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Characterization of Intergeneric Conjugation and *attB* site for Molecular Genetic Studies of *Streptomyces scabies* ATCC 23962

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To facilitate molecular genetic studies of *Streptomyces scabies*, which causes scab diseases in economically important root and tuber crops, an effective gene transfer procedure was established by intergeneric conjugation from *Escherichia coli* ET12567 using an ØC31-derived integration vector harboring the *oriT* and *attP* fragments. High transconjugation efficiency of *S. scabies* was obtained on AS-1 medium containing 40 mM $MgCl_2$ with heat treatment at 30°C for 10 min, with spores as host and 2.5×10^8 *E. coli* as donor. The integration site in the *S. scabies* genome was cloned for the first time and the *attB* site was sequenced. It was located in an open reading frame coding for a pirin homolog as a single site and showed the highest degree of homology with *S. aureofaciens*. The results provide sufficient efficiency to enable conjugal transfer of genetic elements through *attB*/*P*-mediated site-specific integration, and also should facilitate molecular genetic studies for *S. scabies*.

Key words: *Streptomyces scabies* ATCC 23962, *attB* site, intergeneric conjugation, integration site.

Streptomycetes produce a variety of secondary metabolites, including more than two-thirds of all antibiotics employed in the fields of human medicine and agriculture. However, only a few species such as *Streptomyces acidiscabies*, *S. ipomoeae*, *S. scabies*, and *S. turgidiscabies*, are plant pathogens[1, 2]. *S. acidiscabies* and *S. scabies* infect the tap roots of the radish, turnip, and other crops, and *S. ipomoeae* infects the storage and fibrous roots of sweet potato[4]. Among these strains, *S. scabies* is the predominant and best-known of the plant pathogenic species[3,4]. Although many molecular genetic studies aimed at understanding and preventing scab diseases have been conducted, transformation of streptomycetes has many difficulties because of strong restriction barriers, absence of an efficient transformation system, and inherent instability of recombinants[1, 3, 5, 6].

Generally, protoplast and electroporation methods have been used for the transformation of streptomycetes, but their problems include relatively low efficiency and limited application[7, 8]. Therefore, intergeneric conjugation transferring single-stranded DNA has been considered as a new means for the transformation of streptomycetes[9]. In addition, after employment of methylation-deficient *Escherichia coli* as a DNA donor to avoid the methylated-DNA-dependent restriction systems of streptomycetes, this method has been more widely applied for many other streptomycetes, although the detailed condition of intergeneric conjugation for each strain must be newly identified each time[8-10]. While an effective

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gene transfer system for *S. acidiscabies* and *S. ipomoeae* has been optimized by intergeneric conjugation[11,12], no method has yet been conducted on *S. scabies*, although it is the best representative plant pathogenic species.

Therefore, in this study, the optimal procedure for the intergeneric conjugation of *S. scabies* was established using a bacteriophage ØC31 *att/int* system. Furthermore, the integration site (*attB*) in the genomic DNA of *S. scabies* was characterized to secure the molecular genetic study of *S. scabies*.

MATERIALS AND METHODS

Organisms and plasmid

S. scabies ATCC 23962 was used as the recipient, and *E. coli* strain XL10-Gold (Stratagene, La Jolla, CA) was used as the general cloning host. The methylation-deficient *E. coli* strain ET12567 (*dam-13::Tn9*, *dcm-6*, *hsdM*, *hsdS*) containing pUZ8002, a derivative of RK2 with a defective *oriT* (*aph*), was employed as the donor in conjugation. The site-specific integration vector, pSET152 (5.7 kb), harbors ØC31 *int*, *attP*, and *oriT* of RK2, as well as an apramycin-resistant gene for selection in streptomycetes and *E. coli*[9]. This plasmid does not carry out the replicative functions of the streptomycetes plasmid, and can only be maintained in recipient strains in its chromosomally integrated state.

Culture conditions

For spore formation, *S. scabies* was cultivated at 28°C for 12 days in Maltose-Bennett's Agar (g/L, 1 yeast extract, 1 beef extract, 2 N-Z amine type A, 10 maltose, 20 agar, pH 7.3). AS-1 (g/L, 1 yeast extract, 0.2 L-alanine, 0.2 L-arginine, 0.5 L-asparagine, 5 soluble starch, 2.5 NaCl, 10 Na_2SO_4, 20 agar, pH 7.5), ISP 2 (g/L, 4 yeast extract, 10 malt extract, 4 glucose, 20 agar, pH 7.0–7.4), ISP4 (g/L, 10 soluble starch, 1 K_2HPO_4, 1 $MgSO_4 \cdot 7H_2O$, 1 NaCl, 2$(NH_4)_2SO_4$, 2$CaCO_3$, 0.001 $FeSO_4 \cdot 7H_2O$, 0.001 $MnCl_2 \cdot 4H_2O$, 0.001 $ZnSO_4 \cdot 7H_2O$, 15 agar, pH 7.0–7.4), and MS (g/L, 20 mannitol, 20 soya flour, 20 agar) were used for conjugal transfer.

Conjugal transfer

The early intergeneric conjugation was carried out in accordance with the basic transconjugation protocol developed by Kieser *et al.* (2000)[13]. A culture of the donor *E. coli* ET12567/pUZ8002 harboring pSET152 was grown to an OD_{600} of 0.4 in the presence of 50 mg/ L apramycin, 25 mg/L chloramphenicol, and 50 mg/L kanamycin. To remove the antibiotics, the cells were washed twice in an equal volume of LB and resuspended in 0.1 volume of LB. *S. scabies* spores that were not heat treated were resuspended with 0.5 mL of 2×YT broth (1% yeast extract, 1.6% tryptone, 0.5% NaCl) at room temperature (25°C). *E. coli* donor cells (2.5×10^7) were added to the resuspended spores (1×10^4), and the mixtures were spread on solid medium plate containing 10mM $MgCl_2$. The conjugation plates were incubated for 16~18 h at 28°C and overlaid with 1.5 mL water containing 0.5 mg of nalidixic acid and 1 mg of apramycin. The plates were subsequently incubated for 12 day at 28°C. For confirming the chromosomal integration of pSET152, the exconjugants were analyzed via PCR and Southern-blot hybridization.

RESULTS

Parameters affecting conjugal transfer of *Streptomyces scabies* ATCC 23962

The first step was to select an appropriate medium for the conjugal transfer of *S. scabies*. The base medium for growth and spore formation of *S. scabies* was Maltose-Bennett's Agar, but no exconjugants were obtained. Therefore, representative four media (AS-1, ISP2, ISP4, and MS) were selected for *S. scabies* conjugation. AS-1 and MS have been frequently employed in the conjugal transfer of streptomycetes[2, 9, 14]. ISP2 is the most suitable for the conjugal transfer of *S. lavendulae* FRI-5[15]. ISP4 was selected because it is the only medium that allows the conjugal transfer of *Kitasatospora setae*, a non-*Streptomyces* streptomycetes[16]. The transconjugation frequency of AS-1, ISP4, and MS was 3.7×10^{-3}, 5.2×10^{-4}, and 1.1×10^{-3}, respectively, and no exconjugants were obtained with ISP2. In accordance with this result, AS-1 was selected as the most appropriate for the conjugation of *S. scabies* and was used in all subsequent experiments because its transconjugation frequency was 7.1- and 3.4-fold higher than ISP4 and MS, respectively, although they were the optimal media of *S. ipomoeae* and *S. acidiscabies*[11, 12].

Spores of streptomycetes used for conjugation was generally subjected to heat

Table 1. Effects of the number of donor *E. coli* on the number of recipient spores for transconjugation efficiency

Number of recipient spores	Transconjugation frequency[a] Number of *E. coli* donor		
	2.5×10^{6}	2.5×10^{7}	2.5×10^{8}
1×10^{3}	0	$3.5\pm0.3\times10^{-3}$	$7.0\pm0.8\times10^{-3}$[b]
1×10^{4}	$1.4\pm0.1\times10^{-3}$	$3.7\pm0.5\times10^{-3}$	$6.5\pm0.4\times10^{-3}$
1×10^{5}	$1.1\pm0.2\times10^{-3}$	$1.7\pm0.1\times10^{-3}$	$2.7\pm0.5\times10^{-3}$

[a]Exconjugants per recipient spores were counted on AS-1 medium containing 10 mM $MgCl_2$ after incubation at 28°C for 12 days.
[b]Data represent the mean ±standard deviation (SD)(n=5).

treatments at 50°C for 10 min before being mixed with *E. coli* donor for induction of conjugal transfer in accordance with the basic conjugation protocol[13], but in the conjugal transfer of *K. setae*, the heat treatments under the same conditions yielded no exconjugants[16]. To determine the optimal temperature for heat treatment, the heat susceptibility of the *S. scabies*s pores was first tested in a temperature range between 25 (control) to 60°C for 10 min. As shown in Fig. 1, their viabilities decreased with increasing heat treatment temperatures, being rapidly lost at temperatures above 45°C and abrogated at 60°C. However, the transconjugation frequency of spores treated at 30°C was increased 32% rather than that of control although its spore viability decreased 10% (Fig. 1). Therefore, in the case of *S. scabies*, 30°C was judged the most suitable temperature of heat treatment for conjugation.

To facilitate the conjugal transfer of streptomycetes, although no clear data regarding the function and optimal concentration of $MgCl_2$ are currently available, 10 mM $MgCl_2$ was commonly added to medium consistent with the basic conjugation protocol[13]. However, the optimal concentration of $MgCl_2$ added to the AS-1 medium must be surveyed for *S. scabies* conjugation, because the most optimal concentration of added $MgCl_2$ for conjugation efficiency appears to differ according to the strains employed[12,16]. When 0-60 mM $MgCl_2$ was added to AS-1, the transconjugation frequency of *S. scabies* increased by all concentrations of $MgCl_2$ and proportionately improved until 40 mM, although the colony formation of exconjugants was delayed at above 40 mM (Fig. 2). Therefore, 40 mM was identified as

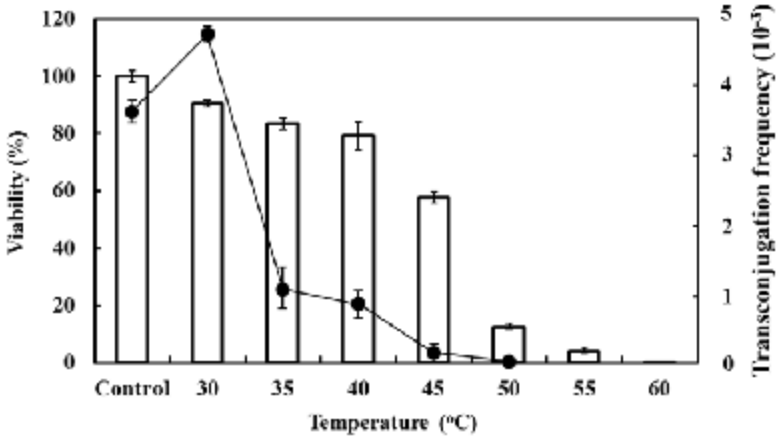

Fig. 1. Effects of temperature on the viability of *S. scabies* spores (bar graph) and the heat treatment of spores on transconjugation efficiency (line graph). For measuring spore viability after the heat treatment of spores, spores (1×10^{2}) in 2 × YT medium were incubated for 10 min at the temperatures indicated. Control means no heat treatment (25°C). The results represent SD (n=5)

the optimal concentration of $MgCl_2$ for addition to the AS-1 medium because its transconjugation frequency was 12- and 3-fold higher than 0 mM and 10 mM, respectively.

The mixing ratio of the number of recipient spores and *E. coli* donor profoundly affects the conjugation efficiency[11, 16]. In the conjugal transfer of *K. setae*, a number of *E. coli* donor used in accordance with the standard conjugation protocol yielded no exconjugants, but a further increase in the number of *E. coli* donor enabled its conjugation[16]. As shown in Table 1, with 1×10^3 recipient spores of *S. scabies*, 2.5×10^6 *E. coli* donor yielded no exconjugants, but the increase in the number of *E. coli* donor ($\geq2.5\times10^7$) made conjugal transfer of *S. scabies* passible and improved the transconjugation frequency of all spores. Contrarily, the increase of the number in recipient spores without increase of *E. coli* donor decreased the transconjugation frequency. This suggests that when increased numbers of recipient spores are used, as well as no exconjugants are present, the number of donor *E. coli* must be increased to increase or maintain transconjugation frequency.

Characterization of *attB* site in *Streptomyces scabies*

Conjugal transfer of streptomycetes was carried out by the integrase (*int*) function that inserts an *attP* site of vector (pSET152) into the *attB* locus in the recipient chromosome. However, some strains have another pseudo-*attB* site or no

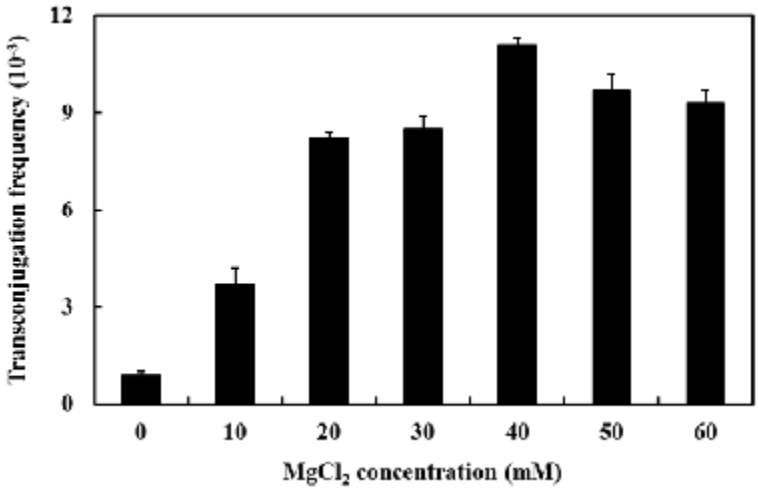

Fig. 2. Effects of $MgCl_2$ concentration added to AS-1 medium on transconjugation efficiency. *E. coli* ET12567 (pUZ8002) (2.5×10^7) and spores (10^4) treated with no heat treatment (25°C) were used. Exconjugants were counted on AS-1 medium containing each concentration of $MgCl_2$ after 12 days of incubationat 28°C. Bars represent SD (n=5)

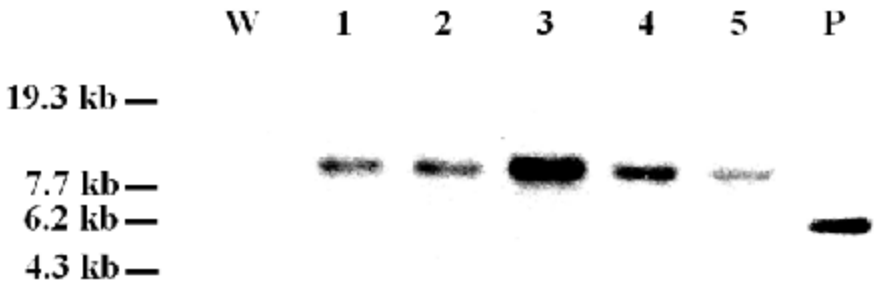

Fig. 3. Southern blot analysis of *Nru*I-digested genomic DNAs of exconjugants. Lane W, wild-type *S. scabies*; lanes 1-5, exconjugants containing pSET152; lane P, plasmid pSET152. The DNA was blotted onto a nylon membrane and hybridized with a DIG-labeled 0.5-kb aparamycin-resistant fragment of pSET152

attB site in their genome[17]. These cause potential problems like mutagenesis, induction of phenotypic changes, or absence of transconjugants[18]. The transconjugation efficiency using the bacteriophage ØC31 *att/int* system depends on the homology of the *attB* site sequence[16,17]. However, no reports have been published regarding the location or sequence of the *attB* site in the *S. scabies* genome. To identify the *attB* site of *S. scabies*, genomic DNAs of exconjugants were digested by *Nru*I that was not included in pSET152, and then confirmed by Southern hybridization using a 0.5-kb apramycin resistance fragment of pSET152 as a probe. As shown in Fig. 3, all of the exconjugants showed an equal single band pattern, suggesting that the *attB*site integrated with the *attP* site of pSET152 is a unique site in the *S. scabies* chromosome.

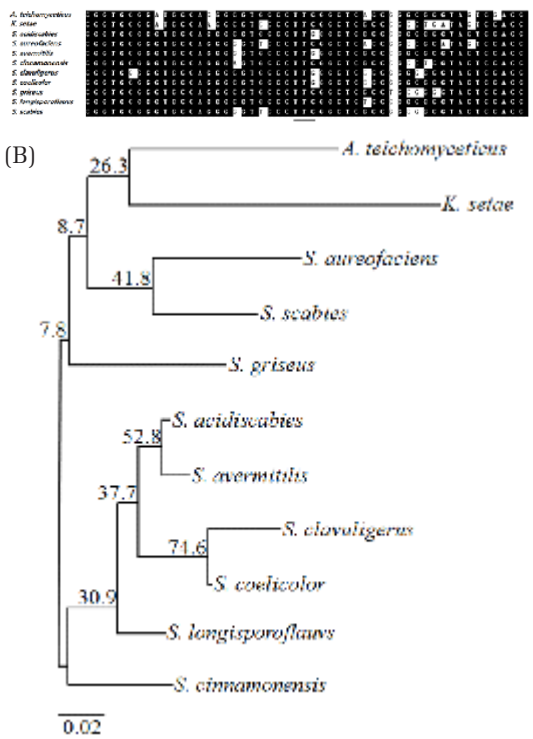

Fig. 4. Alignment of the *attB* site sequences (A) and phylogenetic tree (B) among *S. scabies* and other actinomycetes: *A. teichomyceticus* NBRC 13999, *K. setae* NBRC14216, *S. acidiscabies* ATCC 49003, *S. aureofaciens*, *S. avermitilis* MA-4680, *S. cinnamonensis*, *S. clavuligerus*, *S. coelicolor* A3(2) M145, *S. griseus* ATCC 12475, *S. longisporoflavus* 83E6, *S. scabies* (present study). The phylogenetic tree wasconstructed using GENETYX software (GENETYX Co., Tokyo, Japan). Bootstrap values (based on 1000 replication) are shown as percentages at each node. The scale bar indicates 0.02 substitutions per nucleotide position.

The genomic DNA of single band was obtained as plasmid harboring the genomic *attB* site by *Nru*I digestion and then transformed with *E. coli* XL10-Gold after self-ligation. Plasmid sequencing using the primers ATTPR (5'-CTGGGTGGGTTACACGACGCCCCT-3') and ATTPL(5'-CGTTGGCGCTACGCTGTGTCGCTG-3') revealed that all of the plasmids harbored left- and right-flanking arms of the insertion site in their genomes, and the same insertion endpoints within an open reading frame (ORF) coding for pirin (a newly identified nuclear protein that interacts with Bcl-3 and nuclear factor I). The core sequence (TTS) of *attB* site integrated with the *attP* site was TTC in *S. scabies* (Fig. 4A). In the present study, the 51-bp sequence of the *attB* site in *S. scabies* was determined for the first time and registered as a core region of the *attB* site for the insertion of ØC31 *attP* derived from *S. scabies* ATCC 23962 in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB831110. Also, the *attB* site sequence of *S. scabies* exhibited the highest levels of homology (92.2% nucleotide identity) with that of *S. aureofaciens* (Fig. 4B).

DISCUSSION

Transformation of streptomycetes has many difficulties because of strong restriction barriers, absence of an efficient transformation system, and inherent instability of recombinants. In this study, sufficient efficiency to enable conjugal transfer of genetic elements was provided using a bacteriophage ØC31 *att/int* system for *S. scabies*, which infects the tap roots of crops and causes scab diseases.

E. coli donor can transfer a single-stranded plasmid to recipient spore during its germination, but not in the spore state. Therefore, to facilitate the conjugal transfer of streptomycetes, spores used for conjugation was generally subjected to high heat treatments at 50°C for 10 min before being mixed with *E. coli* donor for induction of conjugal transfer[13]. The heat treatment promotes spore germination to increase efficient conjugation and may be effective in temporarily

reducing the restriction barrier[19, 20]. However, because the spores of *S. scabies* are more sensitive to temperature than other streptomycetes, their viabilities rapidly decreased with increasing heat treatment temperatures (Fig. 1). Therefore, in the case of *S. scabies*, the low temperature of 30°C was the most suitable temperature of heat treatment for conjugation.

From analysis of the phylogenetic tree centering the *attB* site of *S. coelicolor* (Fig. 4B), the transconjugation frequencies of streptomycetes strains such as *S. coelicolor* 5.0×10^{-3}, *S. acidiscabies* 1.2×10^{-3}, and *K. setae* 2.0×10^{-7} seem to be closely related to *attB* site homology[12,16,18]. However, transconjugation frequency of *S. scabies* (3.7×10^{-3}) was higher than that of *S. acidiscabies*, although its *attB* site homology was lower. Therefore, this result indicates that the efficiency of conjugal transfer can be highly dependent on the various conditions of transconjugation experiments.

CONCLUSIONS

In this study, medium, heat treatment, $MgCl_2$, mixing ratio of recipient and donor, and *attB* site affecting the efficiency of intergeneric conjugation for transformation of *S. scabies* were identified and the optimal conditions were determined. These results provide sufficient efficiency to enable conjugal transfer of genetic elements through *attB/P*-mediated site-specific integration for *S. scabies*, and also should facilitate molecular genetic studies in this strain because all pSET152-integrated exconjugants revealed phenotypes identical to those of wild-type *S. scabies* (data not shown).

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