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The Colonization of Peyer's Patches by a Strain of Salmonella typhimurium Cured of the Cryptic Plasmid

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We cured a strain of Salmonella typhimurium of its cryptic plasmid and confirmed that orally administered cured strains lost virulence for mice. Loss of the cryptic plasmid rendered the S. typhimurium strain sensitive to the bactericidal action of normal human serum. However, loss of the plasmid did not change the ability of the strain to associate with HeLa cells in tissue culture. Furthermore, when administered orally to mice, both the plasmid-containing and plasmid-free strains invaded the Peyer's patches of the small intestine to the same extent, and both were capable of inducing resistance to oral challenge with virulent S. typhimurium. When injected intraperitoneally, the cured strain was eliminated rapidly, whereas the parental strain persisted. We also showed that the cured strain did not contain a plasmid copy in the chromosome. We propose that although the plasmid-cured strain of S. typhimurium is able to colonize Peyer's patches, it cannot survive when administered intraperitoneally because it is susceptible to elimination by macrophages.

The course of an infection with Salmonella typhimurium in mice is well established. Following oral administration of a virulent strain to mice, the bacteria invade the Peyer's patches of the small intestine, where they multiply for several days. The bacteria then enter the bloodstream and multiply within reticuloendothelial cells, infective foci, or both, of the spleen and liver. An overwhelming infection results, with large numbers of bacteria present in the blood at the time of death of the infected animals [1, 2].

Although the factor(s) responsible for the virulence of salmonellae has not been well defined, there is some evidence that the large plasmids with no known antibiotic-resistance markers (cryptic plasmids), which are found in S. typhimurium and in other strains of salmonellae, may have a role to play [3-5]. Plasmid-free strains occur spontaneously and have been shown to be less virulent in mice than plasmid-containing strains and to be sensitive to the bactericidal action of normal human serum [3].

Furthermore, Jones et al. [4] cured two plasmid-containing strains of S. typhimurium (CR8500 and CR6600) of their cryptic plasmids and showed that these were less virulent than the parent strains when administered orally to mice. By using monolayers of HeLa cells, these authors also found that the cured strains were less adhesive and less invasive than the parent strains, an observation providing an explanation for the loss of virulence observed.

Jones et al. [4] proposed that one of their two plasmid-containing parent strains also had a copy of the plasmid integrated into the bacterial chromosome. This plasmid copy was thought to be incapable of expressing virulence functions in the integrated state but to be capable of expressing such functions upon excision, which was proposed as a rare event. Because only one of the two cured strains examined by these workers had this property, integration/excision of the plasmid was not thought to be a general phenomenon in S. typhimurium [4].

For some time we have been interested in the adhesins of salmonellae that are required for penetration of the Peyer's patches by these organisms. Because loss of the cryptic plasmid appeared to result in the loss of adhesion to HeLa cells [4], we decided to examine further the effects of plasmid loss. We confirmed that a derivative of the S. typhimurium LT2 strain, cured of its cryptic plasmid, is avirulent when administered to mice either orally or ip. However, our pair of strains (plasmid containing and plasmid
Table 1. Characteristics of the strains used in this study.

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Species</th>
<th>Chromosomal genotype</th>
<th>Origin or reference*</th>
<th>Cryptic plasmid status</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA2305</td>
<td><em>S. typhimurium</em> LT2</td>
<td>Δ his DGBHAFIE 712, arg-501, ser-821</td>
<td>G. Jones +†</td>
<td>+†</td>
</tr>
<tr>
<td>P9144</td>
<td><em>S. typhimurium</em> LT2</td>
<td>leuB129, hisC57, recA1, srl::Tn5</td>
<td>P. Reeves ‡</td>
<td>+</td>
</tr>
<tr>
<td>J41</td>
<td><em>S. typhimurium</em> LT2</td>
<td>as P9144</td>
<td>This study ‡</td>
<td>+‡</td>
</tr>
<tr>
<td>J42</td>
<td><em>S. typhimurium</em> LT2</td>
<td>as P9144</td>
<td>This study ‡</td>
<td>-</td>
</tr>
<tr>
<td>C5</td>
<td><em>S. typhimurium</em></td>
<td></td>
<td>[1]</td>
<td>+</td>
</tr>
<tr>
<td>E381</td>
<td><em>E. coli</em> K12</td>
<td>gyrA96, recA1, endA1, thi-1, hsdR17, supE44</td>
<td>P. Manning</td>
<td></td>
</tr>
</tbody>
</table>

* Dr. Jones is from the University of Michigan, Ann Arbor; Dr. Reeves from the University of Sydney, Australia; and Dr. Manning from the University of Adelaide.  
† Strain AA2305 carries a transposon Tn10 insertion in the cryptic plasmid.  
‡ This strain was constructed by phage P22 transduction of tetracycline resistance from strain AA2305. The cryptic plasmid carries the transposon Tn10 insertion of strain AA2305, as judged by an increase in size of the plasmid relative to that of the plasmid from strain P9144 (not shown).

Materials and Methods

Bacterial strains. We used five strains of *S. typhimurium* and one of *Escherichia coli* (table 1). Strain P9144 contains a cryptic plasmid but is less virulent for mice and has an increased generation time when compared with the parent LT2 strain, partially because of the recA1 mutation that was introduced to enable curing of the cryptic plasmid. We have not been able to effect curing of rec* strains of *S. typhimurium*. The selection on Bochner plates (below) invariably gave rise to tetracycline-susceptible strains that still contained a cryptic plasmid, although markedly reduced in size. The parent strain had an ip LD50 value for mice of $10^5$, whereas the recA1 derivative (P9144) had an LD50 value of $\sim 5 \times 10^4$. Strain J42 is the plasmid-free derivative of strain P9144. Plasmid curing was effected by first marking the cryptic plasmid of P9144 with transposon Tn10 (by phage P22 transduction from strain AA2305 from Jones et al. [4]) to give strain J41 and then by selecting for tetracycline-susceptible cured derivatives of J41 (after growth in novobiocin [100 μg/ml]) on Bochner medium with quinaldic acid [6]. The plasmid profiles of the two strains (P9144 and J42) are shown in figure 1.

*S. typhimurium* C5, a strain virulent for mice, has been described previously [1]. It contains a cryptic plasmid (authors' unpublished data).

Bacterial strains were maintained at -70°C in 30% (vol/vol) glycerol. Cultures were grown at 37°C in nutrient broth (Difco, Detroit). Kanamycin was used in agar medium at 25 μg/ml.

DNA methods. The plasmid of P9144 was prepared as described by Currier and Nester [7]. Small-scale isolation of plasmids was achieved by the method of Kado and Liu [8]. Nick translation of DNA, DNA blotting, restriction enzyme digestions, preparation of chromosomal DNA, and agarose gel electrophoresis were standard methods [9]. Enzymes active on DNA were from Boehringer Mannheim (Indianapolis), whereas label (α-[32P]dCTP) was from BRESA (Adelaide, South Australia).

Animals and animal methods. LACA strain mice were obtained from the breeding facility of the Waite Institute (Adelaide, Australia) and were maintained as previously described [1]. Oral and ip administration of *S. typhimurium*, evaluation of bacterial growth in various organs, and calculation of LD50 doses were performed as reported earlier [1]. Analysis of the fate of bacteria injected into the peritoneal cavity was made by using a previously reported...
Cured S. typhimurium in Mice

method [10]. Peritoneal washouts in 0.9% NaCl (2 ml) were obtained at 0, 15, 30, 60, and 120 min after ip injection of 10^4 test bacteria. Washout samples (0.1 ml) were plated on kanamycin-containing plates. The remainder of each washout was centrifuged at 500 g for 5 min, and samples of the supernatant (0.1 ml) were also plated for evaluation of non-cell-associated bacteria. The pellets were resuspended in 0.9% NaCl to the original washout volume, and samples were plated (0.1 ml) for counts of cell-associated bacteria.

To ensure death of the mice by oral administration of strain P9144, we gave each animal 1 mg of streptomycin in 0.1 ml of 0.9% NaCl orally 24 hr before oral infection.

Assay for HeLa cell invasion. We used a modification of the assay described earlier [4] to measure the invasion of HeLa cells. Here, viable bacteria were enumerated. The earlier assay [4] used fluorescent antibody to detect bacteria. HeLa cells were grown at 37 C for 24 hr in 96-well flat-bottom tissue culture plates (Linbro, New Haven, Conn), with an inoculum of 5 x 10^4 cells per well, by using CRML 1066 medium (Commonwealth Serum Laboratories, Melbourne) supplemented with heat-inactivated fetal calf serum (FCS; 10% vol/vol), penicillin (100 IU/ml), streptomycin (100 µg/ml), and HEPES (10 mM). The monolayers were then washed three times with antibiotic-free HBSS containing 10% FCS and 10 mM HEPES and incubated at 37 C for 1 hr in 0.2 ml of the same medium/well. The medium was removed, and \( \sim 10^8 \) bacteria in 0.1 ml of the washing medium were added to each of four wells for each strain under test. After 30 min or 3 hr at 37 C, unbound bacteria were removed by vortexing the culture plates on a tray shaker (Titertek; Flow Laboratories, Sydney, Australia) at half speed for 40 sec before removing the medium from each well. A 0.1-ml volume of serum-free HBSS was added to each well, and the procedure was repeated another five times. Finally, each well received SDS (0.01% [wt/vol] in water, 0.1 ml), and cell lysis proceeded at 37 C for 30 min. The entire well volume (0.1 ml) was plated on kanamycin agar plates to determine the number of bound bacteria. Control plates did not contain HeLa cells but had been incubated with CRML 1066 medium containing 10% FCS for 24 hr before use. For any experiment, the bacterial counts (which were within 5% of the average well count) were averaged to obtain an adhesion or invasion index.

Test for serum resistance. Sera were obtained from persons in this department and were adsorbed with 10^6 S. typhimurium/ml at 4 C for 1 hr. The cells were pelleted, and the adsorbed serum was sterilized by filtration (pore size, 0.45 µm; Millipore, Kloten, Switzerland). The adsorbed serum was used immediately. Serum (0.45 ml) was mixed with the required number of bacteria (0.05 ml), and each mixture was incubated at 37 C for 1 hr. Suitable dilutions of each mixture were made in 0.9% NaCl and plated for bacterial counts.

Results

Serum sensitivity of the cured strain. Bacteria of strains P9144 and J42 were examined for sensitivity to normal human serum. Strains C5 and E. coli K12 strain E381 were included in the assay as controls because their behavior in fresh normal serum
Table 2. Correlation of resistance to the bactericidal action of normal human serum with the presence of the cryptic plasmid.

<table>
<thead>
<tr>
<th>Strain status</th>
<th>No. of bacteria added at zero time</th>
<th>No. of bacteria present after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5 +</td>
<td>10³</td>
<td>5 x 10⁹</td>
</tr>
<tr>
<td>P9144 +</td>
<td>10³</td>
<td>2 x 10⁹</td>
</tr>
<tr>
<td>J42 -</td>
<td>10⁸</td>
<td>0</td>
</tr>
<tr>
<td>E381 -</td>
<td>10⁸</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE. Bacteria (in 0.05 ml of 0.9% NaCl) were mixed with normal human serum to a concentration of 90% (vol/vol) serum and were incubated at 37 C for 1 hr. Bacterial counts were enumerated at zero time and after incubation.

is known [3]. Strain P9144 was clearly serum resistant, and strain J42 was highly sensitive (table 2). After incubation of 10⁸ bacteria of strain J42 in 90% (vol/vol) serum for 1 hr at 37 C, no viable bacteria could be detected. Strain C5 was confirmed to be serum resistant and strain E381 to be serum sensitive.

Virulence of strains for mice. Neither strain P9144 nor strain J42 was highly virulent when administered orally (see Materials and Methods). To obtain killing of mice by strain P9144 when administered orally, we had to pretreat mice orally with streptomycin. When 10 mice received streptomycin 24 hr before oral administration of 10⁶ P9144, all mice died by day 21 after infection. When J42 was used as the challenge organism, all 10 of the mice appeared healthy at day 21 after infection.

Consistent with these observations, strain P9144 was able to kill mice when administered ip, with an LD₅₀ value of ~5 x 10⁶, whereas strain J42 had lost its virulence. After ip injection of 2 x 10⁹ P9144 organisms, all 10 mice were dead by day 6 after injection, whereas 10 mice that had received 10⁸ bacteria of strain J42 were all healthy at day 21 after injection.

Protective effects of oral administration. Two groups of 15 mice received 10⁹ bacteria of either strain P9144 or J42 orally (streptomycin pretreatment was not used here). A third control group received 0.9% NaCl only. Seven days later, the mice received an oral challenge of 5 x 10⁸ S. typhimurium C5, which was equivalent to ~5 x 10² LD₅₀ doses [1]. The control mice died within five days of challenge, whereas both of the other groups remained healthy to day 21 after infection.

Colonization of Peyer's patches. Groups of 15 mice were infected orally with various doses of strains P9144 and J42, and, at various times after infection, the Peyer's patches were excised and bacterial counts obtained (figure 2). The progress of infection in the Peyer's patches was similar for both strains. The bacterial counts rose to a peak about six days after infection and then gradually declined. The peak count in the Peyer's patches was related to the challenge dose, although a 50-fold increase in dose resulted in only a 30-fold increase in peak count.

Invasion of HeLa cells. Strains P9144, J42, C5, and an E. coli K12 strain (E381) were examined for their ability to adhere to or invade HeLa cells. It had previously been reported that after incubation of HeLa cells and bacteria for 30 min at 37 C, most cell-associated bacteria were still outside the HeLa

![Figure 2](image-url)
Table 3. Adherence of plasmid-containing and plasmid-free strains of *S. typhimurium* to HeLa cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time of incubation (min)</th>
<th>Bacterial counts in control wells</th>
<th>Bacterial counts in wells with HeLa cells</th>
<th>Percent association</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5</td>
<td>30</td>
<td>20</td>
<td>280</td>
<td>0.25</td>
</tr>
<tr>
<td>P9144</td>
<td>30</td>
<td>70</td>
<td>650</td>
<td>0.60</td>
</tr>
<tr>
<td>J42</td>
<td>30</td>
<td>70</td>
<td>520</td>
<td>0.45</td>
</tr>
<tr>
<td>E381</td>
<td>30</td>
<td>50</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>C5</td>
<td>180</td>
<td>20</td>
<td>530</td>
<td>5.10</td>
</tr>
<tr>
<td>P9144</td>
<td>180</td>
<td>110</td>
<td>1,500</td>
<td>13.90</td>
</tr>
<tr>
<td>J42</td>
<td>180</td>
<td>110</td>
<td>1,490</td>
<td>13.80</td>
</tr>
<tr>
<td>E381</td>
<td>180</td>
<td>120</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**NOTE.** Monolayers of HeLa cells in a 96-well Linbro tray received bacteria in 0.1 ml of 0.9% NaCl. After incubation at 37 C, the monolayers were washed, the cells were lysed with SDS, and bacteria were enumerated. Control wells did not contain HeLa cells. For each strain in a particular experiment, four wells of HeLa cells were used, and the individual bacterial counts (which were within 5% of the average) were averaged to give the figures for that experiment. The 30-min incubation experiments were done twice and the 3-hr experiments, three times. The results from the individual experiments were within 5% of the average of the experiments—the averages are given above. The final column gives the percentage of the inoculum that remained associated to the cells after washing; here, the figures were corrected for the counts in the control wells. For 3-hr incubations, the HeLa cells received 10^4 bacteria per well; for the 30-min experiments, the cells received 10^5 bacteria per well.

Persistence of *S. typhimurium* in the mouse peritoneal cavity. A preliminary experiment established that after ip injection, strain P9144 persisted in the peritoneal cavity, whereas strain J42 was eliminated quite rapidly. Some 24 hr after injection of 2 x 10^6 organisms, peritoneal washouts from a group of five mice contained an average of 10^7 P9144 organisms, whereas no bacteria were recovered from the peritoneal cavity of mice injected with J42 bacteria. A detailed analysis of the fate of ip-injected organisms established that strain J42 was phagocytosed and killed very rapidly, whereas most of the P9144 organisms did not associate with peritoneal cells and were not killed (figure 3).

Presence of a plasmid copy in the chromosome. Total DNA was prepared from strains C5, P9144, J41, and J42. The DNA was digested with *EcoRI* and run on an agarose gel. Transfer to nitrocellulose followed, and the DNA was then probed with a nick-translated plasmid prepared from strain P9144 (figure 4, B). Several bands appeared in the digested DNA from strains C5 and P9144; these bands corresponded to those expected from digestion of cryptic-plasmid DNA (figure 4, C). In the DNA from strain J41, several bands also appeared that were generally identical to those seen in the track of strain P9144 DNA. The highest molecular weight band did not appear to be present in the track of strain J41. This may reflect the insertion of transposon Tn10 into this band, followed by a deletion event that resulted in a lowering of the molecular weight of the band to that of the next lower bands, while still retaining the transposon Tn10 [4]. No bands appeared in the track representing the DNA from strain J42. Hence, this strain does not contain a copy of the plasmid in the chromosome.

Discussion

Jones et al. [4] reported that loss of the cryptic plasmid from *S. typhimurium* correlated with a loss of virulence and a loss of adhesive and invasive ability for HeLa cells in vitro. We have confirmed the effect of plasmid curing on virulence but cannot reproduce the HeLa cell findings. Our cured strain (J42) attached to and invaded HeLa cells to the same extent as did its plasmid-containing parent (P9144). More significantly, both strains invaded the Peyer's patches of the mouse small intestine to the same extent.

We have described two virulence-related functions...
of the cryptic plasmid. The cured strain is sensitive to the action of specific antibody-free serum, whereas its parent is resistant. Also, the cured strain is rapidly phagocytosed and killed by the macrophages of the mouse peritoneal cavity, whereas its plasmid-containing parent is not phagocytosed and therefore persists.

Although virulence functions are apparently encoded on the cryptic plasmid, it seems clear that the genetic determinants required for adhesion and invasion of the mouse intestinal wall are chromosomal in location. In this context, it is important to note that plasmid sequences are not present on the chromosome of J42. Jones et al. [4] proposed that their cured strain CR8100 did have a plasmid copy in the chromosome, but another cured strain (CR6260) did not appear to have such a copy. The integration of the cryptic plasmid into the chromosome may thus be a strain-dependent phenomenon, with J42 as a...
further example of a strain in which this does not occur.

We are currently engaged in the molecular cloning of the plasmid determinants responsible for the two virulence-related functions described above.

References