Exposure to bile induces curing of the virulence plasmid in *Salmonella enterica* serovar Typhimurium (pSLT). Disruption of the *ccdB* gene increases pSLT curing, both spontaneous and induced by bile, suggesting that the pSLT *ccdB* genes may encode a homolog of the CcdAB addiction module previously described in the F sex factor. Unlike the F sex factor, synthesis of pSLT-encoded pili does not confer bile sensitivity. These observations may provide insights into the evolution of virulence plasmids in *Salmonella* subspecies I, as well as the causes of virulence plasmid loss in other *Salmonella* subspecies.

Certain *Salmonella* serovars belonging to subspecies I carry a large plasmid of 50 to 90 kb (19). All *Salmonella* virulence plasmids share a 7.8-kb region, *spv*, required for bacterial proliferation in the reticuloendothelial system (10). Other loci of the plasmid, such as the fimbral operon *pef*, the conjugal transfer gene *traT*, and the *rck* and *rsk* genes may play roles in other stages of the infection process (19). The virulence plasmid of *Salmonella enterica* serovar Typhimurium (henceforth, pSLT) is self-transmissible (1); virulence plasmids from other serovars, such as *Salmonella enterica* serovars Enteritidis and Choleraesuis, carry incomplete *tra* operons (19). The presence of virulence plasmids in host-adapted serovars has suggested that virulence plasmid acquisition may have expanded the host range of *Salmonella*. However, *Salmonella* subspecies II, IIIa, IV, and VII do not contain a virulence plasmid and carry the *spv* region on the chromosome (4).

During animal infection, *Salmonella* is exposed to bile salts, which have at least two distinct antibacterial activities, as detergents that disrupt the cell envelope (11) and as DNA-damaging agents that cause DNA rearrangements and point mutations (17). Current evidence suggests that the primary DNA lesions caused by bile salts may involve oxidative damage (18). The bile concentrations encountered by *Salmonella* during the intestinal stage of infection are low and changing (13). However, systemic infection leads to colonization of the hepatobiliary tract, where the concentration of bile is high and steady (15). Furthermore, *Salmonella* can cause chronic infections: for instance, about 3% of humans surviving typhoid fever are chronic, asymptomatic carriers of *S. enterica* serovar Typhi (14), which usually resides in the gall bladder (9). *Salmonella* survival in the presence of bile salts requires a variety of defense functions, including envelope barriers and efflux pumps (11), as well as DNA repair functions able to cope with bile-induced DNA injuries (18).

Because DNA lesions can impair DNA replication, many DNA-damaging agents cause plasmid curing (24). Furthermore, the repertoire of DNA repair functions required for bile resistance suggests that bile salts may impair DNA replication in *S. enterica* (18). On these grounds, we considered the possibility that exposure of *Salmonella* to bile could cure the virulence plasmid. Below we show that bile is a curing agent indeed. We also show that the *ccdB* gene plays a role in virulence plasmid stability. Finally, we describe an unsuspected difference between pSLT and the F sex factor: while synthesis of F pili greatly increases sensitivity to bile salts (3), derepression of the pSLT *tra* operon does not cause bile sensitivity.

**Exposure to bile causes virulence plasmid curing.** Despite its low copy number (6), spontaneous loss of pSLT has not been reported in the literature, indicating that the plasmid is highly stable in *Salmonella* populations. To detect pSLT curing, we designed a positive selection strategy based on selecting tetracycline-sensitive derivatives of a tetracycline-resistant strain (15) (Table 1). For this purpose, a Tn10 insertion (allele zv-6315::Tn10(Tc)) was introduced in pSLT, permitting the selection of Tc derivatives on Bochner-Maloy plates (15). To distinguish plasmid curing from other events causing tetracycline sensitivity (e.g., point mutations and deletions), a kanamycin resistance marker was also introduced in pSLT. The resulting virulence plasmid was thus tagged with two resistance markers, Tc and Km, both located in the *spv* region and separated by 7 kb, approximately (data not shown).

To obtain pSLT-cured derivatives, aliquots of saturated LB-grown cultures of strain LT2 were spread on Bochner-Maloy plates. Tc colonies were then replica printed to LB plates supplemented with kanamycin. Plasmid curing frequency was calculated as the ratio between the number of KmTc isolates and the number of bacterial cells plated (determined by plate counts on LB agar). Most, if not all, Km Tc isolates obtained by this procedure were plasmidless, as indicated by their inability to receive a third, unlinked plasmid marker, *samA*:Cm::19 of 19 independent Km Tc derivatives gave no Cm transductants when tested for receipt of *samA*:Cm by P22 HT-mediated transduction (20).

Spontaneous curing of the virulence plasmid occurred at frequencies below 10^-6 (Fig. 1). The effect of bile on virulence...
plasmid curing was tested by growing *S. enterica* in liquid LB containing different concentrations of ox bile extract. Aliquots from saturated cultures grown in LB-bile were spread on Boehrner-Maloy plates, and Tc\(^+\) colonies were replica printed, as described above, to LB-kanamycin. Exposure to bile increased the frequency of Km\(^+\)Tc\(^+\) isolates in a dose-dependent fashion (Fig. 1), providing evidence that bile is a plasmid-curing agent.

**Effect of ccdB disruption on virulence plasmid stability.** The *Salmonella* virulence plasmid belongs to the F-like family, and contains DNA regions homologous to the F sex factor (19). One such region is ccdAB, which in F encodes an addiction module involved in plasmid stability (8). To investigate whether the ccdAB region of pSLT encoded a functional addiction module, a Ccd\(^-\) mutant of *S. enterica* was constructed by gene targeting. The gene chosen for disruption was ccdB, which in F encodes the toxin of the addiction module (2). Disruption of ccdB was achieved by the procedure of Datsenko and Wanner (7), using the oligonucleotides 5’-CGGATCCTTGCTGACGACAAACAGGAACTGATGATGACTGTGAGCTGGAGCTGGTTGTGCTGACGCA-3’ and 5’-CTGTTCCGTGCACGCATATCAGATCCTGGAGACATCGATATGATGAAATATGCC-3’. Two additional, external PCR primers were used to verify the predicted deletion: 5’-TGAGGTTGCGCAGCTTTATTAG-3’ and 5’-CAGAAAATCCGCACAC

**TABLE 1. Genotypes of the bacterial strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2</td>
<td>Wild type</td>
</tr>
<tr>
<td>SV3000(^a)</td>
<td>dam-201::Tn10dTc</td>
</tr>
<tr>
<td>SV3003(^b)</td>
<td>traB::MudJ</td>
</tr>
<tr>
<td>SV3081(^c)</td>
<td>pSLT</td>
</tr>
<tr>
<td>SV4478(^d)</td>
<td>fimO::Km</td>
</tr>
</tbody>
</table>

\[^a^\] Strain described in reference 22.  
\[^b^\] Strain described in reference 23.  
\[^c^\] Strain described in reference 5.  
\[^d^\] spvA::MudJ allele described in reference 12.

**FIG. 1.** Frequencies of curing of the *Salmonella* virulence plasmid in a Ccd\(^-\) strain (white histograms) and in a Ccd\(^+\) mutant (dark histograms). The strains used were the isogenic pair SV4492 (Ccd\(^-\)) and SV4987 (Ccd\(^+\)). Ox bile extract (sodium cholate) was purchased from Sigma Chemical Co., St. Louis, MO, and used as described elsewhere (17). Data for the Ccd\(^-\) strain are averages of four independent experiments. Data for the Ccd\(^+\) strain are averages of six independent experiments. Bars represent standard errors.

**TABLE 2. MIC of sodium deoxycholate in strain LT2 and mutant derivatives**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>MIC (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2</td>
<td>Wild type</td>
<td>5.3 ± 1.1</td>
</tr>
<tr>
<td>SV3000(^a)</td>
<td>dam-201::Tn10dTc</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>SV3081(^c)</td>
<td>pSLT</td>
<td>4.6 ± 1.4</td>
</tr>
<tr>
<td>SV3003</td>
<td>traB::MudJ</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>SV4478</td>
<td>fimO::Km</td>
<td>5.5 ± 1.0</td>
</tr>
</tbody>
</table>

\[^a^\] Exponential-phase cultures in LB broth were prepared. Samples containing around 3 × 10\(^8\) CFU were transferred to polystyrene microtiter plates (Sorisa Genlab, Valdemoro, Spain) containing known amounts of sodium deoxycholate (Sigma Chemical Co., St. Louis, MO). After 12 h of incubation at 37°C, growth was visually monitored. Data are averages and standard errors of six independent experiments.  
\[^c^\] Strain described in reference 23.

**AGCC 3’.** Primer design was based on the published genome sequence of the LT2 strain (16).

Trials of curing in a CcdB\(^-\) pSLT plasmid were carried out as described above. The spontaneous frequency of pSLT curing increased 1 order of magnitude in a Ccd\(^-\) background (Fig. 1), indicating that the ccdAB genes may encode a functional addiction module that contributes to pSLT stability. Curing of the CcdB\(^-\) plasmid was strongly affected by bile and reached frequencies around or above 10\(^{-4}\) (3 orders of magnitude higher than the spontaneous frequency of curing in wild-type pSLT) in the presence of 15% ox bile extract (Fig. 1).

**Virulence plasmid functions do not affect bile resistance.** To investigate whether the presence of the virulence plasmid affected *S. enterica* survival in the presence of bile, we compared the MICs of sodium deoxycholate (DOC) in the wild type and in a pSLT-cured derivative. Aliquots from exponential cultures in LB broth, each containing around 3 × 10\(^8\) colony-forming units, were transferred to polystyrene microtiter plates containing known amounts of DOC. After 12 h of incubation at 37°C, growth was visually monitored. As a control, a DNA adenine methylase (Dam\(^-\)) mutant was included in these experiments; *S. enterica* Dam\(^-\) mutants are extremely sensitive to bile salts (17). Data shown in Table 2 indicate that curing of the virulence plasmid does not alter sensitivity of *S. enterica* to DOC. However, this observation left open the possibility that virulence plasmid functions which are usually repressed might alter bile sensitivity upon derepression. In fact, the tra operon of the F episome is known to sensitize *Escherichia coli* to bile salts (3). In F, bile salt sensitivity is caused by the tra-encoded type IV secretion system and requires an active F pilus assembly pathway (3). Unlike F, the tra operon of the *Salmonella* virulence plasmid is tightly repressed by the FinOP system (5, 21); hence, we considered the possibility that derepression of tra might confer bile sensitivity to *S. enterica*. Actually, tra operon derepression has been shown to cause bile sensitivity in another F relative, plasmid R100 (3). However, an *S. enterica* strain carrying a tra operon derepressed by a finO mutation did not show increased sensitivity to sodium deoxycholate (Table 2). In turn, a traB mutation, which prevents synthesis of pili, did not alter the MIC of DOC. A complementary observation was that neither a finO mutation nor a traB mutation had any effect on pSLT curing (Fig. 2).
Potential roles of bile in the evolution of salmonellae. A study on the distribution of spv genes among Salmonella subspecies considered that virulence plasmid instability might have favored spv translocation to the chromosome (and concomitant virulence plasmid loss) during the evolution of subspecies II, IIIa, IV, and VII (4). In this study, we suggest that bile could be a factor contributing to virulence plasmid instability in the ancestors of these subspecies. Bile concentrations of 15%, which induce significant rates of virulence plasmid curing under laboratory conditions, are commonly found in the gall bladder of humans and other mammals (13). Hence, bile can be predicted to impair the stability of the virulence plasmid in natural populations of Salmonella during systemic and chronic infections. Because bile can also induce DNA rearrangements (17), an attractive hypothesis is that both spv location and virulence plasmid loss could be caused by bile.

An additional, intriguing observation was that, unlike F and other F-like plasmids (3), synthesis of pSLT-encoded pil does not sensitize the host cell to bile salts. It is also noteworthy that certain pSLT-encoded Tra proteins show high divergence from their F counterparts, despite the overall conservation of tra operon organization and regulation in both plasmids. For instance, amino acid identities are only 68% for TraV, 33% for TraP, 29% for TraY, and 14% for TraS (data not shown). These observations may provide evidence that the type IV secretion apparatus encoded on the virulence plasmid has become bile resistant during the evolution of Salmonella subspecies I.

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