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Transcriptional regulation of *Salmonella enterica* serovar Typhimurium genes by bile

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Abstract

DNA microarrays and two-dimensional (2-D) gel electrophoresis were utilized to analyze the global effect of bile on transcription and protein synthesis in *Salmonella enterica* serovar Typhimurium. Two bile-regulated proteins, YciF and PagC, were identified by 2-D gel electrophoresis and mass spectrometry fingerprinting. The operon *yciGFE-katN* demonstrated increased transcriptional activity in the presence of bile. While this operon has previously been shown to be RpoS-regulated, data from this study suggested that *yciGFE-katN* is regulated by bile independent of RpoS. The PhoP–PhoQ-regulated PagC is decreased in the presence of bile. Characterization of the untranslated leader of *pagC* demonstrated that a 97-bp region is necessary for the bile-mediated repression of this promoter. Analysis of data from the DNA microarray revealed an effect of bile on important global mechanistic pathways in *S. enterica* serovar Typhimurium. Genes involved in type III secretion-mediated invasion of epithelial cells demonstrated an overall repression of transcription in the presence of bile, corroborating previously reported data from this laboratory [Infect. Immun. 68 (2000) 6763]. In addition, bile-mediated transcriptional repression of genes involved in flagellar biosynthesis and motility was observed. These data further demonstrate that bile is an important environmental signal sensed by *Salmonella* spp. and that bile plays a role in regulating bacterial gene expression in multiple virulence-associated pathways.

Keywords: Salmonella; Microarray; Bile; Transcription

1. Introduction

Bile salts are synthesized by the liver, where they are combined with other components, such as cholesterol, phospholipids and bilirubin to form bile. The bile is stored in the gallbladder and released into the duodenum upon consumption of food, particularly fatty meals. Bile aids in digestion through degradation and dispersion of lipids. As a detergent-like substance, bile can also degrade lipid-containing membranes of bacteria. Most enteric organisms possess inherent resistance to bile that involve components such as porins,

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transport proteins, efflux pumps and lipopolysaccharide [1–3]. In addition to resisting the detergent effects of bile, it appears that some bacteria utilize bile as a regulatory environmental signal to affect gene expression within the host [4–8].

Bile has been demonstrated in both bacteria and parasites to regulate genes for bile resistance, as well as genes within pathways important for virulence [4–10]. Bile salt hydrolase (BSH) is a protein found in most gastrointestinal commensals, but not enteropathogens, allowing for continued survival within the host [11]. Recently, BSH was identified in *Listeria monocytogenes*, a pathogenic organism [11]. In *L. monocytogenes*, BSH was demonstrated to be a virulence factor required for survival within the intestine and liver and is regulated by PrfA, a transcriptional activator that responds to signals within the host [11]. Gene regulation by bile has also been observed in the gastrointestinal parasite, *Giardia lamblia*, in which bile can be used as a metabolic substrate and plays a role in promoting encystation, which is critical for pathogenesis [9]. *Shigella flexneri* grown in the presence of bile exhibits increased protein secretion and enhanced invasion of epithelial cells [5]. In *Vibrio cholerae*, bile enhances motility, while simultaneously repressing expression of other virulence factors, such as toxin co-regulated pilus and cholera toxin [4]. While regulation of motility by bile is not understood, it is thought that regulation of the *V. cholerae* virulence factors by bile occurs through modulation of the ToxT protein, the major virulence regulator [7]. All of these organisms depend upon proper expression of virulence factors to effectively cause infection, suggesting that pathogens use bile as a signal for appropriate temporal and spatial regulation of gene expression.

Salmonella enterica serovar Typhimurium, an etiologic agent of gastroenteritis, colonizes the distal ileum of the small intestine. *S. enterica* serovar Typhi, the etiologic agent of typhoid fever, interacts with bile both in the intestine and the human gallbladder. 3%–5% of the population that is infected with *Salmonella typhi* will become asymptomatic carriers of the organism, for which the site of carriage is primarily the gallbladder. Salmonellae have been shown to resist concentrations of bile significantly higher than that of other enteric organisms [8] and genes involved in bile resistance (e.g., the *mar* operon and the *acrAB* efflux pump) are induced in the presence of bile [12]. Additionally, we demonstrated that bile reduced invasion of epithelial cells through transcriptional regulation of genes within the pathogenicity island-1 invasion pathway [6]. Finally, our laboratory has demonstrated that salmonellae form a biofilm on gallstones in vitro [13], consistent with the idea that chronic carriage of *S. typhi* is associated with gallbladder abnormalities, such as gallstones [14,15]. Interestingly, formation of a biofilm on a gallstone requires the presence of bile in the media, suggesting that bile regulates factors necessary for biofilm development [13].

We hypothesize that bile is an important environmental signal for *Salmonella* spp., as it is necessary for environment-responsive gene regulation and protein modulation. Two-dimensional gel electrophoresis was performed to examine the effect of bile on protein levels from whole cell extracts, and DNA microarrays were utilized to determine the effect of bile on gene expression. These techniques allow for identification of genes and proteins specifically affected by bile and provide a more global picture as to the effect of bile on different pathways. From this study, the effect of bile on invasion was corroborated, along with the identification of novel effects on motility. This report more firmly establishes bile as an important regulatory signal in the interaction between salmonellae and their hosts.

2. Material and methods

2.1. Bacterial strains and reagents

JSG210 (ATCC14028s) is the parent of all *S. enterica* serovar Typhimurium strains. Bacterial strains provided for this study include: JSG1741 (*flhC5456*::MudJ), JSG1742 (*flgC5215*::MudJ), JSG1743 (*fliC5050*::MudJ). Strains constructed for this study include: JSG237 (*pagC503*::*luc*), JSG255 (*pagC325*::*luc*), JSG256 (*pagC673*::*luc*), JSG257 (*pagC127*::*luc*), JSG1591 (*pagC407*::*luc*), JSG1639 (*pagC*::*lacZ*), JSG1642 (*pagD*::*lacZ*), JSG1662 (*pmrD*::*lacZ*), JSG1942 (*yciG*::*luc*), JSG1943 (*yciG*::*luc*Δ*rpoS*::FRT). All strains were maintained in Luria–Bertani (LB) broth or agar with appropriate antibiotics (50 μg of ampicillin per ml or 45 μg of kanamycin per ml). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) was used at a concentration of 40 μg/ml. Bile used in this study is labeled ''sodium cholate'' and is a crude ox bile extract containing salts of deoxycholate, taurocholate, glycocholate, and cholate (Sigma Chemical Co.). All primers used in this study are listed as supplementary material and available at http://www.medicine.osu.edu/mvimg/1188.cfm.

2.2. Strain construction

DNA for *yciG*, *pagC127*, *pagC325*, *pagC503*, *pagC407*, and *pagC673* was amplified by PCR using primers JG395–JG396, WPL13–JG14, WPL13–JG12, WPL13–JG24, WPL13–JG381, and WPL13–JG13, respectively. The primers were designed with *Eco*RI and *Kpn*I sites at the 5′ end. The *yciG* fragment was cloned into the firefly luciferase-reporter suicide vector pGPL01 [16]. Recombination of this construct with *yciG* on the

chromosome created a gene fusion and a polar insertion in the *yciG*-containing operon. The *pagC127*, *pagC325*, *pagC503*, *pagC407*, and *pagC673* promoter fragments were cloned into the firefly luciferase-reporter suicide vector pLB02 [17]. Recombination on the chromosome created a gene fusion in which the strain became merodiploid for the *pagC407* promoter region. DNA for *pagC*, *pagD*, and *pmrD* was amplified by PCR using primers WPL13–JG388, JG384–JG389, and JG177–JG390, respectively. The primers were designed with *Eco*RI and *Bam*HI sites at the 5′ end. The *pagC*, *pagD*, and *pmrD* promoter fragments were cloned into the β-galactosidase-reporter vector pRS551 [18]. Linearized pRS551 with the promoter fragments was recombined onto the chromosome of ATCC14028s at the *putPA* locus as previously described, creating a gene fusion in which the strains became merodiploid for the *pagC*, *pagD*, and *pmrD* promoter fragments [19]. Deletion of *rpoS* was accomplished by means of the λ red-mediated site-specific recombination as described by Datsenko and Wanner using primers JG413–JG414 [20]. Colonies were characterized by PCR using primers specific to DNA regions outside of the deletion. The promoter of *yciG* was amplified using primers JG395–JG396 and cloned into the *Eco*RI–*Kpn*I sites of pGPL01. Upon mating into a wild-type *S. typhimurium* background, P22HT-*int* transduction was used to move the *yciG*::*luc* gene fusion into the *rpoS*::FRT deletion strain. Strains JSG1741, JSG1742, and JSG1743 were created by using P22HT-*int* transduction, in which the mutations from a *S. enterica* serovar Typhimurium LT2 wild-type background were transduced into the ATCC14028s background strain.

2.3. Transcriptional assays

Strains carrying *yciG*::*luc* were grown to log phase in LB broth and incubated for 1 h with or without 6% bile. Strains with *pagC127-673*::*luc* promoter fragments were grown to log phase in LB broth and incubated for 1 h with or without 3% bile. Cultures were washed 2× in phosphate-buffered saline (PBS) and firefly luciferase assays were performed as previously described [16].

2.4. Two-dimensional gel assays

Wild-type *S. enterica* serovar Typhimurium was grown overnight at 37 °C with aeration in LB broth, or LB broth plus 3% bile or 15% bile, washed, and pelleted. The whole cells were lysed with sodium dodecyl sulfate (SDS) boiling buffer and the extracts were separated on 2-D (SDS)–polyacrylamide gel electrophoresis gels by Kendrick Labs [21]. At least two gels were run from different samples at each bile concentration, with each gel showing the identical bile-regulated protein spots. Protein spots of interest were excised from the gels by Kendrick Labs and sent to Columbia University Howard Hughes Medical Institute Protein Chemistry Core facility for peptide mass mapping [22,23]. Briefly, the proteins were digested with trypsin and analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The peptide masses obtained by mass spectrometry were entered into search programs, which scan databases, such as NCBI or TIGR, to find significant matches. Numerous peptide matches with significant protein coverage were identified for each spot under examination, leading to full confidence in the protein identification.

2.5. RNA isolation, labeling, and hybridization

JSG210 was grown in LB broth at 37 °C with aeration to an OD_{600} of 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 of lysozyme in Tris–EDTA buffer. RNA was isolated according to the manufacturer's instruction using the Qiagen RNeasy Mini Kit (Qiagen Inc, Valencia, CA). An optional on-column DNase treatment for 1 h was also performed according to the manufacturer's instruction. cDNA was synthesized from the RNA with $pd(N)_6$ Random Hexamer (Amersham Biosciences, Piscataway, NJ) and labeled with Cy3 or Cy5 as previously described [24]. Labeled cDNA was hybridized to DNA microarrays generated from *S. enterica* serovar Typhimurium SL1344 to compare samples grown with or without bile collected at the same timepoint [25]. Labeling and hybridization were repeated three times for each time point. Hybridized slides were scanned and analyzed with a Gene Pix Scanner 4000A and the GENEPIX program (Axon Instruments, Foster City, CA).

2.6. Data analysis

Data was analyzed with the Stanford University Microarray Database, Microsoft Excel and the significance analysis for microarrays (SAM) program (http://www-stat.stanford.edu/~tibs/SAM/index.html) [26]. Three hybridizations from each time point were grouped in an experimental set and were filtered by the Stanford Microarray Database according to their mean $\log_2$–(Cy5/Cy3) ratios. Initial filtering criteria required that at least 80% of the data from a particular spot over three replicate experiments had a regression correlation of 0.6. Data were additionally filtered using Cluster software to remove any genes with missing values in greater than 20% of the columns and those that have standard deviations less than 2. SAM calculates a list of significant

genes and a false discovery rate, which is an estimate of the percentage of false positives. To determine if a common theme was present in the list of significant genes, a lexical analysis using LACK software (http://falkow.stanford.edu/whatwedo/software, script available as Windows executable or source file) was utilized. A list of common terms associated with the theme being examined were imputed into a file. The program searched the significant gene list for the presence of the terms entered. At random, the program also sampled the complete genome annotation for the number of times the terms entered appeared in the entire array list. The output file allowed us an unbiased statistical method for determining if a certain theme, such as invasion, was over-represented in the list of significant genes. Data from all arrays used in this paper are available at http://genome-www.stanford.edu/microarray.

2.7. Motility assays

Motility was measured by tracking the swimming of individual bacteria using a digital video camera attached to an Olympus BX60 phase-contrast microscope [27]. Briefly, overnight cultures were diluted 1:100 and grown to logarithmic phase in LB broth with shaking at 250 rpm at 37 °C. Cultures were then grown for one additional hour with or without 3% bile with shaking at 250 rpm at 37 °C. The cultures were then diluted 1:100 and mounted between a microscope slide and a coverslip, and several fields were analyzed under the microscope. The motion and path of the bacteria were recorded on a Canon Elura 40MC digital video camcorder (Canon Inc. Japan). A total of ca. 5 min of video imagery from two separate fields of view was acquired for each strain. A 1-min segment of each acquired field of view was captured to a computer hard disk using Adobe Premiere (Adobe Systems Inc. San Jose, CA). Still images were taken from this digital file every 0.33 s and exported to Adobe Photoshop as separate TIFF files. The TIFF files, in conjunction with the original digital video file, were subsequently used to locate and measure the position of cells by recording their pixel coordinates. Individual cells were tracked across nine consecutive TIFF files for a total of 2.66 s and the coordinates recorded. The coordinates of each tracked cell were entered into a spreadsheet (Excel, Microsoft Corp. Seattle, WA), from which estimates of the average velocity and tumble frequency were derived for each track. Motility was scored as the distance in micrometers covered per bacterium per second of video recording, while tumbles were assessed as a deviation in angle of direction of greater than 45° from the preceding time segment. For each strain, the motility represents the average of 20 bacteria per sample. The validity of data was assessed using *P* values as calculated using Student's *t*-test.

3. Results and discussion

3.1. Bile repression of pagC is dependent upon a 97-bp region of the leader sequence

Two-dimensional (2-D) gel electrophoresis was performed on whole cell extracts from *S. enterica* serovar Typhimurium grown in LB broth alone, 3% bile or 15% bile. Previously, >15 protein spots were shown to be altered by growth in the presence of bile [8]. Proteins that exhibited a significant, repeatable change, either increased or decreased levels, were excised from the gel, exposed to trypsin and analyzed by mass spectrometry. Database searches were used to identify potential matches. Five protein spots were analyzed, resulting in matches to PagC, OmpD, and FljB (which were all repressed by bile), and YciF and a hypothetical membrane protein STM4242 (which were both activated by bile) (Fig. 1). Transcriptional analysis of *ompD* and STM4242 did not demonstrate bile regulation, suggesting an effect at the posttranscriptional level (data not shown). *ompD*, which is absent in the genome of *S. enterica* seovar Typhi, and STM4242 were not analyzed further in this study.

PagC is a PhoP/PhoQ-regulated outer membrane protein with no currently assigned function, although it is associated with virulence [28,29]. It shows high homology to OmpX from *Escherichia coli*, Ail from *Yersinia enterocolitica*, and Lom from bacteriophage *lambda* [30–35]. Transcriptional activity of *pagC* was measured to examine if the decrease in protein level in the presence of bile was due to repression of transcription. Transcriptional assays demonstrated a 2.5- to 3-fold decrease in activity in the presence of bile (Fig. 2). These results support the data from the 2-D gel electrophoresis and demonstrate that bile represses *pagC* transcription. Because PhoP/PhoQ regulates more than 40 genes, additional PhoP/PhoQ-activated genes, including *pmrD* and *pagD*, were examined to determine if bile specifically regulated *pagC*, or if it has a general effect on PhoP/PhoQ-regulated genes. The results establish that other PhoP/PhoQ-regulated genes were unaffected by the presence of bile (as exemplified by *pagD* and *pmrD*, Fig. 2), suggesting that *pagC* is specifically affected by bile.

pagC has a 558-bp untranslated leader sequence between the promoter and the translational start site [36]. It has been suggested that the leader sequence may be a pseudogene, or possibly required for RNA stability (J.S. Gunn, unpublished results). To determine if the leader sequence was required for bile-mediated repression, fragments including the promoter and 127, 305, 407, 503, or 673 bp of the leader sequence were cloned into a suicide firefly luciferase vector and recombined onto the chromosome (Fig. 3). The vector containing 673-bp of sequence includes the full-length leader region and a

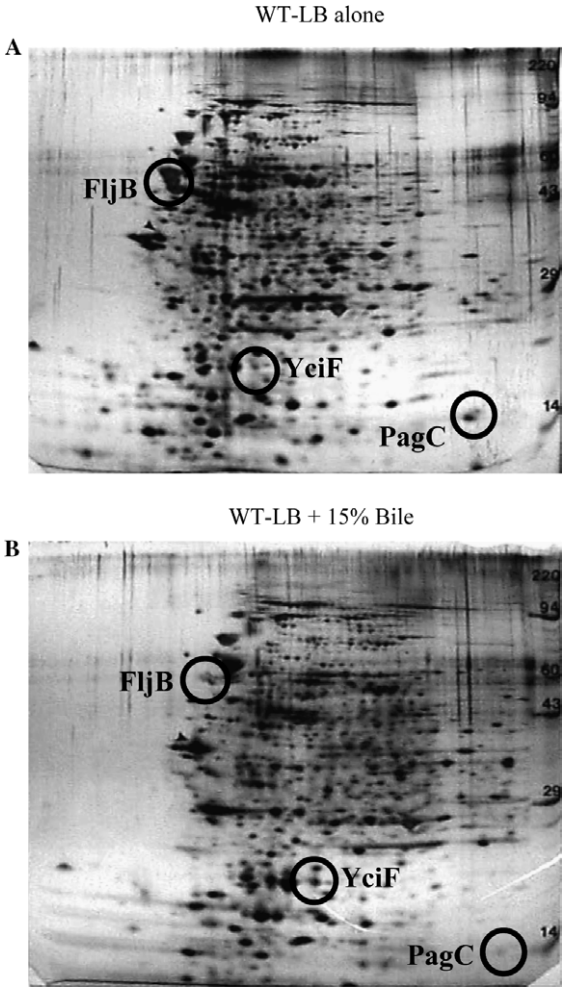

Fig. 1. Examination by 2-D gel electrophoresis of *S. enterica* serovar Typhimurium proteins affected by bile. (A) Wild-type (WT) cells grown in LB broth alone. (B) Wild-type cells grown in LB broth with 15% bile. Proteins discussed in Section 3 are indicated (STM4242 and OmpD not shown). The arrowhead indicates the isoelectric focusing standard (molecular mass, 33 kDa; p*I* 5.2). Molecular mass standards (in kDa) are noted to the right of each panel.

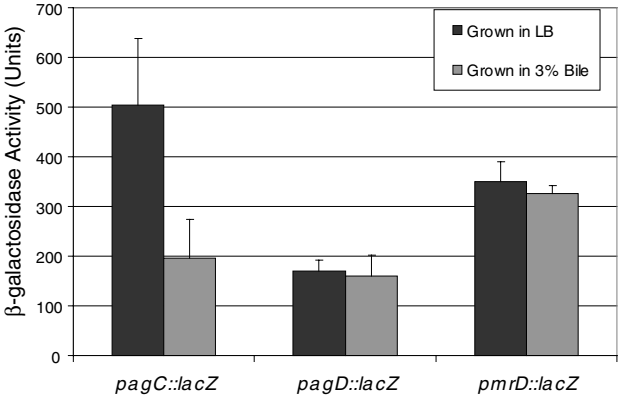

Fig. 2. Effect of bile on the transcription of PhoP-activated genes. β-Galactosidase activity of *pagC*, *pagD*, and *pmrD* was measured in the presence and absence of 3% bile. Results are an average of three separate experiments, and error bars represent standard deviations.

short segment of the *pagC* gene downstream of the translational start site and is representative of the natural promoter and untranslated leader regions (Fig. 3). PhoP exerts its effect on this promoter near the 5′ end of this 673-bp fragment upstream of the untranslated leader. Examination of the strains containing the promoter fragments in the presence and absence of bile established that a region of 97-bp located at position −173 to −269 with respect to the translational start site was required for full-transcriptional activity (Fig. 3). The data also suggest that bile does not necessarily repress *pagC* transcription, but prevents activation of transcription from occurring. Perhaps bile salts prevent binding of a transcriptional activator by either binding to the activator itself or by binding to the DNA within the leader sequence to competitively inhibit binding of the regulator. Interestingly, the same region upstream of the *pagC* translational start has been implicated in binding of the transcriptional regulatory protein SlyA (J. Gunn and

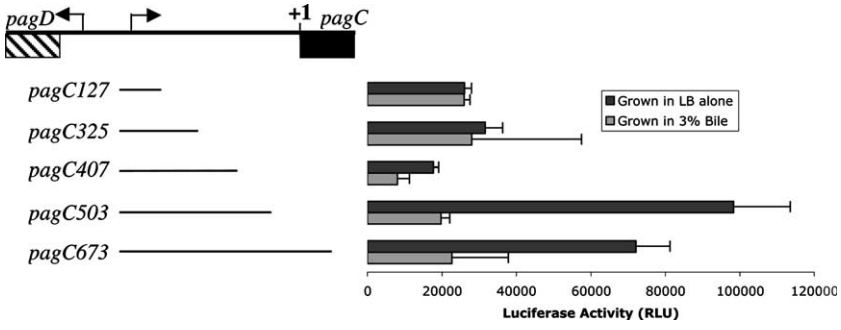

Fig. 3. Gene arrangement of *pagC* including the divergently transcribed *pagD*. The forward arrow indicates the start of transcription for *pagC*, and the reverse arrow indicates the start of transcription for *pagD*. The +1 indicates the translation start site for *pagC*. The promoter/leader sequence fragments (*pagC127*, *pagC325*, *pagC407*, *pagC503*, and *pagC673*) were fused to a luciferase fusion and assayed for transcriptional activity in the presence or absence of bile. Luciferase activity measured from the *pagC673* promoter fragment, which represents the natural promoter and full, untranslated region, was compared to fragments containing the promoter and various lengths of the untranslated region. The transcriptional assay was performed in the presence or absence of 3% bile. Results are an average of three separate experiments, and error bars represent standard deviations. RLU, relative light units.

S. Libby, unpublished observation). While the precise function of *pagC* is unknown, it does appear to affect virulence [28]. A similar protein, Ail, is required for invasion and serum resistance [37]. It is possible that the expression of *pagC* in the presence of bile is detrimental to *Salmonella* spp. viability, possibly due to the involvement (direct or peripheral) of this protein in bile transport. We are currently investigating this hypothesis.

3.2. Bile activates yciF independently of RpoS

The abundance of a second protein from the 2-D gels was observed to increase in the presence of bile and this protein was identified as YciF (Fig. 1). The gene encoding YciF is predicted to be the second gene in the *yciGFE-katN* operon [38]. The functions of *yciG* and *yciF* are unknown [38,39], but *yciE* encodes for an acid shock protein and *katN* encodes for a non-heme catalase [38]. *yciF* was initially identified by a screen for RpoS-regulated genes and a putative RpoS-dependent promoter has been located upstream of *yciG* [39].

To determine if the change in protein levels of *yciF* was due to transcriptional activation of *yciGFE-katN* by bile, a transcriptional fusion to *yciG* was constructed. The results demonstrate a 3-fold increase in transcriptional activity of *yciG*::*luc* in the presence of 6% bile (Fig. 4). Interestingly, 3% bile did not activate transcription of *yciG*, suggesting that this operon may play a role in adapting the cell to more stressful conditions (data not shown). Growth of the strain containing the *yciG*::*luc* polar insertion was not affected in the presence of bile, demonstrating that *yciGFE-katN* operon was not necessary for bile resistance (Fig. 4, data not shown).

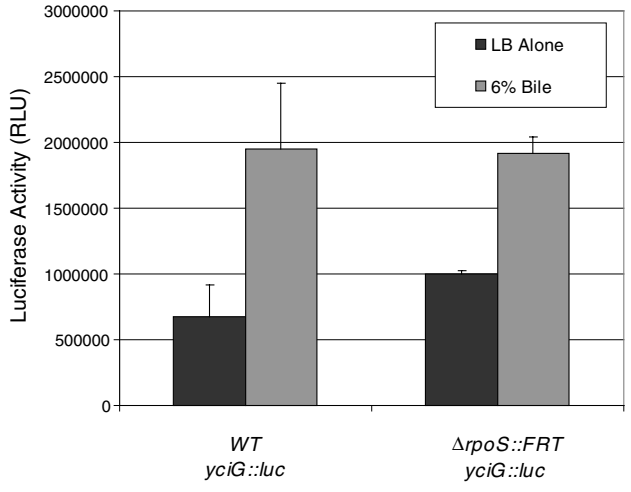

Fig. 4. Transcription of *yciG*::*luc* is activated in the presence of bile independently of RpoS. Luciferase activity from the promoter of *yciGFE-katN* was measured with or without 6% bile and in the presence or absence of *rpoS*. Results are an average of three separate experiments, and error bars represent standard deviations. WT, wild-type. RLU, relative light units.

Because *yciF* was identified in a screen for RpoS-regulated genes, we examined transcription of *yciG*::*luc* with and without RpoS to determine if activation of *yciGFE-katN* by bile was dependent upon RpoS. These data show that bile-mediated activation of *yciG* still occurred in the absence of RpoS (Fig. 4). Also, contradictory to previous findings, we observed that transcriptional activity of *yciG* was not dependent upon RpoS, as luciferase activity of the *yciG*::*luc* fusion was not decreased in a Δ*rpoS* background (Fig. 4). The role for bile activation of *yciGFE-katN* is currently unclear. However, the functions of *yciE* and *katN*, as well as the possible regulation of this operon under certain conditions by RpoS, suggest that this operon may play a role in stress response, possibly including repairs due to the effect of bile salts on the bacterium.

3.3. Microarray data corroborates previous findings that bile represses invasion

In *Salmonella* spp., genes required for type III secretion (TTS)-mediated invasion of epithelial cells are located within pathogenicity island I [40,41]. The genes form a hierarchy, which regulates the formation of the TTS apparatus and production of effector molecules required for induced uptake by epithelial cells [40]. We previously reported that bile represses transcription of the genes within the invasion hierarchy, which led to a reduction in secreted effectors and consequently, decreased invasion of epithelial cells [6]. In the current study, we used *Salmonella* DNA microarrays to identify changes in gene expression when *S. enterica* serovar Typhimurium was exposed to 3% bile for 30 and 60 min. The array data were first analyzed by hierarchical clustering to identify genes that were either repressed or induced by bile. These data showed 230 genes to be >3-fold affected by bile. Of these 230 genes, 101 were activated in the presence of bile while 129 were repressed. The LACK PERL script [42] was then utilized to determine whether particular keywords were over-represented in the list of induced or repressed genes. LACK uses statistical analysis to compare the frequency of occurrence of a particular group of keywords (input by the user) within the list of induced or repressed genes, with the frequency of the same group of words within a list of randomly generated genes. Based on analysis of the DNA microarray by LACK, gene descriptions containing the word "invasion" were over-represented in the group of bile-repressed genes, suggesting that a significant proportion of invasion genes were repressed in the presence of bile (data not shown). This finding corroborated our previously published data demonstrating that bile represses the transcription of genes within the invasion pathway [6].

3.4. Salmonellae show reduced expression of flagellar biosynthesis genes and reduced motility in the presence of bile

Many bacteria exhibit motility by means of flagella, which can also be used for attachment, such as in biofilms [13,43–46]. Expression of genes involved in flagellar biosynthesis is complex and regulation is organized into a tightly controlled hierarchy [43]. *Salmonella* spp. have over 50 genes involved in flagellar synthesis organized into at least 15 operons in three hierarchical classes [43]. Transcription of class II is dependent upon expression of class I and class III is dependent upon expression of class II [43].

Our analysis of the 2-D gel electrophoretic patterns with and without bile showed a significant bile-mediated decrease in the abundance of FljB (Fig. 1), one of two antigenically distinct flagellins expressed by *S. enterica* serovar Typhimurium [47]. From the microarray, genes involved in flagellar biosynthesis (search terms "flagella" or "motility") were shown to be over-represented in the population of bile-repressed genes on the DNA microarray. While *fliD* (6.6-fold) appeared as the most regulated flagellar-related gene in the microarrays, regulation of the majority of flagellar-related genes in the microarray ranged from 3.9-fold to our cutoff value of 2.0. These data supported the hypothesis that bile downregulates transcription of genes within the flagellar hierarchy leading to a decrease in the amount of flagella (data not shown). To directly examine flagellar gene transcription, β-galactosidase assays with and without bile were performed with strains containing MudJ fusions to *flhC*, *flgC*, and *fliC*, representative genes for class I, II, and III, respectively. The results demonstrate a decrease in transcription in the range 26–49% for all three classes (Fig. 5). These results suggest that bile downregulates flagellar expression at a transcription level. The observed regulation, while moderate, may affect flagellation and motility, as it has been shown in *Escherichia coli* that even a 2-fold repression in transcription of genes within the flagellar hierarchy can produce non-flagellated cells [48]. It is also possible that post-transcriptional regulation, which was not examined, may account for some of the regulation of flagellar protein expression.

To determine if the observed regulation by bile results in a decrease in motility, individual bacteria were tracked, and mean distance traveled, mean velocity, and tumble frequency were calculated for bacteria in LB broth alone or with bile. The bacteria exhibited a significant decrease in mean distance traveled and mean velocity in the presence of bile (Table 1). An increase in tumbling frequency was observed but could not be considered statistically significant. These results corroborate regulation observed on 2-D gels and DNA microarrays, and demonstrate that salmonellae are less motile (but not amotile) in bile. Bile has also been demonstrated to regulate motility in *V. cholerae*, but bile appears to increase motility of this organism [4]. Since *S. enterica* serovar Typhimurium flagellar mutants are competent for epithelial cell invasion and *flhD* mutants are still fully virulent in mice [49], bile-mediated repression of motility may be energetically favorable upon entrance into the intestines of a host.

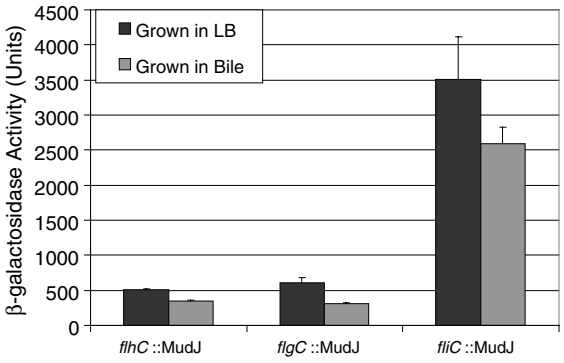

Fig. 5. Effect of bile on transcriptional regulation of the flagellar hierarchy. β-Galactosidase activity from MudJ fusions to *flhC*, *flgC*, and *fliC* (representative genes of Class I, II, and III, respectively) were measured in the presence or absence of 3% bile. Results are an average of three separate experiments, and error bars represent standard deviations.

In this study, two techniques, 2-D gel electrophoresis and DNA microarrays, were utilized to analyze the effect of bile on regulation in the whole cell without using mutagenesis, which could have unrelated effects on the bacteria. In this report, we provide data that implicates bile as an important environmental signal for genetic regulation in the salmonellae. Because many different pathways that do not appear to have any direct interactions with one another are similarly affected by bile, it is likely that bile is an environmental cue that indicates the presence of a host environment, and participates

Table 1
Motility assay

	Distance traveled (μm/s) means ± SD	Velocity (μm/s) means ± SD	Tumble frequency (%) means ± SD
WT-LB alone	11.4 ± 1.3	34.2 ± 3.8	4.8 ± 11.1
WT-LB plus 3% bile	9.9 ± 1.5*	29.8 ± 4.4*	7.5 ± 13.6**

* $P \leqslant 0.001$ compared to WT in LB alone.
** $P > 0.5$ compared to WT in LB alone.

with other host-specific signals to trigger appropriate expression of genes required for pathogenesis.

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