Molecular Cloning and Characterization of Fengycin Synthetase Gene fenB from Bacillus subtilis

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A fengycin synthetase gene, fenB, has been cloned and sequenced. The protein (FenB) encoded by this gene has a predicted molecular mass of 143.6 kDa. This protein was overexpressed in Escherichia coli and was purified to near homogeneity by affinity chromatography. Experimental results indicated that the recombinant FenB has a substrate specificity toward isoleucine with an optimum temperature of 25°C, an optimum pH of 4.5, a $K_m$ value of 922 $\mu$M, and a turnover number of 236 s$^{-1}$. FenB also consists of a thioesterase domain, suggesting that this protein may be involved in the activation of the last amino acid of fengycin.

Fengycin is a lipopeptidic antifungal antibiotic produced by Bacillus subtilis F29-3 (2, 4), consisting of 10 amino acids and having a primary sequence similar to that of plipastatin (10, 16, 24). Mutagenesis and sequencing studies found that fengycin is probably synthesized nonribosomally by peptide synthetases (1, 2). A peptide synthetase may consist of one to several amino acid activation modules for the activation of specific amino acids (9). In each module, there is an amino acid adenylation domain of approximately 500 amino acids, consisting of five highly conserved motifs for ATP binding and for ATPase activity (19). Mutation in the motifs can significantly reduce the activity of amino acid activation (6, 7), indicating that these motifs are indeed essential for peptide synthesis (7).

In a peptide synthetase module, the C-terminal boundary of the activation domain is followed by a thioester formation (20). A transpeptidation step subsequently follows, which transfers the amino acid on ATPase activity (19). In a previous study (2), we 4'-phosphopantetheine, a prerequisite for terminating nonribosomal peptide synthesis (18). In this study, we have cloned, sequenced, and characterized a fengycin synthetase gene, fenB. This gene is involved in the activation of the last amino acid of fengycin.

**Nucleotide sequence of fenB.** In a previous study (2), we identified a 46-kb cosmid clone, pFC660, which contains genes encoding fengycin synthesis. This cosmid consists of three BamHI fragments—B1 (18 kb), B2 (12 kb), and B3 (16 kb) (2). In this study, we have sequenced the entire B2 fragment and found that this fragment is actually 11,459 bp long. In the 3' portion of the fragment, there is a 3,825-bp gene, fenB, which is preceded by a ribosomal binding site and is followed by a putative transcriptional stop signal, which consists of a stem-loop structure and a stretch of T's. The 5' portion of the B2 fragment, ranging from nucleotides (nt) 1 to 6,036, consists of an incomplete open reading frame, which is actually the 3' portion of a 10,488-bp peptide synthetase gene, fenA. The

![Image](https://example.com/image.png)

**FIG. 1.** Expression and purification of His-tagged recombinant FenB. Cell extracts obtained from cells before (lane 1) and after (lane 2) IPTG induction and proteins eluted from His-Bind column (lane 3) were analyzed by SDS-PAGE and stained by Coomassie blue. The top band in lane 2 is overexpressed FenB (140 kDa). The positions of molecular mass markers (M) (in kilodaltons) are shown to the left of the gel.
protein encoded by fenB (FenB) consists of six core sequences (Table 1) and a thioesterase-like domain (GYSAG) which are highly conserved among peptide synthetases (3, 5). The fenB sequence shows 80.6% homology to a gene in the pps operon of B. subtilis 168 (21). Since B. subtilis 168 does not produce fengycin, it is unclear whether the fenB-like gene in strain 168 is functional or whether the proteins encoded by these two genes have the same function.

Expression and purification of FenB. To obtain a sufficient amount of FenB for enzyme analysis, we overexpressed fenB in Escherichia coli M15(pRep4) (Qiagen, Hilden, Germany). This overexpression was accomplished by cloning fenB into an expression vector, pQE60 (Qiagen). The fenB DNA (nt 1 to 3822) was amplified by using primers B1 (5’-ATCCATGGTTAAAAACCAAAAAAAT) and B2 (5’-ACGGATCCATGCTTATTGGGCAGC), which contained an NcoI restriction site and a BamHI restriction site at 5’ ends, respectively. PCR was then performed for 30 cycles, with 1 cycle consisting of 1 min at 94°C, 2 min at 40°C, and 3 min at 72°C. The amplified fragment was cut by BamHI and NcoI and was inserted into the NcoI-BamHI sites of pQE60. FenB expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) treatment. For the purification of FenB, cells were frozen in liquid nitrogen and then were thawed at room temperature. A total of three cycles of freeze-thawing were conducted. Cells were suspended in 4 ml of buffer containing 5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl (pH 7.9) and were sonicated at 0°C for 48 5-s pulses at 10-s intervals with an output control setting at 3 with a sonicator (model UP400A; Ultrasonic Processor Corp., Copiague, N.Y.). Next, cell extract was centrifuged at 15,000 rpm for 60 min at 4°C with a Sorvall SS-34 rotor. FenB in the supernatant was then purified with a His-Bind column (Novagen, Madison, Wis.) (1.5 by 4 cm), and FenB in the fractions was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12) and by staining with Coomassie blue (Merck, Darmstadt, Germany). The expressed FenB has a molecular mass of 140 kDa, as determined by SDS-PAGE (Fig. 1, lanes 2 and 3). The chromatography procedure was able to purify FenB to near homogeneity (Fig. 1, lane 3). In addition, approximately 300 μg of recombinant FenB could be purified from 50 ml of culture.

Substrate specificity. The enzymatic activity of recombinant FenB was determined by an ATP-PPi exchange assay (14) using a reaction mixture containing [32P]tetrasodium pyro-

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**TABLE 1. Comparison of the amino acid sequences conserved in peptide synthetases and FenB**

<table>
<thead>
<tr>
<th>Motif</th>
<th>Conserved sequence</th>
<th>Function</th>
<th>Sequence in fenB</th>
<th>Positions in fenB (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spacer</td>
<td>HHILxDGW</td>
<td>Unknown</td>
<td>HHILMDGGW</td>
<td>435–459</td>
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<tr>
<td>Core 1</td>
<td>LKAGGAYVPID</td>
<td>Unknown</td>
<td>LKAGGTYLPLD</td>
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<tr>
<td>Core 2</td>
<td>YSGTTxGPKGV</td>
<td>ATP binding</td>
<td>SSGTGRPKGV</td>
<td>1,851–1,881</td>
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<tr>
<td>Core 3</td>
<td>GELCIGGxGxARGYL</td>
<td>ATP binding</td>
<td>GELCVGEGVAKGYL</td>
<td>2,409–2,454</td>
</tr>
<tr>
<td>Core 4</td>
<td>YxTDG</td>
<td>ATPase</td>
<td>YRTGD</td>
<td>2,517–2,532</td>
</tr>
<tr>
<td>Core 5</td>
<td>VKIRGxRIELGEIE</td>
<td>ATP binding</td>
<td>IKIRGRKRIEPAIE</td>
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<tr>
<td>Core 6</td>
<td>DNFYxLGGHSL</td>
<td>4’-Phosphopantetheine binding (thioester formation)</td>
<td>DFFALGGHSL</td>
<td>2,982–3,012</td>
</tr>
</tbody>
</table>

* Data taken from Stachelhaus and Marahiel (18, 19).
phosphophangeteinyl group of coenzyme A is responsible for the activation of the last amino acid of fengycin. Our results suggest that FenB is responsible for the activation of the last amino acid of fengycin in the biosynthesis of fengycin (3, 5). A similar system has been isolated from the Chang-Gung Memorial Hospital and by Biological Research Grant NSC-86-2314-B-182-028 from the National Science Council of the Republic of China.

**REFERENCES**


