STUDIES ON THE BIOSYNTHESIS OF FOSFOMYCIN

3. DETECTION OF PHOSPHOENOLPYRUVATE PHOSPHOMUTASE ACTIVITY IN A FOSFOMYCIN HIGH-PRODUCING STRAIN OF *Streptomyces wedmorensis* AND CHARACTERIZATION OF ITS BLOCKED MUTANT NP-7†

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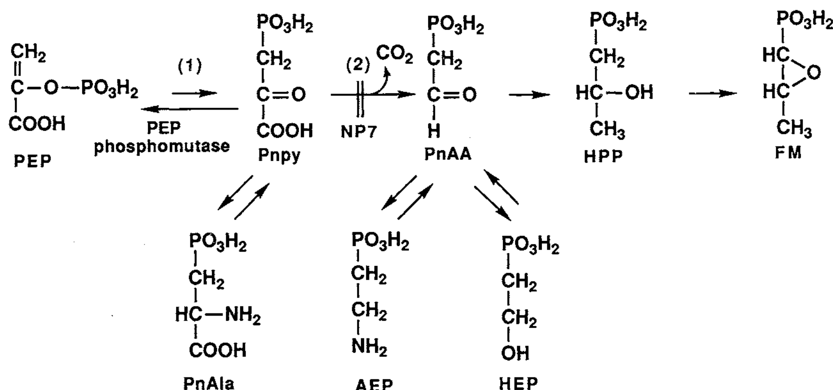
Fosfomycin (FM) is an antibiotic produced by various species of *Streptomyces*[2,3], and is characterized by possessing both a unique C-P bond and an epoxide. It has a broad antibacterial spectrum effective against Gram-positive and Gram-negative bacteria, and is now in clinical use. In a previous paper, we reported that 2-hydroxyethylphosphonic acid (HEP) and 2-aminoethylphosphonic acid (AEP) were transformed to FM by a FM non-producing mutant NP-7 obtained by NTG treatment of the parent strain, *Streptomyces wedmorensis* ATCC 21239[4]. HEP had been isolated from the fermentation broth of a blocked mutant of *Streptomyces hygroscopicus*, a bialaphos (BA)-producing organism, and proven to be an intermediate in the early stage of the BA biosynthesis[5]. Thus, both AEP and HEP, which may be interconvertible each other by the producing strain, are assumed to be common biosynthetic intermediates for BA and FM.

During the biosynthetic studies of BA, we have revealed the reaction sequence from phosphoenolpyruvate (PEP) to HEP (or AEP) to proceed as follows; intramolecular rearrangement of PEP to form phosphonopyruvic acid (Pnpy) catalyzed by PEP phosphomutase (step 1 in Fig. 1, (1))[6] followed presumably by decarboxylation of Pnpy to form phosphonoacetoaldehyde (PnAA) that is easily converted to AEP by transamination (step 2 in Fig. 1, (2)).

We also reported the purification of PEP phosphomutase from BA-producing *Streptomyces hygroscopicus* SF-1293[6] and AEP-producing *Tetrahymena lycheniformis* (in collaboration with the Harvard group)[7], and the detection of the enzyme activity in an FR33289 producer[6] as well as in the HEP-producing *Pseudomonas gladioli* B-1[8]. We could not, however, detect this activity in a FM-producing organism, *Streptomyces wedmorensis* ATCC 21239[6].

Since this enzyme seemed to be common to the

Fig. 1. Proposed biosynthetic pathway of fosfomycin.



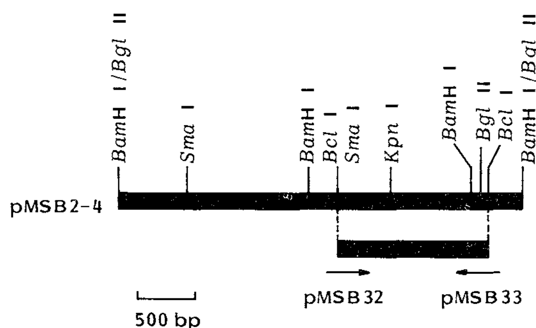
PEP = phosphoenolpyruvic acid, Pnpy = phosphonopyruvic acid, PnAA = phosphonoacetaldehyde, HPP = 2-hydroxypropylphosphonic acid, FM = fosfomycin, PnAla = phosphonoalanine, AEP = 2-aminoethylphosphonic acid, HEP = 2-hydroxyethylphosphonic acid.

† For Part 2[1].

Table 1. Phosphoenolpyruvate (PEP) phosphomutase activity in *Streptomyces wedmorensis* and *Streptomyces hygroscopicus*.

Strain	PEP phosphomutase activity (U/mg protein)
S. wedmorensis 144-91	0.036
S. wedmorensis NP-7 (pMSB32)	0.071
S. wedmorensis NP-7 (pMSB33)	0.005
S. hygroscopicus SF-1293	0.310

Fig. 2. Restriction fragments coding for PEP phosphomutase.



pMSB2-4 complemented PEP phosphomutase deficient mutants of *S. hygroscopicus* as previously described by MURAKAMI *et al.*[9] Small arrows adjacent pMSB32 and pMSB33 indicate the direction of vector promoters with respect to the cloned fragment.

biosynthesis of all C-P compounds, we attempted to detect the PEP phosphomutase activity in other FM high-producing strains. *S. wedmorensis* 144-91, a FM high producer obtained by NTG treatment of the parent strain, was cultivated in 500-ml Erlenmeyer flasks containing 40 ml of the production medium consisting of starch 4%, salad oil 1.5%, Sungrain 5%, wheat germ 2%, K_2HPO_4 0.15% and $CoCl_2 \cdot 6H_2O$ 0.0001% (pH 8.0). Fermentation was carried out on a rotary shaker at 28°C for 3 days and PEP phosphomutase activity was measured as follows. All operations were carried out at 0°C. Mycelia were harvested by centrifugation, washed twice with 50 mM Tris-HCl buffer (pH 7.5) containing 20% glycerol and suspended in the same buffer at a ratio of 1 g (wet weight) per 2 ml of the buffer and then disrupted by sonication. Unbroken cells and cell debris were removed by centrifugation (15,000 × *g*, 20 minutes) and the PEP phosphomutase activity of the supernatant was measured as previously described[6]. As shown in Table 1, the PEP phosphomutase activity was detected in *S. wedmorensis* 144-91, at a far weaker level than that of BA-producing *S. hygroscopicus* SF-1293. It was thus confirmed that the C-P bond of FM was also formed by the PEP phosphomutase reaction in a similar manner as for BA. The previous failure to detect of the activity in *S. wedmorensis* ATCC 21239 might be ascribed to extreme instability of the enzyme. In fact, the PEP phosphomutase activity in a cell free extract of *S. wedmorensis* 144-91 disappeared completely after storage at 4°C for 24 hours.

Now that the involvement of PEP phosphomutase was established in the biosynthesis of FM, it became important for the following reasons to clarify the mutational point of the blocked mutant NP-7, which required the addition of C-P compounds (AEP or HEP) for production of FM. Since the equilibrium of the step 1 reaction is far in favor of the reverse reaction (PEP formation)[6], the reaction product (Pnpy) must be removed from the reaction system by the step 2 enzyme so that step 1 goes in the forward direction. Nothing is, however, known about the step 2 reaction mechanism. Therefore, step 2 blocked mutants which have not been obtained from the BA-producing organism, if available, were expected to be useful to reveal the C-P bond formation mechanism in more detail.

To clarify the mutational point of NP-7, the PEP phosphomutase activity in the cell extract of NP-7 was measured in the same manner as for the high yielding strain 144-91. Based on the results obtained by biotransformation experiments[4], the mutational point of NP-7 is assumed to be at step 1 (PEP phosphomutase reaction) or step 2. However, it could not be determined whether NP-7 was defective in PEP phosphomutase since the enzymatic activity detected in the NP-7 strain was extremely weak.

To clarify this, we used gene manipulation techniques. Recently, we identified the PEP phosphomutase gene of the BA biosynthetic gene cluster of *S. hygroscopicus*[9] and succeeded in its efficient expression in *S. lividans* 66 using plasmid vector pIJ680[10] (unpublished data). Plasmid pMSB32 was constructed by the insertion of a 1.4 kb *Bcl* I fragment coding for PEP phosphomutase into the *aph* gene in pIJ680[11] using the *Bam*H I site (Fig. 2). Transformation of *S. wedmorensis* protoplasts was carried out in the same manner as that of *S. lividans*[10]. As shown in Table 1, the PEP phosphomutase activity was detected in cell extract of NP-7 (pMSB32) at a twice as strong level as that of *S. wedmorensis* 144-91. FM productivity was not detected in the fermentation broth of this strain, but

we recently found that an unidentified region of the BA biosynthetic gene cluster could restore FM productivity to strain NP-7 (unpublished data). We thus conclude that the NP-7 strain is defective not in PEP phosphomutase (step 1) but in the formation of PnAA from Pnpy (step 2). As mentioned above, this reaction is assumed to be the important step for C-P bond formation, therefore, the NP-7 strain is expected to be useful to reveal the step 2 reaction mechanism leading to PnAA from Pnpy and to be a good host for cloning FM biosynthetic genes.

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