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Optimized Transformation of *Streptomyces* sp. ATCC 39366 Producing Leptomycin by Electroporation

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***Streptomyces* sp. ATCC 39366 produces leptomycin derivatives. Leptomycin B, a potent and specific inhibitor against the export of nuclear proteins, is the main product; however, the introduction of DNA into this strain is almost impossible, which has impeded its further use. We developed a *Streptomyces* sp. ATCC 39366 transformation protocol to introduce foreign DNA via electroporation. Various conditions were examined, including treatments of the cell wall with weakening agents, electroporation parameters, and DNA content. We found that only plasmid DNA isolated from a *dam*⁻ ET12567 strain resulted in successful transformation. The mycelium growing in a yeast-peptone-dextrose medium supplemented with 1% glycine at 28°C on a rotary shaker (220 rpm) was more dispersed than those without supplementation and prone to electroporation. The maximum transformation efficiency of 8×10^2 CFU/μg plasmid DNA was obtained at a field strength of 13 kV/cm with a time constant of 13 ms (25-μF capacitor; parallel resistance, 600 Ω) using 1-mm electrocuvettes. The results of the transformations of two other *Streptomyces* species indicated that the optimized conditions established in this study might only be applicable to *Streptomyces* sp. ATCC 39366. However, this is the first report of successful transformation of *Streptomyces* sp. ATCC 39366, and will facilitate the construction of a gene knockout mutant in *Streptomyces* sp. ATCC 39366 to produce series of new leptomycin derivatives.**

***Keywords*:** *Streptomyces* sp. ATCC 39366, leptomycin, electrotransformation, *E. coli*

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Introduction

Streptomyces sp. ATCC 39366 is a Gram-positive, spore-forming aerophilic streptomycete. The strain is capable of producing various leptomycin derivatives, with leptomycin B (LMB) as its primary product, and leptomycin A, kazusamycin A, and kazusamycin B as minor products. As an antibiotic, LMB is an unsaturated branched-chain fatty acid, as well as a potent and specific inhibitor against the export of nuclear proteins (Wolff *et al.*, 1997; Engel *et al.*, 1998; Kancha *et al.*, 2008). In addition, it inactivates the chromosomal region maintenance1 (syn. exportin 1) through covalent modification of a cysteine residue in the central conserved region (Kudo *et al.*, 1999). However, the toxicity of LMB toward mammalian cells has made its clinical use impractical (Newlands *et al.*, 1996). Several attempts have been made to produce leptomycin derivatives that are less toxic, including total synthesis (Bonazzi *et al.*, 2010) and biotransformation (Kuhnt *et al.*, 1998); however, no derivatives have been obtained by modifying the genes involved in the biosynthesis of LMB in the host strain due to the difficulties of introducing DNA into the LMB-producing strain.

Transformation is a basic technique for carrying out mutagenesis and genetic engineering in microorganisms. Since the leptomycin gene cluster was published in 2005 (Hu *et al.*, 2005), no related studies have been reported due to the challenges regarding the transformation of *Streptomyces* sp. ATCC 39366. Electroporation has been shown to be a useful method for introducing DNA into *Streptomyces* (Pigac and Schrempf, 1995; Mazy-Servais *et al.*, 1997); however, no work on the transformation of *Streptomyces* sp. ATCC 39366 by electroporation has been published.

In the present study, we describe the development of a transformation system using electroporation for DNA manipulation that will facilitate the construction of a gene knockout mutant of *Streptomyces* sp. ATCC 39366. Several conditions were optimized, including treatments with different cell wall weakening agents, electroporation parameters, and DNA content. This is the first report of the electrotransformation of *Streptomyces* sp. ATCC 39366 producing leptomycin with plasmid DNA.

Materials and Methods

Bacterial strains and culture media

Streptomyces sp. ATCC 39366 was obtained from the American Type Culture Collection and maintained in a yeast-peptone-dextrose (yeast extract, 1%; peptone, 2%; dextrose, 2%; pH 6.5; YPD) liquid medium. *Streptomyces griseochromo-*

genes and *Streptomyces pulveraceus* were preserved by our laboratory. An ISP2 agar (yeast extract, 0.4%; malt extract, 1%; dextrose, 0.4%; agar, 2%; pH 7.0) plate containing apramycin (25 μg/ml) was used to select transformants. *Escherichia coli* strain DH5α (Sambrook *et al.*, 2001) was used as a host for cloning. The methylation-deficient *E. coli* strain ET12567 (Choi *et al.*, 2004) was used to amplify and maintain the plasmid. All of the *E. coli* strains were cultured in Luria-Bertani medium (Kieser *et al.*, 2000) at 37°C. The final concentration of apramycin for *E. coli* selection was 50 μg/ml.

Construction of plasmid pEGFP152

The *ermE* promoter was amplified by Polymerase Chain Reaction (PCR) (primer1: 5′-GCTCTAGAAGCCCGACCC GAGCACGC-3′, *Xba*I-underlined; primer2: 5′-GGACTAGT TCCTCCTACCAACCGGCACG-3′, *Spe*I-underlined) using the pWHM3-*ermE* plasmid (Xu *et al.*, 2010) as a template. The enhanced green fluorescent protein (eGFP) gene was amplified from the plasmid pEGFP-N2 (Bharali *et al.*, 2005) using the primers 5′-GGACTAGTATG GTGAGCAAGGG CGAGGAG-3′ (*Spe*I site underlined, primer3) and 5′-CCG GAATTCCTTGTACAGCTCGTCCATGCCGAGA-3′ (*Eco*RI site underlined, primer4). Both of the PCR-amplified fragments were cloned into the site-specific integration vector pSET152 (Kieser *et al.*, 2000) to construct the plasmid pEGFP-152. Plasmids pEGFP152 extracted from *E. coli* strains DH5α and ET12567, respectively, were both used for electrotransformation.

Growth conditions and pretreatment of mycelium

Streptomyces sp. ATCC 39366 mycelium was grown in 25 ml of a YPD medium at 28°C on a rotary shaker (220 rpm) for 60 h. It was harvested by centrifugation at 4°C (12,000 rpm). The mycelium was washed three times with an equal volume of 15% ice-cold glycerol, and the pellet was resuspended in 2 ml of electroporation buffer (30% PEG 1000, 10% glycerol, 6.5% sucrose). Samples of 80 μl were dispensed into Eppendorf tubes and stored at -80°C. The effects of several cell wall weakening agents on the mycelium of *Streptomyces* sp. ATCC 39366 were studied, including DL-threonine (0.12 mM and 0.24 mM), glycine (0.5% and 1%), and lysozyme (0.1 and 0.5 mg/ml).

Electroporation protocol

An aliquot (80 μl) containing mycelia fragments was thawed on ice. An initial electroporation protocol was performed as follows: a pEGFP152 plasmid (1 μg) dissolved in double-distilled water was added to the sample, and the mixture was transferred into a 1-mm-gapped electrocuvette (Bio-Rad Laboratories, USA) and subjected to a 1.1 kV electric pulse from the Gene Pulser (Bio-Rad Laboratories) that was connected to a Pulse Controller (25-μF capacitor; parallel resistance, 400 Ω, USA). All electroporation was performed in duplicate at minimum. Following electroporation, the sample was immediately diluted with 1 ml of an ice-cold YPD medium supplemented with 25 mM magnesium chloride. It was then incubated with shaking overnight at 28°C before 500 μl of dilution being plated on the ISP2 agar supplemented with apramycin. The percentage of surviving colonies was estimated from serial dilutions plated on the same medium without apramycin. Transformants could be scored after 5 days. Controls were performed by omitting either the transforming DNA or the electric pulse. The electroporation conditions were then optimized based on the initial electroporation protocol, including time constant (2.5–18.5 ms), field strength (5–15 kV/cm), and DNA content (1 ng –2 μg).

Confirmation of transformation

To confirm the results of the transformation, the PCR primer set (primer1 and primer4) was used to amplify the genomes of the mutants, and an approximate 1,000 bp length of fragment was required for a positive confirmation. Transformants were also examined for eGFP expression by being grown in a YPD medium supplemented with apramycin (50 μg/ml) at 28°C. The culture was examined under an inverted fluorescence microscope (Olympus IX-71, Japan) using a mercury vapor lamp with blue excitation.

Electroporation of two other *Streptomyces* species with the optimized conditions

S. griseochromogenes and *S. pulveraceus* were electroporated with the optimized conditions established here (Kong *et al.*, 2012; Wang *et al.*, 2012). These tests were conducted to help us ascertain whether these conditions can be widely used or are only applicable to *Streptomyces* sp. ATCC 39366.

Results and Discussion

Growth conditions and restriction system

Cultures in the YPD medium with and without supplementation from cell wall weakening agents were harvested after 60 h of incubation when the cultures were just reaching their early logarithmic phase. The results showed that the growth conditions of mycelium were not influenced by any cell wall weakening agent. It has been reported that an R2YE medium could be used to culture *Streptomyces* sp. ATCC 39366 (Hu *et al.*, 2005); however, we found that it grew to a higher density and was more dispersed in the YPD medium, which is compatible with the need to perform electroporation with a finely dispersed culture.

The use of *E. coli* as a host to prepare plasmid DNA was very important for the transformation of *Streptomyces* sp. ATCC 39366. Two kinds of *E. coli* host strains were examined. The results showed that transformation was successful only when the plasmid DNA was isolated from the ET12567 strain, suggesting that there may be a methyl-dependent restriction system in *Streptomyces* sp. ATCC 39366 that acts to decrease the transformation efficiency when plasmids harvested from a *dam*$^+$ *E. coli* strain are used. A previous report showed that the methyl-dependent restriction system is the most important factor in blocking the transformation of foreign DNA (MacNeil, 1988), which was further confirmed by the results of the present study.

The effects of the cell wall weakening agents, including DL-threonine, glycine, and lysozyme, on transformation efficiency were also studied using the initial electroporation

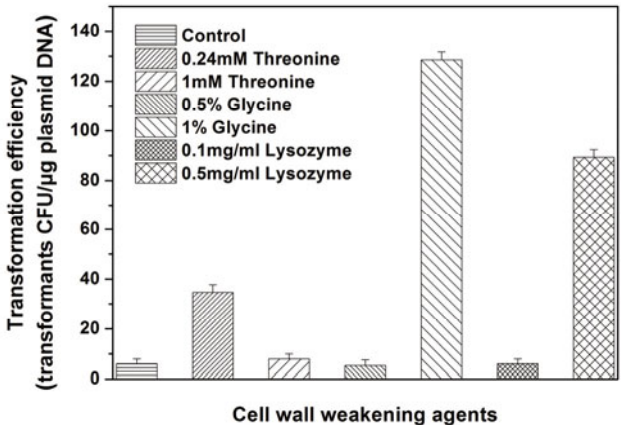

Fig. 1. Effects of different cell wall weakening agents on the transformation of *Streptomyces* sp. ATCC 39366.

protocol. The results (Fig. 1) showed that the use of cell wall weakening agents could increase the transformation efficiency to different extents. The use of 1% glycine gave the best result, with transformation efficiency increasing by 21-fold compared to the control groups. In addition, higher concentrations of glycine and lysozyme appeared to inhibit growth of the mycelium (data not shown). Therefore, mycelia grown in the YPD medium supplemented with 1% glycine were used throughout the following experiments.

Effect of pulse length

The duration of the pulse was a critical parameter. Various time constants τ (τ=R·C) were obtained by modifying the size of the parallel resistor. As shown in Fig. 2A, the transformation efficiency increased from 2 at 100 Ω to 5×10^2 at 800 Ω. In order to avoid arcing, longer pulses were not applied. These results implied that the transformation efficiency at 600 Ω was nearly the same as that at 800 Ω. Longer pulses had no further benefit on the transformation efficiency, but instead, they reduced the number of surviving colonies. Thus, a parallel resistor of 600 Ω (τ=13 ms) was used throughout the subsequent experiments.

Effects of field strength and DNA concentration

Field strength is an important factor that can affect the efficiency of electroporation (Cheong *et al.*, 2008). The efficiency of transformation obtained from field strengths of 5 kV/cm to 15 kV/cm is presented in Fig. 2B. The maximum efficiency of 8×10^2 CFU/μg plasmid DNA was obtained at a field strength of 13 kV/cm. Arcing occurred when a field strength of 15 kV/cm was used. For these reasons, the applied electric field strength was 13 kV/cm at 600 Ω in the following experiments.

The content of DNA used for electroporation was optimized as shown in Fig. 2C, with the electroporation conditions set at 13 kV/cm, 25 μF, and 600 Ω. The number of transformants increased with increasing amounts of DNA. Saturation level was reached when 2 μg of DNA was added, at which point no further increase in the number of transformants was obtained.

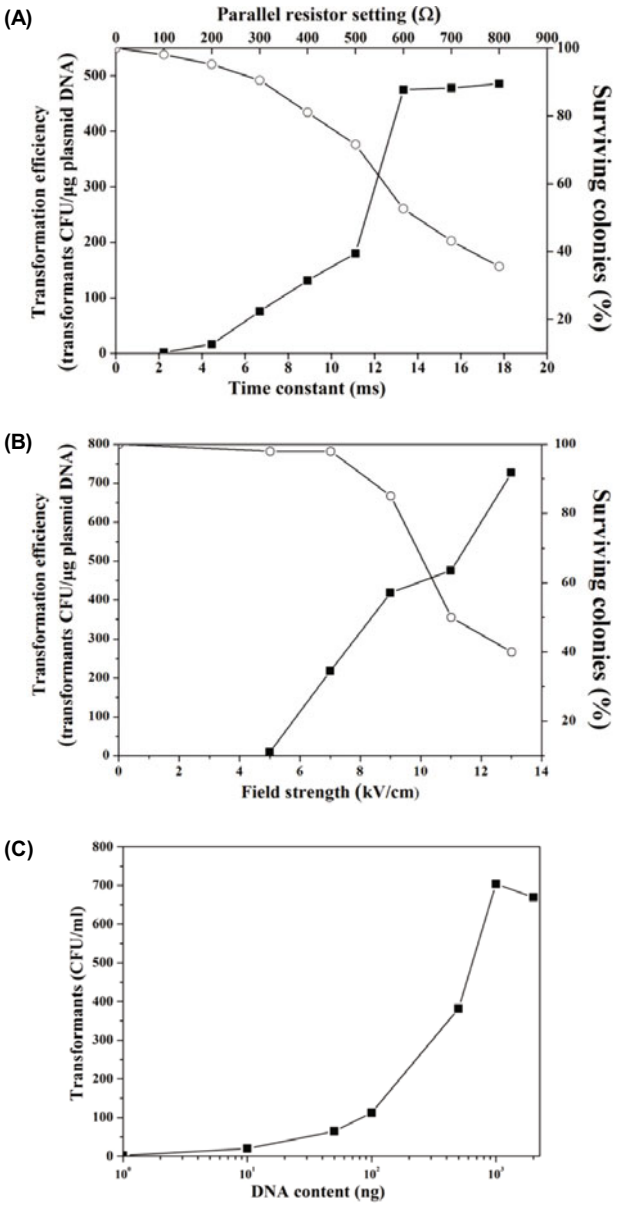

Fig. 2. Effects of different electroporation parameters on transformation efficiency. (A) Effect of external parallel resistance and pulse duration on transformation efficiency at 25 μF and 11 kV/cm. (■) transformation efficiency, (○) surviving colonies. (B) Effect of field strength of the applied pulse on the transformation efficiency at 25 μF and 600 Ω. (■) transformation efficiency, (○) surviving colonies. (C) Effect of DNA content on the number of transformants. Electroporation conditions were 13 kV/cm at 25 μF and 600 Ω.

Based on these results, an optimized electroporation protocol for *Streptomyces* sp. ATCC 39366 was devised. The novel treatment of the methods was that the use of cell wall weakening agents made the transformation of *Streptomyces* sp. ATCC 39366 possible, especially with 1% glycin. The major differences in the conditions of electroporation for this strain were focused on that the mycelium treatment with lysozyme was omitted, and the pulse time was much longer with a higher field strength compared to other previous electroporation methods. Other methods were also considered to

transform this strain (data not shown), including PEG-assisted transformation of protoplasts and conjugation from *E. coli*, but no transformants were produced, mainly due to difficulty in producing spores and non-protoplating lysozyme treatment. Together, these results imply that the transformation of *Streptomyces* sp. ATCC 39366 could not be completed with standard procedures. It has been reported that the addition of lysozyme in the pretreatment of mycelia would assist with electroporation (Pigac and Schrempf, 1995); however, we did not find any significant effects using this process in our study (data not shown). The maximum transformation efficiency achieved here (8×10^2 CFU/μg plasmid DNA) was slightly lower compared to the transformation of some model strains by electroporation (Pigac and Schrempf, 1995; Mazy-Servais *et al.*, 1997). This may be mainly due to differences in strain characteristics and mycelium densities in the samples. The transformation efficiency may be further improved by other techniques such as increasing the density of mycelium and the use of single-stranded DNA (Kieser *et al.*, 2000); however, the electroporation efficiency obtained from the protocol given in this study could be sufficient for genetic manipulations carried out in many research studies such as gene disruption, gene replacement, and so on.

Confirmation of transformation

PCR analysis of the *ermE* and *eGFP* genes in pEGFP152 was used to confirm the transformation results. A fragment of about 1,000 bp in length was obtained as expected. The mycelium of transformants also showed a high level of fluorescence (Fig. 3), confirming the presence of positive transformants.

Electroporation of two other *Streptomyces* species with the optimized conditions

S. griseochromogenes and *S. pulveraceus* were electroporated using the optimized conditions we established. The transformations of these two *Streptomyces* strains have been reported to be very difficult, and they could only be transformed by optimized conjugation methods (Kong *et al.*, 2012; Wang *et al.*, 2012). With the optimized electroporation conditions established in this study, there were almost no transformants obtained for these two *Streptomyces* strains. The results of control tests with the electroporation samples plated on the non-antibiotics plates showed that most of the *Streptomyces* mycelia were dead after electroporation. Therefore, it might be just the longer pulse time and higher field strength that led to the failed transformation of the two strains. It can be concluded that the optimized conditions established in this study will be only applicable to *Streptomyces* sp. ATCC 39366.

In summary, an electrotransformation protocol for *Streptomyces* sp. ATCC 39366 was established for introducing foreign DNA into this bacterium. Only plasmid DNA isolated from a *dam*⁻ ET12567 strain resulted in successful transformation. With *Streptomyces* sp. ATCC 39366 mycelium grown in a YPD medium supplemented with 1% glycine at 28°C on a rotary shaker (220 rpm), a maximum transformation efficiency of 8×10^2 CFU/μg plasmid DNA was obtained at a field strength of 13 kV with a time constant of 13 ms (25-μF capacitor; parallel resistance, 600 Ω) using 1 mm gapped-cuvettes. Unexpectedly, the results of the transformations of *S. griseochromogenes* and *S. pulveraceus* indicated that the optimized conditions established in this study are only applicable to *Streptomyces* sp. ATCC 39366. To our knowledge, this is the first report on the electrotransformation of *Streptomyces* sp. ATCC 39366 with plasmid DNA. The conditions described in the study will permit further investigation on the biosynthesis of leptomycin and the ability to produce leptomycin derivatives by developing knock-out target genes in the leptomycin gene cluster.

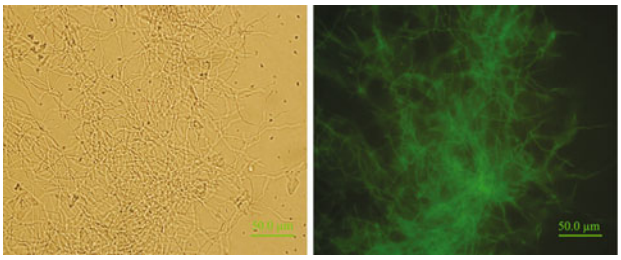

Fig. 3. Transformant of *Streptomyces* sp. ATCC 39366 expressing enhanced green fluorescent protein (eGFP). Mycelia growing in a YPD medium at 3 days were observed using a 100× objective on a fluorescence microscope. The fluorescent image is on the right. Bar, 50.0 μm.

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