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Developmental Expression of Three Proteins from the First Gene of the RNA Polymerase σ^{43} Operon of *Bacillus subtilis*

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The first gene of the *Bacillus subtilis* RNA polymerase σ^{43} operon, *P23*, has a protein-coding capacity of 23,000 daltons. Sequence analysis revealed three potential translational initiation sites within the same reading frame, which could encode proteins of 23,000 (P23), 19,000 (P19), and 9,000 (P9) daltons, respectively. An internal promoter (P3), which is expressed only during the sporulation stage, is located between the second and the third translational start sites. By protein fusion to the *Escherichia coli* β-galactosidase gene, we showed that all three translational initiation sites of the *P23* gene are used in vivo in both *E. coli* and *B. subtilis*, and regulation for differential expression of the three proteins during the development of *B. subtilis* is coupled to the transcriptional promoter switching mechanism. The physiological function of these multiple gene products is unknown and is currently under investigation.

The *Bacillus subtilis* RNA polymerase σ^{43} operon consists of three genes, *P23* (function unknown), *dnaE* (DNA primase), and *rpoD* (σ^{43}) (28) and is similar in several respects to the structure of the *Escherichia coli* σ^{70} operon (3, 15). The major differences occur in the 5′ end of the operon, where the product of the *P23* gene is much higher in molecular weight and has no amino acid homology with the *E. coli* small ribosomal protein S21 encoded by *rpsU*, the first gene of the σ^{70} operon. Furthermore, the transcriptional signals for the σ^{43} operon involve three promoters, two transcribed by the σ^{43} holoenzyme and one transcribed by a σ^{30}-like holoenzyme (4, 6). Analysis of the promoters indicated that the σ^{43} promoters were used during the growth stage and that the σ^{30} promoter was expressed only during early sporulation (29).

Further analysis of this promoter switching phenomenon revealed that the σ^{30} promoter was located within the 5′ region of the *P23* structural gene, which would preclude synthesis of the 23,000-dalton protein encoded by the open reading frame of *P23*. An examination of the base sequence of the *P23* coding region in the mRNA revealed the presence of three potential translational initiation sites that would result in the production of proteins with molecular weights of 23,000 (P23), 19,000 (P19) and 9,000 (P9). The *lacZ* gene fusion has been used to demonstrate in vivo that an open translational reading frame could specify a protein (24). By constructing in-frame fusions of the three translational start sites with the β-galactosidase gene, we found that all three start sites are used in vivo. During growth, P23 and P19 are synthesized. After promoter switching during the early stationary phase, the primary translation product is P9. Thus, a novel mechanism involving both promoter and translation switching regulates expression of the first gene of this important operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* β-galactosidase protein fusion vector pMC1871 (22) (Pharmacia, Inc.) was grown in *E. coli* Mc1061 [Δ(*lacIPOZYA*)*X74 galU galK strA hsdR* Δ(*ala leu*)] (5). *B. subtilis* DB104 (*his nprR2 nprE18* Δ*aprA3*) (12) was used for expression of all of the fusion genes. *B. subtilis* ML1 (*trpC2 sigB*::*cat*) (2) and IS233 (*trpC2 pheA1 spo0H* Δ*Hin*dIII) (6) were kindly provided by R. Losick and I. Smith, respectively. *B. subtilis* plasmid pUB18S was a derivative of pUB110 (16) containing all of the cloning sites (except *Sph*I) present in the pUC18 polylinker.

Construction of *P23-lacZ* gene fusions. Plasmid pMC1871 has a unique *Sma*I site in front of the truncated *lacZ* gene. Blunt-end fragments containing promoter and translational initiation sites of the *P23* gene of the σ^{43} operon were fused in phase with the *lacZ* gene at the *Sma*I site, forming various pMC serial fusion plasmids (see Fig. 2), and the fusion genes were moved to *B. subtilis* plasmid pUB18S. This was done by cutting the pMC derivatives with *Pst*I and subcloning the fused gene (on a *Pst*I fragment) into pUB18S at the unique *Pst*I site in the multiple cloning sites to form pUBZ serial plasmids, which contain the same constructions as their pMC counterparts (see Fig. 1).

Plasmid pMC-HH contains a 1.1-kb *Hae*III fragment insert containing all of the transcription and translation signals (28, 29). The fragment was first cloned into a *Bam*HI site (blunted with Klenow fragment) flanked by two *Sma*I sites in plasmid pSB (29) to form pSB-H1. This plasmid was then digested with *Sma*I, and the 1.1-kb fragment released was subcloned into the unique *Sma*I site in pMC1871 to form pMC-HH so that the reading frame of *P23* was in phase with that of *lacZ*. Plasmid pMC-HL contains the same 1.1-kb *Sma*I insert as that in pMC-HH except that part of an inverted pUC polylinker (*Sma*I-*Bam*HI-*Xba*I-*Bam*HI-*Sma*I) was inserted at the unique *Xmn*I site (Fig. 1, solid bar) so that the first ATG (P23) was forced out of its original phase and subsequently followed by a stop codon in this new reading frame. For construction of pMC-XH, the 1.1-kb *Sma*I fragment was cut by *Xmn*I, and the 430-base-pair *Xmn*I-*Sma*I fragment lacking promoters P1 and P2 and ATG (P23) was subcloned into the *Sma*I site of pUC19 to form pUC19-XH so that the truncated gene was under the control of the *lac* promoter. pUC19-XH was then double digested with *Pvu*II and *Sma*I, and the resulting fragment with blunt ends was directly subcloned into the *Sma*I site of pMC1871. A similar strategy was used for the construction of pMC-TH. After digestion of the 1.1-kb *Sma*I fragment with *Taq*I, the

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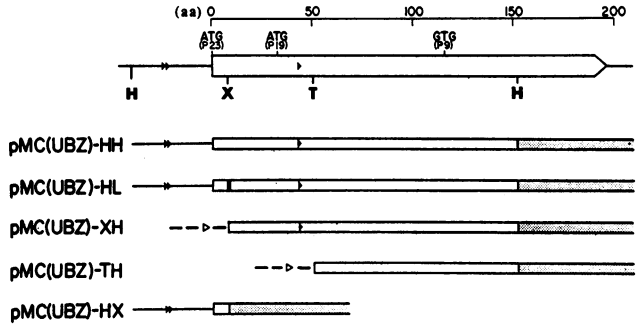

FIG. 1. Construction of *P23-lacZ* gene fusions. The cloning strategy for constructing pMC and pUBZ fusion plasmids is discussed in the legend to Fig. 2. The open reading frame of *P23* is represented by the open box in the upper part of the figure, with its C terminus indicated by an arrow. The locations of three promoters are indicated by small, solid arrowheads. Three potential in-phase translational start sites are shown above the open box (*P23*), and the coding capacities of these open reading frames initiating from different sites are indicated underneath the corresponding start codons in parentheses. In the lower part of the figure, the open triangles and the dashed lines indicate the *lac* promoter and the surrounding sequence in pUC19, respectively. The shaded regions represent the *lacZ* gene portion, which is fused to various parts of the *P23* gene. aa, Amino acids; H, *Hae*III; X, *Xmn*I; T, *Taq*I.

*Taq*I-*Sma*I fragment containing GTG (P9) was first cloned into the *Acc*I-*Sma*I-restricted pUC19 to form pUC19-TH. The following steps were the same as those used for pMC-XH. pMC-HX was constructed simply by digesting the 1.1-kb *Sma*I fragment with *Xmn*I enzyme and directly ligating the *Sma*I-*Xmn*I fragment containing P1, P2, and ATG (P23) with the *Sma*I-digested pMC1871. All of the fusions were made in phase with the *lacZ* gene, and the sequence in the junction regions was confirmed by DNA sequencing (data not shown).

Western blotting. For Western blotting (immunoblotting), cell extracts were prepared simply by lysing of the cells in the sample application buffer (62 mM Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate, 20% glycerol, 5% mercaptoethanol, 0.001% bromphenol blue), denatured by heating for 5 min at 100°C, and electrophoresed in sodium dodecyl sulfate–7.5% polyacrylamide gels for 5 h at 38 mA of constant current. The resolved protein bands were then blotted to nitrocellulose paper (Schleicher & Schuell, Inc.) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% [vol/vol] methanol, pH 8.3). Blotting was carried out at 200 mA for 3 h, and the nitrocellulose paper was blocked with blocking buffer (3% [wt/vol] bovine serum albumin, 0.15 M NaCl, 1 mM EDTA, 0.05 M Tris hydrochloride [pH 7.4], 0.005% [vol/vol] Tween 20) for at least 2 h. After blocking, the nitrocellulose paper was incubated with the primary antibody, rabbit anti-β-galactosidase (1:100 dilution in blocking buffer from the original solution from Cappel Worthington), for 3 h to overnight. The primary antibody was washed away by NET buffer (150 mM NaCl, 5mM EDTA, 50 mM Tris hydrochloride [pH 7.4], 0.25% [wt/vol] gelatin, 0.05% [vol/vol] Nonidet P-40 from Shell). Incubation with the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:500 dilution in blocking buffer from the original solution from Cappel Worthington), followed immediately for 2 h. The above washing process was repeated before development of the nitrocellulose paper in the developing solution (40 mg of 4-chloro-D-naphthanol dissolved in 40 ml of methanol mixed with 200 ml of Tris hydrochloride [pH 7.4], 500 mM NaCl containing 0.8 ml of 30% hydrogen peroxide).

Other Methods. DNA sequencing was conducted by the dideoxy chain termination method of Sanger et al. (21) by the procedures specified in the sequencing kit purchased from Amersham Corp. β-Galactosidase activity was assayed as previously described (17). The protein content of the whole-cell extract was determined by the Folin assay as described by Hanson and Phillips (8).

RESULTS

Effects of promoter switching on transcription and translation of *P23*. We showed previously that switching from promoters P1 and P2 to P3 of the σ^{43} operon occurred during the early stages of sporulation (29). The localization of promoter P3 in the 5′ end of the *P23* gene downstream from the translation start site for synthesis of the 23,000-dalton product indicated that it could no longer be synthesized and that either no product was to be made from *P23* or another translation start site downstream from promoter P3 would be used to synthesize a smaller protein.

An analysis of the σ^{43} transcribed mRNA sequence revealed the presence of three potential translation start sites that could result in production of proteins with masses of 23,000 (P23), 19,000 (P19), and 9,000 (P9) daltons (Fig. 1, upper part). The first two sites are initiated with ATG, whereas the third site is initiated with GTG. All three initiation sites are in the same reading frame. The ribosome-binding sequences (Shine-Dalgarno sequences) of the three initiation sites have free energies ($\Delta G'$) of binding to ribosomes of −9.2, −11.5, and −9.4 kcal/mol, respectively (1 cal = 4.184 J). In comparison with other *Bacillus* ribosome-binding sites, these would be considered as rather poor translation initiation sequences since the average $\Delta G'$ value for most of the characterized *B. subtilis* genes is −17 to −18 kcal/mol (1, 18). Furthermore, since promoter P3 is downstream of the translation initiation sites for P23 and P19, only P9 could be synthesized during the sporulation phase, when the σ^{30} transcript is made from P3.

Construction of *P23-lacZ* fusions for analysis of translation initiation signals. To test whether the three putative translation signals were functional in vivo, protein fusions of the translation start sites with the β-galactosidase gene were used. The lower part of Fig. 1 illustrates the five different protein fusion genes that were constructed in plasmids for this study. All of the fusions were made in frame with the initiation codons, and the sequences in the junction regions were confirmed by DNA sequencing (data not shown). The first construction (HH) contained all three initiation sites in frame with the β-galactosidase gene. Construct 2 (HL) contained a small insertion, resulting in a change in the original open reading frame that prevented a translation product from the P23 initiation site but still allowed the synthesis of products from both the P19 and P9 initiation sites. A deletion removing the P23 site altogether but leaving intact the P19 and P9 sites was used to construct XH. Both the P23 and P19 start sites were eliminated in TH, leaving only the P9 start site intact. In the last construct (HX), the P19 and P9 sites were deleted so that only the P23 start site was functional. Figure 2 schematically shows the cloning strategies used in the construction of the gene fusions in both *E. coli* and *B. subtilis*. Restriction fragments containing the promoter, the ribosomal binding site, and part of the truncated *P23* gene were cloned into the unique *Sma*I site of plasmid pMC1871 to form pMC serial plasmids, which were

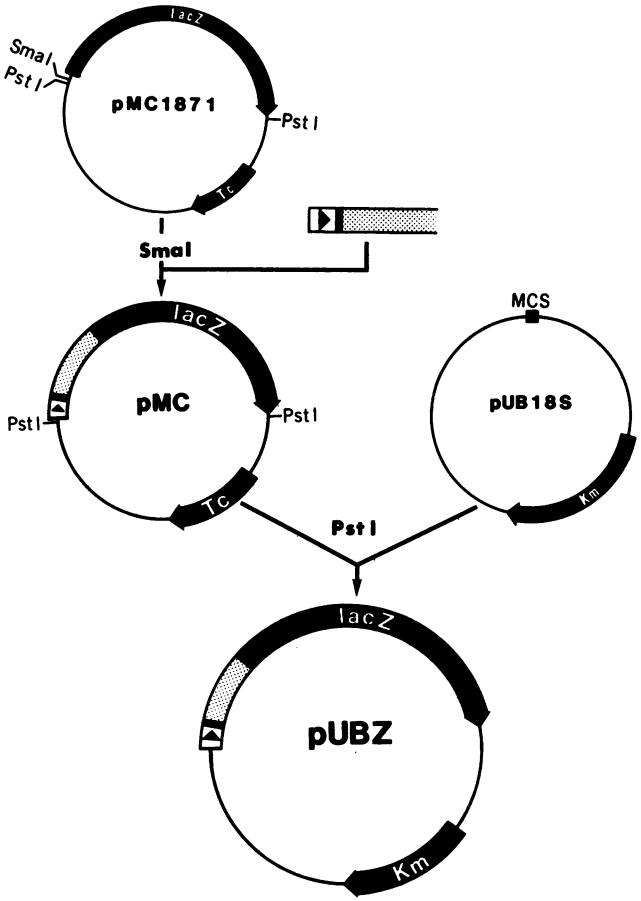

FIG. 2. Strategy for construction of *E. coli* and *B. subtilis* fusion plasmids. Restriction fragments containing a promoter (solid arrowhead), a ribosomal binding site (solid bar), and part of the *P23* gene (shaded region) were cloned into pMC1871 at the unique *Sma*I site. The correct orientation and reading frame were selected by the biological activity, i.e., β-galactosidase activity, of the fused genes. The fusion genes were then moved to *B. subtilis* pUB plasmids in a *Pst*I fragment, as shown, to form pUBZ plasmids, which were expressed in *B. subtilis*. MCS, Multiple cloning sites.

expressed in *E. coli*. Then the entire construction was moved to pUB18S at the unique *Pst*I site, forming the pUBZ plasmid serials, whose expressions in *B. subtilis* were then studied.

Expression of the *P23-lacZ* fusion gene in *E. coli* and *B. subtilis*. The fusion gene products were detected by Western blot analysis with anti-β-galactosidase antibody. The results are shown in Fig. 3 (for *E. coli*) and 4 (for *B. subtilis*); the lanes in both figures are labeled identically. Lane 6 contained *E. coli* β-galactosidase as an authentic marker. Plasmid pUBZ-HX (lane 5), which only has the initiation sequence for P23, produced a fusion product indicating that this ATG (P23) start site was active and that a 23,000-dalton protein could be made in vivo. Similarly, the results in lane 4 indicated that the GTG (P9) start site was active and that a 9,000-dalton product could be made in vivo. Lanes 2 and 3 had virtually the same banding pattern, as expected from the constructs for plasmids pUBZ-HL and pUBZ-XH; these results indicated that the ATG (P19) and GTG (P9) sites were active and that P19 and P9 products could be synthesized from this gene. The construct in lane 1 contained all three

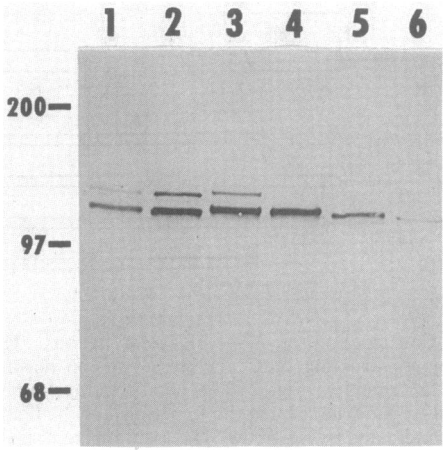

FIG. 3. Western blot of the *P23-lacZ* fusion gene products expressed in *E. coli*. *E. coli* MC1061 containing different pMC fusion plasmids were grown in L broth until mid-log stage. A 0.5-ml volume of each of the cultures was harvested at 4°C. Electrophoresis and Western blotting were carried out as described in Materials and Methods. Lanes 1 to 5 contained samples from cultures of pMC-HH, pMC-HL, pMC-XH, pMC-TH, and pMC-HX, respectively. Lane 6 contained 1 μg of *E. coli* β-galactosidase (Boehringer Mannheim Biochemicals) as an authentic marker. The numbers on the left indicate molecular masses (in kilodaltons) of standards.

potential translational initiation sites. However, because of the similarity in the mobilities of the P23– and P19–β-galactosidase fusion proteins, they were not resolved in the gel system used. Although it is possible that ATG (P19) is blocked in the presence of ATG (P23), a more recent construction containing the same 1.1-kilobase (kb) *Hae*III fragment fused to an N-terminal truncated β-lactamase gene, which is much smaller in size than *lacZ*, generated three well-separated bands (K. S. Sei and R. H. Doi, unpublished data). Thus, these results indicated that the three translation start sites were all active in vivo and that three protein products could be synthesized from gene *P23*. Since these

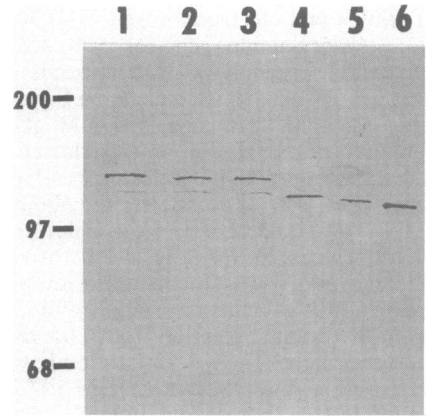

FIG. 4. Western blot of *P23-lacZ* fusion gene products expressed in *B. subtilis*. *B. subtilis* DB104 containing different pUBZ plasmids was grown in sporulation medium 2× SG (13) until mid-log stage. A 1-ml volume of each of the cell cultures was collected at 4°C. The conditions for electrophoresis and Western blotting were as for Fig. 3. Lanes 1 to 5 contained samples from cultures of pUBZ-HH, pUBZ-HL, pUBZ-XH, pUBZ-TH, and pUBZ-HX, respectively. Lane 6 contained 1 μg of β-galactosidase. The numbers on the left indicate molecular masses (in kilodaltons) of standards.

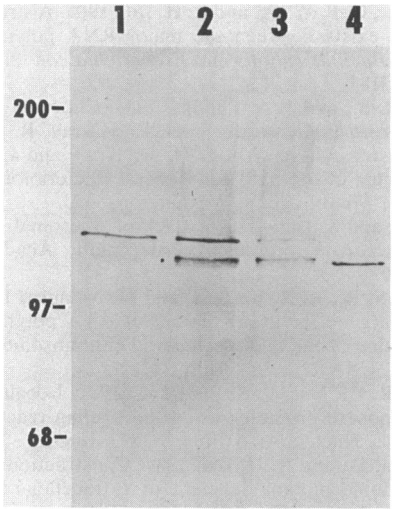

FIG. 5. Time course expression of the *P23-lacZ* fusion gene in *B. subtilis*. DB104 containing plasmid pUBZ-HH (Fig. 1) was grown in sporulation medium 2× SG as described in the legend to Fig. 4, and 10-ml samples of cell cultures were removed at designated stages. The protein contents of the whole-cell extracts were determined as previously described (8). The fusion gene products in 1.5 mg of cell extract from each stage were resolved with a sodium dodecyl sulfate–7.5% polyacrylamide gel and detected by Western blotting with anti-β-galactosidase antibody (from Cooper Biomedical, Inc.). Lanes 1 to 3 contained 1.5 mg each of cell extract from cultures containing plasmid pUBZ-HH at T_0, T_2, and T_4, respectively. Lane 4 contained 1 μg of *E. coli* β-galactosidase. The numbers on the left indicate molecular masses (in kilodaltons) of standards.

proteins were synthesized in the same reading frame, they were partially homologous. One interesting phenomenon was discovered when the Western blot patterns were compared for expression of the fusion genes in *E. coli* and *B. subtilis*. During the growth of *B. subtilis*, P9 was very poorly expressed (Fig. 4), whereas in *E. coli* it was expressed at a much higher level than that of P23 or P19 (Fig. 3). The reason for this is unclear, but the results suggest a difference in the utilization of ribosomal binding sites by *E. coli* and *B. subtilis*.

Time course of expression of the *P23* gene during sporulation. In our previous report, we were able to show that a temporally regulated promoter (P3) was located inside the coding region of the *P23* gene. The promoter had sequence homology with other known σ^{37} promoters (29). However, our recent promoter probe studies conducted with the σ^{37} deletion mutant strain (2) indicated that this promoter was not controlled by *sigB*. Instead, expression from promoter P3 was completely repressed in a *spo0H* deletion mutant strain (6), strongly suggesting that P3 is controlled, directly or indirectly, by the σ^{30} factor, which is the gene product of *spo0H* (4, 6). It was of interest to determine whether there was a direct correlation between promoter switching which occurred at T_1 (29) (the subscript stands for hours after the end of the log phase of growth) and the appearance of the P9 fusion protein product.

Plasmid pUBZ-HH, containing all three translation initiation sites, was grown in the regular sporulation medium, and cell samples were taken at T_0, T_2, and T_4 and analyzed for synthesis of the fusion proteins by Western blotting.

The results indicated that mainly P23 and P19 were synthesized until T_0 (Fig. 5). At T_2, when transcription from promoters P1 and P2, as well as from promoter P3, occurred, translation from GTG (P9) was activated, indicating that this site was suppressed in the promoter P2- and P3-derived early transcripts. By T_4, most of the product synthesized was P9. Thus, the appearance of P9 correlated well with the promoter switching that occurred at T_1 to T_2 (29).

DISCUSSION

The analysis of *P23*, the first gene in the RNA polymerase σ^{43} operon of *B. subtilis*, revealed several unique features of its structure and regulation. The mRNA of this gene can be translated from three in-phase translation initiation sites, resulting in the production of three highly homologous proteins. Two of the translation start sites are used efficiently during the growth phase, whereas the third site is used exclusively during the early sporulation phase. This third site is used poorly during growth, if at all, perhaps because of translational occlusion from use of the two upstream start sites. This is an unusual type of regulation in procaryotic organisms, although a few cases have been reported for the synthesis of two proteins from the same gene. In some cases, the two proteins were derived by modification of a primary translation product through covalent addition of a small group to the primary structure (e.g., see reference 27). Another general mechanism that has been proposed for the production of two homologous proteins from the same gene is dependent on the presence of either (i) one or two mRNAs that overlap a gene or (ii) an internal translation initiation signal in a single mRNA (11, 14, 19, 20, 23, 25). The presence of one mRNA for a gene with an internal translation initiation signal has been demonstrated (14, 19). The mechanism reported in this paper differs from the previous ones in that two different mRNAs are transcribed from gene *P23* at two different stages of growth, and this results in the production of three proteins from the same gene. The large mRNA synthesized during growth codes for all three proteins but is used primarily to synthesize the two larger proteins (P23 and P19). The second, smaller mRNA results from transcription switching and only codes for the third protein, P9. The synthesis of the products are controlled in a temporal fashion and regulated by promoter switching during development. Thus, there is novel regulatory coupling at the transcription and translation levels that allows temporal synthesis of only the third protein of the *P23* gene during the early stage of sporulation.

Another unusual feature of the translation start signals on the mRNA for the *P23*-encoded proteins are the relatively weak ribosome-binding sites. The usual $\Delta G'$ of mRNA and rRNA interaction for *Bacillus* systems is -17 to -18 kcal/mol (1, 18). The $\Delta G'$s for the three ribosome-binding sites present in the *P23* gene range from -9.2 to -11.5 kcal/mol (28). The $\Delta G'$s of the ribosome-binding sites for the *dnaE* and *rpoD* genes (the second and third genes of the operon) are -13.8 (30) and -18.8 kcal/mol (7), respectively. Does this mean that the *P23*-encoded proteins are made less efficiently than most of the proteins, or are there other factors besides the free energies of interaction that determine efficiency of translation? We are currently trying to determine the relative amounts of proteins made from the three genes of the σ^{43} operon.

The comparison of the relative expression levels of P23 and P19 versus P9 in *E. coli* and *B. subtilis* may indicate a basic difference between the translational initiation mechanisms of the two organisms. Although we have not excluded the possibility that the higher-level expression of P9 during mid-log growth in *E. coli* is due to efficient transcription from

the internal promoter P3, previous studies indicating poor expression of *B. subtilis* minor sigma promoter in *E. coli* (31) make this explanation unlikely. On the other hand, the absence of ribosomal protein S1 in *B. subtilis* (9) and the recent discovery of the differences between *B. subtilis* and *E. coli* in two important ribosomal protein-containing operons, i.e., the *rpoD* (3, 15, 28, 29) and *rpoA* operons (10, 26), suggest a possible difference in the ribosome components and functions that might explain the different expression patterns of the same genes as observed in our studies.

The multiple translation start sites result in the production of three partially related proteins whose functions are still unknown. The P23 protein is not homologous to S21. A search of existing amino acid sequence banks has not revealed any closely related proteins from other organisms. This suggests that P23 is a *Bacillus*-specific protein or that the amino acid sequence of an analogous protein that exists in other organisms has not been reported as yet. The difference in operon structures containing the *rpoA* genes (10, 26) also indicates that *B. subtilis* and *E. coli* operons concerned with transcription and translation genes have evolved differently. Thus, it is difficult to predict what the function of P23, P19, and P9 may be. However, it is likely that some translational function may be involved if one considers the functions of the genes in the *E. coli* σ^{70} operon (3, 15).

Since P23 (196 amino acids) and P19 (164 amino acids) differ in size by only 32 amino acids, their functions could be very similar. On the other hand, P9 (81 amino acids) contains only the C-terminal 41% of the amino acid sequence of P23 and its function could be significantly different. However, the functions of all three proteins could be similar if the C-terminal domains of P23 and P19 contain the major functions for these proteins and those functions are retained in P9. We are currently attempting to identify the subcellular location(s) and functions of these proteins to determine whether they are essential gene products and to see whether an analogous protein exists in *E. coli*.

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