Dissecting the regulation of bile-induced biofilm formation in *Staphylococcus aureus*

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Aspiration of bile into the cystic fibrosis (CF) lung has emerged as a prognostic factor for reduced microbial lung biodiversity and the establishment of often fatal, chronic pathogen infections. *Staphylococcus aureus* is one of the earliest pathogens detected in the lungs of children with CF, and once established as a chronic infection, strategies for its eradication become limited. Several lung pathogens are stimulated to produce biofilms *in vitro* in the presence of bile. In this study, we further investigated the effects of bile on *S. aureus* biofilm formation. Most clinical *S. aureus* strains and the laboratory strain RN4220 were stimulated to form biofilms with sub-inhibitory concentrations of bovine bile. Additionally, we observed bile-induced sensitivity to aminoglycosides, which we exploited in a *bursa aurealis* transposon screen to isolate mutants reduced in aminoglycoside sensitivity and augmented in bile-induced biofilm formation. We identified five mutants that exhibited hypersensitivity to bile with respect to bile-induced biofilm formation, three of which carried transposon insertions within gene clusters involved in wall teichoic acid (WTA) biosynthesis or transport. Strain TM4 carried an insertion between the divergently oriented *tagH* and *tagG* genes, which encode the putative WTA membrane translocation apparatus. Ectopic expression of *tagG* in TM4 restored a wild-type bile-induced biofilm response, suggesting that reduced translocation of WTA in TM4 induced sensitivity to bile and enhanced the bile-induced biofilm formation response. We propose that WTA may be important for protecting *S. aureus* against exposure to bile and that bile-induced biofilm formation may be an evolved response to protect cells from bile-induced cell lysis.

**INTRODUCTION**

Chronic infections by pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* are a leading cause of morbidity and mortality in cystic fibrosis (CF) patients. Chronic bacterial infections can rarely be eradicated by antimicrobial treatment, and thus chronic infection of the lung can eventually lead to a fatal decline in lung function (Furukawa et al., 2006). This is becoming an ever increasing clinical issue as many have predicted the onset of a post-antibiotic era (Cooper & Shlaes, 2011). Therefore, innovative and alternative strategies, away from the classical antibiotic approach, are urgently needed. The refractiveness of chronic infections to conventional therapies is largely attributed to bacteria adopting a biofilm-like mode of growth. Microorganisms in biofilms are embedded within a matrix of extracellular polymeric substances that provides a barrier against the host’s immune defences and antimicrobial therapy (Flemming & Wingender, 2010). The biofilm matrix is composed of polysaccharides, proteins, lipids, and genomic

**Abbreviations:** CF, cystic fibrosis; GERD, gastro-oesophageal reflux disease; PIA, polysaccharide intercellular adhesion; SC, sodium cholate; SDC, sodium deoxycholate.

Three supplementary figures and one supplementary table are available with the online Supplementary Material.
DNA that is released by lysed resident bacterial cells. While the molecular mechanisms underlying the formation of biofilms in a broad spectrum of pathogens have been well studied, until recently the molecular triggers that cause lung-colonizers to adopt a chronic lifestyle, and the associated biofilm mode of growth remained largely uncharacterized. Understanding how bacteria adopt this chronic lifestyle in the lung would provide new therapeutic options for prevention and treatment.

The aspiration of bile acids into the lungs arising from gastro-oesophageal reflux disease (GERD) has since emerged as a host-trigger of chronic bacterial infection (Reen et al., 2012, 2014) and chronic inflammation (Legendre et al., 2014), particularly in CF, where up to 40% of child and 80% of adult patients can suffer from this complication (Legendre et al., 2014; Pauwels et al., 2012; Reen et al., 2012, 2014; Stringer et al., 1988). Indeed, the incidence may be underestimated as clinical diagnosis of GERD is often not sufficient to determine bile aspiration. Bile acids have been detected in the sputum and bronchoalveolar lavage fluid of patients who do not present with classical GERD symptoms. This ‘silent aspiration’ phenomenon is particularly severe in CF patients and highlights the urgent unmet need for rapid diagnosis of bile acid profiles in biological samples from respiratory and lung transplant patients (Button et al., 2005). In recent years a number of research publications have suggested that acid and non-acid reflux may negatively influence the progression of respiratory disease (D’Ovidio et al., 2005a, b; el-Serag & Sonnenberg, 1997; Pauwels et al., 2012; Perreg et al., 2007; Wu, 2008; Wu et al., 2009). El-Serag & Sonnenberg showed that patients with erosive oesophagitis, a sign of significant GERD, had increased incidence of pulmonary fibrosis, chronic bronchitis or chronic obstructive pulmonary disease in a case–control study of more than 200,000 patients (el-Serag & Sonnenberg, 1997). A strong correlation between GERD-derived reflux, pulmonary aspiration and increased lung damage also extends to several other respiratory diseases (Navarro et al., 2001), including idiopathic pulmonary disease and advanced lung damage arising from lung transplantation (Sweet et al., 2006, 2007, 2009), ventilator-associated pneumonia (Wu et al., 2009), Barrett’s oesophagus and oesophageal adenocarcinoma (Nassr et al., 2011), and bile acid pneumonia in neonates (Zecca et al., 2004, 2008). Therefore, the implications of elucidating the link between bile aspiration and chronic pathogen behaviour has consequences for a range of clinical conditions.

In this study we examined the effect of bile on virulence-related behaviour in S. aureus, the primary pathogen associated with early stage CF infection. While S. aureus is considered a commensal bacterium, as it is a common colonizer of the human skin and respiratory tract, it is also a frequent cause of clinically important infections (Wertheim et al., 2005). In many cases S. aureus is the earliest colonizer in CF patients, and is the most prevalent CF pathogen in children and adolescents (Kahl, 2010; Souza et al., 2006). First elucidating the impact of physiologically relevant concentrations of bile exposure on this important paediatric pathogen, we focused on antibiotic tolerance and biofilm formation. In order to probe more deeply the regulatory mechanisms governing the bile-mediated biofilm response, we utilized a random transposon mutagenesis screen to isolate S. aureus mutants with an altered bile-response. This uncovered a previously unforeseen switch to a biofilm lifestyle by S. aureus in the presence of bile, with some intriguing insights into the molecular mechanism underpinning this key pathogenic determinant.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are outlined in Table 1. S. aureus strains were cultured at 37°C in tryptic soy broth (TSB; Becton Dickinson) or tryptic soy agar [TSA; TSB containing 1.5% (w/v) agar]. E. coli EP100 was used as a cloning host, and was cultured at 37°C in lysozyme broth (LB), or LB agar. Where required, media were supplemented with the following concentrations of antibiotics unless otherwise stated: 100 µg ml⁻¹ ampicillin, 10 µg ml⁻¹ chloramphenicol, 2.5 µg ml⁻¹ tetracycline, 10 µg ml⁻¹ erythromycin. Media were supplemented with bovine bile (Sigma-Aldrich), sodium cholate (SC; Sigma-Aldrich), sodium deoxycholate (SDC; Sigma-Aldrich) or SDS (AMRESCO) when required. Stock solutions of bile, SC and SDC were prepared in deionized distilled water and filter sterilized prior to addition to media.

DNA manipulations. S. aureus DNA was extracted from broth using the FavorPrep Blood/Cultured Cell Genomic DNA Extraction Mini kit (Favorgen Biotech) following cell lysis with lyostaphin (Sigma-Aldrich) unless otherwise stated. Electroporation of S. aureus strains was carried out using the method described by Schenk & Laddaga (1992). PCR products were amplified using Phusion High-Fidelity DNA Polymerase (New England BioLabs), and purified using the FavorPrep GEL/PCR Purification kit (Favorgen Biotech).

Biofilm attachment assay. Stationary-phase cultures of S. aureus were diluted 1:200 in TSB or TSB supplemented with appropriate treatment, and aliquots transferred to 96-well or 24-well plates. Following incubation at 37°C, the wells were washed twice in water to remove planktonic cells. Attached cells (biofilm) were stained with 0.1% (w/v) crystal violet solution, washed twice to remove unincorporated stain, and solubilized with acetic : ethanol (3:7) before quantification by absorbance at 595 nm. At least three independent biological replicates were performed for each experiment. For each strain of S. aureus, treatment samples were compared to untreated samples using a two-tailed paired or unpaired Student’s t-test.

Growth analysis. To determine the effects of bile on the growth of S. aureus, stationary-phase cultures of S. aureus were diluted 1:500 in 25 ml TSB or TSB supplemented with bovine bile. Cultures were grown at 37°C with agitation at 180 r.p.m. and samples were taken over a 24 h period and the OD measured at a wavelength of 600 nm.

Antibiotic sensitivity determinations. For disc diffusion testing, a single colony of S. aureus RN4220 was resuspended in 1 ml PBS, and evenly spread on TSA plates or TSA plates containing 0.3% bile to prepare a lawn culture. Antibiotic discs, containing gentamicin (10 µg), chloramphenicol (30 µg), ampicillin (10 µg), erythromycin (15 µg) or tetracycline (30 µg), were placed on the agar, and the plates were incubated at 37°C for 18 h, following which the diameter of the inhibition zone was observed.
second PCR was performed using an aliquot of amplified product from PF106, PF107 and PF108) and a transposon-specific primer (GFP1). A PCR was performed using the purified DNA as template, with a random primer mix following cell lysis with lysostaphin. PCR was performed on each of the transposon mutants using PrepMan Ultra Sample Preparation Reagent Oligonucleotide primers are listed in Table 2. DNA was purified from suspension of isolated colonies, adjusted to achieve a turbidity equivalent of a 0.5 McFarland standard, and diluted 1 : 300 in TSB containing 1 mM SC. An inoculum of S. aureus RN4220 was prepared by saline (0.85 % NaCl) suspension of isolated colonies, adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard, and diluted 1 : 300 in TSB containing 1 mM SC and/or antibiotics such that the final inoculum was ~5 × 10^6 f.u. ml⁻¹. Cultures were incubated overnight at 37 °C with shaking. The lowest concentration of antibiotic that inhibited all visually apparent growth was considered the MIC.

**Transposon mutagenesis.** Plasmids pBursa and pFA545 were transformed together into S. aureus RN4220 by electroporation, and transformants were selected on TSA-containing chloramphenicol and tetracycline following incubation at the permissive temperature of 30 °C for 48 h. Resulting colonies were resuspended in sterile water and incubated at the non-permissive temperature of 43 °C for 1 h before spreading on selective media (TSA containing erythromycin, 1 mM SC, and either gentamicin, neomycin or kanamycin) to screen for mutants of interest.

**Determination of site of transposon insertion.** Transposon insertion sites were determined by random-prime PCR as described below. Oligonucleotide primers are listed in Table 2. DNA was purified from transposon mutants using PrepMan Ultra Sample Preparation Reagent (Life Technologies) following cell lysis with lysostaphin. PCR was performed using the purified DNA as template, with a random primer mix (PF106, PF107 and PF108) and a transposon-specific primer (GFP1). A second PCR was performed using an aliquot of amplified product from the first PCR, with primers GFP2 and PF109. Following confirmation of the presence of the amplified product by gel electrophoresis, the PCR reactions were purified and subsequently sequenced using primer GFP3. Sequencing reactions were conducted by the Australian Genome Research Facility. Each sequence was compared using the National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search (BLASTN) tool to the S. aureus NCTC8325 complete genome (CP000253.1). The location at which the query sequence first matched the subject sequence was determined as the transposon insertion site.

### RESULTS

**Bile induces biofilm formation in most S. aureus**

Physiological concentrations of bile or bile acids stimulate in vitro biofilm formation in several lung-colonizing pathogens (Reen et al., 2012). In contrast, bile suppresses biofilm formation by S. aureus strain NCDO 949, a common laboratory strain originally isolated from pleural fluid. To establish whether NCDO 949 bile-response phenotype was representative of S. aureus strains, biofilm formation was analysed for paediatric isolates obtained from the CF unit at Cork University Hospital, Ireland, using the crystal violet attachment assay. In contrast to NCDO 949, biofilm formation was stimulated by bile in these isolates (Fig. 1a). Furthermore, bile-stimulated biofilm formation was observed

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Source/reference</th>
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</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>Type strain, pleural fluid isolate</td>
<td>Shinfield, UK</td>
</tr>
<tr>
<td>NCDO 949</td>
<td>Paediatric clinical isolate, CUH clinic</td>
<td>BRC</td>
</tr>
<tr>
<td>CUHT</td>
<td>Paediatric clinical isolate, CUH clinic</td>
<td>BRC</td>
</tr>
<tr>
<td>CUHE</td>
<td>Dominant CA-MRSA strain in Australia, ST93-IV</td>
<td>Chuai et al. (2010)</td>
</tr>
<tr>
<td>JKDr159</td>
<td>First community-acquired CA-MRSA, ST1-MRSA-IVa (2B) (pMW2)</td>
<td>Baba et al. (2002)</td>
</tr>
<tr>
<td>MW2</td>
<td>Highly transformable restriction-minus derivate of NCTC8325-4</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>E. coli</td>
<td>EPI300</td>
<td>Epicentre</td>
</tr>
<tr>
<td></td>
<td>pFA545</td>
<td>Encode a transposable origin of replication, and a tetracycline resistance selection marker</td>
</tr>
<tr>
<td></td>
<td>pLI50</td>
<td>S. aureus (chloramphenicol resistance selection marker)/E. coli (ampicillin resistance selection marker) shuttle vector</td>
</tr>
<tr>
<td></td>
<td>pLI50 :: int_tagHG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pLI50 containing the tagG-tagH intergenic region amplified from RN4220 genomic DNA using primers TagHGIntFor and TagHGIntRev and cloned as EcoRI–BamHI fragment</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pLI50 :: tagG</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>pLI50 containing tagG amplified from RN4220 genomic DNA using primers TagGForRBS and TagGRev and cloned as EcoRI–BamHI fragment</td>
<td>This study</td>
</tr>
</tbody>
</table>

**CA-MRSA, Community-acquired methicillin-resistant S. aureus; ST, sequence type.**
data suggest that, like other lung pathogens, most strains do not exhibit bile-induced biofilm formation. Therefore these strains are stimulated to form biofilms in the presence of bile, but that there is variation in this response amongst strains.

Since the well-characterized and genetically tractable S. aureus strain RN4220 exhibited a similar response to bile to most clinical S. aureus isolates, we further investigated the effects of the addition of bile to this strain. Analysis of RN4220 growth in TSB broth culture revealed that the log-phase growth rate was uninhibited by the addition of up to 0.3 % bovine bile (Fig. 1c). Bile acids make up over 50 % of the total solute concentration of bile (Kristiansen et al., 2007) and their salt equivalents have been implicated in biofilm formation in P. aeruginosa (Reen et al., 2012). The addition of sodium cholate (SC) (Fig. 2a) or sodium deoxycholate (SDC) (Fig. 2b) at sub-inhibitory concentrations resulted in a dose-dependent increase in S. aureus biofilm formation, indicating that these individual bile components were able to induce a similar response to whole bile in S. aureus. Interestingly, addition of 0.1 mM of the anionic detergent SDS also causes a similar but statistically insignificant increase in biofilm formation, possibly indicating that the biofilm formation by S. aureus may be a response to the common detergent activities of these molecules (Fig. 2c).

### Table 2. Oligonucleotide sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’–3’)</th>
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<tbody>
<tr>
<td>GFP1</td>
<td>TCCACTGAGAAGAAATTGTGCCCATTACA</td>
</tr>
<tr>
<td>GFP2</td>
<td>CATTAAATCGACATCTGACAGAAA</td>
</tr>
<tr>
<td>GFP3</td>
<td>ACAAGAATTGGGACACTCCAGTGA</td>
</tr>
<tr>
<td>PF106</td>
<td>GACCACAGCTGACTAGTGCNNNNNNN</td>
</tr>
<tr>
<td>PF107</td>
<td>GACCACACGTCGACTAGTGCNNNNNNN</td>
</tr>
<tr>
<td>PF108</td>
<td>GACCACACGTCGACTAGTGCNNNNNNN</td>
</tr>
<tr>
<td>PF109</td>
<td>GACCACACGTCGACTAGTGCNNNNNNN</td>
</tr>
<tr>
<td>TagHGIntFor</td>
<td>TAGTAAAGCTGTAATTCCTTGTAGACCTTCC</td>
</tr>
<tr>
<td>TagHGIntRev</td>
<td>TAGTAAAGCTGTAATTCCTTGTAGACCTTCC</td>
</tr>
<tr>
<td>TagForRBS</td>
<td>TAGTAAAGCTGTAATTCCTTGTAGACCTTCC</td>
</tr>
<tr>
<td>TagHGRev</td>
<td>TAGTAAAGCTGTAATTCCTTGTAGACCTTCC</td>
</tr>
</tbody>
</table>

for community-acquired meticillin-resistant S. aureus strains JKD6159 and MW2 and the common laboratory strain RN4220 (Fig. 1b). In contrast, USA300 strain JE2 did not exhibit bile-induced biofilm formation. Therefore these data suggest that, like other lung pathogens, most S. aureus strains are stimulated to form biofilms in the presence of bile, but that there is variation in this response amongst strains.

Development of a bursa aurealis transposon mutagenesis screen to isolate bile-response mutants

Bile acids have been demonstrated to enhance the activity of penicillin and neomycin against staphylococcal strains. Bile has no effect on the efficacy of other antibiotics such as chloramphenicol or erythromycin, but can weaken the activity of some antibiotics including vancomycin (Schneierson & Amsterdam, 1958; Schneierson et al., 1962). In a study which compared the effects of various bile acids in their salt form, as well as other components of bile such as cholesterol, SC and SDC were found to be the most effective at increasing the anti-staphylococcal activity of neomycin (Schneierson et al., 1962). We investigated the effect of SC on antibiotic tolerance in S. aureus RN4220. Disc diffusion antibiotic sensitivity testing revealed that 0.3 % bile increased the sensitivity of S. aureus RN4220 towards gentamicin, but had no effect on chloramphenicol, ampicillin, erythromycin or tetracycline (Fig. S1, available in the online Supplementary Material). We suspected that bile acids may specifically...

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**Fig. 1.** Bile-dependent biofilm formation in S. aureus. (a) Paediatric clinical isolates compared to NCDO 949, and (b) community-acquired meticillin-resistant isolates compared to RN4220. Data are presented as a ratio of the A$_{96}$ from treated vs untreated samples. Graphs show mean (±SEM) relative absorbance of at least three independent experiments, each carried out in triplicate. Statistical analysis was performed by two-tailed paired Student’s t-test (\(P<0.05\), **\(P<0.01\), compared with untreated control). (c) Growth of S. aureus RN4220 in the presence of bovine bile. Graphs show mean (±SD) OD$_{600}$ of two biological replicates.
induce sensitivity to aminoglycosides in S. aureus RN4220. After confirming that SC at a concentration of 1 mM did not have bactericidal or bacteriostatic effects on this strain (Fig. S2), we proceeded to test the MIC of gentamicin, streptomycin, neomycin and kanamycin in the presence of 1 mM SC. The MIC of all aminoglycosides was reduced in the presence of 1 mM SC (Table 3), consistent with a previous study in Lactobacillus species (Elkins & Mullis, 2004), where the authors implicated increased antibiotic uptake following bile exposure.

We hypothesized that the molecular control of bile-induced aminoglycoside sensitivity and bile-induced biofilm formation in S. aureus might be linked at a regulatory level. Following this hypothesis, we predicted that mutant RN4420 strains that overcame bile-induced aminoglycoside sensitivity might also exhibit an altered bile-induced biofilm formation phenotype. In order to isolate genetically marked mutant strains that had overcome bile-induced aminoglycoside sensitivity, we utilized random transposon mutagenesis using the mariner-based transposon bursa aurealis to generate pools of mutant RN4420 and then selected for mutants that had overcome bile-induced aminoglycoside sensitivity in the presence of 1 mM sodium cholate. A range of aminoglycosides was utilized to avoid bias towards mutations that conferred resistance via an antibiotic-specific mechanism. Aminoglycoside concentrations were adjusted in selection plates to a level at which we could observe over ten colonies per plate. Additionally we screened for mutants which displayed resistance to 0.2 mM SDC, as these mutants also may have mutations within regulatory pathways involved in the bile-response that facilitate growth in the presence of inhibitory concentrations of bile acids.

In multiple rounds of mutagenesis with various aminoglycosides, several hundred S. aureus mutants were isolated. For 44 mutants we identified the site of the transposon using random-primed PCR. Table S1 shows the list of transposon mutants, the antibiotic and concentration used to screen for the mutant, and the site of transposon insertion. Thirty-four of the mutants carried the transposon insertion within a defined ORF, while in the remaining 10 mutants the transposon insertion occurred between two coding sequences. Where possible the putative gene and/or gene product associated with the transposon disruption was identified (Table S1).

**Regulatory mutations in wall teichoic acid synthesis stimulate a hypersensitive bile-induced biofilm response**

We screened all 44 mapped mutants for differential biofilm formation in response to bile using the crystal violet assay (Fig. S3). Of these, five exhibited an increased sensitivity to bile, in that they exhibited increased attachment in the presence of 0.03 % bile, compared to wild-type RN4220. As previously discussed, although the parental RN4420 strain displayed a significant increase in biofilm formation in the presence of 0.3 % bile, it failed to respond to bile at the level of 0.03 %. In contrast, five mutants, namely TM4, TM19, TM26, TM28 and TM39, showed an enhanced bile-response, displaying substantially increased biofilm formation in the presence of 0.03 % bile (Fig. 3), a phenotype akin to several of the clinical isolates investigated.

Of the five mutants that exhibited biofilm stimulation in the presence of 0.03 % bile, three carried the transposon insertion within a defined ORF (TM19, TM26, TM28), while two had the transposon insertion occurring in between two coding sequences (TM4, TM39). Interestingly, in both mutants from the latter category, the transposon insertion was between divergently oriented genes where at least one gene was associated with wall teichoic acid (WTA) biosynthesis. TM4 carried an insertion between divergently oriented WTA biosynthesis genes, tagG and tagH (Lazarevic...
In TM39 the insertion was between tagO, which is associated with WTA biosynthesis (Soldo et al., 2002; Xia et al., 2010), and gdpS, the only conserved GGDEF domain protein identified thus far in Staphylococcus (Shang et al., 2009). Furthermore, the transposon insertion in TM26 was within tarJ, another gene involved in the WTA biosynthetic pathway (Brown et al., 2013). It should be noted, however, that although the insertion disrupted tarJ, this gene is duplicated in many S. aureus strains (Qian et al., 2006), and the locus SAOUHSC_00226 in the strain NCTC8325 most likely represents a second copy of tarJ. These data indicated that WTA synthesis or its regulation might be involved in the response to bile and control of biofilm production. The remaining two hypersensitive mutants carried insertions within ctaB (TM19), which encodes for haem O synthase, and a possible D-galactonate transporter (TM28).

The isolation of mutants in WTA-associated genes suggested that WTA synthesis and/or translocation were involved in the bile-induced biofilm response, but because two insertions were within intergenic regions, it was unclear if this was due to increased or decreased WTA. We PCR-amplified and cloned regions of tagG-tagH from RN4220 into the S. aureus shuttle vector pLI50, and introduced each clone into both TM4 and wild-type RN4220. While the introduction of pLI50 into RN4220 did not alter its bile-induced biofilm response, introduction of pLI50 carrying tagG [along with the sod ribosomal binding site (Malone et al., 2009); pLI50::tagG] into TM4 led to the restoration of the wild-type phenotype (Fig. 4). Introduction of a similar construct carrying tagH had no effect (not shown). Interestingly, introduction of pLI50 containing a copy of the non-coding intergenic region located between tagH and tagG (pLI50:: tagHG) into RN4220, produced a strain with a phenotype almost identical to TM4 (Fig. 4). Together, these data suggest that the enhanced bile-induced biofilm phenotype in TM4 may be due to a reduced expression of tagG, caused by the transposon insertion disrupting an operator site for an activator of tagG. The introduction of pLI50::tagG into TM4 likely restores the expression of tagG independent of the activator, producing the observed wild-type phenotype, while the introduction of the intergenic region in RN4220 may sequester a DNA-binding activator of tagG expression.

### DISCUSSION

The pathogenesis of S. aureus in the lungs of respiratory patients is characterized by successful colonization and persistence, particularly in paediatric CF patients, where it dominates the early developing lungs. The environmental factors that cause S. aureus to adopt this pervasive lifestyle are as yet unknown, but our data present a strong case for a role for bile aspiration derived from GERD in the pathogenesis of this organism. In this study we demonstrate that physiologically relevant concentrations of bile, as well as sub-inhibitory concentrations of bile acids, can alter the behaviour of S. aureus, leading to enhanced biofilm formation by this important clinical pathogen. The biofilm mode of growth is frequently associated with chronic infections as it allows the bacterium to evade host defences, and persist for extended periods of time (Furukawa et al., 2006). Our observation is consistent with the increased incidence of S. aureus in paediatric patients suffering from GERD (Palm et al., 2012; van der Doef et al., 2009), the likely source of bile acids in the lungs of these patients (Asee et al., 2012; Pauwels et al., 2012; Reen et al., 2014). This bile-mediated response presented an opportunity to probe more deeply the regulatory mechanisms governing the switch between the phenotypes associated with acute and chronic infection in S. aureus. Using a random transposon mutagenesis approach we identified five S. aureus mutants with an enhanced bile-induced biofilm response. Of these, three mutants had a transposon insertion within or directly upstream of genes involved in WTA biosynthesis. WTAs are surface-exposed anionic glycopolymers present in many Gram-positive species of bacteria, covalently bound to the peptidoglycan layer (Brown et al., 2013). Indeed WTA is the most abundant peptidoglycan bound

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC 0 mM SC (µg ml⁻¹)</th>
<th>MIC 1 mM SC (µg ml⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Neomycin</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

The S. aureus RN4220 MIC of various aminoglycosides in the presence and absence of 1 mM SC is shown.

![Fig. 3. Altered bile-dependent biofilm formation in S. aureus transposon mutants. Graphs show mean ±sd absorbance from three independent experiments each performed in triplicate. Statistical analysis was performed using a two-tailed paired Student's t-test (**P<0.01).](http://mic.microbiologyresearch.org)
glycopolymer in Gram-positive species, making up over half of the dry weight of the cell wall. WTAs have been shown to play key roles in maintenance of cell shape, several aspects of cell division, modulation of antibiotic susceptibility and host tissue colonization (Swoboda et al., 2010; Weidenmaier et al., 2004). Moreover, WTA has been implicated in biofilm formation in staphylococcal species (Holland et al., 2011; Vergara-Irigaray et al., 2008). For example, WTA contains D-alanine modifications that allow S. aureus to modulate its surface charge, aiding its primary attachment to artificial surfaces before the formation of multiple cell layers (Gross et al., 2001).

S. aureus WTA polymers are composed of 30–50 ribitol phosphate (Rbo-P) subunits, connected to peptidoglycan via the murein linkage unit GlcNAc-ManNac-(glycerol phosphate [Gro-P])_2-3 (Yokoyama et al., 1986). WTA biosynthesis is a complex multi-step process that is yet to be fully characterized (Brown et al., 2013). However, it has been shown that tagO, the regulatory region of which is likely disrupted in TM39, is involved in initiating the synthesis of the aforementioned murein linkage unit (Soldo et al., 2002; Xia et al., 2010). Tar (an alcohol dehydrogenase), the function of which is most likely abrogated in TM26, together with Tarl and TarL, catalyses the attachment of Rbo-P to the murein linkage unit. Once WTA polymer formation is complete, it is translocated across the plasma membrane by the two-component ABC (ATP-binding cassette) transporter TagGH (Lazarevic & Karamata, 1995; Schirner et al., 2011), the regulatory region of which was mutated in TM4. TagG presumably facilitates translocation of WTA polymer across the plasma membrane following a conformational change in the transmembrane domain induced by TagH.

The occurrence of three independent mutations within or flanking genes involved in the WTA biosynthesis pathway leading to enhanced biofilm formation in the presence of bile strongly suggests an involvement of WTA in the S. aureus bile-induced biofilm formation response. WTA is essential for biofilm formation and host-colonization by S. aureus (Gross et al., 2001; Weidenmaier et al., 2004), and deletion of tagO impairs biofilm production in Staphylococcus epidermidis (Holland et al., 2011). Interestingly, none of our isolated mutants carried complete knockouts in genes essential for WTA biosynthesis. Mutants TM4 and TM39 carried intergenic insertions, while in TM26 the insertion was in one of two tarJ genes in S. aureus (Qian et al., 2006). This pattern of insertions is consistent with bias against WTA-associated insertions in the Nebraska Transposon Mutant Library, which consists of 1952 strains, each containing a single mutation within a nonessential gene of S. aureus isolate USA300. This collection also lacks mutations within tagO, tagG and tagH, suggesting these may be essential genes (Fey et al., 2013). Thus the mutations within the hyper-biofilm mutants likely do not knock out WTA synthesis, but may merely reduce it and concomitantly induce sensitivity to bile, leading to an increased level of biofilm production on exposure to lower concentrations of bile.

As bile is known to induce cell membrane damage (Begley et al., 2005), and biofilms protect microorganisms from external damaging-agents (Flemming & Wingender, 2010), our observations lead us to propose that the bile-induced biofilm phenotype is an adaptive response to increased cell wall stress. In line with this, the inhibition of WTA synthesis by deletion of LytR-CpsA-Psr proteins has been shown to increase the basal expression of the ‘cell wall stress stimulon’, a collection of genes responsible for mounting a general cell wall stress-response in the presence of cell wall damaging-agents (Dengler et al., 2012). The recently discovered antibiotic targocil, which blocks expression of tagG, has also been shown to induce cell wall stress in S. aureus (Campbell et al., 2012). It is plausible that in the hyper-biofilm mutants identified in the present study, the transposon insertions reduce WTA biosynthesis, thereby stimulating a more profound cell wall stress-response in the presence of bile.

The transposon screen employed by this study utilized an indirect screening approach to identify genes within regulatory pathways involved in the S. aureus bile-response. This approach was limited in that all mutations identified as being important in biofilm formation were a subset of the mutations that conferred aminoglycoside resistance. As such, mutations that alter the bile-modulated biofilm response, but do not affect bile-induced aminoglycoside

![Graph](image)

**Fig. 4.** Complementation of bile-induced biofilm phenotype. Transposon mutant TM4 and wild-type RN4220 strains were transformed with plasmid pLI50 containing parts of the tagG-tagH genomic region from RN4220 (pLI50::int_tagHG, pLI50 containing the intergenic region between tagH and tagG; pLI50::tagG, pLI50 containing tagG). Strains were cultured for 18 h in TSB (untreated), or TSB supplemented with 0.03% bile, following which biofilm attachment was measured. Graphs show mean (±SD) absorbance from three replicates.
sensitivity, were not detected in this study. As the bile-induced aminoglycoside sensitivity is likely caused by cell membrane perturbation by bile, leading to increased uptake of aminoglycosides, it was not surprising to observe a large number of mutations within genes involved in cell envelope integrity. Despite these limitations, the data presented in this study provide several insights into possible regulatory mechanisms governing the bile-response in *S. aureus*. Further investigation of these mutants will provide insights into bile-responsive pathways linking biofilm formation to host-triggers of chronic infection, thereby facilitating the development of innovative strategies for the prevention and treatment of respiratory disease.

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