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High Frequency Spontaneous Deletions within the *IcaADBC* Operon of Clinical *Staphylococcus epidermidis* Isolates.

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Abstract

Staphylococcus epidermidis has been shown to undergo a phase variation correlating with expression of the *icaADBC* operon which contributes to biofilm formation. Biofilm formation of *Enterococcus faecalis* is related to heterogeneity in electrophoretic mobility. Here the relationship between phase variants of clinical isolates of *S. epidermidis*, *icaADBC* presence and electrophoretic mobility distributions is investigated. Of 105 *S. epidermidis* clinical isolates, 5 showed phase variation on Congo Red agar plate. Biofilm forming capability of the black colonies and inability of the red colonies were confirmed using a microtiter plate assay and confocal laser scanning microscopy. Upon analysis of electrophoretic mobility distributions, the black colonies displayed heterogeneity at pH 2 which was absent in the red colonies of the same strain. Surprisingly, it was shown that in all red colonies had lost the *icaADBC* genes. Determination of gene copy number using Real Time PCR targeting *icaA* showed reduction of gene copy within a culture with phase variation. In conclusion, using three fundamentally different approaches phase variation of the five clinical isolates was observed. Variants appeared through loss of *icaA* and *icaC* gens. To our knowledge this is the first report indicating *S. epidermidis* strains irreversible switching from biofilm + to biofilm – phenotype by deletion of *ica* genes.

Key words: deletion, *ica* genes, *Staphylococcus epidermidis, IcaADBC* operon

Introduction

The various physicochemical properties of bacterial cell surfaces, such as surface free energy, ζ-potential, hydrophobicity and elemental and molecular compositions may affect the adherence of *Staphylococcus. epidermidis* (van der Mei *et al*., 1989). The ζ-potential can be calculated from measured electrophoretic mobilities of individual cells within a suspension, allowing the observation of culture heterogeneity. In the determination of a bacterial property, such as cell surface charge or hydrophobicity, pure bacterial cultures are generally considered populations of identical organisms, although it is known that several strains display distinct subpopulations even in pure cultures. Different subpopulations within one culture can differ in flagellation (Streger *et al*., 2002), natural competence (Dubnau, 1991), autofluorescence (Kell *et al*., 1991), or electrophoretic mobility (Cowan *et al*., 1992), (van Merode *et al*., 2006b). Importantly, heterogeneity in electrophoretic mobility is linked to biofilm formation of *Enterococcus faecalis* (van Merode *et al*., 2006a). None of the mechanisms governing the above mentioned heterogeneity have been elucidated.

Staphylococcus epidermidis is a normal constituent of the healthy human skin and mucosal microflora. In recent years, however the bacterium has emerged as a frequent etiologic agent of infection associated with

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indwelling medical devices (Kozitskaya *et al.*, 2004). 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 (Handke *et al.*, 2004), by polysaccharide intercellular adhesin (PIA) which mediates the contact of the bacterial cells with each other, resulting in the accumulation of a multilayered biofilm. PIA, an important virulence factor of *S. epidermidis* (Heilmann *et al.*, 1996), is a sugar polymer consisting of a beta-1,6-linked glucosaminoglycan backbone substituted with different side groups (Mack *et al.*, 1996). The enzymes involved in PIA synthesis were found to be encoded by the *ica* operon comprising the *icaA, icaD, icaB*, and *icaC* genes. PIA is suggested to be an important virulence factor of *S. epidermidis* (Heilmann *et al.*, 1996).

It has been shown that *S. epidermidis* isolates exhibit an extraordinary high phenotypic and genotypic flexibility, which is assumed as an evolutionary advantage that helps staphylococci to adapt to changing environmental conditions. The phenotypic variants can differ in terms of colony morphology, growth rate, hemolysis, biofilm formation, and antibiotic susceptibility (Christensen *et al.*, 1990). In biofilm-producing *S. epidermidis* strains, the expression of the intercellular adhesion genes (*icaADBC*) were found to undergo phenotypic variation that correlates with a change of colony morphology on Congo Red agar (CRA) and an altered biofilm formation (Ziebuhr *et al.*, 1997). Currently several mechanisms have been identified that regulate *ica* expression; insertion of the transposon IS256 in the structural operon (Ziebuhr *et al.*, 1999), or in genes encoding proteins that regulate *icaADBC* expression such as *rsbU* and *sarA* (Conlon *et al.*, 2004). However, all of these are reversible meaning that after repeated subculturing, biofilm + phenotype can be regained from biofilm – inocula.

Because of the link between phenotypic variations and biofilm formation in *S. epidermidis* and culture heterogeneity towards electrophoretic mobility and biofilm formation in *E. faecalis*, we set out to determine the role of phase variation; i.e. *icaADBC* expression, in development of heterogeneous cultures of *S. epidermidis*.

Materials and Methods

Strains and growth conditions

A total of 105 clinical isolates of *S. epidermidis* were obtained from blood culture collection, liquor cerebrospinal, pus and urine, from Microbiology Department, Universitas Gadjah Mada, Yogyakarta, Indonesia. From these samples, five switching isolates were selected, listed in Table 1. Identification of these isolates were confirmed by conventional methods, including Gram staining, colony appearance on blood agar, coagulase test, DNase test and sensitivity test to Novobiocin and polymixin disk antibiotic (Bannerman, 2003). The isolates were stored in -80°C with glycerol to a final concentration of 15 % (v/v). Strains were routinely grown on blood agar plates or in tryptone soy broth (TSB; Oxoid) at 37°C.

Phenotypic characterization

The strains were cultured on Congo Red agar (CRA) plates, prepared by adding 0.8 g of congo red (Sigma), 12 g bacto agar (Becton, Dickinson) and 36 g of saccharose (Merck) to 1 l of brain heart infusion (Oxoid). The plates were subsequently incubated for 24 h at 37°C and additionally overnight at room temperature. We adopted the evaluation of colonies morphology from Freeman et al. (Freeman *et al.*, 1989) a reference six color: very black (vb), black (b), almost black (ab), bordeaux (brd), red (r), and very red (vr). Colonies colored vb and b were considered indicative of normal slime-producer strains, while those colored

ab were indicative of a weak slime production activity, vr to brd colonies were classified as non-producer of slime. The CRA plate test was done in duplicate, and consistent results were obtained.

Biofilm assay

A quantitative biofilm assay was performed as described previously (Christensen *et al.*, 1987), but slightly modified. Briefly, 1:100 dilutions of overnight cultures in TSB were used to inoculate wells in a microtiter polystyrene plate (Falcon). After incubation for 24 h at 37°C, the plates were gently washed two times with phosphate-buffered saline (PBS, composition of the buffer is needed), and stained with 1 % crystal violet solution; the excess stain was washed off with demineralized H_2O. Afterwards the adherent cells were resuspended with acid-isopropanol (5% 1M HCl in isopropanol), and finally, the absorbance (Abs) was measured at 595 nm in an ELISA reader.

Confocal laser scanning microscopy (CLSM)

Biofilm cells were grown in 12-well polystyrene tissue culture plates (Costar; Corning, Inc.) for 24 h in 2 ml TSB medium. The wells with the biofilms were gently washed twice with PBS, the biofilms were stained with the bacterial Live/Dead stain BacLight (Molecular Probes, Leiden, The Netherlands) and incubated for 30 min in the dark. Confocal images were collected through the bottom of the plate using a Leica TCS SP2 microscope with a 40x objective (van Merode *et al.*, 2006a)

Electrophoretic mobility determinations

The electrophoretic mobility of the isolates were measured as described previously (van Merode *et al.*, 2006b). Briefly, each bacterial strain was resuspended in 30 ml 10 mM potassium phosphate, set to a pH of 2, 3, 4, 5, 7 or 9 using either HCl or KOH, to a density of approximately $1x10^9$ cells/ml. The electrophoretic mobility at 150 V of the resuspended bacteria was then measured using a Lazer Zee Meter 501 (PenKem) which uses the scattering of incident laser light to detect the bacteria. Strains were designated to have heterogeneous electrophoretic mobility distributions if two or more populations could be distinguished that comprised at least 10 % of the total number of cells measured. Isoelectric point (IEP) estimations were made by extrapolating the graph to the pH at which the electrophoretic mobility is 0 (i.e. the bacteria do not move in the electric field).

DNA isolation

S. epidermidis strains were grown overnight at 37°C on TSB after which cells were harvested by centrifuging, 1.0 ml of each broth culture at 21.000 x g in an Eppendorf tabletop centrifuge. Cells were resuspended in 75 µl of H_2O and 25 µl of lysostaphin 1 mg/ml in H_2O (Sigma) was added, subsequently the samples were incubated at 37°C for 10 min. Afterwards 10 µl proteinase K solution 1 mg/ml in H_2O (Sigma) and 200 µl of 0.1 M Tris-HCl (pH 7.5) were added, incubation proceeded for a further 30 min at 37°C. Samples were then heated to 100°C for 5 min. The cell debris and unlysed cells were removed by centrifugation (21.000 x g for 10 min), after which 2-3 volume of cold ethanol was added to precipitate the DNA. The DNA was dissolved in TE demineralized H_2O and the concentration was determined using a Nanodrop UV/Vis spectrophotometer (Isogen). Samples were stored at -20°C. For colony DNA isolation the procedure described by (Skow *et al.*, 2005) was used. Briefly, single colonies of *S. epidermidis* were resuspended in 20 µl sterile TE, boiled for 10 min and used directly as PCR template.

PCR and Real Time PCR

To determine the mechanism governing the appearance of heterogeneous cultures, the strains were tested for presence of the IS256 insertional element as described previously (Kozitskaya *et al.*, 2004). None of the tested strains contained this element (not shown). Standard PCR, performed on an ICycler

Table 1A. Primer sequences for PCR used in this study (*=(Handke *et al.*, 2004), #=(Kozitskaya *et al.*, 2004)).

Primer	Sequence (5′-3′)	Product size (bp)
icaA forward*	CAGTATAACAACATTCTATTG	1425
icaA reverse*	GAGAATTGATAAGAGTTCC	
icaC forward*	ATAAACTTGAATTAGTGTATT	1017
icaC reverse*	CCATAGCTTGAATAAGGG	
IS256 forward #	TGAAAAGCGAAGAGATTCAAAGC	1102
IS256 reverse #	ATGTAGGTCCATAAGAACGGC	

Table 1B Primers and their sequences for Real Time PCR used in this study.

Primer	Sequence (5′-3′)	Product size (bp)	Annealing Temp (°C)
icaA-1 forward	GGAAGTTCTGATAATACTGCTG	124	56
icaA-1 reverse	GATGCTTGTTTGATTCCCTC		
gyrB-3 forward	GGAGGTAAATTCGGAGGT	129	57.1
gyrB-3 reverse	CTTGATGATAAATCGTGCCA		

machine (Biorad,) for the *icaA*, *icaC* and *gyrB* genes was performed using the primers listed in Table 1A. Products were analyzed after standard agarose gel electrophoresis. Real Time PCR was performed on a MyCycler PCR machine (Biorad) using SYBR green (Biorad) and the primer sets (Isogen-lifescience, Maarsen, The Netherlands) designed using Perlprimer (Marshall, 2004) listed in Table 1B. For both types of PCR, total genomic DNA isolations were used as templates at 25 ng/ sample. The following program was used for Real Time PCR: 95°C for 3 min, 40 cycles of 95°C for 15 s, primer specific annealing temp (Table 2) for 15 s, elongation at 72°C for 15 s. C_T values were generated by the computer and the gene copy was determined relative to *gyrB* gene copy using the $2^{-\Delta\Delta C}{}_T$ method as described previously (Livak and Schmittgen, 2001).

Results

A total of 105 clinical isolates, identified as *S. epidermidis* were screened for biofilm formation capability using the CRA assay. Five of these isolates showed phase variation, indicated by the appearance of mixed black and red colonies on a single plate. When a black colony was grown overnight in TSB, a mixture of red and black colonies appeared. The frequency of variation was determined by colony counting and ranged from 15-93% (Table 2).

When a red colony was grown overnight in TSB medium, only red colonies appeared on CRA plates. Even prolonged incubation (up to 5 days) or repeated subculturing for 5 days did not result in any black colonies re-appearing from a red inocullum.

Table 2. List of *S. epidermidis* isolates that show phase variation included in this study with site of isolation, phenotype on CRA plates and percentage of variation observed on these plates.

Strain	Origin of isolation	Phenotype on CRA	Percentage of variation (%)
45	pus	black and red	39±5
196	pus	black and red	93±5
493	cerebrospinal fluid	black and red	15±1
906	blood	black and red	79±2
1098	cerebrospinal fluid	black and red	93±11

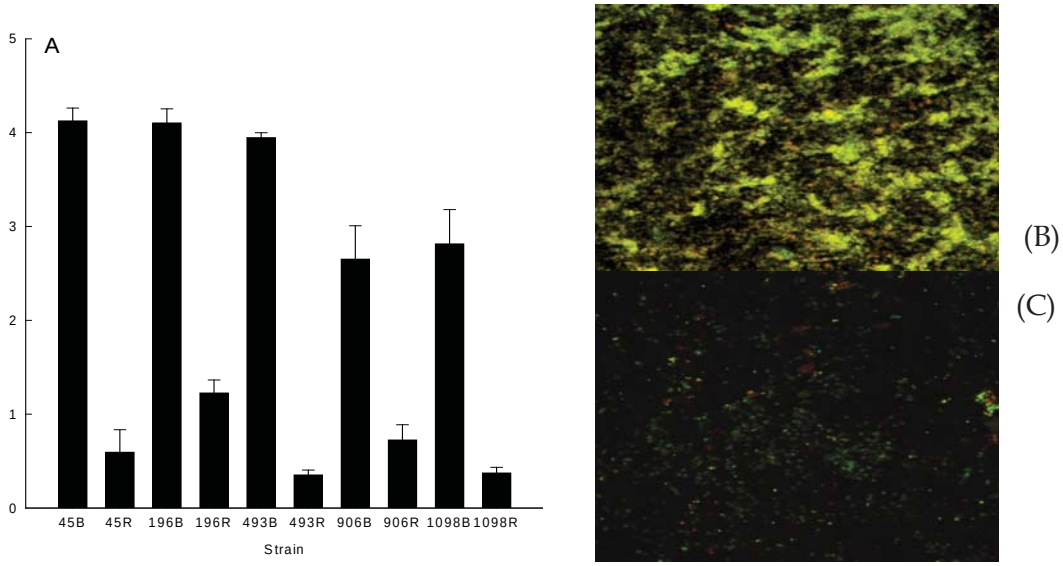

Figure 1. Quantitative biofilm formation of *S. epidermidis* strains that show phase variation. Biofilms were started either with black colonies (indicated with B) or red colonies (indicated with R) derived from the same strain (Panel A). The values are means of triplicate wells (± standard deviations). Panel B and C shows a representative CLSM image of a biofilm started with a black colony (panel B) and a red colony (panel C), of strain 45.

Table 3**.** Percentage distribution of subpopulations of the heterogeneous *S. epidermidis* strains in electrophoretic mobility measurement at pH 2 and (estimated) isoelectric points (IEP) Subpopulations #1 represents the most negative population, while subpopulation #2 represents the less negative population. The population sizes are mean ± standard deviation of three independent experiments.

S. epidermidis strains	Subpopulation #1		Subpopulation #2	
	IEP	Size (%)	IEP	Size (%)
45B	1.5	44±9.5	2	56±9.5
196B	<1.5	78±4.6	2	12±4.6
493B	<1.5	26±4	2	74±4
906B	1.5	85±3.6	2	15±3.6
1098B	1.5	82±3.6	2	18±3.6

Detection of biofilm formation

The biofilm assay performed in this study is a quantitative microtiter assay. Black colonies resulted in high amounts of biofilm formation, in contrast to the red colonies that were poor biofilm formers (Figure 1A). To exclude any effects of crystal violet bound by the PIA (present in black, absent in red colonies) the biofilms were studied by CLSM following Live/Dead staining (Figure 1B and 1C). The CLSM results show that the large difference in crystal violet staining is due to a large difference in biofilm formation and not the presence/absence of PIA.

Electrophoretic mobility measurement

The pH-dependent electrophoretic mobility distributions of the five phase variation colonies (black) and the non-variation (red) colonies were determined (Figure 2). Interestingly, at pH 2 all black colonies showed heterogeneous electrophoretic mobility distributions, which was absent in the red derivatives of the same strain. All phase variation colonies were heterogeneous with regard to their electrophoretic mobility at pH 2 and had one subpopulation with an estimated isoelectric point (IEP) of 2, while the sec subpopulation showed a slightly lower IEP (Figure 2 and Table 3).

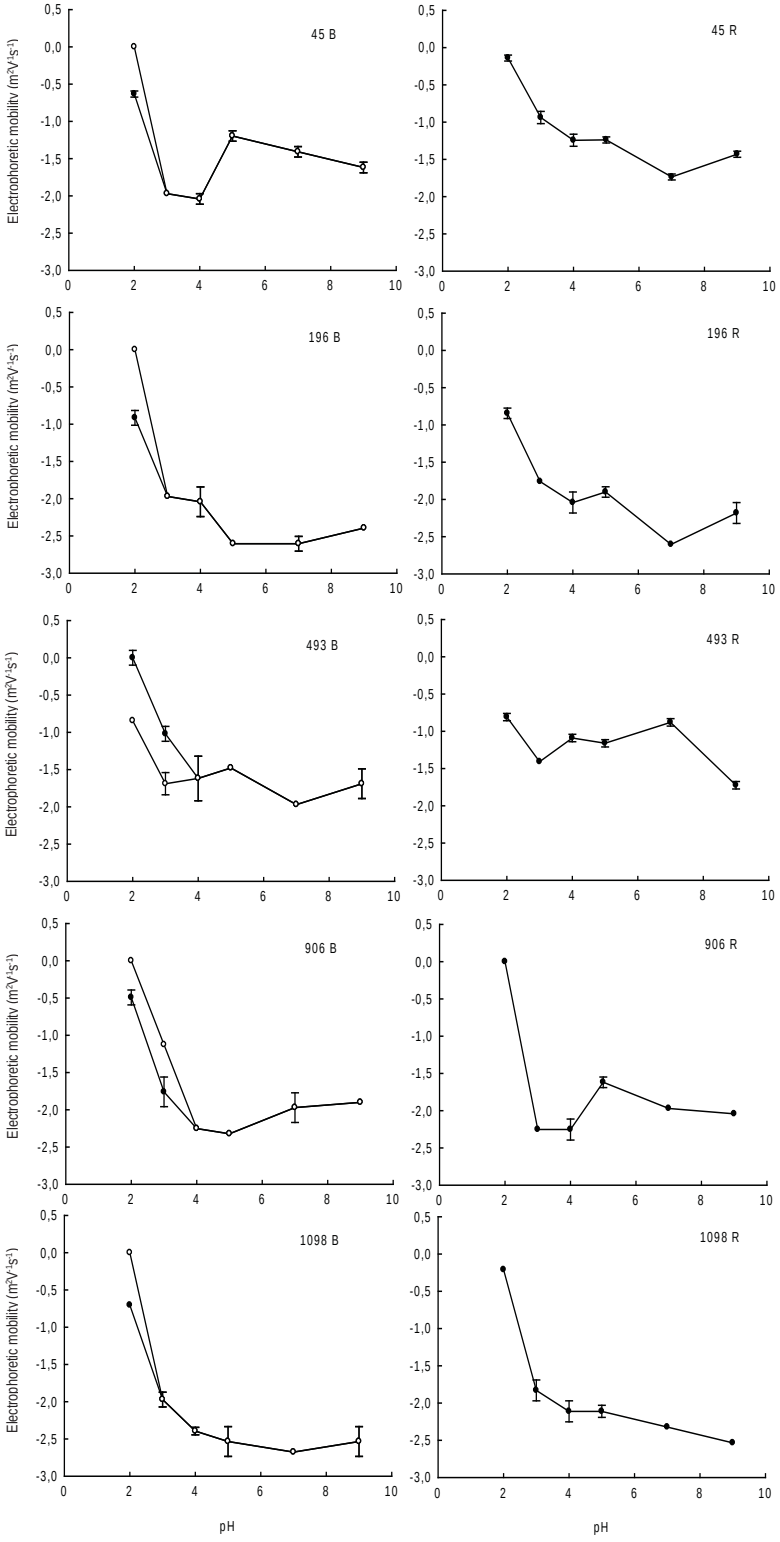

Figure 2. The pH dependence of the electrophoretic mobilities of black (left panel) and red colonies (right panel) of the same strain were determined by in 10mM potassium phosphate. The error bars denote standard deviations over three experiments, with each experiment comprising at least 100 bacteria.

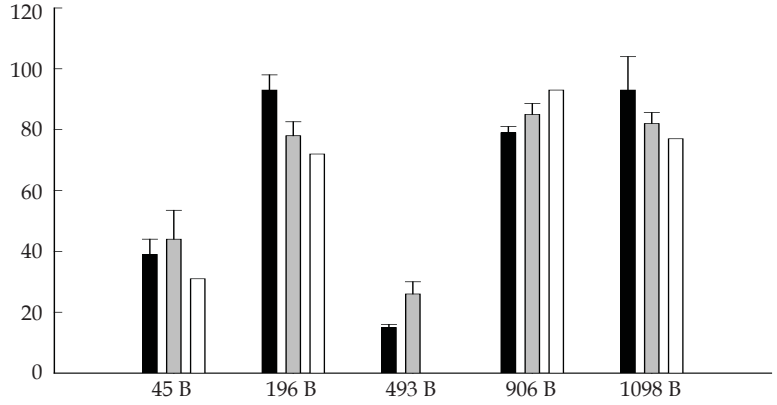

Figure 3. Overview of phase variation as determined using three independent techniques. Black bars show percentage red colonies on CRA plates, grey bars show percentage of the population with lowest IEP and white bars show percentage of cells that lost icaA as determined by Real-Time PCR.

The population with the lowest IEP corresponds to switched cells, in other words to biofilm negative cells, which is in line with a previously report on IEP of *S. epidermidis* strains using Capillary Isoelectric Focusing (Ruzicka *et al.*,). The percentage distribution of switched cells is in line with switching frequencies observed on CRA plates (for example, compare 26% subpopulation 1 with 15% red colonies for strain 493).

Presence of the icaA and icaC genes

Phase variation can occur by insertion of IS256 in *icaA* and *icaC* genes (Ziebuhr *et al.*, 1999). We therefore assayed the presence of the *icaA* and *icaC* genes in black and red colonies of the same strains. Conventional PCR showed presence of *icaA* and *icaC* in all black colonies, while both genes were absent in all red colonies (Table 4). To verify the loss of icaA and icaC as the major reason for phase variation observed in these strains, DNA was isolated from 10 red colonies selected on CRA plates. All of these isolates had lost the *icaA* and *icaC* genes, while all were positive for *gyrB* (data not shown). Apparently, the strains do not regulate expression of the *icaA* and *icaC* genes but somehow the genes are deleted or disrupted.

Gene copy number analysis within cultures

Because the *icaA* and *icaC* genes were deleted during growth of the phase variation strains, it is possible to quantify the occurrence

Table 4. Presence of *icaA* and *icaC* genes cultures showing phase variation determined by PCR, B means black colony, R means red colony. Control experiments using *gyrB* were positive for all DNA isolations

Strain	*icaA*	*icaC*
45B	+	+
196B	+	+
493B	+	+
906B	+	+
1098B	+	+
45R	-	-
196R	-	-
493R	-	-
906R	-	-
1098R	-	-

Table 5. Determination of the gene copy number for *icaA* within a black culture using real time PCR. Amounts were calculated from three replicate wells for *gyrB* (reference gene) and 5 for *icaA* (target gene). As a control the DNA of a non-variation black colony was used. The $2^{-\Delta\Delta C}{}_T$ was calculated from the average C_T values and standard deviations.

Strain	Normalized *icaA* amount relative to *gyrB* $2^{-\Delta\Delta C}{}_T$
Control	1.00 (0.62 – 1.60)
45B	0.69 (0.28 – 1.72)
196B	0.28 (0.23 – 0.35)
493B	1.47 (0.31 – 6.98)
906B	0.07 (0.03 – 0.15)
1098B	0.23 (0.20 – 0.25)

of phase variation by determining gene copy number of *icaA* in a culture. For this reason, real time PCR was performed on the genomic DNA isolations of the black cultures. The gene copy number of the target gene (*icaA*) was quantified relative to the reference gene *gyrB*. For strains 493 and 45 no significant change in *icaA* presence could be observed (presence >0.5 relative to *gyrB*), however, for strain 196, 906 and 1098 significant decreases (presence <0.5 relative to *gyrB*) in *icaA* presence was found (Table 5). Again, this is in line with switching frequencies observed with CRA plating and also with population distribution as determined by zeta potential measurements.

Discussion

Phase variation in biofilm formation is common in clinical isolates of *S. epidermidis*. In the current study, near 5% of all clinical isolates (from the Microbiology Department, Universitas Gadjah Mada, Yogyakarta, Indonesia) were found to undergo phase variation, but with different frequencies. Two strains showed a low frequency of variation (<50%) and 3 showed a high frequency of variation (>50%). In line with previous publications black colony variants (on CRA plates) were good biofilm formers, in contrast to the red derivatives of each strain (Ziebuhr *et al*., 1999), (Conlon *et al*., 2004).

Previous work with *E. faecalis* has shown that the electrophoretic mobility distribution of a culture is also an important determinant for biofilm formation (van Merode *et al*., 2006a) and heterogeneity in electrophoretic mobility distributions is involved in initial and subsequent adhesion (van Merode *et al*., 2006b). Axenic heterogeneous cultures were shown to be excellent biofilm formers while those cultures with homogeneous electrophoretic mobility distributions formed poor biofilms. The electrophoretic mobility distribution of black switching strains was shown to be heterogeneous as well. Two subpopulations could be distinguished at pH 2 for *S. epidermidis* in contrast to *E. faecalis* that showed heterogeneous populations at pH 7 and higher. The heterogeneity at pH 2 is most likely not relevant for the adhesion process as biofilm formation of *S. epidermidis* is initiated at pH 7, and it should be noted that the differences in mobility as observed at pH 2 indicate changes at the surface of the cells, not necessarily charge, but also swelling of slime layers or cell walls (van der Wal *et al*., 1997). However, the observed heterogeneity does indicate that within certain pure culture of *S. epidermidis* two different populations are present that differ in surface composition.

Previous reports have shown that biofilm phase variation is governed by the on/off switching of expression of the *icaADBC* genes in at least two mechanisms; direct through insertion of IS256 into one or more structural genes (Ziebuhr *et al*., 1999), or indirect through insertion of IS256 into the regulatory genes *rsbU* or *sarA* that govern *ica* expression (Conlon *et al*., 2004). Surprisingly, the five strains in the current study show phase variation through a different mechanism. Insertional inactivation of the *icaADBC* genes by IS256 can be ruled out for several reasons, such as : (i) Screening for IS256 of the strains used in this study using PCR (Kozitskaya *et al*., 2004) showed all strains to be IS256 negative (not shown), (ii) Screening for the presence of the *icaADBC* genes in black switching cultures and the corresponding red cultures showed that in all cases *icaA* and *icaC* were absent in the red, but present in the black cultures. This observation was verified by real time PCR for gene copy analysis. It was shown that even when selecting a small fragment of *icaA* in the beginning of the gene, no amplified product could be observed for *icaA*. (iii) Prolonged incubation of red colonies for up to 5 days did not result in any black colonies appearing on CRA (data not shown) indicating that the switch from biofilm + to biofilm – is irreversible, in contrast to insertional inactivation by IS256 which results in reversible switching (Ziebuhr *et al*., 1999). Therefore it should be concluded that the red variants from the five strains studied had permanently lost the *icaA* and *icaC* genes.

It should be noted that previous studies have shown that absence of all *ica* genes is common among clinical *S. epidermidis* isolates (Arciola *et al*., 2005) and that, identical to in our study, in most of these *ica* negative strains IS256 was not present (Arciola *et al*., 2004). Possibly the observation that some clinical isolates rapidly lose these genes might explain that strains isolated from biofilms appear *ica* negative in the laboratory. At this point the mechanism behind this phenomenon remains to be elucidated.

Interestingly, when comparing the population sizes found in the zeta potential and the variation frequencies observed by colony counting, the fraction of *icaA* negative cells nicely fits. Two strains had low frequency of variation as determined with CRA plating, their most negative population (red cells) within the zeta potential distribution was the smallest and no statistical difference in *icaA* presence could be found (< 2-fold reduction). In contrast, the three strains with the highest variation frequencies also showed the largest most negative fraction in the zeta potential measurements (red cells) and a significant decrease in *icaA* presence within the population could be found (>2-fold reduction). Therefore, this study has quantitatively correlated phenotypic observations (heterogeneity on CRA) with physicochemical cell surface analysis (heterogeneity in electrophoretic mobility) and genotypic analysis (decreased gene copy number of *icaA*).

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