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# Survival of *Azotobacter* spp. in Dry Soils

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**Dry soils stored in glass containers in the laboratory and protected from contamination for periods of 22 to 24 years yielded numerous colonies of *Azotobacter chroococcum* and other members of the family *Azotobacteraceae*. These results were compared with those reported in 1974, and the findings are uniformly consistent in terms of surviving populations. The data prove that these bacteria remain viable after prolonged periods of dormancy in much the same way as do the endospores of gram-positive bacteria.**

According to Winogradsky (11), the cyst form of the azotobacter was first observed and described by Prazmowski and by Omeliansky in their early reports on azotobacters. In 1938 (11), Winogradsky described the production of cysts in cultures of *Azotobacter vinelandii* and *A. chroococcum* in which alcohols or organic acids served as the source of carbon and energy. Because of their similarity to the cysts of protozoa, Winogradsky assumed that the azotobacter cysts were the resting stage in a complex life cycle of the organism. Pochon and Tchan (4) confirmed his observations, and later, Socolofsky and Wyss (5) showed that cultures of *A. vinelandii* which contained cysts survived desiccation for 2 years whereas those that contained no cycts died rapidly. In 1974, Vela (7) showed that azotobacters in dry soils remained viable for more than 12 years while cysts survived in dried agar cultures for 10 years. While no reports exist in the readily available literature which show that the azotobacters in nature exist in the cyst form, Bae et al. (1) found structures in situ in the soil which resembled azotobacter cysts.

In 1964, Vela and Wyss (10) found that laboratory cysts were many orders of magnitude more sensitive to gamma rays than were the soil forms of the same organisms. While it was assumed that the organisms in the soil were some form of naturally occurring soil cyst, they were not detected even after rigorous microscopic examination of many soil samples by using a staining procedure specific for azotobacter cysts (9). The survival of azotobacters in dry soil stored in glass containers in the laboratory for extended periods of time suggests that these gram-negative bacteria possess characteristics similar to those of the spores of gram-positive bacteria. This report and the previous report (7) constitute the only evidence of the extended survival of azotobacters in dry soils.

## MATERIALS AND METHODS

**Soils.** Forty-seven soils obtained from various locations in the area of Austin, Texas were stored in sterilized screw-cap glass containers in the years from 1961 to 1963. These have been preserved carefully by one of the authors (G.R.V) since that time. All samples are stored in a cardboard box which is kept at constant temperature (25.5 ± 2.2°C; $P > 0.95$) on a shelf which is never disturbed by staff personnel, students or others. The soil tubes have been opened, aseptically, only in 1973, 1979, and 1985 when the soils were sampled. Some soil samples tested in 1973 or 1979 or both were used in other experiments and were not available for the tests reported here, while others were not tested at this time to save the small quantity of soil remaining for a later time.

**Cultures.** The vials were placed in a UV-sterilized, dust-free hood, and both stoppers and vials were wiped with sterile gauze pads soaked in 70% ethanol. They were opened and flamed, and some of the soil from each was sprinkled on the surfaces of three plates of modified Burk agar medium (8). These were incubated at 26 to 28°C for 3 to 5 days.

**Identification.** Each colony that showed characteristics commonly ascribed to *Azotobacter* spp. was counted, but only two or three colonies from each plate were subcultured on Burk agar and nutrient agar plates; the organism was thus obtained in pure culture. Other tests required to establish the identity of the organisms isolated were performed by the methods of Thompson and Skerman (6). Soils that yielded colonies which were easily recognizable as those of azotobacter were scored as positive while the rest were scored as negative. When the results were ambiguous, subcultures were made on Burk agar and nutrient agar plates. When the identity of *Azotobacter* spp. could not be readily and unambiguously determined, the results were recorded as negative.

## RESULTS

The data in Table 1 show that azotobacters survive in dry soils for periods of 22 to 24 years. Some soils, such as I, 150-Control, 1-Control and 1.1-Control, which yielded viable azotobacter cells in 1973, did not do so in 1979. One soil, A, yielded azotobacters in 1973 and 1979 but not in 1984. As expected, all soils which were previously negative were also negative on subsequent tests, e.g., I, 250 KR, 300 KR, and 3.3. The soils described in Table 2 were exposed to gamma radiation from a $^{60}Co$ source at the time of collection and are included here, not for that reason, but simply to increase the number of samples in the experiment. The number of colonies is given not as an effort to describe the size of the population but simply as an indication that survival was not ascertained on the basis of a few colonies on a given plate. The number shown on the last column of the tables was the average of three plates rounded to the closest increment of 5 (e.g., soil East had 15, 27, and 25 colonies on three plates for an average of 22 and was reported in Table 1 as having 20).

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TABLE 1. Survival of azotobacter in dry soil stored in the laboratory at room temperature

| Original designation | Origin | Soil type[a] | Date of collection | Survival when tested in (year)[b]: 1973 | 1979 | 1984 | Avg no. of colonies per plate[c] |
|---|---|---|---|---|---|---|---|
| East | San Antonio | C | 1961 | + | + | + | 20 |
| Southside | San Antonio | S | 1961 | + | + | + | 15 |
| Mat West | San Antonio | C | 1961 | + | + | + | 25 |
| Cadillac | San Antonio | S | 1961 | + | + | + | 10 |
| Shadywood | Austin | C | 1961 | + | + | + | 5 |
| 12-700 | Austin | C | 1961 | + | + | + | 5 |
| 12-000 | Austin | C | 1961 | | + | | |
| 12-800 | Austin | C | 1961 | + | | − | 0 |
| 12,2,200 | Austin | C | 1961 | + | | + | 15 |
| | Mission | S | 1961 | + | + | + | 15 |
| | Midland | S | 1961 | + | | − | 0 |
| A | Austin | C | 1962 | + | + | − | 0 |
| I | Austin | C | 1962 | + | − | − | 0 |
| J | Austin | C | 1962 | + | | + | 5 |
| 250-Control | Austin | C | 1962 | + | + | + | 5 |
| 300-Control | Austin | C | 1962 | | + | | |
| Control | Austin | C | 1963 | + | + | + | 10 |
| 150-Control | Austin | C | 1963 | + | − | | |
| 3.3-Control | Austin | C | 1963 | + | + | + | 20 |
| 1-Control | Austin | C | 1963 | + | − | | |
| 1.1-Control | Austin | C | 1963 | + | − | | |
| | Waco | C | 1963 | + | | | |
| Downtown | Austin | S | 1963 | + | + | | |
| | Manor | S | 1963 | + | | + | 5 |
| | San Marcos | C | 1963 | + | + | | |
| | Brooks | C | 1963 | + | + | + | 5 |
| 2-8-63 | Austin | S | 1963 | | | + | 20 |
| C | Austin | S | 1963 | | | + | 20 |
| 61-63 | Austin | C | 1963 | | | + | 20 |
| Control 5-23-63 | | | 1963 | | | + | 10 |
| Control -9-63 | | | 1963 | | | + | 10 |

[a] Abbreviations: C, chernozem; S, sandy.
[b] Symbols: +, presence of azotobacter detected; −, no azotobacter detected; blanks in the column indicate that the soil was not tested.
[c] Counts are given to the nearest five to emphasize the fact that these are not population estimates.

TABLE 2. Survival of azotobacters in soils exposed to gamma radiation from $^{60}Co$ on the day of collection and then placed in storage[a]

| Original designation | Origin | Soil type | Date of collection | Radiation (krads) | Survival when tested in (year): 1973 | 1979 | 1984 | Avg no. of colonies per plate |
|---|---|---|---|---|---|---|---|---|
| A | Austin | C | 1962 | 100 | + | + | + | 10 |
| I | Austin | C | 1962 | 100 | + | + | | |
| J | Austin | C | 1962 | 100 | + | + | + | 5 |
| 100 KR | Austin | C | 1962 | 100 | + | + | | |
| 250 KR | Austin | C | 1962 | 250 | − | − | | |
| 300 KR | Austin | C | 1962 | 300 | − | − | | |
| 100 | Austin | C | 1963 | 100 | + | | | |
| 3.3 | Austin | C | 1963 | 330 | + | − | − | 0 |
| | Waco | C | 1963 | 200 | + | | | |
| Downtown | Austin | S | 1963 | 200 | + | | | |
| Manor | Manor | S | 1963 | 200 | + | | + | 5 |
| San Marcos | San Marcos | C | 1963 | 200 | + | | | |
| | Brooks | C | 1963 | 200 | + | | | |
| 350 KR | Austin | C | 1963 | 350 | | − | | |
| M 2.7 KR | | | 1961 | 2.7 | | + | + | 10 |
| 625 KR | | | | 625 | | | + | 5 |

[a] Symbols and abbreviations are described in footnotes *a* and *b* of Table 1.

## DISCUSSION

The data presented here show that *Azotobacter chroococcum* and other soil azotobacters survive in dry soils stored in the laboratory for periods of time as long as 24 years. In this regard, it appears that these organisms can last as long in the dormant state as do the spores of many gram-positive bacteria. While the work of many investigators (4, 5, 11) has indicated that the cyst is the form of the organism which allows it to survive desiccation in the soil, other reports (3, 9) question this conclusion. Although cells resembling the cysts of azotobacter have been seen in the soil (1), the survival form has not been definitively identified by satisfactory experiments. It also seems certain that the survival form is not an endospore since azotobacters cannot be isolated from the soil by heat treatment (5; extensive unpublished data) as is commonly done in the isolation of spore-forming bacteria. While it has been reported by Bisset (2) that spores were found in azotobacter cultures, the observations have not been confirmed by other investigators. Because of this, the ability of bacteria of the genus *Azotobacter* to survive in the dormant state for 24 years brings to light many questions regarding the physiology of dormancy which probably cannot be approached by knowledge derived from the study of bacterial spores.

Viability of the azotobacters in these soils will be assessed again in 1990 and in 1995, and the results will be reported on the latter date.

## ACKNOWLEDGMENTS

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