Activation of the Promoter of the Fengycin Synthetase Operon by the UP Element

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Bacillus subtilis F29-3 produces an antifungal peptidic antibiotic that is synthesized nonribosomally by fengycin synthetases. Our previous work established that the promoter of the fengycin synthetase operon is located 86 nucleotides upstream of the translational initiation codon of fenC. This investigation involved transcriptional fusions with a DNA fragment that contains the region between positions −105 and +80 and determined that deleting the region between positions −55 and −42 reduces the promoter activity by 64.5%. Transcriptional fusions in the B. subtilis DB2 chromosome also indicated that mutating the sequence markedly reduces the promoter activity. An in vitro transcription analysis confirmed that the transcription is inefficient when the sequence in this region is mutated. Electrophoretic mobility shift and footprinting analyses demonstrated that the C-terminal domain of the RNA polymerase α subunit binds to the region between positions −55 and −39. These results indicated that the sequence is an UP element. Finally, this UP element is critical for the production of fengycin, since mutating the UP sequence in the chromosome of B. subtilis F29-3 reduces the transcription of the fen operon by 85% and prevents the cells from producing enough fengycin to suppress the germination of Paecilomyces variotii spores on agar plates.

Bacillus subtilis F29-3 produces an antifungal antibiotic, fengycin, which is a cyclic lipopeptide that contains 10 amino acids with a 14- to 18-carbon fatty acyl residue that is attached to the N terminus of the peptide (47). Previous studies have demonstrated that fengycin is synthesized nonribosomally by an enzyme complex that is formed by five fengycin synthetases, FenC, FenD, FenE, FenA, and FenB (8). These enzymes interlock to form a chain in the order FenC-FenD-FenE-FenA-FenB in interactions between the C-terminal region of an upstream enzyme and the N-terminal region of its downstream partner enzyme (48). These enzymes contain at least one module, and each module activates an amino acid that is incorporated into fengycin (28, 30, 41), allowing the activated amino acids to form a peptide on the enzyme chain (13). The modules typically contain an adenylation domain consisting of about 550 amino acids (10), which recognizes and adenylates an amino acid (13, 42, 44, 51). Following adenylation, the adenylated amino acid is covalently linked through a thioester bond to the cofactor 4′-phosphopantetheine, which is bound to the thiolation domain (27, 40, 43, 45, 46). Meanwhile, in the C-terminal region, every fengycin synthetase except FenB contains an epimerase domain that converts an L-amino acid to a D-amino acid (9, 31). During fengycin synthesis, L-Glu, which is activated by the initiating module FenC1, covalently links to L-Orn, which is activated by the downstream module FenC2 to form a peptide. The epimerase domain that is located in the C-terminal region of FenC2 then converts L-Orn to D-Orn before the peptide is translocated to FenD to generate a peptide with L-Tyr that is activated by the FenD1 module (31). This process continues from one module to the next and from one fengycin synthetase to the next in the fengycin synthetase complex until the peptide reaches FenB, where the peptide is circularized and cleaved by the thioesterase domain of the enzyme (24, 26). Lin et al. (29) showed that the genes in the fengycin synthetase operon (fen operon) encode the five fengycin synthetases. These genes are positioned colinearly with the enzymes in the fengycin synthetase chain and the amino acids in fengycin (48). Lin et al. (29) also established that the promoter (fenp) which transcribes the operon is located 86 nucleotides (nt) upstream of the translation initiation codon of fenC.

It is generally known that the efficient binding of an RNA polymerase holoenzyme to a promoter is crucial for transcription. For instance, the lac promoter in Escherichia coli has two sites in the region between positions −50 and −38 for binding of a cyclic AMP-catabolite activator protein complex (36). The catabolite activator protein is known to interact with the C-terminal domain of the RNA polymerase α subunit (αCTD) to anchor RNA polymerase to the promoter (36). This interaction is required for efficient transcription of the lac operon when glucose is not present in the culture medium. Rather than interacting with a protein, αCTD also binds to a stretch of the 17-bp A- and T-rich sequence called the UP element that is located upstream of the −35 region to facilitate efficient binding of RNA polymerase to a promoter (18). In fact, each of the two αCTDs in RNA polymerase binds to two subsites, which are centered at positions −42 and −52 in the UP element (12). The binding of αCTD to an UP element may enhance the transcription activity by a factor of up to 340 (12). A genetic study revealed that seven amino acid residues, L262,
**MATERIALS AND METHODS**

**Strains and culture media.** *B. subtilis* F29-3 is a wild-type strain that produces fengycin (47). A transcriptional fusion was generated in the *B. subtilis* DB2 (6) chromosome to analyze *fenp* activity. *B. subtilis* F29-3 mutants Fk920 and Fk921 were used to study the function of the UP element in *fenp*. *E. coli* EPI100 was used as a host for gene cloning (Epicentre, Madison, WI). *E. coli* BL21(DE3) was employed for protein expression (37). nHA medium (47), which was used for protein expression, contains 0.5% yeast extract, 2% malt extract broth, 1% glucose, and 0.4% sodium chloride. *B. subtilis* DB2 is a temperature-sensitive strain. *E. coli* BL21 and *B. subtilis* F29-3 were cultured in LB medium (33) to culture both strains. *E. coli* BL21(DE3) was cultured in nHA medium. LB medium (53) was used to culture both *E. coli* and *B. subtilis*.

**Plasmids.** Plasmid pGHL6 (29) was utilized to generate a transcriptional fusion between *fenp* and *luxAB*. A 60-bp double-stranded synthetic oligonucleotide that contained the region from position -60 to position +80, respectively, was constructed using *B. subtilis* F29-3 chromosome. A 186-bp fragment that contains the region from position -105 to position +80 in the *fenp* operon was amplified by PCR with primers P105 (5'-GCGCGAGCTCTCAATCAAGAAAAAATAAATTAATT) and P6+80 (5'-GCCGGTGACCCCTCCTCAATTTATATTATTAAAGAGG) using chromosomal DNA from *B. subtilis* F29-3 (47) as the template. After digestion with SacI and KpnI, the DNA fragment was inserted into the SacI-KpnI sites in pGHL6 to obtain pDFen105 (Fig. 1B). Plasmids pDFen197, pDFen55, and pDFen20 (Fig. 1B), which contain *fenp* regions from position -197 to position +80, from position -75 to position +80, from position -60 to position +80, from position -55 to position +80, from position -40 to position +80, and from position -20 to position +80, respectively, were constructed using pDFen105 and the same strategy. Plasmids pDFen35, pDFen42, and pDFen20 (Fig. 1B), which contain *fenp* regions from position -42 to position +80, and from position -40 to position +80, respectively, were constructed using pDFen105 and the same strategy. Plasmids pDFen35 and pDFen10 contained positions -35 and -10 sequences that were mutated from 5'-`TGTAC` and 5'-`TATAAT` to 5'-`GGTGCA` and 5'-`GCGCGC`, respectively. A temperature-sensitive *B. subtilis* F29-3 chromosome was amplified by PCR with primers P105 (5'-GCCGGAGCTCTCAATCAAGAAAAAATAAATTAATT) and P6+80 (5'-GCCGGTGACCCCTCCTCAATTTATATTATTAAAGAGG) using chromosomal DNA from *B. subtilis* F29-3 (47) as the template. After digestion with SacI and KpnI, the DNA fragment was inserted into the SacI-KpnI sites in pGHL6 to obtain pDFen105 (Fig. 1B). Plasmids pDFen197, pDFen55, and pDFen20 (Fig. 1B), which contain *fenp* regions from position -197 to position +80, from position -75 to position +80, from position -60 to position +80, from position -55 to position +80, from position -40 to position +80, and from position -20 to position +80, respectively, were constructed using pDFen105 and the same strategy. Plasmids pDFen35, pDFen42, and pDFen20 (Fig. 1B), which contain *fenp* regions from position -42 to position +80, and from position -40 to position +80, respectively, were constructed using pDFen105 and the same strategy. Plasmids pDFen35 and pDFen10 contained positions -35 and -10 sequences that were mutated from 5'-`TGTAC` and 5'-`TATAAT` to 5'-`GGTGCA` and 5'-`GCGCGC`, respectively. A 60-bp double-stranded synthetic oligonucleotide that contained the region from position -60 to position -1 and had a SacI site at the 60 end and a KpnI site at the 5' end was synthesized by Bio Basic Inc. (Ontario, Canada). This fragment was inserted into the SacI-KpnI sites in pGHL6 to obtain pDFen601 (Fig. 1B).
Transcription of Fengycin Synthetase Operon

1B. Six pDFen601 mutant derivatives, pDFen601-M1 to pDFen601-M6 (Fig. 1B), which had mutations in the region from position −55 to position −39 (sequence from 5′-AAAAATTTATTTATTTTATTATTGTTCAG-3′), from position −52 to position −50 (5′-AAT to 5′-CCG), from position −49 to position −47 (5′-TTT to 5′-GGG), from position −42 to position −40 (5′-TTT to 5′-GGG), from position −36 to position −31 (5′-TGGTAC to 5′-GGTCCG), and from position −11 to position −6 (5′-TATAAT to 5′-GC CGCG), were formed by inserting synthetic double-stranded oligonucleotides into pGHL6.

Generating transcriptional fusions in the B. subtilis DB2 chromosome. DNA fragments that contained the fepn transcription unit and luxAB were amplified by PCR and then inserted into the BamHI-SacI sites in pDH22. The plasmids were linearized by PstI digestion and then inserted into amyE in the B. subtilis DB2 chromosome by homologous recombination using a method described elsewhere (6).

Mutation of the UP element in fepn in the B. subtilis F293-3 chromosome. A 1-kb fragment that covers the region between positions +86 and +1085 in the fepn operon was amplified by PCR using primers 5′-GCGGGGTACC CTATTGATTTTATTATTATT ATTATGTA-3′ and 5′-CCGGGAGCTTCA CCGAAATATTAAGA AAATACTTATGG-3′. This fragment was digested by KpnI and SacI before it was inserted into the KpnI-SacI sites in pGEC920 to obtain pGEC921. A 277-bp fepn fragment that covered the region between positions −197 and +50 was amplified with primers 5′-GGCGGATCTATCTGAAATATTAAATTAATGGA AAAATACTTATGG-3′ and 5′-CCGGGAGCTTCA CCGAAATATTAAGA AAATACTTATGG-3′. This fragment was digested with BamHI and KpnI and inserted into the BamHI-KpnI sites in pGEC920 to generate pGEC921. The sequence of pGEC920 was identical to that of pGEC921 except that the former plasmid contained a wild-type fepn sequence, which was amplified using pDFen917 as the template. Plasmids pGEC920 and pGEC921 were transformed individually into B. subtilis F293-3.

Cultures were grown in LB broth at 37°C to a mid-log phase and then subcultured using a 1% inoculum in LB broth at 30°C for 5 h to cure the plasmids. The cells were serially diluted with phosphate-buffered saline, plated on LB agar that contained 50 μg/ml of chloramphenicol to the mid-log phase and then subcultured using a 1% inoculum in LB broth.

Luciferase assay. nHA broth that contained 5 μg/ml of chloramphenicol (n-Ha-Cm) was inoculated with an overnight culture using a 1% inoculum. Following inoculation, the culture was aliquoted into test tubes (2 by 15 cm), and the cells in the tubes were cultured at 37°C with constant shaking. At each sampling time, three tubes were removed from the shaker, and the luciferase activity of the cells was measured using a luminometer (model LB953; Berthold, Bad Wildbad, Germany) (29). The A600 of a culture was used as an internal control. The mean difference in the cycle threshold (Ct) value obtained from amplification of FK920 and FK921 was normalized to generate a transcriptional fusion with lucAB.

In vitro transcription. In vitro transcription was performed using a 40-μl reaction mixture and a method described elsewhere (21). The core RNA polymerase and σ8 that were used in the reaction were purified from B. subtilis using methods described previously (21). The DNA templates used in the reactions contained the sequence from −197 to position +50 in fepn and sequences that contained a mutated UP element and the −35 and −10 regions, which were amplified from pDFen197, pDFen197-M1, pDFen35, and pDFen10, respectively. A 57-nt RNA marker was transcribed in vitro from the tns promoter (C. I. Yen and B. Y. Chang, unpublished). Next, a 140-nt RNA marker was transcribed with T7 RNA polymerase from a DNA fragment that contained the T7 promoter and its downstream sequence in pGEM-TZf(+) DNA. This fragment was amplified using primers T7-F (5′-CATGGAATAATGAATGCA CT) and T7-R (5′-ATTTAAGGACCATGATATAAG) and pGEM-TZf(+) as the template. The DNA fragment was also cut by BamHI and used to transcribe an 83-nt transcript. DNA templates for in vitro transcription were purified from a 2% agarose gel using a DNA clean extraction kit (GeneMark, Taiwan). The concentrations of the templates were determined spectrophotometrically at A260 using a spectrophotometer (model Libra S12; Biochrom, United Kingdom).

DNaSE I foot-printing. A DNA probe that contained the region from position −147 to position +50 was amplified by PCR using primers P147 (5′-AATCATTCTTAAATCCTT) and P150 (5′-AATCAGTTAAATAAATGATAA) that had been labeled at the 5′ end with 32P using T4 polynucleotide kinase. Purified His-cfCTD, His-αfNTD, or His-cfCTD (C265A) was incubated with 0.5 nM probe for 30 min on ice in 50 μl of a DNA binding buffer that contained 10 mM Tris-HCl (pH 7.5), 0.2 mM dithiothreitol, 0.02 mM EDTA, 30 mM KCl, 50 mM MgCl2, and 12% glycerol for 30 min at 25°C. Subsequently, 0.15 U of DnaSE I (Roche, Mannheim, Germany) was added to the reaction mixture to cleave the DNA for 30 s. DnaSE I foot-printing was performed by using a procedure described elsewhere (53). DNA Sequencing was performed using a PCR product sequencing kit (version 2.0; USB, Cleveland, OH).

Biassay of fengycin activity. The antifungal activity of fengycin was assayed with spore plates that had been prepared with Paecilomyces variotii Tu137 spores using a method described by Zahnert and Maas (52).

Quantification of fecn mRNA by real-time PCR. Total RNA was prepared from cells using the acid-phenol extraction method of Aiba et al. (1). cDNA was synthesized with 2 μg of RNA using 200 U of Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI) in 25 μl of a reaction mixture that contained 1 μM of cytosine-phosphoguanine dinucleotide at 50°C to generate strains FK920 and FK921.

In vitro transcription was performed using a reaction mixture that contained 2 μl of cDNA, 0.2 μM forward primer 5′-GCCCAAAAG GAGAATGTTGGTCTTACTG, and 0.2 μM reverse primer 5′-GACGCTTACAAA CCAATGCTGAATC and used as an internal control. The mean difference in the cycle threshold (ΔCT) value obtained from amplification of FK920 and FK921 fecn mRNA in three independent experiments was normalized to the ΔCT value for the internal control. The relative amounts of mRNA were determined by a method described elsewhere (14).

RESULTS

Transcription from fepn. Previous work revealed that transcription of the fepn operon starts at nt 86 upstream of the initiation codon of fcnC (29). Accordingly, this study used a DNA fragment that covered the region between positions −105 and +80 in fepn to generate a transcriptional fusion with luxAB in pGHL6 to investigate the regulation of fepn. nHA-Cm broth was inoculated with B. subtilis F293-3(pDFen10S), and the luciferase activity of the cells was monitored over a 32-h period. In our first experiment, luciferase activity was monitored for 32 h at a steady rate with the same time points that were taken from a single flask. B. subtilis may undergo autolysis during the stationary phase when cells experience a drastic temperature change.
shift (49). Hence, repeated sampling of a culture at room temperature using a single flask removed from a 37°C shaker may have influenced cell viability, explaining why the experimental results obtained were frequently erratic and irreproducible, especially after the cells had entered the stationary phase. To overcome this problem, the culture was aliquoted into test tubes following inoculation. At each time point, three tubes were taken from the shaker to determine the luciferase activity by the cells in three tubes at each sampling point was monitored using a luminometer. The relative light unit (RLU) values were averaged. The error bars indicate standard deviations. Cell growth (filled squares), expressed as $A_{600}$, was monitored using a spectrophotometer.

FIG. 2. Activity of fenp. nHA-Cm broth was inoculated with B. subtilis F29-3(pDFen105) (A) and B. subtilis DB2(amyE::Pfen105) (B). Cells were cultured at 37°C, and the luciferase activity (filled circles) of the cells was monitored every hour for 32 h. The culture was aliquoted into test tubes following inoculation. The luciferase activity exhibited by the cells in three tubes at each sampling point was monitored using a luminometer. The relative light unit (RLU) values were averaged. The error bars indicate standard deviations. Cell growth (filled squares), expressed as $A_{600}$, was monitored using a spectrophotometer.

FIG. 3. Deletion analysis of fenp. Plasmid pDFen105 was deleted to identify the region in fenp that was critical for transcription. Deleting the regions from position −105 to position −76 (pDFen75) and from position −105 to position −61 (pDFen60) did not substantially influence the promoter activity; 12 h following inoculation, the luciferase activities of B. subtilis F29-3(pDFen105), B. subtilis F29-3(pDFen55), B. subtilis F29-3(pDFen60), and B. subtilis F29-3(pDFen601) were approximately equal (Fig. 3). Deleting the promoter to position −56 (pDFen55) (Fig. 1) reduced the activity by 12.8% (Fig. 3). However, extending the deletion to position −43 (pDFen42) reduced the fenp activity by 64.5% (Fig. 1B and Fig. 3), indicating that the region between positions −55 and −43 is critical for fenp activity. Further deletion of the promoter to position −21 (pDFen20) (Fig. 1B) reduced the activity by 97.6% (Fig. 3), revealing that the region from position −42 to position −21, which includes the −35 box, is important for fenp activity. Additionally, deletion of the region between positions +1 and +30 from pDFen60 (pDFen601) did not affect the transcription (Fig. 3).

Mutations in fenp and promoter activity. In this study, the regions from position −36 to position −31 and from position −11 to position −6 in pDFen601 were mutated to generate pDFen601-M5 and pDFen601-M6, respectively. At 12 and 24 h after inoculation, the luciferase activity of pDFen601-M5 was 85.4% and 94% lower than that of pDFen601 (Fig. 4), suggesting the importance of this segment of DNA in fenp transcription and confirming that this region contains the −35 box of fenp (29). The activity of pDFen601-M6 at 12 and 24 h following inoculation was reduced by 99.7% and 99.9%, respectively, confirming that the region from position −11 to position −6
contains the −10 box of fenp (29). Meanwhile, mutating the sequence between positions −55 and −39 (pDFen601-M1) reduced the transcription activity by 82.6% and 92.2% at 12 and 24 h (Fig. 4) after inoculation, respectively. In this study, mutations in this region from position −52 to position −50 (pDFen601-M2), from position −49 to position −47 (pDFen601-M3), and from position −42 to position −40 (pDFen601-M4) (Fig. 1B) were generated. The luciferase activities of the resulting plasmids 12 h after inoculation were reduced by 92.2%, 99.7%, and 71.1%, respectively (Fig. 4). Meanwhile, mutating the sequence between positions −50 and −39 was substantially reduced the promoter activity, the expression pattern was similar to that exhibited by B. subtilis DB2(amyE::Pfen601-M1) (data not shown).

In vitro transcription from fenp. To investigate how the region between positions −55 and −39 regulates the transcription of fenp, in vitro transcription was performed using a DNA template that covered the sequence between positions −197 and +80 (Fig. 6B). Additionally, the reverse primer that was used to amplify the DNA fragment contained a KpnI site and a GGCC sequence at the 5′ terminus. Hence, a 90-nt RNA should be transcribed from fenp. In this study, transcripts transcribed from the tms (20) and T7 promoters were used as size markers (Fig. 6A, lanes 1 and 2). The results showed that a 90-nt transcript was transcribed from a DNA template that contained the wild-type fenp sequence. The transcription of this 90-nt transcript was significantly reduced after the region between positions −55 and −39 was mutated (Fig. 6A, lane 6). As expected, mutations in the −35 and −10 regions also markedly reduced the transcription (Fig. 6A, lanes 4 and 5).

Binding of αCTD to the region between positions −55 and −39. The region from position −55 to position −39 in fenp is A and T rich and therefore partially homologous to the corresponding region of the UP element in the rmb P1 promoter (16, 38). Since αCTD binds to the UP element in the rmb P1 promoter, EMSA was employed here to confirm the binding of His-αCTD to the region upstream of the −35 box in fenp. EMSA revealed that 4 μM His-αCTD shifted a 40-nt biotin-labeled probe, WT-45, which contains the sequence between positions −39 and −55, in a gel (Fig. 7A, lanes 1 and 2). However, the protein no longer shifted the DNA when 0.15 or 0.3 μM non-biotin-labeled probe WT-45 was added as a competitor (Fig. 7A, lanes 3 and 4). Meanwhile, adding a G-and C-rich probe, M-45, did not influence the binding of the protein to the WT-45 probe (Fig. 7A, lanes 5 and 6).

FIG. 4. Mutations in the −35, −10, and UP element regions and transcription of fenp. nHA-Cm broth was inoculated with B. subtilis F29-3(pDFen601), B. subtilis F29-3(pDFen601-M1), B. subtilis F29-3(pDFen601-M2), B. subtilis F29-3(pDFen601-M3), B. subtilis F29-3(pDFen601-M4), B. subtilis F29-3(pDFen601-M5), and B. subtilis F29-3(pDFen601-M6). Luciferase activity was measured at 12 and 24 h after inoculation. The luciferase activity values for three experiments were averaged, and levels of activity relative that of B. subtilis DB2(amyE::Pfen601) were calculated. The error bars indicate standard deviations. The relative light unit values for 100% activity at 12 and 24 h were 1.86 × 10^6 and 2.22 × 10^6, respectively.

FIG. 5. Transcription of fenp from fusion constructs in the chromosome of B. subtilis DB2. nHA-Cm broth was inoculated with B. subtilis DB2(amyE::Pfen601) (open circles) and B. subtilis DB2(amyE::Pfen601-M1) (open squares). Cells were cultured at 37°C. Cell growth, expressed as A_600, was monitored using a spectrophotometer. The luciferase activities of B. subtilis DB2(amyE::Pfen601) (filled circles) and B. subtilis DB2(amyE::Pfen601-M1) (filled squares) were monitored every hour following inoculation. RLU, relative light units. The error bars indicate standard deviations.
Analysis of binding of \(\alpha\)CTD to \(fenp\) by DNase I footprinting analysis. A DNase I footprinting study revealed that His-\(\alpha\)CTD protected the regions from position –97 to position –81 and from position –60 to position –39 (Fig. 7B, lane 1). However, these two regions were not protected by His-\(\alpha\)NTD (Fig. 7B, lane 2) or by a \(fenp\) CTD mutant, His-\(\alpha\)CTD(C265A), which was demonstrated to be unable to bind to the UP element (Fig. 7B, lane 3) (17). These results demonstrated that the region between positions –55 and –39 may contain an UP element. Additionally, the presence of two \(\alpha\)CTD-binding sites is not unique to \(fenp\); the presence of two protected regions in several promoters was also revealed by DNase I footprinting analysis (2, 18, 25, 35). Although the site close to the –35 box has the strongest effect on transcription, the function of the distal binding site remains unclear (25, 35).

Effect of mutation in the UP element on transcription of the \(fen\) operon and the synthesis of fengycin by \(B. subtilis\) F29-3. A mutant \(B. subtilis\) F29-3 strain, FK921, which contains a chloramphenicol resistance gene inserted at position –197 and a mutated UP element, and another mutant, FK920, which contains the chloramphenicol resistance gene inserted at position –197 in the wild-type promoter, were tested to determine their capacity to transcribe the \(fen\) operon and produce fengycin. The cells were cultured in nHA-Cm broth to the mid-log phase. The \(fen\) mRNA in the cells was determined by real-time reverse transcriptase PCR using primers that amplified the region between positions +807 and +1113 in the \(fen\) operon. A segment of \(rpoA\) was also amplified and used as an internal control. The mean \(\Delta C_T\) value obtained from amplification of FK920 and FK921 \(fen\) mRNA in three independent experiments was normalized with the \(\Delta C_T\) value of the internal control. The results indicated that the amount of \(fen\) mRNA that was transcribed by FK921 was 85.4% or about seven times control.
time reverse transcriptase PCR was used to determine the and FK921, were cultured in nHA broth to the mid-log phase. Real-
B. subtilis the production of fengycin. Two mutants of
52x481 fenC
52x436 cDNA were calculated from the
52x445 amplified as the internal control. The percentages of the amount of
52x454 for amplification of a segment of fenC
52x490 subtilis
52x289 element in
52x311 ever, mutant strain FK921 did not produce sufficient fengycin
52x322 suppressed the germination of fungal spores (Fig. 8B). How-
52x344 thermore, FK920 produced fengycin in the medium after 24 h
52x355 lower than the amount transcribed by FK920 (Fig. 8A). Fur-
52x391 indicates inhibition of spore germination by fengycin (B).
52x400 celium; the halo around the paper disk where mycelium is not present
52x409 filter paper disk on a spore plate to assay the fengycin produced by the
52x427 cultured for 24 h in nHA broth. The culture medium was spotted on a
52x427 two mutant strains. The background shows the growth of fungal my-
52x418 DISCUSSION
52x430 Fengycin is synthesized nonribosomally by five fengycin syn-
52x441 thetases that are encoded by the fen operon (29, 48). Efficient
52x452 cDNA fragments in the
52x463 promoter region was constructed to investigate the manner in which the transcription of feng is regulated. Analysis of the luciferase activity of B. subtilis DB2(amyE::fen105) and B. subtilis DB2(amyE::fen601) revealed identical expression patterns; transcription by either strain peaks in the late log phase and 14 to 16 h following inoculation in the stationary phase (Fig. 2B and Fig. 5). However, the luciferase activity exhibited by B. subtilis DB2(amyE::fen601) (Fig. 5) is about five to seven times higher than that exhibited by B. subtilis DB2(amyE::fen105) (Fig. 2B) during the first 20 h of cultur-
52x474 ing. Since the amyE::Pfen601 construct lacks the regions from position −105 to position −61 and from position +1 to pos-
52x484 tion +80, the increased luciferase activity of B. subtilis DB2(amyE::fen601) suggests that a negative element may be present in one of these two regions. However, the repression was less obvious when the fusions were in a reporter plasmid than when they were in the chromosome (Fig. 2B). Since pDFen601 has a pC194 ori and a copy number of about 30 (50), B. subtilis F29-3 may not yield enough of the repressor to suppress the transcription from the plasmids. The function of the negative element is now under investigation.
52x494 The UP element, which is a 17-bp A- and T-rich sequence located upstream of the −35 box in a promoter (11, 16, 17, 38), is a docking site for σCTD in RNA polymerase (22). This sequence enhanced transcription of the E. coli rmbP1 prom-
52x504oter (38, 39) and various B. subtilis genes, including spoVG
52x518 (4), the autolysin gene, cwlB (32), the flagellin gene, hag (5),
52x527 and genes in Bacillus phase 629 (32). Additionally, enhance-
52x534 ment by the UP element is not limited to the promoters of the
52x546 σ^A type but extends to the promoters that are recognized by σ^H (15, 19). Although the sequence upstream of the −35 region in σ^A-type promoters is generally enriched for short A and T tracts, only a few promoters contain an UP element (5). This study shows that an UP element between positions −55 and −39 strongly activates the transcription from feng in a plasmid and in the chromosome (Fig. 4, Fig. 5, and Fig. 8A). This sequence is likely to be an UP element since EMSA and DNase I footprinting revealed binding of αCTD to this segment of DNA (Fig. 7). Moreover, feng in the B. subtilis DB2 chromosome is transcribed at two distinct stages of cell growth, the log phase and the stationary phase (Fig. 2B and Fig. 5). The transcription in both of these phases is reduced markedly when the UP element is mutated (Fig. 5), indicating the importance of the UP element in transcription. Our results also showed that mutations in different regions in the UP element, between positions −52 and −50 (pDFen601-M2), positions −49 and −47 (pDFen601-M3), and positions −42 and −40 (pDFen601-M4), substantially reduce the promoter activity (Fig. 4), indicating that binding of two αCTDs to the proximal and distal parts of the UP element simultaneously is critical for activation (12, 18).
52x540 This study demonstrates that the fengcin synthetase operon is transcribed during the log phase and peaks when the cells reach the late log phase (Fig. 5). After the activity decreases, the transcriptional activity increases again and reaches a sec-
52x554 ond peak at 14 to 16 h (Fig. 5). The transcription in these two stages of cell growth is probably driven by the same promoter since the transcription throughout culturing decreased sub-
52x564 stantially after mutation of the −35 and −10 boxes, implying
that the sequences that are required for transcription during both the log phase and the stationary phase are common. Additionally, functioning of an UP element depends on the proper distance from the −35 box (11). The fact that mutation of the UP element reduces the transcription during both the log and stationary phases (Fig. 4) also strongly suggests that a common −35 sequence is involved in transcription in these two phases. If this is true, factors must repress the transcription in the early stationary phase and after 16 h of culturing (Fig. 5).

This study also found that pDFen105 in a spo0A mutant has a transcriptional pattern that is similar to that of B. subtilis 168(pDFen105) (data not shown), indicating that the sporation genes probably do not regulate the transcription of the fen operon.

According to our results, the UP element between positions −55 and −39 in fenp is critical for fengycin synthesis since a B. subtilis F29-3 mutant with a mutated UP element in fenp cannot transcribe the fen operon efficiently or produce sufficient fengycin to inhibit the germination of fungal spores (Fig. 8).

Since factors other than the UP element may regulate the transcription of the fengycin synthetase operon, further work must be performed to elucidate fully how these factors affect the synthesis of fengycin.

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