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

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The *icaADBC*-encoded polysaccharide intercellular adhesin (PIA) and wall teichoic acids (WTA) are structural components of *Staphylococcus epidermidis* biofilms. Deletion of *tagO*, which encodes the first enzymic step in WTA biosynthesis, had pleiotropic effects, including enhanced intercellular aggregation and autolytic activity, and impaired biofilm production. The biofilm-negative phenotype of the *tagO* mutant, named TAGO1, was associated with increased cell surface hydrophobicity, lower rates of primary attachment to polystyrene, and reduced *icaADBC* operon and PIA expression. Mild acid stress induced by growth in BHI glucose media reduced rates of stationary phase autolysis and enhanced aggregation by TAGO1, leading to formation of a pellicle, which unlike a biofilm was only loosely attached to the polystyrene surface. TAGO1 pellicles were dispersed by proteinase K and DNase I but not sodium metaperiodate, implicating protein and extracellular DNA (eDNA) and not PIA in this phenotype. Substantially increased levels of eDNA were recovered from TAGO1 culture supernatants compared with the wild-type. These data indicate that WTA are essential for the primary attachment and accumulation phases of the *S. epidermidis* biofilm phenotype. Furthermore, in the absence of WTA, proteins and eDNA can promote cell aggregation and pellicle formation, which also appear to limit interactions with artificial surfaces.

Received 4 June 2010

Revised 24 September 2010

Accepted 2 November 2010

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 (Hyyrylainen *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

Abbreviations: BATH assay, bacterial adherence to hydrocarbon assay; eDNA, extracellular DNA; PIA, polysaccharide intercellular adhesin; WTA, wall teichoic acids.

A supplementary figure, showing growth curves of CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 cultures, is available with the online version of this paper.

& Peschel, 2008). The *tag* (teichoic acid glycerol) genes were first identified and characterized in *B. subtilis* 168, which contains WTA composed of Gro-P repeating units (Mauël *et al.*, 1991, 1995). The *tagO* gene encodes an enzyme responsible for the first step of WTA biosynthesis (Soldo *et al.*, 2002). Studies of *B. subtilis* W23 and *S. aureus* which contain WTA composed of Rbo-P polymers have identified the *tar* (teichoic acid ribitol) genes, and functions have been identified for these genes based on sequence homologies to the *tag* genes (Brown *et al.*, 2008; Lazarevic *et al.*, 2002; Qian *et al.*, 2006). Similar to *B. subtilis* 168 and W23, WTA biosynthesis in *S. aureus* begins with the *tagO* gene product, and deletion of *tagO* in *S. aureus* impairs WTA biosynthesis (Weidenmaier *et al.*, 2004).

Although the *icaADBC*-encoded exopolysaccharide PIA or polymeric *N*-acetylglucosamine (PNAG) is the best-characterized component of the *S. epidermidis* biofilm matrix (Heilmann *et al.*, 1997; Mack, 1999), WTA also play an important structural role (Sadovskaya *et al.*, 2004, 2005). Recent studies have also revealed the importance of extracellular DNA (eDNA) in the *S. aureus* and *S. epidermidis* biofilm matrix (Eckhart *et al.*, 2007; Izano *et al.*, 2008; Mann *et al.*, 2009; Qin *et al.*, 2007; Rice *et al.*, 2007).

In this study the impact of an *S. epidermidis tagO* deletion mutation on biofilm development and biofilm-related phenotypes was investigated. Autolytic activity, eDNA release and PIA production were examined. Our findings reveal that *tagO* is required for *S. epidermidis* biofilm production. Furthermore, increased cellular aggregation by the *tagO* mutant involving eDNA and protein adhesins can mediate the formation of a pellicle which limits surface interactions.

METHODS

Bacterial strains, phage, plasmids, media and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37 °C on Luria–Bertani (LB) medium (Sigma) supplemented when required with kanamycin, 50 µg ml⁻¹; ampicillin, 50 µg ml⁻¹; Bluogal, 200 µg ml⁻¹. Staphylococcal strains were grown at 37 or 30 °C on brain heart infusion (BHI) media (Oxoid). When required, BHI was supplemented with chloramphenicol (Cm), 10 µg ml⁻¹; tetracycline (Tet), 5 µg ml⁻¹; Bluogal 200 µg ml⁻¹. BHI was supplemented where indicated with 1 % (w/v) glucose. *S. epidermidis* phage KC, a kind gift from Dr Tyrone Pitt, Laboratory of Healthcare Associated Infection, London, UK, was used in phage susceptibility assays, essentially as described elsewhere (Novick, 1991). Briefly, serial dilutions of tryptic soya broth (TSB) containing CSF41498 and TAGO1 cells harvested from BHI agar slants were mixed with phage KC and added to molten

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
CSF41498	Biofilm-positive, cerebrospinal fluid isolate	Conlon <i>et al.</i> (2002)
CSF-2	CSF41498 <i>icaC</i> ::IS256, PIA-negative	Hennig <i>et al.</i> (2007)
TAGO1	CSF41498 derivative, $\Delta tagO$::Tc ^r	This study
RN4220	Restriction-deficient derivative of <i>S. aureus</i> 8325	Kreiswirth <i>et al.</i> (1983)
<i>E. coli</i> TOPO	<i>recA1 endA1 lac[F' proAB lac1q Tn10 (Tc^r)]</i>	Invitrogen
Plasmids		
pCR-Blunt II-TOPO	PCR cloning vector; Km ^r	Invitrogen
pBT2	Temperature-sensitive <i>E. coli</i> – <i>Staphylococcus</i> shuttle vector. Ap ^r (<i>E. coli</i>) Cm ^r (<i>Staphylococcus</i>)	Brückner (1997)
pMAD	Shuttle vector for allele replacement. Ap ^r (<i>E. coli</i>) Em ^r (<i>Staphylococcus</i>). Contains <i>bgaB</i> gene encoding a thermostable β -galactosidase	Arnaud <i>et al.</i> (2004)
pT181	4.45 kb <i>S. aureus</i> plasmid containing <i>tetA</i> gene	Khan & Novick (1983)
pBT2::bga	2803 bp <i>SmaI</i> – <i>SwaI</i> fragment containing the <i>bgaB</i> gene from pMAD cloned into the <i>SmaI</i> site of pBT2	This study
pBlue::tet	pBluescript containing the <i>tetA</i> gene from pT181 on a 2236 bp <i>HindIII</i> fragment	This study
pLI50	<i>E. coli</i> – <i>Staphylococcus</i> cloning vector; Ap ^r (<i>E. coli</i>) Cm ^r (<i>Staphylococcus</i>)	Lee <i>et al.</i> (1991)
pSETAG1	2642 bp PCR product containing the <i>S. epidermidis tagO</i> gene amplified from CSF41498 using primers tagO1 and tagO2 cloned into pCR-Blunt II-TOPO	This study
pSETAG2	Plasmid containing the deleted <i>tagO</i> (1021 bp) gene using the primers SETagOdel1 and SETagOdel2 with incorporated <i>StuI</i> enzyme sites	This study
pSETAG3	2236 bp <i>SwaI</i> – <i>SmaI</i> fragment containing the <i>tetA</i> gene from pBlue::tet cloned into the <i>StuI</i> site of pSETAG2	This study
pSETAG4	3921 bp <i>BamHI</i> – <i>XbaI</i> fragment containing the $\Delta tagO$::Tc ^r allele from pSETAG3 cloned into the <i>BamHI</i> – <i>XbaI</i> sites of pBT2::bga	This study
pSETAG5	1978 bp PCR product containing <i>tagO</i> amplified from CSF41498 using primers SELLm1 and SELLm2 and cloned into pCR-Blunt II-TOPO	This study
pSETAG6	<i>EcoRI</i> fragment from pSETAG5 containing <i>tagO</i> cloned into pLI50	This study

(48 °C) soft agar before being poured onto TSB agar containing 5 mM CaCl₂. The plates were incubated overnight at 37 °C and p.f.u. ml⁻¹ was determined.

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, red, 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 containing *StuI* enzyme sites at the 5' end (Table 2). The resulting PCR fragment was purified and digested with *StuI*, followed by religation to create pSETAG2. The 2236 bp Tc^r gene was cloned from pBlue::tet on a *SmaI*-*SmaI* fragment and ligated into the *StuI* site to create pSETAG3. A 3921 bp *Bam*HI-*Xba*I fragment containing the mutant allele from pSETAG3 was ligated into pBT2::bga digested with *Bam*HI/*Nhe*I 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 Tc^r colonies on BHI agar plates. Blueo-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 Tc^r colonies on BHI agar plates containing 200 µg Blueo-gal ml⁻¹. White, Tc^r 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 *Eco*RI fragment containing the tagO gene was cloned into pLI50 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 A₄₉₂ ≥ 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.

Table 2. Oligonucleotide primers used in this study

Target gene	Primer	Sequence (5'–3')
tagO (for deletion of gene)	tagO1	CCCACTGCACTGACAATACC
	tagO2	CGGTCATAACTGCAATCTTCA
tagO (for inverse PCR)	SEtagOdel1	GAAGGCCTCAGTTCACCTTCATTGAA
	SEtagOdel2	GAAGGCCTTTTACTGGATTAATAGAT
tagO (full-length gene)	SELLm1	CGTTTTGAAATGGGATAGGG
	SELLm2	TAGGTGCAGGCGGTCTTATT
gyrB	GYRB1	TTATGGTGCTGGACAGATACA
	GYRB2	CACCGTGAAGACCGCCAGATA
icaA	KCA1	AACAAGTTGAAGGCATCTCC
	KCA2	GATGCTTGTTTGATTCCCT
icaR	icaRFor2	ATCCAAAGCGATGTGCGTAG
	icaRRev2	TCCATTGACGGACTTTACCAG
atlE	SEatlE_for	TTTGCAATTGGTGCAATTGA
	SEatlE_rev	TACCAGGGTTTGCAGGATTC
aap	AAP1	AAAATTCACGTGTTGGTACGG
	AAP2	AGAAGACATCTGTGGTGCTT

Primary attachment assays. Attachment assays using Nunclon tissue culture-treated (Δ Surface) 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 μ l 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 μ l 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 twice 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 (OD_{t0}), i.e. percentage lysis = $[(OD_{600t0} - OD_{600} \text{ at time } t) / OD_{600t0}] \times 100$. 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 $\times$ PBS and resuspended in 4.5 ml 1 $\times$ 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 $\times$ Dulbecco's PBS (Oxoid) in these experiments. A 500 μ l 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 μ l distilled H₂O. Pellicles were treated with 50 μ l 10 mM sodium metaperiodate (Fluka) in distilled H₂O or 50 μ l 100 μ g ml⁻¹ proteinase K (Sigma) in distilled H₂O for 2 h at 37 °C. The control wells contained 50 μ l distilled H₂O. Following incubation, each well was carefully washed with 50 μ l distilled H₂O and dried for 1 h at 65 °C prior to staining with 0.4 % (w/v) crystal violet. The A₄₉₂ 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 *t*₀ 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 μ l 0.5 M EDTA and boiled for 5 min. The cell debris was again centrifuged, and the supernatant was treated with 200 μ g 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 μ l 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, RelQuant 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 data 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

Fig. 1. (a) Multicellular behaviour of CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 grown in BHI broth at 37 °C for 12 h. TAGO1 pLI50 cultures form macroscopic aggregates which rapidly settle at the bottom of the tube. This phenotype can be fully complemented by the presence of pSETAG6. (b) Colony morphologies of CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 on Congo red agar (0.8 mg ml⁻¹). (c) Electroporation efficiency of pLI50 into CSF41498 and TAGO1 expressed as the number of transformants per microgram of plasmid DNA. The asterisk indicates a significant difference ($P < 0.05$). (d) Triton X-100-induced autolytic activity in exponential phase cultures of CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 grown in BHI. Cells were resuspended in 0.01 % Triton X-100 and incubated at 37 °C. The percentage lysis was determined by measuring the decrease in cell density (OD₆₀₀). Data presented represent the mean of at least three independent experiments.

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.*

Fig. 2. Impact of *tagO* mutation on *S. epidermidis* biofilm formation. (a) Primary attachment phenotypes on tissue culture-treated polystyrene for CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 grown to exponential phase in BHI. Attached cells remaining on the plate after washing were stained with crystal violet and A_{492} was measured. Experiments were repeated three times and SDs are indicated (error bars). (b) Biofilm phenotypes of CSF41498 and TAGO1 strains complemented with pLI50 (control) and pSETAG6 (*tagO*). All strains were grown overnight at 37 °C in BHI. Data are the mean of at least three independent experiments and SDs are indicated (error bars). (a, b) Asterisk indicates a significant difference ($P < 0.05$). (c) Relative hydrophobicity of CSF41498 pLI50, TAGO1 pLI50, TAGO1 pSETAG6 and CSF-2 pLI50 cells. BATH assays were performed on cultures grown to OD₆₀₀ 1.0 in BHI. Data are expressed as percentage relative hydrophobicity. Asterisks indicate significant differences ($P < 0.05$).

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

Fig. 3. (a) Triton X-100-induced autolytic activity in overnight cultures of CSF41498 and TAGO1 grown in BHI and BHI glucose. Cells were resuspended in 0.01% Triton X-100 and incubated at 37 °C. The percentage lysis was determined by measuring the decrease in cell density (OD_{600}). Data presented represent the mean of at least three independent experiments. (b) Comparison of cell aggregation in liquid culture and pellicle formation by CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 grown in BHI and BHI glucose for 24 h at 37 °C. TAGO1 pLI50 cultures undergo less stationary phase lysis in BHI glucose than in BHI, leading to the formation of a pellicle at the base of the well. (c) Pellicle phenotype of TAGO1 grown in tissue culture-treated 96-well plates for 4, 6, 12 and 24 h in BHI glucose at 37 °C. Pellicles, which were very loosely attached to the polystyrene surface, were gently washed to remove planktonic cells before being dried and stained with crystal violet. Pellicle formation was measured at least three times and SDs are indicated (error bars). (d) Pellicle phenotype of TAGO1 grown in tissue culture-treated 96-well plates for 24 h in BHI and BHI adjusted to pH 5.8 at 37 °C. Pellicle formation was measured at least three times and SDs are indicated (error bars). An asterisk indicates a significant difference ($P < 0.05$).

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

Fig. 4. (a) Dispersal of TAGO1 pellicles with sodium metaperiodate and proteinase K added to BHI glucose cultures after 24 h growth compared with an untreated control. The asterisk indicates a significant difference ($P < 0.05$); NS, not significant. (b) Dispersal of TAGO1 pellicles with DNase I (0.5 mg ml^{-1}) added to BHI glucose cultures at t_0 (0 h) or after 24 h growth compared with untreated controls. (c) Comparative analysis of eDNA recovered from culture supernatants of CSF41498 pLI50, TAGO1 pLI50 and TAGO1 pSETAG6 cultures grown to OD_{600} 2.0 in BHI and BHI glucose. eDNA was visualized after electrophoresis on a 1% agarose gel. (d) Dispersal of CSF41498 biofilms with sodium metaperiodate, proteinase K and DNase I (0.5 mg ml^{-1}) added to BHI and BHI glucose cultures after 24 h growth compared with biofilm production in an untreated control. Asterisks indicate significant differences ($P < 0.05$); NS, not significant.

temperatures $>40^\circ\text{C}$, 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 (Hübscher *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 σ^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 σ^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

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 OD₆₀₀ 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 OD₆₀₀ 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.

also been reported in an *S. aureus* $\Delta tagO$ 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

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 intercellular aggregation/pellicle formation is accompanied by a reduced capacity to attach to surfaces and form biofilms.

ACKNOWLEDGEMENTS

This study was funded by the Irish Health Research Board (J. P. O'G.) and the Irish Research Council for Science Engineering and Technology (L. M. H. and B. C.). We are indebted to M. Gomelsky for ongoing support and critical comments on this manuscript. We also thank P. Fey for useful discussion on the findings of this study, T. Pitt for phage KC and W. Ziebuhr for strain CSF-2; rabbit anti-PIA serum was a gift from T. Maira Litran and G. B. Pier (see main text). We are grateful to C. Pozzi, E. O'Neill, P. Houston, S. Rowe, E. dePoire and E. Waters for their support and advice over the course of this study.

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Edited by: J. Lindsay