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Bile-Induced DNA Damage in *Salmonella enterica*

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ABSTRACT

In the absence of DNA adenine methylase, growth of *Salmonella enterica* serovar Typhimurium is inhibited by bile. Mutations in any of the *mutH*, *mutL*, and *mutS* genes suppress bile sensitivity in a Dam$^-$ background, indicating that an active MutHLS system renders Dam$^-$ mutants bile sensitive. However, inactivation of the MutHLS system does not cause bile sensitivity. An analogy with *Escherichia coli*, in which the MutHLS system sensitizes Dam$^-$ mutants to DNA-injuring agents, suggested that bile might cause DNA damage. In support of this hypothesis, we show that bile induces the SOS response in *S. enterica* and increases the frequency of point mutations and chromosomal rearrangements. Mutations in *mutH*, *mutL*, or *mutS* cause partial relief of virulence attenuation in a Dam$^-$ background (50- to 100-fold by the oral route and 10-fold intraperitoneally), suggesting that an active MutHLS system reduces the ability of Salmonella Dam$^-$ mutants to cope with DNA-damaging agents (bile and others) encountered during the infection process. The DNA-damaging ability of bile under laboratory conditions raises the possibility that the phenomenon may be relevant *in vivo*, since high bile concentrations are found in the gallbladder, the niche for chronic Salmonella infections.

ADULT humans produce a daily average of 500 ml of bile, a complex fluid containing bile salts, cholesterol, bilirubin, and other organic molecules (Hofmann 1998). Bile salts aid in the digestion and absorption of fats and fat-soluble vitamins in the small intestine. The most abundant bile salts in humans are cholate and deoxycholate. Bile is stored and concentrated in the gallbladder during fasting and is released through the bile duct into the intestine during food passage.

Because bile salts are detergents, bile possesses strong antimicrobial activity. However, enteric bacteria are intrinsically resistant to high concentrations of bile and individual bile salts (Gunn 2000). Bile salt resistance has in fact permitted the design of traditional microbiological media for the enrichment of enteric bacteria (Maccon-key 1905). Bile resistance is a complex phenomenon, as indicated by the observation that Salmonella cultures can be adapted to resist extremely high bile concentrations by previous exposure to a sublethal dose (Van Velkinburgh and Gunn 1999).

Bile is not merely an antibacterial secretion; Salmonella can sense bile in the intestine and use it as a signal to downregulate secretion and invasion genes of pathogenicity island 1 (Prouty and Gunn 2000). The role of bile as an environmental signal able to influence gene expression is further supported by the observation that growth on bile alters the level of numerous protein species (Van Velkinburgh and Gunn 1999). Furthermore, a recent study has shown that bile induces the formation of Salmonella biofilms on the surface of gallstones (Prouty *et al.* 2003). The potential significance of the latter finding is enormous, because the gallbladder is the niche for chronic Salmonella infections, which are in turn associated with gallstone formation and development of hepatobiliary carcinomas (Dutta *et al.* 2000).

The mechanisms involved in enterobacterial bile resistance are not fully understood. In both *Escherichia coli* and *Salmonella enterica*, the isolation of mutants sensitive to bile has identified cellular functions required for bile resistance. The latter include a variety of envelope structures (Picken and Beacham 1977; Prouty *et al.* 2002; Ramos-Morales *et al.* 2003), porins (Thanassi *et al.* 1997), and efflux pumps required for bile transport outside the cell (Ma *et al.* 1994; Thanassi *et al.* 1997; Nikaido *et al.* 1998). In *S. enterica*, regulatory functions such as MarAB (Sulavik *et al.* 1997) and PhoPQ (Van Velkinburgh and Gunn 1999) are also required for bile resistance.

Mutants of *S. enterica* lacking DNA adenine methyltransferase (Dam) are also bile sensitive (Heithoff *et al.* 2001; Pucciarelli *et al.* 2002), indicating that Dam methylation contributes to bile resistance. Given the complex, pleiotropic effects caused by *dam* mutations on the virulence of *S. enterica* (Garcia-Del Portillo *et al.* 1999; Heithoff *et al.* 1999), the functional relationship between Dam methylation and bile resistance is by no means obvious. Below we describe how the identification of transposon-induced mutations that suppressed bile sensitivity in a Dam$^-$ background led us to the

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TABLE 1

Strains of *S. enterica* serovar Typhimurium

Strain	Genotype
SV1408[a]	*leuA414*
SV1601[a,b]	Dup [*cysG1573* *Mu*dP** *ilvA2642*]
SV1603[b]	Dup [*proA692* *Mu*dQ** *purE2164*]
SV2045[a]	*hisG46*
SV3193[a,b]	Dup [*hisH9962* *Mu*dP** *cysA1586*]
SV4676[c]	*trg-101*::Mu*d*J
SV4392	*dam-201*::Tn*10d*Tc
SV4719	*mutS900*::Mu*d*J *dam-201*::Tn*10d*Tc
SV4721	*mutL111*::Tn*10*
SV4722	*mutL111*::Tn*10* Δ*dam-230*
SV4723	*mutS121*::Tn*10*
SV4802	*mutH101*::Tn*5*
SV4803	*mutH101*::Tn*5* Δ*dam-230*
SV4844	*recB497*::Mu*d*J
SV4869	*recA1 srl-203*::Tn*1od*Cm
SV4870	*srl-203*::Tn*10d*Cm *recA1*/pGE108
SV4930	Δ*dam-230*/pGE108
SV4933	14028 derivative carrying pGE108
SV5006	*leuA414 lexA*(Ind$^-$)
SV5007	*hisG46 lexA*(Ind$^-$)
SV5008	*hisO1242 hisC3072 lexA*(Ind$^-$)
TT10288[a,d]	*hisD9533*::Mu*d*J *hisC9994*::Mu*d*A
TR947[a,d]	*hisO1242 hisC3072*

[a] Strain is an LT2 derivative.
[b] Strain was described by Camacho and Casadesús (2001).
[c] Strain was described by Segura *et al.* (2004).
[d] Strain was obtained from J. R. Roth, Section of Microbiology, University of California, Davis.

unsuspected finding that bile is a DNA-damaging agent. Although our observations have been carried out under laboratory conditions, the bile concentrations used were similar to those found in the gallbladder. We thus discuss the possibility that bile-induced damage of pathogen DNA may occur *in vivo* during chronic Salmonella infection.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophages, and strain construction: All the *S. enterica* strains listed in Table 1 belong to serovar Typhimurium. Unless indicated otherwise, the strains derive from the mouse-virulent strain ATCC 14028. The source of the *lexA*(Ind$^-$) allele used in this study was strain DA6522, obtained from Dan I. Andersson, Swedish Institute for Infectious Disease Control, Solna, Sweden. Transductional crosses using phage P22 HT 105/1 *int201* (Schmieger 1972; 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 as described by Maloy (1990). To obtain phage-free isolates, transductants were purified by streaking on green plates (Chan *et al.* 1972). Phage sensitivity was tested by cross-streaking with the clear-plaque mutant P22 H5. Mu*d*P and Mu*d*Q are P22-Mu hybrids that carry a chloramphenicol-resistance marker (Youderian *et al.* 1988). Plasmid pGE108 (Km^r) is a ColE1 derivative carrying a *cea::lacZ* fusion (Salles *et al.* 1987).

Media and chemicals: Luria broth (LB) was prepared according to Maloy (1990). The carbon source was 0.2% glucose. Solid medium contained agar at 1.5% final concentration. Deoxycholic acid (sodium salt) and sodium choleate (ox bile extract) were both from Sigma (St. Louis). Antibiotics were used at the final concentrations described by Maloy (1990). Green plates were prepared according to Chan *et al.* (1972), except that methyl blue (Sigma) substituted for aniline blue.

Mutagenesis with Mu*d*J: We employed the *cis*-complementation procedure of Hughes and Roth (1988), in which a defective Mu*d*J element is cotransduced with a Mu*d*1 element that transiently provides transposition functions. Mu*d*1 is the specialized transducing phage Mu*d*1(Ap Lac *cts62*) constructed by Casadaban and Cohen (1979). Mu*d*I1734 [KmLac] (Castilho *et al.* 1984) is a transposition-deficient Mu derivative that generates operon fusions upon insertion; the element was renamed Mu*d*J by Hughes and Roth (1988).

Minimal inhibitory concentrations of sodium deoxycholate and ox bile extract: Exponential cultures in LB were prepared. Samples containing ~3×10^2 colony-forming units (CFU) were transferred to polypropylene microtiter plates (Soria Genlab, Valdemoro, Spain) containing known amounts of sodium deoxycholate (DOC) or ox bile extract. After 12-hr incubation at 37°, growth was visually monitored. Assays were carried out in triplicate.

Sensitivity to hydrogen peroxide: Exponential cultures in LB were washed and resuspended in phosphate buffer containing H_2O_2. In parallel, control samples were prepared in phosphate buffer. Treatments were carried out at 37° without shaking. Samples were extracted at various times, washed, diluted as needed, and spread on LB plates. Untreated samples were used for plate counts on LB agar. Survival was calculated according to Heithoff *et al.* (2001).

Cloning and molecular characterization of Mu*d*J inserts: Amplification of DNA sequences close to a Mu*d*J insertion was achieved by inverse polymerase chain reaction, as described by McPherson and Møller (2000). Genomic DNA from each Mu*d*J-carrying isolate was digested with either *Sma*I and *Ssp*I or *Sma*I and *Eco*RV. The resulting fragment was autoligated and used as template in two serial PCR amplifications with primers designed *ad hoc*. The final PCR product was purified with a commercial kit (GFX PCR DNA and band purification kit (Amersham Biosciences Europe GmbH, Cerdanyola, Spain) and sequenced. The primer used for sequencing one boundary of each Mu*d*J insertion was 5′-CGA ATA ATC CAA TGT CCT CC-3′ (Torreblanca and Casadesús 1996).

β-Galactosidase assays: Levels of β-galactosidase activity were assayed as described by Miller (1972), using the $CHCl_3$-sodium dodecyl sulfate permeabilization procedure.

Analysis of duplication segregation: We followed the procedures described by Camacho and Casadesús (2001). The merodiploid strains used carry chromosomal duplications held by a Mu*d*P element (SV1601, SV3193) or a Mu*d*Q (SV1603).

Mixed infection assays: Eight-week-old female BALB/c mice (Charles River Laboratories, Santa Perpetua de Mogoda, Spain) were used for virulence tests. For oral inoculation, bacteria were grown overnight at 37° in LB without shaking. For intraperitoneal inoculation, bacteria were grown overnight at 37° in LB with shaking, diluted into fresh medium (1:100), and grown to an OD_{600} of 0.35–0.6. Oral inoculation was performed by feeding the mice with 25 μl of saline containing 0.1% lactose and 10^8 bacterial CFU. Intraperitoneal inoculation was performed with 0.2 ml of physiological saline containing 10^5 CFU. Bacteria were recovered from the mouse spleen 48 hr after intraperitoneal inoculation or 6 days after

TABLE 2

Minimal inhibitory concentrations of sodium deoxycholate and ox bile extract

Strain	Genotype	Sodium deoxycholate (g/liter)	Ox bile extract (g/liter)
14028s	Wild type	7	16
SV4536	Δ*dam-230*	0.2	5
SV4392	*dam-201*::Tn*10d*Tc	0.2	5
SV4802	*mutH101*::Tn*5*	6	16
SV4721	*mutL111*::Tn*10*	7	16
SV4858	*mutS900*::Mu*d*J	7	16
SV4719	*dam-201*::Tn*10d*Tc *mutS900*::Mu*d*J	4	12
SV4722	Δ*dam-230 mutL111*::Tn*10*	4	12
SV4803	Δ*dam-230 mutH101*::Tn*5*	4	13
SV4869	*recA1*	2	8
SV4844	*recB497*::Mu*d*J	2	8

Values are averages of more than five independent experiments.

oral inoculation, and CFU were enumerated on selective medium. A competitive index for each mutant was calculated as the ratio between the mutant and the wild-type strain within the output divided by their ratio within the input (Freter *et al.* 1981; Taylor *et al.* 1987; Beuzon and Holden 2001).

RESULTS

Trials for suppression of bile sensitivity in DNA adenine methylase mutants: Because a *dam* mutation confers bile sensitivity (Heithoff *et al.* 2001; Pucciarelli *et al.* 2002), the Dam$^-$ strain SV4392 is unable to grow on LB plates containing 1% DOC. To identify genes involved in the bile sensitivity phenotype associated with the *dam* mutation, we sought Mu*d*J insertions that suppressed the DOC-sensitive phenotype of strain SV4392. For this purpose, SV4392 was mutagenized with Mu*d*J. DOC-resistant Km^r mutants were selected on LB-DOC plates supplemented with kanamycin. Putative suppressor-carrying isolates were lysed with P22 HT, and the lysates were used to transduce SV4392, selecting Km^r. A 100% linkage between the Km^r marker and DOC resistance confirmed the existence of a suppressor mutation induced by Mu*d*J.

To identify the loci where the Mu*d*J element had inserted, the boundaries of Mu*d*J insertions were amplified by reverse PCR and sequenced. To our surprise, one such Mu*d*J insertion was found in the mismatch repair gene *mutS*. Use of the collection alleles *mutH101*::Tn*5* and *mutL111*::Tn*10* confirmed that insertions in any of the mismatch repair genes *mutH*, *mutL*, and *mutS* suppressed the bile sensitivity phenotype of Dam$^-$ mutants (Table 2). Suppression by *mutHLS* mutations causes a 20-fold increase in DOC resistance and a 2- to 3-fold increase in resistance to ox bile extract (Table 2).

An additional, relevant observation made in these experiments was that *mutH*, *mutL*, and *mutS* mutations did not cause bile sensitivity on their own, indicating that the bile sensitivity phenotype associated with a *dam* mutation was not caused by lack of Dam-dependent mismatch repair. Thus, an active MutHLS system appeared to render Dam$^-$ mutants bile sensitive. An analogy could be drawn with observations made in *E. coli*, where an active MutHLS system has been shown to sensitize Dam$^-$ mutants to DNA-damaging agents such as O^6-methylguanine (Karran and Marinus 1982) and *cis*-platin (Fram *et al.* 1985). In a Dam$^-$ strain, the absence of DNA strand discrimination results in DNA strand breaks caused by MutHLS activity; this basal level of DNA damage renders the bacterial cell sensitive to agents that cause further DNA injuries (Marinus 2000). On the basis of this analogy, we examined whether inactivation of the MutHLS system was able to suppress the sensitivity of Salmonella Dam$^-$ mutants to hydrogen peroxide (Heithoff *et al.* 2001). The results were clear-cut: mutations in *mutH*, *mutL*, or *mutS* increased 1–2 orders of magnitude the survival of a Dam$^-$ mutant upon H_2O_2 exposure (data not shown). If *mutHLS* mutations were able to suppress the sensitivity of Dam$^-$ mutants to a well-known DNA-damaging agent such as H_2O_2 (Vazquez-Torres and Fang 2001), we tentatively included bile in the same category.

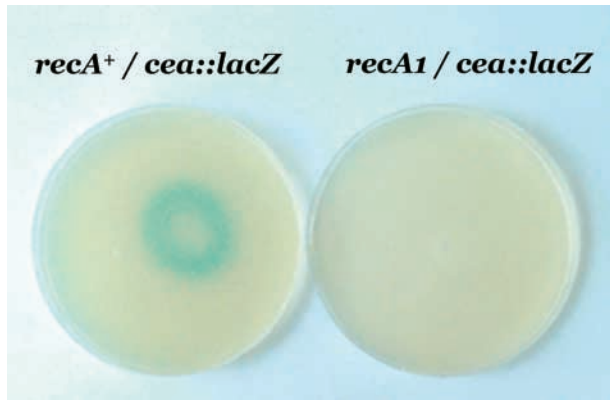

Figure 1.—Plate tests of SOS induction by sodium deoxycholate. Each plate was seeded with $>10^7$ cells (which form a lawn after growth). A small amount of sodium deoxycholate powder was placed in the center of the plate. Plates were incubated overnight at 37°. Left, strain SV4933 (*recA*$^+$/pGE108); right, strain SV4870 (*recA1*/pGE108).

SOS induction by bile: The hypothesis that bile was a DNA-damaging agent was first examined by investigating its ability to induce the SOS system of *S. enterica*. For this purpose, we tested whether sodium deoxycholate was able to turn on the expression of a *cea::lac* fusion carried on plasmid pGE108 (Salles *et al.* 1987). This fusion has been extensively used in our laboratory to monitor SOS induction in Salmonella (Gibert and Casadesús 1990; Torreblanca and Casadesús 1996). A plate test (Figure 1) showed that DOC was able to turn on the colicin E1 gene, thus indicating the occur-

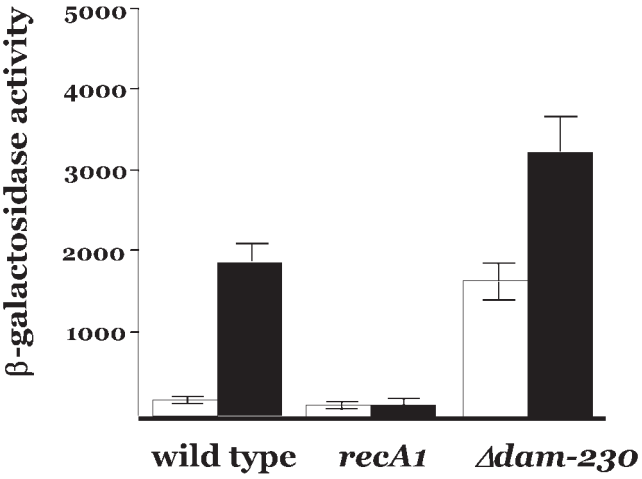

Figure 2.—SOS induction by ox bile extract. All the strains carried the *cea::lacZ* fusion of pGE108. The strains shown are, from left to right, SV4933 (wild type/pGE108), SV4870 (RecA$^-$/pGE108), and SV4930 (Dam$^-$/pGE108). Open bars show β-galactosidase activity of the *cea::lacZ* fusion in LB. Solid bars show β-galactosidase activity of the *cea::lacZ* fusion after exposure to 10% ox bile extract. Because Dam$^-$ mutants are sensitive to 10% ox bile extract, strain SV4930 was preadapted by growth in the presence of LB containing 2% ox bile extract. The data shown are means and standard deviations of more than five independent experiments.

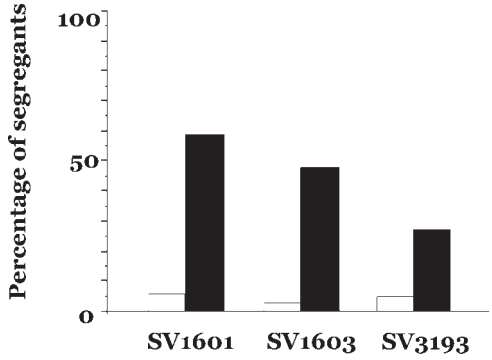

Figure 3.—Effect of ox bile extract on the frequency of duplication segregation. Open bars show the background level of duplication segregation for each merodiploid strain. Solid bars show the percentage of segregants after exposure to 15% ox bile extract. Because of the high numerical dispersion typical of these experiments, the median is shown instead of the average, and error bars are not included (see Camacho and Casadesús 2001). Each median has been calculated from more than eight independent experiments.

rence of SOS induction. DOC was unable to turn on *cea::lac* expression in a RecA$^-$ background (Figure 1), thereby confirming that SOS induction by this bile salt occurred in a canonical, RecA-dependent fashion (Sutton *et al.* 2000). SOS induction by bile was confirmed by comparing β-galactosidase activities in isogenic RecA$^+$ and RecA$^-$ strains carrying the *cea::lac* fusion (Figure 2). In these experiments, ox bile extract was used instead of sodium deoxycholate. Again, SOS induction was observed in the wild type, but not in a RecA$^-$ background (Figure 2). An additional observation was that a *dam* mutation on its own induced the Salmonella SOS system, as previously reported (Torreblanca and Casadesús 1996). In the presence of ox bile extract, further SOS induction occurred in the Dam$^-$ strain (Figure 2). These observations support the idea that the activity of the MutHLS system and the presence of bile are independent sources of DNA damage in a Dam$^-$ mutant and that the combination of both renders Salmonella bile sensitive.

Effect of bile on duplication segregation: The ability of bile to trigger the SOS response suggests that exposure of *S. enterica* to bile may result in DNA strand breaks, either caused directly by bile or generated during repair of bile-related DNA lesions. As a test for the occurrence of DNA strand breaks, we measured segregation of chromosomal duplications. It has been previously shown that duplication segregation is enhanced by DNA-damaging treatments (Galitski and Roth 1997). In our experiments, we used three strains carrying chromosomal duplications with known endpoints (Camacho and Casadesús 2001). Duplication segregation tests were carried out as follows: (i) the duplication-carrying strain was grown on LB-chloramphenicol (to hold the duplication) until saturation; (ii) after washing, aliquots were used to start parallel cultures in LB and LB supplemented with 15% ox bile extract; and (iii) after overnight growth, cultures were washed, diluted, and plated on LB and LB-chloramphenicol. The numbers of segregant colonies were calculated as the colony numbers found in LB plates minus the colony numbers found in LB-chloramphenicol. Although the absolute number of segregants varied among independent experiments, a higher segregation rate was consistently found after exposure to bile (Figure 3). These experiments provided further evidence that bile is a DNA-damaging agent.

Effect of bile on mutation frequencies: To investigate whether the DNA-damaging action of bile resulted in increased frequencies of point mutations, we tested reversion of three alleles: (i) *hisC3072*, a +1 frameshift (Whitfield *et al.* 1966); (ii) *hisG46*, a nucleotide substitution that results in a missense mutation (Hartman *et al.* 1971); and (iii) *leuA414*, a nucleotide substitution that generates an amber codon (Margolin 1963). These alleles revert spontaneously at low frequencies, which can be increased by specific mutagens (Casadesús and Roth 1989). Reversion assays in the presence of bile were carried out as follows: a batch exponential culture was used to start 20 cultures in LB containing either 10% or 15% ox bile extract. As a control, cultures in LB were also prepared. After overnight incubation at 37°, the cultures were washed and plated on selective agar for the detection of revertants. In parallel, dilutions were plated on LB to calculate survival. On the average, cultures grown in the presence of bile contained 10-fold less colony-forming units (data not shown). In contrast,

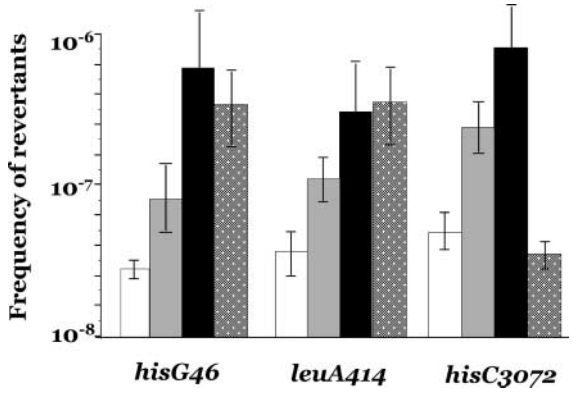

FIGURE 4.—Effect of ox bile extract on the frequency of reversion in strains SV1408 (*leuA414*), SV2045 (*hisG46*), and TR947 (*hisC3072*). Open bars show the frequency of spontaneous reversion. Shaded bars show the frequency of reversion after exposure to 10% ox bile extract. Solid bars show the frequency of reversion after exposure to 15% ox bile extract. Stippled bars show the frequency of reversion in a LexA(Ind$^-$) background after exposure to 15% ox bile extract. In the latter set of experiments the strains used were SV5006 [*lexA*(Ind$^-$) *leuA414*], SV5007 [*lexA*(Ind$^-$) *hisG46*], and SV5008 [*lexA*(Ind$^-$) *hisC3072*]. The data shown are means and standard deviations of 20 independent experiments.

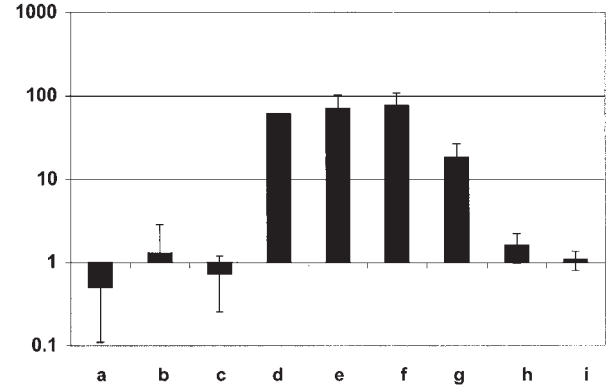

FIGURE 5.—Graphical representation of competitive index analysis of Mut$^-$, Dam$^-$, and Mut$^-$ Dam$^-$ strains, after oral or intraperitoneal infections of BALB/c mice. The mixed infections performed were as follows: (a) MutS$^-$/wild type, oral infection; (b) MutL$^-$/wild type, oral; (c) MutH$^-$/wild type, oral; (d) MutS$^-$ Dam$^-$/Dam$^-$, oral; (e) MutL$^-$ Dam$^-$/Dam$^-$, oral; (f) MutH$^-$ Dam$^-$/Dam$^-$, oral; and (g) MutL$^-$ Dam$^-$/Dam$^-$, intraperitoneal. (h and i) Internal controls. In these infections the wild type was co-inoculated with strain SV4676. The latter carries a *trg*::Mu*dJ* insertion, previously shown to be neutral for virulence (SEGURA *et al.* 2004). (h) Oral infection; (i) intraperitoneal infection. The indexes represented are the means from more than three infections. Error bars represent the standard deviation.

the number of revertants was consistently higher after bile treatment (Figure 4), indicating that the presence of ox bile extract increased the frequencies of reversion of the three alleles tested. Furthermore, a correlation was observed between the frequencies of reversion and the concentration of bile used, suggesting a dose-dependent effect (Figure 4). Hence, we concluded that exposure to ox bile extract caused an increase in mutation frequencies. Notably, bile increased reversion of both nucleotide substitutions and frameshifts. This broad mutational spectrum may reflect the chemical complexity of bile and suggests that bile may cause more than one type of DNA lesion. Bile-induced reversion of the *hisC3072*, *hisG46*, and *leuA414* alleles was likewise detected in a Mut$^-$ background (data not shown).

Because bile induces the SOS response and increases mutation rates, we considered the possibility that bile-induced mutagenesis might require SOS induction. For this purpose, we tested reversion of the *hisC3072*, *hisG46*, and *leuA414* mutations in a LexA(Ind$^-$) background. The ability of the *lexA*(Ind$^-$) allele to prevent SOS induction was confirmed by the observation that the *cea::lac* fusion of pGE108 remained repressed (Lac$^-$) in the presence of either nalidixic acid or ox bile extract (data not shown). Bile-induced reversion was tested as described above, using 15% ox bile extract. The *lexA* (Ind$^-$) mutation caused a significant decrease in the frequency of reversion of the *hisC3072* allele; in contrast, reversion of *hisG46* and *leuA414* was not affected (Figure 4). Hence, a tentative conclusion is that bile-induced reversion of the *hisC3072* frameshift is stimulated by SOS induction, but reversion of the substitution alleles *hisG46* and *leuA414* is not. Again, this result suggests that bile-induced mutagenesis may involve more than one mechanism.

Role of the MutHLS system in Salmonella virulence: Previous studies have shown that inactivation of the MutHLS system does not impair Salmonella virulence (GARCIA-DEL PORTILLO *et al.* 1999; HEITHOFF *et al.* 1999; ZAHRT *et al.* 1999; CAMPOY *et al.* 2000), while lack of DNA adenine methylase causes severe attenuation (GARCIA-DEL PORTILLO *et al.* 1999; HEITHOFF *et al.* 1999). These studies suggested that Dam-directed mismatch repair is dispensable for Salmonella infection and that the pleiotropic virulence defects of *dam* mutations could be attributed to causes other than lack of Dam-directed mismatch repair. However, after observing that an active MutHLS system sensitized Dam$^-$ mutants to bile, we considered the possibility that MutHLS activity might be involved in the attenuation in Dam$^-$ mutants. If this hypothesis was correct, we reasoned, *mut* mutations should relieve attenuation in a Dam$^-$ background. To test the hypothesis, BALB/c mice were subjected to mixed infections with Dam$^-$ and Dam$^-$ Mut$^-$ mutants. Groups of three or four animals were inoculated with a 1:1 ratio of Dam$^-$ and Dam$^-$ Mut$^-$ strains. As a control, the virulence of Mut$^-$ mutants was also compared with that of the wild type. An analysis of the competitive indexes obtained shows that *mut* mutations cause a partial but significant relief of attenuation in a Dam$^-$ background: 50- to 100-fold by the oral route and 10-fold by the intraperitoneal route (Figure 5). The higher in-

crease in virulence after oral inoculation suggests that bile may be a significant hurdle for a Dam$^-$ strain with an active MutHLS system. However, it is also noteworthy that Dam$^-$ Mut$^-$ mutants were more virulent than a Dam$^-$ mutant upon intraperitoneal inoculation. One interpretation is that an active MutHLS system sensitizes Dam$^-$ Salmonellae to additional DNA-damaging agents besides bile. As described above, *mutHLS* mutations also relieve the sensitivity of DNA adenine methylase mutants to hydrogen peroxide, a well-known reactive oxygen species encountered during Salmonella infection (Vazquez-Torres and Fang 2001).

Recombinational repair is required for bile resistance: In both *E. coli* and Salmonella, Dam$^-$ RecA$^-$ and Dam$^-$ RecB$^-$ mutants are inviable unless the MutHLS system is inactivated (Peterson *et al.* 1985; Torreblanca and Casadesús 1996). An explanation is that lack of DNA adenine methylase makes recombination essential for the repair of double-strand breaks caused by the MutHLS system (Marinus 2000). In this framework, we reasoned that recombination might be involved in repair of bile-induced DNA damage and that a Dam$^-$ strain might require recombination functions to survive in the presence of bile. The effect of *recA* and *recB* mutations on the ability of *S. enterica* to grow in the presence of DOC and ox bile extract is shown in Table 1. Both *recA* and *recB* mutations cause a three- to fourfold decrease in the minimal inhibitory concentrations (MICs) of sodium deoxycholate and a twofold decrease in the MICs of ox bile extract. Therefore, *recA* and *recB* mutations cause milder levels of bile sensitivity than a *dam* mutation. The relatively mild bile sensitivity of RecA$^-$ and RecB$^-$ mutants may indicate that *S. enterica* can use other repair procedures besides recombination to cope with bile-induced DNA damage.

DISCUSSION

The DNA-damaging ability of bile was fortuitously discovered. In an attempt to understand why DNA adenine methylation is required for bile resistance in *S. enterica*, we sought suppressors of bile sensitivity in a Dam$^-$ strain. To our surprise, we found that mutations in the mismatch repair genes *mutH*, *mutL*, and *mutS* relieved bile sensitivity in Dam$^-$ mutants. However, *mutHLS* mutations did not impair bile resistance in a Dam$^+$ background. On the basis of the literature on Dam-directed mismatch repair in *E. coli*, in which an active MutHLS system is known to sensitize Dam$^-$ mutants to DNA-damaging agents (Karran and Marinus 1982; Fram *et al.* 1985), we hypothesized that bile might cause DNA damage.

Several lines of evidence support a DNA-damaging activity of bile: (i) the SOS system of *S. enterica* is turned on in the presence of either sodium deoxycholate or ox bile extract; (ii) SOS induction by bile occurs in a canonical, RecA-dependent fashion; (iii) bile increases the frequency of chromosome rearrangements, a property typical of agents causing DNA strand breaks (Galitski and Roth 1997); (iv) *recA* and *recB* mutants of *S. enterica* are bile sensitive, suggesting that bile resistance requires recombinational repair; and (v) bile increases the frequencies of both nucleotide substitutions and frameshifts. It seems unlikely that these activities of bile are exerted specifically on Salmonella: evidence that bile salts turn on *E. coli* SOS promoters has been presented (Bernstein *et al.* 1999).

The fact that bile induces both nucleotide substitutions and frameshifts is consistent with the chemical complexity of bile and suggests that more than one molecular species may cause DNA damage. The observation that sodium deoxycholate alone is able to trigger SOS induction seems to indicate that bile salts have DNA-damaging capacity. Unfortunately, microbiological media containing high concentrations of sodium deoxycholate cannot be prepared, preventing a thorough comparison of the DNA-damaging properties of sodium deoxycholate with those of ox bile extract. An alternative possibility is that DNA damage is an indirect effect, resulting from the interaction of bile with proteins involved in DNA replication and/or DNA repair. A recent study has shown that nitric oxide, a DNA-damaging agent encountered by Salmonella inside macrophages (Vazquez-Torres and Fang 2001), requires the action of specific recombination proteins to cause DNA strand breaks (Schapiro *et al.* 2003). On the other hand, a relevant observation is that bile-induced reversion of the *hisC3072* frameshift is LexA dependent, while bile-induced reversion of the *leuA414* and *hisG46* nucleotide substitutions is not. This finding suggests that bile may cause more than one type of DNA lesion. If such is the case, bile-induced mutagenesis may also involve more than one mechanism at the molecular level.

The significance of bile-induced DNA damage and mutagenesis *in vivo* remains to be established. The concentrations of ox bile extract used in our study are well above the concentration of bile in the small intestine, but similar to that found in the gallbladder of healthy humans (Hofmann 1998). Hence, if the laboratory observations faithfully reproduce the scenario of the gallbladder, DNA damage may be viewed as an additional antibacterial activity of bile. Again, an analogy may be drawn at this point between bile and nitric oxide, which acts as an antimicrobial, DNA-damaging agent aside from playing relevant roles in host physiology (Eisenstein 2001; Vazquez-Torres and Fang 2001).

Bile is not a strong mutagen: note that ox bile extract concentrations of 10–15% cause only a 10- to 20-fold increase in mutation rates. However, mutation induction is often a linear function of mutagen dose, the product of the mutagen concentration by the time of exposure (Jimenez-Sanchez and Cerda-Olmedo 1975; Roncero *et al.* 1984). Hence, the possibility that bile-induced mutagenesis might play a role in chronic Sal-

monella infection can be considered. Studies in other bacterial pathogens have revealed that bacterial adaptation to chronic infection may involve an increase in mutation rates that facilitates adaptation to long-term organ colonization (Oliver *et al.* 2000).

An additional finding of our study concerns DNA adenine methylase mutants of *S. enterica*, which are severely attenuated (Garcia-Del Portillo *et al.* 1999; Heithoff *et al.* 1999). We show that inactivation of the MutHLS system relieves attenuation of Dam$^-$ mutants in the mouse model: 50- to 100-fold orally, 10-fold intraperitoneally. We thus conclude that the possession of an active MutHLS system contributes to the avirulent phenotype of Salmonella Dam$^-$ mutants. In *E. coli*, it is well known that lack of DNA adenine methylation leaves the cell at the mercy of the MutHLS system, which makes the cell sensitive to DNA-damaging agents. In a similar fashion, Salmonella Dam$^-$ MutHLS$^+$ mutants may be unable to cope with bile and other DNA-damaging agents encountered during infection. This view is supported by the observation that *mutHLS* mutations also suppress the sensitivity of Dam$^-$ mutants to hydrogen peroxide, a well-known reactive oxygen species encountered by Salmonella during infection (Vazquez Torres and Fang 2001). Hence, the possession of an active MutHLS system appears to be one component in the pleiotropic virulence defects displayed by Dam$^-$ mutants. In addition, *dam* mutations cause deficient association of Tol, PAL, and Lpp proteins to peptidoglycan (Pucciarelli *et al.* 2002). These envelope defects may contribute to oral attenuation by increasing the sensitivity of Salmonella Dam$^-$ mutants to the detergent action of bile.

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