JB
Journals.ASM.org

Repression of *Salmonella enterica phoP* Expression by Small Molecules from Physiological Bile

L. Caetano M. Antunes,[a] Melody Wang,[b] Sarah K. Andersen,[c] Rosana B. R. Ferreira,[a] Reinhild Kappelhoff,[d] Jun Han,[e] Christoph H. Borchers,[e] and B. Brett Finlay[a,b,c]

Michael Smith Laboratories,[a] Department of Microbiology and Immunology,[b] Department of Biochemistry and Molecular Biology,[c] and Department of Oral Biological & Medical Sciences,[d] The University of British Columbia, Vancouver, British Columbia, Canada, and University of Victoria-Genome BC Proteomics Centre, University of Victoria, Victoria, British Columbia, Canada[e]

Infection with *Salmonella enterica* serovar Typhi in humans causes the life-threatening disease typhoid fever. In the laboratory, typhoid fever can be modeled through the inoculation of susceptible mice with *Salmonella enterica* serovar Typhimurium. Using this murine model, we previously characterized the interactions between *Salmonella* Typhimurium and host cells in the gallbladder and showed that this pathogen can successfully invade gallbladder epithelial cells and proliferate. Additionally, we showed that *Salmonella* Typhimurium can use bile phospholipids to grow at high rates. These abilities are likely important for quick colonization of the gallbladder during typhoid fever and further pathogen dissemination through fecal shedding. To further characterize the interactions between *Salmonella* and the gallbladder environment, we compared the transcriptomes of *Salmonella* cultures grown in LB broth or physiological murine bile. Our data showed that many genes involved in bacterial central metabolism are affected by bile, with the citric acid cycle being repressed and alternative respiratory systems being activated. Additionally, our study revealed a new aspect of *Salmonella* interactions with bile through the identification of the global regulator *phoP* as a bile-responsive gene. Repression of *phoP* expression could also be achieved using physiological, but not commercial, bovine bile. The biological activity does not involve PhoPQ sensing of a bile component and is not caused by bile acids, the most abundant organic components of bile. Bioactivity-guided purification allowed the identification of a subset of small molecules from bile that can elicit full activity; however, a single compound with *phoP* inhibitory activity could not be isolated, suggesting that multiple molecules may act in synergy to achieve this effect. Due to the critical role of *phoP* in *Salmonella* virulence, further studies in this area will likely reveal aspects of the interaction between *Salmonella* and bile that are relevant to disease.

Salmonella enterica is a versatile bacterial pathogen equipped with the machinery required to infect a multitude of hosts (16). Multiple *S. enterica* serovars are able to infect humans, each causing specific clinical manifestations of disease (12, 16). *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) causes localized, self-limiting gastroenteritis in humans, whereas *S. enterica* serovar Typhi (*Salmonella* Typhi) causes systemic, life-threatening typhoid fever (12, 26). Human infection with *Salmonella* Typhi can be modeled in the laboratory through the infection of susceptible mice with *Salmonella* Typhimurium (34). In this model, systemic infection ensues and *Salmonella* can infect multiple organs, such as the gastrointestinal tract, brain, liver, spleen, and gallbladder (19, 42). Many aspects of host colonization in this murine model are reminiscent of human *Salmonella* Typhi infection. In particular, gallbladder infection of patients is common during typhoid fever, and this can also occur in the absence of clinical symptoms; these patients are asymptomatic carriers that aid in the spread of the pathogen through fecal shedding (3, 23, 36, 39). Therefore, the interactions between pathogen and host in the gallbladder are important aspects that must be studied to better understand *Salmonella* infections in humans.

We have recently studied the interactions between *Salmonella* and its host using a gallbladder infection model (19). *Salmonella* can invade and infect the epithelial cells lining the lumen of the gallbladder in a murine host (19). Additionally, *Salmonella* can successfully colonize bile, where it can grow at rates comparable to those achieved in rich culture medium in the laboratory, presumably using phospholipids as carbon and energy sources (1, 19). Therefore, in order to further characterize the interactions between *Salmonella* and the host gallbladder environment, we used DNA microarrays to perform a transcriptome analysis of *Salmonella* growth in bile. We found that many genes are differentially regulated during growth in bile, with the central metabolism being deeply affected. Interestingly, we also found that *phoP*, a major regulator of virulence gene expression in *Salmonella* and many other bacteria (10, 13, 18), was strongly repressed by bile. Follow-up experiments determined that the repression of *phoP* expression does not occur through an effect on PhoPQ signaling; i.e., our evidence suggests that this phenotype is not due to direct sensing of a bile component by the PhoPQ signaling cascade. The repression of *phoP* expression could also be replicated using physiological (but not commercial) bovine bile, and a subset of small molecules from bile with full inhibitory activity was determined. Further studies in this area should reveal more details of the intricate interaction between *Salmonella* and bile and its impact on this pathogen's lifestyle.

MATERIALS AND METHODS

Chemicals. Carbenicillin was purchased from EMD Chemicals (San Diego, CA). Streptomycin, dimethyl sulfoxide (DMSO), dehydrocholic

Received 20 January 2012 Accepted 16 February 2012
Published ahead of print 24 February 2012
Address correspondence to B. Brett Finlay, bfinlay@interchange.ubc.ca.

doi:10.1128/JB.00104-12

acid, cholic acid, chenodeoxycholic acid, deoxycholic acid, glycolithocholic acid ethyl ester, taurocholic acid sodium salt, lithocholic acid, sodium taurodeoxycholate hydrate, sodium tauroursodeoxycholate, sodium taurochenodeoxycholate, methanol, and trifluoroacetic acid were purchased from Sigma-Aldrich (Oakville, Canada).

Commercial bile. Commercial bovine bile was obtained from Sigma-Aldrich and is a crude bile extract from ox gallbladder. It is concentrated through boiling to approximately 50 to 60% total solids, vacuum dried, milled to a powder, and packaged. This powder is soluble in water at 50 mg/ml. For the purpose of comparison, we note that in order to approximate the natural composition of bile, this powder would have to be dissolved in water at a concentration of approximately 80 mg/ml.

Physiological bile. We obtained fresh bile from C57BL/6 and 129Sv/ImJ Nramp1$^{+/+}$ and Nramp1$^{-/-}$ mice from breeding colonies maintained at the Wesbrook Animal Unit at The University of British Columbia. Mice were fed a standard sterile chow diet (Laboratory Rodent Diet 5001; Purina Mills, St. Louis, MO) *ad libitum* throughout the experiments. Mice were sacrificed through CO_2 asphyxiation, and bile was collected immediately. Bile samples were centrifuged for 5 min at 16,000 × *g*, and the supernatant was collected and frozen at −20°C until used. We also obtained fresh cow bile from a small slaughter plant as a kind gift from Neil Rawlyk, Sherry Tetland, Don Wilson, and Andrew Potter (Vaccine and Infectious Disease Organization, University of Saskatchewan). Bile was collected and saved at −20°C until used. In order to distinguish them from commercial bile, the fresh murine bile and bovine bile obtained and used in this study are referred to as "physiological bile."

Microarray experiments. *Salmonella* Typhimurium LT2 glass slide microarrays were obtained through the NIAID's Pathogen Functional Genomics Resource Center (PFGRC), managed and funded by Division of Microbiology and Infectious Diseases, NIAID, NIH, DHHS, and operated by the J. Craig Venter Institute. Two independent cultures of *Salmonella* Typhimurium SL1344 (43) were grown overnight in Luria-Bertani (LB) broth with streptomycin (100 μg/ml) at 37°C with shaking (225 rpm). Cells were pelleted by centrifugation and resuspended in sterile Dulbecco's phosphate-buffered saline (PBS; HyClone, Logan, UT). These suspensions were further diluted 1:50 in PBS and used to inoculate 70 μl of either LB broth or physiological murine bile (in 0.65-ml tubes) at a 1:100 dilution. Cultures were incubated for 24 h at 37°C with agitation (225 rpm). Then, samples were diluted in PBS, and serial dilutions were plated on LB plates containing 100 μg/ml of streptomycin and incubated overnight at 37°C for bacterial growth and enumeration by colony counting. The remaining cells were used in the subsequent steps, as described below. RNA isolation began with the addition of 2 volumes of RNAprotect bacterial reagent (Qiagen, Hilden, Germany) to the bacterial cultures and incubation at room temperature for approximately 5 min. Cells were pelleted by centrifugation, and RNA was isolated using the RNeasy minikit (Qiagen) with on-column DNase digestion, according to the manufacturer's recommendations. Synthesis of cDNA was performed as described herein. The entire RNA sample (≥3 μg) was mixed with 6 μg of random primers (Invitrogen, Burlington, Canada) and 40 U of RNaseOUT recombinant RNase inhibitor (Invitrogen) in a total volume of 18.5 μl and incubated at 70°C for 10 min. Samples were incubated on ice for 30 s, centrifuged to bring down condensation, and incubated with the following components (Invitrogen) at the final concentrations shown in parentheses: First Strand buffer (1×), dithiothreitol (10 mM), dNTPs (dATP, dCTP, dGTP, dTTP; 0.5 mM each) and 100 U of SuperScript II reverse transcriptase in a total volume of 30 μl. The mixture was incubated at 42°C overnight, and the reaction was stopped and the RNA degraded through the addition of 0.5 M EDTA and 1 M sodium hydroxide (10 μl each) and incubation at 65°C for 15 min. Then, 25 μl of 1 M Tris (pH 7) was added to neutralize the pH of the cDNA solution. cDNA was purified using the MinElute PCR purification kit (Qiagen) according to the manufacturer's recommendations, except that the Qiagen wash and elution buffers were substituted for by phosphate buffers, according to the PFGRC microarray protocol. cDNA was then precipitated using ammonium acetate and ethanol, as described elsewhere (17), and labeled with Cy3 (LB cultures) and Cy5 (bile cultures) using the ULS aRNA fluorescent labeling kit (Kreatech Biotechnology, Amsterdam, The Netherlands) according to the manufacturer's instructions. Samples were mixed, dried, and saved at −80°C until used. Prehybridization, hybridization, and washing steps were performed essentially as described in the PFGRC protocols, except that the hybridization buffer contained KREAblock blocking buffer (25%; Kreatech Biotechnology). After hybridization, slides were scanned using an Affymetrix 428 array scanner from Eurofins MWG operon (Huntsville, AL).

TABLE 1 Primers used in this study

Primer	Sequence (5′→3′)[a]
Real-time PCR	
PHOPF	CAATGAAGAGGTCATCAAACTCAC
PHOPR	GAGAACATCAATGGTATGACTTTCC
PMRAF	CATAATAACCAGGGTGAAAGTGAAC
PMRAR	CGTTATCCCAGTTGTAGATATCGTT
MGTAF	AATCCTTTCAACATCTTACTCACGA
MGTAR	ATTTTCATTAATAACCCGCAGTACG
MGTCF	AGGGAGAAAAACGTTATATCCTGAA
MGTCR	ATTTCTTTATAGCCCTGTTCCTGAG
PAGCF	ACATTTAAAGAACATTCCACTCAGG
PAGCR	AGCCGTTTATTTTTGTAGAGGAGAT
Cloning	
PPHOPF	ATTAGAGCTCTCGCGCTGTGACTCTGGTCG
PPHOPR	ATAAGGATCCATCCTCTACAACCAGTACGC
PHILAF	TCCTGAGCTCGATATAATGCCTGGAGCC
PHILAR	CCATGGATCCTATGAAATCATCAAAGACG

[a] Restriction enzyme sites are underlined.

Microarray data analysis. Scanned glass slide images were processed with Spotfinder and MIDAS version 2.22 (32). Each of the two slides was processed independently and contains two spots for each *Salmonella* gene, resulting in a total of 4 possible measurements of transcript abundance per gene. Genes that were detected in at least 3 of the 4 spots and showed average differences in signal intensity of 2.5-fold or more between the two growth conditions as well as individual differences of 2-fold or more in all spots with a detectable signal were selected and are reported.

Real-time PCR. *Salmonella* was cultured in LB broth or physiological murine bile, essentially as described for microarray experiments. RNA was stabilized using RNAprotect bacterial reagent (Qiagen) and extracted using the RNeasy minikit (Qiagen) with the on-column DNase digestion, according to the manufacturer's recommendations. Synthesis of cDNA was performed using the QuantiTect reverse transcription (RT) kit (Qiagen). For RT-PCR, we used the QuantiTect SYBR green PCR kit (Qiagen) and the Applied Biosystems (Foster City, CA) 7500 system. Forward and reverse primers were added to reaction mixtures at a final concentration of 0.4 μM each. All results were normalized using the expression levels of the housekeeping gene *gapA*, encoding the glyceraldehyde-3-phosphate dehydrogenase enzyme (22), as the baseline. Averages of the data obtained with cultures grown in LB broth were normalized to 1, and the data from each sample (LB broth or bile) were normalized accordingly. Primers used for RT-PCR are shown on Table 1.

***Salmonella* growth curves.** We obtained fresh, physiological bovine bile, adjusted its pH to match that of LB broth (around 7.3), and used it to perform *Salmonella* growth assays. LB broth and bile were tested either alone or in 1:1 mixtures with each other or PBS, as indicated. Streptomycin was present in all experiments at a final concentration of 100 μg/ml. Overnight cultures of *Salmonella* Typhimurium SL1344 were used to inoculate each culture medium at 1:200. Cultures were then incubated at 37°C with shaking (225 rpm), and growth was followed through measurements of the optical density at 600 nm.

GFP reporter assays. In order to monitor the effect of bile or bile components on *hilA* or *phoP* expression, we amplified DNA fragments

containing the promoters of *hilA* (−675 through +70, relative to the translational start codon) and *phoP* (−163 through +33, relative to the translational start codon) through PCR and independently cloned them upstream of the promoterless green fluorescent protein (GFP) gene *gfp* of pFPV25 (40) using the SacI and BamHI restriction sites and standard cloning procedures, as described elsewhere (2, 33). The primers used are shown on Table 1. The resulting plasmids were transformed into the appropriate *Salmonella* strains, and GFP production was analyzed through flow cytometry of bacterial cultures using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ), as indicated. All cultures contained carbenicillin (100 μg/ml) and were incubated at 37°C with shaking (225 rpm). In each experiment, between 25,000 and 50,000 events were collected per sample.

Solid-phase extraction of the bioactive compound in physiological bovine bile. In order to purify the active compound from physiological bovine bile, we used Sep-Pak C_{18} resin cartridges (Waters, Milford, MA). Columns were washed with 10 volumes of methanol (relative to the column bed weight) followed by a wash with the same volume of water. Ten volumes of physiological bovine bile was applied to the column, and the flowthrough was collected. Columns were then washed with 20 volumes of water, the molecules bound were eluted with 10 volumes of 50% methanol (in water) followed by 10 volumes of 100% methanol, and both fractions were collected.

RP-HPLC. The fraction from the C_{18} cartridge purification eluted with 100% methanol described above was dried in a centrifuge equipped with a vacuum pump and resuspended in 0.1 volume of 25% methanol (in water). The suspension was filtered through a 0.22-μm pore-size membrane and used for further purification through reverse-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC was performed using a Nucleosil C_{18} column (5-μm particle size, 25 by 0.46 cm; Sigma-Aldrich) with methanol-water mixtures containing 0.1% trifluoroacetic acid as the mobile phase, as indicated.

UPLC-MS. For ultrahigh-performance liquid chromatography coupled with mass spectrometry (UPLC-MS), select fractions from RP-HPLC were dried and the residues were dissolved in 200 μl of methanol and diluted 1 to 500 with 75% methanol. Three microliters was injected into a 10- by 0.21-cm C_{18} UPLC column and run on the UPLC-quadrupole time of flight (QTOF) mass spectrometer in electrospray ionization [ESI(−)] mode.

Statistical analyses. Data were analyzed by unpaired *t* tests with 95% confidence intervals using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA).

Microarray data accession number. Raw microarray data were deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE33604.

RESULTS

Physiological murine bile alters the expression of multiple *Salmonella* genes. We have previously shown that *Salmonella* can successfully multiply in murine bile, despite the well-known antimicrobial properties of this physiological fluid (1, 19). In order to determine the impact of bile on *Salmonella* gene expression, we performed transcriptome comparisons between *Salmonella* grown in LB broth and *Salmonella* grown in physiological murine bile using DNA microarrays. As expected, following the inoculation of bile at an approximate concentration of 8×10^5 cells per ml, *Salmonella* showed signs of growth as early as 2 h postinoculation and displayed robust growth after 24 h of growth, achieving a concentration of 4×10^9 cells per ml (Fig. 1).

To compare transcriptional profiles of cells grown in LB broth or bile, we isolated RNA from these cultures after 24 h of growth and analyzed transcript levels using *Salmonella* Typhimurium LT2 glass slide microarrays obtained through the Pathogen Functional Genomics Resource Center (PFGRC), operated by the J. Craig Venter Institute. By doing so, we found a total of 54 genes whose expression levels were affected 2.5-fold or more during growth in bile. Of these, 17 genes were activated, whereas 37 genes were repressed by bile (Table 2). Most of the genes differentially regulated were involved in bacterial central metabolism and respiration. We found that a significant portion of the genes repressed by bile were involved in the citric acid cycle and the generation of electron donors for aerobic respiration—e.g., succinate dehydrogenase, isocitrate dehydrogenase, aconitate hydratase, and others. Conversely, a number of the genes activated by bile were involved in alternative respiratory systems. For instance, we found that several hydrogenase-related genes (hydrogenase subunits, maturation endopeptidase, and chaperone) were activated by bile, suggesting a shift from the use of NAD(P)H and succinate to H_2 as electron donors. Besides metabolic genes, we found that growth in bile affected the expression of genes involved in iron and copper metabolism and transport of sugars, amino acids, and oligopeptides, as well as virulence regulation.

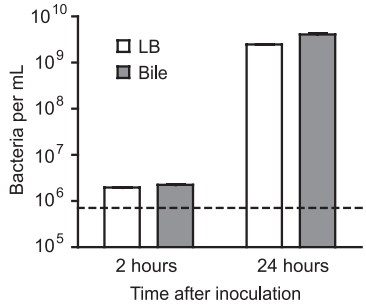

FIG 1 *Salmonella* displays robust growth in physiological murine bile. Cultures were started by pelleting cells from an overnight *Salmonella* culture by centrifugation, resuspending them in one volume of PBS, diluting this solution 1:50 in PBS, and inoculating LB broth or bile with this suspension at a 1:100 dilution. This resulted in an initial bacterial concentration of approximately 8×10^5 cells per ml (indicated by the dashed line). Samples were incubated at 37°C with shaking for 24 h, and aliquots were removed, diluted, and spotted on LB broth plates for bacterial enumeration after 2 and 24 h of growth. Average results of three independent cultures with standard errors of means are shown.

***phoP* expression is strongly repressed by physiological murine bile.** Among the many genes whose expression was repressed by bile, we found one main regulator of virulence gene expression, *phoP*. Because both growth in bile and *phoP* expression are directly linked to *Salmonella* virulence, we chose to focus our studies on the regulation of *phoP* expression by bile, a previously unreported phenomenon with likely implications for *Salmonella* virulence and human disease. Our microarray experiments showed that *phoP* expression was repressed approximately 4-fold during growth in bile. In order to confirm this observation as well as to assess whether the effect of bile on *phoP* expression resulted in the regulation of *phoP*-controlled genes, we used real-time PCR to study transcript levels of *phoP* and several other *phoP*-regulated genes during growth in LB broth or physiological murine bile. Confirming our initial observation of *phoP* repression by bile through microarrays, our RT-PCR results indicated that *phoP* expression was repressed approximately 3.2-fold when *Salmonella* was grown in bile (Fig. 2). We also compared transcript levels of *pmrA*, *mgtA*, *mgtC*, and *pagC*, all of which are regulated by PhoP (14, 21, 37), during growth in LB broth or bile. Supporting our initial observations of *phoP* repression by bile, we found that tran-

TABLE 2 Regulation of *Salmonella* gene expression during growth in physiological murine bile

ORF no.[a]	Common name	Gene	Fold regulation
STM3146	Hydrogenase 2 maturation endopeptidase	*hybD*	10.36
STM3506	Ferrous iron transport protein B	*feoB*	7.02
STM3150	Hydrogenase 2 small subunit	*hypO*	4.55
STM1907	Copper homeostasis protein CutC	*cutC*	4.46
STM3511	Putative DNA uptake protein	*yhgI*	3.98
STM3465	Hypothetical protein	*yhfA*	3.61
STM3505	Ferrous iron transport protein A	*feoA*	3.56
STM1324	Putative cytoplasmic protein		3.53
STM0629	Cold shock protein CspE	*cspE*	3.43
STM3360	Arginine repressor	*argR*	3.17
STM1200	Thymidylate kinase	*tmk*	3.16
STM1121	Putative cytoplasmic protein	*ymdF*	3.15
STM2856	Hydrogenase assembly chaperone	*hypC*	2.88
STM3147	Hydrogenase 2 large subunit	*hybC*	2.87
STM3302	Pseudogene, *yhhE*	*yhbE*	2.84
STM0974	Formate transporter	*focA*	2.71
STM2532	Putative inner membrane lipoprotein		2.55
STM0441	Cytochrome *o* ubiquinol oxidase subunit III	*cyoC*	−10.71
STM0439	Protoheme IX farnesyltransferase	*cyoE*	−7.50
STM3630	Dipeptide transport protein	*dppA*	−6.35
ORF01133	Protein PhoH	*phoH*	−5.60
STM0544	Fimbrial protein	*fimI*	−5.33
STM0154	Dihydrolipoamide dehydrogenase	*lpdA*	−5.14
STM0158	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	*acnB*	−4.79
STM2378	3-Oxoacyl-(acyl carrier protein) synthase I	*fabB*	−4.58
STM4398	d-Alanine/d-serine/glycine permease	*cycA*	−4.56
STM0600	Carbon starvation protein	*cstA*	−4.54
STM3218	Putrescine-2-oxoglutarate aminotransferase	*oat*	−4.43
STM2389	3-ketoacyl-CoA[b] thiolase	*fadI*	−4.20
PSLT052	Plasmid partition protein A	*parA*	−4.18
STM1231	DNA-binding transcriptional regulator PhoP	*phoP*	−4.06
STM4159	Thiamine biosynthesis protein ThiH	*thiH*	−4.05
STM3557	Glycerol-3-phosphate transporter periplasmic binding protein	*ugpB*	−4.03
STM0734	Succinate dehydrogenase flavoprotein subunit	*sdhA*	−3.98
STM0662	Glutamate/aspartate transporter	*gltL*	−3.89
ORF01131	Bifunctional protein PutA	*putA*	−3.84
STM1238	Isocitrate dehydrogenase	*icdA*	−3.83
STM1712	Aconitate hydratase	*acnA*	−3.48
STM0549	Transcriptional regulator FimZ	*fimZ*	−3.45
STM2141	Fructose-bisphosphate aldolase	*fbaB*	−3.37
STM0735	Succinate dehydrogenase iron-sulfur subunit	*sdhB*	−3.25
STM0738	Succinyl-CoA synthetase subunit beta	*sucC*	−3.25
STM1744	Oligopeptide transport protein	*oppC*	−3.21
PSLT046	Putative carbonic anhydrase		−3.20
STM2190	Galactose transport protein	*mglB*	−3.02
STM2799	DNA binding protein, nucleoid associated	*stpA*	−2.97
STM1292	Putative cytoplasmic protein	*yeaC*	−2.94
STM0730	Type II citrate synthase	*gltA*	−2.90
STM3321	Putative σ^{54} modulation protein	*yhbH*	−2.84
STM1742	Oligopeptide transport protein	*oppF*	−2.78
STM3033	Plasmid maintenance protein		−2.72
STM4274	Putative inner membrane protein	*yjcH*	−2.65
STM4126	Soluble pyridine nucleotide transhydrogenase	*udhA*	−2.63
ORF04918	Virulence protein VsdF		−2.59

[a] STM and pSLT designations refer to *Salmonella enterica* serovar Typhimurium LT2. Open reading frame (ORF) designations refer to *Salmonella enterica* serovar Typhimurium 14028.
[b] CoA, coenzyme A.

script levels of both *pmrA* and *pagC* were significantly repressed by bile. Both *pmrA* and *pagC* are activated by PhoPQ (14, 21), and our results suggest that repression of *phoP* by bile causes the repression of these two PhoPQ target genes. Although the other genes tested did not reach statistically significant regulation by bile, *mgtA* displayed an obvious expression trend that suggests it may also be affected by bile through the regulation of *phoP* (Fig. 2).

In order to further confirm the effect of bile on *phoP* expression and study this phenomenon in more detail, we constructed a *phoP* expression reporter strain by inserting the *phoP* promoter upstream of the promoterless *gfp* of pFPV25. The resulting plasmid was then inserted into wild-type *Salmonella*. Overnight cultures of this *phoP* reporter strain were used to inoculate 50 μl of LB broth or physiological murine bile (in 0.65-ml tubes) at a 1:100 dilution. The cultures were incubated for 4 h and then diluted 4-fold in PBS for flow cytometry analysis of GFP expression. Figure 3 shows that *gfp* expression is significantly reduced during *Salmonella* growth in bile compared to LB broth. In these experiments, the average fluorescence intensity per cell was approximately 4.7-fold lower in bile-grown cells than that in LB broth-grown cells.

The effect of bile on *phoP* expression is independent of PhoPQ signaling. As is common with bacterial two-component regulatory systems, PhoPQ signaling controls *phoPQ* gene expression (35). Therefore, we decided to investigate the possibility that the effect of bile on *phoP* expression could be the result of a specific PhoP ligand being present in bile. If such a ligand existed and its binding repressed PhoPQ activity, one would expect that *phoP* expression would be repressed, resulting in a phenotype reminiscent of the one reported here. Therefore, we inserted the *phoP*::*gfp* fusion plasmid into a *phoP* knockout *Salmonella* strain constructed through P22-assisted transduction of a previously described *phoP*::Tn*10* mutation into *Salmonella* Typhimurium SL1344 (9) and measured *gfp* expression. Overnight cultures of the resulting strain were used to inoculate aliquots of LB broth or physiological murine bile at a 1:100 dilution, as described above. The cultures were incubated for 4 h and then diluted 4-fold in PBS for flow cytometry analysis of GFP expression. If PhoPQ signaling were required for bile-mediated *phoP* repression, this repression would not occur in a *phoP* null mutant. Our results suggest that this is not the case. Figure 4 shows that bile can still repress *phoP* expression in a *phoP*::Tn*10* background. Also, a comparison of *phoP* expression levels between wild-type and *phoP*::Tn*10* *Salmonella* revealed that the introduction of a *phoP* mutation reduced *phoP* expression even during growth in bile. Altogether, our data show that bile does not repress *phoP* expression through repression of PhoPQ signaling.

Bile acids are not responsible for the bile-mediated repression of *phoP* expression. Our data suggest that a molecule present in bile can repress the expression of the *phoP* gene without necessarily interfering with PhoPQ signaling. Because bile acids are the most abundant component of bile, accounting for approximately 67% of all of the organic content (8), we decided to study the effect of several bile acids on *phoP* expression. To this end, we prepared solutions of dehydrocholic acid, cholic acid, chenodeoxycholic acid, deoxycholic acid, glycolithocholic acid ethyl ester, taurocholic acid sodium salt, and lithocholic acid in DMSO and added these bile acids to LB broth at the final concentrations indicated. Controls containing only DMSO were also produced. The pH of the medium was not affected by DMSO or any of the bile acids tested. An overnight culture of the *Salmonella phoP*::*gfp* reporter strain was diluted 1:200 in LB broth containing each of the bile acids, and the cultures were incubated for 4 h. Cultures were diluted 1:20 in PBS and analyzed through flow cytometry. Table 3 shows that none of these bile acids, when added to bacterial cultures, repressed the expression of *phoP*. In fact, all bile acids tested caused a slight increase in *phoP* expression, although the change observed was minimal and was unlikely to be biologically relevant.

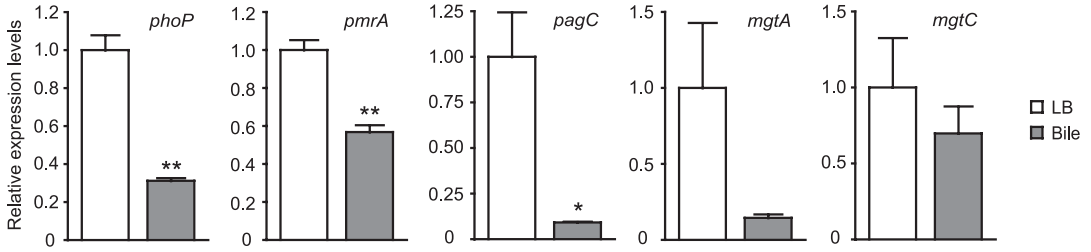

FIG 2 *Salmonella* growth in physiological murine bile modulates transcript levels of *phoP* and *phoP*-regulated genes. *Salmonella* was grown in LB broth or bile for 24 h, RNA was isolated, cDNA was synthesized, and RT-PCR was performed using primers specific for the genes indicated. Expression levels during growth in LB broth were normalized to 1, and expression in bile was adjusted accordingly. All results were normalized using the expression levels of *gapA*. Average expression levels of three independent cultures with standard errors of means are shown. *P* values were >0.05 (no asterisk), <0.03 (*), or <0.003 (**).

Nevertheless, our data suggest that the molecule present in physiological murine bile that represses *phoP* expression is unlikely to be a bile acid.

Commercial bovine bile does not produce the effects of physiological murine bile on *phoP* expression. Commercial bovine bile has been previously shown to repress invasion gene expression in *Salmonella* (30, 31). To investigate whether the components of bile that affect invasion and *phoP* expression are the same, we fused the promoter of the major regulator of invasion gene expression in *Salmonella*, *hilA*, with *gfp* in pFVP25 and studied the production of GFP by *Salmonella* strains containing the *hilA* or *phoP* reporter plasmids during growth in LB broth with or without 3% (wt/vol) bovine bile. We prepared a solution of bovine bile in LB broth and adjusted the pH to match that of LB broth (around 7.3). An overnight culture of the *Salmonella phoP*::*gfp* reporter strain was used to inoculate LB broth, with or without bovine bile, at a 1:200 dilution. Cultures were incubated for 4 h, diluted 1:20 in PBS, and analyzed by flow cytometry. During growth in LB broth, the expression of *hilA* is bimodal, with most cells not producing GFP and a subpopulation of bright cells accounting for approximately 25% of the total number of cells (Fig. 5). In accordance with previous observations, *hilA* expression was strongly repressed by commercial bovine bile. During growth in bile-containing LB broth, the subpopulation of GFP-producing cells accounted for only 3.1% of the total number of cells. The repression of *hilA* by commercial bovine bile could also be clearly seen during the analysis of the average fluorescence values per cell in cells grown in LB broth with or without bile. In LB broth without bile, the average fluorescence per cell was 39.2, whereas in LB broth containing bile, this value was 5.9, accounting for an approximate fold change of 6.7 (Fig. 5). The effect of commercial bovine bile on *phoP* expression, however, was much less pronounced. Growth in LB broth containing bile caused a reduction in the average fluorescence per cell from 164.7 to 109, a 1.5-fold change. Although

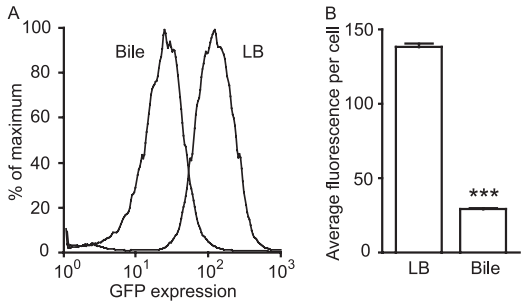

FIG 3 *phoP* expression is strongly repressed by *Salmonella* growth in physiological murine bile. A *Salmonella* strain containing a *phoP*::*gfp* plasmid was grown overnight in LB broth with carbenicillin and subcultured 1:100 in LB broth or bile containing carbenicillin. After incubation at 37°C with shaking for 4 h, cultures were diluted in PBS, and *gfp* expression was analyzed through flow cytometry. (A) Histogram showing the relative proportions of cells (*y* axis) expressing *gfp* at various levels (*x* axis) during growth in LB broth or bile, as indicated. (B) Data from panel A expressed as average fluorescence intensity per cell. The data shown are the average from three independent cultures. Bars represent the standard errors of means. The *P* value was <0.0001 (***).

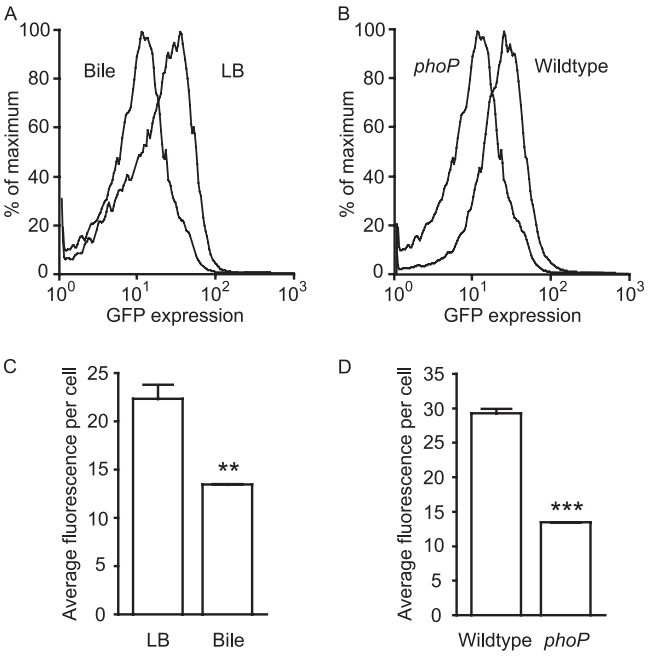

FIG 4 The repression of *phoP* expression by physiological murine bile is independent of PhoPQ signaling. The *phoP*::*gfp* reporter plasmid was inserted into wild-type *Salmonella* as well as a *phoP*::Tn*10* mutant. Strains were grown overnight in LB broth with carbenicillin and subcultured 1:100 in LB broth or bile containing carbenicillin. After incubation at 37°C with shaking for 4 h, cultures were diluted in PBS, and GFP expression was analyzed through flow cytometry. (A) Histogram showing the relative proportions of cells (*y* axis) expressing *gfp* at various levels (*x* axis) during growth of the *phoP*::Tn*10 Salmonella* strain in LB broth or bile, as indicated. (B) Same as in panel A, except that levels of *gfp* production by the wild-type and *phoP*::*Tn10* (*phoP*) *Salmonella* strains were analyzed during growth in physiological murine bile. (C) Data from panel A expressed as average fluorescence intensity per cell. (D) Data from panel B expressed as average fluorescence intensity per cell. The data shown are the average from three independent cultures. Bars represent the standard errors of means. *P* values were <0.005 (**) and <0.0001 (***).

TABLE 3 Effect of individual bile acids on *phoP* expression

Treatment[a]	Relative fluorescence at concn shown[b]		
	0.25 mM	0.5 mM	1 mM
None	100 (2.8)	100 (2.7)	100 (2.1)
Dehydrocholic acid	101 (3.3)	111 (1.2)	101 (1.1)
Cholic acid	101 (2.6)	113 (2.3)	96 (3.0)
Chenodeoxycholic acid	99 (3.3)	111 (0.8)	99 (1.6)
Deoxycholic acid	101 (2.0)	115 (2.3)	102 (0.5)
Glycolithocholic acid	102 (2.0)	113 (1.1)	105 (0.4)
Taurocholic acid	103 (1.7)	116 (0.9)	105 (0.5)
Lithocholic acid	112 (3.7)	121 (2.7)	132 (5.4)

[a] Bile acids were suspended in DMSO and added to LB broth at the concentrations indicated. The controls contained the same concentration of DMSO, but without bile acids.

[b] Arbitrary fluorescence values relative to control. Results shown are the average of three bacterial cultures, with standard errors of means shown within parentheses.

the effects of bile on both *hilA* and *phoP* were statistically significant, it is clear that the activity against *hilA* is distinct from that against *phoP* expression.

Physiological bovine bile alone does not support robust *Salmonella* growth *in vitro*. We have previously shown that physiological murine bile supports *Salmonella* growth at rates comparable to those seen in rich laboratory media (1). In order to begin characterizing the interactions between *Salmonella* and bile from other organisms, we obtained fresh, physiological bovine bile, adjusted its pH to match that of LB broth (around 7.3), and used it to perform *Salmonella* growth assays. Contrary to our findings using physiological murine bile, physiological bovine bile alone did not support *Salmonella* growth (Fig. 6). To determine if this was due to the absence of nutrients required for *Salmonella* growth in bovine bile or the antibacterial action of bile, we mixed LB broth or bile with PBS at a 1:1 ratio and analyzed *Salmonella* growth in these media. If the reason for the lack of *Salmonella* growth in bile were the presence of antibacterial molecules, we expected that diluting bile with PBS would decrease its antibacterial action and perhaps allow *Salmonella* to grow. As can be seen from Fig. 6, this was not the case. Diluting bile with PBS did not improve *Salmonella* growth. These results could be interpreted in two ways. First, it is possible that bile can still repress growth when diluted. The other possibility is that the lack of *Salmonella* growth in bile was solely due to the absence of nutrients in this fluid. To determine which of these was true, we mixed bile and LB broth at a 1:1 ratio and analyzed *Salmonella* growth. If the lack of growth were due to an antibacterial property that is still active in diluted bile, then *Salmonella* would not be able to grow in a 1:1 mixture of LB broth and bile. On the other hand, if the lack of growth were due to the absence of nutrients in bile, we expected *Salmonella* growth in the LB broth-bile mixture to be comparable to growth in the LB broth-PBS mixture (i.e., 50% LB broth in both cases). Although our results confirmed that the lack of growth in bile was not due to its antibacterial action, we found that the addition of bile to LB broth greatly increased *Salmonella* growth (Fig. 6). Altogether, our results indicate that bovine bile is rich in nutrients that can be used by *Salmonella* but lacks one or more nutrients that are required by *Salmonella* to grow; provided that these nutrients are added to the medium (by the addition of LB broth, for example), *Salmonella* can show extremely high growth in bovine bile.

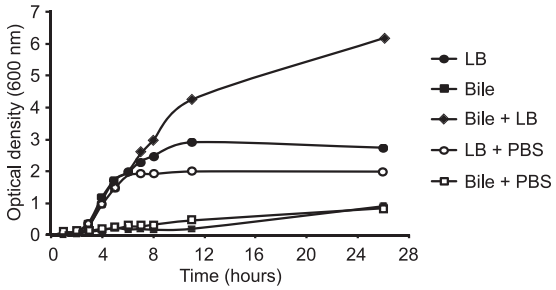

FIG 6 Physiological bovine bile alone does not support *Salmonella* growth. Bile or LB broth samples were kept undiluted, diluted with PBS, or mixed, as indicated, always at a 1:1 ratio. Streptomycin was added to all cultures at a final concentration of 100 μg/ml, and the pH of all solutions was adjusted to approximately 7.3. Overnight cultures of *Salmonella* Typhimurium SL1344 were then subcultured 1:200 in each of the culture media and incubated at 37°C with shaking; growth was monitored at the indicated time points by measuring the optical density of the cultures at 600 nm. The data shown are the average from three independent cultures. Bars represent the standard errors of means.

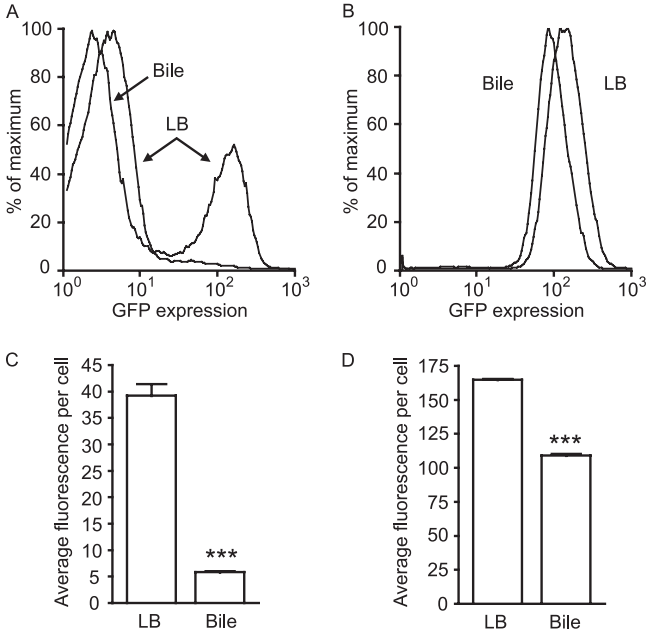

FIG 5 Commercial ox bile does not significantly repress *phoP* while still repressing *hilA* expression. *Salmonella* strains containing *phoP*::*gfp* or *hilA*::*gfp* reporter plasmids were grown overnight in LB broth with carbenicillin and subcultured 1:200 in LB broth containing carbenicillin with or without 3% (wt/vol) bovine bile (pH adjusted). After incubation at 37°C with shaking for 4 h, cultures were diluted in PBS, and *gfp* expression was analyzed by flow cytometry. (A) Histogram showing the relative proportions of cells (*y* axis) expressing *gfp* at various levels (*x* axis) during growth of the *hilA*::*gfp Salmonella* strain in LB broth with or without bovine bile, as indicated. (B) Same as in panel A, but with the *phoP*::*gfp Salmonella* reporter strain. (C) Data from panel A expressed as average fluorescence intensity per cell. (D) Data from panel B expressed as average fluorescence intensity per cell. The data shown are the average from three independent cultures. Bars represent the standard errors of means. *P* values were ≤0.0001 (***).

Physiological bovine bile represses *phoP* expression. The experiments using physiological murine bile described above show that this fluid acts as a strong repressor of *phoP* expression, whereas commercial bovine bile fails to repress *phoP*. This could be due to one of two reasons. First, it is possible that the *phoP* inhibitory activity is a very specific phenomenon and is exclusive

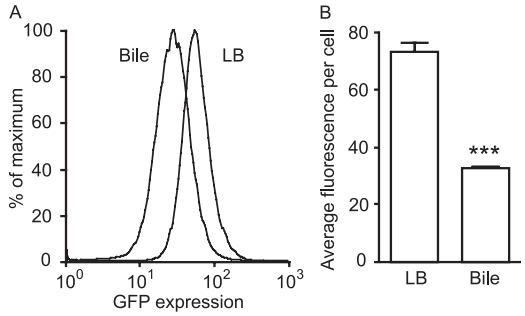

FIG 7 Physiological bovine bile represses *phoP* expression. LB broth was diluted 1:1 with either water or fresh bovine bile. Carbenicillin was added, and the pH of the solutions was adjusted. Overnight cultures of the *Salmonella phoP*::*gfp* reporter strain were then used to inoculate these solutions at a 1:200 dilution. Cultures were incubated at 37°C with shaking for 4 h, diluted in PBS, and analyzed by flow cytometry. (A) Histogram showing the relative proportions of cells (*y* axis) expressing GFP at various levels (*x* axis) during growth of the *phoP*::*gfp Salmonella* strain in LB broth-water (LB) or LB broth-bile (Bile) mixtures, as indicated. (B) Data from panel A expressed as average fluorescence intensity per cell. The data shown are the average from three independent cultures. Bars represent the standard errors of means. The *P* value was ≤0.0002 (***).

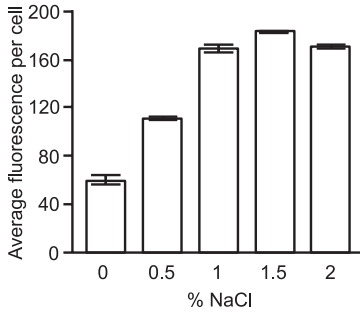

FIG 8 The repression of *phoP* expression by bile is not due to osmolarity. Overnight cultures of the *Salmonella phoP*::*gfp* reporter strain were subcultured at 1:200 in LB broth containing various concentrations of NaCl, as indicated. Cultures were allowed to grow for 4 h at 37°C with shaking, diluted, and analyzed for *gfp* expression through flow cytometry. The data shown are the average from three independent cultures. Bars represent the standard errors of means.

to murine bile. Alternatively, it is possible that commercial bovine bile is not fully representative of physiological bovine bile and that the inhibitory activity is lost during processing or packaging. To determine if the inhibitory activity observed in physiological murine bile is extended to other mammalian species, we used fresh, physiological bovine bile to perform *Salmonella phoP* expression assays. Because we determined that bovine bile alone does not support *Salmonella* growth, we compared levels of *phoP* expression during growth in 1:1 mixtures of LB broth and bile or LB broth and water. The *phoP*::*gfp Salmonella* reporter strain was grown overnight, subcultured 1:200 in the appropriate media, and allowed to grow for 4 h. Samples were diluted, and fluorescence was assayed through flow cytometry, as described above. Figure 7 shows that, like physiological murine bile, physiological bovine bile can also repress *phoP* expression, suggesting that this phenomenon is a conserved property of bile from multiple species. In order to determine if this was due solely to the fact that the pH of physiological bovine bile was adjusted to match that of LB broth, we repeated these experiments comparing *phoP* expression in bile without adjusting its pH (pH 8.5) against expression in LB broth after adjusting its pH to match that of bile (8.5). Our results showed that the pH of bile does not affect its capacity to repress *phoP*; i.e., bovine bile repressed *phoP* expression to a similar extent at pH 7.3 and 8.5 (data not shown).

The repression of *phoP* expression by bile is not due to osmolarity. After determining that both murine bile and bovine bile can repress *phoP* expression and that this is not due to the pH of the solution, we set out to investigate if this phenomenon was caused by a specific molecule present in bile or if it was a more general consequence of the chemical properties of this biological fluid. Bile is rich in organic and inorganic compounds, including salts, and therefore it was possible that differences in osmolarity between bile and the culture media used in our experiments could be responsible for the repression of *phoP*. To determine if this was the case, we measured *phoP* expression during *Salmonella* growth in LB media containing different concentrations of sodium chloride. The concentration of NaCl in LB broth is 1%, and our data show that increased concentrations of NaCl had no effect on *phoP* expression (Fig. 8). This shows that increased osmolarity cannot explain the phenomenon of *phoP* repression by bile. Nevertheless, although bile is rich in salts, it was still a formal possibility that the osmolarity of bile is lower than that of LB broth and that this, in turn, could be responsible for *phoP* repression. Indeed, our results showed that *Salmonella* growth in LB broth containing lower levels of NaCl caused significant repression of *phoP* expression (Fig. 8). However, when we compared *phoP* expression during *Salmonella* growth in a mixture of LB broth and water versus LB broth and bile, we found that expression was significantly lower in the mixture containing bile (Fig. 7), indicating that a potentially lower osmolarity of bile could not explain the phenomenon of *phoP* repression.

The repression of *phoP* by bile is due to specific molecules with hydrophobic properties. To further characterize the *phoP* inhibitory activity present in bile, we attempted to purify the bioactive molecule. Because many hydrophobic organic molecules are present in bile (steroids, bile acids, and phospholipids), we chose to utilize cartridges containing a C_{18} resin for initial purification (Sep-Pak; Waters). We applied bile samples to the cartridges, collected the flowthrough, and eluted the bound molecules with 50% and 100% methanol, sequentially. We then evaporated each fraction, added it to culture media, and analyzed *phoP* expression after *Salmonella* growth. To do so, overnight cultures of the *Salmonella phoP*::*gfp* reporter strain were subcultured 1:200 into the appropriate samples and allowed to grow for 4 h. After this period, samples were diluted in PBS, and *gfp* production was assayed through flow cytometry. Although a significant amount of bioactivity was recovered in the flowthrough, our data show that the bioactivity was mostly retained by the C_{18} resin and was eluted only with 100% methanol, suggesting that the bioactive molecule is of a hydrophobic nature (Fig. 9A). In order to further purify the bioactive molecule, we subjected the active fraction to reverse-phase high-performance liquid chromatography (RP-HPLC) using a linear gradient of 20% to 100% methanol over 90 min, at a flow rate of 1 ml per minute. Bioactivity of fractions was tested essentially as described above. This allowed us to identify three main fractions with *phoP* inhibitory activity, as shown in Fig. 9B. The fraction showing the highest level of activity (fractions 53 and 54) was evaporated and subjected to a second round of RP-

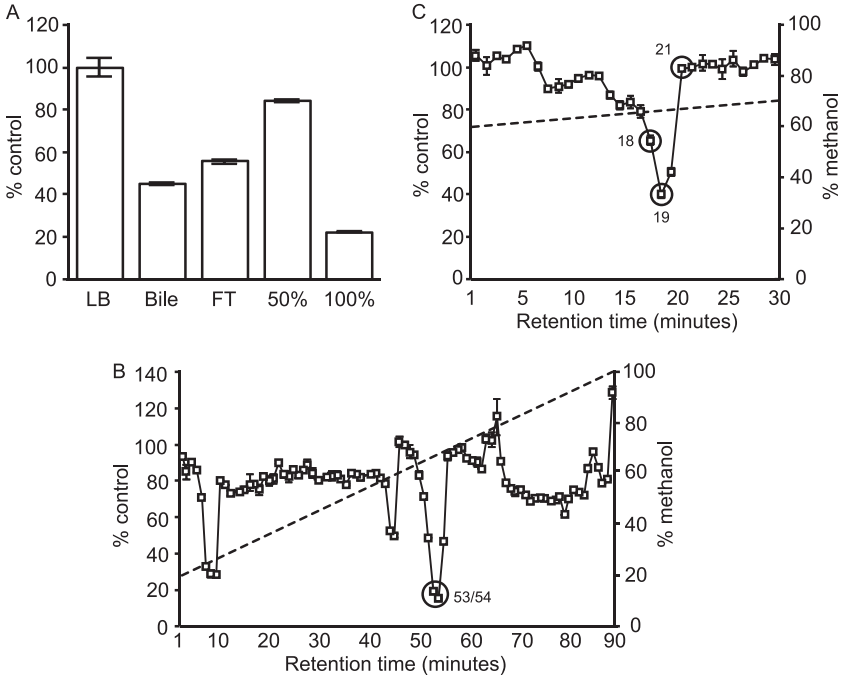

FIG 9 The repression of *phoP* by bile is due to specific molecules with hydrophobic properties. Bovine bile was extracted as described below. Extracts were evaporated and resuspended in LB broth containing carbenicillin. Overnight cultures of the *Salmonella phoP::gfp* reporter strain were subcultured 1:200 in all solutions, which were then incubated for 4 h at 37°C with shaking, diluted, and analyzed for *gfp* production through flow cytometry. The expression level during growth in LB broth (negative control) was set to 100%, and the activity of test fractions was normalized accordingly. (A) Bovine bile was applied to C_{18} resin cartridges, and the flowthrough (FT) was collected. Cartridges were washed with water, and the bound fraction was eluted sequentially with 50% and 100% methanol. Fractions were dried and assayed for *phoP* inhibition as described above. (B) The 100% fraction from panel A was dried, resuspended in 25% methanol, and subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) using a linear gradient of 20% to 100% methanol over 90 min (dashed line), at a flow rate of 1 ml per minute. Fractions were collected every minute, dried, and assayed for *phoP* inhibition as described above. (C) Fractions 53 and 54 from panel B were combined, dried, resuspended in 25% methanol, and subjected to RP-HPLC again using a linear gradient of 60% to 70% methanol over 30 min (dashed line), at a flow rate of 1 ml per minute. Fractions were collected every minute, dried, and assayed for *phoP* inhibition as described above. The data shown are the average from three (A) or two (B and C) independent cultures. Bars represent the standard errors of means.

HPLC, using a linear gradient of 60% to 70% methanol over 30 min, and the bioactivity of the resulting fractions was determined. This revealed a single fraction that concentrated most of the biological activity (fraction 19), and this fraction was selected for further studies (Fig. 9C). Altogether, these results show that the repression of *phoP* by bile is caused by one or more molecules with hydrophobic properties and is not a consequence of the general physicochemical properties of bile.

The most abundant compound in the bioactive fraction is TDC. To try and identify the compound responsible for *phoP* inhibition, we subjected the HPLC fraction with the highest biological activity (fraction 19) to ultrahigh-performance liquid chromatography coupled with mass spectrometry (UPLC-MS). For these experiments, samples were run on a C_{18} column, and UPLC-QTOF MS was performed in ESI(−) mode. Our results showed that a single compound of *m/z* 498.2900756 accounted for over 98% of all signal present in fraction 19 (Fig. 10). We then queried METLIN, the Metabolite and Tandem MS Database (http://metlin.scripps.edu/), with this *m/z* to try and determine putative identities. The database search generated three main hits: taurochenodeoxycholic acid, tauroursodeoxycholic acid, and taurodeoxycholic acid (TDC). To identify which of these compounds was the most abundant compound present in fraction 19, we performed UPLC-MS on standard compounds and compared retention times with the unknown compound of fraction 19 (retention time of 11.91 min). Our results clearly showed that the unknown compound was not tauroursodeoxycholic acid (retention time of 8.37 min) or taurochenodeoxycholic acid (retention time of 11.28). On the other hand, the retention time of the TDC standard matched that of the unknown compound very closely (11.99 min). In order to further confirm that the most abundant compound of fraction 19 was TDC, we performed collision-induced dissociation tandem MS on the standard and unknown compounds. As can be seen in Fig. 10, the fragmentation patterns of both the TDC standard and the unknown compound were identical, indicating that TDC is indeed the most abundant compound in fraction 19.

TDC alone does not repress *phoP* expression. In order to ascertain that TDC was responsible for the repression of *phoP*, we measured *phoP* expression during growth in LB broth containing various concentrations of this bile acid. To our surprise, TDC did not cause any appreciable repression of *phoP*, even at the highest concentration used (100 mM; data not shown). Therefore, TDC alone cannot account for the repression of *phoP* observed during *Salmonella* growth in bile. This suggests that (i) *m/z* 498.2900756 is not TDC, even though it shows the same retention time and fragmentation patterns; (ii) another minor component of the active fraction is responsible for the activity; or (iii) TDC requires a "cofactor" to repress *phoP* expression. To further confirm that the most abundant compound of fraction 19 is indeed TDC, we performed nuclear magnetic resonance (NMR) experiments with the

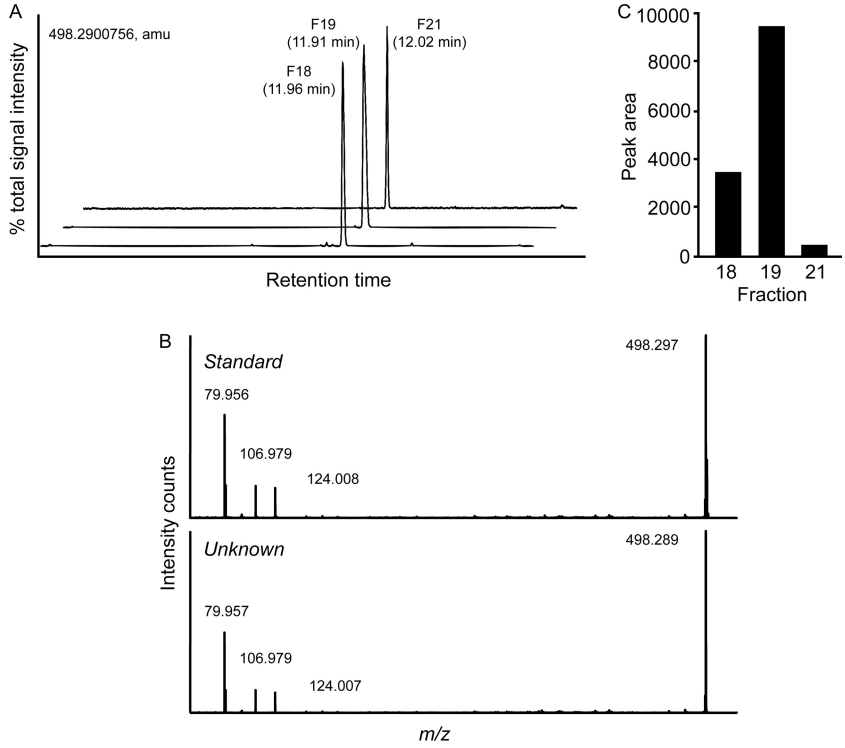

FIG 10 The most abundant compound of the bioactive fraction is taurodeoxycholic acid. Fractions 18, 19, and 21 were dried, dissolved in 200 μl of methanol, diluted 1:500 with 75% methanol, and injected (3 μl) into a 10- by 0.21-cm C_{18} UPLC column and run on UPLC-QTOF MS in ESI(−) mode. (A) Base peak ion chromatograms of fractions 18, 19, and 21. Retention times are shown. The main compound detected in all fractions was *m/z* 498.2900756 (*m/z* determined from fraction 19). amu, atomic mass units. (B) *m/z* 498.2900756 from fraction 19 was subjected to collision-induced fragmentation MS, and chromatograms showing daughter ions of this compound (bottom panel) as well as a taurodeoxycholic acid standard (top panel) are presented. (C) Peak area of *m/z* 498.2900756 from fractions 18, 19, and 21 as an example of the criteria used to select potential culprits of the *phoP* inhibitory activity in the active fractions.

TDC standard and fraction 19 and confirmed the identity of the main compound of fraction 19 as TDC (data not shown). Also, in order to determine if a minor component of fraction 19 was responsible for the bioactivity against *phoP*, we attempted to further purify the active molecule from the active fraction. However, additional rounds of HPLC produced no active fractions (data not shown). Therefore, our data suggest that a mixture of molecules may be necessary for the inhibitory activity of bile against *phoP* expression.

Defining a subset of small molecules responsible for the repression of *phoP* by bile. In order to identify potential culprits of the *phoP* inhibitory activity displayed by bile, we performed UPLC-MS on fractions 18, 19, and 21 (from the last round of HPLC purifications). We chose these fractions because they showed various levels of bioactivity against *phoP*. Fraction 18 showed moderated activity, fraction 19 showed high activity, and fraction 21 showed no appreciable activity (Fig. 9C). This allowed us to compare the signal intensities (as peak areas) of the compounds detected in these fractions with the corresponding biological activity of each fraction, in order to determine the potential bioactive molecules in fraction 19. In fraction 19, the most active of all three, 10 different ions were found (data not shown). We then filtered this list to include only molecules that were also present on fraction 18 and that showed levels at least 1.5-fold higher in fraction 19 compared to those in fractions 18 and 21. This resulted in a list of 4 different ions (Table 4), one of which is *m/z* 498.2900756. Figure 10A and C show the abundance and signal intensity for this ion as an example of the filtering criteria utilized. Aside from *m/z* 498.2900756, none of the other *m/z* values produced any hits when the METLIN database was searched. Therefore, these may represent new molecules, and significant further studies will be required to determine their identities. Nevertheless, the experiments described above allowed us to identify a subset of small molecules, out of the several hundred present in bile (1), that are likely responsible for the biological activity observed.

DISCUSSION

A critical step for the success of microbes during the process of host colonization is the sensing of their surroundings and responding through the regulation of gene expression. This allows the microbe to turn on genes that will aid in host colonization and turn off genes that are not required and whose transcription

TABLE 4 Subset of molecules correlated with biological activity against *phoP* expression

	Peak area in fraction:		
m/z[a]	18	19	21
498.2900756	355,682	824,602	53,377
500.4995894	3,371	5,152	756
598.2227203	1,258	2,806	ND[b]
582.2482633	958	1,759	ND

[a] Determined by UPLC-MS in ESI(−) mode.
[b] ND, not detected.

would represent an unnecessary burden. This is true not only for microbial pathogens during the development of disease but also for symbiotic microorganisms that need to establish stable relationships with their hosts. Environmental sensing by microbes can occur through multiple mechanisms, the most widespread and important one of which involves the use of two-component regulatory systems (two-component systems [TCSs]) (4). TCSs are composed of a membrane-bound histidine kinase that acts as a sensor by binding a specific environmental signal and changing its phosphorylation state in response to it and a response regulator to which the histidine kinase can transfer its phosphate group. Changes in the phosphorylation state of response regulators cause conformational changes that alter their DNA binding capabilities, resulting in changes in gene expression (4).

Salmonella is one of many human pathogens that use TCSs to adapt to the conditions encountered in their host and regulate virulence gene expression in response (4, 28). A search of the *Salmonella* Typhimurium LT2 genome (http://cmr.jcvi.org/) using the terms "histidine kinase" and "response regulator" revealed a high number of TCSs, with 19 histidine kinases and 39 response regulators being found. One of the most important and well-studied TCSs in *Salmonella* is PhoPQ (13, 28). PhoQ is a sensor histidine kinase that responds to a variety of signals, including divalent cations, antimicrobial peptides, and pH (13, 18, 28). PhoP is the cognate response regulator, which translates PhoQ phosphorylation into regulation of gene expression. PhoPQ signaling is an intrinsic part of *Salmonella* virulence, as strains lacking this TCS show reduced virulence (20). Additionally, a strain containing a constitutively active PhoPQ TCS shows virulence defects, illustrating how a balance in PhoPQ activity and regulation of virulence gene expression is required for full virulence (21).

One of the environments that *Salmonella* has evolved to sense and adapt to is bile. *Salmonella* Typhi can colonize the gallbladders of individuals with typhoid fever and use this environment as a deposit of bacteria that constantly reseed the gastrointestinal tract and maintain fecal shedding (23, 36, 39). Infection of mice with *Salmonella* Typhimurium results in similar disease, with the gallbladder being heavily colonized (19). Due to the exposure to bile experienced by *Salmonella* during infection, the effect of bile on *Salmonella* has been studied in detail (6, 29–31). Prouty et al. have shown that bovine bile strongly represses host cell invasion (30, 31). Additionally, it was shown that bile induces *Salmonella* resistance to the antibacterial action of bile as well as antimicrobials through the regulation of transporters and PhoPQ-regulated genes (29, 41). However, in these studies, the authors state that PhoPQ signaling is not required for the induction of bile resistance by growth in bile and that bile does not affect *phoPQ* expression.

To increase our understanding of the interactions between *Salmonella* and bile, we performed transcriptome analyses of *Salmonella* grown in culture medium or physiological murine bile and showed that many genes are differentially regulated. Curiously, one of the genes repressed by bile was the response regulator *phoP*. We confirmed this observation using RT-PCR to show that the expression of *phoP* and other *phoP*-regulated genes is repressed by bile. However, neither commercial bovine bile nor individual bile acids were able to repress *phoP* expression to the same extent as physiological murine bile, explaining why the regulation of *phoP* by bile was missed in previous studies. Commercial bovine bile, however, was able to fully repress the expression of the invasion regulator *hilA*, suggesting that different components of bile may act to repress *phoP* and *hilA*. Regardless of this difference, *Salmonella* growth in bile seems to induce a program of gene expression focused on the repression of virulence genes. Although the reason for this is currently unknown, there are several plausible hypotheses, each focused on a different stage of gallbladder colonization. It is possible that the repression of *hilA* and *phoP* is simply a way to conserve energy when *Salmonella* is swimming in bile in the gallbladder lumen and has not yet reached the lining epithelium, where expression of these genes would be beneficial (the "entry hypothesis"). Support for this hypothesis comes from the fact that *Salmonella* cells can be readily observed inside the epithelial cells lining the gallbladder lumen (19). It is also possible that the repression of these genes is a consequence of adaptation to the gallbladder environment, where *Salmonella* could grow at high rates using bile as a carbon and energy source (the "growth hypothesis"). When encountering a nutritionally rich environment such as that of the gallbladder, *Salmonella* may suppress virulence gene expression to avoid damaging the host so that it can take full advantage of the nutritional status of the gallbladder environment to grow to high densities using bile components. The fact that many metabolic genes were affected by growth in bile may support this hypothesis and suggests that a metabolic rewiring is induced in bile. For instance, we found that many genes repressed by bile are involved in the citric acid cycle and the generation of electron donors for aerobic respiration, whereas genes activated by bile are involved in alternative respiratory systems. The last possibility is that when *Salmonella* encounters bile, it is ready to leave the host organism through excretion in the gastrointestinal tract and fecal shedding. Therefore, the activation of virulence genes would represent an unnecessary expense (the "exit hypothesis").

Some of these hypotheses are not completely novel. Prouty and Gunn have proposed that during *Salmonella* infection of the gastrointestinal tract, an equivalent of our "entry hypothesis" takes place (31). The relatively high concentrations of bile present in the lumen of the small intestine may prompt *Salmonella* to repress invasion gene expression, until it reaches the intestinal epithelium, where the expression of these genes would be required for infection. Additionally, the utilization of bile as an environmental cue is not exclusive to *Salmonella*. For instance, bile salts have been previously shown to induce adherence to and invasion of cultured human cells by *Shigella* (27), a process mediated through the direct interaction of bile salts with the type-three secretion system involved in host cell invasion (7, 38). As with *Salmonella* and *Shigella*, species of *Vibrio* also link bile sensing with the regulation of virulence traits. *Vibrio cholerae*, for example, has been shown to repress the expression of its cholera toxin and toxin-coregulated pilus while upregulating motility in response to bile (15), and some of the molecules involved have been defined (5). *Vibrio parahaemolyticus*, on the other hand, can sense bile acids to induce the production of its thermostable direct hemolysin, capsule polysaccharide, and other virulence traits (11, 24, 25). In many cases, hypotheses for the evolutionary advantage of sensing bile and responding in specific manners can be made, although empirical evidence is lacking in most of these cases. Although for the most part the biological significance of gene regulation by bile in *Salmonella* remains unknown, our study contributes to the understanding of the interactions between *Salmonella* and bile and this pathogen's lifestyle during interactions with its host.

ACKNOWLEDGMENTS

We thank the anonymous reviewers of the manuscript for constructive criticism and insights. We are also greatly thankful to Neil Rawlyk, Sherry Tetland, Don Wilson, and Andrew Potter (Vaccine and Infectious Disease Organization, University of Saskatchewan) for the kind gift of fresh bovine bile, Jingxi Pan (University of Victoria Proteomics Centre) for help with UPLC-MS experiments, Vincent Chen (Michael Smith Laboratories, The University of British Columbia) for help with HPLC, and David Williams and Raymond Andersen (University of British Columbia Chemistry Department) for help with NMR experiments.

This work was funded by the Canadian Institutes of Health Research. L.C.M.A. and R.B.R.F. are supported by postdoctoral fellowships from the Canadian Institutes of Health Research. B.B.F. is the University of British Columbia Peter Wall Distinguished Professor.

REFERENCES

1. **Antunes LC, et al.** 2011. Metabolomics reveals phospholipids as important nutrient sources during *Salmonella* growth in bile *in vitro* and *in vivo*. J. Bacteriol. **193**:4719–4725.
2. **Antunes LC, et al.** 2010. Inhibition of *Salmonella* host cell invasion by dimethyl sulfide. Appl. Environ. Microbiol. **76**:5300–5304.
3. **Bakken K, Vogelsang TM.** 1950. The pathogenesis of *Salmonella typhimurium* infection in mice. Acta Pathol. Microbiol. Scand. **27**:41–50.
4. **Beier D, Gross R.** 2006. Regulation of bacterial virulence by two-component systems. Curr. Opin. Microbiol. **9**:143–152.
5. **Chatterjee A, Dutta PK, Chowdhury R.** 2007. Effect of fatty acids and cholesterol present in bile on expression of virulence factors and motility of *Vibrio cholerae*. Infect. Immun. **75**:1946–1953.
6. **Crawford RW, Gibson DL, Kay WW, Gunn JS.** 2008. Identification of a bile-induced exopolysaccharide required for *Salmonella* biofilm formation on gallstone surfaces. Infect. Immun. **76**:5341–5349.
7. **Dickenson NE, et al. 15 December** 2010, posting date. Conformational changes in IpaD from *Shigella flexneri* upon binding bile salts provide insight into the second step of type III secretion. Biochemistry [Epub ahead of print.]
8. **Esteller A.** 2008. Physiology of bile secretion. World J. Gastroenterol. **14**:5641–5649.
9. **Fields PI, Swanson RV, Haidaris CG, Heffron F.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Nat. Acad. Sci. U. S. A. **83**:5189–5193.
10. **Gooderham WJ, Hancock RE.** 2009. Regulation of virulence and antibiotic resistance by two-component regulatory systems in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **33**:279–294.
11. **Gotoh K, et al.** 2010. Bile acid-induced virulence gene expression of *Vibrio parahaemolyticus* reveals a novel therapeutic potential for bile acid sequestrants. PLoS One **5**:e13365.
12. **Grassl GA, Finlay BB.** 2008. Pathogenesis of enteric *Salmonella* infections. Curr. Opin. Gastroenterol. **24**:22–26.
13. **Groisman EA.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183**:1835–1842.
14. **Gunn JS, Miller SI.** 1996. PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J. Bacteriol. **178**:6857–6864.
15. **Gupta S, Chowdhury R.** 1997. Bile affects production of virulence factors and motility of *Vibrio cholerae*. Infect. Immun. **65**:1131–1134.
16. **Haraga A, Ohlson MB, Miller SI.** 2008. Salmonellae interplay with host cells. Nat. Rev. Microbiol. **6**:53–66.
17. **Kappelhoff R, Overall C.** 2007. The CLIP-CHIP oligonucleotide microarray: dedicated array for analysis of all protease, nonproteolytic homolog, and inhibitor gene transcripts in human and mouse. Curr. Protoc. Protein Sci. Chapter **21**:Unit 21.19.
18. **Kato A, Groisman EA.** 2008. The PhoQ/PhoP regulatory network of *Salmonella enterica*. Adv. Exp. Med. Biol. **631**:7–21.
19. **Menendez A, et al.** 2009. *Salmonella* infection of gallbladder epithelial cells drives local inflammation and injury in a model of acute typhoid fever. J. Infect. Dis. **200**:1703–1713.
20. **Miller SI, Kukral AM, Mekalanos JJ.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. U. S. A. **86**:5054–5058.
21. **Miller SI, Mekalanos JJ.** 1990. Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. **172**:2485–2490.
22. **Nelson K, Whittam TS, Selander RK.** 1991. Nucleotide polymorphism and evolution in the glyceraldehyde-3-phosphate dehydrogenase gene (*gapA*) in natural populations of *Salmonella* and *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **88**:6667–6671.
23. **Nichols HJ.** 1916. Experimental observations on the pathogenesis of gallbladder infections in typhoid, cholera, and dysentery. J. Exp. Med. **24**:497–514.
24. **Osawa R, Arakawa E, Okitsu T, Yamai S, Watanabe H.** 2002. Levels of thermostable direct hemolysin produced by *Vibrio parahaemolyticus* O3:K6 and other serovars grown anaerobically with the presence of a bile acid. Curr. Microbiol. **44**:302–305.
25. **Pace JL, Chai TJ, Rossi HA, Jiang X.** 1997. Effect of bile on *Vibrio parahaemolyticus*. Appl. Environ. Microbiol. **63**:2372–2377.
26. **Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ.** 2002. Typhoid fever. N. Engl. J. Med. **347**:1770–1782.
27. **Pope LM, Reed KE, Payne SM.** 1995. Increased protein secretion and adherence to HeLa cells by *Shigella* spp. following growth in the presence of bile salts. Infect. Immun. **63**:3642–3648.
28. **Prost LR, Miller SI.** 2008. The salmonellae PhoQ sensor: mechanisms of detection of phagosome signals. Cell. Microbiol. **10**:576–582.
29. **Prouty AM, Brodsky IE, Falkow S, Gunn JS.** 2004. Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella typhimurium*. Microbiology **150**:775–783.
30. **Prouty AM, et al.** 2004. Transcriptional regulation of *Salmonella enterica* serovar Typhimurium genes by bile. FEMS Immunol. Med. Microbiol. **41**:177–185.
31. **Prouty AM, Gunn JS.** 2000. *Salmonella enterica* serovar Typhimurium invasion is repressed in the presence of bile. Infect. Immun. **68**:6763–6769.
32. **Saeed AI, et al.** 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques **34**:374–378.
33. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
34. **Santos RL, et al.** 2001. Animal models of *Salmonella* infections: enteritis versus typhoid fever. Microbes Infect. **3**:1335–1344.
35. **Shin D, Lee EJ, Huang H, Groisman EA.** 2006. A positive feedback loop promotes transcription surge that jump-starts *Salmonella* virulence circuit. Science **314**:1607–1609.
36. **Sinnott CR, Teall AJ.** 1987. Persistent gallbladder carriage of *Salmonella typhi*. Lancet **i**:976.
37. **Soncini FC, Garcia Vescovi E, Solomon F, Groisman EA.** 1996. Molecular basis of the magnesium deprivation response in *Salmonella typhimurium*: identification of PhoP-regulated genes. J. Bacteriol. **178**:5092–5099.
38. **Stensrud KF, et al.** 2008. Deoxycholate interacts with IpaD of *Shigella flexneri* in inducing the recruitment of IpaB to the type III secretion apparatus needle tip. J. Biol. Chem. **283**:18646–18654.
39. **Vaishnavi C, et al.** 2005. Prevalence of *Salmonella enterica* serovar Typhi in bile and stool of patients with biliary diseases and those requiring biliary drainage for other purposes. Jpn. J. Infect. Dis. **58**:363–365.
40. **Valdivia RH, Falkow S.** 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol. Microbiol. **22**:367–378.
41. **van Velkinburgh JC, Gunn JS.** 1999. PhoP-PhoQ-regulated loci are required for enhanced bile resistance in *Salmonella* spp. Infect. Immun. **67**:1614–1622.
42. **Wickham ME, Brown NF, Provias J, Finlay BB, Coombes BK.** 2007. Oral infection of mice with *Salmonella enterica* serovar Typhimurium causes meningitis and infection of the brain. BMC Infect. Dis. **7**:65.
43. **Wray C, Sojka WJ.** 1978. Experimental *Salmonella typhimurium* infection in calves. Res. Vet. Sci. **25**:139–143.