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A Simple and Rapid Method of Transformation of *Streptomyces rimosus* R6 and Other Streptomycetes by Electroporation

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Usually plasmid DNA is introduced into *Streptomyces* strains by polyethylene glycol-mediated transformation of protoplasts. However, many *Streptomyces* strains are only poorly or not at all transformable via protoplasts. Therefore, we have optimized the parameters critical for the application of electrotransformation of plasmid DNA into *Streptomyces* species. The most critical parameters evaluated for electrotransformation of the model strain *Streptomyces rimosus* R6 were the pretreatment of mycelia, buffer composition, and electric field strength. The electrocompetent mycelia were prepared from 24-h-old cultures, treated mildly with lysozyme, resuspended in sucrose-glycerol-polyethylene glycol buffer, and stored in aliquots at −70°C. The electric field strength of 10 kV/cm at 400 Ω and a capacitance of 25 μF was applied. The method is simple and rapid, yielding transformant colonies in 48 to 72 h. Efficiencies of 10^5 to 10^6 transformants per μg of plasmid DNA were reproducibly achieved for *S. rimosus* R6 and its mutants, and these numbers were 10^2 to 10^3 higher than those attained by polyethylene glycol-assisted transformation of protoplasts. In addition, we show that electroporation can be applied to other *Streptomyces* species, such as *S. lividans* 66, *S. coelicolor* A3(2), and an *S. venezuelae* strain. This last one could not be transformed by the standard protoplast procedure. Our data suggest that, because of the diversity of streptomycetes, the conditions have to be optimized for each strain.

Streptomycetes are known to degrade numerous macromolecules and synthesize a wide range of antibiotics as well as other commercially important metabolites. The complex life cycle of these bacteria includes formation of substrate mycelia, aerial hyphae, and spores (14). In past years, polyethylene glycol (PEG)-mediated plasmid transformation of protoplasts had allowed the rapid development of gene cloning in various *Streptomyces* species, particularly in *S. lividans* 66 (12), *S. ambofaciens* (18), *S. coelicolor* A3(2) (1), *S. fradiae* (18), and *S. rimosus* (22, 23), as well as in some others. Although this transformation procedure is generally applicable to several *Streptomyces* species, it is necessary to optimize growth and establish the optimal conditions for protoplast formation and regeneration (12). Moreover, the transformation of the fragile protoplasts is tedious and frequently not reproducible; thus, numerous *Streptomyces* strains could not be proven to be transformable.

Within recent years, transformation by electroporation has been rendered possible for several gram-negative (i.e., *Escherichia coli* [6, 26], *Pseudomonas aeruginosa* [5], and *Agrobacterium* spp. [19]) and gram-positive (i.e., *Brevibacterium lactofermentum* [2]; *Corynebacterium glutamicum* [8]; and some species of *Lactobacillus* [3, 11], *Mycobacterium* [9, 10, 27], *Streptococcus* [24], and *Rhodococcus* [4]) bacteria. Electroporation involves the application of a brief, high-voltage pulse to a suspension of cells and DNA, resulting in transient membrane pores and subsequent uptake of DNA. Therefore, this method is less tedious and time-consuming than the transformation of protoplasts and has been proven to be especially useful for strains previously considered untransformable (26). The first attempt to electroporate *S. lividans* protoplasts was made by MacNeil (15). However, this procedure involved the tedious preparation of protoplasts and was much less effective than the PEG-mediated transformation of *S. lividans* protoplasts.

In this paper, we describe for the first time an efficient and rapid method of transforming *Streptomyces* mycelia by electroporation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. rimosus* R6 (21, 22) was cultivated in liquid medium (CRM) containing the following components (grams per liter): glucose, 10; sucrose, 103; $MgCl_2 \times 6H_2O$, 10.12; tryptic soy broth, 15; and yeast extract, 5. The multicopy plasmid pIJ486 (12) was kindly provided by M. J. Bibb, Norwich, United Kingdom, and isolated from an *S. rimosus* R6 transformant. *S. lividans* 66 and *S. coelicolor* A3(2) were obtained (12) from D. A. Hopwood, Norwich, United Kingdom. *S. rimosus* 4018 (25) and *Streptomyces venezuelae* 13S (17) were gifts from I. Hunter, Glasgow, United Kingdom, and L. C. Vining, Halifax, Canada, respectively.

Optimized electrotransformation conditions. The *S. rimosus* R6 mycelium to be electrotransformed was grown in 100 ml of CRM at 30°C on a rotary shaker (250 rpm), harvested after 24 h by centrifugation at 4°C (10,000 rpm), thoroughly resuspended in a total of 100 ml of ice-cold 10% sucrose, centrifuged, resuspended in 50 ml of 15% ice-cold glycerol, and centrifuged. The mycelium was suspended in 10 ml of 15% glycerol containing lysozyme (100 μg/ml). Then the suspension was incubated at 37°C for 30 min and washed twice with ice-cold 15% glycerol. The pellet was resuspended in 1 to 5 ml of 30% (wt/vol) PEG 1000–10% glycerol–6.5% sucrose dissolved in deionized water. The suspension was dispensed in 50-μl aliquots in Eppendorf tubes, immediately frozen on dry ice with methanol, and stored at −70°C. One 50-μl aliquot containing mycelial fragments was thawed at room temperature and placed on ice, and 10 ng to 1 μg of plasmid DNA in 1 to 2 μl of H_2O was added. The mixture was transferred into a 2-mm-gapped electrocuvette (Bio-Rad Laboratories, Richmond, Calif.) and subjected to a 2-kV electric pulse (10 kV/cm) from a gene pulser (Bio-Rad), which was connected to a pulse controller (25-μF capacitor; parallel resistance, 400 Ω). The pulsed mycelium was diluted with 0.75 ml of ice-cold CRM and shaken in small vials for 3 h at 30°C. Prior to plating, liquid CRM was added to a final volume of 1 ml, and dilutions were spread on tryptic soy broth plates containing thiostrepton (30 μg/ml). The percentage of surviving colonies was estimated from serial dilutions plated on the same medium without thiostrepton. Transformants could be scored after 40 h. Controls were performed by omitting either the transforming DNA or the electric pulse.

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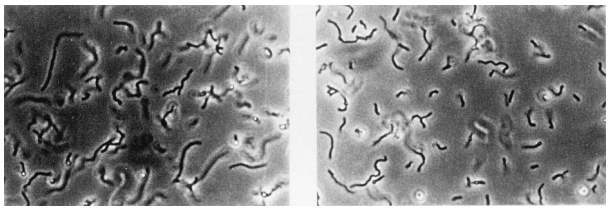

FIG. 1. Microscopy of hyphae grown for 24 h without (left) and after (right) treatment with lysozyme (100 μg/ml).

RESULTS

Growth conditions. *S. rimosus* R6 is an important producer of tetracycline. Since PEG-assisted transformation of this strain resulted in only 10^3 transformants, it was chosen as a model strain (21, 22, 23) to optimize electroporation conditions. The strain was found to grow as finely dispersed mycelia if cultivated in a complete medium containing sucrose (i.e., CRM). The efficiency of transformation attained its highest level when the mycelia were grown for 24 h at 30°C on a rotary shaker at 250 rpm. Many bacteria (including some *Streptomyces* strains) grown with glycine-containing media were shown to be more susceptible to the action of lysozyme (7, 12). However, cultivation in the presence of glycine (0.5 to 2.5%) did not improve the transformation efficiency.

Effect of lysozyme on electrotransformation efficiency. Concentrated mycelia (forming about 10^9 colonies) of *S. rimosus* R6 were either used directly or pretreated with different concentrations of lysozyme (10 to 400 μg/ml). No transformants were obtained without lysozyme treatment. Incubation of the mycelia with lysozyme (up to 100 μg/ml) for 30 min at 37°C was found not to affect the number of CFU. Microscopical inspection (Fig. 1) revealed that the pretreated mycelia resembled the untreated mycelia, and no protoplasts could be detected. In order to optimize the buffer conditions, mycelia (pretreated with lysozyme [100 μg/ml]) were suspended in a mixture containing various ratios of sucrose, glycerol, and PEG 1000, to which 10 ng of the multicopy plasmid vector pIJ486 isolated from *S. rimosus* R6 in its supercoiled conformation had been added. The highest transformation efficiencies were obtained when lysozyme-pretreated mycelia had been suspended in an aqueous solution containing 30% PEG, 10% glycerol, and 6.5% sucrose. The addition of $MgCl_2$ or HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at different concentrations did not increase the transformation efficiency.

Effect of field strength and pulse length. Up to field strengths of 7 kV/cm, the percentage of surviving colonies was nearly 100%. When field strengths ranged between 10 and 12.5 kV/cm, the number of surviving colonies dropped from 80 to 40%. About 2×10^3 transformants were obtained per 1 μg of pIJ486 DNA at 5 kV/cm. This number increased to about 3×10^5 at 10 kV, and it was almost the same at 12.5 kV. However, frequent arcing occurred at 12.5 kV/cm. For these reasons, field strengths of 10 kV/cm were routinely used. The effect of an alteration of the pulse length (time constant), achieved by modifying the size of the resistor placed parallel with the sample in the pulse controller, was also examined. The duration of the pulse impaired the number of surviving colonies, which decreased to about 33% at 800 Ω (Fig. 2). As demonstrated in Fig. 2, the number of transformants increased from 3×10^4 at 200 Ω to 2×10^6 at 800 Ω. Longer pulses were not applied, to avoid arcing. When the mycelium was subjected to two or more consecutive pulses, the efficiency was as high as the one obtained with a single pulse. The mycelium was held on ice for 30 to 60 s between pulses. The routinely applied electric field strength was 10 kV/cm at 400 Ω.

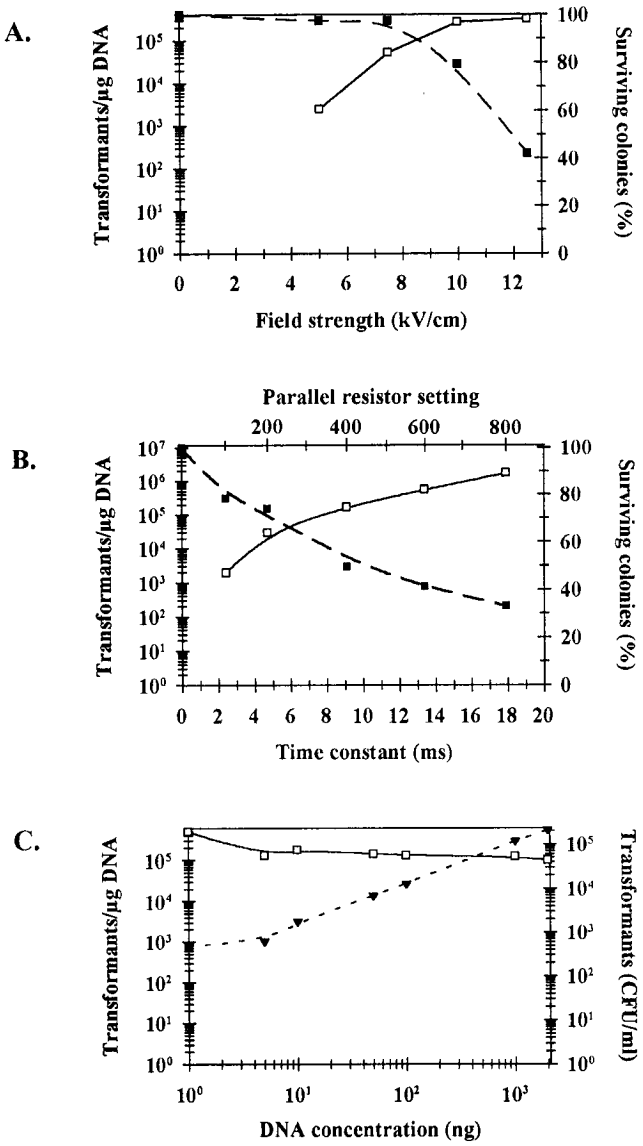

FIG. 2. Effects of different electroporation parameters on transformation and survival of *S. rimosus* R6. Electroporation experiments were carried out with 50 μl of mycelial fragments in the presence of 10 ng of pIJ486. All samples were diluted 1:20 with CRM after the pulse was applied. (A) Effect of the initial voltage of the applied pulse on transformation efficiency and survival of the electroporated cells at 25 μF and 400 Ω. □, transformation efficiency; ■, survival. (B) Effect of the external parallel resistance and the electric pulse duration on transformation efficiency and survival at 25 μF and 10 kV/cm. □, transformation efficiency; ■, survival. (C) Effect of DNA concentration on the number of transformants. Electroporation conditions were 25 μF at 10 kV/cm and 400 Ω. □, transformation efficiency; ▼, number of transformants.

Effect of DNA concentration. With 1 ng of pIJ486 (supercoiled form), approximately 3.2×10^2 transformants were obtained at 10 kV and 400 Ω. The number of transformants rose proportionally when the DNA concentrations ranged between 10 ng and 2 μg. Transformants (1×10^5 to 2×10^5) were obtained with 1 to 2 μg of pIJ486. The saturation level was not reached when 2 μg of DNA was added.

Effect of dilution and selection of transformants. After electroporation, it was essential to dilute a sample with a medium

TABLE 1. Effect of dilution after electric pulse on the efficiency of transformation

Dilution[a]	Number of transformants (CFU/ml)	Efficiency of transformation (transformants/ml/μg of DNA)
1:3	2.2×10^2	2.2×10^4
1:5	2.0×10^3	2.0×10^5
1:10	1.6×10^3	1.6×10^5
1:16	4.8×10^3	4.8×10^5
1:20	2.7×10^3	2.7×10^5

[a] Samples (50 μl) of thawed mycelium were used for each dilution. After a 3-h expression period, all samples were combined with CRM to a final volume of 1 ml prior to plating on medium with thiostrepton. Ten nanograms of pIJ486 DNA in 1 μl of Tris-EDTA was applied in each experiment at 25 μF, 10 kV/cm, and 400 Ω.

containing Mg^{2+} and sucrose to enable a fast regeneration of the damaged cells. This medium had already been successfully used to regenerate protoplasts of *S. rimosus* R6 (22). As *Streptomyces* hyphae form mycelia which tend to aggregate in the presence of PEG, the number of transformants reached its lowest level when the electroporated suspension had been diluted only 1:3. The number of colonies consistently increased by about 20-fold when the suspension had been diluted 1:16 (Table 1). The CRM could be added within 30 min after electrotransformation if the sample was kept on ice. This does not correspond to the data presented for *E. coli* and other gram-positive bacteria (7, 26).

After incubation at 30°C for 3 h with shaking, the mycelia were spread on complete medium supplemented with thiostrepton. When this method is used, transformant colonies develop within 48 h. Thus, this method is advantageous compared with the procedure which uses protoplasts, which require up to a 24-h expression period and regenerate within 2 weeks.

High-efficiency protocol for electroporation. The optimized parameters to efficiently transform *S. rimosus* R6 mycelia with 1 ng of pIJ486 are summarized in Table 2. When using the same batch of mycelia, identical numbers of transformants (10^5 to 10^6/μg of DNA) were repeatedly obtained under comparable conditions. In contrast, transformations of protoplasts are considerably less reproducible.

Electroporation of other *Streptomyces* species. Comparative studies of the electroporation of other strains revealed that several parameters are critical. They need to be optimized for each strain. It was most important to cultivate the strains in such a way (e.g., by modification of media and shaking conditions) that the mycelia did not form extensive pellets during cultivation. The cultivation time varied (16 to 48 h) according to the chosen strain (see below). In addition, some strains (i.e., *S. lividans*) tend to clump considerably more after having been subjected to electroporation; thus, the number of CFU considerably decreases. The concentration of lysozyme had to be altered in some cases, too (see below).

TABLE 2. Summary of transformation efficiencies at optimized conditions[a]

Field strength (kV/cm)	Resistance (Ω)	Time constant (ms)	Number of transformants (CFU/ml)	Transformation efficiency (transformants/ml/μg of DNA)
10	400	9.1	1.7×10^2	1.7×10^5
10	600	13.5	5.2×10^2	6.0×10^5
10	800	17.9	1.7×10^3	1.8×10^6
12.5	400	7.6	3.4×10^2	3.0×10^5
12.5	600	11.0	5.2×10^3	7.5×10^5

[a] The experiments were performed with 1 ng of pIJ486 closed circular DNA. Samples were diluted 1:16 after an electric pulse was applied and combined with CRM to a final volume of 1 ml after 3 h at 30°C with shaking.

It had been shown previously that some mutants (i.e., 554W, 601, and 615) of *S. rimosus* R6 were transformed quite poorly after PEG-assisted transformation of protoplasts, yielding only about 10^2 to 10^3 transformants per μg. However, when they were electroporated under the same conditions as those used for *S. rimosus* R6, 10^5 to 10^6 transformants per μg were achieved. Electroporation of another *S. rimosus* 4018 strain (25) resulted in 10^5 to 10^6 transformants when the mycelia had been grown for 48 h and pretreated with an enhanced concentration of lysozyme (400 μg/ml). Thus, the numbers significantly increased (10 to 100 times for 4018 and 100 to 1,000 times for R6), compared with those yielded by protoplast transformation.

S. venezuelae 13S was subjected to electrotransformation with the vector pIJ486, as an example of a strain whose protoplasts could not be transformed (17a). By using the protocol established for *S. rimosus* R6, 10^3 to 5×10^4 *S. venezuelae* transformants were obtained. With the same protocol, 10^3 transformants could be gained by electroporation of *S. coelicolor* A3(2) mycelia. *S. lividans* mycelia proved to be more sensitive to lysozyme than those of the other strains tested. Nonetheless, it was possible to electrotransform *S. lividans* with the above-mentioned protocol when the lysozyme concentration had been reduced to 50 μg/ml and the mycelia were very young (16 to 20 h); however, only relatively low frequencies were achieved (5×10^2 transformants per μg of DNA).

DISCUSSION

Up to now, transformation of protoplasts has been the only well-established procedure to transform numerous *Streptomyces* strains, including *S. fradiae* (18), *S. coelicolor* A3(2) (1), *S. lividans* 66 (12), *S. rimosus* (22), and several others. Apart from being laborious and time-consuming, the efficiency of the protoplast formation and the regeneration and transformation of protoplasts vary to a great extent among different *Streptomyces* strains. Therefore, effective transformation protocols are still lacking for a lot of *Streptomyces* strains, and many of them are currently not transformable.

An increasing number of different gram-positive and gram-negative bacteria have been shown to become transformable by electroporation. This method has turned out to be simpler, more reproducible, and more efficient than other transformation procedures (26). In this study, *S. rimosus* R6 was chosen to optimize the conditions for electroporation. Since this strain has been used to produce oxytetracycline (21) for a long time, the conditions for its dispersed and rapid growth have already been optimized. Many bacteria grown in the presence of glycine show an increased susceptibility to the action of agents degrading the cell wall. The addition of glycine to the culture medium therefore improved the protoplast formation of various *Streptomyces* species and of several other bacteria (7, 12). Contrary to this, earlier studies had revealed that *S. rimosus* R6 hyphae grown in the absence of glycine can be efficiently converted into protoplasts which regenerate easily (22). Likewise, the electrotransformability of the *S. rimosus* R6 mycelia was shown not to depend on their cultivation in the presence of glycine. Corresponding observations have been made for several other bacteria (i.e., *Bacillus* species [16] and *Acetobacterium woodii* [28]). In contrast, several *Streptococcus* (7) and *Rhodococcus* (29) species as well as *Corynebacterium glutami-*

cum (8) were susceptible to electroporation only if cultivated in the presence of glycine.

As reported for species of *Rhodococcus* (29) and *Corynebacterium glutamicum* (8), *S. rimosus* R6 was most effectively transformed by electroporation at its late logarithmic phase (24 h). Dispersed growth and pretreatment of the *S. rimosus* R6 mycelia with low concentrations of lysozyme were prerequisites for electrotransformation. Preliminary data suggest that the optimal concentration of lysozyme is different for each strain. Mild treatment with lysozyme resulted in *S. rimosus* hyphae which were still osmotically stable but more accessible to take up DNA during electroporation. It has been reported that agents interfering with the synthesis of mureine (i.e., lysozyme and ampicillin) enhance the efficiency of electrotransformation of several gram-positive bacteria (2, 7).

The addition of 30% PEG to the electroporation medium was optimal for *S. rimosus* R6. Similar observations had been made for *Rhodococcus fascians* (4), *Lactobacillus hilgardii* (13), and *Bacillus thuringiensis* (16). The positive effect of PEG has been attributed to volume exclusion, interactions with cell membranes, and the increased survival of electroporated cells.

Depending on the bacterial species studied, the optimal initial field strength of the discharge pulse ranged between 7 and 12.5 kV/cm. The highest transformation efficiency at 10 kV/cm correlated with 80% surviving *S. rimosus* R6 colonies. Thus, as also described for *Campylobacter jejuni* (20) and *R. fascians* (4), the death of a significant portion of the cells was not a prerequisite for efficient transformation. The duration of the electrical discharge was reported to be an important factor for *C. jejuni* (20). The number of *S. rimosus* R6 transformants increased with longer pulses.

A variation of the DNA concentrations over a range of 10 ng to 2 μg/ml led to a significant increase in the number of transformants (up to 10^5 to 10^6), whereas the efficiency dropped by about fivefold. However, the transformation efficiency for *R. fascians* decreased dramatically at higher DNA concentrations (4).

Earlier attempts to electrotransform *S. lividans* protoplasts (15) resulted only in different numbers of transformants, ranging between 10^1 and 10^4. As it is tedious to prepare protoplasts, this approach has never been used again. In contrast, mild lysozyme treatment of *Streptomyces* mycelia can be rapidly performed and does not lead to detectable protoplast formation. As a result, electrotransformation of *S. rimosus* R6 and its mutants or of *S. rimosus* 4018 (25) is considerably more rapid, reproducible, and efficient than PEG-mediated transformation of its protoplasts. Due to the enhanced transformability of these strains, gene disruptions could recently also be carried out quite effectively.

Our results show that the elaborated protocol can be applied to the commonly used strains [*S. coelicolor* A3(2) and *S. lividans* 66]. However, the conditions used for these strains have to be further improved. In addition, we could demonstrate that an *S. venezuelae* strain can be successfully transformed by electroporation, although its protoplasts were formerly not transformable by the standard protocol (17a). These data reveal the power of our method. As soon as other authors apply the described method and optimize it for their individual strains, the number of transformable strains will probably rise quickly.

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