Resistance of Microbial Cells to Antimicrobial Agents by Efflux

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INTRODUCTION

Efflux mechanisms provide microbes with a means of reducing the antibiotic concentration at the target site. As the topic of antimicrobial resistance is expanding in many directions, this review is divided into sections. Efficacy of antimicrobials depends on their ability to reach their target. Factors that influence the accumulation of drugs within the microbial cell can have a big effect on antimicrobial resistance. Efflux mechanisms in microbes have become a huge problem for scientists and clinicians as many of our antimicrobials are becoming useless as a result of these resistance systems.

Many microbes have both intrinsic and acquired resistance to antimicrobials because of their efflux pumps. Acquired resistance comes from the acquisition of plasmids from other microbes or through mutations

whereas intrinsic resistance occurs independently of these.[1] Acquired efflux pumps called multi-drug resistant (mdr) pumps have been found in bacteria. These are very worrying as the genes for these often encode

resistance to a variety of drugs, biocides and other chemicals.[2] They have been found on plasmids (as well as some bacteria having them intrinsically) and these have led to widespread transfer of the mdr phenotype

between bacteria.[3] A number of studies have demonstrated that resistance to almost any antibiotic can be achieved through the activity of these mdr pumps.[4] Acquired resistance highlights that the bacteria can pass these resistance mechanisms from one to another very rapidly.[3,5] Alternative treatments to antimicrobials will have to be evaluated if we are to continue curing people of serious microbial infections.

Intrinsic resistance implies that the efflux pumps have another function in the cell and that the pumps

coincidentally cause the efflux of antimicrobials.[6] The efflux systems have been found in all bacteria, and because of this, it is thought that they may have a "housekeeping role" within the cell.[1,7] Bacteria often encounter substances that are toxic to them. Many of these may be lipophilic and able to penetrate the cell relatively easily. Therefore, in order to survive, the microbe must eliminate these toxic substances. Having a pump that can eject the substance would be a very efficient way for the bacteria to survive.[8]

In studying the efflux pumps of Escherichia coli and Neisseria gonorrhoeae, two systems have been found (the AcrAB system of Escherichia coli and the MtrCDE system of Neisseria gonorrhoeae) that have the natural function of excreting toxic substances from the cell as well as the proposed acquired function of the efflux of antimicrobials. The Escherichia coli AcrAB system pumps various bile acids out of the bacterium. These transporters have been seen to be dispensable in some instances, but this may be because most microbes

have multiple efflux systems each with overlapping functions.[1] Other efflux systems may be able to compensate for the loss of another.

INTRODUCING THE EFFLUX PUMPS

There are 5 classes of efflux pumps: Major Facilitator Superfamily (MFS), ATP-Binding Cassette transporter family (ABC), Resistance-Nodulation-Division family (RND), Small Multidrug Resistance family (SMR), and

Multi-Drug and Toxic Compound family (MATE).[7,9]

Figure 1. The proton-driven drug pumps in Gram-negative bacteria

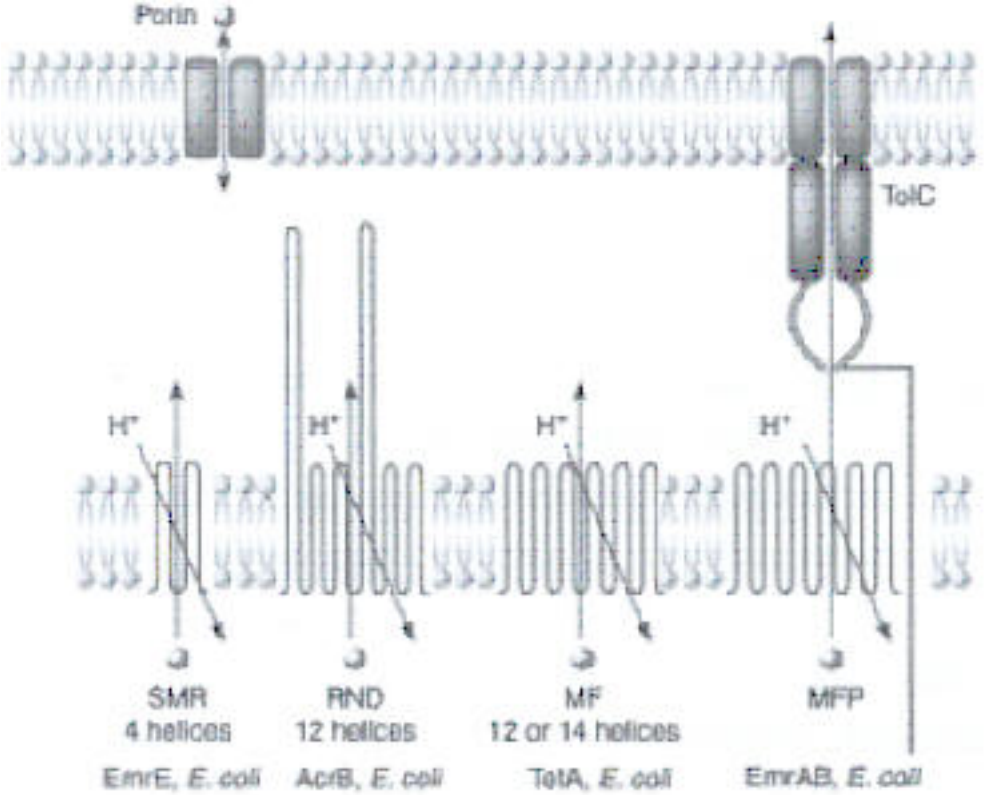

Figure taken from Borges-Walmsley et al.[9]

Most function as drug/proton antiporters although the ABC class uses ATP (adenosine triphosphate) to drive the removal of cationic and lipophilic antimicrobials.[9,10,15] At the moment, no direct knowledge is available about the mechanism of coupling drug transport with the inward transport of protons or with ATP hydrolysis.[11]

The RND family is the most relevant with respect to antimicrobial resistance, so this is the type that will be focused on to illustrate the mechanism of efflux in microbes. RND transporters have been found in all major kingdoms but only have a role in resistance in gram negative bacteria. The RND transporters work with a periplasmic Membrane Fusion Protein (MFP) and an Outer Membrane Factor (OMF) to cause efflux across both the inner and outer membranes (no accumulation occurs in the periplasm).[9,12,13] These transporters are usually chromosomally encoded and they are involved in multidrug resistance, heavy metal ion export, transport of oligosaccharides and extrusion of hydrophobic solvents. It is thought that the RND component of this transporter system is the part that confers substrate specificity, but the cause of this specificity is still unknown.[14,15] RND transporters have a wider range of substrates compared to SMRs or MFSs and RND transporters can expel drugs that are targeted to both the cytoplasm and the periplasm.[11] Studies of the transporter suggest that the only requirement for the drug to be a substrate of these efflux pumps is the presence of a hydrophobic domain capable of insertion into the phospholipid bilayer but they don not tell us how the pump discriminates between its substrates and natural components of the cell membrane. From studies on the BmrR transporter of Bacillus subtilis, it is thought that the hydrophobicity, shape and size of the molecules are important in binding to the transporter.[7,13]

Membrane fusion proteins also form part of this transporter system. Two hydrophobic domains have been identified near the N- and C- termini of the MFPs which are proposed to interact with the inner and outer membrane components of the complex and to transfer the substrate across the periplasm. The MFP's probably function in the tri-partite complex by preventing substrate release into the periplasm. There are two general models of MFP action. Either oligomerisation with the OM component occurs to form a closed channel through the periplasm or the MFP brings the inner and outer membranes together in close apposition.[2,9,13] There is more support for this latter model, as the AcrA protein which is a MFP was shown to promote the close association of the two membranes and to possibly promote a hemi fusion event under conditions similar to those found in the periplasmic space. MFP's seem to be very specific for their own transport efflux systems as even highly homologous MFP's such as MexA and MexC (periplasmic components from Pseudomonas aeruginosa) cannot be interchanged in the tripartite complex.[8,13] This suggests the presence of specific interactions between the MFP and the inner membrane transporter.

In contrast, the formation of the tripartite complex and more specifically the involvement of the outer membrane component, appears to be transient and to be formed only in the presence of the protein substrate. The MFP component is thought to have a role in recruiting the outer membrane component although it is not known how the specificities of the bi- and tripartite complexes are determined.[1]

The outer membrane components are interchangeable between different multidrug efflux systems and a single OMF can function with different pumps.[12] The most widely studied OMF is TolC. It is a porin like protein.[9] It is essential for the activity of the drug transporter AcrB in prokaryotes and protein transporters HlyB and CvaB in eukaryotes.[3] This protein can also work with McaD and MexY from Pseudomonas aeruginosa if these components are expressed in Escherichia Coli.[13] This demonstrates the diversity of this protein.

RND transporter systems are not always tripartite. In some cases, all three components of the efflux pump are coded for in the same gene cluster, e.g. MexAB-OprM from Pseudomonas aeruginosa and MtrCDE from Neisseria gonorrhoeae.[14] Some pumps, however, lack the gene for the outer membrane component in the gene cluster, e.g. MexXY from Pseudomonas aeruginosa and AcrAB from Escherichia coli. There is evidence that AcrAB recruits TolC as the outer membrane component and MexXY appears to share the outer membrane component with MexAB because both of these systems require the presence of a functional OprM channel.[3,14]

SYNERGISTICALLY WORKING WITH THE OUTER MEMBRANE?

The efflux pumps work with exceptional efficiency in gram-negative bacteria. This is thought to be due in part to their synergistic action with the outer membrane. In gram-positive bacteria, the efflux pumps move the substrate across just one membrane. This is rather inefficient, as they have to compete with the rapid spontaneous influx of the lipophilic inhibitor molecule back into the cytoplasm. A high rate of efflux is therefore required to produce significant levels of resistance. The efflux pumps in the gram-negative bacteria traverse both the inner and outer membranes. As the outer membrane is composed largely of LPS, it has different permeability properties to the membrane of gram-positive bacteria. It does allow the penetration of lipophilic molecules but at a rate 50-100 times slower than the phospholipid bilayer.[11,13] The decrease in penetration of lipophilic molecules is responsible for the intrinsic resistance of gram negatives to certain antibiotics (e.g. glycopeptides). Hydrophilic molecules enter the gram-negative cells through porins in the membrane but in the presence of antibiotics or when efflux mechanisms are induced, a decrease in the number of porins in the membrane is also seen. This leads to decreased penetration of the hydrophilic molecules.

The synergistic action between the pumps and the outer membrane is seen in mdr or multiple antibiotic resistant (mar) mutants in Escherichia coli where a simultaneous decrease in porin production and an increase in efflux activity is observed.1 This synergy explains how gram-negative bacteria become hypersusceptible to antimicrobials with the inactivation of the efflux pumps or with permeabilisation of the outer membrane.[13]

The outer membrane itself does not provide resistance to antibiotics as it only has decreased permeability. It is dependent on the other resistance mechanisms such as efflux but these mechanisms have enhanced effectiveness in the presence of the outer membrane. The synergy between the efflux pumps and the outer membrane probably explains the variable effectiveness of related multidrug efflux systems in providing resistance in organisms with differences in intrinsic outer membrane permeability properties.[1,16]

ARE BIOCIDES CONTRIBUTING TO RESISTANCE?

Antibiotics are used predominantly for the treatment of bacterial infections in humans and animals, whereas biocides are employed for their antiseptic, disinfectant and/or preservative properties (Table 1).[17] Efflux pumps can remove therapeutic levels of antibiotics and low but probably not "in use" concentrations of biocides.[18,19] Many mdr systems can also accommodate biocides such that these strains are both antibiotic and biocide resistant·[20] There is a conflict over whether the biocides are contributing to the increasing resistance to antibiotics by activating or increasing the activity of the efflux pumps.[1,21]

Table 1. Biocides to which bacterial resistance may be a problem (taken from A.D. Russell.)[17]

Biocide group	Example(s)	Bacterial resistance
QACs	Cetrimide, Benzalkonium chloride, cetylpyridinium chloride	*Ps. aeruginosa, Proteus* spp., *Providencia* spp., *Staph. aureus* (containing *qac* genes)
Bisbiguanides	Chlorhexidine	
Diamidines	Propamidine, dibromopropamidine	*Staph. aureus* (containing *qac* genes)
Bisphenol (phenylether)	Triclosan	*E. coli* (?), *Staph. aureus* (?), *Ps. aeruginosa*, *Myco. tuberculosis* (?)
Acridines	Acriflavine, proflavine	*Staph. aureus* (containing *qac* genes)

Antibiotics rely on selective toxicity and usually only have one target in a cell whereas a biocide may have many targets. Therefore, resistance development to biocides is highly unlikely because of the multiple targets that the active agent has. However, there has been speculation that the use of a sub-lethal concentration of biocides against microbes might lead to the selection of bacteria that have better efflux or resistance

mechanisms.[22]

Cationic agents (quaternary ammonium compounds, chlorhexidine) and triclosan have been implicated as

possible causes for the selection and persistence of bacterial strains with low level antibiotic resistance.[17,18] It has also been claimed that the emergence of qacA and qacB determinants in clinical isolates of

Staphylococcus aureus mirrors the introduction and usage of cationic biocides.[7,17] If biocides and antibiotics are both pumped out by the same efflux system, then resistance to the antibiotic might develop because of upregulation due to the presence of the biocide. Recent evidence has emerged that sub-lethal concentrations of the antibacterial and antifungal agent triclosan can select for resistant mutants in Escherichia coli. It has been suggested that the triclosan may select for mutants in a target that is shared by diazoborine compounds

and the anti-tuberculosis drug, isoniazid.[17,22]

Sub-lethal treatment with chemical antimicrobial agents has also been shown to induce the expression of multidrug efflux pumps and efflux mutants. The increased or changed efflux pattern does not protect the microbe against "in-use" concentrations of biocides, but is sufficient to confer protection against therapeutic doses of many antibiotics. It has been speculated, therefore, that biocide misuse may have an insidious effect and that it may be contributing to the evolution and persistence of drug resistance within microbial

communities.[22]

The mar operon in Escherichia coli and homologues in other bacteria have been seen to be involved in both

biocide and antibiotic resistance.[17] Mutations in MarR (which regulates expression of MarA) protein of the mar operon upregulates AcrAB and TolC to produce resistance to antibiotics, organic solvents, pine oils, bile salts, antiseptics and disinfectants such as triclosan, quaternary ammonium compounds and chlorhexine (Figure

2).[4,22] The subsequent overexpression in MarA was seen to activate multidrug efflux pumps which were able

to cause the efflux of pine oil as well as antibiotics such as the tetracyclines.[4] Overexpression of the MarA protein gave a 3-4 fold increase in resistance to triclosan (an increase of the same magnitude as that seen for antibiotics). Other evidence reported that Escherichia coli mutants selected for resistance to pine oil overexpressed the marA gene and showed low level resistance to ampicillin, tetracycline, chloramphenicol and nalidixic acid. However, tetracycline-selected mar mutants, which also overexpressed marA (and also showed resistance to pine oil), had much higher resistance to the antibiotics than the pine oil mutants. This would suggest that exposure to antibiotics in this instance was much more important for causing antibiotic resistance than exposure to a biocide. Although some of the evidence suggests that exposure to triclosan may be leading to increased antibiotic resistance, this has not yet been proven in a clinical setting. Likewise, the finding that exposure to triclosar of a triclosan-sensitive Pseudomonas aeruginosa mutant switched on an efflux pump and rendered the cells highly resistant to ciprofloxacin has not as yet been translated to a clinical

situation. [4,17]

Figure 2. Efflux mechanism of Escherichia Coli causing resistance to multiple compounds.

The Mar A protein causes activation of the AcrAB/TolC complex which causes the efflux of many compounds.

Figure taken from S.B. Levy. [4]

There is some evidence that the introduction of biocides into clinical practice might have had an impact on antibiotic resistance. Probably far more significant, however, is the selective pressure exerted by the antibiotics themselves in the treatment of human and animal infections as a result of their incorporation into

animal feedstuff.[19] The issues over biocides and increased resistance is far from being resolved. Also often neglected is that hyper-expressed mutants of efflux pumps are resistant to antibiotics in laboratory cultures, but they pump out key metabolites and therefore, may be relatively non-competitive in mixed microbial communities. This would especially be the case when the antimicrobial selection pressure is removed or

transient.[22]

OVERCOMING THE EFFLUX PUMP RESISTANCE MECHANISMS

As resistance is developing rapidly to antibiotics, biocides and other compounds, there is a need to overcome this resistance in order to effectively combat serious infections. Some approaches are being developed, although most of the research is still in the early stages. Failure encountered with antibiotics designed specifically to resist inactivating enzymes (e.g. beta-lactamases) has shown that chemical improvements are likely to be overcome very quickly by bacteria. Specific and potent inhibitors of antibiotic transporters appear to be the therapeutic approach of most promise. Ligand-based approaches have been tried, but as the transporters displayed non-specificity, this has not successful. There has been some success with target-based approaches. With this method there is more flexibility, as effective inhibitors do not necessarily have to

be directed against the binding site of the natural substrate.[19,23] The first inhibitors of the broadly specific multi-drug efflux system of Pseudomonas aeruginosa (an RND transporter system) have recently been reported and they are effective at both overcoming existing resistance to a certain of antibiotics (i.e. the fluoroquinolones) and also at preventing the emergence of the resistance in the first place. These inhibitors are also likely to be effective at compromising intrinsic and acquired biocide resistance in this organism as

well as antibiotic and biocide resistance in organisms expressing homologous multi-drug efflux systems.[20]

Inhibitors of the NorA multi-drug transporter of Staphylococcus aureus have also been reported and they are effective at enhancing fluoroquinolone susceptibility and preventing the emergence of fluoroquinolone

resistance.[7,24,25] Examples of NorA inhibitors are reserpine and omeprazole (These drugs are already used clinically for the treatment of hypertension and peptic ulcer disease. They inhibit pumps at their site of action in humans). These drugs lack antimicrobial action but are thought to potentiate the antimicrobials. It is thought that these inhibitors may slow down the development of resistance or prolong the life of current quinolones

like the beta-lactam inhibitors did for the beta-lactam antimicrobials. [20,25]

One problem, however, with inhibitors of efflux pumps is that these pumps could be proteins with important physiological functions; use of these inhibitors might have toxic effects in the host. Therefore, inhibitors against antibiotic extruding pumps operating only in prokaryotes may offer significantly greater chances at

therapeutic success.[23] The other interesting group of drugs being studied for potential anti-efflux activity are

the SSRI's (selective serotonin re-uptake inhibitors used in the treatment of depression).[26] They also act as

efflux inhibitors in man and have been shown to help overcome resistance in some bacteria.[25] Little is known on their mode of action in the microbes. They may be acting as efflux inhibitors, which would explain their synergy with tetracyclines and fluoroquinolones in Clostridium urealyticum. However, they also appear to have some anti-microbial activity themselves and may be acting on basic metabolic processes in the microbe. Their

target has not yet been identified.[26] Prudent use of antimicrobials (especially those that leave residue) should also be employed in order to try to curb resistance and preserve the efficacy of these compounds for when

they are needed in life threatening situations.[4,5,19]

CONCLUSION

The purpose of this review was to examine the impact of efflux mechanisms on antimicrobial resistance in

microbial cells. Efflux pumps contributing to resistance in microbes are widespread and are creating major problems with regard to the efficacy of antimicrobials. Efflux pumps are rapidly becoming a very important mechanism of resistance both alone and with the synergistic action of the outer membrane. New drugs targeted at these pumps specifically could have a huge impact in combating antimicrobial resistance. Great care would have to be taken in trying to develop inhibitors that are selective for the microbes as humans have many similar pumps with very important functions in the body. Efforts at inhibiting these pumps are still at the early stages of research, however. Efforts are also being made to try to improve our current practices to prevent further resistance from emerging. This review has potentially outlined one of the ways in which resistance to antimicrobials is increasing. This is through the increasing use of biocides. More prudent and safe use of biocides is required if current resistance figures are to be improved. In the future, new drugs will need to be developed to inhibit the pumps and better usage practices with regard to current antimicrobials will need to be adopted in order for the current antimicrobials to continue to be therapeutically relevant.

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