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NOTES

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 upregulation 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, causes a severe food-borne illness associated with diarrhea, hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS) (17, 29). Upon ingestion, en route to the colon, the 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 bilirubin (15). Bile salts are amphipathic molecules that act as detergents aiding in lipid solubilization and digestion but also play a role in host defense, 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 among Gram-negative bacteria (45). Additionally, the 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 with *Salmonella* (9, 44, 51–54, 57, 59, 68). Since marked differences in gene expression after bile stress have been observed even between *Salmonella enterica* serovar Typhimurium and *S. enterica* serovar Typhi, differences may also exist in EHEC (68). Thus, here we investigated 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 reference 13. Briefly, bacteria were grown in Luria-Bertani medium (LB) at 37°C with shaking overnight and then subcultured in Dulbecco's modified Eagle's medium (DMEM) at pH 7.4 and statically incubated at 37°C in 5% CO_2 until an optical density at 600 nm of 0.4 was reached. Bacteria were then gently pelleted by centrifugation, and the medium was replaced with either DMEM (Wisent) at pH 7.4 or a 0.15% bile salt mixture (BSM; Sigma B-3426) in DMEM at pH 7.4. These cultures were statically incubated at 37°C in 5% CO_2 for 90 min. 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 the absence of BSM. RNA purification and microarray analysis were performed as described by House et al. (27). 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, Canada), and significance was determined by significance analysis of microarrays (SAM) analysis and *t* tests. The complete data set is available under NCBI Gene Expression Omnibus Series accession number GSE22060 (14). Our analysis showed that 30 genes were upregulated (Table 1) and 35 genes were downregulated 1.5-fold or more after exposure to BSM relative to the control (Table 2). Semiquantitative reverse transcriptase PCR (as described in reference 66) was used to confirm several upregulated genes of interest (data not shown). Promoters of genes of interest were identified using the RegulonDB online database (16) and cloned into the promoterless β-galacto-

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TABLE 1. Summary of EHEC 86-24 transcripts with a 1.5-fold or greater increase in expression after bile salt treatment relative to that of the untreated control[a]

Gene	Product and 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 hydrogen lyase 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[b]	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[b]	UDP-glucose 6-dehydrogenase	2.16	<0.001
basS[b]	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 of the C-terminal end of the large subunit of hydrogenase 3	2.05	<0.001
hycG	Component of hydrogenase 3; formate hydrogen lyase 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 hydrogen lyase complex inner membrane protein	1.91	0.026
arnF	Undecaprenyl phosphate-alpha-L-Ara4N exporter; flippase ArnEF subunit	1.90	<0.005
acrA[b]	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 hydrogen lyase complex inner membrane protein	1.72	0.044
yeeF	Putative amino acid/amine transport protein; 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	N^5-Glutamine methyltransferase, modifies release factors RF-1 and RF-2	1.55	0.017
yfbQ	Predicted aminotransferase	1.51	<0.01
yehD[b]	Predicted fimbrial adhesin-like protein; FimA homologue	1.50	<0.01

[a] As determined by SAM analysis. $n = 4$ independent cultures (4 treatment, 4 control), $n = 4$ chips, $n = 2$ replicate spots per chip. *P* values were determined using a one-way Student *t* test. Bolded genes names indicate genes in operons in which increased expression was verified by β-galactosidase reporter assay.
[b] Increased expression verified by semiquantitative reverse transcriptase PCR.

sidase expression vector pMC1403 (5). β-Galactosidase reporter assays (5) were performed under a variety of conditions to further examine the bile responsiveness of promoters of interest (Fig. 1).

Bile salts alter the 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 upregulated 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 (Fig. 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 downregulates the expression of *ompF* (Table 2). Combined, these data demonstrate that EHEC employs several bile resistance mechanisms that are similar to those of 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 the 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 multisubunit toxin were slightly downregulated by bile treatment relative to our control (Table 2). Additionally, five other genes associated with the Stx2 bacteriophage BP-933W were similarly downregulated, indicating that bile treatment does not induce the 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 reference 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 (see Fig. S1 in the supplemental material).

This microarray also indicated no change in the expression of other known EHEC virulence factors, including those in the locus of enterocyte effacement pathogenicity island, after BSM exposure (Table 1). Thus, although bile acts a signal for viru-

TABLE 2. Summary of EHEC 86-24 transcripts with a 1.5-fold or greater decrease in expression after bile salt treatment relative to that of the untreated control[a]

Gene	Product and 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 (*S. enterica* serovar 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 (cryptic prophage 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

[a] As determined by SAM analysis. $n = 4$ independent cultures (4 treatment, 4 control), $n = 4$ chips, $n = 2$ replicate spots per chip. The *P* values presented were determined using a one-way Student *t* test.

lence 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 upregulated 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 upregulated by BSM treatment on our microarray and by our confirmatory methods (Table 1 and Fig. 1B). TCRS sense and respond to environmental signals, producing physiological changes in bacteria (reviewed in reference 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 (Fig. 1B). BasR (PmrA) is known to control the 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 upregulation 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 (Fig. 1C and D). Inactivation of *basS* (12) did not affect the bile response of the *acrAB* promoter (Fig. 1A) but did abrogate that of the *arnB* operon and *ugd* (Fig. 1C and 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 the absence of *basS*, as BSM-induced expression of the reporter gene was lost in the *basS*::Kan^r mutant (Fig. 1B). This suggests that BasS may function in its self-regulation in response to bile.

Exposure to bile salts confers EHEC resistance to PMB. The addition of L-Ara4N to lipid A has been shown to confer on Gram-negative bacteria resistance 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 PMB; however, in neither organism does it appear that these modifications are required for resistance to bile itself (68) (Fig. 2). Therefore, at least in the case of EHEC, bile may be acting

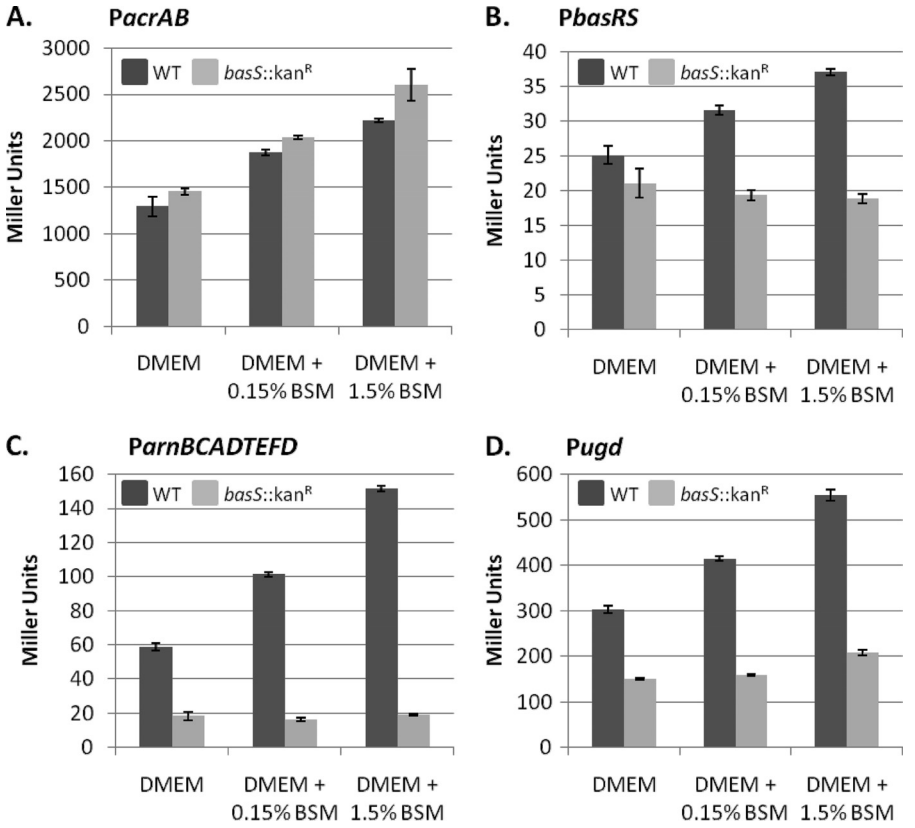

FIG. 1. β-Galactosidase reporter assays demonstrate EHEC promoters of efflux, and lipid A remodeling operons display concentration-dependent responses to bile salts. The activity of the promoters for *acrAB* (A), *basRS* (B), *arnBCADTEFD* (C), and *ugd* (D) were examined in β-galactosidase expression assays in both the wild-type (WT) 86-24 (dark gray bars) and *basS*::Kan^r (light gray bars) backgrounds. In the WT background, all of the promoters tested showed statistically significant and reproducible enhanced responses to exposure to increasing concentrations of the bile salt mixture. 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 responses of P*basRS* (B) and P*arnBCADTEFD* (C) to bile salts were abrogated. The same responses to bile were observed with another base medium (50% LB; with or without 0.15% BSM). Student *t* tests of the difference between the control (DMEM) and each treatment, as well as between both treatments, were done. A statistically significant 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 background. The data shown are for one experiment, but the experiment was repeated four times with similar results (3 independent experiments, 4 replicates within each experiment).

as an environmental signal which triggers outer membrane modifications for resistance to CAMPs 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 negative charge of the outer membrane. Modification of the outer portion of lipid A with L-Ara4N reduces the negative charge, resulting in resistance to several CAMPs. Gunn et al. demonstrated that in *S*. Typhimurium, these lipid A modifications, regulated by PmrA-PmrB, were required for resistance to PMB (18, 20). *Pseudomonas aeruginosa* mutants which constitutively expressed *pmrB* (*basS*) were observed to be not only resistant to PMB but also cross-resistant to α-defensins, β-defensins-1 and -2, α-helical peptides, and protegrin-1 (40). Enteric bacteria encounter defensins within the small intestine. Therefore, since we observed that the genes associated with L-Ara4N modification of lipid A are upregulated by BSM treatment in EHEC, we asked whether BSM treatment could induce resistance to PMB. Using a broth microdilution method, we first determined the MIC of PMB (Sigma, P0972) for EHEC 86-24 in our system to be 0.15 μg/ml. Bacteria were then cultured in LB in the presence or absence of 0.15% BSM overnight, subcultured in the same treatment ("pretreatment"), incubated under static conditions at 37°C in 5% CO_2 for 3 to 4 h, and then washed with PBS. Bacteria (1×10^6 CFU/ml) were resuspended in a "challenge" medium, i.e., LB, LB plus 0.15% BSM, or LB plus 0.15 μg/ml PMB, for 1 h at 37°C with shaking and then quantified by serial dilutions and plating (Fig. 2). Although these growth conditions varied slightly from those of the initial microarray experiment, β-galactosidase expression assays demonstrated that the promoters of our genes of interest displayed similar trends of upregulation (data not shown). Notably, pretreatment with BSM significantly improved the ability of EHEC 86-24 to survive a lethal concentration of PMB (Fig. 2A). Conversely, when the same experiment was performed with an EHEC *basS*::Kan^r

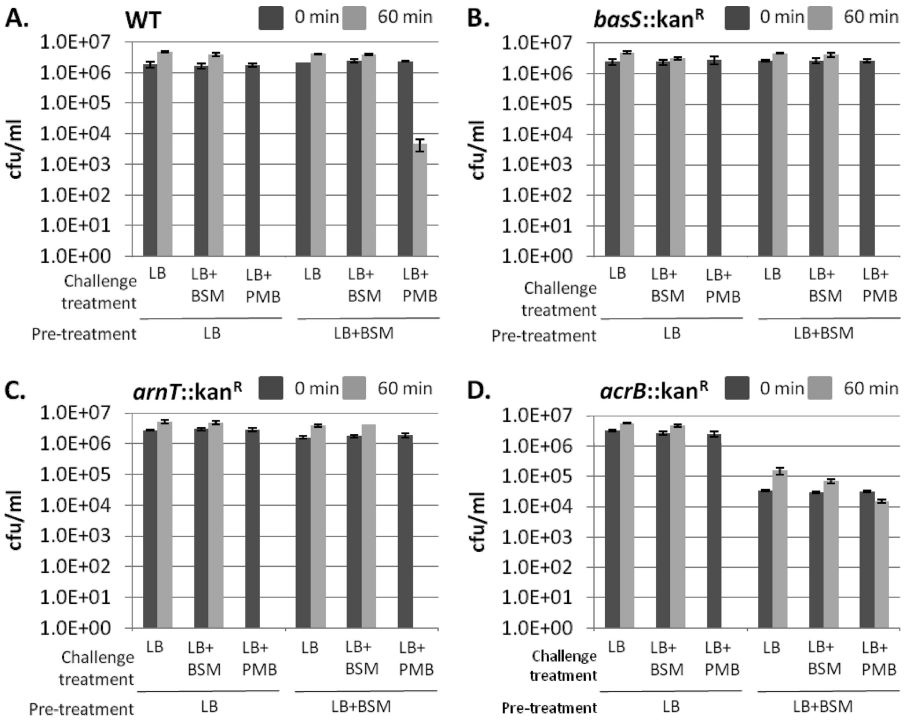

FIG. 2. Pretreatment of EHEC with bile salts induces a *basS*- and *arnT*-dependent resistance to PMB. Bacteria were pretreated with either LB or LB plus BSM (0.15% BSM), and then each was standardized, divided into three samples, and plated for quantification (time, 0 min; dark gray bars). Bacteria were then subjected to one of three challenge treatments (LB, LB plus BSM, or LB plus PMB), incubated for 60 min, and then plated for quantification (light gray bars). Wild-type (WT) 86-24 bacteria (A) pretreated with BSM were able to withstand treatment with PMB, whereas the bacteria pretreated in LB alone were killed by a challenge with PMB. This protection is lost in the *basS*::Kan^r (B) and *arnT*::Kan^r (C) disruption mutants, demonstrating that both *basS* and *arnT* are involved in bile salt-induced resistance to PMB. The *acrB* disruption (D) was able to resist a challenge with PMB when pretreated 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 of bacteria grown in LB. Results are from three independent experiments, with three replicates per experiment.

mutant, BSM pretreatment failed to induce resistance to PMB (Fig. 2B). This is further evidence that BasS is a sensor for bile salts and suggests that, in its absence, EHEC cannot respond with the lipid A modifications that protect it from PMB. As *arnT* encodes the enzyme that transfers L-Ara4N to lipid A (67), the same experiment was performed with an EHEC *arnT* disruption mutant in order to determine if this is the modification that results in BSM-induced PMB resistance and not another downstream BasS target. Significantly, bile-induced resistance to PMB was abrogated by inactivation of *arnT* (12) (Fig. 2C) and restored when the *arnT* mutation was complemented (24) (see Fig. S2 in the supplemental material), providing physiological evidence that this biochemical pathway is induced by BSM and that it results in resistance to PMB, likely due to L-Ara4N modification of lipid A.

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

We have demonstrated increased transcription of BasRS (PmrAB) and their downstream targets, the L-Ara4N lipid A modification genes, in response to bile in EHEC. In contrast, in *Salmonella*, neither PmrAB nor its regulator PhoPQ has been shown to be upregulated in response to bile, although, interestingly, both TCRS appear to be important for bile and antimicrobial peptide resistance (18, 18, 20–22, 68). Merighi et al. demonstrated in an *in vivo* expression system that both the *phoPQ* and *pmrAB* operons of *S. enterica* serovar Typhimurium were upregulated within the 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 TCRS 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 upregulation 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.

Nucleotide sequence accession number. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (14) and are accessible under GEO Series accession number GSE22060 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22060).

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