STUDIES ON THE BIOSYNTHESIS OF FOSFOMYCIN. V. CLONING OF GENES FOR FOSFOMYCIN BIOSYNTHESIS†

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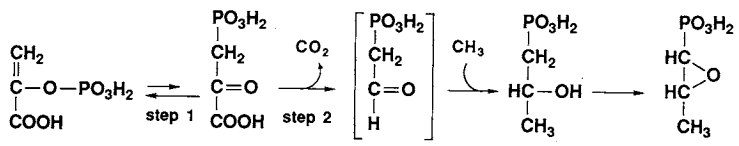
Fosfomycin (FM) is an antibiotic with a broad antimicrobial spectrum and is characterized by the presence of a C-P bond in the molecule. Based on the previous results, we have proposed its biosynthetic pathway consisting of at least four steps as summarized in Fig. 1.[1,2] The first step (step 1) is the intramolecular rearrangement of phosphoenolpyruvate (PEP) to form phosphonopyruvate (PnPy) catalyzed by phosphoenolpyruvate phosphomutase (PEP phosphomutase), that is common to the biosynthesis of all C-P compounds. This unique enzyme was isolated from *Streptomyces hygroscopicus* producing bialaphos (BA)[3], *Tetrahymena pyriformis*, a protozoan producing 2-aminoethylphosphonic acid[4] and *Pseudomonas gladioli* B-1 producing 2-hydroxyethylphosphonic acid[5]. Furthermore the PEP phosphomutase genes of *T. pyriformis*[6] and *S. hygroscopicus*[7] were cloned and sequenced. In *Streptomyces wedmorensis*, a producer of fosfomycin, this enzymatic activity, however, was only detected in cell free extracts[8].

The second step (step 2) following the PEP phosphomutase reaction is assumed to be decarboxylation of PnPy to form phosphonoacetaldehyde (PnAA). This reaction has also been suggested to be common to the biosynthesis of all C-P compounds. The detail mechanism of the step 2 reaction, however, remained unknown. In a previous paper[8], we reported that a FM-nonproducing mutant NP-7 is defective in the step 2 reaction. Thus we have attempted to clone the gene that can complement this mutant. In this paper, we describe the cloning and characterization of the genes encoding step 1 and step 2 enzymes.

pIJ702[9] with thiostrepton resistance and melanin production genes as selection markers was used as a cloning vector for FM biosynthetic genes. pIJ702 prepared from *S. wedmorensis* NP-7 was completely digested by *Bgl*II to cleave the target site in the melanin production gene, and dephosphorylated with bacterial alkaline phosphatase. Genomic DNA prepared from a FM high producer *S. wedmorensis* 144-91 was partially digested with *Bam*HI and size-fractionated by agarose gel electrophoresis to obtain 5-10 kb DNA fragments. The digested genomic DNA and plasmid vector were ligated with T4 DNA ligase at 16°C for 12 hours. This gene library was transformed into *S. wedmorensis* NP-7 protoplasts by the method of THOMPSON *et al.*[10] Melanin-nonproducing and thiostrepton-resistant clones were inoculated onto agar pieces containing the FM-production medium[11] and incubated at 27°C for 5 days. Then these agar pieces were transferred on the nutrient agar seeded with *Proteus* sp. MB 838 for bioassay of FM.

Two clones which restored FM production were obtained among 2600 melanin-nonproducing and thiostrepton-resistant transformants. Recombinant plasmids purified from these two clones, designated pFBG21 and pFBG22 (Fig. 2A), were 5.8 kb and 5.0 kb length, respectively. When these plasmids were introduced into *S. wedmorensis* NP-7, all the thiostrepton-resistant transformants produced FM

Fig. 1. The proposed biosynthetic pathway of fosfomycin.



PEP = phosphoenolpyruvic acid, PnPy = phosphonopyruvic acid, PnAA = phosphonoacetaldehyde, HPP = 2-hydroxypropylphosphonic acid, FM = fosfomycin.

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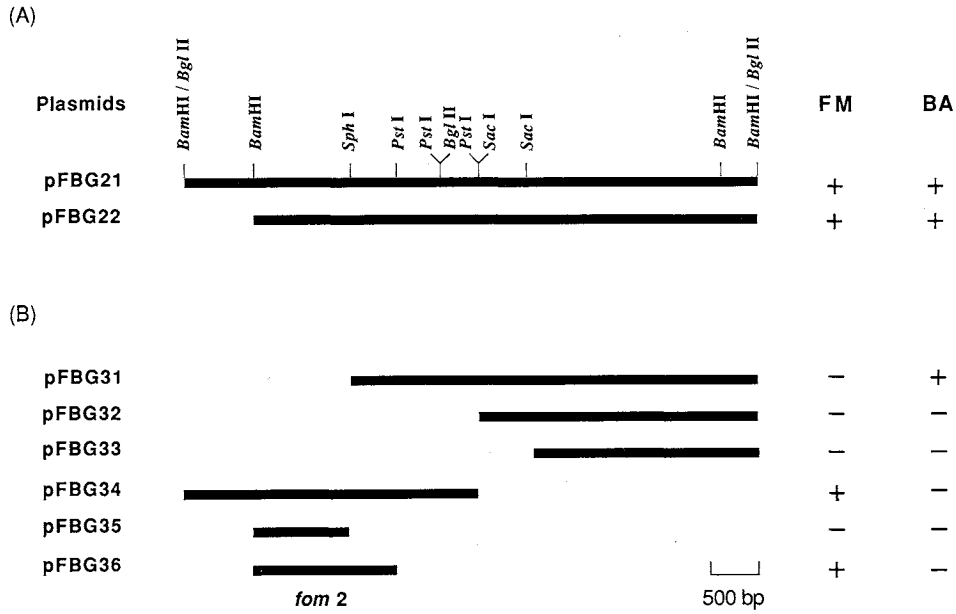
Fig. 2. The physical maps of the cloned fragments containing *fom* 1 and *fom* 2.



Inserted DNA fragments of pFBG21 and pFBG22 (A) and subcloned plasmids (B) are shown. + represents complementation of mutants. The term *fom* 2 indicates the approximate location of the step 2 gene.

(Fig. 2A). The production yields of FM of these transformants remained at about one fifth as compared to that of the parent strain.

A result from restriction mapping showed that these two recombinant plasmids shared a *Bam*H I fragment of 4.7 kb. Furthermore the insert fragments of pFBG21 and pFBG22 were inserted in different direction each other. This indicated that the cloned insert fragments introduced into these recombinant plasmids had a functional promoter for expression of step 2 gene (*fom* 2). The recovery of FM productivity by this transformation may well be explained by recombination occurred between chromosome and plasmids containing homologous DNA.

To examine whether the two cloned DNA carried step 1 gene (*fom* 1), pFBG21 and pFBG22 were introduced into a blocked mutant of BA producing organism *S. hygroscopicus* E26 defective in PEP phosphomutase activity[3]. This mutant was transformed by the method of MURAKAMI *et al.*[12] BA productivity of all the transformants was restored as shown in Fig. 2A.

To localize *fom* 1 and *fom* 2 on these fragments, we next constructed six recombinant plasmids from pFBG21 and pFBG22 (Fig. 2B). The resulting derivatives were introduced into *S. wedmorensis* NP-7 and *S. hygroscopicus* E26. FM and BA productivities of the transformants were assayed by the agar-piece method. As shown in Fig. 2B, plasmid pFBG34 and pFBG36 can efficiently restore the FM production of NP-7. Since these fragments contained a common 1.3 kb *Bam*H I-*Pst* I fragment, *fom* 2 was located within this fragment. On the other hand, BA productivity of E26 was restored by introduction of only pFBG31. Since *fom* 2 is located in 1.3 kb *Bam*H I-*Pst* I fragment, *fom* 1 is located in the neighborhood of *fom* 2.

We succeeded in the first cloning of two fragments encoding *fom* 2. Fortunately, these fragments carried not only *fom* 2 but also *fom* 1. Furthermore, *fom* 1 was located in the neighborhood of *fom* 2. DNA sequencing of the region which could complement mutation of NP-7 and E26 is progressing now.

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