

# Enhanced production of exopolysaccharide matrix and biofilm by a menadione-auxotrophic *Staphylococcus aureus* small-colony variant

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The role of *Staphylococcus aureus* small-colony variants (SCVs) in the pathogenesis of biofilm-associated infections remains unclear. This study investigated the mechanism behind increased biofilm-forming potential of a menadione-auxotrophic *Staphylococcus aureus* SCV compared with the wild-type parental strain, as recently reported by our laboratory. SCVs displayed an autoaggregative phenotype, with a greater amount of polysaccharide intercellular adhesin (PIA), significantly reduced tricarboxylic acid cycle activity and a decreased susceptibility to aminoglycosides and cell-wall inhibitors compared with wild-type. The biofilms formed by the SCV were highly structured, consisting of large microcolonies separated by channels, and contained more biomass as well as significantly more PIA than wild-type biofilms. The surface hydrophobicity of the two phenotypes was similar. Thus, the autoaggregation and increased biofilm-forming capacity of menadione-auxotrophic *Staphylococcus aureus* SCVs in this study was related to the enhanced production of PIA in these variants.

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## INTRODUCTION

*Staphylococcus aureus* small-colony variants (SCVs) represent a naturally occurring, slow-growing, phenotypically variant subpopulation with an increased resistance to antibiotics (Proctor *et al.*, 1998). These variants display a number of characteristics that are atypical of wild-type *Staphylococcus aureus*: small, non-pigmented, non-haemolytic colonies; reduced coagulase and DNase production; failure to utilize mannitol; and increased resistance to aminoglycosides as well as cell-wall inhibitors (Acar *et al.*, 1978; Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). The altered phenotypic traits of SCVs are due to defects in the electron transport chain as a result of a deficiency in haemin, thiamine or menadione biosynthesis (Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). SCVs also exhibit increased expression of protein A (Kahl *et al.*, 2005), surface adhesins (clumping factor and fibronectin-binding proteins) (Moisan *et al.*, 2006; Vaudaux *et al.*, 2002), alternative sigma factor (*sigB*; Moisan *et al.*, 2006) and *Staphylococcus* accessory regulator (*sarA*; Moisan *et al.*, 2006), as well as glycolytic, fermentation and arginine deiminase pathways (Proctor *et al.*, 2006). However, the expression of  $\alpha$  toxin (Kahl *et al.*, 2005) and accessory gene regulator (*agr*) is downregulated (Moisan *et al.*, 2006) in these variants. SCVs may have a stable, inheritable colony type or a transient phenotype that reverts

to wild-type upon subculture (Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). Prolonged exposure to aminoglycosides and cationic peptides may select for these variants *in vivo* (Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006; von Eiff *et al.*, 1997).

SCVs have been implicated in many persistent and antibiotic-resistant infections. *Staphylococcus aureus* SCVs may occur in cases of cystic fibrosis, soft-tissue infections, osteomyelitis, arthritis, sinusitis, brain abscess and many 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). The persistent course of many of these infections has been linked to the formation of biofilms (Costerton *et al.*, 1999; Fux *et al.*, 2005). The relationship between *Staphylococcus aureus* SCVs and biofilm phenotype is unclear, but their similar characteristics suggest that they may have an analogous physiology (Higashi & Sullam, 2006). Both are slow-growing and more resistant to antimicrobials, and the infections with which they are associated overlap considerably. Once adhered, SCVs are almost completely resistant to antibiotics (Chuard *et al.*, 1997). Williams *et al.* (1997) reported the isolation of SCVs from adherent *Staphylococcus 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).

**Abbreviations:** CLSM, confocal laser-scanning microscopy; EPS, exopolysaccharide; HRP, horseradish peroxidase; MBC, minimum bactericidal concentration; PIA, polysaccharide intercellular adhesin; SCV, small-colony variant; TCA, tricarboxylic acid; WGA, wheatgerm agglutinin.

A recent study from our laboratory reported a significantly increased biofilm-forming ability of a menadione-auxotrophic *Staphylococcus aureus* SCV compared with the wild-type parental strain (Singh *et al.*, 2009). The enhanced biofilm-forming potential of SCVs has been linked to an increased production of polysaccharide intercellular adhesin (PIA), the exopolysaccharide (EPS) matrix, in *Staphylococcus epidermidis* (Al Laham *et al.*, 2007) and an increased surface hydrophobicity (Drenkard & Ausubel, 2002; Haussler *et al.*, 2003), as well as EPS production (*psl* and *pek*; Kirisits *et al.*, 2005; Starkey *et al.*, 2009), in *Pseudomonas aeruginosa*. This study was therefore planned to elucidate the mechanism behind the increased biofilm-forming potential of menadione-dependent *Staphylococcus aureus* SCVs.

## METHODS

**Bacterial strains.** *Staphylococcus aureus* ATCC 29213 and its stable SCV, obtained by exposing the parental strain to 100 µg amikacin ml<sup>-1</sup> for 24 h (Singh *et al.*, 2009), were used in this study. The phenotypic properties of this SCV have been characterized and reported previously (Singh *et al.*, 2009). Compared with the wild-type parental strain, colonies of this SCV were approximately 10 times smaller, non-pigmented, non-haemolytic, clumping factor negative, weakly positive for coagulase, negative for DNase and auxotrophic for menadione (Singh *et al.*, 2009). It was stable and did not revert back to the wild-type after 10 repeated subcultures on blood agar (Singh *et al.*, 2009). *Staphylococcus aureus* ATCC 25923 was used as a quality control strain for antibiotic susceptibility testing. *Staphylococcus aureus* ATCC 12598 and *Staphylococcus epidermidis* ATCC 12228 were used as positive and negative controls, respectively, for biofilm formation and PIA production. All strains were preserved in semi-solid agar butts at 4 °C and in brain–heart infusion broth with 15% glycerol at -70 °C until further use.

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

**Determination of MICs and minimum bactericidal concentrations (MBCs).** MICs of antibiotics against the wild-type parental strain, the SCV and the SCV grown in menadione-supplemented (1.5 µg ml<sup>-1</sup>) medium were determined by the broth macrodilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). Ten microlitre drops from tubes containing MIC and higher antibiotic concentrations were plated on nutrient agar and incubated at 37 °C for 24 h to determine the minimum bactericidal concentration (MBC).

**Detection of the autoaggregative phenotype.** Colonies of the wild-type parental strain (grown on blood agar) and SCV (grown on blood agar alone and blood agar supplemented with 1.5 µg menadione ml<sup>-1</sup>) were picked after incubation for 24 h at 37 °C and suspended in normal saline, tryptic soy broth (TSB), Luria broth (LB) and Mueller–Hinton broth (MHB) for detection of the autoaggregative phenotype.

**Detection of PIA in planktonic cultures by fluorescence microscopy.** PIA, also known as poly-*N*-acetylglucosamine, was detected using wheatgerm agglutinin (WGA), a lectin that binds to the sugar residues of PIA. Colonies of the wild-type parental strain

(grown on blood agar) and SCV (grown on blood agar alone and blood agar supplemented with 1.5 µg menadione ml<sup>-1</sup>) were suspended in normal saline and 20 µl drops of these suspensions were applied to glass slides, air dried and heat fixed. The slides were stained with WGA conjugated to Oregon Green (WGA–Green, 0.1 mg ml<sup>-1</sup>) for 15 min in the dark, washed twice with PBS (0.1 M, pH 7.2) and viewed under an Olympus BX51 fluorescence microscope using a ×10 objective lens. Experiments were performed in quadruplicate and four randomly chosen spots were analysed per sample.

**Estimation of biofilm formation on polystyrene and glass.** A significantly ( $P < 0.05$ ) increased biofilm-forming capacity of SCVs on flat-bottomed, 96-well polystyrene microtitre plates compared with the wild-type parental strain has been reported previously (Singh *et al.*, 2009). Biofilm formation by the wild-type, SCV and SCV grown in menadione-supplemented (1.5 µg ml<sup>-1</sup>) medium was also compared on glass using a tube adherence assay according to the method of Heilmann *et al.* (1996). Overnight cultures of the wild-type parental strain grown in 5 ml TSB with 1% glucose (TSBG), SCV grown in TSBG alone and SCV grown in TSBG supplemented with 1.5 µg menadione ml<sup>-1</sup> were adjusted to an OD<sub>600</sub> of 0.3, and 200 µl of this culture was incubated in glass tubes containing 4.8 ml TSBG for 24 h at 37 °C. The biofilms formed were washed twice with PBS, fixed with 5 ml 95% ethanol for 15 min and stained with 0.1% safranin for 30 s. The excess stain was rinsed off in water, and the stain in biofilms was eluted with 5 ml 30% glacial acetic acid and its A<sub>490</sub> was measured. Experiments were performed in quadruplicate.

### Confocal laser-scanning microscopy (CLSM) of biofilms.

Overnight cultures of the wild-type parental strain grown in TSBG, SCV grown in TSBG alone and SCV grown in TSBG supplemented with 1.5 µg menadione ml<sup>-1</sup> were adjusted to an OD<sub>600</sub> of 0.3. Three millilitres of this culture was incubated in 35 mm dishes for 24 h at 37 °C. The biofilms formed were washed twice with PBS, incubated with WGA–Green (0.1 mg ml<sup>-1</sup>) in the dark for 15 min and then washed twice with PBS. CLSM was performed with an LSM 510 META attached to an Axioplan II microscope using a ×10 immersion objective according to the method of Jefferson & Cerca (2006). HFT UV/488/543/633 was used as the excitation beam splitter. Bacterial cells were detected by refraction of light in the red spectrum using an LP650 filter and WGA was detected by fluorescence in the green spectrum using a BP505–530 filter by single-channel analysis with NFT 545 as the beam splitter. Image analysis was carried out using z-series image stacks from four randomly chosen spots of each biofilm and the biofilm architecture and mean thickness (LSM image browser version 4.2.0.121) as well as integrated densities of green and red fluorescence (ImageJ version 1.41o) were determined.

### Semi-quantitative estimation of PIA in biofilms by dot blots.

PIA was extracted from biofilms according to the method of Jefferson & Cerca (2006). Briefly, the biofilms were grown on 35 mm dishes as described previously, scraped off and suspended in PBS. The suspensions were adjusted to an OD<sub>600</sub> of 1.5, centrifuged at 9296 g for 5 min and the pellets were suspended in 50 µl 0.5 M EDTA (pH 8.0). The samples were incubated twice at 100 °C for 5 min with incubation on ice for 5 min in between and then centrifuged at 9296 g for 5 min. The supernatants were treated with proteinase K (2 mg ml<sup>-1</sup>) for 1 h at 60 °C and then incubated at 80 °C for 30 min to inactivate the proteinase K. The samples were stored at -20 °C.

PIA in biofilms of the wild-type, SCV and SCV grown in TSBG supplemented with 1.5 µg menadione ml<sup>-1</sup> was compared semi-quantitatively by dot blot. A series of twofold dilutions of PIA extract were prepared in Tris-buffered saline (TBS) and 5 µl of each suspension was spotted onto nitrocellulose membrane. The membrane was air dried, moistened with TBS and blocked in 50 ml

blocking solution comprising 1% (w/v) BSA in TBST (TBS plus 0.05% Tween 20) for 1 h. Fifty millilitres of WGA-horseradish peroxidase conjugate (WGA-HRP, 130 ng ml<sup>-1</sup>) plus 1% BSA in TBST was added and the membrane was incubated for 30 min. The membrane was then washed three times for 5 min each in TBST, incubated in 50 ml substrate (0.5 mg diaminobenzidine ml<sup>-1</sup> plus 1 µl H<sub>2</sub>O<sub>2</sub> ml<sup>-1</sup> in TBS) for 15 min and rinsed in distilled water. The experiment was performed in quadruplicate.

**Quantification of PIA in biofilms by ELISA.** PIA in biofilms was quantified by ELISA according to the method of Cramton *et al.* (2001). Overnight cultures of the wild-type parental strain grown in TSBG, SCV grown in TSBG alone and SCV grown in TSBG supplemented with 1.5 µg menadione ml<sup>-1</sup> were adjusted to an OD<sub>600</sub> of 0.3, and 200 µl of this culture was incubated per well in flat-bottomed, 96-well polystyrene microtitre plates for 24 h at 37 °C. The resulting biofilms were washed twice with PBS and fixed with 200 µl 95% ethanol for 15 min. An aliquot of 100 µl blocking solution [1%, w/v, BSA in PBST (PBS plus 0.05% Tween 20)] was added per well and the microtitre plate was incubated for 1 h at 37 °C. The blocking solution was then removed, 100 µl WGA-HRP (75 ng ml<sup>-1</sup>) in 1% BSA in PBST was added per well and the microtitre plate was incubated for 30 min at 37 °C. The wells were washed three times for 5 min each in PBST. Substrate [100 µl 0.5 mg *ortho*-phenylenediamine ml<sup>-1</sup> plus 5 µl H<sub>2</sub>O<sub>2</sub> ml<sup>-1</sup> in citrate buffer (pH 5.0)] was then added, the microtitre plate was incubated for 5 min at 37 °C and the reaction was stopped by the addition of 50 µl 12.5% H<sub>2</sub>SO<sub>4</sub> per well. The A<sub>490</sub> was measured. Experiments were performed in quadruplicate.

**Quantification of aconitase activity.** The activity of aconitase was quantified according to the method of Kennedy *et al.* (1983). Overnight cultures of the wild-type parental strain grown in LB, SCV grown in LB alone and SCV grown in LB supplemented with 1.5 µg menadione ml<sup>-1</sup> were adjusted to an OD<sub>600</sub> of 1, centrifuged at 9296 g for 5 min and suspended in lysis buffer containing 100 mM Tris/HCl (pH 8.0), 145 mM NaCl and 200 µg lysostaphin ml<sup>-1</sup>. The suspensions were incubated for 15 min at 37 °C and centrifuged at 20916 g for 30 min at 4 °C. An aliquot of 100 µl of the cell lysate was added to 900 µl assay buffer [100 mM Tris/HCl (pH 8.0), 50 mM trisodium citrate] and incubated for 5 min at 37 °C. The amount of aconitate produced was quantified by measuring the A<sub>240</sub>. A molar absorption coefficient of 3.6 mM<sup>-1</sup> cm<sup>-1</sup> was used and 1 U aconitase was defined as the enzyme activity catalysing the formation of 1 µmol aconitate min<sup>-1</sup>. Experiments were performed in quadruplicate.

**Determination of cell-surface hydrophobicity.** Cell-surface hydrophobicity was determined by a microbial adhesion to hydrocarbons assay (Perez *et al.*, 1998). Overnight cultures of the wild-type parental strain grown in LB, SCV grown in LB alone and SCV grown in LB supplemented with 1.5 µg menadione ml<sup>-1</sup> were pelleted by centrifugation at 9296 g for 5 min, washed twice with phosphate buffer (0.1 M, pH 7.2) and finally diluted in phosphate buffer to an OD<sub>600</sub> of 0.5. Two millilitres of this suspension was mixed with 400 µl xylene and vortexed for 2 min. After 30 min, the aqueous phase was collected carefully and its OD<sub>600</sub> was determined. The OD<sub>600</sub> of the aqueous phase relative to the initial suspension was taken as a measure of cell-surface hydrophobicity. Experiments were performed in quadruplicate.

**Statistical analysis.** One-way analysis of variance with Bonferroni's post-hoc test was used for statistical comparisons.

## RESULTS AND DISCUSSION

*Staphylococcus aureus* SCVs are frequently isolated from biofilm-based infections including cystic fibrosis, osteo-

myelitis and sinusitis as well as those associated with indwelling medical devices (Besier *et al.*, 2007; Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). The SCV and biofilm phenotype have many similar characteristics – both are slow-growing, have an analogous physiology and are more resistant to antimicrobials (Higashi & Sullam, 2006). Williams *et al.* (1997) even suggested that biofilm formation in *Staphylococcus aureus* correlates with this mode of growth. However, the relationship between the *Staphylococcus aureus* SCV and biofilm phenotype remains unclear. A previous study from our laboratory reported the enhanced biofilm-forming capacity of *Staphylococcus aureus* SCVs in comparison with the wild-type parental strain (Singh *et al.*, 2009). This study was therefore planned to elucidate the mechanism behind the increased biofilm-forming potential of *Staphylococcus aureus* SCVs.

### Determination of MICs and MBCs

The MIC and MBC of amikacin for the SCV were 64 and 128 µg ml<sup>-1</sup>, respectively, 32-fold and 4-fold higher than those for the wild-type (Singh *et al.*, 2009). We compared the susceptibility of this variant with another aminoglycoside (gentamicin) as well as β-lactam (oxacillin and cefotaxime), glycopeptide (vancomycin) and fluoroquinolone (ciprofloxacin) antibiotics. The MIC and MBC of gentamicin for the SCV were eightfold and fourfold higher than those for the wild-type, respectively (Table 1). The susceptibility to oxacillin, cefotaxime and vancomycin was also reduced compared with that of the wild-type parental strain (Table 1). However, the susceptibility of the SCV to ciprofloxacin was similar to that of the wild-type parental strain (Table 1). These findings are in agreement with previous reports regarding the reduced susceptibility of SCVs to aminoglycosides and cell-wall inhibitors (Proctor & Peters, 1998; Proctor *et al.*, 1995, 1998, 2006). Furthermore, the MICs and MBCs of antibiotics for the culture obtained by growing the SCV in a menadione-supplemented medium were found to be similar to those for the wild-type, indicating that the SCV phenotype as well as the associated resistance to β-lactams and aminoglycosides was the result of auxotrophy for menadione.

### Detection of the autoaggregative phenotype

The colonies of the wild-type parental strain and the SCV, picked from a blood agar plate after incubation for 24 h at 37 °C, were suspended in normal saline, TSB, LB and MHB. The wild-type colonies were easy to suspend and gave a homogeneous suspension. In contrast, SCV colonies showed increased clumping and autoaggregation and did not give a homogeneous suspension, even after vigorous shaking for several minutes. SCVs of *Staphylococcus epidermidis*, *P. aeruginosa* and *Streptococcus pneumoniae* have also been reported to autoaggregate (Al Laham *et al.*, 2007; Allegrucci & Sauer, 2007; Drenkard & Ausubel, 2002; Haussler *et al.*, 2003; Kirisits *et al.*, 2005; Starkey *et al.*, 2009). However, when the SCV was cultured on blood agar

**Table 1.** MICs and MBCs of antibiotics for *Staphylococcus aureus* ATCC 29213 menadione-auxotrophic SCV and wild-type parental strain as determined by the broth macrodilution method

S, Sensitive; I, intermediate; R, resistant.

Antibiotic	Menadione-auxotrophic SCV		Wild-type parental strain	
	MIC ( $\mu\text{g ml}^{-1}$ )	MBC ( $\mu\text{g ml}^{-1}$ )	MIC ( $\mu\text{g ml}^{-1}$ )	MBC ( $\mu\text{g ml}^{-1}$ )
Oxacillin	2 (S)	8	0.25 (S)	2
Cefotaxime	4 (S)	64	1 (S)	8
Amikacin	64 (R)	128	2 (S)	32
Gentamicin	8 (I)	64	1 (S)	16
Ciprofloxacin	0.5 (S)	2	0.5 (S)	2
Vancomycin	2 (S)	8	1 (S)	4

supplemented with  $1.5 \mu\text{g ml}^{-1}$  menadione, the colonies showed a phenotype similar to that of the wild-type parent – increased colony size, pigmentation and haemolysis, and no autoaggregation upon suspension in normal saline, TSB, LB and MHB. Thus, the altered phenotype as well as the autoaggregation was due to menadione deficiency in these variants.

#### Detection of PIA in planktonic cultures by fluorescence microscopy

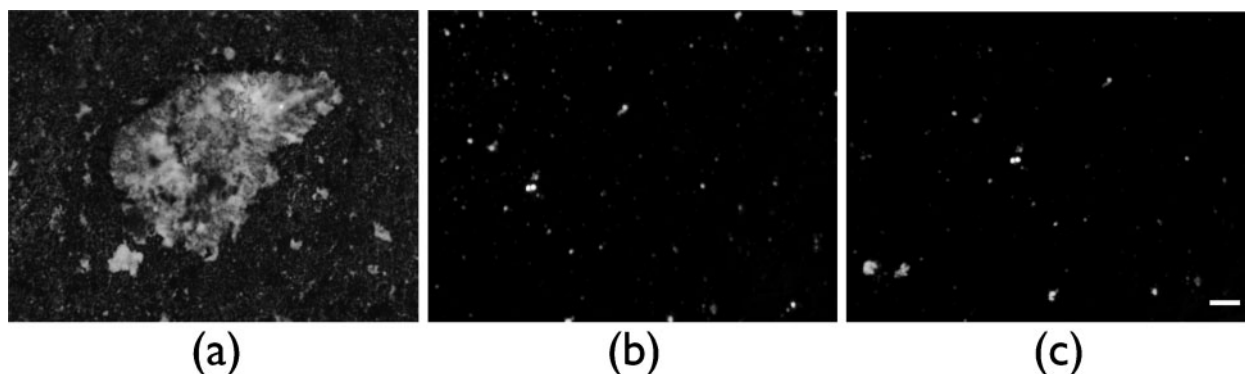
Fluorescence microscopy analysis of the planktonic cultures revealed that SCVs formed large cell clusters, containing substantial amounts of green fluorescence representing PIA, the EPS matrix in *Staphylococcus*, when suspended in liquid medium (Fig. 1). Moreover, the amount of PIA was higher in planktonic SCVs than in the wild-type (Fig. 1), indicating increased production of EPS matrix in these variants. However, when the SCV was grown in the presence of  $1.5 \mu\text{g ml}^{-1}$  menadione, the resulting culture revealed a phenotype similar to that of the wild-type parent (Fig. 1).

#### Estimation of biofilm formation on polystyrene and glass

The biofilm-forming capacity of SCVs on flat-bottomed, 96-well polystyrene microtitre plates has been reported previously to be significantly ( $P < 0.05$ ) increased compared with that of wild-type (Singh *et al.*, 2009). Biofilm formation by the SCV and wild-type parental strain was also compared on glass surfaces using a tube adherence assay. The adherence capacity (mean  $\pm$  SD) of the SCV ( $A_{490} = 0.325 \pm 0.063$ ) on glass tubes was significantly ( $P < 0.01$ ) higher than that of the wild-type parental strain ( $A_{490} = 0.105 \pm 0.006$ ). However, the adherence (mean  $\pm$  SD) of the culture obtained by growing the SCV in a menadione-supplemented medium ( $A_{490} = 0.128 \pm 0.034$ ) was similar to that of the wild-type parental strain ( $P > 0.05$ ) but significantly reduced compared with that of the SCV itself ( $P < 0.05$ ).

#### CLSM of biofilms

PIA is considered to play a critical role in the development and maturation of *Staphylococcus* biofilms (O'Gara, 2007). This cell-surface-associated EPS, being



**Fig. 1.** Detection of PIA in planktonic cultures of *Staphylococcus aureus* ATCC 29213 menadione-auxotrophic SCV (a), wild-type parental strain (b) and a culture obtained by growing the SCV in a menadione-supplemented ( $1.5 \mu\text{g ml}^{-1}$ ) medium (c) by fluorescence microscopy. PIA was detected using WGA–Green. The SCV formed large cell clusters containing considerable amounts of PIA (green fluorescence). Bar,  $20 \mu\text{m}$ .

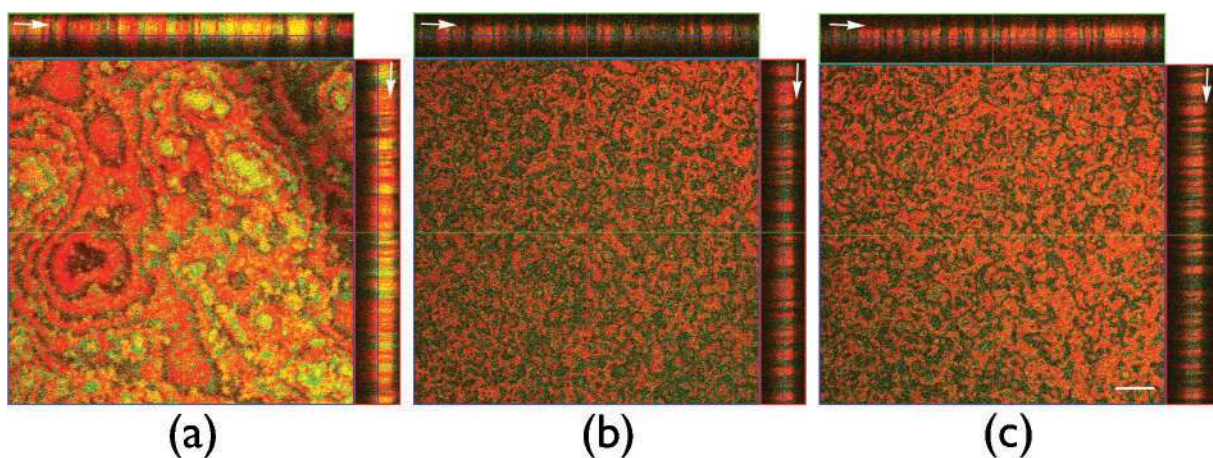
positively charged, acts as an intercellular adhesin and binds the negatively charged bacterial cells together via electrostatic interactions, resulting in increased cellular accumulation (O’Gara, 2007; Otto, 2008). PIA-over-producing mutants have been reported to form highly structured biofilms with dense microcolonies separated by large channels compared with the less-structured biofilms of isogenic *Staphylococcus aureus* parental strains (Jefferson, 2004). The biofilms formed by the *Staphylococcus aureus* SCV and wild-type parental strain were therefore compared for architecture, thickness, relative biomass and EPS matrix (PIA) by confocal microscopy. CLSM analysis indicated that the SCV formed highly structured biofilms, consisting of large microcolonies separated by channels (Fig. 2). Hollow regions were observed in the centre of many large cell clusters (Fig. 2), possibly due to the induction of autolysis as a result of oxygen limitation (Stewart *et al.*, 2007). In contrast, the biofilms of the wild-type parental strain were less structured, with smaller microcolonies and a reduced prevalence of channels (Fig. 2). The integrated green fluorescence density (mean  $\pm$  SD), representing PIA, was significantly higher ( $P < 0.05$ ) in the biofilms formed by the SCV ( $3.10 \times 10^7 \pm 1.03 \times 10^7$ ) than in those formed by the wild-type ( $1.60 \times 10^7 \pm 0.20 \times 10^7$ ). The integrated red fluorescence density (mean  $\pm$  SD), representing biomass, was also greater in the biofilms of the SCV ( $4.42 \times 10^7 \pm 1.66 \times 10^7$ ) than in those of the wild-type parental strain ( $2.76 \times 10^7 \pm 0.34 \times 10^7$ ), although this was not statistically significant ( $P > 0.05$ ). The net amount of PIA per bacterium (integrated green fluorescence density/integrated red fluorescence density) was also higher in the SCV biofilms (0.723 U) than in the wild-type biofilms (0.579 U). However, the thickness (mean  $\pm$  SD) of the

SCV biofilms ( $116.50 \pm 28.07 \mu\text{m}$ ) was similar ( $P > 0.05$ ) to that of the wild-type biofilms ( $139.28 \pm 28.34 \mu\text{m}$ ).

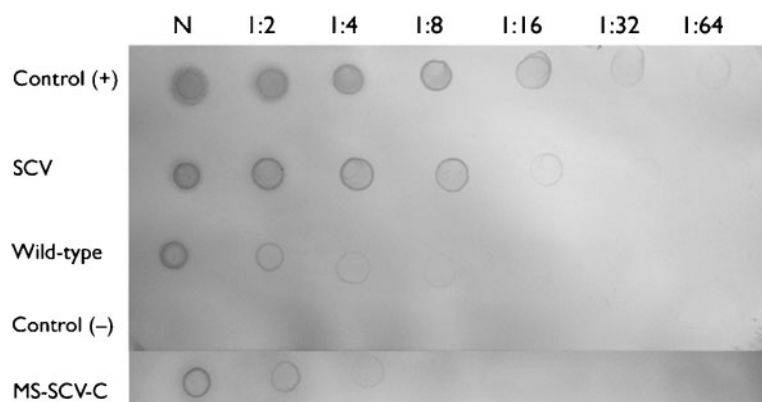
When the SCV was cultured in the presence of  $1.5 \mu\text{g ml}^{-1}$  menadione, the architecture of biofilms formed by the resulting cultures was similar to that of biofilms formed by the wild-type parent (Fig. 2). These biofilms were less structured, with a reduced prevalence of channels (Fig. 2) and an integrated green fluorescence density (PIA;  $1.68 \times 10^7 \pm 0.24 \times 10^7$ , mean  $\pm$  SD) similar to that of the wild-type biofilm ( $P > 0.05$ ) but significantly decreased compared with that of the SCV biofilms ( $P < 0.05$ ). The integrated red fluorescence density (biomass;  $2.85 \times 10^7 \pm 0.33 \times 10^7$ , mean  $\pm$  SD) in these biofilms was also similar to that in the wild-type biofilms ( $P > 0.05$ ) but was less than that in the SCV biofilms, although the difference was not statistically significant ( $P > 0.05$ ). The net amount of PIA per bacterium in these biofilms (0.587 U) was also similar to that in the wild-type biofilms but less than in the SCV biofilms. The thickness (mean  $\pm$  SD) of these biofilms was  $122 \pm 11.31 \mu\text{m}$ .

#### Estimation of PIA in biofilms by dot blot and ELISA

The production of PIA in the biofilms formed by the wild-type parental strain and SCV cultures was compared semi-quantitatively by dot blot and quantitatively by ELISA. In the dot blot, the PIA signal was detected up to a 1:64 dilution in the SCV, up to a 1:16 dilution in the wild-type and up to a 1:8 dilution in the culture obtained by growing the SCV in a menadione-supplemented medium (Fig. 3). In ELISA, the adherent SCVs produced significantly ( $P < 0.001$ ) more PIA ( $A_{490} = 1.99 \pm 0.09$ , mean  $\pm$  SD) than



**Fig. 2.** CLSM (orthogonal views) of biofilms formed by *Staphylococcus aureus* ATCC 29213 menadione-auxotrophic SCV (a), wild-type (b) and a culture obtained by growing the SCV in a menadione-supplemented ( $1.5 \mu\text{g ml}^{-1}$ ) medium (c). Bacterial cells were detected by refraction of light in the red spectrum, and PIA was detected by fluorescence in the green spectrum as a result of the binding of WGA–Green. The central image shows the horizontal ( $x$ – $y$ ) section. Upper and side panels represent the  $x$ – $z$  and  $y$ – $z$  planes, respectively. The lines in horizontal sections indicate the position of vertical sections and the lines in vertical sections indicate the position of horizontal sections. Arrows indicate the top of the biofilm. Bar,  $100 \mu\text{m}$ .



**Fig. 3.** Semi-quantitative estimation of PIA in the biofilms of *Staphylococcus aureus* ATCC 29213 menadione-auxotrophic SCV, wild-type parental strain and a culture obtained by growing the SCV in a menadione-supplemented ( $1.5 \mu\text{g ml}^{-1}$ ) medium (MS-SCV-C) by dot blot. PIA was detected using WGA-HRP. *Staphylococcus aureus* ATCC 12598 and *Staphylococcus epidermidis* ATCC 12228 were used as positive (+) and negative (-) controls, respectively. N, Neat (undiluted) extract.

the wild-type parental strain ( $1.23 \pm 0.08$ ). However, the PIA production in culture obtained by growing the SCV in a menadione-supplemented medium ( $1.20 \pm 0.09$ ) was similar to that of the wild-type ( $P > 0.05$ ) but significantly reduced in comparison with that of the SCV itself ( $P < 0.001$ ).

Thus, the increased biofilm-forming potential of the menadione-auxotrophic *Staphylococcus aureus* SCV as well as the highly structured nature of the SCV biofilms was possibly due to the increased expression of PIA in these variants. This phenotype completely reversed back to the wild-type when the SCV was cultured in a menadione-supplemented medium, indicating that the deficiency in menadione biosynthesis may be the primary factor responsible for the altered phenotype of increased biofilm formation as well as enhanced PIA production observed in the SCV. Enhanced production of EPS has also been reported in *Staphylococcus epidermidis* and *P. aeruginosa* SCVs (Al Laham *et al.*, 2007; Kirisits *et al.*, 2005; Starkey *et al.*, 2009).

### Quantification of aconitase activity

A possible reason for the enhanced production of PIA in menadione-dependent SCVs may be repression of the tricarboxylic acid (TCA) cycle. Menadione is a precursor of menaquinone, whose deficiency results in a defective electron transport chain and thereby represses the TCA cycle (Kohler *et al.*, 2008). Inhibition of the TCA cycle has been reported to upregulate the expression of the *icaADBC* operon encoding PIA in staphylococci (Vuong *et al.*, 2005). The activity of the TCA cycle in the wild-type parental strain and SCV cultures was therefore determined by quantifying the activity of aconitase. The enzyme activity (mean  $\pm$  SD) in cell-lysate extracts from the SCV ( $0.445 \pm 0.013 \text{ U ml}^{-1}$ ) was significantly ( $P < 0.05$ ) reduced in comparison with the wild-type parental strain ( $0.581 \pm 0.075 \text{ U ml}^{-1}$ ) and the cultures obtained by growing the SCV in a menadione-supplemented medium ( $0.584 \pm 0.058 \text{ U ml}^{-1}$ ). Hence, repression of the TCA cycle as a result of a defective electron transport chain may have resulted in augmented expression of PIA in the menadione-auxotrophic SCVs.

### Determination of cell-surface hydrophobicity

Apart from PIA, the enhanced clumping and biofilm-forming ability of SCVs has also been attributed to an increase in cell-surface hydrophobicity, as reported in *P. aeruginosa* and *Streptococcus pneumoniae* (Allegrucci & Sauer, 2007; Drenkard & Ausubel, 2002; Haussler *et al.*, 2003; Kirisits *et al.*, 2005). However, this does not appear to be the case in *Staphylococcus aureus*. The cell-surface hydrophobicity (mean  $\pm$  SD) of the SCV ( $0.731 \pm 0.001$ ), wild-type parental strain ( $0.667 \pm 0.024$ ) and the culture obtained by growing the SCV in a menadione-supplemented medium ( $0.687 \pm 0.041$ ) was similar ( $P > 0.05$ ), as determined by a microbial adhesion to hydrocarbons assay.

In conclusion, our results suggest that the increased autoaggregation and biofilm-forming potential of menadione-auxotrophic *Staphylococcus aureus* SCVs is related to the upregulation of EPS matrix production in these variants. Menadione-dependent SCVs produce considerably more PIA compared with the wild-type parental strain both in the planktonic state and in biofilms. This enhanced production of EPS matrix may also explain the highly structured nature of SCV biofilms. To our knowledge, this is the first report indicating the augmented expression of PIA in menadione-auxotrophic *Staphylococcus aureus* SCVs. However, these findings, carried out with one SCV/wild-type pair, demand further studies involving multiple isolates.

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