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Fate in water of a recombinant *Escherichia coli* K-12 strain used in the commercial production of bovine somatotropin

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SUMMARY

The fate in water of *Escherichia coli* K-12 strain LBB269, both plasmid-free and carrying the recombinant plasmid pBGH1, was studied. *E. coli* K-12 strain LBB269 (pBGH1) is a nalidixic acid resistant derivative of W3110G (pBGH1), the microorganism used by Monsanto Company for the commercial production of bovine somatotropin. Water samples were obtained from the Missouri River and from the Monsanto Life Sciences Research Center aqueous waste basin. Strains LBB269 and LBB269 (pBGH1) were grown in fermentation vessel under bovine somatotropin (BST) production conditions, and inoculated into the water samples. The inoculated water samples were incubated at 26 °C, and the number of viable *E. coli* cells was determined as a function of time. In sterile water from both sources, the two strains remained at a constant level for at least 28 days; LBB269 (pBGH1) remained at a constant level in sterile water for at least 300 days. In non-sterile water from both sources, the two strains declined from an initial concentration of about 3.0×10^6 cells per ml to less than 10 cells per ml in 147 h. The study conditions did not adversely affect the populations of indigenous microorganisms. The selective loss of strains LBB269 and LBB269 (pBGH1) demonstrates that these *E. coli* strains do not survive in environmental sources of water. In addition, it was observed that the presence of pBGH1 had essentially no effect on the disappearance of strain LBB269 from either source of water.

INTRODUCTION

Escherichia coli K-12 strains have been used extensively as hosts for recombinant plasmids, both for research as well as commercial purposes. The existence of an extensive data base on the physiology, biochemistry, and genetics of *E. coli* K-12 have made it popular with microbial genetic engineers. Additionally, the fact that *E. coli* K-12 strains cannot colonize the human intestinal tract [18–20,27,32] has led to approval of their use in recombinant systems. Typically, commercial production of proteins from recombinant strains of *E. coli* involves large-scale fermentation of a strain containing an expression plasmid. These industrial fermentations range in size from hundreds of liters to tens of thousands of liters, with culture densities attaining 1×10^9 to 1×10^{10} cells per ml [4,13]. The prospect of up to 1×10^{17} (or more) recombinant *E. coli* cells being inadvertently released into the environment during some type of catastrophic industrial accident has ignited considerable interest in the determination of the likely consequences of such an event. Such interest has centered on whether recombinant *E. coli* K-12 strains can survive in natural environments and whether the recombinant plasmid they contain can be transferred to indigenous inhabitants of these environments. The natural environments usually studied are water, soil, sewage, and the mammalian intestinal tract.

The fate of *E. coli* K-12 strains in natural sources of water has been examined by a variety of investigators [8,11,23,25], although none of these studies employed strains actually used for commercial production purposes. It was determined that *E. coli* K-12 strains remained viable in sterile water samples for as long as 200 days. In non-sterile water, these strains lost their viability in 15 days or less. The presence of a conjugative or non-conjugative plasmid did not affect the survival of the host in either sterile or non-sterile water. These studies have led to the suggestion that indigenous aquatic microorganisms subject *E. coli* K-12 strains to predation, parasitism, lytic enzymes and toxins [11].

E. coli K-12 strain W3110G [1] containing the pBR322-based plasmid pBGH1 [24] is used by Monsanto Company for the large-scale production of bovine somatotropin (BST) [4,13]. Although a number of systems have been designed to prevent the escape of this recombinant microorganism from fermentation areas, it was of interest to determine the fate of W3110G (pBGH1) in environments outside the production plant. This study

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documents the fate in water of this BST production strain, and as such is the first publication describing the fate in the environment of a recombinant *E. coli* K-12 strain used commercially.

MATERIALS AND METHODS

Media and reagents

Levine eosin Methylene Blue (EMB) agar and plate count agar media were obtained from Difco Laboratories (Detroit, MI). When prepared according to the instructions of the supplier, EMB agar medium contained 10 g of peptone, 10 g of lactose, 2 g of dipotassium phosphate, 15 g of bacto agar, 0.4 g of eosin Y, and 0.065 g of methylene blue/l; plate count agar medium contained 5 g of tryptone, 2.5 g of yeast extract, 1 g of glucose, and 15 g of bacto agar/l. For this study, the plate count agar was referred to as tryptone-yeast extract-glucose (TYG) agar. The antibiotic nalidixic acid (Sigma Chemical Co., St. Louis, MO) was added to EMB medium to a final concentration of 200 mg per l; this medium was referred to as EMBN. L-broth contained 10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and 1 g of glucose/l [17]. L-broth agar plates (LB plates) were prepared from L-broth containing 15 g bacto agar/l. Bottles of sterile 0.1% peptone water were obtained from Fisher Scientific (Pittsburg, PA).

Collection of water

The Missouri River water was collected at river kilometer 86, a point about 19 kilometers upstream of the Monsanto Life Sciences Research Center in Chesterfield, Missouri. The collection site was within the Weldon Spring Wildlife Area, public land owned and operated by the state of Missouri. This site was chosen both because of ease of public access and because this section of the Missouri River is undeveloped. The water was collected in sterile 1-l wide-mouth bottles by dipping them into the river. 4 l of river water were collected in this manner, tightly capped, and brought back to the laboratory. There the bottles of water were wrapped in aluminum foil to keep light out, stored in a 4 °C-refrigerator overnight, and used the next day.

On the grounds of the Monsanto Life Sciences Research Center is an aqueous waste basin, termed an equalization basin. This basin is a 1 200 000 liter open air cement pond with a diameter of 18.6 meters and a water depth of 4.4 meters. The equalization basin holds all of the non-sewage aqueous liquids from the research facility for 8 to 14 h. This holding pattern is designed to achieve pH equalization of the water prior to discharge into municipal sewage lines. The outflow from the equalization basin is mixed with the sanitary stream from the research facility, and the mixed streams enter the municipal sewage system. Water was collected from the equalization basin outflow stream, prior to the point at which the outflow was mixed with the sanitary stream. The water was collected in sterile 1-l wide-mouth bottles by dipping them into the outflow stream. 4 l of equalization basin water were collected in this manner, tightly capped, and brought back to the laboratory. There the bottles of water were wrapped in aluminum foil to keep light out, stored in a 4 °C-refrigerator overnight, and used the next day. Both of these water samples were collected in the late autumn, before any cold weather had occurred.

Water analysis

1-l samples of water from the Missouri River and the equalization basin were sent by overnight express mail to A&L Agricultural Laboratories (Memphis, TN), for analysis. Components measured included pH, total dissolved solids, total nitrogen, total organic carbon, and 12 standard inorganic ions. Indigenous species of bacteria in these water samples were identified using the Vitek AMS identification system (St. Louis, MO). Bacterial isolates which the Vitek system could not positively identify were sent to Five Star Laboratories (Branford, CT) for identification by gas chromatographic analysis of fatty acids using the MIDI Microbial Identification System (Newark, DE).

Growth of E. coli K-12 strains

E. coli K-12 strains LBB269 and LBB269 (pBGH1) were grown in 15-l Biolafitte fermenters containing a medium comprised of: 50 g ammonium sulfate, 60 g dibasic potassium phosphate, 30 g monobasic sodium phosphate, 4.8 g magnesium phosphate, 125 g glucose, 0.7 g thiamine hydrochloride, 54 mg ferric chloride, 4 mg zinc sulfate, 7 mg sodium molybdate, 8 mg copper sulfate, 2 mg boric acid, and 5 mg manganese sulfate in 9 l of deionized water [1]. Each fermenter was inoculated with a 1-l L-broth culture of *E. coli* which was at a density of about 3×10^8 cells per ml. The fermenters were maintained at 37 °C; the pH was maintained at 7.0 by the controlled addition of concentrated ammonium hydroxide. Vigorous aeration was maintained to keep the dissolved oxygen at a level of at least 30% of saturation. Glucose was fed at a controlled rate from a 50% stock solution beginning at an optical density of 10–15 (determined spectrophotometrically at 550 nm) to maintain a glucose level of 0.25%. When the optical density was 20–25 (corresponding to about 1×10^{10} cells per ml), high-level synthesis of BST in the strain LBB269 (pBGH1) was induced by the addition of indole acrylic acid to a final concentration of 25 mg/l [1,4,13]; LBB269 was treated in an identical manner as a mock induction.

10 h after induction, 50-ml aliquots from each of 2 independent fermentations of LBB269 and 2 independent fermentations of LBB269 (pBGH1) were placed at 4 °C. These aliquots were termed I_{final} samples. The cells so collected were used within 10 h to inoculate the water samples.

Inoculation and incubation of water samples

The I_{final} samples from the fermentation samples were diluted prior to inoculating the water samples. 10-ml aliquots from the I_{final} samples of LBB269 (pBGH1) were added to 90 ml of the water that was to be tested, whereas 1-ml aliquots from the I_{final} samples of LBB269 were added to 99 ml of the water that was to be tested. The dilutions were done in this manner to reach approximately equal numbers of cells per ml; the I_{final} samples of LBB269 had roughly 10 times more cells per ml than the I_{final} samples of LBB269 (pBGH1).

To 300-ml Erlenmeyer flasks containing 49.5 ml of water were added 0.5-ml aliquots of the diluted I_{final} samples to give an initial concentration of about $2–5 \times 10^6$ cells per ml. Flasks containing uninoculated samples of water (50 ml) were also prepared. The flasks were placed in the dark in an incubator shaker maintained at 26 °C and 200 rpm.

Dilution and plating of water samples

The sampling time-points used in this study were 0, 8, 20, 26, 32, 44, 50, 56, 68, 119, and 147 h after inoculation for the non-sterile water samples; for the sterile water samples, the sampling time-points were every 24 h for 28 days (672 h) after inoculation. At each sample timepoint, 1-ml aliquots were removed and plated directly or diluted and plated, as appropriate. Dilution of the water samples was made using bottles containing 90 or 99 ml of sterile 0.1% peptone water. The dilutions were carried out to a point where it was estimated that plating of 0.1-ml aliquots of the diluted samples would yield 30 to 300 colonies; each dilution was plated in triplicate. To insure that plates with the desired numbers of colonies were obtained, samples from several dilutions were plated. The dilutions were plated on both EMBN and TYG plates. The TYG plates were incubated at 26 °C for 48 h and the EMBN plates were incubated at 37 °C for 24 h before the number of colonies they contained were counted. Colony counts from the TYG plates indicated the population of total aerobic microorganisms (added *E. coli* plus indigenous microorganisms), while colony counts from the EMBN plates indicated the population of added *E. coli* (the occasional non-*E. coli* colonies observed on this medium were not counted).

Statistical methods

In order to support the aim of comparing strains LBB269 and LBB269 (pBGH1), two independent fermentations of each were used in this study. All four fermentation batches were tested in both Missouri River water and equalization basin water. Uninoculated water samples were included as negative controls. At each sample timepoint, the platings of each dilution were done in triplicate. The entire study was done in duplicate.

Determination of viable count

The method of maximum likelihood [15] was used to estimate the viable counts. The Poisson and binomial models lead to the same estimate. Whenever possible, the method described by Koch [16] was used, that is: "to get the best estimate from a group of plates from the same or different dilutions of the same sample, simply add up the total counts on all the plates and divide it by the total volume of the original solution."

Data analysis

Counts were examined for consistency between plates and dilutions from the same flask using a χ^2 statistic. A mean viable count was determined for each cell type, water type, and time combination as the geometric mean of the corresponding results. This was achieved by taking the antilog of the arithmetic mean of the logarithms of the viable count estimates. This method is appropriate for a variable which is typically measured or reported on a logarithmic scale [29], and aids in stabilization of variance. All subsequent analyses for the response were done on a logarithmic scale.

The estimation of viable count for a given flask was subject to measurement variability. Other researchers have discussed how this determination is impacted by both dilution variability and plating variability [7,16,22]. Since both sources of variation are inevitably present, one would still be limited in precision by the variability of the dilution, regardless of the number of platings done. Statistical methods for the allocation of units in a hierarchical scheme are well known [5,28], and can be applied to determine the appropriate number of dilutions and plates to achieve objectives.

The dilutions were targeted to achieve counts in the 30–300 range. Plates containing numbers outside this range were not discarded, but were included in the maximum likelihood estimation procedure since it was considered that every plate provided useful information.

RESULTS

Isolation of a nalidixic acid resistant strain

Environmental sources of water contain substantial populations of indigenous bacteria. In order to facilitate

tracking of an *E. coli* K-12 strain in an environment populated with other species of bacteria a nalidixic acid resistant derivative of strain W3110G was utilized. The main advantage to using resistance to nalidixic acid as the marker is that it is rarely encountered among indigenous bacterial inhabitants of aquatic environments [26]. Nalidixic acid resistance has been used to mark *E. coli* K-12 strains for studies on their fate in river water [8], soil [6], and the mammalian intestinal tract [32,33].

E. coli strain W3110G was marked with a chromosomal mutation in the *gryA* locus [30] that resulted in resistance to nalidixic acid. An overnight L-broth culture of W3110G was plated on LB plates containing 200 μg nalidixic acid per ml of medium. This concentration of nalidixic acid was found to completely inhibit the growth of strain W3110G, and permitted selection of resistant derivatives. The plates were incubated at 37 °C for 24 h, to allow resistant cells to develop into colonies. Several of these colonies were streaked on the same medium. As all had the same morphology, one isolate was retained and designated LBB269. The mutation resulting in nalidixic acid resistance was shown by transductional analysis to be linked to the *gryA* locus (data not shown). This strain was then transformed with the plasmid pBGH1. In this manner, an isogenic pair of nalidixic acid resistant derivatives of W3110G were obtained, one containing the plasmid pBGH1 and the other being plasmid free. The use of a marked strain of *E. coli* permitted direct plating of 0.1-ml aliquots from the flasks containing the mixtures of water and cells onto the EMBN plates, since essentially all of the indigenous bacteria in the water samples were inhibited by nalidixic acid. Furthermore, on EMB-type media, Lac+ *E. coli* K-12 strains such as LBB269 form dark purple to black colonies with a distinctive metallic green sheen, permitting easy visual discrimination between colonies of *E. coli* and other microorganisms. Thus, the limit of detection for the added *E. coli* cells in water samples was approximately 10 cells per ml of water (i.e., from the counting of one *E. coli* colony forming unit in 0.1 ml of water).

TABLE 1

Compositions of Missouri River water, equalization basin water, and Vogel-Bonner medium.

Component[a]	Missouri River	Equalization basin	Vogel-Bonner medium[b]
pH	7.2	7.4	7.0
Total dissolved solids	512	744	19200
Total nitrogen	0.4	71.4	234
Total organic carbon	35.7	5.25	2690
Sodium	43	91	385
Calcium	66	21	1
Magnesium	21	19	20
Chloride	32	80	126
Sulfate	115	135	78
Nitrate	0.39	0.46	1.19
Bicarbonate	256	432	5980
Phosphorus	0.13	1.75	2300
Potassium	4	9	4500
Boron	0.17	0.16	0.11
Copper	0.05	0.09	0.04
Iron	0.83	0.12	0.27
Zinc	0.02	0.09	0.10
Manganese	0.08	0.01	0.03

[a] All of the components except pH are reported as μg/ml.

[b] Vogel-Bonner medium contains $MgSO_4 \cdot 7H_2O$ at 200 μg/ml, citric acid monohydrate at 2 mg/ml, anhydrous K_2HPO_4 at 10 mg/ml, $NaNH_4HPO_4 \cdot 4H_2O$ at 3.5 mg/ml, and glucose at 5 mg/ml (Vogel and Bonner, 1956).

Characteristics of water samples

When compared to a typical minimal medium used to support the growth of *E. coli*, both the Missouri River water and the equalization basin water contained relatively low levels of most key components required for the growth of *E. coli* K-12 (Table 1). For instance, Vogel-Bonner medium [31], commonly employed as a chemically defined medium for the growth of *E. coli*, contains much higher levels of nitrogen, organic carbon, phosphorus and potassium than either Missouri River water or equalization basin water. The two types of water contained magnesium, sulfate, and trace metals at levels comparable to those found in Vogel-Bonner medium.

Preliminary investigations revealed that water from the equalization basin inflow was essentially free of bacteria (less than 10 cells per ml). However, the equalization basin itself supports a rich bacterial population (approximately 1.5×10^6 cells per ml) and the subsurface walls of the basin are covered with a biofilm. The Missouri River water was found to contain about 4×10^3 bacterial cells per ml.

The most common indigenous species of bacteria found in the Missouri River water were (in order of abundance): *Pseudomonas fluorescens*, *P. indigoflora*, *Acinetobacter calcoaceticus* biotype *lwoffi*, *Micrococcus luteus*, and *Janthinobacterium lividum*. The most common bacteria in the equalization basin water were: *P. fluorescens*, *P. putida*, *P. stutzeri*, and *Aeromonas hydrophila*. Direct phase-contrast microscopic examination of cell suspensions from both sources of water did not reveal the presence of protozoa at any time during these studies.

Growth of LBB269 and LBB269 [pBGH1] in fermenters

The two fermentation runs of strain LBB269 (pBGH1) exhibited essentially identical growth curves, as did the

two fermentation runs of strain LBB269 (data not shown). When compared to each other, however, strain LBB269 reached higher final cell densities (1.1–3.3 × 10^{10} cells per ml) than strain LBB269 (pBGH1) (1.1–2.5 × 10^{9} cells per ml). These results probably reflect the detrimental effects the plasmid pBGH1 has on the growth of *E. coli*, particularly after induction of high-level BST synthesis. As previously described, BST accumulates in the cell within inclusion bodies [1,2,13,14]; microscopic examination revealed that over 98% of the LBB269 (pBGH1) cells from the I_{final} samples contained inclusion bodies. It is thought that formation of inclusion bodies inhibits cell division [2,13].

Dilution of cells and inoculation of water samples

Since the final cell density of LBB269 was about 10-fold higher than LBB269 (pBGH1), the I_{final} fermentation samples of LBB269 were diluted 100-fold and those of LBB269 (pBGH1) were diluted 10-fold prior to inoculating the flasks of Missouri River water and equalization basin water. This was done so that after inoculation, the flasks of water contained essentially identical populations of added *E. coli* cells. The diluent used in each case was the same water as that in the flasks to be inoculated, that is, *E. coli* cells diluted with Missouri River water were used to inoculate flasks of Missouri River water. Following this initial step, 0.5 ml of the diluted cells were used to inoculate 49.5 ml of water in the flasks. Taken together, these two dilution steps represented a 10000-fold dilution of the LBB269 fermenter samples and a 1000-fold dilution of the LBB269 (pBGH1) fermenter samples, thus minimizing any alteration of the water composition due to carry-over of nutrients from the fermentation medium.

Viable counts of LBB269 and LBB269 (pBGH1) in sterile water

Both strains LBB269 and LBB269 (pBGH1) were inoculated in Missouri River water and equalization basin water that had been sterilized by autoclaving. Viable counts of the added *E. coli* remained at the initial level of 3.0–5.0 × 10^{6} cells per ml for 28 days (672 h), at which point the experiment was terminated. In a separate study with autoclaved Missouri River water and equalization basin water, LBB269 (pBGH1) remained at the initial level of 2.0–4.0 × 10^{6} cells per ml for over 300 days, at which point the experiment was terminated.

Viable counts in non-sterile water at zero time

To determine the hour zero viable counts, samples were removed from the flasks at the start of the study and plated on TYG and EMBN plates. With the flasks of water that had been inoculated with LBB269 or LBB269 (pBGH1), the *E. coli* cells in the water samples far outnumbered the cells of indigenous bacteria. For strain LBB269, the initial population of *E. coli* ranged from 3.1–3.6 × 10^{6} cells per ml in Missouri River water and from 2.8–4.2 × 10^{6} cells per ml in equalization basin water. For strain LBB269 (pBGH1), the initial population of *E. coli* ranged from 3.0–4.3 × 10^{6} cells per ml in Missouri River water and from 3.1–3.4 × 10^{6} cells per ml in equalization basin water. These cell concentrations were about 10- to 1000-fold higher than the indigenous microbial populations in the equalization basin water and Missouri River water, respectively. In the uninoculated water samples, the initial populations of aerobic bacteria were about 4.1–5.0 × 10^{3} cells per ml in the Missouri River water and about 1.2–2.3 × 10^{5} cells per ml in the equalization basin water.

Selective loss of LBB269 and LBB269 (pBGH1) in Missouri River water

With strain LBB269 in Missouri River water, the number of viable cells remained essentially constant for the first 30 h, with equivalent numbers of colonies found on EMBN and TYG plates (Fig. 1). Subsequently, the viable counts dropped on both media, and after 147 h less than 10 viable cells of LBB269 per ml were detected by plating on EMBN plates. The correlation between the counts on TYG and EMBN was lost once the population of inoculated *E. coli* dropped below the level of the indigenous microorganisms, at about 60 h. At this point the indigenous microbes became the major fraction of the bacterial population, and remained so for the duration of

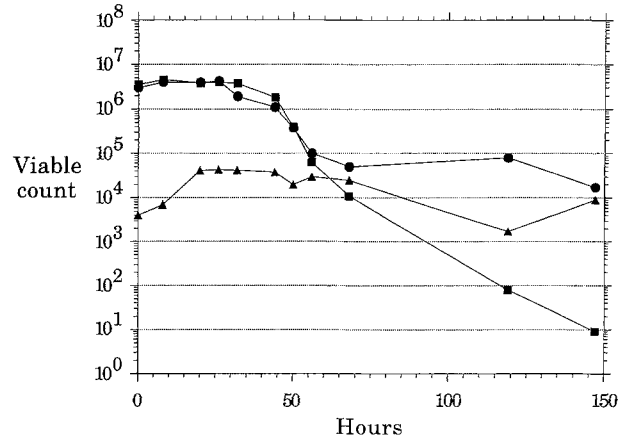

Fig. 1. Microbial counts in Missouri River water with and without added *E. coli* strain LBB269. (-■-), viable counts of LBB269 in inoculated water samples; (-●-), total aerobic viable counts (added *E. coli* plus the indigenous aerobic microorganisms) in inoculated water samples; (-▲-), viable counts of indigenous aerobic microorganisms in uninoculated water samples.

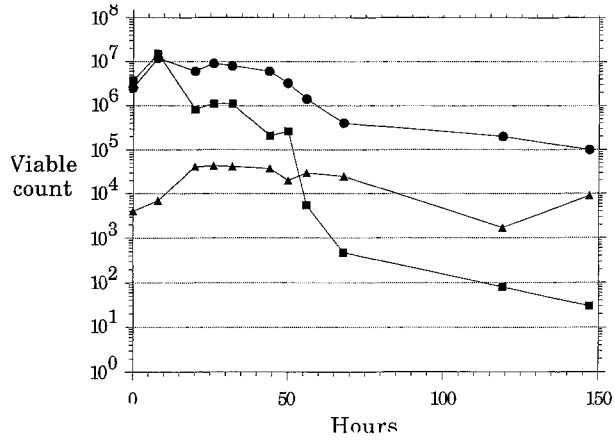

Fig. 2. Microbial counts in Missouri River water with and without added *E. coli* strain LBB269 (pBGH1). (-■-), viable counts of LBB269 (pBGH1) in inoculated water samples; (-●-), total aerobic viable counts (added *E. coli* plus the indigenous aerobic microorganisms) in inoculated water samples; (-▲-), viable counts of indigenous aerobic microorganisms in uninoculated water samples.

the experiment. The viable counts of strain LBB269 (pBGH1), shown in Fig. 2, behaved in an essentially identical manner.

Selective loss of LBB269 and LBB269 [pBGH1] in equalization basin water

The viable counts for strain LBB269 continually decreased from 8 h through 119 h (Fig. 3) after which no colonies of LBB269 were found. Similar results were

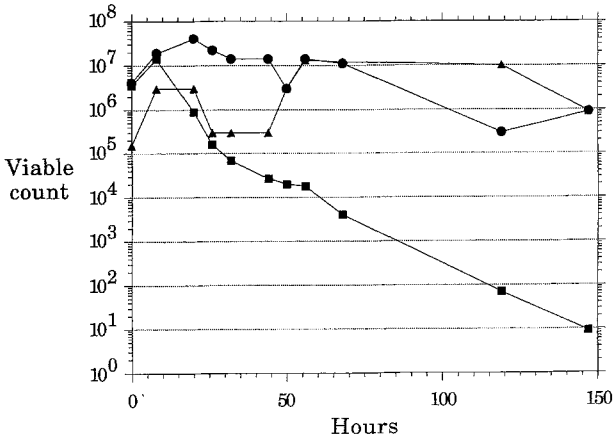

Fig. 3. Microbial counts in equalization basin water with and without added *E. coli* strain LBB269. (-■-), viable counts of LBB269 in inoculated water samples; (-●-), total aerobic viable counts (added *E. coli* plus the indigenous aerobic microorganisms) in inoculated water samples; (-▲-), viable counts of indigenous aerobic microorganisms in uninoculated water samples.

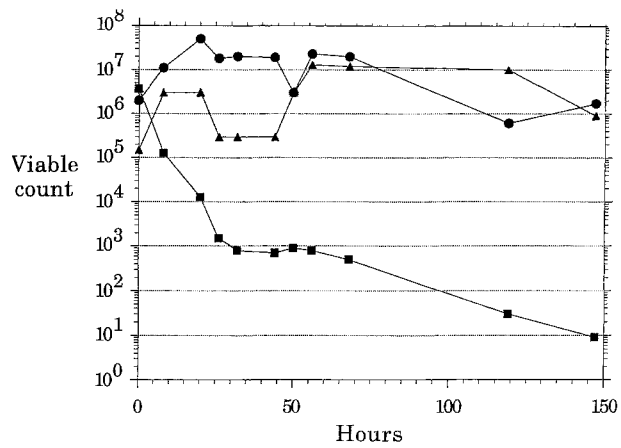

Fig. 4. Microbial counts in equalization basin water with and without added *E. coli* strain LBB269 (pBGH1). (-■-), viable counts of LBB269 (pBGH1) in inoculated water samples; (-●-), total aerobic viable counts (added *E. coli* plus the indigenous aerobic microorganisms) in inoculated water samples; (-▲-), viable counts of indigenous aerobic microorganisms in uninoculated water samples.

found with LBB269 (pBGH1) (Fig. 4). No colonies of LBB269 (pBGH1) were detected after 119 h. When compared to the results obtained with Missouri River water, it can be seen that both LBB269 and LBB269 (pBGH1) declined more rapidly in equalization basin water (Fig. 1–4).

Comparison of the plasmid-free and the plasmid-containing strains

The viable cell counts of LBB269 and LBB269 (pBGH1) in the flasks of Missouri River water and equalization basin water are shown in Figs. 5 and 6. The two strains had similar patterns of decline in both types of

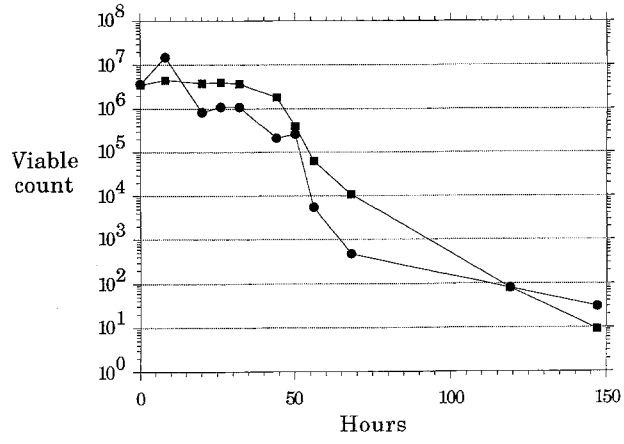

Fig. 5. Comparison of *E. coli* strains LBB269 (pBGH1) and LBB269 in Missouri River water. (-■-), viable counts of LBB269 (pBGH1); (-●-), viable counts of LBB269.

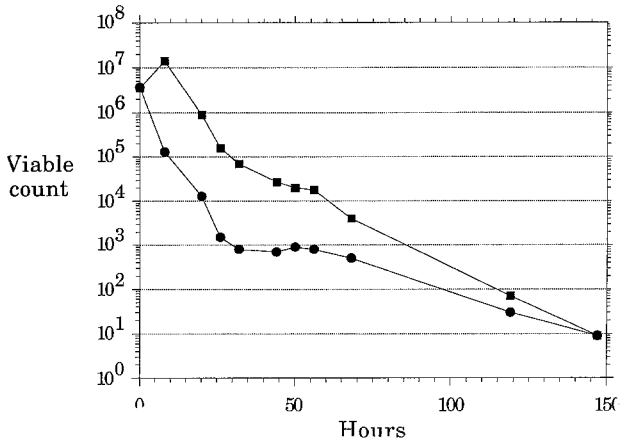

Fig. 6. Comparison of *E. coli* strains LBB269 (pBGH1) and LBB269 in equalization basin water. (-■-), viable counts of LBB269 (pBGH1); (-●-), viable counts of LBB269.

water. These results indicate that the presence of the plasmid pBGH1 had essentially no effect on the survival of *E. coli* strain LBB269 in these two types of water.

Reliability of colony counts from EMBN plates

If after prolonged incubation in non-sterile Missouri River water or equalization basin water strains LBB269 and LBB269 (pBGH1) had somehow lost the ability to grow on the selective EMBN plates, then using colony counts from EMBN plates to ascertain the viable counts of *E. coli* in the water samples would lead to artificially low results. Use of a nonselective medium such as TYG plates would circumvent this potential problem, but with samples containing high populations of indigenous bacteria it would not be possible to directly discern low levels of *E. coli* cells. To test the possibility that the EMBN plates were unreliable, five pairs of plates (TYG plates and EMBN plates) from the 56-h sampling time-point were examined. These five pairs of plates had high numbers of colonies on TYG plates, but on the EMBN plates some of them had many *E. coli* colonies while others had few or none (Table 2). A replica-plating test was employed to determine whether the *E. coli* colony counts on EMBN were an accurate reflection of the number of colonies of *E. coli* on the TYG plates. By replica-plating the colonies from TYG plates to EMBN plates, the number of colonies of *E. coli* on the TYG plates could be determined; as mentioned previously, EMB-type media permit easy visual discrimination between colonies of Lac+ *E. coli* strains and colonies of other species of bacteria. The original colony counts were similar on both the TYG and EMBN plates of the 10^3 dilutions from the 56-h samples of Missouri River water inoculated with *E. coli* from three different fermenters. When the colonies on the TYG plates were replica-plated to EMBN plates, it was found

TABLE 2

Results of replica plating colonies from TYG plates to EMBN plates

Sample ID[a]	Dilution	Colonies on TYG	Colonies on EMBN	Replica plate results
A56MO2	10^3	48	13	Almost all *E. coli*
A56MO2	10^3	>300	>300	Almost all *E. coli*
C56MO1	10^3	>300	>300	Almost all *E. coli*
D56MO1	10^3	>300	1	No *E. colli*
B56EQ2	10^4	>300	3	Few *E. coli*

[a] The sample ID code is organized as follows. The first letter refers to the fermenter, with A and B being the fermentation runs of LBB269 and C and D being the fermentation runs of LBB269 (pBGH1). The '56' refers to the sample time, in this case 56 h. The next two letters refer to the water source, with 'MO' referring to the Missouri River and 'EQ' referring to the equalization basin. The last number, 1 or 2, refers to which replicate of the experiment the sample was derived from.

that the colonies on the TYG-plates were comprised almost entirely of *E. coli* (Table 2, lines 1–3). Thus, in these samples the numbers of colonies of *E. coli* on the two types of media were comparable. The TYG plate of the 10^3 dilution from the 56-h sample of Missouri River water inoculated with *E. coli* from the fourth fermenter contained over 300 colonies, but the original EMBN plate of the same dilution from this sample had only one colony of *E. coli*. Replica-plating of the colonies on the TYG plate to an EMBN plate revealed that none were colonies of *E. coli* (Table 2, line 4). The TYG plate of the 10^4 dilution from the 56-h sample of equalization basin water inoculated with *E. coli* had over 300 colonies, but only about 3 colonies of *E. coli* were seen on the corresponding EMBN plate. Replica-plating onto an EMBN plate revealed that the TYG plate also had few colonies of *E. coli* (Table 2, line 5). These results show that the original EMBN plates gave an accurate representation of the number of *E. coli* cells present in the samples of water.

DISCUSSION

In non-sterile Missouri River water and equalization basin water the viable populations of added *E. coli* K-12 strains LBB269 and LBB269 (pBGH1) decreased from about 3×10^6 cells per ml to about 10 cells or less per ml after 147 h of incubation. In two sets of studies with sterile water, by contrast, the viable populations of both strains

remained at their initial levels for at least 28 days (672 h) or 300 days, respectively. These results indicated that the loss of viability was dependent on the presence of the indigenous inhabitants of the water samples. This conclusion was further supported by the observation that the populations of added *E. coli* decreased more rapidly in equalization basin water than in Missouri River water, given that the indigenous microbial population in the equalization basin water was 100-fold higher than in Missouri River water. Furthermore, it is important to note that while the study conditions resulted in the loss of the added *E. coli*, there was no adverse effect on the populations of indigenous microorganisms.

For these studies, two separate fermentations of each strain were used to obtain samples for testing. The plating of each dilution was performed in triplicate. In addition, each study was done in duplicate. The data illustrated in Figs. 1–6 are a composite of these multiple measurements, although in every case there were remarkably uniform results.

Strains LBB269 and LBB269 (pBGH1) were obtained at the end of fermentation runs when the cell concentration was the highest. The cells were diluted 10000- or 1000-fold, respectively, with sample water in order to reduce carryover of nutrients and still provide for added *E. coli* populations that ranged from 10- to 1000-fold higher than the indigenous microbial populations. It was reasoned that these conditions would approximate a 'worst case scenario' wherein material from a fully grown fermenter was accidentally spilled into an environmental source of water and, although naturally undergoing some dilution, overwhelmed the indigenous microbial population.

An analysis of the composition of the water samples (Table 1) indicated that the added *E. coli* cells likely would have undergone nitrogen starvation in the Missouri River water and carbon starvation in equalization basin water. The Missouri River water contained only 0.4 μg/ml total nitrogen, versus 71.4 μg/ml total nitrogen in the equalization basin water; the equalization basin contained only 5.25 μg/ml total organic carbon, versus 35.7 μg/ml total organic carbon in the Missouri River water. Both sources of water contained only low levels of potassium and phosphate. Despite this paucity of key nutrients, two sets of studies revealed that both strains remained viable in sterile water from either source for at least 28 days (672 h) or 300 days, respectively. The experiments were terminated at this point, although the *E. coli* cells could possibly have been maintained at these levels in the sterilized waters for much longer. Flint [8] also reported that an *E. coli* K-12 strain maintained a constant viable count in sterile river water for over 200 days.

These results indicate that starvation for carbon or nitrogen did not lead to a 'dormant' condition in which the added *E. coli* entered a 'viable but non-culturable' state, as has been reported by others [3,10,34]. The concept that cells can enter a such a state was developed to explain the apparent discrepancy between number of colony-forming units, and number of viable cells as determined by direct counting methods, in sterile water samples. One technique usually employed involves staining with acridine orange and directly counting cells which appear red or orange under a fluorescent microscope [12]. Acridine orange staining discriminates between cells on the basis of their RNA levels; under the fluorescent microscope, acridine orange stained cells with high levels of RNA appear red or orange, while stained cells with low levels of RNA appear green. An essential assumption in this concept is that all non-viable cells have low levels of RNA, and therefore appear green rather than red or orange. This critical assumption has not been unequivocally established. Nevertheless, data have been generated with such methods and used to propose that *E. coli* and other microorganisms can exist in a type of viable state in which they are unable to form colonies on media which can normally support their growth. It has been claimed that after culturable colony-forming units have dropped below detectable limits, the cells could be 'resuscitated' by the addition of nutrients [21]; the degree of resuscitation depended on the level of nutrient added, and after too long a 'dormancy' no cells could be resuscitated. This latter effect was said to indicate that "longer periods of 'dormancy' appear to require conditions other than simple nutrient addition for resumption of cell growth" [21]. It is puzzling that these investigators did not consider a more parsimonious explanation of such observations, one not invoking the 'viable but non-culturable' phenomenology. The alternative explanation is simply that shortly after the number of viable cells fell below detectable limits, but while there were still some viable cells left, addition of nutrient could have enabled these remaining cells to grow. At a point much longer after the number of viable cells fell below detectable limits, however, the added nutrient would have had no effect since there were no viable cells left at all. It also is puzzling that these studies showed a rapid loss of viability of organisms added to sterile water, a result in sharp contrast with those presented here and by others [8]. Most of the conflicting studies were done in seawater [3,10,34]; the one study in river water was with *Salmonella enteritidis* rather than *E. coli* [21]. These differences could account for the conflicting results. In any event, the *E. coli* K-12 strains used in this study, starved for either carbon or nitrogen in sterile water, did not exhibit any loss of viability for at least 300 days.

In a slightly different approach to the question of reliability of plate counts, it has been argued that added *E. coli*

cells in non-sterile soil or sterile water enter, over time, a state of 'stress' wherein they are unable to form colonies on selective media such as agar plates containing antibiotics [6,9]. In order to obtain reliable plate counts, it is claimed that the cells must first be plated on a non-selective medium and then transferred to the selective medium. We did not observe such effects, but rather observed similar numbers of colonies of *E. coli* on both non-selective (TYG plates) and selective (EMBN plates) media.

What is the mechanism by which added *E. coli* K-12 strains are lost from non-sterile water? It has been suggested that indigenous aquatic microorganisms subject *E. coli* K-12 strains to predation, parasitism, lytic enzymes or toxins [8,11,23,25]. In a recent publication Gurijala and Alexander argue that protozoa are responsible for the decline of bacteria, including *E. coli*, introduced into lake water [11]. However, we did not detect any protozoa in either source of water utilized in this study. Such questions have not been addressed in a systematic manner and the matter remains unsettled. What emerges from the studies presented here is a clear indication that either living indigenous aquatic microorganisms or a heat-labile component produced by them, or both, are required for the decline of introduced *E. coli.*

The presence of the plasmid pBGH1 had essentially no effect on the loss of LBB269 cells from either Missouri River water or equalization basin water. The decreases in viable counts for both strains yielded nearly superimposable curves (Figs. 5,6). In sterile water, the presence of pBGH1 also had no effect on the survival of LBB269. Apparently, the presence of a high copy number plasmid did not result in any significant advantage or disadvantage to the host strain, at least in terms of surviving in sterile or non-sterile water.

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