Formation and properties of *in vitro* biofilms of *ica*-negative *Staphylococcus epidermidis* clinical isolates

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Coagulase-negative *Staphylococcus epidermidis* has become the leading cause of foreign-body infections due to its biofilm formation on all kinds of medical-device surfaces. The biofilm development of *S. epidermidis* includes two steps: the initial attachment phase and the accumulative phase. In the accumulative phase, the polysaccharide intercellular adhesin (PIA), encoded by the *icaADBC* locus, is the major component mediating intercellular adhesion. However, recent studies have revealed the emergence of biofilm-positive/*ica*-negative staphylococcal clinical isolates. In this report, two *ica*-negative *S. epidermidis* clinical strains, SE1 and SE4, exhibited their heterogeneity in biofilm architecture under static and flow conditions, compared with the biofilm-positive/*ica*-positive RP62A strain. Strains with this type of absence of PIA from biofilms also displayed intermediate resistance to vancomycin. More importantly, the cells of both SE1 and SE4 strains were more tolerant than those of RP62A to exposure to lysostaphin and vancomycin. Based on the results, it is suggested that the biofilm-positive/*ica*-negative strain represents a newly emergent subpopulation of *S. epidermidis* clinical strains, arising from selection by antibiotics in the nosocomial milieu, which displays a survival advantage in its host environment. Recent epidemiological data support this suggestion, by showing a tendency towards an increasing proportion of this subpopulation in staphylococci-associated infections.

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INTRODUCTION

In recent years, coagulase-negative *Staphylococcus epidermidis* has become the leading cause of infections related to indwelling medical devices such as vascular catheters, prosthetic joints and artificial heart valves (Raad & Bodey, 1992; Inman *et al.*, 1984; Rupp & Archer, 1994). The major pathogenicity of *S. epidermidis* is attributed to its biofilm formation on the surface of medical devices, which enhances bacterial resistance to antibiotics and host-defence mechanisms (von Eiff *et al.*, 2002; Vadyvaloo & Otto, 2005). Numerous studies have shown that *S. epidermidis* biofilm

formation is a two-step process, in which bacteria first adhere to the surface (initial attachment phase), and subsequently form cell–cell aggregates and a multilayered architecture (accumulative phase) (Gotz, 2002; Mack *et al.*, 2004). One autolysin protein, AtlE, is thought to play a role in bacterial attachment to the surface of medical devices, and this autolysin is also involved in the pathogenesis of *S. epidermidis* biofilm-mediated infection *in vivo* (Heilmann *et al.*, 1997; Rupp *et al.*, 2001). In the accumulative phase, the polysaccharide intercellular adhesin (PIA), a linear poly-*N*-acetyl-1,6- β -glucosamine (PNAG) is the major component mediating intercellular adhesion (Mack *et al.*, 1996; Mack, 1999). PIA biosynthesis is encoded by the *icaADBC* locus, which is widespread in *S. epidermidis* clinical isolates (Heilmann *et al.*, 1996; Galdhart *et al.*, 2000). In an animal model of foreign-body infection, an *ica* mutant exhibits reduced virulence compared with its parental strain, indicating that PIA expression is important for the pathogenesis of *S. epidermidis* (Rupp *et al.*, 1999a, b). However, recent studies also indicate the presence of PIA-independent mechanisms mediating biofilm formation in clinical isolates of *S.*

Abbreviations: CLSM, confocal laser-scanning microscope; GISA, glycopeptide-intermediate *S. aureus*; MBC, minimal bactericidal concentration; PIA, polysaccharide intercellular adhesin; PNAG, poly-*N*-acetyl-1,6- β -glucosamine; PI, propidium iodide; TRITC, tetramethyl rhodamine isothiocyanate.

Figures showing DNase I inhibition of *S. epidermidis* strains, the effect of vancomycin on *S. epidermidis* biofilms, and the effect of vancomycin on *S. epidermidis* planktonic cells, are available as supplementary data with the online version of this paper.

epidermidis and *Staphylococcus aureus* (Rohde *et al.*, 2005; Fitzpatrick *et al.*, 2005; Toledo-Arana *et al.*, 2005).

In our previous study, a total of 101 *S. epidermidis* clinical isolates were investigated for the presence and transcription of the *ica* operon, and association with biofilm formation (Jiang *et al.*, 2006). Our analysis indicated that 23.8 % (24/101) isolates were biofilm positive, among which 22 isolates were *ica* positive. Interestingly, two of the 101 isolates, named SE1 and SE4, were identified as biofilm positive/*ica* negative, and they were isolated from different patients and sites. SE1 was collected from a blood culture of a female patient who carried a central venous catheter (CVC) and had symptoms of bacteraemia. SE4 was isolated from a bone marrow culture of a male patient who was a bone marrow transplant recipient and also displayed symptoms of infection after the operation. However, it remains to be answered whether the presence of PIA influences biofilm architecture and responses to antibiotics and other agents. More importantly, it is interesting to explore whether the planktonic cells of biofilm-positive/*ica*-negative strains have developed some distinct individual characters, e.g. those related to antibiotic resistance. In the present study, the biofilm development of SE1 and SE4 was compared with that of RP62A, a model biofilm-positive/*ica*-positive strain (Gill *et al.*, 2005), in the static-chamber and flow-chamber systems. The resistance of biofilms and planktonic cells to antibiotics and other agents was also compared among the three strains. Based on these results, a hypothesis was developed to explain the derivation and evolutionary orientation of these *S. epidermidis* biofilm-positive/*ica*-negative clinical isolates under the antibiotic selection pressure of the nosocomial environment.

METHODS

Bacterial strains, media and reagents. *S. epidermidis* strain RP62A was purchased from the American Type Culture Collection (ATCC). *S. epidermidis* strains SE1 and SE4 are two clinical isolates from Ruijin Hospital, Shanghai, China, and were verified by biochemical characterization using Gram stain and the API 20 Staph system (bioMérieux). Tryptic soy broth (TSB; Oxoid) medium containing 0.25 % glucose was used for biofilm formation in a 96-well plate and static-chamber system. AB medium [1.51 mM $(\text{NH}_4)_2\text{SO}_4$, 3.37 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.2 mM KH_2PO_4 , 5.1 mM NaCl, 1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.02 mg $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ l^{-1} , 0.02 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ l^{-1} , 0.002 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ l^{-1} , 0.002 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ l^{-1} , 0.002 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ l^{-1} , 0.001 mg $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ l^{-1} , 0.001 mg $\text{NaMoSO}_4 \cdot \text{H}_2\text{O}$ l^{-1} , 0.0005 mg H_3BO_3 l^{-1}] (Clark & Maaloe, 1967) supplemented with 0.3 mM glucose and 3 % TSB medium was used for biofilm cultivation in the flow-chamber system. If not mentioned specifically, biofilms and batch cultures were grown at 37 °C. SYTO9 and propidium iodide (PI; Molecular Probes) were used at a concentration of 1 μM for staining. Tetramethyl rhodamine isothiocyanate (TRITC)-labelled wheat germ agglutinin (Molecular Probes) was used at a concentration of 0.1 mg ml^{-1} to stain the PIA in *S. epidermidis* biofilms.

Cultivation of biofilms

In polystyrene microtitre plates. Biofilm cultivation on polystyrene microtitre 96-well plates (Nunc) was carried out as described

elsewhere (Christensen *et al.*, 1985). Briefly, overnight cultures of *S. epidermidis* strains grown in TSB (0.25 % glucose) medium were diluted 1 : 200. The diluted cultures were transferred to wells of polystyrene microtitre plates (200 μl per well) and incubated for 24 h at 37 °C. In order to observe the influence of biofilm formation, DNase I (2 mg ml^{-1} , Sigma) was added to the bacterial suspension in the wells prior to incubation. Biofilm stability against proteinase K (Sigma) or sodium metaperiodate treatment was tested as published elsewhere (Kogan *et al.*, 2006). The 24-hour-old biofilms were washed with 200 μl 0.9 % NaCl and then treated for 2 h at 37 °C with 100 μl 10 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5), or 100 μl proteinase K (100 μg ml^{-1} in 20 mM Tris, pH 7.5, 100 mM NaCl). Control wells were filled with an appropriate buffer. After treatment, the biofilms were washed with 0.9 % NaCl, air-dried and stained with 2 % crystal violet for 5 min. Then, the plate was rinsed off under running tap water, air-dried, and the A_{590} was determined. Each assay was repeated three times.

In the static-chamber system. Biofilms were developed in the coverglass cell-culture chamber (Nunc), as described elsewhere (Jager *et al.*, 2005). Briefly, overnight cultures of *S. epidermidis* strains grown in TSB (containing 0.25 % glucose) medium were diluted to OD₆₀₀ 0.001, inoculated into wells of a chamber (1.5 ml per well) and incubated at 37 °C for 24 h. After that, the chamber was washed gently four times with 1 ml sterile PBS, then stained by using SYTO9 and PI for 15 min, and observed under the microscope. For further treatment by antibiotics, the incubated chamber was washed gently four times with 1 ml sterile PBS to remove planktonic cells, and then fresh medium was added containing antibiotics for 24 h incubation at 37 °C. After that, the chamber was washed, stained and observed under the microscope, as described above.

In the flow-chamber system. Biofilms were grown in a flow chamber with individual channel dimensions of 1 × 4 × 40 mm. The flow-chamber system was assembled and prepared as described previously (Moller *et al.*, 1998). The flow chambers were inoculated by injecting 350 μl overnight culture diluted to OD₆₀₀ 0.001 into each flow channel with a small syringe. After inoculation, flow channels were left without flow for 1 h, then medium flow (0.2 mm s^{-1}) was started using a Watson-Marlow 205S peristaltic pump at 37 °C.

Microscopy and image acquisition. All microscopic observations and image acquisitions were performed with a Zeiss LSM 510 confocal laser-scanning microscope (CLSM; Carl Zeiss) equipped with detectors and filter sets for monitoring SYTO9, PI and TRITC fluorescence. Images were obtained using a ×63/1.4 objective or a ×40/1.3i objective. Simulated 3D images and sections were generated using the IMARIS software package (Bitplane).

MIC and minimal bactericidal concentration (MBC) assays.

MIC assays for the antibacterial activities of the compounds were performed according to the broth microdilution (in tubes) method of the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 1997). The MBC was obtained by subculturing 100 μl from each negative (no visible bacterial growth) tube from the MIC assay onto substance-free Mueller–Hinton agar plates. The plates were incubated at 35 °C for 24 h, and the MBC was defined as the lowest concentration of substance which produced subcultures growing no more than five colonies on each plate.

Cell-autolysis assays. Autolysis assays were performed as described elsewhere (Brunskill & Bayles, 1996). Cell samples (50 ml) were collected from exponential-phase cultures growing in TSB medium (OD₅₈₀ 0.7) containing 1 M NaCl, and cells were pelleted by centrifugation. The cells were washed twice with 50 ml ice-cold water and resuspended in 50 ml 0.05 M Tris/HCl, pH 7.2, containing 0.05 % (v/v) Triton X-100 (Sigma). The cells were then incubated at 30 °C with shaking, and the OD₅₈₀ was measured at 30-minute intervals.

Time-killing assay. The time-killing assay was performed by the broth macrodilution method, as described in an earlier study, with some small modifications (Petersen *et al.*, 2004). Briefly, a starting inoculum of approximately 10^6 c.f.u. ml⁻¹ and a final concentration of the antibiotic or compound equal to the MBC were used in the assay. Flasks containing 25 ml Mueller–Hinton II broth (Sigma) with the appropriate antimicrobial agent were inoculated with 25 ml of the test organism in the exponential-growth phase. Test flasks were incubated with shaking (160 r.p.m.) in a 35 °C water bath. Samples were taken at 0, 2, 4, 6 and 24 h for the determination of viable counts. Serial dilutions were prepared in sterile 0.9 % sodium chloride. The diluted samples (0.1 ml) were plated onto trypticase soy agar (TSA) and incubated at 35 °C for 24 h. The number of colonies on plates was determined on a ProtoCOL plate reader plater (Don Whitley Scientific). Time-killing curves were constructed by plotting the log₁₀(c.f.u. ml⁻¹) versus time over 24 h, and the change in bacterial concentration was determined.

Statistical analysis. The two-tailed Student's *t* test was performed with a computer (Excel 8.0), and *P* < 0.05 was considered significant; *P* < 0.01 was considered highly significant.

PCR detection of *aap* and *bhp* genes in staphylococcal strains. The genomic DNA of staphylococcal strains was extracted by using the Genomic DNA Extraction Midi kit (Qiagen) according to the manufacturer's protocol. The sequences of *aap* primers were 5'-ATGGGCAAACGTAGACAAG-3' (sense) and 5'-ACCGTAAAA-ATCGTAATTATCTC-3' (anti-sense). The sequences of *bhp* primers were 5'-ATGAAAAATAACAAGGATTTC-3' (sense) and 5'-GCC-TAAGCTAGATAATGTTTG-3' (anti-sense). The primers were designed according to the published sequences of the staphylococcal *aap* and *bhp* genes (GenBank accession nos AJ249487 and AY028618, respectively). The PCR was performed in a DNA thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) under conditions of 94 °C for 5 min, 30 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 90 s.

Detection of Aap expression by immuno-dot-blot assay. The cell proteins of *S. epidermidis* strains were extracted as described in our previous study (Yang *et al.*, 2006). Protein concentrations of all the samples were determined by the Bradford method. After that, 5 µl aliquots (30 ng cell-extraction proteins from *S. epidermidis* strains RP62A, SE1 and SE4) were spotted onto nitrocellulose membranes, then immersed in blocking buffer (5 % skimmed milk in PBS) and developed by standard immuno-blotting procedures to detect the expression of Aap by these *S. epidermidis* strains. Aap production was detected by using an anti-Aap antiserum (Rohde *et al.*, 2005) diluted 1:5000. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sino-American Biotech) diluted 1:50.

RESULTS

Biofilm development of *ica*-negative *S. epidermidis* clinical isolates

In our previous study, the two biofilm-positive/*ica*-negative strains SE1 and SE4 were identified among 101 *S. epidermidis* clinical isolates collected from Ruijin Hospital, Shanghai, China (Jiang *et al.*, 2006). The biofilm phenotype was validated by the microtitre plate assays described in Methods, while the *ica*-negative genotype was confirmed by PCR and Southern blotting with *ica*-specific primers and probes, respectively (Jiang *et al.*, 2006). Here the capabilities for biofilm development of SE1 and SE4 were compared

with that of *S. epidermidis* RP62A, a reference biofilm-positive/*ica*-positive strain (Gill *et al.*, 2005). In the static-chamber system, both SE1 and SE4 displayed variant morphology compared with RP62A in the initial attachment phase after incubation for 1 h (Fig. 1a, d, g). SE1 formed small cellular clumps on the surfaces, and SE4 developed much bigger clumps, whereas RP62A exhibited single-cell scattering on the surfaces. The attachment morphologies of the three strains were also associated with their behaviour under planktonic conditions. In the samples of suspension used to inoculate the chambers, it was observed that both SE1 and SE4 had a tendency to cell aggregation, while RP62A did not (Fig. 2a–c). In addition, planktonic cells of SE1 and SE4 deposited more readily on the bottom of tubes than those of RP62A (Fig. 2d). After incubation for 6 h, RP62A exhibited a greater number of microcolonies than SE1 and SE4 on the chamber surface (Fig. 1b, e, h). In mature biofilms (24 h old), RP62A developed more compact biofilm architecture than that of SE1 and SE4 (Fig. 1c, f, i). SE1 displayed similar microcolony structure to that of RP62A, whereas SE4 formed relatively loose and thin biofilms. A common characteristic of biofilms formed by the three strains was that almost no dead cells (yellow colour shown by PI stains) were observed in mature biofilm architecture.

In the flow-cell system, both RP62A and SE1 developed microcolonies of different sizes in mature biofilms (Fig. 3a, c). Although the number of microcolonies formed by SE1 was much smaller than that of RP62A, the thickness of the big microcolonies was similar (60–70 µm) in both strains. In contrast, SE4 formed a relatively flat and loose biofilm architecture, and the thickness of biofilms was about 30–40 µm (Fig. 3e). Interestingly, far fewer dead cells were observed in mature biofilms formed by SE1 and especially SE4 than those of RP62A (Fig. 3a, c, e). In biofilms formed by RP62A, a large number of dead cells were located in the centre and bottom of almost all the microcolonies (Fig. 3a), whereas fewer dead cells were found in the bottom of the large-sized microcolonies of SE1 (Fig. 3c). In contrast, almost no dead cells were found in mature biofilms formed by SE4 (Fig. 3e). TRITC-labelled wheat germ agglutinin was used to stain PIA in biofilms, as described elsewhere (Jager *et al.*, 2005). As expected, both SE1 and SE4 exhibited an absence of PIA in mature biofilms (Fig. 3d, f), despite their heterogeneity in biofilm architecture, whereas RP62A displayed a ring-like distribution of PIA around the microcolonies in mature biofilms (Fig. 3d).

Biofilm detachment by different agents

It has been shown that metaperiodate (HIO₄ and NaIO₄) can disrupt biofilms containing PIA (or PNAG) formed by *Escherichia coli* or *S. epidermidis* (Wang *et al.*, 2004; Kogan *et al.*, 2006). Because SE1 and SE4 are both PIA-negative strains, their biofilms should be resistant to the PNAG-degrading agents. The PIA-positive strain RP62A was used as a positive control, while sodium acetate buffer treatment was used as a negative control. Compared with the negative

Fig. 1. Biofilms of *S. epidermidis* RP62A (a–c), SE1 (d–f) and SE4 (g–i) strains were grown in static chambers with TSB medium for 1, 6 and 24 h, then stained with SYTO9 (green) and PI (red). Microscopic investigation was performed with a CLSM. Bars, (a), (d) and (g), 20 μ m; (b), (c), (e), (f), (h) and (i), 30 μ m.

controls, the preformed biofilm of RP62A was partially disrupted by metaperiodate ($\sim 60\%$), but metaperiodate had no effect on the biofilms of the two *ica*-negative clinical

isolates SE1 and SE4 (Fig. 4a). We also observed detachment of the preformed biofilms caused by proteinase K. Compared with the Tris-buffer treatment group (negative

Fig. 2. Cells of *S. epidermidis* RP62A (a), SE1 (b) and SE4 (c) strains under planktonic growth conditions. Planktonic suspensions (5 μ l) used to inoculate the static chamber (see Methods) were dropped onto a glass slide, and observed immediately under the CLSM. RP62A cells exhibited single-cell morphology, while those of SE1 and SE4 showed cell clumping. In culture tubes, cells of SE1 and SE4 were easier to precipitate onto the bottom of the tube than those of RP62A (d).

Fig. 3. Biofilms of *S. epidermidis* RP62A (a, b), SE1 (c, d) and SE4 (e, f) strains were grown for 24 h in flow chambers irrigated with glucose minimal medium supplemented with 3% TSB, then stained with SYTO9 (green) and PI (red) (left column), or SYTO9 (green) and TRITC (red) (right column). Microscopic investigation was performed with a CLSM. Bars in the images of horizontal optical sections, 50 µm.

control), proteinase K had almost no effect on biofilms formed by RP62A, whereas it detached preformed biofilms of SE1 and SE4 (~50 and ~95 %, respectively) (Fig. 4b). Interestingly, DNase I could inhibit the biofilm formation of all three *S. epidermidis* strains in microtitre plates (Fig. 4c), but was not able to cause detachment of preformed biofilms formed by the three strains (data not shown), in accordance with observations with *Pseudomonas aeruginosa* (Whitchurch *et al.*, 2002). Further investigations indicated that DNase I inhibition of *S. epidermidis* biofilm formation was related to the inhibition of bacterial initial attachment (Supplementary Fig. S1A–C in JMM online). Additionally, heat-inactivated DNase I lost its inhibitory effect, while DNase I-free RNase did not cause inhibition (data not shown). Taken together, these results indicate that the major components of the biofilm matrix are protein and DNA in both SE1 and SE4, whereas PIA predominates in the biofilm matrix of RP62A.

Fig. 4. Dispersal of biofilms of *S. epidermidis* RP62A and clinical strains by sodium metaperiodate, proteinase K and DNase I. Mature biofilms (24 h old) were treated with NaIO₄ in sodium acetate buffer (a) and proteinase K in Tris buffer (b) for 2 h at 37 °C. Control wells were filled with appropriate buffers. DNase I (2 mg ml⁻¹) was added to the bacterial suspension in wells prior to incubation for biofilm formation (c). Mean results ± SD for six wells for each strain are shown. The experiments were repeated three times with similar results.

Biofilms response to vancomycin treatment

The glycopeptide vancomycin has been considered to be one of the most reliable therapeutic agents against infections caused by multidrug-resistant staphylococci (Hiramatsu, 2001). However, the emergence of vancomycin-resistant *S. epidermidis* (VRSE) strains has been reported, and increased resistance to vancomycin has also been observed in *S. epidermidis* biofilms (Schwalbe *et al.*, 1987; Hiramatsu *et al.*, 1997; Evans & Holmes, 1987; Khardori *et al.*, 1995).

Table 1. MIC and MBC values of *S. epidermidis* RP62A, SE1 and SE4 strains for vancomycin and lysostaphin

ND, Not determined.

<i>S. epidermidis</i> strain	MIC ($\mu\text{g ml}^{-1}$)		MBC ($\mu\text{g ml}^{-1}$)	
	Vancomycin	Lysostaphin	Vancomycin	Lysostaphin
RP62A	2	2	8	> 32
SE1	2	> 32	4	ND
SE4	2	> 32	8	ND

Therefore, it is interesting to know whether biofilms with or without PIA exhibit different responses to vancomycin treatment. In this assay, vancomycin was used at concentrations of $8\text{ }\mu\text{g ml}^{-1}$ (the MBC to *S. epidermidis* planktonic cells) and $128\text{ }\mu\text{g ml}^{-1}$ (a high concentration), since all three strains, RP62A, SE1 and SE4, displayed similar MICs and MBCs to vancomycin (Table 1). Interestingly, instead of killing the cells, exposure to $8\text{ }\mu\text{g vancomycin ml}^{-1}$ stimulated RP62A to develop a thicker and more compact

biofilm structure, and even exposure to $128\text{ }\mu\text{g vancomycin ml}^{-1}$ only killed a few scattered cells in the biofilm (Fig. 5a–c, Supplementary Fig. S2A–F in JMM online). In SE1, treatment with $8\text{ }\mu\text{g vancomycin ml}^{-1}$ had no significant effect on biofilm development, but exposure to the high concentration ($128\text{ }\mu\text{g ml}^{-1}$) disrupted the normal micro-colony structure obviously, resulting in only small cell aggregations remaining on the chamber surface (Fig. 5d–f). Biofilms formed by SE4 exhibited a similar response to

Fig. 5. Treatment of 24-hour-old biofilms of *S. epidermidis* RP62A (a–c), SE1 (d–f) and SE4 (g–i) strains by medium as control (left column), $8\text{ }\mu\text{g vancomycin ml}^{-1}$ (middle column) and $128\text{ }\mu\text{g vancomycin ml}^{-1}$ (right column) in static chambers. After exposure to vancomycin for 24 h, the biofilms were stained with SYTO9 (green) and PI (red), and observed with a CLSM. Bars in the images of horizontal optical sections, $30\text{ }\mu\text{m}$. The experiments were repeated twice with similar results.

Fig. 6. Time-killing curves for vancomycin against *S. epidermidis* RP62A (●), SE1 (▲) and SE4 (■) planktonic cells. Vancomycin was used at the MBC of $8 \mu\text{g ml}^{-1}$ against all three strains in this assay (solid lines). Medium without vancomycin was used as control (dotted lines). The assay was repeated three times and no significant difference was seen among the experiments. The arrows represent the samples chosen to stain with SYTO9 (green) and PI (red) for observation of bacterial viability (shown in Supplementary Fig. S3 in JMM online). * $P < 0.05$; ** $P < 0.01$ (SE1 or SE4 versus RP62A at the same time point, two-tailed t test).

vancomycin treatment to that of SE1, so that $8 \mu\text{g ml}^{-1}$ vancomycin had little disruptive effect on biofilm structure (Fig. 5g–i). However, one finding common to SE1 and SE4 was that almost all the bacterial cells remaining in biofilms on the chamber surfaces remained alive, and could completely resist vancomycin (low or high concentration) after 24 h treatment (Fig. 5e, f, h, i).

Planktonic cell response to vancomycin treatment

The growth of both SE1 and SE4 showed no significant difference compared with that of RP62A in TSB medium, except that in the exponential phase, SE1 and SE4 exhibited growth that was a little more rapid than that of RP62A (data not shown). In order to investigate whether variant responses to vancomycin are present among planktonic cells of the three strains, time-killing kinetic assays of vancomycin ($8 \mu\text{g ml}^{-1}$) against *S. epidermidis* cells were performed. Interestingly, both SE1 and SE4 cells displayed a temporary resistant phenotype in the initial treatment phase (from 2 to 6 h); in particular, the samples at 4 h displayed a tendency to proliferation compared with those at 2 h (Fig. 6). In contrast, RP62A planktonic cells continuously reduced in viable counts after exposure to vancomycin. Statistical analysis indicated that samples of SE1 and SE4 showed significant differences from those of RP62A at 4 h (two-tailed t test; $P = 0.036$ and 0.041 , respectively). At 6 h,

SE1 samples also displayed significant differences from those of RP62A ($P = 0.014$), while SE4 samples exhibited highly significant differences from those of RP62A ($P = 0.003$). However, after exposure to vancomycin for 24 h, samples of both SE1 and SE4 showed no significant differences compared with those of RP62A ($P = 0.263$ and 0.656 , respectively). In the control group, both SE1 and SE4 still showed a growth rate a little more rapid than that of RP62A without vancomycin treatment. After exposure to vancomycin for 24 h, five single colonies of each strain on agar plates were randomly selected for subculture and tested for their MICs and MBCs to vancomycin. The results indicated that after exposure to vancomycin, both SE1 and SE4 cells increased their MBCs from 8 to $\geq 64 \mu\text{g ml}^{-1}$, but their MICs remained unchanged ($2 \mu\text{g ml}^{-1}$). In contrast, RP62A cells displayed similar MICs and MBCs before and after vancomycin treatment (2 and $8 \mu\text{g ml}^{-1}$, respectively) (Table 2). Reduced autolytic properties have been reported in some glycopeptide-intermediate *S. aureus* (GISA) strains (Utaida *et al.*, 2006; Sieradzki & Tomasz, 2006). The whole-cell autolysis rates of RP62A, SE1 and SE4 were compared under the same conditions. As demonstrated in Fig. 7, both SE1 and SE4 displayed reduced autolysis compared with RP62A. After 4 h incubation, the extents of whole-cell autolysis were 95, 65 and 30 % in RP62A, SE1 and SE4, respectively.

Detection of *aap* and *bhp* genes in SE1 and SE4 strains

In a previous study, it has been reported that Aap (accumulation-associated protein) and Bhp [Bap (biofilm-associated protein) homologue protein] are two factors that mediate the independence of staphylococcal biofilm formation from PIA (Rohde *et al.*, 2005; Tormo *et al.*, 2005). Accordingly, we first investigated whether Aap and

Table 2. MIC and MBC values of *S. epidermidis* strains after exposure to vancomycin for 24 h

<i>S. epidermidis</i> strain	MIC ($\mu\text{g ml}^{-1}$)	MBC ($\mu\text{g ml}^{-1}$)
SE1-1	2	64
SE1-2	2	64
SE1-3	2	> 64
SE1-4	2	> 64
SE1-5	2	> 64
SE4-1	2	> 64
SE4-2	2	> 64
SE4-3	2	> 64
SE4-4	2	64
SE4-5	2	> 64
RP62A-1	2	8
RP62A-2	2	4
RP62A-3	2	8
RP62A-4	2	8
RP62A-5	2	8

Fig. 7. Autolysis of whole cells of *S. epidermidis* RP62A (◆), SE1 (■) and SE4 (▲) strains. Mid-exponential-phase cultures were resuspended in 0.05 M Tris/HCl (pH 7.2) containing 0.05 % Triton X-100 and incubated at 30 °C. The changes in A_{580} were determined as described in Methods.

Bhp are present in the SE1 and SE4 strains, employing PCR to amplify the specific fragments of both genes. The results indicated that no specific bands were seen in samples using genomic DNA of SE1 or SE4 as template, while the specific fragments of both *aap* (~1.1 kb) and *bhp* (~1.3 kb) genes were observed in samples of strain RP62A (Fig. 8). We also detected the expression of Aap in these three strains by the immuno-dot-blot assay with anti-Aap antiserum (Rohde *et al.*, 2005). The absence of Aap expression in both SE1 and

Fig. 8. PCR detection of *aap* and *bhp* genes in *S. epidermidis* strains. PCR was carried out on purified genomic DNA of *S. epidermidis* strains with primers targeting the specific fragments of *aap* and *bhp*. The specific bands of *aap* (~1.1 kb) and *bhp* (~1.3 kb) were seen in RP62A, but were absent in both SE1 and SE4 strains.

SE4 strains was confirmed, compared with the high expression of this protein in strain RP62A (Fig. 9).

DISCUSSION

In previous studies, PIA was considered the major component required for biofilm formation, bacterial pathogenesis and immune evasion in staphylococcal strains (Mack, 1999; Rupp *et al.*, 1999a, b; Vuong *et al.*, 2004). Further evidence supporting this viewpoint is that one commensal *S. epidermidis* strain, ATCC 12228, has been found by our group to be biofilm negative/*ica* negative and less invasive in an animal model of catheter-associated infection (Zhang *et al.*, 2003). Furthermore, it has been reported that the bacteria can recover the ability to form biofilms when a plasmid containing the *ica* locus is transferred to *ica*-negative *S. epidermidis* strains (Li *et al.*, 2005). However, recent data have reported the presence of biofilm-positive/PIA-negative staphylococcal clinical isolates (Rohde *et al.*, 2005; Kogan *et al.*, 2006). In the present study, two biofilm-positive/*ica*-negative *S. epidermidis* clinical isolates, SE1 and SE4, were also identified from a large-scale epidemiological investigation. In flow-chamber systems, SE1 exhibited similar biofilm architecture (round microcolonies) to that of the PIA-positive RP62A strain, whereas SE4 formed a relatively flat and thin biofilm structure, indicating a heterogeneity in the mechanisms of biofilm development between the two strains. Recently, Rohde *et al.* (2005) reported a 140 kDa exoprotein, Aap, mediating biofilm formation in PIA-negative *S. epidermidis* clinical isolates. However, this PIA-independent mechanism cannot be used to explain biofilm formation of all the PIA-negative *S. epidermidis* clinical isolates, since it has been reported that not all biofilm-positive *S. epidermidis* strains produce this protein (Hussain *et al.*, 1997). Another novel

Fig. 9. Relative expression of Aap determined by immuno-dot-blot analysis. Cell proteins were extracted from cultures of *S. epidermidis* strains ($OD_{600} = 1.5$). Aap production was detected with an anti-Aap antiserum by immuno-dot-blot assay. The protein Aap was produced in large amounts in RP62A, but was absent in both SE1 and SE4 strains. An equal amount of BSA protein was used as a negative control.

cell-wall-associated protein, Bap, has been shown to be involved in initial attachment, intercellular adhesion and biofilm formation of *S. aureus* by the transposon–insertion mutation (Cucarella *et al.*, 2001). Interestingly, the Bap homologue protein Bhp is present in human strains of *S. epidermidis*, and induces an alternative mechanism of biofilm formation that is independent of the PIA (Tormo *et al.*, 2005). To our surprise, the two genes *aap* and *bhp* are not present in the SE1 and SE4 strains, although they are both present in the reference strain RP62A. We therefore suppose that a novel molecular mechanism mediates the biofilm formation of SE1 and SE4 clinical isolates, which needs further investigation.

In the time-killing assay, both SE1 and SE4 planktonic cells display a temporary phase of resistance to vancomycin, which is absent in RP62A cells. In accordance with this, both SE1 and SE4 cells exhibit lower autolysis rates than RP62A under the same conditions. In *S. aureus*, recent studies reveal reduced autolysis in some heterogeneous GISA compared with glycopeptide-susceptible *S. aureus* (GSSA) strains, which may be associated with reduced expression of the *atl* autolysin gene (Utaida *et al.*, 2006; Sieradzki & Tomasz, 2006; Wootton *et al.*, 2005). Nunes *et al.* (2006) report that induction with subinhibitory concentrations of glycopeptides can obviously increase the cell wall thickness of clinical staphylococcus strains. Thus, in future work, it needs to be determined whether the increased cell wall thickness of SE1 and SE4 is attributable to their resistance to vancomycin. In addition, in our study, both SE1 and SE4 cells displayed total resistance to lysostaphin, an endopeptidase that cleaves the pentaglycine cross-bridges in the cell wall (Table 1). Lysostaphin has been successfully used to treat endocarditis in a rabbit model of *S. aureus* infection, and is thought to be a promising therapeutic agent against staphylococci infection (Climo *et al.*, 1998; Patron *et al.*, 1999). The resistance to lysostaphin also indicates the architectural change of cell walls in SE1 and SE4, compared with those of lysostaphin-susceptible strains, and further investigation is now in progress within our group.

Taking the results together, we raise a hypothesis to explain the evolutionary origin of these biofilm-positive/*ica*-negative staphylococcal clinical isolates (Fig. 10). The ancestor of such strains is thought to be biofilm negative/*ica* negative,

since this phenotype has been reported in several epidemiological studies of staphylococcal clinical isolates (Jiang *et al.*, 2006; Frebourg *et al.*, 2000; Klug *et al.*, 2003). More importantly, *S. epidermidis* is a normal inhabitant of human skin and mucous membranes, and most commensal strains are usually biofilm negative/*ica* negative (Ziebuhr *et al.*, 1997; Frebourg *et al.*, 2000; Galdart *et al.*, 2000). However, cells of biofilm-negative/*ica*-negative strains are more easily killed by antibiotics and the host immune system than those embedded in biofilms (Vuong *et al.*, 2004). Therefore, under antibiotic selection pressure on entering the nosocomial milieu, some commensal strains need to develop the biofilm-positive/*ica*-negative phenotype, although this type represents a very small subpopulation of *S. epidermidis* clinical strains. In our previous study, the proportion of biofilm-positive/*ica*-positive versus biofilm-positive/*ica*-negative strains was 22:2 % (Jiang *et al.*, 2006). In another study of *S. epidermidis* clinical isolates from blood cultures, this proportion is reported to be 85:2 % (Ziebuhr *et al.*, 1997). The much higher proportion of biofilm-positive/*ica*-positive strains in the latter study may be attributed to different sources of the strains, since our clinical strains were collected from multiple sources, such as sputum, catheters, blood and wounds. Another possible strategy used by bacteria for survival in the human body is an increase in the resistance of planktonic cells to antibiotics. As shown in this study, the cells of biofilm-positive/*ica*-negative strains exhibit more tolerance to lysostaphin and vancomycin than those of biofilm-positive/*ica*-positive strains. Noticeably, evidence provided here also indicates that this kind of evolution is still at an early stage, for the following reasons. (1) The absence of PIA depresses the resistance of biofilms to vancomycin treatment (both SE1 and SE4), although biofilm formed by SE1 displays a similar architecture to that of RP62A. This kind of biofilm (PIA[−]) only represents one ‘incomplete’ biofilm in evolution under the pressure of antibiotics. (2) The cells of both SE1 and SE4 display temporary resistance to vancomycin treatment in the time-killing kinetic assay, and increasing MBC values after exposure to vancomycin; these characteristics are all absent in strain RP62A. In contrast, the original MIC/MBC values of SE1 and SE4 are similar to those of RP62A, indicating that the resistance of planktonic cells of both strains is still developing. At present, therefore, we cannot

Fig. 10. Model of the evolution of biofilm-positive/*ica*-negative *S. epidermidis* clinical isolates under the pressure of antibiotics. The solid curve represents biofilms hyper-resistant to antibiotics, and the dashed curve represents biofilms of intermediate resistance to antibiotics. The green, black and red circles represent antibiotic-susceptible cells, dead cells and antibiotic-resistant cells of *S. epidermidis*, respectively.

define them as glycopeptide-intermediate *S. epidermidis* (GISE) strains. Another possible explanation is that a biofilm-positive/*ica*-negative strain may be derived from a biofilm-positive/*ica*-positive ancestor through natural passage or antibiotic selection. However, we think such spontaneous mutation is rare, because of its disadvantage to bacterial survival in the human body, and also because we know that no antibiotics currently in use are specific to staphylococcal PIA synthesis. Under the selection of widely used antibiotics, it is believed that the biofilm-positive/*ica*-negative strains will eventually develop a type of 'complete' biofilm that possesses a resistance to antibiotics similar to those of 'classical' biofilms mediated by PIA. On the other hand, the planktonic cells of biofilm-positive/*ica*-negative strains will also become more resistant to antibiotics under such selection pressure. Therefore, it is reasonable to consider that the more resistant strain will greatly increase its proportion of the subpopulation of cases of staphylococci-related infection. Interestingly, recent data seem to support this tendency of evolution in staphylococcal clinical isolates. In their study, Ninin *et al.* (2006) found that the subpopulation of biofilm-positive/*ica*-negative strains had increased to ~10% of the total of 109 *S. epidermidis* clinical isolates that caused bacteraemia in bone marrow transplant recipients. The intensity of the surveillance of this subpopulation of staphylococcal clinical isolates should be increased when carrying out epidemiological studies, especially those related to antibiotic resistance.

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REFERENCES

- Brunskill, E. W. & Bayles, K. W. (1996). Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus*. *J Bacteriol* **178**, 611–618.
- Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M. & Beachey, E. H. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* **22**, 996–1006.
- Clark, J. D. & Maaloe, O. (1967). DNA replication and the cell cycle in *Escherichia coli* cells. *J Mol Biol* **23**, 99–112.
- Climo, M. W., Patron, R. L., Goldstein, B. P. & Archer, G. L. (1998). Lysostaphin treatment of experimental methicillin-resistant *Staphylococcus aureus* aortic valve endocarditis. *Antimicrob Agents Chemother* **42**, 1355–1360.
- Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I. & Penades, J. R. (2001). Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. *J Bacteriol* **183**, 2888–2896.
- Evans, R. C. & Holmes, C. J. (1987). Effect of vancomycin hydrochloride on *Staphylococcus epidermidis* biofilm associated with silicone elastomer. *Antimicrob Agents Chemother* **31**, 889–894.
- Fitzpatrick, F., Humphreys, H. & O'Gara, J. P. (2005). Evidence for *icaADBC*-independent biofilm development mechanism in methicillin-resistant *Staphylococcus aureus* clinical isolates. *J Clin Microbiol* **43**, 1973–1976.
- Frebourg, N. B., Lefebvre, S., Baert, S. & Lemeland, J. F. (2000). PCR-based assay for discrimination between invasive and contaminating *Staphylococcus epidermidis* strains. *J Clin Microbiol* **38**, 877–880.
- Galdart, J. O., Allignet, J., Tung, H. S., Ryden, C. & El Solh, N. (2000). Screening for *Staphylococcus epidermidis* markers discriminating between skin-flora strains and those responsible for infections of joint prostheses. *J Infect Dis* **182**, 351–355.
- Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., Deboy, R. T., Ravel, J., Paulsen, I. T., Kolonay, J. F., Brinkac, L. & other authors (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* **187**, 2426–2438.
- Gotz, F. (2002). *Staphylococcus* and biofilms. *Mol Microbiol* **43**, 1367–1378.
- Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D. & Gotz, F. (1996). Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* **20**, 1083–1091.
- Heilmann, C., Hussain, M., Peters, G. & Gotz, F. (1997). Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol Microbiol* **24**, 1013–1024.
- Hiramatsu, K. (2001). Vancomycin-resistant *Staphylococcus aureus*: a new model of antibiotic resistance. *Lancet Infect Dis* **1**, 147–155.
- Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., Fukuchi, Y. & Kobayashi, I. (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet* **350**, 1670–1673.
- Hussain, M., Herrmann, M., von Eiff, C., Perdreau-Remington, F. & Peters, G. (1997). A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. *Infect Immun* **65**, 519–524.
- Inman, R. D., Gallegos, K. V., Brause, B. D., Redecha, P. B. & Christian, C. L. (1984). Clinical and microbial features of prosthetic joint infection. *Am J Med* **77**, 47–53.
- Jager, S., Mack, D., Rohde, H., Horstkotte, M. A. & Knobloch, J. K. (2005). Disintegration of *Staphylococcus epidermidis* biofilms under glucose-limiting conditions depends on the activity of the alternative sigma factor sigmaB. *Appl Environ Microbiol* **71**, 5577–5581.
- Jiang, J., Sun, J. Y., Ou, Y. Z., Qin, Z. Q., Chen, J. M. & Qu, D. (2006). Influence of *ica* transcription on biofilm phenotype of *Staphylococcus epidermidis* clinical isolates. *Shanghai Med J* **29**, 40–44 (in Chinese).
- Khardori, N., Yassien, M. & Wilson, K. (1995). Tolerance of *Staphylococcus epidermidis* grown from indwelling vascular catheters to antimicrobial agents. *J Ind Microbiol* **15**, 148–151.
- Klug, D., Wallet, F., Kacet, S. & Courcol, R. J. (2003). Involvement of adherence and adhesion *Staphylococcus epidermidis* genes in pace-maker lead-associated infections. *J Clin Microbiol* **41**, 3348–3350.
- Kogan, G., Sadovskaya, I., Chaignon, P., Chokr, A. & Jabbouri, S. (2006). Biofilms of clinical strains of *Staphylococcus* that do not contain polysaccharide intercellular adhesin. *FEMS Microbiol Lett* **255**, 11–16.

- Li, H., Xu, L., Wang, J., Wen, Y., Vuong, C., Otto, M. & Gao, Q. (2005). Conversion of *Staphylococcus epidermidis* strains from commensal to invasive by expression of the *ica* locus encoding production of biofilm exopolysaccharide. *Infect Immun* 73, 3188–3191.
- Mack, D. (1999). Molecular mechanisms of *Staphylococcus epidermidis* biofilm formation. *J Hosp Infect* 43 (Suppl.), S113–S125.
- Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H. & Laufs, R. (1996). The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol* 178, 175–183.
- Mack, D., Becker, P., Chatterjee, I., Dobinsky, S., Knobloch, J. K., Peters, G., Rohde, H. & Herrmann, M. (2004). Mechanisms of biofilm formation in *Staphylococcus epidermidis* and *Staphylococcus aureus*: functional molecules, regulatory circuits, and adaptive responses. *Int J Med Microbiol* 294, 203–212.
- Moller, S., Sternberg, C., Andersen, J. B., Christensen, B. B., Ramos, J. L., Givskov, M. & Molin, S. (1998). In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. *Appl Environ Microbiol* 64, 721–732.
- NCCLS (1997). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*, 4th edn. Approved standard M7-A4. Villanova, PA: National Committee for Clinical Laboratory Standards.
- Ninin, E., Caroff, N., Espaze, E., Marailac, J., Lepelletier, D., Milpied, N. & Richet, H. (2006). Assessment of *ica* operon carriage and biofilm production in *Staphylococcus epidermidis* isolates causing bacteraemia in bone marrow transplant recipients. *Clin Microbiol Infect* 12, 446–452.
- Nunes, A. P., Teixeira, L. M., Iorio, N. L., Bastos, C. C., de Sousa Fonseca, L., Souto-Padron, T. & dos Santos, K. R. (2006). Heterogeneous resistance to vancomycin in *Staphylococcus epidermidis*, *Staphylococcus haemolyticus* and *Staphylococcus warneri* clinical strains: characterisation of glycopeptide susceptibility profiles and cell wall thickening. *Int J Antimicrob Agents* 27, 307–315.
- Patron, R. L., Climo, M. W., Goldstein, B. P. & Archer, G. L. (1999). Lysostaphin treatment of experimental aortic valve endocarditis caused by a *Staphylococcus aureus* isolate with reduced susceptibility to vancomycin. *Antimicrob Agents Chemother* 43, 1754–1755.
- Petersen, P. J., Wang, T. Z., Dushin, R. G. & Bradford, P. A. (2004). Comparative in vitro activities of AC98-6446, a novel semisynthetic glycopeptide derivative of the natural product mannopeptimycin alpha, and other antimicrobial agents against Gram-positive clinical isolates. *Antimicrob Agents Chemother* 48, 739–746.
- Raad, I. I. & Bodey, G. P. (1992). Infectious complications of indwelling vascular catheters. *Clin Infect Dis* 15, 197–208.
- Rohde, H., Burdelski, C., Bartscht, K., Hussain, M., Buck, F., Horstkotte, M. A., Knobloch, J. K., Heilmann, C., Herrmann, M. & Mack, D. (2005). Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol Microbiol* 55, 1883–1895.
- Rupp, M. E. & Archer, G. L. (1994). Coagulase-negative staphylococci: pathogens associated with medical progress. *Clin Infect Dis* 19, 231–243.
- Rupp, M. E., Ulphani, J. S., Fey, P. D., Bartscht, K. & Mack, D. (1999a). Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. *Infect Immun* 67, 2627–2632.
- Rupp, M. E., Ulphani, J. S., Fey, P. D. & Mack, D. (1999b). Characterization of *Staphylococcus epidermidis* polysaccharide intercellular adhesin/hemagglutinin in the pathogenesis of intravascular catheter-associated infection in a rat model. *Infect Immun* 67, 2656–2659.
- Rupp, M. E., Fey, P. D., Heilmann, C. & Gotz, F. (2001). Characterization of the importance of *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. *J Infect Dis* 183, 1038–1042.
- Schwalbe, R. S., Stapleton, J. T. & Gilligan, P. H. (1987). Emergence of vancomycin resistance in coagulase-negative staphylococci. *N Engl J Med* 316, 927–931.
- Sieradzki, K. & Tomasz, A. (2006). Inhibition of the autolytic system by vancomycin causes mimicry of vancomycin-intermediate *Staphylococcus aureus*-type resistance, cell concentration dependence of the MIC, and antibiotic tolerance in vancomycin-susceptible *S. aureus*. *Antimicrob Agents Chemother* 50, 527–533.
- Toledo-Arana, A., Merino, N., Vergara-Irigaray, M., Debarbouille, M., Penades, J. R. & Lasa, I. (2005). *Staphylococcus aureus* develops an alternative, *ica*-independent biofilm in the absence of the *arlRS* two-component system. *J Bacteriol* 187, 5318–5329.
- Tormo, M. A., Knecht, E., Gotz, F., Lasa, I. & Penades, J. R. (2005). Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? *Microbiology* 151, 2465–2475.
- Utaida, S., Pfeiltz, R. F., Jayaswal, R. K. & Wilkinson, B. J. (2006). Autolytic properties of glycopeptide-intermediate *Staphylococcus aureus* Mu50. *Antimicrob Agents Chemother* 50, 1541–1545.
- Vadyvaloo, V. & Otto, M. (2005). Molecular genetics of *Staphylococcus epidermidis* biofilms on indwelling medical devices. *Int J Artif Organs* 28, 1069–1078.
- von Eiff, C., Peters, G. & Heilmann, C. (2002). Pathogenesis of infections due to coagulase-negative staphylococci. *Lancet Infect Dis* 2, 677–685.
- Vuong, C., Voyich, J. M., Fischer, E. R., Braughton, K. R., Whitney, A. R., DeLeo, F. R. & Otto, M. (2004). Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell Microbiol* 6, 269–275.
- Wang, X., Preston, J. F., III & Romeo, T. (2004). The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J Bacteriol* 186, 2724–2734.
- Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. (2002). Extracellular DNA required for bacterial biofilm formation. *Science* 295, 1487.
- Wootton, M., Bennett, P. M., MacGowan, A. P. & Walsh, T. R. (2005). Reduced expression of the *atl* autolysin gene and susceptibility to autolysis in clinical heterogeneous glycopeptide-intermediate *Staphylococcus aureus* (hGISA) and GISA strains. *J Antimicrob Chemother* 56, 944–947.
- Yang, X. M., Li, N., Chen, J. M., Ou, Y. Z., Jin, H., Lu, H. J., Zhu, Y. L., Qin, Z. Q., Qu, D. & Yang, P. Y. (2006). Comparative proteomic analysis between the invasive and commensal strains of *Staphylococcus epidermidis*. *FEMS Microbiol Lett* 261, 32–40.
- Zhang, Y. Q., Ren, S. X., Li, H. L., Wang, Y. X., Fu, G., Yang, J., Qin, Z. Q., Miao, Y. G., Wang, W. Y. & other authors (2003). Genome-based analysis of virulence genes in a non-biofilm-forming *Staphylococcus epidermidis* strain (ATCC 12228). *Mol Microbiol* 49, 1577–1593.
- Ziebuhr, W., Heilmann, C., Gotz, F., Meyer, P., Wilms, K., Straube, E. & Hacker, J. (1997). Detection of the intercellular adhesion gene cluster (*ica*) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. *Infect Immun* 65, 890–896.