JOURNAL OF BACTERIOLOGY, May 1989, p. 2553–2562
0021-9193/89/052553-10$02.00/0

Vol. 171, No. 5

Gene Encoding the Alpha Core Subunit of *Bacillus subtilis* RNA Polymerase Is Cotranscribed with the Genes for Initiation Factor 1 and Ribosomal Proteins B, S13, S11, and L17

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Received 20 October 1988/Accepted 8 February 1989

We describe the genetic and transcriptional organization of the promoter-distal portion of the *Bacillus subtilis* alpha operon. By DNA sequence analysis of the region surrounding *rpoA*, the gene for the alpha core subunit of RNA polymerase, we identified six open reading frames by the similarity of their products to their counterparts in the *Escherichia coli* transcriptional and translational apparatus. Gene order in this region, given by gene product, was IF1-B-S13-S11-alpha-L17. Gene order in *E. coli* is similar but not identical: SecY-B-S13-S11-S4-alpha-L17. The *B. subtilis* alpha region differed most strikingly from *E. coli* in the presence of IF1 and the absence of ribosomal protein S4, which is the translational regulator of the *E. coli* alpha operon. In place of the gene for S4, *B. subtilis* had a 177-base-pair intercistronic region containing two possible promoter sequences. However, experiments with S1 mapping of in vivo transcripts, gene disruptions in the alpha region, and a single-copy transcriptional fusion vector all suggested that these possible promoters were largely inactive during logarithmic growth, that the major promoter for the alpha operon lay upstream from the region cloned, and that the genes in the IF1 to L17 interval were cotranscribed. Thus, the transcriptional organization of the region resembles that of *E. coli*, wherein the alpha operon is transcribed primarily from the upstream *spc* promoter, but the absence of the S4 gene suggests that the translational regulation of the region may differ more fundamentally.

Studies of operons encoding RNA polymerase subunits and ribosomal proteins have contributed to understanding fundamental mechanisms of gene regulation in *Escherichia coli*. The coordinate synthesis of 52 ribosomal proteins, the genes for which lie in 12 operons, is largely mediated by an autogenous feedback mechanism wherein certain ribosomal proteins, when unbound by rRNA, prevent the translation of their own mRNAs (19, 26). This feedback mechanism may also play a role in both stringent control and growth rate regulation of these operons (7, 19). However, the details of the autogenous translational control and the mechanism of transcriptional regulation differ somewhat among the *E. coli* operons studied thus far (19).

Ten of the 12 ribosomal protein operons also contain nonribosomal protein genes whose products are involved in RNA and protein synthesis. In the alpha ribosomal protein operon, which contains the gene for the alpha core subunit of RNA polymerase, a novel form of translational coupling has been suggested to coordinate synthesis of the ribosomal proteins independently from the alpha protein (41).

Studies of the ribosomal protein operons of *Bacillus subtilis*—a gram-positive, spore-forming bacterium evolutionarily divergent from *E. coli*—may indicate which mechanisms of growth rate regulation are common among procaryotes. Earlier we isolated the gene encoding the alpha subunit of *B. subtilis* RNA polymerase, mapped its locus within the major ribosomal protein gene cluster at 12° on the *B. subtilis* chromosome, and by DNA sequence analysis identified the genes encoding *B. subtilis* ribosomal proteins S13 and S11 immediately preceding the alpha gene (39). This organization is similar to that of the *E. coli* alpha operon, which in addition to the alpha gene contains four ribosomal protein genes in the order (given by gene product) S13, S11, S4, alpha, and L17 (2). In *E. coli*, more of the alpha operon transcript originates from the promoter of the upstream *spc* operon than from the alpha promoter immediately preceding S13 (6; L. Lindahl, R. H. Archer, and J. M. Zengel, personal communication), but the translational regulation of the alpha operon by free ribosomal protein S4 is independent of *spc* translational control (46).

Here we report the characterization of the region surrounding the *B. subtilis* alpha gene, a region physically comparable to the entire *E. coli* alpha operon. DNA sequencing of a 3.2-kilobase (kb) fragment in the alpha region indicated a gene order of *infA* (IF1), *rpmJ* (ribosomal protein B), *rpsM* (S13), *rpsK* (S11), *rpoA* (alpha), and *rplQ* (L17). S1 nuclease mapping experiments, disruptions of the region with integrative plasmids, and studies with a single-copy transcriptional fusion vector all suggest that the genes in the *B. subtilis* alpha region constitute a single transcriptional unit whose major promoter lies upstream of the region cloned.

MATERIALS AND METHODS

Bacteria and phage. *E. coli* Y1090 (48) was used as a host for λgt11, strain JM101 was host for M13mp18 and M13mp19 (45), and strain DH5α (14) was the host for plasmid constructions. *B. subtilis* PB2 (39) served as the transformation host; transformations were done as described previously (31), selecting for drug resistance on tryptose blood agar base (TBAB; Difco Laboratories) plates containing chloramphenicol (5 μg/ml). The AmyE$^-$ phenotype of *B. subtilis* transformants was scored after iodine staining of TBAB plates containing 1% starch (36). λgt11 phage were grown in liquid culture and on plates as described by Davis et al. (8).

DNA methods. Chromosomal and plasmid DNA isolations, restriction endonuclease digestions, agarose gel electrophoresis, ligations, and transformations of *E. coli* strains were

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done as described previously (39). Miniscreens of lambda clones were done with LambdaSorb Phage Adsorbant (Promega Biotec) according to the manufacturer's instructions. Restriction fragments were purified for subcloning by electrophoresis in Seaplaque low-gelling-temperature agarose (FMC Bioproducts). The visualized fragments were cut from the gel, remelted at 65°C, and added to the ligation reaction mix. After 24 h of incubation at room temperature, the reaction mix was heated to 65°C, diluted 10-fold with 0.1 M Tris hydrochloride (pH 8), and then added to competent cells (38). Restriction fragments used as probes were purified by electroelution from an agarose gel (22). The DNA was purified from contaminants with an Elutip-d column (Schleicher & Schuell, Inc.).

We labeled DNA probes used in Southern analyses and λgt11 library screenings with [α-^{32}P]dATP (Amersham Corp.) by the random-primed DNA method of Feinberg and Vogelstein (11). Reaction mixtures contained 400 ng of DNA; 12.5 μg of random hexanucleotide primer (Pharmacia, Inc.); 3 nmol each of dCTP, dGTP, and dTTP; 100 pmol of dATP; 50 μCi of [α-^{32}P]dATP (3,000 Ci/mmol); and 2 U of Klenow fragment (Boehringer Mannheim Biochemicals). After 1 h of incubation at room temperature, the reaction was terminated by the addition of 5 μl of 0.5 M EDTA. Unincorporated nucleotides were removed by chromatography on a 1-ml Sephadex G-50 column.

For hybridization screening of the λgt11 banks, we made plaque lifts with nitrocellulose filters (Schleicher & Schuell) and hybridized them for 6 h at 65°C in an aqueous reaction mix containing 2×10^6 dpm of ^{32}P-labeled probe (8). For Southern analyses, DNA was transferred from an agarose gel to a Zetaprobe (Bio-Rad Laboratories). Alkaline blotting and hybridization conditions were those described by the supplier.

Restriction fragments were cloned into M13mp18 and M13mp19 for sequencing by the dideoxynucleotide chain termination method of Sanger et al. (35). We used Sequenase (U.S. Biochemicals Corp.) to label sequencing reactions with [α-^{35}S]dATP (Amersham). Reaction conditions were those described by the manufacturer.

S1 nuclease mapping. We isolated RNA from *B. subtilis* PB2 grown to mid-log phase (100 Klett units) in Schaeffer 2XSG medium (18). RNA was prepared essentially as described by Gilman and Chamberlin (12), with the following exceptions. Before cell lysis, vanadyl-ribonucleoside complex (Bethesda Research Laboratories, Inc.) was added to a final concentration of 10 mM. The first series of phenol-chloroform extractions were done at 50°C, and proteinase K was self-digested for 2 h at 37°C before use.

Restriction fragments were labeled at their 3′ ends with Klenow fragment as described by Maniatis et al. (22), except 50 μCi of [α-^{32}P]dATP (3,000 Ci/mmol) was added to the reaction mixture. Fragments were labeled at their 5′ ends as described by Maxam and Gilbert (23); after treatment with calf intestinal alkaline phosphatase (Boehringer Mannheim), we end-labeled them with T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP. After labeling, we then digested all DNA probes with a second restriction enzyme and purified the desired fragment by electroelution from an agarose gel as described above. In this manner, only the template strand of the double-stranded probe was labeled at the appropriate 5′ or 3′ end.

We mixed labeled DNA fragments (10,000 to 20,000 dpm) with either 120 μg of *B. subtilis* in vivo RNA or 50 μg of *E. coli* tRNA and then precipitated the fragments with ethanol. The precipitated nucleic acids were dried and dissolved in 35 μl of 40 mM, 1,4-piperazine-diethanesulfonic acid buffer, pH 6.4, containing 0.4 M NaCl, 1 mM EDTA, and 80% formamide (3). Samples were incubated at 80°C for 10 min and then rapidly transferred to a heat block for a 3-h hybridization at the temperatures indicated in the figure legends. We terminated the hybridization reactions by diluting the samples into 350 μl of chilled buffer containing 5% glycerol, 0.25 M NaCl, 30 mM sodium acetate, 1 mM $ZnSO_4$, 8 μg of denatured salmon sperm DNA, and 160 U of S1 nuclease (Boehringer Mannheim). After 1 h of incubation at 37°C, the nucleic acids were precipitated with ethanol. Dried samples were dissolved in 5 μl of Sequenase stop solution (U.S. Biochemicals), heated at 95°C for 5 min, and then analyzed by electrophoresis on an 8% polyacrylamide gel containing 8.3 M urea.

Computer analysis of protein sequence data. To compare the predicted products of open reading frames with those in the National Biomedical Research Foundation library, we used the FASTP program (21) and the National Biomedical Research Foundation VAX computer. Statistical evaluation of the relationships was done by the criteria of Lipman and Pearson (21), including (i) the initial and optimized similarity score determined from identical residues and from conserved replacements derived from an amino acid replaceability matrix and (ii) a z value calculated by the program RDF. The z value is defined as the similarity score minus the mean similarity score of randomly shuffled permutations of the sequence divided by the standard deviation about the randomly shuffled mean. Highly related sequences are considered those with initial similarity scores greater than 50 that increase to over 100 after optimization and that have a z value greater than 10 (21).

RESULTS

Isolation of λgt11 phages containing the alpha region. We previously isolated from a λgtll expression vector library four unique clones carrying *rpoA*, the gene encoding the alpha subunit of *B. subtilis* RNA polymerase (39). Together these clones carried part of the S13 gene, all of S11 and alpha, and about 3.0 kb of DNA downstream of alpha (Fig. 1). In *E. coli*, the alpha operon is largely transcribed from the upstream *spc* promoter in vivo, but a secondary promoter has recently been detected immediately upstream of S13 (L. Lindahl, R. H. Archer, and J. M. Zengel, personal communication), at the site suggested by earlier work (16, 30). Consequently we used chromosome walking to isolate the promoter-proximal part of S13 from *B. subtilis*, together with additional upstream DNA that might contain an alpha operon promoter, in order to directly compare this entire region with the corresponding operon in *E. coli*.

As shown in Fig. 1, we used the 0.9-kb *Eco*RI fragment carried by clone 11-3 as a probe to isolate six phages from our λgtll *Eco*RI library (described previously [39]). Restriction analysis indicated that all of the new isolates contained a 1.8-kb *Eco*RI fragment which completely overlapped the 0.9-kb fragment and which contained additional upstream DNA (clone 10-1 in Fig. 1). Southern analysis of chromosomal DNA with the 0.9-kb *Eco*RI fragment as a probe showed that the restriction map of the *B. subtilis* genome in this region was entirely consistent with the restriction analysis of the fragments isolated in λgtll (data not shown).

Nucleotide sequence of the alpha region. Earlier we sequenced the 0.9-kb *Eco*RI fragment from clone 11-3 to identify the 3′ end of the gene for ribosomal protein S13, the

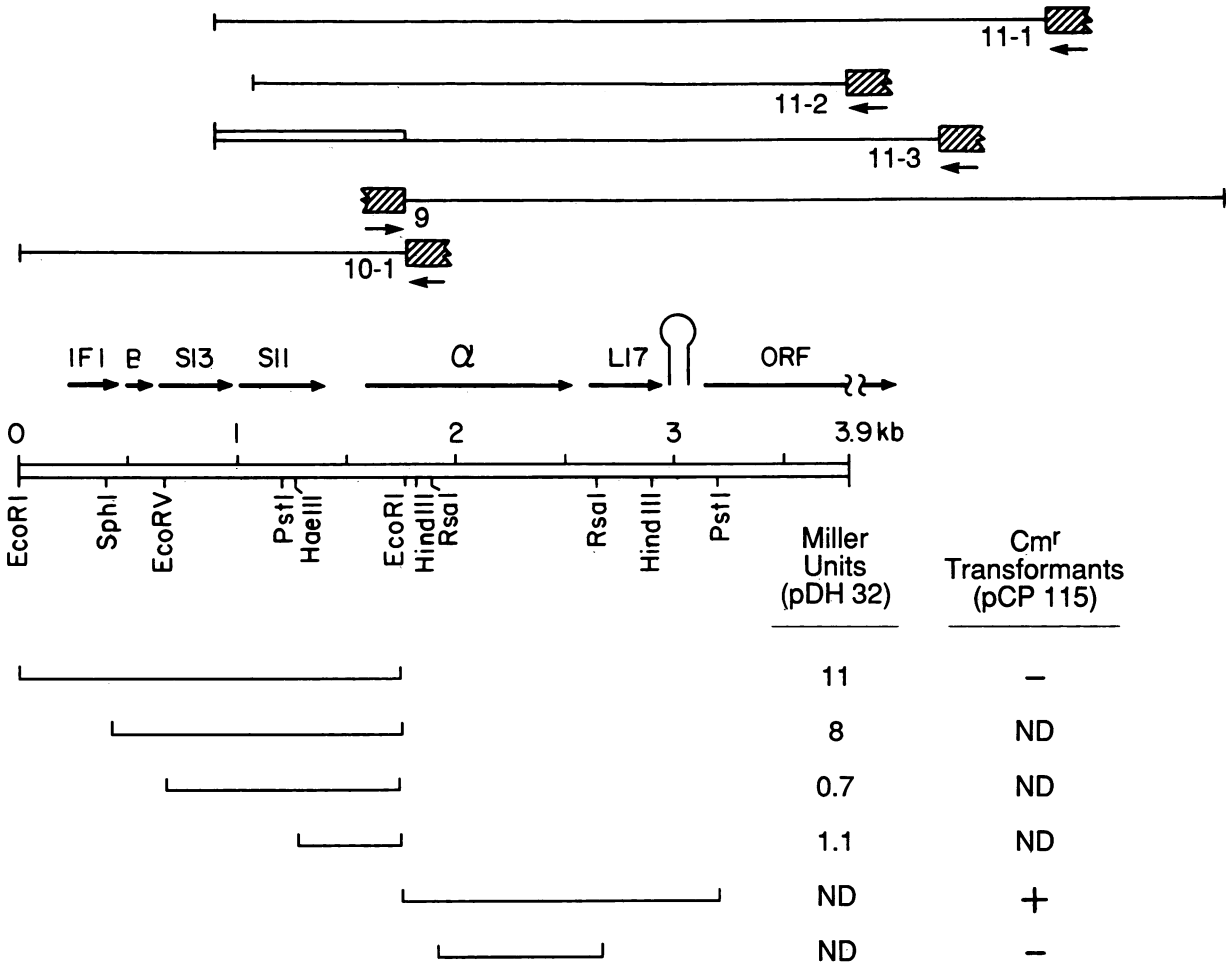
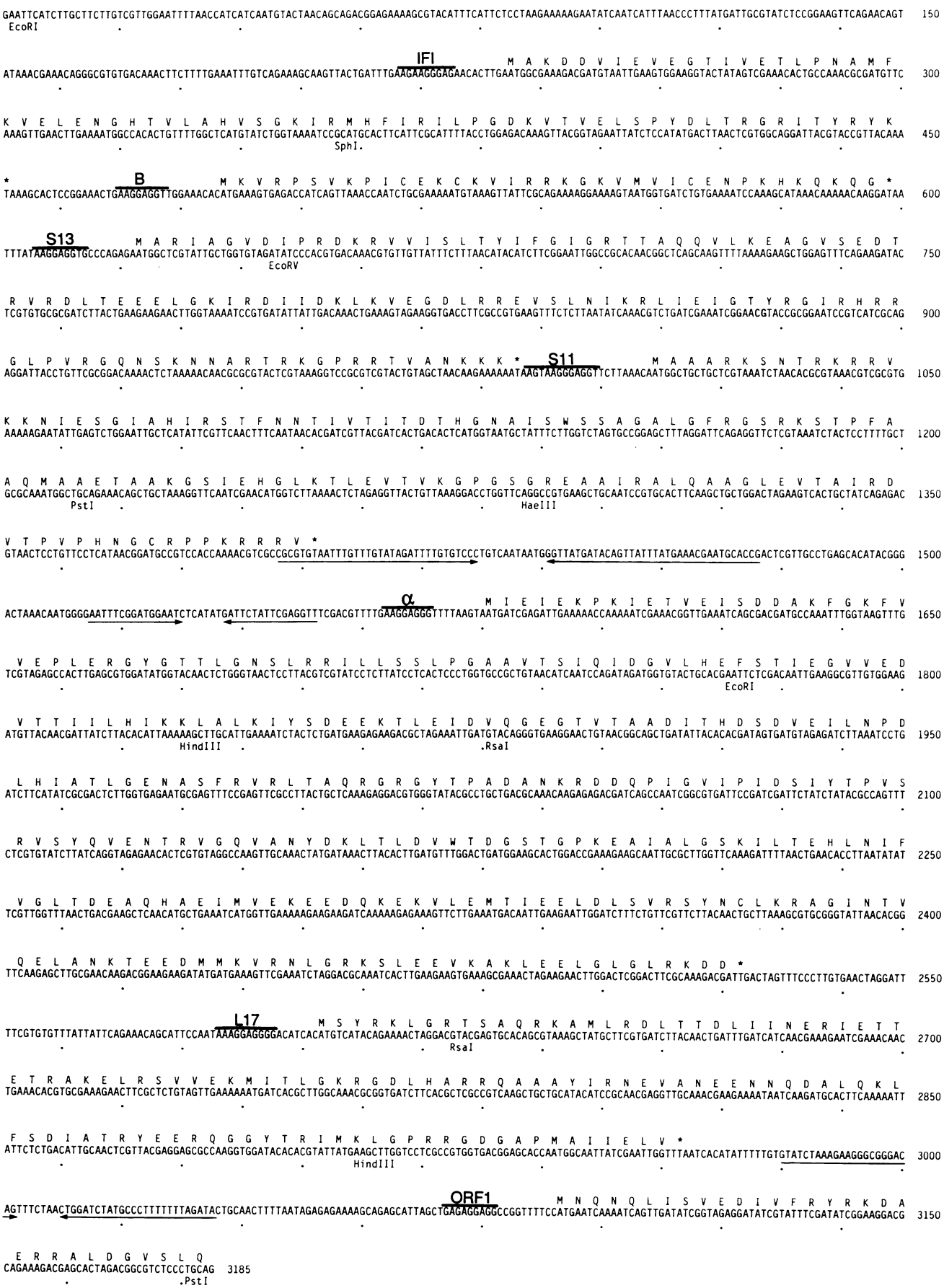


FIG. 1. Physical map of the alpha region. The upper portion shows the recombinant phages used to derive the restriction map. Phages 11-1, 11-2, 11-3, and 9 were isolated previously (39), and phage 10-1 was isolated in this study. The *lacZ* gene (hatched box with arrow denoting direction of transcription) indicates orientation of cloned fragments with respect to the right arm of λgtll (47). The inserts of phages 11-1, 11-2, and 11-3 are bounded by the *Eco*RI linkers used during library construction, whereas the inserts of phages 9 and 10-1 are bounded by *Eco*RI sites native to the *B. subtilis* genome. The 0.9-kb *Eco*RI probe used to isolate phage 10-1 is denoted by the open box on the left end of the phage 11-3 insert. The coding regions and direction of transcription of the genes identified are represented by arrows above the restriction map, and the proposed transcription terminator of the alpha operon is represented by the loop between the L17 gene and the downstream open reading frame (ORF). Results of plasmid integration experiments and β-galactosidase activity (Miller units) from transcriptional fusions are shown beneath the physical map. The thin lines denote the restriction fragments from the alpha region cloned into the *Eco*RI site of the single-copy transcriptional fusion vector pDH32. *Eco*RI linkers (New England BioLabs) were attached to the restriction fragments as necessary, and fragments were oriented so that transcription would extend into the promoterless *lacZ* gene of pDH32. The 1.8-kb *Eco*RI fragment was cloned in the reverse orientation to serve as a negative control, whereas the strong promoter for the tRNA gene cluster internal to the *rrnB* operon (43) was used as a positive control. These pDH32 plasmids were linearized and transformed into the *amyE* locus of *B. subtilis* PB2 *trpC2* and then grown in Schaeffer 2XSG medium (18) for assay of β-galactosidase activity (24) at the mid-log phase of growth, as shown in the left-hand column (ND, not determined). Under these conditions, the negative control gave 0 Miller units, whereas the strong tRNA promoter gave 418 Miller units. The other fragments shown beneath the physical map were subcloned into the appropriate sites of the pCP115 integration vector (31). *B. subtilis* PB2 was transformed with these plasmids with selection for chloramphenicol resistance. A + in the right-hand column indicates 1,000 to 4,000 colonies per ml of transformed cells, whereas a – indicates 0 to 10 colonies per ml. (Only the *Eco*RV, *Hae*III, and *Rsa*I sites used in the subcloning are shown on the restriction map. Additional sites were found for *Eco*RV at nt 3112, 3125, and 3138; for *Hae*III at nt 320, 699, 2137, and 3082; and for *Rsa*I at nt 47, 72, 267, 439, 878, 943, 964, 1679, and 1762.)

S11 gene, and the 5′ end of the *B. subtilis* alpha gene (39). Here we report the sequence of 3.2 kb in the alpha region, extending from the *Eco*RI site at 0 kb to the *Pst*I site at 3.2 kb (Fig. 1). By subcloning appropriate fragments from λgtll clones 10-1 and 11-2 and making a set of nested exonuclease III deletions in M13mp18, we sequenced the entire region on both strands and through all deletion endpoints, with the exception of the 900 base pairs (bp) sequenced earlier, which we sequenced here on one strand only.

The new sequence data confirmed and extended our previous data, completing the sequences of the alpha and S13 genes and identifying three additional genes whose products are involved in translation. The two open reading frames upstream of the S13 gene probably encode initiation factor 1 (IF1) and ribosomal protein B, whereas the frame downstream of alpha probably encodes ribosomal protein L17 (Fig. 1 and 2). Following a sequence at nucleotides (nt) 217 to 225 representative of strong ribosome-binding sites in *B. subtilis* (25), an AUG initiation codon at nt 235 to 237 began a 216-bp open reading frame (Fig. 2). The predicted


EcoRI
IFI
M A K D D V I E V E G T I V E T L P N A M F
SphI.
B
M K V R P S V K P I C E K C K V I R R K G K V M V I C E N P K H K Q K Q G *
S13
M A R I A G V D I P R D K R V V I S L T Y I F G I G R T T A Q Q V L K E A G V S E D T
EcoRV
R V R D L T E E E L G K I R D I I D K L K V E G D L R R E V S L N I K R L I E I G T Y R G I R H R R
G L P V R G Q N S K N N A R T R K G P R R T V A N K K K *
S11
M A A A R K S N T R K R R V
K K N I E S G I A H I R S T F N N T I V T I T D T H G N A I S W S S A G A L G F R G S R K S T P F A
A Q M A A E T A A K G S I E H G L K T L E V T V K G P G S G R E A A I R A L Q A A G L E V T A I R D
PstI
HaeIII
V T P V P H N G C R P P K R R R V *
α
M I E I E K P K I E T V E I S D D A K F G K F V
V E P L E R G Y G T T L G N S L R R I L L S S L P G A A V T S I Q I D G V L H E F S T I E G V V E D
EcoRI
V T T I I L H I K K L A L K I Y S D E E K T L E I D V Q G E G T V T A A D I T H D S D V E I L N P D
HindIII
.RsaI
L H I A T L G E N A S F R V R L T A Q R G R G Y T P A D A N K R D D Q P I G V I P I D S I Y T P V S
R V S Y Q V E N T R V G Q V A N Y D K L T L D V W T D G S T G P K E A I A L G S K I L T E H L N I F
V G L T D E A Q H A E I M V E K E E D Q K E K V L E M T I E E L D L S V R S Y N C L K R A G I N T V
Q E L A N K T E E D M M K V R N L G R K S L E E V K A K L E E L G L G L R K D D *
L17
M S Y R K L G R T S A Q R K A M L R D L T T D L I I N E R I E T T
RsaI
E T R A K E L R S V V E K M I T L G K R G D L H A R R Q A A A Y I R N E V A N E E N N Q D A L Q K L
F S D I A T R Y E E R Q G G Y T R I M K L G P R R G D G A P M A I I E L V *
HindIII
ORF1
M N Q N Q L I S V E D I V F R Y R K D A
E R R A L D G V S L Q
PstI

72-residue product (8,213 daltons [Da]) was very similar to *E. coli* initiation factor IF1 (69% identical and 29% conserved residues) (Fig. 3) and was also 57% identical to spinach chloroplast IF1 (37). Because this open reading frame very likely encodes the *B. subtilis* homolog of *E. coli* IF1, we will refer to it as *infA*.

At 19 bp after *infA*, another possible strong ribosome-binding site at nt 469 to 477 was followed by an AUG codon at nt 487 to 489. Of the 37 amino acid residues predicted from the succeeding open reading frame (4,305 Da), 22 were identical to the residues of *E. coli* ribosomal protein B and 12 were conserved substitutions (Fig. 3). The *B. subtilis* protein was even more closely related to the corresponding *Bacillus stearothermophilus* (40) and spinach chloroplast (37) proteins, with 35 and 26 identical residues, respectively. The *rpmJ* gene encoding *E. coli* ribosomal protein B (44), previously called gene X, is the last gene in the *spc* operon and precedes the *E. coli* S13 gene by 149 bp (6). This interval contains the secondary alpha promoter detected in vitro (30) and in vivo (L. Lindahl, R. H. Archer, and J. M. Zengel, personal communication) and also encodes the mRNA-binding site of ribosomal protein S4, the translational regulator of the *E. coli* alpha operon (9, 10, 46).

In contrast, our DNA sequence indicated that the *B. subtilis* S13 gene began only 23 bp after the B gene (Fig. 2). At 6 bp after the B termination codon was a possible strong ribosome-binding site (nt 606 to 614) followed by an AUG initiation codon (nt 623 to 625). The predicted product of the succeeding open reading frame (13,786 Da) was similar (56% identical and 39% conserved residues) to the *E. coli* S13 protein (Fig. 3) and also very similar (88% identical) to *B. stearothermophilus* S13 (4). Our predicted amino acid sequence closely matched the amino-terminal sequence chemically determined for the *B. subtilis* S13 protein by Higo et al. (15). The mature protein analyzed by Higo and his colleagues lacked the first methionine residue, but the remaining 41 residues are identical to our sequence save for residue 29, which they described as an arginine. Overlapping the termination codon of S13 was the proposed ribosome-binding site for the S11 gene identified earlier (39).

In the *E. coli* alpha operon, the gene for S4 immediately follows S11. In place of S4, *B. subtilis* has a 177-bp intercistronic region between S11 and alpha (39). We noted that this intercistronic region contained two possible promoter sequences, but experiments reported below suggest that neither putative promoter is active during logarithmic growth. We also had noted a region of dyad symmetry of unknown function (nt 1515 to 1552 in Fig. 2). Here we report a second region of dyad symmetry in the intercistronic region, at nt 1396 to 1476, also of unknown function.

From a partial DNA sequence (nt 1581 to 1775 in Fig. 2), we identified the open reading frame 177 bp downstream of S11 as the *B. subtilis* alpha gene (39). We have now completed the sequence of this frame. The *B. subtilis* alpha gene (nt 1581 to 2523) encoded a 314-residue product (34,815 Da) that had 46% identical and 38% conserved residues in common with *E. coli* alpha (Fig. 3) and 24% identity with the predicted alpha product of the spinach chloroplast genome (37).

In *E. coli*, the coding sequence for ribosomal protein L17 lies 41 bp downstream of alpha (2). At 64 bp downstream of the termination codon of *B. subtilis* alpha were sequences suggestive of a strong ribosome-binding site, followed by an AUG initiation codon (nt 2586 to 2595 and 2603 to 2605, respectively; Fig. 2). The predicted product of the succeeding 360-bp open reading frame (13,750 Da) shared 45% identical and 37% conserved residues with *E. coli* L17 and thus probably encodes *B. subtilis* L17 (Fig. 3). Beginning 15 bp downstream of the *B. subtilis* L17 termination codon, mRNA transcribed through nt 2980 to 3036 (Fig. 2) can form a structure similar to that of rho-independent terminators of *E. coli* (33). S1 mapping experiments described in the next section indicated that alpha operon mRNA either terminated or was processed in this region.

A strong ribosome-binding site downstream of the L17 gene (nt 3074 to 3082) preceded an open reading frame which has been partially sequenced (Fig. 2 and unpublished data). The predicted amino acid sequence (J.-W. Suh, S. M. Thomas, and C. W. Price, manuscript in preparation) was similar to that of *E. coli* HisP and MalK, conserved inner membrane proteins involved in periplasmic transport systems (1).

S1 nuclease mapping of in vivo transcripts. We used three different approaches—S1 mapping of in vivo mRNAs, plasmid integration, and single-copy transcriptional fusions—to determine the basic transcriptional organization of the alpha region. We previously noted two possible σ^A promoter recognition sequences (25) in the 177-bp intercistronic region between S11 and alpha (−10 hexamers ending at nt 1439 and 1510 of Fig. 2). Here we first used long DNA probes and low-resolution S1 mapping (5) to determine whether these regions defined promoters active during logarithmic growth and whether the genes in the IF1-L17 interval were cotranscribed. DNA probes were the 1.8-kb *Eco*RI fragment from clone 10-1 (0 to 1.8 kb in Fig. 1) and the 0.9-kb *Eco*RI fragment from clone 11-3 (0.9 to 1.8 kb in Fig. 1), which were 5′-end labeled at their identical *Eco*RI ends lying within the alpha gene. In vivo mRNAs isolated from logarithmically growing cells completely protected both probes from S1 nuclease digestion (data not shown), suggesting that the two possible promoters immediately upstream from alpha were largely inactive during logarithmic growth and that most of the transcription of the alpha region initiated upstream from IF1.

In order to determine whether the alpha transcript originated within or beyond the cloned region just upstream of IF1, we did high-resolution S1 mapping experiments with an upstream probe, the 275-bp *Eco*RI-*Taq*I fragment (nt 1 to 275 in Fig. 2) which we had cloned into the *Eco*RI and *Acc*I sites of M13mp18. The resulting *Sal*I site created at the junction of the *B. subtilis* and M13 DNA was cleaved with *Sal*I and then 5′-end labeled. After the labeled M13 clone was cut

FIG. 2. DNA sequence of the alpha region. Nucleotides are numbered from the 5′ end, and intervals of 20 bp are marked. The sequence of nt 877 to 1775 was reported previously (39). The predicted amino acid sequence for each gene is given in single-letter code above the DNA sequence. The names of the gene products are given above the proposed ribosome-binding sites, overlined; free energies (ΔG) of binding between 16S RNA and the proposed Shine-Dalgarno sequences (25), calculated from the rules of Tinoco et al. (42), are: −15.0 kcal (IF1), −20.0 kcal (B), −22.2 kcal (S13), −19.2 kcal (S11), −19.8 kcal (alpha), −19.2 kcal (L17), and −18.8 kcal (ORF1, the designation of the open reading frame following L17). Regions of proposed dyad symmetry are underlined with arrows at nt 1396 to 1476 (−14.5 kcal), nt 1515 to 1552 (−11.8 kcal), and nt 2980 to 3036 (−27.6 kcal). We list in Fig. 1 the locations of sites for *Eco*RV, *Hae*III, and *Rsa*I in addition to those shown here.

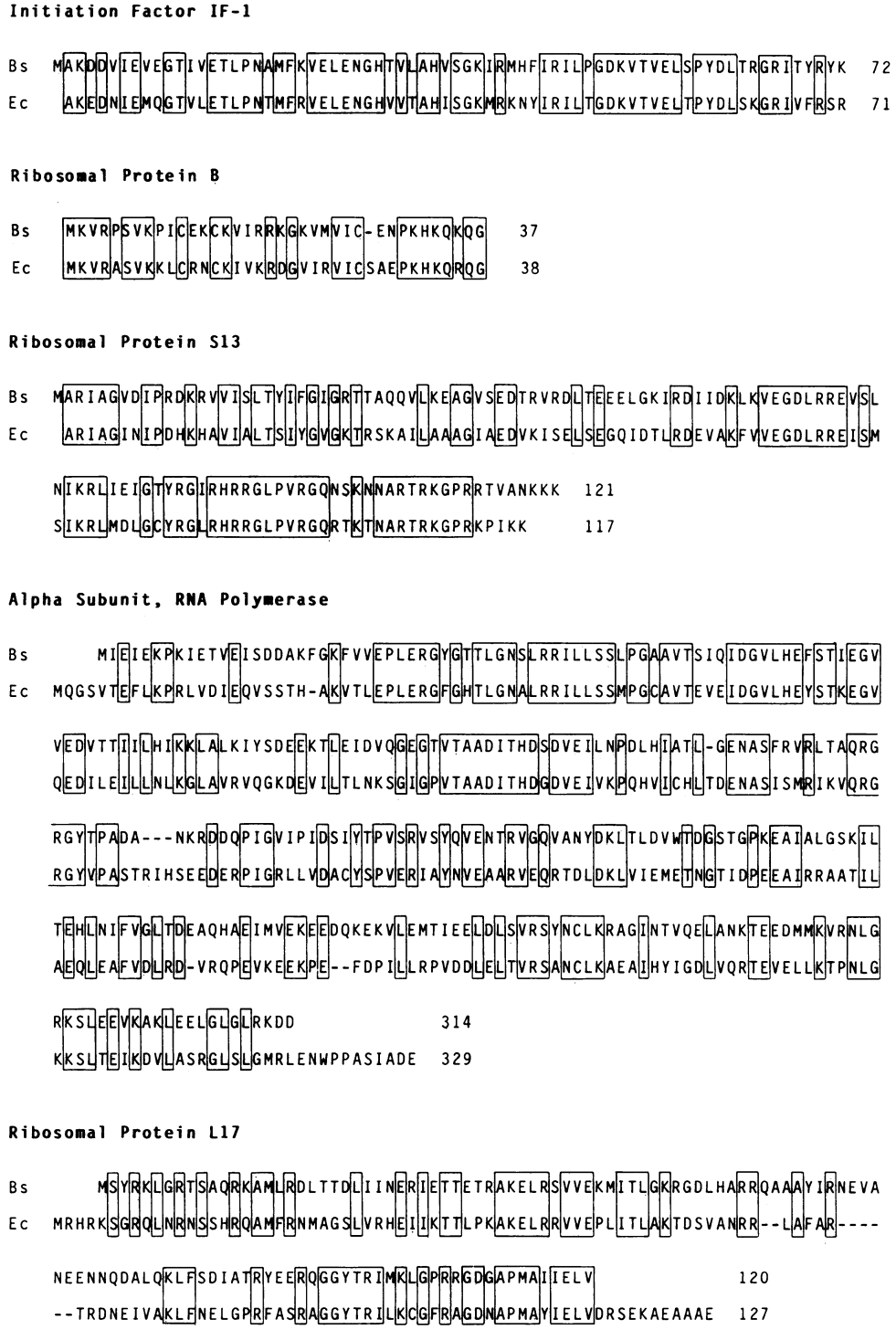


FIG. 3. Alignment of identical residues (boxed) between the predicted amino acid sequences for *B. subtilis* (Bs) IF1, B, S13, alpha, and L17 and the corresponding *E. coli* (Ec) proteins, determined by the FASTP program of Lipman and Pearson (21). The *E. coli* IF1 (29, 34), B (6, 44), S13 (2, 20), alpha (2, 27), and L17 (2, 32) sequences are from the literature, and the *B. subtilis* sequences are from Fig. 2. The statistical significance of these comparisons, given as optimized alignment scores and z values, respectively, are: 258 and 32.0 (IF1), 135 and 14.4 (B), 388 and 38.6 (S13), 708 and 50.1 (alpha), and 250 and 27.4 (L17). We described previously the comparison of *B. subtilis* and *E. coli* S11, the carboxy termini of S13, and the amino termini of alpha (39).

with *Ava*II, the 595-bp *Ava*II-*Sal*I fragment was gel purified and used as the probe (Fig. 4A). We made this double-stranded probe larger than necessary, with 278 bp of *B. subtilis* DNA and 317 bp of nonhomologous M13 DNA, in order to clearly distinguish undigested or reannealed probe from the shorter fragment protected by *B. subtilis* mRNA. The 275- to 280-nt protected fragments shown in Fig. 4A indicated that the majority of mRNA originated upstream of

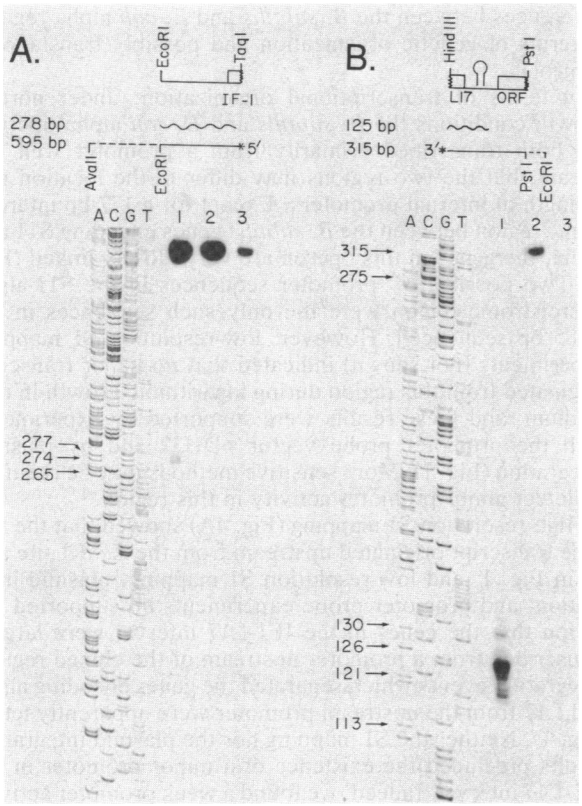


FIG. 4. (A) High-resolution S1 nuclease mapping of the 5′ end of the alpha transcript. Conditions used for S1 mapping are described in Materials and Methods; hybridization temperature was 48°C. (Top) The double-stranded 595-bp *Ava*II-*Taq*I fragment shown beneath the physical map was 5′-end labeled with [γ-^{32}P]ATP at the *Taq*I site indicated. The solid line represents *B. subtilis* DNA, and the dotted line represents the 317 bp of M13mp18 DNA sequence that constituted part of the 595-bp probe. The portion of the probe protected from S1 nuclease by *B. subtilis* RNA is represented by a wavy line, with the sizes of the probe and the protected region indicated to the left. (Bottom) Autoradiograph of the high-resolution S1 nuclease gel. Lanes A, C, G, and T show the dideoxynucleotide sequencing ladder of the probe, obtained with a 17-mer sequencing primer. Because the probe was cloned into the *Eco*RI and *Acc*I sites of M13mp18, the ladder was 39 nt longer than the probe at the 5′ end. Fragment sizes (including primer and the intervening polylinker region) for selected bands in the A lane are given to the left (in nt). Lane 1, Probe plus 120 μg of *B. subtilis* RNA; lane 2, no S1 nuclease (control); lane 3, probe plus 50 μg of *E. coli* tRNA. (B) High-resolution mapping of the 3′ end of the alpha transcript. Reaction conditions are given in Materials and Methods; hybridization temperature was 50°C. (Top) The 315-bp *Hin*dIII-*Eco*RI fragment shown beneath the physical map was 3′-end labeled with [α-^{32}P] dATP at the *Hin*dIII site indicated and used as a probe. The solid line represents *B. subtilis* DNA, and the dotted line represents an additional 40 bp of pUC19 DNA from the polylinker region. The portion of probe protected from S1 nuclease by *B. subtilis* RNA is indicated by the wavy line. Numbers to the left of the diagram indicate the sizes of the probe and protected fragment. (Bottom) Autoradiograph of the high-resolution S1 nuclease gel. The 2,500-bp *Hin*dIII fragment (nt 2910 in Fig. 2 to approximately nt 5500, not shown) was subcloned into the *Hin*dIII site of M13mp18. This clone was used to generate the dideoxynucleotide sequencing ladder shown in Lanes A, C, G, and T. Due to the presence of the polylinker region and the 17-mer primer, the sequencing ladder is 21 nt longer than the probe at the 3′ end. Fragment sizes (including primer and polylinker region) for selected bands in the A lane are given to the left (in nt). Lane 1, Probe plus 120 μg of *B. subtilis* RNA; lane 2, no S1 nuclease (control); lane 3, probe plus 50 μg of *E. coli* tRNA.

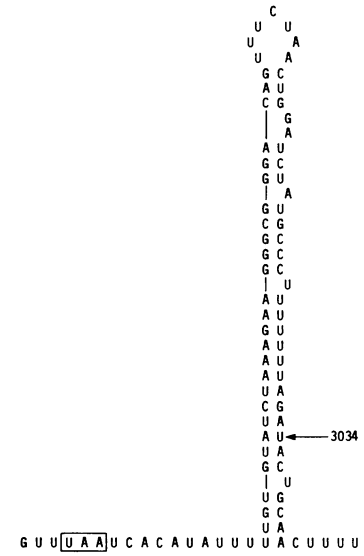


FIG. 5. Terminator region of the alpha operon. This structure has a free energy of −27.6 kcal calculated by the rules of Tinoco et al. (42). The UAA termination codon of the L17 gene is boxed, and the 3′ end of alpha operon mRNA, determined by S1 mapping (Fig. 4B), is indicated by the arrow at nt 3034.

the *Eco*RI site (nt 1 in Fig. 2). However, in three separate experiments, this protected band was weaker than expected, and most of the probe retained its 595-nt undigested size in all S1 reactions done with it, probably because the M13mp18-containing portion (53.6 mol% G+C) facilitated rapid probe reannealing at the hybridization temperature optimal for the *B. subtilis* portion of the probe (36.6 mol% G+C).

The 3′ end of the alpha mRNA was also first located by low-resolution S1 mapping and then more accurately positioned by a high-resolution experiment. For the low-resolution mapping, we used the 1.4-kb *Eco*RI-*Pst*I fragment from clone 9 (1.8 to 3.2 kb in Fig. 1) as the probe, 3′-end labeled at the *Eco*RI site within the alpha gene. A 1.2-kb fragment was protected by in vivo mRNA (data not shown), indicating that the alpha transcript ended near the putative terminator sequence shown in Fig. 5. In order to better locate the 3′ end of the transcript, the 275-bp *Hin*dIII-*Pst*I fragment (nt 2910 to 3184 in Fig. 2) was subcloned into the *Hin*dIII and *Pst*I sites of pUC19 (45). We used the 315-bp *Hin*dIII-*Eco*RI fragment as the probe (Fig. 4B). The 125-nt protected fragment shown in Fig. 4B indicated that the transcript terminated or was processed at nt 3028 to 3034, immediately following the putative terminator sequence. The major band of the band set corresponded to the T at nt 3034 (Fig. 2 and 5). The minor band corresponding to the A at nt 3040 may be the result of in vivo processing of a longer mRNA to the stem of the loop shown in Fig. 5.

Plasmid integration into the alpha region. Plasmid integration is a standard procedure to define transcriptional units in *B. subtilis* (13, 28). As an additional test to determine whether the genes in the IF1 to L17 interval were cotranscribed, we disrupted this region with the integrative plasmid pCP115 (31) carrying the fragments shown in Fig. 1. Because pCP115 cannot replicate in *B. subtilis*, it confers chloramphenicol resistance only via a Campbell insertion into the chromosome at the region of homology supplied. An integration separating an essential gene from its promoter would be lethal, and no transformants should arise.

As shown in Fig. 1, transformation of wild-type *B. subtilis* with pCP115 plasmids carrying either the 1.8-kb *Eco*RI fragment (0 to 1.8 kb) or the 0.75-kb *Rsa*I fragment (1.88 to 2.63 kb) each gave about 100-fold fewer chloramphenicol-resistant colonies than the control transformation, which used the 1.4-kb *Eco*RI-*Pst*I fragment (1.8 to 3.2 kb) spanning the alpha-open reading frame (ORF) interval. We interpret these results to indicate that the genes for both alpha and L17 are primarily transcribed from a promoter upstream of the *Eco*RI site at 0 kb. These results further suggest that the *Pst*I site at 3.2 kb is downstream of the 3′ end of the alpha transcriptional unit or that the gene defined by the open reading frame is not essential for growth.

Analysis of alpha region for internal promoters. We also used the single-copy transcriptional fusion vector pDH32 (D. Henner, personal communication) to locate possible promoters in the alpha region. This vector is similar to the ptrpBG1 translational fusion vector of Shimotsu and Henner (36). pDH32 carries a split *amyE* gene flanking a chloramphenicol resistance element and a promoterless *spoVG-lacZ* fusion, with the *spoVG* portion providing the *B. subtilis* ribosome-binding site. Fragments of *B. subtilis* DNA are cloned immediately upstream from the promoterless fusion, the plasmid is linearized at a site outside the *amyE*-flanked region, and upon transformation the construction integrates into the chromosomal *amyE* gene by a double crossover event.

We constructed a *B. subtilis* strain containing at the *amyE* locus a single copy of the 1.8-kb *Eco*RI fragment from the alpha region fused to the pDH32 *spoVG-lacZ* hybrid reporter gene. Southern analyses of this construct (and those described below) showed that it contained a single copy of the vector at the *amyE* locus (data not shown). As shown in Fig. 1, during logarithmic growth this strain had very low but significant β-galactosidase levels, 11 Miller units versus 0 Miller units for the negative control. By contrast, the strong promoter for the tRNA gene cluster internal to the *rrnB* operon (43), kindly supplied before publication by Barbara Vold, gave 418 Miller units.

In order to locate sequences necessary for the minor promoter activity, we constructed a series of deletions at the 5′ end of the 1.8-kb *Eco*RI fragment by subcloning into the pDH32 vector the restriction fragments indicated in Fig. 1. The 285-bp region between the *Sph*I and *Eco*RV sites was required for the weak activity (Fig. 1). There were no obvious promoter recognition sequences for σ^A holoenzyme in the *Sph*I-*Eco*RV region. Indeed, the only likely σ^A promoter sequences in the alpha region were found between the genes for S11 and alpha, and these were apparently not active under the conditions tested, with either the transcriptional fusions (Fig. 1) or the low-resolution S1 analysis described above.

DISCUSSION

We have characterized a 3.2-kb region of the *B. subtilis* genome that is comparable to the entire *E. coli* alpha operon. The *B. subtilis* alpha region contains the gene for the alpha core subunit of RNA polymerase and the adjacent genes for initiation factor IF1 and ribosomal proteins B, S13, S11, and L17. The products of these genes—all part of the transcriptional and translational machinery—are highly conserved between *E. coli* and *B. subtilis* (Fig. 3) and are generally less conserved between *B. subtilis* and the spinach chloroplast genome (37). However, despite the overall similarity in gene order and transcriptional organization, there are striking differences between the *B. subtilis* and *E. coli* alpha regions in terms of genetic organization and possibly translational control.

In terms of transcriptional organization, under normal growth conditions the *B. subtilis* and *E. coli* alpha operons are both transcribed primarily from a promoter well upstream, but the two regions may differ in the location and strength of internal promoters. Except for a 177-bp intercistronic region between the *B. subtilis* genes encoding S11 and alpha, the genes in this operon are very closely linked (Fig. 2). Two possible σ^A promoter sequences in the S11-alpha intercistronic region were the only such sequences in the 3,185 bp sequenced. However, low-resolution S1 mapping experiments (not shown) indicated that no major transcript originated from this region during logarithmic growth in rich medium, and these results were supported by experiments with the promoter probe vector pDH32 and by plasmid integration (Fig. 1). More sensitive methods may be required to detect minor promoter activity in this region.

High-resolution S1 mapping (Fig. 4A) showed that the IF1 gene transcript originated upstream from the *Eco*RI site at 0 kb in Fig. 1, and low-resolution S1 mapping, plasmid integration, and promoter probe experiments all supported the notion that the genes in the IF1-L17 interval were largely transcribed from a promoter upstream of the cloned region. Integration events which separated the genes encoding alpha and L17 from the upstream promoter were apparently lethal (Fig. 1). Neither the S1 mapping nor the plasmid integration results precluded the existence of a minor promoter in the IF1-L17 interval. Indeed, we found a weak promoter activity in logarithmically growing cells when the 1.8-kb *Eco*RI fragment was cloned into the single-copy promoter probe vector pDH32 (Fig. 1). Under different growth conditions, such as during the stringent response, carbon starvation, or sporulation, this minor promoter may become more active.

Sequences necessary for this weak promoter activity lay within the closely coupled IF1, B, and S13 genes, between the *Sph*I and *Eco*RV sites (nt 364 to 649). This region did not contain any obvious σ^A (σ^{70}-like) promoter sequences (Fig. 2), whereas the σ^{70} recognition sequences of the *E. coli* alpha promoter do lie in the 148-bp intercistronic region between the B and S13 genes (2, 30). S1 mapping of *E. coli* mRNA indicates that most of the alpha mRNA originates from the strong *spc* promoter further upstream (6), but a significant fraction of alpha mRNA originates from the alpha promoter as well (L. Lindahl, R. H. Archer, and J. M. Zengel, personal communication).

Our preliminary evidence indicates that the major promoter for the *B. subtilis* alpha operon also lies well upstream, more than 3.5 kb upstream of the *Eco*RI site at 0 kb in Fig. 1. By chromosome walking, we isolated the adjacent 3.5-kb upstream fragment which in the pCP115 integrative vector was unable to transform *B. subtilis* to chloramphenicol resistance, suggesting that this fragment also lies within the alpha transcriptional unit (J.-W. Suh, S. A. Boylan, S. M. Thomas, and C. W. Price, unpublished). From a partial DNA sequence of the 3.5-kb upstream region, we identified the genes encoding part of the secretory apparatus, SecY, and ribosomal proteins S5, L15, and L30 (unpublished results). In *E. coli*, these genes are part of the *spc* operon, which lies immediately upstream of the alpha operon (6). Thus, as in *E. coli*, under logarithmic growth conditions the *B. subtilis* alpha operon may be read primarily from the *spc* operon promoter.

Downstream from the *B. subtilis* L17 gene was an open reading frame whose partial amino acid sequence was similar

to that of the family of conserved inner membrane proteins involved in *E. coli* periplasmic transport systems (1). However, this open reading frame did not appear to be part of the alpha operon. Sequences similar to a rho-independent terminator (33) lay between the genes for L17 and the open reading frame (Fig. 5). High-resolution S1 mapping experiments (Fig. 4B) indicated the majority of mRNA terminated or was processed at bp 3034, near the end of the predicted terminator structure (Fig. 5).

In terms of gene organization, there was substantial overall similarity between the *B. subtilis* and *E. coli* alpha regions. The most striking differences were in the presence in the *B. subtilis* region of IF1, which maps directly opposite from the alpha region on the *E. coli* chromosome (34), and in the absence of ribosomal protein S4, the translational regulator of the *E. coli* alpha operon. In these respects, the genetic organization of the *B. subtilis* alpha region was more similar to that of the spinach chloroplast genome, which also contains IF1 and lacks ribosomal protein S4, but which in addition lacks ribosomal protein S13 (37).

The absence of S4 from the *B. subtilis* alpha operon may signal a divergence in translational control mechanisms. In *E. coli*, the synthesis of ribosomal proteins is coordinately regulated at the translational level, primarily by a feedback mechanism involving key ribosomal proteins (19, 26). The mechanism is also thought to adjust ribosomal protein synthesis to match growth rate and to regulate synthesis during the stringent response (7, 19). For the alpha operon, S4 binds to a site on the 95-nt leader mRNA preceding the S13 coding sequence (10). In contrast, the corresponding region in *B. subtilis* contains nothing resembling the proposed S4-binding site.

The question remains whether *B. subtilis* ribosomal protein operons are regulated by the translational feedback mechanism found in *E. coli* (19, 26). Although the *B. subtilis* S4 gene does not lie in the alpha region, its gene product could still regulate expression in *trans* (17). However, the site of S4 regulation probably would not immediately precede the S13 coding region, as is the case in *E. coli*. Future studies will address this question of feedback regulation and also identify promoters that may become more active under special growth conditions, such as during the stringent response, slow growth, or the sporulation process.

ACKNOWLEDGMENTS

We thank Dennis Henner for providing the pDH32 fusion vector and the *amyE* probe for Southern analysis and Barbara Vold for providing the strain carrying the tRNA promoter fusion.

This research was supported by Public Health Service grants AI22459 from the National Institute of Allergy and Infectious Diseases and GM42077 from the National Institute of General Medical Sciences to C.W.P. and by a Jastro-Shields Graduate Student Research Award to J.-W.S.

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