J. Microbiol. Biotechnol. (2011), **21**(7), 675–678
doi: 10.4014/jmb.1102.02038
First published online 14 May 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Improvement of Transformation Efficiency by Strategic Circumvention of Restriction Barriers in *Streptomyces griseus*

Suzuki, Hirokazu[1,2]*, Shunji Takahashi[1], Hiroyuki Osada[1], and Ken-ichi Yoshida[3]

[1]*Chemical Biology Department, Advanced Science Institute, RIKEN, Saitama 351-0198, Japan*
[2]*Organization of Advanced Science and Technology, Kobe University, Hyogo 657-8501, Japan*
[3]*Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, Hyogo 657-8501, Japan*

Received: February 28, 2011 / Revised: April 12, 2011 / Accepted: April 13, 2011

DNA methylation in *Streptomyces griseus* IFO 13350 was analyzed by high-performance liquid chromatographic analysis and bisulfite-based analysis to reveal two methylation sites, 5'-GC^{5m}CGGC-3' and 5'-GAG^{5m}CTC-3'. The methylation was reconstituted in *Escherichia coli* by simultaneous expression of *S. griseus SGR4675* and *S. achromogenes M.SacI*. The *E. coli* cells produced plasmids that mimicked the methylation profile of *S. griseus* DNA, which was readily introduced into *S. griseus*. The results of this study raise the possibility of a promising approach to establish efficient transformation in several streptomycetes.

Keywords: DNA methylation, DNA restriction, host-mimicking DNA, *Streptomyces griseus*

Genetic transformation plays important roles in microbiological and biotechnological studies of *Streptomyces* species, but it is often hampered by a DNA restriction–modification (R-M) system to cut exogenous DNA in the organisms. Among the four types of R-M systems [11], type II consists of a restriction endonuclease and DNA methylase. The endonuclease digests exogenous DNA selectively at specific sites but not endogenous DNA that has already been methylated by the methylase. Type IV are methyl-specific restriction systems that restrict heterologously methylated DNA. Although type IV restriction barriers have been observed in several streptomycetes, they can be readily circumvented by using methyl-free DNA [2, 5, 8, 14]. However, methyl-free DNA is restricted in streptomycetes that have R-M systems other than type IV [7]. Here, we report the efficient transformation of the streptomycin-producing *Streptomyces griseus* IFO 13350 that has a putative type II R-M system [10]. This was achieved by using a plasmid that mimicked the DNA methylation profile of *S. griseus* to circumvent the restriction barrier.

Table 1 lists the *Escherichia coli* strains and plasmids used in this study. To determine the DNA methylation profile of *S. griseus*, deoxynucleosides were prepared from the chromosome using nuclease P1 and alkaline phosphatase, and analyzed by reversed-phase high-performance liquid chromatography [4]. This analysis indicated 5-methyl-2'-deoxycytidine but not N^6-methyl-2'-deoxyadenosine or N^4-methyl-2'-deoxycytidine. The composition ratio of 5-methyl-2'-deoxycytidine to 2'-deoxycytidine was 0.66 mol%. These results suggested that *S. griseus* possesses approximately one 5-methylcytosine (^{5m}C) per 0.5 kb of the chromosome with 67% GC content. A 31 kb sequence from the *S. griseus* genome was then analyzed by bisulfite-based analysis using a Methylamp DNA Modification Kit (Epigentek, NY, USA). Bisulfite treatment converts methyl-free cytosine to uracil without affecting ^{5m}C; therefore, the ^{5m}C positions can be determined by comparing the bisulfite-treated and untreated DNA sequences [9]. This analysis revealed two consensus sites, 5'-GC^{5m}CGGC-3' and 5'-GAG^{5m}CTC-3'. Methylation at these two sites was also confirmed by bisulfite-based analysis of an *E. coli–Streptomyces* shuttle plasmid pMS103 that was propagated in *S. griseus* (Table 2). Moreover, the resistance of the *S. griseus* chromosome to *Nae*I and *Sac*I digestion (data not shown) supported methylation, because *Nae*I cuts 5'-GCCGGC-3' but not 5'-GC^{5m}CGGC-3' and *Sac*I cuts 5'-GAGCTC-3' but not 5'-GAG^{5m}CTC-3'. The abundance of the consensus sites was estimated to be approximately one site per 0.5 kb in the genome sequence, which was in good agreement with the results of reversed-phase high-performance liquid chromatographic analysis. These results clarified the 5'-

*Corresponding author
Phone: +81-92-642-7609; Fax: +81-92-642-3053;
E-mail: hirokap@xpost.plala.or.jp
Present address: Hirokazu Suzuki, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka 812-8581, Japan

Table 1. *E. coli* strains and plasmids used in this study.

Strain or plasmid	Feature[a]
E. coli strains	
IR27	F^-, *fhuA*2, Δ(*lacZ*)r1, *glnV*44, *e*14$^-$(*mcrA*), *trp*-31, *his*-1, *rpsL*104(Str^R), *xyl*-7, *mtl*-2, *metB*1, Δ(*mrr-hsdRMS-mcrBC*)114::*IS10*, Δ*dam*::*metB*, Δ*dcm*::*lacZ*
IR500	The strain IR27 carrying pIR500
IR391	The strain IR27 carrying pIR391
IR539	The strain IR27 carrying pIR539
Plasmids	
pMS103	Derivative of an *E. coli*–*Streptomyces* shuttle plasmid pWHM3 [13], carrying a 182 bp fragment with 5'-GCCGGC-3' and 5'-GAGCTC-3' sites, pIJ101 origin, Tsr^R, pUC origin, and Amp^R
pIR201	Derivative of pACYCDuet-1, carrying the *lac* promoter expression cassette, p15A origin, and Cm^R
pIR500	pIR201 carrying *SGR4675*
pIR391	pIR201 carrying *M.SacI*
pIR539	pIR201 carrying *SGR4675* and *M.SacI*

[a]Amp^R, Cm^R, Tsr^R, and Str^R denote resistance genes for ampicillin, chloramphenicol, thiostrepton, and streptomycin, respectively.

GC^{5m}CGGC-3' and 5'-GAG^{5m}CTC-3' methylation in *S. griseus* and also implied that *S. griseus* might harbor R-M systems involved in the restriction of methyl-free 5'-GCCGGC-3' and 5'-GAGCTC-3' sites.

To produce DNA that mimics the methylation profile of *S. griseus*, putative methylase genes from the *S. griseus* genome [10] were cloned into plasmid pIR201 and expressed in *E. coli* IR27. Fig. 1 shows a summarized schematic representation of the construction of plasmids pIR201, pIR391, pIR500, and pIR539. *E. coli* IR27 was constructed by *dam* and *dcm* disruptions in *E. coli* ER1793 (New England Biolabs, Ipswich, USA). pMS103 was propagated in the recombinant *E. coli* strains by culturing them at 30°C overnight in LB medium supplemented with 50 μg/ml ampicillin, 12.5 μg/ml chloramphenicol, and 10 g/l lactose, and characterized for DNA methylation by bisulfite-based analysis (Table 2). Among the pIR201 derivatives examined, pIR500 to express *SGR4675* was responsible for 5'-GC^{5m}CGGC-3' methylation of pMS103. The product of *SGR4675* showed moderate homologies to uncharacterized proteins, such as putative methylases from *Streptomyces* sp. SPB78, *Streptomyces ghanaensis*, and *Rhodococcus erythropolis* SK121 (identities, 47–49%). Although *SGR3359* encoded the M.*Sac*I homolog (identity, 44%), no gene was responsible for 5'-GAG^{5m}CTC-3' methylation in *E. coli* IR27. This may be attributable to the difficulty in achieving heterologous expression in *E. coli*. Therefore, pIR391 to express the *M.SacI* gene of *Streptomyces achromogenes* ATCC12767 (JCM4121) was constructed and used for 5'-GAG^{5m}CTC-3' methylation. Functional expression of *M.SacI* in *E. coli* IR27 was confirmed by bisulfite-based analysis (Table 2). *E. coli* IR539, which was strain IR27 harboring pIR539 to express both of *SGR4675* and *M.SacI*, produced pMS103 with 5'-GC^{5m}CGGC-3' and 5'-GAG^{5m}CTC-3' methylation. In fact, this plasmid completely mimicked *S. griseus* DNA with regard to the DNA methylation profile.

The host-mimicking pMS103 was used for transformation of *S. griseus* (Table 2). Protoplast transformation was performed according to Hopwood *et al*. [6]. As expected, pMS103 from *E. coli* IR539 was effectively introduced

Table 2. Characterization of pMS103 propagated in hosts.

Host	Cytosine 5-methylation frequency[a]					Transformation efficiency[b]
	5'-GcCGGC-3'	5'-GCcGGC-3'	5'-GCCGGc-3'	5'-GAGcTC-3'	5'-GAGCTc-3'	
S. griseus	5/63	63/63	2/63	63/63	1/63	7.7×10^3
E. coli IR500	5/59	51/59	5/59	1/59	0/59	2.0×10^2
E. coli IR391	3/57	3/57	1/57	49/57	2/57	3.1×10^1
E. coli IR539	9/59	57/59	1/59	47/59	1/59	3.1×10^3
E. coli IR27	6/61	8/61	4/61	2/61	0/61	N.D.[c]

[a]The 182 bp insert of pMS103 was treated with bisulfite using a Methylamp DNA Modification Kit and amplified by PCR. PCR fragments were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, USA) and sequenced to determine cytosine 5-methylation in the 182 bp sequence. Several plasmids were analyzed. The numerator denotes the number of plasmids that were methylated at the position shown by lower-case letters. The denominator denotes the number of plasmids analyzed.
[b]Number of *S. griseus* transformants per microgram of supercoiled plasmid. Results are means of duplicate experiments.
[c]No transformant was obtained.

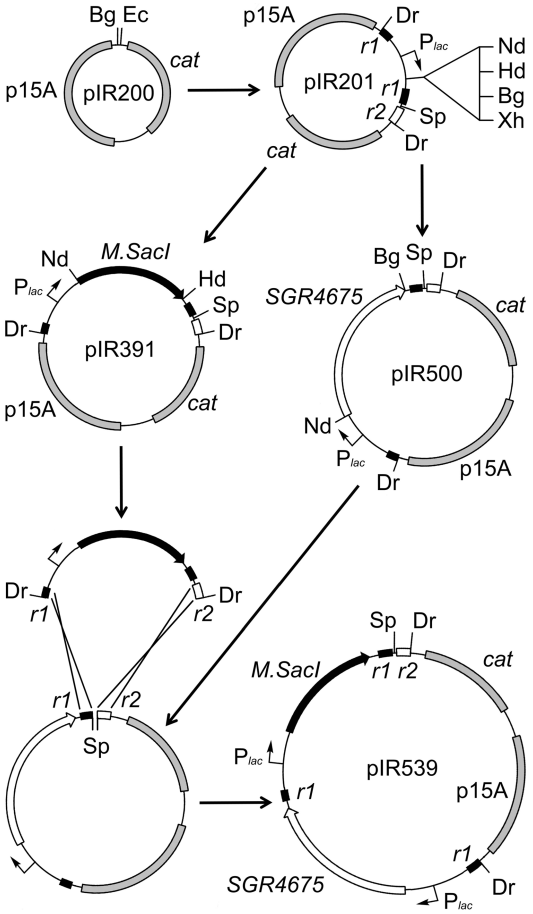

Fig. 1. Construction of pIR201, pIR391, pIR500, and pIR539.
The region including the p15A origin and chloramphenicol acetyltransferase gene on pACYCDuet-1 (Merck, Darmstadt, Germany) was PCR-amplified and self-ligated. Two *Dra*I sites in the resulting plasmid were sequentially disrupted using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) to give pIR200. The *lac* promoter region of pUC19 (Takara, Otsu, Japan) was amplified and introduced between the *Bgl*II and *Eco*RI sites of pIR200 to give pIR201. pIR201 was used for construction of pIR391 and pIR500. *SGR4675* was amplified and cloned between the *Nde*I and *Bgl*II sites of pIR201 to give pIR500. The *M.SacI* gene of *S. achromogenes* was amplified and cloned between the *Nde*I and *Hin*dIII sites of pIR201 to obtain pIR391. The expression cassette for *M.SacI* was extracted from pIR391 by *Dra*I digestion and ligated with *Spe*I-digested pIR500 by a recombinase reaction using the In-Fusion PCR Cloning Kit (Takara) between recombination site 1 (*r1*, 5'-AAACCTGGTCACTAG-3') and site 2 (*r2*, 5'-CTAGTCCCATCTTTT-3') of the cassette and vector, to give pIR539. The restriction sites of *Nde*I, *Hin*dIII, *Spe*I, *Dra*I, *Bgl*II, *Eco*RI, and *Xho*I are abbreviated as Nd, Hd, Sp, Dr, Bg, Ec, and Xh, respectively. P_{lac}, *lac* promoter; *cat*, chloramphenicol resistance gene; p15A, p15A origin of replication.

into *S. griseus*, whereas methyl-free pMS103 from *E. coli* IR27 was not. The transformation efficiency by pMS103 from *E. coli* IR539 was comparable to that from *S. griseus*. Either 5'-GC^{5m}CGGC-3' or 5'-GAG^{5m}CTC-3' methylation partially improved the transformation efficiencies. The transformation efficiency of pMS103 with 5'-GC^{5m}CGGC-3' was approximately 10-fold higher than that with 5'-GAG^{5m}CTC-3'. The difference was attributable to the number of restriction sites of pMS103, which possesses five 5'-GCCGGC-3' and two 5'-GAGCTC-3' sites. These results indicated that *S. griseus* had restriction systems for methyl-free 5'-GCCGGC-3' and 5'-GAGCTC-3' sites, and the host-mimicking pMS103 circumvented both restriction barriers. Although the restriction system in *S. griseus* remains unclear, *SGR3358* encoding type II restriction endonuclease is probably involved in the restriction [10].

The host-mimicking strategy improved the transformation efficiency in *S. griseus*. Because restriction systems should not restrict host-mimicking DNA as well as host DNA, this approach has the potential to improve the transformation efficiency in other streptomycetes. As demonstrated, reversed-phase high-performance liquid chromatographic analysis of deoxynucleosides is one of the most efficient methods for estimating R-M systems in streptomycetes. The absence of methylated DNA indicates that the streptomycete has only type IV or no R-M system. In such cases, methyl-free DNA corresponds to host-mimicking DNA. Meanwhile, the presence of methylated DNA implies that the streptomycete has a functional R-M system other than type IV. In such cases, the production of host-mimicking DNA requires determination and reconstitution of the DNA methylation profile of the *Streptomyces* strain. Determining the wide-ranging methylation states by restriction mapping, bisulfite-based methods, and DNA sequencing technologies is possible [1, 3, 9]. Information on DNA methylases is readily available from the REBASE database [12], along with their gene sequences, methylation sites, and organism sources. Plasmid pIR201 and *E. coli* IR27 could also facilitate production of host-mimicking DNA. pIR201 contains an expression cassette driven by a *lac* promoter, which can be extracted by *Dra*I digestion and integrated with *Spe*I-digested pIR201 by a recombinase-mediated reaction to produce a pIR201 derivative containing two tandem expression cassettes (Fig. 1). Because the resulting plasmid has another integration site originating from the integrated cassette, such integration can be repeated further. The p15A origin of pIR201 allows compatible propagation of any ColE1 plasmids in *E. coli* cells. Meanwhile, *E. coli* IR27 is deficient in all intrinsic genes involved in methylation (*dam*, *dcm*, and *hsdM*) and restriction (*mcrABC*, *mrr*, and *hsdR*) of DNA. Because methylation due to *dam* and *hsd* has been reported to decrease the transformation efficiency in several *Streptomyces* spp. [2, 5, 7, 8, 14], considering endogenous methylation in the *E. coli* host to produce host-mimicking DNA is important. Moreover, this strain is deficient in the type IV restriction systems of *E. coli*, which prevent its heterologously methylated DNA from being restricted by its cells. Thus, the results of this study not only provide an efficient method for *S. griseus* transformation but also suggest a promising approach to establishing efficient transformation in several streptomycetes.

Acknowledgments

This work was supported by KAKENHI (20780080) and in part by funding from the Special Postdoctoral Researchers Program of RIKEN and Special Coordination Funds for Promoting Science and Technology under the project Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan.

References

1. Bart, A., M. W. J. van Passel, K. van Amsterdam, and A. van der Ende. 2005. Direct detection of methylation in genomic DNA. *Nucleic Acids Res.* **33:** e124.
2. Enríquez, L. L., M. V. Mendes, N. Antón, S. Tunca, S. M. Guerra, J. F. Martín, and J. F. Aparicio. 2006. An efficient gene transfer system for the pimaricin producer *Streptomyces natalensis*. *FEMS Microbiol. Lett.* **257:** 312–318.
3. Flusberg, B. A., D. R. Webster, J. H. Lee, K. J. Travers, E. C. Olivares, T. A. Clark, J. Korlach, and S. W. Turner. 2010. Direct detection of DNA methylation during single-molecule, real-time sequencing. *Nat. Methods* **7:** 461–465.
4. Gehrke, C. W., R. A. McCune, M. A. Gamasosa, M. Ehrlich, and K. C. Kuo. 1984. Quantitative reversed-phase high-performance liquid-chromatography of major and modified nucleosides in DNA. *J. Chromatogr*. **301:** 199–219.
5. González-Cerón, G., O. J. Miranda-Olivares, and L. Servin-González. 2009. Characterization of the methyl-specific restriction system of *Streptomyces coelicolor* A3(2) and of the role played by laterally acquired nucleases. *FEMS Microbiol. Lett.* **301:** 35–43.
6. Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, *et al*. 1985. *Genetic Manipulation in Streptomyces: A Laboratory Manual*. The John Innes Foundation, Norwich.
7. Kwak, J., H. Jiang, and K. E. Kendrick. 2002. Transformation using *in vivo* and *in vitro* methylation in *Streptomyces griseus*. *FEMS Microbiol. Lett.* **209:** 243–248.
8. MacNeil, D. J. 1988. Characterization of a unique methyl-specific restriction system in *Streptomyces avermitilis*. *J. Bacteriol.* **170:** 5607–5612.
9. Oakeley, E. J. 1999. DNA methylation analysis: A review of current methodologies. *Pharmacol. Ther.* **84:** 389–400.
10. Ohnishi, Y., J. Ishikawa, H. Hara, H. Suzuki, M. Ikenoya, H. Ikeda, A. Yamashita, M. Hattori, and S. Horinouchi. 2008. Genome sequence of the streptomycin-producing microorganism *Streptomyces griseus* IFO 13350. *J. Bacteriol.* **190:** 4050–4060.
11. Roberts, R. J., M. Belfort, T. Bestor, A. S. Bhagwat, T. A. Bickle, J. Bitinaite, *et al*. 2003. A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes. *Nucleic Acids Res.* **31:** 1805–1812.
12. Roberts, R. J., T. Vincze, J. Posfai, and D. Macelis. 2010. REBASE – a database for DNA restriction and modification: Enzymes, genes and genomes. *Nucleic Acids Res*. **38:** D234–D236.
13. Vara, J., M. Lewandowska-Skarbek, Y.-G. Wang, S. Donadio, and C. R. Hutchinson. 1989. Cloning of genes governing the deoxysugar portion of the erythromycin biosynthesis pathway in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). *J. Bacteriol*. **171**: 5872–5881.
14. Zotchev, S. B., H. Schrempf, and C. R. Hutchinson. 1995. Identification of a methyl-specific restriction system mediated by a conjugative element from *Streptomyces bambergiensis*. *J. Bacteriol.* **177:** 4809–4812.