REVIEW ARTICLE

Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems

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Abstract

Multidrug efflux pumps have emerged as relevant elements in the intrinsic and acquired antibiotic resistance of bacterial pathogens. In contrast with other antibiotic resistance genes that have been obtained by virulent bacteria through horizontal gene transfer, genes coding for multidrug efflux pumps are present in the chromosomes of all living organisms. In addition, these genes are highly conserved (all members of the same species contain the same efflux pumps) and their expression is tightly regulated. Together, these characteristics suggest that the main function of these systems is not resisting the antibiotics used in therapy and that they should have other roles relevant to the behavior of bacteria in their natural ecosystems. Among the potential roles, it has been demonstrated that efflux pumps are important for processes of detoxification of intracellular metabolites, bacterial virulence in both animal and plant hosts, cell homeostasis and intercellular signal trafficking.

Introduction

Efflux as a mechanism for antibiotic resistance was first described in 1980 (McMurry *et al.*, 1980). This seminal work demonstrated the presence of plasmid-encoded tetracycline efflux pumps in *Escherichia coli*. It was assumed that these antibiotic resistance determinants were likely acquired through horizontal gene transfer (HGT) from tetracycline producers, thus resembling classical antibiotic resistance genes (Benveniste & Davies, 1973; Davies, 1994, 1997). However, it soon became evident that antibiotic-efflux elements were neither exclusively plasmid encoded nor specific for a given antibiotic (George & Levy, 1983a, b). Genes encoding for these elements were found in the chromosome of other prokaryotes and also in the chromosomes of archeae and eukaryotes (Saier *et al.*, 1998; Paulsen *et al.*, 2001; Ninio & Schuldiner, 2003; Gbelska *et al.*, 2006; Stavrovskaya & Stromskaya, 2008). For instance, a relevant role in the resistance of tumour cells to chemotherapy was established for the P-glycoprotein previously shown to be involved in colchicine resistance (Juliano & Ling, 1976; Kartner *et al.*, 1983). Over the years, the contribution of antibiotic-efflux elements towards the antibiotic resistance exhibited by relevant bacterial pathogens has been highlighted in several studies (Li & Nikaido, 2004; Piddock, 2006b; Poole, 2007). Nevertheless, the main physiological function of multidrug resistance (MDR) pumps in natural bacterial populations is less well understood (Neyfakh, 1997).

MDR pumps differ from classical antibiotic resistance determinants in many aspects. As mentioned above, the general assumption has been that antibiotic resistance genes originate in antibiotic-producing organisms (Benveniste & Davies, 1973; Pang *et al.*, 1994) and that they spread to bacteria through HGT as a result of the selective pressure exerted by the intensive use of antibiotics against pathogens (Pang *et al.*, 1994; Davies, 1997; D'Acosta *et al.*, 2006; Wright, 2007). In contrast, we now know that the presence of MDR pumps is not restricted to antibiotic producers. In the case of bacteria, most of the genes coding for MDR pumps analyzed so far are found within the chromosome and exhibit a conserved structure and arrangement (Fig. 1) as well as a tightly regulated expression (Grkovic *et al.*,

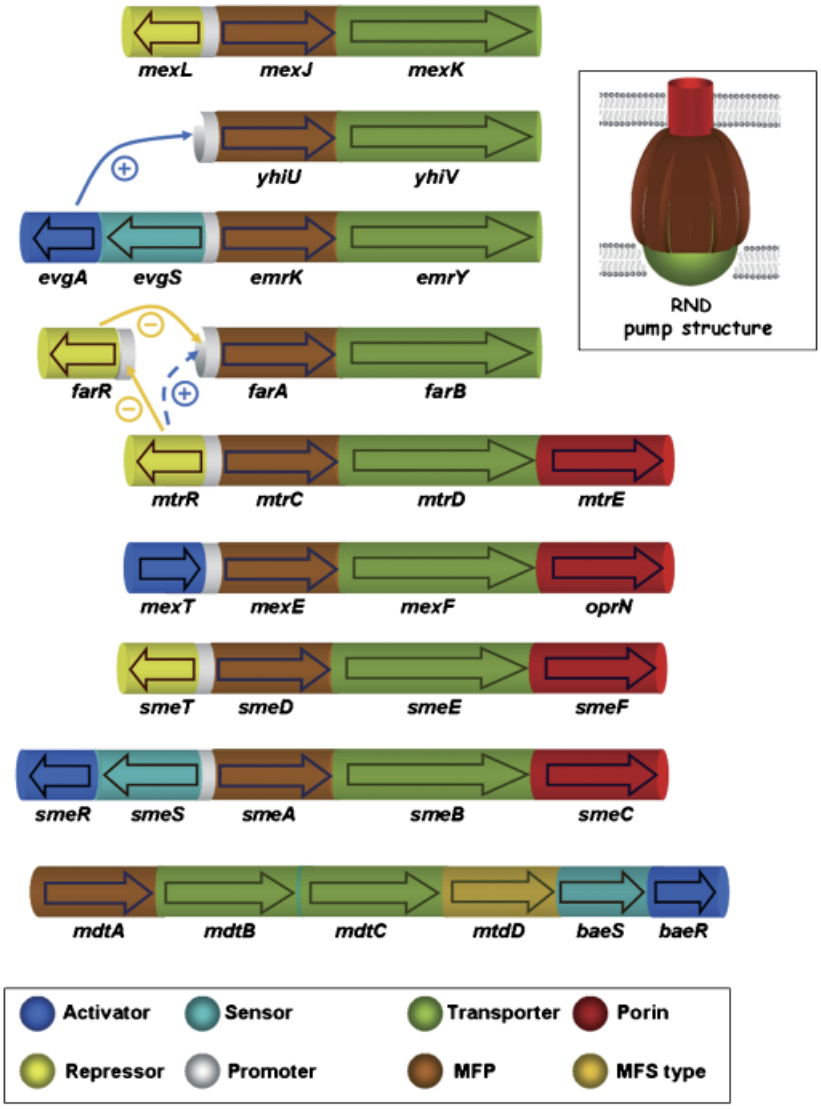

Fig. 1. RND efflux pump gene structure and regulation. The genes encoding RND tripartite efflux pumps are usually forming operons that follow a transcriptional order of membrane fusion protein (MFP) gene, resistance-nodulation-cell division (RND) gene and outer-membrane protein (OMP) gene (smeDEF in *Stenotrophomonas maltophilia*) (Alonso & Martinez, 2000). In some cases, the operon lacks the gene coding for the porin, and the pump is completed by assemblage to an OMP that forms part of another MDR operon (MexJK-OprM in *Pseudomonas aeruginosa*) (Chuanchuen *et al*., 2002) or is encoded by an independent gene (EmrKY-TolC in *Escherichia coli*) (Nishino & Yamaguchi, 2002). Expression of the MDR operons is tightly regulated by nearby transcriptional repressors (SmeT in *S. maltophilia*) (Sanchez *et al*., 2002a), activators (MexT in *P. aeruginosa*) (Kohler *et al*., 1999) or two-component systems (EvgAS in *E. coli*) (Eguchi *et al*., 2003). Some of these local regulators can modify the expression of faraway operons in a direct (EvgA on *yhiU* in *E. coli*) (Nishino & Yamaguchi, 2002) or an indirect way (MtrR on the efflux pump *farAB* through FarR in *Neisseria gonorrhoeae*) (Lee *et al*., 2003a).

2002). In addition, all the strains of a given species carry the same conserved genes coding for MDR pumps in their chromosome (Alonso *et al*., 1999). The incidence of bacteria carrying MDR pumps is not limited to environments with a high antibiotic load. In fact, the organisms with the largest number of MDR pumps are found in the soil or in association with plants (Konstantinidis & Tiedje, 2004). Even more importantly, some of the most intrinsically resistant MDR-containing bacteria with clinical relevance have an environmental origin where the antibiotic pressure is unlikely to be as high as that of clinical settings (Alonso *et al*., 2001; Martinez & Baquero, 2002). These characteristics suggest that chromosomally encoded MDR pumps are not antibiotic resistance genes recently acquired by bacterial pathogens in response to antimicrobial chemotherapy. In contrast, they appear to be evolutionarily ancient elements, highly relevant for the physiology and ecological behavior of all living beings, including bacteria. The fact that bacterial MDR determinants are functional upon expression in mammalian cells (van Veen *et al*., 1998) suggests an important conservation of the function of these elements along the evolutionary tree (Neyfakh *et al*., 1991).

Different studies have demonstrated that MDR pumps are capable of extruding not only antibiotics but also antiseptics (Chuanchuen *et al*., 2001; Sanchez *et al*., 2005; Pumbwe *et al*., 2007), heavy metals (Silver & Phung, 1996, 2005), solvents (Ramos *et al*., 2002) and detergents (Zgurskaya & Nikaido, 2000), among other toxic molecules (Fig. 2). Therefore, when considering their putative physiological role in bacteria, it is tempting to conclude that they mainly function as detoxification elements. In spite of their contribution to the efflux of toxic molecules, a closer look at the

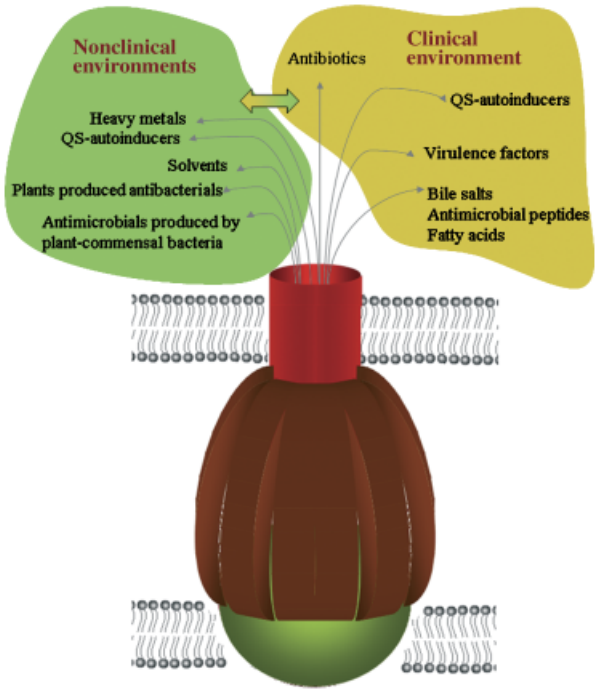

Fig. 2. Functional role of MDR pumps in clinical and nonclinical environments. MDR pumps are involved in resistance to antimicrobial compounds present on mucosal surfaces (Lacroix *et al.*, 1996; Lee & Shafer, 1999; Jerse *et al.*, 2003). This resistance allows bacteria to grow on these surfaces and can thus be considered as a colonization factor. Besides this, MDR pumps might efflux virulence factors (Hirakata *et al.*, 2002) and are involved in the QS-regulated expression of virulence determinants (Pearson *et al.*, 2000; Kohler *et al.*, 2001; Aendekerk *et al.*, 2005). All these roles, together with antibiotic resistance, which is a key element in the treated patient (Martinez & Baquero, 2002), are relevant for the survival, colonization and pathogenic outcome of virulent bacteria in clinical environments. In the case of nonclinical environments, MDR pumps may be involved in resistance to heavy metals (Silver & Phung, 1996) and organic solvents (Ramos *et al.*, 2002) and in the colonization of plants, because they have a main role in resistance to antimicrobials produced by the plants (Burse *et al.*, 2004a) and their commensal bacteria (Burse *et al.*, 2004b). The fact that efflux pumps, which have been selected to cover a relevant function in the environment, may also have a very important role in clinical environments is highlighted with a double arrow. The scheme in the figure represents an RND pump from Gram-negative bacteria.

number and substrate specificity of chromosomally encoded MDR pumps suggests that they are not just committed to detoxification. Genome sequence analyses reveal that, on average, efflux pumps constitute at least 10% of the transporters in several bacterial species, and they usually have the capability of extruding a broad range of structurally different compounds (Paulsen, 2003). As reviewed by Poole (2007), several toxic molecules serve as substrates for more than one efflux pump in the same organism; therefore, if detoxification were the sole function of efflux pumps, there would be no need to carry a large number of MDR pump paralogs in the chromosome. The fact that the synthetic antimicrobials quinolones are a favorite substrate of bacterial MDR pumps (Kohler *et al.*, 1997a; Hooper, 1999; Piddock, 1999; Poole, 2000a, b), together with the finding that environmental strains of *Pseudomonas aeruginosa* isolated before the discovery of quinolones are capable of extruding these drugs (Alonso *et al.*, 1999), further supports the notion that, at least in some cases, antibiotic resistance is not the primary function of MDR pumps.

The number of MDR pumps is proportional to the genome size of a given organism (Ren & Paulsen, 2005). In turn, the genome size of bacteria is dependent on their ecological behavior (Mira *et al.*, 2001). As a result, free-living organisms tend to have larger genomes carrying all the genes required for colonization of different environments including a vast array of transcription factors that enhance adaptability, whereas intracellular bacteria such as endosymbionts as well as some pathogens display a reductive evolution process of their genome (Sokurenko *et al.*, 1999; Cases *et al.*, 2003). The larger repertoire of MDR pumps among free-living organisms suggests a wide variety of physiological functions beyond detoxification. Just as in the case of transcription factors, efflux pumps are likely to contribute to adaptation to different environments by responding to assorted signals.

Several efflux transporters have been described so far, some of them involved in resistance to antibiotics. There are studies indicating that MDR pumps typically involved in the resistance to antibiotics can be important elements in different aspects of microbial physiology. In this article, we will review the role that these elements may play in the ecological behavior of bacterial populations not only during infection but also in nonclinical, natural environments.

Common characteristics of MDR pumps

Each bacterial MDR system described to date belongs to one of the following five families (Fig. 3): ATP-binding cassette (ABC) (Lubelski *et al.*, 2007), major facilitator superfamily (MFS) (Law *et al.*, 2008), resistance/nodulation/cell division (RND) (Tseng *et al.*, 1999), small multidrug resistance (SMR) (Chung & Saier, 2001) or multidrug and toxic-compound extrusion (MATE) (Moriyama *et al.*, 2008). In terms of energy source, ABC transporters are dependent on ATP hydrolysis; MFS, RND and SMR are proton-driven efflux pumps and MATE transporters consist of a Na^+/H^+ drug antiporter system (Piddock, 2006b). Although ABC transporters are relevant in anaerobes and Gram-positive bacteria, the best studied prokaryotic MDR efflux pumps belong to MFS and RND families (Poole, 2007). In the case of Gram-negative bacteria, these transporters consist of cytoplasmic, periplasmic and outer-membrane proteins that associate to form multicomponent efflux systems (Piddock,

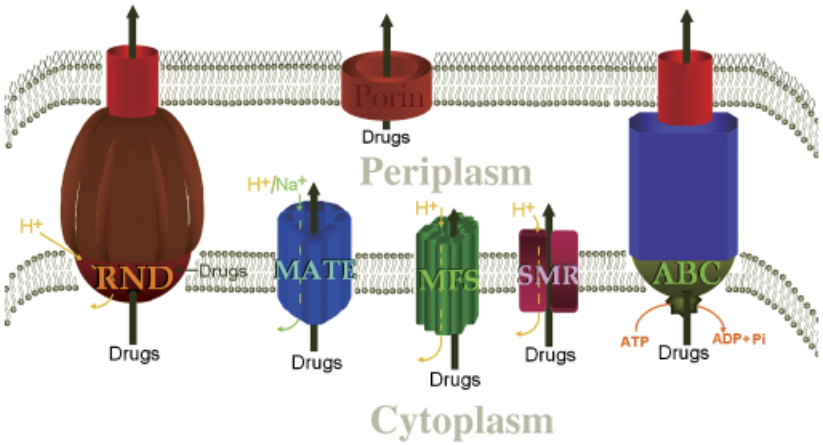

Fig. 3. Schematic representation of the five MDR families assembled on a Gram-negative bacterial membrane. MDR pumps of Gram-positive bacteria have only one component, whereas efflux pumps expressed by Gram-negative bacteria usually have several components. The figure shows these multicomponent structures for the ABC and RND families. The transporter component is connected to an MFP and an OMP that allow pumping of the drugs directly outside the bacteria. The drug efflux can be driven either by a gradient of protons (RND, SMR, MATE and MFS) or sodium ions (MATE) or by energy from ATP hydrolysis (ABC). Note that the RND family can extrude drugs from the cytoplasm or from the inner membrane. In the RND family, the tripartite pump involves a trimeric RND transporter protein [AcrB (Murakami *et al.*, 2006)], a tridecamer of the MFP [MexA (Akama *et al.*, 2004)] and a trimer of OMP [TolC (Koronakis *et al.*, 2000; Murakami *et al.*, 2006)]. In the MFS [EmrD (Yin *et al.*, 2006)] and MATE [NorM (Singh *et al.*, 2006)] families, the drugs are extruded by passing through a 12 helices monomer [although some members with 14 helices have been described (Paulsen *et al.*, 1996)]. In the SMR [EmrE (Ubarretxena-Belandia *et al.*, 2003)] family, the functional unit comprises a homodimer, four helices per monomer of oppositely oriented subunits. In the ABC (Dawson & Locher, 2006) family, a homodimer of the transporter protein delimits two regions: a cytoplasmic nucleotide-binding domain and a transmembrane domain. Some examples of MDR efflux pumps described in the review are as follows: RND family: AcrAB-TolC, MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexGHI-OpmD, SmeDEF, MtrCDE, CzcCBA, TtgABC, TtgGHI, IfeABR, BpeAB-OprB, BmeB or CmeABC. MATE family: NorM and DinF. MFS family: EmrAB-TolC, RmrAB, EbrB, EbrC, RosAB, QepA, NorB, NorC, NorA, Blt or FarAB. SMR family: EbrA. ABC family: YbiT and Msr. For an exhaustive classification of transporters, see http://www.tcdb.org/index.php and http://www.membranetransport.org.

2006b). AcrAB-TolC in *E. coli* (Ma *et al.*, 1993), MexAB-OprM in *P. aeruginosa* (Gotoh *et al.*, 1995) or SmeDEF in *Stenotrophomonas maltophilia* (Alonso & Martinez, 2000) are three examples of tripartite efflux pumps of the RND family involved in resistance to antibiotics.

The phylogenetic profiling of membrane transport systems from 141 organisms with published genomes has shown that the type of transporters and their substrates display a strong correlation with the physiology of the corresponding organism (Ren & Paulsen, 2005). Although in some species, chromosomally encoded transporters might be variable, likely depending on the bacterial ecotype, on several occasions, bacterial species from the same genus share most transporters (Ren & Paulsen, 2005). This is a feature characteristic of genes belonging to the core bacterial genome (Morales *et al.*, 2004). Furthermore, some works indicate that the protein sequences along with the promoter regions of MDR efflux pumps are highly conserved among strains belonging to the same species (Gould *et al.*, 2004; Sanchez *et al.*, 2004). All this suggests that (1) MDR pumps are evolutionarily ancient elements; (2) they have been selected as a function of the bacterial physiology and ecological behavior and hence should have physiologically relevant roles; (3) because their structures are highly conserved in all strains of a bacterial species, they are not dispensable elements; and (4) given the strong conservation of the promoter regions, their expression is probably tightly regulated and integrated in bacterial transcriptional networks.

As mentioned in the Introduction, one characteristic feature of MDR pumps is their ability to efflux a broad range of structurally different compounds (Fig. 2). Constitutive and/or uncontrolled expression of these elements might therefore impose a metabolic burden on bacteria. Experimental data showing that overproduction of MDR pumps may impair bacterial physiology support this notion (Sanchez *et al.*, 2002c; Ruiz-Diez *et al.*, 2003; Alonso *et al.*, 2004; Linares *et al.*, 2005). Furthermore, the level of expression of these pumps can significantly impact bacterial *in vivo* fitness in experimental infection models (Cosson *et al.*, 2002; Hirakata *et al.*, 2002; Warner *et al.*, 2007, 2008). As expected, expression of the MDR pumps is usually regulated by a complex network, including specific regulators such as AcrR in *E. coli* (Ma *et al.*, 1996), MexR in *P. aeruginosa* (Poole *et al.*, 1996; Sanchez *et al.*, 2002b) or SmeT in *S. maltophilia* (Sanchez *et al.*, 2002a), and global regulators such as MarA, SoxS and Rob in *E. coli* and other *Enterobacteriaceae* (Martin *et al.*, 2008), or MgrA in *Staphylococcus*

aureus (Truong-Bolduc *et al.*, 2005). More recently, it has been described that the expression of MDR systems can be further modulated by antirepressor elements that bind the specific regulatory proteins (Daigle *et al.*, 2007; Wilke *et al.*, 2008).

In some cases, transcriptional regulation of the MDR pumps involves a certain degree of crosstalk. For instance, the *Neisseria gonorrhoeae* MtrR regulator was first described as a local repressor of the MtrCDE efflux pump (Pan & Spratt, 1994; Hagman & Shafer, 1995). Further studies demonstrated that MtrR is part of a regulatory cascade that triggers the expression of *farAB*, another *N. gonorrhoeae* efflux pump. By coordinating repression with transcriptional activation, MtrR prevents simultaneous overexpression of both *farAB* and *mtrCDE* (Lee *et al.*, 2003a). Similarly, a study comparing the expression levels of the *P. aeruginosa* MexAB-OprM, MexCD-OprJ and MexEF-OprN efflux pumps in wild-type strains and defined mutants showed an inverse correlation between *mexAB-oprM* and *mexEF-oprN* expression (Li *et al.*, 2000). The authors propose that the overall expression of MDR pumps is closely monitored, and whenever the levels of one of these systems are altered, compensatory changes in the levels of the other MDR pumps follow. Another example is the *S. aureus* NorR protein, the local activator of the NorA transporter. NorR is a multifunctional regulator responsible for NorA activation, repression of unidentified efflux pump(s) and, possibly, regulation of the *S. aureus* clumping phenotype (Truong-Bolduc *et al.*, 2003). Altogether, these data reinforce the perception that the expression of different MDR efflux systems in bacteria must be coordinated in order to maintain cellular homeostasis at any given time.

Efflux of toxic compounds in nonclinical environments

A number of toxic compounds may be found in nonclinical environments. Some have a natural origin, such as antibiotics, antimicrobial agents produced by plants and heavy metals derived from the earth's crust. Others are xenobiotic compounds produced by human industrial activity. Industrial activity has also increased the amount of toxic derivatives (e.g. some ionic forms) of natural compounds found in the environment such as heavy metals, which are among the main anthropogenic pollutants (Srivastava & Majumder, 2008).

Resistance to toxic metals

Efflux pumps are part of the complex system that confers heavy metal resistance to bacteria (Nies, 2003). Although in some cases a particular efflux pump may be involved in both heavy metal and antibiotic resistance (Hernandez *et al.*, 1998; Aendekerk *et al.*, 2002; Perron *et al.*, 2004; Nishino *et al.*, 2007), heavy metal efflux pumps are usually substrate specific (Saier *et al.*, 1998) and therefore have little impact on antibiotic resistance. Hence, we will restrict our discussion to those aspects of heavy metal efflux that may contribute to the understanding of the physiological role that MDR efflux pumps play in bacteria. As mentioned above, toxic heavy metals are abundant in the environment, and bacteria have been exposed to them presumably since the beginning of life (Silver & Phung le, 2005). *Cupriavidus metallidurans* is mostly found in environments polluted with metallurgic wastes and is considered a prototype of heavy metal-resistant bacteria. It carries genes coding for heavy metal efflux pumps in plasmids and in the chromosome (Mergeay *et al.*, 2003). The chromosomal location of these efflux pumps suggests that *C. metallidurans* is specialized to grow in biotopes rich in heavy metals (perhaps volcanic biotopes), and that a considerable part of this specialization took place long before human-driven contamination of natural ecosystems with heavy metals (Mergeay *et al.*, 2003). The same appears to be true for other heavy metal-resistant bacteria, as the availability of sequenced genomes has revealed that similar systems are encoded not only by plasmidic genes but also by chromosomal genes with an ancient evolutionary origin (Nies, 2003; Silver & Phung le, 2005). Similar to plasmid-encoded antibiotic resistance genes found in nonclinical environments (Cattoir *et al.*, 2008), anthropogenic pollution has likely enriched for plasmid-encoded heavy metal resistance genes that were originally found in the chromosome of bacteria adapted to grow in the presence of heavy metals.

Heavy metals are required in trace amounts as cofactors of several bacterial proteins; therefore, the intracellular concentration probably needs to be finely tuned in order to maintain metal homeostasis. Bacteria might utilize efflux pumps in order to regulate their intracellular metal concentration even if they are not specialized to grow in the presence of high concentrations of heavy metals. Indeed, the expression of genes encoding for efflux determinants in *P. aeruginosa* has been found to increase in response to increasing levels of heavy metals (Perron *et al.*, 2004; Teitzel *et al.*, 2006), although this bacterial species is a free-living organism without a specialization to grow in environments with a high metal load. It has been found that increased levels of heavy metal resistance correlate with overexpression of the efflux pump CzcCBA (Perron *et al.*, 2004). Expression of this pump is regulated by a two-component regulator system that senses heavy metal concentration. This system not only regulates CzcCBA (and thus the intracellular concentration of metals) but also regulates the expression of the OprD porin (Perron *et al.*, 2004), responsible for the transport of basic amino acids and carbapenem antibiotics inside *P. aeruginosa* (Fukuoka *et al.*, 1993). It is worth noting that OprD expression is also

cross-regulated regulated by MexT, the activator of the MDR efflux pump MexEF-OprN (Kohler *et al.*, 1997b, 1999). Altogether, this suggests that the expression of CzcCBA (and thus heavy-metal resistance) is part of a complex response network that mediates the adaptation of *P. aeruginosa* to grow in different environments.

Resistance to solvents

Organic solvents constitute another example of toxic compounds whose bacterial tolerance is partly mediated by efflux pumps (Ramos *et al.*, 2002). Solvent-extruding pumps are not as substrate specific as heavy metal efflux pumps because they can also accommodate antibiotics. Some examples include TtgABC and TtgGHI from *Pseudomonas putida* (Rojas *et al.*, 2001), AcrAB-TolC from *E. coli* (White *et al.*, 1997; Aono, 1998; Tsukagoshi & Aono, 2000) and MexAB-OprM from *P. aeruginosa* (Li *et al.*, 1998; Muller *et al.*, 2007). Several organic solvents are present in petroleum, a mixture of compounds with nonanthropogenic origin, and aromatic hydrocarbons can be found in soil and water as a product of the biosphere activity. However, unlike ecosystems that are naturally rich in heavy metals, environments with high organic solvent concentrations originate from recent human-driven pollution (Ramos *et al.*, 2002). It is thus improbable that efflux systems have evolved to specifically protect bacteria from these toxic agents.

The ecological behavior of the bacterial species cited above further supports this view: *P. putida* is a saprophytic free-living organism with an important role in biodegradation processes. *Pseudomonas aeruginosa* is both a biodegrader and an opportunistic pathogen. Finally, *E. coli* is regularly found in the intestine, where it does not encounter high concentrations of solvents such as *n*-hexane, a good AcrAB-TolC substrate (White *et al.*, 1997). Organic solvents as well as other xenobiotic compounds possibly share physicochemical characteristics with the natural substrates of MDR efflux pumps. This would explain why bacteria that have not been exposed to organic solvents during their evolution are able to extrude the latter using chromosomally encoded efflux systems. It is worth noting that AcrAB-TolC (Sulavik *et al.*, 2001) and MexAB-OprM (Li *et al.*, 1994, 2003; Morita *et al.*, 2001) are the most relevant MDR pumps contributing to intrinsic antibiotic resistance in *E. coli* and *P. aeruginosa*, respectively. This highlights the versatility of MDR pumps in responding to different molecules that bacteria encounter in their surrounding environment. Solvent-extruding efflux pumps are also found in plasmids (Rodriguez-Herva *et al.*, 2007). Predictably, industrial contamination will contribute to the dissemination of the mentioned plasmids among bacterial populations, thus speeding up their evolutionary process.

Ecological role of bacterial MDR pumps in the soil biosphere including bacteria/plant interactions

Soils likely constitute the environment with the largest and more diverse bacterial population (Curtis *et al.*, 2002). The interactions of bacteria with the chemicals present in soil and with other members of the soil biosphere shape the structure of bacterial populations in this ecosystem. A good example of such interactions can be found in the rhizosphere. The rhizosphere is a complex ecosystem where different microorganisms form a community in close contact with plant roots. Root and other plant exudates contain a large number of compounds that include nutrients and effectors that mediate positive and negative plant/bacteria interactions (Bais *et al.*, 2006). Among these are antimicrobial toxins secreted to shield plants against microbial pathogens (Dixon, 2001). The role of MDR efflux pumps in the plant/microorganism interaction, including antimicrobial evasion by bacterial phytopathogens, has been addressed. It has been found that MDR pumps might have a relevant role in the interaction of bacteria with their plant hosts, from the first steps of colonization (Espinosa-Urgel *et al.*, 2000) to the survival in plant tissues (Barabote *et al.*, 2003). A recent analysis of *P. putida* genes differentially expressed during the interaction of this bacterial species with maize roots revealed that several efflux pumps are induced when *P. putida* adjusts its genetic program to the colonization of roots (Matilla *et al.*, 2007), suggesting a role of such MDR pumps in plant/bacteria interactions.

Studies on some pathogenic species indicate that loss or inactivation of efflux pumps compromises initial colonization and virulence. *Erwinia amylovora* is the causative agent of fire blight disease on rosaceous plants (Eastgate, 2000). Mutants in the *E. amylovora* AcrAB efflux pump were susceptible to the apple plantlet toxins phloretin, naringenin, quercetin and (+)-catechin, and exhibited dramatically reduced virulence (Burse *et al.*, 2004a). The same study also showed that naringenin and phloretin induce *acrAB* expression. *Agrobacterium tumefaciens* is a plant pathogen that can be found on alfalfa roots. Palumbo *et al.* (1998) demonstrated that *A. tumefaciens* cells utilize the IfeABR system to extrude coumestrol, an antimicrobial isoflavonoid present in root exudates capable of inducing *ifeABR* expression. *ifeABR* mutants accumulated coumestrol and were impaired in competitive root colonization.

Erwinia chrysanthemi constitutes yet another example of efflux pumps required to effectively cause virulence in plants. The *E. chrysanthemi* TolC outer-membrane efflux component participates in extrusion of the antimicrobial compound berberine (Barabote *et al.*, 2003). A *tolC* mutant was severely impaired in *in planta* multiplication and infection of plant tissue (Barabote *et al.*, 2003). The availability of

the *E. chrysanthemi* complete genome allowed the identification of additional efflux systems that contribute to virulence with some degree of host specificity (Llama-Palacios *et al.*, 2002; Barabote *et al.*, 2003; Maggiorani Valecillos *et al.*, 2006). For instance, both the Acr1AB and the Acr2AB system were required for full virulence on chicory leaves. However, in Saintpaulia plants, the Acr1AB system seemed to contribute more to virulence, while Acr2AB had no effect whatsoever. Mutants in *acr1AB* and *acr2AB* were susceptible to a number of plant antimicrobials and also to some antibiotics. For all the phytopathogens cited above, the compromised ability to infect and/or colonize a particular host results from the successful antimicrobial effect that plant-derived toxins have on efflux pump mutants. The case of *E. amylovora* (Burse *et al.*, 2004a) and *A. tumefaciens* (Palumbo *et al.*, 1998), where plant-derived toxins induce the expression of genes encoding for efflux pumps able to extrude the very same compounds, suggests that these elements were naturally selected in order to evade the plant defence system.

Plant-derived antimicrobials are not the only compounds capable of inducing the expression of efflux pumps relevant to plant/bacteria interactions. Salicylic acid is an important signalling molecule that triggers resistance to phytopathogens (Loake & Grant, 2007). In *E. chrysanthemi*, a combination of salicylic acid and its precursors induces the expression of genes encoding the AcrAB and EmrAB efflux pumps (Ravirala *et al.*, 2007). The ability of bacteria to utilize defence-signalling compounds to induce the expression of efflux systems also suggests adaptation to the plant defence system. As stated by Palumbo *et al.* (1998), while the physiological functioning of these efflux pumps remains undefined, these results define their ecological significance in plant/bacteria interactions. Interestingly, salicylate also induces the expression of MDR pumps in human pathogens such as *Burkholderia cepacia*, which has an environmental origin (Nair *et al.*, 2004). Also, the *P. aeruginosa* MexAB-OprM efflux system not only mediates intrinsic antibiotic resistance, or resistance to solvents, but also tolerance to the tea tree oil antimicrobial produced by the leaves of *Melaleusca alternifolia* and to its monoterpene components terpinen-4-ol, 1,8-cineole and α-terpineol (Papadopoulos *et al.*, 2008). This indicates that some of the mechanisms that allow bacteria to survive in nature can also be useful in clinical environments. The relevance of efflux pumps in bacterial interactions with plants is also observed in symbiotic bacteria. Mutants of *Rhizobium etli*, a mutualistic symbiont of the *Phaseolus vulgaris* bean, with a defective RmrAB efflux pump formed on average 40% less nodules than the wild-type strain (Gonzalez-Pasayo & Martinez-Romero, 2000).

Any phytopathogen must compete with the microbial epiphytic community for space and nutrients besides colonizing its host plant. Because several epiphytes synthesize antimicrobials, a potential role of efflux pumps in intermicrobial competition can be envisaged. *Erwinia chrysanthemi* carries a putative ABC transporter, YbiT, that might play such a role. A *ybiT* mutant retained virulence in potato tubers and chicory leaves, but was less infectious than the wild-type strain in coculture experiments with saprophytic bacteria such as *P. putida* or *Pseudomonas fluorescens* (Llama-Palacios *et al.*, 2002). A possible explanation for this phenotype is that YbiT is capable of extruding toxic compounds produced by *P. putida* and *P. fluorescens*, and in its absence *E. chrysanthemi* is outcompeted. A more conclusive result was obtained with the NorM single-component efflux system from *E. amylovora* (Burse *et al.*, 2004b). A *norM* mutant was fully virulent on apple rootstock. However, it was susceptible to antimicrobial compounds produced by *Pantotea agglomerans*, an apple and quince blossom epiphyte. This suggests that *E. amylovora* would require NorM in order to establish successful infections *in planta*. Antibiotic resistance has been considered previously as a colonization factor in clinical environments in the presence of antibiotics (Martinez & Baquero, 2002). Similarly, resistance to antimicrobial compounds produced by epiphytic flora might be considered as a colonization factor in plant pathogens.

Two other elements are relevant for the ecological behavior of bacterial populations in soil. It has been described that chromosomally encoded MDR pumps enhance the ecological fitness of *Shewanella oneidensis* in aquifer sediments, probably because these determinants are capable of effluxing not just antibiotics but also other compounds, such as humic acids, present in these ecosystems (Groh *et al.*, 2007). The structure of microbial populations in natural soil and water environments is also highly dependent on the activity of bacteriovorus nematodes and protozoans. The prey–predator relationship of these eukaryotes with bacteria is not unidirectional, because bacteria can kill both nematodes and unicellular eukaryotes. Furthermore, it has been stated that some virulence determinants relevant for infecting humans are also relevant for infecting plants, animals and amoebae (Rahme *et al.*, 1995, 2000; Gao *et al.*, 1997; Mahajan-Miklos *et al.*, 2000; Steinert & Heuner, 2005; Mylonakis *et al.*, 2007). This suggests that virulence determinants of some opportunistic pathogens may have been initially selected to play an ecological role in nonhuman environments (Navas *et al.*, 2007). Perhaps because neither nematodes nor protozoans release such a high amount of chemical compounds as plants do, the role of bacterial efflux pumps in the bacterial relationship with these organisms has not been explored thoroughly. Nevertheless, a few results suggest that efflux pumps may also be significant in such interactions. *Pseudomonas aeruginosa* is capable of killing the *Caenorhabditis elegans* nematode (Tan *et al.*, 1999) and

the *Dictyostelium discoideum* amoeba (Cosson *et al*., 2002). It has been shown that constitutive overexpression of MDR pumps changes the behavior of *P. aeruginosa* with respect to these organisms from virulent to nonpathogenic (Cosson *et al*., 2002; Sanchez *et al*., 2002c; Ruiz-Diez *et al*., 2003). Similar results have been observed with *S. maltophilia* mutants overexpressing the SmeDEF MDR pump (Alonso *et al*., 2004). As we will see later on, in some cases the function of MDR pumps may not be the efflux of host-produced compounds, but possibly extrusion of signal molecules that mediate intercellular communication and regulate bacterial virulence.

Bacterial homeostasis and detoxification

MDR pumps belong to a large family of transport systems. Several of them are highly conserved among different bacterial species (Ren & Paulsen, 2005). Because of this conservation, many proteins have been designated as multidrug transporters based on sequence homology, with little or no evidence for such a function or even when a different physiological role has been identified (Higgins, 2007). Often, the so-called MDR pumps extrude drugs only when overexpressed, but play no role in MDR at physiological expression levels. Some *P. aeruginosa* MDR pumps are a good example of this behavior. Constitutive overexpression of any of the following MDR pumps makes *P. aeruginosa* less susceptible to antibiotics: MexAB-OprM, MexCD-OprJ, MexEF-OprN or MexXY. However, only MexXY and MexAB-OprM contribute to intrinsic resistance in this opportunistic pathogen (Piddock, 2006b).

Even in the case of bacterial antibiotic producers, it is not clear whether some genes that confer resistance when they are expressed at a high level in a heterologous host have the same role in the original organism. For instance, *Streptomyces* species are antibiotic producer prototypes and carry a large arsenal of determinants capable of conferring antibiotic resistance in a heterologous host; yet, under routine laboratory conditions, they are susceptible to a wide variety of drugs used in antimicrobial therapy (Lee *et al*., 1996, 2003b). As shown for the *Streptomyces lividans* transporters encoded by *ebrA*, *ebrB* and *ebrC* (Lee *et al*., 2003b, 2007) and the *Streptomyces rochei msr* MDR ABC transporter (Fernandez-Moreno *et al*., 1998), the observed susceptibility most likely results from low or no expression of antibiotic resistance determinants during growth in the laboratory. This does not mean that MDR pumps in *Streptomyces* species are not involved in endogenous antibiotic resistance. The intracellular antibiotic concentration in producers may probably reach high levels if the cells did not eliminate these toxic compounds through transport systems. A role in extrinsic antibiotic resistance would be secondary for these transporters. Lee *et al*. (2003b, 2007) have suggested that this is the case in *Streptomyces* species where the primary role of MDR pumps does not seem to be the removal of external drugs, but rather the elimination of endogenous toxic compounds.

Increasing evidences suggest that some MDR pumps are involved in the elimination of endogenous toxic compounds generated by bacterial metabolism not only in antibiotic producers but in other bacteria as well (Neyfakh, 1997). For instance, the *P. aeruginosa* MexGHI-OpmD MDR pump appears to be implicated in the extrusion of anthranilate, a toxic precursor of the *Pseudomonas* quinolone signal (PQS) (Aendekerk *et al*., 2002, 2005; Sekiya *et al*., 2003).

MDR pumps are linked to bacterial metabolism not just through detoxification. Expression of MDR pumps must be co-ordinately regulated in order to maintain cellular homeostasis; therefore, it is not surprising that in some cases they have been directly implicated in bacterial homeostasis. For instance, MdfA confers antibiotic resistance to *E. coli* when overexpressed; however, deletion of *mdfA* does not affect antibiotic susceptibility. Instead, at physiological expression levels, MdfA functions as a $Na^+(K^+)/H^+$ antiporter that enables cells to maintain intracellular pH homeostasis under alkaline conditions (Lewinson & Bibi, 2001). Likewise, overexpression of the *Bacillus subtilis* Blt MDR determinant results in antibiotic resistance; yet, at normal expression levels, Blt is involved in spermidine transport (Ahmed *et al*., 1995).

Multidrug efflux pumps and bacterial virulence

It has been suggested that MDR pumps are not only important antibiotic resistance elements but that they also play a role in bacterial pathogenicity relevant to animal diseases (Piddock, 2006a). This is not surprising, given the significant role MDR pumps play in bacteria/plant interactions, as discussed previously. As we will see later on, some of the phenotypes are directly due to MDR efflux pumps, whereas on other occasions the effect of MDR pumps on virulence is likely indirect. The first step of microbial infection is colonization: the establishment of a pathogen at the appropriate portal of entry such as the skin or the different mucosal surfaces of the body. Colonization of mucosal surfaces can be inhibited by host-produced compounds such as bile salts, long-chain fatty acids and antimicrobial peptides (Begley *et al*., 2005; Laube *et al*., 2006; Drake *et al*., 2008). Bacteria require mechanisms to avoid the effect of these compounds in order to successfully colonize a tissue and subsequently cause infection. Both commensal and pathogenic bacteria carry multiple MDR efflux pumps that may accomplish this task.

Several MDR efflux systems in Gram-negative bacteria confer resistance to bile salts *in vitro*, the best-studied one

being the *E. coli* AcrAB system (Thanassi *et al.*, 1997). AcrAB was first described as an antibiotic resistance determinant that plays a major role in the multiple antibiotic resistance (*mar*) phenotype of *Enterobacteriaceae* (Ma *et al.*, 1995; Okusu *et al.*, 1996). The AcrAB system is present both in virulent and in avirulent *E. coli* strains that inhabit the animal intestinal tract, where it traps and pumps out toxic bile salts. Because AcrAB homologs are widely distributed, they possibly play a similar role in other *Enterobacteriaceae*, as in the case of *Salmonella enterica* serovar Typhimurium, in which this system confers resistance to antibiotics, bile salts and detergents (Lacroix *et al.*, 1996). Furthermore, inactivation of *acrAB* impairs *Salmonella typhimurium* intestinal colonization in a murine model, indicating that this system is required for full virulence (Lacroix *et al.*, 1996). Similar results have been obtained with *Francisella tularensis* and probably apply to other bacterial species (Bina *et al.*, 2008a). The *Campylobacter jejuni* CmeABC efflux pump confers resistance to a broad range of antimicrobials including bile salts, fatty acids and detergents, and is required to successfully colonize the chicken intestinal track (Lin *et al.*, 2003). *cmeABC* is expressed in chicken, suggesting that factors encountered during *in vivo* growth trigger the expression of this efflux system. Lin *et al.* (2003) proposed that functional inhibition of this efflux pump could control antibiotic resistance as well as prevent *C. jejuni* host colonization. This may also apply to other MDR efflux pumps with a relevant role in both antibiotic resistance and virulence (Vila & Martinez, 2008).

As mentioned above, long-chain fatty acids can also prevent bacteria from colonizing mucosal surfaces. In some cases, the same efflux system is involved in resistance to both bile salts and fatty acids. For example, AcrAB protects *E. coli* from decanoate found in mucosal surfaces (Ma *et al.*, 1995). The FarAB efflux pump from *N. gonorrhoeae* is homologous to AcrAB and is also involved in protection against fatty acids (Lee & Shafer, 1999). Many gonococcal isolates from men with rectal infections have been found to be resistant to fatty acids, a phenotype attributed to FarAB-mediated efflux (Morse *et al.*, 1982; McFarland *et al.*, 1983; Lee & Shafer, 1999). MtrCDE is another MDR efflux pump with a relevant role in gonococcal virulence (Warner *et al.*, 2007). An *mtrCDE* mutant was cleared more rapidly than the wild-type strain in competitive genital tract infection experiments on female mice (Jerse *et al.*, 2003). The aforementioned FarAB efflux pump did not seem to be important in this model system. This indicates that assorted MDR pumps of a given bacteria may have different roles during colonization depending on the host or the target tissue, a situation that resembles the above-discussed host-specific role of MDR pumps in bacterial phytopathogens (Maggiorani Valecillos *et al.*, 2006). For instance, it is possible that MtrCDE, which is also involved in resistance to antimicrobial peptides, has a more significant role than FarAB during colonization of host tissues with relevant concentrations of these antimicrobials, whereas FarAB, which can efflux fatty acids, seems to be more important for the colonization of fatty acid-rich environments. Whichever the specific role for each efflux pump may be, the fact that MtrR positively regulates *farAB* expression and represses *mtrCDE* expression suggests that each of these efflux pumps is expressed only when (and where) needed (Lee *et al.*, 2003a). In *Yersinia enterocolitica*, the RosAB system mediates antimicrobial peptide efflux and constitutes another example of MDR systems that appear to be expressed only when necessary (Bengoechea & Skurnik, 2000). Induction of *rosAB* expression in the presence of antimicrobial peptides at 37 °C allows *Y. enterocolitica*, an important pathogen with an environmental habitat, to tolerate these compounds during host colonization.

Besides their involvement in resisting the antimicrobial compounds present in host surfaces, MDR efflux pumps may also have a direct role in bacterial virulence. The contribution of the four best-studied *P. aeruginosa* MDR pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprM and MexXY) to virulence has been tested in two experimental models: invasion of epithelial cells and septicaemia in a murine model (Hirakata *et al.*, 2002). All except the *mexCD-OprJ* knock-out mutant exhibited a decreased ability to invade MDCK cells. Invasion levels were restored when culture supernatants from MDCK cells infected with the wild-type strain were added to cells infected with the MDR-defective mutants. This effect was the highest in the case of the *mexA* mutant. In fact, the MexAB-OprM system proved to be essential for inducing lethal endogenous septicaemia in the murine model. This led the authors to conclude that this efflux system mediates the release of virulence factors essential for pathogenesis. Interestingly, a *mexA* null mutant is also avirulent in a *C. elegans* model (Mahajan-Miklos *et al.*, 2000). Mutants overproducing MexAB-OprM were found to be avirulent in the same model (Sanchez *et al.*, 2002c; Ruiz-Diez *et al.*, 2003); therefore, it seems that the expression of MDR pumps must be finely tuned in order to observe full virulence in *P. aeruginosa*.

Virulence is a complex phenomenon that requires the coordinate expression of different sets of genes (Lee *et al.*, 2006). If MDR efflux pumps were involved in this phenomenon, we would expect to observe, at least in some cases, some degree of cross-regulation along with established virulence factors. Indeed, this has been demonstrated for a few systems. The *E. coli* transcriptional activator MarA (from multiple antibiotic resistance) regulates the expression of > 60 genes (Barbosa & Levy, 2000). Among these are genes encoding MDR efflux determinants such as *acrAB* as well as genes involved in oxidative stress (for instance, *zwf*, *fpr* and *sodA*) and in iron metabolism (e.g. *hemB*, *fumC*, *fecA* and *acnA*), which have a potential role in survival inside

the host. As we mentioned above, *N. gonorrhoeae* MtrR regulates the expression of both *farAB* and *mtrCDE* efflux pumps (Lee *et al.*, 2003a). In addition, MtrR positively regulates *ponA* expression, which encodes penicillin-binding protein 1, and represses the *pilMNOPQ* operon, which encodes components of the type IV pilus secretion system that has been implicated in bacterial virulence (Folster *et al.*, 2007). MgrA regulates the expression of around 350 genes in *S. aureus* (Luong *et al.*, 2006), including MDR pumps (Truong-Bolduc *et al.*, 2003, 2005) and virulence factors (Ingavale *et al.*, 2005). Recent work has shown that expressions of the efflux pumps *norB* and *tet38* are upregulated *in vivo* in a mouse subcutaneous abscess model, whereas *norA* and *norC* are downregulated (Ding *et al.*, 2008). These changes in the level of expression were associated with an increase in *mgrA* expression. Notably, both *norB* and *tet38* mutants were impaired for *in vivo* growth in mice abscesses. Because overexpression of these efflux determinants is not due to the staphylococcal general stress response, it was suggested that specific elements of the abscess environment trigger the expression of specific efflux pumps that are important for the bacterial growth in this environment. This pattern of expression of *S. aureus* MDR pumps is mediated at least in part by changes in the level of expression of the global regulator MgrA (Ding *et al.*, 2008).

A connection between MDR efflux pumps and expression of the type III secretion (T3S) system has also been reported. T3S is an important virulence factor for several bacterial pathogens (Coburn *et al.*, 2007). Using this system, bacteria are able to inject a battery of effectors directly into the cytoplasm of eukaryotic host cells. It has been shown that constitutive overproduction of *P. aeruginosa* MexCD-OprJ or MexEF-OprN efflux pumps affects the expression of the T3S system negatively (Linares *et al.*, 2005). As we will see later, some *P. aeruginosa* MDR pumps may have a direct role in the bacterial response to quorum-sensing (QS) signals, and consequently in bacterial virulence. However, the effect of these MDR pumps on T3S was independent of the QS response and was due to the lack of expression of the *exsA* gene encoding the transcriptional activator of T3S in *P. aeruginosa*. Whether this is a direct or an indirect effect remains to be established. Recent studies suggest that the connection between T3S and MDR pump expression is not exclusive to *P. aeruginosa*. It has been described that the MDR pump DinF contributes to *Ralstonia solanacearum* virulence, and that expression of this efflux determinant is negatively regulated by the T3S regulator HrpB (Brown *et al.*, 2007). The fact that MDR efflux pumps are involved in protection against host-produced antimicrobial compounds and belong to regulatory networks that encompass virulence factors supports the notion that some MDR pumps play a crucial role in the interactions of commensal and pathogenic bacteria with their host. It is possible that some of these functions emerged as a result of bacterial coevolution with their host.

Cell-to-cell communication

Intercellular communication has an important role in bacterial behavior in different environments. One of the most relevant systems of bacterial communication is the QS response. QS was earlier described in the luminescent marine bacterium *Vibrio fischeri* (Nealson *et al.*, 1970). Since then, it has been studied in a large number of Gram-positive and Gram-negative microorganisms (Keller & Surette, 2006; Williams *et al.*, 2007; Jayaraman & Wood, 2008). In the QS response, bacteria utilize self-produced diffusible signalling molecules to sense and respond to their population density. As the population density increases, the signals, commonly referred to as autoinducers, accumulate until a threshold concentration is reached. At this point, the autoinducers bind specific receptors that activate the expression of a particular set of genes (Miller & Bassler, 2001). Some of the functions controlled by QS include biosynthesis of antimicrobial peptides, motility, polysaccharide synthesis and a number of virulence factors such as elastases and proteases, among others (Jayaraman & Wood, 2008).

QS has been well characterized in the opportunistic pathogen *P. aeruginosa*. Similar to many Gram-negative bacteria, *P. aeruginosa* utilizes *N*-acylated homoserine lactones (acyl-HSL) as autoinducers synthesized by two different systems: *las* and *rhl* (Schuster & Greenberg, 2006). In the *las* system, the LasI synthase produces the autoinducer *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL), which binds its cognate receptor LasR to activate the transcription of a specific set of genes (Gambello & Iglewski, 1991; Passador *et al.*, 1993; Pearson *et al.*, 1994). Similarly, the *rhl* system comprises the RhlI synthase, which produces the autoinducer *N*-butanoyl-homoserine lactone (C4-HSL) and its signal receptor, RhlR (Ochsner *et al.*, 1994; Ochsner & Reiser, 1995; Pearson *et al.*, 1995). The two systems interact with each other in a hierarchical way, where the *las* system controls the transcription of the *rhl* system (Latifi *et al.*, 1996; Pesci *et al.*, 1997). *Pseudomonas aeruginosa* produces a third signal: 2-heptyl-3-hydroxy-4-quinolone, the PQS (Pesci *et al.*, 1999). PQS synthesis is regulated by the *las* and *rhl* systems and in turn the role of PQS in the QS network includes regulating the expression of a subset of *rhl* and *las*-controlled genes (Diggle *et al.*, 2006).

A key aspect of QS is how the autoinducers come out into the extracellular milieu where they accumulate as the cell density increases. Although some autoinducers diffuse freely across the cell membrane, some molecules such as PQS and 3OC12-HSL are not readily diffusible due to their hydrophobic nature (Mashburn & Whiteley, 2005), and they traverse the bacterial envelope using membrane vesicles or

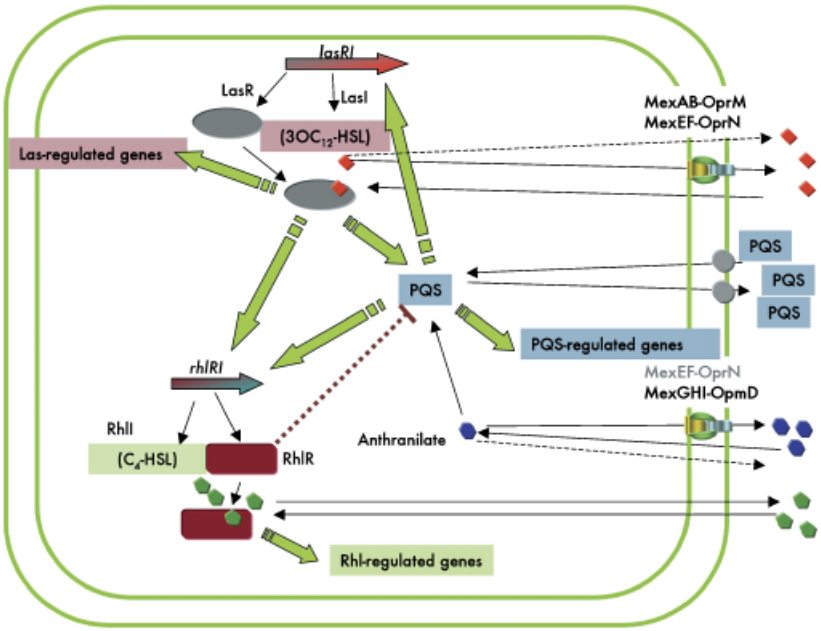

Fig. 4. Multidrug efflux pumps and QS in *Pseudomonas aeruginosa*. The QS system of *P. aeruginosa* presents a hierarchical organization with two main branches. The Las system is formed by the autoinducer 3OC12-HSL and the regulator LasR, whereas the Rhl system is formed by the autoinducer C4-HSL, and the regulator RhlR. In both cases, the autoinducer binds the regulator on reaching a critical intracellular concentration, leading to induction of transcription of specific subsets of genes. As shown in the figure, the two systems interact with each other and with the PQS signalling system [for a more detailed description of the PQS signalling network, see Diggle *et al.* (2006)]. Because the intracellular autoinducer concentration is the key factor for triggering *P. aeruginosa* QS response, any change in the expression of the systems involved in signal trafficking through the cell envelope may impact QS. It has been proposed that the three signals can freely diffuse through the cell membrane, and that they make use of specific efflux systems as well. Whereas the PQS signal is transported inside membrane vesicles (Mashburn & Whiteley, 2005), 3OC12-HSL is transported by the efflux pumps MexAB-OprM and MexEF-OprN, and anthranilate, one toxic precursor of PQS, is transported by MexGHI-OpmD and likely (although this has not been formally demonstrated) by MexEF-OprN.

membrane transporters. Among those transporters, we will discuss the role of MDR efflux pumps in *P. aeruginosa* autoinducers traffic and in the QS response (Fig. 4). It has been shown that 3OC12-HSL can be a substrate for the MexAB-OprM efflux pump and that mutations affecting *mexAB-oprM* expression impact 3OC12-HSL transport to the extracellular environment, and as a result, the production of some QS-controlled virulence factors decreases (Evans *et al.*, 1998; Pearson *et al.*, 1999). MexEF-OprN and MexGHI-OpmD play an indirect role in QS presumably by exporting anthranilate, a toxic metabolite and PQS precursor. Mutations that affect *mexEF-oprN* and *mexGHI-opmD* expression and that either increase or decrease efflux have an impact on PQS levels; this in turn affects the production of a subset of QS-controlled virulence factors due to the interaction of PQS with the *las* and *rhl* systems (Kohler *et al.*, 2001; Aendekerk *et al.*, 2005). The finding that the expression of MexGHI-OpmD is induced by PQS and by phenazines, together with the suggestion that phenazines might be involved in *P. aeruginosa* signalling, also indicates that some *P. aeruginosa* MDR pumps may be involved in signal trafficking (Bredenbruch *et al.*, 2006; Dietrich *et al.*, 2006). This putative role is further supported by the fact that C4-HSL enhances the expression of MexAB-OprM (Maseda *et al.*, 2004).

A potential role of MDR pumps in QS has also been proposed for other bacterial species. For instance, it has been described that the *Burkholderia pseudomallei* MDR pump BpeAB-OprB effluxes six different homoserine lactone signals involved in the QS response of this bacterial species (Chan & Chua, 2005; Chan *et al.*, 2007). The expression of the *bmeB* efflux pump of the human commensal bacteria *Bacteroides fragilis* is regulated by QS, and it has been proposed that it could transport acyl-HSL signals besides antibiotics (Pumbwe *et al.*, 2008). AcrAB-TolC, the most important MDR pump of *Enterobacteriaceae*, constitutes another example. Expression of this efflux pump is controlled by the QS regulator SdiA in *E. coli* (Rahmati *et al.*, 2002). Studies suggest that both AcrAB-TolC and NorE can efflux *E. coli* autoinducers, thus contributing to the QS response in this bacterial species (Yang *et al.*, 2006).

It is important to mention that MDR pumps may play a dual role, affecting the virulence of bacterial pathogens. A work with *Vibrio cholerae* has shown that MDR systems in

this bacterial species are involved in resistance to antimicrobial compounds (bile salts and antimicrobial peptides) present in the host. In addition, loss of MDR pumps resulted in decreased expression of the virulence gene regulator *tcpP*, possibly by affecting cell-to-cell signalling (Bina *et al*., 2008b).

Although the main physiological function of MDR pumps might not be to efflux QS signals, they definitely seem to play a role in the QS response. In the particular case of *P. aeruginosa*, the QS network interacts with other regulatory systems, presumably to integrate and respond to a wide variety of environmental signals (Schuster & Greenberg, 2006). It is possible that *P. aeruginosa* alters the expression of certain efflux pumps to fine-tune the QS response when faced with as yet unidentified signals. Interestingly, mutants defective in the QS response are often isolated from environmental and clinical samples, and this suggests that the loss of the this cell-to-cell communication system may be advantageous to *P. aeruginosa* under some circumstances (Heurlier *et al*., 2006). It has been shown that QS-null *P. aeruginosa* mutants are not defective in autoinducer production, but are rather signal-blind. The reason for this may be the energetic cost of one or another type of defect. It has been calculated that only 0.01% of the total cellular amount is needed to make *P. aeruginosa* autoinducers, whereas the QS response consumes at least 5% of the total energy supply in this bacterial species (Haas, 2006). In this scenario, the presence, in the bacterial cells, of systems capable of modulating the QS response even when the extracellular autoinducers concentrations are high may confer a selective advantage for free-living bacteria, sequentially faced with environments in which the QS response is either adaptive or has a cost. In such situations, efflux pumps may be useful to enable a quick environmental adaptation by shutting down the QS response through an increase in the efflux of autoinducers and/or autoinducer precursors.

The putative role of MDR pumps in regulating QS homeostasis links efflux pumps to virulence regulation as QS controls the production of a variety of virulence factors in *P. aeruginosa* (Schuster *et al*., 2003; Wagner *et al*., 2003), and mutants defective in QS exhibit reduced virulence in different models (Rasmussen & Givskov, 2006). Indeed, Aendekerk *et al*. (2005) reported that *mexI* and *opmD* mutants were attenuated in virulence in animal and plant infection models due to perturbations of the QS response. *Pseudomonas aeruginosa* is a free-living opportunistic pathogen, and as such it utilizes an overlapping repertoire of virulence factors to infect mammals, plants and insects (Mahajan-Miklos *et al*., 2000). Assuming that MDR pumps contribute to virulence regulation in all these instances, efflux pumps appear relevant in the environment as well as in the colonization of human hosts, where they also contribute to resistance to antibiotic therapy. The same might be true for MDR pumps from other free-living opportunistic pathogens.

Antibiotic resistance: a recent functional role of MDR pumps as a consequence of anthropogenic activity?

In this review, we have highlighted the different roles that bacterial MDR pumps may have, including resistance to antimicrobial compounds produced by hosts or other bacterial species, virulence, detoxification and intercellular signal trafficking. Even in the case of antibiotic-producing bacteria, the main role of MDR pumps appears to be detoxification of intracellular antibiotics rather than resistance to external ones. Some studies have suggested that antibiotics regularly used in clinical settings have a different role in nature (Davies, 2006; Linares *et al*., 2006; Yim *et al*., 2007; Fajardo & Martinez, 2008). Yim *et al*. (2007) have recently proposed that the majority of low-molecular-weight organic compounds secreted by microorganisms function as cell signalling molecules. These authors state that cell-signalling mediated by low concentrations of these molecules controls gene expression in microbial populations and possibly the interactions with surrounding organisms. According to this perspective, high (toxic) concentrations of antibiotics will be rarely found in nature and will mostly be the consequence of human activity, mainly in medicine and farming. Resistance against toxic concentrations of these compounds would not have an adaptive value in natural environments, and the resistance determinants should serve other purposes in nature (Martinez, 2008; Martinez *et al*., 2009).

Does this mean that MDR pumps do not have a functional role as antibiotic resistance elements? Obviously, this is an overstatement. As described throughout the review, several studies have demonstrated that resistance mediated by MDR pumps is important for survival during competition with epiphytic bacteria and in the presence of host-produced antimicrobials. Indeed, we would like to go one step further. We cannot ignore that human activity is shaping the evolution of bacterial populations, and that the introduction of antibiotics for therapeutic purposes at concentrations much higher than those present in nature has largely contributed to this process. It has been stated that 'To pathogenic microparasites (viruses, bacteria, protozoa, or fungi), we and other mammals (living organisms at large) are little more than soft, thin-walled flasks of culture media' (Levin & Antia, 2001). If we consider ourselves as ecosystems colonized by bacteria, the recent presence of antibiotics introduces a new strong selective pressure for the microorganisms colonizing these habitats (Martinez & Baquero, 2002). Being antibiotic resistant will be an ecological advantage in an antibiotic-rich environment. In fact, antibiotic

treatment is considered as a risk factor for being infected by intrinsically resistant bacteria. It is worth mentioning that acquisition of an antibiotic resistance phenotype may lead to drastic changes in bacterial metabolism (Andersson & Levin, 1999; Morosini *et al.*, 2000; Andersson, 2006) to the point that it has been suggested that antibiotics operate as accelerators of bacterial evolution (Blazquez *et al.*, 2002).

The term exaptation was coined to indicate an evolutionary process in which some functional features evolved by natural selection to cover a given function are used to play a different role that becomes the most important one (Gould & Vrba, 1982). An example of this could be feathers that were initially selected for thermoregulation and were later co-opted for flight. In this regard, antibiotic resistance is a clear example of exaptation. One protein previously selected for detoxification, homeostasis or signal trafficking is co-opted to serve as an antibiotic resistance element in an environment with a high antibiotic load. Take for instance the tetracycline resistance determinants *otrA* and *otrB* found in *Streptomyces rimosis* and in mycobacteria (Pang *et al.*, 1994). These determinants have an intracellular detoxification function in the former; however, they are solely committed to external antibiotic resistance in the latter. The *mefA* gene coding for a macrolide efflux determinant is another good example because its role in the original organism is unknown, and once acquired by bacterial pathogens, it functions exclusively in resistance to external antibiotics (Clancy *et al.*, 1996).

Just as discussed previously for determinants involved in tolerance to solvents, efflux pumps implicated in antibiotic resistance are increasingly being detected in association with transferable elements. Different from chromosomally encoded efflux pumps, the determinants present in mobile elements can spread and evolve rapidly as a consequence of antibiotic selective pressure. A few examples of efflux pumps (not necessarily MDR determinants) include the *tet* and *mef* genes mentioned above, which confer resistance to tetracycline and macrolides, respectively, and the newly described *qepA* responsible for fluoroquinolone resistance (Yamane *et al.*, 2007). Introduction of high concentrations of antibiotics in different environments such as infected humans, farming and aquaculture (Baquero *et al.*, 2008; Martinez, 2008) will undoubtedly favor the dissemination of plasmids containing MDR efflux determinants whose sole ecological function in these antibiotic-rich environments will be resistance to antibiotics.

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