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Do Biocides Select for Antibiotic Resistance?*

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Abstract

Some similarities exist between bacterial resistance to antibiotics and to biocides, and gram-negative bacteria that have developed resistance to cationic biocides may also be insusceptible to some antibiotics. Outer membrane changes are believed to be responsible for this non-specific increase in resistance. Efflux, another important resistance mechanism, is associated with the *qacA/B* gene system in staphylococci that confers low-level resistance to cationic agents including chlorhexidine salts and quaternary ammonium compounds. It has been proposed that the introduction into clinical practice of chlorhexidine and quaternary ammonium compounds has resulted in the selection of staphylococci containing *qacA* genes on multiresistance plasmids. A linkage between low-level resistance to triclosan and to antibiotics has recently been claimed to occur in *Escherichia coli*, with the bisphenol selecting for chromosomally-mediated antibiotic resistance. A key issue in many studies has been the use of biocides at concentrations significantly below those used clinically. It remains to be determined how an increase to low-level resistance to cationic biocides can be held responsible for the selection of antibiotic-resistant bacteria.

When applied to antibiotics, the term 'bacterial resistance' refers to a strain that is not killed or inhibited by a concentration attainable in-vivo (e.g. blood level, urine concentration); to a strain that is not killed or inhibited by a concentration to which most strains of that organism are susceptible; or to bacterial cells that are not killed or inhibited by a concentration that acts upon the majority of cells in that culture. There are usually clear cut off-points by relating minimum inhibitory concentrations (MICS) or minimum bactericidal concentrations (MBCS) to blood or serum levels (Thornsberry 1991).

The term 'bacterial resistance' applied to biocides (antiseptics, disinfectants, preservatives; Russell et al 1999) can be readily used in the context of the second and third aspects, but not the first, described above. This situation then refers to a strain that is not killed or inhibited by a concentration of biocide used in practice (Russell et al 1986). Tolerance (to a preservative) has been defined (Chapman 1998; Chapman et al 1998) as a situation in which a formerly effective (preservative) system no longer controls microbial growth. Potential causes of tolerance were then considered to be destabilization of preservative, establishment of biofilms and the development of resistance. Unfortunately, with antibiotics, the term 'tolerance' has a highly specific meaning, being used to refer to organisms (e.g. enterococci) that are not lysed by penicillin (Handwerger & Tomasz 1985).

It is not an easy matter, therefore, to relate antibiotic and biocide resistance. To avoid confusion, 'tolerance' will not be used hereafter but 'resistance' to antibiotics and biocides will be employed.

This paper will discuss the mechanisms of bacterial resistance to antibiotics and biocides, explore the possibility of a link between the two, and consider whether biocides can select for antibiotic resistance.

Mechanisms of Bacterial Resistance to Antibiotics

Bacterial resistance to antibiotics is generally considered under two categories, intrinsic and acquired (Russell & Chopra 1996). Intrinsic resistance (intrinsic susceptibility; Courvalin 1996) is a nat-

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ural or innate property of a bacterial cell and usually results from impermeability associated with outer cellular layers, for example the outer membrane in Gram-negative bacteria (Russell 1998). Acquired resistance, on the other hand, arises by mutation or by the acquisition of plasmids or transposons. In the clinical context, whilst the former conveys a natural lack of susceptibility to an antibiotic, it is the latter that causes more concern.

The biochemical mechanisms of acquired resistance have been widely studied and have been categorized as follows: alteration of antibiotic; change in target site; decreased antibiotic accumulation; duplication of target site; overproduction of target; and absence of an enzyme-metabolic pathway (Russell & Chopra 1996; Russell 1998). Examples of these mechanisms are provided in Table 1. Resistance is not necessarily the result of a single mechanism but can arise from combined expression of more than one mechanism.

Some of these are worthy of further comment in so far as the general aspects apply also to biocides. Inactivation of many β-lactam antibiotics is a much-studied phenomenon; β-lactamases may be chromosomally-mediated or plasmid-encoded (Table 1). Decreased accumulation via impaired uptake or enhanced efflux has been found with several antibiotics (Chopra 1992; Levy 1992; Nikaido 1994; George 1996) and is being increasingly studied with biocides also, as described below.

Table 1. General categorization of acquired antibiotic resistance mechanisms.

Acquired resistance mechanism	Examples of antibiotics
Inactivation/modification of antibiotic	β-Lactams, aminoglycoside-aminocyclitol antibiotics, chloramphenicol
Insensitive target site	β-Lactams, aminoglycoside-aminocyclitol antibiotics, glycopeptides, macrolides, tetracyclines, quinolones
Decreased antibiotic accumulation	
Impaired uptake	β-Lactams, fusidic acid
Enhanced efflux	Tetracyclines, chloramphenicol, macrolides, quinolones
By-pass of antibiotic-sensitive step	Methicillin, sulphonamides, trimethoprim, mupirocin
Overproduction of target	Trimethoprim
Absence of enzyme-metabolic pathway	Isoniazid (in mycobacteria)

Based on Russell & Chopra (1996) and Russell (1998).

Mechanisms of Bacterial Resistance to Biocides

As with antibiotics, bacterial resistance to biocides can be considered as being either intrinsic or acquired (Table 2). Intrinsic resistance is usually manifested by a decreased uptake, as found with bacterial spores, mycobacteria and various types of gram-negative bacilli, notably *Pseudomonas aeruginosa*, *Proteus* spp. and *Providencia stuartii* (Russell & Russell 1995; Russell & Chopra 1996; Russell et al 1997; McDonnell & Russell 1999; Stickler & King 1999). It has also been proposed that chlorhexidine-resistant bacteria can degrade the bisbiguanide (Ogase et al 1992). Physiological (phenotypic) adaptation can modulate the intrinsic insusceptibility of cells to biocides, e.g. of sessile cells contained within a biofilm (Costerton et al 1987; Brown & Gilbert 1993; Brown et al 1995; Westland & Stewart 1996).

Acquired resistance to biocides (Table 2), involving outer cell changes, may occur as a result of mutation or adaptation (Prince et al 1978; Gandhi et al 1993; Nicoletti et al 1993; Russell et al 1998; Tattawasart et al 1999). Plasmid-mediated changes in the outer membrane of Gram-negative bacteria

Table 2. Mechanisms of resistance to biocides.

Resistance mechanism	Examples of biocides
Intrinsic insusceptibility	
impermeability	Bacterial spores: quaternary ammonium compounds, chlorhexidine salts, phenolics, mercurials, alcohols Gram-negative bacilli: quaternary ammonium compounds, diamidines Biofilms: quaternary ammonium compounds, chlorhexidine salts, iodine
inactivation	Chlorhexidine salts?
Acquired resistance	
mutation	Gram-negative bacteria: quaternary ammonium compounds, chlorhexidine salts, triclosan
plasmid-encoded outer membrane changes	Gram-negative bacteria: quaternary ammonium compounds
efflux	Antibiotic-resistant staphylococci: quaternary ammonium compounds, chlorhexidine salts, diamidines, acridines
enzymatic	*Serratia marcescens*: formaldehyde Gram-positive/Gram-negative: inorganic and organic mercury compounds

Table 3. Examples of efflux as a resistance mechanism to antibiotics and biocides.

Efflux system	Resistance expressed to	Comment
NorA	4-Quinolones, chloramphenicol, cationic dyes	Plasmid-mediated
TetK	Tetracyclines	Plasmid-mediated
qac	Cationic biocides	Common ancestry with tetracycline- and sugar-transport processes
Acr[a]	Hydrophobic antibiotics, dyes, detergents	Chromosomally-mediated
robA	Multiple antibiotics and heavy metals (including Ag^+)	Chromosomally-mediated
soxRS	Several antibiotics and superoxide-generating agents	Chromosomally-mediated
Mar	Tetracyclines, chloramphenicol, 4-quinolones; some biocides?	Chromosomally-mediated

[a]Mutation at an *acr* locus confirms hypersensitivity.

can reduce sensitivity to quaternary ammonium compounds (Roussow & Rowbury 1984). Inorganic and organic mercury compounds may be detoxified by bacterial enzymes (hydrolases, reductases; Silver et al 1989; Nies & Silver 1995).

An increasingly important mechanism associated with bacterial resistance to biocides is efflux (Table 3; Russell 1997, 1998, 1999a; Day & Russell 1999) which is responsible for low-level resistance to cationic biocides in antibiotic-resistant cocci and to dyes, detergents and heavy metals (George 1996) in Gram-negative bacteria.

Possible Link between Antibiotic and Biocide Resistance

Certain similarities appear to exist between antibiotic resistance on the one hand and biocide resistance on the other (Table 4). It is now pertinent to consider this possible relationship by examining the following aspects.

Susceptibility to biocides of antibiotic-resistant bacteria

In-use concentrations of antiseptics and disinfectants are well described (Rutala 1996). It has been shown by several laboratories that antibiotic-resistant staphylococci, enterococci and other hospital pathogens are not more resistant to biocides than are antibiotic-sensitive strains (Alqurashi et al 1996; Anderson et al 1997; Haines et al 1997; Rutala et al 1997). Thus, on the evidence prevented in these studies no relationship could be found between antibiotic and biocide resistance and Rutala (1996) has pointed out that the United States Centers for Disease Control does not recommend any special strategies for biocides being used against antibiotic-resistant bacteria.

Table 4. Possible link between antibiotic and biocide resistance.

Aspect	Antibiotic resistance
Isolation of chlorhexidine/quaternary ammonium compound-resistant Gram-negative bacteria	Multiple
Training of Gram-negative bacteria to chlorhexidine or quaternary ammonium compound resistance	Possible resistance to antibiotics
qac genes on plasmids	Multiple

Plasmid-mediated resistance to antibiotics and biocides

Plasmid-mediated resistance, involving different mechanisms, has been described for several antibiotics (Russell & Chopra 1996). Such resistance is also known for some types of biocides, e.g. mercurials (as pointed out above), and formaldehyde resistance in *E. coli* may be plasmid-linked (Kummerle et al 1996). Plasmid-mediated resistance to cationic biocides such as chlorhexidine, quaternary ammonium compounds, diamidines and acridines has been found in staphylococci (Lyon & Skurray 1987; Behr et al 1994; Heir et al 1995, 1999; Paulsen et al 1996; Russell 1997, 1999b; Sundheim et al 1998; Day & Russell 1999).

Resistance to biocides in *S. aureus* is encoded by several multidrug resistance determinants, *qacA–G*, of which the *qacA/B* gene family is probably the most important. These encode proton-dependent export proteins and demonstrate significant homology to other energy-dependent transporters, for example the tetracycline exporters found in tetracycline resistant bacteria (Rouche et al 1990). Behr et al (1994) used the polymerase chain reaction (PCR) to detect the *qacA* gene encoding anti-

septic resistance in a large number of clinical *S. aureus* isolates; the gene was not found in sensitive strains.

Coagulase-negative staphylococci (e.g. *S. epidermidis*) contain either or both *qacA* or *qacC* genes, encoding low-level resistance to intercalating agents, quaternary ammonium compounds, chlorhexidine, diamidines and to ethidium bromide and some quaternary ammonium compounds, respectively (Leelaporn et al 1994). A selective advantage may be obtained by possessing both genes rather than *qacA* only. These coagulase-negative staphylococci strains and *S. aureus* may share a common pool of resistance determinants.

There is evidence to show that resistance to cationic biocides in antibiotic-resistant *S. aureus* strains arises by efflux (Paulsen et al 1996).

It is appropriate to explore further the relationship between biocide and antibiotic resistance in *S. aureus*. The term 'nucleic acid-binding' (NAB) compound has been coined to describe cationic biocides that bind strongly to DNA (Townsend et al 1984). Methicillin- and gentamicin-resistant *S. aureus* (MGRSA) strains without NAB plasmids have been shown to be more sensitive to chlorhexidine than methicillin-sensitive *S. aureus* (MSSA) strains, whereas methicillin-resistant *S. aureus* (NMSA) isolates with GNAB plasmids that confer resistance to NAB compounds and to gentamicin were more resistant to chlorhexidine (Cookson et al 1991a). Curing of GNAB plasmids produced a fall in the minimum inhibitory concentration (MIC), but not a consistent decrease in the lethal activity, of chlorhexidine from which Cookson et al (1991a) wondered whether chlorhexidine resistance in MRSA was of significance or merely an increased MIC value. It is of considerable relevance to point out that increased resistance to chlorhexidine, quaternary ammonium compounds and other cationic biocides is low-level in nature with an increase of some 2–8 fold in MIC values. The elevated MIC values are considerably below the concentrations of these agents used in practice. Nevertheless, the presence of *qac* genes might confer enhanced survival on those strains possessing such genes (Leelaporn et al 1994), a point that will be re-examined below.

Transferable resistance to triclosan has also been demonstrated in MRSA strains, but is again only low-level (Cookson et al 1991b).

Multidrug resistance (MDR) in Gram-negative bacteria

MDR, a term used to describe a resistance mechanism by genes that comprise part of the normal cell genome, is quite distinct from plasmid-mediated resistance. The genes are activated by induction or mutation caused by some types of stress. Exposure to a drug leads to resistance not only to that drug but also to chemically unrelated drugs, e.g. chromosomal multiple-antibiotic-resistant (Mar) mutants of *E. coli* selected on agar containing low concentrations of tetracycline or chloramphenicol are much less sensitive than wild-type cells to fluoroquinolones; further, the frequency of emergence is at least 1000-times higher than with norfloxacin (Cohen et al 1989). Antibiotic inactivation does not occur and efflux by membrane transporters comprises the resistance mechanism.

Other MDR systems present in Enterobacteriaclae include: the Acr system in which mutations at an acr locus render *E. coli* more sensitive to hydrophobic antibiotics, dyes and detergents; the *robA* gene in which over-expression of the RobA protein confers multiple antibiotic and heavy metal (including Ag^{+}) resistance; the *E. coli* multidrug resistance (*emr*) locus in *E. coli* that specifies a transporter system induced by several drugs; and the *soxRS* regulon in *E. coli* induced in response to oxidative stress (George 1996; Miller & Sulavik 1996).

Plasmids, genes and enhanced survival

The presence of a specific resistance mechanism may contribute to the long-term selection of antibiotic-resistant mutants under in-vivo conditions despite large differences in MIC values and the expected drug concentration at the site of infection (Bacquero et al 1991). Rouche et al (1990) believed that the *qacA* determinant evolved well before the widespread use of antiseptics and disinfectants and that its presence in *S. aureus* cells might aid their survival in the hospital environment. It has, in fact, been proposed that the prevalence of multidrug export *qac* genes in *S. aureus* and *S. epidermidis* is a consequence of the selective pressure exerted by the use of antiseptics and disinfectants (Leelaporn et al 1994), although Bacquero et al (1991) could find no association between chlorhexidine usage in a variety of circumstances in the hospital environment and chlorhexidine resistance.

Selection by biocides of antibiotic-resistant bacteria

Strains of *Ps. aeruginosa*, *Prov. stuartii* and *Proteus* spp. isolated from urinary tract infections in paraplegic patients have been shown to be resistant not only to quaternary ammonium compounds and

chlorhexidine but also to several chemically-unrelated antibiotics (Stickler et al 1983). It was suggested that the widespread usage of these cationic biocides could select for the presence of antibiotic-resistant strains, although no plasmid link was observed.

The disinfectant pine oil has been found (Moken et al 1997) to select Mar mutants in *E. coli* that showed low-level resistance to several antibiotics. These mutants over-expressed the *marA* gene. Deletion of the *mar* or *acrAB* locus (which encodes a protonmotive force-dependent efflux pump) rendered the cells sensitive to pine oil. Deletion of *acrAB*, but not of *mar*, increased susceptibility to a quaternary ammonium compound and to chloroxylenol. These findings clearly suggest that a disinfectant, pine oil, can select for chromosomal low-level antibiotic resistance, although the concentration of pine oil was only one-tenth of that employed in practice.

Several interesting studies have recently been undertaken on the bisphenol, triclosan (Heath et al 1998, 1999; McDonnell & Pretzer 1998; McMurry et al 1998a, b; Schweizer 1998; Levy et al 1999; Stewart et al 1999). Low concentrations of triclosan inhibit an enoyl-reductase involved in fatty acid synthesis in sensitive but not in triclosan-resistant mutants of *E. coli* (McMurry et al 1998a, b) from which it was proposed that triclosan had a single target site in *E. coli*. This thus suggests that resistance arises by a single mutation at this site. However, it is clear from other studies that additional effects are needed to produce a bactericidal effect (Heath et al 1998; McDonnell & Pretzer 1998). As pointed out earlier, chromosomally-encoded efflux systems, e.g. *marRAB*, *acrAB*, *soxRS*, play an important role in conferring resistance in Gram-negative bacteria. McMurry et al (1998b) proposed that a small increase in resistance of *E. coli* to triclosan could select for antibiotic resistance, with the mechanism involving drug efflux. Again, however, the concentrations of triclosan involved in this study were low and do not approach those much higher levels used in clinical practice.

This is analogous to the *qacA/B* gene systems in *S. aureus*, where it has been proposed (Paulsen et al 1996, 1998) that the presence of a *qac* gene in staphylococci results in selection for antibiotic-resistant bacteria, that the widespread introduction of chlorhexidine into clinical practice has been responsible for the selection of strains containing *qacA* that show multiple antibiotic resistance, that a quaternary ammonium compound, benzalkonium chloride, can induce the expression of both *qacA* and *qacB*, and that the chronological emergence of these genes on multiresistance plasmids in clinical isolates of *S. aureus* has followed the introduction of cationic biocides into clinical practice.

A key aspect of all of these studies has been biocide concentration. Low-level increases in MIC values (ca 2–8-times) are considerably below the concentrations of biocides used clinically as antiseptics and disinfectants (Russell et al 1999). Furthermore, stepwise development of resistance to chlorhexidine in antibiotic-sensitive and -resistant *S. aureus* strains is very difficult to achieve; only a slight increase in chlorhexidine resistance arises, which is unstable (Suller & Russell 1999). Chlorhexidine-resistant mutants of *S. aureus* have not been produced by exposure to high concentrations of chlorhexidine (Fitzgerald et al 1992; Suller & Russell 1999).

Conclusions

Resistance to antiseptics and disinfectants varies widely in non-sporulating bacteria. Generally, Gram-negative bacilli are more resistant than Gram-positive cocci by virtue of a permeability (outer membrane barrier) in the former. However, low-level resistance to cationic biocides has been demonstrated in *S. aureus* and to triclosan in *E. coli*. It has been proposed that the widespread usage of these antimicrobial agents has contributed to the selection of antibiotic-resistance strains in hospitals with a potential for the same problem to arise in domiciliary environments, when triclosan is used (Levy et al 1999). It has recently been found (McMurry et al 1999) that InhA of *M. smegmatis* is a target not only for an important chemotherapeutic drug, isoniazid, but also for triclosan. This is undoubtedly a matter for concern.

However, several questions remain to be answered satisfactorily before any conclusions can be unequivocally reached.

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