

# Homologs of the *Acinetobacter baumannii* *AceI* Transporter Represent a New Family of Bacterial Multidrug Efflux Systems

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**ABSTRACT** Multidrug efflux systems are a major cause of resistance to antimicrobials in bacteria, including those pathogenic to humans, animals, and plants. These proteins are ubiquitous in these pathogens, and five families of bacterial multidrug efflux systems have been identified to date. By using transcriptomic and biochemical analyses, we recently identified the novel *AceI* (*Acinetobacter* chlorhexidine efflux) protein from *Acinetobacter baumannii* that conferred resistance to the biocide chlorhexidine, via an active efflux mechanism. Proteins homologous to *AceI* are encoded in the genomes of many other bacterial species and are particularly prominent within proteobacterial lineages. In this study, we expressed 23 homologs of *AceI* and examined their resistance and/or transport profiles. MIC analyses demonstrated that, like *AceI*, many of the homologs conferred resistance to chlorhexidine. Many of the *AceI* homologs conferred resistance to additional biocides, including benzalkonium, dequalinium, proflavine, and acriflavine. We conducted fluorimetric transport assays using the *AceI* homolog from *Vibrio parahaemolyticus* and confirmed that resistance to both proflavine and acriflavine was mediated by an active efflux mechanism. These results show that this group of *AceI* homologs represent a new family of bacterial multidrug efflux pumps, which we have designated the proteobacterial antimicrobial compound efflux (PACE) family of transport proteins.

**IMPORTANCE** Bacterial multidrug efflux pumps are an important class of resistance determinants that can be found in every bacterial genome sequenced to date. These transport proteins have important protective functions for the bacterial cell but are a significant problem in the clinical setting, since a single efflux system can mediate resistance to many structurally and mechanistically diverse antibiotics and biocides. In this study, we demonstrate that proteins related to the *Acinetobacter baumannii* *AceI* transporter are a new class of multidrug efflux systems which are very common in *Proteobacteria*: the proteobacterial antimicrobial compound efflux (PACE) family. This is the first new family of multidrug efflux pumps to be described in 15 years.

Received 15 October 2014 Accepted 23 December 2014 Published 10 February 2015

**Citation** Hassan KA, Liu Q, Henderson PJF, Paulsen IT. 2015. Homologs of the *Acinetobacter baumannii* *AceI* transporter represent a new family of bacterial multidrug efflux systems. *mBio* 6(1):e01982-14. doi:10.1128/mBio.01982-14.

**Editor** Karen Bush, Indiana University Bloomington

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Multidrug efflux is a ubiquitous mechanism of drug resistance in bacterial pathogens that is mediated by integral membrane transport proteins. These proteins are typically very promiscuous, recognizing a range of antimicrobial substrates that differ in both structure and valency. To date, five distinct families of transport proteins have been shown to include multidrug efflux systems: the major facilitator superfamily, the resistance/nodulation/division superfamily, the ATP-binding cassette superfamily, the multidrug and toxic compound extrusion family, and the small multidrug resistance family.

Recently, we identified the *aceI* (*Acinetobacter* chlorhexidine efflux) gene in *Acinetobacter baumannii*, which is involved in adaptive resistance to the widely used biocide chlorhexidine (1). This gene was overexpressed more than 10-fold in response to a subinhibitory shock of chlorhexidine in *A. baumannii* ATCC 17978. The *aceI* gene encodes a membrane protein that is approximately 150 amino acid residues in length and contains two tandem bacterial transmembrane pair (BTP; Pfam accession number PF05232) domains (2). Heterologous expression of *aceI* increased *Escherichia coli* resistance to chlorhexidine (1) and, conversely,

deletion of *aceI* from the *A. baumannii* genome increased its susceptibility to chlorhexidine (3). The *AceI* protein was shown to interact directly with chlorhexidine and to mediate its efflux via an energy-dependent mechanism (1). However, resistance to other antimicrobial compounds was not observed (1).

Genes that encode BTP domain proteins homologous to *aceI* are carried by diverse bacterial species but are particularly common among proteobacterial lineages. Similar to *A. baumannii*, genes encoding BTP domain proteins were upregulated in the human pathogens *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* J2315 in response to chlorhexidine and were able to mediate resistance to this biocide (1). Furthermore, related BTP domain protein genes from the soil bacterium *Acinetobacter baylyi* ADP1 and the plant commensal bacterium *Pseudomonas protegens* Pf-5 were also shown to mediate resistance to chlorhexidine when expressed in *E. coli* (1). Deletion of this gene from *A. baylyi* ADP1 increased its susceptibility to chlorhexidine (1).

In addition to *aceI*, the *A. baumannii* genome harbors a second gene that encodes a BTP family protein, A1S\_1503, that does not confer chlorhexidine resistance and whose expression is not in-

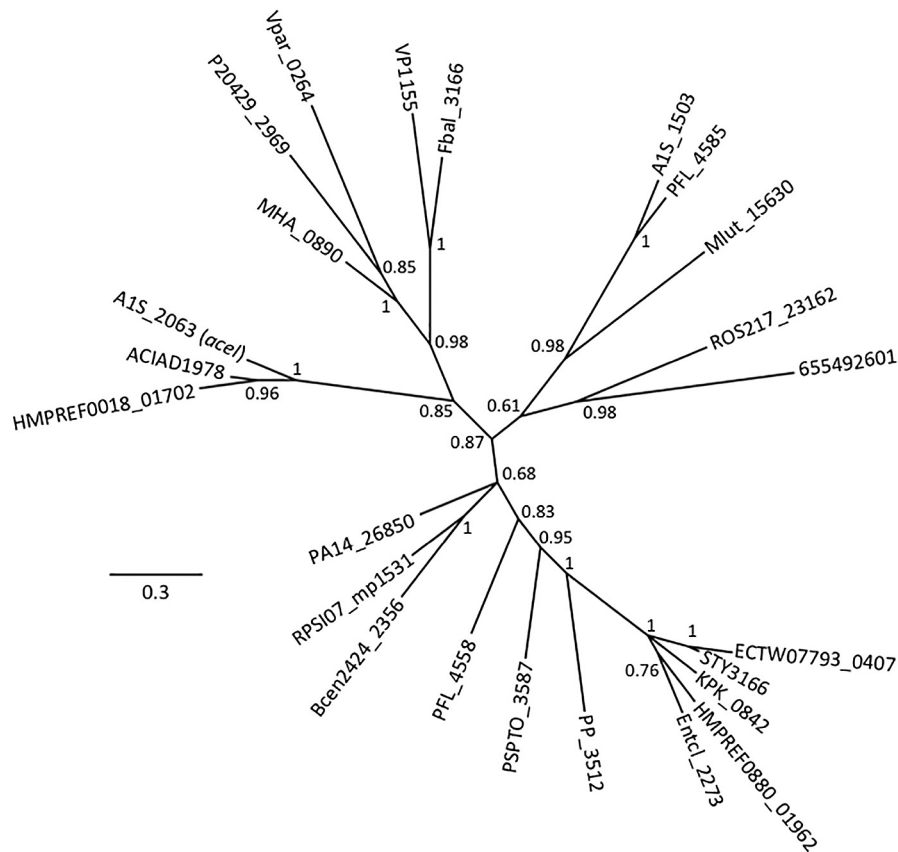


FIG 1 Tree showing the phylogenetic relationships of BTP family proteins included in this study. The tree was generated using MrBayes 3.2.1 (14) from a ClustalX2 alignment of protein sequences obtained from the National Center for Biotechnology Information database. Interior node values are clade credibility values (posterior probabilities) generated and assigned by MrBayes.

duced by chlorhexidine. Similarly, *P. protegens* harbors a second gene encoding a BTP domain protein that appears to be nonfunctional with respect to chlorhexidine resistance (1), and the *P. aeruginosa* and *B. cenocepacia* genomes carry one or two BTP domain protein genes that are not induced by chlorhexidine (4, 5).

Here, we sought to identify alternative drug substrates for BTP domain proteins. We demonstrate that, in addition to chlorhexidine, many BTP domain proteins are able to mediate resistance to other biocides, as well as fluorescent dyes. The protein from *Vibrio parahaemolyticus* VP1155 provided particularly strong resistance to biocides and dyes and mediated rapid transport of acriflavine and proflavine. These results indicate that BTP domain proteins represent a new family of transport proteins that includes multi-drug efflux systems, which we have designated the proteobacterial antimicrobial compound efflux (PACE) family.

**BTP protein gene cloning and expression.** At the time of writing, the Pfam database (version 27.0) listed close to 800 proteins that contain BTP domains from more than 600 bacterial species (2). The majority (95%) of these proteins were predicted to have the same tandem BTP domain architecture as AceI and were encoded by *Proteobacteria*, particularly the gamma, beta, and alpha subdivisions (although this may be biased by the species for which genome sequence data are available). In addition to *Proteobacteria*, the genomes of several *Veillonella* and *Micrococcus* species (*Firmicutes* and *Actinobacteria*, respectively) also carried BTP do-

main protein genes. We have not, however, detected these genes in the genomes of archaeal or eukaryotic organisms.

In this study, we examined the drug resistance/transport capabilities of 24 BTP domain proteins, including AceI. These proteins were selected to encompass the full spectrum of currently sampled phylogenetic diversity within this group (Fig. 1) and included 18 gammaproteobacterial proteins, 3 betaproteobacterial proteins, and 1 representative protein from each of *Alphaproteobacteria*, *Firmicutes*, and *Actinobacteria* (Table 1). Seven of the genes encoding these proteins were previously cloned into the *E. coli* pTTQ18 expression vector via conventional methods (1). The remaining 17 were synthetically designed *E. coli* codon-optimized sequences and were synthesized in single gBlock gene fragments (Integrated DNA Technologies) and then cloned into the pTTQ18 plasmid vector (6). With the exception of Vpar\_0264, ROS217\_23162 and MHA\_0890, the proteins under investigation were expressed at levels detectable in Western blot assays of whole-cell lysates (see Fig. S1 in the supplemental material). This level of expression success (87.5%) is in line with our previous experiences using this expression system for the heterologous production of transport systems (7–9).

**Chlorhexidine resistance mediated by AceI homologs.** Previously, we demonstrated that BTP domain proteins homologous to the *A. baumannii* AceI protein from *A. baylyi* ADP1 (ACIAD1978), *P. aeruginosa* PA14 (PA14\_26850), *P. protegens*

TABLE 1 Drug resistance conferred by BTP family proteins<sup>a</sup>

Organism	Gene or protein (locus tag)	MIC or MIC range ( $\mu\text{g/ml}$ ) <sup>b</sup>				
		CH	DQ	BK	PF	AF
NA (negative control)	NA <sup>c</sup> (vector only)	0.195–0.39	50	0.39–0.78	6.25	3.125
<i>Acinetobacter baumannii</i> ATCC 17978	A1S_2063 ( <i>aceI</i> )	<b>1.56</b>	12.5–25	0.39–0.78	6.25	3.125
<i>Acinetobacter radioresistens</i> SH164	HMPREF0018_01702	<b>0.78</b>	25	0.39–0.78	6.25	3.125
<i>Acinetobacter baylyi</i> ADP1	ACIAD1978	<b>0.78</b>	50	0.78	6.25	3.125
<i>Mannheimia haemolytica</i> PHL213	MHA_0890	0.39	50	0.78	6.25	3.125
<i>Pseudoalteromonas</i> sp. BSi20429	P20429_2969	0.195	50	0.78	6.25	<b>6.25</b>
<i>Veillonella parvula</i> DSM 2008	Vpar_0264	0.195	50	0.39–0.78	6.25	3.125
<i>Vibrio parahaemolyticus</i> RIMD 2210633	VP1155	<b>1.56</b>	50	<b>3.125</b>	<b>25</b>	<b>12.5</b>
<i>Ferrimonas balearica</i> DSM 9799	Fbal_3166	<b>0.78–1.56</b>	50	0.78	3.125	<b>6.25</b>
<i>Micrococcus luteus</i> NCTC 2665	Mlut_15630	0.195	50	0.39–0.78	6.25	3.125
<i>Acinetobacter baumannii</i> ATCC 17978	A1S_1503	0.195	25	0.78	6.25	<b>6.25</b>
<i>Pseudomonas protegens</i> Pf-5	PFL_4585	0.195	<b>100</b>	<b>1.56</b>	3.125–6.25	3.125
<i>Tepidiphilus margaritifera</i> DSM 15129	655492601 <sup>d</sup>	0.195	50–100	0.78–1.56	1.56–3.125	3.125
<i>Roseovarius</i> sp. 217	ROS217_23162 <sup>e</sup>	0.195	50	0.78	6.25	3.125
<i>Pseudomonas aeruginosa</i> PA14	PA14_26850	<b>0.78</b>	<b>100</b>	<b>1.56</b>	6.25	3.125
<i>Ralstonia solanacearum</i> PSI07	RPS107_mp1531	0.195–0.39	50–100	<b>1.56–3.125</b>	3.125	<b>6.25</b>
<i>Burkholderia cenocepacia</i> HI2424	Bcen2424_2356	<b>1.56</b>	25	<b>1.56–3.125</b>	<b>12.5–25</b>	<b>6.25</b>
<i>Pseudomonas protegens</i> Pf-5	PFL_4558	<b>1.56</b>	12.5–25	0.39–0.78	3.125–6.25	3.125
<i>Pseudomonas syringae</i> pv. Tomato strain DC3000	PSPTO_3587	<b>0.78</b>	50	0.78	<b>12.5–25</b>	<b>6.25</b>
<i>Pseudomonas putida</i> KT2440	PP_3512	<b>0.78</b>	50	0.78–1.56	6.25	3.125
<i>Enterobacter cloacae</i> SCF1	Entcl_2273	0.39	25	<b>1.56</b>	6.25	3.125
<i>Yokenella regensburgei</i> ATCC 43003	HMPREF0880_01962	<b>0.78</b>	50	0.78–1.56	3.125–6.25	3.125
<i>Klebsiella pneumoniae</i> 342	KPK_0842	<b>0.78</b>	25	0.78–1.56	<b>12.5</b>	3.125
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhi strain ct18	STY3166	0.195	25–50	0.39	6.25	3.125
<i>Escherichia coli</i> TW07793	ECTW07793_0407	0.39–0.78	50	0.78	6.25–12.5	<b>6.25</b>

<sup>a</sup> None of the cloned BTP family genes conferred reproducible resistance to tetracycline, chloramphenicol, tetraphenylphosphonium, ethidium, Hoechst 33342, pyronin Y, acridine yellow, or 4',6-diamidino-2-phenylindole.

<sup>b</sup> The values given are from at least two independent biological replicates, and those indicating reproducible increases in resistance are shown in boldface. Abbreviations: CH, chlorhexidine; DQ, dequalinium; BK, benzalkonium; PF, proflavine; AF, acriflavine.

<sup>c</sup> NA, not applicable.

<sup>d</sup> The GenBank protein ID is given for the *Tepidiphilus margaritifera* DSM 15129 protein.

<sup>e</sup> The cloned *Roseovarius* sp. 217 gene ROS217\_23162 contains a single base change that results in a serine-to-phenylalanine mutation at position 139 (C-terminal tail).

Pf-5 (PFL\_4558), and *B. cenocepacia* HI2424 (Bcen2424\_2356) were able to confer resistance to chlorhexidine, whereas the phylogenetically distinct (Fig. 1) proteins from *A. baumannii* (A1S\_1503) and *P. protegens* (PFL\_4585) did not confer resistance (1). Chlorhexidine MIC analyses were conducted as previously described (8) to gauge the level of resistance provided by the 17 newly cloned BTP domain protein homologs. These assays were conducted in medium containing 0.05 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) to induce basal levels of expression. Among the 24 cloned genes, we saw reproducible increases in chlorhexidine resistance from 12 genes (Table 1). This result confirmed that chlorhexidine is a common substrate of this group of transporters.

**BTP domain proteins are multidrug efflux systems.** To explore the possibility that BTP domain proteins represent a new family of multidrug efflux transporters, we tested their capacities to confer resistance to a range of additional antimicrobial compounds in MIC analyses. These compounds included: the biocides dequalinium, tetraphenylphosphonium, and benzalkonium, the antibiotics tetracycline and chloramphenicol, and a number of fluorescent antimicrobial dyes that are common substrates of multidrug efflux systems, such as proflavine, acriflavine, ethidium, Hoechst 33342, pyronin Y, acridine yellow, and 4',6-diamidino-2-phenylindole (Table 1).

Resistance to a variety of antimicrobials was common, particularly among the *Pseudomonas* and betaproteobacterial genes

(Table 1). Six BTP domain protein genes conferred reproducible resistance to the biocide benzalkonium. Additionally, two *Pseudomonas* proteins, PA14\_26850 and PFL\_4585, mediated resistance to dequalinium. Notably, PFL\_4585 is a paralog of the chlorhexidine resistance protein PFL\_4558, demonstrating that both BTP domain proteins encoded by *P. protegens* Pf-5 mediate resistance to selected biocides. Nine of the BTP domain protein genes also conferred resistance to one or both of the DNA-intercalating antimicrobial dyes acriflavine and proflavine (Table 1). Only 6 genes out of the 24 examined in this study did not provide resistance to any of the antimicrobials tested. Of these, three were not expressed at levels detectable by Western blotting (see Fig. S1 in the supplemental material). The *V. parahaemolyticus* gene VP1155 provided the highest and most consistent increases in resistance to the greatest number of compounds, with at least 4-fold increases in resistance to chlorhexidine, benzalkonium, proflavine, and acriflavine. To determine whether resistance mediated by these proteins was dependent on a TolC-like outer membrane, we examined resistance mediated by *AceI* and VP1155 in a TolC-inactivated background (10). Both of these proteins mediated resistance in this mutant strain.

To explore further the substrate recognition profile of the VP1155 protein, we conducted Biolog OmniLog Phenotype microarray (PM) experiments (11). The resistance levels of *E. coli* BL21 carrying pTTQ18-VP1155 were compared to those of *E. coli* BL21 carrying pTTQ18 for 240 different antimicrobials in the

PM11-20 plate series, as previously described (1, 11). These tests confirmed that VP1155 provides resistance to the substrates identified by conventional MIC assays: chlorhexidine, proflavine, and acriflavine (see Fig. S2 in the supplemental material; benzalkonium is not included in the PM11-20 panel of compounds). Furthermore, the Biolog PM tests indicated that VP1155 also provides resistance to 9-aminoacridine, domiphen bromide, 3,5-diamino-1,2,4-triazole (guanazole), and plumbagin (see Fig. S2).

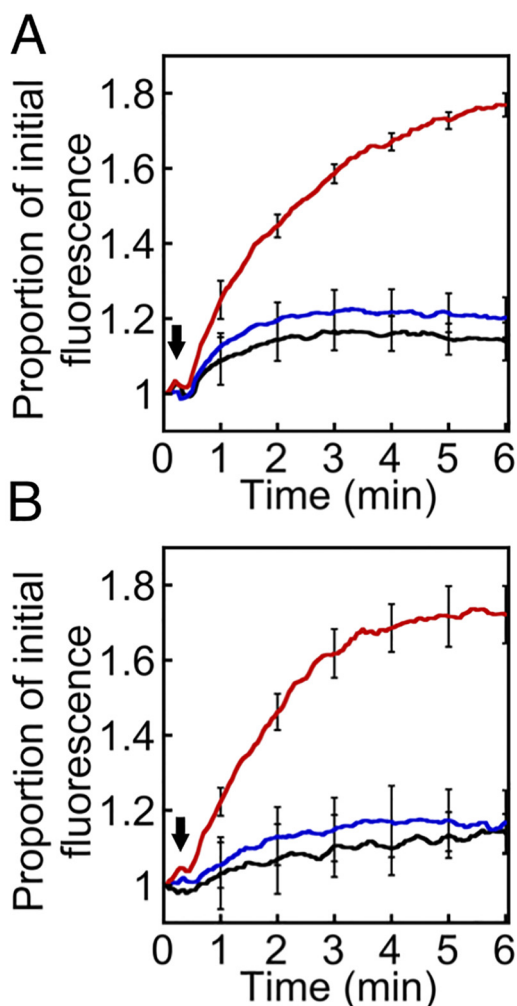
**Fluorimetric transport assays demonstrate efflux mediated by VP1155.** The observations of resistance to the DNA-intercalating antimicrobial dyes proflavine and acriflavine from several of the BTP domain protein genes presented the opportunity to assay directly the efflux of these substrates in real-time fluorimetric transport assays. We applied these assays to cells expressing VP1155, exploiting the capacity of this protein to mediate resistance to both dyes. The assays were conducted in the *E. coli* triple deletion mutant strain BW25113 ( $\Delta acrB \Delta emrE \Delta mdxA::kan$ ), which is defective in the three major *E. coli* multidrug efflux system genes (12) and provides a sensitive background for these assays. Cells carrying the pTTQ18-VP1155 expression plasmid were assayed both pre- and postinduction of VP1155 expression by using 0.2 mM IPTG (see Fig. S3 in the supplemental material). Cells carrying “empty” pTTQ18 treated with IPTG were also included as a negative control. The transport assays were conducted essentially as described previously (13), except that cells were grown in glycerol-supplemented medium and reenergized by using glycerol to initiate transport from substrate-loaded cells.

The fluorescence intensity of both proflavine and acriflavine is lower when intercalated into DNA, so efflux from the cell was characterized by an increase in fluorescence over time. In our transport experiments we observed a rapid increase in fluorescence in cells that expressed the VP1155 protein but not in control cells lacking this protein (Fig. 2; see also Fig. S3 in the supplemental material). These results provide additional evidence that efflux is the mechanism of resistance operating in this group of proteins.

**Conclusions.** In this study, we examined a large panel of genes encoding proteins related to the AclE chlorhexidine efflux system for their ability to confer resistance to a set of 12 different biocides, antibiotics, and antimicrobial dyes. To facilitate this broad survey of phylogenetically diverse proteins, we adopted a synthetic cloning approach; the majority of genes were codon optimized for expression in *E. coli* and chemically synthesized for cloning into our expression system. Of the 24 transport proteins studied, 21 were expressed at levels detectable in Western blot assays of whole-cell lysates, and 18 conferred resistance to one or more antimicrobial compounds.

Our results demonstrate that this group of proteins is a new family of bacterial multidrug efflux systems, which we have designated the proteobacterial antimicrobial compound efflux (PACE) family. The PACE family is only the sixth family of bacterial multidrug efflux systems to have been described and the first new family for more than 15 years. Multidrug efflux systems encoded by nosocomial pathogens are particularly problematic. Antimicrobial pressures selecting for increased expression of multidrug exporters can promote resistance to not only the selecting compound but also to a swath of otherwise-effective compounds.

Using a radiolabeled substrate, we previously demonstrated that the AclE protein within the PACE family is able to mediate the active efflux of chlorhexidine. In this work, we have identified fluorescent substrates for several members of the PACE family



**FIG 2** Acriflavine and proflavine efflux mediated by VP1155. *E. coli* BW25113  $\Delta acrB \Delta emrE \Delta mdxA::kan$  cells carrying pTTQ18 only (black line) or pTTQ18-VP1155 (blue [noninduced] and red [induced]) were grown in Luria-Bertani medium containing 0.5% glycerol to an optical density at 600 nm of 0.6. Samples of pTTQ18-VP1155 cells were taken and assayed as non-induced controls (blue lines). The cultures were then grown for a further 1 h, after which 0.2 mM IPTG was added to induce expression directed by the PtaC promoter in pTTQ18. The cells were washed in assay buffer (HEPES, pH 7.0) and loaded with 20  $\mu$ M acriflavine (A) or proflavine (B) in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The loaded cells were again washed and suspended in assay buffer (37°C). Transport was initiated with the addition of 1% glycerol at the point marked with an arrow. The assays were performed in biological triplicates, and the error bars show the standard errors of the means at 1-min intervals. Expression of the RGS6-tagged VP1155 protein in the samples was assessed by Western blotting (see Fig. S3 in the supplemental material) and was positively correlated with efflux.

that facilitate the development of rapid fluorimetric efflux assays. These assays will be highly valuable in future studies to define the molecular transport mechanism operating in members of this family, including the mode of energization, which is likely to involve an electrochemical gradient.

Notably, PACE family proteins are encoded in the core genomes of many proteobacterial species that are separated by hundreds of millions of years of evolution. Given that the substrates we have now defined for these efflux systems—chlorhexidine, de-

qualinium, benzalkonium, proflavine, and acriflavine—are synthetic biocides that have only been widely used within the last century, it seems unlikely that these compounds are the physiological substrates of these transporters. Nonetheless, as with other multidrug efflux systems, the substrate promiscuity of PACE efflux systems is likely to have enhanced the success of proteobacterial pathogens in clinical settings.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01982-14/-/DCSupplemental>.

Figure S1, TIF file, 1.4 MB.

Figure S2, TIF file, 1.8 MB.

Figure S3, TIF file, 0.6 MB.

## ACKNOWLEDGMENTS

This work was supported by a project grant from the Australian National Health and Medical Research Council (1060895) to I.T.P., K.A.H., and P.J.F.H. and the award of a Leverhulme Trust emeritus fellowship to P.J.F.H.

We thank Shimon Schuldiner from the Hebrew University of Jerusalem for the gift of *E. coli* strain BW25113  $\Delta$ acrB  $\Delta$ emrE  $\Delta$ mdfA::kan.

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