Mutation of tagO reveals an essential role for wall teichoic acids in Staphylococcus epidermidis biofilm development

Linda M. Holland, Brian Conlon and James P. O’Gara

INTRODUCTION

Teichoic acids are a group of polyanionic cell surface molecules that have been implicated in maintaining cation homeostasis (Hughes et al., 1973; Lambert et al., 1977), anchoring of cell surface proteins (Navarre & Schneewind, 1999), modulation of autolytic enzymes (Calamita & Doyle, 2002; Schlag et al., 2010), and biofilm development (Gross et al., 2001). Staphylococci contain two types of teichoic acid, peptidoglycan-bound wall teichoic acids (WTA) and membrane-anchored lipoteichoic acids (LTA). WTA of staphylococci are mainly composed of ribitol phosphate groups, whereas LTA contain glycerol phosphate groups (Endl et al., 1983; Vinogradov et al., 2006). Cell surface teichoic acids can be further modified by the addition of positively charged D-alanine residues by the dltABCD operon (Gertz et al., 1999). A Staphylococcus aureus dltA mutant is unable to produce biofilm on polystyrene or glass. This phenotype has been attributed to a decrease in primary attachment and not accumulation, as levels of the extracellular polysaccharide polysaccharide intercellular adhesin (PIA) were unaffected (Gross et al., 2001). A loss of D-alanine residues on surface teichoic acids as a result of the dltA mutation may lead to an increase in the net negative charge of the cell surface increasing the strength of repulsive forces, which in turn prevents adherence of the bacteria to a surface. There is also evidence that alterations of the teichoic acid net charge in Bacillus subtilis may affect adhesive properties in an indirect manner, by causing changes in the folding of exo-proteins, thereby altering cell surface interactions (Hyryrälainen et al., 2000).

The composition of peptidoglycan-bound WTA varies among organisms and species, but the most common structures contain either glycerol phosphate (Gro-P) or ribitol phosphate (Rbo-P) repeating units (Neuhaus & Baddiley, 2003; Swoboda et al., 2010). In Staphylococcus epidermidis RP62A, WTA are composed of Gro-P repeating units, substituted with glucose, galactose and N-acetylated aminosugars, as well as D-alanyl, L-lysyl or acetyl residues (Sadovskaya et al., 2004). S. aureus WTA comprise two to three units of Gro-P followed by approximately 40 units of Rbo-P polymers substituted with D-alanine and N-acetylglucosamine (Vinogradov et al., 2006; Weidenmaier...
Table 1. Strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
<th>Relevant characteristic(s)</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>CSF41498</td>
<td>Biofilm-positive, cerebrospinal fluid isolate</td>
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<td>CSF-2</td>
<td>CSF41498 icaC::IS256, PIA-negative</td>
<td>Hennig et al. (2007)</td>
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<td>TAGO1</td>
<td>CSF41498 derivative, ΔtagO::Tc′</td>
<td>This study</td>
</tr>
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<td>RN4220</td>
<td>Restriction-deficient derivative of S. aureus 8325</td>
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<td>E. coli TOPO</td>
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<td>pBT2</td>
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<td>pBlue::tet</td>
<td>pBluescript containing the tetA gene from pT181 on a 2236 bp HindIII fragment</td>
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<td>pL150</td>
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<td>Lee et al. (1991)</td>
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<td>pSETAG1</td>
<td>2642 bp PCR product containing the S. epidermidis tagO gene amplified from CSF41498 using primers tagO1 and tagO2 cloned into pCR-Blunt II-TOPO</td>
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<td>pSETAG5</td>
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<td>pSETAG6</td>
<td>EcoRI fragment from pSETAG5 containing tagO cloned into pL150</td>
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AGGCCTTTTACTGGATTAATAGAT
AGGCCTCAGTTCACCTTCATTGAA

Table 2. Oligonucleotide primers used in this study

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<td>tagO2</td>
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<td></td>
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Detection of biofilm formation on Congo red agar. The morphology of staphylococcal isolates on Congo red agar (CRA) has been used to detect biofilm production and has been shown to correlate with a biofilm-positive phenotype (Handke et al., 2004). Biofilm-forming isolates produce black, dry colonies with irregular morphology on CRA. Biofilm-negative isolates produce smooth, red colonies. Single colonies were subcultured from BHI agar onto CRA containing 0.8 mg Congo red ml⁻¹ and 5% (w/v) sucrose. Plates were incubated for 24 h at 37 °C and a further 72 h at room temperature. Plates were examined after 48 and 72 h incubation at room temperature, and strains were noted to produce either black, dry colonies with irregular morphology (biofilm-forming colonies) or smooth, convex colonies.

Construction of an S. epidermidis tagO deletion mutant. The S. epidermidis CSF41498 tagO deletion mutant TAGO1 was constructed using the following procedure. A 2642 bp fragment containing the tagO gene was amplified by PCR using Phusion high-fidelity DNA polymerase (NEB) and the primers tagO1 and tagO2 (Table 2), and was cloned into the pCR-Blunt II-TOPO plasmid (Invitrogen) to create pSETAG1. A 1021 bp portion of the tagO gene was subsequently deleted by inverse PCR using Phusion high-fidelity DNA polymerase and the primers SEtagOdel1 and SEtagOdel2 (Table 2). The resulting PCR fragment was purified and digested with Stul, followed by religation to create pSETAG2. The 2236 bp Tc² gene was cloned into pBlue::tet on a SwaI–Stul fragment and ligated into the Stul site to create pSETAG3. A 3921 bp BamHI–Xbal fragment containing the mutant allele from pSETAG3 was ligated into pBT2::bga digested with BamHI/Nhel to create pSETAG4. The temperature-sensitive pSETAG4 was electroporated into RN4220 and subsequently CSF41498.

The S. aureus tagO mutation results in a temperature-sensitive phenotype (Vergara-Irigaray et al., 2008); therefore, allele replacement was achieved using a variation of the normal heat-shock protocol. CSF41498 containing plasmid pSETAG4 was grown at 30 °C in the presence of Cm and Tet for 24 h, followed by 6 h growth at 42 °C without antibiotic selection and selection of Tc² colonies on BHI agar plates. Bluo-gal, 200 µg ml⁻¹ (Invitrogen), was included in the media to facilitate blue/white screening. A mixture of dark-blue and pale-blue colonies was observed following incubation for 24 h at 37 °C. Dark-blue colonies presumably contain extra-chromosomal pSETAG4, whereas pale-blue colonies have undergone a single recombination event and have incorporated the entire plasmid onto the chromosome. Plasmid DNA was prepared from the pale-blue colonies and digested to confirm the loss of the extra-chromosomal pSETAG4. These colonies were then grown for several subcultures in BHI without antibiotic selection at 37 °C to encourage plasmid excision via a second recombination event. This was followed by selection of Tc² colonies on BHI agar plates containing 200 µg Bluo-gal ml⁻¹. White, Tc² colonies were then screened for sensitivity to Cm to confirm plasmid loss, and PCR analysis was used to verify the presence of the mutant allele on the chromosome (data not shown).

To complement the tagO deletion mutant, a 1978 bp fragment containing the S. epidermidis tagO gene was amplified from CSF41498 genomic DNA using Phusion high-fidelity DNA polymerase (NEB) and the primers SEllm1 and SEllm2 (Table 2), and was cloned into the pCR-Blunt II-TOPO plasmid (Invitrogen) to create pSETAG5. From pSETAG5 an EcoRI fragment containing the tagO gene was cloned into pLJ50 to create pSETAG6.

Biofilm and pellicle formation assays. Semiquantitative measurements of biofilm formation under static conditions were determined using Nunclon tissue culture-treated (ΔSurface) 96-well polystyrene plates (Nunc), as described previously (Conlon et al., 2002). Tissue culture-treated polystyrene is more negatively charged and hydrophilic than untreated polystyrene, which is hydrophobic. Each strain was tested at least three times and mean results are presented. A biofilm-positive phenotype was defined as A560 ≥ 0.17.

Measurement of pellicle formation was performed using a modified biofilm assay in which the pellicle, which is defined here as an adherent mat of biomass only loosely attached to the polystyrene surface, was very gently washed to prevent its detachment from the plate prior to staining. In contrast, for biofilm measurements, the plates were vigorously washed three times by submersion in water. Vigorous washing caused the pellicle to rapidly detach and wash away.
Primary attachment assays. Attachment assays using NuncLab tissue culture-treated (ΔSurface) 96-well polystyrene plates (Nunc) were carried out using overnight cultures adjusted to OD600 1.0 and exponential phase cultures (OD600 1.0). A 200 μl volume of each suspension was used to inoculate the microtitre plate wells prior to incubation at 37 °C for 2 h. Following incubation, the wells were washed gently three times with distilled H2O, dried at 65 °C for 1 h and stained for 10 min with 0.4 % crystal violet. After staining, the plates were washed gently three times with distilled H2O and the remaining crystal violet was solubilized using 100 μl 33 % (v/v) acetic acid. The absorbance of the solubilized crystal violet was measured at 492 nm using a Multiskan Ascent plate reader (Thermo Electron Corp.). Each experiment was carried out at least three times.

Autolysis assays. Triton X-100-induced autolysis was assayed in exponential cultures grown to OD600 2.0 and in overnight cultures adjusted to OD600 1.0. Cells were pelleted by centrifugation and washed with 30 ml cold PBS (Sigma). The cells were then resuspended in 30 ml 0.01 % (v/v) Triton X-100 (Sigma) in PBS and incubated at 37 °C with shaking. The OD600 was taken at regular time intervals. Results were normalized to time zero (OD600), i.e. percentage lysis=[(OD600t−OD600) at time t]/(OD600t0)×100. All results presented are the mean of at least three independent experiments.

Stationary phase lysis was measured by following the OD600 of cultures grown in BHI and BHI glucose media for 24 h.

Bacterial adherence to hydrocarbon (BATH) assay. Cultures were grown with shaking in BHI and BHI glucose at 37 °C to OD600 2.0 before being washed twice in 1 × PBS and resuspended in 4.5 ml 1 × PBS (OD600 1.0). As noted elsewhere (Rosenberg, 2006), the buffer in which the cells are suspended is an important variable in BATH assays, and in our experiments we found that different results were obtained using different commercial PBS buffers. We have used 1 × Dulbecco’s PBS (Oxoid) in these experiments. A 500 μl volume of p-xylene (Sigma) was added to the cell suspension and vortexed vigorously for 1 min. After 20 min (to allow for phase separation) the percentage of cells (as determined spectrophotometrically at OD600) retained in the aqueous phase was measured. Each experiment was repeated three times.

Pellicle dispersal by sodium metaperiodate, proteinase K and DNase I. Pellicle stability against proteinase K or sodium metaperiodate treatment was tested as described elsewhere (Mack et al., 1994; Rohde et al., 2005a), with the following modifications. Bacteria were grown at 37 °C overnight in BHI and BHI supplemented with 1 % (w/v) glucose in 96-well microtitre plates. Supernatants were carefully removed and each well was washed with 50 μl distilled H2O. Pellicles were treated with 50 μl 10 mM sodium metaperiodate (Fluka) in distilled H2O or 50 μl 100 μg ml−1 proteinase K (Sigma) in distilled H2O for 2 h at 37 °C. The control wells contained 50 μl distilled H2O. Following incubation, each well was carefully washed with 50 μl distilled H2O and dried for 1 h at 65 °C prior to staining with 0.4 % (w/v) crystal violet. The A692 of the adhered, stained pellicles was measured using a Multiskan Ascent plate reader. Each strain was tested at least three times and mean results are presented.

Strains were grown in media supplemented with 0.5 mg ml−1 DNase I (Sigma). DNase I was added at t0 unless otherwise stated, and plates were incubated for 24 h at 37 °C before pellicle formation was measured as described above.

PIA assays. PIA assays were performed as described previously (Holland et al., 2008). Briefly, 5 ml of an exponential culture (OD600 2.0) was collected by centrifugation, resuspended in 250 μl 0.5 M EDTA and boiled for 5 min. The cell debris was again centrifuged, and the supernatant was treated with 200 μg proteinase K at 37 °C for 1 h. The proteinase K was inactivated by boiling for 5 min and the samples were diluted as appropriate. To measure PIA in the culture supernatant, 500 μl samples of supernatant were treated with proteinase K as described above. Samples were applied to a nitrocellulose membrane [pre-wetted in Tris-buffered saline (TBS)] using a vacuum blotter (Bio-Rad). The blots were dried, rewetted with TBS, and blocked for 1 h in 1 % (w/v) BSA. The primary antibody (1:5000 dilution of rabbit anti-PIA (a kind gift from Tomas Maira Litran and Gerald Pier, Brigham and Women’s Hospital, Boston, MA, USA) in TBS-Tween-0.1 % BSA) was applied to the membrane for 1 h. Horseradish peroxidase-linked anti-rabbit IgG secondary antibody (1:5000 dilution in TBS-Tween-0.1 % skimmed milk) was then incubated with the membrane for 1 h. A chemiluminescence kit (Amersham) was used to generate light via the horseradish peroxidase-catalysed oxidation of luminol and detected using a Bio-Rad Fluor-S MAX charge-coupled device (CCD) camera system.

RNA purification. RNA purification was performed as described previously (Conlon et al., 2002) and the concentration was determined using a Nano-Drop spectrophotometer. Real-time RT-PCR was performed on a LightCycler instrument using an RNA Amplification Kit SYBR Green I (Roche Biochemicals) following the manufacturer’s recommended protocol (Holland et al., 2008). Reverse transcription was performed at 61 °C for 20 min, followed by a denaturation step at 95 °C for 30 s and 35 amplification cycles of 95 °C for 2 s, 50 °C for 5 s and 72 °C for 8 s. Melting curve analysis was performed at 45–95 °C (temperature transition, 0.1 °C s−1) with stepwise fluorescence detection. For LightCycler RT-PCR, ReQuant software (Roche Biochemicals) was used to measure relative expression of target genes. The gyrB gene was used as an internal standard in real-time RT-PCR experiments. Each experiment was performed at least three times and mean and SDs are presented. The primers used for RT-PCR are listed in Table 2.

Statistical analysis. Two-tailed, two-sample equal variance Student’s t tests (Microsoft Excel 2007) were used to determine statistically significant differences in assays performed during this study. A significant difference was indicated as a P value <0.05.

RESULTS

Mutation of the S. epidermidis tagO gene has pleiotropic effects, including impaired biofilm production and increased autolytic activity

Allele replacement was used to construct a tagO deletion mutation in the biofilm-forming clinical isolate CSF41498. The TAGO1 mutant exhibited a range of phenotypic changes, including sensitivity to temperatures of greater than 40 °C, resistance to bacteriophage KC and a significantly lower turbidity in overnight liquid cultures (data not shown). TAGO1 liquid cultures were characterized by macroscopic aggregates that rapidly settled at the bottom of the tube, indicative of enhanced intercellular aggregation (Fig. 1a). The TAGO1 mutant was unable to grow on Congo red agar (Fig. 1b) and interestingly exhibited a two-log increase in plasmid electroporation efficiency (Fig. 1c). Triton X-100-induced autolysis was also significantly increased in the mutant (Fig. 1d).

We used static 96-well plate assays to measure primary attachment and biofilm formation of TAGO1 grown in BHI media. Primary attachment to hydrophilic, tissue culture-treated polystyrene by TAGO1 cells was significantly

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Role of tagO in S. epidermidis biofilm phenotype

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impaired (P<0.05) and could be complemented by the tagO gene (Fig. 2a). Biofilm production by TAGO1 on tissue culture-treated polystyrene after 24 h was significantly decreased (P<0.05) and was also successfully complemented by tagO (Fig. 2b). Because hydrophobic interactions are important for cell interactions and biofilm development we used a BATH assay to measure the hydrophobicity of TAGO1 cells. This experiment revealed a significant increase in the hydrophobicity of TAGO1 compared with CSF41498 (Fig. 2c). This finding was consistent with the recent finding that an S. aureus tagO mutant is also more hydrophobic than its wild-type parent strain (Kohler et al., 2009). The increased surface hydrophobicity of TAGO1 cells may contribute to the observed changes in intercellular aggregation, attachment to polystyrene and biofilm production.

Pellicle phenotype of the S. epidermidis tagO mutant

Given that enhanced intercellular aggregation by TAGO1 cells appears to limit interactions with surfaces, we compared macroscopic cell clustering in BHI media supplemented with NaCl or glucose, both of which can promote biofilm development. The TAGO1 mutant was unable to grow in BHI media supplemented with >1% NaCl (data not shown), indicating an important role for WTA in osmotic stress tolerance. Growth of TAGO1 in BHI glucose was associated with reduced levels of stationary phase lysis (Supplementary Fig. S1). Consistent with this, Triton X-100-induced autolytic activity was reduced in both the wild-type and TAGO1 strains grown in BHI glucose compared with BHI (Fig. 3a). Reduced autolytic activity in TAGO1 BHI glucose cultures correlated with the formation of a dense cell aggregate that settled to the bottom of the culture tube (Fig. 3b). Modified biofilm assays in which adherent cells were only gently washed to remove planktonic cells (see Methods for details) revealed that TAGO1 produced a pellicle in BHI glucose (Fig. 3b). This pellicle can be described as an aggregation of cells on the polystyrene surface that was easily detached during washing of the plate. We have chosen the term pellicle to be consistent with the nomenclature used by Vergara-Irigaray et al. (2008) in their description of an S.
aureus tagO mutant. In contrast, biofilms are tightly attached to the plate and can withstand vigorous washing. Growth in BHI glucose is associated with acidification of the culture medium (to ~pH 5.5) (O’Neill et al., 2008). BHI acidified with acetic acid to pH 5.8 also induced significant pellicle production by TAGO1 after 24 h ($P<0.05$) (Fig. 3d). Interestingly, time-course assays revealed the absence of pellicle production in the early stages of growth followed by significant pellicle formation after 12 and 24 h growth. This growth phase-dependent pellicle formation correlated with the acidification of the growth media over time (Fig. 3c). Taken together, these data indicated that pellicle formation by TAGO1 in BHI glucose media is induced by mild acid stress and is associated with reduced autolytic activity.

Role of protein adhesins and eDNA in acid-induced TAGO1 pellicles

TAGO1 pellicles were significantly dispersed by proteinase K ($P<0.05$) but unaffected by treatment with sodium metaperiodate (Fig. 4a), implicating protein adhesin(s) and not PIA in this phenotype. Glucose-induced TAGO1 pellicle formation was inhibited by the addition of DNase I to the growth medium at $t_0$ (Fig. 4b). In addition, pellicles formed by TAGO1 were also dispersed with DNase I added after 24 h growth (Fig. 4b). Heat-inactivated DNase I, used as a control, had no effect on pellicle formation or dispersal (data not shown). These data suggest that release of eDNA is required for formation of TAGO1 pellicles and that eDNA acts as a structural component of the TAGO1 pellicle matrix.

Ethanol precipitation was used to precipitate and visualize eDNA present in supernatants from exponential phase cultures of CSF41498, TAGO1 and the complemented mutant. Substantially more high-molecular-mass eDNA was recovered from TAGO1 culture supernatants compared with CSF41498 and the complemented mutant in both BHI and BHI glucose (Fig. 4c). This observation is consistent with eDNA playing an important role in TAGO1 pellicle formation, but suggests that it is not so important for wild-type biofilm formation.

For comparison, the role of PIA, protein adhesins and eDNA was also examined in the biofilms produced by the wild-type CSF41498. These experiments revealed that periodate significantly dispersed CSF41498 biofilms grown in BHI but not in BHI glucose (Fig. 4d), whereas proteinase K dispersed wild-type biofilms grown in BHI and BHI glucose (Fig. 4d). These data suggested that PIA may play a variable role in CSF41498 biofilm production and are supported by an earlier study which demonstrated that this strain is capable of producing PIA- and proteinaceous-type biofilms (Hennig et al., 2007). DNase I also dispersed CSF41498 biofilms grown in BHI but not in BHI glucose (Fig. 4d), indicating that eDNA can contribute to biofilm production in this strain under specific growth conditions.
Mutation of tagO reduces icaADBC expression and PIA production in S. epidermidis

The increased autolytic activity and impaired primary attachment/biofilm phenotypes of the tagO mutant prompted us to examine the transcriptional activity of the major autolysin gene atlE and the icaADBC operon. Real-time RT-PCR revealed that atlE expression was similar in CSF41498 and TAGO1 grown in BHI or BHI glucose (data not shown). However, icaA transcript levels in TAGO1 were reduced approximately six- and 16-fold in BHI and BHI glucose, respectively, compared with the wild-type (Fig. 5a). Decreased icaA expression in TAGO1, in turn, corresponded to an approximately 2.5- and 6.6-fold increase in icaR transcription in BHI and BHI glucose, respectively, compared with CSF41498 (Fig. 5b). Growth of CSF41498 and the complemented mutant in BHI glucose was associated with a 2.8-fold increase in icaA expression and a 1.5-fold decrease in icaR expression (Fig. 5a, b), suggesting that mild acid stress may activate ica operon expression via repression of the negative regulator icaR. Immuno-dot blots revealed that deletion of tagO led to a reduction in PIA levels in both whole-cell extracts and supernatants from cultures grown in BHI and BHI glucose media (Fig. 5c). To examine whether the reduced PIA production by TAGO1 was involved in increased cell surface hydrophobicity, we compared CSF41498 and its isogenic ica mutant CSF-2 using the BATH technique. This revealed that surface hydrophobicity was increased in CSF-2 (Fig. 2c), indicating that extracellular PIA also influences surface hydrophobicity in this strain. Taken together, these data reveal that mutation of tagO is associated with activation of icaR, decreased icaADBC expression and reduced PIA production. In addition, diminished PIA levels and the loss of WTA combine to increase surface hydrophobicity. Thus, these data indicate that the biofilm-negative phenotype of the tagO mutant can be attributed to impaired PIA production and increased surface hydrophobicity.

DISCUSSION

Deletion of tagO in S. epidermidis had pleiotropic effects which were consistent with the loss of WTA. These included increased sensitivity to osmotic stress and...
temperatures >40 °C, increased susceptibility to Congo red, resistance to bacteriophage infection, macroscopic clumping during growth in liquid culture media and increased autolytic activity. Increased sensitivity to Congo red, which is known to be toxic, may be due to increased cell envelope penetration. A recent study of *S. aureus* found that increased sensitivity to Congo red in an *msrR* mutant was associated with reduced levels of WTA (Hu¨bscher et al., 2009). To our knowledge, this study is the first report of increased plasmid electroporation efficiencies in a *tagO* mutant and perhaps suggests that changes in WTA composition influence genetic exchange and transfer of antibiotic and virulence genes among staphylococci. In the laboratory, *tagO* mutants may also have potential as a tool in genetic manipulation of *S. epidermidis* strains, which are notoriously difficult to transform.

Increased autolytic activity in TAGO1 was consistent with earlier studies of *S. aureus* *tagO* and *dltA* mutants (Gross et al., 2001; Koprivnjak et al., 2008; Maki et al., 1994; Vergara-Irigaray et al., 2008). The mechanistic basis for increased autolytic activity in WTA mutants has recently been described. Schlag et al. (2010) have revealed that WTA prevents binding of the major autolysin Atl to the cell wall and that binding of the Atl-derived amidase enzyme is increased at the cross wall region, presumably due to a lower WTA concentration. Thus, in the absence of WTA, increased binding and activity of Atl-derived peptidoglycan hydrolases leads to increased rates of autolysis.

The *tagO* mutant was biofilm-negative, a phenotype which correlated with increased cell surface hydrophobicity, impaired primary attachment, increased autolytic activity and defective PIA production. Deletion of *tagO* in *S. aureus* has also been shown to impair biofilm formation, although it does not affect PIA expression or anchoring to the cell wall (Gross et al., 2001; Vergara-Irigaray et al., 2008). Decreased PIA production was due, at least in part, to activation of the transcription of icaR, which encodes a negative regulator of the *ica* operon (Conlon et al., 2002), with the concomitant repression of the *icaADBC* operon. Interestingly, Knobloch et al. (2004) have reported that activation of the alternative sigma factor $\sigma^B$ results in repression of *icaR* and increased *icaADBC* transcription. Thus, although it remains to be determined precisely how the transcriptional activity of the *ica* locus is altered in the *tagO* mutant, one possible mechanism is that cell surface changes associated with loss of WTA influence $\sigma^B$-dependent *icaADBC* regulation.

Under mild acidic growth conditions in BHI glucose media, the *S. epidermidis* TAGO1 mutant produced a pellicle, which we define here as an aggregation of cells easily detached from a surface. Pellicle formation by TAGO1 in BHI glucose correlated with reduced levels of TAGO1 stationary phase lysis as a result of decreased autolytic activity. Thus, higher cell densities in BHI glucose cultures correlate with pellicle formation. An increased tendency for cell aggregation and pellicle formation has

![Graph and images](http://mic.sgmjournals.org)
also been reported in an *S. aureus* ΔtagO mutant grown in tryptone soya media supplemented with glucose (Vergara-Irigaray et al., 2008). In contrast to TAGO1 pellicles, CSF41498 biofilms are tightly attached to the surface and can withstand vigorous washing. Acid-induced TAGO1 pellicles were dispersed with DNase I and proteinase K but not sodium metaperiodate, implicating both protein and eDNA adhesins and not PIA in this phenotype. Autolytic activity can also influence cell aggregation by controlling the release of eDNA, which has recently been shown to play a role in early *S. epidermidis* biofilm formation (Qin et al., 2007). Our analysis revealed that increased autolytic activity in TAGO1 during the exponential phase of growth correlated with increased release of eDNA, and that eDNA was important for pellicle formation in BHI glucose. However, eDNA levels in exponential phase culture supernatants of TAGO1 grown in BHI and BHI glucose were similar, suggesting that eDNA alone cannot explain enhanced pellicle production by TAGO1 in BHI glucose. Consistent with this, acid-induced TAGO1 pellicles were also significantly dispersed with proteinase K, implicating a protein adhesin(s) in this phenotype. One candidate for this adhesin is the accumulation-associated protein (Aap), which has previously been implicated in both *S. epidermidis* (Rohde et al., 2005b) and *S. aureus* (Corrigan et al., 2007) biofilm production. Significantly, a CSF41498 *icaC::IS256* mutant which spontaneously switches to PIA-independent biofilm production is associated with upregulation of *aap* transcription (Hennig et al., 2007). However, our data revealed that *aap* expression was unaffected in the TAGO1 mutant grown in BHI or BHI glucose. Staphylococcal surface proteins may also be anchored to the cell wall via interactions with teichoic acids (Navarre & Schneewind, 1999). Although earlier analysis of WTA-deficient mutants has revealed only minor effects on cell surface proteins (Gross et al., 2001; Weidenmaier et al., 2004), electron

\[\text{Fig. 5. Comparative measurements of icaA (a) and icaR (b) transcription by real-time RT-PCR in CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6. Total RNA was extracted from cultures grown at 37 °C to OD}_{600} \text{ 2.0 in BHI and BHI glucose. RelQuant software (Roche) was used to compare the relative expression of icaA and icaR transcript levels with respect to the constitutively expressed gyrB gene. Transcript levels of icaA and icaR in strains grown in BHI and BHI glucose were compared with transcript levels in CSF41498 pLI50 grown in BHI, which was assigned a value of 1. Data represent the mean of three independent experiments and SDs are indicated (error bars). Asterisks indicate significant differences (P<0.05). (c) Immunoblots of PIA production in whole-cell lysates and supernatants of CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 cultures grown to OD}_{600} \text{ 2.0 in BHI and BHI glucose. PIA immunoblots of whole-cell extracts of wild-type CSF41498 and its isogenic PIA-negative derivative CSF-2 are also shown.}\]
microscopy of S. aureus tagO mutants reveals a rough outer surface compared with the wild-type (Koprivnjak et al., 2008; Schlag et al., 2010), possibly as a result of cell wall disintegration (Schlag et al., 2010). Therefore, it is also possible that cell surface physical changes in strains lacking WTA may be accompanied by the unmasking of an acid-activated cell wall protein(s) which has not been previously implicated in cell aggregation.

Overall, our data reveal that WTA are required for S. epidermidis biofilm formation and that mutation of tagO is associated with increased cell surface hydrophobicity and repression of icaADBC and PIA expression. Interestingly, enhanced autolytic activity in the TAGO1 mutant is associated with increased eDNA release, which, together with protein adhesins, promotes the formation of a pellicle. Unlike a biofilm, this pellicle is only loosely attached to surfaces, indicating that enhanced intracellular aggregation/pellicle formation is accompanied by a reduced capacity to attach to surfaces and form biofilms.

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