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

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Abstract:	<p>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. <i>Staphylococcus aureus</i> 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 <i>S. aureus</i> biofilm formation. Most clinical <i>S. aureus</i> strains and the laboratory strain RN4220 were stimulated to form biofilms with sub-inhibitory concentrations of 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-tagG genes, encoding 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 bile-induced biofilm formation. We propose that WTA may be important for protecting <i>S. aureus</i> against exposure to bile and that bile-induced biofilm formation may be an evolved response to protect cells from bile-induced cell lysis.</p>



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26 **ABSTRACT**

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

46 **INTRODUCTION**

47 Chronic infections by pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*
48 are a leading cause of morbidity and mortality in cystic fibrosis (CF) patients. Chronic bacterial
49 infections can rarely be eradicated by antimicrobial treatment, and thus chronic infection of the
50 lung can eventually lead to a fatal decline in lung function (Furukawa *et al.*, 2006). This is

51 becoming an ever increasing clinical issue where many have predicted the onset of a post-
52 antibiotic era (Cooper & Shlaes, 2011). Therefore, innovative and alternative strategies are
53 urgently needed, away from the classical antibiotic approach. The refractory nature of chronic
54 infections to conventional therapies is largely attributed to bacteria adopting a biofilm-like
55 mode of growth. Microorganisms in biofilms are embedded within a matrix of extracellular
56 polymeric substances which provides a barrier against host immune defenses and antimicrobial
57 therapy (Flemming & Wingender, 2010). The biofilm matrix is composed of polysaccharides,
58 proteins, lipids, and genomic DNA that is released by lysed resident bacterial cells. While the
59 molecular mechanisms underlying the formation of biofilms in a broad spectrum of pathogens
60 has been well studied, until recently the molecular triggers which cause lung-colonizers to
61 adopt a chronic lifestyle and the associated biofilm mode of growth remained largely
62 uncharacterized. Understanding how bacteria adopt this chronic lifestyle in the lung would
63 provide new therapeutic options for prevention and treatment.

64 The aspiration of bile acids into the lungs arising from gastro-esophageal reflux disease
65 (GERD) has since emerged as a host-trigger of chronic bacterial infection (Reen *et al.*, 2012;
66 Reen *et al.*, 2014) and chronic inflammation (Legendre *et al.*, 2014), particularly in CF, where
67 up to 40% of children and 80% of adult patients can suffer from this complication (Legendre
68 *et al.*, 2014; Pauwels *et al.*, 2012; Reen *et al.*, 2012; Reen *et al.*, 2014; Stringer *et al.*, 1988).
69 Indeed, the incidence may be underestimated as clinical diagnosis of GER is often not sufficient
70 to determine bile aspiration. Bile acids have been detected in the sputum and bronchoalveolar
71 lavage fluid of patients that do not present with classical GER symptoms. This ‘silent
72 aspiration’ phenomenon is particularly severe in CF patients and highlights the urgent unmet
73 need for rapid diagnosis of bile acid profiles in biological samples from respiratory and lung
74 transplant patients (Button *et al.*, 2005). Over recent years a number of research publications
75 have suggested that acid-and non-acid reflux may negatively influence the progression of

76 respiratory disease (D'Ovidio *et al.*, 2005a; D'Ovidio *et al.*, 2005b; el-Serag & Sonnenberg,
77 1997; Pauwels *et al.*, 2012; Perng *et al.*, 2007; Wu, 2008; Wu *et al.*, 2009). el-Serag and
78 Sonnenberg showed that patients with erosive esophagitis, a sign of significant GER, had
79 increased incidence of pulmonary fibrosis, chronic bronchitis or chronic obstructive pulmonary
80 disease in a case control study of more than 200,000 patients (el-Serag & Sonnenberg, 1997).
81 A strong correlation between GER-derived reflux, pulmonary aspiration, and increased lung
82 damage also extends to several other respiratory diseases (Navarro *et al.*, 2001), including
83 idiopathic pulmonary disease and advanced lung damage arising from lung transplantation
84 (Sweet *et al.*, 2006; Sweet *et al.*, 2007a; Sweet *et al.*, 2007b), ventilator associated pneumonia
85 (Wu *et al.*, 2009), Barrett's esophagus and esophageal adenocarcinoma (Nassr *et al.*, 2011) and
86 Bile Acid Pneumonia in neonates (Zecca *et al.*, 2004; Zecca *et al.*, 2008). Therefore, the
87 implications of elucidating the link between bile aspiration and chronic pathogen behavior has
88 consequences for a range of clinical conditions.

89 Apart from an association between GERD and increased colonization by *P. aeruginosa*
90 and *S. aureus* (Palm *et al.*, 2012; van der Doef *et al.*, 2009), bile has been shown to influence
91 the behavior of *P. aeruginosa* and other respiratory pathogens *in vitro*, suppressing phenotypes
92 associated with acute infection, while up-regulating phenotypes associated with chronic
93 infection, including biofilm formation (Reen *et al.*, 2012). Microbial diversity has also shown
94 to be significantly reduced in bile-aspirating patients compared with non-aspirating patients,
95 suggesting that aspirated bile may be a major factor in shaping the pervasive lung microbiota
96 signature in CF patients (Blainey *et al.*, 2012; Reen *et al.*, 2014). Furthermore, bile acids have
97 been shown to trigger the IL-6 pro-inflammatory cytokine *in vitro*, suppressing HIF-1 signaling
98 in *P. aeruginosa* infected cells (Legendre *et al.*, 2014), while reflux and aspiration have been
99 shown to correlate with increased levels of cytokines and neutrophils *in vivo* (D'Ovidio *et al.*,
100 2005a). Therefore, the possibility that the aspiration of bile into the lungs of pediatric patients

101 must be considered as a potential underlying factor in the emergence of chronic microbial
102 infections.

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

116 **MATERIALS AND METHODS**

117 **Bacterial strains, plasmids, and growth conditions**

118 The bacterial strains and plasmids used in this study are outlined in Table 1. *S. aureus* strains
119 were cultured at 37°C in Tryptic Soy Broth (TSB; Becton Dickinson) or Tryptic Soy Agar
120 (TSA; TSB containing 1.5% (w/v) agar). *Escherichia coli* EPI300 was used as a cloning host,
121 and was cultured at 37°C in Lysogeny Broth (LB), or LB agar. Where required, media were
122 supplemented with the following concentrations of antibiotics unless otherwise stated: 100
123 µg/mL ampicillin, 10 µg/mL chloramphenicol, 2.5 µg/mL tetracycline, 10 µg/mL
124 erythromycin. Media were supplemented with bovine bile (Sigma-Aldrich), sodium cholate

125 (SC; Sigma-Aldrich), sodium deoxycholate (SDC; Sigma-Aldrich), or sodium dodecyl sulfate
126 (SDS; Amresco) when required. Stock solutions of bile, SC and SDC were prepared in
127 deionized distilled water and filter sterilized prior to addition to media.

128 **DNA manipulations**

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

135 **Biofilm attachment assay**

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

144 **Growth analysis**

145 To determine the effects of bile on the growth of *S. aureus*, stationary-phase cultures of *S.*
146 *aureus* were diluted 1:500 in 25 mL TSB or TSB supplemented with bovine bile. Cultures were
147 grown at 37°C with agitation at 180 rpm and samples were taken over a 24 hour period and the
148 optical density (OD) measured at a wavelength of 600 nm.

149 **Antibiotic sensitivity determinations**

150 For disc diffusion testing, a single colony of *S. aureus* RN4220 was resuspended in 1 mL of
151 phosphate buffered saline, and evenly spread on TSA plates or TSA plates containing 0.3%
152 bile to prepare a lawn culture. Antibiotic discs, containing gentamicin (10 µg),
153 chloramphenicol (30 µg), ampicillin (10 µg), erythromycin (15 µg), or tetracycline (30 µg),
154 were placed on the agar, and the plates were incubated at 37°C for 18 hours, following which
155 the diameter of the inhibition zone was observed.

156 The minimum inhibitory concentrations (MICs) of the aminoglycosides gentamycin,
157 streptomycin, neomycin, and kanamycin were determined in duplicate by broth macrodilution
158 method. Antibiotic solutions (0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µg/mL) were prepared in
159 TSB or TSB containing 1 mM SC. An inoculum of *S. aureus* RN4220 was prepared by saline
160 (0.85% NaCl) suspension of isolated colonies, adjusted to achieve a turbidity equivalent to a
161 0.5 McFarland standard, and diluted 1:300 in TSB containing 1 mM SC and/or antibiotics such
162 that the final inoculum was $\sim 5 \times 10^5$ colony-forming units (CFU)/mL. Cultures were incubated
163 overnight at 37°C with shaking. The lowest concentration of antibiotic which inhibited all
164 visually apparent growth was considered the MIC.

165 **Transposon mutagenesis**

166 Plasmids pBursa and pFA545 were transformed together into *S. aureus* RN4220 by
167 electroporation, and transformants were selected on TSA containing chloramphenicol and
168 tetracycline following incubation at the permissive temperature of 30°C for 48 hours. Resulting
169 colonies were resuspended in sterile water and incubated at the non-permissive temperature of
170 43°C for 1 hour before spreading on selective media (TSA containing 1 erythromycin and SDC,
171 or TSA containing erythromycin, SC, and either gentamycin, neomycin, or kanamycin) to
172 screen for mutants of interest.

173 **Determination of site of transposon insertion**

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

187 **RESULTS**

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

189 Physiological concentrations of bile or bile acids stimulate *in vitro* biofilm formation in several
190 lung-colonizing pathogens (Reen *et al.*, 2012). In contrast, bile suppresses biofilm formation
191 by *S. aureus* strain NCDO949, a common laboratory strain originally isolated from pleural
192 fluid. To establish whether NCDO949 bile-response phenotype was representative of *S. aureus*
193 strains, biofilm formation was analyzed for pediatric isolates obtained from the CF unit at Cork
194 University Hospital, Ireland, using the crystal violet attachment assay. In contrast to
195 NCDO949, biofilm formation was stimulated by bile in these isolates (Fig. 1a). Furthermore,
196 bile-stimulated biofilm formation was observed for community-acquired MRSA strains

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

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

214 **Development of a *bursa aurealis* transposon mutagenesis screen to isolate bile-response** 215 **mutants**

216 Bile acids have been demonstrated to enhance the activity of penicillin and neomycin against
217 staphylococcal strains. Bile has no effect on the efficacy of other antibiotics such as
218 chloramphenicol or erythromycin, but can weaken the activity of some antibiotics including
219 vancomycin (Schneierson & Amsterdam, 1958; Stanley Schneierson *et al.*, 1962). In a study
220 which compared the effects of various bile acids in their salt form, as well as other components
221 of bile such as cholesterol, SC and SDC were found to be the most effective at increasing the

222 anti-staphylococcal activity of neomycin (Stanley Schneierson *et al.*, 1962). We investigated
223 the effect of SC on antibiotic tolerance in *S. aureus* RN4220. Disc diffusion antibiotic
224 sensitivity testing revealed that 0.3% bile increased the sensitivity of *S. aureus* RN4220
225 towards gentamicin, but had no effect on chloramphenicol, ampicillin, erythromycin or
226 tetracycline (Fig. S1). We suspected that bile acids may specifically induce sensitivity to
227 aminoglycosides in *S. aureus* RN4220. After confirming that SC at a concentration of 1 mM
228 did not have bactericidal or bacteriostatic effects on this strain (Fig. S2), we proceeded to test
229 the MIC of gentamycin, streptomycin, neomycin and kanamycin both in the presence of 1mM
230 SC. The MIC of all aminoglycosides was reduced in the presence of 1 mM SC (Table 3),
231 consistent with a previous study in *Lactobacillus* species (Elkins & Mullis, 2004), where the
232 authors implicated increased antibiotic uptake following bile exposure.

233 We hypothesized that the molecular control of bile-induced aminoglycoside sensitivity
234 and bile-induced biofilm formation in *S. aureus* might be linked at a regulatory level. Following
235 this hypothesis, we predicted that mutant RN4220 strains that overcame bile-induced
236 aminoglycoside sensitivity might also exhibit an altered bile-induced biofilm formation
237 phenotype. In order to isolate genetically marked mutant strains that had overcome bile-
238 induced aminoglycoside sensitivity, we utilized random transposon mutagenesis using the
239 mariner-based transposon *bursa aurealis* to generate pools of mutant RN4220 and then
240 selected for mutants that had overcome bile-induced aminoglycoside sensitivity in the presence
241 of 1 mM sodium cholate. A range of aminoglycosides was utilized to avoid bias towards
242 mutations which conferred resistance via an antibiotic-specific mechanism. Aminoglycoside
243 concentrations were adjusted in selection plates to a level at which we could observe over ten
244 colonies per plate.

245 In multiple rounds of mutagenesis with various aminoglycosides, several hundred *S.*
246 *aureus* mutants were isolated. For 44 mutants we identified the site of the transposon using

247 random-primed PCR. Table S1 shows the list of transposon mutants, the antibiotic and
248 concentration used to screen for the mutant, and the site of transposon insertion. Thirty four of
249 the mutants carried the transposon insertion within a defined open reading frame (ORF), while
250 the remaining 10 mutants had the transposon insertion occurring at between two coding
251 sequences. Where possible the putative gene and/or gene product associated with the
252 transposon disruption were identified (Table S1).

253 **Regulatory mutations in wall-teichoic acid synthesis stimulate a hypersensitive bile-** 254 **induced biofilm response**

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

263 Of the five mutants that exhibited biofilm stimulation in the presence of 0.03% bile,
264 three carried the transposon insertion within a defined ORF (TM19, TM26, TM28), while two
265 had the transposon insertion occurring at between two coding sequences (TM4, TM39).
266 Interestingly, in both mutants from the latter category, the transposon insertion was between
267 divergently oriented genes where at least one gene was associated with wall teichoic acid
268 (WTA) biosynthesis. TM4 carried an insertion between divergently oriented WTA
269 biosynthesis genes, *tagG* and *tagH* (Lazarevic & Karamata, 1995; Schirner *et al.*, 2011). In
270 TM39 the insertion was between *tagO*, which is associated with WTA biosynthesis (Soldo *et*
271 *al.*, 2002; Xia *et al.*, 2010), and *gdpS*, the only conserved GGDEF domain protein identified

272 thus far in *Staphylococcus* (Shang *et al.*, 2009). Furthermore, the transposon insertion in TM26
273 was within *tarJ*, another gene involved in the WTA biosynthetic pathway (Brown *et al.*, 2013).
274 It should be noted however, that although the insertion disrupted *tarJ*, this gene is duplicated
275 in many *S. aureus* strains (Qian *et al.*, 2006), and the locus SAOUHSC_00226 in the strain
276 NCTC8325 most likely represents a second copy of *tarJ*. These data indicated that WTA
277 synthesis or its regulation might be involved in the response to bile and control of biofilm
278 production. The remaining two hypersensitive mutants carried insertions within *ctaB* (TM19),
279 which encodes for heme O synthase, and a possible D-galactonate transporter (TM28).

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

297 **DISCUSSION**

298 The pathogenesis of *S. aureus* in the lungs of respiratory patients is characterized by successful
299 colonization and persistence, particularly in pediatric CF patients where it dominates the early
300 developing lungs. The environmental factors that cause *S. aureus* to adopt this pervasive
301 lifestyle are as yet unknown, but our data presents a strong case for a role for bile aspiration
302 derived from GERD in the pathogenesis of this organism. In this study we demonstrate that
303 physiologically relevant concentrations of bile, as well as sub inhibitory concentrations of bile
304 acids, can alter the behavior of *S. aureus*, leading to enhanced biofilm formation by this
305 important clinical pathogen. The biofilm mode of growth is frequently associated with chronic
306 infections as it allows the bacterium to evade host defenses, and persist for extended periods
307 of time (Furukawa *et al.*, 2006). Our observation is consistent with the increased incidence of
308 *S. aureus* in pediatric patients suffering from GERD (Palm *et al.*, 2012; van der Doef *et al.*,
309 2009), the likely source of bile acids in the lungs of these patients (Aseeri *et al.*, 2012; Pauwels
310 *et al.*, 2012; Reen *et al.*, 2014).

311 This bile-mediated response presented an opportunity to probe more deeply the
312 regulatory mechanisms governing the switch between the phenotypes associated with acute
313 and chronic infection in *S. aureus*. Using a random transposon mutagenesis approach we
314 identified five *S. aureus* mutants with an enhanced bile-induced biofilm response. Of these,
315 three mutants had a transposon insertion within or directly upstream of genes involved in WTA
316 biosynthesis. WTA are surface-exposed anionic glycopolymers present in many gram positive
317 species of bacteria, covalently bound to the peptidoglycan layer (Brown *et al.*, 2013). Indeed
318 WTA are the most abundant peptidoglycan bound glycopolymer in gram positive species,
319 making up over half of the dry weight of the cell wall. WTA have been shown to play key roles
320 in maintenance of cell shape, several aspects of cell division, modulation of antibiotic
321 susceptibility, and host tissue colonization (Swoboda *et al.*, 2010; Weidenmaier *et al.*, 2004).

322 Moreover, WTA have been implicated in biofilm formation in staphylococcal species (Holland
323 *et al.*, 2011; Vergara-Irigaray *et al.*, 2008). For example, WTA contain D-alanine modifications
324 which allow *S. aureus* to modulate its surface charge, aiding its primary attachment to artificial
325 surfaces before the formation of multiple cell layers (Gross *et al.*, 2001).

326 *S. aureus* WTA polymers are composed of 30-50 ribitol phosphate (Rbo-P) subunits,
327 connected to peptidoglycan via the murein linkage unit GlcNAc-ManNAc-(glycerol phosphate
328 [Gro-P])₂₋₃ (Yokoyama *et al.*, 1986). WTA biosynthesis is a complex multi-step process which
329 is yet to be fully characterized (Brown *et al.*, 2013). However, it has been shown that *tagO*, the
330 regulatory region of which is likely disrupted in TM39, is involved in initiating the synthesis
331 of the aforementioned murein linkage unit (Soldo *et al.*, 2002; Xia *et al.*, 2010). TarJ (an
332 alcohol dehydrogenase), the function of which is most likely abrogated in TM26, together with
333 TarI and TarL, catalyzes the attachment of Rbo-P to the murein linkage unit. Once WTA
334 polymer formation is complete, it is translocated across the plasma membrane by the two-
335 component ABC (ATP-binding cassette) transporter TagGH (Lazarevic & Karamata, 1995;
336 Schirner *et al.*, 2011), the regulatory region of which was mutated in TM4. TagG presumably
337 facilitates translocation of WTA polymer across the plasma membrane following a
338 conformational change in the transmembrane domain induced by TagH.

339 The occurrence of three independent mutations within or flanking genes involved in the
340 WTA biosynthesis pathway leading to enhanced biofilm formation in the presence of bile,
341 strongly suggests an involvement of WTA in the *S. aureus* bile-induced biofilm formation
342 response. WTA are essential for biofilm formation and host-colonization by *S. aureus* (Gross
343 *et al.*, 2001; Weidenmaier *et al.*, 2004), and deletion of *tagO* impairs biofilm production in
344 *Staphylococcus epidermidis* (Holland *et al.*, 2011). Interestingly, none of our isolated mutants
345 carried complete knockouts in genes essential for WTA biosynthesis. Mutants TM4 and TM39
346 carried intergenic insertions, while in TM26 the insertion was in one of two *tarJ* genes in *S.*

347 *aureus* (Qian *et al.*, 2006). This pattern of insertions is consistent with bias against WTA-
348 associated insertions in the Nebraska Transposon Mutant Library, which consists of 1,952
349 strains, each containing a single mutation within a nonessential gene of *S. aureus* isolate
350 USA300. This collection also lacks mutations within *tagO*, *tagG*, and *tagH*, suggesting these
351 may be essential genes (Fey *et al.*, 2013). Thus the mutations within the hyper-biofilm mutants
352 likely do not knock out WTA synthesis, but may merely reduce it and concomitantly induce
353 sensitivity to bile, leading to an increased level of biofilm production on exposure to lower
354 concentrations of bile.

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

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

372 sensitivity is likely caused by cell membrane perturbation by bile, leading to increased uptake
373 of aminoglycosides, it was not surprising to observe a large number of mutations within genes
374 involved in cell envelope integrity. Despite these limitations, the data presented in this study
375 provides several insights into the possible regulatory mechanisms governing the bile-response
376 in *S. aureus*. Further investigation of these mutants will provide insights into bile-responsive
377 pathways linking biofilm formation to host-triggers of chronic infection, thereby facilitating
378 the development of innovative strategies for the prevention and treatment of respiratory
379 disease.

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382

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394

395 **ABBREVIATIONS**

396 CF, cystic fibrosis; GERD, gastro-esophageal reflux; disease; LB, lysogeny broth; MIC,
397 minimum inhibitory concentration; ORF, open reading frame; PIA, polysaccharide
398 intercellular adhesion; SC, sodium cholate; SDC, sodium deoxycholate; TSA, tryptic soy agar;
399 TSB, tryptic soy broth.

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621

622 **FIGURES**

623 **Fig. 1 Bile-dependent biofilm formation in *Staphylococcus aureus*.** (a) Pediatric clinical isolates
624 compared to NCDO949, and (b) Community acquired methicillin-resistant isolates compared to
625 RN4220. Data is presented as a ratio of the Abs595 nm from treated vs untreated samples. Graphs show
626 mean (+/- SEM) relative absorbance of at least three independent experiments, each carried out in
627 triplicate. Statistical analysis was performed by two-tailed paired Student's t-test (* P < 0.05, ** P <
628 0.01, compared with untreated control). (c) Growth of *S. aureus* RN4220 in presence of bovine bile.
629 Graphs show mean (+/- SD) OD600 nm of two biological replicates.

630 **Fig. 2 Biofilm formation in *Staphylococcus aureus* RN4220 in response to bile and bile salts.** *S.*
631 *aureus* strain RN4220 cultured for 18 h in TSB (untreated), or TSB supplemented with (a) sodium
632 cholate (SC), (b) sodium deoxycholate (SDC), and (c) sodium dodecyl sulphate (SDS). Graphs show
633 mean (+/- SD) relative absorbance of three biological replicates. Statistical analysis was performed a
634 two-tailed unpaired Student's t-test (* P < 0.05, ** P < 0.01 compared with untreated).

635 **Fig. 3 Altered bile-dependent biofilm formation in *Staphylococcus aureus* transposon mutants.**
636 Graphs show mean (+/- SD) absorbance from three independent experiments each performed in
637 triplicate. Statistical analysis was performed a two-tailed paired Student's t-test (** P < 0.01).

638 **Fig. 4 Complementation of bile induced biofilm phenotype.** Transposon mutant TM4 and wild type
639 RN4220 strains were transformed with plasmid pLI50 containing parts of the *tagG*-*tagH* genomic
640 region from RN4220 (pLI50::int_tagHG, pLI50 containing intergenic region between *tagH* and *tagG*;
641 pLI50::tagG, pLI50 containing *tagG*). Strains were cultured for 18h in TSB (untreated), or TSB
642 supplemented 0.03% bile, following which biofilm attachment was measured. Graphs show mean (+/-
643 SD) absorbance from 3 replicates.

644

645 TABLES

646 Table 1 Bacterial strains and plasmids

Strain/ Plasmid	Description	Source/ Reference
<i>S. aureus</i>		
NCDO949	Type strain, pleural fluid isolate	Shinfield, UK
CUHT	Pediatric Clinical Isolate, CUH clinic	BRC
CUHE	Pediatric Clinical Isolate, CUH clinic	BRC
JKD6159	Dominant CA-MRSA strain in Australia, ST93-IV	(Chua <i>et al.</i> , 2010)
MW2	First community acquired CA-MRSA, ST1-MRSA-IVa (2B) (pMW2)	(Baba <i>et al.</i> , 2002)
RN4220	Highly transformable restriction-minus derivative of NCTC8325-4	(Kreiwirth <i>et al.</i> , 1983)
<i>E. coli</i>		
EPI300	Restriction-minus [<i>mcrA</i> , Δ (<i>mcrCB-hsdSMR-mrr</i>)], recombination-minus (<i>recA1</i>), endonuclease-minus (<i>endA1</i>)	Epicentre
Plasmids		
pBursa	Encodes <i>bursa aurealis</i> transposable element, a temperature sensitive origin of replication, and chloramphenicol resistance selection marker	(Bae <i>et al.</i> , 2004)
pFA545	Encodes a transposase, origin of replication, and a tetracycline resistance selection marker	(Bae <i>et al.</i> , 2004)
pLI50	<i>S. aureus</i> (chloramphenicol resistance selection marker) / <i>E. coli</i> (ampicillin resistance selection marker) shuttle vector	(Lee <i>et al.</i> , 1991)
pLI50::int_tagHG	pLI50 containing the <i>tagG-tagH</i> intergenic amplified from RN4220 genomic DNA using primers TagHGIntFor and TagHGIntRev and cloned as EcoRI-BamHI fragment	This study
pLI50::tagG	pLI50 containing <i>tagG</i> amplified from RN4220 genomic DNA using primers TagGForRBS and TagHGRev and cloned as EcoRI-BamHI fragment	This study

647 CA-MRSA, Community-acquired Methicillin-resistant *Staphylococcus aureus*; ST, sequence type.

648

649 **Table 2** **Oligonucleotide sequences**

Primer name	Sequence (5' - 3')
GFP1	TCCACTGACAGAAAATTTGTGCCCATTAAC
GFP2	CATTAACATCACCATCTAATTCAACAAGAA
GFP3	ACAAGAATTGGGACAACCTCCAGTGA
PF106	GACCACACGTCGACTAGTGCNNNNNNNNNNAGAG
PF107	GACCACACGTCGACTAGTGCNNNNNNNNNNACGCC
PF108	GACCACACGTCGACTAGTGCNNNNNNNNNNGATAC
PF109	GACCACACGTCGACTAGTGC
TagHGIntFor	TAGTAAAGCTTGAATTCTTGTAGACCTTCCTTATTCACATT
TagHGIntRev	TAGTAGGATCCTCCATTAACCACACTTTCAAATGT
TagGForRBS	TAGTAGAATTCTTAGGAGGATGATTATTTATGTCAGCAATAGGAACAG TTTTT
TagHGRev	TAGTAGGATCCTTACAAGAAGTCTGCAAATTGATCTC

650

651 **Table 3** **Effect of SC on the minimum inhibitory concentration of aminoglycosides in *S.***
 652 ***aureus*.**

Antibiotic	MIC	
	0 mM SC	1 mM SC
Gentamycin	4 µg/mL	0.5 µg/mL
Streptomycin	16 µg/mL	8 µg/mL
Neomycin	4 µg/mL	1 µg/mL
Kanamycin	8 µg/mL	2 µg/mL

653 The *S. aureus* RN4220 minimum inhibitory concentration of various aminoglycosides in the presence
 654 and absence of 1 mM SC is shown.

655

656 **SUPPLEMENTARY FIGURES**

657 **Fig. S1** **Effect of 0.3% bile on antibiotic sensitivity in *Staphylococcus aureus* measured by**
658 **disc diffusion testing.**

659 **Fig. S2** **Effect of sodium cholate on the growth of *Staphylococcus aureus* RN4220.** Growth
660 of *S. aureus* RN4220 in the presence of increasing concentrations of sodium cholate revealed that a
661 concentration of 1 mM did not have any bacteriostatic or bactericidal effects on this strain. To determine
662 the bacteriostatic effects of sodium cholate, a single colony of *S. aureus* was resuspended in 1 mL of
663 TSB or TSB supplemented sodium cholate, and 200 μ L aliquots transferred to a 96-well plate. The
664 density of the *S. aureus* cells were quantified by measuring the absorbance at 600 nm following
665 incubation at 37°C for 24 hours with agitation at 250 rpm. Two μ L of the culture was subsequently
666 dotted on to TSA plates and incubated at 37°C for 24 hours to determine the bactericidal effects of
667 sodium cholate. Graph shows mean (+/- SD) absorbance of three replicates. Photographs show the
668 bacterial growth resulting from the 2 μ L culture on TSA.

669 **Fig. S3** **Mutational analysis of the bile-induced biofilm response in *S. aureus*.** *S. aureus*
670 transposon mutants were cultured in TSB (untreated) or TSB supplemented with 0.3% bile. For each
671 mutant biofilm formation is presented as Abs_{595nm} in both treated and untreated samples. Graph shows
672 the mean (+/- SD) absorbance of four biological replicates.

673

674 SUPPLEMENTARY TABLES

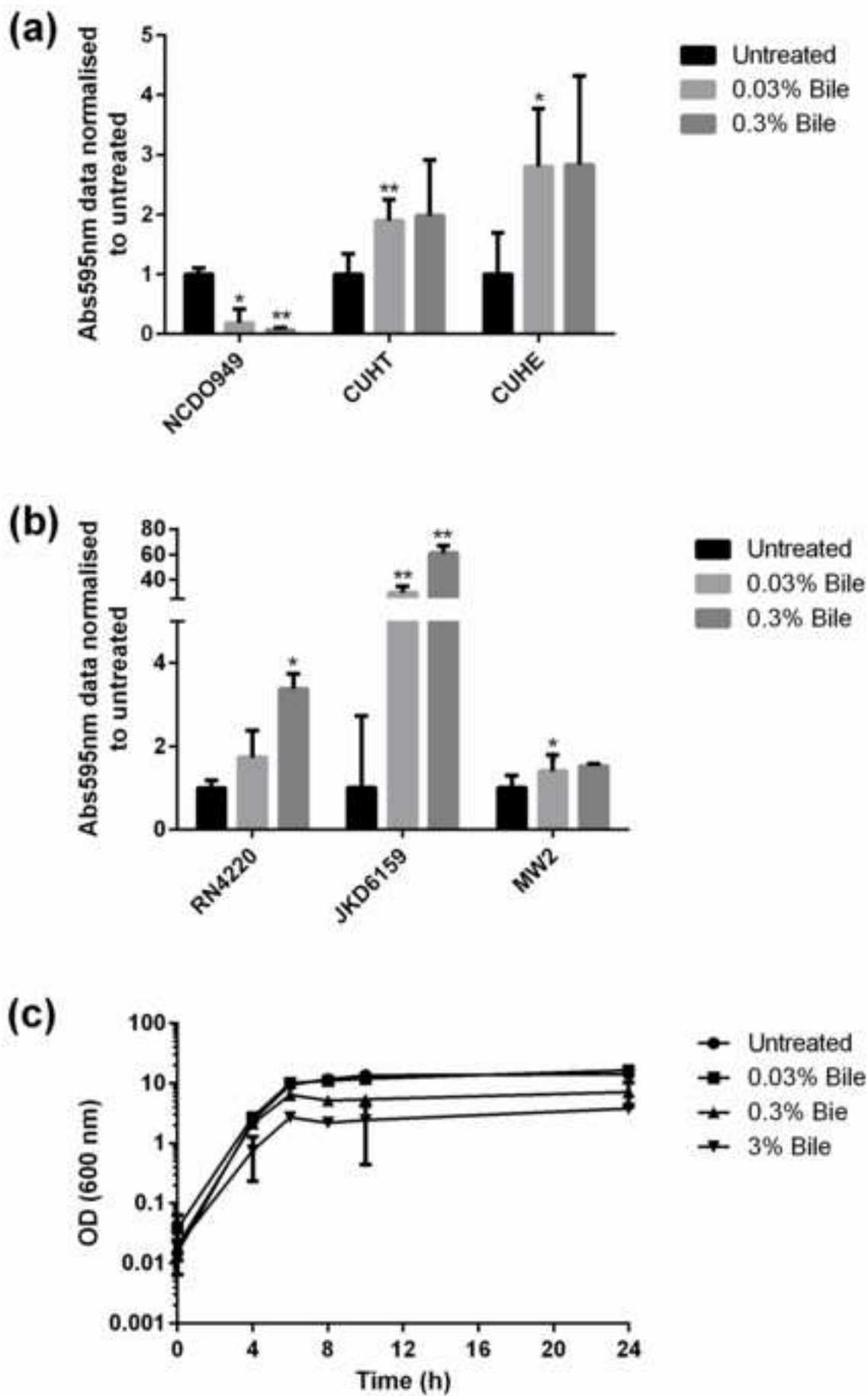
675 Table S1 Genes disrupted in *Staphylococcus aureus* by transposon mutagenesis.

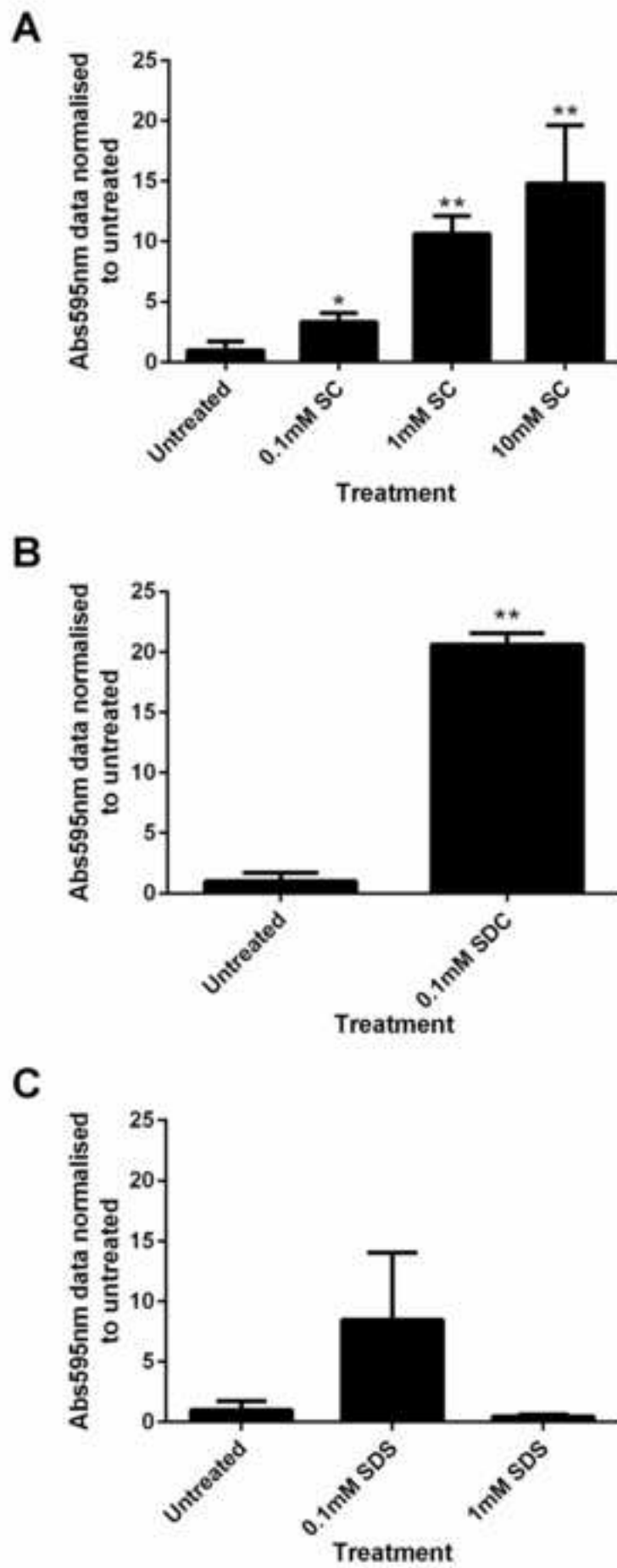
Mutant	Insertion Site ^a	ORF ^b	Putative Gene/Gene Product
Gentamicin (0.5 µg/mL) and SC (1 mM)			
TM1	1775393	SAOUHSC_01868	Dipeptidase PepV
TM2	1676550	SAOUHSC_01776	<i>hemA</i> ; Glutamyl-tRNA reductase
TM3	1444671	SAOUHSC_01488	Heptaprenyl pyrophosphate synthase subunit A
Gentamicin (0.35 µg/mL) and SC (1 mM)			
TM4	630715	SAOUHSC_00641- SAOUHSC_00642	<i>tagH</i> <i>tagG</i>
Gentamicin (0.3 µg/mL) and SC (1 mM)			
TM5	252438	SAOUHSC_00230	<i>lytS</i> ; two-component sensor His-kinase
TM6	205584	SAOUHSC_00186- SAOUHSC_00187	ABC transporter substrate-binding Pyruvate formate lyase
TM7	814365	SAOUHSC_00843	ABC-transporter interface protein
TM8	302717	SAOUHSC_00290	PTS system transporter
TM9	1602302	SAOUHSC_01691	Competence protein ComEC/Rec2
TM10	2605235	SAOUHSC_02826	MarR HTH regulator
TM11	2385772	SAOUHSC_02595	Sodium bile acid transporter
TM12	2083628	SAOUHSC_02247- SAOUHSC_02248	<i>ktrB</i> ; Potassium transporter GNAT family acetyltransferase
TM13	135192	SAOUHSC_00129	UDP-N-acetylglucosamine 2-epimerase
TM14	1691106	SAOUHSC_01793- SAOUHSC_01794	<i>nrdR</i> Glyceraldehyde 3-phosphate dehydrogenase
TM15	43138	SAOUHSC_00039	Dihydrouridine synthase
TM16	1233041	SAOUHSC_01275	<i>glpF</i> ; Glycerol uptake facilitator
TM17	433362	SAOUHSC_00435	Glutamate synthase subunit
TM18	2805147	SAOUHSC_03033	<i>nixA</i> ; Nickel transporter
TM19	1033768	SAOUHSC_01066	<i>ctaB</i> ; heme O synthase
TM20	2084622	SAOUHSC_02249	Membrane protein
TM21	974315	SAOUHSC_01001	<i>qoxB</i> ; Quinol oxidase subunit I

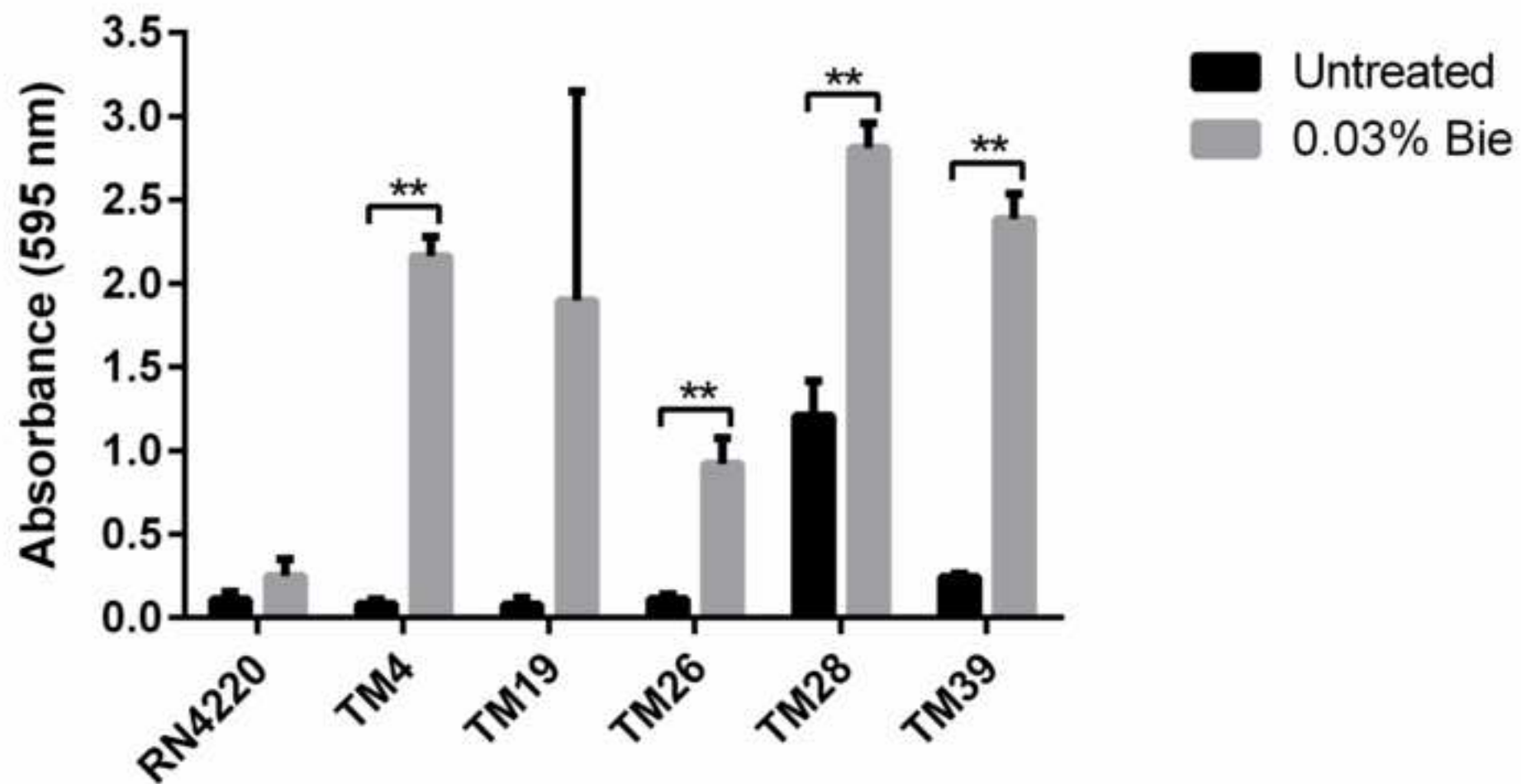
TM22	2807414	SAOUHSC_03035	Membrane protein
Streptomycin (4 µg/mL) and SC (1 mM)			
TM23	2536967	SAOUHSC_02760	Glutamate synthase
TM24	2007589	SAOUHSC_02133-	<i>nadC</i> ; Nicotinate phosphoribosyltransferase
		SAOUHSC_02134	Nitric oxide synthase oxygenase
TM25	2604892	SAOUHSC_02825	Glyoxalase
SDC (0.2 mM)			
TM26	241577	SAOUHSC_00221	<i>tarJ</i> ; Ribitol-5-phosphate dehydrogenase
TM27	2296822	SAOUHSC_02474	hypothetical protein
TM28	2554107	SAOUHSC_02777	Probable D-galactonate transporter
TM29	2004986	SAOUHSC_02131	hypothetical protein
		SAOUHSC_02132	NAD synthetase
TM30	860206	SAOUHSC_00895	Glutamate dehydrogenase
TM31	495319	SAOUHSC_R0007	Cell wall-associated hydrolase
Neomycin (0.5 µg/mL) and SC (1 mM)			
TM36	136894	SAOUHSC_00131-	Membrane spanning protein
		SAOUHSC_00132	Aldehyde dehydrogenase
Kanamycin (1.5 µg/mL) and SC (1 mM)			
TM39	744341	SAOUHSC_00760-	<i>gdpS</i>
		SAOUHSC_00762	<i>tagO</i>
TM40	44147	SAOUHSC_00040	Membrane protein
TM41	1325036	SAOUHSC_01380-	<i>nikB</i> ; Nickel ABC transporter permease
		SAOUHSC_01381	Membrane protein
TM42	2387891	SAOUHSC_02597	<i>ptsG</i> ; Phosphotransferase system
TM43	2075304	SAOUHSC_02239-	Phage integrase
		SAOUHSC_02241	Leukocidin/Hemolysin toxin family
TM44	2735026	SAOUHSC_02972	<i>isaB</i> ; Immunodominant surface antigen B
TM45	2598814	SAOUHSC_02820	ABC-2 transporter family protein
TM46	71816	SAOUHSC_00067	<i>lldP</i> ; L-lactate permease
TM47	876020	SAOUHSC_00906	Fumarylacetoacetate (FAA) hydrolase family
TM48	226300	SAOUHSC_00202-	Uroporphyrinogen-III C-methyltransferase
		SAOUHSC_00203	Membrane protein
TM49	1787295	SAOUHSC_01873	Hypothetical Protein

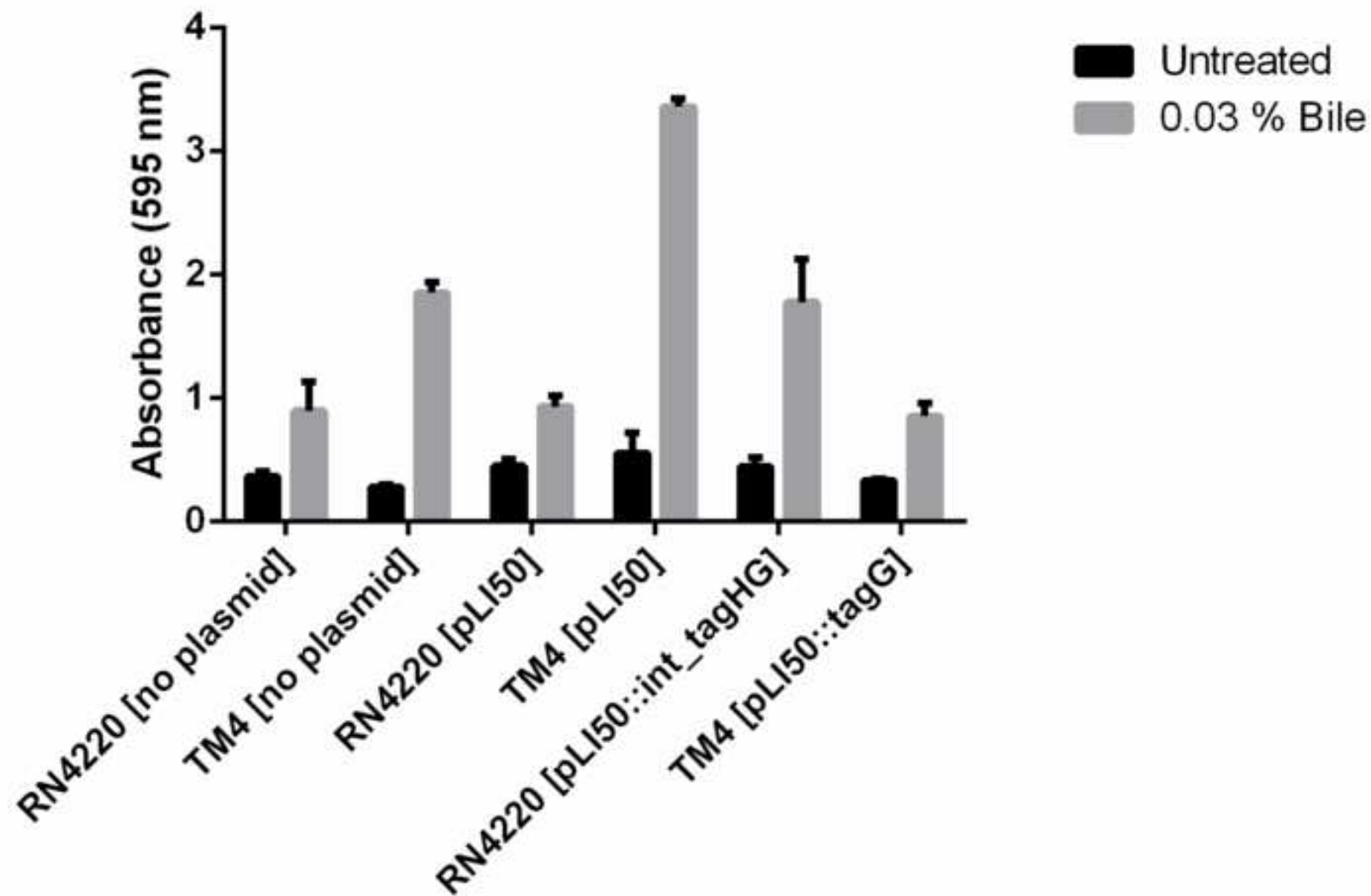
TM50	549173	SAOUHSC_00544	<i>sdrC</i> ; Serine-aspartate repeat protein
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676 Transposon mutants are grouped according to antibiotic and/or bile acid concentrations used to screen
677 for the mutant. ^a Genomic position in the NCTC8325 genome is presented. ^b Corresponding ORF in the
678 NCTC8325 genome is presented.









Gentamycin
10 μ g

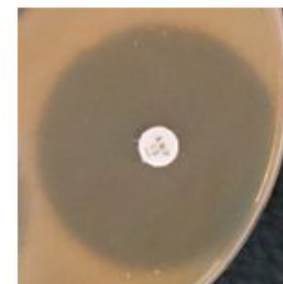
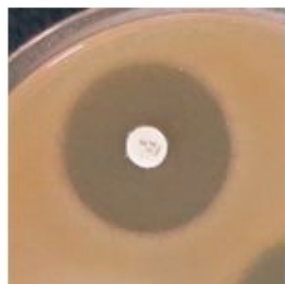
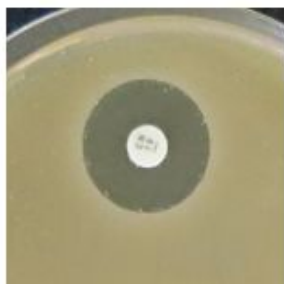
Erythromycin
15 μ g

Chloramphenicol
30 μ g

Tetracycline
30 μ g

Ampicillin
10 μ g

0% Bile



0.3% Bile

