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# Quantitative PCR analysis of genes expressed during biofilm development of methicillin resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

*Staphylococcus aureus* biofilm associated infections remains a major clinical concern in patients with indwelling devices. Quantitative real-time PCR (qPCR) can be used to investigate the pathogenic role of such biofilms. We describe qPCRs for 12 adhesion and biofilm-related genes of four *S. aureus* isolates which were applied during *in vitro* biofilm development. An endogenous control (16S rRNA) was used for signal normalization. We compared the qPCR results with structural analysis using scanning electron microscopy (SEM). The SEM studies showed different cellular products surrounding the aggregated cells at different times of biofilm formation. Using qPCR, we found that expression levels of the gene encoding fibronectin binding protein A and B and clumping factor B (*fnb*A/B and *clf*B), which involves in primary adherence of *S. aureus*, were significantly increased at 24 h and decreased slightly and variably at 48 h when all 4 isolates were considered. The elastin binding protein (*ebps*) RNA expression level was significantly enhanced more than 6-fold at 24 and 48 h compared to 12 h. Similar results were obtained for the intercellular adhesion biofilm required genes type C (*icaC*). In addition, qPCR revealed a fluctuation in expression levels at different time points of biofilm growth of other genes, indicating that different parameter modes of growth processes are operating at different times.



## 1. Introduction

*Staphylococcus aureus* is one of the most common bacterial pathogens, notorious for its ability to cause foreign-body infections. The ability to form biofilms on such biomaterials is probably the major contributing factor to associated infections.

Biofilm formation involves bacterial attachment to solid surfaces and subsequent assembly of matrix-embedded cellular communities (Rohde et al., 2010; Costerton et al., 1999; Cramton et al., 1999). In this way, *S. aureus* infections are becoming more chronic in wound infections and on catheters, shunts, implants, etc. (Donlan, 2001; Christensen et al., 1994; Locci et al., 1981). Adhesion is the fundamental step in the formation of biofilm communities which is facilitated by the expression of various microbial surface components which recognize adhesive matrix molecules (MSCRAMMs). These MSCRAMMs can bind to one or more host extracellular matrix factors including elastin (*ebpS*), laminin (*eno*), collagen (*cna*), fibronectin A and B (*fnbA* and *fnbB*), fibrinogen (*fib*), bone sialoprotein (*bbp*) and bacterial ligands clumping factors A and B (*clfA* and *clfB*) (Seo et al., 2008). These proteins share a common signal sequence for secretion and as well as for anchoring to the cell wall. Such a classification is useful for *S. aureus* surface molecules as host matrix proteins rapidly coat medical devices after implant. MSCRAMMs have to be imported from the inner part of the bacteria followed by successive attachment to the bacterial surface, thus playing a pivotal role for *S. aureus* pathogenesis (Mazmanian et al., 1999). This process involves the sec pathway and the sortase in *S. aureus* (Siboo et al., 2008). The development of an actual biofilm is mainly facilitated by polysaccharide intercellular adhesion (PIA); PIA production results in elaborate multilayered cell clustering. PIA is a polysaccharide composed of $\beta$-1, 6-linked *N*-acetylglucosamine with partially deacetylated residues. It surrounds the cells and protects them against both host immune defenses and antibiotic treatment (Götz, 2002). The products of the *ica*ADBC operon are the proteins necessary for the synthesis of

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PIA and capsular polysaccharide/adhesion (PS/A) in staphylococcal species (Chaieb et al., 2005). The *ica*A, *ica*C and *ica*D proteins are located in the membrane fraction; *ica*B gene is mainly found in the culture supernatant and deacetylates PIA when it is localized on the cell surface (Gerke et al., 1998; Vuong et al., 2004). Co-expression of *ica*A and *ica*D increases *N*-acetylglucosaminyl transferase activity and slime production (Arciola et al., 2006). *ica*B encodes an extracellular protein whose function is still unknown, whereas *ica*C encodes a membrane protein believed to have a receptor function for polysaccharides. Recently, the role of microbial genes essential to the development of biofilms during infection has received much interest (Cucarella et al., 2001, 2002). However, we still lack in understanding the differential expression of adhesion and biofilm related genes during biofilm growth. In addition, changes in the phenotype of biofilm cells over time are not precisely documented. In the present study, we investigated the transcriptional profiles of specific staphylococcal genes encoding MSCRAMMs during biofilm production on polystyrene plates. We also attempted to correlate these findings with SEM-defined changes in biofilm morphology.

## 2. Methods

### 2.1. Bacterial strain and culture conditions

In this study, 4 distinct *S. aureus* clinical isolates were selected from a total of 30 different clinical MRSA isolates studied previously and well known for their ability to form stable biofilms (Salman et al., 2011). These clones as one of the highly spread MRSA types in Malaysia, and frequently isolated from different clinical infected with MRSA. The isolates were received in the form of stock culture from the Medical Microbiology Laboratory at which was previously garnered from Kuala Lumpur General Hospital (HKL). The sources of the isolates were from different infection sites of clinically ill patients. These isolates were previously characterized as different clones {t037-ST-239-CC8 (527,524) and t138-ST-1283-CC8 (139,199)}, using SCCmec typing, spa sequencing, and MLST in our earlier investigation. All the isolates belong to the same agr type I (Ghaznavi-Rad et al., 2010). In this study, the isolates were selected and characterized based on the presence of the 12 adherence and biofilm target genes (*finb*A,B, *clf*A,B, *fi*b, *cna*, *eno*, *epbs*, and *ica*A,D,B,C), and negative for the presence of the *bbp* and biofilm associated protein (*Bap*) by PCR method. Selected isolates were grown in enriched tryptic soy broth supplemented with 1% glucose (TSBG; Merck, Darmstadt, Germany Baker, UK) and for all experiments; bacterial cultures were grown aerobically in 6-well polystyrene tissue culture plates (Roskilde, Denmark) at 37 °C.

### 2.2. Quantification of MRSA biofilms by Safranin assay

The ability to form biofilm formation was investigated for all four MRSA isolates using the safranin microtiter plate assay according to our previously described method (Salman et al., 2012).

### 2.3. Preparation of biofilm samples for scanning electron microscopy (SEM)

To determine the appropriate time point for total RNA isolation, the morphological changes of surface structures in each isolates were initially observed using SEM along the course of bacterial growth. Biofilm cells for SEM analysis were performed on 6-well polystyrene tissue culture plates following the previously described method by Stepanovic et al. (2007). Briefly, cells of MRSA isolates grown overnight were diluted 1:10 in TSBG and 5-mL aliquots were placed into each well of polystyrene tissue culture plate covered with a lid (Roskilde, Denmark) and incubated statically at 37 °C for 6, 12, 24 and 48 h. Bacterial growth was monitored by measuring the absorbance at 600 nm. After incubation, the plate was carefully washed with sterile double-distilled water ($ddH_2O$) in order to remove all planktonic cells. After washing, adhering bacterial cells were fixed with 4% buffered glutaraldehyde for 5 h according to the previously described method (Ganderton et al., 1992). At each time point, the bottom of the polystyrene plates was cut to a length suitable for electron microscopy. The cut polystyrene piece was rinsed three times with 0.1 M sodium cacodylate buffer, then fixed in 0.1 M osmium tetroxide for 1 h at 4 °C and dehydrated with 35–100% ethanol in double distilled water. The samples were mounted using double-sided tape and then gold-coated using a gold sputtering unit (spray coating, Bal-Tec SCD 005). All experiments were performed in duplicates at different voltages for SEM.

### 2.4. RNA isolation from biofilms

For RNA isolation, the same culture plate model and growth condition for SEM was used. In brief, cells on 6-well polystyrene plates at each time point as described above were washed thrice with ddH2O. The adhering bacterial cells in each well were disrupted and re-suspended in cold sterile double distilled water by rapidly scraping them from the plate surface until no visible adherent layer was left. This was achieved using sterile micropipette tips and the suspensions were immediately incubated with an appropriate volume of RNA protect (Qiagen). Vortexed for 5 s and incubated for 5 min at room temperature. The mixture was pelleted by centrifugation at 10000*g* for 10 min and the cell pellets were stored at −80 °C awaiting RNA isolation.

#### 2.4.1. RNA isolation

Cell pellets obtained after centrifugation were re-suspended in 100 μL of 4 °C sterile RNase-free distilled water (Qiagen). Cell densities were adjusted to obtain comparable values and total RNA was subsequently isolated from 4 samples at each incubation time points using a RNeasy mini kit (Qiagen) according to our previous described method (Atshan et al., 2011). The RNA quality and quantity was determined by agarose gel electrophoresis and by measuring the absorbance at 260 and 280 nm using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Residual DNA present in the RNA preparation was removed using "RNase-free DNase I and gDNA wipeout buffer" (Qiagen). Further, total RNA samples were analyzed for the presence of DNA contamination by PCR targeting the 16S rRNA gene – forward (5′-CGGTCCAGACTCCTACGGGAGGCAGCA-3′) and reverse (5′-GCGTGGACTACCAGGGTATCTAATCC-3′) without the reverse transcriptase step (no *c*DNA) (Silver et al., 2006), using the same condition as the one used for qPCR (see below). Purified RNA was immediately converted to cDNA to avoid RNA degradation using RevertAid™ first strand *c*DNA synthesis kit (Fermentas) with random hexamer primers according to the manufacturer's instructions.

### 2.5. Primers and their specificities for qPCR

Gene-specific primers were designed using the free primer-design software (NCBI) and were synthesized by Bio-RP (Table 1). Annealing temperatures were optimized for each primer pair by the use of melting curve analysis and by post-PCR agarose gel electrophoresis for the products obtained. The identities of all PCR products were confirmed by sequencing and the amplification efficiency for each primer set was determined by a RT-qPCR assay in order to evaluate the linearity of target amplification.

**Table 1**
Sequences of oligonucleotide primers used for qPCR.

| Genes | Nucleotide sequence of primers (5'-3') | Accession numbers | Annealing temperature | Amplicon size (bp) |
|---|---|---|---|---|
| *ica*A (intercellular adhesion gene) | 5-GAGGTAAAGCCAACGCACTC-3<br>5-CCTGTAACCGCACCAAGTTT-3 | AF086783 | *60 | 151 |
| *ica*D (intercellular adhesion gene) | 5-ACCCAACGCTAAAATCATCG-3<br>5-GCGAAAATGCCCATAGTTTC-3 | AF086783 | 60 | 211 |
| *ica*B (intercellular adhesion gene) | 5-ATACCGGCGACTGGGTTTAT-3<br>5-TTGCAAATCGTGGGTATGTGT-3 | AF086783 | 60 | 140 |
| *ica*C (intercellular adhesion gene) | 5-CTTGGGTATTTGCACGCATT-3<br>5-GCAATATCATGCCGACACCT-3 | AF086783 | 60 | 209 |
| *fnb*A (fibronectin binding protein A) | 5-AAATTGGGAGCAGCATCAGT-3<br>5-GCAGCTGAATTCCCATTTTC-3 | X95848.1 | 60 | 121 |
| *fnb*B (fibronectin binding protein B) | 5-ACGCTCAAGGCGACGGCAAAG-3<br>5-ACCTTCTGCATGACCTTCTGCACCT-3 | X62992.1 | 60 | 197 |
| *clf*A (clumping factor A) | 5-ACCCAGGTTCAGATTCTGGCAGCG-3<br>5-TCGCTGAGTCGGAATCGCTTGCT-3 | Z18852.1 | 60 | 165 |
| *clf*B (clumping factor B) | 5-AACTCCAGGGCCGCCGGTTG-3<br>5-CCTGAGTCGCTGTCTGAGCCTGAG-3 | AJ224764.1 | 60 | 159 |
| *fib* (fibrinogen binding protein) | 5-CGTCAACAGCAGATGCGAGCG-3<br>5-TGCATCAGTTTTCGCTGCTGGTTT-3 | X72014.1 | 60 | 239 |
| *ebps* (elastin binding protein) | 5-GGTGCAGCTGGTGCAATGGGTGT-3<br>5-GCTGCGCCTCCAGCCAAACCT-3 | U48826.2 | 60 | 191 |
| *eno* (laminin binding protein) | 5-TGCCGTAGGTGACGAAGGTGGTT-3<br>5-GCACCGTGTTCGCCTTCGAACT-3 | AF065394.1 | 60 | 195 |
| *cna* (collagen binding protein) | 5-AATAGAGGCGCCACGACCGT-3<br>5-GTGCCTTCCCAAACCTTTTGAGCA-3 | M81736.1 | 60 | 156 |
| 16S rRNA | 5-GGGACCCGCACAAGCGGTGG-3<br>5-GGGTTGCGCTCGTTGCGGGA-3 | L37597.1 | 60 | 191 |

* Optimized PCR annealing temperature program for all the genes at 60 °C.

### 2.6. Quantitative real-time PCR

To see what kind of gene expression events are correlated with phenotypic differences, we measured the transcript levels of the 12 selected genes for 4 different isolates of *S. aureus* at different time points using qPCR. The reaction was carried out in a Mastercycler® Realplex system (Eppendorf) utilizing the Power SYBR Green Master Mix (Bio-RP) following the manufacturer's recommended protocol. Reactions were performed in triplicates using 96-well plates and the reaction volume was set at 20 μL per sample. All reactions contained 2 μL of *c*DNA, 10 μL of SYBR Green Master Mix, 0.5 μL of 100 μM of each primer, and 7 μL of sterile double RNase treated water. The reaction was started with an initial denaturation at 95 °C for 5 min and 40 amplification cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s.

### 2.7. Data analysis using relative standard curve method

The expression of 12 selected genes at 12, 24, and 48 h was calculated relative to the calibration sample at 6 h and an endogenous control (16S rRNA) to normalize the sample input. The 6 h time point was chosen as calibrator, since at this point of growth none of the 4 isolates showed cell-associated fibrous materials, in contrast with growth at 12 h, 24 h and 48 h which demonstrated cell surface-associated meshwork-like structures in SEM analysis. The X-fold change of the transcription level was calculated by a relative standard curve method because the PCR efficiencies of the target and endogenous controls do not have to be equivalent (Eleaume and Jabbouri, 2004). For each experimental sample, standard curves were generated for both the target and endogenous references. The data were then subjected to analysis using the Relative Expression Software Tool (REST) program (Qiagen). REST 2009 is a

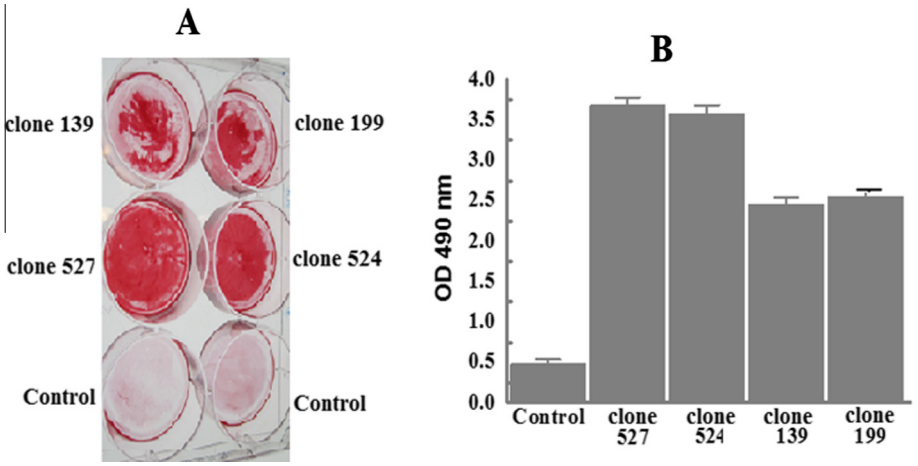


**Fig. 1.** Biofilm formation on microtiter plates. A representative biofilm production of *S. aureus* clone 527,524,139 and 199 on 6-well polystyrene microtiter plates surface stained with safranin after 48 h of incubation (A). The quantitative analysis of biofilm production by measuring the optical density of destained biofilms at 490 nm (B). Control: indicates reference strains, negative biofilm producer ATCC 12228 (right and left). Bars indicate standard deviations.

software tool used to determine changes in target gene expression standardized by non-regulated reference genes. The experiment was performed twice and the representative values were the means of triplicates.

### 2.8. Statistical analysis

Two-tailed, two-sample equal variance Student's *t*-tests (Microsoft Excel 2007) were used to determine statistically significant differences in biofilm forming capacity and relative gene expression.

## 3. Results

### 3.1. Biofilm quantitative assay

Fig. 1A, compares the varying adherence levels of 4 different MRSA isolates grown on flat-bottomed polystyrene microtiter plates. As indicated in Fig. 1B, all the 4 isolates (527, 524, 139 and 199) of two *spa* types (t037 and t138) were strongly adherent with $OD_{490}$ values of >3.0 and $OD_{490}$ values of >2.0 respectively. The cut end point was $OD_{490} \leqslant 0.5$.

### 3.2. Scanning electron microscopy

As shown in Fig. 2A, the growth of isolates 527 and 524 was slightly faster than isolates 139 and 199, entering into an exponential phase at 6 h and reaching plateau after 22 h of growth. Morphological observation of cultures at different stages of growth revealed that all isolates of *S. aureus* exhibited cell surface-associated meshwork-like structures at 12 h and the structures became denser with fibrous materials at 24 and 48 h (see Fig. 2B and Fig. S5–S8).

### 3.3. Stability of RNA, cDNA and specificity of primers for qPCR

The quality and quantity of RNA extracted at different time points of growth i.e., 6 h, 12 h, 24 h and 48 h respectively (see Table S1.A–D and Fig. S1.A–D) was found to be satisfactory. The *c*DNA synthesis generated large amounts of product and all the primers utilized in this study showed high specificities with a single peak and a single band at optimized annealing temperature 60 °C (Fig. 3).

### 3.4. Expression levels of biofilm-associated genes quantified by qPCR

The expressions of 12 selected genes involved in biofilms were compared using the Ct values which showed significant differences among all genes at every time intervals (mean Ct values ranging from 16.55 to 31.82). The standard deviation of Ct values for each gene was <0.5 and the comparative relative expression of 12 selected genes at each different time points of biofilm formation for the 4 MRSA isolates were calculated relative to the calibration samples as illustrated in Table 2.

Three out of 12 genes tested (*fnb*A, *fnb*B, *clf*B) showed the highest up-regulation at 12 h of incubation (Fig. S2), and the annotations of these genes are described in Table 2. Expression of *fnb*A, *fnb*B, *clf*B, *ebps* and *ica*C was 6-fold up-regulated at 24 h of growth, with *ica*C and *fnb*B being the most up-regulated genes (Fig. S3 and Table 2). Prolonged incubation for up to 48 h showed an up-regulation of *fnb*A, *fnb*B, *clf*B, *ebps* and *ica*C genes among the MRSA isolates and the data obtained are explained in Fig. S4 and Table 2. Overall analysis of the 12 "biofilm-involved" genes showed that several of them fluctuated in gene expression level over time: initial up-regulation was followed by a down-regulation at the early adherence phase (12 h), further increased during the mid-adherence phase (24 h) followed by a slight down-regulation (48 h). This pattern of gene regulation was observed in all the 4 MRSA isolates tested with variation in expression levels (Fig. 4).

## 4. Discussion

The mechanism and/or process of biofilm formation in *S. aureus* is poorly understood and the studies on the expression profiles of genes involved in biofilm mechanism are still limited in number. To ascertain the molecular mechanism of biofilm formation, we attempted to define the expression levels of 12 selected genes involved in biofilm formation including the *ica*ADBC genes. A

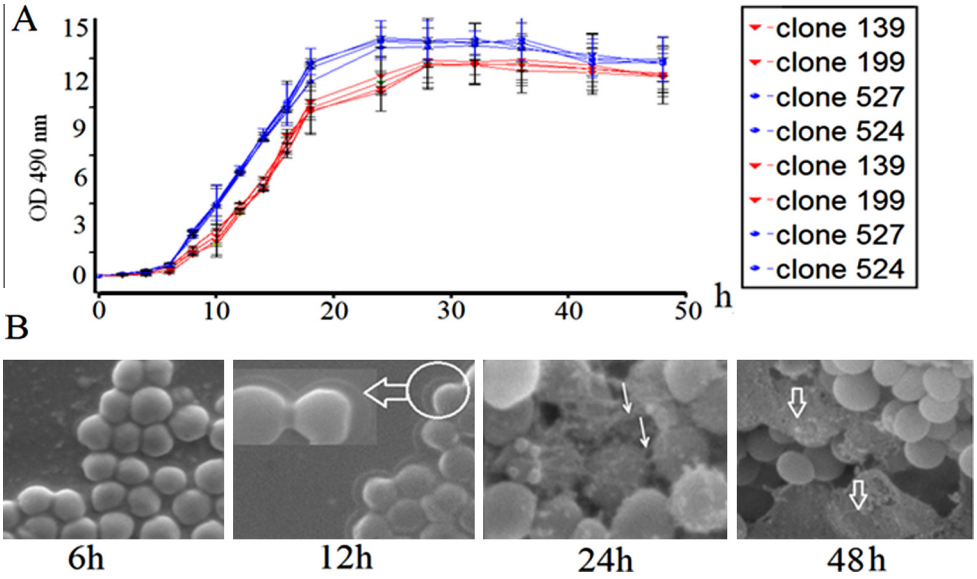


**Fig. 2.** Growth of *S. aureus* in enriched-tryptic soy broth and scanning electron micrographs showing morphological changes associated with growth. Clone 527 and 524 (blue) show a faster growth rate than clone 139 and 199 (red) as shown in image A (The clone names printed twice indicates the experiment was performed in duplicates). Meshwork-like structures around cells were observed at 12 h and became denser containing "arrow heads" with time. No such fibrous materials were observed at 6 h for all strains at this stage of the growth cycle (B). MRSA clone number 527 showing morphological structures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

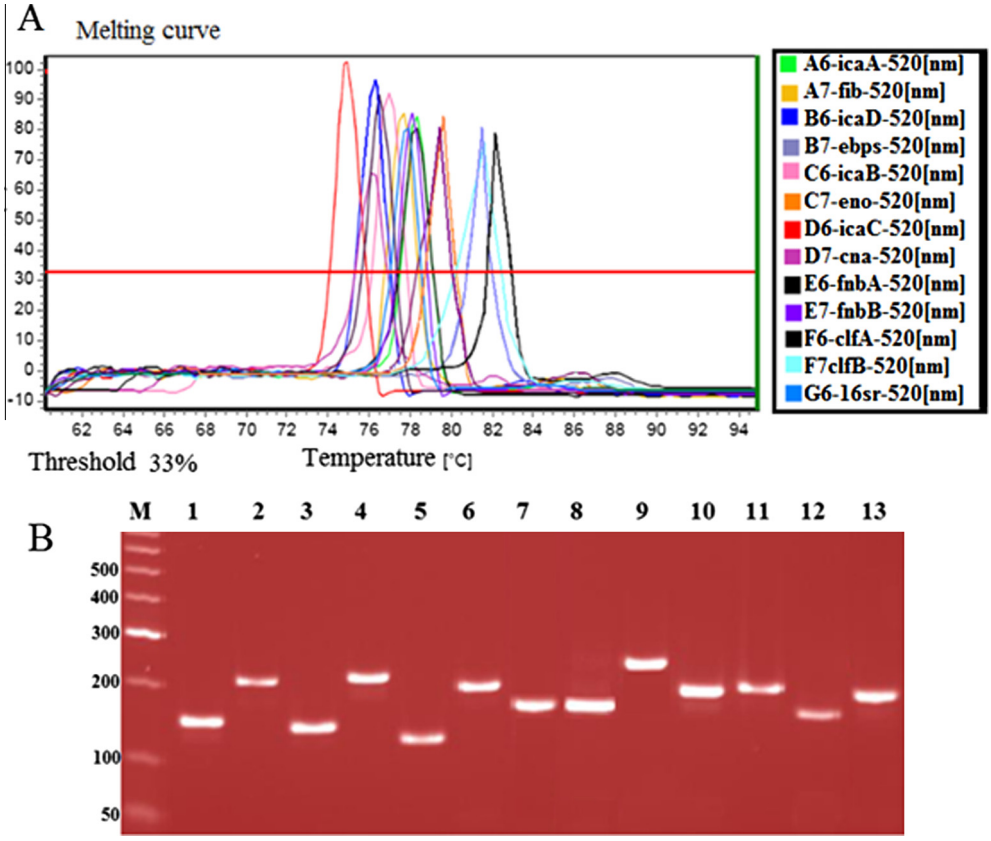


**Fig. 3.** The specificity of each primer set for the amplification of adhesion and biofilm genes in this study. (A) Melting curve analyses was used at the end of qPCR products amplifications to confirm that there was only one peak and one product. (B) End amplified products of real-time RT-PCR were visualized after separation on an agarose gel, showing all primer pairs resulted in amplification of a single product of *ica* (1), *ica*D (2), *ica*B (3), *ica*C (4), *fnb*A (5), *fnb*B (6), *clf*A (7), *clf*B (8), *fib* (9), *ebps* (10), *eno* (11), *can* (12), and *16s* (13), M, molecular weight marker.

**Table 2**
Fold change in mRNA levels of adhesion and biofilm target genes in cultures of different S. aureus isolates grown at 12, 24 and 48 h.

| Gene | Type | Fold change in level of biofilm genes | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Isolate 527 | | | Isolate 524 | | | Isolate 139 | | | Isolate 199 | | |
| | | 12 h | 24 h | 48 h | 12 h | 24 h | 48 h | 12 h | 24 h | 48 h | 12 h | 24 h | 48 h |
| *16s* | REF | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| *fnb*A | TRG | 15.8 ↑ | 20.5 ↑ | 7.8 ↑ | 18.6 ↑ | 21.1 ↑ | 8.2 ↑ | 13.3 ↑ | 18.7 ↑ | 7.2 ↑ | 12.9 ↑ | 19.0 ↑ | 7.3 ↑ |
| *fnb*B | TRG | 9.9 ↑ | 169.1 ↑ | 15.0 ↑ | 10.0 ↑ | 159.8 ↑ | 14.5 ↑ | 10.2 ↑ | 194.8 ↑ | 16.6 ↑ | 10.4 ↑ | 214.1 ↑ | 17.8 ↑ |
| *clf*A | TRG | 0.9* | 0.2 ↓ | 0.1 ↓ | 0.9* | 0.1 ↓ | 0.1 ↓ | 0.9* | 0.2 ↓ | 0.1 ↓ | 0.9* | 0.2 ↓ | 0.1 ↓ |
| *clf*B | TRG | 3.6 ↑ | 12.7 ↑ | 2.6 ↑ | 3.7 ↑ | 13.2 ↑ | 2.8 ↑ | 3.7 ↑ | 12.7 ↑ | 2.4 ↑ | 3.7 ↑ | 13.0 ↑ | 2.4 ↑ |
| *fib* | TRG | 0.9* | 1.0* | 0.5 ↓ | 1.0* | 1.1* | 0.6 ↓ | 1.2* | 1.7 ↑ | 1.0* | 0.8* | 1.3 ↑ | 0.8* |
| *eno* | TRG | 2.8 ↑ | 0.2 ↓ | 1.0* | 2.8 ↑ | 0.1 ↓ | 0.9* | 2.1 ↑ | 0.1 ↓ | 0.7 ↓ | 2.3 ↑ | 0.1 ↓ | 0.7 ↓ |
| *cna* | TRG | 1.4* | 3.3 ↑ | 0.7* | 1.4 ↑ | 3.3 ↑ | 0.9* | 1.4* | 3.2 ↑ | 0.6* | 1.4* | 3.2 ↑ | 0.6* |
| *ebps* | TRG | 0.5 ↓ | 6.0 ↑ | 26.5 ↑ | 0.5 ↓ | 6.0 ↑ | 25.9 | 0.4 ↓ | 7.3 ↑ | 36.3 ↑ | 0.3 ↓ | 9.2 ↑ | 45.1 ↑ |
| *ica*A | TRG | 0.2 ↓ | 1.7 ↑ | 0.2 ↓ | 0.2 ↓ | 1.6 ↑ | 0.2 ↓ | 0.2 ↓ | 1.9 ↑ | 0.2 ↓ | 0.2 ↓ | 2.0 ↑ | 0.2 ↓ |
| *ica*D | TRG | 0.2 ↓ | 1.7 ↑ | 1.1* | 0.2 ↓ | 1.8 ↑ | 0.7* | 0.2 ↓ | 1.7 ↑ | 0.7 ↓ | 0.1 ↓ | 1.5 ↑ | 0.7 ↓ |
| *ica*B | TRG | 0.8* | 3.4 ↑ | 0.8* | 0.8* | 3.2 ↑ | 0.9* | 0.8* | 5.6 ↑ | 1.1* | 0.5 ↓ | 10.2 ↑ | 1.9 ↑ |
| *ica*C | TRG | 0.7 ↓ | 1440 ↑ | 153 ↑ | 0.8* | 1855 ↑ | 239 ↑ | 0.4 ↓ | 1530 ↑ | 91.4 ↑ | 0.6 ↓ | 1419 ↑ | 113 ↑ |

REF indicates reference gene, TRG target gene, *fnb*A and B to fibronectin binding proteins A and B, *clf*A and B to clumping factors A and B, *fib* to fibrinogen binding protein, *eno* to laminin binding protein, *cna* to collagen binding protein, *ebps* to elastin binding protein, *ica*ADBC to intercellular adhesion biofilm required genes. Green downward arrows indicates to significantly decreased and red upward arrows to significantly increased if $P$ value is <0.05 (Target sample is different to control).
* indicates the target sample is not different from control ($P$ value is >0.05).

better understanding of the dynamic *S. aureus* genes other than *ica*ADBC involved in biofilm environment may shed some light and innovative information on the functions of certain genes in superbugs like MRSA. We used the SEM study to show that our 6-well microtiter plate model for the biofilm development at 6 h, 12 h, 24 h and 48 h was satisfactory as a comparative analysis (gradual changes in complexity of the biofilm are shown in Fig. 2B and Fig. S5–S8) with the gene expression at four different time points of growth. Despite the DNA microarray analysis and RT-PCR results of the *S. aureus* biofilm gene expression reported elsewhere (Resch et al., 2005; Dunman et al., 2001; Beenken et al., 2004), the timing and the relative quantification of the expression of these genes in clinical MRSA isolates in largely unknown. Production of intercellular adhesion molecules such as *ica*ADBC and slime plays an important role in staphylococcal biofilms (Götz, 2002). However, molecular insights on the *ica*ADBC

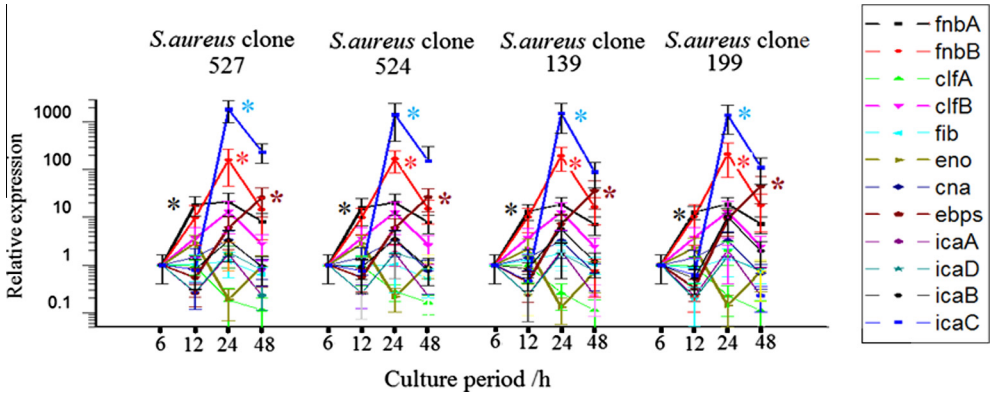


**Fig. 4.** Fluctuation of transcriptional levels of 12 adhesion and biofilm genes through a culture period in four *S. aureus* clones (527,524,139, 199). Total RNA was isolated from 6, 12, 24, and 48 h-old cultures of all clones, and the relative expression levels of 12 genes were compared by real-time RT-PCR (Relative plotted against log10). The expression levels of these genes were fluctuating in all four clones of *S. aureus* at different point times. Data are representative of two independent experiments. ✻ indicates the *ica*C was significantly expressed at 24 and 48 h with higher levels compared to 12 h ( $P \leqslant 0.05$). ✻ indicates the *fnb*B was significantly expressed at 12, 24 and slightly decreased at 48 h compared to other genes ( $P \leqslant 0.05$). ✻ indicates the *fnb*A was significantly expressed at 12, 24 and slightly decreased at 48 h compared to other genes ($P \leqslant 0.05$). ✻ indicates the *ebps* was significantly expressed at 24 and 48 h compared to 12 h ($P \leqslant 0.05$).

proteins and its associated genes in biofilm formation have not yet been well established. One of the main goals of this investigation was to determine whether the genes encoding *ica*ADBC and other biofilm related genes are expressed at varying levels along the course of biofilm formation.

In an earlier investigation, Resch et al. (2005) compared biofilm cells with planktonic cells, showing that ica gene expression is required for biofilm formation at the beginning of biofilm development between 6 and 8 h of growth. However, the present study results are contradictory, where the expression level of ica genes were found to be low between 6 and 12 h among biofilm forming strains. It has been reported that surface characteristics determines the adherence properties and varies with different polymer materials (Tebbs et al., 1994). At 24 and 48 h, *ica*C was the only gene found to be highly expressed of all the *ica* gene family (see Fig. S2, S3 and Table 2). Similarly, it has been shown that *icaC* expression was the highest for the *icaADBC* operon (Beenken et al., 2004; Vandecasteele et al., 2003). However, it also showed a significant decrease in *ica*C gene expression but with a high level still observed at 48 h suggesting that growth media, environmental stimuli, and the nature of the strain play major roles in the regulation of biofilm gene expression (Beloin et al., 2006). Overall, the expression of the *ica* operon, involved in adhesion and biofilm formation was shown to fluctuate over time (Fig. 4). This pattern of gene regulation was observed in all the 4 MRSA isolates tested but with variation in expression levels. *ica* genes are mainly responsible for the initial colonization phase of biofilm formation rather than maturation and persistence (Vandecasteele et al., 2003; Blevins et al., 1999), which is the main reason for us to investigate whether other binding factors were also assisting in the accumulating in biofilm cells at different time points of bacterial growth.

A series of MSCRAMMs that interact with host extracellular ligands showed a very high expression at 12 h and the level of expression of the 8 non *ica* genes remained high during the entire biofilm growth period. In particular, the expression level of *clf*B was high at 12 h of growth, followed by *fnb*A, B and *ebps* which showed more than 6-fold increased at 24 h and 48 h in all the 4 isolates tested. The expression level of the *clf*A gene in the *S. aureus* Newman strain has also been reported to be high after 24 of growth (Ythier et al., 2012). Interestingly, the gene corresponding to fibronectin binding protein-A and B was still highly expressed even in early and in mature stages of biofilm cells at 12, 24 and 48 h. Thus, it is clear that the fibronectin-binding protein A gene was continually expressed throughout the course of growth during biofilm development. This property of biofilms will remain important in differentiating biofilms at different time points either directly or indirectly. However, a more detailed study will aid in defending this hypothesis. The expression levels of the genes *ica*C, *fnb*A, *fnb*B, and *ebps* were found to be significantly higher after 24 and 48 h of biofilm growth when compared to other genes. Hence, it can be assumed that these genes are important for the perpetuation of the biofilm and the survival of cells in a dense and nutrient-scarce community. These genes include those involved in the synthesis of binding factors and PIA and their functions are possibly correlated with the presence of extracellular and intercellular products of cells in a biofilm observed in SEM at different time points. The present findings strongly underscores that a single gene or a subset of genes cannot be utilized as indicator(s) for biofilm morphology and maturity unless additional studies are performed to document the physiological impact caused by differentiating gene expression. This may help to better understand the molecular mechanism of biofilm formation over time. Additionally, a knockout study eliminating the highly expressed genes could possibly fine-type the activity of certain genes that are responsible for establishment and differentiation of biofilms. Despite the high expression levels of certain genes observed in this study, other genes showed down-regulation at 12, 24, and 48 h of biofilm development when compared to cells grown at the exponential phase. It is clear that microorganisms undergo alterations during their transition from exponential-phase to cells that are part of complex, surface-attached communities at 48 h. Therefore, the most interesting point in this study is the morphological imaging coupled to qRT-PCR which allowed correlating morphological modifications to gene expression profile.

## 5. Conclusion

Although this study was mainly geared towards investigation of the expression of 12 genes, microarray or next generation sequencing analysis of more genes will provide more detailed insights in the molecular mechanisms of how bacteria build their homes. Such determinants may explain why some of the genes are expressed in both the early and mature stages of biofilm growth. To conclude, qPCR and SEM analyses demonstrates that the expression of adhesion- and biofilm-related genes in comparison with the phenotypic biofilm morphology can be utilized as a model in order to study the up- and down- regulation of such genes. Delayed expression of cer-

tain genes after 24 and 48 h at significantly higher levels are considered important for biofilm development and also for the survival of composing cells in a nutrient-scarce niche.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.05.002.

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