Effects of erythromycin on the phenotypic and genotypic biofilm expression in two clinical Staphylococcus capitis subspecies and a functional analysis of Ica proteins in S. capitis

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The ica operon encoding polysaccharide intercellular adhesion, which facilitates biofilm formation in staphylococci, has been extensively studied in Staphylococcus epidermidis and Staphylococcus aureus. Based on in silico analysis, we suggest the following functional model for Ica proteins in S. capitis. IcaA is responsible for polysaccharide synthesis. IcaA and IcaD complete transferring the growing sugar chain to the cell surface; IcaB is a deacetylase, with the same function as IcaB of S. epidermidis. IcaC mainly modifies the synthesized glucan by acetylation. We also examined the effects of subinhibitory concentrations of erythromycin on phenotypic biofilm expression and transcription of biofilm-related genes, using isolates representing the two subspecies of Staphylococcus capitis and different biofilm and resistance phenotypes. On induction with erythromycin, biofilm density was strongly elevated in two erythromycin-resistant S. capitis, but not in three susceptible isolates. In the representative erythromycin-resistant S. capitis subsp. urealyticus, there were significant upregulations of the icaA gene and its positive regulator sarA on transition to the stationary phase without erythromycin induction. There were also significant increases in the transcription levels of icaA, rsbU and sigB corresponding to a very strong biofilm phenotype in the stationary phase on erythromycin stress. In contrast, the representative erythromycin-susceptible S. capitis subsp. capitis displayed upregulation only of altE on entry into the stationary phase with erythromycin induction, but this change was not associated with enhancement of biofilm production. These findings suggest that the two subspecies of S. capitis adopt different pathogenesis and survival strategies to adapt to a hostile environment.

INTRODUCTION
Recent studies indicated the emergence of Staphylococcus capitis as a significant pathogen that causes late-onset sepsis in very-low-birth-weight infants (<1500 g) (Ng et al., 2006). The pathogenic potential of S. capitis has been attributed to the ability to form biofilms on polymer surfaces (Nalmas et al., 2008). Biofilm formation in staphylococci proceeds in two stages (Heilmann et al., 1996). Primary attachment of bacterial cells to a polymer surface is followed by accumulation of bacteria in a multilayered biofilm. The synthesis of polysaccharide intercellular adhesin (PIA), which mediates cell-to-cell adhesion, is essential for cell accumulation by Staphylococcus epidermidis. The icaADBC operon, responsible for the synthesis of PIA (Cramton et al., 1999), has been detected in Staphylococcus aureus and a range of other coagulase-negative staphylococci, and was confirmed in a recent study to be present and the same length in all 60 clinical S. capitis isolates examined (52 S. capitis subsp. urealyticus and eight S. capitis subsp. capitis) (Cui et al., 2013). The ica operon in S. capitis contains four structural genes and a regulatory gene located upstream of icaA with ≥65 % identity to the corresponding genes of Staphylococcus caprae, S. epidermidis and S. aureus (icaA, icaD, icaB, icaC and icaR, respectively) (Cui et al., 2013). Moreover, the amino acid sequences deduced from the genes exhibited 66–94 % identity to those of the same three species. Preliminary examination of the sequences of five isolates representing both subspecies, as well as biofilm-positive and biofilm-negative phenotypes,
indicated the sequences of two biofilm-positive isolates were identical, but the biofilm-negative phenotype appeared to be associated with different mutations: a deletion mutation in the promoter element, a stop codon in the icaB gene or an in-frame 3 aa deletion in the icaA gene (Cui et al., 2013).

The complete DNA sequence of the ica operon of S. capitis was also determined (Cui et al., 2013); however, a clear picture of the structural basis for functions of the enzymes encoded by this operon has not yet emerged. Recently, online bioinformatics tools have become available, making biological discoveries in silico in a highly time- and cost-efficient manner. By utilizing various bioinformatics prediction and analysis tools, hypotheses and theoretical models can be generated quickly, which could then guide the design of experiments for further validation.

The phenotypic expression of biofilm in staphylococci is affected by various environmental conditions (Knobloch et al., 2001; Dobinsky et al., 2003; Cotter et al., 2009). Although there is limited knowledge on the regulation of the ica operon, recent data suggest the involvement of genes such as positive sigma factors rsbU and sigB (Knobloch et al., 2004), two quorum-sensing elements, RNAII and luxS (Kong et al., 2006), a negative regulatory gene of the ica operon, icaR, and a global regulatory gene, sarA (Weiss et al., 2009).

It has been shown that subinhibitory antibiotic concentrations influence the expression of important virulence factors of bacteria, such as adhesins and toxins (Bernardo et al., 2004; Cummins et al., 2009). In addition, several reports showed subinhibitory concentrations of tetracycline and erythromycin enhanced biofilm formation in S. epidermidis (Rachid et al., 2000; Wang et al., 2010); however, there are no reported studies on the effect of antibiotics on biofilm expression by S. capitis.

In this study, we investigated (i) the effects of subinhibitory concentrations of erythromycin on biofilm expression by S. capitis and (ii) the transcriptional profile of biofilm-related genes in the two subspecies of S. capitis (S. capitis subsp. urealyticus and S. capitis subsp. capitis) in the presence and absence of erythromycin. The results provided an insight into the mechanisms regulating the icaADBC operon. Moreover, the individual and collective functions of Ica proteins encoded by S. capitis were deduced, based on in silico analysis.

**METHODS**

**Bacteria and growth conditions.** Five clinical S. capitis isolates, 6, 17, 44, 65 and 70, obtained from blood cultures of newborn babies suspected of having catheter-associated bloodstream infections were used in this study. Isolates 6, 17 and 70 were determined to be members of S. capitis subsp. urealyticus by biochemical assays (Cui et al., 2013). These three isolates were oxacillin resistant, and displayed the same DNA profiles of ica operons as assessed by PCR-RFLP. Isolates 6 and 70 had biofilm-positive phenotypes, whilst isolate 17 was biofilm-negative. Isolates 44 and 65 were members of S. capitis subsp. capitis, and were biofilm negative. These two isolates were sensitive to oxacillin and their ica operons showed variable RFLP patterns (Cui et al., 2013). The ica operons of the five isolates have been sequenced (GenBank accession numbers JF930147, KJ544504, KJ544505, KJ544506 and KJ544507).

For phenotypic biofilm assays, the bacteria were grown in Trypticase Soy Broth (TSB; Oxoid) in the presence of various concentrations of erythromycin, depending on the MICs of the isolates. A control in the absence of erythromycin was set up for each isolate at the same time.

For gene expression assays, because the bacterial cell pellets in TSB were too sticky for RNA isolation, Brain Heart Infusion Broth (BHI; Oxoid) was used as the growth medium. Cultures were incubated at 37 °C, aerobically with gentle shaking.

**Phenotypic characterization of biofilm formation by microtitre plate assay.** Quantification of biofilm was achieved by the previously described microtitre plate assay with minor modification (Christensen et al., 1985). Briefly, overnight cultures in TSB were diluted 1:100 with TSB supplemented with NaCl to make the final concentration to 0.85 % or 4% and with various concentrations of erythromycin. Aliquots of 200 μl per well were seeded into 96-well flat-bottomed microtitre tissue culture plates (Asahi Glass). The plates were incubated at 37 °C and examined for biofilm formation at 4–6 (exponential phase) and 20 h (stationary phase). After four washes in PBS (pH 7.2), biofilms were fixed by drying for 1 h at 55 °C and stained with Hückner crystal violet. Finally, the plates were rinsed under running tap water, air-dried and OD600 determined using a POLARstar Omega (BMG). Strong biofilm formation was defined as OD600 ≥ 0.24; weak biofilm formation was OD600 0.12–0.24. OD600 ≤ 0.12 was considered as indicating a biofilm negative result (Deighton et al., 2001). For each isolate the microtitre plate test was repeated in duplicate.

**Growth curve assessment.** Two isolates (6 and 44), as typical representatives of biofilm-positive S. capitis subsp. urealyticus and biofilm-negative S. capitis subsp. capitis, respectively, were compared for transcription of biofilm genes and their regulators under erythromycin stress. These isolates were chosen because previous studies had shown that the growth rates on agar media of the three members of S. capitis subsp. urealyticus were similar to each other as were the two members of S. capitis subsp. capitis (data not shown). The isolates were first examined for growth in the presence and absence of erythromycin to determine the time to reach the mid-exponential phase and the onset of the stationary phase, as required for harvesting cells for RNA quantification. Bacteria were pre-cultured in 10 ml BHI in 37 °C for 16 h with shaking at 150 r.p.m. Then, 500 μl of each culture was inoculated into 50 ml pre-warmed BHI (final NaCl concentration 0.85 %) with 16 mg erythromycin l⁻¹ for isolate 6 (representing 1/32 MIC and equivalent to the highest serum level following standard dose) or 0.25 mg erythromycin l⁻¹ for isolate 44 (representing 1/2 MIC) (Balitch et al., 1998). Cultures were incubated at 37 °C with gentle shaking. The amount of growth of bacterial cultures was measured as OD600. To construct growth curves, viable counts were performed in duplicate by standard plate counts every 2 h for the first 8 h and then at 24 h for isolate 6 or 48 h for isolate 44.

**Extraction of total bacterial RNA and real-time quantitative reverse transcription (RT)-PCR assays.** S. capitis cells were harvested at the mid-exponential phase and at the onset of the stationary phase according to the isolate’s growth curve. RNA was extracted using a RNA protect Bacteria Reagent kit and a RNeasy Mini kit (Qiagen) following the manufacturer’s instructions. A TURBO DNA-free kit (Ambion) was used for the removal of DNA in the samples following the instructions of the manufacturer. cDNA synthesis was performed using a Omniscript RT kit (Qiagen) according to the
The manufacturer’s instruction with modification. RNA for RT was isolated from two separate cultures of isolates of 6 and 44. Briefly, 1 µg RNA and 0.4 µM primer in a total volume of 14 µl were heated at 65 °C for 10 min and immediately chilled on ice for at least 3 min. The RT reaction master mix was prepared in a total volume of 5 µl, which consisted of 2 µl of 1 × RT buffer, 2 µl 5 mM dNTP and 2 U RNase inhibitor. The mixture was gently mixed and added to the RNA/primer mixture followed by addition of 1 µl reverse transcriptase (200 U µl⁻¹). Reverse transcriptase was substituted by RNase-free water for the negative control. The reaction mixture was gently mixed and incubated at 37 °C for 1 h. After cDNA synthesis, 230 µl molecular-grade water was added to the samples.

For each gene, a dilution series of newly synthesized cDNA was included in quantitative PCR to examine the efficiency of PCR and subsequent relative quantitative calculations.

Quantitative PCR was performed with a MiniOpticon (Bio-Rad) using IQ SYBR Green Supermix (Bio-Rad) to compare gene expression of the icaA gene and putative regulators in the presence and absence of erythromycin. The manufacturer’s instruction was applied in with the kits were followed. The primers designed using Clone manager version 7 (Scientific and Educational Software) are shown in Table 1. Each PCR was performed in triplicate. PCR conditions were as follows: cycle 1, one cycle at 95 °C for 5 min; cycle 2, 40 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; cycle 3, 100 cycles at 95 °C for 10 s. The settemperature was decreased after cycle 2 by 0.5 °C to enable melting curve data collection and analysis. Each specific amplicon was verified by the presence of a single melting temperature peak and by the presence of a single band of expected size on a 2.5 % agarose gel after electrophoresis. Cycle threshold (Ct) values were determined by CFX software (Bio-Rad).

Expression of the icaA gene and its regulators. Isolates 6 and 44 were chosen for the gene expression investigations as they differed most in biofilm-producing capacity with and without induction by erythromycin. The icaA gene was chosen to represent the ica gene cluster in transcription studies, as icaA is essential for phenotypic biofilm production in staphylococci. In addition, we quantified transcription of several other genes which have been well studied in S. epidermidis and S. aureus for their involvement in the process of biofilm regulation. These genes were the negative regulatory gene icaR, altE which encodes a major autolysin that promotes binding to hydrophobic surfaces for initial attachment and possibly biofilm accumulation, several global regulatory genes (sara, rsbU and sigB), and the quorum-sensing genes RNAIII and luxS.

To determine whether erythromycin altered the expression of regulatory and structural genes, a transcriptional profile of icaA and potential regulatory genes was developed, after choosing the most appropriate reference gene for normalization (Cui et al., unpublished). Normalization was performed using the relative quantitative method (Cui et al., unpublished). Five staphylococcal Reference genes from different functional classes (recA, gyrB, rpoB, gyrB and tuf) were used as internal controls for normalization of the real-time quantitative RT-PCR data for the following reasons. On induction with erythromycin, the geometric mean expression of recA and gyrB was identified as the most suitable normalizer in the exponential phase in isolate 6, whilst sodA was identified as the most stably expressed gene in isolate 44. In the stationary phase, the geometric mean of tuf and gyrB was identified as the most appropriate normalizer with erythromycin induction in isolate 6, and that of rpoB and sodA was recognized as the most suitable normalizer in isolate 44 under the same condition. Without erythromycin induction, the gyrB gene was stably expressed during transition in isolate 6; however, none of five reference genes displayed stable expression between the two growth phases in isolate 44 (Cui et al., 2015).

The amplification efficiency and the relative changes in gene transcription between the included (presence of erythromycin) and calibrated (without erythromycin) samples were calculated using the method described by Qiagen (2010). The genes were considered as down- or upregulated if their relative change in expression was >2 or <2⁻¹ (Livak & Schmittgen, 2001).

Statistical analysis. ANOVA was conducted, followed by Dunnett’s post hoc analysis to examine significant differences in the biofilm formation between experimental conditions. P < 0.05 was considered statistically different. Calculations were performed using SPSS version 16 (SPSS).

DNA and protein primary sequence alignment of the ica operon of S. capitis. Clone Manager version 7 (Scientific and Education Software) was used to compare the primary DNA and deduced protein sequences of the ica operon of five isolates (6, 14, 45, 65, and 70). The sequence of isolate 6 (GenBank accession number JF930147) was used as the master sequence for comparison. DNA sequences were inspected for deletions, insertions and substitutions, whilst the encoded protein sequences were examined for amino acid changes. Intergenic regions between the icaA and icaR genes in the ica operon of isolates 6 and 44 were aligned by using CLUSTAL W 2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default parameters.

Analysis of protein secondary structure–structure relationships to identify proteins. The conserved domain database was used for searching functional domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). PSI-BLAST was used to find distant evolutionary relationships for IcaA and IcaB proteins of isolate 6. A list of related known Protein Data Base (PDB) structures was generated as potential templates for protein modelling. The related sequences with a comparably high degree of similarity (about 15–27 % identity) were aligned using CLUSTAL Omega, with default parameters. CLUSTAL Omega is a multiple sequence alignment program that uses seeded guide trees and hidden Markov models as profile techniques to generate alignments (Sievers et al., 2011). Key functional residues for the family were determined. Next, key residues in the query sequence were determined by comparison with the potential relatives present in the PDB. The putative relatives were verified to be carrying known functional motifs that were curated previously from the literature. Remote homologues were therefore identified. PSORTb version 3.0 was used for predicting the cellular location of IcaD (Yu et al., 2010). The IcaC protein sequence of S. capitis isolate 6 was queried against the UniProt database using BLAST to identify functional homologues.

Protein secondary structures were derived from the PDB coordinates of the representative proteins. Hydrophobicity analysis was performed using the web-predictor TopPred II (von Heijne, 1992). All the possible topologies that included certain transmembrane (TM) segments and either included or excluded each of the candidate segments were automatically generated. SignalP 3.0 was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences (Bendtsen et al., 2004). The UniProt database was used to determine the location of the helix-turn-helix motif in IcaR of S. epidermidis.

RESULTS

Growth curves of isolates 6 and 44 in the presence and absence of subinhibitory concentrations of erythromycin

Growth curves were generated in order to determine optimal time points for harvesting cells for RNA analysis.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward/reverse primer (5’→3’)</th>
<th>GenBank accession no.</th>
<th>Function encoded</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>icaA</td>
<td>F: GGGCTATGGAAACAACG</td>
<td>AY146582</td>
<td>An enzyme that exhibits a sugar transferase activity in <em>S. epidermidis</em></td>
<td>Gerke <em>et al.</em> (1998)</td>
</tr>
<tr>
<td></td>
<td>R: TGTCGGGATACCAACTCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>icaR</td>
<td>F: CAACAACGATTCTAACTCCT</td>
<td>AY146584</td>
<td>IcaR, a negative regulator in <em>S. epidermidis</em></td>
<td>Jeng <em>et al.</em> (2008)</td>
</tr>
<tr>
<td></td>
<td>R: GGATAATTGGGCTAATAACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sarA</td>
<td>F: CGAAACAGATGAAAAGACAG</td>
<td>NZ_ACFR01000002</td>
<td>SarA, a global regulator which positively regulates biofilm formation in <em>S. aureus</em></td>
<td>Chien <em>et al.</em> (1999)</td>
</tr>
<tr>
<td></td>
<td>R: TTCGCCCTCAGTATACGTTTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rsbU</td>
<td>F: CTCATATTTCTGCTTCAG</td>
<td>AFX01000024</td>
<td>RsbU, a positive sigma factor, which positively regulates biofilm formation in <em>S. epidermidis</em></td>
<td>Knobloch <em>et al.</em> (2004)</td>
</tr>
<tr>
<td></td>
<td>R: GTGTGCTTGGAGTAAGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sigB</td>
<td>F: AGTCGAAATCAGCTAGTGAAG</td>
<td>AF274004</td>
<td>SigB, a positive sigma factor, which positively regulates biofilm formation in <em>S. epidermidis</em></td>
<td>Knobloch <em>et al.</em> (2004)</td>
</tr>
<tr>
<td></td>
<td>R: GCACCTATAGACACCAACATA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>atlE</td>
<td>F: CTGGGAACGGGGAATCAGAAGC</td>
<td></td>
<td><em>atlE</em> gene in <em>S. capitis</em> SK14</td>
<td>Qin <em>et al.</em> (2007)</td>
</tr>
<tr>
<td></td>
<td>R: GCGCCACCATAATTGCTTTAC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>RNAI1H</td>
<td>F: GGAAGGATGAAATGCTAATG</td>
<td>223044475: 8492–9045</td>
<td>Quorum sensing factor, which divergently regulates biofilm formation in <em>S. aureus</em></td>
<td>Coelho <em>et al.</em> (2008)</td>
</tr>
<tr>
<td></td>
<td>R: TTGGGATGCTAACAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>luxS</td>
<td>F: TTAATGTCGTACGTAGGAAG</td>
<td>luxS gene of <em>S. capitis</em> SK14</td>
<td>Quorum sensing factor, which negatively regulates biofilm formation in <em>S. epidermidis</em></td>
<td>Kong <em>et al.</em> (2006)</td>
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<tr>
<td></td>
<td>R: CCAGTTGCGAACCACCATAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gyrB</td>
<td>F: CCTCCACCATGTTAATACC</td>
<td>CP000029</td>
<td>B subunit of the DNA gyrase</td>
<td>Duquenne <em>et al.</em> (2010)</td>
</tr>
<tr>
<td></td>
<td>R: GAGGCTTGCACCTTTAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TGACCAGCCACCATTTAC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>recA</td>
<td>F: TAAAGTAGCACCCTCCCTAC</td>
<td>FN554702</td>
<td>RecA, which contributes to homologous recombination, DNA repair and the SOS response</td>
<td>Duquenne <em>et al.</em> (2010)</td>
</tr>
<tr>
<td></td>
<td>R: CCAAGCACCAGATTTATCAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AATGGCAGTACAGGAACAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AGTCCACCTGTCCTTCTAG</td>
<td></td>
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</table>
(exponential phase and onset of stationary phase). As expected (Bannerman & Kloos, 1991), isolate 6 (S. capitis subsp. urealyticus) grew more rapidly in the absence of erythromycin than isolate 44 (S. capitis subsp. capitis). The growth rate of isolate 44 was ~20% lower than that of isolate 6 (Fig. S1, available in the online Supplementary Material).

Isolate 6, which was resistant to erythromycin (MIC 512 mg l⁻¹), grew in the presence of 16 mg erythromycin l⁻¹ (1/32 MIC), although the time to reach both the mid-exponential and the stationary phase was delayed by ~2 h. Isolate 44, which was susceptible to erythromycin (0.5 mg l⁻¹), displayed a different growth pattern in the presence of 0.25 mg erythromycin l⁻¹ (1/2 MIC), reaching the mid-exponential phase after ~4 h with and without erythromycin, but only reaching the stationary phase after 24 h of growth in the presence of erythromycin, compared with ~12 h in medium without erythromycin (Fig. 1).

Phenotypic characterization of biofilm formation in S. capitis induced by erythromycin

The five clinical S. capitis isolates (6, 17, 44, 65 and 70) were examined for biofilm formation in the presence and absence of erythromycin. As for growth curves, 1/32 MIC (16 mg l⁻¹) of erythromycin was employed for isolate 6, whilst 1/2 MICs were employed on all the other isolates (1, 0.25, 0.125 and 0.25 mg l⁻¹ for isolates 17, 44, 65 and 70, respectively).

At the mid-exponential phase, the biofilm-positive phenotype was displayed only by isolate 6, both in the presence and absence of erythromycin. Although biofilms appeared to be denser in the presence of erythromycin than in its absence, the difference was not statistically significant. The other isolates either produced no biofilm or very small amounts at this stage of the growth cycle.

At the stationary phase, in the absence of erythromycin, isolates 6 and 70 produced biofilms with OD₆₀₀ values of 1.16 ± 0.042 and 0.39 ± 0.028, respectively. The other three isolates (17, 44 and 65) failed to produce significant amounts of biofilm without erythromycin. Erythromycin induced biofilm formation in isolates 6 (P<0.01) and 17 (P<0.05). Biofilm production by isolate 6 occurred in a dose-dependent manner (Fig. S2). The other two biofilm-negative isolates, 44 and 65 (both S. capitis subsp. capitis), remained biofilm-negative in the presence of erythromycin (Fig. 2). When stressed by growth in TSB with 4% NaCl, isolates 6 and 70 also produced strong biofilms.

Gene expression profile in response to growth phase and erythromycin induction

When isolate 6, representing S. capitis subsp. urealyticus, was grown in BH1 (0.85% NaCl) without erythromycin, the expression levels of icaA and a positive global regulatory gene, sarA, were elevated in the stationary phase compared with the mid-exponential phase (Table 2). In the presence of erythromycin, only icaR, the negative ica regulator, was overexpressed between these stages. In addition, transcription of the icaA and sarA genes slightly increased, but not significantly according to the criteria used (Livak & Schmittgen, 2001). The quorum-sensing factors RNAIII and luxS, as well as altE, were downregulated between the two growth phases, both in the presence or absence of erythromycin.

The relative expression of icaA of isolate 6 and its potential regulatory genes in the presence of erythromycin compared with no induction at the exponential phase and the stationary phase is presented in Fig. 3. At the exponential phase, mRNAs for icaA, the positive regulator sarA and the positive sigma factor rsbU showed the greatest differences in levels between the two phases. From the onset of the stationary phase, all the genes studied exhibited an almost equal or increased transcription, except for the luxS gene (a negative regulator of biofilm), which showed reduced expression in the presence of erythromycin. At this stage, transcription of the positive regulator sigB was 10-fold greater under

Fig. 1. Growth curves of (a) isolate 6 (S. capitis subsp. urealyticus) and (b) isolate 44 (S. capitis subsp. capitis) in the absence and presence of erythromycin (ERY). Each data point represents the mean ± SEM. The data shown were from duplicate independent experiments, which showed similar results.
There were also significant increases in the transcription of icaA and rsbU (positive regulator) at this stage of growth.

Isolate 44 displayed quite different transcriptional profiles from those of isolate 6 during growth. In both growth phases, all genes studied exhibited downregulation or almost unchanged transcription levels in the presence of erythromycin, compared with its absence. The only exception was altE, which showed 4.7-fold higher expression during the stationary phase in the presence of erythromycin, compared with its untreated control treatment (Fig. 4). This increase in the expression of altE was not associated with an increase in biofilm production. With erythromycin induction, the transcription of the sarA gene showed the most upregulated expression on the transition from exponential to the stationary phase, with ~13.4-fold increase; however, this elevation did not result in biofilm formation in this isolate.

### Structural–functional analysis of Ica proteins in S. capitis

Sequence alignments showed that the ica operons in the two S. capitis subsp. urealyticus isolates either showed 100 % identity to those of isolate 6 (isolate 70) or one 9 base deletion (isolate 17), the two S. capitis subsp. capitis isolates (44 and 65) demonstrated many nucleotide substitutions, some of which were associated with amino acid sequence changes (Cui et al., 2013; Table S1).

Sequence analysis showed that IcaB of S. capitis was a polysaccharide N-deacetylase with 71 % identity to the corresponding acetylase of S. epidermidis (Fig. S3), IcaR belonged to the tetracycline repressor family of proteins that function as transcription activators and repressors (Fig. S4), and the intergenic region between IcaACBD and IcaR acted to control the adjacent genes (Figs S5 and S6).

### Table 2. Relative gene expression on the transition from the exponential to the stationary phase in isolate 6 with and without erythromycin induction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative gene expression ratio*</th>
<th>95 % Lower confidence limit</th>
<th>95 % Upper confidence limit</th>
<th>Relative gene expression ratio*</th>
<th>95 % Lower confidence limit</th>
<th>95 % Upper confidence limit</th>
</tr>
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<tbody>
<tr>
<td>icaR</td>
<td>1.36</td>
<td>0.91</td>
<td>1.73</td>
<td>3.68</td>
<td>2.67</td>
<td>6.30</td>
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<tr>
<td>icaA</td>
<td>3.30</td>
<td>0.19</td>
<td>5.05</td>
<td>1.58</td>
<td>0.73</td>
<td>2.30</td>
</tr>
<tr>
<td>altE</td>
<td>0.13</td>
<td>0.10</td>
<td>0.14</td>
<td>0.15</td>
<td>0.11</td>
<td>0.19</td>
</tr>
<tr>
<td>sarA</td>
<td>3.56</td>
<td>2.63</td>
<td>4.27</td>
<td>1.32</td>
<td>0.52</td>
<td>2.68</td>
</tr>
<tr>
<td>rsbU</td>
<td>1.12</td>
<td>0.017</td>
<td>1.15</td>
<td>0.98</td>
<td>0.75</td>
<td>1.16</td>
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<td>sigB</td>
<td>0.02</td>
<td>0.003</td>
<td>0.02</td>
<td>0.95</td>
<td>0.86</td>
<td>2.41</td>
</tr>
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<td>RNAIII</td>
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<td>0.18</td>
<td>0.35</td>
<td>0.24</td>
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<td>0.50</td>
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<tr>
<td>luxS</td>
<td>0.002</td>
<td>0.002</td>
<td>0.005</td>
<td>0.21</td>
<td>0.16</td>
<td>1.13</td>
</tr>
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*Normalized gene expression in the stationary phase was divided by normalized gene expression in the exponential phase (exponential phase gene expression was used as calibrator).
Sequence alignments showed that IcaA of isolate 6 was homologous to known glycotransferases (Figs S7 and S8a). IcaD was likely to be involved in translocating the glycan across the membrane (Figs S7 and S8b). The TM region of IcaA of S. capitis included five TM (1–5) helices, indicating that it was a hydrophobic protein. In contrast, the protein sequence for isolate 17 was predicted to have values below the threshold for hydrophobicity segments and so did not support a TM location, possibly leading to abolition of biofilm production in this isolate (Fig. S9). Most TM regions of IcaA and IcaD proteins were conserved with those of BcsA–BcsB of Rhodobacter sphaeroides in the alignment (Fig. S8b), e.g. the locations of TM1, 3, 4 and 5 in IcaA are conserved with those of TM4–7 in BcsA–BcsB, respectively (Fig. S8a).

The motif QRxRW (Gln379, Arg380, x381, Arg382 and Trp383) is the sugar accepter in BcsA–BcsB, which forms a binding site for the terminal disaccharide of the glucan. This sequence is a part of the cytoplasmic entry to the glucan channel and is well conserved between BcsA–BcsB and IcaA (Fig. S8a). In BcsA–BcsB, Gln379, forms a hydrogen bond with the guanidine group of the neighbouring

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**Fig. 3.** Relative gene expression of isolate 6 in the presence of erythromycin (ERY; 16 mg l⁻¹): (a) exponential phase and (b) stationary phase. Mean values are marked by filled circles; confidence limits are shown by vertical bars. The dotted lines represent the threshold value of 100 % gene expression in the presence of erythromycin relative to that without erythromycin induction. LCL, lower confidence limit; UCL, upper confidence limit.
Arg380, which in turn forms a salt bridge with Glu297. Also, Arg382 most likely coordinates the diphosphate of the UDP co-substrate. The adjacent Trp383 produces a van der Waals force with the ultimate glucose molecule present at the acceptor site, and packs against the Gln379 and Arg380 pair towards the distal site of the catalytic pocket (Morgan et al., 2013).

In the case of BcsA–BcsB, the channel formed accommodates 10 glucose units of the translocating glucan. The glucan enters the channel through the cytoplasmic opening, crosses the membrane parallel to TM5 and 6, and exits on the periplasmic side between TM loops 5/6 and 7/8. In the absence of glucan, TM7 and 8 might move towards the N-terminal TM helices to close the channel (Morgan et al., 2013).

Although the sequence alignment in this region is weak, a cluster of conserved residues which interact with the translocating glucan is well conserved between the two structures of BcsA–BcsB and IcaA (Fig. S8a). These residues include Tyr149 (Tyr56, IcaA), Trp383 (Trp267, IcaA), Phe426 (Phe313, IcaA) and Tyr433 (Tyr325, IcaA) in TM5 of BcsA–BcsB, and Phe441 (Phe333, IcaA) in the TM5/6 loop. A proposed model for PIA synthesis and translocation in S. capitis is presented in Fig. 5.

**Function of the IcaC protein of S. capitis**

Sequence analysis showed that IcaC was an acetyltransferase (Fig. 6). The conserved functional domain search revealed that IcaC was homologous to the Acyl_transf_3 ( Pfam 01757) acetyltransferase family that contains >1000 confirmed or hypothetical acetyltransferases with activity on a variety of substrates. Fig. 6(a) shows an alignment of IcaC from S. capitis isolate 6 with three acetyltransferases. All four structures had 10 predicted TM regions, which were, in most cases, at similar positions within the
Partially conserved motifs between alignments suggested the corresponding functions of the terminal region of the protein. Another motif, SYGxY, TM2 and 3 segments, respectively, are situated in the N-terminal region of the protein. Shigella flexneri O-acetyltransferase (OAT) of S. capitis, including the close proximity of a critical Arg80 to Phe85 and Pro87, suggested that the three residues may be involved in forming one of the active sites. The conserved histidine residue His23 in S. capitis, highlighted in the alignment, is likely to be responsible for the deprotonation of the substrates as observed in other acetyltransferases (Wu et al., 2003; Govindasamy et al., 2004; Lee et al., 2009).

**Fig. 5.** Proposed roles of IcaA and IcaD in PIA synthesis and translocation. Conserved residues of IcaA and IcaD may interact with the translocating glucan. The outlines represent the two proteins. IcaA mediates the synthesis of the glucan in the cytoplasm; the glucan is transferred through a channel formed by IcaA and IcaD to the outside of the cell membrane.

**DISCUSSION**

The expression of biofilm by S. capitis was investigated under erythromycin stress. Isolate 6 (S. capitis subsp. urealyticus; MIC 512 mg erythromycin l⁻¹) showed strong biofilm formation under all conditions examined; in particular, there was significant enhancement of biofilm production when stressed with salt (TSB with 4 % NaCl) (Cui et al., 2013) or with subinhibitory concentrations of erythromycin (1/32 MIC). This pattern of enhancement in the presence of erythromycin has also been shown for S. epidermidis, which exhibited enhanced biofilm formation in a dose-dependent manner (Wang et al., 2010). These similarities between S. capitis and S. epidermidis suggest that the Ica proteins of the two species, which we have shown to have similar structures and functions, are also regulated in a similar manner.

Isolate 70, also identified as S. capitis subsp. urealyticus, behaved in a different manner from isolate 6, although the ica operon of the two isolates is 100 % identical (Cui et al., 2013). Biofilm density of isolate 70 was only marginally enhanced in the presence of 1/2 MIC (0.25 mg l⁻¹) of erythromycin. This was probably due to the low MIC value (0.5 mg l⁻¹), which may not be sufficiently stressful to promote a significant increase in biofilm density. The third isolate belonging to S. capitis subsp. urealyticus (isolate 17) failed to produce biofilm in TSB, even with supplementation with 4 % NaCl, but showed weak enhancement in the presence of 1/2 MIC (1 mg l⁻¹) (Cui et al., 2013). The extremely weak biofilm production by isolate 17 can be explained by three deletions of amino acids that occurred in the IcaA protein (Gln192, Ser193 and Leu194) and substitution of Ala195 with Pro (Cui et al., 2013; Table S1). The insertion of Pro residue in the predicted z-helices (Fig. S6) might lead to the disruption of the secondary structure (Woolfson & Williams, 1990). Moreover, changes in membrane protein topology (four TM locations) compared with that of isolate 6 (five TM locations) might demonstrate the inability to produce biofilm by this isolate (Fig. S9). However, isolate 17 did produce weak biofilm in the presence of 1/2 MIC of erythromycin, possibly suggesting an alternative biofilm formation pathway, e.g. mediated by protein or DNA (Qin et al., 2007).
Fig. 6. (a) Multiple sequence alignment of the IcaC protein of *S. capitis*. CapiC, the IcaC protein from *S. capitis* 6; YFIQ_BACSU, a membrane-bound acyltransferase yfiQ from *Bacillus subtilis*. MDMB_STRMY, an acyltransferase MdmB from *Streptomyces mycarofaciens*; OAC_BPSFV, an O-acetyltransferase from *Enterobacteria* phage Sf6. Identical residues are shown in white with a black background; conserved amino acids are coloured black with a grey background. The membrane-spanning segments are underlined; the predicted TM regions of CapiC are indicated with black dashed lines on the top of the sequence. The key residues involved in catalytic reactions are indicated with black dots under the sequences. (b) Multiple partial sequence alignment of IcaC of *S. capitis*. ARY1_HUMAN, arylamine NAT1 (GenBank accession number X17059); U17260, rat arylamine NAT1 (GenBank accession number U17260); ARY1_RABBIT, arylamine NAT1; AAB33787, *Salmonella* Typhimurium OAT (GenBank accession number AAB33787); CapiC, IcaC protein in *S. capitis* 6. Two conserved Arg residues are indicated by a black background with white letters.
Sequence analysis of the ica operon and icaR gene of the two S. capitis subsp. capitis isolates (44 and 65) revealed numerous differences compared with the same regions of isolate 6 (Table S1). Both isolates exhibited a biofilm-negative phenotype under all conditions. The data together led us to the hypothesis that erythromycin induction of biofilm formation in S. capitis is substantially dependent on the ica pathway; however, more data are required to determine the exact regulatory pathway.

After showing significant differences in phenotypic expression of biofilm between isolate 6 (S. capitis subsp. urealyticus) and isolate 44 (S. capitis subsp. capitis), in response to growth phase and erythromycin, transcriptional analysis of IcaA and its putative regulators was conducted, using real-time quantitative RT-PCR with RNA prepared from mid-exponential-phase and stationary-phase planktonic cells (Table 2). In the absence of erythromycin, the increased level of icaA expression from the exponential to the stationary phase is consistent with the findings for S. epidermidis and S. aureus, and other staphylococcal species (Bischoff et al., 2001; Nielsen et al., 2011), in which the role of IcaA in biofilm formation was established (Götz, 2002; O’Gara, 2007). The elevated level of SarA expression is also in agreement with other studies, in which this protein was shown to function as a positive regulator of icaA in S. aureus and S. epidermidis (Valle et al., 2003; Tormo et al., 2005). The overexpressed values of the icaA and sarA genes in S. capitis correspond to an increase in biofilm formation from OD600 0.18 ± 0.03 to 1.16 ± 0.04 (Fig. 2) from the exponential phase to the stationary phase. In the present study, a conserved SarA-binding site was identified, located in the primary sequence of the intergenic region between the icaR and icaA genes in S. capitis (Fig. S5). This suggested that SarA may have a direct effect on transcription of ica. The positive sigma factor RsbU showed a small elevation in its expression at the beginning of the stationary phase, whilst another positive sigma factor, sigB, was strongly downregulated, consistent with a previous study on S. aureus (Valle et al., 2003). This downregulation of sigB in S. capitis, however, did not repress icaA gene expression; possibly it occurred at a different time point, e.g. at the end of the exponential phase, which suggests that the combination of the alternative positive sigma factor RsbU and SarA modulates biofilm development in S. capitis as it progresses into the stationary phase.

In the presence of erythromycin, relative gene expression ratios were similar to those in media without erythromycin, but were generally lower. Only icaR exhibited strongly increased expression in the stationary phase; however, this did not result in the inhibition of icaA expression. Together with the weak enhancement of icaA and sarA expression in isolate 6, this result suggests that different factors are involved in the regulation of biofilm formation depending on the conditions.

Further analysis was conducted to assess the influence of erythromycin on the expression of these genes. In the exponential phase, erythromycin caused increased expression of icaA, sarA and rsbU, and decreased expression of icaR. The results further demonstrate that SarA and RsbU positively regulate expression of icaA in isolate 6. It is difficult to say whether the repression of icaR contributes to the increased icaA expression at this stage, because icaR appears to be a weak regulator and biofilm expression in this isolate is regulated by several other global regulators, e.g. sigma factors. Expression of the sigB gene was downregulated during the exponential phase in the presence of erythromycin; however, interestingly, when entering into the stationary phase, most of the genes studied, with the exception of luxS, exhibited increased transcription. The increased transcription of sigB gene was the most pronounced (~10-fold) compared with the untreated control. This result suggests SigB may start regulation at the onset of the stationary phase when induced with erythromycin.

No SigB-binding site was identified in the intergenic region between the icaA and icaR genes in the present study or in a previous study involving S. epidermidis (Knobloch et al., 2004), suggesting that SigB functions indirectly alter expression of icaA, through an unknown regulatory gene. The strongly elevated expression level, at the stationary phase, of atLE, which encodes a major autolysin for primary staphylococcal adherence, suggests that erythromycin-induced gene expression leads only to initial attachment to a surface. Quorum-sensing factors were apparently not expressed either in the absence and presence of erythromycin, possibly because the planktonic cells did not reach sufficient densities for their activation. Taking the data together, we speculate that SigB plays an important role in the regulation of ica-dependent biofilm formation in clinical isolate 6 in the presence of erythromycin; nevertheless, this remains to be tested with isogenic mutants. The findings from the transcriptional analysis of icaA and its related regulatory factors are consistent with the phenotypic biofilm assays in this study; biofilm production was not significantly increased in the mid-exponential phase, whilst a pronounced increase was observed in the stationary phase with the induction of erythromycin, along with increased expression of icaA and positive regulators, rsbU and sigB.

The genes that were examined for isolate 6 displayed a different transcriptional profile in S. capitis subsp. capitis (isolate 44, biofilm-negative phenotype with or without erythromycin) under the same conditions. The presence of a single nucleotide deletion mutation in the −10 promoter element of the icaA operon, compared with that of biofilm-positive isolates (Fig. S6), might prevent the transcription of the ica genes; however, as icaA mRNA was abundant when isolate 44 was grown in the presence of erythromycin, the RNA polymerase must have bypassed this mutation. Alternatively, biofilm formation might be regulated by different mechanisms in these two subspecies. Further work is necessary to determine the basis of differential regulation of the ica locus in the two subspecies, and in biofilm-positive and -negative isolates, e.g. using isogenic strains to examine the role of a particular pathway.
in biofilm formation. In addition, the investigation of more isolates with a similar genetic background would provide more detailed information on gene regulation relevant for biofilm formation in *S. capitis*.

In this study, based on sequence similarity, we suggest an alternative proposal for the functions of Ica proteins in *S. capitis* (Fig. 7). Previous models for Ica protein function in *S. epidermidis* suggested that IcaA and IcaD were TM proteins (Gerke *et al.*, 1998); IcaA is a glucotransferase that is involved in the synthesis of the sugar polymer, whilst it was proposed that the IcaD increases the enzymic activity of IcaA. The IcaB protein has been shown experimentally to be a deacetylase. Another integral protein, IcaC, is required for further elongation of PIA and can be recognized by a polyclonal antibody raised against PIA purified from WT cells. This protein is believed to be involved in the export of the growing PIA chain; however, the route through which the growing polymer is transferred from the cytoplasm to outside the membrane was not clearly addressed in this model.

Data presented here suggests that the IcaA and IcaD proteins in *S. capitis* are TM proteins that play roles in both synthesis and translocation of the polysaccharide. It is suggested that the IcaA protein is involved in sugar donation and acceptor binding, and synthesis of the glucan. Subsequently, IcaA and IcaD coordinate in forming a transport channel parallel to the TM regions to complete the process of transferring the synthesized sugar from the cytoplasmic opening to outside the cell membrane (Fig. 7a), similar to the activity of BcsA–BcsB of *R. sphaeroides*. Based on previous research (Gerke *et al.*, 1998), combined with the current analysis in this study, we suggest the translocation of the sugar polymer is important for extending the sugar chain and that it positions the synthesized glucan outside the membrane. IcaB of *S. capitis* may exhibit a de-N-acetylase function similar to that in *S. epidermidis*, in which IcaB protein has been characterized as a de-N-acetylase, involved in the introduction of positive charges in the PIA polymer, to facilitate adhesion to the cell surface. The IcaB protein may also play a role in immune evasion by modification of the cell wall to significantly increase its resistance to lysozyme, which is present in high concentrations at the sites of infection. Based on our new analysis, IcaC in *S. capitis* mainly catalyses the acetylation of polysaccharide and this

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**Fig. 7.** Function of Ica proteins in the biosynthesis of polysaccharide in *S. capitis*. IcaA is required for glucan synthesis in cytoplasm; the growing sugar polymer is transported through a channel formed by IcaA and IcaD. IcaB is involved in deacetylation of the growing chain and introduces positive charges which are crucial for the growing chain to adhere on the outer membrane. IcaC transfers the acetyl groups to the polysaccharide from the cytoplasm to the outer membrane; this process needs CoA.
structural modification might aid the synthesized polysaccharide to evade the host immune system in vivo.

Acetyltransferases generally use the acetyl donor and acetyl-CoA to transfer the acetyl group from one molecule to another, with specificity for the acetyl acceptor. Thus, IcaC may be required for modifying the growing polysaccharide chain according to the reaction:

\[
\text{acetyl-CoA + polysaccharide} \rightarrow \text{acetyl-polysaccharide + CoA}
\]

The conservation of these key residues in IcaC and other acetyltransferases strongly suggests that they may be involved in similar functions. It is, therefore, likely that IcaC requires acetyl-CoA for its activity in which an acetyl group from the acetyl-CoA in cytoplasm is transferred to polysaccharide located outside the membrane, after which CoA returns to the cell cytoplasm (Bera et al., 2005), and leaves a more acidic polysaccharide chain.

In addition, according to the multiple alignment of IcaC, one of these conserved motifs (Ile84, Pro87 and Leu89) contains a Pro residue. Pro residues are not normally favoured in \(\alpha\)-helices because they are thought to produce a ‘kink’ in the TM helix (Woolfson & Williams, 1990). A TM Pro might be required for the correct conformation of a bent helix that may be critical for protein function or to increase conformational flexibility of the helix (Fig. 6a) (Van Arnam et al., 2011).

In summary, the findings of a previous study (Cui et al., 2013) that \(S.\) capitis subsp. urealyticus is generally multiply antibiotic resistant and produces biofilm, whilst \(S.\) capitis subsp. capitis is susceptible to most antibiotics and biofilm-negative, together with the results presented here on major differences in the regulation of the ica operon stressed by erythromycin, are consistent with the differing prevalence of the two species in neonatal intensive care units. Different pathogenesis and survival strategies are apparently adopted by the two different subspecies in adapting to hostile environments. Whilst \(S.\) capitis subsp. urealyticus has developed traits that enhance its ability to persist in the neonatal intensive care unit environment, it is most likely that \(S.\) capitis subsp. capitis isolates are derived from skin flora and are not well adapted to persistence. Experiments are still required to confirm the new Ica protein functional model identified in this study. Given that biofilm formation by PIA is an important virulence determinant in staphylococci, knowledge of the biosynthesis of PIA could lead to the discovery of new therapeutic targets specific to these bacteria.

ACKNOWLEDGEMENTS

We thank Vennessa Fleming and Nerida Thurbon for excellent technical assistance. This study was supported by an Australian Postgraduate Award to B. C.

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Biofilm formation is mediated via the alternative sigma factor implicated in biofilm formation in 36 depends on functional RsbU, an activator of the expression data using real-time quantitative PCR and the 2


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