenes* and *B. subtilis* harboring plasmids pLm74, pMK4, and pMa39

Strain	MIC[a]	
	Cd^{2+} (μM)	Zn^{2+} (mM)
L. monocytogenes		
Lm74	512	7
Lm74 cured	16	7
Lm74 cured(pLm74)	512	7
LO28	16	3.5
LO28(pMK4)	16	3.5
LO28(pMa39)	128	3.5
B. subtilis		
No plasmid	8	3.5
pMK4	8	3.5
pMa39	256	3.5
pLm74	256	3.5

[a] MICs were determined after 48 h of incubation of plates at 37°C.

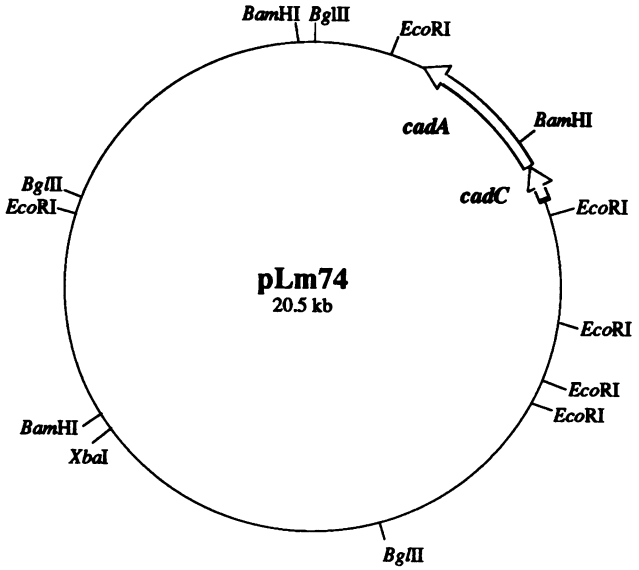

FIG. 1. Physical and genetic map of pLm74. The cadmium resistance genes *cadA* and *cadC* are indicated. The direction of transcription of the genes is given by the arrows.

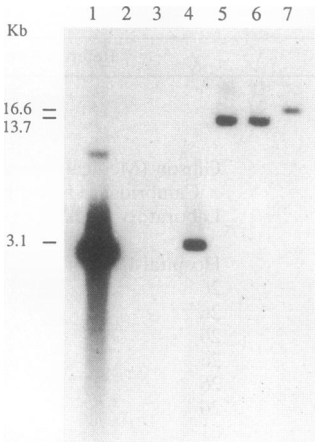

FIG. 2. Identification of restriction fragments of *L. monocytogenes* plasmids hybridizing with an *S. aureus cadAC*-specific probe under low-stringency conditions. The 3.0-kb *Xba*I fragment of plasmid pKPY11 was used as a probe. Lanes: 1, 3.0-kb *Xba*I probe DNA, control; 2 and 3, pLm24 and pLm162, respectively, from cadmium-sensitive *L. monocytogenes* strains, digested with *Eco*RI; 4 through 7, pLm74, pLm40, pLm101, and pLm106, respectively, from cadmium-resistant *L. monocytogenes* strains, digested with *Eco*RI.

(Amersham) and purified on a G50 column (Pharmacia) as recommended by the manufacturer. Southern blot hybridization was performed under conditions of low stringency as described previously (10).

***L. monocytogenes* electroporation and *B. subtilis* transformation.** Electroporation of *L. monocytogenes* LO28 was performed as described previously (30), and competent *B. subtilis* cells were transformed with recombinant plasmid DNA or native plasmid pLm74 as described elsewhere (24).

MICs. The agar dilution method (42) was used to determine the MIC to $3CdSO_4 \cdot 8H_2O$ and $ZnSO_4 \cdot 7H_2O$.

Plasmid curing. Strain Lm74 was cured of pLm74 by high-temperature treatment as described previously (26).

Nucleotide sequencing. The sequence of the cadmium resistance determinants was obtained from pMa4, a pUC18 vector that contained the 3.1-kb *Eco*RI fragment of pLm74. pMa4 was digested with exonuclease III (12) with the double-stranded nested deletion kit from Pharmacia. Appropriate clones for sequence determinations were chosen. The plasmids used as templates were purified with the Qiagen kit (Qiagen, Inc.), and sequencing reactions were performed by the dideoxynucleotide chain termination method of Sanger et al. (47) with [^{35}S]dATP (600 Ci/mmol), the T7 sequencing kit from Pharmacia, and the universal primer. The sequence of the second DNA strand of pMa4 was determined by directly sequencing plasmid pMa4 with oligonucleotide primers (18-mer) derived from the first strand of DNA. Oligonucleotides were supplied by the Unité de Chimie Organique, Institut Pasteur, Paris.

Computer analysis of sequences. A translated gene bank (Genpept; release 64.3) and the Swiss-Prot data bank (release 17.0) were searched for amino acid sequence similarities with the BLAST program (1).

RNA preparation and slot blot DNA-RNA hybridization. A 100-ml culture of *L. monocytogenes* Lm74 was grown at 37°C in BHI supplemented with 1% glucose to an optical density at 600 nm (OD_{600}) of 1.3. The culture was split into five aliquots: 2 or 20 μM Cd^{2+} was added to the cells, which were then incubated for 5 or 30 min at 37°C. The fifth aliquot was a Cd^{2+}-free control. Cultures were harvested and total cellular RNA was extracted as described elsewhere (28). RNA concentrations were determined spectrophotometrically at 260 nm, and purity was evaluated by the A_{260}/A_{280} ratio. RNA samples (1 and 5 μg) were denatured with 3 volumes of denaturing buffer (1× MOPS [morpholinepropanesulfonic acid], 50% deionized formamide, 2.2 M formaldehyde) at 65°C for 5 min, and SSC was added to a final concentration of 10× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate). RNA samples were deposited onto an Immobilon N membrane with a Bio-dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.). RNA was then fixed on the membrane by 20 min of incubation at 80°C. Prehybridization (1 h) and hybridization (2 h) at 65°C were performed under high-stringency conditions with the rapid hybridization system of Amersham. The membrane was then washed at 65°C twice in 2× SSC–0.1% sodium dodecyl sulfate (SDS) for 30 min, once in 1× SSC–0.1% SDS for 10 min, and twice in 0.7× SSC–0.1% SDS for 30 min and autoradiographed.

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 3 has been deposited in GenBank under accession number L28104.

RESULTS AND DISCUSSION

Characterization of plasmid pLm74. The cadmium-resistant *L. monocytogenes* strain harboring the smallest plasmid is Lm74: the plasmid was designated pLm74 (26). *L. monocytogenes* Lm74 is a strain of serotype 1, isolated in France from a food product. To determine whether cadmium resistance in Lm74 is plasmid borne, Lm74 was cured of its plasmid.

Loss of pLm74 caused a 32-fold decrease in the MIC of cadmium for Lm74. Reintroduction of pLm74 into the cured strains restored full resistance (Table 2). Thus, pLm74 confers cadmium resistance.

Plasmid pLm74 DNA was digested with restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, and *Xba*I. Its restriction map is given in Fig. 1. pLm74 is 20.5 kb long, smaller than the previous estimate of 29 kb (26).

Genes homologous to *S. aureus cadA* and *cadC* are present on plasmids conferring cadmium resistance in *L. monocytogenes*. The 3.0-kb *Xba*I fragment of plasmid pKPY11, which carries the *cadA* and *cadC* genes of pI258 of *S. aureus* (61), was used as a probe to identify similar sequences in *L. monocytogenes* plasmids conferring cadmium resistance. The probe hybridized to pLm74 and three other plasmids isolated from cadmium-resistant *L. monocytogenes* strains (pLm40, pLm101, and pLm106, chosen arbitrarily among cadmium resistance plasmids) but did not hybridize to two plasmids isolated from the cadmium-sensitive strains (pLm24 and pLm162) (Fig. 2). Thus, plasmids from *L. monocytogenes* strains that are resistant to cadmium contain genes similar to the *cadA* and *cadC* genes of *S. aureus*, whereas those from sensitive strains do not.

A 3.1-kb fragment of pLm74 is sufficient to confer cadmium resistance on cadmium-sensitive *L. monocytogenes* and *B. subtilis* strains. The *cadAC* probe of *S. aureus* hybridizes to a 3.1-kb *Eco*RI fragment of plasmid pLm74 (Fig. 2). To evaluate the role of this fragment in cadmium resistance, we cloned it in the *E. coli-Listeria* shuttle vector pMK4 (53), creating pMa39, and introduced pMK4 and pMa39 into cadmium-sensitive *L. monocytogenes* LO28 and into *B. subtilis*. We also introduced pLm74 into *B. subtilis*. Neither transformation of LO28 with pLm74 nor transformation of Lm74 cured of pLm74 with pMa39 could be obtained. The susceptibility to cadmium of all transformed strains was tested (Table 2). *L. monocytogenes* LO28 and *B. subtilis* transformed with pMK4 were as sensitive to Cd^{2+} as cells without plasmids. In contrast, pMa39 con-

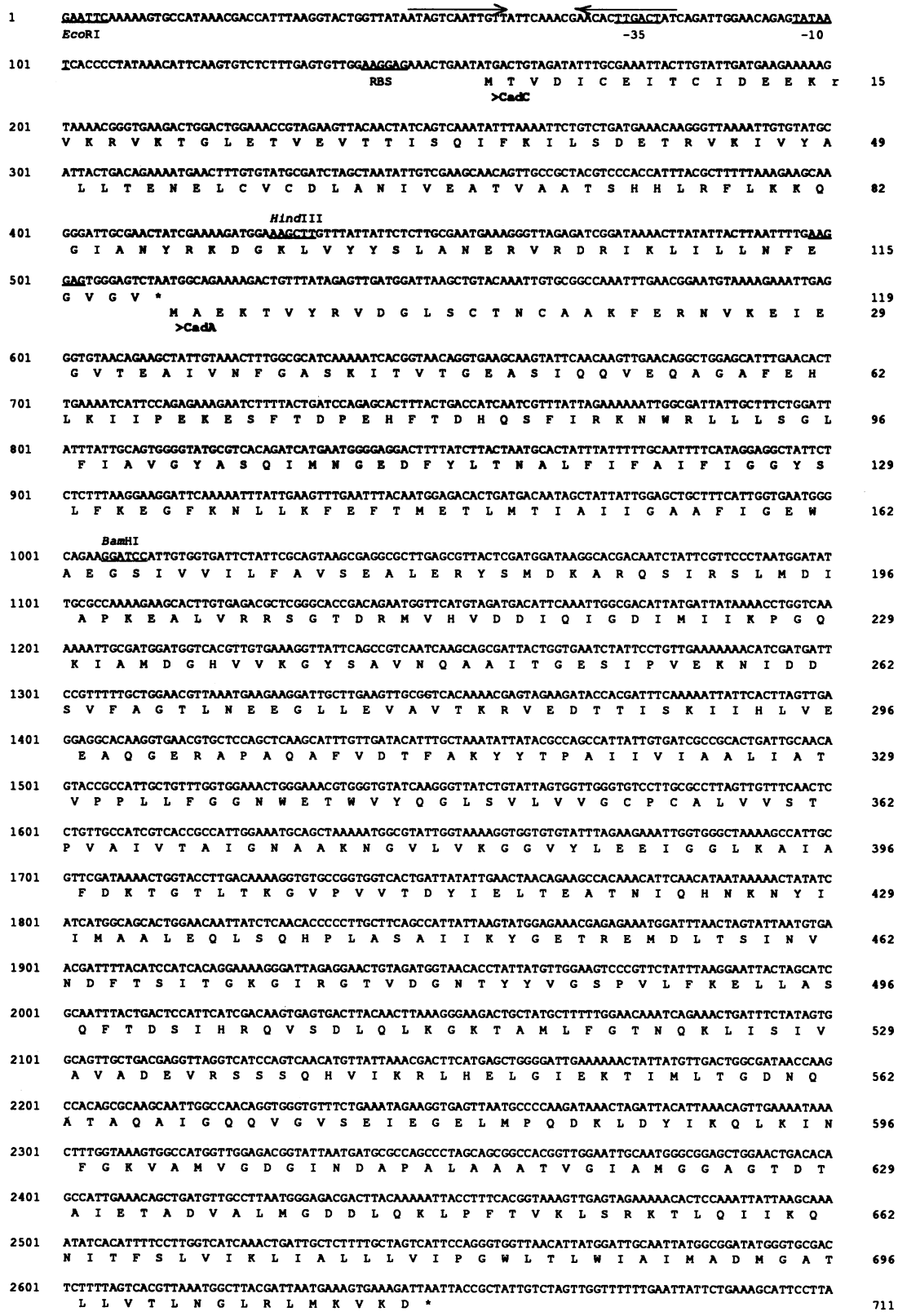

FIG. 3. Nucleotide sequence of the cadmium resistance determinant of pLm74. The deduced amino acid sequences of the cadmium resistance genes *cadA* and *cadC* are shown. Asterisks indicate stop codons. The ribosome-binding site (RBS), −10 and −35 consensus sequences, and *Eco*RI, *Bam*HI, and *Hin*dIII sites are indicated. Inverted repeat sequences are shown by arrows, and arrowheads indicate the direction of the transcription of each gene. Numbering starts at the first nucleotide of the *Eco*RI fragment.

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CadC L. monocytogenes     1   MTV----DICEITCIDEEKVKRVKTGLETVEV-TTISQIFKILSDETRVK
CadC B. firmus                MNK---KDTCEIFCYDEEKVNRIQGDLKTIDI-VSVAQMLKAIADENRAK
CadC S. aureus                MKK---KDTCEIFCYDEEKVNRIQGDLQTVDI-SGVSQILKAIADENRAK
CadX S. aureus.               MSY---ENTCDVICVHEDKVNNALSFLEDDKS-KKLLNILEKICDEKKLK
SmtB Synechococcus            MTKPVLQDGETVVCQGTHAAIASELQAIAPEVAQSLAEFFAVLADPNRLR
ArsR S. xylosus               MS----------------------------YKELSTILKVLSDPSRLE
ArsR E. coli                  M-----------------------LQLTPLQLFKNLS-------DETRLG
ArsR S. aureus                MS----------------------------YKELSTILKILSDSSRLE
NolR Rhizobium meliloti       MN---FRMEHTMQPLPPEKHEDAEI----------AAGFLSAMANPKRLL
HlyU Vibrio cholerae          MP---YLKGAPMNLQEMEKNSAKAV----------V--LLKAMANERRLQ
                              *                                     . .

CadC L. monocytogenes     46  IVYALLTENELCVCDLANIVEATVAATSHHLRFLKKQGIANYRKDGKLVY
CadC B. firmus                ITYALCQDEESCVCDIANIIGITAANASHHLRTLHKQGIVRYRKEGKLAF
CadC S. aureus                ITYALCQDEELCVCDIANILGVTIANASHHLRTLYKQGVVNFRKEGKLAL
CadX S. aureus.               IILSLIKEDELCVCDISLILKMSVASTSHHLRLLYKNEVLDFYKDGKMAY
SmtB Synechococcus            LLSLLAR-SELCVGDLAQAIGVSESAVSHQLRSLRNLRLVSYRKQGRHVY
ArsR S. xylosus               ILDLLSC-GELCACDLLEHFQFSQPTLSHHMKSLVDNELVTTRKNGNKHM
ArsR E. coli                  IVLLLREMGELCVCDLCMALDQSQPKISRHLAMLRESGILLDRKQGKWVH
ArsR S. aureus                ILDLLSC-GELCACDLLEHFQFSQPTLSHHMKSLVDNELVTTRKDGNKHW
NolR Rhizobium meliloti       ILDSLVK-EEMAVGALAHKVGLSQSALSQHLSKLRAQNLVSTRRDAQTIY
HlyU Vibrio cholerae          ILCMLLD-NELSVGELSSRLELSQSALSQHLAWLRRDGLVNTRKEAQTVF
                              .  *   *    .     . .  *...  *  .   .   ...

CadC L. monocytogenes     96  YSLANERVRDRIKLI---LLNFE---GVG----------------V
CadC B. firmus                YSLDDEHIRQ-IMMI---VLEHKKEVNVN----------------V
CadC S. aureus                YSLGDEHIRQ-IMMI---ALAHKKEVKVN----------------V
CadX S. aureus.               YFIKDDEIRE--------FFSKNHE---G----------------F
SmtB Synechococcus            YQLQDH------HIVALY----QNALDHLQE------CR--------
ArsR S. xylosus               YQL-NH------EFLDYI----NQNLDIINTSDQGCACKNMKSGEC-
ArsR E. coli                  YRLSPHIPSWAAQIIEQAWLSQQDDVQVIARKLASVNCSGSSKAVCI
ArsR S. aureus                YQL-NH------AILDDI----IQNLNIINTSNQRCVCKNVKSGDC-
NolR Rhizobium meliloti       YSSSSDAVLKILGALSDIYGDDTDAVEEKPLVRKSA-----------
HlyU Vibrio cholerae          YTLSSTEVKAMIELLHRLYCQANQ-----------------------
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FIG. 4. Comparison of *L. monocytogenes* CadC protein with related proteins. CadC of *L. monocytogenes* plasmid pLm74 was aligned with the program Clustal (14) with CadC of *B. firmus* OF4 (19), CadC of *S. aureus* plasmid pI258 (37), CadX of *S. aureus* plasmid pOX4 (7), SmtB of *Synechococcus* sp. strain PCC7942 (17), ArsR of *E. coli* plasmid R773 (46), ArsR of *S. aureus* plasmid pI258 (20), ArsR of *S. xylosus* plasmid pSX267 (41), NolR of *R. meliloti* (23), and HlyU of *V. cholerae* (57). Dashes represent gaps introduced to optimize similarity. Asterisks indicate identical residues in all sequences shown, and dots represent similar residues. Three putative metal-binding motifs identified in CadC of *L. monocytogenes* are overlined, and the putative helix-turn-helix motifs identified in CadC of *L. monocytogenes*, HlyU, SmtB, and NolR are underlined (see Results).

ferred cadmium resistance on *B. subtilis* and on *L. monocytogenes* LO28. In *B. subtilis*, pMa39 conferred the same level of resistance as plasmid pLm74, strongly suggesting that the 3.1-kb fragment of pLm74 carries all of the cadmium resistance genes. For *L. monocytogenes* LO28, the MIC of cadmium for cells harboring pMa39 was lower than that for strain Lm74. This difference in the degree of cadmium resistance conferred by pMa39 and pLm74 in two different *L. monocytogenes* strains could be due to differences in plasmid copy number. On the basis of plasmid DNA extraction, the difference in estimated plasmid copy number was much greater between the two plasmids extracted from the two *L. monocytogenes* strains Lm74 and LO28(pMa39) than between the two plasmids extracted from the two *B. subtilis* strains (data not shown).

Sequence of the cadmium resistance genes from pLm74 suggests that they encode a cadmium efflux system. The 3.1-kb *Eco*RI fragment conferring cadmium resistance was cloned into pUC18 to give plasmid pMa4. The nucleotide sequence of 2,700 bp of pMa4 was determined on both strands of the DNA and is shown in Fig. 3. Plasmid pMa4 contains two large open reading frames (ORFs) in the same direction. The smaller ORF, starting at position 155 and ending at position 514, is 360 bp long and encodes a 119-amino-acid protein. The largest ORF, beginning at position 514 and ending at base 2649, overlaps the 360-bp ORF by 1 bp. This 2,136-bp ORF could encode a protein of 711 amino acids. The first ORF is preceded by a putative ribosome-binding sequence (AAGG AG) complementary to the 3′-terminal sequence of *L. monocytogenes* 16S rRNA (27). It is preceded 53 bp upstream by a potential transcriptional start signal (Fig. 3). A 13-bp palindromic sequence was detected in the −35 region. Its calculated free energy was $\Delta G = -9.9$ kcal (3). A role for this palindrome is discussed below. The smaller ORF encodes a 13.5-kDa protein that has 50.4 and 47.8% sequence similarity with CadC of *S. aureus* (37) and CadC of *B. firmus* (19), respectively. It is also similar to CadX (33.9% identity), the product of the ORF in the sequence of another cadmium resistance system of *S. aureus*, designated *cadB* (7). By analogy with other CadC proteins, three putative metal-binding motifs can be proposed in the CadC homolog of *L. monocytogenes* (Fig. 4).

The CadC polypeptide of *L. monocytogenes* and those of *S. aureus* and *B. firmus* show similarity to transcriptional regulators, including ArsR of the arsenate-arsenite-antimony resistance system of *E. coli*, *S. aureus*, and *Staphylococcus xylosus* (20, 41, 46); SmtB, a polypeptide involved in the regulation of cadmium, copper, and zinc resistance in *Synechococcus* sp. strain PCC7942 (17); NolR, a factor controlling the *nod* regulon in *Rhizobium meliloti* (23); and HlyU, a regulator of expression of the hemolysin in *Vibrio cholerae* (57). As detected previously in the other regulators, a helix-turn-helix motif can be predicted in the *L. monocytogenes* CadC sequence

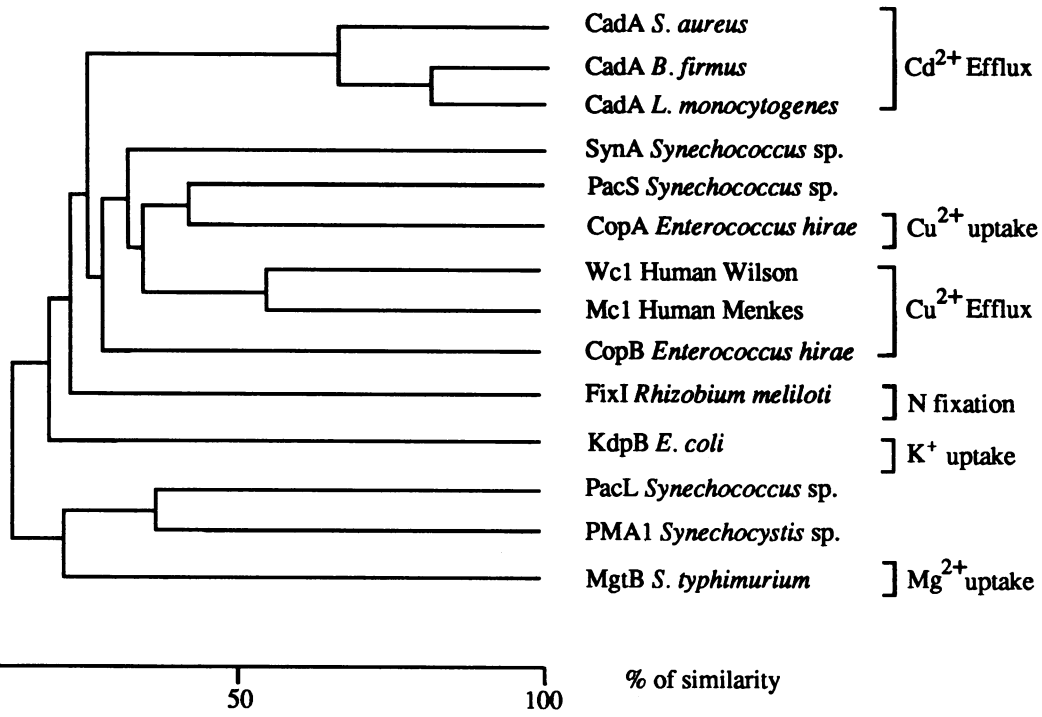

FIG. 5. Tree of sequence relationships among currently known procaryotic P-type ATPases and the Cu^{2+} P-type ATPase sequence involved in human Menkes' and Wilson's diseases. The length of each branch in the tree is a measure of relatedness as determined by the program Clustal (14). The primary amino acid sequences are CadA of *S. aureus* (37), CadA of *B. firmus* (19), SynA of *Synechococcus* sp. strain PCC7942 (accession number U04356), PacS and PacL of *Synechococcus* sp. strain PCC7942 (22), Mc1 of human Menkes' disease (6, 56), Wc1 of human Wilson's disease (4), CopA and CopB of *Enterococcus hirae* (38), FixI of *R. meliloti* (21), KdpB of *E. coli* (13), PMA1 of *Synechocystis* sp. strain PCC6803 (11), and MgtB of *Salmonella typhimurium* (51).

(Fig. 4). It is thus likely that CadC is a regulator of cadmium resistance. Interestingly, a putative cadmium-binding motif occurs close to the DNA-binding motif in the primary sequence at CadC. This could have implications for regulation of the expression of cadmium resistance.

The 2,136-bp ORF encodes a 711-amino-acid polypeptide of 77.1 kDa, showing 67.5 and 65.8% amino acid sequence similarity with the CadA polypeptides of *B. firmus* (19) and *S. aureus* (37), respectively. CadA is member of the family of P-type ATPases, which includes bacterial Cu^{2+}-, Mg^{2+}-, and K^+-ATPases, fungal H^+-ATPases, and ATPases in higher eucaryotes. ATP-driven active transport systems involving such ATPases have recently been reviewed (43, 48). The CadA homolog of *L. monocytogenes* is also similar to recently sequenced procaryotic and eucaryotic Cu^{2+}-transporting ATPases (4, 6, 29, 38, 56). CadA has 29 and 22.9% similarity with the Cu^{2+}-transporting ATPases CopA and CopB, respectively, of *Enterococcus hirae* (38), 27% similarity with the product of the Mc1 gene, which is a Cu^{2+}-transporting ATPase implicated in human genetic X-linked Menkes' disease (6, 29, 56), and 24.7% similarity with the product of the Wc1 gene, which is a Cu^{2+}-transporting ATPase implicated in human genetic Wilson's disease (4). Twelve sequences of closely related procaryotic P-type ATPases have been identified to date (Fig. 5).

L. monocytogenes CadA has the basic structural elements and regions of similarity observed for the P-type ATPases. It contains all the recognizable motifs and key conserved residues necessary for P-type ATPase function (50): (i) a region possibly involved in cation binding; (ii) the phosphatase site; (iii) a Cd^{2+} channel region; (iv) the aspartyl phosphorylation site; and (v) the ATP-binding site. Figure 6 shows sequence alignments of the metal-binding domains and conserved regions of presumed functional significance among P-type ATPases. CadA of *L. monocytogenes* has a low cysteine content (4 of 711 residues), like *S. aureus* and *B. firmus* CadA. The cysteines are probably implicated in metal binding and are found at positions 14, 17, 354, and 356. The last pair of cysteines flank an invariant proline residue, characteristic of virtually all P-type ATPases, and may be involved in substrate affinity. A conserved Thr-Gly-Glu-Ser tetrapeptide believed to be involved in removing phosphate from its covalently bound position on Asp-398 is present at positions 250 through 253, and the seven-amino acid kinase stretch (Asp-Lys-Thr-Gly-Thr-Leu-Thr) which is always conserved in P-class ATPases is present at positions 398 through 404: Asp-398 is expected to be phosphorylated by ATP. CadA also has 10 residues (603-Val-Gly-Asp-Gly-Ile-Asn-Asp-Ala-Pro-Ala-612) which fix ATP.

In conclusion, the sequence of the cadmium resistance determinant suggests that *L. monocytogenes* is resistant to cadmium by an energy-dependent efflux mechanism encoded by the genes *cadA* and *cadC*, which are carried on plasmids and prevent accumulation of cadmium in the cell.

***L. monocytogenes* cadmium resistance determinant does not confer zinc resistance.** In *S. aureus*, the *cadAC* efflux system confers cadmium and zinc resistance (61). Zinc resistance in *L. monocytogenes* has never been investigated. *L. monocytogenes* and *B. subtilis* transformed with pLm74 and pMa39 were tested for zinc susceptibility. The MIC of zinc did not change in the presence of either pLm74 or pMa39 (Table 2). This suggests that the plasmid-borne cadmium resistance determinant of *L. monocytogenes* does not confer zinc resistance.

The chromosomal cadmium resistance determinant from methicillin-resistant *S. aureus* confers resistance to cadmium but not to zinc (58). The DNA sequence of this chromosomally

A. Metal binding locus

```
Cd2+, L. monocytogenes     9-VDGLSCTNCAAKFERNVKEIEGV-31
Cd2+, B. firmus              VQGFTCANCAGKFEKNVKQLSGV
Cd2+, S. aureus              VQGFTCANCAGKFEKNVKKIPGV
Cu2+, Human                  VEGMTCNSCVWTIEQQIGKVNGV
Cu2+, E. hirae, CopA         ITGMTCANCSARIEKELNEQPGV
Hg2+, T. ferrooxidans        ITGMTCAHCAHSVEKALLGIHGI
Hg2+, MerP, Tn501            VPGMTCSACPITVKKAISEVEGV
Hg2+, MerA, Tn501            ITGMTCDSCAAHVKEALEKVPGV
                               *  *  *          *
```

B. ATPase domains

	Phosphatase	Ion channel	Aspartyl kinase
Cd^{2+}, *L. monocytogenes*	232-AMDGHVVKGYSA---VNQAAITGESIPVEKNID--DS-263	354-CPCALVVSTP-363	387-EEIGGLKAIAFDKTGTLTKG-406
Cd^{2+}, *B. firmus*	AMDGVVVSGYSA---VNQTAITGESVPVEKTVD--NE	CPCALVISTP	EEMGALKAIAFDKTGTLTKG
Cd^{2+}, *S. aureus*	AMDGIIVNGLSA---VNQAAITGESVPVSKAVD--DE	CPCALVISTP	EKLGAIKTVAFDKTGTLTKG
Cu^{2+}, Human	PVDGRVIEGHSM---VDESLITGEAMPVAKKPG--ST	CPCSLGLATP	EMAHKVKVVVFDKTGTITHG
Cu^{2+}, *E. hirae*, CopA	PTDGRIIAGTSA---LDESMLTGESVPVEKKEK--DM	CPCALGLATP	EGAAHLNSIILDKTGTITQG
FixI, *R. meliloti*	PVDGRVLSGTSD---LDRSVVNGESSPTVVTTG--DT	CPCALGLAVP	ERLAEIDTVLLDKTGTLTIG
Cu^{2+}, *E. hirae*, CopB	PTDGTIDKGHTI---VDESAVTGESKGVKKQVG--DS	CPHALGLAIP	EQANDLDVIMLDKTGTLTQG
H^+/K^+, *E. coli*	PCDGEVIEGGAS---VDESAITGESAPVIRESG--GD	IPTTIGGLLS	EAAGDVDVLLLDKTGTITLG
Na^+/K^+, human	PADLRIISANGC--KVDNSSLTGESEPQTRSPDFTNE	VPEGLLATVT	ETLGSTSTICSDKTGTLTQN
Ca^{2+}, rabbit	PADIRILSIKSTTLRVDQSILTGESVSVIKHTEPVPD	IPEGLPAVIT	ETLGCTSVICSDKTGTLTTN
	* **	*	* ***** *

	ATP binding
Cd^{2+}, *L. monocytogenes*	583-PQDKLDYIKQLKINFGKVAMVGDGINDAPALAAATVGIAM-622
Cd^{2+}, *B. firmus*	PQDKLDFIKQLRSEYGNVAMVGDGVNDAPALAASTVGIAM
Cd^{2+}, *S. aureus*	PQDKLDYIKKMQSEYDNVAMIGDGVNDAPALAASTVGIAM
Cu^{2+}, Human	PSHKVAKVKQLQEEGKRVAMVGDGINDSPALAMANVGIAI
Cu^{2+}, *E. hirae*, CopA	PEEKANYVEKLQKAGKKVGMVGDGINDAPALRLADVGIAM
FixI, *R. meliloti*	PREKVQVCAAAAEAGHKALVVGDGINDAPVLRAAHV---M
Cu^{2+}, *E. hirae*, CopB	PDDK-AIVQRYLDQGKKVIMVGDGINDAPSLARATIGMAI
H^+/K^+, *E. coli*	PEAKLALIRQYQAEGRLVAMTGDGTNDAPALAQADVAVAM
Na^+/K^+, human	PQQKLIIVEGCQRQGAIVAVTGDGVNDSPALKKADIGVAM
Ca^{2+}, rabbit	PSHKSKIVEYLQSYDEITAMTGDGVNDAPALKKAEIGIAM
	* * *** ** * *

FIG. 6. Alignments of the key features of CadA of *L. monocytogenes* with related proteins. (A) Alignment of Cd^{2+}-binding domains of CadA from *L. monocytogenes*, *S. aureus* (37), and *B. firmus* (19) with protein motifs involving metal ion binding (Cu^{2+} [6, 29, 38, 56] and Hg^{2+} [18, 31, 32]). (B) Functional-domain alignment of procaryotic and eucaryotic P-type ATPases. The primary references for sequences shown can be found in reference 56. Asterisks indicate identical residues.

encoded system shows that it is a P-type ATPase, closely related to CadA of *S. aureus* and *B. firmus* (49) and therefore also similar to the *cadAC* system in *L. monocytogenes*.

Cadmium resistance determinants are induced by Cd^{2+}. Plasmid-encoded ion transport mechanisms are generally regulated at the transcriptional level and are induced by their substrates (50). We investigated cadmium resistance inducibility by two different approaches.

The amounts of *cadA* and *cadC*-specific mRNA in strain Lm74 in the presence of subinhibitory concentrations of cadmium were measured by slot blot hybridization with a *cadA*/*cadC*-specific probe. In the absence of cadmium, *cadA*/*cadC*-specific RNA was barely detected (Fig. 7, lane 1), whereas in the presence of cadmium, *cadA* and *cadC* RNA signals were intense (Fig. 7, lanes 2 to 5). *cadA*/*cadC*-specific RNA was detected after 5 min in the presence of 2 μM cadmium, and the signals were not significantly different with a higher concentration of Cd^{2+} (20 μM) or longer incubation in the presence of Cd^{2+} (30 min) (Fig. 7, lanes 2 to 5). Thus, the transcription of the *cadA* and *cadC* genes is induced immediately after the addition of subinhibitory concentrations of cadmium to the culture medium.

To confirm this finding, *L. monocytogenes* Lm74 cells were grown to an OD_{600} of 0.6. The culture was split into three aliquots, which were treated in three different ways: (i) the cells were incubated with 2 μM Cd^{2+} for 30 min (induction) and challenged with 384 μM Cd^{2+}; (ii) the cells were not induced but were challenged with 384 μM Cd^{2+}; and (iii) the cells were

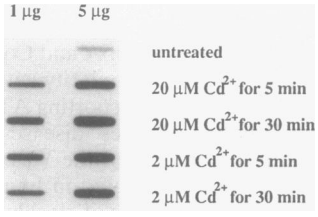

FIG. 7. Effect of cadmium on transcription of the *cadAC* genes. Aliquots of exponentially growing *L. monocytogenes* Lm74 were treated with cadmium-free medium (control), 20 μM Cd^{2+} for 5 min, 20 μM Cd^{2+} for 30 min, 2 μM Cd^{2+} for 5 min, or 2 μM Cd^{2+} for 30 min. RNA was then isolated and probed for hybridization with a ^{32}P-labeled *cadA-cadC* probe consisting of a 578-bp *Bam*HI-*Hin*dIII fragment of pLm74 (Fig. 2), a region spanning the 3′ end of *cadC* and 5′ end of *cadA*, and exposed for 2 h at −80°C. The same blot was reprobed with a 16S rRNA probe to assess the total amount of RNA loaded on the membrane (data not shown).

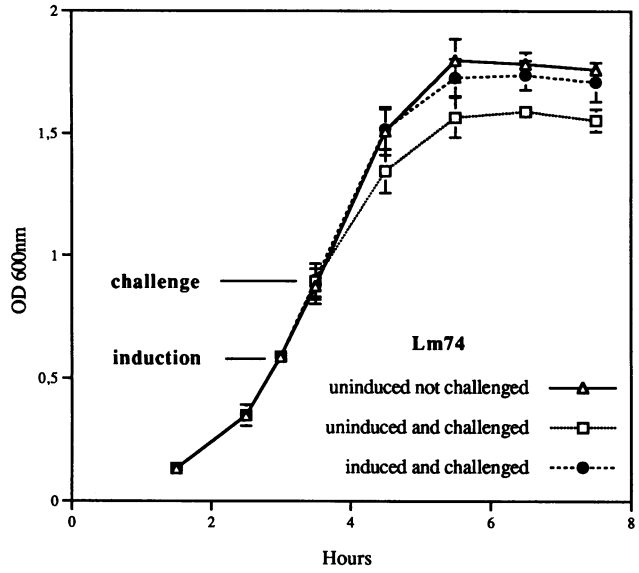

FIG. 8. Induction of cadmium resistance by Cd^{2+}. *L. monocytogenes* Lm74 cells harboring pLm74 were neither induced nor challenged (△), induced with 2 μM Cd^{2+} and challenged with 384 μM Cd^{2+} (●), or not induced and challenged with 384 μM Cd^{2+} (□). Similar curves were obtained in at least three separate experiments. Bars show standard errors of the mean.

not induced or challenged (control). Induced cells grew as well in presence of 384 μM Cd^{2+} as the control uninduced cells (Fig. 8). Uninduced cells grew more slowly. These results show that cadmium resistance is induced by trace amounts (2 μM) of Cd^{2+} ions in the culture medium.

The *cadAC* cadmium efflux system in *S. aureus* is also inducible by Cd^{2+} (60), and a 7-bp inverted repeat, centered on the *cadAC* transcriptional start signal, was shown to be required for induction of cadmium resistance (60). Deletion of the distal portion of the inverted repeat of *S. aureus* leads to low-level constitutive activity of the promoter (60). This inverted repeat was suggested to be a binding site for an unidentified cadmium-regulatory protein. No similar inverted repeat is present at the putative +1 position in pLm74, but an imperfect inverted repeat of 13 bp is present in the putative −35 region of the *L. monocytogenes cadAC* genes (see above). It may have a role in the transcriptional regulation of *cadC* and *cadA*.

This study demonstrates that in *L. monocytogenes*, cadmium resistance is mediated by two genes similar to the *cadAC* determinants of *S. aureus*, which prevent accumulation of Cd^{2+} in the bacteria by an ATP efflux mechanism. Sequences hybridizing to *cadAC* of *S. aureus* are found on various plasmids in several cadmium-resistant strains of *L. monocytogenes*. The accompanying article (25) describes evidence that the spread of the *cadAC* sequences has been mediated by transposon Tn*5422*.

ACKNOWLEDGMENTS

We are indebted to S. Silver for providing plasmid pKPY11. We thank K. Dyke for communicating results prior to publication, T. Msadek for help in transformation of *B. subtilis*, and J. McLauchlin and A. Edelmam for critical reading of the manuscript. M.L. is particularly grateful to all members of the Laboratoire de Génétique Moléculaire des *Listeria* for advice and discussions.

This work was supported by a grant from the Conseil Régional de la Région Centre, CNRS (URA 1300), from the EEC Programme Science (SCI CT91 0682), the Ministère de l'Agriculture (R91-37), and the Pasteur Institute.

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