Development and horizontal gene transfer of triclosan resistance in *Staphylococcus aureus*

Thesis presented for the Degree of Philosophiae Doctor

by

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If we knew what it was we were doing, it would not be called research, would it?

Albert Einstein

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Scientific Publications and Presentations

Parts of this work have been published in scientific journals and presented orally as detailed below:

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Seaman, PF & Day MJ. (2007) Isolation and characterization of a bacteriophage with an unusually large genome from the Great Salt Plains National Wildlife Refuge, Oklahoma, USA. *FEMS Microbiol Ecol.* 60, 1-13.

Summary

Staphylococcus aureus is a major cause of hospital-acquired infections that are becoming increasingly difficult to treat because of the organism's ability to acquire resistance to current antimicrobial agents. Particular attention has been focussed on the evolution of methicillin-resistant *S. aureus* (MRSA) – strains of *S. aureus* that are, in some cases, resistant to almost all known antibiotic classes. One method used to control the spread of MRSA has been the use of topical washes that include triclosan, a potent antimicrobial with particular activity against Gram-positive organisms. Triclosan has traditionally been classed as a biocide and is also used in a broad spectrum of consumer healthcare products, including toothpastes and deodorants. It has also been used to prevent bacterial growth through incorporation into plastics used during food preparation or sutures used to close wounds following surgery. However, in 1991 resistance to triclosan was reported and was described to transfer in association with mupirocin resistance. This was followed by reports that resistance was present in 7.5% of *S. aureus* isolates and that MRSA was less susceptible to triclosan than methicillin-sensitive *S. aureus* (MSSA). It later emerged that, contrary to previous thinking, triclosan targets a specific bacterial protein, FabI. We aimed to characterize the development of reduced susceptibility to triclosan in MSSA and MRSA and to identify whether triclosan does have a single, specific target. We also set out to elucidate the potential for triclosan resistance to be disseminated by horizontal gene transfer (HGT). By using extensive microbiological and genetic techniques we found that *S. aureus* can evolve reduced susceptibility to triclosan through spontaneous mutation. MICs of 1-4 mg/L were achieved by a C284T mutation of *fabI*, compared to wild-type MICs of ~0.03 mg/L. However, reduced susceptibility was also observed in non-*fabI* mutants, implying that other mechanisms of resistance are available (and that triclosan has targets other than FabI). We have shown that triclosan induces the leakage of potassium ions from cells, an indication that triclosan targets the cytoplasmic membrane. However, whilst reduced susceptibility to triclosan did confer reduced susceptibility to the lethal effects of 7.5 mg/L triclosan, this effect was ameliorated by higher concentrations of triclosan. Indeed, in-use concentrations of the commercial preparation of triclosan, Irgacide LP10, are equally active against reduced susceptibility *S. aureus* and wild-type. Therefore, the evolution of reduced-susceptibility to triclosan is of ambiguous clinical significance. We found that spontaneous mutation to reduced susceptibility was not associated with a significant fitness cost, augmenting its potential for emergence in nature. Evolution of reduced susceptibility did not confer co-resistance to other antimicrobials and MRSA and MSSA strains were equally susceptible. An assessment of commensal *S. aureus* carried amongst the student population of Cardiff revealed that reduced susceptibility to triclosan is rare in this population. However, coagulase-negative staphylococci (CoNS) showed consistently higher MICs for triclosan and may represent an amenable reservoir of resistance. There was no indication that mupirocin and triclosan resistance have co-transferred in the past. Indeed, there appeared to be no relationship between resistance to either of these compounds in *S. aureus*. Reduced susceptibility to triclosan could not be disseminated amongst *S. aureus*, or related Gram-positive bacteria by transduction, conjugation or transformation. Importantly, during the course of this work we discovered that triclosan could select for *S. aureus* small-colony variants (SCVs) that were coincidently resistant to gentamicin and penicillin. These were slow growing and illustrated the typical SCV phenotype. SCVs were more readily transformable than wild-type cells and may represent an enduring reservoir of resistance determinants.

In conclusion, we found that *S. aureus* could develop reduced susceptibility to triclosan by spontaneous mutation or the evolution of SCVs. However, the level of resistance is of ambiguous significance. We propose that triclosan does target FabI, but also has other cellular targets, particularly at higher concentrations. Whilst the evolution of reduced susceptibility and the occurrence of SCVs should be monitored these should not preclude the use of triclosan as part of infection control procedures. However, to reduce the opportunities for resistance, infection control procedures should not rely upon a single antimicrobial to provide the panacea for nosocomial infections.

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List of Abbreviations

ACP	acyl carrier protein
AER	alternative excision repair
AIP	autoinducing peptide
Amp	ampicillin
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BA	blood agar
BER	base-excision repair
BHIA	brain-heart infusion agar
BHIB	brain-heart infusion broth
bhr	broad host range
BrAA	brucella agar albimi
BSAC	British Society for Antimicrobial Chemotherapy
BZK	benzalkonium chloride
CA-MRSA	community-associated methicillin-resistant *Staphylococcus aureus*
Cec	cefaclor
CFU	colony forming units
Chl	chloramphenicol
chlR	chloramphenicol resistant
CHX	chlorhexidine diacetate
CIP	ciprofloaxcin
CoNS	coagulase-negative staphylococci
CPC	cetylpyridinium chloride
CRA	chlorine-releasing agent
Cro	ceftriaxone
CTAB	cetyltrimethylammonium bromide
DMSO	dimethlysulfoxide
DNase	deoxyribonuclease
EMRSA	epidemic methicillin-resistant *S. aureus*
Ery	erythromycin

ETC	electron transport chain
Fd	fusidic acid
Gen	gentamicin
genR	gentamicin resistant
GISA	glycopeptide-intermediate *S. aureus*
HGT	horizontal gene transfer
HGT-DB	Horizontal Gene Transfer Database
HL-mupR	high-level mupirocin resistance
IleS	isoleucyl tRNA synthetase
IS	insertion sequence
ISA	Iso-Sensitest agar
ISB	Iso-Sensitest broth
kb	kilobase
LZD	linezolid
M	molar
MACA	microassay culture agar
mg/L	milligrams per litre
MGE	mobile genetic element
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
MLST	multi-locus sequence typing
MMR	methyl-directed mismatch repair
MRSA	methicillin-resistant *S. aureus*
MSSA	methicillin-sensitive *S. aureus*
Mup	mupirocin
mupR	mupirocin resistant
MupRSA	mupirocin-resistant *S. aureus*
MupSSA	mupirocin-sensitive *S. aureus*
NA	nutrient agar
NaClO	sodium hypochlorite
NaDCC	sodium dichlorocyanurate
NB	nutrient broth

NCTC	National Collection of Type Cultures
NER	nucleotide-excision repair
nhr	narrow host range
NNIS	National Nosocomial Infections Surveillance System
Oxa	oxacillin
PBP	penicillin binding protein
PBS	phosphate buffered saline
PCMX	parachlorometaxylene
PCR	polymerase chain reaction
Pen	penicillin
PFU	plaque forming units
phage	bacteriophage
PHLS	Public Health Laboratory Service
PHMB	polyhexamethylenebiguanide
QAC	quaternary ammonium compound
Rif	rifampicin
rif^R	rifampicin resistant
SaPI	*S. aureus* pathogenicity island
SCC*mec*	staphylococcal chromosome cassette *mec*
SCV	small colony variant
SD	standard deviation
SDGH	Singleton District General Hospital
SE	standard error of the mean
SEM	scanning electron microscopy
SMA	Sabouraud maltose agar
Tec	teicoplanin
TEM	transmission electron microscopy
Tet	tetracycline
$tric^r$	triclosan resistant
$tric^s$	triclosan sensitive
UHW	University Hospital of Wales
UV	ultra violet

Van	vancomycin
VISA	vancomycin-intermediate *S. aureus*
VJA	Vogel-Johnson agar
VRE	vancomycin-resistant enterococci
w/v	weight/volume
WCA	Wilkins-Chalgren-Bouillon agar
YA	Youman's agar
$\Delta\Psi$	transmembrane potential

Chapter 1

Introduction

1.0 Introduction

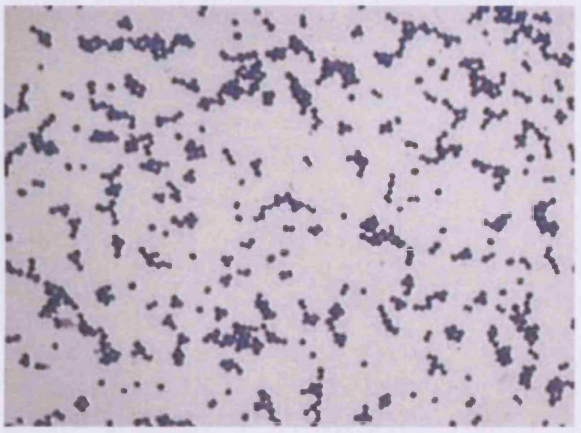
Staphylococcus aureus is the leading cause of nosocomial infection in the United Kingdom (Griffiths *et al.*, 2004) and United States (Pfaller *et al.*, 1998). Infection with *S. aureus* is associated with substantial morbidity and mortality – a trend that is increasing due to widespread dissemination of methicillin-resistant *S. aureus* (MRSA) (NNIS, 2004). The introduction and widespread use of antibiotics to treat these infections has led to increased resistance among bacteria, particularly in the clinical setting (Livermore, 2003). Recently antimicrobials other than antibiotics have also been implicated in bacterial resistance (Russell, 2002b). Such antimicrobials include disinfectants and antiseptics which, along with preservatives, are known as biocides. Biocides are an important group of antimicrobials which are used extensively throughout both the clinical and community settings. Consequently, any development of resistance is of great importance to healthcare. *S. aureus* has proved adept at developing resistance to antimicrobials, and it is the evolution of biocide resistance in this organism that this report will concentrate on.

1.1 Phylogeny, structure and function of the staphylococci

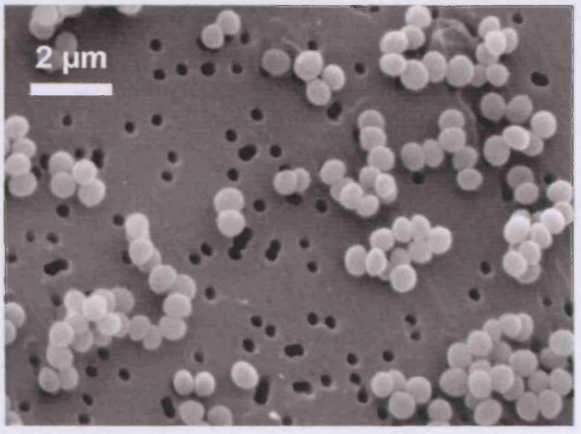
The staphylococci are Gram-positive coccoid cells that divide in two planes, resulting in characteristic 'grape-like' clusters when stained and viewed by microscopy (Figure 1.1). Pasteur and Koch were the first to observe and culture staphylococci, but the earliest detailed studies on staphylococci were performed by Alexander Ogston in 1881 and Rosenbach in 1884 (Ogston, 1882; Cookson *et al.*, 2003). Ogston coined the name *Staphylococcus* to describe grape-like clusters of bacteria he observed in pus from human abscesses and by 1884 a scientist named Rosenbach was able to isolate and grow these microorganisms in pure culture. He described two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (golden) and *Staphylococcus albus* (white) (Kloos & Schleifer, 1986). The latter species is now known as *Staphylococcus epidermidis.* Although nineteen species of *Staphylococcus* are described in Bergey's Manual, (Holt *et al.*, 1994) only *S. aureus* and *S. epidermidis* have been thought of as significant in their interactions with humans, but this appears to be changing with other coagulase-negative staphylococci (CoNS) species becoming increasingly prominent (Tan *et al.*, 2006).

Figure 1.1. The coccoid cells of *S. aureus* divide in two planes causing the cells to form clusters resembling grapes. (a) Light microscopy can resolve this characteristic feature and is a simple way to distinguish staphylococcal cells from those of streptococci, which divide in a single plane and as a result form chains of cells. (b) Scanning and (c) transmission electron microscopy provide higher resolution images of *S. aureus* cells, enabling visualization of the polysaccharide capsule, cell wall and intracellular moieties.

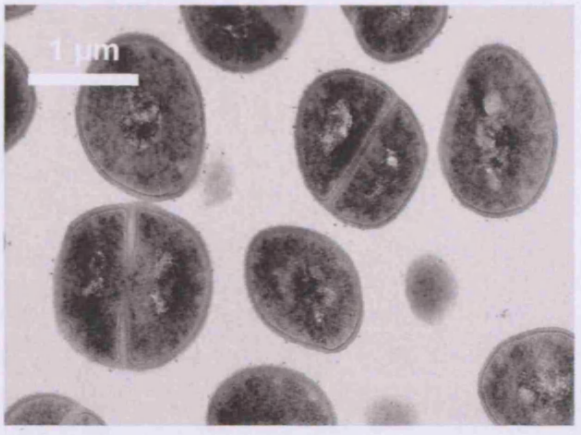
(a)



(b)



(c)



Humans are a natural reservoir of *S. aureus*. Although figures vary, about ten to twenty percent of the healthy adult population are intermittent carriers and twenty five to forty percent are persistently colonized (VandenBergh *et al.*, 1999; Bishop *et al.*, 2006). *S. aureus* colonizes mainly the moist squamous epithelium of the anterior nares (Lowy, 1998; Massey *et al.*, 2006) and throat (Nilsson & Ripa, 2006), but it may be found in most other anatomical locales (Cookson *et al.*, 2003). *S. epidermidis* is a ubiquitous colonizer of human and animal skin (Tan *et al.*, 2006). Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are also catalase-positive and oxidase-negative. *S. aureus* can grow at temperatures ranging from 15 to 45°C and are characteristically tolerant of salt, being able to grow when exposed to sodium chloride concentrations as high as 15% (w/v). Nearly all strains of *S. aureus* produce the enzyme coagulase, hence the presence of extracellular coagulase is a commonly used diagnostic test for the differentiation of *S. aureus* from other, non-coagulase producing, staphylococci.

The differentiation of *S. aureus* from CoNS is important in the clinic, since *S. aureus* is substantially more pathogenic (Gill *et al.*, 2005b). Indeed coagulase is itself a virulence factor; it is an adhesin that reacts with prothrombin in the blood. The resulting complex is called staphylothrombin, which helps blood to clot by converting fibrinogen to fibrin (Kawabata *et al.*, 1985). Some coagulase is tightly bound to the surface of the bacteria and so *S. aureus* coats itself with fibrin upon contact with blood. Fibrin-coated staphylococci resist phagocytosis making the bacteria more virulent (Lowy, 1998).

CoNS include *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. lugdunensis* and a number of other species. Most are normal skin commensals and all are much less pathogenic than *S. aureus*. However, they have recently gained a role as pathogens as advances in other fields of medicine have provided an increasing supply of debilitated patients, highly prone to infection (von Eiff *et al.*, 2002). CoNS are important causes of line- and device-associated infections in the immunosuppressed and account for about 7–9% of bacteraemias reported to the Public Health Laboratory Service (Reacher *et al.*, 2000). They are important also as causes of prosthetic valve endocarditis (Livermore, 2001) and are the major causative microorganisms in neonatal nosocomial sepsis (Krediet *et al.*, 2004).

Taxonomically, the genus *Staphylococcus* is in the bacterial family *Bacillaceae,* although the staphylococci are distantly related phylogenetically to other genera in the family. A variety of genetic criteria specify that the genus *Staphylococcus* forms a coherent and well-defined natural group that is broadly divergent from others within the family. On the basis of 16s RNA analysis, the genus *Staphylococcus* belongs to the broad *Bacillus-Lactobacillus-Streptococcus* cluster. The closest relatives of staphylococci are the enterococci and bacilli (Cole *et al.*, 2003).

1.1.1 Genomic and genetic background of *S. aureus.*

Phylogenetic classification indicated that over 50% of predicted proteins encoded by the *S. aureus* genome are most similar to those in *Bacillus subtilis* and *Bacillus halodurans* (Kuroda *et al.*, 2001). These typically contain house-keeping genes that perform essential functions such as absorption of nutrients from the environment, synthesis of metabolic intermediates and bacterial multiplication. A Basic Local Alignment Search Tool (BLAST) homology search for the most closely related orthologues indicates that the genetic background of *S. aureus* has been vertically transmitted from a common ancestor that subsequently diverged to *Bacillus* and *Staphylococcus* species. With this perspective, it can be predicted that the corresponding domain of the chromosome will also be well conserved in other staphylococcal species, such as *S. epidermidis* (Ito *et al.*, 2003).

Whole genome sequences are now available for nine *S. aureus* strains and four CoNS strains (Table 1.1). The genomes of the MRSA strains have low G+C content (32.8–32.9%). Three sequenced MRSA strains possess high nucleotide sequence identities (Table 1.2). Table 1.3 displays some general features of seven MRSA genomes, including mobile genetic elements (MGE). Indeed, the majority of the nucleotide sequence disparity is due to the insertion of DNA regions that consist of putative exogenous genes. These regions are judged to be exogenous due to the absence of homologues in different *S. aureus* strains. Further nucleotide sequence differences are reported as a consequence of different preferences in codon usage and different G+C content within the ORFs (Kuroda *et al.*, 2001). This points towards a belief that the *S. aureus* chromosome is composed of two categories of DNA: background DNA,

Table 1.1. Description of staphylococcal genomes that have been sequenced to date.

Strain	Refseq	Genome		Strain features	Reference
		size (bp)	G+C (%)		
S. aureus RF122	NC_007622	2742531	32	Associated with bovine mastitis	(Herron *et al.*, 2002)
S. aureus COL	NC_002951	2809422	32	Early methicillin-resistant isolate	(Gill *et al.*, 2005a)
S. aureus MRSA252	NC_002952	2902619	32	Epidemic MRSA-16 clone	(Holden *et al.*, 2004)
S. aureus MSSA476	NC_002953	2799802	32	Invasive MSSA clone	(Holden *et al.*, 2004)
S. aureus MW2	NC_003923	2820462	32	Community-acquired MRSA strain isolated in mid-west USA	(Baba *et al.*, 2002)
S. aureus Mu50	NC_002758	2878529	32	Nosocomial MRSA strain, also shows intermediate vancomycin resistance	(Kuroda *et al.*, 2001)
S. aureus N315	NC_002745	2814816	32	Nosocomial MRSA	(Kuroda *et al.*, 2001)
S. aureus NCTC 8325	NC_007795	2821361	32	Typical laboratory strain	(Gillaspy *et al.*, 2006)
S. aureus USA300 FPR3757	NC_007793	2872769	32	Epidemic clone of community-acquired MRSA	(Diep *et al.*, 2006)
S. epidermidis ATCC 12228	NC_004461	2499279	32	Non-biofilm forming, non-infection associated strain	(Zhang *et al.*, 2003)
S. epidermidis RP62A	NC_002976	2616530	32	Biofilm-producing methicillin-resistant *S. epidermidis*	(Gill *et al.*, 2005a)
S. haemolyticus JCSC1435	NC_007168	2685015	32	Opportunistic pathogen with a highly antibiotic-resistant phenotype	(Takeuchi *et al.*, 2005)
S. saprophyticus ATCC 15305	NC_007350	2516575	33	Uropathogenic CoNS	(Kuroda *et al.*, 2005)

Table 1.2. Nucleotide sequence homology between genome sequenced strains of MRSA. Data taken from Baba *et al.*, (2002).

Strain	Mu50	N315
Mu50	-	-
N315	99.7%	-
MW2	94.7%	94.8%

Table 1.3. General features of seven staphylococcal genomes. Data collected from (Baba *et al.*, 2002; Gill *et al.*, 2005a; Diep *et al.*, 2006).

Feature	*S. aureus*					*S. epidermidis*	
	MW2	N315	Mu50	COL	MRSA300	RP62A	ATCC12228
Chromosome							
Genome size (bp)	2820462	2814816	2878040	280942	2872769	2616530	2499279
G+C content (%)	32.8	32.8	32.9	32.8	32.8	32.1	32.1
Open reading frames	2849	2797	3028	2721	2560	2553	2381
Percentage coding (%)	83.5	83.4	83.8	82	81	82	83
Ribosomal RNAs							
16S	6	5	5	6	5	6	6
23S	6	5	5	6	5	6	6
5S	7	6	6	7	6	7	7
Insertion Sequences							
IS*1181*	0	8	10	2	-	0	0
IS*1181* (degenerate)	0	0	0	1	-	0	0
IS*431* (on SCC*mec*)	1	1	1	1	-	1	0
IS*431*	0	1	1	0	-	4	3
IS*431* (degenerate)	0	0	0	0	-	5	0
IS*200*	1	0	0	1	-	2	3
IS*200* (degenerate)	0	0	0	1	-	1	1
IS*256*	0	0	0	0	-	5	0
IS*3*	0	0	0	1	-	0	0
ISep*1*	0	0	0	0	-	11	15
ISep*1* (degenerate)	0	0	0	0	-	2	0
IS*1272* (on SCC*mec*)	1	0	0	1	-	0	0
IS*1272*	1	0	0	0	-	0	1
IS*1272*-related (degenerate)	6	5	3	9	-	26	34
Transposons							
Tn*554*	0	4	2	0	-	3	0
Tn*4001*	0	0	0	0	-	1	0
TN*5801* (conjugative)	1	0	1	0	-	0	0
Transposases	3	6	6	0	2	3	6
Degenerate transposases	1	6	9	1	-	5	0

Table 1.3. General features of seven staphylococcal genomes, continued.

Feature	*S. aureus*					*S. epidermidis*	
	MW2	N315	Mu50	COL	MRSA300	RP62A	ATCC12228
Genomic islands							
Prophage	2	1	2	1	2	1	0
SCC*mec* (type)	1 (IVa)	1 (II)	1 (II)	1 (I)	1 (IVa)	1 (II)	0
SSC*pbp4*	0	0	0	0	0	0	1
vSa islands	5	4	5	5	3	0	0
vSe islands	0	0	0	0	0	2	3
ACME (type)	0	0	0	0	1 (1)	0	0
Plasmids							
Length (bp)	20654	24653	25107	4440	3125-37136[b]	28080	4439-24370[a]
G+C content	28.3	28.7	28.9	30.0	-	32	28-35.6
Open reading frames	-	35	-	3	-	35	3-22
Percentage coding	75.6	78.0	80.9	-	-	-	-

[a] Contains 6 plasmids

[b] Contains 3 plasmids

transmitted from ancestral bacteria closely related to *Bacillus* species and MGE, which originated from other bacteria and were acquired by horizontal genetic transfer.

The paradigm of bacterial genomes is increasingly being viewed as having a core of essential genes (or housekeeping genes) that are accessorized with the acquisition or loss of dispensable genetic elements (Frost *et al.*, 2005). Whilst this is a truism it fails to take in to account the different evolutionary processes employed by bacterial species. Evolution is believed to involve the movement of MGE, homologous recombination and the gradual accumulation of point mutations. Whilst bacterial species utilize these, they tend to show bias. For example the divergence of *Escherichia coli* and *Salmonella enterica* has been driven predominantly by the acquisition of horizontally transferred genes (Lawrence & Ochman, 1998). Examination of *S. aureus* using multilocus sequence typing (MLST) shows that point mutations give rise to new alleles at least fifteen-fold more frequently than does recombination. This contrasts with the naturally transformable species *Neisseria meningitidis* and *Streptococcus pneumoniae*, in which alleles change between five- and ten-fold more frequently by recombination than by mutation (Feil *et al.*, 2003). However, phylogenetic analysis suggests that gene transfer does contribute toward the evolution of *S. aureus* over the long term (Feil *et al.*, 2001) and Robinson & Enright (2004) described how two large recombinatorial events founded two lineages of *S. aureus*. So we see that the evolution of this species is a combination of macro- and microevolution, providing both the constancy that is required for the preservation of essential functions and the plasticity that is necessary for rapid phenotypic changes and the consequent ability to exploitation of new niches.

The clinical significance and global spread of MRSA has resulted in the evolution and epidemiology of this organism being the subject of much scrutiny. Interestingly, MLST analysis, along with SCC*mec* typing, has established that there are relatively few major epidemic MRSA (EMRSA) clones (Enright *et al.*, 2002). Our present state of knowledge indicates that, rather than a single historical transfer of the genetic determinant for methicillin resistance (SCC*mec*) into *S. aureus* as suggested previously (Kreiswirth *et al.*, 1993), this event has occurred several times (Enright *et al.*, 2002). Furthermore, the emergence of major MRSA clones has come from the transfer of SCC*mec* into epidemic methicillin-sensitive *S. aureus* (MSSA) strains

(Enright *et al.*, 2002). This finding suggests the evolution of MRSA involved *S. aureus* strains that are well adapted to transmission within hospitals repeatedly receiving the *mec* determinant after the introduction of methicillin and then becoming the successful EMRSA clones within hospitals that we see today. As discussed in section 1.2.1, these same successful EMRSA clones are now proving their genetic resourcefulness by responding to the increasing use of vancomycin by becoming less susceptible to glycopeptides.

1.1.2 Colonization and infection with *S. aureus.*

S. aureus is a common coloniser of nasal passages and skin, and in this role the bacterium is commensal and exhibits no virulence. However, *S. aureus* is an opportunistic pathogen and can, in certain circumstances, initiate an infection. For this reason persons colonized with *S. aureus* are at an increased risk for subsequent infections (Wenzel & Perl, 1995). Rates of staphylococcal colonization are high among patients with type 1 diabetes (Tuazon *et al.*, 1975), patients undergoing haemodialysis (Kirmani *et al.*, 1978), surgical patients (Kluytmans *et al.*, 1995; Herwaldt *et al.*, 2004), intravenous drug users (Tuazon & Sheagren, 1974) and patients with acquired immunodeficiency syndrome (Weinke *et al.*, 1992).

Analysis of the transmission properties of staphylococcal species reveals a number of factors suggesting that *S. epidermidis* is more efficient at transferring between hosts than *S. aureus*. Firstly, there are no known host barriers preventing colonization by *S. epidermidis*; current research indicates that all humans are colonized by this organism whilst only a sub-population of humans are colonized by *S. aureus* (Kluytmans *et al.*, 1997). Secondly, the staphylococcal mode of transmission between healthy hosts is by direct contact (Bauer *et al.*, 1990). Since *S. epidermidis* resides on the skin, direct transfer between two hosts can be considered as a relatively simple process and is more likely to occur on a regular basis. In contrast, transmission of *S. aureus* requires the transfer of the microorganism from the nose to skin, subsequent transfer to the skin of a new host and, finally, colonization of the anterior nares of the new host. This extra degree of complexity is likely to make each transmission event less probable compared with the mechanism used by *S. epidermidis* (Massey *et al.*, 2006).

Studies on the phenomenon of *Agr* interference between genetically diverse *S. aureus* strains have revealed a third factor that contributes to *S. epidermidis* being more transmissible than *S. aureus*. *Agr* is a two-component signal-transduction system in staphylococci that uses a secreted octapeptide (autoinducing peptide; AIP) to communicate information on population density (Ji *et al.*, 1997). In *S. aureus* four different *Agr* groups have been identified and interference between the strains expressing these different *Agr* groups has been demonstrated (Ji *et al.*, 1997). Consequently, when a host is colonized by *S. aureus* of one *Agr* type, the competitive nature of *Agr* interference will inhibit the colonization of any newly transmitted strains with a different *Agr* type (Massey *et al.*, 2006). However, whilst studies on the *Agr* locus in *S. epidermidis* have revealed sequence diversity, no interference has been demonstrated between different *Agr* sequence types in this species (Dufour *et al.*, 2002; Lina *et al.*, 2003). So these three factors indicate that *S. epidermidis* is more readily transmissible than *S. aureus*. Interestingly, this has been argued as one reason for the greater virulence of *S. aureus*; virulence has evolved to overcome the complex transmission pathway (Massey *et al.*, 2006).

S. aureus can cause a wide variety of infections including cutaneous infections, e.g., furunculosis, impetigo and abscesses, organ infections, e.g., osteomyelitis, endocarditis and arthritis and the toxinoses such as food poisoning, bacteraemia, scalded skin syndrome and toxic shock syndrome. *S. aureus* is also commonly isolated from the sputum of cystic fibrosis patients (Besier *et al.*, 2007) and has a significant role in veterinary medicine, including the commercially important bovine mastitis (Taverna *et al.*, 2007). *S. aureus* is able to invoke the associated symptoms of infection by the production of a variety of extracellular proteins, some cell-bound, others secreted, which participate in the initiation and establishment of an infection and by these criteria are defined as virulence factors (Novick *et al.*, 2001).

1.1.2.1 Pathogenicity *S. aureus.*

S. aureus possesses many virulence factors which it is able to utilize to initiate an infection. *S. aureus* infections generally occur in those who are immunodeficient and have provided a means of entry into the body, i.e. a cut or wound (Lowy, 1998). It is

for this reason that *S. aureus* is highly prevalent within post-operative infections. Some of the diverse virulence factors of *S. aureus* are shown in Table 1.4.

S. aureus infections are complex and require intricate combinations of the factors shown in Table 1.4, hence no single virulence determinate will lead to pathogenicity. Some global regulatory genes that coordinate the expression of various groups of *S. aureus* virulence genes have been identified (Peng *et al.*, 1988; Dufour *et al.*, 2002; Novick, 2003b; Lindsay *et al.*, 2006). Of these the most extensively studied is *Agr*, which induces the expression of extracellular protein whilst suppressing the expression of surface protein. *Agr* and the other regulators appear to coordinate the pathogenesis of staphylococcal infections a sequential nature; surface proteins are predominantly synthesized during the exponential growth phase and secreted proteins during the stationary phase. So it seems that different stages of infection require different selections of virulence determinants, a hypothesis that is supported by evidence that the expression of surface proteins that bind extra-cellular-matrix molecules aids colonization of host tissues whereas subsequent synthesis of extracellular proteins favours the spread to adjacent tissues (Lowy, 1998).

Antimicrobial resistance is an important feature in *S. aureus*. Indeed, *S. aureus* has proven very successful at developing antimicrobial resistance and these antimicrobial-resistant strains are now causing considerable problems in both the clinical and community settings.

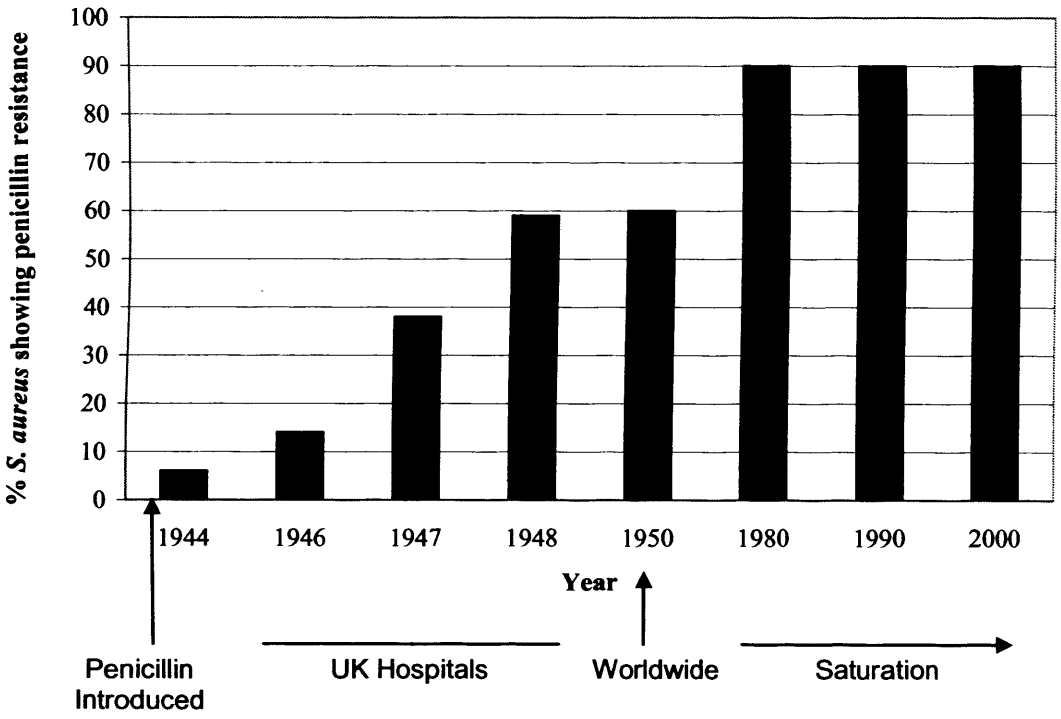
1.2 Antimicrobial resistance in *S. aureus*

Drug-resistant *S. aureus* strains have appeared following the introduction of new antimicrobials. At the time of introduction of penicillin for therapeutic use in the early 1940s less than 1% of *S. aureus* isolates showed resistance to the antibiotic. However, a few years later the incidence of penicillin resistance in *S. aureus* had increased such that by 1946 approximately 60% of UK hospital isolates were penicillin resistant (Barber & Rozwadowska-Dowzendo, 1948). Levels of penicillin-resistant *S. aureus* continued to rise, Figure 1.2, and owing to the high incidence of penicillin-resistance it was necessary to introduce other antibiotics to control the infections.

Table 1.4. Virulence factors of *S. aureus*. The genes encoding *S. aureus* pathogenicity are located in a variety of chromosomal locations, often associated with genomic islands and other mobile genetic elements.

Virulence Factor	Role	Examples
Adhesins	Surface proteins that promote attachment to host proteins such as laminin and fibronectin.	Coagulase (Moreillon *et al.*, 1995) and fibronectin-binding proteins (Menzies, 2003).
Invasins	Enable invasion by damage of host membranes.	α-toxin, β-toxin, δ-toxin, γ-toxin (Novick, 2003a) and leukocidin (Gillet *et al.*, 2002).
	Promote bacterial spread by dissolution of fibrin clots.	Staphylokinase (Collen, 1998)
	Enhance survival through nutrient provision.	Proteases, lipases, haemolysins and deoxyribonucleases (Urban *et al.*, 2006)
Avoidance of host defences	Prevention of opsonization and phagocytosis by immunological disguise.	Capsule polysaccharide (O'Riordan & Lee, 2004), protein A (Peterson *et al.*, 1977) and leukocidin.
	Enhance survival in phagocytes.	Carotenoids, catalase production.
Exotoxins	Disrupt host membranes or otherwise promote symptoms of disease.	Toxic shock syndrome toxin (TSST-1), enterotoxins A-M and other superantigen toxins (Novick *et al.*, 2001).
Intrinsic and acquired antimicrobial resistance	Prevent infection control by chemotherapeutics.	β-lactamses, penicillin binding protein 2a (PBP2a) (Matsuhashi *et al.*, 1986) or drug efflux (Kaatz *et al.*, 2005; Ojo *et al.*, 2006).

Figure 1.2. The accumulation of penicillin-resistance in *S. aureus*. Adapted from Livermore (2001).



Antibiotics such as streptomycin, tetracycline, chloramphenicol, and erythromycin were introduced but these were similarly followed by the emergence of resistant strains (Shanson, 1981). Throughout the 1950s the introduction of new antibiotics lead to the development of multi-drug resistant *S. aureus*. In 1959 the β-lactamase stable agent methicillin was made available for treatment of these multi-drug resistant strains, however by 1960 the first methicillin-resistant *S. aureus* was reported (Jevons, 1961; Jevons *et al.*, 1963). An increasing number of MRSA strains were subsequently isolated and by the mid 1980s MRSA had become common in many parts of the world (al-Masaudi *et al.*, 1991a). The mechanisms of resistance to several key antibiotics in *S. aureus* are shown in Table 1.5.

1.2.1 The Emergence of MRSA

After first appearing in the early 1960s, MRSA was able to spread to such an extent that by 1970-71 it accounted for nearly 10% of *S. aureus* at a major general hospital in Birmingham and for 15% of *S. aureus* from infective sources in Denmark (Livermore, 2001). Interestingly however, their prevalence fell to almost zero later that decade and into the early 1980s. This decline has been attributed to the combination of several factors; strain displacement, the use of gentamicin and better infection control (Ayliffe, 1997). Unfortunately, this decline did not continue indefinitely and gentamicin resistance began to emerge in *S. aureus*, including MRSA, in the late 1970s and early 1980s. Gentamicin-resistant MRSA became a problem by the mid-1980s and a strain designated epidemic MRSA 1 (EMRSA 1) achieved wide occurrence in the United Kingdom, the Irish Republic, and Australia (Ayliffe, 1997). EMRSA 1 was followed by further 'epidemic' MRSA strains, defined as those that had spread to two or more patients at two or more hospitals. Molecular epidemiological studies conducted in several countries since the late 1980s plainly indicate that a key factor for the huge geographic spread of MRSA is the dissemination of a relatively few highly epidemic clones (Crisóstomo *et al.*, 2001). In the late 1980s EMRSA 3 became prominent in the UK and was followed in the mid-1990s by EMRSA 15 and 16. These latter strains have achieved major spread and are often associated with severe infections. MRSA continues to increase in prevalence and cause severe clinical complications. Bacteraemia data for England and Wales demonstrate that the proportion of MRSA among *S. aureus* bacteraemia increased

Table 1.5. Mechanism of resistance to several key antibiotics and there associated genetic location in *S. aureus*. Adapted from al Masaudi *et al.* (1991a).

Antibiotic	Main resistance mechanism	Genetic location of resistance			Comments
		Chromosome	Plasmid	Transposon	
β-lactam (penicillins & cephalosporins)	Enzymatic hydrolysis of β-lactam ring.	MRSA	Common	Tn*552* Tn*4002* Tn*3852* Tn*4201*	Always linked with other antimicrobial determinants.
Methicillin	Production of new PBP (PBP2' or 2a) with reduced affinity to β-lactams.	Exclusively	Not found	Probably	MRSA always show resistance to other β-lactam antibiotics.
Mupirocin	Alteration to or acquisition of novel isoleucyl-tRNA synthetase (*MupA*)	Low-level resistance (common) high-level resistance (rare)	High-level resistance (common)		Chromosomal high-level resistance may be due to integration of a mupirocin resistance plasmid (Udo *et al.*, 2003).
Glycopeptides (vancomycin & teicoplanin)	Intermediate resistance is not fully understood, but thickened cell wall and decreased peptidoglycan cross-linking are implicated. High-level resistance is via alteration in cell wall precursors with low affinity for vancomycin/teicoplanin.	Probably	Yes	Tn*1546*	Resistance is still fairly rare. No universal genetic marker for intermediate glycopeptide resistance has been discovered. High-level resistance is likely to have come from vancomycin-resistant enterococci (Howe *et al.*, 1998; Smith *et al.*, 1999; McCallum *et al.*, 2006; Weigel *et al.*, 2007)
Streptomycin	Alterations in the structure of the ribosome. Sometimes resistance can be through enzymatic modification of streptomycin.	Common	Infrequently		
Erythromycin	Target modification by adenine methylation of domain V of the 23S rRNA conferred by *erm* gene.	Infrequently	Common		The product of *erm*, adenine-N^6-methyltransferase, also confers resistance to other macrolides and also lincosamides (Smith, 2004).

Table 1.5. Mechanism of resistance to several key antibiotics and there associated genetic location in *S. aureus* continued.

Antibiotic	Main resistance mechanism	Genetic location of resistance			Comments
		Chromosome	Plasmid	Transposon	
Gentamicin	Enzymatic modification of the antibiotic frequently by the enzymes AAC(6') and APH(2").	Often	Frequently	*Tn*4001 *Tn*3851 *Tn*4201	Plasmids often encode resistance to other antimicrobial agents and may be conjugative. Small colony variants (SCV) of *S. aureus* are often gentamicin resistant (Proctor *et al.*, 2006).
Tetracycline	Efflux; ribosomal protection proteins.	Infrequent	Common	Several incl. Tn*916* & Tn*1545*	Conjugative transposons have been implicated in the dissemination of tetracycline resistance (Roberts, 2005).
Chloramphenicol	Chloramphenicol acetyl-transferase.	Not found	Exclusively	Not found	
Fusidic acid	Decreased affinity of the G factor for the antibiotic; decreased permeability.	Common	Often		Penicillin/ fusidic acid plasmid, aminoglycoside/ fusidic acid.
Rifampicin	DNA polymerase with reduced affinity.	Exclusively	Not found	Not found	
Fluoroquinolones (ciprofloxacin & levofloxacin)	Modification of type II and IV topoisomerases; efflux.	Often			Efflux is mediated by the NorA system, which is expressed weakly in wild-type strains, but mutations leading to over-expression can confer resistance (Ohshita *et al.*, 1990).
Sulphonamide	Increased production of *p*-aminobenzoic acid.	Common	Infrequent		Second mechanism of resistance through the synthesis of dihydropteroate synthase (DHPS) with reduced affinity.
Trimethoprim	Overproduction of or mutation in host dihydrofolate reductase.				
Linezolid	Mutation in the central loop domain V of the component 23S rRNA subunit.	Exclusively			A G2576T mutation of the 23S rRNA has been described in three linezolid-resistant MRSA isolates (Tsiodras *et al.*, 2001).
Daptomycin	Mechanism of resistance is currently under investigation but may involve mutations affecting lysylphosphatidylglycerol synthetase, histidine kinase YycG and RNA polymerase	Exclusively			Although resistance is low-level, treatment failure can result due to poor tissue penetration (Hayden *et al.*, 2005).

from under 2% in 1990 to around 40% in 2004 (Reacher *et al.*, 2000; Department of Health, 2004). The number of deaths for which the underlying cause of death was reported as MRSA increased from 15 in 1993, to 360 in 2004 (Griffiths *et al.*, 2004; Office for National Statistics, 2006). Rates for deaths involving MRSA in males increased by 66 per cent from 12.2 per million population in 2000 to 20.2 in 2004. In females, rates increased by 51 per cent, from 5.9 to 9.0 per million population, over the same period (Office for National Statistics, 2006). Further to this, laboratory reports of bacteraemia caused by MRSA also increased from 210 in 1993 to 5,309 in 2002 (Griffiths *et al.*, 2004).

Current therapies of choice for MRSA infections are the glycopeptides vancomycin and teicoplanin. Patients unable to tolerate vancomycin have been treated with fluoroquinolones, trimethoprim-sulfamethoxasole or clindamycin, which although effective, are not as popular as vancomycin because they have less antistaphylococcal activity or because resistance develops during therapy (Lowy, 1998). Fusidic acid and rifampicin are also used, but only in combination either with each other or with a glycopeptide due to the risk of selecting resistant mutants. More recently, two new drugs with anti-MRSA activity have been developed, the oxazolidinone antimicrobial, linezolid (Swaney *et al.*, 1998) and the cyclic lipopeptide antimicrobial, daptomycin (Jeu & Fung, 2004).

However, *S. aureus* with reduced susceptibility to glycopeptides have been reported. These are known as glycopeptide-intermediate *S. aureus* (GISA) (Hiramatsu *et al.*, 1997) and fears that high-level resistance could be acquired from vancomycin-resistant enterococci (VRE) appear to have been fulfilled (Gonzalez-Zorn & Courvalin, 2003; Weigel *et al.*, 2003; Tenover *et al.*, 2004; Mater *et al.*, 2005; Weigel *et al.*, 2007). Low-level resistance to daptomycin and linezolid leading to treatment failure has also been described in MRSA strains (Tsiodras *et al.*, 2001; Hayden *et al.*, 2005). GISA, high-level vancomycin resistant strains and MRSA resistant to new therapies represent a major gap in antimicrobial chemotherapeutics.

Efforts to control MRSA infection rates have also focussed upon reducing transmission of the organism from person to person. The key mechanism for transmission of MRSA is by skin to skin contact between a carrier and a new host

(Massey *et al.*, 2006), where they are available to initiate a new infection or be passed on to another host. Hence, targeting transient and asymptomatic carriage of MRSA is theorized to reduce infection rates by reducing the number of infection sources. Techniques for the decolonization of non-sterile skin sites are centred on alcohol based handwashes, washing with antimicrobial preparations (see section 1.4.1.1) and the use of topical antibiotics to remove colonization of the nose (see section 1.4.2).

The isolation of MRSA infected or colonised patients by means of single rooms or cohort isolation systems is also used to reduce spread. However the value of isolation has been the subject of much discussion; Verhoef *et al.* (1999) strongly favoured the use of isolation, whilst Cooper *et al.* (2004) found there was a paucity of well designed studies to allow the role of isolation measures alone to be assessed, but that there is evidence that concerted efforts that include isolation can reduce MRSA. However, Cepeda *et al.* (2005) have since found that moving MRSA-positive patients into single rooms or cohorted bays does not reduce cross-infection. Gloves and other personal protective equipment are also used as barriers to transmission.

Whilst the above are the main procedures used in healthcare facilities, the public, particularly in the UK, has focused on cleaning up "dirty" hospitals. It has been proposed that an increase in order and cleanliness does probably foster an increase in overall attention to detail within an organisation, but house-keeping programmes are unlikely to have a substantial impact on MRSA transmission (Huskins & Goldmann, 2005; Eaton, 2005; Green *et al.*, 2006).

Hence, the challenge for healthcare professionals is to develop a multifaceted approach to MRSA control, involving the skilled choice of antibiotic therapy in combination with efforts to reduce transmission.

1.2.2 Mechanism of methicillin resistance in *S. aureus*

MSSA utilize three penicillin-binding proteins (PBPs), known as PBP1, 2, and 3, to catalyse cross-linking of peptidoglycan. As discussed in the previous section (1.2.1) *S. aureus* has evolved following several successful MSSA strains acquiring an additional component, PBP 2′ or 2a, which has low affinity for β-lactams. As a consequence

MRSA are resistant to all β-lactams. The genetic determinant of methicillin resistance, *mec,* has long been known to be localized on the chromosome of *S. aureus* (Sjostrom *et al.*, 1975). *mec* was mapped to a locus between the genes encoding the virulence factor protein A (*spa*) and a purine biosynthesis protein (*purA*) (Kuhl *et al.*, 1978). PBP 2′ or 2a is encoded by the *mecA* gene which has the regulatory genes *mecI* and often *mecR*1 all of which have been cloned and their nucleotide sequences determined (Ito *et al.*, 2003). Comparative genomics of MRSA strains in the 1980s revealed that a substantial length of the chromosomal DNA segment (greater than 30 kb) carrying *mec* had no allelic equivalence in methicillin-susceptible *S. aureus* strains, therefore the segment was called additional DNA or *mec*DNA (Beck *et al.*, 1986). Subsequent work showed this region to be a genomic island that acted as a specific structure for the genetic trait of methicillin resistance. Further to this it was later shown that the entire island was precisely excised from *S. aureus* N315 chromosome by the function of two site-specific recombinases (ccrA and ccrB) encoded by the island. This led to naming the island staphylococcal cassette chromosome *mec* or SCC*mec* (Ito *et al.*, 1999). Direct and inverted repeat sequences are found at the boundary of SCC*mec*. One of which is present on the *S. aureus* chromosome and the other inside SCC*mec*. SCC*mec* is present at a fixed site on the chromosome inside *orfX* (with unknown function) located near the origin of replication, which is 10 kb downstream of *purA* and 66–89 kb upstream of the *spa* gene (Ito *et al.*, 1999). So SCC*mec* consists of *mecA* and intact or truncated sets of regulatory genes, *mecR1* and *mecI*, the *ccr* gene complex and also 'junkyard' or J regions (J1, J2, and J3) that are located between and around the *mec* and *ccr* complexes and include a variety of genes or pseudogenes which, in general, do not appear to be essential or useful for the bacterial cell, although noteworthy exceptions include plasmid- or transposon-mediated resistance genes for non-β-lactam antibiotics or heavy metals (Zhang *et al.*, 2005).

Several types of SCC*mec* elements have been described. These are classified as types I-VI on the basis of the *mec* and *ccr* gene complexes they contain and are further classified into subtypes according to their junkyard region DNA segments. Thus far, there are three classes (A, B, and C) of *mec* complex and five allotypes (1 to 5) of *ccr* complex. Different combinations of these complex classes and allotypes generate the various SCC*mec* types (Table 1.6). SCC*mec* types have their own epidemiology.

Table 1.6. Classification of current SCC*mec* types found in MRSA.

SCC*mec* type[a]	*mec* complex[b]	*ccr* allotype[c]	Original strain	Reference
I	Class B	Type 1	NCTC10442	(Ito *et al.*, 2001)
II	Class A	Type 2	N315	(Ito *et al.*, 2001)
III	Class A	Type 3	85/2082	(Ito *et al.*, 2001)
IVa	Class B	Type 2	CA05	(Ma *et al.*, 2002)
IVb	Class B	Type 2	8/6-3P	(Ma *et al.*, 2002)
IVc	Class B	Type 2	MR108	(Ito *et al.*, 2003)
IVd	Class B	Type 2	JCSC4469	(Ma *et al.*, 2006)
V	Class C	Type 5	WIS [WBG8318]-JCSC3624	(Ito *et al.*, 2004)
VI	Class B	Type 4	HDE288	(Oliveira *et al.*, 2006)

[a] Subtypes of SCC*mec* IV differ based on junkyard (J) region DNA.

[b] Class A *mec* contains the complete *mec* complex (*mecI-mecR1-mecA*) and classes B and C have the *mecA* regulatory genes disrupted due to the presence of insertion sequences (ΨIS*1272*-Δ*mecR1- mecA* and IS*431*-Δ*mecR1-mecA*, respectively).

[c] *ccr* type 1 contains *ccrB1-ccrA1*, type 2 contains *ccrB2-ccrA2*, type 3 contains *ccrB3-ccrA3*, type 4 is a sporadic allotypes that contains *ccrB4-ccrA4* and type 5 contains *ccrC*.

For example, SCC*mec* types IV or V are associated with newly emerging community-acquired MRSA (CA-MRSA) strains. These SCC*mec* types possess novel, small, mobile SCC*mec* genetic elements which contain the *mecA* gene with or without additional antibiotic resistance genes and are more easily transferred to other strains of *S. aureus* than larger SCC*mec* (types I, II, and III) elements (O'Brien *et al.*, 2004).

Interestingly, *mecA*-determined resistance to β-lactams has been found to be present in up to 50% of CoNS isolates and resistance to other antibiotics, including aminoglycosides, quinolones, macrolides and tetracyclines is widespread amongst these species. The mechanisms responsible for resistance are identical to those prevalent in *S. aureus* indicating a role for horizontal gene transfer in the dissemination of methicillin resistance. Indeed, it has been proposed that SCC*mec* has been dispersed amongst staphylococci by transduction; *mecA* has never been found on mobile DNA but has spread among several *S. aureus* lineages and among different coagulase-negative species (Livermore, 2001).

1.2.3 Development of reduced susceptibility to antimicrobials

Two main categories exist for the development of antimicrobial resistance in *S. aureus*: intra-cellular diversification i.e. mutation or recombination within an already present bacterial gene, and inter-cellular diversification, mediated by the acquisition of a resistance gene from other organisms by some form of genetic exchange (horizontal gene transfer). Mutations can be caused by chemically- or physically-induced DNA damage, or by polymerase error during DNA replication, Figure 1.3(a). Consequently, *in vitro* spontaneous mutants resistant to agents generally arise at a frequency of between 10^{-6} and 10^{-8} per bacterium per generation (al-Masaudi *et al.*, 1991a). This value is inline with bacterial basal mutation rate. Recombination can also create new combinations of genes, as outlined in Figure 1.3(b).

Resistance to certain antimicrobials can be achieved through mutation including streptomycin, fusidic acid, rifampicin and novobiocin. Resistance to rifampicin, for example, is conferred by a mutation in RNA polymerase (*rpoB*) gene (Aubry-Damon *et al.*, 1998). Mutations confer resistance often by changing the amino acid sequence

Figure 1.3(a). Processes that generate intra-cellular diversity: mutagenesis. Mutagenesis and DNA repair are intimately related. Native DNA can be subjected to endogenous and exogenous sources of DNA damage. The nature of the damage determines the repair pathways activated to counteract these lesions. Failure to accurately restore the wild-type sequence has potentially lethal consequences, but may also bring about a phenotypic change that facilitates the colonization of a new niche. Adapted from Battista & Earl (2004). AER, alternative excision repair; BER, Base-excision repair; MMR, methyl-directed mismatch repair; NER, Nucleotide-excision repair.

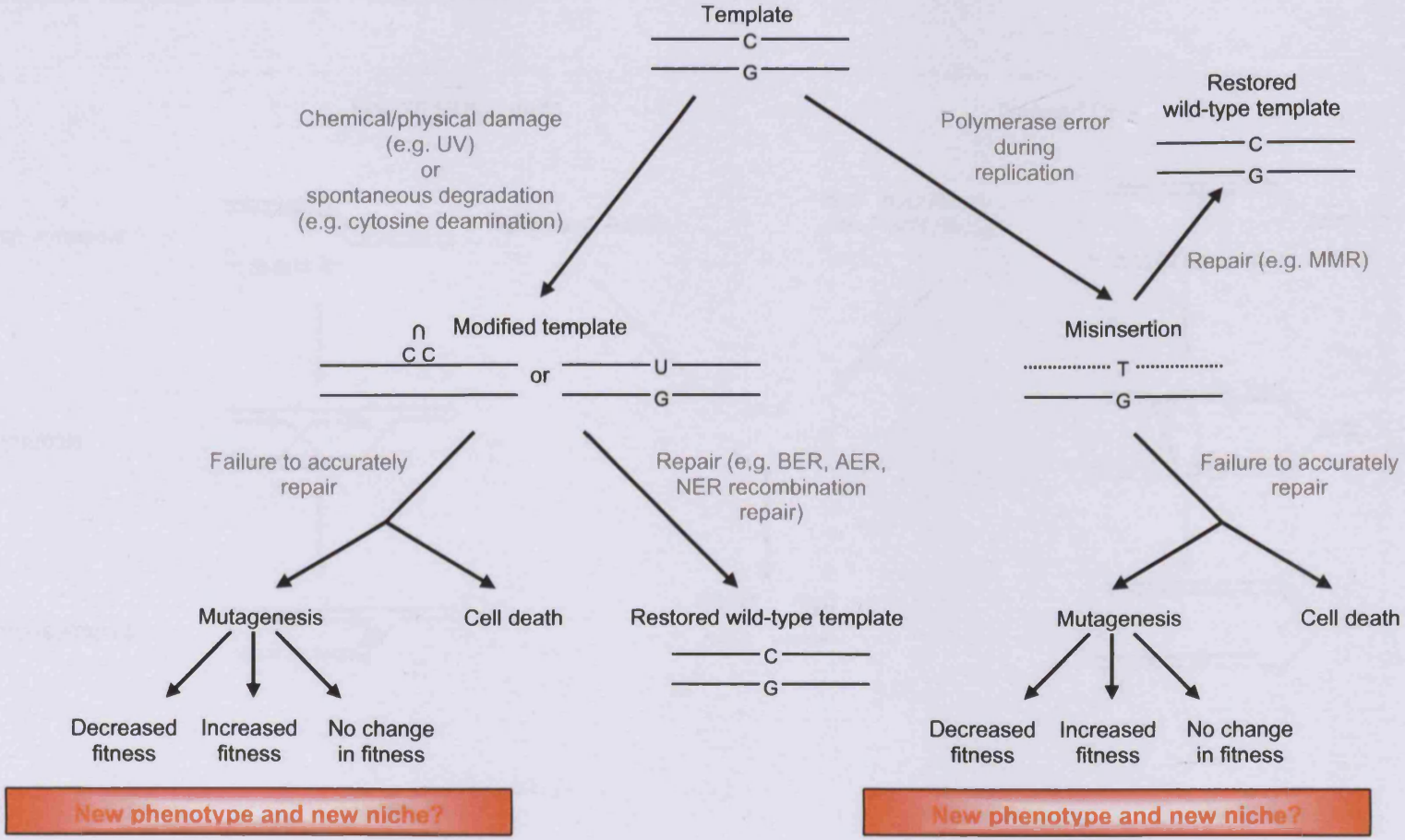
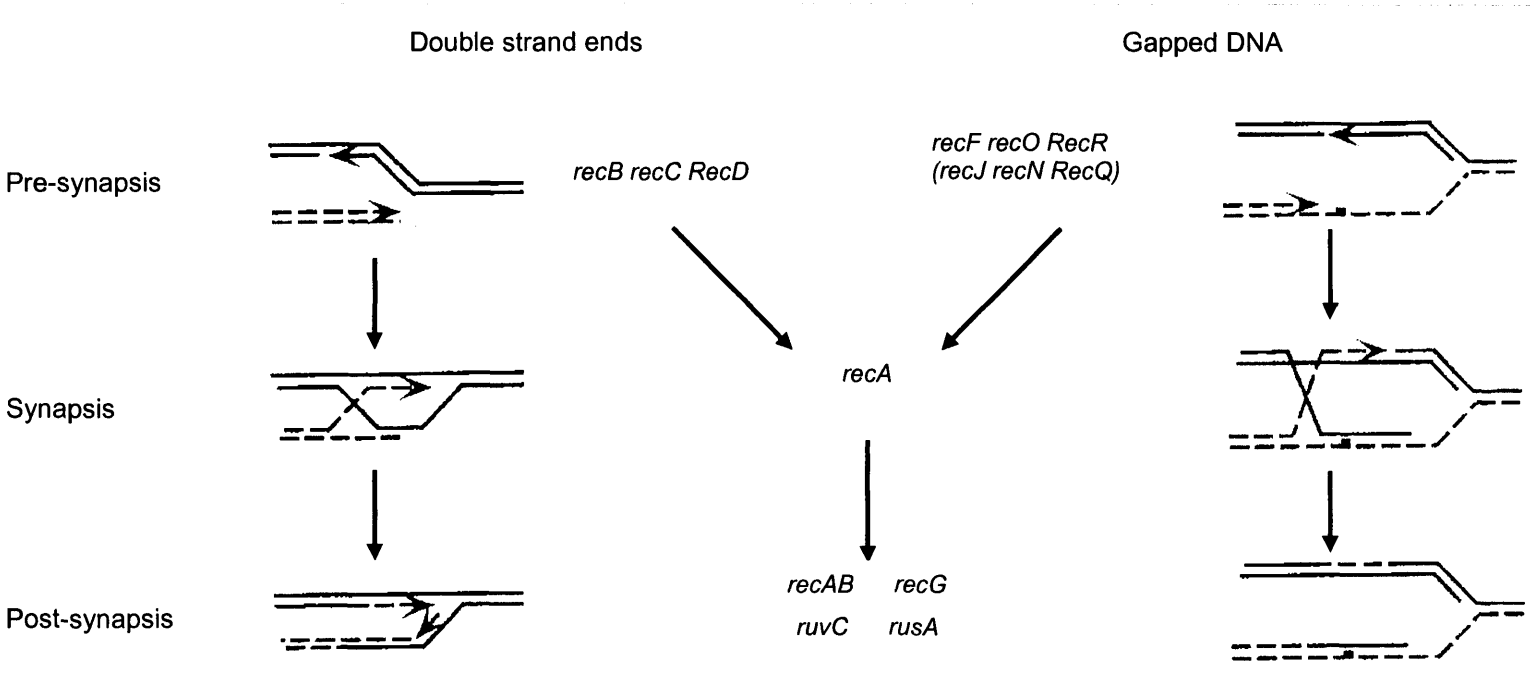


Figure 1.3(b). Processes that generate intra-cellular diversity: recombination. Diversity can also be created by the process of recombination. Two different types of DNA substrates could be formed as a result of a replication fork running into a nick (formation of a double-strand end; also the substrate generated during processes of conjugation and transduction) or a non-coding lesion (lesion square on the DNA) to form a gap. The genes and the particular stage of recombination at which their products are predicted to function are indicated. Adapted from Sandler & Nüsslein (2004).



of the target protein, resulting in a protein with altered properties, such as reduced binding affinity to the antimicrobial. This is frequently conferred by mutations within the active site of the protein. Alternatively, mutations can affect the expression of a gene; up-regulation or over expression of a target gene will reduce the potency of an antimicrobial (Fan *et al.*, 2002). However, it seems unlikely that these would be an overly common occurrence *in vivo* since chromosomal mutations conferring antibiotic resistance can cause loss of fitness in the mutant organisms (Andersson & Levin, 1999).

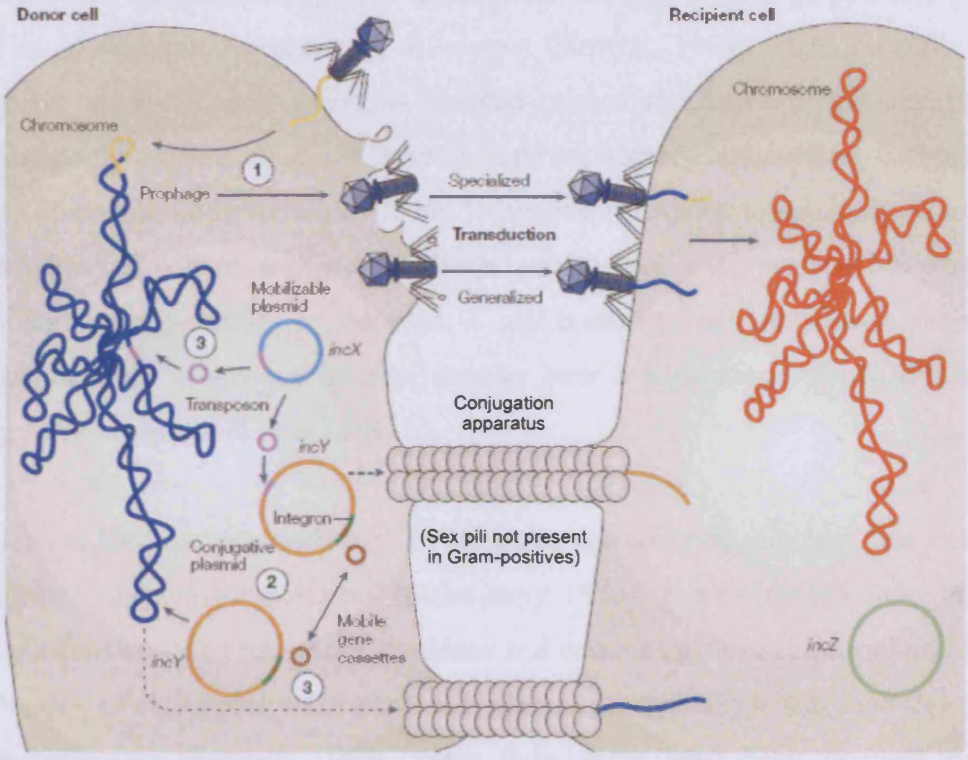
Nonetheless, this is not always the case, for example low-level mupirocin resistance conferred by a V588F substitution in isoleucyl-tRNA synthetase has been shown not to be associated with a loss in fitness (Hurdle *et al.*, 2004). Furthermore, it is possible that the original fitness costs can be ameliorated by subsequent evolution (Andersson & Levin, 1999). The acquisition of resistance genes has little or no effect on the fitness of the recipient and would therefore be expected to have a raised prevalence amongst *S. aureus* in the natural environment. Indeed it is now generally accepted that horizontal gene transfer plays a major role in the mediation and accumulation of antimicrobial resistance among the staphylococcal population (al-Masaudi *et al.*, 1991a).

Several mobile genetic elements carrying antimicrobial resistance genes in staphylococci have been discussed in depth (Novick, 1989; Novick *et al.*, 2001). Exogenous resistance genes are found on various mobile genetic elements (plasmids, insertion sequences (IS), transposons, bacteriophages (phages) and genomic islands). These mobile genetic elements can be transferred between staphylococci by several methods, facilitating the dissemination of resistance and virulence genes.

1.3 Gene transfer systems in staphylococci

There are three fundamental mechanisms for the transfer of resistance genes between staphylococci. These are conjugation, transduction and transformation, Figure 1.4.

Figure 1.4. Transfer of DNA between bacterial cells. **Transduction (1).** The DNA genome (yellow) of a temperate phage inserts into the chromosome (dark blue) as a prophage; it later replicates, occasionally packaging host DNA alone (generalized transduction) or with its own DNA (specialized transduction), lyses the cell, and infects a naive recipient cell in which the novel DNA recombines into the recipient host cell chromosome (red). **Conjugation (2).** Large, low copy number conjugative plasmids (orange) use a protein structure to establish contact with a recipient cell and to transfer themselves to the recipient cell. Alternatively, a copy of a small, multicopy plasmid or defective genomic island or a copy of the entire bacterial chromosome can be transferred to a naive cell, in which these genetic elements either insert into the chromosome or replicate independently if compatible with the resident plasmids (light green). **Transposition (3).** Transposons (pink) integrate into new sites on the chromosome or plasmids by non-homologous recombination. Integrons (dark green) use similar mechanisms to exchange single gene cassettes (brown). Adapted from Frost *et al.*, 2005.



1.3.1 Conjugation

Bacterial conjugation is a highly specific process whereby DNA (plasmid) is transferred from donor to recipient bacteria by a specialized multi-protein complex, named the conjugation apparatus. A key prerequisite for conjugative transfer is an intimate association between the cell surfaces of the donor and recipient cells. In Gram-negative bacteria, this physical contact is established by complex extracellular filaments, the sex pili. For the majority of Gram-positive bacteria, the means to achieve this intimate cell to cell contact have not yet been fully identified, but a strong level of homology has been found with type IV secretion systems (Grohmann *et al.*, 2003).

Staphylococcal conjugation was first described in 1982 with the description of the transfer of class III plasmids carrying gentamicin resistance (Jaffe *et al.*, 1982). These plasmids occur very commonly in *S. epidermidis* and may well be responsible for the spread of gentamicin resistance to *S. aureus* (Novick, 1989). Until recently most conjugative staphylococcal plasmids isolated carried resistance to gentamicin and other aminoglycosides, ethidium bromide and quaternary ammonium compounds. Some of these plasmids were also found to encode resistance to penicillin. Conjugal plasmids have also been shown to mobilize smaller non-self-transmissible plasmids (Grohmann *et al.*, 2003). In addition a self-transmissible gentamicin resistance plasmid was able to mediate its own transfer over a wide range of pH values and temperatures (al-Masaudi *et al.*, 1991b).

The view of the role plasmids take in bacterial resistance to biocides has changed significantly since research started in the early 1980s. It was initially thought that, whilst plasmid-encoded resistance to anions and cations (such as mercurial and silver compounds) existed, plasmids were not normally responsible for high levels of biocide resistance (Russell, 1997). Since then, there have been frequent reports linking the presence of plasmids with resistance to biocides such as chlorhexidine gluconate (CHX) (Cookson *et al.*, 1986; al-Masaudi *et al.*, 1988; Cookson *et al.*, 1991a) and quaternary ammonium compounds (QACs) (Paulsen *et al.*, 1996a; Bjorland *et al.*, 2001; Chapman, 2003; Noguchi *et al.*, 2005). These findings show

that conjugation systems are probably of significant importance in the dissemination of antimicrobial resistance genes among the *Staphylococcus* genus.

In addition to plasmids, *S. aureus* strains are also known to possess conjugative transposons. Conjugative transposons are mobile DNA elements that encode all the necessary functions for intracellular transposition and intercellular conjugation. The first identified was an 18 kb Tn*916* transposon from *E. faecalis* (Jaworski & Clewell, 1995). Tn*916* and the closely related element Tn*1545* from *S. pneumoniae* form the foundation of a family of conjugative transposons with an extremely broad host range. Members of the Tn*916*-Tn*1545* family have been found naturally in, or have been introduced into, more than 50 different species and more than 20 genera of bacteria including *S. aureus*. All members of the family encode a tetracycline determinant of the TetM type and many also carry genes encoding resistance to further antimicrobial agents (Salyers *et al.*, 1995).

1.3.2 Transduction

Phages are widely distributed in staphylococcal populations. Moreover, most clinical isolates harbour at least one prophage, illustrated by the genome sequences of *S. aureus* strains, Table 1.3. It appears probable that transduction is the mechanism by which the *mecA* determinant has spread amongst staphylococci; m*ecA* has never been found on mobile DNA but has spread among several *S. aureus* lineages and among different coagulase-negative species (Livermore, 2001). Phage-mediated transduction has also been postulated to be the primary mechanism by which β-lactamase genes have spread among staphylococci. Supportive of this mechanism is the fact that most staphylococcal class I, β-lactamase-producing plasmids are 35-40 kb in size, roughly the size of a phage genome. As such, the mistaken incorporation of the plasmid into the phage head (generalized transduction) can be facilitated (Novick, 1989; Rice, 2000). DNA mobilized by specialized transduction arises from illegitimate recombination during excision of a prophage from the host genome, leading to the packaging of a phage 'genome' lacking some phage genes but gaining some host traits. The fate of the transferred DNA is independent of the transduction process and depends on the recipient cell's mechanisms of recombination and DNA stabilisation (Miller, 2004). However, the requirement for the death of the donor cell, the need to

protect the recipient from destruction by normal phage particles in addition to the artificial conditions needed for the transduction process *in vitro* raises doubts as to whether transduction could be a significant process in nature (al-Masaudi *et al.*, 1991a). To this end, transfer of other resistance determinants by transduction is not well documented, although is has been implicated as a mechanism for the dissemination of virulence determinants (Rice, 2000).

1.3.3 Transformation

The ability to absorb free DNA from the environment is relatively rare in species of Gram-positive bacteria. However *S. aureus*, under specific conditions, is able to take up 'naked' DNA from the environment. The natural competence system of *S. aureus* is induced only in very early exponential growth (Novick, 1989). Either chromosomal or plasmid DNA can be transformed into *S. aureus*, although competence requires high calcium concentrations (0.1 M). Indeed, the requirement for a non-physiological calcium concentration and a short period of competence (when nucleases are absent) are reported to dramatically reduce the likelihood of transformation in nature (Lacey, 1984). There are several stages to the process of taking up DNA from the environment. Firstly, potential recipients must encounter a DNA molecule, and this must be positioned immediately adjacent to uptake sites on the cell surface. Molecules may drift to the organism, if it is attached in an aquatic environment, or unlike *S. aureus* the organism might have motility and locate/interact with a DNA source. Secondly, the fragment must be of the right size and conformation to be recognized by the cell. DNA must be double stranded and, ideally, have a size greater than five kb (*Bacillus subtilis* and *S. pneumoniae* most efficiently bind duplex DNA of about 10 to 18 kb). Once a DNA fragment has been recognized it can be transported in to the cell. This process requires several genes and slightly different pathways are adopted by Gram-positive and Gram-negative bacteria. Due to the complexity and thickness of the Gram-positive cell wall an intricate collection of transport and accessory proteins are required to achieve successful DNA transport. In Gram-positive bacteria DNA binds rapidly to cell surface uptake sites, where an ordered process appears to take place; the DNA is reduced enzymatically into fragments of a predetermined optimal size for translocation (averaging 15 to 18 kb). The newly generated ends are adjacent to uptake sites, and uptake can start. In *B. subtilis* and *S. pneumoniae* a nuclease

(EndA) degrades one strand (5` to 3`), allowing the 3` end of a single-stranded molecule to travel into the cytoplasm (Day, 2004). Finally, once in the cell the DNA molecule is reliant upon the host organism's recombination (generally recA), stabilization and expression systems to be assimilated into the host genome and, ultimately, expressed.

1.4 Antimicrobial agents

Antibiotics are used as chemotherapeutic drugs whilst biocides are chemicals used as antiseptics, disinfectants and preservatives. Biocides have a range of uses such as disinfection of surfaces (disinfectants), control/prevention of infection (antiseptics and topical antimicrobials) or incorporated into pharmaceutical, cosmetic, consumer healthcare or other types of product to prevent microbial contamination (preservatives), Table 1.7.

No single antimicrobial agent is ideal for use in all situations. When selecting an antimicrobial for use in a particular setting several factors are taken into consideration including the active ingredient, concentration, user acceptability, rapidity of action, persistence, cost, inactivation, safety and toxicity (Rutala & Weber, 2001). Some characteristics of topical antimicrobial agents are discussed in Table 1.9. Biocide activity is affected by several factors, notably concentration, period of contact, pH, temperature, presence of inactivants and the nature, numbers, location and condition of the microorganisms (Russell, 2006). Biocide concentration is of prime importance. The effect of concentration, or dilution, on the activity of a biocide is measured by the concentration exponent (n or η). Biocides with high η-values, such as phenolics and alcohols, rapidly lose activity upon dilution. QACs, chlorhexidine, glutaraldehyde and triclosan have a low η-value and hence retain much of their activity upon dilution (Russell, 2003b). Many biocides also have an optimum pH range. Glutaraldehyde and cationic biocides, such as chlorhexidine and QACs, are most active at high pH whilst the potency of hypochlorites and phenolics is increased at low pH. Generally the activity of biocides increases with increased temperature, although this is of little practical use. Inactivation readily reduces the efficacy of biocides. Inactivation can occur through interaction with organic matter (blood, serum, pus, dirt) and non-ionic

Table 1.7. Clinical and other uses of biocides. Adapted from Russell (2003).

Biocide type	Example(s)	General examples of use(s)
Alcohol	Ethanol	Hand sanitising
Aldehyde	Formaldehyde	Virucidal agent
	Formaldehyde-releasing agents	Topically; irrigation solutions
	Glutaraldehyde	Endoscope disinfection
Biguanide	Chlorhexidine	Antiseptic, disinfectant, pharmaceutical preservative
	PHMB (polymeric)	Swimming pool disinfection, contact lens solutions
CRA	NaClO, NaDCC industrial	Disinfection of blood spillages; sanitisation compounds
Isothiazolon	Chloromethyl and methyl derivatives	Cosmetic and pharmaceutical preservatives
Peroxygen	Hydrogen peroxide	Antiseptic, disinfectant, deodorant
	Peracetic acid	Endoscope disinfection
Phenylether	Triclosan	Preoperative disinfection, body washes, dental hygiene, consumer healthcare
Phenol and cresol		Preservatives, disinfectants
QAC	BZK, CPC, CTAB	Skin disinfection, preoperative disinfection, antiseptics, pharmaceutical preservatives
Vapour phase	Ethylene oxide	Low-temperature sterilisation of non-thermostable materials

PHMB, polyhexamethylenebiguanide; NaClO, sodium hypochlorite; NaDCC, sodium dichlorocyanurate; BZK, benzalkonium chloride; CPC, cetylpyridinium chloride; CTAB, cetyltrimethylammonium bromide; CRA, Chlorine-releasing agent.

surfactants, and also through adsorption to containers. Microorganisms show substantial differences in their response to biocidal treatment, for example the differences between Gram-positive and Gram-negative bacteria, Table 1.8. The condition of the microorganisms, such as biofilm cells, also has a marked outcome on biocide susceptibility (Russell, 2003b). There are several similarities and differences between biocide and antibiotic action. Antibiotics inhibit a specific target in a biosynthetic process, such as peptidoglycan synthesis. By contrast biocides have multiple, concentration-dependent targets, with subtle effects occurring at low concentration and more damaging effects at higher concentrations. However, some similarities do also exist, including cellular penetration of cationic agents, both antibiotic and biocide. Some biocides and antibiotics also share membrane-damaging effects and morphological changes induced (Russell, 2003a). There is also a shared target site between one biocide (triclosan) and an antibiotic (isoniazid) in some mycobacteria (McMurry *et al.*, 1999).

1.4.1 Triclosan

Triclosan (2,4,4'-trichloro-2'-hydroxyphenyl ether, Irgasan® DP300, Irgacare® MP and Irgacide® LP10) is a broad spectrum biocide that has been in use for more than 35 years. It was developed in the 1960s by J.R. Geigy AG (Basel, Switzerland) and is now manufactured predominantly by Ciba Speciality Chemicals GmbH (Grenzach-Wyhlen, Germany). Usage was increasing, but has now stabilized, with about 350 tonnes of triclosan being used within countries of the European Union every year. Of this, the UK is estimated to use 60-90 tonnes (Environment Agency, 2004).

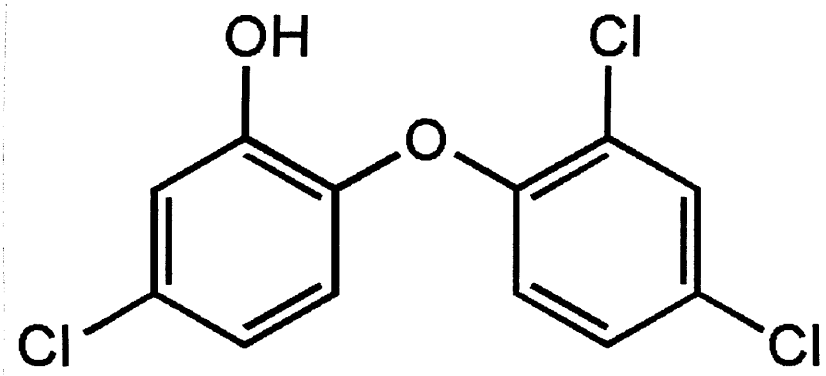
Triclosan is the most potent of the bis-phenol class of antimicrobials and, as with all biocides of this class, possesses the hydroxy-halogenated derivatives of two phenolic groups connected by a bridge, Figure 1.5. It is an off-white, tasteless and odourless, crystalline powder with a molecular weight of 289.5. Triclosan is practically insoluble in water, but moderately soluble in alkaline solutions and readily soluble in most non-polar organic solvents. The thermal stability of triclosan powder is relatively good up to 150°C or even up to 200°C, if not heated for more than 2 hours (Bhargava & Leonard, 1996).

Table 1.8. Characteristics of topical antimicrobial agents. Adapted from Rutala & Weber (2001).

	Alcohols	CHX	Iodophores	PCMX	Triclosan
Gram-positive bacteria	Excellent	Excellent	Excellent	Good	Good
Gram-negative bacteria	Excellent	Good	Good	Fair	Good
M. tuberculosis	Excellent	Fair	Good	Fair	Fair
Fungi	Good	Fair	Good	Fair	Poor
Viruses	Good	Good	Good	Fair	Unknown
Rapidity of action	Most rapid	Intermediate	Intermediate	Intermediate	Intermediate
Residual activity	None	Excellent	Minimal	Good	Excellent
Concentration (%)	70-92	4, 2; 0.5 in alcohol	10, 7.5, 2, 0.5	0.5-.3.75	0.3-2.0
Inactivation	No data	Minimal	Yes	Minimal	Minimal
Safety	Drying, volatile	Ototoxicity, keratitis	Skin irritation	Need data	Skin and eye irritant

M. tuberculosis, *Mycobacterium tuberculosis*; CHX, Chlorhexidine gluconate; PCMX, parachlorometaxylene.

Figure 1.5. The chemical structure of triclosan. The synthetic bisphenol consists of hydroxy-halogenated derivatives of two phenolic groups connected by a bridge.



Triclosan is a broad spectrum antimicrobial, but exhibits particular activity against Gram-positive bacteria. However, efflux systems and a non-susceptible enoyl reductase in *Pseudomonas aeruginosa* confer an innate resistance (Heath *et al.*, 2001; Levy, 2002; Chuanchuen *et al.*, 2003). The susceptibilities of some Gram-positive and Gram-negative bacteria, including several antibiotic resistant strains, are described in Table 1.9. Susceptibility of Gram-negative bacteria and yeasts can be significantly enhanced by formulation effects. For example, triclosan in combination with EDTA caused increased permeability of the outer membrane (Leive, 1974). Indeed, during clinical or domestic use, triclosan is delivered typically as part of complex formulations containing various combinations of surfactants, detergents, chelating agents and wetting agents, which affect the antimicrobial activity of triclosan and ultimately affect the susceptibility of microorganisms exposed to it.

Depending on the type and concentrations of constituents in the formulation, triclosan products with the same levels of antimicrobial may demonstrate varying levels of effectiveness; efficacy may be affected by pH, detergent base, emollients and humectants, ionic nature of the formulation and type of surfactants (Larson, 1995). Triclosan exhibits minimal pH sensitivity and interference from skin conditioners (Jones *et al.*, 2000). It is also non-ionic, as a consequence of this it would be unlikely that the inclusion of lotions or moisturizers would affect the effectiveness of triclosan formulations, unlike products containing cationic biocides, such as chlorhexidine, which are readily inactivated by anionic lotions. However, the activity of triclosan is influenced by the amount and type of surfactant in the formulation, a key ingredient in handwash products (Jones *et al.*, 2000). Surfactants are surface active agents that lower surface tension (allowing the cleanser to wet the skin), emulsify soils and remove transient microorganisms. Their interaction with triclosan starts when surfactant molecules aggregate in water to form micelle structures. These structures are important to the emulsification process but can also lead to an unwanted consequence; molecules of triclosan can be sequestered in these micelles and thus become less bioavailable. Recently this problem has been avoided by optimizing the ratios of certain surfactants and other common skin care ingredients, which can reduce the interaction between triclosan and the micelles. This technology is referred to as "de-blocking" or "activated triclosan" (Fuls & Fischler, 2004).

Table 1.9. The susceptibilities of some Gram-positive and Gram-negative bacteria to triclosan. The MICs were calculated by the agar dilution method. Data are taken from Vischer & Regos, (1973), Regos *et al.*, (1979) and Bhargava & Leonard (1996).

Organism	Strain	Medium[a]	Triclosan MIC (mg/L)
Gram-positive bacteria			
Bacillus subtilis	NCTC 8236	NA	0.1
Bacillus megaterium		NA	3
Bacillus cereus		NA	3
Bacillus cereus var. mycoides		NA	3
Clostridium botulinum	NCTC 3805	BHIA	3
Clostridium tetani	NCTC 9571	BHIA	3
Corynebacterium diphtheriae	ATCC 6917	BHIA	3
Corynebacterium diphtheriae	NCTC 3984	BHIA	3
Corynebacterium diphtheriae		BHIA	3
Corynebacterium minutissimum	ATCC 6501	BHIA	5
Diplococcus pneumoniae	NCTC 7465	BHIA	3
Lactobacillus arabinosus	CITM 706	MACA	33
Lactobacillus arabinosus	ATCC 8014	MACA	33
Lactobacillus fermenti	CITM 707	MACA	33
Listeria monocytogenes	ATCC 15313		1
Mycobacterium tuberculosis		YA	
Mycobacterium smegmatis	NCTC 8152	BHIA	1
Mycobacterium phlei		BHIA	0.3
Propionibacterium acnes	ATCC 6919	BHIA	3
Sarcina lutea	NCTC 196	BHIA	3
Sarcina ureae	ATCC 6473	BHIA	0.1
Staphylococcus aureus	NCTC 6571	NA	0.03
Staphylococcus aureus	NCTC 7447	NA	0.01
Staphylococcus aureus	NCTC 6966	NA	0.1
Staphylococcus aureus	NCIMB 9518	NA	0.03
Staphylococcus aureus (MRSA)	MRSA 11	NA	0.075
Staphylococcus aureus (MRSA)	MRSA 11777	NA	0.5
Staphylococcus aureus (MRSA)	MRSA 9543	NA	1
Staphylococcus epidermidis		NA	0.03
Streptococcus agalactiae	NCTC 8181	BHIA	33
Streptococcus mutans	NCTC 10832	BHIA	21
Streptococcus pyogenes	NCTC 8322	BHIA	3
Streptococcus sanguis	NCTC 7863	BHIA	2.6
Streptococcus oralis	NCTC 11427	BHIA	5.2
Enterococcus faecalis	ATCC 8619	BHIA	10

Table 1.9. The susceptibilities of some Gram-positive and Gram-negative bacteria to triclosan continued.

Organism	Strain	Medium	Triclosan MIC (mg/L)
Gram-negative bacteria			
Aerobacter aerogenes	CITM 413	NA	1
Alcaligenes faecalis	ATCC 8750	MHA	0.5
Bacteroides denticola	ATCC 33185	NA	3.1
Bacteroides intermedius	ATCC 25612		1.6
Burkholderia mallei	NCTC 9674	NA	0.3
Burkholderia pseudomallei	NCIMB 10230	NA	1
Burkholderia cepacia		NA	256
Brucella intermedia		BrAA	0.1
Brucella melitensis		BA	1
Brucella suis		BrAA	0.03
Enterobacter cloacae	ATCC 13047	MHA	0.5
Escherichia coli	NCTC 86	NA	0.1
Escherichia coli	NCTC 8196	NA	0.03
Escherichia coli	NCTC 8739	MHA	0.2
Escherichia coli (serotype 0157)	ATCC 35150	MHA	0.2
Escherichia coli (serotype 0157)	ATCC 43888	MHA	0.1
Escherichia coli (serotype 0157)	ATCC 43894	MHA	0.2
Haemophilus influenzae	ATCC 33391	MHA	2
Klebsiella pneumoniae	ATCC 11296	MHA	0.3
Klebsiella pneumoniae	ATCC 4352	BHIA	0.06
Legionella pneumophila	ATCC 33152		1
Moraxella catarrhalis	NCTC 3622	BA	33
Morganella morganii		NA	10
Pasteurella septica	NCTC 948	NA	0.1
Porphyromonas gingivalis		WCA	2.5
Prevotella oralis	ATCC 33573		6.3
Proteus vulgaris	NCTC 8313	NA	0.1
Proteus vulgaris	NCTC 4175	NA	0.01
Proteus mirabilis	NCTC 8309	NA	0.3
Proteus mirabilis	ATCC 14153	MHA	0.5
Pseudomonas aeruginosa	NCTC 1999	NA	>100
Pseudomonas aeruginosa	ATCC 12055	NA	>1000
Pseudomonas aeruginosa	NCTC 8060	NA	>1000
Pseudomonas fluorescens	NCTC 4755	NA	>100
Pseudomonas oleovorans	ATCC 8062	BHIA	0.5
Pseudomonas stutzeri	ATCC 17588	MHA	1

Table 1.9. The susceptibilities of some Gram-positive and Gram-negative bacteria to triclosan continued.

Organism	Strain	Medium	Triclosan MIC (mg/L)
Gram-negative bacteria continued			
Salmonella enteritidis		NA	0.3
Salmonella paratyphi A	NCTC 5322	NA	0.3
Salmonella paratyphi B	NCTC 5704	NA	0.1
Serratia marcescens	ATCC 14756	MHA	>512
Serratia marcescens	ATCC 13880	BHIA	>1000
Shigella dysenteriae	NCTC 2249	NA	0.1
Shigella flexineri	NCTC 8192	NA	0.3
Shigella sonnei	NCTC 7240	NA	0.1
Vibrio cholerae		NA	10
Vibrio eltor	NCTC 8457	NA	10
Fungi and yeasts			
Aspergillus niger	ATCC 6275	M	30
Aspergillus fumigas	ATCC 9197	SMA	10
Candida albicans		NA	3
Candida albicans	ATCC 10259	M	3
Epidermophyton floccosum	ATCC 10227	SMA	1-10
Trichophyton ajelloi		SMA	10
Trichophyton mentagrophytes	ATCC 9533	SMA	1
Trichophyton rubrum		SMA	10
Trichophyton tonsurans		SMA	10

[a] BA, blood agar; BHIA, Brain-heart infusion agar; BrAA, Brucella agar albimi; M, mycophil agar; MACA, microassay culture agar; MHA, Mueller-Hinton agar; NA, nutrient agar; SMA, Sabouraud maltose agar; WCA, Wilkins-Chalgren-Bouillon agar; YA, Youman's agar.

1.4.1.1 Clinical and domestic use of triclosan

For many years triclosan has been included in products such as hand soaps, lotions, toothpastes, deodorants and oral rinses (Russell, 2004). It has also been incorporated into a range of items including tea towels, chopping boards and toys (Levy, 2001). These applications have been used to prevent the development and/or spread of infection and microorganism-associated odours in the community setting. However, triclosan has an important role in infection control within the clinical setting (Jones *et al.*, 2000).

Although the efficacy of triclosan in surgical scrub studies has been limited, it has shown effectiveness against transient bacteria in health care personnel handwash studies (Jones *et al.*, 2000). The efficacy of various triclosan formulations, in comparison with 4% chlorhexidine formulations, was demonstrated in a healthcare personnel handwash test (Jampani *et al.*, 1998). Additionally, Bartzokas *et al.* (1983) demonstrated the effectiveness of several variations of triclosan detergents and 60% isopropyl alcohol on *E. coli.* In two similar studies the effectiveness of triclosan formulations were demonstrated against *E. coli* and *Pseudomonas fluorescens* mixed in a ground beef slurry to reproduce conditions found in meat and poultry processing facilities (Sheena & Stiles, 1983; Stiles & Sheena, 1987). The aforementioned studies confirm the efficacy of various triclosan formulations to remove transient microbial flora that may be acquired by medical staff in the health care environment and in food handling or preparation settings.

Importantly, in 1987 triclosan was successfully used to control on outbreak of MRSA (Tuffnell *et al.*, 1987) and more recently it was used successfully to control MRSA outbreaks in a neonatal nursery (Zafar *et al.*, 1995), a cardiothoracic surgical unit (Brady *et al.*, 1990) and one outbreak of a strain also resistant to mupirocin (Irish *et al.*, 1998). Subsequently, in 1998 it was recommended for the control of nosocomial MRSA (Ayliffe *et al.*, 1998) and 2% triclosan baths are now one of the recommended rationales for skin decolonisation of MRSA carriers (Coia *et al.*, 2006).

Another benefit of using triclosan in the regimen of decolonising hospital patients is that it has long been thought of as a non-specific biocide (Chuanchuen *et al.*, 2001)

and as a consequence the development of resistance was seen as highly unlikely (Russell, 2004). Recently this previous dogma has been challenged by evidence that triclosan may act on a specific cellular target, much like an antibiotic.

1.4.1.2 Triclosan: biocide or antibiotic?

Biocides are generally broad-spectrum antimicrobials which act by many non-specific means, targeting numerous intracellular, cytoplasmic and cell wall sites. Meanwhile, antibiotics have single and specific cellular targets. Triclosan was previously seen as a biocide, but a growing amount of research challenges this point of view. Evidence now indicates that triclosan may act on a specific target, the highly conserved enzyme enoyl-ACP reductase, which is also known as FabI (Heath *et al.*, 1998; McMurry *et al.*, 1998; Heath *et al.*, 1999; Heath & Rock, 2000; Fan *et al.*, 2002). The enoyl reductase, InhA, in *Mycobacterium smegmatis* was also found to be a target for triclosan action (McMurry *et al.*, 1999). However the question still remains whether inhibition of a single enzyme by triclosan is indeed responsible for all its inhibitory and lethal actions (Russell, 2003b).

1.4.1.3 Reduced susceptibility to triclosan in *S. aureus*

Following the introduction of triclosan into clinical practice there has been an increased interest in reduced susceptibility of microorganisms to the antimicrobial. Several reports now describe the isolation of reduced susceptibility *S. aureus* strains. In 1991 *S. aureus* strains with minimum inhibitory concentrations (MICs) of 2-4 mg/L were isolated from patients treated with daily triclosan baths (Cookson *et al.*, 1991b). The MICs of sensitive *S. aureus* are 0.01-0.1 mg/L (Table 1.10). Furthermore in 1997 7.5% of *S. aureus* clinical isolates were found to have triclosan MICs of at least 1 mg/L, although they did not find any differences between MRSA and MSSA strains (Bamber & Neal, 1999). However, Al-Doori *et al.* maintain that there has not been a significant increase in reduced triclosan susceptibility amongst nosocomial MRSA in Scotland (Al-Doori *et al.*, 2003).

As mentioned in section 1.4.1.1 triclosan has been reported to achieve its growth-inhibitory activities by blocking bacterial fatty-acid synthesis and more specifically by

explicitly inhibiting an NADH-dependent enoyl-acyl carrier protein (ACP) reductase, encoded by the *fabI* gene, Figure 1.6.

Triclosan has been shown to bind strongly to, and inhibit, *S. aureus* FabI (Ward *et al.*, 1999; Fan *et al.*, 2002; Sivaraman *et al.*, 2004). Consequently, FabI has been scrutinised for its role in reduced triclosan susceptibility and two main mechanisms of FabI mediated resistance have been proposed. Comparison of *fabI* sequences from susceptible and reduced susceptibility *S. aureus* strains indicated that a mutation within *fabI* resulting in a F204C alteration in the FabI protein conferred greatly reduced triclosan susceptibility (1-4 mg/L; Figure 1.7) (Fan *et al.*, 2002).

Since the first description of *fabI* mutations several more predicted alterations to the FabI amino acid sequence that confer reduced triclosan susceptibility have been described (Brenwald & Fraise, 2003). It is also proposed that a moderate reduction in susceptibility in *S. aureus* can be conferred by the over production of FabI (Fan *et al.*, 2002).

However, some authors maintain that triclosan has cellular targets other than FabI. Brenwald and Fraise (2003) did not find mutations in the *fabI* gene of three clinical *S. aureus* isolates with triclosan MICs of 4.0 mg/L. This lack of *fabI* mutations suggests that genetic loci other than *fabI* are also involved in triclosan resistance. Indeed, other targets, principally in the cytoplasmic membrane, have been suggested for triclosan (McDonnell & Pretzer, 1998). Additionally triclosan has been shown to intercalate into the cell wall causing it to become destabilised and compromising its functional integrity (Villalain *et al.*, 2001; Guillen *et al.*, 2004). Triclosan resistance in other genera is conferred by alternative enoyl-ACP reductase enzymes and/or drug efflux. Efflux has been reported as the sole source of high-level triclosan resistance in *P. aeruginosa* (Chuanchuen *et al.*, 2003).

In practice triclosan is normally utilized at concentrations much greater than the MICs associated with reduced triclosan susceptibility, see section 1.1.2. At such concentrations triclosan is rapidly bactericidal. This level of lethality has been shown to extend to 'triclosan-resistant' strains of *E. coli* (McDonnell & Pretzer, 1998) and *S. aureus* (Suller & Russell, 2000). Membrane damage has been shown to occur at

Figure 1.6. Reactions in type II bacterial fatty acid biosynthesis. Key enzymes are shown but the distribution of particular components varies in a species-specific manner. For example, FabA occurs only in proteobacteria, whereas FabZ is ubiquitous. Both function interchangeably in elongation cycles up to 10 carbons and both function in the synthesis of saturated fatty acids. FabB and FabF both occur in proteobacteria whereas only a FabF synthase occurs in pathogenic Gram-positive bacteria. Three different enoyl-ACP reductase enzymes have been discovered in different pathogens, FabI (*S. aureus*) and FabK (Heath & Rock, 2000) and FabL (Heath *et al.*, 2000). Adapted from Payne *et al.* (2001).

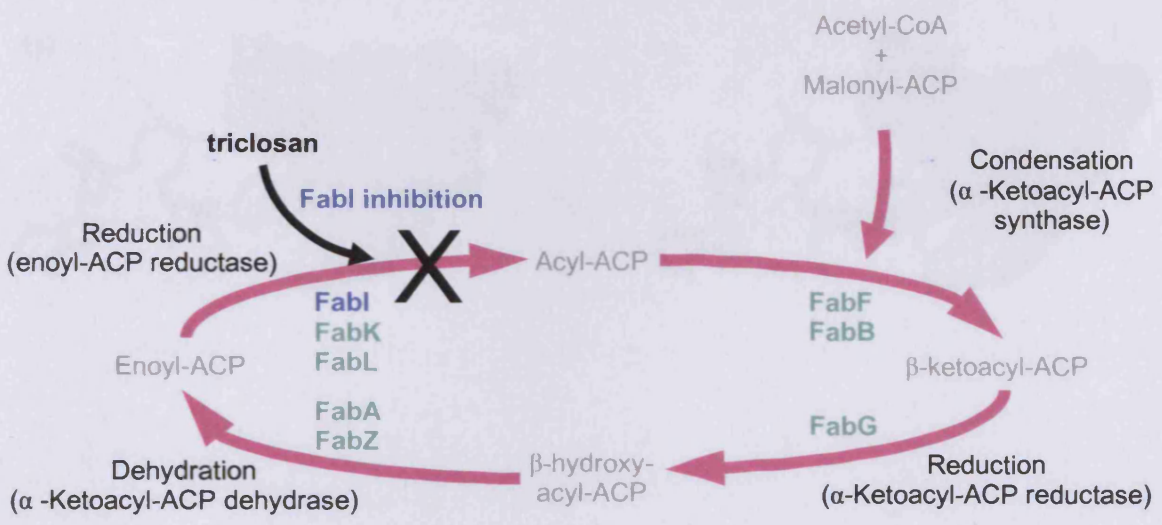
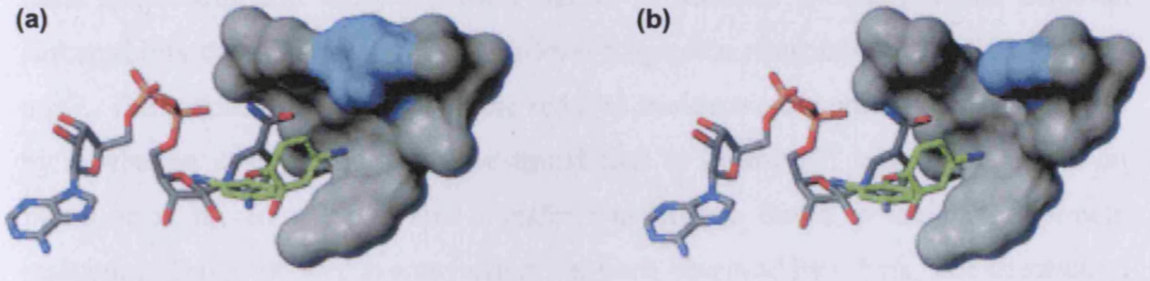


Figure 1.7. Structural consequences of mutations within *fabI* conferring a F204C alteration in the FabI protein. (a) X-ray crystal structure of triclosan bound to wild-type *E. coli* FabI and (b) model of triclosan bound to F204C *E. coli* FabI. The side-chain atoms of F204 and C204 are highlighted in cyan. Adapted from Fan *et al.*, 2002.



bactericidal levels by triclosan-induced potassium ion leakage and other membrane-destabilizing effects have also been demonstrated during exposure to high level triclosan (Russell, 2004). Triclosan demonstrates a Z-pattern type of adsorption, which is indicative of the breakdown of a structure, presumably the membrane, and the generation of new adsorbing sites (Denyer & Maillard, 2002). Therefore, whilst low concentrations of triclosan may target FabI, at in use concentrations triclosan possesses multiple modes of action.

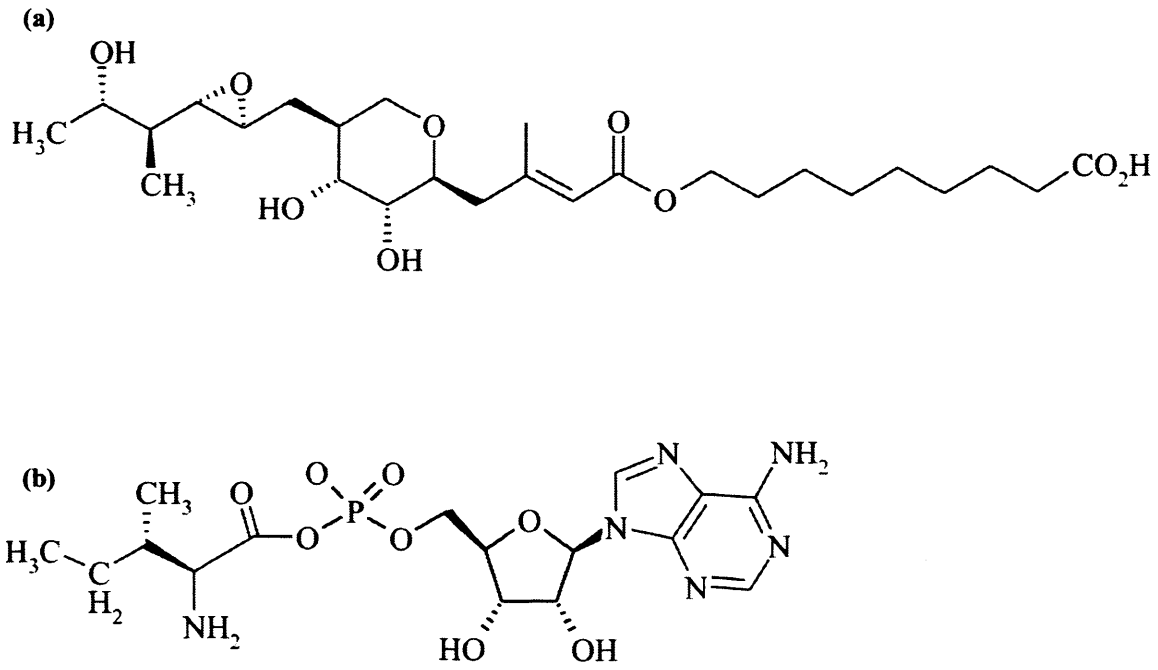
1.4.1.4 Transfer of reduced triclosan susceptibility amongst MRSA strains

As mentioned in the previous section in 1991 Cookson *et al.* isolated several MRSA strains with reduced susceptibility to triclosan (MICs between 2-4 mg/L). These strains were isolated from patients who had undergone 2 weeks of treatment with nasal mupirocin and daily triclosan baths. In addition to the reduced triclosan susceptibility the isolates showed high-level mupirocin resistance with MICs of >512 mg/L. The authors also report that the reduced triclosan susceptibility could be cured by incubation at 42°C and could be transferred to sensitive *S. aureus* recipients on filters or in mixed culture. This transfer was always found to include mupirocin resistance. These transfer events have never been observed by others. The description of development of and co-transfer of triclosan and mupirocin resistance would be of great significance with regards the dissemination of triclosan resistance determinants within MRSA.

1.4.2 Mupirocin

Mupirocin is a topical antibiotic with no systemic formulation (it is rapidly hydrolyzed to an inactive form by blood plasma). It is a natural product of *Pseudomonas fluorescens* and has a novel structure that classifies it as member of the pseudomonic acid family, Figure 1.8. Mupirocin is not readily classified with any known group of antibiotics and has a unique mode of action. It was first introduced to clinical practice in the UK in 1985 and has since proved effective in the treatment of skin infections and in particular the elimination of nasal MSSA and MRSA carriage (Hurdle *et al.*, 2005). Consequently, it is the world's most widely used topical antibiotic for the control of MRSA (Boyce, 2001).

Figure 1.8. Mupirocin is a member of the pseudomonic acid family. (a) It consists of a short fatty acid (non-anoic acid) ester linked to monic acid. (b) The tail portion closely resembles the isoleucyl moiety of the isoleucyl-adenylate reaction intermediate required for polypeptide synthesis (Hurdle *et al.*, 2005).



Mupirocin is bacteriostatic, but at lower pH (approximating that of parts of the skin) appears to be bactericidal. Mupirocin is in part an analogue of isoluecine and exerts an antimicrobial effect by irreversibly and specifically binding bacterial isoleucyl-tRNA synthetase (IleS) causing the interruption of polypeptide chain elongation and the abolition of protein synthesis (Hughes & Mellows, 1980; Yanagisawa *et al.*, 1994). This is followed by a response that results in the biosynthesis of guanosine tetra- and pentapeptides. These in turn inhibit the activity of RNA polymerase and down-regulate several key processes, including the biosynthesis of stable RNA, DNA, protein, and peptidoglycan. So by perturbing several metabolically important processes, the action of mupirocin leads to the cessation of bacterial growth and the attenuation of virulence *in vivo* (Hurdle *et al.*, 2005).

1.4.2.1 Emergence of mupirocin resistance

The definitions of mupirocin resistance have varied, but with an increasing number of resistant strains having been investigated, it appears that there are 2 resistant populations; those showing low-level resistance (MIC = 8-256 mg/L) and those showing high-level resistance (MIC >256 mg/L). The former is of tentative clinical significance whilst high-level resistance has been implicated in treatment failure (Cookson, 1998).

High-level resistance was described shortly after mupirocin was introduced into clinical practice (Rahman *et al.*, 1987) and is mediated by the *mupA* gene, which is almost always located on plasmids that vary in size, restriction pattern and ability to transfer by conjugation (Thomas *et al.*, 1999; Udo *et al.*, 2001). *mupA* encodes a second, novel staphylococcal IleS and was in existence before the introduction of mupirocin (Rahman *et al.*, 1990). This second IleS has a 50% inhibitory concentration (IC_{50}) of 7000-10,000 μg/L in comparison to 0.9-2.5 μg/L for the sensitive enzyme (Gilbart *et al.*, 1993). Whilst it is well established that *mupA* encodes plasmid-borne high-level resistance a recent report discusses the identification of a chromosomal location for *mupA*. It is surmised that this chromosomal *mupA* has resulted from the integration of a mupirocin resistance plasmid into the *S. aureus* chromosome (Udo *et al.*, 2003).

Low-level mupirocin resistance is more common than high-level and arises from point mutations within the chromosomal isoleucyl-tRNA gene (*IleS*) of staphylococci. Comparison of *ileS* nucleotide sequences from *S. aureus* strains expressing mupirocin sensitivity and low-level mupirocin resistance has identified mutations correlated with low-level resistance. Mutations conferring valine to phenylalanine alterations at either residue 588 or 631 were found to impart low-level mupirocin resistance (Antonio *et al.*, 2002). Low-level mupirocin resistance has not been found to be transferable amongst staphylococci.

1.4.3 Cationic biocides

The broad grouping of cationic biocides include the widely used chlorhexidine (CHX) compounds and cetylpyridinium chloride (CPC). The chemical structures of these two biocides are quite different with CHX being a bisbiguanide and CPC a quaternary ammonium compound (QAC). Both show substantial bactericidal effects against *S. aureus*.

CHX is probably the most widely used biocide in antiseptic products, in particular in handwashing and oral products. It is bactericidal and has a broad spectrum of activity. CHX damages outer cell wall layers, crosses the cell wall and subsequently attacks the cytoplasmic membrane, resulting in cell death (El-Moug *et al.*, 1986). Importantly, CHX activity is highly pH dependent and is greatly reduced in the presence of organic matter, which precludes its use in some preparations.

Antiseptics and disinfectants based on QACs are used extensively in hospitals and other health care settings, as well as in the food industry and consumer healthcare products (McDonnell & Russell, 1999; Bjorland *et al.*, 2001). CPC has long been known to be a membrane-active agent, with the target site predominantly the cytoplasmic membrane (Hugo & Frier, 1969). The cytoplasmic membrane is essential many key cellular processes, hence the disruption of the structural organization and integrity of the membrane by CPC causes cell death.

Plasmid-encoded resistance to QACs has been found in staphylococci. Resistance to QAC among *S. aureus* and CoNS was first studied and reported in human clinical

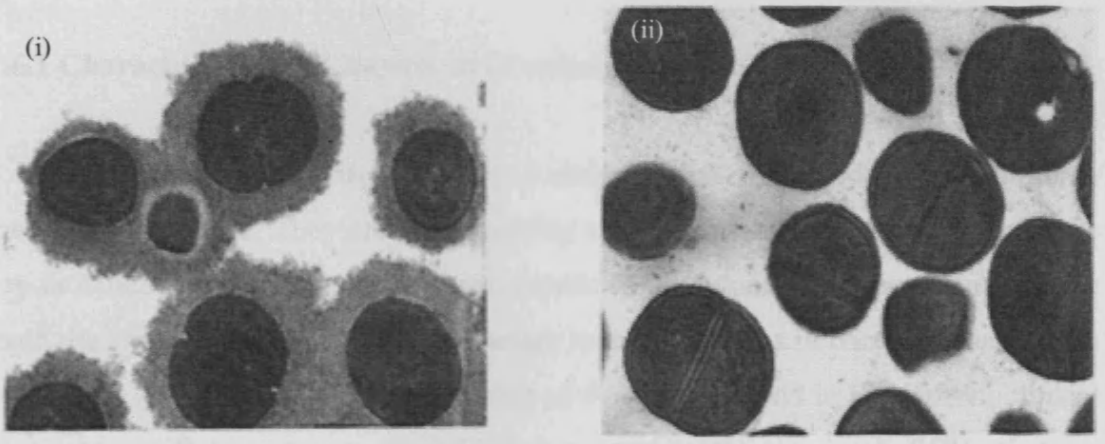
isolates in which three QAC resistance genes have been characterized: *qacA*, *qacB*, and *smr* (formerly *qacC*) (Paulsen *et al.*, 1996a; Paulsen *et al.*, 1996b; Bjorland *et al.*, 2001). Additionally, plasmid encoded resistance to CPC has been found in *S. aureus* (Thomas & Archer, 1989). CHX shares similarities in mode of action with CPC and there is often cross resistance between the compounds. Resistance to CHX has also been linked with nucleic acid binding proteins that are frequently plasmid encoded (Cookson *et al.*, 1991a; Russell, 1997).

1.5 *S. aureus* capsule polysaccharide

Microorganisms that cause invasive disease frequently produce extracellular capsular polysaccharides. Capsule production in *S. aureus* was first described in 1931 (Gilbert, 1931) although this was restricted to the large, mucoid capsules. *S. aureus* microcapsules (non-mucoid) and the current capsular polysaccharide typing scheme were described some 50 years later (Karakawa & Vann, 1982). Since the introduction of this scheme approximately 90% of *Staphylococcus aureus* isolates have been shown to produce one of 11 serotypes of capsular polysaccharides. Serotypes CP5 and CP8 account for approximately 25% and 50%, respectively, of isolates recovered from humans, offering support for their pathogenic significance (O'Riordan & Lee, 2004). CP5 and CP8 serotypes are considered as microcapsules because they are much smaller than those produced by the mucoid serotypes 1 and 2. The microcapsules of CP5 and CP8 are extracellular, uronic acid-containing polysaccharides that are too small to be visualized by negative staining with India ink, Figure 1.9. These two capsules are very similar and differ only in the position of *O*-acetyl groups and the linkages between the amino sugars. The function of CP5 and CP8 in *S. aureus* virulence has been investigated in great depth especially with regard their role they take in impeding phagocytosis. Consequently *S. aureus* capsules have been at the centre of research aimed at developing a *S. aureus* vaccine (Fattom *et al.*, 2004). In addition to their role in avoiding host defence one author has also reported upon the potential for the capsule polysaccharide of mucoid *S. aureus* to provide a level of protection from chemotherapeutic treatments by posing a permeability barrier to antimicrobial agents (Kolawole, 1984).

Figure 1.9. *S. aureus* microcapsules are too small to be viewed by negative staining. (a) Hence transmission electron microscopy (TEM) is used to view the capsule polysaccharide. (i) CP5 microcapsule and (ii) acapsular *S. aureus*. Adapted from O'Riordan and Lee (2004) (b) The structures of *S. aureus* capsule polysaccharide types 5 and 8.

(a)



(b)

CP5; →4)-β-D-Man*p*NAcA3Ac(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-(1→

CP8; →3)-β-D-Man*p*NAcA4Ac(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Fuc*p*NAc-(1→

1.6 Small colony variants of *S. aureus*

The first description of a bacterial small colony variant (SCV) was in 1910, when an abnormal form of *Eberthella typhosa* (now known as *Salmonella enterica*) was reported (Jacobsen, 1910). Subsequently, SCVs have been described for a broad variety of genera and species, including the 1957 description of an *S. aureus* SCV (Jensen, 1957). Since their discovery *S. aureus* SCVs have been associated with persistent, recurrent and antibiotic resistant infections. In particular isolates from these unusually tenacious infections have harboured mutations in genes responsible for biosynthesis of thymidine and electron transport chain (ETC) electron carriers.

1.6.1 Characteristics of *S. aureus* small colony variants

S. aureus SCVs appear during exposure to antimicrobial compounds, most commonly the aminoglycosides. They are slow growing and appear as small colonies on tryptic soy or Mueller Hinton agar. The characteristic small colonies are approximately one tenth the size of wild type strains. Although many alterations in bacterial metabolism cause slow growth, only a limited number of defects are found in SCVs from clinical specimens, with two groups of SCVs being consistently recovered: SCVs that are deficient in electron transport and SCVs that are deficient in thymidine biosynthesis (Proctor *et al.*, 2006). Electron transport-deficient SCVs are menaquinone or haemin auxotrophs. Menaquinone and the prosthetic group haemin are essential components of staphylococcal ETC; menaquinone is the first electron acceptor, accepting electrons from $FADH_2$ or the NADH oxidase complex, whilst haemin is the component of cytochromes that receive electrons from menaquinone. Menaquinone and heme are synthesized using enzymes encoded by the *men* and *heme* operons, respectively (Tien & White, 1968).

Thymidine-auxotrophic SCVs are less well understood. Their phenotype is very similar to ETC-deficient SCVs, yet the link between genotype and phenotype is less explicit. It is possible that thymidine-auxotrophs are double mutants, because they can be recovered from pus, which is a plentiful source of thymidine for *S. aureus*. The mechanisms of thymidine-dependent SCVs persistence have not been elucidated fully,

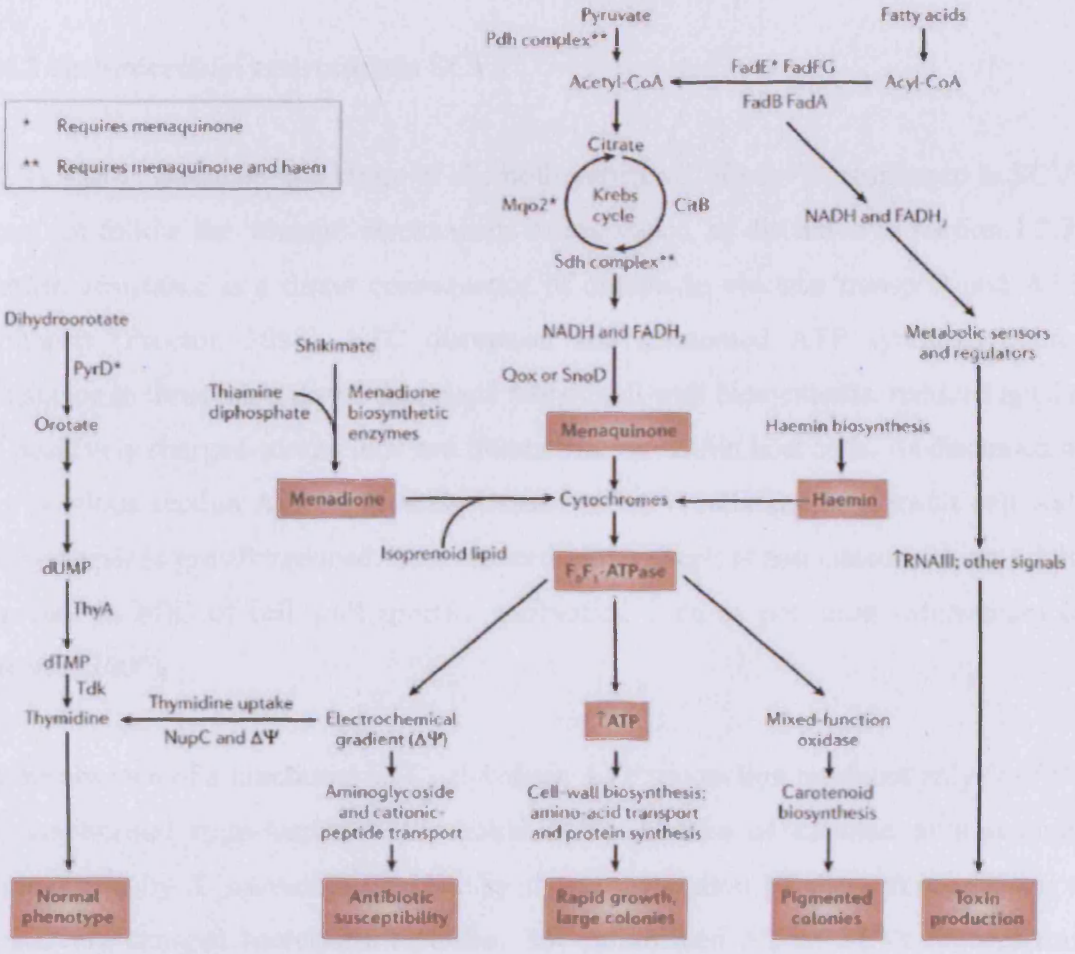
however they have been reported to show altered tricarboxylic acid cycle and acetic acid metabolism (Chatterjee *et al.*, 2007).

A typical pleiotropic phenotype is associated with SCVs, including slow growth rate, absence of pigmentation, reduced range of carbohydrate utilization and a lack of exoenzyme production (including virulence factors). In ETC-defective SCVs this phenotype can be directly related to the interruption of electron transport (McNamara & Proctor, 2000), Figure 1.10. Without oxidative respiration the *S. aureus* cells can produce ATP only through fermentation. This process is significantly less efficient, resulting in a severe shortage in ATP. Biosynthesis of cell components essential for growth, such as cell wall teichoic acid, require ATP. In addition, ATP is required for many cellular functions such as uptake of amino acids and carbohydrates and in the structure and maintenance of nucleic acids. Therefore, a lower capacity to produce ATP slows growth and results in bacteria that produce small colonies. Interestingly anaerobic growth of *S. aureus* mimics the SCV phenotype, because under such conditions menaquinone is not synthesized. This is in contrast to *E. coli* which has two quinines, menaquinone for anaerobic conditions and ubiquinone which is used when oxygen is present, enabling wild-type growth under anaerobic conditions.

ATP is required for carotenoid biosynthesis, hence SCVs are non-pigmented (McNamara & Proctor, 2000). Alteration of carbohydrate utilization, including manitol, can also be related to defects in electron transport. *S. aureus* cells devoid of an ETC can generate ATP directly from glucose; however, the utilization of other sugars requires an intact tricarboxylic acid cycle. The interruption of electron transport results in down-regulation of the tricarboxylic acid cycle, blocking the uptake of complex carbohydrates. The reduction in cellular ATP levels indirectly blocks the first step of the phosphotransferase system inhibiting the use of manitol, xylose, lactose, sucrose, maltose, and glycerol (Reizer *et al.*, 1988).

In general, interruption of electron transport alters bacterial virulence factor production. For example, staphylococcal SCVs show a consistent reduction in α-toxin production. Interestingly, this phenomenon occurs even though SCV cells are able to enter into stationary phase, a situation when *agr* (see section 1.1.2) is active and toxins are produced (von Eiff *et al.*, 1997b). Furthermore, a *hemB* mutant of *S. aureus*

Figure 1.10. Relationship between electron transport and the small colony variant phenotype in *S. aureus*. Defects in electron transport limit the amount of ATP that is used for is available for cell wall biosynthesis, which leads to a slower growth rate and, subsequently, smaller colonies. The dearth of energy also decreases pigment formation and reduces the membrane potential ($\Delta\Psi$), which results in decreased uptake of cationic compounds. Reproduced from Proctor *et al.* (2006).



(used as a model for studying ETC-deficient SCVs) displayed more fibrinogen binding sites than the parent strain. SCV internalization is mediated by fibronectin bridging between the bacterial fibronectin-binding proteins and the receptor $\alpha_5\beta_1$-integrin, which is present at the surface of eukaryotic cells. Importantly, the lack of α-toxin production can work in combination with the greater number of fibrinogen binding sites to enable SCVs to enter, rather than lyse, host cells (Dziewanowska *et al.*, 1999; Sinha *et al.*, 1999; Proctor *et al.*, 2006). Survival within host cells shields the bacteria from host defence systems and antibiotics unable to cross eukaryotic cell membranes, providing conditions highly conducive for persistent colonization.

1.6.2 Antimicrobial resistance in SCVs

SCVs show resistance to a range of chemotherapeutics. However, resistance in SCVs does not follow the 'classic' mechanisms of resistance, as discussed in section 1.2.3. Rather, resistance is a direct consequence of defects in electron transport and ATP synthesis (Proctor, 1998). ETC disruption and attenuated ATP synthesis confer resistance in three main ways: decreased rate of cell wall biosynthesis, reduced uptake of positively charged compounds and internalization within host cells. As discussed in the previous section ATP is severely limited in SCV cells and as a result cell wall biosynthesis is greatly reduced. This reduced biosynthesis is associated with an 4-fold increase in MIC of cell wall specific antibiotics, such as penicillin (McNamara & Proctor, 2000).

In the absence of a functional ETC, glycolytic ATP production produces only 60-70% of the normal trans-membrane potential ($\Delta\Psi$). Uptake of cationic antimicrobial compounds by *S. aureus* is enabled by the accumulation of the compounds on a negatively charged bacterial membrane. The diminished $\Delta\Psi$ of SCVs reduces this accumulation, and hence uptake, of positively charged antimicrobials. Reduced uptake by SCVs results in a 10-30 fold increase in MIC of aminoglycosides, lantibiotics, and cationic peptides (Kahl *et al.*, 1996; Koo *et al.*, 1996; McNamara & Proctor, 2000; Proctor *et al.*, 2006). The potency of other antimicrobials may also be affect by changes in $\Delta\Psi$, however this process affects only those compounds that require a large membrane electrochemical gradient for uptake.

The survival of SCVs within host cells also reduces the efficacy of antimicrobial therapeutics through the limited ability of these compounds to cross eukaryotic membranes; the host cell acts as a barrier against the drug. Of further concern is the substantial decrease in susceptibility SCVs show when adhering to a surface. SCVs adhering to fibronectin covered surfaces were shown to exhibit a >100-fold increase in MIC of oxacillin, vancomycin and fleroxacin. And when SCVs were both adhering to a surface and in stationary phase of growth, two conditions that reduce metabolic activity, they were found to be fully resistant to oxacillin and vancomycin, with fleroxacin exhibiting very limited effects (Chuard *et al.*, 1997).

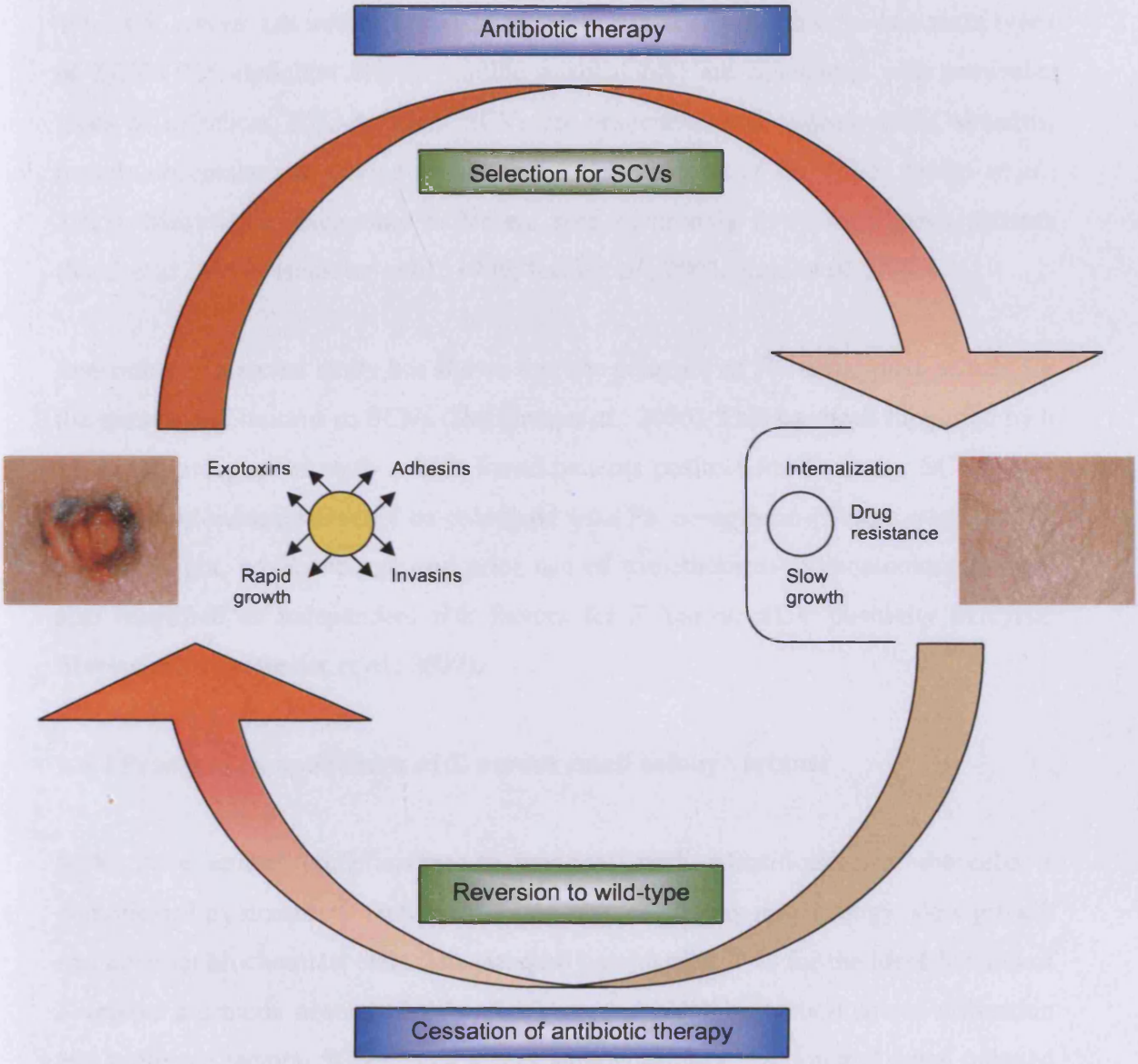
In addition to the widely studied aminoglycosides, a variety of other compounds can be used to select for *S. aureus* SCVs. These include inhibitors of cell-wall synthesis (vancomycin, cycloserine, and bacitracin), other antibiotics (e.g. chloramphenicol, trimethoprim-sulfamethoxazole, streptomycin), crystal violet, and barium chloride (Seligman, 2006).

1.6.3 Clinical complications of SCVs

It is postulated that SCVs are spontaneous mutants that are selected *in vivo* by antimicrobial exposure, their ability to survive within host cells and the low levels of free menaquinone and haemin. Patients infected with SCVs often have a history of long-term antibiotic treatment. *S. aureus* SCVs can cause tenacious infections. Individuals with SCV infections can experience prolonged disease-free periods that are punctuated by recurrences of normal colony morphology revertants, that are susceptible to previously used antimicrobial chemotherapeutics. Hence, patients can enter a prolonged cycle of infected and seemingly infection-free periods, Figure 1.11.

SCVs further complicate infection control and antibiotic prescribing practice by obscuring the aetiology of the infection and by their altered antibiogram. As discussed below, the altered phenotype of SCVs greatly complicates the bacterial isolation and identification procedures required to make a diagnosis and implement the appropriate therapy. Whilst the incidence of SCVs in clinical specimens has been found to range from 1% to more than 70% in different studies (Acar *et al.*, 1978; von Eiff *et al.*,

Figure 1.11. The cycle of recurrent SCV infections. Following diagnosis of an *S. aureus* infection antibiotics are administered. Whilst the therapy appears to clear the infection, the antibiotic is actually selecting for the SCV form of the organism. Due to reduced expression of virulence factors SCV infections have few symptoms. Consequently, clinicians believe the infection has cleared and antibiotic therapy is stopped. This removes the selective pressure that maintains the *S. aureus* cells in their SCV form, and they are free to revert to wild-type. The symptoms recur and the cycle starts again.



1997a; Kahl *et al.*, 2003), it is thought that SCVs are often overlooked or misidentified during routine clinical microbiology procedures. Although data concerning the virulence of SCVs are ambiguous, infections have proven fatal (Pelletier *et al.*, 1979; Seifert *et al.*, 1999; Jonsson *et al.*, 2003). Hence, misdiagnosis may have severe repercussions.

Whilst *S. aureus* can initiate a variety of infections, it appears that the two main types of SCV (ETC-deficient and thymidine auxotrophic) are associated with particular types of infection. ETC-deficient SCVs are associated with osteomyelitis, sinusitis, muscle abscesses and device related infections (Proctor *et al.*, 1995; Spanu *et al.*, 2005). Meanwhile, thymidine SCVs are seen commonly in cystic fibrosis patients (Kahl *et al.*, 1998; Haussler *et al.*, 1999; Kahl *et al.*, 2003; Kahl *et al.*, 2005).

Interestingly, a recent study has shown that the presence of *Ps. aeruginosa* selects for the growth of *S. aureus* as SCVs (Hoffman *et al.*, 2006). This has been supported by a 12-month prospective study which found patients positive for *S. aureus* SCVs were significantly more commonly co-colonized with *Ps. aeruginosa* (Besier *et al.*, 2007). Lower weight, advanced age and prior use of trimethoprim-sulfamethoxazole were also identified as independent risk factors for *S. aureus* SCV positivity in cystic fibrosis patients (Besier *et al.*, 2007).

1.6.4 Practical complications of *S. aureus* small colony variants

SCVs pose several complications to practical work. Identification of the cells is complicated by numerous factors including atypical colony morphology, slow growth and aberrant biochemical tests. The standard biochemical tests for the identification of *S. aureus* are made obsolete by the change in antibiogram, carbon source utilization and virulence factors; SCVs show a very slow coagulase reaction and much reduced haemolytic activity – two factors regularly used in *S. aureus* identification. As a result staphylococcal SCVs are difficult to recognize and are often misidentified as CoNS or varidans streptococci (McNamara & Proctor, 2000). Many selective agars and commercially available systems lack the reliability to identify and characterize *S. aureus* SCVs (Kipp *et al.*, 2005; Seligman, 2006). Further ambiguity is provided by the instability of their phenotype. The doubling time of a reverted bacterium,

depending on the growth medium, is six to nine times faster than that of SCVs, allowing revertants to out compete the SCVs during culture.

SCVs, depending on the mutation, revert to a wild type phenotype at various rates. For example, slow growing variants that follow penicillin treatment often revert on a single passage through fresh media. In contrast, streptomycin-induced SCVs are often stable through more than ten passages on fresh media (McNamara & Proctor, 2000).

The elucidation of antimicrobial susceptibility is also problematic with SCVs; slow growth often invalidates disc diffusion or automated methods. Currently, detection of the *mecA* gene by PCR and the MRSA-Screen latex agglutination test using higher colony numbers are the only methods shown to reliably and rapidly detect methicillin resistance in SCVs (Kipp *et al.*, 2004).

1.7 Aims and objectives of this study

The information provided in this chapter demonstrates clearly that the subject of biocide resistance in bacteria is an area where much further research is required. Required areas of study are the development of resistance, mechanisms of biocide action and mechanisms of resistance (McDonnell & Russell, 1999; Russell, 1999; White & McDermott, 2001; Russell, 2002a). The possibility of linked biocide and antibiotic resistance remains contentious (Walsh *et al.*, 2003; Braoudaki & Hilton, 2004; Sattar, 2006; Yazdankhah *et al.*, 2006), but given the scale of the problem with antibiotic resistance, it is clear that continued study in this area is also essential (Fraise, 2002).

The objective of this study, involving the important biocide triclosan and clinically significant organisms, was to investigate aspects of triclosan resistance and the potential role of horizontal gene transfer in *S. aureus*. This was achieved by the following aims:

- Investigate the role of clinically significant *S. aureus* capsule polysaccharides upon biocide susceptibility.

- Characterize the development of reduced susceptibility to triclosan in *S. aureus*.

- Identify whether reduced susceptibility to triclosan involves a single mechanism and if this is associated with Darwinian fitness effects.

- Investigate the potential for a reduction in triclosan susceptibility affecting resistance to other antimicrobials.

- Assess the incedence of reduced triclosan susceptibility in the community.

- Identify whether triclosan susceptibility in *S. aureus* is typical of staphylococci, or if there are differences amongst these species.

- Identify if there is a relationship between mupirocin resistance and reduced susceptibility.

- If a relationship is identified, is the resistance transferable amongst *S. aureus*?

- Investigate how triclosan affects gene transfer in staphylococci.

Whilst completing the work necessary to achieve these aims we discovered an intriguing feature of *S. aureus* during exposure to triclosan: the development of SCVs. Therefore, we also aimed to characterize this phenomenon as follows:

- Characterize the development of *S. aureus* SCVs in the presence of triclosan.

- Investigate the phenotypic and lifestyle properties of triclosan-induced SCVs.

- Identify the potential for SCVs to acquire and disseminate antimicrobial resistance by horizontal gene transfer.

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Chapter 2

Susceptibility of capsular *Staphylococcus aureus* strains to some antibiotics, triclosan and cationic biocides

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Seaman PF, Day MJ, Russell AD & Ochs D (2004) Susceptibility of capsular *Staphylococcus aureus* strains to some antibiotics, triclosan and cationic biocides. *Journal of Antimicrobial Chemotherapy* **54**: 696-698.

Sir,

Approximately 90% of *Staphylococcus aureus* isolates produce one of 11 serotypes of capsular polysaccharides. Serotypes CP5 and CP8 account for ~25% and 50%, respectively, of isolates recovered from humans, offering support for their pathogenic significance (O'Riordan & Lee, 2004). The importance and relevance of these capsule types is confirmed by the development of a conjugate vaccine, StaphVAX™ that includes type 5 and 8 capsule polysaccharides (Fattom *et al.*, 2004). CP5 and CP8 serotypes are considered as microcapsules because they are much smaller than those produced by the mucoid serotypes 1 and 2. The microcapsules of CP5 and CP8 are extracellular, uronic acid-containing polysaccharides that are too small to be visualized by negative stains such as India ink. These two capsules are very similar and differ only in the position of *O*-acetyl groups and the linkages between the amino sugars. The function of CP5 and CP8 in *S. aureus* virulence has been investigated in great depth, especially with regard to their role in impeding phagocytosis (O'Riordan & Lee, 2004). However, few authors have reported upon the potential for a capsule polysaccharide to present a permeability barrier to antimicrobial agents. Gram-positive bacteria possess a cell wall that is usually permeable and does not limit the incursion of antimicrobials. However, resistance through reduced penetration has been shown to occur, for example vancomycin-intermediate resistant *S. aureus* (VISA) strains produce a distinctly thickened cell wall (Lambert, 2002). Furthermore, in 1984 Kolawole discussed the effect of a mucoid capsule upon disinfectant and antiseptic susceptibility in *S. aureus*. He concluded that the thick capsule, as associated with serotypes CP1 and CP2, does provide a permeability barrier to common biocides but fell short of testing the more common and clinically important serotypes. We wish to report how CP5 and CP8 microcapsules affect the susceptibility of *S. aureus* to several antibiotics and three widely used biocides: triclosan, chlorhexidine gluconate and cetylpyridinium chloride.

A range of 14 antibiotic discs were purchased from Oxoid (Basingstoke, UK) and used to analyse *S. aureus* Reynolds and two isogenic capsule mutants. Antibiotic susceptibility was established as per the BSAC standardized disc susceptibility testing methodology (Andrews, 2001a) (Table 1). The MICs of nine of these clinically relevant antibiotics were elucidated by Etest strips (AB Biodisk, Sweden) according to the manufacturer's recommendations. MICs for the three biocides were calculated

Table 1. Antibiotic susceptibility of the three *S. aureus* capsule strains alongside the control strain NCTC 6571 for which the antibiogram is known.

			Antibiotic susceptibility											
Strain	Pen	Oxa	Van	Mup	Chl	Tet	Ery	Gen	Fd	Amp	Cec	Cro	Rif	Tec
CP5	R	S	S	S	S	S	S	S	S	R	R	S	S	S
CP8	R	S	S	S	S	S	S	S	S	R	R	S	S	S
CP-	R	S	S	S	S	S	R	S	S	R	R	S	S	S
6571	S	S	S	S	S	S	S	S	S	S	S	S	S	S

R, resistant; S, susceptible; Pen, penicillin; Oxa, oxacillin; Van, vancomycin; Mup, mupirocin; Chl, chloramphenicol; Tet, tetracycline; Ery, erythromycin; Gen, gentamicin; Fd, fusidic acid; Amp, ampicillin; Cec, cefaclor; Cro, ceftriaxone; Rif, rifampicin; Tec, teicoplanin.

using Iso-Sensitest agar (Oxoid, Basingstoke, UK), multipoint inoculator (Denley; Mast Diagnostics, Bootle, UK) and incubation in air at 37°C for 18–20 h, in accordance with BSAC guidelines (Andrews, 2001b). Triclosan (Irgasan DP300) was a gift from Ciba Speciality Chemicals; chlorhexidine gluconate and cetylpyridinium chloride were purchased from ICN Biomedicals Inc. (Ohio, USA). Of the three *S. aureus* Reynolds strains, one was wild-type, expressing a serotype CP5 capsule, one a mutant expressing CP8 and the third a second mutant, lacking a capsule (CP–) (Nilsson *et al.*, 1997). The acapsular Reynolds strain was constructed by replacing the serotype-specific capsule genes cap5HIJK on the bacterial chromosome with an *erm*(B) gene, conferring erythromycin resistance. *S. aureus* NCTC 6571 (Oxford) was included alongside the capsule strains as a control. The MICs were defined as the lowest concentration of antimicrobial with which there was no visible growth of the organism and are shown in Table 2, along with the Etest data. In addition, all strains were investigated for β-lactamase production by nitrocefin stick (Oxoid, Basingstoke, UK; Table 2).

The antibiogram for NCTC 6571, as deduced by antibiotic disc susceptibility testing, was as expected. No difference in antibiotic susceptibility was observed between the capsule strains other than for erythromycin, resistance to which was found in CP–. This was as expected due to the construction of the CP– strain by disruption of the serotype-specific capsule genes with an *erm*(B) gene. The nitrocefin test implies that penicillin and ampicillin resistance is conferred by β-lactamase production in *S. aureus* Reynolds. MICs for NCTC 6571 were within plus or minus one two-fold dilution of the expected MIC (Andrews, 2001b). The capsule strains all demonstrated MICs for each antimicrobial within plus or minus one two-fold dilution of each other. The only exception to this was—once again—erythromycin, confirming the results of the susceptibility disc test. From this we deduced that there is no significant difference between the antibiotic and biocide susceptibilities of the strains investigated herein. Consequently, we conclude that the capsule polysaccharide serotypes CP5 and CP8 do not present a permeability barrier to the antibiotics used in this investigation, or to triclosan, chlorhexidine gluconate or cetylpyridinium chloride.

Table 2. MICs for *S. aureus* Reynolds expressing either capsule serotype CP5, CP8 or CP– (acapsular).

		MIC (mg/L)											
Strain	β-lactamase	Pen[a]	Oxa[a]	Van[a]	Mup[a]	Chl[a]	Tet[a]	Ery[a]	Gen[a]	Fd[a]	Tric[b]	CHX[b]	CPC[b]
CP5	+	16	0.75	1	0.125	6	0.28	0.38	0.25	0.064	0.13	2	2
CP8	+	16	0.75	1	0.125	6	0.38	0.38	0.25	0.064	0.13	2	2
CP-	+	16	0.75	1	0.125	4	0.5	>256	0.19	0.064	0.13	2	2
6571	-	0.023	0.125	1	0.19	4	0.064	0.19	0.19	0.094	0.13	2	2

[a] Calculated by Etest (AB Biodisk, Sweden).

[b] Calculated in accordance with BSAC guidelines.

Pen, penicillin; Oxa, oxacillin; Van, vancomycin; Mup, mupirocin; Chl, chloramphenicol; Tet, tetracycline; Ery, erythromycin; Gen, gentamicin; Fd, fusidic acid; Tric, triclosan; CHX, chlorhexidine gluconate; CPC, cetylpyridinium chloride.

These results indicate that whereas *S. aureus* capsule polysaccharides are implicated in virulence, they are not involved in conferring reduced antibiotic or biocide susceptibility. Clinical implications are pertinent through the choice of clinically relevant antibiotics and biocides regularly used in the hospital setting.

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Chapter 3

FabI mutations, drug resistance and fitness costs of reduced triclosan susceptibility in *Staphylococcus aureus*

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Synopsis

Objectives: To evaluate the impact of mutations conferring reduced triclosan susceptibility upon antimicrobial susceptibility and fitness in *Staphylococcus aureus*.
Methods: We evaluated the effects of mutation towards reduced triclosan susceptibility upon antimicrobial susceptibility and fitness in methicillin-susceptible and methicillin-resistant *S. aureus*. Log_{10} reductions in viable cell numbers and potassium leakage were used to assess susceptibility to the bactericidal effects of triclosan. The *fabI* gene of reduced susceptibility mutants was sequenced and their fitness analyzed by investigating growth and relative competitive fitness.
Results: Several mutants with reduced susceptibility to triclosan (MIC of 1-2 mg/L) were isolated, most of which possessed a novel triclosan resistance mutation in the *fabI* gene, C284T. This mutation confers an alanine to valine substitution at the 95^{th} residue of the FabI protein. A reduced susceptibility strain with an unaltered *fabI* gene was also isolated. These mutants showed reduced susceptibility to the lethal effects of triclosan at 7.5 mg/L, but were as susceptible as the wild type at ≥20 mg/L. These mutations did not significantly affect bacterial fitness and did not affect susceptibility to a range of antimicrobials.
Conclusions: A lack of fitness cost supports the potential for development of low-level triclosan resistance. However, this level of resistance is of ambiguous significance and does not affect susceptibility to other antimicrobials. The description of a reduced susceptibility mutant without an associated *fabI* mutation and evidence of triclosan-induced cytoplasmic membrane damage indicates that the fabI protein is not the sole target for triclosan, providing weight to the argument that whilst fabI is undoubtedly a target for low-level triclosan, it is also has other modes of action.

Introduction

The biocide triclosan (Irgasan DP300; 2,4,4'-trichloro-2'-hydroxydiphenyl ether) is an antimicrobial used broadly in personal care and household applications, mainly in bar soap, toothpastes and mouthwashes, but also in hand disinfectants and other consumer health care products. Additionally, triclosan has found some use in the home through incorporation in domestic plastics such as chopping boards. It has activity against a broad spectrum of Gram-negative and Gram-positive microorganisms, including the

common nosocomial pathogens *Escherichia coli, Staphylococcus aureus* and *Enterobacter* spp. Following a successful application in 1987[1] and more recent reports, triclosan has been shown to be effective for the control of methicillin-resistant *S. aureus* (MRSA).[2-5] Consequently, 2% triclosan baths are now one of the recommended rationales for skin decolonisation of MRSA carriers.[6]

Triclosan is an important antimicrobial and due to its classification as a biocide, it was with some furore that resistance to the compound was reported.[7] MRSA strains were isolated with MICs of 2-4 mg/L from patients treated with daily triclosan baths; this compares with MICs of 0.001-0.1 mg/L for sensitive strains. Subsequently, 7.5% of *S. aureus* clinical strains were found to have triclosan MICs of at least 1 mg/L.[8] In 1998 McMurry *et al.*[9] postulated that, in contradiction to the biocidė dogma, triclosan acts on a specific cellular target; traditionally biocides have been viewed to act through multiple cytoplasmic and membranous targets.[10] The lack of a discrete mechanism of action makes the acquisition of cellular resistance unlikely. However, it has now been shown that a mode of action of triclosan is via inhibition of enoyl-acyl carrier protein (ACP) reductase (FabI) in organisms such as *S. aureus* and *E. coli* that rely on this enzyme to execute the final step in the elongation cycle of bacterial fatty acid biosynthesis.[9,11-13] In fact it appears that the mode of action of triclosan is concentration dependent;[14-16] low-level triclosan targets FabI but at bactericidal concentrations it appears to act upon multiple non-specific targets and has been shown to intercalate into the cell wall causing it to become destabilised, compromising its functional integrity.[17,18]

Two reduced triclosan susceptibility phenotypes are mediated by alterations to FabI; MICs raised above 0.016 mg/L result from a three- to five-fold increase in the levels of FabI production, whereas further reduced susceptibility (MIC = 1 to 2 mg/L) results from alteration of the native FabI as a consequence of spontaneous mutations in the *fabI* gene in combination within increased enzyme production.[19] A number of predicted alterations to the FabI amino acid sequence that confer reduced triclosan susceptibility have been described.[19-21] The significance of such increases in MIC is uncertain. These MICs are far below the in-use concentrations of triclosan in hand wash, bath formulations (0.1 to 0.3%, or 1000 to 3000 mg/L, respectively) and surface disinfectants (0.1 to 1%, or 1000 to 10,000 mg/L, respectively), for hospital wash

applications for MRSA eradication even 2% (20,000 mg/L) triclosan is recommended.[6] These in-use concentrations are magnitudes higher than the concentrations of triclosan needed to inhibit raised MIC isolates described to date. Additionally, during clinical or domestic use, triclosan is delivered typically as part of complex formulations containing various combinations of surfactants, detergents, chelating agents and wetting agents, which support the antimicrobial activity of triclosan and ultimately affect the susceptibility of microorganisms exposed to it. However, in nature bacteria often grow as complex biofilms and this can impact upon antibacterial susceptibility,[22] residual levels of triclosan may be closer to the MICs of reduced susceptibility strains and the presence of triclosan provides a selection pressure for acquisition of resistance by horizontal transfer, if this is possible. More recently, low-level resistance to triclosan has been described in small colony variants (SCV) of *S. aureus*.[23] The mode of reduced susceptibility to triclosan in SCVs is yet to be fully understood, however their *fabI* gene sequences were unchanged compared to their parent, implying that alterations in the FabI protein were not involved.

Mutations in drug targets that confer resistance to antimicrobials can impose fitness costs on the organism.[24] The occurrence of some *fabI* mutations in clinical staphylococcal isolates showing reduced triclosan susceptibility appears to reflect a low-cost of these mutations in terms of bacterial fitness. In contrast, other *fabI* mutations may impose fitness costs, precluding their appearance.

There is a paucity of data discussing the impact of reduced susceptibility upon the clinical efficacy of triclosan and to what extent resistant strains will impact upon healthcare. Herein we discuss the molecular basis of reduced triclosan susceptibility in *S. aureus* and investigate its associated fitness costs. Furthermore, we describe the lethal effects of triclosan upon wild type and reduced susceptibility *S. aureus* and discuss whether triclosan does truly have a single cytoplasmic target.

Materials and Methods

Unless otherwise stated, all concentrations are % weight/volume. All experiments were performed in triplicate (n=3) and unless otherwise stated results are presented as mean ± standard error of the mean (s.e.).

S. aureus strains and growth conditions. Table 3.1 describes the 37 *S. aureus* strains used within this investigation. Prior to experimentation all strains were confirmed as *S. aureus* by streaking on Vogel-Johnson agar (VJA; Oxoid) and latex-agglutination test for extra-cellular coagulase (Staphylase test; Oxoid). Strains were stored in 50% glycerol at -80°C and, when required, maintained on either nutrient agar (NA; Oxoid), Iso-Sensitest agar (ISA; Oxoid) or Mueller Hinton agar (MHA; Oxoid). Over-night cultures were prepared by inoculation of 10 mL nutrient broth (NB; Oxoid) or Iso-Sensitest broth (ISB; Oxoid) with colonies from a streak plate and incubation in an orbital shaker at 37°C for 15 h.

S. aureus mutants with reduced susceptibility to triclosan were isolated by the growth of *S. aureus* strains to mid-log phase in 10 mL ISB at 37°C. At this point pre-warmed triclosan solution (100 mg/L) was added to achieve a final concentration of 0.01 mg/L, further incubation was performed for 6 h at 37°C. Cells were harvested by centrifugation (5000 rev/min for 15 min), washed and resuspended in 10 mL quarter-strength Ringer's solution (Oxoid). 100 µL volumes of 10^9 cells/mL were spread on to the surface of ISA plates containing 0-10 mg/L triclosan. Plates were incubated for 48 h at 37°C. Suspected reduced triclosan susceptibility mutants were observed as pigmented colonies on plates containing triclosan and confirmed as *S. aureus* by streaking onto VJA, coagulase test and multiplex PCR, as described previously.[25] Such colonies were maintained on NA supplemented with 1 mg/L triclosan, from which over-night cultures prepared in NB or Mueller Hinton broth (MHB; Oxoid) with 1 mg/L triclosan were made when required. Stability of reduced triclosan susceptibility was examined by sub-culture in triclosan-free NB with sporadic testing for triclosan MIC. The phenotype was designated as stable if no reduction in MIC was seen following a course of 10 sub-cultures. Triclosan powder (Irgasan DP300) was a gift from Ciba Spezialitätenchemie Grenzach GmbH (Grenzach-Wyhlen, Germany) and was dissolved in sterile dimethylsulphoxide (DMSO). DMSO concentrations were maintained below 1% in growth media and DMSO controls were run alongside experiments where required.

Biocide and antibiotic susceptibility. MICs for three biocides, triclosan, chlorhexidine gluconate (CHX; ICN Biomedicals Inc., USA) and cetylpyridinium chloride (CPC;

Table 3.1. *S. aureus* strains used during this investigation.

S. aureus strain	Description	Reference/Source
NCTC6571	Oxford strain, isolated in 1940. Highly susceptible to many antimicrobials	NCTC
Mu50	Genome sequenced VISA strain	[60]
NCIMB9518	Triclosan susceptible industrial strain	[61]
9518T1	Low-level reduced triclosan susceptibility mutant of NCIMB9518	[61]
9518T2	Low-level reduced triclosan susceptibility mutant of NCIMB9518	[61]
9518T3	Low-level reduced triclosan susceptibility mutant of NCIMB9518	[61]
9518T4	Low-level reduced triclosan susceptibility mutant of NCIMB9518	This study
9518Rif	Rifampicin resistant mutant of NCIMB9518	This study
F89	High-level mupirocin resistant strain	[62]
F89T1	Low-level reduced triclosan susceptibility mutant of F89	This study
24500	Clinical MRSA isolate	UHW, Cardiff
24500T1	Low-level reduced triclosan susceptibility mutant of 24500	This study
27343	Clinical MRSA isolate	UHW, Cardiff
27343T1	Low-level reduced triclosan susceptibility mutant of 27343	This study
24532	Clinical MRSA isolate	UHW, Cardiff
27393	Clinical MRSA isolate	UHW, Cardiff
27368	Clinical MRSA isolate	UHW, Cardiff
24497	Clinical MRSA isolate	UHW, Cardiff
24467	Clinical MRSA isolate	UHW, Cardiff
22373	Clinical MRSA isolate	SDGH, Swansea
223065	Clinical MRSA isolate	SDGH, Swansea
223021	Clinical MRSA isolate	SDGH, Swansea
224035	Clinical MRSA isolate	SDGH, Swansea
224005	Clinical MRSA isolate	SDGH, Swansea
223232	Clinical MRSA isolate	SDGH, Swansea
223278	Clinical MRSA isolate	SDGH, Swansea
223058	Clinical MRSA isolate	SDGH, Swansea
269263	Clinical MRSA isolate with low-level mupirocin resistance	UHW, Cardiff
260379	Clinical MRSA isolate with low-level mupirocin resistance	UHW, Cardiff
266261	Clinical MRSA isolate with low-level mupirocin resistance	UHW, Cardiff
260556	Clinical MRSA isolate with low-level mupirocin resistance	UHW, Cardiff
247378	Clinical MRSA isolate with high-level mupirocin resistance	UHW, Cardiff
259658	Clinical MRSA isolate with high-level mupirocin resistance	UHW, Cardiff
260711	Clinical MRSA isolate with high-level mupirocin resistance	UHW, Cardiff
260989	Clinical MRSA isolate with high-level mupirocin resistance	UHW, Cardiff
269026	Clinical MRSA isolate with high-level mupirocin resistance	UHW, Cardiff
B17637	Clinical MRSA isolate with high-level mupirocin resistance	UHW, Cardiff

UHW, University Hospital of Wales; SDGH, Singleton District General Hospital.

ICN Biomedicals Inc., USA), were calculated following the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC).[26] Etest® strips (Bio-stat Ltd, UK) were utilized to attain the MIC for antibiotics, as per the manufacturer's directions. Significant effect of mupirocin resistance upon triclosan MIC was analysed by Kruskal-Wallis test. Significant effect of mutation to reduced triclosan-susceptibility upon antimicrobial susceptibility was analysed by analysis of variance (ANOVA).

Calculation of mutation rate. The rate of mutation towards reduced triclosan susceptibility was assessed by growth of *S. aureus* strains to late-log phase in 10 mL NB at 37°C. At this point cells were harvested by centrifugation (5000 rev/min for 15 min), washed, resuspended in quarter-strength Ringer's solution and adjusted to 10^{10} cells/mL. 100 µL volumes of 10^{10} cells/mL were spread on to the surface of NA agar plates containing 0.1, 0.5, 1.0, 2.0 and 10 mg/L triclosan. Additionally, dilutions of the cultures were plated onto NA in order to calculate the number of wild type CFU. Plates were incubated for 48 h at 37°C. Colonies that grew on triclosan plates were counted and a selection streaked on VJA and analysed for coagulase to confirm as *S. aureus*. Frequencies were expressed as numbers of mutants per CFU.

Sequencing of the fabI gene. Chromosomal DNA was extracted as described previously[27] and the *fabI* gene sequenced as per Seaman *et al.*[23] Sequence construction and analyses were performed using BioEdit version 7.0.5.3.[28]

Bactericidal effects of triclosan. The bactericidal effects of triclosan on wild-type *S. aureus* and their reduced susceptibility mutants were compared as previously reported[29] except the inoculum was adjusted to 10^7 cfu/mL and sampling was continued up to 40 min. Log reduction was then calculated from $\log_{10}N_c - \log_{10}N_t$, where N_c and N_t represent the numbers of cfu/mL in the control and triclosan solutions, respectively. The same method was used when assessing the lethal effects of Irgacide LP10 except a stock solution of Irgacide LP10 (pH 5.5, 10% triclosan, 4% sodium lauryl sulphate; gift from Ciba Spezialitätenchemie Grenzach GmbH) was used to achieve final triclosan concentrations of 7.5×10^{-5}, 2.0×10^{-4}, 0.2 and 2.0%.

To investigate potassium ion leakage from cells during exposure to triclosan, cultures were grown on the surface of an NA plate at 37°C for 24 h. Cells were then emulsified

in 5 mL of 0.9% NaCl (Sigma), washed three times by centrifugation and resuspended in 100 mL of 0.9% NaCl, with the appropriate concentration of triclosan, to give a bacterial concentration of 10^9 cfu/mL. At timed intervals, 10 mL was removed and filtered through a 0.2 µm cellulose nitrate filter to remove cellular material. The potassium concentration in the supernatant was then measured using an atomic absorption spectrophotometer (Varian Spectra AA100). As control experiments, cells were incubated in triclosan-free NaCl solution (negative control) and treated with lysostaphin 100 mg/L (Sigma) for 30 min at 30°C and heated at 100°C for 10 min (positive control). Significant effect of triclosan or Irgacide LP10 on the reduction in cell density and potassium leakage was tested with ANOVA.

Efficacy and toxicity of triclosan neutralizing solution. The neutralizer used here was based on the British Standard EN 1499.[30] The solution was made fresh in distilled water and contained 30 g/L Tween 80 (Sigma), 3 g/L lecithin from eggs (Sigma), 1 g/L L-histidine (Sigma) and 5 g/L sodium thiosulphate (Fisher). Both the efficacy and the toxicity of the neutralizer were investigated prior to testing with methods described by Walsh *et al.* (1999)[31] and Langsrud and Sundheim (1998)[32] respectively. Briefly, 8 mL of neutralizer, 100 µL of triclosan (10 mg/mL) and 900 µL sterile de-ionized water was inoculated with 1 mL of an 18 h culture of *S. aureus* NCIMB9518 grown at 37°C. The viable cell count was enumerated after 5 min contact time at ambient temperature. As controls, sterile de-ionized water was added to the incubation mixture instead of the triclosan solution or the neutralizer. During exposure of the cells to triclosan without the neutralizer no viable cells were detected in the sample after the 5 min incubation period. There was no significant difference ($P = 0.869$) between the viable cell count of cultures exposed to triclosan and the neutralizer and where water was substituted for triclosan. This confirmed the ability of the neutralizer to quench bactericidal activity of triclosan at 100 mg/L. When examining the possible toxicity of the neutralizer, there was no significant difference ($P = >0.05$) between the viable cell counts of cultures exposed to sterile water and those exposed to the neutralizer for 15 min, confirming that the neutralizer was non-toxic to those strains under investigation.

Analysis of fitness. Growth curves were attained by spectrophotometric analysis (580 nm) of MHB cultures using a DYNEX Technologies MRX® Microplate Absorbance

Reader with Revelation™ application programme. In each well of a sterile 96-well microtitre plate (Fisher Scientific, UK) 100 µL of ISB or ISB containing 2× final concentration of triclosan was inoculated with 100 µL of ISB culture, diluted with broth to 10^6 cells per mL. Plates were sealed with adhesive plate seals (ABgene, UK) and incubation was performed at 37°C with agitation for 48 h. Significant effect of mutation towards reduced triclosan susceptibility and effect of triclosan on bacterial growth was tested with ANOVA or Mann-Whitney test. All data are presented as the mean ± s.e.

The relative competitive fitness of reduced triclosan susceptibility mutants and parental strains was measured in pair-wise competition experiments. ISB was inoculated with a mixture of overnight cultures of triclosan-susceptible parental strain ($tric^s$) and reduced-susceptibility mutant ($tric^r$) in a 1:1 ratio. The number of viable $tric^s$ and $tric^r$ were determined by plating dilutions of the mixed cultures onto both non-selective ISA and selective ISA containing triclosan at a concentration of 4 x MIC for the parental strain at time 0 and after 24 h incubation at 37°C. A minimum of three independent cultures were used and the relative competitive fitness, W, was calculated according to Lenski (1988):[33]

$$W = \frac{\ln(a_1/a_0)/day}{\ln(b_1/b_0)/day}$$

where a_0 and a_1 are the cell density of the parent strain at time 0 and 1 day, respectively. Likewise for b_0 and b_1, but representing the cell density of the mutant strain. A control culture of just the parent strain was run alongside and plated on the selective ISA to control against spontaneous $tric^r$ mutants of NCIMB9518.

To assess the fitness of the strains during exposure to triclosan the experiment was repeated with the presence of 0.5 mg/L triclosan in the growth media. Due to the exposure to triclosan the number of viable parent cells was expected to diminish during the course of the mixed infection. Hence, to enumerate this shrinking population it was necessary to use rifampicin resistant (Rif^R) mutants of the parent strain and to adjust the selective media accordingly (ISA containing 10 mg/L rifampicin and ISA containing triclosan at a concentration of 0.5 mg/L). Rif^R mutants were isolated[34] and investigated for affect upon fitness as described previously.[35] This

ensured bacterial fitness was not affected by Rif^R. Under conditions of antimicrobial stress it was preferable to express the performance of the strains in terms of selection rates, *r*:[33]

$$r = \frac{\ln(a_1/a_0) - \ln(b_1/b_0)}{day}$$

where, a_0 and a_1 are the cell density of the mutant strain at time 0 and 1 day, respectively. Likewise for b_0 and b_1, but representing the cell density of the parent strain.

Results

Triclosan resistance in S. aureus and reduced susceptibility mutants

Triclosan MICs ranged between 0.03 and 0.06 mg/L for clinical isolates and lab strains, whilst mutants selected for reduced triclosan susceptibility showed MICs of 1-2 mg/L, Table 3.2.

Reduced triclosan susceptibility was stable in all mutants selected for culturing. Reduced susceptibility mutants were isolated at rates of between $3.5\times10^{-9} \pm 1.05\times10^{-10}$ and $\sim1.0\times10^{-11}$ per CFU (level of detection) (Figure 3.1). There was no significant difference between the rates of mutation in MRSA and MSSA or between mupirocin-resistant (MupRSA) and mupirocin-susceptible (MupSSA) strains (P = 0.754 and 0.917, respectively). However, rate of mutation was dependent upon the concentration of triclosan used for selection. The rate of selection for mutants was similar on 0.1, 0.5 and 1.0 mg/L triclosan (P = 0.122), but was significantly reduced when the selection medium contained 2 or 10 mg/L triclosan (P = 0.001 and <0.001, respectively).

Antibiotic and biocide resistance in reduced-triclosan susceptible S. aureus.

The MICs of several antibiotics and biocides before and after mutation to reduced-triclosan susceptibility are shown in Table 3.3. No significant difference was found between triclosan MICs of mupirocin-susceptible and mupirocin-resistant *S. aureus*

Table 3.2. MICs of triclosan and antibiotic resistance profiles for *S. aureus* strains.

S. aureus strain	Antibiotic resistance profile	Triclosan MIC (mg/L)
NCTC6571	-	0.03
Mu50	Pen, Oxa, Van^{int}, Tet, Ery, Cip, Amp, Cec, Cro, Rif	0.06
NCIMB9518	-	0.03
9518T1	-	1.0
9518T2	-	2.0
9518T3	-	2.0
9518T4	-	1.0
9518Rif	Rif	0.03
F89	Pen, Mup^{H}, Tec, Amp, Cec	0.03
F89T1	Pen, Mup^{H}, Tec, Amp, Cec	1.0
24500	Pen, Oxa, Cip, Amp, Cec, Cro	0.03
24500T1	Pen, Oxa, Cip, Amp, Cec, Cro	1.5
27343	Pen, Oxa, Cip, Amp, Cec, Cro	0.06
27343T1	Pen, Oxa, Cip, Amp, Cec, Cro	1.0
24532	Pen, Oxa, Ery, Tec, Cip, Amp, Cec, Cro	0.03
27393	Pen, Oxa, Ery, Cip, , Amp, Cec, Cro	0.03
27368	Pen, Oxa, Ery, Cip, Amp, Cec, Cro	0.06
24497	Pen, Oxa, Ery, Cip, , Amp, Cec, Cro	0.03
24467	Pen, Oxa, Cip, Amp, Cec, Cro	0.06
22373	Pen, Oxa, Cip, Amp	0.06
223065	Pen, Oxa, Cip, Amp	0.03
223021	Pen, Oxa, Ery, Cip, Amp	0.13
224035	Pen, Oxa, Ery, Cip, Amp	0.03
224005	Pen, Oxa, Cip, Amp	0.06
223232	Pen, Oxa, Ery, Cip, Amp	0.03
223278	Pen, Oxa, Cip, Amp	0.06
223058	Pen, Oxa, Cip, Amp	0.13
269263	Pen, Oxa, Gen, Cip, Amp	0.03
260379	Pen, Oxa, Mup^{L}, Ery, Cip, Amp, Cec, Cro, Rif	0.06
266261	Pen, Oxa, Mup^{L}, Ery, Cip, Amp, Cec, Cro	0.06
260556	Pen, Oxa, Mup^{L}, Ery, Cip, Amp, Cec, Cro	0.13
247378	Pen, Oxa, Mup^{H}, Ery, Cip, Amp, Cec, Cro	0.03
259658	Pen, Mup^{H}, Ery, Amp, Cec, Cro	0.06
260711	Pen, Mup^{H}, Ery, Amp, Cec, Cro, Rif	0.06
260989	Pen, Mup^{H}, Ery, Amp, Cec, Cro, Rif	0.03
269026	Pen, Mup^{H}, Amp, Cec, Cro, Rif	0.13
B17637	Pen, Mup^{H}, Ery, Amp, Cec, Cro, Rif	0.03

Pen, Penicillin; Oxa, Oxacillin; Van^{int}, Intermediate-Vancomycin; Mup^{L}, Low-level Mupirocin; Mup^{H}, High-level Mupirocin; TET, Tetracycline; ERY, Erythromycin; GEN, Gentamicin; TEC, Teicoplanin; CIP, Ciprofloxacin; Amp, Ampicillin; Cec, Ceflacor; Cro, Ceftriaxone; Rif, Rifampicin.

Figure 3.1. Affect of genetic background upon rate of mutation to triclosan resistance. There was no significant difference between the mean rate of mutation to reduced triclosan susceptibility for MSSA (filled squares), MRSA (filled triangles) and MupRSA (filled circles) strains (P = >0.05). Points represent mean ± s.e.

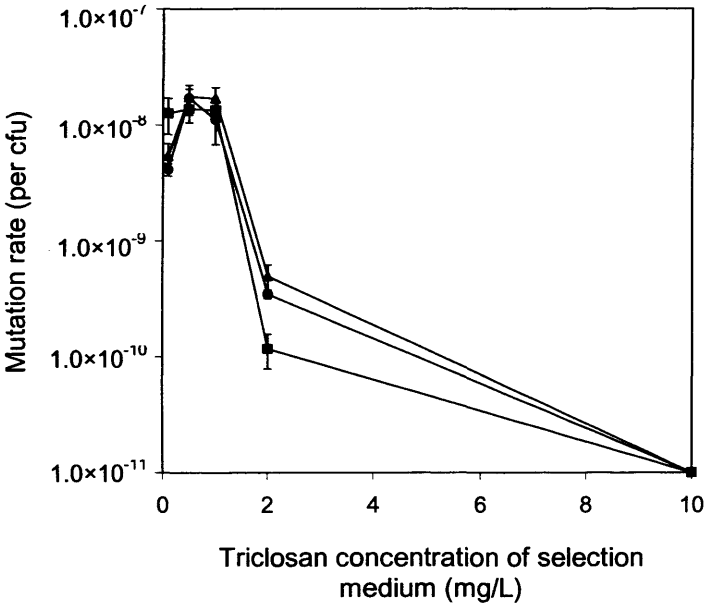


Table 3.3. Antibiotic and biocide MICs before and after mutation to reduced triclosan susceptibility.

Strain	Minimum Inhibitory Concentration (mg/L)													
	PEN†	OXA†	VAN†	MUP†	CHL†	TET†	ERY†	GEN†	LZD†	TEC†	CIP†	Tric‡	CHX‡	CPC‡
NCIMB9518	<0.016	0.125	1	0.19	8	0.19	0.38	0.19	0.5	0.38	0.19	0.03	1	1
9518T1	0.023	0.125	1	0.38	8	0.5	0.38	<0.064	0.5	0.38	0.19	1	1	1
9518T2	0.023	0.125	1.5	0.19	6	0.25	0.5	0.125	0.5	0.38	0.19	2	1	1
9518T3	0.023	0.125	1.5	0.125	6	0.25	0.38	0.125	0.5	0.38	0.19	2	1	1
9518T4	0.023	0.125	1	0.19	8	0.25	0.38	0.125	0.5	0.38	0.19	1	1	1
F89	1	0.75	1.5	>1024	4	0.5	>256	0.19	0.5	0.75	0.38	0.03	1	1
F89T1	1	0.38	1.5	>1024	4	0.5	>256	0.25	0.5	0.75	0.19	1	1	1
24500	48	64	1.5	0.25	4	0.5	0.38	0.19	0.5	0.25	>32	0.063	3	3
24500T1	48	48	1	0.38	4	0.5	0.5	0.125	0.5	0.25	>32	1.5	3	3
27343	32	>256	1	0.38	8	0.5	0.38	0.19	0.38	0.5	>32	0.063	2	2
27343T1	48	>256	1	0.38	8	0.25	0.38	0.19	0.5	0.5	>32	1	2	2

†Calculated by Etest (AB Biodisk, Sweden). ‡Calculated in accordance with BSAC guidelines. PEN, Penicillin; OXA, Oxacillin; VAN, Vancomycin; MUP, Mupirocin; CHL, Chloramphenicol; TET, Tetracycline; ERY, Erythromycin; GEN, Gentamicin; LZD, Linezolid; TEC, Teicoplanin; CIP, Ciprofloxacin; Tric, triclosan; CHX, chlorhexidine gluconate; CPC, cetylpyridinium chloride.

(P = 0.712). Mutation to reduced triclosan susceptibility was not shown to affect susceptibility to another antimicrobial, including mupirocin, which has previously been associated with low-level triclosan resistance.[7] MRSA and MSSA strains were equally susceptible to triclosan.

Bactericidal effects of triclosan

We investigated the effect of a raised triclosan MIC upon the bactericidal effects of this biocide. Lethality of triclosan at 7.5 and 20 mg/L was examined on reduced susceptibility strains and their progenitor. In an effort to model the effects of triclosan in a complex formulation, as employed in the clinic, this experiment was repeated with 7.5×10^{-4}, 2.0×10^{-3}, 2 and 20 % Irgacide LP10 (equivalent to 7.5, 20, 2000 and 20,000 mg/L triclosan respectively). Our results show that, contrary to previous reports, reduced susceptibility to triclosan does affect the bactericidal effects of 7.5 mg/L triclosan (Figure 3.2). However, all mutants were as susceptible as their parent strain to the lethal effects of triclosan at 20 mg/L. 2 and 20% Irgacide LP10 exhibited very high bactericidal efficacy; both reduced cultures by 7 Logs within 0.5 min and there was no significant difference between the killing of wild type or reduced susceptibility strains (data not shown). At much lower concentrations of Irgacide LP10 the killing was not so rapid and comparable to that of standard triclosan (Figure 3.2). We found no significant difference between the bactericidal effects of triclosan or Irgacide LP10 in exponentially growing cells, stationary phase cells and non-growing cells (P = >0.05; data not shown).

The leakage of potassium from cells is an indication of cytoplasmic membrane damage and consequently cell death. We measured triclosan-induced potassium leakage in NCIMB9518 and reduced susceptibility mutants to identify if there was a correlation between MIC and susceptibility to the cytoplasmic effects of triclosan. Triclosan caused significant potassium leakage in all strains and at all concentrations (Figure 3.3). However, 7.5 mg/L triclosan and 7.5×10^{-4} % Irgacide LP10 induced significantly less membrane damage than higher concentrations (P = <0.05) and this was dependent upon triclosan MIC; potassium leakage was less in mutants with reduced susceptibility to triclosan. However, this correlation between MIC and

Figure 3.2. The bactericidal effects of triclosan or Irgacide LP10 on *S. aureus* NCIMB9518 (solid line) and four mutants with reduced susceptibility to triclosan (dashed lines; T1, filled diamond; T2, filled triangle; T3, filled circle ; T4, cross). **(a)** Treatment with 7.5 mg/L triclosan or **(b)** equivalent Irgacide LP10 produced a kill rate dependent upon strain MIC. At these concentrations the lethal effect of triclosan is significantly different between wild type and mutants (P= <0.05). The times for a five $\log_{10}$ reduction in cell density were increased from 13 min for wild type *S. aureus* to 20 min for C284T mutant strain 9518T1, 34 and 33 for double mutants (C284T and T-108G) 9518T2 and T3 respectively and 36 min for 9518T4. However, at higher concentrations of triclosan, **(c)** 20 mg/L triclosan and **(d)** 2.0×10^{-3}% Irgacide LP10, no significant difference was observed between the lethal effect upon wild type and mutants (P = >0.05). Data are the mean of three measurements ± s.e.

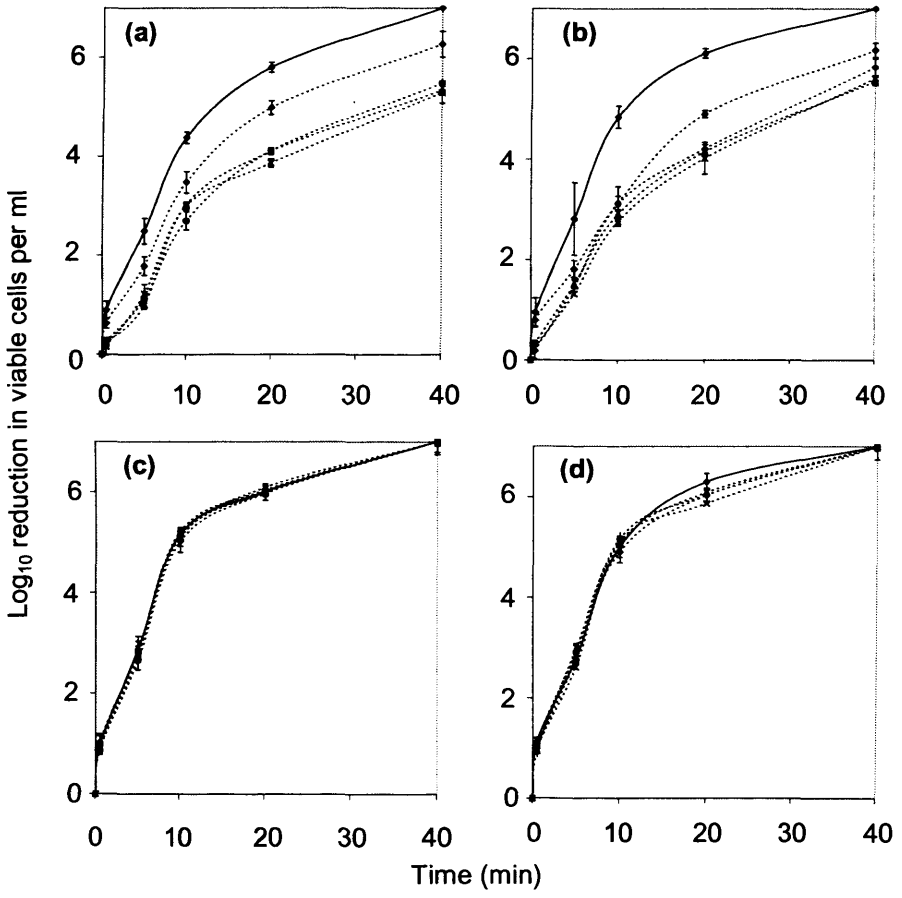
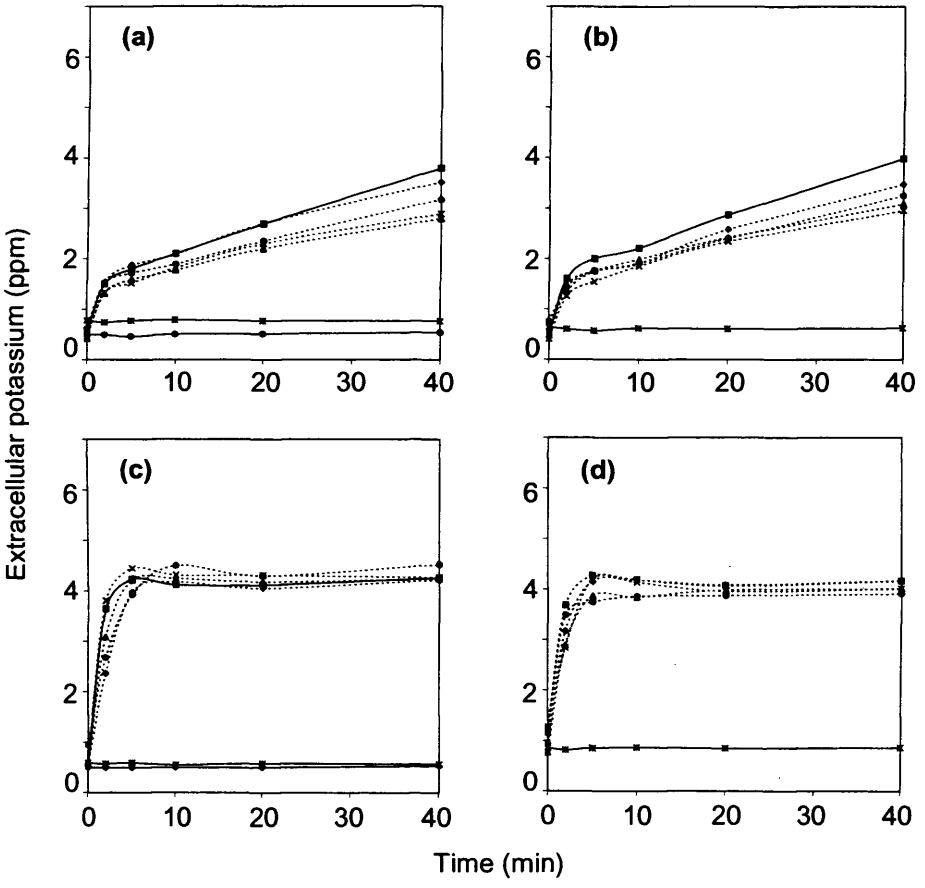


Figure 3.3. Estimation of cytoplasmic membrane damage by measurement of extracellular potassium concentration. (a) The leakage of potassium from NCIMB9518 (solid line) and reduced susceptibility mutants (dashed lines; T1, filled diamond; T2, filled triangle; T3, filled circle ; T4, cross) was assessed when exposed to 7.5 mg/L triclosan and **(b)** 7.5×10^{-4}% Irgacide LP10. **(c)** and **(d)** There was no significant difference in triclosan-induced cytoplasmic membrane damage between wild type and mutants at 20 mg/L triclosan or equivalent Irgacide LP10 ($P = >0.05$).



potassium leakage was not statistically significant, unlike when assessing cell death described above (Figure 3.4).

FabI mutation detection

The *fabI* genes of all triclosan mutants and their progenitor strains were amplified and sequenced, including the putative promoter region *(see appendix 9.1)*. The sequence was highly conserved amongst all strains. F89 showed the greatest sequence diversity, with a total of 5 base pairs difference, none of which altered the amino acid sequence. Most triclosan mutants (9518T1, 9518T2, 9518T3, 24500T1 and 27343T1) had a previously un-described C284T mutation, conferring an alanine to valine substitution at the 95th residue. Furthermore, 9518T2 and 9518T3, mutants with the highest MICs, also possessed a mutation in the putative promoter region (T-108G). 9518T4 showed no change in its *fabI* gene sequence.

Fitness costs of fabI mutation

Fitness of reduced susceptibility mutants was compared to that of their parent strain by analysis of their growth curves and in competitive fitness experiments. There was no significant difference between the doubling times of C284T mutants and their parent strain (P = 0.47) or between those carrying both the C284T and T-108G mutation (P = 0.48) whilst under conditions free from triclosan, Table 3.4. The mutations also had no significant effect upon the competitive fitness of the strains (P = 0.867). The results showed a significant difference between the doubling time and competitive fitness of mutants and their parent strain in the presence of 0.5 mg/L triclosan (P = 0.014). Parent strains were unable to grow in triclosan media and as a result were vastly out competed by mutant strains that were unaffected by the presence or absence of 0.5 mg/L triclosan (P = 0.848).

Discussion

Triclosan resistance and antimicrobial susceptibility. Wild type *S. aureus* strains were highly susceptible to triclosan, exhibiting MICs far below the concentrations expected to be encountered *in situ*. In congruence with some reports, MRSA and MSSA strains

Figure 3.4. Correlation between susceptibility to bactericidal effects and triclosan MIC. (a) The rate of kill when exposed to 7.5 mg/L triclosan (filled triangles) or 7.5×10^{-5} % Irgacide LP10 (open triangles) was significantly effected by triclosan MIC. **(b)** This was not true for concentrations ≥20 mg/L or 2.0×10^{-3}% or when measuring potassium leakage. Filled squares represent 20 mg/L triclosan; open squares represents 2.0×10^{-3}% Irgacide LP10; Open circles represent 2.0% Irgacide LP10; crosses represent 20% Irgacide LP10.

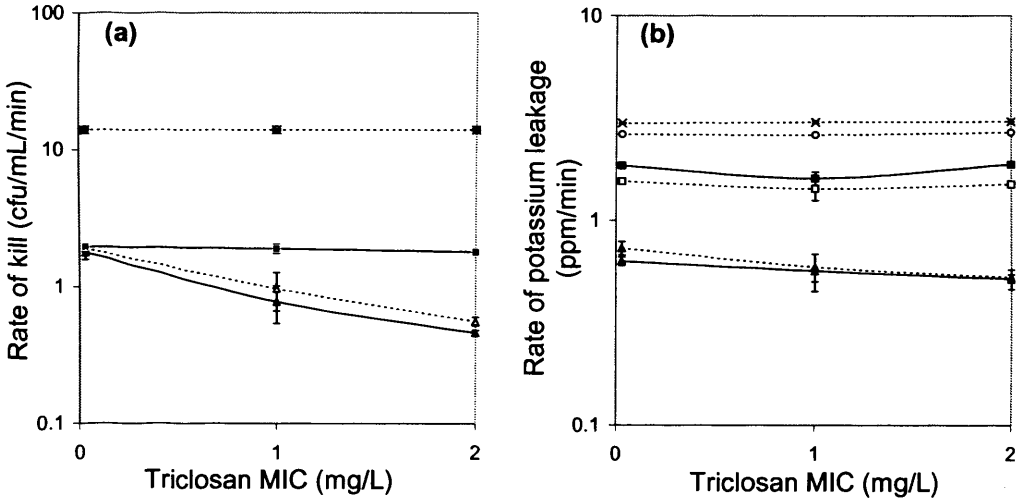


Table 3.4. Mutations associated with reduced triclosan susceptibility and their effects on bacterial growth and fitness with and without exposure to triclosan.

Strain	Triclosan MIC (mg/L)	MHB[a]		MHB with 0.5 mg/L triclosan[a]		Substitutions	
		Doubling time (min)	Competitive Fitness, *W*	Doubling time (min)	Selection rate, *r* (/day)[b]	Nucleotide substitutions in *fabI* region[c]	Amino acid substitution in FabI
NCIMB9518	0.03	39.6 ± 0.26	1.00		0.03 ± 0.003 [d]	NA	NA
9518T1	1	51.4 ± 1.02	1.38 ± 0.02	59.3 ± 1.12	15.73 ± 0.56	C284T	A95V
9518T2	2	38.4 ± 0.30	0.98 ± 0.01	37.2 ± 0.45	14.92 ± 0.44	C284T, T-108G	A95V
9518T3	2	37.9 ± 0.86	1.09 ± 0.02	36.8 ± 0.77	17.83 ± 0.49	C284T, T-108G	A95V
9518T4	1	40.2 ± 0.68	1.02 ± 0.01	38.7 ± 0.39	15.27 ± 0.16	None detected	None detected
F89	0.03	37.3 ± 0.21	1.00		0.98 ± 0.002 [d]	NA	NA
F89T1	1	38.6 ± 0.44	1.03 ± 0.01	40.2 ± 0.52	16.74 ± 0.23	C284T	A95V
24500	0.063	44.7 ± 0.71	1.00		0.05 ± 0.003 [d]	NA	NA
24500T1	1.5	42.8 ± 0.48	0.96 ± 0.01	44.5 ± 0.82	15.93 ± 0.41	C284T	A95V
27343	0.063	40.1 ± 0.58	1.00		0.02 ± 0.006 [d]	NA	NA
27343T1	1	40.9 ± 0.90	0.94 ± 0.01	39.4 ± 1.06	15.66 ± 0.73	C284T	A95V

[a] Mean ± s.e. [b] In the presence of triclosan it was preferable to express fitness in terms of selection rates. [c] Includes structural *fabI* gene and putative promoter region. [d] Value shows selection rate for wild type parent versus rifampicin resistant parent (in the absence of triclosan). NA, Not applicable.

were equally susceptible to triclosan.[8,36,37] If, as previously reported, triclosan resistance is transferable in association with mupirocin resistance[7] then we would expect an association between these resistances in our strain collection. However, no correlation was found between resistance to mupirocin and resistance to triclosan, implying that no co-transfer of these resistances had occurred in the recent history of our collection. Several other authors have also expressed surprise at finding a lack of congruence between these resistances.[8,20,38]

However, we were able to isolate mutants with low-level resistance to triclosan. There was no evidence for mutation conferring co-resistance to other antimicrobials, a possibility discussed at length with regards triclosan.[15,38-45] Nonetheless, this may remain a prospect, especially if resistance to triclosan can be conferred by alteration of other, yet undescribed, targets or processes, such as efflux. Indeed, it should be considered that the use of antimicrobials not only selects for the evolution of antimicrobial resistance. It also selects for the infrastructure required to develop resistance. Hence, although the mutation to reduced triclosan susceptibility described herein does not appear to confer resistance to any other antimicrobials, it may assist future evolution of drug resistance. Furthermore, low-level antimicrobial resistance in *S. aureus* is often overlooked and may be associated with greater clinical risks than we might expect, such as increased mutation frequencies to higher resistance, increased virulence, improved *in vivo* fitness and resistance to unrelated compounds.[46]

The frequency of mutation was close to that expected from spontaneous mutation ($\sim 1.0 \times 10^{-8}$). This rate was not affected by the genetic background of the strain; there was no significant difference between the rates of mutation in MRSA, MSSA, MupRSA or MupSSA. However, the triclosan concentration did bias the selection of those strains with reduced susceptibility to triclosan. 1 mg/L triclosan biased towards the selection of those strains with either the single or double mutation, whilst 2 mg/L selected for the subset of these with the double mutation; reflecting the fact that resistance to triclosan concentrations of ~1 mg/L requires a single point mutation, but progression to higher levels of resistance (2-4 mg/L) requires additional alterations.[19]

A previous report suggests that the multi-target nature of biocides detaches susceptibility as measured by MIC from bactericidal susceptibility, unlike antibiotics,

which exhibit an explicit link between MIC and bactericidal susceptibility.[38] Hence we aimed to establish whether there was or was not a link between triclosan MIC and susceptibility to their lethal effects in our collection. Our findings contradict this previous report, suggesting that at low concentrations of triclosan, reduced susceptibility mutants are more resistant to the lethal effects of triclosan. However, higher concentrations proved equally lethal. This provides further evidence that it is only at low concentrations that triclosan acts upon a discrete target. This is further supported by our evidence that the growth state of the cells does not affect the bactericidal activity of triclosan; as proposed by Suller and Russell,[38] if FabI is the sole target for triclosan, then should exponentially growing organisms be more susceptible to it?

During clinical or domestic use, triclosan is delivered typically as part of complex formulations containing various combinations of surfactants, detergents, chelating agents and wetting agents, all of which will affect their action, and ultimately, efficacy. Furthermore, typical in-use concentrations of triclosan in hand wash, bath formulations (0.1 to 0.3%) and surface disinfectants are 0.1 to 1%, for hospital wash applications for MRSA eradication up to 2% triclosan is recommended.[6] Hence, in an attempt to better reflect *in situ* conditions, we also assessed the antimicrobial activity of Irgacide LP10, a commercial preparation of triclosan intended for clinical use. As with standard triclosan, reduced triclosan-susceptibility mutants did display an increased tolerance towards Irgacide LP10 at very low concentrations, however all strains were very rapidly killed by recommended in-use concentrations of Irgacide LP10.

Potassium ion leakage from cell suspensions, used to assess triclosan-induced cytoplasmic membrane damage, occurred at similar rates for all *S. aureus* strains, including reduced susceptibility mutants. Mutants do show some reduction in susceptibility to lethal effects when measured by potassium leakage, but less than when measured by viable counts. Hence, mutation of *fabI* does not offer any statistically significant reduction in susceptibility to triclosan induced cytoplasmic membrane damage. The correlation between MIC and insusceptibility to killing does not hold true for potassium leakage. We propose that FabI mutation does confer a greater tolerance towards the lethal effects of triclosan, but only at low concentrations

where there is insufficient triclosan available to bring about significant membrane, DNA, RNA or protein synthesis related effects. Hence we provide further evidence that triclosan causes a multitude of effects (including membrane perturbations) culminating in cell lysis, but this is concentration dependent.

Fitness costs of fabI mutation. Sequencing the *fabI* gene and preceding promoter of triclosan mutants revealed two novel mutations conferring reduced triclosan susceptibility; C284T and T-108G. C284T confers an alanine to valine substitution at the 95th residue, although not previously described in *S. aureus* this is in close proximity to the previously reported G93V alteration described in *E. coli* by Heath *et al.* in 2000. Binding studies have demonstrated that triclosan inhibits FabI via the formation of a stable triclosan-NAD^+-FabI complex.[19] Hence, we hypothesize that the location of the A95V substitution affects the formation of this complex, reducing the inhibitory effect of triclosan on the protein.

Triclosan MICs were increased further by development of a mutation in the putative promoter region, feasibly by conferring the over expression of the mutated FabI, as suggested by Fan *et al.*[19] Interestingly, we also isolated a mutant showing reduced susceptibility to triclosan without any sequence alterations within its *fabI*. Implying that some other target has been altered and hence FabI is not the sole target for triclosan; genetic loci other than *fabI* may also be involved in triclosan resistance. Indeed a recent study has shown that in *Streptococcus mutans*, an organism that lacks triclosan-susceptible FabI, triclosan is lethal by acting upon multiple targets including several enzymes of the glycolysis pathway.[47]

Evolution of reduced susceptibility to triclosan by mutation did not diminish the fitness of strains. Hence mutations such as these may be expected *in situ*, especially in locations exposed to triclosan where reduced susceptibility mutants may out-compete the wild type. However, current data have not indicated any explicit link between triclosan exposure and resistance in *ex-situ* isolates, microcosms, domiciliary environment or triclosan manufacturing site.[48-52]

Although the experiments reported here did not reveal a statistically significant difference in the *in vitro* fitness of strain 9518T1 there is, nevertheless, an indication

that the C284T mutation in 9518T1 is associated with decreased fitness when compared to the parental strain NCIMB9518. 9518T1, isolated after the first round of triclosan exposure showed a longer doubling time and was less able to compete with its parent than 9518T2 and T3, strains isolated from second and third serial exposures to triclosan. This indicates that in addition to reducing their susceptibility 9518T2 and T3 have restored their fitness, possibly through compensatory chromosomal mutations.

Triclosan remains an important and efficacious antimicrobial and bacterial fatty acid biosynthesis is a major target for development of novel antimicrobials, including the potential MRSA targeting platensimycin that inhibits FabF.[19,53-59] However, we should be aware of the development of reduced susceptibility through mutation, altered gene expression and the development of SCVs. Using a combination of antimicrobials for control of microorganisms is a favourable way to reduce the chances of resistance and triclosan can be an important tool as part of this control.

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Transparency declarations

PFS and MJD have nothing to declare. DO is an employee of Ciba Spezialitätenchemie Grenzach GmbH, Grenzach-Wyhlen, Germany.

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Chapter 4

Carriage and antimicrobial susceptibility of staphylococci in an undergraduate teaching class

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Abstract

We aimed to evaluate the level of antimicrobial resistance in staphylococci isolated from a representative of the student population of Cardiff. Microorganisms were recovered from a group of undergraduate students (n = 93) by nasal and skin swab. Identification was performed using selective agars, multiplex PCR and coagulase test. Isolated *S. aureus* and coagulase negative staphylococci (CoNS) where subsequently assessed for susceptibility to a selection of antibiotics, cationic biocides and triclosan. Students also completed a short questionnaire providing details of risk factors associated with *S. aureus* carriage. CoNS were isolated from 81 students (87.1%) whilst *S. aureus* was isolated from 30 (32.3%). CoNS were found to carry significantly more antimicrobial resistance traits than *S. aureus*, including methicillin, penicillin and triclosan resistance (P <0.05). One MRSA strain was isolated, the source of which was found to be a family member. In conclusion we find that CoNS represent an important reservoir of antimicrobial resistance genes, providing a resource for evolution of resistance to a broad spectrum of antimicrobial agents. Hence, the reduced susceptibility of CoNS should be considered when delivering antimicrobial therapies. We also discuss how familial transfer of MRSA may play a significant role in the spread of MRSA.

Introduction

Staphylococcus aureus is a major cause of hospital-acquired infection and the persistent increase in the numbers of methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA; Office for National Statistics, 2006) is causing considerable concern amongst healthcare professionals and general public. The emergence and spread of microorganisms with reduced susceptibility to antimicrobials in the community now presents a new challenge to public health. Increasing attention is being paid to community-associated MRSA (C-MRSA), for which infection rates are rising in many areas around the world (Lu *et al.*, 2005). In contrast, whilst coagulase-negative staphylococci (CoNS) are frequently isolated in clinical microbiology laboratories and are becoming increasingly important causative agents of hospital-acquired infections (von Eiff *et al.*, 2002) they attract little interest

(Shittu *et al.*, 2006). CoNS have particular association with prosthetic devices and other invasive apparatus and regularly harbour multi-drug resistance (von Eiff *et al.*, 2002).

The relationship between the use of antibiotics and the development of antibiotic resistance has been discussed at length (Livermore, 2003). More recently the increased use of biocides as disinfectants, antiseptics and preservatives has also created discussion about their role in the development of resistance (Russell, 1999, Russell, 2000, Levy, 2001, Levy, 2002, Russell, 2002). Particular attention has been paid to the broad-spectrum antimicrobials triclosan (Irgasan DP300; 2,4,4'-trichloro-2'-hydroxydiphenyl ether), chlorhexidine gluconate (CHX) and cetylpyridium chloride (CPC). All are used widely in mouthwashes, hand disinfectants and also in surgical scrubs and during pre-operative skin preparation. Moreover, triclosan and CHX have been found to be useful for the control of MRSA (Webster, *et al.*, 1994, Zafar, *et al.*, 1995, Wenisch, *et al.*, 2006, Simor, *et al.*, 2007) and are part of the recommended rationale for skin decolonisation of MRSA carriers in the United Kingdom (Coia *et al.*, 2006).

Resistance to biocides has traditionally been viewed as unlikely, principally due to their multiple mechanisms of action. However reduced susceptibility to all three of these biocides has been reported. Triclosan has been found to target enoyl-acyl carrier protein reductase, the product of the *fabI* gene which catalyzes the final elongation step during bacterial fatty acid biosynthesis (Heath & Rock, 2000; Fan *et al.*, 2002). Over expression or mutation of this gene can confer reduced susceptibility to triclosan, however triclosan has also been shown to have other targets and resistance is limited to low levels (MIC 1-4 mg L^{-1}, compared to 0.01- 0.1 mg L^{-1} for susceptible strains; Russell, 2004; Seaman *et al.*, 2007b). Plasmid encoded resistance to CPC, a quaternary ammonium compound, has been found in *S. aureus* (Thomas & Archer, 1989). Resistance to CHX, a bisbiguanide, has been linked with nucleic acid binding proteins that are often plasmid encoded (Cookson *et al.*, 1991; Russell, 1997).

Staphylococci are normal skin commensals and as such are often ignored when isolated from non-sterile sites (Tan *et al.*, 2006). However, commensal staphylococci may contain an important 'pool' of antimicrobial resistance genes, which could

impact severely upon public health by being transferred to other, more virulent, species or strains or by a shift in the commensal to a more pathogenic existence. Therefore, it is important to quantify the resistance gene pool of both the CoNS and *S. aureus* flora of a unique community-based population, whilst considering the role of biocides in antimicrobial chemotherapy and resistance. We calculated the incidence of skin carriage of staphylococci among 93 university students. Isolates were investigated for their susceptibility to a selection of antibiotics, cationic biocides and triclosan, and this was collated along with data on risk factors for *S. aureus* and MRSA carriage.

Materials and Methods

Study population and sample collection

The samples were collected from undergraduate university students attending a series of practical classes at Cardiff University, Cardiff, Wales, in the period from 7th to 17th March 2005. Students were chosen at random and asked to participate in the study. Each subject underwent swabbing of the axilla and the anterior vestibule of both nares with a sterile swab. A questionnaire was also completed by each student to ascertain their exposure to known risk factors for MRSA carriage (Charlebois *et al.*, 2004; Lu *et al.*, 2005) including age, visitation to a hospital within last 30 days, admission to a hospital for ≥two days in the last six months or living with a healthcare professional. A total of 93 (53 male, 40 female) students underwent the same swabbing procedure and completed the questionnaire as part of the study.

Each swab specimen was streaked on to a Vogel-Johnson agar plate (VJA; Oxoid, Basingstoke, UK) and two Mueller Hinton agar plates (MHA; Oxoid), one of which was supplemented with triclosan (0.5 mg L^{-1}). Plates were incubated at 37°C for 48 h, after which morphological and Gram stain examinations were performed. Colonies of interest were streaked on MHA supplemented with 5% defibrinated sheep blood (Oxoid) and incubated at 37°C for 24 h.

Bacterial strains and growth conditions

S. aureus NCTC6571, *S. aureus* NCIMB9518, MRSA Mu50 and *S. epidermidis* 12228 were used as control strains during this investigation. All control and test organisms were confirmed as *S. aureus* or coagulase-negative staphylococci (CoNS) by streaking on VJA, latex-agglutination test for extra-cellular coagulase (Staphylase test; Oxoid) and multiplex PCR, as described previously (Zhang *et al.*, 2004; Seaman *et al.*, 2007a; Table 4.1). Strains were stored in 50% glycerol at -80°C and, when required, maintained on either nutrient agar (NA; Oxoid), Iso-Sensitest agar (ISA; Oxoid) or MHA. Over-night cultures were prepared by inoculation of 10 ml nutrient broth (NB; Oxoid) or Iso-Sensitest broth (ISB; Oxoid) with colonies from a streak plate and incubation in an orbital shaker at 37°C for 15 h. Triclosan-susceptible isolates were preliminarily identified by growth on MHA and the absence of growth on the plate supplemented with triclosan.

Biocide and antibiotic susceptibility

MICs for three biocides, triclosan (gift from Ciba Spezialitätenchemie Grenzach GmbH, Grenzach-Wyhlen, Germany), CHX (ICN Biomedicals Inc., Costa Mesa, USA) and CPC (ICN Biomedicals Inc.) were calculated for all organisms according to the guidelines of the British Society for Antimicrobial Chemotherapy (Andrews, 2001). Etest strips (Bio-stat Ltd, Stockport, UK) were utilized to attain the MIC for penicillin, oxacillin, mupirocin, vancomycin, teicoplanin, linezolid, ciprofloxacin, gentamicin, chloramphenicol, tetracycline and erythromycin. Isolates were categorized as either susceptible or resistant according to MIC breakpoints described previously (Andrews, 2004; Brown *et al.*, 2005). Methicillin resistance, as indicated by oxacillin MIC, and high-level mupirocin resistance were confirmed by amplification of the *mecA* and *mupA* genes, respectively, as part of the multiplex PCR described above. In addition, all isolates were investigated for β-lactamase production by nitrocefin stick (Oxoid).

Table 4.1. Primers used during multiplex PCRs used in identifying *S. aureus* and CoNS and in typing of SCC*mec* elements. The concentration of each primer used in the reaction is given.

Primer	Oligonucleotide sequence (5'-3')	Final concentration (μM)	Target	Reference
Staph756F	aactctgttattagggaagaaca	0.24	*Staphylococcus* 16S rRNA gene	(Zhang *et al.*, 2004)
Staph750R	ccaccttcctccggtttgtcacc	0.24		(Jaffe *et al.*, 2000)
Nuc1	gcgattgatggtgatacggtt	0.08	*S. aureus*-specific *nuc* gene	(Shortle, 1983)
Nuc2	agccaagccttgacgaactaaagc	0.08		
MupA1	tatattatgcgatggaaggttgg	0.1	*mupA* (high-level mupirocin resistance)	(Anthony *et al.*, 1999)
MupA2	aataaaatcagctggaaagtgttg	0.1		
MecA1	gtagaaatgactgaacgtccgataa	0.24	*mecA* (methicillin resistance)	(Ryffel *et al.*, 1990)
MecA2	ccaattccacattgtttcggtctaa	0.24		
Type I-F	gctttaaagagtgtcgttacagg	0.048	SCCmec I	Zhang *et al.* (2005)
Type I-R	gttctctcatagtatgacgtcc	0.048		
Type II-F	cgttgaagatgatgaagcg	0.032	SCC*mec* II	Zhang *et al.* (2005)
Type II-R	cgaaatcaatggttaatggacc	0.032		
Type III-F	ccatattgtgtacgatgcg	0.04	SCC*mec* III	Zhang *et al.* (2005)
Type III-R	ccttagttgtcgtaacagatcg	0.04		
Type IVa-F	gccttattcgaagaaaccg	0.104	SCC*mec* IVa	Zhang *et al.* (2005)
Type IVa-R	ctactcttctgaaaagcgtcg	0.104		
Type IVb-F	tctggaattacttcagctgc	0.092	SCC*mec* IVb	Zhang *et al.* (2005)
Type IVb-R	aaacaatattgctctccctc	0.092		
Type IVc-F	acaatatttgtattatcggagagc	0.078	SCC*mec* IVc	Zhang *et al.* (2005)
Type IVc-R	ttggtatgaggtattgctgg	0.078		
Type IVd-F5	ctcaaaatacggaccccaataca	0.28	SCC*mec* IVd	Zhang *et al.* (2005)
Type IVd-R6	tgctccagtaattgctaaag	0.28		
Type V-F	gaacattgttacttaaatgagcg	0.06	SCC*mec* V	Zhang *et al.* (2005)
Type V-R	tgaaagttgtacccttgacacc	0.06		
MecA147-F	gtgaagatataccaagtgatt	0.046	*mecA*	Zhang *et al.* (2005)
MecA147-R	atgcgctatagattgaaaggat	0.046		

SCC*mec* typing by multiplex PCR

Chromosomal DNA was extracted as described previously (Enright *et al.*, 2000). Nine pairs of primers, designed by Zhang *et al.*, (2005), were used in 25 µL PCRs intended to characterize the SCC*mec* type of MRSA isolates (Table 4.1). Reactions contained 0.5 µL of chromosomal DNA (approximately 0.5 µg), 0.032 to 0.104 µL of each primer (see Table 4.1), 1 U of *Taq* DNA polymerase (Bioline Ltd, London, UK), 5 µL of 10× buffer (Bioline Ltd), and 0.2 mM deoxynucleoside triphosphates (Bioline Ltd). The PCR was performed in a PTC-200 DNA engine (MJ Research, Waltham, USA) with an initial denaturation step at 94°C for 5 min followed by 10 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 90 s and a further 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s, ending with a final extension step at 72°C for 10 min before a hold at 4°C. All PCR assay runs incorporated a reagent control (template DNA replaced with molecular grade water). PCR amplicons were analyzed using UV transillumination after electrophoresis on a 2% agarose gel containing 0.5 µg mL^{-1} ethidium bromide. 1 kb Plus DNA ladder™ (Invitrogen, Carlsbad, USA) was run alongside the samples as a molecular weight marker.

Questionnaire and data analysis

Each participant completed a standardized questionnaire. Students' age, gender, exposure to healthcare personnel and medical history over the preceding 6 months, including previous hospitalization and medication history were collated. Data were analyzed with SPSS release 12.0.2 (SPSS Inc, Chicago, USA) and correlated with *S. aureus* and CoNS carriage data. Comparison of categorical variables was done by the Pearson chi-square test or Fisher's exact test, as appropriate. Minitab release 14 software (Minitab Inc., Pennsylvania, USA) was used to test for significant difference between the susceptibility of *S. aureus* and CoNS using Student's t-test, carried out at the 95% confidence interval. If the assumptions required to perform this test were violated, the Mann-Whitney test was performed at 95% confidence intervals.

Results

93 students were recruited for this study, of which 53 (57%) were male and 40 (43%) were female. Participant age ranged from 18-24 years, with a mean age 19.03 years (males, 19.13 years; females, 18.90 years). Of the 93 students 89 (95.7%) were found to be colonized with staphylococci. CoNS were the most readily recovered organisms, being isolated from 81 students (87.1%), whilst *S. aureus* was isolated from 30 students (32.3%). 26 *S. aureus* isolates (86.67%) were recovered from the anterior nares, whilst the majority of CoNS, 78 (96.3%), were isolated from the axilla.

Antimicrobial resistances were significantly more prevalent in CoNS than *S. aureus* (P <0.01; Table 4.2). 77 CoNS (95.1%) showed resistance to at least one antimicrobial whilst 6 *S. aureus* (20.0%) showed the same number of resistance traits. CoNS were also more likely to show multi-drug resistance (Fig. 4.4.1). Strikingly, 77 (95.06%) of the CoNS isolated showed triclosan MICs >0.25 mg L^{-1}, causing 82.80% of participants to be identified as carrying staphylococci with reduced susceptibility to triclosan. There was no significant difference in the triclosan susceptibility of methicillin-resistant or susceptible CoNS (P = 0.687). Reduced susceptibility to triclosan was not found amongst *S. aureus* isolates, including one MRSA isolate. No resistance to the cationic biocides CHX or CPC was found amongst the isolates. Penicillin resistance was most prevalent, followed by methicillin and then ciprofloxacin resistance (Table 4.2).

β-lactamase production was detected in all isolates that showed penicillin resistance, implying that this was the mechanism of resistance. The *mecA* gene was detected in all staphylococci with MICs of oxacillin ≥1 mg L^{-1}, confirming that methicillin resistance was associated with this element.

Correlation of participants' questionnaire answers with the microbiological data revealed that there was no significant difference between the frequency of *S. aureus* or CoNS carriage in males and females (P = 0.570 and 0.413, respectively). There

was also no significant difference in resistance to penicillin or methicillin between genders ($P > 0.05$). Neither *S. aureus* or CoNS carriage were significantly different

Fig. 4.1 Frequency distribution of number of resistance traits per staphylococcal isolate. The median number of resistance traits per isolate was zero for *S. aureus* (■) and two for CoNS (■).

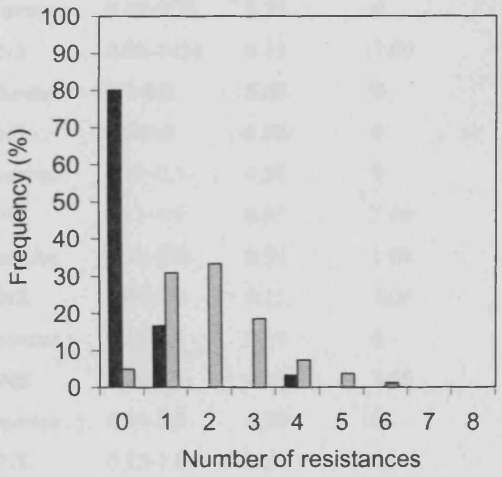

Table 4.2. Antimicrobial susceptibility of staphylococci isolated from 93 undergraduate students.

Antimicrobial[a]	Organism	MIC (mg L^{-1})		Resistance		
		Range	Median	Frequency	% isolates	% students
Triclosan	*S. aureus*	0.03-0.13	0.03	0	0	0
	CoNS	0.13-8.0	4.00	77.00	95.06	82.80
CHX	*S. aureus*	1.0-2.0	1.00	0	0	0
	CoNS	0.5-2.0	1.50	0	0	0
CPC	*S. aureus*	1.0-2.0	1.00	0	0	0
	CoNS	0.5-2.0	2.00	0	0	0
Penicillin	*S. aureus*	0.02-48	0.02	6.00	20	6.45
	CoNS	0.13-64	8.00	52.00	64.20	55.91
Oxacillin	*S. aureus*	0.13-64	0.38	1.00	3.33	1.08
	CoNS	0.13-32	0.13	22.00	27.16	23.66
Vancomycin	*S. aureus*	1.0-1.5	1.00	0	0	0
	CoNS	1.0-1.5	1.00	0	0	0
Mupirocin	*S. aureus*	0.19-0.75	0.25	0	0	0
	CoNS	0.06-1024	0.19	7.00	8.64	7.53
Chloramphenicol	*S. aureus*	4.0-8.0	5.00	0	0	0
	CoNS	4.0-8.0	6.00	0	0	0
Tetracycline	*S. aureus*	0.19-0.5	0.38	0	0	0
	CoNS	0.13-4.0	0.25	2.00	2.47	2.15
Erythromycin	*S. aureus*	0.38-256	0.50	1.00	3.33	1.08
	CoNS	0.06-2.0	0.12	2.00	2.47	2.15
Gentamicin	*S. aureus*	0.13-0.5	0.19	0	0	0
	CoNS	0.064-16	0.25	3.00	3.70	3.23
Linezolid	*S. aureus*	0.19-0.5	0.50	0	0	0
	CoNS	0.25-1.0	0.50	0	0	0
Teicoplanin	*S. aureus*	0.19-0.75	0.44	0	0	0
	CoNS	0.19-0.75	0.38	0	0	0
Ciprofloxacin	*S. aureus*	0.19-32	0.19	1.00	3.33	1.08
	CoNS	0.13-48	0.25	4.00	4.94	4.30
β-lactamase[b]	*S. aureus*	-	-	3.00	10	3.23
	CoNS	-	-	55.00	67.90	59.14
mecA[c]	*S. aureus*	-	-	1.00	3.33	1.08
	CoNS	-	-	22.00	27.16	23.66
mupA[d]	*S. aureus*	-	-	0	0	0
	CoNS	-	-	5.00	6.17	5.38

[a] CHX, Chlorhexidine gluconate; CPC, cetylpyridinium chloride;

[b] β-lactamase production was assessed by nitrocefin stick.

[c] The presence of *mecA* was used to confirm the genetic determinant for methicillin resistance

[d] Presence of *mupA* was used to confirm the genetic determinant for mupirocin resistance and indicated high-level resistance.

between participants who had visited a hospital in the last 30 days (P = 0.612 and 0.430, respectively), been hospitalized for >2 days (P = 0.694 and 0.343) or lived with a member of the healthcare profession (P = 0.513 and 0.308). Similarly, carriage of CoNS resistant to methicillin or penicillin could not be correlated with prior hospitalization (P = 0.967 and 0.8, respectively) or recent use of antibiotics (P = 0.619 and 0.409).

Whilst 22 methicillin-resistant CoNS were isolated (27.16%) only a single MRSA strain was detected. This MRSA isolate was recovered from an 18 year old female student who had no history of hospitalization within the last 6 months, had not used any prescribed antimicrobial products and did not live with any healthcare professionals. However, during a follow-up session it was revealed that the girl's mother was currently undergoing treatment for an MRSA infection acquired whilst attending a South Wales hospital. Subsequently, a swab specimen was recovered from the mother, which yielded coagulase-positive cocci with resistance to penicillin, methicillin, erythromycin and ciprofloxacin, the same resistance profile to the strain isolated from her daughter. Multiplex PCR confirmed both isolates as *S. aureus* and as *mecA* positive. SCC*mec* typing using a second multiplex PCR revealed both MRSA isolates to harbour a type III SCC*mec* element.

Discussion

Whilst variations in sampling techniques make comparison between studies difficult, staphylococcal carriage amongst this student population was similar to that of previous community studies (Abudu *et al.*, 2001; Sá-Leão *et al.*, 2001; Maudsley *et al.*, 2004). Our results clearly show that commensal CoNS are often multiply-resistant and generally carry more resistance traits than *S. aureus*. Consequently, CoNS infections, which are largely from a patients own microflora (Tan *et al.*, 2006), may provide a greater challenge during antimicrobial treatment.

We were interested to find that CoNS appear to show reduced susceptibility to triclosan, when compared with *S. aureus*. One previous report that found similar results also found that methicillin-resistant CoNS were more likely to show reduced susceptibility to triclosan (Schmid & Kaplan, 2004). In contrast we found no significant difference in triclosan MIC between methicillin-resistant and methicillin-susceptible CoNS ($P = 0.687$). This could be a consequence of variations between the strain collections and testing methods. Alternatively, our results may indicate that there is little relationship between methicillin resistance and triclosan susceptibility, as has been reported previously in *S. aureus* (Bamber & Neal, 1999; Suller & Russell, 2000; Russell, 2004). If there is indeed a relationship between these two resistances we are unaware of any biochemical of physical reason as to why they may be related and propose that is possibly a reflection of the augmented ability of multiply-resistant isolates to acquire and maintain resistance traits.

The median MIC of triclosan in CoNS (4 mg L^{-1}) is similar to the MIC of *S. aureus fabI* mutants, which show reduced susceptibility to triclosan (1-4 mg L^{-1}; Fan *et al.*, 2002; Seaman *et al.*, 2007b). Triclosan inhibits FabI (enoyl-acyl carrier protein reductase) via the formation of a stable triclosan-NAD^+-FabI complex (Fan *et al.*, 2002). Hence, the reduced susceptibility in CoNS may be due to sequential and structural differences between CoNS and *S. aureus* FabI and these differences affect the formation of the triclosan-NAD^+-FabI complex, reducing the inhibitory effect of triclosan on the protein. However, triclosan is known to act through several mechanisms (Seaman *et al.*, 2007b), so future work should be directed towards characterizing the mechanism of action of triclosan in CoNS.

It is notable that CoNS were predominantly isolated from the axilla, whilst *S. aureus* were more commonly recovered from nasal swabs. The reduced susceptibility seen in CoNS may reflect the presence of antimicrobial compounds in underarm preparations such as deodorants, whilst the exposure of the nares to antimicrobials is limited. It should also be remarked that the clinical significance of the MICs for triclosan seen in the CoNS of this study is ambiguous; they are still far below the in-use concentrations of triclosan (0.1 to 2%) and in addition, specific ratios of surfactants, wetting agents, detergents and chelating agents are included in commercial preparations of triclosan, which affect the overall antimicrobial activity of the formulation.

Our study highlights the genetic resource for antimicrobial resistance that CoNS maintain. Furthermore, *S. aureus* and CoNS are closely related, and as such share similar genetic systems, they also often colonize the same locale. As a consequence of this, we theorize that CoNS may represent a potential reservoir of resistance that *S. aureus* could exploit. In particular they may be a source for the development of *S. aureus* isolates with reduced susceptibility to triclosan. However, recent work failed to detect any transfer of triclosan resistance amongst *S. aureus* isolates or from *S. epidermidis* (Seaman PF, Day MJ & Ochs D, 2007, unpublished work). Hence it remains to be shown what role CoNS may play in the evolution of triclosan resistance in the more pathogenic *S. aureus*.

Our study did not identify any risk factors for staphylococcal carriage. However, we report a suspected example of familial transfer of MRSA. Both mother and daughter specimens recovered MRSA with identical resistances profiles and both were found to be SCC*mec* type III (Class A *mec* complex and type 2 *ccr* complex). These data lead us to conclude that both isolates are the same strain. Hence it appears that the daughter had acquired it from her mother, following the mother developing the initial infection whilst hospitalized. Indeed, it has been demonstrated previously that family members can serve as reservoirs of MRSA and that familial transfer of MRSA can prolong the eradication (Huijsdens *et al.*, 2006). This may amplify the problems already associated with containing the spread of MRSA and provides an example of how previously nosocomial strains can become community-based.

In conclusion we have identified that CoNS show reduced susceptibility to triclosan than *S. aureus* and that familial transfer of MRSA strains is an ongoing phenomenon that has the potential to severely impact upon public health. Although the susceptibilities of CoNS are below the in-use concentrations of triclosan, these organisms should be closely monitored for susceptibility, particularly in light of their emerging clinical significance.

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Chapter 5

Gene transfer of biocide resistance in staphylococci

Submitted for publication in the Journal of Antimicrobial Chemotherapy

Synopsis

Objectives: To identify the potential for gene transfer of triclosan resistance by conjugation, transduction and transformation in *S. aureus* and related species.
Methods: A collection of low-GC, Gram-positive organisms were investigated for ability to transfer plasmid- and chromosome-encoded antimicrobial resistance during bi- and tri-parental filter matings, transduction with bacteriophages 80α and K and transformation experiments. These were supported by analysis of the *fabI* gene and surrounding region from 10 staphylococcal genomes for %GC; GC frame plot; Karlin Signature Difference as indicators of horizontal acquisition.
Results: Plasmid-encoded resistances including mupirocin, quaternary ammonium compound and chloramphenicol resistance could be transferred by conjugation and could also be mobilized by transduction. However, no transfer of triclosan resistance could be detected and no association between mupirocin and triclosan resistance was found. Triclosan was also found to significantly effect the gene transfer of antimicrobial resistance (P = >0.05), with concentrations between 1 and 10 mg/L generally reducing transfer, whilst lower concentrations had less effect. No indication that *fabI* had been horizontally acquired in the staphylococcal genomes was found.
Conclusions: Whilst some antimicrobial resistances are readily transferable amongst staphylococci and other genera our data question the significance of this phenomenon in the dissemination of low-level triclosan resistance. Furthermore, triclosan does not appear to promote the transfer of other resistances and triclosan resistance does not readily form associations with mupirocin resistance.

Introduction

Bacteria can develop resistance to antimicrobial compounds either by spontaneous mutation of their genome or by acquisition of exogenous genetic material. Whilst mutations are generally associated with development of resistance to a single, or very closely related antimicrobials, acquisition of new genes by horizontal gene transfer (HGT) often confers resistance to multiple, frequently unrelated, antimicrobials. Hence HGT can facilitate the rapid evolution of a micro-organism, indeed it has been proposed that it is HGT that drives bacterial speciation; basal mutation events such as point mutations rarely confer a phenotype enabling a bacterium to colonize new

environments, however HGT can more often provide the genetic information for this goal. Take for example the divergence of *Escherichia coli* and *Salmonella enterica*; all those phenotypic characteristics that distinguish these two species originate from horizontally transferred genes.[1] Comparative analysis of staphylococcal genomes has revealed that genomic islands in nonsyntenic regions are the principal source of diversity in pathogenicity and resistance,[2] and that the distribution of mobile cassettes carrying virulence and drug-resistance determinants may explain the phenotypic differences, and variation in clinical importance, between an epidemic methicillin-resistant *Staphylococcus aureus* (MRSA) clone and a community-acquired methicillin-susceptible *S. aureus* (MSSA) clone.[3] The transfer of genes between staphylococci and low-GC-content Gram-positive bacteria appears to have moulded their virulence and ability to resist antimicrobial chemotherapy.

HGT of antibiotic resistance has been a hot topic for study, especially since *Staphylococcus aureus* showed itself to be so adept at acquiring antibiotic resistance by this mechanism.[4] Many mobile genetic elements (MGE; plasmids, genomic islands, transposons, insertion sequences (IS) and phage-associated elements) can harbour antibiotic resistance genes. Examples include staphylococcal cassette chromosome mec (SCCmec), the key genetic component of methicillin resistance,[5] multiresistance plasmid pSK41 which, along with other resistances, confers resistance to aminoglycosides[6] and *ileS2*-carrying plasmids which play a significant role in the dissemination of high-level mupirocin resistance among pandemic MRSA lineages.[7-9]

Bacterial genomes are highly plastic, being able to donate and receive DNA (generally MGE) to and from each other. MGE can move between cells by several routes; conjugation, transduction, phage-mediated conjugation or transformation. Bacterial conjugation is a highly specific process whereby DNA (plasmid, or less familiarly transposon) is transferred from donor to recipient by a specialized multi-protein complex present on the cell surface, named the conjugation apparatus. A key prerequisite for conjugative transfer is an intimate association between the cell surfaces of the donor and recipient cells. In Gram-negative bacteria, this physical contact is established by the sex pili. For the majority of Gram-positive bacteria the means to achieve this intimate cell-cell contact have not yet been fully identified, but a strong level of homology has been found with type IV secretion systems.[10] Some

plasmids carry all the genes necessary for self-transmission and are known as conjugative plasmids. Others lack these genes but are able to be mobilized by conjugative plasmids resident in the same organism.[11,12] Interestingly, *S. aureus* strains have been shown to secrete a peptide, staph-cAM373, that stimulates *in vitro* conjugative transfer of plasmids from *Enterococcus faecalis*.[13] Conjugal plasmids are able to mediate transfer over a wide range of pH values and temperatures.[14]

Bacteriophages (phages) are widely distributed in staphylococcal populations and many integrated phage have been identified in sequenced staphylococcal genomes, with at least one prophage in every genome, except that of *S. epidermidis* ATCC 12228.[2] It appears probable that transduction is the mechanism by which the *mecA* determinant has spread amongst staphylococci; *mecA* has never been found on mobile DNA but has spread among several *S. aureus* lineages and among different coagulase-negative species.[15] Gene transfer by transduction can be either generalized or specialized, the former entails a mis-incorporation of host DNA (chromosomal or plasmid) into the phage head which is then released during cell lysis and infects another cell, injecting the DNA from the previous host. The genetic material to be transferred by specialized transduction arises from an illegitimate recombinational event occurring during excision of a prophage from the host genome, leading to the packaging of a phage 'genome' lacking some phage functions but gaining some host traits. The fate of the transferred DNA is independent of the process of transduction and depends on the recipient cell's mechanisms of recombination and DNA stabilisation.[16] The requirement for the death of the donor cell, the need to protect the recipient from destruction by normal phage particles in addition to the artificial conditions needed for the transduction process *in vitro* has raised doubts as to whether transduction could be a significant process in nature.[17] However, transduction *in situ* has been described[18-20] and the potential for transduction to greatly affect evolution of bacteria is apparent.[16] Furthermore, induction of staphylococcal prophage, triggered by β-lactam antibiotics, can induce the horizontal transfer of virulence factors.[21]

The ability to take up free DNA is relatively rare in species of Gram-positive bacteria. However *S. aureus*, under specific conditions, is able to take up 'naked' DNA from the environment.[22] The natural competence system of *S. aureus* is induced only in very early exponential growth.[23] Either chromosomal or plasmid DNA can be

transformed into *S. aureus*, although competence requires high calcium concentrations (0.1 M). Indeed, the requirement for a non-physiological calcium concentration and a short period of competence (when nucleases are absent) are likely to reduce the likelihood of staphylococcal transformation *in situ*.[24]

Mupirocin (Bactroban; pseudomonic acid) and triclosan (Irgasan DP300; 2,4,4'-trichloro-2'-hydroxydiphenyl ether) are important antimicrobials for the control of MRSA.[25-37] However, in 1991 transferable low-level resistance to triclosan (MIC = 2-4 mg/L) was linked to mupirocin resistance in isolates of *S. aureus* isolated after exposure to both of these compounds.[38] No further evidence has been offered in support of this phenomenon and several investigations have failed to identify any link between reduced susceptibility to triclosan and mupirocin resistance.[39-42] Herein we discuss the gene transfer of reduced triclosan susceptibility in *S. aureus* and coagulase-negative staphylococci (CoNS), investigate possible associations with other resistances and the effect of triclosan on the transfer of resistance genes.

Materials and Methods

Unless otherwise stated, all concentrations are % weight/volume. All experiments were performed in triplicate (n=3) and unless otherwise stated results are presented as mean ± standard error of the mean (s.e.).

Bacterial strains and growth conditions. Table 5.1 describes the bacterial strains, plasmids and bacteriophages used within this investigation. Prior to experimentation strains were confirmed as *S. aureus* or coagulase-negative staphylococci (CoNS) by streaking on Vogel-Johnson agar (VJA; Oxoid), latex-agglutination test for extra-cellular coagulase (Staphylase test; Oxoid) and multiplex PCR, as described previously.[43,44] Staphylococcal strains were stored in 50% glycerol at -80°C and, when required, maintained on either nutrient agar (NA; Oxoid), Iso-Sensitest agar (ISA; Oxoid) or Mueller Hinton agar (MHA; Oxoid). Over-night cultures were prepared by inoculation of 10 mL nutrient broth (NB; Oxoid) or Iso-Sensitest broth (ISB; Oxoid) with colonies from a streak plate and incubation in an orbital shaker at 37°C for 15 h. *E. faecalis* and *Streptococcus pneumoniae* strains were stored similarly

Table 5.1. Bacterial strains, plasmids and bacteriophage used in the study of transfer of antimicrobial resistance amongst staphylococci.

Name	Description[a]	Resistance phenotype[b,c]	Phage susceptibility	Reference/Source[d]
S. aureus NCTC6571	Oxford strain, isolated in 1940. Widely used control strain	-	Φ80α	NCTC
S. aureus 6571Rif	Rifampicin resistant mutant of NCTC6571	Rif	Φ80α	This study
S. aureus RN450	Plasmid-free recipient strain cured of Φ11, Φ12 & Φ13	Nov, Rif	Φ80α, ΦK	78
S. aureus NCIMB9518	Triclosan susceptible industrial strain	-	Φ80α, ΦK	41,79
S. aureus 9518T1	Reduced triclosan susceptibility mutant of NCIMB9518	Tric (1)	Φ80α, ΦK	41,79
S. aureus 9518T2	Reduced triclosan susceptibility mutant of NCIMB9518	Tric (2)	Φ80α, ΦK	41,79
S. aureus 9518T3	Reduced triclosan susceptibility mutant of NCIMB9518	Tric (2)	Φ80α, ΦK	41,79
S. aureus 9518T3Mup	9518T3 with *ileS2*-carrying plasmid from *S. aureus* Eagles	Mup^H, Tric (2)	Φ80α, ΦK	This study
S. aureus 9518T3pG01	9518T3 with pG01 plasmid from *S. aureus* 661	Gen, Kan, Neo, Tob, Tmp, QAC, Tric (2)	Φ80α, ΦK	This study
S. aureus 9518T3pIP501	9518T3 with pIP501 plasmid from *S. pneumoniae* T4N514	Chl, MLS, Tric (2)	Φ80α, ΦK	This study
S. aureus 9518T4	Reduced triclosan susceptibility mutant of NCIMB9518	Tric (1)	Φ80α, ΦK	41
S. aureus 9518Rif	Rifampicin resistant mutant of NCIMB9518	Rif	Φ80α, ΦK	41
S. aureus 24500	Clinical MRSA isolate	Pen, Oxa, Cip, Amp, Cec, Cro	Φ80α, ΦK	41
S. aureus 24500T1	Reduced triclosan susceptibility mutant of 24500	Pen, Oxa, Cip, Amp, Cec, Cro, Tric (1.5)	Φ80α	41
S. aureus 24532	Clinical MRSA isolate	Pen, Oxa, Ery, Tec, Cip, Amp, Cec, Cro	Φ80α	41
S. aureus 247378	Clinical MRSA isolate with high-level mupirocin resistance	Pen, Oxa, Mup^H, Ery, Cip, Amp, Cec, Cro	Φ80α, ΦK	41
S. aureus Eagles	Carries plasmid-borne, high-level mupirocin resistance	Pen, Mup^H, Ery, Tec, Amp, Cec	Φ80α, ΦK	9
S. aureus EaglesT4	Reduced triclosan susceptibility mutant of *S. aureus* Eagles	Pen, Mup^H, Ery, Tec, Amp, Cec, Tric (1.5)	Φ80α, ΦK	This study
S. aureus EaglesT4Mup$^-$	*S. aureus* EaglesT4 cured of *ileS2*-carrying plasmid	Pen, Ery, Tec, Amp, Cec, Tric (1.5)	Φ80α, ΦK	This study
S. aureus 661	Donor strain carrying pGO1 (pWBG613)	Gen, Kan, Neo, Tob, Tmp, QAC, Etbr	ΦK	80
S. aureus WBG541	Plasmid-free recipient	Rif, Fus	Φ80α, ΦK	81
S. epidermidis 12228	Non-biofilm forming, non-infection associated strain	Oxa, Tet	ΦK	82

Table 5.1 cont. Bacterial strains, plasmids and bacteriophage used in the study of transfer of antimicrobial resistance amongst staphylococci.

Name	Description[a]	Resistance phenotype[b,c]	Phage susceptibility	Reference/Source[d]
S. epidermidis LTN	Clinical isolate from UHW, Cardiff, Wales	Pen, Oxa, Cip, Amp, Mup^H, Fus, Tric (4)	ΦK	This study
S. pneumoniae T4N514	Apathogenic donor strain carrying pIP501	Chl and MLS		EG
E. faecalis JH2-2	Recipient strain	Rif, Fus		83
pG01	pWBG613, 52 kb plasmid (nhr)	Gen, Kan, Neo, Tob, Tmp, QAC		10,80
pIP501	30.2 kb plasmid originally identified in *S. agala*	Chl and MLS		10,84
Bacteriophage 80α	Mosaic genome related to Φ11and known to mc	-		46,85
Bacteriophage K	Polyvalent *Myoviridae* with a 127 kb genome (bhr)	-		86

[a] UHW, University Hospital of Wales; nhr, narrow host range; bhr, broad host range; SaPI1, staphylococcal pathogenicity island 1.

[b] Amp, ampicillin; Cec, ceflacor; Chl, chloramphenicol; Cip, ciprofloxacin; Cro, ceftriaxone; Ery, erythromycin; Fus, fusidic acid; Gen, gentamicin; Kan, kanamycin; MLS, macrolide, lincosamide, streptogramin B antibiotics; Mup^H, high-level mupirocin; Neo, neomycin; Nov, novobiocin; Oxa, oxacillin; Pen, penicillin; QAC, quaternary ammonium compounds; Rif, rifampicin; Tec, teicoplanin; Tet, tetracycline; Tmp, trimethoprim; Tob, tobramycin; Tric, triclosan.

[c] MIC of triclosan (mg/L) is given in brackets if resistance is indicated.

[d] NCTC, The National Collection of Type Cultures; EG, Dr Elisabeth Grohmann, University of Technology, Berlin, Germany.

but brain heart infusion agar (BHIA; Oxoid) and brain heart infusion broth (BHIB; Oxoid) were used as growth media. Due to the requirement for calcium during transduction and transformation experiments all media contained 400 mg/L $CaCl_2$.

Biocide and antibiotic susceptibility. MICs for three biocides, triclosan (gift from Ciba Speciality Chemicals, Germany), chlorhexidine gluconate (CHX; ICN Biomedicals Inc.) and cetylpyridinium chloride (CPC; ICN Biomedicals Inc.), were calculated according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC).[45] Etest® strips (Bio-stat Ltd) were utilized to attain the MIC for antibiotics, as per the manufacturer's directions.

Curing of mupirocin resistance. High-level mupirocin resistance was cured by incubation in antibiotic free-media at 43°C for 24 h. Curing of the *ileS2*-carrying plasmid was confirmed by assessing the reduction in mupirocin MIC and by loss of the mupirocin resistance gene as assessed by multiplex PCR.[43,44]

Isolation of resistant mutants. Mutants of *S. aureus* resistant to rifampicin, for use as recipients in gene transfer experiments, were isolated as described previously. Low-level reduced triclosan susceptibility mutants, used as donors, were also isolated as described previously.[41]

Conjugation. Mating experiments were performed by a method adapted from Thomas *et al.*. (1999)[9]. Briefly, donor and recipient cells were grown overnight at 37 °C in 10 ml of the appropriate broth. 1 ml of the donor cell culture and 3 ml of the recipient cell culture were mixed and the mixture filtered through a 0·2 μm cellulose nitrate membrane filter (Millipore) held in a sampling manifold (Millipore). Using sterile forceps the membranes were placed with the bacterial cells facing upwards onto a NA plate and incubated overnight at 37 °C (ISA agar was used for transfers involving coagulase-negative staphylococci or BHIA for those with *E. faecalis* or *S. pneumoniae*). Following incubation, the membranes were removed and vortex mixed in 1 ml NB (or appropriate media). These suspended mating mixtures were serially diluted with phosphate buffered saline (PBS; Oxoid) and spread onto plates containing appropriate selective antimicrobials to select for growth of either recipient

or transconjugant cells. Antimicrobials were used at the following concentrations: chloramphenicol, 10 mg/L; CPC, 10 mg/L; erythromycin, 100 mg/L; gentamicin, 10 mg/L; mupirocin, 100 mg/L; rifampicin, 50 mg/L; triclosan, 1 mg/L. The lithium salt of mupirocin was a gift from GlaxoSmithKline (Harlow, UK) whilst, unless previously stated otherwise, all other antimicrobials were purchased from Sigma. Separate donor and recipient cultures were run alongside as controls. Colonies were counted after incubation for 48 h at 37°C and the transfer frequencies calculated as the number of transconjugants per recipient cell. Each cross was repeated three times and the results represent mean values. When required, the direction of transfer was determined by replica plating and utilizing secondary markers not used for selection in the original cross. Additionally, when examining transfer of high-level mupirocin resistance (HL-mupR) suspected transconjugants were confirmed as carrying the *ileS2* (mupirocin resistant) gene by multiplex PCR.[43,44] This PCR was also employed to confirm the species identification of a selection of transconjugants from each experiment. Tri-parental matings were performed similarly, except 1 mL of each donor and 3 mL of recipient was used.

Transduction. The ability of staphylococcal transducing phages 80α and K to transfer resistance traits by transduction was investigated by a method adapted from Jiang & Paul, (1998)[18] and Novick (1991)[46]. Briefly, transducing particles were produced by infecting a donor strain with Φ80α by the overlay method.[47] If either donor or recipient were unsusceptible to Φ80α, phage K was used. Phages were purified from the plates after overnight incubation by flooding overlay plates, showing near confluent lysis, with 10 mL of warm 0.5 M Tris-HCl and incubating at room temperature for 20-30 min. The liquid was recovered, filtered (0.2 μm Minisart syringe units; Sartorius) and a second round of high-titre phage lysate was produced with the same donor strain to ensure that the transducing particles contained only donor strain DNA. Lysates were stored at 4°C until required. Phage lysates were digested with 50 U of DNase I per mL before use in transduction assays to remove any free DNA that would be available for transformation.

0.5 mL of phage lysate was mixed with an equal volume of mid-log phase recipient culture (~10^{10} cells) at multiplicities of infection (MOI) ranging from 0.01 to 1.

Control experiments contained the recipient cell culture but 0.5 mL of quarter-strength Ringers solution (Oxoid) replaced the lysate. Following incubation at 30°C for 30 min to allow for adsorption, unadsorbed phages were removed by three rounds of centrifugation and washes with broth. The final washed cell pellet was resuspended in 1 mL of broth. The cells were allowed to recover for 10 to 20 min before they were plated onto selective plates containing appropriate antimicrobials at concentrations described above. The recipient-free transducing phage lysate was also plated onto selective plates as a control. After 48 h incubation at 37°C, transductants were counted. The recipient titre at the start of each transducing period was determined using the drop plate method[48] allowing transfer frequency to be calculated as transductants per recipient. This was performed in triplicate for each lysate/recipient combination and the results presented as mean ± s.e.

Transformation. Cell lysates were prepared from overnight cultures of donor bacteria washed 3 times by centrifugation at 13,000 rpm for 5 min to remove any extracellular nuclease. Following a final centrifugation the pellet was resuspended in 0.5 mL sterile saline citrate/detergent solution (0.15 M NaCl, 0.015 M Sodium citrate and 0.05% Sodium dodecyl sulphate) and incubated for 1 hour at 60°C with agitation every 15 min. Recipient cells were prepared by 3 washes in broth to remove extracellular nucleases, followed by resuspension in broth to ~10^{10} cells/mL. 100 µL of the resulting cell suspension was added to an equal volume of cell lysate and mixed thoroughly. This mixture was subsequently transferred to a sterile 47 mm nitrocellulose filter membrane (Millipore; pore size 0.2 µm) placed on the surface of a NA plate. Transformation membranes were incubated for 3, 6, 12 or 24 hours at 37°C. Lysate-free controls were prepared in parallel, containing 100 µL of recipient and 100 µL of sterile Ringer's solution. Following incubation membranes were removed with sterile forceps and vortex mixed in 1 mL NB (or appropriate media) for 1 minute to re-suspend the filter bound bacteria. Serial dilutions of the resuspended cells were plated on to the appropriate selective agar and, following incubation at 37°C for 24-48 h the transformation frequency was calculated as transformants per recipient. This was performed in triplicate and the results presented as mean ± s.e.

Effect of triclosan on gene transfer. The effect of sub-inhibitory triclosan on gene transfer was investigated for all three mechanisms of transfer. Conjugation experiments were performed exactly as described above except 0.01, 0.1, 1 or 10 mg/L triclosan was included in the agar upon which the filter and bacteria were incubated. Triclosan-free experiments were run alongside as controls. The effects of triclosan on transduction were examined by three approaches: pre-exposure of the transducing particles; pre-exposure of the recipient; presence of triclosan in the infection mixture. 1 mL of transducing lysate was mixed with triclosan to give final concentrations of 0.01, 0.1, 1 or 10 mg/L. The triclosan was then neutralized with 4 mL of neutralizing solution and the remainder of the transduction process continued as described above. This method was repeated but using 1 mL of bacterial culture, creating recipients pre-exposed to triclosan. To assess the effects of triclosan exposure in the infection mixture the mixed recipient and lysate cultures were adjusted to 0.01, 0.1, 1 or 10 mg/L triclosan, incubated as described above, followed by the addition of 4 mL of neutralizing solution. Each sample was then taken to completion with the standard protocol detailed above. The transformation protocol described above was adapted to assess the effect of triclosan by supplementing the growth media used during the incubation of bacterial cells and free DNA with 0.01, 0.1, 1 or 10 mg/L triclosan. Triclosan-free controls were run alongside all transfer experiments.

Efficacy and toxicity of triclosan neutralizing solution. The neutralizer used here was based on the British Standard EN 1499.[49] The solution was made fresh in distilled water and contained 30 g/L Tween 80 (Sigma), 3 g/L lecithin (Sigma), 1 g/L L-histidine (Sigma) and 5 g/L sodium thiosulphate (Fisher). The efficacy and toxicity of the neutralizer to bacteria were confirmed as satisfactory as described previously.[41] This method was adapted to examine the suitability of the solution to neutralize triclosan in phage lysates by replacing the bacterial culture with a high titre lysate of Φ80α or phage K. There was no significant difference ($P = 0.798$) between the viable phage count of lysates exposed to triclosan and the neutralizer and where water was substituted for triclosan, confirming the ability of the neutralizer to quench the virucidal activity of triclosan at 100 mg/L. When examining the possible toxicity of the neutralizer, there was no significant difference ($P = >0.05$) between the viable phage counts of lysates exposed to sterile water and those exposed to the neutralizer

for 15 min, confirming that the neutralizer was non-toxic to the phages under investigation.

Bioinformatic analyses. The evolutionary histories of staphylococcal *fabI* genes and surrounding sequences were investigated for potential horizontal acquisition with two bioinformatic programmes: Artemis[50] and alien_hunter[51]. Sequence files of prokaryotic genomes were retrieved from Entrez Genome.[52] Artemis was used to view genome sequences and to display parameters used for inferring horizontal acquisition: %GC; GC frame plot; Karlin Signature Difference and data output from alien_hunter. Two databases containing predictions of the likelihood that a gene has been horizontally acquired were also used: The Horizontal Gene Transfer Database (HGT-DB)[53] and Horizontal Gene Transfer Predictions in Archaea and Bacterial Genomes by IBM's Bioinformatics & Pattern Discovery Group.[54] These databases were searched to identify if they predicted *fabI* or any gene within 20 kb either side of *fabI* to be horizontally acquired. Artemis and the sequence resources of Entrez Genome were also used to identify any putative MGE (prophage, transposons, IS and remnants thereof) located within this ~41 kb region.

Statistical analyses. All experiments were performed in triplicate (n=3) and unless otherwise stated results are presented as mean ± standard error of the mean (s.e.; calculated by dividing the standard deviation of the mean by the square root of n). Significant effect was investigated by t test or one-way ANOVA, carried out at 95% confidence interval. This was performed using Minitab® release 14 software (Minitab Inc.). If the assumptions required to perform these tests were violated, the Mann-Whitney or Kruskal-Wallis tests were performed at 95% confidence intervals.

Results

Bioinformatic analysis of fabI gene. Bioinformatic analysis of the gene which, when mutated can confer reduced susceptibility to triclosan, revealed it to be highly conserved amongst *S. aureus* strains and no obvious signs of horizontal acquisition. Querying the HGT-DB and Horizontal Gene Transfer Predictions in Archaea and Bacterial Genomes databases did not indicate that any of the staphylococcal genomes stored in these databases contained *fabI* sequences that may have been horizontally

acquired. Moreover, neither database was able to identify any notable regions within 20 kb (up and down stream) as being horizontally acquired. This was supported by our own analyses; we detected no major variation in %GC, GC frame plot or Karlin Signature Difference amongst the same ~41 kb locus in all staphylococcal genomes available currently, including CoNS (data not shown). Similarly, no major area of horizontally acquired DNA was identified when viewing the data from alien_hunter analyses. Additionally, no putative MGE where identified within the proximity of *fabI*. To corroborate this approach these analyses were also performed on a known horizontally acquired gene, *mecA*, the genetic determinant for methicillin resistance. The sequence environment of *mecA* was found to be quite different to the of *fabI*; the HGT-DB identified 5 genes within 20 genes of *mecA* in MRSA strains Mu50 and N315, whilst the same region of the community-associated MRSA, MW2, contained 7. As expected, further analysis identifies a high density of resistance genes and a large number of MGE associated sequences, including 5 transposases, 3 recombinases (including the cassette chromosome recombinases A and B) and plasmid-associated genes.

Gene transfer of triclosan resistance. Although bi-parental filter matings of plasmid-bearing *S. aureus* and *S. pneumoniae* with various recipients resulted in transfer of antimicrobial resistance traits, no transfer of reduced triclosan susceptibility was detected (Table 5.2). Mupirocin resistance was transferred from *S. aureus* Eagles and clinical mupirocin resistant strain *S. aureus* 247378 at low frequencies (10^{-8} to 10^{-10}), whilst pGO1 from *S. aureus* 661 transferred at higher frequencies (10^{-6} to 10^{-9}). EaglesT4, a mutant of *S. aureus* Eagles showing reduced susceptibility to triclosan, was still able to transfer MupR at similar frequencies to it's parent (data not shown) however, no transfer of mupirocin and triclosan resistance in combination was detected. Similarly, transconjugants of *S. aureus* 9518T3, now carrying MupR, were able to transfer Mupirocin resistance, but not in association with triclosan resistance. This was repeated with 9518T3 transconjugants carrying pGO1 or pIP501, again these were able to transfer the plasmid-associated resistance (GenR and ChlR, respectively; Table 5.2) but transfer in combination with reduced susceptibility to triclosan was below the level of detection. No transfer of reduced-triclosan susceptibility was detected from mutated strains with a range of genetic backgrounds; MRSA, MupR-SA and an industrial strain (NCIMB9518; Table 5.1).

Table 5.2. Conjugative transfer of antimicrobial resistance during bi-parental matings. Transfer frequencies are presented as transconjugants per recipient.

Donor	Trait(s) transferred[a]	Recipient					
		S. aureus 9518Rif	*S. aureus* 9518T3	*S. aureus* RN450	*S. aureus* 6571Rif	*S. aureus* WBG541	*E. faecalis* JH2-2
S. aureus Eagles	Mup^r	1.92×10^{-9}	4.29×10^{-9}	5.14×10^{-8}	9.94×10^{-10}	2.86×10^{-8}	$<6.04\times10^{-11}$
S. aureus 9518T3	$Tric^r$	$<2.31\times10^{-11}$	-	$<8.33\times10^{-11}$	$<9.18\times10^{-11}$	$<2.71\times10^{-11}$	$<4.70\times10^{-11}$
S. aureus EaglesT4	Mup^r	7.23×10^{-10}	-	2.35×10^{-8}	7.88×10^{-10}	4.41×10^{-9}	$<3.81\times10^{-11}$
S. aureus EaglesT4	Mup^r & $Tric^r$	$<9.07\times10^{-11}$	-	$<1.87\times10^{-10}$	$<5.48\times10^{-11}$	$<3.25\times10^{-11}$	$<2.12\times10^{-11}$
S. aureus 9518T3Mup	Mup^r	6.74×10^{-9}	-	1.33×10^{-8}	2.93×10^{-10}	5.47×10^{-9}	$<7.43\times10^{-11}$
S. aureus 9518T3Mup	Mup^r & $Tric^r$	$<1.77\times10^{-11}$	-	$<2.31\times10^{-11}$	$<8.45\times10^{-11}$	$<7.91\times10^{-11}$	$<2.24\times10^{-11}$
S. aureus 24500T1	$Tric^r$	$<1.64\times10^{-11}$	-	$<1.64\times10^{-11}$	$<1.64\times10^{-11}$	$<1.64\times10^{-11}$	$<8.61\times10^{-11}$
S. aureus 247378	Mup^r	4.03×10^{-9}	1.37×10^{-8}	7.41×10^{-8}	5.48×10^{-9}	8.47×10^{-8}	$<4.27\times10^{-11}$
S. aureus 661	Gen^r	8.98×10^{-7}	5.58×10^{-8}	2.12×10^{-7}	5.22×10^{-9}	2.31×10^{-6}	$<5.35\times10^{-11}$
S. aureus 9518T3pGO1	Gen^r	6.07×10^{-9}	-	1.17×10^{-8}	5.84×10^{-10}	9.36×10^{-9}	$<8.71\times10^{-11}$
S. aureus 9518T3pGO1	Gen^r & $Tric^r$	$<6.35\times10^{-11}$	-	$<4.29\times10^{-11}$	$<3.12\times10^{-11}$	$<6.33\times10^{-11}$	$<4.24\times10^{-11}$
S. pneumoniae T4N514	Chl^r	8.53×10^{-8}	6.86×10^{-9}	7.39×10^{-9}	2.02×10^{-10}	9.67×10^{-7}	3.11×10^{-6}
S. aureus 9518T3pIP501	Chl^r	3.47×10^{-9}	-	6.68×10^{-9}	7.89×10^{-10}	4.58×10^{-8}	$<5.41\times10^{-11}$
S. aureus 9518T3pIP501	Chl^r & $Tric^r$	$<5.50\times10^{-11}$	-	$<1.09\times10^{-12}$	$<6.24\times10^{-11}$	$<8.94\times10^{-11}$	$<7.84\times10^{-11}$
S. epidermidis LTN	$Tric^r$	$<3.06\times10^{-11}$	-	$<5.66\times10^{-11}$	$<1.87\times10^{-11}$	$<7.11\times10^{-11}$	$<4.49\times10^{-11}$

[a] Mup^r, mupirocin resistance; $Tric^r$, triclosan resistance.

Strains of *S. epidermidis* are less susceptible to triclosan than their coagulase-positive relatives.[55] *S. epidermidis* has also been described as a source of a mupirocin resistance plasmid in an MRSA strain.[56] As a consequence of this we theorized that *S. epidermidis* may represent a potential reservoir of resistance that *S. aureus*, being closely related, found often in the same locale, could exploit. Hence, we investigated *S. epidermidis* LTN as a potential triclosan resistance donor. However, no transfer of triclosan resistance from *S. epidermidis* was detected (level of detection was $<1.87\times10^{-11}$ to $<7.11\times10^{-11}$ transconjugants per recipient).

It is known that some plasmids have the ability to mobilize other, non-self-transmissible, plasmids and MGE.[10] Consequently, we used tri-parental matings to examine this phenomenon in our strain collection. These matings involved mixing a primary donor, carrying a conjugative plasmid, with a secondary donor, carrying another resistance trait and a recipient strain. We were specifically looking for the ability of three conjugative plasmids, Eagles' mupirocin resistance plasmid, pGO1 and pIP501, to mobilize the genetic determinant for reduced triclosan susceptibility from a selection of secondary donors. However, none of these plasmids were able to transfer triclosan resistance from the secondary donor into either *S. aureus* 9518Rif or WBG542 (data not shown). Remarkably, when we used strains of *S. aureus* that carried mupirocin resistance in addition to triclosan resistance as secondary donors the frequency of mupirocin resistance transfer was significantly increased ($P = <0.05$), but the frequency of triclosan resistance transfer remained below our levels of detection (level of detection, $<4.17\times10^{-11}$ to $<6.25\times10^{-11}$; Table 5.3). Neither triclosan or mupirocin resistance could be transferred into *E. faecalis* JH2-2 at frequencies above the level detection (level of detection, $<2.04\times10^{-11}$ to $<5.70\times10^{-11}$).

Interestingly, we found that the MIC for triclosan in *S. aureus* EaglesT4 before and after curing of the *ileS2*-carrying plasmid was 0.03 mg/L. Similarly, when 9518T3 received and expressed plasmid-borne mupirocin resistance, from either *S. aureus* Eagles or clinical isolate 247378, it's triclosan MIC (2 mg/L) was unaffected. Indeed, the triclosan susceptibility of all *S. aureus* 9518T3 transconjugants was unchanged from the parent and subsequent curing did not alter this (data not shown). So we found no evidence for plasmid carriage or strain background affecting the MIC to triclosan.

Table 5.3. The ability of plasmids pG01 and pIP501 to mobilize resistance to triclosan in association with mupirocin resistance during tri-parental matings. Transfer frequencies are transconjugants per recipient. For each mating, selective media were used to calculate the transfer of mupirocin resistance (Mup^r) on its own and mupirocin resistance in combination with reduced susceptibility to triclosan (Mup^r, $Tric^r$).

		Donor 2							
		S. aureus EaglesT4		*S. aureus* 9518T3Mup		*S. aureus* EaglesT4		*S. aureus* 9518T3Mup	
Donor 1	Trait(s) transferred	Mup^r	Mup^r, $Tric^r$	Mup^r	Mup^r, $Tric^r$	Mup^r	Mup^r, $Tric^r$	Mup^r	Mup^r, $Tric^r$
S. aureus 661	Gen^r	2.83×10^{-8}	$<6.25\times10^{-11}$	8.66×10^{-8}	$<4.17\times10^{-11}$	$<5.70\times10^{-11}$	$<5.70\times10^{-11}$	$<2.64\times10^{-11}$	$<2.64\times10^{-11}$
S. pneumoniae T4N514	Chl^r	5.06×10^{-9}	$<4.32\times10^{-11}$	7.37×10^{-10}	$<5.21\times10^{-11}$	$<2.04\times10^{-11}$	$<2.04\times10^{-11}$	$<4.25\times10^{-11}$	$<4.25\times10^{-11}$
		S. aureus WBG542		*S. aureus* WBG542		*E. faecalis* JH2-2		*E. faecalis* JH2-2	
		Recipient							

To investigate the potential for reduced susceptibility to triclosan to be disseminated by transduction, we created lysates of bacteriophages 80α and K propagated on a selection of resistance bearing staphylococci. Whilst examining transduction we found a MOI of 0.1 to be optimum for gene transfer (data not shown). Consequently, all results presented herein were obtained using this ratio of phages to bacteria. *S. aureus* strains NCIMB9518, RN450, NCTC6571 and WBG541 were able to receive and express resistance traits transferred by transduction (Table 5.4). Mupirocin resistance was transferred to *S. aureus* recipients at frequencies of between 3.02×10^{-9} and 1.34×10^{-10} transductants per recipient, whilst transfer of rifampicin resistance was transferred at higher frequencies (9.35×10^{-8} to 6.79×10^{-9} transductants per recipient). Gentamicin resistance was transferred at similar frequencies to mupirocin resistance, whilst no transfer of triclosan resistance was detected. As above, we investigated the potential of *S. epidermidis* LTN to donate triclosan resistance. However, no CoNS to *S. aureus* transfer of triclosan resistance was detected and neither was it able to transfer this resistance to *S. epidermidis* 12228.

Due to the rarity of the event, transformation is difficult to study in species of Gram-positive bacteria and particularly *S. aureus*, which produces large amounts of extracellular DNase. These enzymes digest any free DNA which would otherwise be available for transformation. The process is further complicated by the window of competency being fairly short and reliance upon high calcium concentrations. In an effort to reduce digestion of the free DNA all recipients were washed extensively to remove as much extracellular staphylococcal DNase as possible. However, we were unable to identify any frequency of transformation above our level of detection (between 3.76×10^{-10} and 5.02×10^{-11} transformants per recipient).

Effects of triclosan on gene transfer. Several studies had shown that the presence of antibiotics or biocides can affect the frequency of gene transfer events.[14,21,57-61] Consequently, we aimed to model the effects of triclosan upon gene transfer of antimicrobial resistance traits amongst staphylococci and some other low-GC, Gram-positive bacteria.

Triclosan had noticeable effects on gene transfer of antimicrobial resistance by conjugation; concentration of triclosan had a significant effect upon conjugal transfer

Table 5.4. The transfer of antimicrobial resistance traits by transduction. Both chromosomally encoded (rifampicin resistance; Rif^r) and plasmid encoded (mupirocin resistance and gentamicin resistance; Mup^r and Gen^r, respectively) resistance traits were transferred between *S. aureus* by transduction. However, no transduction of triclosan resistance was detected. Transduction frequency was optimal when using an MOI of 0.1. Bacteriophage 80α was used for all transductions, unless either donor or recipient was unsusceptible to this phage and bacteriophage K was used instead. Transfer frequencies are shown as transductants per recipient.

Donor	Trait(s) transferred	Recipient				
		S. aureus 9518	*S. aureus* RN450	*S. aureus* 6571	*S. aureus* WBG541	*S. epidermidis* 12228
S. aureus Eagles	Mup^r	9.86×10^{-10}	1.47×10^{-9}	1.34×10^{-10}	3.02×10^{-9}	$<1.78\times10^{-10}$
S. aureus 9518Rif	Rif^r	1.93×10^{-8}	9.35×10^{-8}	6.79×10^{-9}	8.59×10^{-8}	$<9.68\times10^{-11}$
S. aureus 9518T1	$Tric^r$	$<5.02\times10^{-11}$	$<1.32\times10^{-11}$	$<9.02\times10^{-11}$	$<8.43\times10^{-11}$	$<8.67\times10^{-11}$
S. aureus 9518T3	$Tric^r$	$<7.08\times10^{-11}$	$<7.49\times10^{-11}$	$<5.40\times10^{-11}$	$<5.46\times10^{-11}$	$<4.12\times10^{-11}$
S. aureus 9518T4	$Tric^r$	$<8.11\times10^{-11}$	$<1.24\times10^{-10}$	$<8.89\times10^{-11}$	$<3.93\times10^{-11}$	$<1.55\times10^{-10}$
S. aureus EaglesT4	Mup^r & $Tric^r$	$<3.77\times10^{-11}$	$<8.21\times10^{-11}$	$<5.25\times10^{-11}$	$<8.91\times10^{-11}$	$<3.61\times10^{-11}$
S. aureus 9518T3Mup	Mup^r & $Tric^r$	$<9.08\times10^{-11}$	$<5.32\times10^{-11}$	$<2.50\times10^{-11}$	$<9.11\times10^{-11}$	$<7.35\times10^{-11}$
S. aureus 24500T1	$Tric^r$	$<7.67\times10^{-11}$	$<6.96\times10^{-11}$	$<9.65\times10^{-11}$	$<6.20\times10^{-11}$	$<1.50\times10^{-11}$
S. aureus 661	Gen^r	6.44×10^{-9}	1.07×10^{-9}	2.13×10^{-10}	6.74×10^{-9}	$<7.96\times10^{-11}$
S. aureus 9518T3pGO1	Gen^r & $Tric^r$	$<3.92\times10^{-11}$	$<8.56\times10^{-11}$	$<6.09\times10^{-11}$	$<7.52\times10^{-11}$	$<9.24\times10^{-11}$
S. epidermidis LTN	$Tric^r$	$<7.94\times10^{-11}$	$<4.21\times10^{-11}$	$<2.00\times10^{-11}$	$<8.96\times10^{-11}$	$<2.82\times10^{-11}$

of mupirocin resistance (P = 0.001; Figure 5.1a). The presence of 0.1 mg/L triclosan increased significantly the frequency of mupirocin transfer (P = 0.09), but this increase did not hold true for 1 mg/L, and when exposed to 10 mg/L of triclosan conjugation was decreased significantly (P = <0.001). However, no transfer of reduced triclosan susceptibility was detected during exposure to any level of triclosan (Figure 5.1b). Triclosan significantly affected the conjugal transfer of other plasmids and their associated resistances (P = 0.034; Figure 5.1c). No significant difference in the transfer of pGO1 or pIP501 was observed with 0 to 0.1 mg/L triclosan (P = <0.05), but 10 mg/L significantly reduced the frequency of transfer of these MGE (P = 0.008).

We were keen to investigate the effects of triclosan upon the interspecies transfer of resistance determinants by conjugation and found that triclosan also significantly affected the transfer of pIP501 from *S. pneumoniae* T4N514 to *S. aureus* WBG542 (P = 0.391; Figure 5.1d). Interestingly, 0.1 mg/L triclosan appeared to slightly, but significantly, increase the transfer of pGO1 from *S. aureus* 661 to *S. epidermidis* 12228 (P = 0.041). However, the presence of triclosan failed to induce neither the transfer of pGO1 to *E. faecalis* JH2-2 nor the transfer of triclosan resistance from *S. epidermidis* LTN to *S. aureus* WBG542. The presence of DMSO, the solvent in our triclosan solutions, had no significant effect upon conjugation when compared with water (P = <0.05).

Whilst the pre-exposure of the phages to triclosan did not induce the transduction of triclosan resistance to detectable levels, it was shown to affect transduction frequencies of other resistance traits (Figure 5.2a). Transduction of rifampicin resistance and pGO1-encoded QAC and Gentamicin resistance were significantly reduced by pre-exposure of the transducing phages to 1 or 10 mg/L triclosan (P = >0.05). We were unable to test the effects of exposing recipient cells to 1.0 and 10 mg/L triclosan since this was lethal to the recipient. However, pre-exposure of the recipient to 0-0.1 mg/L triclosan had no significant effect upon transduction frequency (P = <0.05). Interestingly, the presence of triclosan in the transducing mixture had the most potent effect upon transduction. Whilst 0 and 0.01 mg/L triclosan did not significantly effect transduction (P = <0.05), 0.1, 1 and 10 mg/L triclosan did

Figure 5.1. Effect of triclosan on gene transfer of antimicrobial resistance by conjugation. (a) Conjugal transfer of mupirocin resistance was significantly affected by 10 mg/L triclosan (black bar, Eagles × 9518Rif; grey bar, EaglesT4 × 9518Rif; white bar, 247378 × 9518Rif). **(b) Transfer of triclosan resistance** was not induced by the addition of triclosan during conjugation, either singularly or in combination with mupirocin resistance (black bar, 9518T3 × 9518Rif; grey bar, EaglesT4 × 9518Rif; white bar, 9518T3mup × 9518Rif). **(c) The conjugative transfer of other resistances** was also significantly affected by 10 mg/L triclosan ($P = 0.008$). *S. aureus* 661 was used as the donor for gentamicin resistance and *S. pneumoniae* T4N541 the donor of chloramphenicol resistance (black bar, 661 × WBG542; grey bar, 661 × 9518Rif; white bar, T4N514 × JH2-2). **(d) Interspecies transfer of resistance** was affected by the presence of triclosan, however no transfer of triclosan resistance from *S. epidermidis* LTN was induced. Again *S. aureus* 661 and *S. pneumoniae* T4N541 were used as donors, additionally LTN was employed as a potential donor of triclosan resistance (black bar, T4H514 × WBG542; grey bar, 661 × JH2-2; white bar, 661 × 12228; chequered bar, LTN × WBG542). The dashed line represents the limit of detection.

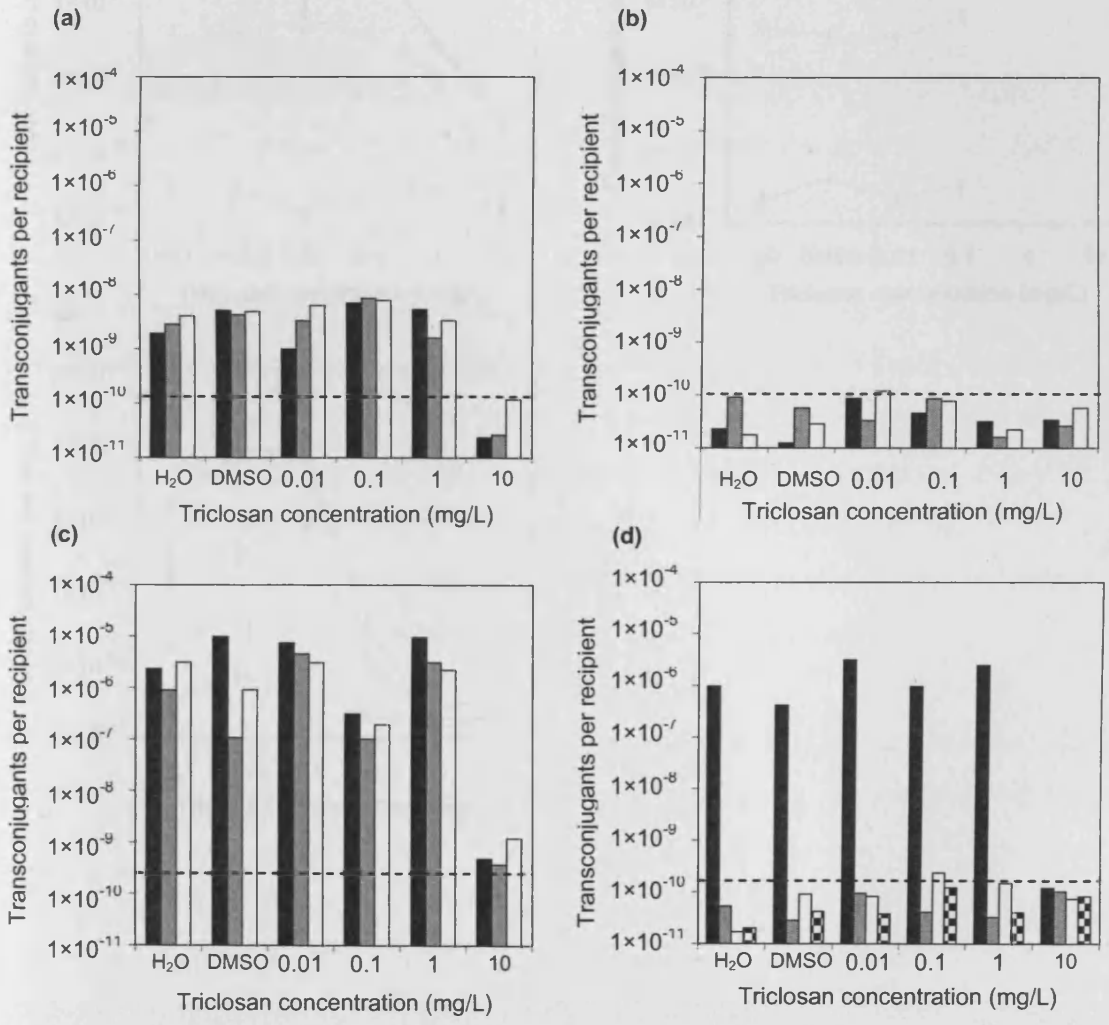
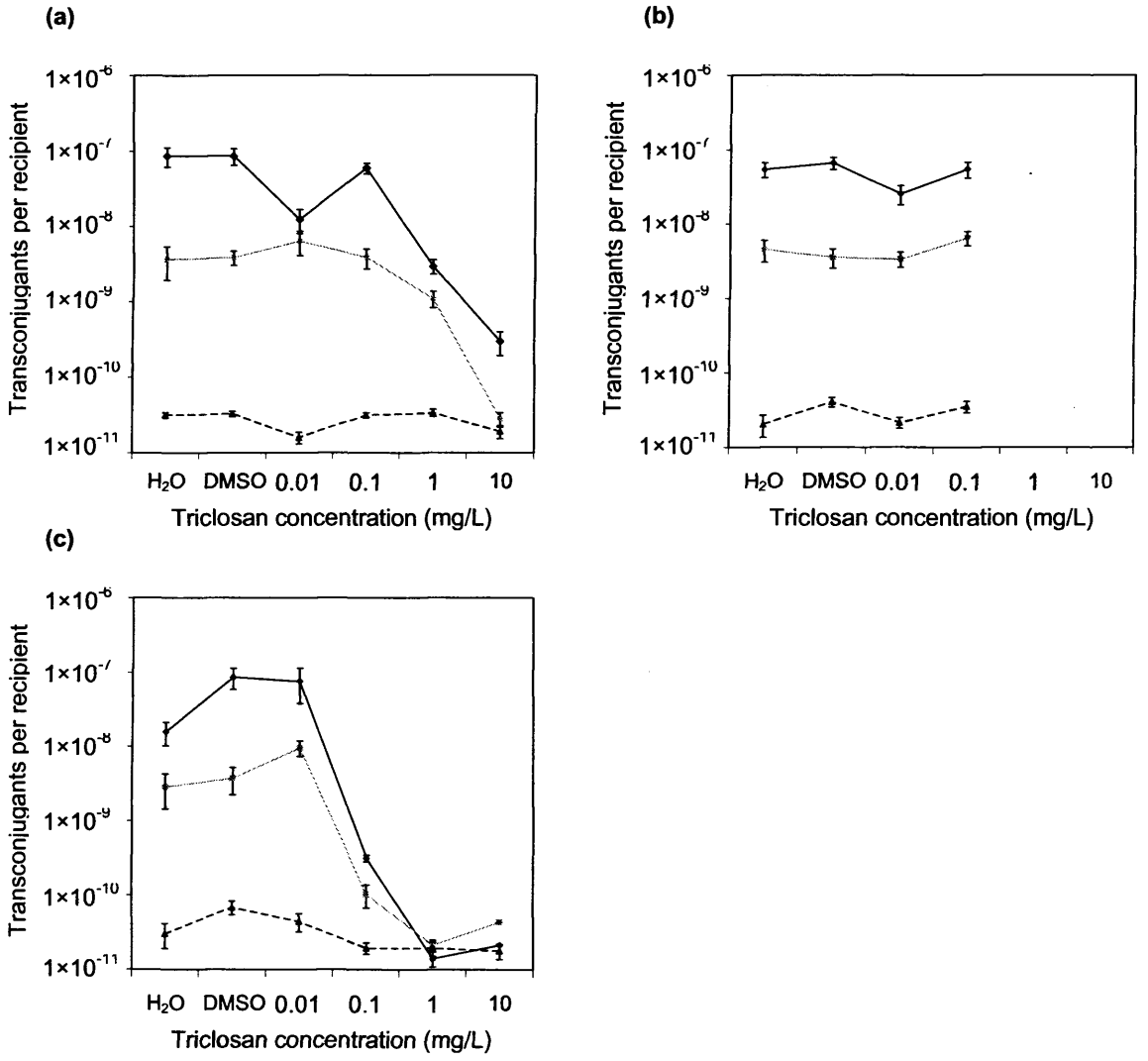


Figure 5.2. Effect of triclosan on transduction. Triclosan was shown to affect transduction during **(a)** pre-exposure of the transducing particles **(b)** Pre-exposure of the recipient had the least effect upon transduction, **(c)** whilst the presence of triclosan during infection significantly reduced the frequency of transduction (P = >0.05). Black line, rifampicin resistance; grey line, CPC resistance; dashed line, triclosan resistance.



significantly reduce the frequency of transduction of both rifampicin resistance and pGO1-associated resistances (P = 0.808 and 0.415, respectively). The presence of DMSO had no significant effect upon transduction when compared against water (P = <0.05).

Although we had been unable to detect the gene transfer of antimicrobial resistance genes by transformation, as described above, we hypothesized that the presence of triclosan may induce the transformation of reduced susceptibility to this antimicrobial by providing a strong selective pressure for its transfer. However, no transformation of *S. aureus* was detected; triclosan did not induce the transformation of the determinants for reduced susceptibility to triclosan amongst *S. aureus*.

Discussion

If horizontal transfer of triclosan resistance has occurred, it may reasonably be hypothesized that a gene associated with this resistance would show signs of horizontal acquisition. However, our analyses of eight *S. aureus* and four CoNS genomes revealed no such indication. We conclude that the chromosomal location, high-level of conservation and essentiality for survival, in combination with this bioinformatic analysis, signify that the likelihood of horizontal transfer of triclosan resistance, as mediated by *fabI*, is very low. This is also important in light of the knowledge that bacterial fatty acid biosynthesis is becoming a popular target for experimental chemotherapeutics.[62-68] However, the high-level of conservation of the genes that encode the fatty acid biosynthetic pathway does provide the homology required for effective recA-mediated recombination, if transfer was to occur.

Previous transfer of triclosan resistance was associated with a mupirocin resistance plasmid[38] and presumably the mode of transfer was conjugation. Therefore, we aimed to study the conjugative transfer of triclosan resistance, both singularly and in combination with mupirocin resistance. Whilst transfer of mupirocin and other antimicrobial resistances was mediated by conjugation, no transfer of triclosan resistance was detected. Indeed, when *S. aureus* strain Eagles, carrying plasmid-borne mupirocin resistance, was mutated to triclosan resistance, only mupirocin resistance was transferable. Similarly, whilst a triclosan resistant mutant, which had received the

mupirocin resistant plasmid by conjugation, was able to disseminate the plasmid, we did not detect any transfer of triclosan resistance. This indicates that within these strains no 'transfer relationship' was developed between the mupirocin and triclosan resistances and thus co-transfer is unlikely. This occurred in a variety of *S. aureus* strains, indicating that transfer was not reliant upon the evolutionary history of the host. This was also seen with resistance plasmids pGO1 and pIP501.

We hypothesized that the movement of plasmids through a secondary donor may provide the apparatus necessary for transfer and facilitate the dissemination of triclosan resistance. We found that mupirocin resistance transfer was increased; the presence of the more promiscuous plasmids, pGO1 and pIP501, augmented the conjugation of *ileS2*-carrying plasmids. Yet, tri-parental matings failed to yield any detectable transfer of triclosan resistance. This also held true for inter-species transfer, which may play a significant role in the evolution of *S. aureus* and its clinical impact. For example, *Enterococcus faecium* has been identified as a source of high-level vancomycin resistance in MRSA, following conjugative transfer of an 100 kb plasmid in a poly-microbial biofilm.[69] So other species appear to be a source of resistance genes for *S. aureus* and since *S. epidermidis* is less susceptible to triclosan than *S. aureus* we investigated whether this organism could be a donor for triclosan resistance. A lack of detectable transfer does not support this theory, by conjugative transfer at least. However, our *in vitro* model does not accurately replicate the complex interactions of a natural biofilm, which may affect this process.

Evidence that removing or introducing an *ileS2*-carrying plasmid from or into a triclosan resistant strain had no effect upon triclosan susceptibility reiterates the lack of association between these two resistances. Indeed, it appears that several other groups have failed to find an association between these resistances in *S. aureus*.[39-42]

A MOI of 0.1 was optimum for transfer of rifampicin and mupirocin resistance by transduction. However, no intra- or inter-species transduction of triclosan resistance was detected at any MOI (0.01 to 1). We thus conclude that whilst transduction may be an important factor in the dissemination of other resistance phenotypes, it is unlikely to be involved in the transfer of triclosan resistance amongst staphylococci.

Furthermore, an alternative mode of transfer, transformation, also failed to facilitate triclosan resistance transfer.

As discussed above, we did not detect any transfer of triclosan resistance by conjugation, transduction or transformation. These transfer experiments were performed in the absence of triclosan, so we wanted to investigate the effects of triclosan on these interactions. We aimed to identify whether the presence of triclosan can induce the transfer of its resistance, in addition to elucidating the effects of triclosan upon gene transfer of other resistances, including resistances to other biocides. Triclosan did affect resistance transfer; a low concentration of triclosan (0.1 mg/L) appeared to stimulate the transfer of mupirocin resistance, whilst higher concentrations reduced the efficiency of transfer. This also occurred, with lower significance, during the transfer of pGO1 from *S. aureus* 661 to *S. epidermidis* 12228. We are unable to explain why low levels of triclosan had this promotional effect on conjugation. We could hypothesise it influences the surface properties and hence promotes the interaction between the mating cells. Such an interaction would reflect the complex nature of the conjugation process. More expectedly, the conjugative transfer of pGO1 and pIP501 amongst most strains was diminished during exposure to triclosan, a phenomenon which may be a result of the cell wall effects of triclosan; previous hypotheses have speculated that cell wall targeting antimicrobials reduce conjugation efficiency by disrupting the cell wall-associated conjugation apparatus.[57,60]

Transduction frequencies were most notably affected by introducing triclosan in to the infection mixture. Pre-exposure of the transducing particles did also have a significant effect, although this was probably due to triclosan-induced inactivation of phages. The potent nature of triclosan exposure during infection was possibly a combination of the biocidal effects upon the virus, the host and again there is the possibility that, again, cell wall-associated aspects of the process were disrupted by the activity of triclosan on the cell wall.

Neither transduction nor transformation facilitated the transfer of triclosan resistance, and transfer was not induced by the presence of triclosan. The lack of transfer in the

face of such strong selective pressure leads to the conclusion that the transfer of triclosan resistance is rare.

Several authors have also questioned the significance of low-level triclosan resistance in *S. aureus*, where MICs of 1-4 mg/L are magnitudes lower than the in-use concentrations of this antimicrobial.[41,43,70] It should also be considered that, whilst most *in vitro* studies have used pure triclosan preparations, clinical and domestic formulations contain complex ratios of surfactants, detergents, chelating agents and wetting agents, which have their own antimicrobial effects and will affect the efficacy of the formulation. Although, low-level antimicrobial resistance in *S. aureus* may be associated with greater clinical risks than we might expect, such as increased mutation to higher resistance, augmented virulence, enhanced *in vivo* fitness and resistance to unrelated compounds.[71]

We have shown that, whilst mupirocin, QAC, chloramphenicol and rifampicin resistance could be transferred by conjugation and transduction, transfer of triclosan resistance was not detected. Additionally, the lack of historical transfer of the triclosan resistance-associated gene, *fabI*, draws us to question the significance of just one previous description of transferable triclosan resistance,[38] which has attracted many citations and thus may have lead to it having become an accepted phenomenon. This is supported by a lack of significant triclosan resistance amongst clinical, domestic and industrial staphylococci exposed to triclosan,[72-76] which would not be unexpected if resistance was readily disseminated.

Although triclosan has been shown to inhibit FabI, it is not the sole target, consequently, other modes of resistance may exist.[41,43,70,77] Therefore it should be considered that the transferable resistance described by Cookson *et al.*[38] may have been conferred by one of these alternative, possibly more easily mobilized, mechanisms. Indeed, this previous report reminds us of the need to use a selection of antimicrobial 'tools' in our approaches to control the spread of infection.

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Transparency declarations

PFS and MJD have nothing to declare. DO is an employee of Ciba Specialty Chemicals, Grenzach-Wyhlen, Germany.

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Chapter 6

Small colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*

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Synopsis

Objectives: A little understood mode of antimicrobial resistance in *Staphylococcus aureus* is the evolution of a sub-population of small-colony variants (SCVs). SCVs are a cause of persistent and recurring infections refractory to antimicrobial chemotherapy. Following the inadvertent isolation of suspected SCVs growing in the presence of triclosan we set out to evaluate the formation of these colonial mutants and assess their antimicrobial susceptibility.

Methods: SCVs were isolated on Mueller-Hinton agar supplemented with 1 mg/L triclosan. SCV formation frequency was calculated using a selection of both clinical meticillin resistant *S. aureus* (MRSA) isolates and meticillin-susceptible *S. aureus* (MSSA) strains. Antimicrobial susceptibility was assessed and the *fabI* gene of SCVs was sequenced to ensure resistance was not mediated by mutation of this gene.

Results: We have found *in vitro* that triclosan can select for *S. aureus* colonies showing the characteristic SCV phenotype with low-level triclosan resistance and which coincidently have reduced susceptibility to penicillin and gentamicin. Additionally, triclosan isolated SCVs were shown to have an increased tolerance to the lethal effects of triclosan.

Conclusions: We propose the formation of SCVs by *S. aureus* is a novel mechanism of resistance to low concentrations of triclosan, which for twenty five years has been used widely in the domestic setting in various consumer healthcare products. More recently it has been recommended for the control of MRSA. Consequently, our results identify the potential for treatment failure in infections already notoriously difficult to eradicate. It remains unclear to what extent isolates with decreased susceptibility to triclosan would develop and have the fitness to survive under real world conditions.

Introduction

SCVs of *S. aureus* arise as a drug resistant sub-population during exposure to antimicrobial chemotherapeutics.[1] Two types of SCV are regularly isolated; those with an interrupted electron transport chain (ETC) and those showing thymidine auxotrophy. ETC deficient SCVs can be further divided into those with mutations affecting menaquinone (vitamin K), haemin, thiamine or heme A biosynthesis. ETC SCVs are associated with persistent and relapsing osteomyelitis, sinusitis, muscle

abscesses and device related infections[2,3] whilst thymidine SCVs are seen in cystic fibrosis patients.[4] Although data concerning the virulence of SCVs is ambiguous, infections have proven fatal.[5-7] Mutations knocking out menaquinone, haemin or heme A biosynthesis block the *S. aureus* ETC. The subsequent energy deficiency bestows a characteristic pleiotropic phenotype including slow growth (hence small colonies), lack of pigmentation, non-production of virulence factors and reduced spectrum of carbohydrate utilisation.[8] The basis for thymidine-auxotrophic SCVs is not currently understood.[1]

The ability to form a variant subpopulation affords *S. aureus* a number of survival advantages. SCV persistence intracellularly within nonprofessional phagocytes shields them from host defences and decreases exposure to antimicrobial agents and SCVs show reduced antimicrobial susceptibility. It is well documented that *S. aureus* has been adept at developing resistance to antimicrobials.[9] Exposure of *S. aureus* to antimicrobials through prophylaxis and treatment has contributed to the isolation of multi-drug resistant staphylococci. These resistances have generally been mediated by spontaneous chromosomal mutations and acquisition of genes encoded on plasmids or other mobile genetic elements that modify drugs, induce their efflux, or alter the target molecule.[9,10] However, resistance in SCVs does not follow one of these 'classic' mechanisms, but is instead a direct consequence of the SCV phenotype.

Reduced antimicrobial susceptibility in SCVs is conferred through three known processes. Firstly, reduced growth rate has been shown to reduce the susceptibility to cell wall targeting antimicrobials by up to 4-fold.[8] Secondly, the break down in bacterial ETC reduces the transmembrane potential ($\Delta\Psi$) resulting in reduced uptake of cationic compounds.[11] Lastly, subsistence within host cells confers resistance to those chemotherapeutics unable to pass into host cells. Interestingly it has been shown that the appearance of SCVs following exposure to gentamicin results from a rapid switch, and those bacteria exposed to cycles of gentamicin followed by antibiotic-free medium repeatedly switched between a resistant SCV and a susceptible parental phenotype (revertants). The fitness of revertants relative to *S. aureus* with stable gentamicin resistance was greater in drug-free media, which suggests that the SCV phenotype has evolved as an inducible and reversible resistance mechanism that evades a permanent fitness cost.[12]

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a synthetic bisphenol antimicrobial agent, which shows activity against a wide range of Gram-positive and Gram-negative bacteria. For over twenty five years it has been included efficaciously in many hygiene and consumer health products, such as soaps, skin cleansers and mouthwashes, and is increasingly incorporated into a range of domestic plastics including toys, tea towels and chopping boards.[13] In 1998, it was recommended for the control of MRSA[14] after being successfully used to control outbreaks in a neonatal nursery,[15] cardiothoracic surgical unit[16] and to provide an alternative to expensive vancomycin administration.[17] Subsequently, 2% triclosan baths are now a recommended rationale for skin decolonisation of MRSA carriers in the United Kingdom.[18]

Since the introduction of triclosan into clinical practice, strains of *S. aureus* exhibiting low-level resistance to triclosan have been isolated. MRSA strains with minimum inhibitory concentrations (MICs) of 2-4 mg/L have been isolated from patients treated with daily triclosan baths; this compares with MICs of 0.01-0.1 mg/L for susceptible strains.[19] Traditionally triclosan has been regarded as a biocide rather than an antibiotic and, as such, has been thought to have numerous intracellular and cytoplasmic target sites.[20-23] However, this view has been challenged by evidence that triclosan may act on a specific target, FabI, an enoyl reductase enzyme involved in bacterial fatty acid biosynthesis.[24-26] Notably, fabI mediated resistance to triclosan in *S. aureus* remains low-level and of ambiguous clinical significance. In addition studies have failed to demonstrate any explicit link between triclosan exposure and resistance in *ex-situ* isolates, microcosms or the domiciliary environment.[27-30]

Thus, although the development of *S. aureus* SCVs upon exposure to antibiotics, and in particular aminoglycosides, is well documented, this is not the case with biocides. So the small, non-pigmented colonies which arose on Mueller Hinton (MH) agar, containing 1 mg/L triclosan, inoculated with *S. aureus* were initially suspected to be contaminants. However, these atypical colonies proved to be *S. aureus* SCVs. Here, we aimed to study these organisms to see if they represent a novel resistance mechanism to this antimicrobial and to further examine their biology.

Materials and Methods

Experimental conditions. Unless otherwise stated, all experiments were performed in triplicate (n=3) and results are presented as mean ± standard deviation (s.d.). *Staphylococcus aureus* strains NCTC6571,[31] NCIMB9518, F89 (high-level mupirocin resistant) and clinical MRSA isolates 24500 and 27343 were maintained on nutrient agar (Oxoid Ltd, UK) from which cultures in nutrient broth (Oxoid Ltd, UK) were prepared when required. SCVs were isolated by the growth of *S. aureus* strains to mid-log phase in 10 mL Mueller-Hinton (MH) broth (Oxoid Ltd, UK) at 37°C. At this point pre-warmed triclosan solution (100 mg/L) was added to achieve a final concentration of 0.01 mg/L, further incubation was performed for 6 h at 37°C. Cells were harvest by centrifugation (5000 rev/min for 15 min), washed and resuspended in 10 mL quarter-strength Ringer's solution. 100 µL volumes of 10^9 cells/mL were spread on to the surface of MH agar (Oxoid Ltd, UK) plates containing 0-10 mg/L triclosan. Plates were incubated for 48 h at 37°C. Suspected reduced triclosan susceptibility SCVs were observed as tiny, non-pigmented colonies on plates containing triclosan. Such colonies were maintained on MH agar supplemented with 1 mg/L triclosan, from which over-night cultures prepared in MH broth with 1 mg/L triclosan were made when required. Triclosan powder (Irgasan DP300) was a gift from Ciba Specialty Chemicals (Grenzach-Wyhlen, Germany) and was dissolved in sterile dimethylsulphoxide (DMSO). DMSO concentrations were maintained below 1% in growth media and DMSO controls were run alongside experiments where required.

Electron microscopy. Cells in logarithmic growth phase were prepared for SEM by fixing in 2% glutaraldehyde in 0.1% sodium phosphate buffer, pH 7.4, for 2 h and subsequently treated with 1% osmium tetroxide for 2 h at 4°C. Cells were dehydrated with graded concentrations of ethanol and sputter coated with gold. Samples were analysed with a Philips XL-20 high vacuum scanning electron microscope (Phillips, Netherlands). Samples for TEM were prepared as for SEM, but following ethanol dehydration were embedded in SPI-Chem™ Araldite® CY212. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined with transmission electron microscope JEM-1210 (JEOL, Japan).

Species confirmation. All suspected SCVs were confirmed as *S. aureus* by multiplex-PCR as previously reported.[32]

SCV characterisation. SCVs were analysed for haemolysis by streaking on MH agar supplemented with 5% defibrinated sheep blood (Oxoid Ltd, UK) and incubation at 37°C for 48 h. The presence of coagulase production was investigated by Staphylase test kit (Oxoid Ltd, UK) according to the manufacturers directions. 4-5 colonies were transferred to a microscope slide followed by the addition of hydrogen peroxide, the presence of bubbling was taken as an indication of catalase production. Auxotrophy for haemin, menaquinone and thiamine was examined as described previously.[33] Growth curves were attained by spectrophotometric analysis (580 nm) of MH broth cultures using a DYNEX Technologies MRX® Microplate Absorbance Reader with Revelation™ application programme. In each well of a sterile 96-well microtitre plate (Fisher Scientific, UK) 100 µL of MH broth, containing 2× final concentration of triclosan was inoculated with 100 µL of MH broth culture, diluted with broth to 10^3 cells/mL. Plates were sealed with adhesive plate seals (ABgene, UK) and incubation was performed at 37°C with agitation for 48 h. Significant effect of triclosan on bacterial growth was tested with analysis of variance (ANOVA). All data are presented as the mean ± s.d.

SCV formation and reversion rates. The rate of mutation towards the SCV phenotype was assessed by growth of *S. aureus* strains to mid-log phase in 9 mL MH broth at 37°C. At this point either MH broth or pre-warmed triclosan solution was added to give final concentrations of 0, 0.01, 0.1 and 1 mg/L, and further incubation was performed for 6 h at 37°C. Cells were harvested by centrifugation (5000 rev/min for 15 min), washed, resuspended in quarter-strength Ringer's solution and adjusted to 10^{10} cells/mL. 100 µl volumes of 10^{10} cells/mL were spread on to the surface of MH agar plates containing 1 mg/L triclosan. Additionally, dilutions of the cultures were plated onto MH agar in order to calculate the number of wild type cfu. Plates were incubated for 48 h at 37°C. Non-pigmented colonies that grew to less than 1 tenth of the size of wild type colonies were recorded as SCVs. The frequencies were expressed as numbers of SCVs per cfu. Reversion rates were calculated by inoculating a MH agar plate with cells from ten SCV colonies suspended in 3 mL of 0.9% NaCl. After 48 h growth at 37°C SCVs and wild-type colonies were counted and the frequency of reversion was determined as number of wild-type cfu per SCV.

Antimicrobial susceptibility. MICs for the three biocides, triclosan, chlorhexidine gluconate (CHX; ICN Biomedicals Inc., USA) and cetylpyridinium chloride (CPC; ICN Biomedicals Inc., USA), were calculated according to the guidelines of the

British Society for Antimicrobial Chemotherapy (BSAC).[34] Due to the slow growth of the SCVs MIC plates were incubated for 48 h. Etest® strips (Bio-stat Ltd, UK) were utilized to attain the MIC for antibiotics, as per the manufacturer's directions. The bactericidal effects of triclosan on wild-type *S. aureus* and their SCVs were compared as previously reported[35] except the inoculum was adjusted to 10^7 cfu/mL, 2% Azolectin and 5% Tween 80 in molecular grade de-ionized water was used as the neutralizing solution and sampling was continued up to 2 h. Log_{10} reduction was then calculated from $\log_{10}N_c$- $\log_{10}N_t$ where N_c and N_t represent the numbers of cfu/mL in the control and triclosan solutions, respectively. Significant effect of triclosan on the reduction in cell density was tested with ANOVA. All data are presented as the mean ± s.d.

Validation of triclosan neutralizer. 2% Azolectin and 5% Tween 80 in molecular grade de-ionized water was used as the triclosan neutralizer. Its ability to neutralize triclosan without toxicity to bacterial cells was examined as described previously.[36,37] Briefly, 8 mL of neutralizer, 100 µL of triclosan (10 mg/mL) and 900 µL sterile de-ionized water was inoculated with 1 mL of an overnight culture of *S. aureus* NCIMB9518 grown at 37°C. The viable cell count was enumerated after 5 min contact time at ambient temperature. As controls, sterile de-ionized water was added to the incubation mixture instead of the triclosan solution or the neutralizer. When the cells were exposed to triclosan without the neutralizer no viable cells were detected in the sample after the 5 min incubation period. There was no significant difference (P = 0.879) between the viable cell count of cultures exposed to triclosan and the neutralizer and where water was added instead of triclosan. This confirmed the ability of the neutralizer to quench bactericidal activity of triclosan at 100 mg/L. When examining the possible toxicity of the neutralizer, there was no significant difference (P = >0.05) between the viable cell counts of cultures exposed to sterile water and those exposed to the neutralizer for 15 min, confirming that the neutralizer was non-toxic.

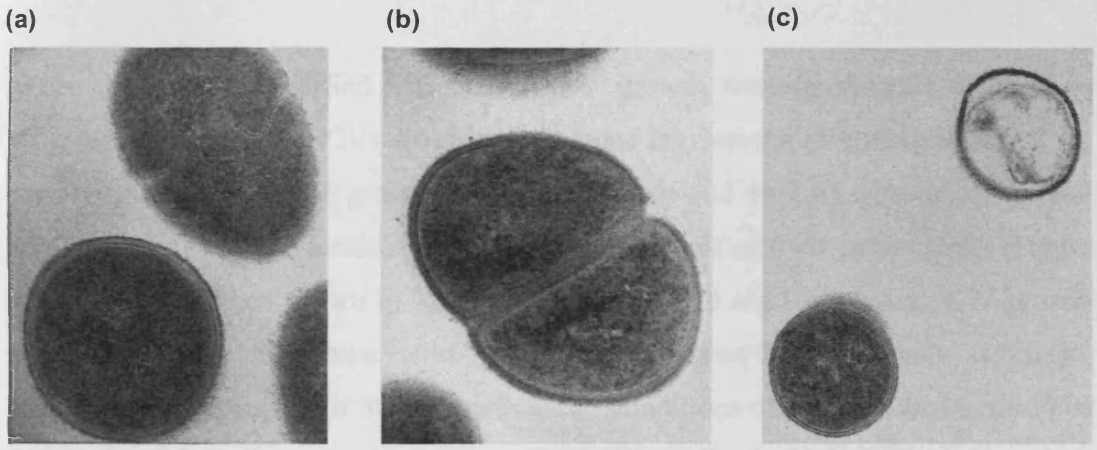
FabI gene sequencing. Chromosomal DNA was extracted as described previously.[38] Primers adapted from those designed for the amplification of the staphylococcal *fabI* gene and putative *fabI* promoter region[39] were used in 50 µL PCRs, (Fab1F, tgttccgcatggagatacac and Fab1R, taaggactaattctgtggatgt). Reactions contained 0.5 µL of chromosomal DNA (approximately 0.5 µg), 0.5 µg of each primer, 1 U of *Taq* DNA polymerase (Bioline Ltd, UK), 5 µL of 10× buffer (Bioline Ltd, UK), and

0.2 mM deoxynucleoside triphosphates (Bioline Ltd, UK). The PCR was performed in a PTC-200 DNA engine (MJ Research, USA) with an initial 5-min denaturation at 94°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 45 s, followed by a final extension step at 72°C for 5 min. The amplified products were purified by QIAquick PCR purification kit (Qiagen Ltd, UK) and the sequences of both strands determined with an ABI Prism 377 DNA sequencer, BigDye fluorescent terminators and primers used in the initial PCR amplification. To ensure complete sequence coverage a further set of primers, again adapted from Slater-Radosti *et al.* (2001)[39] were also used (Fab2F, atgttaaatcttgaaaacaaaac and Fab2R, ttatttaattgcgtggaatccgc).

Results

Suspected SCVs were isolated from MH agar plates containing 1 mg/L triclosan and spread with a heavy inoculum of *S. aureus* strains NCTC6571 (Oxford strain), NCIMB9518, F89 (high-level mupirocin resistant) and clinical MRSA isolates 24500 and 27343. Non-pigmented colonies were approximately one tenth the size of wild type *S. aureus* colonies and non-haemolytic on sheep blood agar. Most appeared coagulase negative during examination by latex agglutination test, although SCVs of F89 and 24500 showed very weak positive reactions. Upon addition of hydrogen peroxide the colonies were shown to be weakly catalase positive. The cells generally appeared as Gram-positive cocci, although some cultures of suspected SCVs appeared to show a heterogeneous cellular morphology; apparently normal *S. aureus* mixed with larger cell morphotypes. Subsequent scanning electron microscopy (SEM) of SCV samples revealed bacterial cells of different sizes, in distinction to homogeneous cocci of the isogenic wild type *S. aureus* (data not shown). Kahl *et al.* (2003)[40] suggests the larger cells are due to impaired cell separation. Transmission electron microscopy (TEM) disclosed this to a greater extent, by revealing SCV cultures to contain 48.7% large cells with incomplete cross walls and 2.3% 'empty' cells (Figure 6.1), significantly more than were found in wild type cultures ($P = <0.05$, for both phenomena).

Figure 6.1. Heterogeneous SCV cultures containing a mixture of large and small cell morphologies. Analysis of a triclosan induced SCV culture revealed a large number of unusually large cells (2.13:1), attributed to interrupted cell division. **(a)** TEM analysis of a triclosan induced a SCV showing a heterogeneous culture of cells, some (48.7%) contained incomplete cell walls. **(b)**, in comparison, wild type cultures contained only homogeneous cocci with clear single cell walls. **(c),** Also observed in cultures of SCVs were a smaller number (2.3%) of 'empty' cells, which were unseen in wild-type cultures.



Species confirmation of *S. aureus* SCVs is notoriously challenging and can often lead to misidentification as coagulase negative staphylococci (CoNS).[6] Consequently, the suspected colonies were ultimately identified as *S. aureus* by genomic analysis. Suspected SCVs were interrogated for the presence of four genes (Staphylococcal 16s rRNA, *nuc*, *mecA* and *mupA*) by multiplex PCR.[32] The presence of a Staphylococcal 16s rRNA gene and *S. aureus* specific *nuc* gene was recorded for all suspected SCVs analysed, (Figure 6.2). This procedure also indicated that the SCVs maintained the *mecA* and *mupA* resistance determinants associated with their parents.

None of the SCVs were identified as haemin, menaquinone or thymidine auxotrophs and neither did any show a combination of menaquinone, haemin or thymidine auxotrophy. Thus the reason for their expression of the SCV phenotype remains uncharacterised.

When grown in unmodified MH broth, SCV growth was significantly different to wild type ($P = <0.05$). SCVs exhibited extended lag periods (3 h compared to 1 h), greatly reduced maximal growth rates (0.23 compared to 1.65 generations/h) and achieved maximal cell densities approximately one half of their parent strain (Figure 6.3). However, when grown in MH broth adjusted to 1 mg/L triclosan, SCV growth remained unaffected, whilst wild-type *S. aureus* growth was greatly restricted, indicating the advantage of SCV growth under conditions of antimicrobial stress. The SCVs showed similar antimicrobial susceptibilities, all of which differed from their wild type parent (Table 6.1). Susceptibility to triclosan was decreased by between 24- and 60-fold and to gentamicin by between 2.5- and 10-fold. The MIC of penicillin was raised by up to 4-fold in all SCVs for which the progenitor were penicillin susceptible (MSSA). The penicillin MIC for the two MRSA strains that had existing penicillin resistance was either unaffected (24500) or lowered (27343).

Additionally, SCV susceptibility to two other biocides was investigated. Chlorhexidine (CHX) a bis-biguanide biocide used widely in mouthwashes, toothpastes and clinical hand washes and cetylpyridinium chloride (CPC), a quaternary ammonium compound (QAC). Both are cationic, and as such the uptake of these compounds may be affected by a reduction in $\Delta\Psi$, reducing their antimicrobial effect[11]. However, none of the SCV strains showed any reduction in susceptibility to

Figure 6.2. SCVs were confirmed as *S. aureus* by amplification of the staphylococcal 16s rRNA (756 bp) and *nuc* (279 bp) genes. Additionally *mecA* (310 bp) and *mupA* (457 bp) were also targeted to identify meticillin and high-level mupirocin resistance respectively. 1, no template control; 2, *E. coli*; 3; *S. epidermidis* 1228; 4 & 5, 27343 and 27343SCV; 6 & 7, 24500 and 24500SCV; 8 & 9, F89 and F89SCV; 10 & 11, NCIMB9518 and 9518SCV; 12 & 13, NCTC6571 and 6571SCV.

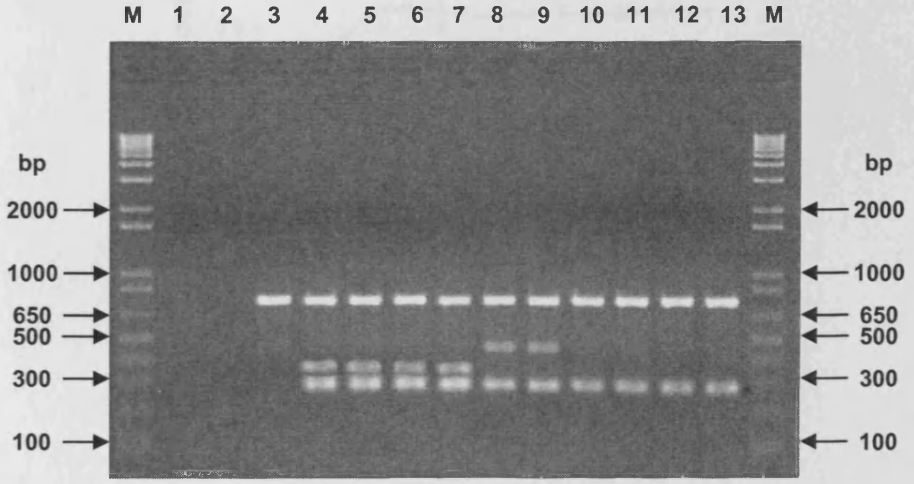


Figure 6.3. Typical SCV and wild-type growth dynamics. SCV (grey lines) and their wild type *S. aureus* parent (black lines) were grown in broth with (dashed lines) and without (solid lines) 1 mg/L triclosan. Typical SCV growth was significantly retarded in comparison to typical wild-type *S. aureus* (P = <0.05). SCVs characteristically exhibited extended lag phases and reduced maximal growth rate and cell density. Data are the mean of three measurements ± s.d.

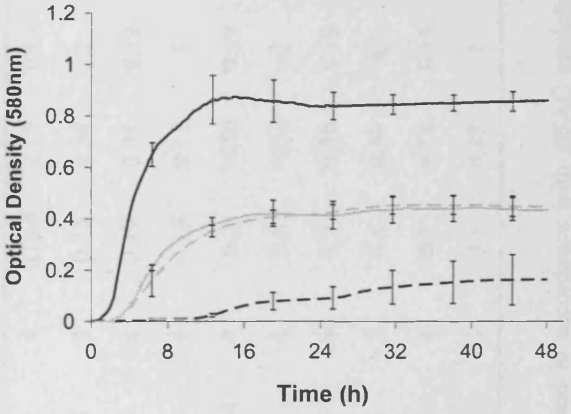


Table 6.1. MICs for *S. aureus* SCVs and their corresponding wild type parent.

	Minimum Inhibitory Concentration (mg/L)													
Strain	PEN[†]	OXA[†]	VAN[†]	MUP[†]	CHL[†]	TET[†]	ERY[†]	GEN[†]	LZD[†]	TEC[†]	CIP[†]	Tric[‡]	CHX[‡]	CPC[‡]
6571	0.023	0.13	1.5	0.19	4	0.064	0.19	0.19	0.25	0.19	0.25	0.063	1	1
6571SCV	1.0	0.25	2	0.19	4	0.75	0.38	2	1	0.5	1	4	1	1
9518	<0.016	0.13	1	0.19	8	0.19	0.38	0.19	0.5	0.38	0.19	0.063	1	1
9518SCV	0.5	0.05	0.75	0.19	4	0.19	0.13	1	1	1	0.25	3	1	1
F89	1	0.75	1.5	>1024	4	0.5	>256	0.19	0.5	0.75	0.38	0.063	1	1
F89SCV	1.5	0.38	1.5	512	4	0.5	>256	1	0.5	2	1	2	1	1
24500	48	64	1.5	0.25	4	0.5	0.38	0.19	0.5	0.25	>32	0.063	3	3
24500SCV	48	64	1.5	0.25	2	0.5	0.38	0.5	1	1	>32	1.5	3	3
27343	32	>256	1	0.38	8	0.5	0.38	0.19	0.38	0.5	>32	0.063	2	2
27343SCV	16	>256	1	0.38	4	0.5	0.19	1	0.50	1	>32	3	2	2

[†]Calculated by Etest (AB Biodisk, Sweden). [‡]Calculated in accordance with BSAC guidelines. PEN, Penicillin; OXA, Oxacillin; VAN, Vancomycin; MUP, Mupirocin; CHL, Chloramphenicol; TET, Tetracycline; ERY, Erythromycin; GEN, Gentamicin; LZD, Linezolid; TEC, Teicoplanin; CIP, Ciprofloxacin; Tric, triclosan; CHX, chlorhexidine gluconate; CPC, cetylpyridinium chloride.

either of these compounds in comparison to their isogenic wild type, perhaps indication that a reduction in $\Delta\Psi$ is not associated with these SCVs, or that the permeability of these biocides remains unaffected.

Commonly, antibiotics possess single target sites, and consequently increased MICs and reduced bactericidal effectiveness are linked. In contrast, this correlation is not so for biocides; biocides have multiple targets and increased MICs often do not correlate with decreased bactericidal activities of that compound.[23] It has been shown previously that increases in triclosan MIC in *S. aureus* have resulted in little to no increase in bactericidal effectiveness of the compound.[21] Contrary to these data, our SCVs showed significantly increased resistance to the lethal effects of 7.5 mg/L triclosan ($P = <0.05$). Five $\log_{10}$ reductions in cell density by 7.5 mg/L triclosan were achieved in times ranging from 15 to 20 min for wild type *S. aureus*, this compares to times of over 2 h for SCV strains (Figure 6.4a). However, the bactericidal efficacy of triclosan at 20 and 40 mg/L was comparable between wild type and SCV strains (Figure 6.4 b, c).

DNA sequences for the *fabI* gene and preceding promoter region were ascertained for all SCVs and compared to those of their wild type parent. Sequence comparison failed to identify any mutations within the gene, showing that the reduced susceptibility within the SCVs was not mediated by mutation of *fabI*. This implies that fabI is not the sole target for triclosan.

SCV formation occurs at rates as high as 1 in 1000 when selected by gentamicin.[12,39] The rate of reversion can also be very high,[12] although some SCVs can show stability under non-selective conditions. Triclosan was shown to select for SCVs at rates between 1.86×10^{-11} and 9.46×10^{-10} SCV/cfu. Interestingly, these frequencies were increased by up to four orders of magnitude when strains were subject to a pre-exposure to triclosan (Figure 6.5). Subsequent reversion occurred at variable frequencies, with some SCVs of strains 9518, F89 and 24500 exhibiting a stable phenotype (e.g. less than 10^{-10}). Revertants exhibited antimicrobial susceptibilities comparable to their wild type parent i.e. upon reversion all strains lost any SCV-associated reduction in antimicrobial susceptibility.

Figure 6.4. Typical death curves for wild type and SCV *S. aureus* exposed to triclosan. Reduction in viable cell count following exposure to various concentrations of triclosan was measured for a wild type *S. aureus* (solid line) and its isogenic SCV (dashed line) **(a)** The lethal effects of 7.5 mg/L triclosan where shown to be greatly reduced against SCVs. The lethal effect of triclosan is significantly different between typical wild type and typical SCV (P= <0.05). The times for a five $\log_{10}$ reduction in cell density were increased from 15-20 min for wild type *S. aureus* to >2 h for SCVs strains. However, at higher concentrations of triclosan, **(b)** 20 and **(c)** 40 mg/L, no significant difference was observed between the lethal effect upon wild type and SCV (P = >0.05). Data are the mean of three measurements ± s.d.

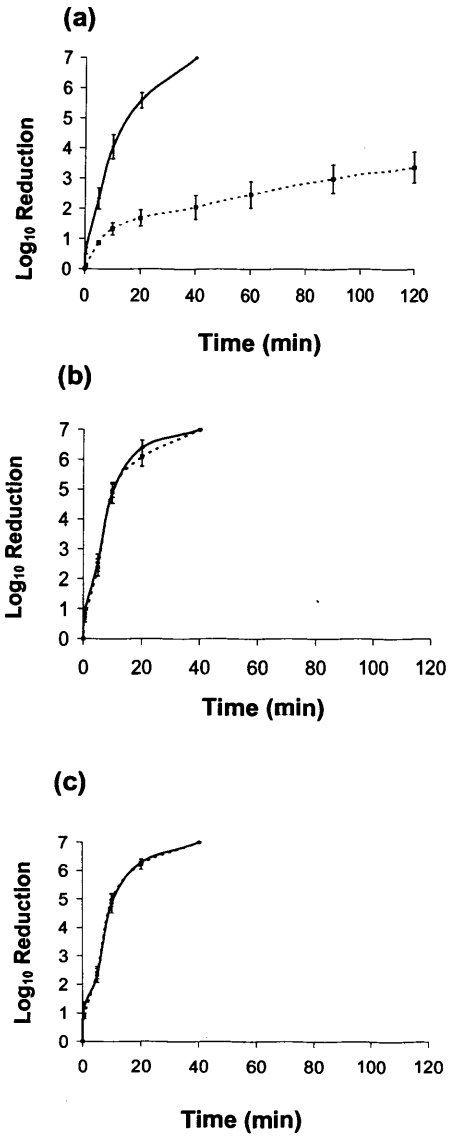
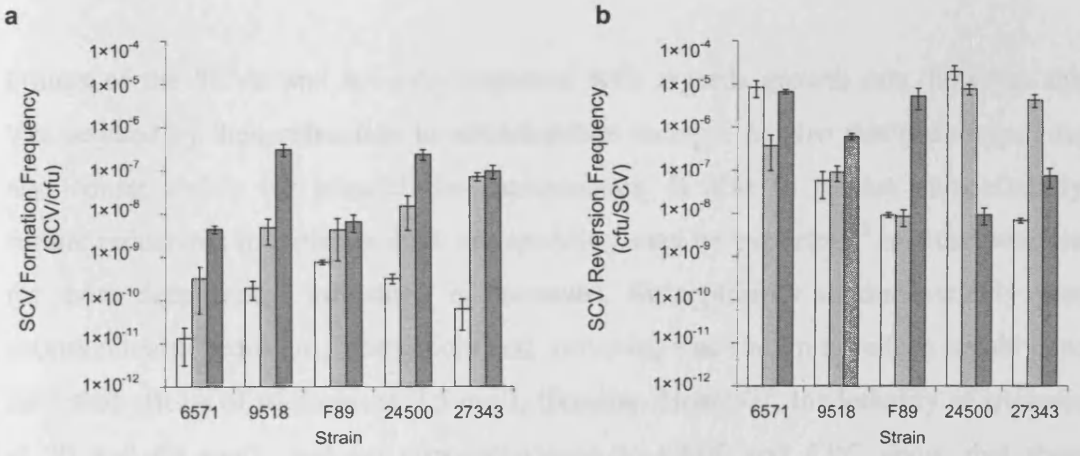


Figure 6.5. (a) Frequency of mutation to the SCV phenotype following exposure to zero (open bar), sub-MIC (0.01 mg/L; light grey bar) and higher than MIC (0.1 mg/L; dark grey bar) concentrations of triclosan and **(b) the rate of reversion to wild-type of SCVs** selected on various concentrations of triclosan. Data are the mean of three measurements ± s.d.



Discussion

We have shown that *in vitro* exposure to triclosan can trigger the emergence of reduced drug-susceptible SCVs, and this was augmented by a pre-exposure to sub-MIC triclosan. These SCVs showed the characteristic SCV phenotype, as a consequence of this the organisms were difficult to culture and identify by classical methods. These findings raise potential issues in healthcare; although clinical evidence for failure of triclosan has not yet been shown, the findings create the possible scenario that selection of SCVs during skin decolonization with triclosan may cause patients to be misidentified as MRSA free. These missed SCVs may then be transmitted between patients and/or revert to the wild type state and initiate more familiar MRSA infections. Additionally, we have shown that MRSA SCVs retain the genetic determinants for meticillin resistance; hence these may provide a longstanding reservoir of resistance genes to be shared amongst the microbial community, although it remains to be shown that these variants are able to partake in horizontal gene transfer.

Fitness of the SCVs was severely impaired with regards growth rate, however this was negated by their refraction to antimicrobial therapy. *In vivo* this phenotype may also confer ability for intracellular maintenance. If able to persist intracellularly, further reductions in antimicrobial susceptibility may be expected,[42] and the potential for more deep seated infections is increased. Susceptibility to three widely used antimicrobials (penicillin, gentamicin and triclosan) was shown to reduce notably and the lethal effects of triclosan at 7.5 mg/L likewise. However, the lethality of triclosan at 20 and 40 mg/L and no cross-resistance to CHX and CPC show that these staphylococcal variants can be controlled by pertinent use of antimicrobial chemotherapeutics. Reductions in triclosan susceptibility were comparable to those achieved through alteration of the *fabI* gene product, and as such are hypothetically of equal clinical significance. It is important to understand that in laboratory studies susceptibility is not comparable to that *in situ*. During clinical or domestic use, triclosan is delivered often as part of a complex formulation. These formulations contain various combinations of surfactants, detergents, chelating agents and wetting agents, all of which will affect their action, and ultimately the susceptibility of microorganisms exposed to them. Moreover, typical in-use concentrations of triclosan

in hand wash, bath formulations and surface disinfectants are 0.3% to 1%, for hospital wash applications for MRSA eradication even 2% triclosan is recommended.[18] These in-use concentrations are magnitudes higher than the concentrations of triclosan needed to inhibit SCV isolates of MRSA reported herein.

We were unable to identify any auxotrophy towards haemin, menaquinone or thymidine; hence the reason for the expression of the SCV phenotype remains uncharacterised.

This investigation highlights a microbial phenomena (SCV formation) affecting antimicrobial chemotherapy. Despite this, it is important to appreciate the differences between *in vitro* susceptibility to triclosan in pure culture conditions and those *in situ* where mixed microbial populations are exposed to complex formulations. Further investigations should be targeted to elucidate the existence of this phenomenon under conditions better reflecting the environments in which triclosan is used.

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We would like to acknowledge the contribution of Professor A. Denver Russell, Welsh School of Pharmacy, Cardiff, who sadly died in September 2004, for his invaluable contribution to our work. The authors would also like to thank Mr Alan Paull, PHLS, Cardiff, for the gift of the clinical MRSA isolates. We are also grateful to Dr Ant Hann, Cardiff School of Biosciences, for his help with the electron microscopy. This work was supported by Ciba Specialty Chemicals, Grenzach-Wyhlen, Germany, including a research studentship to PFS.

Transparency declarations

PFS and MJD have nothing to declare. DO is an employee of Ciba Specialty Chemicals, Grenzach-Wyhlen, Germany.

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On March 2nd 2007 the Journal of Antimicrobial Chemotherapy published an article by Roger Bayston et al. entitled 'Triclosan resistance in methicillin-resistant* Staphylococcus aureus *expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics' (doi:10.1093/jac/dkm03). We believe that this article has several shortcomings in the methods used to collect the data and during their subsequent interpretation. Consequently, we have submitted a letter to the Journal of Antimicrobial Chemotherapy to raise these issues and add to the discussion on triclosan-induced SCVs. The full text of this letter is included in appendix 9.2.

Chapter 7

Horizontal gene transfer in triclosan-resistant small colony variants of *Staphylococcus aureus*

Synopsis

Objectives: Following the isolation of triclosan-resistant small colony variants (SCVs) of *Staphylococcus aureus* we aimed to elucidate their potential involvement in the evolution of *S. aureus* by horizontal gene transfer.

Methods: SCVs were isolated on Mueller-Hinton agar supplemented with 1 mg/L triclosan. Phage-mediated transfer (transduction) of antibiotic resistance genes was assessed with bacteriophages 80α and K. Transformation experiments were performed, investigating the transfer of resistance genes between wild-type and SCV forms of *S. aureus* and filter matings were employed to assess gene transfer by conjugation.

Results: Triclosan SCVs showed the same phage susceptibility as their parent strains but no gene transfer by transduction was detected. Multiplex PCR confirmed the presence of antibiotic resistance plasmids in SCVs, however no conjugal transfer was detected. Transformation of antibiotic resistance markers was detected, indicating a higher level of competency in *S. aureus* SCVs compared to wild-type.

Conclusions: Whilst many studies have investigated the direct impact of *S. aureus* SCVs upon patient morbidity the data presented here demonstrate their potential as a critical source of resistance genes. We propose that SCVs can act as a genetic 'middleman', capable of surviving antibiotic treatments and host defences whilst receiving and donating mobile DNA.

Introduction

We previously isolated small colony variants (SCVs) of *Staphylococcus aureus* during exposure to triclosan (Seaman *et al.*, 2007a). Triclosan-induced SCVs showed the characteristic SCV phenotype with low-level triclosan resistance and coincidently had reduced susceptibility to penicillin and gentamicin. In addition to raised MICs, triclosan isolated SCVs were also shown to have an increased tolerance to the bactericidal effects of triclosan. SCVs are an intriguing form of *S. aureus*; the phenotype enables them to persist intracellularly within several human cell types including keratinocytes, non-professional phagocytes and cultured endothelial cells (Balwit *et al.*, 1994; von Eiff *et al.*, 1997; von Eiff *et al.*, 2001; Bayles & Bohach, 2002) and also confers a level of resistance to various antimicrobials, especially

aminoglycosides (Proctor *et al.*, 1995; Proctor, 1998; Proctor *et al.*, 2006; Seaman *et al.*, 2007a). They are also able to switch rapidly between SCV and wild-type and so each form will amplify when conditions suit them, e.g. when antibiotic treatment has ceased SCVs can switch to wild-type growth (Massey *et al.* 2001). These qualities enable SCVs to persist and cause recurring infections that can return several weeks or even years later (McNamara & Proctor, 2000).

The biochemical differences that lead to the distinction of SCVs from wild-type *S. aureus* and the role that SCVs play in human infection have attracted most attention. Consequently, there is a paucity of data regarding the role of SCVs in the dissemination of resistance and virulence genes by horizontal gene transfer.

Horizontal gene transfer in staphylococci is discussed in depth in chapter 5. However, we do not yet know the extent to which SCVs can partake in gene transfer, and consequently what their involvement in the evolution of *S. aureus* is. SCVs are physiologically very different to wild-type *S. aureus*, hence their ability to partake in conjugation, transduction and transformation may also be impacted. For example transduction relies upon the ability of phages to attach to and infect host cells, however the phenotypic alterations encountered in SCVs may also include modification of phage receptors conferring resistance to phage infection. Transformation and conjugation might be effected similarly if the uptake sites and related factors are impacted. Herein we describe for the first time the potential for SCVs to mediate gene transfer of antibiotic resistance and discuss their role within staphylococcal evolution.

Materials and Methods

Experimental conditions. Unless otherwise stated, all experiments were performed in triplicate (n=3) and results are presented as mean ± standard error (SE). *Staphylococcus aureus* strains NCTC 6571 (Heatley, 1944), NCIMB 9518, RN450 (Novick, 1967), Eagles (high-level mupirocin resistant) and F89 (high-level mupirocin resistant) were maintained on nutrient agar (NA; Oxoid Ltd, UK) from which cultures in nutrient broth (NB; Oxoid) were prepared when required. Triclosan-

induced SCVs were isolated as described previously (Seaman *et al.*, 2007a). Following isolation SCVs were maintained on Mueller-Hinton agar (MHA; Oxoid) supplemented with 1 mg/L triclosan, from which over-night cultures prepared in Mueller-Hinton broth (MHB; Oxoid) with 1 mg/L triclosan were made when required. Triclosan powder (Irgasan DP300) was a gift from Ciba Specialty Chemicals (Grenzach-Wyhlen, Germany) and was dissolved in sterile dimethylsulphoxide (DMSO). To circumvent DMSO related effects solvent concentrations were maintained below 1% in growth media and DMSO controls were run alongside experiments where required.

Species confirmation. All suspected SCVs were confirmed as *S. aureus* by multiplex-PCR as reported previously (Seaman *et al.*, 2007a). This multiplex PCR was also used to confirm the presence of plasmid-encoded mupirocin resistance.

SCV characterization. The haemolytic ability of SCVs was evaluated by streaking on MH agar supplemented with 5% defibrinated sheep blood (Oxoid) and incubation at 37°C for 48 h. The presence of coagulase production was investigated by Staphylase test kit (Oxoid) according to the manufacturer's directions. 4-5 colonies were transferred to a microscope slide followed by the addition of hydrogen peroxide, the presence of bubbling was taken as an indication of catalase production. The manufacturer's directions were adapted to use DNase agar (Oxoid) to estimate the secretion of extracellular nucleases from SCV and wild type *S. aureus*. Briefly, Deoxyribonuclease (DNase) enzymes were detected by spotting of over night cultures on to DNase agar, which contains 2% DNA, supplemented with 1 mg/L triclosan. 10 µl spots were applied in triplicate and allowed to dry before incubation at 37°C for 24-48 hours. Once a thick plaque of growth was achieved for each spot the plates were flooded with 1 M hydrochloric acid (HCl). Following incubation at room temperature for ~3 minutes the spots were examined for surrounding areas of clear agar; HCl hydrolysis of DNA results in the agar turning opaque, the presence of DNase digests the DNA and as a consequence none is available to be hydrolyzed. Hence, well defined clear zones indicated a presence of DNase enzymes whilst no clear zone indicated a lack of DNase. Width of clear zones was measured from the edge of bacterial growth to the boundary between clear and opaque agar. Triplicate spots were compared to ensure reliability and any ambiguous isolates were repeated. Significant difference between the width of clear zones was tested with Student's t-test using Minitab® release 14 software (Minitab Inc.).

Conjugation. Bi-parental matings were performed as described previously (Seaman *et al.*, 2007b) except, due to the low cell density of SCV cultures, when SCVs were used as donor or recipient cells their volume filtered on to the filter was doubled. Additionally, incubation times were increased to 72 h to enable the slower growing SCVs to be visualized effectively.

Transduction. Prior to performing transduction experiments strains were examined for their susceptibility to phage infection. This was performed as described previously (Seaman & Day, 2007) using bacteriophages 80α, K and NCTC 9319 and MHA as the growth medium. Transduction experiments were performed using bacteriophages 80α and K as described previously (Seaman *et al.*, 2007b), except incubation times were increased to allow for the slow growth of SCVs.

Transformation. Transformation experiments were performed as described previously (Seaman *et al.*, 2007b).

Results

SCVs with reduced triclosan susceptibility were observed as tiny, non-pigmented colonies on plates containing triclosan (Figure 7.1). All SCVs were non-haemolytic on sheep blood agar (Figure 7.2) and were not found to produce extracellular coagulase (Figure 7.3). As with previous triclosan-induced SCVs colonies were found to be weakly catalase positive (Seaman *et al.*, 2007a). The level of extracellular DNase secretion had not previously been tested in SCVs. The use of DNase agar revealed SCVs to produce significantly less extracellular DNase than wild-type colonies ($P = <0.001$; Table 7.1). DNase diffusion around SCVs was seen on average only 11.5% of the distance achieved by wild-type cells. Since diffusion through agar is logarithmic, we can estimate that the actual amount of DNase secreted by SCVs is ~1/100 of that produced by wild-type cells.

Prior to conjugation experiments being performed SCVs were checked for the presence of plasmid-encoded mupirocin resistance. This was confirmed in SCVs of both strains known to carry this plasmid, *S. aureus* Eagles and *S. aureus* F89. To test the ability of SCVs to act as recipients during conjugal transfer of antibiotic resistance

Figure 7.1. SCVs with reduced triclosan susceptibility were observed as tiny, non-pigmented colonies on plates containing triclosan. As illustrated with NCIMB 9518, SCV colonies were approximately one tenth the size of wild-type colonies. *S. aureus* cells that had mutated to low-level triclosan resistance by means other than through SCV formation remained as characteristic large, gold/yellow colonies.

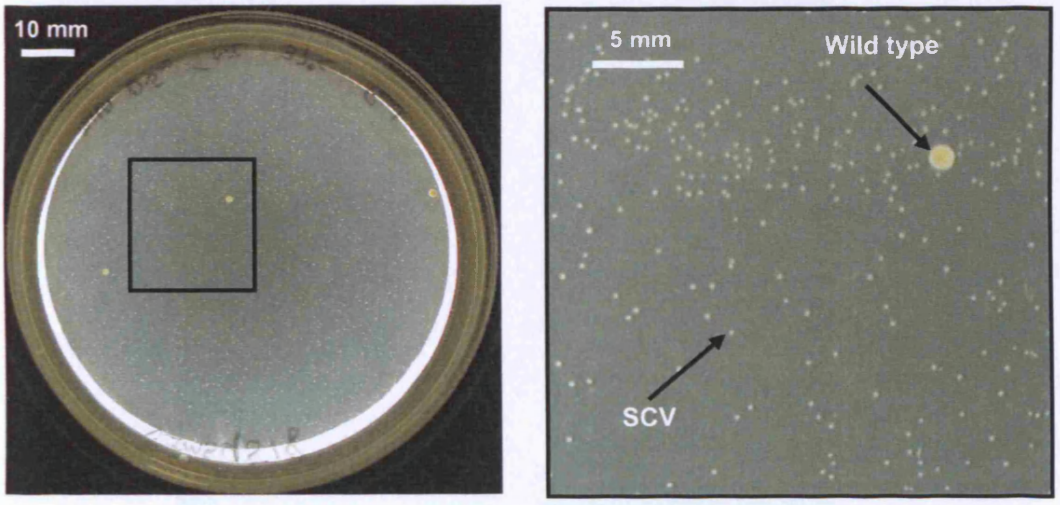


Figure 7.2. All SCVs were non-haemolytic on sheep blood agar. The picture shows 9518SCV and NCIMB 9518 streaked on a blood agar plate. The clear zone around the wild-type cells indicates haemolysis, whilst no haemolysis was shown by the SCV.

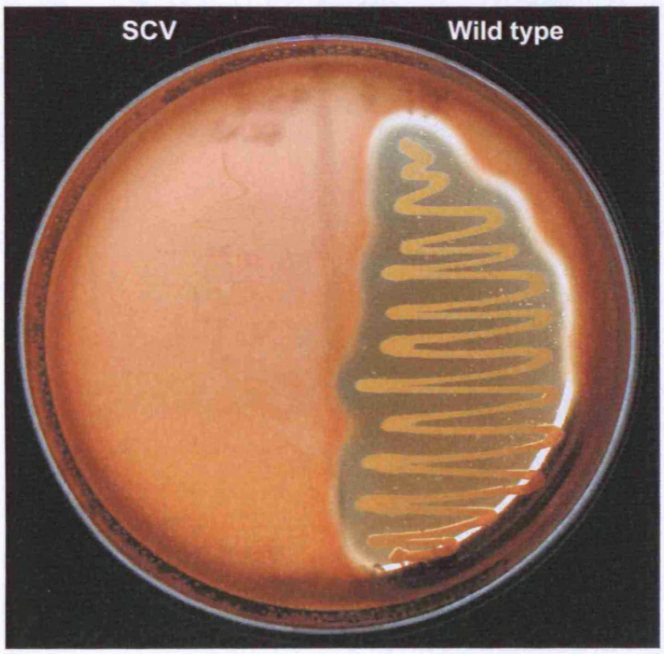


Figure 7.3. latex-agglutination test did not detect the production of extracellular coagulase in SCVs. Clumping indicates a positive reaction, as seen with the three wild type cultures.

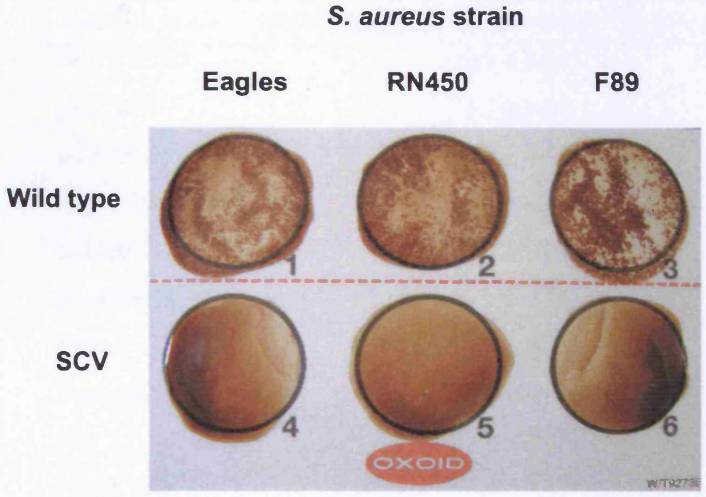


Table 7.1. SCVs secreted significantly less DNase than wild-type *S. aureus* ($P = 0.001$).

S. aureus strain	Mean width of DNA-free zone ± SE ($n = 3$, mm)
NCTC 6571	10.7 ± 0.04
6571SCV	1.1 ± 0.09
NCIMB 9518	9.7 ± 0.03
9518SCV	1.2 ± 0.10
RN450	9.1 ± 0.04
RN450SCV	1.0 ± 0.18
Eagles	5.7 ± 0.15
EaglesSCV	0.9 ± 0.09
F89	12.1 ± 0.07
F89SCV	1.2 ± 0.12

genes bi-parental matings we performed using either wild-type *S. aureus* Eagles and *S. aureus* F89 as donors and RN450SCV as recipient the recipient. Conversely, to examine whether *S. aureus* SCVs can disseminate genes by conjugation, EaglesSCV and F89SCV were used as donors and wild-type *S. aureus* RN450 was used as the recipient. The experiment was also repeated using SCV isolates as both donor and recipient. However, no conjugative transfer either out of or into SCVs was detected (levels of detection were between $<1.21\times10^{-9}$ and $<3.25\times10^{-11}$ transconjugants per recipient).

SCVs were found to have the same phage susceptibility pattern as their parent strain (Figure 7.4 and Table 7.2). Subsequently, the appropriate bacteriophage was used in attempts to transfer rifampicin resistance from *S. aureus* RN450 to 9518SCV, from RN450SCV to *S. aureus* NCIMB 9518 and from RN450SCV to 9518SCV. However, once again no transfer was detected (levels of detection were between $<3.68\times10^{-9}$ and $<4.74\times10^{-11}$ transductants per recipient).

The last method of gene transfer we investigated was the uptake of free DNA or transformation. Free DNA was provided by lysing cells with antibiotic resistance genes (mupirocin and rifampicin) and mixing this with recipient (SCV) cells on a filter paper support. Whilst transformation was not detected in wild-type NCIMB 9518 it was successfully detected in the SCV of this strain (Figure 7.5). Both mupirocin resistance and rifampicin resistance could be transferred by transformation in to the SCV form at frequencies of between 1.48×10^{-6} transformants per recipient and 2.35×10^{-5} transformants per recipient. Control plates indicated that spontaneous mutation of SCVs to rifampicin or mupirocin resistance was below the level of detection (6.48×10^{-8} to 3.75×10^{-9} transformants per recipient).

Figure 7.4. Triclosan-induced SCVs shared the same phage susceptibility profile as their progenitor. Susceptibility to bacteriophages 80α, K and NCTC 9319 was tested. (a) Both F89 and (b) F89SCV were susceptible to all three phages. (c) NCIMB 9518 and (d) 9518SCV were susceptible to 80α and K, but not NCTC 9319. Interestingly, with closer inspection one could see the lawns of (e) F89SCV and (f) 9518SCV also contained revertants. The revertants did not occur in the presence of the phages, indicating their susceptibility.

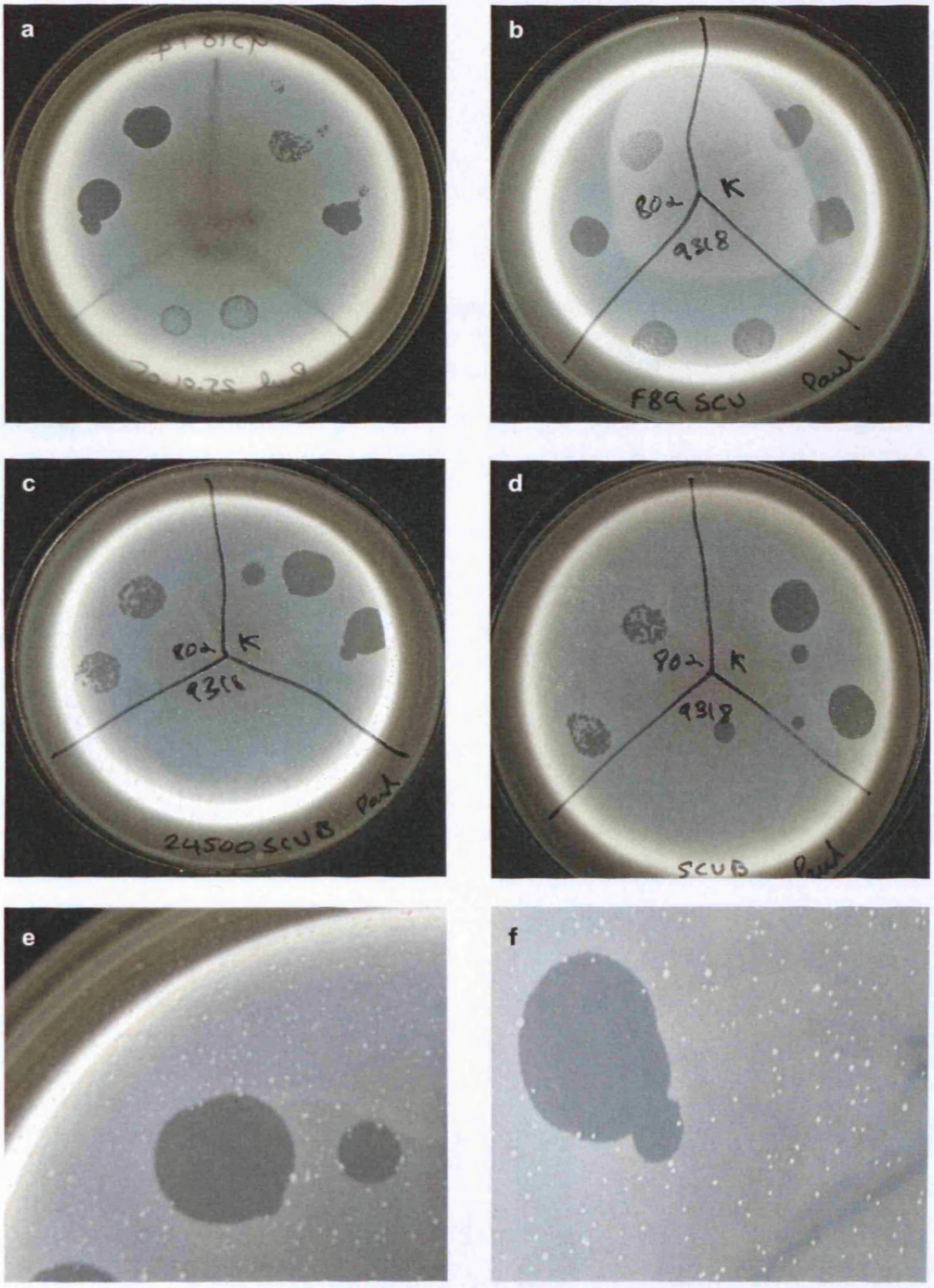
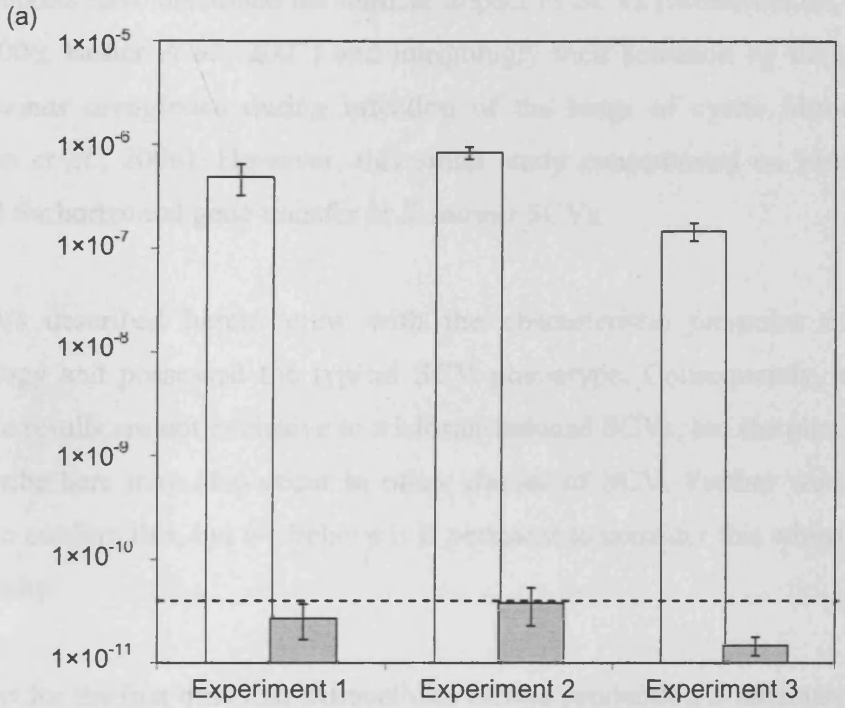
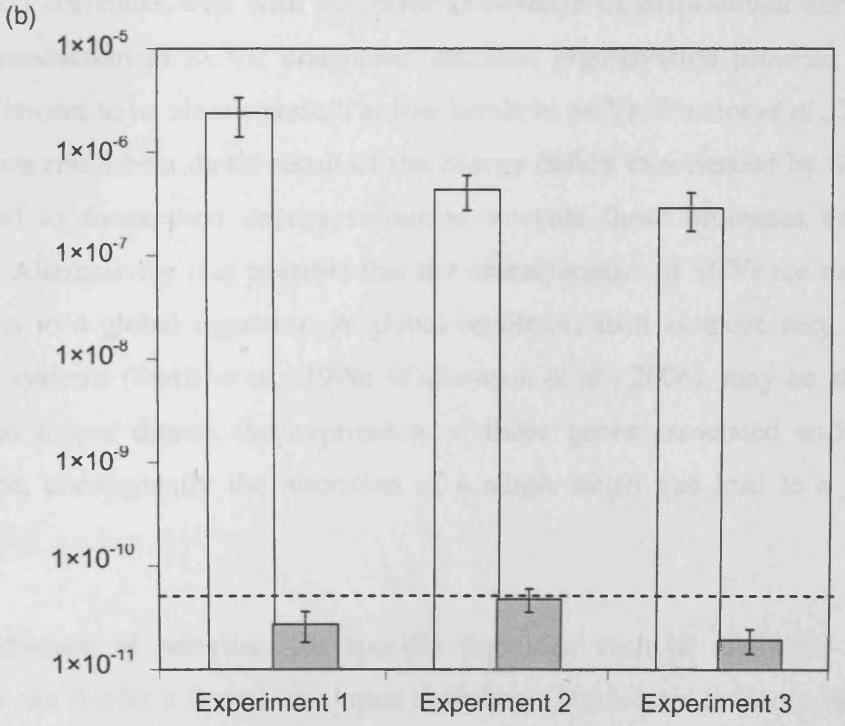


Table 7.2. Phage susceptibility of *S. aureus* strains and their SCV progeny.

S. aureus strain	Bacteriophage		
	80α	K	NCTC 9319
NCTC 6571	+	-	-
6571SCV	+	-	-
NCIMB 9518	+	+	-
9518SCV	+	+	-
RN450	+	+	+
RN450SCV	+	+	+
Eagles	+	+	+
EaglesSCV	+	+	+
F89	+	+	+
F89SCV	+	+	+

Figure 7.5. Horizontal gene transfer of antibiotic resistance by transformation in *S. aureus* SCVs. (a) Transformation of mupirocin resistance in *S. aureus* NCIMB 9518 (■) and its SCV (□) (b) and transformation of rifampicin resistance in the same strains. Dashed horizontal line represents highest level of detection. Each transformation experiment was repeated three times. Values are mean ±SE.





Discussion

S. aureus SCVs have been recognised for several decades (Proctor *et al.*, 2006), however recently increasing attention is being paid to this intriguing phenotype. Recent reports have discussed the clinical impact of SCVs (Moisan *et al.*, 2006; Sifri *et al.*, 2006; Besier *et al.*, 2007) and intriguingly their selection by the presence of *Pseudomonas aeruginosa* during infection of the lungs of cystic fibrosis patients (Hoffman *et al.*, 2006). However, this small study concentrated on identifying the potential for horizontal gene transfer in *S. aureus* SCVs.

All SCVs described herein grew with the characteristic pin-point SCV colony morphology and possessed the typical SCV phenotype. Consequently, we propose that these results are not exclusive to triclosan-induced SCVs, but the phenomena that we describe here may also occur in other classes of SCV. Further work would be needed to confirm this, but we believe it is pertinent to consider this whilst discussing these results.

We report for the first time that extracellular DNase production is attenuated in SCVs. This result correlates well with our prior knowledge of extracellular and accessory protein production in SCVs; coagulase, catalase, pigmentation proteins are among proteins known to be absent/present at low levels in SCVs (Proctor *et al.*, 2006). This attenuation could be a direct result of the energy deficit experienced by SCVs, since they need to focus their energy resources towards those processes essential for survival. Alternatively it is possible that the characteristics of SCVs are as a result of alterations to a global regulator. A global regulator, such as those seen in quorum sensing systems (Swift *et al.*, 1996; Williamson *et al.*, 2006), may be altered such that it no longer directs the expression of those genes associated with the SCV phenotype, consequently the alteration of a single target can lead to a pleiotropic effect.

In the absence of selection for specific functions such as antibiotic resistance, plasmids can confer a fitness cost upon their host (Dykhuizen & Hartl, 1983; Lee & Edlin, 1985; Cooper *et al.*, 1987; Lenski & Bouma, 1987; Lenski, 1988; Lenski, 1997); energy is required to sustain the extra-chromosomal DNA. Plasmids generally

encoded accessory functions that are not essential for survival. Consequently, we hypothesized that when *S. aureus* mutate to the SCV form they would lose their plasmids in an effort to conserve energy. Furthermore, we know that cell division of SCVs is severely impaired (Kahl *et al.*, 2003; Seaman *et al.*, 2007a). Hence, upon cell separation the inheritance of plasmids by daughter cells may be affected. However, we found that SCVs were able to maintain plasmids, namely the *ileS2*-carrying plasmid that confers high-level mupirocin resistance in *S. aureus* Eagles and F89. So *S. aureus* SCVs maintain the potential to disseminate important resistance and virulence traits by conjugation.

When we investigated conjugation in SCVs, using them as both donors and recipients, we were unable to detect any transfer. This was surprising, since we had shown they were able to sustain plasmids, but our results may be a consequence of experimental problems. Due to the nature of SCV cultures it was difficult to attain the desired cell densities during filter matings. Hence, the ratio of donor to recipient cells may not have been at the optimum, a variable known to impact upon conjugation frequencies (al-Masaudi *et al.*, 1991). Likewise, the growth phase of cells strongly influences the frequency of plasmid transfer (al-Masaudi *et al.*, 1991). Again, the abnormal growth dynamics of SCVs in culture, which show extended lag phases, short log phase growth and retarded maximum growth rates (Seaman *et al.*, 2007a) may have been detrimental to conjugation. Alternatively, the physiological state of SCVs may have precluded conjugation. Although plasmid transfer in Gram-positive organisms doesn't require the synthesis of complex conjugation apparatus (sex pili) seen in Gram-negative bacteria, they still need to achieve intimate cell-cell contact. The processes for this have not been fully identified, but a strong level of homology has been found with type IV secretion systems (Grohmann *et al.*, 2003). The synthesis of these elements requires energy, which SCVs have in very short supply. Consequently, SCVs may have been unable to synthesize the proteins necessary for the conjugation apparatus, impacting severely upon their ability to act as either donors or recipients.

It is possible that the plasmids were transferred, but were not expressed in the recipient cells. However, this appears unlikely; SCVs of plasmid-bearing *S. aureus* are able to express plasmid genes (EaglesSCV and F89SCV are high-level mupirocin resistant). Furthermore, whilst a plasmid may impose a fitness cost upon its host, this

is negated when a strong selective pressure for their maintenance is applied, such as antibiotic treatment. This could be checked by avoiding the selection of transconjugants on selective agar, but using genetic analysis, PCR or pulsed field gel electrophoresis (PFGE), to scrutinize recipient cells for the presence of plasmids. Indeed, further work is required to better identify whether SCVs can undergo conjugation. A wider selection of plasmids, donors and recipients should be used in conjunction with a broader set of experimental methods, adapting the method to one that better suits SCVs.

The fact that the SCVs were susceptible to phage infection tells us that they are able to express the cell surface features used by these phages as attachment sites. Furthermore, the formation of visible plaques implies that phage replication occurred; if phage replication did not occur the infection would not pass further than the initial cell infected and the number of cells lysed would not be enough to form visible plaques. Consequently, we know that genes introduced in to SCV cells by phage particles were expressed and so we can identify SCVs as potential donors and recipients for gene exchange by transduction.

We were unable to detect the transfer of resistance traits by transduction with SCVs. This is little harder to explain than why conjugation may have failed. Transduction is a more passive process for the cells, during the early stages at least. It doesn't require synthesis of specialized proteins and we know that those genes pass into cells by phages are expressed. Again the attenuated growth of SCVs may have caused practical problems. Whilst it was possible to adjust the multiplicity of infection by controlling the number of phages added to the transduction mixture it was difficult to achieve the high cell densities required for satisfactory levels of detection. Further work should be targeted at analyzing transduction in SCVs. This should encompass a broader selection of selective markers and will require further optimization for use with SCVs.

Interestingly, transformation was detected in SCVs whilst wild-type strains were unable to take up and express free DNA at frequencies above our level of detection. In fact the transformation frequencies seen with SCVs was high (2.38×10^{-5} to 1.48×10^{-6} transformants per recipient) in comparison to other gene transfer events in *S. aureus*

(Seaman *et al.* 2007b). Although transformation does occur in *S. aureus* it is a generally rare event; transformation requires high calcium concentrations (0.1 M) and the natural competence system of *S. aureus* is induced only in very early exponential growth (Novick, 1989). Either chromosomal or plasmid DNA can be transformed into *S. aureus*, however the production of nucleases by *S. aureus* further complicates the situation since these digest DNA that would otherwise be available for transformation (Lacey, 1984). Hence it is pertinent that, as discussed above, SCVs produce significantly less DNase (over 100-fold) than wild type *S. aureus*. Although the method employed herein involved several wash steps designed to minimize the effects of DNA digestion by nucleases, wild-type cells would have continued to produce these enzymes, degrading the free DNA. As a consequence more intact DNA would have been available to SCVs than wild-type. This may help explain why SCVs are capable of higher rates of transfer by transformation than wild-type *S. aureus*.

These transformation results indicate that recA was present in SCV cells. Thus the failure of conjugation and transduction to yield recombinants was not due to the absence or shortage of this enzyme.

The role of SCVs in gene transfer is yet to be fully gauged, however it appears they are able to partake in transformation and the potential for conjugation and transduction exists. The role of SCVs as mediators of gene transfer is of importance due to their unique lifestyle; their persistence during infections, even during antimicrobial chemotherapy means that they are in turn a persistent genetic resource and are available to accept and donate DNA under strong selective pressure. Hence, SCVs could act as a 'DNA dealers', trading DNA with other microorganisms that they encounter during the course of long-term infection. For this reason more experimentation should be performed to understand their role in the evolution of *S. aureus* with an ultimate aim to model this *in vivo*.

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Chapter 8

General Discussion and Perspectives

8.0 General discussion and perspectives

Since each practical-based chapter (2-7) contains a discussion pertinent to the findings of that investigation this chapter will mainly relate information across the thesis in a more general manner and will discuss future perspectives and further research.

S. aureus has a long history of causing disease and advances in medicine have created further opportunities for infections. Surgery and the wide spread use of intravenous cannulae and catheters facilitate entry in to the body of potential hosts. A huge increase in the use of in-dwelling devices, such as prosthetic joints and heart valves, has also provided new niches for *S. aureus* to exploit (Dickinson & Bisno, 1989). Additionally, patients are living longer and surviving trauma or diseases that would previously have been fatal. This is resulting in an increased population of immunocompromised individuals, often with the other risk factors for infection mentioned above. So whilst these advances caused an increase in the number of hosts for *S. aureus* infections modern medicine also promised the answer: antibiotics.

However, evolution has facilitated the development of antibiotic resistance. Consequently, we are now faced with the enormous problem of multiply-resistant bacteria, the most significant of which is MRSA. Our hospitals, and to some extent homes, represent an environment populated by a high density of suitable hosts with a strong selective pressure for the growth of antimicrobial resistant organisms. Consequently, large numbers of patients are contracting MRSA, which is causing substantial morbidity and mortality. Moreover, MRSA infections have been estimated to be costing the UK National Health Service (NHS) £1 billion per year (Bourn *et al.*, 2004). Indeed MRSA is endemic in many UK hospitals (Coia *et al.*, 2006). There has also been a substantial increase in the incidence of community-associated infections (Ribeiro *et al.*, 2005; Huang *et al.*, 2006; Johnson *et al.*, 2006).

Consequently, an extensive list of procedures to reduce the incidence of MRSA infections has been introduced (Coia *et al.*, 2006; Pratt *et al.*, 2007). These include systematic surveillance of MRSA, avoiding the inappropriate or unnecessary use of antibiotics, which should also reduce the likelihood of the emergence and spread of strains with reduced susceptibility to glycopeptides. Patients and clinical areas are

screened for MRSA carriage, patient isolation is often employed and strict handwashing regimes have been put in place. Nasal and skin decolonization are also an important part of infection control procedures. Consequently, triclosan, as one of the antimicrobials used during skin decolonization, has a major in reducing the transmission and initiation of MRSA infections.

Hence, when Cookson *et al.*, (1991) described the appearance of triclosan resistance in *S. aureus* the potential for decolonization failure and increased infection rates became apparent. This was made worse by the report also stating that triclosan resistance was transferable - increasing the likelihood and speed of dissemination. Moreover, triclosan is used widely in other applications, particularly in consumer healthcare products and domestic goods. Therefore, there several authors have expressed concern that exposure of *S. aureus* to triclosan in domestic and industrial settings may provide additional selective pressure for resistance (Fraise, 2002; Beumer *et al.*, 2003; Cole *et al.*, 2003; Aiello *et al.*, 2005; Weber & Rutala, 2006). Indeed, triclosan has been at the centre of controversy for some time. Of particular interest has been the potential for triclosan to select for resistance to other antimicrobials (Russell, 1999; Russell *et al.*, 1999; Russell, 2000; Levy, 2001; Russell, 2002; Aiello & Larson, 2003; Aiello *et al.*, 2005).

Whilst one mechanism of 'resistance' to triclosan in *S. aureus* has been elucidated and studies have investigated the effect of triclosan exposure upon antimicrobial resistance, there is a paucity of data regarding the evolutionary effects and consequences of reduced susceptibility to triclosan. There have also been few data on the gene transfer of reduced triclosan susceptibility. Consequently, we have investigated several factors surrounding the development and gene transfer of reduced susceptibility to triclosan in *S. aureus*.

8.1 Mechanisms of triclosan resistance in *S. aureus*

8.1.1 A role for capsule polysaccharide in antimicrobial resistance?

There are 11 known serotypes of *S. aureus* capsular polysaccharides. Serotypes CP5 and CP8 are by far the most common amongst isolates recovered from humans

(O'Riordan & Lee, 2004). These serotypes are considered as microcapsules because they are much thinner than the mucoid serotypes 1 and 2. Capsule polysaccharides, and particularly CP5 and CP8, are involved in impeding phagocytosis (O'Riordan & Lee, 2004). Moreover, they may also present a permeability barrier to antimicrobial agents, reducing the susceptibility of certain strains (Kolawole, 1984). However, we found that strains that expressed either CP5 or CP8 were equally as susceptible to a range of antimicrobials as an isogenic strain that was engineered to be deficient in capsule formation. This indicates that whereas *S. aureus* capsule polysaccharides are implicated in virulence, they are not involved in conferring reduced antibiotic or biocide susceptibility.

Subsequent work has found that the vancomycin-intermediate MRSA strain Mu50 was as susceptible to triclosan, CHX and CPC as vancomycin susceptible MRSA strains. This is interesting, since the reduced susceptibility to vancomycin in Mu50 and other clinical VISA and heteroVISA strains has been attributed to thickened cell walls (which can reduce permeability) and reduced cross linking of glycan chains (Hiramatsu *et al.*, 1999; Cui *et al.*, 2000; Cui *et al.*, 2006). Hence, there appears to be no co-resistance between triclosan and vancomycin, and thickening of *S. aureus* cell walls does not confer reduced susceptibility to common biocides; triclosan and CHX should remain efficacious against these infections.

After elucidating the role of the major capsule serotypes upon triclosan susceptibility we aimed expound other methods of reduced susceptibility to triclosan in *S. aureus*. These centred around chromosomal mutations affecting fatty acid biosynthesis.

8.1.2 FabI and triclosan susceptibility

We found that we could isolate *S. aureus* mutants with reduced susceptibility to triclosan at rates of between 3.5×10^{-9} and $\sim1.0\times10^{-11}$ per CFU (Chapter 4, Figure 4.1). These mutants had triclosan MICs that were generally 100-200 times higher than their parent. Following previous reports that mutations affecting the *fabI* gene may lead to reduced triclosan susceptibility (Heath & Rock, 2000; Fan *et al.*, 2002), we sequenced the entire *fabI* gene and preceding promoter region. Comparison of parent and mutant sequences revealed a novel mutation, C284T. This mutation confers an alanine to

valine substitution at the 95th residue of the FabI protein. Due to the position of this substitution, we hypothesize that the location of the A95V affects the formation of the inhibitory triclosan-NAD^+-FabI complex. Binding and inhibition studies would be needed to test this hypothesis; analyzing the ability of wild-type and mutant FabI to form the complex with triclosan and NAD^+ and calculating the inhibitory effects of this upon the enzyme (such as I_{50}; concentration of triclosan than produces 50% inhibition). Similar studies have been performed previously with other *fabI* mutants and protein targets (Stewart *et al.*, 1999; Thomas *et al.*, 1999; Heath & Rock, 2000; Fan *et al.*, 2002). This could be aided by X-ray crystallographic analysis of the complex, which would provide data on the structure of the protein, its binding sites and structural interactions within triclosan and NAD^+ (Qiu *et al.*, 1999).

Possession of C284T conferred an increase in triclosan MIC to ~1 mg/L, however we found that when this occurred in combination with a further mutation, T-108G, within the putative *fabI* promoter region, the MIC was further increased (~2 mg/L). Following from prior evidence that increased expression of *fabI* can reduce triclosan susceptibility (Fan *et al.*, 2002) we hypothesize that this mutation is affecting the expression of *fabI* and increasing the bioavailability of enoyl-ACP reductase. Confirmation of increased FabI levels in T-108G cells would be needed to support this hypothesis. This could be performed by examination of FabI abundance in T-108G and wild-type *S. aureus* strains by Western immunoblotting as described by Fan *et al.*, (2002). Alternatively, reverse transcription, real-time PCR could be used to calculate the abundance of *fabI* mRNA, and subsequently the level of expression of *fabI*.

Further work should also be aimed at confirming the role of the identified mutation(s) in triclosan resistance by transfer to a triclosan-sensitive host via transduction, cloning or allelic replacement. This would identify whether these known *fabI* mutations are the sole explanation for the reduced susceptibility of mutants, or whether other, yet unidentified, alterations are also involved. One possible method for the construction of isogenic strains to evaluate effects of FabI over expression and C284T on triclosan susceptibility would involve the cloning of the wild-type fabI gene from *S. aureus* into an *E. coli-S. aureus* shuttle vector, that allows expression of *S. aureus* genes in *E. coli* (such as pYH4; Huang *et al.*, 2004). Site-directed mutagenesis could then be used

to change the *fabI* contained within this plasmid to contain the mutations described by us in chapter 4. Following the introduction of this mutant-*fabI* bearing plasmid into a triclosan susceptible *S. aureus* the effects of C284T and T-108G could be elucidated.

Indeed, we also isolated a reduced susceptibility strain with an unaltered *fabI* gene, indicating that there are mutations other than within *fabI* that can confer reduced triclosan susceptibility. So it remains a possibility that the reduced-susceptibility strains identified as C284T and T-108G mutants may also carry other mutations. However, it could be argued that since the identified and unidentified mutations appear to involve separate mechanisms their effects would be cumulative – it could be expected that whereas possession of C284T and T-108G or unidentified mutations confer triclosan MICs of ~2 mg/L, possession of both would result in yet higher MICs.

Furthermore, the frequency of mutation to triclosan resistance was close to that expected from spontaneous mutation (~1.0×10^{-8}; see section 1.2.3). If multiple sites were subjected to spontaneous mutation, then the evolution of mutants resistant to triclosan would be expected to occur at much lower rates (Battista & Earl, 2004). This was not found, providing further support for the hypothesis that it was solely the C284T and T-108G mutations conferring resistance. However, it is possible that if a small increase in resistance occurs in one bacterium it will have an increased growth rate/survival rate and thus be a larger proportion of the population. Thus further mutations occurring in this ever increasing population will occur. Ultimately, the 'hitchhiking' of one mutation on another could cause multiple mutations to appear at an increased rate; the chance of a double mutation is not simply the sum of the the probabilities.

8.1.3 Alternative mechanisms of resistance

We isolated reduced susceptibility mutants with no obvious FabI alteration, hence an alternative method of resistance must have evolved. Similar phenomena have been reported previously, but have failed to offer an explanation for the reduced susceptibility (Brenwald & Fraise, 2003). So what are the possible candidates? Firstly, we must look at the known sites that triclosan targets in *S. aureus*; FabI and the

cytoplasmic membrane. Although no mutation was found within *fabI*, the abundance of FabI protein could have been altered by gene duplication or changes to a global expression control system. An increase in *fabI* copy-number could bring about an increased abundance of FabI, reducing the susceptibility of the strain. This would not have been detected by the sequence analysis, but could be identified by quantitative PCR and enumeration of FabI.

S. aureus cells utilise a global regulatory network, constituents of which include the loci *agr*, *sar*, *sigB*, *rot*, *arlR/S*, *svrA*, and *saeR/S* (Arvidson & Tegmark, 2001; Beenken et al., 2004; Benton et al., 2004; Cheung et al., 2004; Goerke et al., 2005). Consequently, an up-regulation of the expression of fabI could have mediated by a global regulator, circumventing the involvement of fabI or its promoter. This would possibly have led to other physiological changes, but these would have gone undetected; we were only looking for a narrow range of phenotypic changes. If exposure to triclosan does affect the global control systems, then this could have many interesting effects. Virulence and/or resistance could be up- or down-regulated and many other metabolic processes could be affected, such as induction of stress response.

An interesting experiment would be to compare the expression of *S. aureus* genes with and without triclosan exposure. This could be facilitated by using a *S. aureus* microarray, such as the one described previously by Witney *et al.*, (2005). This microarray contains 3,623 PCR products representing every predicted open reading frame in seven genome sequencing projects. The microarray can be used to scrutinise gene expression and hence those genes affected by triclosan exposure and could be identified. Subsequent bioinformatic analysis would give clues as to what processes and functions are affected by triclosan, perhaps providing evidence of previously unknown resistance mechanisms and associated effects. However, microarrays are limited in providing quantitative data; they can discriminate between on and off, but resolution between up-regulated and down-regulated is poor. Hence, to quantify the expression of genes of interest quantitative PCR could be performed on individual genes (which are compared to controls with constant expression rates). This will provide a greater level of discrimination, identifying discrete changes in gene expression, such as the possible up-regulation of *fabI*.

A review by Russell suggested that the bacterial cytoplasmic membrane was the major target for triclosan action (Russell, 2003b). Indeed, we have shown that triclosan-induced potassium ion leakage, indicative of membrane damage, occurs at bactericidal levels. Additionally, membrane-destabilizing effects have been demonstrated by Guillen *et al.*, (2004) and Villalain *et al.*, (2001). Triclosan has also been reported to demonstrate a Z-pattern type of adsorption, which is suggested to be due to the breakdown of the membrane (Denyer & Maillard, 2002). Recent studies have demonstrated that triclosan arrests the growth of the parasites *Trypanosoma brucei*, *Plasmodium falciparum* and *Toxoplasma gondii* by inhibition of their enoyl-acyl carrier protein reductase (Paul *et al.*, 2004; Muench *et al.*, 2007). However triclosan has also been found that to act on these organisms by a non-specific perturbation of the membrane structure, which leads to disruption of sensitive membrane-resident biochemical pathways (Paul *et al.*, 2004). In Gram-negative bacteria these effects can be reduced by the reduced permeability of their cell wall and removal of the antimicrobial by efflux.

Gram-negative bacteria have demonstrated an ability to decrease their susceptibility to antimicrobials by enhancing the impermeability barrier function of their outer membrane. Lipopolysaccharides (LPS) of Gram-negative bacteria are responsible for part, if not most, of the cell impermeability characteristics. Hence, any changes in the expression or structure of LPS are likely to have an effect on permeability (Russell & Furr, 1986). Altered LPS composition in *Proteus* spp., *P. aeruginosa* and *Burkholderia cepacia*, has been linked to resistance to cationic biocides (Tattawasart *et al.*, 1999; Denyer & Maillard, 2002; Maillard, 2002). Additionally, loss of porin proteins can be important in the permeability of the outer membrane of Gram-negative bacteria, particularly to hydrophilic molecules. Alteration of porin size or loss of porin proteins implicated in the uptake of antimicrobial agents has been shown to result in an increase in bacterial resistance (Achouak *et al.*, 2001). Furthermore, Gram-negative bacteria have developed other mechanisms to reduce the intra-cellular concentration of antimicrobials, such as xenobiotic degradation processes, particularly in the periplasm (Beveridge, 1995; Heinzel, 1998).

The intrinsic resistance of *P. aeruginosa* to triclosan has been shown to be a combination of outer membrane permeability and an active efflux system (Levy, 2002; Poole, 2002; Chuanchuen et al., 2003; Champlin et al., 2005). Efflux removes intracellular triclosan, lowering its concentration and causing the organism to become less susceptible. Efflux pumps may be specific for one substrate or may transport a range of structurally disparate compounds. Pumps that are associated with multiple drug resistance are termed MDR efflux pumps. Whilst these have been most extensively studied in Gram-negative bacteria, efflux pumps have been described in *S. aureus* (Huang *et al.*, 2004; Kaatz *et al.*, 2005; McAleese *et al.*, 2005; Piddock, 2006). No efflux system for the removal of triclosan has been described in *S. aureus*. However, some authors have speculated that triclosan could select for the up-regulation or mutation of *S. aureus* MDR efflux pumps, resulting in augmented resistance to a variety of antimicrobials (Levy, 2001; Fraise, 2002; McBain *et al.*, 2002). There is currently no evidence to support these perspectives, but it remains that this is a potential mechanism for triclosan resistance in *S. aureus*. As research into staphylococcal efflux systems progresses we may learn the full implications of this phenomenon, this would certainly be of interest due to the potential for co-resistance.

Triclosan was found to directly inhibit glycolysis in *Streptococcus mutans* by irreversibly inhibiting the glycolytic enzymes pyruvate kinase, lactic dehydrogenase, aldolase and the phosphoenolpyruvate:sugar phosphotransferase system (Phan & Marquis, 2006). The authors concluded that triclosan is a multi-target inhibitor for mutans streptococci, which lack a triclosan-sensitive FabI enoyl-ACP reductase. This raises the possibility that glycolysis is also s target in *S. aureus*, providing further mutated loci that may arise when grown under selective pressure for resistance to triclosan.

8.1.4 Triclosan resistant SCVs

A major finding during the course of this research was the identification that triclosan can select for small colony variants (SCVs) of *S. aureus*. We have found that triclosan can select for *S. aureus* SCVs with low-level triclosan resistance and which coincidently had reduced susceptibility to penicillin and gentamicin. Additionally, triclosan isolated SCVs were shown to have an increased tolerance to the lethal effects

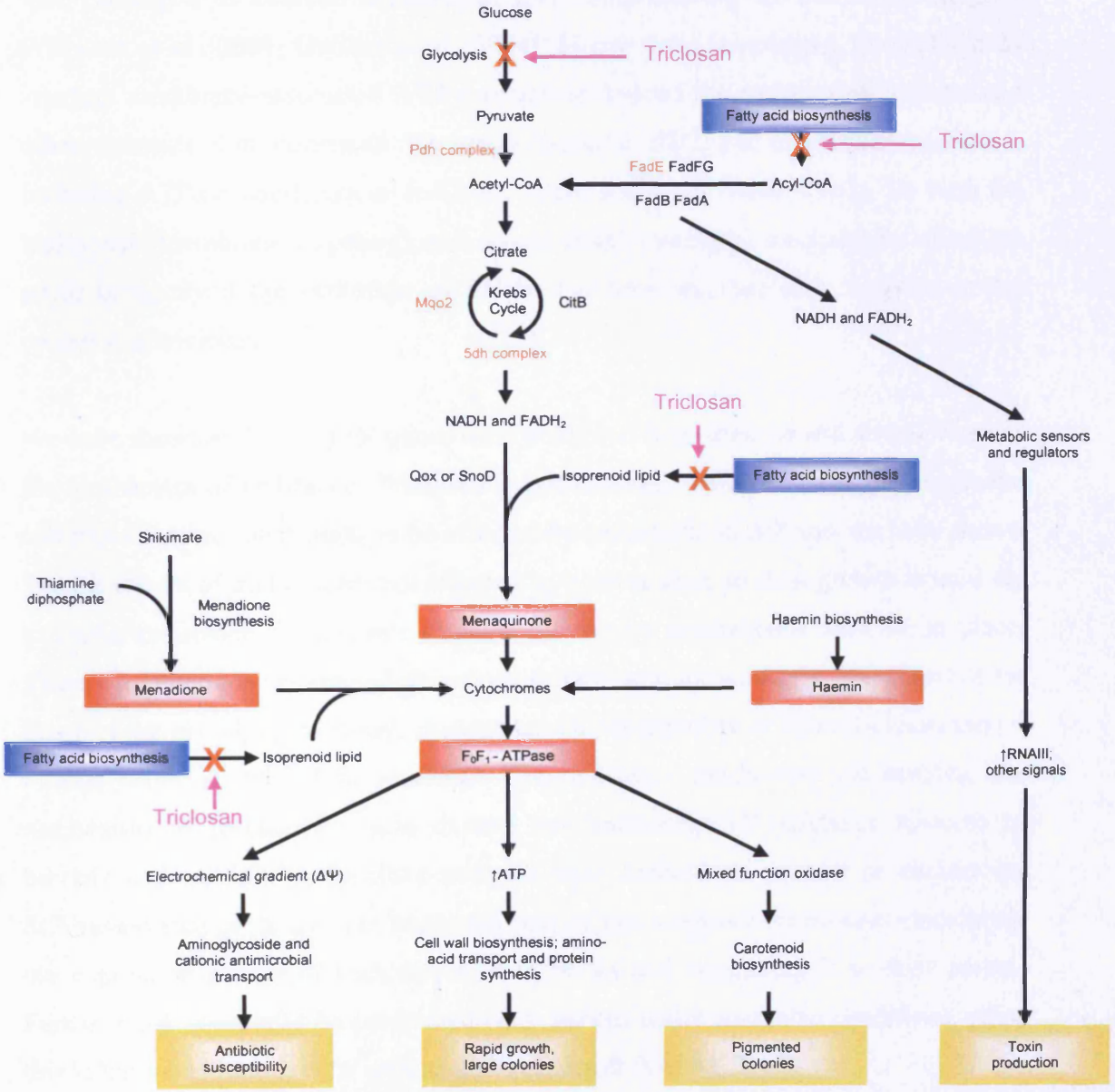
of triclosan. SCV status has previously been associated with a reduction in bacterial energy generation and/or transport, accounting for the down-regulation of such functions as synthesis of cell walls and pigments and toxin production (Proctor, 1998; McNamara & Proctor, 2000; Proctor *et al.*, 2006). A number of loci have been identified as causing SCV growth, namely those involved in electron transport (menaquinone, haemin and haem A), thymidine biosynthesis and processes of the Krebs cycle (TCA cycle). Whereas the source of resistance to aminoglycosides and cationic compounds in SCVs has been elucidated, the mechanism of resistance to triclosan is unknown.

Our current knowledge of SCVs provides several candidates for how explaining how triclosan selects for SCVs and how their reduced triclosan susceptibility is facilitated. Firstly, we will consider the ATP producing process of electron transport. As briefly discussed above several constituents of the staphylococcal ETC can mutate, blocking ATP production by this process (a more comprehensive analysis is provided in section 1.6). These electron carriers are not known targets for triclosan, however biosynthesis of *S. aureus* cytochromes requires isoprenoid – a lipid based organic molecule (Collins, 1981; Collins & Jones, 1981; Bentley & Meganathan, 1982; Lange *et al.*, 2000; Voynova *et al.*, 2004). Since isoprenoid contains lipid it is reliant upon bacterial fatty acid synthesis, a target for triclosan. It is possible that the presence of triclosan perturbs the synthesis of isoprenoid and prevents the production of functional cytochromes, resulting in the isolation of SCVs, Figure 8.1.

As discussed above glycolysis may also be a target for triclosan. This provides another putative mechanism for the selection of SCV growth by triclosan. Glycolysis is a key ATP and NADH producing process in *S. aureus* and is also involved in the provision of substrate (pyruvate) for ATP production by the Krebs cycle. Hence, perturbation of glycolysis could also have severe ATP limiting effects upon the cell, resulting in SCVs, Figure 8.1.

It is intriguing that all the antimicrobial compounds described as selecting for SCVs are bacteriostatic (vancomycin, cycloserine, bacitracin, methicillin, gentamicin, kanamycin, chloramphenicol, sulfamethoxazole-trimethoprim) and it is only at bacteriostatic concentrations that triclosan selected for SCVs. We hypothesise that the

Figure 8.1. Potential mechanisms for selection of SCV growth by triclosan. Triclosan is known to target fatty acid biosynthesis, a process that is key to several constituents of cellular ATP production. Isoprenoid biosynthesis will be perturbed, as will synthesis of Acyl-CoA and the conversion of menadione. Consequently, the cell will be unable to produce functional cytochromes, electron carriers that are key to the staphylococcal ETC. Several other enzymes involved in conversion of pyruvate and fatty acids to provide Acetyl-CoA to the Krebs cycle may also be affected by FabI inhibition by triclosan (red text). The combination of these effects would cause an ATP deficiency and bring about the characteristic SCV phenotype. Additionally, glycolysis is a potential target for triclosan that would have a clear ATP and NADH limiting outcome.



difference in action between bacteriostatic and bactericidal compounds allows for the selection of SCVs. This should be investigated further by directly comparing the propensity for SCV formation when exposing *S. aureus* so a selection of bacteriostatic and bactericidal compounds.

Finally, we hypothesize that those parts of ATP production that are membrane-associated may be affected by triclosan. Triclosan is known to intercalate into the cell wall causing it to become destabilised and compromising its functional integrity (Villalain *et al.*, 2001; Guillen *et al.*, 2004). Hence there is potential for triclosan to interrupt membrane-associated ATP production. Indeed the majority of proteins and other elements that constitute the staphylococcal ETC are membrane-associated, including ATPase itself (Gross & Coles, 1968; Kubak & Yotis, 1981). So both the traditional (membrane targeting) and recent (FabI targeting) mechanisms of action could bring about the evolution of SCVs, but how are they able to grow in the presence of triclosan?

We have shown that SCV *fabI* genes were unaltered from their parent, so this was not the mechanism of resistance. Triclosan is not cationic, therefore transport across the cell membrane is not thought to be affected by reductions in $\Delta\Psi$ and we have shown that the effects of triclosan are not affected by growth state, so slow growth is unlikely to confer resistance. Hence, one or more alternative mechanisms must be in place. These could include undetected alterations to fatty acid biosynthesis, alterations in the genes of the glycolytic pathway, changes to cell permeability or other triclosan target. Further work is needed to elucidate this resistance mechanism. Identifying the mechanism of resistance would expand our knowledge of resistance systems in bacteria and would also facilitate research in to methods to prevent or circumvent SCV-associated problems. An important part of this work would involve elucidating the expression profile of triclosan-induced SCVs and comparing it to their parent. Further work could also be performed on *S. aureus* under anaerobic conditions, since this is known to induce SCV growth (McNamara & Proctor, 2000).

8.2 Triclosan resistance in the environment

There have been a number of conflicting reports on the incidence of reduced triclosan susceptibility outside of the laboratory. Cookson *et al.* described the isolation of triclosan resistant staphylococci in 1991 and Bamber & Neal, (1999) reported that 7.5% of *S. aureus* clinical isolates they investigated had triclosan MICs of at least 1 mg/L. Fan *et al.* (2002) identified *fabI* mutations in clinical isolates with reduced susceptibility to triclosan and (Brenwald & Fraise, (2003) reported that 2% of clinical *S. aureus* show reduced susceptibility to triclosan. Conversely, Al-Doori *et al.*, (2003) maintain that there has not been a significant increase in reduced triclosan susceptibility amongst nosocomial MRSA in Scotland. A study by (Ledder *et al.*, 2006) failed to indicate any explicit link between triclosan exposure and resistance in *ex-situ* bacterial isolates. Triclosan resistance was not found in sink drain microcosms or domiciliary environments repeatedly exposed to triclosan (Cole *et al.*, 2003; McBain *et al.*, 2003a; McBain *et al.*, 2003b; Aiello *et al.*, 2004; McBain *et al.*, 2004). Lear *et al.*, (2002) found that residual biocide concentrations in a triclosan manufacturing site did not promote the emergence of bacterial tolerance for them. Suller & Russell, (2000) found a low incidence of triclosan resistance amongst antibiotic resistant *S. aureus* isolates. Finally, the results presented in chapter 3 indicate that the frequency of resistance to triclosan is very low amongst MRSA and MSSA isolates from South Wales hospitals.

The disparity between these findings may reflect differences in sampling and testing techniques. Various methods were used to assess triclosan susceptibility and a selection of sampling methods were employed, some possibly more effective than others. Alternatively, the results may be accurate, but reflect the differences between the individual test locations; some hospitals may rely more heavily upon CHX or other antimicrobials as part of their infection control policy, rather than triclosan. Most of the investigations included a large selection of *S. aureus* isolates, so bias towards clones with reduced susceptibility to triclosan appears unlikely – although this would be an interesting route of investigation.

In an attempt to investigate the carriage of triclosan resistant staphylococci in a specific community setting we analyzed samples recovered from 93 undergraduate

students attending a practical teaching class at Cardiff University. We found no evidence of reduced susceptibility to triclosan amongst the recovered *S. aureus* isolates, indicating that prevalence of triclosan resistance is low in this population. This and previous reports of low prevalence of triclosan resistance in the community are encouraging. They fail to support the hypothesis that triclosan exposure in the home can lead to the development of antimicrobial resistance as mooted by several authors (Russell, 1999; Russell, 2000; Levy, 2001; McBain & Gilbert, 2001; White & McDermott, 2001; McBain *et al.*, 2002; Poole, 2002; Tan *et al.*, 2002; Russell, 2003a; White *et al.*, 2005; Sattar, 2006). Carriage of *S. aureus* was found to be inline with previous studies (Abudu *et al.*, 2001; Sá-Leão *et al.*, 2001; Maudsley *et al.*, 2004). CoNS carriage was more prevalent than *S. aureus* and these organisms were also significantly less susceptible to triclosan, showing a median triclosan MIC of 4 mg/L (133-fold higher than the median MIC for *S. aureus*). However, CoNS MICs were still far below the typical in-use concentrations of triclosan (0.1 to 2%).

8.3 Significance of low-level triclosan resistance

Analysis of staphylococcal triclosan susceptibility by MIC has identified that some strains of *S. aureus* and most CoNS can demonstrate low-level resistance to this antimicrobial. There has been some speculation on the clinical significance of this resistance, but the data available currently appear ambiguous (Cookson *et al.*, 1991; Suller & Russell, 2000; Brenwald & Fraise, 2003; Russell, 2004; Seaman *et al.*, 2007).

There are several factors that lead to this ambiguity: MIC may not be appropriate for assessment of triclosan susceptibility; triclosan formulations differ; there are differences between triclosan uses; physiological state of the bacteria may vary; there is a paucity of data on the clinical efficacy of triclosan against low-level resistant organisms.

8.3.1 Biocide MIC: an indication of efficacy?

Assessing an antibiotic's MIC for a particular organism is a useful method for examining susceptibility. It is used widely by clinical microbiologists to aid their

formulation of treatment plans for patients and to monitor resistance levels within the environment. It is also used widely in the laboratory to assess susceptibility for research purposes. Whereas MIC is appropriate for the assessment of susceptibility to antibiotics, this may not be the case for biocides, where MIC is does not always correlate with bacterial killing (Russell, 2004). A lack of correlation between MICs and lethal effects has been demonstrated previously (Cookson *et al.*, 1991; Suller & Russell, 2000; Russell, 2004; Gomez Escalada *et al.*, 2005) and by the data presented in chapter 3. This has been hypothesised to be a consequence of the mechanisms of action of triclosan (Suller & Russell, 2000); generally, antibiotics have single target sites and consequently increased MICs and reduced bactericidal effectiveness are linked. In contrast, biocides have multiple targets and increased MICs often do not correlate with decreased bactericidal activities (McDonnell & Pretzer, 1998). It has been proposed that additional triclosan-induced cellular changes are required to produce a bactericidal effect whether the strain is resistant or sensitive to triclosan, as judged purely by the MICs (Russell *et al.*, 1999). Consequently, whilst clinical strains may be identified as triclosan-resistant by laboratory MIC, they may be killed *in-situ* just as effectively as triclosan-susceptible strains. More research is required to develop better methods for assessing bacterial susceptibility to biocides.

8.3.2 Formulation effects of triclosan products

In many instances triclosan is incorporated into products that have other ingredients that are bactericidal or bring about cellular changes. Such ingredients include detergents, surfactants, chelators or alcohols, the inclusion of which depends on the product application. For example, the commercial triclosan preparation, Irgacide LP10, as used in this study, contains sodium lauryl sulphate, a surfactant with not inconsiderable antimicrobial activity (Jenkins *et al.*, 1991; Babich & Babich, 1997). It is reasonable to assume that these ingredients will increase the antimicrobial activity of the product. They may also affect the development of resistance by placing additional stress on the bacteria and providing yet further targets for activity.

Additionally, these ingredients may affect the efficacy of the product by more indirect methods. For example surfactants aid the physical removal of dirt from soiled surfaces and the inclusion of moisturisers increase the frequency of use of handwash products.

As mentioned above, the inclusion of additional ingredients in a triclosan product depends on the intended function. Triclosan has a broad spectrum of applications, from bar soap to toothpastes and from preoperative washes to chopping boards, therefore each formulation must be adapted to its particular function. This may cause the efficacy to vary significantly (Russell & McDonnell, 2000; Suller & Russell, 2000). The nature of the application will also have an effect, for example bathing of hospital patients in 2% triclosan provides a highly controlled exposure to a high concentration of triclosan in combination with other ingredients, whereas inclusion of triclosan in polymers, such as chopping boards, will confer chronic, low-level exposure to triclosan. Clearly these will provide different opportunities for the development of resistance. So studies into the efficacy of triclosan should also consider both the direct and indirect effects of additives included in triclosan preparations and also the intended use of the formulation.

8.3.3 Slime problems: the effects of biofilm upon susceptibility

The vast majority of investigations that have linked triclosan exposure to resistance development have been conducted in planktonic pure culture (McBain *et al.*, 2003a), however natural communities of bacteria are often polymicrobial and biofilm-based. Biofilms can confer antimicrobial resistance, mainly by physically blocking the compound from coming into contact with cells; antimicrobial diffusion through biofilms can be limited so that only those cells close to the surface are exposed to lethal concentrations of antimicrobial (Stewart & Costerton, 2001; McBain *et al.*, 2003a). Other hypotheses for the mechanism of resistance in biofilms include the theory that some of the community differentiate into a protected phenotypic state and that in zones of nutrient depletion or waste accumulation antimicrobial action may be antagonised (Stewart & Costerton, 2001).

So the mechanisms of resistance in natural biofilms are different to those studied in the majority of laboratory experiments. However, some studies have looked at the effects of triclosan upon biofilms. Interestingly, these have failed to show the development of resistance in biofilms such as those found in the domestic drain and in dental plaque communities. Indeed, a study by Jones *et al.*, (2006) found that loading

the retention balloons of urinary catheters with a triclosan solution (10 g/L) provided sufficient antibacterial activity to prevent catheter encrustation by *Proteus mirabilis* and biofilm formation by several other common pathogens of the catheterized urinary tract.

8.3.4 Small... but insignificant? Triclosan resistant SCVs in healthcare.

Another form that *S. aureus* cells may take is that of SCVs. We have shown that triclosan can induce the growth of SCVs with reduced susceptibility to triclosan, gentamicin and penicillin. They also displayed attenuated production of the extracellular proteins coagulase and DNase. SCV growth was significantly slower than wild-type. SCV Growth rate was significantly reduced and cultures demonstrated extended lag periods. This caused sizeable practical problems, especially when attempting experiments that required high cell densities or when assessing antimicrobial susceptibility. Practical problems were compounded by poor genetic stability of SCVs. Hence it was necessary to consistently apply a selective pressure for the maintenance of the SCV phenotype. These problems are associated with growth *in vitro*, however the full impact of SCVs can only be realised *in vivo*.

Several features of SCVs are pertinent only during infection of a host. These include intracellular survival and the impact of attenuated virulence factor production. We were able to demonstrate that SCVs had a reduced susceptibility to several antimicrobials. Subsequently, we are able to hypothesise that this could cause treatment problems if these colonial mutants initiated/arose during an infection. However, we do not know whether these SCVs can support intracellular growth and, if it is possible, what the impact of this would be upon antimicrobial susceptibility and virulence. Therefore further work, either performed in animal models or cell culture (principally macrophages) should be performed, such as that carried out on other SCVs by Bates *et al.*, (2003), Jonsson *et al.*, (2003) and Barcia-Macay *et al.*, (2006). Alternatively, advances made by Sifri *et al.*, (2006), who were able to assess the virulence of SCVs in a *Caenorhabditis elegans* model, could be used. These would help to better understand the interaction between triclosan-induced SCVs and host cells – a key feature of infections. It appears that there are several genetically/biochemically different SCV types, so it is reasonable to hypothesise that

these may have different capacities to survive in cells. Hence, these models could also be used to distinguish between the SCV types and identify the potential clinical significance of each type.

A significant difference between triclosan and other antimicrobials known to select for SCVs is that it is only used topically. It is thought that it is not the SCV form that initiates an infection, rather, once a wild-type infection has been established antibiotic treatment selects for mutation to SCV form (section 1.6, Figure 1.11). The significance of this is that selection for SCVs by systemic antibiotics occurs once the bacterium has broached the skin and is at the site of infection, whereas triclosan-induced SCVs will presumably be on the skin surface. It is not known whether these would then be capable of initiating an infection. Data on the virulence of SCVs are unclear; characteristics of SCV phenotypes, such as attenuation of virulence factors, can seemingly reduce and increase the chance of pathogenesis. Virulence factors aid the infection process but also increase the host's immune response (Pelletier *et al.*, 1979; Jonsson *et al.*, 2003; Moisan *et al.*, 2006; Sifri *et al.*, 2006).

It is an obvious step to assess the prevalence of SCVs in the environment, most likely starting with hospitals. This would provide evidence for or against the existence of triclosan-induced SCVs in real-life situations. However, such a study is not as straightforward as it may seem. Firstly, SCVs are difficult to isolate, identify and work with, so specialised techniques would need to be developed (especially to avoid confusion with CoNS). Secondly, it is desirable to distinguish triclosan-induced SCVs from others. How may this be achieved? Even SCVs isolated during the same experiment can often vary in their phenotypic (and presumably genotypic) characteristics and at present we known too little about the mechanisms behind SCVs needed to distinguish between types. This could be surmounted partially by using a randomized controlled trial in which the recovery of SCVs was compared between individuals who had undergone triclosan therapy and those who had not (an appropriate control should be used).

Since, as discussed above, triclosan-induced SCVs are assumed to be at the skin surface they may be transmissible by skin-skin contact, as wild-type *S. aureus*. This would leave them vulnerable to the current mechanisms of preventing transfer of

infection, such as alcohol hand-washes and barrier systems such as gloves. The transfer of SCVs should be investigated, along with an assessment of the efficacy of procedures such as alcohol hand washes. It remains to be shown whether triclosan commercial triclosan formulations, as applied in the clinic, are capable of eliminating SCV carriage.

Although triclosan is generally applied topically it is also impregnated into polymers such as plastics or silicone for in-dwelling devices. This provides a unique environment that is possibly more worrying than selection of SCVs by topical preparations. For example, the use of triclosan-impregnated sutures (Rothenburger *et al.*, 2002; Edmiston *et al.*, 2004; Edmiston *et al.*, 2006) will provide a selective pressure for SCV status in close proximity to a mode of entry into the body. The release of triclosan is less controlled so the opportunity that triclosan concentrations are closer to those that select for SCVs is greater. Furthermore, during this method of triclosan delivery the antimicrobial is not combined with surfactants or other ingredients found in topical formulations, hence triclosan will be acting without the synergistic effects of these chemicals. A recent paper has reported that silicone discs containing triclosan selected for SCVs of 3 MRSA strains (Bayston *et al.*, 2007). Hence, there is clear potential for device-related triclosan-induced SCVs. Future work should be aimed at assessing the clinical impact of this phenomenon.

8.3.5 Requirement for clinical studies

At present most hypotheses surrounding triclosan resistance are based on the plethora of *in vitro* investigations. Whilst we can speculate on the impact of low-level resistance (Goldstein, 2007) clinical studies are required to assess the real-life impact of low-level triclosan resistance in staphylococci. Particular attention should be paid to assessing formulation effects, assessing the prevalence of resistance and examining the efficacy of triclosan treatments against reduced susceptibility organisms. Studies should also include CoNS and SCVs as these may represent a little studied reservoir of resistance. The effects of triclosan exposure on the development of resistance to other antimicrobials requires clarification, and this includes establishing the role of gene transfer.

8.4 Horizontal gene transfer of triclosan resistance in staphylococci

8.4.1 Transferable triclosan resistance: is their a relationship with mupirocin resistance?

An article published by Cookson *et al.*, (1991) implies that triclosan resistance was found on a plasmid alongside mupirocin resistance. This article has attracted much attention and is now cited by more than 40 papers that have published in major journals. This may have lead to it having become an accepted phenomenon. However, no further work has been published regarding the issue and in the light of more recent research these findings appear tenuous. We report in chapters 3, 4 and 5 that there is no correlation between resistance to triclosan and resistance to mupirocin in our strain collection or novel isolates. This has also been reported by Suller & Russell, (2000), Brenwald & Fraise, (2003) and Bamber & Neal, (1999). The combined breadth of these studies indicates that if triclosan resistance is transferable, in combination with mupirocin resistance, then it is a rare event. Indeed, we were unable to transfer low-level triclosan resistance amongst *S. aureus*, either singularly or in association with mupirocin resistance.

8.4.2 Gene transfer and the dissemination of low-level triclosan resistance

Our inability to transfer low-level triclosan resistance by three mechanisms of gene transfer implies that the evolution of triclosan resistance amongst *in situ S. aureus* is unlikely to be involved this process. These results are encouraging and provide further evidence that triclosan resistance is not transferable. Consequently, we hypothesise that the main routes for the spread of low-level triclosan resistance are clonal expansion and mutation. Indeed, *S. aureus* is highly clonal, an indication that mutation has proved important in the evolution of *S. aureus*, whereas recombination has had negligible impact on the diversification of the core genome of this species (Feil *et al.*, 2003).

So the most likely mechanism for the spread of triclosan resistance is development of a triclosan-resistant mutation in a successful strain that is able to out compete others. This clone would then become dominant, a possibility that is supported by our

evidence that triclosan resistance is not associated with a significant fitness cost (disregarding SCV-associated resistance). However, the lack of such a clone in reports to date indicates that this has not happened, even after 30 years of use. This is probably due to the high concentration and formulation effects of commercial triclosan products and the combinatorial use of other antimicrobials, such as CHX.

8.5 Effects of triclosan on gene transfer

Triclosan did not induce the transfer of resistance and generally caused a reduction in transfer frequencies. We found it to be active against bacteriophages, preventing gene transfer by transduction. Consequently, we hypothesise that exposure to triclosan does not increase the dissemination of resistance traits.

8.6 Gene transfer in SCVs

The discovery that triclosan-induced SCVs did not secrete extracellular DNase led us to hypothesise that they may be more amenable for gene transfer, especially by transformation. This hypothesis was supported by evidence that *S. aureus* SCVs were more easily transformable than wild-type. So the SCV phenotype appears to confer competence, in addition to reduced susceptibility and alterations to virulence and growth. Hence, we see that SCVs represent a complex and evolutionarily significant stage in *S. aureus*. Switching between SCV and wild-type phenotypes affords *S. aureus* an inducible and reversible resistance mechanism that circumvents a permanent cost to fitness (Massey *et al.*, 2001) and also allows it to better exploit the genetic resources of the surrounding environment. If *S. aureus* is clonal then the SCV phenotype offers a process of gene exchange (in this subpopulation) allowing for a parasexual cycle to occur in environments where such organisms are normally genetically isolated by mechanisms such as conjugation and transduction.

It makes clear evolutionary sense for SCVs to show augmented gene exchange properties. They are under a strong environmental pressure from the antimicrobial and are restricted in their growth. However, increasing the ability to accept exogenous DNA boosts their chances or acquiring other mechanisms of resistance that would enable them to revert to wild-type and grow at the significantly faster rate associated

with this phenotype. Furthermore, SCVs may represent a persistent reservoir of genes that is not adversely affected by antimicrobial treatment.

8.7 Conclusions

- The clinically dominant *S. aureus* capsule polysaccharide serotypes CP5 and CP8 do not represent a permeability barrier to antimicrobials, including triclosan.
- *S. aureus* can evolve reduced susceptibility to triclosan through spontaneous mutation. MICs of 1-4 mg/L are achieved by mutation of the *fabI* gene, which encodes an enzyme essential for type 2 bacterial fatty acid biosynthesis.
- However, reduced susceptibility has also been observed in non-FabI mutants, implying that other mechanisms of resistance are available (and that triclosan has targets other than fabI).
- Furthermore, triclosan is shown to induce the leakage of potassium ions from cells, an indication of membrane damage. Hence, there is increasing evidence that whilst fabI is a target for triclosan, it is not the sole target.
- Reduced susceptibility to triclosan, as measured by MIC, also confers reduced susceptibility to the lethal effects of 7.5 mg/L triclosan, but this effect is ameliorated by higher concentrations of triclosan.
- In-use concentrations of the commercial preparation of triclosan, Irgacide LP10, are equally active against reduced susceptibility *S. aureus* and wild-type.
- Spontaneous mutation to triclosan resistance is not associated with a significant fitness cost.
- Evolution of triclosan resistance does not confer co-resistance to other antimicrobials, including oxacillin, vancomycin, gentamicin, penicillin, CHX or CPC.
- MRSA and MSSA strains are equally susceptible to triclosan.
- Reduced susceptibility to triclosan is rare amongst the commensal *S. aureus* strains of the student population of Cardiff. However, CoNS showed consistently higher MICs for triclosan and may represent an amenable reservoir of resistance.

- Familial transfer of MRSA can occur, causing the organism to persist in the community setting.
- There is no indication that mupirocin and triclosan resistance have co-transferred in the past. Indeed, there appears to be no relationship between resistance to either of these compounds in *S. aureus*.
- Reduced susceptibility to triclosan cannot be disseminated amongst wild-type *S. aureus*, or related Gram-positive bacteria by transduction, conjugation or transformation.
- There is no bioinformatic evidence to support the historical acquisition of *fabI* by horizontal gene transfer in *S. aureus*.
- Triclosan can select for *S. aureus* SCVs that are coincidently resistant to gentamicin and penicillin. These are slow growing and illustrate the typical SCV phenotype. When they revert they are as susceptible as their parent.
- The mechanism of resistance to triclosan in triclosan-induced SCVs has not been elucidated, however there are close links between fatty acid biosynthesis and cellular ATP production.
- Triclosan-induced SCVs show attenuated production of coagulase, haemolysins and DNase.
- Triclosan-induced SCVs show a higher level of competency than wild-type. Hence, they were more readily transformable and may represent an enduring reservoir of resistance determinants.

8.8 Epilogue

The significance of reduced susceptibility to triclosan in *S. aureus* is ambiguous. The development of resistance has clear repercussions for the efficacy of triclosan treatments, however low-level resistant strains remain susceptible to the in-use concentrations of triclosan, especially when these are delivered with other ingredients such as surfactants. It is also pertinent that most infection control strategies employ a selection of control measures, ensuring that efficacy is not reliant upon a single procedure.

The description of transferable triclosan resistance by Cookson *et al.*, (1991) is a unique observation, and the uniqueness is confirmed by the fact that the widespread dissemination of triclosan resistance predicted by the authors has not occured. This may reflect the poor significance of low-level resistance in *S. aureus* in the presence of the combination of measures that constitute infection control strategies.

The use of triclosan in the home does not seem to have selected for increased resistance. However, it remains to be seen whether new antimicrobials directed towards bacterial fatty acid biosynthesis, such as platensimycin, will be undermined by the increasing use of triclosan-containing products.

Triclosan is highly efficacious against MRSA and forms an important part of current and future infection control procedures. However, it is imperative that research is continued to fully elucidate the role of triclosan in the formation of SCVs. The level of resistance within the environment should be monitored and careful control of the types of products that triclosan is incorporated in to is required to reduce the possibility of increased resistance.

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Chapter 9

Appendix

9.1 *fabI* gene sequences

Codons corresponding to 1st amino acid of the FabI protein and stop codon are highlighted in green and red, respectively. Triclosan-resistant mutations are highlighted in blue (affected codon is in **bold**).

>9518 *fabI*

TTAAAATGCAGACGTATTTTAGTACGACGTAAAATTAATGATTTTAAAATGCTAGTATGTATATGATTT
TGATAAATAAATGCTTTTTACGTAAATCAAGTTTGATACAGAAAGGACTAAATCAAACATTTATCGTTG
TAATACGTTTAAATAACTTTATTAAAAAGTCATAATAGTGTTAAAATGTATTGACGAATAAAAAGTTAG
TTAAAACTGGGATTAGATATTCTATCCGTTAAATTAATTATTATAAGGAGTTATCTTACATGTTAAATC
TTGAAAACAAAACATATGTCATCATGGGAATCGCTAATAAGCGTAGTATTGCTTTTGGTGTCGCTAAAG
TTTTAGATCAATTAGGTGCTAAATTAGTATTTACTTACCGTAAAGAACGTAGCCGTAAAGAGCTTGAAA
AATTATTAGAACAATTAAATCAACCAGAAGCGCACTTATATCAAATTGATGTTCAAAGCGATGAAGAGG
TTATTAATGGTTTTGAGCAAATTGGTAAAGATGTTGGCAATATTGATGGTGTATATCATTCAATCGCAT
TTGCTAATATGGAAGACTTACGCGGACGCTTTTCTGAAACTTCACGTGAAGGCTTCTTGTTAGCTCAAG
ACATTAGTTCTTACTCATTAACAATTGTGGCTCATGAAGCTAAAAAATTAATGCCAGAAGGTGGTAGCA
TTGTTGCAACAACATATTTAGGTGGCGAATTCGCAGTTCAAAATTATAATGTGATGGGTGTTGCTAAAG
CGAGCTTAGAAGCAAATGTTAAATATTTAGCATTAGACTTAGGTCCTGATAATATTCGCGTTAATGCAA
TTTCAGCTGGTCCAATCCGTACATTAAGTGCAAAAGGTGTGGGTGGTTTCAATACAATTCTTAAAGAAA
TCGAAGAGCGTGCACCTTTAAAACGTAACGTTGATCAAGTAGAAGTAGGTAAAACAGCGGCTTACTTAT
TAAGTGACTTATCAAGTGGCGTTACAGGTGAAAATATTCATGTAGATAGCGGATTCCACGCAATTAAAT
AATATCATTCAACAGCTTTGTTCACGTTATCATATATGTGAGCAAAGCTTTTTTGCTTTTATAATAATC
GGACTGATGGAAAATTATTTGATATTTCATCTGACTGATTTTTTTTATGCACAAAAAAATCTCCTCAAA
AGCTGTAGTTTCAAC

>9518T1 *fabI*

TTAAAATGCAGACGTATTTTAGTACGACGTAAAATTAATGATTTTAAAATGCTAGTATGTATATGATTT
TGATAAATAAATGCTTTTTACGTAAATCAAGTTTGATACAGAAAGGACTAAATCAAACATTTATCGTTG
TAATACGTTTAAATAACTTTATTAAAAAGTCATAATAGTGTTAAAATGTATTGACGAATAAAAAGTTAG
TTAAAACTGGGATTAGATATTCTATCCGTTAAATTAATTATTATAAGGAGTTATCTTACATGTTAAATC
TTGAAAACAAAACATATGTCATCATGGGAATCGCTAATAAGCGTAGTATTGCTTTTGGTGTCGCTAAAG
TTTTAGATCAATTAGGTGCTAAATTAGTATTTACTTACCGTAAAGAACGTAGCCGTAAAGAGCTTGAAA
AATTATTAGAACAATTAAATCAACCAGAAGCGCACTTATATCAAATTGATGTTCAAAGCGATGAAGAGG
TTATTAATGGTTTTGAGCAAATTGGTAAAGATGTTGGCAATATTGATGGTGTATATCATTCAATC**G**TAT
TTGCTAATATGGAAGACTTACGCGGACGCTTTTCTGAAACTTCACGTGAAGGCTTCTTGTTAGCTCAAG
ACATTAGTTCTTACTCATTAACAATTGTGGCTCATGAAGCTAAAAAATTAATGCCAGAAGGTGGTAGCA
TTGTTGCAACAACATATTTAGGTGGCGAATTCGCAGTTCAAAATTATAATGTGATGGGTGTTGCTAAAG
CGAGCTTAGAAGCAAATGTTAAATATTTAGCATTAGACTTAGGTCCTGATAATATTCGCGTTAATGCAA

TTTCAGCTGGTCCAATCCGTACATTAAGTGCAAAAGGTGTGGGTGGTTTCAATACAATTCTTAAAGAAA
TCGAAGAGCGTGCACCTTTAAAACGTAACGTTGATCAAGTAGAAGTAGGTAAAACAGCGGCTTACTTAT
TAAGTGACTTATCAAGTGGCGTTACAGGTGAAAATATTCATGTAGATAGCGGATTCCACGCAATTAAAT
AATATCATTCAACAGCTTTGTTCACGTTATCATATATGTGAGCAAAGCTTTTTTGCTTTTATAATAATC
GGACTGATGGAAAATTATTTGATATTTCATCTGACTGATTTTTTTTATGCACAAAAAAATCTCCTCAAA
AGCTGTAGTTTCAAC

>9518T2 *fabI*

TTAAAATGCAGACGTATTTTAGTACGACGTAAAATTAATGATTTTAAAATGCTAGTATGTATATGATTT
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TAATACGTTTAAATAACTTGATTAAAAAGTCATAATAGTGTTAAAATGTATTGACGAATAAAAAGTTAG
TTAAAACTGGGATTAGATATTCTATCCGTTAAATTAATTATTATAAGGAGTTATCTTACATGTTAAATC
TTGAAAACAAAACATATGTCATCATGGGAATCGCTAATAAGCGTAGTATTGCTTTTGGTGTCGCTAAAG
TTTTAGATCAATTAGGTGCTAAATTAGTATTTACTTACCGTAAAGAACGTAGCCGTAAAGAGCTTGAAA
AATTATTAGAACAATTAAATCAACCAGAAGCGCACTTATATCAAATTGATGTTCAAAGCGATGAAGAGG
TTATTAATGGTTTTGAGCAAATTGGTAAAGATGTTGGCAATATTGATGGTGTATATCATTCAATCGTAT
TTGCTAATATGGAAGACTTACGCGGACGCTTTTCTGAAACTTCACGTGAAGGCTTCTTGTTAGCTCAAG
ACATTAGTTCTTACTCATTAACAATTGTGGCTCATGAAGCTAAAAAATTAATGCCAGAAGGTGGTAGCA
TTGTTGCAACAACATATTTAGGTGGCGAATTCGCAGTTCAAAATTATAATGTGATGGGTGTTGCTAAAG
CGAGCTTAGAAGCAAATGTTAAATATTTAGCATTAGACTTAGGTCCTGATAATATTCGCGTTAATGCAA
TTTCAGCTGGTCCAATCCGTACATTAAGTGCAAAAGGTGTGGGTGGTTTCAATACAATTCTTAAAGAAA
TCGAAGAGCGTGCACCTTTAAAACGTAACGTTGATCAAGTAGAAGTAGGTAAAACAGCGGCTTACTTAT
TAAGTGACTTATCAAGTGGCGTTACAGGTGAAAATATTCATGTAGATAGCGGATTCCACGCAATTAAAT
AATATCATTCAACAGCTTTGTTCACGTTATCATATATGTGAGCAAAGCTTTTTTGCTTTTATAATAATC
GGACTGATGGAAAATTATTTGATATTTCATCTGACTGATTTTTTTTATGCACAAAAAAATCTCCTCAAA
AGCTGTAGTTTCAAC

>9518T3 *fabI*

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>27343 *fabI*

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>27343T1 *fabI*

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>24500 *fabI*

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>F89 *fabI*

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>F89T1 *fabI*

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Corresponding amino acid sequences for *S. aureus* strains. Triclosan-resistant substitutions are highlighted in blue.

>9518 FabI

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>T1 FabI

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>T2

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>27343 FabI

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>24500 FabI

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9.2 Reply to Bayston *et al.* published in the Journal of Antimicrobial Chemotherapy (Seaman *et al.* 2007, *J Antimicrob Chemother* 60: 175-176)

Comment on: Triclosan resistance in methicillin-resistant *Staphylococcus aureus* expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics

Sir,

In their recent article, Bayston *et al.*[1] provide an interesting report on the occurrence of small colony variants (SCV) of *Staphylococcus aureus* during exposure to silicone impregnated with triclosan. In parallel with our prior report[2] Bayston *et al.* describe the isolation of SCVs with low-level resistance to triclosan, reduced coagulase production, reduced haemolytic activity and did not detect auxotrophy. They recognise, as others have done, the possible clinical importance of SCVs and the potential role of triclosan in their generation. However we would like to urge some caution in their interpretation of their data and consequently on the significance of the issues raised.

A significant difference between triclosan and other antimicrobials known to select for SCVs (principally aminoglycosides) is that it is used topically. It is thought that SCVs are unlikely to initiate infections, rather, once a wild-type infection has been established, antibiotic treatment selects for mutation to give the SCV phenotype/form. Hence, selection for SCVs by systemic antibiotics occurs once the bacteria have broached the skin and are at the site of infection, whereas triclosan-induced SCVs will presumably be on the skin surface. So an important question is whether these SCVs would then be capable of ever initiating an infection in healthy individuals.

Extending this logic means the triclosan-induced SCVs formed at the skin surface may be transmissible by skin-skin contact, as wild-type *S. aureus*. This could provide a mechanism for them coming into contact with wounds or finding other routes through the epidermis. However, it would also leave them vulnerable to the current mechanisms of preventing transfer of infection, such as alcohol hand-washes and barrier systems such as gloves.

The association of SCVs with triclosan-impregnated polymers is possibly more worrying than selection of SCVs by topical preparations. For example, the use of triclosan-impregnated sutures[3,4] will provide a selective pressure for SCV status in close proximity to a site of entry into the body, and the release of triclosan is less controlled, so the opportunity that triclosan concentrations are closer to those that select for SCVs is greater. Furthermore, during this method of triclosan delivery the antimicrobial is not combined with surfactants or other ingredients found in topical formulations, hence triclosan will be acting without the synergistic effects of these chemicals.[5] It has also been reported that surface-bound SCVs were highly resistant to the bactericidal action of oxacillin or vancomycin[6], consequently it would be interesting to know the susceptibility of these SCVs whilst adhered to the silicone discs. However, in contrast to the authors' comment, we believe that the use of materials impregnated with triclosan is not as widely established as some authors report. Triclosan is not used as a typical product protectant in plastic articles. Indeed, its use in plastic is limited to special articles that represent a niche market. Thus the frequency and impact of these events is likely to be very low.

We found previously that the selection of SCVs by triclosan was concentration dependent.[2] It would be very interesting to know what the triclosan concentration in the discs was and at what rate it was released. This is also key to any speculation on their clinical impact.

Due to the abnormal characteristics of *S. aureus* SCVs they are easily confused with coagulase negative staphylococci[7] and potentially with various other slow growing bacterial genera. Additionally, they require extended incubation times and their slow growth rate leaves them liable to be 'overgrown' by faster growing organisms. For these reasons we feel it is necessary to unambiguously confirm that putative SCVs are indeed *S. aureus* and not contaminants. We have found nucleic acid-based methods useful for this.[2]

Bayston *et al.* state that triclosan acts by inhibiting FabI, an enzyme that executes the final step in the elongation cycle of bacterial fatty acid biosynthesis. It should be noted that whilst FabI is undoubtedly a target for low-levels of triclosan, the antimicrobial also has other targets, such as the cytoplasmic membrane.[8] Thus we feel it would have

been appropriate to have investigated the possibility that the triclosan resistance found in their SCVs was as a result of this phenotype and not through coincidental alterations to FabI or other targets.

Finally, we were concerned to see that the title of the article states that SCVs are 'a novel mode of evasion of susceptibility to antiseptics', however their results report only on susceptibility to triclosan. As the paper presents no evidence that SCVs demonstrate resistance to other antiseptics the title of the article is misleading. It clearly implies that SCV status, obtained as a result of triclosan exposure, confers resistance to multiple antiseptics when there is no evidence to support this hypothesis.

The role of *S. aureus* SCVs in morbidity and mortality is coming under increasing scrutiny. Whilst we welcome all research that aids our understanding of these curious infections, there is a danger that too much speculation on the clinical impact of SCVs based only on *in vitro* findings could restrict the use of an antimicrobial that has to date performed well during infection management situations. We would also recommend that studies unambiguously confirm that putative SCVs are indeed *S. aureus* and investigate alternative mechanisms of resistance in addition to making the association with the SCV phenotype.

Transparency declarations

PFS and MJD have nothing to declare. DO is an employee of Ciba Spezialitätenchemie Grenzach GmbH, Grenzach-Wyhlen, Germany.

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