Note

Cloning and Expression in Escherichia coli of 2-Hydroxypropylphosphonic Acid Epoxidase from the Fosfomycin-producing Organism, Pseudomonas syringae PB-5123

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The fosfomycin resistance gene, fosC, has been cloned from the fosfomycin-producing organism, Pseudomonas syringae PB-5123. Sequence analysis upstream of this gene found a new ORF showing significant homology to 2-hydroxypropylphosphonic acid epoxidase from fosfomycin-producing Streptomyces wedmorensis. The purified recombinant protein of this ORF converted 2-hydroxypropylphosphonic acid to fosfomycin. This result clearly showed the ORF to encode 2-hydroxypropylphosphonic acid epoxidase in PB-5123.

Key words: fosfomycin; biosynthesis; Pseudomonas syringae; 2-hydroxypropylphosphonic acid epoxidase

Fosfomycin (FM) is a medically important antibiotic produced by various species of Streptomyces,1-2 Pseudomonas syringae,3 and Pseudomonas viridiflava.4 We have proved that FM was synthesized from phosphoenolpyruvate via at least four steps in S. wedmorensis.5-8 Furthermore, we have cloned four genes corresponding to all the enzymes necessary for the FM biosynthesis in S. wedmorensis.9,10 The FM biosynthesis by S. wedmorensis and S. fradiae include the epoxide formation not by the addition of molecular oxygen to a chemically most plausible olefinic compound, cis-propenylphosphonic acid,11,12 but by dehydrogenation of the secondary alcohol, 2-hydroxypropylphosphonic acid (HPP).13,14 This unique mechanism is in sharp contrast with the ubiquitous formation of the epoxide functions by the addition of molecular oxygen to double bonds in most natural products. Therefore, the dehydrogenation of HPP for FM production is very specific for the FM-producing organisms. This chemically unique epoxidation was proved to be catalyzed by HPP epoxidase, which is coded by the fom4 gene in S. wedmorensis.10,11

One approach to obtain important information on the reaction mechanism of HPP epoxidase is to clone its corresponding gene from other FM-producing organisms. Highly conserved amino acid residues to be found among HPP epoxidases would give a clue to analyze the essential moieties of the enzyme. Therefore, we tried to clone the HPP epoxidase gene from P. syringae PB-5123 by using the FM self-resistance gene, fosC, which had already been cloned from PB-5123 and sequenced.15 The nucleotide sequence of a 1721 bp SacI-EcoRV fragment containing the fosC (formerly orf1) gene had been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number Z33413 (Fig. 1). The DNA fragment also contained an undefined ORF (orf2) and an incomplete ORF, which is newly named orf3 in this paper. We found that the deduced amino acid sequence of this incomplete ORF showed significant homology to the C-terminal portion of the fom4 gene product. By assuming that orf3 encoded an HPP epoxidase, we then tried to clone the complete ORF from a gene library of P. syringae PB-5123.

On the basis of the nucleotide sequence databases with the accession number just mentioned, two oligonucleotide primers, 5'-GAGCTCAAGGAAGATGTGTTGAAATGGG-3' (5' of the SacI-EcoRV fragment) and 5'-TCGAAACATGATCAGTGTCATCATCGGC-3' (5' of the fosC gene) were synthesized (Amersham Pharmacia Biotech) and used together with total DNA from PB-5123 to amplify the DNA fragment upstream of the fosC gene. PCR was done in PCR buffer (Boehringer) of 20 μl total volume containing 50 ng of total DNA from PB5123, 0.2 μM of each deoxy NTP, 2.5 pmol of each primer, and 1.8 units Tag polymerase (Boehringer) using 25 cycles (0.5 min of 95°C; 0.5 min 60°C; 1 min 72°C). In this PCR, a single DNA fragment of 0.4-kb was amplified (Fig. 1). By using this 0.4-kb DNA fragment as the probe for the colony hybridization method, a 1.6 kb SphI-SphI fragment was obtained (Fig. 1). The DNA sequence of this 1.6-kb DNA fragment was analyzed by the dideoxy chain termination method16 with a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech) and an automated sequencer (model 4000L; Li-cor).

The nucleotide sequence of the 1.6-kb fragment identified one complete ORF (orf3), one incomplete ORF (orf4), and the N-terminal portion of the fosC gene (Fig. 1). The orf3 consists of 573 bp starting with an initiation codon, ATG at position 396, and a termination

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Abbreviations: FM, Fosfomycin; HPP, 2-hydroxypropylphosphonic acid
codon, TGA at position 966. A putative Shine-Dalgarno sequence, GGGAG, was found 10 bp upstream of the initiation codon. The deduced amino acid sequence of the ORF3 was similar to that of the *fom4* gene product of *S. wedmorensis* (32.4% in 148 amino acids overlap, Fig. 2), suggesting that the *orf3* encodes HPP epoxidase. On the other hand, ORF4 upstream of the ORF3 showed no significant similarity to any other amino acid sequences. The nucleotide sequence data of the 1.6-kb *SphI*-SphI fragment was deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D82818.

To verify the catalytic function of the ORF3, the corresponding gene was overexpressed in *E. coli*. The QIAexpress system (Qiagen) was used because of the advantages of high-level expression and easy purification. On the basis of the total nucleotide sequence of the *orf3* from PB5123, two oligonucleotide primers, 5'-GGGCGATCGCAGCTTGGACATTTACG-3' (5' of the *orf3*) and 5'-CCGCGATCGACCATATGCAGATGCGAAAT-3' (3' of the *orf3*), including SphI restriction sites (underlined) were synthesized (Amersham Pharmacia Biotech) and used together with total DNA from PB5123 to amplify *orf3*. PCR was done in PCR buffer (Boehringer) of 20 μl total volume containing 50 ng of total DNA from PB5123, 0.2 mM of each deoxy NTP, 2.5 pmol of each primer, and 1.8 units *Taq* polymerase (Boehringer) using 25 cycles (0.5 min of 95°C; 0.5min 55°C; 1 min 72°C). A single DNA fragment of 0.6-kb was amplified. The PCR fragment was cleaved with *SphI*, and cloned into the *SphI* site in pUC118 (Takara Shuzo). Strain JM109 (Takara Shuzo) was used as a recipient in this transformation. DNA sequencing as described above cloned analyzes for a correct insert DNA, and then the correct fragment was cloned into the *SphI* site present in the multicloning site of the expression vector pQE30 (Qiagen) to give pQ42P. This plasmid, pQ42P, was designed to have an affinity tag consisting of just six consecutive histidine residues in the N-terminal region of the recombinant enzyme. Nitriolitriacetic acid agarose resin has a strong affinity for protein that has such histidine residues. *E. coli* M15 harboring pREP4 [*neo, lacI*] (Qiagen) was used as a host for expression of *orf3*. M15 (pREP4, pQ42P) was cultured at 37°C in 100 ml of LB medium containing 25 μg/ml kanamycin (Nacalai, Kyoto) and 200 μg/ml ampicillin (Sigma) for 5 hours with the addition of 0.1 mM isopropyl β-D-thiogalactoside upon reaching an optical density at 660 nm of 0.8. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5). After brief sonication, the lysate was centrifuged at 10,000 g for 20 min and the supernatant was collected. The crude extract was applied to a Ni-nitriolitriacetic acid agarose column (1.3 × 20 mm) (Qiagen) previously equilibrated with 50 mM Tris-HCl (pH 7.5). The resin was washed with 50 mM imidazole in 50 mM Tris-HCl (pH 7.5) and then the protein that bound to the resin was eluted with 200 mM imidazole in 50 mM Tris-HCl.

![Fig. 1. Restriction Map of the DNA Fragments Containing the fosC Gene and the *orf3*, and Fosfomycin Biosynthesis by *Ps. syringae* PB5123.](image)

The SacI-EcoRV fragment had previously been sequenced. The 1.6-kb *SphI*-SphI fragment was cloned and sequenced in this study. The double-ended arrow represents the 0.4-kb DNA probe to clone the *SphI*-SphI fragment. The enzymatic function of the ORF3 was confirmed as HPP epoxidase in this study. The function of the *orf2* just downstream of the *fosC* gene is undefined. HPP, 2-hydroxypropylphosphonic acid; FM, fosfomycin.

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ORF3  M-DVRLAVGKATL-ATRKM-TELEHDLQVRHDATQVY-F-D-NG-LEHLQVNA  49
FOM4  MSNKTAESTGFAELKDKREQVQMKDAALASLLGETPETVAENEGGELTTLQGR1A  60

ORF3  QYLAIPLEFFAYQGSDLDGGYKIAVRGCVHYTTYEHLVTTNQDPLMA  109
FOM4  HLHGTCSGA-LLPPACNDLLLDDQVLIQMPDERPIKGLVRDNVDVYCVNCVTRKRPALSVP  119

ORF3  LRLDLSDEQLPLRLNLGGCSSAREIVVTRCAVRVMVGNDELKEDVLNEGDSIFILPNV  169
FOM4  LHDVTVTNDPNPPDKFAKNQSGHQQENFLVEGLEHMKW-GQDENPKELALPTSGMFEEHV  178

ORF3  PHSFTNVGHGKASEI1AINYG  190
FOM4  PHAFACKTGSAKLI1AVNF  198
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![Fig. 2. Alignment of the Deduced Amino Acid Sequences of the HPP Epoxidases of *P. syringae* PB-5123 (ORF3) and *S. wedmorensis* (FOM4).](image)

A highly conserved sequence Asp Leu Asp Asp Gly Val is underlined. Dashes indicate gaps introduced for the optimization of the alignment.
(pH 7.5).

The molecular mass of the recombinant orf3 product was estimated by gel filtration on a Superdex 200 (1.6 × 60 cm) column (Amersham Pharmacia Biotech), which was equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl. Under these conditions, the molecular mass of the orf3 product was estimated to be 68 kDa, while the molecular mass of the product was estimated to be 26 kDa by SDS-PAGE. These results suggested that the protein is most likely to be a dimer.

Incubation of the purified recombinant orf3 product at 30°C for 60 min with chemically synthesized racemic HPP (7) (4 mm) in 100 ml of 50 mM Tris-HCl (pH 7.5) containing 1 mM FeCl3 resulted in production of an antibacterial compound. After removal of the protein with activated carbon, this product was partially purified from the incubation mixture by Dowex-1 (Cl− type) eluting with 1.0% NaClaq.

In the 31P NMR (A500 NMR spectrometer; JEOL) spectrum of the reaction product, a signal was observed at 10.1 ppm, which is identical to that of authentic FM. Furthermore, the product was identified as FM by bioautography using E. coli MB838 as an FM-sensitive organism (data not shown). The enzymatic function of ORF3 was thus confirmed as HPP epoxidase in P. syringae PB-5123 (Fig. 1).

As just mentioned, sequence analysis upstream of the fosC gene has identified orf3 encoding HPP epoxidase in P. syringae PB-5123. Alignment of the deduced amino acid sequences of HPP epoxidases from the two FM-producing organisms, P. syringae and S. wedemoresii, showed a highly conserved sequence, Asp Leu Asp Asp Gly Val, which might be important for the catalytic properties of the enzyme (Fig. 2). Our findings will be useful for a more detailed examination of this unusual enzyme-catalyzed epoxidation.

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