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–ToIC to boost efflux (Alekshun & Levy, 1997).

Abbreviation: Cm, chloramphenicol.
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 Aec C-3')/JG135 (5'–ggg gTA Ccg gTT Aaa gTg gTT ggT gCg-3'), JG423 (5'–cgg gTA Ccg CAT TTg gAg gAg c-g-3')/JG424 (5'–cgg gTA cgg CAT TTg gTg gTT cTT cgc-3') and JG581 (5'–cgg gAT TcT ggg gGG gAA gAA gTT ccc cgg c-3')/JG582 (5'–cgg gTA Ccc gTC AgT TcA ggg ATA TtC g-3'), respectively. The primers were designed with EcoRI or KpnI sites at their 5' ends. The *mar*

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<td><strong>Strains or plasmid</strong></td>
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*FRT, FLP recognition target."
(GI34/GI35) 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 acrB 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 acrB promoter region. Gene deletions were accomplished by means of the \( \lambda \) red-mediated site-specific recombination as described by Datsenko & Wanner (2000). Deletions were constructed with the following primer pairs: marKRA, JG546 (5'-TTT cgc TAC Tct gCA aGG TTA cTA TcG Tag Gct gAg Gct gCT gcT gcT-3')/JG547 (5'-cgc ATA Aaa aCA cTA gTA gTc GcT gAT CAT ATG AAT Atc cTc cTT Ag-3'); marRB, JG548 (5'-gcc gAA xTT AAT TAC TtG ccG gCA cTG Tag gCT gAg GCT gcT gcT-3')/JG549 (5'-ggT gcT AgC AgT gAA ATG AAT Atc cTc cTT Ag-3'); marRAB, JG546/JG593 (5'-gga aAg TTA AAg gTg Ttg gTc gTT ATA cAT Atg AAT AAT cTc cTc cTT Ag-3'); rob, JG577 (5'-gCt ggc ATA ATT cGC cAg cTg TTA tATg Ttg Tgt cAg ctg gAg ctG cTt cTc cTc cgc-3')/JG578 (5'-cgg cGC aTC ATT gcG Agc AGa AAT TcG cAg cCA tAt gAa TAT cCt cTc cTAg-3'). Colonies were characterized for the presence of the deletion by PCR with primers of the outer deleted allele. An insertion in acrB was accomplished by recombination of pGPL01 carrying an internal fragment of the acrB gene amplified using primers JG136 (5'-ggc ATc ggg ATc AgA) and JG550 (5'-gcc ATT cca Tat gAt gAa AAC cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cAg cA...
the results from microarray chips comparing samples from cells grown in LB broth alone or LB broth plus 3% bile, marK, 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. in the absence of Rob was somewhat reduced (r.l.u., relative light units; 31 389 r.l.u.), 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 promote the binding of MarR to marO, indicating that bile, not detergent, was responsible for the activation of the mar operon.
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. 2.** Electrophoretic mobilities of *mar* promoter complexes with MarR. (a) A 110 bp Cy5-labelled wild-type *mar* promotor 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.

**Fig. 3.** Pre-incubation in sublethal concentrations of bile allows *S. typhimurium* to adapt and survive in lethal concentrations of bile. ●, *OD*<sub>600</sub> readings of wild-type (WT, JSG210) bacteria that were exposed to LB broth before being resuspended in 30% bile. ■, *OD*<sub>600</sub> 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.
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 (Schumacher & 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 *mar* (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*...
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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