Roles of the Outer Membrane Protein AsmA of *Salmonella enterica* in the Control of *marRAB* Expression and Invasion of Epithelial Cells

Ana I. Prieto, † Sara B. Hernández, † Ignacio Cota, † M. Graciela Pucciarelli, ‡ Yuri Orlov, † Francisco Ramos-Morales, † Francisco García-del Portillo, ‡ and Josep Casadesús †*

Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, Seville 41080, † Departamento de Biología Molecular, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, ‡ and Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, C. S. I. C., Darwin 3, Cantoblanco, 28049 Madrid, Spain

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A genetic screen for suppressors of bile sensitivity in DNA adenine methylase (*dam*) mutants of *Salmonella enterica* serovar Typhimurium yielded insertions in an uncharacterized locus homologous to the *Escherichia coli* *asmA* gene. Disruption of *asmA* suppressed bile sensitivity also in *phoP* and *wec* mutants of *S. enterica* and increased the MIC of sodium deoxycholate for the parental strain ATCC 14028. Increased levels of *marA* mRNA were found in *asmA, damA, phoP, and wec* strains of *S. enterica*, suggesting that lack of AsmA activates expression of the *marRAB* operon. Hence, *asmA* mutations may enhance bile resistance by inducing gene expression changes in the *marRAB*-controlled Mar regulon. In silico analysis of AsmA structure predicted the existence of one transmembrane domain. Biochemical analysis of subcellular fractions revealed that the *asmA* gene of *S. enterica* encodes a protein of ~70 kDa located in the outer membrane. Because AsmA is unrelated to known transport and/or efflux systems, we propose that activation of *marRAB* in *asmA* mutants may be a consequence of envelope reorganization. Competitive infection of BALB/c mice with *asmA* and *asmA* isogenic strains indicated that lack of AsmA attenuates *Salmonella* virulence by the oral route but not by the intraperitoneal route. Furthermore, *asmA* mutants showed a reduced ability to invade epithelial cells in vitro.

Bile is a fluid containing cholesterol, bile salts, phospholipids, proteins, bilirubin, and a variety of electrolytes (23). A fraction of the bile synthesized in the liver flows directly into the small intestine, while another fraction is stored in the gallbladder and released into the duodenum during food passage. About two-thirds (dry weight) of bile is made of bile salts, a family of molecules with steroid structure which derive from cholesterol (23). Aside from their role in digestion, bile salts have two distinct antibacterial activities, as detergents that disrupt the bacterial envelope and as DNA-damaging agents (5, 18, 39).

The mechanisms employed by *Escherichia coli* and *Salmonella enterica* to survive in the presence of bile are diverse and only partially understood (18) and involve a variety of cell functions: envelope structures that provide physical barriers to bile salts (31, 45, 55), efflux pumps that transport bile salts outside the cell (34, 38, 46, 52), and DNA repair functions that maintain genome integrity (39, 40). Resistance to bile is especially relevant in *Salmonella* physiology, since systemic infection leads to colonization of the hepatopancreatic tract (35), where the concentration of bile is high and steady (23). Furthermore, the gallbladder is a major niche for *Salmonella* in chronic carriers of *Salmonella enterica* serovar Typhi (14). The ability of *Salmonella* to survive in the mammalian gallbladder reflects its ability to adapt to virtually any concentration of bile (18) and to form biofilms on the surface of gallstones (9, 42, 43).

A strategy that has proven useful to identify cellular functions required for bile resistance in *S. enterica* is the isolation of bile-sensitive mutants and the subsequent identification of the mutations involved. In certain cases, however, mutation identification does not permit straightforward inference of the mechanisms whose disruption causes bile sensitivity. An example of this kind is found in *S. enterica* mutants lacking DNA adenine methylase (Dam), which suffer pleiotropic virulence effects including extreme bile sensitivity (15, 21, 22, 44). However, a functional relationship between DNA methylation and bile resistance is by no means obvious. In such circumstances, a strategy of classical genetics that can identify genetic partners is suppressor analysis: mutations that suppress a mutant phenotype often affect genes involved in the process under study (20). In the case of *Salmonella dam* mutants, their extreme bile sensitivity makes suppressor analysis easy: plating of a *dam* null mutant on a medium containing ox bile produces bile-resistant revertants that carry extragenic suppressor mutations (39). Using this strategy, a previous study showed that bile sensitivity in *Salmonella dam* mutants was suppressed by inactivation of the Dam-dependent mismatch repair system, MutHLS (39). Below we describe the characterization of a second, unsuspected class of suppressors of bile sensitivity in *Salmonella dam* mutants, involving loss of function in an *S. enterica* locus homologous to the *asmA* gene of *Escherichia coli*.

In *E. coli*, AsmA appears to be involved in preventing mis-
We propose that backgrounds, presumably by increasing outer membrane enhances bile resistance in diverse genetic encodes an OMP. The absence of AsmA in the S. enterica derivatives TT1704 and TT10288, obtained from J. R. Roth, University of California, used in this study (Table 1) belong to oral route, which can be tentatively correlated with the rearrangement associated with loss of this OMP. Another consequence of AsmA may be an indirect consequence of an envelope asmA folding of outer membrane proteins (OMPs), but its precise role has not been established. Mutations in asmA were initially described as suppressors that permitted assembly of mutant OmpF proteins (29), a role later extended to mutant OmpC proteins (56). The idea of involvement of AsmA in the assembly of wild-type OMPs was, however, discarded (30). An interpretation was that the presence of AsmA might create an environment refractory to the assembly of mutant (misfolded) OMPs (11). In the absence of AsmA, a more permissive environment would thus permit mutant OMP assembly (11). For Salmonella enterica, the only AsmA reference in the literature is a recent study that found asmA among the genes required for survival of Salmonella enterica serovar Typhimurium in the swine gastric environment (4). We show that the asmA gene of S. enterica encodes an OMP. The absence of AsmA in the outer membrane enhances bile resistance in diverse genetic backgrounds, presumably by increasing marRAB expression. We propose that marRAB transcriptional activation in the absence of AsmA may be an indirect consequence of an envelope rearrangement associated with loss of this OMP. Another consequence of AsmA absence is attenuation of virulence by the oral route, which can be tentatively correlated with the reduced ability of S. enterica asmA mutants to invade cultured epithelial cells.

### MATERIALS AND METHODS

#### Bacterial strains, bacteriophages, and plasmids. The strains of Salmonella enterica used in this study (Table 1) belong to Salmonella serovar Typhimurium and derive from the mouse-virulent strain ATCC 14028. Exceptions are the LT2 derivatives TT1704 and TT10288, obtained from J. R. Roth, University of California, Davis, CA. E. coli DH5α was the standard host for recombinant plasmids (57). Plasmid pGE108 (Km) is a CoElI derivative carrying a cew::lacZ fusion (47). pLP1581 is a pBAD18 derivative expressing the S. enterica asmA gene from the arabinose-dependent BAD promoter (see below). pLP253 is a pUC19 derivative carrying the internal HindIII fragment of Tn5; this fragment includes the kanamycin resistance gene (26). Transductional crosses using phage P22 HT1051 and 2041 (48; G. Roberts, unpublished data) were used for strain construction operations involving chromosomal markers and for transfer of plasmids among Salmonella strains. The transduction protocol was described elsewhere (16). To obtain phage-free isolates, transductants were purified by being streaked on green plates (7). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant phage P22 H2.

#### Construction of pLP1581. The asmA gene of S. enterica ATCC 14028 was PCR amplified using primers 5′CATGGAGCTCTTTAGACTGCGC5′ (G. Roberts, unpublished data) for strain construction operations involving chromosomal markers and for transfer of plasmids among Salmonella strains. The transduction protocol was described elsewhere (16). To obtain phage-free isolates, transductants were purified by being streaked on green plates (7). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant phage P22 H2.

#### Construction of asmA, dcd, udk, tolC, and marA mutants. The strains of Salmonella serovar Typhimurium in the swine gastric environment (4) were initially sequenced at the facilities of Sistemas Genómicos SL, Paterna, Valencia, Spain.

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HindIII fragment of plasmid p253 as a probe; this fragment contains the Tn5 kanamycin resistance gene, which is the same Km\(^{-}\) determinant carried by the MduJ element. The DNA fragments obtained, all 2 to 3 kb long, were treated overnight with T4 ligase at 15°C and PCR amplified with primers derived from the MduJ DNA sequence: 5′ AAG TGT GCT CGA CGT TGT CA 3′ and 5′ CGA ATA ATC CAA TGA CCT CTT CC 3′. If unsuccessful amplification had occurred, its products were used as templates for a second round of PCR amplification using two additional MduJ primers: 5′ GAT CTC GAG GAA GAG CAT C 3′ and 5′ ATT GCA CTA CAG GTT GCA AG 3′. Whenever PCR amplification was successful, the amplification product was purified with the GFX PCR DNA and gel band purification kit (Amersham Biosciences) and cloned onto pGEM-T Easy (Promega Corporation, Madison, WI). Standard DNA sequencing was performed with T3 and T7 primers.

Tagging of the AsmA protein with a 3′ FLAG epitope. Addition of a 3′ FLAG epitope tag at the 3′ end of the asmA gene was carried out using plasmid pSUB11 (Km\(^{-}\), 3′ FLAG) as template (45). An S. enterica chromosomal fragment containing the appropriate region of the asmA gene was PCR amplified using primers 5′ GGA ATT TTT CGG CTA AAT AAC GAG GAA GTC TAT GTG GTA GGG TGC ACG TTC TCT 3′ and 5′ CCA TAG ACG CCA TGT CTT CAC TCT GAG TCT GAA TCA TAT GAA TAT GTC TAT GGT GTA GGC TGG AGC TGC TTC 3′. The resulting PCR fragment was purified and used to electroporate an ATCC 14028 derivative carrying pKD46. Transformants were selected on LB-kanamycin. Incorporation of the 3′ FLAG tag was verified by PCR amplification and DNA sequencing. The primers used for this amplification were 5′ AAT GGA TGG CTA CGG CCA TC 3′ and 5′ GTG GCC AGT AAC GTC TTT CG 3′.

Subcellular fractionation. Bacteria were fractionated as described elsewhere (44). Briefly, bacteria were grown overnight in LB medium at 37°C with vigorous shaking (200 rpm) and spun down by centrifugation at 15,000 × g for 15 min at 4°C. These bacteria were suspended in cold phosphate-buffered saline (PBS; pH 7.4) buffer and either mixed with Laemmli buffer (total protein extract) or disrupted by sonication. Unbroken cells were further removed by low-speed centrifugation, 5,000 × g for 5 min at 4°C. The supernatant was centrifuged at high speed (200,000 × g, 15 min, 4°C), and the new supernatant was recovered as cytosol fraction. The pellet containing envelope material was suspended in PBS containing 1% Triton X-100. Upon incubation of this material for 1.5 h at 4°C, the sample was centrifuged at 15,000 × g for 5 min. The supernatant contained mostly soluble inner membrane proteins. The insoluble fraction enriched in OMPs was prepared upon suspension of the pellet in PBS, pH 7.4. An appropriate volume of Laemmli buffer was added to all fractions, and upon heating (100°C, 5 min) and clearing by centrifugation (15,000 × g for 5 min at room temperature), samples were analyzed for protein content by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in Tris-Tricine buffer by using Merck's 10% Tricine gel system.

RESULTS

**Virulence assays in mice.** Groups of three to four 8-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were inoculated with a 1:1 ratio of two strains. For oral inoculation, bacterial cultures were grown overnight at 37°C in LB without shaking. Each strain was grown overnight at 37°C in LB with shaking, diluted into fresh medium (1:100), and grown to an optical density at 600 nm of 0.3 to 0.6. Oral inoculation was performed by feeding the mice with 25 μl of saline containing 0.1% lactose and 10\(^{-9}\) bacterial CFU. Intrapерitoneal inoculation was performed with 0.2 ml of saline containing 10\(^{6}\) CFU. Salmonellae were recovered from spleen 48 h after inoculation, and CFU were enumerated on LB and on selective medium. A competitive index (CI) for each mutant was calculated as the ratio between the mutant and the wild-type strain in the output (bacteria recovered from the host after infection) divided by their ratio in the input (initial inoculum) (6). The “canceled out” CI (COI) is the CI corresponding to mixed infections of double mutants with corresponding single mutant strains and is defined as the ratio between a double mutant strain and the corresponding single mutant strain in the output divided by their ratio in the input (6).

**Invasion assay in HeLa epithelial cells with mixed bacterial strains.** HeLa cells (ATCC CCL2) were seeded with 5 × 10\(^{4}\) to 8 × 10\(^{5}\) CFU the day before the infection, using 24-well plates (Costar; Corning, New York, NY) and grown at 37°C and 5% CO\(_2\). Bacteria were grown overnight in LB at 37°C without shaking. A 1:1 or 10:1 mix of two bacterial strains was prepared in Dulbecco modified Eagle medium. The CFU of the two strains in the input were enumerated by plating a dilution series of the inoculum, using the appropriate antibiotic or the colony color to distinguish the strains. The bacterial mixture was added to HeLa cells to reach a multiplicity of infection of 50 bacteria per eukaryotic cell. Thirty minutes after the infection, cells were washed twice with PBS and incubated in fresh Dulbecco modified Eagle medium containing 100 μg/ml gentamicin for 90 min. Numbers of viable intracellular bacteria were obtained after lysis of infected cells with 1% Triton X-100 and plating on appropriate medium. Statistical analysis was achieved as described for the input. Infections were carried out in triplicate. The CI in invasion is defined as the ratio between the two strains in the output (intracellular bacteria recovered 2 h after infection) divided by their ratio in the input (49).

**Statistical analyses.** Each CI or COI value is the mean of at least three independent infections ± standard error. Student's t test was used to analyze every CI or COI. The null hypothesis was that mean CI was not significantly different from 1. Every COI was analyzed with two null hypotheses: (i) mean is not significantly different from 1 and (ii) mean COI is not significantly different from the CI of the corresponding single mutant. P values of 0.05 or less were considered significant. Student's t test was likewise used to analyze differences in Mics and in mRNA levels detected by quantitative RT-PCR.

**β-Galactosidase assays.** Levels of β-galactosidase activity were assayed as described by Miller (27), using the CHCl\(_3\)-sodium dodecyl sulfate permeabilization procedure.

**Bioinformatic analysis.** Sequence alignment was carried out at http://www.ncbi.nlm.nih.gov/BLAST/. PPSearch (http://www2.ebi.ac.uk/ppsearch/) was used to search for motifs in the asmA sequence against patterns in the PROSITE database. The SignalP 3.0 algorithm (http://www.cbs.dtu.dk/services/SignalP/) predicts the location of potential signal cleavage sites in amino acid sequences. Secondary structure prediction (helix, sheet, and coil) was carried out at http://www.bork.embl-heidelberg.de/SSCP. Transmembrane segment predictions were made using both a sliding window hydropathy plot (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and the TMpred algorithm (http://www.ch.embnet.org/software/TMPRED_form.html) based on the analysis of naturally occurring transmembrane-spanning segments.

**Trials for MduJ-induced, DOC-resistant revertants of a dam mutant.** The inability of dam strains of S. enterica to grow on plates containing 1% DOC permitted the positive selection of isolates carrying extragenic suppressor mutations. MduJ insertions that suppressed the DOC-sensitive phenotype of a dam mutant (SV4392) were sought using this strategy. SV4392 was mutagenized with MduJ, and DOC-resistant Km\(^{-}\) mutants were selected on LB-DOC-kanamycin plates. Putative suppressor-carrying isolates were lysed with P22 HT, and the lysates were used to transduce SV4392, selecting Km\(^{-}\). A 100% linkage between DOC resistance and Km\(^{-}\) resistance con-
 firms the existence of a suppressor mutation induced by MuJ. To identify the loci where the MuJ element had inserted, the boundaries of MuJ insertions were amplified by reverse PCR and sequenced. This procedure provided us with three independent MuJ insertions in asmA. In both E. coli and S. enterica, the asmA locus lies between yegH and dcd. DNA analysis in silico indicated that asmA is transcribed in the same orientation as that of the udk and dcd genes encoding uridine/cytidine kinase and dihydrouridine triphosphatase, respectively (Fig. 1). However, in silico analysis did not provide evidence that asmA, udk, and dcd might be part of the same transcriptional unit (see below).

Disruption of asmA caused a 20-fold increase in DOC resistance (Table 2). Only one asmA::MuJ insertion allele is included in Table 2 because all three showed identical suppressor abilities. Strains carrying null asmA alleles constructed by gene targeting (SV5056 and SV5057) had identical phenotypes (Table 2).

**Suppression of bile sensitivity in dam mutants is specifically caused by asmA disruption.** Although no evidence exists that the clustered dcd, udk, and asmA genes might be part of the same transcriptional unit (30), the dcd and udk genes were disrupted, and the ability of dcd and udk mutations to suppress bile sensitivity in dam strains was tested. Neither mutation was able to increase resistance to DOC in a dam background (Table 2). In fact, disruption of dcd caused moderate sensitivity to DOC (Table 2). Lack of dUTPase is known to induce the SOS response as a consequence of replication impairment (37). Because bile salts may also impair DNA replication (39), it is not surprising that lack of dUTPase renders the cell bile sensitive. For the purpose of this study, however, the relevant observation was that neither udk nor dcd mutations were suppressors of bile sensitivity. A corollary was that udk and dcd knockouts were not polar on asmA, thus supporting the view that asmA may be part of an independent transcriptional unit as previously proposed for E. coli (30).

Additional support for the conclusion that the lack of AsmA suppressed bile sensitivity in a dam background was provided by complementation analysis using a previously described “drop” assay (44). A plasmid carrying the asmA gene under the control of the arabinose-inducible pBAD promoter (pIZ1581) was constructed for this purpose. Arabinose-induced asmA expression did not impair growth of the wild type (ATCC 14028/pIZ1581) in LB (data not shown). Plasmid pIZ1581 was introduced in a dam asmA strain (SV4704), and growth on LB-DOC plates containing either glucose or arabinose was tested. As shown in Fig. 2, the dam asmA derivative carrying pIZ1581 grew in the presence of glucose but not in the presence of arabinose, indicating that expression of the plasmid-borne asmA gene restored bile sensitivity.

**Expression of the asmA gene is not under Dam methylation control.** Because the asmA gene conferred bile sensitivity when expressed from a heterologous promoter, we considered the possibility that asmA expression might be under Dam methylation control. Specifically, repression of asmA by Dam might be required for bile resistance, and uncontrolled expression might occur in dam mutants, resulting in bile sensitivity. This possibility was examined by comparing the expression of a MuJ-induced transcriptional asmA::lac fusion in dam" and dam hosts (strains SV4708 and SV4704, respectively). The fusion showed similar β-galactosidase activities in the two backgrounds: 120 ± 18 Miller units in SV4708 and 138 ± 28 Miller units in SV4704.
asmA unambiguously indicated that we investigated whether lack of AsmA increased the level of multiple toxic substances including bile (1). On these grounds, transcriptional activation of the grounds. One response of this kind in enteric bacteria is tran-
scription able to exert its effects in a variety of genetic back-
grounds. Lack of AsmA reduces SOS induction by DOC. Previous studies had shown that exposure of S. enterica to bile salts induces the SOS response (39, 40). To investigate whether suppression of bile sensitivity by asma mutations was accompanied by reduced bile-induced DNA damage, induction of the SOS system was monitored using a cea::lacZ fusion carried on pGE108 (47). The β-galactosidase activities of the cea::lacZ fusion were 749 ± 45 Miller units in ATCC 14028/pGE108 versus 319 ± 20 Miller units in an isogenic asma derivative, SV5426 (means and standard deviations of three independent experiments). Reduced SOS induction in the presence of DOC further supports the view that lack of AsmA enhances bile resistance.

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In silico analysis of AsmA protein structure. Sequence alignment using BLAST (NCBI database) detected no relatives of the AsmA protein with known function up to an E

Miller units in SV4704. Hence, asma transcription does not seem to be under Dam methylation control. This conclusion is further supported by a transcriptomic study which detected similar levels of asma mRNA in dam⁻ and dam hosts (2).

Lack of AsmA suppresses bile sensitivity in phoP and wec mutants. The ability of asma mutations to suppress bile sen-
sitivity in dam mutants raised the question of whether suppres-
sion was dam specific or broader. This issue was investigated by construc-
ting asma derivatives in other S. enterica mutants known to be bile sensitive, such as phoP (55) and wec (45) strains. Albeit it was less efficient than in dam asma mutants, suppression was observed in both phoP asma and wec asma strains (Table 2). Introduction of an asma mutation in the wild type also caused a small but significant increase in the MIC of DOC (Table 2).

Lack of AsmA causes transcriptional activation of the marRAB operon but does not affect acrAB expression. The ob-
servation that asma mutations behaved as broad suppressors of bile sensitivity suggested the potential involvement of a cell response able to exert its effects in a variety of genetic back-
grounds. One response of this kind in enteric bacteria is tran-
scriptional activation of the marRAB operon, which in turn controls the so-called “Mar regulon” involved in resistance to multiple toxic substances including bile (1). On these grounds, we investigated whether lack of AsmA increased the level of marA mRNA. Quantitative RT-PCR data shown in Fig. 3 unambiguously indicated that asma mutants contained higher levels of marA mRNA than did the wild type (Fig. 3A). In-
creased marA mRNA levels were also found in pairwise compari-
sions between dam, phoP, and wec mutants and isogenic asma derivatives (Fig. 3B). These observations provided evidence that lack of AsmA activates marRAB expression. Because marRAB activation is known to enhance bile resistance in S. enterica (41), we tentatively correlated marRAB activation with the ability of asma mutations to behave as suppressors and enhancers of bile resistance. Furthermore, the ability of asma mutations to suppress bile sensitivity in dam mutants was greatly reduced in the absence of MarA (Table 2), thus pro-
viding additional evidence that asma mutations confer bile resistance by activating the Mar regulon.

Quantitative RT-PCR experiments were likewise performed to monitor expression of the acrAB operon in asma⁺ and asma strains. AcrAB-TolC is an efflux pump regulated by MarRAB-dependent and MarRAB-independent mechanisms (41, 46) and previously shown to enhance bile resistance in S. enterica (41). The amounts of acrA mRNA in the wild type and in an asma mutant were thus compared. Statistical analysis indicated that the small differences found were not significant. Even more clear was the absence of differences in acrA mRNA content in pairwise comparisons between dam and dam asma, phoP and phoP asma, and wecD and wecD asma strains (data not shown). Altogether, these results indicated that lack of AsmA does not increase acrAB transcription and suggested that bile resistance mediated by asma mutations may involve MarRAB-regulated genes other than acrAB. The observation that suppression of bile sensitivity by asma mutations requires TolC (Table 2) suggests that TolC-dependent efflux pumps other than AcrAB may be involved in MarRAB-mediated bile resistance. An alternative possibility is that tolC mutations may cause an envelope defect that does not permit suppression by asma mutations.

Lack of AsmA does not increase resistance to tetracycline. The finding that asma mutations activated expression of marRAB but not acrAB was at first sight perplexing, given the ability of the AcrAB efflux pump to enhance bile resistance (41). Hence, we examined whether asma mutations affected tetracycline resistance, another well-known phenotype associated with AcrAB expression (36). The MIC of tetracycline hydrochloride was 0.5 mg/liter for ATCC 14028 and 0.4 mg/liter for an isogenic asma mutant (SV5057). This observation provided further evidence that lack of AsmA does not activate acrAB expression.

Lack of AsmA reduces SOS induction by DOC. Previous studies had shown that exposure of S. enterica to bile salts induces the SOS response (39, 40). To investigate whether suppression of bile sensitivity by asma mutations was accompanied by reduced bile-induced DNA damage, induction of the SOS system was monitored using a cea::lacZ fusion carried on pGE108 (47). The β-galactosidase activities of the cea::lacZ fusion were 749 ± 45 Miller units in ATCC 14028/pGE108 versus 319 ± 20 Miller units in an isogenic asma derivative, SV5426 (means and standard deviations of three independent experiments). Reduced SOS induction in the presence of DOC further supports the view that lack of AsmA enhances bile resistance.

FIG. 3. (A) Relative amounts of marA mRNA in exponential and stationary cultures of the wild type (ATCC 14028, gray histograms) and of an asma mutant (SV5057, black histograms). RNA amounts were normalized using ompA mRNA as a control. (B) Relative amounts of marA mRNA, normalized to ompA mRNA, in exponential cultures of dam, phoP, and wecD mutants and in isogenic asma derivatives. The strains used were as follows: dam (SV4536), dam asma (SV4704), phoP (SV4699), phoP asma (SV5736), wecD (SV4429), and wecD asma (SV4929) strains. In both panels, bars represent averages and standard deviations from three independent experiments.
value of $e^{-8}$ (data not shown). To characterize structural features of the *S. enterica* AsmA gene product (accession number gi29141254, NCBI), secondary structure content (percentages of alpha-helix, beta-sheet, and coil) was predicted based on both amino acid sequence and composition. The results indicated that the secondary structure of AsmA is characterized by high alpha-helix and coil content (47.0 and 41.8%, respectively) and low beta-sheet content (11.8%). In turn, in silico analysis of AsmA supersecondary structure (transmembrane segments, signal peptides, and motifs) suggested that AsmA has one transmembrane segment, from amino acid 5 to amino acid 26; an N-terminal signal peptide; and one cleavable site between amino acids 25 and 26 (VLL-VN). These results imply that AsmA may be a secreted protein. A signal typical of secreted proteins has been also described for the AsmA amino acid sequence of *E. coli* (30).

The *Salmonella enterica* AsmA protein localizes in the outer membrane. Construction of an AsmA protein derivative tagged with a 3× FLAG epitope permitted the detection of AsmA by Western immunoblot analysis. The tagged AsmA variant proved to be functional, since the MIC of DOC for AsmA by Western immunoblot analysis. The tagged AsmA protein and the prestained molecular mass standards in kDa are indicated.

![FIG. 4. Distribution of the AsmA protein tagged with a 3× FLAG epitope in subcellular fractions of *S. enterica* serovar Typhimurium (strain SV5061). Coomassie blue-stained proteins (left) and anti-FLAG Western hybridization (right) are shown for the following fractions: total protein (TP), cytosol (Cyt), inner membrane (IM), and outer membrane (OM). The volume loaded for all fractions was normalized to the same number of bacteria (5 × 10^5 CFU). The positions of the AsmA-FLAG protein and the prestained molecular mass standards in kDa are indicated.](http://jb.asm.org/)
brane may be required to trigger efficient bacterial uptake by nonphagocytic cells.

**DISCUSSION**

The ability of *asmA* mutations to enhance bile resistance in various genetic backgrounds suggested the involvement of a general mechanism such as mutational activation of a defense response. Note that the causes of bile sensitivity in *dam, phoP,* and *wec* mutants of *S. enterica* are diverse, perhaps unrelated (31, 39, 44, 45), and that enhanced bile resistance is also observed upon introduction of *asmA* null alleles in the wild type (Table 2). We provide evidence that the general mechanism by which *asmA* mutations enhance bile resistance in diverse genetic backgrounds involves transcriptional activation of the *marRAB* operon (Fig. 3). Initially identified in *E. coli* (17) and later in *S. enterica* (51), the *marRAB* operon plays a major role in the response of enteric bacteria to toxic substances (1). One of its products, MarA, is a transcriptional regulator that controls multiple genes which constitute the so-called “Mar regulon” (3). In *S. enterica*, transcription of *marAB* is activated by bile in a dose-dependent manner (41). This activation confers bile resistance, presumably via MarA-mediated activation of *acrAB* and other unidentified genes of the Mar regulon (41).

Our observation that *asmA* mutants undergo increased *marRAB* expression can likewise explain their ability to increase bile resistance.

The mechanism of *marRAB* activation in *asmA* mutants remains to be studied. Bioinformatic analysis indicates that AsmA is unrelated to any known transport protein. A dual role of AsmA as a membrane component and a transcriptional regulator cannot be ruled out (32) but seems a priori unlikely because a putative DNA-binding domain is not found in AsmA. Hence, a tentative hypothesis is that lack of AsmA may cause an outer membrane reorganization that directly or indirectly results in *marRAB* activation. The possibility that AsmA absence causes changes in the outer membrane has been previously proposed to explain the phenotypes of *E. coli asma* mutants (11, 56). On the other hand, the Mar regulon is known to respond to many kinds of stimuli, including intake of antibiotics, oxidative stress, and metabolic signals (3, 12, 28). Hence, it is conceivable that the absence of AsmA in the outer membrane may generate a signal that results in Mar regulon activation. An analogy may be found in the RcsBCD signaling system (25), which is activated by external stimuli but also responds to envelope perturbations caused by *mdo* mutations (13).

The mechanism by which *marRAB* activation in *asmA* mutants enhances bile resistance remains also to be identified. MarA regulates, either up or down, at least 60 genes (3). Hence, bile resistance in *asmA* mutants might result from activation or repression of genes of the Mar regulon. Although OmpF is known to be under MarRAB control (1), the possibility that bile resistance in *asmA* mutants might result from reduced OmpF synthesis was ruled out since a previous study had shown that lack of AsmA does not affect assembly of wild-type OMPS in *E. coli* (30). The list of genes under MarA control includes also the components of an efflux pump belonging to the RND family (*AcrAB-ToIC*), which is known to transport bile salts (46). However, *asmA* mutations do not increase transcription of *acrA*, suggesting that increased bile resistance in *asmA* mutants is caused by altered expression of Mar-regulated genes other than *acrAB*. The fact that *asmA* mutations activate transcription of *marAB* but not of *acrAB* is curious but not unusual: regulation of *acrAB* expression is known to be complex and multilayered (33, 46). A tentative hypothesis is that *asmA* mutations might activate a MarA-regulated efflux pump hitherto unknown to transport bile salts. A more speculative possibility is that lack of AsmA might activate one or more genes shared by the SoxRS and MarRAB regulons (28). In support of this view, the SoxRS regulon has been previously shown to be activated by bile salts (40), and *asmA* mutations reduce SOS induction by DOC.

A priori, any mutation that restores bile resistance in bile-sensitive mutants can be expected to favor survival of infecting *Salmonella* populations during intestinal passage, thus increasing their virulence by the oral route. However, *asmA* mutations failed to suppress oral attenuation in *dam* mutants (Fig. 5). This paradox was, however, solved when we observed that *asmA* mutations caused attenuation on their own: the CI of an *asmA* mutant by the oral route was found to be 30- to 40-fold lower than that of the wild type (Fig. 5). Hence, we tentatively infer that the benefits derived from increased bile resistance had been compensated (in fact, overrun) by other consequences associated with *asmA* mutations. In tests for invasion of epithelial cells, *asmA* mutants displayed a fivefold reduction in invasion capacity, suggesting that AsmA is required for optimal invasion of the intestinal epithelium.

If lack of AsmA causes indeed a major outer membrane rearrangement as proposed above and in a previous *E. coli* study (11), reduced invasion of epithelial cells might be a direct consequence of envelope alteration. Involvement of outer membrane components in *Salmonella* invasion has been previously described (8, 50). An alternative possibility is that uncontrolled activation of the Mar regulon might impair the interaction between *Salmonella* and epithelial cells. However, a previous study has shown that the *marRAB* operon is dispensable for virulence in the mouse model (51).

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