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Cloning, Sequencing, and Disruption of the *Bacillus subtilis* σ^{28} Gene

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Bacillus subtilis **contains multiple forms of RNA polymerase holoenzyme, distinguished by the presence of different specificity determinants known as σ factors. The σ^{28} factor was initially purified as a unique transcriptional activity in vegetatively growing *B. subtilis* cells. Purification of the σ^{28} protein has allowed tryptic peptides to be prepared and sequenced. The sequence of one tryptic peptide fragment was used to prepare an oligonucleotide probe specific for the σ^{28} structural gene, and the gene was isolated from a *B. subtilis* subgenomic library. The complete nucleotide sequence of the σ^{28} gene was determined, and the cloned σ^{28} gene was used to construct a mutant strain which does not express the σ^{28} protein. This strain also failed to synthesize flagellin protein and grew as long filaments. The predicted σ^{28} gene product is a 254-amino-acid polypeptide with a calculated molecular weight of 29,500. The σ^{28} protein sequence was similar to that of other sequenced σ factors and to the *flbB* gene product of *Escherichia coli*. Since the *flbB* gene product is a positive regulator of flagellar synthesis in *E. coli*, it is likely that σ^{28} functions to regulate flagellar synthesis in *B. subtilis*.**

Bacterial RNA polymerase is a multisubunit enzyme central to the process of gene expression. Although the catalytic activity resides in the core subunits of the enzyme, the promoter specificity of a particular holoenzyme is determined by the nature of the associated σ factor (31). The majority of cellular transcription is dependent on the primary σ factor, which exhibits a conserved promoter recognition specificity throughout the eubacteria (43). Many bacterial species also contain alternative σ factors that are specific for transcription of distinct regulons of coordinately regulated genes. These alternative σ factors normally recognize promoter sequences that are different from those recognized by the primary σ factor. Examples of alternative σ factors in the enteric bacteria include σ^{32} (15), specific for the transcription of heat shock genes, and σ^{54} (19, 20), specific for transcription of nitrogen-regulated genes. For *Bacillus subtilis*, at least six alternative factors have been described (26, 31).

In *B. subtilis*, alterations in cellular transcription, mediated at least in part by alternative σ factors, effect the precise temporal changes in gene expression necessary for endospore formation (26). The products of the *spo0H* (σ^{30}) and *spoIIGB* (σ^{29}) genes are sporulation-specific σ factors that have been characterized both genetically and biochemically (6, 22, 24, 26, 40, 41). In addition, the *spoIIAC* gene product is homologous to other sequenced σ factors (9) and may also function as a σ factor. The σ^{37} and σ^{28} factors are found in vegetatively growing cells and are dispensable for sporulation, since disruption of these genes does not impair sporulation (3, 8; see below). To define the biological function of the *B. subtilis* σ^{28} factor, we have begun a genetic and structural analysis of the σ^{28} structural gene (*sigD*) based on the cloning of that gene.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* W168 (originally from T. Leighton, University of California, Berkeley) was used for the preparation of chromosomal DNA for library construction. The parent strain for the construction of plasmid integrants was *B. subtilis* BG-2 (*trpC2*) (obtained from E. Ferrari). For immunochemical (Western blot) analysis, *B. subtilis* was grown on Penassay broth media (Difco Laboratories) with chloramphenicol added to a final concentration of 5 μg/ml when appropriate. For *B. subtilis* transformation, cells were grown on Spizizen minimal salts (36) supplemented with 0.1% yeast extract, 0.02% Casamino Acids (Difco), 1 μM $FeSO_4$, 0.1 mM $MnSO_4$, and 0.5% glucose. For *Escherichia coli* transformation, the strains used were JM83 [*ara* Δ(*lac pro*) *strA thi* φ80 F'*lacZ*ΔM15], HB101 [*hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44* $F^-\lambda^-$], and 71-18 [Δ(*lac pro*) F' *lacI*^{q} *lacZ*ΔM15 *pro*$^+$ *supE*].

Materials. Antisera to the *B. subtilis* σ^{28} factor were prepared in this laboratory (18). Antisera to *B. subtilis* σ^{43} were a gift from Roy Doi, and antisera to the *B. subtilis* flagellin protein were obtained from George Ordal. Nitrocellulose was from Schleicher & Schuell, Inc., [γ-^{32}P]ATP was from ICN Pharmaceuticals, Inc., and [α-^{32}P]CTP was from Amersham Corp. The oligonucleotide primer for dideoxy sequencing was obtained from Pharmacia, Inc., and dideoxy nucleotides were from P-L Biochemicals, Inc. Horseradish peroxidase coupled to goat anti-rabbit immunoglobulin G was from Bio-Rad Laboratories, and 4-chloro-1-naphthol was from Sigma Chemical Co.

Oligonucleotide probe preparation. Synthetic oligonucleotides were synthesized on an Applied Biosystems model 380A DNA Synthesizer and purified by gel electrophoresis. Radioactively labeled probes were prepared by incubation of the single-stranded DNA with [γ-^{32}P]ATP and T4 polynucleotide kinase. Probe A has the sequence 5'-AA(T/C)TA(T/C)GA(A/G)GA(T/C)CA(A/G)GT-3', where equimolar mixtures of the nucleotides indicated in parentheses were incorporated at each position. Thus, probe A is a 32-fold degenerate, 17-mer probe. Probe B has the sequence 5'-GGCGCAATCATAGACGG-3'.

Southern blots. Southern blots were performed essentially as described previously (28, 35). Chromosomal DNA from

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B. subtilis was digested with the appropriate restriction enzymes and electrophoretically separated on a 0.7% agarose gel. Following transfer to nitrocellulose, the membrane was blocked with 15× Denhardt solution (1× Denhardt solution is 0.02% [wt/vol] Ficoll, polyvinylpyrrolidone, and bovine serum albumin)–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM sodium phosphate buffer (pH 7) for 2 h at 37°C. Hybridizations were performed for 12 to 36 h at 37°C by using a buffer of 5× Denhardt solution–6× SSC–50 mM sodium phosphate (pH 7) with radioactively labeled oligonucleotide added to a final concentration of 12 μg/liter (probe A) or 1 μg/liter (probe B). Nitrocellulose membranes were rinsed five times with 6× SSC–0.1% sodium dodecyl sulfate, and the nonspecifically bound probe was eluted with a buffer containing 3 M tetramethylammonium chloride as described previously (44). For both 17-mer probes, a wash temperature of 50°C was found to be optimal. Hybridizing bands were visualized by autoradiography.

Screening of plasmid libraries. Chromosomal DNA from *B. subtilis* W168 was digested with the appropriate restriction enzymes, size fractionated by agarose gel electrophoresis, and electroeluted from gel slices. Size-fractionated inserts were then ligated to restriction enzyme-cleaved and alkaline phosphatase-treated pUC18 vector DNA, and the ligation mixture was used to transform competent *E. coli* JM83 or HB101 cells to ampicillin resistance. Pools of ampicillin-resistant transformants were screened by preparation of plasmid DNA by an alkaline lysis procedure (28) followed by spot blot hybridization analysis. Typically, 2 μl of plasmid DNA was denatured by addition of 1 μl of 1 N NaOH for 10 min at room temperature. The DNA solution was then neutralized with 0.7 μl of 5 M ammonium acetate and spotted onto nitrocellulose membranes. The nitrocellulose was air dried, rinsed with 2× SSC, and baked in a vacuum oven at 80°C for 2 h. The procedure for prehybridization, hybridization, and washing was the same as that described for Southern blot hybridizations.

DNA sequencing. *B. subtilis* DNA was prepared by gel purification and electroelution of insert DNA from plasmids pJH6-2 and pJH1-1 (see below). Purified insert DNA was restricted, ligated into M13mp18 or M13mp19, and transformed into *E. coli* 71-18 cells, and single-stranded bacteriophage DNA was prepared. The enzymatic sequencing technique of Sanger et al. was used to determine the nucleotide sequence (32).

Construction of plasmid integrants. The indicated DNA fragments were cloned into the integrational plasmid vector pJM102 (originally prepared by Marta Perego, laboratory of J. Hoch, Research Institute of Scripps Clinic, La Jolla, Calif.; obtained from E. Ferrari). The resulting plasmid subclones were oligomerized (5) and used to transform naturally competent *B. subtilis* BG-2 (*trpC2*) cells to chloramphenicol resistance. To confirm that the expected integration event had occurred, Southern blot analysis was performed.

Immunochemical analysis. Immunochemical analysis of protein samples was performed following electrophoretic transfer to nitrocellulose as described previously (39). Whole-cell lysates of *B. subtilis* were prepared by suspension of frozen cell pellets (representing 8 ml of cell culture grown to an optical density of 1.0 at 600 nm) in 0.3 ml of 25 mM Tris (pH 8)–1% glucose–50 mM EDTA containing 0.5 mg of lysozyme per ml and incubation at room temperature for 10 min. Lysates were sonicated for 20 to 30 s each and centrifuged at 12,000 × *g* for 5 min, and the soluble supernatant fraction was assayed for protein by using the Bio-Rad protein assay kit. A 20-μg portion of protein from each lysate was electrophoresed on 15% polyacrylamide gels and electrophoretically transferred to nitrocellulose (39). The nitrocellulose was stained with Ponceau S (Sigma) to check transfer efficiency and blocked with 10 mM Tris (pH 8)–100 mM NaCl (TBS) containing 2% nonfat dried milk. The primary antibody incubation typically contained a 1:1,000 dilution of antiserum in TBS–1% bovine serum albumin incubated for 2 h at room temperature. Nitrocellulose filters were then rinsed with TBS, TBS–0.05% Nonidet P-40, and again with TBS for ca. 5 min each. The secondary antibody incubation contained a 1:2,000 dilution of horseradish peroxidase coupled to goat anti-rabbit immunoglobulin G incubated in TBS–1% bovine serum albumin at room temperature for 60 min. To visualize reactive bands, the nitrocellulose was rinsed as described above and incubated in a solution made by mixing 1 part of 0.5 mg of 4-chloro-1-naphthol per ml of methanol with 5 parts of TBS–0.02% H_2O_2.

RESULTS

Isolation of the σ^{28} structural gene (*sigD*). Purified σ^{28} protein was used to prepare and sequence a series of tryptic peptide fragments (18). The sequence of one tryptic peptide was of appropriate length and amino acid composition to be useful in preparing an oligonucleotide probe specific for the σ^{28} gene. This sequence, MQSLNYEDQVL, allowed the preparation of a 32-fold degenerate, 17-mer oligonucleotide probe (probe A). To determine whether this probe would be of sufficient selectivity to isolate the σ^{28} structural gene, we performed Southern blot hybridization. At high stringency, the synthetic oligonucleotide probe hybridized to a single band in chromosomal digests of *B. subtilis* DNA. This experiment suggested that the σ^{28} gene resided, at least in part, on a 1.5-kilobase-pair *Hin*dIII fragment.

To isolate this 1.5-kilobase-pair *Hin*dIII fragment, we used size-fractionated *B. subtilis* DNA to prepare a subgenomic library in *E. coli*. This library was screened with oligonucleotide probe A to isolate a plasmid, designated pJH6-2 (Fig. 1). DNA sequence analysis of the insert from plasmid pJH6-2 supported the idea that this *Hin*dIII fragment contains the 5′ portion of the σ^{28} structural gene. An open reading frame was identified that contains sequences corresponding to five of the six identified σ^{28} tryptic fragments (18). A second oligonucleotide probe (probe B) was designed to isolate the remaining portion of the σ^{28} gene as an

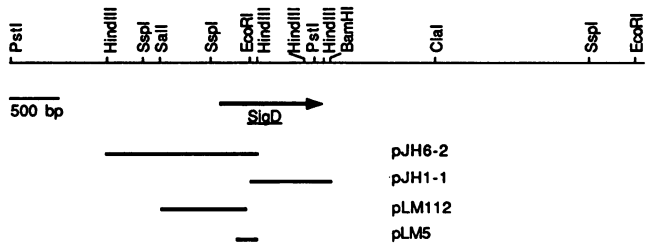

FIG. 1. Genomic map of the *sigD* region and plasmid subclones. Restriction sites near the *sigD* gene are indicated above the top line. The extent and direction of the *sigD* open reading frame are indicated by the arrow. Plasmid subclones contain the indicated inserts cloned into the polylinker cloning sites of pUC18 (pJH6-2 and pJH1-1) or pJM102 (pLM112 and pLM5) and were constructed as described in Materials and Methods.

*Eco*RI-*Bam*HI fragment (Fig. 1, plasmid pJH1-1). The insert in plasmid pJH1-1 overlaps the insert in plasmid pJH6-2 by 95 base pairs (bp). Together, these two plasmids contain the complete coding information for a 29.5-kilodalton (kDa) protein. Final confirmation of the identity of this sequence as the σ^{28} gene was obtained by disruption of the gene and immunochemical analysis (see below). We designate this as the *sigD* gene in accordance with current nomenclature for these factors (26).

Nucleotide sequencing and structure of the *sigD* region. The dideoxy sequencing technique was used to determine the nucleotide sequence of the *sigD* gene and its flanking DNA (32). Insert DNA from plasmids pJH6-2 and pJH1-1 was isolated, digested with restriction enzymes, and cloned into M13mp18 or M13mp19. The nucleotide sequence was determined from both strands and across all restriction enzyme sites used in cloning (Fig. 2).

The σ^{28} open reading frame is preceded by a potential ribosome-binding site (16) (Fig. 3; $\Delta G^{25} = -13$ kcal/mol [−54.4 kJ/mol]). In addition, there is a sequence similar to the consensus sequence for σ^{43}-dependent promoters (13) that may function as a σ^{43}-dependent promoter element in *B. subtilis*. However, gene disruption experiments suggest that normal σ^{28} transcription depends on promoters well upstream of this region (see below). In the 1,000 bp preceding the start point for translation of the σ^{28} structural gene, there are four other open reading frames that may represent upstream members of a larger operon. These open reading frames overlap by 1 to 8 nucleotides, as is commonly observed with genes that are translationally coupled (29). (The sequence of this entire 2-kilobase region has been deposited in the GenBank database.)

Structural features of the σ^{28} factor. The σ^{28} structural gene encodes a polypeptide of 254 amino acids with a calculated molecular mass of 29.5 kDa (Fig. 3). Sequences corresponding to all of the chemically sequenced tryptic peptide fragments are present (boxed amino acids in Fig. 3). The tryptic peptide sequence used for the construction of probe A corresponds to the amino terminus of the mature σ^{28} polypeptide, suggesting that the amino-terminal methionine is not posttranslationally removed.

The σ^{28} protein is homologous to other sequenced σ factors (20 to 30% amino acid identity). All σ factors share an amino-terminal region, perhaps involved in core binding (region 2), and most have one or two carboxy-terminal regions that may be involved in promoter recognition (regions 3 and 4) (14, 37). The sequence alignment in the most highly conserved regions (regions 2 and 4) is shown in Fig. 4. When the algorithm of Lipman and Pearson (25) is used for pairwise comparisons, σ^{28} is most similar to σ^{37}, σ^{43}, the *spoIIAC* gene product, and *E. coli* σ^{70}.

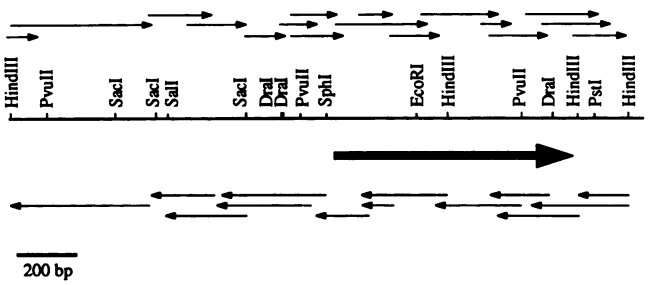

FIG. 2. Sequencing strategy for *sigD*. Each thin arrow represents the actual sequence determined by using the dideoxy sequencing technique. The thick arrow represents the σ^{28} structural gene. The sequence was determined from both strands throughout the 2-kilobase region indicated.

The finding that disruption of the σ^{28} structural gene eliminates *B. subtilis* flagellin synthesis led us to compare its structure with those of genes implicated in regulation of flagellar synthesis in other bacteria. We found significant similarity between the σ^{28} gene product and the *E. coli flbB* gene product (2) in the carboxy-terminal region of both proteins (Fig. 4, region 4). In this region, σ^{28} is as similar to *flbB* as it is to σ^{37}. The amino acid residues of *flbB* that are conserved relative to the sequenced σ factors and σ^{28} are shown in bold face in Fig. 4.

When the *flbB* protein sequence was used to search the National Biomedical Research Foundation protein database (>4,000 sequences) and a database containing sequenced σ factors, the score for alignment with σ^{28} was 3.7 standard deviations above the mean ($z = 3.7$). Indeed, only one other bacterial protein (*E. coli trp* repressor) has an alignment score greater than that for σ^{28} ($z = 5.8$). Although *flbB* may be homologous to σ^{28}, no significant similarity ($z > 2$) was detected between *flbB* and other sequenced σ factors.

Mutational analysis of the *sigD* locus in *B. subtilis*. To assess the biological role of the σ^{28} polypeptide in *B. subtilis*, we used an integrational plasmid vector to generate a disruption in the σ^{28} structural gene. A 189-bp internal *Sau*3A-*Hin*dIII fragment of the σ^{28} structural gene was cloned into the integrational vector pJM102 to generate plasmid pLM5 (Fig. 1). This plasmid was integrated into competent *B. subtilis* BG-2 cells, and a chloramphenicol-resistant integrant was isolated (disruptional plasmid integrant, strain DP-I). This integration event disrupts the resident copy of the σ^{28} gene to generate a strain that cannot synthesize full-length σ^{28} protein.

To facilitate the genetic mapping of the σ^{28} structural gene, an 890-bp *Sal*I-*Eco*RI fragment spanning the amino terminus of the gene was cloned into pJM102 to generate plasmid pLM112 (Fig. 1). This plasmid was integrated into *B. subtilis* BG-2 to generate a chloramphenicol-resistant strain, MP-I. Upon integration, a functional copy of the σ^{28} gene was regenerated, together with 637 bp of upstream flanking DNA. These two strains (MP-I and DP-I) were analyzed by Southern blot analysis to verify that the expected integration events had occurred (data not shown).

To determine whether the disruptional integrant (strain DP-I) had lost the ability to synthesize σ^{28} polypeptide, we performed immunoblot analysis on whole-cell extracts. The parent strain, *B. subtilis* BG-2, contains levels of σ^{28} protein similar to those observed in *B. subtilis* W168 cells. We have estimated, by using quantitative immunoblot analysis, that there are approximately 220 molecules of σ^{28} per cell during vegetative growth (18). In contrast, *B. subtilis* DP-I failed to synthesize detectable amounts of σ^{28} protein (Fig. 5). Interestingly, strain MP-I synthesized low but detectable levels of σ^{28} protein. Since this strain retains a functional copy of the σ^{28} gene flanked by 637 bp of upstream sequences, this result suggests that a promoter element greater than 637 bp upstream may be required for normal expression of σ^{28}.

Each of these cell extracts was also analyzed for proteins recognized by anti-σ^{43} antiserum and anti-flagellin antiserum (Fig. 5). All three strains contain approximately equal levels of σ^{43} protein. However, the disruptional integrant (strain DP-I) failed to synthesize detectable amounts of flagellin protein. Strain MP-I appeared to have normal levels of flagellin protein, despite the marked reduction in σ^{28} protein levels. This suggests that σ^{28} RNA polymerase is normally present in excess with respect to expression of this particular gene product.

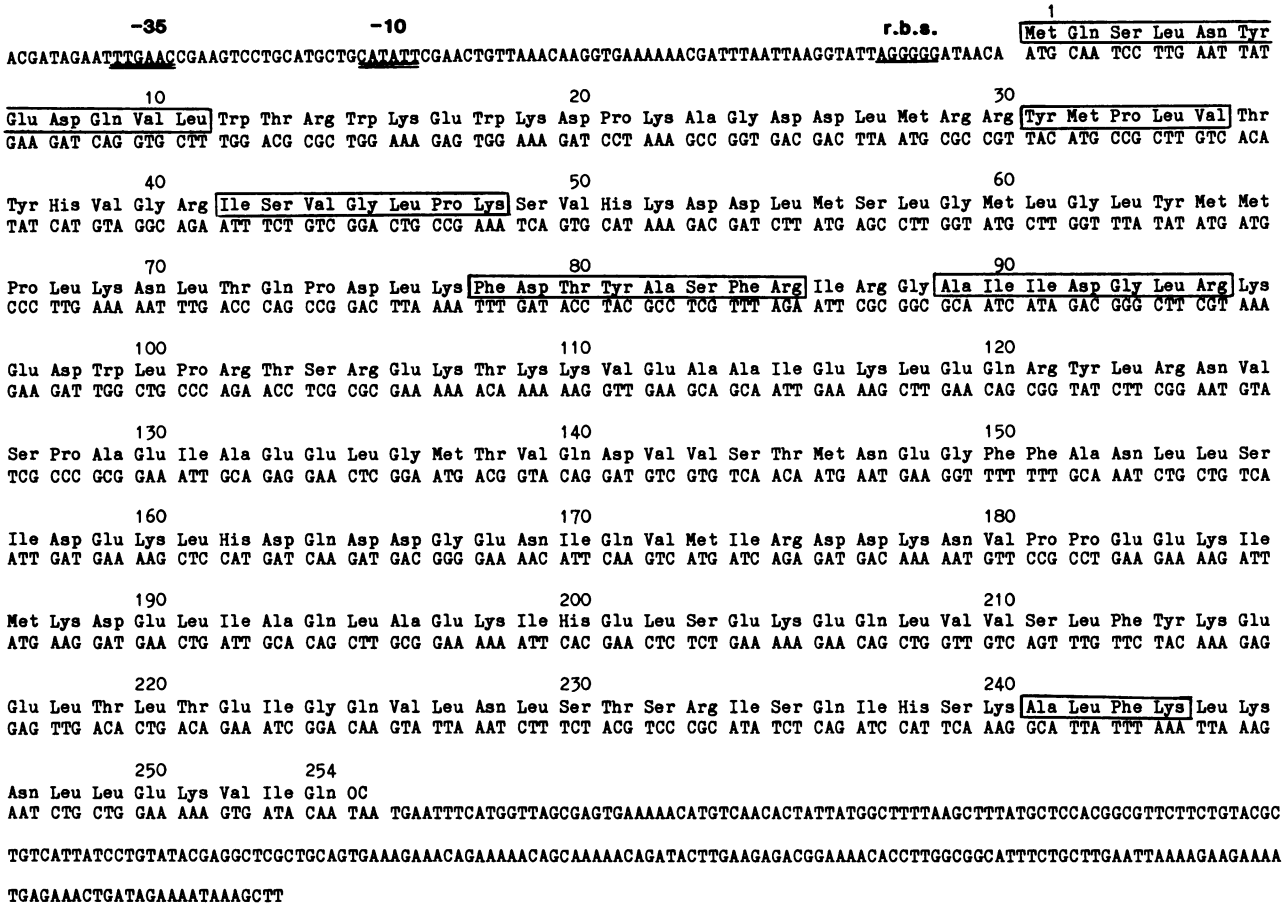

FIG. 3. Nucleotide and amino acid sequences of the *sigD* gene. The complete nucleotide sequence of the σ^{28} structural gene is shown, together with the predicted amino acid sequence of the σ^{28} gene product. The −35 and −10 region homologies which may function as a σ^{43}-dependent promoter in *B. subtilis* are underlined twice. Single underlines identify the potential ribosome-binding site. Amino acid sequences enclosed in boxes were independently determined by chemical sequencing of tryptic peptide fragments of the purified σ^{28} protein.

Strain DP-I was nonmotile when observed by light microscopy, as expected from its lack of flagellin protein. In addition, it grew as long filaments. Although electron microscopy revealed apparently normal septum formation, the mutant cells failed to separate following cell division. A filamentous phenotype is usually associated with reduced levels of autolysin activity in *B. subtilis* (10, 30). This phenotype is often also linked with a lack of flagellar synthesis, suggesting that autolysin synthesis may be under the control of some of the same regulatory mechanisms that control flagellar synthesis (30). In contrast, strain MP-I was motile, had apparently normal levels of flagellin protein, and grew as short filaments in rich media. Further characterization of these strains will be described elsewhere (L. M. Márquez, J. D. Helmann, E. Ferrari, and M. J. Chamberlin, manuscript in preparation).

DISCUSSION

The structural gene for the *B. subtilis* σ^{28} factor (*sigD*) was cloned and sequenced by oligonucleotide hybridization. The predicted protein product is a 254-amino-acid acidic protein that is similar in sequence to other σ factors (Fig. 4). The predicted protein sequence includes all of the tryptic peptide fragments determined by chemical sequencing of the protein (18). The translation initiation codon is preceded by a ribosome-binding site with a calculated stability of −13 kcal/mol. This is expected to be a weak, but potentially active ribosome-binding site in *B. subtilis* (16).

The transcriptional control signals that regulate expression of the σ^{28} structural gene have not yet been thoroughly characterized. Although there is a sequence similar to σ^{43}-dependent promoters located upstream of the start of the σ^{28} structural gene, this site is probably not responsible for the majority of σ^{28} expression in vivo. A strain which retains a full-length copy of the σ^{28} structural gene flanked by 637 bp of upstream sequences (MP-I) produced only low levels of σ^{28} protein (Fig. 5). This suggests that regions farther upstream are required for normal expression of σ^{28}.

The simplest interpretation of these results is that the σ^{28} gene is part of an operon that is transcribed primarily from an upstream promoter element. The residual levels of σ^{28} protein in *B. subtilis* MP-I may reflect the use of a weaker promoter site immediately upstream of the structural gene, although this has not been tested directly. DNA sequence analysis suggests that there are four other open reading frames in the 1,000 bp preceding the σ^{28} gene, which may form an operon. It would not be surprising if σ^{28} were part of a polycistronic transcription unit; the genes for many bacterial σ factors appear to reside in operons. These include the σ^{43} (42), σ^{70} (4), σ^{29} (22), σ^{37} (8), and *spoIIAC* (11) factors.

An understanding of the *B. subtilis* σ^{28} regulon will require the identification of genes under σ^{28} control and elucidation

SEQUENCE ALIGNMENT IN REGION 2

FACTOR		AMINO ACID SEQUENCE
		<----2.1----> <-----2.2------>
SIGMA-28	21	PKAGDDLMRRYMPLVTYHYGRISVGLPKSVHKDDLMSLGMLGLYMMP
SIGMA-37	39	TNLVDMLAKKYS.....KGKSF...HEDLRQVGMIGLLGAI
SIGMA-70	384	LRLVISIAKKYTN.....RGLQF....LDLIQEGNIGLMKAV
SIGMA-43	143	LRLVVSIAKRYVG.....RGMLF....LDLIHEGNMGLMKAV
SIGMA-32	58	LRFVVHIARNYAG.....YGLPQ....ADLIQEGNIGLMKAV
SIGMA-29	67	LRLVVYIARKFEN.....TGINI....EDLISIGTIGLIKAV
SPO1GP28	28	LITFAARQQMENN.....GADTMMS.RQDLEQEGLLKLYDCW
SPO1GP34	25	LRKSVY..KKFKD.....KMINQSD.REDLMGTIDQIFLQLV
SPOIIAC	42	MRLVWSVVQRFLN.....RGYEP....DDLFQIGCIGLLKSV
T4 GP55	48	MLIAEGLSKRFNF.....SGYTQSW.KQEMIADGIEASIKGL
FLAI(2.2)	17	MELITLGA.RLQMLE
SIGMA-28	21	PKAGDDLMRRYMPLVTYHYGRISVGLPKSVHKDDLMSLGMLGLYMMP
		<-------2.3------><--------2.4------->
SIGMA-28	68	LKNLTQPDLKFDTYASFRIRGAIIDGLRKEDWLPRTSREKTKKVEAAI
SIGMA-37	72	KRYDPVVGKSFEAFAIPTIIGEIKRFLRDKTWSVHVPRRIKELGPRIK
SIGMA-70	417	DKFEYRRGYKFSTYATWWIRQAITRSIADQARTIRIPVHMIETINKLN
SIGMA-43	176	EKFDYRKGYKFSTYATWWIRQAITRAIADQARTIRIPVHMVETINKLI
SIGMA-32	91	RRFNPEVGVRLVSFAVHWIKAEIHEYVLRNWRIVKVATTKAQRKLFFN
SIGMA-29	100	NTFNPEKKIKLATYASRCIENEILMYLRRNNK.IRSEVSFDEPLNIDW
SPO1GP28	64	EKWCFKENKQMDEFGPIF.RKSLFRKVKQSGGTGRALGPVAIDDEDNP
SPO1GP34	59	SEYNPNRGVDFPYYIKRMLELRTYHHITKYLKRINGETSLYVKNEDGE
SPOIIAC	75	DKFDLTYDVRFSTYAVPMIIGEIQRFIRDDG.TVKVSRSLKELGNKIR
T4 GP55	84	HNFDETKYKNPHAYIT...QACFNAFVQRIKKERKEVAKKYSYFVHNV
FLAI(2.3)	143	GNFITHADQPVGSFACSL
SIGMA-28	68	LKNLTQPDLKFDTYASFRIRGAIIDGLRKEDWLPRTSREKTKKVEAAI
FLAI(2.4)	33	TQLSRGRLILKLYKELRGSPPP

SEQUENCE ALIGNMENT IN REGION 4

	<-------4.1------><----------4.2---------------->
SIGMA-28	LSEKEQLVVSLFY....KEELTLTEIGQVLNLSTSRISQIHSKALFKLKNLLEKVIQ*
SIGMA-37	LSDREKQIIDLTYIQN....KSQKETGDILGISQMHVSRLQRKAVKKLREALIEDPSMELM*
SIGMA-70	LTAREAKVLRMRFGIDMNTDYTLEEVGKQFDVTRERIRQIEAKALRKLRHPSRSEVLRSFLDD*
SIGMA-43	LTDREENVLRLRFGLDDGRTRTLEEVGKVFGVTRERIRQIEAKALRKLRHPSRSKRLKDFLE*
SIGMA-32	LDERSQDIIRARW.LDEDNKSTLQELADRYGVSAERVRQLEKNAMKKLRAAIEA*
SIGMA-29	LNEREKQIMELRFGLVGEEEKTQKDVADMMGISQSYISRLEKRIIKRLRKEFNKMV*
SPOIIAC	LEEREKLIVYLRYYKD....QTQSEVAERLGISQVQVSRLEKKILKQIKVQMDHTDG*
SPO1GP28	MLKSQGHRVNVPKDT....TVRMKHIDQTLGISNKQYDSELKKFVKRLTI*
SPO1GP34	LGEKHRNLMIGLFIR....KKTLQELAQEEGVPLDRLHARLYFLIRKFEKEHQIDTEIFGEDLY*
FLBB	LAETNQLVCHFRFD...SHQTITQLTQD..SRVDDLQQIHTGIMLSTR.LLNDVNQPEEALRTKKRA*
SIGMA-28	LSEKEQLVVSLFY....KELTLTEIGQVLNLSTSRISQIHSKALFKLKNLLEKVIQ*

FIG. 4. Similarity between σ^{28}, FlbB, FlaI, and other sequenced σ Factors. The amino acid sequence of σ^{28} is aligned with other sequenced σ factors in regions 2 and 4 as defined by Gribskov and Burgess (14). Amino acids are represented by the one-letter code, and an asterisk identifies the carboxy terminus of each protein. Residues in boldface type are identical or chemically conserved in at least 6 of the 10 sequenced σ factors compared in region 2 or at least 7 of the 9 sequences compared in region 4. Conservative amino acid changes were defined as any within the following groupings: (G, A), (E, D, Q, N), (K, R, H), (L, I, M, V), (F, Y, W), and (S, T). σ^{70}, σ^{32} (HtpR), and the T4 phage σ^{gp55} normally function in *E. coli*. The remaining σ factors are all from *B. subtilis* or the *B. subtilis* phage SPO1. The number of the starting amino acid for each alignment is as indicated to the left of each alignment in region 2. The alignments in region 4 are all at the carboxy terminus of the indicated proteins. Sequence data are adapted from the references cited in Gribskov and Burgess (14), except for σ^{28} (this work), σ^{37} (3), *spoIIAC* (9), and FlbB and FlaI (2).

of the mechanisms regulating expression of these genes. Studies of the in vivo expression from σ^{28}-controlled promoters have shown that these promoters are active during vegetative growth, transiently induced at the end of logarithmic growth (T_0), and completely repressed by the second hour of sporulation (T_2) (12; V. Singer, unpublished results). These results suggest that σ^{28} controls functions that are active during vegetative growth and during the transition from vegetative growth to sporulation.

To define the biological role of the σ^{28} factor, we used the cloned gene to construct a mutant strain that can no longer express functional σ^{28} protein. This strain is filamentous and fails to synthesize detectable amounts of flagellin protein. This phenotype is similar to those reported for *B. subtilis lyt*

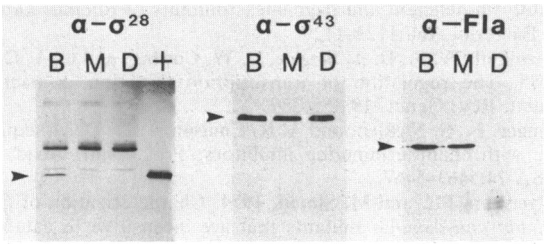

FIG. 5. Immunoblot analysis of plasmid integrants. Cell lysates from *B. subtilis* BG-2 (lanes B), MP-I (lanes M), and DP-I (lanes D) were analyzed for antigenically reactive proteins as described in Materials and Methods. For the anti-σ^{28} immunoblot (left panel), purified σ^{28} RNA polymerase was used as a positive control (lane +). Lysates were also analyzed for proteins reactive with anti-σ^{43} antiserum (middle panel) and anti-flagellin antiserum (right panel).

(10) and *sacU*^{h} (1) mutants. Analysis of these and other *fla* mutants suggests that autolysin synthesis may be regulated by some of the same factors that control flagellar synthesis (30). The relationship between the σ^{28} gene and known *fla* mutations is under investigation.

The factors regulating flagellar synthesis in *B. subtilis* have not been thoroughly characterized, at least in part because standard mapping techniques for *B. subtilis* rely upon the flagellotropic bacteriophage PBS1 (21). In contrast, the flagellar regulon of the enteric bacteria has been the subject of intensive study.

In the enteric bacteria, mutations in any of 33 discrete genes can prevent the synthesis of morphologically intact flagella, and many of these mutations block flagellin synthesis (23, 27, 34). Expression of the flagellar regulon in *E. coli* is absolutely dependent on the products of two cotranscribed regulatory genes, *flbB* and *flaI*, which are themselves under catabolite repression (33). Many *fla*, *che*, and *mot* genes of *E. coli* and *Salmonella typhimurium* are in operons preceded by σ^{28}-like promoter elements (17), and these sites are effective promoter elements for purified σ^{28} RNA polymerase (D. Arnosti, V. Singer, and M. Chamberlin, unpublished results). This suggests that there may be an alternative σ factor in the enteric bacteria that is specific for the transcription of flagellar and chemotaxis genes.

We have compared the σ^{28} protein sequence with the protein sequence of the two *E. coli* regulatory proteins, FlbB and FlaI (2). We found that the carboxy-terminal half of the FlbB protein of *E. coli* was similar in sequence to the carboxy terminus (region 4) of the σ^{28} protein (Fig. 4). In addition, some similar regions could be detected between the FlaI protein sequence and the highly conserved amino-terminal domain (region 2) common to many σ factors (Fig. 4). Since both FlbB and FlaI are required for flagellar gene expression, these proteins may function together as a σ factor in *E. coli*. Alternatively, the FlaI protein may function as a σ factor, with the FlbB protein acting as a positive activator for transcription. However, protein sequence comparisons led us to favor a model in which these proteins function together as a σ factor.

The regulatory role and molecular masses of the FlbB (13-kDa) and FlaI (22-kDa) gene products are reminiscent of the two regulatory proteins required for late gene expression in the *B. subtilis* phage SPO1, gene product 33 (12 kDa) and gene product 34 (24 kDa) (7). In *B. subtilis*, these two proteins appear to bind simultaneously to the core RNA polymerase to determine promoter recognition at the late phage promoter sites (38). Determination of whether the FlbB and FlaI gene products act as a σ factor in *E. coli* will require purification of the protein factors involved and reconstitution of transcriptional specificity in an in vitro transcription system.

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