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

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Abstract:	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 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 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.

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ABSTRACT

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 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 *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 where many have predicted the onset of a post-antibiotic era (Cooper & Shlaes, 2011). Therefore, innovative and alternative strategies are urgently needed, away from the classical antibiotic approach. The refractory nature 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 which provides a barrier against host immune defenses and antimicrobial therapy (Flemming & Wingender, 2010). The biofilm matrix is composed of polysaccharides, proteins, lipids, and genomic DNA that is released by lysed resident bacterial cells. While the molecular mechanisms underlying the formation of biofilms in a broad spectrum of pathogens has been well studied, until recently the molecular triggers which 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-esophageal reflux disease (GERD) has since emerged as a host-trigger of chronic bacterial infection (Reen *et al.*, 2012; Reen *et al.*, 2014) and chronic inflammation (Legendre *et al.*, 2014), particularly in CF, where up to 40% of children and 80% of adult patients can suffer from this complication (Legendre *et al.*, 2014; Pauwels *et al.*, 2012; Reen *et al.*, 2012; Reen *et al.*, 2014; Stringer *et al.*, 1988). Indeed, the incidence may be underestimated as clinical diagnosis of GER is often not sufficient to determine bile aspiration. Bile acids have been detected in the sputum and bronchoalveolar lavage fluid of patients that do not present with classical GER 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). Over 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; D'Ovidio *et al.*, 2005b; el-Serag & Sonnenberg, 1997; Pauwels *et al.*, 2012; Perng *et al.*, 2007; Wu, 2008; Wu *et al.*, 2009). el-Serag and Sonnenberg showed that patients with erosive esophagitis, a sign of significant GER, 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 GER-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; Sweet *et al.*, 2007a; Sweet *et al.*, 2007b), ventilator associated pneumonia (Wu *et al.*, 2009), Barrett's esophagus and esophageal adenocarcinoma (Nassr *et al.*, 2011) and Bile Acid Pneumonia in neonates (Zecca *et al.*, 2004; Zecca *et al.*, 2008). Therefore, the implications of elucidating the link between bile aspiration and chronic pathogen behavior has consequences for a range of clinical conditions.

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

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

In this study we examined the effect of bile on virulence related behavior 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 pediatric 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.

MATERIALS AND 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). *Escherichia coli* EPI300 was used as a cloning host, and was cultured at 37°C in Lysogeny Broth (LB), or LB agar. Where required, media were supplemented with the following concentrations of antibiotics unless otherwise stated: 100 µg/mL ampicillin, 10 µg/mL chloramphenicol, 2.5 µg/mL tetracycline, 10 µg/mL erythromycin. Media were supplemented with bovine bile (Sigma-Aldrich), sodium cholate

(SC; Sigma-Aldrich), sodium deoxycholate (SDC; Sigma-Aldrich), or sodium dodecyl sulfate (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 Corp) following cell lysis with lysostaphin (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 Corp).

Biofilm attachment assay

Stationary-phase cultures of *S. aureus* cultures 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 acetone:ethanol (3:7) before quantification by measuring the 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 rpm and samples were taken over a 24 hour period and the optical density (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 of phosphate buffered saline, 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 hours, following which the diameter of the inhibition zone was observed.

The minimum inhibitory concentrations (MICs) of the aminoglycosides gentamycin, streptomycin, neomycin, and kanamycin were determined in duplicate by broth macrodilution method. Antibiotic solutions (0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 µg/mL) were prepared in TSB or 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 x 10^5 colony-forming units (CFU)/mL. Cultures were incubated overnight at 37°C with shaking. The lowest concentration of antibiotic which 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 hours. Resulting colonies were resuspended in sterile water and incubated at the non-permissive temperature of 43°C for 1 hour before spreading on selective media (TSA containing 1 erythromycin and SDC, or TSA containing erythromycin, SC, and either gentamycin, neomycin, or kanamycin) to screen for mutants of interest.

Determination of site of transposon insertion

Transposon insertion sites were determined by random-primed 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 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 NCDO949, a common laboratory strain originally isolated from pleural fluid. To establish whether NCDO949 bile-response phenotype was representative of *S. aureus* strains, biofilm formation was analyzed for pediatric isolates obtained from the CF unit at Cork University Hospital, Ireland, using the crystal violet attachment assay. In contrast to NCDO949, biofilm formation was stimulated by bile in these isolates (Fig. 1a). Furthermore, bile-stimulated biofilm formation was observed for community-acquired MRSA 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 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.

Since the well-characterized and genetically tractable *S. aureus* strain RN4220 exhibited a similar response to bile as most clinical *S. aureus* isolates, we further investigated the effects of the addition of bile on 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).

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; Stanley 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 (Stanley 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). We suspected that bile acids may specifically 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 gentamycin, streptomycin, neomycin and kanamycin both in the presence of 1mM 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 RN4220 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 RN4220 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 which 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.

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

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

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 heme 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 containing 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::int_tagHG) into in RN4220, produced a strain with an almost identical phenotype 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* independently 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 pediatric 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 presents 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 behavior 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 defenses, and persist for extended periods of time (Furukawa *et al.*, 2006). Our observation is consistent with the increased incidence of *S. aureus* in pediatric 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 (Aseeri *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. WTA 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 are the most abundant peptidoglycan bound glycopolymer in gram positive species, making up over half of the dry weight of the cell wall. WTA 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 have been implicated in biofilm formation in staphylococcal species (Holland *et al.*, 2011; Vergara-Irigaray *et al.*, 2008). For example, WTA contain D-alanine modifications which 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])$_{2\text{-}3}$ (Yokoyama *et al.*, 1986). WTA biosynthesis is a complex multi-step process which 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). TarJ (an alcohol dehydrogenase), the function of which is most likely abrogated in TM26, together with TarI and TarL, catalyzes 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 are 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 1,952 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 its 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 which conferred aminoglycoside resistance. As such, mutations which alter the bile-modulated biofilm response, but do not affect bile-induced aminoglycoside sensitivity, were not able to be 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 provides several insights into the 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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ABBREVIATIONS

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

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FIGURES

Fig. 1 Bile-dependent biofilm formation in *Staphylococcus aureus*. (a) Pediatric clinical isolates compared to NCDO949, and **(b)** Community acquired methicillin-resistant isolates compared to RN4220. Data is presented as a ratio of the Abs595 nm 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 presence of bovine bile. Graphs show mean (+/- SD) OD600 nm of two biological replicates.

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

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

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 intergenic region between *tagH* and *tagG*; pLI50::tagG, pLI50 containing *tagG*). Strains were cultured for 18h in TSB (untreated), or TSB supplemented 0.03% bile, following which biofilm attachment was measured. Graphs show mean (+/- SD) absorbance from 3 replicates.

TABLES

Table 1 Bacterial strains and plasmids

Strain/ Plasmid		Description	Source/ Reference
S. aureus			
	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 *et al.*, 2010)
	MW2	First community acquired CA-MRSA, ST1-MRSA-IVa (2B) (pMW2)	(Baba *et al.*, 2002)
	RN4220	Highly transformable restriction-minus derivate of NCTC8325-4	(Kreiswirth *et al.*, 1983)
E. coli			
	EPI300	Restriction-minus [*mcrA*, *Δ(mcrCB-hsdSMR-mrr)*], recombination-minus (recA1), endonuclease-minus (*endA1*)	Epicentre
Plasmids			
	pBursa	Encodes *bursa aurealis* transposable element, a temperature sensitive origin of replication, and chloramphenicol resistance selection marker	(Bae *et al.*, 2004)
	pFA545	Encodes a transposase, origin of replication, and a tetracycline resistance selection marker	(Bae *et al.*, 2004)
	pLI50	*S. aureus* (chloramphenicol resistance selection marker) / *E. coli* (ampicillin resistance selection marker) shuttle vector	(Lee *et al.*, 1991)
	pLI50::int_tagHG	pLI50 containing the *tagG-tagH* intergenic amplified from RN4220 genomic DNA using primers TagHGIntFor and TagHGIntRev and cloned as EcoRI-BamHI fragment	This study
	pLI50::tagG	pLI50 containing *tagG* amplified from RN4220 genomic DNA using primers TagGForRBS and TagHGRev and cloned as EcoRI-BamHI fragment	This study

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

Table 2 Oligonucleotide sequences

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

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

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

SUPPLEMENTARY FIGURES

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

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

Fig. S3 Mutational analysis of the bile-induced biofilm response in *S. aureus*. *S. aureus* transposon mutants were cultured in TSB (untreated) or TSB supplemented with 0.3% bile. For each mutant biofilm formation is presented as Abs595nm in both treated and untreated samples. Graph shows the mean (+/- SD) absorbance of four biological replicates.

SUPPLEMENTARY TABLES

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	*hemA*; 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-	*tagH*
		SAOUHSC_00642	*tagG*
Gentamicin (0.3 μg/mL) and SC (1 mM)			
TM5	252438	SAOUHSC_00230	*lytS*; two-component sensor His-kinase
TM6	205584	SAOUHSC_00186-	ABC transporter substrate-binding
		SAOUHSC_00187	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-	*ktrB*; Potassium transporter
		SAOUHSC_02248	GNAT family acetyltransferase
TM13	135192	SAOUHSC_00129	UDP-N-acetylglucosamine 2-epimerase
TM14	1691106	SAOUHSC_01793-	*nrdR*
		SAOUHSC_01794	Glyceraldehyde 3-phosphate dehydrogenase
TM15	43138	SAOUHSC_00039	Dihydrouridine synthase
TM16	1233041	SAOUHSC_01275	*glpF*; Glycerol uptake facilitator
TM17	433362	SAOUHSC_00435	Glutamate synthase subunit
TM18	2805147	SAOUHSC_03033	*nixA*; Nickel transporter
TM19	1033768	SAOUHSC_01066	*ctaB*; heme O synthase
TM20	2084622	SAOUHSC_02249	Membrane protein
TM21	974315	SAOUHSC_01001	*qoxB*; 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-	*nadC*; Nicotinate phosphoribosyltransferase
		SAOUHSC_02134	Nitric oxide synthase oxygenase
TM25	2604892	SAOUHSC_02825	Glyoxalase
SDC (0.2 mM)			
TM26	241577	SAOUHSC_00221	*tarJ*; 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	Aldyhyde dehydrogenate
Kanamycin (1.5 μg/mL) and SC (1 mM)			
TM39	744341	SAOUHSC_00760-	*gdpS*
		SAOUHSC_00762	*tagO*
TM40	44147	SAOUHSC_00040	Membrane protein
TM41	1325036	SAOUHSC_01380-	*nikB*; Nickel ABC transporter permease
		SAOUHSC_01381	Membrane protein
TM42	2387891	SAOUHSC_02597	*ptsG*; Phosphotransferase system
TM43	2075304	SAOUHSC_02239-	Phage integrase
		SAOUHSC_02241	Leukocidin/Hemolysin toxin family
TM44	2735026	SAOUHSC_02972	*isaB*; Immunodominant surface antigen B
TM45	2598814	SAOUHSC_02820	ABC-2 transporter family protein
TM46	71816	SAOUHSC_00067	*lldP*; 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	*sdrC*; Serine-aspartate repeat protein

Transposon mutants are grouped according to antibiotic and/or bile acid concentrations used to screen for the mutant. [a] Genomic position in the NCTC8325 genome is presented. [b] Corresponding ORF in the NCTC8325 genome is presented.

Figure 1

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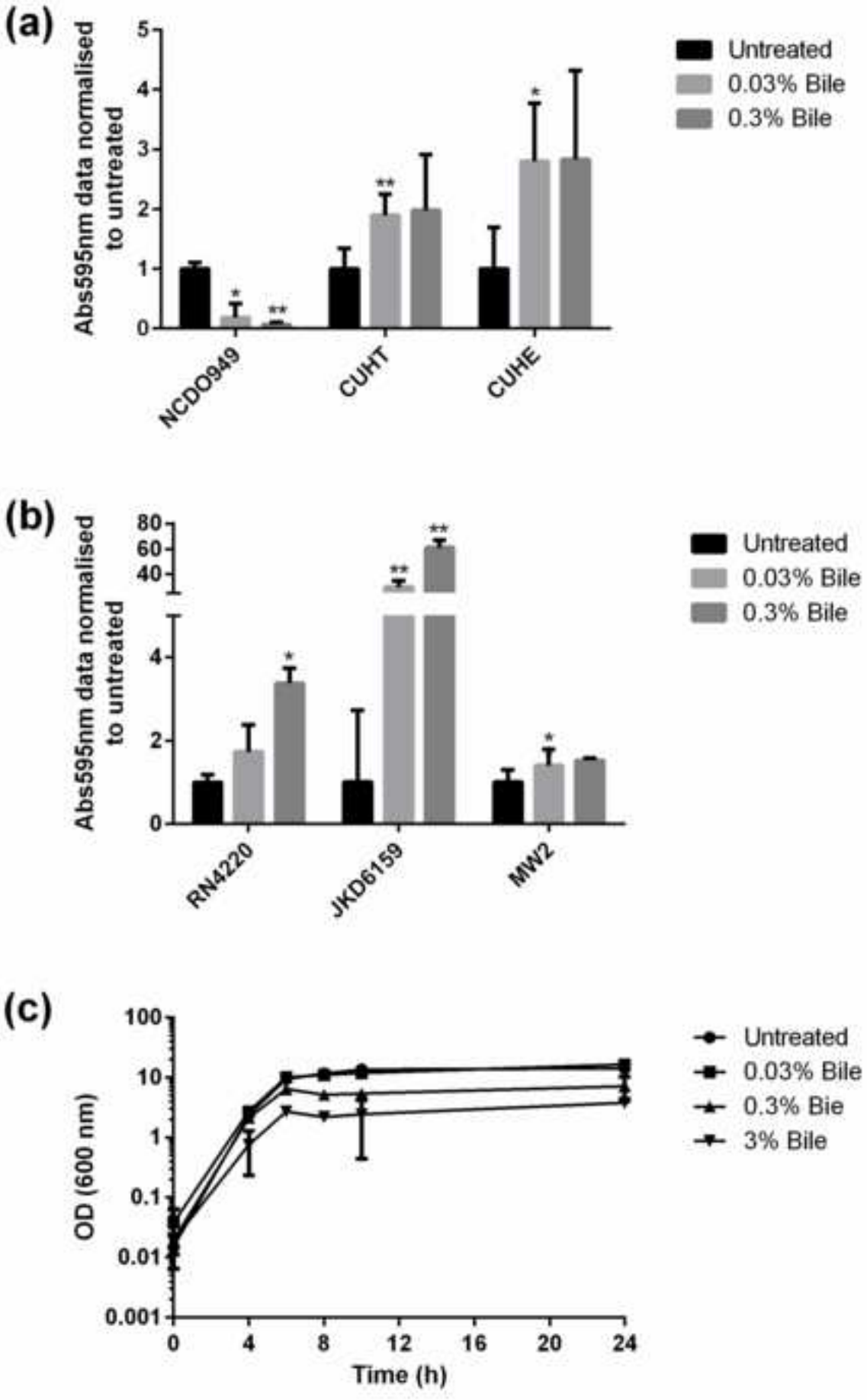

Figure 2

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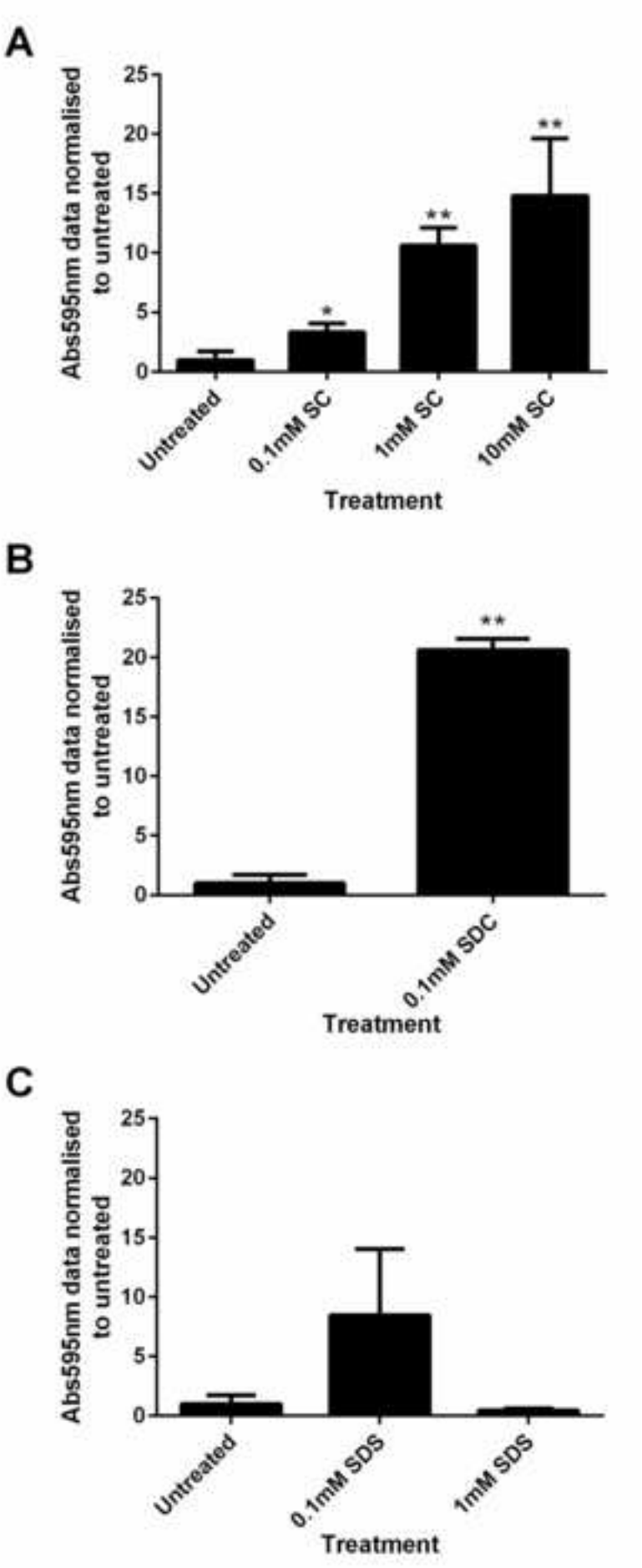

Figure 3

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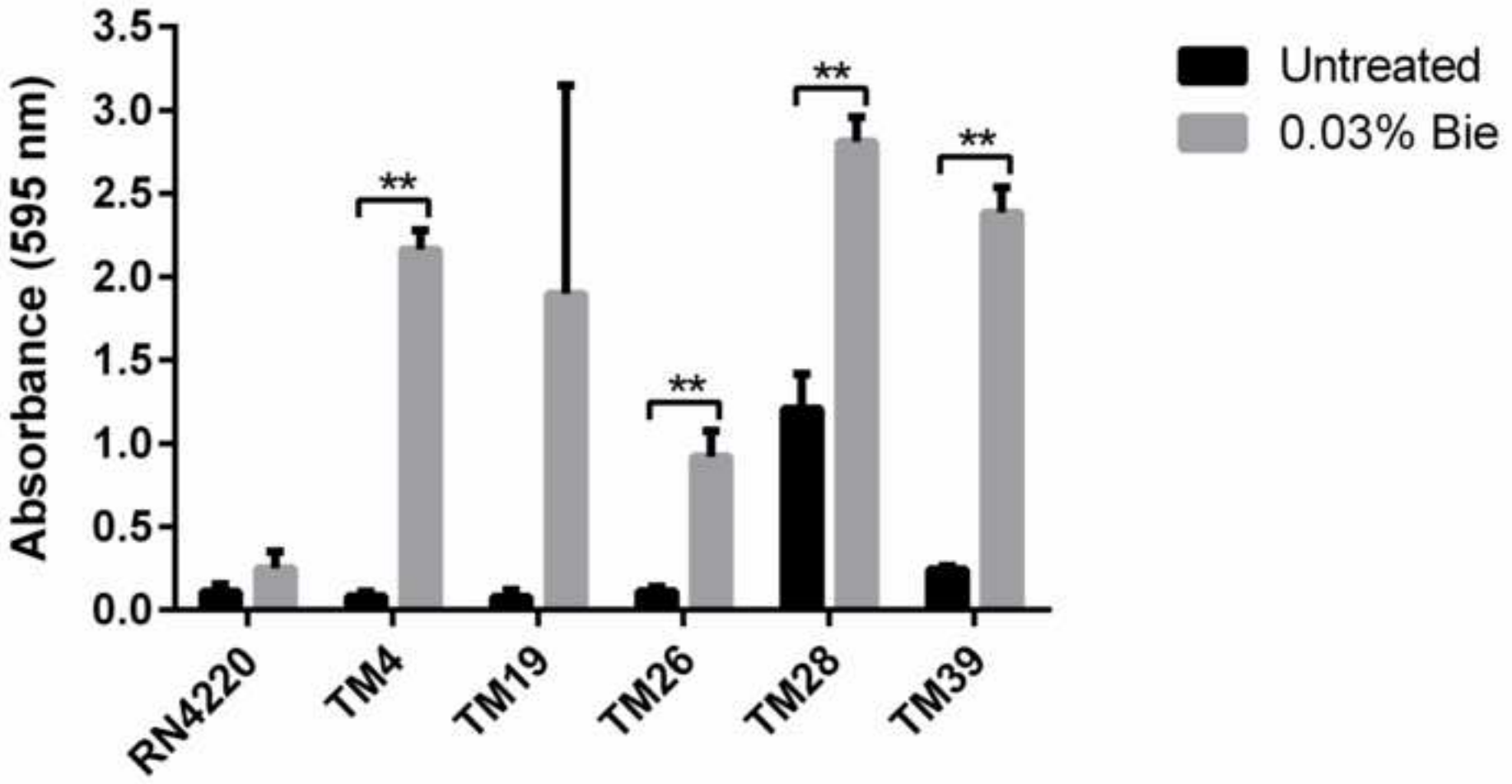

Figure 4

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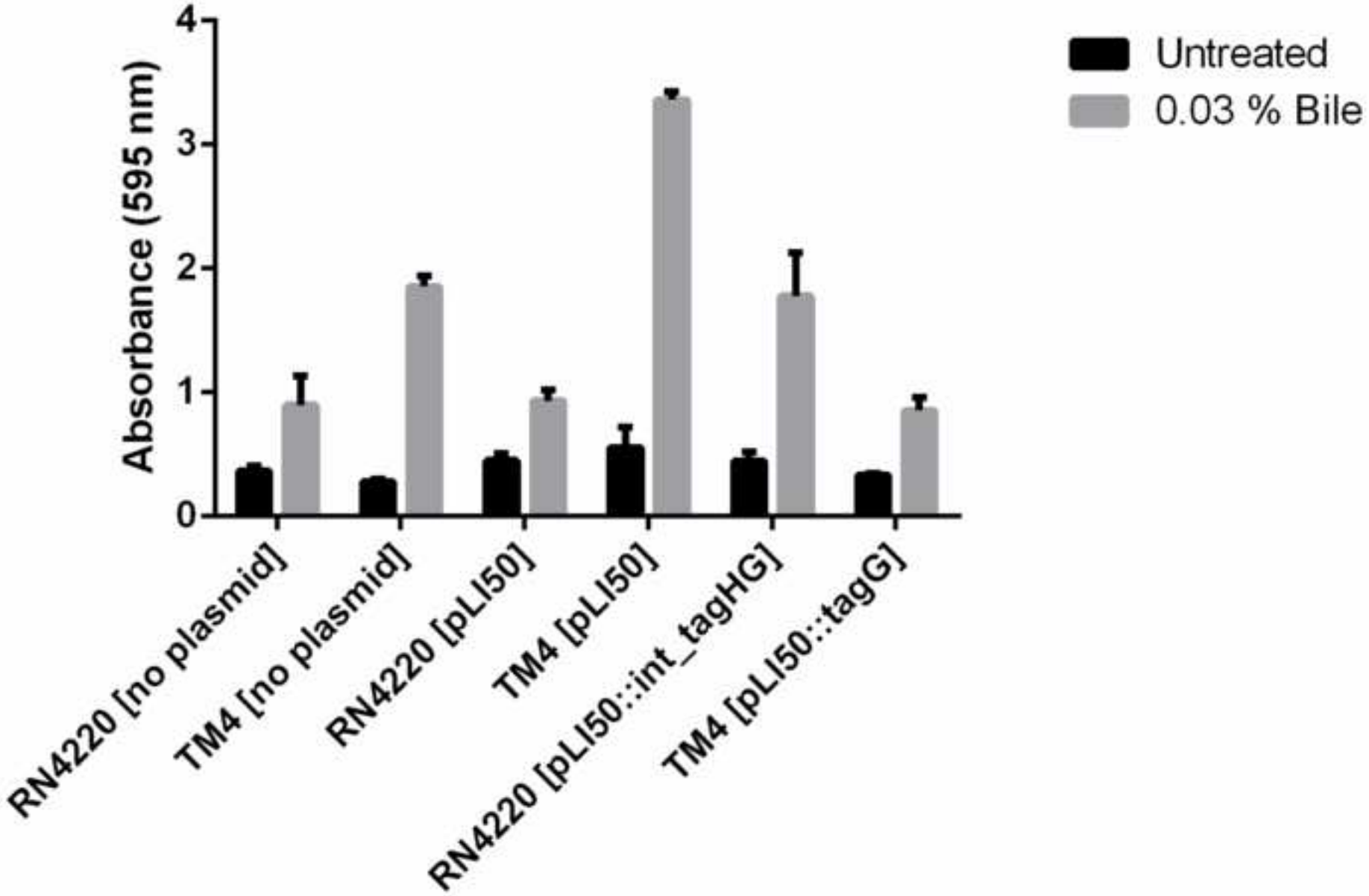
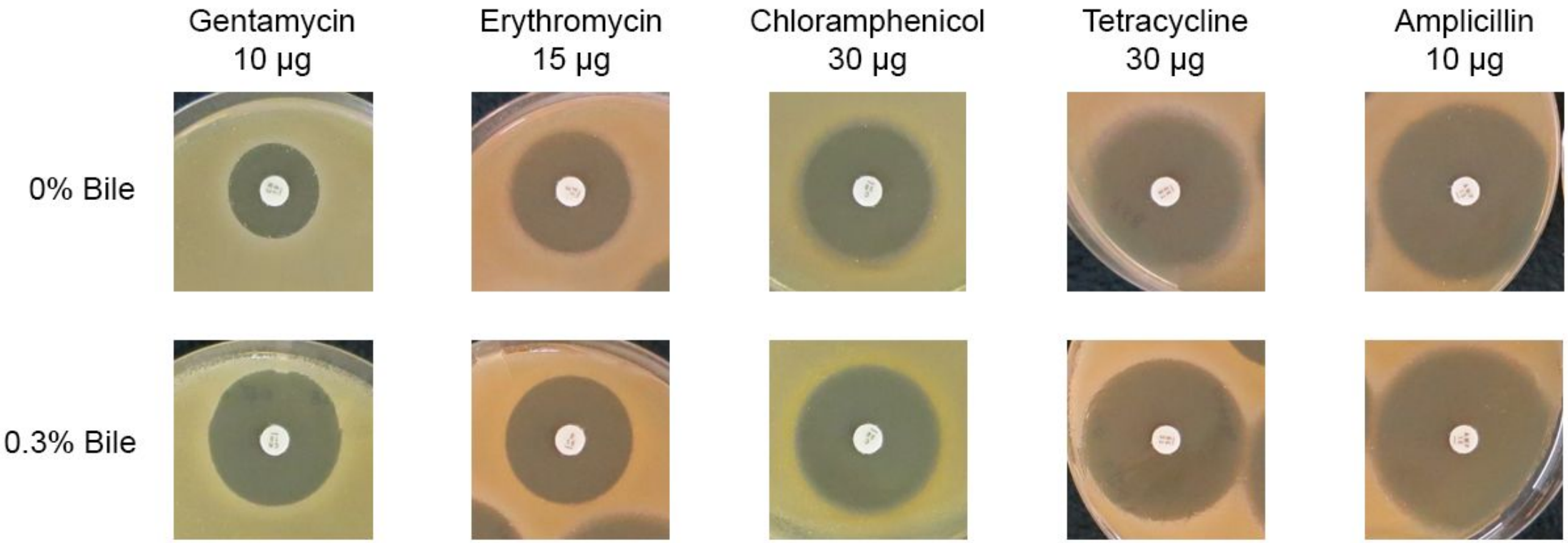
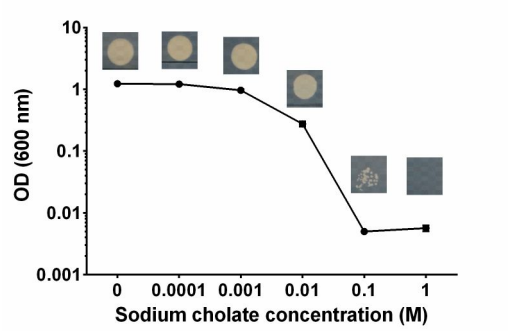
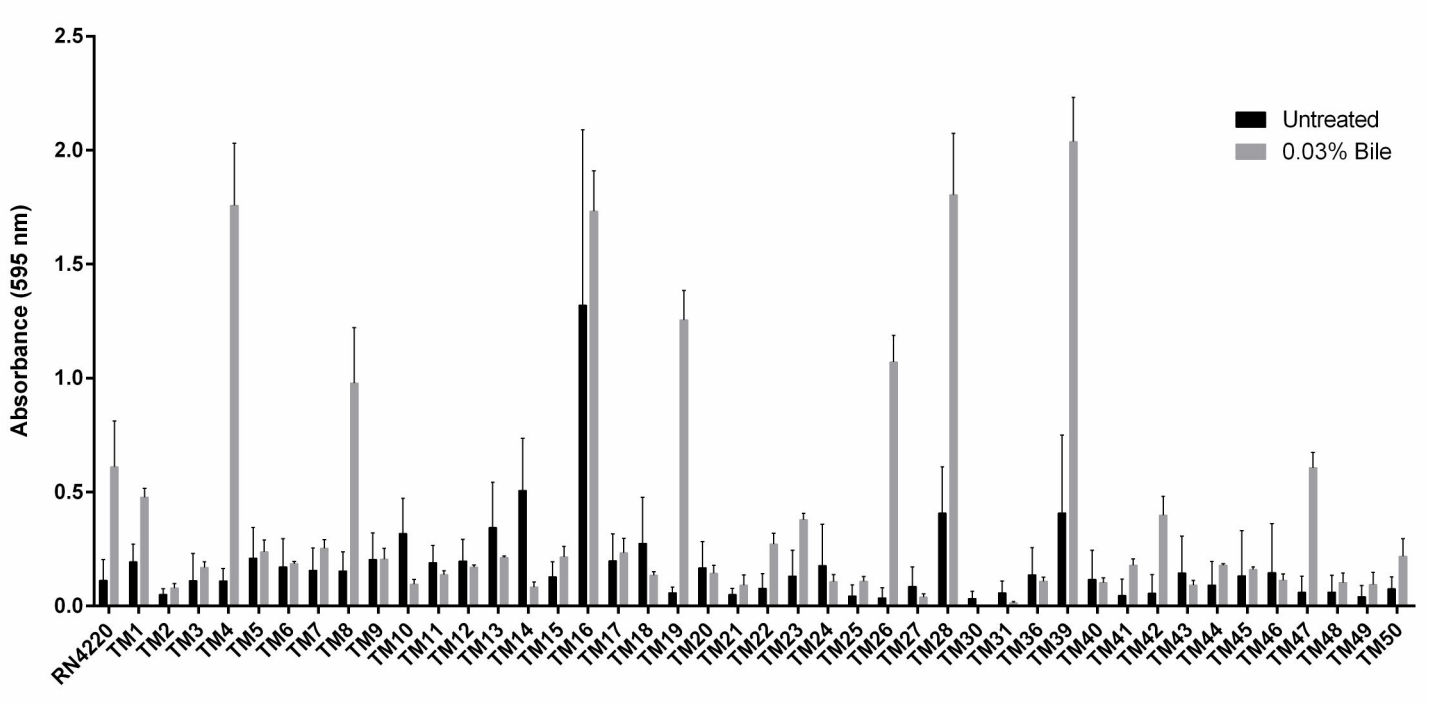





10
1
0.1
0.01
0.001
OD (600 nm)
0
0.0001
0.001
0.01
0.1
1
Sodium cholate concentration (M)


Absorbance (595 nm)
2.5
2.0
1.5
1.0
0.5
0.0
Untreated
0.03% Bile
RN4220
TM1
TM2
TM3
TM4
TM5
TM6
TM7
TM8
TM9
TM10
TM11
TM12
TM13
TM14
TM15
TM16
TM17
TM18
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TM50