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*Staphylococcus aureus* MnhF mediates cholate efflux  
and facilitates survival under human colonic conditions

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1 **Abstract**

2 Resistance to the innate defences of the intestine is crucial for the survival and  
3 carriage of *Staphylococcus aureus*, a common coloniser of the human gut. Bile salts  
4 produced by the liver and secreted into the intestines are one such group of molecules  
5 with potent anti-microbial activity. The mechanisms by which *S. aureus* is able to  
6 resist such defences in order to colonize and survive in the human gut are unknown.  
7 Here we show that *mnhF* confers resistance to bile salts, which can be abrogated by  
8 efflux pump inhibitors. MnhF mediates efflux of radiolabelled cholic acid in both *S.*  
9 *aureus* and when heterologously expressed in *Escherichia coli*, rendering them  
10 resistant. Deletion of *mnhF* attenuated survival of *S. aureus* in an anaerobic three-  
11 stage continuous culture model of the human colon (gut model), which represent  
12 different anatomical areas of the large intestine.

13 **Introduction**

14 *Staphylococcus aureus* is a ubiquitous and highly adaptable human pathogen  
15 responsible for a significant global burden of morbidity and mortality. The bacterium  
16 lives as a commensal in the nares of 20-25% of the population at any one time (1, 2).  
17 While nasal colonisation is a well-established risk factor for most types of *S. aureus*  
18 infections, several recent studies have suggested that colonisation of the intestine,  
19 which occurs in *c.* 20% of individuals and which by and large has been overlooked,  
20 could have important clinical implications (3). Patients with *S. aureus* intestinal  
21 colonisation can serve as an important source of transmission, as they often  
22 contaminate the adjacent environment (4). Similarly, such patients display an  
23 increased frequency of skin colonisation (5). A study in intensive care and liver  
24 transplant units showed that patients with both rectal and nares colonisation by MRSA  
25 had a significantly higher risk of disease (40%) than did patients with nasal  
26 colonisation alone (18%) (6). Furthermore, a study of hospitalised patients in the  
27 United States reported co-colonisation by *S. aureus* and vancomycin-resistant  
28 enterococci in >50% of the individuals studied (7). Thus it is likely that intestinal  
29 colonisation by *S. aureus* provides the pathogen with a potential opportunity to  
30 acquire new antibiotic resistance genes.

31

32 While the clinical implications of intestinal colonisation by *S. aureus* are still  
33 relatively ill-defined, it is assumed that carriage is a risk for intestinal infection; *S.*  
34 *aureus* can induce pseudomembranous colitis that is histologically distinct from that  
35 caused by *Clostridium difficile* (8). Multiple studies have demonstrated frequent  
36 intestinal colonisation in infants, particularly in those that were breast-fed and that a  
37 positive correlation exists with development of allergies (9-13). While a role for *S.*

38 *aureus* intestinal carriage in development of systemic *S. aureus* disease has not been  
39 established, colonisation of the intestinal lumen of mice can lead to the pathogen  
40 crossing the intestinal epithelial barrier and subsequent spread to the mesenteric  
41 lymph nodes (14, 15).

42

43 As a common commensal and pathogen, *S. aureus* must resist the human host's innate  
44 defences that have evolved to limit its *in vivo* growth and spread. In particular, bile  
45 represents a major challenge to bacteria that survive transit through the stomach and  
46 enter the intestines. Bile is a digestive secretion that plays an essential role in  
47 emulsification and solubilisation of lipids. We have previously demonstrated survival  
48 of *S. aureus* in a human colonic model fed with physiological levels of bile (16).  
49 Resistance to bile salts has been demonstrated to be important for intestinal survival  
50 of several enteric pathogens, but in *S. aureus* such an understanding is lacking. The  
51 role of the *S. aureus* *mnhABCDEFG* locus in bile resistance was identified using a  
52 Tn917 library screened for bile-sensitive mutants. MnhF is homologous to  
53 mammalian bile salt transporters, thus we hypothesized that it was involved in bile  
54 resistance and therefore survival of *S. aureus* in conditions modeling the human  
55 colon.

56

57 Here we provide molecular proof that a cause of bile salt resistance in *S. aureus* is  
58 efflux, catalysed by MnhF. This represents the first description of an intestinal  
59 colonisation factor in this pathogen.

60

61

62

63 **Materials and Methods**

64 **Bacteria, plasmids and growth conditions**

65 The strains and plasmids used in this work are listed in Tables 1 and 2, respectively.  
66 *Escherichia coli* strains were grown on Luria–Bertani medium, using selection with  
67 the antibiotic ampicillin (100 µg/mL) where appropriate. *S. aureus* was grown on  
68 Brain Heart Infusion (BHI) (Oxoid) at 37°C. Where appropriate, antibiotics were  
69 added at the following concentrations: erythromycin 5 µg/mL, lincomycin 25 µg/mL.  
70 Phage transductions were as described previously (23).

71

72 **Determination of minimum inhibitory concentration (MIC)**

73 The MICs of selected bile salts, sodium cholate (CA), sodium chenodeoxycholate  
74 (CDCA), sodium deoxycholate (DCA), sodium glycocholate (GCA), and sodium  
75 taurocholate (TCA) were determined by broth dilution. MICs were determined by  
76 doubling dilutions and MICs were reproduced in 3 independent experiments.

77

78 **Time-course measurement of bacterial viability upon exposure to bile salts.**

79 Overnight cultures were grown to mid-exponential phase in BHI broth at 37°C with  
80 shaking. After harvesting, cells were washed twice with sterile 5 mM HEPES buffer  
81 (pH 7.2) containing 10 mM glucose, then resuspended in the same buffer to an OD<sub>600</sub>  
82 0.5. Cells were incubated with various concentrations of bile salt for 30 minutes at  
83 37°C. At 10 minute intervals, dilutions from each of the bile salt treated groups were  
84 made with sterile peptone saline diluent. Dilutions were plated onto tryptic soy agar  
85 plates and incubated for overnight at 37°C. Colonies were counted, and percentage  
86 viabilities calculated based on the initial untreated cell suspension.

87

88 **Generation of an in-frame *mnhF* mutant.**

89 For the  $\Delta mnhF$ , DNA fragments corresponding to *c.* 0.7 kb upstream and downstream of  
90 *mnhF* were amplified using Pwo polymerase (Roche) with primers  
91  $\Delta mnhFLFor/\Delta mnhFLRev$  and  $\Delta mnhFRFor/\Delta mnhFRRev$  (Table 3). Following  
92 purification, PCR products were digested with *Bam*HI/*Eco*RI and cloned into pMAD.  
93 The resulting plasmid was used to transform electrocompetent *S. aureus* RN4220 (24).  
94 Plasmids were transduced into SH1000 using  $\phi 11$  phage. The temperature sensitive  
95 nature of plasmid replication was exploited to integrate the plasmid into the bacterial  
96 chromosome, by plating cells on media containing erythromycin and lincomycin at 42°C.  
97 After further rounds of plating, erythromycin and lincomycin sensitive colonies were  
98 isolated and the loss of *mnhF* confirmed by PCR.

99

100 **Cloning and expression of *mnhF*.**

101 The *mnhF* gene was amplified by PCR with *S. aureus* SH1000 DNA. For cloning into *S.*  
102 *aureus*, *mnhFFor2* and *mnhFRev* (Table 3) were used. PCR products were digested with  
103 *Eco*RI and *Bam*HI and ligated into similarly digested pRMC2. This created pMnhF2,  
104 where *mnhF* is fused to  $P_{xyl/tetO}$ , which is under the control of TetR and induced with  
105 anydrotetracycline. For cloning into *E. coli*, oligonucleotides *mnhFFor1* and *mnhFRev*  
106 (Table 3) were used. PCR products were digested with *Eco*RI and *Bsp*HI and ligated into  
107 similarly digested pBAD/His A. This created pMnhF1, where *mnhF* is fused to  $P_{BAD}$ ,  
108 which is under tight control of AraC.

109

110 **Bile salt accumulation assay.**

111 Accumulation of cholic acid in *S. aureus* was quantified using a previously described  
112 method (25). Briefly, *S. aureus* and *E. coli* were grown in BHI and LB broth  
113 respectively, at 37°C to an  $OD_{600}$  *c.* 0.6. Cells were centrifuged (5 mins, 16,000g),



114 washed twice in 25mM potassium phosphate buffer (pH 7.0) containing 1mM MgSO<sub>4</sub>  
115 and resuspended in same buffer to a concentration of 100 OD units/mL. One μCi of  
116 <sup>14</sup>C labelled cholic acid (American Radiolabelled Chemicals) with specific  
117 radioactivity of 55 mCi/mmol was added, to a final concentration of 18 μM, cells  
118 were incubated at 37°C for 2 h. Cells were then diluted to 10 OD units/ml in 25 mM  
119 potassium phosphate buffer (pH 7.0) containing 1 mM MgSO<sub>4</sub>, 20 mM glucose and  
120 0.2 mM non-radiolabelled cholic acid, and incubated at 37°C. Incorporation of  
121 radiolabelled cholic acid was measured by scintillation counting. At the indicated  
122 time, 250 μl cells were centrifuged at 16,000g for 2 min, and the pellets resuspended  
123 in 500 μl of sterile water and 3 ml of Ulitma Gold scintillation cocktail (Perkin  
124 Elmer). CPM were counted in a Beckman LS 6500 Coulter liquid scintillation  
125 counter.

126

### 127 **Quantitative real-time PCR.**

128 mRNAs from mutant and wild type strains were quantified using quantitative real-  
129 time PCR (qRT-PCR). Cells were grown in triplicate as described above, then treated  
130 with RNA protect (Qiagen) and RNA was isolated using the Qiagen RNeasy Mini kit.  
131 DNA was removed using Turbo DNase-free (Life Technologies). Purified RNA was  
132 quantified using a nonodrop ND-1000 spectrophotometer (Thermo Scientific). 0.5 μg  
133 of RNA was reverse transcribed using the Tetro cDNA synthesis kit (Bioline). qRT-  
134 PCR was performed using the Aligent qPCR System and iQ SYBR Green Supermix  
135 (Biorad). The relative amounts of RNAlII mRNA in parental wild type and mutant  
136 cells was determined by relative quantification using *gyrB*, based on consistent levels  
137 observed in previous studies (26, 27, 28, 29). The oligonucleotides used for qRT-  
138 PCR are listed in Table 3.

139

140 **Three-stage continuous culture colonic model system (human gut model).**

141 The three-stage continuous culture model of the human colon has been described  
142 previously (16, 30). The experiment was carried out in triplicate using faecal samples  
143 from three different volunteers. After obtaining verbal informed consent, a standard  
144 questionnaire to collect information regarding the health status, drugs use, clinical  
145 anamnesis, and lifestyle was administrated before the donor was ask to provide a  
146 faecal sample. No volunteers had received antibiotics, probiotics, steroids or other  
147 drugs with a proven impact on gut microbiota for at least 3 months before sampling.  
148 None of them had any history of gastrointestinal disorder. All healthy faecal donors  
149 had the experimental procedure explained to them and were given the opportunity to  
150 ask questions. The University of Reading research Ethics Committee exempted this  
151 study from review because no donors were involved in any intervention and waived  
152 the need for written consent due to the fact the samples received were not collected by  
153 means of intervention. All faecal samples were collected on site, kept in an anaerobic  
154 cabinet (10 % H<sub>2</sub>, 10 % CO<sub>2</sub> and 80 % N<sub>2</sub>) and used within a maximum of 15 minutes  
155 after collection. Samples were diluted 1/10 w/v in anaerobic PBS (0.1 mol/l phosphate  
156 buffer solution, pH 7.4) and homogenized (Stomacher 400, Seward, West Sussex,  
157 UK) for 2 minutes at 460 paddle-beats.

158

159 Samples were plated onto BHI agar containing 0.01% (w/v) potassium tellurite as a  
160 selective agent at different dilutions in PBS (from 10<sup>2</sup> to 10<sup>9</sup> CFU/ml) in triplicate for  
161 each time point to measure bacterial counts.

162

163 **Statistical analysis**

164 All experiments were repeated three times and data were presented as  $\pm$  standard error  
165 of mean. Analysis was performed using GraphPad Prism 5 software. Experimental  
166 data were analysed by One-Way Anova and Two-Way Anova method, using  
167 Bonferroni post-test analysis.

168

169 **Results**

170 **Identification of a bile salt resistance locus.**

171 Genes conferring resistance to bile were identified by replica plating *S. aureus*  
172 SH1000 Tn917 insertion libraries on BHI agar and onto BHI agar containing 18%  
173 (w/v) bile salts (Oxoid), which represented  $0.8 \times$  MIC. Six colonies were unable to  
174 grow in the presence of bile salts, but exhibited no growth defect on BHI agar in the  
175 absence of bile. Sequencing of the genomic DNA flanking the transposon insertion  
176 site of bile sensitive strains was carried out in order to identify the DNA insertion  
177 sites of Tn917, revealing that all six strains were siblings containing the transposon  
178 inserted in the same gene, namely the previously described *mnhA*, the first gene in the  
179 polycistronic *mnhABCDEFG* operon which encodes a  $\text{Na}^+/\text{H}^+$  antiporter (31).  
180 *Bacillus subtilis* contains the orthologous *mrpABCDEFG* operon that has an identical  
181 function, however *mrpF* and by extension *mnhF*, are homologous to mammalian bile  
182 transporters and *mrpF* mediates cholic acid efflux (32, 33).

183

184 **MnhF mediates resistance to bile salts.**

185 We hypothesized that MnhF was responsible for the observed bile salt resistance  
186 phenotype. To test this, an in-frame  $\Delta mnhF$  strain was created in *S. aureus* SH1000.  
187 The mutant strain had no growth defect when grown on BHI solid or liquid media in

188 the absence of bile salts (results not shown). Compared to the parental wild type, the  
189  $\Delta mnhF$  strain had a reduced MIC for unconjugated bile salts and, in particular, cholic  
190 acid (Table 4). Complementation of mutation with *mnhF* under the control of an  
191 inducible promoter restored the bile resistance phenotype to that observed in the  
192 parent strain in the presence of anhydrotetracycline as an inducer (Table 4), whereas  
193 there was no such resistance in the absence of the inducer (results not shown). In  
194 killing assays, the  $\Delta mnhF$  strain was significantly more sensitive than the parent. In  
195 the presence of 1  $\mu\text{g/mL}$  anhydrotetracycline, the complemented strain exhibited a  
196 similar rate of cell death as the parental wild type (Fig 1). The increased sensitivity of  
197 the mutant strain was only observed with unconjugated bile salts. However it should  
198 be noted that we were unable to determine the MIC of conjugated bile salts for *S.*  
199 *aureus*, as they were insoluble at concentrations greater than 200 mM.

200

201 To confirm the role of *mnhF* in bile salt resistance, it was cloned under the control of  
202 the arabinose-inducible  $P_{\text{BAD}}$  promoter of plasmid pBAD/HisA, which enabled  
203 arabinose-dose dependent expression of MnhF in *E. coli* TG1 and TOP10 strains.  
204 Expression of MnhF increased the MICs to both conjugated and unconjugated bile  
205 salts in both background strains and in the case of cholic acid, the increased resistance  
206 was arabinose-dose dependent (Table 5). Similarly, expression of MnhF in *E. coli*  
207 decreased the bacteriostatic effects of bile salts on that bacterium (Fig 2). Thus MnhF  
208 was sufficient to enable bile salt resistance in the absence of the rest of the  
209 *mnhABCDEFG* operon.

210

211 **The effect of efflux pump inhibitors on bile salt resistance.**

212 Given the ability of MnhF to confer bile salt resistance and its similarity to other  
213 known and putative bile efflux systems, its ability to mediate removal of cholic acid  
214 from bacteria was tested. Both Phe-Arg- $\beta$ -naphthylamide (PA $\beta$ N), a synthetic  
215 dipeptide that inhibits bacterial efflux pumps, including bile salt efflux pumps of  
216 Gram negative bacteria, and reserpine, a plant alkaloid which can inhibit multidrug  
217 efflux pumps in Gram positive bacteria, were tested for their ability to reduce bile salt  
218 MICs in *S. aureus*. Both inhibitors caused reductions in the *S. aureus* MIC for cholic  
219 acid and PA $\beta$ N reduced the MIC for all three unconjugated bile salts (Table 6A),  
220 however the reduction was much smaller in the  $\Delta mnhF$  strain than the parental wild-  
221 type, possibly indicating the presence of other bile salt efflux systems in the pathogen.  
222 Similarly, in *E. coli* (pMnhF1), PA $\beta$ N reduced bile salt MICs to levels lower than that  
223 for untreated *E. coli* (pBAD His A) (Table 6B). Thus in both *S. aureus* and *E. coli*,  
224 inhibitors of efflux pumps abrogated bile salt resistance in an MnhF dependent  
225 manner.

226

#### 227 **MnhF transports cholic acid.**

228 Given the ability of efflux pump inhibitors to reduce the MICs of certain bile salts in  
229 *S. aureus*, the capacity of the MnhF to transport cholic acid was determined *in*  
230 *vitro* using a  $^{14}\text{C}$ -radiolabelled cholic acid substrate, similar to previous efflux assays  
231 (25, 34, 35). *S. aureus* SH1000 and  $\Delta mnhF$  strains were incubated with  $^{14}\text{C}$ -cholic  
232 acid (uptake period) and then diluted in buffer containing excess of non-radiolabelled  
233 cholic acid (efflux period). Initial  $^{14}\text{C}$ -cholic acid uptake was the same for both  
234 strains ( $10962 \pm 550$  cpm for *S. aureus* SH1000 and  $10278 \pm 278$  cpm for *S. aureus*  
235  $\Delta mnhF$ ), but throughout the efflux period *S. aureus*  $\Delta mnhF$  retained significantly  
236 more of the radiolabel than the parental wild-type (Fig 3A). To further corroborate

237 these findings, efflux assays were also carried out on *E. coli* expressing MnhF. *E. coli*  
238 TG1, *E. coli* TG1 (pBAD) and *E. coli* TG1 (pMnhF1) were grown overnight in LB  
239 supplemented with 1% arabinose at 37°C, then incubated with <sup>14</sup>C- cholic acid. All  
240 the *E. coli* TG1 strains incorporated similar levels of <sup>14</sup>C-cholic acid during uptake  
241 period (20774 ± 363 for TG1, 23274 ± 386 for TG1: pBAD and 22435 ± 460 CPM  
242 for TG1: pMnhF1). At various points after the initial incorporation of radiolabelled  
243 cholic acid, cells were centrifuged and cell-associated radioactivity was determined  
244 by liquid scintillation method. *E. coli* TG1 cells expressing MnhF retained  
245 significantly (P<0.05) lower levels of <sup>14</sup>C-radiolabelled cholic acid than parental TG1  
246 and TG1 cells with the empty pBAD vector (TG1: pBAD) (Fig 3B). In both sets of  
247 experiments the reason for increasing cell-associated radiolabel during the efflux  
248 period, after which cells have been diluted in excess non-labelled cholic acid, is  
249 unclear, but has also been observed in previous studies on *Listeria monocytogenes*  
250 and may reflect continued incorporation of <sup>14</sup>C-cholic acid during the efflux period  
251 after dilution (25).

252

### 253 **Bile salt resistance is not affected by *agr*.**

254 To examine whether *agr* quorum sensing system is involved in bile salt resistance, the  
255 MICs for CA, DCA and CDCA in *S. aureus* SH1001 (*agr*) were determined and  
256 found to be indistinguishable from those of the wild type (results not shown).  
257 Furthermore, the *agr* system is not inhibited by the *mnhF* mutation as the RNAIII  
258 effector molecule is still produced (Fig 4). Thus we were unable to demonstrate a role  
259 for *agr* in bile resistance.

260

### 261 **MnhF is required for survival of *S. aureus* in a human gut model.**

262 To examine the role of MnhF in survival of *S. aureus* in conditions found in the  
263 human colon, we used a three-stage continuous culture gut model system, designed to  
264 reproduce the spatial, temporal, nutritional and physicochemical characteristics of the  
265 microbiota in the human colon. *In vivo* studies of colonic bacteria are hampered by  
266 the lack of suitable animal models, as these do not correctly simulate the microbiota  
267 and physicochemical conditions of the human colon (36). We have previously used  
268 this *in vitro* model to study survival of *S. aureus* and the impact of infection on the  
269 host's intestinal microflora (16).

270

271 Mutational inactivation of the whole *mnhABCDEFG* operon does not affect the ability  
272 of *S. aureus* to grow at a range of pH levels (37). In order to exclude the possibility  
273 that the normal pH range (5.5 to 7.5) found in the colon, influenced survival of the  
274  $\Delta mnhF$  mutant, we corroborated the previous observation at pH 5.5 to 8.5 using this  
275 strain (results not shown).

276

277 After inoculating vessel 1 (which models the proximal colon) of the colonic models  
278 with *S. aureus* to a concentration of *c.*  $2 \times 10^{10}$  cfu/mL, as a single dose, the *S. aureus*  
279 populations stabilised at 6 to 7 Log<sub>10</sub> units over a period of up to 8 hours. Survival of  
280 *S. aureus*  $\Delta mnhF$  was significantly attenuated compared to its parental strain in all  
281 three vessels (Fig 5A-C).

282

## 283 **Discussion**

284 A complex set of interactions exists between *S. aureus* and its human host as the  
285 bacterium is able to colonize several niches, both as an opportunist pathogen of great  
286 medical importance and as a common commensal. In order to defend against

287 colonization by microorganisms, the host produces a range of antimicrobials such as  
288 peptides, fatty acids and bile. Bile represents one significant challenge to the gut  
289 microflora; in humans the liver secretes up to one liter of bile per day into the  
290 intestines (38). Furthermore molecules secreted by bacteria, including *S. aureus*,  
291 during infection are an important cause of metabolic cholestasis; an inability of  
292 hepatocytes to produce bile (39). Bile is a complex cocktail composed principally of  
293 bile salts, phospholipids, cholesterol, proteins and bilirubin (40). Originally  
294 characterised as digestive molecules, bile salts have antimicrobial activity, which has  
295 been attributed to their ability to damage cell membranes (41). Additionally, they  
296 cause intracellular acidification, induce formation of secondary structures in RNA,  
297 DNA damage and misfolding and denaturation of proteins. Thus bile salts represent a  
298 serious challenge to bacterial cells in the gastrointestinal tract and bacteria that are  
299 able to colonise the gut should therefore be able to overcome their toxicity.

300

301 Bile salts which pass into the large intestine undergo modification by the normal  
302 microbiota (42). The major modifications include deconjugation, oxidation of  
303 hydroxyl groups at C-3, C-7 and C-12, and  $7\alpha/\beta$ -dehydroxylation (43, 44). Thus the  
304 normal commensal inhabitants of the human gastrointestinal tract such as  
305 *Lactobacillus*, *Propionibacterium* and *Bifidobacterium*, are required by the host for  
306 maintenance of gut health and the ecological balance by influencing the composition  
307 of the bile acids in the large intestine and by extension, the gut microbiome (45, 46).  
308 Their ability to survive in the presence of bile salts indicates the existence of inherent  
309 bile resistance mechanisms. Indeed, colonic commensals deploy various different  
310 strategies for resisting bile. *Lactobacillus plantarum* produces a bile salt hydrolase,  
311 which detoxifies bile salts by deconjugating bile salts inside the cell, turning them into



312 weaker acids, thus negating the drop in pH that they cause (47). Bifidobacteria  
313 possess a number of characterised bile salt resistance mechanisms. In addition to  
314 multiple efflux pumps, exposure to bile salts results in a modification of the cell  
315 envelope. Increased concentrations of membrane fatty acids and altered  
316 phospholipids increase membrane rigidity and reduce the permeability to lipophilic  
317 bile salts (48). Similarly, exposure of *Bifidobacterium animalis* ssp *lactis* to bile salts  
318 induces increased expression of exopolysaccharides, which are proposed to form a  
319 protective layer around the bacterium (49).

320

321 Bile salts represent a physiological challenge for bacteria and an environmental cue;  
322 *Salmonella enterica* and *Vibrio cholera* regulate intestinal colonisation and virulence  
323 in response to bile (50, 51). However pathogens that inhabit the human intestines are  
324 also exposed to the bactericidal nature of bile salts and hence must also exhibit  
325 resistance in order to survive. Generally, Gram-negative bacteria are more innately  
326 resistant than Gram positives, due to the presence of an outer membrane, which acts  
327 as a barrier (38). Indeed maintenance of membrane integrity by lipopolysaccharide  
328 (LPS) in the cellular envelope of Gram-negative bacteria imparts protection against  
329 the actions of bile salts (52, 53). *Salmonella typhi* and *Salmonella typhimurium* are  
330 able to grow at bile concentrations that are much higher than those encountered *in*  
331 *vivo*. This is due, at least in part, to the presence of outer membrane efflux pumps  
332 such as AcrAB (54). Similarly, HefC is an AcrB homologue that confers bile salt  
333 resistance in *Helicobacter pylori* (55). The multidrug efflux pump CmeABC, of  
334 *Campylobacter jejuni* mediates bile salt resistance and is required for colonisation of  
335 chickens (56). Gram-positive pathogens such as *Enterococcus faecalis* and *L.*  
336 *monocytogenes* also exhibit bile resistance. In addition to bile salt hydrolase

337 activities, both bacteria possess multiple bile efflux systems. Exposure of *E. faecalis*  
338 to bile results in up-regulation of two open reading frames EF0420 and EF1814,  
339 which are homologous to the QacA family of efflux pumps (57). *L. monocytogenes*  
340 OpuC, an osmolyte transporter, as well as specialist bile transporters BilE and MdrT,  
341 all confer bile salt resistance to the pathogen (58).

342

343 We demonstrated that the *mnhABCDEFG* operon in *S. aureus* confers bile salt  
344 resistance to the pathogen. Previous studies have shown this operon to encode a  
345 multi-subunit hetero-oligomeric antiporter system involved in efflux of monovalent  
346 cations such as Na<sup>+</sup>, K<sup>+</sup> and Li<sup>+</sup> in exchange for H<sup>+</sup> (59). Transposon insertion into  
347 *mnhD* (also called *snoD*) resulted in reduced susceptibility to platelet microbicidal  
348 protein 1 (37), thus the operon also has the ability to sensitize the pathogen to other  
349 host innate antimicrobials. The function of individual components remains to be  
350 determined, however *mnhF* is homologous to a hamster ileal bile salt transporter (60)  
351 and rat liver organic anion transporter that was shown to efflux cholic acid (61). A  
352 transposon insertion at *mnhA*, which presumably had a polar effect on the rest of the  
353 operon and in-frame deletion of *mnhF*, rendered the bacterium equally susceptible to  
354 bile salts. Together with our observation that cloning of *mnhF* in *E. coli* increased the  
355 bile salt MIC, demonstrated that MnhF alone is sufficient to confer bile salt  
356 resistance. Furthermore, MnhF acted to exclude cholic acid from both *S. aureus* and  
357 *E. coli*.

358

359 In order to confirm that this increase sensitivity of *S. aureus* translated into a  
360 decreased ability of *S. aureus* to survive under conditions found in the human colon,  
361 we studied survival of the mutant in a well characterised *in vitro* three-stage system

362 which models the microbial and physicochemical conditions of the in the proximal,  
363 transverse and distal colon (30). The  $\Delta mnhF$  strain was attenuated in its ability to  
364 survive in the model, compared to the parental wild type. To date, no suitable *in*  
365 *vivo* models have been developed to study carriage and survival of *S. aureus* in the  
366 human intestine. Laboratory mouse models of infection do not reproduce the  
367 complex microbial ecosystem or the human gut's physicochemical defences (36).

368

369 The physiology of *S. aureus* in the human gut is very poorly understood, relative to  
370 other niches. A recent study to determine *S. aureus* genetic traits associated with  
371 observed higher rectal carriage rates was inconclusive (62), thus this is the first report  
372 of an *S. aureus* intestinal colonisation factor. Given the complex nature of the gut as a  
373 niche, it seems highly likely that other loci are similarly required. Indeed it would  
374 appear from our data that other bile resistance factors also exist. As such much  
375 remains to be discovered about the behaviour and survival of *S. aureus* in the human  
376 gut.

377

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380

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631 **Table 1. Bacterial strains**

Strain	Description/Genotype	Source or Reference
<i>S. aureus</i> SH1000	Wild type	(17)
<i>S. aureus</i> SH1001	<i>agr</i> mutation in SH1000	(17)
<i>S. aureus</i> RN4220	Accepts <i>E. coli</i> DNA	(18)
<i>S. aureus</i> <i>mnhA::Tn917</i>	Tn917 inserted into <i>mnhA</i> in SH1000	This study
<i>S. aureus</i> $\Delta$ <i>mnhF</i>	$\Delta$ <i>mnhF</i> mutation in SH1000	This study
<i>E. coli</i> Top10	<i>F- mcrA</i> $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ) $\phi$ 80 <i>lacZ</i> $\Delta$ <i>M15</i> $\Delta$ <i>lacX74 nupG recA1</i> <i>araD139</i> $\Delta$ ( <i>ara-leu</i> )7697 <i>galE15</i> <i>galK16 rpsL(Str<sup>R</sup>) endA1 <math>\lambda^-</math></i>	Invitrogen
<i>E. coli</i> TG1	<i>F'</i> [ <i>traD36 proAB<sup>+</sup> lacI<sup>q</sup> lacZ</i> $\Delta$ <i>M15</i> ] <i>supE thi-1</i> $\Delta$ ( <i>lac-proAB</i> ) $\Delta$ ( <i>mcrB-</i> <i>hsdSM</i> )5, ( <i>r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>-</sup></i> )	Lucigen

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634 **Table 2. Plasmids**

<b>Plasmid name</b>	<b>Description</b>	<b>Antibiotic resistance</b>	<b>Source or Reference</b>
pLTV1	Carries Tn917	Em <sup>R</sup> /Tc <sup>R</sup>	(19)
pMAD	Temperature sensitive (30°C) <i>E. coli</i> – <i>S. aureus</i> shuttle vector. pE194 <sup>ts</sup> ::pBR322	Em <sup>R</sup>	(20)
pBAD His A	Expression vector containing <i>araBAD</i> promoter	Ap <sup>R</sup>	(21)
pRMC2	<i>S. aureus</i> expression vector	Ap <sup>R</sup> /Cm <sup>R</sup>	(22)
pΔ <i>mnhF</i>	Vector for Δ <i>mnhF</i> mutation	Em <sup>R</sup>	This study
pMnhF1	pBAD His A containing <i>mnhF</i> internal fragment	Ap <sup>R</sup>	This study
pMnhF2	pRMC2 containing <i>mnhF</i> internal fragment	Ap <sup>R</sup> /Cm <sup>R</sup>	This study

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638 **Table 3. Oligonucleotides.** Restriction endonuclease sites are underlined

Name	Sequence 5'-3'
<i>ΔmnhFL</i> For	CCAAAAGGATCCGATCTTAATAAC
<i>ΔmnhFL</i> Rev	CATTAGAATTCATTATATTTTCGCCACC
<i>ΔmnhFR</i> For	TATGGAATTCGGTAAGGTGATTGAAC
<i>ΔmnhFR</i> Rev	GCGATTGCGGATCCCTGTATGCC
<i>mnhFF</i> For1	GGGCGAAATATCATGAATCATAATG
<i>mnhFF</i> For2	GGGCGAAATAGGATCCATCATAATG
<i>mnhF</i> Rev	TGATGAATTCGATAAGTGCAAGACTAATC
RNAIIIFor	ACATGGTTATTAAGTTGGGATGG
RNAIIIRev	TAAAATGGATTATCGACACAGTGA
<i>gyrB</i> For	ATCGACTTCAGAGAGAGGTTTG
<i>gyrB</i> Rev	CCGTTATCCGTTACTTTAATCCA

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644 **Table 4. MICs of bile salts for *S. aureus* SH1000 and  $\Delta mnhF$**

Bile salt	Wild type (mM)	$\Delta mnhF$ (mM)	$\Delta mnhF$ [pMnhF2] (mM)	$\Delta mnhF$ [pRMC2] (mM)
CA	22	5	22	5
DCA	1.2	0.6	1.2	0.6
CDCA	1.2	0.6	1.2	0.6
GCA	>200	>200	ND	ND
TCA	>200	>200	ND	ND

645  
646 **NOTE.** CA, sodium cholate; DCA, sodium deoxycholate; CDCA, sodium  
647 chenodeoxycholate; GCA, sodium glycocholate; TCA, sodium taurocholate. ND, not  
648 determined.

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662 **Table 5. MICs of bile salts for wild type and recombinant *E. coli* strains**  
 663 **expressing MnhF at different levels of arabinose induction**

Bile salt	Wild type		Vector control		Recombinants					
	TG1	TOP10	TG1	TOP10	TG1 pMnhF1			TOP10 pMnhF1		
			pBAD	pBAD	0%	0.02%	2%	0%	0.02%	2%
					Arabinose	Arabinose	Arabinose	Arabinose	Arabinose	Arabinose
CA	30	30	30	30	30	60	90	30	60	90
DCA	4	4	4	4	4	>4	>4	4	>4	>4
CDCA	4	4	4	4	4	>4	>4	4	>4	>4
GCA	50	50	50	50	50	100	100	50	100	100
TCA	50	50	50	50	50	100	100	50	100	100

664  
 665 **NOTE.** CA, sodium cholate; DCA, sodium deoxycholate; CDCA, sodium  
 666 chenodeoxycholate; GCA, sodium glycocholate; TCA, sodium taurocholate.

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674 **Table 6.** Effect of efflux pump inhibitors on MICs of bile salts to (A) *S. aureus* and  
 675 (B) *E. coli*.

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**A.**

Bile salt	<i>S. aureus</i> SH1000 (mM)			<i>S. aureus</i> $\Delta$ <i>mnhF</i> (mM)		
	Control	<sup>a</sup> PA $\beta$ N	<sup>a</sup> Reserpine	Control	<sup>a</sup> PA $\beta$ N	<sup>a</sup> Reserpine
CA	22	2.5	10	5	2.5	2.5
DCA	1.2	0.3	1.2	0.6	0.3	0.3
CDCA	1.2	0.3	1.2	0.6	0.3	0.3
GCA	>200	200	>200	>200	200	>200
TCA	>200	200	>200	>200	200	>200

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**B.**

Bile salt	<i>E. coli</i> TG1 (mM)			<i>E. coli</i> TG1 pMnhF1 (mM)		
	Control	<sup>a</sup> PA $\beta$ N	<sup>a</sup> Reserpine	Control	<sup>a</sup> PA $\beta$ N	<sup>a</sup> Reserpine
CA	30	2.5	30	90	2.5	90
DCA	4	0.6	>4	>4	0.6	>4
CDCA	4	0.6	>4	>4	0.6	>4
GCA	50	10	50	100	10	100
TCA	50	10	50	100	10	100

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**NOTE.** CA, sodium cholate; DCA, sodium deoxycholate; CDCA, sodium chenodeoxycholate; GCA, sodium glycocholate; TCA, sodium taurocholate; PA $\beta$ N, Phe-Arg- $\beta$ -naphthylamide. <sup>a</sup>PA $\beta$ N at 20  $\mu$ g/ml and Reserpine at 40  $\mu$ g/ml.

690 **Figure Legends**

691 **Figure 1. MnhF protects *S. aureus* against the bactericidal activity of bile salts.**

692 Viability of *S. aureus* SH1000 [■],  $\Delta mnhF$  [▲],  $\Delta mnhF$  (pMnhF2) [◆],  $\Delta mnhF$   
693 (pRMC2) [×] treated with (A) 2 mM CA, (B) 0.25 mM DCA, and (C) 20 mM GCA.

694 Data represents mean  $\pm$  standard error of mean from three independent experiments.

695 \*P<0.01, †P>0.05.

696

697 **Figure 2. Heterologous expression of MnhF in *E. coli* protects against the**

698 **bacteriostatic effects of bile salts.** Viability of wild type *E. coli* TG1 and *E. coli*

699 TG1 pMnhF1 cells in LB medium containing CA (10 and 20 mM), DCA (2 and 4

700 mM) and GCA (25 and 50 mM) and then grown for overnight at 37°C. Cell counts

701 were then determined by viable plate counting. Data represents mean  $\pm$  standard error

702 of mean from three independent experiments. \* P<0.001

703

704 **Figure 3. MnhF exports cholic acid.** (A) *S. aureus* SH1000 wild type [■] and

705  $\Delta mnhF$  [▲] cells were loaded with 1  $\mu$ Ci of <sup>14</sup>C-cholic acid, and then diluted into a

706 buffer containing excess of non-radiolabelled cholic acid (0.2 mM). (B) *E. coli* TG1

707 parental type (TG1) [▲], *E. coli* TG1 expressing pBAD (TG1: pBAD) [◆] and *E.*

708 *coli* TG1 expressing pMnhF1 (TG1: pMnhF1) [■] cells grown overnight in LB under

709 1% arabinose induction, were loaded with 1  $\mu$ Ci of <sup>14</sup>C- cholic acid, and then diluted

710 into a buffer containing excess of non-radiolabelled cholic acid (0.2 mM) and 1%

711 arabinose. At indicated times, the amount of retained <sup>14</sup>C-cholic acid in cell pellets

712 were determined by liquid scintillation counting. Data represents mean  $\pm$  standard

713 error of mean of three independent experiments. \*P<0.05

714

715 **Figure 4. Mutation of *mnhF* does not affect *agr*.** qRT-PCR was performed in  
716 order to quantify amounts of RNAIII in *S. aureus* strains during exponential and  
717 stationary phases of growth. Data represents mean  $\pm$  standard error of mean of three  
718 independent experiments. \* $P > 0.05$ .

719

720 **Figure 5. MnhF is required for *S. aureus* survival in the human colonic model.**  
721 Survival of *S. aureus* SH1000 [■] and  $\Delta mnhF$  [▲] in the human colonic model. (A)  
722 V1, models the ascending colon, (B) V2 model the transverse colon and (C) V3  
723 models the descending colon. Samples were taken at inoculation (0 h) and 4, 8, 24,  
724 48, 72, and 96 hours post infection. Results are reported as means ( $\text{Log}_{10}$  CFU/mL)  
725 of the data of three colonic models  $\pm$  standard error of mean. \* $P < 0.05$ ; \*\* $P < 0.001$ .

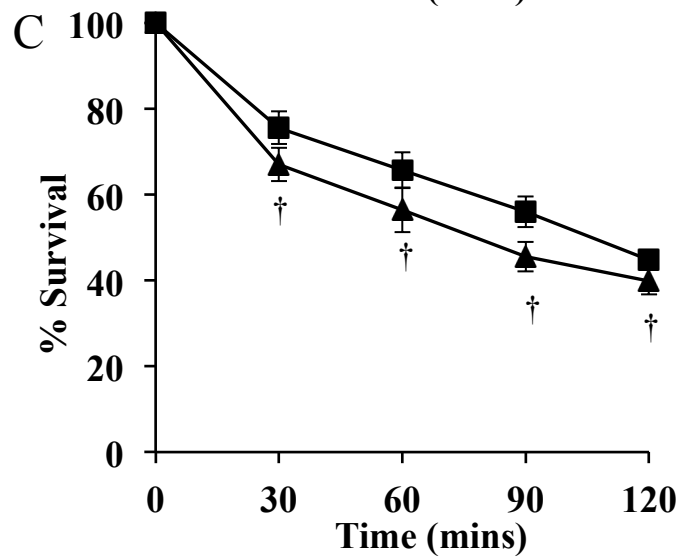
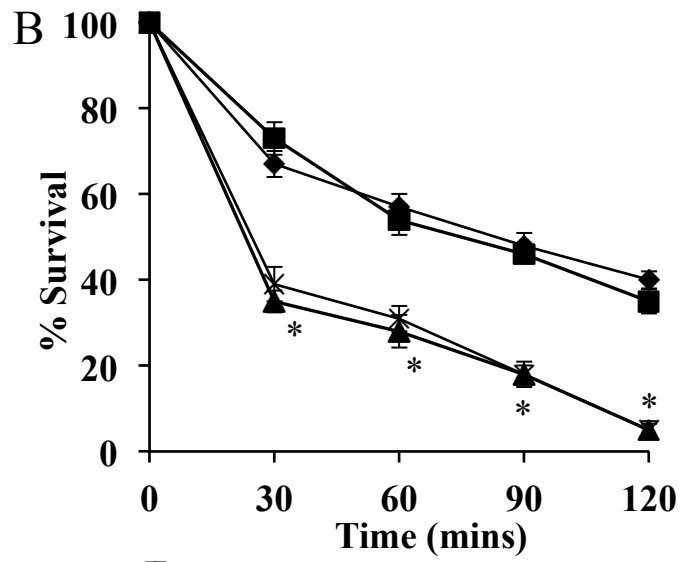
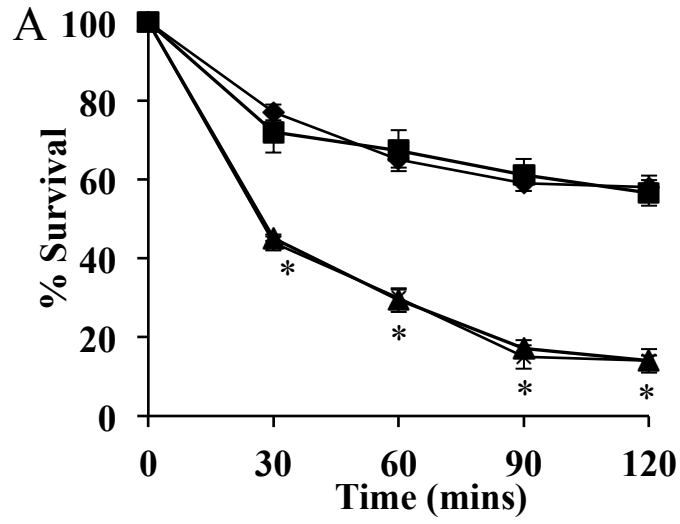


Figure 1

□ TG1: pMnhF    ▨ TG1: pMnhF@0.02% Arabinose    ■ TG1: pMnhF@2% Arabinose

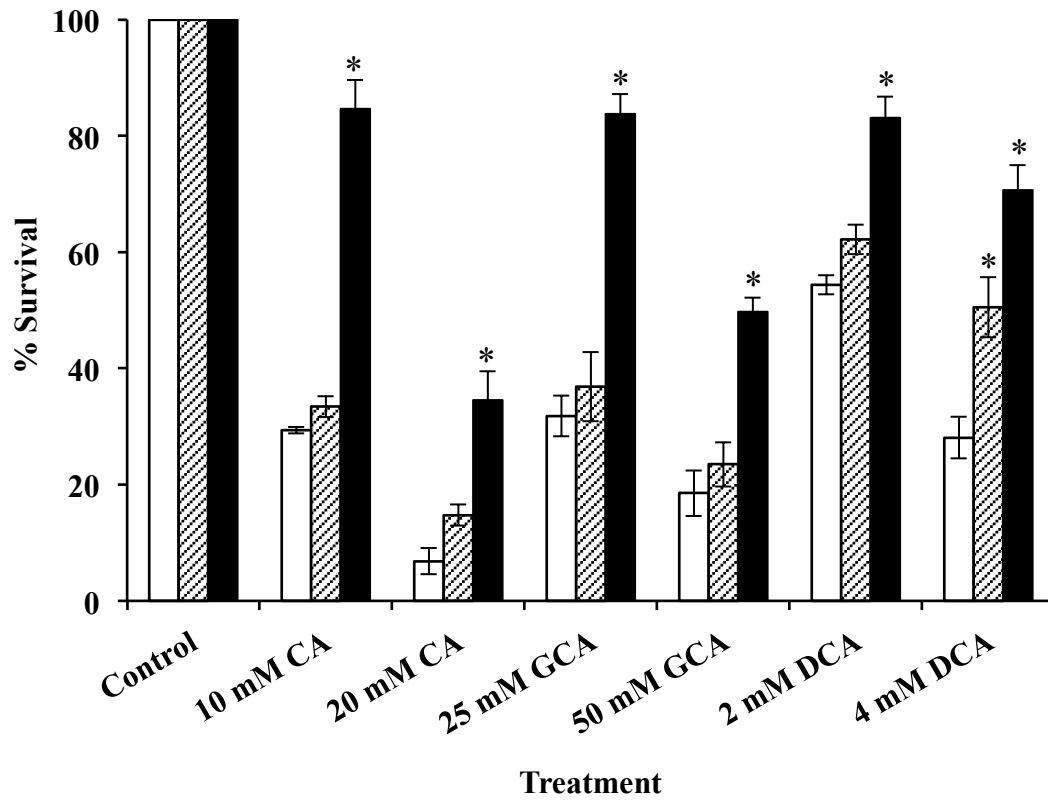


Figure 2

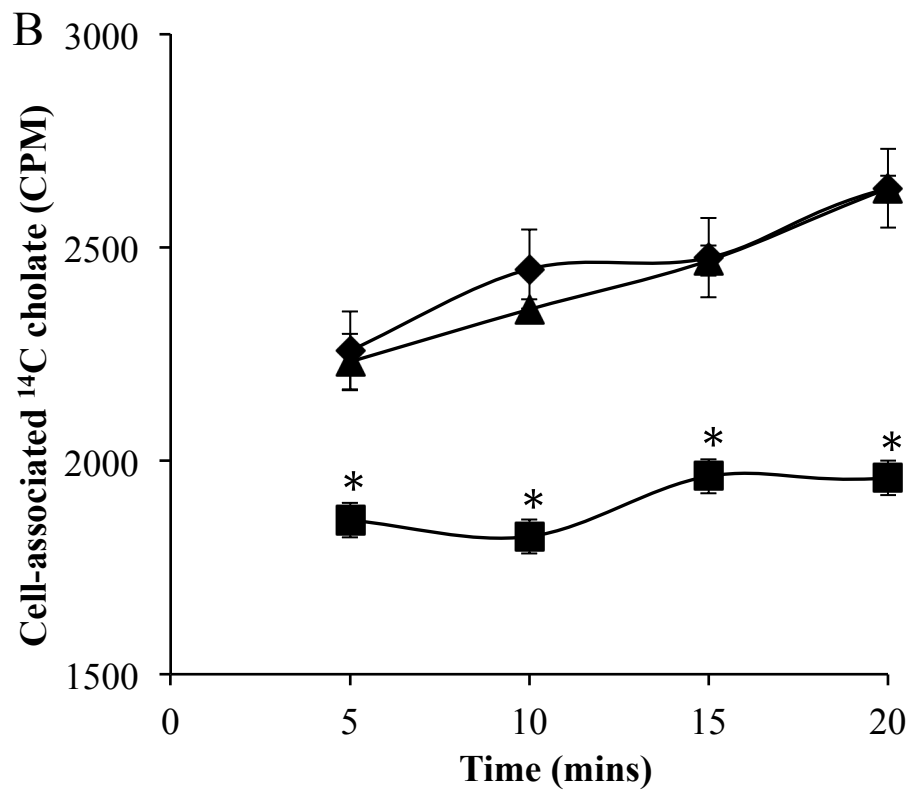
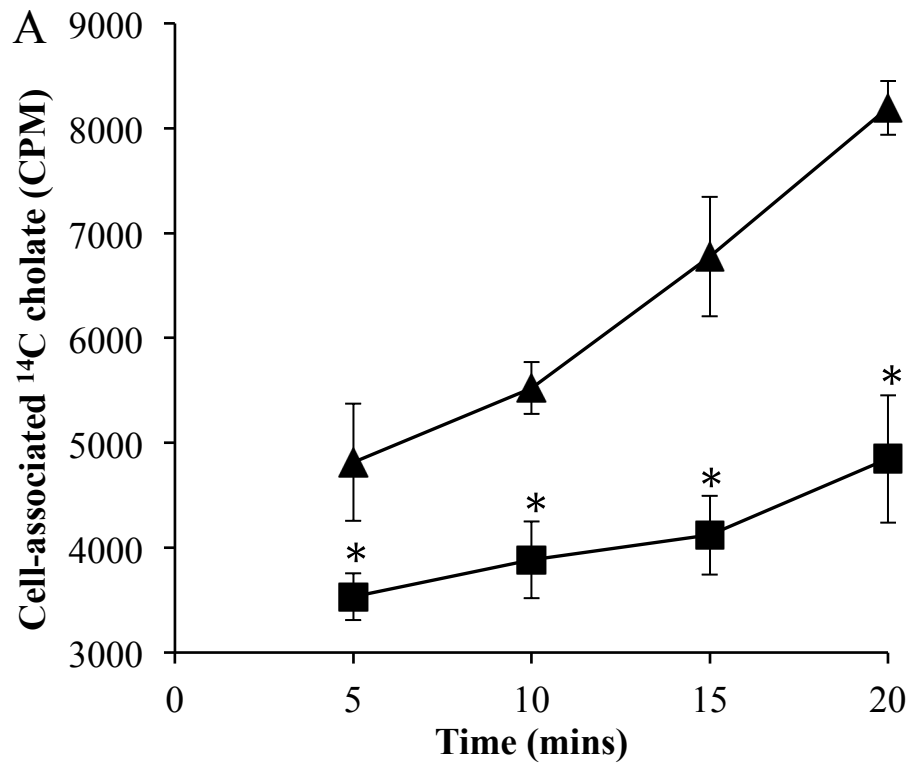


Figure 3



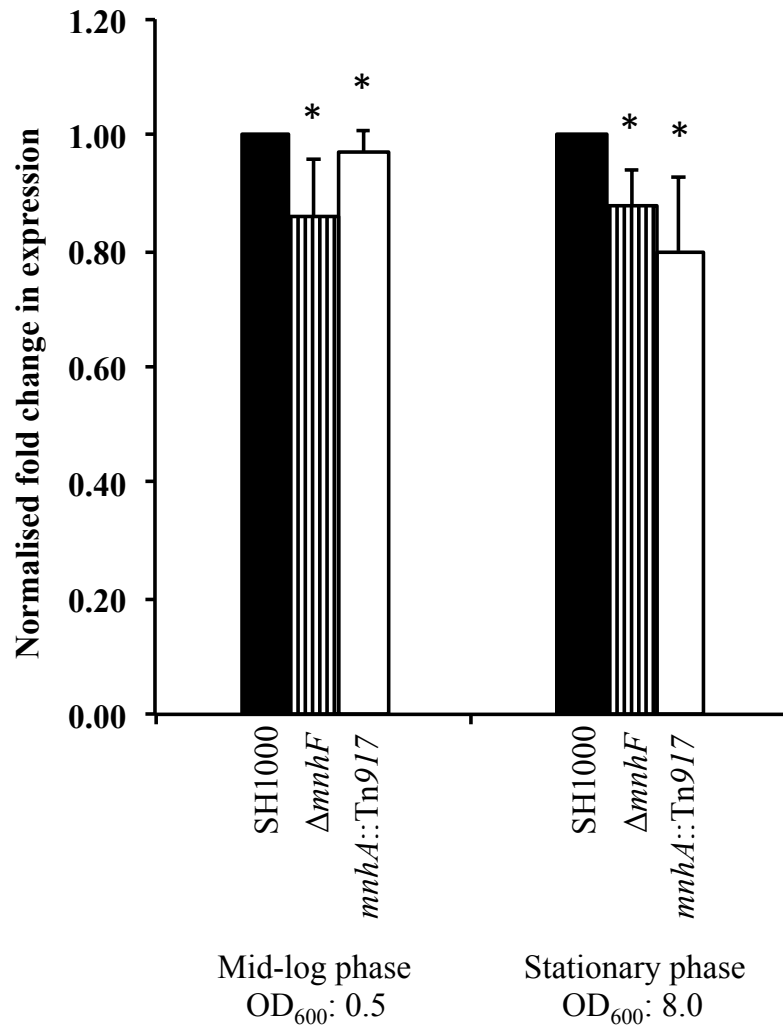


Figure 4

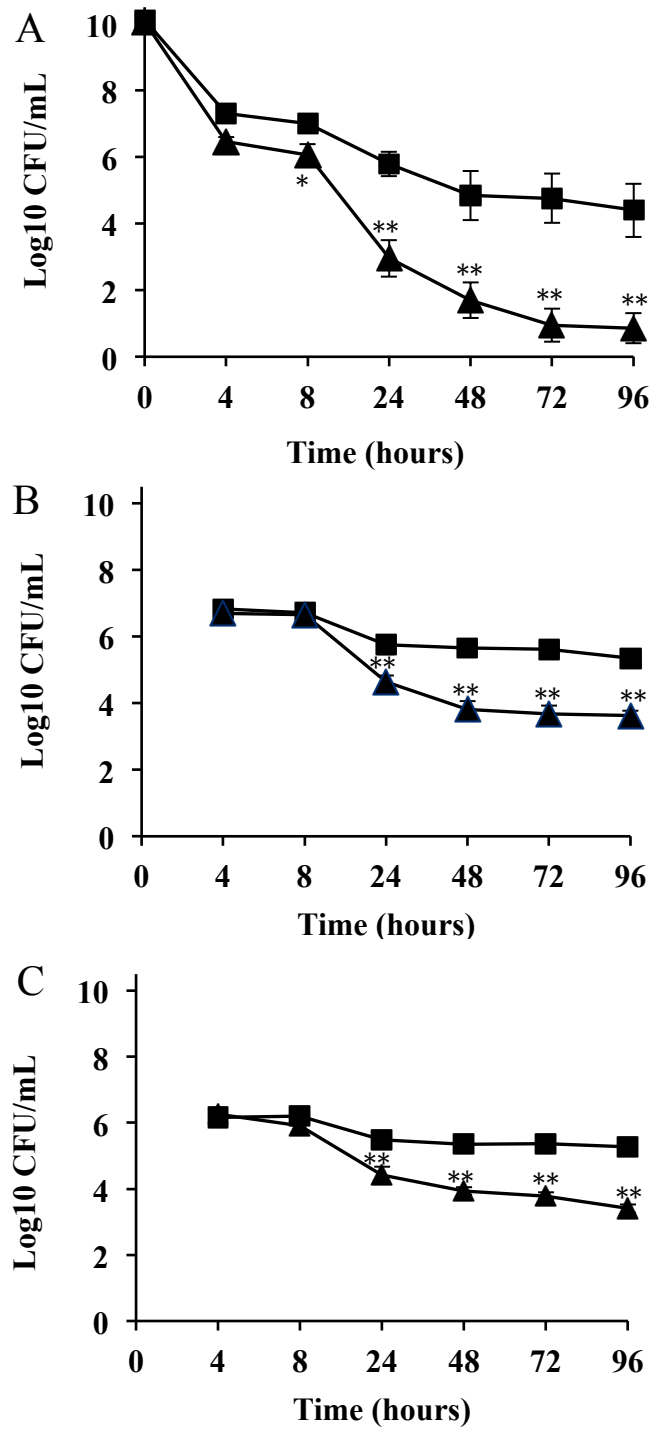


Figure 5