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Formation and Regeneration of Protoplasts of the Actinorhizal Nitrogen-Fixing Actinomycete *Frankia*

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Procedures for forming and regenerating protoplasts of four *Frankia* strains are described. Cells obtained from growth medium containing 0.1% glycine were digested with lysozyme (250 μg/ml) in a medium containing 0.5 M sucrose, 5.0 mM $CaCl_2$, and 5.0 mM $MgCl_2$. Protoplasts were formed during 15 to 120 min of digestion at 25°C. Optimum conditions for protoplast regeneration involved placing protoplasts on a layer of complex growth medium containing 0.3 M sucrose, 5.0 mM $CaCl_2$, and 5.0 mM $MgCl_2$ which was overlaid with a layer of 0.8% low-melting-point agarose containing 0.5 M sucrose, 5.0 mM $MgCl_2$, and 5.0 mM $CaCl_2$. The maximum regeneration efficiency was 36.9% for strain CpI1, 1.3% for strain ACN1^{AG}, 27% for strain EAN1pec, and 20% for strain EuI1c.

Genetic studies of the symbiotic nitrogen-fixing actinomycete genus *Frankia* are impractical at present because of the filamentous mode of growth of the organism. Prior to the procedures reported in this communication, it has not been possible to obtain single genomic cell units for study. Although it is possible that some protoplasts of a coenocytic organism such as *Frankia* may contain more than one copy of the chromosome, it is likely that most protoplasts will contain only one copy. Genetic studies of other filamentous actinomycetes are practical because it is easy to obtain and germinate the haploid spores. Most *Frankia* strains produce spores as clusters inside a sporangium (4, 12), but there are no published reports of germination of the spores. A comprehensive study of spore germination in several *Frankia* strains was undertaken in this laboratory (M. McBride, unpublished observations). We never observed more than a few of the spores to germinate and form germ tubes following incubation in a variety of simple and complex growth media. Addition of hot water extracts of roots and nodules of alders did not enhance germination. Attempts to activate by heat and cold shock, by treatment with chemicals such as detergents and solvents, and by repeated water washing also did not enhance germination.

Some *Frankia* strains harbor plasmids (7, 10, 11). The plasmids have yet to be exploited in genetic studies. A multicopy plasmid of *Frankia* sp. strain ArI3 was cloned into an *Escherichia coli* plasmid and transformed into *Streptomyces lividans* protoplasts (5), but the recombinant plasmid did not replicate. There are no reports of conjugation, transformation, or transduction in these bacteria (6).

Important tools in genetic studies of *Streptomyces* spp. are fusion and transformation of protoplasts (3). We report in this communication conditions necessary for producing and regenerating protoplasts of several *Frankia* strains. This information may prove useful in developing techniques for genetic studies of *Frankia*.

MATERIALS AND METHODS

Organisms and growth conditions. Four *Frankia* strains were used in this study. Strains ACN1^{AG} and EAN1pec were obtained from M. Lalonde, Quebec. Strains CpI1 and EuI1c were obtained from J. Torrey, Petersham, Mass. These strains will be referred to hereafter as ACN, EAN, CPI, and EUI, respectively. The basal growth medium for CPI and ACN contained 20 mM MOPS (morpholinopropanesulfonic acid) and 10 mM KH_2PO_4 (MP buffer), pH 6.5. The following constituents were sterilized separately and added to MP buffer to the indicated final concentrations: 10 mM NH_4Cl, 2.0 mM $MgSO_4$, 20 μM $FeCl_3$ dissolved in 100 μM nitrilotriacetic acid, and 1.0 mM $Na_2MoO_4 \cdot 2H_2O$. A 1.0-ml sample of trace salts mixture was added per liter of medium. The trace salts mixture contained (in grams per liter): 5.0 $MnCl_2 \cdot 2H_2O$, 0.25 $CuCl_2 \cdot 2H_2O$, 0.2 $CoCl_2 \cdot 6H_2O$, 0.5 H_3BO_3, 1.0 $ZnSO_4 \cdot 7H_2O$, and 10.0 $CaCl_2 \cdot 2H_2O$. The basal medium was the same for strains EAN and EUI except that the pH of the MP buffer was 6.8. The carbon sources, added after separate sterilization, were (final concentrations): 20 mM succinate for CPI and ACN, 20 mM mannitol, fructose, or succinate for EAN, and 5 mM propionate for EUI.

The cultures were incubated stationary in 1-liter flasks containing 250 ml of medium. Temperatures of incubation were 33°C for EUI, CPI, and ACN and 25°C for EAN.

Protoplast formation. Growth media supplemented with 0.1% glycine were inoculated with a 10% (vol/vol) suspension of fragmented mycelia. Inocula were grown for 14 days, harvested by centrifugation at 6,000 × *g*, and fragmented by grinding in a sterile glass tissue homogenizer. The cultures were incubated statically for 4 to 6 days as described in the preceding paragraph. The cells were then harvested by centrifugation at 6,000 × *g* at 20°C and washed twice with a solution of 0.5 M sucrose–5.0 mM $CaCl_2$–5.0 mM $MgCl_2$ (SCM). The washed mycelia were incubated at 25°C in SCM containing lysozyme (250 μg/ml) (0.5 mg/ml of cells [dry weight]). The suspensions were mixed by titurating at 15-min intervals with a wide-bore pipette.

Protoplast regeneration. Protoplasts were diluted 10^3- to 10^7-fold in SCM, and 0.5-ml samples were pipetted onto the surface of a medium containing 20 mM succinate, 0.3 M sucrose, 1.0 mM NH_4Cl, 0.005% KH_2PO_4, 5.0 mM $MgCl_2$, 5.0 mM $CaCl_2$, 1.0 mM Na_2MoO_4, 20 μM $FeCl_3$, trace salts (as described above), 0.1% yeast extract, 25 mM MOPS buffer, pH 7.0 (except for strain CPI, pH 6.5), and 2.2% Bacto-Agar (Difco Laboratories). Glucose at 20 mM replaced succinate in the medium for strain EUI. A 3.0-ml

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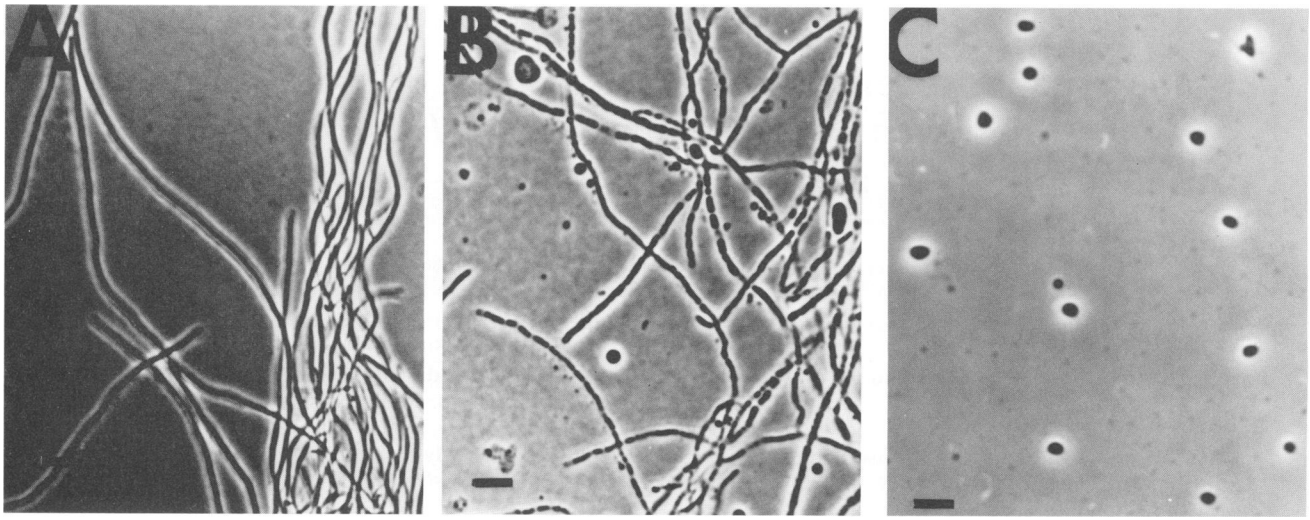

FIG. 1. Phase-contrast photomicrographs of *Frankia* sp. strain CPI during protoplast formation. Cells grown for 8 days in medium containing 0.1% glycine were suspended in SCM containing lysozyme (250 μg/ml). Samples were taken at zero time (A) and after 15 min (B) and 120 min (C) of digestion. Bars, 10 μm.

sample of 0.8% low-melting-point agarose in SCM at 37°C was poured onto the surface of each dilution plate. Mixing was done by tipping the plates from side to side before the agarose hardened. Spreading the protoplasts over agar media with a glass rod or mixing them directly with soft agarose or agar before pouring onto medium caused a significant reduction in protoplast regeneration. The plates were incubated at 25°C until colony growth was evident.

To determine the efficiency of protoplast regeneration, serial dilutions in SCM were plated. The numbers of colonies were counted and compared with the numbers of protoplasts obtained by microscope counting with a Petroff-Hauser counter. To determine whether colonies arose from mycelial fragments and not protoplasts, the protoplasts were diluted in water before plating.

Materials. Lysozyme and MOPS buffer were from Sigma Chemical Co. Low-melting-point agarose was from Bethesda Research Laboratories. Bacto-Agar, casein hydrolysate, and yeast extract were from Difco Laboratories.

RESULTS

Protoplast formation. Protoplasts were formed from each of the four *Frankia* strains following incubation with lysozyme (250 μg/ml) for 30 min in hypertonic stabilizing solution. The photomicrographs (Fig. 1) illustrate the time course of protoplast formation for strain CPI. In normal undigested hyphae after 15 min of lysozyme digestion, cytoplasm appeared to coalesce inside the hyphae walls (Fig. 1A), and protoplasts began to bleb out from the disintegrating hyphae (Fig. 1B). The maximum number of protoplasts was reached after 1 to 2 h of incubation. Spherical protoplasts formed at 120 min. (Fig. 1C). The protoplast preparations were sufficiently free of intact hyphae to make subsequent purification unnecessary. Passing the protoplasts through a cotton wool plug, as is recommended for *Streptomyces* protoplast preparations (3), removed some hyphal debris but significantly reduced the number of protoplasts. Unfiltered protoplast preparations were used in subsequent experiments.

The optimum concentration of lysozyme for each organism was 250 μg/ml (data not shown). Smaller or larger amounts of lsozyme decreased the protoplast yields.

The number of protoplasts of each organism was greater when 5.0 mM $MgCl_2$ and 5.0 mM $CaCl_2$ were present during lysozyme digestion. Sucrose, sorbitol, mannitol, and succinate at 0.5 to 1.0 M were equally effective in stabilizing protoplasts with respect to morphology and regeneration efficiency. The stabilization medium used in most experiments contained 0.5 M sucrose, 5.0 mM $MgCl_2$, and 5.0 mM $CaCl_2$.

Protoplast yields for each of the four isolates were increased by growing the cells in medium containing 0.1% (the optimal concentration) glycine. Growth was repressed and morphology was abnormal with higher concentrations of glycine in the medium. The effect of cell age on efficiency of protoplast formation is shown for strain EAN in Fig. 2. The number of protoplasts obtained per milligram of mycelium (dry weight) increased progressively as the cells grew for 10 days. The efficiency of protoplast formation then declined rapidly during further incubation. The same pattern of increased followed by decreased protoplast efficiency was similar for the other three isolates; the optimum age for protoplasting was 4 to 11 days (data not shown).

Regeneration of protoplasts. Protoplasts did not grow into colonies following inoculation onto the complex medium generally used for regeneration of protoplasts of *Streptomyces* (3). Initial success in obtaining regeneration of *Frankia* protoplasts was obtained by spreading cells onto the surface of a medium containing 0.1% Casamino Acids, 0.1% yeast extract, 1.0 mM NH_4Cl, 0.005% KH_2PO_4, 25 mM MOPS buffer, pH 7.0, 0.5 M sucrose, 20 mM succinate (except for strain EUI, for which succinate was replaced by 20 mM glucose), trace salts, and 2.0% agar. Overlaying the protoplasts on this medium with a layer of medium which contained 0.8% low-melting-point agarose, 5.0 mM $CaCl_2$, 5.0 mM $MgCl_2$, and 0.5 M sucrose, applied at 37°C, increased the number of colonies formed from the protoplasts 10^2 to 10^3 times. Bacto-Agar at 0.8%, cooled to 42°C, could replace the low-melting-point agar, but the efficiency of regeneration was slightly reduced.

Growth that occurred following inoculation of protoplast

preparations onto regeneration medium resulted from regenerated protoplasts and not from growth of fragments of mycelia. Evidence for this statement is based on three control experiments. No colonies were formed when protoplast preparations were diluted 1:10 with water just prior to inoculation onto regeneration medium. Vortexing protoplast suspensions in SCM medium prior to inoculation also resulted in no growth. Finally, no growth occurred following inoculation of protoplast preparations onto growth medium not containing an osmotic stabilizing agent.

To define the optimum conditions for protoplast regeneration, the effect of varying the sucrose and Mg and Ca ion concentrations was tested. Other components of the medium were as described above. Regeneration of protoplasts of the four isolates was optimal with 0.3 M sucrose, 5.0 mM $CaCl_2$, and 5.0 mM $MgCl_2$ (Table 1). This medium is referred to subsequently as preliminary regeneration medium (PRM). The optimal temperature for regeneration was 25°C for all isolates (data not shown).

Regeneration of protoplasts was slow. Visible growth for each isolate appeared only after 14 days of incubation. An example of the time course for regeneration of protoplasts of the four *Frankia* strains in PRM is illustrated in Fig. 3. Growth of none of the four isolates visible until after 14 days of incubation. The efficiency of regeneration (percentage of protoplasts forming colonies) increased for strain EAN to a maximum value of 27% at 40 days. The maximum efficiency for EUI of 12% was reached at 35 days. Maximum efficiency for CPI and ACN was 18.3 and 0.9%, respectively. The colonies formed from all four isolates originated from protoplasts because plating of each onto appropriate growth medium without osmotic stabilizer yielded no growth during the 35-day incubation period (data not shown).

A detailed study was made of optimal conditions for regeneration of protoplasts of strains CPI and EAN, representatives of two different host compatibility groups. The optimum pH and buffer conditions for regeneration of strain CPI in PRM was 25 mM MOPS buffer, pH 6.0 to 6.5. The percentage of regeneration was reduced by half at pH 7.0 (the values were 14.4% at pH 6.5 and 7.1% at pH 7.0). The optimum for EAN was 25 mM MOPS, pH 7.0. The percentage of regeneration was 13.9% at pH 6.5, 24.0% at pH 7.0, and 16.1% at pH 7.5.

Several osmotic stabilizing agents were tested at 0.5 and 0.3 M for their effect on efficiency of regeneration for CPI and EAN. Sucrose proved superior to sorbitol, mannitol, succinate, and NaCl for both organisms. The percent regeneration for CPI and EAN with PRM containing 0.5 M sucrose was 13.5 and 4.7%, respectively; the values at 0.3 M were 36.9 and 11.2%, respectively.

PRM contained NH_4Cl, Casamino Acids, and yeast extract. The requirements for each were tested. Protoplast regeneration did not occur in medium containing NH_4Cl without yeast extract and Casamino Acids. The percent regeneration was essentially the same in medium containing

TABLE 1. Effect of concentration of sucrose, calcium, and magnesium on regeneration of protoplasts of four *Frankia* strains[a]

Medium Components			% Regeneration[b]			
Sucrose (M)	$CaCl_2$ (mM)	$MgCl_2$ (mM)	CPI	ACN	EAN	EUI
0.3	—	—	1.7	0.6	3.0	2.6
0.3	5.0	5.0	17.2	1.3	25.0	20.0
0.3	25	25	1.8	0.2	8.1	0.1
0.5	—	—	0	0	0.2	0
0.5	5.0	5.0	3.2	0.2	0.4	0
0.5	25	25	1.3	0	0	0

[a] Protoplasts of each organism were spread onto the medium described in Materials and Methods, except for the variations in concentrations of sucrose, $CaCl_2$, and $MgCl_2$ shown, and overlaid with 0.8% Bacto-Agar containing SCM. Incubation was at 25°C for 35 days.

[b] Percentage of protoplasts (microscope counts) forming colonies. The values shown are the averages of two or more determinations.

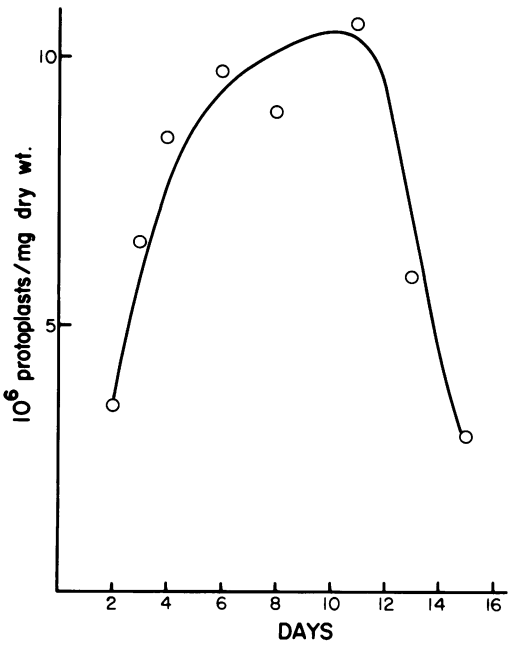

FIG. 2. Effect of culture age on protoplast formation by *Frankia* sp. strain EAN. Mycelia were incubated in succinate-NH_4Cl medium supplemented with 0.1% glycine. Protoplasts were prepared at the times shown. Protoplast numbers were obtained by microscope counts and standardized to the mycelial dry weight.

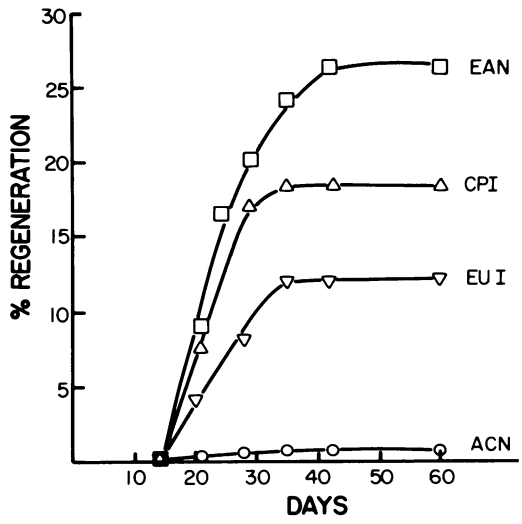

FIG. 3. Effect of incubation time on regeneration of protoplasts of four *Frankia* strains. Protoplast preparations of each organism were spread onto PRM and overlaid with 0.8% Bacto-Agar containing SCM. The number of colony-forming units was counted at various times of incubation. Data are expressed as percentage of the initial protoplasts (microscopic counts) that formed colonies.

NH_4Cl and yeast extract with and without Casamino Acids. The optimal concentration of NH_4Cl and yeast extract was 1.0 mM and 0.1%, respectively. Yeast extract was required for regeneration of protoplasts but not for growth, since colonies formed from protoplasts grew well when transferred to growth medium containing NH_4Cl as the nitrogen source and no yeast extract.

DISCUSSION

This report describes the first successful procedure for isolation and regeneration of protoplasts of *Frankia* strains. Faure-Raymaud et al. (2) described protoplast formation of four *Frankia* strains but did not report their regeneration. These workers used lysozyme and achromopeptidase, both at a concentration of 7.5 mg/ml, to prepare the protoplasts. The four strains of *Frankia* studied in this investigation were converted efficiently into protoplasts by lysozyme (0.25 mg/ml). It was necessary to grow the organisms in medium containing glycine to render the walls suceptible to the lower level of lysozyme. Efficient conversion of streptomycetes to protoplasts by lysozyme also requires growth in medium containing glycine (8).

For optimum regeneration, the *Frankia* protoplasts had to be sandwiched between a layer of a nutritionally rich osmotically stabilizing medium and a layer of low-melting-point agarose containing 0.3 M sucrose and calcium and magnesium ions. The maximum efficiency of regeneration of the four *Frankia* strains varied widely: 27% for EAN, 20% for EUI, 37% for CPI, and 1.3% for ACN. Conditions for maximum regeneration were studied in detail only with strains CPI and EAN. The regeneration efficiency for strains EUI and ACN might be improved by further investigation. The percent regeneration efficiency that we observed for these *Frankia* strains is within the range of values reported for protoplasts of *Streptomyces* spp. (1, 8, 9).

Frankia research is hindered by the lack of a genetic transfer system. The possibility of forming and regenerating protoplasts of the organisms is an important step toward developing a feasible genetic system. The kinds of protoplast transformation systems that work efficiently in *Streptomyces* spp. and other organisms should also be applicable to *Frankia* sp.

ACKNOWLEDGMENTS

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