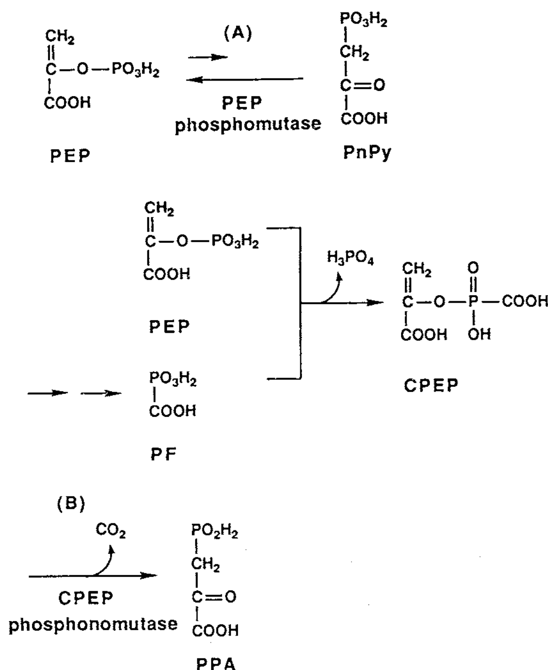
STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293). 14. NUCLEOTIDE SEQUENCE OF PHOSPHOENOLPYRUVATE PHOSPHOMUTASE GENE ISOLATED FROM A BIALAPHOS PRODUCING ORGANISM, *Streptomyces hygroscopicus*, AND ITS EXPRESSION IN *Streptomyces lividans*†

Sir:

In a previous paper[2], we reported that the first step of the bialaphos (BA) biosynthesis is the intramolecular rearrangement of phosphoenolpyruvate (PEP) to form the carbon-phosphorus (C-P) bond of phosphonopyruvate (PnPy) catalyzed by phosphoenolpyruvate phosphomutase (Fig. 1(A)). Due to the extreme instability of PEP phosphomutase of *Streptomyces hygroscopicus* SF1293, a bialaphos producing organism, we have not yet succeeded in obtaining the enzyme in a pure form.

Fig. 1. C-P bond formation steps in the bialaphos biosynthetic pathway.

PEP = phosphoenolpyruvate, PnPy = phosphonopyruvate, PF = phosphonoformic acid, CPEP = carboxyphosphonoenolpyruvate, PPA = phosphinopyruvate.



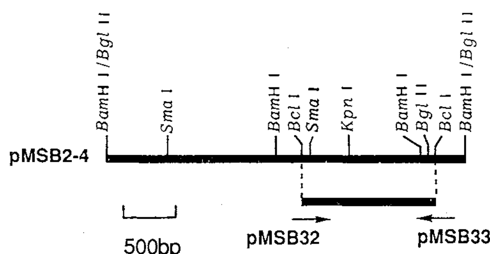
This enzyme is considered to be common to the biosynthesis of all C-P compounds[3]. It is characterized by the very strong activity to catalyze the reverse reaction, and the equilibrium between PEP and PnPy favors of the phosphate ester (in the ratio of more than 100 : 1)[2]. Since the investigation of this unique enzyme is very important to reveal the C-P bond formation mechanism, we have determined the nucleotide sequence of the PEP phosphomutase gene.

In this communication, we describe the cloning and nucleotide sequence determination of the PEP phosphomutase gene and its expression in *Streptomyces lividans* 66.

In a previous report[4], MURAKAMI *et al.* showed that a 3.6-kb *Bam*H I fragment in the BA biosynthetic gene cluster could restore BA productivity to mutant NP47 that is defective in PEP phosphomutase (Fig. 2, pMSB2-4). Introduction of frame shift mutation into a *Bgl* II site of this fragment gave a new mutant E26 which showed no PEP phosphomutase activity[2].

A 1.3-kb *Bcl* I fragment containing this *Bgl* II site was inserted into the *Bam*H I site of pIJ680, downstream of the promoter of the aminoglycoside phosphotransferase (*aph*) gene[5], as shown in Fig. 2. The constructed plasmids (pMSB32 and pMSB33) were introduced into *S. lividans* 66 by transformation[6], and expression of the PEP phosphomutase activity was tested as follows. The *S. lividans* 66 harboring pMSB32 or pMSB33 was cultivated using 60 ml of the medium, glucose 7%, wheat germ 3.5%, Sungrain (Suntory Ltd.) 3.5%, KH_2PO_4 2.25%,

Fig. 2. Restriction map of pMSB2-4, pMSB32 and pMSB33.



pMSB2-4 complemented PEP phosphomutase deficient mutants of *S. hygroscopicus* as previously described by MURAKAMI *et al.*[4] Arrows indicate the direction of the transcription from *aph* promoter with respect to the inserted fragments.

† For part 13[1].

Fig. 3. Nucleotide sequence of 1,314-bp *Bcl* I fragment containing PEP phosphomutase gene.

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        10        20        30        40        50        60        70        80        90       100       110       120
TGATCAGCGGATCGATCCTGGAACAGGCAAAGCCGGGCGCCTTCCTCGTCAACACCGCCCGTGGCGCCCTCGTCGACCCGGGGTGCGTGCTGCGGGCCCTGGAGAGCGAACAGCTCGGCG
   I  S  G  S  I  L  E  Q  A  K  P  G  A  F  L  V  N  T  A  R  G  A  L  V  D  P  G  C  V  L  R  A  L  E  S  E  Q  L  G  G

       130       140       150       160       170       180       190       200       210       220       230       240
GCTTCGCCTCGGACGTCTTCTCCCCCGAGGACCCCAACCAGTCGCCGGTGGCCCGCAAGCTCCTGGAACGCGACGACGTCGTTGTCAGCTCCCACCGCGCGTTCCTTTCCGCCGAGTCGG
   F  A  S  D  V  F  S  P  E  D  P  N  Q  S  P  V  A  R  K  L  L  E  R  D  D  V  V  V  S  S  H  R  A  F  L  S  A  E  S  E

       250       260       270       280       290       300       310       320       330       340       350       360
AGGAGAGCCTGCGCCGCCGTGTGGCCGAGGGGGTTCGCTCGGTGCTCCGGGACGGTAACCCGCCCGCCGAGGGCCGCGTGGCATGACCCCAACCAACACAGCACCGCACGTCCCCCATTG
   E  S  L  R  R  R  V  A  E  G  V  R  S  V  L  R  D  G  N  P  P  A  E  G  R  V  A  ***

       370       380       390       400       410       420       430       440       450       460       470       480
GAGGCACTCATGAACGCGACGGAGCAGGCAGCGAACGGCGACCGGGGCACCACACGATCGGCCGGCGGCCGGCTCAGGTACCTGCTGCACGCCCCAGGCGCCTGTCAGCTGATGGGTGTG
SD        M  N  A  T  E  Q  A  A  N  G  D  R  G  T  T  R  S  A  G  G  R  L  R  Y  L  L  H  A  P  G  A  C  Q  L  M  G  V

       490       500       510       520       530       540       550       560       570       580       590       600
CACGACGGACTGAGCGCGCGGATTGCCGTCGCCGAGGGGTTCGAGGCGCTGTGGGCGTCCGGCCTGTGCATGTCCACGGCGCGTGGCGTCCGGGACAGCGATGAAGCGTCCTGGACGGAA
H  D  G  L  S  A  R  I  A  V  A  E  G  F  E  A  L  W  A  S  G  L  C  M  S  T  A  R  G  V  R  D  S  D  E  A  S  W  T  E

       610       620       630       640       650       660       670       680       690       700       710       720
CTGCTCACGCTGGTCGGCACCATGACCGACGCCGTCCCCGGCGTGCCCGTCCTCGTCGACGGTGACACCGGATACGGCAACTTCAACACCGCCCGCCGGTTCGCCGGCCGCGCGGAGCGG
L  L  T  L  V  G  T  M  T  D  A  V  P  G  V  P  V  L  V  D  G  D  T  G  Y  G  N  F  N  T  A  R  R  F  A  G  R  A  E  R

       730       740       750       760       770       780       790       800       810       820       830       840
GTCGGCGCGGCCGGGGTGTGCTTCGAGGACAAGGTCTTCCCGAAGATGAACTCCTTCTTCGGCGACGGCCACCAGCTGGCCCCGGTCGCCGAGTTCTGCGGAAAGATCCGGGCCTGCAAG
V  G  A  A  G  V  C  F  E  D  K  V  F  P  K  M  N  S  F  F  G  D  G  H  Q  L  A  P  V  A  E  F  C  G  K  I  R  A  C  K

       850       860       870       880       890       900       910       920       930       940       950       960
GACGCCCAGCGTGACCCGGACTTCGTCGTGGTGGCGCGGACCGAGGCACTGATCTCCAAACTGCCGATGGAAGAGGCGCTGGACCGGGCCGCGGCCTACGCCGAGGCCGGCGCCGACGCC
D  A  Q  R  D  P  D  F  V  V  V  A  R  T  E  A  L  I  S  K  L  P  M  E  E  A  L  D  R  A  A  A  Y  A  E  A  G  A  D  A

       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
CTGTTCATCCACTCCCGGATGAACACCCCGCAGCAGATCGCCACCTTCATGGAGCGCTGGGAGGGCAGTACTCCCGTCCTGATCGCGCCCACCACCTACCACACGCCGTCCGTCGACGAC
L  F  I  H  S  R  M  N  T  P  Q  Q  I  A  T  F  M  E  R  W  E  G  S  T  P  V  L  I  A  P  T  T  Y  H  T  P  S  V  D  D

      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
TTCGCCGCGCTCGGCATCGCCGGCTGCATCTGGGCCAACCACAGCATGCGGGCCGCGTTCGCCGCGATGCGTGACGTCTGCCAGCGGATCCGCACCGACCGCGGCATCTACGGCATCGAG
F  A  A  L  G  I  A  G  C  I  W  A  N  H  S  M  R  A  A  F  A  A  M  R  D  V  C  Q  R  I  R  T  D  R  G  I  Y  G  I  E

      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310
GATCAGGTGGCGCCCCTGAAGGAGATCTTCGGCCTCTTCGACTACGAGGGCCTGGAGAAGGACGAGAACTGCTACACACAGGCCCCCGACCTGGCCGCGGTGCAGGGATGATCA
D  Q  V  A  P  L  K  E  I  F  G  L  F  D  Y  E  G  L  E  K  D  E  N  C  Y  T  Q  A  P  D  L  A  A  V  Q  G  ***
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The deduced amino acid sequence of the enzyme is shown under the nucleotide sequence. The partial amino acid sequences of an other predicted gene is written in italic type. This nucleotide sequence data is in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under the accession number D10016.

$CoCl_2$ 0.0001%, and Silicon KM-72 antifoam (Shinetsu Chem. Co.) 0.01%, (pH adjusted to 7.0) in 500-ml Erlenmeyer flasks at 27°C for 6 days with shaking. The mycelium collected by centrifugation was suspended in 50 mM Tris-HCl buffer (pH 7.5) at a ratio of 1 g wet weight of mycelium/2 ml buffer and then disrupted by sonication for 10 minutes at 4°C. 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[2].

S. lividans 66(pMSB32) exhibited high PEP phosphomutase activity (0.62 unit/mg protein, see ref.[2] for the definition of the unit), comparable to that in BA-producing *S. hygroscopicus* SF1293. It thus seems that the 1.3-kb *Bcl* I fragment of pMSB32 encodes PEP phosphomutase. On the other hand, *S. lividans* 66(pMSB32) with the PEP phosphomutase gene oriented oppositely to the direction of *aph* gene transcription did not show any of the enzymatic activity. The subcloned 1.3-kb fragment probably lacks a functional promoter region and the PEP phosphomutase gene is expressed under the control of the *aph* gene promoter in *S. lividans* 66(pMSB33). Of course, another possibility is that the *S. hygroscopicus* promoter is not functional in *S. lividans*.

The 1,314-bp fragment was subcloned into pUC18 and pUC19 and its nucleotide sequence was determined by the dideoxy chain termination method[7]. As shown in Fig. 3, an open reading frame of 313 codons, starting at 370th nucleotide and terminating at 1,308th nucleotide, was identified. The molecular weight of the encoded protein was calculated to be 33,700 dalton. A ribosomal binding site-like sequence, GGAGG, is present at the position 6-bp upstream from the putative initiation codon, ATG. In the region upstream of the PEP phosphomutase gene, the *C*-terminal portion of another open reading frame can be seen (presumably the step 4 gene[4] of BA biosynthesis).

Recently, a Harvard group reported the nucleotide sequence of a PEP phosphomutase gene of *Tetrahymena pyriformis*[8] producing 2-aminoethylphosphonic acid. There is significant similarity between the PEP phosphomutases from *Tetrahymena pyriformis* and *S. hygroscopicus* (50.3% identity over a 180 residue overlap, data not shown).

Fig. 4. Homology between the amino acid sequences of PEP phosphomutase (PEP PM) and CPEP phosphonomutase (CPEP PM).

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PEPPM   MNATEQAANGDRGTTRSAGGRLRYLLHAPGACQLMGVHDGLSARIAVAEGFEALWASGLC   60
                             .* *..**.   . ...*.***..  ..** *.  .*
CPEPPM               MAVTKARTFRELMNAPEILVVPSAYDALSAKVIQQAGFPAVHMTG--   45

PEPPM   MSTARGVRDSDEASWTELLTLVGTMTDAV--PGVPVLVDGDTGYGNFNTARRFAGRAERV  118
         .*. .. .  . ..*.. . . .... *   .***..*.*.****  ...* .   ***
CPEPPM  SGTSASMLGLPDLGFTSVSEQAINLKNIVLTVDVPVIMDADAGYGNAMSVWRATREFERV  105

PEPPM   GAAGVCFEDKVFPKMNSFFGDGHQLAPVAEFCGKIRACKDAQRDPDFVVVARTEALISKL  178
        * .*  .**.* **. . . .*..* ...*. *** *  .*. * **...***.*  ...
CPEPPM  GIVGYHLEDQVNPKRCGHL-EGKRLISTEEMTGKIEAAVEAREDEDFTIIARTDA-RESF  163

PEPPM   PMEEALDRAAAYAEAGADALFIHSRMNTPQQIATFMERWEGS-TPVLIAPTTYHTPSVDD  237
         ..**. *. .*..**** .*......  ...    .. ...  . ... .. .  ....
CPEPPM  GLDEAIRRSREYVAAGADCIFLEAMLDV-EEMKRVRDEIDAPLLANMVEGGKTPWLTTKE  222

PEPPM   FAALGIAGCIWANHSMRAAFAAMRDVCQRIR---TDRGIYGIEDQVAPLKEIFGLFDYEG  294
        ....* .  *..  . .** ...*..  .*    *.. ...   .  .. *.*..*.*.
CPEPPM  LESIGYNLAIYPLSGWMAAASVLRKLFTELREAGTTQKFWDDMGLKMSFAELFEVFEYSK  282

PEPPM   LEKDENCYTQAPDLAAVQG                                          313
        ... *. .....*
CPEPPM  ISELEARFVRDQD                                                295
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The conserved amino acids are marked by asterisks. The numbers indicate the positions of the last amino acid in each line.

As shown in Fig. 4, the amino acid sequence of PEP phosphomutase deduced from its nucleotide sequence showed approximately 26% homology to carboxyphosphonoenolpyruvate phosphomutase[1,9], another C-P bond forming enzyme that catalyzes a reaction analogous to the one carried out by PEP phosphomutase in the BA biosynthetic pathway (Fig. 1, B). The derived amino acid sequence of the PEP phosphomutase gene of *S. hygroscopicus* has no significant similarity to any other sequences in the SWISS Prot protein sequences database.

High expression of this enzyme in *S. lividans* will facilitate the studies on the unique reaction mechanism(s) of C-P bond formation.

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