

Chapter 2

Bacterial Resistance to Hospital Disinfection

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Abstract With the number of infections caused by bacteria that are multidrug resistant (MDR) increasing, it is imperative that these infections can be controlled. Biocides are used to prevent contamination in a variety of topical and hard-surface applications and are an essential component of infection control in hospitals. They are also used for disinfection and preservation of foodstuffs and within the home environment for a range of applications which again includes hard-surface disinfection. Whilst antibiotic resistance has been well studied in a variety of bacteria, there is little known about potential resistance mechanisms to biocides. We have yet to see well-documented outbreaks caused by biocide-resistant bacteria, although this is rarely looked for. Development of resistance to biocides is thought to be more difficult than for antibiotics for several reasons including the high concentration of biocides used in many applications and that biocides act on multiple targets. However, the increased use of biocides has led to speculation of development of resistance to biocides and whether this leads to cross resistance to antibiotics. This chapter will consider the different resistance mechanisms to biocides in bacteria, the possible link between biocide and antibiotic resistance and whether we should be concerned about bacterial biocide resistance.

2.1 Introduction

Biocides have been used for centuries mainly as preservative agents for water (e.g. the use of copper and silver vessels) and foodstuffs as well as the use of vinegar and honey for cleansing wounds. The nineteenth century saw the onset of antiseptic surgery and the introduction of disinfectants into clinical use. Iodine was used as a wound disinfectant, phenol (carbolic acid) in wound dressings and water containing chlorine used in general disinfection and obstetrics. In the early part of the twentieth century, many biocides were introduced including other chlorine-releasing agents (CRAs) and some quaternary ammonium compounds (QACs)

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(Hugo 1999). The mid-twentieth century saw the introduction of biguanides (chlorhexidine, alexidine), amphoteric surfactants, bisphenols (triclosan), aldehydes (glutaraldehyde, ortho-phthalaldehyde, succinaldehyde-based products), CRAs (isocyanurates), iodine-releasing agents (iodophors), isothiazolones and peracetic acid (Russell 2002). Whilst biocides have been in use for centuries as disinfectants, the ability of bacteria to survive and adapt to the presence of biocides has only been documented within the last 50 years. Comparison of strains of *Klebsiella pneumoniae* isolated pre (Murray) and post (modern) the widespread introduction of many biocides showed that more modern strains, which have presumably been more exposed to particular biocides, have become less susceptible to biocides such as triclosan, chlorhexidine and benzalkonium chloride (Wand et al. 2015) (Table 2.1). Although more work is needed, this suggests that whilst bacteria are still highly susceptible to in-use concentrations of biocides, they are beginning to adapt to lower concentrations. In hospitals and the environment, many bacteria are now resistant to several in-use antibiotics which places an even greater burden on biocides for infection prevention and control. This means that bacteria which have adapted (become more resistant) to particular biocides will be increasingly problematic for infection control and could lead to outbreaks which are difficult to combat. Still more problematic is whether biocide adaptation leads to increased resistance to antibiotics and other antimicrobial agents.

2.2 Types of Biocides and Mode of Action

2.2.1 Cationic Antimicrobial Agents

The surface of bacterial cells is usually negatively charged which is stabilized by the presence of divalent cations such as Mg^{2+} and Ca^{2+} . For Gram-positive bacteria, this is associated with the teichoic acid and polysaccharide components of the cell wall, for Gram-negative bacteria, the lipopolysaccharide and the cytoplasmic membrane. Therefore, antimicrobial agents which are cationic have a high binding affinity for bacterial cells. Cationic antimicrobials interact initially with the bacterial cell wall by displacing the divalent cations with subsequent interactions with membrane proteins and the lipid bilayer dependent upon the actual biocide.

2.2.2 Quaternary Ammonium Compounds

The QACs are amphoteric surfactants with activity towards specific species of bacteria dependent upon the hydrophobicity and chain length of the *n*-alkyl chain (Tomlinson et al. 1977). Those QACs with a C_{16} hydrophobic tail length have more activity against Gram-negative bacteria than do shorter-chain compounds, possibly

Table 2.1 Comparison of susceptibility to disinfectants between Murray and modern *K. pneumoniae* strains

		Murray		Modern	
		Range	Mode	Range	Mode
Disinfectants	CHX	0.25 to 32	8	8 to 32	16
	BAC	1 to 16	4	8 to 32	16
	HDPCM	1 to 8	2	4 to 32	8
	TRI	0.007 to 0.25	0.06 to 0.125	0.125 to 1	0.5
	H ₂ O ₂ (%)	0.03 to 0.06	0.03	0.03 to 0.06	0.06
	Per acid (%)	0.04 to 0.08	0.08	0.04 to 0.08	0.08
	HSD1 (% WC)	1.56 to 3.125	3.125	1.56 to 3.125	3.125
	EtoH (%)	1.56 to 6.25	6.25	3.125 to 6.25	6.25
	Chlorine (ppm)	250 to 500	500	250 to 500	500
	HSD2 (% WC)	1.56 to 3.125	3.125	1.56 to 3.125	3.125
Antibiotics	AMK	1 to 4	2	4 to >64	8
	GEN	0.5 to 4	2	4 to >64	>64
	PIP	0.25 to >64	0.5	8 to >64	>64
	TZP	0.25 to 8	0.5	1 to >64	>64
	AMX	0.5 to >64	0.5 to 1	64 to >64	>64
	AMC	0.5 to 8	0.5	1 to >64	>64
	CTX	≤0.06 to 0.125	≤0.06	1 to >64	>64
	MEM	≤0.06 to 0.125	≤0.06	≤0.06 to 0.125	≤0.06
	CIP	≤0.06 to 0.125	≤0.06	≤0.5 to 128	≤0.5
	CHL	2 to 4	2	1 to >512	4

Potential adaptation of *K. pneumoniae* isolates through exposure. Several *K. pneumoniae* isolates ($N = 39$) from the Murray Collection (isolated pre-1950) were compared to more modern *K. pneumoniae* isolates (isolated post-2000) ($N = 39$). For certain disinfectants, particularly the antiseptics, e.g. BAC, there was a clear significant shift towards decreased susceptibility between the Murray isolates and the modern isolates. This did not occur for all disinfectants, e.g. Per acid and chlorine. Several antibiotics were tested against the same panel and are shown for comparison. The large increase in resistance to antibiotics such as AMX and CTX is likely due to the presence of antibiotic resistance genes, e.g. *bla*_{SHV} in the majority of modern isolates

All values are given as mg/L unless otherwise stated (adapted from Wand et al. 2015)

CHX chlorhexidine, BAC benzalkonium chloride, HDPCM hexadecylpyridinium chloride monohydrate, TRI triclosan, H₂O₂ hydrogen peroxide, Per acid peracetic acid, HSD1 (2) hard-surface disinfectant 1 (2), EtoH ethanol, AMK amikacin, GEN gentamicin, PIP piperacillin, TZP piperacillin/tazobactam (TZP), CTX cefotaxime, MEM meropenem, CIP ciprofloxacin, CHL chloramphenicol, WC working concentration

due to the increased affinity of the C₁₆ chain with the fatty acid portion of lipid A (Ahlström et al. 1999). Their mode of action is also dependent on the concentration of the biocide. Low concentrations of QAC cause cellular leakage of potassium and hydrogen ions and loss of osmoregulatory capability by binding to anionic sites on the surface of the bacterial cell membrane (Lambert and Hammond 1973). At in use, high-concentration QACs kill bacterial cells by solubilization of the cellular membrane and rapid cell leakage occurring at bactericidal concentrations (Ioannou et al. 2007).

As a group, QACs have been in use since the 1930s, and although some changes in resistance have been noted, these are generally increases in MIC (minimum inhibitory concentration) level which is still far below the in-use concentration level (Gilbert and McBain 2003). Some bacteria such as *Pseudomonas aeruginosa* are relatively resistant to QACs possibly due to the inability of the compounds to penetrate the outer membrane. Examples of QACs include benzalkonium chloride and cetrimide.

2.2.3 Biguanides

Bisbiguanide antiseptics (e.g. chlorhexidine) have a mode of action which is similar to QACs in that the biguanide groupings strongly associate with anionic sites on the cell surface, particularly acidic phospholipids and proteins (Chawner and Gilbert 1989a). Chlorhexidine has both bacteriostatic (inhibits bacterial growth) and bactericidal (kills bacteria) mechanisms of action, depending on its concentration. The chlorhexidine cation forms a bridge between pairs of adjacent phospholipid head groups and displaces the associated divalent cations (Mg^{2+} and Ca^{2+}) (Davies 1973). This results in reduction of membrane fluidity and osmoregulation as well as a change in metabolic capability of the enzymes associated with the cell membrane (Hugo and Longworth 1966). At higher concentrations the interaction between chlorhexidine and the cellular membrane causes the membrane to lose its structural integrity, and the membrane adopts a liquid crystalline state which leads to a rapid loss of cellular contents (Longworth 1971; Chawner and Gilbert 1989b). Again, increased resistance to chlorhexidine has been described, mainly resulting from increased efflux; this is mainly far below in use concentrations, although some bacteria have been isolated from chlorhexidine-containing solutions (Brooks et al. 2004).

For polyhexamethylene biguanides (PHMB), the mode of action is similar to the biguanide antiseptics except that the bridging is not restricted to adjacent phospholipid pairs which results in the formation of a mosaic of individual phospholipid domains. These each have a different phase transition temperature causing the membrane to separate into liquid and fluid crystalline regions. This again results in generalized cellular leakage (Broxton et al. 1983; Gilbert and Moore 2005). Again, PHMB is bacteriostatic at low concentrations, but bactericidal at higher concentrations and increased resistance has been associated with upregulation of Rhs (recombinational hot spots) elements which suggests that PHMB may interact directly with DNA (Allen et al. 2006).

2.2.4 Peroxygens

2.2.4.1 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) works as an oxidant by the production of hydroxyl free radicals which then attack essential cellular components such as membrane lipids, DNA and proteins. Exposed sulphhydryl groups and double bonds are particularly targeted (Block 1991). Hydrogen peroxide is active against a wide range of bacteria and has been shown to be sporicidal (Block 1991; Turner 1983) but has greater activity against Gram-positive bacteria compared to Gram-negative bacteria. It is widely used in hospitals and other environments as a whole room decontaminator. The presence of bacterial enzymes called catalases can help to protect against low concentrations of hydrogen peroxide.

2.2.4.2 Peracetic Acid

Its mode of action is thought to be similar to other oxidizing agents, i.e. it functions by denaturation of proteins, disruption of the cell wall permeability and oxidation of sulphhydryl and sulphur bonds in enzymes and other proteins (Baldry and Fraser 1998; Block 1991). Again it has a broad spectrum of activity and has been shown to be especially penetrative against bacterial biofilms (Perumal et al. 2014).

2.2.5 Alcohols

It is generally thought that alcohols are membrane disruptors and act by denaturation of proteins. Alcohol can destroy the dehydrogenases in *E. coli* (Sykes 1939). They have increased activity in water with proteins being denatured more quickly in the presence of water rather than absolute alcohol (Ali et al. 2001; Morton 1983). Ethanol is able to cause the rapid release of intracellular components and membrane disruption by interaction with the hydrocarbon component of the phospholipid bilayer. Other examples of alcohols include phenylethanol and phenoxyethanol which induce generalized loss of cytoplasmic membrane function (Fitzgerald et al. 1992). Ethanol has also been shown to inhibit DNA, RNA, protein and peptidoglycan synthesis in *E. coli* (Nunn 1975). Alcohols exhibit rapid broad spectrum antimicrobial activity against vegetative bacteria but are not sporicidal. Low concentrations of alcohol are often used in conjunction with other biocides, e.g. chlorhexidine.

2.2.6 Aldehydes

Glutaraldehyde has a broad spectrum of activity against both vegetative bacteria and bacterial spores. For vegetative bacteria glutaraldehyde strongly associates with the outer layers of the cell wall, specifically with unprotonated amines on the cell surface (Bruck 1991) which causes cross-linking of amino groups within proteins and subsequent inhibition of transport processes into the bacterial cell. The action of glutaraldehyde against bacterial spores is dependent upon its concentration. At low concentrations spore germination is inhibited, whereas at high concentrations glutaraldehyde strongly interacts with the outer spore layers (Thomas and Russell 1974a, b).

Formaldehyde is also sporicidal and can penetrate the outer spore layers. It is an extremely reactive chemical and works to inactivate bacteria by alkylating the amino and sulphhydryl groups of proteins and ring nitrogen atoms of purine bases (Favero and Bond 1991).

2.2.7 Phenolics

Phenol derivatives or phenolics are derivatives of phenol where a functional group (e.g. alkyl, phenyl, benzyl, halogen) replaces one of the hydrogen atoms on the aromatic ring. Many have improved biocide activity over phenol. Phenolic compounds in high concentrations are gross protoplasmic poisons which penetrate and disrupt the cell wall and precipitating cellular proteins. At low concentrations phenol can induce the progressive leakage of intracellular constituents and inactivation of essential enzyme systems (Prindle 1983).

Bisphenols are hydroxyl-halogenated derivatives of two phenolic groups connected by a variety of bridges (Gump 1977). They have a broad host range although some bacteria, e.g. *P. aeruginosa*, exhibit intrinsic resistance and they only show sporostatic activity. Examples of bisphenols include hexachlorophene and 2,4,4'-trichloro-2'-hydroxydiphenylether (triclosan). Triclosan is unusual for a biocide in that, although it has been shown to have a variety of targets, it primarily appears to target fatty acid synthesis by inhibition of the enzyme enoyl reductase FabI by competitive inhibition of the enzyme's natural substrate (McMurry et al. 1998a; Heath et al. 1998). However, this inhibition process is relatively slow, and the rapid killing of bacteria by high concentrations of triclosan cannot be explained solely by inhibition of fatty acid synthesis (Gomez Escalada et al. 2005). Studies have shown that insertion of triclosan into the bacterial cell membrane will comprise the membrane functional integrity (Villalain et al. 2001; Guillén et al. 2004).

2.2.8 *Halogen-Releasing Agents*

2.2.8.1 Chlorine-Releasing Agents

The exact mechanisms of microbial destruction by free chlorine are still not fully understood. Oxidation of sulphhydryl enzymes and amino acids, ring chlorination of amino acids, decreased uptake of nutrients and oxygen, inhibition of protein synthesis, oxidation of respiratory components and decreased ATP production and effects on DNA synthesis have all been shown to be factors resulting from inactivation by chlorine (Dychdala 2001). Low pH is thought to increase activity. Examples of chlorine-releasing agents include sodium hypochlorite, chlorine dioxide and sodium dichloroisocyanurate (NaDCC). Low concentrations of these agents are active against vegetative bacteria, but higher concentrations are necessary to have an effect against mycobacteria or bacterial spores (Rutala et al. 1991; Bloomfield and Arthur 1992; Ungurs et al. 2011). The activity of CRAs is affected by the presence of organic matter, e.g. blood (Coates 1991).

2.2.8.2 Iodophors

Iodophors are a combination of iodine and a solubilizing agent or carrier, which acts as an active ‘free’ iodine reservoir (Gottardi 1991). Similar to chlorine, iodine acts by rapidly penetrating into bacteria and attacking cysteine and methionine amino acids as well as nucleotides and fatty acids (Kruse 1970; Gottardi 1991). This results in disruption to protein and nucleic acid structure and synthesis. They are generally used as antiseptics but have been used for disinfection of endoscopes and other medical equipment. Dilution is a critical factor with contamination of iodophor solutions reported (Craven et al. 1981; Parrott et al. 1982).

2.2.9 *Others*

There are many other types of biocide which have not been discussed here including silver-containing biocides (silver nitrate, silver sulphadiazine), diamidines which are used for wound disinfection and anilides which show good activity against Gram-positive bacteria but are poor against Gram-negative bacteria. These are reviewed in the article by McDonnell and Russell (1999).

2.3 Resistance to Biocides

Bacterial resistance to biocides is not a new phenomenon having been described over 50 years ago (Russell 2004; Maher et al. 1961). However, there are a now growing number of reports of resistance to biocides, both within clinical and laboratory conditions, suggesting that biocide resistance is increasingly problematic. The response of bacteria to particular biocides, and the nature of their resistance to it, is driven primarily by the nature of the biocide itself and the type of organism. Biocide activity is also influenced by a variety of factors including pH, presence of organic matter, temperature, concentration and contact time. Changes to the environment can result in alterations to biocide susceptibility. The outer cell wall generally plays a critical role in determination of intrinsic bacterial susceptibility (or insusceptibility) to biocides. Bacteria can also develop resistance to particular biocides (acquired) through various mechanisms, which will be discussed below.

2.3.1 *Intrinsic Resistance to Biocides*

Although biocides usually have a low degree of selectivity in their action against different types of microorganism, they do show varying degrees of activity against different species of bacteria. Some bacterial species are intrinsically resistant to certain biocides. This is defined as the innate (natural) ability of a bacterial species to resist the activity of a particular antimicrobial agent (biocide) through its inherent structural or functional characteristics. This can be due to one or more factors which include (a) the lack of affinity of the biocide for the bacterial target, (b) the inaccessibility of the biocide into the bacterial cell, (c) the extrusion of the biocide by chromosomally encoded active exporters and (d) innate production of enzymes which inactivate the biocide (Fig. 2.1). Production of specific enzymes is rare, and since biocides have multiple targets (one exception being triclosan), lack of affinity is also uncommon.

Bacterial endospores are considered more resistant to biocides than vegetative bacteria (Fig. 2.2). Gram-negative bacteria are intrinsically more resistant to biocides than Gram-positive bacteria due to their outer membrane composition. The exception is mycobacteria which, for vegetative bacteria, are the most resistant, again due to their cell wall composition which limits access of biocides. There are also additional factors, such as whether the bacteria are within a biofilm, which will also affect and generally decrease the effectiveness of biocides.

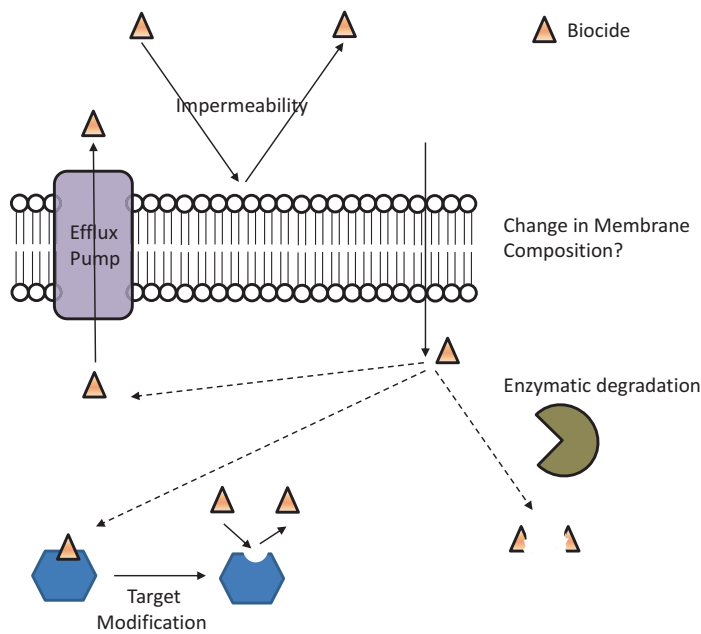


Fig. 2.1 Examples of mechanisms of resistance to biocides found in bacteria. Membrane impermeability and increased bacterial efflux are the most common forms of resistance mechanisms found in bacteria. Target alteration and enzymatic degradation of biocides are limited to a few examples

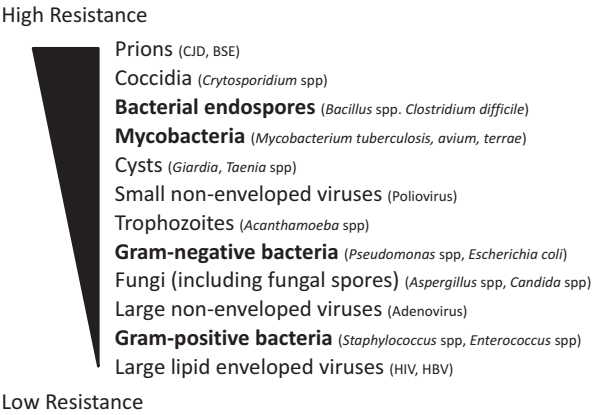


Fig. 2.2 Classification of micro-organisms according to their resistance to biocides. Groups containing bacteria are highlighted in *bold*. Adapted from Maillard (2002)

2.3.1.1 Spore-Forming Bacteria

Many biocides are active against non-sporulating bacteria but have little sporicidal activity; for others sporicidal activity is only achieved at high concentrations. *Clostridium* and *Bacillus* spores are the most resistant of all types of bacteria to biocides (Russell 1990). Examples of biocides which are described as sporicidal include glutaraldehyde and formaldehyde. Resistance of bacterial endospores is mainly due to their structure with factors such as the spore coat and the cortex all providing biocide protection due to inaccessibility of the target site. The biochemical composition of the spore coat varies between species and can even vary between different strains of the same species (Driks 1999; Henriques and Moran 2007). It has been shown to play an essential role in protection against many chemicals including hydrogen peroxide (Young and Setlow 2004) and hypochlorite (Young and Setlow 2003). *Bacillus subtilis* spores which lack several spore coat layers due to mutations in genes which code for a protein essential for outer spore coat formation (*cotE*) and a regulator of coat protein formation (*gerE*) are more susceptible to hypochlorite as compared to wild-type spores produced by this species (Ghosh et al. 2008). Superoxide dismutase (SOD) when present on the spore surface was shown in *Bacillus anthracis* spores to protect against oxidative stress (Cybulski et al. 2009); however, this was not shown to be the case for *B. subtilis* (Casillas-Martinez and Setlow 1997).

Water availability within the spore core and core water content may also play a role in the ability of bacterial spores to resist biocides. *B. subtilis* spores which had a higher core water content appeared to be more susceptible to killing by liquid hydrogen peroxide (Popham et al. 1995). The presence of small acid-soluble spore proteins (SASPs) has been shown to protect nucleic acids within the spore by binding directly to and saturating potential binding sites on the DNA. This prevents access of the DNA to many biocides which are DNA damaging, e.g. hydrogen peroxide (Imlay and Linn 1988; Setlow 2007). Mutants lacking particular types of SASPs in *B. subtilis* correspondingly have been found to be more sensitive to hydrogen peroxide (Setlow et al. 2000).

There have been a number of studies on the sporicidal activity of various biocides and how their activity is affected by various factors including temperature, level of soiling, type of surface, etc. (Reviewed by Maillard 2011). For surface disinfection, the most widely used sporicidal agents are chlorine-based disinfectants, which include hypochlorites (e.g. bleach) and sodium dichloroisocyanurate (NaDCC) (Maillard 2011). The latter appeared to be more effective against *Clostridium difficile* spores on stainless steel rather than PVC surfaces (Block 2004), and the activity of NaDCC is improved when coupled with rigorous surface pre-cleaning using detergent (Ungurs et al. 2011). There is a distinct lack of methodological clarity when testing disinfectants which claim to have sporicidal activity, with different procedures used to evaluate different disinfectants. This has led to the proposed standardized test for testing sporicidal activity of disinfectants against *C. difficile* spores (Fraise et al. 2015).

2.3.1.2 Mycobacteria

The mycobacterial cell wall is more complex than other Gram-positive bacterial cell walls. It has an abundance of high molecular weight lipids, whilst the inner region contains peptidoglycan linked to another polysaccharide polymer, arabinogalactan (Lambert 2002). Anchored to this skeleton are mycolic acids which combine to give the cell a thick waxy coat which acts as an efficient permeability barrier (Brennan and Nikaido 1995). This waxy layer has been shown to contribute towards increased biocide resistance. Spanning the outer layer are porin proteins, and deletion of Msp group of porins decreases the susceptibility of *Mycobacterium smegmatis* to many widely used biocides, e.g. octenidine and polyhexamethylene biguanide (PHMB) (Frenzel et al. 2011). One biocide which is effective against *Mycobacterium* is triclosan which inhibits the action of the enoyl reductase InhA (McMurry et al. 1999; Parikh et al. 2000) though there are reports suggesting that this is not the only site of action (Boshoff et al. 2004). In response to triclosan, *Mycobacterium* significantly differentially expressed several hundreds of genes and these included potential drug detoxification and efflux mechanisms (Betts et al. 2003; Boshoff et al. 2004). Ligand-binding studies have shown which residues are important in triclosan binding to InhA (Cohen et al. 2011). Presumably, mutations at these residues would affect the binding affinity and therefore effectiveness of triclosan against *Mycobacterium*.

2.3.1.3 Other Gram-Positive Bacteria

For bacteria, Gram-positive micro-organisms are highly susceptible to many biocides. Their cell wall is essentially peptidoglycan and teichoic acid which does not act as an effective barrier against the entry of biocides. This is in part due to the ability of high molecular weight substances to readily traverse the cell wall of staphylococci and vegetative *Bacillus* spp. which renders these organisms highly sensitive to QACs and chlorhexidine (Russell 1991, 1995).

The plasticity of the bacterial cell envelope is a well-known phenomenon (Poxton 1993). The cell physiological state will be affected by growth rate and limiting nutrients which will alter the thickness and degree of peptidoglycan cross-linking, altering the cell's biocide sensitivity. Chlorhexidine sensitivity was altered in *Bacillus megaterium* following changes in nutrient availability (Gilbert and Brown 1995). Mucoïd strains of *S. aureus*, where the cells are surrounded by a slime layer, are less sensitive to killing by chlorhexidine than non-mucoïd strains, and removal of this slime layer rendered the cells more sensitive (Kolawole 1984). This indicates that the slime layer plays a role in protection either as a physical barrier or by absorption of biocide molecules (Kolawole 1984). This is different from experimental work on the Gram-negative species *K. pneumoniae* where there was no difference observed in sensitivity to several biocides between non-mucoïd and mucoïd isolates (M. Wand, unpublished results).

There are differences in susceptibility within Gram-positive bacteria with *Enterococci* being, in general, less sensitive to biocides than *Staphylococci*. There is, to date, no correlation between the carriage of antibiotic resistance and biocide resistance, e.g. vancomycin-resistant *Enterococcus* (VRE) are not more resistant to biocides than those *Enterococcus* strains which are vancomycin sensitive (VSE) (Sakagami and Kajimura 2002). MRSA (methicillin-resistant *Staphylococcus aureus*) does not appear to have a selective advantage over MSSA (methicillin-sensitive *Staphylococcus aureus*) with respect to biocide resistance (Wootton et al. 2009).

2.3.1.4 Gram-Negative Bacteria

As already mentioned, Gram-negative bacteria are, in general, more resistant to biocides than Gram-positive bacteria due to their cell wall composition. The outer membrane acts as a barrier which helps either to limit or prevent the entry of biocides into the cell. Some cationic biocides are able to damage the outer membrane which may help to promote their own uptake (Hancock 1984). Gram-negative bacteria which are particularly resistant to biocides are *Pseudomonas* species (especially *P. aeruginosa*) due to a number of efflux systems (Morita et al. 2014) and *Burkholderia cepacia*. *B. cepacia* has been readily isolated as a contaminant of disinfectants and other anti-infective solutions (Romero-Gomez et al. 2008; Frank and Schaffner 1976). Biocide testing showed that *B. cepacia* is able to remain viable in several commercial biocide formulations (Rose et al. 2009). *B. cepacia* is also able to biodegrade and inactivate benzyldimethylalkylammonium chloride (benzalkonium chloride) by cleavage of the C-alkyl-N bond (Ahn et al. 2016). Other species highly resistant to disinfectants, especially chlorhexidine, are *Providencia stuartii* and *Proteus* spp. (Strickler and Thomas 1976; Martin 1969; Strickler 1974). This is linked to their hydrophobicity of the cell surface and especially for *Proteus* spp. the membrane lipid content (el Moug et al. 1985; Thomas and Strickler 1979).

2.3.2 Acquired Resistance to Biocides

Increased tolerance to biocides can arise through several strategies including mutation of existing genes, expression of previously silent genes or acquisition of new genetic information by horizontal gene transfer on extrachromosomal elements, e.g. plasmids and transposons. Within the laboratory organisms can be 'adapted' to the presence of particular biocides by continuous exposure (MacGregor and Elliker 1958), whilst residue disinfectant can remain on equipment which could lead to a selective pressure for more biocide-resistant organisms (Cousins 1963). There is now a prevalence to generate biocide-tolerant bacteria

by exposure to gradually increasing concentrations of biocide, e.g. chlorhexidine (Furi et al. 2013; Bock et al. 2016).

Biocide resistance, unlike antibiotic resistance, is rarely associated with acquisition of genes, and relatively few ‘disinfectant resistance’ genes have been identified. For Gram-positive organisms, particularly *S. aureus*, the plasmid-encoded *qacA/B* genes (Gillespie and Skurray 1986; Lyon and Skurray 1987) and for Gram-negative organisms *qacE* and *qac Δ E1* (Paulsen et al. 1993) have been linked to disinfectant resistance. These genes are often carried on plasmids which also contain either antibiotic or heavy metal resistance genes, which leads to the debate of whether acquired biocide resistance can also lead to antibiotic resistance.

2.3.2.1 Impermeability

As already described, target sites for biocides are usually situated within the cytoplasm or for Gram-negative bacteria at the cytoplasmic membrane. Changes in the outer membrane, in particular LPS (lipopolysaccharide) and loss of porin proteins, are the principal methods used to change bacterial permeability in response to biocides (Denyer and Maillard 2002). Chlorhexidine-resistant strains of *Pseudomonas stutzeri* have been found which possessed alterations in their OMP (outer membrane proteins) profiles (Tattawasart et al. 2000a) and also had reduced uptake of chlorhexidine associated with decreased cellular amounts of magnesium, calcium and phosphorous (Tattawasart et al. 2000b). Outer membrane fatty acid composition was observed to have changed in *P. aeruginosa* in response to treatment to quaternary ammonium compounds (Guérin-Méchin et al. 2000). Downregulation of porin expression has been noticed in *Salmonella typhimurium* and *E. coli* in response to disinfectant exposure (Karatzas et al. 2008; Zhang et al. 2011). Increased expression of *rarA*, an AraC-type regulator in *K. pneumoniae*, following challenge with disinfectants has resulted in decreased porin expression (De Majumdar et al. 2013).

2.3.2.2 Target Alteration

This method of resistance is uncommon due to the ability of biocides to act on multiple cellular components. The most widely described target alteration in response to a biocide is mutations in the *fabI* gene resulting in increased resistance to triclosan. At low concentrations, triclosan functions to inhibit the action of the enoyl-acyl carrier protein (ACP) reductase (FabI) and therefore prevents fatty acid synthesis (Heath et al. 1998). Mutations in this gene were first described in *E. coli* (Heath et al. 1999) but have since been found in other organisms including *P. aeruginosa* (Hoang and Schweizer 1999), *S. aureus* (Heath et al. 2000), *Acinetobacter baumannii* (Chen et al. 2009) and *Rhodobacter sphaeroides* (Lee et al. 2002). Current *Staphylococcus epidermidis* isolates have also been shown to have mutations in the *fabI* gene or its putative promoter region coupled to increased

triclosan resistance when compared to isolates from the 1960s suggesting adaptation to the widespread use of triclosan (Skovgaard et al. 2013). Other studies have shown that high levels of triclosan resistance are not just dependent on mutations in *FabI*, but other distinct pathways are involved. In *P. aeruginosa* a $\Delta fabI$ strain retained high levels of resistance to triclosan, but a deletion of another enoyl reductase, *fabV*, resulted in increased susceptibility to triclosan (Zhu et al. 2010). In *Salmonella* substitutions within a gene not directly related to fatty acid synthesis *gryA* also led to a small decrease in triclosan susceptibility (Webber et al. 2008).

2.3.2.3 Efflux Systems

Efflux systems commonly have a wide range of structurally unrelated substrates, and those that are linked to biocide resistance (Table 2.2) often are involved in the efflux of antibiotics. Efflux pumps have a number of different structures and may consist of single proteins which are either ATP driven, e.g. VcaM from *Vibrio cholera* (Huda et al. 2003), HorA from *Lactobacillus brevis* (Sakamoto et al. 2001) and LmrA in *Lactococcus lactis* (van Veen et al. 1999), or are proton-motive force (PMF) driven, e.g. Qac proteins in *S. aureus*. More complex efflux pumps include three-component systems which are composed of an outer membrane and an inner membrane protein and a protein that traverses the periplasm to connect the two membrane proteins. Examples include the AcrAB-TolC transporter in *E. coli* and the MexAB-OprM transporter in *P. aeruginosa*.

Efflux pumps are broadly grouped into five major classes (Putman et al. 2000) (Fig. 2.3): (1) the ATP (adenosine triphosphate)-binding cassette (ABC) family, (2) the major-facilitator superfamily (MFS), (3) the resistance-nodulation-division (RND) family, (4) the small multidrug resistance (SMR) family (a member of the drug/metabolite transporter (DMT) superfamily) and (5) the multidrug and toxic compound extrusion (MATE) family. Most of the multidrug transporters, apart from the ABC transporters which are powered by ATP hydrolysis, utilize the transmembrane H^+ or Na^+ gradient to catalyse drug extrusion.

Insusceptibility to several biocides including benzalkonium chloride, triclosan and chlorhexidine can be attributed to expression of several MATE transporters in a variety of bacteria. These include, for Gram-negative bacteria, PmpM from *P. aeruginosa* (He et al. 2004), NorM from *Neisseria* species (Rouquette-Loughlin et al. 2003) and its homologue VmrA from *Vibrio* species (Chen et al. 2002) and AbeM in *A. baumannii* (Su et al. 2005). From Gram-positive bacteria the most well-characterized MATE transporter is MepA in *S. aureus* (DeMarco et al. 2007) where exposure to sublethal levels of numerous biocides led to the appearance of mutants overexpressing MepA (Huet et al. 2008).

ABC transporters contributing to biocide resistance include EfrAB from *Enterococcus faecalis* (Lee et al. 2003) which have also been detected in *Staphylococcus* and *Bacillus* species (Fernández-Fuentes et al. 2014) where overexpression of this pump led to increased resistance to chlorhexidine and triclosan (Lavilla Lerma et al. 2014).

Table 2.2 Efflux determinants of biocide resistance

Efflux determinant	Type ^a	Biocide ^b	Organism	Reference
<i>Gram-positive</i>				
MepA	MATE	CHX, QAC	<i>S. aureus</i>	DeMarco et al. (2007)
EfrAB	ABC	CHX, TRI	<i>E. faecalis</i> , <i>S. aureus</i> , <i>Bacillus</i> spp.	Lee et al. (2003), Fernández-Fuentes et al. (2014)
QacA	MFS	QAC, BG	<i>S. aureus</i> , <i>E. faecalis</i>	Brown and Skurray (2001), Bischoff et al. (2012)
QacB	MFS	QAC	<i>S. aureus</i> , <i>E. faecalis</i>	McDonnell and Russell (1999), Bischoff et al. (2012)
QacC/D smr	SMR	QAC	<i>S. aureus</i>	Noguchi et al. (1999)
QacEΔ1	SMR	QAC	<i>S. aureus</i> , <i>E. faecalis</i>	Kazama et al. (1998)
QacG	SMR	QAC	<i>S. aureus</i>	Heir et al. (1999a)
QacH	SMR	QAC	<i>S. aureus</i>	Heir et al. (1998)
QacJ	SMR	QAC	<i>S. aureus</i>	Bjorland et al. (2003)
NorA	MFS	QAC, CTM, CHX	<i>S. aureus</i>	Noguchi et al. (1999), DeMarco et al. (2007)
NorB	MFS	QAC, CTM, CHX	<i>S. aureus</i>	Truong-Bolduc and Dunman (2005), DeMarco et al. (2007)
MdeA	MFS	QAC, CHX	<i>S. aureus</i>	DeMarco et al. (2007)
EmeA	MFS	QAC	<i>E. faecalis</i>	Schwaiger et al. (2014)
Mmr	SMR	CTAB	<i>M. tuberculosis</i>	Rodrigues et al. (2013)
<i>Gram-negative</i>				
QacE	SMR	QAC	Widespread	Paulsen et al. (1993)
QacEΔ1	SMR	QAC	Widespread	Paulsen et al. (1993)
QacF	SMR	QAC	<i>Enterobacter</i> spp., <i>P. aeruginosa</i>	Ploy et al. (1998), Jeong et al. (2009)
QacG	SMR	QAC	<i>P. aeruginosa</i>	Laraki et al. (1999)
PmpM	MATE	QAC	<i>P. aeruginosa</i>	He et al. (2004)
NorM	MATE	BAC	<i>N. gonorrhoea</i> , <i>N. meningitis</i>	Rouquette-Loughlin et al. (2003)
VmrA	MATE	TPPCI	<i>Vibrio</i> spp.	Chen et al. (2002)
AbeM	MATE	TRI	<i>A. baumannii</i>	Su and Chen (2005)
MexAB- OprM	RND	PHN, TRI	<i>P. aeruginosa</i> , <i>P. azelaica</i>	Chuanchuen et al. (2001), Czechowska et al. (2013)
MexCD- OprJ	RND	BAC, CHX, TRI	<i>P. aeruginosa</i>	Morita et al. (2003), Chuanchuen et al. (2001)
MexEF- OprN	RND	TRI	<i>P. aeruginosa</i>	Chuanchuen et al. (2001)
SmeDEF	RND	TRI	<i>S. maltophilia</i>	Sanchez et al. (2005)
SdeXY	RND	TRI	<i>S. marcescens</i>	Chen et al. (2003)
AdeABC	RND	CHX	<i>A. baumannii</i>	Rajamohan et al. (2010)
AdeIJK	RND	TRI	<i>A. baumannii</i>	Fernando et al. (2014)
AceI	Novel	CHX	<i>A. baumannii</i>	Hassan et al. (2013)

(continued)

Table 2.2 (continued)

Efflux determinant	Type ^a	Biocide ^b	Organism	Reference
CmeABC	RND	TRI	<i>C. jejuni</i>	Pumbwe et al. (2005)
CmeDEF	RND	TRI	<i>C. jejuni</i>	Pumbwe et al. (2005)
AbuO	RND	TRI, CHX, BAC	<i>A. baumannii</i>	Srinivasan et al. (2015)
AdeF	MFS	CHX	<i>A. baumannii</i>	Hassan et al. (2011)
AbeS	SMR	CHX	<i>A. baumannii</i>	Srinivasan et al. (2009)
OqxAB	RND	TRI, CHX, BAC, CTM	<i>E. coli</i>	Hansen et al. (2007)
CepA	SMR	CHX	<i>K. pneumoniae</i>	Fang et al. (2002)
KpnEF	SMR	BAC, CHX, TRI	<i>K. pneumoniae</i>	Srinivasan and Rajamohan (2013)
KpnGH	MFS	BAC, CHX, TRI	<i>K. pneumoniae</i>	Srinivasan et al. (2014)
SmvA	MFS	CHX	<i>K. pneumoniae</i>	Wand et al. (2017)
AcrAB-TolC	RND	QAC, TRI	<i>E. coli</i> , <i>S. enterica</i> serovar Typhimurium	Piddock (2006), Webber et al. (2008)
MdtM	MFS	QAC	<i>E. coli</i>	Holdsworth and Law (2013)
YhiUV-TolC	RND	BAC	<i>E. coli</i>	Nishino and Yamaguchi (2001)
TriABC-OpmH	RND	TRI	<i>P. aeruginosa</i>	Mima et al. (2007)
MexJK-OpmH	RND	TRI	<i>P. aeruginosa</i>	Chuanchuen et al. (2003)

^a*MATE* multidrug and toxic compound extrusion (efflux pump type), *ABC* ATP (adenosine triphosphate)-binding cassette, *MFS* major-facilitator superfamily, *SMR* small multidrug resistance, *RND* resistance-nodulation-division, *Novel* not characterized

^b*BAC* benzalkonium chloride, *BG* biguanides, *CTM* cetrimide, *CHX* chlorhexidine, *QAC* quaternary ammonium compounds, *PHN* phenolics, *TRI* triclosan, *TPPCI* tetraphenylphosphonium chloride, *CTAB* cetyltrimethylammonium bromide (adapted and updated from Poole 2005)

For the RND efflux pumps, MexCD-OprJ in *P. aeruginosa* has been found to be induced by tetraphenylphosphonium chloride (TPPCI) in mutants lacking other Mex pumps (MexAB-OprM, MexEF-OprN and MexXY) (Morita et al. 2001). It is also induced in subinhibitory concentrations of benzalkonium chloride and chlorhexidine gluconate (Morita et al. 2003) in a process that is dependent on the stress response factor, AlgU (Fraud et al. 2008). Other genes affected in subinhibitory concentrations of benzalkonium chloride include mutations in the Mex efflux regulator gene, *nfxB*, which, in turn, led to overexpression of MexCD-OprJ and MexAB-OprM (McCay et al. 2010). MexCD-OprJ is central to the response of *P. aeruginosa* when exposed to chlorhexidine diacetate being, along with another RND efflux pump *oprH-phoPQ*, upregulated (Nde et al. 2009). MexCD-OprJ is also important in the development of chlorhexidine-tolerant subpopulations within *P. aeruginosa* biofilms (Chiang et al. 2012). In *A. baumannii* chlorhexidine tolerance is linked to a broad range of efflux systems including the RND efflux pump

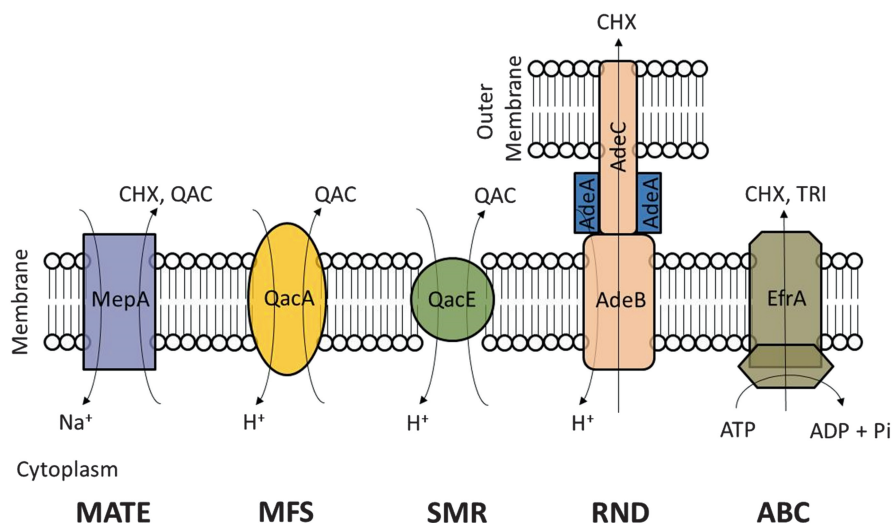


Fig. 2.3 Representatives of the five known families of efflux pumps thought to be involved in bacterial resistance to biocides. The MATE, MFS and SMR families are driven by chemiosmotic energy, with H^+ or Na^+ ions being pumped into the cell whilst pumping biocides out. The RND family spans the inner and outer membranes in Gram-negative bacteria and usually consists of multisubunits. The ABC superfamily utilizes ATP to drive efflux of biocides from the cell. Biocides shown in the diagram are CHX (chlorhexidine), QAC (quaternary ammonium compounds) and TRI (triclosan)

AdeABC (Rajamohan et al. 2010) with exposure to chlorhexidine leading to elevated levels of expression of AdeAB (Hassan et al. 2013). A recently described efflux pump, AceI, from a novel family of bacterial drug efflux transporters was found to be an active chlorhexidine efflux pump (Hassan et al. 2013). This transporter was also found to be present in other bacterial species including *Pseudomonas* sp. and *Burkholderia cenocepacia* (Hassan et al. 2015). Other efflux pumps involved in resistance to chlorhexidine in *A. baumannii* include the MFS efflux pump AedF (Hassan et al. 2011) and an efflux pump, AbeS, belonging to the SMR family (Srinivasan et al. 2009). Although many of these efflux pumps are found on the bacterial chromosome, a plasmid-encoded RND efflux pump, OqxAB, from *E. coli* which confers resistance to a number of biocides, e.g. triclosan, was able to be successfully transferred to other members of the Enterobacteriaceae (Hansen et al. 2007).

Many RND family pumps associated with resistance to antibiotics are also associated with resistance to triclosan including the Mex pumps in *P. aeruginosa* (Chuanchuen et al. 2003), SmeDEF of *Stenotrophomonas maltophilia* (Sanchez et al. 2005), SdeXY of *Serratia marcescens* (Chen et al. 2003), AdeIJK in *A. baumannii* (Fernando et al. 2014) and CmeABC and CmeDEF of *Campylobacter jejuni* (Pumbwe et al. 2005). Enhanced triclosan resistance has been linked to overexpression of AcrAB-TolC and the global regulators of active efflux, MarA and SoxS in *E. coli* and *Salmonella* (McMurry et al. 1998b; Bailey et al. 2009;

Karatzas et al. 2007). Inactivation of AbuO (a TolC homologue) in *A. baumannii* led to decreased survival in subinhibitory concentrations of triclosan, chlorhexidine and benzalkonium chloride (Srinivasan et al. 2015).

Resistance to quaternary ammonium compounds (QACs) has been readily described in Gram-positive bacteria, particularly *S. aureus*. QAC resistance in *S. aureus* is predominantly linked to plasmid-encoded MFS (QacA/B) or SMR family exporters (Smr (QacC/D), QacE Δ 1, QacG, QacH, QacJ) with resistance arising from plasmid acquisition. The most prevalent pump is QacA which can mediate resistance to a number of organic cations including lipophilic monovalent cations such as benzalkonium chloride and cetrime and divalent cations such as chlorhexidine. The seven nucleotide differences between *qacA* and its homologue *qacB* are distributed throughout the gene and result in only one amino acid change (Asp273 in QacA and Ala273 in QacB). However, this change means that QacB offers negligible protection from divalent cations, being primarily associated with resistance to monovalent cations (Paulsen et al. 1996). There is conflicting evidence to suggest that the presence of QacA/B actually plays a role in biocide tolerance. Several studies, in *S. aureus*, have shown that the presence of *qacA* does not always correlate with increased resistance to chlorhexidine; isolates which are *qacA* positive may have comparable chlorhexidine susceptibility levels to isolates which are negative for the presence of *qacA* (Horner et al. 2012). Analysis of over 1600 staphylococcal isolates was unable to show a clear breakpoint between susceptible and non-susceptible populations to benzalkonium chloride and chlorhexidine and the presence of *qacA* and *qacB* genes (Furi et al. 2013). In contrast, there was a clear relationship between resistance to ethidium bromide and the detection of *qacA* and *qacB* (Patel et al. 2010). QacA/B have also been detected in other Gram-positive bacteria, e.g. *Enterococcus faecalis* (Bischoff et al. 2012).

Other efflux pumps contributing to QAC resistance in *S. aureus* are chromosomally encoded and include NorA, NorB and the MFS family efflux protein MdeA (Huang et al. 2004; Noguchi et al. 1999). NorA is a chromosomally encoded MDR (multidrug resistant) efflux pump in *S. aureus* (Patel et al. 2010) and is implicated in resistance to fluoroquinolones including norfloxacin and ciprofloxacin (Yoshida et al. 1990; Ng et al. 1994). Mutations in the promoter of NorA, leading to increased expression, resulted in a slight increase in resistance to ethidium bromide, benzalkonium chloride and chlorhexidine (Furi et al. 2013). Homologues to NorA exist in other Gram-positive bacteria and include EmeA (*Enterococcus faecalis*) (Jonas et al. 2001) with variants in this gene leading to increased resistance to QAC (Schwaiger et al. 2014).

For Gram-negative organisms efflux-mediated biocide resistance is generally chromosomally encoded. The exceptions are *qacE*, *qacE* Δ 1, *qacF* and *qacG* which are widely disseminated and are associated with mobile genetic elements. However, again there is conflicting evidence as to whether the presence of the *qacE* and *qacE* Δ 1 genes correlates with increased QAC resistance. Isolates from a range of Gram-negative organisms showed similar MIC values to benzalkonium chloride whether *qacE* or *qacE* Δ 1 was detected or not (Kücken et al. 2000). In *K. pneumoniae*, chlorhexidine resistance has been linked to another SMR family

efflux pump, *cepA* (Fang et al. 2002), with several resistant strains to chlorhexidine and benzalkonium chloride showing carriage of this gene and *qacEΔ1* (Abuzaid et al. 2012). Homologues to *cepA* have been found in other bacteria, e.g. *P. aeruginosa*, indicating that this mechanism is widely distributed within Gram-negative pathogenic bacteria (Poole 2005). It is again worth noting that there were several isolates which showed elevated levels of antiseptic resistance, in which *cepA* and *qacEΔ1* were not detected, and isolates which were positive for *cepA* had low MIC values to the disinfectants tested (Abuzaid et al. 2012). Another study indicated that reduced susceptibility to chlorhexidine appeared to be independent of the expression of *cepA* and two other efflux pumps, *acrA* and *kdeA* (Naparstek et al. 2012). Therefore, the role, if any, of these genes in antiseptic resistance is unclear. Several *K. pneumoniae* strains which were highly susceptible to chlorhexidine have shown insertions within the *cepA* gene (Wand et al. 2015) which suggests that investigating the genetic sequence of these genes is more important than looking at their presence/absence. Subsequent studies showed that these isolates also lacked the MFS pump SmvA (Wand et al. 2017) which has been implicated in methyl viologen resistance in *S. enterica* (Santiviago et al. 2002). SmvA also has high homology to QacA from *S. aureus* (Santiviago et al. 2001). When strains of *K. pneumoniae* were cultured in sublethal levels of chlorhexidine, increased tolerance was found to be associated with deletion of *smvR* (a Tet repressor acting on *smvA*) and increased expression of *smvA* (Wand et al. 2017).

Other efflux pumps which have been implicated in biocide resistance in *K. pneumoniae* include the SMR-type pump KpnEF (Srinivasan and Rajamohan 2013) and the MFS pump KpnGH (Srinivasan et al. 2014) where the removal of these genes led to a slight increase in susceptibility to benzalkonium chloride, chlorhexidine and triclosan.

2.4 Biofilms

Biofilms are an important source of infection, particularly in clinical environments where an estimated 80% of all infections are linked to biofilms (National Nosocomial Infections Surveillance 1999). Therefore, particularly with surface disinfectants, biocides need to be effective against bacterial biofilms. There is a plethora of literature describing increased antibiotic resistance when bacteria are in a biofilm or when they are attached to surfaces. The same is true for biocides; microbes which are attached to a surface have reduced biocide susceptibility when compared to planktonic cells (Condell et al. 2012; Leung et al. 2012). When bacteria are part of an established biofilm, the biocide resistance is greatly increased which can render certain biocides ineffectual (Smith and Hunter 2008). There have been many studies which have shown that biocides have reduced efficacy against biofilms when compared to planktonic cells, but most are simple observational studies without exploring the reasons for this reduced efficacy. Several studies have also demonstrated that multispecies biofilms are more resistant to biocides than mono-

species biofilms (Luppens et al. 2008; Van der Veen and Abee 2010). Within a mixed-species biofilm, *P. aeruginosa* and *K. pneumoniae* were able to survive challenge with above in clinical use concentrations of chlorhexidine (Touzel et al. 2016), and a multispecies biofilm was more resistant to hydrogen peroxide than each individual mono-species biofilm alone (Burmølle et al. 2006). Why multispecies biofilms are more resistant than mono-species biofilms is unclear, but several theories have been hypothesized. Firstly an association or aggregation with more resistant isolates may help protect more susceptible species, e.g. the presence of *Kocuria* sp., which are more resistant to chlorine, was found to protect *Staphylococcus sciuri* from disinfection (Leriche et al. 2003). There also may be a synergistic interaction between different bacterial species within the same biofilm, whether this is production of different enzymes which complement each other to inactivate toxic compounds (Shu et al. 2003) or the production of a more highly viscous matrix due to chemical interactions between different bacterial species polymers (von Canstein et al. 2002). Mechanisms within biofilms which confer reduced susceptibility to antimicrobial agents include reduced penetration (Szomolay et al. 2005). Chlorine dioxide was unable to completely penetrate a mixed-species biofilm, possibly due to interactions between the biocides and components of the biofilm (Jang et al. 2006). The mean penetration time of alkaline hypochlorite into a mixed *P. aeruginosa* and *K. pneumoniae* biofilm was eight times longer than for chlorosulphamate (Stewart et al. 2001). This was potentially due to the greater capacity of alkaline hypochlorite to react with matrix constituents within the biofilm. Cell density and biofilm accumulation invariably play a role in biocide effectiveness with older thicker biofilms being less susceptible than younger less dense biofilms (Stewart 2015).

Other factors influencing biocide effectiveness against biofilms include alteration to growth, modulation of metabolic processes including the stress response and changes in quorum sensing. The expression of *rpoS*, the principal regulator of the general stress response, was over threefold upregulated in a 3-day-old *P. aeruginosa* biofilm when compared to stationary phase planktonic cells (Xu et al. 2001). There are also several experimental factors which have been indicated in biofilm formation which include surface type and growth temperature. An increase in growth temperature has been noted to increase resistance of *S. aureus* biofilm to polyhexamethylene biguanide and QAC disinfectants (Abdallah et al. 2014).

Some biocidal agents are thought to be more effective than others at killing bacteria within biofilms. The oxidizing agents sodium hypochlorite and peroxygens were found to be the most successful at eradication of *P. aeruginosa* and *S. aureus* biofilms (Toté et al. 2010). Sodium hypochlorite was found to be more effective over chlorhexidine at the removal of *E. faecalis* and MRSA in biofilms (Lee et al. 2009; Williamson et al. 2009). Oxidizing agents have multiple targets within the biofilm, whereas other biocides such as chlorhexidine specifically target cell wall components which may explain the high level of efficacy for oxidizing agents

against biofilms (Otter et al. 2015). Benzalkonium chloride was shown to have delayed penetration into a *P. aeruginosa* biofilm when compared to peracetic acid (Bridier et al. 2011). Peracetic acid appears to be very effective against bacterial biofilms with multiple strains of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* having low MBEC (minimum biofilm eradication concentration) against a disinfectant containing peracetic acid (Perumal et al. 2014). Investigation of the ability of certain biocides, e.g. chlorhexidine digluconate and triclosan to actively penetrate and remove oral biofilms, was found to range between 86.8 and 99.5% efficacy after 1 h with chlorhexidine being the most effective (Corbin et al. 2011). Therefore, clearly the ability of a biocide to penetrate biofilms is of paramount importance to eradication of these structures.

Within biofilms, the gene expression profile is thought to be different from planktonic cells. Induction of genes involved in the oxidative stress response has been shown in biofilms in multiple bacteria, e.g. *P. aeruginosa* and *E. coli* (Sauer et al. 2002; Ren et al. 2004). Analysis of gene expression of sessile *A. baumannii* showed changes in expression of genes involved in amino acid and fatty acid metabolism, motility, active transport, DNA methylation, iron acquisition, transcriptional regulation and quorum sensing (Rumbo-Feal et al. 2013). When *B. cenocepacia* biofilms were exposed to chlorhexidine, the expression of several genes including RND and MFS efflux systems, membrane proteins and chaperonins was increased (Coenye et al. 2011). Studies have also shown that subinhibitory concentrations of biocides can lead to upregulation of genes involved in biofilm formation and therefore actively promote biofilm formation (Dong et al. 2012). *Bacillus subtilis* biofilm formation was stimulated in response to sublethal doses of chlorine dioxide by upregulation of the major operons *epsA-epsO* and *yqxM-sipW-tasA* responsible for matrix production (Shemesh et al. 2010). This suggests that biofilm formation is a defensive mechanism employed to help protect the bacteria from the toxic effects of the biocide.

Within biofilms there is thought to be a greater concentration of bacterial persister cells (Lewis 2007; Fauvart et al. 2011). Persister cells are characterized by their ability to survive exposure to antimicrobial agents, yet do not develop resistance to these agents (Lewis 2007) and thus are not true 'mutants'. The persistence of these cells is linked to their metabolic inactivity and slow growth, both of which are characteristic in biofilms. These persister cells are also increasingly likely to survive exposure to a biocide (Simões et al. 2011) though it is not known if these cells remain metabolic inactive or alter their gene expression profile in response to biocide exposure. When *S. aureus* cells were challenged with triclosan, they were observed to completely shut down the *agr* quorum-sensing system but they also increased the expression of *fabI* (Nielsen et al. 2013).

Other phenotypic resistance mechanisms linked to biocide resistance include swarming motility. Swarming has similar aspects to biofilm communities, and swarm cells from a variety of Gram-negative organisms including *P. aeruginosa* showed increased resistance to biocides, e.g. triclosan (Lai et al. 2009).

2.5 Link to Antibiotic Resistance

Recent evidence has shown that the indiscriminate use of biocides can select for particular bacterial pathogens which have increased resistance to both biocides and antibiotics with many studies showing links between increased biocide and antibiotic resistance (e.g. Schwaiger et al. 2014; Russell et al. 1998). However, often these studies showed no clear mechanism. In addition, there are many other studies which were unable to find evidence for co-resistance to biocides and antibiotics (e.g. Suller and Russel 2000; Loughlin et al. 2002). There is also little evidence to suggest that isolates that are multidrug resistant are also more resistant to biocides (Shinoda et al. 2016) or that the carriage of specific antibiotic resistance genes, e.g. *bla_{SHV}*, is linked to biocide resistance.

Since biocide usage is less regulated than antibiotic usage and biocides are widely used in a variety of settings, there is a danger that this will lead to outbreaks of pathogens which are both multidrug resistant and resistant to a number of biocides. The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) published a strategy to investigate the antimicrobial resistance effects of biocides (SCENIHR 2010). Within this document there were several recommendations for future research. These covered areas such as the effect of biofilms and exposure to sublethal levels of biocides on biocide and antibiotic resistance.

Horizontal transfer of antibiotic resistance genes and genetic elements between bacteria is common and occurs through a variety of methods including cell to cell conjugation, use of a bacteriophage intermediate or transformation of DNA. Many of these mobile antibiotic resistance genes are found on plasmids. *QacA/B* genes which are thought to confer increased resistance to benzalkonium chloride were found on multi-resistance plasmids containing *bla* and *tet* resistance genes in *S. aureus* (Heir et al. 1999b; Sidhu et al. 2002). Thus, acquisition of these plasmids will invariably lead to increased resistance to benzalkonium chloride, penicillin and tetracycline. There is also reported genetic linkage between *qac* genes and antibiotic resistance genes (*bla_Z*, *aacA-aphD*, *dfrA* and *ble*) on the same plasmids in *staphylococcal* species isolated from clinical and food environments (Sidhu et al. 2001). The higher frequency of antibiotic resistance among benzalkonium chloride-resistant strains indicates that the presence of either resistance determinant selects for the other during selective pressure caused by either antimicrobial therapy or clinical disinfection (Sidhu et al. 2002). The presence of these resistant determinants on plasmids leads to the spread of biocide and antibiotic ‘multi-resistance’ to other strains or species which are independent of their original carrier (Bjorland et al. 2005).

Upregulation of efflux pumps, as already discussed, is a common resistance mechanism associated with increased antibiotic and biocide tolerance. In *Salmonella* increased triclosan tolerance was associated with increased resistance to ampicillin, tetracycline and kanamycin. This was probably down to overexpression of the *acrAB* efflux pump (Karatzas et al. 2007). Exposure to triclosan also led to

cross resistance to antibiotics in *S. maltophilia* (Sanchez et al. 2005) through its binding to the transcriptional repressor SmeT; this in turn causes overexpression of the SmeDEF efflux pump and reduced susceptibility to quinolones (Hernandez et al. 2011). However, another study showed that increased expression of SmeDEF caused by exposure to another biocide, benzalkonium chloride, did not show any changes in susceptibility in *S. maltophilia* to antibiotics. This may be due to the fact that the concentration of benzalkonium chloride required to observe changes in antibiotic susceptibility is lethal (Sanchez et al. 2015).

In *K. pneumoniae* exposure to sublethal levels of chlorhexidine led to mutations in the two-component regulator PhoPQ which in turn led to increased resistance to the last resort antibiotic colistin (Wand et al. 2017). Although these mutations on their own do not appear to increase chlorhexidine tolerance, the fact that they were regularly observed following exposure to chlorhexidine is cause for concern. Similarly, exposure to MIC levels of chlorhexidine led to increased expression of *vanHAX* and *liaXYZ* (involved in vancomycin and daptomycin resistance, respectively) in *Enterococcus faecium* (Bhardwaj et al. 2016). Increased expression of these genes, however, again did not provide any increased protection against chlorhexidine. For *Salmonella* culture in sublethal levels of several commercially available biocides resulted in mutants which had elevated levels of resistance to nalidixic acid, ciprofloxacin, chloramphenicol and tetracycline as well as triclosan but not to the biocides themselves (Webber et al. 2015). Mutations observed with exposure to multiple biocides were in genes *gyrA* (involved in fluoroquinolone resistance), *ramR* (involved in regulation of *acrAB*) and *fabI* (implicated in triclosan resistance) (Webber et al. 2015).

2.6 Problems and Challenges Associated with Testing Biocide Efficacy

Although the number of reports showing an increase in bacterial resistance to biocides is growing and there are outbreaks caused by a failure in disinfection (Gillespie et al. 2007; Gamble et al. 2007; Duarte et al. 2009), there are many factors which should be taken into account with regard to biocide efficacy against bacteria. Often biocide resistance is defined in terms of MIC/MBC values, and whilst an increase in these values is seen, the levels are still far below the in-use concentration. Therefore, are these increased MIC/MBC values clinically relevant? Many studies will show laboratory adaptation to biocides but make little attempt to understand the mechanism of increased resistance. Often bacterial adaptation in the laboratory to biocides occurs over a prolonged period of time; within a clinical environment, most biocides only have a contact time of a few minutes; therefore, is this enough time for bacterial adaptation to occur? The contact time is important since again with susceptibility to biocides being measured by MIC/MBC over a period of 20–24 h, this does not mirror contact time of many biocides.

There is some debate as to whether adaptation of bacteria to biocides using a 'stepwise' method is clinically relevant. Although this method regularly produces bacteria with increased MIC/MBC values to particular biocides, again these are rarely in-use concentration levels (Walsh et al. 2003; Lear et al. 2006). Also, we must consider the question of 'Is this likely to occur within a clinical environment such that adaptation seen in the laboratory would be relevant to an in situ setting?' Exposure to high levels of biocide, performed so as to mirror 'in-use' concentrations, has also produced adaptation, and bacteria can be isolated as a contaminant from biocides, e.g. chlorhexidine (Ko et al. 2015; Brooks et al. 2004). Also exposure to low concentrations of biocide which is indicative of residual concentrations has been shown to induce low-level resistance (Thomas et al. 2000).

Many bacteria will be found as a contaminant on surfaces, and indeed, as already discussed, bacteria either attached to surfaces or in the form of biofilms are more resistant to biocides. The majority of biocide efficacy testing occurs against planktonic bacteria which are invariably more susceptible. Where biofilms are used to test disinfectant efficacy, again the exposure is invariably for 24 h although some studies have done a range of contact times to attempt to mimic biocide use (Perumal et al. 2014). Often the biofilms in the laboratory are grown as mono-species and in rich media. In hospital environments the bacteria may be starved of such nutrients, be in mixed populations or alternatively mixed with organic matter (e.g. blood) (so-called biological soil) which will affect biocide killing. The presence of organic matter has been shown to decrease biocide effectiveness (Condell et al. 2012) with chlorine in particular reacting with organic matter, thus reducing effectiveness (Nou and Luo 2010). Mechanical wiping is often important in the removal of bacterial contamination (Ungurs et al. 2011; Sattar and Maillard 2013), and this is often critical in the removal of biofilms yet is seldom taken into account. Less well understood is the growth of bacteria on surfaces with residual biocide concentrations or the effect of low concentrations of biocide on biofilms. Microbial diversity was reduced following exposure to triclosan but not quaternary ammonium-based formulations (Dunne 2002; McBain et al. 2004). There are also a lack of studies highlighting the link between gain of biocide resistance and biological fitness. It is assumed that following removal of selective pressure, the mutations that arise following biocide adaptation are transient. One study on *S. enterica* serovar Typhimurium SL1344 showed that adaptation to various biocides rendered those isolates 'less fit' (Curiao et al. 2016). Those 'biocide-adapted' cells which are less fit will be outcompeted by a more susceptible population following removal of the selective pressure provided by the biocide. However, triclosan-resistant mutants in *S. typhimurium* had comparable fitness with wild-type sensitive strains (Webber et al. 2008). Another study showed that fitness was impaired in some strains of *K. pneumoniae* but not others following adaptation to chlorhexidine (Wand et al. 2017). This shows that retention of fitness is dependent upon a number of factors, e.g. strain, the individual mutation and particular biocide used. Those mutations which show no impact on fitness are perhaps more clinically relevant especially if they occur in MDR isolates. Perhaps more worryingly is a recent study which links the carriage of a plasmid containing the benzalkonium

chloride tolerance efflux pump gene, *emrC* and clinical outcomes of meningitis linked to infections with ST6 *Listeria monocytogenes* (Kremer et al. 2016). This is one of the first studies to link the use of biocides with an increase in disease outcome.

Bacterial resistance to biocides has been well documented in vitro, but concrete evidence of clinical resistance is lacking. There are also issues surrounding the lack of consensus on the methodologies used to study the emergence of bacterial resistance to biocides and also the lack of guidance on the use of biocides within clinical environments. Especially for certain species of bacteria which are multidrug resistant, the use of biocides is critical to combat the spread of these infections. Since antibiotics appear to have failed, it is imperative that biocides do not.

Compliance with Ethical Standards

Conflict of Interest: Matthew E. Wand declares that he has no conflict of interest.

Ethical approval: This chapter does not contain any studies with human participants or animals performed by any of the authors.

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