# COSYNTHESIS AND PROTOPLAST FUSION BY MUTANTS OF BIALAPHOS (AMPBA) PRODUCING *STREPTOMYCES HYGROSCOPICUS*

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Cosynthesis in mixed culture and protoplast fusion of non-producing mutants of *Streptomyces hygroscopicus* which may produce biosynthetic intermediates of bialaphos (AMPBA) were studied. Non-producing mutants were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) treatment, and six stable non-producing mutants were used for cosynthesis and protoplast fusion studies. The biosynthetic block sites of the non-producers were consistent with studies of the biosynthetic pathway of AMPBA.

Bialaphos (AMPBA) is a metabolite of *Streptomyces hygroscopicus* ATCC 21705 having strong herbicide activity[1]. The structure of this substance was determined to be 2-amino-4-(hydroxy)(methyl)-phosphinoylbutyrylalanylalanine[2], and is the first natural product to have the unique C–P–C bond of the phosphinothricyl moiety[3]. In previous papers[4,5], we have described non-producing mutants of AMPBA producer obtained by mutagenesis with NTG treatment; these mutants produced derivatives or intermediates of AMPBA. We have further investigated the biosynthetic pathway of AMPBA by the technique of mixed culture cosynthesis and protoplast fusion using six additional AMPBA non-producing mutants of *S. hygroscopicus*. The present paper deals with the determination of blocked sites in non-producers and the results of a complementation study by cosynthesis and protoplast fusion.

## Materials and Methods

### Organisms

*Streptomyces hygroscopicus* ATCC 21705 was used parental strain. *Proteus* sp. MB-838 was used for the assay of AMPBA.

### Media

Seed culture medium (S-1) was prepared with 2.0% soluble starch, 1.0% peptone, 0.3% meat extract, 0.05% $K_2HPO_4$, pH 7.0. Agar medium (A-4: 0.4% glucose, 0.35% wheat germ, 0.23% soluble vegetative protein, 0.03% $KH_2PO_4$, 0.0001% $CoCl_2 \cdot 6H_2O$, 2.0% agar, pH 7.0) was used for cosynthesis experiments and selection of non-producing mutants by the use of agar piece method.[6]

### Cosynthesis on an Agar Plate

Cosynthesis on agar plates was carried out by modifying the procedure derived by DELIC *et al.*[7] and FURUMAI *et al.*[8,9]. The mycelial suspension of two non-producing mutants was harvested from S-1 medium (0.1 ml each) and spread about 1 ~ 2 mm apart on opposite halves of an A-4 medium plate. The plate was incubated for 7 days at 28°C. An agar strip, 5 × 60 mm was cut from the plate at right angles to the line of separation between the two mutants. This strip was plated on the surface of an agar plate seeded with *Proteus* sp. MB-838, and incubated at 32°C overnight. The appearance of an inhibition zone along the agar strip was considered a sign of the production of AMPBA.

Protoplast Formation and Regeneration

Protoplasts were prepared by treatment with lysozyme (Sigma Chemical Co.) and achromopeptidase (Wako Pure Chemical Co.) and regenerated to colonies as previously described for *S. hygroscopicus*[10].

Fusion of Protoplast

Protoplasts (0.25 ml: about $4 \times 10^8$/ml) from each of two strains were mixed, added to 1.5 ml of 30% polyethylene glycol (PEG) 1500 in medium P3[11], and incubated at 30°C for 5 minutes. The PEG treated protoplast mixture was diluted with P3 solution, and plated on the regeneration medium. The regenerated colonies were inoculated to each agar pieces of medium A-4 and incubated for 7 days at 28°C, then placed on an agar plate seeded with *Proteus* sp. MB-838 and incubated at 32°C overnight. Colonies which showed inhibition zone were selected.

## Results and Discussion

Among numerous AMPBA non-producing mutants obtained by NTG treatment, six stable non-producing mutants were selected for the cosynthesis and protoplast fusion studies.

### Cosynthesis

Cosynthesis was carried out as described above, and Fig. 1 shows the result of mixed culture of the non-producing strains NP-221 and NP-213. The upper and lower strips shown on the Petri dish were cultures of the strains NP-221 and NP-213 respectively. In the central strip the left half was inoculated with NP-221 and right half with NP-213. Using this pair of strains, an inhibition zone appeared around the central strip on the side of NP-221. This may indicate that NP-213 was producing one or several intermediates which were converted into AMPBA by the strain NP-221. Strain NP-213 was a secretor, and NP-221 was a converter. Therefore, it was concluded that NP-221 and NP-213 were blocked at different biosynthetic steps; NP-221 being blocked at an earlier step than NP-213. In a similar fashion, all combinations of the six non-producing mutants were tested. As shown in Table 1, mutant NP-221 always acted as a converter in co-culture with strains NP-213, NP-42, NP-3 and NP-43. Mutant NP-

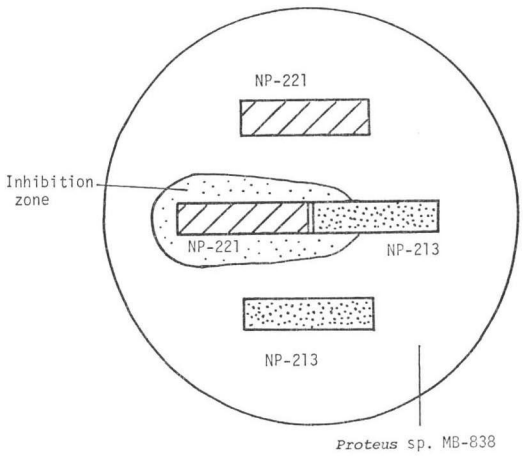
Table 1. Cosynthesis and protoplast fusion test by AMPBA non-producing mutants.

| Strains | | Cosynthesis test | | Protoplast fusion test |
|---|---|---|---|---|
| | | Recovery of productivity | Converter | Recombinants |
| NP-221 | NP-213 | + | NP-221 | 2 / 400 (0.5%) |
| NP-221 | NP-42 | + | NP-221 | 0 / 400 (0%) |
| NP-221 | NP-3 | + | NP-221 | 0 / 400 (0%) |
| NP-221 | NP-43 | + | NP-221 | 0 / 400 (0%) |
| NP-213 | NP-42 | + | NP-213 | 4 / 400 (1.0%) |
| NP-213 | NP-3 | + | NP-213 | 5 / 400 (1.3%) |
| NP-213 | NP-43 | − | | 0 / 400 (0%) |
| NP-42 | NP-3 | − | | 0 / 400 (0%) |
| NP-42 | NP-43 | − | | 0 / 400 (0%) |
| NP-3 | NP-43 | − | | 0 / 400 (0%) |
| NP-30 | NP-221 | + | − | 1 / 500 (0.2%) |
| NP-30 | NP-213 | + | − | 2 / 400 (0.5%) |
| NP-30 | NP-42 | + | − | 6 / 500 (1.2%) |
| NP-30 | NP-3 | + | − | 5 / 400 (1.3%) |
| NP-30 | NP-43 | + | − | 6 / 500 (1.2%) |

No revertant was obtained in each NP strain alone with PEG treatment.

213 acted as a converter with NP-42 and NP-3. As NP-43 was derived from NP-213 by mutation, ocsynthesis between NP-213 and NP-43 was not observed. The intermediates on AMPBA biosynthetic pathway (Fig. 4) had no inhibitory activity against *Proteus* sp. MB-838 on agar plate assay. Mutants NP-42 and NP-3 always acted as secretors only. Mutant NP-30 showed cosynthetic ability with all the other mutants. Vitamin $B_{12}$ was required for the biosynthesis of AMPBA probably at the step of transmethylation and since NP-30 produced AMPBA in combination with all other mutants it appears that it was a vitamin $B_{12}$ idiotroph.[12]

Fig. 1. Cosynthesis between AMPBA non-producing mutants NP-221 and NP-213.




## Protoplast Fusion

In order to investigate protoplast fusion of AMPBA non-producing mutants of *S. hygroscopicus*, the optimal molecular weight and concentration of polyethylene glycol (PEG) were examined. As shown in Fig. 2, maximum frequency of recombinants between NP-221 and NP-213 was obtained with PEG 1500, at a concentration of 30 w/v%. Recombinant frequency was calculated as the appearance of AMPBA producing colonies among the total colonies regenerated after PEG treatment.

We carried out protoplast fusion between six AMPBA non-producing mutants. As shown in Table 1, recombinants were obtained with NP-221 × NP-213, NP-213 × NP-42, NP-213 × NP-3 and NP-30 × (all the other five blocked mutants). Recombinants could not be obtained with NP-221 × NP-42, NP-221 × NP-3 and NP-221 × NP-43.

We were not able to obtain recombinants in case of NP-221 × NP-42, NP-221 × NP-3 and NP-221 × NP-43. This fact might be due to either low regeneration efficiency of recombinant protoplast or location of each blocked sites on chromosomal genes. However, in case of NP-213 × NP-43, NP-42 × NP-3, NP-42 × NP-43 and NP-3 × NP-43, these pairs of NP-strains had common blocked sites each other, therefore recombinants were not obtained.

Fig. 2. Effect of concentration and molecular weight of PEG on recombination frequency with *Streptomyces hygroscopicus* NP-221 × NP-213.

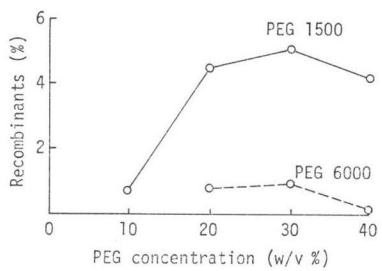



Fig. 3. Complementation pattern of blocked mutants for the biosynthesis of AMPBA.

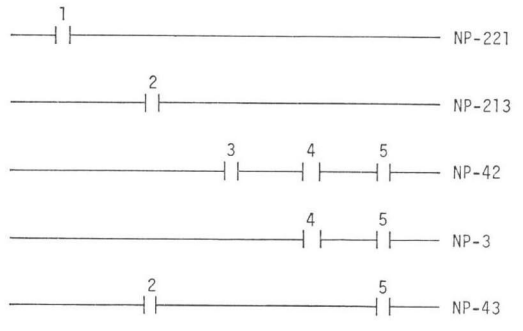
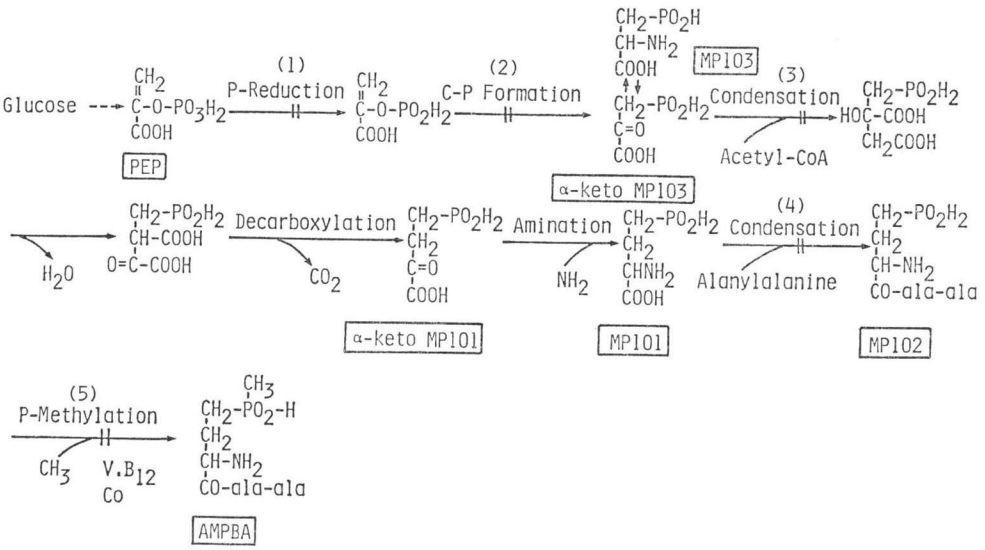


Fig. 4. Scheme in AMPBA biosynthesis pathway and blocked sites.




## Blocked Sites of AMPBA Biosynthesis

We have determined the blocked sites in the AMPBA non-producing mutants based on the results of protoplast fusion and cosynthesis as shown in Fig. 3.[4,5] Site 1 refers to P-reduction, site 2 to C–P formation, site 3 to acetyl condensation, site 4 to alanylalanine condensation and site 5 to methylation. The biosynthetic pathway and blocked sites of mutants are summarized in Fig. 4.

BALTZ *et al.* have reported a genetic and biological study of a macrolide antibiotic, tylosin.[13] The structural genes of tylosin were lost at high frequency after cell fusion and regeneration of protoplasts, and they were found to be transmissible. However, in case of AMPBA production, productivity was not lost after cell fusion and regeneration. Moreover, its productivity was expressed after cell fusion by some pairs of non-producing mutants that exhibited productivity by cosynthesis. This may indicate that recombination occurs by cell fusion in some chromosomal genes contributing to the biosynthesis of AMPBA.

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