UNIVERSIDADE NOVA DE LISBOA

INSTITUTO DE HIGIENE E MEDICINA TROPICAL

The role played by efflux systems on the resistance to antibiotics

Marta Sofia Lopes Martins

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ABSTRACT

Multidrug resistance (MDR) to antibiotics presents a serious therapeutic problem in the treatment of infections. The importance of this mechanism of resistance in clinical settings is reflected in the increasing number of reports of MDR isolates. The most common mechanisms of resistance to antibiotics in bacteria are: i) the inactivation of the antibiotic by bacterial enzymes; ii) mutations in the structural or regulatory genes of the target protein; iii) alterations in the outer membrane that will contribute to decreased drug permeability, being this more preponderant on Gram-negative bacteria, due to their outer membrane structure; and iv) extrusion of the antibiotic from the cell by the activation of the efflux systems. These last systems are often associated with the over-expression of transporters (efflux pumps) that recognize and efficiently expel from the cells a wide gamut of structurally unrelated compounds. These transporter proteins involved in the extrusion of toxic substrates are found in both Gram-positive and -negative bacteria as well as in eukaryotic organisms. They can be specific for one substrate or may transport a range of structurally distinct compounds, including antibiotics of multiple classes. There are five major families of efflux transporters, described until the present date, namely, the: (1) Major Facilitator Superfamily (MFS); (2) Small Multidrug Resistance (SMR) family; (3) Multidrug And Toxic compound Extrusion (MATE) family; (4) Resistance Nodulation Division (RND) superfamily; and (5) Adenosine Triphosphate (ATP)-Binding Cassette (ABC) superfamily. All these systems utilize the proton motive force as an energy source, apart from the ABC family, which utilizes ATP hydrolysis to drive the export of substrates. One of the recent challenges in this area is to develop new compounds that inhibit these efflux systems and subsequently potentiate the activity of co-administered antibiotics thus extending the clinical utility of existing antibiotics. Unfortunately and although several efflux pump inhibitors (EPIs) have been characterized, none of them has yet resulted in a clinical useful compound that could be applied in the clinical setting to treat MDR infections. However, the search continues and among the distinct types of EPIs we can find a large and distinct number of compounds, such as: peptidomimetics; phenothiazines; a class of natural products produced by Streptomyces spp., the benastatins; tetracycline derivatives/homologues; compounds

isolated from plant extracts; quinoline and its derivatives; arylpiperidines and arylpiperazines; microbial-derived EPIs and a distinct group of compounds, the energy uncouplers. If these EPIs be used as "helper compounds" in combination with antibiotics to which the organism is initially resistant, then the required cure may be achieved. This new approach will bring back to action the re-use of various antibiotics that are affected by the efflux systems as well as the control of the emergence and the dissemination of MDR-associated efflux strains. However, we know very little about the mechanisms and function of these efflux systems. New methods to assess this efflux-mediated resistance are therefore needed. In the last few years, a series of methods have been developed and may contribute to the rapid screening of MDR strains. Among the commonly used methods, one has received particular relevance, and it is based on the efflux of ethidium bromide. Ethidium bromide is a common substrate of efflux pumps and due to its fluorescent properties, it allows the monitoring in a real time basis of the efflux systems that are activated on a bacterial strain. The design and improvement of this and other methods is therefore one important tool to screen large collections of clinical isolates showing an MDR phenotype. The combined approaches, *i.e.*, screening of MDR-efflux mediated isolates and the search for new and effective EPIs can bring to date the control and treatment of MDR infectious. The future will show us the results…

RESUMO

A resistência a várias classes de antibióticos, *i.e.*, multi-resistência (MDR), constitui um dos maiores problemas a nível terapêutico, no tratamento de diversas infecções. A importância que este mecanismo de resistência adquiriu no contexto hospitalar, reflecte-se no elevado número de casos relativos a multi-resistência em isolados clínicos. Os mecanismos de resistência mais comuns em bactérias são: i) a inactivação do antibiótico pelas enzimas bacterianas; ii) mutações em genes estruturais ou reguladores da proteína alvo; iii) alterações na membrana externa, que podem provocar um decréscimo da permeabilidade aos diversos compostos, sendo este caso mais relevante em bactérias Gram-negativas, dada a sua estrutura membranar; e iv) extrusão do antibiótico da célula por activação de sistemas de efluxo. Estes últimos sistemas encontram-se normalmente associados a uma sobre-expressão de transportadores proteicos, designados bombas de efluxo, que reconhecem e expelem eficientemente uma vasta gama de compostos estruturalmente distintos. Estes transportadores, que se encontram envolvidos na extrusão de substratos tóxicos, encontram-se quer em baterias Gram positivas, quer em bactérias Gram negativas, bem como em células eucariotas. Estes sistemas podem ser específicos para um substrato ou podem transportar uma série de compostos estruturalmente distintos, incluindo antibióticos de classes diferentes. Os sistemas de efluxo descritos até à presente data podem ser classificados em cinco famílias distintas, nomeadamente: (1) "Major Facilitator Superfamily" (MFS); (2) "Small Multi-drug Resistance (SMR) family"; (3) "Multidrug And Toxic compound Extrusion (MATE) family" (21); (4) "Resistance-Nodulation-Division (RND) superfamily"; and (5) "Adenosine Triphosphate (ATP)-Binding Cassette (ABC) superfamily". Estes sistemas utilizam a força motriz de protões, como fonte de energia, com a excepção da família ABC, que utiliza a hidrólise do ATP para fazer a extrusão dos substratos. Um dos mais recentes desafios nesta área tem sido o desenvolvimento de novos compostos que inibam estes sistemas de efluxo e consequentemente possam potenciar a actividade de antibióticos que sejam co-administrados na terapêutica, podendo desta forma dar uma nova utilidade clínica aos antibióticos já existentes. Infelizmente e apesar de vários inibidores de bombas de efluxo (aqui designados como EPIs – "efflux pump inhibitors") terem sido sintetizados, até à

presente data, nenhum destes inibidores resultou num composto com utilidade clínica, que pudesse ser aplicado no tratamento de infecções provocadas por bactérias multi-resistentes. No entanto, a procura continua e de entre os vários tipos de EPIs caracterizados, podemos encontrar uma grande e variada gama de compostos, como: análogos peptídicos; as fenotiazinas; um grupo de produtos naturais produzidos por Streptomyces spp, ("benastatins"); compostos derivados ou homólogos da tetraciclina; compostos isolados de extractos de plantas; a quinolina e alguns dos seus derivados; arilpiperidinas and arilpiperazinas; EPIs produzidos por microrganismos e um grupo distinto de compostos, os desacopladores de energia. Se estes EPIs puderem ser utilizados como "helper compounds", em combinação com os antibióticos aos quais o microrganismo é resistente, então o tratamento destas infecções poderá ser bem sucedido. Esta nova abordagem pode permitir a re-utilização de vários antibióticos que são substratos de bombas de efluxo, bem como permitir o controlo do aparecimento e disseminação de estirpes que apresentam uma multi-resistência mediada por sistemas de efluxo. No entanto, ainda pouco se sabe acerca dos mecanismos e função destes sistemas. Desta forma, torna-se necessário desenvolver novos métodos que permitam caracterizar esta resistência, mediada pelos sistemas de efluxo. Nos últimos anos, uma série de métodos têm sido desenvolvidos com este intuito e podem contribuir para a rápida identificação de estirpes multi-resistentes. De entre a metodologia usualmente utilizada, o método que tem recebido particular destaque, tem sido o que se baseia no efluxo do brometo de etídeo. O brometo de etídeo é um conhecido substrato de bombas de efluxo e dadas as suas propriedades fluorescentes, permite a monitorização em tempo real dos sistemas de efluxo que se encontram activados numa dada estirpe bacteriana. A criação e o desenvolvimento deste e de outros métodos, torna-se portanto uma importante ferramenta para estudar e caracterizar grandes colecções de isolados clínicos que apresentam um fenótipo multi-resistente. A combinação das novas abordagens descritas anteriormente, *i.e.*, a caracterização de isolados que apresentam um fenótipo multi-resistente mediado por sistemas de efluxo, aliada à busca de novos e efectivos EPIs, pode contribuir para o controlo e tratamento eficaz de infecções multi-resistentes. O futuro o dirá…

TABLE OF CONTENTS

INDEX OF FIGURES

LIST OF ABREVIATIONS

13-CPTC	13-cyclopentylthio-5-OH tetracycline
50-MHC-D	50-methoxyhydnocarpin-D
ABC	ATP-Binding Cassette
ATCC	American Type Cell Culture
ATP	Adenosine Triphosphate
BSAC	British Society of Antimicrobial Chemotherapy
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CLSI	Clinical Laboratory Standards Institute
CPZ	Chlorpromazine
DNP	Dinitrophenol
EPIs	Efflux Pump Inhibitors
FDA	Food and Drug Administration
MATE	Multidrug And Toxic compound Extrusion
MDR	Multidrug Resistance
MFP	Membrane Fusion Protein
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MRP	Multidrug Resistance Protein
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
OMP	Outer Membrane Protein
PAβN	Phenylalanyl arginyl-β-naphthylamide
P-gP	P-glycoprotein
RND	Resistance Nodulation Division
SDS	Sodium Dodecyl Sulphate
SMR	Small Multidrug Resistance
TMS	Transmembrane Segment

1. Antibiotic resistance

One of the major scientific achievements of the 20th century was the discovery and use of antibiotics. During the early period of antibiotic usage, bacterial infections were considered tamed since antibiotics were being used to cure potentially lethal infections. However, widespread use and misuse of antibiotics has promoted the emergence of antibiotic-resistant pathogens. The most common mechanisms of resistance to antibiotics in bacteria are: i) alteration/modification of the target site (*e.g.* by mutating DNA gyrase in fluoroquinolone resistance or by producing methicillin-resistant transpeptidase in methicillin-resistant *Staphylococcus aureus*); ii) degradation of the antibiotic molecule by inactivating drugs by hydrolysis (*e.g.* via β-lactamase) or modification (*e.g.* aminoglycoside resistance) and iii) prevent access of drugs to the target or reduce the effective intracellular concentration of the antibiotic. Antibiotic resistance is wide spreading rapidly, especially in the hospital setting, where the bacterium is exposed to a constant antibiotic pressure that contributes to the development and emergence of multidrug resistant strains. Multidrug resistance (MDR) is defined as the resistance to three or more distinct classes of antibiotics (Piddock, 2006a; Tenover, 2006). One bacterium can become resistant to several distinct classes of antibiotics by genetic or physiologic mechanisms. In the last years, efflux-mediated resistance has been extensively studied and in many cases is attributable to the synergy between reduced drug uptake (mainly due to changes in outer membrane permeability) and active drug export (via efflux pumps) (Kumar and Schweizer, 2005; Langton *et al.*, 2005; Ryan *et al.*, 2001). The next sections will be focused on the relevance of the efflux systems on the resistance to antibiotics in MDR bacteria.

2. Components that prevent antibiotic from reaching their target

Some bacteria are unusually successful in surviving in the presence of toxic compounds due to the combining of several mechanisms of resistance. Some of the first components that prevent toxic compounds, such as antibiotics, from reaching their targets, involve: i) the down-regulation of porins; ii) efflux systems; or iii) increase of the

lipopolysaccharide component of the cell envelop.

2.1. The outer membrane permeability barrier of Gram-negative bacteria

Intrinsic resistance of Gram-negative bacteria has often been attributed entirely to the presence of the outer membrane barrier. The outer membrane, located outside the cytoplasmic membrane and the periplasm (Figure 1) is known to serve as a general permeability barrier that slows down the diffusion of various types of solutes, including drugs. This barrier contributes to the intrinsic drug resistance that is found on these bacteria (Nikaido, 1998c). Hydrophilic drugs cross the outer membrane barrier through water-filled channels of pore-forming proteins, called porins. The porin channels, however, impose several restrictions for the influx of various solutes (Nikaido, 2001).

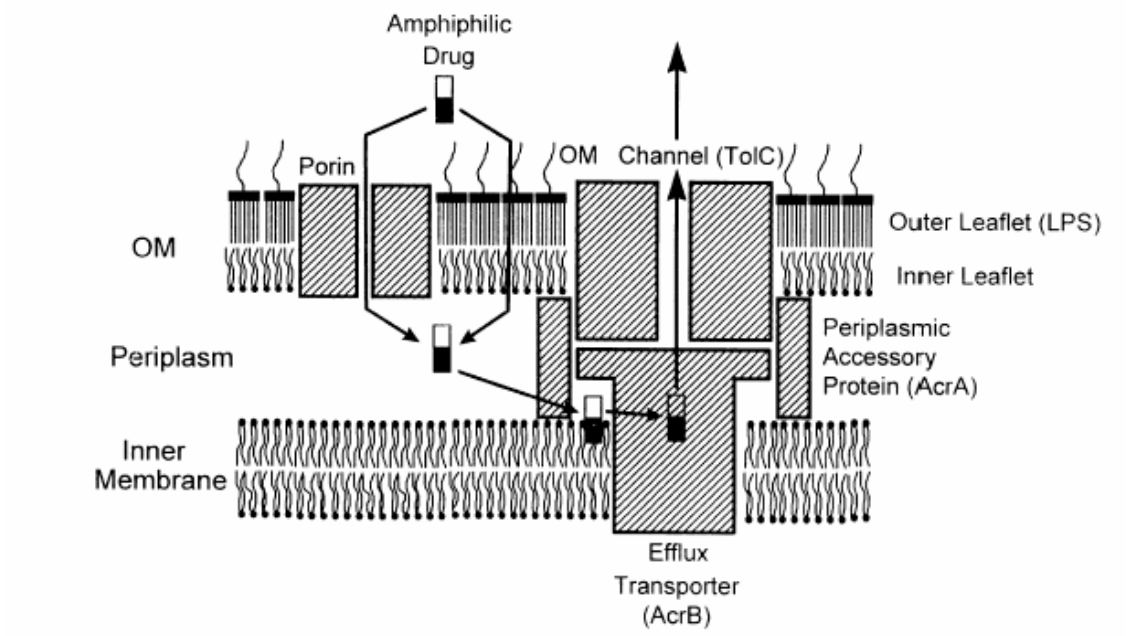


Figure 1. Outer membrane permeability barrier in Gram-negative bacteria. Drugs penetrate across the outer membrane, the more hydrophilic passing through the narrow porin channel with difficulty, and the more lipophilic penetrating through the lipid bilayer domain slowly because the outer leaflet, consisting entirely of lipopolysaccharides, has low fluidity due to the absence of unsaturated fatty acids (wavy lines). Once in the periplasm, amphiphilic drugs partition spontaneously into the cytoplasmic membrane; are captured by a transporter and pumped back into the medium by a multi-subunit complex containing a periplasmic accessory protein and an outer membrane channel (*source:* Nikaido, 2001).

One of the major porins described to date in *Escherichia coli* is an outer membrane protein, OmpF that contains a channel with the size of 8 × 10 Å at its narrowest point. If we consider that the length of a single C-C bond is 1.54 Å, we can understand that antimicrobial agents can barely go through this channel, and that even the penetration of small agents such as fluoroquinolones or chloramphenicol occurs only slowly (Nikaido, 1998a). In other Gram-negative bacteria, such as *Pseudomonas aeruginosa*, which lack the OmpF-like porins, only a very slow influx of solutes through extremely inefficient porins is possible (Nikaido, 1996; Sen and Nikaido, 1991). Lipophilic drugs, in contrast, should be able to dissolve into the hydrocarbon interior of the lipid bilayer domains of the outer membrane, and traverse the membrane by redissolving into the aqueous phase on the other side. However, even this mechanism is made difficult, because the outer half of bilayer is made of unusual lipids, lipopolysaccharides, which do not allow the easy entry of extraneous lipophilic molecules (Nikaido and Rosenberg, 1981). Several studies suggest that very lipophilic molecules, such as steroids, traverse the bilayers of the outer membrane at rates that are about two orders of magnitude lower than the rates at which they traverse the usual cytoplasmic membranes (Nikaido, 1998c; Nikaido and Rosenberg, 1981; Thanassi *et al.*, 1995).

What are the mechanisms involved in bestowing bacteria with intrinsic resistance to antibiotics and what are the mechanisms that account for MDR of bacteria? Are they the same for both types of resistance? These questions will be discussed on the next sections.

2.2. Efflux systems

In the mid-1970s, P-glycoprotein (P-gp), the transporter protein of an efflux pump of mammalian cells, was implicated as the cause for the MDR phenotype on cancer cells (Kumar and Schweizer, 2005). A similar efflux system that accounted for resistance of some bacteria to tetracycline was recognized by Stuart Levy and his associates in the 1980's (Levy and McMurry, 1978), and is now known as a major mechanism of tetracycline resistance in bacteria (Nikaido, 1998a,c). Since these studies, efflux-mediated resistance to a wide range of antibacterial agents, including antibiotics, biocides and

solvents, has been reported in many bacteria (Levy, 2002; McBain *et al.*, 2002; Piddock, 2006a,b; Poole, 2002, 2005, 2007; Russell, 2002, 2003; Schweizer, 2003). Although some of these efflux systems are drug-specific, some can be considered to be non-specific since they recognise a large gamut of structurally unrelated drugs. These latter efflux systems contribute significantly to intrinsic and acquired MDR of bacteria (Kumar and Schweizer, 2005).

Efflux systems are found in Gram-negative and Gram-positive bacteria, however, efflux mediated resistance in Gram-negative bacteria is a more complex problem due to the molecular architecture of the cell envelope (Kumar and Schweizer, 2005; Nikaido, 1998a,b,c).

2.2.1 Classes of bacterial MDR efflux systems

Efflux pumps have a marked role in the resistance of bacteria since these systems pump out a broad range of chemically and structurally unrelated noxious compounds from the bacteria, in an energy-dependent manner, without drug alteration or degradation (Webber and Piddock, 2003). It is important to note that an antibiotic is a noxious substance to bacteria and of no special significance to the bacterium other than its noxious quality. Analysis of several available bacterial genome sequences has shown that known and putative drug efflux transporters constitute from 6 to 18% of all transporters found in any given bacterial cell (Kumar and Schweizer, 2005). Efflux pumps can be classified into single- or multi-component pumps. Single-component pumps transport their substrates across the cytoplasmic membrane. Multi-component pumps, found in Gram-negative bacteria, consist of three proteins: a fusion protein which attaches the transporter protein to the surface of the lipid component of the plasma membrane; the transporter protein which recognizes a substrate present in the periplasmic space or cytoplasm immediately below the internal margin of the plasma membrane, and TolC, a tribarrel protein that is contiguous with the transporter protein and provides a channel that traverses the cell envelop through which the substrate reaches the outside (Nikaido, 1998a) (Figure 2).

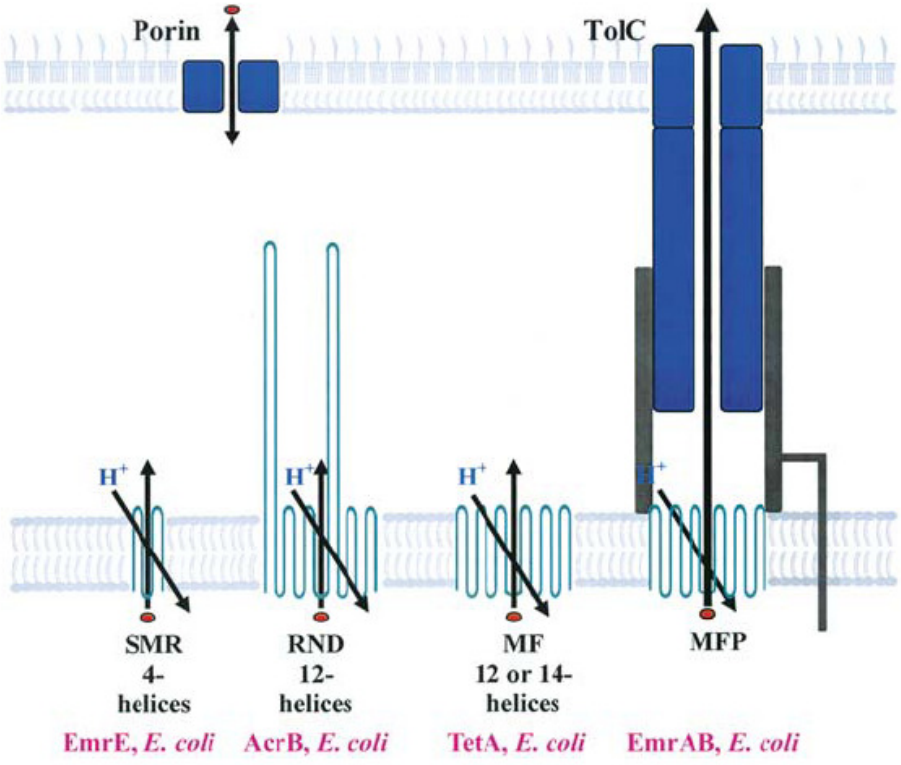


Figure 2. Diagrammatic representation of the membrane topology of single- or multi-component proton-driven pumps in Gram-negative bacteria. The three classes of antiporters shown are: SMR, RND, MFS. These transporters are shown utilizing the proton motive force generated by respiration to expel antibiotics and other drugs into the periplasmic space between the inner and outer membranes. SMR, Small Multidrug Resistance; RND, Resistance-Nodulation-Division superfamily; MFS, Major Facilitator Superfamily (*reproduced from* Borges-Walmsley *et al.*, 2003).

Single-component efflux pumps are also found in Gram-positive bacteria. The cell envelope of Gram-positive bacteria is a structure that contains a single-component efflux pumps in the cytoplasmic membrane (Marquez, 2005; Piddock, 2006a). These bacteria possess drug-specific and multidrug efflux pumps that also contribute to drug resistance (Kumar and Schweizer, 2005).

Bacterial drug efflux transporters are currently classified into five families, the: (1) Major Facilitator Superfamily (MFS); (2) Small Multidrug Resistance (SMR) family; (3) Multidrug And Toxic compound Extrusion (MATE) family; (4) Resistance-Nodulation-Division (RND) superfamily; and (5) Adenosine Triphosphate (ATP)-Binding Cassette (ABC) superfamily (Kumar and Schweizer, 2005; Piddock, 2006b) (Figure 3). Of these, the ABC and MFS superfamilies are very large and the other three are smaller families (Kumar and Schweizer, 2005).

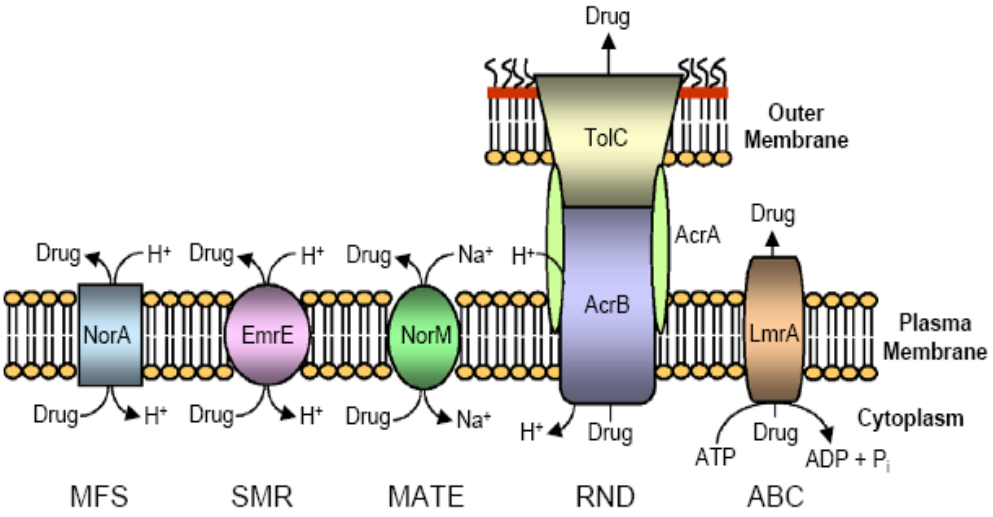


Figure 3. Schematic illustration of the main types of bacterial drug efflux pumps. Illustrated are *Staphylococcus aureus* NorA, a member of the MFS; *Escherichia coli* EmrE, a member of the SMR superfamily; *Vibrio parahaemolyticus* NorM, a member of the MATE superfamily; *E. coli* AcrAB–TolC, a member of the RND superfamily; and *Lactococcus lactis* LmrA, a member of the ABC superfamily (*reproduced from* Kumar and Schweizer, 2005).

2.2.1.1 Major Facilitator Superfamily (MFS)

The major facilitator superfamily (MFS) of transporters is an ancient superfamily that probably dates back through evolutionary time of more than three billion years (Kumar and Schweizer, 2005). This large and diverse superfamily consists of more than 300 sequenced proteins that fall into seventeen recognized, distantly related families that are specific for a different type of solute (Saier *et al.*, 1998). These families either catalyze uniport, solute/cation (H^+ or Na^+) symport, solute/H^+ antiport or solute/solute antiport

(Kumar and Schweizer, 2005) and include four families specific for various types of sugars, a fifth that catalyzes uptake of phosphorylated glycolytic intermediates, a sixth that catalyzes uptake of Krebs cycle intermediates and other metabolites, two families that catalyze drug efflux, and several that transport organic and inorganic anions (Saier *et al*., 1998). These transporters usually function as single-component pumps, *e.g.*, NorA of *Staphylococcus aureus*. However, in some Gram-negative bacteria they function with membrane fusion proteins (MFP) and OMP components, *e.g.*, EmrAB–TolC of *E. coli* (Kumar and Schweizer, 2005). MFS transporters are typically composed of approximately 400 amino acids that are putatively arranged into twelve membrane-spanning helices (domains) (Figure 4A), with a large cytoplasmic loop between helices six and seven (Borges-Walmsley *et al.*, 2003). It is most likely that this structure has arisen by gene duplication, as the two halves of the transporter usually have related sequences. A smaller number of these transporters have a putative fourteen membrane-spanning domains topology (Figure 4B); however, MFS transporters of this type tend to have a much smaller cytoplasmic loop (Borges-Walmsley *et al*., 2003; Saier *et al.*, 1998).

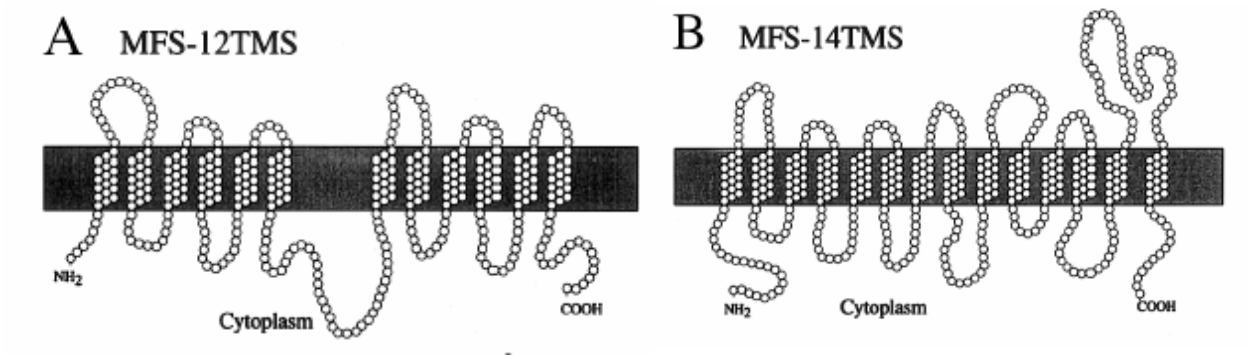


Figure 4. Structural model for the 12-transmembrane segment (A) and the 14-transmembrane segment (B) drug efflux pumps of the MFS. TMS, transmembrane segment (*reproduced from* Saier *et al*., 1998).

The MFS proteins that catalyze drug efflux are from three subfamilies: i) DHA1 that are drug/H^+ antiporters (*e.g.*, Bmr of *Bacillus subtilis*); ii) DHA2 (*e.g.*, QacA of *S. aureus* and iii) DHA3 (*e.g.*, MefA of *Streptococcus pyogenes*) (Kumar and Schweizer, 2005). The DHA1 and DHA2 family of proteins are ubiquitous among prokaryotes and

eukaryotes, and are known to efflux a very broad range of structurally distinct drugs. Members of the DHA1 family export sugars, polyamines, uncouplers, monoamines, acetylcholine, paraquat and methylglyoxal. In contrast, members belonging to the DHA2 family exhibit more restricted substrate specificity, and transported substrates include bile salts and dyes. Members of the DHA3 family are only found in prokaryotes, and are known to efflux antibiotics, including macrolides and tetracycline. Tetracycline efflux pumps constitute some of the best-characterized members of the MFS family. These pumps are found in both Gram-negative and Gram-positive bacteria. Most of them confer resistance to tetracycline, but not to minocycline or glycylcyclines. However, some Gram-negative tetracycline proteins confer resistance to both tetracycline and minocycline, but not to glycylcyclines (Kumar and Schweizer, 2005).

2.2.1.2 Small Multidrug Resistance (SMR) family

SMR transporters are the smallest of the known bacterial efflux pumps, and it is difficult to imagine how a single SMR protein could comprise a functional drug transporter unit. SMR transporters are much smaller than those belonging to the MFS and RND families (Borges-Walmsley *et al*., 2003). SMR family transporters are normally composed of approximately 110 amino acid residues putatively arranged into four domains (Figure 5) and are energized by the proton motive force (Saier *et al*., 1998).

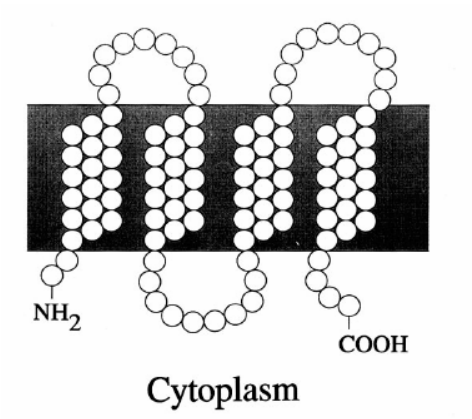


Figure 5. Structural model for members of the SMR family (*reproduced from* Saier *et al*., 1998).

The SMR family consists of two phylogenetic subfamilies. Members of one subfamily confer multidrug resistance and catalyze drug efflux via a drug:H^+/antiport mechanism, as do the corresponding MFS drug resistance proteins (Rotem and Schuldiner, 2004) However, members of the other subfamily apparently do not confer drug resistance or catalyze drug:H^+/ antiport (Kumar and Schweizer, 2005). Subdivision of the SMR family into two phylogenetic clusters and the observation that the members of only one of these clusters apparently catalyze drug extrusion argue strongly that, for the SMR family, drug resistance permeases arose only once during its evolutionary history (Saier *et al*., 1998). Some of the well characterized pumps of this family include the Smr pump of *S. aureus* and the EmrE pump of *E. coli*, which efflux dyes, drugs and cations (Kumar and Schweizer, 2005). EmrE from *E. coli* is a multidrug transporter that contributes to resistance to ethidium bromide and methyl viologen (Yelin *et al*., 1999; Yerushalmi *et al.*, 1995). Another SMR efflux pump from *P. aeruginosa* with close identity with EmrE has also been characterized and is shown to play an important role in the intrinsic resistance of *P. aeruginosa* to ethidium bromide, acriflavine and aminoglycoside antibiotics (Li *et al.*, 2003).

2.2.1.3 Multidrug And Toxic compound Extrusion (MATE) family

It is important to stress that in prokaryotes, even though H^+-driven antiport is the major mechanism of drug efflux, other important transport mechanisms have also been described, like those described for a group of transport proteins, the MATE family. Previously thought to be members of the MFS, the proteins belonging to the MATE family are now recognized as a separate family of transporters because, despite similar membrane topology, they show no sequence homology to MFS proteins (Kumar and Schweizer, 2005). Since they are a relatively new family among bacterial drug transporters they are the least well characterized. However, this situation is changing rapidly, especially because they appear to play an important role in drug resistance to clinically relevant antibiotics in pathogenic organisms. The common premise shared by this group of proteins is that drug efflux is coupled to Na^+ influx (Kumar and Schweizer, 2005). MATE transporters are similar in size to the MFS transporters, and are typically

composed of approximately 450 amino acid residues in length which are putatively arranged into twelve predicted transmembrane segments (Omote *et al.*, 2006). Proteins belonging to this family use the Na^+ gradient as the energy source to efflux cationic dyes and fluoroquinolones (Burse *et al.*, 2004) (Figure 6). Examples of proteins belonging to this family include NorM, a multidrug Na^+-antiporter from of *Vibrio parahaemolyticus* which confers resistance to dyes, fluoroquinolones and aminoglycosides (Otsuka *et al.*, 2005). Homologues of NorM have been found and characterized in *E. coli*, *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Long *et al.*, 2008; Rouquette-Loughlin *et al.*, 2003; Yang *et al.*, 2003). In *E. coli,* YdhE, was shown to confer resistance to cationic antimicrobials (Long *et al.*, 2008) and in *Neisseria gonorrhoeae* and *Neisseria meningitidis* deletions of these pumps resulted in an increased susceptibility to cationic compounds (Rouquette-Loughlin *et al.*, 2003).

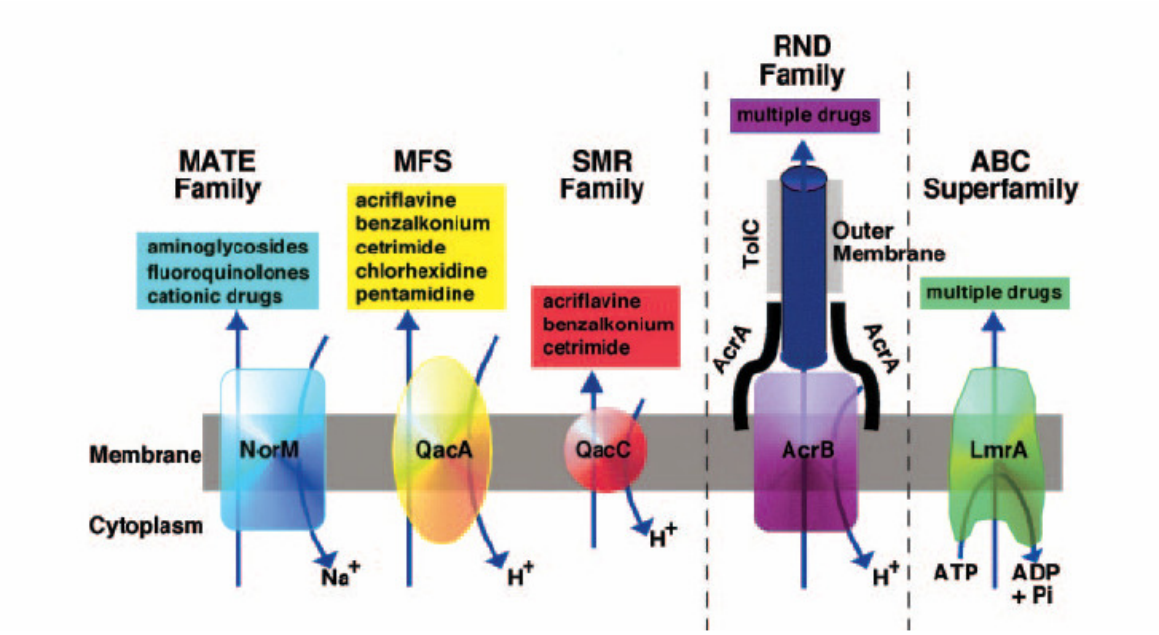


Figure 6. Diagrammatic comparison of the five families of efflux pumps and the specificity of the substrates extruded from the cell. The RND and ABC families are involved on the extrusion of multiple drugs. The MATE family extrudes mainly aminoglycosides, fluoroquinolones and cationic drugs. The MFS and SMR families are involved on the extrusion of more specific substrates, such as acriflavine, benzalkonium and cetrimide (*reproduced from* Piddock, 2006a).

2.2.1.4 Resistance Nodulation Division (RND) superfamily

It was originally thought that proteins from the RND superfamily were exclusively found in eubacteria (Kumar and Schweizer, 2005). However, several studies have also reported their presence in eukaryotes and archaea (Paulsen *et al.*, 1996a; Tseng *et al.*, 1999). RND transporters are typically encoded by chromosomal genes (Kumar and Schweizer, 2005), but a plasmid-encoded RND drug transporter has already been reported (Hansen *et al.*, 2004, 2007). Like the SMR family, the RND family is a small, bacterial-specific family. However, RND transporters are much larger than MFS transporters, being composed typically of approximately 1000 amino acid residues (Saier *et al*., 1998). Even with this obvious disparity in size, they are predicted to adopt a similar twelve-helical structure. However, unlike MFS transporters, they possess large periplasmic or extra-cytoplasmic domains between helices 1 and 2 and between helices 7 and 8 (Borges-Walmsley *et al*., 2003; Saier *et al*., 1998). They possess an unusual putative topology characteristic of the family (Figure 7).

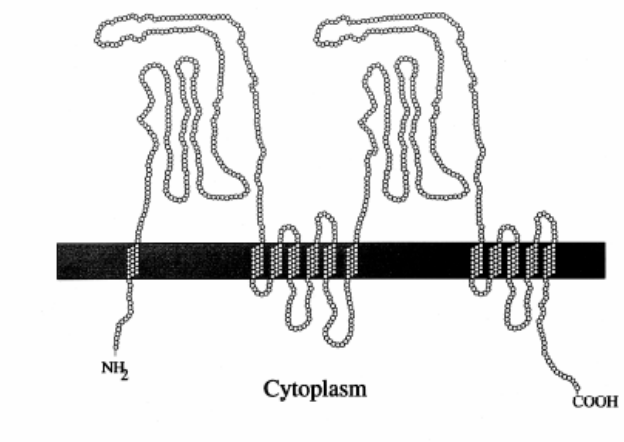


Figure 7. Structural model for representative members of the RND family (*reproduced from* Saier *et al*., 1998).

At the amino-terminal end of each protein, the polypeptide chain probably traverses the cytoplasmic membrane once from cytoplasm to periplasm, and this spanner is followed by a large water soluble domain localized to the periplasmic or extra-cytoplasmic space.

The polypeptide chain then spans the membrane six more times before it again emerges into the periplasm as another water-soluble domain of the same size as the first one. The carboxyl-terminal end of the permease is again embedded in the membrane with five additional spanners. Thus, each permease has twelve putative spanners as well as two large, presumably extra-cytoplasmic domains (Figure 7) (Saier *et al*., 1998). Phylogenetic studies performed on members of the RND family revealed that these proteins fall into three subfamilies. Members of one subfamily are specific for divalent heavy metal ions; those of the second are probably specific for lipooligosaccharides (example, a single putative three-component transporter), and those of the third subfamily all catalyze efflux of multiple drugs (Paulsen *et al.*, 1996b; Saier *et al.*, 1994). All members characterized to date catalyze substrate efflux via a substrate/H^+ antiport mechanism. RND pumps play an important role in acquired and intrinsic resistance of Gram-negative bacteria to a variety of antimicrobials and all RND pumps studied to date are multidrug transporters (Kumar and Schweizer, 2005). In Gram-negative bacteria, RND pumps function by forming complexes consisting of an RND membrane transport protein with twelve transmembrane segments, a membrane fusion protein and an outer membrane protein (Figure 8). A characteristic feature of RND transporter topology is the presence of 2 large periplasmic loops between transmembrane segment 1 and 2 and transmembrane segment 7 and 8. The N-terminal halves of RND family proteins are homologous to the C terminal halves and, as such, these proteins are believed to have arisen from an gene duplication event that occurred before the divergence of the family members (Borges-Walmsley *et al*., 2003). There is, however, one example of an RND homologue from *Mycobacterium jannaschii* that has only six transmembrane segments and no internal duplication (Kumar and Schweizer, 2005). It is possible that this protein functions either as a homodimer or as a heterodimer, or by association with another protein. The best-studied members of RND pumps are the AcrAB–TolC system of *E. coli* (Elkins and Nikaido, 2002, 2003a,b; Zgurskaya and Nikaido, 1999, 2000) and the MexAB–OprM system of *Pseudomonas aeruginosa* (Evans *et al.*, 1998; Köhler *et al.*, 1997; Li *et al.*, 1995; Masuda *et al.*, 1999; Nakae *et al.*, 1999) that are known to efflux antibiotics, heavy metals, dyes, detergents, solvents, plus many other substrates (Kumar and Schweizer, 2005).

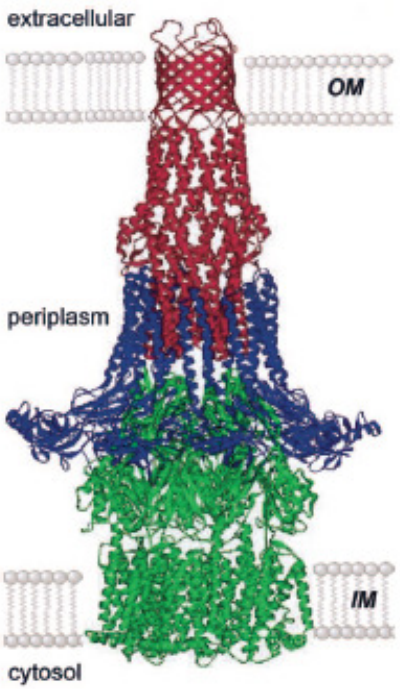


Figure 8. Model of a tripartite efflux pump. This hypotetical model of an efflux pump belonging to the RND family is based on the open-state model of TolC (represented in red) forming a minimal contact interface with the six hairpins at the apex of AcrB (represented in green). A ring of nine MexA molecules (represented in blue) is modelled to form a sheath around AcrB and the α-barrel of TolC. IM, inner membrane; OM, outer membrane (*reproduced from* Piddock, 2006a).

2.2.1.5 ATP-Binding Cassette (ABC) superfamily

The ABC transporters superfamily are ubiquitous membrane systems (Marquez, 2005), that shows both uptake and efflux transport systems and consists of numerous families, each specific for one of a tremendous variety of substrates (Davidson and Chen, 2004). These substrates include small molecules that may be taken up or expelled from the cell, depending on the transporters, and also macromolecules such as proteins and complex carbohydrates that are synthesized in the cytoplasm and secreted to the cell envelope or the external milieu (Davidson and Maloney, 2007; Marquez, 2005). For all bacterial ABC transport systems, ATP provides the source of protons that drives transport. Members of this superfamily use energy derived from ATP hydrolysis to transport this variety of substances (Davidson and Chen, 2004; Davidson and Maloney, 2007). Transporters of

the ABC-type are multi-protein complexes consisting of: 1) integral membrane proteins (presumably forming a transport pore through the cytoplasmic membrane and 2) energy-coupling cytoplasmic proteins with ATPase activity (Kumar and Schweizer, 2005). Bacterial ABC permeases generally contain 6 transmembrane segments each and associate in the membrane in pairs as either homo- or hetero-dimers. Two ATPase subunits associate with the permeases on the cytoplasmic face of the inner membrane to form functional transporters (Davidson and Maloney, 2007) (Figure 9).

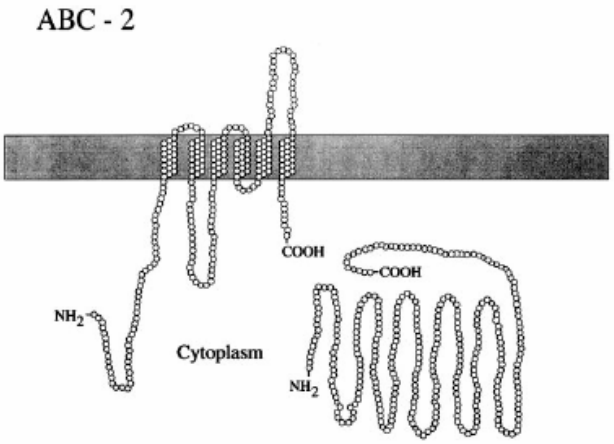


Figure 9. Generalized structural model for ATP binding cassette-2 (ABC-2) family permeases (*reproduced from* Saier *et al*., 1998).

Drug efflux pumps belonging to the ABC superfamily are rare in bacteria, however, a few have been identified, namely, the LmrA, multidrug transporter from *Lactococcus lactis* (Poelarend *et al.*, 2000); the DrrAB, doxorubicin/daunorubicin transporter from the anthracycline-producing actinomycete *Streptomyces peucetius* (Gandlur *et al.*, 2004), EfrAB of *Enterococcus faecalis* (Lee *et al.*, 2003), and the MacB transporter from *E. coli* that is involved in the efflux of macrolides (Kobayashi *et al.*, 2001). From all these, probably the LmrA pump of *Lactococcus lactis* being the most studied system (Borges-Walmsley, *et al*., 2003; Poelarend *et al.*, 2000). In contrast with prokaryotes, the major mechanism of efflux in eukaryotes is dependent on proteins that belong to the ABC superfamily of membrane transporters. Members of this family include the clinically significant MDR pump, P-gp (Davidson and Maloney, 2007; Marquez, 2005) and multidrug resistance protein (MRP) (Borges-Walmsley, *et al*., 2003), both of which

confer resistance to anticancer drugs. Related transporters are also found in a number of pathogenic fungi and parasitic protozoa, where they confer resistance to antimicrobial drugs. One of these examples is P-gpA, a MRP homologue, which is an arsenic/antimony pump that is responsible for resistance to the antimonial drug Pentostam in *Leishmania* (Borges-Walmsley, *et al*., 2003; Légaré *et al.*, 2001). Although most ABC transporters were discovered as drug transporters, they frequently transport a wide range of substrates, including dyes, ionophoric peptides, lipids and steroids (Borges-Walmsley, *et al*., 2003). Phylogenetic studies of the families of the ABC superfamily showed that members of the ABC-2 family include proteins that catalyze the export of cell surface carbohydrates synthesized within the bacterial cell. It seems that substrate specificity has been a well conserved trait during evolution of the ABC-2 family. Moreover, drug resistance apparently evolved only once during the early evolution of this ABC subfamily, and all members of the family that catalyze drug resistance were therefore probably derived from a single primordial permease (Davidson and Maloney, 2007). Whether these ABC-2 drug resistance pumps are drug-specific or capable of transporting multiple drugs has not been tested.

In summary, the efflux systems previously described contribute to the increased resistance of some bacterial strains. Some of these systems are specific for a single drug or substrate while others are capable of transporting multiple and unrelated compounds (Piddock, 2006a,b; Poole 2007). The over-expression of these systems results in sub-therapeutic intracellular concentrations of antibiotics and the subsequent therapeutic failure. The wide variety of efflux systems and their plasticity to extrude antimicrobials increases the bacterial intrinsic resistance to a wide spectrum of structurally and functionally unrelated antibiotics and is undoubtedly a major problem in the treatment of multidrug resistant infections (Rouveix, 2007). In clinical isolates the resistance-mediated by enhanced efflux is increasing and this will have an impact on the therapeutic choices that are available (Rouveix, 2007; Webber and Piddock, 2003). By this manner, these efflux systems need to be identified as early as possible as drug resistance develops in a patient under treatment in order to adjust the therapeutic strategies and minimize the selection of genetically resistant variants. The inappropriate use of antibiotics will

increase the risk of selecting genetically resistant bacteria, since sub-therapeutic drug levels may only suppress bacteria, but not eliminate them.

3. Clinical relevance of MDR efflux pumps

Since one of the causes that contribute to the MDR in clinical isolates is the over-expression of bacterial efflux pumps, the assessment of these systems on a clinical isolate has been the subject of intense research on the last few years (Giske *et al.*, 2008; Kern *et al.*, 2006; Kriengkauykiat *et al.*, 2005; Pannek *et al.*, 2006; Piddock, 2006a). The creation of new agents which have the capacity to inhibit the MDR efflux pumps and hence render the organism susceptible to the antibiotic(s) to which it was once resistant is one of the main goals of research (Kamicker *et al.*, 2008; Lomosvskaya and Watkins, 2001a,b; Lomovskaya *et al.*, 2001; Mahamoud *et al.*, 2007; Nguyen and Thompson, 2006). However, prior to the evaluation of an efflux pump inhibitor (EPI), the existence of an efflux pump responsible for a MDR phenotype of a clinical isolate must first be shown (Piddock, 2006b; Poole, 2007). In the clinical setting, there are some particular species that show an increasing predisposition towards resistance to drugs, being considered among the most infectious organisms (Piddock, 2006b; Webber and Piddock, 2003). Some of the efflux systems present on these bacteria will be the subject of discussion in the next sections.

3.1 Gram-positive bacteria

The cell envelope of Gram-positive organisms shows a relatively simple structure when compared to that of Gram-negative organisms in that the cytoplasmic membrane is surrounded by a single thick layer of peptidoglycan (Marquez, 2005; Piddock, 2006a). Drug-specific and multidrug efflux pumps have been described in Gram-positive bacteria, some of which make important contributions to drug resistance, especially to macrolides and fluoroquinolones (Kumar and Schweizer, 2005). Some of the more clinically important Gram-positive drug efflux pumps will therefore be briefly discussed in the following paragraphs.

3.1.1 *Staphylococcus aureus*

Staphylococcus aureus is a major cause of hospital-acquired infections (Moreillon, 2008; Shorr, 2007). Efflux pumps characterized in this organism include QacA (MFS family) (Paulsen *et al.*, 1996b), Smr (SMR family) and NorA (MFS family) (Piddock, 2006a). QacA and Smr are examples of plasmid-encoded efflux pumps, while NorA is chromosomally encoded. QacA has been shown to efflux acriflavine, crystal violet, diamidines, ethidium bromide and quaternary ammonium compounds (Brown and Skurray, 2001; DeMarco *et al.*, 2007). The NorA efflux pump has been shown to be responsible for moderate fluoroquinolones resistance of *S. aureus* (Sabatini *et al.*, 2008), due to a weakly expressed *norA* gene (Piddock, 2006a). This pump is responsible for resistance of *S. aureus* to hydrophilic fluoroquinolones only and does not extrude lipophilic substrates (Kumar and Schweizer, 2005). NorA is present in both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). The agents used to treat infections by MSSA include flucloxacillin, nafcillin, and ciprofloxacin. The agents used to treat MRSA include ciprofloxacin (where the MRSA strain has been shown to be susceptible), vancomycin, and linezolid (Piddock, 2006a). It has been shown that the minimum inhibitory concentration (MIC) of nafcillin and vancomycin are unaffected by the over-expression of NorA (Kaatz *et al.*, 2003). Other efflux pump genes are also present on the *S. aureus* genome and have been investigated. Over-expression of NorB confers decreased susceptibility to fluoroquinolones, tetracycline, disinfectants, and dyes (Truong-Bolduc *et al.*, 2005). Over-expression of Tet38 confers resistance to tetracycline only (Truong-Bolduc *et al.*, 2006). Over-expression of MepA confers resistance to fluoroquinolones and biocides (DeMarco *et al.*, 2007; Li and Nikaido, 2004). Further studies are needed on other putative transporters to see if other transporters are also involved in antimicrobial resistance in *S. aureus*. In addition, any clinical relevance of these new transporters has yet to be defined. *S. aureus* NorA has been shown to have 44% amino acid identity and 67% similarity with Bmr. Bmr and NorA are structurally similar to the plasmid-encoded efflux proteins TetA, TetB, and TetC, with 24 to 25% sequence identity with these proteins (Piddock, 2006a). Plasmid-encoded over-expression of both Bmr and NorA confers MDR to fluoroquinolones,

chloramphenicol, antiseptics, dyes, and disinfectants (Kaatz and Seo, 1997, 2004; Kaatz *et al.*, 1993; Neyfakh *et al.*, 1993; Piddock, 2006a).

3.1.2 *Streptococcus pneumoniae*

Over the last decade, considerable effort has been expended by pharmaceutical companies to develop anti-pneumococcal agents, so there has been considerable focus on *Streptococcus pneumoniae* and the presence of efflux pump proteins that could confer MDR, including to new agents (Piddock, 2006a). *S. pneumoniae* is a major cause of respiratory tract infections. Some isolates are resistant to a wide range of antibiotics that include β-lactams, macrolides, quinolones and tetracycline (Li and Nikaido, 2004; Piddock, 2006a). Efflux mechanisms have been shown to play a role in quinolone resistance of this organism, though target mutations are believed to be the main contributors to this resistance (Kumar and Schweizer, 2005). In 1999, Gill *et al*. identified PmrA (Gill *et al.*, 1999). This protein has 43% amino acid similarity with NorA and 42% similarity with Bmr (Piddock, 2006a) and its expression causes a 2- to 4-fold increase in resistance to several fluoroquinolones. PmrA knockouts do not exhibit changes in antibiotic susceptibility patterns, suggesting that this pump is not expressed in wild-type strains (Brenwald *et al.*, 2003). Macrolide resistance in *S. pneumoniae* is conferred by the MefE pump of the MFS family (Klaassen and Mouton, 2005). MefE is 90% identical to MefA of *S. pyogenes*, and together these two pumps are referred to as Mef(A) (Tait-Kamradt *et al.*, 1997). They have been shown to efflux both 14- and 15-membered macrolides and are responsible for approximately 70% of the macrolide resistance of *S. pneumoniae* observed in the United States (Zhong and Shortridge, 2000). The *mef(A)* gene of *S. pneumoniae* is part of a mobile element that is transferable by transformation (Kumar and Schweizer, 2005).

3.1.3 *Bacillus subtilis*

Two different MFS-type efflux pumps have been identified and characterized in *B. subtilis*: Bmr and Blt (Kumar and Schweizer, 2005). There is little clinical significance of

the Bmr efflux pump in human and veterinary medicine, but it has been shown that the NorA pump of *S. aureus* and PmrA of *S. pneumoniae* have significant similarity and identity at the DNA and amino acid levels (Piddock, 2006b). Therefore, a considerable number of analogies have been made between the properties of Bmr and of NorA and PmrA (Piddock, 2006a). Despite the fact that *B. subtilis* is not a clinically important organism, these two pumps (Bmr and PmrA) provide an excellent model system for mechanistic studies of MFS-type multidrug efflux systems. Substrates for these two pumps include fluoroquinolones, ethidium bromide and energy inhibitors (Kumar and Schweizer, 2005; Piddock, 2006a).

3.1.4 Mycobacteria

Mycobacteria, among which are the important human pathogens *Mycobacterium tuberculosis* and *Mycobacterium leprae*, are Gram-variable bacteria, often considered as Gram-positive, that display marked intrinsic resistance to a variety of antimicrobial agents, and this property is caused by their unique cell wall structure, which is rich in long-chain fatty acids such as C60 to C90 mycolic acids (Brennan, 2003). Mycolic acids are covalently linked to the peptidoglycan-associated polysaccharide arabinogalactan. Moreover, mycobacterial porins, the water-filled channel proteins which form the hydrophilic diffusion pathways through the cell wall, are sparse (Brennan and Nikaido, 1995; Draper, 1998). A major porin of *Mycobacterium smegmatis*, MspA, forms a tetrameric complex with a single central pore, but the density of this protein is 50-fold lower than that of porins of Gram-negative bacteria (Hillmann *et al.*, 2007). Thus, the mycobacterial cell wall functions as an even more efficient protective barrier than the outer membrane of Gram-negative bacteria and limits the access of drug molecules to their cellular targets (Nguyen and Thompson, 2006). The cell wall barrier alone, however, is not sufficient to explain the intrinsic drug resistance of these bacteria. Drug efflux, is now known to contribute to intrinsic or acquired resistance in mycobacteria (Li *et al.*, 2004; Viveiros *et al.*, 2003). Several efflux pumps of different classes have been described for *Mycobacterium tuberculosis* and/or *M. smegmatis* (Danilchanka *et al.*, 2008; De Rossi *et al.*, 2002, 2006; Escribano *et al.*, 2007; Gupta *et al.*, 2006; Jiang *et al.*,

2008; Li *et al.*, 2004; Montero *et al.*, 2001). All of the five classes of drug efflux transporters can be identified in the genome sequences of several mycobacteria, including *M. tuberculosis* (http://www.sanger.ac.uk/Projects/M_tuberculosis). Indeed, drug efflux pumps have been described in several mycobacteria to date. For example, *M. smegmatis* LfrA, an MFS transporter homologous to the QacA multidrug pump of *S. aureus*, was the first multi-drug efflux pump reported for mycobacteria (Liu *et al.*, 1996; Takiff *et al.*, 1996). Since then, several other mycobacterial drug efflux pumps have been reported. When expressed on a plasmid, LfrA mediates low-level resistance to fluoroquinolones and other toxic compounds such as ethidium bromide (Liu *et al.*, 1996). EfpA, Tap, and P55 are three other MFS pumps reported for several mycobacterial species, and of these pumps, Tap and P55 are known to produce low-level resistance to aminoglycosides and tetracyclines when introduced on multi-copy plasmids (Aínsa *et al.*, 1998). In addition to the MFS pumps, Mmr (an SMR pump) and DrrAB (an ABC exporter) were reported in *M. tuberculosis* (Choudhuri *et al.*, 2002; De Rossi *et al.*, 1998). Despite the presence of a large number of putative drug efflux genes in the genomes of *M. tuberculosis*, *M. bovis*, and *M. smegmatis*, the role of these drug exporters in intrinsic drug resistance of mycobacteria remains largely unknown, except for the study of *lfrA* gene disruption strain in *M. smegmatis* (Sander *et al.*, 2000). Several other pumps have also been shown to be involved in the transport of several different antibiotics, including fluoroquinolones, aminoglycosides, tetracycline, rifampin, and possibly isoniazid and ethambutol. However, it is not completely clear which of these are associated with antibiotic resistance in mycobacterial clinical isolates (Piddock, 2006b).

3.2 Gram-negative bacteria

Although Gram-negative bacteria contain efflux pumps representing the five superfamilies, RND pumps are the most prominent (Poole, 2007). They not only play a major role in both intrinsic and acquired resistance of many Gram-negative bacteria to a variety of clinically significant antibiotics, but also export biocides, dyes, detergents and organic solvents (Denyer and Maillard, 2002; Kumar and Schweizer; 2005; Piddock, 2006b). These tripartite pumps span the entire Gram-negative cell envelope and are thus

uniquely suited to synergize with reduced outer membrane permeability to impart drug resistance. This may be the main reason why fewer non-RND family MDR efflux systems promote resistance to clinically relevant antibiotics. Notable exceptions may be the following: NorA (MFS) in *Bacteroides fragilis* (Miyamae *et al.*, 1998); MdfA (MFS) (Adler and Bibi, 2002), MacAB–TolC (ABC) (Kobayashi *et al.*, 2001) in *E. coli*; NorM (MATE) in *Vibrio parahaemolyticus* (Morita *et al.*, 1998); BcrA (MFS) in *Burkholderia cepacia* (Wigfield *et al.*, 2002); NorM (MATE) in *Burkholderia vietnamensis* (Fehlner-Gardiner and Valvano, 2002); and VcmA (MATE) in *Vibrio cholerae* (Huda *et al.*, 2001). One of the major problems of multi-resistance in Gram-negative bacteria is that most of these bacteria are among the most naturally resistant (intrinsically resistant) organisms even in the absence of antimicrobial selective pressure. *P. aeruginosa, Acinetobacter baumanni* and *Stenotrophomonas maltophilia* are three examples of important pathogens that are resistant to many commonly used antibiotics (Kumar and Schweizer, 2005). Moreover, in the presence of selective pressure they can emerge resistant to even the relatively few antibiotics that have activity (Rice, 2006). It is worthwhile to consider the common characteristics of these and other clinically representative Gram-negative bacteria in the following sections.

3.2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic human pathogen that causes infections in patients who suffer from burn or cystic fibrosis. It shows a high level of intrinsic resistance to a very large number of antimicrobial agents (Rouveix, 2007). This resistance has historically been attributed to the presence in this organism of an outer membrane of low permeability (Mesaros *et al.*, 2007), but it is increasingly clear that resistance owes much to the operation of broadly specific, so-called multidrug efflux systems (Poole, 2007; Schweizer, 2003) that work synergistically with limited outer membrane permeability (Nehme *et al*, 2004). Several multi-drug efflux systems have been described to date (Chuanchuen *et al.*, 2002; Hirakata *et al.*, 2002; Hocquet *et al.*, 2007; Quale *et al.*, 2006), although the major system contributing to intrinsic MDR is encoded by the *mexAB-oprM* operon (Lim *et al.*, 2002). MexAB-OprM accommodates a broad range of

structurally diverse antimicrobials, including dyes, detergents, inhibitors of fatty acid biosynthesis, organic solvents, disinfectants, and clinically relevant antibiotics (Poole, 2001; Schweizer, 2003), and is implicated in the export of homoserine lactones involved in quorum sensing (Evans *et al.*, 1998; Poole, 2001) and, possibly, virulence factors (Hirakata *et al.*, 2002). In addition to the MexAB-OprM system, other RND efflux pumps have also been characterized: MexXY-OprM, MexCDOprJ, and MexEF-OprN (Linares *et al.*, 2005; Wolter *et al.*, 2004), but either are not expressed at high levels in wild-type strains or show only a limited substrate range (Babayan and Nikaido, 2004). Like the MexAB-OprM system, MexXY-OprM is constitutively expressed in wild-type cells and confers intrinsic MDR. However, MexCD-OprJ and MexEF-OprN are inducible by some of their substrates. In addition to exporting fluoroquinolones, tetracyline, chloramphenicol, and some β-lactams, these pumps also export ethidium bromide, acriflavine, sodium dodecyl sulphate, triclosan, organic solvents, and acylated homoserine lactones involved in quorum sensing. Infections by *P. aeruginosa* are usually treated with ceftazidime, ciprofloxacin, imipenem, gentamicin, tobramycin, ticarcillin-clavulanate, or piperacillin-tazobactam in combination or alone. Some of these agents are substrates of the Mex efflux pumps. However, despite the increase in MIC when these pumps are over-expressed, for agents such as ciprofloxacin the increase may not take the MIC above the recommended breakpoint concentration (Piddock, 2006a; Poole, 2001). Recently, a MATE transporter, PmpM has also been described (He *et al.*, 2004). PmpM transports fluoroquinolones, benzalkonium chloride, ethidium bromide, acriflavine, and tetraphenylphosphonium chloride. This system uses hydrogen ions, but not sodium ions, as an energy source (Piddock, 2006a).

3.2.2 *Escherichia coli*

Analysis of the *E. coli* genome has revealed the presence of many RND transporters (Piddock, 2006b). To date, some of these have been functionally characterized and confirmed to participate in drug efflux, such as: AcrAB, AcrEF, AcrD, YhiUV and MdtABC (Piddock, 2006a; Poole, 2007). All *E. coli* RND pumps studied so far have been found to be associated with the TolC outer membrane protein channel. The AcrAB-TolC

system *in E. coli* has been identified as the predominant drug efflux pump of this organism (Piddock, 2006a) and is the more studied and described MDR mechanism (Elkins and Nikaido, 2002). The two genes of this system, *acrA* and *acrB*, encode a MFP and a cytoplasmic membrane efflux pump of the RND family, respectively. They confer resistance in *E. coli* to a variety of lipophilic and amphiphilic drugs, dyes, and detergent molecules that include tetracycline, chloramphenicol, fluoroquinolones, β-lactams, erythromycin, fusidic acid, ethidium bromide, crystal violet, sodium dodecyl sulphate (SDS), and bile acids. Genetic studies showed that both genes were required for this resistance (Piddock, 2006a). Further mutational analysis suggested that this process also required TolC and therefore that the system probably functions as a tripartite complex: the AcrAB-TolC (Elkins and Nikaido, 2002) (Figure 10).

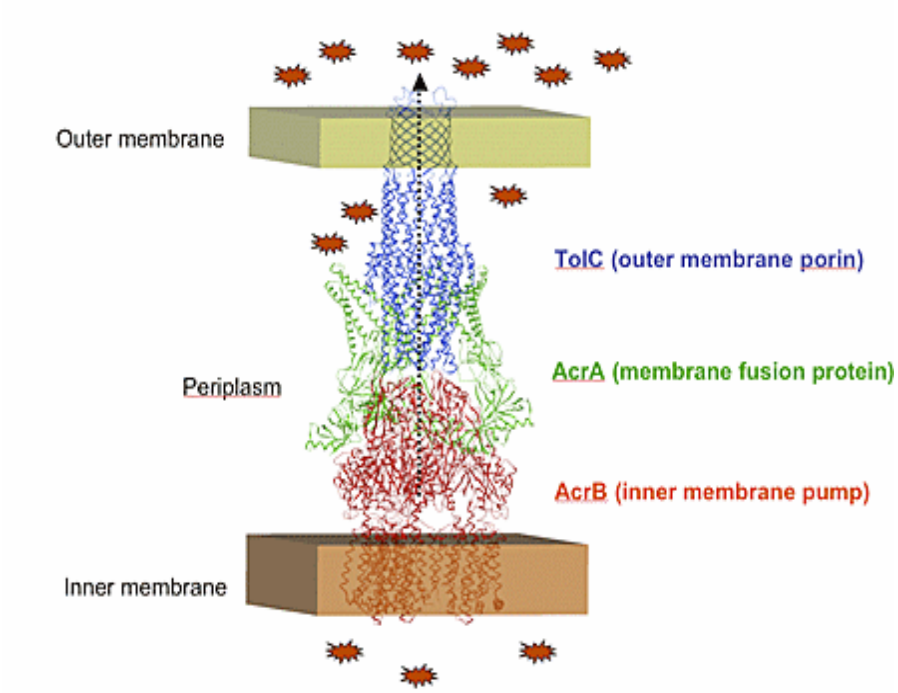


Figure 10. Structural organization of the tripartite efflux system, AcrAB-TolC from *E. coli*. The AcrAB-TolC is the major, constitutively expressed, tripartite multidrug efflux system in *E. coli* that recognizes various structurally unrelated molecules, including many antibiotics, dyes, and steroids. The AcrB inner membrane pump portion is thought to bind substrates at multiple sites, suggesting that particular substrate may compete for efflux by interfering with the binding site (*reproduced from* www.mpexpharma.com/efflux.html).

This tripartite complex is highly homologous to the MexAB-OprM RND system in *P. aeruginosa* (Borges-Walmsley, *et al*., 2003; Piddock, 2006a; Poole, 2007). AcrA is a 397-amino-acid protein that interacts with AcrB, a much larger protein that contains 1,048 amino acids. TolC, a 506-amino-acid protein, is also associated with AcrA (Piddock, 2006a). AcrB is thought to capture its substrates preferentially from within the outer leaflet of the cytoplasmic membrane (Sennhauser *et al.*, 2007; Tikhonova and Zgurskaya, 2004; Yu *et al.*, 2003). The drugs are extruded across the periplasmic space and the outer membrane via the combined action of AcrA and the TolC channel. This system is advantageous over simple cytoplasmic membrane pumps because it can extrude drugs directly into the extracellular medium (Elkins and Nikaido, 2002). The AcrAB–TolC system demonstrates a very broad substrate specificity that includes: chloramphenicol, lipophilic β-lactams, fluoroquinolones, tetracycline, rifampin, novobiocin, fusidic acid, nalidixic acid, ethidium bromide, acriflavine, bile salts, short-chain fatty acids, SDS, Triton X-100 and triclosan (Piddock, 2006a; Poole, 2007). Not all MDR efflux pumps export exclusively lipophilic and amphiphilic substrates. In *E. coli*, *acrD* and the *acrEF* operon also encode efflux pumps. The AcrD pump was originally believed to function as a single component pump for the efflux of a variety of aminoglycosides, a very hydrophilic class of drugs, and its gene does not form an operon with a membrane fusion protein gene (Nishino and Yamaguchi, 2001; Piddock, 2006a). Several studies showed that it actually requires AcrA and TolC to efflux bile salts, novobiocin and aminoglycosides (Rosenberg *et al.*, 2003) and can also mediate resistance to a limited range of amphiphilic compounds such as SDS, deoxycholate, and novobiocin (Elkins and Nikaido, 2002). The AcrEF pump is not expressed in wild-type cells, but is expressed in fluoroquinolone-resistant mutants that lack the AcrAB pump (Jellen-Ritter and Kern, 2001). AcrE and AcrF are 80 and 88% similar to AcrA and AcrB, respectively (Piddock, 2006a). This could predict a similar role for these pumps; however, knockout experiments with AcrEF, YhiUV and MdtABCD did not change drug susceptibilities of the wild-type strain of *E. coli*, suggesting that these pumps do not play a significant role in the antimicrobial resistance of this organism (Elkins and Nikaido, 2003). However it is known that this system confers resistance to solvents (Ramos *et al.*, 2002). Interestingly, the AcrF protein was shown to function with AcrA and TolC for solvent efflux (Elkins

and Nikaido, 2003), suggesting the components of the RND complex may be interchangeable. When over-expressed, the YhiUV pump is responsible for resistance to doxorubicin, erythromycin, deoxycholate and crystal violet, while the MdtABC system confers resistance to bile salts and novobiocin (Piddock, 2006a). The MdtABC system contains two different RND transporters, MdtB and MdtC and both are required for drug extrusion. An MFS transporter-encoding gene, *mdtD*, has been found downstream of the mdtABC operon, but does not appear to play a role in antibiotic resistance (Elkins and Nikaido, 2003). While recognized as a commensal organism, *E. coli* is also the most common cause of urinary tract infections, and treatment is usually with a fluoroquinolone, trimoxazole or nitrofurantoin. Enteropathogenic and enterotoxigenic *E. coli* are a common cause of diarrhoea in developing countries and for travellers to these locations, and if antimicrobial therapy is indicated, the same agents are often used as for the treatment of urinary tract infections. In children and in immunocompromised individuals, *E. coli* can cause more serious infections, associated with higher morbidity and mortality (Campos *et al.*, 2004). For these groups, antimicrobial therapy is required; treatment may be with a broad-spectrum cephalosporin (*e.g.*, ceftriaxone) or a fluoroquinolone (Marcos and DuPont, 2007). While some of these agents are substrates of the AcrAB-TolC system, over-expression alone is unlikely to give rise to clinical levels of resistance. For fluoroquinolones, a mutation(s) in a topoisomerase gene is also unlikely to give rise to clinical levels of resistance; however, when combined with enhanced efflux, such isolates are resistant to the breakpoint concentration of ciprofloxacin (Piddock, 2006a,b). Of current concern is the increasing number of *E. coli* isolates expressing an extended spectrum β-lactamases. Infections with such *E. coli* isolates are often treated with second- and third-line agents, which are often substrates of efflux pumps; therefore, the selective pressure on this species toward selection of highly MDR strains is increasing (Coque *et al.*, 2008; Piddock, 2006b).

3.2.3 *Salmonella enterica*

Another area in which MDR efflux pumps are thought to play an important role is in the antibiotic resistance of food-borne pathogens. Over the last two decades there has been an

increase in the numbers of antibiotic-resistant bacteria isolated, both from humans and from animals (Baucheron *et al.*, 2004; Piddock, 2006a,b). Particular concern has been expressed about antibiotic-resistant foodborne zoonoses such as *Campylobacter jejuni* and various serovars of *S. enterica* (Baucheron *et al.*, 2004; Ge *et al.*, 2005). For both of these species, poultry meat consumption is a significant route of transmission of these bacteria to humans. Bacteria isolated from both animals and humans have been shown to be cross resistant to antibiotics used both in veterinary and human medicine (Piddock, 2006a; Thorrold *et al.*, 2007). Agents used in treating infections in poultry include fluoroquinolones, β-lactams, macrolides and tetracycline. All of these agents are substrates for MDR efflux pumps. *S. enterica* serovar Typhimurium AcrA and AcrB are very similar to AcrA (94%) and AcrB (97%), respectively, of *E. coli* (Eaves *et al.*, 2004). Mutants of *S. enterica* serovar Typhimurium lacking AcrB were shown to be hyper-susceptible to quinolones, tetracycline, chloramphenicol, bile salts, SDS, Triton-X100, acriflavine, ethidium bromide, cetyltrimethylammonium bromide, and triclosan (Webber *et al.*, 2008). Over-expression of AcrB has also been associated with MDR in human clinical and veterinary isolates (and laboratory mutants) of *S. enterica* serovar Typhimurium (Piddock, 2006a). Two other RND efflux pumps AcrD and AcrF, are present on the genome of *S. enterica*. Genomic analysis reveals that *S. enterica* serovar Typhimurium LT2 AcrF is 88% similar to *E. coli* AcrF. Furthermore, *E. coli* AcrB is 90% similar to *S. enterica* AcrF. *S. enterica* serovar Typhimurium LT2 AcrD is 79 and 78% similar to *S. enterica* serovar Typhimurium AcrB and AcrF, respectively (Eaves *et al.*, 2004). Deletion of *acrD* or *acrF* from *S. enterica* serovar Typhimurium had little effect on the MIC of clinically relevant antibiotics (Eaves *et al.*, 2004; Piddock, 2006a). However, it was shown that when either of these genes was deleted, AcrB expression was increased (Eaves *et al.*, 2004); likewise, when *acrB* was deleted, expression of *acrD* or *acrF* increased (Eaves *et al.*, 2004; Ricci *et al.*, 2006). It may be that the bacterium can compensate for the lack of AcrD or AcrF, and consequently there is no effect on the MIC. However, a double-knockout mutant lacking AcrB and AcrF was no more hyper-susceptible than a construct lacking AcrB alone (Eaves *et al.*, 2004). These data suggest that the major efflux pump protein in *S. enterica* serovar Typhimurium, and probably all serovars of *S. enterica*, is the AcrAB-TolC pump.

3.2.4 *Campylobacter* spp.

It has been shown that CmeABC mediated efflux in *C. jejuni* conferred MDR (Pumbwe *et al.*, 2005). CmeA and CmeB have some similarity to AcrA (51%) and MexA (49%) and to AcrB (63%) and MexB (62%), respectively, of *E. coli* and *P. aeruginosa*. Deletion of *cmeB* revealed that the substrates of CmeABC include ciprofloxacin and erythromycin, both common first-line agents used to treat a human campylobacter infection. In addition, over-expression of CmeB confers resistance to ciprofloxacin, ampicillin, tetracycline, and chloramphenicol and decreased susceptibility to triclosan, bile salts, SDS, and Triton X-100 (Piddock, 2006a). A second efflux pump system, CmeDEF, has also been identified, but this system does not appear to confer resistance to ciprofloxacin or erythromycin (Pumbwe *et al.*, 2005).

3.2.5 *Acinetobacter baumannii*

A. baumannii is a multidrug resistant bacillus that is causing increasing problems in the nosocomial setting, particularly on the intensive care units (Giamarellou *et al.*, 2008). This MDR is commonly due to chromosomally mediated fluoroquinolone resistance (due to mutations in *gyrA*) and a species-specific cephalosporinase. It can also possess plasmid- or transposon-encoded genes encoding β-lactamases and aminoglycoside inactivating enzymes. In addition to these mechanisms of resistance, an RND MDR tripartite efflux pump, AdeABC, has been described (Vila *et al.*, 2007). AdeA and AdeB have some similarity to AcrA (55%) and MexA (58%) and to AcrB (68%) and MexB (67%), respectively, of *E. coli* and *P. aeruginosa*. When *adeB* was deleted in a clinical isolate, BM4454, the organism became susceptible to gentamicin, ofloxacin, cefotaxime, and tetracycline, with MIC below the recommended breakpoint concentration (Piddock, 2006a). Over-expression of AdeABC confers resistance to aminoglycosides and decreased susceptibility to fluoroquinolones, tetracycline, chloramphenicol, erythromycin, trimethoprim, and ethidium bromide, as well as to netilmicin and meropenem (Bratu *et al.*, 2008). Treatment of *A. baumannii* infection typically includes aminoglycosides, such as gentamicin, in combination with a β-lactamase-stable β-lactam,

such as piperacillin or imipenem. An alternative therapy would be another β-lactam, a fluoroquinolone, rifampin, or colistin, but these alternative therapies are relatively new and have not been supported by much clinical data (Giamarellou *et al.*, 2008). By this manner, over-expression of the AdeABC efflux pump reduces the therapeutic options.

3.2.6 *Neisseria gonorrhoeae*

MtrCDE mediates MDR and resistance to certain antimicrobial peptides produced at the host mucosal surfaces. Compared with homologies between other RND pump systems, MtrC has low similarity with *E. coli* AcrA and *P. aeruginosa* MexA (47% and 49%, respectively), whereas similarity of MtrD with *E. coli* AcrB and MexB is higher (67% and 68%, respectively). MtrE corresponds to TolC. In penicillin- resistant strains, it has been shown that the MtrCDE efflux pump interacts synergistically with other mechanisms of β-lactam resistance, including porins (penB) and low-affinity penicillin binding proteins (Veal *et al.*, 2002). Increased expression of MtrCDE alone does not increase the MIC of antimicrobial agents sufficiently to be resistant to the recommended breakpoint concentration. Ciprofloxacin is an alternative agent for the treatment of gonorrhoea, and this agent is not a substrate of the Mtr system (Piddock, 2006b).

3.2.7 Other Gram-negative bacteria

Homologues of the RND Mex and Acr efflux systems associated with MDR have also been found in other *Enterobacteriaceae*, including *Enterobacter aerogenes*, *Klebsiella* spp., *Proteus mirabilis*, *Serratia marcescens*, *Morganella morganii*, *Haemophilus influenzae*, and *Helicobacter pylori* (Piddock, 2006a). MDR pumps of the MATE family have been described for several Gram-negative bacteria: *V. parahaemolyticus* (NorM), *B. thetaiotaomicron* (BexA), *V. cholerae* (VcmA, VcrM), *Brucella melitensis* (NorMI), *N. gonorrhoeae* (NorM), *H. influenzae* (HmrM), and *P. aeruginosa* (PmpM) (Saier and Paulsen, 2001). The substrate profile typically includes a fluoroquinolone (norfloxacin and ciprofloxacin), DNA intercalating dyes, and detergents. However, the clinical relevance of these systems has not been completely established (Piddock, 2006b).

4. Strategies to reduce/inhibit efflux

Given the significance of efflux mechanisms, particularly MDR efflux mechanisms, as regards antimicrobial resistance in important human pathogens there is a need to address efflux in the design and development of new antimicrobials and in using existing agents.

In order to address the problem of efflux pumps and their consequences on decreasing the intracellular active concentration of antibiotics, it is necessary to search for and develop new molecules to circumvent efflux activity (Mahamoud *et al.*, 2007; Poole, 2007). Different strategies could be adopted to reach this objective, such as: i) by-passing efflux activity through the improvement of the molecular design of old antibiotics to reduce their efflux; ii) direct action on the permeability of the bacterial cell envelope: decreasing the efficacy of the membrane barrier; iii) blocking the efflux capacity of bacterial cell: alteration of pump function (*see* Figure 11) (Mahamoud *et al.*, 2007; Webber and Piddock, 2003). This last approach will be discussed in more detail in the next sections.

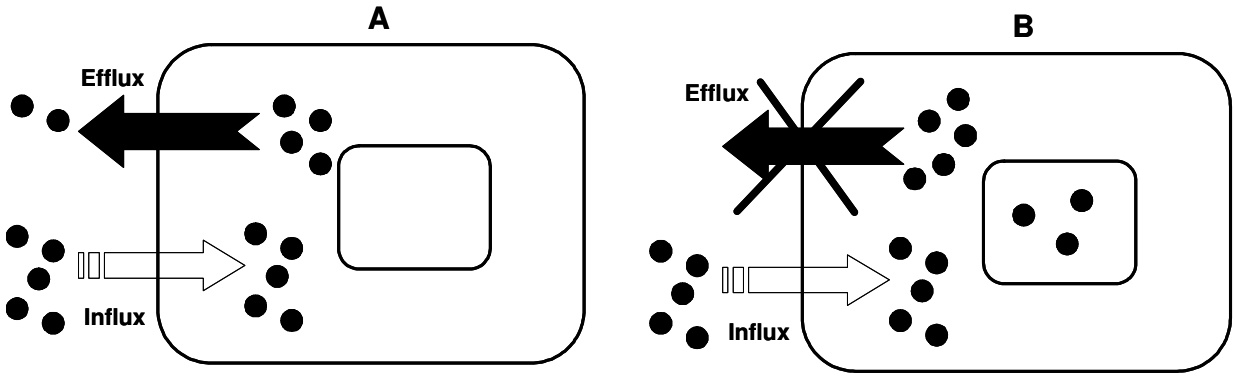


Figure 11. Strategy to inhibit the efflux capacity of a bacterial cell by alteration of the efflux pumps function. Blocking the efflux capacity of a bacterial cell will cause the alteration of the pump function and the antibiotic will be entrapped and accumulated inside (*sources:* Carryn *et al.*, 2003; Martins *et al.,* 2008).

The development of novel molecules that overcome the resistance mechanisms involving enzymes, mutations or efflux appear to be a suitable strategy to bypass the restricted number of new antibiotics. By this manner, the efflux activity and pump components are putative targets for the development of new molecules (Mahamoud *et al.*, 2007; Marquez, 2005; Piddock, 2006b). To specifically block the activity of drug efflux pumps, several approaches could be developed: i) create a jam in the outer membrane channel; ii) inhibit the interactions of different components of a multi-component pump; iii) inhibit the drug binding to the inner membrane pumps generating competition in the inner membrane pump; iii) alter the pump assembly; iv) target the energy source of pumps by collapsing the energy component of the mechanism; or v) target the regulatory networks that control the expression of the efflux pumps (Mahamoud *et al.*, 2007). The demonstration of drug capture in the periplasm and outer-membrane pumping out, termed "periport", by Lomovskaya and Totrov (Lomovskaya and Trotov, 2005; Lomovskaya *et al.*, 2007) offers the possibility to use periplasmic transit blockers to obstruct the AcrB-pump central cavity. From the structural data, few amino acid residues are involved in interactions with the transported substrate and these strategic sites are well preserved in the various efflux pumps belonging to the AcrB family. This multiplicity of sites might be the basis for the difference observed in the efflux rate of drugs. To date, the inhibitory activities of several structurally unrelated compounds have been assessed on diverse actively effluxing drug resistant Gram-negative bacteria, including *E. coli*, *Enterobacter aerogenes*, *K. pneumoniae*, *C. jejuni*, *P. aeruginosa* and *S. enterica* (Lomovskaya *et al.*, 2007; Mahamoud *et al.*, 2007; Piddock, 2006a; Poole, 2007). To address the effect of efflux pumps inhibition and its consequence on decreasing the intracellular active concentration of antibiotics, it is necessary to search and develop new molecules that can circumvent this efflux activity. This provides strong support for research and development of new compounds; namely, efflux pumps inhibitors (EPIs) that by inhibiting the efflux systems contribute to preserve antibacterial potency of antibiotics.

4.1 Efflux pumps inhibitors (EPIs)

There are compounds that are used for the therapy of non-infectious pathogens and that have antimicrobial properties, having been termed "non-antibiotics" (Kristiansen *et al.*, 2007; Martins *et al.*, 2008). Due to their potential for the therapy of some MDR infections, they may eventually achieve an antimicrobial status (Hendricks *et al.*, 2003). The group of the so called "non-antibiotics" can be further divided into two subgroups: i) antimicrobial non-antibiotics and ii) "helper compounds". Each of these subgroups has distinct modes of action. The antimicrobial non-antibiotics have direct antimicrobial activity while the "helper compounds" are known to alter the permeability of the microorganism to a given antibiotic (Kristiansen *et al.*, 2007; Martins *et al.*, 2008). Over the past decade, a series of EPIs have been identified. However, only a few number of new and effective molecules obtained the Food and Drugs Administration (FDA) approval and few antibacterial are in pre- or clinical developments (Mesaros *et al.*, 2007). When discussing this subject several questions arise: what is an EPI? How many types of EPIs are known to date? This will be discussed in the next sections.

4.1.1 Peptidomimetics

The first broad-spectrum RND pump inhibitor identified was phenylalanyl arginyl-β-naphthylamide (PAβN). This EPI enhanced the activity of levofloxacin against wild-type *P. aeruginosa* 8-fold and against a MexAB–OprM over-expressing strain 64-fold (Lomovskaya and Bostian, 2006). Several improved EPIs exhibited antibiotic potentiation activity for *P. aeruginosa* strains expressing MexAB–OprM, MexCD–OprJ and MexEF–OprN, and for *E. coli* expressing AcrAB–TolC (Mahamoud *et al.*, 2007). Importantly, EPIs dramatically reduced the emergence of spontaneously levofloxacin-resistant bacteria and were effective in animal models of *P. aeruginosa* infections. Similar compounds have been shown to inhibit efflux pumps in *Enterobacter aerogenes* and *Campylobacter* (Lomovskaya and Bostian, 2006; Mahamoud *et al.*, 2007). It is believed that these compounds act by inhibiting the specific binding sites of antibiotics within the pump molecule (Poole, 2007). These molecules significantly and rapidly

increase the intracellular accumulation of different efflux sensitive drugs, such as quinolones, chloramphenicol or macrolides, in clinical isolates expressing different levels of efflux pumps, without affecting the integrity of the membrane under the conditions used (Piddock, 2006b). However, some EPIs, such as PAβN, can affect the membrane integrity when used at high concentrations (Lomovskaya *et al.*, 2007). This point is of special interest because it might induce the emergence of resistance profiles, such as those described for certain cationic antimicrobial peptides or polymyxines (modification of the lipopolysaccharide structure inducing a change in drug penetration). In the case of PAβN the analyses of the inhibitory mechanism demonstrated that this EPI is a substrate of efflux pumps that may act as a competitive inhibitor. Some derivatives of this compound, such as pyridopyrimidines, have also being developed as EPIs for *P. aeruginosa*. However, the disadvantage of the members of this EPI family is their toxic properties, which prevent their clinical applications (Mahamoud *et al.*, 2007).

4.1.2 Phenothiazines

The work of Paul Ehrlich, in the 19th century, demonstrated the antimicrobial activity of methylene blue, shown to obviate the mobility of microorganisms and the increased interest on its properties lead to the synthesis of a group of heterocyclic compounds called phenothiazines (Martins *et al.*, 2008) (Figure 12). These compounds demonstrate a wide gamut of antibacterial activities (Amaral *et al.*, 2004; Bettencourt-Viveiros and Amaral, 2001; Hendricks *et al.*, 2005; Kaatz *et al.*, 2003; Kawase *et al.*, 2001; Thanacoody, 2007; Vitale *et al.*, 2007). However, with few exceptions the concentrations of these agents needed to inhibit *in vitro* growth of the bacteria are well beyond those that can be clinically achievable (Amaral and Kristiansen, 2001; Amaral *et al.*, 2004; Kristiansen and Amaral, 1997). Because antibiotic resistance, especially MDR, has become common, a number of investigators turned their interest to chlorpromazine (CPZ) and other derived phenothiazines as potential agents against MDR infections, acting as "helper compounds" (Amaral and Kristiansen, 2000; Amaral *et al.*, 2001, 2006; Crowle *et al.*, 1992; Hendricks *et al.*, 2005; Kristiansen and Amaral, 1997; Kristiansen *et al.*, 2007; Ordway *et al.*, 2002).

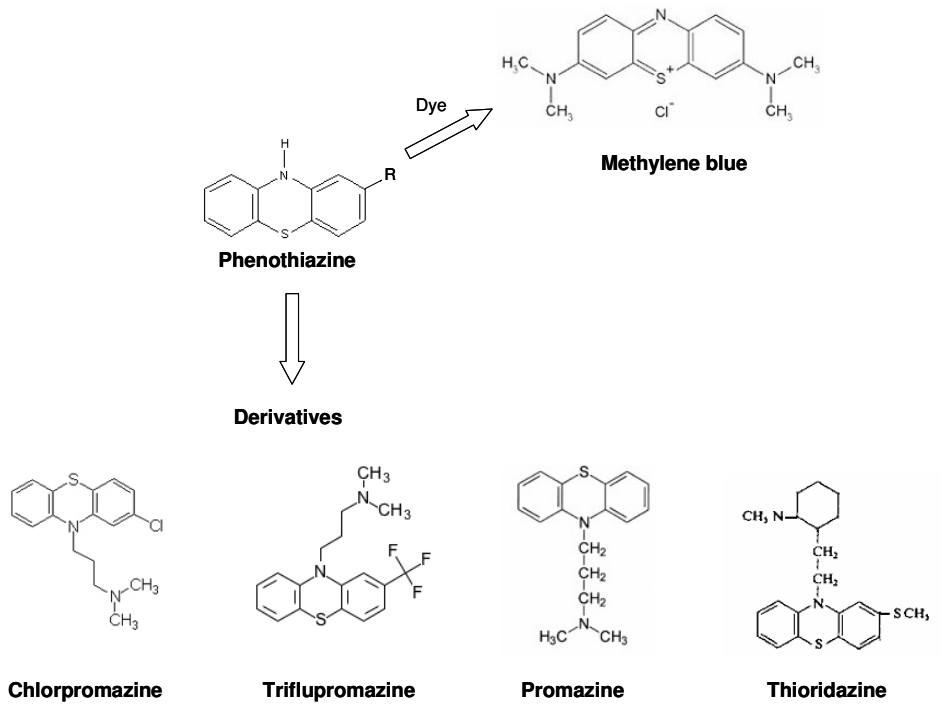


Figure 12. Structure of phenothiazines and its derivatives. Phenothiazines are a group of heterocyclic compounds characterized by a three-ring structure in which two benzene rings are joined by a sulfur and nitrogen atom at nonadjacent positions (*reproduced from* Martins *et al.*, 2008).

Phenothiazines have been shown to reverse MDR phenotypes of bacteria and therefore render these bacteria susceptible to antibiotics to which they were initially resistant (Kristiansen and Amaral, 1997; Kristiansen *et al.*, 2007). Phenothiazines are known to inhibit the transport of calcium (Ca^{2+}) by preventing its binding to Ca^{2+}-binding proteins such as calmodulin (Bhatnagar and Singh, 2003, 2004; Dhople, 1999; Sinha and Dick, 2004). By this manner, enzyme systems which are dependent upon Ca^{2+}, such as those involved in generating cellular energy from hydrolysis of ATP, could also be inhibited (Bhatnagar and Singh, 2003). Because MDR is due to the over-expression of efflux pumps and these efflux systems are driven by energy provided by the proton-motive force which is dependent upon Ca^{2+}-dependent enzyme systems, the inhibition of Ca^{2+} binding to Ca^{2+}-dependent enzymes will render the bacterium susceptible to that which they were initially resistant (Bhatnagar and Singh, 2003, 2004; Dhople, 1999; Sinha and Dick, 2004).

4.1.3 Benastatins

Benastatins obtained from fermentation of an actinomycete are another group of compounds that were found to be active against *P. aeruginosa* expressing MexAB–OprM (Fronko *et al.*, 2000; Qureshi *et al.*, 2001). EPIs have been identified for other bacteria and from other sources. 2,8-Dimethyl-4-(2V-pyrrolidinoethyl)-oxyquinolone, an alkoxyquinolone derivative, was shown to inhibit efflux pumps in *E. aerogenes* and *K. pneumoniae*. This EPI potentiated the efficacy of chloramphenicol, norfloxacin, tetracycline and cefepime by up to 8-fold (Chevalier *et al.*, 2004).

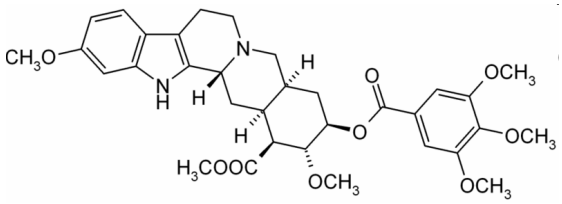
4.1.4 Tetracycline derivatives/homologues

For specific efflux pumps, many putative inhibitors that share tetracycline-analogue structural properties have been screened to inhibit the tetracycline efflux mechanism (Chopra, 2002). Several tetracycline derivatives were described that inhibit the TetB efflux pump. Some of them demonstrate an interesting reversal capacity and these studies indicate that tetracycline derivatives, identified by their ability to block the Tet(B) efflux protein, can restore tetracycline activity against resistant bacteria bearing either of the two known resistance mechanisms, namely, efflux and a ribosomal-protection mechanism (Sudano *et al.*, 2004). The most potent of these is a derivative of doxycycline, 13-cyclopentylthio-5-OH tetracycline (13-CPTC) (Dean *et al.*, 2003). Contrary to the EPIs described above, 13-CPTC has antibacterial properties of its own against lipopolysaccharide-deficient *E. coli* and *S. aureus*. Combination of 13-CPTC with doxycycline resulted in reduction of MIC values for doxycyline by 4- to 10-fold (Nelson and Levy, 1999). Blocking specific drug efflux and increasing intracellular drug concentrations constitute an effective approach to reverse tetracycline resistance in Gram-negative bacteria (Piddock, 2006a,b). Various antibiotic analogues have also been developed and among them are tetracycline analogues which were first described by Levy and McMurry (Levy and McMurry, 1978). Initially focused on *S. aureus* and tetracyclines, these molecules have been tested with other antibiotics and several bacterial pathogens. One of the main problems with this class of modified antibiotics is its high

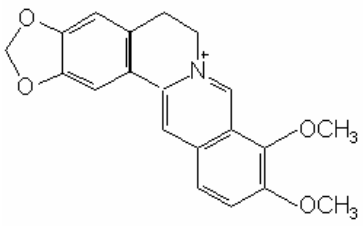
structural similarity with the true antibiotic molecules and its residual activity on bacterial targets; an adverse consequence of these properties is the reinforcement of the selection of resistance mechanisms directed against the antibiotic (Mahamoud *et al.*, 2007).

4.1.5 Compounds isolated from plant extracts

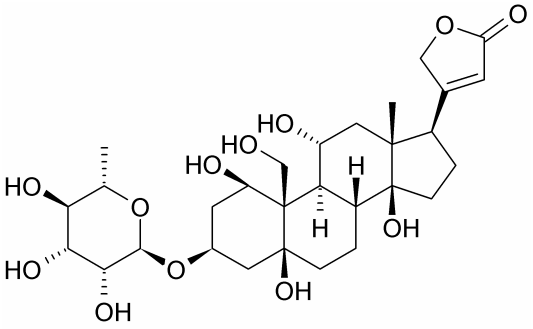
Plant extracts constitute one of the major sources of compounds with antibacterial activity (Marquez, 2005). One of these examples is the antihypertensive plant alkaloid, reserpine (Figure 13). This indole alkaloid was first isolated from the roots of *Rauwolfia vomitoria* Afz (Poisson *et al.*, 1954). Its EPI activity was originally demonstrated against the Bmr efflux pump, which mediates tetracycline efflux in *Bacillus subtilis*. It also potentiates the activity of fluoroquinolones on MDR Gram-positive bacteria and of tetracycline on MRSA strains (Piddock, 2006a). Neyfakh *et al*. showed that reserpine inhibited NorA (Neyfakh *et al.*, 1993), while Kaatz and Seo showed that reserpine potentiated the activity of norfloxacin for *S. aureus* (Kaatz and Seo, 1997). It has been also described that decreases the emergence of MDR *S. aureus* and *S. pneumoniae* strains *in vitro* (Marquez, 2005) and its inhibition of the P-gp is largely known (Piddock, 2006a). Unfortunately, reserpine cannot be used in combination with antibiotics for the treatment of staphylococcal infections, since the concentrations required for the treatment are neurotoxic (Michalet *et al*., 2007). The natural products isolated from *Berberis* plants have been identified as inhibitors of bacterial efflux pumps (Michalet *et al.*, 2007). Interestingly, they potentiate the antibacterial activity of berberine, an alkaloid isolated from *Berberis fremontii*, what may suggest that plants have evolved to produce weak antibacterial compounds associated with EPIs, to enhance their activity (Marquez, 2005; Michalet *et al.*, 2007; Stavri *et al.*, 2007). Studies conducted with berberine showed that this compounds has weak antibacterial activity (MIC = 256 mg/L) against a wild-type strain of *S. aureus*. However, the isolation of the flavonolignan 50-methoxyhydnocarpin-D (50-MHC-D) and a synergistic study between these two compounds led to a 16-fold increase in the antibacterial activity of this alkaloid (MIC =16 mg/L). 50-MHC-D also had a synergistic effect with several other NorA substrates, including norfloxacin.



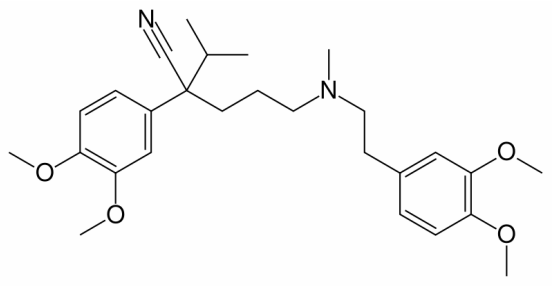
Reserpine



Berberine



Ouabain



Verapamil

Figure 13. Structure of EPIs obtained from plant extracts (*sources*: Marquez, 2005; Stermitz *et al.*, 2000).

There have also been a number of methoxylated flavones and isoflavones described as putative inhibitors of the MDR pump NorA in the presence of sub-inhibitory concentrations of berberine and norfloxacin (Stavri *et al.*, 2007). However, one important aspect that should be carefully looked at when interpreting results is to ensure that the activity of these compounds is solely due to potentiation of the compounds and not by direct inhibition of the bacterial growth.

A group of phenolic metabolites that have provided interest are the catechin gallates. It has been shown that these compounds are able to reverse the methicillin resistance in MRSA (Stavri *et al.*, 2007).

Many other plants and herbs have been tested for their EPI activity on the last few years. Among these are the abietane diterpenes carnosic acid and carnosol, isolated from Rosemary (*Rosmarinus officinalis*) (Oluwatuyi *et al.*, 2004). These diterpenes were identified as potentiators of tetracycline and erythromycin against *S. aureus* strains possessing the Tet(K) and Msr(A) efflux pumps, respectively (Smith *et al.*, 2007). Several projects have been developed in the last years to obtain natural compounds with modulatory activity. One of such example is an extract of *Lycopus europaeus* (*Lamiaceae*). The lipophilic extract of *L. europaeus*, commonly known as Gipsywort in Britain, was shown to potentiate the activity of tetracycline and erythromycin against strains of *S. aureus* possessing the MDR efflux pumps Tet(K) and Msr(A) (Stavri *et al.*, 2007).

A biological evaluation of grapefruit oil, isolated from *Citrus paradisi*, has highlighted some of the components as potential modulators of efflux pumps in MRSA strains. Fractionation of the grapefruit oil led to the characterization of a coumarin derivative, a bergamottin epoxide derivative and a coumarin epoxide derivative able to enhance the activity of ethidium bromide and norfloxacin (Abulrob *et al.*, 2004). Work on plants belonging to the family Euphorbiaceae has resulted in the isolation of inhibitors of the P-gp, including a jatrophane diterpene that caused a 2-fold greater inhibition of daunomycin efflux, with respect to cyclosporin A, at a concentration of 5 μM (Marquez *et al.*, 2005). Piperine, a major plant alkaloid within the family Piperaceae including black pepper (*Piper nigrum*) and long pepper (*Piper longum*), has been reported to enhance the accumulation of ciprofloxacin by *S.* aureus (Stavri *et al.*, 2007). This compound is a substrate of NorA and therefore it is plausible that piperine acts as an inhibitor of this transporter (Kumar *et al.*, 2008).

When conducting a more extensive analysis, some studies reveal that a common feature of these compounds is that they are highly lipophilic, suggesting that this characteristic could be exploited for the development of clinically useful inhibitors of MDR efflux pumps of Gram-positive bacteria (Stavri *et al.*, 2007).

Members of the plant family Aizoaceae, considered as one of southern Africa's most diverse and abundant plant families, but also the least studied in terms of its medicinal potential (van der Watt and Pretorius, 2001) also contain alkaloids known to have narcotic-anxiolytic properties and strong synergism with psychomimetics (Smith *et al.*, 1996). These properties are consistent with those presented by other neuro-active compounds whose activities reside primarily at the level of the plasma membrane (Williams *et al.*, 2001). Although some of these alkaloids have been reported to have anticancer properties as well (Smith *et al.*, 1996), they have received little attention, perhaps due to their reported toxicity. One such member of the Aizoaceae family is *Carpobrotus edulis* a plant that besides been found in southern Africa (van der Watt and Pretorius, 2001) is also found along the coast of Portugal and that is so prolific that it is considered a nuisance (Ordway *et al.*, 2003). Due to the antimicrobial activity of this family, *C. edulis* has also become the subject of research for antimicrobial activity (Ordway *et al.*, 2003; van der Watt and Pretorius, 2001).

An interesting compound found in the ripe seeds of the African plants *Strophanthus gratus* and *Acokanthera ouabaio*, is ouabain. It is used extensively worldwide for *in vitro* studies to block the Na+ pump (Na+/K+-ATPase) (Clausen, 2003). The blockage of this pump is associated with high concentrations of the compound that are attainable *in vitro* whereas low concentrations stimulate the Na+/K+-ATPase (Gao *et al.*, 2004). Interestingly, this compound was also identified in the human, as an endogenous hormone (Schoner and Scheiner-Bobis, 2005), being probably an isomer in the hypothalamus, that is augmented in conditions of oxygen deficiency. However, its exact mode of action and physiological significance is not yet determined (Schoner and Scheiner-Bobis, 2007). In France and Germany, ouabain has a long history in the treatment of heart failure, and some continue to advocate its use in angina pectoris and myocardial infarction (Gao *et al.*, 2004). Some phenols, present in green tea extracts, also possess numerous biological activities, including antimicrobial activity (Bandyopadhyay *et al.*, 2005; Marquez, 2005), reversal of methicillin resistance in MRSA strains (Michalet *et al.*, 2007) or inhibition of P-gp (Marquez, 2005; Michalet *et al.*, 2007).

4.1.6 Quinoline and its derivatives

Various quinolines, which block drug efflux in MDR clinical isolates, have also being pursued for application as an EPI. This is a novel class of compounds that was discovered by using several screening procedures with *Enterobacter aerogenes* strains (Chevalier *et al*., 2001; Mahamoud *et al*., 2006). Several studies demonstrate the potential of these compounds, and several quinoline derivatives are being considered as broad-spectrum EPIs for rendering antibiotic-resistant *Enterobacter aerogenes* and *K. pneumoniae* susceptible to chloramphenicol, tetracycline and norfloxacin. Their ability to increase the accumulation of the antibiotic has been compared to that resulting from the addition of carbonyl cyanide-m-chlorophenylhydrazone (CCCP) or PAβN to the culture (Chevalier *et al.*, 2004). These compounds affect the electrochemical gradient across the membrane used by some efflux pumps as a source of energy, thus inhibiting efflux (Ramón-García *et al.*, 2006). However, in this class of EPIs, additional studies are needed to define the role of pharmacophoric groups and their reactivity with the affinity pockets reported in AcrB. Alkylamino-, alkoxy-, thioalkoxy-, chloro-quinoline derivatives present two advantages: their similarity with the quinolone family, which greatly argues for an efficient pharmacokinetic profile and a negligible intrinsic activity, and no additional side-effect (permeabilization or alteration) on the membrane. However, studies, such as toxicity assays and pharmacodynamics tests are still needed to determine the therapeutic potency of these compounds (Kumar *et al.*, 2008; Mahamoud *et al.*, 2007).

4.1.7 Arylpiperidines and arylpiperazines

Several compounds belonging to these two families have been tested as potential EPIs (Kern *et al.*, 2006). Among arylpiperidines, some derivatives are able to restore linezolid susceptibility and accumulation in *E. coli*. In the case of arylpiperazine, screening of a limited library of low-molecular-weight N-heterocyclic compounds in *E. coli* has led to the discovery of several arylpiperazines with potency to reverse MDR in cells over-expressing RND-type efflux pumps (Thorarensen *et al.*, 2001). The mechanism of action of these and related compounds has not been completely elucidated. Unlike PAβN, the

low intrinsic antibacterial activity of these compounds and its cellular accumulation are not enhanced in cells with inactivated efflux pumps. One of the main disadvantages is that these compounds show serotonin agonist properties that most likely will be toxic for use in man and animals (Mahamoud *et al.*, 2007)

4.1.8 Microbial-derived EPIs

EPIs derived from microbial sources have been relatively scarce to date. The ability of microorganisms to produce antimicrobial compounds as part of their "chemical arsenal" needs to be combated by susceptible microbes through the evolution of drug resistance (Stavri *et al.*, 2007). MDR pumps are an example, with an ability to extrude a number of chemically diverse antibiotics with the expression of just a single efflux mechanism (Rouveix, 2007). It would therefore seem logical that, as is the case with plants, microorganisms would evolve to produce a second compound that could nullify the effect of MDR pumps in a competing microorganism resulting in the accumulation of the antimicrobial compound to a level that would be static or cidal. Screening of microbial fermentations has resulted in the characterization of new natural EPIs. The MDR inhibitors were isolated from *Streptomyces* MF-EA-371-NS1, which is a new strain closely related to *Streptomyces vellosus*. EA-371a and EA-371d (Qureshi *et al.*, 2001) inhibited the MDR pump MexAB-OprM of *P. aeruginosa* PAM1032, which over-expresses this pump. At a concentration of 0.625 mg/L both compounds caused a 4-fold reduction in the MIC of levofloxacin. An 8-fold reduction of this fluoroquinolone was effected at 1.25 and 2.5 mg/L of EA-371d and EA-371a, respectively (Stavri *et al.*, 2007).

4.2 Energy uncouplers

Most efflux pump systems, except for the ABC family, which utilizes ATP hydrolysis, utilize the proton motive force as an energy source to drive the export of substrates (Piddock, 2006a,b; Poole, 2007). Compounds that seriously affect the energy level of the bacterial membrane such as CCCP and dinitrophenol (DNP) dissipate the proton motive force, thereby inhibiting efflux (Mahamoud *et al.*, 2007). However, these compounds are

not considered inhibitors of efflux systems. These compounds reduce the viability of the bacterium and cause cell death via the dissipation of the proton motive force of the membrane (Pagès *et al.*, 2005). Consequently, there is always the question of whether it is their effect on the efflux pump that is the cause of an increase in the penetration of the antibiotic, or whether it is due to the alteration of the cell envelope itself that results in the death of the bacterium (Marquez, 2005; Pagès *et al.*, 2005). In addition, some uncouplers, like CCCP, are recognized as highly noxious and cytotoxic and are also substrates of bacterial efflux pumps. Neyfakh *et al.* showed that efflux-mediated MDR in *B. subtilis* had some similarities with that of P-gP of mammalian cells (Neyfakh, 1992), in that MDR was reversed in the presence of reserpine and verapamil, known inhibitors of the P-gP (Mahamoud *et al.*, 2007). Several other EPIs have been reported, such as globomycin, an inhibitor of lipoprotein-precursor-processing enzyme (Malléa *et al.*, 2002). Despite all the studies on this area, no molecule belonging to the energy-blocker family has been developed for clinical use or has been patented until this date (Mahamoud *et al.*, 2007).

In summary, EPIs clearly show promising for developing combination therapies with existing antimicrobials to restore their antibacterial activity against resistant bacteria. However, this approach presents its own challenges because of the potential undesirable effects on eukaryotic cells, for example toxicity and inhibition of eukaryotic transporters that are structurally and functionally similar. Bypassing efflux pumps may be an available alternative to EPIs. This could be achieved by development of newer drugs that are poor substrates for these pumps. Indeed, some of the newer fluoroquinolones seem to be poor substrates for certain pumps found in Gram-positive bacteria (Mahamoud *et al.*, 2007). However, it is not yet clear if the increased activity is due to higher affinity for the target or lower affinity for these pumps. As mentioned before, there is always the risk of inducing an alternate pump in response to exposure to an antibiotic that is a poor substrate for a particular pump. The glycylcycline tigecycline (GRA-936) is an example of a substrate that is a poorer substrate for tetracycline pumps (Felmingham, 2005). However, glycylcyclines are substrates for RND pumps of many Gram-negative bacteria, including *P. aeruginosa*, *K. pneumoniae*, *P. mirabilis* and *Morganella morganii*, which render them ineffective against Gram-negative pathogens (Chopra, 2002).

4.3 The use of EPIs in conjunction with antibiotics – the "helper compounds" approach

The search for compounds that have been shown to be EPIs have being extensively studied on the last few years, as previously demonstrated (Mahamoud *et al.*, 2007; Pagès *et al.*, 2005; Piddock, 2006a,b; Poole, 2007). If these compounds are to be used in combination with antibiotics to which a given bacteria is initially resistant, they may increase the susceptibility of this bacteria to the given antibiotic and be used as "helper compounds".

It has been demonstrated that exposing an antibiotic-sensitive bacteria to increasing concentrations of a given antibiotic will induce an increased resistance to this same antibiotic (Martins *et al.*, 2007; Viveiros *et al.*, 2002, 2005, 2007). Exposure of *E. coli* to increasing concentrations of tetracycline results in significantly increased activity of genes that regulate genes coding for transporters of the RND superfamily efflux pumps (Viveiros *et al.*, 2005, 2007). However, concomitantly and accompanying this induced resistance are increases in resistance to other distinct classes of antibiotics (Viveiros *et al.*, 2005, 2007). This induced resistance can be reversed with the transfer of the bacterium to a drug-free medium or by exposure of the bacterium to an EPI, such as PAβN, phenothiazines, reserpine, or to proton un-couplers, such as CCCP, *etc*. (Kern *et al.*, 2006; Mahamoud *et al.*, 2007; Pagès *et al.*, 2005; Piddock, 2006a). By this manner, exposing a given bacteria to an antibiotic at concentrations that do not completely inhibit its replication induce the bacterium to withstand the action of the antibiotic even though its concentration has been increased. This poses the question if the development of a MDR infection in patients treated with sub-inhibitory doses of the antibiotic occurs via the same mechanism.

Among the commonest Gram-negative bacteria involved in nosocomial respiratory and urinary tract infections are *Enterobacter aerogenes* and *Klebsiella pneumoniae* (Pagès *et al.*, 2005; Piddock, 2006a) that show a marked decrease in antibiotic susceptibility. These modifications of the envelope permeability contribute to a high level of resistance for structurally unrelated molecules such as β-lactams, quinolones, macrolides, tetracyclines and chloramphenicol (Poole, 2007). In various clinical isolates, the MDR phenotype is strongly associated with a marked decrease in the synthesis of non-specific porins and the overproduction of active drug efflux systems (Webber and Piddock, 2003). Since this MDR phenotype involves over-expression of efflux pumps and down regulation of porins, both systems work together to reduce the permeability of the organism to that antibiotic as well as to other unrelated antibiotics (Piddock, 2006; Thanassi *et al.*, 1995). Because the MDR efflux pump can be inhibited by a variety of agents, the use of EPIs as "helper compounds" to the antibiotic mode of action to which the bacterium is initially resistant has become a focus of interest and intensive research (Amaral *et al.*, 2001; Kern *et al.*, 2006; Kristiansen *et al.*, 2006; Mahamoud *et al.*, 2007; Martins *et al.*, 2008; Pagès *et al.*, 2005; Piddock, 2006). The synthesis and characterization of new compounds that are capable of circumventing efflux activity and restore the internal concentration of common antibiotics that are substrates of efflux pumps is one of the main challenges that man is facing. An additional aspect that should be considered is that these compounds must be devoid of any intrinsic antibacterial activity at the concentration commonly used.

4.4 Practical applications and feasibility of EPIs in the clinical context

The ability of the "helper compounds" to reduce or reverse the resistance of the bacterium to a given antibiotic is of grateful application, since these compounds could be used as adjuvant with the conventional antibiotic to which the bacterium is resistant. This would be an important achievement since that if these "helper compounds" reach the clinical use many antibiotics that have fallen by the wayside due to MDR phenotypes of clinically relevant bacteria may again be used. But what is the implication in the clinical setting?

4.4.1 The establishment of MIC breakpoints and the interpretation of the *in vitro* susceptibility assays

The need to know whether an organism is likely to respond to antimicrobial therapy is as old as chemotherapy itself. All methods used attempt to integrate the pattern of susceptibility of a population of bacteria with the pharmacokinetics of the antimicrobial and then, possibly, to review this relationship in the light of clinical experience. All have many problems in common (MacGowan and Wise, 2001; Piddock, 2006a). These include: (i) the need for antimicrobial group testing; namely, can just one agent be taken as representative of others? Commercially and scientifically this is a difficult problem; (ii) how to take into account the changing dosing regimens (for example, penicillin and ampicillin dosing for *pneumococci* with intermediate susceptibility); (iii) infections at specific sites, including the urinary tract, and the possible need for site-specific breakpoints; (iv) the role of the intermediate category between susceptible and resistant populations; (v) how to integrate the newly emerging knowledge on pharmacodynamics with breakpoint determination; (vi) how to deal with organism–antimicrobial combinations where a substantial proportion of the distribution of susceptibility straddles the pharmacological breakpoint (MacGowan and Wise, 2001). Different countries should produce guidance on methodology and breakpoints that reflect clinical and laboratory practice in that country (Webber and Piddock, 2003). The primary function of *in vitro* antimicrobial susceptibility testing in clinical laboratories is to provide information to clinicians on the choice of appropriate chemotherapy, whether it is for therapy or prophylaxis in specific patients, or to help in antimicrobial policy formulation. Increasingly, routine susceptibility testing is also seen as having public health significance, in that the data generated can be used to track the occurrence and prevalence of antimicrobial resistance in the geographical area served by the laboratory (Tenover, 2006). The definition of a strain according to the susceptibility profile is as follows: i) susceptible: when a infection due to the bacteria tested will probably respond to the antibiotic; ii) intermediate, when indeterminate or uncertain response is likely given standard dosing (in some circumstances increased doses would be effective); and iii) resistant when the infection due to bacteria tested will probably not respond to the

antibiotic. To categorize strains as susceptible, intermediate or resistant, breakpoint antibiotic concentrations are used. Breakpoints are discriminatory antimicrobial concentrations that are used in the interpretation of results of susceptibility testing to define isolates (MacGowan and Wise, 2001). However, clinical, pharmacological and microbiological considerations are important in setting breakpoints, and the ideal mixture of these factors is unknown. Three features of both antimicrobial and pathogen must be considered when deciding upon a breakpoint: (i) the distribution of the profile of susceptibilities of the bacterial population; (ii) pharmacological properties of the antimicrobial; and (iii) clinical outcome data. Some times there are difficulties than can arise when trying to reconcile these three parameters (Tenover, 2006).

Until now, the MIC continues to be considered the gold standard for assessing an antibiotic's potency, however, one must keep in mind that it is a crude measure with several limitations. However, all other susceptibility test methods should be validated against an MIC determined by a standard methodology. Breakpoints that fall in the troughs of bimodal or polymodal MIC distribution are most likely to yield a reproducible categorization of susceptible, intermediate or resistant. In the opposite, those breakpoints that lie in the middle of a continuous distribution will result in poor reproducibility (MacGowan and Wise, 2001). In some cases it may be necessary to shift breakpoints or to introduce two breakpoints to help diminish the impact of this problem. Different species differ in their MIC distributions, and therefore it may be necessary to choose breakpoints that relate to the more common and/or important organisms (Piddock, 2006a,b). The choose of breakpoints considering the majority of clinical isolates may result in a classification of "susceptible" for organisms with specific resistance mechanisms that affect clinical outcomes. It may consequently be necessary to shift breakpoints to reduce this problem.

In most cases, the distribution of susceptibilities (or distribution of MIC) for a bacterial population to an antimicrobial can be considered: i) unimodal, when the bacteria are innately susceptible or resistant (Figure 14A); or ii) bimodal, when two populations are obtained, for instance, a susceptible population and a population possessing a mechanism or mechanisms of resistance (Figure 14B) (MacGowan and Wise, 2001).

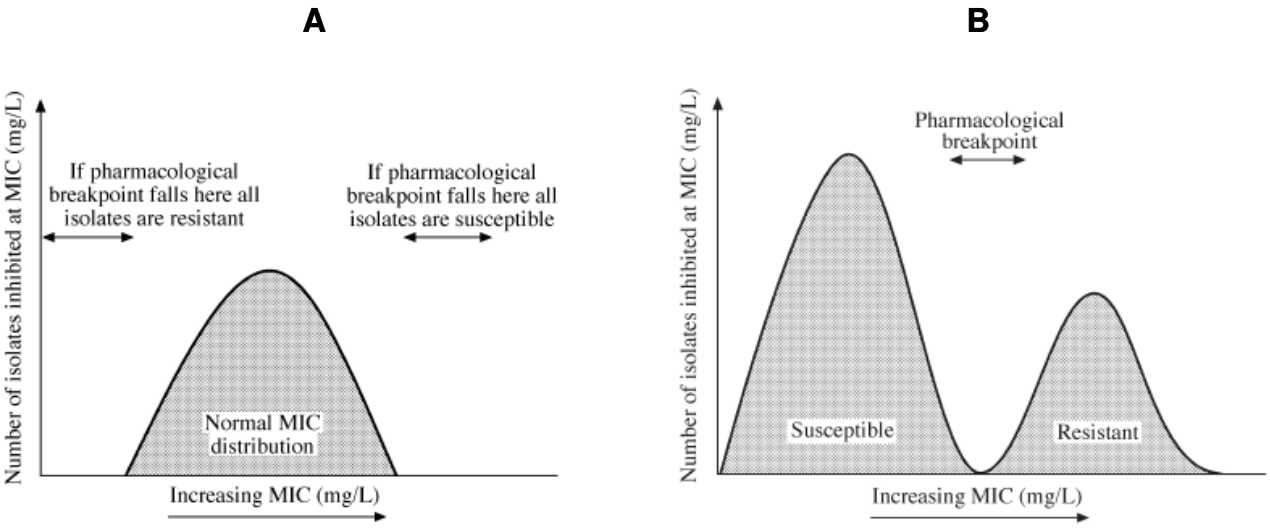


Figure 14. Unimodal and bimodal MIC distribution (*Adapted from* MacGowan and Wise, 2001).

However, it must be understood that for certain drug-pathogen interactions that have the susceptibility profile distributed over a wide range of MIC values it can be difficult, and sometimes almost impossible, to choose a meaningful breakpoint that will yield consistent results in an acceptably high proportion of tests. For example, *Klebsiella* and *Serratia* spp. are less susceptible than other Enterobacteriaceae to some fluoroquinolones, but may well respond to therapy, while "wrongly" being reported as resistant relative to breakpoints that have been optimized for other Enterobacteriaceae (MacGowan and Wise, 2001; Piddock, 2006a). Vancomycin resistance in *S. aureus* is another example, where changes may need to be made in the future.

Implicit in choosing a breakpoint is the assumption that the concentration of an antimicrobial at the site of infection is one of the important features likely to determine the outcome of therapy. For these reasons serum concentrations are used as surrogates for those in tissue. A feature of certain antimicrobials, particularly some of the macrolide group, is their high tissue concentrations yet low serum levels (MacGowan and Wise, 2001). A feature not yet addressed is the impact that a particular choice of a breakpoint might have upon the emergence of resistance among pathogens to a particular antimicrobial. There is increasing evidence that the use of an antimicrobial when MIC for infecting organisms are very close to the MIC breakpoint may be associated with the emergence of resistance. It is possible that this should be factored into future breakpoint determinations.

4.4.2 Clinical issues and the necessity to establish a breakpoint

Determining a breakpoint implies that an organism designated as "susceptible" should respond to the standard dose of the agent. A "resistant" organism should not respond and an "intermediate" may or may not respond to standard doses, yet would have an increased chance of responding to a greater dose if the infection is at a site where the antimicrobial is actively concentrated (MacGowan and Wise, 2001; Tenover, 2006). Usually, the MIC ant its distribution for a relevant number of pathogens is determined and according with the guidelines of each country or societies it is considered acceptable a limits established between <5% and <1%, believing that false-resistant reporting to be of lesser clinical consequence than false susceptible. Occasionally it is found that certain groups of pathogens and antimicrobials (for example *P. aeruginosa* and the fluoroquinolones) give consistently high rates of false reporting (Wise and Andrews, 1999). By this manner, the use of one agent to represent a family of closely related compounds is not a consensual subject. If one chooses one antibiotic that is the least active representative of the family of compounds, increased "false resistance" reports to a more active member can emerge. This is of less clinical danger than predicting the susceptibility of a less active agent based upon information obtained by susceptibility testing of a more active compound. However, if a particular agent in a group is used locally, that agent should be tested.

There can be some exceptions as is the case of the "third-generation cephalosporins", where the least active compound may not be the best indicator of resistance for that particular class (MacGowan and Wise, 2001).

4.4.3 The clinical relevance of the MIC reduction

Using an EPI to reduce or reverse the resistance to a given antibiotic continues to be a subject of intense discussion and research (Amaral *et al.*, 1992, 2007; Kern *et al.*, 2006; Kristiansen *et al.*, 2006; Mahamoud *et al.*, 2007; Martins *et al.*, 2008; Pagès *et al.*, 2005; Piddock, 2006). To be considered clinically relevant an EPI should reduce the MIC of a strain to a given antibiotic, in order to change the susceptibility profile of the strain. For example, an EPI can reduce the MIC of a given strain but it only has a clinical impact if the MIC of the isolate identifies that strain as "susceptible" (Webber and Piddock, 2003). Hundreds of studies have been published with results on the MIC reduction of a given strain to several antibiotics; however, not always the change of the susceptibility profile according to the breakpoint for that strain is discussed. For example, for an AcrB-overexpressing strain of *Salmonella enterica* serovar Typhimurium the MIC of nalidixic acid, tetracycline, and chloramphenicol are above the recommended breakpoint concentrations (Piddock *et al.*, 1993). The MIC of ciprofloxacin is usually 0.5 mg/L for an AcrB-overexpressing strain, *i.e.*, below the Clinical and Laboratory Standards Institute (CLSI) and the British Society of Antimicrobial Chemotherapy (BSAC) recommended breakpoint concentrations for this agent and for this organism. However, serovars of *S. enterica* with mutations in *gyrA* are inhibited by 0.25 mg/L, but such strains have been shown to fail therapy with a fluoroquinolone (Piddock *et al.*, 1993; Ricci *et al.*, 2006). There has been considerable discussion in the literature that the recommended breakpoint concentration of ciprofloxacin should be lowered to 0.25 mg/L. If this were the recommended value, then the MIC of ciprofloxacin for an AcrB-overexpressing strain would be above this concentration and so would be deemed clinically resistant.

When using an EPI as a "helper compound" to test the effect on the resistance to a given antibiotic, several results can be obtained, namely, the EPI: i) shows no effect (full growth is obtained); ii) reduces the resistance of the strain to that antibiotic (partial growth is observed); or iii) reverses the resistance to the antibiotic (no growth is observed). This reduction or reversal of resistance can be confirmed by a new MIC determination in the presence of the EPI. If the MIC is reduced to the same level of an ATCC strain (considered "susceptible"), then, one can consider that the resistance of the strain to that particular antibiotic was reversed, to a level considered clinically significative. This demonstrates the relevance of using an antibiotic in conjunction with a "helper compound" that contributes to the reversal of resistance to a given antibiotic of the tested strain. Therefore, new methods that can rapidly and eficiently detect efflux activity on MDR clinical isolates are needed.

5. Methods to access efflux pump activity in bacteria

Assessment of efflux pump has been primarily conducted by the use of ethidium bromide as the extruded substrate of the efflux pumps and its increased retention by the bacterium after an uncoupler of the proton motive force has been added (Kamicker *et al.*, 2008). The baseline of ethidium bromide associated with the bacteria (retained) prior to the addition of the uncoupler and the increase of ethidium bromide retained after the uncoupler has been added is determined with the use of specialised and expensive fluorometric instrumentation that are not readily found in a clinical bacteriology laboratory (Viveiros *et al.*, 2008). These fluorometry assays are based on the movement of ethidium bromide through the cell. Ethidium bromide traverses the bacterial cell envelope via porin channels and once inside, it is concentrated to a point where it fluoresces when excited by U.V. light. This substrate is recognized by efflux pumps of MDR bacteria and is extruded to the medium as long as its concentration in the medium does not overcome the capacity of the pump. Therefore, the loading of bacteria with ethidium bromide at a concentration that is well below that which inhibits replication can be continuously recorded under defined conditions, such as time, temperature and contents of the medium (Jernaes and Steen, 1994). The activity of an efflux pump is

controlled by energy provided from calcium dependent enzymes and hence, it is temperature dependent (Moreau *et al.*, 2005). Although efflux of ethidium bromide can be readily shown by the use of a standard fluorometer or cytometer (Jernaes and Steen, 1994; Looser *et al.*, 2005) when efflux is due to over-expressed efflux pumps, it cannot be easily shown when efflux is due to intrinsic efflux activity (Viveiros *et al.*, 2007). The current fluorometric systems used to access efflux pumps activity are non-physiological; usually the control of the temperature is restricted, are cumbersome and do not yield data that can be subjected to standardization for intra-laboratory comparison. Moreover, not one single aspect of these assays have been standardised due to variety of instrumentation, reagents, media, *etc.*, used by the many laboratories active in this area (Kamicker *et al.*, 2008). There is thus a strong and obvious need to establish new and optimized assays for the assessment of efflux pumps of distinct bacteria, especially those of clinical origin. These assays should be reproducible from laboratory to laboratory, yielding a value that can be used for establishing reference ranges of efflux pump activity that can be used by a clinical laboratory and does not need any specialised instrumentation for its conductance. Assessment of efflux pumps activity of MDR bacteria can therefore establish the basis by which agents can be examined for their ability to inhibit efflux pumps activity rendering the organism susceptible to one, if not all of the antibiotics to which it was initially resistant (Piddock, 2006a,b). If and when such agents are available for patient use, it would be expected that the assay would have extensive use within the clinical laboratory and provide much of the guidance needed for the administration of the agent to patients presenting with the MDR bacterial infection. If such agents are eventually available and implemented in therapeutic regimens, one can see how this would result in the opportunity to use those outdated, inexpensive and safe antibiotics that had fallen by the wayside as a consequence of MDR bacteria. If the activity of an EPI is to be firmly described, the method by which that activity is defined and quantified must be one that lends itself to standardization.

Concluding remarks

It is now well established that bacterial resistance to antibiotics has become a serious problem of public health. The role that efflux pumps play in antibiotic resistance in MDR bacteria is an important subject that has been extensively discussed on the last years. Although high-level resistance may not occur as a result of MDR efflux pumps alone, the association of over-expression of specific genes among highly resistant clinical isolates cannot be mistreated. Therefore, one should not forget that the intrinsic antibiotic resistance of some isolates may be largely due to efflux systems. Synergic increases in resistance seen with over-expression of efflux systems, as well as target site mutations can lead to highly resistant bacteria that are difficult to treat with the antibiotics that are currently available. The contribution of efflux pumps to the resistance of clinical strains needs to be considered in the design of future antibiotics or other compounds. Alterations on the structure of a given antibiotic should be made to reduce the ability of that same antibiotic to be extruded from the bacterial cell, without compromising its activity. Another approach could be the use of EPIs that can improve and potentiate the activity of antibiotics. The development of inhibitors that can reduce the impact of efflux pumps on the activity of some antibiotics will be of clinical interest and of great impact on the clinical setting. Since many efflux systems share a structural homology, one main goal is to discover an EPI that will be active against a range of distinct efflux pumps from different bacterial species. Among the current collection of EPIs, only a few compounds have been studied taking into account the structure–activity relationships and the spectrum of activity in terms of antibiotics, pumps and bacteria. Therefore, there is an acute need for new active agents in order to overcome this emergence of MDR infections. When tested in conjunction with an antibiotic, these EPIs, used as "helper compounds" could contribute to the reduction of the MIC to the antibiotic at a clinical significant level, *i.e.*, rendering the bacteria susceptible to the tested antibiotic. If this approach is successful, the group of "helper compounds" can constitute an important alternative in the therapy of some of the most serious MDR infections (Martins *et al.*, 2008).

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Glossary

- **Adenosine Triphosphate (ATP)-Binding Cassette (also called ABC) superfamily:** group of proteins which bind and hydrolyse ATP to transport substances across membranes. They are prevalent in bacteria but are also present in humans, being involved in tumour resistance, cystic fibrosis, bacterial multidrug resistance and a range of inherited human diseases. ABC proteins have two nucleotide binding domains (areas where ATP binds to the protein and are hydrolysed to ADP) and two transmembrane domains (parts of the protein which span the membrane through which the substrate that's to be transported passes, the substrate translocation pathway).

- **Aryl:** any functional group or substituent derived from a simple aromatic ring, such as, phenyl, thiophenyl, indolyl, *etc*.

- **Arylpiperazines:** organic compounds that present an aryl group and that consists of a six-membered ring containing two opposing nitrogen atoms. The piperazines exist as small alkaline deliquescent crystals with a saline taste and are a broad class of chemical compounds, many with important pharmacological properties, which contain a core piperazine functional group. They also bind to serotonin receptors with moderate to high affinity.

- **Arylpiperidines:** piperidine is an organic compound with the molecular formula (CH2)5NH that presents an aryl group. This heterocyclic amine consists of a six-membered ring containing five methylene units and one nitrogen atom. It is a colorless fuming liquid with an odor described as ammoniacal, pepper-like, or similar to strong pungent cheese. Piperidine is a widely used building block in the synthesis of organic compounds, including pharmaceuticals.

- **Benastatins:** class of polyketide natural products that are produced by *Streptomyces* spp. They are a structurally and functionally diverse group of long-chain polyphenols.

- **Cassette:** a gene cassette is a DNA sequence encoding one or more genes for a single biochemical function.

- **Domain:** an independently folded unit within a protein, often joined by a flexible segment of the polypeptide chain. It can also be defined as a region of special biological interest within a single protein chain. This term also has been used with many different meanings; in particular, it has been used to characterize a region within the three-dimensional structure of a protein that may encompass regions of several distinct protein chains.

- **Efflux pump inhibitor:** compound that inhibit one or multiple bacterial efflux pumps and that can reverse efflux-mediated resistance to many classes of antibiotics in bacteria. In combination with an antibiotic this compound can increase the antibacterial potency against clinical isolates of some bacteria.

- **Efflux pumps:** transporters proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. These proteins are found in the membrane of both Gram-positive and -negative bacteria as well as in eukaryotic organisms.

- **Efflux systems:** function via an energy-dependent mechanism (active transport) to pump out unwanted toxic substances through specific efflux pumps. Some efflux systems are drug-specific while others may accommodate multiple drugs, and thus contribute to bacterial multidrug resistance.

- **Efflux:** mechanism responsible for extrusion of toxic substances and antibiotics outside the cell, being considered a vital part of the xenobiotic metabolism. This mechanism can contribute to bacterial antibiotic resistance.

- **Energy uncouplers:** chemical agents that uncouple oxidation from phosphorylation in the metabolic cycle so that ATP synthesis does not occur. These compounds usually are ionophores that disrupt the electron transfer by short-circuiting the proton gradient across the membranes, blocking the energy involved in the efflux process. Ex: carbonyl cyanide m-chlorophenylhydrazone (CCCP), dinitrophenol, *etc*.

- **Family:** group of evolutionarily related proteins. Proteins belonging to the same family descend from a common ancestor and typically have similar three-dimensional structures, functions, and significant sequence similarity.

- **"Helper compounds":** non-antibiotic compounds that have antimicrobial properties and which alter the permeability of a microorganism to a given antibiotic. If co-administered with conventional antibiotics to which an organism is initially resistant they can reverse or reduce its resistance.

- **Major Facilitator Superfamily (also called MFS):** Group of secondary carriers proteins that transport small solutes in response to chemiosmotic ion gradients.

- **Membrane fusion protein:** membrane proteins which cause more than one membrane to combine.

- **Microbial-derived EPIs:** compounds derived from microbial sources and that act as efflux pump inhibitors.

- **Multidrug And Toxic compound Extrusion (also called MATE) family:** Multidrug And Toxic compound Extrusion family. Family of bacterial drug transporters that play an important role in drug resistance to clinically relevant antibiotics in pathogenic organisms. On this group of proteins efflux is coupled to Na^+ influx. They are present in bacteria as well as in mammalian cells.

- **Multi-drug resistance:** condition that defines a bacterium resistant to three or more distinct classes of antibiotics, except in Mycobacteria where resistance to two or more classes is considered.

- **Outer membrane proteins (also called OMP):** family of proteins that form trimeric channels that allow the export of a variety of substrates in Gram-negative bacteria. Each member of this family is composed of two repeats. The trimeric channel is composed of a 12 stranded all beta sheet barrel that spans the outer membrane, and a long all helical barrel that spans the periplasm.

- **Peptidomimetics:** compounds which mimic the biological activity of peptides while offering the advantages of increased bioavailability, biostability, bioefficiency, and bioselectivity against the natural biological target of the parent peptide. Examples are compounds isolated as natural products, synthesized as libraries from novel subunits, and designed on the basis of X-ray crystallographic studies and through an intricate knowledge of the biological mode of action of natural peptides. They offer challenging synthetic targets and are increasingly important medicinal agents and biological probes.

- **Phenothiazine:** it is a three-ring structure compound in which two benzene rings are joined by a sulfur and nitrogen atom at nonadjacent positions. It is obtained by fusing diphenylamine with sulfur. It can be also called dibenzothiazine or thiodiphenylamine since it is a benzo derivative of thiazine, although thiazine itself is not used as a starting point in the manufacturing of this molecule. It is commonly used as an intermediate chemical in the manufacture of various antipsychotic neuroleptic psychotropic drugs.

- **Porins:** beta barrel proteins that cross a cellular membrane and act as a pore through which molecules can diffuse. Unlike other membrane transport proteins, porins are large enough to allow passive diffusion, acting as channels that are specific to different types of molecules. They are present in the outer membrane of Gram-negative bacteria, the mitochondria, and the chloroplast.

- **Quinoline:** is a heterocyclic aromatic organic compound, also known as 1-azanaphthalene, 1-benzazine, or benzo[b]pyridine. It is mainly used as a building block to other specialty chemicals. Its principal use is as a precursor to 8-hydroxyquinoline, which is a chelating agent and precursor to pesticides. Its 2- and 4-methylderivatives are precursors of cyanine dyes.

- **Resistance-Nodulation-Division (also called RND) superfamily:** Group of secondary membrane transporters that use energy derived from electrochemical gradients across the cell membrane.

- **Small Multidrug Resistance (also called SMR) family:** Group of bacterial multidrug membrane transporters composed of four transmembrane alpha-helices of approximately 100-140 amino acids in length.

- **Superfamily:** term introduced in 1974 by Margaret O. Dayhoff. Originally defined as a group of evolutionarily related proteins, it has also been used in the published literature to refer to a group of structurally or functionally related proteins not necessarily of common evolutionary origin.

- **Transmembrane domain:** single transmembrane alpha helix of a transmembrane protein.