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Fate in Model Ecosystems of Microbial Species of Potential Use in Genetic Engineering

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The changes in populations of *Staphylococcus aureus, Bacillus subtilis, Salmonella typhimurium, Klebsiella pneumoniae, Agrobacterium tumefaciens, Rhizobium meliloti*, and *Saccharomyces cerevisiae* were measured after their introduction into samples of sewage, lake water, and soil. Enumeration of small populations was possible because the strains used were resistant to antibiotics in concentrations and combinations such that few species native to these ecosystems were able to grow on agar containing the inhibitors. Fewer than 2 cells per ml of sewage or lake water and 25 cells per g of soil could be detected. *A. tumefaciens* and *R. meliloti* persisted in significant numbers with little decline, but *S. aureus, K. pneumoniae, S. typhimurium, S. cerevisiae*, and vegetative cells of *B. subtilis* failed to survive in samples of sewage and lake water. In sterile sewage, however, *K. pneumoniae, B. subtilis, S. typhimurium, A. tumefaciens*, and *R. meliloti* grew; *S. cerevisiae* populations were maintained at the levels used for inoculation; and *S. aureus* died rapidly. In sterile lake water, the population of *S. aureus* and *K. pneumoniae* and the number of vegetative cells of *B. subtilis* declined rapidly, *R. meliloti* grew, and the other species maintained significant numbers with little or a slow decline. The populations of *S. aureus, K. pneumoniae, A. tumefaciens, B. subtilis*, and *S. typhimurium* declined in soil, but the first four species grew in sterile soil. It is suggested that some species persist in environments in which they are not indigenous because they tolerate abiotic stresses, do not lose viability readily when starved, and coexist with antagonists. The species that fails to survive need only be affected by one of these factors.

Considerable effort is currently being directed to the genetic engineering of microorganisms, and such new genotypes are believed to be of considerable value for many areas of basic and applied research, for producing useful products, and for destroying chemical wastes. These unique genotypes may or will be released into sewage, lake water, or soils as a result of laboratory accidents, inadvertent industrial discharges, or planned introductions. Such introductions may come about to enhance industrial waste treatment, destroy pollutants in aquatic environments, or aid in crop production.

Knowledge is lacking on the persistence, decline, or growth of such microorganisms in ecosystems into which they may be thus introduced. However, considerable attention has been given to the occurrence and potential survival of a few species of human, animal, and plant pathogens in environments in which they are not indigenous. Thus, the frequency of recovery of *Salmonella* has been used as a basis for suggesting the greater survival of members of the genus in freshwater sediments than in the water column (9). McFeters et al. (11) noted a rapid decline of *Salmonella* in well water. Temple et al. (16) found that *Salmonella typhimurium* persisted in a soil amended with feces, whereas Chandler and Craven (2) observed a decrease in *S. typhimurium* in an unamended soil. *Agrobacterium tumefaciens* declined in abundance more readily in nonsterile than in sterile soil (6). It has also been noted that *Staphylococcus aureus* died rapidly after its introduction into marine waters (14).

In view of the paucity of information on the fate in natural ecosystems of a number of the microbial species currently used in research in genetic engineering, a study was initiated to assess the behavior of seven species in samples of sewage, lake water, and soil. For this purpose, a highly sensitive technique was used to assess the population changes.

MATERIALS AND METHODS

A rifampin-resistant isolate of *S. aureus* (strain RN450) was provided by G. M. Dunny, a streptomycin-resistant isolate of *Bacillus subtilis* (strain CU155) by S. A. Zahler, and *S. typhimurium* NK613 and a cycloheximide-resistant isolate of *Saccharomyces cer-*

evisiae (strain GRF104) by G. R. Fink, all of Cornell University. *Klebsiella pneumoniae* KNo was supplied by A. A. Szalay, Boyce Thompson Institute for Plant Research, and a streptomycin-resistant isolate of *A. tumefaciens* ATCC 23308 was provided by M. A. Cole, University of Illinois. A strain of *Rhizobium meliloti* (102F34) resistant to streptomycin and erythromycin was from this laboratory. The major details of the methods used for enumerating these organisms after their introduction into samples of natural ecosystems have been described before (4), but the procedure was modified to give far greater sensitivity in counting than was available heretofore. Each organism was resistant to two or more antibiotics, and selective chemicals were added to media used to count *S. cerevisiae* and *S. typhimurium*. The detection limits were generally 2 cells per ml of sewage or lake water and 17 to 24 cells per g of soil.

Antibiotic-resistant isolates of the bacteria were obtained by serial transfer of the original cultures in liquid media containing increasing concentrations of antibiotics. Stability of the resulting antibiotic-resistant isolates was demonstrated by growing the bacteria in antibiotic-free liquid media for three sequential transfers and observing that plate counts of the final subculture were identical on agar media, which are described below, with and without the antibiotics.

S. cerevisiae was grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.); *S. aureus* in nutrient broth (BBL Microbiology Systems, Cockeysville, Md.); *B. subtilis* and *S. typhimurium* in Trypticase soy broth (BBL Microbiology Systems); *K. pneumoniae* in nutrient broth containing 100 μg of novobiocin, 300 μg of kasugamycin, and 250 μg of cycloheximide per ml; *A. tumefaciens* in yeast extract-mannitol broth containing 25 μg of congo red, 100 μg of erythromycin, and 1.0 mg of streptomycin per ml; and *R. meliloti* in yeast extract-mannitol broth. The yeast extract-mannitol medium contained 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. The organisms were grown at 30°C on a rotary shaker. The incubation periods were 24 h for *S. aureus, B. subtilis, S. typhimurium*, and *S. cerevisiae*, 72 h for *K. pneumoniae* and *A. tumefaciens*, and 120 h for *R. meliloti*. Even after 3 days, when the culture was turbid, this strain of *B. subtilis* produced less than 10 spores per ml in the growth medium. The cells were collected by centrifugation at 4°C for 10 min at 1,470 × *g* for *S. cerevisiae* and 10,400 × *g* for the bacteria, and before their addition to samples of sewage, freshwater, or soil, the cell suspensions were washed with sterile distilled water for *K. pneumoniae* and *A. tumefaciens* and sterile phosphate buffer (580 mg of Na_2HPO_4 and 368 mg of NaH_2PO_4 per liter, pH 7.0) for the other organisms.

The soil, Canadaigua silty loam (pH 6.7, 4.7% organic matter), was dried in air and passed through a 2-mm sieve, and 10-g portions were placed in sterile milk dilution bottles. In instances in which sterile soil was used, the soil was sterilized by irradiation with 2.5 Mrad from a ^{60}Co source. The soil samples were moistened to 90% of field capacity by adding 3.0 ml of the cell suspension, and they then were mixed with the inoculum. The bottles were incubated at 30°C in the dark.

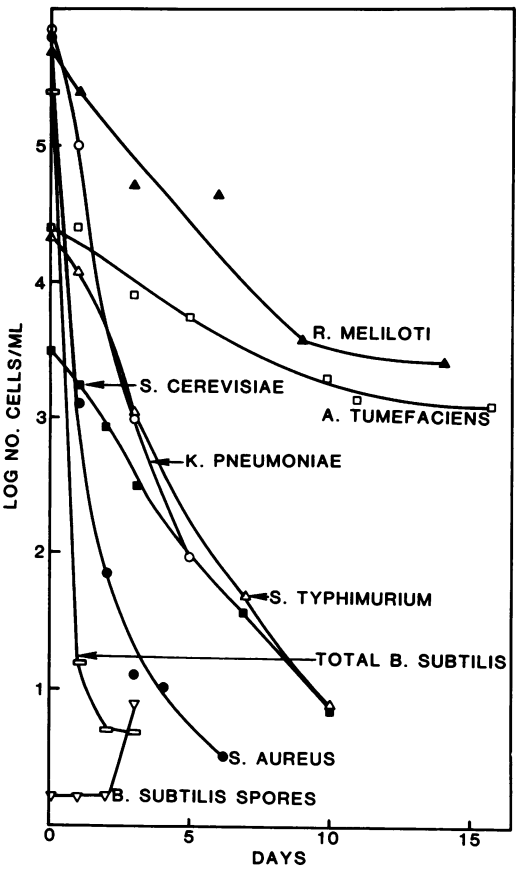

FIG. 1. Changes in numbers of seven microbial species after their addition to sewage.

Samples of raw sewage from the Ithaca, N.Y. sewage treatment plant and water samples from Beebe Lake, Ithaca, N.Y., were taken immediately before the experiments were conducted. If sterile sewage or lake water was desired, portions were sterilized either by filtration of fresh samples through a 0.22-μm membrane filter (Sybron Corp., Rochester, N.Y.) or by irradiation of frozen samples with 2.5 Mrad from a ^{60}Co source. Frozen samples were used to prevent microbial destruction of the organic matter before sterility was achieved. After irradiation of samples of soil, sewage, and lake water, subsamples inoculated into a glucose-asparagine broth (1.0% glucose, 0.05% asparagine, 0.05% K_2HPO_4) failed to show growth in 7 days. Portions of sewage and lake water (250 ml for *S. typhimurium* and *S. cerevisiae* and 100 ml for the other species) were placed in either 250- or 500-ml Erlenmeyer flasks, and the liquids were inoculated with the test organisms. The flasks containing *S. aureus, B. subtilis, K. pneumoniae, A. tumefaciens*, and *R. meliloti* were incubated at 30°C in the dark on a rotary shaker operating at 120 rpm. The samples of *S. typhimurium* and *S. cerevisiae* were incubated at 18°C in the dark without shaking.

Each treatment was replicated three times, except that treatments involving *S. cerevisiae* and *S. typhi-*

murium were replicated twice. Counts were made by the spread-plate technique with 250 μg of cycloheximide per ml of medium to inhibit fungi, except that the agar for counting *S. cerevisiae* contained 5 μg of cycloheximide per ml. *S. cerevisiae* was counted after incubation for 96 h at 30°C on agar containing 6.7 μg of yeast nitrogen base (Difco), 5.0 mg of glucose, 30 μg of rosaniline, 100 μg of erythromycin, and 1.0 mg of streptomycin per ml. *B. subtilis* was counted after incubation at 37°C for 48 h on half-strength Trypticase soy agar (BBL Microbiology Systems) with 50 μg of rifampin and 1.0 mg of streptomycin per ml. Agar containing rifampin was prepared the day it was used, and the plates were dried for 2 h at 50°C. Spore counts of *B. subtilis* were made by heating appropriate dilutions at 80°C for 10 min before plating. *S. aureus* was counted after 72 h at 30°C on nutrient agar with 100 μg of streptomycin and 500 μg rifampin per ml; *S. typhimurium* after 48 h at 30°C on half-strength Trypticase soy agar with 12.5 μg of brilliant green, 100 μg of erythromycin, and 1.0 mg of streptomycin per ml; *K. pneumoniae* after 48 h at 30°C on nutrient agar (BBL Microbiology Systems) with 100 μg of novobiocin and 300 μg of kasugamycin per ml; *A. tumefaciens* after 72 h at 30°C on yeast extract-mannitol agar with 100 μg of erythromycin, 1.0 mg of streptomycin, and 25 μg of congo red per ml; and *R. meliloti* after 120 h at 30°C on yeast extract-mannitol agar with 125 μg of congo red, 10 μg of erythromycin, and 1.0 mg of streptomycin per ml. The antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

After addition of the microorganisms to separate samples of raw sewage at densities of 3.3×10^3 to 6.3×10^5 cells per ml, the populations of *B. subtilis, S. aureus, S. typhimurium, K. pneumoniae*, and *S. cerevisiae* declined rapidly, and 78 cells or fewer per ml were detected at the end of the test periods (Fig. 1). On the other hand, *A. tumefaciens* and *R. meliloti* persisted in significant numbers, and little decline was evident in the last 5 or 6 days of the test periods. The sensitivity of the counting technique is evident from the few cells of the test species that were detected. The population of *B. subtilis* had fallen to 15 cells per ml at day 1, 78 per ml of *K. pneumoniae* at day 5, 10 per ml of *S. aureus* at day 4, and less than 10 per ml of *S. typhimurium* and *S. cerevisiae* by day 10. In contrast, more than 10^3 cells per ml of *R. meliloti* and *A. tumefaciens* were present at the end of the incubation period.

These species behaved differently in sterile sewage. The sewage was sterilized by irradiation for *K. pneumoniae* and by filtration for the other organisms. The initial population of *S. aureus* was maintained for 2 days and then declined to 98 cells per ml by day 8 (Fig. 2). The size of the *S. cerevisiae* population was not appreciably altered in the incubation period. In contrast, the abundance of *A. tumefaciens, K. pneumoniae, R. meliloti*, and *S. typhimurium* increased by 1

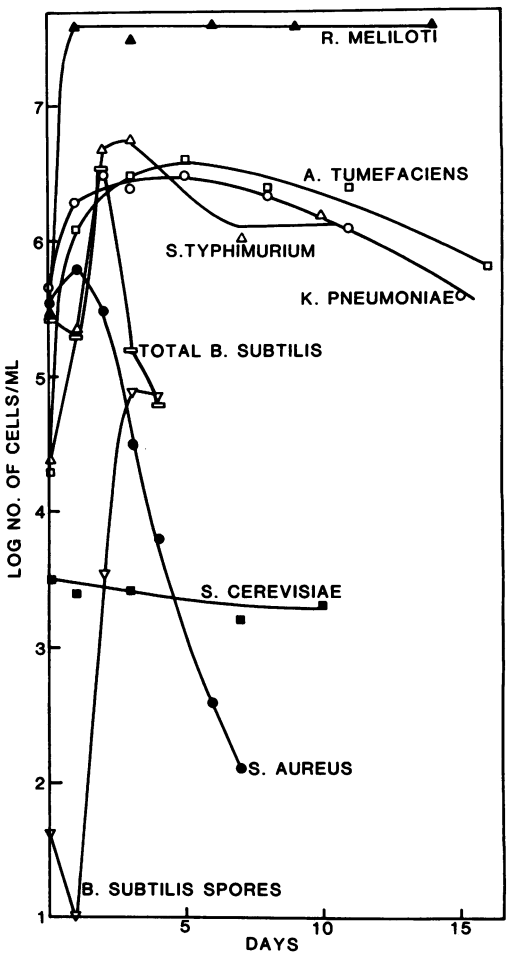

FIG. 2. Changes in numbers of seven microbial species after their addition to sterile sewage.

to 2 logarithmic orders of magnitude, and reasonably large numbers were maintained thereafter. *B. subtilis*, on the other hand, increased in abundance, and then the number of vegetative cells declined; however, the spore density rose so that all cells that remained at the end of the incubation period were spores.

In nonsterile lake water, *A. tumefaciens* declined during the first 4 days, but its population size thereafter remained almost constant at somewhat more than 10^2 cells per ml (Fig. 3). The abundance of *R. meliloti* also fell initially, but its numbers subsequently remained unchanged. None of the other species persisted. The population of *K. pneumoniae* declined rapidly until only 22 cells per ml remained at day 3. *S. cerevisiae* fell to levels at 7 days that were no longer detectable, and similarly, none of the 2.0×10^5 cells of *S. aureus* added per ml could be detected at day 3. *S. typhimurium*, added at an

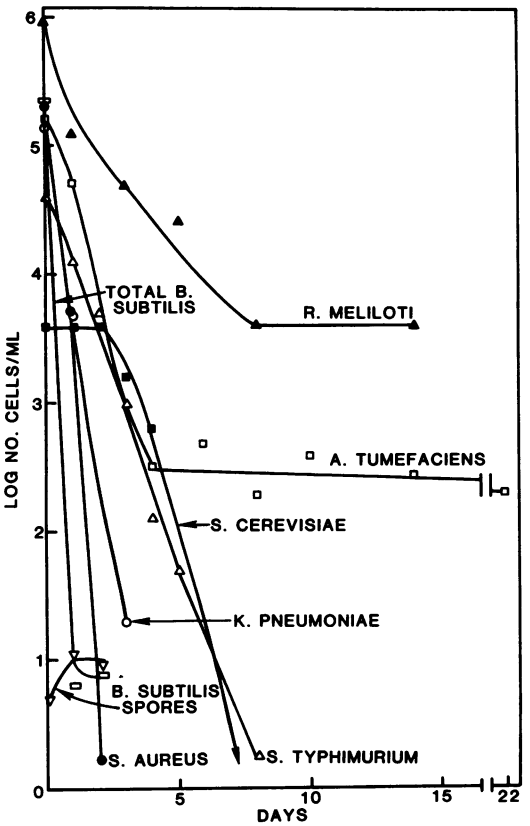

FIG. 3. Changes in numbers of seven microbial species after their addition to lake water.

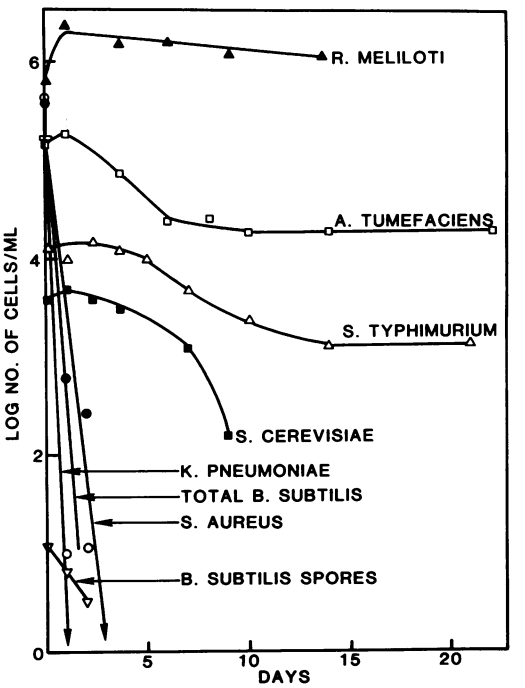

FIG. 4. Changes in numbers of seven microbial species after their addition to sterile lake water.

initial density of 4.2×10^4 cells per ml, fell to less than 33 cells per ml by day 6. The population of *B. subtilis* fell from an initial value of 4.1×10^5 per ml to 8 cells per ml at day 2, whereas the spore density increased from 5 to only 8/ml in the same period.

The behavior of *A. tumefaciens*, *R. meliloti*, *S. typhimurium*, and *S. cerevisiae*, but not of *S. aureus*, *B. subtilis*, and *K. pneumoniae*, differed in sterile lake water from that noted in nonsterile lake water (Fig. 4). Sterilization was accomplished by irradiation in studies of *A. tumefaciens*, *K. pneumoniae*, and *S. aureus* and by filtration in tests of the other organisms. *R. meliloti* grew somewhat in the first day in sterilized lake water, and then it persisted at a level of about 10^6 cells per ml for 2 weeks. *S. typhimurium* maintained its initial number of about 10^4 cells per ml for 5 days and then declined to about 10^3 cells per ml. The decline of *A. tumefaciens* in sterile lake water was slight, and no decline was detected after 8 days. The population of *S. cerevisiae* fell slowly to 2.2×10^3 cells per ml at day 4 and then sharply declined. The density of vegetative cells of *B. subtilis* fell to 12/ml at day 2, and spores were not produced under these conditions. Thus, *S. typhimurium* and *S. cerevisiae* were essentially eliminated from the nonsterile, but not from the sterile, lake water, whereas *S. aureus*, *B. subtilis*, and *K. pneumoniae* died quickly in both nonsterile and sterile lake water.

The survival of *S. aureus*, *K. pneumoniae*, *A. tumefaciens*, *B. subtilis*, and *S. typhimurium* populations introduced into nonsterile soil is shown in Fig. 5. The abundance of *S. aureus* declined rapidly to 3.9×10^2 cells per g on day 2, and this phase was followed by a period of slow decline until no viable cells were found on day 6. *S. typhimurium* declined steadily with time until only 2.5×10^3 cells per g were found at day 14. The size of the populations of *K. pneumoniae* and *B. subtilis* also fell with time, reaching 170 cells per g at day 10 for the first species and 24/g at day 3 for the second. The number of *A. tumefaciens* appeared to increase slightly in the first day and then decreased to 40 cells per g by day 25.

S. aureus, *K. pneumoniae*, *B. subtilis*, and *A. tumefaciens* grew in sterile soil, each reaching populations in excess of 10^7/g (Fig. 6). Thereafter, the number of viable cells of the first three species fell slowly with time, but large numbers were still present at the end of the test period. Under these conditions, *B. subtilis* sporulated profusely.

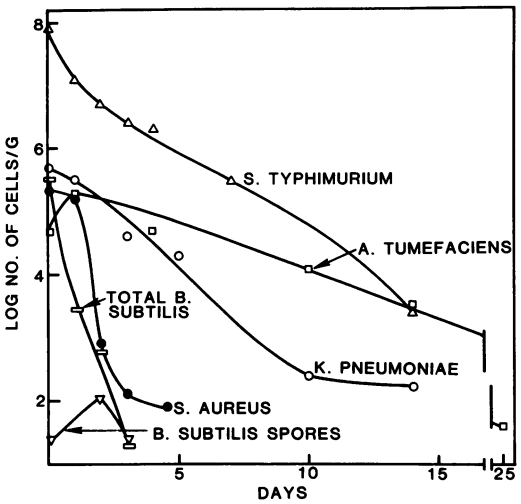

FIG. 5. Survival of five bacterial species in nonsterile soil.

DISCUSSION

Three patterns of behavior are evident from these data. (i) Some species declined rapidly in nonsterile and sterile samples of the natural ecosystems. This pattern was noted for *K. pneumoniae* in lake water and for *S. aureus* and *B. subtilis* in sewage and lake water, although *B. subtilis* grew briefly in sterile sewage. Such organisms probably owe their short persistence either to their sensitivity to abiotic factors (e.g., pH or the presence of inorganic or organic inhibitors), their inability to obtain nutrients from the environment coupled with an intrinsic susceptibility to elimination by starvation, or both. (ii) Some of the organisms declined rapidly in nonsterile samples but persisted or grew in sterile samples. This pattern was evident for *K. pneumoniae*, *S. typhimurium*, and *S. cerevisiae* in sewage, for the latter two organisms in lake water, and *S. aureus* and *B. subtilis* in soil. These species are not destroyed by abiotic stresses. Those that grow are evidently able to obtain organic nutrients from the habitat in the absence of competition, but they may fail to do so in the presence of other species. Their elimination in nonsterile samples indicates that they either are susceptible to predation, parasitism, or lytic enzymes or toxins produced by the indigenous community or are eliminated because they are prone to rapid loss of viability during starvation and are not getting the nutrients they require in the presence of an established community. (iii) A few species declined slowly in natural environments and often reached stable population sizes that were not further reduced in size, and these organisms also grew or persisted in sterile samples. This type of behavior was evident for *A. tumefaciens* and *R. meliloti* in all three environments and for *K. pneumoniae* in soil. Although *R. meliloti* was not tested in soil in this study, published evidence exists showing that this species behaves this way in soil (5). Such organisms are tolerant of abiotic stresses and do not die out readily when starved. Their slow decline may be a reflection of their being under attack by protozoa, as has been shown for species of *Rhizobium* and for *Xanthomonas campestris* (5, 7), but the number of cells coexisting with protozoa is less than expected (1). Such organisms presumably would not be eliminated by protozoa. In view of the hypothesis that bacteria may coexist with their protozoan predators by reproducing to replace the prey cells that are consumed (1), a species unable to obtain organic nutrients and thus one unable to replace the consumed cells would be eliminated; these are organisms of the second type.

The strain of *B. subtilis* persisted in environments in which the supply of organic nutrients permitted the formation of a resistant structure, but not in environments where the nutrient level was reduced by other inhabitants so that the resistant structure was not produced. Thus, this bacterium formed spores in large numbers in the nutrient-rich sterile sewage and soil but not in nutrient-poor sterile lake water or in nonsterile samples of all three environments.

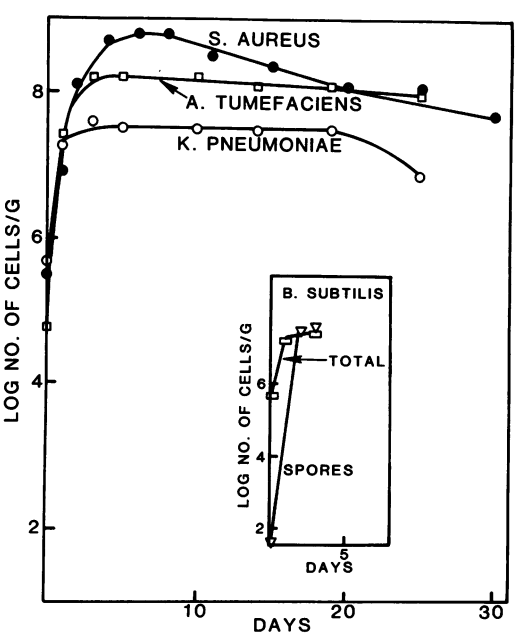

FIG. 6. Growth of four bacterial species in sterile soil.

A large literature exists on the decline or occurrence in soils, sewage, and natural waters of individual species of bacteria of importance in public health and of plant pathogenic fungi. It was not the purpose of this study to enlarge the data base on populations in environments in which they are not indigenous, but rather to seek explanations for the persistence or decline of species introduced into sewage, freshwater, and soil, and this report represents the first phase of an inquiry designed to establish the reasons for persistence or elimination. In addition, the approach used here has the advantage of relying on a far more sensitive counting technique than is commonly used.

A comparison of these data with published information is worthwhile. For example, McFeters et al. (11) reported a rapid decline of *Salmonella* in well water, whereas Temple et al. (16) found that *Salmonella* introduced into soil with feces survived for some time. It has long been accepted that species of *Bacillus* occur in soil principally as spores (3) so that it is not surprising that the vegetative cells lost viability readily in the present study. The lack of survival of *S. aureus* in all three test environments is in agreement with the finding of Saz et al. (14) of a rapid decline of this species in marine waters. A decline of *Klebsiella* sp. in drinking water distribution systems has also been noted (12). *A. tumefaciens* was found to decline more markedly in nonsterile than in sterile soil (6).

A decline in abundance in nonsterile but not in sterile soil has been reported for several species of *Rhizobium* (5). The decline apparently is not a result of an attack by *Bdellovibrio*, but rather by protozoa (8, 10). The rhizobia are not eliminated but rather remain in reasonably large numbers, apparently because the protozoa feeding on them are unable to reduce the size of this prey population when its density is low (1). In addition to protozoa, other inhabitants of natural ecosystems, such as *Bdellovibrio* (17), have been postulated as having a role in the decline of introduced species; however, neither *Bdellovibrio* nor bacteriophages are believed to eliminate low densities of the species they attack (1). Toxins are believed to suppress certain species introduced into natural waters (12, 14). Starvation associated with a paucity of organic nutrients may result in a loss of viability of some organisms (13, 15), and extremes of light intensity, salinity, temperature, or pH may cause reductions in the number of cells of some introduced species.

These studies have a bearing on assessments of the risks associated with the deliberate or inadvertent release of new genotypes into natural waters or soils. Components of such risk analyses include the probabilities of release, survival, growth, and bringing about some undesirable environmental or ecological modification. For species deliberately introduced into these ecosystems, the value of the first probability is 1.0. If the value of any of these probabilities is 0.0, then no risk exists. The present inquiry is directed at survival only, and the data show that some of the organisms of interest in genetic engineering do in fact survive in large numbers. For organisms of these types, an analysis of risk must therefore be extended to include the other probabilities. This is particularly true of an organism, such as *A. tumefaciens*, that is known to be a plant pathogen, and hence the probability of its causing a deleterious change in a suitable host population cannot be assumed to be nil.

ACKNOWLEDGMENT

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