

# In Search of Natural Substrates and Inhibitors of MDR Pumps

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## Abstract

The function of microbial MDRs remains a hotly debated subject. Given the very broad substrate specificities of some MDRs, like the RND pumps that can extrude all classes of amphipathic compounds (cationic, neutral, and anionic), it seems difficult to develop a rationale for pinpointing possible natural substrates of these translocases. At the same time, several clues can be used to guide our search for natural MDR substrates. One is the fact that amphipathic cations appear to be the preferred substrates of MDRs. These substances are extruded by MDRs of all 5 known families and are the almost exclusive substrates of SMR and MF family MDRs. The universal nature of amphipathic cations as MDR substrates suggests that these were the substances that fueled the evolution of MDR pumps. Two factors apparently favored this particular class of molecules for the role of original MDR substrates – need and opportunity. Unlike other substances, amphipathic cations accumulate in the cell driven by the membrane potential, which makes cations potentially the most dangerous toxins. At the same time, amphipathic cations are highly hydrated and do not permeate the membrane as readily as neutral compounds, making it feasible to design a defense based on an efflux pump. The paucity of known cationic (non-basic) antimicrobials might be a result of using MDR-expressing microbial cells for antibiotic discovery. Plant amphipathic cations, the berberine alkaloids, are good MDR substrates. The *Berberis* plants produce 5'-methoxyhydnocarpin-D, an MDR inhibitor that potentiates the action of berberine. It is suggested that the further evolution of MDR pumps was determined largely by the barrier function of the membrane they reside in. Thus Gram negative bacteria have an outer membrane barrier that slows the penetration of virtually all amphipathic molecules, and transenvelope MDRs of the RND and EmrAB-type extrude their substrates across this barrier. A low permeability of the cytoplasmic membrane of yeast similarly allows for the operation of broad-specificity ABC and MF MDRs. The presence of MDR sensors that regulate the expression of some MDR pumps strongly suggests

that defense against external toxins is the function of these MDRs. The BmrR transcriptional activator of the MerR family induces expression of the Bmr pump in *B. subtilis* and is a sensor specifically designed to recognize amphipathic cations. Similarly, the QacR repressor binds chemically unrelated cations, which leads to the expression of the QacA pump in *S. aureus*. In *E. coli*, the EmrR sensor of the MarR repressor family binds unrelated neutral molecules, allowing for expression of the transenvelope EmrAB pump.

## Introduction

Even though MDR pumps have the potential to protect cells from antimicrobials and do this both in the laboratory and in a clinical setting, this does not necessarily mean that drug resistance is the natural function of MDRs.

For example, the Blt pump of *B. subtilis* that protects cells from amphipathic cationic antimicrobials is part of an operon that codes for a putrescine acetyl transferase (Woolridge *et al.*, 1997). The Blt pump extrudes putrescine from the cell, suggesting that this might be its natural function, and drug resistance is a coincidental consequence of the protein being a “sloppy translocase”. Yet even in this seemingly clear case of a specific transporter behaving like a true MDR, there are indications that Blt might after all having multidrug resistance as one of its functions. A global regulator Mta, a member of the MerR family of transcriptional activators, was found to activate expression of both Blt and a closely homologous “true” MDR Bmr (Baranova *et al.*, 1999). The activating ligand(s) for Mta is unknown.

A considerable body of data indicate that drug resistance is the natural function of MDRs. The context of *mdr* gene location, the nature of their substrates, and the mode of regulation provide important clues to the function. For example, the QacA MDR pump is found on broad host range plasmids that also carry specific gentamicin and trimethoprim resistance genes (Rouch *et al.*, 1990). This context suggests QacA is a dedicated drug resistance component of the plasmid as well. The substrate spectrum of MDRs with little exception is limited to amphipathic substances. This in itself suggests these translocases are aimed at protecting the cell from external toxins. Indeed, a toxin must be sufficiently apolar in order to cross the lipid bilayer and gain access to intracellular targets. By the same token, all cellular compounds must be hydrophilic in order to stay in the cytoplasm. This difference in polarity distinguishes between toxins and cell compounds and might be the basis for drug recognition by MDRs that do not discriminate substances on the basis of structure (Higgins and Gottesman, 1992; Lewis, 1994). But the most straightforward indication regarding MDR function comes from the finding of MDR sensors that control expression of the pumps.

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## Multidrug Sensors

The induction of MDRs by their substrates acting through MDR sensors is probably the strongest argument for multidrug resistance being the natural function of at least some of these translocases. Three such proteins have been described so far.

In *E. coli*, we identified a regulator of the EmrAB pump, EmrR, an 18Kd protein that is coded by the first gene of the *emrRAB* operon (Lomovskaya *et al.*, 1995) and belongs to the MarR family of transcriptional repressors (Miller and Sulavik, 1996). EmrR binds to such EmrAB substrates as uncouplers of oxidative phosphorylation and binding releases repression and activates transcription. Experiments with purified EmrR show that it directly binds its ligands *in vitro*. Scatchard analysis of equilibrium dialysis data showed a 1 ligand per monomer of dimeric EmrR with good affinity -  $K_S$  around 1  $\mu$ M for FCCP and CCCP, and  $K_S$  around 10  $\mu$ M for the more hydrophilic DNP (Brooun *et al.*, 1999). The central region of the protein is fairly well conserved within the family and corresponds to a helix-turn-helix motif (Miller and Sulavik, 1996).

Efforts to localize a ligand binding site were successful in the study of the *B. subtilis* BMR multidrug sensor and the crystal structure of this C-terminal domain has recently been resolved (Zheleznova *et al.*, 1999; 1997). BmrR is a transcriptional activator of the MF BMR pump (Ahmed *et al.*, 1994; Markham *et al.*, 1996). BmrR binds chemically unrelated hydrophobic cations, such as TPP<sup>+</sup> and ethidium bromide (EtBr), substrates of the BMR pump, and activates the BMR transcription. BmrR is a 32 kDa protein that forms a dimer and binds one ligand molecule per dimer. The 18.4 kDa C-terminal ligand binding domain still forms a dimer, and this peptide was crystallized both with and without the ligand TPP<sup>+</sup>. The binding site is rather unusual - it is not obviously present in the apoprotein and is formed in the process of ligand binding. TPP<sup>+</sup> apparently aids unfolding and displacement of an  $\alpha$ -helix, which exposes a hydrophobic binding pocket with a buried glutamate residue. Once pass the gate, the ligand is bound by stacking and van der Waals interactions with residues of hydrophobic amino acids and by electrostatic interaction with the glutamate. This interesting and unusual structure explains the main features of selectivity. Apparently, hydrophilic molecules will not gain access to the hydrophobic site that is not even open in the apoprotein; once inside the pocket, the amphipathic ligand will be retained by hydrophobic interactions; and the presence of a strong negative charge in this hydrophobic environment will select for cationic species.

A third multidrug sensor has been described that regulates expression of the QacA MF MDR found on multidrug resistance plasmids of *S. aureus*. It is coded by a *qacR* gene located immediately upstream of *qacA* and is divergently transcribed from its own promoter. The QacR repressor binds a wide range of amphipathic cations (Grkovic *et al.*, 1998), exclusive substrates of QacA, and belongs to the TetR family of repressors. It is interesting to note that BmrR does not bind planar molecules like ethidium bromide and berberine. Planar molecules will not produce high-affinity interaction with a rigid cone-shaped binding site of BmrR. The BMR pump, like other MDRs,

extrudes both planar and aplanar hydrophobic cations perfectly well. This probably suggests that the MDR has a flexible hydrophobic binding pocket that will properly envelope the incoming toxin. The QacR sensor binds both the planar ethidium bromide and the aplanar rhodamine, also suggestive of a more accommodating hand-glove type of flexible interaction with ligands. The nature of possible natural ligands of Bmr remains an intriguing question.

## MDR Substrates

If there are dedicated MDRs, what are their natural substrates? RND pumps extrude natural antibiotics, but handle artificial substrates just as well or better, and have such a broad substrate spectrum that it is unclear whether antibiotic extrusion plays a leading role outside of the clinical setting. The AcrAB pump was shown to strongly protect *E. coli* from bile acids, and it was proposed that bile acid extrusion might be the natural function of this pump (Thanassi *et al.*, 1997). This is an appealing hypothesis, and bile acids might indeed be among the many natural substrates of the extremely broad-spectrum AcrAB pump. Protection from bile salts and fatty acids appears to be one of the functions of the Mtr RND pump of *N. gonorrhoeae* (Hagman *et al.*, 1995) and of VceAB which is a *Vibrio cholerae* homolog of the *E. coli* EmrAB pump (Colmer *et al.*, 1998). The Mtr pump of *N. gonorrhoeae* has been reported to protect the cell from small defensin peptides protegrin-1, a cationic peptide from porcine leukocytes, and peptide LL-37 expressed in human granulocytes and other cell types (Shafer *et al.*, 1998). Mtr is not a universal transporter of defensins, and it remains unclear whether its ability to extrude these two peptides is accidental, or part of its natural function. More to the point, it does not seem that RND pumps that are widely distributed among Gram-negative bacteria evolved to cope with bile acids or defensins of animals. Another possible example of a natural substrate has been described in a study of an IfeAB RND pump from *Agrobacterium tumefaciens* (Palumbo *et al.*, 1998). Mutation of the pump decreased accumulation of an isoflavonoid coumestrol that is produced by its host, the alfalfa plant. Coumestrol induced expression of the pump and the mutant was outcompeted by the wild type in colonizing the plant. However, neither the wild type nor the mutant were sensitive to coumestrol. As the authors note, coumestrol might be acting as an inducing signal for the pump, rather than its natural substrate. The issue of natural MDR substrates in most cases remains very much an open question.

## Amphipathic Cations as Universal MDR Substrates

Even though MDR pumps extrude structurally unrelated compounds, a general theme emerges if one considers the preferred artificial substrates of most MDRs. These substances with little exception are amphipathic cations (Figure 1). This observation suggests that amphipathic cations represent the prototypical and existing natural substrates of MDR pumps (Hsieh *et al.*, 1998; Lewis, 1999b). The simplest MDRs of the SMR family are a group of MDRs that have amphipathic cations as their exclusive substrates, at least in Gram positive bacteria. There are

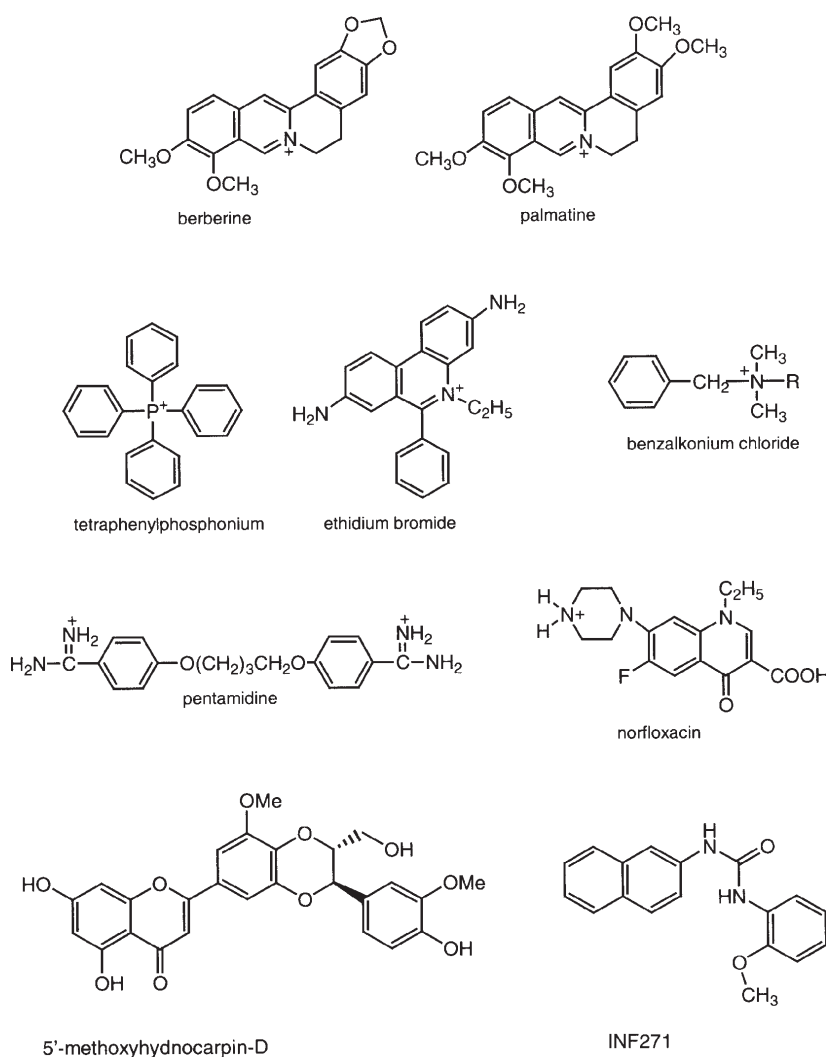


Figure 1. Cationic MDR substrates, and inhibitors of NorA. Berberine and palmatine are plant isoquinoline alkaloids. Tetraphenyl phosphonium has been used as a probe to measure the membrane potential. Benzalkonium chloride is an antiseptic and disinfectant. Substrates that are weak bases are shown in their cationic form - pentamidine is a systemic antiyeast antimicrobial, chlorhexidine is an antiseptic, and norfloxacin is a fluoroquinolone antibiotic. 5'-methoxyhydrocarpin-D is a natural MF MDR inhibitor produced by *Berberis* plants that synthesize berberine. INF271 is a synthetic MDR inhibitor.

many different MDR pumps in the MF family, and most of them exclusively extrude amphipathic cations. For example, the QacA pump only extrudes cations; the NorA pump of *S. aureus* extrudes cations and to a lesser extent quinolones; the BMR pump of *B. subtilis* extrudes primarily cations and neutral chloramphenicol (Review, (Paulsen *et al.*, 1996a)). The BmrR regulator that activates BMR transcription has a design suggesting it specifically evolved to detect a wide range of amphipathic cations. MDRs of the RND family have a broad substrate spectrum, and all tested RND pumps extrude amphipathic cations. The preferred substrates for ABC MDRs LmrA and P-glycoprotein are amphipathic cations, but some neutral compounds can be extruded as well. There are many ABC MDRs in yeast, and at least 8 functional ABC MDRs are present in *S. cerevisiae* alone. The substrates of these pumps are amphipathic cations, and neutral substances such as anti-yeast azoles (Kolaczowski and Goffeau, 1997; Paulsen *et al.*, 1998).

The following picture emerges from this analysis. Simple MDRs like SMRs only export amphipathic cations; MF and MATE MDRs export mainly cations; RND and ABC are the most complex of the MDRs and have broad spectra

of specificity that include amphipathic cations as preferred substrates. MDRs clearly prefer amphipathic cations to other substances, even though they belong to 5 unrelated protein families. Not only are MDRs unrelated, but even the general mechanisms of drug transport are likely different for different MDRs (Lewis, 1999a). It appears that a similar need to protect the cell from amphipathic cations evolved in different groups of MDRs (and in different organisms) in spite of a lack of overall homology or similarity in the mechanism of action.

Quite surprisingly, one does not find amphipathic cations in a general list of natural antimicrobials. (The known cationic antibiotics of the aminoglycoside group such as streptomycin and kanamycin are hydrophilic substances that get smuggled into the cell via specific translocases and are not general substrates of MDRs). At the same time, amphipathic cations should be among the most potent antimicrobials. A positive charge will lead to a considerable accumulation of a substance in the cell. According to the Nernst equation, there is a 10-fold accumulation of a cation (cat) for every 60 mV of the membrane potential:

$$\Delta\phi(\text{mV}) = (RT/nF)\ln\{[\text{cat}]_{\text{in}}/[\text{cat}]_{\text{out}}\} = 60\lg\{[\text{cat}]_{\text{in}}/[\text{cat}]_{\text{out}}\}$$

The  $\Delta\phi$  in bacteria is around 140mV and around 180mV in yeast plasma membrane (Skulachev, 1988), which would result in a 100-1000 fold accumulation of an antibiotic. Note that weak amphipathic bases (such as chlorhexidine) are also MDR substrates, but one would not expect these compounds to be among natural antibiotics, since they are extruded from the cell by the pH gradient and are therefore intrinsically less potent than neutral compounds or cations. The fact that amphipathic cations are conspicuously absent from known natural antibiotics is especially puzzling given that these substances are the preferred substrates for most MDRs. We have argued that it is precisely the existence of MDR pumps that is responsible for this apparent paradox (Hsieh *et al.*, 1998). If MDRs evolved in response to natural antimicrobial amphipathic cations, then these substances would be difficult to discover in standard screens that employ cells carrying MDR pumps. In the process of drug discovery, the concentration of antimicrobials is prone to be low, and MDR substrates will be overlooked. MDR mutants can therefore be used as sensitive tools for drug discovery (See papers by Davies *et al.* and Rogers *et al.* in this issue).

While using MDR mutants is a reasonable (if somewhat unpredictable) way to discover possible cationic antimicrobials, another approach is to search for possible MDR substrates among known compounds. Many natural substances have been described as a result of systematic chemical analysis of organisms, rather than in particular bioassay-driven purifications. One would then look for substances that are amphipathic cations of natural origin that have little or no antimicrobial activity. Using these criteria, we identified a group of plant alkaloids whose members have little or no antimicrobial activity. These are the isoquinoline alkaloids (Figure 1) that are widely spread among the plant world and are found among many *Ranunculales* species, for example (Colombo and Bosisio, 1996). These substances bear a resemblance to artificial MDR substrates such as ethidium bromide (Figure 1). They are amphipathic and have a strong positive charge which is delocalized by the conjugated ring structure, an essential feature of a good permeant cation (Skulachev, 1988).

We have chosen two representative substances of this group, berberine and palmatine, to test whether they are MDR substrates. Palmatine had very low (> 200  $\mu\text{g/ml}$  MIC) and berberine poor (240-120  $\mu\text{g/ml}$ ) activity against wild type *S. aureus* (Hsieh *et al.*, 1998). The antimicrobial activity of the alkaloids increased sharply in a *norA* mutant, with an MIC of 50  $\mu\text{g/ml}$  for palmatine and 7.5 for berberine. Sensitivity to alkaloids increased further in the presence of an MDR inhibitor INF271 (provided kindly by Dr. P.N. Markham of Influx, Inc.; Lewis, unpublished). It appears that the inhibitor disables both *NorA* and possible additional unknown MDR(s). Thus in the presence of the MDR inhibitor berberine becomes an extremely potent antibiotic (MIC 0.5  $\mu\text{g/ml}$ ), about 10 times stronger than streptomycin. Our preliminary experiments show that berberine is also the substrate of the plasmid-borne *QacA* pump of *S. aureus* (MIC 500  $\mu\text{g/ml}$  vs. 1  $\mu\text{g/ml}$  in the presence of INF271). In the presence of the MDR inhibitor, yeast become very sensitive to berberine as well (MIC 120  $\mu\text{g/ml}$  vs. 1  $\mu\text{g/ml}$ ).

Why should plants keep on making isoquinoline

alkaloids if microorganisms have MDR pumps that can render these substances essentially ineffective? One possibility is that isoquinoline alkaloids are not antimicrobial compounds *in vivo* and have a different function, such as antiherbivoral. A more interesting possibility is that in response to bacterial resistance mechanisms, plants have developed MDR inhibitors that act synergistically with isoquinoline alkaloids. We have tested this hypothesis using a berberine-producing *Berberis fremontii*. An extract of the plant has at least two different MDR inhibitors that act synergistically with berberine in inhibiting the growth of *S. aureus* (Stermitz *et al.*, 2000a; Guz and Stermitz, 2000; Stermitz *et al.*, 2000b; Guz *et al.*, 2001). The first one identified is 5'-methoxyhydrnocarpin-D (5'-MHC-D), a flavonolignan. 5'-MHC-D had no activity on its own, but at 1  $\mu\text{g/ml}$  completely inhibited growth of *S. aureus* in the presence of sub-inhibitory berberine. 5'-MHC-D was also found to completely inhibit MDR-dependent efflux of ethidium bromide and berberine from the cells, and the level of berberine accumulation by cells was sharply increased in the presence of the inhibitor. *S. aureus* is likely to encounter natural cationic antimicrobials like berberines when the microbe is persisting in the environment. Berberis species are not known to be infected by bacterial pathogens, apparently due to the presence of effective antimicrobials like berberine and 5'-MHC-D. Unlike antibiotics produced by bacteria or fungi, plant antimicrobials are weak or narrow-spectrum, which is puzzling. It will be interesting to learn whether plants commonly employ anti-MDR strategies to potentiate their antimicrobials.

### The Nature of the Permeability Barrier Determines the Nature of MDR Substrates?

In the process of studying adaptation of *E. coli* to uncouplers of oxidative phosphorylation, we cloned a translocase *EmrAB* that produced resistance to CCCP and an array of other chemically unrelated compounds. This seemed puzzling, since the very mechanism of uncoupling depends on the ability of a protonophore like CCCP to shuttle back and forth across the membrane carrying protons. Attempts to "extrude" CCCP across the cytoplasmic membrane by a pump would only increase the activity of uncoupling. We therefore proposed that the pump extrudes its substrates all the way across the outer membrane, "taking advantage" of the barrier function of the outer membrane to hydrophobic substances (Lomovskaya and Lewis, 1992; Lewis, 1994). Subsequently it was found that another type of MDR pump in *E. coli*, *AcrAB-ToIC*, also has a transenvelope design (Ma *et al.*, 1993; Nikaido, 1996; Zgurskaya and Nikaido, 1999b).

The outer membrane of Gram negative bacteria is a barrier to a broad range of amphipathic compounds, and to all large molecules, which allowed for the evolution of broadly specific MDRs that pump their various substrates across this barrier. By the same token, Gram positive species that lack an outer membrane have MDRs with a reported substrate spectrum largely limited to amphipathic cations. Apart from the usefulness of protecting the cell from this class of compounds that actively accumulate in the cell, evolution of "cation-pumping" MDRs was made

possible by the relatively slow permeation of amphipathic cations across the cytoplasmic membrane, as compared to neutral compounds of similar polarity. Not everything however fits into this seemingly neat scheme of things.

In this volume, Davies and co-authors describe a systematic effort of disabling all putative MDRs of *Enterococcus faecalis*. This particular bacterium is known for its elevated “intrinsic resistance” to antimicrobials (Lynch *et al.*, 1997). Mutants with knockouts in ABC transporters *abc16* and *abc23* were shown to have a considerably increased susceptibility to neutral antibiotics. *abc16* had an increased susceptibility to macrolides, which is not surprising – this is probably a macrolide-specific pump (which have been described in Gram positive species) rather than an MDR. Strain *abc23* on the other hand showed greatly increased susceptibility to some neutral compounds with completely unrelated chemical structures – lincosamides lincomycin and clindamycin; and streptogramins of the virginiamycin complex, as well as semi-synthetic streptogramins quinupristin and dalopristin, constituents of Synercid. Interestingly, *abc23* did not show increased susceptibility to the other components of the 40-odd member antimicrobial panel (Davies *et al.*). Macrolides and streptogramins are very large molecules that are likely to have a low rate of permeability across the membrane.

The fact that the cytoplasmic membrane is generally not a good barrier for amphipathic neutral compounds explains why antibiotics are effective in the first place, and why extrusion via a specific pump is such a rare type of a resistance mechanism in Gram positive species. We indeed know of only a handful of cases. Extrusion of tetracycline is a most common mechanism of resistance by efflux, and tet pumps come in several different families (See the review by Krulwich and colleagues in this volume). Tetracycline forms a complex with a divalent metal like magnesium, and is actually a cation *in vivo*, which would explain its lowered capability to cross the membrane, providing an opportunity for a pump to expel it effectively. Chloramphenicol is another example of an antibiotic that is effluxed by specific pumps across the cytoplasmic membrane. This is a fairly hydrophilic compound, which suggests that it is a relatively poor membrane permeator. Large and fairly hydrophilic macrolides represent another type of antibiotics that are exported by specific resistance pumps in Gram positive bacteria.

One may expect that Gram positive bacteria like Streptomyces producing antibiotics and employing specific pumps for their export will have a membrane that is a good barrier for these substances. This in turn suggests that antibiotic producers may also have broadly specific MDRs to protect the cell from an antibiotic attack from their neighbors. Gram positive Mycobacteria have of course evolved the most impenetrable barrier to all substances in the form of a “cell wall” which is actually analogous in its general design to the outer membrane of Gram negative bacteria. This cell wall is in fact a proper second membrane, a very thick lipid bilayer made of C60-C90 mycolic acid bound to arabinogalactan and a number of additional lipid components (Brennan and Nikaido, 1995). Porins traverse this membrane, providing a channel for nutrients, and an opportunity for toxins like antibiotics to enter the cell. Porins are sparse, accounting for the slow rate of growth of species

like *M. tuberculosis*, and for high resistance to antimicrobials. Is the outer membrane of Mycobacteria traversed by MDRs? This is currently an intriguing open question. Not much is known about Mycobacterial MDRs – there is only one report of an *M. smegmatis* MF pump of the 14 TMS family which confers low level resistant to cations and fluoroquinolones when overexpressed from a multicopy plasmid (Takiff *et al.*, 1996). Perhaps a systematic deletion of Mycobacterial putative MDRs will show something more interesting. One thing to keep in mind is the redundancy of MDRs – knocking out a single pump will have little effect if a set of several MDRs with similar substrate spectra is operating in the cell. Multidrug resistant strains of *M. tuberculosis* have so far been shown to carry several mutations each conferring specific resistance to a given antibiotic. Whether *intrinsic* resistance of *M. tuberculosis* is due to a combination of the “outer membrane” barrier and MDRs that pump substrates across it is an open question.

Barrier properties of the cytoplasmic membrane explain why the useful range of Gram positive MDRs is limited to amphipathic cations in most cases. An exception that questions this reasoning has been recently reported. Seven homologs of SMR proteins are found in the genome of *B. subtilis*. Interestingly, six of these are grouped in three operons, each carrying two different *smr* open reading frames (Jack *et al.*, 2000). Attempts to functionally express these single ORFs in *E. coli* were not successful. However, coexpression in *E. coli* of two genes from one of these operons, *ykkCD* (but not *ykkC* or *ykkD* alone), gave rise to an MDR phenotype. The MDR appeared to have a specificity spectrum broader than previously examined homo-oligomeric SMRs, that included the usual set of amphipathic cations, but also streptomycin, tetracycline, chloramphenicol, and fosfomycin (currently known as fosfomycin). As we have mentioned above, tetracycline is likely to exist in the form of a metal chelate and is a cation, and chloramphenicol is frequently found to be a substrate of otherwise cation-restricted MDRs like Bmr. Streptomycin is a hydrophilic cation, is not membrane-permeant on its own (aminoglycosides are smuggled into the cell via specific transporters) and is only extruded by several recently discovered MDRs (See (Mao and Lomovskaya, 2001) for a discussion). A substantial, 100 fold resistance to the anionic fosfomycin conferred by *YkkCD* is unexpected. Is it possible that this broader than expected spectrum of activity can only be observed when *YkkCD* is expressed in a Gram negative bacterium? A weak net pumping activity by an SMR into the periplasm can be amplified if the substrate is then picked up by a transenvelope MDR and extruded from the cell. This tandem arrangement has been described for several cases, including, for example, a chloramphenicol pump located in the cytoplasm of *E. coli*, and the transenvelope AcrAB-TolC (Lee *et al.*, 2000). The tandem pair works synergistically. But even if the broad functionality of the *B. subtilis* SMR is an artifact of expressing these proteins in a Gram negative host, these interesting experiments still show that the recognition potential for these SMR is broader than what has been reported for Gram positive bacteria.

The majority of studies to identify an MDR and determine its substrate spectrum follow a common pattern

– a strain overexpressing a recombinant putative MDR is tested for susceptibility with a panel of antimicrobials, and increased resistance indicates the capabilities of the pump. Similar approaches are to test a regulatory mutant overexpressing the pump, or a deletion strain lacking the locus. In the case of the NorA pump of *S. aureus*, for example, such studies lead to the finding of amphipathic cations representing the main if not the single class of substrates of this MDR (Hsieh *et al.*, 1998). A screen of a compound library to identify NorA inhibitors produced a surprising result – a hit rate of an amazing 4% (Markham *et al.*, 1999). This library of 10,000 randomly assembled synthetic compounds produced around 400 substances that completely inhibited the pump when screened at a fairly low concentration of 20  $\mu\text{g/ml}$ . Many of these were neutral compounds, and from these hits inhibitors of high activity were further identified. INF271 (Figure 1) is an example of such an inhibitor, which completely blocks the pump at 1  $\mu\text{g/ml}$ . It would seem that the simplest possibility is that all these substances, cationic and neutral, are competitive inhibitors, and thus substrates of the pump. This in turn would mean that NorA (and other MDRs?) has an extremely broad substrate specificity. But having a capability to recognize substrates that are not restricted by the membrane barrier is not only useless, but might be detrimental to the cell. Pumping out a substance that effectively leaks back in will result in an energy-consuming futile cycle. So why does NorA not have a narrower recognition spectrum, which would limit the substrates to amphipathic cations that the pump could handle in a useful way? One possibility is that *S. aureus* is capable of producing a membrane that is a better barrier, and that is when NorA, and other MDRs get a chance to show their full capability. A general possibility is a decreased permeability due to a temperature downshift. For example, *S. aureus* growing at 37C will experience sharply lower ambient temperature when expelled from its host into the environment. The external environment will also present a variety of toxins produced by microorganisms and plants. A shift from a liquid to a more solid state below the membrane transition state temperature is likely to create a permeability barrier that might allow the MDRs to employ their full protective capacity.

There actually are a number of interesting well-documented cases of changes in bacterial membranes that help MDRs in their efflux activity. The most straightforward example is the simultaneous upshift in the expression of the AcrAB-TolC pump and a downshift in the expression of the larger outer membrane porin OmpF by the *marA* (multiple antibiotic resistance) global regulator (Cohen *et al.*, 1988; Okusu *et al.*, 1996). This creates a better outer membrane barrier and more pump to extrude toxins across this barrier. MarA is a transcriptional activator that is controlled by the MarR repressor. MarR ligands are phenolic compounds like salicylate (Alekshun and Levy, 1999), and it was proposed that the increased drug resistance is activated when *E. coli* finds itself in the gut of omnivores rich in plant antimicrobials (Sulavik *et al.*, 1995).

In *P. putida*, the cytoplasmic membrane composition and physical properties differ strongly depending on the environment. In the presence of toluene, cells produced more *trans*, rather than *cis* unsaturated fatty acids (Weber

*et al.*, 1994), and synthesized more unsaturated fatty acids (Heipieper and de Bont, 1994). This resulted in a more “solid-state” membrane which had a 7C higher transition temperature, producing increased resistance to organic solvents that are extruded by an RND-type pump SrpABC (Kieboom *et al.*, 1998a). Importantly, organic solvents also induce the expression of SrpABC (Kieboom *et al.*, 1998b). This dual effect of improving the barrier and inducing the pump is similar to the porin shutdown/AcrAB-TolC induction controlled by the *mar* operon.

Yeast like Gram positive bacteria have a single cytoplasmic membrane, yet show a capability for extruding a broad range of chemical substances resembling that of Gram negative bacteria (Kolaczowski *et al.*, 1998). Apparently, the yeast membrane is a fairly good general barrier for amphipathic substances. The barrier function of the membrane is likely the result of the presence of large amounts of ergosterol that is not found in prokaryotes. Null ergosterol mutations are lethal, accounting for the effectiveness of azole-based antifungals that are inhibitors of ergosterol biosynthesis. Mutants of *S. cerevisiae* with decreased expression of ergosterol have been long used for “increased penetration” of various substances, and functionally resemble yeast MDR mutants. It is not clear how ergosterol makes the membrane less permeable to solutes; a decreased fluidity is probably part of the mechanism. The presence of a similar cholesterol lipid in higher eukaryotes is probably responsible for the barrier function of membranes as well, accounting for the very broad functional capability of MDRs like P-glycoprotein and MRP. What remains unclear is why Gram positive bacteria do not have ergosterol or a functional equivalent imparting a constitutive good barrier function to the cytoplasmic membrane. One possibility is that a barrier function comes at a price of increased membrane rigidity that would slow down enzymatic processes, such as translocation of nutrients or electron transport.

Remarkably, plants make saponins that specifically “extract” ergosterol from the membrane (Polacheck *et al.*, 1991). Saponins are glycosylated sterols that form a complex with ergosterol. Some saponins have the ability to directly kill yeast or fungal pathogens (Polacheck *et al.*, 1991). What has not been considered so far is the interesting possibility of saponins potentiating the penetration of other antimicrobial compounds. This in fact might turn out to be the main function of saponins, especially in plants that do not make large amounts of these substances, or make saponins that have weak antifungal activity. Indeed, saponins seem to act much better *in vivo* than *in vitro*. Mutant oats that lack saponin avenacin A-1 were sensitive to a range of fungal pathogens (Papadopoulou *et al.*, 1999). However, fungal pathogens were resistant to avenacin A-1 *in vitro* (Carter *et al.*, 1999). These findings strongly suggest that *in vivo*, the function of avenacin A-1 is to facilitate the penetration of other antimicrobial compounds into cells of pathogens. Destroying the permeability barrier of a pathogen might be the plant’s solution to the formidable problem of disabling a suite of numerous MDRs. Whether a plant can make MDR inhibitor(s) to act against all MDRs of its often numerous yeast and fungal pathogens is problematic. *S. cerevisiae* alone has at least 8 different functional MDRs

(Kolaczowski *et al.*, 1998), and judging by the genomic sequence, may have more than 20 (Paulsen *et al.*, 1998). Employing a saponin to destroy the permeability barrier would be a much smarter approach to disabling MDR resistance than going after each and every conceivable MDR pump. ERG mutants of *S. cerevisiae* were recently shown to have a diminished function of the ABC MDR transporter Pdr5 (Kaur and Bachhawat, 1999). This suggests that ergosterol is indeed part of the barrier that prevent Pdr5-exported drugs from leaking back in.

An interesting practical consequence of this hypothesis is a possible new approach to circumventing multidrug resistance. Saponins can be tested as potentiators of antimicrobials that are substrates of yeast/fungal MDRs. Yeast resistance is largely due to the overexpression of MDRs, and saponins could be employed to circumvent their action and potentiate the main antiyeast/antifungals currently in use – azole compounds like fluconazole; and amphotericin B. The same approach can be employed for circumventing the MDRs responsible for multidrug resistance of human cancer – P-glycoprotein and MRP. Note that saponins are abundantly present in many plant foods such as soy beans, indicating their low toxicity.

#### Acknowledgements

The work from the author's Laboratory reported in this paper has been supported by NIH grants RO1GM54412 and RO1 GM61162.

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