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Role of Resistance to Starvation in Bacterial Survival in Sewage and Lake Water

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A study was conducted to determine the significance of starvation resistance to the ability of a species to survive in sewage and lake water. Tests were conducted for periods of up to 14 days. *Rhizobium meliloti* and one fluorescent and one nonfluorescent strain of *Pseudomonas* were resistant to starvation because their population sizes did not fall appreciably in buffer and sterile lake water, and the first two maintained high numbers after being added to sterile sewage. Cell densities of these bacterial species dropped slowly in nonsterile sewage, and more cells of these three organisms than of the other test organisms remained in nonsterile lake water. *Rhizobium leguminosarum* was moderately resistant to starvation because its numbers fell slowly in buffer and sterile lake water and did not change appreciably in sterile sewage. The abundance of *Micrococcus flavus* added to buffer and sterile lake water did not change, but the density of *M. flavus* declined in nonsterile lake water. The abundance of *R. leguminosarum* fell in nonsterile lake water and nonsterile sewage. *Streptococcus faecalis*, *Staphylococcus aureus*, an asporogenous strain of *Bacillus subtilis*, and *Streptococcus* sp. were susceptible to starvation because their populations were markedly reduced in buffer. Populations of the last three species declined rapidly in nonsterile and sterile samples of lake water and sewage. *S. faecalis* declined rapidly when added to nonsterile lake water and sewage and sterile lake water but not when added to sterile sewage, the persistence in the last instance probably being associated with the availability of organic nutrients. In a comparison of random isolates from nutrient-poor and nutrient-rich habitats, the percentage of survival of 17 of 19 bacteria from lake water was found to be greater than that of all 11 isolates from human skin and mouth. It is suggested that starvation-susceptible bacteria will not persist in environments that are nutrient poor or in which they fail to compete for organic nutrients and that starvation resistance is a necessary but not sufficient condition for persistence in environments that are nutrient poor or that support intense interspecific competition.

The survival of bacteria in natural environments is of practical importance in public health and in plant pathology, and this subject also has been of interest to microbial ecologists. Recently, the possible detrimental consequences arising from the long-term survival of new genotypes has aroused concern.

One factor that may influence the period of survival of bacteria is their ability to survive starvation. The effect of starvation resistance of bacteria on their survival in pure culture has been studied by a number of investigators. In a study of *Enterobacter aerogenes* in buffered physiological saline, Postgate and Hunter (12) have reported that the population declines at a rate of 8% per h for the first 7 to 10 h, and the viable count at 24 h is less than 2% of the original count. Strange (15) has noted that starved cells of *E. aerogenes* and *Escherichia coli* die more slowly if they contain glycogen than if free of glycogen reserves, but *Sarcina lutea* dies more readily if the starved cells contain the reserve material. Nelson and Parkinson (10) have observed marked dissimilarities in the starvation resistance of *Pseudomonas*, *Arthrobacter*, and *Bacillus* strains in a carbon-limited medium, and Boylen and Ensign (2) have shown that *Arthrobacter crystallopoietes* is especially tolerant of starvation stress in phosphate buffer. Wu and Klein (16) have evaluated the interaction between starvation and secondary warming stresses affecting *E. coli* and *Aeromonas* sp. Novitsky and Morita (11) have reported that large numbers of a vibrio from the marine environment, which is typically poor in nutrients, survive for 6 to 7 weeks and some cells survive for at least a year in a salts solution.

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In these investigations, the test organisms were usually added to sterile buffer or saline in batch or carbon-limited continuous culture, and the population declines were noted. However, little attention has been given to assessing the potential relationship between the ability of bacteria to resist starvation and their survival in natural habitats. A notable exception is the investigation of Klein and Casida (6), who report that the decline of *E. coli* in soil was markedly diminished by the addition of glucose; they suggest that the decrease in population of the bacterium may result from its inability to reduce its rate of metabolism in an environment with low levels of readily available organic nutrients. Morita (9) has recently reviewed the problem of starvation and survival in the marine environment.

This study was designed to determine whether starvation resistance is significant in the survival of bacteria that are introduced into environmental samples. For this purpose, species that differed in their ability to withstand starvation and random isolates from lake water, human skin, and human mouths were used.

MATERIALS AND METHODS

Streptococcus faecalis OG1B10-6 and *Staphylococcus aureus* RN 450 were obtained from G. M. Dunny and an asporogenous strain of *Bacillus subtilis* was provided by S. A. Zahler (both researchers are at Cornell University). *Rhizobium meliloti* 102F34, *Rhizobium leguminosarum* 128C53, and *Micrococcus flavus* 557 were from this laboratory. A nonfluorescent pseudomonad (*Pseudomonas* sp. strain B4) was isolated from Beebe Lake, Ithaca, N.Y. A fluorescent *Pseudomonas* (designated *Pseudomonas* sp. strain S1) was isolated from the Ithaca, N.Y., sewage

treatment plant. The pseudomonads were identified by the API 20 system (13). *Streptococcus* sp. strain 4T3 was isolated from human mouths.

These organisms were enumerated by the method of Mallory et al. (8). Mutants of the isolates were obtained that were resistant to two or more inhibitors. Stability of the resistance was shown by growing the bacteria for three serial transfers in antibiotic-free media and then plating the final dilution on the antibiotic-supplemented and antibiotic-free media; under these conditions, the counts were identical. When the antibiotics were added to the counting medium, recovery of small populations of the test organisms from environmental samples was possible while indigenous microbial populations were suppressed by the inhibitors in the medium. Antibiotic resistance was developed as described by Liang et al. (7).

All bacteria except the rhizobia, *S. faecalis*, and *Streptococcus* sp. were grown in half-strength Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The rhizobia were grown in a solution containing 1.0 g of yeast extract, 5.0 g of mannitol, 0.5 g each of K_2HPO_4 and KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.12 g of $CaCl_2 \cdot 2H_2O$, and 0.1 g of NaCl per liter of distilled water. *S. faecalis* and *Streptococcus* sp. were grown in half-strength brain heart infusion broth (BBL Microbiology Systems) supplemented with 5.0 g of yeast extract and 2.0 g of glucose per liter of distilled water. The bacteria were grown in the dark at 28°C in 100 ml of medium contained in 250-ml Erlenmeyer flasks placed on a rotary shaker operating at 120 rpm. The cultures were incubated for 24 h, except that the rhizobia were incubated for 120 h. The cells were collected by centrifugation at 10,400 × *g* for 10 min at 4°C, washed, and resuspended in a buffer (pH 7.0) containing 0.3 g of KH_2PO_4 and 0.7 g of K_2HPO_4 per liter of distilled water.

Washed cells were added to 500-ml Erlenmeyer flasks to give densities of 10^5 to 10^6 cells per ml. Raw sewage was obtained from the Ithaca, N.Y., sewage treatment plant, and water was obtained from Beebe Lake within an hour of use in the experiments. For sterile sewage or lake water, samples were sterilized by passage through Nalgene filters (pore size, 0.22 μm). The buffer used in tests of survival was the phosphate buffer described above. Each 500-ml Erlenmeyer flask contained 100 ml of the environmental sample or buffer solution and was shaken at 120 rpm at 28°C in the dark.

Three replicate flasks were used for each treatment. Bacteria in each flask were counted by the spread-plate technique, with two replicate plates per dilution. The plating media were the same as those used to grow the bacteria, but they contained agar and 250 μg of cycloheximide per ml to inhibit eucaryotes and antibacterial antibiotics to which the test organisms were resistant. The antibiotics (and dye, in some cases) were 1.0 mg of streptomycin and 100 μg of erythromycin per ml for *Pseudomonas* sp. strain S1 and *Pseudomonas* sp. strain B-4; 1.0 mg of streptomycin, 75 μg of erythromycin, and 25 μg of Congo red per ml for *R. meliloti*; 250 μg of streptomycin and 100 μg of bacitracin per ml for *B. subtilis*; 500 μg of streptomycin and 50 μg of spectinomycin per ml for *S. faecalis*; 1.0 mg of streptomycin and 50 μg of rifampin per ml for *S. aureus* and *Streptococcus* sp. and the same two antibiotics plus 25 μg of Congo red per ml for *R. leguminosarum*; and 200 μg of rifampin per ml for *M. flavus*. Before the colonies were counted, the plates were incubated at 28°C for 48 h for all organisms, except that plates used to count *R. meliloti* and *R. leguminosarum* were incubated for 144 h. As few as 2 cells of *S. aureus*, *S. faecalis*, or *R. leguminosarum* per ml of lake water or sewage and as few as 100 to 1,000 cells of *R. meliloti* or the pseudomonads per ml of sewage could be detected. To determine whether spores were produced by the strain of *B. subtilis*, samples were heated at 80°C for 10 min and then plated.

Survival of each bacterium was tested one to three times. No significant differences were noted in the various tests of the same species.

Samples of water from Cayuga Lake, Ithaca, N.Y., taken from sites away from the shore were plated on a medium containing 0.05% yeast extract and 1.5% agar in lake water. Random isolates with different colony morphologies were selected and were categorized by Gram reaction and by whether they were rods, individual cocci, or streptococci. The isolates were grown in Trypticase soy broth or in sterile lake water amended with 0.05% yeast extract. The cells were collected, washed with sterile phosphate buffer, and added to fresh buffer. The number of viable cells was determined on days 0 and 14. Bacteria from the human skin or mouth were isolated in half-strength brain heart infusion broth supplemented with 0.5% yeast extract and 1.5% agar, and random isolates with different colony morphologies were

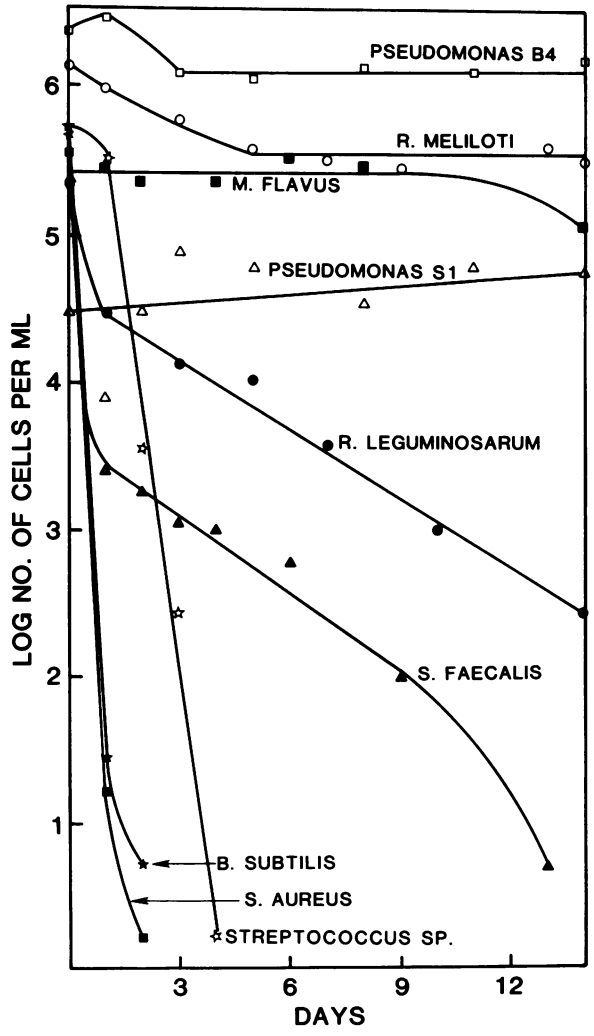

FIG. 1. Survival of several bacterial species suspended in phosphate buffer.

grown in the same medium but without agar. The cells were washed, added to sterile phosphate buffer, and counted on days 0 and either 7 or 14.

RESULTS

An assessment was made of the changes in numbers of viable cells suspended in sterile phosphate buffer. *R. meliloti*, *M. flavus*, *Pseudomonas* sp. strain B4, and *Pseudomonas* sp. strain S1 showed little or no decline in 14 days (Fig. 1). The other bacteria showed various degrees of decline. The numbers of *R. leguminosarum* fell slowly, and its density decreased by 3 logarithmic orders in 14 days. *S. faecalis* also declined, although a few cells were still present at day 13. In contrast, populations of *S. aureus*, *B. subtilis*, and *Streptococcus* sp. rapidly lost viability, and few viable cells were present on day 2 for the first two species and on day 4 for the third organism. Because they persisted in buffer, *R. meliloti*, *M. flavus*, *Pseudomonas* sp. strain B4, and *Pseudomonas* sp. strain S1 appear to be tolerant to the stress of starvation.

Before assessing the significance in sewage and lake water of the differences in susceptibility to starvation among these organisms, a test was conducted to determine the persistence of the bacteria in sterile sewage and lake water, in which the biotic stresses of the natural environment, such as predation and parasitism, would not affect the test species. In sterile sewage, *R. meliloti*, *Pseudomonas* sp. strain S1, and *Pseudomonas* sp. strain B4 grew and reached cell densities of about 10^7 cells per ml (Fig. 2). Thereafter, *Pseudomonas* sp. strain S1 maintained its numbers, *R. meliloti* declined somewhat, and the numbers of *Pseudomonas* sp. strain B4 fell by more than 2 orders of magnitude. The populations of *R. leguminosarum* and *S. faecalis* did not change greatly in the sterile sewage. In contrast, the numbers of *S. aureus*, *B. subtilis*, and *Streptococcus* sp. fell markedly. The arrows in this and subsequent figures extend to the limit of detection of that organism, and the counts on the day indicated by arrows were less than this sensitivity limit.

In sterile lake water, no appreciable increase in cell density of any of the species was noted in the test period, although a small increase in numbers of one pseudomonad was evident (Fig. 2). However, *R. meliloti*, *M. flavus*, and the two pseudomonads did not decline significantly in abundance in the test periods, but the numbers of *R. leguminosarum* slowly fell. *S. faecalis*, *S. aureus*, and *Streptococcus* sp. were quite different, however, and fewer than 10 cells per ml were detectable by day 3. The numbers of *B. subtilis* also fell appreciably, and only 18 cells per ml, none of which was a spore, remained on day 6. These observations suggest that the two pseudomonads, *M. flavus*, and *R. meliloti* are able to maintain their viability in the absence of nutrients or when the supply of nutrients is depleted.

The behavior of these organisms in nonsterile samples of the natural environments was then evaluated. In nonsterile sewage, the two pseudomonads and *R. meliloti* declined only slightly (Fig. 3). These organisms were previously found to be starvation resistant. *R. leguminosarum*, which was moderately sensitive to starvation in buffer and also persisted in sterile sewage and sterile lakewater, declined at a moderate rate and fell below the limit of detection in 10 days. The abundance of *S. faecalis*, which declined markedly in sterile

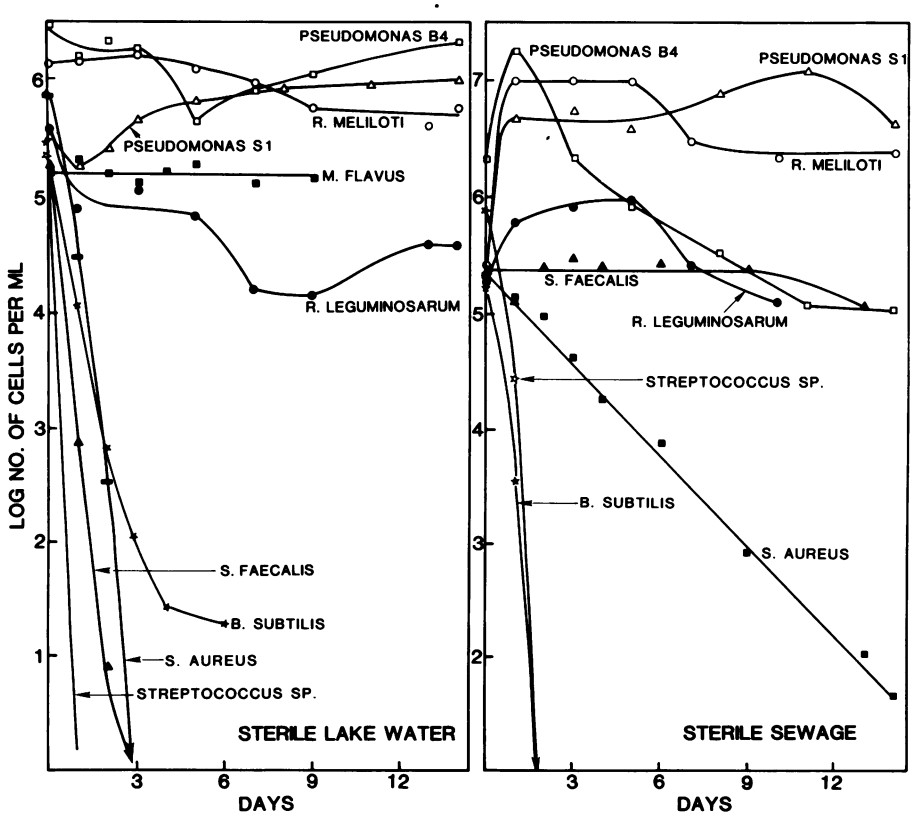

FIG. 2. Changes in populations of several bacterial species after their introduction into sterile sewage or lake water.

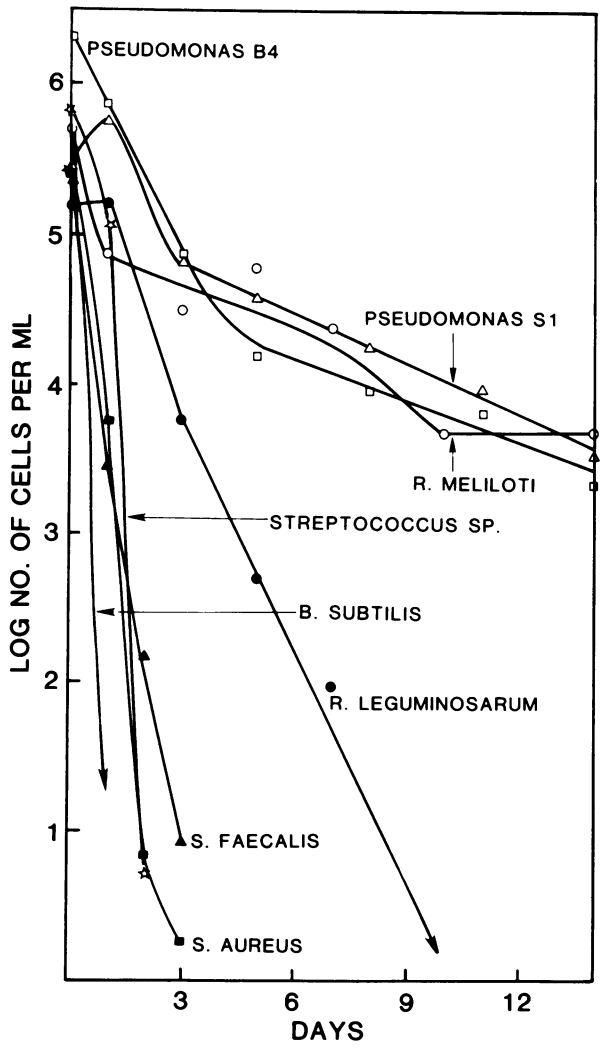

FIG. 3. Persistence and decline of bacteria in nonsterile sewage.

lake water but not in sterile sewage, fell to fewer than 10 cells per ml remained on day 14. *R. leguminosarum* and *M.* declines in nonsterile sewage were evident for *S. aureus*, *B. subtilis*, and *Streptococcus* sp., bacteria whose populations also fell markedly in buffer.

All of the organisms declined considerably in nonsterile lake water, but the rate and extent of decline varied greatly (Fig. 4). The populations of *R. meliloti* declined the least, and the population remained essentially constant after day 7. *Pseudomonas* S1 fell by about 3 orders of magnitude, but essentially no decline was evident after day 11. The numbers of *Pseudomonas* B4 dropped steadily with time, and only 62 cells per ml remained on day 14. *R. leguminosarum* and *M. flavus* declined steadily up to day 9. *S. faecalis*, *S. aureus*, *B. subtilis*, and *Streptococcus* sp. died rapidly, and fewer than 10 cells per ml remained viable after 3 or fewer days.

The survival in buffer of isolates from Cayuga Lake, human skin, and human mouth was then tested. Of the 19 lake water bacteria, 5 grew in the buffer, and the survival percentage of 8 of the remaining 14 exceeded 1.0% after 14 days (Table 1). In contrast, the percent survival of none of the 11 strains obtained from human skin or mouths exceeded 1.0% even after 7 days.

DISCUSSION

The results show that *S. faecalis*, *S. aureus*, *B. subtilis*, and *Streptococcus* sp. readily lost viability in the absence of organic nutrients. The abundance of the last three fell by 4 or more and of the first by 2 orders of magnitude in 1 to 4 days in sterile buffer. The density of the last three also declined markedly in samples of all environments tested. In the nutrient-poor sterile lake water, *S. faecalis* also failed to survive, but its persistence in sterile sewage probably is a result of its ability to use organic compounds of this nutrient-rich environment when competitors are absent. The rapid disappearance of these four bacteria from lake water and sewage probably results from their inability to obtain organic nutrients, which are in low concentrations in lake water and for which there is intense competition in sewage. It is of interest that at least some starvation-sensitive bacteria may be less able to withstand other stresses than resistant organisms (16).

R. meliloti and the two pseudomonads were resistant to starvation. This is evident from their persistence in large numbers in buffer and in sterile lake water. Although popula-

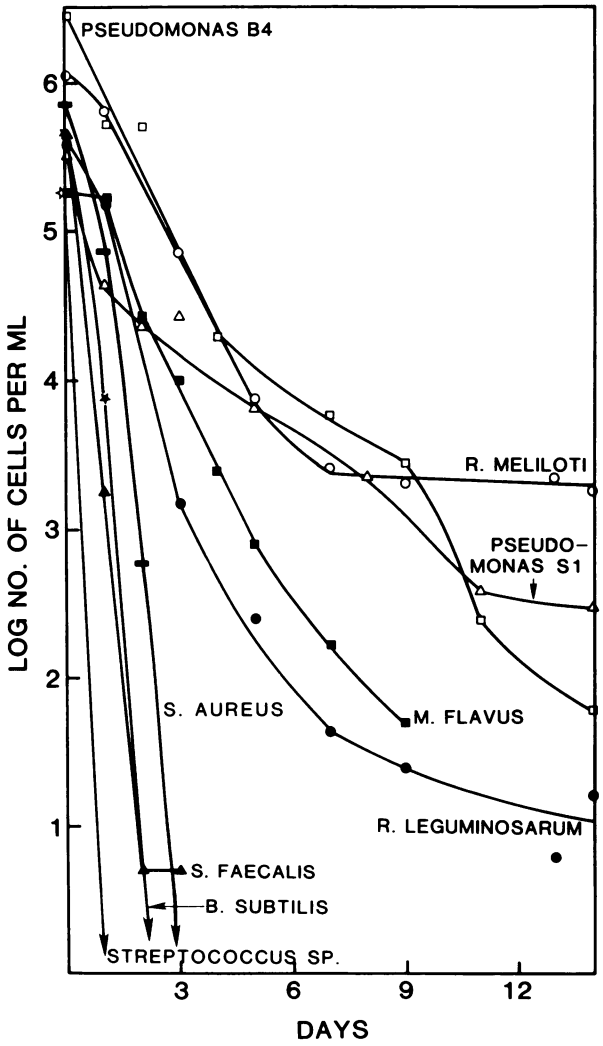

FIG. 4. Persistence and decline of bacteria in nonsterile lake water.

tions of two of these bacteria also did not decline after their addition to sterile sewage, the abundance of *Pseudomonas* sp. strain B4 did drop after its initial period of growth, possibly a consequence of toxins the bacterium generated as it proliferated using organic constituents of the sewage. None of these three starvation-resistant species was eliminated from nonsterile sewage or lake water, and thus it seems that starvation resistance is a necessary trait of a species that is to endure in a habitat poor in organic nutrients or one in which it fails to compete for the supply of available organic carbon. Nevertheless, the populations of all three were reduced in sewage and lake water. This is not surprising because toxins may be important in natural environments (7), and predators or parasites may feed on the bacterial population. Indeed, the rapid initial fall in numbers followed by a period with little or no decline, as was evident in the cases of *R. meliloti* and *Pseudomonas* sp. strain B4 in nonsterile sewage, is characteristic of the bacterial prey of protozoan predators (1). Thus, resistance to starvation is a necessary but not sufficient condition for survival of species in nutrient-poor environments or of species that do not compete well for supplies of limiting nutrients.

The data suggest that *R. leguminosarum* is intermediate in its tolerance to starvation. Thus, it was not surprising that its rate of decline in samples of natural environments fell between those of the species that were highly susceptible or highly resistant to this stress in axenic culture. The comparison is particularly pertinent for the two species of *Rhizobium* tested, because their decline rates in nonsterile sewage and lake water were directly related to their susceptibility to starvation stress in culture. *M. flavus*, which was tested only in some environmental samples, is apparently starvation resistant, as is evident by its behavior in buffer and sterile lake water. This resistance is not sufficient to ensure its survival, because its population was reduced in nonsterile lake water. The greater survival of the lake water isolates than the skin and mouth isolates supports the view that bacteria from a nutrient-poor environment are better able to withstand starvation than those from a nutrient-rich habitat, in which starvation resistance is not a necessary trait. The two lake water strains that survived poorly, moreover, were isolated at a time when algal growth was extensive in the water.

TABLE 1. Bacterial isolates obtained from three environments: survival in phosphate buffer

Source	Strain no.	Gram reaction	Morphology	Initial no. ($\times 10^3$)/ml	% Surviving
Cayuga Lake	6L	−	Rod	41	1,400
	2L	−	Rod	40	1,400
	10L	−	Rod	89	980
	9L	−	Rod	96	600
	13L	+	Rod	3.0	600
	18L	+	Rod	120	77
	12L	+	Rod	170	71
	14L	+	Rod	95	55
	17L	+	Rod	4.0	46
	11L	+	Rod	270	37
	29L	−	Rod	4,500	23
	19L	+	Rod	170	22
	28L	+	Rod	690	6.4
	3L	−	Rod	850	0.97
	15L	+	Rod	2,000	0.87
	25L	−	Rod	3,300	0.58
	21L	−	Rod	8,100	0.29
	1L	−	Rod	120	0.0041
	4L	−	Rod	690	<0.00023
Skin	2S	−		860	0.29[a]
	2S	−		860	0.018
	1S	−		800	0.042[a]
	1S	−		800	0.0087
	4S	+	Coccus	230	0.018[a]
	4S	+	Coccus	230	<0.00071
	9S	+	Coccus	470	0.0074[a]
	3S	+	Coccus	440	0.0011[a]
	11S	+	Coccus	200	0.00080[a]
	7S	+	Rod	350	0.00046[a]
	8S	+	Coccus	470	0.00034[a]
	10S	+	Coccus	81	0.00020[a]
Mouth	6M	+	Streptococcus	95	0.0017[a]
	5M	−	Rod	310	0.00052[a]

[a] These values were obtained after isolates were incubated for 7 days; all other isolates were incubated for 14 days.

The persistence in nature or in samples of natural environments of some of the bacterial species or genera we used has been measured by other investigators. Thus, *Rhizobium phaseoli* was noted to decline in nonsterile soil, but then the population density stabilized at about 10^3 cells per g (4). Saz et al. (14) observed a rapid drop in numbers of *S. aureus* in sea water, and although they attributed much of this decline to a bactericidal compound in the water, a significant decrease in viable cells occurred in 72 h in 2.5% NaCl. Kibbey et al. (5) studied the decline of *S. faecalis* in natural soil. *P. aeruginosa* was shown to decline in nonsterile soil as well as in buffer, and the data suggested that predators and parasites were not important but starvation was apparently important in the death of this bacterium (17). With *Micrococcus luteus*, on the other hand, predators rather than starvation may be the chief cause of the loss of viability of much of the population (3).

Only a few species and only samples of two environments have been investigated. Additional study is thus needed to determine whether the generalizations proposed here are valid. Such information is of importance in predicting whether a new genotype deliberately or inadvertently introduced into a natural ecosystem will persist.

ACKNOWLEDGMENT

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LITERATURE CITED

1. **Alexander, M.** 1981. Why microbial predators and parasites do not eliminate their prey and hosts. Annu. Rev. Microbiol. **35**:113–133.
2. **Boylen, C. W., and J. C. Ensign.** 1970. Long-term starvation survival of rod and spherical cells of *Arthrobacter crystallopoietes*. J. Bacteriol. **103**:569–577.
3. **Casida, L. E., Jr.** 1980. Death of *Micrococcus luteus* in soil. Appl. Environ. Microbiol. **39**:1031–1034.
4. **Chao, W. L., and M. Alexander.** 1981. Interaction between protozoa and *Rhizobium* in chemically amended soil. Soil Sci. Soc. Am. J. **45**:48–50.
5. **Kibbey, H. J., C. Hagedorn, and E. L. McCoy.** 1978. Use of fecal streptococci as indicators of pollution in soil. Appl. Environ. Microbiol. **35**:711–717.
6. **Klein, D. A., and L. E. Casida, Jr.** 1967. *Escherichia coli* die-out from normal soil as related to nutrient availability and the indigenous microflora. Can. J. Microbiol. **13**:1461–1470.
7. **Liang, L. N., J. L. 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.
8. **Mallory, L. M., J. L. Sinclair, L. N. Liang, and M. Alexander.**

1982. A simple and sensitive method for assessing survival in environmental samples of species used in recombinant DNA research. Recomb. DNA Tech. Bull. **5**:5–6.
9. **Morita, R. Y.** 1982. Starvation-survival of heterotrophs in the marine environment. Adv. Microb. Ecol. **6**:171–198.
10. **Nelson, L. M., and D. Parkinson.** 1978. Effect of starvation on survival of three bacterial isolates from an arctic soil. Can. J. Microbiol. **24**:1460–1467.
11. **Novitsky, J. A., and R. Y. Morita.** 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl. Environ. Microbiol. **33**:635–641.
12. **Postgate, J. R., and J. R. Hunter.** 1962. The survival of starved bacteria. J. Gen. Microbiol. **29**:233–263.
13. **Robertson, E. A., and J. D. MacLowry.** 1974. Mathematical analysis of the API Enteric 20 Profile Register using a computer diagnostic model. Appl. Microbiol. **28**:691–695.
14. **Saz, A. K., S. Watson, S. R. Brown, and D. L. Lowery.** 1963. Antimicrobial activity of marine waters. I. Macromolecular nature of antistaphylococcal factor. Limnol. Oceanogr. **8**:63–67.
15. **Strange, R.E.** 1968. Bacterial "glycogen" and survival. Nature (London) **220**:606–607.
16. **Wu, S.-Y., and D. A. Klein.** 1976. Starvation effects on *Escherichia coli* and aquatic bacterial responses to nutrient addition and secondary warming stresses. Appl. Environ. Microbiol. **31**:216–220.
17. **Zechman, J. M., and L. E. Casida, Jr.** 1982. Death of *Pseudomonas aeruginosa* in soil. Can. J. Microbiol. **28**:788–794.