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Characterization of the RND family of multidrug efflux pumps: *in silico* to *in vivo* confirmation of four functionally distinct subgroups

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Summary

We have developed a generalized profile that identifies members of the root-nodulation-cell-division (RND) family of efflux pumps and classifies them into four functional subfamilies. According to Z-score values, efflux pumps can be grouped by their metabolic function, thus making it possible to distinguish pumps involved in antibiotic resistance (group 1) from those involved in metal resistance (group 3). *In silico* data regarding efflux pumps in group 1 were validated after identification of RND efflux pumps in a number of environmental microbes that were isolated as resistant to ethidium bromide. Analysis of the *Pseudomonas putida* KT2440 genome identified efflux pumps in all groups. A collection of mutants in efflux pumps and a screening platform consisting of 50 drugs were created to assign a function to the efflux pumps. We validated *in silico* data regarding efflux pumps in groups 1 and 3 using 9 different mutants. Four mutants belonging to group 2 were found to be more sensitive than the wild-type to oxidative stress-inducing agents such as bipyridyl and methyl viologen. The two remaining mutants belonging to group 4 were found to be more sensitive than the parental to tetracycline and one of them was particularly sensitive to rubidium and chromate. By effectively combining *in vivo* data with generalized profiles and gene annotation data, this approach allowed the assignment, according to metabolic function, of both known and uncharacterized RND efflux pumps into subgroups, thereby providing important new insight into the functions of proteins within this family.

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Introduction

The analysis of protein sequences makes it possible to identify conserved domains in proteins, such as enzyme catalytic sites, cofactor binding sites, small molecule ligand domains, DNA binding domains and many others (Corpet *et al.*, 1999; Bradley *et al.*, 2008). Proteins or protein domains belonging to a particular family often share functional attributes, and therefore the grouping of proteins has been used in turn to characterize them at the functional level (Holm and Sander, 1996; Tatusov *et al.*, 2001). Several approaches are available to define domains and protein families, and the availability of semi-automatic methods for profile construction, as well as their high sensitivity, has improved the efficiency and eased the process involved in the definition of protein families (Bucher and Bairoch, 1994; Bucher *et al.*, 1996; Bateman *et al.*, 2004; Ramos *et al.*, 2005; Tobes and Ramos, 2005; Hulo *et al.*, 2006). Profiles are not necessarily confined to small regions with high sequence similarity, but rather they attempt to characterize a protein family (or domain) based on full-length sequences (Rigali *et al.*, 2002; Hulo *et al.*, 2006; Molina-Henares *et al.*, 2009).

Microorganisms in the environment are exposed to a large number of drugs of natural and xenobiotic origin and, as such, have developed strategies to cope with toxic compounds. The extensive use of some drugs in medicine, such as biocides and antibiotics, has lead to a major therapeutic problem as bacteria have developed resistance to multiple antibiotics (Zhang and Mah, 2008; Aminov, 2009; Baquero *et al.*, 2009; Daniels and Ramos, 2009). Resistance-Nodulation-cell Division (RND) efflux pumps are common elements in multidrug resistance, and their wide substrate specificity explains cross-resistance between antibiotics, biocides, dyes and solvents in laboratory strains (Nikaido, 1996; 1998; 2000; Daniels and Ramos, 2009; Nikaido and Takatsuka, 2009). A number of RND efflux pumps have also been described that extrude heavy metals, and represent a major determinant in the proliferation of microorganisms at sites polluted with zinc, lead, mercury, cobalt and other metals (Checa *et al.*, 2007). Although the entire suite of physiological functions of RND pumps has not yet been well established, a number of recent studies have shown that these pumps

may be involved in the extrusion of intracellularly generated toxic compounds. These RND pump-excluded compounds may include, for example, formaldehyde produced from the metabolism of histidine and methoxylated chemicals (Roca *et al*., 2008); amino acids to maintain amino acid homeostasis (Herrera *et al*., 2010); and quorum sensing molecules (Pearson *et al*., 1999; Ueda *et al*., 2009). As well, RND pumps may also be important for protecting cells against toxic compounds in the cell's environment (Levy, 1998; Nikaido and Takatsuka, 2009).

A typical RND efflux pump consists of three components, with one of these components being the inner membrane protein, which acts as the extrusion element, and which is often more than 1000 amino acids long, consisting of 12 transmembrane helices (Murakami *et al*., 2002; Yu *et al*., 2005; Törnroth-Horsefield *et al*., 2007; Nikaido and Takatsuka, 2009). A second component of RND efflux pumps is the outer membrane protein that penetrates into the periplasmic space to form a channel (Koronakis *et al*., 2000; Nikaido, 2000; Wong *et al*., 2001). The third component is a lipoprotein that is linked to the inner membrane, and which plays a role in stabilizing the interactions between the two other elements (Zgurskaya and Nikaido, 1999; Mikolosko *et al*., 2006; Takatsuka and Nikaido, 2009). The best-studied antibiotic-extruding RND pump in structural and functional terms is the AcrAB-TolC system in *Escherichia coli* (Nikaido and Takatsuka, 2009). This pump was initially described as a transporter for the topical antiseptic acriflavin (hence the name Acr), but it was later shown to transport a large variety of other substrates (Nikaido, 1998; Fábrega *et al*., 2009). Another well-characterized RND pump is the CzcABC metal-extruding pump, which is involved in the extrusion of heavy metals such as zinc, cadmium and cobalt (Nies, 1999; 2003).

In this study we have constructed a stringent profile for RND efflux pumps and have used it to search for and identify members of this family within annotated genomes. With the protein sequences that we found we were able to group them, using Z-score values and phylogenetic analysis into four groups, which represents a novel method regarding the application of profiles. Based on gene annotation data, those within groups 1 and 3 appeared to be involved in extrusion of antibiotic and metals respectively. Using a series of *P. putida* KT24440 mutants, we were able to validate that the efflux pumps found in group 1 do in fact extrude a wide range of antibiotic compounds, and that those in group 3 extrude heavy metals. The paucity of information available for proteins in groups 2 and 4 made it necessary to carry out screens, using mutants, in order to assign functional activities to these groups. Our results show that the efflux pumps found in group 2 are involved in the extrusion of oxidative stress-causing agents, while the pumps in group 4 appear to be involved in the extrusion of organic and inorganic chemicals.

Results and discussion

Construction of the RND profile

We have constructed a stringent profile for RND efflux pumps using Provalidator (Molina-Henares *et al*., 2009), a web-based tool that combines nearly full automation, being able to search for and identify family members in databases, as well as validate results. This tool is freely available at http://www.bacTregulators.com. A detailed explanation of the method of profile construction is provided as Appendix S1. Here we describe some key steps in the construction of the RND profile. For its construction of the RND profile, based on literature search, we chose a set of 16 of well-characterized sequences of RND efflux pumps able to extrude antibiotics, solvents and heavy metals (Table S1) to be considered as seed sequences for the profile. Provalidator clustered these sequences using BLASTCLUST without rejecting any of them since they were found to be non-redundant (less than 60% identical). The generation of a profile requires a multi-alignment of the seed sequences as input, which was done by CLUSTALW. The results revealed conserved sequence identity throughout the full length of the proteins (Fig. S1). This is in contrast with most multi-alignments for which high-sequence conservation is only observed between functional domains, e.g. helix–turn–helix motifs in DNA binding proteins or cofactor binding pockets in enzymes (Ramos *et al*., 2005). In using the full length of the RND efflux pump proteins to construct the profile, we are the first to report a profile being generated using the whole protein sequence rather than a region of conserved sequences. Provalidator uses PFMAKE to translate the multi-alignment into a matrix table of positions and converts frequency distributions into positive specific amino acid weights and gap costs according to the algorithms developed by Sibbald and Argos (1990) and by Lüthy and colleagues (1994). Next, Provalidator calibrates the profile using PFSEARCH, which runs the profile against a random database to produce a list of high-scoring profile matches that are sorted by scores (raw scores). Thereafter, Provalidator normalizes the scores (Z-scores) using the Pearson and Lipman (1998) algorithm in order to provide statistical values for comparison. It has been empirically determined that cut-off values of Z-scores equal to or greater than 8.5 are biologically significant and guarantee the correct assignment of a protein to a given family (Gallegos *et al*., 1997).

The generalized profile for RND efflux pumps, generated automatically using the 16 seed sequences (shown in Table S1), was run, by Provalidator, against all entries in the SwissProt and TrEMBL databases (Siezen and Wilson, 2008). Around 2000 proteins ranging in length from 950 to 1200 amino acids were identified as members of the RND efflux pump family (Table S2). The profile

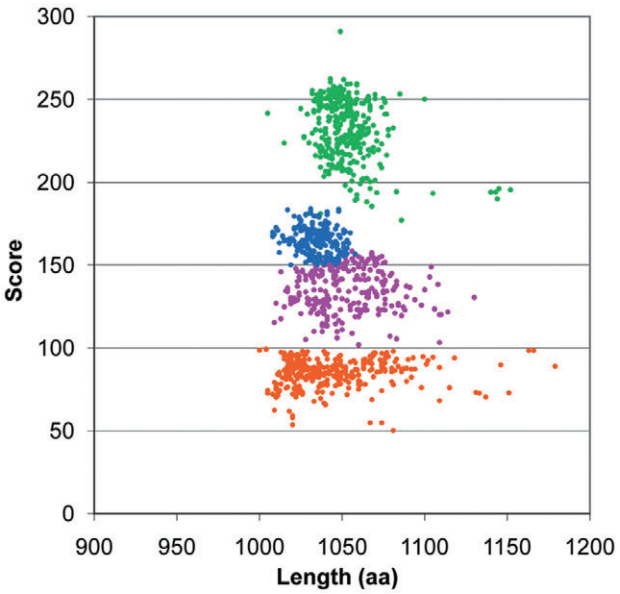

Fig. 1. Grouping of efflux pumps based on Z-score. All proteins identified by the RND efflux pump profile were at least 950 residues long. Based on Z-scores we established four groups of proteins. Each group corresponds to a set of 50-unit intervals.

yielded a Z-score of > 50 for all of these proteins, a value well above the empirically determined value of 8.5 that guarantees biological relevance for family member identification. Z-scores were used to classify the proteins into four well-defined groups based on intervals of about 50 arbitrary units on the Z-score scale (Fig. 1). A phylogenetic tree was also constructed, and the sequences of each Z-score group tended to cluster together (Fig. 2), suggesting that protein function within these groups may be related. When proteins in these clusters were analysed with regard to their function, we found that those in group 1 were antibiotic/drug efflux pumps such as the Mex/Ttg pumps of *Pseudomonas* (Li *et al*., 1995; 1998; Köhler *et al*., 1997; Ramos *et al*., 1998; Rojas *et al*., 2001; Poole, 2004; Jeannot *et al*., 2008; Fernández *et al*., 2009), the Acr pumps of enteric bacteria (Ma *et al*., 1993; Zgurskaya and Nikaido, 1999; Kobayashi *et al*., 2001) and the Cme pump of *Acinetobacter* spp. (Magnet *et al*., 2001; Chau *et al*., 2004). Group 3 included metal resistance efflux pumps such as Czc/Cnr from *Ralstonia* (Stähler *et al*., 2006), and Sil from *Salmonella* (McHugh *et al*., 1975; Gupta *et al*., 1999; Silver, 2003), among others. No known function was found to be previously assigned to members of the proteins in groups 2 and 4.

Wet testing of the efflux pump profile groups

We then hypothesized that it would be possible to validate the Z-score groups of the RND pumps by identifying efflux pumps in environmental microorganisms that exhibited tolerance to drugs. To test this hypothesis we isolated environmental microorganisms tolerant to ethidium bromide (EtBr). We choose EtBr because it has been reported that a number of pathogenic and non-pathogenic microorganisms, such as *Acinetobacter braumannii*, *Chromohalobacter* sp., *Lactococcus lactis* and several species of the genus *Pseudomonas* (Bolhius *et al*., 1994; Li *et al*., 1995; Poole *et al*., 1996; Köhler *et al*., 1997; Magnet *et al*., 2001; Rojas *et al*., 2001; Chau *et al*., 2004; Poole, 2004; Tokunaga *et al*., 2004; Ramos *et al*., 2005), are tolerant to the compound thanks to efflux pumps. Subsequently, we planned to identify (by PCR amplification and DNA sequencing) genes that encoded efflux pumps in these microorganisms related to the phenomenon under scrutiny.

To isolate environmental microorganisms tolerant to EtBr, 20 ml of waste water samples from the city of Granada's wastewater treatment plant was mixed with 80 ml M9 minimal medium (Abril *et al*., 1989) containing glucose (0.5% w/v) as a carbon source and EtBr at a final concentration of 10 mg ml^{-1}, and the samples were used for enrichment, as described in *Experimental procedures*. Based on differences in colony morphology, size and

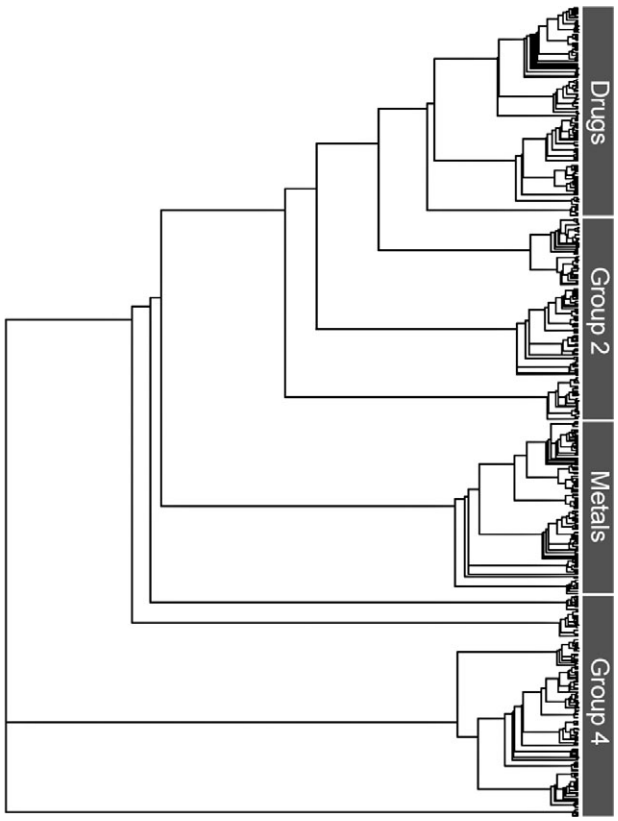

Fig. 2. Phylogenetic tree of efflux pumps and assigned function. The phylogenetic tree was constructed using the TREE program. The function of the characterized pumps in each of the four groups is indicated to the right of the cluster.

colour we retained 11 clones from these enrichments. The clones were taxonomically identified based on Gram staining, the API growth test, metabolic profiles and sequencing of the 16S rRNA gene. Three clones belonged to the genus *Klebsiella*, two to the genus *Kluyvera,* and one of each belonged to the genera *Shewanella*, *Empedobacter* and *Pseudomonas*. Two others exhibited high similarity to uncultured microorganisms and one clone exhibited significant homology to Bacterium G3 Greenlake. The predominance of enteric bacteria among the isolates and their ability to use a wide variety of sugars (Table S3) was not surprising, considering that the microorganisms were isolated from wastewater. We characterized all these strains with regard to EtBr and antibiotic resistance using Minimal Inhibitory Concentration assays (Amsterdam, 1991). The results showed that all EtBr-resistant clones exhibited high resistance to certain antibiotics, but we did not find a regular pattern of antibiotic resistance, except that clones resistant to EtBr were often highly resistant to penicillin-derived antibiotics (Table S4).

We next tested whether these environmental isolates contained genes that encoded efflux pumps. To this end, and based on the alignment in Fig. S1 and previous studies by Meguro and colleagues (2005), we used two oligoprimers to amplify by PCR a region of about 500 nucleotides of the RND efflux pumps within the isolates. As a control for amplification we included *P. putida* DOT-T1E, a strain that is known to be resistant to EtBr due to its extrusion through *ttgABC*-encoded gene products (Terán *et al*., 2003; Bernal *et al*., 2007). As expected, the *ttgB* gene was amplified from the DOT-T1E strain using the above primers. We also found that a related gene was also present in eight of the 11 strains (including two *Kluyvera* strains, the three *Klebsiella* strains, one of the *Pseudomonas* strains, *Bacterium* G3 and one of the strains exhibiting 16S rRNA similar to the EV821 uncultured bacterium). These amplified fragments were cloned, sequenced and clustered within the sequences used to construct the phylogenetic tree in Fig. 2. Six of the sequences clustered with the RND efflux pumps assigned to group 1, which includes extrusion pumps for acriflavine, acridine and other drugs. Two of the new sequences, which belong to fragments amplified from *Pseudomonas* sp., clustered with pumps in Group 4, suggesting that this phylogenetically separate group of RND pumps may also include antibiotic/dyes efflux pumps (Fig. S2). It should be noted that the fact that an RND efflux pump gene was amplified from a tolerant microorganisms is not enough evidence to suggest that the gene is responsible for the resistant trait observed and therefore isolation of mutants is necessary. To gain further insights we decided to use microbes whose entire genome was available, as described below.

Identification of extrusion pumps in the genome of P. putida *KT2440 and characterization of mutants using a phenomics platform*

Using the RND family profile that we constructed, we screened the genome of *P. putida* KT2440 (Nelson *et al*., 2002) and identified 14 RND efflux pumps corresponding to genes PP0043, PP0906, PP1385, PP1517, PP2065, PP2410, PP2818, PP3302, PP3426, PP3456, PP3583, PP3584, PP5173 and PP5387 (Table S5). Three of these efflux pumps were grouped as being potentially involved in metal extrusion in group 3 (PP0043, PP2410 and PP5387); four of these efflux pumps were placed within group 1 (PP1385, PP3456, PP2818 and PP3426), while another four pumps fell within group 2 (PP2065, PP0906, PP3584, PP3583). The three remaining pumps were clustered within group 4 (PP1517, PP5173, PP3302) (see Fig. S3). Mutants for two of the pumps in group 1 [TtgABC (Siezen and Wilson, 2008) and MexEF/OprN (Roca *et al*., 2008)] have been isolated before and partially characterized. As such, it is known that the TtgABC pump (PP1384-PP1386) is an antibiotic extrusion pump, and that it is able to extrude ampicillin, chloramphenicol, tetracycline and flavonoids (Duque *et al*., 2001; Terán *et al*., 2003; Roca *et al*., 2008). The MexEF/OprN (PP3425-PP3427) has been shown to be involved in formaldehyde detoxification and in phenylalanine homeostasis (Roca *et al*., 2008; Herrera *et al*., 2010). No information was available for the pumps annotated as potential 'Metal Resistance pumps,' nor was information available for the other pumps. In order to characterize the function of these putative efflux pumps, we used a number of mini-Tn*5*, including mutants for four pumps within group 2 (PP2065, PP0906, PP3583 and PP3584), two mutants in pumps within group 3 (PP0043 and PP5387) and two pumps within group 4 (PP1517 and PP5173). This set of mutants was subjected to a systematic phenotype screen that allowed us to test for growth under 190 different conditions (see *Experimental procedures*). The platform allows for growth under different carbon, nitrogen, sulfur and phosphate sources to be tested, as well as growth in the presence of 47 different stressors. The results showed that none of the mutants exhibited significant differences with respect to growth under different carbon, nitrogen, sulfur or phosphate sources, as expected. However, significant differences in growth curves were observed for some of the mutants when compared with the parental strain in the presence of certain stressors (Fig. 3). The mutants were found to exhibit two different growth patterns. One of these growth patterns corresponded to a deep inhibition of growth so that turbidity of the cultures did not increase with time. This indicated to us that the knocked-out efflux pump was an essential element for tolerance to the stressor. This is clearly exemplified in Fig. 3A, which shows

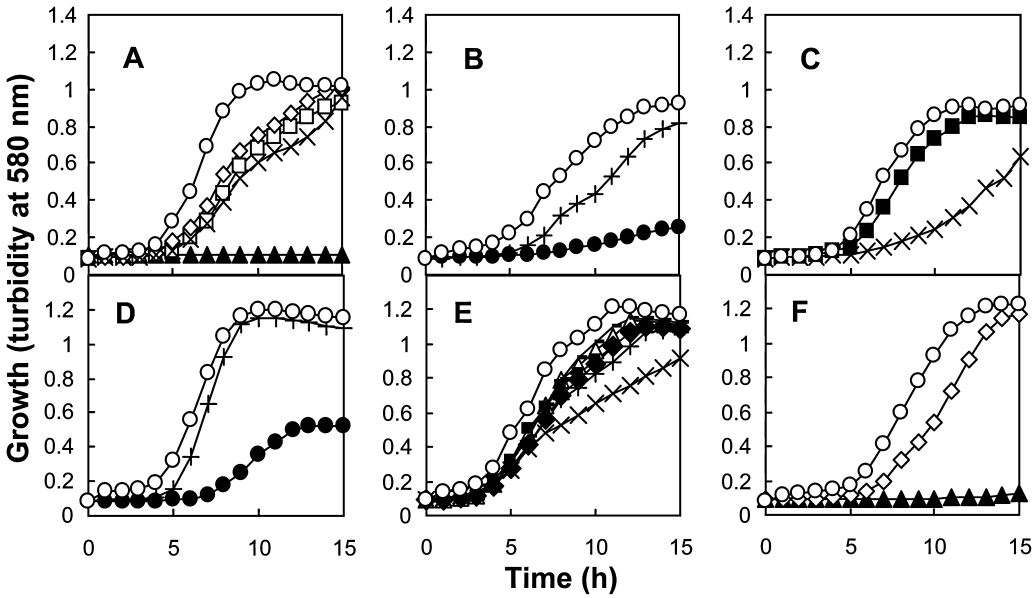

Fig. 3. Growth characteristics of a set of knock-out mutants in efflux pumps in *Pseudomonas putida*. Wild-type *Pseudomonas putida* and mutants strains were grown overnight in M9 minimal medium with glucose as carbon source. Cultures were diluted 100-fold in the same medium and 180 μl of resulting culture was placed in 100-well polystyrene plates and incubated at 30°C in a Bioscreen C incubator that agitated the plates while measuring growth every 60 min, in the presence of either 20 μg ml^{-1} of tetracycline (A), 0.25 mM Cd^{2+} (B), 0.25 M Rb^{+} (C), 0.5 mM Zn^{2+} (D), 1 mM $Cr_2O^{2-}{}_7$ (E) or 30 μg ml^{-1} chloramphenicol (F). Symbols: ○, wild-type; ●, mutant PP0043; △, mutant PP0906; ▲, mutant PP1385; □, mutant PP1517; ■, mutant PP2065; ◆, mutant PP3583; —, mutant PP3584; ×, mutant PP5173; +, mutant PP5387; ◇, mutant PP3426.

that novobiocin strongly inhibits growth of the *ttgB* mutant. The second observed growth pattern was different in that growth was not fully inhibited, but rather that it was significantly decreased. This indicated to us that more than one efflux pump was required in order to extrude the chemical. Table 1 summarizes the results organized according to placement of the pumps within the four groups identified by the RND profile. As expected efflux pumps in group 1 were found to be involved in the extrusion of antibiotics. TtgB was the most critical efflux pump for antibiotic resistance as its deficiency led to growth inhibition in the presence of tetracycline, novobiocin, gentamicin, ampicillin, chloramphenicol, cefotaxime, erythromicin and the detergent deoxycholate. These results raise the profile of the TtgB efflux pump, which has previously only been shown to be able to extrude EtBr, ampicillin, chloramphenicol and a number of flavonoids. We also found that MexEF/OprN plays a role in extrusion of tetracycline and chloramphenicol, as shown by delayed growth of the corresponding mutant in the presence of these compounds (Fig. 3F). Since deficiency in TtgABC fully prevents growth (Fig. 3F), we propose that the MexEF OprN efflux pump is of less relevance to the removal of these antibiotics. In group 3 the tested mutants were found to exhibit inhibited growth in the presence of certain metals. As such, mutants deficient in PP0043 showed increased sensitivity to Cd^{2+} and Zn^{2+} (see Fig. 3D), while the mutant deficient in PP5387 was more sensitive to Cd^{2+}, Ni^{2+} and chromate (Table 1). These results show that metal efflux pumps within *P. putida* KT2440 have overlapping specificity regarding removal of Cd^{2+}, but that they also show specificity regarding other metals, such as Ni^{2+} and Zn^{2+} (Table 1 and Fig. 3D). The presence of multiple efflux pumps with similar specificity is advantageous to cells in that, although some redundancy exists, they are provided with a wider total spread of chemicals that can be extruded.

Table 1. Potential substrates for efflux pumps of *Pseudomonas putida* KT2440 as deduced from growth inhibition of mutants in the presence of the indicated drugs.

Number	Group	Proposed name	Substrates
PP1385	1	TtgB	Tc, Nov, Gm, Sm, Ap, Cm, Ctx, Ery, DOC
PP3426	1	MexF	Tc, Cm
PP0906	2	OapB	Bip, Mv
PP2065	2	OapE	Bip, Mv
PP3584	2	OapH, MtdC	Bip, Mv
PP0043	3	CZPB	Cd^{2+}, Zn^{2+}, $Cr_2O_7^{2-}$
PP5387	3	CNCB	Cd^{2+}, Ni^{2+}, $Cr_2O_7^{2-}$, MV
PP1517	4	MEXK	Tc
PP5173	4	TRCB	Tc, RB^{+}, $Cr_2O_7^{2-}$

Tc, Nov, Gm, Sm, Ap, Cm, Ctx, Ery, Bip, DOC, Mv stand for resistance to tetracycline, novobiocin, gentamicin, streptomycin, ampicillin, chloramphenicol, cefotaxime, bipyridyl, deoxycholate and methyl viologen respectively.

The four mutants within group 2 have a common phenotypic background, and appear to be preferentially involved in the extrusion of organic compounds that generate oxidative stress. In fact, all four mutants exhibited retarded growth in the presence of bipyridyl and methyl viologen (Table 1). Additionally, the mutant lacking PP3583 may also be involved in the extrusion of Ni^{2+} and chromate (Table 1 and Fig. 3E). Mutants in the pumps within group 4 exhibited certain sensitivity to tetraycycline, and the mutant deficient in PP5173 showed striking inhibition of growth in the presence of rubidium and chromate (Table 1 and Fig. 3C and E). This group, then, may include a number of efflux pumps with broader substrate specificity and therefore capable of removing heavy metals and antibiotics.

Our results support that, based on a limited number of sequences derived from a literature search, it is possible to construct a profile for RND efflux pumps, which not only serves to identify members of the family in databases but that can also be used to group them into functional subfamilies based on Z-score values. Most importantly, the subfamilies exhibit differential specificity with regards to substrate specificity. As a proof of concept we isolated environmental microbes tolerant to EtBr and found that, in many of these microorganisms, it was possible to amplify genes that encode pumps that are specifically assigned to the expected Z-score groups, as confirmed with *in vivo* and *in vitro* assays against EtBr. The profile also allowed us to identify new efflux pumps within *P. putida* KT2440. These results were combined with *in vivo* experiments using mutants in these genes, which consisted of stringent phenomics screens that allowed us to assign functions and to reveal the metabolic context within which these previously uncharacterized efflux pumps operate. These results provide new foundations for further studies in the area of substrate specificity of RND efflux pumps in environmental isolates and metagenomes.

Experimental procedures

Seed sequences for profile construction

The 16 sequences that were used to create the profile were chosen based on literature searches of well-characterized RND efflux pumps (Table S1). First sequences were clustered using BLASTCLUST, which established that they were non-redundant (identity below 60%) and all sequences were subsequently aligned using CLUSTALW (http://align.genome.jp), revealing that there exists a high degree of sequence conservation along the whole sequence of the proteins (Fig. S1). The final multi-alignment was then used as input for the construction of a conventional profile using the PFMAKE program, which is part of the PFTOOLS package of programs, available from the Swiss Institute of Bioinformatics (see Appendix S1 for calculation of weight matrix and profile generation). The RND profile was subsequently calibrated by Provalidator by running it against shuffled sequences in the database (http://www.isrec.isb-sib.ch/pub/databases/shuffled/). This provided raw score values that were normalized (Z scores) by Provalidator using the Lipman and Pearson algorithm as described (Hulo *et al.*, 2006). The constructed RND profile then was used to search for members of this family in the UNIPROT database. PFTOOLS proposes an empirically tentative threshold score of 8.5 for a protein to be considered a member of the family of interest. A set of almost 2000 members of the RND family was identified (Table S2).

Bacterial strains and growth conditions

Pseudomonas putida KT2440 (Nelson *et al.*, 2002) and its derived Tn*5*-Km mutant strains were obtained from the *Pseudomonas* Reference Culture Collection established at Estación Experimental del Zaidín in Granada (Spain) (Duque *et al.*, 2001). Strains were grown at 30°C with shaking at 200 r.p.m. in an orbital platform in Luria–Bertani (LB) medium supplemented with rifampicin (30 μg ml^{-1}) or kanamycin (50 μg ml^{-1}) respectively. When required, M9 minimal medium (Abril *et al.*, 1989) was used and supplemented with the appropriate carbon source.

Isolation of ethidium bromide-resistant microorganisms

Twenty millilitre of waste water samples from the wastewater treatment plant of city of Granada was mixed with 80 ml M9 minimal medium (Silver, 2003), with glucose (0.5% w/v) as a carbon source, and EtBr was added at a final concentration of 10 mg ml^{-1}. Flasks were shaken (200 r.p.m.) at 30°C for 24 h, at which point the samples were diluted 50-fold in the same medium. After incubating 24 h more, serial dilutions were spread on solid M9 minimal medium with glucose (0.5% w/v) as the C-source and 10 mg ml^{-1} EtBr as a counter selective agent. The clones were retained based on colony morphology, size and colour. To identify taxonomically these clones, we sequenced the whole length 16S rRNA gene and used the API strip test system. Ethidium bromide-resistant strains were grown according to routine procedures in LB medium. When required, M9 minimal medium (Abril *et al.*, 1989) was used and supplemented with different carbon sources at 10 mM.

DNA techniques

Preparation of chromosomal DNA was carried out using standard methods (Zhang and Mah, 2008). To amplify HAE-1 RND efflux pumps, we used the A24f2 (5′-CCSRTITTY GCITGGGT-3′) and A577r2 (5′-SAICCARAIRCGCATSGC-3′) primers, as described in Meguro and colleagues (2005) to PCR-amplify a 500 bp fragment. The PCR reaction procedure was as follows: an initial step at 94°C for 10′ min., was followed by 30 cycles at 94°C for 1′ min., then 50°C for 1′ min., and a final step at 72°C for 1′ min. was run. Amplification products were visualized on a 1.5% (w/v) agarose gel stained with ethidium bromide, and the corresponding band was extracted with the QUIAEX II Gel Extraction kit (Quiagen). The PCR products were sequenced using an ABI-PRISM Dye Terminator Cycle Sequencing Ready Reaction kit

with Amplitaq DNA polymerase in an automatic DNA sequencer (model ABI-PRISM 3100; Applied Biosystems, USA).

Phenotypic characterization

Pseudomonas putida strains were grown on solid M9 minimal medium (Ausubel *et al.*, 1991; Li *et al.*, 1995) supplemented with 0.1% (w/v) glucose. The following day cells were recovered with a loop and resuspended in M8 minimal medium (Ausubel *et al.*, 1991). The wild-type and the mutant strains were inoculated in micro-well plates in M9 minimal medium with different carbon (5 mM), nitrogen (5 mM) and sulfur (5 mM) sources, and grown at 30°C with continuous shaking while at 60 min intervals the turbidity was measured by a Bioscreen C (ThermoFisher Scientific) at 420–580 nm for 24 h (Daniels *et al.*, 2010). Minimal M9 medium was prepared as in Abril and colleagues (1989), but when appropriate $MgSO_4$ or NH_4Cl was replaced with other sulfur or nitrogen sources. All data recordings were performed using a Bioscreen C MBR analyser type system FP-1100-C. At least three independent experiments were performed for each condition. Growth curves were drawn using the average values of the three experiments (standard deviations were always less than 0.1 units of the OD value).

Carbon sources: D-glucose, D-fructose, D-glucuronic acid, glycerol, sodium acetate, trisodium citrate, fumaric acid, sodium succinate, sodium lactate, malic acid, sodium pyruvate, methyl pyruvate, propionic acid, sodium benzoate, sodium 4-hydroxybenzoate, quinic acid, aminobutyric acid, 5-amino-*n*-valeric acid, 2,4-dihydroxyphenylacetic acid, sodium decanoate, Tween 20, 2-phenylethanolamine, L-Leu, L-Lys, L-His, L-Gln, L-Glu, L-Phe, L-Arg, L-Asn, L-Ala, L-Pro, L-Tyr, L-Ile, L-Cys, L-Met, L-Val, glutaric acid, and xylose. *Nitrogen sources*: D- and L-Arg, D- and L-Lys, D- and L-Pro, D- and L-Val, D- and L-Ala, D- and L-Asn, D-Met, D-Leu, L-Asp, L-Cys, L-Phe, L-Glu, L-Gln, L-Gly, L-His, L-homoserine, L-Ile, L-*trans* hydroxyproline, L-Ser, L-Tyr, Ala-Glu, Ala-Gly, Ala-His, Ala-Leu, Ala-Phe, Gly-Gln, Gly-Gly, Gly-Leu, Gly-Ser, Gly-Val, Tyr-Ala, adenine, agmatine sulfate, hypoxanthine, phenylethanolamine, ethanolamine, putrescine, D,L-ornithine, NH_4Cl and NH_4NO_3, and urea. *Sulfur sources:* L-Cys, D- and L-Met, L-cystine, cysteamine, D,L-ethionine, D,L-homocysteine, taurine, thiourea, 2-thiouracil, *N*-acetyl-cysteamine, 2-thiohidanthoin, sodium taurocholate, agmatine sulfate, Na_2SO_4, Na_2SO_3.

Toxic compound resistance assays

Individual colonies of *P. putida* KT2440 and mutant strains were picked from freshly cultured LB plates, and streaked onto LB medium plates supplemented with the suitable antibiotic and grown overnight at 30°C. The biomass of this overnight plate was recovered from the plate surface and resuspended in 15 ml of LB liquid medium to an OD_{660} of 0.1. Micro-well plate wells were filled with 180 μl of the above cell suspension and 20 μl of the different 10× concentrated solutions of stressors. Micro-well plates were incubated and data recordings were processed using the Bioscreen C MBR analyser type system as described above. *Toxic compounds:* $AgNO_3$ (3 μM), $CdCl_2$ (0.156 mM), $CoCl_2$ (0.156 mM), $CuSO_4$ (1 mM), $HgCl_2$ (2.5 μg ml^{-1}), LiCl (0.25 M), $MnSO_4$ (1 mM), $NiCl_2$ (1 mM), RbCl (0.25 M), K_2TeO_3 (0.9 μg ml^{-1}), $ZnCl_2$ (0.5 mM), non-detergent sulfobetaine (NDSB-201) (1%), cetyl trimethylammonium bromide (CTAB) (0.002%), *N*-lauryl sarcosine (0.3%), SDS (0.06%), deoxycholate (DOC) (1%), Triton X-100 (1%), ethylenediaminetetraacetic acid (EDTA) (0.125 mM), 2,2′-Bipyridine (1 mM), NaCl (0.5 M), $K_2Cr_2O_7$ (12.5 μg ml^{-1}), H_2O_2 (0.004%), NH_2OH (5 y 2.5 mM), Methyl viologen (100 μM), Tert-butyl hydroperoxide (0.00078%), ethidium bromide (0.1 mg ml^{-1}), KSCN (100 mM), KCN (0.325 mg ml^{-1}), K_2HAsO_4 (0.9 mg ml^{-1}), NaBr (0.25 M), ampicillin (20 μg ml^{-1}), carbenicillin (160 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), cefotaxime (0.375 μg ml^{-1}), erythromycin (30 μg ml^{-1}), gentamicin (4 μg ml^{-1}), kanamycin (0.195 μg ml^{-1}), nalidixic acid (0.0125 mg ml^{-1}), neomycin (1 μg ml^{-1}), norfloxacin (0.05 μg ml^{-1}), novobiocin (20 μg ml^{-1}), piperacillin (10 μg ml^{-1}), streptomycin (2 μg ml^{-1}), spectomycin (0.1 mg ml^{-1}), tellurite (0.5 μg ml^{-1}) and tetracycline (1 μg ml^{-1}).

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Supporting information

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

Fig. S1. Multi-alignment of the 16 RND efflux pumps initially chosen for the construction of an RND efflux pump profile. Sequences were aligned using the ALIGN programme.
Fig. S2. Location of RND efflux pumps amplified from EtBr-resistant clones within the general phylogenetic tree of efflux pumps. The pumps identified were included in the phylogenetic tree according to their sequence identity. They are named on the basis of the microorganism from which they

were amplified. TtgB and TtgH are RND efflux pumps of the *P. putida* DOT-T1E.

Fig. S3. Location of RND efflux pumps present in the genome of *P. putida* KT2440 within the general tree of efflux pumps. Pumps are named based on the PP number.

Table S1. RND Efflux pumps chosen for construction of the seed sequence. All of the chosen pumps had been characterized through *in vivo* or *in vitro* assays.

Table S2. RND efflux pumps identified in databanks with the corresponding identification number, score and length. Data obtained on 14 July 2009.

Table S3. Isolated ethidium bromide-resistant bacteria strains.

Table S4. Growth of the isolated EtBr-resistant strains in the presence of different carbon sources.

Table S5. Susceptibility of EtBr-resistant clones and *P. putida* DOT-T1E strains to different antimicrobial agents.

Table S6. *Pseudomonas putida* KT2440 RND efflux pumps identified in the genome.

Appendix S1. Methodology for the development of profile entries.

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