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Improvement of Transformation and Electroduction in Avermectin High-Producer, *Streptomyces avermitilis*

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ABSTRACT. Factors affecting the PEG-mediated transformation and electrotransformation of *Streptomyces avermitilis* protoplasts, an industrial avermectin high-producer, were evaluated. The maximum protoplast transformation efficiency under optimum conditions with PEG was 3×10^6 transformants per μg plasmid pIJ702 DNA. The efficiency of electrotransformation with the same plasmid the intact cells grown in medium with 0.5 mmol/L $CaCl_2$, suspended in buffer with 0.5 mol/L sucrose +1 mmol/L $MgCl_2$, and pulsed at an electric field strength of 10 kV/cm, 800 Ω, 25 μF, was of 2×10^3 transformants per μg DNA. When the cells were electroporated after mild lysozyme-treatment, the efficiency was up to 10^4 transformants per μg DNA. Electroporation of protoplasts and germlings had a lower efficiency (10^2 transformants per μg DNA). We report that electroporation under optimum conditions can be used for direct transfer of nonconjugative plasmid pIJ699 between two different *Streptomyces* species, *S. avermitilis* and *S. lividans*.

Avermectins (Avm) produced by *Streptomyces avermitilis* are composed of 8 components of 16-membered macrocyclic lactones. Because of its excellent anthelminthic and insecticidal activities, avermectin is widely used for treatment of diseases caused by nematodes and arthropods in veterinary and agricultural fields (Burg *et al*. 1979). The biosynthetic pathway of Avm was elucidated (Ikeda and Omura 1997) and the gene cluster for its biosynthesis was cloned and sequenced (Ikeda *et al*. 1999); this provided favorable conditions for further strain improvement. The development of methods for gene transfer may play an important role in the genetic manipulation of *S. avermitilis.* Since Bibb *et al*. (1978) reported a transformation procedure for *Streptomyces coelicolor* using protoplasts and polyethylene glycol (PEG), the PEG-mediated transformation of protoplasts has become a common technique applicable for introducing exogenous DNA into the cells of many *Streptomyces* spp. (Pigac and Schrempf 1982; Hopwood *et al*. 1985; Matsushima and Baltz 1985; MacNeil and Klapko 1987). However, this procedure has not provided the satisfactory transformation frequency in our avermectin high-production strain MMR630; we obtained less than 1000 transformants per μg of plasmid DNA. Moreover, Lee *et al*. (2000) and Hwang *et al*. (2001) indicated that the formation and regeneration of protoplasts severely decreased avermectin production in avermectin high-producers.

Electroporation, which is a much faster and simpler method used widely for transformation of bacterial cells in the presence of exogenous DNA (Pigac and Schrempf 1995; Tyurin *et al*. 1995; Mazy-Servais *et al*. 1997) and for the direct transfer of plasmid DNA from a plasmid-bearing strain to plasmid-free one (Pfau and Youderian 1990; Summers and Withers 1990; Vujaklija and Davies 1995), was suggested as an alternative way. In order to obtain desired results, however, it is necessary to establish the optimum conditions for a given strain, particularly in the case of industrial antibiotic-producer.

Here we aimed at determining the optimum conditions for PEG-mediated transformation of protoplasts and electrotransformation of *S. avermitilis* MMR630, the industrial producer of avermectin; this is also the first report on electroduction of nonconjugative plasmid DNA between *S. lividans* and *S. avermitilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids used are given in Table I.

Media. *E. coli* JM110 was grown in Luria–Bertani (LB) broth. *S. lividans* 1326 and TK24 were grown in YEME at 30 °C; cells for the preparation of protoplasts and electrocompetent cells were grown in

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YEME with 0.5 % glycine. *S. avermitilis* MMR630 protoplasts were regenerated in R10 – solution A (in g/L): sucrose 200, dextrin 10, yeast extract 5, casamino acids 0.1, $MgCl_2 \cdot 6H_2O$ 10.12, K_2SO_4 0.25, 2 mL trace element solution; solution B (in g/L; sterilized separately and added to A): TES buffer 5.72, proline 3, $CaCl_2 \cdot 2H_2O$ 2.94, NaOH 0.28, KH_2PO_4 0.05 (final pH 6.8). Putative transformants were purified on YMS medium (Ikeda *et al*. 1988) supplemented with appropriate antibiotic. YEME and double strength germination medium were prepared according to Hopwood *et al*. (1985) except for the concentration of sucrose which was reduced to 30 % of original YEME to obtain more turbid cultures.

Table I. Strains and plasmids[a]

Name		Characteristics	Source
		Strains	
S. avermitilis	MMR630	high producer of avermectin, used for commercial production of avermectin	*our laboratory*
	G-1	streptomycin-resistant derivative of MMR630, used as recipient in electroduction	*this work*
S. lividans 66	1326	wild-type strain, used as the host for plasmid pIJ702	D.A. Hopwood, *John Innes Center* (Norwich Research Park, Colney, UK)
	TK24	*str-6* $SLP2^-$ $SLP3^-$, used as recipients in electroduction	*ditto*
E. coli	JM110	*dam dcm*, used for the preparation and isolation of shuttle plasmid pIJ699 for *E. coli* and streptomycetes	*our laboratory*
		Plasmids	
pIJ702		pIJ101 derivative; *tsr mel* Ltz^-	E. Katz, *John Innes Center* (Norwich Research Park, Colney, UK)
pIJ699		pIJ101 derivative; *tsr vph* Ltz^-	T. Kieser, *John Innes Center* (Norwich Research Park, Colney, UK)

[a]*Abbreviations*: *dam* DNA-adenine N^6-methyltransferase; *dcm* DNA-cytosine N^4-methyltransferase; Ltz lethal zygosis; *mel* tyrosinase gene; SLP plasmid of *S. lividans*; *str* streptomycin resistance gene; *tsr* thiostrepton resistance gene; *vph* viomycin resistance gene

Transformation of protoplasts of S. avermitilis *MMR630.* Spores were inoculated into 50 mL YEME in a 250-mL flask and grown for 2 d at 30 °C; then 1.5 mL was transferred to another 250-mL flask containing 50 mL YEME supplemented with 0.5 % glycine. After 25–30 h, mycelia were harvested and washed once with 10.3 % sucrose. The cells were suspended in 10 mL modified P medium (in g/L): sucrose 200, $MgCl_2 \cdot 6H_2O$ 1.22, K_2SO_4 0.25, 2 mL trace elements solution; added after sterilization: TES buffer 5.72, $CaCl_2 \cdot 2H_2O$ 0.44, KH_2PO_4 0.05 (final pH 6.8) with 1 mg/mL lysozyme and incubated at room temperature for 1 h with slow shaking. The resulting protoplasts were filtered through glass wool and centrifuged (1500 ***g***, 10 min). The pellet was washed once with modified P medium, resuspended in 2 mL of the same medium, quick frozen, and stored at –70 °C.

For transformation, DNA was added to 50 µL of protoplast suspension followed by 200 µL of T medium (40 % PEG in modified P medium, pH adjusted to 9.0) and mixed by gentle pipetting. The mixture was incubated at room temperature for 30 s and then quickly diluted in modified P medium. The transformed protoplasts were rapidly spread on R10 agar plates and incubated at 30 °C for 18 h, then the plates were overlaid with R10 soft agar with appropriate antibiotics. The plates were further incubated at the same temperature for 3–5 d.

Electrotransformation of intact cells. Cells of strain MMR630 from 50 mL of late exponential phase culture were harvested by centrifugation (1500 ***g***, 10 min, 4 °C) and washed thrice with ice-cold distilled water and twice with cold electroporation medium (0.5 mol/L sucrose + 1 mmol/L $MgCl_2$). Finally, the cells were 20-fold concentrated in the same medium. Competent cells were either used directly for electroporation or stored at –70 °C. Electroporation was done using a *Bio-Rad* Gene Pulser™ with pulse controller. Before

electroporation, 50 μL competent cells was mixed with 1 μL DNA in a 1-mm cuvette and preincubated at 50 °C for 5 min. The mixture was subjected to an electric pulse (voltage 1 kV, resistance 800 Ω, capacitance 25 μF) for various time periods and, immediately, R10 medium was added. The pulsed cells were incubated for 4–6 h at 30 °C, then plated on YMS supplemented with the appropriate antibiotic and incubated at 30 °C for 3–5 d.

Electrotransformation of protoplasts. The protoplasts were pelleted by centrifugation (1500 ***g***, 10 min) and resuspended in 0.6 mol/L sucrose. Fifty μL protoplast solution was mixed with 1 μL DNA in an ice-cold 1-mm cuvette. After the electroporation (1 kV, 800 Ω, 25 μF), the suspension was diluted with modified P medium and spread on R10 regeneration medium. The regeneration plates were overlaid after an 18-h incubation at 30 °C with 3 mL R10 soft agar containing an antibiotic.

Electrotransformation of germlings. The spores from frozen glycerol stock were pelleted by centrifugation (1500 ***g***, 10 min) and washed with 1.5 mL TES buffer (50 mmol/L, pH 8). Then the pelleted spores were resuspended in 0.5 mL TES buffer and heat shocked at 50 °C for 10 min. After cooling under cold tap water, an equal volume of double strength germination medium was added and the sample was incubated at 37 °C with shaking for 2–3 h. The germinated spores were pelleted by centrifugation (1500 ***g***, 10 min) and washed with electroporation medium (0.5 mol/L sucrose +1 mmol/L $MgCl_2$). Germinated spores were resuspended in the same way as intact cells.

Electroduction. The cells of donor and recipient for electroduction were prepared as mentioned above. Twenty-five μL aliquots of the cells were mixed in a microfuge tube before being transferred to an ice-cold 1-mm cuvette. The sample was electroporated at a field strength of 10 kV/cm and the resistance of 800 Ω, with capacitance of 25 μF. The pulsed cells were spread on R2YE (Hopwood *et al*. 1985) or YMS medium containing streptomycin (25 μg/mL) and thiostrepton (50 μg/mL) (only streptomycin-resistant recipient cells which had acquired the plasmid should grow on these plates).

RESULTS

Transformation of protoplasts

Effect of growth stage on transformation of protoplasts. In preliminary experiments, cells were harvested at different stages from the early exponential to the stationary phase and protoplasts were prepared. The most competent for transformation were protoplasts from the exponential phase (25–30 h culture, A_{600} = 6–7) (Fig. 1).

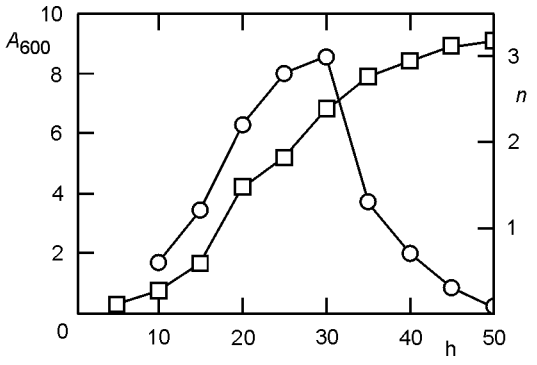

Fig. 1. Growth of *S. avermitilis* MMR630 (*squares*; A_{600}) and the transformation efficiency (*circles*; *n* – number of transformants, 1/pg DNA) of protoplasts of this strain.

Optimization of protoplast preparation. The regeneration rate was perceptibly increased when 20 % sucrose was added to P buffer. The highest efficiency was obtained when Ca^{2+} concentration was 3 mmol/L and Mg^{2+} reached 6 mmol/L (Fig. 2). The modified P medium gave 10–20-fold increase in transformation efficiency compared with the original P medium.

Various buffers with different buffering pH-zones, including Tes, Mes and Mops were used to determine the optimum pH. The highest regeneration efficiency (15 %) was obtained when Tes was used both in the modified P and regeneration medium. The pH optimum for protoplast regeneration was 6.8. Using Mops at the same concentration and pH, the regeneration rate decreased 3-fold.

Optimization of regeneration medium and regeneration conditions. If the standard R2YE medium was used for protoplast regeneration in the MMR630 strain, only 10 ppm of total protoplast counts were shown to be regenerated. We therefore tested the effect of osmotic stabilizers (sucrose, mannitol, glucitol, succinic acid, NaCl) on the regeneration and improvement of the regeneration efficiency. Regeneration increased 10–20-fold when using R2YE medium supplemented with sucrose than on the same medium supplemented with other osmotic stabilizers; the optimum sucrose concentration was 0.5–0.6 mol/L. On modified regeneration medium the maximum protoplast regeneration efficiency reached 15 %.

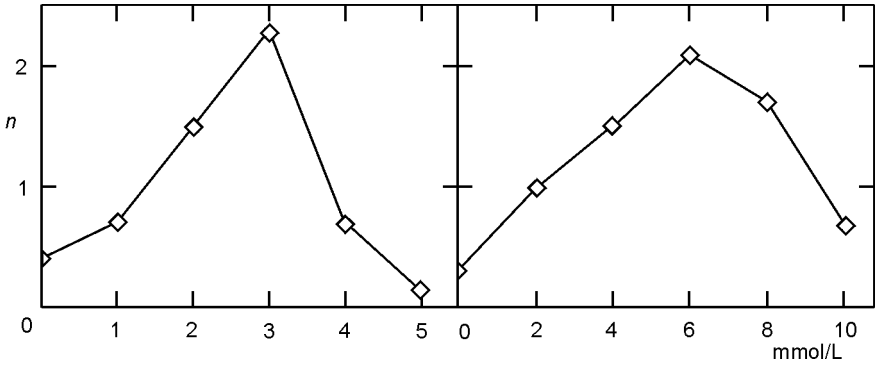

Fig. 2. Effect of Ca^{2+} (*left*) and Mg^{2+} (*right*) concentration (mmol/L) in modified P medium on the transformation efficiency (*n* – number of transformants, 1/pg DNA) of protoplasts of *S. avermitilis* MMR630.

Transformation of protoplasts. Effect of PEG type and concentration on transformation efficiency was compared in experiments with PEG-mediated transformation of the MMR630 protoplasts with pIJ702 plasmid DNA. Much better than PEG 2000 or 4000 was PEG 1000; the highest efficiency of transformation was obtained at a concentration of 40 % (*W*/*V*) (Fig. 3).

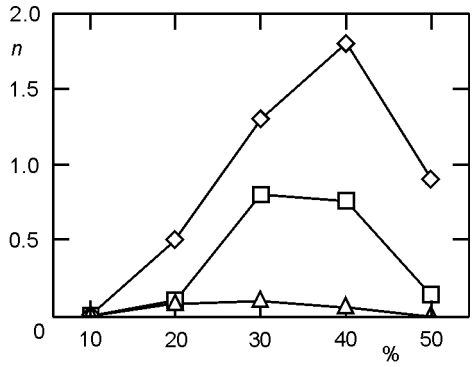

Fig. 3. Effect of PEG type (*diamonds* – PEG 1000; *squares* – PEG 2000; *triangles* – PEG 4000) and concentration (%) on the transformation efficiency (*n* – number of transformants, 1/pg DNA) of protoplasts of *S. avermitilis* MMR630.

Effect of protoplast concentration. In the range of protoplast concentrations between 2×10^6/μL and 2×10^8/μL the maximum efficiency (3×10^6 transformants per μg pIJ702 DNA) was obtained at a concentration of 10^8/μL (Fig. 4).

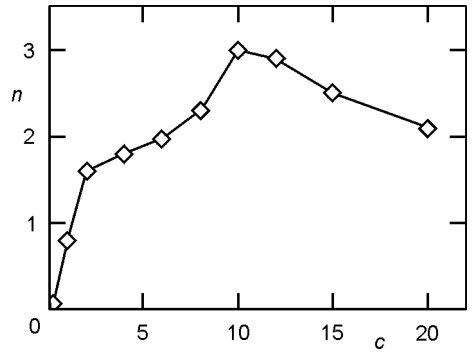

Fig. 4. Effect of protoplast concentration (*c*, 10/pL, *i.e.* 10^7 per μL) on the transformation efficiency (*n* – number of transformants, 1/pg DNA) of protoplasts of *S. avermitilis* MMR630.

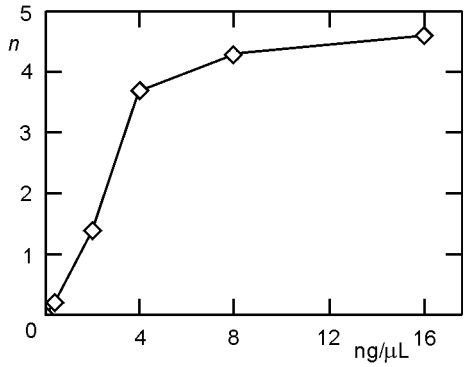

Fig. 5. Effect of the concentration of plasmid pIJ702 DNA (ng/μL) on the transformation efficiency (*n* – number of transformants, 1/pg DNA) of protoplasts of *S. avermitilis* MMR630.

Effect of plasmid DNA concentration. Similarly, using the range of plasmid pIJ702 DNA concentration of 0.004 to 16 ng/μL, the highest efficiency was obtained at a concentration of 4 ng/μL (Fig. 5).

Electrotransformation of *S. avermitilis* MMR630

Intact cells. Effect of the growth medium. With respect to the transformation of protoplasts, nearly no difference was observed among cells cultivated prior to protoplasting in YEME with various concentrations of Ca^{2+} or Mg^{2+}. A remarkably different result was observed in electrotransformation which gave a 10-fold increase in efficiency after addition of 5 mmol/L $CaCl_2$.

Effect of the electroporation medium. The presence of glycerol in electroporation medium decreased significantly the electrotransformation efficiency. When this medium was prepared with 1 mmol/L $MgCl_2$ and 0.5 mol/L sucrose, a twice higher efficiency than with 10.3 % sucrose and 15 % glycerol was obtained.

Effect of electric parameters. The most efficient electrotransformation of MMR630 intact cells with the pIJ702 DNA was at the field strength of 10 kV/cm, 800 Ω and pulse duration of 14–15 ms (Fig. 6). About 2×10^3 transformants per μg DNA were obtained using these conditions.

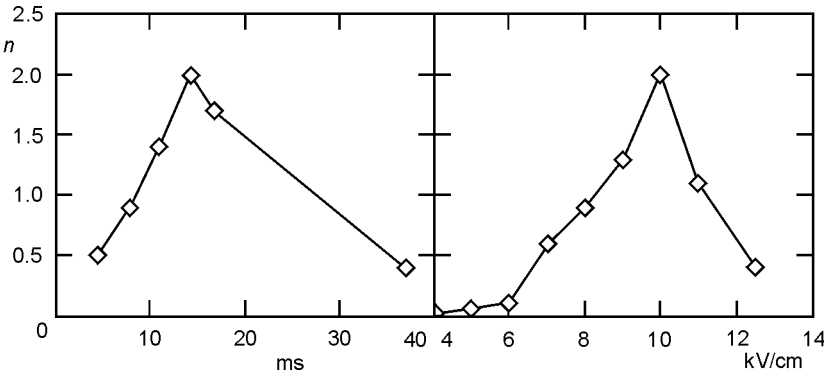

Fig. 6. Effect of pulse time (*left*; ms) and field strength (*right*; kV/cm) on the electrotransformation efficiency (*n* – number of transformants, 1/ng DNA) of *S. avermitilis* MMR630 with plasmid pIJ702.

Effect of the plasmid concentration. The transformation efficiency in the PEG-mediated transformation remained constant in a plasmid pIJ702-concentration range of 0.004 to 4 ng/μL. The efficiency increased linearly with pIJ702 DNA concentration in the range of 0.02–0.2 ng/μL (Fig. 7).

Different cell forms. Three different cell forms (germlings, protoplasts, lysozyme-treated cells) were electrotransformed with pIJ702 DNA using the electroporation procedure. The electrotransformation of germlings of *S. avermitilis* MMR630 gave only 100 or less transformants per μg DNA. For the protoplasts, the highest efficiency was 5×10^2 per μg DNA. There was a 5-fold increase in efficiency when mycelia pretreated with lysozyme (200 μg/mL) for 10 min at room temperature were electrotransformed (giving the efficiency of 10^4 transformants per μg DNA). The transformation efficiency decreased after longer time periods. When testing different electric parameters, no further increase in efficiency was observed.

The *electroduction of nonconjugative plasmid* pIJ699 between *S. avermitilis* and *S. lividans* was shown. The recipient strains included *S. avermitilis* G-1, a streptomycin-resistant mutant of strain MMR630, and *S. lividans* TK24 (Table II). A total of 10–100 electroductants per one electric pulse for transfer of plasmid pIJ699 DNA was obtained between the same species (*S. lividans* 1326 and TK24 or *S. avermitilis* MMR630 and G-1). Less than 10 electoductants were obtained between different species (*S. lividans* 1326 and *S.avermitilis* G-1 or *S. avermitilis* MMR630 and *S. lividans* TK24) which demonstrated that the method is more efficient for direct transfer of nonconjugative plasmid intraspecifically than interspecifically.

DISCUSSION

We demonstrated that protoplasts prepared from the mycelia grown in YEME medium to the late exponential phase and suspended in modified P medium with 6 mmol/L Mg^{2+} and 3 mmol/L Ca^{2+} gave the

maximum transformation efficiency of 3×10^6 transformants per μg DNA when transformed with the plasmid pIJ702 in the presence of 40 % PEG 1000. Matsushima and Baltz (1985) described that the addition of low levels of protamine sulfate or heterologous DNA (*e.g.*, calf thymus DNA) to the transformation mixture substantially enhance protoplast transformation efficiency in *S. fradiae* and *S. ambofaciens*; a similar effect has not been found in *S. avermitilis* MMR630.

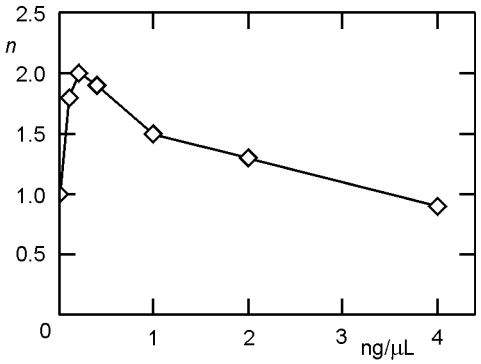

Fig. 7. Effect of pIJ702 DNA concentration (ng/μL) on the electrotransformation efficiency (n – number of transformants, 1/ng DNA) of *S. avermitilis* MMR630.

Table II. Efficiency of electroduction of pIJ699 between *S. lividans* and *S. avermitilis*

Donor/pIJ699 (Thio^r Str^s)	Recipient (Thio^s Str^r)	Electroductant (Thio^r Str^r)[a]
S. lividans 1326	*S. avermitilis* G-1	1–10
S. avermitilis MMR630	*S. lividans* TK24	10
S. lividans 1326	*S. lividans* TK24	10–100
S. avermitilis MMR630	*S. avermitilis* G-1	10–100

[a]Thio – thiostrepton, Str – streptomycin.
[a]Number of colony forming units obtained after regeneration of cells from 100 μL of suspension seeded onto regeneration agar.

Brief pulses of electric fields cause the formation of pores in cell membrane and provide a local driving force for ionic and molecular transport through the pores (Weaver 1990). Electroporation has been used for electrorelease of DNA in *E. coli* (Süleymanoğlu 2002) and for introducing of plasmid DNA into protoplasts of *Bacillus cereus* (Shivarova *et al*. 1983), *Streptococcus lactis* (Harlander 1987) and also into intact cells of *Lactobacillus delbrueckii* (Serror *et al*. 2002), *Streptococcus thermophilus* (O'Sullivan and Fitzgerald 1999), *etc*. However, the use of electroporation had only a limited effect in several *Streptomyces* strains (MacNeil 1987; Pigac and Schrempf 1995; Tyurin *et al*. 1995; Mazy-Servais *et al*. 1997). We showed that intact cells of *S. avermitilis* MMR630 harvested from a culture grown in YEME medium with 5 mmol/L $CaCl_2$ were electrotransformed with a transformation efficiency of up to 2×10^3 per μg DNA while in germinated spores or protoplasts pulsed under optimized conditions the efficiency decreased to 10^2 per μg DNA or less. A major improvement in the efficiency was achieved in MMR630 cells by using a mild treatment with lysozyme (without protoplast formation) which gave the efficiency of 10^4 per μg DNA.

Electroporation as a method for introducing plasmid DNA into bacterial cells and transfer of plasmid DNA from donor to recipient cells is considered to function both intraspecifically and interspecifically. Summers and Withers (1990) reported a simple and reliable electroporation method allowing the direct transfer of plasmid pBR322 between two strains of *E. coli*. This method was then successfully applied to the transfer of shuttle vectors between *E. coli* and *S. typhimurium* (Pfau and Youderian 1990), *E. coli* and *Mycobacterium* sp. (Baulard *et al*. 1992), and *E. coli* and *Streptomyces* sp. (Vujaklija and Davies 1995). We were the first to achieve the electroduction of the nonconjugative plasmid pIJ699 between two species of *S. avermitilis* MMR630 and *S. lividans* TK24 by electroporation.

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