Transformation using in vivo and in vitro methylation in *Streptomyces griseus*

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Abstract

*Streptomyces griseus* does not readily take up foreign DNA isolated from other *Streptomyces* species or *Escherichia coli*, presumably due to its unique restriction-modification systems that function as a barrier for interspecific DNA transfer. To efficiently transform *S. griseus* by avoiding the restriction barriers, we methylated incoming DNA in vivo and in vitro and treated protoplasts with heat prior to transformation. Whereas heat treatment of protoplasts or methylation of the *E. coli*-*Streptomyces* shuttle vectors (pXE4 and pKK1443) did not prominently improve the transformation efficiency, *Hpa*\(^II\) methylation of the vectors from any *E. coli* strain tested in this study highly increased the transformation efficiency. The highest transformation efficiency was observed when the shuttle vectors were isolated from the *dam, hsd* strain of *E. coli* (GM161) and methylated by *Alu* and *Hpa*\(^II\) methyltransferases, and the efficiency was approximately the same as that of the vectors from *S. griseus*. We identified several restriction-modification systems that decrease the transformation efficiency. This research also led us to understand methylation profiles and restriction-modification systems in *S. griseus*. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Transformation; Methylation; Restriction-modification enzyme; *Streptomyces griseus*

1. Introduction

*Streptomyces griseus* has been intensively studied for genetic and biochemical aspects of streptomycin [1] and cycloheximide production [2]. *S. griseus* is also an attractive host for sporulation studies because, unlike other *Streptomyces* species, *S. griseus* can be induced to sporulate in liquid culture [3]. The production of relatively synchronous and homogeneous sporulating cultures by the submerged sporulation facilitates the characterization of physiological and biochemical events such as transcript and protein analyses, as well as the observation of delicate morphological changes during sporulation [4,5]. However, genetic manipulations in *S. griseus* including gene cloning and transformation have been problematic, due to difficulties in introducing foreign DNA.

It is a general belief that the transformation problem is caused by the presence of restriction-modification (R-M) systems in the organisms rather than physical factors involved in transformation procedures. R-M systems are also widespread among *Streptomyces* species. Most of them have been classified as type II R-M systems composed of two structural genes, an endonuclease and a methyltransferase. Restriction enzymes cleave incoming foreign DNA but not the self-DNA protected by the endogenous methylation by the methyltransferases. These include *Sac*\(^I\) and *Sac*\(^II\) from *S. achrornogenes* [6], *Sal*\(^I\) from *S. albus* [7], *Sgr*\(^AI\) from *S. griseus* [8], and *Sph*\(^I\) from *S. phaeochromogenes* [9]. The presence of methyl-specific R-M systems has been demonstrated in *S. bambergeriensis* [10] and *S. avermitilis* [11], suggesting that the methyl-specific R-M systems may also function as a major hindrance to interspecific DNA transfer in *Streptomyces* species. Methyl-specific R-M systems are found in many bacterial species. The restriction enzyme, *Dpn*\(^I\), which cleaves the sequence GATC when it is methylated at the adenine, was first identified from *Streptococcus pneumonia* [12]. *Escherichia coli* K-12 contains three methyl-specific restriction systems [13]: McrBC catalyzing cleavage of...
DNA containing 5-hydroxymethylcytosine, 5-methylcytosine or 4-methylcytosine preceded by a purine [14]. McrA restricting DNA methylated by HpaII and SssI methylases [14], and the Mrr system cleaving DNA methylated by HhaII or PstI methylation enzyme [15]. In this research, we report an efficient and easily applicable transformation method using in vivo and in vitro methylation DNA, which completely overcomes the restriction barriers of S. griseus.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

S. griseus NRRL B-2682 obtained from the Northern Regional Research Laboratory (Peoria, IL, USA) was used as the wild-type strain. The S. griseus strain Rfs2, which is transformed approximately 10 times more efficiently than the wild-type strain, was used as an intermediate host in introducing plasmids isolated from S. lividans to S. griseus wild-type. S. lividans TK 24 [16] was also used as an intermediate host prior to transformation of S. griseus Rfs2. E. coli strains DH5αMCR (hsd) [17], GM48 (dam, dcm) [18], GM161 (dam, hsd) [18], and ET12567 (dam, dcm, hsd) [11] were used as methylation-deficient hosts for plasmids prior to in vitro methylation. The plasmids isolated from the modification-deficient strains of E. coli were methylated in vitro by each methyltransferase and mixed with the protoplasts. E. coli cultures were grown in LB supplemented with ampicillin (100 μg ml⁻¹) or apramycin (100 μg ml⁻¹) as needed. SpM agar supplemented as needed with apramycin (20 μg ml⁻¹) or thioestrept (5 μg ml⁻¹) was used for maintenance of S. griseus strains. SpMR [19] was used for protoplast regeneration.

2.2. Transformation of Streptomyces

Transformation of Streptomyces protoplasts with plasmid DNA was as described [19]. For the soft agar overlay method, instead of P buffer, 2.5 ml of SpMR soft agar (containing 0.6% agar and 20 mM CaCl₂), which had been pre-incubated at 25°C, 30°C, 40°C, 45°C, or 50°C for 30 min, was mixed with the transformation mixture containing plasmid DNA, 0.05 ml of protoplast, and 0.2 ml of T buffer. The transformation efficiency was calculated as the number of transformants per 1 μg of DNA averaged from four or five transformation experiments.

2.3. Methylation of plasmid DNA

Plasmids for transformation were isolated by Qiagen Prep kit (Qiagen) as recommended by the manufacturer. The amount of DNA was spectrophotometrically quantified by measuring OD at 260 nm. The plasmid DNA isolated from E. coli strains was methylated in vitro with AluI, Dam, MspI, HpaII, and SssI DNA methylases purchased from New England Biolabs. 160 μM of S-adenosylmethionine (SAM) was added for SssI methylation reaction and 80 μM of SAM was added for AluI, Dam, MspI, and HpaII methylation reactions. After the methylation reaction, the methylases were inactivated by heating at 65°C for 15 min.

2.4. Preparation of protein extracts containing endogenous S. griseus methylases

The DNA modification experiments were performed in a buffer containing EDTA and no Mg²⁺ to minimize the endogenous restriction endonuclease activity. S. griseus was inoculated into 250 ml of TSB containing 1% glycine and 5 mM MgCl₂ and was grown to OD of 8–9 (approximately 24 h). The cell was harvested by centrifugation at 10000 rpm for 10 min, washed with PENP buffer (10 mM potassium phosphate, 10 mM EDTA, 50 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, pH 7.0), and suspended in 5 ml of PENP buffer. The cell extract was centrifuged at 12000 rpm for 15 min. The protein extract was combined 50% glycerol and 0.1 mg ml⁻¹ of nuclease-free bovine serum albumin (BSA; BRL) and stored at −20°C. Methylation reaction with endogenous methylases was performed in 0.1 ml of TNE (500 mM Tris-HCl, pH 7.5, 500 mM NaCl, 100 mM EDTA) buffer containing 0.16 mM SAM, 0.1 mg ml⁻¹ nuclease-free BSA, 10 μg of plasmid DNA from each E. coli strain. After overnight incubation at 30°C, the reaction mixture was extracted once with phenol–chloroform and twice with chloroform, then precipitated with ethanol.

3. Results and discussion

3.1. Increase in transformation efficiency by AluI and HpaII methylation

We have used two Streptomyces vectors, pX4E, a low copy number Streptomyces-E. coli shuttle vector [20], and pIJ702, a high copy number Streptomyces vector [21]. However, the pX4E derivatives isolated from the E. coli DH5αMCR strain and pIJ702 derivatives containing E. coli DNA as inserts cannot be directly introduced into S. griseus protoplasts. We passed the transforming DNA through two intermediate hosts, S. lividans and a derivative of S. griseus, Rfs2. The transformation efficiency of S. lividans with the vectors is relatively high (approximately 3.5×10³–6.9×10³ transformants per μg of DNA). However, when the plasmids isolated from S. lividans were introduced into S. griseus Rfs2, the transformation efficiency of the vectors was 5–20. Approximately 90% of the recovered vectors show restriction digestion profiles different from those of the original vectors,
indicating that the incoming DNA is rearranged during entry. To further improve the transformation of \textit{S. griseus}, we exploited previous observations to improve transformation efficiency. The genomic and plasmid DNAs from \textit{S. griseus} NRRL B-2682 are not digested with \textit{SacI} restriction enzyme recognizing the hexanucleotide sequence 5'-G/AGCTC-3', indicating that \textit{S. griseus} contains DNA modification activity at the site. A class II restriction endonuclease, SgrAI, has been isolated from \textit{S. griseus} recognizing the palindromic sequence 5'-CR/CCGGYG-3' [8]. We used \textit{AluI} methyltransferase which modifies AGCT at A to methylate the \textit{SacI} recognition site (5'-G/AGCTC-3'). To modify the SgrAI recognition site (5'-CR/CCGGYG-3'), we selected \textit{HpaII} methylase modifying CCGG at the internal C site, \textit{MspI} methylase modifying CCGG at the external C site, and \textit{SssI} methylase methylating CG at the C site.

We used two \textit{E. coli}-\textit{Streptomyces} shuttle vectors, pXE4, the low copy number vector, and pKK1443, the high copy number constructed in this experiment by combining pIJ2925 and pIJ702, after digestion with \textit{BglII}. No \textit{S. griseus} transformants were detected in the transformation experiments performed with the pXE4 extracted from any \textit{E. coli} strains tested and methylated in vitro with \textit{AluI}, \textit{Dam}, \textit{HpaII}, \textit{MspI}, and \textit{SssI} methyltransferases, respectively. However, substantially high numbers of transformants were found from the pXE4 isolated from all \textit{E. coli} strains and methylated with \textit{HpaII} or \textit{SssI} methylase (Fig. 1A–D). The pXE4 showed the maximum transformation efficiency.
Alu DNAmethylated at the cytosine of AGCT by (GAGCTC). The DNA from the wild-type strain or the possess a unique R-M system recognizing the vented the restriction barrier.

containing CCGG and methylating at the internal cytosine contain a R-M system recognizing the DNA sequence¢les of the genomic DNA of S. griseus derivatives.

through two intermediate hosts using pXE4 and pIJ702 and plasmid rearrangement on each step when passing reliability in transformation experiments by overcoming

ases (Fig. 1). However, Alu generated relatively high numbers of transformants (Fig. 3). The transformation e¢ciency of pXE4 isolated from GM161 after heat treatment. and designate the transformation e¢ciency of the pXE4 isolated from GM161 and methylated with HpaII and SssI methylases, respectively. and indicate the transformation e¢ciency of the pXE4 from S. griseus and GM161 without any methylat, respectively.

and the transformation efficiency was approximately the same as that of the pXE4 from S. griseus when it was extracted from GM161 and methylated with HpaII methylase (Fig. 1D). In the case of pKK1443, a similar pattern of increase in transformation efficiency was observed when they were methylated with HpaII or SssI methyltransferases (Fig. 1). However, Alu methylation of the pKK1443 generated relatively high numbers of transformants (Fig. 1D). The maximum transformation efficiency was observed when it was methylated with both AluI and HpaII methylases (Fig. 2). The transformation efficiency of pKK1443 was also increased by AluI and SssI methylation (Fig. 2). The direct DNA transfer from E. coli to S. griseus has strong advantages. We can take advantage of the shuttle vector systems such as rapid cloning and e¢cient isolating of the plasmid from E. coli, compared to the previous way in which the pXE4 and pIJ702 derivatives should have been passed through two intermediate hosts, S. lividans and S. griseus Rf62. This method increased reliability in transformation experiments by overcoming the difficulties caused by low transformation efficiency and plasmid rearrangement on each step when passing through two intermediate hosts using pXE4 and pIJ702 derivatives.

This study revealed R-M systems and methylation pro¢les of the genomic DNA of S. griseus. S. griseus seems to contain a R-M system recognizing the DNA sequence containing CCGG and methylating at the internal cytosine of the CCGG sequence, since HpaII methylation circumvented the restriction barrier. S. griseus also appeared to possess a unique R-M system recognizing the SacI site (GAGCTC). The DNA from the wild-type strain or the DNA methylated at the cytosine of AGCT by AluI methylase was not digested with SacI. Consistently, AluI methylation of the shuttle vector pKK1443 highly increased the transformation efficiency. However, it is not clear why AluI methylation did not observably have any effect on the transformation efficiency of pXE4, whereas the transformation efficiency of pKK1443 substantially increased after AluI methylation. It seems to be related to the fact that pKK1443 contains two SacI sites whereas pXE4 contains one SacI recognition site.

3.2. Effect of SssI methylation on transformation

SssI methylation of both vectors from any E. coli tested in this experiment substantially increased the transformation efficiency (Fig. 1A–D). However, the same methylation of S. griseus DNA decreased the transformation efficiency by more than 100-fold (Fig. 1E). The decrease in transformation efficiency was also observed in several double methylation experiments (Fig. 2). The vectors isolated from GM161 and methylated with both HpaII and SssI methylases transformed S. griseus approximately 50–100 times less ef®ciently than the DNA methylated with only HpaII methylase (Fig. 2). These observations suggest that SssI methylation bypasses the restriction barrier through methylating a CCGG-containing sequence, but the DNA is restricted by a methyl-speci®c restriction system recognizing the CG-containing sequence methylated at the cytosine.

3.3. Transformation using modification-de®cient E. coli hosts

Based on the previous result that S. avermitilis was more ef®ciently transformed when vectors were isolated from a modification-de®cient E. coli strain, ET12567 (dam, dcm, hsd) [16,17], we tested four E. coli strains including DH5zMCR (hsd), GM161 (dam, hsd), GM48 (dam, dcm), and ET12567. The highest transformation efficiency was observed when the vectors were isolated from GM161 (Fig. 1). The vectors from ET12567 showed the second highest transformation ef®ciency (Fig. 1). The vectors from GM48 transformed two to four times more ef®ciently than those from DH5zMCR (Fig. 1A,B). The observation that the dam strains, GM161 and ET12567, showed much higher transformation ef®ciency than DH5zMCR and GM48 demonstrated that Dam methylation of the transforming DNA decreased the transformation ef®ciency. Consistent with the result is that the S. griseus plasmids methylated with Dam methylase transformed S. griseus at least 200 times less ef®ciently than those without Dam methylation did (Fig. 1E). The vectors double-methylated with Dam and HpaII methylases also showed much less transformation ef®ciency than the vectors methylated with HpaII only (Fig. 2). Based on these results, we speculate that S. griseus possesses a methyl-speci®c restriction system recognizing the dam-recognized sequence, GATC.

The vectors from the E. coli strains carrying Dem activity (GM161) were introduced into S. griseus more ef®ciently than those from the dcm strains (ET12567 and
GM48), suggesting that there could exist another S. griseus R-M system recognizing a DNA sequence overlapping the dcm site (CCWGG). We also analyzed the data to evaluate the effect of hsd methylolation. The pXE4 and pKK1443 from the hsd\textsuperscript{+} strain (GM48) transformed S. griseus 4–10 times less efficiently than those from the hsd strain (ET12567) (Fig. 1). Thus in vivo hsd methylolation appeared to decrease the transformation efficiency. There could be another methyl-dependent restriction system recognizing the hsd site (AACNNNNNNGTG, where N is any nucleotide) [22], when N\textsuperscript{6}-methyladenosine exists in the sequence.

3.4. Transformation after heat treatment and incubation with S. griseus protein extracts

In previous research, heat treatment of S. clavuligerus protoplasts increased the transformation efficiency of pIJ702 by 100-fold [23]. To test the heat treatment effect on the transformation of S. griseus, S. griseus protoplasts were mixed with the plasmid and T buffer, mixed with preheated soft agar medium at 25°C, 30°C, 40°C, 45°C, or 50°C, and plated on SpMR. Heat treatment of the pXE4 from GM161 did not increase the transformation efficiency when it was not modified by any methylase (Fig. 3). The heat treatment at 45°C resulted in approximately 10 times more transformants than the treatment at room temperature, when pXE4 was prepared from the GM161 strain and modified by SsI methylase (Fig. 3). However, the heat treatment of pXE4 prepared from S. griseus, or prepared from GM161 and methylated by HpaII methyltransferase, decreased the transformation efficiency by 2–200-fold, perhaps because the viability of S. griseus protoplasts was impaired by the heat treatment. The existence of a methyl-specific system is also supported by heat treatment experiment. Heat treatment increased the transformation efficiency of SsI-methylated E. coli DNA, perhaps because the treatment inactivates the methyl-specific restriction system recognizing CG-containing sequences. However, heat treatment had a negative effect on the transformation efficiency of S. griseus DNA and the E. coli DNA modified by HpaII methylase.

As suggested in the transformation experiments of Sacccharopolyspora spinosa [24] and Helicobacter pylori [25] to test whether S. griseus protein extract contains DNA modification activities effective in transforming S. griseus, we incubated pKK1443 and pXE4 with S. griseus protein extract prior to transformation. However, the transformation efficiency of pXE4 and pKK1443 incubated with the S. griseus protein extracts was not higher than that of the vectors without any treatment (data not shown). When pKK1443 and pXE4 were double-methylated with S. griseus protein extracts and HpaII or SsI methylases, the transformation efficiency decreased by approximately two-fold compared to the data without the incubation with the protein extract.

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References


