Molecular Characterization of Cadmium Resistance in *Streptococcus thermophilus* Strain 4134: an Example of Lateral Gene Transfer

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Two genes (*cadC_{St}* and *cadA_{St}* [subscript *St* represents *Streptococcus thermophilus*]), located on the chromosome of *S. thermophilus* 4134, were shown to constitute a cadmium/zinc resistance cassette. The genes seem to be organized in an operon, and their transcription is cadmium dependent *in vivo*. The proposed product of the *cadA* open reading frame (*CadA_{St}*) is highly similar to P-type cadmium efflux ATPases, whereas the predicted protein encoded by *cadC_{St}* (*CadC_{St}*) shows high similarity to ArsR-type regulatory proteins. The observed homologies and G+C content of this cassette and surrounding regions suggest that this DNA was derived from *Lactococcus lactis* and may have been introduced relatively recently into the *S. thermophilus* 4134 genome by a lateral gene transfer event. The complete cassette confers cadmium and zinc resistance to both *S. thermophilus* and *L. lactis*, but expression of *cadA_{St}* alone is sufficient to give resistance. By using electrophoretic mobility shift assays it was shown that the *CadC_{St}* protein is a DNA binding protein that binds specifically to its own promoter region, possibly to two copies of an inverted repeat, and that this *CadC_{St}*-DNA interaction is lost in the presence of cadmium. Using *lacZ* fusion constructs it was shown that the cadmium-dependent expression of *CadA_{St}* is mediated by the negative regulator *CadC_{St}*. A model for the regulation of the expression of cadmium resistance in *S. thermophilus* is discussed.

Lactic acid bacteria (LAB) have been used for centuries in food preservation processes and are used with increasing intensity for specific industrial food fermentation processes. The efforts to create functional foods that have additional beneficial properties for human health, like increased vitamin or amino acid content, necessitate the development of food-grade marker systems as well as the discovery of regulated promoters that can be used for targeted protein expression during food fermentation. Antibiotic resistance has been rejected as a selectable marker for microorganisms destined for use in food production because of the risk of spreading the antibiotic resistance in the human intestinal microflora and ultimately to pathogenic bacteria. Hence, efforts have focused on the identification of alternative selectable markers such as bacteriocin immunity or heavy metal resistance (15, 30).

The heavy metal cadmium is toxic in its ionized form to many organisms, including microbes and humans. It is believed to enter the bacterial cell via transport systems normally used for essential divalent cations and to exert its toxic effect by inhibiting respiration through binding to sulfhydryl groups of essential proteins (39). Bacteria carrying cadmium resistance mechanisms have a selective advantage for survival in the environment (17). Unlike resistance systems to mercury and arsenic, which are highly homologous in all bacteria studied, cadmium resistance seems to have evolved at least three times, having given rise to the ATP-dependent cadmium efflux transporters found in gram-positive bacteria, the unrelated chemiosmotic cation-proton antiporters present in gram-negative bacteria, and the metallothionein system used by cyanobacteria (34).

The cadmium resistance system found in gram-positive bacteria is encoded by two adjacent genes, *cadA* and *cadC*, and was first identified in *Staphylococcus aureus*, the only organism in which the function of the two genes was studied in detail (10, 27, 28). Since then, homologues of these genes have been identified in many other bacterial species, e.g., *Bacillus firmus*, *Listeria monocytogenes*, *Lactococcus lactis*, *Listeria innocua*, *Stenotrophomonas maltophilia*, and *Geobacillus stearothermophilus* (1, 12, 14, 18, 20, 25a). However, only for a few organisms (e.g., *S. aureus*, *L. monocytogenes*, and *L. lactis*) were they actually shown to confer cadmium resistance (18, 20, 28).

The cadmium resistance system of *S. aureus* is specified by two adjacent genes, *cadA* and *cadC*, both of which are necessary for cadmium resistance *in vivo* (42). The *CadA* protein has been shown to be a cadmium efflux ATPase of the P type, i.e., a transport ATPase that has a covalent phospho-protein intermediate (38). *CadC* was identified by *in vitro* assays as a *trans*-acting DNA binding negative regulatory protein, whose DNA binding capacity is diminished *in vitro* in the presence of cadmium (10). The binding properties of *CadC* from *S. aureus* on the *cadA* operator/promoter DNA region were investigated by DNase I footprinting experiments, which showed that the recognition site of *CadC* contains an inverted repeat sequence (10).

Genes highly homologous to the *cadA* and *cadC* genes of *S. aureus* and *L. monocytogenes* have been identified on plasmids of *L. lactis* (20, 30), but gene regulation or the mechanism of their action has never been investigated. This may be of interest in view of recent reports, which showed that the cadmium resistance cassette can be used as a selectable marker in *Lactococcus* (30) and that it can be transferred into industrially relevant *L. lactis* strains (37).

Thermophilic starter strains are used for the production of

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TABLE 1. Plasmids used in this study

Plasmid	Relevant features	Reference or Source
pNZ8048	Cloning vector containing nisin-inducible promoter P _{nisA} , Cm ^r	8
pNZ44	Cloning vector containing constitutive P ₄₄ promoter, Cm ^r	22
pNZcadCA	pNZ8048 containing <i>cadCA</i> , Cm ^r	This study
pNZcadA	pNZ8048 containing <i>cadA</i> , Cm ^r	This study
pNZcadC	pNZ44 containing <i>cadC</i> , Cm ^r	This study
pIR12	Low copy number, P _{R-lacZ} fusion, Em ^r	25
pIRcadCA	pIR12 containing P _{cadCA} , <i>cadC</i> , and <i>cadA-lacZ</i> , Em ^r	This study
pIRcadC	pIR12 containing P _{cadCA} and <i>cadC-lacZ</i> , Em ^r	This study

certain fermented milk products. *Streptococcus thermophilus* is an important species of LAB in the dairy industry, mainly used as a starter culture component in yogurt fermentation and cheese making (11). It is also present as a major microbial component in combination with other LAB species in natural thermophilic starters used for the production of certain cheeses (3). This paper reports the identification of a cadmium resistance cassette on the genome of an industrial *S. thermophilus* strain. We demonstrate the functionality of this cassette in two LAB, *S. thermophilus* and *L. lactis*, and present a model for the regulation of the expression of cadmium resistance in LAB. Throughout the text the expression “cadmium” or “zinc” refers to cadmium or zinc in its ionized form (Cd²⁺ or Zn²⁺).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. thermophilus* strains, which are or have been used in industrial fermentations, were taken from the University College Cork collection, Cork, Ireland (strains 4035, 4038, 4039, 4044, 4116, 4134, 4141, 90461, 90726, 90728, and 90731), or were provided by Nestlé, Lausanne, Switzerland (ST11 [24]). *L. lactis* strains NZ9800 (8) and 302 (19) and *Escherichia coli* JM101 (New England Biolabs) were also used. Bacterial plasmids used in this study are listed in Table 1. *S. thermophilus* strains were grown in Elliker medium (Difco) supplemented with 10 g of beef extract and 9.5 g of β-glycerophosphate per liter at 37°C. *L. lactis* strains were grown in M17 broth (Oxoid) supplemented with 5 g of glucose per liter (GM17) at 30°C. *E. coli*

strains were grown in Luria-Bertani broth as described earlier (33) at 37°C. Erythromycin and chloramphenicol were used at 100 and 10 μg/ml, respectively, for *E. coli* or at 5 μg/ml for each of these antibiotics in *S. thermophilus* and *L. lactis*. If simultaneous selection for two antibiotics was desired, the final concentration of each of the antibiotics was 2.5 μg/ml. Nisin was used at a final concentration of 2 nM.

Plasmid DNA isolation, transformation, and cloning strategies. Plasmid DNA was isolated from *E. coli* and *S. thermophilus* using the Wizard Plus Miniprep Kit (Promega) following the manufacturer's instructions, except that, for isolation of plasmid DNA from *S. thermophilus*, an incubation step at 37°C for 30 min in the presence of 30 mg of lysozyme/ml in the “cell resuspension solution” was applied prior to the standard isolation procedure. Plasmid DNA was isolated from *L. lactis* using a previously described method (29). Electroporation of *E. coli* (33), *S. thermophilus* (31), or *L. lactis* (41) was performed as previously described, except that for the latter GM17 broth supplemented with 2% (wt/vol) glycine was used as growth medium. Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals and were used according to the manufacturer's instructions. PCR products were amplified using either the Expand Long Template PCR System (Roche Molecular Biochemicals) or *Taq* PCR Mastermix (Qiagen) with a Gene Amp PCR System 2400 Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Primers used in this study are listed in Table 2. The *cadC_{St}* (subscript *St* represents *S. thermophilus*) and/or *cadA_{St}* gene was amplified by PCR from the genome of *S. thermophilus* strain 4134 (Table 2). Appropriate restriction enzyme sites were incorporated in the PCR primers to facilitate cloning of the *cadA_{St}* gene on its own or the *cadCA_{St}* genes, including the assumed promoter region, downstream of the nisin-inducible promoter P_{nisA} of pNZ8048 (8) to generate pNZcadA or pNZcadCA, respectively. The *cadC_{St}* gene was cloned downstream of the constitutive promoter P₄₄ of pNZ44 (22) to generate p44cadC. To measure in vivo expression of *cadC_{St}* and *cadA_{St}*, two different translational fusions to the *E. coli lacZ* gene on plasmid pIR12 (25) were constructed. To generate a *cadC_{St}-lacZ* fusion, the assumed *cadC_{St}* promoter region and the first nine codons of the *cadC_{St}* gene were amplified by PCR (Table 2) and were introduced into pIR12 to produce plasmid pIRcadC. For the construction of a *cadA_{St}-lacZ* fusion plasmid, the *cadC_{St}* promoter region, the complete *cadC_{St}* gene, and the first nine codons of the *cadA_{St}* gene were amplified by PCR (Table 2) and were introduced into pIR12 to produce pIRcadCA. All plasmid constructions involving PCR were verified by DNA sequencing of relevant regions.

β-Galactosidase assays. Cells were grown in GM17 containing the appropriate antibiotics at 30°C to an optical density at 600 nm of 0.2 to 0.3. The culture was halved, and to one half of it CdCl₂ was added to a final concentration of 5 μM. Cells were grown for an additional 4 h, after which 4 ml was collected by centrifugation and resuspended in 1 ml of cold Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, and 1 mM MgSO₄, pH 7 [23]). Cells were then disrupted using a bead beater in the presence of 200 μl of glass beads (0.1-mm diameter). Glass beads and cell debris were removed by centrifugation at 14,000 rpm for 10 min in a tabletop centrifuge. β-Galactosidase activity was measured

TABLE 2. Primers used in this study

Sequence (5'–3') ^a	Location ^b	Use ^c
AATGGCGTCTAGATTGCTTCATAACTAGCGTTC	+2573–+2542	pNZcadCA, pNZcadA
CTTAATCCTTCTATTCTAGAGGTTTTTCGG	+402–+373	pNZcadC, pIRcadCA
AAGAGCTGCAGGCATGAGTGAGAAAATGACC	+344–+374	pNZcadA
CAAAGTTTGCTAGTTTAGATTGACAC	+79–+53	Fragments 1, 2, 3, and 4
ATGATCCCATGGAATAATGAAATTTGTGAAATAACTTGATTC	–8–+34	pNZcadC
ACATTCAAATGTTTGTGTTGAATATA	–15––39	Fragments 5 and 7
GAAATGTTCAAAAATGGTCATTTCTGAACACTC	–78––111	Fragment 6
TATATTCAAACAACATTTGAATGT	–39––15	Fragments 4 and 5
GAGTGTTCAAAAATGACCATTTTATGAACATTC	–111––78	Fragment 3
GTGTATATAAATGAACAAAGAATAAATTACGGG	–194––162	Fragment 2
CAATTTGAATTCAGTTGAAACTCGTGC	–279––252	Fragments 1, 6, and 7 and pIRcadCA and pIRcadC
CGCAATCTGCAGTCCAGTTGAAACTCG	–281––255	pNZcadCA
CTTCATGCTCTAGAAGTTATTTACAAATTTCAATTATTC	+40–+3	pIRcadC

^a Introduced restriction sites are shown in boldface; mismatches to the *S. thermophilus* strain 4134 genomic sequence are underlined.

^b Relative location to the start codon of the *cadC_{St}* ORF. –, upstream; +, downstream.

^c For generation of plasmids or fragments 1 to 7 from Fig. 4A.

FIG. 1. Genomic context of the cadmium resistance cassette of *S. thermophilus* 4134. (A) Schematic representation of the gene organization of the complete sequenced 15,874-bp fragment from the genome of *S. thermophilus* 4134 (St4134 [GenBank accession no. AJ315964]) in comparison to the relevant plasmid sequences of pAH82 (AF243383) and pNZ4000 (NC_002137) from *L. lactis*, pLI100 (NC_003383) from *L. innocua*, pER35 (NC_000937) from *S. thermophilus*, and the relevant genome region of *S. thermophilus* LMG18311 (LMG18311 [http://www.biol.ucl.ac.be/gene/genome/], Genemark prediction of ORFs of contig C112). ORFs are denoted by arrows, while insertion sequence elements are denoted by boxes. DNA sequence similarity to St4134 is indicated by color shading of ORFs, with the exception of LMG18311, where only protein sequences could be compared. Homologous regions are connected by a gray background. The *hsdR* ORF of St4134 contains an in-frame stop codon (denoted by a black bar within the ORF) and may not be expressed (Schirawski et al., unpublished). The figure is to scale. (B) Detail of the DNA sequence upstream of *cadC*, comprising nucleotides -85 to +10 (nucleotides 12140 to 12045 of sequence represented by GenBank accession no. AJ315964). The -10 and -35 promoter regions are underlined, the ribosomal binding site (rbs) is in italics, and the inverted repeats (IR1 and IR2) are boldfaced and denoted with an arrow above the sequence. The start codon of *cadC* is in boldface and is indicated by a bent arrow.

at 23°C in Z buffer containing 0.8 mg of *o*-nitrophenyl- β -D-galactopyranoside/ml. The reaction was stopped by the addition of an equal volume of 0.5 M Na₂CO₃. Specific β -galactosidase activity was calculated as described previously (23).

Electrophoretic mobility shift assays (EMSA). For the preparation of cell extracts, an overnight *L. lactis* cell culture (20 ml) was collected by centrifugation and resuspended in 1 ml of buffer containing 20 mM Tris, pH 8.0, and 1 mM dithiothreitol and was ruptured in a bead beater in the presence of 200 μ l of glass beads (0.1-mm diameter). Samples were centrifuged at 14,000 rpm for 5 min in a tabletop centrifuge to remove cell debris. Protein concentrations of the supernatants were determined using the Bio-Rad protein assay kit (Bio-Rad). Gel retardation assays were performed as outlined below. PCR fragments were labeled with polynucleotide kinase using [γ -³²P]ATP (Amersham). DNA binding assays were carried out in 40- μ l reaction volumes containing 50 mM Tris, pH 8.0, 10% (vol/vol) glycerol, 1 mM EDTA, 5 mM MgCl₂, 500 mM KCl, 2 mM dithiothreitol, 50 μ g of bovine serum albumin/ml, 50 μ g of calf thymus DNA/ml, 1 μ l of labeled probe, and 10 μ g of total protein of a particular cell lysate. Samples were incubated for 10 min at room temperature and, following the addition of 10 μ l of 50% glycerol, were loaded on a 4% polyacrylamide gel

containing 2.5% glycerol. Gels were run in Tris-acetate-EDTA buffer (0.04 M Tris-acetate, pH 7.5, and 2 mM EDTA) at 120 V for 3 h, dried, and exposed overnight at -70°C to X-Omat film (Kodak).

Sequence analysis. DNA sequence determination was executed by MWG-Biotech (Ebersberg, Germany). Sequence assembly and alignments were performed using the DNASTAR 1996 release software package. Analysis of protein sequences for coiled-coil motifs was done using the Network Protein Sequence program @nalysis, found at the website http://npsa-pbil.ibcp.fr. Database searches were performed using the BLASTN or BLASTP program (2). Transcriptional terminator sequences were identified with the program mfold, version 3.1 (21, 43). Sequence data for *S. thermophilus* strain LMG18311, presented in Fig. 1A, were obtained from the Université catholique de Louvain Life Sciences Institute website at http://www.biol.ucl.ac.be/gene/genome/. (Sequencing of the *S. thermophilus* strain LMG18311 genome was supported by Walloon Region [BIOVAL grant no. 9813866].)

Nucleotide sequence accession number. The sequence determined from the *S. thermophilus* strain 4134 genome containing the *cadC*_{St} and *cadA*_{St} genes (15,874 bp) was deposited in GenBank (accession number AJ315964).

TABLE 3. Comparison of the putative CadA_{St} and CadC_{St} protein sequences of *S. thermophilus* strain 4134 to the respective CadA and CadC protein sequences from other bacteria

Species	Location	Protein	Amino acid identity (%) to CadA (CadC) of <i>S. thermophilus</i>	Accession no.
<i>L. innocua</i>	Plasmid pLI100	CadA (CadC)	100 (100)	NC_003383
<i>L. lactis</i> subsp. <i>lactis</i>	Plasmid pAH82	CadA (CadC)	100 (100)	AF243383
<i>L. lactis</i> M17	Plasmid pND302	CadA (CadC)	100 (100)	U78967
<i>L. monocytogenes</i> EGD-e	Chromosome	CadA (CadC)	73 (48)	NC_003210
<i>L. monocytogenes</i>	Plasmid (transposon)	CadA (CadC)	68 (52)	L28104
<i>S. aureus</i>	Chromosome (transposon)	CadA (CadC)	64 (49)	L10909
<i>S. aureus</i>	Plasmid pI258	CadA (CadC)	64 (43)	J04551
<i>S. maltophilia</i>	Chromosome	CadA (CadC)	63 (43)	AJ251015
<i>B. firmus</i> OF4	Chromosome	CadA (CadC)	63 (43)	M90750
<i>G. stearothermophilus</i>	Chromosome	CadA (CadC)	52 (37)	AF098974

RESULTS

Identification of a cadmium resistance cassette on the chromosome of *S. thermophilus* strain 4134. In search of a type I restriction/modification system, a 15,874-bp fragment from the chromosome of *S. thermophilus* strain 4134 was completely sequenced (Fig. 1A). It contained three open reading frames (ORFs) that putatively code for the three subunits of a type I restriction/modification system (*hsdR*, *hsdM*, and *hsdS*) (J. Schirawski, G. F. Fitzgerald, and D. van Sinderen, unpublished data), a complete copy of the insertion element *IS1191* (13), a partial copy each of the insertion elements *ICES1* (6) and *IS1295L* (GenBank accession no. AJ278471), two partial ORFs, and two ORFs with high sequence similarity to proteins of unknown function derived from lactococcal plasmids (30, 40). Furthermore, an ORF was identified (Fig. 1A) whose putative amino acid sequence of 705 amino acids is identical in sequence to the plasmid-encoded CadA proteins of *L. lactis* and *L. innocua* and showed significant similarity to CadA of *S. aureus* (64% amino acid identity), as well as to those of other bacteria (Table 3). Immediately upstream of this ORF, designated here *cadA_{St}*, a second ORF was located on the chromosome of *S. thermophilus* strain 4134, whose putative amino acid sequence of 129 amino acids is identical in sequence to the AsrR-type regulatory protein CadC from *L. lactis* and *L. innocua* and is highly similar to CadC from *L. monocytogenes* (52% amino acid identity) as well as to those from other bacteria (Table 3). The identified *S. thermophilus* homologue of CadC was therefore designated CadC_{St}. Although the cadmium resistance cassette described here has been identified earlier on plasmids of *L. lactis* and *L. innocua*, gene regulation or the mechanism of action of the cassette has never been studied.

The high sequence homology of the two ORFs *cadC_{St}* and *cadA_{St}* to their respective ORFs from plasmids identified in *L. lactis* and *L. innocua* (Table 3) induced us to investigate whether the sequence similarity would extend beyond the coding regions of these two ORFs. We found that the sequence similarity to the lactococcal plasmid pAH82 (30) extended over a stretch of about 10 kb of the sequenced genomic DNA from *S. thermophilus*. This homologous DNA region encompassed the three ORFs of the type I restriction/modification system, the *cadCA_{St}* genes, and two more ORFs (*orf3* and *orf4*) (Fig. 1A). With the exception of the variable sequences of the *hsdS*

gene (Schirawski et al., unpublished), the 10-kb stretch from *S. thermophilus* showed >95% DNA sequence homology to the corresponding stretch of the lactococcal plasmid pAH82. Downstream of the *cadA* gene from pLI100 (12) of *L. innocua*, an ORF was located that showed 87% DNA sequence homology to *orf4*, which is located upstream of *cadC_{St}* on the *S. thermophilus* 4134 genome (Fig. 1A). The two ORFs of unknown function (*orf3* and *orf4*) and about 700 bp of upstream sequence also showed high sequence similarity to the lactococcal plasmid pNZ4000 (40) (Fig. 1A), while the three ORFs encoding the type I restriction/modification system showed (with the exception of the variable regions of the *hsdS* gene) high sequence similarity to the streptococcal plasmid pER35 (35) (Fig. 1A). These data suggest that the *cadCA_{St}* genes and surrounding DNA were introduced into the *S. thermophilus* genome by a lateral gene transfer event.

The potentially plasmid-derived sequences discovered on the genome of *S. thermophilus* 4134 are flanked by insertion elements previously identified for this organism (6, 13) (GenBank accession no. AJ278471) (Fig. 1A). These elements, in turn, are flanked by two only partially sequenced ORFs that have also been identified during the sequencing project of *S. thermophilus* strain LMG18311 (<http://www.biol.ucl.ac.be/gene/genome/>) (Fig. 1A). However, on the LMG18311 genome, these genes are situated within 2 kb of each other, while on the genome of strain 4134 they are separated by about 15 kb of sequence. This suggests that, in the genome of *S. thermophilus* strain 4134, which has been used in industrial fermentations, a sequence of about 2 kb has been deleted and replaced by insertion elements from *S. thermophilus* and by sequences derived from one or several plasmids of lactococcal, streptococcal, or listerial origin.

Analysis of the DNA sequence upstream of *cadC_{St}* revealed the presence of a typical promoter sequence found in LAB (9, 16), i.e., a –35 (TTGAAT) and an extended –10 region (TGATACAAT) (Fig. 1B). The stop codon of *cadC_{St}* partially overlaps the start codon of *cadA_{St}*, indicating that the two genes are translationally coupled. Downstream of *cadA_{St}*, a putative rho-independent transcriptional terminator was identified (not shown). These findings suggest that a cadmium resistance cassette (*cadCA_{St}*), consisting of the *cadC_{St}* and *cadA_{St}* genes, is present on the chromosome of *S. thermophilus*

strain 4134 and that the two genes appear to be organized in an operon.

***S. thermophilus* strains containing the *cadCA_{St}* genes are cadmium resistant.** A number of *S. thermophilus* strains were tested for their ability to grow in the presence of cadmium. Of 12 different *S. thermophilus* strains tested, only two (strains 4134 and 4116) were able to grow in the presence of 1 mM CdCl₂, while 10 strains (strains 4035, 4038, 4039, 4044, 4141, 90461, 90726, 90728, 90731, and ST11) proved to be cadmium sensitive (not shown). The cadmium-sensitive *S. thermophilus* strain 4035 was unable to grow in the presence of 0.25 mM CdCl₂, whereas strain 4134 grew well in the presence of up to 1.6 mM CdCl₂ (not shown). The strains were tested for presence of the *cadCA_{St}* cassette by PCR, using *cadC_{St}*- and *cadA_{St}*-specific primer combinations. PCR products of the expected sizes were obtained for strains 4134 and 4116 but not for any of the cadmium-sensitive strains (not shown), indicating that only strains containing the *cadCA_{St}* genes are able to grow in the presence of elevated levels of CdCl₂.

The *cadCA_{St}* cassette from *S. thermophilus* confers cadmium resistance to both *L. lactis* and *S. thermophilus*. The putative cadmium resistance cassette from the chromosome of *S. thermophilus* 4134, including 271 bp of upstream sequence, was cloned into pNZ8048. The resulting plasmid, pNZcadCA, was introduced into the cadmium-sensitive strains *L. lactis* NZ9800 and *S. thermophilus* 90728. While the control strains containing pNZ8048 were unable to grow in the presence of 0.5 mM CdCl₂, the strain *L. lactis* NZ9800 containing pNZcadCA was cadmium resistant and was capable of growth in the presence of as much as 1.5 mM CdCl₂, while *S. thermophilus* 90728 containing pNZcadCA grew well in the presence of up to 1 mM CdCl₂ in the growth medium (not shown). This confirms that the *cadCA* cassette from *S. thermophilus* strain 4134 confers resistance against CdCl₂ to both *S. thermophilus* and *L. lactis*. Cadmium-dependent expression of the *cad-lacZ* fusions confirmed the apparent presence of all regulatory elements on the cloned fragment (see below).

The *cadCA_{St}* cassette also confers resistance to zinc. Growth of the cadmium-resistant *S. thermophilus* strain 4134 was compared to growth of the cadmium-sensitive *S. thermophilus* strain 4035 in the presence of various concentrations of different metal ions. No difference in the growth capacities of the two strains was observed in the presence of various concentrations of the metal ions Cu²⁺, Ni²⁺, Co²⁺, Ag⁺, and Hg²⁺. However, strain 4134 was resistant to higher concentrations of Zn²⁺ (2 mM) than was strain 4035 (0.5 mM [data not shown]). Growth of the two *L. lactis* NZ9800 strains, containing pNZ8048 or containing pNZcadCA, was compared. The pNZcadCA-containing strain was able to grow in the presence of 5 mM Zn²⁺, whereas the control strain could not grow above Zn²⁺ concentrations of 1 mM (not shown). This demonstrates that the presence of the *cadCA_{St}* cassette not only confers resistance against cadmium but is also responsible for increased zinc resistance.

CadA_{St} protein is sufficient for cadmium resistance in *L. lactis*. The *cadA_{St}* gene was cloned under the control of the nisin-inducible promoter of pNZ8048 to generate pNZcadA. pNZcadA was introduced into *L. lactis* strain NZ9800. In the absence of nisin, *L. lactis* NZ9800 containing pNZcadA was not able to grow in the presence of cadmium (0.5 mM),

FIG. 2. β -Galactosidase activity of *L. lactis* 302 containing pIRcadCA in the absence (open circles) or presence (solid squares) of 10 μ M CdCl₂.

whereas the addition of nisin to the medium allowed growth of the strain in the presence of up to 1 mM Cd²⁺ (not shown). This is in line with the prediction that the CadA_{St} protein functions as a cadmium efflux transporter and shows that the CadC_{St} protein is not necessary for cadmium resistance when CadA_{St} is expressed.

CadC_{St} regulates CadA_{St} expression in response to cadmium in vivo. A *cadA_{St}-lacZ* fusion was constructed by replacing the P_R promoter derived from bacteriophage r1t of pIR12 (25) by the putative *cadCA_{St}* promoter, the *cadC_{St}* gene, and the 5' end of the *cadA_{St}* gene, generating pIRcadCA and thus placing the expression of the *cadA_{St}-lacZ* fusion under the control of the *cadCA_{St}* promoter. pIRcadCA was introduced into *L. lactis* 302, and β -galactosidase activity during growth of the strain was monitored in the absence or presence of 10 μ M CdCl₂ (Fig. 2). β -Galactosidase activity is at a basal, low level in the absence of cadmium but increases within 2 h after the addition of cadmium chloride, indicating that CadA expression in vivo is induced by the presence of cadmium.

When β -galactosidase activity levels of *L. lactis* 302 containing pIRcadCA and of *L. lactis* 302 containing pIR12 were compared during growth of the strains in the absence of cadmium, a higher β -galactosidase activity was measured for the strain containing pIRcadCA (Table 4). The P_R promoter present on pIR12 is derived from bacteriophage r1t and constitutes a very stringently regulated early lytic promoter, which is very tightly repressed by the r1t repressor, also encoded on pIR12 (25). In contrast, the promoter of the *cadCA_{St}* genes apparently allows a low-level basal transcription of the *cadCA_{St}* genes.

A *cadC_{St}-lacZ* fusion was constructed in pIR12, generating pIRcadC. In contrast to strain *L. lactis* 302 containing

TABLE 4. β -Galactosidase activity (Miller units) of *L. lactis* 302 containing different plasmids^a

Cadmium concn (μ M)	Activity of different plasmids			
	pIRcadCA	pIRcadC	pIRcadC and p44cadC	pIR12
5	54	99	23	0
0	11	97	3	0

^a Cells were grown to an optical density at 600 nm of 0.3 and were harvested 4 h later after growth in the absence or presence of 5 μ M CdCl₂.

FIG. 3. EMSA of promoter DNA fragment 1 (A) or fragment 3 (B) by a CadC_{St}-containing protein extract prepared from an overnight culture of *L. lactis* NZ9800 containing p44cadC. (A) Promoter fragment 1 (40 ng) was mixed with no protein extract (lane 1), CadC_{St}-containing protein extract (lane 2), or CadC_{St}-containing protein extract in the presence of 0.04, 0.2, 0.4, 1.0, 2.0, or 3.5 µg, respectively, of unlabeled promoter DNA (lanes 3 to 8). Lanes 9 and 10, same as lanes 1 and 2, respectively. Lane 11, labeled promoter fragment 1 in the presence of cell extract prepared from an overnight culture of *L. lactis* NZ9800 containing pNZ44. Lanes 12 and 13, 40 ng of a labeled 400-bp DNA fragment derived from an unrelated plasmid in the absence (lane 12) or presence (lane 13) of the CadC_{St}-containing protein extract. (B) Promoter fragment 3 (40 ng, lane 1) with the CadC_{St}-containing cell extract in the absence (lane 2) or presence (lanes 3 to 5) of 10, 100, or 1,000 µM CdCl₂, respectively, in the binding reaction.

pIRcadCA, which showed high β -galactosidase activity only in the presence of cadmium, the strain containing pIRcadC, which lacks a functional CadC_{St} protein, showed high β -galactosidase activity regardless of the presence or the absence of cadmium (Table 4), suggesting that CadC_{St} is a negative regulator of *cadCA*_{St} expression. In line with this interpretation is the finding that, in strain *L. lactis* 302 containing both pIRcadC and p44cadC (the latter plasmid expresses CadC constitutively), cadmium-dependent expression of *cadC*_{St}-*lacZ* is restored (Table 4). The activity of β -galactosidase of *L. lactis* 302 containing both pIRcadC and p44cadC is lower than that of *L. lactis* 302 containing pIRcadCA, both in the presence and in the absence of cadmium. This probably reflects high expression of CadC_{St} from the constitutive promoter in p44cadC. CadC_{St} in high concentrations may bind more efficiently to the *cadCA*_{St} promoter and thus repress *cadCA*_{St} transcription more effectively, so that the negative regulatory effect cannot be completely alleviated by the presence of 5 µM cadmium.

CadC_{St} is a sequence-specific DNA binding protein. The CadC_{St} protein was expressed in *L. lactis* NZ9800 under the control of the constitutive P₄₄ promoter in p44cadC. A cell extract from this strain served as a source of CadC_{St} protein for use in an EMSA. An EMSA was performed with a 358-bp fragment (fragment 1) containing 279 bp of sequence upstream of *cadC*_{St} (Fig. 3A). The mobility of this DNA fragment was retarded by the CadC_{St}-containing cell extract (Fig. 3A, lane 2) and was not retarded by a cell extract lacking CadC_{St} prepared from an overnight culture of *L. lactis* NZ9800 pNZ44 (Fig. 3A,

lane 11), showing that CadC_{St} is a DNA binding protein. An unrelated control DNA fragment of 400 bp was not retained by the CadC_{St}-containing cell extract (Fig. 3A, lanes 12 and 13), confirming that CadC_{St}-directed DNA binding is sequence specific. Furthermore, retardation of the labeled promoter fragment by the CadC_{St} protein could be effectively reversed by competitive binding using increasing amounts of unlabeled DNA fragment 1 (Fig. 3A, lanes 2 to 8).

CadC_{St} binds its own promoter region at a repeated inverted repeat. In search for a potential binding site of the CadC_{St} protein to its own promoter region, the sequence upstream of *cadC*_{St} was examined. The analysis revealed the presence of a 7-bp inverted perfect repeat (IR1) (ATTCAAaacaacTTTG AAT) spaced by 6 nt (lowercase letters). A second copy of this inverted repeat (which was imperfect at 1 nucleotide) was detected 45 bp upstream of the first (IR2) (ATTCAAacattca CTTGAAT) (Fig. 1B). An analysis of the complete 15,874-bp sequenced fragment from the *S. thermophilus* 4134 chromosome did not reveal any other copy of this inverted repeat. To ascertain whether the inverted repeats could serve as potential binding sites for the CadC_{St} protein, six fragments (fragments 2 to 7 [Table 2]) were generated by PCR, which represented 5'- and/or 3'-truncated versions of the 358-bp promoter fragment 1 and contained both (fragments 1, 2, 3, and 7), one (fragments 4 and 5), or no (fragment 6) copy of the inverted repeats (Fig. 4A). All seven fragments were tested in an EMSA by using the CadC_{St}-containing cell extract of *L. lactis* NZ9800 p44cadC (Fig. 4B). The mobility of fragments 1, 2, 3, and 7 was retarded by the presence of the cell extract, and fragment 4 exhibited only a partial mobility shift, while the presence of the CadC_{St}-containing cell extract did not influence the migration behavior of fragments 5 and 6 (Fig. 4B). The 5' end of the CadC_{St} binding site could be located to position -111 upstream of *cadC*_{St}, since the mobility of fragments 1, 2 and 3 but not of fragment 4 was efficiently retarded by CadC_{St}-containing cell extract, while the 3' end was mapped to position -15, since fragment 7, but not fragment 6, exhibited mobility shifts in the presence of CadC_{St}. Thus, the CadC_{St} binding region was localized to the promoter region of *cadC*_{St} (positions 12166 to 12070 of the sequence represented by GenBank accession no. AJ315964) and contains both inverted repeats.

The CadC_{St}-DNA interaction is lost in the presence of cadmium. An EMSA was performed using promoter fragment 3 and the CadC_{St}-containing cell extract (Fig. 3B). The fragment was retarded by the presence of the cell extract in the absence of cadmium. However, the presence of 1 mM CdCl₂ in the binding assay completely abolished the capacity of the cell extract to retard the fragment (Fig. 3B). Thus, the CadC_{St} protein seems to lose its DNA affinity in the presence of cadmium.

DISCUSSION

This work is the first description of a cadmium resistance cassette from the genome of *S. thermophilus*. This cadmium resistance cassette, which consists of the two adjacent genes *cadC*_{St} and *cadA*_{St}, was identified on a 15,874-bp fragment from the genome of *S. thermophilus* strain 4134. A stretch of about 10 kb of this sequence, which includes the *cadCA*_{St} genes, shows very high DNA sequence homology (>90%) to

FIG. 4. EMSA of truncated promoter DNA fragments with a CadC_{St}-containing cell extract. (A) Representation of promoter fragments 1 to 7 used for CadC_{St}-DNA binding studies. Fragment names and their sizes are indicated on the left. On the right, the results of the EMSA (B) are stated. Gray rectangle, cadC_{St} ORF; black triangles, repeats; IR1, inverted repeat 1; IR2, inverted repeat 2; and -35 and -10, -35 and -10 promoter sequences. (B) EMSA of the labeled promoter DNA fragments 1 to 7 (A) as specified below the lanes. -, absence; +, presence of a CadC_{St}-containing cell extract. Lanes 1 to 12 were run for 3 h; lanes 13 and 14 were run for 1.5 h.

plasmid pAH82 from *L. lactis*. The G+C content of the cassette is lower (34.5%) than the average G+C content of *S. thermophilus* (37.2 to 40.3%) (36) or that of *L. monocytogenes* and *L. innocua* (39 and 37%, respectively) (12) but is in line with the G+C content of *L. lactis* (35.4%) (4). Although the cadmium resistance cassette is widespread among different (mainly gram-positive) bacteria, it does not seem to be common in *S. thermophilus*. Out of 12 strains tested, only two appeared to contain the cassette. It is thus possible that the cadmium resistance cassette has originated from *L. lactis* and has entered the genome of *S. thermophilus* 4134 through a plasmid that was exchanged during industrial coculture. Intergeneric transfer of DNA from *L. lactis* to *S. thermophilus* during coculture in the manufacture of cheese was suggested to explain the presence of the insertion element iso-IS981 on the genome of *S. thermophilus* (13). Possessing the cadmium resistance cassette may have such a selective advantage for the bacteria (17) that some species acquire it through intergeneric transfer from only distantly related organisms, as has been proposed for the gram-negative bacterium *S. maltophilia*, which contains the cadmium resistance cassette usually found

in gram-positive bacteria (1). In the case of *S. thermophilus* strain 4134, the selective advantage may even have been larger, because the acquired DNA also contains a type I restriction/modification system that is thought to act as a phage resistance cassette. Sequences adjacent to the 10-kb stretch showing homology to lactococcal plasmids contained the complete or partial *S. thermophilus* insertion sequences IS1191 (13), ICES_{St}1 (6), and IS1195L (GenBank accession no. AJ278471). Sequence analysis of the regions next to the indicated IS elements identified potential ORFs with counterparts in the genome sequence of *S. thermophilus* LMG18311. This may indicate that the genomic region sequenced may be a result of multiple DNA integration events. Whether integration of the insertion sequences occurred independently or at the same time or even allowed insertion of lactococcal plasmid sequences is a matter of speculation.

The two genes of the cassette are putatively organized as a transcriptional unit in *S. thermophilus*, with a promoter sequence upstream of the cadC_{St} gene and a transcriptional terminator downstream of the cadA_{St} gene. Thus, gene organization seems similar to that found in other bacteria, with the exception of *L. monocytogenes*, where the gene order is reversed (18) and where the two chromosomally located genes are not adjacent (12).

The cadCA_{St} cassette including 279 bp of upstream sequence was shown to confer cadmium and zinc resistance to both *S. thermophilus* and *L. lactis*. The system is thus similar to the one identified on *S. aureus* plasmid p1258, which confers cadmium and zinc resistance (42), but is different from that of *L. monocytogenes*, which does not confer zinc resistance (18).

Expression of CadA_{St} was shown to be sufficient for cadmium resistance, confirming that CadA_{St} functions as the cadmium resistance protein. CadC_{St} was shown to be a trans-acting, sequence-specific, DNA binding and cadmium-dependent regulatory protein of cadCA_{St} expression. The specific CadC_{St}-DNA interaction is completely alleviated in the presence of cadmium in vitro. Thus, cadmium seems to have a more stringent role in preventing or reversing the CadC_{St}-DNA interaction than in affecting the interaction of CadC from *S. aureus* with its promoter DNA, which is only partly diminished in the presence of cadmium (10). It seems possible that CadC_{St} can either bind DNA or cadmium and can function thus as a cadmium sensor protein. The amino acid sequence contains three motifs with similarity to domains involved in binding to metal ions (26). Two of the metal binding motifs overlap the proposed helix-turn-helix motif involved in DNA binding (18). It has been suggested that one of the functions of the CadC protein is to deliver the metal ion to the CadA protein for export (42) or to function as a structural component for the cadmium efflux complex (42). Since we have shown that CadA_{St} is sufficient for cadmium resistance, we propose that CadC_{St} functions as a sensor of cadmium concentration. Metal binding to CadC_{St} may influence its capacity to act as a negative regulator through DNA-protein interactions. It may therefore be that the interaction of CadC_{St} with cadmium or with DNA is mutually exclusive.

CadC_{St} binds specifically to the cadCA_{St} promoter region at positions -111 to -15 upstream of cadC_{St} (positions 12166 to 12070 of the complete sequenced fragment [GenBank accession no. AJ315964]). The CadC_{St} DNA binding region includes

the presence of two inverted repeats. Only fragments containing both inverted repeats were efficiently retarded in an EMSA (Fig. 4). It is likely that these inverted repeats constitute the core recognition sequence that is specifically recognized by the CadC_{St} protein. CadC_{St} might bind these elements independently or cooperatively and/or in a multimeric form. An analysis of the protein sequences of CadC of *S. thermophilus* and *S. aureus* indicated the presence of putative coiled-coil motifs (amino acids 10 to 25 of CadC_{St} and 12 to 27 of *S. aureus* CadC). These sequences may therefore represent a part of the CadC protein required for protein-protein interaction (5), potentially between CadC molecules. Since CadC_{St} does not bind efficiently to fragments that contained only IR1, it is tempting to speculate that CadC_{St} binds to both IR1 and IR2 as a dimer of dimers. The EMSA did not show the formation of two or more bound forms of the promoter fragments, as reported by Endo and Silver (10) for the interaction of CadC from *S. aureus* plasmid pI258 with the *cadC* promoter. It is possible that the difference in retardation behavior of the two systems is a result of the different purification states of the respective proteins. Additional bound forms of promoter DNA may be observed when purified CadC_{St} protein is used for the EMSA. Alternatively, CadC_{St} might bind as a multimer formed in solution, and multimer formation might be stabilized by other proteins present in the crude cell extracts.

Endo and Silver (10) reported for the CadC protein of pI258 of *S. aureus* sequence-specific binding to an inverted repeat (TCAAATAaaTATTTGA). We analyzed their sequence and found a second copy of the inverted repeat 45 bp upstream of the first (TCAAATAtTGCTTGC), which, as is the case for *S. thermophilus*, is an imperfect repeat. The CadC protein of *S. aureus* might only be able to bind to the imperfect repeat when a CadC dimer is already bound to the perfect inverted repeat. Alternatively, CadC binding affinity to the imperfect repeat might be lower.

We propose the following model for the regulation of expression of cadmium resistance in *S. thermophilus* (Fig. 5): at low cadmium concentrations, a low basal level of transcription allows the production of CadC_{St} protein, which binds (potentially as a dimer of dimers) to the inverted repeats IR1 and IR2 of the *cadCA*_{St} promoter region. This binding prevents the efficient establishment of a functional RNA polymerase-*cadCA*_{St} promoter DNA interaction and thus inhibits further transcription. At high cadmium concentrations, the binding of cadmium to the CadC_{St} protein leads to a conformational change, which results in the inability of CadC_{St} to bind to DNA. The CadC_{St}-promoter DNA complex is thus destroyed, making room for binding of RNA polymerase. This results in the production of *cadCA*_{St} transcript and the expression of CadA_{St}, which is responsible for the establishment of cadmium resistance.

The most widely accepted approach for precise genetic improvement of starter cultures destined for consumer products is the conjugal transfer of naturally occurring plasmids responsible for key industrial traits, like lactose catabolism, citrate transport, proteinase production, polysaccharide production, bacteriocin production and immunity, and bacteriophage resistance. Bacteriophage attack of starter cultures is still one of the most eminent problems in the dairy industry and leads to significant economic losses worldwide. Hence, the transfer of

FIG. 5. Working model for the regulation of the expression of cadmium resistance in *S. thermophilus*. At low cadmium concentrations in the growth medium, most of the CadC_{St} binding sites (black triangles) will be occupied by CadC_{St} protein (dark gray circles), which possibly binds as a dimer of dimers and inhibits most of the *cadCA*_{St} transcription. At high cadmium concentrations, binding of cadmium to the CadC_{St} protein is proposed to cause a conformational change in the CadC_{St} protein (gray triangles), which interferes with its DNA binding properties. As a consequence, CadC_{St} will release the promoter DNA and make room for binding of the RNA polymerase complex. Thus, transcription of the *cadCA*_{St} cassette is activated, which leads to the production of CadA_{St} protein and the establishment of cadmium resistance.

plasmids conferring bacteriophage resistance into industrially relevant strains is of key interest (37). A difficulty with this approach is the paucity of easily selectable markers for phage resistance plasmids. Bacteriocin immunity has been used successfully for the transfer of phage resistance plasmids (7, 32) but resulting transconjugants produced bacteriocin, which could be undesirable in some industrial situations, particularly where a combination of starter strains is used. Recently, it was shown that the cadmium resistance cassette identified on the *L. lactis* plasmid pNP-40 could be used as a selectable marker for the introduction of phage resistance plasmids into industrial starter strains (37). It should be possible to use the cadmium resistance cassette identified here as a selectable marker to also improve thermophilic starter strains like *S. thermophilus*.

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