JB Accepts, published online ahead of print on 1 July 2011
J. Bacteriol. doi:10.1128/JB.00200-11

Bile salts induce resistance to polymyxin in Enterohemorrhagic *Escherichia coli* O157:H7

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Many enteric bacteria use bile as an environmental cue to signal resistance and virulence gene expression. Microarray analysis of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) treated with bile salts revealed up-regulation of genes for an efflux system (*acrAB*), a two-component signal transduction system (*basRS/pmrAB*) and lipid A modification (*arnBCADTEF* and *ugd*). Bile salt treatment of EHEC produced a *basS*- and *arnT*-dependent resistance to polymyxin.

Enterohemorrhagic *Escherichia coli* (EHEC), including serotype O157:H7, cause severe food-borne illness associated with diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (17, 29). Upon ingestion, on route to the colon, bacteria encounter a variety of antimicrobial stresses, including gastric acids in the stomach (27), and bile in the duodenum and small intestine. Bile is a complex mixture, composed mainly of bile salts, as well as phospholipids, cholesterol, proteins, and bilirubrin (15). Bile salts are amphipathic molecules that act as detergents aiding in lipid solubilisation and digestion, but also play a role in host defence as they have potent antimicrobial properties (26). For this reason, bile-resistance is an essential characteristic of enteric bacteria, and is achieved primarily via active efflux mechanisms (6, 32, 35, 53, 64) and altered permeability of the outer membrane (64, 70). The RND efflux systems have been well described as playing a significant role in bile-resistance amongst Gram-negative bacteria (45). Additionally, use of two-component regulatory systems (TCRS) (52, 68) and alterations of the lipopolysaccharide (LPS) layer have been shown to be involved in resistance to bile in several bacteria (8, 42, 43, 49, 71).

Bile has also been demonstrated to be an environmental signal that controls the expression of colonization and virulence factors of several enteric bacteria (13, 27, 28, 31, 36, 50, 51, 54, 55, 65). Much of the work on Gram-negative bacteria's response to bile has been performed in *Salmonella* (9, 44, 51-54, 57, 59, 68). Since marked differences in gene expression after bile stress have been observed even between *Salmonella typhimurium* and *Salmonella typhi,* differences may also exist in EHEC (68). Thus, here we sought to investigate the response of *E. coli* O157:H7 to bile salt stress and the influence bile salts have on bile resistance mechanisms and virulence gene expression.

Transcriptional analysis of bile salt-treated EHEC. The bile salt-stress protocol used here was modified from de Jesus et al. (2005) (13). Briefly, bacteria were grown in Luria Bertani media at 37˚C with shaking overnight, followed by sub-culturing into DMEM pH 7.4 and static incubation at 37˚C, in 5% CO_2 until OD_{600}=0.4. Bacteria were then gently pelleted by centrifugation and the media was replaced with either Dulbecco's Modified Eagle's Medium (DMEM; Wisent) pH 7.4, or 0.15% bile salt mixture (BSM; Sigma B-3426) in DMEM pH 7.4. These cultures were statically incubated at 37˚C, in 5% CO_2 for 90 minutes. Bacteria were then harvested for analysis or additional treatments. Initially, we used microarray-based expression profiling of EHEC strain 86-24 (MWG *E. coli* O157:H7 array GenBank accession number GPL533, (27)) in both the presence and absence of BSM. RNA purification and microarray analysis were performed as in House *et al*. (2009). Computational analysis of four control and four BSM-treated EHEC RNA samples, on four microarrays was performed by the University Health Network Microarray Center (Toronto, Ontario) and significance was determined by Significance Analysis of Microarrays (SAM) analysis and t-tests. The complete data set is available at NCBI Gene Expression Omnibus Series accession number GSE22060 (14). Our analysis showed that 30 genes were up-regulated (Table 1) and 35 genes were down-regulated 1.5-fold or more, after exposure to BSM relative to the control (Table 2). Semi-quantitative reverse transcriptase PCR (as in (66)) was used to confirm several up- regulated genes of interest (data not shown). Promoters of genes of interest were identified using the RegulonDB online database (16), and cloned into the promoter-less β-galactosidase expression vector pMC1403 (5). β-galactosidase reporter assays (5) were performed using a variety of conditions to further examine the bile-responsiveness of promoters of interest (Figure 1).

Bile salts alter expression of genes for efflux systems and porins. Microarray analysis revealed that genes encoding the AcrA-AcrB RND efflux pump and its regulator (*acrA*, *acrB* and *acrR*) were up-regulated in EHEC by BSM (Table 1). This efflux system has been shown to be a crucial component of bile resistance in *E. coli* K-12 and *S. typhimurium*, as it actively pumps bile out of the cell (32, 44, 45, 53, 64). Using β-galactosidase assays (2) we further demonstrated that the *acrAB* promoter showed a concentration-dependent response to BSM (Figure 1A). Bile has previously been demonstrated to pass into the periplasm of *E. coli* via the OmpF outer membrane porin channel (64). Our microarray results show that BSM-treatment down-regulates expression of *ompF* (Table 2). Combined, these data demonstrate that EHEC employs several bile resistance mechanisms that are similar to other Gram-negative bacteria and that our bile salt treatment is effective at eliciting a bona-fide physiological response to bile.

Bile salts do not induce Shiga-toxin expression or release. Bile has been demonstrated to induce expression of *Vibrio cholerae* cholera toxin in the small intestine (28). This toxin is responsible for the severe dehydrating diarrhea associated with cholera (48). EHEC produces similar toxins, known as verotoxins or Shiga toxins (Stx1 and Stx2), which are key virulence factors of the pathogen and are associated with the diarrhea, HC, and HUS characteristic of EHEC infection (4, 10, 58, 61). These toxin genes are located on lambdoid prophages integrated into the bacterial genome (41, 60). Our microarray analysis showed that the genes which encode both subunits (*stx2A*, *stx2B*) of this multi-subunit toxin were slightly down-regulated by bile treatment relative to our control (Table 2). Additionally, five other genes associated with the Stx2 bacteriohage BP-933W were similarly down-regulated, indicating that bile treatment does not induce expression of these phage genes in EHEC. This result was supported by an experiment in which we exposed EHEC to various bile salt treatments (glycocholate,

deoxycholate, chenodeoxycholate, ursodeoxycholate, and BSM) and evaluated periplasmic and secreted levels of Stx2 using a well-established Vero cell cytotoxicity assay (as in (27)). We found no increase in periplasmic or secreted Stx2 after treatment of EHEC with individual bile salts (2.5 mM) or the 0.15% BSM relative to the untreated control (Figure S1).

This microarray also indicated no change in the expression of other known EHEC virulence factors, including those in the locus of enterocyte effacement (LEE) pathogenicity island, after BSM exposure (Table 1). Thus, although bile acts a signal for virulence gene expression in other bacteria, it does not appear to do so in EHEC under the conditions used in this study.

The BasR-regulated genes for lipid A modification are up-regulated by bile salts. While efflux is a vital means of resisting the deleterious effects of bile, limiting penetration by altering the composition of the outer membrane is an additional strategy used by many bacteria (42, 49, 57). The genes encoding the BasR-BasS (also known as PmrA-PmrB) histidine kinase TCRS were up-regulated by BSM-treatment on our microarray, and by our confirmatory methods (Table 1, Figure 1B). TCRSs sense and respond to environmental signals producing physiological changes in bacteria (reviewed in (30)). Regulation of *basR-basS* expression has not previously been linked to bile in *E. coli* or *Salmonella spp.,* but has been associated with other stresses including metal ion stress (7, 23, 33, 46, 62, 73) and mild acid stress (25, 62). Here, we established that the *basRS* promoter follows a concentration-dependent response to BSM treatment (Figure 1B). BasR (PmrA) is known to control expression of the *arnBCADTEFD* (also known as *pmrHFIJKLM*) operon, members of which along with *ugd* are responsible for the synthesis and transfer of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A (56). Our

transcriptome analysis showed up-regulation of all members of the *arn* operon and *ugd* by treatment with BSM (Table 1). Additionally, a concentration-dependent response was also observed for promoters of *arnB* and *ugd* using a β-galactosidase reporter assay (Figure 1C, D). Inactivation of *basS* did not affect bile-response of the *acrAB* promoter (Figure 1A) but did abrogate that of the *arnB* operon and *ugd* (Figure 1C, D), providing further evidence that the BSM is eliciting the expression of these lipid A modification genes. Interestingly, the *basRS* promoter lost the ability to respond to BSM in absence of *basS* as BSM-induced expression of the reporter gene was lost in the *basS*::Kan^{R} mutant (Figure 1B). This suggests that BasS may function in its self-regulation in response to bile.

Exposure to bile salts confers EHEC resistance to polymyxin B. The addition of L-Ara4N to lipid A has been shown to confer resistance to Gram-negative bacteria to several cationic antimicrobial peptides (CAMPs), including polymyxin B (PMB), a peptide antibiotic, often used to study antimicrobial peptide resistance (19, 20, 37, 38, 40, 63, 67, 74). The lipid A modifications, controlled through the BasRS (PmrAB) TCRS, in both *E. coli* and *Salmonella spp*. are essential for resistance to polymyxin B (PMB), however in neither organism does it appear that these modifications are required for resistance to bile itself (68) (Figure 2). Therefore, at least in the case of EHEC, bile may be acting as an environmental signal which triggers outer membrane modifications for resistance to CAMPs of the within the small intestine.

Paneth cells within the small intestine produce CAMPs known as defensins, as part of the innate immune system (1, 3, 11, 47). CAMPs are attracted to negative charges of the outer membrane; in Gram-negative bacteria they function by penetrating this membrane and disrupting the inner membrane (1, 34, 69, 72). Lipid A is an anionic molecule that contributes to the

130 negative charge of the outer membrane. Modification of the outer portion of Lipid A with of L-
131 Ara4N reduces the negative charge resulting in resistance to several CAMPs. Gunn *et al*.
132 demonstrated that in *S. typhimurium* these lipid A modifications, regulated by PmrA-PmrB, were
133 required for resistance to PMB (18, 20). *Pseudomonas aeruginosa* mutants which constitutively
134 expressed *pmrB* (*basS*) were observed to not only be resistant to PMB, but also be cross-resistant
135 to α-defensins, β-defensins-1 and -2, α-helical peptides and proteginl (40). Enteric bacteria
136 encounter defensins within the small intestine. Therefore, since we observed the genes associated
137 with L-Ara4N modification of lipid A are up-regulated by BSM-treatment in EHEC, we asked
138 whether BSM treatment could induce resistance to PMB. Using a broth microdilution method,
139 we first determined the minimum inhibitory concentration (MIC) of PMB (Sigma, P0972) for
140 EHEC 86-24 in our system to be 0.15 µg/ml. Bacteria were then cultured in LB in the presence
141 or absence of 0.15% BSM overnight then sub-cultured in the same treatment ("pre-treatment"),
142 and incubated under static conditions at 37˚C, 5% CO_2 for 3-4 hours, then washed with PBS.
143 Bacteria ($1x10^6$ cfu/ml) were resuspended in a "challenge" media, either LB, LB + 0.15% BSM,
144 or LB + 0.15 µg/ml PMB for one hour at 37˚C with shaking, then quantified by serial dilutions
145 and plating (Figure 2). Although these growth conditions varied slightly from the initial
146 microarray experiment, beta-galactosidase expression assays demonstrated that the promoters of
147 our genes of interest displayed similar trends of up-regulation (data not shown). Notably, pre-
148 treatment with BSM significantly improved the ability of EHEC 86-24 to survive a lethal
149 concentration of PMB (Figure 2A). Conversely, when the same experiment was performed with
150 an EHEC *basS*::Kan^R mutant, BSM pre-treatment failed to induce resistance to PMB (Figure
151 2B). This is further evidence that BasS is a sensor for bile salts, and suggests that in its absence
152 EHEC cannot respond with the lipid A modifications that protect it from PMB. As *arnT* encodes

153 the enzyme that transfers L-Ara4N to lipid A (67) the same experiment was performed in an
154 EHEC *arnT* disruption in order to determine if this is the modification that results in BSM-
155 induced PMB resistance and not another downstream BasS target. Significantly, bile-induced
156 resistance to PMB was abrogated by inactivation of *arnT* (Figure 2C), and restored when the
157 *arnT* mutation was complemented (Figure S2B), providing physiological evidence that this
158 biochemical pathway is induced by BSM and that it results in resistance to PMB likely due to L-
159 Ara4N modification of lipid A.

160 To establish that the bile induced PMB resistance is not a consequence of increased
161 efflux by AcrA-AcrB, we performed the same experiment in an *acrB* disruption. We observed
162 BSM-induced resistance to PMB was not affected (Figure 2D), however the BSM pre-treatment
163 was observed to effect overall bacterial viability, pointing to the significant role this efflux
164 system has in bile-resistance. Interestingly, in *S. typhimurium acrAB* mutants are killed by even
165 low concentrations of bile (53), however here we see this is not the case in EHEC. Thanassi *et al.*
166 also observed that while an E.coli K-12 *acrA* mutant was hypersensitive to bile, this and an *acrA-*
167 *emrB* double mutant were still able to survive under bile stress (64). The authors remarked that
168 an additional, unknown efflux system(s) must be in place in *E. coli* for managing bile.

169 We have demonstrated increased transcription of BasSR (PmrAB) and their down-stream
170 targets, the L-Ara4N lipid A modification genes, in response to bile in EHEC. In contrast, in
171 *Salmonella,* neither PmrAB or its regulator PhoPQ have been shown to be up-regulated in
172 response to bile, although interestingly both TCRSs appear to be important for bile and
173 antimicrobial peptide resistance (18, 18, 20-22, 68). Merighi *et al.* demonstrated in an *in vivo*
174 expression system that both the *phoPQ* and *pmrAB* operons of *S. enteric*a serovar typhimurium

were up-regulated within mouse intestinal lumen and spleen in response to an unidentified signal (39). Since the authors controlled for known inducers of these operons it is possible that bile is a signal to which at least one of these TCRSs is responding.

Our data are consistent with a model where bile salts in the small intestine serve as an environmental signal for EHEC, one that triggers changes in gene expression which result in protective alterations of the outer membrane, thereby permitting successful transit through the small intestine. We report, for the first time, that bile causes up-regulation of the BasR-BasS TCRS, the L-Ara4N LPS alteration pathway and concomitant antimicrobial resistance in EHEC. These findings offer insights into potential strategies used by EHEC to resist the antimicrobial effects of bile and CAMPs of the small intestine.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE22060 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22060).

Acknowledgements: This work was supported by funding to JVK through a NSERC Post Doctoral Fellowship and a Ryerson University Post Doctoral Fellowship; to VD through a NSERC-USRA, and to DBF through NSERC Discovery Grant #238684. We thank Dr. Victor Gannon (Public Health Agency of Canada) for the microarray chips, Dr. Jorge Giron for EHEC strain 86-24 (University of Florida), Dr. Brett Finlay (University of British Columbia) for

196 pMC1403, Dr. Lori Burrows (McMaster University) for pBADGr, and Dr. Jeffery Fillingham for
197 helpful discussions and suggestions for the manuscript.

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406

Table 1. Summary of EHEC 86-24 transcripts with 1.5-fold or more increase in expression after bile salt treatment relative to untreated control (N=4 independent cultures (4 treatment, 4 control), N=4 chips, n=2 replicate spots per chip) as determined by SAM analysis. P-values were determined using one-way Student's t-Test. Bolded genes names indicate gene in operons in which increased expression was verified with β-galactosidase reporter assay; * indicates increased expression verified with semi-quantitative RT-PCR.

Gene Symbol/ID	Name & Predicted Function	Fold-Change	p-value
ais	protein induced by aluminum; function unknown	6.85	<0.005
arnC	undecaprenyl phosphate-L-Ara4FN transferase	6.39	<0.001
arnD	undecaprenyl phosphate-alpha-L-ara4FN deformylase	4.46	<0.001
hycF	formate hydrogenlyase complex iron-sulfur protein	3.43	<0.005
hycB	hydrogenase 3, Fe-S subunit	3.21	<0.005
hydN	putative electron transport protein HydN/Iron-sulfur protein required for Hyd-3 activity	2.90	0.022
arnT*	4-amino-4-deoxy-L-arabinose (L-Ara4N) transferase	2.65	0.005
arnB	UDP-4-amino-4-deoxy-L-arabinose synthase; UDP-4"-ketopentose aminotransferase; L-glutamate is the amine donor	2.63	0.028
hycA	transcriptional repression of *hyc* and *hyp* operons	2.59	0.011
arnF	undecaprenyl phosphate-alpha-L-ara4N exporter; flippase ArnEF subunit	2.45	0.0066

acrR	*acrAB* operon repressor	2.37	0.015
fdhF	formate dehydrogenase	2.36	0.014
ugd*	UDP-glucose 6-dehydrogenase	2.16	<0.001
basS*	sensory histidine kinase in two-component regulatory system with BasR	2.13	0.0057
eptA	predicted metal-dependent hydrolase/Lipid A phosphoethanolamine transferase, associated with polymyxin resistance	2.12	0.031
hycI	protease involved in processing C-terminal end of the large subunit of hydrogenase 3	2.05	<0.001
hycG	component of hydrogenase 3; formate hydrogenlyase complex	2.04	0.01
arnA	UDP-glucuronate dehydrogenase and UDP-ara4N formyltransferase	2.01	0.031
basR	DNA-binding response regulator in two-component regulatory system with BasS	2.00	<0.005
hycD	Formate hydrogenlyase complex inner membrane protein	1.91	0.026
arnF	undecaprenyl phosphate-alpha-L-ara4N exporter; flippase ArnEF subunit	1.90	<0.005
acrA*	membrane fusion protein/Component of AcrAB-TolC multidrug efflux system/acridine efflux pump	1.82	<0.005

acrB	AcrB RND-type permease/Component of AcrAB-TolC multidrug efflux system	1.75	<0.005
hycC	formate hydrogenlyase complex inner membrane protein	1.72	0.044
yeeF	putative amino acid/amine transport protein; *yeeF*; required for swarming phenotype, function unknown	1.65	<0.005
gatY	D-tagatose 1,6-bisphosphate aldolase 2, catalytic subunit	1.56	0.019
hycE	hydrogenase 3, large subunit	1.56	0.016
prmC	N5-glutamine methyltransferase, modifies release factors RF-1 and RF-2	1.55	0.017
yfbQ	predicted aminotransferase	1.51	<0.01
yehD*	predicted fimbrial-like adhesin protein; FimA homologue	1.50	<0.01

413

414 **Table 2.** Summary of EHEC 86-24 transcripts with 1.5-fold or more decrease in expression after
415 bile salt treatment relative to untreated control (N=4 independent cultures (4 treatment, 4
416 control), N=4 chips, n=2 replicate spots per chip) as determined by SAM analysis. P-values
417 presented were determined using one-way Student's t-Test.

Gene Symbol/ID	**Name & Predicted Function**	**Fold-Change**	**p-value**
Z1540	hypothetical protein	-3.21	<0.05
ymfP	pseudogene, e14 prophage	-2.77	<0.05
ompF	outer membrane protein 1a	-2.54	<0.005
ECS2038	similar to putative membrane transport protein B1433 (*E. coli*)	-1.91	<0.05
Z0273	hypothetical protein	-1.81	<0.05
yrbL	hypothetical protein	-1.80	<0.05
ECS3219	similar to B2335 (*E. coli*), minor fimbrial subunit StfE protein (*Salmonella typhimurium*)	-1.75	0.016
Z5401	hypothetical protein	-1.71	0.013
yciO	hypothetical protein	-1.71	<0.05
ydfZ	conserved protein	-1.70	0.0011
yaiS	conserved protein	-1.67	<0.005
exoP	putative exodeoxyribonuclease (crypticprophage CP-933P)	-1.67	0.012

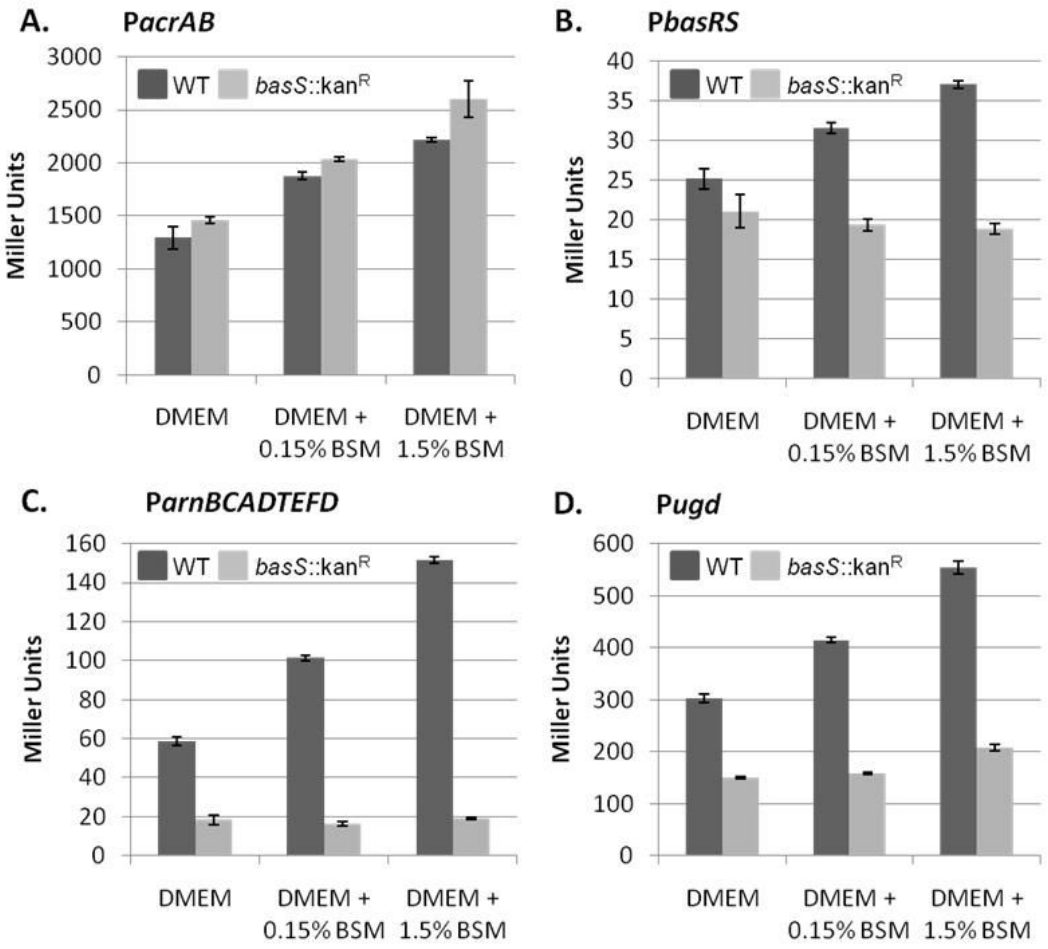
Z4067	hypothetical protein	-1.64	<0.05
proW	glycine betaine transporter membrane protein	-1.64	0.0057
ECS1528	similar to hypothetical protein (bacteriophage 933W)	-1.60	0.012
moaA	molybdenum cofactor biosynthesis protein A	-1.60	<0.05
ECS1219	similar to putative small subunit terminase (bacteriophage 933W)	-1.60	0.017
Z5162	hypothetical protein	-1.58	<0.05
terA2	putative phage inhibition, colicin resistance and tellurite resistance protein	-1.58	<0.005
ECS2283	hypothetical protein	-1.58	<0.05
ECS1211	similar to hypothetical protein (bacteriophage 933W)	-1.56	<0.05
Z1466	unknown protein (bacteriophage BP-933W)	-1.56	<0.05
Z2042	unknown protein (prophage CP-933O)	-1.56	<0.05
yaiY	predicted inner membrane protein	-1.56	<0.05
yajO	2-carboxybenzaldehyde reductase, function unknown	-1.55	<0.05
modD	molybdenum transport protein	-1.54	<0.05
Z1491	unknown protein (bacteriophage BP-933W)	-1.53	<0.05
B2640	hypothetical protein	-1.53	<0.005
ECS0337	similar to probable transcription regulator YkgA	-1.52	<0.05
terD	putative tellurium resistance protein TerD	-1.50	0.012
engA	GTP-binding protein EngA	-1.50	<0.05
stx2B	Shiga toxin 2 B subunit	-1.50	<0.05

Z2087	unknown protein (prophage CP-933O)	-1.50	0.0066
stx2A	Shiga toxin 2 A subunit	-1.50	0.017
ECS1329	hypothetical protein	-1.50	<0.05

418

419 **Figure 1.**

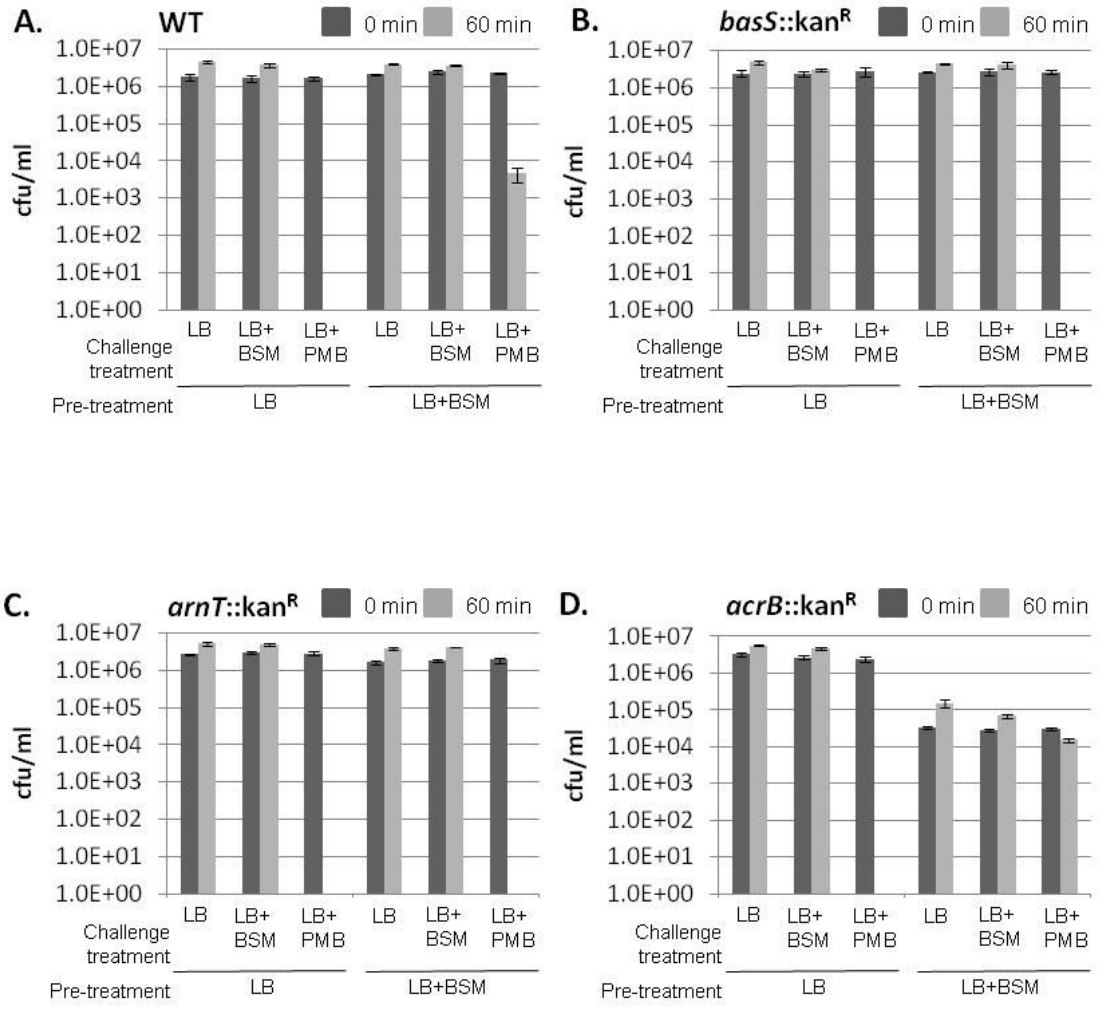
420



421

Figure 1. β-galactosidase reporter assays demonstrate EHEC promoters of efflux and lipid A remodelling operons display concentration-dependent responses to bile salts.

The activity of the promoters for *acrAB* (**A**), *basRS* (**B**), *arnBCADTEFD* (**C**) and *ugd* (**D**) were examined in beta-galactosidase expression assays in both wild-type 86-24 (WT; dark grey bars) and *basS*::kan^R (light grey bars) backgrounds. In the WT background all promoters tested showed statistically significant and reproducible enhanced responses to exposure to increasing concentrations of bile salt mix. In the *basS*::kan^R background P*acrAB* (**A**) remained responsive to the presence of bile salts, however, the activity of P*ugd* (**D**) was significantly diminished and the response of the P*basRS* (**B**) and P*arnBCADTEFD* (**C**) to bile salts was abrogated. The same responses to bile were observed with other base media (50% LB; N=3, n=4). Student's t-tests were done between control (DMEM) and each treatment, as well as between both treatments. Statistical difference ($p<0.01$) was observed between all compared treatments within the same background strain with the exception of P*basRS* and P*arnBCADTEFD* in the *basS*::Kan^R. Data shown is for one experiment, but was repeated four times with similar results; N=3, n=4.



436 **Figure 2.**

437

Figure 2. Pre-treatment of EHEC with bile salts induces a *basS* and *arnT* – dependent resistance to polymyxin B.

Bacteria were pre-treated with either LB or LB+BSM (0.15% BSM), then each was standardized and divided into three samples, and plated for quantification (time 0 minutes; dark grey bars). Bacteria were then subjected to one of three challenge treatments (LB, LB+BSM or LB+PMB), incubated for 60 minutes then plated for quantification (light grey bars). Wild-type 86-24 bacteria (A) pre-treated with BSM were able to withstand treatment with PMB, whereas the bacteria pre-treated in LB-alone were killed by challenge with PMB. This protection is lost in the *basS*::Kan^R (B) and the *arnT*::Kan^R (C) disruptions demonstrating that both *basS* and *arnT* are involved in bile salt-induced resistance to PMB. The *acrB* disruption (D) was able to resist challenge with PMB when pre-treated with bile salts, although these bacteria were more susceptible to the deleterious effects of bile salts as demonstrated by reduced levels of growth in the bile salt-treated bacteria relative to those grown in LB. N=3, n=3.