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Identification of Flagellar Synthesis Regulatory and Structural Genes in a σ^D-Dependent Operon of *Bacillus subtilis*

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The σ^D form of RNA polymerase from *Bacillus subtilis* has been shown previously to direct the synthesis of several transcription units bearing genes for flagellin, motility proteins, and autolysins. In this report, we describe an operon of genes transcribed from the σ^D-dependent promoter P_{D-1}. We have identified three complete open reading frames and one partial one downstream of this promoter; immediately upstream is the previously identified *comF* locus. The P_{D-1} operon encodes the presumptive *B. subtilis* homologs of two *Salmonella typhimurium* late flagellar genes, *flgM* and *flgK*. Also present in this operon are two genes of unknown function, *orf139* and *orf160*, whose products show similarities to the eukaryotic cytoskeletal proteins myosin and vimentin, respectively. *orf139* and *orf160* may encode proteins that form extended α-helical secondary structures and coiled-coil quaternary structures which may be filamentous components of the gram-positive bacterial flagellum. We have characterized the *B. subtilis flgM* gene further by constructing an in-frame deletion mutation, *flgM*Δ80, and creating strains of *B. subtilis* in which this allele has replaced the wild-type copy. By primer extension analysis of cellular RNA, we have shown that the *flgM*Δ80 mutation relieves the block to transcription of two other σ^D-dependent operons imposed by an unlinked mutation in a gene directing early flagellar synthesis. We conclude that, as in the case of *S. typhimurium*, early flagellar synthesis in *B. subtilis* is coupled to late flagellar synthesis through repression of σ^D-dependent transcription by the *flgM* gene product.

The specificity of promoter recognition by RNA polymerase, often a key step in bacterial gene expression, is determined by proteins known as sigma (σ) factors that confer recognition of distinct promoter sequences. The gram-positive bacterium *Bacillus subtilis* possesses many alternative sigma factors and employs them in the temporal and developmental regulation of specific sets of genes, or regulons. *B. subtilis* σ^D is a secondary sigma factor that controls a regulon of genes expressed in exponential and early postexponential growth (19, 20, 42, 46; see reference 26 for a review). Mutations in the structural gene for σ^D lead to defects in flagellation, motility, and/or chemotaxis functions (28, 42). σ^D is the structural homolog of the enteric flagellar-specific sigma factor σ^F, and each factor appears to control the transcription of a similar regulon of genes required for flagellar assembly (*fla*), motility (*mot*), and potentially chemotaxis (*che*) functions (for a review, see reference 50). Sigma factors having this same promoter specificity are implicated in flagellar gene expression in a wide variety of bacterial species (26) and appear to form a family of sigma factors with related promoter specificities.

The gene expression hierarchy that controls flagellar morphogenesis in the enteric bacteria, the *fla/che/mot* regulon, has been depicted primarily as a linear pathway (17, 37; see reference 40 for a review). The expression of all flagellar genes and operons requires positive regulation by two master regulators, the *flhD* and *flhC* genes (which compose a catabolite-repressible operon); these are the sole class I flagellar genes. The remainder of the flagellar genes then fall into two other classes, II and III. Whereas class II genes require only the positive action of the class I genes, the class III genes require the correct expression of all class II genes as well. Genetic, molecular, and structural studies have uncovered further distinctions between class II and class III flagellar genes. Class II genes include those that encode flagellar basal-body or hook components, flagellar products that are known to be required early in the process of assembly of a flagellum. Class III operons encode the gene products required later during flagellar assembly, e.g., flagellin, hook-associated proteins, flagellar motor components, and the chemotaxis apparatus. In addition, class III gene transcription is dependent on the *fliA* gene. *fliA* is a class II gene that encodes σ^F, the sigma factor that recognizes class III operon promoters (5, 48).

The flagellar gene hierarchy can also be viewed from the standpoint of flagellar assembly (40). Since late flagellar genes (class III) require the correct expression of the earlier flagellar genes (class II), it has been proposed that they also require the correct assembly of class II gene products into the basal-body and hook structures. Recent studies indeed show that early flagellar gene product synthesis precedes late synthesis (35). It thus appears that class III gene expression (namely, σ^F-dependent transcription) is subject to regulation by a morphological cue that determines that the flagellar structure encoded by class II genes (namely, the basal body and hook) has been assembled correctly. There is now some physical evidence that a morphological signal recognizing completion of the early flagellum activates class III gene expression. Gillen and Hughes isolated a mutation defining a new gene, *flgM*, that allowed class III gene expression to occur in a cell that was otherwise mutant for a class II gene (with the exception of *fliA*) (17). The *flgM* locus has since been cloned and found to encode a 97-amino acid-peptide (16).

It is very likely that members of the σ^D regulon of *B. subtilis* are homologs of the enteric class III flagellar genes. One of the ways we have obtained information about the genes controlled by σ^D, and thus determined the role of σ^D, is by sequencing *B.*

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TABLE 1. *B. subtilis* strains used in this study

Strain[a]	Relevant genotype	Reference
CB25		19
CB100	*sigD*	27
CB139	*flaA*::Tn*917*Ω1836	This work
CB149	*flgM*Δ80	This work
CB150	*flgM*Δ80 *flaA*::Tn*917*Ω1836	This work

[a] All of the strains except CB100 are derived from the parent CB25 (originally JH642; Fla$^+$ Mot$^+$ Che$^+$ Lyt$^+$ *trpC2 pheA1*) and were constructed as described in the text. CB100 is derived from I168 (*trpC2*) as described in reference 27.

subtilis DNA fragments each known to contain a strong σ^D promoter, denoted a P_D (20, 21, 58). This approach has led previously to identification of the *B. subtilis hag* gene, encoding the flagellar filament structural protein flagellin, and the cotranscribed *motA* and *motB* genes, which are required for the function of the flagellum and motility of the cell (46, 47). In this report, we continue using this approach towards the characterization of the transcription unit controlled by one of the first σ^D-dependent promoters identified, P_{D-1} (58). We have found that the P_{D-1} operon contains structural and regulatory flagellar genes, including the *B. subtilis* homologs of *Salmonella typhimurium flgM* and *flgK*. We have used mutational analysis to show that *B. subtilis flgM* acts in a manner similar to that of its enteric counterpart in coupling early and late flagellar synthesis.

MATERIALS AND METHODS

Bacterial strains, media, growth, and transformation. The *Escherichia coli* host for growth of recombinant plasmid and M13 DNA was either strain TG-2 [Δ(*lac-proAB*) *supE thi hsdD5* Δ(*srl-recA*)*306*::Tn*10*(Tet^r) EcoK$^-$ F′ *traD36 proAB lacI^q*ZΔM15] or DH5α [F$^-$ φ80 *dlacZ*ΔM15 Δ(*lacZYA-argF*) *lacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA relA1*λ$^-$]. For oligonucleotide-directed mutagenesis, we used strain CJ236 [F$^-$ *dut-1 ung-1 thi-1 relA1*/pCJ105 (Cm^r)] and the f1 helper phage R408 (52). *E. coli* was grown in Luria broth medium supplemented with ampicillin at 50 μg/ml when appropriate (41). Transformation of *E. coli* was performed as described in standard published procedures (41).

The *B. subtilis* strains used in this study are listed in Table 1. Strains were grown in Penassay broth and on tryptose blood agar base plates. Transformation of strain CB25 by plasmid or chromosomal DNA was performed by standard procedures (4). Cm^r strains were maintained with 5 μg of chloramphenicol per ml, and Ery^r strains were maintained with 1 μg of erythromycin per ml and 25 μg of lincomycin per ml. Strain CB139 was created by transformation of strain CB25 with DNA from a strain bearing the transposon insertion *fla*::Tn*917* Ω1836 (63), with selection for Ery^r. Similarly, strain CB150 was created by transformation of this same DNA into CB149 with selection for Ery^r. The motility (Mot) and filamentous (Lyt) phenotypes of the various strains were checked by light microscopy.

DNA manipulations. Small quantities of plasmid DNA were prepared by the alkaline lysis method of Birnboim and Doly (7). Large quantities of DNA were prepared by a modification of this procedure involving an additional LiCl precipitation. Restriction, ligation, and related manipulations of plasmid DNA were performed by published procedures (41). DNA fragments were isolated from polyacrylamide gels via overnight elution into buffer consisting of 2 mM Tris-HCl (pH 8.0)–0.2 mM EDTA and from agarose gels by treatment with NaI and Glassmilk with the Geneclean kit (Bio 101, Inc.). *B. subtilis* chromosomal DNA was prepared by a modification of published procedures (6).

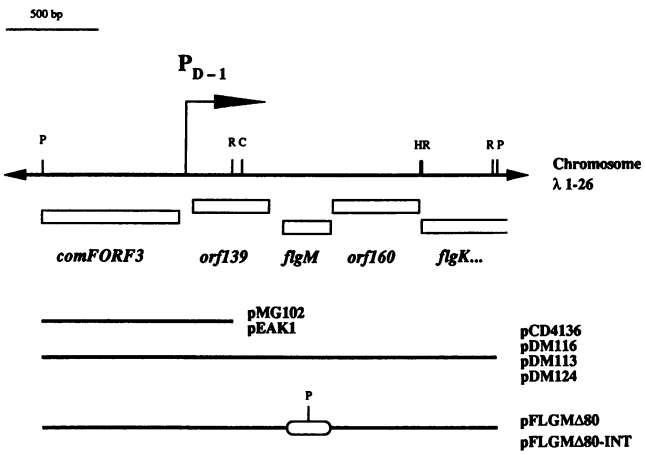

FIG. 1. Physical map of the beginning of the *B. subtilis* P_{D-1} operon. The segment of the *B. subtilis* chromosome known to contain the σ^D-dependent promoter P_{D-1} is shown as a thick horizontal line; this DNA segment is also contained on the phage λ1-26. The *degSU* and *comF* loci are located to the left in this figure; the *hag* locus is located to the right. Several restriction sites are indicated: P, *Pst*I; R, *Eco*RI; C, *Cla*I; H, *Hin*dIII. The location and direction of transcription of promoter P_{D-1} are indicated by the arrow. The locations and names of the putative open reading frames are shown as empty boxes below the line. Only the N terminus of *flgK* is shown and is so marked by the ellipsis. The relevant plasmid subclones, containing portions of this segment of the *B. subtilis* chromosome, are shown at the bottom of the figure. The derivations of these plasmids are described in more detail in the text. For the plasmids containing in-frame deletions (pFLGMΔ80 and pFLGMΔ80-INT), the deleted region is shown as a "bubble"; the new *Pst*I site created is also shown.

Plasmids. The plasmids used for sequencing and other manipulations are shown in Fig. 1. The original plasmid which contains promoter P_{D-1} is pCD4136; it consists of a 2.5-kb *Pst*I fragment of *B. subtilis* DNA cloned in the vector pHV14 (58). A 1.1-kb *Pst*I-*Eco*RI fragment from pCD4136 had been subcloned previously into pBR322 and pJH101 to yield pMG102 and pEAK1, respectively (21, 30, 54).

We also obtained the same 2.5-kb *Pst*I fragment of *B. subtilis* that contains P_{D-1} from the phage DNA of λ1-26; this member of a phage library of *B. subtilis* DNA also contains the promoter for the *hag* gene, P_{D-6} (20, 46). Plasmids pDM113 and pDM116 contain this 2.5-kb *Pst*I fragment cloned (in either orientation) in pUC18, whereas in pDM124 this fragment is inserted into pBS-KS$^+$ (Stratagene). The construction of pFLGMΔ80 and pFLGMΔ80-INT is described below.

DNA sequencing and analysis. DNA sequencing was performed by the chain termination method of Sanger et al. (53). Sequencing reactions were carried out with Sequenase 2.0 (United States Biochemical) as recommended by the manufacturer. Our initial sequencing of the P_{D-1} operon was performed by the shotgun subcloning of small restriction fragments of pMG102 into M13mp18 and mp19 (60). Subsequently, the sequence of both strands of the 2,497 bp of pDM113 and pDM116 was obtained by the creation of nested deletions generated by digestion with exonuclease III and S1 nuclease by modified versions of published procedures (6, 29, 41). Primers for sequencing were the universal M13 primer (25) and 20- to 22-nt oligonucleotides synthesized on a Bio-

search 8750 DNA synthesizer (MilliGen/Biosearch) and purified by polyacrylamide gel electrophoresis, followed by elution and ethanol precipitation. DNA sequences were compiled, analyzed, and searched against GenBank and EMBL databases by using IntelliGenetics programs and the BLAST algorithm (3, 9, 10). We searched the nonredundant DNA databases that included GenBank release 78.0 and EMBL data library release 35.0. Protein secondary-structure analysis was performed by using the IntelliGenetics PEP program, employing the algorithm of Chou and Fasman (13).

PCR procedures. The PCR was performed by methods described by Innis et al. (34). Ten nanograms of *B. subtilis* chromosomal DNA and 20 pmol each of two oligonucleotide primers were used in a 100-μl reaction mixture containing 0.2 mM (each) of all four deoxynucleoside triphosphates, 10 mM Tris (pH 8.2), 30 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg of gelatin per ml, and 2.5 U of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus). The reaction was overlaid with washed light mineral oil, heated to 94°C for 5 min, and then subjected to 25 cycles in a thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of 1 min of denaturation at 94°C, 2 min of annealing at 40°C, and 3 min of extension at 72°C. Subsequently, the mineral oil was removed by chloroform extraction, and the reaction products were analyzed by gel electrophoresis.

Construction of an in-frame deletion mutation of *flgM*. To create an in-frame deletion of the *flgM* open reading frame (ORF), oligonucleotide-directed mutagenesis without phenotypic selection was performed (6, 36). An oligonucleotide, designated FLGMDELP, having the sequence 5′-GGAATC CTATGAAAATCAATCAACTGCAGAAAAAGCAAT AAAAAAGGAG-3′, was designed to delete nucleotides 1,343 through 1,582 inclusive of the P_{D-1} operon region (as numbered in Fig. 2) and replace then with a *Pst*I restriction site (underlined above). The result is to replace FlgM amino acid residues 6 through 85, inclusive, with the dipeptide Leu-Gln, as indicated in Fig. 3.

The mutagenesis procedure is outlined briefly as follows. Uridine-containing single-stranded DNA of plasmid pDM124 was prepared by propagation of this plasmid in *E. coli* CJ236 infected with f1 helper phage R408 (52). The purified single-stranded DNA was annealed at a 1:1 molar ratio to 5′-phosphorylated FLGMDELP. Second-strand synthesis was achieved by using Klenow fragment DNA polymerase (New England Biolabs) and T4 DNA ligase (United States Biochemical) as described previously (6, 36). The resultant gapped-duplex DNA was transformed into *E. coli* TG-2. Candidate transformant plasmids were checked by restriction mapping and by sequencing in both directions across the deletion or replacement region, using appropriate primers. The plasmid pFLGMΔ80 was thus identified and confirmed to have the intended mutation in the *flgM* gene, the *flgM*Δ80 deletion allele.

To introduce this mutation into *B. subtilis*, an integrational vector was made. The entire insert of pFLGMΔ80 was cloned as a *Sst*I-*Sal*I fragment in pJM102 (27). The resultant plasmid construct was named pFLGMΔ80-INT.

Introduction of the *flgM* in-frame deletion into *B. subtilis*. Creation of a *B. subtilis* strain bearing an in-frame deletion of the *flgM* gene was accomplished by the gene replacement technique first used for the alkaline and neutral protease genes (55, 59) and used by our laboratory for the *motA* and *motB* genes (47). In short, a nonreplicating plasmid containing a gene deletion is integrated at the gene locus by a Campbell-type, single-crossover recombination, such that both a wild-type and a deleted copy of the gene as well as selectable antibiotic resistance and vector sequences are inserted. In some cases, a double-crossover event can occur so that two deleted copies of the gene of interest replace the wild-type version. Subsequently, the replacement of the wild-type gene is achieved by homologous recombination of the directly repeated plasmid sequences, with the concomitant loss of the Cm^r marker, as described previously (55).

To create a strain bearing the *flgM*Δ80 gene deletion, *B. subtilis* CB25 was transformed to Cm^r by integration of plasmid pFLGMΔ80-INT. All transformants tested were found to be motile (Mot^+) by light microscopy. By using PCR analysis with the appropriate primers, a Cm^r strain bearing two copies of *flgM*Δ80 and no *flgM*$^+$ allele was identified. Reversion of this integrant strain from Cm^r to Cm^s was achieved by growth and passage for several days in PA broth without chloramphenicol and screening for Cm^s colonies on replica plates. Cm^s revertants were found and shown to possess only the *flgM*Δ80 allele by PCR analysis. One such revertant was named CB149; this strain is Mot^+.

Strain growth, RNA extraction, and primer extension analysis. Cultures of the *B. subtilis* strains listed in Table 1 were grown to late log phase in SPIM (4) supplemented with tryptophan and phenylalanine, the cells were harvested, and the total cell RNA was extracted as described previously (46).

Quantitative primer extension analysis, using 5′-^{32}P-labeled primers, was performed essentially as described previously (46). Flagellin gene transcripts were detected by using the *hag* primer (46). Transcription of the *motAB* operon was detected by using the primer VL4 (5′-GGGAACGCAATAACGACT GCTGAG-3′). Five micrograms of total cell RNA, or an appropriate amount of RNA produced by in vitro transcription by σ^D RNA polymerase of plasmids containing the promoters for *hag* or *motAB*, were used for primer extension analysis. The amount of extended primer was quantitated with an AMBIS scintillation scanner (AMBIS Corp., San Diego, Calif.).

Nucleotide sequence accession number. The nucleotide sequence has been submitted to GenBank under the accession number L14437.

RESULTS

Nucleotide sequence analysis of the beginning of the P_{D-1} operon. We have determined the nucleotide sequence of the 2,497 bp of the *B. subtilis* chromosome surrounding the σ^D-dependent promoter P_{D-1}. The 2.5-kb *Pst*I fragment sequenced is present on pCD4136, one of the first plasmids isolated on the basis of possession of a σ^D-dependent promoter (formerly P_{28-1}, now P_{D-1}), as shown in Fig. 1 (58). This *Pst*I fragment is also present as part of the cloned insert of the lambda phage λ1-26, independently isolated on the basis of possession of promoter P_{D-6}, the *B. subtilis* flagellin gene (*hag*) promoter (20, 46). The subclones of λ1-26 containing P_{D-1}, pDM113 and pDM116, were also sequenced (Fig. 1). We have thus characterized a segment of the *B. subtilis* chromosome between *degSU* and *hag* (30, 46). No transcription terminator was found to be located on pCD4136 (19, 58), and so it is believed that the transcription unit continues beyond the right *Pst*I site shown in Fig. 1, proceeding towards P_{D-6} and the *hag* gene. Earlier studies showed that this region of DNA is transcribed only from the P_{D-1} promoter, and therefore this operon is entirely dependent on σ^D for expression (19, 58).

The nucleotide sequence in the vicinity of the beginning of the P_{D-1} transcription unit is presented in Fig. 2. Three complete ORFs and the start of a fourth ORF are found downstream of P_{D-1}. Each of these ORFs has a strong consensus to the gram-positive Shine-Dalgarno sequence coupled with an AUG translational start codon in the correct position

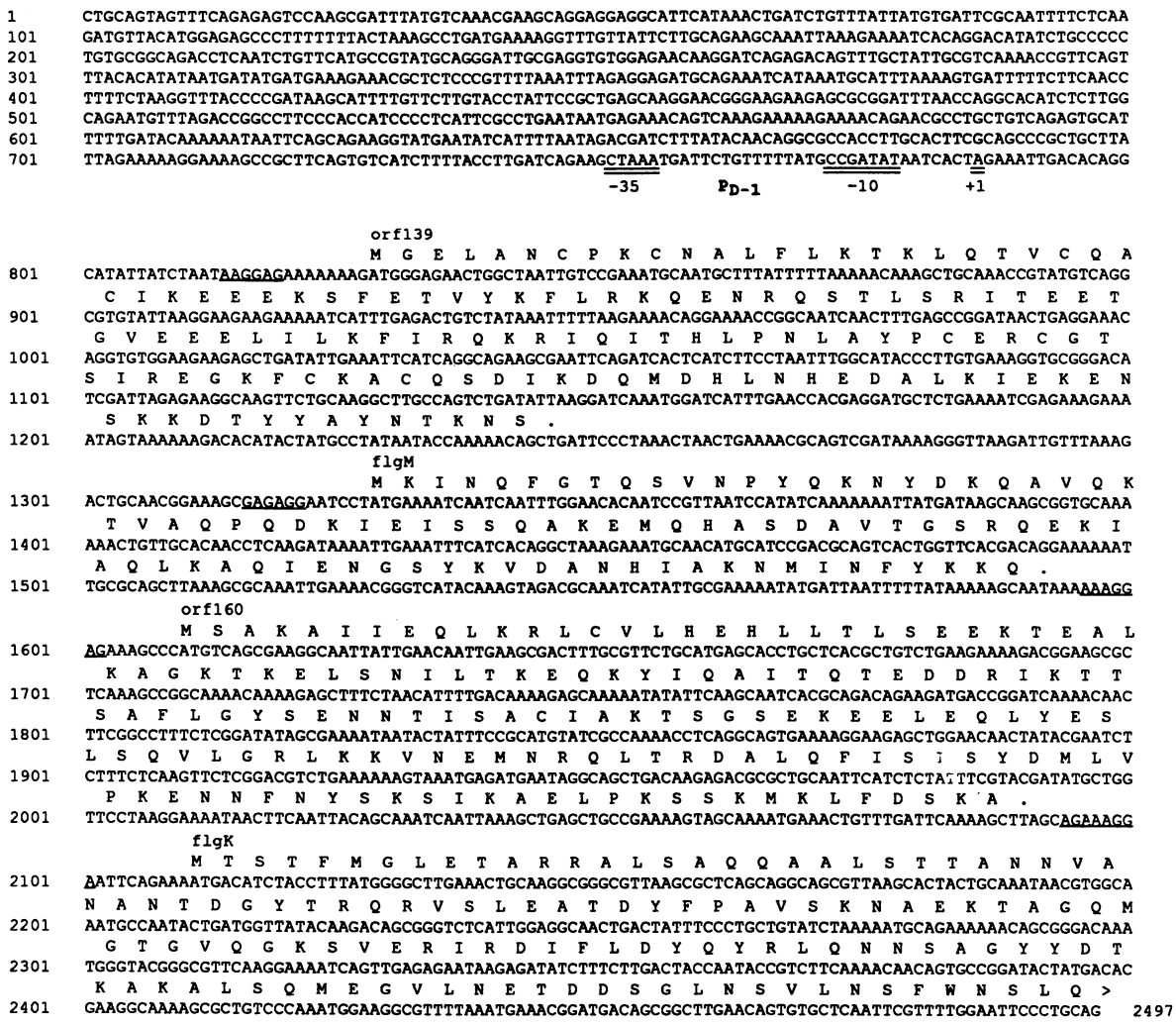

FIG. 2. Nucleotide sequence of the beginning of the *B. subtilis* P_{D-1} operon. The 2,497 nucleotides of pDM113 and pDM116 are shown in the orientation indicated in Fig. 1. The features of the σ^D-dependent promoter P_{D-1} known to transcribe this operon (the −35 and −10 promoter consensus regions and transcription start site [+1]) are indicated by double lines below the nucleotide sequence. The putative translation initiation signals (ribosome binding site) for the four ORFs are designated by a single underline. The amino acid sequences of the conceptual translations of the several putative ORFs are shown above the nucleotide sequence by a one-letter code. The translation of the *comFORF3* coding region is not shown (39). The *flgK* reading frame is presumed to continue downstream, beyond the sequence presented here.

(23), and hence each is a likely protein coding sequence (Fig. 2). We propose that P_{D-1} directs the synthesis of a polycistronic message, and so we will refer to the entire transcription unit as the P_{D-1} operon.

The sequence presented here overlaps that of the 3′ end of the *comF* locus presented by Londoño-Vallejo and Dubnau (39). Specifically, the sequence of nucleotide bases 1 through 1,050 has been published previously; this region of DNA includes the *comFORF3* gene (39) (Fig. 1).

Identification of ORFs. The beginning of the P_{D-1} operon potentially encodes gene products involved in flagellar synthesis as well as other proteins of yet-unknown function. The first ORF downstream of P_{D-1} encodes a 139-amino-acid polypeptide (calculated mass, 18.2 kDa), which we temporarily call the product of the *orf139* gene. On the basis of comparisons with other proteins and similarities in primary structure to other proteins, we have found that Orf139 is similar to the C terminus of eukaryotic myosin heavy-chain polypeptides (data not shown). The C terminus of myosin proteins (the tail region) folds into an extended α-helical secondary structure which forms coiled-coils in association with other myosin molecules and subsequently can form large filamentous networks (57). By protein secondary structure prediction analysis (13), we have determined that the Orf139 polypeptide can form at least two long α-helices (data not shown). It is plausible to predict that the Orf139 peptide polymerizes to form a filamentous structure that is part of the gram-positive flagellum. Orf139 also possesses an incomplete similarity to eukaryotic zinc-finger protein motifs (data not shown); it is possible that one or more Zn^{2+} ions can bind to this peptide (14).

The next ORF encodes an 88-amino-acid polypeptide (calculated mass, 10.0 kDa) which is homologous to the *S. typhimurium flgM* gene (16), on the basis of sequence similarities (Fig. 3A) and by genetic analysis described in detail below. Overall, these two *flgM* gene products share 30% identical

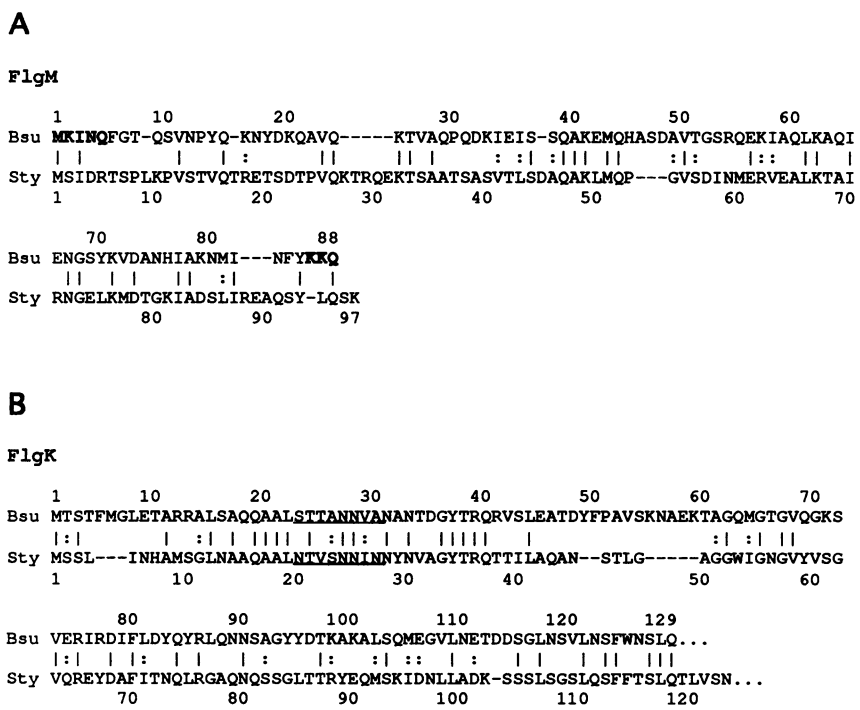

FIG. 3. Comparison of the complete *flgM* and part of the *flgK* genes of *B. subtilis* and *S. typhimurium*. The predicted amino acid sequences of *B. subtilis* (Bsu) and *S. typhimurium* (Sty) flagellar genes are shown. Alignment of FlgM proteins (A) and that of the N terminus of the FlgK proteins (B) are shown. Amino acid identities between the two sequences are indicated by |, whereas alignments of similar amino acids (as defined by McLachlan [44]) are shown by a colon (:). Gaps that were introduced into the sequences to maximize alignment are shown by a dash (–). The distance from the N terminus of the proteins is indicated by numerals. In panel A, the amino acid residues of *B. subtilis flgM* that are shown in bold type indicate the ones that are maintained in the *flgM*Δ80 allele; the rest of the residues are deleted and replaced by the dipeptide Leu-Gln (LQ). In panel B, the extended ANNLAN consensus sequence (31, 32) is indicated by underlining.

residues; the shared identical residues increase to 39% if conservative substitutions are considered as well. This degree of sequence similarity is well within the range found for other homologous flagellar proteins of *B. subtilis* and *S. typhimurium* (50). We note also the long (80-nucleotide) untranslated spacer region between *orf139* and *flgM* (Fig. 2), which may provide some regulatory function.

Downstream of *flgM* is an ORF, tentatively titled *orf160*, directing the synthesis of a 160-residue polypeptide with a calculated mass of 16.2 kDa. By comparing its primary sequence with that of other proteins, we have found that the predicted Orf160 protein shares similarity with eukaryotic intermediate filament proteins (data not shown). Specifically, Orf160 shares sequence conservation with α-helical domains 1A and 1B of class III intermediate filament proteins such as vimentin and desmin (1). These proteins form homopolymeric filaments as a result of interactions of two extended α-helices to form a coiled-coil tertiary structure (1). By protein secondary structure prediction analysis (13), we have determined that the Orf160 polypeptide has the capacity to form a long α-helix structure at its N terminus and another one internally (data not shown). As for Orf139, it is plausible to predict that the Orf160 peptide also polymerizes to form a filamentous structure that is part of the gram-positive flagellum.

Finally, further downstream of *orf160* is the 5′ end of a coding region that continues beyond the sequence presented in this report. This last ORF potentially directs the synthesis of a protein with marked similarity to the enteric flagellar hook-filament junction protein HAP1, which is encoded by the *flgK* gene (31). A comparison of this potential protein product and that of *S. typhimurium* FlgK is shown in Fig. 3B. We have tentatively designated this gene *flgK* because of its apparent homology to the enteric gene. These two proteins possess 32% amino acid identity along their primary sequence; the similarity is 43% when conservative amino acid substitutions are considered. As can be seen in Fig. 3B, *B. subtilis flgK* possesses the structural motif ANNLAN, which is common to many axially arranged flagellar proteins (31, 32), as well as other specific primary sequence similarities to *S. typhimurium flgK*.

Creation of a *flgM* deletion allele and *B. subtilis* mutant. We were especially interested in determining if the *B. subtilis flgM* ORF encoded a regulatory protein that was involved in the control of transcription of flagellar genes of *B. subtilis*. We therefore wanted to characterize the phenotypes resulting from a disruption of the *flgM* gene. As the evidence suggests that *flgM* is part of an operon of genes, we wished to ensure that a mutation in *flgM* would not have a polar effect on other genes of the polycistronic message. We therefore chose to create an in-frame deletion of most of the *flgM* coding sequence; such a deletion should not affect transcription or translation of downstream cistrons.

We used oligonucleotide-directed mutagenesis to create an in-frame deletion of the *B. subtilis flgM* gene present on plasmid pDM124 and then used a gene replacement technique to substitute the mutant allele (called *flgM*Δ80) for the wild-type copy on the *B. subtilis* genome (see Materials and Methods). As indicated in Fig. 3A, the product of the $flgM^+$ allele is a protein of 88 amino acids, whereas the *flgM*Δ80 allele produces a protein of only 10 amino acids in length. In this mutation, the first five and the last three amino acid residues of

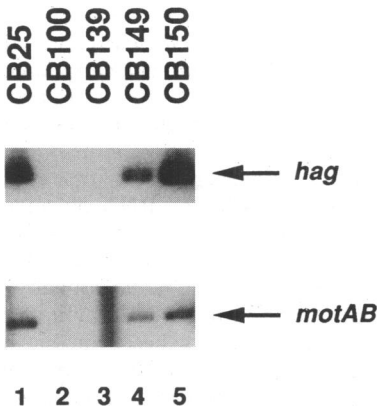

FIG. 4. Utilization of σ^D-dependent promoters in *B. subtilis sigD*, *flgM*, and *flaA* strains. Primer extension analysis was performed on RNA from various *B. subtilis* strains, using primers specific for transcripts derived from the promoter for the *B. subtilis hag* gene, P_{D-6} (top), or for the *motAB* operon, P_{D-2} (bottom). The arrows indicate the location of fully extended primer and, thus, levels of mRNA from each promoter. Lanes: 1, RNA from strain CB25; 2, RNA from CB100; 3, RNA from CB139; 4, RNA from CB149; 5, RNA from CB150.

$FlgM^+$ are preserved; the central 80 residues are replaced by the dipeptide Leu-Gln. We then created strains in which the *flgM*Δ80 allele replaced the wild-type gene (see Materials and Methods) (Table 1).

Analysis of σ^D-dependent promoter utilization in *fla* and *flgM* backgrounds. In *S. typhimurium*, mutations in *flgM* relieve the repression of late flagellar gene expression indirectly caused by a mutation in an early flagellar gene (17). If *B. subtilis flgM* is functionally homologous to its enteric counterpart, then a *flgM* mutant strain should also uncouple late flagellar synthesis from early flagellar assembly.

We examined the phenotype of a *B. subtilis flgM*Δ80 mutant by assessing the transcription of the late flagellar genes and operons *hag* and *motAB* by primer extension analysis. The promoters for these two transcription units are recognized by the *B. subtilis* σ^D form of RNA polymerase (46, 47). We examined isogenic *B. subtilis* strains bearing the four combinations of *flgM*$^+$ versus *flgM*Δ80 alleles and *fla*$^+$ versus *flaA*::Tn*917*Ω1836 alleles (Table 1). The *flaA*::Tn*917*Ω1836 mutation is a transposon insertion within the large operon of early flagellar structural genes and confers a Fla^- phenotype (50, 63). We also compared these four strains with one bearing an insertion-disruption mutation in the σ^D structural gene *sigD* (27). The primer extension analysis is presented in Fig. 4. We note that on the *motAB* primer extension panel, the streak in lane 3 does not occlude the entire lane and thus does not interfere with visual interpretation of the data. The absence of extended *motAB* primer in this lane was confirmed by analysis using an AMBIS scintillation scanner.

Figure 4 demonstrates that the repression of σ^D-dependent transcription caused by a block to early flagellar synthesis is relieved by a deletion of the *flgM* gene. As expected, σ^D-dependent transcription from the *hag* and *motAB* gene promoters seen in a wild-type strain (CB25; lane 1) is abolished in the absence of σ^D protein in the *sigD*-disrupted strain (CB100; lane 2). Transcription from these promoters is also abolished in the strain bearing the *flaA*::Tn*917*Ω1836 mutation (CB139; lane 3), although it is known that σ^D protein is still present (43). The presence of the *flgM*Δ80 mutation in an otherwise wild-type strain (CB149; lane 4) does not qualitatively affect the level of σ^D-dependent transcription. However, the presence of the *flgM*Δ80 mutation does restore the transcription of *hag* and *motAB* that was repressed by the effects of the *flaA*::Tn*917*Ω1836 mutation (CB150; lane 5). These data demonstrate that the *B. subtilis flgM* gene product functions genetically in a manner identical to that of the same gene of *S. typhimurium* and strongly suggests that the two are homologs. We are uncertain as to the statistical significance of the quantitative differences in *hag* and *motAB* transcription in the samples of exponentially growing cultures of strains CB25, CB149, and CB150 shown in Fig. 4. This uncertainty is a consequence of the rapid change in the levels of mRNA from σ^D-dependent transcription units during exponential growth, such that small variations in culture growth time or cell density can result in large changes in transcript levels (19, 45, 46).

B. subtilis autolytic activity can serve as an additional reporter of σ^D-dependent promoter utilization. Strain CB100 has a filamentous (Lyt^-) phenotype due to a reduction in autolytic activity (42). We note that CB139 is also Lyt^-, whereas CB150 grows as single cells and thus is phenotypically Lyt^+. These observations suggest that the *flgM*Δ80 mutation also relieves the block to expression in a Fla^- background of σ^D-dependent autolytic activity, presumably including expression of the *lytABC* operon (38).

DISCUSSION

We have sequenced 1.7 kb of DNA transcribed from the σ^D-dependent promoter P_{D-1} and have characterized the beginning of an operon containing late flagellar genes. Three complete ORFs and one partial one have been identified within the transcribed region (Fig. 1). Searches of the nucleotide and protein databases have revealed similarities between the second and fourth ORFs with the enteric flagellar genes *flgM* (30% identity) and *flgK* (32% identity), respectively (Fig. 3). We have characterized the *B. subtilis flgM* gene and its product in the greatest detail and discuss its role and relevance below. We will discuss the potential roles of the other P_{D-1} operon genes and gene products later.

Role of *B. subtilis flgM* in regulation of flagellar synthesis. We believe that we have identified the *B. subtilis* homolog of the enteric flagellar regulatory gene *flgM*. First, the two gene products are similar in primary structure (Fig. 3) (16). Second, *flgM* mutations produce the same phenotype. Mutation of *flgM* in *S. typhimurium* leads to relief of repression of flagellin gene expression brought about by a mutation in an early (i.e., class II) flagellar gene, identifying *S. typhimurium* FlgM protein as a negative regulator of flagellar class III gene expression (17). The *B. subtilis flgM*Δ80 mutation described herein has an identical effect on *hag* and *motAB* gene expression in its relief of repression of the transcription of these genes caused by the class II *flaA*::Tn*917*Ω1836 mutation (Fig. 4). Third, we have shown that *B. subtilis flgM* is transcribed from a σ^D-dependent promoter; in *S. typhimurium flgM* is at least partially σ^F dependent (16, 33).

This and previous studies make it clear that in *B. subtilis* the repression of late flagellar gene expression by a mutation in an early flagellar gene is a result of inactivation of σ^D and not due directly to changes in its cellular levels (43). Recently, the mechanism of action of *S. typhimurium* FlgM was examined biochemically. Ohnishi and coworkers showed that FlgM represses transcription of the *fliC* gene in vitro by acting as an anti-sigma factor, binding to σ^F and preventing its association with core RNA polymerase (49). We do not yet know if *B. subtilis* FlgM inactivates σ^D in a similar way; biochemical experiments with purified components are under way. Recently, Hughes et al. have shown that in *S. typhimurium* the

FlgM protein is in fact exported through the flagellum-specific export pathway upon the correct completion of assembly of the hook and basal body; thus, the concentration of FlgM is lowered by its expulsion from the cell, allowing σ^F to associate with core RNA polymerase and transcribe the class III flagellar genes (33). We do not yet know how the sensing of hook-basal body completion occurs in *B. subtilis* and if it occurs by a mechanism similar to that of enteric bacteria.

We propose that *flgM* is allelic to the previously identified *ifm* locus, which maps very close to *hag* (22, 51). *ifm* mutant cells are more motile and more flagellated than are wild-type *ifm* cells (22). The *ifm* mutation can arise spontaneously and can partially suppress the Fla$^-$ phenotype of the *flaA4* or the *flaD1* mutations (51). The *flaA4* mutation lies within the *fla/che* operon and is likely to be a class II flagellar mutation (2, 50). The partial suppression of the *flaA4* mutation is akin to the ability of the *flgM*Δ80 mutation to restore *hag* gene expression but not restore flagellation in the *flaA*::Tn*917*Ω1836 mutant. We have not yet examined the phenotype of a *flaA4-flgM*Δ80 double mutant with regard to σ^D-dependent gene expression nor have we localized an *ifm* mutation to the *flgM* gene by DNA sequencing or by complementation. Furthermore, we have not rigorously quantitated the differences in motility or flagellation or σ^D-dependent gene expression between *flgM*$^+$ and *flgM*Δ80 strains of *B. subtilis*.

It is useful to review what is known about the transcription unit that controls FlgM expression in *B. subtilis*. The P_{D-1} promoter was originally identified as a strong in vitro promoter used by σ^D RNA polymerase and located on the 2,497-bp *Pst*I fragment cloned in plasmid pCD4136 (58). This fragment was shown to contain no other in vitro promoters for either *B. subtilis* σ^D or σ^A RNA polymerases. There is no evidence for the presence of a rho-independent terminator downstream of P_{D-1} on the *Pst*I fragment, on the basis of in vitro transcription and examination of the DNA sequence. The start site for P_{D-1} in vitro and in vivo was mapped by the S1 nuclease method, and the P_{D-1} transcription unit was shown to be regulated temporally in *B. subtilis* (19). Those experiments rule out the possibility of any significant amount of transcription in vivo from promoters upstream of P_{D-1}. Thus, the *flgM* operon in *B. subtilis* appears to be a polycistronic operon controlled by the single strong P_{D-1} promoter, in contrast with the *flgM* operon in *S. typhimurium*, which is only partially dependent on σ^F RNA polymerase (16, 18). However, since we have not yet been able to detect the full-length *flgM*-containing transcript in Northern (RNA) blot experiments, we do not know the size of the complete operon nor can we rule out the possible presence of weak promoters within the operon that may play some secondary role in its regulation.

We envision the use of the *flgM*Δ80 mutant in a potential test to distinguish class II flagellar genes. We expect that any and all class II (i.e., early) flagellar mutations will repress σ^D-dependent promoter utilization, and this repression can be relieved by the compensatory *flgM*Δ80 mutation. Specific transposon insertions in the *fla/che* operon are known to inactivate σ^D RNA polymerase (43). The gene disrupted by the *flaA*::Tn*917*Ω1836 insertion (probably *fliE* or *fliF* [62]) is a class II flagellar gene, as expected by homology to the enteric paradigm. It is likely that the other *flaA*::Tn*917* insertions that behave like the Ω1836 allele also define class II genes (2, 43, 50, 64). Currently, as individual genes from the *fla/che* operon are identified and characterized, the effects of mutations in each are noted in terms of changes in *hag* or *motAB* gene expression, often in relation to that of an (unaffected) σ^A-dependent transcription unit. By this criterion alone, one can infer that the *fla/che* operon genes *flhA*, *fliM*, *fliR*, *fliF*, and *flhF* are class II flagellar genes (11, 12, 43, 45, 61). Further proof would entail determination that *flgM*Δ80 relieved the observed repression of class III flagellar gene expression. From the arguments above, it is clear that *cheA*, *cheB*, and *cheW* are not class II flagellar genes, although they reside in the *fla/che* operon (8, 15, 24). It is not even clear if the *B. subtilis che* genes are in fact class III flagellar genes; at least one, *cheX*, is evidently not transcribed by σ^D RNA polymerase (63). We emphasize that the distinction between a class II and a class III flagellar gene is based on analysis of the effects of a mutation in the gene itself (thus defining its functional role in flagellar assembly or operation) on the transcription of other flagellar operons. The distinction is not based on the transcriptional regulation of the operon to which the gene belongs. In this way, it is not at all surprising that chemotaxis gene products are not class II flagellar genes; since these genes are presumably not σ^D dependent, we also cannot classify them as class III genes.

We also envision the use of the *flgM*Δ80 mutant as a potential means to distinguish true *lyt* mutants from those which are Lyt$^-$ because they are *fla* mutants. In the latter case, the added presence of the *flgM*Δ80 mutation should result in a reversion to Lyt$^+$, as we have seen for strain CB150. This test would be applicable both to evaluating existing Lyt$^-$ strains and to facilitating genetic screens for new *lyt* mutants of *B. subtilis*.

Function and role of P_{D-1} operon genes. In *S. typhimurium*, the *flgK* gene encodes the hook-associated protein HAP1, a 553-amino-acid late flagellar protein present at the hook-filament junction (31). The sequenced portion of *B. subtilis flgK* is only 120 codons long, and so if it is the *B. subtilis* homolog of HAP1, it would be expected to continue further. In addition, in *S. typhimurium*, the *flgL* gene (encoding HAP3) follows, and is thought to be cotranscribed with, *flgK* (31); a homolog of the latter gene may also be transcribed by P_{D-1}. Indeed, the *S. typhimurium flgKL* operon is preceded by a σ^F-dependent promoter consensus sequence and is likely to be transcribed by this form of RNA polymerase (31). We are currently sequencing and further characterizing the region of DNA between *flgK* and *hag* (56).

At this point, we can only speculate on the functions of the products of the *orf139* and *orf160* genes. Being members of a σ^D-dependent operon, these proteins are likely important to the assembly, structure, or function of the flagellum of *B. subtilis*. As these peptides do not share similarity with known gram-negative flagellar genes, they may in fact be flagellar proteins specific to gram-positive bacteria. We find it interesting that these two proteins appear similar to filament-forming eukaryotic proteins like myosin and vimentin (1, 57) and that both possess long stretches of amino acid residues capable of folding into extended α-helices. To our knowledge, there are few examples of bacterial proteins possessing extended α-helices or that are capable of forming coiled-coil quaternary structures. Many other flagellar proteins are arranged axially on the flagellum (31, 32); it is possible that a gram-positive flagellum contains components or structural elements that are quite different from its gram-negative counterpart.

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