Conjugal Transfer of the *Salmonella enterica* Virulence Plasmid in the Mouse Intestine

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Six decades after the discovery of bacterial conjugation, the underlying genetic and biochemical mechanisms are known in great detail, especially in model plasmids like the F sex factor and its relatives (15, 16), the Ti plasmid of *Agrobacterium tumefaciens* (47), the IncP-1 group of broad-host-range plasmids (2), R388 (19), R1162 (30), and pIPS01 (1). In contrast, we have a more limited understanding of the environmental and physiological factors that affect conjugal transfer and of the natural habitats where conjugation occurs. Exceptions are transfer of *A. tumefaciens* Ti plasmids to plant cells in response to compounds present in the rhizosphere (47), pheromone-triggered mating in *Enterococcus faecalis* (20), and transfer of *Yersinia* plasmids in response to quorum sensing (4).

Animals harbor numerous bacterial species, which can establish a wide and complex range of interactions with their hosts. The possibility that plasmid transfer can occur inside animals has been considered since the 1960s, usually as a potential scenario for antibiotic resistance dissemination. Pioneering studies showed that transfer of drug resistance determinants in *Escherichia*, *Shigella*, and *Klebsiella* could be detected in the mouse intestine (26). A later, relevant observation was that drug resistance plasmids of *Salmonella* and *Escherichia* are clustered in an ~34-kb region (28). A single transcriptional unit (the *tra* operon) includes homologs of the F factor genes involved in pilus synthesis and assembly, surface exclusion, stabilization of mating aggregates, DNA nicking, DNA strand displacement, and DNA transport and the regulatory gene *finO* (28). As in F, transcription of the pSLT *tra* operon requires activation by the TraJ transcription factor, encoded by a nearby gene (7). In turn, *traJ* expression is under the control of the FinOP system of fertility inhibition, which in pSLT is fully functional and keeps conjugation tightly repressed (7, 37, 43). The *finP* gene is located in the antisense DNA strand of *traJ* and overlaps the 5′ region of *traJ* (10). The product of *finP* is an antisense RNA that acts together with the RNA chaperone FinO to prevent *traJ* mRNA translation and to promote *traJ* mRNA degradation (25, 45, 46).

Below, we describe surveys of pSLT transfer in the common house mouse, *Mus musculus* L., the natural host of *S. enterica* serovar Typhimurium. We show that pSLT is transferred at high frequencies during animal infection and present evidence that pSLT-promoted mating occurs in the distal portion of the small intestine (the ileum). Virulence plasmid transfer in the ileum might compensate for plasmid loss during intestinal passage. This view is consistent with the fact that bile salts, which have plasmid-curing capacity (18), are found at high concentrations in the mammalian small intestine, especially in the duodenum (24).
MATERIALS AND METHODS

Bacterial strains. The strains of S. enterica used in this study (SV5534 [isp:Mud/Q(Cmr)] pSTL7, SV5553 [ΔΔam-320)pSLT finO::Kmr]), SV5556 [ispR::Trn(Kmr)]), and SV5557 [ispR::Trn(Kmr)] belong to serovar Typhimurium and derive from the mouse-virulent strain ATCC 10428. The ΔΔam-230 allele was constructed by in-frame deletion of the ΔΔ entE dam gene encoding DNA adenine methylase (31). The ΔΔ finO and finO::Kmr alleles have been described elsewhere (7, 18). The ΔΔ:Mud/Q allele is neutral for Salmonella virulence and provides a marker for strain discrimination in experimental infections of mice (35). The spvR::Trn allele was described by Hensel et al. (23).

Media and chemicals. Luria-Bertani (LB) broth was used as rich medium. Solid LB medium contained agar at a 1.5% final concentration. Microaerophilic growth was achieved in GasPak incubation jars (Becton Dickinson Biosciences, San Agustín de Guadalix, Spain). Deoxycholic acid (sodium salt) and sodium propionate were both from Sigma Chemical Co., St. Louis, MO. Antibiotics were used at final concentrations described previously (42).

Bacterial matings in LB plates. Cultures of the donor and the recipient were grown overnight in LB broth. Cells were harvested by centrifugation and washed with LB broth (supplemented with 4% sodium deoxycholate [DOC] or 2% sodium propionate when necessary). Aliquots of both strains (500 µl each) were sucked onto a 0.45-µm-pore-size membrane filter. The donor/recipient ratio was 1:1. The filters were placed on LB plates and incubated at 37°C for 4 h. In certain experiments, LB plates contained 4% DOC or 2% sodium propionate (see below). After mating took place, the mixtures were diluted in 10 mM MgSO4 and spread on selective plates. As controls, 0.1 ml of the donor and the recipient cultures was also spread on selective plates. Conjugation frequencies were calculated per donor bacterium. Matings under the so-called “Salmonella pathogenicity island 1 (SPI-1)-inducing conditions” involved slightly different conditions. Donor and recipient strains were grown overnight in LB broth without shaking. The LB plates contained 0.3 M NaCl, and the membrane-bound mating mixtures were incubated for 4 h at 37°C in GasPak microaerophilic jars.

Bacterial matings in LB broth. Overnight cultures of the donor and the recipient were prepared in LB broth. Aliquots of 500 µl were mixed to obtain a donor/recipient ratio of 1:1. Each mixture was centrifuged for 2 min at 13,000 rpm, and the supernatant was discarded. The pellet was resuspended in 50 µl of either LB broth or LB broth supplemented with feces, which had been previously homogenized in LB broth to obtain a final concentration of 1 g/liter. Mating mixtures were incubated at 37°C for 4 h. Diluted and undiluted aliquots were then spread on selective plates.

Bacterial matings in mice. Feeding of mice was suspended 1 day before inoculation. The donor and recipient strains were grown overnight in LB broth at 37°C without shaking. Under these conditions, the cultures reached saturation in the morning and contained around 3 × 109 bacterial cells/ml. The cultures were pelleted and resuspended in an aqueous solution containing 0.4% lactose and 0.9% NaCl. Each mouse was orally inoculated with 25 µl of donor bacterial suspension and with the same amount of recipient suspension. Each suspension contained 1 × 106 CFU. Inoculation of the second suspension (usually the donor) was performed 30 min after inoculation of the first (usually the recipient). Mouse sacrifice, extraction of liver and spleen, harvest and lysis of eukaryotic cells, and recovery of bacterial cells followed the procedures described by Shea et al. (36).

Bacterial matings in ileal loops. BALB/c mice were starved for 24 h and anaesthetized before surgery by intraperitoneal injection of 300 µl of 2.5% avertin. A stock of 100% avertin was prepared by mixing 10 g of 2,2,2-tribromoethyl alcohol with 10 ml of tert-amyl alcohol (Sigma-Aldrich Quimica SA, Tres Cantos, Spain). Before use, this 100% stock was diluted to 2.5% in isotonic saline with 0.3 M NaCl. A stock of 100% avertin was prepared by mixing 10 g of 2,2,2-tribromoethyl alcohol with 10 ml of tert-amyl alcohol (Sigma-Aldrich Quimica SA, Tres Cantos, Spain). Before use, this 100% stock was diluted to 2.5% in isotonic saline with 0.3 M NaCl. A stock of 100% avertin was prepared by mixing 10 g of 2,2,2-tribromoethyl alcohol with 10 ml of tert-amyl alcohol (Sigma-Aldrich Quimica SA, Tres Cantos, Spain). Before use, this 100% stock was diluted to 2.5% in isotonic saline with 0.3 M NaCl. A stock of 100% avertin was prepared by mixing 10 g of 2,2,2-tribromoethyl alcohol with 10 ml of tert-amyl alcohol (Sigma-Aldrich Quimica SA, Tres Cantos, Spain). Before use, this 100% stock was diluted to 2.5% in isotonic saline with 0.3 M NaCl. A stock of 100% avertin was prepared by mixing 10 g of 2,2,2-tribromoethyl alcohol with 10 ml of tert-amyl alcohol (Sigma-Aldrich Quimica SA, Tres Cantos, Spain). Before use, this 100% stock was diluted to 2.5% in isotonic saline with 0.3 M NaCl.

Experimental conditions for the detection of conjugation in mice. Initial trials for the detection of S. enterica transconjugants after infection of mice were carried out using strain SV5535 ([ΔΔam-230)pSLT finO::Kmr]) as the donor and SV5534 ([isp:Mud/Q pSLT −]) as the recipient. Both strains are attenuated (by a dam mutation and by lack of the virulence plasmid, respectively), while transconjugants can be expected to be virulent. Use of a FinO− pSLT derivative was intended to increase the chances of detecting conjugation. The mice were sacrificed 6 days after infection. Km− Cm− transconjugants were recovered from the ileum and the spleen, indicating that conjugation inside mice had indeed occurred. However, we observed that donor cells (Km− Cm−) were rare or absent in organs, suggesting that conjugation had occurred at an infection stage prior to organ colonization. Detection of transconjugants in feces provided additional evidence that conjugation occurs in the gut (see below). Albeit informative, the above-mentioned experiments presented a caveat: because transconjugants are more virulent than donors, transconjugants might have a growth advantage inside the animal; as a consequence, conjugation frequencies could be overestimated. To sort out this potential flaw, we devised crosses in which the donor, the recipient, and the transconjugants were equally virulent. Also, because the transconjugants were more abundant in the mouse feces than in deep organs, we chose the feces as the standard material for the calculation of conjugation frequencies. Hence, in subsequent experiments, we employed the following materials and conditions. (i) We used a donor strain whose virulence plasmid carried a Tn5 insertion in spvR (23). The plasmidless recipient carried a chromosomal [isp:Mud/Q marker, which is neutral for virulence (35). Lack of spv attenuates the virulence of donors, recipients, and transconjugants 100- to 1,000-fold (data not shown). As mentioned above, each genotype could be easily identified on plates: donors were Km−, recipients were Cm−, and transconjugants were Km− Cm−. (ii) Oral inoculation with a mixture of the donor and the recipient was avoided, since control experiments indicated that conjugal transfer readily occurred in the mixture: if aliquots from the mixture were spread on selective plates, Cm− Km− transconjugants were detected. Hence, the two strains were inoculated separately (30-min difference) by the oral route.

Detection of transconjugants in mouse feces. BALB/c mice were inoculated with strains SV5536 ([ispO::Trn(dam)]) and SV5534 ([ispR::Trn(Kmr)]). As a control, we confirmed that Cm−, Km−, and Cm− Km− bacterial CFU were absent from mouse feces prior to inoculation. Feces from infected mice were collected at 24-h intervals, homogenized in saline, diluted, and spread on antibiotic agar plates for the detection of Cm− Km− transconjugants. Colonies formed by Km− Cm− donors were also counted. Data from five matings are shown in Fig. 1. The frequency of transconjugants increased from day 2 to day 4 and seemed to reach a plateau after that. The mice were sacrificed at day 8.

The conjugation frequencies detected in these experiments were surprisingly high, even for a FinO− pSLT derivative (around 10−5). Note that under optimal laboratory conditions, pSLT FinO− transfer occurs at frequencies between 10−2 and 10−3 (7). The donor and the recipient strains have similar

RESULTS

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growth rates under laboratory conditions, and they are equally virulent; hence, they can be expected to grow at similar rates inside the animal. It seems unlikely, therefore, that the frequencies found could be skewed by a growth advantage of the transconjugants. It is likely, however, that the frequencies found on later days were higher because conjugal transfer had increased the number of donors relative to the number of recipients.

Matings with a FinO\(^+\) pSLT plasmid (strain SV5556) caused a delay in the detection of transconjugants, which started to appear at day 5 after inoculation, and the frequencies of transconjugants were lower than those obtained for pSLT FinO\(^-\). Low frequencies made statistical analysis difficult, but a rough estimation was that the frequency of pSLT transfer decreased 100-fold in a FinO\(^+\) background, a difference similar to that found between FinO\(^+\) and FinO\(^-\) pSLT under laboratory conditions (7).

A caveat for the above-mentioned experiments was that conjugation could occur not only inside the animal, but also in the feces. In other words, the total number of transconjugants might be the sum of those formed in the gut and those formed in the feces. To investigate this possibility, two kinds of experiments were carried out. (i) Feces collected on a given day were divided into aliquots. One aliquot was homogenized and immediately used to score transconjugants on selective plates. The other aliquots were kept at room temperature and used to score transconjugants after 24, 48, and 72 h. The numbers of transconjugants per donor did not increase over time (data not shown), suggesting that conjugation did not occur in the feces. (ii) Feces of uninfected mice were homogenized and added to LB broth at a final concentration of 1 g/liter. A 1:1 mixture of the donor and the recipient (SV5536 and SV5534, respectively) was added, and conjugation was allowed to proceed at 37°C for 4 h. Aliquots were spread on selective plates (LB broth supplemented with kanamycin and chloramphenicol) for the detection of transconjugants. Km\(^+\) Cm\(^+\) donors were enumerated by plating them on LB-kanamycin and replica printing to LB-chloramphenicol. Data from seven independent crosses are summarized in Fig. 2 compared with their corresponding controls (matings in LB broth). Lower frequencies of conjugal transfer in liquid cultures are typical of pSLT; for this reason, the standard protocol for pSLT transfer employs conjugation on membrane filters (7). Conjugation frequencies decreased 50-fold in the presence of feces, suggesting an inhibitory effect upon conjugation. Transfer of FinO\(^+\) pSLT was likewise inhibited in the presence of feces: 5 \times 10^{-7} in liquid LB broth versus 10^{-8} or less in liquid LB broth plus feces. We thus ruled out the possibility that conjugal transfer in feces might have skewed the calculation of conjugation frequencies inside mice. The evidence that feces strongly inhibit conjugation is discussed further below.

**Effects of bile and propionate on pSLT transfer.** The evidence that pSLT promoted *Salmonella* mating in the gut raised the question of which was the section of the gut where mating occurred. This issue was initially investigated using laboratory conditions that are known to mimic (obviously only to a certain extent) specific gut environments. (i) The proximal portion of the small intestine, especially the duodenum, contains high concentrations of bile (24). Previous studies in our laboratory had shown that, besides their detergent activities, bile salts are DNA-damaging agents that cause point mutations, gene rearrangements, and plasmid curing (18, 31, 32). To investigate whether bile salts affected pSLT-mediated conjugation, crosses were performed in the presence of DOC. The concentration used (4%) was lower than the DOC MIC in vitro (7%) and thus did not impair *S. enterica* viability. The donor strain was either SV5536 (FinO\(^-\)) or SV5556 (FinO\(^+\)). The recipient was SV5534 in all cases. Donor and recipient cultures were prepared and mixed (1:1) using the standard protocol for conjugation on a solid support (a membrane filter on top of agar). The presence of DOC caused an ~10-fold decrease in the frequency of pSLT transfer (Fig. 3), suggesting that conjugation is inhibited in the presence of bile. Despite the extreme reductionism of these experiments, a tentative conclusion is that conjugation is unlikely to occur in the duodenum. (ii) The large intestines of mammals contain metabolites that are rare or absent in the small intestine. For instance, high concentrations of propionate and butyrate are produced as a consequence of \(\alpha\)-mannitol utilization by intestinal bacteria (50). We investigated whether the presence of 2% sodium propionate (a concentration that does not inhibit growth) affected the frequency of pSLT transfer under laboratory conditions. An ~10-fold decrease in the frequency of transconjugants was found for both wild-type pSLT and its FinO\(^-\) derivative (Fig. 3). Albeit cautiously, we propose that inhibition of conjugal trans-
brane filters. The donors were either SV5536 (FinO\(^+/+\)) or SV5556 (FinO\(^-/+\)), and the recipient was SV5534. The data are averages and standard deviations from six independent matings. Statistical analysis indicated that the conjugation frequencies in LB broth were significantly different from those in LB broth plus DOC, LB broth plus propionate, and LB broth plus 0.3 M NaCl. *P* values of 0.017, 0.022, and <0.0001, respectively, were obtained for crosses involving a FinO\(^+\) donor. *P* values were all <0.0001 for crosses involving a FinO\(^-\) donor.

The results described above faithfully reproduce conditions in laboratory conditions stimulate conjugal transfer. The high frequencies (3, 7, 37) of fertility inhibition and promotes conjugation at very low frequencies (3, 7, 37). This contradiction may have more than one resolution. There is the possibility that pSLT is a rare system of fertility inhibition and promotes conjugation at very low frequencies. The donors were SV5536 (FinO\(^+/+\)) or SV5556 (FinO\(^-/+\)), and the recipient was SV5534. The data are averages and standard deviations from three independent matings.

**Effects of SPI-1-inducing conditions on pSLT transfer.** If the results described above faithfully reproduce conditions found in specific sections of the mouse intestine, the hypothetical scene of conjugal transfer should be an intestinal section where the concentrations of bile and propionate are low. These conditions are met by the distal portion of the small intestine, especially the ileum. Additional traits of the ileum are microaerophilia and relatively high osmolarity (29). In fact, microaerophile incubation in LB broth containing 0.3 M NaCl is a standard procedure to reproduce in vitro the conditions that induce expression of SPI-1 in the ilea of infected animals (13).

On these grounds, we tested the effect of microaerophilic growth in the presence of 0.3 M NaCl on pSLT transfer. Matings were performed as described above, with the differences that the mating mixtures were incubated in a GasPak microaerophilic jar. An 8- to 10-fold increase in the number of transconjugants was found (Fig. 3), indicating that ileum-like laboratory conditions stimulate conjugal transfer. The high conjugation rates found (between \(10^{-3}\) and \(10^{-2}\) per donor cell for FinO\(^-\) pSLT and nearly \(10^{-2}\) for the wild-type virulence plasmid) suggest that pSLT transfer may be stimulated under the same conditions that trigger invasion of the intestinal epithelium by *Salmonella*. Interestingly, high rates of conjugal transfer in anaerobiosis have also been described for the *E. coli* F sex factor (38).

**Conjugal transfer of pSLT in murine ileal loops.** Because several lines of evidence suggested that pSLT transfer inside mice might occur in the distal portion of the small intestine, we devised conjugation trials in the ilea of BALB/c mice. Donor and recipient suspensions were prepared in phosphate-buffered saline to avoid injection of LB medium (which obviously supports conjugation) into the mouse intestine. Control matings indicated that both pSLT FinO\(^+\) and pSLT FinO\(^-\) were transferred at low frequencies in phosphate-buffered saline. Further by propionate may indicate that mating inside mice does not occur in the large intestine. Propionate, which is likewise present in feces, may also contribute to inhibition of mating in the presence of feces (Fig. 2).

**FIG. 3.** Effects of DOC, sodium propionate, and SPI-1-inducing conditions on conjugal transfer of pSLT FinO\(^-\) (black histograms) and pSLT FinO\(^+\) (gray histograms). Matings were carried out on membrane filters. The donors were either SV5536 (FinO\(^-\) ) or SV5556 (FinO\(^+\) ), and the recipient was SV5534. The data are averages and standard deviations from six independent matings. Statistical analysis indicated that the conjugation frequencies in LB broth were significantly different from those in LB broth plus DOC, LB broth plus propionate, and LB broth plus 0.3 M NaCl. *P* values of 0.017, 0.022, and <0.0001, respectively, were obtained for crosses involving a FinO\(^+\) donor. *P* values were all <0.0001 for crosses involving a FinO\(^-\) donor.

**FIG. 4.** Transfer of FinO\(^-\) and FinO\(^+\) pSLT plasmids in ileal loops of mice. The donors were SV5536 (FinO\(^-\) ) and SV5556 (FinO\(^+\) ), and the recipient was SV5534. The data are averages and standard deviations from three independent matings.

**DISCUSSION**

Phylogenetic analysis suggests that *S. enterica* virulence plasmids are transferred at high rates in nature (6). However, the model *Salmonella* virulence plasmid (the pSLT plasmid of *S. enterica* serovar Typhimurium) carries a functional FinOP system of fertility inhibition and promotes conjugation at very low frequencies (3, 7, 37). This contradiction may have more than one resolution. There is the possibility that pSLT is a rare exception and that many natural *Salmonella* virulence plasmids are derepressed for transfer, like the *E. coli* F episome (15). However, it is also conceivable that transfer in nature may follow rules different from those observed in the laboratory. In support of the latter view, an intriguing feature of F-like plasmids is the fact that their transfer functions are under the control of an ample variety of host-encoded regulators. The current list includes the redox sensor ArcA (41), the global transcriptional regulators Lrp (7, 9, 40) and CRP (39), the nucleoid protein H-NS (10, 49), the RNA chaperone Hfq (48), the GroEL heat shock chaperone (51, 52), DNA adenine methylation (43), and the extracytoplasmic stress response CpxAR system (21). Certain regulators have slightly different
functions depending on the plasmid, suggesting that each plasmid may have adapted to the particular lifestyle of its host (8, 9). Even under laboratory conditions, different F-like plasmids seem to follow different transfer rules: for instance, F transfer is inhibited in stationary-phase cultures (17) while pSLT transfer is not (7). In turn, pSLT transfer occurs at lower frequencies in LB broth than in minimal medium, a trait not found in F (3). A tentative interpretation for the existence of so many controls is that F-like plasmids may promote mating in response to specific physiological and environmental signals and increase their conjugation rates in appropriate environments.

Conjugal transfer in the mouse intestine may be an example of activation of conjugal transfer under convenient circumstances. The gut provides a rich supply of nutrients, which permit rapid multiplication of Salmonella cells. However, certain sections of the gut may not be suitable for conjugation. For instance, conditions that mimic the duodenum (LB broth plus DOC) inhibit mating. Although synthesis of pSLT Tra pilis does not cause bile sensitivity as it does in F (18), it is conceivable that cell-to-cell interactions can be disturbed by the presence of bile salts. Transfer of pSLT is also inhibited in LB broth plus propionate, suggesting that the large intestine may likewise be inappropriate to support conjugation. In fact, Salmonella is known to be at a disadvantage when it competes with the bacterial flora of the large intestine, apparently as a consequence of inefficient nutrient uptake (44). Because conjugation is an energetically expensive process (11), its inhibition may make full sense under such conditions.

Can our laboratory observations be extrapolated to nature? In principle, the BALB/c mouse can be expected to be a reliable model for the study of conjugation in the intestines of animals. Lack of the macrophage-associated protein N-RAMP renders BALB/c mice especially sensitive to bacterial infections; however, the effects of N-RAMP loss are much less pronounced at the intestinal stage of infection than during systemic infection (12). On the other hand, Salmonella colonization of animals requires competition with the established intestinal flora. For this reason, treatment of mice with antibiotics prior to Salmonella administration is an old practice used to boost experimental Salmonella infections (5). However, we detected high frequencies of conjugal transfer without antibiotic pretreatment of mice (in other words, under circumstances that do not especially favor Salmonella proliferation). Altogether, these considerations suggest that intestinal transfer of virulence plasmids may indeed occur in house mice, and perhaps in other animals, during Salmonella infection.

The detection of Salmonella conjugation in the mouse intestine does not exclude the possibility that other environments may also provide suitable conditions for virulence plasmid transfer. In fact, transfer of a close relative of pSLT, the F sex factor, has been detected inside cultured epithelial cells (14). However, the population sizes and cell densities attained by Salmonella in the small intestine (29) are unlikely to be found in other environments, either inside animals or outside. Transfer of the virulence plasmid in the intestines of animals might thus account for the high conjugation rates predicted by phylogenetic analysis (6).

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