Inactivation, Complementation, and Heterologous Expression of \textit{encP}, a Novel Bacterial Phenylalanine Ammonia-Lyase Gene*\textsuperscript{a}

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The enzyme phenylalanine ammonia-lyase, which catalyzes the nonoxidative deamination of \(\text{L-phenylalanine} \rightarrow \text{trans-cinnamic acid}\), is ubiquitously distributed in plants. We now report its characterization for the first time in a bacterium. The phenylalanine ammonia-lyase homologous gene \textit{encP} from the \textit{“Streptomyces maritimus”} enterocin biosynthetic gene cluster was functionally characterized and shown to encode the first enzyme in the pathway to the enterocin polyketide synthase starter unit benzoyl-coenzyme A pathway intermediates or with the wild-type gene \textit{encP} restored the formation of the benzoate-primed polyketide antibiotic enterocin. Heterologous expression of the \textit{encP} gene under the control of the \textit{ermE}\textsuperscript{*} promoter in \textit{Streptomyces coelicolor} furthermore led to the production of cinnamic acid in the fermented cultures, confirming that the \textit{encP} gene indeed encodes a novel bacterial phenylalanine ammonia-lyase.

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5)\textsuperscript{1} is a ubiquitous higher plant enzyme that catalyzes the nonoxidative deamination of the primary amino acid \(\text{L-phenylalanine} \rightarrow \text{trans-cinnamic acid}\). Its product is the precursor of several important classes of plant phenylpropanoids including lignins, flavonoids, and coumarins. These cinnameate-derived natural products are relatively nonexistent in bacteria; however, because of the rarity of PAL in prokaryotes.

Bacteria and animals do carry out a similar enzymatic reaction, the conversion of \(\text{L-histidine} \rightarrow \text{trans-urocanic acid}\) by the highly homologous histidine ammonia-lyase (HAL, EC 4.3.1.3). The recent crystal structure of HAL from \textit{Pseudomonas putida} (1) along with numerous biochemical studies on both enzymes (2, 3) established the reaction mechanism of this novel deamination reaction. The structure revealed that the novel prosthetic group 4-methylidene imidazol-5-one (4, 5) which is formed autocatalytically from the cyclization and dehydration of the active site tripeptide Ala-Ser-Gly, serves as the essential catalytic electrophile of this Friedel-Crafts-type enzymatic reaction (6). Although HALs and PALs have analogous mechanisms of action, PALs are considerably larger homotetrameric proteins (312 versus 215 kDa), suggesting that these related enzymes evolved independently.

We recently cloned and sequenced the putative PAL gene \textit{encP}, which is associated with the enterocin biosynthetic gene cluster, from the sediment-derived bacterium \textit{“Streptomyces maritimus”} (7, 8).\textsuperscript{2} Although the product of \textit{encP} is more homologous in sequence and size to bacterial HALs than to plant PALs, circumstantial evidence suggested that EncP functions as a PAL. The bacteriostatic agent enterocin is biosynthesized by a unique type II polyketide synthase pathway that utilizes a novel cinnameate-derived benzoyl-coenzyme A (CoA) starter unit (Fig. 1) (9). A sequence analysis of the 20 open reading frame \textit{enc} cluster and feeding experiments with labeled precursors demonstrated that benzoyl-CoA is produced in this bacterium in a plantlike manner from phenylalanine via cinnamic acid followed by \(\beta\)-oxidation (10, 11).

To functionally assign \textit{encP} and its role in benzoyl-CoA biosynthesis in \textit{“S. maritimus,”} we developed a genetics system in this bacterium. Here, we report the inactivation, complementation, and heterologous expression of \textit{encP}, a novel bacterial phenylalanine ammonia-lyase-encoding gene.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions—All of the strains and plasmids used in this work are listed in Table I. \textit{“S. maritimus”} strain BD26T was grown as described previously (8). AI medium was used for sporulation, and R2YE medium was used for isolation of genomic DNA. Mutant strains were grown at 37 °C on plates containing 100 \(\mu\text{g/ml} \) apramycin for ~24–30 h until sporulation, whereas the complemented mutant strains were similarly grown with added thiostrepton (50 \(\mu\text{g/ml}\) ). \textit{Escherichia coli} XL1-Blue was used for subcloning and grown on LB plates or in LB liquid medium. \textit{E. coli} S17-1 was used as the host for \textit{E. coli}-“\textit{S. maritimus}” conjugation (12).

DNA Manipulations—\textit{“S. maritimus”} total genomic DNA was isolated as described previously (7). Recombinant DNA procedures were performed by standard techniques (13, 14). Biotin labeling and detection of chemiluminescent positives were performed with the DNA-Detector\textsuperscript{TM} HPR Southern blotting kit (KPL, Inc.). Oligonucleotides were obtained from Sigma Genosys. PCR was carried out on a PTC-2000 thermal cycler (MJ Research) with Taq (Invitrogen) or \textit{Pfu}\textsuperscript{Turbo} (Stratagene) DNA polymerase. DNA sequencing by BigDye terminator cycle sequencing reaction using an ABI 377 sequencer was performed at the Laboratory of Molecular Systematics and Evolution at the University of Arizona. The gene sequence of the enterocin biosynthetic gene cluster including \textit{encP} has been deposited at GenBank under accession number AF259495.

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\textsuperscript{1} The abbreviations used are: PAL, phenylalanine ammonia-lyase; CoA, coenzyme A; HAL, histidine ammonia-lyase; HPLC, high performance liquid chromatography; kb, kilobase pair(s).

\textsuperscript{2} The bacterium \textit{“S. maritimus”} strain BD26T, originally described in Ref. 21, is pending as a new species within the genus \textit{Streptomyces} on the basis of cultural and physiological characteristics and 16 S rDNA analysis (GenBank\textsuperscript{TM} accession number AF233338). The type strain is deposited at the National Center for Agricultural Utilization Research (NRRL B-24122).
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Construction, Analysis, and Complementation of the encP Mutant KP—The encP disruption vector pBM4 was constructed as follows. A 800-bp internal fragment of encP was PCR-amplified from pJP15F11 with the primers 5'-GTCTGCGCCGGCTTGGC-3' and 5'-GTCTGC-CCGGCGGAATTCCTCAG-3' and ligated into pCR2.1-TOPO. The EcoRI fragment from the resulting plasmid was cloned into pKC1139 to create pBM4, which was conjugated into “S. maritimus” as described previously (15). The encP single crossover mutant KP was selected after propagating trans-conjugants on SGGP (16) plates at 37 °C, and apramycin-resistant colonies were confirmed by Southern hybridization with biotinylated encP.

Polyketide production in S. maritimus KP was analyzed by high performance liquid chromatography (HPLC) and electrospray-mass spectrometry as described previously (7). Feeding experiments involved overlaying cinnamic or dβ-benzoic acids (0.5–1 mg/plate).

To facilitate the genetic complementation of the encP mutant, we constructed the conjugal expression plasmid pBM6 by first inserting the 0.8-kb encP promoter DNA fragment. The gene encP was PCR-amplified, blunt end-cloned into pCR2.1-Blunt to yield pBM7, and cloned into the BamHI-XbaI cloning site of pBM6 to create pBM8. The sequence of the 1.679-kb encP PCR product was confirmed by sequencing. The primers for the amplification of encP were 5'-GTCTAGAGGTCTCTCCCTCAGG-3' (reverse) and 5'-GACTTAATTGGGTCGGACG-3' (forward). Plasmids pBM6 (negative control) and pBM8 were introduced into “S. maritimus” KP using the plasmid conjugal transfer method. Transformants were directly selected on A1 plates containing 100 μg/ml apramycin and 50 μg/ml thiolstrepton at 37 °C.

**Production of Cinnamic Acid in Streptomyces coelicolor**—Plasmids pBM6 and pBM8 were introduced into S. coelicolor A3 (2) and S. coelicolor YU105 by protoplast transformation as described previously (14). The nonmethylation E. coli strain ET12567 (17) was used to obtain DNA for transformation of S. coelicolor. The strains were grown on R2YE agar plates containing 20 μg/ml thiolstrepton at 30 °C for up to 7 days. The cultured agar was chopped and extracted with 95:5 EtOAc:MeOH, and the dried crude extract was analyzed by HPLC as described above.

**RESULTS**

**DNA Sequence Analysis of encP**—The encP sequence starting with a methionine start codon encodes a 523-amino acid protein and is preceded by a putative ribosome binding site (5'-AGGGA-3') at -10 to -6. EncP shows greater sequence homology to prokaryotic HALs than to eukaryotic PALs and contains a conserved motif around Ser-143 (Fig. 2), which is the probable precursor of the modified dehydroalanine residue in the 4-methylidene imidazol-5-one prosthetic group (1). Although most HALs and PALs contain either an alanine or cysteine residue adjacent to the active site serine, EncP instead uniquely harbors a threonine residue at this position. Most other HAL active site residues are conserved in EncP. However, a notable exception is Val-83, which in HALs is a conserved histidine residue that coordinates its imidazole group through a hydrogen bond with that of the bound histidine substrate (3). On the other hand, plant PALs carry aliphatic residues such as valine and isoleucine at this position, which is consistent with that of EncP, to provide a hydrophobic environment for the benzene ring of the substrate phenylalanine.

**Construction and Complementation of the encP Knock-out Mutant “S. maritimus” KP**—To establish the in vivo function of the EncP gene product, we developed a genetics system in “S. maritimus” and disrupted encP by single crossover homologous recombination. The pKC1139-based temperature-sensitive plasmid pBM4 was constructed with an internal 0.8-kb fragment of the targeted encP gene. The conjugal transfer of pBM4 from E. coli to “S. maritimus” and growth of the resulting trans-conjugant under selective conditions resulted in the mutant strain KP (Fig. 3). The single crossover event resulted in the tandem duplication of truncated encP genes with vector containing an apramycin resistance gene between the sequences. Southern blot hybridization of genomic DNA from the wild type and mutant with a biotinylated DNA probe carrying encP verified the gene disruption. Predicted band shifts were detected in SphI digests of the total DNA. “S. maritimus” KP did not exhibit any different phenotypes in comparison with the wild-type strain in A1 medium at 37 °C.

**HPLC analysis of an organic extract from the encP-inactivated strain “S. maritimus” KP demonstrated that cinnamic acid and the benzoxo-prime polyketides enterocin and the**

**Bacterial strains and plasmids**

| Strains | Properties or product | Sources or references |
|---------|-----------------------|----------------------|
| “S. maritimus” BD-26T | Wild-type, enterocin | (21) |
| “S. maritimus” KP | BD-26T exconjugant containing pBM4, enterocin+; Am+ | This study |
| S. coelicolor A3(2) | Wild-type | A. Hopwood |
| S. coelicolor YU105 | proA1 argA1 redE60 Δact(ermE) ΔsctE(hyg) | (22) |

**Plasmids**

| Plasmids | Properties or product | Sources or references |
|----------|-----------------------|----------------------|
| pCR2.1-Blunt and -TOPO | Vectors for cloning PCR products | Invitrogen |
| pKC1139 | E. coli-streptomyces conjugal transfer vector; Am+ | (23) |
| pOJ446 | Source of oriT | (23) |
| pWHM3 | E. coli-streptomyces shuttle plasmid | (24) |
| pJP15F11 | pOJ446 cosmid clone containing enc gene cluster | (7) |
| pBM3 | 0.8-kb amplified encP internal fragment cloned into pCR2.1-TOPO | This study |
| pBM4 | 0.8-kb EcoRI fragment from pBM3 cloned into pKC1139 | This study |
| pBM5 | 0.8-kb FsiI fragment containing oriT from pOJ446 cloned into pWHM3 | This study |
| pBM6 | 0.4-kb EcoRI-XbaI fragment containing ermA+ was cloned into pBM5 | This study |
| pBM7 | 1.679-kb amplified encP fragment cloned into pCR2.1-Blunt | This study |
| pBM8 | 1.7-kb BamHI-XbaI fragment containing encP from pBM7 cloned into pBM6 | This study |

**Fig. 1. Conversion of phenylalanine to trans-cinnamic acid and biosynthesis of benzoyl-CoA-derived enterocin in “S. maritimus.”**
wailupemycins were not produced (Fig. 4). Upon supplementation of cinnamate and benzoate to the culture medium, enterocin production was restored albeit at 5 and 14%, respectively, of wild-type levels. The administration of \( {\text{d}}_5 \)-labeled benzoic acid resulted in no dilution of the deuterium label in the resultant enterocin as detected by HPLC mass spectrometry, verifying the complete abolishment of benzoate biosynthesis in the mutant strain.

The \( \text{encP} \) mutant strain KP was next complemented with the wild-type \( \text{encP} \) gene to measure the level of pathway restoration.

The pWHM3-based \( \text{E. coli} \) streptomyecete expression vector pBM8 was constructed with wild-type \( \text{encP} \) under the strong constitutive \( \text{ermE}^* \) promoter with \( \text{oriT} \) to allow conjugation from \( \text{E. coli} \). The complementation of mutant KP with pBM8 resulted in the biosynthesis of cinnamic acid and the complete restoration of enterocin production to wild-type levels (Fig. 4).

**DISCUSSION**

Our data clearly show that the “\( S. maritimus \)” gene \( \text{encP} \), which is associated with the enterocin biosynthesis gene cluster, encodes a rare bacterial phenylalanine ammonia-lyase whose product cinnamic acid is converted to benzoyl-CoA during the biosynthesis of enterocin. Although ubiquitous in plants and found in some fungi, PAL activity has only once before been identified in a bacterium where it appears to catalyze the first step in the biosynthesis of cinnamamide in the actinomycete \( \text{Streptomyces verticillatus} \) (18). More recently, a bacterial tyrosine ammonia-lyase from \( \text{Rhodobacter capsulatus} \) was characterized and shown to be 150 times more catalytically efficient toward L-tyrosine than L-phenylalanine as the substrate (20). Like EncP, there are two amino acid substitutions...
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in the conserved active site residues in the *R. capsulatus* tyrosine ammonia-lyase when compared with HAL enzymes, His-83 to Leu and Glu-414 to Gln (Fig. 2). A biochemical analysis of all aromatic amino acid ammonia-lyases to date reveals that these proteins are homotetramers and that the prokaryotic enzymes are considerably smaller than their eukaryotic counterparts (Fig. 2).

Downstream of the disrupted *encP* gene in mutant KP and separated by 26 nucleotides is the methionine start codon of *encO*. As this gene may be co-transcribed with *encF*, insertional inactivation of *encP* via a single crossover may have given rise to polar effects on the expression of *encO*. The results from the complementation and expression experiments suggest that EncO does not serve a biosynthetic role in the EncP-catalyzed conversion of l-phenylalanine to cinnamic acid. The inability of cinnamate and benzoate supplementation to fully restore wild-type levels in the *encO* mutant rather suggests that *encO* may serve a regulatory role in either cinnamic acid or enterocin biosynthesis. Preliminary data on the inactivation of *encP* indicate that cinnamic acid and enterocin production is dramatically reduced. The gene *encO* may exert feedback control on *encP* whose product funnels the proteinogenic amino acid l-phenylalanine to the dedicated secondary metabolic pathway to polyketide antibiotic enterocin.

Thus, *encO* may represent a novel regulatory gene as its putative 130 amino acid gene product does not resemble any protein in the databases. We are currently examining the *in vivo* and *in vitro* function of this novel gene.

In summary, we have characterized the first phenylalanine ammonia-lyase-encoding gene from a bacterium whose protein product is more homologous in sequence and size to bacterial HALs than to plant PALs. The enzymatic product of the "*S. maritimus*" PAL EncP is *trans*-cinnamic acid, which is the first dedicated pathway intermediate to the *enc* polyketide synthase primer unit benzoyl-CoA.

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