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Absence of persistence and transfer of genetic material by recombinant *Escherichia coli* in conventional, antibiotic-treated mice

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SUMMARY

Strain BST-1 is a derivative of *Escherichia coli* K-12 that carries a plasmid designated pURA-4 and is the expression system used by The Upjohn Company in the production of recombinant bovine somatotropin (rbSt). This plasmid also encodes an ampicillin resistance gene. The plasmidless carrier strain, BST-1C, contains a gene for tetracycline resistance which is provided by the chromosomal insertion of the transposon Tn*10*. Therefore, BST-1 is resistant to ampicillin and tetracycline, while BST-1C is resistant only to tetracycline. The Food and Drug Administration requested that we conduct an environmental assessment study to monitor the 'persistence of the recombinant live K-12 *E. coli* organism compared to the host *E. coli* organism'. In addition, we were requested to monitor 'the potential transfer of genetic material from (our) recombinant organism to the indigenous microflora' of the mouse gastrointestinal (GI) tract. The differences in persistence were determined by monitoring shedding of BST-1 and BST-1C in the feces of conventionally reared, outbred mice inoculated with either of the two strains. Even with antibiotic selective pressure applied (tetracycline in the water), BST-1 did not persist as well as the non-plasmid carrying parental stain, BST-1C. In the gene transfer experiments, transfer of pURA-4 was monitored by the appearance of the ampicillin resistance marker and/or by hybridization assays for the rbSt gene in indigenous, mouse-colonizing *E. coli* strains which had been made streptomycin resistant. At the limit of detection, no transfer of pURA-4 was detected either in vitro or in vivo. These data support an interpretation that BST-1 does not present an environmental hazard as measured by colonization/persistence in the gut of conventionally reared mammals.

INTRODUCTION

Escherichia coli BST-1, carrying the plasmid pURA-4, is the expression system used by The Upjohn Company for the production of recombinant bovine somatotropin (rbSt). This plasmid is a derivative of pBR322 [5,6] from which the tetracycline (Tet^r) region was deleted but which still retains the β-lactamase coding region for ampicillin resistance (Amp^r). The bacterial strain BST-1 was derived from *E. coli* K-12. Both BST-1 and its pURA-4-cured derivative, BST-1C, are Tet^r due to a chromosomal insertion of the transposon Tn*10*. Therefore, BST-1 is Amp^r and Tet^r, while BST-1C, is simply Tet^r.

As part of the environmental assessment study requested by the Food and Drug Administration (FDA) to monitor the 'persistence of the recombinant live K-12 *E. coli* organism compared to the host *E. coli* organism' it was suggested that our studies should address 'survival and colonization in the (rodent) gut' and 'the potential transfer of genetic material from (our) recombinant organism to the indigenous microflora' of the mouse gastrointestinal (GI) tract. The objectives of this study were two-fold. The first objective was to assess the survival and persistence of *E. coli* BST-1 in the gut of adult mice being treated with tetracycline (Tet) compared to the survival of BST-1C in the gut of mice which have not been treated with antibiotic. The purpose of the Tet supplementation of the water in the BST-1 challenged mice was, as personnel at the FDA requested, to provide 'selection pressure favoring gastrointestinal tract colonization by this organism'. The second objective of the study was to determine whether the gene encoding rbSt would be transferred to *E. coli* strains colonizing the gut of mice. The mice were pre-colonized for 1 week with streptomycin-resistant (Str^r) *E. coli* strains and then challenged with BST-1. Mice also were fed ampicillin in the water beginning shortly after challenge with BST-1 to provide selective pressure for transfer of pURA-4. This report is a summary of those results and the complications that arose in respect to the experiments.

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MATERIALS AND METHODS

Bacterial strains

All cultures were maintained as frozen stocks at −70 °C. *Escherichia coli* strains BST-1 and BST-1C were derived from *E. coli* K-12 ATCC 23716. Strain C600(pR751) was obtained from N.B. Shoemaker, University of Illinois. Strain HB101 [25] was obtained from the Upjohn culture collection.

The day prior to inoculation of the mice, storage vials of *E. coli* BST-1 and BST-1C were withdrawn from the freezer, thawed, and a 0.1-ml sample from each vial was inoculated into separate tubes containing 10 ml of brain-heart infusion broth (BHIB). In the case of BST-1, the BHIB was supplemented with 100 μg Amp/ml to insure maintenance of pURA-4. The two cultures were incubated 16–18 h at 30 °C and used to inoculate the mice.

Two *E. coli* strains were isolated from the feces of 5–6-week-old, CD-1, female mice and were made Str^r by selection on Str-containing, agar medium plates. These Str^r strains (UC12699 and UC12700) were stored on 3-mm glass beads at −70 °C in trypticase soy broth containing 10% glycerol. Inocula for the colonization experiments were prepared by removing one bead from the frozen stock and placing it into 10 ml of BHIB supplemented with 100 μg Str/ml. These cultures were incubated 16–18 h at 37 °C and used to inoculate the mice.

Plasmid and strain constructions

The 5.6-kb plasmid pURA-4 (pUC1195) expresses rbSt using the *trp* promoter, the *trpL* ribosome-binding site, the rbSt M4 gene [43], and the *rpoC* transcription terminator [3,38]. This expression unit is located from base pairs 1 to 1219 on the plasmid. The R1 runaway replicon was isolated from the pKN402 derivative, pBEU-17 [44–46], and is located from base pair 1210 to 3711. A portion of R1 was cloned into the *Nar* I and *Nde* I sites of the pBR322 vector as a 2.5-kb *Aha*II–*Nde*I fragment [31] containing the entire replication region of the vector. The remainder of the vector contains the pBR322 origin of replication and the β-lactamase gene.

The bacterial strain BST-1 was derived from *E. coli* K-12 (ATCC 23716). The ATCC strain was cured of bacteriophage λ and the fertility factor F, was made Tet^r by insertion of Tn*10*, and was transformed with pURA4. A derivative of strain BST-1 which was cured of pURA4 was designated BST-1C. The minimal inhibitory concentration (MIC) of Tet for BST-1 and BST-1C was 128 μg/ml. The MIC of ampicillin was > 1 mg/ml and 4.0 μg/ml for BST-1 and BST-1C, respectively.

The self-transmissible plasmid pR751 was introduced into various strains by mating with strain C600(pR751). This plasmid contains two transposons, Tn*4321* and Tn*402* [35]. Transposon Tn*402* encodes trimethoprim resistance (Tp^r). The plasmid pBR322 was introduced into BST-1C by transformation.

Bacterial transformation

The transformation procedure of Maniatis et al. [25] was used with minor modifications. *E. coli* strains were grown to mid-log and a culture sample was then centrifuged (6000 rpm, Sorvall SS-34 rotor (Sorvall), 4 °C, 5 min). The resulting cell pellet was suspended in ice-cold, sterile transformation buffer (50 mM $CaCl_2$ without (BST-1) or with (all other strains) 10 mM Tris hydrochloride (pH 8)). After incubation on ice, the cells were centrifuged, the pellet was suspended in a smaller volume of fresh of ice-cold, sterile transformation buffer and plasmid DNA was added to the cell suspension. This mixture was held on ice for 30 min to 1 h, followed by heat shock at 37 °C for 7 min (BST-1) or 42 °C for 2 min (all other transformations). After the mixture was cooled to room temperature it was either: plated directly on the appropriate selective agar media (pURA-4); incubated in Luria-Bertani (LB) broth 1 h at 37 °C (pBR322), diluted, and plated on selective media; or, incubated in LB broth for 1 h at 30 °C (all other DNA preps), diluted, and plated on selective media.

Media

The media used for the animal experiments included Bacto MacConkey agar (MA) medium (Difco; 50 g/l) unsupplemented or supplemented with either: 100 μg Str per ml; 100 μg Str and 100 μg Amp per ml; or, 12.5 μg Tet and 100 μg Amp per ml medium. Bacto BHIB (Difco; 37 g/l) with or without Amp or Str supplementation (100 μg/ml) was also used.

For the in vitro plasmid transfer experiments, LB broth medium consisting of 10 g of Bacto Tryptone (Difco), 5 g Bacto Yeast Extract (Difco) and 10 g NaCl per 1000 ml of deionized water was used. Bacto agar (15 g/liter; Difco) was added to LB broth for LB agar medium. Bacto MA supplemented with the various concentrations of antibiotic described above was also used.

Antibiotic stock solutions

Stock solutions of Amp, Str, and Tet were prepared by dissolving the appropriate amount of antibiotic in deionized water and by filter sterilizing (0.22-μm filter) the resulting solution. Trimethoprim was dissolved in 0.009 M HCl and filter sterilized.

Mice

Five to six-week-old, female, Crl:CD-1(ICR)BR Swiss mice weighing 18–22 g each from Charles Rivers Breeding Laboratories, Portage, MI, were used. This strain is an

outbred strain that was caesarean derived in 1959 by Charles Rivers Breeding Laboratories from HaM/ICR (Hauschka and Mirand-Roswell Park Memorial-Swiss) mice.

Unless otherwise indicated, mice were caged individually in plastic cages with wood-chip litter, fitted with isolator tops. Mice were allowed food and water (sometimes supplemented with either 100 μg Amp/ml, 100 μg Str/ml, or 32 μg Tet/ml) ad libitum. Mice were acclimated to the cages and room for a period of at least 1 week prior to inoculation.

In vivo testing

For the colonization persistence experiments, the mice were assigned randomly to two treatment groups of ten mice each per replication of the experiment by a computer generated randomization schedule. The experiment was replicated four times, providing a total of 40 mice per treatment group at the end of the study. This was a randomized complete block design with the time of the experiment considered a block and the ten mice per treatment group considered samples. Mice inoculated with BST-1 were treated with Tet by its addition to the drinking water (32 μg/ml). The Tet dosing began 1 week prior to and continued throughout the experiment. Tetracycline water was freshly prepared and the old water was replaced at 7 day intervals beginning 8 days prior to inoculation. Mice inoculated with BST-1C received unsupplemented water throughout the experiment.

On the day of mouse inoculation the cultures were harvested by centrifugation at $6000 \times g$ for 10 min and resuspended by vortex mixing in 10 ml of fresh BHIB. Each mouse was inoculated once intragastrically using a 22-G, 1-inch, straight animal feeding needle affixed to a 1-cc syringe. Mice received 0.2 ml of undiluted, resuspended culture. Preliminary experiments had shown that this would provide an inoculum size of approx 2×10^8 colony forming units (CFU)/mouse. Plate counts were conducted to determine the exact inoculum size.

On day −1 (the day prior to inoculation), and on days 0.25, 0.5, 1, 2, 3, 7, 14, 21, and 28 post-inoculation, 2–5 fresh fecal pellets were obtained from each mouse. These were weighed and homogenized in 1 ml saline (0.9% sodium chloride) with a micro-tissue grinder pestle (No. 189460, Spectrum Medical Industries, Inc.; sterilized by flaming with 95% ethanol) and a mortar consisting of sterile 12 × 75 mm polypropylene tubes. The resulting suspension was serially diluted in saline and plated in triplicate on MA medium plates containing 100 μg Amp/ml and 12.5 μg Tet/ml (group 1, BST-1) or MA containing only 12.5 μg Tet/ml (group 2, BST-1C). The colony count was determined after incubation of the plates at 30 °C for 18–24 h.

For the gene transfer experiments, on the day of the pre-colonization portion of the experiment (day −7), the cultures (UC12699 or UC12700) were harvested by centrifugation at $6000 \times g$ for 10 min and resuspended by vortex mixing in 10 ml of fresh BHIB. Each mouse was inoculated once intragastrically receiving 0.2 ml of undiluted, resuspended culture resulting in an inoculum size of approx. 2×10^8 CFU/mouse. On day 0, the mice were inoculated with BST-1, as described above. Ampicillin supplementation (100 μg/ml) of the drinking water of these mice was begun 6 h post-inoculation with BST-1. On day −7 (the day of pre-colonization), and on days 0, 1, 2, 3, 4, and 7 (and sometimes 10 and 18) post-inoculation with BST-1, 2–5 fresh fecal pellets were obtained from each mouse. These were weighed, homogenized in 1 ml saline, diluted in saline and plated on MA medium containing 100 μg Str/ml, 100 μg Amp and 12.5 μg Tet/ml, or 100 μg Amp and 100 μg Str/ml in order to enumerate *E. coli* UC12699 or UC12700, BST-1, or putative transcipients (defined as DNA recipients by any genetic transfer mechanism including conjugation, transformation or transduction), respectively. The colony count was determined after incubation of the plates at 20–25 °C for 18–24 h. Any colonies which were suspected of being transcipients (growth on Amp/Str plates) were confirmed by transfer to secondary plates containing Amp/Str, Amp/Tet and Str. Colonies growing on the secondary Amp/Str plates and certain of colonies from the Str (only) plates were probed for rbSt cDNA as described below. In addition, the genus and species identification of organisms arising on the secondary plates was determined using a commercial identification system (API20E, Analytab Products, Inc.) according to the manufacturer's instructions.

In vivo mating experiments

Strains were first subcultured by streaking them individually on agar media containing antibiotics appropriate for their plasmid and chromosomal antibiotic resistance markers. After incubation overnight at 30 °C, bacterial growth was removed from the plate, suspended in 5 ml of sterile LB broth. The OD_{550} of the cell suspension was measured and used to determine the appropriate volume of the suspension to add as inoculum to 100 ml of LB broth to yield a final OD_{550} of 0.04. The recipient cultures were incubated at 30 °C with shaking (130 rpm) until the culture OD_{550} was 0.5–0.7 and then were stored on ice until their use. Donor cultures were treated similarly, except that after the culture OD_{550} reached 0.3 the cultures were incubated statically.

Standard methods for filter mating (2-h incubations) were used to detect conjugative genetic transfer between bacterial strains [12,47]. In mating experiments involving *E. coli* C600(pR751), culture samples of the donor (0.2 to

0.6 ml) and recipient strain (9.5 ml) were mixed and filtered onto a 25-mm, 0.45-μm pore size nitrocellulose filter (Millipore Corp.). In all other mating experiments, 100 ml each of donor and recipient cultures were mixed together and centrifuged (7500 rpm in GSA rotor (Sorvall Instruments), 22–24 °C, 5 min). The supernatant solution was decanted and the cells were suspended in the fluid remaining with the cell pellet. The resulting cell suspension was spotted on a 100-mm, 0.45-μm pore size nitrocellulose filter (Millipore), resting on an LB agar plate.

The mating mixtures were incubated at 30 °C for 2 h and then were removed from the filter by vigorous mixing in 1.6 or 2 ml of PBS (phosphate-buffered saline: 0.85% NaCl in 0.066 M sodium phosphate buffer (pH 6.8)). Serial dilutions of the mating mixtures and each parental strain were plated on MA plates supplemented with appropriate antibiotic(s). The ratios of donor to recipient cells were monitored and were approx. 1:10 at the end of the incubation period.

To check putative transconjugants for phenotype, colonies were picked with sterile applicator sticks and spotted onto a series of MA plates supplemented with appropriate antibiotics and sugars (lactose, galactose or sucrose) to determine antibiotic resistance and sugar utilization. If there was growth on the last plate (containing no antibiotic), then it was assumed that each of plates received inoculum and that lack of growth on an antibiotic-supplemented plate was due to sensitivity to that antibiotic.

The frequency of genetic transfer of antibiotic resistance(s) to the recipient cells was calculated based on the number of donor cells added to the mating mixture. The populations of Amp^r and Amp^rTp^r transconjugants in mating mixtures were estimated differently. Plates, selective for Amp^r transconjugants, were inoculated with low dilutions (10^0, 10^{-1}, and 10^{-2}) of mating mixtures. After 48 h incubation, the lawns of bacterial growth were replica plated onto agar supplemented with the same antibiotics. If no growth or colonies appeared on the replica plate, the dilution from which the inoculum came originally was used to give an '*approximation*' of the limit of detection for Amp^r transconjugants. All matings were conducted two to three times.

Detection of transcipients

Potential transcipients were probed by cDNA hybridization assay by the method of Sambrook et al. [32]. Screening of colonies for rbSt cDNA employed the *Cla* I/*Bam* HI restriction fragment of plasmid pURA-4 containing the rbSt coding sequence. This fragment was radiolabelled to a specific activity of $1–2 \times 10^9$ cpm/μg DNA using the random primer method of Feinberg and Vogelstein [13] as modified for use in the Prime-It Kit™ (Stratagene).

Data analysis

The survival times of the inoculated bacteria in the GI tract of the mice were investigated using a summary variable of the log (base 10) CFU/g of the bacteria in the fecal pellets of the mice. Values below the limits of detection of the laboratory procedure were estimated by the limit of detection. The analysis method used was a repeated measures analysis of variance with treatment, replicate and the treatment by replicate interaction as independent variables and the repeated (over time) bacterial counts as multivariate dependent variables. The treatment by replicate interaction was significant ($P < 0.25$) in univariate tests at several measurement times and was consequently left in the model and used to test for treatment differences.

RESULTS

In vivo persistence studies

An important consideration for defining the conditions to monitor the survival of BST-1 relative to BST-1C in the mouse gut was to characterize the *E. coli* population in the gut of CD-1 mice from Charles River Breeding Laboratories. This strain of mouse was chosen because previous reports had suggested that CD-1 mice had very low levels ($< 10^3$ CFU/g) or lacked culturable *E. coli* in their GI tracts [9,17]. The mice from the Charles River breeding colony in Portage, MI, however, were found to be colonized with moderate numbers of coliforms which, on further identification, were found to be exclusively *E. coli*. In preliminary experiments (data not shown) fresh fecal pellets were cultured for coliforms at the time the mice arrived (4–5 weeks of age) and at weekly intervals thereafter. *E. coli* had colonized these mice in a stable population at $1–5 \times 10^5$ CFU/g of fecal material. This resident population contained no detectable Tet^r or Amp^r/Tet^r *E. coli* even when the mice were placed on water which had been supplemented with Tet.

In the persistence experiment, the indigenous coliform populations of the mice were monitored at day −1, before inoculation but after half of the mice (group 1) had been on Tet containing water for 7 days (Table 1). The purpose of these counts was to determine the impact of Tet on the resident coliform population and to determine whether, before inoculation, Amp^r/Tet^r or Tet^r coliforms were present in the feces of group 1 (BST-1 inoculated) or group 2 (BST-1C inoculated) mice, respectively.

The counts were conducted on either plain MA plates or on MA plates which had been supplemented with Amp/Tet or Tet. Coliforms able to grow on the antibiotic supplemented plates were assumed to be resistant to that

TABLE 1

Least-square means of coliform counts ($\log_{10} \pm$ SE CFU/g) from CD-1 mice 1 day before inoculation

Replication	Least-squares mean $\log_{10}$ coliform count ($\pm$ SE; $n = 10$) in CFU/g of mouse feces on medium			
	Tet in water		unsupplemented water	
	MacConkey	M + T/A[a]	MacConkey	M + T[b]
1	4.48 ± 0.26	$< 1.73 \pm 0.44$	5.30 ± 0.26	$< 1.90 \pm 0.44$
2	4.77 ± 0.26	$< 1.84 \pm 0.44$	5.96 ± 0.26	$< 1.79 \pm 0.44$
3	5.03 ± 0.26	$< 1.80 \pm 0.44$	5.62 ± 0.26	$< 1.72 \pm 0.44$
4	4.37 ± 0.26	$< 1.73 \pm 0.44$	4.72 ± 0.26	$< 1.79 \pm 0.44$
Mean[c]	4.66 ± 0.13	–	5.40 ± 0.13	–

[a] M + T/A; MacConkey agar supplemented with Tet and Amp.
[b] M + T; MacConkey agar supplemented with Tet.
[c] Differences among the treatment means were significant ($P < 0.05$).

(those) antibiotics and would have complicated monitoring of persistence of BST-1 and BST-1C. The results from the plain MA plates were analyzed using the $\log_{10}$ CFU/g of fecal material as the dependent variable. The F ratio for the test of significance of the treatment by replication interaction was 0.93 ($P = 0.43$), thus this term was pooled with the residual error for testing treatment and replicate differences. The count differences were significant across treatments ($P < 0.01$) and replicates ($P < 0.01$). Mice in group 1 had significantly less coliforms than those in group 2. No Amp^r/Tet^r or Tet^r coliforms were detected in any of the animals before inoculation.

The concentrations of Tet in the feces of group 1 mice are summarized in Table 2. Based on a report in the literature [10] the 32 μg Tet/ml concentration in the water was expected to provide a concentration in the feces of 12.3 ppm. The concentrations of Tet in the feces of the mice at 29 days post-inoculation (36 days on Tet water) were considerably higher than expected, however, ranging from a low of 31.3 ppm to a high of 113.2 ppm for individual mice. There were no significant differences ($P > 0.05$) among the mean replicate levels of Tet in the feces at day 29 (Table 2). No antimicrobial activity was measured in the feces group 2 mice.

The differences in persistence (presence in the feces) with the combined data from the four replications is summarized in Table 3. In the data summary and for the analysis, the bacterial count from mice which had no detectable BST-1 or BST-1C in the fecal material was taken as the limit of detection (LOD). The LOD was based on the amount of fecal material and the dilution plated. The LOD was generally 50–100 CFU/g of fecal material. Therefore, the mean counts in Table 3 are an overestimation of the mean number of bacteria present. Although there was greater shedding of BST-1C than BST-1 (especially at 24–72 h post-inoculation), univariate analysis of the variance at each sample time showed that these differences were not significant ($P > 0.05$) for all times. The pattern of shedding between the two strains did, however, differ significantly ($P < 0.0001$) over time as demonstrated by a significant multivariate strain·time interaction. Strain BST-1 was eliminated more quickly than BST-1C. In addition, there were more animals shedding BST-1C for longer periods of time than those shedding BST-1 (Table 3). At 7 days post-inoculation one of the 40 mice had

TABLE 2

Assay of Tet[a] in the feces at day 29 of mice receiving Tet-treated water (n = 10/replication except rep. 3 which had five mice).

Replication	Tet concentration (ppm)	
	least-squares mean $\pm$ SE	range
1	54.9 ± 6.2	31.3 – 97.1
2	68.6 ± 6.2	42.3 – 113.2
3	71.5 ± 8.8	37.5 – 90.0
4	79.2 ± 6.2	60.0 – 109.3

[a] The concentration of Tet in the feces of group 1 mice was determined from fecal pellets collected at day 29, post-inoculation. Fecal pellets were homogenized and diluted as needed in 0.1 M phosphate buffer (pH 4.5). This solution was then assayed by the microbiological cylinder-late method with *Bacillus cereus* UC225 as the assay organism. This procedure was adapted from published procedures for analysis of chlortetracycline HCl [1]. All samples were stored at $-20°C$ until processed for assay.

TABLE 3

Fate of *Escherichia coli* strains BST-1 and BST-1C in mice

Days post-inoculation	Challenge strain of *Escherichia coli*				*P* value
	BST-1		BST-1C		
	Mean[a] ± SD of $\log_{10}$ CFU/g	No. culture positive/ No. tested	Mean[a] ± SD of $\log_{10}$ CFU/g	No. culture positive/ No. tested	
0.25	7.757 ± 0.407	40/40	7.973 ± 0.401	40/40	0.054
0.50	6.339 ± 0.519	40/40	6.650 ± 0.549	40/40	0.129
1.0	3.674 ± 1.074	39/40[c]	4.771 ± 0.635	40/40	0.057
2.0	1.932 ± 0.437	9/40	2.536 ± 0.854	27/40	0.052
3.0	1.800 ± 0.319	3/40	2.259 ± 0.831	15/40	0.110
7.0	1.788 ± 0.251	1/40	1.884 ± 0.484	6/40	0.446
14–28	ND[b]	0/40	ND	0/40	–

[a] $n = 40$.

[b] ND, none detected.

[c] When no challenge strain *E. coli* were detected in an animal, the count was calculated based on the limit of detection for that animal (see text for further discussion).

detectable BST-1 populations in its feces, while six of the 40 mice had detectable BST-1C. Neither strain was detected in the feces at 14 days post-inoculation.

In vivo gene transfer experiments

Determining transfer of DNA to all or even most of the >400 different species of the microbiota in the GI tract [20] would be an impossible task. Therefore, it was decided in our conference with the CVM that murine GI coliforms such as *E. coli* could be monitored as the potential gene recipient in the in vivo transfer experiments. In preliminary experiments it was necessary to demonstrate colonization of the recipient strain. To enhance colonization by the Str^r recipient strains, mice (housed in groups of six) were pretreated for 1 week with Str in the drinking water before challenge with strain UC12699 (Table 4). As determined by the colony counts at day 0 on plain MA plates, Str pretreatment eliminated all detectable coliforms

TABLE 4

Colonization of streptomycin pretreated mice with *Escherichia coli* strain UC12699

Week post-inoculation	Geometric mean $\log_{10}$ coliform count in CFU/g feces on MacConkey agar with:		% of coliform count Str^r	Animals with Strep^{r}coliforms
	Streptomycin	no antibiotic		
0	<2.09 ± 0.01	<2.09 ± 0.01	0	0/3
1	5.34 ± 0.15	5.51 ± 0.48	68	6/6
2	5.58 ± 0.15	5.56 ± 0.30	105	6/6
3	5.06 ± 0.59	5.10 ± 0.65	91	6/6
4	5.65 ± 0.32	5.69 ± 0.51	91	5/6
5	4.76 ± 0.60	4.75 ± 0.67	104	6/6
6	5.42 ± 0.34	5.33 ± 0.41	123	6/6
7	6.65 ± 0.10	6.57 ± 0.13	120	6/6
8	5.52 ± 0.56	5.50 ± 0.62	105	6/6
9	6.13 ± 0.49	6.19 ± 0.55	87	6/6
10	5.99 ± 0.57	5.94 ± 0.73	112	6/6
Mean	5.61 ± 0.54	5.61 ± 0.52	100	5.9/6

from these animals compared to an expected count of $> 10^5$ CFU/g of feces. No Str^r coliforms were detected in any of the mice before inoculation as determined by the colony counts at day 0 on Str-containing MA. Unlike preliminary studies using non-antibiotic treated mice, strain UC12699 was found to stably colonize the Str-pretreated mice at a level of 10^5 to 10^6 CFU/g of feces for up to 10 weeks post-inoculation (Table 4). Also, it was noted that the Str^r strain, UC12699, comprised essentially 100% of the coliform flora in these mice as the viable counts on the plain MA plates were nearly identical to the counts on the Str-supplemented plates. In addition, except for one sampling (week 4), all Str-pretreated mice were culture positive for the inoculated strains at every sampling time. Results with strain UC12700 were similar (data not shown).

As described in Materials and Methods, our initial strategy for monitoring in vivo transfer of pURA-4 was to eliminate the normal *E. coli* flora of the mice with Str, colonize the gut with the characterized Str^r mouse-isolate derivatives UC12699 and UC12700, challenge with BST-1 supplying selective pressure for the transfer event with Amp supplementation of the drinking water, and to detect possible but unlikely transcipients in the feces by colony enumeration on MA containing Amp/Str. The populations of the donor and recipients were also monitored by selection on Amp/Tet or Str MA plates, respectively. Table 5 summarizes an experiment with the two colonizing strains. When 100 μg Amp/ml water was provided after BST-1 inoculation, a negative impact on the colonizing strain populations was noted at the first sampling. Additionally, with this concentration of Amp supplementation, BST-1 (as measured by growth of coliforms on the Amp/Tet MA plates) appeared to persist in the gut of some of the mice for up to 10 days post-BST-1 inoculation. This occurred despite the fact that ampicillin was removed from the water at day 7. Moreover, coliform colonies and patches of growth were detected on the Amp/Str plates in one of the mice colonized with strain UC12700 beginning at day 4 and persisting through day 7. In additional experiments, similar observations were made. Strain BST-1 was discovered to persist in large numbers in mice pro-

TABLE 5

Fate of BST-1, UC12699, UC12700 and appearance of putative transcipients with Amp in the drinking water as selection pressure

Treatment regimen (group No.)[a]	Days post BST-1 inoculation	Geometric mean $\log_{10} \pm$ SD coliform count in CFU/g feces on MacConkey agar containing (No. animals positive):				Animals with confirmed Str^r/Amp^r *E. coli*
		Str	Amp/Tet	Amp/Str	no antibiotic	
(1)	−7	$< 1.56 \pm 0.03$ (0/5)	ND[b]	ND	$< 1.56 \pm 0.03$ (0/5)	0/5
UC12699	0	6.18 ± 1.15 (5/5)	$< 1.71 \pm 0.01$ (0/5)	$< 1.71 \pm 0.01$ (0/5)	ND	0/5
	1	4.28 ± 1.69 (4/5)	4.52 ± 0.67 (5/5)	$< 1.72 \pm 0.03$ (0/5)	ND	0/5
	2	3.82 ± 1.34 (4/5)	1.72 ± 0.03 (1/5)	$< 1.72 \pm 0.03$ (1/5)	ND	0/5
	3	4.71 ± 0.96 (5/5)	$< 1.76 \pm 0.03$ (0/5)	$< 1.76 \pm 0.03$ (0/5)	ND	0/5
	4	4.78 ± 3.14 (3/5)	$< 1.71 \pm 0.03$ (0/5)	$< 1.71 \pm 0.03$ (0/5)	ND	0/5
	7	5.34 ± 2.07 (5/5)	$< 1.70 \pm 0.01$ (0/5)	1.70 ± 0.01 (0/5)	ND	0/5
(2)	−7	$< 1.54 \pm 0.05$ (0/5)	ND	ND	1.54 ± 0.05 (1/5)	0/5
UC12700	0	5.96 ± 0.72 (5/5)	$< 1.77 \pm 0.11$ (0/5)	$< 1.77 \pm 0.11$ (0/5)	ND	0/5
	1	4.13 ± 1.89 (5/5)	4.30 ± 0.55 (5/5)	$< 1.72 \pm 0.03$ (0/5)	ND	0/5
	2	2.71 ± 1.08 (4/5)	4.27 ± 3.45 (3/5)	$< 1.75 \pm 0.04$ (0/5)	ND	0/5
	3	2.76 ± 0.97 (3/5)	4.50 ± 3.80 (2/5)	$< 1.72 \pm 0.01$ (0/5)	ND	0/5
	4	3.23 ± 1.64 (3/5)	4.17 ± 3.39 (2/5)	2.21 ± 1.15 (1/5)	ND	0/5
	7	3.64 ± 2.02 (3/5)	4.35 ± 3.56 (2/5)	2.19 ± 1.04 (1/5)	ND	0/5
	10	6.04 ± 2.85 (5/5)	5.15 ± 0.37 (2/5)	$< 1.72 \pm 0.02$ (0/5)	ND	0/5
	18	3.70 ± 1.94 (3/5)	$< 1.73 \pm 0.04$ (0/5)	$< 1.73 \pm 0.04$ (0/5)	ND	0/5

[a] Treatments: (1) Str (100 μ g/ml) in drinking water on days −14 to −7; inoculation with UC12699 (2.4×10^8 CFU/mouse) and unsupplemented H_2O at days −7 to 0; at day 0, BST-1 (1.3×10^8 CFU/mouse); at 6 h post-inoculation, Amp (100 μg/ml) water and for the duration of the experiment. (2) Str (100 μg/ml) in drinking water on days −14 to −7; inoculation with UC12700 (1.7×10^8 CFU/mouse) and unsupplemented H_2O at days −7 to 0; at day 0, BST-1 (1.3×10^8 CFU/mouse); at 6 h post-inoculation to day +7, Amp (100 μg/ml) water; day +7 and for the duration of the experiment, plain H_2O.

[b] ND, not determined.

vided 100 μg Amp/ml of drinking water (12 of 39 mice at day 7; geometric mean count in culture positive animals = $\log_{10}$ 7.31 $\pm$ 1.43).

Because preliminary in vitro experiments showed that UC12699 and UC12700 would "grow" on Amp/Str plates in patches as an artifact of β-lactamase from BST-1 cells present in mating mixture samples spread on the primary plates, it was deemed appropriate to determine the actual antibiotic resistance phenotype and identify randomly selected colonies from these Amp/Str plates. Any colonies which were suspected of being transcipients (growth on Amp/Str plates) were confirmed by transfer to fresh plates containing Amp/Str, Amp/Tet and Str. Genus and species identification of clones from the subculture plates was accomplished by the use of API20E strips. Similar to the in vitro experiments, the predominant isolate on the Amp/Str plates was UC12699 or UC12700 growing as an artifact of carry-over of β-lactamase from BST-1. None of the colonies picked from the Amp/Str plates in the experiment summarized in Table 5 could grow upon subculture to Amp/Str or Amp/Tet containing media, but all grew on Str-containing MA.

In one experiment, an additional complication emerged. Colonies of non-*E. coli*, lactose-positive, facultative Gram-negative rods (coliforms) which were multiply antibiotic resistant were detected. These eventually became the predominant coliform species from the mice. Identification of these non-*E. coli* coliforms by API20E strips revealed that they were: *Enterobacter cloacae* (analytical profile index {API} 2305573; 43/49 isolates); *Ent. amnigenus 1* (API 0105173; 1/49); and *Citrobacter freundii* (API 3104532; I/49). In addition, four of the isolates were identified as *Providencia alcalifaciens* (API 0224000; 4/49). All of the clones (62 tested) on the secondary plates that were probed for the rbSt M4 gene were negative, implying that the strains' resistance was due to a resistance factor other than that encoded by pURA-4.

In vitro gene transfer experiments

Since conditions for transfer of DNA in the GI tract were less than optimal for BST-1, it was deemed useful to estimate the transfer frequency of pURA-4 under the more controlled environment which in vitro conditions would allow. Accordingly, we tested for direct transfer of pURA-4 from BST-1 to the Str-resistant recipient strains, UC12699 and UC12700. Additionally, we examined the potential for transfer of pURA-4 via mobilization by the conjugative plasmid, pR751 and compared pURA-4's potential for mobilization to that of pBR322 and Tn*10* in the BST-1 and BST-1C genomic background.

For pR751-mediated mobilization of pURA-4 to occur, these plasmids must be compatible in BST-1. Moreover, pR751 must be able to transfer from BST-1 to the final recipients, UC12699 and UC12700. *E. coli* C600(pR751) was mated individually with BST-1(pURA-4), UC12699, and UC12700 and their closely related derivatives. Recipient strains BST-1(pURA-4), BST-1C and BST-1C(pBR322) were selected for these matings to determine whether the frequency of pR751 transfer to BST-1(pURA-4) was affected by the presence of pURA-4 or one of the plasmids from which it was derived (i.e., pBR322). C600(pR751) was also mated with the *E. coli* recipient strains UC12699 and UC12700 as well as pURA-4 transformants of UC12699 and UC12700, to determine whether these gut-colonizing strains could act as recipients and maintain pR751. The transfer frequency of pR751 was high (10^0 to 10^{-1} transconjugants per added donor) for recipients BST-1(pURA-4), BST-1C, and BST-1C(pBR322) (data not shown). Transfer to UC12700 and UC12699, regardless of the presence of pURA-4, was also high (10^{-1} to 10^{-2} per donor) but lower than that observed for the BST-1(pURA-4) related recipients. Clearly, pR751 could be maintained with pURA-4 or pBR322 in all hosts.

In the experiments summarized in Table 6, transfer of the Amp^r determinant located on pURA-4 or pBR322 was estimated by plating mating mixtures on agar containing Amp/Str. Only the data with strain UC12699 as the recipient are shown although the results with UC12700 were similar. As observed in the in vivo experiments, lawns and patches of bacterial growth appeared on these plates when they were inoculated with low dilutions (10^0 to 10^{-2}) of mating mixtures containing Amp^r donors, such as BST-1(pURA-4), BST-1C(pR751, pBR322), and BST-1(pR751, pURA-4). This pattern of growth was an artifact of the high population density of the Amp^r donors (containing either pURA-4 or pBR322) whose production of β-lactamase permitted growth of the recipients. To get some indication whether there were any true Amp^{r}Str^r transconjugants in the mating mixtures, the lawns of growth were replica plated onto plates containing Amp/Str. For the majority of the replica plates, no growth appeared after 48 h. For the few plates that did show growth, the growth was patchy again and could not be subcultured onto agar plates containing Amp/Str. If there had been transconjugants formed that harbored pBR322, then the Tet/Str-supplemented plates that were inoculated with samples of the 10^0 dilutions of the mating mixtures also would have had lawns of growth. This was not the case. Instead, these plates contained isolated colonies of Tet^r-Str^r bacteria that, upon subculture were found also to be Tp^r but not Amp^r. Based on these observations, our conclusion was that true Amp^{r}Str^r transconjugants were not present in the 0.1-ml samples of the 10^0 dilutions of the original mating mixtures of: BST-1(pR751, pURA-4) with UC12699; BST-1(pR751, pURA-4) with UC12700; BST-

TABLE 6

Frequency of antibiotic resistance transfer from BST-1(pURA-4) related derivatives to UC12699

Donor strain and description							
		Tet^r location		Antibiotic resistance transfer frequency[a]			
Strain	pR751	plasmid	chromosome	Amp[b]	AmpTp[b]	Tp	Tet
BST-1C	–	–	+(Tn*10*)	–[c]	–	–	$<3\times10^{-10}$
BST-1 (pURA-4)	–	–	+(Tn*10*)	$<10^{-9}$	$<10^{-9}$	–	$<4\times10^{-10}$
BST-1 (pR751, pURA-4)	+	–	+(Tn*10*)	$<10^{-9}$	$<10^{-9}$	$0.20-1.0\times10^{-5}$	$0.3-9.0\times10^{-10}$
BST-1C (pR751)	+	–	+(Tn*10*)	–	–	$0.07-9.0\times10^{-3}$	$2.0-4.0\times10^{-9}$
BST-1C (pR751, pBR322)	+	+	+(Tn*10*)	$<10^{-9}$	$<10^{-9}$	$0.06-1.0\times10^{-4}$	$0.2-4.0\times10^{-9}$

[a] UC12699 is Str^r. Therefore, the antibiotic resistance transfer frequency was calculated as the number of Amp^{r}Str^r, Amp^{r}Tp^{r}Str^r, Tp^{r}Str^r or Tet^{r}Str^r CFU recovered from the amting mixture divided by the number of donor (Tet^r) CFU added to the mating mixture. Two replications of each mating were perrformed. Transfer frequencies when UC12700 was the recipient gave similar frequencies (data not shown).

[b] Values are approximations due to the necessity of replica plating of primary plates. See text.

[c] Not determined.

1C(pR751, pBR322) with UC12699; BST-1C(pR751, pBR322) with UC12700; BST-1(pURA-4) with UC12699; and BST-1(pURA-4) with UC12700. Since there were roughly 10^9 to 10^{10} donor CFU/ml in a 2-ml mating mixture and our detection limit for transconjugants in the 2-ml reaction mixture was 1 colony per 0.1 ml of a 10^0 dilution of any of these mating mixtures, our sensitivity for detecting a transconjugant was approx. $\leq 10^{-9}$. This number can be only a rough approximation of the detection limit, since the estimate was derived from replica plates of the primary enumeration plates.

To determine whether pR751 would mobilize either pURA-4 or pBR322 or Tn*10*, we set up matings between recipient strains (UC12699 or UC12700) and donor strains BST-1(pR751, pURA-4), BST-1C(pR751, pBR322), and BST-1C(pR751) obtained from matings of C600(pR751) with BST-1(pURA-4), BST-1C(pBR322) and BST-1C, respectively. Plasmid pBR322 was used as a prototype for determining potential transfer of pURA-4 because it has been documented to be mobilized by cointegrate formation with a mobilizing plasmid, F1 [19] and importantly, it has a Tet^r marker which is more selective than Amp^r in mating mixtures of bacteria with high population densities [12,28,47]. Mating experiments using BST-1C(pR751) as the donor were conducted to compare the rates of pR751 transfer in the presence or absence of pURA-4 and pBR322. Finally, BST-1(pURA-4) was mated with the recipient strains to confirm that Tet^r transfer would not occur without pR751 present. Transfer of Tp^r was detected (10^{-2} to 10^{-5} transconjugants/added donor) in all matings that had donors harboring pR751. Tetracycline resistant transconjugants were detected at very low frequencies (10^{-9} to 10^{-10}) for all matings in which both pR751 and Tn*10* was present in the donor strain (i.e., BST-1(pR751, pURA-4), BST-1C(pR751), BST-1C(pR751, pBR322)). No Tet^r transconjugants were detected ($<4\times10^{-10}$ transconjugants/added donor) when either of these two genetic elements were absent in the donor (i.e., BST-1C or BST-1(pURA-4)). Therefore, it appeared that Tn*10* was transferred and that its transfer was dependent on pR751.

One of the Tet^r transconjugants from a mating between BST-1C(pR751, pBR322) and UC12699 was found to be Tp^r and Amp^r and it had the sugar utilization profile of the recipient. Although we can not rule out the possibility of Tet^r transfer occurring as a result of Tn*10* insertion into pR751 (as described above for the other transconjugants), this event would not explain the inheritance of Amp^r. A more likely scenario in this case was that pBR322 was mobilized into this transconjugant. Although complete characterization of this transconjugant was beyond the scope of this study, it was found that plasmid DNA obtained from this transconjugant could transform *E. coli* strain HB101 to yield Amp^{r}Tet^r transformants. Ampicillin resistant transformants were obtained only from the Tet^{r}Str^{r}Tp^{r}Amp^r transconjugant of the BST-1C(pR751, pBR322) UC12699 mating. All of the Amp^r transformants were Tet^r ($n=42$). Of these, seven were Tp^r. No Amp^r

transformants were obtained from any of the transformations involving the other transconjugants, regardless of the antibiotic selection for the transformant. The success in transforming HB101 to these various antibiotic resistances would support the conclusion that the transconjugants were truly resistant to the antibiotic and that the genes encoding antibiotic resistance were likely to be located on plasmid DNA.

DISCUSSION

For efficient transfer of recombinant DNA in vivo in the GI tract, BST-1 must be able to colonize that environment in high numbers at least transiently [41]. However, BST-1 exhibited a limited ability to persist in the GI tract of mice. Even with Tet supplementation in the water of the mice to apply selective pressure for its colonization, BST-1 was shed for only a short time period in the fecal pellets of mice. Shedding peaked within 6 h of inoculation and declined steadily to detection limits within 7–14 days post-inoculation. There were no significant differences in the numbers of BST-1 and BST-1C in fecal pellets of the two treatment groups. However, the patterns of shedding of BST-1 and BST-1C were different, in that BST-1 was eliminated from the gut tract significantly more rapidly than BST-1C.

The reason for the discrepancy between the concentrations of Tet in the feces found in this study (mean concentration approx. 68 ppm) and that of Corpet et al. (mean concentration 12.3 ppm) [10] is unclear. It is suspected, however, that these differences may have been related to Corpet's use of gnotobiotic mice vs. our use of conventional mice. It is also possible that mouse strain, dietary differences, or daily water consumption differences were responsible. Why the Tet did not reduce the *E. coli* population more dramatically is unclear since these indigenous bacteria were inhibited by the 12.5 μg Tet/ml present in the antibiotic supplemented MA (Table 1). Our studies showed that this concentration of Tet in the water resulted in Tet levels in the feces in excess of 30 ppm for all group 1 mice, while the MIC of Tet for the mouse gut *E. coli* was found to be 8 μg/ml (data not shown). It is obvious that ppm of antibiotic in the feces is not an accurate refection of Tet's antimicrobial activity in the gut.

Although an in vivo genetic transfer event is unlikely to occur with BST-1 as the donor, the objective of subsequent studies was to determine whether the gene encoding rbSt would be transferred to *E. coli* strains UC12699 or UC12700 colonizing the GI tracts of mice. The mice were pre-colonized for 1 week with the Str-resistant *E. coli* strains and then challenged with BST-1. The mice also were fed Amp in the water beginning shortly after challenge with BST-1 to provide selective pressure for pURA-4 transfer, since β-lactamase is encoded by pURA-4. Only in the mice in which Amp was added to the drinking water at 100 μg/ml was there a suggestion that persistence of BST-1 would occur. In the occasional animals in which colonization of BST-1 exceeded 10^7 CFU/g, technical difficulties occurred in detecting Amp^r transcipients due to overproduction of β-lactamase (generated from the pURA-4) which in turn protected otherwise Amp sensitive strains from this antibiotic. Ampicillin resistance was the only selectable phenotype that could be used to detect pURA-4 transfer.

From both the in vivo and in vitro studies it was clear that a number of aspects of the genotype of BST-1 worked against its use in a murine GI tract model for detection of pURA-4 transfer. First, BST-1 did not consistently colonize the GI tracts of CD-1 mice in appreciable numbers, regardless of the selective antibiotic pressure that was applied to enhance persistance. BST-1 was derived from *E. coli* K-12 (ATCC 23716) which had had both the bacteriophage λ and the fertility factor removed for BST-1 construction. Repeatedly, other investigators have shown that *E. coli* K-12 strains and their derivatives lack the ability to colonize the GI tracts of various animals, including mice and humans [2,5,21–24,36,37,48]. This was corroborated by most of the persistence experiments for BST-1. Secondly, the pURA-4 plasmid is nonconjugative [6,44]. It was derived from fragments of plasmid DNA which did not contain sequences necessary either for its own self-transmission or that would permit efficient mobilization by a transmissible plasmid that might enter BST-1 if it were able to persist in the gut. Thus, it was not surprising that in vitro transfer of the pURA-4 plasmid from BST-1 to UC12699 or UC12700 was not detected. Finally, for those animals in which colonization was appreciable ($> 10^7$ CFU/g) technical difficulties arose which precluded detection of true transconjugants due to the cross-protection by β-lactamase. The Amp^r marker was of little use as a selectable marker for detection of low frequency genetic transfer events ($< 10^{-5}$ per BST-1{pURA-4} donor) because the β-lactamase produced by the pURA-4 permitted the otherwise Amp-sensitive recipients in these matings to grow in the presence of Amp added to the selective media. This resulted in high backgrounds of false-positive growth on plates of mating mixtures [12,47] and prevented detection of low level populations of true Amp^r transconjugants.

Because of the difficulty in establishing high populations of BST-1 in vivo in the mouse model, the potential for pURA-4 transfer was examined in vitro so that the ratios of donor to recipient cells could be more easily manipulated to increase the likelihood of detecting a pURA-4 transfer event. Although pURA-4 is nonconjugative, it is conceivable that, in the gut, a self-transmissible

plasmid might enter a BST-1 cell and mobilize the pURA-4 plasmid to a potential recipient. Therefore, in vitro studies were conducted to identify the frequency with which pURA-4 could be mobilized.

Efficient mobilization of a nonconjugative plasmid is dependent on the presence of mobilization gene products and functional *nic* and *bom* genetic loci on the nonconjugative plasmid [7,15]. Plasmid pURA-4, however, does not contain functional *nic* and *bom* genetic loci. However, if the self-transmissible plasmid were to carry a transposable genetic element and entered the cell, pURA-4 could be transferred as a cointegrate with the self-transmissible plasmid [4,8,47]. Accordingly pR751 was selected for study of possible pURA-4 mobilization because it is promiscuous, i.e., it can transfer (at varying frequencies) to a number of bacterial genera and species [34,35]. It encodes two transposons, Tn*4321* and Tn*402* [35], which increase the possibility of pURA-4 transfer by cointegrate formation with pR751. The frequency of pR751 transfer into all of these strains was high, confirming that the pR751 plasmid could enter the strains used in the in vivo studies in mice. It also confirmed that the host bacterial strains, BST-1, BST-1C, UC12699 and UC12700, could carry pR751 and either one of the nonconjugative plasmids (pURA-4 or pBR322) simultaneously. Thus, no plasmid stability, plasmid incompatibility or DNA restriction barriers were evident among any of the plasmids or hosts used in these studies.

When one of the transconjugants resulting from a mating of C600(pR751) and BST-1 {BST-1(pURA-4, pR751)} was used as a donor in matings with UC12699 or UC12700, the Tp^r encoded by pR751 was transferred at readily detected frequencies. Although pURA-4 mobilization did not occur at detectable levels in these matings, the Tet^r determinant was transferred consistently at low frequencies. The Tet^r transfer was most likely due to a transpositional event involving Tn*10* insertion in the conjugative pR751 plasmid and subsequent transfer to the recipient strains by conjugation. The Tet^r transconjugants were invariably Tp^r (an unselected marker for these transconjugants) and carried a plasmid whose electrophoretic migration in agarose was characteristic of pR751 (data not shown). Furthermore, in matings with various derivatives of BST-1 used as donors (i.e., BST-1 derivatives with/without pR751, pURA-4, or pBR322) and strains UC12699 or UC12700 used as recipients, transfer of Tet^r was observed only when both pR751 and Tn*10* were in the donor strain and was not dependent on the presence of pURA-4 or pBR322. The conclusions from these experiments were that Tn*10* could be mobilized at very low frequency (10^{-9} to 10^{-10} transconjugants/donor) to *E. coli* strains UC12699 and UC12700 isolated from mouse feces. Only one of the Tet^r transconjugants obtained from matings of BST-1C(pR751, pBR322) with UC12699 was also Amp^r. It appeared to be a transconjugant that resulted from mobilization of pBR322. Thus, the transfer frequency of pBR322 was at or below the detection limits of in vitro conjugation ($<10^{-9}$ transconjugants/donor). No transfer of pURA-4 was ever detected.

Because pURA-4 is nonconjugative and nonmobilizable, it seems highly unlikely that the mouse model could be used to detect direct transfer of pURA-4 in particular without using tremendous numbers of animals. Moreover, transfer of the nonconjugative cloning vector, pBR322 and its derivatives has been difficult to demonstrate in the mammalian intestine by other investigators without the use of vigorously colonizing, non-K-12 strains of *E. coli* [5,16,21–24,27,36,41]. Transformation or transduction would appear to be even less likely mechanisms by which pURA-4 transmission might occur in the GI tract. Transfer of large conjugative plasmids by generalized bacteriophage transduction has been demonstrated [26], but this type of genetic transfer of pURA-4 is unlikely in a setting other than the laboratory. Unless it were somehow inserted into the chromosome, the 5.6-kb pURA-4 is too small to be packaged by known transducing phages [26]. In cases where small cloning vectors have been transduced by phages, either there were sequences encoded by the plasmid that were homologous to phage DNA, a mutant phage mediated the transduction or concatemers of the cloning vector were needed [26,29,33,42].

In vivo transfer of pURA-4 by natural transformation (i.e., DNA uptake by a cell as a part of its normal physiology [39]) also would appear to have a lower frequency than pURA-4 transfer by conjugation. Natural transformation of bacteria has been demonstrated to occur at low frequencies in aquatic and terrestrial ecosystems [11,30,40]. Bacteria which take up DNA as part of their normal cell physiology include certain species of *Bacillus*, *Streptomyces*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Neisseria*, *Achromobacter*, *Methylobacterium*, *Synechoccus*, *Azotobacter*, *Micrococcus* and *Mycobacterium*, *Streptococcus* and *Haemophilus* [39]. Of these, only *Streptococcus sanguis* is present in the oral cavity of the GI tract and is infrequently isolated from the colon [14,18]. *E. coli* is not an organism that is naturally competent for DNA uptake. Rather, it must be subjected to temperatures near 0 °C and exposure to divalent cations to ensure competency. Thus, transfer by transformation would most likely occur by intergenus transfer. Because natural transformation (unlike conjugation or transduction) involves uptake of extracellular DNA, the presence of DNA-hydrolyzing bacteria in the GI tract could preclude any high efficiency transformation in this densely populated ecosystem. From this viewpoint, it follows that, among these three modes of

genetic transfer, the most likely mechanism of pURA-4 transfer that might occur in the gut would be by conjugation, especially since conjugative plasmid transfer has been demonstrated in many ecosystems [39,41]. Based on our observations that pURA-4 transfer by conjugation was not demonstrable in the laboratory, there is even less likelihood that such transfer by transduction or transformation should be detectable in the mouse model.

In summary, BST-1 did not persist in the GI tract of a mouse under normal conditions or when Tet selection pressure was used, although the strain did persist in some animals under high-level Amp selection pressure. In addition, transfer of pURA-4 from BST-1 to other strains of *E. coli* could not be demonstrated either in vitro or in vivo. Based on the inability to detect transfer in vitro under optimal transfer conditions even in a facilitated triparental mating system, we are justified in saying that transfer of pURA-4 from BST-1 in vivo is highly unlikely.

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