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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 μM, 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 domain which contains a conserved DNFYxLGGHSL motif for the binding of cofactor 4′-phosphopantetheine (9, 19). After adenylation, the amino acid is transferred to the 4′-phosphopantetheine at the carrier domain (20). A transpeptidation step subsequently follows, which transfers the amino acid on the cofactor of the initiating module to the activated amino acid at the thioester formation domain in the next module to form a peptide (9). This condensation step continues from one module to the other until a complete peptide is synthesized (9). It is thought that peptide synthetases may form a complex in vivo and the amino acid activation modules among the enzymes are connected and aligned colinearly with the sequence of the amino acids in the antibiotic (8, 18), thereby allowing an antibiotic with the correct sequence to be sequentially synthesized. A peptide synthetase also consists of a conserved spacer domain which is present at the N-terminal region, upstream from the adenylation domain of each module (4), except for the module activating the initiating amino acid in which the spacer domain is located in the C-terminal end, downstream from the thioester carrier domain (20). In addition, the C terminus of the last module of a peptide synthetase may contain an epimerization domain for the conversion of L-amino acid to D-amino acid (4) and a spacer domain which may be essential for the elongation of peptide. The peptide synthetases involved in the activation of the last amino acid of a peptide usually consist of a thioesterase-like domain in the C-terminal region (3). This domain may be responsible for the release of the peptide from 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 *Bam*HI 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

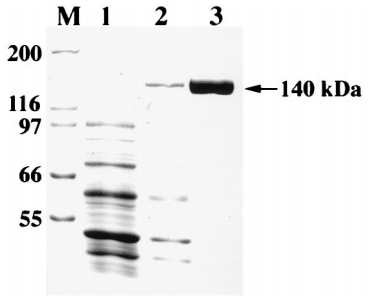

FIG. 1. Expression and purification of His-tagged recombinant FenB. Cell extracts obtained from cells before (lane 1) and after (lane 2) IPTG induction and proteins eluted from His-Bind column (lane 3) were analyzed by SDS-PAGE and stained by Coomassie blue. The top band in lane 2 is overexpressed FenB (140 kDa). The positions of molecular mass markers (M) (in kilodaltons) are shown to the left of the gel.

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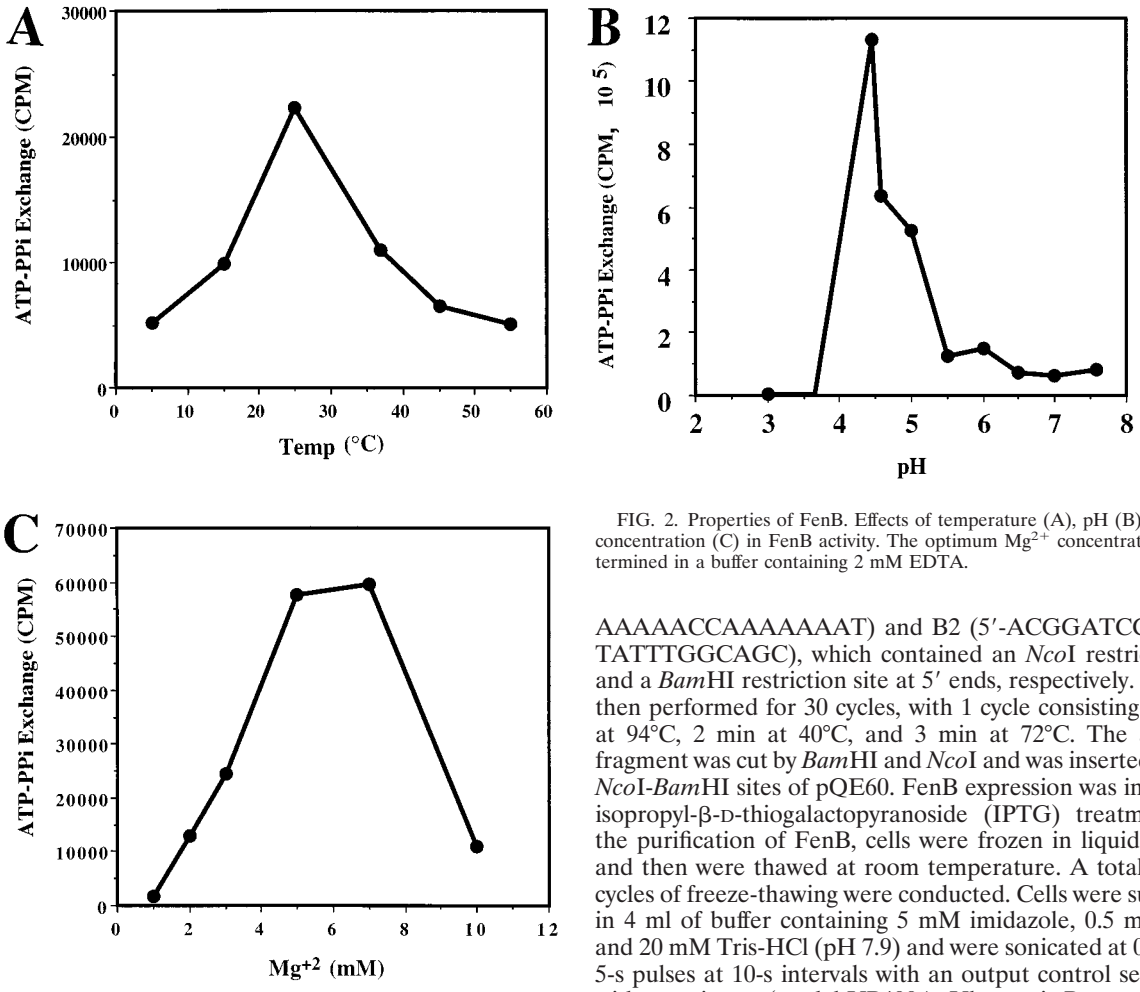

FIG. 2. Properties of FenB. Effects of temperature (A), pH (B), and Mg^{2+} concentration (C) in FenB activity. The optimum Mg^{2+} concentration was determined in a buffer containing 2 mM EDTA.

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′-ATCCATGGTT AAAAACCAAAAAAAT) and B2 (5′-ACGGATCCATGCT TATTTGGCAGC), which contained an *Nco*I restriction site and a *Bam*HI 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 *Bam*HI and *Nco*I and was inserted into the *Nco*I-*Bam*HI 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-PP_i exchange assay (14) using a reaction mixture containing [^{32}P]tetrasodium pyro-

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

Motif[a]	Conserved sequence[a]	Function[a]	Sequence in FenB	Positions in *fenB* (nt)
Spacer	HHILxDGW	Unknown	HHILMDGGW	435–459
Core 1	LKAGGAYVPID	Unknown	LKAGGTYLPLD	1,617–1,650
Core 2	YSGTTGxPKGV	ATP binding	SSGSTGRPKGV	1,851–1,881
Core 3	GELCIGGxGxARGYL	ATP binding	GELCVGGEGVAKGYL	2,409–2,454
Core 4	YxTGD	ATPase	YRTGD	2,517–2,532
Core 5	VKIRGxRIELGEIE	ATP binding	IKIRGKRIEPAEIE	2,559–2,631
Core 6	DNFYxLGGHSL	4′-Phosphopantetheine binding (thioester formation)	DFFALGGHSL	2,982–3,012

[a] Data taken from Stachelhaus and Marahiel (18, 19).

TABLE 2. ATP-PP_i exchange activity of FenB

Amino acid	Exchange activity (cpm)[a]	% Activity
Isoleucine	107,038	100
Glutamic acid	284	0.26
Alanine	3,370	3.1
Proline	1,384	1.2
Ornithine	1,582	1.4
Threonine	2,171	2.0
Tyrosine	1,834	1.7
Valine	13,630	12.7
Blank	1,497	1.4

[a] The reaction mixture consisted of 84 μg of recombinant FenB. The reaction was allowed to proceed for 10 min.

phosphate (9,120 Ci/mmol) (NEN, Boston, Mass.), 2 mM ATP, and 2 mM amino acid. The amino acids used for the assay included the eight amino acids present in the fengycin molecule (Table 2) (10, 25) as well as other common amino acids. Experimental results indicated that adding isoleucine to the reaction mixture produced the highest ATP-PP_i exchange activity (Table 2), i.e., approximately 8- to 70-fold higher than the binding to the other amino acids. Above results suggested that FenB has a substrate specificity toward isoleucine. Previous reports have demonstrated that isoleucine is the last amino acid of the fengycin molecule (10, 25). This finding suggests that FenB is not only responsible for activating the last amino acid of fengycin but also involved in releasing the fengycin molecule from the peptide synthetase. Actually, the amino acid sequence of FenB also reveals that this is indeed the case. In the C-terminal region of FenB, the protein consists of a thioesterase-like domain instead of an epimerase domain, a feature shared by all the peptide synthetases involved in activating the last amino acid of antibiotics (3, 5).

Binding of amino acid to FenB. Covalent binding of amino acid to FenB was examined with 1 μCi of L-[^{14}C]isoleucine (315 mCi/mmol) (Amersham, Buckinghamshire, England) and 11 μg of purified recombinant FenB by the method described by Ullrich et al. (23). The reaction was allowed to proceed for 30 min at 37°C and then was stopped by adding 2 ml of ice-cold 10% trichloroacetic acid. Our results demonstrated that [^{14}C]-isoleucine could bind to FenB covalently and gave a radioactivity reading of 6,725 cpm, whereas the negative control, which lacked ATP in the reaction mixture, had a value of 98 cpm. This binding is specific, since nonradioactive isoleucine, when added in an excessive amount in the reaction mixture, could compete with the binding of radioactive isoleucine to FenB. In theory, binding of isoleucine to FenB requires a prior binding of 4′-phosphopantetheine to the enzyme (11, 17). In the case of surfactin synthetases, this binding is catalyzed by the enzyme encoded by the *sfp* gene in *B. subtilis* (13). A similar gene is also involved in plipastatin synthesis (22). A previous study has demonstrated that approximately 14% of the peptide synthetase expressed in *E. coli* has a phosphopantetheinyl group attached to the enzyme (19). This binding is catalyzed by an *E. coli* enzyme, phosphopantetheinyl transferase (17, 19). Presumably, the phosphopantetheinyl group of coenzyme A is transferred to FenB by the same mechanism and subsequently results in the binding of the [^{14}C]isoleucine to the enzyme. We found that approximately 13% of FenB expressed in *E. coli* bound to the amino acid.

Biochemical characterization of recombinant FenB. The recombinant FenB enzyme had optimum activity at 25°C (Fig. 2A), at pH 4.5 (Fig. 2B), and with a Mg^{2+} concentration between 5 and 8 mM in a buffer containing 2 mM EDTA (Fig. 2C). The activity of FenB at pH 7.0 is approximately 18-fold lower than the activity exhibited under pH 4.5 (Fig. 2B). The low optimum pH for FenB may allow the enzyme to function efficiently in the acidic intracellular environment. Although many peptide synthetases have been isolated from *Bacillus* spp. and characterized biochemically (11, 15, 19), the optimum pHs of these enzymes were not determined in those studies. The activity also decreased when the Mg^{2+} concentration exceeded 10 mM (Fig. 2C). It is likely that a high concentration of Mg^{2+} affects the amount of EDTA, which may be critical in maintaining the enzyme's stability. The recombinant FenB, under optimum conditions, exhibited Michaelis-Menten kinetics, with a K_m for isoleucine of 922 μM and a turnover number of 236 s^{-1}.

In summary, we have sequenced and characterized the fengycin synthetase gene *fenB* from *B. subtilis* F29-3. Experimental results demonstrate that the FenB protein functions as a peptide synthetase which is involved in the nonribosomal synthesis of fengycin. This enzyme is responsible for the adenylation of isoleucine and for the binding of the amino acid to its cofactor, 4′-phosphopantetheine. Evidence presented herein suggests that FenB is involved in the activation of the last amino acid of the fengycin peptide. Our results should provide a valuable reference for future studies involving fengycin synthesis.

Nucleotide sequence accession numbers. The nucleotide sequences of the 3,825-bp *fenB* gene and the 10,488-bp *fenA* gene have been deposited in GenBank under accession no. L42523 and AF023464, respectively.

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