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Long-Term Storage of Plant-Pathogenic Bacteria in Sterile Distilled Water

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This study was made to determine the effectiveness of the preservation of plant-pathogenic bacteria in sterile distilled water. After 20 or 24 years of storage in distilled water, a very high percentage (90 to 92%) of the isolates of *Agrobacterium tumefaciens and Pseudomonas* spp. were still alive. Moreover, 12 of 13 viable (after 24 years) isolates of *P. syringae* subsp. *syringae* maintained their ability to produce syringomycin and were pathogenic to bean seedlings. The water-stored cells of two isolates of *P. syringae* subsp. *syringae*, when observed by electron microscopy, were smaller than cells of 24-h-old subcultures of bacterial cells grown in nutrient broth; the water-stored cells appeared plasmolysed with an electron-dense cytoplasm and thickened cell wall.

A severe problem for microbiologists is the maintenance and preservation of bacterial cultures. Convenient methods for maintaining the viability of cultures in a stable genetic form are necessary. The choice of storage methods is related to the nature of the microorganism, to the period of storage desired (short or long term), and to the facilities available. It is well known that the best methods for long-term preservation of many microorganisms are freeze-drying (lyophilization) and cryogenic storage; however, not all laboratories have these facilities. Additionally, there are some other simple but well-proven methods to consider, such as storage of cultures under mineral oil and preservation of cells in sterile soil, in distilled water, on porcelain beads, in gelatin disks, or over phosphorus pentoxide in vacuo (8). Preservation in distilled water was found to be suitable for the storage of important plant pathogens such as *Corynebacterium insidiosum*, *Agrobacterium tumefaciens*, *Pseudomonas* spp., and *Rhizobium* spp. (2, 4, 5, 9).

In 1961, more than 100 single-celled isolates or strains of oxidase-negative fluorescent *Pseudomonas* spp. from soil and diseased plant tissues were stored in sterile distilled water. The colonies were allowed to develop on an agar surface, and then a loopful of cells was transferred to 5 ml of sterile glass-distilled water in a screw-cap tube. The cells were dispersed in the water, and then the tubes were stored at 10°C. At the time of storage and again after 6 and 20 months the viable cells in the water suspensions were determined (4). Moreover, the phatogenicity on peach seedlings of 15 representative isolates of *Pseudomonas syringae* subsp. *syringae* and their ability to produce a broad-spectrum antibiotic (syringomycin) was confirmed after 20 months of storage. In this note we report results on the viability of 75 isolates of fluorescent *Pseudomonas* spp. and 30 isolates of *A. tumefaciens* stored in sterile distilled water for 24 and 20 years, respectively (water cultures). After this period of storage, 92% of the *Pseudomonas* isolates and 90% of the *A. tumefaciens* isolates were still alive. The populations of viable cells of 14 representative isolates of *P. syringae* subsp. *syringae* are reported in Table 1. After appropriate serial dilutions of the original culture in sterile water, samples were plated in triplicate onto Difco nutrient agar. Plates were incubated at 26°C, and colonies that formed were counted after 3 to 4 days.

After 24 years the bacterial populations of *P syringae* subsp. *syringae* isolates have not greatly decreased; it appears that after an initial decrease of viable cells, the remaining population underwent a much slower decline in viable cells.

All viable isolates of *P. syringae* subsp. *syringae*, except B124, were high producers of syringomycin, as determined by the method of DeVay et al. (3). Single colonies of each strain were transferred on potato dextrose agar plates and allowed to grow at 26°C for 6 days. The plates were then sprayed with a suspension of arthrospores of *Geotricum candidum*, and after 24 h the growth inhibition of *G. candidum* around the colonies was determined. All isolates were still pathogenic on bean seedlings when cells from a 24-h-old culture in nutrient broth (NB) were washed in water and injected into bean hypocotyls. Bacterial cells of the

TABLE 1. Viability of cells of *P. syringae* subsp. *syringae* after storage in distilled sterile water at approximately 10°C

Isolate	CFU/ml after:			
	Zero time[a]	6 mo[a]	20 mo[a]	24 yr
B3	1.0×10^8	1.0×10^5	8.1×10^5	6.6×10^2
B15	5.5×10^7	1.3×10^5	9.5×10^5	0
B59	1.9×10^8	5.4×10^6	1.3×10^6	1.4×10^6
B60	2.7×10^8	3.0×10^6	8.8×10^6	6.4×10^5
B61	1.1×10^8	1.4×10^6	1.3×10^6	3.9×10^6
B68	3.5×10^8	2.9×10^6	1.9×10^6	1.0×10^5
B70	3.6×10^8	8.4×10^6	8.6×10^7	2.9×10^5
B98	7.4×10^8	5.8×10^5	9.3×10^7	5.0×10^5
B99	1.0×10^9	1.2×10^6	4.1×10^8	8.0×10^5
B100	9.6×10^8	2.4×10^6	4.2×10^7	8.9×10^5
B101	6.7×10^8	1.3×10^6	3.4×10^7	5.3×10^5
B104	1.2×10^9	2.8×10^7	5.0×10^8	2.6×10^6
B105	8.7×10^8	2.3×10^7	7.6×10^7	3.5×10^6
B124	7.7×10^7	2.0×10^4	2.4×10^5	1.0×10^5

[a] Data from reference 4.

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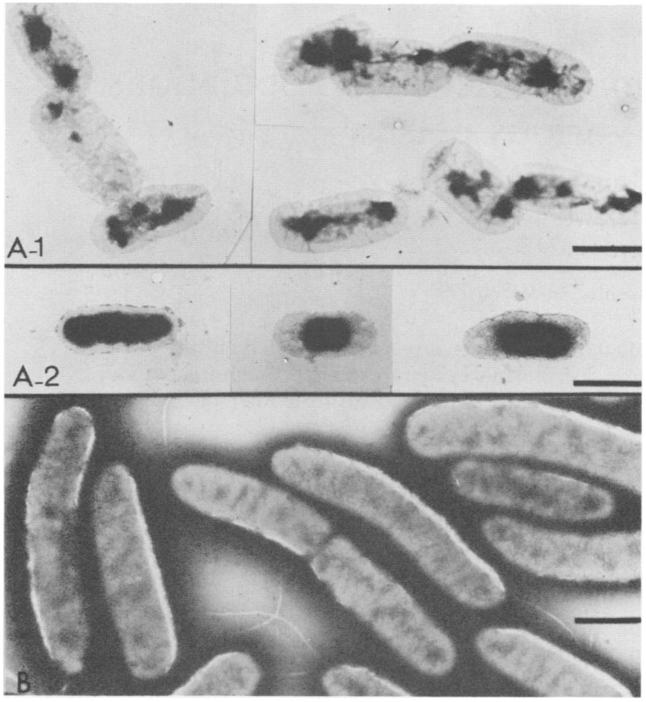

FIG. 1. Electron micrographs of cells of *P. syringae* subsp. *syringae* negatively stained with phosphotungstic acid. (A) Cells stored in distilled water for 24 years (A-1, isolate B59; A-2, isolate B100). (B) Water-stored cells of isolate B100 regrown in NB for 24 h. Scale bar, 1 μm.

water cultures were also observed under an electron microscope (7) and compared with cells of the same isolates regrown in NB for 24 h. The bacterial cells from either water or NB culture were pelleted by centrifugation at 7,000 × *g* for 10 min, suspended in sterile distilled water, serially diluted, and negatively stained with phosphotungstic acid. For each sample being observed four or five grids were prepared. Bacterial cells stored in sterile water for 24 years appeared smaller than cells regrown in NB for 24 h. The water-stored cells of isolates B59 and B100 were on average 0.7 by 1.86 μm, whereas the respective NB culture cells were 0.7 by 2.8 and 0.7 by 2.7 μm, respectively.

Although the morphology of NB cultures cells resembled that reported for *Pseudomonas* spp. (1), the water culture cells were smaller and presented an electron-dense cytoplasm (Fig. 1). The cells were plasmolyzed, and the cell walls appeared thickened. In some cells it was possible to observe the apparent origin of polar flagella in the cytoplasmic membrane (Fig. 1). The finding that bacterial cells stored in water were smaller than cells cultured in NB was not surprising, since it is known that the size of bacterial cells is related to their nutritional status. The decrease in cell size of bacteria is an evident morphological event during starvation (6).

The finding that a high percentage of water-stored isolates of *Pseudomonas* spp. was alive after 24 years is noteworthy, but it is also significant that isolates of *P. syringae* subsp. *syringae* maintained their pathogenicity and their ability to produce syringomycin, indicating a genetic stability for these traits. Thus, the water method of maintaining bacteria cultures for long-term storage is effective and simple: the cells maintain their viability and genetic stability, and the method provides easy access for reestablishing original culture types.

It is not clear how these bacteria survived in distilled water, since this group of *Pseudomonas* spp. do not accumulate polyhydroxybutyrate as an intracellular carbon reserve; the reduced metabolic rate of these starved bacteria must be maintained by the utilization of other cell constituents.

The appearance of these cells after long-term storage suggests that they might represent a resting form, in contrast to spores formed by certain other genera of bacteria.

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