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

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Received 19 August 2006; returned 22 September 2006; revised 26 September 2006; accepted 9 October 2006

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 methicillin-resistant *S. aureus* (MRSA) isolates and methicillin-susceptible *S. aureus* 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 25 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.

Keywords: atypical growth, co-resistance, biocide resistance, mechanisms of resistance

Introduction

Small-colony variants (SCVs) of *Staphylococcus 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), haem, thiamine or haem A biosynthesis. ETC SCVs are associated with persistent and relapsing osteomyelitis, sinusitis, muscle abscesses and device-related infections²,³ 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 haem 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 utilization.⁸ The basis for thymidine-auxotrophic SCVs is not currently understood.¹

The ability to form a variant sub-population affords *S. aureus* a number of survival advantages. SCV persistence intracellularly within non-professional 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

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of multidrug-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. 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. First, reduced growth rate has been shown to reduce the susceptibility to cell-wall-targeting antimicrobials by up to 4-fold. Secondly, the break down in bacterial ETC reduces the transmembrane potential (ΔΨ) resulting in reduced uptake of cationic compounds. Finally, 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.

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 25 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 growth media and DMSO controls were run alongside experiments where required.

**Electron microscopy**

Cells in logarithmic growth phase were prepared for scanning electron microscopy (SEM) by fixing in 2% glutaraldehyde in 0.1% sodium phosphate buffer, pH 7.4, for 2 h and were 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 (Philips, The Netherlands). Samples for transmission electron microscopy (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.

**SCV characterization**

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. Colonies (4–5) were transferred to a microscope slide follow by the addition of hydrogen peroxide; the presence of bubbling was taken as an indication of catalase
Triclosan-selected *S. aureus* SCVs

production. Auxotrophy for haemin, menaquinone and thiamine was examined as described previously. Growth curves were attained by spectrophotometric analysis (580 nm) of MH broth cultures using a DYNEX Technologies MRX<sup>®</sup> Microplate Absorbance Reader with Revelation<sup>®</sup> 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<sup>3</sup> cells/mL. Plates were sealed with adhesive plate seals (ABgene, UK) and further incubation was performed at 37°C.

Plates were incubated for 48 h. Etest<sup>®</sup> incubation was performed at 37°C. 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 ± SD.

**SCV formation and reversion rates**

The rate of mutation towards the SCV phenotype was assessed by the growth of *S. aureus* strains to mid-log phase in 9 mL of 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 rpm for 15 min), washed, resuspended in quarter-strength Ringer’s solution and adjusted to 10<sup>10</sup> cells/mL. Volumes (100 μL) of 10<sup>10</sup> cells/mL were spread onto 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 one-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 an MH agar plate with cells from ten SCV colonies suspended in 3 mL of 0.9% NaCl. After 48 h of 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 of the three biocides, triclosan, chlorhexidine gluconate (ICN Biomedicals Inc., USA) and cetylpyridinium chloride (ICN Biomedicals Inc., USA) were calculated according to the guidelines of the BSAC.<sup>34</sup> Due to the slow growth of the SCVs, MIC plates were incubated for 48 h. Etest<sup>®</sup> strips (Bio-stat Ltd, UK) were utilized to determine the MICs of antibiotics, as per the manufacturer’s directions. The *bactericidal* effects of triclosan on wild-type *S. aureus* and their SCVs were compared as reported previously.<sup>35</sup> Except that the inoculum was adjusted to 10<sup>7</sup> cfu/mL, 2% azolecin and 5% Tween 80 in molecular grade de-ionized water was used for the neutralizing solution and sampling was continued up to 2 h. Log<sub>10</sub> reduction was then calculated from log<sub>10</sub>N<sub>t</sub> - log<sub>10</sub>N<sub>c</sub> where N<sub>t</sub> and N<sub>c</sub> represent the numbers of cfu/mL in the control and triclosan solutions, respectively. The significant effect of triclosan on the reduction in cell density was tested with ANOVA. All data are presented as the mean ± SD.

**Validation of triclosan neutralizer**

Aliquots of 2% azolecin and 5% Tween 80 in molecular grade de-ionized water were used as the triclosan neutralizer. Its ability to neutralize triclosan without toxicity to bacterial cells was examined as described previously.<sup>36,37</sup> Briefly, 8 mL of neutralizer, 100 μL of triclosan (10 mg/mL) and 900 μL of sterile de-ionized water was inoculated with 1 mL of an overnight culture of *S. aureus* NCIMB 9518 grown at 37°C. The viable cell count was enumerated after 5 min of 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 the 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.<sup>38</sup> Primers adapted from those designed for the amplification of the staphylococcal *fabI* gene and putative *fabI* promoter region<sup>39</sup> were used in 50 μL PCRs (FabI1F, tgtgcgcattaagctatcc; and FabI1R, taaggactaatctgtagt). Reactions contained 0.5 μL of chromosomal DNA (~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) and 0.2 mM deoxyribonucleoside triphosphates (Bioline Ltd). 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 using a QIAquick PCR purification kit (Qiagen Ltd, UK) and the sequences of both strands were 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<sup>39</sup> were also used (Fab2F, agttaaatctgggaataacaac; and Fab2R, ttatattgaatggagt).

**Results**

Suspected SCVs were isolated from MH agar plates containing 1 mg/L triclosan and spread with a heavy inoculum of *S. aureus* strains NCTC 6571 (Oxford strain), NCIMB 9518 and F89 (high-level mupirocin-resistant) and clinical MRSA isolates 24500 and 24 500 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 heterogeneity of cell morphology; apparently normal *S. aureus* mixed with larger cell morphotypes. Subsequent SEM of SCV samples revealed bacterial cells of different sizes, in distinction to homogeneous cellular morphology; apparently normal *S. aureus* mixed with larger cell morphotypes. 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 1), significantly more than were found in wild-type cultures (*P* = <0.05, for both phenomena).

Species confirmation of *S. aureus* SCVs is notoriously challenging and can often lead to misidentification as coagulase-negative...
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.\(^3\) The presence of a staphylococcal 16S rRNA gene and *S. aureus* specific nuc gene was recorded for all suspected SCVs analysed (Figure 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 uncharacterized.

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 with 1 h), greatly reduced maximal growth rates (0.23 compared with 1.65 generations/h) and achieved maximal cell densities approximately one-half of their parent strain (Figure 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 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 was penicillin susceptible (methicillin-susceptible *S. aureus*). 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, a bis-biguanide biocide used widely in mouthwashes, toothpastes and clinical hand washes, and cetylpyridinium chloride, a quaternary ammonium compound.
Both are cationic, and as such the uptake of these compounds may be affected by a reduction in Dy, reducing their antimicrobial effect. However, none of the SCV strains showed any reduction in susceptibility to either of these compounds in comparison with their isogenic wild-type, perhaps an indication that a reduction in Dy 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. 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. 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 with times of over 2 h for SCV strains (Figure 4a). However, the bactericidal efficacy of triclosan at 20 and 40 mg/L was comparable between wild-type and SCV strains (Figure 4b and c).

DNA sequences for the fabI gene and preceding promoter region were ascertained for all SCVs and compared with 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. The rate of reversion can also be very high, although some SCVs can show stability under non-selective conditions. Triclosan was shown to select for SCVs at rates between $1.86 \times 10^{-11}$ and $9.46 \times 10^{-10}$ 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 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.

**Discussion**

We have shown that in vitro exposure to triclosan can trigger the emergence of reduced drug-susceptibility 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

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**Table 1. MICs for S. aureus SCVs and their corresponding wild-type parent**

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<td>0.19</td>
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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.

*Calculated by Etest (AB Biodisk, Sweden).

*Calculated in accordance with BSAC guidelines.
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 methicillin 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 regard to growth rate, however, this was negated by their refractoriness to antimicrobial therapy. In vivo this phenotype may also confer the ability for intracellular maintenance. If able to persist intracellularly, further reductions in antimicrobial susceptibility may be expected, 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 similarly the lethal effects of triclosan at 7.5 mg/L. However, the lethality of triclosan at 20 and 40 mg/L and no cross-resistance to chlorhexidine and cetylpyridinium chloride 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 formulations and surface disinfectants are 0.3–1%; for hospital wash applications for MRSA eradication even 2% triclosan is recommended. These in-use concentrations are magnitudes of wild-type.
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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 uncharacterized.

This investigation highlights a microbial phenomenon (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.

Acknowledgements

We would like to acknowledge the contribution of Professor A. Denver Russell, Welch School of Pharmacy, Cardiff, who died in September 2004, for his invaluable contribution to our work. We 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 P. F. S.

Transparency declarations

P. F. S. and M. J. D. have nothing to declare. D. O. is an employee of Ciba Specialty Chemicals, Grenzach-Wyhlen, Germany.

References

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