RESEARCH LETTER

Differing ability to transport nonmetal substrates by two RND-type metal exporters

Otakuye Conroy[1,2], Eun-Hae Kim[1], Megan M. McEvoy[2] & Christopher Rensing[1]

[1]Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, AZ, USA; and [2]Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA

Correspondence: Christopher Rensing, Department of Soil, Water, and Environmental Science, University of Arizona, Shantz Building 38, Rm 429, Tucson, AZ 85721, USA. Tel.: +1 520 626 8482; fax: +1 520 626 6782; e-mail: rensingc@cals.arizona.edu

Received 12 November 2009; revised 5 March 2010; accepted 16 April 2010.
Final version published online 21 May 2010.

DOI:10.1111/j.1574-6968.2010.02006.x

Editor: Skorn Mongkolsuk

Keywords
Escherichia coli; RND transporters; metal resistance; copper efflux.

Abstract

The metal-exporting systems CusCFBA of *Escherichia coli* and GesABC of *Salmonella* are resistance-nodulation-division (RND)-type multiprotein systems responsible for detoxification during metal stress. In this study, the substrate range was determined for each metal transport system and possible amino acid residues important in substrate specificity were identified. The Ges system, previously identified as a gold-efflux system, conferred resistance to the greatest number and variety of organic chemicals including chloramphenicol, not recognized previously as a substrate. Phylogenetic analysis showed that GesB is most closely related to a class of RND transporters including MexF that have been shown to be responsible for exporting fluoroquinolones, chloramphenicol, and biocides. However, many of the closest homologs of GesABC appear to play a role in metal resistance judging from the genetic context. In contrast, CusCFBA belongs to a distinct family of RND-type monovalent metal-exporter systems containing a number of essential metal-binding methionines, resulting in a much narrower substrate range.

Introduction

Efflux is the most common widespread mechanism to regulate the concentration of a myriad of substances in all organisms. The substrate specificities of transporters vary widely and the mechanisms governing substrate recognition and subsequent transport are not well understood. Multiprotein complexes of the resistance-nodulation-division (RND) family in Gram-negative bacteria are both of medical and environmental importance. Within the genome of *Escherichia coli*, there are seven genes belonging to the RND family; *acrB*, *acrD*, *acrF*, *cusA*, *mdtB*, *mdtC*, and *mdtF*. Together with a membrane fusion protein (MFP) and an outer membrane factor (OMF), these inner membrane proteins form a complex responsible for the extrusion of a large variety of substrates mainly from the periplasm in a proton-gradient-dependent manner. The best-characterized member in *E. coli*, AcrB, forms a complex with the MFP AcrA and the outer membrane protein TolC. Aptly termed periplasmic 'vacuum cleaners' (Lomovskaya *et al.*, 2007), the broad specificity for AcrAB-TolC varies from hydrophilic to hydrophobic, and includes bile salts, antibiotics, ethidium bromide, sodium dodecyl sulfate (SDS), and crystal violet (Pos, 2009). The substrates of AcrEF-TolC are similar to that of AcrAB-TolC, while AcrDA-TolC confers resistance to more hydrophilic substances such as SDS and aminoglycoside antibiotics (Elkins & Nikaido, 2002). MdtF substrates include fluoroquinolones, macrolides, oxacillin, novobiocin, and ethidium bromide (Bohnert *et al.*, 2007). Complexed with MFP protein MdtA and OMF protein MdtB, the RND pair MdtBC (YegNO) can shuttle out bile salts, norfloxacin, and kanamycin, among others (Baranova & Nikaido, 2002).

Other RND-type transporters are involved in conferring resistance to metals such as copper, zinc, cadmium, and gold (Nies, 2003; Pontel *et al.*, 2007). *Escherichia coli* possesses the *cusCFBA* determinant, which is proposed to extrude copper and silver from the periplasm to the extracellular environment (Franke *et al.*, 2003). The inner membrane RND protein CusA interacts with both the MFP CusB and the OMF CusC. Additionally, the small periplasmic protein CusF binds copper and silver (Kittleson *et al.*, 2006) and subsequently transfers it to CusB (Bagai *et al.*, 2008). Several essential, conserved methionine residues have been

FEMS MICROBIOLOGY LETTERS

identified both in CusB and in CusA (Franke *et al.*, 2003; Bagai *et al.*, 2008). The recently discovered gold-efflux determinant *gesABC* in *Salmonella* encodes the inner-membrane RND transporter GesB, the membrane-fusion protein GesA, and the OMF GesC. GesABC is able to pump organic molecules including methylene blue and crystal violet, after induction by gold ions (Pontel *et al.*, 2007). The OMF GesC can be substituted by TolC, and so *gesAB* alone can be functionally expressed in *E. coli* (Nishino *et al.*, 2006).

Here, three strains of *E. coli* with different gene deletions encoding RND transporters were transformed with plasmids containing *cusCFBA* and *gesAB* and tested for sensitivity to approximately 240 chemicals. Following initial screening, select compounds were tested further on liquid and solid media. While GesAB was shown to have broad substrate specificity typical for other RND-type systems, the CusCFBA was found to have limited substrate specificity.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth at 37 °C. To determine substrates of the efflux pumps, strains were grown overnight from a single colony, diluted, and tested for growth as described below. All experiments were performed at least three times. Antibiotic concentrations for ampicillin were 100 μg mL^{-1}.

Biolog assay

Biolog (Biolog Inc., Hayward, CA) has developed a rapid screen to determine the phenotypic classifications of bacteria and fungi. Simply, the growth rates of tested strains are compared after exposure to essential nutrients, carbon and nitrogen sources, or metals and complex organics (Bochner *et al.*, 2001; Bochner, 2003). Detection and analysis is performed colorimetrically, which represents bacterial growth. A tetrazolium dye is introduced into the medium and acts as the terminal electron acceptor during growth. Once reduced, the colorless dye turns violet, with a λ_{max} of 590 nm. The intensity of dye is directly proportional to the amount of bacteria in the wells.

To verify the results from the rapid screening method, positive compounds (i.e. chemicals conferring resistance) were tested using both solid and liquid media. All stock solutions were stored at −20 °C in the dark.

Additional strains containing their respective plasmids were tested simultaneously (Table 2). These included wild-type *E. coli* W3110, 5X RND, and W4680AE carrying pCusCFBA, pGesAB, pUH21, or pGEM-T. For liquid tests, all strains were precultured in LB (containing 100 μg mL^{-1} ampicillin when necessary) to an $OD_{600\,nm} = 0.6–1.0$. Bacteria were then diluted to a final concentration of 5×10^5 cells mL^{-1} in LB and exposed to different levels of the test chemical. Dose–response curves were created by

Table 1. Strains and plasmids used in this study

	Relevant genotype	Sources or References
Strains		
W3110	Wild type	Bachmann (1972)
W4680AD	W3110 Δ*acrA/B* Δ*acrD*	Krishnamoorthy *et al.* (2007)
W4680AE	W3110 Δ*acrA/B* Δ*acrE/F*	Krishnamoorthy *et al.* (2007)
5X RND	W3110 Δ*acrB*::*kan*, Δ*acrD*, Δ*acrF*, Δ*yegNO*, Δ*mdtF*	Personal gift from D. Nies
Plasmids		
pGEM-T	Empty vector control, Ap^R	Promega
pUH21	Empty vector control, Ap^R	Soncini *et al.* (1995)
pCusCFBA	pGEM-T-*cusCFBA*, Ap^R	Franke *et al.* (2001)
pGesAB	pUH21-*gesAB*, Ap^R	Pontel *et al.* (2007)

Table 2. Substrates to which *Escherichia coli* W4680AD expressing pGesAB shows resistance, as determined by the Biolog assay

Resistance level	Chemical	Class/function
Strong	Chlorquinaldol	Antiseptic
	Acriflavine	Antiseptic
	Alexidine	Disinfectant (found in mouthwash)
	Chelerythrine	Protein kinase C inhibitor
	Chloramphenicol	Bacteriostatic antibiotic
	Cloxacillin	β-Lactam antibiotic
	Nafcillin	β-Lactam antibiotic
	Niaproof	Anionic detergent
	Plumbagin	Antimicrobial
	Proflavine	Flavone, antibacterial
	Sanguinarine	Antimicrobial
	Thiamphenicol	Bacteriostatic antibiotic
Moderate	1-Chloro-2,4-dinitrobenzene	Oxidation, glutathione
	5,7-Dichloro-8-hydroxyquinoline	Antibacterial; antifungal
	Amikacin	Aminoglycoside antibiotic
	Bleomycin	Glycopeptide antibiotic
	Captan	Fungicide
	Cefoxitin	β-Lactam antibiotic
	Cephalothin	β-Lactam antibiotic
	Chlortetracycline	Tetracycline antibiotic
	Dichlofluanid	Fungicide
	Dodine	Fungicide
	Domiphen bromide	Antimicrobial (cosmetics)
	Hydroxylamine	DNA mutagen
	Oxacillin	β-Lactam antibiotic
Weak	Sulfamethazine	Veterinary antibacterial

recording $OD_{600\,nm}$ vs. concentration after 16 h of exposure. In solid media tests, compounds were diluted into cooling agar at different concentrations reflective of the levels present in liquid media tests. *Escherichia coli* strains W3110, W4680AD, W4680AE, or 5X RND carrying no plasmid, vector control, pCusCFBA, or pGesAB were streaked onto an agar plate, and minimum inhibitory concentrations (MICs) were determined.

The responses to different classes of chemicals varied in the Biolog assay. Certain levels and/or chemicals were toxic to both strains (empty vector vs. vector containing), creating no response in the growth curves. For chemicals that had no effect on growth, the empty vector control and metal-exporter growth curves were identical, indicating no resistance exhibited by expression of the respective RND-type metal export system. The growth rates of the expression of the RND-type metal export system exceeded that of the empty vector strain were recorded as conferring resistance. It was possible to approximate the MICs of an individual chemical using the Biolog assay based on the level of response. No metals were added to overexpress pCusCFBA and pGesAB in these experiments, and consequently, expression levels are likely to be low. Thus, it is possible that some potential substrates may not have been identified.

Escherichia coli strain W4680AD (Δ*acrA*/*B*, Δ*acrD*) containing the control vectors (pGEM-T, pUH21) or metal exporters (pCusCFBA and pGesAB) were grown in LB medium supplemented with ampicillin, 100 μg mL^{-1}, overnight at 37 °C. The inoculum was then diluted in IF-10 Base (Biolog part number 72264) to a concentration of 5×10^6 cells mL^{-1} (Bochner *et al.*, 2001). A solution containing the cell suspension (1.2 mL), sterile water (18.8 mL, IF-10 Base (98.8 mL), and Dye Mix D (Biolog part number 74224, 1.2 mL) was mixed and dispensed (100 mL per well) to each of the ten 96-well assay plates (Biolog panels PM11–PM20, part numbers 12 211–12 220). Each plate contained 24 chemicals of varying structures and functions at concentrations spanning orders of magnitude (Supporting Information, Table S1). The plates were incubated at 37 °C and the absorbance of the reduced tetrazolium dye, an indicator of cell growth, was recorded at $A_{590\,nm}$ periodically over 48 h. Absorbance vs. time was plotted for each chemical at four concentrations, comparing the strain containing the metal exporter with the strain containing an empty vector (control). The Biolog assay was repeated in triplicate on three different occasions.

Phylogenetic analysis

Protein sequences for the two metal-exporting pumps described thus far were aligned with 60 other RND proteins with known function and substrates using CLUSTALW (Higgins *et al.*, 1994). RND pumps were first identified through a search of the NCBI and SwissProt databases using CusA and GesB as the queries.

Genomic sequence resources and analysis

We examined fully sequenced bacterial genomes in the *Gammaproteobacteria* class (195 unique genomes were available as of September 22, 2009). Sequenced genomes that were used in this study can be found on the NCBI website.

CusF (gi:16128556) and CusB (gi:16128557) were queried against all 195 *Gammaproteobacteria* sequenced genomes using BLASTP with default parameters (Altschul *et al.*, 1990). Sequence alignment hits with *E*-values < 0.001 and sequence percent identity $> 25\%$ were further analyzed. Subsequently, these sequences were scanned for metal-binding motifs, $M_{21}M_{36}M_{38}$ for CusB and $W/M_{36}H_{44}M_{47}M_{49}$ for CusF.

Results and discussion

Novel substrates for two metal-transporting RND-type systems could be identified via the Biolog assay

Our aim in this study was to determine additional potential substrates of two RND-type transport systems: the gold transporter GesAB and the copper and silver transporter CusCFBA. Biolog assay plates were used for the initial screening of approximately 240 organic and inorganic compounds (Table S1). The level of resistance due to the expression of metal exporter was then classified as weak, moderate, or strong. Resistance was classified as strong when the strain expressing an RND-type exporter attained log growth, while the empty vector strain failed to grow, or grew only slightly, over 48 h. When the growth rate of the empty vector strain was within 50% of the metal-exporting strain, the resistance was classified as moderate. Resistance was classified as weak when the growth rate of the metal-exporting strain was only slightly greater than the control. Compounds to which resistance was observed for strains expressing pGes or pCusCFBA were identified (Tables 2 and 3). Chemicals to which moderate or strong resistance was exhibited were selected for further testing with liquid and solid media.

Potential substrates were identified for *E. coli* W4680AD (Δ*acrA*/*B*, Δ*acrD*) expressing pCusCFBA or pGesAB, suggesting that the RND transporter is responsible for increased resistance (data not shown). Subsequently, a few promising substrates were further tested in other *E. coli* strains. The strains *E. coli* W4680AE (Δ*acrA*, Δ*acrB*, Δ*acrE*, and Δ*acrF*) and *E. coli* strain 5X RND (Δ*acrB*, Δ*acrD*, Δ*acrF*, Δ*mdtF*, and Δ*mdtBC*) were used for further analysis in addition to *E. coli* W4680AD.

GesAB has a broad substrate range

Escherichia coli W4680AD expressing the gold-efflux system from pGesAB exhibited strong resistance toward 12 chemicals (Table 2). The classes of compounds included β-lactams, the bacteriostatics chloramphenicol and thiamphenicol, several other antimicrobials, a surfactant, and a protein kinase C inhibitor. Although the cloning vector contained coding sequences for β-lactamase and chloramphenicol acetyltransferase (Soncini *et al.*, 1995), the resistance observed for β-lactams, chloramphenicol, and thiamphenicol was much greater than the empty vector control. Moderate resistance was detected in approximately the same number of chemicals and consisted mostly of antibiotics, fungicides, a cationic surfactant, and a DNA mutagen. Of the chemicals initially identified from the Biolog screen, chloramphenicol, chlorquinaldol, and dichlofluanid were chosen for further analysis. Methylene blue and crystal violet were previously reported to be substrates of GesABC in *Salmonella typhimurium* (Nishino *et al.*, 2006; Pontel *et al.*, 2007), and were also tested here.

Table 3. Substrates to which *Escherichia coli* W4680AD expressing pCusCFBA shows resistance, as determined by the Biolog assay

Resistance level	Chemical	Class/function
Strong	2,4-Dintrophenol	Oxidative phosphorylation uncoupler
	1,3-Dinitrobenzene	Proton ionophore, H^+
	Ethionamide	Used to treat tuberculosis
Moderate	2, 2′-Dipyridyl	Iron chelator
	4-Hydroxycoumarin	Rodenticide
	Benserazide	Used to treat Parkinson's disease
	Boric acid	Insecticide
	Chloroxylenol	Pesticide
	Cytosine-1-β-D-arabinofuranoside	Inhibits DNA polymerase
	FCCP	Disrupts mitochondrial membrane potential
	Fusaric acid	Mycotoxin
	Sodium arsenate	Metal
	Sulfamethoxazole	Sulfonamide antibiotic
Weak	3-Amino-1,2,4-triazole	Herbicide
	Cefmetazole	β-Lactam antibiotic
	Cobalt chloride	Cetal
	Guanidine hydrochloride	Denatures proteins
	Lincomycin	Lincosamide antibiotic
	Oxolinic acid	Quinolone antibiotic
	Sodium metaborate	Insecticide

All three *E. coli* strains expressing GesAB showed chloramphenicol resistance in the liquid media tests (Fig. S3). MIC analysis showed that the level of resistance increased fourfold in *E. coli* strain 5X RND and eightfold in strains W4680AD and W4680AE (Table 4). Chloramphenicol had not been identified as a substrate of the Ges system previously. Moreover, chlorquinaldol resistance was detected in all the tested strains expressing GesAB in liquid media tests (data not shown). *Escherichia coli* strains W4680AD and W4680AE carrying pGesAB were resistant to chlorquinaldol with a twofold increase MIC value, via the agar results. The discrepancy between growth in the Biolog assay and MIC assays in LB medium could be attributed to differing growth conditions (media, incubation time, detection method). Crystal violet and methylene blue, which was not present in the Biolog panels, were tested because GesABC conferred resistance to both compounds in *Salmonella* (Nishino *et al.*, 2006; Pontel *et al.*, 2007). Previous studies have shown that the MIC values for crystal violet and methylene blue are 8- and 16-fold greater when the gold efflux system is overexpressed in a Δ*gesABC*, Δ*acrB* knockout (Nishino *et al.*, 2006). Here, only the MIC value of *E. coli* W4680AD containing pGesAB exposed to crystal violet was greater than the control, but *gesAB* expressed in *E. coli* strains W4680AE and 5X RND did not show any difference from the vector control. It is possible that the level of expression of *gesAB* in these backgrounds is not sufficient to detect a difference in the MIC values when compared with the vector controls. Crystal violet is a polyaromatic compound and is the largest of the chemicals studied with the Ges system. Liquid media results show that *E. coli* strain W4680AD containing pGesAB conferred resistance to crystal violet, while *E. coli* strains W4680AE and 5X RND containing pGesAB did not (Fig. S4). In liquid media tests,

Table 4. MIC (mg L^{-1}) for five compounds in different *Escherichia coli* strains expressing pGesAB or pUH21

	MIC (mg L^{-1})				
E. coli strain	Chloramphenicol	Chlorquinaldol	Crystal violet	Dichlofluanid	Methylene blue
W4680AD pUH21	2.5	0.25	0.5	> 100	4
W4680AD pGes	20	0.5	0.6	> 100	> 4
W4680AE pUH21	2.5	0.25	0.5	> 100	3
W4680AE pGes	20	0.5	0.5	> 100	4
5XRND pUH21	2.5	0.5	0.5	> 100	4
5XRND pGes	10	0.5	0.5	> 100	4

the *E. coli* strains containing pGesAB did not display a significant difference in the resistance to methylene blue (data not shown). Agar results showed that low-level resistance was conferred by pGesAB in *E. coli* strains W4680AD ($> 1\times$) and W4680AE ($1.3\times$) when exposed to methylene blue (Table 4).

CusCFBA has a narrow substrate range

To determine whether *cusCFBA* is functionally expressed in pCusCFBA, the growth of the copper-sensitive strain GR10 (Δ*cueO* Δ*cusCFBA*; Grass & Rensing, 2001) containing either pGEM-T or pCusCFBA was monitored for growth on LB medium containing different concentrations of copper. Only pCusCFBA, but not pGEM-T, was able to confer copper resistance in strain GR10, confirming that *cusCFBA* was functionally expressed (data not shown). During initial Biolog screening, pCusCFBA conferred strong resistance to dinitrophenol, dinitrobenzene, and ethionamide in W4680AD (Table 3 and Fig. S1). Both dinitrophenol and dinitrobenzene are similar in structure with a single aromatic ring. Ethionamide contains a heterocycle and two uncommon side chains. All three compounds are relatively small. The chemicals classified as moderate (10 in total) and weakly resistant (seven in total) covered a wide range of functionalities and structures and included antibiotics, metals, a metal chelator, and other biologically active compounds (Table 3).

Additional testing in liquid media revealed that the presence of pCusCFBA in *E. coli* W4680AD conferred resistance to dinitrobenzene and dinitrophenol, but the results obtained from exposure to ethionamide were inconclusive. For dinitrobenzene, a 1.2–1.4-fold-increase in the MIC value was observed for the three mutant strains expressing *cusCFBA* (Table 5). Liquid tests verified the results for strain W4680AD, but increased sensitivity was not observed between the control and the metal-exporting strains in W4680AE and 5X RND (Fig. S2). These results show that dinitrobenzene may be exported by AcrE/F, which is present in W4680AD and not W4680AE or 5X RND. For dinitrophenol, the MIC levels varied depending on the strain (Table 5) (threefold for W4680AD, 1.5-fold for W4680AE and 0.63-fold for 5X RND in metal exporter vs. control). Liquid results were similar for dinitrobenzene in that differences were observed between W4680AD pCusCFBA and control, but not for W4680AE and 5X RND. Dinitrophenol may be exported by AcrE/F. Finally, no difference was observed in any mutants exposed to ethionamide. The three strains and controls responded similarly to different concentrations of ethionamide in both liquid and agar tests (Table 5). Concentrations beyond 200 $\mu g\,mL^{-1}$ ethionamide were not evaluated due to solubility issues. In conclusion, only a limited number of potential substrates could be identified in the initial screenings. However, in an extended analysis using different strains and growth conditions, the ability of CusCFBA to confer resistance to these substances could not be verified. These results strongly suggest a narrow substrate specificity for the CusCFBA system.

Table 5. MIC ($mg\,L^{-1}$) for three compounds in different *Escherichia coli* strains expressing pCusCFBA or pGEM-T

	MIC ($mg\,L^{-1}$)		
E. coli strain	Dinitrobenzene	Dinitrophenol	Ethionamide
W4680AD pGEM-T	60	50	> 200
W4680AD pCusCFBA	80	150	> 200
W4680AE pGEM-T	60	100	> 200
W4680AE pCusCFBA	70	150	> 200
5XRND pGEM-T	70	200	> 200
5XRND pCusCFBA	100	125	> 200

Differing results between the Biolog and the MIC assays may be due to differences in the preparation of the tested compounds. The native Biolog multiwell plate contained dry deposited chemicals and the concentration range of the chemicals covered orders of magnitude. It is possible that in the Biolog assay certain hydrophobic analytes were not fully soluble, such that the bacteria were not exposed to the intended concentration. In the MIC assays, organic solvents or ionic mixtures were used to solubilize the compounds to a particular concentration. Thus, some differences may be seen if the end concentrations are different between the two assays.

Thioethers constitute the signature sequence in putative monovalent RND-type efflux pumps

Narrow substrate specificity can be attributed to the metal-binding sites of CusB and CusF. X-ray absorption spectroscopy data show that Cu(I) is bound to CusB in a three-coordinate environment, indicative of Cu–S interactions (Bagai *et al.*, 2007). CusB does not contain any cysteine residues; consequently, the sulfur-containing species in CusB that coordinate Cu(I) are methionine residues. Through site-directed mutagenesis and subsequent isothermal titration calorimetry data, Bagai and colleagues showed that three methionine residues, $M_{21}M_{36}M_{38}$, are important in metal binding and subsequent copper efflux. Moreover, CusF, a metallochaperone of the Cus complex, has been shown to bind metal via a primarily three-coordinate metal-binding site (Loftin *et al.*, 2007) and directly transfers the metal ion to the periplasmic component, CusB (Bagai *et al.*, 2008). Here, Cu(I) is coordinated with two sulfurs from M_{47} and M_{49} and a nitrogen from H_{36}, with W_{44} capping the metal site. These methionine residues in CusB and CusF are essential in the extrusion of copper and silver from the periplasm to the extracellular space.

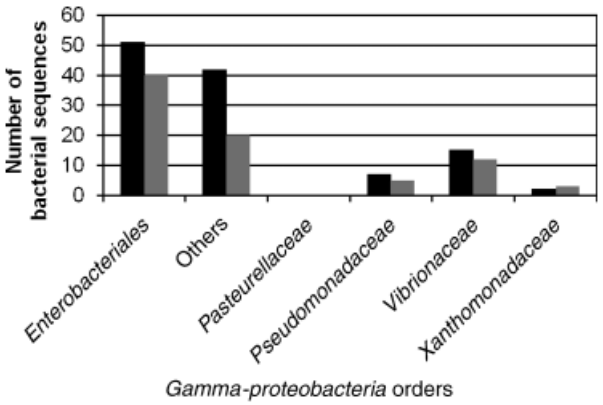

Fig. 1. Prevalence of CusB- and CusF-like proteins containing metal-binding motifs among *Gammaproteobacteria* sequences. Black bars represent CusB-like proteins with the metal-binding motif, $M_{21}M_{36}M_{38}$. Gray bars represent CusF-like proteins with the metal-binding motif, $W_{36}H_{44}M_{47}M_{49}$.

To determine the prevalence of these metal-binding motifs, BLAST analysis was performed against all sequenced gammaproteobacterial genomes (Altschul *et al.*, 1990). The number of sequences that contained these specific metal-binding motifs is shown in Fig. 1. All orders within the *Gammaproteobacteria* class, except one, *Pasteurellaceae*, contain genes encoding CusB- and CusF-like proteins with the metal-binding motifs. Interestingly, when performing BLAST analysis on the MFP GesA, no highly conserved residues were found. Consequently, the narrow substrate specificity for the CusCFBA complex may be attributed to the conserved residues for metal binding in CusB and CusF.

Analysis of CusA showed that it belongs specifically to a group of efflux pumps responsible for the extrusion of heavy metals. CusA shares high sequence identity to SilA (*S. typhimurium*, 86% identity), S0480 (*Photobacterium profundum*, 56% identity), and CebA (*Legionella pneumophila*, 46% identity). All four proteins possess three methionines that may be responsible for copper/silver binding and export. Interestingly, the three essential methionines present in CusA (Franke *et al.*, 2003) are located in a periplasmic cleft shown to be important for substrate binding and function in AcrB (Takatsuka & Nikaido, 2007).

CLUSTALW alignments showed that GesB belongs to the class of RND proteins containing MexQ (*Pseudomonas aeruginosa*, 69% identity), MexF (*P. aeruginosa*, 62% identity), BpeF (*Burkholderia mallei*, 59% identity), SdeB (*Serratia marcescens*, 55% identity), and LmxF (*L. pneumophila*, 41% identity). Both MexQ and MexF export macrolides, biocides, fluoroquinolones, tetracycline, and chloramphenicol (Mima *et al.*, 2007). SdeB is known to pump fluoroquinolones (Begic & Worobec, 2008). Chloramphenicol and trimethoprim are substrates of BpeF (Kumar *et al.*, 2006).

Further analysis of GesB showed that it may possess methionine residues capable of coordinating with metals. Like MexB of *P. aeruginosa* (Guan *et al.*, 1999), GesB (42% identity) has two periplasmic loops that interact with substrates. Within loop 2 of GesB (residues 567–881) resides three Met residues, M_{636}, M_{639}, and M_{864}, and a potential metal ligand H_{826}. Both H_{826} and M_{864} are conserved in proteins with high sequence identity to GesB, MexQ, and MexF, while M_{636} and M_{639} are conserved only in proteins with high sequence identity to GesB and MexQ. GesB, MexQ, and MexF have $>62\%$ sequence identity to each other, which is higher than the CusA homologues stated above. As gold lies within the same transition metal group as copper and silver (Group IB), it is expected that efflux will occur through interaction with metal-coordinating residues such as methionine and histidine, although the exact pathway is yet to be determined. In *Salmonella*, *gesABC* is adjacent to an operon encoding a Cu(I)-translocating P-type ATPase and a CueR-like regulator. Similarly, a GesB homolog (RPD_2310) in *Rhodopseudomonas palustris* is encoded adjacent to a GesA homolog (RPD_2311) and a CueR-regulated Cu(I)-translocating P-type ATPase and a putative Cu(I) chaperone (RPD_2307, RPD_2308, and RPD_2309). In contrast, GesB-like proteins are encoded adjacent to genes encoding putative Cd(II), Zn(II), and Pb(II)-translocating P-type ATPases in *P. aeruginosa* LESB58 (CadA is PLES_26261; GesB is PLES_26281), *Diaphorobacter* sp. TPSY (CadA is Dtpsy_1151; GesB is Dtpsy_1153), and *Shewanella* sp. W3-18-1 (CadA is Sputw3181_1126; GesB is Sputw3181_1130). These examples show that the GesABC system is possibly not the only RND-type complex related to the broader MexQ family involved in the efflux of metals. However, at this time, the substrate range of these related transporters is not known and awaits further studies.

Conclusions

The extended substrate spectrum of two metal-exporting RND systems was determined. The gold-transporting GesAB system was confirmed to have a broad substrate spectrum, with chloramphenicol identified as an additional substrate. In contrast, CusCFBA had a narrow substrate spectrum, transporting Cu(I) and Ag(I) almost exclusively. Three conserved residues in these metal exporters might be responsible for substrate recognition and specificity.

Acknowledgements

We greatly appreciate our colleagues for the supply of the strains and plasmids. Helen Zgurskaya provided the *E. coli* deletion strains W4680AD and W4680AE, as well as valuable correspondence. The deletion strain *E. coli* 5X RND and plasmid pCusCFBA were supplied by Dietrich Nies.

Fernando Soncini provided plasmids pUH21 and pGesAB. E.H.-K. is a scholar of the Alfred P. Sloan Foundation. This work was supported by National Institutes of Health Grant GM079192 to M.M.M. and C.R.

References

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Bachmann BJ (1972) Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriol Rev* **36**: 525–557.

Bagai I, Liu W, Rensing C, Blackburn NJ & McEvoy MM (2007) Substrate-linked conformational change in the periplasmic component of a Cu(I)/Ag(I) efflux system. *J Biol Chem* **282**: 35695–35702.

Bagai I, Rensing C, Blackburn NJ & McEvoy MM (2008) Direct metal transfer between periplasmic proteins identifies a bacterial copper chaperone. *Biochemistry* **47**: 11408–11414.

Baranova N & Nikaido H (2002) The BaeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. *J Bacteriol* **184**: 4168–4176.

Begic S & Worobec EA (2008) The role of the *Serratia marcescens* SdeAB multidrug efflux pump and TolC homologue in fluoroquinolone resistance studied via gene-knockout mutagenesis. *Microbiology* **154**: 454–461.

Bochner BR (2003) New technologies to assess genotype–phenotype relationships. *Nat Rev* **4**: 309–314.

Bochner BR, Gadzinski P & Panomitros E (2001) Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res* **11**: 1246–1255.

Bohnert JA, Schuster S, Fähnrich E, Trittler R & Kern WV (2007) Altered spectrum of multidrug resistance associated with a single point mutation in the *Escherichia coli* RND-type MDR efflux pump YhiV (MdtF). *J Antimicrob Chemoth* **59**: 1216–1222.

Elkins CA & Nikaido H (2002) Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominately by two large periplasmic loops. *J Bacteriol* **184**: 6490–6498.

Franke S, Grass G & Nies DH (2001) The product of the ybdE gene of the *Escherichia coli* chromosome is involved in detoxification of silver ions. *Microbiology* **147**: 965–972.

Franke S, Grass G, Rensing C & Nies DH (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol* **185**: 3804–3812.

Grass G & Rensing C (2001) Genes involved in copper homeostasis in *Escherichia coli*. *J Bacteriol* **183**: 2145–2147.

Guan L, Ehrmann M, Yoneyama H & Nakae T (1999) Membrane topology of the xenobiotic-exporting subunit, MexB, of the MexA,B-OprM extrusion pump in *Pseudomonas aeruginosa*. *J Biol Chem* **274**: 10517–10522.

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.

Kittleson JT, Loftin IR, Hausrath AC, Engelhardt KP, Rensing C & McEvoy MM (2006) Periplasmic metal-resistance protein CusF exhibits high affinity and specificity for both CuI and AgI. *Biochemistry* **45**: 11096–11102.

Krishnamoorthy G, Tikhonova EB & Zgurskaya HI (2007) Fitting periplasmic membrane fusion proteins to inner membrane transporters: mutations that enable *Escherichia coli* AcrA to function with *Pseudomonas aeruginosa* MexB. *J Bacteriol* **190**: 691–698.

Kumar A, Chua K-L & Schweizer HP (2006) Method for regulated expression of single-copy efflux pump genes in a surrogate *Pseudomonas aeruginosa* strain: identification of the BpeEF-OprC chloramphenicol and trimethoprim efflux pump of *Burkholderia pseudomallei* 1026b. *Antimicrob Agents Ch* **50**: 3460–3463.

Loftin IR, Franke S, Blackburn NJ & McEvoy MM (2007) Unusual Cu(I)/Ag(I) coordination of *Escherichia coli* CusF as revealed by atomic resolution crystallography and X-ray absorption spectroscopy. *Protein Sci* **16**: 2287–2293.

Lomovskaya O, Zgurskaya HI, Totrov M & Watkins WJ (2007) Waltzing transporters and 'the dance macabre' between humans and bacteria. *Nat Rev Drug Discov* **6**: 56–65.

Mima T, Joshi S, Gomez-Escalada M & Schweizer HP (2007) Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. *J Bacteriol* **189**: 7600–7609.

Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev* **27**: 313–339.

Nishino K, Latifi T & Groisman EA (2006) Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* **59**: 126–141.

Pontel LB, Pérez Audero ME, Espariz M, Checa SK & Soncini FC (2007) GolS controls the response to gold by the hierarchical induction of *Salmonella*-specific genes that include a CBA efflux-coding operon. *Mol Microbiol* **66**: 814–825.

Pos KM (2009) Drug transport mechanism of the AcrB efflux pump. *BBA-Proteins Proteom* **1794**: 782–793.

Soncini FC, Vescovi EG & Groisman EA (1995) Transcriptional autoregulation of the *Salmonella typhimurium* phoPQ operon. *J Bacteriol* **177**: 4364–4371.

Takatsuka Y & Nikaido H (2007) Site-directed disulfide cross-linking shows that cleft flexibility in the periplasmic domain is needed for the multidrug efflux pump AcrB of *Escherichia coli*. *J Bacteriol* **189**: 8677–8684.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Biolog plots for dinitrobenzene (top), dinitrophenol (middle), and ethionamide (bottom).

Fig. S2. Growth of *Escherichia coli* strains W4680AD (top), W4680AE (middle), and 5X RND (bottom) expressing pCusCFBA (dashed line) or the control vector pGem-T (solid line) in liquid media containing different concentrations of dinitrobenzene.

Fig. S3. Biolog results for chlorquinaldol (top), chloramphenicol (middle), and dichlofluanid (bottom).

Fig. S4. Growth of *Escherichia coli* strains W4680AD (top), W4680AE (middle), and 5X RND (bottom) harboring pGesAB (dashed line) or pUH21 (solid line) in liquid media containing different concentrations of crystal violet.

Table S1. Chemicals in the Biolog Chemical Sensitivity Panels PM11–PM20.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.