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Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella typhimurium*

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By DNA microarray, the *Salmonella typhimurium marRAB* operon was identified as being bile-activated. Transcriptional assays confirm that *marRAB* is activated in the presence of bile and that this response is concentration-dependent. The bile salt deoxycholate is alone able to activate transcription, while there was no response in the presence of other bile salts tested or a non-ionic detergent. Deoxycholate is able to interact with MarR and interfere with its ability to bind to the *mar* operator. In addition, incubation of salmonellae in the presence of sublethal concentrations of bile is able to enhance resistance to chloramphenicol and bile, by means of both *mar*-dependent and *mar*-independent pathways. To further characterize putative *marRAB*-regulated genes that may be important for the resistance phenotype, *acrAB*, which encodes an efflux pump, was analysed. In *S. typhimurium*, *acrAB* is required for bile resistance, but while transcription of *acrAB* is activated by bile, this activation is independent of *marRAB*, as well as Rob, RpoS or PhoP–PhoQ. These data suggest that bile interacts with salmonellae to increase resistance to bile and other antimicrobials and that this can occur by *marRAB*- and *acrAB*-dependent pathways that function independently with respect to bile activation.

INTRODUCTION

During infection, *Salmonella* spp. must sense and respond to harsh environments within the host, such as the small intestine and gallbladder. Bile, found in these two environments and consisting primarily of bile salts, degrades and disperses lipids during digestion and, as such, is a potent antimicrobial (Gunn, 2000). Both *Salmonella typhimurium* and *Salmonella typhi* demonstrate particularly high resistance to bile (minimal bactericidal concentrations: >60 and 18 %, respectively), which exceeds bile concentrations encountered during infection (van Velkinburgh & Gunn, 1999). High-level resistance to bile has been shown to be dependent upon PhoP–PhoQ, a two-component regulatory system necessary for virulence in mice and humans (Fields *et al.*, 1986; Miller *et al.*, 1989). However, the mechanism for PhoP–PhoQ-mediated resistance to bile is currently unknown (Prouty *et al.*, 2002; van Velkinburgh & Gunn, 1999). Bile, like other environmental signals, can regulate genes and modulate proteins in salmonellae and other enteric bacteria, as demonstrated by regulation of genes involved in *Shigella flexneri* and *Salmonella typhimurium* invasion, and modulation of *Vibrio cholerae* ToxT and *Escherichia coli* Rob (Pope *et al.*, 1995; Prouty & Gunn, 2000; Rosenberg *et al.*, 2003; Schuhmacher & Klose, 1999).

The *marRAB* operon is involved in multiple antibiotic resistance to structurally unrelated antimicrobials including chloramphenicol (Cm), tetracycline and quinolones (for a review, see Alekshun & Levy, 1997, 1999). The *mar* operon was first identified in *E. coli* and is prevalent in many bacterial species, including *S. typhimurium* (Alekshun & Levy, 1999; Kunonga *et al.*, 2000; Sulavik *et al.*, 1997). *marR* encodes a DNA-binding protein that functions as a repressor of the *marRAB* operon by binding the promoter region (*marO*) to prevent transcription (Martin & Rosner, 1995). *marA* encodes a DNA-binding protein of the XylS/AraC family that is a positive global regulator (Ariza *et al.*, 1995; Martin *et al.*, 1996). *marB* encodes a small protein with unknown function that does not appear to play a significant role in antibiotic resistance (Martin *et al.*, 1995). Activation of *marRAB* is thought to induce a variety of phenotypes, such as a decreased level of the OmpF porin to reduce influx and an increased level of AcrAB–TolC to boost efflux (Alekshun & Levy, 1997).

Abbreviation: Cm, chloramphenicol.

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The system for multiple antibiotic resistance has been well investigated in *E. coli* (Alekshun & Levy, 1997; Ariza *et al.*, 1995; Martin *et al.*, 1996; Rosner, 1985; Seoane & Levy, 1995a). The presence of antibiotics and phenolic compounds such as salicylate induces transcription of the *mar* operon, which leads to low-level antibiotic resistance (Cohen *et al.*, 1993; Hachler *et al.*, 1991). The mechanism of induction by phenolic compounds, specifically salicylate, is by the binding of salicylate to MarR, which inhibits binding of MarR to the *marRAB* promoter (Martin & Rosner, 1995). Hypersusceptibility to antibiotics can be observed in *E. coli* strains with a *marRAB* deletion (Cohen *et al.*, 1993). Strains exhibiting a *mar* phenotype (mutants with high-level resistance to antibiotics) demonstrate an increase in *acrAB* activity, and if *acrAB* is deleted, these mutants become highly sensitive to antibiotics (Okusu *et al.*, 1996). However, the genetic basis for high-level resistance can only be partially attributed to *marRAB* as suggested by Alekshun & Levy (1997). Furthermore, in *E. coli*, Rob, a global regulator with homology to MarA, independently regulates genes also regulated by MarA. These data suggest the possibility of *mar*-dependent and *mar*-independent pathways of antibiotic resistance (Ariza *et al.*, 1995).

Here we demonstrate that *S. typhimurium marRAB* is activated in the presence of bile and that deoxycholate interacts with MarR to prevent DNA binding. In addition, bile activates transcription of the AcrAB efflux pump, but independently of MarA. While Rob appears to be an important positive regulator of *acrAB* in *E. coli* (Rosenberg *et al.*, 2003), we present data suggesting that Rob does not play a role in bile-mediated activation of *acrAB* in *S. typhimurium*. This work further supports evidence that bile is an important environmental signal for enteric organisms and that even closely related enteric organisms have developed unique pathways to utilize bile as a host-derived signal.

METHODS

Strains, plasmids and reagents. The *S. typhimurium* and *E. coli* strains and plasmids used in this study are listed in Table 1. All strains were maintained in Luria–Bertani (LB) broth or on agar with appropriate antibiotics: 50 μg ampicillin ml^{-1}, 25 μg Cm ml^{-1}, 45 μg kanamycin ml^{-1}. X-Gal was used at a concentration of 40 μg ml^{-1}. Bile was used at various concentrations throughout the study ranging from 0·5 to 30 %. The specific concentrations used were experiment-dependent.

Sodium salicylate, bile (sodium cholate) and conjugated and unconjugated bile salts were purchased from Sigma Chemical. Triton X-100 was purchased from Fisher Chemical/Fisher Scientific.

Strain construction. DNAs specific to *marAB*, *marRA* and the *acrAB* promoters or internal regions were amplified by PCR using primer pairs JG134 (5′-ggA ATT CAT gAC gAT gTC Cag Acg C-3′)/JG135 (5′-ggg gTA Ccg gTT AAA ggT gTT ggT Cg-3′), JG423 (5′-cgg AAT TcT AAA gTg AAA TTg ccc Agg c-3′)/JG424 (5′-ggg gTA ccg cAT TTT cAT ggT gcT cTT cgc-3′) and JG581 (5′-cgg AAT TcT gTc ggT gAA TTT AcA ggc g-3′)/JG582 (5′-ggg gTA ccc gTc AgT TcA gcg ATA TTc g-3′), respectively. The primers were designed with *Eco*RI or *Kpn*I sites at their 5′ ends. The *marA*

Table 1. Strains and plasmid used in this study

Strains or plasmid	Properties*	Source or reference
***Salmonella* strains**		
JSG210	14028s (wild-type, parent of all *Salmonella* strains)	ATCC
JSG782	*marRAB*::*luc*	van Velkinburgh & Gunn (1999)
JSG1939	*marRA*::*luc*	This study
JSG1945	Δ*marRA*::FRT	This study
JSG1996	*acrB*::*luc*	van Velkinburgh & Gunn (1999)
JSG2047	*acrAB*::*luc*	This study
JSG2048	Δ*rob*::Kan *acrAB*::*luc*	This study
JSG2049	Δ*rob*::Kan *marB*::*luc*	This study
JSG2050	Δ*marRAB*::Kan	This study
JSG2060	Δ*marRAB*::Kan *acrAB*::*luc*	This study
JSG2061	Δ*marRAB*::FRT	This study
JSG2067	Δ*marRAB*::*lacZ*	This study
JSG2079	Δ*rpoS*::FRT *acrAB*::*luc*	This study
JSG2080	*phoP*::Tn*10*d–cam *acrAB*::*luc*	This study
JSG2081	$PhoP^c$ (*pho24*) *acrAB*::*luc*	This study
***E. coli* strain**		
BL21(DE3)		Stock strain
Plasmid		
pAMP53	pET15b with *marR* cloned	This study

*FRT, FLP recognition target.

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(JG134/JG135) fragment was cloned into the firefly luciferase-reporter suicide vector pGPL01 (Gunn & Miller, 1996). Recombination on the chromosome accomplished a gene fusion and a disruption in the operon (*marRAB*::*luc*, JSG782). The *acrAB* promoter fragment was cloned into the firefly luciferase-reporter suicide vector pLB02 creating JSG2047 (Gunn *et al.*, 1996). Recombination on the chromosome created a gene fusion in which the strain became merodiploid for the *acrAB* promoter region. Gene deletions were accomplished by means of the λ red-mediated site-specific recombination as described by Datsenko & Wanner (2000). Deletions were constructed with the following primer pairs: *marRA*, JG546 (5′-TTT cgc cAg TgT gcA AgT TAA TAT ccT cTA gTg TAg gcT ggA gcT gcT Tcg-3′)/JG547 (5′-cgc ATA AAc AAA cTA gTA gTT gcc ATg gTT cAT ATg AAT ATc cTc cTT Ag-3′); *marR*, JG548 (5′-gAc gAA aTT AAT TAc TTg ccg ggg cAA ccA gTg TAg gcT ggA gcT gcT Tcg-3′)/JG549 (5′-gTT AAT Tcc TgA Tgc Agg TcT Tgc ccT ggT cAT ATg AAT ATc cTc cTT Ag-3′); *marRAB*, JG546/JG593 (5′-gcA AAg Agg TTA AAg gTg TTg gTc gTT ATA cAT ATg AAT ATc cTc cTT Ag-3′); *rob*, JG577 (5′-gcT ggc ATA ATT cgc gAc cTg TTA ATc Tgg TgT Agg cTg gAg cTg cTT cg-3′)/JG578 (5′-cgg cgA ATc ggg ATc AgA AAT Tcg cAg ccA TAT gAA TAT ccT ccT TAg-3′). Colonies were characterized for the presence of the deletion by PCR with primers outside of the deleted region. An insertion in *acrB* was accomplished by recombination of pGPL01 carrying an internal fragment of the *acrB* gene amplified using primers JG136 (5′-ggA ATT CgA CgC gCA AAT CCA Tg-3′)/JG566 (5′-ggg gTA ccg cAA ATc Agc gAT gTT cTg Tcg-3′). The β-galactosidase gene fusion to the *marRAB* promoter region was constructed as described by Ellermeier *et al.* (2002). Briefly, JSG2061 with FLP (flip) plasmid pCP20 was transformed by electroporation with pCE36 and plated on LB/kanamycin/X-Gal at 37 °C.

RNA isolation, labelling and hybridization. JSG210 was grown in LB broth at 37 °C with aeration to OD_{600} 0·25. Cultures were then incubated in LB broth with or without 3 % bile. Samples were collected at 30 and 60 min after the addition of bile. Cells from each sample were pelleted and resuspended in 100 µl of 400 µg ml^{-1} lysozyme in Tris/EDTA buffer. RNA was isolated according to manufacturer's instructions using the Qiagen RNeasy Mini Kit (Qiagen). An optional on-column DNase treatment for 1 h was also performed according to the manufacturer's instructions. cDNA was made from the RNA with $pd(N)_6$ Random Hexamer (Amersham Biosciences) and labelled as described previously (Eisen & Brown, 1999). Labelled cDNA was hybridized to compare samples grown with or without bile collected at the same time-point. Labelling and hybridization was repeated multiple times for each time-point. Hybridized slides were scanned and analysed with a Gene Pix Scanner 4000A and the GENEPIX program (Axon Instruments).

Data analyses. Data were analysed with the Stanford University Microarray Database, Microsoft EXCEL and the SIGNIFICANCE ANALYSIS OF MICROARRAYS (SAM) program (http://www-stat.stanford.edu/~tibs/SAM/index.html) (Tusher *et al.*, 2001). Three hybridizations from each time-point were grouped in an experimental set and were filtered by the Stanford University Microarray Database according to their mean log_2 (Cy5/Cy3) ratios. Initial filtering included keeping data for mRNAs in which at least 80 % of the spots had a regression correlation of 0·6. Missing data points were estimated with a K-Nearest-Neighbor Imputator, where K equals 10 (Tusher *et al.*, 2001). Data were additionally filtered using CLUSTER software to remove any genes with missing values in greater than 20 % of the columns and those that had standard deviations of observed values greater than 2. SAM calculates a list of significant genes and a false discovery rate, which is an estimate of the percentage of false-positives.

Transcriptional assays. Strains carrying *marA*::*luc* or *marB*::*luc* were grown to exponential phase in LB broth and incubated for 1 h with or without bile (concentrations indicated in Results). Strains with *acrA*::*luc* were grown overnight in LB broth and incubated in LB broth with or without 3 % bile. Cultures were washed twice in PBS (Fisher Scientific), and firefly luciferase assays were performed as described previously (Gunn & Miller, 1996). Strains for β-galactosidase assays were grown to exponential phase and incubated in LB broth with or without bile for 1 h. Cultures were washed twice in PBS and assayed for β-galactosidase activity as described previously (Gunn & Miller, 1996).

Gel electrophoretic mobility shift assays. To overexpress and purify MarR, the coding sequence of *marR* was amplified with primers JG550 (5′-ggA ATT ccA TAT gAA AAg cAc cAg TgA TcT gTT c-3′)/JG551 (5′-cgg gAT ccc TAc ggc AgA ATT TTc TTg Agc-3′) and cloned into pET15b (an IPTG-inducible vector with an N-terminal histidine tag) and introduced into *E. coli* BL21(DE3) (Novagen). Near-native MarR was purified as described previously (Martin & Rosner, 1995). A 110 bp region of *marO* containing both MarR-binding sites was amplified with primers JG552 (5′-cgg ccA ATT cAT TTA gTT gAc-3′)/JG565 (5′-cAT TgA AcA gAT cAc Tgg Tgc-3′) and labelled with Cy5-dCTPs (Amersham Biosciences) incorporated by PCR. Tris/glycine 6 % polyacrylamide gels were used for gel shift experiments. Cy5-labelled *marO* and various concentrations of MarR (indicated in Results) were incubated at 37 °C for 15 min in 10 mM sodium phosphate pH 7·2/30 mM sodium chloride/1 mM sodium azide/10 mM DTT/5 % glycerol/0·3 mg poly(dI-dC) ml^{-1}. Where indicated, gels and buffers contained 1 mM deoxycholate or 1 mM glycocholate (Martin & Rosner, 1995). Gels were run at 120 V. Gels were scanned and fluorescence detected at 670 nm using the Typhoon 9400 (Amersham Biosciences). Data were analysed using IMAGEQUANT 5.2 software (Molecular Dynamics).

Resistance assays. Adaptation assays were performed using cultures grown to exponential phase (OD_{600} 0·6). Exponential-phase cultures were then incubated in the presence or absence of 10 % bile for 2 h at 37 °C. Cultures were next pelleted and resuspended in 30 % bile in LB broth. OD_{600} readings were taken every 15 min for 1 h beginning at the time of resuspension. Cultures were washed twice in PBS prior to reading. Plating on solid agar was also performed to confirm cell viability.

For MIC assays, bacterial cells were challenged with Cm either in the presence or in the absence of inducers, bile and salicylate. Inducers (5 % bile or 5 mM salicylate) or LB broth alone was added to stationary-phase cultures, which were then incubated for 1 h. Cultures were diluted into the same growth media such that 2×10^3 to 5×10^3 c.f.u. ml^{-1} were subjected to concentrations of Cm ranging from 0·25 to 3 µg ml^{-1}. MIC assays were performed in polypropylene microtitre plates and incubated overnight at 37 °C. The pellet in each well was resuspended and an OD_{600} reading was taken. Data points represent the ratio of each well to that of the well with no antibiotic. Plating on solid agar was also performed to confirm cell viability.

RESULTS

Microarray data demonstrate activation of *marR*, *marA* and *marB* in the presence of bile

To better understand the effect of bile on salmonellae, potential bile-regulated genes were identified using DNA microarrays. Three per cent bile was added to exponential-phase cultures of *S. typhimurium*, and samples were collected at 30 and 60 min following the addition of bile. RNA, isolated from the samples, was used to create cDNA, which was hybridized to oligonucleotide microarrays of *S. typhimurium* SL344 (Chan *et al.*, 2003). Upon analysing

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the results from microarray chips comparing samples from cells grown in LB broth alone or LB broth plus 3 % bile, *marR*, *marA* and *marB*, co-transcribed genes of the *mar* operon, were among the most regulated genes in the presence of bile (data not shown). Bile activation of the *mar* operon provides a potential link between bile regulation and resistance.

Bile activates transcription of *marRAB*

Results from the microarray suggest that the *marRAB* operon is activated in the presence of bile. To confirm the effect of bile on the transcription of the *marRAB* operon, a chromosomal fusion of the *marB* gene to the firefly luciferase gene (*luc*) was constructed (creating JSG782, *marRAB*::*luc*). Transcriptional activity was measured at concentrations of bile ranging from 1 to 9 % and compared to activity of a culture with no bile added. A 2·9-fold increase in activity was observed in 1 % bile increasing to a maximum 5·3-fold induction in 5 % bile. These assays were also performed at 30 °C, a temperature at which *mar* transcription has been shown to be increased (Seoane & Levy, 1995b). While *mar* transcription was slightly elevated at 30 versus 37 °C, the relative fold induction observed in bile at both temperatures was nearly identical (e.g. 5·5-fold induction in 5 % bile at 30 °C). These data demonstrate that activity of *marRAB* is regulated by bile in a concentration-dependent manner and that bile-mediated induction is not temperature-dependent.

Deoxycholate specifically induces transcription of *marRAB*

To test whether bile induction of the *marRAB* operon is due to a specific component of bile or to general detergent effects on the bacterium, transcription of *marA*::*luc* was measured in the presence of individual bile salts or Triton X-100, a non-ionic detergent. Of the four bile salts tested – deoxycholate, taurocholate, glycocholate and glycochenodeoxycholate – deoxycholate was the only bile salt that activated transcription to a level similar to that observed in the presence of ox bile (Fig. 1). In addition, Triton X-100 did not activate transcription of *marRAB*, demonstrating that the *mar* operon does not simply respond to the presence of detergent.

Bile-mediated activation of the *mar* operon occurs independently of Rob

Rob, a known activator of *marRAB* in *E. coli* (Martin & Rosner, 1997), was examined for its role in bile activation of the *mar* operon in *S. typhimurium*. The transcription of *marB*::*luc* was measured with or without 3 % bile in a *rob* deletion strain. While transcriptional activity of *marRAB* in the absence of Rob was somewhat reduced (r.l.u., relative light units; 31 389 r.l.u. ± 4305 vs 18 072 r.l.u. ± 1502 with bile and 3958 r.l.u. ± 732 vs 2057 r.l.u. ± 294 without bile), the loss of Rob had no major effect on the relative bile-mediated activation of *marRAB* (7·93-fold in a wild-type background; 8·78-fold in the absence of Rob). This experiment demonstrates that Rob is not required for activation of the *mar* operon by bile.

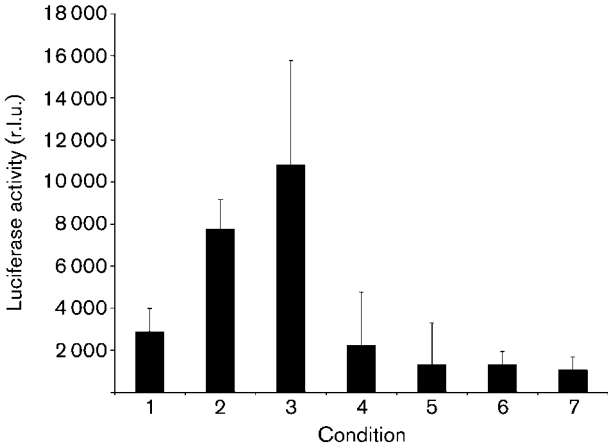

Fig. 1. Transcription of *marRA*::*luc* (JSG1939) is specifically induced in the presence of the bile salt deoxycholate. Transcriptional activity of *marRAB* in (1) LB broth alone was measured and compared to transcriptional activity after 1 h incubation in 0·5 % of (2) bile, (3) deoxycholate, (4) taurocholate, (5) glycocholate, (6) glycochenodeoxycholate or (7) Triton X-100. Results are the mean of three different experiments; error bars represent standard deviations. r.l.u., Relative light units.

Deoxycholate specifically interacts with MarR

Salicylate, an inducer of *marRAB* activity, has been shown to bind the repressor MarR, leading to an increase in transcriptional activity of the operon (Cohen *et al.*, 1993; Martin & Rosner, 1995). Because transcriptional activation of *marRAB* is specific for deoxycholate and because MarR has been observed to bind a variety of structurally different compounds (Schumacher & Brennan, 2002), studies concerning interactions of deoxycholate with the repressor MarR were initiated. To test whether deoxycholate disrupts the binding of MarR to its binding sites at *marO*, gel electrophoretic mobility shift assays (GEMSA) were performed. Initially, MarR and *marO* of *S. typhimurium* were tested to determine if they interacted in a manner similar to that shown for *E. coli*. Incubation of MarR with *marO*, analysed by GEMSA, resulted in four retarded complexes similar to what is observed in the *E. coli* MarR–*marO* interaction (Fig. 2a). The binding was sequence-specific as demonstrated by the ability of unlabelled *marO*, but not non-specific DNA, to compete for binding to MarR (data not shown). Incubation of samples in the presence of 1 mM deoxycholate abolished MarR–*marO* complexes. To account for the detergent effects that could occur with deoxycholate, glycocholate, a bile salt and detergent that did not activate *marRAB* transcription, was used as a control (Fig. 2b). One millimolar glycocholate was unable to

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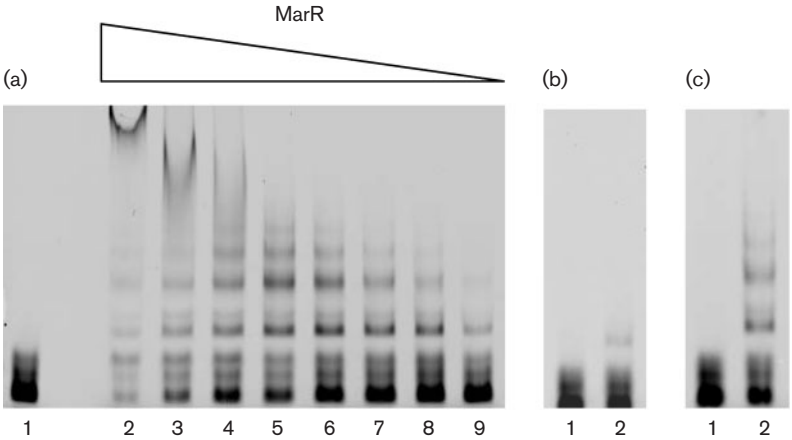

Fig. 2. Electrophoretic mobilities of *mar* promoter complexes with MarR. (a) A 110 bp Cy5-labelled wild-type *mar* promoter fragment was incubated alone (lane 1) or with 63·75, 31·9, 15·5, 8, 4, 2, 1 and 0·5 ng of MarR (lanes 2–9, respectively). (b, c) The labelled *marO* fragment either alone (lane 1) or with 8 ng MarR (lane 2) was incubated with (b) 1 mM deoxycholate or (c) 1 mM glycocholate.

disrupt the complexes, suggesting a specific interaction of deoxycholate with MarR (Fig. 2c).

Bile promotes increased resistance to bile and antibiotics

Previous studies analysing the phenotype associated with induced *marRAB* activity in *E. coli* have demonstrated a low-level increase in antibiotic resistance (Cohen *et al.*, 1993; Rosner, 1985). Salmonellae can adapt to growth in high concentrations of bile salts by pre-incubation with sublethal amounts of bile. To determine if the ability of *Salmonella* to adapt to lethal concentrations of bile is dependent on *marRAB*, a wild-type *S. typhimurium* strain and a *marRA* deletion strain were incubated either in LB broth alone or in a sublethal concentration of bile, followed by exposure to a lethal concentration of bile. Viability was determined through measurement of optical density and colony counting on solid agar. Bacteria pre-incubated in bile were able to both survive and sustain growth at a lethal concentration of bile, while those pre-incubated in LB broth alone demonstrated a sharp decline in viability (Fig. 3). Interestingly, the *marRA* deletion strain grown with or without bile exhibited the same viability patterns as the wild-type strain that is shown in Fig. 3 (data not shown). Therefore, adaptation to high levels of bile is not dependent upon *marRAB*.

It has been shown that activation of the *mar* operon in *E. coli* by salicylate leads to increased resistance to other antimicrobial agents (Cohen *et al.*, 1993). MICs of Cm for *S. typhimurium* were measured to examine if the presence of bile could induce increased resistance to antibiotics and if such resistance would be dependent on the presence of *marRAB*. Wild-type or a *marRAB* deletion strain of *S. typhimurium* were pre-incubated in LB broth alone or with LB broth plus 5 % bile and diluted into microtitre plate wells with various concentrations of Cm. Strains pre-incubated in bile exhibited increased resistance to Cm as compared to incubation in LB broth alone (Fig. 4). The observed increase in resistance appears to be partially

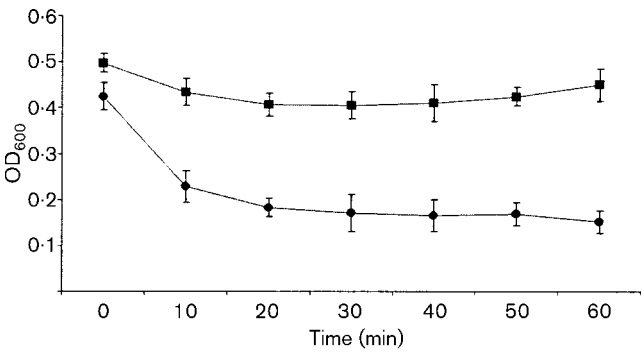

Fig. 3. Pre-incubation in sublethal concentrations of bile allows *S. typhimurium* to adapt and survive in lethal concentrations of bile. ●, OD_{600} readings of wild-type (WT, JSG210) bacteria that were exposed to LB broth before being resuspended in 30 % bile. ■, OD_{600} readings of WT bacteria pre-incubated in 10 % bile in LB broth, then resuspended in 30 % bile. Time-points represent time of spectrophotometer reading after resuspension in 30 % bile; error bars represent standard deviations.

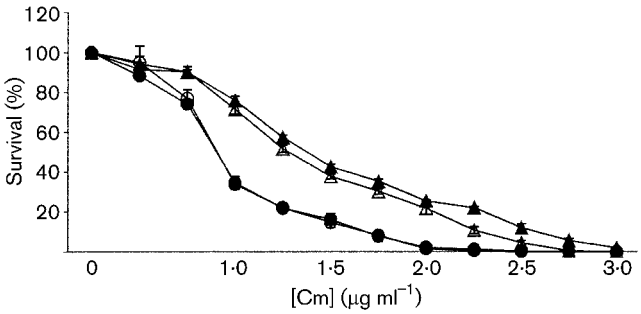

Fig. 4. Relative survival in the presence of Cm. Comparison of *S. typhimurium* 14028s (JSG210; solid symbols) and Δ*marRAB*::FRT (JSG2061; open symbols) grown either in LB broth (circles) or in 5 % bile (triangles) and exposed to Cm. Error bars represent standard deviations.

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dependent on *marRAB*, as the *marRAB* mutant incubated in the presence of bile is more sensitive than the wild-type strain grown in bile, but sensitivity does not drop to levels observed for incubation in LB broth alone. It is also interesting to note that deletion of *marRAB* does not make the bacterium more susceptible to Cm in the absence of an inducer. These results suggest there is another pathway, independent of *marRAB*, involved in bile-mediated resistance to antibiotics.

acrAB activation by bile is independent of MarA, Rob, PhoP–PhoQ and RpoS

MarA is believed to mediate enhanced resistance to antimicrobials through activation of the genes encoding the AcrAB efflux pump (White *et al.*, 1997). AcrAB is known to efflux bile salts and play a role in bile resistance in *E. coli* and *S. typhimurium* (Lacroix *et al.*, 1996; Ma *et al.*, 1995; Thanassi *et al.*, 1997). A strain with a disruption in *acrB* confirmed the necessity for AcrAB in bile resistance, as a concentration of 0·5 % effectively killed exponential- or stationary-phase cultures of this strain (data not shown). Transcription of *acrAB* was also examined by creating a single copy chromosomal *acrAB* promoter fusion to the *luc* gene (without disrupting the *acrAB* genes). Transcriptional activity in the presence and absence of bile demonstrated an approximate eightfold induction by bile (data not shown). These results indicate that AcrAB is absolutely required for bile resistance and that the transcription of *acrAB* increases in the presence of bile.

The *marRAB* products are proposed to be involved in *acrAB* transcriptional activation in *E. coli* (Ma *et al.*, 1995; Okusu *et al.*, 1996). Therefore, *marRAB* was investigated for its role in bile activation of *acrAB* in *S. typhimurium*. *acrAB* promoter activity was measured in a *marRAB* deletion strain (JSG2060) in the presence of bile. There was no observed alteration of bile-induction of *acrAB* in strains with or without *marRAB*, demonstrating that the *mar* operon is not required for activation of *acrAB* by bile (data not shown).

Additional regulators were examined for potential roles in bile activation of *acrAB*. Rob has been demonstrated to directly regulate transcription of *acrAB* in *E. coli* (Rosenberg *et al.*, 2003). Transcriptional activity of *acrAB* was measured in the presence and absence of Rob. The results demonstrate that bile-mediated activation of *acrAB* in *S. typhimurium* occurs independently of Rob (data not shown). PhoP–PhoQ, an important virulence regulator implicated in bile resistance, and RpoS, a global stationary-phase regulator, were also examined for their potential role in bile-mediated activation of *acrAB*. Analysis of transcriptional data demonstrated that there is no observed role for PhoP–PhoQ or RpoS in bile activation of *acrAB* (data not shown). These results suggest the presence of a novel pathway for bile-mediated regulation of the AcrAB efflux pump in *S. typhimurium*.

DISCUSSION

This work employed the use of a DNA microarray to identify genes regulated by bile. From this screen, the *mar* operon was found to be upregulated in the presence of bile. We hypothesized that salmonellae might use this compound as a means to detect the presence of a host environment and to activate *marRAB* to increase resistance to antimicrobials in the host. It has been proposed that *mar* operon activation could be a mechanism for bile resistance (Sulavik *et al.*, 1997) and that bile could be the *in vivo* signal to activate *mar* genes (Rosenberg *et al.*, 2003). The work presented here supports these assertions by providing the first evidence of the *mar* operon specifically responding to bile or bile salts.

marRAB has been shown to be regulated both directly by salicylate and indirectly by antibiotics (Cohen *et al.*, 1993; Hachler *et al.*, 1991; Randall & Woodward, 2001). Salicylate is able to interact with MarR, which prevents MarR from binding DNA, which in turn derepresses *marRAB* transcription (Martin & Rosner, 1995). MarR has also been shown to bind other anionic compounds, including 2,4-dinitrophenol, plumbagin and menadione (Schumacher & Brennan, 2002). The two binding sites for salicylate on MarR are within the DNA-binding motif, suggesting a mechanism as to how salicylate derepresses the *mar* operon (Schumacher & Brennan, 2002). Based on our microarray and reporter fusion results, we hypothesized that deoxycholate would interact with MarR similarly to salicylate. Our results indicate that deoxycholate does interact with MarR to prevent DNA-binding and that this interaction is specific for deoxycholate. In *E. coli*, deoxycholate and chenodeoxycholate are able to bind to the C-terminal domain of Rob, which affects *acrAB* transcription in *E. coli* (Rosenberg *et al.*, 2003). In addition, bile has been demonstrated to affect host cell invasion in both *Salmonella* and *Shigella*, and in the former, through transcriptional repression of key invasion determinants (Pope *et al.*, 1995; Prouty & Gunn, 2000). Furthermore, in *V. cholerae*, bile is believed to repress ToxT-dependent transcription of virulence factors through modulation of the ToxT protein by an unknown mechanism (Schuhmacher & Klose, 1999). These results support the hypothesis that enteric organisms have adapted to use bile salt as a regulatory signal, most likely by direct interactions between key regulatory proteins and bile salts.

The involvement of the *mar* locus in multidrug resistance was initially identified when spontaneous, highly resistant strains were shown to have mutations in *marR* (Cohen *et al.*, 1989; George & Levy, 1983; Kunonga *et al.*, 2000). However, in a wild-type strain, transcriptional induction of the *mar* operon causes non-heritable low-level increases in resistance (Rosner, 1985). Based on our observations that *marRAB* transcription increases in the presence of bile, and the known role of *marRAB* in gene regulation, we hypothesized that the *mar* operon could play a role in increased resistance to bile. Interestingly, though, *marRAB*

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did not have an effect on the ability of salmonellae to adapt to lethal concentrations of bile. We were initially surprised by these results, but it has been observed in *E. coli* that salicylate does not induce resistance to higher levels of salicylate. This suggests the possibility of gratuitous induction of the *mar* operon by bile in the salmonellae (Cohen *et al.*, 1993). This induction then leads to the observed phenotypes of increased antibiotic resistance. Because *Salmonella* spp. possess an inherently high-level resistance to bile, pathways other than the *mar* regulon, which is predominantly involved in low-level resistance, may have evolved to compensate for major changes in bile concentrations.

Incubation of either *S. typhimurium* or *E. coli* in salicylate leads to increased resistance to a variety of antibiotics including tetracycline and Cm (Cohen *et al.*, 1993; Hachler *et al.*, 1991). We hypothesized that bile, like salicylate, would enhance resistance to antibiotics and that *marRAB* would play a role. Results from MIC assays demonstrated that bile did increase resistance to Cm but *mar*-independent pathways were mainly involved in antimicrobial resistance. Similar results have been observed in *E. coli* and *S. typhimurium* DT104, in which salicylate can still induce antimicrobial resistance in a *mar* mutant (Randall & Woodward, 2001). It is interesting to note that while an *E. coli mar* mutant demonstrates greater sensitivity to antibiotics than its parental strain, an *S. typhimurium mar* mutant is roughly equally as sensitive to antimicrobials as its parent (Cohen *et al.*, 1993; Randall & Woodward, 2001). This may indicate that while *E. coli* still depends on the *mar* regulon for low-level resistance, *Salmonella* spp. may rely on other pathways that better suit their environment. The results of this study support the presence of *mar*-dependent and *mar*-independent pathways of antimicrobial resistance and demonstrate that the *marRAB* of *S. typhimurium* and *E. coli* are not necessarily isofunctional.

MarA is believed to be a transcriptional activator of unrelated genes necessary for antimicrobial resistance (Alekshun & Levy, 1999). Studies in *E. coli* indicate that MarA indirectly represses *ompF*, reducing the number of porins in the outer membrane and activates transcription of *acrAB* to increase the number of efflux pumps in the membrane (Alekshun & Levy, 1997). Groups have observed that transcriptional activity of *acrAB* is increased in a MarR mutant exhibiting high-level resistance to antibiotics (Ma *et al.*, 1995; Okusu *et al.*, 1996). Furthermore, deletion of *acrAB* renders the MarR mutant hypersusceptible to the same antibiotics (Ma *et al.*, 1995; Okusu *et al.*, 1996). These observations have led researchers to conclude that *acrAB* is a part of the *mar* regulon (White *et al.*, 1997). However, Piddock *et al.* (2000) also suggest that salmonellae may regulate *acrAB* through pathways other than *mar*, but did not test this hypothesis. To further elucidate the *mar* regulon in the salmonellae, we examined the effect of bile on *acrAB* transcription and whether the presence of MarA was necessary for *acrAB* regulation. Our studies show that the effect of bile on *acrAB* transcription is not dependent upon MarA or MarR. These results indicate that either *acrAB* is not a gene of the *mar* regulon in *S. typhimurium* or elimination of the *mar* operon alone is not sufficient to observe an effect on *acrAB*, suggesting the presence of additional regulators. While Rosenberg *et al.* (2003) presented evidence that *mar* is not necessary for bile-salt-mediated activation of *acrAB* in *E. coli*, they observed that induction of *acrAB* by bile salts is dependent upon Rob. However, work presented here demonstrates that activation of *acrAB* by bile was not dependent upon Rob. These conflicting results indicate an interesting divergence between *E. coli* and *S. typhimurium* that is likely to be related to the organisms' differing response and resistance to bile and suggest that bile either directly regulates *acrAB* or signals through a currently unidentified mechanism.

Previous studies in *E. coli* demonstrate that *acrAB* mutants are highly sensitive to bile salts, but that these compounds are only weak inducers of *acrAB* transcription (Ma *et al.*, 1995). Studies from both Ma *et al.* (1995) and Lacroix *et al.* (1996) demonstrate that an *S. typhimurium acrAB* mutant exhibits hypersusceptibility to bile salts. Our studies further confirm the necessity for AcrAB in bile resistance, as an *acrAB* mutant was effectively eliminated in 0·5 % bile. Transcriptional studies from this report also indicate that bile is a major inducer of *acrAB* transcription in salmonellae, inducing greater than eightfold, while only 1·5- to 1·7-fold induction has been reported in *E. coli* (Rosenberg *et al.*, 2003). Interestingly, activation of *acrAB* transcription in the presence of bile was most easily observed in cells in the stationary phase of growth. Transcriptional activity of *acrAB* was dramatically elevated in exponential phase even in the absence of bile, which made further activation difficult to observe.

In this study, we initially hypothesized that bile activates transcription of *marRAB*, which would lead to activation of *acrAB* and, subsequently, higher resistance to bile. However, while bile does regulate both *marRAB* and *acrAB*, it appears to do so through independent pathways. Even though the role of *marRAB* in antimicrobial resistance is not clearly defined for *S. typhimurium*, the unique interaction of deoxycholate with MarR indicates that this operon may play a role in the host that is not observable *in vitro*. We propose a model in which bile salts enter the bacterial cell and deoxycholate interacts with MarR to regulate gene expression of the *mar* operon. This regulation then affects currently unknown genes that play a role in survival within host microenvironments. Concurrently, deoxycholate activates transcription of the AcrAB efflux pump independently of MarA to allow for efficient removal of bile salts from inside the bacterium. Studies of the functional consequences of *marRAB* activation by bile, as well as the mechanism by which bile activates *acrAB*, will aid in the further elucidation of the role of bile in *Salmonella* spp. pathogenesis.

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