Liquid Nitrogen Storage of Phytopathogenic Bacteria

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ABSTRACT

A simple, uniform method of culture storage in liquid nitrogen was developed to preserve selected species of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas,* and *Xanthomonas*. Survival of cells was generally enhanced by suspending them in 10% skim milk before freezing and storage. However, the most convenient procedure was to freeze and store all species except the "soft-rotting" types in the culture medium in which they were grown. Regardless of the nature of the suspending fluid, the viability of rapidly-frozen cells usually declined immediately after freezing, but remained stable thereafter through 12 and 30 months of storage at −172 to −196 C. By contrast, viability dropped rapidly over a 6-month period for several species stored at −20 C, but survival was enhanced in 10% skim milk. Pathogenicity was unaffected by storage under liquid nitrogen.

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Bacterial cultures have been preserved in a variety of ways because of inherent differences between species (4, 5, 17, 20–cf. 15 for a summary of methods). Although no single procedure has been successful with all species, storage in liquid nitrogen may have broad application to bacterial cultures because of successful preservation of a wide range of biological materials (1, 2, 6, 8, 14, 16, 18, 19, 21). Several procedural variables greatly influence the viability of organisms preserved in liquid nitrogen; e.g., choice of freezing medium (3, 10, 12, 15) and adjuvants (1, 3, 7, 8, 9, 13, 18), pH of the medium (3, 9, 10) growth stage of the bacterial cultures when frozen (9, 10, 12), population levels (3, 10, 12, 15), freezing and thawing time (1, 7, 9, 14, 15, 19) and the medium used to enumerate the thawed bacteria (2, 3, 9, 12). This study was initiated to evaluate the potential for long-term storage in liquid nitrogen to preserve a wide range of phytopathogenic bacteria. A preliminary report has been published (11).

MATERIALS AND METHODS.—*Organisms, media, and cultural conditions.*—The following bacterial isolates were used in this study; *Agrobacterium tumefaciens* (Smith and Townsend, 1907) Conn, 1942; *Corynebacterium flaccumfaciens* (Hedges, 1922) Dowson, 1942; *Erwinia amylovora* (Burril, 1882) Winslow et al., 1920; *E. carotovora* (Jones, 1901) Holland, 1920; *Pseudomonas morsprunorum* Wormald, 1931; *P. phaseolicola* (Burkholder, 1926) Dowson, 1943; *P. syringae* van Hall, 1902; and unidentified *Pseudomonas* sp. "soft-rotter"-type from diseased iris *(Iris xiphium* L. 'Wedgewood'); *Xanthomonas corylina* (Miller et al., 1940) Starr and Burkholder, 1942; *X. incanae* (Kendrick and Baker, 1942) Starr and Weiss, 1943; and *X. pruni* (E. Smith, 1903) Dowson, 1939.

The bacteria were grown in a liquid medium (YDP) containing 0.4% yeast extract, 2% dextrose, 0.4% peptone, and 0.5% ammonium sulfate and adjusted to pH 7.0. Fifty ml of medium in 250-ml flasks fitted with Klett-tube side arms were inoculated with cells from exponentially growing cultures. The flasks were incubated on a rotary shaker at 30 C, except for the *Corynebacterium* species which were grown at 25 C.

Suspending fluids, freezing, storage, and thawing.—The bacterial cultures in their exponential growth phase [10^8-10^9 colony-forming units (CFU)/ml] were centrifuged (12,000 *g* for 10 minutes), and the pellets resuspended in one of the following sterile fluids (pH 7.0) at 4 C: YDP medium, YDP medium containing 1% dimethylsulfoxide (DMSO), 10% skim milk, or distilled water. Aliquots (0.5 ml) of each cell suspension were dispensed in sterile 1-ml ampules (Kimble Neutraglas), the ampules were flame-sealed (Kahlenberg Globe Bench Model 161 Ampul Sealer), cooled, examined for leaks, fitted into aluminum ampule canes (Shur-Bend Mfg.), and rapidly frozen by plunging the cane into liquid nitrogen. Samples of the original cultures were similarly frozen. The canes were coded with indelible ink, and the frozen cultures were stored in a liquid nitrogen container (Linde LD-30) at −172 to −196 C. Periodically, ampules were thawed quickly by swirling in water at 40-45 C until the bacterial suspension was liquified (10-15 seconds), and viability of the bacterial cells was determined on YDP agar plates.

Some ampules initially frozen and stored in liquid nitrogen were placed in a freezer at −20 C and bacterial viability was determined monthly for 6 months.

Colony counts.—The number of CFU per milliliter of suspending fluid was determined before and after freezing for each isolate. Two samples from each cultivar were diluted serially, and selected dilutions were plated in triplicate by spreading 0.1 ml of the dilution with a bent glass rod over the surface of YDP agar in petri plates. The plates were incubated at 25 C for the *Corynebacterium* species and at 30 C for all other species. Colony counts were made 5 and 2 days later, respectively.

The data were analyzed statistically using a three-way balanced analysis of variance when all observations were present, and by a three-way unbalanced analysis of variance when observations occasionally were missing due to contamination. When the F-test ($P = 0.05$) was significant, a least significant difference (LSD) analysis ($P = 0.05$) was used to compare sample means. All mean colony reductions following freezing of tenfold or greater were statistically significant, $P = 0.05$. Following freezing, colony reductions of less than tenfold, and differences between the results obtained in various suspending fluids, are presented in the text as significant only when significant at $P = 0.05$.

Pathogenicity after freezing and storage at ⩽ −172

TABLE 1. Population changes in bacterial cultures after storage for 6 months at −20 C. The bacteria were initially frozen and maintained for 1 year in liquid nitrogen before being stored at −20 C

Bacterial species and treatment	Survivors (colony-forming units) frozen and stored in various fluids				
	Culture growth medium[a]	Sterile distilled water	Skim milk (10%)	Fresh YDP[a] Broth	Fresh YDP Broth + 1% DMSO[b]
Agrobacterium tumefaciens, CG-1					
Before freezing	2.7×10^9	2.6×10^9	4.6×10^9	4.2×10^9	4.0×10^9
After 6 months	1.5×10^5	1.4×10^3	2.3×10^9	3.0×10^7	5.0×10^6
Erwinia amylovora					
Before freezing	3.0×10^9	5.0×10^9	3.8×10^9	1.8×10^9	1.8×10^9
After 6 months	$< 10^3$	2.2×10^5	2.0×10^7	5.0×10^5	2.8×10^5
carotovora					
Before freezing	...	...	2.6×10^8	...	...
After 6 months			4.6×10^5		
Pseudomonas phaseolicola					
Before freezing	9.1×10^7	...	...	...	...
After 6 months	7.7×10^3				
"soft-rotter"-type					
Before freezing	...	...	...	...	10.0×10^9
After 6 months					$< 10^3$
Xanthomonas pruni					
Before freezing	3.0×10^8	...	...	...	...
After 6 months	7.7×10^3				

[a] YDP = liquid medium containing 0.4% yeast extract, 2.0% dextrose, 0.4% peptone, and 0.5% ammonium sulfate, adjusted to pH 7.0.
[b] DMSO = dimethylsulfoxide.
[c] Not tested.

TABLE 2. Changes in bacterial populations after storage in liquid nitrogen (≤ −172 C) for 12 or 30 months

Bacterial genera species isolates	Storage time (months)	Reduction in viability (%) in various suspending fluids				
		Growth medium	Sterile distilled water	10% Skim milk	Fresh YDP[a] Broth	Fresh YDP Broth + 1% DMSO
Agrobacterium tumefaciens						
CG-1	12	30	31	0	14	20
B-233	12	59	29	24	47	36
U-11	30	68	33	0	52	0
B-234	30	61	3	0	0	0
Corynebacterium flaccumfaciens	12	36	19	15	24	2
Erwinia	30	2	13	0	0	0
amylovora	12	95	96	99.3	99.7	0
	30	98	99	99.5	98	0
carotovora	12	>99.99	>99.99	21	>99.99	>99.99
	30	>99.99	>99.99	91	>99.99	>99.99
Pseudomonas						
morsprunorum	12	81	55	6	66	55
phaseolicola	12	0	88	77	26	90
"soft-rotter"-type	12	99.99	89	83	91	92
syringae	12	50	57	26	73	88
Xanthomonas						
corylina	12	53	80	81	91	95
incanae	12	93	74	29	87	96
pruni	12	0	83	88	99.1	99.2

[a] YDP = liquid medium containing 0.4% yeast extract, 2.0% dextrose, 0.4% peptone, and 0.5% ammonium sulfate, adjusted to pH 7.0.

TABLE 3. The influence of freezing and thawing on pathogenicity of bacterial isolates

Bacteria	Host[a]	Symptom development following inoculation with bacteria never frozen or frozen and stored in liquid nitrogen[b]		
		Never Frozen	Thawed and Recultured	Thawed Only
Agrobacterium tumefaciens	Tomato seedling	+ + +	+ + +	+ + +
Erwinia	Pear			
amylovora	seedling	+ + +	+ + +	+ + +
	fruit	+ + +	+ + +	+ + +
carotovora	Potato slice	+ + +	+ + +	+ + +
	Carrot slice	+ + +	+ + +	+ + +
Pseudomonas	Cherry			
morsprunorum	seedling	+ +	+ +	+ +
	fruit	+ +	+ +	+ +
phaseolicola	Bean leaves	+ + +	+ + +	+ + +
"Soft rot"-type	Potato slice	+ +	+ +	+ +
	Carrot slice	±	±	±
syringae	Cherry			
	seedling	+ + +	+ + +	+ +
	fruit	+ + +	+ + +	+ +
Xanthomonas	Filbert			
corylina	seedling	+ + +	+ + +	+ + +
incanae	Stock			
	seedling	+ + +	+ + +	+ + +
pruni	Cherry			
	seedling	+	+	+
	fruit	+	+	+

[a] Plant tissues were inoculated with 10^6 to 10^7 bacteria per milliliter in either a droplet applied to the tissue (a sharp, thin needle was repeatedly jabbed through the drop into the host tissue) or atomized over the leaf.

[b] Disease rating scale: + + + = severe symptoms of water soaking, cankering, soft rotting, or necrosis, depending on the type of pathogen; + + = intermediate between weak and severe symptoms; + = weak symptoms; ± = symptom appearance nearly the same as the control. Wounded, noninoculated control plants were all rated "0" = no visible symptoms.

C.—Pathogenicity of the thawed bacterial suspensions was determined. Inoculum consisted of: (i) exponentially growing bacteria never subjected to freezing; (ii) bacteria frozen in their YDP culture medium, stored 4-6 months, thawed, and immediately applied to plant tissues; and (iii) bacteria similarly frozen, but cultured on fresh YDP medium after being thawed. Host plant tissue included slices of potato *(Solanum tuberosum* L.) and carrot *(Daucus carota* L. var. *sativa* DC.); immature fruits of pear *(Pyrus communis* L.) and cherry *(Prunus avium* L.); and potted seedlings of pear, cherry, filbert *(Corylus avellana* L. 'Barcelona'), stock *(Matthiola incana* L. Voss), and tomato *(Lycopersicon esculentum* Mill. 'Bonny Best'). Droplets of inoculum containing 10^6-10^7 bacteria were placed on the pear and cherry fruits, slices of potato and carrot, or at the junction of a leaf petiole with the stem (near the shoot apex) of cherry, filbert, stock, and tomato seedlings. A sharp needle was repeatedly jabbed through the drop of inoculum and into the host tissue. Bean plants *(Phaseolus vulgaris* L.) were preconditioned for 24 hours in a moist chamber before being sprayed with inoculum.

Three host plants were inoculated with each bacterial species and the plants were incubated in a greenhouse at 22-27 C. The inoculated potato and carrot slices, and pear and cherry fruits were incubated in deep covered dishes at 100% relative humidity (RH). After symptoms developed, samples of diseased tissue were placed in sterile water for 30-90 minutes and then streaked onto potato-dextrose agar (Difco) plus 0.5% calcium carbonate and King's medium B to determine the presence of the pathogenic bacteria.

RESULTS.—Bacterial populations of all species assayed prior to freezing in liquid nitrogen averaged 2.8×10^9 CFU/ml (range of 10^8-10^{10} CFU, except for *P. phaseolicola* and the "soft-rotter" isolates which had 9×10^7 and 2.7×10^6 CFU, respectively.

Recovery of viable bacteria after storage at −20 C.—In general, storage of bacteria (initially frozen in liquid nitrogen and then placed at −20 C) resulted in a progressive reduction in the bacterial population over a 6-month period (Table 1). However, viability of some species was enhanced in certain suspending fluids. For example, recovery of *A. tumefaciens* (CG-1) stored in skim milk at −20 C was similar to recovery after storage at ≤ −172 C (Table 1 and 2). In contrast, the numbers of viable *E. carotovora* stored in skim milk at −20 C dropped to one-thousandth of the initial population after 6 months. Populations of several *Pseudomonas* spp. and *X. pruni* similarly dropped sharply at −20 C, even though suspended in fluids that minimized viability losses when the bacteria were stored at −172 C (Table 1 and 2).

Recovery of viable bacteria after storage at ≤ −172 C.—The viability of bacterial cultures after 12 months of

storage at ≤ -172 C was similar to that determined immediately after freezing, but generally was lower than counts made before freezing (Table 1 and 2). This suggests that the freezing process was lethal to some cells. The reductions in populations generally were similar regardless of the suspending fluid in which the bacteria were frozen (Table 2). However, viability following freezing in skim milk was significantly higher (at $P=0.05$) for *A. tumefaciens* (CG-1, E-233), *P. morsprunorum, E. carotovora, Pseudomonas* sp. "soft-rotter"-type, *P. syringae,* and *X. incanae*. Recovery of *C. flaccumfaciens* surprisingly was good regardless of the suspending medium (Table 2).

Significant population reductions (at $P = 0.05$) (from 10^9 to 10^7 CFU) occurred when *A. tumefaciens* (B-233, B-234), *E. carotovora, P. morsprunorum,* and the *Pseudomonas* "soft-rotter" were frozen directly in their growth medium (Table 2). However, in every instance (except for the two soft-rotting types) enough bacteria survived to begin new working cultures. Because these population reductions occurred primarily as the bacteria were frozen, and the populations remained relatively stable thereafter, processing bacteria directly in their growth medium is a simple method for prolonged storage.

Pathogenicity after freezing and storage.—Symptom development for each pathogen-host combination was the same regardless of whether thawed bacteria were immediately used for inoculum or they were first allowed to grow on fresh medium before host inoculation (Table 3). Bacteria isolated from infected host tissues were phenotypically similar to those in the inoculum.

DISCUSSION.—Storage in liquid nitrogen was used successfully to preserve species of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas,* and *Xanthomonas*. Survival of plant pathogenic bacteria in liquid nitrogen is excellent after the initial freezing shock as is the case with a variety of other organisms (7, 8, 14, 20). Although survival was usually enhanced by resuspending centrifuged bacteria in fluids containing protectants, it is questionable whether the extra processing is necessary when the bacteria are stored at low temperatures e.g. ≤ -172 C (cf. also 1, 9). The bacterial isolates we tested (except for the "soft-rotting" types of *Erwinia* and *Pseudomonas* spp.) could be successfully preserved in the medium in which they were cultured. Colony counts remained at 10^7/ml or greater after 12 months of storage, which is more than adequate to start new working cultures.

At the higher storage temperature (−20 C), the protectants significantly enhanced survival of bacteria. Similarly, others (1, 3, 7, 12) have demonstrated a beneficial effect of protectants such as glycerol, casein hydrolysate, yeast extract, and egg white at this temperature.

Pathogenicity was not altered by freezing the bacteria in liquid nitrogen, suggesting that physiological and genetic stability for pathogenicity were maintained. Similar findings have been reported for *Penicillium chrysogenum* (19), *Streptococcus* species used in cheese-making (1, 9), L-cell suspensions (18), neuron cells (6), and bacteria for antibiotic assays (16).

Processing phytopathogenic bacteria for storage in liquid nitrogen is fast and convenient. Twenty-four sterile ampules can be routinely filled, sealed, and frozen in 20 min. Ampules stored in coded aluminum canes can be readily retrieved, thawed in 10 to 15 seconds, and the contents used immediately as inoculum. Although in our study only 0.5-ml aliquots were frozen in each ampule, 4- to 10-ml volumes have been frozen and stored successfully (7, 10).

Our experience with other methods of culture preservation such as lyophilization, storage in sterile water blanks, covering cultures grown on agar slants with mineral oil, or freezing samples of diseased leaves in water at −20 C has frequently resulted either in loss of some species or culture contamination. Although numerous species can be preserved by the above methods, we have found it more convenient and reliable to use liquid nitrogen storage for all our isolates which included many diverse species. To our knowledge, this is the first report of the use of liquid nitrogen for long-term preservation of virulent phytopathogenic bacteria.

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