Genetic and phenotypic analysis of biofilm phenotypic variation in multiple Staphylococcus epidermidis isolates


INTRODUCTION

Staphylococcus epidermidis is the pre-eminent cause of infections involving prosthetic heart valves, intravascular catheters and other biomaterial-based devices. A fundamental step in the pathogenesis of S. epidermidis-mediated foreign body infections is the ability of the organism to adhere to and to produce biofilm on the surface of the biomaterial. Adherence of S. epidermidis to prosthetic devices is thought to occur in two distinct steps: (i) initial attachment to the biomaterial surface, and (ii) subsequent accumulation of bacterial cells involving intercellular adhesion. Factors involved in the initial attachment to the polymer surface include non-specific interactions as well as specific adhesins including SSP-1 (Veenstra et al., 1996), SSP-2 (Veenstra et al., 1996) and AtlE (Heilmann et al., 1997). In addition, the biomaterial rapidly becomes coated with cellular matrix proteins such as fibronectin, fibrinogen and vitronectin. Specific bacterial adhesins that interact with these proteins may play a role in initial adherence to the biomaterial (Nilsson et al., 1998; Williams et al., 2002). After initial adherence, certain strains of S. epidermidis produce an extracellular biofilm, or polysaccharide intercellular adhesin (PIA), which is synthesized by gene products of a four-gene (icaA, icaD, icaB, icaC) operon, ica. The pathogenic significance of biofilm formation has been established in a rat model (Rupp et al., 1999b) and a mouse foreign body infection model (Rupp et al., 1999a), where an ica transposon mutant was significantly less virulent than its isogenic wild-type parent. In addition, PNAG (previously termed PS/A), a compound closely related to PIA, has been demonstrated to...
have potential as a vaccine candidate against staphylococcal disease (Joyce et al., 2003; Maira-Litran et al., 2002; McKenney et al., 1999, 2000; Tojo et al., 1988).

Several studies have shown that levels of biofilm production differ among variants of the same strain (Christensen et al., 1987, 1990; Deighton et al., 1992a, b; Rupp et al., 1995; Ziebuhr et al., 1997, 1999). These variants have typically been isolated after overnight growth and plating on specialized media formulated to detect differences in ability to produce biofilm. Christensen et al. (1990) observed that S. epidermidis phenotypic variants arise at a frequency of approximately $10^{-3}$. These investigators indicated that S. epidermidis strain RP62A produced a spectrum of distinct colonial forms on a medium called Memphis agar. Each member of this series of colony phenotypes was found to have a corresponding level of slime, or biofilm, synthesis. In addition, all colonies within the RP62A biofilm spectrum were able to give rise to other members of the spectrum with differing biofilm-forming capabilities.

More recently, Ziebuhr and colleagues demonstrated that, in S. epidermidis RP62A, phenotypic variation of biofilm production was due to inactivation of icaC by IS256 in 30% of the variants (Ziebuhr et al., 1999). This process was also shown to be reversible. However, in the remaining 70% of phenotypic variants isolated from RP62A, and in strains lacking IS256, the mechanism by which phenotypic variation occurs remains unknown. Further research demonstrated that phenotypic variants isolated from S. epidermidis strains 161 and CSF41498 were associated with loss of ica transcription (Ziebuhr et al., 1997; Conlon et al., 2002a).

The present study sought to fulfill two primary objectives: (i) to determine the prevalence of phenotypic variation among multiple unrelated isolates of S. epidermidis and (ii) to quantify levels of ica and icaR transcription in these variants. Much of the previous research into variation of biofilm expression has focused upon one or two specific isolates. For these experiments, ten strains were chosen, six of which were IS256-negative. Furthermore, we tested the ability of these variants to give rise to other phenotypic forms on Congo red agar (CRA). To explore the possibility that alterations in the levels of ica transcription may be responsible for phenotypic variation within these isolates, and to exclude the known involvement of IS256, transcriptional studies were performed on variants from three strains lacking IS256. Interpretation of the data obtained from these studies suggests that phenotypic variation of biofilm formation is a widespread phenomenon within S. epidermidis, and mutations within ica and transcriptional regulation are primary mechanisms by which phenotypic variation of biofilm expression occurs within S. epidermidis.

### METHODS

**Strains used in this study.** Staphylococcal strains used in the study are shown in Table 1. Ability to form a biofilm (crusty phenotype on CRA, 

<table>
<thead>
<tr>
<th>Strain</th>
<th>IS256</th>
<th>Crusty (%)</th>
<th>Intermediate (%)</th>
<th>Smooth (%)</th>
<th>Colonies counted, n (x)</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU13652</td>
<td>+</td>
<td>1.846* (88.0)</td>
<td>0.553 (9.7)</td>
<td>0.296 (2.3)</td>
<td>1 810 (2)</td>
<td>This study/native valve endocarditis</td>
</tr>
<tr>
<td>DU17174</td>
<td>+</td>
<td>1.103* (97.6)</td>
<td>0.391 (2.1)</td>
<td>0.251 (0.2)</td>
<td>1 614 (3)</td>
<td>This study/blood</td>
</tr>
<tr>
<td>DU6331</td>
<td>+</td>
<td>2.104* (63.0)</td>
<td>0.592 (36.6)</td>
<td>0.409 (0.3)</td>
<td>2 303 (3)</td>
<td>This study/blood</td>
</tr>
<tr>
<td>RP62A</td>
<td>+</td>
<td>0.831 (1.6)</td>
<td>0.792 (14.4)</td>
<td>0.407* (84.1)</td>
<td>33 976 (10)</td>
<td>This study/native valve endocarditis</td>
</tr>
<tr>
<td>1457</td>
<td>–</td>
<td>1.918* (39.2)</td>
<td>0.689 (56.2)</td>
<td>0.313 (4.6)</td>
<td>3 742 (3)</td>
<td>Mack et al. (1995)/catheter-related sepsis</td>
</tr>
<tr>
<td>SE5</td>
<td>–</td>
<td>1.172* (40.8)</td>
<td>1.156 (54.7)</td>
<td>0.293 (4.6)</td>
<td>6 136 (10)</td>
<td>Rupp et al. (1995)/prosthetic valve endocarditis</td>
</tr>
<tr>
<td>PVE1</td>
<td>–</td>
<td>1.360* (90.0)</td>
<td>1.333 (8.7)</td>
<td>0.132 (1.3)</td>
<td>1 116 (3)</td>
<td>This study/prosthetic valve endocarditis</td>
</tr>
<tr>
<td>PVE6</td>
<td>–</td>
<td>1.405* (67.4)</td>
<td>0.691 (25.4)</td>
<td>0.211 (7.1)</td>
<td>2 982 (3)</td>
<td>This study/prosthetic valve endocarditis</td>
</tr>
<tr>
<td>SE55</td>
<td>–</td>
<td>1.233* (96.3)</td>
<td>0.226 (0.3)</td>
<td>0.152 (3.4)</td>
<td>2 089 (3)</td>
<td>This study/blood</td>
</tr>
<tr>
<td>DU14765</td>
<td>–</td>
<td>1.477* (60.4)</td>
<td>0.808 (33.6)</td>
<td>0.394 (5.3)</td>
<td>15 140 (10)</td>
<td>This study/native valve endocarditis</td>
</tr>
</tbody>
</table>

*Colonty phenotype of the strain at the outset of the 5 day incubation study.
see below) was considered the wild-type phenotype in all isolates except RP62A. In contrast to other studies, our RP62A isolate does not produce significant biofilm (Christensen et al., 1990). All isolates were found to be divergent as assessed by PFGE (methods described below). *Staphylococcus carnosus* TM300 (Götz & Schumacher, 1987) was used as a negative control.

**Detection of biofilm-negative variants on CRA.** Phenotypic variation of biofilm formation in *S. epidermidis* was detected on CRA [21 g Mueller–Hinton broth (Difco), 15 g granulated agar; 36 g sucrose (Sigma) and 0.8 g congo red (Sigma) per litre distilled water]. Initially, strains were cultured in 10 ml tryptic soy broth (TSB; Difco-Becton Dickinson) at 37 °C for 5 days without shaking, and were then plated onto CRA for 24 h at 37 °C and an additional 24 h at room temperature (Freeman et al., 1989). Phenotypic variants were selected based upon colony appearance following incubation (Supplementary Fig. A).

**Biofilm and haemagglutination assays.** Assays for the production of biofilm and haemagglutination were respectively performed according to the methods outlined by Christensen et al. (1990) and Fey et al. (1999).

**PFGE.** Genomic DNA suitable for PFGE was prepared using previously described methods (van Belkum et al., 1998) and digested with *SmaI* (New England Biolabs). Isolates were analysed on a Bio-Rad CHEF DR-III (Bio-Rad) using the following parameters: 6 V cm⁻¹; initial pulse time, 1 s; final pulse time, 30 s, for 20 h at 14 °C.

**Molecular methods.** Southern and Northern blots were performed according to established protocols (Sambrook et al., 1989). For Northern blots, total RNA was harvested from a culture in mid-exponential phase using a combination of the FastPrep FP120 (Bio 101) and RNeasy mini preps (Qiagen) and was subsequently treated with DNase (DNA-free; Ambion). A 1.29 kb probe encompassing the 3′ end of icaB and the 5′ end of icaD for Southern and Northern blot hybridization was prepared using primers 1 (5′-CAACAGTGAAGGCCATCTCC-3′) and 4 (5′-CCGAAATTTGTAATTTT-3′). Probes were labelled using DIG-dUTP (Roche). For RT-PCR, bacterial cells were harvested from a culture in mid-exponential phase and were stored in RNA later (Ambion) solution to prevent degradation of cellular RNA. After treatment with 50 µg lysorphin in 100 µl 0.5 M EDTA, total RNA was extracted using the method outlined in the GenElute Total RNA purification kit (Sigma). The DNA-free DNase treatment and DNase removal reagents (Ambion) were used to remove contaminating DNA from the RNA samples. RNaseque resuspension solution (Ambion) was used to store eluted RNA. For cDNA synthesis, the OneStep RT-PCR kit (Qiagen) was used according to the manufacturer’s recommendations. Reverse transcription was allowed to occur for 30 min at 55 °C, followed by 23–26 cycles of amplification with the following parameters: 94 °C for 20 s, 50 °C for 20 s and 72 °C for 20 s. Reactions were performed with the following oligonucleotides: for the detection of gyrB transcripts: 5′-TTATGTTGCGTAGAGTAGACA-3′ and 5′-CCGCTGAGTCGCCGAC-3′; for icaA transcripts: 5′-AAC AAGTGGAAGCCATCTCC-3′ and 5′-GATCTGTGTCATTCCCT -3′ and for icaR transcripts: 5′-GTGAAAGTTCGCTAATGGA-3′ and 5′-CCGAAATTTGTAATTTT-3′. In these reactions, gyrB was used as an internal standard, as it has been shown previously to be expressed constitutively (Conlon et al., 2002b). Each reaction was performed in triplicate with RNA made from separate broth flasks.

Chromosomal DNA was prepared from *S. epidermidis* strains using previously published protocols (Galetto et al., 1987). All restriction enzymes were purchased from Roche. Strains were tested for the presence of IS256 by PCR using an established primer set (Ziebuhr et al., 1999). Full-length sequencing of the ica operon from phenotypic variants (encompassing icaR) was performed using primers derived from the RP62A ica sequence (GenBank accession no. U43366). The PCR products were sequenced using an Applied Biosystems 377 sequencer. Primers used to amplify the specific sequences of icaR, icaA, icaD, icaB and icaC are as follows: icaR forward 5′-CTGAAATGTTGTA CATACTAG-3′; icaR reverse 5′-CTTACCTTTCTGTTAGTTAGG-3′; icaA forward 5′-CTGATAACAACATGTATGTCG-3′; icaA reverse 5′-GAAATGTAAGAGTTCCC-3′; icaD forward 5′-TTGACAC TGCTAGCAAAAG-3′; icaD reverse 5′-CTCCGAGTATGATGTTGATG -3′; icaB forward 5′-GGCTGATGACATATGGAACC-3′; icaB reverse 5′-GGCTGATTGCAGTGTCGAC-3′; icaC forward 5′-ATAAACCT GAATTAGTGTATT-3′; icaC reverse 5′-CCATAGCTTGAATAAGGG -3′. The PCR primers above amplified products of 743 bp (icaR), 1425 bp (icaA), 646 bp (icaD), 945 bp (icaB) and 1017 bp (icaC).

**Enrichment assay.** Enrichment assays for biofilm-positive revertants were performed as described by Christensen et al. (1990). Briefly, phenotypic variants of biofilm-positive strains were inoculated into 5 ml TSB and incubated overnight at 37 °C. The next morning, the broth was decanted and the culture tube was refilled with 5 ml fresh TSB. After 6 h incubation at 37 °C, the tube was vortex-mixed, diluted and plated on CRA. After incubation, the phenotype of the colonies was scored based upon appearance on CRA.

**RESULTS AND DISCUSSION**

Phenotypic variation is a widespread phenomenon in bacterial gene regulation. These mechanisms, which include slipped-strand mispairing, site-specific DNA rearrangements, and gene conversion, are thought to have evolved to evade the host immune response (Hallet, 2001; Henderson et al., 1999; Robertson & Meyer, 1992). In addition to having a possible role in the natural dispersal of *S. epidermidis* in its native niche, phenotypic variation of biofilm production within the staphylococci may have pathogenic significance in facilitating dispersal of non-adherent cells and colonization of other fertile areas, resulting in bacteraemia and metastatic disease. Results from the current studies demonstrated the extent to which phenotypic variation of biofilm expression occurs in strains of *S. epidermidis*. Further, the amount of variation that is attributable to downregulation of ica transcription was determined.

**Detection of phenotypic variants on CRA**

*S. epidermidis* strains used in this study were chosen on the basis of production of biofilm and presence or absence of IS256 (Table 1). Phenotypic variants were detected on CRA after inoculation of at least two tubes containing TSB with a single colony possessing the wild-type colony phenotype. These tubes were incubated for 5 days at 37 °C without shaking. Prolonged incubation enriches strains for the presence of phenotypic variants (P. D. Fey, unpublished observation). Upon growth on CRA, various colony phenotypes emerged (Supplementary Fig. A). Crusty colonies tended to appear dry, black and filamentous on CRA, while smooth colonies were red and circular with entire edges. Numerous colonies termed intermediate, comprising a spectrum from near-crusty to near-smooth, were judged to have appearances between these two classes. At least one colony phenotype was chosen from each strain for a biofilm assay (Table 1). With strains DU14765, 1457, DU13652, PVE1, SE5, PVE6 and RP62A, multiple [DU14765 (n = 48);
1457 (n = 5); PVE1 (n = 11); SE5 (n = 32); PVE6 (n = 16); RP62A (n = 17) colonies of crusty, intermediate and smooth phenotypes were tested. Biofilm values given in these cases are means. It has been previously demonstrated that production of PIA strongly correlates with the production of biofilm in the Christensen biofilm assay (Cramton et al., 1999; Helmann et al., 1996; Mack et al., 1996). The biofilm assays demonstrated that the three colony phenotype classes produced different amounts of biofilm and, thus, PIA. Crusty phenotypes were shown to be highly biofilm-positive, while smooth colonies were biofilm-negative. Intermediate phenotypes were found to have biofilm levels between these values.

In order to determine how the population had changed upon 5 days incubation, numbers of colonies possessing each colony phenotype were counted, and percentages are listed in Table 1. Half of the strains under investigation, including DU6331, 1457, SE5, PVE6 and DU14765, frequently gave rise to colony phenotypes other than the wild-type. The other half, composed of DU13652, DU17174, RP62A, PVE1 and SE5, were more ‘phase-locked’ in that over 80% of the colonies isolated retained the wild-type phenotype.

Detection of a spectrum of biofilm production among phenotypic variants

As outlined by Christensen et al. (1990), a spectrum of phenotypic variants with differing levels of biofilm production was isolated from an IS256-positive strain, RP62A, on Memphis agar. To see whether a similar spectrum could be isolated from strains of S. epidermidis that lacked IS256, four S. epidermidis isolates were further characterized. As a reference, the IS256-positive strain RP62A was included, as well as four IS256-negative strains, DU14765, PVE1, SE5 and PVE6. After 5 days incubation in TSB as described above, and growth on CRA, a minimum of three colonies from each of the three colony phenotypes were chosen for biofilm assays. These results are shown in Fig. 1. RP62A produced a spectrum of colonies on CRA with varying levels of biofilm production when tested in the biofilm assay. The colonies sampled from the four IS256-negative isolates were found to resemble similar, yet wider, spectra. It was also found that, in some cases, colonies judged to be intermediate in appearance actually produced more biofilm than that of the crusty phenotype. Invariably, however, smooth colonies were always found to produce little biofilm. The ica-negative S. carnosus strain TM300 was used as a negative control.

Analysis of phenotypic switching frequency between colonial forms

To explore the possibility that members of each colonial form could give rise to the other two forms, one crusty (SE5 Cr), two intermediate (SE5 Int-1 and SE5 Int-2) and one smooth (SE5 Sm-1) colonies from S. epidermidis strain SE5 were inoculated into TSB tubes without shaking at 37 °C. Arithmetic means from biofilm assays conducted in triplicate indicated that these isolates had readings of 1-910 (SE5 Cr), 1-602 (SE5 Int-1), 0-671 (SE5 Int-2) and 0-251 (SE5 Sm-1). Aliquots were plated on CRA after 1 and 5 days of incubation (Supplementary Fig. B). After 1 day of incubation, the phenotype of all colonies was identical to that of the parent isolate. By the fifth day, however, each colony phenotype showed the ability to produce the other two colony forms. In particular, the intermediate forms were found to be more likely to give rise to more intermediate colony phenotypes, with a smaller percentage of crusty and smooth forms. The crusty isolate was shown to produce intermediate phenotypes with the greatest frequency, while just over 50% of the colonies isolated from the smooth phenotype after 5 days were smooth.

ica transcriptional studies on phenotypic variants

To investigate the possibility that lowered levels of biofilm synthesis within the intermediate and smooth colony forms were due to decreased transcription of ica, transcriptional studies were performed on various colonial forms from the IS256-negative strains, 1457, DU14765 and SE5. Biofilm readings (performed in triplicate) for strains under investigation are shown in Table 2. Isolates used in these studies were shown to lack large insertions or deletions within ica as assessed by specifically amplifying icaA, icaA, icaD, icaB and icaC (data not shown). Furthermore, PFGE demonstrated that each phenotypic variant was derived from the parental strain (data not shown). Using conventional RT-PCR analysis on total cellular RNA from these strains, it was found that, in general, there was an association between decreased biofilm synthesis and lowered transcription of ica in relation to wild-type (Fig. 2a–c). For example, in 1457 Int-1 and Int-2, although equal amounts of transcript from the constitutively expressed gyrB gene were observed, significantly less ica
transcript was noted when compared with the Cr wild-type strain. This pattern also held for DU14765 Int-1, Int-2 and Sm; and SE5 Int-1, Sm-1, Sm-2 and Sm-3, when compared with wild-type. In contrast, the 1457 Int-3 isolate, though found to produce less biofilm than its crusty counterpart, produced equal amounts of transcript to wild-type. Further, SE5 Int-2 was shown to generate approximately twice the amount of ica transcript of SE5 Cr, yet it made one-third the amount of biofilm. Conversely, though the DU14765 Cr-1 isolate made more biofilm than DU14765 Cr-2, its ica transcript levels were lower.

### Phenotypic variation in strain SE5

Smooth phenotypic variants from SE5 were studied further. An additional seven smooth variants were isolated (from seven separate broth cultures) from SE5. RT-PCR demonstrated that ica transcription was significantly decreased in three of these isolates; however, ica transcription was similar to wild-type expression in the remaining four smooth variants (data not shown). Three of these seven phenotypic variants, SE5 PV2, SE5 PV3 and SE5 PV10, were chosen for further study. These phenotypic variants were determined to be of SE5 lineage as assessed by PFGE (data not shown). The biofilm assay demonstrated that the three SE5 phenotypic variants produced significantly less biofilm, and thus PIA, compared to SE5 (Supplementary Table). In addition, because PIA has been shown to confer haemagglutination (Fey et al., 1999; Mack et al., 1999), all three phenotypic variants were tested for the ability to haemagglutinate sheep red blood cells. All phenotypic variants had a significantly lower haemagglutination titre compared with SE5, confirming the results of the biofilm experiments (data not shown). ica RT-PCR demonstrated that two of these isolates, SE5 PV3 and SE5 PV10, produced wild-type quantities of transcript, whereas SE5 PV2 produced significantly reduced ica transcript (Fig. 2d). To confirm these data, Northern blot analysis was performed using an icaAD DNA probe. SE5 PV3 and SE5 PV10 produced a 3-4 kb ica transcript at levels similar to that of wild-type SE5, whereas SE5 PV2 did not produce detectable ica transcript (data not shown). As assessed by PCR, the ica operon did not have detectable insertions or deletions (data not shown). In addition, Southern blot analysis using an icaB DNA probe against chromosomal DNA digested with BamHI demonstrated that the ica operon had not translocated to another region of the chromosome (data not shown).

### Sequencing and enrichment assay of SE5 phenotypic variants

Full-length sequencing of the ica operon (icaR–icaC) was performed on SE5 (GenBank accession no. AY138959) and the three phenotypic variants under investigation. The ica region from SE5 was found to have a 99.7 % amino acid identity to the ica region from RP62A. DNA sequencing revealed that SE5 PV3 had an 8 bp deletion at the 3’ end of icaA (RP62A ica sequence bp 1839–1846), which presumably caused a frame shift (Supplementary Fig. C). SE5 PV10 had a single base pair change at RP62A ica sequence bp 1968 (C→A) compared with wild-type SE5. This causes a silent mutation [GUC (valine)→GUA (valine)] and is the second coding triplet in icaD. No sequence divergence was found within the ica region of SE5 PV2. Since mutations were found within both SE5 PV3 and SE5 PV10, it was hypothesized that

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**Table 2. Colony phenotype and biofilm production of isolates used in transcriptional studies**

Mean biofilm values obtained represent absorbance readings at 490 nm after staining with crystal violet. Assays used in calculating the mean biofilm were performed at least in triplicate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colony phenotype on CRA</th>
<th>Mean biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1457 Cr</td>
<td>Crusty</td>
<td>1.918</td>
</tr>
<tr>
<td>1457 Int-1</td>
<td>Intermediate</td>
<td>0.920</td>
</tr>
<tr>
<td>1457 Int-2</td>
<td>Intermediate</td>
<td>0.834</td>
</tr>
<tr>
<td>1457 Int-3</td>
<td>Intermediate</td>
<td>0.312</td>
</tr>
<tr>
<td>DU14765 Cr-1</td>
<td>Crusty</td>
<td>1.463</td>
</tr>
<tr>
<td>DU14765 Cr-2</td>
<td>Crusty</td>
<td>1.154</td>
</tr>
<tr>
<td>DU14765 Int-1</td>
<td>Intermediate</td>
<td>0.530</td>
</tr>
<tr>
<td>DU14765 Int-2</td>
<td>Intermediate</td>
<td>0.360</td>
</tr>
<tr>
<td>DU14765 Sm</td>
<td>Smooth</td>
<td>0.181</td>
</tr>
<tr>
<td>SE5 Cr</td>
<td>Crusty</td>
<td>1.910</td>
</tr>
<tr>
<td>SE5 Int-1</td>
<td>Intermediate</td>
<td>1.602</td>
</tr>
<tr>
<td>SE5 Int-2</td>
<td>Intermediate</td>
<td>0.671</td>
</tr>
<tr>
<td>SE5 Sm-1</td>
<td>Smooth</td>
<td>0.251</td>
</tr>
<tr>
<td>SE5 Sm-2</td>
<td>Smooth</td>
<td>0.355</td>
</tr>
<tr>
<td>SE5 Sm-3</td>
<td>Smooth</td>
<td>0.185</td>
</tr>
</tbody>
</table>

**Fig. 2.** ica transcriptional analysis of phenotypic variants isolated from 1457 (a), DU14765 (b), SE5 (c) and additional smooth variants from SE5 (d). The constitutively expressed gene gyrB was used as a control.
wild-type (crusty) or intermediate colonies would not arise from these strains, in contrast to SE5 PV2. To test this hypothesis, an enrichment assay was used in order to detect biofilm-positive, crusty revertants (biofilm-negative to biofilm-positive) from the three smooth phenotypic variants under study, SE5 PV2, SE5 PV3 and SE5 PV10. Approximately 100 colonies from each strain were analysed per day on CRA for 17 days to detect biofilm-positive colonies. Crusty colonies were readily detected for strain SE5 PV2 by day 4 and reached a maximum of 90% of the population by day 13 (Supplementary Fig. D). In contrast, no biofilm-positive revertants (intermediate or crusty phenotype) were found using strains SE5 PV3 and SE5 PV10. After further enrichment assays were conducted, one biofilm-positive revertant strain (SE5 PV10-R1) (Supplementary Table) was isolated from SE5 PV10. Sequencing of the icaD region from SE5 PV10-R1 revealed that the variant adenine residue was replaced with the wild-type cytosine residue.

**icaR expression in smooth phenotypic variants**

To explore the possibility that increased levels of IcaR, a known repressor of ica transcription, may act to suppress ica expression in smooth phenotypic variants, icaR transcription levels were investigated by RT-PCR. For these studies, smooth phenotypic variants with decreased ica operon expression (SE5 PV2-like) from five strains (SE5, RP62A, 1457, DU13652 and DU17174) were compared to the wild-type. Results from the RT-PCR studies are shown in Fig. 3. Interestingly, transcription of the icaR gene was unaffected in ica transcriptional variants. We previously reported the absence of icaR gene expression in two biofilm-negative variants of RP62A (IS256-positive) (Conlon et al., 2002b). However, wild-type levels of icaR gene expression were detected in transcriptional variants produced by all of the strains in this study, including RP62A. These data further suggest that altered icaR transcriptional regulation does not contribute to the biofilm-negative phenotype of phenotypic variants. As expected, icaR transcription was also not affected in smooth phenotypic variants with wild-type levels of icaA transcript (SE5 PV3/PV10-like, data not shown) These data suggest that, in ica transcriptional variants isolated from IS256-negative and IS256-positive strains, icaR transcription is not significantly affected, whereas ica operon expression is significantly diminished.

Interpretation of the data presented within this study suggests that phenotypic variation of biofilm synthesis is a prevailing phenomenon within strains of *S. epidermidis*. This was shown in both IS256-positive and -negative strains. These observations suggest that phenotypic variation of biofilm expression is not merely an ON—OFF switch, but is reversible and may be fine-tuned to generate various levels of biofilm synthesis. We have noted that phenotypic variants are more difficult to isolate (frequency ranging from $10^{-3}$ to $10^{-6}$) when incubated for only 24 h in TSB (P. D. Fey, unpublished observation). In contrast, if *S. epidermidis* isolates are incubated for up to 5 days at 37 °C, phenotypic variants are a recognizable portion of the population (see Table 1). These findings may suggest that intermediate and smooth types arise only through maturation of biofilm on the surface of the glass tubes. It is well documented that differential gene regulation occurs during biofilm maturation within *Pseudomonas aeruginosa* and other Gram-negative bacteria (Stoodley et al., 2002; Whiteley et al., 2001). Further experimentation is necessary to explore whether phenotypic variation within *S. epidermidis* is due to cell–cell signalling within a mature biofilm. However, this report demonstrates that intermediate and smooth phenotypic variants are stable even after 24 h of incubation in broth, which is not consistent with the concept that phenotypic variants were derived through a quorum sensing or cell–cell signalling event.

Phenotypic variation was shown to be mediated in several cases through down-regulation of ica transcript. Nine of eleven phenotypic variants isolated from three strains lacking IS256 and displaying decreased amounts of biofilm production were found to have a corresponding decrease in ica transcription. In contrast, in 1457 Int-3 and SE5 Int-2, comparable (1457 Int-3) or increased (SE5 Int-2) levels of ica transcript compared with wild-type were detected despite the lower levels of biofilm production. Reasons for the decreased amounts of biofilm in these isolates are open to speculation, but may occur as the result of mutations within ica or icaR. Also unclear is the observation that one phenotypic variant, DU14765 Cr-1, produced less ica transcript than wild-type despite producing more biofilm.

Since smooth phenotypic variants consistently produced
little biofilm compared with intermediate phenotypic variants, smooth phenotypic variants from strain SE5 were further studied to determine whether a correlation existed between lack of ica transcription and biofilm production. These studies suggested that there are at least two types of phenotypic variants found in IS256-negative S. epidermidis. Class I phenotypic variants, represented by SE5 PV2, can be considered true phenotypic variants as they revert readily to wild-type biofilm production as demonstrated in the enrichment assay. In addition, we found that SE5 PV2 did not produce detectable ica transcript, and ica sequence data revealed no sequence divergence in comparison to SE5. Knobloch et al. (2001) have demonstrated that the icaB regulon plays a role in regulation of baseline ica transcription in S. epidermidis strain 1457. These investigators demonstrated that insertional inactivation of rsbU results in a biofilm-negative phenotype and lack of ica transcription when grown in TSB and TSB supplemented with 3 % NaCl. However, Pla production and ica transcript could be induced when the rsbU mutant was grown in TSB supplemented with 4 % ethanol, suggesting that ethanol and NaCl activate ica via different regulatory pathways. Interestingly, ica transcription and biofilm formation can be induced in SE5 PV2 when grown in TSB supplemented with either 3 % NaCl or 4 % ethanol (data not shown). At this point, it remains unclear whether icaB is involved in class I phenotypic variation. Alternatively, a series of sequential mutations may be acquired in the transcriptional regulatory genes of phenotypic variants, leading to the wide ranges of biofilm expression and the intermediate levels of ica transcript observed in some variants. Finan et al. (2002) recently demonstrated that acquisition of mutations was found to govern the switch from heterotypic to homotypic resistance to oxacillin within the staphylococci. The second class of phenotypic variants (class II), represented by SE5 PV3 and SE5 PV10, cannot be considered true phenotypic variants as they do not revert readily to biofilm-forming cells and seem to be caused by apparently random mutation or deletion within the ica operon.

The observation that icaR expression levels are similar in biofilm-positive and class I phenotypic variants suggests that altered regulation of icaR transcription is not responsible for repression of ica operon expression in phenotypic variants produced by these strains (Conlon et al., 2002a, b). We have previously reported that alterations in the transcriptional and post-transcriptional activity of IcaR, which is a member of the TetR family of transcriptional regulators, contribute to environmental activation of ica operon expression. The carboxy-terminal domain of TetR family proteins can interact with compounds that modulate their regulatory activity (Aramaki et al., 1995; Grkovic et al., 1998; Rouch et al., 1999). Therefore, even though icaR transcription was unaffected in class I phenotypic variants and there was no nucleotide sequence variation in the icaR genes of the variants, it is still possible that altered activity of the IcaR protein, perhaps in the presence or absence of an inducing compound(s), may contribute to ica operon repression.

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