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Chromosomal Locus for Cadmium Resistance in *Pseudomonas putida* Consisting of a Cadmium-Transporting ATPase and a MerR Family Response Regulator

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Pseudomonads from environmental sources vary widely in their sensitivity to cadmium, but the basis for this resistance is largely uncharactarized. A chromosomal fragment encoding cadmium resistance was cloned from *Pseudomonas putida* 06909, a rhizosphere bacterium, and sequence analysis revealed two divergently transcribed genes, *cadA* and *cadR*. CadA was similar to cadmium-transporting ATPases known mostly from gram-positive bacteria, and to ZntA, a lead-, zinc-, and cadmium-transporting ATPase from *Escherichia coli*. CadR was related to the MerR family of response regulators that normally control mercury detoxification in other bacterial systems. A related gene, *zntR*, regulates *zntA* in *E. coli*, but it is not contiguous with *zntA* in the *E. coli* genome as *cadA* and *cadR* were in *P. putida*. In addition, unlike ZntA and other CadA homologs, but similar to the predicted product of gene PA3690 in the *P. aeruginosa* genome, the *P. putida* CadA sequence had a histidine-rich N-terminal extension. CadR and the product of PA3689 of *P. aeruginosa* also had histidine-rich C-terminal extensions not found in other MerR family response regulators. Mutational analysis indicated that *cadA* and *cadR* are fully responsible for cadmium resistance and partially for zinc resistance. However, unlike *zntA*, they did not confer significant levels of lead resistance. The *cadA* promoter was responsive to Cd(II), Pb(II), and Zn(II), while the *cadR* promoter was only induced by Cd(II). CadR apparently represses its own expression at the transcriptional level. However, CadR apparently does not repress *cadA*. Homologs of the cadmium-transporting ATPase were detected in many other *Pseudomonas* species.

The cadmium cation is toxic to most microorganisms, probably by binding to essential respiratory proteins (54) and through oxidative damage by production of reactive oxygen species (50). Cadmium enters bacterial cells by the transport systems for essential divalent cations such as Mn^{2+} (53) or Zn^{2+} (22). Microbial resistance to cadmium is usually based on energy-dependent efflux mechanisms (46).

One of the best-characterized bacterial cadmium resistance mechanisms is determined by the cadmium-transporting ATPase found initially in gram-positive bacteria (47). The cadmium-transporting ATPase is a P-type ATPase, a member of the cation-transporting ATPases found in both *Bacteria* and *Eucarya* (48). It is widespread in *Staphylococcus aureus* (36) and *Listeria monocytogenes* (23). The ATPase is encoded by *cadA*, which is usually plasmid-borne and associated with transposons in *L. monocytogenes* (23, 24). The cadmium efflux genes in *S. aureus* are both plasmid-borne and chromosomal. The chromosomal locus of *S. aureus* is similar to *cadAC* of the plasmid-borne genes but confers resistance to low concentrations (MIC of 128 μg/ml) of cadmium nitrate (56). CadC, encoded immediately downstream of *cadA*, is a regulatory protein, which is also required for cadmium resistance in gram-positive bacteria. CadC binds to the promoter-operator area of the *cadA* gene and works as a transcriptional repressor in vitro (12).

Another class of cadmium resistance genes in *S. aureus* includes *cadB* or the *cadB*-like *cadD*, which confers a different mechanism of resistance (11, 39). The function of CadB is not well defined, but it may protect bacterial cells by binding cadmium in the membrane (39). A positive response regulator gene, *cadX*, was found in the *cadB*-like operon on plasmid pLUG10 in *Staphylococcus lugdunensis*. CadX is similar to CadC of the *cadA* operon but acts as a positive regulator (7). CadD of *S. aureus* is similar to CadB of *S. lugdunensis*. Hydropathy analysis of the CadD from plasmid pRW001 revealed transmembrane domains with potential cadmium cation-binding motifs in the cytosolic domain (11).

A well-characterized cadmium resistance system in gram-negative bacteria is the cadmium, zinc, and cobalt (*czc*) resistance determinant of *Alcaligenes eutrophus*. The CzcC, CzcB, and CzcA proteins comprise an active efflux mechanism driven by a cation-proton antiporter, rather than a cation-transporting ATPase (35). Homologs of the *czc* genes, called *czr*, which conferred cadmium and zinc resistance, were recently identified in the chromosome of *Pseudomonas aeruginosa* and appear to be highly conserved in environmental isolates of that species (14). In addition, a homolog of the *cadAC* operon, found previously only in gram-positive bacteria, was identified in the gram-negative bacterium *Stenotrophomonas maltophilia* (1). The flanking insertion sequences and unusual G+C content of the locus was suggestive of its transfer from gram-positive bacteria (10).

Recently, the genome sequences of several gram-negative

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	F⁻ *recA1* Δ(*lacZYA-argF*) U169 *hsdR17 thi-1 gyra66 supE44 endA1 relA1* Δ(*lacZ*)M15	44
GM2163	F⁻ *ara-14 leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 supE44 hisG4 rpsL 136* (Str^r) *xyl-5 mtl-1 dam13::*Tn*9* (Cam^r) *dcm-6 mcrB1 hsdR2 mcrA*	New England Biolabs
HB101	F⁻ *hsdS20* (r⁻m⁻) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ⁻	5
P. putida		
06909	Amp^r, wild type	59
06909s21x	Kan^r, kanamycin cassette inserted into the LysR family response regulator in wild-type strain 06909 chromosomal locus	This study
06909s22x	Kan^r, kanamycin cassette inserted into cadmium-transporting ATPase in wild-type strain 06909 chromosomal locus	This study
06909s23	Gen^r, gentamicin cassette inserted into *cadR* in wild-type strain 06909 chromosomal locus	This study
Plasmids		
pUC119	Ap^r, cloning vector	60
pRK415	Tc^r, RK2 derived broad-host-range cloning vector	21
pRK2013	Km^r, mobilization helper	13
pHRP311	Gm^r Sm^r, an RSF1010 derivative, broad-host-range transcriptional fusion vector	38
pRKL1	Tc^r, a 3.5-kb *Hin*dIII fragment carrying a promoterless *lacZ* gene from pHRP311 cloned into pRK415	This study
pMGm	Ap^r Gm^r, plasmid carrying a gentamicin resistance gene cassette	33
pMKm	Ap^r Km^r, plasmid carrying a kanamycin resistance gene cassette	33
pRIVS2	Tc^r, pRIV16 carrying a clone expressed on cultured *Phytophthora parasitica*	25
pUIVS2	Ap^r, pUC119 carrying a 5.5-kb *Eco*RI fragment from pRIVS2	25
pUIVS21	Ap^r, pUC119 carrying a 2-kb *Sph*I fragment from pUIVS2	25
pUIVS22	Ap^r, pUC119 carrying a 1-kb *Sph*I fragment from pUIVS2 with a partial cadmium-transporting ATPase gene	This study
pUIVS21K	Ap^r Km^r, pUIVS21 carrying a 1.8-kb kanamycin cassette from pMKm cloned in a blunt-ended *Xho*I site of *lysR*	This study
pUIVS22K	Ap^r Km^r, pUIVS22 carrying a 1.8-kb kanamycin cassette from pMKm cloned in a blunt-ended *Xho*I site of *cadA*	This study
pRS21k	Tc^r Km^r, pRK415 carrying a 3.8-kb *Sph*I fragment from pUIVS21K	This study
pRS22k	Tc^r Km^r, pRK415 carrying a 2.8-kb *Sph*I fragment from pUIVS22K	This study
pRS23s	Tc^r Gm^r, pRCD12 carrying a 1.9-kb *Pst*I fragment blunt-ended gentamicin cassette from pMGm cloned in a blunt-ended *Cla*I site of *cadR*	This study
pRCD12	Tc^r, a 4.2-kb *Pst*I fragment carrying cadmium resistance genes in pRK415	This study
pRCD22	Tc^r, the 4.2-kb *Pst*I fragment in pRCD12 is reverse oriented against *lac* promoter in pRK415	This study
pRCD13	Tc^r, a 3.0-kb *Pst*I-*Hin*dIII fragment of pRCD12 cloned in pRK415	This study
pRCD14	Tc^r, a 1.0-kb *Pst*I-*Xho*I fragment was removed from pRCD13	This study
pUCD12	Ap^r, a 3.0-kb *Pst*I-*Hin*dIII fragment of pRCD12 in pUC119	This study
pUCD30	Ap^r, pUC129 carrying a 2-kb *Cla*I-*Xho*I fragment of pRCD12	This study
pRCD31	Tc^r, pRKL1 carrying *cadA* promoter region, an 800-bp *Eco*RI-*Kpn*I fragment of pUCD30	This study
pRCD32	Tc^r, pRKL1 carrying *cadR* promoter region, an 800-bp *Pst*I-*Kpn*I fragment of pUCD30	This study

[a] Amp^r, chromosomal ampicillin resistance; Kan^r, chromosomal kanamycin resistance; Gen^r, chromosomal gentamicin resistance; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Sm^r, streptomycin resistance.

bacteria have revealed homologs of *cadA*. Functional analysis of their role in metal resistance has been conducted in *Helicobacter pylori* (16) and with the *Escherichia coli cadA* homolog, *zntA* (42). ZntA was originally described as a zinc-transporting ATPase, but it also confers resistance to cadmium and lead. Recent studies proposed that CadA of *S. aureus* and ZntA of *E. coli* are Pb(II)-transporting ATPases (40, 41, 45). In contrast to *cadA* of gram-positive bacteria, *zntA* expression is regulated by *zntR*, encoding a MerR homolog, but located in another region of the *E. coli* chromosome from *zntA* (6, 37).

In this study, and in previous reports (14, 18, 28, 43), *Pseudomonas* spp. from the soil and other environments have been shown to vary widely in sensitivity to cadmium. In addition to the finding of *czc* homologs in *P. aeruginosa*, one report identified *cadA*-encoded cadmium resistance in a *Pseudomonas* sp. from river sediment (61). For most pseudomonads, the basis of cadmium resistance has not been characterized, but the recent finding of *cadA* homologs in several bacterial genome sequences suggests that CadA may play a broader role in cadmium resistance in gram-negative bacteria. We report here homologs of CadA-ZntA and ZntR that are adjacent in the chromosome of a rhizosphere strain of *Pseudomonas putida* 06909 (59) and confer higher levels of cadmium resistance than ZntA of *E. coli* but not lead resistance. In addition, we investigated the specificity of metal ion induction of *cadA* and *cadR* transcription and the presence of *cadA* homologs in various *Pseudomonas* species.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are listed in Table 1. *E. coli* strains were cultured at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with the appropriate antibiotics (29). Antibiotic concentrations used for *E. coli* strains were as follows: tetracycline, 15 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; or gentamicin, 20 μg/ml. *E. coli* strain GM2163 (*dam* mutant) was a host strain used for manipulation of the plasmid carrying a *Cla*I site that was resistant to the restriction enzyme due to an overlapping Dam methylation site. *P. putida* strains were grown at 28°C on mannitol-glutamate medium (MG) (20) supplemented with yeast extract (0.25 g/liter) (MGY) or in MGY broth. Antibiotic concentrations used in MGY were as follows: tetracy-

cline, 20 μg/ml; kanamycin, 30 μg/ml; or gentamicin, 20 μg/ml. To conduct maker exchange mutagenesis, *P. putida* strains were cultured in LB broth under the same conditions without antibiotics.

General DNA manipulations and DNA sequencing. Standard recombinant DNA techniques were carried out for restriction endonuclease digestion, ligation, transformation of plasmid DNA, and isolation of total DNA (44). A DNA sequencing facility at the University of California, Berkeley, was used for DNA sequencing of cadmium resistance genes. The DNA sequences were analyzed with a software package from the Genetics Computer Group of the University of Wisconsin and the BLAST programs provided by the National Center for Biotechnology Information. The primers used for DNA sequencing were synthesized commercially (Genosys Biotechnologies, Inc., Woodlands, Tex). Preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org.

Plasmid construction for marker exchange mutagenesis. Insertional mutations in *cadA* and the gene downstream from *cadA* encoding a putative LysR family response regulator were constructed by cloning a kanamycin resistance gene cassette into *Xho*I sites in each of these genes, with the cassette in the opposite orientation relative to the transcription of the target genes (Table 1). The *cadR* gene was similarly mutated by insertion of a gentamicin resistance gene cassette. After subcloning of these constructs into the broad-host-range plasmid pRK415, the plasmids were introduced into the wild-type strain *P. putida* 06909 by triparental mating with pRK2013 as a helper plasmid. Marker exchange mutagenesis was carried out as described previously (57). Mutants sensitive to tetracycline but resistant to kanamycin or gentamicin were selected, and the correct gene replacement was confirmed by Southern blot hybridization. *P. putida* 06909s21x, 06909s22x, and 06909s23 were selected as LysR, CadA, and CadR mutants, respectively.

Plasmid construction to measure promoter activity. A low-copy-number broad-host-range transcriptional fusion vector, pRKL1, was constructed and used to analyze promoter activity from *cadA* and *cadR*. A promoterless *lacZ* gene was cloned into pRK415 in the opposite orientation to the *lac* promoter, and the promoter regions of divergently transcribed *cadA* and *cadR* were cloned in front of *lacZ* in this vector (Table 1). The resulting pRCD31 carries the *cadA* promoter, and pRCD32 carries the *cadR* promoter, as transcriptional fusions with *lacZ*.

MIC determination. The MICs of several metals, along with cadmium chloride, were determined on mannitol-glutamate agar (20) supplemented with yeast extract at 0.25 g/liter (MGY agar) and one of the following chemicals, at various concentrations, as described previously (9): $CdCl_2 \cdot 2.5H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $HgCl_2$, $AgNO_3$, $CoCl_2 \cdot 6H_2O$, $NiSO_4$, KCl, NaCl, $FeCl_3 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, $Pb(C_2H_3O_2) \cdot 3H_2O$, and $MnCl_2 \cdot 4H_2O$.

β-Galactosidase assays. *P. putida* 06909 or 06909s23 transconjugants carrying various plasmids were grown at 28°C overnight in 5 ml of MGY broth supplemented with appropriate antibiotics. A 0.3-ml sample of each culture was transferred into a fresh 5-ml portion of MGY broth supplemented with appropriate antibiotics and one of the metal salts at a subinhibitory concentration. The subinhibitory concentration chosen for each metal was the highest level that allowed the same growth rate of the strain as obtained without metals added, based on the MIC studies described above. The supplemented concentration of each metal for the induction studies was as follows: $CdCl_2 \cdot 2.5H_2O$, 12.5 μM; $CuSO_4 \cdot 5H_2O$, 25 μM; $ZnSO_4 \cdot 7H_2O$, 50 μM; $HgCl_2$, 0.5 μM; $CoCl_2 \cdot 6H_2O$, 5 μM; $NiSO_4$, 10 μM; $Pb(C_2H_3O_2) \cdot 3H_2O$, 10 μM; and $MnCl_2 \cdot 4H_2O$, 25 μM. The bacterial culture was further shaken under the same conditions for 6 h. Finally, the grown cells were resuspended into sterile water and lysed to measure the β-galactosidase activity as described by Miller (29) with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate.

Detection of *cadA* homologs from *Pseudomonas* species. Total genomic DNA from various bacteria was isolated as described previously (25). The *Sph*I fragment of pUIVS22 was gel purified and labeled by random primed labeling with a digoxigenin-dUTP DNA-labeling kit (Boehringer GmbH, Mannheim, Germany). Southern blot analysis of *Bam*HI-digested genomic DNA from various bacteria was performed on nylon membranes (MSI, Westboro, Mass.). Posthybridization washes were carried out at relatively low stringency (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate at 62°C). The hybridization was detected with a chemiluminescent substrate, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo(3.3.1.1^{3,7})decan}-4-yl)phenyl phosphate (CSPD; Boehringer), as described in the manufacturer's instructions.

Nucleotide sequence accession number. The DNA sequence containing the *P. putida* 06909 *cadA* and *cadR* genes has been assigned GenBank accession no. AF333961.

TABLE 2. MICs of different metals for *P. putia* 06909, *P. putida* 06909s22x, *P. putida* 06909s21x, and *P. putida* 06909s23

Metal	MIC (mM) for *P. putida* strain:			
	06909	06909s22x	06909s21x	06909s23
$CdCl_2 \cdot 2.5H_2O$	1.7	0.05	1.7	1.3
$CuSO_4 \cdot 5H_2O$	2.0	2.0	2.0	2.0
$ZnSO_4 \cdot 7H_2O$	11.5	7.0	11.5	10.5
$HgCl_2$	0.03	0.03	0.03	0.03
$AgNO_3$	<0.01	<0.01	<0.01	<0.01
$CoCl_2 \cdot 6H_2O$	0.3	0.3	0.3	0.3
$NiSO_4$	1.0	1.0	1.0	1.0
KCl	>100	>100	>100	>100
NaCl	>100	>100	>100	>100
$FeCl_3 \cdot 6H_2O$	>2.0	>2.0	>2.0	>2.0
$CaCl_2 \cdot 2H_2O$	>25	>25	>25	>25
$Pb(C_2H_3O_2) \cdot 3H_2O$	2.4	2.3	2.4	2.3
$MnCl_2 \cdot 4H_2O$	>2.5	>2.5	>2.5	>2.5

RESULTS AND DISCUSSION

Identification and mutagenesis of a cadmium-transporting ATPase. We have previously cloned genes from *P. putida* 06909 induced during colonization of a plant pathogenic fungus, *Phytophthora parasitica* (25). One of the clones, pUIVS2, carried at its 5′ end a partial sequence of a heavy-metal-transporting ATPase gene. The nucleotide sequence was determined for 1,043 bp of the pUIVS22 clone. The deduced amino acid sequence of the 1,030-bp open reading frame (ORF) was highly similar to the C-terminal half of cadmium-transporting ATPases and other heavy-metal-transporting ATPases from many bacterial species. Comparison of the deduced amino acid sequences showed that the ORF in clone pUIVS22 is not complete, missing the N-terminal sequences and its promoter. Based on the 342-amino-acid peptide sequence, our clone was the most similar to the cadmium-transporting ATPases of gram-positive bactaria. Since experiments to detect plasmids from wild-type strain *P. putida* 06909 were unsuccessful (data not shown), the ORF is likely to reside in the bacterial chromosome. An insertional mutation of the partial clone was made by inserting a kanamycin resistance gene cassette in the bacterial chromosome through marker exchange mutagenesis as described in Materials and Methods. Mutational analysis of the partial clone showed that the ATPase mutant, 06909s22x, was highly sensitive to cadmium and had moderately decreased zinc resistance (Table 2). The analysis of the 06909s22x strain, along with other mutants, is further described in the next section. Therefore, we designated the gene *cadA*, encoding a cadmium-transporting ATPase. The ATPase was not important for fungal hyphae colonization (data not shown).

Organization and nucleotide sequences of genes required for cadmium resistance. Since we had only a partial fragment of the cadmium-transporting ATPase gene, the clone carrying the full-length gene was obtained by complementing the cadmium-sensitive mutant 06909s22x with a subgenomic library of wild-type strain *P. putida* 06909 DNA constructed in a broad-host-range plasmid pRK415. The subgenomic library was constructed with *Pst*I inserts of ca. 4 to 6 kb, since Southern hybridizations showed that a 4.2-kb *Pst*I fragment of the wild-type strain 06909 hybridized with the *cadA* probe from pUIVS22 (data not shown). The plasmid pRCD12 complemented the cadmium sensitivity of mutant 06909s22x. When the orientation of

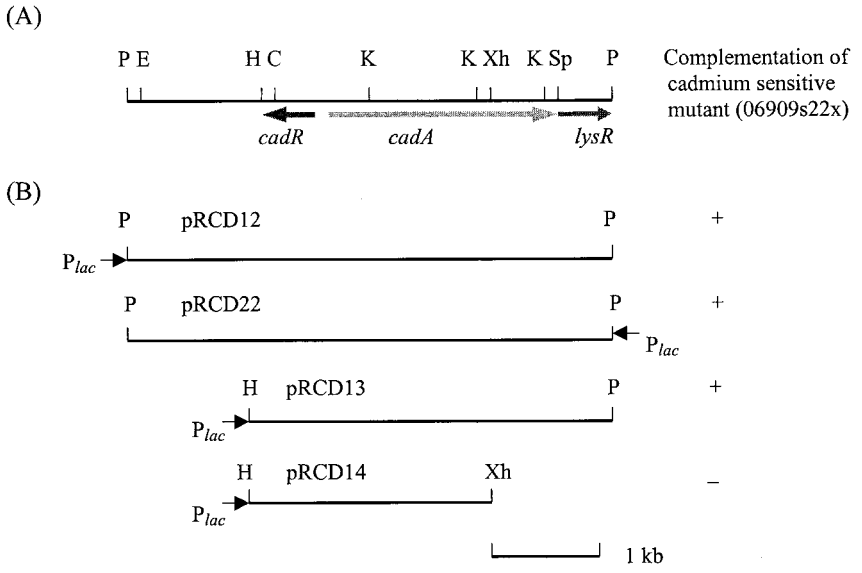

FIG. 1. Complementation of cadium-sensitive mutant 06909s22x (*cadA*::*km*). (A) Map of a 4.2-kb *Pst*I fragment carrying *cadA* and *cadR*. (B) Complementation of cadmium sensitivity by different subclones. P_{lac} indicates *lac* promoter in pRK415. Restriction endonuclease sites of the subclones are not indicated. A "+" indicates complementation of cadmium sensitivity to cadmium resistance, but a "−" indicates no complementation. Abbreviations: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I; Xh, *Xho*I.

the 4.2-kb insert of pRCD12 was reversed in pRCD22, the clone still complemented the mutation in 06909s22x. A subcloned 3-kb *Pst*I-*Hind*III fragment in pRCD13 also complemented the cadmium sensitivity of 06909s22x, but a 2-kb subclone in pRCD14 did not (Fig. 1B). This indicated that the 3-kb DNA fragment was sufficient for cadmium resistance in *P. putida* 06909. DNA sequence analysis of the insert in pUCD12 revealed two ORFs (Fig. 1A). One was *cadA*, encoding the cadmium-transporting ATPase, and the other was *cadR*, named after sequence comparison and mutational analysis. *cadR* was divergently transcribed from *cadA*, indicating the presence of separate promoters for the two genes. Another ORF found downstream of *cadA* was a partial fragment of a LysR family response regulator, which may be involved in bacterium-fungus interactions (25).

The space between the ATG codons for *cadA* and *cadR* was 84 bp, which should contain the promoter regions for the two transcriptional units. The organization and nucleotide sequence of this area was similar to a region in contig 10704 of the unfinished microbial genome of bioremediation strain *P. putida* KT2440 (32). A similar region was also found in the recently published sequence of *P. aeruginosa* (51). However, to our knowledge, the role of these homologs in cadmium resistance has not been determined. No significant similarities were found in the unfinished genome sequences of *P. putida* RPS1. The organization of *cadA* and *cadR* in *P. putida* 06909 is different from cadmium resistance determinants reported from other bacterial systems. The most similar system may be *zntA* and *zntR* of *E. coli*, but these two genes are separated from each other in the bacterial chromosome (6, 37). The divergent transcriptional orientation of *cadR* with respect to the adjacent *cadA* in *P. putida* is reminiscent of the organization of *merR* and the genes encoding mercury detoxification in other bacteria (47).

Similarity to genes for heavy metal resistance. *cadA* encodes 737 amino acids, and the deduced amino acid sequence shared strong similarity with other known heavy-metal-transporting ATPases, especially cadmium-transporting ATPases and zinc-transporting ATPases (Fig. 2). Alignment of CadA of *P. putida* 06909 showed the conserved motifs and residues for P-type ATPase function, including metal binding (Fig. 2), ATP binding, and aspartyl phosphorylation sites (data not shown) (49). Initially, ZntA was identified as a zinc-transporting ATPase in *E. coli*. However, it was also shown that ZntA transports Cd(II) and Pb(II) (40, 41). CadA of pI258 in *S. aureus*, a homolog of ZntA, also transports Cd(II), Zn(II), and Pb(II) (40). In contrast, CadA of *P. putida* 06909 provided more specific resistance to cadmium. It was partially responsible for zinc resistance, but its contribution to lead resistance was negligible. The level of cadmium resistance in *P. putida* 06909 was also 17-fold higher than the cadmium resistance of *E. coli* K-12 carrying *zntA*.

An unusual feature of CadA of *P. putida* 06909 and of *P. aeruginosa*, compared with other known CadA sequences, was a histidine-rich N-terminal extension (Fig. 2). It will be interesting to test whether this histidine-rich motif (HEHKHDH HAH) contributes to the higher levels of cadmium resistance conferred by CadA in *P. putida* or to the differences in the specificity of metal resistance.

cadR is predicated to encode a 147-amino-acid protein that is similar to MerR family response regulators (Fig. 3), which control the expression of mercury-detoxifying genes both positively and negatively (27, 47, 52). It was also similar to ZntR of *E. coli* (6) and to the predicted product of gene PA3689 of *P. aeruginosa* (51). The conserved helix-turn-helix motif for DNA binding, probably binding to the promoter-operator region of *cadA* and *cadR*, was found in the CadR sequence. There were also three conserved cysteine residues (Cys77,

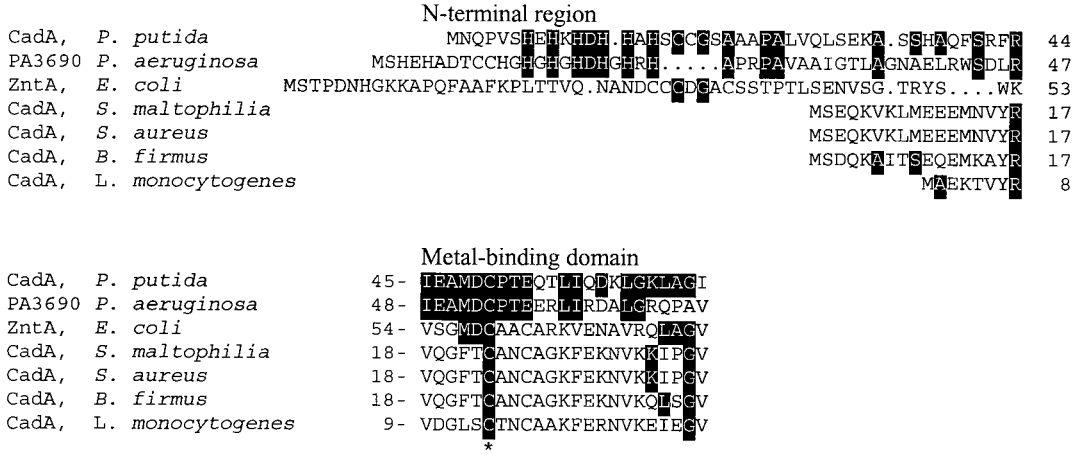

FIG. 2. Alignment of the N-terminal region and metal-binding domains of CadA or ZntA from *P. putida, P. aeruginosa* (51), *E. coli* (48), *S. maltophilia* (1), *S. aureus* (42), *B. firmus* (19), and *L. monocytogenes* (27). Identical bases are shown as white letters on a dark background. Asterisks indicate identical residues among all seven proteins.

Cys112, and Cys119 of CadR), which are putative cation-binding sites (62). The five heptad repeats between Cys77 and Cys 112 form a potential helical strand, which is an interface to form homodimers of MerR family response regulators (6). MerR represses expression of the *mer* operon in the absence of Hg(II) but activates expression in the presence of Hg(II) (15). MerR is expressed irrespective of Hg(II) (15). In contrast, our results indicate that CadR represses its own expression in the absense of Cd(II), but it is induced in the presence of Cd(II). Induction of ZntR by cadmium or other metal ions has not been reported, to our knowledge. Also, in contrast to MerR and ZntR, CadR has an unusual histidine-rich C-terminal extension (HSHVGRSHGH) (Fig. 3). It will be interesting to test if the histidine-rich C-terminal of CadR contributes to the metal specificity of the cadmium operon in *P. putida* 06909.

Mutation of *cadA* and *cadR* and heavy metal sensitivity. While the MIC of cadmium chloride for the wild-type *P. putida* 06909 was 1.7 mM, the MICs of cadmium chloride for the mutant *P. putida* 06909s22x (*cadA*::*km*) and 06909s23 (*cadR*::*gm*) were 0.05 and 1.3 mM, respectively (Table 2). The MIC of zinc sulfate was also different among mutant strains. The 06909s22x (*cadA*::*km*) mutant showed less resistance to zinc sulfate, although the MIC (7.0 mM) was still high. The mutant 06909s23 (*cadR*::*gm*) also showed only a slight decrease in its MIC (10.5 mM) of zinc sulfate from the wild-type MIC of 11.5 mM. A trivial decrease in the MIC of lead acetate was observed from the two mutant strains. The MIC to lead acetate was 2.4 mM for the wild type and 2.3 mM for both the *cadA* and the *cadR* mutants. However, the MICs of other heavy metals tested for mutant *P. putida* 06909s22x and 06909s23 strains were not different from that of wild-type *P. putida* 06909 (Table 2). The mutational analysis was consistent with *cadA* encoding a cadmium-specific transporting ATPase that is partially responsible for zinc resistance in *P. putida* 06909. *cadR* is also necessary for full resistance to zinc and cadmium. The MIC of cadmium chloride for *E. coli* K-12 strain carrying *zntA*

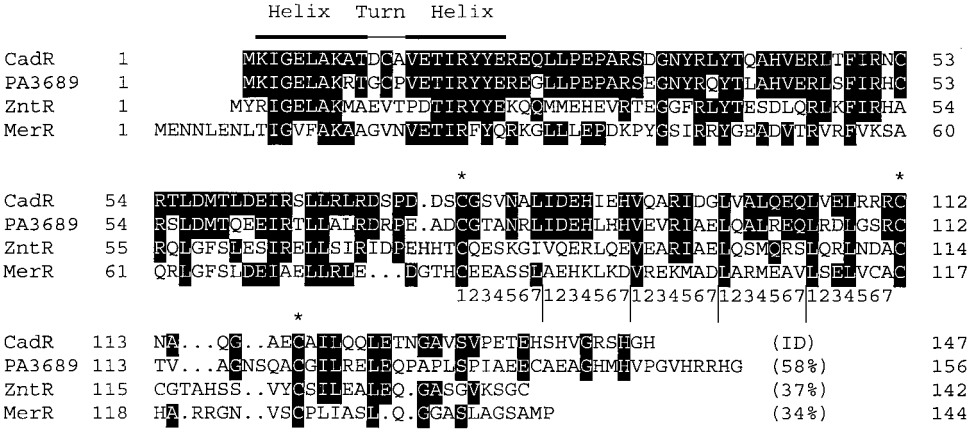

FIG. 3. Alignment of CadR of *P. putida* 06909 with the MerR family of activator-repressors, including the predicted product of gene PA3689 of *P. aeruginosa* (51), ZntR of *E. coli* (6), and MerR of Tn*501* in pVS1 of *P. aeruginosa* (31). Identical bases are shown as white letters on a dark background. Asterisks are Hg(II)-binding cysteine residues of MerR (62), and numbers 1 to 7 are the heptad repeats that form helical structures to promote homodimer formation (6). The identities (ID) of the amino acid sequences to CadR are indicated.

TABLE 3. Specificity of expression of β-galactosidase activity from the promoter regions of *cadA* and *cadR* in wild-type 06909 with different metal ions

Metal	Concn (μM)	β-Galactosidase activity (Miller units)[a] with plasmid:		
		pRKL1	pRCD31 (P_{cadA})	pRCD32 (P_{cadR})
No metal		196	690	735
$CdCl_2 \cdot 2.5H_2O$	12.5	235	8,417	1,652
$CuSO_4 \cdot 5H_2O$	25	NT	802	656
$ZnSO_4 \cdot 7H_2O$	50	NT	1,015	745
$ZnSO_4 \cdot 7H_2O$	500	NT	1,531	NT
$HgCl_2$	0.5	NT	1,007	592
$CoCl_2 \cdot 6H_2O$	5	NT	709	493
$NiSO_4$	10	NT	683	639
$MnCl_2 \cdot 4H_2O$	25	NT	761	605
$Pb(C_2H_3O_2) \cdot 3H_2O$	10	NT	1,033	672
$Pb(C_2H_3O_2) \cdot 3H_2O$	500	NT	3,250	NT

[a] NT, not tested.

was 0.1 mM on MGY plates (data not shown), while the MIC of cadmium acetate for this strain on LB medium was 1.5 mM (42). This indicated that *cadA* of *P. putida* 06909 is responsible for much higher resistance to Cd(II) than *zntA* of *E. coli*.

A LysR family response regulator was located immediately downstream from *cadA* with nine bases between the start codon of the LysR family response regulator and the stop codon of CadA. The Shine-Dalgarno box of the LysR regulator overlaps with the stop codon of *cadA*, which suggests that *cadA-lysR* may be part of a single transcriptional unit. However, disruption of the response regulator in the wild-type chromosome (06909s21x) did not affect cadmium resistance and showed the same resistance to all of the tested metals at the level of the wild-type strain *P. putida* 06909 (Table 2). Thus, the LysR response regulator does not appear to be involved in cadmium resistance in *P. putida* 06909, but the loss of LysR did impair bacterial growth rate and colony morphology (26).

Induction of the promoter region by cadmium and its specificity. The 84-bp space between the divergently transcribed *cadA* and *cadR* genes was assumed to contain two promoters with different orientations (Fig. 1A). A transcriptional fusion vector, pRKL1, with a promoterless *lacZ* gene was constructed. Since the vector pRKL1 alone showed a low background level of β-galactosidase activity in the presence of various metals in our strain (data not shown), it was used to construct a transcriptional fusion between the *cadA* or *cadR* promoter and the promoterless *lacZ*.

Both the *cadA* promoter (P_{cadA}) and the *cadR* promoter (P_{cadR}) were inducible by cadmium at subinhibitory concentrations in the wild-type strain 06909 background, but induction of *cadA* by Cd(II) was stronger than that of *cadR* (Table 3). Cd(II) was the most effective divalent cation for induction of *cadA*, but Pb(II) was also an effective inducer at a high concentration. Zn(II) and Hg(II) only induced *cadA* slightly. These results are similar to the induction of *ZntA* in *E. coli*, which was induced with the following order of effectiveness: Cd(II) > Pb(II) > Zn(II) (4). *cadA* of pI258 in *S. aureus* is induced with the following order of effectiveness: Pb(II) > Cd(II) > Zn(II) (41). However, only cadmium induced P_{cadR} (Table 3). The lack of induction of *cadR* by lead and zinc also suggests differences in the transcriptional apparatus at the *cadA* and *cadR* promoters.

TABLE 4. β-Galactosidase activity from promoter regions of *cadA* and *cadR* in wild-type strain 06909 and $CadR^-$ mutant 06909s23 backgrounds

Plasmid (promoter)	β-Galactosidase activity (Miller units)[a] in strain:			
	06909		06909s23	
	−Cd	+Cd	−Cd	+Cd
pRKL1 (control)	208	235	341	340
pRCD31 (P_{cadA})	690	8,417	636	8,129
pRCD32 (P_{cadR})	776	1,994	3,560	4,127

[a] Activity was determined without (−) or with (+) cadmium (Cd).

In the *cadR* mutant 06909s23 (*cadR*::*gm*) background, P_{cadA} was still cadmium inducible, but P_{cadR} was constitutive (Table 4). P_{cadA} was induced more than 10-fold in the presence of 12.5 μM cadmium chloride both in the wild-type and in the *cadR* mutant background. However, the induction of P_{cadR} by cadmium was only about 2.5-fold in the wild-type background. The constitutive expression of P_{cadR} in the *cadR* mutant background suggests that CadR represses its own expression. However, it is not clear if CadR is a repressor for *cadA* expression. The *cadR* mutant 06909s23 may still produce a truncated CadR, which maintains the N-terminal DNA binding domain and the first Cys77 residue for cation binding but missing the C-terminal 50 amino acids. The mutated CadR may thus retain some activity as an activator of *cadA*. However, the helical domain to form homodimers of CadR should have been dis-

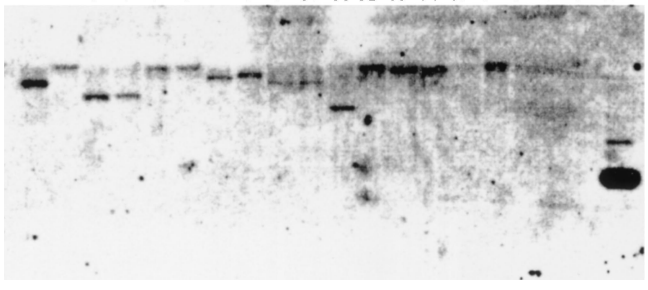

FIG. 4. Southern blot hybridization of the *cadA* gene of *P. putida* 06909 to total DNA from cadmium-resistant *Pseudomonas* species and other gram-negative bacteria. The MICs (mM) of cadmium chloride for each strain are indicated in parentheses with the original strain reference in brackets as follows: lane 1, *P. putida* 06909 (1.7 [59]); lane 2, *P. putida* 08891 (4.0 [9]); lane 3, *P. fluorescens* 09906 (0.7 [58]); lane 4, *P. fluorescens* 2-79 (0.7 [55]); lane 5, *P. fluorescens* 08908 (>4.0 [8]); lane 6, *P. fluorescens* 0785-17 (1.0 [30]); lane 7, *P. fluorescens* 08892 (0.1 [9]); lane 8, *P. fluorescens* 513 (0.1); lane 9, *P. aeruginosa* PAO1 (4.0 [17]); lane 10, *P. stutzeri* ATCC 17588 (4.0); lane 11, *P. cichorii* 07881 (0.1 [9]); lane 12, *P. syringae* pv. syringae PS61 (0.5 [3]); lane 13, *P. syringae* pv. tomato (0.4 [2]); lane 14, *Pseudomonas* sp. strain 02894 (2.0 [8]); lane 15, *Pseudomonas* sp. strain 07887 (1.0 [8]); lane 16, *Pseudomonas* sp. strain 07888 (1.2 [8]); lane 17, *Xanthomonas axonopodis* pv. vesicatoria 07882 (0.2 [9]); lane 18, *Agrobacterium radiobacter* K84 (0.05 [34]); lane 19, *E. coli* DH5α (0.2 [44]); and lane 20, plasmid DNA of pUIVS22 carrying the *cadA* gene. The DNA in each lane was completely digested with *Bam*HI, and a total of 2 μg of DNA per lane was loaded for each sample. A total of 100 ng of plasmid DNA was loaded for the plasmid in the lane 20.

rupted. Alternatively, another regulatory gene may contribute to *cadA* induction.

Conservation of cadmium-transporting ATPase genes among *Pseudomonas* species. Southern hybridizations were carried out to determine whether the cadmium-specific P-type ATPase is widespread in various *Pseudomonas* species. The bacterial strains, their characteristics, and respective cadmium chloride MICs are summarized in Fig. 4. Most of the pseudomonads tested showed various degrees of hybridization signals with the cadmium-transporting ATPase of *P. putida* 06909 except *Pseudomonas* sp. strain 07887 isolated from tomato (Fig. 4). No hybridization was observed from cadmium-sensitive *Xanthomonas axonopodis* pv. vesicatoria, *Agrobacterium radiobacter*, or *E. coli* DH5α. Southern hybridizations suggested that the CadA ATPase is conserved among many *Pseudomonas* species and strains. It will be interesting to investigate whether variations in the CadA sequence or in its expression contribute to the observed variation in cadmium resistance among *Pseudomonas* species and strains.

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