MrdH, a Novel Metal Resistance Determinant of *Pseudomonas putida* KT2440, Is Flanked by Metal-Inducible Mobile Genetic Elements[▽][†]

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We report here the identification and characterization of *mrdH*, a novel chromosomal metal resistance determinant, located in the genomic island 55 of *Pseudomonas putida* KT2440. It encodes for MrdH, a predicted protein of ~40 kDa with a chimeric domain organization derived from the RcnA and RND (for resistance-nodulation-cell division) metal efflux proteins. The metal resistance function of *mrdH* was identified by the ability to confer nickel resistance upon its complementation into *rcnA* mutant (a nickel- and cobalt-sensitive mutant) of *Escherichia coli*. However, the disruption of *mrdH* in *P. putida* resulted in an increased sensitivity to cadmium and zinc apart from nickel. Expression studies using quantitative reverse transcription-PCR showed the induction of *mrdH* by cadmium, nickel, zinc, and cobalt. In association with *mrdH*, we also identified a conserved hypothetical gene *mreA* whose encoded protein showed significant homology to NreA and NreA-like proteins. Expression of the *mreA* gene in *rcnA* mutant of *E. coli* enhanced its cadmium and nickel resistance. Transcriptional studies showed that both *mrdH* and *mreA* underwent parallel changes in gene expression. The mobile genetic elements Tn*4652* and IS*1246*, flanking *mrdH* and *mreA* were found to be induced by cadmium, nickel, and zinc, but not by cobalt. This study is the first report of a single-component metal efflux transporter, *mrdH*, showing chimeric domain organization, a broad substrate spectrum, and a location amid metal-inducible mobile genetic elements.

Bacterial efflux systems for inorganic metal cations and anions play an imperative role in the regulatory network governing metal homeostasis. These efflux systems are also important for the environmental adaptability of the bacteria thriving in metal-rich serpentine environments. Bioinformatic and functional genomic analyses have revealed that the efflux systems belonging to the resistance-nodulation-cell division (RND), cation diffusion facilitator (CDF), and P-type ATPases constitute the majority of the multiple layers of heavy metal resistance in an organism (36). Members of the RND protein family include group of bacterial transport proteins involved in heavy metal resistance, nodulation, and cell division. The best-characterized RND family members include the efflux systems CzcCBA (Cd^{2+}, Zn^{2+}, and Co^{2+} resistance), CnrCBA (Co^{2+} and Ni^{2+} resistance), and NccCBA (Ni^{2+}, Co^{2+}, and Cd^{2+} resistance) from *Cupriavidus metallidurans* (*Alcaligenes metallidurans* or *Ralstonia metallidurans*) and the CznCBA efflux system (Co^{2+}, Zn^{2+}, and Ni^{2+} resistance) from *Helicobacter pylori* (11, 14, 23, 29, 49, 52). CDF family members serve as secondary cation filters in bacteria. The characterized CDF family members CzcD, ZntA, and ZitB were found to have a substrate spectrum consisting of Co^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+}(1, 26, 57). P-type ATPase exporters that detoxify heavy metal cations by efflux include Cu-CPx-type ATPases (exporting Cu^+ and Ag^+) and Zn-CPx-type ATPases (export Zn^{2+}, Cd^{2+}, and Pb^{2+}) (4, 37, 43–45). Other heavy metal export

systems include the CHR protein family, NreB, and CnrT-like proteins (36). Recently, a new class of efflux proteins RcnA that confer Ni^{2+} and Co^{2+} resistance was identified in *Escherichia coli* (46).

Simultaneously, analyses of annotated bacterial genomes indicated the increased prevalence of mobile genetic elements (e.g., insertion sequences, integrons, and transposons) in disseminating metal resistance and drug resistance traits. Recent studies provided support for the prominent role of horizontal gene transfer (HGT) in the evolution of metal homeostasis in proteobacteria and also identified putative genomic islands (GIs) containing metal resistance genes in environmental bacteria like *C. metallidurans* and *Pseudomonas putida* KT2440 (8, 9, 34, 56). GIs are defined as horizontally acquired genetic elements that share the same structural features of pathogenicity islands (PAIs) and contribute to a microorganism's fitness, metabolic versatility, and adaptability. Most of the novel genes in a prokaryotic genome are more likely to be present in GIs and related horizontally acquired regions (19). Features frequently found to be associated with GIs include the presence of flanking repeats, mobility genes (e.g., integrases and transposases), proximal transfer RNAs (tRNAs), atypical guanine, and cytosine content. More recently, a putative GI was defined as a region containing eight (or more) consecutive open reading frames (ORFs) with dinucleotide bias, or eight consecutive ORFs with dinucleotide bias plus at least one mobility gene in the vicinity (19). The global features of *P. putida* KT2440 revealed 105 GIs of ≥4,000 bp with atypical GC contents and oligonucleotide signature. Most of these islands enhance the metabolic proficiency of *P. putida* as a saprophytic omnivore by endowing the organism with determinants of resistance and defense or with constituents and appendages of the cell wall. Major features are the uptake and degrada-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or characteristics	Source or reference
Strains		
P. putida		
KT2440	Wild type	35
HMR020	KT2440 *mrdH*::*ltrB*	This study
E. coli		
DH5α	F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r$_K^-$ m$_K^+$) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Lab collection
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r$_K^-$ m$_K^+$) Δ*el4*(Δ*mcrA*) *supE44 relA1* Δ(*proAB lac*) F′ [*proAB*$^+$ *traD36 lacI*^{q} *lacZ*ΔM15]	58
ARY023	F *supE thi* Δ(*lac-proAB*) *hsd5*/F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 *yohM*::*uidA*-Kan^r	46
Plasmids		
pTZ57R/T	Amp^r, cloning vector	Fermentas
pUC18	ColE1 replicon; Amp^r; 2.7 kb	58
pUCmrdH	pUC18 containing *mrdH* gene of *P. putida* KT2440	This study
pUCrcnA$_{Psy}$	pUC18 containing the *rcnA*$_{Psy}$ gene of *P. syringae* DC3000	This study
pUCmreA	pUC18 containing *mreA* gene of *P. putida* KT2440	This study
pJB653	Amp^r; 7.0 kb, derivative of pJB652 in which the PstI fragment containing the *Pm* promoter was cloned in the opposite orientation	3
pJBmrdH	pJB653 containing the *mrdH* gene of *P. putida* KT2440	This study

^{a} Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

tion of organic chemicals, ion transport, and the synthesis and secretion of secondary metabolites (56). Thirteen metal transporters were found to be distributed in GIs of *P. putida* KT2440 (16, 56).

The genus *Pseudomonas* encompasses the most diverse group of bacteria thriving on the planet with widespread interactomes and has acquired functions that enable them to exhibit remarkable metabolic versatility and adaptability. The best-studied species include *P. aeruginosa* PA01, which acts as an opportunistic human pathogen; the soil bacterium *P. putida* KT2440, which is capable of degrading a wide range of environmental pollutants; the plant pathogen *P. syringae* DC3000; and the plant growth-promoting *P. fluorescens* Pf-5 (6, 35, 40, 53). The availability of complete genomic sequences of *P. putida* KT2440 and *P. putida* CD2 enabled researchers to study the capacity of these species to endure exposure to heavy metals (7, 20). Functionally well-characterized metal resistance determinants in this genus include the CzrCBA transporter from *P. putida* CD2 and *P. aeruginosa*, which confers resistance to Zn^{2+}, Cd^{2+}, and Co^{2+} (17, 20). Through expression studies this transport system was found to be induced by Zn^{2+} and Cu^{2+} (20). Cadmium P-type ATPases (CadA) that confer Cd^{2+} and Zn^{2+} resistance were characterized in *P. putida* 06909, *P. fluorescens* ATCC 13525, and *P. putida* CD2 (20, 25, 47). A copper-inducible *cin* operon encoding an unusual methionine-rich azurin-like protein and a Pre-Q$_0$ reductase was identified in *P. putida* KT2440 (41). A recent report showed the interplay of the P-type ATPases (CadA1 and CadA2) and RND proteins (CzcCBA1 and CzcCBA2) in mediating the resistance of *P. putida* KT2440 to divalent heavy metals (27). Except for the studies mentioned above, the diversity of metal resistance determinants in this genus has not been investigated extensively.

In our previous in silico study of *Pseudomonas* metal transportomes, we carried out data set cataloging of 262 metal ion transporters from metabolically versatile *P. putida* KT2440, *P. fluorescens* Pf-5, *P. aeruginosa* PA01, and *P. syringae* DC3000

(16). Significantly, 13 metal ion transporters, which were not annotated in their respective genome databases, were identified, and all of the metal transporter genes with GI-associated features were dissected from the metal transportomes of four *Pseudomonas* species (16). Two of the novel metal resistance determinants identified from *P. putida* KT2440 (PP_2968) and *P. syringae* DC3000 (PSPTO_4280) displayed high levels of sequence identity (63%) with the prototypical Ni^{2+} and Co^{2+} resistance determinant *rcnA* from *E. coli* (*rcnA*$_{Eco}$) (46).

We report here the identification and characterization of PP_2968, as a novel metal resistance determinant *mrdH*, located in the GI of *P. putida* KT2440. In the present study, we demonstrate that the *mrdH*-encoded resistance determinant is a novel metal efflux transporter. In addition, the heavy metal response of the associated cytoplasmic gene *mreA* and of the flanking mobile genetic elements was also demonstrated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains, plasmids, and oligonucleotides used in the present study are listed in Table 1 and 2. For the construction and maintenance of plasmids, the *E. coli* strain DH5α was used. Complementation experiments were performed with strain ARY023, a Ni^{2+} and Co^{2+} sensitive, *rcnA* mutant of *E. coli* (46). Bacteria were routinely grown in Luria-Bertani (LB) or M9 minimal medium (MM; without yeast extract supplementation) (48). Cultures were grown at 37°C (*E. coli* strains) or 30°C (*P. putida* KT2440 and its derivatives) on a rotary shaker at 150 rpm. Antibiotics were added to the medium when appropriate at the following concentrations: ampicillin, 100 μg/ml (*E. coli*) and 500 μg/ml (*P. putida*); chloramphenicol, 50 μg/ml (*P. putida*); and kanamycin, 30 μg/ml (*P. putida*).

DNA manipulations. The genomic DNA of *P. putida* KT2440 was isolated with Himedia columns according to the instructions of the manufacturer. Qiagen Mini-Prep and gel purification kits were used for plasmid isolation and for the purification of digested DNA. The enzymes used for DNA manipulation were obtained from Fermentas. PCRs were carried out using Fermentas PCR Master Mix. Plasmids were constructed by using standard recombinant DNA procedures, and *E. coli* cells were transformed by the routine CaCl$_2$ procedure (48). Competent cells of *P. putida* KT2440 and the mutant strain HMR020 were prepared by the MgCl$_2$-CaCl$_2$ method, followed by chemical transformation (30).

TABLE 2. Sequences of the primers used in PCR amplifications

Primer pair	Sequence (5′–3′)[a]
A1	CCCGGG ATGTCGAGCTTCGCTGAACTG
	AAGCTT AGGCCGATCCAGCCATGGA
A2	CCCGGG ATGCCCGACTTTGCCAGTCTG
	AAGCTT ACAGACCATTCCAGCCGTG
A3	CCCGGG ATGAGCGATCACGAACACG
	AAGCTT TAGAGGTACTTAGTGATTTGCTTGAA
A4	CCCGGG ATGTCGAGCTTCGCTGAACTG
	GGATCC AGGCCGATCCAGCCATGGA
A5	GTAAGCGTTCGGTCGAGAAG
	CCACCGATGTACGAGGAAAC
A6	ATGCTGAAGCACAACACCAG
	TTTAGCAATCGCAACGTCAC
A7	TGGTCTTGGATAAGCACACG
	GCCATCAGCACCAAAGTCAT
A8	GTGTTTGCCTTCACCCATCT
	CCAGTGGGTTTCGATCAAGT
A9	GATTCTGTTGCAGCGACTCA
	GACCAATTCGATGCCTGTCT
A10	TAACTGGAAGCCAGCCAAAC
	GAAGTGCTTGCTGTCATGGA

[a] Restriction sites are indicated by underlining.

Complementation of *rcnA* mutant of *E. coli* (ARY023 strain) by the hypothetical metal transporters *mrdH* and *rcnA*$_{Psy}$. The genomic DNA of *P. putida* KT2440 was used as a template to PCR amplify the 1.1-kbp *mrdH* gene (PP_2968) with the primer pair A1. The genomic DNA of *P. syringae* DC3000 was used as a template to amplify the *P. syringae rcnA* (*rcnA*$_{Psy}$) gene (PSPTO_4280) of 879 bp with the primer pair A2. Restriction sites for SmaI and HindIII were incorporated into the primers. The PCR products were cloned into pTZ57R/T (Fermentas), and the constructs were verified by DNA sequencing. Subsequently, these constructs in T/A (pTZ57R/T-mrdH and pTZ57R/T-rcnA$_{Psy}$) were digested with SmaI and HindIII and then subcloned into pUC18 (ampicillin resistant [Amp^r]) at the same restriction sites under the control of the *lacZ* promoter. The ability of these pUC18 derivatives (pUCmrdH and pUCrcnA$_{Psy}$) to confer Ni^{2+} and Co^{2+} resistance was tested by transforming them into ARY023 strain. Cultures of the ARY023 transformants (ARY023, ARY023/pUC18, ARY023/pUCmrdH, and ARY023/pUCrcnA$_{Psy}$) grown overnight in M9 MM supplemented with 0.4% glucose were diluted 1:100 into fresh M9 medium with the indicated metal ion concentrations (Zn^{2+}, Cd^{2+}, Ni^{2+}, and Co^{2+}). Bacterial growth was monitored as the optical density at 600 nm (OD$_{600}$) after 16 h of incubation at 37°C. The results are presented as the mean values from three independent experiments.

Expression of the associated cytoplasmic gene *mreA* in the *rcnA* mutant of *E. coli* (ARY023 strain). The cytoplasmic 288-bp gene, *mreA*, was PCR amplified from the genomic DNA of *P. putida* KT2440 with primer pair A3. Restriction sites for SmaI and HindIII were incorporated into the PCR primers. The amplified product was gel purified, cloned into the vector pTZ57R/T, and sequenced. The *mreA* gene was then subcloned into SmaI- and HindIII-digested pUC18, under the control of the *lacZ* promoter. The resulting plasmid pUCmreA was then transformed into ARY023 strain of *E. coli*. Cultures of the ARY023 transformants (ARY023/pUC18 and ARY023/pUCmreA) grown overnight in M9 MM supplemented with 0.4% glucose were diluted 1:100 into fresh M9 medium with various metal ions (Zn^{2+}, Cd^{2+}, Ni^{2+}, and Co^{2+}). Bacterial growth was monitored as the OD$_{600}$ after 16 h of incubation at 37°C.

Construction of the *mrdH* gene knockout mutant of *P. putida* KT2440 (HMR020 strain) using group II introns. The TargeTron gene knockout system (Sigma-Aldrich) based on the modified group II intron (*L1.LtrB* intron) was used specifically to disrupt *mrdH* gene in the chromosome of *P. putida* KT2440. Group II introns insert themselves via the activity of an RNA-protein complex expressed from the pACD4KC vector (21, 59). To target the intron to *mrdH*, the group II intron sequence in pACD4K-C vector was modified based on the sequences of predicted insertion sites in the *mrdH* gene by using Targetron Design (Sigma-Aldrich). The program predicted nine intron insertion sites across the 1,134-bp *mrdH* gene. The E values (expected number of false positives) of the nine target sites ranged from 0.050 to 0.467. For optimal gene interruption and stable insertion, the insertion site in the antisense strand at position 627 or 628 from the initial ATG with the lowest E value of 0.05 was chosen for intron modification, and primers were designed to retarget the RNA portion of the RNA-protein complex. Subsequently, the retargeted 350-bp PCR gene was ligated into a linearized pACD4K-C (chloramphenicol resistant [Cm^r]) under the control of the T7 promoter resulting in pACD4K-mrdHC construct. Since *P. putida* KT2440 does not express T7 polymerase to drive the expression of the intron component, the pAR1219 vector (Amp^r) that expresses T7 polymerase under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible LacUV5 promoter (10) was used for the functional expression of the retargeted intron. Finally, the retargeted intron containing pACD4K-mrdHC and pAR1219 vectors were cotransformed into competent *P. putida* KT2440 cells. The transformation mixture was added to LB medium containing ampicillin, chloramphenicol, and glucose (1%). After incubation for 18 h at 30°C, the culture was further diluted into a fresh medium (LB) containing antibiotics and glucose (1%), incubated until the OD$_{600}$ reached 0.2 at 30°C. These early-log-phase cells were induced with IPTG (0.1 M) for 30 min at 30°C. The cells were centrifuged, resuspended in fresh medium with glucose (1%), and again incubated for 1 h at 30°C. Selection was performed on LB plates with kanamycin (25 μg/ml) that were maintained at room temperature for 3 days. The resulting colonies were then plated onto LB plates containing kanamycin and 1 mM nickel. Colonies unable to grow on final selection plates containing 1 mM Ni^{2+} were recovered and colony PCRs were performed across the intron-gene (*mrdH*) junction to verify the correct target site insertion.

Complementation of the HMR020 by expression of *mrdH* in the broad-range-host vector pJB653. The *mrdH* gene was PCR amplified from the genomic DNA of *P. putida* KT2440 using the primer pair A4. Restriction sites for SmaI and BamHI were incorporated into the primers. The PCR product was cloned into pTZ57R/T, and the construct was verified by DNA sequencing. The *mrdH* gene was isolated by digesting the pTZ57R/T-mrdHC plasmid with SmaI and BamHI and then subcloned into the SmaI/BamHI-digested pJB653 vector (Amp^r), under the control of the *Pm* promoter, which is induced by *m*-toluic acid. The resulting plasmid, pJBmrdH, was transformed into HMR020 competent cells by chemical transformation. Part of the transformation reaction was added to LB containing ampicillin and *m*-toluic acid (0.1 M). After incubation for 1 h at 30°C, the cells were plated onto LB agar containing ampicillin, *m*-toluic acid (0.1 M), and various concentrations of Ni^{2+} (0.5 to 3 mM). Nickel was used for the selection of HMR020/pJBmrdH transformants since HMR020 strain has intrinsic ampicillin resistance.

Estimation of metal content. Overnight cultures of *P. putida* KT2440 (wild type), HMR020, and HMR020/pJBmrdH were diluted in 50 ml of LB; grown to exponential phase; and then supplemented with 0.5 mM Cd^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}. These bacterial suspensions were incubated at 30°C with shaking at 150 rpm for 12 h. The cells were harvested by centrifugation and washed three times with Milli-Q water. The resulting pellet was suspended in Millipore water, and cellular metal was extracted with an acid digestion procedure (55). The Cd^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} concentrations were measured on an Atomic absorption spectrophotometer (GBC). Metal content is expressed as μg of metal/100 mg of dry bacteria.

Isolation of RNA and quantitative real-time PCR. The cells of *P. putida* KT2440 were grown at 30°C in conical flasks with 10 ml of LB/M9 MM and shaken at 120 rpm on an orbital shaker until the OD$_{600}$ reached 0.2 to 0.3 (early log phase). The media were then supplemented with various heavy metals such as Cd^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} and incubated further at 30°C for 40 min. The total RNA was isolated by the Triton X-100 boiling and chloroform extraction method (22). Heavy metal induction and RNA extraction were performed in duplicate using two independent cultures. The quality of RNA was monitored on a 2% agarose gel, followed by spectrophotometric quantification at A_{260}. The material was then digested with RNase-free DNase (Fermentas) to remove the residual DNA. The total RNA (2 μg) was reverse transcribed to cDNA by using Revert Aid H minus Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Fermentas). The target genes selected in the present study were *mrdH*, *mreA*, *tnpA*, *tnpC*, and IS*1246* transposases, and the corresponding primer pairs used were A4, A5, A6, A7, and A8. The expression levels of the target genes were normalized to the housekeeping RPSL gene levels (16S rRNA gene). Real-time PCR was performed on Bio-Rad iCycler machine using Real Master Mix SYBR ROX from 5 PRIME. Both target and normalizer reactions were run in triplicate. The following PCR conditions were used: initial denaturation at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 20 s, and extension at 68°C for 20 s. The reaction specificity was determined for each reaction by using the melting-curve analysis of the PCR product. To calculate the fold change in gene expression, the $2^{-\Delta\Delta CT}$ method was used (31).
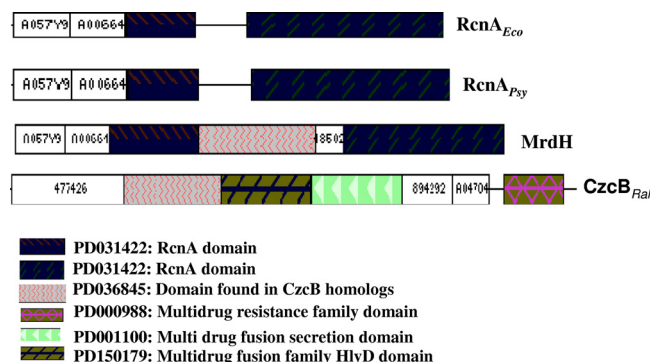
FIG. 1. Domain organization of putative *Pseudomonas* metal efflux proteins. ProDom browsing using the MrdH protein sequence as the query gave the domain arrangements of prototypical metal efflux proteins RcnA$_{Eco}$ (*E. coli*) and CzcB$_{Ral}$ (*Ralstonia* species). Their architectures were compared to the output obtained for domain arrangements of putative *Pseudomonas* metal efflux proteins MrdH (*P. putida* KT2440) and RcnA$_{Psy}$ (*P. syringae* DC3000).

RESULTS

In silico identification of the novel metal resistance genes *mrdH* and *mreA* from *P. putida* KT2440. Our previous in silico study identified PP_2968 from *P. putida* and PSPTO_4280 from *P. syringae* as RcnA efflux members (nickel/cobalt efflux) exhibiting six transmembrane domains and signature sequences characteristic of the prototypical *rcnA*$_{Eco}$ (16). In the present study we focused on the domain analysis of the proteins encoded by PP_2968 and PSPTO_4280 using ProDom software (release 2005.1/CG171) (50) to identify the compositional differences between them (Fig. 1). The 1,134-bp PP_2968 gene encodes a predicted protein of 41 kDa, composed of an RcnA domain characteristic of RcnA efflux family and a putative domain commonly found in CzcB homologs of the RND efflux family. In contrast, the 879-bp PSPTO_4280 gene from *P. syringae*

DC3000 encodes a predicted protein of 31 kDa, with a protein size and domain composition resembling that of the experimentally characterized RcnA$_{Eco}$ (with a molecular mass of 30 kDa and exhibiting only an RcnA domain). Thus, PP_2968 has a significantly different composition due to the presence of an additional domain, which has also been observed in CzcB homologs. The region of PP_2968 encoded protein that comprises the additional domain is located between amino acids 139 and 222, whereas this region is absent in the proteins encoded by PSPTO_4280 and *rcnA*$_{Eco}$. Due to its novel chimeric domain organization and unidentified substrate spectrum, PP_2968 was annotated as metal resistance determinant *mrdH*, whereas PSPTO_4280 was annotated as *rcnA*$_{Psy}$.

We also identified a conserved hypothetical protein, PP_2969, associated with MrdH. This protein composed of 95 amino acids was found to possess a DUF156 domain (PFAM accession no. PF02583). The three protein families found for the DUF156 domain in the SYSTERS database were P151469 (includes hypothetical RcnR proteins), N151470 (includes hypothetical YrkD protein), and P15471 (includes NreA/NreA-like proteins). Among these three families, PP_2969 is homologous to P15471 family members (pairwise identity ranging from 62 to 66%) that include NreA/NreA-like proteins from *Sinorhizobium meliloti* (accession no. Q92YI5), *Legionella pneumophila* (accession no. Q93M45), and *Achromobacter xylosoxidans* (accession no. Q44590) (12, 15). Hence, it was annotated as metal resistance-associated cytoplasmic gene *mreA*.

Functional complementation of *rcnA* mutant of *E. coli* (ARY023 strain) by *Pseudomonas* metal efflux transporters. To assess whether the *Pseudomonas* transporter genes *mrdH* and *rcnA*$_{Psy}$ could complement the nickel cobalt-sensitive phenotype of *E. coli rcnA* mutant (ARY023 strain), these genes were expressed in *trans* from the multicopy plasmids pUCmrdH and pUCrcnA$_{Psy}$. Expression of *mrdH* conferred Ni^{2+} resistance (fivefold) to the *E. coli* mutant strain, while Co^{2+} resistance was least affected. In contrast, the expression of *rcnA*$_{Psy}$
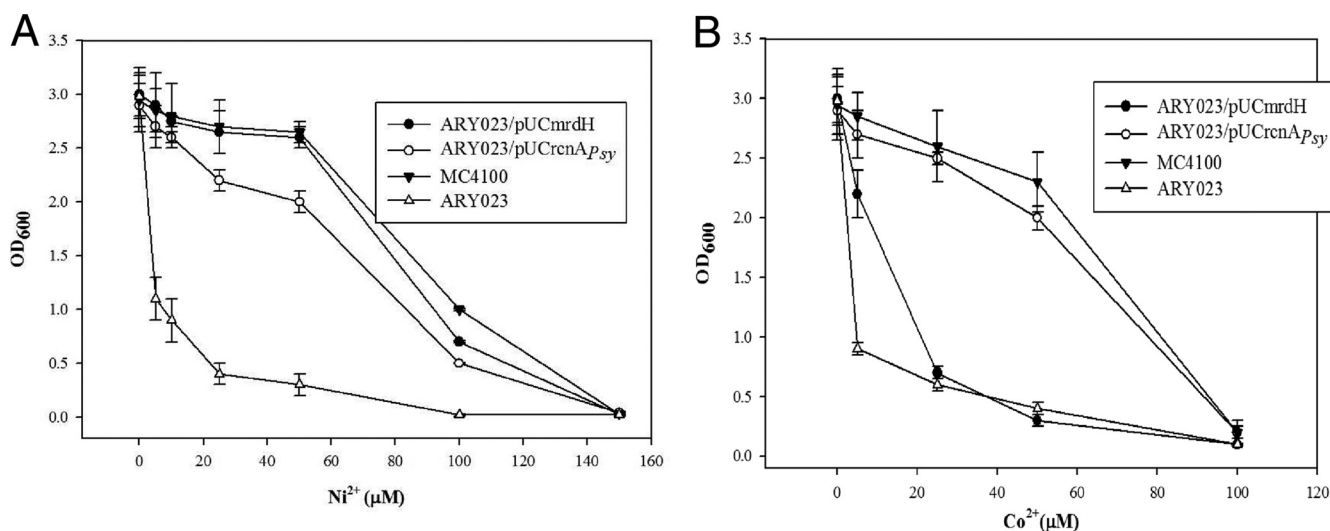

FIG. 2. Effect of nickel and cobalt on growth of ARY023 strains of *E. coli*. Overnight cultures of ARY023 (open triangle), MC4100 (filled triangle), ARY023/pUCmrdH (filled circle), and ARY023/pUCrcnA$_{Psy}$ (open circle) in M9 MM supplemented with 0.4% glucose were diluted into 1:100 fresh identical medium with the indicated concentrations of Ni^{2+} (A) and Co^{2+} (B). Cell growth was monitored as the OD$_{600}$ after 16 h of incubation at 37°C. Experiments were performed in triplicate, and the values given are averages.

conferred both Ni^{2+} and Co^{2+} resistance (~3-fold and ~5-fold, respectively) to the *E. coli* mutant strain. Compared to ARY023/pUCrcnA$_{Psy}$, the curve obtained for ARY023/pUCmrdH reflected an increased nickel resistance of the *E. coli* strain and reached a level similar to that found for the MC4100 (wild-type) strain (Fig. 2). The sensitivity of the ARY023 strain transformed with empty pUC18 vector was similar to that displayed by ARY023 strain alone (data not shown).

To further support the data obtained in liquid medium, nickel and cobalt viability analysis of the *E. coli* strains described above was performed on LB plates. Viable counts of ARY023 strain and the complemented strains (ARY023/pUCmrdH and ARY023/pUCrcnA$_{Psy}$) were measured on LB agar plates after incubation in liquid medium (LB) containing different Ni^{2+} and Co^{2+} concentrations. When cells were grown in the absence of metal, viable counts were similar for all of the strains tested. However, with increasing concentrations of Ni^{2+} and Co^{2+}, the viable counts of the ARY023 strain decreased, indicating its sensitivity to the metals due to the lack of an *rcnA* efflux system. In contrast, the viable counts of the complemented strains (ARY023/pUCmrdH and ARY023/pUCrcnA$_{Psy}$) were not affected significantly by increasing concentrations of Ni^{2+} due to the expression of Ni^{2+} resistance by *mrdH* and *rcnA*$_{Psy}$ genes. However, in the presence of increasing concentrations of Co^{2+}, the viable counts obtained for the *mrdH* complemented strain (ARY023/pUCmrdH) were relatively less than those obtained for the *rcnA*$_{Psy}$ complemented strain ARY023/pUCrcnA$_{Psy}$ (see the supplemental material). These data are in accordance with the liquid medium data and show that *mrdH* confers more Ni^{2+} resistance than Co^{2+} resistance.

Thus, the heterologous complementation studies in *E. coli* provided preliminary evidence for the functional divergence of *mrdH* from its homologs, *rcnA*$_{Eco}$ and *rcnA*$_{Psy}$.

Construction of *mrdH* mutant strain in *P. putida* KT2440 with the *mrdH* targetron. The targeted disruption of *mrdH* was accomplished by the cotransformation of pACD4K-mrdC (harboring a retargeted intron) and pAR1219 (to drive the expression of pACD4K-mrdC) into the host strain. Twenty-five colonies were obtained on the final LB selection plates containing kanamycin, and these colonies were further tested for their ability to grow on LB plates containing Ni^{2+} (1 mM). After 48 h of incubation, the 15 Ni^{2+}-sensitive colonies were further screened for insertion of the targetron and inactivation of *mrdH* gene by colony PCR. Ten of the fifteen Ni^{2+}-sensitive colonies showed the presence of targetron (in the middle of the *mrdH* gene) in the PCR analysis, performed using *mrdH*-specific primer (forward primer) and intron-specific primer (EBS universal) to amplify an 846-bp gene-intron junction (data not shown). Sequence analysis of the 846-bp PCR product from these two colonies indicated that the intron had inserted in the antisense strand at the 627/628 site of the *mrdH* gene. The stability of the *mrdH* gene knockout mutant, HMR020, was evaluated after 20 rounds of subculture.

The transporter gene *mrdH* was cloned into the pJB plasmid under the *m*-toluic acid inducible *Pm* promoter and transformed into the HMR020 strain. The HMR020/pJBmrdH transformants were finally selected on a LB agar containing *m*-toluic acid (0.1 M) and Ni^{2+} (2 mM) selection plates. The

TABLE 3. Determination of MTCs for *P. putida* KT2440 and derivative strains[a]

Metal	MTC (mM) for *P. putida* strain:		
	KT2440	HMR020/pJB653	HMR020/pJBmrdH
Cd^{2+}	0.2	0.01	0.2
Ni^{2+}	4	1	4
Zn^{2+}	4	1	4
Co^{2+}	0.6	0.4	0.6
Cu^{2+}	2	2	2
Pb^{2+}	>1.0	>1.0	>1.0
Mn^{2+}	>1.5	>1.5	>1.5

[a] *P. putida* KT2440 (wild type), HMR020/pJB653. and HMR020/pJBmrdH strains were grown till early log phase (OD$_{600}$ = 0.3) in LB medium at 30°C, diluted 1:100 in fresh medium, and spotted onto LB agar plates containing the indicated concentrations of Cd^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}.

pJB653 vector, without an inserted *mrdH* gene, was also transformed into HMR020 strain and used as a control in complementation tests.

Disruption of the *mrdH* gene in *P. putida* KT2440 conferred sensitivity to cadmium and zinc in addition to nickel and cobalt. The functionality and substrate range of the *mrdH* gene was studied by determining the maximum tolerable concentrations (MTCs) of divalent heavy metals for the wild-type, HMR020/pJB653, and HMR020/pJBmrdH strains of *P. putida* KT2440 on LB plates. Disruption of the *mrdH* gene affected the resistance to Cd^{2+}, Zn^{2+}, and Ni^{2+}, but Co^{2+} resistance was only marginally affected (Table 3). The sensitivity of the HMR020/pJB653 strain to the metal ions of interest compared to wild-type and HMR020/pJBmrdH strains was Cd^{2+} (20-fold) > Zn^{2+} (4-fold) $\geq Ni^{2+}$ (4-fold) > Co^{2+} (2-fold). However, the HMR020/pJB653 strain did not show any sensitivity toward copper, lead, or mercury (Table 3). These data demonstrate the substrate spectrum of *mrdH* to be Cd^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}. In addition, we observed that the HMR020/pJB653 strain had a high level of sensitivity in liquid media to Cd^{2+} (100-fold) and to a lesser extent to Ni^{2+} (3-fold) and Zn^{2+} (3-fold); the levels of HMR020/pJB653 strain sensitivity to Co^{2+} did not alter significantly from wild-type *P. putida* KT2440 and HMR020/pJBmrdH strains (data not shown).

***mrdH* encodes a heavy metal efflux transporter in *P. putida* KT2440.** To substantiate the role of *mrdH* as a metal resistance determinant, we examined the metal accumulation in wild-type *P. putida* and HMR020 strains of KT2440 (Fig. 3). The HMR020 strain showed a remarkably higher accumulation of Cd^{2+} (ninefold more) compared to the wild type. Similarly, the accumulation of Ni^{2+} and Zn^{2+} was also increased (twofold) in the HMR020 strain. However, the accumulation of Co^{2+} was twofold higher in the wild-type *P. putida* than in the HMR020 strain. The preceding results suggests that MrdH can function as an efflux system.

A 63 Ni^{2+} uptake assay was performed to monitor the in vivo activity of MrdH. If Cd^{2+}, Ni^{2+}, and Zn^{2+} compete for the same efflux system, then the concentration of Ni^{2+} in the wild-type strain should be higher when competing metal is present than when it is absent. Our results showed an increased Ni^{2+} accumulation in wild-type and HMR020/pJBmrdH strains of *P. putida* in the presence of Cd^{2+} and Zn^{2+} (Fig. 4).
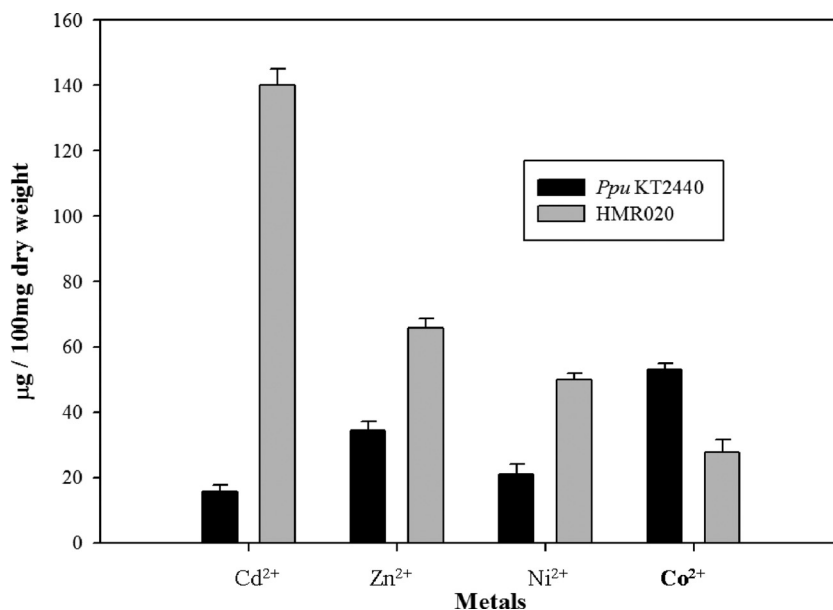
FIG. 3. Estimation of metal content in *P. putida* KT2440 and HMR020 strains. Cultures of *P. putida* KT2440 and HMR020 strains were grown in LB until the exponential phase and then supplemented with 0.5 mM Cd^{2+}, Zn^{2+}, Ni^{2+}, or Co^{2+}. Cells were harvested after 12 h, and the pellet was suspended in Millipore water. Cellular metal was extracted by acid digestion procedure. The concentrations of Cd^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} were measured by atomic absorption spectroscopy.

However, no change was observed for Ni^{2+} accumulation in the presence or absence of competing metals in the mutant strain, HMR020. These findings also support the proposed role of *mrdH* as a broad-spectrum metal efflux transporter with a substrate range including Cd^{2+}, Zn^{2+}, and Ni^{2+}.

The expression pattern of *mreA* parallels that of *mrdH*. Expression of *mreA* gene in ARY023 strain of *E. coli* enhanced its Cd^{2+} and Ni^{2+} resistance (Fig. 5), whereas the resistance levels of Zn^{2+} and Co^{2+} were not affected.

Expression patterns of *mrdH* and *mreA* by Cd^{2+}, Zn^{2+}, and

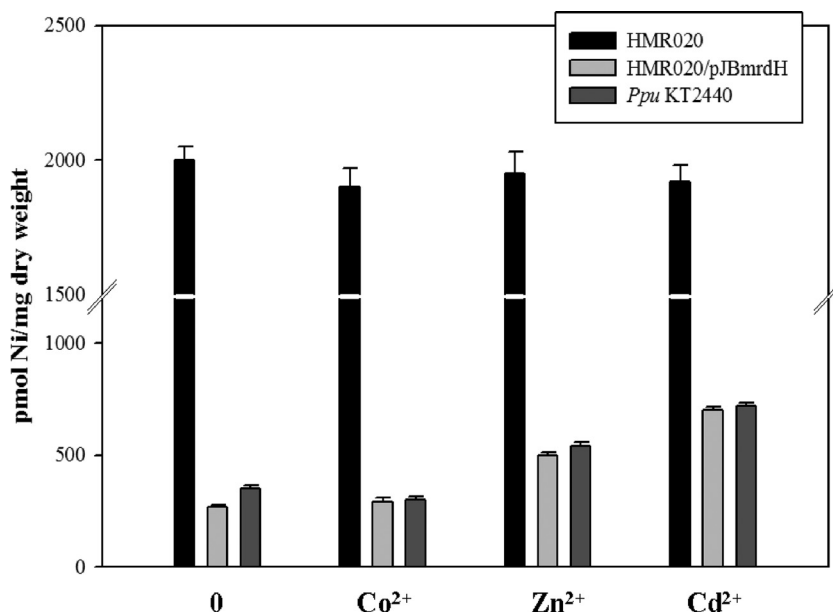FIG. 4. ^{63}Ni uptake analysis. *P. putida* KT2440, HMR020, and HMR020/pJBmrdH strains were grown in LB medium in the presence of 0.5 mM Ni^{2+} at 30°C under aerobic conditions until mid-log phase (OD_{600} = 0.5), harvested, and then washed with buffer consisting of 66 mM KH_2PO4-K_2HPO4 (pH 6.8) and 0.4% glucose. Cells were resuspended in the same buffer with a fivefold concentration factor. The uptake assay was performed in the presence of 5 μM $^{63}Ni^{2+}$ or 5 μM $^{63}Ni^{2+}$ and 50 μM Cd^{2+}, Zn^{2+}, or Co^{2+}. Aliquots (100 μl) were filtered after a time period of 45 min (this time point was standardized and kept constant). The intracellular concentration of ^{63}Ni per milligram of bacterial dry weight was determined.
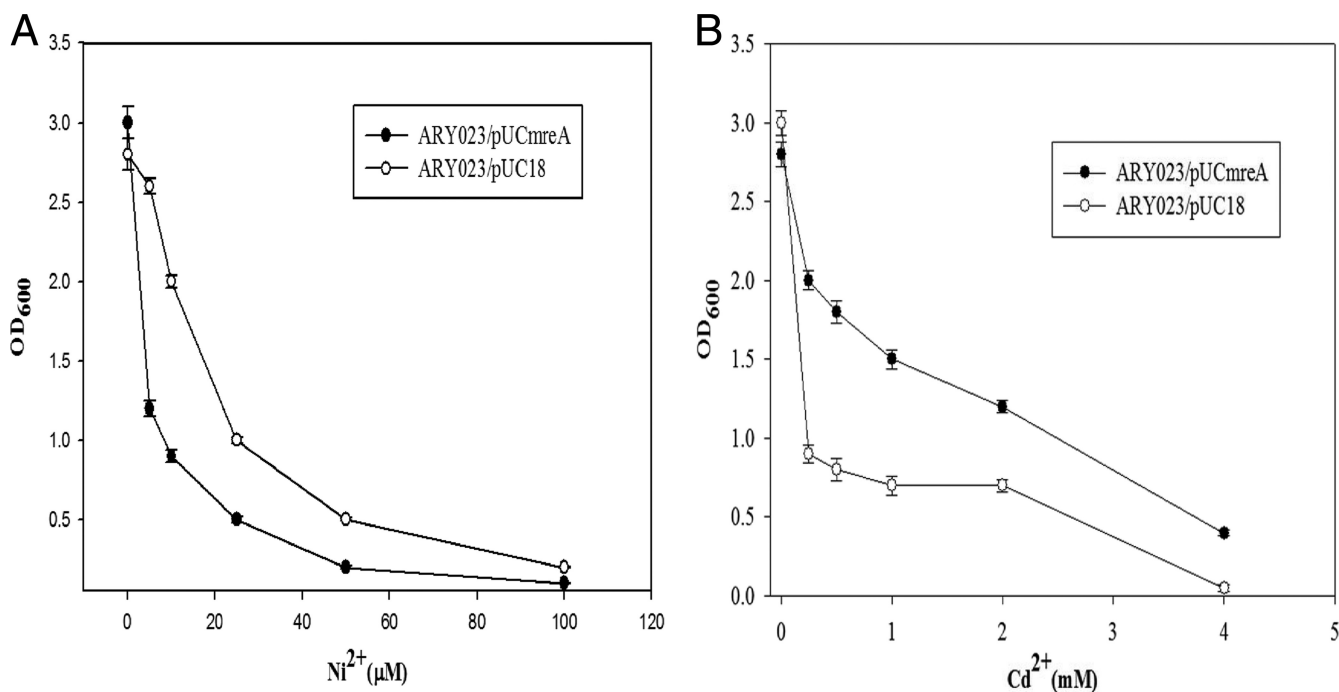
FIG. 5. Expression of *mreA* in *E. coli* ARY023. Overnight cultures of ARY023/pUC18 (open circle) and ARY023/pUCmreA (filled circle) in M9 MM supplemented with 0.4% glucose were diluted into 1:100 fresh identical medium with the indicated concentrations of metals (Cd^{2+} and Ni^{2+}). Cell growth was monitored as the OD_{600} after 16 h of incubation at 37°C.

Ni^{2+} and Co^{2+} were analyzed in both rich and minimal media by quantitative reverse transcription-PCR (Table 4). The induction of *mrdH* and *mreA* genes by different metals was dependent on the growth medium used.

In LB media, the *mrdH* gene was induced in the presence of Cd^{2+} (2.5-fold), Ni^{2+} (2.2-fold), and Co^{2+} (2.0-fold). The expression pattern of the *mreA* gene was similar to that of *mrdH* and was induced by Cd^{2+} (4.0-fold), Ni^{2+} (3.0-fold), and Co^{2+} (2-fold). In MM, both the *mrdH* and the *mreA* genes were induced by Cd^{2+} (2.58- and 2.27-fold), Zn^{2+} (7.8- and 4.14-fold), Ni^{2+} (5.5- and 4.3-fold), and Co^{2+} (2.29- and 2.14-fold).

The flanking mobile genetic elements Tn*4652* and IS*1246* respond to heavy metal stress. The genetic determinants

TABLE 4. Expression analysis of *mrdH* and *mreA* in *P. putida* KT2440

Metal[a]	Concn (mM)	Mean fold change ± SD[b]			
		mrdH		*mreA*	
		LB	M9 MM	LB	M9 MM
Cd^{2+}	0.02	2.2 ± 0.15	1.18 ± 0.10	3.7 ± 0.10	1.40 ± 0.15
	0.2	2.5 ± 0.10	2.58 ± 0.15	4.0 ± 0.15	2.27 ± 0.10
Ni^{2+}	0.1	1.0 ± 0.20	3.89 ± 0.15	1.27 ± 0.2	2.4 ± 0.20
	1.0	2.2 ± 0.21	5.5 ± 0.20	3.0 ± 0.15	4.28 ± 0.30
Zn^{2+}	0.05	1.03 ± 0.05	1.19 ± 0.15	1.03 ± 0.2	1.10 ± 0.15
	0.5	1.36 ± 0.10	7.78 ± 0.25	1.36 ± 0.2	4.14 ± 0.25
Co^{2+}	0.1	1.56 ± 0.15	2.29 ± 0.10	1.27 ± 0.05	2.14 ± 0.15
	1.0	2.0 ± 0.05	1.00 ± 0.20	2.0 ± 0.20	1.40 ± 0.20

[a] *P. putida* KT2440 was grown till early log phase in LB/M9 MM and then induced with the indicated metals for 40 min.
[b] The values indicate the fold change in expression of the *mrdH* and *mreA* genes in the presence of various metals.

(*mrdH* and *mreA*) involved in the heavy metal resistance of *P. putida* KT2440 were found to be clustered in a genomic region flanked by Tn*4652* and IS*1246* elements (Fig. 6). Three other hypothetical proteins, which clustered along with *mrdH* and *mreA*, were PP_2966, PP_2967, and PP_2970. Two of these hypothetical genes, PP_2966 and PP_2970, did not have any hits in the database, whereas the third gene, PP_2967, encoded a protein showing homology (69.7% identity) to conserved hypothetical proteins of unknown function from *P. fluorescens* Pf0-1 (accession no. Pfl_2552) and *P. entomophila* L48 (accession no. PSEEN3504).

The mobile genetic element Tn*4652* belongs to the Tn*3* family of transposons and is well characterized in the *P. putida* strain PaW85. It is a 17-kb derivative of Tn*4651* that contains a deletion of its catabolic region, including the *xyl* gene, likely resulting from the recombination of two copies of IS*1246*. The other element, IS*1246*, belongs to the IS*5* family and is located within Tn*4652* (18, 42).

Expression analysis of the flanking mobile genetic elements showed that their genes were induced by heavy metals, in spite of the fact that they code for transposition functions (Table 5). The *tnpA* component of the Tn*4652* element was induced by Zn^{2+} (5.3-fold), Ni^{2+} (5-fold), and Cd^{2+} (2.5-fold) in LB medium. The same metals caused the induction of *tnpA* in MM, but a stronger response was observed for Zn^{2+} (16-fold) than for Ni^{2+} and Cd^{2+} (2.5-fold). No induction of the *tnpC* component was observed with any of the metals in LB, but Ni^{2+} alone caused the induction of this component in MM (2.5-fold). The other flanking element, IS*1246*, was induced by Zn^{2+} (2.5-fold) alone in LB, whereas Zn^{2+} (8-fold), Ni^{2+} (4-fold), and Cd^{2+} (2.6-fold) caused induction in MM.
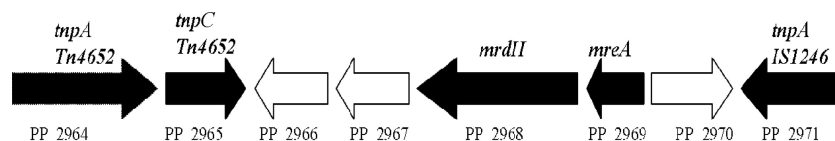
FIG. 6. Genomic organization of *mrdH* and *mreA* in *P. putida* KT2440. Arrows in the figure indicate the transcriptional orientation of the respective genes. Tn*4652* components: *tnpA* and *tnpC* (PP_2964 and PP_2965 [transposition function] and PP_2966, PP_2967, and PP_2970 [unknown function]), *mrdH* (PP_2968 [heavy metal efflux transporter]), *mreA* (PP_2969 [metal resistance determinant]), and IS*1246* *tnpA* (PP_2971 [transposition function]).

An increased transcriptional response to heavy metals was observed for these flanking elements in MM relative to rich medium. However, Co^{2+} did not induce the expression of Tn*4652* or IS*1246* in either of the tested media.

Phylogeny of MrdH and related homologs from environmental bacteria. BLAST searching uncovered several homologs of MrdH that are hypothetical membrane transporters from various environmental bacteria, and most of them were annotated as high-affinity Ni^{2+} transporters or as members of the RcnA efflux family. All of the MrdH homologs exhibited six transmembrane segments and signature sequences characteristically observed for RcnA efflux proteins. However, phylogenetic analysis of the 36 identified MrdH homologs led to their classification into three major subgroups of orthologous genes (Fig. 7). The differences in their core-conserved domains also enabled us to predict distinct domain organizations for the three major subgroups identified. Therefore, we propose division of the MrdH homologs from gram-negative bacteria into three subgroups of efflux proteins: subgroup 1, RcnA proteins (825 bp, 274 amino acids); subgroup 2, cation efflux-related membrane (CEM) proteins (1,270 bp, 422 amino acids); and subgroup 3, MrdH proteins (1,134 bp, 377 amino acids). The RcnA proteins are mainly composed of transmembrane RcnA domain, annotated as PD031422 in the PRODOM database (50). The CEM proteins are composed of three putative domains: the cation efflux transporter system domain (domain identification no. PD001602), the domain found in CzcB homologs (domain PD036845), and the zinc transporter efflux cation system domain (domain PD001369). However, the MrdH proteins (MrdH and related proteins) exhibited chimeric domain organization due to the presence of RcnA domain and the putative domain PD036845, found in CzcB homologs. Intriguingly, all of the MrdH subgroup proteins exhibited a chimeric domain organization and were flanked by mobile genetic elements.

DISCUSSION

This report has demonstrated the presence of a novel metal resistance determinant *mrdH*, in association with *mreA*, amid the metal-inducible mobile genetic elements of *P. putida* KT2440. MrdH was found to contain 377 amino acid residues, with six predicted transmembrane segments, and it contained all of the conserved signature sequences characteristic of an RcnA efflux protein. However, our conserved domain analysis of MrdH showed a chimeric architecture formed by RcnA domain and putative domain found in CzcB homologs. The predicted RcnA domain was found largely in the transmembrane region, whereas the domain derived from CzcB homologs was identified in the cytosolic portion. Such a chimeric domain organization has not been reported for any of the characterized efflux proteins to date. In contrast, $RcnA_{Psy}$, the putative ortholog of MrdH, is composed entirely of a RcnA domain, as is observed for the prototypical member of the family, $RcnA_{Eco}$ (46).

Previously, *yohM* (*rcnA*) gene in *E. coli* possessing RcnA domain conferred increased nickel and cobalt resistance (46). In the *czc* efflux system (*czcABC*) of *Alcaligenes eutrophus* CH34, a component exhibiting the CzcB domain is thought to funnel zinc cations to the CzcA transport protein and facilitate zinc efflux (23). Therefore, the RcnA domain in MrdH could be hypothesized to confer the nickel resistance, whereas the domain derived from CzcB homologs accounts for the observed cadmium and zinc resistance of *P. putida* KT2440.

A functional status was assigned to the hypothetical membrane transporters *mrdH* and *rcnA_Psy* by complementation

TABLE 5. Expression analysis of *tnpA* (Tn*4652*), *tnpC* (Tn*4652*), and IS*1246* in *P. putida* KT2440

Metal[a]	Concn (mM)	Mean fold change ± SD[b]					
		tnpA (Tn*4652*)		*tnpC* (Tn*4652*)		IS*1246*	
		LB	M9 MM	LB	M9 MM	LB	M9 MM
Zn^{2+}	0.05	2.8 ± 0.30	8 ± 0.35	1 ± 0.2	1.10 ± 0.15	1.1 ± 0.25	8 ± 0.15
	0.5	5.3 ± 0.40	16 ± 0.40	1.4 ± 0.2	1.14 ± 0.20	2.42 ± 0.2	8 ± 0.2
Ni^{2+}	0.1	2.46 ± 0.18	1.4 ± 0.15	1.27 ± 0.10	2.3 ± 0.10	1.31 ± 0.2	2.5 ± 0.1
	1.0	4.92 ± 0.10	2.5 ± 0.10	1.62 ± 0.15	2.5 ± 0.25	1.50 ± 0.15	4 ± 0.2
Cd^{2+}	0.02	1.1 ± 0.10	1.62 ± 0.10	1.0 ± 0.15	1.40 ± 0.15	1.5 ± 0.1	2.2 ± 0.15
	0.2	2.51 ± 0.23	2.5 ± 0.25	1.15 ± 0.12	1.27 ± 0.13	1.62 ± 0.1	2.54 ± 0.10
Co^{2+}	0.1	1.51 ± 0.15	1.40 ± 0.30	1.27 ± 0.15	1.14 ± 0.10	1.40 ± 0.15	1.6 ± 0.15
	1.0	1.60 ± 0.25	1.75 ± 0.45	1.51 ± 0.2	1.40 ± 0.2	1.60 ± 0.2	1.8 ± 0.2

[a] *P. putida* KT2440 was grown till early log phase in LB/M MM and then induced with the indicated metals for 40 min.
[b] The values indicate the fold change in expression of the *tnpA*, *tnpC*, and IS*1246* genes in the presence of various metals.
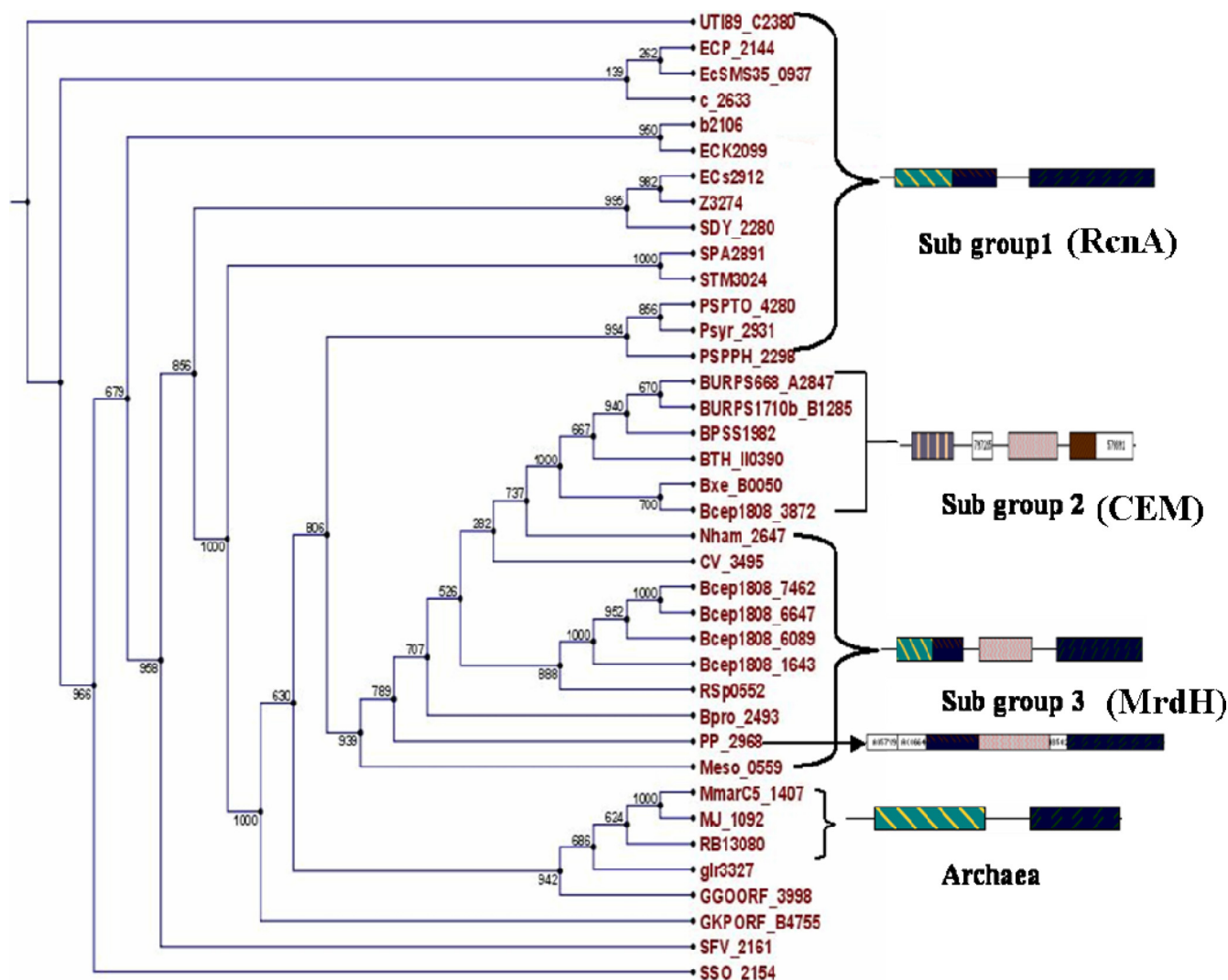
FIG. 7. Phylogeny of MrdH and related homologs. The neighbor-joining phylogram showed the clustering of MrdH and 35 other homologous proteins into three distinct subgroups. Bootstrap support values are indicated by percentages at the nodes. The accession codes of all of the homologs are indicated in the phylogenetic tree. The domain organizations of the corresponding subgroups are shown on the right side. RcnA proteins constituting subgroup 1 were found to be composed of a cyanobacterial marine plasmid hydrogenase domain (PD129330) and RcnA domains (PD031422 and PD031424). CEM proteins constituting subgroup 2 were predominantly composed of the cation efflux transporter system domain (PD001602), the cation efflux system domain found in CzcB homologs (PD036845), and the zinc transporter efflux cation system domain (PD001369). MrdH proteins constituting subgroup 3 exhibited chimeric domain organization derived from the RcnA domain (PD031422) and the cation efflux system domain found in CzcB homologs (PD036845).

into the ARY023 strain ($rcnA$) of *E. coli*. In cross-complementation studies, the *mrdH* gene was found to confer more Ni^{2+} resistance than Co^{2+} resistance, whereas the $rcnA_{Psy}$ gene conferred equal resistance to Ni^{2+} and Co^{2+}. This result is in agreement with the domain analysis data and strongly suggests that $rcnA_{Psy}$ is an ortholog of $rcnA_{Eco}$, whereas *mrdH* showed functional divergence from the above homologs. Further, disruption of *mrdH* in *P. putida* KT2440 caused increased sensitivity of the strain to Cd^{2+}, Zn^{2+}, and Ni^{2+} compared to Co^{2+}. To confirm the functionality data of the *mrdH* transporter derived from the mutant studies, HMR020 strain was complemented with the deleted *mrdH* transporter (HMR020/pJBmrdH), and the complementation assay confirmed the role of *mrdH* as a metal resistance

determinant. Concomitantly, increased accumulation of Cd^{2+}, Zn^{2+}, and Ni^{2+} was also observed in the HMR020 strain, indicating the loss of metal efflux activity of the MrdH transporter upon disruption of the corresponding *mrdH* gene. Transporter studies of MrdH showed that Cd^{2+} and Zn^{2+} indeed served as substrates for this transporter and thus competed with $^{63}Ni^{2+}$ for metal efflux.

Metal-dependent expression analysis of $rcnA_{Eco}$ (by β-glucuronidase assay) showed that the gene was specifically induced by Ni^{2+}/Co^{2+}, and the addition of either Cd^{2+} or Zn^{2+} had no effect on the transcription of *yohM* (46). In the present study, we analyzed the expression of *mrdH* by real-time PCR and demonstrated the induction of genes by Cd^{2+}, Ni^{2+}, and Co^{2+} in LB and by all of the four metals in M9 MM. Such

growth medium-dependent inducer specificities and expression patterns of the metal transporter genes were reported earlier for the *cadA* and *czcCBA* genes of *P. putida* KT2440 (27).

Most of the previously characterized metal efflux transporters with a broad substrate scope were shown to be members of the tripartite RND family and showed no sequence homology to MrdH. Although MrdH has a high degree of conservation with the archetypal RcnA efflux member (RcnA$_{Eco}$), the presence of an additional domain derived from CzcB homologs may be responsible for conferring Cd^{2+} or Zn^{2+} resistance apart from Ni^{2+} and Co^{2+} resistance. Thus, *mrdH* showed a broad substrate profile and compositional differences from the previously reported nickel cobalt efflux protein, *rcnA$_{Eco}$*. Therefore, we report MrdH as a novel single component metal efflux transporter exhibiting chimeric domain structure and a broad substrate scope.

In the phylogenetic analysis of MrdH homologs, three distinct subgroups comprised of RcnA, CEM, and MrdH proteins were identified in the phylogram with variations in their domain architecture (Fig. 7). Among these three subgroups, MrdH subgroup was predominantly composed of efflux proteins from environmental bacteria such as *P. putida* KT2440, *Burkholderia vietnamiensis*, *Ralstonia solanacearum*, *Polaromonas* species, *Mesorhizobium* species, *Nitrobacter* species, and *Chromobacter violaceum* that colonize the rhizosphere. Two hits (35 and 37% sequence identities) were also observed from the methanogenic archaea *Methanococcus jannaschii* DSM2661 (MJ_1092) and *Methanococcus maripaludis* C5 (Mmarc5_1407), which differed from the other homologs of gram-negative bacteria in possessing five TMDs. The chimeric domain organization of all of the MrdH subgroup proteins and the presence of the mobile genetic elements in the vicinity indicate their acquisition through HGT (except for Nham_2647 and CV_3495). The features described above are apparently absent from members of the RcnA and CEM subgroups. Moreover, the presence of five copies of the *mrdH* gene in the genome of *B. vietnamiensis* suggests its pivotal role as a resistance determinant, which might have fostered its evolution and persistence.

We identified, in association with *mrdH*, a conserved hypothetical cytoplasmic protein encoding gene (PP_2969) that belongs to the DUF156 group of proteins that are widely distributed in bacteria and are speculated to be regulators of gene expression. The reported DUF156 proteins include NreA from *Legionella pneumophila* (NreABC), NcrB from *Serratia marcescens* (NcrABC), NirB from *Klebsiella oxytoca* (NirABCD), and NcrB from *Leptospirillum ferriphilum* (NcrABCY) (15, 32, 33, 39, 54). Functionally, expression of the NreA gene (*L. pneumophila*) in *E. coli* conferred resistance to Ni^{2+} and Co^{2+} but not to Cd^{2+} or Zn^{2+} (15). In *L. ferriphilum* the deletion of *ncrB* gene reduced Ni^{2+} resistance, suggesting that this gene might assist the associated transporter *ncrA* in Ni^{2+} efflux (54). In the present study, expression of *mreA* endowed *rcnA* mutant of *E. coli* with Cd^{2+} and Ni^{2+} resistance. However, expression studies of *mreA* at the transcriptional level demonstrated that transcription was significantly induced by Cd^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+} depending on the growth medium used. Moreover, the gene expression of *mreA* parallels that of *mrdH*.

The phenomenon of HGT driven by mobile genetic elements, such as plasmids, insertion sequences, integrons, transposons, and phages, has been shown to provide microbes with a wide variety of adaptive traits such as antibiotic resistance, heavy metal resistance, and xenobiotic compound degradation for microbial survival and proliferation (38). Recently, detectable HGT of heavy metal translocating P_{IB}-type ATPases (*zntA*/*cadA*/*copA*-like genes) has been reported in bacterial isolates from pristine, as well as heavy metal-contaminated subsurface environments (8, 9). In fact, most of the novel genes in gammaproteobacteria were found to be distributed in GIs, indicating their acquisition through HGT from distant sources (19). Our previous in silico studies also show HGT to be a source of gene innovation in *Pseudomonas* metal transportomes (16). In this study, the characterized novel metal resistance determinants *mrdH* and *mreA* were found to be located within a GI flanked by mobile genetic elements. Further, the absence of GI-associated features in MrdH homologs from other *Pseudomonas* (Pmen_2243, PSPTO_4280, PSPPH_2298, and Psyr_2931) and the phylogenetic incongruence observed (Fig. 7) is compelling enough to hypothesize that the *mrdH* gene in *P. putida* KT2440 could have been acquired through HGT. Intriguingly, complete sequence of the IncP-9 TOL plasmid pWW0 from *P. putida* also revealed the presence of two genes (ORF144 and ORF145) encoding putative NreA and RcnA-like metal resistance determinants amid mobile genetic elements (IS*1246* and Tn*4652* transposase genes) (13). Since they shared 100% identity with the chromosomal metal resistance determinants MrdH and MreA (encoded by PP_2968 and PP_2969) identified here, there is a strong possibility of transposition events in disseminating metal resistance traits from plasmid to chromosome under acute metal stress conditions.

Recent studies have explored the capacity of transposable elements to respond to metal stress by enhancing their transcriptional or transpositional activity. The whole-genome transcriptional profile of a metal-tolerant *E. coli* strain exhibited an increase in the transcript level of the IS elements, particularly *insA*, whose expression in *E. coli* confers tolerance to Zn^{2+} (5). Transcriptomic analyses of pMOL28 and pMOL30, which encode metal resistance in *C. metallidurans*, showed metal-mediated upregulation of some transposases (Tn*4378* and Tn*4380*) and truncated IS elements (34). However, not all of the mobile genetic elements flanking metal resistance determinants respond to metal stress. For instance, on the pMOL30 plasmid, an incomplete *mer* cluster (*merRTP*) contains genes encoding proteins with significant similarity to Tn*4378* and Tn*4380*, but they were not induced under any of the experimental conditions examined and thus were reported as nonfunctional (34). In *Listeria* species, Cd^{2+} resistance genes were found on the Tn*5422* transposon, but the flanking elements were not induced by Cd^{2+} under any of the tested conditions (24). In *Pseudomonas* spp., mercury resistance determinants were often found to reside on transposons Tn*5041*, Tn*5053*, and Tn*5056*, but there is no functional evidence available for the metal-induced response of the transposable elements in this genus (51). We have demonstrated here a strong induction of the flanking Tn*4652* and IS*1246* elements by Zn^{2+} and a moderate induction by Ni^{2+} and Cd^{2+}. Therefore, Tn*4652* and IS*1246* in *P. putida* KT2440 are not remnants of evolutionary rearrangements but are functional genes that have entrapped novel metal resistance genes *mrdH* and *mreA*. Since the role of

transposons in the coselection of antibiotic and metal resistance has been well documented for Tn*21* and Tn*21*-like transposons, the identification of novel metal inducible mobile genetic elements here provides future research opportunities in metal-antibiotic coselection (2, 28).

Given this body of evidence, we conclude that *mrdH*, is novel heavy metal efflux transporter exhibiting broad substrate specificity. In association with *mrdH*, *mreA* encoding a putative cytoplasmic protein has been identified, with a role in Cd^{2+}/Ni^{2+} resistance and an expression pattern parallel to that of *mrdH*. The location of both of these metal-responsive genes amid functional mobile genetic elements implicates their acquisition through HGT. Further investigations are required to understand the molecular mechanism of cation efflux by *mrdH*, the potential interactions between *mrdH* and *mreA*, and the full substrate profile of MrdH subgroup of proteins.

ACKNOWLEDGMENTS

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