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# *Bacillus subtilis* Subtilisin Gene (*aprE*) Is Expressed from a $\sigma^A$ ($\sigma^{43}$) Promoter In Vitro and In Vivo

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**In vitro studies demonstrated that the *Bacillus subtilis* subtilisin gene (*aprE*) could be transcribed by RNA polymerase holoenzyme reconstituted from core and $\sigma^A$ factor obtained from vegetative cells. Upstream deletions (from −45) reduced the amount of transcription from the promoter. A deletion downstream of the promoter that overlapped a putative downstream minor promoter did not affect transcription from the $\sigma^A$ promoter, which indicated that the putative downstream promoter is not utilized in vivo. S1 nuclease mapping studies showed that there was a low level of transcription from the subtilisin promoter during the growth phase and that the site of transcription initiation was the same during log and stationary phases. We conclude from these findings that there is only one promoter for the subtilisin gene and that it can be transcribed by the $\sigma^A$ form of RNA polymerase in vitro.**

The *Bacillus subtilis* subtilisin gene, *aprE*, is expressed maximally during the early stages of sporulation and is regulated in part by the *spo0A* and other *spo0* genes (6, 7) and by several additional regulatory genes, such as *prtR* (26, 30, 36), *sacQ* (1, 35), *sacU* (14), *sacV* (22), *hpr* (15), *sin* (9), and *sen* (31a, 34). Thus, a complex mechanism involving the products of early sporulation genes and transcriptional regulatory factors controls the expression of *aprE*. These properties make this gene a good model system for the study of gene expression in *B. subtilis*.

Ferrari et al. (6) recently provided strong evidence that the subtilisin promoter was located upstream of the promoter site originally proposed by Wong et al. (33). Furthermore, the minor RNA polymerase holoenzyme containing $\sigma^B$, proposed by Wong et al. (33) as the enzyme responsible for subtilisin gene expression, was shown by the use of a *sigB* null mutant not to be involved in expression of *aprE*. The conclusions of Wong et al. (33), which were based on S1 nuclease mapping and in vitro transcription data, were therefore in error. The most probable causes for this misinterpretation were the presence of a degradation product during S1 mapping which made the initiation point appear to be further downstream of the promoter and the use of a $\sigma^B$ RNA polymerase preparation that contained some $\sigma^A$ enzyme.

To reinvestigate the promoter location and determine the form of RNA polymerase involved in transcription of the subtilisin gene, we analyzed the promoter region by deletion analysis, S1 nuclease mapping experiments, and RNA polymerase holoenzyme reconstitution studies. Our findings (i) confirmed the location and presence of a single promoter as reported by Ferrari et al. (6), (ii) demonstrated a low level of transcription of the gene during the growth phase from the same promoter as that used during the stationary phase, and (iii) showed that the subtilisin promoter could be transcribed by a holoenzyme reconstituted from core enzyme and $\sigma^A$ factor isolated from vegetative cells.

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## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Unless otherwise stated, the *B. subtilis* strains and plasmids used are described in Table 1.

**Plasmid and strain construction.** For deletion of the subtilisin promoter region, two *B. subtilis* plasmids, pKWZ (Fig. 1A) and pWWP (Fig. 1B), were constructed. To construct pKWZ, the *Eco*RI site of pUBHR (pUB110 containing the M13mp9 polylinker; 18) was removed by cutting with *Eco*RI enzyme and filling in with Klenow fragment to form plasmid pUBHRΔE. Then a 2.7-kilobase pair (kbp) *Sma*I-*Pst*I DNA fragment from pLP1 (32) containing the whole *B. subtilis* subtilisin gene was inserted into pUBHRΔE to form pKWZ. In the construction of pWWP, a *Bam*HI linker was attached to the *Dra*I blunt end of an *Eco*RI-*Dra*I DNA fragment (0.75 kbp) from the S fragment (11, 33) containing the regulatory region of the subtilisin gene; this fragment was then inserted into pWP18, which is a promoter probe plasmid (31) containing only the structural part of the subtilisin gene and the M13mp18 polylinker.

To prepare the promoter template for in vitro transcription, a recombinant *Escherichia coli* plasmid, pSP1, was constructed. A *Taq*I-*Hpa*I fragment (160 bp) from pWWPΔ1 was blunted by Klenow filling and subcloned to the *Sma*I site of pUC19.

To examine the effect of deletions on expression of the subtilisin promoter Ps, a single-copy integration plasmid, pEK, was constructed from pUC19. The 1.25-kbp *Taq*I fragment of pC194 containing the chloramphenicol acetyltransferase gene (16) was subcloned into the *Acc*I site of pUC19 to form pEK106. The 0.91-kbp *Hpa*I-*Pvu*II fragment containing the 3′ end of the subtilisin gene was attached with *Pst*I linkers and then subcloned into the *Pst*I site of pEK106 to form pEK107. The *Xba*I site of pEK107 was converted to a *Sal*I site to form pEK108. The promoters and the N-terminus of *aprE* in pKWZ (wild type), pWWPΔ1, and pKWZΔ1 (see below) were translationally fused to the β-galactosidase gene at amino acid residue 14 of the signal peptide of the subtilisin gene. The *Eco*RI-*Sal*I fragment of each fusion on the plasmid was subcloned into pEK108 to construct pEK112, pEK113, and pEK114, respectively. The structures of these plasmids are described in the legend to Fig. 5. With the use of these plasmids, strains DB508,

TABLE 1. *B. subtilis* strains and plasmids

| Strain (plasmid) | Relevant genotype |
|---|---|
| DB104 | *hisH nprR2 nprE18 ΔaprE* |
| DB501(pKWZ) | *hisH nprR2 nprE18 ΔaprE* |
| DB502(pWWP) | *hisH nprR2 nprE18 ΔaprE* |
| DB503(pKWZΔ1) | *hisH nprR2 nprE18 ΔaprE* |
| DB504(pKWZΔ2) | *hisH nprR2 nprE18 ΔaprE* |
| DB505(pWWPΔ1) | *hisH nprR2 nprE18 ΔaprE* |
| DB506(pKWZΔ3) | *hisH nprR2 nprE18 ΔaprE* |
| DB507(pKWZΔ4) | *hisH nprR2 nprE18 ΔaprE* |
| DB508 | *trpC2 aprE*(pKWZ)::*lacZ cat* |
| DB509 | *trpC2 aprE*(pWWPΔ1)::*lacZ cat* |
| DB510 | *trpC2 aprE*(pKWZΔ1)::*lacZ cat* |

DB509, and DB510 were constructed. Single-copy integration plasmids pEK112 and pEK113 were cut with *Sph*I to linearize the plasmids and then transformed into DB2 to obtain the recombinant strains DB508 and DB509 by a double crossover at the homologous site of the subtilisin gene (29); chloramphenicol-resistant ($Cm^r$) strains that showed a blue color and reduced halo size on 2×SG plates (20) containing 1% skim milk were selected. Strain DB510 was constructed with intact plasmid pEK114 by a Campbell-type recombination since, if this plasmid is linearized and transformed into DB2, the same type of fusion as that in pEK112 (wild-type promoter to *lacZ* fusion) would be constructed by a double crossover at the 5′ and 3′ ends of the subtilisin gene between the chromosome and the plasmid. After selecting for $Cm^r$ strains, we compared these strains against DB2 with respect to blue color intensity and halo size of DB2 on X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) and skim milk plates, respectively, and then selected for light blue colonies with normal-size halos.

**Promoter deletion.** *Bal* 31 enzyme (fast, mixed, and slow types; IBI Co.) deletions were performed on plasmids pKWZ (Fig. 1A) and pWWP (Fig. 1B). The plasmids, purified by CsCl density gradient centrifugation, were cut with *Eco*RI (pKWZ) and *Bam*HI (pWWP) and electroeluted to remove the residual RNA and single-stranded DNA that interfere with the synchronized rate of deletion. The amount of *Bal* 31 enzyme used and the reaction time were controlled as described by the manufacturer and by Legerski et al. (19). The samples were removed from each reaction mixture as a function of time. The sticky ends caused by the uneven deletion rate were filled with Klenow fragment, and *Eco*RI and *Bam*HI linkers were attached to pKWZ and pWWP, respectively. The deletion strategy is shown in Fig. 2A. One deletion directed toward the promoter region started from the *Eco*RI site of pKWZ (Fig. 1A); the other started from the *Bam*HI linker site (at the *Dra*I site) of pWWP (Fig. 1B) toward the promoter. In both cases, the deletions were screened in vivo on the basis of the halo size of the transformants on 2×SG medium (20) plates containing 1% skim milk and by rough determination of the deletion size as judged by 1% agarose gel electrophoresis. To determine the exact position of the deletions, pKWZΔ1, pKWZΔ2, and pKWZΔ4 were digested with *Eco*RI and pWWPΔ1 was digested with *Bam*HI. The 5′ protruding ends of the digested plasmids were labeled with [$\gamma$-$^{32}$P]ATP (23), and the plas-

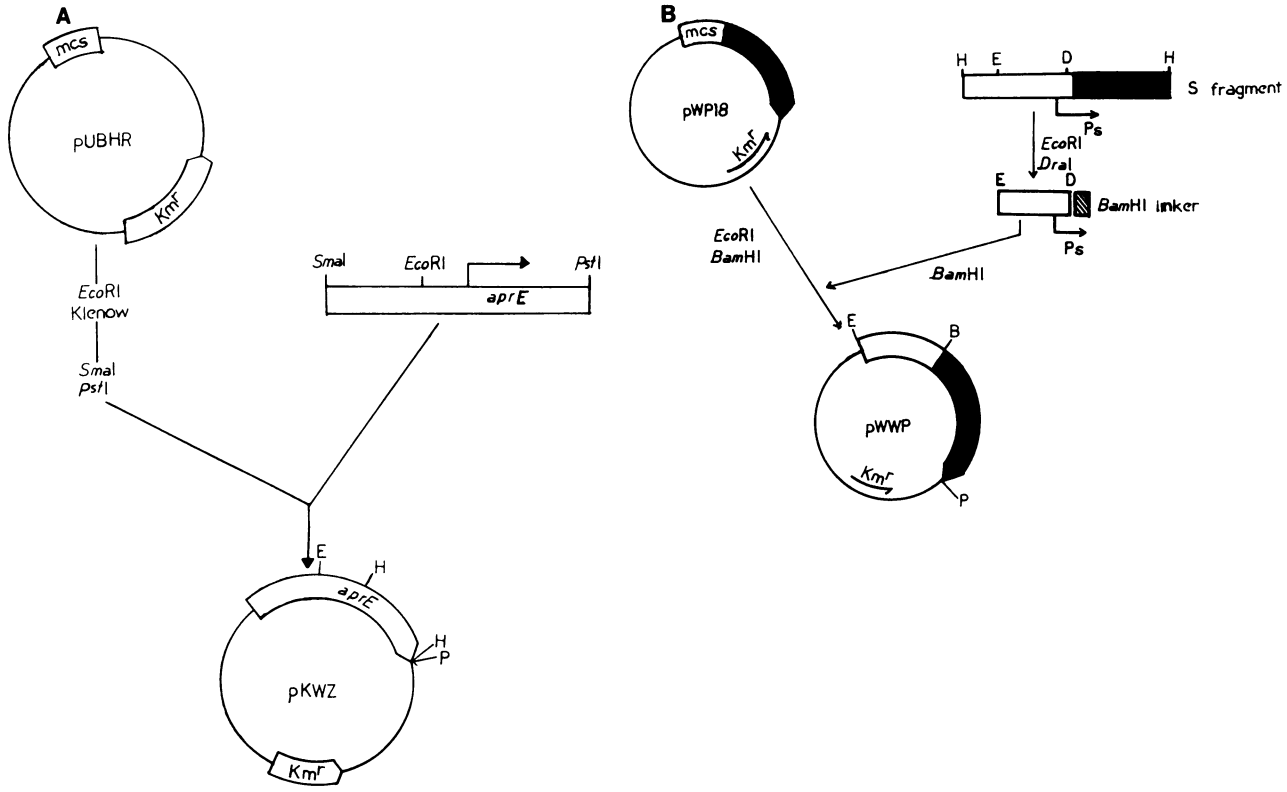


FIG. 1. (A) Construction of pKWZ. Construction started from pUBHR, which is a derivative of pUB110 (18). mcs, Multiple cloning site of M13mp9; H, *Hin*dIII. The available single restriction enzyme sites are *Eco*RI (E), *Sma*I (S), and *Pst*I (P) in the subcloned *aprE* gene. The size of pKWZ is about 7.0 kbp. (B) Construction of pWWP. Construction started from pWP18 (31). The available single restriction enzyme sites are *Eco*RI, *Bam*HI, and *Pst*I. D, *Dra*I; other abbreviations are as given above. The size of pWWP is about 6.7 kbp. For details, see text.

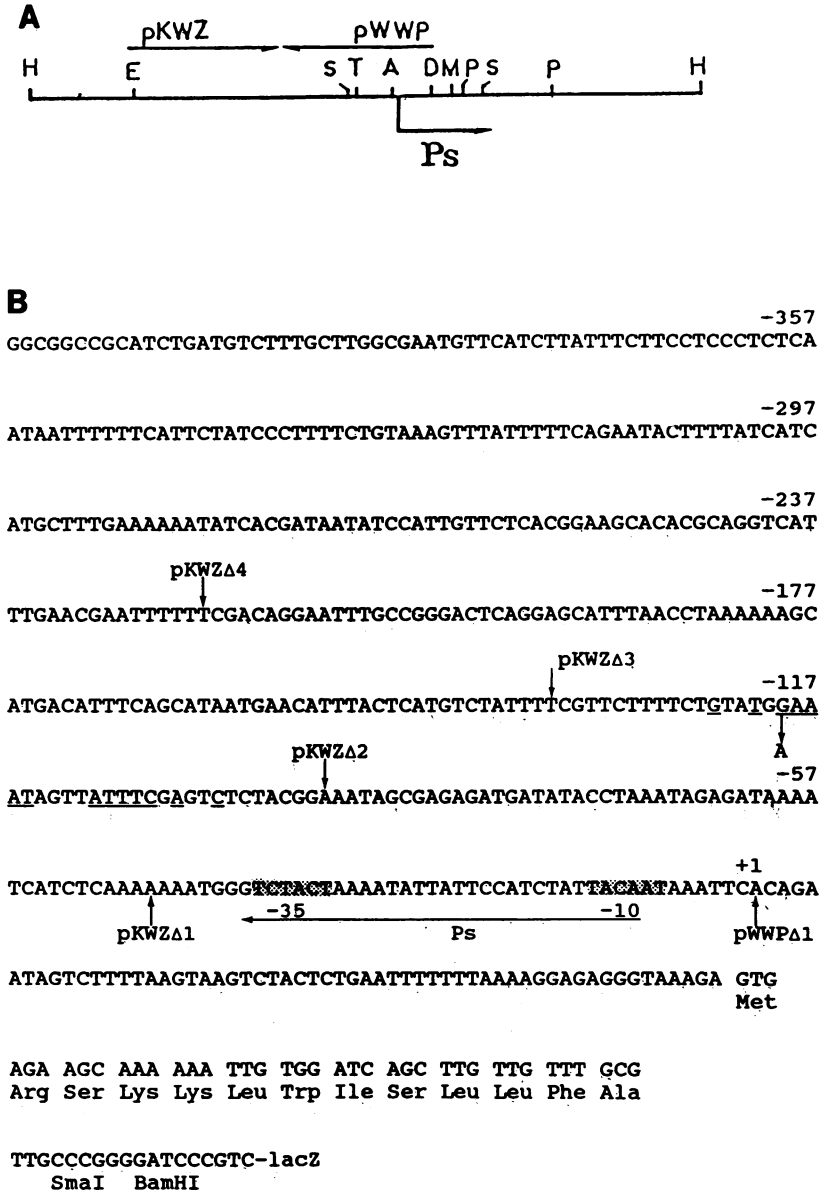


FIG. 2. (A) Restriction enzyme sites, promoter region, and start points and direction of *Bal* 31 deletion of the S fragment. Arrowed lines designated pKWZ and pWWP show the direction of *Bal* 31 treatment from the *Eco*RI and *Dra*I sites. H, *Hin*dIII; R, *Eco*RI; T, *Taq*I; D, *Dra*I; M, *Mbo*I; P, *Hpa*I. (B) Sequences around the promoter Ps region. The location (←) and consensus sequences (−35 and −10) (stippled area) of promoter Ps are indicated. The arrow pointing to the A residue indicates the transcription start site of promoter Ps. The in-frame fusion of *lacZ* to the subtilisin gene is indicated with sequences around the junction. Vertical arrows point to deletion endpoints of pKWZΔ1 to -Δ4 and pWWPΔ1. Sequence data are from Henner et al. (14) and Wong et al. (33).

mids were digested again as follows: pKWZΔ1 and pKWZΔ2 with *Mbo*I, pKWZΔ4 with *Acc*I, and pWWPΔ1 with *Taq*I (Fig. 2A). These end-labeled DNA fragments were loaded onto an 8% sequencing gel with a sizing ladder prepared by using bacteriophage M13mp18 (37) containing the *Eco*RI-*Hin*dIII (1.2-kbp) fragment from pKWZ. The deleted promoter region of pKWZΔ3, *Eco*RI-*Hin*dIII, was subcloned to M13mp19 and sequenced by the method of Sanger et al. (28).

**Protease (subtilisin) activity assay.** *B. subtilis* DB501, DB503, DB504, DB505, and DB506 were cultured in 2×SG sporulation broth, and the amounts of protease produced at various stages of growth and sporulation were analyzed. From $T_0$ (beginning of the log phase), 1.0 ml of culture was removed at 1-h intervals, centrifuged for 1 min in a microcentrifuge (Eppendorf Co.), and suspended in 1 ml of supernatant medium from a parallel culture of DB104 harboring the vector (pUB110) only, and then cultured for 10 min at 37°C with rotary shaking. Portions (0.5 ml) of these cultures were centrifuged, and 0.3 ml of supernatant of each sample was used for the assay reaction. All other steps were carried out as described by Rinderknecht et al. (27). One unit of specific activity was defined as the change in $A_{595}$ per minute per Klett unit × $10^5$.

**Quantitative S1 nuclease mapping.** The quantitative S1 nuclease mapping experiment was performed by the method of Berk and Sharp (2), with some modifications. To prepare the DNA probe, the *Pvu*II-*Bam*HI fragment from pEK114 (the site of *Pvu*II is in the *lacZ* fragment of pUC19, and the

*Bam*HI site is at the junction of the subtilisin promoter-to-*lacZ* fusion) was labeled at both ends with [$\gamma$-$^{32}$P]ATP (23) and cut with *Eco*RI to purify the *Eco*RI-*Bam*HI (150-bp) fragment containing the promoter region and the 5′ end of the β-galactosidase gene (Fig. 2B). The mRNA was isolated from strains DB508 and DB510 at different stages of growth and sporulation by the method of Gilman and Chamberlin (10), and the concentration of mRNA used was normalized on the basis of its $A_{260}$ (21). The concentration of the DNA probe hybridizing with mRNA isolated from DB510 was three times higher than that of the probe hybridized with mRNA from DB508, since the latter strain contains wild-type subtilisin transcripts which lack the 5′ end of the β-galactosidase gene (*Sma*I-*Bam*HI, 10 bp; Fig. 2B) and interfere with hybridization.

**β-Galactosidase assay.** DB508, DB509, and DB510 were cultured in 2×SG sporulation broth. β-Galactosidase activities were assayed every hour from $T_0$ as described by Miller (24).

**Purification of RNA polymerase holoenzyme and core enzyme.** *B. subtilis* DB104 was cultured in superrich broth (8) for isolation of vegetative RNA polymerase and in 2×SG sporulation broth for isolation of $T_3$ (3 h after the log phase) RNA polymerase. In both cases, 50 g (wet weight) of cells was used for isolation as described by Fukuda and Doi (8), Halling et al. (13), and Davison et al. (4). The core enzyme was purified by the method of Fukuda and Doi (8).

**Reconstitution of E$\sigma^A$ holoenzyme.** E$\sigma^A$ was reconstituted by the method of Hager and Burgess (12), with some modifications. E$\sigma^A$ holoenzyme from the DNA-cellulose fraction was electrophoresed on a 12% polyacrylamide-sodium dodecyl sulfate gel. The $\sigma^A$ band was visualized by 0.25 M KCl staining and sliced out; the protein was then electroeluted in elution buffer (25 mM Tris, 192 mM glycine [pH 8.3]) overnight at 4°C (17) in an Elutrap (Schleicher and Schuell, Inc., Keene, N.H.). The protein in the elution buffer (400 μl) was kept at −70°C for 30 min and precipitated with 1.6 ml of acetone. The pellet was washed with 500 μl of 80% acetone and dried briefly under $N_2$ gas. The eluted protein was denatured by suspension in 10 μl of dilution buffer (20 mM Tris hydrochloride [pH 7.9], 10 mM $MgCl_2$, 0.1 M KCl, 0.2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol) containing 6 M guanidine hydrochloride, incubated at room temperature for 15 min, diluted 50-fold with 490 μl of dilution buffer, and allowed to renature at room temperature for 1 h. Renatured $\sigma^A$ protein (5 μl, or 1% of the total) was added to *B. subtilis* RNA polymerase core enzyme (0.1 μg) purified as described by Fukuda and Doi (8), and holoenzyme was reconstituted by incubation at 0°C for 15 min.

**In vitro transcription.** The *Eco*RI-*Sal*I (190-bp) fragment of pSP1, which contains the subtilisin promoter, was used as the template for in vitro transcription. ϕ29 DNA digested with *Hin*dIII was transcribed by reconstituted E$\sigma^A$ RNA polymerase to prepare the RNA size markers and to assay RNA polymerase activity. All other steps of in vitro transcription were carried out by the method of Goldfarb et al. (11).

**S1 nuclease mapping with in vitro and in vivo mRNAs.** In vivo RNA was prepared from sporulating cells at $T_3$ as described above. To prepare in vitro mRNA of the subtilisin gene, 1 μl (3 μg) of pUC9-S (pUC9 containing the S fragment; see above) and 3 μl (15 μg) of vegetative RNA polymerase were mixed in 125 μl of in vitro transcription mixture (40 mM Tris hydrochloride [pH 7.9], 10 mM $MgCl_2$, 100 mM KCl, 0.4 mM dithiothreitol, 12.5 nmol each ATP, CTP, GTP, and UTP). The reaction mixture was incubated at 37°C for 15 min and then extracted once with 2 volumes of phenol-chloroform (1:1). Protease K-treated DNase I solution (250 μl) was added to remove the DNA template, pUC9-S, and the preparation was incubated at 37°C for 30 min. Two volumes of phenol-chloroform was added again to deproteinate the reaction mixture. RNA was precipitated with 2 volumes of ethanol (200 proof) in the presence of 20 μg of *E. coli* tRNA as a carrier. The precipitate was dried under vacuum and dissolved in 20 μl of water. A 10-μl amount (50% of the total) was used for S1 nuclease mapping as described above.

## RESULTS

**Analysis of the putative downstream promoter of the subtilisin gene.** The deletion studies of the *aprE* promoter region reported by Ferrari et al. (6) indicated that removal of the upstream bases from −26 caused a total abolishment of promoter activity. However, since the deletion to −26 could affect the putative promoter located at +25 bp, we decided to delete the downstream promoter region from +41 to −1 (Fig. 2B) and test whether transcription was altered; this deletion included the putative promoter and eliminated any possibility of transcription initiation from this locus.

Since we had mapped the location of the subtilisin promoter in the S fragment (33), the deletions were made in the promoter region of the subtilisin gene present in plasmids pWKZ and pWWP, harbored by host strain DB104. Four clones, DB503(pKWZΔ1), DB504(pKWZΔ2), DB506 (pKWZΔ3), and DB507(pKWZΔ4), containing plasmids with deletions starting at the upstream *Eco*RI site of the *aprE* gene (Fig. 1A and Fig. 2A), were selected. The deletions reached the −92 position in strain DB504(pKWZΔ2) and the −45 position in strain DB503(pKWZΔ1) (Fig. 2B). From the deletion starting at the *Bam*HI linker site (at *Dra*I) of pWWP and progressing upstream, one clone, DB505(pWWPΔ1), which was deleted up to the −1 position, was selected. The effect of the deletions on promoter activity was detected initially by determining the relative sizes of halos on skim milk agar plates: the less active the promoter, the smaller the halo. Deletion strains DB504, DB505, and DB507 produced normal-size halos comparable to the halo of the wild type, DB501(pKWZ), whereas DB503 and DB506 produced a very small and extremely large halos, respectively.

To examine these strains more quantitatively, we assayed the protease activity of each strain (Fig. 3). We selected only three strains, DB503, DB504, and DB506, from the upstream region deletions, since the halo size of the other deletion strain, DB507, was the same as that of the wild type (DB501). As expected, the protease activity of DB504, containing a deletion to −92, corresponded to that of DB501. The protease activity began to appear after $T_1$, reached a maximum at $T_3$, and declined thereafter. The protease activity of DB505, containing a deletion up to −1 from +41, was similar to the activities of DB501 and DB504, even though the deletion in this strain removed completely the previously reported P1 and P2 promoters (33). The deletion in DB503, which removed bases upstream to position −45 of the promoter Ps, reduced the activity significantly compared with that of DB504. The deletion in DB506(pKWZΔ3), containing a deletion up to −136 and a base change of G to A (at −119 in Fig. 2B) in the inverted region upstream of promoter Ps, accelerated expression of the subtilisin gene by 2 h, and its activity was highly detectable even during the growth stage (Fig. 4). The region bounded by the deletions of

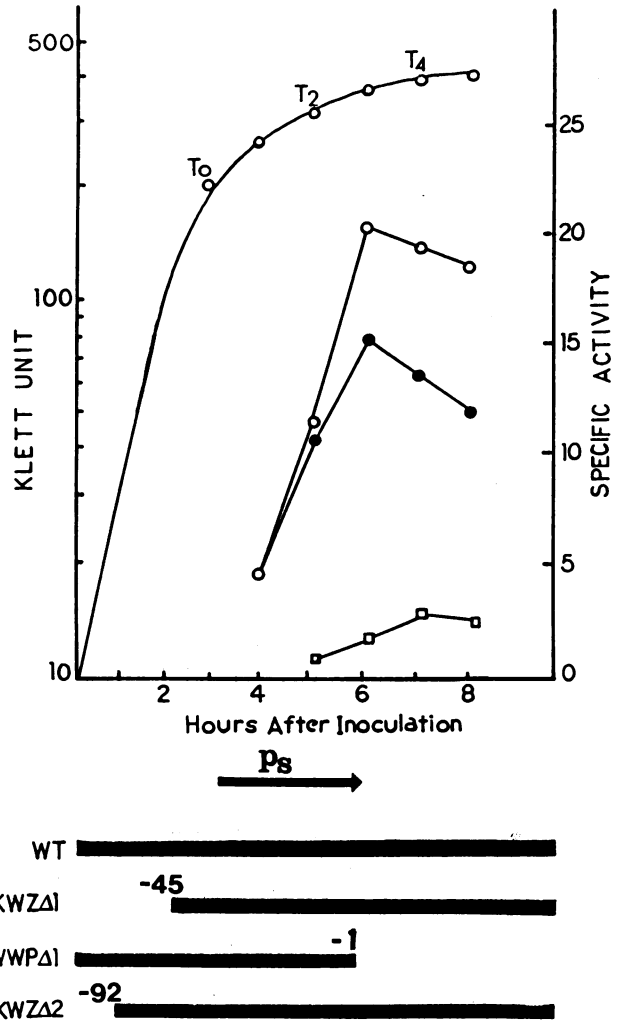


FIG. 3. Protease activity after deletion of the subtilisin promoter. The four lines below the relative position of promoter Ps indicate the relative size of each deletion in comparison with that of the wild type (WT) promoter; numbers indicate endpoints of deletions upstream of the +1 site of promoter Ps. Growth is given in Klett units. Specific activities of subtilisin during the sporulation stage for the three deletion strains: ○, wild type (DB501) and DB504(pKWZΔ2; ●, DB505(pWWPΔ1); □, DB503(pKWZΔ1).

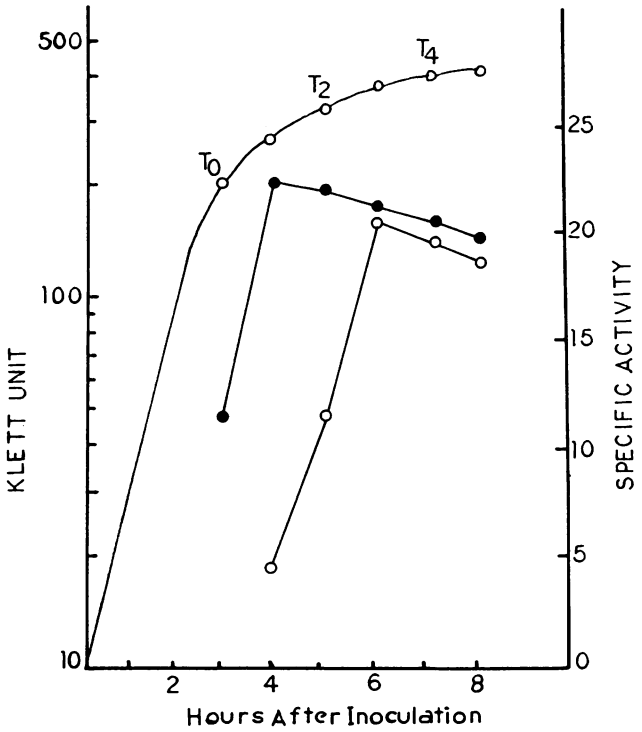


FIG. 4. Protease activity of DB506(pKWZΔ3). Specific activity was calculated as described in Materials and Methods. Growth is given in Klett units. Symbols: ○, DB501(pKWZ); ●, DB506 (pKWZΔ3).

pKWZΔ1 and pWWPΔ1 contained a $\sigma^A$-like promoter sequence (Fig. 2B).

**Transcription initiation and temporal expression of the subtilisin gene.** To test the exact point of transcription initiation and the temporal expression of the subtilisin gene in the wild type and the deletion mutant (DB503), S1 nuclease mapping experiments were carried out. We obtained the mRNA from strains DB508 and DB510, which contained a single copy of the fusions of the wild type and the −45 deletions, respectively, of the subtilisin promoter with the β-galactosidase gene. These strains, which were constructed by use of integration plasmids pEK112 and pEK114 (Fig. 5), showed identical patterns of β-galactosidase and subtilisin expression (data not shown), although the amount of β-galactosidase synthesized in DB510 was very low and similar to the reduced protease activity of pKWZΔ1 (Fig. 3). We found that the subtilisin gene was transcribed from a single transcription initiation site with mRNA isolated from the vegetative stage (Fig. 6, lane 3) to $T_5$ (lane 6). Two faint lower bands could be seen at $T_3$ (lane 5). We believe that these two minor bands were degradation products from the upstream transcript. Levels of the transcripts seen even during the vegetative stage (lane 3) were low until $T_1$, increased up to $T_3$, and decreased thereafter. The temporal pattern and intensity of the band followed the typical expression pattern for the subtilisin gene, which is expressed at low levels even during growth. Since protease activity was severely reduced in DB503(pKWZΔ1), which was deleted to −45, and S1 nuclease mapping with strain DB510, which was constructed with the promoter of pKWZΔ1, showed reduced transcription from Ps (Fig. 6, lane 7), we concluded that the low protease activity of DB503 was due to the almost complete inactivation of promoter Ps with the upstream deletion and not to expression from a putative downstream promoter. On the basis of the calculated size of the protected band and the +1 position of transcription, a $\sigma^A$-like promoter sequence was found (Fig. 2B).

**In vitro transcription of the subtilisin promoter with E$\sigma^A$ RNA polymerase.** Since subtilisin mRNA was present in vegetative cells (Fig. 6, lane 3) and a $\sigma^A$-like promoter sequence was found in the promoter Ps region (6; Fig. 2B), the E$\sigma^A$ RNA polymerase was isolated from vegetative cells to determine whether the promoter Ps could be utilized by this form of the enzyme. We purified the E$\sigma^A$ holoenzyme (Fig. 7, lane A) from vegetative-stage cells, reconstituted E$\sigma^A$ holoenzyme from the core enzyme (lane B) and purified $\sigma^A$ (lane C), and carried out runoff transcription assays. To ensure the sizes of transcripts produced and the specificity of the E$\sigma^A$ holoenzyme, φ29 DNA digested with *Hin*dIII was used as a template for in vitro transcription (Fig. 8, lane 1).

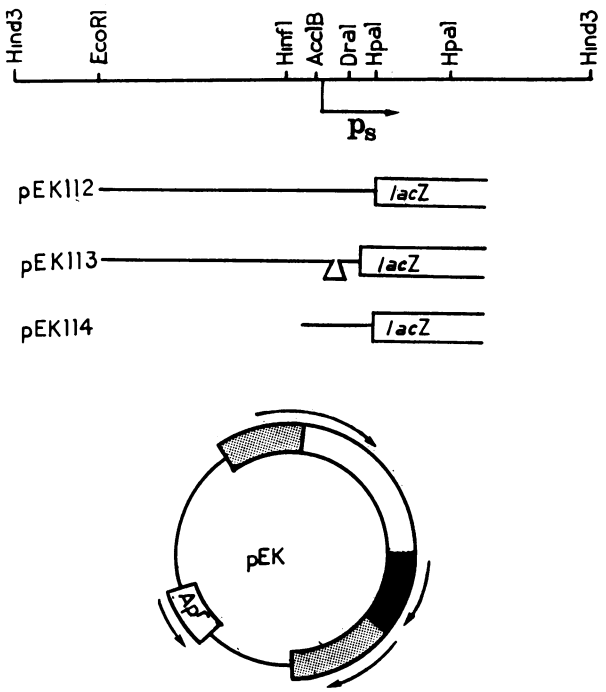


FIG. 5. Construction of the single-copy integration plasmid pEK. The relative size of the 5′ end of the subtilisin gene promoter region after deletion and fusion to *lacZ* is indicated (for details, see Materials and Methods and legend to Fig. 2B). Symbols: ▨, *aprE*; ▭, *lacZ*; ▬, $Cm^r$; —, pUC19.

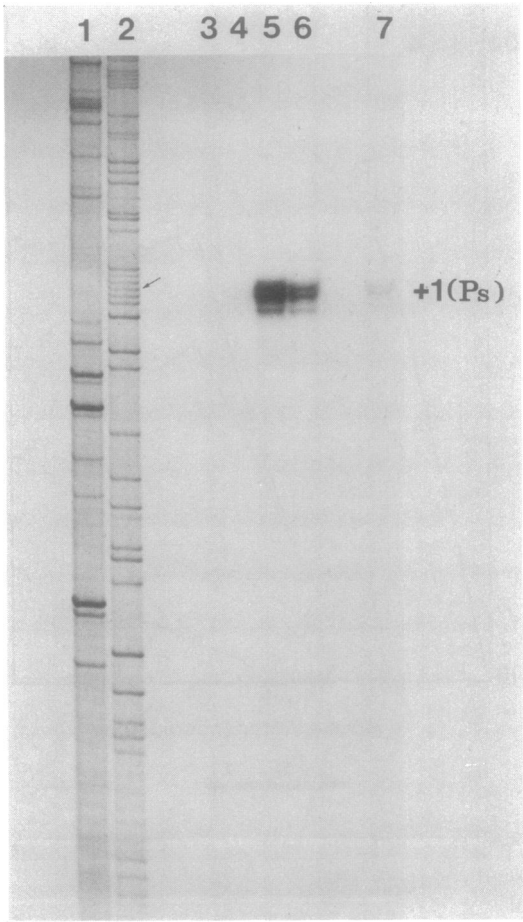


FIG. 6. Determination of the transcription start point for the subtilisin gene. Lanes: 1 and 2, sizing ladder (C and T); 3 to 7: RNA from log-phase ($T_{-1}$) (lane 3), $T_1$ (lane 4), $T_3$ (lane 5), $T_5$ (lane 6), and $T_3$ (lane 7) cells. RNA was purified from DB508 (lanes 3 to 6) and DB510 (lane 7). Arrow indicates the transcription start point of promoter Ps. The sizing ladder was prepared by the method of Sanger et al. (28) with a previously determined sequence (*B. subtilis senS* gene; 34). The size of the T band (lane 2) corresponding to the +1 site was calculated from the M13 sequencing primer. The transcription start site of the promoter was mapped by calculating the same number of bases from the 5′ end of the DNA probe. We used the same strategy with a different sizing ladder to determine the identical +1 site in Fig. 9.

The reconstituted $E\sigma^A$ holoenzyme produced the same sizes of $\phi 29$ $\sigma^A$ transcripts as were reported earlier by Murray and Rabinowitz (25). Only the reconstituted (Fig. 8, lane 4) and purified (lane 7) $E\sigma^A$ holoenzyme transcribed promoter Ps and generated the expected 70-nucleotide runoff transcripts and a 190-nucleotide transcript. The larger band (190 nucleotides) must be the end-to-end transcript generated nonspecifically from the 190-bp template by core enzyme alone, since core enzyme (lane 5) produced only the 190-nucleotide transcript.

On the basis of the size of the in vitro transcripts and sequences around the promoter region, we found good agreement between these results and those obtained from S1 nuclease mapping (Fig. 6).

**Determination of the transcription start site in vivo and vitro.** To determine whether $E\sigma^A$ holoenzyme in vitro recognizes and starts transcribing promoter Ps at the same site as in vivo, we did S1 nuclease mapping with in vivo and in vitro RNA. In vitro RNA was produced by using circular pUC9-S and purified vegetative-cell $E\sigma^A$ holoenzyme (Fig. 7, lane 1), since the circular plasmid was a more efficient template for in vitro transcription than was the linearized one, and the purified RNA polymerase generated the same size of transcript as did the reconstituted enzyme (Fig. 8, lanes 4 and 7). $E\sigma^A$ RNA polymerase started transcription at the same site both in vivo and in vitro (Fig. 9, lanes 5 and 7).

## DISCUSSION

The deletion analyses and S1 nuclease mapping data indicate that a single promoter, Ps, controls expression of the *aprE* gene during growth and sporulation. The deletion downstream of promoter Ps (downward from −1), which removed the region previously thought to contain a $\sigma^B$ promoter (33), did not affect normal transcription or the pattern of subtilisin production. Moreover, deletions that removed regions upstream of promoter Ps (upward from −45) reduced the extent of transcription from Ps significantly, but the reduced transcription was still initiated from only Ps. Deletions upward from −92 allowed expression of the *aprE* gene at the wild-type level. Therefore, promoter Ps appears to be the only promoter present for the *aprE* gene. This finding confirms a recent demonstration of the location of the *aprE* promoter (6) and is in contrast to the location reported previously (33).

Henner et al. (14) have shown that discrete regions that affect maximal expression and temporal expression of the gene are present in the promoter region and that there is a region upstream of the promoter which is required by the products of the *sacQ*, *sacU*, and *hpr* genes. Different effects are observed for deletions upstream of the promoter. Deletions upward from −52 do not affect the wild-type activity of

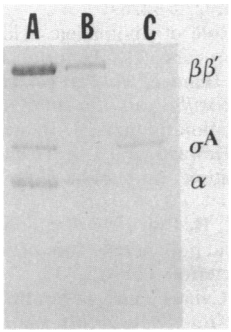


FIG. 7. RNA polymerase profiles on sodium dodecyl sulfate-polyacrylamide gel. Lanes: A, $E\sigma^A$; B, core enzyme; C, $\sigma^A$. $E\sigma^A$ was purified from vegetative cells, and core enzyme was purified from the vegetative holoenzyme shown in lane A as described in Materials and Methods. $\sigma^A$ was cut from the sodium dodecyl sulfate-polyacrylamide gel run of vegetative $E\sigma^A$ (lane A) as described in Materials and Methods. The α and ββ′ subunits of core enzyme are indicated.

the gene (6). However, deletions upward from −45 cause a drastic reduction in Ps activity. Therefore, the 7 bp between −52 and −45 must carry significant information for utilization of the promoter. Mutations downstream of the promoter

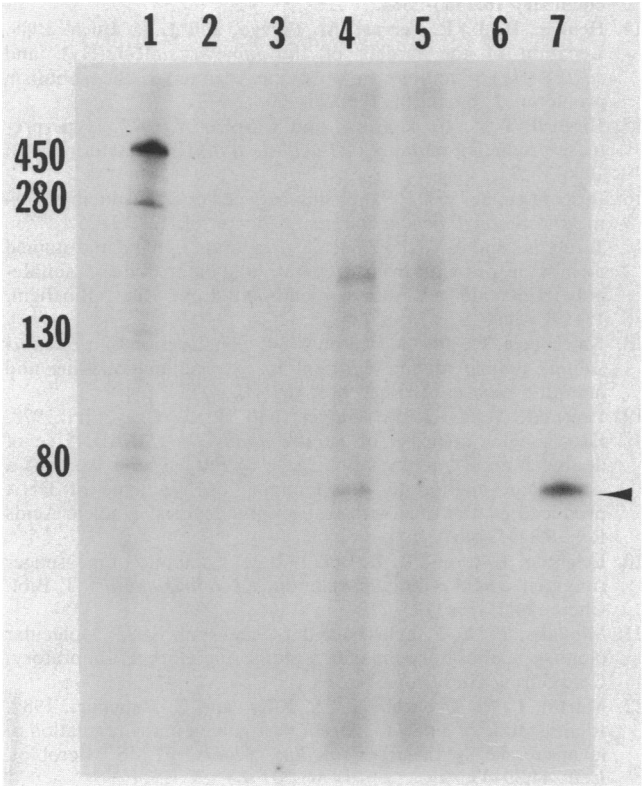


FIG. 8. In vitro runoff transcription from promoter Ps with the reconstituted and purified $E\sigma^A$ RNA polymerase. Lanes: 1, φ29 DNA digested with *Hin*dIII and transcribed with reconstituted $E\sigma^A$; 2, φ29 DNA digested with *Hin*dIII and transcribed with core enzyme; 3, φ29 DNA digested with *Hin*dIII and transcribed with $\sigma^A$; 4 to 7, promoter Ps transcribed with reconstituted $E\sigma^A$ (lane 4), core enzyme (lane 5), $\sigma^A$ (lane 6), and purified vegetative $E\sigma^A$ (lane 7). Arrowhead indicates the transcription start site of promoter Ps. Numbers on the left are sizes (in nucleotides) of standard markers.

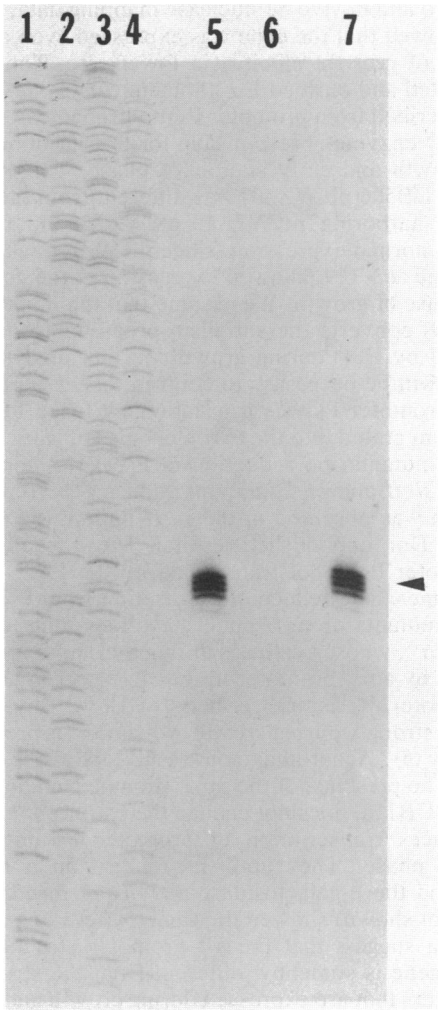


FIG. 9. S1 nuclease mapping of promoter Ps with RNAs prepared in vivo and in vitro. Lanes: 1 to 4, size markers (A, C, G, and T, prepared with M13mp19 [37]); 5, in vivo RNA isolated from $T_3$ cells; 6, *Hpa*II DNA probe without RNA; 7, in vitro RNA produced from pUC9-S with purified $E\sigma^A$. Arrowhead indicates the transcription start site of promoter Ps.

from −1 have no significant effect on wild-type expression of the promoter, which indicates that a 52-bp region between −52 and −1 contains all information required for correct temporal expression of the gene as well as for the wild-type level of expression of the gene.

On the other hand, the region upstream of the promoter, −141 to −164, is required for full stimulation by the *sacU32*(Hy) and *sacQ36*(Hy) mutations, and a region upstream of −200 is necessary for full stimulation by an *hpr-97* mutation (14). These factors control *aprE* expression at the transcription level (14). However, it is not known how these regulatory factors stimulate transcription of the *aprE* gene, nor is it known what physiological conditions regulate expression of the regulatory factor genes. Since the mutations in the regulatory factor genes cause increased production of these factors, the normal cell must be able to elevate the expression of these genes. It is of great interest to determine what physiological conditions can stimulate the production of these regulatory factors.

Previous studies had indicated that the *aprE* gene was expressed only from the early stationary phase of growth.

The in vitro and in vivo S1 nuclease mapping data, however, clearly showed that the gene was expressed even during the log phase of growth, albeit at a lower rate. The fact that reconstituted and purified $E\sigma^A$ holoenzyme from vegetative cells transcribed from promoter Ps in vitro indicates strongly that the $\sigma^A$ enzyme is responsible for transcription from Ps during growth and early stationary phase. The expression pattern of DB506(pKWZΔ3) was different from the patterns of strains harboring pKWZΔ1, pKWZΔ2 and pKWZΔ4, which had normal expression, since pKWZΔ3, with a single base change at −119, allowed expression of the gene during the log phase of growth. We assume that the mutation (G to A at −119) converts the subtilisin promoter to one that is recognized by $E\sigma^A$ during growth; further analysis of this construct will be necessary to confirm this assumption.

When promoter Ps was translationally fused to the *lacZ* gene and integrated into the chromosomes of *sigB*, *sigE*, and sigH null mutants, no reduction in Ps expression was observed in *sigB* and *sigE* mutants, and a 60% reduction of expression was observed in the *sigH* mutant (6; S.-S. Park and R. H. Doi, unpublished results). These results indicate that promoter Ps is not transcribed by the $E\sigma^B$, $E\sigma^E$, and $E\sigma^H$ enzymes. The reduced transcription from Ps observed with null mutants of *sigH* could have been due to reduced levels of $E\sigma^A$, whose synthesis during the stationary phase is controlled by a $\sigma^H$ promoter in the *sigA* operon (3).

If promoter Ps is transcribed by $E\sigma^A$, why is it not expressed strongly during growth, when $E\sigma^A$ is present most abundantly (5)? According to our results and those of Ferrari et al. (7), the presence of the *aprE* promoter on a multicopy plasmid (pUB110) does not change the pattern of expression but enhances transcription of promoter Ps during early stationary phase. The subtilisin activity on a multicopy plasmid and the β-galactosidase activity of fused promoter Ps (data not shown) showed the same pattern of expression. These data suggest that the $\sigma^A$ promoter structure of the subtilisin gene is somehow different from the structures of $\sigma^A$ promoters that are expressed during growth and that $E\sigma^A$ holoenzyme may need another factor to recognize the subtilisin promoter more efficiently at the early stationary phase.

Both the data of Henner et al. (14) and our results suggest that the upstream region of the subtilisin promoter contains information critical for regulation of the gene. It is of great interest to investigate how these factors interact with $E\sigma^A$ holoenzyme and promoter Ps and how they enhance and regulate transcription of the subtilisin gene.

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