# Survival of Soil Bacteria during Prolonged Desiccation

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A determination was made of the kinds and numbers of bacteria surviving when two soils were maintained in the laboratory under dry conditions for more than half a year. Certain non-spore-forming bacteria were found to survive in the dry condition for long periods. A higher percentage of drought-tolerant than drought-sensitive bacteria were able to grow at low water activities. When they were grown in media with high salt concentrations, bacteria generally became more tolerant of prolonged drought and they persisted longer. The percent of cells in a bacterial population that remained viable when exposed to drought stress varied with the stage of growth.

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Most microorganisms which are capable of surviving for long periods in dry soil form specialized resistant structures such as endospores, cysts, conidia, chlamydospores, or sclerotia. However, certain bacteria, though unable to form endospores, can also retain their viability during prolonged droughts. Thus, Arthrobacter and selected rod-shaped bacteria withstand long periods of desiccation, while other bacteria soon die out (10,11). Although occasional reports have been concerned with the decrease in bacterial cell numbers during the drying of soils (5,13,14), studies of microbial survival in such environments and explanations for the persistence of these long-lived species are few.

The present study was designed to isolate and characterize bacteria susceptible and resistant to prolonged drying. An attempt was also made to modify the ability of the isolates to endure the stress of protracted desiccation.

## MATERIALS AND METHODS

Two soil samples, a Collamer silt loam and a Honeoye silt loam, were collected from sites near Ithaca, N.Y. The former soil contained 2.6% organic matter, 0.14% nitrogen, and had a pH of 6.2, while the latter contained 3.4% organic matter, 0.18% nitrogen, and had a pH of 5.9. The soil samples were passed through a 1.0 mm diameter sieve immediately after collection. Five grams of each soil was placed in a 35 mm diameter petri dish. Replicate dishes for each sample were incubated at 30 C in a desiccator over calcium chloride, and dishes of each sample were removed at fixed intervals for an assessment of the number and identity of the

survivors. The numbers of viable bacteria were determined by plating dilutions of the soils on soil extract agar amended with 0.2% yeast extract (7), asparagine-mannitol agar (17), and nutrient agar containing 0.0002% crystal violet (A. J. Holding, J. Appl. Bacteriol., 17:XVI, 1954). Three replicate plates were poured for each sample, and the plates were incubated at 30C and examined daily for 14 days. Random colonies were selected and their cellular and colonial morphologies recorded. The microorganisms were categorized by the procedure of Taylor & Lochhead (16).

Soil isolates were grown in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth. Rhizobium strains were grown in yeast extract-mannitol solution (2). The cultures were incubated at 30 C on a rotary shaker operating at 120 rpm. The cells were collected during the stationary phase by centrifugation and washed three times with 0.10 M phosphate buffer (pH 7.2), except as otherwise stated. A 0.25 ml portion of this cell suspension was applied uniformly to 6.0 g of sterilized quartz sand contained in a 35 mm diameter petri dish. About $2 \times 10^8$ viable cells were thus added to each dish. Six to ten plates for each organism were maintained at 30 C in a desiccator containing calcium chloride. Dishes were withdrawn from the desiccator at regular intervals, and the numbers of survivors were enumerated by the dilution technique on triplicate plates of nutrient agar or, for Rhizobium, on yeast extract-mannitol agar.

Bacteria were isolated from the soils during the period of drying. Isolates which survived a 10 min heating at 85 C and those which were found by microscopic examination to be actinomycetes were discarded. Strains differing in either colonial or cell morphology and capable of surviving the dry conditions for more than 15 days (considered as drought

resistant) and dissimilar strains which perished in 1 to 4 days of exposure to the dry circumstances (considered as drought susceptible) were collected in this way from the soils, all the isolates being non-spore-forming bacteria. In addition, three drought-susceptible Rhizobium strains were investigated.

For studies of the ability of bacteria to withstand dry conditions after the osmotic tension of the cell had apparently been raised, five drought-resistant and five sensitive bacteria were selected from among 17 resistant and 15 susceptible isolates. The lower limit of the water activity ($a_w$) that permitted growth of these microorganisms was ascertained. The cultures were then grown in either nutrient broth or, for Rhizobium, in yeast extract-mannitol broth containing a mixture of NaCl, KCl, and $Na_2SO_4$ in a molar ratio of 5:3:2. The salts were added to the medium to give the desired water activity. The $a_w$ values of the media were derived from the chart of Scott (12), and the liquid media were adjusted to an $a_w$ value of 0.999 by the techniques given by Stokes (15). The tolerance of the bacteria to drought stress was determined using cells grown in nutrient broth or yeast extract-mannitol broth and in the medium with the minimum $a_w$ just permitting growth of the organism. The cells were washed three times with a salts solution having the same $a_w$ as the growth medium, and they were then suspended in a salts solution (0.335% NaCl, 0.255% KCl and 0.327% $Na_2SO_4$) with an $a_w$ value of 0.995. The initial cell densities were approximately the same. The survival was determined by plate dilution on triplicate plates of nutrient agar or yeast extract-mannitol agar. The bacteria were counted after exposure to drought stress for varying periods of time.

# RESULTS

The changes in the microbial community induced by drying are presented in Table 1 for Honeoye silt loam and in Table 2 for the Collamer silt loam. The absence of a value from the table indicates that the cell density was too small to be detected by the procedures used. The non-spore-forming bacilli were categorized into sub-groups according to their length and pigmentation as well as gram reaction, but these data are omitted inasmuch as all categories were about equally susceptible to drought stress as measured by the techniques employed.

The results show that all groups were affected by the absence of water, but marked differences existed in the resistance of the various microbial types. Although the abundance of propagules of actinomycetes and Bacillus declined rapidly, a significant number of these organisms persisted for the 7 or 11 month period, presumably by virtue of the conidia and endospores they form, so that the community after several months of drought was composed solely of these two groups. The asporogenous gram-positive rods are eliminated within the first two weeks, but a few of the cocci and gram-negative bacilli endure somewhat longer. Particularly interesting is the longevity of Arthrobacter propagules, a few surviving for two to three months in these dry conditions. The same trends as listed in the tables with two media were observed in asparagine-mannitol agar, but the counts in this medium were lower than those observed in soil extract-yeast extract agar but higher than in the crystal violet-containing medium.

A short gram-positive rod, strain R1, was used to study the effect of culture age on survivability. The bacterium was grown on a shaker at 30 C in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth, the

cells were collected at three time intervals, washed three times with 0.1 M phosphate buffer, pH 7.0, and then added to sterilized quartz sand contained in petri dishes. The dishes were incubated for 20 days at 30 C in a desiccator over $CaCl_2$. Using 4.0, 2.8, and 2.5 x $10^8$ cells from 17, 66, and 144 hour cultures, a total of 2,500, 270, and less than 10 surviving cells were found after 20 days. Hence, the age of the culture affects the ability of the cells to endure drought stress.

Non-spore-forming bacteria apparently resistant and susceptible to drying were isolated and grown in nutrient broth. The cells were collected during the stationary phase, they were washed three times in phosphate buffer, and added uniformly over the surface of sterile quartz sand contained in petri dishes. Samples were taken at regular intervals from replicate dishes of each organism that had been kept in a desiccator over calcium chloride. Representative data are given in Fig. 1. The three susceptible strains endured for less than 4 days, while the two resistant cultures maintained themselves in reasonable, although modest, abundance for more than 10 days. The curves with these five pure cultures are in agreement with the persistence of the organisms in natural soil.

The minimum $a_w$ value for growth of the bacteria was determined by inoculating the organisms into nutrient broth or yeast extract-mannitol broth adjusted to various water activities, and the presence or absence of turbidity was recorded after a 7-day incubation at 30 C. The organisms studied were 17 strains resistant and 15 strains susceptible to drought stress. The data of Table 3 show that of the 17 resistant strains, 10 were capable of growing in a medium with an $a_w$ value of 0.96 or lower, whereas only 2 of the 15 susceptible bacteria were able to multiply in these solutions.

A comparison was made of the survivability of bacteria grown in nutrient broth or yeast extract-mannitol broth at an $a_w$ value of 0.999 and in the same medium adjusted with salts so that it had an $a_w$ level which just permitted growth of the organism. The minimum $a_w$ value allowing for growth of the five drought-susceptible bacteria used was 0.985, 0.960, 0.995, 0.990, and 0.985 for Flavobacterium $S_1$, Pseudomonas $S_3$, Rhizobium $S_4$, and gram-negative rods $S_2$ and $S_5$, respectively, and the minimum $a_w$ values for the five drought-resistant bacteria were 0.880, 0.940, 0.980, 0.960, and 0.960 for gram-positive rod R1, Arthrobacter R2, and gram-negative rods R3, R4, and R5. Growth of all five drought-susceptible bacteria in media with the high salt levels, a treatment which presumably increased their internal osmotic pressure, allowed the isolates to survive longer in dry conditions. Among the drought-resistant bacteria, growth of strains R1, R3, and R4 in salt-rich fluids increased their ability to survive in dry circumstances, but the persistence of propagules of strains R2 and R5 was the same whether grown in salt-rich or salt-poor media. Representative data for three strains are shown in Fig. 2.

Attempts were made to isolate resistant variants from the susceptible strains by using the individuals remaining viable longest in the dry conditions. The survivors were then grown in nutrient broth and re-exposed to the drought stress. Though many attempts were made, no increase in drought resistance was observed. Moreover, resistant mutants were not obtained when the susceptible population was exposed to UV light or chemical mutagens.

To determine whether the ability to withstand drought could be increased by acclimating the culture to low $a_w$ values, the 10 strains

listed above were cultured initially in a medium with the lowest $a_w$ permitting their growth. When proliferation was evident, aliquots were transferred to solutions of slightly lower $a_w$ values, and when growth was noted, the cells were inoculated into a medium with still lower $a_w$ values. By these means, it was found possible to grow $S_1$ and $S_2$ in nutrient broth with an $a_w$ of 0.980. The organisms were then propagated in nutrient broth with various water activities. The cells were collected, washed three times with a salts solution of the same ionic strength as the growth medium, and then suspended in a salts solution with an $a_w$ of 0.995. After adjusting the cell suspensions to the same optical density, 0.25 ml was added to 6 g sterile quartz sand contained in 35 mm diameter petri dishes. The dishes were incubated in a desiccator over $CaCl_2$ at 30 C, and dishes were removed at regular intervals for viable counts using nutrient agar as a counting medium.

The results of Fig. 3 show that the survival of strain $S_2$ was directly related to the salt concentration in the medium, cultures grown in solutions with increasing salt levels persisting for longer periods. Longevity was even shorter when the $a_w$ level of the medium was 0.999. In this test, the organism grown at $a_w$ of 0.980 and 0.985 was the salt-acclimated strain, whereas the original culture was used in broth with water activities of 0.990, 0.995, and 0.999.

Essentially the same results were obtained with strain $S_1$, the period required for eliminating all survivors being 12, 7, and 4 days for the original strain grown in solutions with $a_w$ values of 0.990, 0.995, and 0.999, whereas $10^2$ cells of the acclimated culture grown in medium with $a_w$ of 0.980 were still present at 12 days. Hence, the higher the salt concentration of the medium in which the cells were propagated,

the more likely was a proportion of the population to endure extremely dry conditions.

When tested by the method of Christian and Waltho (3), the cell water content of strain $S_1$ was found to have changed with cultural conditions. Thus, the cell water content was 1.82 g water per g dry wt after 2 days of growth in nutrient broth, but the figure was 1.41 g water per g dry wt when the organism was grown 8 days in salt-amended nutrient broth having an $a_w$ value of 0.985. The 8-day incubation was used in the latter instance because of the organism's slow growth in that medium.

## DISCUSSION

The results shed additional light on the behavior of soil microorganisms exposed to extremely dry conditions. Certain soil bacteria are capable of withstanding drought for a long period, and yet they may not be protected by colloids, or they may be unable to form endospores. Understanding the physiological basis for their durability poses a challenging problem. It is possible that drought resistance is associated with a cell surface relatively impervious to water loss, so that the bacterium is able to retain its internal water, or with intracellular colloids not irreversibly affected by water loss. The data presented herein suggest that there is a relationship between high internal osmotic tension or the capacity to develop at low water activities and the ability to persist through prolonged drought.

It is known that the osmotic tension of bacterial cells can be raised if the organisms are placed in appropriate solutions (1, 6, 9). In the present report, it has been shown that cultural conditions may

indeed alter the cell water level. The decrease in cell water content and the concomitant change in internal solute concentration of Staphylococcus aureus in relation to water activity has already been well studied (3, 4), and on the basis of such published observations, it seems quite likely that growth of the bacteria in media with low $a_w$ values increases the cell's internal osmotic tension as it increases the organisms resistance to drought stress.

Though the present data demonstrate that a greater percentage of drought-tolerant bacteria exhibit resistance to high salt concentrations or possibly have a higher internal osmotic tension than is found among the susceptible bacteria, some of the drought-intolerant isolates did indeed grow in media with low $a_w$ values. Conversely, some resistant bacteria multiplied only in media with high $a_w$ levels. Therefore, the ability to multiply in salt-rich circumstances or intracellular osmotic tension is not the sole factor protecting cells from drought inactivation, although it seems to be a significant determinant. The importance of this factor is further supported by the observation that all of the drought-susceptible isolates became more tolerant to drying as the cells were grown in media with low $a_w$ values.

The influence of growth stage on the osmotic tension of the organisms used in the present inquiry is unknown. However, Mitchell and Moyle (9) noted that Escherichia coli remained viable in distilled water if collected in the stationary phase, whereas many of the cells became nonviable if they were harvested in the exponential phase, and they proposed that the effect of age on the osmotic fragility and viability of the organism may result from differences in its internal osmotic pressure. Similar age-controlled differences in osmotic tension may account for the present findings, too. Age of the culture from which the

cells are taken also affects the resistance of the diatom Stauroneis anceps to dehydration (8).

## ACKNOWLEDGMENT

This investigation was supported by research grant NGR-33-010-013 from the National Aeronautics and Space Administration.

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TABLE 1. Survival of bacteria in desiccated Honeoye silt loam

| Microbial group | Counts (X $10^3$/g) at various days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 7 | 15 | 30 | 60 | 90 | 150 | 210 |
| | Nutrient agar with crystal violet | | | | | | | | |
| Total count | 18,000 | 140 | 119 | 13 | 15 | 21 | 14 | 20 | 23 |
| Actinomycetes | 10,000 | 33 | 2.3 | 4.5 | 9.5 | 6.8 | 9.0 | 15 | 18 |
| Spore formers | 670 | 53 | 7.0 | 8.1 | 4.6 | 10 | 5 | 5 | 5 |
| Arthrobacter | 670 | | 6.7 | | 0.33 | 2.0 | | | |
| Gram-neg. rods | 6,000 | 54 | 84 | 0.2 | 0.1 | 2.2 | | | |
| Gram-pos. rods, asporogenous | | | 19 | | | | | | |
| Cocci | 670 | | | | | | | | |
| | Soil extract-yeast extract agar | | | | | | | | |
| Total | 47,700 | 11,000 | 6,300 | 5,800 | 4,800 | 4,700 | 3,000 | 1,500 | 1,100 |
| Actinomycetes | 24,900 | 3,800 | 2,500 | 2,900 | 3,200 | 2,500 | 1,400 | 300 | 110 |
| Spore formers | 6,000 | 2,700 | 1,600 | 2,200 | 1,300 | 1,600 | 1,300 | 1,200 | 1,000 |
| Arthrobacter | 670 | 900 | 500 | 600 | 270 | 400 | 300 | | |
| Gram-neg. rods | 8,900 | 1,200 | 500 | | | 200 | | | |
| Gram-pos. rods, asporogenous | 4,100 | 1,300 | 1,000 | | | | | | |
| Cocci | 3,100 | 1,100 | 200 | 100 | | | | | |

TABLE 2. Survival of bacteria in desiccated Collamer silt loam

| Microbial group | Counts ($X 10^3$/g) at various days | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 7 | 15 | 30 | 60 | 90 | 150 | 210 | 330 |
| **Nutrient agar with crystal violet** | | | | | | | | | | |
| Total count | 1,150 | 340 | 105 | 115 | 15 | 13 | 7.9 | 4.5 | 3.4 | 3.0 |
| Actinomycetes | 500 | 100 | 2.7 | 84 | 8 | 8 | 5.8 | 2.5 | 2.4 | 2.5 |
| Spore formers | 50 | 25 | 15 | 16 | 6 | 5 | 2.1 | 2.0 | 1.0 | 0.5 |
| Arthrobacter | 50 | 45 | 20 | 15 | 0.6 | | | | | |
| Gram-neg. rods | 550 | 130 | 54 | | 0.6 | | | | | |
| Gram-pos. rods, asporogenous | | 30 | 13 | | | | | | | |
| Cocci | | 10 | | | | | | | | |
| **Soil extract-yeast extract agar** | | | | | | | | | | |
| Total | 43,000 | 5,400 | 4,600 | 3,000 | 3,300 | 2,700 | 1,600 | 1,200 | 1,000 | 700 |
| Actinomycetes | 18,000 | 2,500 | 2,100 | 1,600 | 2,100 | 1,500 | 900 | 200 | 300 | 200 |
| Spore formers | 7,000 | 1,500 | 1,200 | 1,300 | 1,100 | 1,000 | 690 | 1,000 | 700 | 500 |
| Arthrobacter | 6,000 | 540 | 400 | | 130 | 100 | | | | |
| Gram-neg. rods | 3,500 | 670 | 100 | 67 | | 100 | | | | |
| Gram-pos. rods, asporogenous | 5,500 | 130 | 400 | | | | | | | |
| Cocci | 3,000 | 100 | 400 | 67 | | | | | | |

TABLE 3. Minimum water activities allowing for microbial growth

| Minimum $a_w$ value for growth | Bacteria growing at the $a_w$ value | |
|---|---|---|
| | Drought-resistant strains | Drought-susceptible strains |
| 0.880 | 1 Gram-pos. rod | 0 |
| 0.920 | 1 *Micrococcus* | 0 |
| | 1 Gram-neg. rod | |
| 0.940 | 2 *Arthrobacter* strains | 1 Gram-neg. rod |
| | 1 Gram-neg. rod | |
| 0.960 | 3 Gram-neg. rods | 1 *Pseudomonas* |
| | 1 Gram-pos. rod | |
| 0.980 | 4 Gram-neg. rods | 1 Gram-pos. rod |
| | 1 *Arthrobacter* | |
| | 1 Gram-pos. rod | |
| 0.985 | 0 | 2 Gram-neg. rods |
| | | 1 *Flavobacterium* |
| 0.990 | 1 Gram-neg. rod | 3 Gram-neg. rods |
| | | 1 *Rhizobium* |
| 0.995 | 0 | 3 Gram-neg. rods |
| | | 2 *Rhizobium* strains |

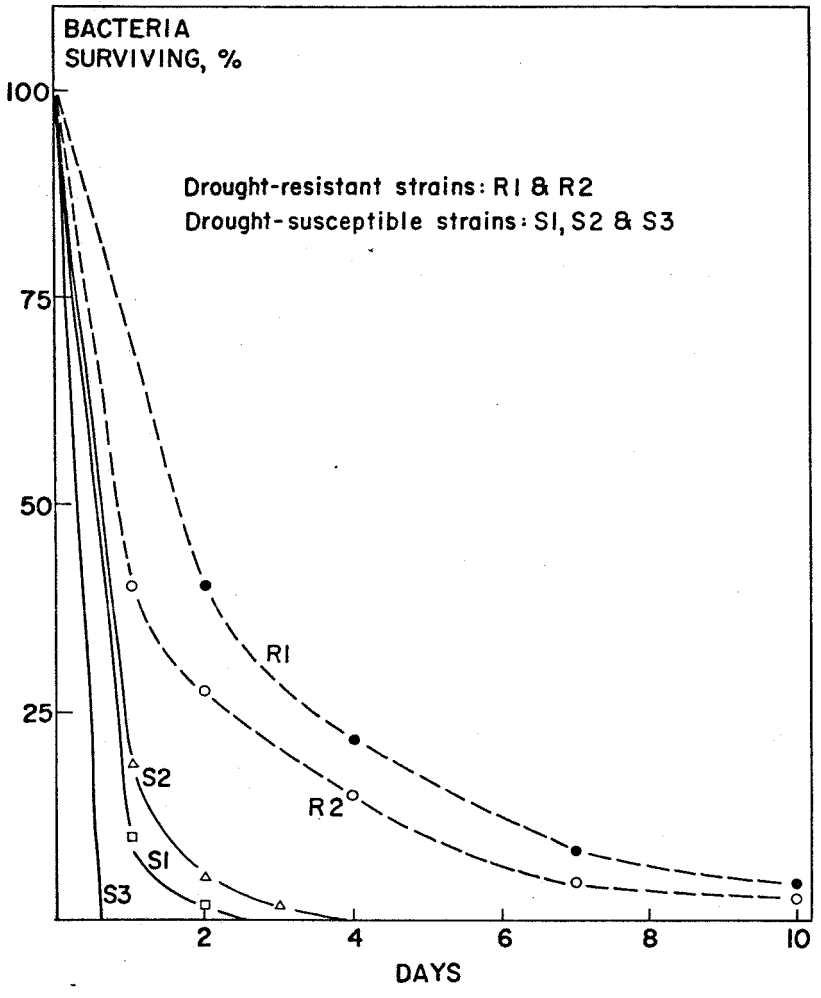


FIG. 1. Survival of drought-resistant and drought-susceptible soil bacteria in dry quartz sand.

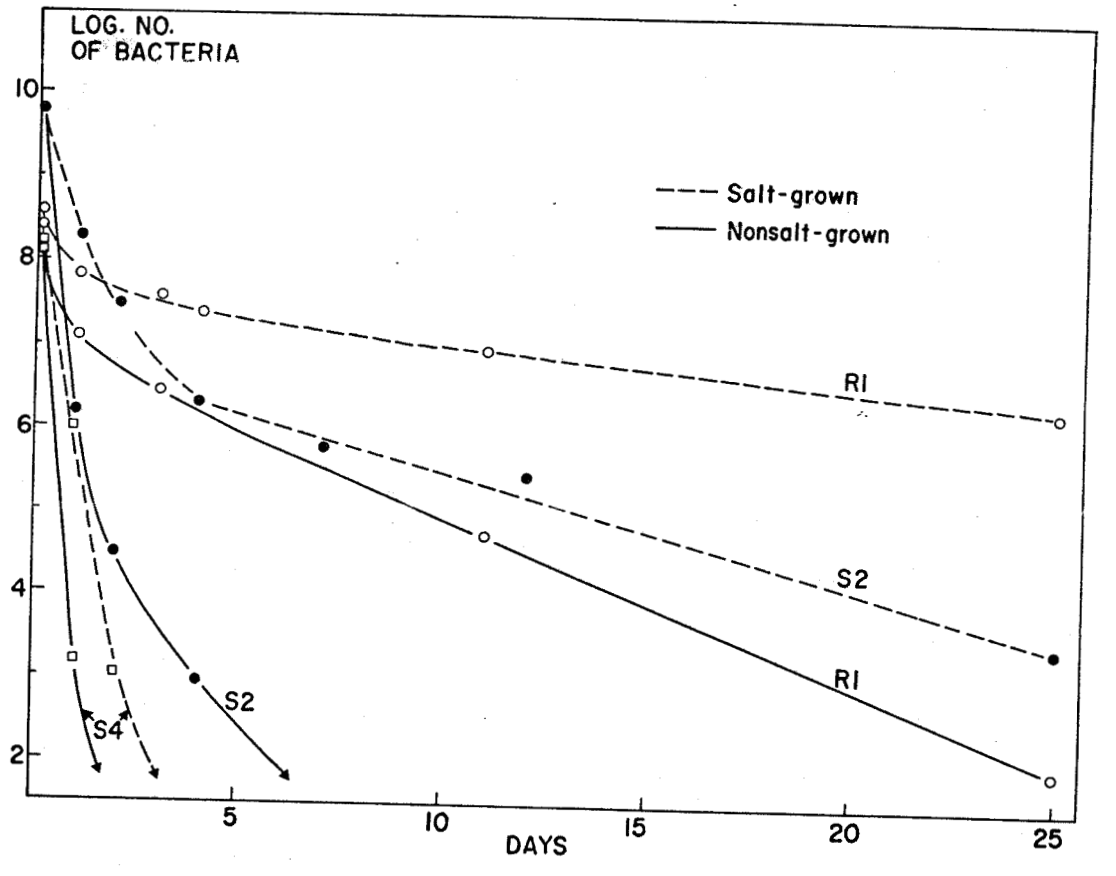


FIG. 2. Survival of three bacterial strains grown in solutions rich in salt or in media containing no supplemental salt.

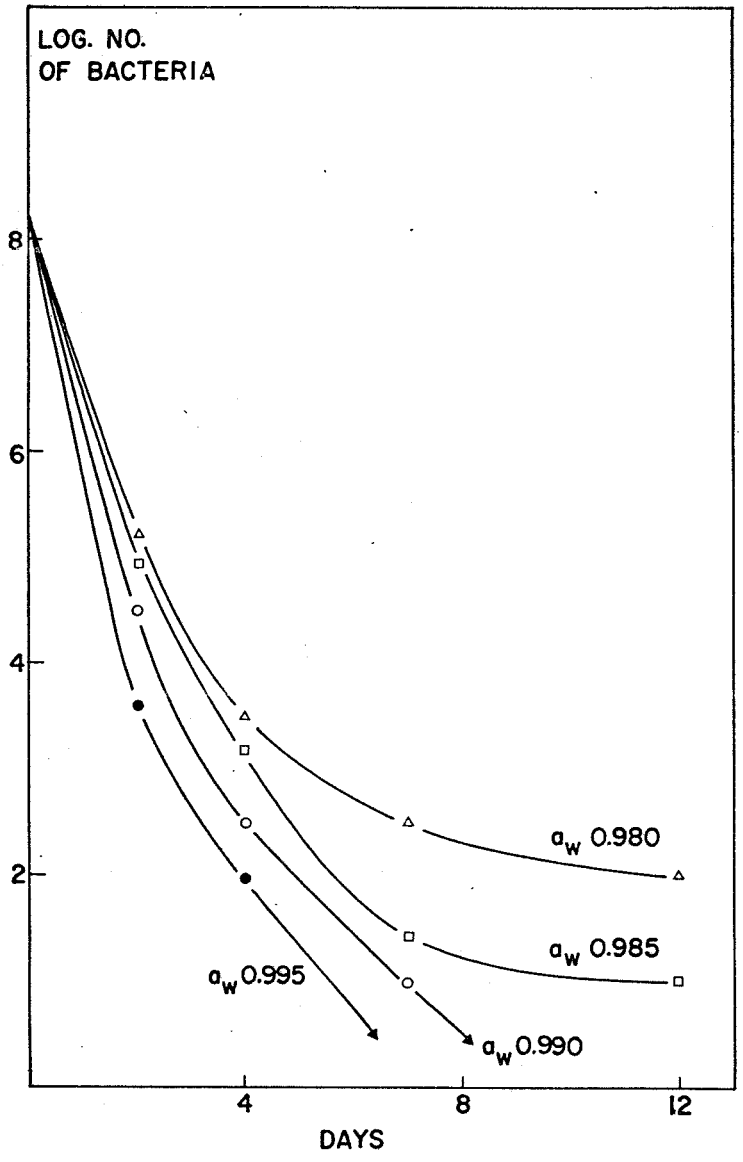


FIG. 3. Effect of growing strain $S_2$ in media of different $a_w$ levels on its ability to survive dry conditions.