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Gene Disruption Using *In Vivo* and *In Vitro* Methylation in *Streptomyces griseus*

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Abstract Previous study demonstrated that the restriction barrier of *Streptomyces griseus* is almost completely bypassed by the *Streptomyces-E. coli* shuttle vectors passed through the *E. coli* GM161 strain and methylated with AluI and HpaII methyltransferases. The same DNA methylation of the genomic DNA fragments cloned the nonreplicative vectors generated integrative transformation and gene disruption of their chromosomal counterparts at high efficiencies in *S. griseus*. This result indicated that the efficiency of gene disruption depends on the efficient transfer of the incoming DNA into bacterial hosts.

Key words: Transformation, methylation, gene disruption, *Streptomyces griseus*, homologous recombination

Streptomyces is a Gram-positive soil bacterium intensively studied for its production of secondary metabolites [7, 10, 25]. *Streptomyces* is also an attractive host, because *Streptomyces* undergoes a complex life cycle of morphological differentiation that includes formation of uninucleoidal spores from multinucleoidal, and filamentous hyphae [4, 17]. Recent technical development in bacterial genome sequences and bioinformatics led to accumulation of a plethora of information about *Streptomyces* genes whose functions are not well understood. Thus, more systematic and efficient inactivation methods are required to understand the functional roles of individual genes, although several gene disruption methods have been used in *Streptomyces* species. In *Streptomyces hygroscopicus*, elimination of plasmids by consecutive protoplasting and regeneration efficiently replaces an antibiotic production gene with the disrupted counterpart of the gene on a plasmid having a *Streptomyces* replicon [1]. Introduction of DNA as single-stranded DNA drastically increases the integrative transformation efficiency in *Streptomyces viridochromogenes* [6] and in *Streptomyces coelicolor* [21]. Recently, in *S. coelicolor*, the PCR-mediated gene replacement methods have facilitated the construction of plasmid-borne disruptions by introducing PCR fragments containing a selectable marker and flanking DNA homologous to the cloned gene in the plasmids [5]. The subsequent transformation or conjugal transfer with the disrupted plasmids greatly enhanced construction of knockout mutants in many genes [5, 29].

In *Streptomyces griseus* IFO13350, several genes including *amfC* [14], *amfR* [28], *adpA* [23], *arpA* [22], and *strR* [27] have been disrupted by introduction of disrupted constructs on an unstable plasmid and subsequent selection. Another *S. griseus* strain, NRRL B-2682 obtained from the Northern Regional Research Laboratory (Peoria, IL, U.S.A.), has been intensively studied for submerged sporulation, because the strain can be induced to sporulate in liquid culture, unlike other *Streptomyces* species [11]. The production of relatively synchronous and homogenous sporulating cultures by the submerged sporulation facilitates the characterization of physiological and biochemical events, as well as the observation of delicate morphological changes [15, 17]. However, genetic manipulations, including transformation and gene disruption, have rarely been successful, because of difficulties in introducing foreign DNA, despite of repeated attempts.

In the previous study, we exploited a predicted DNA methylation profile to improve transformation in *S. griseus* NRRL B-2682 [11, 16]. The genomic and plasmid DNA of *S. griseus* NRRL B-2682 are not digested with SacI restriction enzyme, which recognizes the hexanucleotide sequence 5'-G/AGCTC-3', and SgrAI has been isolated from *S. griseus* recognizing the palindrome sequence 5'-CR/CCGGYG-3' [26], indicating that *S. griseus* contains

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Table 1. Vectors used in this study.

Plasmid	Insert size (kb)	Vector	Gene	Source
pIJJ30				Gift from H. Kieser
pIJ2920				[8]
pIJ2926				[8]
pKK535	3.5	pIJ2926	*hutH*	[30]
pKK574		pIJ2926	*hutH*	[30]
KK847	2.4	pIJ2926	*eshA*	[18]
pKK1410	8.0	pIJ2926	*eshA*	[18]
pKK1400		pIJ2920		In this study
pKK1441		pIJ2926		In this study
pKK1417	8.0	pKK1400	*eshA*	In this study
pKK2202	5.4	pKK1400	*eshA*	In this study
pKK2208	3.5	pKK1441	*hutH*	In this study
pKK2205	2.4	pKK1400	*eshA*	In this study
pKK1466	0.8	pKK1441	*hutH*	In this study
pKK1469	3.5	pKK1400	*hutH*	In this study
pKK1470	4.9	pKK1400	*hutH::apr*	In this study

DNA modification activities at the restriction sites. We used AluI methylase modifying AGCT at A and HpaII methylase modifying CCGG at the internal C site to avoid the SacI recognition site (5'-G/AGCTC-3') and the SgrAI recognition site (5'-CR/CCGGYG-3'), respectively. The methylation to mimic the DNA methylation profile drastically increases the transformation efficiency by overcoming the restriction barrier of *S. griseus* [11, 16]. In the present study, the same DNA methylation method was applied for generating integration and chromosomal disruptants between the chromosome and the incoming DNA on nonreplicative vectors. We used two nonreplicative vectors, pKK1400 and 1441, that harbor only an *E. coli* replicon, so that the vectors do not replicate in *Streptomyces* (Table 1). pKK1400 is a pIJ2920 derivative in which the 1.8 kb DNA fragment containing a thiostrepton-resistant cassette from pIJ30 (a gift of H. Kieser) was inserted into pIJ2920 [8] at the BglII site. pKK1441 is a pIJ2926 [8] derivative in which the ampicillin resistance cassette was replaced with the apramycin resistance cassette after DraI digestion and blunt-end production. Into these nonreplicative vectors, five genomic DNA fragments, ranging from 0.8 to 8.0 kb, were cloned (Table 1). The 2.4 kb BglII fragment from pKK847, the 5.4 kb BglII fragment from pKK1410, and the 8.0 kb BamHI-HindIII fragment from pKK1410 [18] harboring the *eshA* gene were cloned into pKK1400 to generate pKK2205, pKK2202, and pKK1417, respectively. The 0.8 kb HindIII-SalI fragment from pKK574 containing the truncated *hutH* open reading frame and the 3.5 kb EcoRI-HindIII fragment from pKK535 [30] containing the *hutH* gene were cloned into pKK1441, generating pKK1466 and 2208, respectively (Table 1). pKK1469 was generated by ligating the 1.4 kb apramycin resistance cassette into the MluI site of pKK535 containing the 3.5 kb *hutH* gene, and the resulting 4.9 kb insert was ligated into pKK1400, generating pKK1470 (Table 1).

Table 2. Integration efficiency of *S. griseus* with vectors containing different sizes of inserts with or without *in vitro* methylation.

Plasmid	Integration efficiency without methylation[a]	Integration efficiency with methylation
pKK1417	0	2,014
pKK2202	0	734
pKK2208	0	234
pKK2205	0	184
pKK1466	0	34
pKK1400	0	0
pKK1441	0	0

[a]The integration efficiency was calculated as the number of transformants per 1 μg of DNA averaged from 3–4 times of transformation experiments. Transformation of *Streptomyces* protoplasts with plasmid DNA was performed as previously described [16].

The nonreplicative vectors were isolated from the *E. coli* GM161 strain according to the previous method [12], a methylation-deficient host for plasmids [24] and methylated with HpaII and AluI methyltransferases [16]. When the methylated vectors were introduced into *S. griseus* protoplasts prepared according to the previous method [2], a relatively high number of transformants grew well on SpM plates containing 20 μg/ml of apramycin or 5 μg/ml of thiostrepton, whereas no transformant was found from the same vectors without any methylation or from pKK1400 and 1441 (Table 2). These observations indicate that the vectors were integrated into the chromosome and homologous recombination occurred at least at single sites, since the vector contained no *Streptomyces* replicon. To determine whether the integration efficiency is influenced by the size of the incoming genomic DNA, the efficiency was compared among the nonreplicative vectors containing five genomic DNA fragments ranging in size from 0.8 to 8.0 kb (pKK1417, 1466, 2205, 2202, and 2208). The maximum efficiency was observed when the insert size in the nonreplicative vector was 8.0 kb (Table 2). This observation confirmed that the integration efficiency depends on the size of the cloned genomic DNA, but not on the markers or vectors. When pKK1466 containing a 0.8 kb genomic DNA fragment was introduced to *S. griseus* after the same methylation, 34 apramycin-resistant transformants were isolated per mg of DNA (Table 2).

We confirmed the single crossover event with pKK1466 containing the truncated 0.8 kb *hutH* open reading frame by Southern hybridization using digoxigenin (DIG) DNA labeling kit (Boerhinger Mannheim). Southern hybridization of several apramycin-resistant transformants revealed that the transformants contained two truncated copies of the open reading frame (3.3 kb and 1.5 kb fragments), as

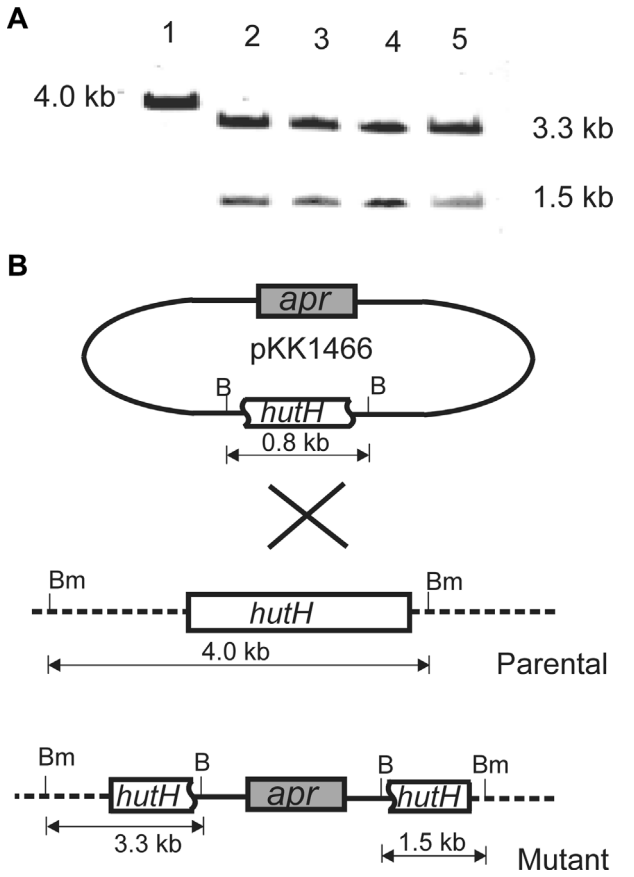

Fig. 1. Generation of single crossover null mutants by pKK1466. **A**. Southern hybridization of genomic DNA from the wild-type strain (lane 1), and the expected single crossover strains in which the null mutations were generated by introducing the truncated *hutH* open reading frame (lanes 2, 3, 4, and 5). The numbers alongside the bands mark the molecular sizes in kilobases (kb). **B**. Diagramatic presentation of the pKK1466, parental chromosome, and the chromosome of the expected double crossover strains. B and Bm mark BglII and BamHI restriciton sites, respectively. a*pr* represents the apramycin-resistant cassette.

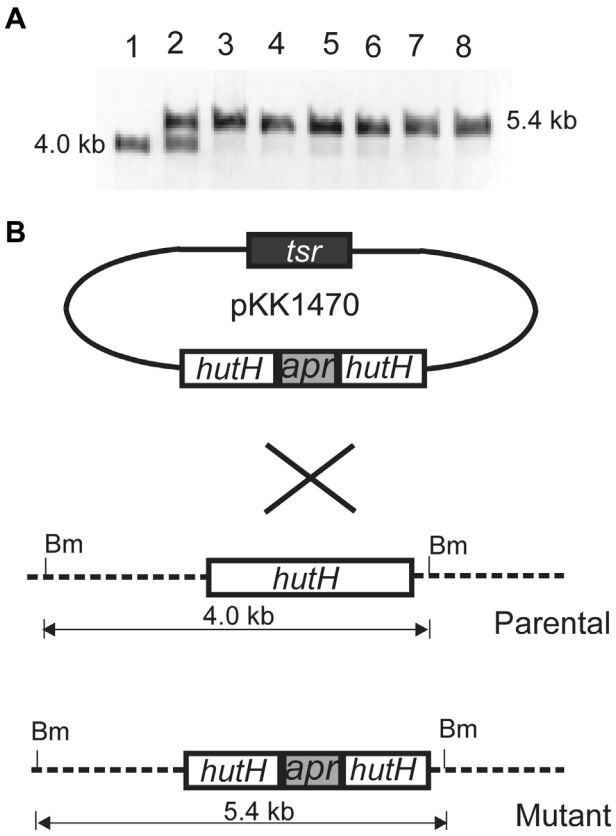

Fig. 2. Generation of double crossover null mutants by pKK1470. **A**. Southern hybridization of genomic DNA from the wild-type strain (lane 1), the expected single crossover strain (lane 2), and the expected double crossover strains further selected from the single crossover strain (lanes 3, 4, 5, 6, 7, and 8). The numbers alongside the bands mark the molecular sizes in kilobases (kb). **B**. Diagrammatic presentation of the pKK1466, parental chromosome, and the chromosome of the expected double crossover strain. Bm marks BamHI restriction sites. a*pr* and *tsr* represent the apramycin- and thiostrepton-resistant cassettes, respectively.

shown in Fig. 1. The transformants with pKK1466 did not grow on histidine-deficient minimal media, since the *hutH* gene encodes histidine ammonia-lyase (histidase), which is involved in the first step of histidine utilization.

To examine whether the transformation method is efficient enough to generate double crossover events, we used the vector pKK1470, containing a 4.9 kb insert ligated into pKK1400, where the 3.5 kb DNA fragment of the *hutH* open reading frame was disrupted by the 1.4 kb apramycin resistance cassette (Fig. 2). The plasmid was passed through *E. coli* GM161 and methylated with HpaII and AluI methyltransferases [16], and the methylated DNA was introduced into *S. griseus* protoplasts prepared according to the previous method [2]. The transformants were selected for apramycin resistance and screened for thiostrepton sensitivity. Replica plating showed that 17 out of 730 apramycin-resistant transformants were sensitive to thiostrepton, indicating that double crossover homologous recombination occurred. We further isolated putative double crossover strains from a single crossover transformant after subsequent selection. To isolate double crossover strains, a single crossover strain was incubated in SpM containing 25 μg of apramycin for 3–4 days, and the culture was spread on SpM agar containing apramycin and screened for thiostrepton sensitivity. A single crossover strain was incubated for 5 days in SpM containing apramycin and the spore suspension was plated on SpM agar plates containing apramycin. Twenty-two thiostrepton-sensitive strains were screened out of 1,000 apramycin-resistant colonies by replica plating. Southern hybridization analysis showed that all putative double crossover transformants contained the 5.4-kb BamHI fragment, which was expected for the disrupted *hutH* gene, but lacked the 4.0 kb BamHI fragment characteristic of the intact *hutH* gene (Fig. 2). All double crossover mutants did not grow on histidine-deficient minimal medium. Southern hybridization analyses were used to confirm whether the

gene disruption occurred between the incoming DNA and the genomic counterparts. Southern hybridization using the 1.7 kb DNA fragment of the *hutH* open reading frame confirmed the disrupted restriction profiles of the *hutH* gene; all the putative single crossover transformants contained the 5.4 kb BamHI fragment expected for the disrupted *hutH* gene and the 4.0 kb BamHI fragment characteristic of the intact *hutH* gene (Fig. 2).

The present study also revealed that efficient gene disruption and replacement depend on the interspecific transfer of cloned vectors from cloning hosts to *Streptomyces*, since homologous recombination is known to occur at high frequencies [13, 20]. The major hindrance against the interspecific DNA transfer is the existence of restriction-modification systems that cleave the incoming foreign DNA, but not the self-DNA protected by the endogenous methyltransferases [3]. Several pieces of evidence showed that methyl-specific restriction-modification systems also play an important role as a restriction barrier in *Streptomyces bambergiensis* [31], *Streptomyces avermitilis* [19], and *S. griseus* [16]. The direct DNA transfer from *E. coli* to *S. griseus* has strong advantages. First, we can take advantage of the *E. coli* vectors such as rapid cloning and efficient isolation. Second, 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. The effectiveness of our DNA transfer method has been proven by recent generation of almost 30 disrupted alleles in several genes of *S. griseus* including *hutH* [30], *ssfR* and *ssgA*, [9], *eshA* [18], *ftsZ* [15], *adpA* [Kwak, unpublished], and *whiG* [Lezhava, unpublished].

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