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Dual Promoters Are Responsible for Transcription Initiation of the *fla*/*che* Operon in *Bacillus subtilis*

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The *fla*/*che* region contains more than 30 genes required for flagellar synthesis and chemotaxis in *Bacillus subtilis*, including the gene for the flagellum-specific σ^D factor, *sigD*. Sequence and primer extension data demonstrate that a P_A promoter immediately upstream of *flgB*, henceforth referred to as the *fla*/*che* P_A, and the P_{D-3} promoter are active in vivo. Transcription from the P_{D-3} element is dependent on σ^D activity and is regulated by the flagellum-specific negative regulator, FlgM. In a strain containing a deletion of *fla*/*che* P_A ($P_A\Delta$), σ^D protein was not detected, demonstrating that the *fla*/*che* P_A is necessary for wild-type expression of the *sigD* gene. Thus, *sigD* is part of the >26-kb *fla*/*che* operon. Consistent with a lack of detectable σ^D protein, the $P_A\Delta$ strain grows as long filaments and does not express a σ^D-dependent *hag*::*lacZ* reporter construct. These phenotypes are indicative of a lack of *sigD* expression or complete inhibition of σ^D activity by FlgM. However, σ^D activity is found in a double mutant containing the $P_A\Delta$ and a null mutation in *flgM*. The double mutant no longer grows as long filaments, and expression of *hag*::*lacZ* is partially restored. These data demonstrate that a low level of σ^D activity does exist in the $P_A\Delta$ mutant but can be detected only in the presence of a null mutation in *flgM*. Therefore, normal expression of *sigD* may also involve another promoter(s) within the *fla*/*che* operon.

Gene expression in *Bacillus subtilis* is principally regulated by alternate forms of RNA polymerase differing in composition by the association of alternate σ factors, which determine the promoter specificity of the resulting holoenzyme (25, 34, 44). The σ^D holoenzyme is directly responsible for transcription of the genes for flagellin (30), several hook-associated proteins (4), the MotA and MotB motor proteins (31), the CheV chemotaxis protein (7), the methyl-accepting chemotaxis proteins (12), and the FlgM negative regulator (33). The σ^D regulon in *B. subtilis* is therefore composed of genes encoding proteins for flagellar synthesis, motility, and chemotaxis.

The structural gene for the σ^D factor, *sigD*, is located at the distal end of the *fla*/*che* region of DNA (29). The *fla*/*che* region of DNA was originally isolated as two overlapping lambda clones, containing *B. subtilis* genomic DNA, that complemented chemotaxis mutations mapping between *pyrD* and *thyA* on the bacterial chromosome (39). The genes residing in this region of DNA have been sequenced, and many of the predicted protein products have been shown to be homologous to structural proteins that form the hook-basal body (HBB), as well as several chemotaxis proteins in the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* (38). The homologous genes in these bacteria, however, are found in 13 different operons (21, 22).

In the enteric bacteria, a hierarchy of transcription of three classes of genes (21, 26) ensures that the expression of the gene encoding the major flagellar protein flagellin (*hag*) is tightly regulated and is dependent on the functional assembly of the HBB complex (14, 20). The master regulators FlhD and -C are class I gene products and are transcriptional activators that are required for expression of class II genes (24). Class II genes encode the structural proteins that form the HBB complex (36) and include the *fliA* locus, which encodes the alternate sigma factor σ^F, a homolog of σ^D (36). Class III genes possess σ^F-dependent promoters and are transcribed by this form of RNA polymerase (27). Furthermore, class III gene expression is regulated by FlgM and is dependent on the expression of all class II genes (15). FlgM has been described as an anti-sigma factor that inhibits σ^F binding to RNA polymerase (37). Once the HBB complex is functionally assembled, the FlgM regulator is specifically exported and σ^F is able to associate with RNA polymerase (14, 20) and initiate transcription of the class III genes, including *hag*.

A similar hierarchy of flagellar gene expression seems to exist in *B. subtilis*, although homologs to the class I master regulators have not been identified. The *fla*/*che* operon appears to be primarily a class II transcription unit. Mutations within the *fla*/*che* operon that disrupt HBB open reading frames eliminate flagellin synthesis (29, 46). This morphogenetic repression of class III gene expression is controlled by the FlgM homolog in *B. subtilis*, since the lack of this factor restores expression of the *hag* gene in strains lacking an HBB complex (32). Therefore, *hag* gene expression in *B. subtilis* is dependent upon the expression of the HBB genes and on σ^D RNA polymerase as it is in the enteric bacteria.

Interestingly, the genes encoding the HBB complex appear to be part of a single operon in *B. subtilis*. Sequence analyses of the region demonstrate the existence of consecutive open reading frames that appear to be translationally coupled (1, 38). Additionally, genetic analyses of this region suggest that it is transcribed from a single upstream promoter region, since insertions of heterologous DNA within *fla*/*che* result in decreased expression of downstream genes (29, 45, 46). Although much of the DNA that makes up the *fla*/*che* region has been cloned, sequenced, and characterized genetically, the transcription initiation signals have not been identified. Therefore, to better understand the molecular mechanisms that govern flagellar gene expression in *B. subtilis*, this study has centered on the characterization of the promoter region for the *fla*/*che* transcription unit. This work provides a foundation for studying the molecular machinery that governs expression of a very large transcription unit encoding flagellar and chemotaxis

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

Strain	Genotype	Source or derivation and reference[a]
LMB1	*trpC2*	E. Ferrari, I168[b]
LMB10	*trpC2 sigD*::pLM5 (Cm^r)	M. J. Chamberlin, CB100[b]; 13
LMB24	*trpC2 phe hag*::*lacZ* (Cm^r)	M. J. Chamberlin, CB25[b]; 32
LMB213	*trpC2 flgM*::mini-Tn*10* (Sp^r)	Transform [LMB1:HB4229, Sp^r]; reference 6 and this study
LMB214	*trpC2 fla/che* $P_A\Delta$	Transform [LMB1:pWE4-int, Cm^r][c]; this study
LMB216	*trpC2 fla/che* $P_A\Delta$ *flgM*::mini-Tn*10* (Sp^r)	Transform [LMB214:LMB213, Sp^r]; this study
LMB219	*trpC2 hag*::*lacZ* (Cm^r)	Transform [LMB1:LMB24, Cm^r]; this study
LMB220	*trpC2 fla/che* $P_A\Delta$ *hag*::*lacZ* (Cm^r)	Transform [LMB214:LMB24, Cm^r]; this study
LMB221	*trpC2 flgM*::mini-Tn*10* (Sp^r) *hag*::*lacZ* (Cm^r)	Transform [LMB219:LMB213, Sp^r]; this study
LMB222	*trpC2 flgM*::mini-Tn*10* (Sp^r) *fla/che* $P_A\Delta$ *hag*::*lacZ* (Cm^r)	Transform [LMB220:LMB213, Sp^r]; this study

[a] Transformation of [first (recipient) strain: with chromosomal DNA from second strain, and selecting for resistance indicated] is shown for some strains.
[b] Previous designation of strain.
[c] This strain was subsequently cured of the chloramphenicol resistance marker.

functions in *B. subtilis* which, in enteric bacteria, are encoded in more than 13 operons (21, 22).

MATERIALS AND METHODS

Bacterial strains, media, growth, and transformation. The *E. coli* host for growth of recombinant plasmid was the strain Epicurian Coli XL1-Blue {*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]} (Stratagene). For site-directed oligonucleotide mutagenesis, *E. coli* CJ236 [F^- *dut-1 ung-1 thi-1 relA1*/pCJ105 (Cm^r)] (Bio-Rad Laboratories), *E. coli* Epicurian Coli XLmutS {Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 gyrA96 relA1 lad mutS*::Tn*10* (Tet^r)[F′ *proAB lacI*^{q}ZΔM15 Tn*5* (Kan^r)]} (Stratagene), and the M13 helper phage R408 (Promega) were used. Transformation of competent *E. coli* cells was performed as described in the suppliers' protocols. Electrocompetent *E. coli* CJ236 (Bio-Rad Laboratories) cells were transformed by electroporation with a Gene-Pulser. *E. coli* cells containing plasmids were selected by growth in LB (Luria-Bertani) broth supplemented with 50 μg of ampicillin (Sigma) per ml. In cases where a blue-white screen was used to identify transformants containing the desired recombinant plasmids, transformants were plated on LB agar with 32 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma) per ml, 32 μg of IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) per ml, and the appropriate antibiotic.

The *B. subtilis* strains used in this study are listed in Table 1. Strains were grown in 2× SG medium [2× nutrient broth, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnSO_4$, 0.1% glucose] and tryptose blood agar base (TBAB) and LB plates. Transformation of plasmid or chromosomal DNA into *B. subtilis* was accomplished as previously described (5). Chloramphenicol-resistant (Cm^r) strains were maintained by adding 5 μg of chloramphenicol (Sigma) per ml to the appropriate medium, whereas spectinomycin-resistant (Sp^r) strains were maintained by adding 100 μg of spectinomycin (Sigma) per ml.

Manipulations of DNA. Plasmid DNA propagated in *E. coli* was purified by a modification of the alkaline lysis method (41), which included isopropanol and lithium chloride precipitations. *B. subtilis* chromosomal DNA was isolated as described previously (28). The recovered plasmid and chromosomal DNA was resuspended in TE (10 mM Tris-Cl [pH 8.0], 1 mM EDTA), and the yield and concentration were quantified by use of a spectrophotometer (UV-1201; Shimadzu).

Restriction digests for preparatory or diagnostic purposes were performed by standard procedures (41). Agarose gels were photographed with a GDS 7500 gel documentation system (UVP) or a Polaroid camera and Polaroid 667 black-and-white film. DNA fragments of interest were gel purified with the Prep-A-Gene DNA purification system (Bio-Rad Laboratories) under the manufacturer's recommended conditions. The 5′ phosphates were removed from the digested vector DNA by using shrimp alkaline phosphatase (United States Biochemical), and the subsequent ligation was accomplished by using the Takara ligation kit (Panvera) as recommended by the manufacturer.

DNA sequencing and analysis. Sequencing reactions were carried out with Sequenase version 2.0 (United States Biochemical), by using the following primers: PD3 (5′-CACCCTCAATATCCTTGTCG-3′), OWE1 (5′-CATAGAAAGA CTTTCAA-CCCAGG-3′), and OWE2 (5′-GAGGGTTCTTTTTTTATTTC-3′). Primers were synthesized by Cruachem, Inc. (Dulles, Va.). The nucleotide sequences obtained were compiled and analyzed by using MacVector (Kodak).

Construction of plasmids. The 2.6-kb *Pst*I fragment from pAZ210 (5), which contains the *fla/che* promoter region, was subcloned into pGEM5Zf(+) (Promega) to yield the plasmid pWE1. pWE4, a derivative of pWE1, contains a 29-bp deletion of the *fla/che* P_A promoter ($P_A\Delta$) created by oligonucleotide-directed mutagenesis (see below). The 2.6-kb *Pst*I fragment from pWE4 containing the $P_A\Delta$ was subcloned into the *B. subtilis* integrational vector pJM102 (41) to generate the plasmid pWE4-int.

RNA extraction and primer extension analysis. To determine if promoter consensus sequences identified by computer analyses are functional in vivo, total RNA was purified from the appropriate strains and subjected to primer extension analyses. *B. subtilis* LMB1 and LMB10 were grown to late log phase in 2× SG medium. The cells were harvested at two time points, T_0, marking the point when the *B. subtilis* culture breaks from logarithmic-phase growth, and $T_{0.5}$, marking 30 min after T_0. Total RNA was extracted by using the RNeasy Maxi RNA isolation kit (Qiagen) as recommended by the manufacturer except that cells were disrupted by sonication. Total RNA was precipitated and resuspended in double-distilled water. The integrity of the total RNA was verified by resolution on a formaldehyde-agarose gel under standard conditions (41).

Primer extension reactions were performed as described previously (33). Oligonucleotides PD3 and OWE3 (5′-AATATCCGCTCTGCTCAAGGCAT-3′; Cruachem, Inc.) were end labeled with ^{32}P to high specific activity (~10^8 cpm/μg). Primer extension reactions were resolved by electrophoresis alongside sequencing reactions of pWE1 which were generated by using the same oligonucleotide used in the primer extension reactions. Autoradiography was carried out at room temperature without an intensifying screen for 5 to 22 days. Exposed X-ray film was scanned with an Epson 2.01 scanner into Adobe Photoshop 3.0 (Adobe Systems, Inc.).

Deletion of the *fla/che* P_A. To determine the importance of the *fla/che* P_A promoter in initiating transcription of *sigD*, the promoter sequence was deleted from the *B. subtilis* chromosome by using oligonucleotide-directed mutagenesis as described by others (19). Oligonucleotide OWE4 (5′-GGACATTTTTTTAC ACGAACTTCAGAATTCAAGCATATAGTTTTACAATTC-3′) lacks the 29 bp comprising the −10 and −35 P_A consensus sequences as well as the 17-bp spacer region for the *fla/che* P_A (see Fig. 1) and in their place bears an *Eco*RI restriction site (underlined in the sequence above). In the in vitro synthesis reaction, OWE4 was extended with T4 DNA polymerase (Promega), and the newly synthesized strand was joined with T4 DNA ligase (Promega). The plasmid containing the $P_A\Delta$ was identified by restriction map analysis and named pWE4. This plasmid was sequenced by use of an ABI Prism 377 automatic sequencer with the PD3, OWE1, and OWE2 primers; besides the $P_A\Delta$, no new mutation was introduced as a result of the mutagenesis procedure.

A fragment bearing the $P_A\Delta$ was then subcloned into the *B. subtilis* integrational vector pJM102 (40) under standard conditions. The resultant plasmid, pWE4-int, was transformed into a wild-type *B. subtilis* strain, LMB1. Transformant colonies were selected by growth on LB plates containing 5 μg of chloramphenicol per ml. Transformants generated from a double-crossover event, in which two deleted copies of the *fla/che* P_A replaced the wild-type sequence, were identified by single-colony PCR as described below. Loss of the plasmid sequences was accomplished by repeatedly growing the cells in PA (Penassay or antibiotic medium 3; Difco) broth without chloramphenicol and screening for Cm^s colonies by replica plating. This regimen allows for homologous recombination between directly repeated $P_A\Delta$ sequences, resulting in the loss of the Cm^r gene and thus antibiotic resistance. Curing of the inserted plasmid from the integrant strain was verified by single-colony PCR as described below.

Single-colony PCR. To identify the *B. subtilis* cells harboring the double-crossover event and the $P_A\Delta$, DNA from single colonies was subjected to PCR analysis. Cell pellets derived from saturated 2-ml cultures were lysed in 0.1 mg of lysozyme per ml at 37°C. Cell debris was collected by centrifugation in a microcentrifuge for 1 min, and the supernatant was collected for PCR amplification. One microliter of the supernatant collected was added to a 20-μl reaction mixture containing 1× *Taq* buffer, 2.5 mM $MgCl_2$, 0.18 mM deoxynucleoside triphosphates, 1 μM concentrations of oligonucleotide OWE7 (5′-GTGAGGA CATTTTTTTACACG-3′) and OWE8 (5′-ACCCTCAATATCCTTGTCGAG-3′), and 0.3 U of *Taq* polymerase. PCRs were done in an MJR thermal cycler by using the following parameters: 1 min at 95°C, 1.5 min at 52°C, and 1 min at 72°C for 30 cycles. The PCR products were resolved on a 4% agarose gel and stained with 5 μg of ethidium bromide per ml. The gel was photographed with Polaroid

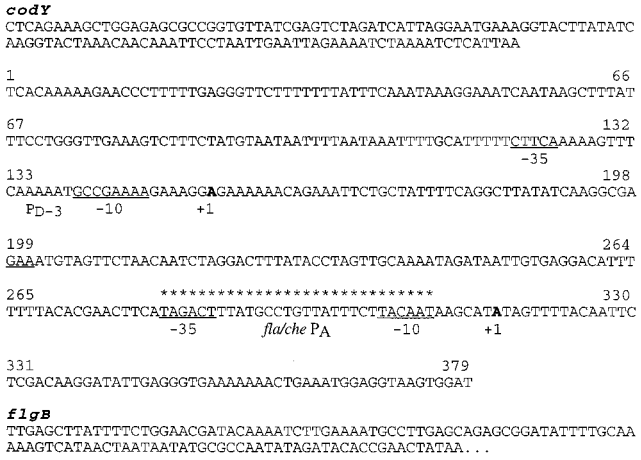

FIG. 1. *fla*/*che* promoter region. The intergenic region between *codY* and *flgB* has been defined as the *fla*/*che* promoter region. Two promoter elements were found to function in vivo: a σ^A-dependent promoter (*fla*/*che* P_A, present study) and P_{D-3}, a previously identified σ^D-dependent promoter (11). The −10 and −35 sequences for both of these promoters are underlined, and the +1 transcription initiation sites appear in boldface type. The 29 bp that were deleted in the *fla*/*che* $P_A\Delta$ strain and replaced with the *Eco*RI restriction site (GAATTC) are indicated by asterisks.

667 black-and-white film, and the photograph was scanned and the image was processed as described above.

Anti-σ^D immunoblot. The level of σ^D protein was determined by immunoblot analysis. Strains to be analyzed were grown in 2× SG medium containing the appropriate antibiotic. Thirty minutes after the end of logarithmic growth ($T_{0.5}$), 20 ml of cell culture was collected by centrifugation and washed in ice-cold STE (150 mM NaCl, 10 mM Tris-Cl, 100 mM EDTA). Total protein was then extracted and quantified as described previously (28), and 50 μg of protein per sample was resolved by electrophoresis on a sodium dodecyl sulfate–12.5% polyacrylamide gel. The protein was electrophoretically transferred to nitrocellulose, and the filter was blocked, incubated in hybridization buffers, and washed as described previously except that the secondary goat anti-rabbit antibody was conjugated to alkaline phosphatase (29). Reactive proteins were visualized by using BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium) substrate (Sigma) in distilled deionized water. The developed nitrocellulose filter was scanned, and the image was processed as described above.

Light microscopy. Strains were grown in 2× SG medium, and samples were collected 30 min after the break from logarithmic growth ($T_{0.5}$). Cells were viewed by differential interference microscopy at ×1,250 with a Nikon Optiphot-2 scope with Nomarski optics and photographed with a Nikon Microflex UFX-DX attachment and Ektachrome black-and-white film for slides. Slides were subsequently scanned with a Polaroid Sprint Scan 35, and the image was processed as described above.

β-Galactosidase assay. Flagellin gene expression was monitored by measuring the β-galactosidase activity produced in strains bearing a *hag*::*lacZ* translational fusion. Strains to be tested were grown in 2× SG sporulation medium containing 5 μg of chloramphenicol per ml when appropriate. Growth was monitored by spectrophotometer at 600 nm, and samples were collected at optical densities ranging from 0.2 to 3.0 and stored on ice. The β-galactosidase assay was conducted as described previously (8), except that β-galactosidase activity in the supernatant was measured at 420 nm. The calculation and manipulation of specific activity as Miller units, average values, and standard deviations were performed with Excel version 5.0 (Microsoft).

RESULTS

Analysis of the nucleotide sequence upstream of the *fla*/*che* region. pAZ210 contains a 5.2-kb segment of *B. subtilis* DNA bearing *flgB*, the first gene of the *fla*/*che* region, and 1.8 kb of DNA upstream of this gene, including the region of DNA thought to be responsible for initiating transcription of the *fla*/*che* operon (46). Directed sequencing was used to obtain 620 nucleotides of sequence data upstream of *flgB* (Fig. 1). Computer analysis of the data generated two interesting findings.

First, the DNA sequence furthest upstream of the *flgB* translational start site was found to be identical to the 3′ terminus of the *cod* operon, an operon in *B. subtilis* that was recently characterized (42). The *cod* and *fla*/*che* operons are transcribed in the same direction and are separated by a 379-bp sequence. We have defined the intergenic region between *codY* (the last gene in the *cod* operon) and *flgB* (the first gene in the *fla*/*che* transcription unit) as the *fla*/*che* promoter region. It is possible, however, that one or more promoters within the *cod* operon, or read-through from this transcription unit, may contribute to *fla*/*che* expression.

Second, sequence analysis of this promoter region demonstrated that part of the *fla*/*che* promoter sequence is identical to a segment of *B. subtilis* DNA previously shown to contain a σ^D-dependent promoter (11). This particular σ^D-dependent promoter, originally and hereinafter called P_{D-3}, was shown to be a sequence of DNA specifically recognized by the σ^D holoenzyme in an in vitro transcription reaction (11). Whether this promoter was active in vivo, however, had not been determined; the nature of the gene(s) it potentially regulated was also unknown.

Primer extension analysis of *fla*/*che* promoter region. Primer extension analyses reveal that transcription of the *fla*/*che* operon initiates from a P_A immediately upstream of *flgB* and from P_{D-3}. The P_A immediately upstream of *flgB* has therefore been renamed *fla*/*che* P_A. Expression from this promoter is found at T_0 and at $T_{0.5}$ in both the wild-type and *sigD* mutant strains (Fig. 2A, lanes 5 to 8). Primer extension products from P_{D-3}, however, are generated from RNA derived from the wild-type strain (Fig. 2B, lanes 5 and 6) but not from the *sigD* mutant (Fig. 2B, lanes 7 and 8). Therefore, transcription initiation from P_{D-3} is dependent on the σ^D holoenzyme. Furthermore, there appears to be an increase in primer extension products produced from RNA isolated from a *flgM* mutant strain (Fig. 2B, lanes 9 and 10). This result demonstrates that P_{D-3} is subject to regulation by the FlgM negative regulator; the absence of FlgM protein may allow for increased σ^D activity and thus increased transcription from P_{D-3} in this strain.

Construction of *fla*/*che* P_A deletion mutant. After the *fla*/*che* P_A was shown to be active in vivo, it was deleted from the *B. subtilis* chromosome to determine its importance in promoting expression of *sigD*. The *fla*/*che* P_A was first deleted by oligonucleotide-directed site mutagenesis of the plasmid pWE1, resulting in plasmid pWE4. To introduce this deletion into the *B. subtilis* chromosome, the 2.6-kb *Pst*I fragment containing the *fla*/*che* P_A deletion was subcloned into the integrational vector pJM102 (40), generating pWE4-int. pJM102 lacks a *B. subtilis* origin of replication and cannot be maintained as an episome. Concatemers of pWE4-int, however, can integrate into the *fla*/*che* promoter region by homologous recombination, by using either a single- or double-crossover mechanism (Fig. 3A, panels b and c). These integration events can be distinguished by the single-colony PCR method (see Materials and Methods). Only a strain bearing the double crossover can be used to obtain an exact deletion of the endogenous *fla*/*che* P_A without the introduction of plasmid sequences (43). This strain was grown under nonselective conditions to allow for homologous recombination of the duplicated regions and the concomitant loss of the intervening plasmid sequence (Fig. 3A, panel d). Transformants containing a double-crossover event were identified by using a PCR-based assay (Fig. 3B, lane 6), and strains bearing the exact deletion of *fla*/*che* P_A were verified by the same assay (Fig. 3B, lanes 7 and 8). Strains deleted for the endogenous *fla*/*che* P_A will henceforth be referred to as $P_A\Delta$ strains, and the deletion mutation will be referred to as $P_A\Delta$.

Characterization of *fla*/*che* P_A deletion strain. (i) Anti-σ^D immunoblot. Computer analyses of sequences 5′ and 3′ of the

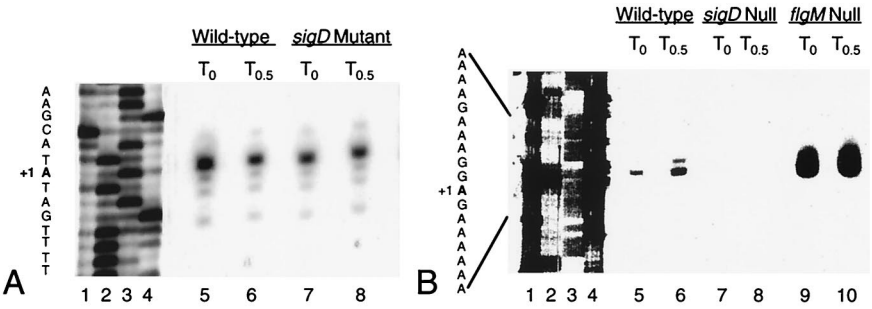

FIG. 2. Primer extension products from *fla/che* P_A and P_{D-3}. Total RNA from *B. subtilis* wild-type (LMB1), *sigD* null (LMB10), and *flgM* null (LMB213) strains that was isolated at the end of logarithmic growth (T_0) and 30 min later ($T_{0.5}$) was subjected to primer extension analysis. (A) Lanes 1 to 4, sequencing reactions of pWE1 (G, A, T, and C, respectively); lanes 5 to 8, primer extension products from total RNA, isolated by using primer OWE3, from *B. subtilis* LMB1 at T_0 (lane 5) and $T_{0.5}$ (lane 6) and from *B. subtilis* LMB10 at T_0 (lane 7) and $T_{0.5}$ (lane 8). (B) Lanes 1 to 4, sequencing reactions of pWE1 (G, A, T, and C, respectively); lanes 5 to 10, primer extension products from total RNA, isolated by using primer PD3, from *B. subtilis* LMB1 at T_0 (lane 5) and $T_{0.5}$ (lane 6), from *B. subtilis* LMB10 at T_0 (lane 7) and $T_{0.5}$ (lane 8), and from *B. subtilis* LMB213 at T_0 (lane 9) and $T_{0.5}$ (lane 10). The sequence given to the left of each panel corresponds to the nontemplate sequence, where +1 represents the nucleotide at which transcription initiates. Reactions were performed with 98 μg of LMB1 RNA isolated at T_0, 131 μg of LMB1 RNA at $T_{0.5}$, 153 μg of LMB10 RNA at T_0, 128 μg of LMB10 RNA at $T_{0.5}$, 84 μg of LMB213 RNA at T_0, and 92 μg of LMB213 RNA at $T_{0.5}$.

sigD gene suggest that *sigD* is the penultimate gene in the *fla/che* region, >26 kb downstream of the *fla/che* P_A (4a). To determine the effect of the $P_A\Delta$ on *sigD* expression, total protein was isolated from the appropriate strains and analyzed by anti-σ^D immunoblot. While σ^D protein was found in a wild-type and an *flgM* null mutant strain, there was no detectable σ^D protein in either the *fla/che* $P_A\Delta$ strain or the *sigD* null mutant (Fig. 4, lanes 2 and 3). Interestingly, the level of σ^D protein found in the *flgM* null mutant is comparable to the level found in the wild-type strain, even though transcription from the P_{D-3} promoter elements is dramatically increased in the mutant lacking the negative regulator (Fig. 2B, lanes 9 and 10).

(ii) Microscopic observation. Having demonstrated that there is no detectable σ^D protein in the $P_A\Delta$ strain, we examined cell morphology since the lack of σ^D has been shown to result in a filamentous phenotype (28). This is due to the fact that the genes encoding the major autolysins in *B. subtilis* responsible for hydrolyzing the cell wall after chromosomal replication are preceded by σ^D-dependent promoters (23). The lack of σ^D protein results in a significant decrease in autolysin production and thus a filamentous phenotype. The results obtained by microscopic observation are shown in Fig. 5. The wild-type strain grows as short rods (Fig. 5A), whereas the *sigD* null mutant grows as long filaments (Fig. 5B). The

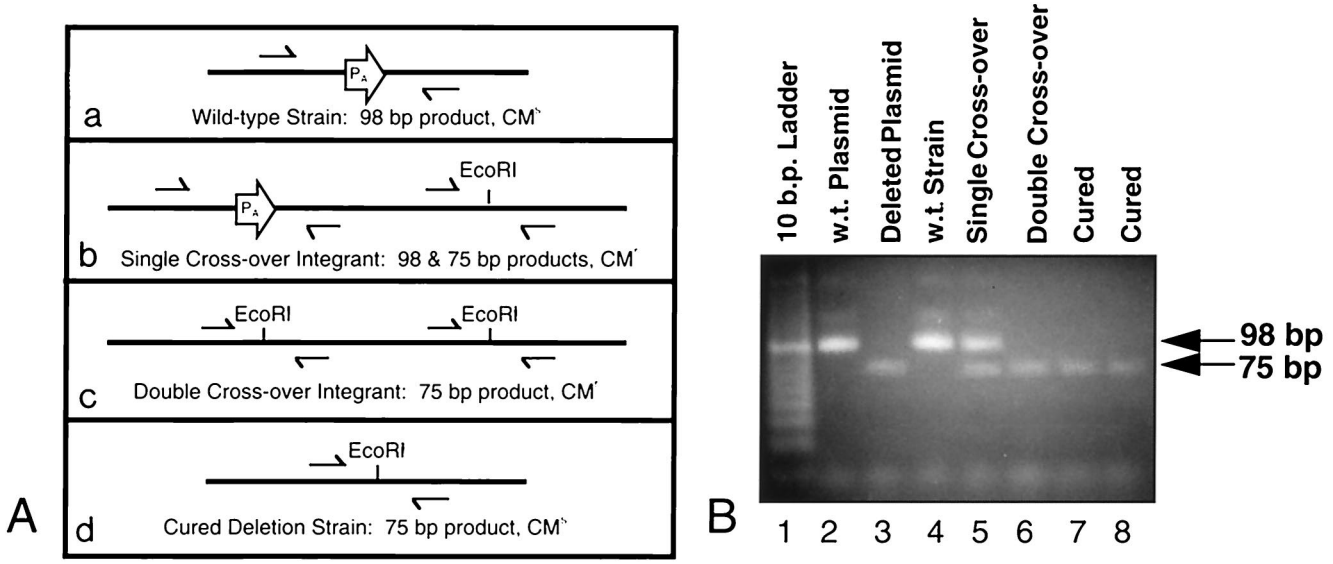

FIG. 3. PCR-based assay for the analysis of chromosome structure at the *fla/che* promoter region. (A) (a) The wild-type strain is sensitive to chloramphenicol, and its chromosome contains a single *fla/che* P_A, resulting in a 98-bp PCR product upon amplification. Introduction of pWE4-int gives rise to chloramphenicol-resistant transformants due to integration of plasmid sequences by homologous recombination. (b) DNA from a transformant generated by a single-crossover event (Campbell-like recombination) gives rise to PCR products of 98 and 75 bp. (c) DNA from a transformant generated by a double-crossover event with concatemers of pWE4-int (gene replacement) results in a single 75-bp product. (d) Once the strain bearing the double crossover is cured of chloramphenicol resistance, with a concomitant loss of the plasmid sequences, a single 75-bp fragment is produced. Half-arrows indicate primers used for PCR amplification. (B) Agarose gel of PCR products from wild-type (w.t.), transformant, and deleted strains. PCR-amplified products obtained from plasmid and chromosomal DNA with primers OWE7 and OWE8 were resolved by agarose electrophoresis. Lane 1, 10-bp ladder; lane 2, amplification of pWE1; lane 3, amplification of pWE4; lane 4, amplification of chromosomal DNA from LMB1; lane 5, amplification of chromosomal DNA from a chloramphenicol-resistant strain bearing a single crossover; lane 6, amplification of chromosomal DNA from a chloramphenicol-resistant strain bearing a double crossover; lanes 7 and 8, amplification of chromosomal DNA from chloramphenicol-sensitive strains bearing an exact deletion of *fla/che* P_A.

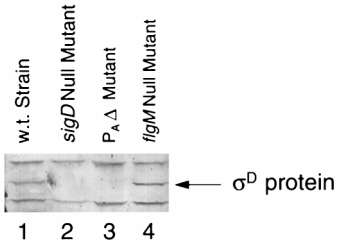

FIG. 4. Anti-σ^D immunoblot. The reactive σ^D protein was visualized in total protein extracts isolated from a wild-type and three mutant strains. Lane 1, wild-type strain (LMB1); lane 2, *sigD* null mutant (LMB10); lane 3, *fla*/*che* $P_A\Delta$ strain (LMB214); lane 4, *flgM* null mutant (LMB213). Fifty micrograms of total protein was loaded into each lane.

$P_A\Delta$ strain also grows as long filaments (Fig. 5C), which is consistent with a lack of detectable σ^D protein in this strain (Fig. 4, lane 3). This phenotype can also be explained by a complete inhibition of σ^D activity by FlgM (33). A double mutant bearing the $P_A\Delta$ and a null mutation in *flgM* ($P_A\Delta$/*flgM* null strain) no longer grows as long filaments (Fig. 5D) but as short rods like the wild-type cells.

(iii) *hag*::*lacZ* expression. To further assess σ^D activity, we examined the expression of a *hag*::*lacZ* reporter construct in the wild-type, $P_A\Delta$, and $P_A\Delta$/*flgM* null strains. The *hag* gene, which encodes the flagellin protein in *B. subtilis*, is preceded by a strong σ^D-dependent promoter element that is at least partially responsible for its high level of expression (30). In fact, 7% of the normal amount of σ^D protein is sufficient to produce nearly 70% of the wild-type levels of flagellin protein (28). Expression of the *hag*::*lacZ* fusion, as measured by β-galactosidase assay, is therefore a sensitive measure of σ^D activity in the cell.

We found that expression of the *hag*::*lacZ* reporter is greatly reduced in a strain bearing the $P_A\Delta$ (Fig. 6B). The pattern of expression is nearly identical to that found for an isogenic strain lacking the *hag*::*lacZ* fusion and for a strain bearing the fusion as well as a null mutation in *sigD* (data not shown). The low levels of expression found early in logarithmic growth appears to result from an error introduced by very low cell density during this time. In the $P_A\Delta$/*flgM* null double mutant, *hag*::*lacZ* expression is restored to approximately 50% of the wild-type level and exhibits an altered pattern (Fig. 6C). Specifically, *hag*::*lacZ* expression in the double mutant appears higher earlier in vegetative growth yet never displays the significant increase at the end of logarithmic growth that is found in the wild-type strain (Fig. 6A).

DISCUSSION

The 620-nucleotide fragment upstream of *flgB*, the first gene in the *fla*/*che* transcription unit, has been sequenced and characterized by primer extension and genetic analyses. Computer analysis of the sequence data obtained has allowed for (i) the demarcation of the putative limits of the *fla*/*che* promoter region as the intergenic region between the *cod* operon and *flgB* and (ii) the identification of a σ^D promoter consensus sequence, $P_{D\text{-}3}$, a previously described sequence of DNA shown to be recognized by the σ^D holoenzyme in vitro (11). However, whether this promoter was functional in vivo and the function of the gene(s) dependent on this element for transcription initiation were unknown. Primer extension data demonstrate that the P_A immediately upstream of *flgB* (*fla*/*che* P_A)

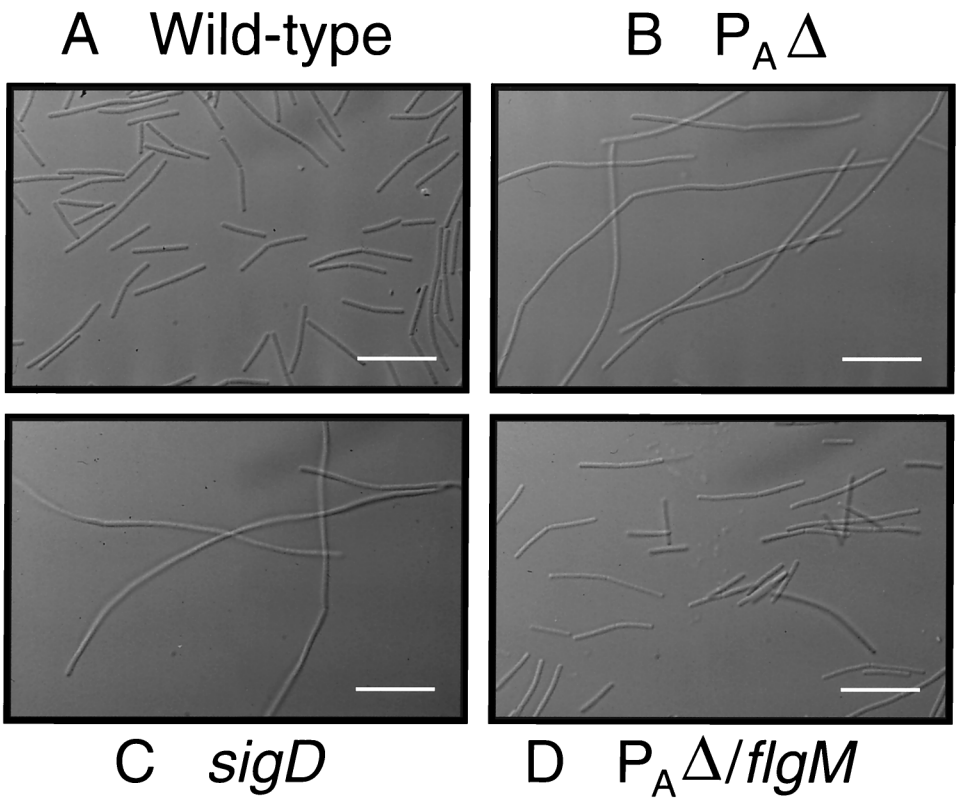

FIG. 5. Microscopic observation of wild-type and mutant strains. (A) Wild-type strain (LMB1); (B) *fla*/*che* $P_A\Delta$ mutant (LMB214); (C) *sigD* null mutant (LMB10); (D) *fla*/*che* $P_A\Delta$/*flgM* double mutant (LMB216). White scale bar = 5 μm.

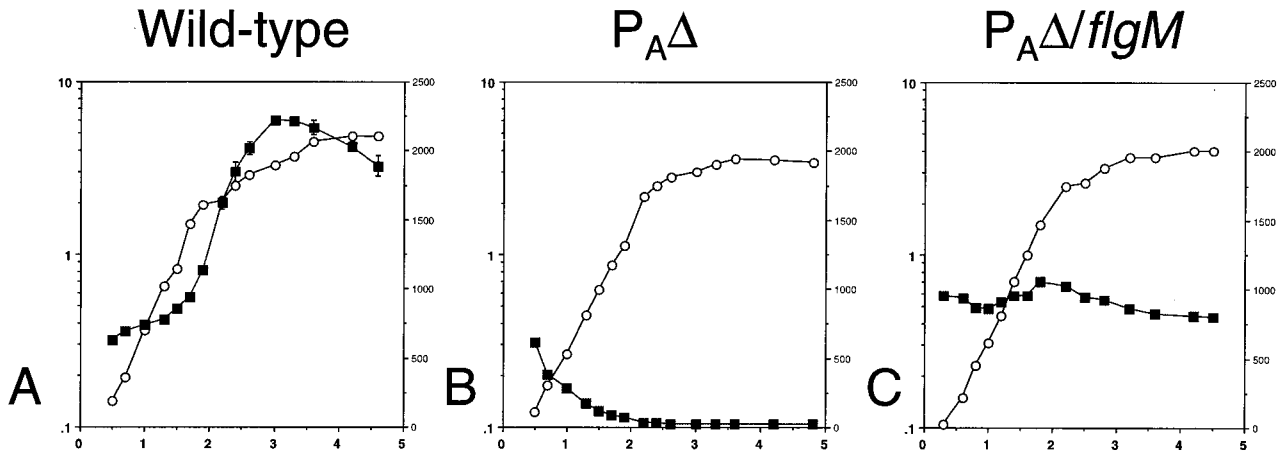

FIG. 6. *hag*::*lacZ* expression in wild-type and mutant strains. β-Galactosidase activity was monitored in strains bearing a *hag*::*lacZ* reporter construct throughout growth in sporulation medium. For each panel, the left *y* axis is absorbance at 600 nm, the right *y* axis is β-galactosidase activity as expressed in Miller units, and the *x* axis is time expressed in hours. Symbols: ○, growth; ■, β-galactosidase activity. (A) Wild-type strain (LMB219); (B) *fla*/*che* $P_A\Delta$ strain (LMB220); (C) *fla*/*che* $P_A\Delta$/*flgM* double mutant (LMB222).

and the P_{D-3} promoter are active in vivo (Fig. 2A, lanes 5 to 8, and 2B, lanes 5 to 6). Expression from the P_{D-3} element is absent in a *sigD* null mutant and is increased in a *flgM* null mutant (Fig. 2B, lanes 7 to 10), demonstrating that this promoter is σ^D dependent and is regulated by the flagellum-specific regulator, FlgM. The presence of two functional promoters upstream of a single transcription unit, recognized by different forms of the holoenzyme, is common in *B. subtilis* and allows for precise control of gene expression depending upon the availability and activation of the alternate sigma factors (25). Interestingly, the structural gene for σ^F, the enteric homolog of σ^D, has been shown to be transcribed from two overlapping promoters, one recognized by the major form of the holoenzyme and a second recognized by the σ^F holoenzyme (35). The spacing of the *fla*/*che* P_A and P_{D-3} elements, however, differs from the enteric system in that the P_{D-3} is 133 bp upstream of *fla*/*che* P_A.

The functional P_A promoter, identified above and referred to as *fla*/*che* P_A, was deleted to analyze its role in *sigD* expression and σ^D activity. In *B. subtilis* cells lacking the *fla*/*che* P_A ($P_A\Delta$), no σ^D protein was detected, in contrast to σ^D levels in wild-type or *flgM* null mutant backgrounds (Fig. 4, lanes 1 and 4). Microscopic observation of the strain bearing the $P_A\Delta$ demonstrates that this mutant grows as long filaments, which is consistent with a lack of σ^D expression or activity (Fig. 5B). However, a mutant strain containing both the $P_A\Delta$ and a null mutation in *flgM* no longer grows as long filaments. This suggests that a low level of σ^D protein is present in the $P_A\Delta$ strain (which is not detectable by immunoblot) but activity of the remaining sigma factor is inhibited by the FlgM regulator. It is likely that deletion of the *fla*/*che* P_A results in a lack of HBB gene expression, preventing the functional assembly of the HBB complex, which would allow FlgM to inhibit σ^D activity. This inference is further supported by the lack of expression of a *hag*::*lacZ* reporter construct in the $P_A\Delta$ mutant, whereas expression is partially restored in a $P_A\Delta$/*flgM* null double mutant (Fig. 6B and C).

These data demonstrate that the *sigD* structural gene, located >26 kb downstream of the *fla*/*che* P_A, is part of the *fla*/*che* operon, since the *fla*/*che* P_A is necessary for wild-type *sigD* expression. While the *fla*/*che* P_A is necessary for the production of detectable σ^D protein, expression of *sigD* may also involve another promoter(s) within the *fla*/*che* operon, since strains lacking the *fla*/*che* P_A and bearing a *flgM* null mutation have the σ^D function partially restored. In the absence of the FlgM regulator, low levels of σ^D protein produced from an internal promoter(s) could bind to RNA polymerase and initiate transcription from the P_{D-3} promoter. Expression from the P_{D-3} promoter would then result in increased expression of the *fla*/*che* transcription unit, including *sigD*. A minor σ^A-dependent promoter immediately upstream of *sigD*, P_{sigD}, has been identified recently, and deletion of this element results in a modest decrease in *fla*/*che* expression (2). Studies are in progress to determine the relative importance of P_{D-3}, *fla*/*che* P_A, and a putative promoter(s) within the *fla*/*che* operon in initiating transcription of *sigD*.

In this work, we have demonstrated that dual promoters recognized by the σ^A and σ^D holoenzymes initiate transcription of the *fla*/*che* operon, including *sigD*. In a previous study, however, it was concluded that the *fla*/*che* transcription unit (and *sigD* in particular) is not expressed from a σ^D-dependent promoter (3). This inference was based on the observation that σ^D protein levels in a *flgM* mutant are not elevated with respect to a wild-type control (3), since a lack of the FlgM regulator has been shown to increase the expression of σ^D-dependent genes 10- to 15-fold (6). We have also found the level of σ^D protein in the *flgM* mutant (Fig. 4, lane 4) to be comparable to the amount found in a wild-type strain, despite the significant increase in mRNA production from P_{D-3} found in this strain (Fig. 2B, lanes 9 and 10). Our results suggest that either (i) P_{D-3} drives transcription of *fla*/*che* including *sigD* and there is posttranscriptional regulation of σ^D expression, (ii) the P_{D-3} transcript does not extend 26 kb and does not encode σ^D protein, or (iii) P_{D-3} is a very weak promoter, in comparison to *fla*/*che* P_A, such that even increased σ^D activity cannot increase overall *fla*/*che* gene expression. We favor the first hypothesis and postulate that there is a general mechanism for posttranscriptional regulation of flagellar gene expression. As shown for *sigD* mRNA and σ^D protein in this study, it has been demonstrated that *hag* mRNA production in a *flgM* null mutant is significantly increased but flagellin protein levels in the cell are nearly identical to flagellin protein levels found in a wild-type strain (9).

Our working model for transcriptional regulation of *fla*/*che* is diagrammed in Fig. 7. Transcription of the *fla*/*che* operon, including *sigD*, is initiated from the *fla*/*che* P_A. The importance

FIG. 7. Model for *fla*/*che* expression. Transcription of the *fla*/*che* operon, including *sigD*, is initiated from the *fla*/*che* P_A. Once σ^D protein is synthesized, expression from P_{D-3} is initiated and may account for the rapid accumulation of this transcription factor. The importance of the minor P_A immediately upstream of *sigD* under normal conditions is unclear.

of P_{sigD}, the minor P_A immediately upstream of *sigD* (2), in allowing expression of σ^D protein under normal conditions is unclear. Expression of σ^D protein and its association with RNA polymerase allows for transcription from P_{D-3}, which may account for the rapid accumulation of σ^D protein during logarithmic growth (29a) and the rapid increase in σ^D activity found during this period of growth (10, 30). In fact, several alternate sigma factors are encoded in transcription units preceded by a cognate promoter sequence, allowing for a rapid accumulation of the factor (16–18). The dependence of wild-type *fla*/*che* expression on the P_{D-3} element would also allow for a sensitive monitor of HBB assembly, since σ^D activity is intimately tied to FlgM regulation (32).

Although the flagellar regulons in both *B. subtilis* and the enteric bacteria are highly homologous (38), the physical organizations of the genes that comprise the HBB complex and the alternate sigma factors are very different. The genes encoding the HBB and σ^D in *B. subtilis* are found within the *fla*/*che* operon (38). In contrast, the genes encoding the HBB in the enteric bacteria are found in 13 operons located throughout the bacterial chromosome (21, 22) and the gene for the σ^D homolog, σ^F, is found in yet another transcription unit (27). Our studies demonstrate that the precise expression of *fla*/*che* operon gene products is dependent on at least two forms of the holoenzyme and morphogenetic control by FlgM. Moreover, our data suggests that posttranscriptional regulation of the operon occurs.

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