

# A Phosphonate-Induced Gene Which Promotes *Penicillium*-Mediated Bioconversion of *cis*-Propenylphosphonic Acid to Fosfomycin

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*Penicillium decumbens* is able to epoxidize *cis*-propenylphosphonic acid (cPA) to produce the antibiotic fosfomycin [FOM; also referred to as phosphonomycin and (–)-*cis*-1,2-epoxypropylphosphonic acid], a bioconversion of considerable commercial significance. We sought to improve the efficiency of the process by overexpression of the genes involved. A conventional approach of isolating the presumed epoxidase and its corresponding gene was not possible since cPA epoxidation could not be achieved with protein extracts. As an alternative approach, proteins induced by cPA were detected by two-dimensional gel electrophoresis. The observation that a 31-kDa protein (EpoA) was both cPA induced and overaccumulated in a strain which more efficiently converted cPA suggested that it might take part in the bioconversion. EpoA was purified, its amino acid sequence was partially determined, and the corresponding gene was isolated from cosmid and cDNA libraries with oligonucleotide probes. The DNA sequence for this gene (*epoA*) contained two introns and an open reading frame encoding a peptide of 277 amino acids having some similarity to oxygenases. When the gene was subcloned into *P. decumbens*, a fourfold increase in epoxidation activity was achieved. *epoA*-disruption mutants which were obtained by homologous recombination could not convert cPA to FOM. To investigate the regulation of the *epoA* promoter, the bialaphos resistance gene (*bar*, encoding phosphinothricin acetyltransferase) was used to replace the *epoA*-coding region. In *P. decumbens*, expression of the *bar* reporter gene was induced by cPA, FOM, and phosphorous acid but not by phosphoric acid.

Microbial conversion of precursors obtained by chemical synthesis is an attractive method for drug manufacture because it allows efficient stereoselective biosynthesis. Moreover, if genes involved in microbial conversion are identified, the converting activity can be markedly increased by gene manipulation. This study was undertaken to identify a *Penicillium decumbens* epoxidase that converts the chemically synthesized substrate *cis*-propenylphosphonic acid (cPA) into the commercially important antibiotic fosfomycin (FOM) (Fig. 1).

FOM, first discovered in cultures of *Streptomyces fradiae* ATCC 21096, is structurally characterized by a phosphonate group, a carbon-phosphorus (C-P) bond, and an epoxy ring (2, 5). FOM inhibits the initial reaction in the biosynthesis of prokaryotic peptidoglycans (11) and thus has broad-spectrum antibiotic activity against gram-positive and gram-negative bacteria (24). FOM prepared by chemical epoxidation of cPA (2) yields a racemic mixture and thus requires a costly separation process to isolate the L-stereoisomer. In 1971, White et al. discovered that many *Penicillium* species could catalyze epoxidation of cPA (23). The microbial process had the advantage that the L-stereoisomer could be produced selectively.

We wanted to employ *P. decumbens* for commercial application of this bioconversion. However, the natural isolate had low conversion activities which could not be easily improved by mutation and screening. Furthermore, the classical approach of isolating the presumed rate-limiting enzyme and using re-

verse genetics to identify and overexpress the corresponding gene could not be used since cPA epoxidase was not detected in vitro. As an alternative strategy, we presumed that the enzyme(s) involved would accumulate in cultures most actively carrying out the conversion. Such proteins were identified by two-dimensional (2D) gel analyses. When the corresponding gene, *epoA*, was cloned by reverse genetics and reintroduced into *P. decumbens*, the goal of increasing cPA conversion yields was achieved. The similarity of the predicted EpoA protein sequence to those of other oxidases and induction of the corresponding promoter by phosphorous acid suggested that it was the cPA oxidase.

## MATERIALS AND METHODS

**Purification of the proteins induced by cPA and determination of their amino acid sequences.** A spore stock of *P. decumbens* was inoculated into 30 ml of SM-1 medium (glucose, 3%; nutrient broth, 0.8%; yeast extract, 0.2%; malt extract, 0.3%; pH 7.0) and cultured in a 250-ml flask at 28°C for 48 h with shaking. This culture (1 ml) was transferred to 30 ml of P6-1 medium (wheat germ, 3.3%; corn steep liquor, 0.8%; NH<sub>4</sub>NO<sub>3</sub>, 0.2%; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 0.025%; glucose, 10%), and cultured in a 250-ml flask at 28°C for 48 h. Mycelia were harvested by centrifugation and resuspended in 20 mM Tris-HCl buffer (pH 7.5). A cell extract was obtained by sonication (with a Sonifier Cell Disruptor 350; Branson Sonic Power Company) and subjected to 2D gel electrophoresis with a Multiphor II (Pharmacia Biotech) system. Electrophoresis was performed with Immobiline Dry Strip (pH 4 to 7) (Pharmacia Biotech) in the first dimension and with ExcelGelR sodium dodecyl sulfate gradient 8-18 (Pharmacia Biotech) in the second dimension. The protein was transferred to a polyvinylidene difluoride membrane (ProBlott; Perkin-Elmer) and applied to a model 492 protein sequencer (Perkin-Elmer), and the N-terminal amino acid sequence was determined by Edman degradation. The internal amino acid sequence of the protein was determined after digesting the protein adsorbed to the polyvinylidene difluoride membrane with lysyl endopeptidase (Wako Pure Chemical Industries, Ltd.) and fractionating peptides with a model 172  $\mu$ -Preparative high-performance liquid chromatography (HPLC) chromatograph (Perkin-Elmer).

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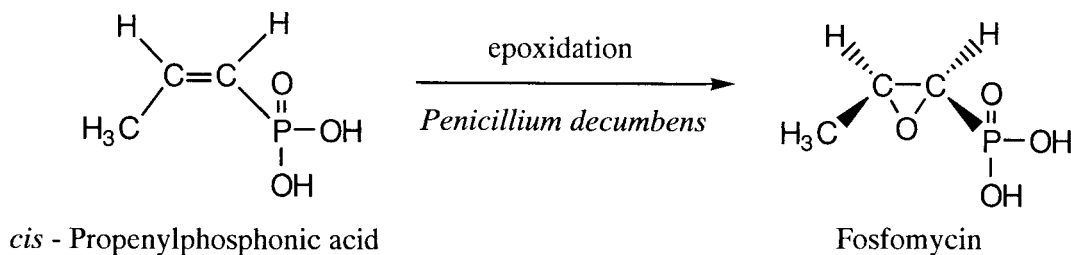


FIG. 1. Conversion of cPA to fosfomycin.

**Design of the synthetic DNA primer for PCR.** The mixed oligonucleotides N-1 (5'-AC[T/A]CCXGA[G/A]CA[G/A]AT[C/T/A]GC-3') and N-2 (5'-AC[C/G]C-CXGA[G/A]CA[G/A]AT[C/T/A]GC-3'), corresponding to the N-terminal amino acid sequence of *epoA* (T-P-E-Q-I-A), were used as forward primers. The mixed oligonucleotides 15-1 (5'-GC[C/T]TG[G/A]AAXCC[G/A]TT[T/A]CC-3') and 15-2 (5'-GC[C/T]TG[G/A]AAXCC[G/A]TT[C/G]CC-3'), corresponding to the internal sequence (G-N-G-F-Q-A), were used as reverse primers for PCR amplification of the nucleotide sequence corresponding to peptide 15. X is inosine.

**Cosmid cloning of genomic DNA.** *P. decumbens* HP147 mycelia (about 10 g) grown in SM-1 medium were washed twice with 20 mM Tris-HCl buffer (pH 7.5), frozen with liquid nitrogen, and fragmented with a homogenizer (Nihonseiki Kaisha, Ltd.). Chromosomal DNA was extracted (18), digested partially with *Sau3A*I, and ligated into the *Bam*HI site of cosmid pCRB8 (constructed and provided by K. Gomi [4a]). Packaging was done with a lambda DNA in vitro packaging kit (Amersham), and libraries were screened with an ECL direct nucleic acid labelling and detection kit (Amersham).

**cDNA library construction, screening, and DNA sequencing.** Cultures were grown and lysed as described above in "Purification of the proteins induced by cPA and determination of their amino acid sequences." Poly(A)<sup>+</sup> RNA was prepared with a QuickPrep mRNA purification kit with procedure C provided by the supplier (Pharmacia Biotech). The cDNA library was prepared in lambda gt11 with 5 µg of poly(A)<sup>+</sup> RNA isolated from cPA-induced cultures. cDNA was made with a TimeSaver cDNA synthesis kit (Pharmacia Biotech). Screening was performed with ECL direct nucleic acid labelling and detection systems with a PCR product as probe. The target fragment from the positive clone was subcloned into the *Eco*RI site of pUC118 (pFOC2), and its nucleotide sequence was determined.

**Transformation of *P. decumbens*.** *P. decumbens* LP3 spores were inoculated into 40 ml of S-1 medium (glycerol, 3%; nutrient broth, 0.8%; malt extract, 0.3%; yeast extract, 0.2%; sodium glutamate, 0.1%) and cultured in a 250-ml flask at 28°C for 48 h. This culture (2 ml) was diluted into a similar flask containing 40 ml of S-1 medium and grown for an additional 24 h. Mycelia in the culture were collected by centrifugation (this and subsequent centrifugations were carried out at 2,500 × *g* for 10 min), suspended in 35% sorbitol, and centrifuged again. Protoplasts were prepared by incubating the mycelia at 34°C for 3 h with gentle agitation in a solution containing 3 mg of achromopeptidase (Wako Pure Chemical Industries, Ltd.) per ml, 3 mg of lysing enzyme (Sigma) per ml, and 1 mg of chitinase (Sigma) per ml. After digestion, mycelia were removed by filtration through cotton, and protoplasts were recovered by centrifugation and suspended in 10 ml of 0.5 M sucrose containing 10 mM Tris-HCl buffer (pH 7.5) and 10 mM CaCl<sub>2</sub> (0.5 M sucrose buffer). This protoplast suspension (100 µl) was mixed with 10 µl of DNA (10 µg) and 400 µl of polyethylene glycol solution (60% polyethylene glycol 4000, 10 mM Tris-HCl buffer [pH 7.5], 10 mM CaCl<sub>2</sub>). Ten milliliters of 0.5 M sucrose buffer was then added, and the mixture was stirred and centrifuged. The pellet obtained was resuspended in 1 ml of 35% sorbitol and spread on protoplast regeneration medium (potato dextrose agar [Difco], 3.9%; raffinose, 30%; hygromycin B, 25 µg/ml). A soft agar medium (2 ml; potato dextrose agar, 1.3%; raffinose, 30%) was poured over the surface, and the culture was incubated at 26°C for 5 days.

**Construction and verification of *epoA*-disruption mutants.** *P. decumbens* LP3 was transformed (see "Transformation of *P. decumbens*," above) with pFOM13, a plasmid containing its homologous *epoA* locus in which the structural gene was disrupted with the hygromycin resistance cassette (*hgt*). Transformants were selected with hygromycin (see "Transformation of *P. decumbens*," above), purified, and grown in S-1 liquid medium (see "Purification of the proteins induced by cPA and determination of their amino acid sequences," above). Genomic DNA was extracted and analyzed by Southern hybridization (see "Southern blot hybridization," below).

**Evaluation of the cPA-converting activity of transformed strains.** *P. decumbens* was grown as described above ("Purification of the proteins induced by cPA and determination of their amino acid sequences"). After cPA was added to a final concentration of 4 mg/ml, cultivation was continued at 28°C for an additional 7 days. The culture was centrifuged, and FOM was assayed in the super-

natant by ion chromatography (column, TSKgel IC-Anion-PWXL; eluent, TSKeluent IC-Anion-A [Tosoh Co.]).

**Southern blot hybridization.** For Southern hybridization (20), DNA probes were labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by random priming. Hybridization was carried out at 42°C in DIG Easy Hyb solution buffer (Boehringer Mannheim). Membranes were washed twice at 68°C in 2× SSC (1× SSC is 0.15 M NaCl and 15 mM C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>) and twice in 0.1× SSC containing 0.1% sodium dodecyl sulfate. The hybridized probes were immunodetected with antidigoxigenin according to the procedure provided (Boehringer Mannheim).

**Measurement of the *bar*-inducing activity.** A spore stock of *P. decumbens* LP3/pFOM8 was used to inoculate 30 ml of SM-1 medium. The culture was incubated at 28°C for 48 h with shaking, diluted 1/30 into P6-1 medium, and cultured at 28°C for 48 h. Phosphonates were added, and mycelia were collected 24 h later and used to prepare a cell extract as described above ("Purification of the proteins induced by cPA and determination of their amino acid sequences"). Phosphinothricin acetyltransferase (PAT) encoded by the *bar* gene was measured with phosphinothricin as the substrate at room temperature (21), and the specific activity (micromoles per minute per milligram of protein) was calculated from the rate of change in molar absorption coefficient of 5,5'-dithio-bis(2-nitrobenzoic acid) (13.6 × 10<sup>6</sup>).

**Bacterial strains and reagents.** cPA was chemically synthesized and used after purification (98.5% homogeneity). Other phosphonates were provided by Sigma Chemical Company. Concentrated aqueous stock solutions of these compounds were neutralized with NaOH and sterilized with a 0.45-µm-pore-size Millex filter unit.

*P. decumbens* LP3, a soil isolate, was subjected to repeated rounds of mutagenesis and screening to isolate a derivative able to convert cPA to FOM more efficiently (HP147).

**Nucleotide sequence accession number.** The nucleotide sequence described in this paper (2,329-bp *Hind*III-*Xba*I fragment) has been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases and assigned the accession number D73371.

## RESULTS

**Identification of proteins induced by cPA and determination of their amino acid sequences.** Although repeated attempts to assay cPA-epoxidizing activity in crude extracts were fruitless, two observations suggested a novel approach to identifying the enzyme(s). Bioconversion rates observed in *P. decumbens* cultures pregrown in cPA were apparently higher than those observed in cultures without cPA preinduction. Secondly, cPA conversion was much more efficient in the improved strain, HP147, than in the original soil isolate. Therefore, 2D gels were used to identify proteins which either were induced by cPA or accumulated at higher levels in HP147 than in its parent (wild-type *P. decumbens* LP3). Representative gels are shown in Fig. 2.

Comparison of uninduced HP147 (Fig. 2b) with induced HP147 (Fig. 2a) revealed three cPA-induced proteins (A, B, and C). All had the same apparent molecular mass of 31 kDa but different isoelectric points. Two of these cPA-induced spots (B and C) were also present in higher concentrations in the more efficient converter strain HP147 (Fig. 2a) than in its wild-type parent, *P. decumbens* LP3 (Fig. 2c).

Peptide sequence analysis was carried out as a first step toward isolation of the gene(s) corresponding to these proteins. The first 10 amino acid residues of the N termini of these

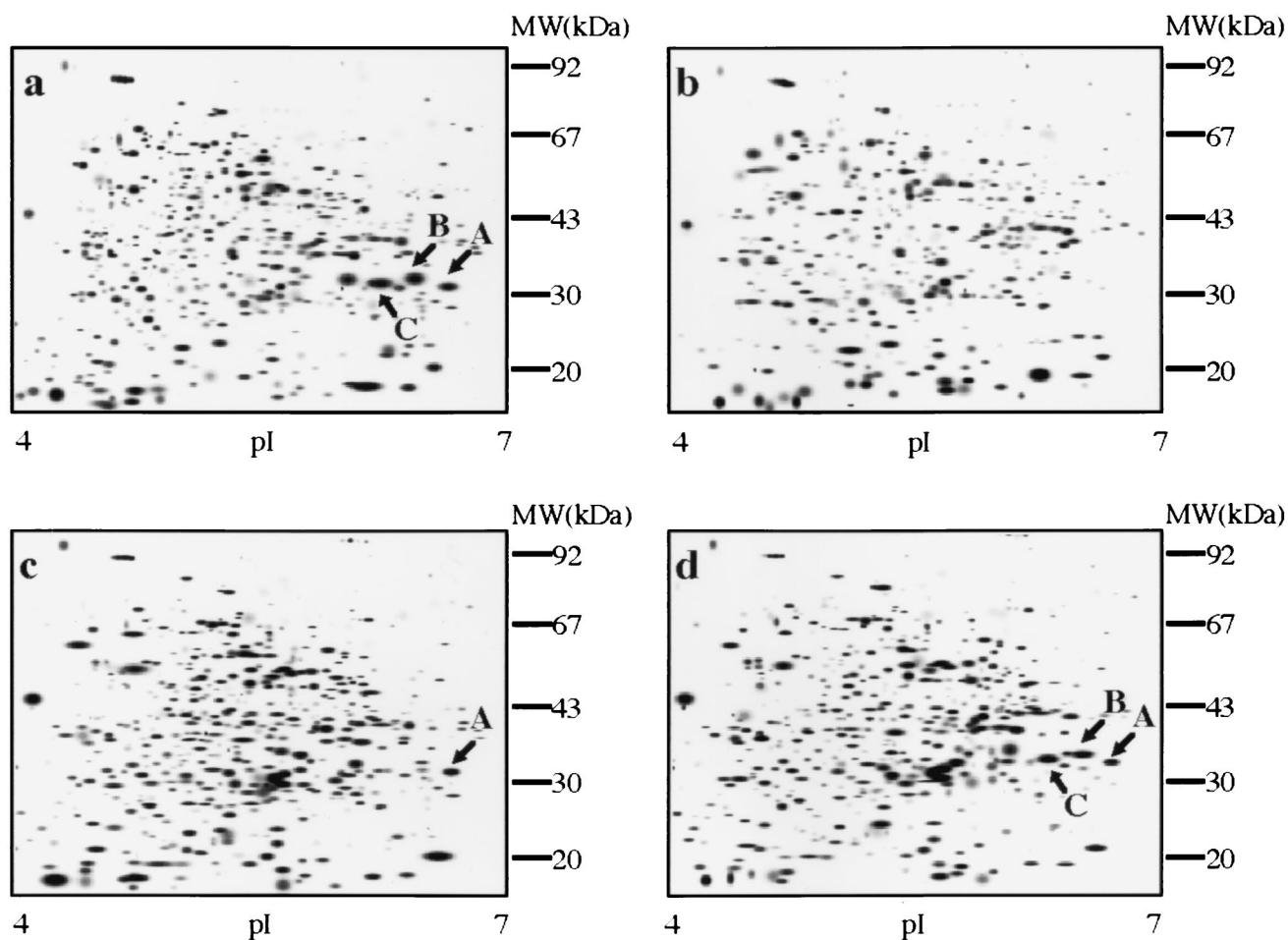


FIG. 2. cPA induction of *P. decumbens* protein spots. *P. decumbens* wild-type LP3 (c), a higher-producing derivative (HP147) (a and b), and LP3 transformed with a plasmid containing *epoA* (pFOM4) (d) were induced with cPA (a, c, and d) and compared to an untreated HP147 control (b). In other words, panels a to d show HP147 plus cPA, HP147, LP3 plus cPA, and LP3/pFOM4 plus cPA, respectively. Protein spots were silver stained and then scanned to produce the smoothed gel image with an Image Master system (Pharmacia Biotech) (4). cPA-induced proteins are labelled A, B, and C. MW, molecular mass.

three proteins were identical (MKPLTPEQIA). When spots A and C were digested with lysyl endopeptidase and fractionated by HPLC, the peptide profiles of the two proteins were identical (data not shown). Four of the eluted peptides were isolated, and their amino acid sequences were determined (peptide 3, INYK; peptide 7, DYSEGYRT; peptide 13, SEGLDLRE; and peptide 15, QPHGNGFQAHL DAPAY-DHIG).

To prepare long probes for cloning the corresponding gene (designated *epoA*), PCR was performed with chromosomal DNA of *P. decumbens* HP147 and oligonucleotide probes based on the N-terminal and peptide 15 amino acid sequences. A DNA fragment of about 500 bp was amplified, and its nucleotide sequence was determined (Fig. 3). The fact that this sequence (designated "pep-3 probe") encoded peptide 3 confirmed the amplification of the target gene.

**Cloning and sequencing of *epoA* and corresponding cDNA.** A cosmid bank was prepared from HP147 genomic DNA and screened with the pep-3 probe. Four hybridization-positive cosmid clones were obtained from about 10,000 colonies. Southern hybridization of cosmid DNA showed that the target gene was located on a *HindIII-XbaI* fragment of about 2.3 kb (Fig. 4). This was subcloned into pUC119 to generate plasmid pFOM3 (Fig. 4).

mRNA isolated from *P. decumbens* HP147 was used to prepare a cDNA bank with phage vectors. Three clones which hybridized to the *HindIII/XbaI* fragment were isolated from about 8,000 plaques. All contained a fragment of about 1 kb which was subcloned into pUC118 (pFOC2). Analysis of the nucleotide sequences of pFOC2 and pFOM3 showed that the *HindIII-XbaI* fragment consisted of 2,329 bp and contained an open reading frame encoding 277 amino acids including two introns. This open reading frame encoded amino acid sequences corresponding to the N terminus and various peptides (peptides 3, 7, 13, and 15) in the cPA-induced protein (Fig. 3). A TATAAAT sequence was immediately upstream of the gene, and two CCAAT sequences were in series further upstream (Fig. 3).

**Improved cPA-converting activity of strains transformed with *epoA*.** To examine whether multiple copies of *epoA* isolated from the efficiently converting strain HP147 would improve the cPA-converting activity in its parental strain, wild-type *P. decumbens* LP3 was transformed with pFOM4 (containing the intact *epoA* gene) or pFOM13 (containing an insertionally inactivated *epoA* allele [see below, "Insertional mutagenesis of *epoA*," or Fig. 4]). Ten recombinants were cultured, and their cPA-converting activities were compared with that of the parental strain and *P. decumbens* containing



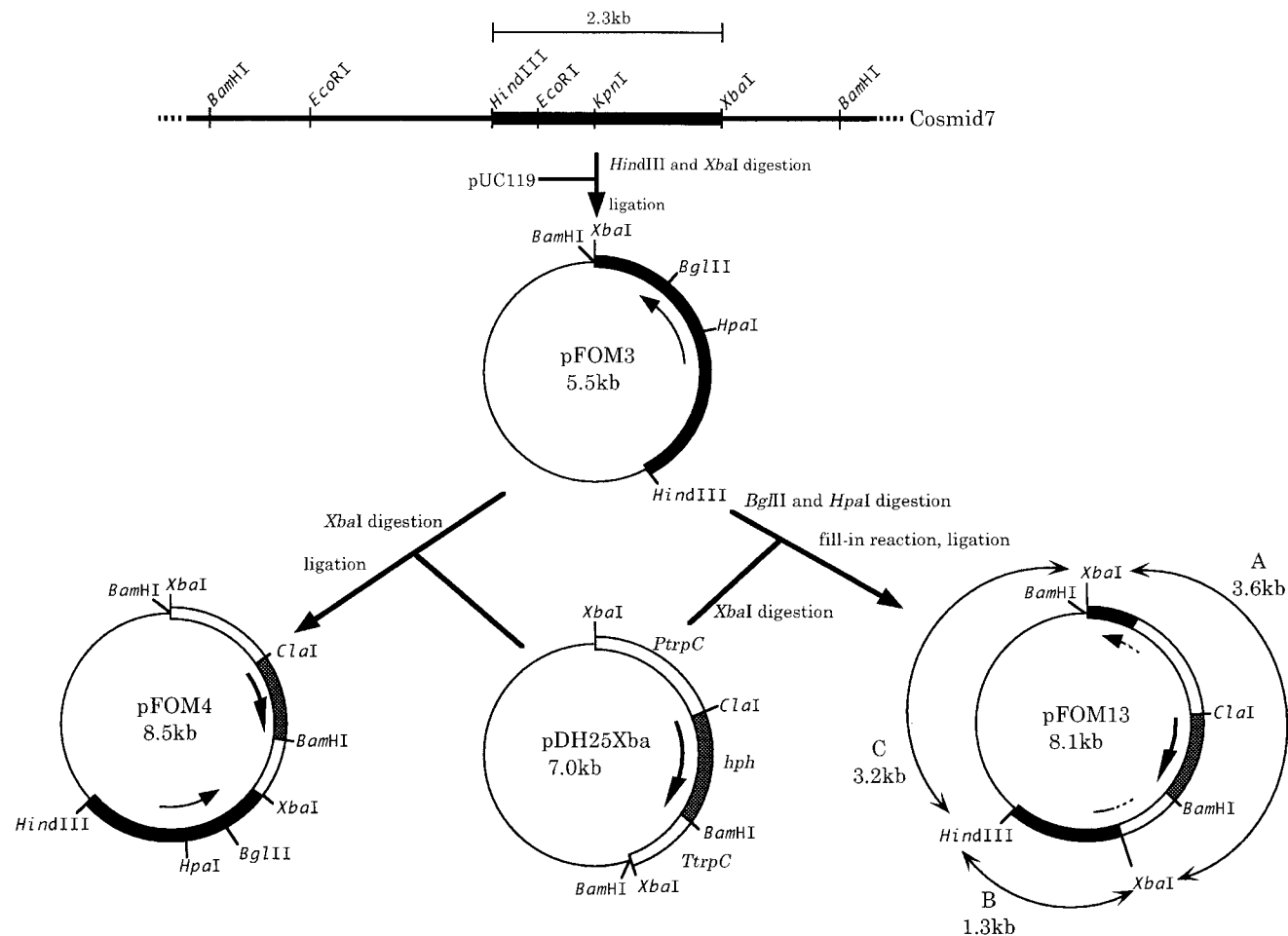


FIG. 4. Construction of the plasmid for cloning and disruption of *epoA*. A HindIII-XbaI fragment (2.3 kb) containing *epoA* was isolated from cosmid 7 and inserted into pUC119 to generate pFOM3. pFOM4 was prepared by inserting the *hph* cassette [on an XbaI fragment isolated from pDH25Xba after EcoRI/XbaI linkers were added to pDH25 (3)] into the XbaI site of pFOM3 located downstream of *epoA*. To prepare pFOM13, pFOM3 was digested to eliminate an HpaI-BglII fragment encoding about 400 bp of the *epoA* gene. The digested plasmid was filled in and ligated to the *hph* cassette (isolated from pDH25Xba as a filled-in XbaI fragment). pFOM XbaI/HindIII fragments are labelled A, B, and C (observed in Southern blot of Fig. 5). Abbreviations: P<sub>trpC</sub>, the *A. nidulans trpC* promoter region; T<sub>trpC</sub>, the *A. nidulans trpC* terminator region; *hph*, the *E. coli* hygromycin B phosphotransferase gene.

pFOM13 (Fig. 4) as a control. The converting activities of the recombinants were 1.43- to 4.28-fold higher than that of the parental strain (*P. decumbens* LP3). *P. decumbens* LP3/pFOM13 was comparable to the parental strain (Table 1).

**Overexpression of cPA-induced protein in transformed strains.** To examine whether cPA-induced proteins were expressed in large amounts of pFOM4-containing strains with improved converting activities, *P. decumbens* LP3 and the recombinant that showed the greatest increase in the converting activity (*P. decumbens* LP3/pFOM4 TF7) were analyzed by 2D gel electrophoresis. Compared to the untransformed host (Fig. 2c), larger amounts of 31-kDa protein spots A, B, and C accumulated in the recombinant (Fig. 2d). Northern blots showed that induction of *epoA* by cPA was at the transcriptional level (data not shown).

**Insertional mutagenesis of *epoA*.** To further elucidate its function, the *P. decumbens* LP3 chromosomal *epoA* gene was inactivated by homologous recombination by the following strategy. First, the HpaI-BglII region (about 400 bp) of *epoA* (Fig. 3) cloned in pFOM3 was deleted and replaced with an *hph* cassette (pFOM13) (Fig. 4). This plasmid was used to

TABLE 1. cPA conversion efficiency of recombinants carrying multiple copies of the cloned *epoA* gene<sup>a</sup>

<i>P. decumbens</i> strain	FOM ( $\mu\text{g/ml}$ )	Increase (fold)
LP3	800	1.00
LP3/pFOM13	760	0.95
LP3/pFOM16	780	0.98
LP3/pFOM4 TF1	1,140	1.43
LP3/pFOM4 TF2	1,150	1.44
LP3/pFOM4 TF3	2,460	3.08
LP3/pFOM4 TF4	1,960	2.45
LP3/pFOM4 TF5	2,020	2.53
LP3/pFOM4 TF6	2,120	2.65
LP3/pFOM4 TF7	3,420	4.28
LP3/pFOM4 TF8	3,090	3.86
LP3/pFOM4 TF9	2,040	2.55
LP3/pFOM4 TF10	2,500	3.13

<sup>a</sup> The efficiency of cPA conversion in *P. decumbens* LP3 was compared to that in transformants containing pFOM4 (TF1 to -10, containing *epoA*), pFOM13 (containing disrupted *epoA*), or pFOM16 (vector control containing the *hph* cassette cloned into the XbaI site of pUC119). cPA was added (final concentration, 4 mg/ml) to *P. decumbens* cultures prepared as described in Materials and Methods. Incubation was continued under the same conditions for 7 days. FOM was assayed by HPLC analysis as described in Materials and Methods.

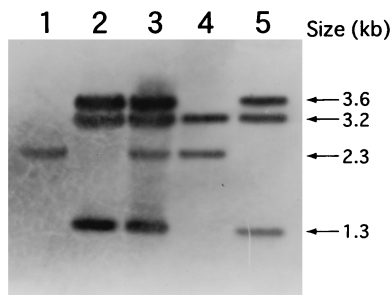


FIG. 5. Southern blot analysis of *P. decumbens* genomic DNA. Genomic DNA from *P. decumbens* transformants was double digested with *Hind*III/*Xba*I, blotted, and probed with pFOM13. Lane 1, genomic DNA from *P. decumbens* LP3; lane 2, genomic DNA from *P. decumbens* LP3/pFOM13 TF1 (cPA bioconversion-negative strain); lane 3, genomic DNA from *P. decumbens* LP3/pFOM13 (cPA bioconversion-positive strain); lane 4, pFOM13 double digested with *Hind*III/*Xba*I; lane 5, pFOM13 double digested with *Hind*III/*Xba*I. Four different *Hind*III/*Xba*I-sized bands were observed: 3.6 kb, identified in Fig. 4 as *hph-epoA* (fragment A); 3.2 kb, the pUC119 vector fragment (Fig. 4, fragment C); 2.3 kb, the nondisrupted *epoA* locus (Fig. 4); and 1.3 kb, *hph* (Fig. 4, fragment B).

transform *P. decumbens* LP3, and 50 hygromycin-resistant transformants were examined for their ability to convert cPA. Four of these could no longer epoxidize cPA to FOM.

Genomic DNA from representative conversion-positive (*P. decumbens* LP3/pFOM13) or conversion-negative (*P. decumbens* LP3/pFOM13 [TF-1]) strains was digested with enzymes that do not cleave the pFOM3 *epoA* locus (*Hind*III/*Xba*I) and analyzed by Southern blot hybridization with pFOM13 as a probe (representative hybridization is shown in Fig. 5; refer to Fig. 4, where fragments are described and labelled A, B, and C). In the conversion-negative strain (*P. decumbens* LP3/pFOM13 TF1 [Fig. 5, lane 2]), the 2.3-kb fragment (Fig. 4) containing the native *epoA* gene (Fig. 5, lane 1) was not detected. Transformants which retained the wild-type conversion activity contained an intact 2.3-kb fragment (Fig. 5, lane 3). This provided unambiguous evidence that the *epoA* gene was mutagenized in conversion-negative strains. Both cPA bioconversion-active and inactive strains contained all pFOM13 fragments (Fig. 5, lane 5; 3.6, 3.2, and 1.3 kb). This indicated the presence of multimeric forms of pFOM13 inserted as tandem copies into the genome (22) at an undefined location. The precise molecular topological changes associated with this mutational event were not studied further.

**Investigation of the function of EpoA.** To further investigate the function of EpoA from the molecular evolutionary point of view, proteins having similar sequences were identified by using BLASTA. These analyses drew our attention to the similarity of EpoA to L-proline 4-hydroxylase of *Dactylosporangium* sp. (DDBJ/EMBL/GenBank accession no. D78338). The alignment was refined by careful visual examination (Fig. 6). We then computed homology between the two aligned sequences. Of 240 positions available for comparison, 75 were occupied by the same amino acids. Taking into account evolutionary constraints (Poisson correction), 37% of the sites were conserved. This value indicates that the two sequences had evolved from a common ancestor. Moreover, as the figure shows, there were many sets of continuous residues at which the two sequences have the same amino acid. In addition, both sequences have an H-DXXXXXH motif (Fig. 6), which forms the active site of a family of oxygenases (17). These results together provide independent supporting evidence that *epoA* encodes an oxygenase.

BLASTA searches did not detect significant homology between EpoA and peroxidases; however, the two most similar

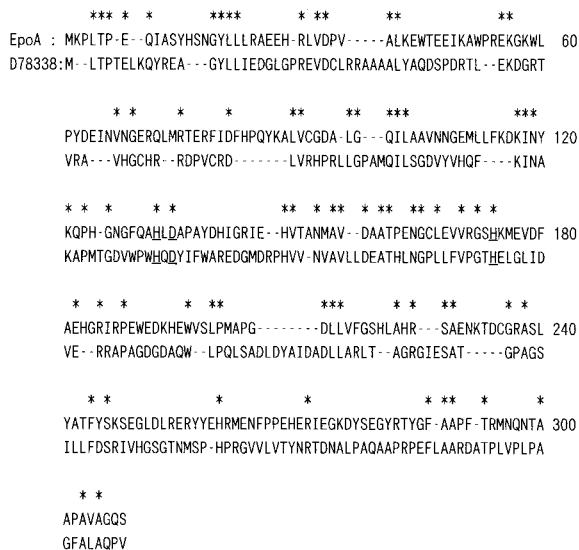


FIG. 6. Alignment of EpoA and L-proline-4-hydroxylase (D78338) sequences. The sequences are represented by the one-letter amino acid code, and a dash refers to a gap. An asterisk above a residue indicates the same amino acid in both sequences. The underlined H-D...H residues in the two sequences form a motif that resembles the active site of oxygenase.

proteins of known function found were hydroxylases (BLAST alignments; L-proline 4-hydroxylase,  $P = 0.06$ ; phytanoyl-coenzyme A alpha hydroxylase [15],  $P = 0.99$ ).

**Regulation of the *epoA* promoter by phosphorus-containing compounds.** To study the regulation of the *epoA* promoter, the structural gene cloned in pFOM4 was replaced with a reporter gene (*bar*) which confers bialaphos resistance (*bar*) to generate pFOM8 (Fig. 7). *bar* gene expression was measured by the activity of its corresponding enzyme, PAT. *P. decumbens* LP3/pFOM8 was used to screen compounds for their ability to induce *bar* expression (Table 2). PAT activity was markedly induced by cPA, FOM, and phosphorous acid (also demonstrated by unpublished Northern blots). Inductions with phosphonoacetic acid, DL-2-amino-3-phosphonopropionic acid, and 2-aminoethylphosphonic acid (9) were much lower (21, 13, and 2%, respectively). No induction of PAT activity was detected with phosphoric acid or phosphocreatine (Table 2). When the putative *epoA* promoter region was deleted (pFOM15 [Fig. 7]), PAT activity was not induced by cPA (*P. decumbens* LP3/pFOM15; Table 2).

## DISCUSSION

In the fermentation industry, empirical approaches involving repeated rounds of mutation and screening are often required to create modified strains for efficient manufacturing of microbial products. Although the activities of some rate-limiting enzymes or transport proteins are undoubtedly increased in these modified strains (1), corresponding biochemical assays can be difficult to establish. In such cases, 2D gel electrophoresis can be used to identify these proteins (8).

We were not able to assay the enzyme system that converts cPA to FOM in *P. decumbens*. To overcome this problem, our approach was based on the principle that enzymes for bioconversions are often induced by corresponding substrates. We focused on three protein spots, apparently products of the same gene, which were induced by cPA under conditions where it was being converted to FOM. The fact that these spots

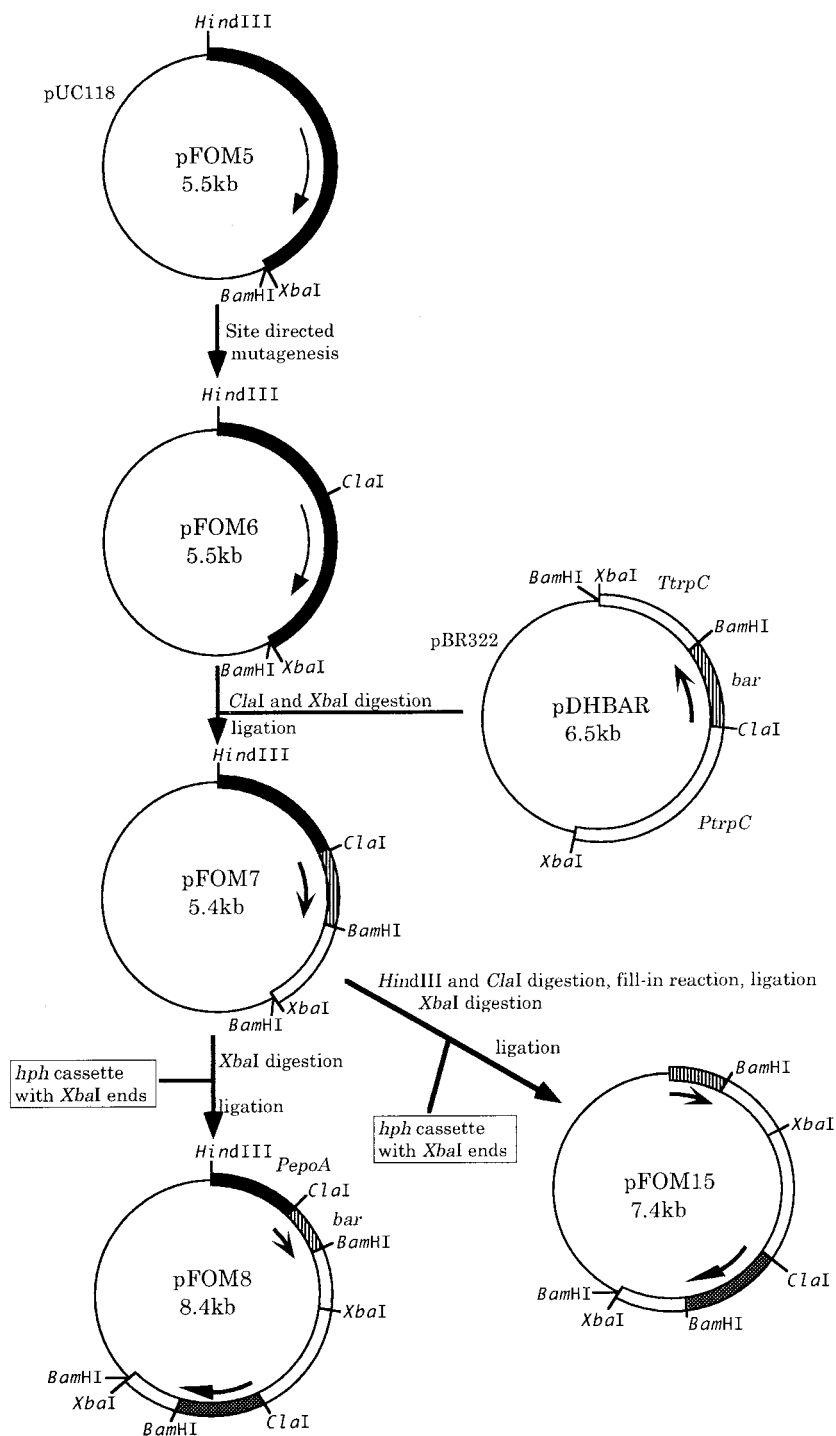


FIG. 7. Construction of transcriptional fusions linking the *epoA* promoter region to the *bar* reporter gene. Plasmid pFOM5 was prepared from pFOM4 (Fig. 4) by ligating a 2.3-kb *Hind*III-*Xba*I fragment including *epoA* into *Hind*III-*Xba*I sites of pUC118. Plasmid pFOM6 was obtained from pFOM5 by introducing a *Cla*I site upstream of the ATG start codon of *epoA* by site-specific mutation (12) with a primer (5'-AAATCCCCCATCGATGAAGCCTC-3'). The *Streptomyces hygroscopicus bar* gene was PCR amplified from pARK22 (19) with two primers, *barU* (5'-AAGGATCGATATGAGCCCAGAACGACGCC-3') and *barR* (5'-GCTTGGATCC TCAGATCTCGGTGACGGGC-3'), which included *Cla*I and *Bam*HI sites. The termini were cut with *Cla*I and *Bam*HI and replaced with *hph* from pDH25 (3) (pDHBAR). Next, a *Cla*I-*Xba*I fragment including *bar* and the *trpC* terminator (*TtrpC*) was removed from pDHBAR and inserted into pFOM6 cleaved with *Cla*I/*Xba*I so as to replace the *epoA* structural gene and put the *bar* reporter gene under the control of the *epoA* promoter (pFOM7). pFOM8 was prepared by inserting a cassette containing the *hph* gene into the *Xba*I site of pFOM7. pFOM15 was prepared by first deleting the putative *epoA* promoter region from pFOM7 by digestion with *Hind*III and *Cla*I and then by using the same procedure to insert the *hph* cassette. Abbreviations: *PepoA*, the *P. decumbens epoA* promoter region; *bar*, the *S. hygroscopicus* bialaphos resistance gene.

TABLE 2. Induction of the *epoA* promoter with phosphonates<sup>a</sup>

<i>P. decumbens</i> strain	Inducer	<i>bar</i> activity
LP3	NA	0
LP3	cPA	0
LP3/pFOM15	NA	0
LP3/pFOM15	cPA	0
LP3/pFOM8	NA	0
LP3/pFOM8	cPA	470
LP3/pFOM8	FOM	192
LP3/pFOM8	Phosphorous acid	365
LP3/pFOM8	Phosphonoacetic acid	98
LP3/pFOM8	DL-2-Amino-3-phosphonopropionic acid	61
LP3/pFOM8	2-Aminoethylphosphonic acid	9
LP3/pFOM8	N,N-Bis(phosphonomethyl)-glycine	9
LP3/pFOM8	Phosphocreatine	0
LP3/pFOM8	Phosphoric acid	0

<sup>a</sup> *P. decumbens* LP3 strains containing pFOM8 (described in Fig. 7; the *epoA* promoter region upstream of the *bar* reporter gene) or pFOM15 (a vector control similar to pFOM8 but without the *epoA* promoter) were cultured and induced (48 h) with phosphonates (10 mM) as described in Materials and Methods. Protein extracts were prepared and assayed for reporter gene (*bar*) expression. *bar* activity, PAT specific activity, expressed as nanomoles of phosphinothricin converted/min/mg of protein. NA, not added.

also overaccumulated in strains that carry out the bioconversion more efficiently further indicated the important role of the corresponding gene, *epoA*. When *epoA* was reintroduced to *P. decumbens* LP3, the converting activity increased as much as 4.3-fold. *P. decumbens* lost the ability to convert cPA to FOM when the *epoA* gene was destroyed by homologous gene replacement (16). Thus, EpoA plays an important role in the epoxidation of this phosphonic acid derivative. cPA can also be converted to FOM in *Pseudomonas* and *Flavobacterium* spp.; however, two enzymes (bromoperoxidase and bromohydrin epoxidase) are required (10). Epoxidation of cPA is not the biosynthetic pathway used in the biosynthesis of FOM in *Streptomyces wedmorensis* (6). Transcriptional regulation of *epoA* gave some clues as to its role in phosphonate catabolism or anabolism.

In order to study cPA-induced transcriptional control, a construction was made in which the *epoA* promoter directed expression of the *bar* gene. This reporter gene was chosen because its product, PAT, is both easy to assay and selectable. The *bar* gene confers resistance to phosphinothricin and is widely employed as a selectable marker, primarily in plants but also in fungi. Thus, selection for increased phosphinothricin resistance in *P. decumbens* containing pFOM8 could be used to select for mutants which overexpress the *epoA* promoter. A subset of these mutants may be second-site, *trans*-acting alleles which also increase expression of the native *epoA* promoter directing transcription of this putative epoxidizing activity.

Here, we used the *bar* reporter construct to screen various substances for their ability to induce expression of the *epoA* promoter. This is the first report of a promoter whose expression can be regulated by phosphorous acid, phosphonates, and diverse derivatives. cPA-like phosphonates which induced the promoter included those that have no double bonds (FOM), one having a shorter carbon chain (phosphonoacetic acid), and several having amino radicals such as 2-aminoethylphosphoric acid.

Interestingly, while phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) did not induce expression, phosphorous acid (H<sub>3</sub>PO<sub>3</sub>) was active. It is reasonable that the reduced, rather than the more oxidized, form of phosphorus induced expression of this promoter, apparently regulating an oxidative conversion. However, we cannot easily

rationalize the fact that both the substrate (cPA) and the putative product of *epoA* (FOM) induced the promoter. Future studies of these curious observations may provide clues to the regulation of phosphonate metabolism, a field where little information is currently available (7, 14).

The TATAAAT sequence characteristic of eukaryotic promoters was found immediately upstream of *epoA*; the regulatory motif CCAAT appeared repeatedly further upstream. These sequences are likely to be involved in expression and induction by cPA. They play a similar role in regulation of *amdS* in *Aspergillus nidulans* (13).

The cPA-converting activity was markedly improved simply by amplifying one gene (*epoA*). Derivatives containing the cloned *epoA* gene (Fig. 2d) accumulated increased levels of three related proteins. Although these three proteins had slightly different molecular weights and isoelectric points, comparison of their N-terminal amino acid sequences and peptide maps (data not shown) suggested that they represented post-translationally modified derivatives of EpoA.

The increase in the cPA-converting activity varied among the recombinants transformed with the same plasmid (Table 1, 1.43- to 4.28-fold). Southern hybridization of genomic DNAs (data not shown) indicated that this phenomenon was probably related to the site of plasmid insertion (a phenomenon commonly observed in fungi) rather than to differences in the number of copies of the plasmid. Further understanding of the complete enzyme system and its coordinated synthesis is expected to allow even greater increases in bioconversion efficiency.

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