Conditions for Protoplasting, Regenerating, and Transforming the Calicheamicin Producer, *Micromonospora echinospora*

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We report methods to generate protoplasts, to regenerate mycelia, and to transform *Micromonospora echinospora*. This actinomycete produces the unusual antitumor antibiotics, the calicheamicins. These protocols may be applied to other actinomycetes that have been difficult to transform. These methods also may facilitate the cloning of calicheamicin biosynthetic genes by genetic complementation.

*Micromonospora echinospora* (NRRL 15839) is an actinomycete which produces the calicheamicins (9, 10), a family of antitumor antibiotics of distinct structure that cause site-specific double-stranded cleavage of DNA (20). *M. echinospora* is also interesting because developmental regulation of unusual promoters has been documented (1, 2). We describe protocols for the protoplasting, regeneration, and transformation of *M. echinospora*. Because mutants blocked in the calicheamicin pathway have been isolated (14), a transformation system is an important step in cloning calicheamicin biosynthetic genes, as described for other antibiotic pathways (3, 5, 18).

Transformation of *Streptomyces* protoplasts with plasmid DNA has been well documented (4). Procedures have been described for protoplasting and regenerating *Micromonospora* species (7, 8, 12, 13, 15-17). Successful transformation of *Micromonospora* sp., however, is rare. Recently it has been found that both *Micromonospora rosaria* (12) and *Micromonospora purpurea* (7) could be transformed by pIJ702, the broad-host-range plasmid derived from *Streptomyces* sp. (6). Unfortunately, *M. echinospora* is naturally resistant to thiostrrepton, precluding the use of many common *Streptomyces* plasmids. To maximize the possibility of expressing another selectable marker, encoding kanamycin resistance, we constructed plasmids that contained *M. echinospora* promoters expressing the kanamycin resistance gene.

To form protoplasts, *M. echinospora* mycelia were inoculated into 50 ml of GER medium (8) supplemented with 0.15% glycine and CaCl₂ to 20 mM. The culture was incubated for 48 to 60 h at 28°C, vigorously aerated in 250-ml baffled flasks (Bellco) containing three glass beads (4 mm). The cells were harvested by centrifugation, washed with 10.3% sucrose, and resuspended in 4 ml of L buffer (4) (pH 7.6) containing 2 mg of lysozyme. Effective protoplasting usually occurred after 1 to 2 h of incubation at 30°C. Protoplast formation, monitored by microscopy, was generally greater than 95% of cells.

To stabilize the protoplasts after incubation, the final concentration of MgCl₂ and CaCl₂ was adjusted to 25 and 50 mM, respectively. In order to separate protoplasts from mycelia and to enhance the frequency of regeneration, the suspension was filtered through cotton wool (4) and then through Schleicher and Schuell Spartan 5-μm-pore-size filters that had been rinsed with modified P buffer (4). Protoplasts were harvested by centrifugation (IEC HNSII clinical centrifuge; 3,000 rpm for 10 min at room temperature), washed twice with modified P buffer, and resuspended in a final volume of 4 ml.

To test for regeneration, protoplasts were diluted, mixed with 3 ml of soft regeneration agar medium, and inoculated onto regeneration agar medium plates (8) modified to contain 0.15 M sucrose. Protoplasts were incubated at 30°C for 10 to 14 days. The CFU were compared with the number of protoplasts observed with the microscope by using a Petroff-Hauser chamber; from 1 to 4% of protoplasts regenerated to form visible colonies. Protoplasts were incapable of forming colonies in the absence of an osmotic stabilizer. However, mycelium that escaped the protoplasting and filtration treatment were detected as faster growing colonies which could grow in the absence of 0.15 M sucrose. They represented less than 1% of protoplasts counted in the microscope. A high proportion of protoplast formation and efficient filtration steps are important to minimize mycelial contamination.

Several modifications of the regeneration medium were tested. The Casamino Acids were omitted from the regeneration medium without visible effect on regeneration. Thus, it is possible to select for prototrophic markers among auxotrophic mutants of *M. echinospora* (13a) by using regeneration medium lacking Casamino Acids. Disodium succinate, glycerol, and sorbitol were substituted as osmotic stabilizers without success. We did not test the effect of adding alternative carbon sources, because sucrose, the osmotic stabilizer, is a favored carbon source for *M. echinospora* (data not shown). Other nitrogen sources, yeast extract or NZ amine (enzymatic digest of casein [Sheffield Products]) at 0.5%, were substituted for asparagine. Mucoid colonies that appeared to contain L forms rather than mycelia were observed. These colonies could not replicate onto media lacking an osmotic stabilizer. We also attempted to regenerate protoplasts on complex medium (GER medium containing 0.15 M sucrose as the osmotic stabilizer); again mucoid colonies grew on the plates. Apparently, regeneration of protoplasts to mycelia was enhanced in medium containing a growth rate-limiting nitrogen source.

In order to transform *M. echinospora*, a vector containing a selectable marker was required. *M. echinospora* contains no detectable plasmids. We therefore considered plasmids from the related genera, *Streptomyces*. The *tsr* gene (6), which encodes the thiostrrepton resistance determinant, is a frequently used selectable marker. However, *M. echinospora* is resistant to 150 μg of thiostrrepton per ml. The promoter-probe plasmid, pIJ486 (19), contains the *aphII*

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structural gene, encoding a kanamycin resistance determinant, in addition to the *tsr* gene. When a promoter-bearing fragment is inserted upstream of *aphIII*, kanamycin resistance is then conferred. In order to maximize the possibility that the *aphIII* gene would be expressed in *M. echinospora* transformants, BamHI fragments derived from *M. echinospora* were inserted into the BamHI site preceding *aphIII*. The ligation was transformed into *Streptomyces lividans*, and thiostrepton-resistant transformants were selected for as previously described (2). One transformant was resistant to 200 μg of kanamycin per ml contained in minimal medium (4) supplemented with histidine and leucine. It contained a plasmid called pPP8, which consisted of the 6.2-kb vector and a 1.8-kb insert derived from *M. echinospora* (data not shown) and was used in our transformation studies.

*M. echinospora* protoplasts were mixed with 4 μg of purified pPP8 DNA as previously described (4), and then a portion containing 7 × 10⁷ protoplasts and 1 μg of DNA was poured onto a regeneration plate in a soft agar overlay. After incubation for 6 days at 30°C, the regeneration plate was overlaid with 1/10 volume of soft agar containing kanamycin to a final plate concentration of 150 μg/ml. Two weeks after the transformation, colonies were tested for growth at 30°C in GEAR agar medium containing 5 μg of kanamycin per ml and subsequently in medium containing 50 μg of kanamycin per ml. Six putative transformants were resistant, in GEAR medium, to 50 μg of kanamycin per ml. The frequency was one transformant per 10⁷ protoplasts.

In order to prove that kanamycin resistance was due to the transformation of *M. echinospora* by plasmid pPP8, DNA minipreps of putative transformants were prepared as previously described (4) and subjected to agarose gel electrophoresis. Although the *M. echinospora* chromosomal band was visible after staining with ethidium bromide, no plasmid band was detectable. The plasmid supercoiled and nicked bands, however, were clearly visible by Southern analysis (11), with plasmid pPIJ486 DNA nick translated with [α-³²P]dCTP as the probe (Fig. 1A). When the plasmid was digested with BamHI restriction enzyme, the 6.2-kb band was then detectable within the *M. echinospora* transformants (Fig. 1B). In order to confirm the presence of plasmid pPP8 within the *M. echinospora* transformants by an independent method, *S. lividans* TK54 was transformed with the *M. echinospora* miniprep used for Fig. 1. Plasmid pPP8 was recovered from *S. lividans* transformants, confirming that *M. echinospora* had been successfully transformed. Despite its high copy number in *S. lividans*, the plasmid was found in very low copy number within *M. echinospora*, indicated by the Southern analysis (Fig. 1) and the inability to confirm its presence by ethidium bromide staining (data not shown).

In summary, we have developed useful protocols for protoplasting, regenerating, and transforming *M. echinospora* that may be applicable to other unusual actinomycetes. Regeneration of protoplasts to mycelia required 0.15 M sucrose, and not other osmotic stabilizers, and a growth rate-limiting source of nitrogen. The plasmid pPP8 could transform *M. echinospora* at low frequency. Thiostrepton resistance, a good selectable phenotype for *M. rosaria* (12) and *M. purpurea* (7), could not be utilized for *M. echinospora*, because of its natural resistance to the drug. Our strategy was to utilize promoters from *M. echinospora* to assure expression of the kanamycin resistance marker. Finally, because replication and/or maintenance of the plasmid pPP8 was probably defective in *M. echinospora*, the transformant system could be improved after identifying a more effective plasmid replicon for this host. These methods may prove useful in cloning calicheamicin biosynthetic genes by genetic complementation of blocked mutant strains (14).

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REFERENCES


