Pumping out biocides – cause for concern?

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Bacteria employ a number of resistance mechanisms against antimicrobials, commonly including target site modification, antimicrobial degradation and active efflux. Of these mechanisms, efflux is unique in that a single efflux system can confer resistance to a remarkably broad range of structurally dissimilar antibiotics and biocides that have different intracellular targets and modes of action. For example, in the opportunistic pathogens *Staphylococcus aureus* and *Acinetobacter baumannii*, single multidrug efflux systems facilitate the extrusion of agents as broad and diverse as quaternary ammonium compounds, intercalating dyes, diamidines, biguanidines, anionic detergents, aminoglycosides, β-lactams, chloramphenicol, tetracyclines, trimethoprim and fluoroquinolones (Table 1). This promiscuity of substrates, coupled with the genetic linkage of exporter genes with other resistance determinants on transferable elements such as plasmids, raises the possibility of cross and co-resistance to biocides and antibiotics. Furthermore, data generated by our and other research groups clearly demonstrate a remarkable propensity of multidrug efflux systems to bind new substrates following only minor amino acid changes to their active sites.

Staphylococcus aureus and *Acinetobacter baumannii* are endemic in hospitals worldwide and reports of community-acquired infections by these pathogens are on the rise. To prevent or limit infections caused by these bacteria, considerable effort has gone into improving practices relating to infection control and prevention within hospitals[1]. One such practice involves the use of chemical biocides including quaternary ammonium compounds (QACs) such as dequalinium chloride, biguanides such as chlorhexidine, and phenylesters such as triclosan[2,3], in disinfectants, antiseptics and preservatives[4]. Active efflux, where a membrane protein transports antimicrobial agents out of the cell to reduce their intracellular concentration, is a primary mechanism of biocide resistance in bacteria. *S. aureus* and *A. baumannii* provide excellent examples of Gram-positive and Gram-negative pathogens, respectively, that maintain a multitude of efflux systems in their biocide resistance arsenals. There are five main families of transporters that have been shown to participate in antimicrobial resistance (Figure 1). The systems most extensively studied in *S. aureus* include a number of major facilitator superfamily (MFS) transporters, as well as members of the small multidrug resistance (SMR), multidrug and toxin extrusion (MATE) and ATP-binding cassette (ABC) superfamilies (Table 1). In contrast, in *A. baumannii* resistance nodulation division (RND) efflux systems have received considerable

attention (Table 1). In Gram-negative bacteria, export systems are of particular concern due to their exceptionally broad substrate recognition profiles and their assembly into tripartite complexes that span the entire cell envelope. This organisation potentiates higher levels of resistance since exported antimicrobials must negotiate both the inner membrane and less permeable outer membrane to regain access to the cell (Figure 1).

Studies using both *S. aureus* and *A. baumannii* have shown that a single antimicrobial can select for overexpression of multidrug efflux systems and thus for resistance to a broad range of compounds. For example, *A. baumannii* strains overexpressing the RND efflux systems AdeABC, AdeIJK or AdeFGH, have been isolated by selecting for resistance to a single antimicrobial substrate[20]. Although the strains were selected using only one antimicrobial, they displayed resistance phenotypes in line with the substrate recognition profiles of the overexpressed efflux pumps (Table 1). Similarly, mutant *S. aureus* strains selected for resistance to a single biocide have displayed multidrug resistance phenotypes as a result of overexpression of the NorA export protein [21].

Several lines of evidence suggest that this phenomenon extends beyond the research laboratory and that strains overexpressing multidrug export systems have been selected for within clinical environments. In the absence of selective pressures, the maintenance and high-level expression of drug efflux systems could put strains at a competitive disadvantage. Nonetheless, quantitative PCR studies have shown that multidrug-resistant clinical strains often express chromosomally-encoded efflux system genes at elevated levels, for example, the *adeABC* and *adeIJK* systems in *A. baumannii* strains[14, 22] and *norA*, *mepA* and *mdeA* in *S. aureus* strains[23]. Furthermore, a large proportion of strains isolated from clinical settings worldwide have been found to carry plasmids encoding efflux pumps, for example, the *qacA/B* and *qacC* determinants in clinical *S. aureus* isolates[24-26]. Studies conducted in intensive care facilities have demonstrated the clear advantage of these strains over others in evading controlled antiseptic/disinfectant protocols[27]. Exporter genes encoded on plasmids or other mobile elements may further compound the problem of multidrug resistance, since such mobile elements often carry additional resistance genes that are co-selected with the exporter gene. For example, the *qacA* determinant can be found on large multi-resistance plasmids such as pSK1, that in addition to *qacA* encodes resistance to aminoglycosides and trimethoprim[28].

The remarkable promiscuity of bacterial multidrug export proteins stems from the architecture of their drug binding sites. Given the high hydrophobicity of membrane transport proteins, their structural analyses have proven problematic. Nonetheless, significant insight into multidrug-binding sites has been achieved through a combination of biochemical analyses and studies of the transcriptional regulators that control expression of drug exporter genes. Typically, these regulatory proteins bind a similar spectrum of compounds to their cognate multidrug exporter using seemingly similarly organised binding sites. One of the best studied examples of a multidrug regulator/exporter system is the staphylococcal QacR/QacA system. The QacR transcriptional repressor was the first multidrug binding protein to be co-crystallised with multiple substrates[29]. These crystal structures demonstrated that QacR contains a large binding region lined primarily by aromatic, hydrophobic and polar amino acid side chains, as well as a few key acidic residues that help to 'steer' the compounds into their optimum binding site. This organisation facilitates binding of the typically amphiphilic biocide ligands via an induced fit mechanism. Mutagenic studies of the QacR regulator have shown that multidrug binding regions are remarkably resistant to functional attenuation. Manipulation of the binding pocket in this protein does not prevent binding

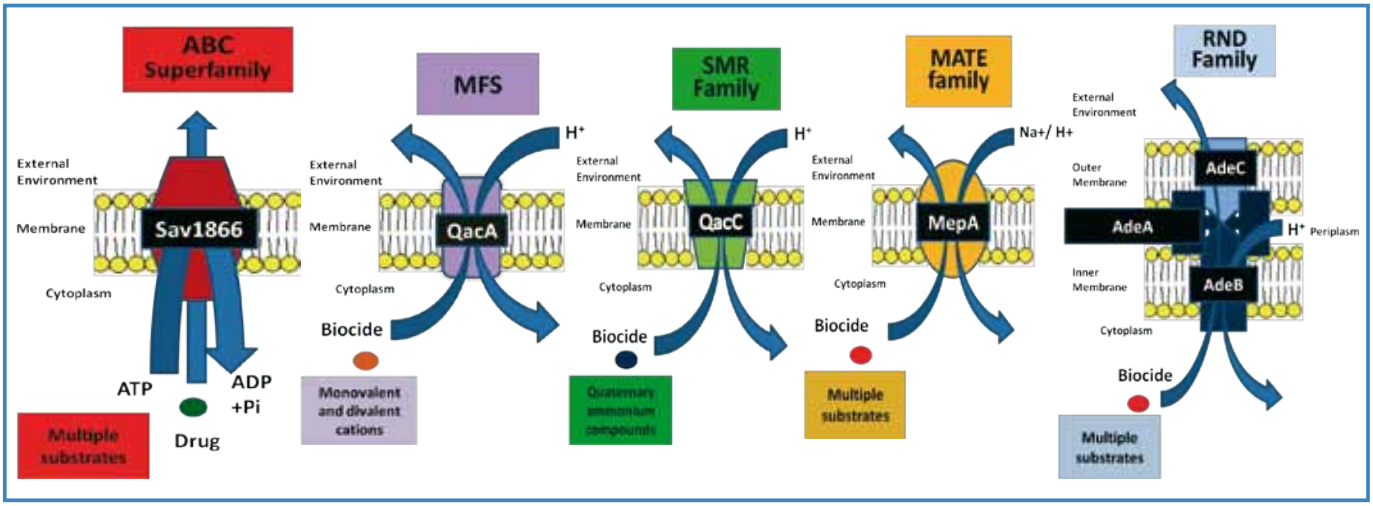

Figure 1. Diagrammatic representation of representative transporters from the five major multidrug transporter families. A summarised substrate recognition profile for each representative transport system is given (further details are listed in Table 1), along with their energy-coupling mechanisms (i.e., ATP hydrolysis for the ABC superfamily export system and the use of electrochemical gradients for the remaining proteins). The export systems classified within the ABC, MFS, SMR and MATE families typically transport their substrates across the cytoplasmic membrane. However, in Gram-negative bacteria some MFS and ABC systems, as well as, most commonly, RND transporters, assemble with periplasmic and outer membrane-bound proteins to form tripartite systems to expel substrates across both the inner and outer membranes.

Table 1. Important biocide efflux pumps identified in *S. aureus* and *A. baumannii.*

Organism	Efflux pump	Transporter family	Resistance against*	Determinant location	References
Staphylococcus aureus	Sav1866	ABC	dyes, anti-cancer agents	Chromosome	5, 18
	MepA	MATE	dyes, QACs, diamidines, glycyclines	Chromosome	6
	NorA	MFS	dyes, QACs, quinolones	Chromosome	7, 8
	QacA	MFS	dyes, QACs, diamidines, biguanidines	Plasmid	9, 10
	QacB	MFS	dyes, QACs	Plasmid	10
	QacC	SMR	dyes, QACs	Plasmid	11
	SepA	SMR	dyes, QACs	Chromosome	12
Acinetobacter baumannii	AdeABC	RND	aminoglycosides, β-lactams, chloramphenicol, tetracyclines, tigecycline, trimethoprim, dyes, fluoroquinolones, QACs, diamidines, biguanidines, anionic detergents	Chromosome	13, 14
	AdeFGH	RND	tetracyclines, chloramphenicol, fluoroquinolones, trimethoprim, dyes, anionic detergents	Chromosome	15
	AdeIJK	RND	β-lactams, chloramphenicol, fluoroquinolones, tetracyclines, dyes, diamidines, biguanidines, anionic detergents	Chromosome	16, 14
	AbeM	MATE	dyes, QACs, quinolones, aminoglycosides	Chromosome	19
	Aed2F	MFS	dyes, diamidines, biguanidines	Chromosome	**
	AbeS	SMR	dyes, QACs, erythromycin, chloramphenicol	Chromosome	17

*Pumps may not efflux all compounds classified within the group of agents listed. For full range refer to references. ABC: ATP-binding cassette superfamily; MATE: Multidrug and toxic compound extrusion family; MFS: Major facilitator superfamily; RND: Resistance-nodulation-division family; SMR: Small multidrug resistance family; QACs: Quaternary ammonium compounds.
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or even reduce the binding affinity of most QacR ligands, but in some cases shifts their preferred binding site, illustrating the plasticity of this binding mechanism[30-32].

Genetic and biochemical analyses of the QacA efflux protein have suggested that it contains characteristics similar to the drug binding region to QacR. One of the most fascinating, yet troubling features of the QacA multidrug binding site is its capacity to bind new substrates in response to only minor amino acid changes. The first evidence of this phenomenon came from comparisons of the QacA and QacB export proteins[33]. These proteins differ by only seven amino acid residues; however, the substrate recognition profile of QacB is limited to monovalent cations, whereas QacA is able to recognise both monovalent and bivalent cationic antimicrobials (Table 1). Remarkably, genetic analyses determined that the extended drug recognition spectrum of QacA was the result of only a single amino acid difference, an acidic residue within the transmembrane region that is thought to stabilise the binding of compounds carrying an additional positive charge[33]. Even more remarkable, recent studies of QacA have shown that this negative charge can be moved to other locations within the predicted binding region and result in a similar expansion to the drug recognition spectrum of the protein[34,35]. Interestingly, the location of key negatively charged residues in QacA has an influence on the subset of bivalent cations recognised[35]. Naturally occurring QacA/B variants carrying negatively-charged groups in various locations around the predicted binding region have been identified [26], suggesting that QacA/B proteins are actively evolving, possibly in response to the use of different biocides within clinical settings. The adaptability of the drug binding region in QacA highlights the ease with which multidrug transporters may be able to accommodate new classes of biocides after only minor mutational changes, emphasising the importance of the prudent use of these compounds.

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Biographies

Karl Hassan is a Postdoctoral Fellow at Macquarie University where he is engaged in microbial genomics projects of both medical and agricultural significance. Prominent among his broad research interests are the roles of bacterial efflux systems in drug resistance, virulence and the maintenance of stable cell physiology.

Sylvia Baltzer is a PhD student at Flinders University. She is currently investigating the small multidrug resistance efflux system in *Staphylococcus aureus*.

Ian Paulsen is Professor of Genomics at Macquarie University and is an ISI Highly Cited Researcher. As a former faculty member at The Institute for Genomic Research, he has led the sequencing of many microbial genomes. His research interests are focused on a) using genome sequencing, metagenomics and functional genomics to understand how lateral gene transfer in bacteria enables them to adapt to different environmental niches; and b) using bioinformatics and functional genomics to characterise the function of novel membrane transport systems, particularly multidrug efflux pumps.

Melissa Brown is an Associate Professor in Microbiology at Flinders University. She has a long-standing interest in bacterial antimicrobial resistance mediated by membrane-bound transport proteins. In particular she has focused on multidrug binding proteins to understand how such proteins can recognise multiple ligands.