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Escherichia coli K12 does not colonize the gastrointestinal tract of Fischer-344 rats

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SUMMARY

The colonizing potential of *Escherichia coli* K12 containing a vector coding for somidobove (bovine somatotropin) was determined. Treated male and female Fischer-344 rats were given a single oral gavage inoculum of sucrose with/without tetracycline (15 μg/ml). Untreated control animals received similar drinking water regimes. All animals survived until termination. There were no clinical signs of toxicity observed and no treatment-related effect upon body weight, food consumption, or efficiency of food utilization. Fresh fecal samples were collected from each rat every 24 h following inoculation and the population of the marked strain was quantitated until no bacterial colonies were observed for two consecutive days. While all inoculated rats were positive at 24 h, by 72 and 96 h all had become negative for the test (marked) strain, as were the corresponding control group throughout the test. The frozen stock of the marked strain used as the positive control demonstrated that the agar plates were selective for the test strain. Fourteen days following inoculation, all groups of rats were killed and the gastrointestinal tracts removed and treated to recover the marked strain. There was no evidence of the marked strain in the gastrointestinal tract of any rat from any group. Thus, the *E. coli* K12 host/vector system used in this experiment does not colonize the gastrointestinal tract of Fischer-344 rats.

INTRODUCTION

The original *E. coli* K12 strain was isolated over 60 years ago from the feces of an in-hospital patient. The strain has been cultured more or less continuously since then and has become a microbial genetics workhorse since Tatum and Lederberg began using the strain 45–50 years ago [12]. The original strain has been used in many labs, and has been repeatedly mutated to a large number of novel genotypes. An excellent attempt has been made by Bachman [1] to identify and trace the lineage.

The strain of *E. coli* K12 we have used for the production of BST, RV308, was first identified as such in a publication by Mauer et al. [7] in 1980 and is genetically distinct from its predecessors. RV308 is now in common use in many laboratories around the world. In spite of the containment prepared for the production of BST [8,9], which included a contained fermentation system and a contained heat inactivation system, some concerns were expressed about the safety of the host/vector system we contemplated using.

A comparison of many of the characteristics which might differentiate an enteropathogenic strain of *E. coli* from either *E. coli* K12 or more specifically *E. coli* K12 strain RV308 is seen in Table 1. More generally, Gorbach [3] has sited six criteria for pathogenicity in a microorganism. The microorganism must: (i) survive outside the host; (ii) have a mechanism for penetrating the mucosal surface of the bowel; (iii) be able to systemically spread within the host; (iv) be able to multiply within the host; (v) be resistant to host defense mechanisms; and (vi) produce toxin(s). He concludes that *E. coli* K12 is more or less deficient in all six areas. Freter [2] additionally has pointed out that deficiency in any one of these six areas is probably sufficient to render the microbe avirulent. With this information, we concluded that the RV308 was a safe, non-pathogenic host suitable for use with a variety of vector systems.

The more specific literature and our own early data on colonization of *E. coli* K12, in general, and *E. coli* K12 strain RV308, in particular, seemed to be just as conclusive. Levy et al. [5] fed *E. coli* K12 (both with and without plasmid pBR322) to mice and human volunteers and was unable to recover the test organism from feces and concluded that no colonization had taken place. Smith [11] showed that thy$^-$ mutants (RV308 is thy$^-$) are significantly less hardy than the prototrophic parent. Thus, they disappear sooner from the recoverable fecal spectrum

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TABLE 1

Comparison of a typical enteropathogenic strain of *E. coli* with *E. coli* K12 and RV308

Trait	Pathogenic strain	Nominal K12	RV308
1 K12 restriction/ modification	−	+	+
2 Galactose util.	+/−	+/−	−
3 Lactose util.	+	+/−	−
4 Thiamine auxotrophy	−	−/+	+
5 Lambda lysogeny	+/−	+/−	−
6 R or F (fertility)	+	+/−	−
7 Surface antigen (K-1)	+	−	−
8 Lipopolysaccharide production (O-ant.)	+	−	−
9 Enterotoxin production (inc. Shiga-like toxin)	+	−	−
10 Colicin production	+/−	−	−

of microorganisms, making colonization even less likely. Marshall et al. [6] reported that when a K12 strain was used in intestinal survival studies and it contained a plasmid with a Tc resistance marker, the number of recoverable cells did not go up when oral tetracycline was administered. They concluded that the K12 strain was indeed under a strong selective disadvantage. In two early experiments completed at Lilly using RV308 containing a derivative of plasmid pBR322 in both normal and gnotobiotic mice and rats, complete clearing of the test organism occurred in all cases after GI inoculation of approximately 10^{10} viable cells in milk within a few days.

In spite of the wealth of data available in the literature and our own early experiments, there was still some question about the potential for colonization of the GI tracts of mammals with a production host/vector system after accidental ingestion. Human volunteers would have been the ideal mammalian host; however, this was not feasible. The rat was chosen as the host since *E. coli* are part of its normal intestinal flora [4,10]. Furthermore, Ruijs and van der Waaij [10] demonstrated that exogenous *E. coli* could colonize the GI tract of rats. Therefore, rats were a suitable model for colonization determinations of *E. coli*-based recombinant host/vector systems. Thus, we set out, in a GLP, QA-audited study, to determine rigorously whether our production host/vector system could colonize the intestinal tract of Fischer-344 rats.

MATERIALS AND METHODS

Host and vector

The host, RV308, is genetically streptomycin resistant and is unable to grow on lactose. The vector is a reasonably low copy derivative of the col E1 replicon and pBR322, is mob$^-$ and in addition to the gene encoding the BST molecule, contains a selectable tetracycline resistance marker (Fig. 1). The combination of this host and vector have been designated ELBGH for the purpose of this study.

Test animal

Fischer rats (344/Crl) were separated by sex, caged in groups of five to seven animals, and acclimated for 15 days prior to study initiation. After acclimation, rats were randomly distributed to test groups according to weight with no more than a 10% difference between individual body weights within and between dose groups. Sick or deficient animals were not assigned to the test. Each animal was assigned an ID number to identify sex and dose level. The rats were approximately 6–7 weeks old at the initiation of the study. The mean body weights ($\pm$ SD) were 154.1 ± 8.2 g for males and 117.8 ± 4.2 g for females.

Test animal housing and care

The rats were individually caged in ventilated units and were allowed free access to a standard mash diet. Drinking water containing 5% sucrose or 5% sucrose plus 15 μg/ml tetracycline was continuously available to the animals. The animals were maintained in a temperature and humidity controlled room with a photoperiod of 12 h light and 12 h dark.

Treatment groups and study duration

This study consisted of two control groups and two host/vector treatment groups, with each control group having five rats per sex and each treated group having ten rats per sex. The treatment period was started 3 days prior to inoculation. The study ran a total of 14 days after the oral administration of the culture suspension. Treatment groups 1 and 3 were administered sucrose water (5%) ad libitum during the entire study. Groups 2 and 4 were administered sucrose water (5%) containing tetracycline HCl

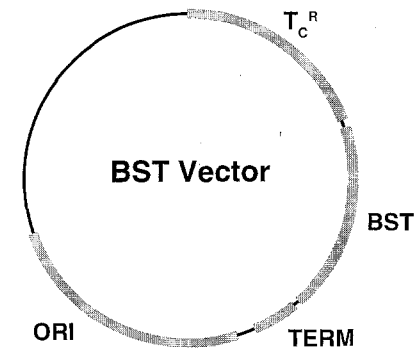

Fig. 1. Vector used in the production of bovine somatotropin.

(15 μg/ml) ad libitum during the entire study. Groups 3 and 4 were administered the oral culture suspension.

Preparation and administration of ELBGH

The culture of ELBGH used for the inoculation of Fischer-344 rats in this study was started from a frozen (−80 °C) stock by inoculating fresh L-broth containing both dextrose (0.2%) and tetracycline HCl (5 μg/ml) with 1% of the stock. After incubation at 30 °C the cells were centrifuged at 25 °C and resuspended in homogenized vitamin D milk at a cell concentration of 5×10^9 to 5×10^{10} bacteria per ml. The actual viable cell counts was 1.6×10^{10} per ml. The one time dose given via oral gavage to each rat in groups 3 and 4 was 0.5 ml containing 8.0×10^9 cells.

Survival and clinical signs

Test animals were examined daily for general physical condition and behavior. A detailed examination was performed two times each week in which muscle tone, condition of pelage, color and appearance of eyes, respiration, posture, excreta, locomotion, and the presence of external lesions or growths were evaluated.

Body weight and food consumption

Test animals were weighed and food consumption was determined twice weekly. An efficiency of food utilization value was determined using these data.

Fecal sample and gastrointestinal tract assay for ELBGH

Fresh fecal samples were collected daily from all rats directly into individual collection tubes for quantitative bacterial (ELBGH) enumeration following inoculation with the host/vector. Fecal samples were collected until two consecutive negative isolations were obtained per animal. At the termination of the study, all animals were killed and the gastrointestinal tract was collected and submitted for quantitative bacterial isolation of ELBGH. Each sample was weighed and diluted in dilute peptone broth containing 0.1% Triton X-100. Dispersal of the fecal material without killing the bacteria present was effected with an ultrasonic cleaning bath. The gastrointestinal tract was cut into two approximately equal sections and each was diluted with diluent and ground in a Ten Broeck tissue grinder. The halves were recombined and diluted appropriately.

EMB selective plates

To be able to pick out the test organism, a differential and highly selective agar medium was designed and used. EMB basal agar medium fortified with 2% glucose, tetracycline (10 μg/ml), and streptomycin (25 μg/ml) was prepared. ELBGH readily grew out on this medium within 18–24 h, producing typical flat dark colonies with a green sheen, while most of the other microorganisms present were selected against by the use of two antibiotics. A few non-test organisms were recovered as small pink shiny colonies. Dilutions of the appropriate samples were plated in triplicate and averaged as an estimation of number of viable ELBGH cells present in each sample. Positive and negative controls were provided at each time point and consisted of the diluted original frozen culture and the diluent (containing no sample), respectively.

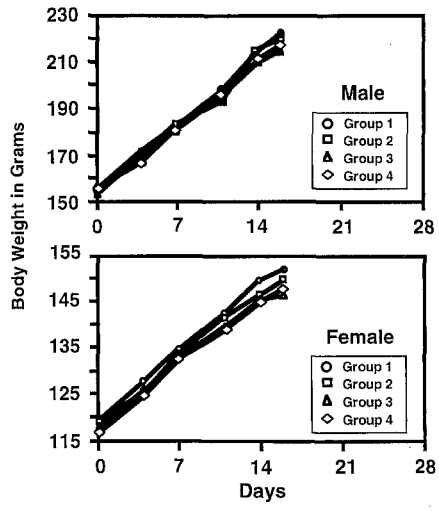

Fig. 2. Mean body weights for groups 1–4 separated by sex.

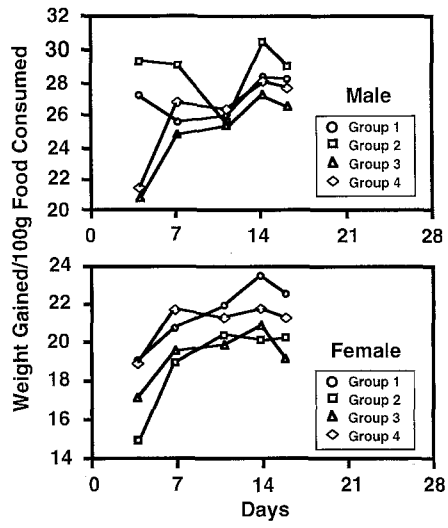

Fig. 3. Mean cumulative efficiency of food utilization for groups 1–4 separated by sex.

RESULTS AND DISCUSSION

Survival and clinical signs

All animals survived to necropsy and showed no clinical signs of toxicity at any point in the study.

Body weight, food consumption, and feed efficiency

Body weights are summarized in Fig. 2. Mean body weights and mean body weights gains were similar between control and inoculated animals. The average amount of food consumed on a cumulative basis was not significantly different between treatment groups. The efficiency of food utilization (EFU) expressed as grams of body weight gained per 100 g of feed is shown in Fig. 3. Although there is some scatter, there was no significant difference between treatment groups.

Fecal and intestinal culture

Starting at 24 h after inoculation, fecal samples were collected and the presence or absence of ELBGH was quantitated. All control groups (1 and 2) at every time point showed no evidence of ELBGH growth on the EMB selective plates (Table 2). The positive and negative controls showed the corresponding presence or absence of ELBGH as expected.

The ELBGH inoculated groups (3 and 4) all clearly showed the presence of the host/vector at 24 h after inoculation (20/20) (Table 2). In these rats the number of marked cells recovered ranged from 8 per mg to 1.01×10^7 per mg. There did not appear to be any correlation between the number of selected cells per mg feces, the weight of the feces, or the presence of tetracycline in the drinking water. At 48 h after inoculation, 32 of the 40 inoculated rats showed no evidence of ELBGH in the feces (Table 2). Eight of these 40 rats were still positive for ELBGH, with one of these in group 3 and seven in group 4. At 72 h following inoculation, all rats were negative for ELBGH, including the eight which were still positive at 48 h. Therefore, for 32 of the 40 inoculated rats the criterion of two consecutive negative was met. For those eight rats which were positive at 48 h and for the corresponding control rats, fecal samples were obtained at 96 h and plated to determine the presence or absence of ELBGH. These rats were all negative for ELBGH, and thus the criteria for two consecutive negative samples was met.

TABLE 2

Recovery of ELBGH from rat feces as a function of time after inoculation

Treatment group	Hours post-inoculation			
	24	48	72	96
1	0/10*	0/10	0/10	0/10
2	0/10	0/10	0/10	0/10
3	20/20	1/20	0/20	0/20
4	20/20	7/20	0/20	0/20

* Number of rats from which ELBGH was recovered in feces over the total number of rats in treatment group.

TABLE 3

Recovery of ELBGH from the entire GI tract of rats at termination of experiment

Group No.	Average weight GI tract (g)	Colony count	ELBGH/g GI tract
1	12.01	0	0
2	11.89	0	0
3	11.48	0	0
4	11.78	0	0

It did seem that the tetracycline treatment attenuated the shedding of ELBGH since seven of those rats which shed for 48 h were from group 4. However, those rats which shed for 48 h were not necessarily the rats which had the highest marked cell count after 24 h, which increased the likelihood that the initial observation was coincidental.

After two consecutive negative days, fecal samples were no longer collected; however, the rats were maintained on sucrose water or sucrose-tetracycline water until the 14th day following inoculation. At that time the rats were euthanized with CO_2 and the entire gastrointestinal tract was removed. The presence or absence of ELBGH in each GI tract was quantitated using the EMB-selective plates. All control groups (1 and 2), as well as all ELBGH inoculated groups (3 and 4), showed no evidence of ELBGH (Table 3).

In summary, this study demonstrated that 8.0×10^9 viable ELBGH, when given as a single oral gavage, did not colonize the gastrointestinal tract of Fischer-344 rats. Furthermore, pretreatment and maintenance of rats on tetracycline in the water did not provide an environment in the gastrointestinal tract which resulted in colonization of ELBGH. These results corroborate previous work which indicated that the parental strain of RV308, *E. coli* K12 and *E. coli* K12 carrying derivatives of pBR322, were not able to be recovered from the feces of mice, rats, and humans [5,6,11].

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