REPLACEMENT OF *STREPTOMYCES HYGROSCOPICUS* GENOMIC SEGMENTS WITH *IN VITRO* ALTERED DNA SEQUENCES

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We have developed a method for gene replacement in *Streptomyces hygroscopicus* which permits introduction of an *in vitro* derived mutation carried on a plasmid into the chromosome. We constructed the plasmid pMSB212 which can replicate in *S. hygroscopicus* and contains the step5 gene of the bialaphos biosynthetic pathway which was inactivated by a frame-shift mutation caused by filling in the cohesive ends of the *Eco*R I site in the structural gene. pMSB212 was introduced into a bialaphos producer strain and by protoplast regeneration of the primary thiostrepton-resistant transformants, non-producing mutants, were obtained. Biochemical and genetical analyses indicated that these mutants were specifically blocked by introduction of the frame-shift mutation in the step5 gene on the chromosome. This method will enable us to obtain isogenic mutants of known genes and to identify new genes encoded on a cloned fragment.

Streptomycetes are very important microorganisms which produce many antibiotics and enzymes of commercial value. Recent developments in *Streptomyces* gene cloning have resulted in cloning of the resistance, biosynthetic, and regulatory genes of antibiotics, and elucidation of *Streptomyces* gene organization and regulation[1~7].

Mutant analysis gives us much information about gene function and regulation. Mutants usually have been obtained by using chemical or physical mutagens, but there have been reports recently about another mutagenic approach, gene replacement techniques in *Escherichia coli*[8], *Bacillus subtilis*[9~12] or *Saccharomyces cerevisiae*[13,14].

It is desirable to devise a method for generating specific mutations in the chromosomes of streptomycetes. CHATER and BRUTON have developed the mutational cloning method using derivatives of temperate phage and applied it to *Streptomyces coelicolor* and *Streptomyces lividans*[1].

In this paper we describe a method that allows the introduction of an *in vitro* derived mutation carried on the plasmid into the chromosome of *Streptomyces hygroscopicus*. This approach is based on our finding that recombination between the host chromosome and a plasmid containing a homologous region to it occurred in *S. hygroscopicus*[6,7], which produces a tripeptide antibiotic, bialaphos[15].

Materials and Methods

Bacterial Strains and Plasmids

S. lividans 66 was kindly supplied by Dr. N. D. LOMOVSKAYA and Dr. K. F. CHATER. *S. hygroscopicus* strains are listed in Table 1. Plasmid pMSB72[6] has a 670 bp *Sph* I-*Bgl* II fragment containing a portion of the step5 gene in pIJ702.

DNA Manipulation and Transformation

Chromosomal DNA was isolated as described by SMITH in HOPWOOD *et al.*[16]. Plasmid preparations from streptomycetes were carried out as described by MURAKAMI *et al.*[17]. Restriction enzymes and T4 DNA-Ligase (Takara Shuzo Co., Ltd., or Toyobo Co., Ltd.), and calf intestinal alkaline phosphatase were used according to the recommendations of the suppliers. DNA polymerase I (Klenow fragment, Takara Shuzo Co., Ltd.) was used to fill in the site of *Eco*R I according to the protocol of MANIATIS *et al.*[18]. Gel electrophoresis of DNA was done according to HOPWOOD *et al.*[16]. Transformation of *S. lividans* protoplasts was carried out by the method of THOMPSON *et al.*[19]. *S. hygroscopicus* was transformed according to MURAKAMI *et al.*[6].

Table 1. Strains of *Streptomyces hygroscopicus*.

Designation	Defective step	Source
NP8	Step13	IMAI *et al.*[25]
NP44	Step6	IMAI (unpublished)
NP45	Step12	IMAI *et al.*[25]
NP46	Step3	IMAI *et al.*[26]
NP47	Step1	IMAI *et al.*[26]
NP213	Step5	SETO *et al.*[27]
NP221	Step4	IMAI *et al.*[26]
HP5-29	None	ANZAI *et al.*[7]

Protoplast Regeneration and Curing of Plasmids

Protoplast regeneration of *S. hygroscopicus* was carried out in the same manner as its transformation, but in the absence of thiostrepton. Thiostrepton-sensitive clones were scored on Nutrient agar (Difco) containing 20 μg/ml of thiostrepton.

Bialaphos Productivity and Co-synthesis Tests

The production of bialaphos was detected by a conventional agar plug assay. Agar plugs of A4 medium[20] were inoculated and incubated for 5 days at 28°C. The plugs were then placed on the surface of the bialaphos assay agar medium of OGAWA *et al.*[20] seeded with lawns of the bialaphos-sensitive indicator bacterium, *Bacillus subtilis* ATCC 6633. For the co-synthesis tests, mycelial suspensions of two non-producing mutants were spread about 2 mm apart on opposite halves of a Petri dish containing A4 medium. After 5 days of growth at 28°C, an agar strip was removed from the center of the plate and placed on a bialaphos assay plate.

Preparation of Cell Extracts

Cultures of *S. hygroscopicus* were grown at 28°C for 4 days in 30 ml of liquid production medium[7]. Mycelium was collected by centrifugation and then washed twice in cold TM buffer (50 mM Tris-HCl, 5 μM 2-mercaptoethanol, pH 7.0). Washed mycelium was suspended in 20 ml of TM buffer and disrupted by sonication. Cell debris was removed by centrifugation (17,000$\times g$, 20 minutes, 4°C).

SDS-polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was carried out according to LAEMMLI[21]. Samples were denatured at 100°C for 5 minutes in the presence of 1% SDS and 10% 2-mercaptoethanol before loading on a 12.5%-polyacrylamide gel. We used the SDS-PAGE Standard [Low] (Bio-Rad) as protein size markers.

Southern Hybridization

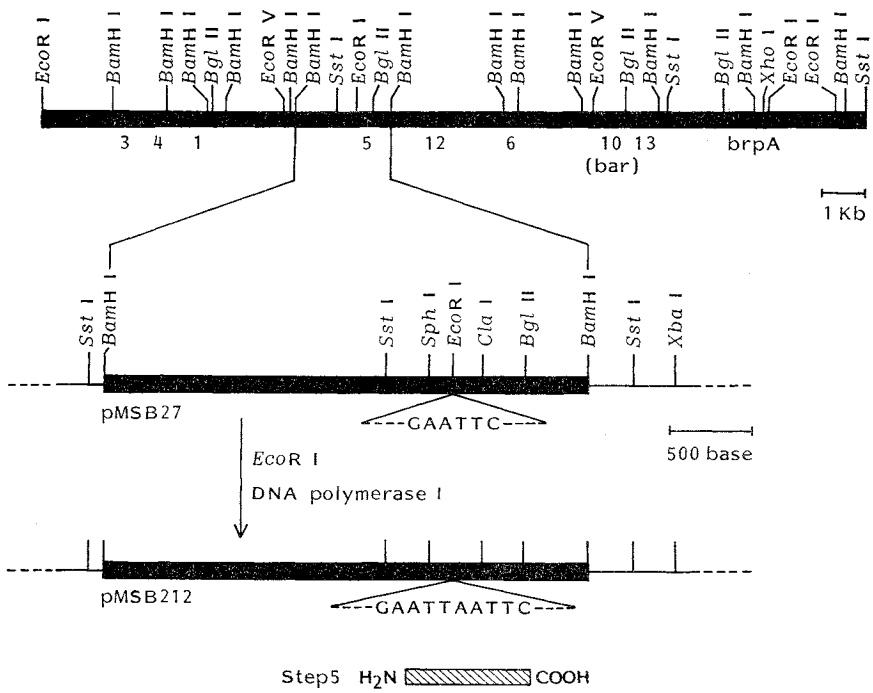
Transfer of DNA from agarose gels to nylon membranes (Hybond-N, Amersham, Ltd.) were carried out by the method of SOUTHERN[22] following the recommendation of the suppliers. DNA probes were labeled by the Nick Translation Kit (Amersham, Ltd.). Pre-hybridization and hybridization were done as described in HOPWOOD *et al.*[16].

Results

Construction of Plasmids Carrying the Inactivated Step5 Gene

pMSB27 (Fig. 1) contains the entire step5 gene of the bialaphos biosynthetic pathway, and has a unique *Eco*R I site located within this gene. pMSB27 DNA was digested with *Eco*R I and the cohesive ends were filled in by treating it with DNA polymerase I (Klenow fragment). The ligated plasmid DNA was digested with *Eco*R I again and used to transform *S. lividans*. Thiostrepton-

Fig. 1. Construction of plasmids carrying the inactivated step5 gene.



Above map indicates the bialaphos biosynthetic gene cluster. Numbers under the map represent steps of the biosynthetic pathway.

bar: Bialaphos-resistant gene, brpA: bialaphos regulatory gene. pMSB27 was constructed by the insertion of a 3.0-kb *Bam*H I fragment into the *Bam*H I site of pIJ680. Thick lines, *Streptomyces hygroscopicus* sequences; thin lines, vector sequences.

Table 2. Isolation of non-producing mutants from HP5-29 containig pMSB212 and pMSB27.

	Producer		Non-producer		Total
	Thio^r	Thio^s	Thio^r	Thio^s	
pMSB212	181	14	4	1	200
pMSB27	153	47	0	0	200

Thio: Thiostrepton, ^{r}: resistant, ^{s}: sensitive.

resistant transformants were selected. Most of the transformants contained the same plasmid, pMSB212, lacking the *Eco*R I site (Fig. 1). The digestion pattern of pMSB212 with other restriction endonucleases was the same as pMSB27.

Isolation of Mutants Blocked at Step5

pMSB212 and pMSB27 were introduced into *S. hygroscopicus* HP5-29 and thiostrepton-resistant clones were selected. Protoplasts prepared from these clones were spread on regeneration plates without thiostrepton. Two hundred colonies from each regenerated clone were picked onto Nutrient agar without thiostrepton and replicated to Nutrient agar containing thiostrepton, and to agar plugs containing A4 medium to measure thiostrepton sensitivity and bialaphos productivity. Five bialaphos non-producing mutants from HP5-29 containing pMSB212 were obtained but none from the transformants containing pMSB27 (Table 2). Four out of 5 non-producing mutants still contained the

Table 3. Co-synthesis test of the generated mutants.

Mutant	NP47 (1)[a]	NP46 (3)	NP221 (4)	NP213 (5)	NP44 (6)	NP45 (12)	NP8 (13)
VM1	+	+	+	−	+	+	+
VM2-1	+	+	+	−	+	+	+
VM2-2	+	+	+	−	+	+	+
VM2-3	+	+	+	−	+	+	+
VM2-4	+	+	+	−	+	+	+
NP213	+	+	+	−	+	+	+

[a] Number in parenthesis shows blocked step.
+, −: Bialaphos production.

plasmid, pMSB212. We designated the thiostrepton-sensitive non-producer as VM1 and the thiostrepton-resistant ones as VM2-1, 2-2, 2-3 and 2-4.

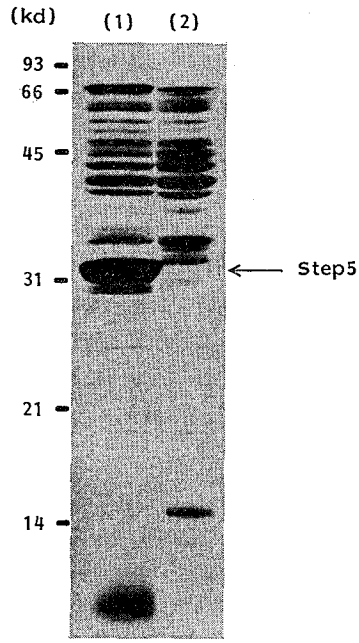
Co-synthesis Test of the Generated Mutants

To determine which step was defective in VM1, 2-1, 2-2, 2-3, and 2-4, a co-synthesis test was done. All mutants could convert intermediates secreted by NP44, NP45, and NP8, which are blocked at step6, 12 and 13, respectively (Table 3). Futhermore, these mutants secreted intermediates which NP47, NP46 and NP221 could convert to bialaphos. However, NP213, which is blocked at step5, was not able to produce bialaphos in a co-synthesis test with these mutants.

Analysis of Intracellular Protein

SDS-PAGE analysis of intracellular proteins showed that a 32K-dalton protein, which is the product of the step5 gene, could not be detected in mutant VM1. The amounts of other proteins were the same as those of the parent strain HP5-29 (Fig. 2). Demethylphosphinothricin (DMPT) acetyltransferase activity, which is the product of step10, was about the same in strains VM1 and HP5-29 (0.155 and 0.170 U/mg protein, respectively).

Fig. 2. SDS-PAGE profiles of VM1 and its parent HP5-29 during production phase. (1) HP5-29, (2) VM1.



Numbers at the left of the figure are the MW of size markers.

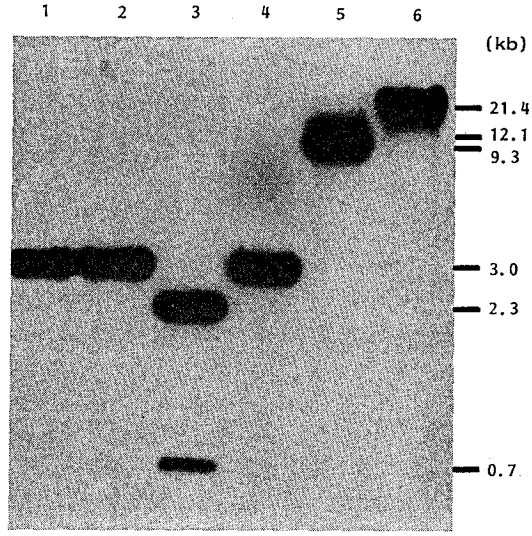
Southern Hybridization

Southern analysis was used to confirm the genotype of VM1. Chromosomal DNAs, obtained from VM1 and its parent HP5-29, was digested with *Bam*H I, *Eco*R I and both *Bam*H I and *Eco*R I, then electrophoresed on agarose gels, and transferred to nylon membranes. The hybridization pattern of these DNAs, when probed with ^{32}P-labeled pMSB27, is shown in Fig. 3. The hybridization data in lane 4 compared with lane 3 confirm the restriction pattern expected for replacement of the

Table 4. Recovery of bialaphos productivity in VM1.

Strain	Relative productivity (%)
HP5-29/none	100
VM1/none	0
VM1/pMSB27	90
VM1/pMSB72	0.5
VM1/pMSB212	0

Fig. 3. Southern hybridization of ^{32}P-labeled pMSB27 DNA to HP5-29 (lanes 1, 3 and 5) and VM1 (lanes 2, 4 and 6) chromosomal DNAs digested with *Bam*H I (lanes 1 and 2), *Eco*R I (lanes 5 and 6) and both *Bam*H I and *Eco*R I (lanes 3 and 4).



normal DNA fragment containing the step5 gene with one lacking the internal *Eco*R I site (Fig. 1). There are two bands, 0.7 kb and 2.3 kb in size, in lane 3 but only one band, 3.0 kb in size, in lane 4. This observation suggests the lack of the *Eco*R I site in the step5 gene locus within the 3.0-kb *Bam*H I fragment on the VM1 chromosome.

Restoration of Bialaphos Production by Re-transformation

VM1 has already been cured of the plasmid pMSB212. Therefore we re-transformed VM1 with pMSB27, pMSB212 and pMSB72 which contained the wild-type, mutant-type and a portion of step5 gene, respectively. As shown in Table 4, pMSB27 and pMSB72 could restore the bialaphos productivity in VM1 but pMSB212 could not. VM1 containing pMSB72 produced a low level of bialaphos compared with the parent strain HP5-29, because this restoration might be caused by recombination between the plasmid and the chromosome[6].

Discussion

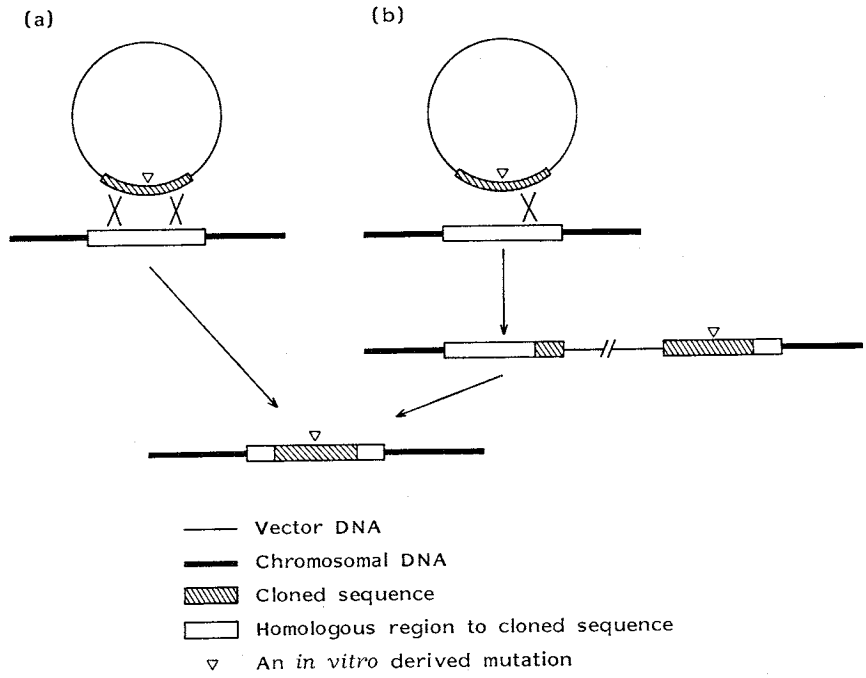
In this report, we have described a method for replacing a specific chromosomal locus in *S. hygroscopicus* with altered DNA sequences constructed *in vitro*. Other examples of gene replacement in the *Streptomyces* have been reported: i) Recombination between the mutant chromosome and the homologous region of cloned *act* gene cluster in *S. coelicolor*[2], ii) the gene homogenetization of *bald* genes in *S. coelicolor*[23].

We assume that the replacement of the genomic segments with altered DNA sequences on the plasmid depended on *in vivo* homologous recombination. Thus the location of the base substitution mutation and the size of a deletion or an insertion within the cloned fragment should be important. Since it is presumed that the frequency of homologous recombination between a plasmid and chromosome increases with the size of homologous regions on both sides of a mutation, large insertions or deletions will decrease the efficiency of gene replacements.

In the case of the step5 gene, we could not obtain non-producing mutants directly by the transformation of a bialaphos producer strain with pMSB212. By protoplast regeneration of a producer strain containing pMSB212, we were able to isolate the desired mutants, and to cure the strain of the resident plasmid at a frequency of *ca.* 7%. However, if the mutant obtained still possesses a plasmid, it is possible to cure it by repeated protoplast regeneration (Y. KUMADA *et al.*, and O. HARA *et al.*; in preparation). Therefore the protoplast regeneration procedure was indispensable to both single colony isolation of mutants and plasmid curing.

Co-synthesis tests showed that the non-producing mutants, VM1 and others, isolated by our gene

Fig. 4. Putative pathways of gene replacement in *Streptomyces hygroscopicus*.



(a) A double crossover replacement of the wild-type gene with an altered sequence occurs.
(b) A single crossover between a plasmid and a chromosome occurs and follows by a loss of the plasmid sequence. Resulting that an altered sequence remains on the chromosome.

replacement method and NP213, obtained by the conventional mutation method, are blocked at step5 of the bialaphos biosynthetic pathway and are isogenic. Moreover VM1 accumulated phosphinoformic acid and could convert phosphinomethylmalic acid to bialaphos like NP213 (data not shown).

We have already reported that the product of the step5 gene is a 32K-dalton protein and is produced in a large amount by a producer strain[7]. SDS-PAGE analysis indicated the loss of a 32K-dalton protein mutant in VM1, as in NP213[7], and the restoration of its lesion by introduction of plasmid pMSB27 containing all of the step5 gene. Therefore, VM1 is a step5 blocked mutant like NP213.

Southern hybridization analysis showed that VM1 lacks the *Eco*R I site in the step5 structual gene on the chromosome. Moreover pMSB212 could not restore the bialaphos productivity of VM1, although both pMSB27 and pMSB72 containing an entire or a portion of this gene, respectively, could do so. These observations indicate that the mutation on the chromosome of VM1 is located at an *Eco*R I site as expected.

The pathway for replacement of a chromosomal segment with an altered sequence in *E. coli*, *B. subtilis* and *S. cerevisiae* has been elucidated[9~14]. Since we used a replicating vector in *S. hygroscopicus*, pIJ680, instead of a suicide vector, it is thought that the mechanism of replacement in this strain differed from that of *E. coli*[8] and *S. cerevisiae*[13,14].

We assume that there are two possibilities for the mechanism of the gene replacement in *S. hygroscopicus* as shown in Fig. 4. One is by a double crossover and another is by a single crossover leading to integration and a subsequent excision. Because both systems result in the same product, we must perform further experiments to determine which mechanism is operating in *S. hygroscopicus*.

This gene replacement method should be applied to other *Streptomyces* species and would be useful for the following purposes: i) The isolation of an isogenic mutant of a known gene already defined; for example, a blocked mutant of an antibiotic or an auxotroph mutant, ii) the improvement of a host-vector system by the inactivation of unfavorable genes; for example, protease or restriction

enzyme genes, iii) the identification of an unknown gene located on the cloned DNA fragment by the loss of an identifiable function. We emphasize the possible application to the last purpose. Gene cloning experiments have revealed that the genes which code for the resistance to and biosynthesis of antibiotics are closely linked[1,4,6,24], and moreover that regulatory genes can be located in the gene cluster[1,4,7]. Thus this new technique should enable one to elucidate whether unknown biosynthetic or regulatory genes of antibiotics are located close to the production or resistance genes which have already been cloned. For example, some new genes of the bialaphos biosynthetic pathway encoded on a cloned DNA fragment have been identified recently using this method (Y. KUMADA *et al.*, and O. HARA *et al.*; in preparation).

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