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A Second Gene in the *Staphylococcus aureus cadA* Cadmium Resistance Determinant of Plasmid pI258

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Two open reading frames on a 3.7-kb *BglII-XbaI* fragment which encodes the *Staphylococcus aureus cadA* cadmium (and zinc) resistance determinant of plasmid pI258 were identified (G. Nucifora, L. Chu, T. K. Misra, and S. Silver, Proc. Natl. Acad. Sci. USA 86:3544–3548, 1989). The [^{35}S]methionine-labelled protein products of the 727-amino-acid CadA ATPase and of the 122-amino-acid CadC polypeptide in *Escherichia coli* were identified by using the T7 RNA polymerase-promoter expression system. A truncated CadA polypeptide (402 amino acids) did not confer resistance in *S. aureus* but was expressed in *E. coli* under control of the T7 RNA polymerase-promoter. Removal of 678 nucleotides from the 5′ end of the published sequence (which includes the *cadA* promoter) abolished resistance to cadmium, whereas a 146-nucleotide-shorter deletion was without effect. The *cadC* gene is needed in addition to *cadA* for full resistance to cadmium in *S. aureus* and *Bacillus subtilis*. *cadC* functions both in *cis* and in *trans*.

Bacterial cells have plasmid-determined mechanisms for resistances to antimicrobial substances including toxic heavy metals. The mechanisms and molecular genetics of heavy metal resistances in a wide range of bacteria have been studied extensively (for reviews, see references 24, 25, 27, and 30). Hg^{2+} is reduced by the enzyme mercuric reductase to Hg^0, which volatilizes. For AsO_4^{3-}, AsO_2^-, Cd^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} resistances, plasmid-governed systems of membrane proteins pump toxic ions out of the cells (10, 15, 24, 30, 32).

A 3.7-kb DNA fragment containing the *cadA* cadmium resistance determinant of *Staphylococcus aureus* plasmid pI258 was sequenced, and it contains two open reading frames (ORFs) (13). The product of the longer ORF shows strong sequence homology with the P class of ATPases (14, 20, 21, 26, 27), such as the *Escherichia coli* KdpB polypeptide (5, 34). These highly conserved ATPases, which are found in all living cells from bacteria to humans (2, 14, 20, 26), were previously called E_1E_2 ATPases because they are found in two conformational forms during the ATPase transport cycle.

Given the existence of two ORFs, there arose the questions of what the role of the second ORF was and of whether it was needed for resistance to the toxic cations Cd^{2+} and Zn^{2+}, much as the KdpA and KdpC polypeptides are needed for function of the KdpB ATPase in K^+ uptake (5, 34). An alternative possibility for the role of the second ORF in gene regulation was eliminated by experiments described in the accompanying paper (36). The results presented here establish that the second ORF is required for full Cd^{2+} and Zn^{2+} resistance. Therefore, the short ORF of Nucifora et al. (13) has been renamed *cadC* (*cadB* has been used for a second, quite separate Cd^{2+} and Zn^{2+} resistance system found on plasmids, including pII147 [11, 22, 28]).

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MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the bacterial strains and plasmids used. Cells were grown in 2XNY medium (17) containing ampicillin (100 μg/ml), kanamycin (40 μg/ml), erythromycin (10 μg/ml), or chloramphenicol (5 μg/ml), all from Sigma Chemical Co. (St. Louis, Mo.), as necessary. Procedures for manipulating DNA were as described by Sambrook et al. (17).

Materials. $^{109}Cd^{2+}$, scintillation counting fluid, [^{35}S]methionine, ^{35}S-dCTP and ^{14}C-labelled protein molecular weight markers were obtained from Amersham Corp. (Arlington Heights, Ill.). Kodak XAR film was from Eastman Kodak (Rochester, N.Y.). Restriction nuclease enzymes, calf intestinal phosphatase, and the *Nhe*I nonsense codon linker, deoxy(CTAGCTAGCTAG), were from Bethesda Research Laboratories (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and New England Biolabs (Beverly, Mass.), respectively. Lysostaphin was from Bristol-Meyers Pharmaceuticals (Evansville, Ind.), and rifampin was from Sigma Chemical Co.

DNA sequencing. The nucleotide sequences of the ends of fragments used for subcloning were determined by dideoxy chain termination sequencing with DNA polymerase I (Klenow fragment) (19).

DNA fragment purification. To isolate DNA fragments from agarose gel slices, the Geneclean kit purchased from Bio 101, Inc. (La Jolla, Calif.) was used according to the manufacturer's instructions.

***S. aureus* protoplast transformation and regeneration.** After transformation of lysostaphin-generated protoplasts (12) with ligated DNA, transformants were selected on regeneration medium containing either 5 μg of chloramphenicol per ml or 10 μg of erythromycin per ml or both, when necessary.

***Bacillus subtilis* transformation.** Transformation of competent *B. subtilis* cells with plasmid DNA was done as described elsewhere (1).

Construction of 5′ and 3′ deletion mutants of the cadmium and zinc resistance operon. The 3.7-kb *Xba*I fragment containing the intact *cadA* resistance determinant was subcloned from phage M13 (used for sequencing [13]) into the *Xba*I site of pSK265 (7). In the resulting plasmid, *cadC* and

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference
Strains		
E. coli		
K38	HfrC(λ)	28
JM101	Δ(*lac pro*) *thi supE* F'*traD36 proAB lacI*^{q} Δ*lacZM15*	33, 35
JM83	*ara* Δ(*lac proAB*) *rpsL* φ80 Δ*lacZM15*	33, 35
S. aureus RN4220	Mutant that is an efficient acceptor of *E. coli* DNA	9
B. subtilis BD224	*trpC2 recE4 thr-5*	3
Plasmids		
pT7-5	Multiple cloning site downstream of the T7 phage RNA polymerase promoter	29
pGP1.2	T7 phage RNA polymerase gene under control of lambda p_L promoter; cI857ts	29
pSK265	pC194 derivative with multiple cloning site from pUC19	7
pSK270	pE194 derivative with multiple cloning site from pUC19	8, 23

cadA were in the orientation opposite to the direction of transcription of ORFD of pSK265 (plasmid pKPY1) (Fig. 1). ORFD is transcribed constitutively and thus provides a transcriptional origin to fragments cloned into the site (7). A 3.0-kb *Xba*I fragment isolated from a phage M13 deletion which was used for the sequencing of *cadA* (13) was cloned into the *Xba*I site of pSK265 and named pKPY2 (Fig. 1).

To generate a deletion mutant in the operator-promoter region, the 3.0-kb fragment used to make pKPY2 was digested with *Ssp*I. (There are two *Ssp*I sites in the 3.0-kb *Xba*I fragment, at nucleotide [nt] 679 [in the inverted repeat in the *cadA* operator-promoter] [36] and 86 nt downstream from the end of the *cadA* gene.) The resultant 2,660-nt *Ssp*I fragment was subcloned into the *Sma*I site of pSK265 in both orientations, and the plasmids were named pKPY3 and pKPY4, respectively (Fig. 1).

A 2.6-kb *Xba*I fragment which contained the 3′ part of the *cadC* gene and intact *cadA* was subcloned from a phage M13 used for sequencing (13) into the *Xba*I site of pSK265 in both orientations (Fig. 1), and the plasmids were named pKPY5 and pKPY6.

pKPY7 with both the intact *cadC* determinant and the *Nhe*I nonsense codon linker, deoxy(CTAGCTAGCTAG), inserted after the 402nd codon of *cadA* cloned into pSK265, was prepared by digestion of pKPY13 (described below for expression studies) with *Xba*I. The 3.0-kb fragment containing the mutated cadmium resistance determinant was inserted into the *Xba*I site of pSK265 in the orientation shown in Fig. 1.

pKPY8 was prepared by subcloning a 584-nt *Bam*HI fragment (nt 533 to 1116) carrying the *cadA* operon promoter and *cadC* into the *Bam*HI site of pSK265. This fragment was isolated from *Bam*HI-digested DNA from pKPY22 carrying the 584-nt fragment at the *Bam*HI site of pUC19. pKPY9 was prepared by inserting the same 584-nt *Bam*HI fragment into the *Bam*HI site of pSK270.

pKPY20 is a derivative of pUC19, with the *Eco*RI and *Hin*dIII sites in the multicloning site removed. The *Eco*RI site of pUC19 was converted to an *Xmn*I site by being cut with *Eco*RI, blunt-ended with DNA polymerase I (Klenow enzyme), and self-religated with T4 DNA ligase. The *Hin*dIII site was lost in a parallel way.

pKPY21 was prepared as follows. The 3.0-kb *Xba*I fragment used to make pKPY2 was subcloned into the *Xba*I site of pKPY20, in which *cadC* and *cadA* were in the orientation opposite to the direction of transcription of the *lac* promoter of pUC19.

pKPY22 was constructed as follows. pKPY21 was digested with *Xmn*I. The 1.35-kb fragment was purified from an agarose gel and digested with *Nla*IV. After ligation with *Sma*I-digested pUC19, the desired clone, which contains the 0.6-kb fragment, was identified by restriction enzyme digest pattern and named pKPY22.

pKPY25 was prepared by subcloning the 2,660-nt *Ssp*I fragment used to construct pKPY3 into the *Sma*I site of pUC19, with *cadC* and *cadA* in the orientation opposite to the direction of transcription of the *lac* promoter of pUC19.

Construction of vectors for expression of *cadC* and *cadA* gene products in *E. coli* under control of the T7 promoter. pKPY11 and pKPY12 were prepared by subcloning the same 3.0-kb fragment used to make pKPY2 into the *Xba*I site of the T7 expression promoter of plasmid pT7-5 (29) in both orientations (Fig. 2).

pKPY13 was prepared by cutting pKPY11 with nuclease *Xho*I and blunting the ends with DNA polymerase I (Klenow enzyme). Ligation was carried out to incorporate the 12-nt *Nhe*I nonsense codon linker, deoxy(CTAGCTAGCTAG), into the blunted *Xho*I site.

pKPY14 and pKPY15 were prepared by cloning the 2.6-kb *Xba*I fragment used to generate pKPY5 into the *Xba*I site of T7 expression vector pT7-5 in both orientations.

pKPY16 was prepared by subcloning the 0.45-kb *Eco*RI-*Sal*I fragment (nt 679 to 1116 of the published sequence) into the *Eco*RI and *Hin*dIII sites of pT7-5. The fragment carries the distal half of the operator region and an intact *cadC*.

Expression of *cadC* and *cadA* gene products in *E. coli* under control of the T7 promoter. The Tabor and Richardson (29) method was used. The *cadA* genes cloned into the T7-promoter-containing vector pT7-5 were transformed into *E. coli* K38 containing the gene for T7 RNA polymerase, under control of a temperature-sensitive repressor on plasmid pGP1-2 (29). Cells were grown at 30°C and transferred to the basal salts medium M9 supplemented with 20 μg of thiamine per ml and 100 μg each of 18 amino acids (except methionine and cysteine) per ml. After incubation for 1.5 h at 30°C, T7 RNA polymerase synthesis was induced at 42°C for 15 min. Rifampin (200 μg/ml) was added, and the cells were incubated for an additional 10 min at 42°C. The temperature was then shifted to 30°C for 40 min. The cells were labelled with 10 μCi of [^{35}S]methionine (1,000 Ci/mmol) for 5 min at 30°C. The labelled proteins were analyzed (33) by electrophoresis followed by autoradiography.

Growth inhibition. Overnight cultures were diluted 100-fold in 5 ml of 2XNY medium supplemented with ampicillin (100 μg/ml) or chloramphenicol (5 μg/ml), when necessary, plus variable amounts of toxic cations. After growth at 37°C in a shaker for 12 to 20 h, turbidity measurements were made with a Klett-Summerson colorimeter (Klett Manufacturing Co., Long Island City, N.Y.) with a no. 54 Kodak Wratten filter (Eastman Kodak Co.).

MICs. The lowest $CdCl_2$ and $ZnCl_2$ concentrations that

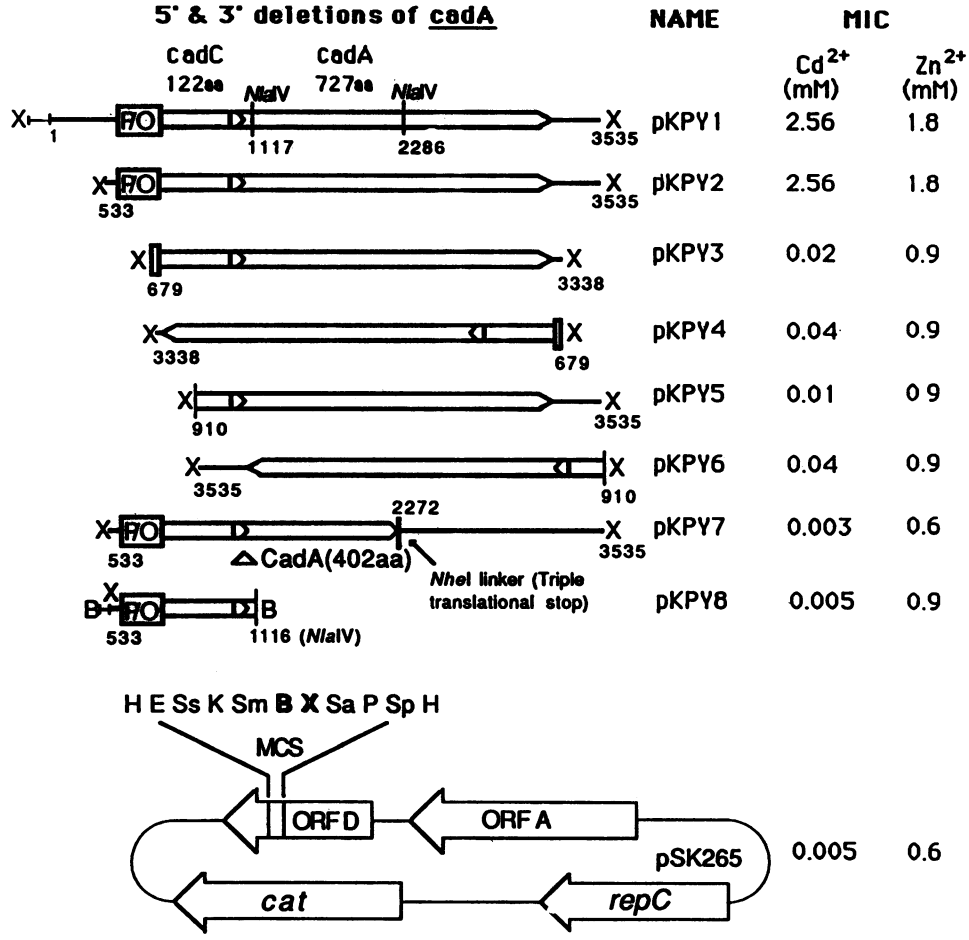

FIG. 1. Structures of 5′ and 3′ deletions and associated cadmium and zinc resistance levels. Numbering of base pair positions is from the published 3,535-bp sequence (13). Open bars indicate the operator-promoter region (P/O) and *cadC* and *cadA* genes, with arrows showing the direction of transcription. The sizes of CadC and CadA (in amino acids [aa]) are given at the top. The thin line starting the pKPY1 diagram indicates additional cloned DNA outside the genes (about 0.2 kb before the numbering of the sequence starts [13]). A thick vertical bar at nt 2272 of pKPY7 represents the *Nhe*I nonsense mutation linker. H, E, Ss, K, Sm, B, X, Sa, P, and Sp, restriction nuclease sites for *Hin*dIII, *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Pst*I, and *Ssp*I, respectively, within the plasmid pSK265 multicloning site. *cat* (encoding chloramphenicol resistance), *repC* (encoding replication control), ORFA, and ORFD (of unknown function) are from the vector plasmid pSK265. See Materials and Methods for the determination of MICs.

inhibited cell growth completely were determined on GL agar plates (12). Fresh cultures were grown for 3 h in 2XNY broth from overnight stationary-phase cultures and diluted 1,000-fold in 2XNY. Drops (3 μl) of each culture were placed on a series of plates which contained twofold dilutions of $CdCl_2$ (0, 0.1 μM to 2.5 mM) or $ZnCl_2$ (0, 0.3 to 1.8 mM). After a 20-h incubation at 37°C, the lowest concentration at which no growth was seen was recorded as the MIC.

Growth inhibition zone. Overnight cultures of *S. aureus* and *B. subtilis* (200 μl each) were spread on GL agar plates containing 5 μg of chloramphenicol per ml, and 0.6-cm-diameter paper disks (Schleicher and Shuell, Inc., Keene, N.H.) preloaded with 0.05 or 1.0 μmol of $CdCl_2$ were placed on the plates. The plates were incubated at 37°C for 24 h, and the diameters of zones of growth inhibition surrounding the disks were measured.

RESULTS

Deletion analysis. A series of deletion mutants were prepared. Fragments (3.7 and 3.0 kb) which contained the promoter and two ORFs were cloned in plasmid pSK265 (pKPY1 and pKPY2, respectively, in Fig. 1), and a deletion in *cadC* was generated by cloning the 2.6-kb DNA fragment (which is missing the 5′ half of the *cadC*) (plasmids pKYP5 and pKPY6 in Fig. 1). Intact *cadC* under its own promoter (0.6 kb) was cloned into both pSK265 (pKPY8) and pUC19 (pKPY22).

In both *S. aureus* and *B. subtilis*, pKPY1 and pKPY2 conferred equal and maximum resistance levels. Cells with the vector pSK265 or pKPY8 were as sensitive to $CdCl_2$ as cells without plasmids were (Fig. 3; Table 2). The other deletion variants gave intermediate resistance levels lower than those of pKPY1 or pKPY2 (Fig. 3; Table 2).

For cells with the 2.6-kb fragment expressing only *cadA* (pKPY5 in Fig. 1), the MIC of cadmium increased twofold, and cells showed no noticeable decrease in the size of the inhibition zone (Table 2), compared with that of cells harboring pSK265. The intact *cadA* determinant in pKPY1 or pKPY2 caused a 512-fold increase in MIC in *S. aureus*, a 16-fold increase in MIC in *B. subtilis*, and large decreases in

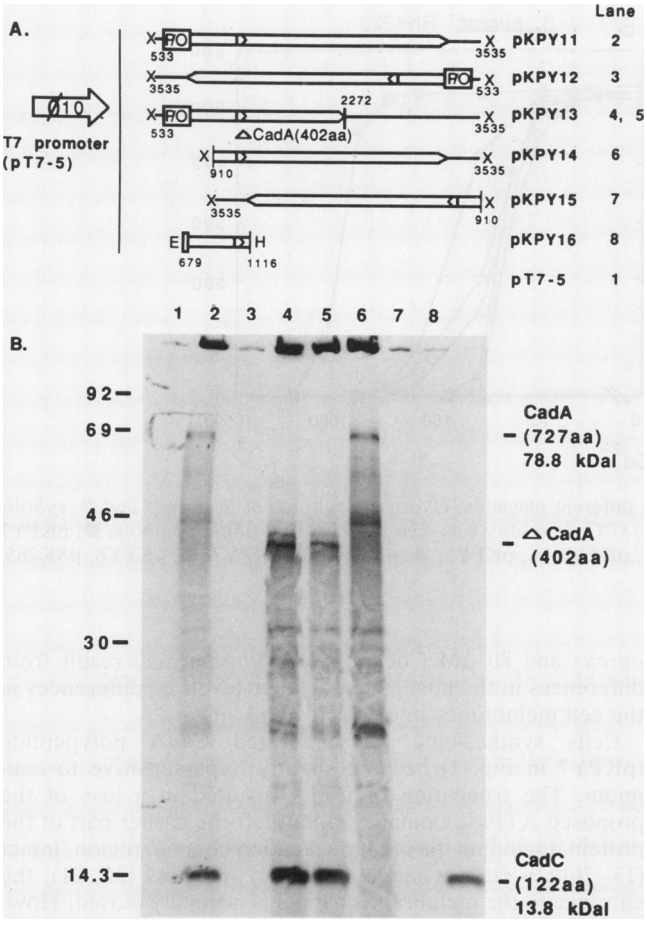

FIG. 2. CadA and CadC proteins expressed in *E. coli* under T7 polymerase control. (A) Plasmid constructs (the legend to Fig. 1 describes the fragments used). Restriction enzyme sites: X, *Xba*I; E, *Eco*RI; H, *Hin*dIII. The arrow of the T7 promoter shows the direction of transcription. (B) Autoradiography of SDS-polyacrylamide gel with [^{35}S]methionine-labelled proteins. Molecular weight marker sizes (in thousands) are indicated at the left. The identified positions (and sizes, in amino acids [aa]) of the expressed proteins are indicated at the right side of gel. Lanes: 1, pT7-5 vector with no insert; 2, pKPY11 (the 3.0-kb intact operon insert in the T7 promoter orientation); 3, pKPY12 (the 3.0-kb insert in the opposite orientation); 4 and 5, pKPY13 (the 3.0-kb with the nonsense mutations at the 402nd codon of *cadA* and in the T7 promoter orientation); 6, pKPY14 (the 2.6-kb insert with the intact *cadA* gene in T7 promoter orientation); 7, pKPY15 (the 2.6-kb insert in the opposite orientation); 8, pKPY16 (the 0.45-kb insert with *cadC* in the T7 promoter orientation).

the sizes of inhibition zones on petri dishes (Fig. 3; Table 2). In contrast, the effects of the *cadA* gene alone (in plasmids pKPY5 and pKPY6) were smaller than the effects in cells harboring pKPY1 (Fig. 1 and 3; Table 2). Cells harboring pKPY3 or pKPY4 (lacking the *cadA* promoter) lost most resistance to cadmium and zinc (Fig. 1 and 3; Table 2). Cells harboring pKPY7 (the nonsense mutation within *cadA*) became slightly hypersensitive to cadmium (Fig. 3; Table 2). These results showed that the 3.0 kb containing the promoter, *cadC*, and *cadA* were essential for full cadmium and zinc resistance.

When a pUC19-derived plasmid with the 3.0 kb containing the *cadC* and *cadA* genes was transformed into *E. coli*, the cells showed increased sensitivity to cadmium (data not shown). *E. coli* cells harboring a plasmid with only the promoter plus the *cadC* gene showed a slight hypersensitivity to cadmium, compared with cells harboring the *cadC* and *cadA* genes but no promoter (data not shown). These results suggest that the *S. aureus cadA* cadmium resistance determinant promoter is functional in *E. coli* and that the *cadA* and *cadC* gene products make *E. coli* hypersensitive but not resistant to cadmium.

Expression of *cadC* and *cadA* polypeptides in *E. coli*. The *cadC* and *cadA* genes were cloned into the bacteriophage T7 expression system (Fig. 2). The gene products made under control of the T7 promoter were labelled with [^{35}S]methionine and analyzed by autoradiography of sodium dodecyl sulfate (SDS)-polyacrylamide gels (Fig. 2). The 3.0-kb fragment, which contains the *cadC* and *cadA* genes, produced two polypeptides of appropriate sizes in one orientation only (compare lanes 2 and 3 in Fig. 2). The upper (calculated molecular weight, 78,800 [CadA]) and lower (molecular weight, 13,800 [CadC]) radioactive bands migrated as if they had 68-kDa and 14-kDa molecular masses, respectively. Cells carrying only the *cadA* gene yielded the upper band (Fig. 2, lane 6), and cells which carried only the *cadC* gene produced the lower band (Fig. 2, lane 8). Cells expected to produce the truncated 402-amino-acid CadA polypeptide yielded a corresponding band of the expected reduced molecular weight (Fig. 2, lanes 4 and 5). These results show that both ORFs can be translated, that the upper band is indeed CadA, and that the lower band is CadC. Four additional bands at the 46-kDa, 32-kDa, 25-kDa, and 20-kDa positions might be degraded products of CadA, since cells carrying a cloned fragment in the orientation opposite to that of the T7 promoter or cells carrying the pT7-5 vector without an insert did not show such bands (Fig. 2, lanes 1, 3, and 7). A Coomassie blue-stained band migrating at a location corresponding to CadC was visualized after pT7-5 induction with the intact 3.0-kb fragment, but the band corresponding to CadA was not present in amounts sufficient to be visible (data not shown).

Complementation between *cadA* and *cadC* on compatible plasmids. pKPY6, which expressed the CadA protein under control of the constitutive ORFD promoter of vector pSK265, showed significantly reduced resistances to Cd^{2+} and Zn^{2+} (Fig. 1 and 3; Table 2). pKPY6 was complemented to essentially full cadmium resistance by an intact *cadC* gene in *trans* under control of its own promoter (Fig. 4). With MIC determinations on petri dishes, a twofold difference in MICs between the intact cadmium resistance determinant (with plasmid pKPY2) and the complementing pair (pKPY6 and pKPY9) with *cadA* under pSK265 ORFD control was observed (data not shown). This difference might be caused by the reduced production of the CadA efflux protein by the heterologous ORFD promoter of pSK265.

Uptake of radioactive $^{109}Cd^{2+}$. With the intact cadmium resistance determinant, uninduced cells accumulated more $^{109}Cd^{2+}$ than the induced cells did and as much as the sensitive cells with the vector plasmid did (data not shown). A similar inducible reduction in net cadmium accumulation was seen with the complementing pair of plasmids, with *cadC* under its own promoter but *cadA* being expressed constitutively under vector pSK265 ORFD promoter control (data not shown). Cells expressing the *cadA* gene alone under the ORFD promoter did not show the reduced Cd^{2+} accumulation of cells with the intact system or the two genes in *trans* (data not shown). Therefore the reduced net cadmium accumulation requires both genes (*cadC* and *cadA*)

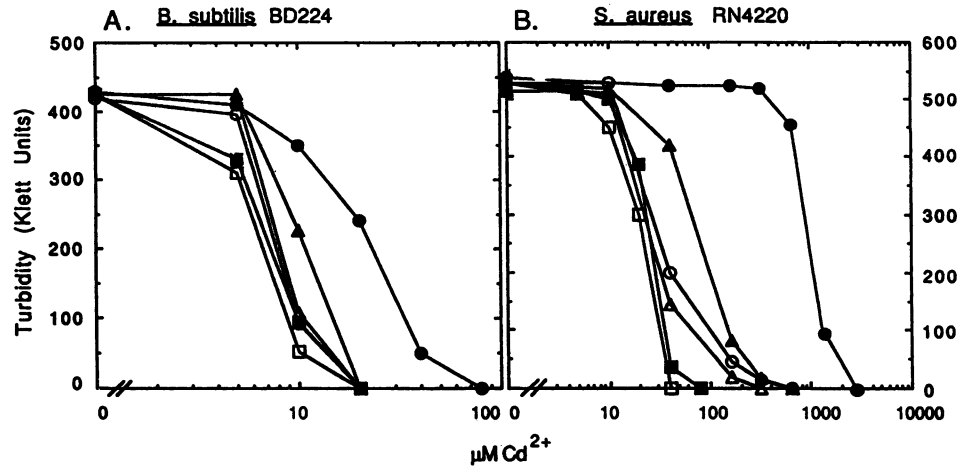

FIG. 3. Growth inhibition of *S. aureus* and *B. subtilis* cells harboring different plasmids. Overnight cultures of *S. aureus* and *B. subtilis* were diluted in 2XNY broth containing $CdCl_2$ and were grown for 12 h at 37°C. Turbidity was measured in Klett units. Symbols: ●, pKPY1 (pKPY2 gave indistinguishable turbidities at all Cd^{2+} concentrations); ○, pKPY3; △, pKPY5; ▲, pKPY6; □, pKPY7; ■, pKPY8, pSK265, or no plasmid.

and induction of the system. The accompanying paper (36) describes an experiment showing the need for both *cadA* and *cadC* and induction for efflux of radioactive Cd^{2+} from preloaded cells.

DISCUSSION

The sequence of the cadmium resistance determinant of *S. aureus* plasmid pI258 (13) contains two ORFs. We present evidence that both ORFs are functional genes (i.e., they encode proteins) and are needed for resistance to cadmium. Deletion analysis showed that neither *cadA* nor *cadC* alone is enough to confer full cadmium and zinc resistance. The cadmium resistance level of *B. subtilis* cells harboring the same sets of deletion fragments showed the same pattern of resistance and the same need for both genes for full resistance. The difference in the MICs (2.5 mM $CdCl_2$ for *S. aureus* and 80 μM $CdCl_2$ for *B. subtilis*) may result from differences in the host cell resistance levels or differences in the cell membranes into which CadA inserts.

Cells synthesizing the truncated CadA polypeptide (pKPY7 in Fig. 1) became slightly hypersensitive to cadmium. The truncation of CadA resulted in a loss of the proposed ATPase domain (26) but left the earlier part of the protein, including the presumed cation channel region, intact (13, 26). In current models of E_1E_2 ATPases (20, 21), the entrance to the membrane channel is normally closed. However, with the truncated CadA protein the channel might be transiently open and this might cause hypersensitivity.

In *E. coli*, quite surprisingly, the 3.0-kb *cadA* fragment conferred cadmium sensitivity instead of cadmium resistance to strain JM83. CadC alone made *E. coli* slightly sensitive to cadmium (data not shown).

With *cadA* and *cadC* on two compatible plasmids, com-

TABLE 2. Resistance levels for *S. aureus* and *B. subtilis* harboring plasmids with different 5′ and 3′ deletions of the cadmium resistance determinant region

Plasmid	MIC on plates (μM $CdCl^{2+}$)		Zone of inhibition (cm)[a]	
	B. subtilis BD224	*S. aureus* RN4220	*B. subtilis* BD224	*S. aureus* RN4220
None	5	5	1.6	1.7
pSK265	5	5	1.6	1.7
pKPY1	80	2,560	1.0	0.4
pKPY2	80	2,560	1.0	0.4
pKPY3	10	20	1.7	1.5
pKPY4	ND[b]	40	ND	1.0
pKPY5	10	10	1.6	1.6
pKPY6	20	40	1.2	1.0
pKPY7[c]	2	3	2.1	2.0
pKPY8	5	5	1.6	1.7

[a] A. 0.05-μmol amount of CdCl per disk for *B. subtilis* and a 1.0-μmol amount of CdCl per disk for *S. aureus* were used. After 24 h of incubation at 37°C, the diameters of the inhibition zones surrounding the disks (minus the 0.65-cm diameter of the paper disks) were measured.

[b] ND, not done.

[c] Hypersensitivity of pKPY7 to cadmium is shown.

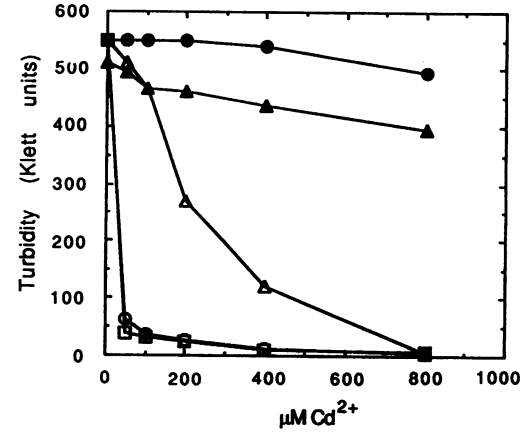

FIG. 4. Complementation between *cadC* and *cadA* for resistance to cadmium. Overnight cultures of *S. aureus* RN4220 cells with the plasmids listed were diluted and grown in 2XNY medium at 37°C for 20 h, and turbidities were measured as described in Materials and Methods. Symbols: ○, pSK265; ●, pKPY2; △, pKPY6; □, pKPY9; ▲, pKPY6 and pKPY9.

plementation between two proteins provided full cadmium resistance (Fig. 4). These results showed that both CadA and CadC are needed for full resistance. Measurements of MICs supported the need for both CadA and CadC for full resistance to Cd^{2+} and Zn^{2+}. The MIC for cells producing only CadA was 40 μM; the MIC for cells producing CadC alone was only 5 μM (the same as that found with the vector plasmid pSK265). These cadmium resistance levels were very low compared with the MIC of 2.56 mM for the intact promoter with the whole cadmium resistance determinant of plasmid pKPY2.

The *cadA* cadmium resistance system functions as an energy-dependent pump (26, 27, 32), causing the efflux of $^{109}Cd^{2+}$ from the resistant cells. The energy coupling for the CadA system has recently been directly shown to be ATP in experiments on the uptake of radioactive cadmium by everted membrane vesicles (31). The need for CadC for efflux was shown directly in efflux measurements (36). Although the role of CadC in efflux is not clear, polypeptides in addition to the primary ATPase subunit have been found repeatedly with other E_1E_2 ATPases (5, 20, 26).

The CadC amino acid sequence is significantly related to the ArsR proteins of plasmids from both *E. coli* (18) and *Staphylococcus* species (6, 16) and to CadX, the product of an ORF that is part of the *cadB* cadmium resistance system of a different *S. aureus* plasmid (4). CadC has 29 to 32% identical amino acids when optimally aligned with different versions of ArsR and 41% identical amino acids with CadX (analysis not shown). ArsR is the regulatory protein of the arsenic resistance system (6, 15, 18) but CadX is thought to be a required structural component of the CadB system (4), as CadC is required for the CadA system. Although CadC, ArsR, and CadX have sequence homologies, how these relate to the functional roles and evolutionary histories of the genes is uncertain.

ACKNOWLEDGMENTS

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REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. **Brandl, C. J., N. M. Green, B. Korczak, and D. H. MacLennan.** 1986. Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. Cell **44**:597–607.
3. **Dubnau, D., R. Davidoff-Abelson, B. Scher, and C. Cirigliano.** 1973. Fate of transforming deoxyribonucleic acid after uptake by competent *Bacillus subtilis*: phenotype characterization of radiation-sensitive recombination-deficient mutants. J. Bacteriol. **114**:273–286.
4. **Dyke, K. G. H., J. A. Walters, and S. P. Curnock (Oxford University).** 1991. Personal communication.
5. **Epstein, W., M. O. Walderhaug, J. W. Polarek, J. E. Hesse, E. Dorus, and J. M. Daniel.** 1990. The bacterial Kdp-ATPase and its relationship to other transport ATPases, such as the Na^+/K^+- and the Ca^{2+}-ATPases in higher organisms. Philos. Trans. R. Soc. Lond. B **326**:479–487.
6. **Ji, G., and S. Silver.** Unpublished data.
7. **Jones, C. L., and S. A. Khan.** 1986. Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. J. Bacteriol. **166**:29–33.
8. **Khan, S. A. (University of Pittsburg).** Personal communication.
9. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. M. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature (London) **305**:709–712.
10. **Nies, D. H., and S. Silver.** 1989. Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus*. J. Bacteriol. **171**:896–900.
11. **Novick, R. P., E. Murphy, T. J. Gryczan, E. Baron, and I. Edelman.** 1979. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. Plasmid **2**:109–129.
12. **Novick, R. P., C. Sanchez-Rivas, A. Gruss, and I. Edelman.** 1980. Involvement of the cell envelope in plasmid maintenance: plasmid curing during the regeneration of protoplasts. Plasmid **3**:348–358.
13. **Nucifora, G., L. Chu, T. K. Misra, and S. Silver.** 1989. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium efflux ATPase. Proc. Natl. Acad. Sci. USA **86**:3544–3548.
14. **Pedersen, P. L., and E. Carafoli.** 1987. Ion motive ATPases. I. Ubiquity, properties and significance to cell function. Trends Biochem. Sci. **12**:146–150.
15. **Rosen, B. P., C. M. Hsu, C. E. Karkaria, J. B. Owolabi, and L. S. Tisa.** 1990. Molecular analysis of an ATP-dependent anion pump. Philos. Trans. R. Soc. Lond. B **326**:455–463.
16. **Rosenstein, R., A. Peschel, B. Wieland, and F. Götz.** Unpublished data.
17. **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.
18. **San Francisco, M. J. D., C. L. Hope, J. B. Owolabi, L. S. Tisa, and B. P. Rosen.** 1990. Identification of the metalloregulatory element of the plasmid-encoded arsenical resistance operon. Nucleic Acids Res. **18**:619–624.
19. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
20. **Serrano, R.** 1988. Structure and function of proton translocating ATPase in plasma membranes of plants and fungi. Biochim. Biophys. Acta **947**:1–28.
21. **Serrano, R.** 1989. Structure and function of plasma membrane ATPase. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**:61–94.
22. **Shalita, Z., E. Murphy, and R. P. Novick.** 1980. Penicillinase plasmids of *Staphylococcus aureus*: structural and evolutionary relationships. Plasmid **3**:291–311.
23. **Shivakumar, A. G., T. J. Gryczan, Y. I. Kozlov, and D. Dubnau.** 1980. Organization of the pE194 genome. Mol. Gen. Genet. **179**:241–252.
24. **Silver, S., and R. A. Laddaga.** 1990. Molecular genetics of heavy metal resistance systems in *Staphylococcus aureus* plasmids, p. 531–549. *In* R. P. Novick (ed.), Molecular biology of the *Staphylococci*. VCH Publications, New York.
25. **Silver, S., and T. K. Misra.** 1988. Plasmid-mediated heavy metal resistances. Annu. Rev. Microbiol. **42**:717–743.
26. **Silver, S., G. Nucifora, L. Chu, and T. K. Misra.** 1989. Bacterial resistance ATPases: primary pumps for exporting toxic cations and anions. Trends Biochem. Sci. **14**:76–80.
27. **Silver, S., and M. Walderhaug.** Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev., in press.
28. **Smith, K., and R. P. Novick.** 1972. Genetic studies on plasmid-linked cadmium resistance in *Staphylococcus aureus*. J. Bacteriol. **112**:761–772.
29. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
30. **Tisa, L. S., and B. P. Rosen.** 1990. Transport systems encoded by bacterial plasmids. J. Bioenerg. Biomembr. **22**:493–507.
31. **Tsai, K.-J., K.-P. Yoon, and A. R. Lynn.** ATP-dependent cadmium transport by the staphylococcal *cadA* determinant in everted membrane vesicles of *Bacillus subtilis*. Submitted for publication.
32. **Tynecka, Z., Z. Gos, and J. Zajac.** 1981. Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in

Staphylococcus aureus. J. Bacteriol. **147**:313–319.
33. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
34. **Walderhaug, M. O., D. C. Dosch, and W. Epstein.** 1987. Potassium transport in bacteria, p. 84–130. *In* B. P. Rosen and S. Silver (ed.), Ion transport in prokaryotes. Academic Press, Inc., San Diego, Calif.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
36. **Yoon, K. P., T. K. Misra, and S. Silver.** 1991. Regulation of the *cadA* cadmium resistance determinant of *Staphylococcus aureus* plasmid pI258. J. Bacteriol. **173**:7643–7649.