THE BIALAPHOS BIOSYNTHETIC GENES OF *STREPTOMYCES HYGROSCOPICUS*: CLONING AND ANALYSIS OF THE GENES INVOLVED IN THE ALANYLATION STEP

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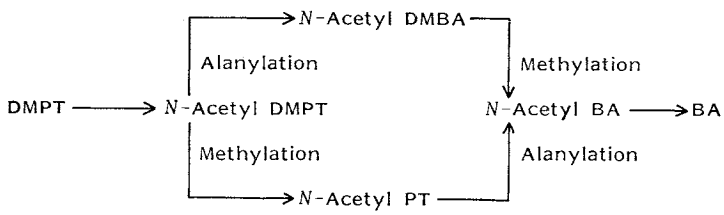
We have isolated and studied the genes involved in the alanylation step in the biosynthesis of a herbicide, bialaphos which is produced by *Streptomyces hygroscopicus*. Three bialaphos-nonproducing mutants, NP60, NP61 and NP62, isolated from *S. hygroscopicus* by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were defective for the alanylation step and were not restored to productivity by any locus of the gene cluster previously cloned. Three plasmids were isolated using NP60, NP61 and NP62 as recipients. The genes which restored productivity to NP61 and NP62 hybridized to the contiguous region of the bialaphos biosynthetic gene cluster. The gene cluster involved in the bialaphos production was about 35 kb long. The gene which restored productivity to NP60 did not hybridize to the bialaphos biosynthetic gene cluster. VM3 and VM4, putative alanylation blocked mutants, were derived from a bialaphos producer by gene replacement of an unidentified region of the biosynthetic gene cluster with an *in vitro* altered DNA sequence. The genes which restored productivity to VM3 and VM4 were located between the genes which code for phosphinomethylmalic acid synthase and demethylphosphinothricin acetyltransferase in the cluster. These results suggest that multiple genes are involved in the alanylation step.

Bialaphos is a tripeptide herbicide consisting of two L-alanine molecules and phosphinothricin which is produced by *Streptomyces hygroscopicus*[1,2]. The biosynthetic pathway of bialaphos has been determined by the analysis of products that are accumulated and converted to bialaphos by a series of nonproducing mutants[3~9]. Although it has been thought that the bialaphos biosynthetic pathway consists of at least 13 steps, the mechanism of the linkage of a phosphinothricin and two L-alanine molecules is not clear. IMAI *et al.*[8] proposed two routes of biosynthetic sequence from demethylphosphinothricin to bialaphos shown in Fig. 1. As previously reported[10,11], the bialaphos production genes and its regulatory gene were cloned, but the gene(s) involved in the alanylation

Fig. 1. Proposed biosynthetic pathway from demethylphosphinothricin to bialaphos.
DMPT: Demethylphosphinothricin, DMBA: demethylbialaphos, PT: phosphinothricin, BA: bialaphos.



step(s) was not well elucidated.

In the course of genetic studies on bialaphos production by *S. hygroscopicus*, we obtained five alanylation related mutants by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) or by introducing the *in vitro* derived mutation of an unidentified region of the biosynthetic gene cluster into the chromosome. Cloning experiments revealed that at least five genes related to alanylation lie in or out of the bialaphos biosynthetic gene cluster. In this paper we describe the cloning and characterization of the genes involved in the alanylation step(s).

Materials and Methods

Bacterial Strains and Plasmids

S. hygroscopicus strains were obtained from the Meiji Seika Kaisha Culture Collection. *Streptomyces lividans* 66 was supplied by N. D. LOMOVSKAYA and K. F. CHATER and used as recipient for the construction of plasmids. Plasmids pIJ702[12] and pIJ680[13] were obtained from the John Innes Culture Collection. Cosmid pHC79[14] and host LE392[15] were supplied by B. HOHN. Plasmids pMSB2-4 and pMSB12, and cosmid pMSB13-3 which contain the bialaphos biosynthetic genes have been described[10]. Plasmid pMSB35[11] contains the regulatory gene (*brpA*) of the bialaphos production.

DNA Manipulation and Transformation

Chromosomal DNA was isolated as described by SMITH in HOPWOOD *et al.*[13]. Plasmid preparation was carried out as described by MURAKAMI *et al.*[16]. Introduction of an *in vitro* derived mutation on the plasmid into the chromosome of *S. hygroscopicus* was carried out as described by ANZAI *et al.*[17]. Restriction endonucleases and T4 ligase (Takara Shuzo Co., Ltd. or Toyobo, Ltd.), and calf intestinal alkaline phosphatase (Boehringer-Manheim) were used according to the recommendations of suppliers. DNA polymerase I (Klenow fragment, Takara Shuzo Co., Ltd.) was used to fill up the site of *Bam*H I according to the protocol of MANIATIS *et al.*[18]. S1 nuclease (Takara Shuzo Co., Ltd.) was used to remove single-stranded tails from DNA fragments to produce blunt ends according to the recommendations of suppliers. Gel electrophoretic analysis of DNA was done according to HOPWOOD *et al.*[13]. 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.*[10].

Preparation of Genomic Libraries

The preparation of genomic libraries using streptomycete plasmids was described by THOMPSON *et al.*[19]. Genomic libraries of DNA from HP662 were made using cosmid pHC79, according to the protocol of MANIATIS *et al.*[18]. DNA which was partially digested with *Sau*3AI was ligated to *Bam*H I- and phosphatase-treated cosmid, packaged *in vitro*, and used to infect *Escherichia coli*. Transformants which contained bialaphos production genes were identified by colony hybridization.

Protoplast Regeneration and Curing of Plasmids

Protoplast regeneration of *S. hygroscopicus* was carried out in the same manner as the transformation, but in the absence of thiostrepton. For the curing experiments, strains containing a plasmid were incubated in S medium[20] at 33°C instead of 28°C. After protoplast preparation and regeneration, thiostrepton-sensitive colonies were selected on Nutrient agar (Difco) plate containing 20 μg/ml thiostrepton.

Bialaphos Productivity and Cosynthesis Tests

The production of bialaphos by transformants of nonproducing mutants was detected using a conventional agar plug assay. Agar plugs of A4 medium[21] were inoculated and incubated for 5 days at 28°C. The plugs were then placed on the surface of bialaphos assay agar medium[11] seeded with lawns of the bialaphos-sensitive indicator bacterium, *Bacillus subtilis* ATCC 6633. Bialaphos production was indicated by a zone of growth inhibition. Cosynthesis tests were carried out in the same manner as the assay of transformants, except that two nonproducing mutants were inoculated on one agar plug.

Analysis of Intermediates Accumulated and Converted to Bialaphos by Bialaphos-nonproducing Mutants

Cultures were grown at 28°C for 5 days in 30 ml of liquid production medium[11]. Broth filtrates of bialaphos-nonproducing mutants were analyzed by HPLC. For the conversion experiments, a nonproducing mutant was cultured in the presence of an intermediate of bialaphos biosynthesis.

Southern Hybridization

Transfer of DNA from agarose gels to nylon membranes (Hybond-N, Amersham, Ltd.) was done by the method of SOUTHERN[22] and the recommendation of the suppliers. DNA probes were labeled with the Multiprime DNA labeling system (Amersham, Ltd.). Prehybridization and hybridization were done as described in HOPWOOD *et al.*[13].

Results

Characterization of Five Bialaphos-nonproducing Mutants

Bialaphos-nonproducing mutants NP60, NP61 and NP62 were isolated from bialaphos producer strains, HP644, HP662 and HP13-1, respectively after treatment with NTG. Bialaphos-nonproducing mutants, VM3 and VM4 were obtained from a bialaphos producer, HP782, by introducing the *in vitro* derived mutation of an unidentified region of the bialaphos biosynthetic gene cluster into the chromosome as described below. These high producer strains (HP644, HP662, HP13-1 and HP782) were derived from *S. hygroscopicus* ATCC 21705 by successive treatments with UV light and chemical mutagens, and are used for industrial production of bialaphos.

In order to characterize the five mutants, analysis of products that were accumulated and converted by these mutants was carried out and the results are shown in Table 1. All mutants accumulated *N*-acetyl demethylphosphinothricin (*N*-acetyl DMPT). NP61 and NP62 also accumulated a small amount of *N*-acetyl phosphinothricin (*N*-acetyl PT). Purification of *N*-acetyl PT was carried out from filtered broth of NP61 and the purified material was identical with chemically prepared *N*-acetyl PT (S. IMAI; unpublished data). *N*-Acetyl DMPT and *N*-acetyl PT are intermediates in bialaphos biosynthesis. Moreover, all of the mutants converted *N*-acetyl bialaphos to bialaphos, but did not convert *N*-acetyl PT and *N*-acetyl DMBA to bialaphos. These five mutants did not produce bialaphos by cosynthesis on agar plug in any combination.

The biosynthetic step of bialaphos following the formation of *N*-acetyl DMPT has been proposed as shown in Fig. 1[8]. Our results indicate that these mutants have defects in the alanylation step in any route of bialaphos biosynthesis.

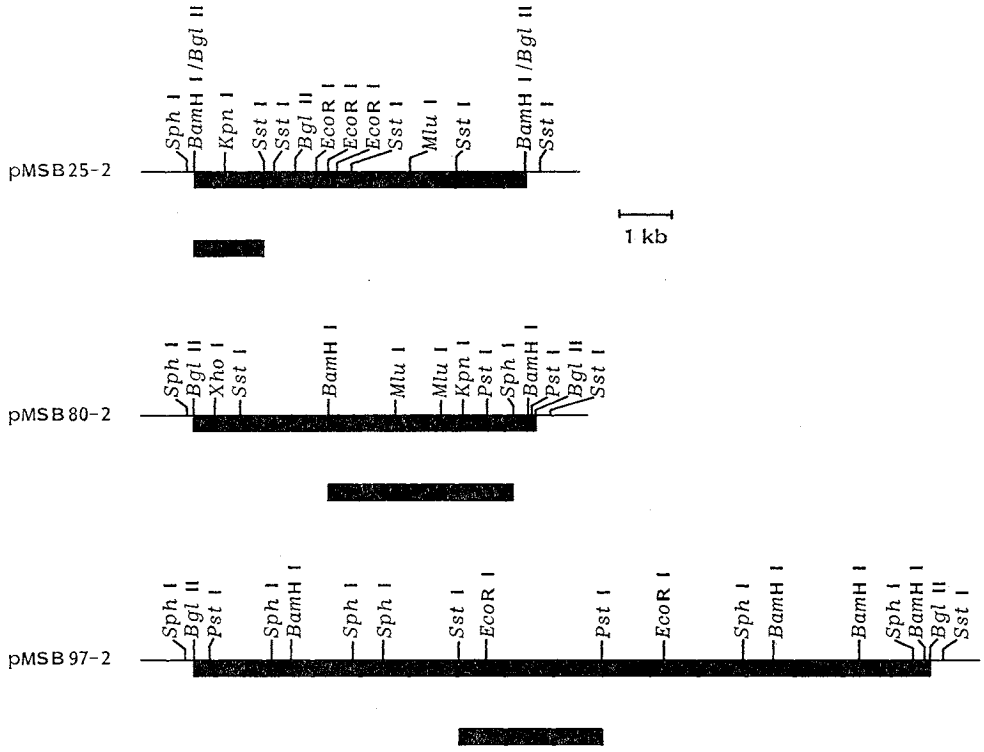
Table 1. Accumulation and conversion of *N*-acetylated form intermediates by bialaphos-nonproducing mutants.

Mutants	Accumulation		Conversion of bialaphos			
	N-Acetyl DMPT	*N*-Acetyl PT	*N*-Acetyl DMPT	*N*-Acetyl PT	*N*-Acetyl DMBA	*N*-Acetyl BA
NP60	+	−	−	−	−	+
NP61	+	+	−	−	−	+
NP62	+	+	−	−	−	+
VM3	+	−	−	−	−	+
VM4	+	−	−	−	−	+

+: Detected, −: not detected.

Fig. 2. Restriction fragments which restored productivity to NP60, NP61 and NP62.

pMSB25-2, pMSB80-2 and pMSB97-2 restored productivity to NP60, NP61 and NP62, respectively. Solid bars below the physical maps show the minimum locus which can restore productivity to the nonproducing mutants.



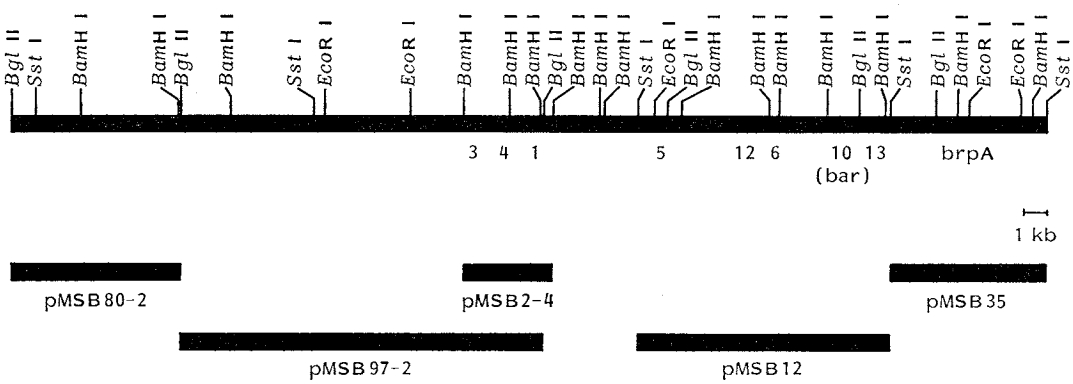
Cloning the Genes Which Restore Productivity to NP60, NP61 and NP62

NP60, NP61 and NP62 could not be restored to productivity by any of the bialaphos production genes[10] or the bialaphos regulatory gene[11] previously isolated.

In order to isolate a DNA fragment which could restore productivity to NP60, chromosomal DNA from HP644 was cloned into NP60. *Bam*H I-digested HP644 genomic DNA was inserted into the *Bgl* II site of pIJ702. The ligated DNA was used to transform NP60. Thiostrepton-resistant and white (indicating insertional inactivation of the melanin gene in pIJ702) transformants were picked individually onto agar plugs containing production medium. The plugs were then transferred to bialaphos-sensitive indicator lawns of *B. subtilis*. Two bialaphos-producing transformants were obtained from about 1,600 transformants tested. The recombinant plasmids pMSB25-1 and pMSB25-2 containing the same 6.3 kb *Bam*H I fragment were obtained from the transformants. The orientation of the insertions in pMSB25-1 and pMSB25-2 was different. From the result of subcloning experiments, the minimum locus which restored productivity to NP60 was a 1.3-kb *Bam*H I/*Sst* I fragment as shown in Fig. 2.

In the case of NP61, *Bgl* II-digested HP662 genomic DNA was inserted into the *Bgl* II site of pIJ702 and the ligation mixture was used to transform it. Thiostrepton-resistant transformants were screened for the melanin production and the bialaphos production. About 900 transformants were white and six produced bialaphos (pMSB80-1, 2, 3, 4, 5 and 6). All contained the same 6.5 kb *Bgl* II

Fig. 3. Organization of the bialaphos biosynthetic gene cluster.
Bar: Bialaphos resistance gene, brpA; bialaphos regulatory gene.



The locations of inserts contained in plasmids pMSB80-2, pMSB97-2, pMSB2-4, pMSB12 and pMSB35 are indicated by solid bars. Figures below the map show the bialaphos biosynthetic steps[10].

fragment. The orientation of the insertions in pMSB80-1, 3, 4, 6 and pMSB80-2, 5 was different. Subcloning experiments showed that the minimum locus which restored productivity to NP61 was a 3.6-kb *Sph* I/*Bam*H I fragment as shown in Fig. 2.

Bgl II-digested HP13-1 genomic DNA was inserted into the *Bgl* II site of pIJ702 and the ligation mixture was used to transform NP62. Four out of about 600 transformants which were thiostrepton-resistant and white, restored bialaphos productivity (pMSB97-1, 2, 3 and 4). All contained the same 14 kb *Bgl* II fragment. The insertions in pMSB97-1, 3 and pMSB97-2, 4 were of different orientation. Subcloning experiments showed that the minimum locus which restored productivity to NP62 was a 2.7-kb *Sst* I/*Pst* I fragment as shown in Fig. 2.

Genetic Linkage of Three Cloned Genes and the Bialaphos Biosynthetic Gene Cluster

The possible linkage of three cloned genes (pMSB25-2, pMSB80-2 and pMSB97-2) and the bialaphos biosynthetic gene cluster was investigated. The restriction map of the 14-kb insert found in pMSB97-2 overlapped with the restriction map of pMSB2-4, previously reported, as shown in Fig. 3. Southern blot experiments confirmed that cosmid pMSB13-3 hybridized to pMSB97-2 (data not shown). As pMSB13-3 did not hybridize to pMSB25-2 and pMSB80-2, cosmids which hybridized to pMSB25-2 and pMSB80-2 were isolated, respectively. HP662 DNA was partially digested with *Sau*3 A and inserted into the *Bam*H I site of cosmid pHC79. Three colonies isolated which hybridized to pMSB80-2 also hybridized to pMSB97-2. Southern blot experiments confirmed that a 6.5-kb *Bgl* II fragment in pMSB80-2 was located adjacent to the *Bgl* II fragment in pMSB97-2 (data not shown). The gene cluster involved in the bialaphos production is about 35-kb long as shown in Fig. 3.

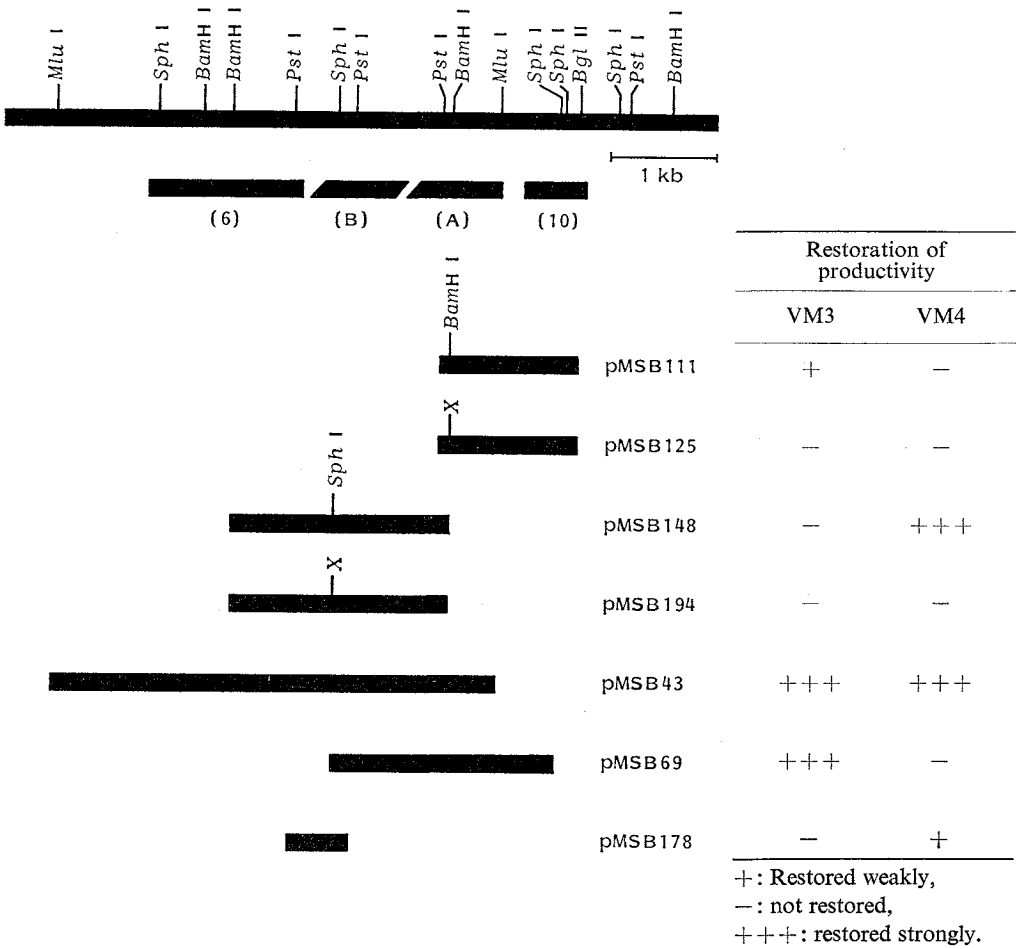
Five clones which hybridized to pMSB25-2 were isolated in the same way as described for pMSB80-2. However, these cosmids did not hybridize to any regions of the bialaphos biosynthetic gene cluster, *i.e.*, pMSB80-2, pMSB12 or pMSB35.

Isolation of Bialaphos-nonproducing Mutants, VM3 and VM4 by the Gene Replacement Method

pMSB111 subcloned from pMSB12 containing bialaphos biosynthetic genes has a unique *Bam*H I

Fig. 4. Analysis of the genes which complement VM3 and VM4.

(6): The genes which codes phosphinomethylmalic acid (PMM) synthase, (10): the genes which codes demethylphosphinothricin (DMPT) acetyltransferase, (A): the genes which restores productivity to VM3, (B): the genes which restores productivity to VM4.



pIJ702 was used as a vector for construction of pMSB43 and pMSB69, and pIJ680 for construction of pMSB111, pMSB125, pMSB148, pMSB178 and pMSB194. pMSB125 and pMSB194 lack the *Bam*H I site and the *Sph* I site, respectively. Solid bars below the map show the possible location of the genes which code PMM synthase and DMPT acetyltransferase and the genes which restored productivity to VM3 and VM4, respectively.

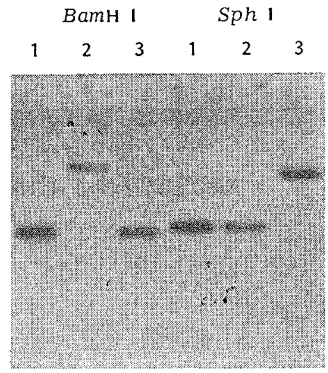
site as shown in Fig. 4. pMSB125, derived from pMSB111 by filling up the cohesive ends of the *Bam*H I site using DNA polymerase I, was introduced into a bialaphos-producing strain, HP782 and thiostrepton-resistant clones were selected. By a protoplast regeneration experiment, two nonproducing mutants of bialaphos were obtained out of 300 colonies tested. By a curing experiment, one thiostrepton-sensitive mutant, named VM3, was isolated from one of two thiostrepton-resistant nonproducing mutants.

pMSB148 subcloned from pMSB12 has a unique *Sph* I site as shown in Fig. 4. pMSB194, derived from pMSB148 by removing the cohesive ends of the *Sph* I site using S1 nuclease, was transformed into HP782 and thiostrepton-resistant clones were selected. Three nonproducing mutants of bialaphos were obtained out of 200 colonies tested after protoplast regeneration and showed the same character.

VM4, which was thiostrepton-sensitive, was isolated from one of three mutants by a curing experiment.

VM3 and VM4 has a defect in the alanylation step as described above.

Fig. 5. Southern hybridization of ^{32}P-labeled pMSB69 DNA to HP782 (lane 1), VM3 (lane 2) and VM4 (lane 3) chromosomal DNAs digested with *Bam*H I or *Sph* I.



Analysis of Mutation Site in VM3 and VM4 by Southern Hybridization

Southern analysis was carried out to confirm the genotypes of VM3 and VM4. Chromosomal DNAs prepared from VM3, VM4 and their parent strain HP782 were digested with *Bam*H I or *Sph* I, then electrophoresed on agarose gels, and transferred to nylon membranes. The hybridization pattern of these DNAs, when probed with the labeled pMSB69, is shown in Fig. 5. *Bam*H I digested VM3 chromosomal DNA gave one band (4.0 kb), but those of VM4 and HP782 gave two bands of the same size (2.0 kb). In the case of *Sph* I digestion, although VM3, VM4 and HP782 gave one band, that of VM4 (3.8 kb) was larger than that of VM3 and HP782 (2.1 kb). These results show the lack of the *Bam*H I site within the 2.1-kb *Sph* I fragment on the VM3 chromosome and the lack of the *Sph* I site within the 2.0-kb *Bam*H I fragment on the VM4 chromosome.

Restoration of Bialaphos Productivity by Retransformation

Fig. 4 shows restoration level of bialaphos in VM3 and VM4 by retransformation. pMSB111 and pMSB125 were introduced into VM3, and pMSB148 and pMSB194 were introduced into VM4. pMSB111 and pMSB148 could restore productivity to VM3 and VM4, respectively, but pMSB125 and pMSB194 could not. VM3 containing pMSB111 could produce a very low level of bialaphos as compared with the parent strain or VM3 containing pMSB43 or pMSB69. Also VM4 containing pMSB178 produced a very low level of bialaphos as compared with the parent strain or VM4 containing pMSB43 or pMSB148. It seemed that restoration by pMSB111 in VM3 and by pMSB178 in VM4 was caused by the recombination between a plasmid and chromosome. These results suggest the existence of two genes related to the alanylation step in this region.

Discussion

This paper describes the cloning and characterization of alanylation related genes in bialaphos biosynthesis. As previously reported[10,11], we have cloned about a 20-kb bialaphos biosynthetic gene cluster, and have defined eight structural genes and one regulatory gene. It has been thought that other bialaphos biosynthetic genes may exist on the outside of the bialaphos gene cluster, or that regions of the gene cluster which do not correspond to any of the nonproducing mutants may contain unidentified genes. For the cloning and characterization of all genes involved in the bialaphos biosynthesis, especially alanylation related genes which have not been identified yet, we used two strategies; first, isolation of alanylation defective bialaphos-nonproducing mutants and then cloning using those as hosts; second, analysis of the unidentified region of the gene cluster using a new method for the isolation of mutants introduced by an *in vitro* derived mutation on the plasmid into the chromosome by means of *in vivo* genetic recombination[17].

According to the first strategy, among many bialaphos-nonproducing mutants isolated by NTG treatments, three were estimated to be alanylation defective mutants, and three different alanylation related genes were cloned. Because pMSB80-2 and pMSB97-2 hybridized to the contiguous region of the bialaphos biosynthetic gene cluster, while pMSB25-2 did not, at least two regions related to bialaphos production exists on the chromosome at a distance. Moreover, hybridization experiment showed that the gene cluster extends to about 35 kb long. Recent molecular cloning experiments have shown that biosynthetic genes of methylenomycin[23], actinorhodin[24], undecylprodigiosin[25] and streptomycin[26] are clustered as in the case of bialaphos. In the case of actinorhodin[24,27], the cloning and further characterization of the genes, both at the genetic and molecular levels, has shown an orderly organization of all the biosynthetic genes, including at least one regulatory gene over 26 kb of a contiguous DNA.

On the other hand, by the gene replacement method several bialaphos-nonproducing mutants were isolated which were different from any bialaphos-nonproducing mutants previously isolated by conventional mutagenesis. Two of them are the alanylation defective mutants, VM3 and VM4. From these mutants, it seems that the bialaphos biosynthetic genes within the cluster are very closely linked. There are the step 6 gene encoding phosphinomethylmalic acid synthase (K. SHIMOTOHNO; personal communication) at the left adjacent region of *Sph* I and the step 10 gene encoding DMPT acetyltransferase[10,28] at the right adjacent region of *Bam*H I as shown in Fig. 4. These results show that this method is very useful, especially for the functional analysis of the unidentifiable region on the cloned DNA fragment. Recently, molecular cloning have showed that genes which code for the resistance and biosynthesis of antibiotics are closely linked[10,26,29], and moreover that the regulatory genes of antibiotics are located in the clusters[11,26,29]. Thus, after cloning of the resistant gene, it may be possible to identify whether unknown biosynthetic or regulatory genes involved in antibiotic production are located close to the resistance gene by using this gene replacement method.

In the previous paper[8], it was reported that NP45 was a bialaphos-nonproducing mutant which lacks the ability to catalyze methylation of phosphinic acid derivatives, since NP45 could convert *N*-acetyl PT to bialaphos and accumulated DMPT, *N*-acetyl DMPT, DMBA[8] and *N*-acetyl DMBA (S. IMAI *et al.*; unpublished). Furthermore NP61 and NP62 accumulated *N*-acetyl DMPT and *N*-acetyl PT. Thus two pathways from *N*-acetyl DMPT to *N*-acetyl bialaphos are proposed as described in Fig. 1. But NP61 and NP62 accumulated small amounts of *N*-acetyl PT, while NP60, VM3 and VM4 accumulated only *N*-acetyl DMPT. Therefore, the alanylation step and the methylation step may be simultaneously regulated. Although all mutants could convert *N*-acetyl BA to bialaphos, they could not convert *N*-acetyl DMBA to bialaphos. This result may be caused by a difference in permeability of *N*-acetyl BA and *N*-acetyl DMBA or because the deacetylation rate of *N*-acetyl DMBA may be faster than the methylation rate. On the other hand, NP45 is a bialaphos-nonproducing mutant which lacks the ability to catalyze methylation of phosphinic acid derivatives[8]. Although NP45 did not accumulate *N*-acetyl PT, it could convert *N*-acetyl PT to bialaphos. Therefore, NP60, NP61, NP62, VM3 and VM4 are at least believed to lack the ability to catalyze alanylation.

The result that at least five genes related to alanylation exist on the chromosome, suggests that the alanylation step is very complicated and under the control of multiple genes. It may be that these genes are structural genes of alanylation, regulatory genes, or the genes which encode a cofactor indispensable for the alanylation step. Furthermore they might be transfer RNA related genes or subunits of these genes. In order to investigate whether or not unidentified genes exist on the contiguous regions of these genes which complement NP60, NP61 and NP62, respectively, it will be necessary to continue the gene replacement experiment. Biochemical and genetic analyses are now required for better understanding of the mechanism of the alanylation step.

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