

## Functional and Transcriptional Analyses of a Fengycyn Synthetase Gene, *fenC*, from *Bacillus subtilis*

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Received 25 January 1999/Accepted 21 May 1999

**A 37-kb DNA fragment containing five fengycyn synthetase genes, including *fenC*, *fenD*, *fenE*, *fenA*, and *fenB*, was cloned and sequenced. Among these genes, *fenC* encodes a fengycyn synthetase 2,560 amino acids long with an estimated molecular mass of 287 kDa. This protein contains two amino acid activation modules, FenC1 and FenC2, which activate L-glutamic acid and L-ornithine, respectively. Primer extension, using mRNA isolated from the log-phase cells, identified a transcription start site located 86 nucleotides upstream from the initiation codon of *fenC*, implying that a promoter is located upstream from the start site. Primer extension using total RNA isolated from stationary-phase cells also identified a transcription start site located 61 nucleotides upstream from the initiation codon of *fenC*. Gene fusion studies demonstrated that in nHA medium, the cells transcribe the fengycyn synthetase genes at two different stages of cell growth. The promoter is active during the log phase, and the activity reaches the highest level during the late log phase. The activity decreases sharply but is maintained at a low level for approximately 24 h after cells enter the early stationary phase. The results of this investigation also suggest that the transcription of *fenC* is positively regulated during the late log phase. Results presented herein provide further insight into fengycyn synthesis by *B. subtilis* F29-3.**

Fengycyn, an antifungal antibiotic produced by *Bacillus subtilis* F29-3 (39), is a cyclic lipopeptide with the sequence fatty acid · L-Glu · D-Orn · L-Tyr · D-*allo*-Thr · L-Glu · D-Ala (D-Val) · L-Pro · L-Glu · D-Tyr · L-Ile, with a lactone bond connecting L-Tyr and L-Ile (19). Many peptide antibiotics produced by *Bacillus* spp., including fengycyn, gramicidin S, surfactin, and tyrocidine, are synthesized nonribosomally by peptide synthetases (9, 14, 17, 18, 20, 27). These enzymes typically contain one or several amino acid activation modules approximately 1,000 amino acids long. In each peptide synthetase module, an adenylation domain recognizes and adenylates a specific amino acid (17, 31–33). The adenylated amino acid then forms a thioester bond with the cofactor 4'-phosphopantetheine at the thiolation domain (18, 32, 35, 40). A transpeptidation process then transfers the activated amino acid in the initiating module to the activated amino acid in the thiolation domain in the next module, ultimately forming a peptide. This process continues from one module to another until an antibiotic is completely synthesized (18). Kleinkauf and von Döhren postulated that peptide synthetases are connected to each other in a specific order in cells, thereby allowing the amino acids to link sequentially to form a peptide (18). In addition to the adenylation domain and the thiolation domain, each module, except for the module involved in initiation of peptide synthesis, also contains a region called the condensation domain (12, 18, 32–34), which is present in the N-terminal region upstream from the adenylation domain (20, 25, 41). The final module of a peptide synthetase, except for the one involved in the termination process, typically contains an epimerization domain (12) which converts an L-amino acid to a D-amino acid. The module in-

involved in the termination of peptide synthesis contains a thioesterase domain in the C-terminal region (11, 24, 30, 32, 33).

In a previous study, we cloned a fengycyn synthetase gene, *fenB*. This gene encodes a fengycyn synthetase which activates L-isoleucine (24). This enzyme also contains a thioesterase domain, indicating that FenB participates in terminating fengycyn synthesis (24). The entire chromosome of *B. subtilis* has recently been sequenced (21). According to this sequence, *B. subtilis* 168 contains a *pps* cluster. Among the five *pps* genes in this cluster, *ppsE* encodes a protein with 74% sequence identity to FenB (37, 38). Based on this sequence identity, Tosato et al. postulated that the proteins encoded by the *pps* genes are fengycyn synthetases (38). Because the *pps* genes are not transcribed in *B. subtilis* 168 (38), the functions of these *pps* genes have not been demonstrated experimentally. Recently, Steller et al. (36) characterized the enzymes involved in the synthesis of plipastatin, an antibiotic similar to fengycyn. They also showed that the genes encoding fengycyn, plipastatin, and Pps synthetases are highly homologous (36). In light of above developments, we analyze the function of the first gene (*fenC*) of the fengycyn synthetase operon and examine how this gene is transcribed in *B. subtilis* F29-3.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture media.** *Escherichia coli* HB101 (5) was used for gene cloning. *E. coli* M15(pREP4) and pQE60 (Qiagen, Hilden, Germany) (6, 43) were used for overexpressing the adenylation domains of FenC. Plasmids pGEM-3Zi(+), pGEM-5Zi(+), and pGEM-7Zi(+) were purchased from Promega Corp. (Madison, Wis.). Plasmid pGHL6 (7.9 kb) is a *B. subtilis*-*E. coli* shuttle vector carrying a pC194 *ori*, an ampicillin resistance gene, a chloramphenicol resistance gene, and *luxAB* genes of *Vibrio harveyi*. Plasmid pD917lux, a derivative of pD917, was constructed by inserting the *luxAB* genes into the *NotI* site in pD917 (8). This insertion created a transposon, Tn917lux, capable of generating transcriptional fusion on the chromosome of *B. subtilis*. LB medium (26) was used as a general-purpose medium; nHA medium (39) was used for analyzing the activity of the *fenC* promoter. An nHA-spore plate was prepared as previously described (9). Culture media were supplemented with ampicillin (100 µg/ml), chloramphenicol (5 µg/ml), and erythromycin (1 µg/ml) to select antibiotic-resistant colonies.

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**DNA techniques.** Plasmids in *E. coli* were screened as described by Kado and Liu (16) and purified by the alkaline lysis method described by Sambrook et al. (29). Plasmids in *B. subtilis* were screened by using an alkaline lysis method described elsewhere (9). A cosmid library of *B. subtilis* F29-3 was constructed with pHC79 in *E. coli* HB101 as previously described (9). Plasmids were isolated from each cosmid clone and blotted onto Zeta-Probe membrane (Bio-Rad, Richmond, Calif.) by using a dot blot apparatus (Bio-Rad) as recommended by the manufacturer. The DNA probe was prepared with [ $\alpha$ - $^{32}$ P]dCTP (3,000 Ci/mmol; Amersham, Little Chalfont, Buckinghamshire, England), using a Rediprime labeling kit (Amersham). Finally, hybridization was performed as described elsewhere (29).

**Transformation.** *E. coli* was transformed by the  $\text{CaCl}_2$  transformation method of Cohen and Chang (10). *B. subtilis* F29-3 was transformed by the protoplast transformation method of Imanaka et al. (15).

**DNA sequencing.** DNA fragments to be sequenced were subcloned into pGEM-5Zf(+) or pGEM-7Zf(+). The sequencing reaction was performed with dye-labeled T7 and SP6 primers (Li-Cor, Lincoln, Neb.) and a SequiTherm Excel Long Read DNA sequencing kit (Epicentre Technologies, Madison, Wis.). The reaction mixtures were initially incubated at 95°C for 5 min; PCR was performed at 95°C for 30 s, 60°C for 30 s, and 70°C for 1 min, for 30 cycles. Finally, the DNA sequence was analyzed with an automated DNA sequencer (model 4000; Li-Cor).

**Cloning of the adenylation domains of FenC1 and FenC2.** A DNA fragment encoding the adenylation domain (AD-FenC1) of FenC1 was amplified with primers *fenCAN* (5'-GCGCCATGGATATGGCAGAAAAACGTGAG) and *fenCAC* (5'-ACAGATCCCTGGGAAAGCTTCATCTCTGTC). A DNA fragment encoding the adenylation domain (AD-FenC2) of FenC2 was amplified with primers *fenCB5* (5'-TATCCATGGTTTCACAAGTGGATATACT) and *fenCBC* (5'-ATAGGATCCCGCTGTCATTCCTGCAA). A DNA fragment encoding ADS-FenC2 was amplified with *fenCBN2* (5'-CCGGATCCATGGATATACTAGCGAAAAAGAA) and *fenCBC*. PCR was performed at 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min, for a total of 30 cycles. PCR fragments were digested with *NcoI* and *BamHI* and inserted into the *NcoI*-*BamHI* sites of pQE60. Finally, the plasmids were transformed into *E. coli* M15(pREP4).

**Purification of AD-FenC1 and ADS-FenC2.** Cells were cultured at 37°C in LB-ampicillin broth to mid-log phase. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM was added to the culture medium to induce gene expression. Cells were cultured for 3 h, harvested by centrifugation, and lysed by a method previously described (24). Cell lysate was centrifuged at 15,000 rpm for 60 min with a Sorvall SS-34 rotor. Recombinant protein in the supernatant was purified by affinity chromatography with a His-Bind column (Novagen, Madison, Wis.) as previously described (24). Finally, proteins were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) and staining with Coomassie blue (Merck, Darmstadt, Germany).

**ATP-PP<sub>i</sub> exchange assay.** Activities of AD-FenC1 and ADS-FenC2 were determined by an ATP-PP<sub>i</sub> exchange assay (23). Each reaction mixture contained 1  $\mu\text{Ci}$  of [ $^{32}$ P]tetrasodium pyrophosphate (14.5 Ci/mmol) (NEN, Boston, Mass.), 2 mM amino acid, and 20 mM HEPES-morpholine ethanesulfonic acid buffer (pH 4.5). Reaction mixtures were incubated at 25°C for 10 min.

**Primer extension.** *B. subtilis* F29-3 was inoculated in nHA broth at a density of  $5 \times 10^7$  CFU/ml and cultured at 37°C for 6 h and for 20 h. Total RNA was prepared from the cells according to an acid-phenol extraction method described by Aiba et al. (1) except that the cells were homogenized with acid-treated glass beads (425 to 600- $\mu\text{m}$  diameter; Sigma) in hot phenol. Primer *fenp-4* (5'-CCC TCCAATTCTAATTATAAGAGG) was end labeled with 0.3  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}$ P]ATP (6,000 Ci/mmol; Amersham) and 8 U of T4 polynucleotide kinase (Promega) (29). Primer extension was performed with a kit purchased from Promega. Finally, labeled cDNA was analyzed on an 8% urea-polyacrylamide gel (29).

**Generation of transcriptional fusion in pGHL6.** A 2,076-bp *EcoRI* fragment containing the sequence immediately upstream from the initiation codon of *fenC* was cloned into the *EcoRI* site of pGEM-3Zf(+). A fragment (2,083 bp) containing this region was then isolated from the plasmid by *SacI* digestion and cloned into the *SacI* site of pGHL6 to generate a transcriptional fusion between the promoter of *fenC* and *luxAB*.

**Isolation of mutants containing Tn917lux insertion in fenC.** Plasmid pD917lux was transformed into *B. subtilis* F29-3. Transformants were selected on LB agar containing chloramphenicol. Cells were then cultured at 37°C for 3 h in LB broth containing 0.1  $\mu\text{g}$  of erythromycin per ml to induce transposition (42). Cells were plated on LB-chloramphenicol agar and were incubated at 45°C overnight to eliminate pD917lux from the cells. Colonies resistant to chloramphenicol but sensitive to erythromycin were selected. The colonies were then spotted on an nHA-spore plate (9) to screen mutants incapable of synthesizing fengycin. Chromosomal DNA fragments adjacent to the transposons were cloned as previously described (8). Finally, the fragments adjacent to the transposon were sequenced to determine the locations of Tn917lux insertions.

**Luciferase assay.** Cells expressing *luxAB* genes were cultured overnight in LB-ampicillin broth. Cells were inoculated into nHA-ampicillin broth at a density of  $5 \times 10^7$  CFU/ml and cultured under constant shaking at 37°C. Luciferase activity exhibited by *B. subtilis* (measured in relative light units [RLU]) was monitored at different stages of cell growth with a luminometer (model LB953; Berthold, Bad Wildbad, Germany) as described elsewhere (7).

**Nucleotide sequence accession numbers.** The nucleotide sequences of *fenC*, *fenE*, *fenA*, and *fenB* have been deposited in GenBank under accession no. AF087452, AF023465, AF023464, and L42523, respectively. The sequence of *fenD* was deposited in the EMBL database under accession no. AJ011849.

## RESULTS

**Isolation of cosmid clones containing fengycin synthetase genes.** Genes for nonribosomal peptide synthesis are often clustered. If this is also the case for fengycin synthetase genes, it was estimated that a DNA fragment 37 kb long would be necessary to accommodate all of the fengycin synthetase genes. We have previously isolated a cosmid clone, pFC660, from a genomic library of *B. subtilis* F29-3 (9). Sequencing and Southern hybridization studies revealed that this cosmid contains only the 3' region, approximately 17 kb in length, of the gene cluster (9). To isolate the cosmid clones containing the 5' region of the gene cluster, we screened a genomic library of *B. subtilis* F29-3, using a 2.4-kb *KpnI*-*ClaI* fragment in pFC660 as a probe (Fig. 1). This screening identified an overlapping cosmid, pFC3-1 (Fig. 1). However, this clone did not extend far enough into the 5' region to cover the entire gene cluster (Fig. 1). Therefore, the library was screened again, using a 4.2-kb *NcoI*-*DraIII* fragment in pFC3-1 as a probe (Fig. 1). This screening identified the cosmid clone pFC6A5. Restriction mapping depicted that the distance between the 5' end of pFC6A5 and the end of the gene cluster (*fenB*) in pFC660 was approximately 45 kb (Fig. 1), a distance long enough to accommodate all of the fengycin synthetase genes.

**Organization of fengycin synthetase genes.** The sequences of pFC6A5, pFC3-1, and pFC660 were determined. These three clones cover a 64-kb segment of the *B. subtilis* F29-3 chromosome that can be arbitrarily divided into four regions according to the *BamHI* sites on the map (Fig. 1). Computer analysis revealed that the B1 and the B2 regions contain five genes, *fenC*, *fenD*, *fenE*, *fenA*, and *fenB* (Fig. 1). Proteins encoded by these genes contain sequences highly conserved among peptide synthetases, suggesting that these proteins are peptide synthetases. Earlier studies demonstrated that inserting Tn917 or Tn917*ac1* into these genes results in cells defective in fengycin synthesis (8, 9), suggesting that these five genes are involved in fengycin synthesis and that the enzymes encoded by these genes are fengycin synthetases.

**Nucleotide sequence of fenC.** Sequence analysis revealed that *fenC* is the first gene in the cluster. This gene, 7,680 bp long, is present within the two *NcoI* fragments in the B1 region (Fig. 1). This gene starts from a TTG codon which is preceded by a ribosome-binding sequence, GGAGG (Fig. 2). The protein (FenC) encoded by this gene has an estimated molecular mass of 287,233 Da. FenC contains two sets of 10 core sequences that are highly conserved among peptide synthetases (Table 1) (32, 33), suggesting that FenC contains two amino acid activation modules, FenC1 and FenC2. In addition to these core sequences, FenC1 and FenC2 contain a condensation domain at N-terminal regions. FenC2 contains an epimerization domain with seven conserved motifs marking the end of the enzyme (Table 1).

**Expression and purification of the amino acid adenylation domains of FenC.** The N-terminal and C-terminal regions of a peptide synthetase can be deleted without significantly affecting the catalytic activity of the adenylation domain of the enzyme (32, 33). It is also known that the region required for amino acid adenylation in a peptide synthetase resides in the region from the 100th amino acid upstream from the core A2 sequence to the 100th amino acid downstream from the core A8 sequence of a peptide synthetase (Table 1) (27). Therefore, we cloned the DNA fragment (nucleotides 1389 to 2996 of

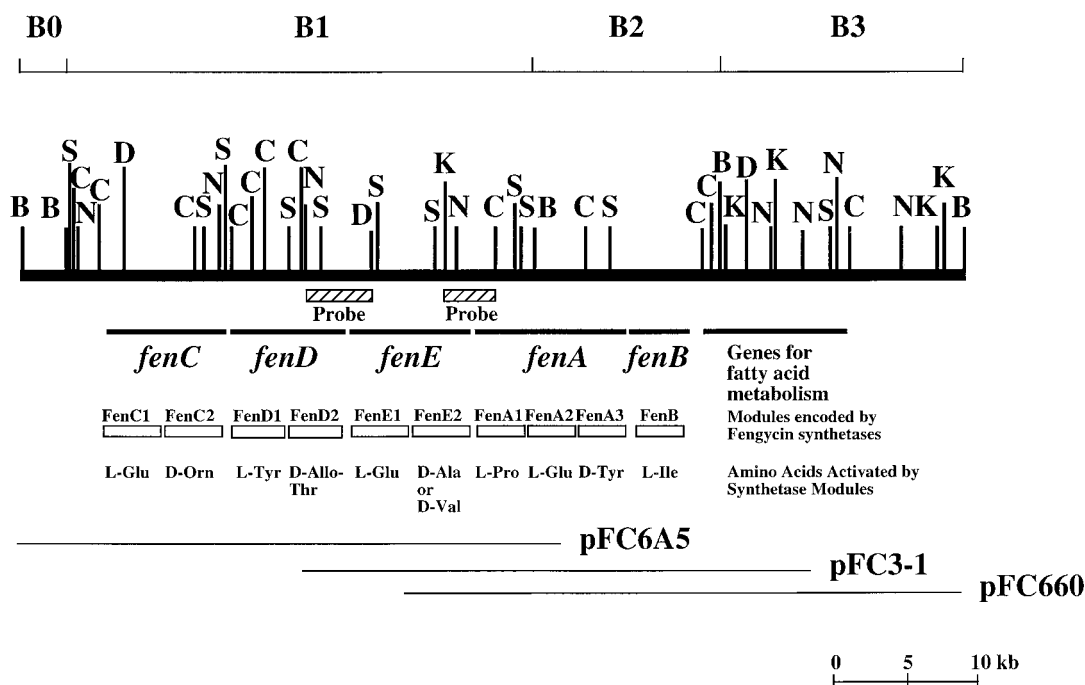


FIG. 1. Map of the *B. subtilis* F29-3 chromosome fragment containing the genes involved in fengycin synthesis. This fragment is arbitrarily divided into four regions (B0, B1, B2, and B3) according to the four *Bam*HI sites on the map. The fragments used as probes for library screening are indicated. Plasmids pFC660, pFC3-1, and pFC6A5 are cosmid clones. B, *Bam*HI; C, *Cla*I; D, *Dra*III; K, *Kpn*I; N, *Nco*I; S, *Sac*I.

*fenC*) encoding this region in FenC1 into pQE60 and overexpressed the adenylation domain in *E. coli* M15(pREP) (Fig. 3, lanes 1 and 2). The overexpressed recombinant protein (AD-FenC1) was present in both supernatant and pellet fractions of cell lysate (lanes 3 and 4). AD-FenC1 was purified from the supernatant fraction by affinity chromatography with a His-Bind column. This single purification step purified AD-FenC1 to near homogeneity (lane 5). A DNA fragment (nucleotides 4483 to 6134 of *fenC*) encoding the same region (AD-FenC2) in FenC2 was also cloned and overexpressed (lanes 6 to 9). However, the recombinant protein was insoluble and was present in the pellet fraction (lane 8) but not in the supernatant fraction (lane 9) of the cell lysate. AD-FenC2 was also shortened from the N-terminal end. A recombinant protein, ADS-FenC2 (lanes 10 to 14), which was three amino acids shorter than AD-FenC2, was present in both supernatant and pellet fractions of the cell homogenate (lanes 12 and 13). This allowed purification of the recombinant protein from the supernatant fraction by affinity chromatography with a His-Bind column (lane 14).

**Substrate specificity.** The enzymatic activities of recombinant AD-FenC1 and ADS-FenC2 proteins were determined by an ATP-PP<sub>i</sub> exchange assay (23). The amino acids used for the assay included the eight amino acids in the fengycin molecule (Fig. 4) as well as other common amino acids. Adding L-glutamic acid to the reaction mixture containing 6 μg of AD-FenC1 produced the highest ATP-PP<sub>i</sub> exchange activity (Fig. 4A), approximately 16- to 207-fold higher than the activities exhibited by the other amino acids. This finding suggests that FenC1 has a substrate specificity toward L-glutamic acid. The result also demonstrated that FenC2 specifically activates L-ornithine. Adding L-ornithine to the reaction mixture containing 20 μg of ADS-FenC2 also produced the highest ATP-PP<sub>i</sub> exchange activity (Fig. 4B), approximately 3- to 51-fold higher than the activities exhibited by the other amino acids. Adding

the other 13 common amino acids to the reaction mixtures produced only background levels of ATP-PP<sub>i</sub> exchange activity (data not shown). Notably, ADS-FenC2 was less active than AD-FenC1. Approximately three times more ADS-FenC2 was necessary to achieve roughly the same level of ATP-PP<sub>i</sub> exchange activity exhibited by AD-FenC1.

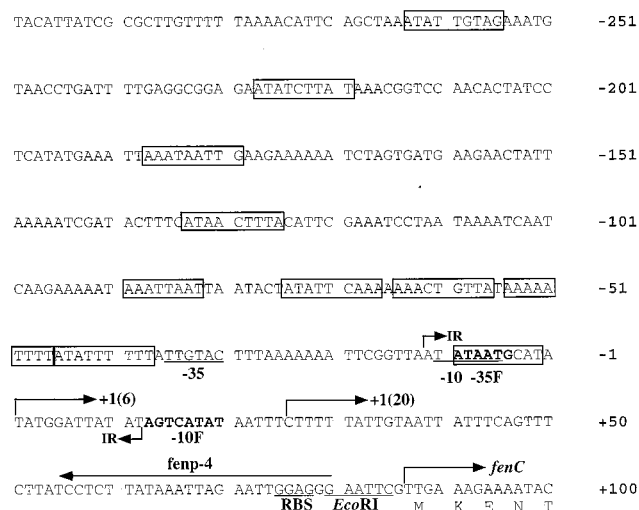


FIG. 2. Sequence of the region upstream from *fenC*. -10 and -35 denote a  $\sigma^A$ -type promoter; -10F and -35F denote sequences homologous to the -10 and -35 sequences of  $\sigma^F$  promoters. Sequences homologous to the 9-bp direct repeats in the *gltA* and *gltC* promoters are boxed. 1(6) and 1(20), transcription start sites identified with RNA prepared from cells cultured for 6 and 20 h, respectively; IR, 24-bp region of dyad symmetry; RBS, ribosome-binding site; *fenp*-4, primer used to identify the transcription start site.

TABLE 1. Comparison of conserved sequences in peptide synthetases and FenC

Domain	Core	Sequence conserved in peptide synthetases <sup>a</sup>	Sequence in FenC	Module in FenC	Encoding region in <i>fenC</i> (nucleotides)
Adenylation	A1	L(T/S)YxEL	LTYAQL ISYRHL	C1 C2	1542-1559 4650-4667
	A2	LKAGxAYL(V/L)P(L/I)D	LKAGGAYVPLD MKAGGVYIPID	C1 C2	1673-1705 4785-4817
	A3	LAYxxYTSG(S/T)TGxPKG	LAYVIYTSGSTGQPKG SAYIIYTSGTTGAPKG	C1 C2	1887-1934 5010-5057
	A4	FDxS	FDAS FDVF	C1 C2	2037-2048 5163-5174
	A5	NxYGPTE	HGYGPTE NSYGVTE	C1 C2	2319-2339 5454-5474
	A6	GELIxGxG(V/L)ARGYL	GELYIAGAGVARGYL GELCIAGAGVAKGYH	C1 C2	2481-2525 5616-5660
	A7	Y(R/K)TGDL	YKTGDL YRTGDL	C1 C2	2589-2606 5724-5741
	A8	GRxDxQVKIRGxRIELGEIE	GRLDDQVKIRGYRIEPECE GRIDHQVKINGYRIETEEIE	C1 C2	2643-2702 5778-5837
	A9	LPxYM(I/V)P	LPGYMP LPAYMVP	C1 C2	2835-2855 5979-5999
	A10	NGK(V/L)DR	SGKLDL NGKLDL	C1 C2	2905-2922 6039-6056
Thiolation	T	DxFFxxLGG(H/D)S(L/I)	DNFFDRGGNSL DSFFELGGDSI	C1 C2	3039-3071 6180-6212
Epimerization	E1	PIQxWF	PIQSRF	C2	6369-6386
	E2	HHxiSDG(W/V)S	HHLAVDGVV	C2	6768-6794
	E3	DxLLxAxG	ELLLTALG	C2	7113-7136
	E4	EGHGRE	EGHGGV	C2	7185-7202
	E5	RTVGWFTxxYP(Y/V)PFE	RTVGWFTSIYPILLD	C2	7230-7274
	E6	PxxGxGYG	PHKGTGYG	C2	7350-7373
	E7	FNYLG(Q/R)	FNYLQOL	C2	7422-7442
Condensation	C1	SxAQxR(L/M)(W/Y)xL	THAQRVWFT SSAQKRMVYL	C1 C2	111-140 3244-3273
	C2	RHExLRTxF	RHESLRTSF	C2	3381-3408
	C3	MHHxiSDG(W/V)S	FHHIIMDDIS MHHIISDGVV	C1 C2	492-521 3606-3635
	C4	YxD(F/Y)AVW	YKDYAVW	C2	3695-3715
	C5	(I/V)GxFVNT(Q/L)(C/A)xR	LGMFVSSLPIR LGMFVNTLALR	C1 C2	949-981 4050-4082
	C6	(H/N)QD(Y/V)PFE	HQDYVFE	C2	4157-4177
	C7	RDxSRNPL	RDMSRNPV	C2	4206-4229

<sup>a</sup> From reference 25.

**Biochemical characteristics.** The catalytic activities of recombinant AD-FenC1 and ADS-FenC2 were examined under different reaction conditions. Similar to FenB (24), these two enzymes had an optimum activity at 25°C, at pH 4.5 to 5.0, and with an Mg<sup>2+</sup> concentration at 10 mM in a buffer containing 2 mM EDTA.

**Determination of the transcription start site of *fenC* mRNA.** The presence of a promoter in the region immediately upstream from *fenC* could be predicted because *fenC* is the first gene of the gene cluster. Therefore, a primer complementary to the region between +56 and +80 (Fig. 2) was used to identify the transcription start site of the mRNA by primer extension. We use total RNA prepared from the cells cultured for 6 h and identified a start site that was located 86 nucleotides upstream from the initiation codon of *fenC* (Fig. 2 and 5). Sequence analysis identified -10 and -35 sequences typical for a  $\sigma^A$  promoter. A 24-bp region of dyad symmetry was found between nucleotides -12 and +12. The function of this structure is unknown. We also performed a primer extension experiment with total mRNA prepared from cells cultured for 20 h and identified another transcription start site located 61 nucleotides upstream from the initiation codon of *fenC* (Fig. 2 and 5). Sequences that are homologous to the nine conserved

9-bp direct repeats (5'-ATATTGTTT) in *gltA* and *gltC* promoters (4) were also identified (Fig. 2).

**Isolation of mutants containing Tn917lux insertion in *fenC*.** We have generated mutants defective in fengycin synthesis with Tn917lux. Among these mutants, five contained the Tn917lux insertion in *fenC*. Tn917lux in these mutants, including FX12, FX17, FX18, FS26, and FS41, was inserted at nucleotides 769, 1871, 5056, 5563, and 6989 in *fenC*, respectively. Among these five mutants, only the *luxAB* genes in mutant FS41 were oriented facing the promoter of *fenC*; this allowed the mutant to produce light. Tn917lux in the other mutants was inserted in the opposite orientation.

**Transcription of *fenC*.** Mutant FS41 was cultured in nHA medium, and the transcription of *fenC* was examined by monitoring the luciferase activity exhibited by the cells. Cells were inoculated at a density of approximately  $5 \times 10^7$  CFU/ml and cultured under constant shaking at 37°C for a total of 44 h. Under these conditions, cells reached late log phase approximately 7 h after inoculation. Cells exhibited low levels of luciferase activity during early log phase (Fig. 6A). The activity started to increase from h 6 and reached the highest level at h 7, when the cells were at late log phase (Fig. 6A). After cells entered early stationary phase, the activity immediately de-

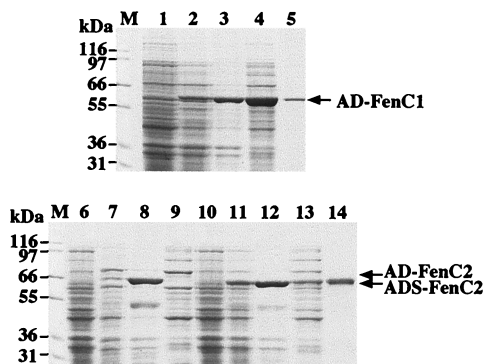


FIG. 3. Overexpression and purification of recombinant FenC adenylation domains. Proteins were purified from cells overexpressing AD-FenC1 (lanes 1 to 5), AD-FenC2 (lanes 6 to 9), and ADS-FenC2 (lanes 10 to 14). Cell extracts were prepared before (lanes 1, 6, and 10) or after (lanes 2, 7, and 11) IPTG induction. Cell extracts were centrifuged and separated into pellet (lanes 3, 8, and 12) and supernatant (lanes 4, 9, and 13) fractions. Recombinant AD-FenC1 (lane 5) and ADS-FenC2 (lane 14) were further purified with a His-Bind column. Proteins were analyzed by SDS-PAGE and stained with Coomassie blue. Arrows indicate overexpressed recombinant proteins. The positions of molecular mass markers (M) are shown at the left.

creased by 75% (Fig. 6A). However, transcription of *fenC* was maintained at this level for approximately a day and then decreased to the basal level. A 2.1-kb fragment containing the promoter of *fenC* was also cloned into a fusion vector, pGHL6

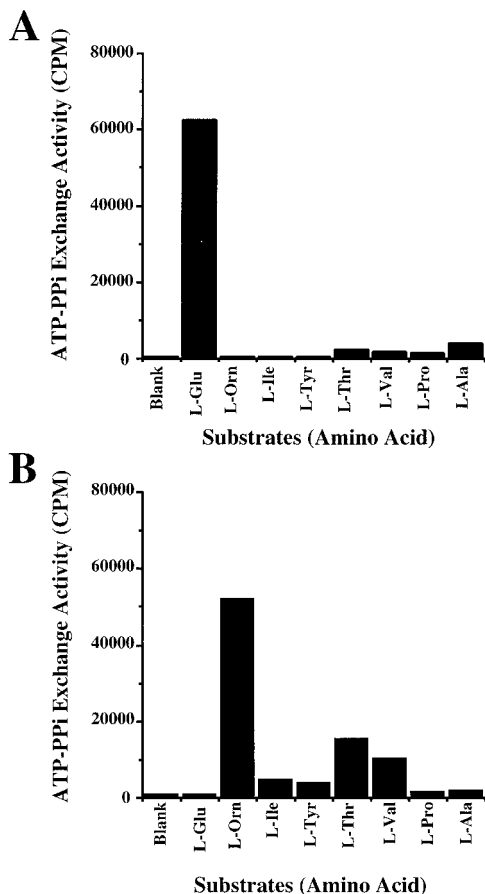


FIG. 4. ATP-PP<sub>i</sub> exchange activities of AD-FenC1 (A) and ADS-FenC2 (B).

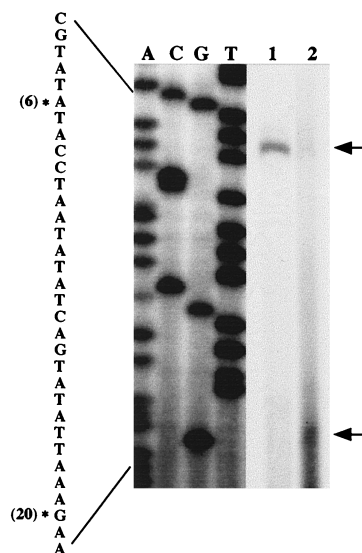


FIG. 5. Determination of the transcription start site. A, C, G, and T denote the dideoxynucleotides used to terminate the reactions. Asterisks indicate the 5' terminus of RNA; arrows indicate the cDNA products of primer extension; (6) and (20) denote RNA prepared from cells cultured for 6 and 20 h, respectively.

(Fig. 2). In *B. subtilis* F29-3, this plasmid (pGFS) exhibited a level of luciferase activity higher than that displayed by mutant FS41 (Fig. 6B), presumably due to the copy numbers of *luxAB* genes in the cells. *B. subtilis* F29-3(pGFS) had a growth rate lower than that of mutant FS41. As in the experiment with mutant FS41, the cells were also inoculated at a concentration of  $5 \times 10^7$  CFU/ml. The cells reached late log phase approximately 9 h after inoculation (Fig. 6A). *B. subtilis* F29-3(pGFS) exhibited the highest level of luciferase activity at late log phase; the activity decreased by 75% at the early stationary phase and then remained at the 50% level for approximately 24 h (Fig. 6B).

## DISCUSSION

This study identified five fengycin synthetase genes clustered in a 37-kb region on the chromosome of *B. subtilis* F29-3. These genes, in the order *fenC-fenD-fenE-fenA-fenB* (Fig. 1), are separated by short intercistronic regions of 16 to 32 nucleotides. Two transcription start sites, situated 86 and 61 nucleotides upstream from the initiation codon of *fenC*, were also identified by primer extension (Fig. 2). Neither subcloning experiments with a promoter-probe vector (pGHL6) nor computer analysis revealed any additional promoter sequence within the gene cluster, implying that these five fengycin synthetase genes are organized in an operon and are transcribed from the identified promoter. An earlier study identified a sequence resembling a transcription stop signal located immediately downstream from *fenB* (24), suggesting that *fenB* is the last gene of the operon. This study demonstrated that the two amino acid activation modules encoded by *fenC* (the first gene of the operon) activate the first two amino acids of the fengycin molecule (Fig. 4). An earlier study also demonstrated that *fenB*, the last gene in the operon, activates the last amino acid of fengycin molecule (24). In addition to FenC and FenB, the functions of several other fengycin synthetase modules, including FenD2, FenE1, FenA1, FenA2, and FenA3, are now known (unpublished data). From these results, it seems likely

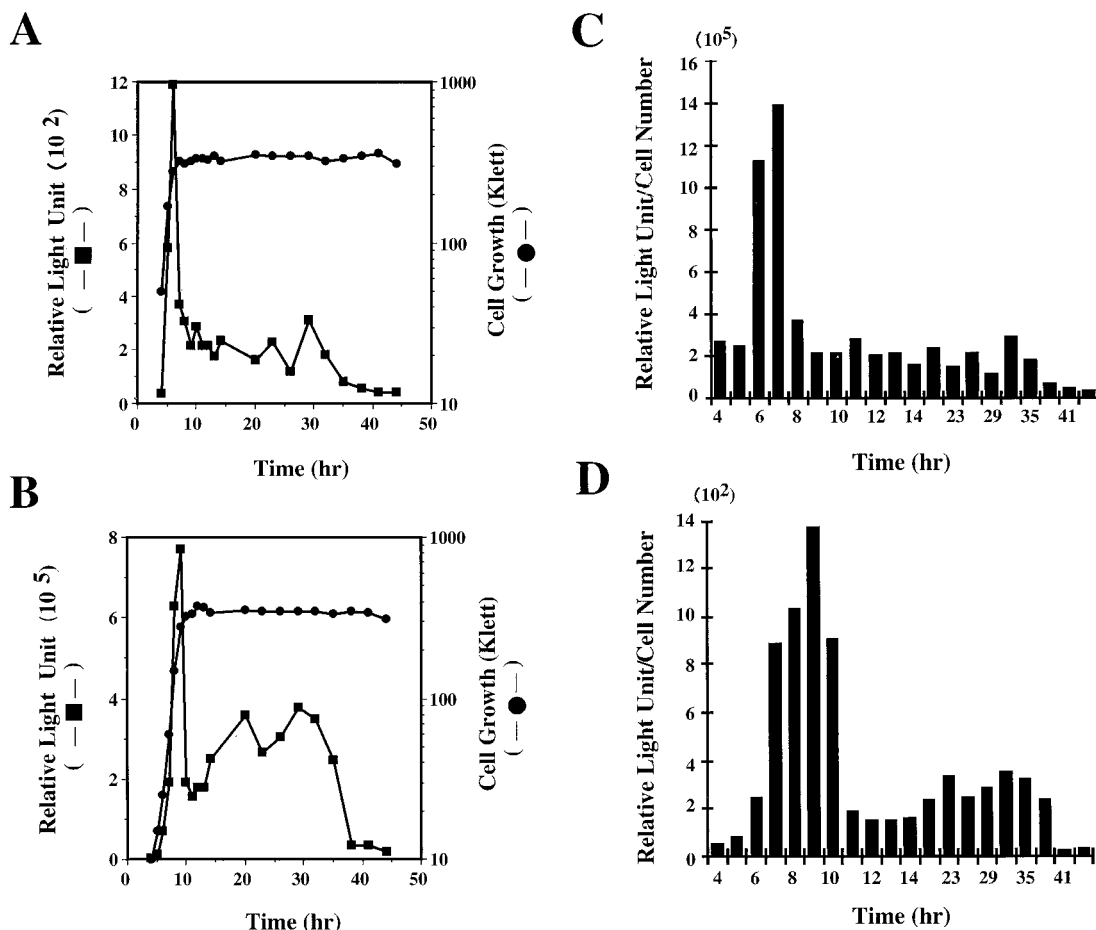


FIG. 6. Transcription of *luxAB* genes by mutant FS41 (A) and by *B. subtilis* F29-3(pGFS) (B). Cells were cultured in nHA broth. Cell growth was monitored with a Klett-Summerson photoelectric colorimeter; luciferase activity was measured with a luminometer. Luciferase activities shown in panels A and B were also calculated on a per-cell basis (C and D) to demonstrate that increases in RLU during late log phase were unrelated to cell growth.

that fengycin synthetase genes and the amino acid sequence in fengycin are colinear.

Tosato et al. have sequenced five *pps* genes of *B. subtilis* 168 (38). They reported that the sequences between FenB and PpsE, a protein encoded by *ppsE*, were 90% identical (38). Although this percentage was calculated incorrectly and the actual sequence identity is 73.9%, they postulated that PpsE may have the same enzymatic function as FenB. Owing to the sequence identity, that investigation postulated that the other four *pps* genes may also encode fengycin synthetases (38). From the sequence of the fengycin synthetase operon, this may indeed be the case because the extents of identity are as follows: between FenC and PpsA, 78.9%; between FenD and PpsB, 80.2%; between FenE and PpsC, 81.3%; between FenA and PpsE, 72.3%; and between FenB and PpsE, 73.9%. However, since *B. subtilis* 168 does not transcribe *pps* genes and does not synthesize fengycin (38), it is not clear whether these Pps proteins are still functional. Tosato et al. (38) did not demonstrate the functions of Pps proteins experimentally but did predict the functions of Pps proteins, although they predicted the functions of several enzymes incorrectly (24, 38). Interestingly, we have found a gene cluster in *B. subtilis* F29-3 which resembles the surfactin synthetase operon of *B. subtilis* 168 (unpublished data). The fact that strain F29-3 does not produce surfactin (39) raises the possibility that strains F29-3 and 168 evolved from a common ancestor that produced both

fengycin and surfactin. The fengycin synthetase genes in strain 168 probably lost their functions during evolution and became pseudogenes. We also compared the sequences located upstream of *fenC* and *ppsA*. We found that the sequences in the *fenC* and the *ppsA* promoters differ by one nucleotide in the -10 region: TATAAT in the *fenC* promoter and TATAGT in the *pps* promoter. It is not known if this single-nucleotide change results in a lack of transcription of *pps* genes.

When the two amino acid adenylation domains of FenC were expressed in *E. coli*, it was found that recombinant AD-FenC1 was a soluble protein that could be purified from the cell homogenate (Fig. 4). However, AD-FenC2 was insoluble in *E. coli* and was difficult to refold. The solubility of the enzyme could be changed by deleting the three N-terminal amino acids of AD-FenC2 (Fig. 3). However, deleting these three amino acids may have influenced the catalytic activity of the protein since approximately three times more ADS-FenC2 was necessary to achieve the same level of PP<sub>i</sub>-exchange activity exhibited by AD-FenC1 (Fig. 4). This finding is unsurprising since changing the length of an adenylation domain has been demonstrated to influence enzymatic activity (13). This study found that like FenB (24), AD-FenC1 and ADS-FenC2 have a low pH optimum between 4.5 and 5.0 and a temperature optimum at 25°C.

A primer extension study, using total RNA prepared from log-phase cells, identified a transcription start site located 86

nucleotides upstream from the initiation codon of *fenC*. Also found was a  $-10$  and a  $-35$  sequence typical of a  $\sigma^A$  promoter, explaining why *fenC* is transcribed during log phase (Fig. 6). Primer extension study also revealed that a second promoter which has a transcription start site located 61 nucleotides upstream from the initiation codon of *fenC* (Fig. 2 and 5) is involved in the transcription of fengycin synthetase genes during stationary phase. Upstream from this start site, we found a  $-10$  sequence of AGTCATAT and a  $-35$  sequence of ATAATG with a spacing of 16 nucleotides. These sequences show a low degree of homology with the  $-10$  and  $-35$  sequences of  $\sigma^F$  promoters. Whether  $\sigma^F$  regulates this promoter is not known. Fusion studies revealed that *fenC* transcription increases during log phase. We have calculated the RLU on a per-cell basis. The RLU-to-cell number ratios were relatively constant during early and mid log phase (Fig. 6C and D). However, the ratios increased significantly after mid log phase (Fig. 6C and D), indicating that the increase in RLU (Fig. 6A and B) is not simply due to cell growth. This increase also cannot be attributed to accumulation of *luxAB* mRNA in the cells, because the half-life of the mRNA at this stage is less than 3 min (data not shown). Therefore, it is likely that transcription of *fenC* is positively regulated during late log phase. When cells enter stationary phase, this positive regulatory mechanism is probably turned off, resulting in a 75% decrease in transcription activity (Fig. 6A and B). The  $\sigma^A$  promoter, which is active during stationary phase (28), may continue to transcribe the fengycin synthetase operon at this stage, albeit at low levels (Fig. 5, lane 2), and the downstream  $\sigma^F$ -like promoter may play an important role in transcribing the mRNA during stationary phase.

One of the fengycin synthesis mutants that we have isolated, FX28, contains a Tn917*lux* insertion in a gene highly homologous to the *gltC* of strain 168 (unpublished data). GltC is a transcription activator that regulates the transcription of *gltA* and *gltC* (4); this is the reason why we compared the sequence of the promoter of *fenC* with the sequences of *gltA* and *gltC* promoters. Although no GltC-binding sequence (2, 3) was found in the promoter of *fenC*, copies of 9-bp direct repeats, which were present in the promoters of *gltA* and *gltC* (4), were found. Because the functions of these repeats are unknown, it is not certain whether GltC regulates the *fenC* promoter. Furthermore, a nitrogen source in a culture medium can affect the timing and amount of fengycin synthesis (39). Therefore, investigation is under way to determine whether the genes involved in nitrogen metabolism, including *gltC*, regulate fengycin synthesis. Fengycin synthesis apparently involves large numbers of genes, and their expression probably involves complex regulatory mechanisms. Results presented herein provide valuable reference for future studies on the mechanisms involved in fengycin synthesis.

#### ACKNOWLEDGMENTS

This research was supported by Medical Research Grant CMRP759 from the Change-Gung Memorial Hospital and by Biological Research Grant NSC-88-2314-B-182-004 from the National Science Council of the Republic of China.

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