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

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Abstract

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

Introduction

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

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

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

As a common commensal and pathogen, *S. aureus* must resist the human host's innate defences that have evolved to limit its *in vivo* growth and spread. In particular, bile represents a major challenge to bacteria that survive transit through the stomach and enter the intestines. Bile is a digestive secretion that plays an essential role in emulsification and solubilisation of lipids. We have previously demonstrated survival of *S. aureus* in a human colonic model fed with physiological levels of bile (16). Resistance to bile salts has been demonstrated to be important for intestinal survival of several enteric pathogens, but in *S. aureus* such an understanding is lacking. The role of the *S. aureus mnhABCDEFG* locus in bile resistance was identified using a Tn*917* library screened for bile-sensitive mutants. MnhF is homologous to mammalian bile salt transporters, thus we hypothesized that it was involved in bile resistance and therefore survival of *S. aureus* in conditions modeling the human colon.

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

Materials and Methods

Bacteria, plasmids and growth conditions

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

Determination of minimum inhibitory concentration (MIC)

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

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

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

Generation of an in-frame *mnhF* mutant.

For the Δ*mnhF*, DNA fragments corresponding to *c*. 0.7 kb upstream and downstream of *mnhF* were amplified using Pwo polymerase (Roche) with primers Δ*mnhF*LFor/Δ*mnhF*LRev and Δ*mnhF*RFor/Δ*mnhF*RRev (Table 3). Following purification, PCR products were digested with *Bam*HI/*Eco*RI and cloned into pMAD. The resulting plasmid was used to transform electrocompetent *S. aureus* RN4220 (24). Plasmids were transduced into SH1000 using φ11 phage. The temperature sensitive nature of plasmid replication was exploited to integrate the plasmid into the bacterial chromosome, by plating cells on media containing erythromycin and lincomycin at 42ºC. After further rounds of plating, erythromycin and lincomycin sensitive colonies were isolated and the loss of *mnhF* confirmed by PCR.

Cloning and expression of *mnhF*.

The *mnhF* gene was amplified by PCR with *S. aureus* SH1000 DNA. For cloning into *S. aureus, mnhF*For2 and *mnhF*Rev (Table 3) were used. PCR products were digested with *Eco*RI and *Bam*HI and ligated into similarly digested pRMC2. This created pMnhF2, where *mnhF* is fused to $P_{xyl/tetO,}$ which is under the control of TetR and induced with anydrotetracycline. For cloning into *E. coli*, oligonucleotides *mnhF*For1 and *mnhF*Rev (Table 3) were used. PCR products were digested with *Eco*RI and *Bsp*HI and ligated into similarly digested pBAD/His A. This created pMnhF1, where *mnhF* is fused to $P_{BAD,}$ which is under tight control of AraC.

Bile salt accumulation assay.

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

washed twice in 25mM potassium phosphate buffer (pH 7.0) containing 1mM $MgSO_4$ and resuspended in same buffer to a concentration of 100 OD units/mL. One μCi of ^{14}C labelled cholic acid (American Radiolabelled Chemicals) with specific radioactivity of 55 mCi/mmol was added, to a final concentration of 18 μM, cells were incubated at 37°C for 2 h. Cells were then diluted to 10 OD units/ml in 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$, 20 mM glucose and 0.2 mM non-radiolabelled cholic acid, and incubated at 37°C. Incorporation of radiolabelled cholic acid was measured by scintillation counting. At the indicated time, 250 μl cells were centrifuged at 16,000g for 2 min, and the pellets resuspended in 500 μl of sterile water and 3 ml of Ulitma Gold scintillation cocktail (Perkin Elmer). CPM were counted in a Beckman LS 6500 Coulter liquid scintillation counter.

Quantitative real-time PCR.

mRNAs from mutant and wild type strains were quantified using quantitative real-time PCR (qRT-PCR). Cells were grown in triplicate as described above, then treated with RNA protect (Qiagen) and RNA was isolated using the Qiagen RNeasy Mini kit. DNA was removed using Turbo DNase-free (Life Technologies). Purified RNA was quantified using a nonodrop ND-1000 spectrophotometer (Thermo Scientific). 0.5 μg of RNA was reverse transcribed using the Tetro cDNA synthesis kit (Bioline). qRT-PCR was performed using the Aligent qPCR System and iQ SYBR Green Supermix (Biorad). The relative amounts of RNAIII mRNA in parental wild type and mutant cells was determined by relative quantification using *gyrB*, based on consistent levels observed in previous studies (26, 27, 28, 29). The oligonucleotides used for qRT-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_2, 10 % CO_2 and 80 % N_2) 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 10^2 to 10^9 CFU/ml) in triplicate for
161 each time point to measure bacterial counts.

162

Statistical analysis

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

Results

Identification of a bile salt resistance locus.

Genes conferring resistance to bile were identified by replica plating *S. aureus* SH1000 Tn*917* insertion libraries on BHI agar and onto BHI agar containing 18% (w/v) bile salts (Oxoid), which represented 0.8 × MIC. Six colonies were unable to grow in the presence of bile salts, but exhibited no growth defect on BHI agar in the absence of bile. Sequencing of the genomic DNA flanking the transposon insertion site of bile sensitive strains was carried out in order to identify the DNA insertion sites of Tn*917*, revealing that all six strains were siblings containing the transposon inserted in the same gene, namely the previously described *mnhA*, the first gene in the polycistronic *mnhABCDEFG* operon which encodes a Na^+/H^+ antiporter (31). *Bacillus subtilis* contains the orthologous *mrpABCDEFG* operon that has an identical function, however *mrpF* and by extension *mnhF*, are homologous to mammalian bile transporters and *mrpF* mediates cholic acid efflux (32, 33).

MnhF mediates resistance to bile salts.

We hypothesized that MnhF was responsible for the observed bile salt resistance phenotype. To test this, an in-frame Δ*mnhF* strain was created in *S. aureus* SH1000. 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 Δ*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 Δ*mnhF* strain was significantly more sensitive than the parent. In
195 the presence of 1 μg/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_{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-β-naphthylamide (PAβN), 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 PAβN reduced the MIC for all three unconjugaged bile salts (Table 6A),
220 however the reduction was much smaller in the Δ*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), PAβN 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}C-radiolabelled cholic acid substrate, similar to previous efflux assays
231 (25, 34, 35). *S. aureus* SH1000 and Δ*mnhF* strains were incubated with ^{14}C-cholic
232 acid (uptake period) and then diluted in buffer containing excess of non-radiolabelled
233 cholic acid (efflux period). Initial ^{14}C-cholic acid uptake was the same for both
234 strains (10962 ± 550 cpm for *S. aureus* SH1000 and 10278 ± 278 cpm for *S. aureus*
235 Δ*mnhF*), but throughout the efflux period *S. aureus* Δ*mnhF* retained significantly
236 more of the radiolabel than the parental wild-type (Fig 3A). To further corroborate

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

Bile salt resistance is not affected by *agr*.

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

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

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

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

After inoculating vessel 1 (which models the proximal colon) of the colonic models with *S. aureus* to a concentration of *c.* 2×10^{10} cfu/mL, as a single dose, the *S. aureus* populations stabilised at 6 to 7 Log_{10} units over a period of up to 8 hours. Survival of *S. aureus* Δ*mnhF* was significantly attenuated compared to its parental strain in all three vesels (Fig 5A-C).

Discussion

A complex set of interactions exists between *S. aureus* and its human host as the bacterium is able to colonize several niches, both as an opportunist pathogen of great 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 ant C-3, C-7 and C-12, and 7α/β-dehydroxylation (43, 44). Thus the
304 normal commensal inhabitants of the human gastrointestinal tract such as
305 *Lactobacillus, Propionibacterium* and *Bifodobacterium*, 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

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

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

In order to confirm that this increase sensitivity of *S. aureus* translated into a decreased ability of *S. aureus* to survive under conditions found in the human colon, 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 Δ*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
S. aureus SH1000	Wild type	(17)
S. aureus SH1001	*agr* mutation in SH1000	(17)
S. aureus RN4220	Accepts *E. coli* DNA	(18)
S. aureus mnhA::Tn*917*	Tn*917* inserted into *mnhA* in SH1000	This study
S. aureus Δ*mnhF*	Δ*mnhF* mutation in SH1000	This study
E. coli Top10	*F- mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) φ80*lacZ*Δ*M15* Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL*(*Str*^{R}) *endA1* λ$^-$	Invitrogen
E. coli TG1	F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*] *supE thi-1* Δ*(lac-proAB)* Δ(*mcrB-hsdSM*)*5,* ($r_K^-m_K^-$)	Lucigen

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633

634 **Table 2. Plasmids**

Plasmid name	Description	Antibiotic resistance	Source or Reference
pLTV1	Carries Tn*917*	Em^R/Tc^R	(19)
pMAD	Temperature sensitive (30°C) *E. coli – S. aureus* shuttle vector. $pE194^{ts}$::pBR322	Em^R	(20)
pBAD His A	Expression vector containing *araBAD* promoter	Ap^R	(21)
pRMC2	*S. aureus* expression vector	Ap^R/Cm^R	(22)
p∆*mnhF*	Vector for ∆*mnhF* mutation	Em^R	This study
pMnhF1	pBAD His A containing *mnhF* internal fragment	Ap^R	This study
pMnhF2	pRMC2 containing *mnhF* internal fragment	Ap^R/Cm^R	This study

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

Name	**Sequence 5'-3'**
Δ*mnhF*LFor	CCAAAAGGATCCGATCTTAATAAC
Δ*mnhF*LRev	CATTAGAATTCATTATATTTCGCCCACC
Δ*mnhF*RFor	TATGGAATTCGGTAAGGTGATTGAAC
Δ*mnhF*RRev	GCGATTGCGGATCCCTGTATGCC
*mnhF*For1	GGGCGAAATATCATGAATCATAATG
*mnhF*For2	GGGCGAAATAGGATCCATCATAATG
*mnhF*Rev	TGATGAATTCGATAAGTGCAAGACTAATC
RNAIIIFor	ACATGGTTATTAAGTTGGGATGG
RNAIIIRev	TAAAATGGATTATCGACACAGTGA
*gyrB*For	ATCGACTTCAGAGAGAGGTTTG
*gyrB*Rev	CCGTTATCCGTTACTTTAATCCA

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644 **Table 4. MICs of bile salts for *S. aureus* SH1000 and Δ*mnhF***

Bile salt	Wild type (mM)	Δ*mnhF* (mM)	Δ*mnhF* [pMnhF2] (mM)	Δ*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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Table 5. MICs of bile salts for wild type and recombinant *E. coli* strains expressing MnhF at different levels of arabinose induction

Bile salt	Wild type		Vector control		Recombinants					
	TG1	TOP10	TG1 pBAD	TOP10 pBAD	TG1 pMnhF1			TOP10 pMnhF1		
					0% Arabinose	0.02% Arabinose	2% Arabinose	0% Arabinose	0.02% Arabinose	2% 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

NOTE. CA, sodium cholate; DCA, sodium deoxycholate; CDCA, sodium chenodeoxycholate; GCA, sodium glycocholate; TCA, sodium taurocholate.

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

A.

Bile salt	***S. aureus* SH1000 (mM)**			***S. aureus* Δ*mnhF* (mM)**		
	Control	[a]PAβN	[a]Reserpine	Control	[a]PAβN	[a]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

B.

Bile salt	***E. coli* TG1 (mM)**			***E. coli* TG1 pMnhF1 (mM)**		
	Control	[a]PAβN	[a]Reserpine	Control	[a]PAβN	[a]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

NOTE. CA, sodium cholate; DCA, sodium deoxycholate; CDCA, sodium chenodeoxycholate; GCA, sodium glycocholate; TCA, sodium taurocholate; PAβN, Phe-Arg-β-naphthylamide. [a]PAβN at 20 μg/ml and Reserpine at 40 μg/ml.

690 **Figure Legends**

691 **Figure 1. MnhF protects *S. aureus* against the bactericidal activity of bile salts.**
692 Viability of *S. aureus* SH1000 [■], Δ*mnhF* [▲], Δ*mnhF* (pMnhF2) [◆], Δ*mnhF*
693 (pRMC2) [×] treated with (A) 2 mM CA, (B) 0.25 mM DCA, and (C) 20 mM GCA.
694 Data represents mean ± standard error of mean from three independent experiments.
695 $^{*}P<0.01$, $^{\dagger}P>0.05$.

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 ± 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 Δ*mnhF* [▲] cells were loaded with 1 μCi of ^{14}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 μCi of ^{14}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 ^{14}C-cholic acid in cell pellets
712 were determined by liquid scintillation counting. Data represents mean ± 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 ± 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 Δ*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 (Log_{10} CFU/mL)
725 of the data of three colonic models ± standard error of mean. $^{*}P<0.05$; $^{**}P<0.001$.

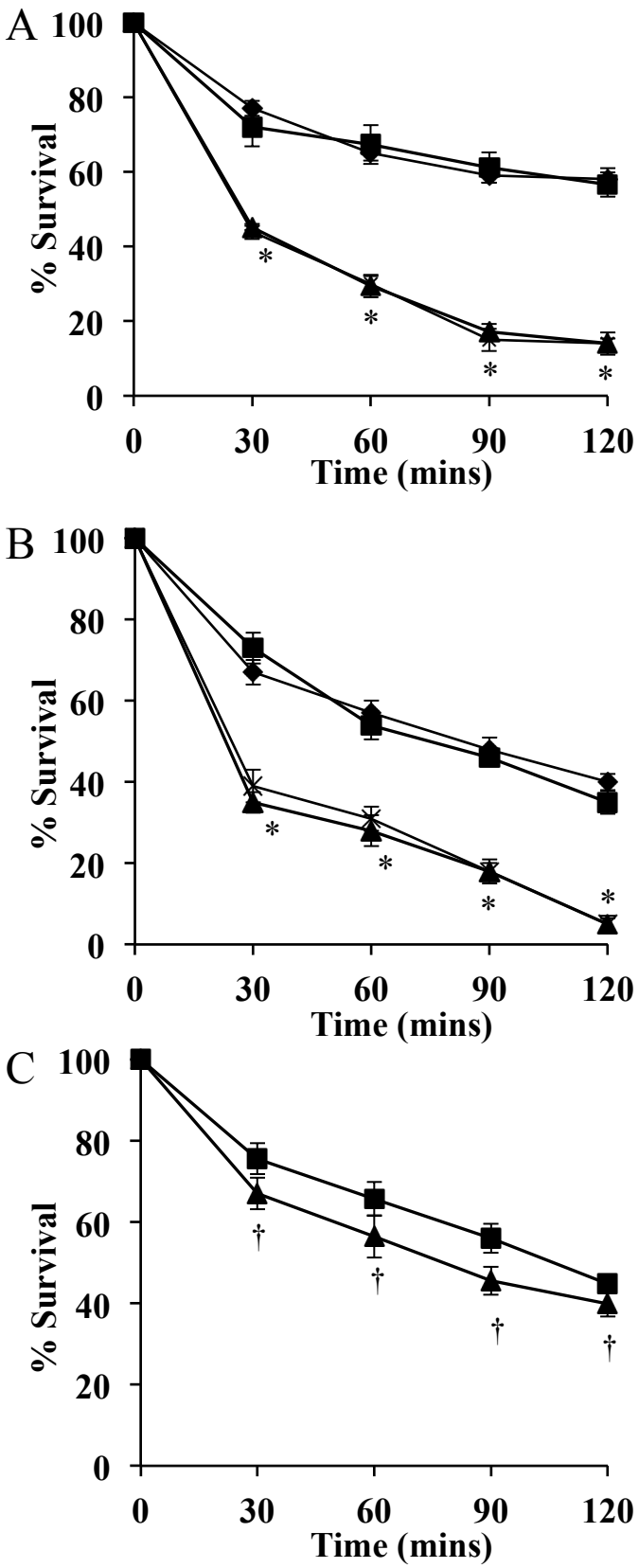
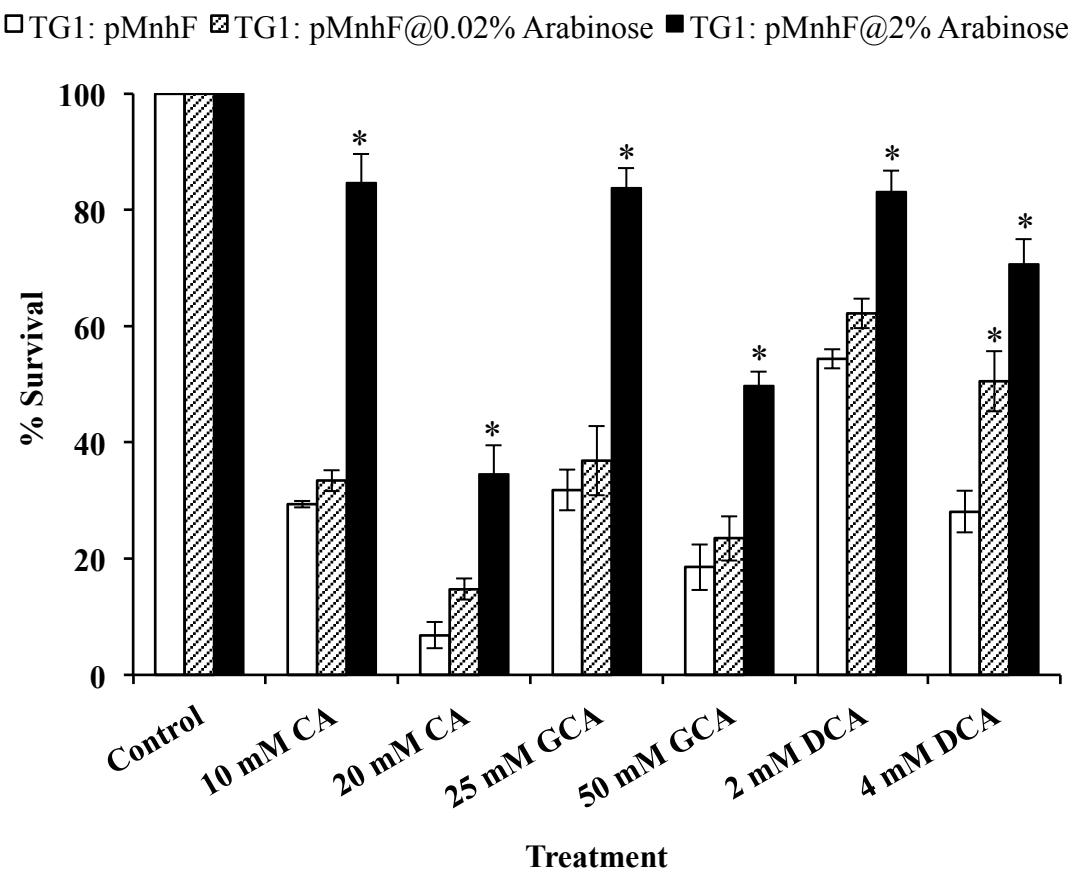


Figure 1


TG1: pMnhF
TG1: pMnhF@0.02% Arabinose
TG1: pMnhF@2% Arabinose
% Survival
100
80
60
40
20
0
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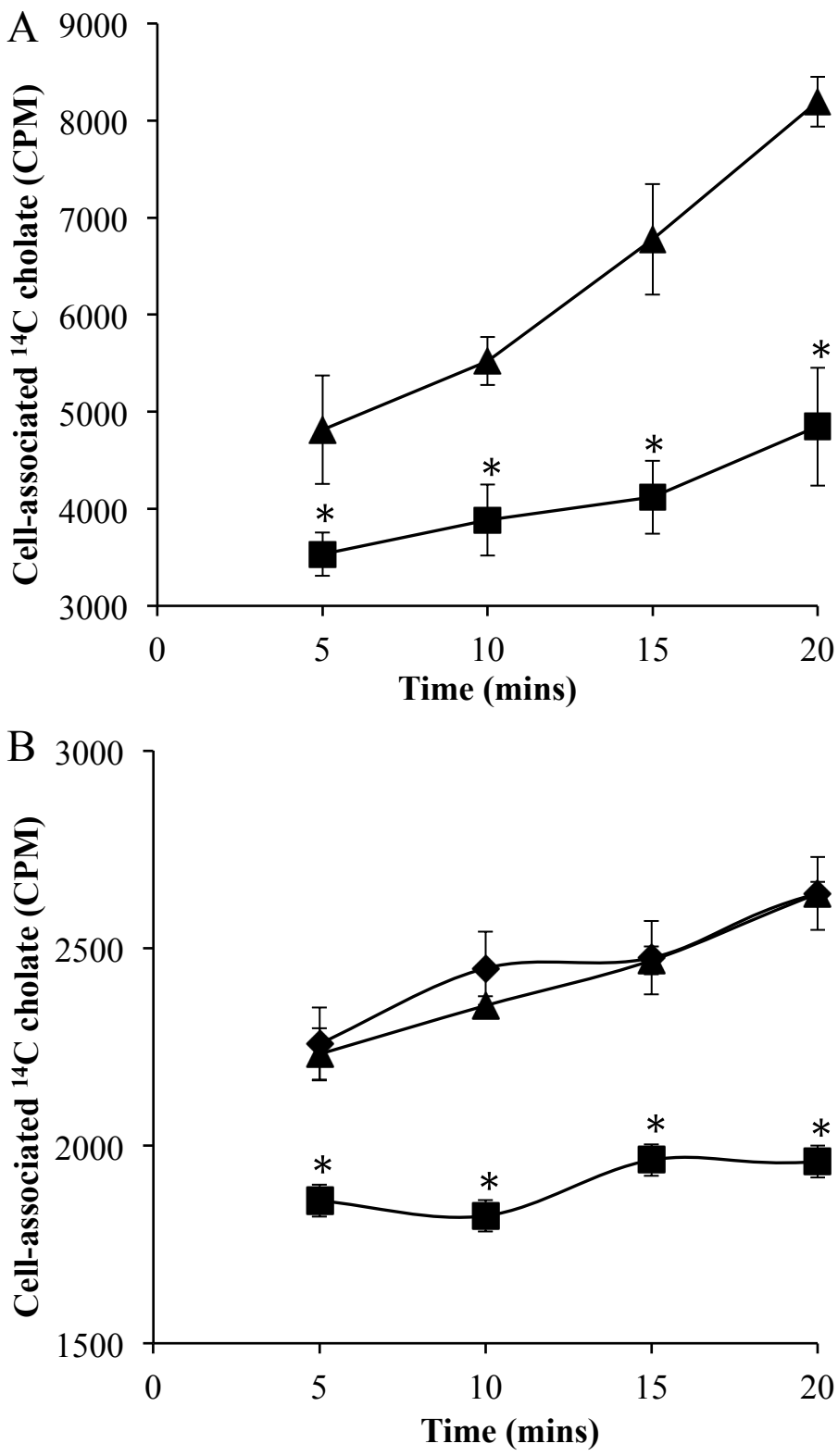
Control
10 mM CA
20 mM CA
25 mM GCA
50 mM GCA
2 mM DCA
4 mM DCA
Treatment

Figure 2


A
9000
8000
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Cell-associated ^{14}C cholate (CPM)
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Time (mins)
B
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1500
Cell-associated ^{14}C cholate (CPM)
0
5
10
15
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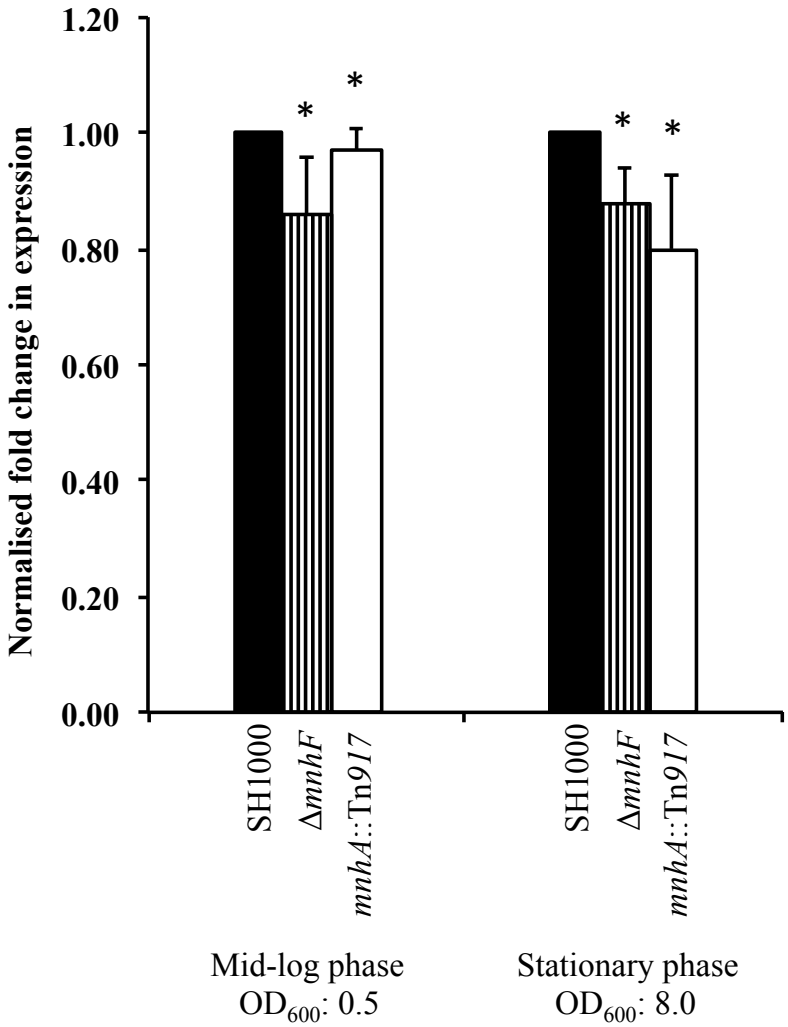
Time (mins)

Figure 3


1.20
1.00
0.80
0.60
0.40
0.20
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Normalised fold change in expression
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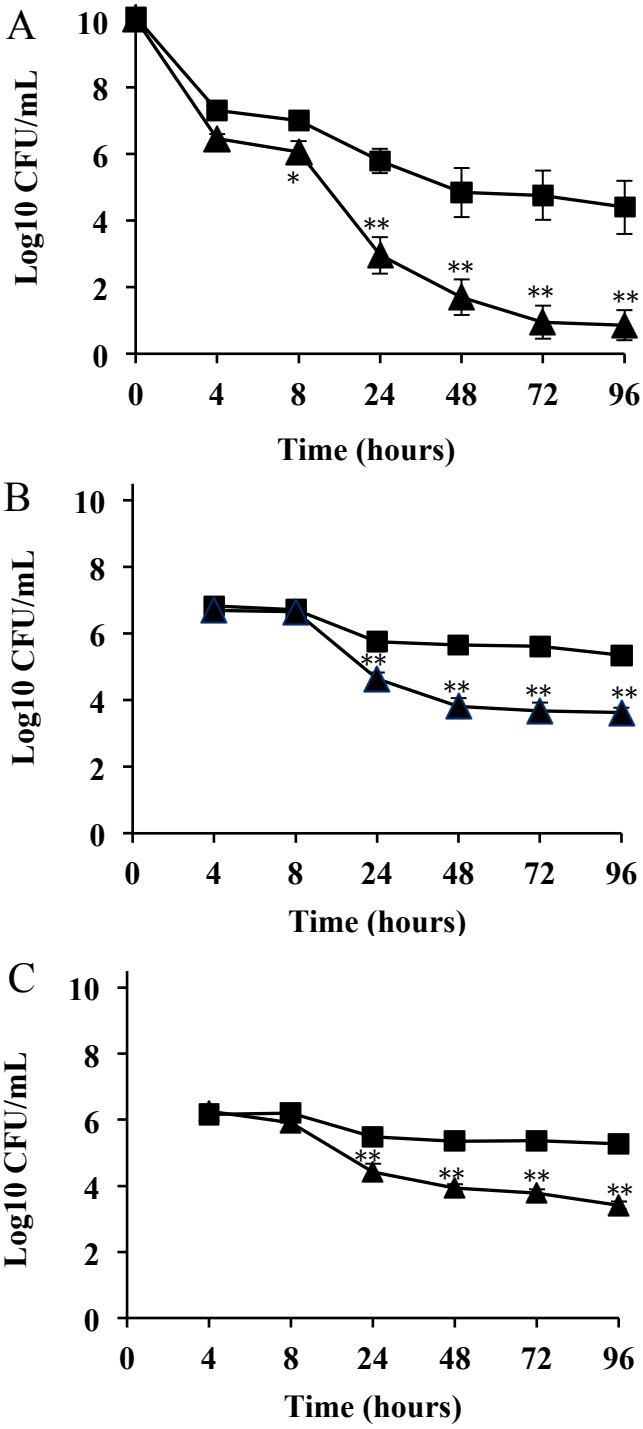
SH1000
ΔmnhF
mnhA::Tn917
SH1000
ΔmnhF
mnhA::Tn917
Mid-log phase
OD600: 0.5
Stationary phase
OD600: 8.0

Figure 4


A
Log10 CFU/mL
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6
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Time (hours)
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B
Log10 CFU/mL
Time (hours)
C
Log10 CFU/mL
Time (hours)

Figure 5