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Plasmid Expression and Maintenance during Long-Term Starvation-Survival of Bacteria in Well Water†

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Strains of enteric bacteria and pseudomonads containing plasmid R388::Tn*1721* (Tp^r, Tc^r) or pRO101 (Hg^r, Tc^r) were starved for over 250 days in sterile well water to evaluate effects of starvation-survival on plasmid expression and maintenance. Viable populations dropped to between approximately 0.1 and 1% of the initial populations. *Escherichia coli*(pRO101) and *Pseudomonas cepacia*(pRO101) lost both viability and plasmid expression at a lower rate than strains containing R388::Tn*1721*. Three patterns of host-plasmid interaction were detected: (i) no apparent loss of plasmid expression, (ii) loss of plasmid expression on initial recovery with subsequent expression upon resuscitation, and (iii) loss of capability to produce functional plasmid resistance.

Under conditions of nutrient starvation, bacteria have been found to alter cellular nucleic acid concentrations as well as protein concentration and composition (2, 3, 10, 11; C. L. Moyer, M.S. thesis, Oregon State University, Corvallis, 1988). Since plasmid DNA is relatively autonomous from the chromosomal genome, there are several ways in which bacteria can regulate plasmid DNA and its products during starvation. First, if the plasmid is maintained intact, the products can either be present in active form or be absent. Alternatively, the host can degrade the plasmid, either partially or completely, as a source of energy or nucleic acids for biosynthesis to elevate cellular levels of RNA, as has been noted during starvation of a marine *Vibrio* sp. (3; Moyer, M.S. thesis). This mechanism could prime the cell for rapid protein synthesis during recovery from starvation. Plasmid-borne resistances to antibiotics and/or heavy metals can provide a convenient system for examining and studying plasmid and protein changes during long-term starvation-survival. These involve synthesis and maintenance both of specific proteins whose functions can include detoxification and of cell membrane components to exclude the agent or modified biosynthetic pathways (8).

While Jain et al. (12) demonstrated that *Pseudomonas putida* and indigenous groundwater isolates stably maintained plasmid genotype through an 8-week period, the DNA-DNA colony hybridization method used would not establish whether the plasmid product is actively expressed throughout the starvation interval. In addition, other starvation studies have indicated no apparent effect of plasmid presence or type on bacterial survival in fresh water (6, 7, 9), although plasmid changes have been reported in several nutrient-limited-growth studies (4).

Because of the potential importance of plasmid-borne traits, either as markers or in performing specific functions in microorganisms of potential biotechnological use, we have undertaken a study of changes in plasmid expression and maintenance in various hosts during long-term starvation in a sterile well water menstruum.

MATERIALS AND METHODS

Organisms and media. Strains of bacteria containing various plasmids encoding antibiotic and heavy-metal resistances (Table 1) were routinely maintained on Luria-Bertani (LB) broth or agar (15) (made up with 5 g instead of 10 g of NaCl per liter) at 30°C with or without the appropriate selective agents in combination. The number of CFU was also determined by using the same selective and nonselective agar plates. Because the organisms were grown under selective pressure, the zero time CFU counts on selective and nonselective plates were essentially identical.

Survival studies. Log-phase cultures were harvested from LB broth containing selective agents by centrifugation (5,000 × *g*, 10 min) and suspended in sterile well water obtained from a 12-m well supplying the Oregon State University Fish Hematoma Laboratory. One- to two-liter batches, with concentrations of approximately 10^7 cells per ml, were prepared in acid-washed Wheaton bottles or aspirator bottles containing Teflon-coated magnetic stir bars to suspend cells prior to sampling. These starvation menstruums were then incubated at room temperature.

At various intervals, subsamples were serially diluted and plated onto nonselective (LB) and selective (LB plus antibiotics or mercury) agar. After incubation at 30°C, colonies were counted and the number of CFU per milliliter was calculated. Plasmid expression, the presence of active plasmid products responsible for the observed resistances, was calculated from the ratio of CFU counts on selective and nonselective LB agar. Plasmid maintenance, the presence of plasmid genome capable of producing effective gene products, was periodically determined by restreaking approximately 100 colonies from the initial nonselective dilution plates on selective media and then calculating the percentage of isolates growing on the selective media.

RESULTS AND DISCUSSION

Recovery of bacterial strains containing R388::Tn*1721* and pRO101 on selective and nonselective media after various periods of starvation is shown in Fig. 1 and 2, respectively. Table 2 lists the plasmid expression and maintenance percentages at sampling times when both were determined. For members of the family *Enterobacteriaceae* containing R388::Tn*1721* (Fig. 1A through C), there was an initial decrease in

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TABLE 1. Bacterial strains and plasmids used

Plasmids	Resistance[a]	Host species and strain
R388::TN*1721*	Tc (100), Tp (50)	*Enterobacter agglomerans* 70[b]
	Tc (100), Tp (50)	*Escherichia coli* ED 8654[c]
	Tc (100), Tp (50)	*Klebsiella pneumoniae* 64A[b]
	Tc (100), Tp (50)	*Pseudominas cepacia* PCO 1215[c]
pRO101	Hg (50), Tc (30)	*Escherichia coli* HB 101[c]
	Hg (50), Tc (50)	*Pseudomonas aeruginosa* PAO 4032[c]
	Hg (50), Tc (50)	*Pseudomonas cepacia* DB01[c]
	Hg (50), Tc (50)	*Pseudomonas putida* PPO 2042[c]

[a] Hg, Mercury; Tc, tetracycline (Sigma Chemical Co.); Tp, trimethoprim (Sigma). Numbers in parentheses are concentrations in micrograms per milliliter.

[b] John Armstrong and Michael Walters, U.S. Environmental Protection Agency, Corvallis, Oreg.

[c] Ron Olsen, University of Minnesota.

viability (growth on nonselective media), with or without a partial recovery, followed by a final stabilization at between approximately 0.1 and 1% of the initial population. Similarly, the *Pseudomonas cepacia* population, after a sharp initial drop and partial recovery (Fig. 1D), also stabilized with some oscillation out to 624 days. Viability losses of similar magnitude have been reported for *Escherichia coli* after 55 days in river water (9) and by Morita and co-workers for marine bacteria (1, 13; Moyer, M.S. thesis). In contrast, Liang et al. (14) found that *Klebsiella pneumoniae* rapidly died off in lake water, while another study of environmental isolates (21) suggested two catagories: survivors, including *K. pneumoniae*; and nonsurvivors, including *E. coli* and *Enterobacter* species.

Both *Enterobacter agglomerans* and *P. cepacia* exhibited a partial recovery of both viable cells and cells expressing plasmid products after approximately 30 days (Fig. 1A and D). This could be a result of cryptic growth (19) if the initial drop in viable cells was the result of cell death. Subsequent leakage of cytoplasmic contents could provide limited energy and nutrients to the remaining viable cells for protein synthesis (including re-establishment of antibiotic resistances) and possible cell division. *K. pneumoniae* demonstrated no distinct drop and recovery as described above, although there were two intervals (days 25 to 38 and 65 to 89) in which both the rate of viability loss and loss of plasmid expression leveled out (Fig. 1C).

Although a drop and recovery also appeared to occur on day 11 with *Escherichia coli* (Fig. 1B), this may be unrelated to the phenomenon in the *E. agglomerans* and *P. cepacia* menstruums because (i) it occurred well before 25 to 30 days, (ii) there was no appreciable loss of expression associated with the drop in recoverable cells, and (iii) a break in the rate of both viability and expression loss, similar to the *K. pneumoniae* pattern, occurred between days 32 and 65.

In all four strains containing R388::Tn*1721*, the population still expressing the plasmid phenotype (growth on selective media) followed the total plate counts during the initial rapid decline and then dropped to our limit of detection. When we

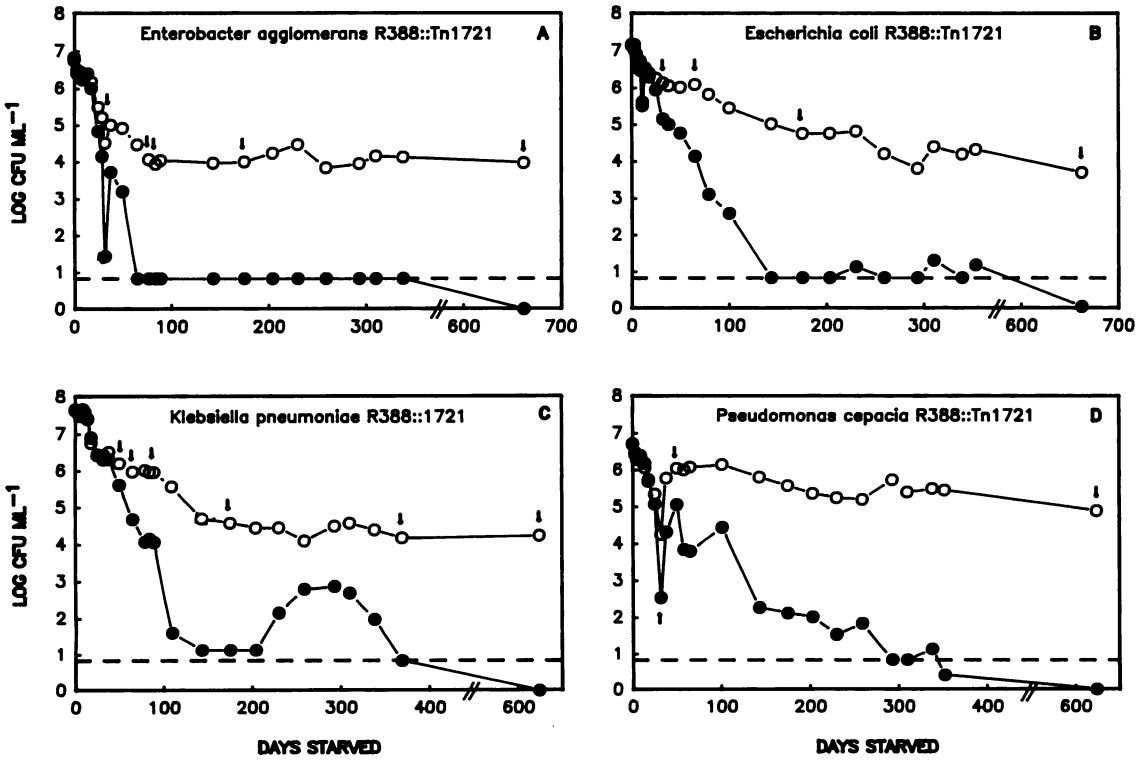

FIG. 1. Population changes and initial expression of plasmid R388::Tn*1721* phenotype in four bacterial species during long-term starvation-survival in well water. Symbols: ○, growth on nonselective media; ●, growth on media selective for plasmid-borne phenotype; ↓, sampling of plasmid maintenance (Table 2); – – –, limit of detection. Points below the limit of detection were determined by filtering the starved-cell menstruums (10 or 100 ml) through a sterile membrane filter and then placing the membrane onto the appropriate selective agar medium.

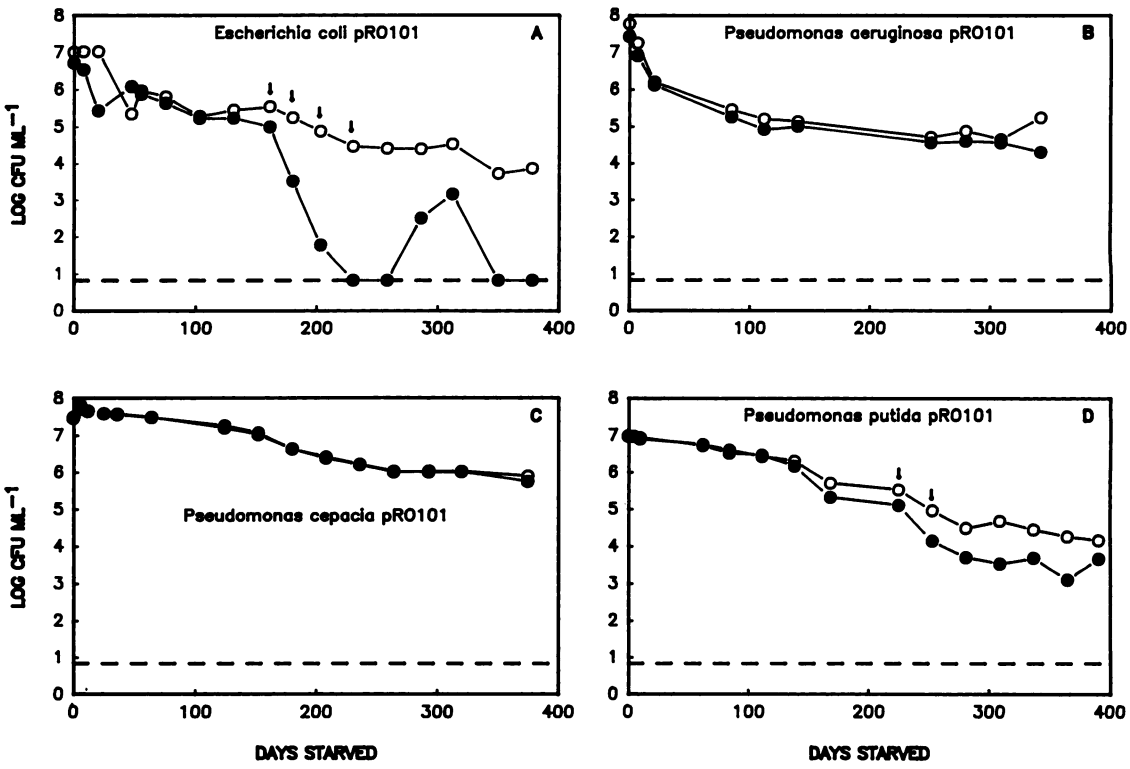

FIG. 2. Population changes and initial expression of pRO101 phenotype in four bacterial species during long-term starvation-survival in well water. Symbols are as in Fig. 1.

periodically tested isolates for the presence of an intact plasmid capable of producing active product (Table 2), both *Escherichia coli* and *E. agglomerans* demonstrated 95 to 100% plasmid maintenance, while *K. pneumoniae* plasmid maintenance slowly declined with time. In contrast, plasmid maintenance in *P. cepacia* dropped to 8.3% of the viable population within 32 days and appeared to be reduced to 1% by 50 days. However, this latter value is in apparent conflict with the concurrent 10.3% rate of expression. This could happen if the plasmid is inherently unstable in the host or becomes unstable as a result of starvation of the host. In that case, during initial plating on nonselective medium which lacks selective pressure, the plasmid may not be maintained or may be maintained at too low a copy number to grow upon subsequent transfer to selective media.

Agarose gel electrophoresis of plasmid preparations from isolates of *K. pneumoniae* and *P. cepacia* no longer capable of producing active plasmid-encoded proteins showed either reductions in plasmid size (in the former) or no visible change or apparent loss of plasmid (in the latter, where the bands were frequently faint and difficult to detect). These starvation-associated plasmid alterations, with R388::Tn*1721* and other plasmids, are under further study and are the subject of a subsequent paper now in preparation (R. P. Griffiths, C. L. Moyer, B. A. Caldwell, C. Ye, and R. Y. Morita).

Four different bacterial strains carrying pRO101 responded differently in long-term starvation (Fig. 2) than did bacteria containing R388::Tn*1721* (Fig. 1) and continued to express and maintain plasmid-borne resistances for a much longer period. While *Escherichia coli*(R388::Tn*1721*) showed an initial rapid decrease in viability and plasmid expression after approximately 30 days (Fig. 1A), another strain of *Escherichia coli*(pRO101) showed a much lower rate of viability loss with an initial loss of expression and then a return to full expression between days 50 and 130.

P. cepacia(pRO101) (Fig. 2C) showed only slight loss of viability and no change in plasmid expression during the course of the experiment, in marked contrast to the pattern observed in Fig. 1D, in which R388::Tn*1721* expression and capacity to produce a functional plasmid product even after resuscitation were lost. Such differences in loss of expression and viability for the same species with different plasmids may be due in part to intraspecies strain differences (7); however, the contrasts between R388::Tn*1721* and pRO101 suggest a plasmid role, particularly if the resistance is manifested as a membrane modification (8) that may have an additional survival advantage. The membrane of *Escherichia coli* become more fluid (16) upon nutrient deprivation, which would suggest that antibiotics and/or mercury would be more readily transported into the cell. If the gene product is induced by the selective media, the antibiotics and/or mercury will probably be present. However, it could be argued that the expression on selective media could be the result of the status of the cells.

Pseudomonas aeruginosa (Fig. 2B) also continued to express resistance, while *Escherichia coli* and *P. putida* (Fig. 2A and D) showed a loss of expression after 150 days. A significant dip and recovery in plasmid expression but not in viability was seen in *Escherichia coli* (Fig. 2A) from days 8 and 47; however, this pattern was not observed in the other bacteria carrying pRO101. Since these survival menstruums were not monitored with the same initial frequency as the R388::Tn*1721* menstruums, this rapid event may have been missed. For the two survival menstruums that began to show loss of expression, plasmid maintenance (Table 2) remained

TABLE 2. Expression and maintenance of plasmid gene product

Plasmid and host	Figure	No. of days starved	% Expressed	% Maintained
R388:Tn*1721*				
Enterobacter agglomerans	1A	32	5.25	100
		77	—[a]	99
		84	—	99
		175	—	100
		662	—	64
Escherichia coli	1B	32	10.9	98
		65	1.06	95
		175	—	100
		662	—	59
Klebsiella pneumoniae	1C	50	25.9	95
		65	5.06	93
		84	1.56	88
		175	0.036	88
		369	—	83
		624	—	19
Pseudomonas cepacia	1D	32	1.92	8.3
		50	10.3	1
		624	—	0
pRO101				
Escherichia coli	2A	161	28.0	98
		180	1.87	98
		203	—	100
		230	—	100
Pseudmonas putida	2D	225	17.3	100
		253	6.88	81

[a] —, Counts on selective media were below limits of detection.

high in *Escherichia coli*, while that in *P. putida* began to decline, as did R388::Tn*1721* maintenance in *K. pneumoniae*. *P. cepacia* viability declined much less than that of the members of the *Enterobacteriaceae* tested.

The ability of cells to remain viable under starvation conditions for a long period is probably the result of metabolic arrest (17), a type of dormancy due to a lack of available energy. During this period, the cells are able to reorganize both the plasmid product and genome in several ways, as well as to become ultramicrocells. The survival patterns observed in both *P. aeruginosa*(pRO101) and *P. cepacia*(pRO101) suggest that a functional plasmid product is maintained and expressed by the cells through starvation. The plasmid product can also disappear, possibly being recycled into new proteins or catabolized for energy, as was the case with *E. agglomerans*(R388::Tn*1721*), *Escherichia coli*(R388::Tn*1721*), and initially *K. pneumoniae*(R388::Tn*1721*). Alternatively, the plasmid can be altered by partial or complete deletions, resulting in the loss of the ability to produce a functional product after resuscitation. This can happen either early [*P. cepacia*(R388::Tn*1721*) and *K. pneumoniae*(R388::Tn*1721*)] or well into the survival state [*P. putida*(pRO101)]. Reports that plasmids are present in indigenous microflora of groundwater (18), where the hosts are under nutrient stress (23) but not under the grazing pressures present in surface waters (5), suggest that long-term plasmid maintenance occurs naturally.

Our results have shown that under long-term starvation-survival conditions, the same plasmid in different hosts behaves differently in terms of expression and maintenance. Short-term experiments would not have yielded the data obtained in this study. While we have focused on starvation effects on plasmid-borne traits, similar changes may also occur in engineered chromosomal traits. The range of results encountered in this study strongly indicates that examination of the starvation dynamics affecting the maintenance and expression of the desired marker or function gene(s), whether plasmid or chromosomal, should be a primary step when biological agents are developed for environmental usage. This is particularly relevant for areas where groundwater may represent an ultimate reservoir for organisms that have been applied to soil and have migrated through root channels and soil macropores (20, 22).

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LITERATURE CITED

1. **Amy, P. S., and R. Y. Morita.** 1983. Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. Appl. Environ. Microbiol. **45**:1109–1115.
2. **Amy, P. S., and R. Y. Morita.** 1983. Protein patterns of growing and starved cells of a marine *Vibrio* sp. Appl. Environ. Microbiol. **45**:1748–1752.
3. **Amy, P. S., C. Pauling, and R. Y. Morita.** 1983. Starvation-survival processes of a marine vibrio. Appl. Environ. Microbiol. **45**:1041–1048.
4. **Caulcott, C. A., A. Dunn, H. A. Robertson, N. S. Cooper, M. E. Brown, and P. M. Rhodes.** 1987. Investigation of the effect of growth environment on the stability of low-copy-number plasmids in *Escherichia coli*. J. Gen. Microbiol. **133**:1881–1889.
5. **Chao, W.-L., R.-J. Ding, and R.-S. Chen.** 1988. Survival of *Yersinia enterocolitica* in the environment. Can. J. Microbiol. **34**:753–756.
6. **Cruz-Cruz, N. E., G. A. Toranzos, D. G. Ahearn, and T. C. Hazen.** 1988. In situ survival of plasmid-bearing and plasmidless *Pseudomonas aeruginosa* in pristine tropical waters. Appl. Environ. Microbiol. **54**:2574–2577.
7. **Devanas, M. A., D. Rafaeli-Eshkol, and G. Stotzky.** 1986. Survival of plasmid-containing strains of *Escherichia coli* in soil: effect of plasmid size and nutrients on survival of hosts and maintenance of plasmids. Curr. Microbiol. **13**:269–277.
8. **Foster, T. J.** 1983. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. **47**:361–409.
9. **Grabow, W. O. K., O. W. Prozesky, and J. S. Burger.** 1975. Behaviour in a river and dam of coliform bacteria with transferable or non-transferable drug resistance. Water Res. **9**:777–782.
10. **Hood, M. A., J. B. Gluckert, D. C. White, and F. Deck.** 1986. The effect of nutrient (carbon) deprivation on the levels of lipids, carbohydrates, DNA, RNA, and proteins in *Vibrio cholerae*. Appl. Environ. Microbiol. **52**:788–793.
11. **Jaan, Å. J., B. Dahllöf, and S. Kjelleberg.** 1986. Changes in protein composition of three bacterial isolates from marine waters during short periods of energy and nutrient deprivation. Appl. Environ. Microbiol. **52**:1419–1421.
12. **Jain, R. K., G. S. Sayler, J. T. Wilson, L. Houston, and D. Pacia.** 1987. Maintenance and stability of introduced genotypes in groundwater aquifer material. Appl. Environ. Microbiol. **53**: 996–1002.
13. **Kurath, G., and R. Y. Morita.** 1983. Starvation-survival physiological studies of a marine *Pseudomonas* sp. Appl. Environ.

Microbiol. **45**:1206–1211.

14. **Liang, L. N., J. S. Sinclair, L. M. Mallory, and M. Alexander.** 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. Environ. Microbiol. **44**:708–714.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Massa, E. M., A. L. Viñals, and R. N. Farías.** 1988. Influence of unsaturated fatty acid membrane component on sensitivity of an *Escherichia coli* fatty acid auxotroph to conditions of nutrient depletion. Appl. Environ. Microbiol. **54**:2107–2111.
17. **Morita, R. Y.** 1988. Bioavailability of energy and its relationship to growth and starvation survival in nature. Can. J. Microbiol. **34**:436–441.
18. **Ogunseitan, O. A., E. T. Tedford, D. Pacia, K. M. Sirotkin, and G. S. Saylor.** 1987. Distribution of plasmid in groundwater bacteria. J. Ind. Microbiol. **1**:311–317.
19. **Postgate, J. R.** 1967. Viability measurements and the survival of microbes under minimum stress. Adv. Microb. Physiol. **1**:2–23.
20. **Rahe, T. M., C. Hagedorn, E. L. McCoy, and G. F. Kling.** 1978. Transport of antibiotic-resistant *Escherichia coli* through western Oregon hillslope soils under conditions of saturated flow. J. Environ. Qual. **7**:487–494.
21. **Sjogren, R. E., and M. J. Gibson.** 1981. Bacterial survival in a dilute environment. Appl. Environ. Microbiol. **41**:1331–1336.
22. **Smith, M. S., G. W. Thomas, R. E. White, and D. Ritonga.** 1985. Transport of *Escherichia coli* through intact and disturbed soil columns. J. Environ. Qual. **14**:87–91.
23. **White, D. C., G. A. Smith, M. J. Gehron, J. H. Parker, R. H. Finflay, R. F. Martz, and H. L. Fredrickson.** 1983. The groundwater aquifer microbiota: biomass, community structure and nutritional status. Dev. Ind. Microbiol. **24**:201–211.