Wall teichoic acids are dispensable for anchoring the PNAG exopolysaccharide to the Staphylococcus aureus cell surface

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INTRODUCTION

The Gram-positive bacterium Staphylococcus aureus is a major worldwide cause of community-acquired and nosocomial infections. S. aureus can cause a wide spectrum of diseases, ranging from superficial wound infections to life-threatening infections such as septicemia, endocarditis, osteomyelitis and toxic shock syndrome. In addition to acute diseases, S. aureus can also cause chronic infections, many of which are associated with the use of medical devices (Mack et al., 2004). This property is related to the capacity of S. aureus to adhere and form multilayered communities embedded in a self-produced matrix, termed a biofilm, that survives on the surfaces of implanted devices (Gotz, 2002).

S. aureus biofilm formation is generally mediated by the production of the extracellular polysaccharide adhesin termed both polysaccharide intercellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG), whose synthesis depends on the expression of the biosynthetic enzymes encoded by the icaADBC operon (Cramton et al., 1999; Heilmann et al., 1996b; Mckenney et al., 1999). The exopolysaccharide PNAG is a linear β-(1-6)-linked N-acetylglucosaminoglycan with a high proportion of amino groups substituted with N-linked acetate, and a small amount of the hydroxyl groups esterified with acetate and succinate (Joyce et al., 2003; Mack et al., 1996; Maira-Litrán et al., 2002). It has been shown recently that the IcaB protein is involved in the production of positive charge in the PNAG polymer by deacetylation of a proportion of the

Abbreviations: LTA, lipoteichoic acid; PIA, polysaccharide intercellular adhesin; PNAG, poly-N-acetylglucosamine; TA, teichoic acid; WTA, wall teichoic acid.
N-acetylglicosammines (GlcNAcs). In the absence of IcaB, N-acetylated PNAG cannot be retained on the bacterial cell surface and is released into the liquid media, probably due to the loss of its cationic character (Cerca et al., 2002, 2004). Accordingly, in an animal model of implant infection, a Staphylococcus epidermidis icaB mutant exhibits deficiencies in biofilm formation, immune evasion, adhesion to epithelial cells and virulence similar to those of the PNAG-deficient mutants (Vuong et al., 2004). Similar phenotypes in terms of biofilm formation and immune evasion, as well as a decreased ability to survive in the blood of infected mice, have been found for an icaB-deletion mutant of S. aureus (Cerca et al., 2007).

Among the cell wall-associated components that could mediate ionic interactions with PNAG are the teichoic acids (TAs), which represent the most abundant polyanions associated with the bacterial cell envelope. TAs include wall teichoic acids (WTAs), which are covalently linked to the peptidoglycan, and lipoteichoic acids (LTAs), which are membrane-anchored (for a review see Neuhaus & Baddiley, 2003). Both polymers are assembled via different pathways and have different chemical structures (Neuhaus & Baddiley, 2003; Ward, 1981). With the exception of some unusual strains, it is considered that WTAs in S. aureus are composed of ribitol phosphate repeating units, while LTAs are polyglycerolphosphate chains (Endl et al., 1983; Vinogradov et al., 2006). Both polymers have a highly variable content of $\alpha$-alanine ester substitutions that reduce the net negative charge of TAs by introduction of basic amino groups (Baddiley et al., 1961; Fischer, 1994; Neuhaus & Baddiley, 2003). The absence of $\alpha$-alanine esters in dltA mutants of S. aureus causes a deficiency in their capacity to form biofilms on polystyrene or glass surfaces (Gross et al., 2001). This is likely to be due to the increased net negative charge on the bacterial surface causing electrostatic repulsions between the cell and the surface that consequently inhibit the initial steps of the biofilm formation process. TAs have been shown to affect adherence of S. aureus to host tissues. For example, Aly et al. (1980) showed that treating human nasal epithelial cells with TAs extracted from S. aureus significantly decreased the binding of the bacteria to the epithelial cells. More recently, the importance of WTAs for the bacterial interaction with human nasal epithelial cells as well as with endothelial cells was demonstrated using a rat nasal colonization and a rabbit endocarditis model, respectively (Weidenmaier et al., 2004, 2005). However, the precise function of TAs within the wall matrix remains speculative.

As the net charge of both WTAs and PNAG polymers seems to be important for their functionality, we hypothesized that attachment of PNAG to the bacterial cell surface could be mediated by ionic interactions between the positively charged free amino groups on partially deacetylated PNAG and the negatively charged WTAs. In favour of this hypothesis, it has been shown that TAs can complex with different polysaccharides in a relatively non-specific way (Doyle et al., 1975). In addition, TAs are usually found as contaminants in PNAG purification processes, although no covalent linkage between them has been found (Joyce et al., 2003; Mairal-Litrán et al., 2002; McKenney et al., 1998).

In this study, we generated mutants deficient in WTA synthesis in two genetically independent S. aureus strains and studied the influence of the mutations on PNAG production, localization and biofilm formation. Our mutants showed an increased cell-to-cell aggregation and a defect in biofilm formation, though the levels, location and strength of PNAG attachment were similar to those of the wild-type strains. These findings indicate that WTAs are dispensable for the association of PNAG with the bacterial cell surface.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* XL1-Blue cells were grown in Luria–Bertani (LB) broth or on LB agar (Pronadisa). Staphylococcal strains were cultured on trypticase soy agar (TSA) or in trypticase soy broth (TSB) supplemented with 0.25% glucose (TSB-gluc). Media were supplemented with appropriate antibiotics at the following concentrations: erythromycin (1.5 μg ml$^{-1}$); ampicillin (100 μg ml$^{-1}$); chloramphenicol (20 μg ml$^{-1}$). In order to compare growth kinetics, overnight cultures of the test strains were diluted (1:200) in TSB-gluc and incubated with shaking at 37°C while OD$_{650}$ was regularly monitored.

**DNA manipulations.** DNA plasmids were isolated from E. coli using the Bio-Rad plasmid miniprep kit (Bio-Rad Laboratories) according to the manufacturer’s protocol. Plasmids were transformed into staphylococci by electroporation, using a previously described protocol (Cucarella et al., 2001). Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer’s instructions. DNA polymerase was purchased from Biotools. Oligonucleotides were obtained from Thermo Electron Corporation (Table 2).

**Allelic exchange of chromosomal genes.** To generate the deletion mutants ΔtagO and ΔicaB we amplified by PCR two fragments of 500 bp that flanked the left (primers A and B) and right sequences (primers C and D) of the two genes targeted for deletion (Table 2). The PCR products were purified and cloned separately in the pGEM-T Easy vector (Promega). Fragments were then fused by ligation into the shuttle plasmid pMAD and the resulting plasmids were transformed into S. aureus by electroporation. pMAD contains a temperature-sensitive origin of replication and an erythromycin-resistance gene (Arnaud et al., 2004). Homologous recombination experiments were performed as previously described (Valle et al., 2003), but in the case of the ΔtagO mutation, the final temperature of incubation of double crossover mutants on agar plates was reduced from 44 to 37°C. This change was introduced to avoid negative selection of mutants due to their susceptibility to high temperatures. Erythromycin-sensitive white colonies, which no longer contained the pMAD plasmid, were tested by PCR using primers E and F (Table 2). The Δica deletion mutant was constructed as described previously (Toledo-Arana et al., 2005).

**Complementation experiments.** The tagO gene under the control of its own promoter was amplified by PCR from S. aureus 15981 and S. aureus 10833 with primers tagO-1 and tagO-2 (Table 2). The PCR
products were cloned into pCU1 (Augustin et al., 1992), and the resulting plasmid, pCU1tagO, was transformed by electroporation into ΔtagO mutants.

**Biofilm formation and primary attachment assays.** The biofilm formation assays in microtitre wells were performed as described previously (Heilmann et al., 1996a). Briefly, 5 μl of a culture of *S. aureus* grown overnight in TSB-gluc at 37°C was inoculated into the wells of microtitre plates containing 195 μl TSB-gluc medium. Sterile 96-well polystyrene microtitre plates from the same manufacturer (Iwaki) were used throughout the study. After 24 h incubation at 37°C, the plates were washed twice with 200 μl H2O and stained with 200 μl crystal violet for 5 min at room temperature. Then, the microtitre plates were rinsed twice with H2O, dried in an inverted position and stained with crystal violet. The wells were rinsed again, and the crystal violet was solubilized in 200 μl ethanol/acetone (80:20, v/v). OD595 was determined using a microplate reader.

For adherence assays in glass tubes, a single colony was transferred to 5 ml TSB-gluc and incubated at 37°C in an orbital shaker (250 r.p.m.) for 12 h. To analyse biofilm formation under flow conditions we used 60 ml microfermenters (Pasteur Institute, Laboratory of Fermentation) with a continuous flow of 40 ml TSB-gluc h^{-1} and constant aeration with sterile compressed air (0.3 bar). Submerged Pyrex slides served as the growth substratum. Approximately 10^8 bacteria from an overnight culture of each strain grown in TSB-gluc were used to inoculate 96-well polystyrene microtitre plates (Iwaki). After 1 h at 37°C, the cultures were resuspended in 20 ml TSB-gluc. OD650 of the suspensions was then measured.

Biofilm inhibition and detachment assays with DNase I, Dispersin B and wall teichoic acids in *S. aureus* biofilm development was recorded with a Nikon Coolpix 950 digital camera. To quantify the biofilm formed, bacteria adherent to the Pyrex slides were air-dried before staining.

Table 1. Strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tr>
<td><strong>S. aureus strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>15981</td>
<td>Clinical strain; biofilm-positive</td>
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<tr>
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<td>15981 with deletion of the tagO gene</td>
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<tr>
<td>RN4220</td>
<td>A mutant of 8325-4 S. aureus strain that accepts foreign DNA</td>
<td>Novick (1990)</td>
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<td>E. coli–S. aureus shuttle vector with a thermosensitive origin of replication for Gram-positive bacteria</td>
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<tr>
<td>pCU1</td>
<td>Vector for complementation experiments</td>
<td>Augustin et al. (1992)</td>
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Table 2. Oligonucleotides used in this study

Restrictions sites are underlined.

<table>
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<td>icaB-B</td>
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<td>icaB-C</td>
<td>AAGCTTGATCAACTCGAACACTCC</td>
</tr>
<tr>
<td>icaB-D</td>
<td>ACTGTGATAGTTATAG</td>
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<td>icaB-E</td>
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<td>icaB-F</td>
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<td>tagO-A</td>
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<td>tagO-B</td>
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<td>tagO-D</td>
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<tr>
<td>tagO-E</td>
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<td>tagO-F</td>
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<td>tagO-1</td>
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</tr>
<tr>
<td>tagO-2</td>
<td>GGATCCCATATTCTCTCTTATGAGATGA</td>
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</table>
of cells for each strain, grown to mid-exponential phase, were
M Tris-HCl (pH 6.4), 4% SDS, 20% glycerol, 10% removed and placed on top of a 10
the grids were placed on a 10
guinea pig serum in PBS (blocking buffer) for 30 min. After blocking,
described previously (Ingavale
hydrolases, SDS-PAGE zymographic analysis was performed as
PNAG detection.
Surface-located PNAG was detected as described
Zymographic analysis.
Autolysis assays. Bacteria were grown in TSB-gluc to OD650 ~0.6.
Cells were collected by centrifugation (10 000
10
1/100 at 37
culture were resuspended in 50
1000 at 37
Microscopy
Scanning microscopy. Scanning electron microscopy was performed on biofilms grown in microfermenters on Thermoxan slides (Nalgene Nunc International) fixed on the internal removable glass slides, as described by Prigent-Combaret et al. (2000) at the Laboratoire de Biologie Cellulaire et Microscope Electronique, UFR Médecine (Tours, France).
Immunoelectron microscopy. The bacterial strains were grown overnight in TSB. A 1 ml volume of the cultures was centrifuged (15 000 g for 5 min) and washed by mild vortexing in sterile PBS. Electronic microscope grids (200-mesh Formvar-carbon-coated copper grids, Electron Microscopy Sciences) were placed on top of a 10 μl drop of the bacterial suspension for 10 min. The grids were removed and placed on top of a 10 μl drop of 1% BSA and 10% guinea pig serum in PBS (blocking buffer) for 30 min. After blocking, the grids were placed on a 10 μl drop of immune rabbit antiserum raised against deacetylated PNAG conjugated to diphtheria toxoid (diluted 1:25 in 1% BSA in PBS) for 45 min (Maira-Litran et al., 2005). Then, the grids were washed three times for 2 min with PBS. The secondary antibody was then applied for 30 min. For all the strains, the secondary antibody used was a 10 μl drop of 12 nm colloidal gold-labelled donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) at a 1:60 dilution in 1% BSA in PBS. Each grid was then washed three times for 2 min in PBS and then twice in deionized water. The grids were examined with a transmission electron microscope (Phillips Tecnai 12) and photographs were taken at ×11 000 magnification.

Autolysis assays. Bacteria were grown in TSB-gluc to OD650 ~0.6. Cells were collected by centrifugation (10 000 g, 4 °C, 10 min), washed once with cold water and resuspended in the same volume of PBS (pH 7.5) containing 0.05 % (v/v) Triton X-100 (USB). Autolysis was measured during incubation with shaking at 37 °C as a decrease in the OD650. The experiment was repeated at least three times.

Zymographic analysis. For the detection of cell-associated murein hydrolases, SDS-PAGE zymographic analysis was performed as described previously (Ingavale et al., 2003). In brief, equal numbers of cells for each strain, grown to mid-exponential phase, were centrifuged, washed and resuspended in SDS/gel loading buffer [0.125 M Tris-HCl (pH 6.4), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and bromophenol blue], heated for 3 min at 100 °C and centrifuged to obtain supernatants. Supernatants were separated on a 12% SDS-polyacrylamide gel containing Micrococcus luteus or S. aureus RN4220 cells (one milligram wet weight of heat-killed cells per millilitre of gel). After electrophoresis, gels were washed with water and incubated for 12–16 h in 25 mM Tris·HCl (pH 8.0) containing 1% Triton X-100 at 37 °C. Gels were stained with 1% methylene blue, and clear zones of hydrolysis were observed against a dark background.

PNAG detection. Surface-located PNAG was detected as described elsewhere (Cramton et al., 1999). Cells were grown overnight in TSB-gluc, the OD650 was determined and the same number of cells of each culture were resuspended in 50 μl 0.5 M EDTA (pH 8.0), H2O, NaCl (0.8 M), NaCl (1 M) or urea (4 M) when indicated. Then, cells were incubated for 5 min at 100 °C unless indicated and centrifuged to pellet them. A 40 μl volume of each supernatant was incubated with 10 μl proteinase K (20 mg ml−1; Sigma) for 30 min at 37 °C. After addition of 10 μl Tris-buffered saline [20 mM Tris/HCl, 150 mM NaCl (pH 7.4)] containing 0.01% bromophenol blue, 5 μl of 1/50, 1/75 and 1/100 dilutions was spotted onto a nitrocellulose filter using a Bio-Dot microfiltration apparatus (Bio-Rad), blocked overnight with 5% skimmed milk in PBS with 0.1% Tween 20, and incubated for 2 h with rabbit antibodies raised against S. aureus deacetylated PNAG conjugated to diphtheria toxoid diluted 1:10 000 (Maira-Litran et al., 2005). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories; diluted 1:10 000) and the Amersham ECL Western blotting system.

Far-dot-blot analysis. Staphylococcal peptidoglycan (PG) was isolated as described by Herbert et al. (2007). Similar amounts of PG obtained from wild-type strain 15981 and its ΔtagO mutant were resuspended in water and dilutions of the samples (1/5, 1/10, 1/50 and 1/100) were spotted onto nitrocellulose membranes. Then, membranes were incubated with pure PNAG (5 μg ml−1) extracted from S. aureus MN8m as described previously (Maira-Litran et al., 2005). PNAG association was detected with rabbit antiserum raised against deacetylated PNAG conjugated to diphtheria toxoid.

Statistical analysis. Statistical analyses were performed with the SPSS program. A non-parametric test (Mann–Whitney U test) was used to assess significant differences in bacterial density between the wild-type and ΔtagO strains recovered from the glass spatulas from the microfermenters. For analysis of primary attachment a one-way ANOVA test with Tukey’s pairwise comparisons was used. Differences were considered statistically significant for P <0.05.

RESULTS

Mutants in WTAs are sensitive to high temperatures and display increased autolysis rates
To investigate the role of WTAs in PNAG association with the cell surface, we constructed mutants in the tagO gene (ΔtagO), which encodes the first enzyme involved in the WTA biosynthetic pathway, using markerless allelic exchange facilitated by the pMAD plasmid (Arnaud et al., 2004) in 15981 (strong biofilm former) and 10833 (weak biofilm former) S. aureus clinical isolates. To favour the selection of mutants that had lost the plasmid after the resolution of the second recombination event, we incubated the mutants at 44 °C, a restrictive temperature, preventing plasmid replication. After several failed trials, in which we were unable to obtain ΔtagO mutants, we hypothesized that deletion of the tagO gene might cause an increased sensitivity to high temperatures. To test this hypothesis, we repeated the double recombination process without the subsequent incubation step at 44 °C. By this modified protocol many ΔtagO mutants were obtained in both strains, strongly suggesting that tagO deletion in S. aureus caused an inability to grow at 44 °C. This observation was confirmed when we analysed the growth of ΔtagO mutants on agar plates at 37 and 44 °C. The ΔtagO mutants had a seriously compromised capacity for growth at 44 °C. The temperature sensitivity of ΔtagO mutants was lost when they were complemented with the pCU1 plasmid carrying a PCR-amplified 1349 bp fragment containing the tagO gene under the control of its own promoter (Fig. 1a). The absence of WTAs in ΔtagO mutants and their presence in the complemented mutants was confirmed by subjecting WTA
extracts to gel electrophoresis and combined alcian blue and silver staining (data not shown).

To study the effect of tagO deletion on bacterial growth, we compared the growth curves of ΔtagO and wild-type strains at 37 °C in rich media. The results showed that ΔtagO mutants exhibited growth rates similar to those of wild-type strains. However, the turbidity of the cultures began to decrease in ΔtagO mutants during prolonged incubation at stationary phase (Fig. 1b), suggesting that the absence of WTAs could induce spontaneous autolysis. To test this hypothesis, we monitored bacterial cell lysis in the presence of Triton X-100. As shown in Fig. 1(c), the ΔtagO strains showed increased autolysis compared to wild-type strains and complemented mutants. In order to study whether the increased autolysis rate of ΔtagO mutants was a consequence of a higher content of cell wall murein hydrolases, we compared the autolysin pattern of wild-type and ΔtagO strains in a zymogram gel. The zymograms revealed that the cell lysate of the ΔtagO mutant displayed zones of increasing clearance corresponding to areas of increased murein hydrolytic activity (Fig. 1d). Taken together, these results indicate that the absence of WTAs increases both the sensitivity of S. aureus to high temperatures and the autolysis rate, which must be at least partially attributable to a higher murein hydrolase activity.

**Multicellular behaviour of ΔtagO strains**

As bacterial surface components play an important role in cell-to-cell interactions and adherence to surfaces, we analysed the effect of the absence of WTAs on the formation of multicellular structures by analysing three different phenotypes: production of a ring of cells adherent to the glass wall at the air–liquid interface, and biofilm formation in static microtitre plates and under continuous-flow conditions in microfermenters.

The ΔtagO strains retained the capacity to produce a ring of cells adherent to the glass wall when growing overnight in liquid medium under shaking conditions. However, the majority of the cells of ΔtagO mutants precipitated and settled at the bottom of the tubes (Fig. 2a). This phenotype was related to the absence of WTAs, because the complemented strains exhibited a much greater turbidity throughout the culture tube. We also noted that when the
Parental strains were grown to stationary phase they also formed aggregates in the tubes (Fig. 2a); however, these aggregates were very stable to vortexing, unlike those formed by the ΔtagO strains, which were readily dispersed by mild vortexing.

Analysis of the biofilm-forming capacity of the bacterial strains in microtitre plate assays revealed that compared to the parental strains, ΔtagO strains displayed a reduced capacity to produce biofilms (Fig. 2b). However, observation of the microtitre plates wells before washing revealed that ΔtagO strains were still able to form a pellicle loosely associated with the polystyrene surface that was easily removed when rinsed. The presence of this pellicle in ΔtagO strains was visualized both macroscopically after air-drying of the microtitre plates before staining (Fig. 2c) and by observation of the biofilm with a scanning electron microscope (Fig. 3). The scanning electron micrographs revealed that wild-type cells were interconnected by an extracellular matrix mesh (Fig. 3a, c). This mesh was more irregular, containing numerous aggregates of material, in the ΔtagO mutant. In agreement with the macroscopic aggregative phenotype of ΔtagO mutants in liquid media, scanning electron micrographs also revealed that ΔtagO mutant cells grew in highly dense bacterial aggregates with very little void space (Fig. 3b, d).

The reduced ability of ΔtagO mutants to form polystyrene-attached biofilms suggested that these strains could have a deficiency in the initial attachment to the polystyrene surface. To test this possibility, primary attachment experiments with static incubation periods of 60 min were carried out. The results showed that the ΔtagO mutants exhibited a reduced level of initial binding, indicating that the absence of WTAs affects the initial steps in the biofilm formation process (Fig. 2d).

Finally, analysis of the biofilm-forming capacity of a ΔtagO mutant grown under continuous-flow conditions in microfermenters showed that the biofilm biomass produced by the ΔtagO strain was significantly reduced in comparison to the parental strain, and only a thin and weak layer of cells covered the surface of the slide (Fig. 4). Taken together, these results indicate that modifications in the physico-chemical properties of the bacterial cell surface in the absence of WTAs provoke bacterial aggregation and reduce the capacity of bacteria to adhere to abiotic surfaces, thereby affecting the development of biofilms firmly attached to surfaces.

**Role of WTAs in PNAG localization**

To investigate whether the reduced ability to form biofilms of ΔtagO mutants was due to altered PNAG production, we monitored the levels of surface-attached polysaccharide in overnight cultures of wild-type and ΔtagO strains by dot-blot analysis, using specific antibodies raised against

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**Fig. 2.** Biofilm formation and aggregative phenotypes of two genetically unrelated *S. aureus* strains and their respective ΔtagO mutants. (a) Biofilm-forming capacity on glass surfaces of overnight cultures, incubated in TSB-gluc medium with shaking at 37 °C, of *S. aureus* wild-type strains 15981 (strong biofilm former) and 10833 (weak biofilm former), their isogenic ΔtagO mutants, the complemented ΔtagO mutants and the control ΔtagO strains carrying empty pCU1 plasmid. (b) Biofilm-forming capacity on microtitre plates after 24 h static incubation at 37 °C in TSB-gluc medium. (c) Biofilm formation on microtitre plates after 24 h static incubation at 37 °C in TSB-gluc medium. The pellicles formed on the bottom of the plates were air-dried before staining. (d) Comparison of the primary attachment ability of *S. aureus* 15981, its ΔtagO mutant, the complemented mutant and the control ΔtagO strain carrying pCU1. Data represent the means of at least nine counts from three independent experiments. Error bars show SD. Very significant differences were detected between the wild-type strain and ΔtagO mutant (ns, not significant versus wild-type; ***, P<0.001 versus wild-type; one-way ANOVA test with Tukey’s pairwise comparisons).
deacetylated PNAG. The results showed that the levels of PNAG attached to the ΔtagO bacterial cell surface were only slightly reduced in comparison to those of the wild-type strain. As an internal control for the procedure, we used a Δica mutant that is unable to produce PNAG (Fig. 5a).

Next, we analysed whether the absence of WTAs could affect the strength of PNAG interaction with the bacterial cell surface. To test this hypothesis, we compared the efficiency of different PNAG extraction conditions ranging from harsher to milder solvents (4 M urea to 1 M urea).

![Fig. 3. Scanning electron microscopy analysis of ΔtagO mutant. Scanning electron micrographs of S. aureus 15981 (a, c) and its ΔtagO mutant (b, d) at ×5000 and ×20000 magnifications. Scale bars: (a, b), 5 μm; (c, d), 2 μm.](http://mic.sgmjournals.org)

![Fig. 4. Biofilm formation of S. aureus 15981 and ΔtagO mutant under continuous-flow conditions. (a) Biofilm development of wild-type S. aureus strain 15981 and ΔtagO mutant grown under continuous-flow conditions in microfermenters with TSB-gluc after 24 h at 37 °C. The microfermenters (upper panels) contain the glass slides on which bacteria form biofilms (lower panels). The results of a representative experiment are shown. (b) Quantification of the biofilm mass adherent to the glass slides. The cells were removed from the glass slides into 20 ml TSB-gluc by vortexing, and the OD650 of the resulting suspensions was measured. Significant differences were detected between the wild-type and the ΔtagO mutant (n=4; P<0.05, Mann–Whitney U test).](http://mic.sgmjournals.org)
NaCl>0.8 M NaCl; and EDTA, 100 °C>EDTA, room temperature>water, 100 °C>water, room temperature). No significant differences in the levels of extracted PNAG between the ΔtagO mutant and the wild-type strain were detected with any of the extraction protocols (Fig. 5b). As we had observed previously for overnight cultures, we detected slightly reduced PNAG levels in the ΔtagO mutant as compared to the parental strain. These results indicate that the strength of PNAG interaction with the bacterial cell surface in ΔtagO mutants is similar to that of the wild-type strain, strongly suggesting that WTAs are not implicated in the attachment of PNAG to the bacterial cell surface.

We reasoned that the absence of WTAs could be compensated for by LTAs, the other type of TA, which also contribute to the negative charge of the bacterial cell surface. To investigate this possibility, we carried out a ‘far-dot-blot’ experiment with purified peptidoglycan, partially deacetylated PNAG and anti-PNAG antibodies. The results revealed that purified PNAG binds with similar affinity to peptidoglycan of both wild-type and ΔtagO mutant strains, suggesting that PNAG is able to interact with the bacterial cell wall in the absence of WTA and LTA (data not shown).

Finally, to confirm the presence of PNAG on the cell surface of the ΔtagO mutants, we studied, by immunoelectron microscopy, the localization of PNAG using specific polyclonal rabbit antiserum raised against deacetylated PNAG and gold-labelled donkey anti-rabbit IgG as secondary antibody. As shown in Fig. 6, PNAG was located on the bacterial cell surface in both wild-type and ΔtagO strains, while it was absent from the surface of a Δica mutant and mostly released to the supernatant in a ΔicaB strain. In summary, these results indicate that PNAG interacts with the bacterial cell surface in the absence of WTAs.

**Aggregation phenotype of ΔtagO mutants is not dependent on PNAG**

To investigate the possible role of PNAG in the aggregative phenotype observed in ΔtagO mutants, we constructed ΔtagO Δica double mutants. As shown in Fig. 7(a, b) ΔtagO Δica double mutants displayed a similar aggregative phenotype to that of ΔtagO strains, indicating that this phenotype is independent of the presence of PNAG. In contrast to Δica strains, the double ΔtagO Δica mutants retained the capacity to produce both a ring of cells adherent to the glass wall (Fig. 7a) and the loosely associated pellicle formed by ΔtagO mutants that was only visible during air-drying of the microtitre plates before staining (Fig. 7b). These results suggest that although PNAG is associated with the bacterial surface in ΔtagO mutants, the multicellular behaviour of ΔtagO mutants seems not to be dependent on its presence.

In order to determine the nature of the matrix produced by ΔtagO mutants, pellicles produced on polystyrene microtitre plates were treated with proteinase K, Dispersin B [an enzyme of Actinobacillus actinomycetemcomitans (formerly Actinobacillus actinomycetemcomitans)] that is able to hydrolyse β-(1-6) glycosidic linkages (Kaplan et al., 2004a)] and DNase I. Neither proteinase K nor Dispersin B produced any effect on the pellicles formed by ΔtagO and ΔtagO Δica mutants. Only incubation with DNase I slightly decreased pellicle formation. As expected, treatments with Dispersin B completely disrupted the PNAG-dependent biofilm formed by wild-type S. aureus strain 15981 (Fig. 7c). These results suggest that the aggregation observed in ΔtagO mutants is independent of the production of PNAG, proteins or the relative amount of extracellular DNA present in the medium.

Fig. 5. Dot-blot analysis of PNAG accumulation. (a) Dot-blot analysis of surface-localized PNAG levels of S. aureus wild-type strain 15981, its ΔtagO mutant, the complemented mutant and the control ΔtagO strain carrying the empty pCU1 plasmid. Cell-surface extracts of overnight cultures were treated with proteinase K and dilutions of the samples (1:50, 1:75 and 1:100) were spotted onto nitrocellulose membranes. PNAG production was detected with rabbit antiserum raised against deacetylated PNAG conjugated to diphtheria toxoid. A Δica mutant unable to produce PNAG was used as a negative control. (b) Dot-blot analysis of PNAG cell-surface levels of overnight cultures using different extraction conditions ranging from harsher to milder solvents (4 M urea>1 M NaCl>0.8 M NaCl; and EDTA, 100 °C>EDTA, room temperature>water, 100 °C>water, room temperature).
DISCUSSION

Although multiple bacterial and external factors influence biofilm formation, production of an extracellular polysaccharide adhesin, here termed PNAG but also referred to as PIA, by biosynthetic enzymes encoded in the ica operon is currently the best understood molecular factor involved in biofilm formation by *S. aureus*. Many efforts have been made to understand how transcription of the ica locus and PNAG biosynthesis are regulated. In contrast, few studies have focused on how PNAG interacts with bacterial cell surfaces and remains in contact with them giving shape to the biofilm matrix.

In comparison to other known exopolysaccharides that are neutral, negatively charged or, on occasion, zwitterionic with both positive and negative charges (Cobb *et al.*, 2004; Cobb & Kasper, 2005), PNAG represents a very unusual bacterial exopolysaccharide molecule, as some GlcNAc residues become deacetylated by the IcaB protein (Vuong *et al.*, 2004), which provides a positive net charge to the polymer (Joyce *et al.*, 2003). The deacetylation process, and therefore the cationic character of PNAG, has been shown to be crucial for the attachment of PNAG to the negatively charged bacterial surface and the biofilm formation process in both *S. epidermidis* (Vuong *et al.*, 2004) and *S. aureus* (Cerca *et al.*, 2007).

Fig. 6. Immunoelectron microscopy. Images of cells probed first with rabbit antiserum raised against deacetylated PNAG conjugated to diphtheria toxoid and then with gold-labelled donkey anti-rabbit IgG. (a, b) *S. aureus* 15981, (c, d) *S. aureus* 15981 ΔtagO mutant, (e) *S. aureus* 15981 Δica mutant, (f) *S. aureus* 15981 ΔicaB mutant.
WTA polymers are major contributors to the negative charge of the bacterial cell wall, and consequently were good candidates for anchoring PNAG exopolysaccharide through ionic interactions. However, using WTA-deficient ΔtagO mutants we obtained evidence that WTAs are not implicated in the attachment of PNAG exopolysaccharide to the S. aureus cell surface. In the absence of WTAs, both the levels of PNAG on the bacterial surface and the strength of interaction between PNAG and the bacterial cell surface are similar to those of the wild-type strain. The simplest explanation for these results is that WTAs are not required for anchoring PNAG. Alternatively, it is possible that the absence of WTAs is compensated by other cell surface compounds, such as LTAs. Due to the impossibility of generating LTA-deficient mutants because LTAs are essential for S. aureus viability (Grundling & Schneewind, 2007), we decided to evaluate this possibility by analysing the capacity of purified PNAG to interact with purified peptidoglycan. The results showed that PNAG was able to bind in vitro to peptidoglycan of both wild-type and ΔtagO mutant strains, suggesting that LTAs are not essential for the attachment of PNAG to the bacterial cell surface. Other alternative candidates for anchoring PNAG could be any of the numerous surface proteins linked to the peptidoglycan. However, we have observed that sortase A mutants of S. aureus, which lack the family of LPXTG-anchored proteins, contain levels of surface PNAG identical to those of wild-type S. aureus strains and retain the capacity to form biofilms on microtitre polystyrene plates, strongly suggesting that LPXTG proteins are also dispensable for PNAG binding to the S. aureus cell surface (our unpublished results).

In this work we also have analysed the impact of the ΔtagO mutation on the biofilm formation process of S. aureus. Our data showed that depletion of WTAs significantly reduced the in vitro capacity of S. aureus to form biofilms under both steady-state and flow conditions, and promoted cell-to-cell interactions, making cells aggregate and sediment. The reduced biofilm-forming capacity of ΔtagO mutants could be due to a decreased capacity of the mutants to establish initial interactions with the polystyrene or glass surfaces. In fact, primary attachment experiments showed that ΔtagO mutants exhibit a reduced capacity in the initial binding to polystyrene plates in comparison to wild-type strains. This phenotype is in accordance with the less efficient capacity for adherence to human epithelial and endothelial cells observed in a ΔtagO mutant by Weidenmaier et al. (2004, 2005), and is consistent with that of dltA mutants lacking D-alanine esters in TAs, which form cell pellets unable to adhere to abiotic surfaces (Gross et al., 2001). Those authors explain this phenotype by hypothesizing that the absence of D-alanine esters causes a stronger negative charge on the bacterial cell surface that leads to a pronounced increase in the repulsive forces and therefore to inhibition of the interaction between bacteria and surfaces. However, a similar explanation cannot be applied to ΔtagO mutants because WTA-deficient bacteria should not display a strong negative charge on the cell surface, since cell wall
phosphate content is vastly reduced in tagO mutants (D’Elia et al., 2006b; Weidenmaier et al., 2004).

The role of TAs in cell aggregation has already been described for several bacterial isolates and has been related to the content of alanine ester groups as well as to bound divalent metal cations, especially Ca$^{2+}$ (Baddiley, 2000; Neuhaus & Baddiley, 2003). In fact, the increased tendency for cell aggregation of ΔtagO mutants has been reported in Bacillus subtilis (D’Elia et al., 2006a; Soldo et al., 2002a) but not in S. aureus. The deficiencies in primary attachment to abiotic surfaces observed in the ΔtagO mutants could be a consequence of this aggregation, since ΔtagO mutant cells seem to prefer to interact between themselves rather than with artificial surfaces. Interestingly, this phenomenon of coaggregation seems to be independent of the presence of PNAG or proteins, since it also occurs in double ΔtagO Δica mutants and it is not affected by Dispersin B or proteinase K treatment. It seems that the alteration caused by the absence of WTAs in the bacterial cell wall enables new interactions between components of the cell wall that are not usually possible and in the absence of WTAs lead to the aggregative phenotype observed here.

The cell wall of Gram-positive bacteria has been shown to be essential for the survival, shape and integrity of the cells. The lack of WTAs must have a strong impact on the structure of the bacterial cell envelope, since WTAs make up a significant portion of the cell wall, and it is likely that their absence results in a cell wall more sensitive to external conditions and environmental changes. This fact could explain the increased autolysis rate of the mutant as well as its inability to survive at temperatures of 44 °C. The temperature sensitivity of TA-deficient mutants has already been described for an mnaA mutant of B. subtilis, which lacks an enzyme involved in the synthesis of the linkage unit of TAs to the peptidoglycan, and consequently has a reduced WTA content (Soldo et al., 2002b). Increased autolysis due to tagO deficiency has also been reported previously among tagO transposon insertion mutants of meticillin-resistant S. aureus clinical isolates (Maki et al., 1994). This higher degree of autolysis could be due to a lower content of D-alanyl esters. It is well known that the absence of D-alanyl esters from TAs leads to increased sensitivity toward cationic antimicrobial peptides (Peschet et al., 1999), and although the basis for this is not fully known, it is thought that the presence or absence of these esters within the cell wall matrix at specific locations might constitute a targeting mechanism for proteins such as autolysins that require a specific ionic microenvironment for function (Neuhaus & Baddiley, 2003). Accordingly, zymographic analysis revealed enhanced urease hydrolase activity in the ΔtagO mutant as compared to the wild-type strain. However, these results contradict those observed by Bera et al. (2007), which show no increased endogenous autolysis activity in an S. aureus strain SA113 ΔtagO mutant, and suggest that a strain-specific behaviour might exist.

The presence of genes required for the synthesis of the PNAG-homologous exopolysaccharides in both Gram-positive and Gram-negative bacteria (Wang et al., 2004) suggests that the interaction between deacetylated PNAG and the negatively charged bacterial cell wall is mediated by non-specific electrostatic interactions. WTAs and LPSs are the two main contributors to the negative charge on the bacterial cell surface. We have shown that WTAs are not required for PNAG anchoring, but it remains to be determined whether LPS is also dispensable for PNAG localization on the bacterial surface.

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