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Construction of intergeneric conjugal transfer for molecular genetic studies of *Streptomyces mobaraensis* producing transglutaminase

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To facilitate molecular studies of *Streptomyces mobaraensis* producing transglutaminase, an effective transformation method was established via intergeneric conjugal transfer using *Escherichia coli* ET12567 harboring the ØC31-derived integration vector, pSET152. The highest frequency was attained on ISP4 medium containing 20 mM $MgCl_2$, using 2.5 × 10^8 *E. coli* as donor and spore treated with heat treatment at 35°C for 10 min as host. The *attB* integration site in the *S. mobaraensis* genome was detected as a single *attB* site within an open reading frame coding for a pirin homolog. Its sequence showed the highest degree of homology with *S. aureofaciens*.

Key words: *Streptomyces mobaraensis*, conjugal transfer, integration site, exoconjugant, transglutaminase.

INTRODUCTION

Transglutaminase (TGase, EC 2.3.2.13) catalyzes an acyl transfer reaction between the primary grade amine and protein or γ-carboxyamide group of peptide-bound glutaminyl residues (Lorand and Conrad, 1984). TGases are used in food processing material, specifically to gel protein-rich foods after the formation of cross-links, and are present in most animal tissues and body fluids. TGases have also been discovered in microorganisms including *Bacillus subtilis* (Kobayashi et al., 1996), *Candida albicans* (Ruiz-Herrera et al., 1995), *Escherichia coli* (Schmidt et al., 1998), *Physarum polycephalum* (Klein et al., 1992), and actinobacteria (Duran et al., 1998). Among them, TGases of actinobacteria have several advantages as they are extracellular enzymes, calcium-independent, and can be produced by general fermentation at low cost (Yokoyama et al., 2004). Although *Streptomyces mobaraensis* is well known producer in actinobacteria, many approaches for increasing production of TGases have been restricted to the mutation of *S. mobaraensis*, $MgCl_2$ stress, and high level expression in heterogeneous host (Date et al., 2004; Yokoyama et al., 2010; Zhang et al., 2012),

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Abbreviations: ORF, Open reading frame; ***int*,** integrase gene; **ATTPR**, *attP* right; **ATTPL**, *attP* left; **ISP,** international *streptomyces* project medium; **AS-1,** amino acid soluble starch medium; **MS,** mannitol soya flour medium.

because actinobacteria has strong restriction barriers, absence of an efficient transformation system, and the inherent instability of recombinants (MacNeil, 1988; Baltz, 1998). The protoplast technique has generally been used for actinobacteria transformation. However, it suffers from low efficiency and minimal application (Matsushima and Baltz, 1996; Voeykova et al., 1998). Therefore, there has been considerable interest in the use of intergeneric conjugation as a means of transferring single-stranded DNA (Flett et al., 1997; Stegmann et al., 2001). In the present study, to promote the molecular genetic study of *S. mobaraensis*, which is well known as a representative strain for TGases production, we established an efficient transformation procedure using intergeneric conjugation as a means of transferring single-stranded DNA (Stegmann et al., 2001).

METHODOLOGY

The method used for the intergeneric conjugation was the bacteriophage ϕC31 *att*/*int* system, integrase-mediated site-specific integration, conjugally transferring a single-stranded DNA from *E. coli* into *S. mobaraensis*. In addition, the *attB* site in *S. mobaraensis* chromosome integrated with the *attP* site of a site-specific integration vector was cloned from several exconjugants of *S. mobaraensis*, and its properties were identified through sequencing and alignment.

Conjugal transfer of *S. mobaraensis*

S. mobaraensis ATCC29032 was used as a 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 intergeneric conjugations. The site-specific integration vector, pSET152 (5.7 kb), harbors ØC31 *int*, *attP*, and *oriT* of RK2, as well as an aparamycin-resistant gene for selection in actinobacteria and *E. coli* (Bierman et al., 1992). This plasmid does not carry out the replicative functions of the actinobacteria plasmid, and can only be maintained in recipient strains in its chromosomally integrated state. The early intergeneric conjugation was carried out in accordance with the basic transconjugation protocol developed by Kieser et al. (2000). *E. coli* donor cells (0.5 mL; 2.5×10^6 - 2.5×10^8 cells) were added to the spores (1×10^4 - 1×10^6), and the mixtures were spread on MS plates containing 0 - 50 mM $MgCl_2$. For confirming the chromosomal integration of pSET152, the exconjugants were analyzed via PCR and Southern-blot hybridization.

RESULTS AND DISCUSSION

Confirmation of parameters and the *attB* site affecting efficiency of conjugal transfer

As the medium were used, it profoundly affects the successful conjugal transfer of actinobacteria, four representative media (AS-1, ISP2, ISP4, and MS) for the conjugal transfer in actinobacteria were tested to select a medium promoting the conjugal transfer of *S. mobaraensis* (Bierman et al., 1992; Flett et al., 1997; Choi et al., 2004). Of the four media, no exconjugants were obtained with AS-1 and ISP2, although AS-1 has been frequently employed in the conjugal transfer of streptomycetes and ISP2 also was the base media used for the growth and spore formation of *S. mobaraensis*. ISP4 and MS were suitable for the conjugation of *S. mobaraensis*; the transconjugation efficiencies of ISP4 and MS were 3.4×10^{-4} and 8.1×10^{-5}, respectively. ISP4 and MS could be employed for the conjugation of *S. mobaraensis*. However, ISP4 medium was selected as the most appropriate medium in the present work and was employed in all subsequent experiments due to its 4.2-fold higher conjugation efficiency than that observed in the MS medium. Recipient spores employed for conjugation are often subjected to heat treatments before being mixed with *E. coli* donor cells for conjugal transfer in actinobacteria (Kieser et al., 2000), because the heat treatment promotes spore germination to increase efficient conjugation (Mazodier et al., 1989) and may be effective in temporarily reducing the restriction barrier (Bailey and Winstanley, 1986). To determine the appropriate temperature for spore germination, the heat tolerance of *S. mobaraensis* spores was first examined in a temperature range between 25 (control) to 60°C. As shown in Figure 1, although the spores incubated at 55°C for 10 min quickly lost viability, viability of spore was the highest by the heat treatment at 45°C as it increased spore germination, and was maintained over 100% until 50°C, appeared to be more tolerance to temperature than other actinobacteria. Based on this result, the heat treatment of spores for conjugal transfer was performed below 50°C. The transconjugation frequency was increased by the heat treatment of spores until 45°C compared with control (Figure 1). Specifically, because heat treatment at 35°C gave the highest conjugation efficiency, it was selected as the most suitable temperature for the heat treatment of spores. Since $MgCl_2$ added to the medium for conjugal transfer affects the conjugation efficiency (Kieser et al., 2000) and its optimal concentration differs with strains (Choi et al., 2004), the optimal concentration of $MgCl_2$ added to the ISP4 medium used for conjugation of *S. mobaraensis* was surveyed. When 0 ~ 50 mM $MgCl_2$ was added to ISP4 medium, 20 mM yielded the highest efficiency for *S. mobaraensis*, and its conjugation efficiency was 3.5-fold higher than that of 10 mM, although all concentration of added $MgCl_2$ increased the transconjugation frequency (data not shown). The mixing ratio of the number of recipient spores and *E. coli* donor cells is important in actinobacteria conjugation (Choi et al., 2004). In the conjugal transfer of *Kitasatospora setae*, a standard number of *E. coli* donor cells (2.5×10^7) yielded no exconjugants, but transconjugation was possible with a further increase in the number of *E. coli* donor cells ($\geq 1.25 \times 10^8$) (Choi et al., 2004). As shown in Table 1,

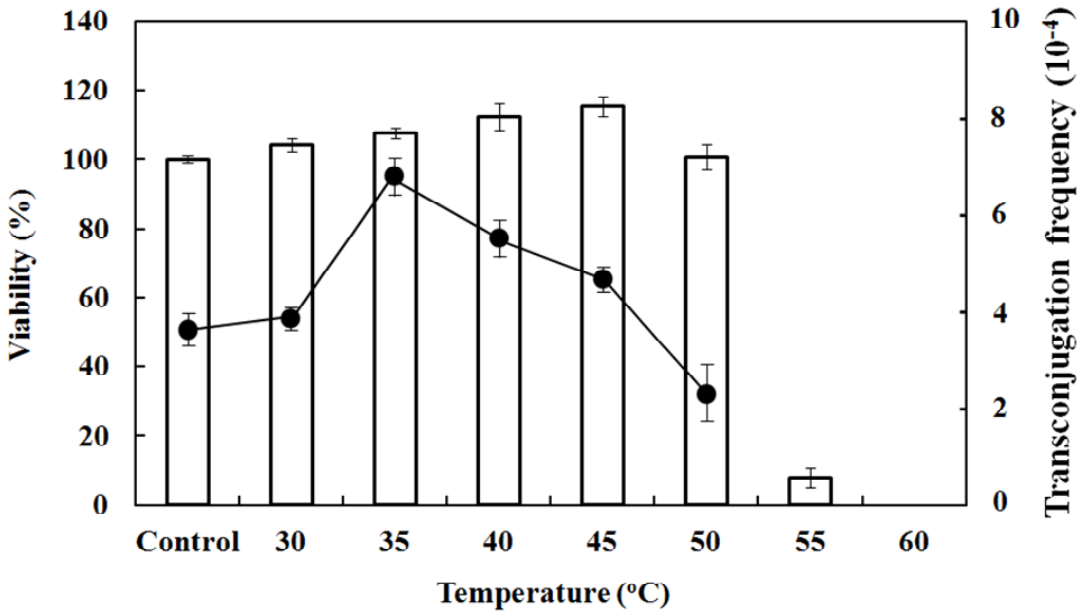

Figure 1. Effects of temperature on the viability of *S. mobaraensis* spores (bar graph) and the heat treatment of spores on transconjugation efficiency (line graph). For measuring spore viability after the heat treatment of spores, spores (1×10^3) that were incubated for 10 min at the temperatures indicated were resuspended with 0.5 mL of 2 × YT broth (1% yeast extract, 1.6% tryptone, 0.5% NaCl) at room temperature (25°C). Viability was determined by counting colonies on ISP2 medium after 12 days of incubation at 28°C. Control means no heat treatment (25°C). For measuring transconjugation efficiency after the heat treatment of spores, 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 aparamycin, 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. *E. coli* (2.5×10^7) were added to the resuspended spores, and the mixtures were spread on ISP4 plates containing 10 mM $MgCl_2$. 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 aparamycin. The plates were subsequently incubated for 12 day at 28°C. The results represent SD of three experiments (n=3 per experiment).

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]		
	2.5×10^6 *E. coli*	**2.5×10^7 *E. coli***	**2.5×10^8 *E. coli***
1×10^4	0	4.5×10^{-4}	1.3×10^{-2}
1×10^5	1.1×10^{-4}	3.5×10^{-4}	2.2×10^{-3}
1×10^6	1.3×10^{-4}	3.9×10^{-4}	7.1×10^{-4}

[a]Data are the average of three independent experiments.

with 1×10^4 recipient spores of *S. mobaraensis*, 2.5×10^6 *E. coli* donor cells yielded no exconjugants, but the increase in the number of *E. coli* donor cells ($\geq 2.5 \times 10^7$) made conjugal transfer of *S. mobaraensis* possible. The increase of the number of recipient spores without increase of *E. coli* donor cells did not affect the transconjugation frequency. Therefore, this suggests that when increased numbers of recipient spores are used and no exconjugants are present, the number of *E. coli* donor cells must be increased for transconjugation efficiency.

Conjugal transfer of actinobacteria was carried out by integration of vector (pSET152) having an *attP* site into the *attB* locus in the recipient chromosome via the integrase (*int*) function. All of the *attB* sites of actinobacteria lay within an open reading frame (ORF) coding for pirin (a newly identified nuclear protein that interacts with Bcl-3 and nuclear factor I) (Choi et al., 2004). However, some strains have another pseudo-*attB* site or no *attB* site in their genome (Combes et al., 2002). These cause potential problems such as mutagenesis, inducing phenotypic changes, or no transconjugant

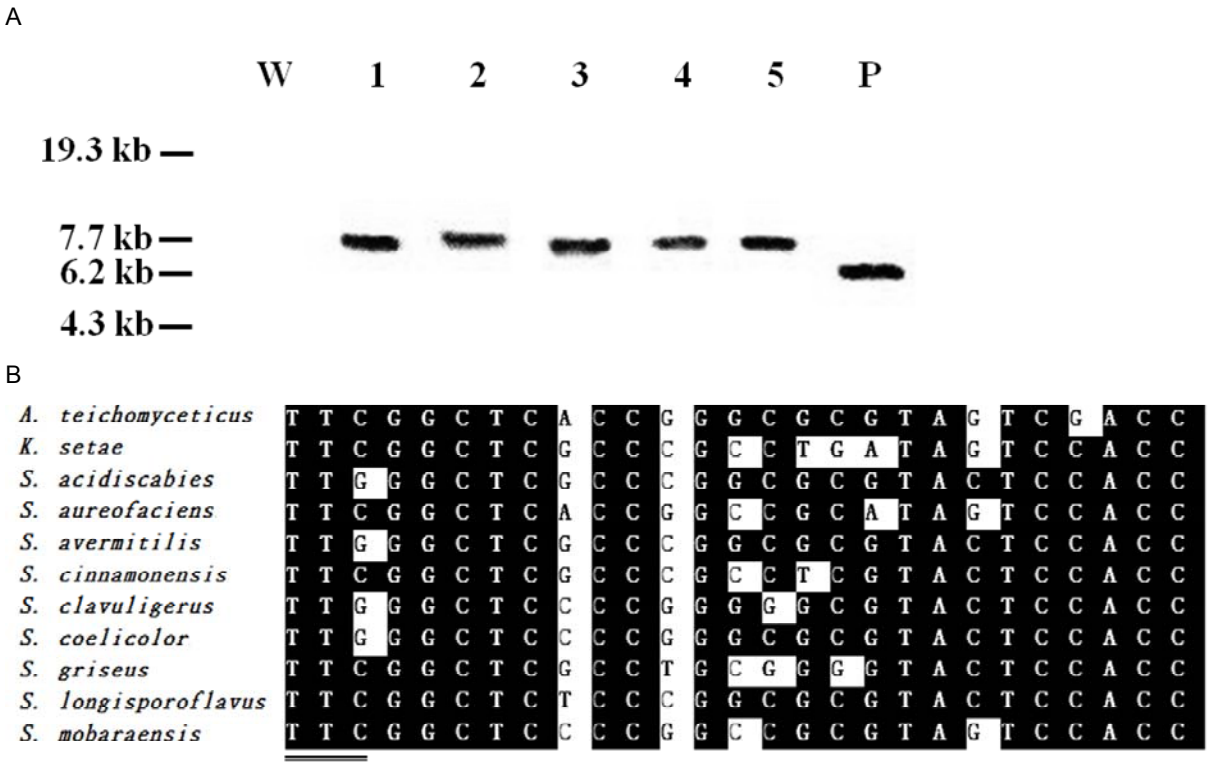

Figure 2. Southern blot analysis of *Kpn*I-digested genomic DNAs of exconjugants (a) and Alignment of the *attB* site sequences of *S. mobaraensis* and other actinomycetes (b). (a) Lanes: lane W, wild-type *S. mobaraensis*; lanes 1 ~ 5, exconjugants containing pSET152; lane P, plasmid pSET152. Genomic DNA from the wild-type strain and exconjugants were digested with *Kpn*I. pSET152 was digested with *Bam*HI (lane P). The DNA was blotted onto a nylon membrane and hybridized with a DIG-labeled 0.5-kb aparamycin-resistant fragment of pSET152. (b) *Actinoplanes teichomyceticus* NBRC13999, *K. setae* NBRC14216, *S. acidiscabies* ATCC49003, *S. aureofaciens*, *S. avermitilis* MA-4680, *S. cinnamonensis*, *S. clavuligerus*, *S. coelicolor* A3(2) M145, *S. griseus* ATCC12475, *S. longisporoflavus* 83E6, *S. mobaraensis* ATCC29032 (present study). Under double line represents the core sequence of *attB* site integrated with the *attP* site.

(Gregory et al., 2003). To identify the *attB* site of *S. mobaraensis*, genomic DNA of exconjugants were prepared, digested by *Kpn*I that was not included in pSET152, and then confirmed by Southern hybridization using a 0.5 kb aparamycin resistance fragment of pSET152 as a probe. As shown in Figure 2a, all of the exconjugants showed an equal single band pattern with Southern hybridization, suggesting that the *attB* site integrated with the *attP* site of pSET152 is a unique site in the *S. mobaraensis* chromosome. Genomic DNA fragments bearing the integration site were rescued as plasmids from pSET152-integrated exconjugants following *Kpn*I digestion by self-ligation and transformation into *E. coli*. Plasmid sequencing using the primers ATTPR (5'-CTGGGTGGGTTACACGACGCCCCT-3') and ATTPL(5'-CGTTGGCGCTACGCTGTGTCGCTG-3') revealed that all of the plasmids harbored a right-flanking region of the insertion site in their genomes, and the same insertion endpoints within the ORF coding for pirin (data not shown). Presently, the sequence of the right-flanking region of the *attB* site in *S. mobaraensis* 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. mobaraensis* ATCC49003 in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB819044. However, although no clear data are available regarding the reason, the left-flanking region of the *attB* site in *S. mobaraensis* genomes was not detected in this study, unlike the case of other actinobacteria, despite high transconjugation efficiency. The core sequence (TTS) of *attB* site integrated with the *attP* site was found to be TTC in *S. mobaraensis* (Figure 2b). Also, the right-flanking region of the *attB* site of *S. mobaraensis* exhibited the highest levels of homology (92.6% nucleotide identity) with that of *S. aureofaciens*. The observations recorded in this investigation provide sufficient efficiency to enable conjugal transfer of genetic elements through *attB/P*-mediated site-specific integration for *S. mobaraensis*, 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).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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