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Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated *Staphylococcus aureus*: an *in vitro* study

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The presence of persister cells and small-colony variants (SCVs) has been associated with enhanced antibiotic resistance of many organisms in biofilms. This study investigated whether persisters and/or SCVs contribute to the antibiotic resistance of *Staphylococcus aureus* biofilms. A detailed dose-dependent killing of biofilms and planktonic cells with five antibiotics (oxacillin, cefotaxime, amikacin, ciprofloxacin and vancomycin) was analysed by treating them with each antibiotic at a concentration of 0–100 μg ml^{-1} at 37 °C for 48 h. The killing of biofilm cells by all of the antibiotics showed the presence of persister cells – most cells in the population died, leaving a fraction that persisted, even at higher concentrations of the antibiotics. These persisters represented a transient resistant phenotype and reverted to a killing curve resembling that of the wild-type parent upon re-exposure to the antibiotics. SCVs were observed in biofilms only after treatment with ciprofloxacin, and these SCVs were of a transient nature. The treatment of planktonic cells with oxacillin, cefotaxime, ciprofloxacin and vancomycin killed the entire population and no persisters were detected. Transient SCVs, observed in planktonic cells following exposure to these antibiotics, were killed at higher antibiotic concentrations. The treatment of planktonic cells with amikacin yielded a small subpopulation of survivors that included persisters (at numbers significantly lower than for the biofilms) and highly resistant, stable SCVs with an increased biofilm-forming capacity in comparison with the wild-type parent. Thus the high resistance of *S. aureus* biofilms to multiple unrelated antibiotics is largely dependent on the presence of persister cells. Biofilms harbour a large number of persisters in comparison with planktonic cultures, which either do not harbour persisters or harbour only a small number. SCVs, although not specifically associated with *S. aureus* biofilms, have an increased biofilm-forming capacity and this may explain the frequent isolation of SCVs from biofilm-associated infections. The intrinsic resistance of these variants may in turn contribute to the enhanced antibiotic resistance of the biofilms thus formed.

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INTRODUCTION

Staphylococcus aureus biofilms have been widely implicated in many implant-based and chronic infections including those associated with prosthetic heart valves, central venous catheters, urinary catheters, orthopaedic prostheses, penile prostheses and contact lenses, endocarditis, otitis media, osteomyelitis and sinusitis (Bendouah *et al.*, 2006; Costerton *et al.*, 1999; Fux *et al.*, 2005). These biofilm infections are difficult to treat, and *in vitro* susceptibility tests have shown that biofilms are considerably more resistant than planktonic cells to the action of antibiotics (Amorena *et al.*, 1999; Ceri *et al.*, 1999). The enhanced antibiotic resistance of biofilms has been attributed to the presence of exopolysaccharide matrix, a slow growth rate, spatial heterogeneity and biofilm-specific drug-resistant or drug-tolerant physiologies including the presence of persister cells and small-colony variants (SCVs) (Gilbert *et al.*, 2002; Mah & O'Toole, 2001; Stewart & Costerton, 2001).

Persisters are small subpopulations of bacteria that survive lethal concentrations of antibiotics without any specific resistance mechanisms (Lewis *et al.*, 2006). These cells are not drug-resistant mutants; rather, hyper-resistance in these persisters is a transient phenotypic switch and, on reculturing, they revert to wild-type with a new population of persisters (Bigger, 1944; Broun *et al.*, 2000). The presence of persisters within a population is indicated by killing data that show most cells in a population dying,

Abbreviation: SCVs, small-colony variants.

with a subpopulation (0.1–10 %) persisting, even on prolonged exposure (Balaban *et al.*, 2004; Keren *et al.*, 2004a) or at higher concentrations of the antibiotics (Brooun *et al.*, 2000; Spoering & Lewis, 2001). Persisters pre-exist in a population and arise independently of the use of antibiotics (Keren *et al.*, 2004a). The formation of these cells is not dependent on quorum sensing (Lewis *et al.*, 2006). Persisters survive high concentrations of antibiotics by overexpression of genes such as the chromosomal toxin–antitoxin modules that shut down cellular functions and hence the antibiotic targets, resulting in a dormant cell that is tolerant to the lethal action of antibiotics (Keren *et al.*, 2004b). Persisters have been implicated in the antibiotic resistance of *Escherichia coli*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus* and *Gardnerella vaginalis* biofilms (Ashby *et al.*, 1994; Brooun *et al.*, 2000; Muli & Struthers, 1998; Roberts & Stewart, 2005; Spoering & Lewis, 2001). However, the literature has been relatively silent about the role of persisters in the antibiotic resistance of *S. aureus* biofilms although *S. aureus* planktonic cells have been shown to harbour persisters (Bigger, 1944; Keren *et al.*, 2004a).

The generation of resistant phenotypic variants such as SCVs during growth and infection by *S. aureus* has been well described. *S. aureus* SCVs may occur in cases of cystic fibrosis, soft tissue infections, osteomyelitis, arthritis, sinusitis, brain abscess and device-related infections including those associated with prosthetic heart valves, pacemakers, ventriculoperitoneal shunts and prosthetic joints (Besier *et al.*, 2007; Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). They differ from the normal phenotype in their small colony size, reduced growth rate, pigmentation and haemolysis, altered expression of virulence factors and auxotrophy for haemin, menadione, thiamine or thymidine, and they are more resistant to the action of aminoglycosides and cell-wall inhibitors and have a tendency to persist (Acar *et al.*, 1978; Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). This complex phenotype of SCVs is due to defects in the electron transport chain. These variants may represent a stable, inheritable change or a transient colony type (Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). The relationship between *S. aureus* SCVs and biofilm phenotype is unclear, but their shared characteristics suggest that they may have a similar underlying physiology (Higashi & Sullam, 2006). Both are slow-growing and more resistant to antimicrobials, and the diseases with which they are associated overlap considerably (Higashi & Sullam, 2006). Once adhered, SCVs are almost completely resistant to antibiotics (Chuard *et al.*, 1997). Williams *et al.* (1997) reported isolation of SCVs from adherent *S. aureus* cultures even in the absence of antibiotics, suggesting that biofilm formation may correlate with this mode of growth. However, following antibiotic treatment, these variants appeared with equal frequency in both biofilms and planktonic cells (Williams *et al.*, 1997). The specific role of SCVs in conferring antibiotic resistance to *S. aureus* biofilms thus remains unclear.

Given the frequency of *S. aureus* biofilm-associated infections, the involvement of persisters in the antibiotic resistance of biofilms in many organisms and the regular isolation of *S. aureus* SCVs from patients with biofilm infections, we sought to determine whether persisters and/or SCVs contribute to the antibiotic resistance of *S. aureus* biofilms.

METHODS

Bacterial strain. *S. aureus* ATCC 29213, a biofilm-forming strain, was used in this study. The strain was preserved in semi-solid agar butts at 4 °C and in brain–heart infusion broth with 15 % glycerol at −70 °C until use.

Antibiotics. Oxacillin, cefotaxime, amikacin sulfate, ciprofloxacin hydrochloride and vancomycin hydrochloride representing members of the isoxazolyl penicillin, third-generation cephalosporin, aminoglycoside, fluoroquinolone and glycopeptide classes, respectively, were used.

Biofilm preparation. The method of Anderl *et al.* (2000) was followed to prepare colony biofilms. An overnight culture of the strain was grown in tryptic soy broth (TSB) and diluted in the same medium to an OD_{600} of 0.05 with 0.1 M phosphate buffer (PB; pH 7.2). A 10 µl drop of this diluted culture was used to seed black polycarbonate membrane filters (13 mm diameter; pore size 0.4 µm) placed on tryptic soy agar (TSA) plates. The plates were inverted and incubated at 37 °C for 48 h, with the membrane-supported biofilms transferred to fresh culture medium every 24 h. The biofilms were washed with PB by agitation at 180 r.p.m. for 1 min to remove non-adherent cells. For determination of the mean number of c.f.u., the biofilm cells were suspended in 0.9 ml PB, the mixture was vortexed at high speed for 2 min and viable bacteria were enumerated by drop plating (Miles *et al.*, 1938). Biofilm structure was confirmed by transmission and scanning electron microscopy.

Determination of MIC and minimum bactericidal concentration (MBC) of planktonic cells. The MICs of antibiotics against planktonic cells were determined by a broth macrodilution method according to CLSI (2006) guidelines. The tubes containing MICs and higher concentrations of the antibiotics were plated on nutrient agar and incubated at 37 °C for 24 h for determination of the MBC of the antibiotic.

Susceptibility testing of biofilms and planktonic cells to antibiotics. The method of Walters *et al.* (2003) was followed with modifications. Colony biofilms were transferred to TSA plates containing antibiotics at concentrations representing MIC multiples corresponding to the clinically achievable level of the antibiotic and a twofold higher concentration (the MICs of antibiotics determined by the agar dilution method in TSA were the same as those obtained in Mueller–Hinton broth by broth macrodilution). As the maximum clinically achievable concentrations of oxacillin, cefotaxime, amikacin, ciprofloxacin and vancomycin are 40, 45, 40, 4.5 and 40 µg ml^{-1}, respectively (intravenously) (Yao & Moellering, 2003), the concentrations used in the susceptibility assay were 32 and 64 µg ml^{-1} for oxacillin (128 and 256 times the MIC), 32 and 64 µg ml^{-1} for cefotaxime (32 and 64 times the MIC), 32 and 64 µg ml^{-1} for amikacin (16 and 32 times the MIC), 4 and 8 µg ml^{-1} for ciprofloxacin (eight and 16 times the MIC), and 32 and 64 µg ml^{-1} for vancomycin (32 and 64 times the MIC). The plates were incubated at 37 °C for 24 h. The antibiotic-treated biofilms and control biofilms (initial or time zero control and untreated or 24 h control) were sampled and viable bacteria were enumerated by drop plating (Miles *et al.*, 1938). Experiments were performed in duplicate.

Planktonic cultures were prepared by inoculating the strain in 5 ml TSB and incubating overnight at 37 °C. One millilitre of an overnight culture was mixed with 1 ml fresh TSB, and 1 ml antibiotic solution or PB (control) was added. The tubes were incubated at 37 °C for 24 h. The bacteria in antibiotic-treated and control tubes (initial or time zero control and untreated or 24 h control) were pelleted by centrifugation at 4500 ***g*** for 5 min, washed twice with PB and finally suspended in 1 ml PB. The resulting cell suspension was serially diluted, and the viable count was enumerated by drop plating (Miles *et al.*, 1938). Experiments were performed in duplicate.

Dose-dependent killing of biofilms and planktonic cells: detection of persister cells and/or SCVs. The method of Brooun *et al.* (2000) was followed with modifications for studying the dose-dependent killing of planktonic cells and biofilms.

Planktonic cultures were prepared as described above. Fifty microlitres of this culture was added to 1.5 ml TSB containing antibiotics at the desired concentration (0, 0.1, 1, 10, 20, 40, 60, 80 and 100 μg ml^{-1}) and incubated for 48 h at 37 °C. The suspension was then centrifuged at 4500 ***g*** for 5 min, washed twice with PB and finally suspended in 1 ml PB. The number of c.f.u. ml^{-1} was determined by drop plating (Miles *et al.*, 1938). The cells surviving the highest concentration (100 μg ml^{-1}) of the antibiotic were picked and streaked on blood agar plates. These survivors were grown overnight in 5 ml TSB at 37 °C and subjected again to dose-dependent killing with the same antibiotic used to isolate them. The experiments were performed in duplicate. The SCVs obtained were characterized for stability, haemolysis, catalase production, clumping factor and coagulase production, DNase production, and auxotrophy for haemin, thiamine and menadione by using the method of Bayston *et al.* (2007). The susceptibility (MIC and MBC) of these SCVs to the antibiotic used to isolate them was determined by the broth macrodilution method as described above. The adherence capacity of SCVs to flat-bottomed polystyrene microtitre plates was compared with that of the wild-type (in quadruplicate) by using the method of Vuong *et al.* (2000).

To study the dose-dependent killing of biofilms by antibiotics, colony biofilms were transferred to TSA plates containing antibiotics at the desired concentrations (0, 0.1, 1, 10, 20, 40, 60, 80 and 100 μg ml^{-1}) and incubated at 37 °C for 48 h. The biofilms were sampled and the number of viable bacteria was enumerated by drop plating (Miles *et al.*, 1938). The cells surviving the highest concentration (100 μg ml^{-1}) of the antibiotic were picked and streaked on blood agar plates. These survivors were grown overnight in 5 ml TSB at 37 °C and the biofilms formed by these cells were again subjected to dose-dependent killing with the same antibiotic that was used to isolate them. Experiments were performed in duplicate.

Statistical analysis. The results were analysed statistically using GraphPad Prism 5 software. A two-way analysis of variance with Tukey's multiple comparison test was applied to compare the antibiotic susceptibility of biofilms and planktonic cells and to determine the effect of various antibiotic treatments. A two-tailed, unpaired *t*-test assuming unequal variances was used to compare the adherence capacity of the wild-type parent and SCVs as well as the percentage survival of biofilms versus planktonic cells after treatment with 100 μg amikacin ml^{-1}.

RESULTS AND DISCUSSION

Biofilm preparation

Colony biofilms of *S. aureus* ATCC 29213 were grown on black polycarbonate membrane filters. The $\log_{10}$ c.f.u. per membrane (mean ± SD) after 48 h growth was 9.59 ± 0.24. Mean biofilm thickness and area were 0.206 ± 0.02 mm and 31.93 ± 1.77 mm^2, respectively and the cell density within the biofilms was in the order of 10^8 c.f.u. mm^{-3}.

MICs and MBCs of planktonic cells

The MICs of oxacillin, cefotaxime, amikacin, ciprofloxacin and vancomycin for planktonic cells of *S. aureus* ATCC 29213 were 0.25, 1, 2, 0.5 and 1 μg ml^{-1}, respectively. The MBCs were 2, 8, 32, 2 and 4 μg ml^{-1}, respectively.

Susceptibility testing of biofilms and planktonic cells to antibiotics

Colony biofilms and planktonic cells at similar viable counts were treated with antibiotics at concentrations representing MIC multiples corresponding to the clinically achievable level of the antibiotic and a twofold higher concentration. Intact biofilms of *S. aureus* were significantly ($P < 0.001$) more resistant than planktonic cells to the action of antibiotics (Fig. 1). Planktonic cells were effectively killed on treatment with the antibiotics. However, the viability of biofilm cells was not significantly ($P > 0.05$) affected after 24 h of exposure to antibiotics at MIC multiples representing clinically achievable concentrations (Fig. 1). At these concentrations of antibiotics, no statistically significant difference was observed between the treated and untreated control ($P > 0.05$). Similar results were obtained on exposure of biofilms to antibiotics at MIC multiples representing a concentration higher than the clinically achievable levels, except for vancomycin at 64 μg ml^{-1}, which resulted in a modest but statistically insignificant reduction ($P > 0.05$) in viable counts (Fig. 1). Many earlier studies have also reported increased resistance of biofilms to antibiotics (Amorena *et al.*, 1999; Ceri *et al.*, 1999; Gilbert *et al.*, 2002; Mah & O'Toole, 2001; Stewart & Costerton, 2001).

Dose-dependent killing of biofilms and planktonic cells: detection of persister cells and/or SCVs

The enhanced antibiotic resistance of biofilms has been attributed to the presence of exopolysaccharide matrix, slow growth, spatial heterogeneity and biofilm-specific drug-resistant or drug-tolerant physiologies (Gilbert *et al.*, 2002; Mah & O'Toole, 2001; Stewart & Costerton, 2001), including the presence of persister cells (Ashby *et al.*, 1994; Brooun *et al.*, 2000; Muli & Struthers, 1998; Roberts & Stewart, 2005; Spoering & Lewis, 2001) and SCVs (Allegrucci & Sauer, 2007; Drenkard & Ausubel, 2002; Haussler *et al.*, 2003; Williams *et al.*, 1997). We assessed the role of persisters and SCVs in the antibiotic resistance of *S. aureus* biofilms. A detailed comparative dose-dependent killing of biofilms and planktonic cells with five different antibiotics was studied by treating them with each antibiotic at a concentration of 0–100 μg ml^{-1} at 37 °C for 48 h.

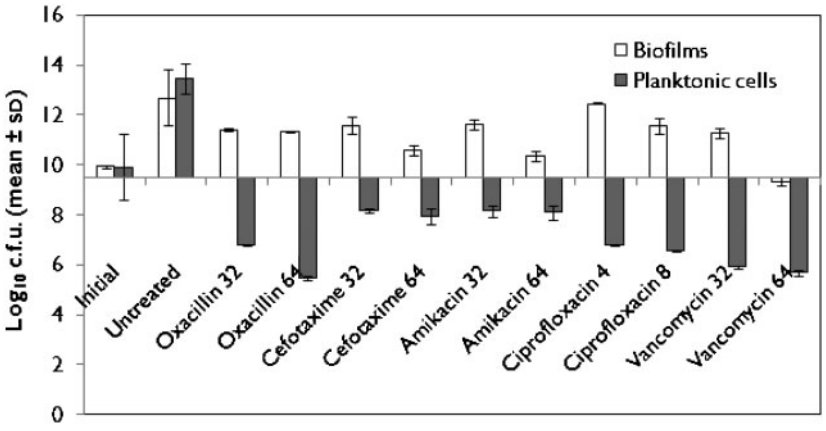


Fig. 1. Susceptibility of *S. aureus* ATCC 29213 biofilms and planktonic cells to oxacillin (32 and 64 μg ml^{-1}), cefotaxime (32 and 64 μg ml^{-1}), amikacin (32 and 64 μg ml^{-1}), ciprofloxacin (4 and 8 μg ml^{-1}) and vancomycin (32 and 64 μg ml^{-1}). The antibiotic concentrations chosen represent MIC multiples corresponding to clinically achievable levels of the antibiotic and a twofold higher multiple. Data are represented as log_{10} c.f.u. ml^{-1} for planktonic cells and log_{10} c.f.u. per membrane for biofilms. Experiments were performed in duplicate. Error bars indicate standard deviation.

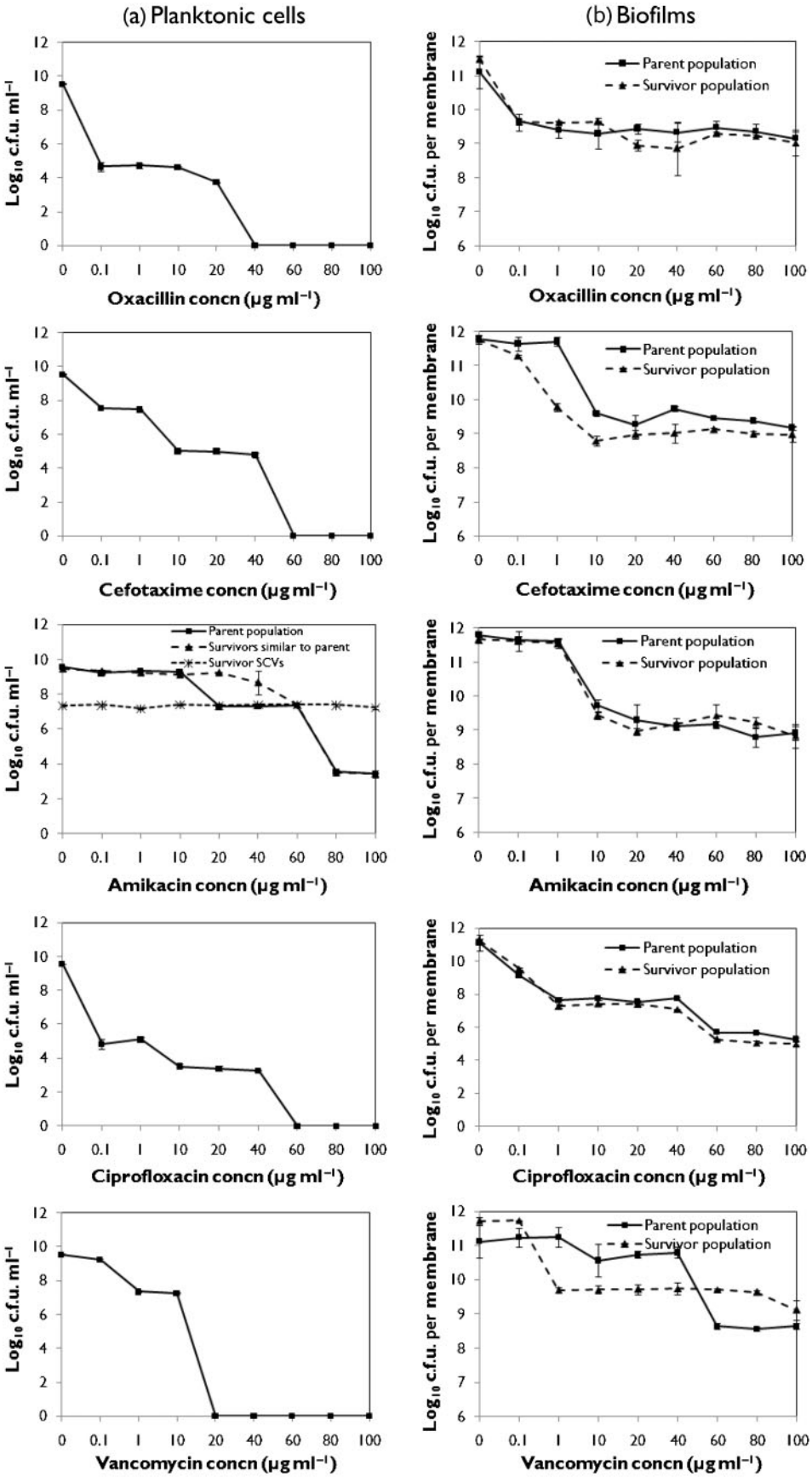
Oxacillin, a β-lactam antibiotic, effectively eliminated planktonic cells and no persisters were detected (Fig. 2). As the antibiotic concentration increased, SCVs were observed among the survivors but a higher concentration (40 μg ml^{-1}) completely eliminated the entire planktonic population including the SCVs. These SCVs were transient and reverted to the parental strain upon subsequent culture on blood agar. With reversion, the susceptibility returned to the MIC and MBC of the parental strain. Reversion of *S. aureus* SCVs to wild-type has been reported in many studies (Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). Dose-dependent treatment of biofilms with oxacillin resulted in an initial reduction followed by a plateau and a fraction of cells (1.86 %) survived, even at higher concentrations of the antibiotic (Fig. 2). This type of killing kinetics is a distinctive characteristic of persisters (Brooun *et al.*, 2000; Spoering & Lewis, 2001). The plateau observed at increasing oxacillin concentrations indicated that persisters are not vulnerable to killing by this antibiotic (Brooun *et al.*, 2000; Spoering & Lewis, 2001). Persisters are not mutants, and reculturing a population of persisters produces a wild-type with a new population of persisters (Bigger, 1944; Brooun *et al.*, 2000). We confirmed this by examining the sensitivity of cells obtained from biofilms after treatment with the highest concentration (100 μg ml^{-1}) and re-exposing the biofilms formed by these survivor cells to the dose-response killing with oxacillin. A graph similar to that for the wild-type parental strain was obtained (Fig. 2), confirming the 'transient' resistance phenotype of these cells. No SCVs were observed in biofilms following exposure to oxacillin.

As with oxacillin, dose-dependent treatment of planktonic cultures with another β-lactam antibiotic, cefotaxime, eradicated the entire population and no persisters were detected (Fig. 2). Transient SCVs observed following antibiotic treatment were killed at a higher cefotaxime concentration (60 μg ml^{-1}). In contrast, the killing kinetics of biofilms with cefotaxime again indicated the presence of persisters – the majority of cells in the biofilm died, except for a fraction (0.24 %) that persisted, even at higher cefotaxime concentrations (Fig. 2). These persisters represented a transient resistant phenotype and reverted to a killing curve resembling that of the wild-type parent upon re-exposure to cefotaxime. No SCVs were observed in biofilms following exposure to cefotaxime.

Thus *S. aureus* biofilms survived the action of oxacillin and cefotaxime due to the presence of persisters. SCVs were not observed in biofilms. In contrast to biofilms, no persisters were detected in planktonic cultures and the transient SCVs observed in these cultures following exposure to the two antibiotics were eliminated at higher antibiotic concentrations.

Unlike oxacillin and cefotaxime, treatment of planktonic cells with amikacin, an aminoglycoside antibiotic, resulted in a distinct killing curve with two survivor subpopulations: the persisters, which represented a transient resistance phenotype and reverted to a killing curve similar to that of the wild-type parent upon re-exposure to amikacin; and the stable SCVs, which were highly resistant to amikacin upon re-exposure (Fig. 2). Compared with the wild-type, the colonies of this SCV were approximately 10 times smaller, non-pigmented, non-haemolytic, clumping factor negative, weakly positive for coagulase and negative for DNase. The SCV was stable and did not revert to the wild-type after 10 repeat subcultures on blood agar. The MIC and MBC values of amikacin for this variant were 64 and 128 μg ml^{-1}, respectively, which were 32-fold and 4-fold higher than those of the wild-type. It has been

Fig. 2. Dose-dependent killing of *S. aureus* ATCC 29213 planktonic cells (a) and biofilms (b) by oxacillin, cefotaxime, amikacin, ciprofloxacin and vancomycin. Cells were treated with each antibiotic at a concentration of 0–100 μg ml^{-1} for 48 h at 37 °C. Survivors, if any, were re-exposed to dose-dependent killing by the same antibiotic that was used to isolate them. The limit of detection was 100 c.f.u. Experiments were performed in duplicate. Error bars indicate standard deviation.


(a) Planktonic cells
(b) Biofilms
Log10 c.f.u. ml−1
Log10 c.f.u. per membrane
Parent population
Survivor population
Survivors similar to parent
Survivor SCVs
Oxacillin concn (μg ml−1)
Cefotaxime concn (μg ml−1)
Amikacin concn (μg ml−1)
Ciprofloxacin concn (μg ml−1)
Vancomycin concn (μg ml−1)

suggested that such properties of the SCV phenotype are due to reduced electron transport owing to defects in biosynthesis of menadione, haemin or thiamine (Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). In the present study, the SCV was found to be auxotrophic for menadione, and growth around the discs containing menadione (1.5 μg) showed increased colony size, pigmentation and haemolysis. The adherence capacity of this SCV to polystyrene microtitre plates ($A_{490}=1.70\pm0.40$, mean ± SD) was significantly ($P<0.05$) higher than that of the wild-type parent ($A_{490}=0.82\pm0.28$, mean ± SD). In contrast to the planktonic cultures, only one type of survivor population, the persisters, was obtained upon exposure of biofilms to amikacin. No SCVs were observed. Nevertheless, the percentage of survivors in biofilms (0.15 ± 0.07 %) following exposure to 100 μg amikacin ml^{-1} was significantly ($P<0.05$) higher in comparison with planktonic cells (0.00009 ± 0.00003 %).

Our results suggest that the increased resistance of *S. aureus* biofilms to amikacin is largely dependent on the presence of persister cells. Biofilms harbour a greater number of persisters than the planktonic culture. Moreover, planktonic persisters, even if they exist, are liable to be eliminated by the immune system *in vivo*, whereas biofilm persisters are protected by the exopolysaccharide matrix (Lewis, 2007). SCVs were not observed in biofilms following exposure to amikacin. However, stable SCVs, observed in planktonic cells following exposure to amikacin, had a significantly higher biofilm-forming capacity. This increased adherence capacity of SCVs may explain the frequent isolation of SCVs from biofilm-associated infections, whilst the intrinsic resistance of these variants may in turn contribute to the antibiotic resistance of the biofilms thus formed.

Dose-dependent treatment of planktonic cells with ciprofloxacin, a fluoroquinolone, eliminated the entire population and no persisters were detected (Fig. 2). Transient SCVs observed were killed at a higher concentration (60 μg ml^{-1}). In contrast, treatment of biofilms with ciprofloxacin resulted in two survivor subpopulations: persisters, which represented a transient resistance phenotype and reverted to a killing curve similar to that of the wild-type parent upon re-exposure to ciprofloxacin; and transient SCVs, which reverted to the wild-type on subsequent culture, with MIC and MBC values the same as those of the parental strain (Fig. 2). Thus the resistance of biofilms to fluoroquinolones such as ciprofloxacin is again mediated by persisters. SCVs were observed in both planktonic cultures and biofilms following ciprofloxacin treatment and hence were not specifically associated with biofilm resistance.

Treatment with vancomycin, a glycopeptide antibiotic, again effectively killed the planktonic population and no persisters were detected (Fig. 2). As the antibiotic concentration increased, transient SCVs were observed but a higher vancomycin concentration (20 μg ml^{-1}) killed the entire planktonic population including the SCVs. Dose-dependent killing of biofilms by vancomycin indicated the presence of persisters – most of the cells in the biofilm died except for a fraction (0.4 %) that survived, even at higher concentrations (Fig. 2). These persisters represented a transient resistant phenotype and reverted to a killing curve resembling that of the wild-type parent upon re-exposure to vancomycin. No SCVs were observed in biofilms upon treatment with vancomycin.

In conclusion, our results suggest that, in contrast to planktonic cultures, which either do not harbour persisters (as observed following exposure to oxacillin, cefotaxime, ciprofloxacin and vancomycin) or harbour only a small number (as observed following exposure to amikacin), *S. aureus* biofilms harbour large numbers of persister cells. To our knowledge, this is the first report indicating the role of persisters in conferring drug resistance to *S. aureus* biofilms. This study also indicates that SCVs, although not specifically associated with *S. aureus* biofilms, have an increased biofilm-forming capacity and this may explain the frequent isolation of SCVs from biofilm-associated infections. The enhanced resistance of SCVs may in turn contribute to the adverse therapeutic outcome in these infections. However, these findings, carried out with one reference strain, demand further studies using more isolates.

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REFERENCES

Acar, J. F., Goldstein, F. W. & Lagrange, P. (1978). Human infections caused by thiamine- or menadione-requiring *Staphylococcus aureus*. *J Clin Microbiol* **8**, 142–147.

Allegrucci, M. & Sauer, K. (2007). Characterization of colony morphology variants isolated from *Streptococcus pneumoniae* biofilms. *J Bacteriol* **189**, 2030–2038.

Amorena, B., Gracia, E., Monzón, M., Leiva, J., Oteiza, C., Pérez, M., Alabart, J. L. & Hernández-Yago, J. (1999). Antibiotic susceptibility assay for *Staphylococcus aureus* in biofilms developed *in vitro*. *J Antimicrob Chemother* **44**, 43–55.

Anderl, J. N., Franklin, M. J. & Stewart, P. S. (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother* **44**, 1818–1824.

Ashby, M. J., Neale, J. E., Knott, S. J. & Critchley, I. A. (1994). Effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. *J Antimicrob Chemother* **33**, 443–452.

Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* **305**, 1622–1625.

Bayston, R., Ashraf, W. & Smith, T. (2007). Triclosan resistance in methicillin-resistant *Staphylococcus aureus* expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. *J Antimicrob Chemother* **59**, 848–853.

Bendouah, Z., Barbeau, J., Hamad, W. A. & Desrosiers, M. (2006). Biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. *Otolaryngol Head Neck Surg* **134**, 991–996.

Besier, S., Smaczny, C., von Mallinckrodt, C., Krahl, A., Ackermann, H., Brade, V. & Wichelhaus, T. A. (2007). Prevalence and clinical significance of *Staphylococcus aureus* small-colony variants in cystic fibrosis lung disease. *J Clin Microbiol* **45**, 168–172.

Bigger, J. W. (1944). Treatment of staphylococcal infections with penicillin. *Lancet* **244**, 497–500.

Brooun, A., Liu, S. & Lewis, K. (2000). A dose–response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* **44**, 640–646.

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D. & Buret, A. (1999). The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* **37**, 1771–1776.

Chuard, C., Vaudaux, P. E., Proctor, R. A. & Lew, D. P. (1997). Decreased susceptibility to antibiotic killing of a stable small colony variant of *Staphylococcus aureus* in fluid phase and on fibronectin-coated surfaces. *J Antimicrob Chemother* **39**, 603–608.

CLSI (2006). *Performance Standards for Antimicrobial Susceptibility Testing; 16th Informational Supplement*. CLSI document M100-S16. Wayne, PA: Clinical and Laboratory Standards Institute.

Costerton, J. W., Stewart, P. S. & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.

Drenkard, E. & Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**, 740–743.

Fux, C. A., Costerton, J. W., Stewart, P. S. & Stoodley, P. (2005). Survival strategies of infectious biofilms. *Trends Microbiol* **13**, 34–40.

Gilbert, P., Allison, D. G. & McBain, A. J. (2002). Biofilms *in vitro* and *in vivo*: do singular mechanisms imply cross-resistance? *Symp Ser Soc Appl Microbiol* **92**, 98S–110S.

Haussler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B. & Steinmetz, I. (2003). Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol* **52**, 295–301.

Higashi, J. M. & Sullam, P. M. (2006). *Staphylococcus aureus* biofilms. In *Biofilms, Infection, and Antimicrobial Therapy*, pp. 81–108. Edited by J. L. Pace, M. E. Rupp & R. G. Finch. Boca Raton, FL: Taylor & Francis.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y. & Lewis, K. (2004a). Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230**, 13–18.

Keren, I., Shah, D., Spoering, A., Kaldalu, N. & Lewis, K. (2004b). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol* **186**, 8172–8180.

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**, 48–56.

Lewis, K., Spoering, A. L., Kaldalu, N., Keren, I. & Shah, D. (2006). Persisters: specialized cells responsible for biofilm tolerance to antimicrobial agents. In *Biofilms, Infection, and Antimicrobial Therapy*, pp. 241–256. Edited by J. L. Pace, M. E. Rupp & R. G. Finch. Boca Raton, FL: Taylor & Francis.

Mah, T. F. & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34–39.

Miles, A. A., Misra, S. S. & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *J Hyg (Lond)* **38**, 732–749.

Muli, F. W. & Struthers, J. K. (1998). The growth of *Gardnerella vaginalis* and *Lactobacillus acidophilus* in Sorbarod biofilms. *J Med Microbiol* **47**, 401–405.

Proctor, R. A. & Peters, G. (1998). Small colony variants in staphylococcal infections: diagnostic and therapeutic implications. *Clin Infect Dis* **27**, 419–422.

Proctor, R. A., van Langevelde, P., Kristjansson, M., Maslow, J. N. & Arbeit, R. D. (1995). Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin Infect Dis* **20**, 95–102.

Proctor, R. A., Kahl, B., von Eiff, C., Vaudaux, P. E., Lew, D. P. & Peters, G. (1998). Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. *Clin Infect Dis* **27** (Suppl. 1), S68–S74.

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M. & Peters, G. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* **4**, 295–305.

Roberts, M. E. & Stewart, P. S. (2005). Modelling protection from antimicrobial agents in biofilms through the formation of persister cells. *Microbiology* **151**, 75–80.

Spoering, A. L. & Lewis, K. (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **183**, 6746–6751.

Stewart, P. S. & Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135–138.

Vuong, C., Saenz, H. L., Gotz, F. & Otto, M. (2000). Impact of the *agr* quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J Infect Dis* **182**, 1688–1693.

Walters, M. C., III, Roe, F., Bugnicourt, A., Franklin, M. J. & Stewart, P. S. (2003). Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* **47**, 317–323.

Williams, I., Venables, W. A., Lloyd, D., Paul, F. & Critchley, I. (1997). The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus*. *Microbiology* **143**, 2407–2413.

Yao, J. D. C. & Moellering, R. C., Jr (2003). Antibacterial agents. In *Manual of Clinical Microbiology*, 8th edn, pp. 1039–1073. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, J. H. Jorgensen & R. H. Yolken. Washington, DC: American Society for Microbiology.