EFFICIENT TRANSFORMATION OF MICROMONOSPORA PURPUREA WITH pIJ702 PLASMID

GABRIELLA H. KELEMEN, ISTVÁN FINANCSEK and MIKLÓS JÁRAI

Chinoin Pharmaceutical and Chemical Works Ltd., Budapest, H-1045, Hungary

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The aminoglycoside antibiotic gentamicin is produced by *Micromonospora purpurea* strains[1,2]. A gene cloning system for these organisms would be useful both in the study of the organization and regulation of gentamicin biosynthesis genes and, hopefully, for the improvement of the productivity of the strains. Efficient host-vector system and transformation methods have already been developed for several *Streptomyces* species[3~7] but in the experiments of *Micromonospora* strains with plasmid DNA relatively low transformation frequencies were observed[8,9]. In order to develop an efficient transformation method for *M. purpurea* we studied and optimized the parameters affecting the formation, regeneration and transformation of protoplasts.

Optimization of Protoplast Formation and Regeneration

In preliminary experiments none of the transformation procedures developed for *Streptomyces* strains[3~7] proved to be suitable for efficient transformation of *Micromonospora* strains with the broad host range vector pIJ702[10]. Our *M. purpurea* (MNG 00209)[11] did not grow well in YEME medium[12] that is generally used for *Streptomyces* protoplasting. Optimal growth was obtained in JM medium containing soluble starch 2.5%, Tryptone 0.5%, peptone 0.5%, powdered boullion 0.5%, $CaCO_3$ 0.1% (pH 7.2). The effect of the glycine concentration on the efficiency of protoplast formation was examined in the range of 0.05% to 0.8%. More than 95% of the mycelia grown in the presence of 0.1% glycine and 5 mM $MgCl_2$ were converted into protoplasts within 30 minutes of lysozyme treatment at 37°C.

The temperature during both the cell growth and the protoplast regeneration was found to greatly affect the results. Cells grown at 28, 31 or 35°C were protoplasted and regenerated at 26, 28 or 35°C. The most efficient protoplast formation was obtained if the cells were grown at 30~32°C. More than 50% of the mycelia remained unchanged after 1.5~2 hours of lysozyme treatment if the cells were grown at 28 or 35°C. The optimal initial temperature for protoplast regeneration was 26°C for the first 40~44 hours, 4~6°C lower than the optimal growing temperature for cells. The number of regenerated protoplasts was 1~2 orders of magnitude lower at temperatures higher than 26°C (Table 1). After the initial 40 hours regeneration phase the plates were further incubated at 35°C. The lower initial regeneration temperature was found to be essential for the efficient protoplast regeneration and transformation of *Streptomyces clavuligerus*[7].

Using different buffers (Tris, TES, CHES, HEPES, MOPS, PIPES) in the regeneration medium the changes in the efficiency of the regeneration were not significant (1~3-fold differences).

Optimization of the Protoplast Transformation

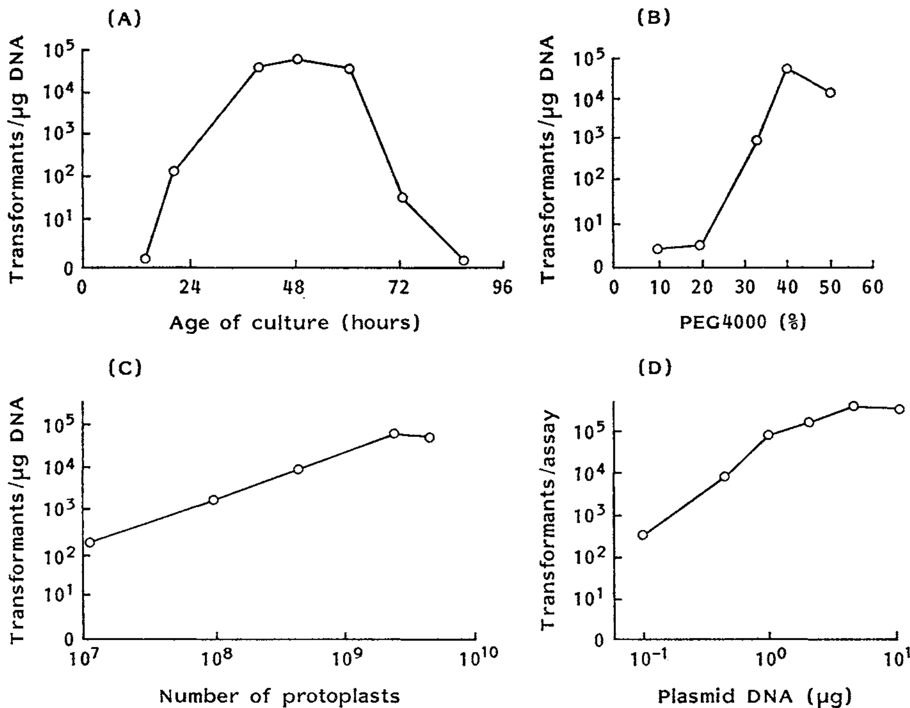
The growth phase of the cells at the time of protoplasting greatly influenced the efficiency of transformation as in *Streptomyces* strains[3~7]. Cells in the late log phase or at the early sta-

Table 1. The effect of the temperature on the yield of regenerated protoplasts.

Temperature of cell growth (°C)	Protoplasted cells (%)	Regeneration frequency		
		Regeneration temperature (°C)		
		26	28	35
28	20~30	1.8×10^{-2}	1.3×10^{-3}	1.2×10^{-4}
31	98~100	2.5×10^{-2}	1.2×10^{-3}	1.0×10^{-4}
35	30~40	2.0×10^{-2}	1.1×10^{-3}	9.8×10^{-5}

The regeneration frequencies shown in the table were defined as the number of regenerated protoplasts/formed protoplasts counted in haemocytometer.

Fig. 1. Parameters affecting the transformation efficiency.



Effects of growth phase (A), PEG4000 concentration (B), number of protoplasts (C) and the amount of plasmid DNA (D) on the transformation frequency. The data of typical experiments are shown.

tionary phase were the most transformable as shown in Fig. 1A.

For different *Streptomyces* species PEG1000 is generally used for transformation. 20% for *Streptomyces lividans*[3,12], 30% for *Streptomyces wadayamensis*[5], 40% for *Streptomyces clavuligerus*[7] and 55% for *Streptomyces ambofaciens*[4]. For transfection of *Micromonospora* sp. strain IMET 8002 20% of PEG1000 was the most effective[13]. Depending on the type of PEG (PEG1000, 4000 and 6000) during the DNA uptake of *M. purpurea* protoplasts the transformation frequencies varied between 2~4-fold, the best result was obtained with PEG4000. The dependence of the transformation efficiency on PEG4000 concentration is demonstrated on Fig. 1B. A short exposure of the protoplasts with plasmid DNA in the presence of 40% PEG4000 gave the most effective DNA uptake.

By increasing the number of protoplasts in the transformation mixture containing constant amount of plasmid DNA the number of the transformants increased linearly from 1×10^8 to 2×10^9 protoplasts per assay and then reached a plateau (Fig. 1C).

The dependence of the number of transformants on the amount of plasmid DNA in the transformation of constant number (2×10^9) of protoplasts is shown on Fig. 1D, where a plateau was reached at about 5 μg DNA per assay. Since there were slight variations in the absolute numbers of the transformation experiments the results of single tipical experiments are presented in Fig. 1.

As a result of these experiments we describe a new transformation method that have routinely yielded 10^5 transformants/μg plasmid DNA: Cells of *M. purpurea* were grown in JM in the presence of 0.1% glycine and 5 mM $MgCl_2$ at 30~32°C for 48 hours. The mycelia were harvested, washed twice with 10.3% sucrose and resuspended in P medium[14] containing 4 mg/ml lysozyme. The mixture was incubated at 37°C for 30~40 minutes until the completion of protoplast formation followed by microscopic observation. The protoplasts were filtered through cotton wool, centrifuged (1,000×*g*, 10 minutes) washed twice with P medium. Plasmid

pIJ702 in 10 mM Tris-HCl, 1 mM EDTA (pH 7.4) of max 20 μl (average 5 μl) was added to *ca.* 2×10^9 protoplasts pelleted by centrifugation and immediately 0.5 ml 40% PEG4000 (Fluka) in P medium was added into the transformation mixture. After 1 minute at room temperature the mixture was diluted with 5 ml of P buffer and was centrifuged. Aliquots of the transformation mixture suspended in 3.0 ml of R2 medium[15] containing 0.6% agar were spread on R2 regeneration plates. After 40 hours incubation at 26°C the regeneration plates were overlayered with soft R2 agar containing thiostrepton at 10 μg/ml final concentration. Thiostrepton-resistant transformants appeared after incubation at 35°C for 10~12 days. Under these conditions up to 2×10^3 transformants/μg DNA were obtained with pIJ702 isolated from *S. lividans*. If pIJ702 DNA derived from *M. purpurea* was used the transformation frequency was 5×10^4~10^5 transformants/μg DNA, indicating the presence of a restriction-modification system in *M. purpurea*.

The functional expression of pIJ702 in *M. purpurea* was limited. While the preservation of the plasmid and the expression of thiostrepton-resistance gene are functioned in *M. purpurea* but no melanin formation in the thiostrepton-resistant transformants could be detected. Similar observations were made in *Streptomyces coelicolor*[10] and *Streptomyces jumonjinensis*[16]. Plasmids isolated from *M. purpurea* transformants and transformed into *S. lividans* gave brown pigmented colonies, indicating that other sequences needed for the melanin production are not present in *M. purpurea* strains.

Streptomyces plasmids have already been successfully transformed into species of other genera of actinomycetes: *Amycolatopsis*[17] and *Thermomonospora*[18]. Using the efficient transformation procedure developed in the present work the pIJ702 will be useful for the cloning and studying of genes involved in the antibiotic biosynthesis in *Micromonospora* host strains.

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