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***Frankia* strain HFP ArI3 which had been preserved for 27 months by being lyophilized, frozen in glycerol, or stored in complex medium was successfully used as an inoculum after being subcultured for inducing nodulation and nitrogen fixation of *Alnus rubra*. Glycerol-preserved HFPArI3 produced significantly lower rates of nitrogenase activity than did lyophilized or complex-medium-preserved inocula. Bacteria that had been preserved by all three methods were successfully induced to fix atmospheric nitrogen by being cultured in nitrogen-free medium. Subculturing of these cells in nitrogen-free medium a second and third time yielded increasing rates of nitrogenase activity. Initial nitrogenase activity was detected on days 5, 4, and 3 during the first, second, and third subcultures after preservation, respectively. Maximum activity was observed on days 11, 10, and 8 during the first, second, and third subcultures, respectively. A description is given of standard culture techniques used in our laboratory for *Frankia* isolates, and methods used to distribute *Frankia* cultures by mail are described.**

Actinomycetous, nitrogen-fixing bacteria of the genus *Frankia* associate symbiotically within root nodules of nonleguminous, woody, dicotyledonous plants. Since the first isolation of *Frankia* spp. from root nodules of *Comptonia* spp. (9), hundreds of isolations have been achieved in various laboratories, allowing successful culturing of many strains of these filamentous bacteria in vitro.

Progress in defining the culture conditions necessary for successful growth and nitrogen fixation in vitro has allowed advances to be made in studies of the physiology (2, 22, 23), enzymology (20), genetics (24, 26), and systematics (17, 18) of free-living *Frankia* strains. Recently, methods for large-scale inoculations of actinorhizal plants with cultured *Frankia* strains have been applied in land reclamation and revegetation programs (24a).

Research on and practical interest in *Frankia* strains have led to many requests for cultures of *Frankia* isolates from our laboratory. In the past 5 years, we have successfully shipped *Frankia* isolates to over 50 laboratories throughout the world. These activities have made clear the necessity for long-term preservation of isolates. The objectives of our study were to evaluate techniques for preserving *Frankia* isolates and to assess these storage methods in relation to the subsequent ability of the bacteria to nodulate host plants (infectivity) and fix atmospheric nitrogen within nodules (effectivity). We also wished to determine the importance of repeated subculturing of *Frankia* strains after preservation and before use of the bacteria as inocula for in vitro nitrogenase production. A further objective was to record standard conditions used in our laboratory for culturing and handling *Frankia* isolates.

MATERIALS AND METHODS

Bacterial strain. All of the experiments were done with *Frankia* strain HFPArI3 (catalog no. HFP013003 [17]; hereafter referred to as ArI3) which had been isolated from *Alnus rubra* (3) and preserved for 27 months by one of the techniques described below. The performance of the preserved samples was compared with that of our stock culture of strain ArI3. For each separate experiment a common inoculum was used.

Preservation techniques. (i) Glycerol. Sterile glycerol (3 ml; Sigma Chemical Co.) in 10-ml vials was inoculated with a 0.1-ml packed-cell volume of late-log-phase ArI3 which had been homogenized with a Potter-Elvehjem tissue grinder in 5 ml of medium. The culture was then aspirated several times by being mixed with a sterile pipette. The caps were tightened and sealed with Parafilm. Samples were stored at −15°C. The final concentration of glycerol was approximately 35%; the preparation produced a liquid suspension at −15°C.

(ii) Lyophilization. Sterile 10% skim milk (1 ml; Carnation Instant Dry Milk) in 10-ml screw-cap vials was inoculated with a 0.1-ml packed-cell volume of late-log-phase bacteria which had been homogenized with a Potter-Elvehjem tissue grinder in 1 ml of medium. The vials, with caps attached loosely, were frozen for ca. 24 h in ethanol-sterilized, fast-freezing lyophilization flasks (300 ml) with silicone rubber snap-on caps (Labconco Co.). The frozen samples were then lyophilized with a Labconco model FD3 freeze-dry apparatus. After lyophilization, the screw caps of the vials were tightened and sealed with Parafilm, and the samples were stored with desiccants at −15°C.

(iii) Complex medium. Sterile M6B medium (5 ml; see below for ingredients) in 18-mm test tubes was inoculated with a 0.1-ml packed-cell volume of late-log-phase bacteria which had been homogenized with a Potter-Elvehjem tissue grinder. The tubes were capped with Kaputs, sealed with Parafilm, and stored stationary in the dark at 27°C.

Stock culture techniques. We currently maintain 30 *Frankia* strains in our laboratory. All strains are cultured in 125-ml Erlenmeyer flasks containing 50 ml of medium. Flasks are capped with aluminum foil, sealed with Parafilm, and stored stationary in the dark at 27°C. For subculturing, log-phase cells from one of these flasks are homogenized with a sterile homogenizer and used to inoculate two flasks. If cells are transferred from complex to defined medium, they are first washed twice in distilled water by centrifugation.

When cells are not immediately required for experimenta-

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tion, they are grown in complex medium, e.g., QMOD medium (16), Czapek medium (Difco Laboratories, Detroit, Mich.) supplemented with 0.4% yeast extract or M6B medium, which is a modification of *Frankia* broth (1) that contains the following (per liter): yeast extract (Difco) (5 g); glucose (10 g); Casamino Acids (Difco) (5 g); KH_2PO_4 (1 g); $MgSO_4$ (0.1 g); $CaCl_2$ (0.01 g); $CoCl_2$ (0.001 g); 0.02 mM ferric disodium EDTA; the trace elements H_3BO_3 (2.86 mg), $MnCl_2$ (1.81 mg), $ZnSO_4$ (0.22 mg), $CuSO_4$ (0.08 mg), and $NaMoO_4$ (0.025 mg); and the vitamins thiamine hydrochloride (10 μg), nicotinic acid (50 μg), and pyridoxine hydrochloride (50 μg). Cells grown in these complex media are subcultured every 3 to 4 months.

When substantial volumes of *Frankia* strains are needed for experimental purposes, we grow cells in defined BAP medium, which is a modification of the medium of Murry et al. (23) that contains 0.2 mM $MgSO_4$, 0.07 mM $CaCl_2$, 5 mM NH_4Cl, and 0.02 mM ferric disodium EDTA (from a stock containing 5.56 g of $FeSO_4$ and 7.45 g of disodium EDTA per liter; pH 6.3); the micronutrients (per liter) H_3BO_3 (2.86 mg), $MnCl_2$ (1.81 mg), $ZnSO_4$ (0.22 mg), $CuSO_4$ (0.08 mg), $NaMoO_4$ (0.025 mg), and $CoSO_4$ (0.001 mg); and the vitamins (per liter) thiamine hydrochloride (10 μg), pyridoxine hydrochloride (50 μg), nicotinic acid (50 μg), biotin (22.5 μg), folic acid (10 μg), calcium pantothenate (10 μg), and riboflavin (10 μg). Sterile phosphate buffer (10 mM) is added after autoclaving. The buffer is prepared by titrating 1 M K_2HPO_4 with 1 M KH_2PO_4 until a pH of 6.7 is achieved. Filter-sterilized 1 M propionate is added as the sole carbon source to a final concentration of 5 mM after autoclaving, unless the isolate is from *Casuarina* sp., in which case 10 mM pyruvate is used as the sole carbon source (Z. Zhang, M. A. Murry, and J. G. Torrey, Plant Soil, in press). *Frankia* cells are grown in 100 ml of this medium in 250-ml Erlenmeyer flasks on a rotary shaker (80 rpm). These cultures can serve as inocula for larger-volume cultures grown with aeration and mechanical stirring (23).

When a newly isolated strain is received from another laboratory, we culture it in the medium recommended by those who isolated it. When the strain has been grown to a substantial volume, we transfer it to defined BAP medium, if that is as effective for growth as the original medium.

Distributing *Frankia* isolates. *Frankia* isolates are mailed in VACUTAINER (Becton Dickinson Vacutainer Systems, Rutherford, N.J.) tubes containing a homogenate of a 0.1- to 0.3-ml packed-cell volume of log-phase bacteria in 1 ml of the medium in which the cells had been growing. The VACUTAINER tubes are mailed in cardboard cylinders. We recommend that, upon receipt of a mailed *Frankia* isolate, the isolate be pipetted into sterile 18-mm test tubes containing 5 ml of the medium in which the isolate had been grown. When substantial growth is observed, the bacteria may be transferred to larger containers of medium. *Frankia* isolates generally do not grow well on agar plates. We recommend growing all isolates in liquid cultures.

Plant culture techniques. Fruits of *A. rubra* were sown in flats of moist sand and maintained in a growth chamber with a cycle of 16 h of light and 8 h of dark, a daytime temperature of 24°C, and a nighttime temperature of 19°C. Seedlings were watered twice weekly with one-quarter-strength Hoagland solution (14). Six-week-old seedlings were transferred to 450-ml water culture jars containing one-quarter-strength Hoagland solution minus nitrogen at pH 6.0. Each jar contained three seedlings anchored with nonabsorbent cotton through holes in the jar caps. Roots were entirely immersed within the Hoagland solution. The water culture jars were maintained in greenhouse facilities and topped off daily with deionized water. The one-quarter-strength Hoagland solution minus nitrogen was changed weekly after nodules appeared.

Plant inoculation techniques. Sample of *Frankia* strain ArI3 which had been preserved for 27 months by the above-described techniques were used to inoculate 6-week-old seedlings. The preserved cultures were washed by centrifugation with sterile distilled water (the lyophilized cultures first having been rehydrated with sterile M6B medium). Bacteria were then transferred to 125-ml flasks containing 50 ml of M6B medium, grown to the mid-log phase, and used as inocula after being washed twice in distilled water by centrifugation. A suspension of a 0.03-ml packed-cell volume was used as the inoculum for each of the plant culture jars. Nine seedlings were inoculated with ArI3 prepared by each of the preservation methods. The experiment was repeated three times. We also inoculated seedlings with ArI3 which had been under continuous routine subculturing in our laboratory in M6B medium. Control seedlings which received no inoculum showed no nodulation.

Plants were carefully observed each day for the presence of nodules. At 8 weeks, nodules were harvested by excision with a scalpel and assayed for nitrogenase activity with the acetylene reduction assay (8) under the conditions described by VandenBosch and Torrey (28). After being assayed, the nodules were dried to a constant weight and weighed on a Mettler balance.

In vitro culturing techniques. Cells from each of the preservation techniques were washed and transferred to 1-liter, air-sparged bottles of defined BAP medium. The cells were grown to the mid-log phase, washed twice in nitrogen-free medium, and transferred to nitrogen-free B medium (23) to induce vesicle formation and nitrogenase activity. This induction process was completed after each of three subcultures in defined BAP medium of cells from the three preservation techniques. We compared the nitrogenase activity of the inocula from the three preservation techniques to the activity of strain ArI3 which had been cultured routinely in our laboratory in BAP medium in 1-liter, air-sparged bottles.

Nitrogenase activity was monitored with the acetylene reduction assay (8) in standardized 10-ml vials with serum stoppers and containing 10% (vol/vol) acetylene. Samples were placed on a rotary shaker at 28°C and assayed after 2 h. Ethylene production was assayed with a Carle model 9500 gas chromatograph with a 1.2-m stainless steel column containing a 25:75 mixture of Porapak R (80/200 mesh) and N (50/80 mesh). Duplicate 100-μl samples were injected directly into the column. Four replicate assays were completed each day for each treatment.

Inoculum samples were taken on the day of maximum acetylene reduction activity for vesicle counts. These samples were sonicated for 15 s at 100 W with a Braunsonic model 1510 sonicator, and the vesicles were counted in a Petroff-Hausser counting chamber under phase optics at 400×.

The protein concentration of the cells was determined with the Bradford (5) procedure on cells that had been sonicated for 15 s at 100 W and boiled for 10 min in 0.3 N NaOH (10).

RESULTS

Plant infectivity and effectivity. All three preservation methods resulted in inocula which caused seedlings of *A. rubra* to develop nodules and acetylene reduction (=nitrogenase) activity (Table 1). No significant differences

TABLE 1. Effects of prior preservation treatments on the infectivity and effectivity of *Frankia* strain ArI3 used as an inoculum for *A. rubra*[a]

Treatment	Days ± SD to first nodulation	No. of nodules ± SD per plant	Nodule wt (mg ± SD)	Acetylene reduction (μmol of C_2H_4 produced per g of fresh nodule wt per h ± SD)
Stock culture[b]	15.0 ± 3.6[a]	2.29 ± 1.04[a]	206.2 ± 198.9[a]	1.61 ± 1.55[a]
Lyophilization	13.3 ± 4.0[a]	2.97 ± 1.63[a]	190.1 ± 161.6[a]	1.22 ± 1.04[a]
Complex medium	13.3 ± 2.5[a]	1.62 ± 0.54[a]	87.7 ± 55.9[b]	1.41 ± 0.89[a]
Glycerol	16.3 ± 3.1[a]	1.03 ± 0.33[b]	31.7 ± 11.2[c]	0.16 ± 0.05[b]

[a] For each column of data, means followed by the same letter are not significantly different at the 0.05 level of probability (Duncan's new multiple-range test).
[b] *Frankia* strain ArI3 routinely subcultured in M6B medium.

existed in the time required for the first nodules to appear on plants inoculated with *Frankia* cells from the four sources tested. A large variation (up to 50% standard deviation) was observed in the number of nodules produced per plant. The glycerol-preserved inoculum produced significantly fewer nodules than did the other inocula. These nodules also weighed significantly less and had significantly lower rates of acetylene reduction than did those from the other treatments. The lyophilized inoculum and the complex-medium-preserved inoculum produced nodules which fixed atmospheric nitrogen at rates comparable to those for nodules from the stock culture inoculum. The growth of the seedlings attributable to the symbiotic fixation of atmospheric nitrogen was not quantitatively determined during these experiments; however, seedlings inoculated with stock culture, complex-medium-preserved, or lyophilized *Frankia* cells generally grew more vigorously than did seedlings inoculated with glycerol-preserved *Frankia* cells.

In vitro nitrogenase activity. When cultured in nitrogen-free medium, the inocula from the three preservation methods produced vesicles and displayed acetylene reduction activity (Table 2). However, the rates of acetylene reduction obtained with the preserved inocula were significantly lower than the rates obtained with the stock culture inoculum. These reduced rates could not be attributed to a lack of vesicles (the proposed site of nitrogenase production [28]), since the glycerol-preserved inoculum was the only inoculum which showed significantly reduced vesicle production. The differences in rates between the preserved inocula and the stock culture inoculum decreased as the preserved *Frankia* cells were subcultured a second and third time (Fig. 1). After three subcultures were done under standard conditions, the inocula preserved by lyophilization and by the complex medium achieved rates of acetylene reduction equal to those of the stock culture inoculum. The glycerol-preserved inoculum also achieved increasing rates of nitrogenase activity; however, after three subcultures were done under standard culture conditions, this activity reached only ca. 65% of the activity of the inoculum preserved by lyophilization or by the complex medium.

The kinetics of nitrogenase induction also changed as the inocula were subcultured a second and third time (Fig. 2). For the lyophilized inoculum, initial nitrogenase activity was detected on days 5, 4, and 3 for the first, second, and third subcultures, respectively. The time required to attain maximum activity decreased from 11 to 10 to 8 days for the first, second, and third subcultures, respectively. The kinetics observed for the third subculture were similar to the kinetics usually observed for ArI3 nitrogenase production (11, 23). During each subculture, maximum nitrogenase activity was followed by a sharp decline in activity over 1 to 2 days. This decline was probably a manifestation of carbon starvation, a phenomenon observed previously for *Frankia* strain ArI3 (19, 23). Figure 2 represents the kinetics for the lyophilized inoculum only; however, time courses for nitrogenase induction for the other preservation methods showed the same trends.

TABLE 2. Effects of prior preservation treatments on in vitro nitrogenase activity and vesicle production by *Frankia* strain ArI3 after induction in B medium[a]

Treatment	nmol of C_2H_4 produced per mg of protein per h ± SD	No. of vesicles produced per mg of protein (10^7) ± SD	nmol of C_2H_4 produced per vesicle (10^{-6}) ± SD
Stock culture[b]	398.8 ± 51.9[a]	3.67 ± 0.88[a]	10.87 ± 3.40[a]
Lyophilization	144.8 ± 37.0[b]	2.94 ± 0.65[a]	4.92 ± 0.73[b]
Complex medium	120.9 ± 36.4[b]	2.80 ± 0.14[a]	4.31 ± 1.29[b]
Glycerol	88.7 ± 14.1[b]	2.17 ± 0.64[b]	4.09 ± 0.93[b]

[a] For each column of data, means followed by the same letter are not significantly different at the 0.05 level of probability (Duncan's new multiple-range test).
[b] *Frankia* strain ArI3 routinely subcultured in defined BAP medium before induction in B medium.

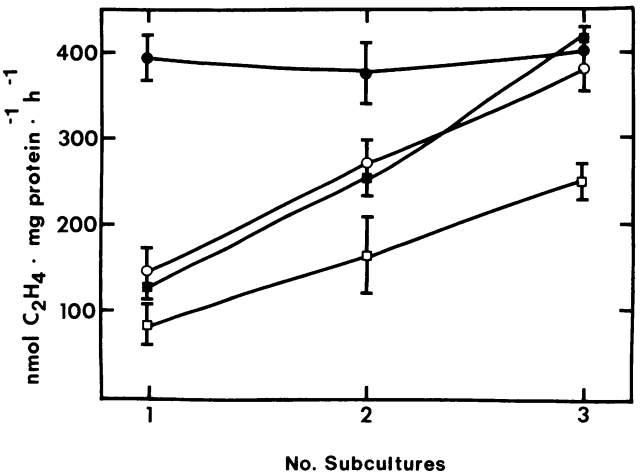

FIG. 1. Maximum nitrogenase activity of *Frankia* strain ArI3 as affected by subculturing. Symbols: ●, stock culture (grown continuously in defined BAP medium before induction); ○, lyophilization; ■, complex medium; □, glycerol. See the text for experimental methods. Bars represent standard deviations.

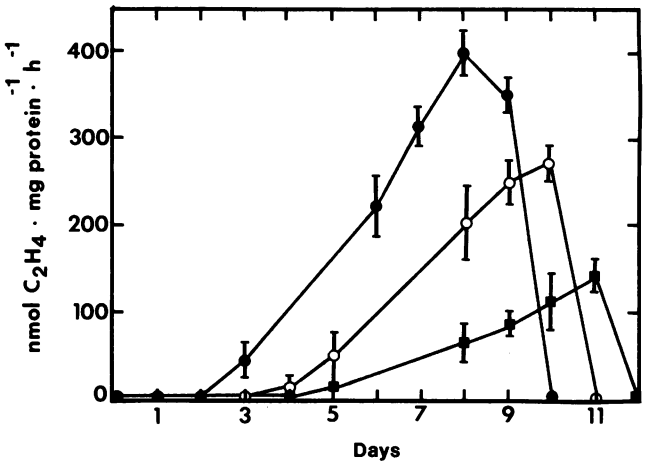

FIG. 2. Time course of nitrogenase activity for lyophilized *Frankia* strain ArI3 after first (■), second (○), and third (●) subcultures under standard culture conditions. Bars represent standard deviations.

DISCUSSION

Lyophilization, freezing in glycerol, and long-term storage in complex medium all appear to be acceptable techniques for the long-term preservation of *Frankia* strain ArI3. Each of these methods produced an inoculum that was effective and infective for *A. rubra* and also produced cells that fixed nitrogen in vitro when cultured in nitrogen-free medium. Lyophilization produced the best results, and freezing in glycerol produced the poorest. It is important to note that repeated subculturing under standard conditions improved the in vitro production of nitrogenase by inocula preserved by all three methods. Repeated subculturing after storage allows the vigor of the culture to return to its prestorage level. The effects of repeated subculturing after preservation on the ability of the inocula to form an effective symbiosis were not evaluated in these trials.

Lyophilization is a common method for the long-term preservation of microorganisms. Many species of bacteria preserved by this method have remained viable for 30 years (13). Nodulation and nitrogenase activity have been obtained from *Comptonia peregrina* HFPCpI1 (9) lyophilized for 6 years (P. Young, unpublished data).

Storing cultures in stationary tubes of complex medium was a convenient, inexpensive method for the long-term preservation of ArI3. The inoculum preserved in this fashion proved to be a good source for inducing nodulation of *A. rubra*. It was necessary to subculture the inoculum several times before obtaining in vitro nitrogenase activity rates comparable to the rates produced by our stock cultures.

Glycerol, used for freezing bacterial cultures, readily passes into cells and provides a cryoprotective action which provides both intracellular and extracellular protection against freezing (13). Glycerol concentrations ranging from 5 to 50% at freezing temperatures from −10 to −80°C have been tested for different microorganisms (15). This technique, under the conditions tested, proved to be the least favorable of the methods evaluated in our experiments. It is not known if *Frankia* strain ArI3 can metabolize glycerol, although another *Frankia* strain, LDAgp1, isolated from an *Alnus* sp. can utilize glycerol as a growth substrate (25). It is possible that the metabolism of glycerol-preserved ArI3 was affected by glycerol in such a way to make the inoculum less effective for nodulation and in vitro nitrogenase activity. Further experimentation involving a range of glycerol concentrations and storage temperatures seems warranted.

Frankia isolates have traditionally been cultured in complex media (1, 4, 6, 16). Cells grown in these complex media are typically subcultured every 3 to 4 months (12, 18). The development of a defined medium (7, 23, 27) has allowed progress to be made in studies of the nutritional and culture requirements of these bacteria. The time between subcultures has been reduced to 4 to 6 days under optimal conditions (21, 23). Despite the well-defined conditions used to culture these isolates, substantial variation remains in rates of growth and nitrogenase activity of *Frankia* strain ArI3. This variation may be due to the state of the inoculum used (23). Long-term preservation of *Frankia* stock may help to eliminate some of this variation and provide a common inoculum source less likely to be affected by mutations.

The results of this study indicate that *Frankia* strain ArI3 can be effectively preserved for long periods of time by a variety of techniques. The use of *Frankia* sp. as a commercial or research organism should benefit from the use of long-term preservation methods which maintain the bacteria in a condition as close as possible to that of the original isolates.

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