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Relative Roles of the *fla/che* P_A, P_{D-3}, and P_{sigD} Promoters in Regulating Motility and *sigD* Expression in *Bacillus subtilis*

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Three promoters have been identified as having potentially important regulatory roles in governing expression of the *fla/che* operon and of *sigD*, a gene that lies near the 3′ end of the operon. Two of these promoters, *fla/che* P_A and P_{D-3}, lie upstream of the >26-kb *fla/che* operon. The third promoter, P_{sigD}, lies within the operon, immediately upstream of *sigD*. *fla/che* P_A, transcribed by $E\sigma^A$, lies ≥24 kb upstream of *sigD* and appears to be largely responsible for *sigD* expression. P_{D-3}, transcribed by $E\sigma^D$, has been proposed to participate in an autoregulatory positive feedback loop. P_{sigD}, a minor σ^A-dependent promoter, has been implicated as essential for normal expression of the *fla/che* operon. We tested the proposed functions of these promoters in experiments that utilized strains that bear chromosomal deletions of *fla/che* P_A, P_{D-3}, or P_{sigD}. Our analysis of these strains indicates that *fla/che* P_A is absolutely essential for motility, that P_{D-3} does not function in positive feedback regulation of *sigD* expression, and that P_{sigD} is not essential for normal *fla/che* expression. Further, our results suggest that an additional promoter(s) contributes to *sigD* expression.

Motility and chemotaxis functions in *Bacillus subtilis* are encoded within the *fla/che* operon. This large (>26-kb) operon includes both structural and regulatory components required for motility (6, 19, 32). The proximal region of the operon includes genes that encode the hook and basal body (HBB) complex, a structure that is required for tethering the flagellar filament to the cell. The distal-most region of the *fla/che* operon encodes the flagellum-specific sigma factor, σ^D (19). σ^D activity is required for transcription of the genes encoding flagellin (*hag*) and for the *motA* and *motB* genes, which encode the motor proteins that drive flagellar rotation (21, 22). In addition, σ^D is also partially responsible for expression of the anti-sigma factor, FlgM (14, 18). FlgM antagonizes σ^D activity in vivo (3, 8) and binds directly to σ^D in vitro (2). FlgM may inhibit σ^D activity either by binding to σ^D protein in a manner that precludes it from forming a stable complex with core RNA polymerase or by binding to the σ^D-holoenzyme and interfering with its function (4). In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, it has been demonstrated that FlgM is exported through the HBB to allow activation of the σ^D homolog (11, 15). It is believed that FlgM activity is also controlled by export through the HBB in *B. subtilis* (5, 21, 24). Expression of the *fla/che* operon thus controls motility in a complex manner. First, HBB components are expressed concurrently with σ^D. Subsequent assembly of the HBB structure allows export of FlgM. This activates σ^D to promote transcription of σ^D-dependent motility genes.

Recent studies have implicated three promoters in the expression of the *fla/che* operon and the *sigD* gene (1, 6) (see schematic, Fig. 1A). One of these promoters, P_{sigD}, is located within the *fla/che* operon, immediately upstream of the *sigD* gene. Transcription from P_{sigD} is dependent upon σ^A, the major, vegetative sigma factor in *B. subtilis*. Previous studies indicated that P_{sigD} contributed only weakly to overall expression of the *sigD* gene (1). However, genetic data suggested that this slight level of expression might be required to control temporal regulation of the entire *fla/che* operon (1). This requirement would presumably be indirect, since the location of P_{sigD} precludes it from directly promoting transcription of the *fla/che* operon (see schematic in Fig. 1A).

Two additional promoters, *fla/che* P_A and P_{D-3}, have been identified. These promoters lie upstream of the entire *fla/che* operon (Fig. 1A). Deletion of *fla/che* P_A, a σ^A-dependent promoter eliminates motility (6). Moreover, the *fla/che* $P_A\Delta$ strain exhibits a dramatic reduction in σ^D protein levels. Additionally, σ^D-dependent gene expression is abolished (6). These phenotypes apparently occur, in part, because the loss of *fla/che* P_A-dependent transcription of the operon gives rise to impaired export of FlgM. When *flgM* is deleted concurrently with the *fla/che* P_A, expression of a σ^D-dependent reporter fusion is restored (6). P_{D-3}, the other promoter upstream of the operon, is thought to be involved in this restoration of σ^D-dependent motility gene expression (6). P_{D-3}, located approximately 130 bp upstream of *fla/che* P_A, is a σ^D-dependent promoter. Its activity is induced, by as much as 10-fold, in the absence of FlgM (6). Based on these data, it has been suggested that inactivation of FlgM triggers an autoregulatory positive feedback loop at P_{D-3}. Specifically, activation of transcription from P_{D-3} has been proposed to increase *sigD* gene expression, resulting in an accumulation of σ^D protein, which gives rise to increased expression from σ^D-dependent promoters (including P_{D-3}).

We sought to clarify the roles of each of the three promoters in regulating expression of the *fla/che* operon and the *sigD* gene. Our approach was to construct strains that carry chromosomal deletions of *fla/che* P_A, P_{D-3}, and P_{sigD}, either singly or in combination. Moreover, we generated strains that carry insertional disruptions of *flgM* in addition to the promoter deletion(s). Our results indicate that the *fla/che* P_A is the major promoter responsible for *fla/che* expression and that *fla/che* P_A is essential for motility. In our experiments P_{D-3} did not exhibit autoregulatory positive feedback control over *sigD* gene expression. Moreover, P_{D-3}-mediated transcriptional activation of the *fla/che* operon is insufficient to promote motility. Fur-

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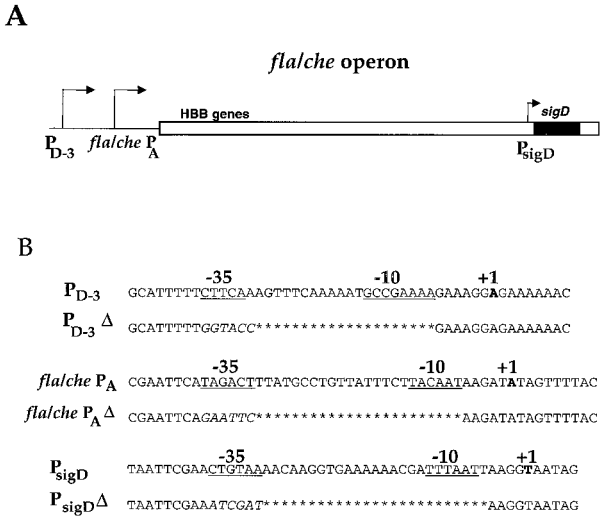

FIG. 1. (A) Schematic of the *fla/che* operon. The intergenic region upstream of the operon is indicated by the black line. The white box indicates *fla/che* operon sequences; the *sigD* gene, at the distal end of the operon, is indicated by a black bar. The location of the HBB genes, in the proximal portion of the operon, is indicated. The relative positions of each promoter are indicated by arrows. The identity of the promoter is indicated, in boldface, below the arrows. *fla/che* P_A and P_{sigD} are both σ^A-dependent promoters. P_{D-3} is a σ^D-dependent promoter. It has been suggested (6) that initial expression of the *fla/che* operon involves transcription from *fla/che* P_A. Such expression would allow HBB assembly and export of FlgM, yielding active σ^D. Thus, $E\sigma^D$ could then initiate transcription from P_{D-3} to upregulate *sigD* expression, contributing to peak levels of σ^D activity observed at the end of exponential growth. P_{sigD} has been implicated as having a regulatory role in *fla/che* expression (1). (B) Sequences of the three promoters are indicated. The −35 and −10 regions are underlined. The +1 position is indicated in boldface. For the promoter deletions, sequences which have been deleted are indicated by asterisks. Sequences that have been replaced by restriction sites are indicated in italics.

ther, we found that P_{sigD} is not essential for normal expression of the *fla/che* operon or for σ^D-dependent motility functions. Finally, our results suggest that there is an additional promoter(s) that mediates *sigD* expression.

MATERIALS AND METHODS

Construction of strains. Strains of *B. subtilis* are listed in Table 1. Strains LMB214 and LMB216 have been described previously (6). However, because the original strains were lost, they were reconstructed for this study. The procedure for reconstructing the strains was identical to the one used in the original construction (6) and is essentially the same as the procedure described below for the construction of LMB226 and LMB228. For LMB214 and LMB216, however, the integrational plasmid pWE4-int, which contains the *fla/che* P_A deletion and a wild-type version of P_{D-3}, was utilized. PCR primers OWE7 and OWE8B were utilized, as described below, to identify *fla/che* P_A deletion strains.

Strains bearing deletions of both *fla/che* P_A and P_{D-3} (LMB226 and LMB228) were constructed by integration and subsequent curing of the plasmid pSS7-1. Beginning with plasmid pWE4 (6), which contained the *fla/che* P_A deletion, oligonucleotide-mediated site-directed mutagenesis was employed to delete P_{D-3}, using primer OWE5B (5′-GTATAATTTAATAAATTTTGCATTTTT GGTACCGAAAGGAGAAAAACAGAATTCTGC-3′). This results in replacement of P_{D-3} with a *Kpn*I site (see Fig. 1B). The resulting double-deletion sequences were subcloned as a *Pst*I fragment into pJM102 (26) to yield pSS7-1. The procedures utilized were essentially identical to those described elsewhere (6). pSS7-1 was digested and concatemerized and then transformed into LMB1 according to standard procedures (23); finally, it was plated onto Luria-Bertani medium (LB) plus 7 μg of chloramphenicol per ml to select for transformants. Double-crossover integrants were identified by PCR. For detection of the *fla/che* P_A deletion, primers OWE7 (5′-GTGAGGACATTTTTTTACACTG-3′) and OWE8B (5′-CCCTCAATATCCTTGTCGAG-3′) were used. The deletion yields a product of 75 bp, while the wild-type product is approximately 100 bp. The P_{D-3} deletion was detected with primers OSS4 (5′-GCAGAATTTCTGTT TTTTCTCC-3′) and OSS5 (5′-CCTGGGTTGAAAGTCTTTCTATG-3′). The deletion and wild-type products also differ by approximately 25 bp. The plasmid sequences were "cured" from the strain by growing them without selection for several passages. Chloramphenicol-sensitive candidates were identified by replica patching onto selective and nonselective plates. Chloramphenicol-sensitive isolates were screened by PCR to identify cured deletion strains. To generate LMB228 (*flgM*::mini-Tn*10*, Spc^r) LMB226 was transformed with chromosomal DNA (100 ng) from strain LMB213 (6) according to standard protocols (23). Spc^r candidates were rescreened by PCR, with the OWE7-OWE8B and OSS4-OSS5 primer pairs, to ensure that they retained the promoter deletions. Strains were also microscopically examined for σ^D-dependent autolysin activity to confirm the presence of the *flgM* mutation.

$P_{sigD}\Delta$ strains were generated by integration and subsequent curing of plasmid pJW14. The *Sph*I-*Eco*RI fragment of pLM112 (9) that contains P_{sigD} was subcloned into pGEM7zf+ (Promega) and then subjected to oligonucleotide-mediated site-directed mutagenesis using primer OJW7 (5′-CCCGACGTCGCATG CTGCATATTCGAATCGATTAAGGTATTAGGGGGATACATGC-3′). This results in the replacement of P_{sigD} with a *Cla*I restriction site (Fig. 1B). This deletion fragment was subcloned, replacing the wild-type version of the *Sph*I-*Eco*RI portion of pLM112 to yield the plasmid pJW9. pJW9 includes an approximately 900-bp fragment from the *Sal*I site upstream of the *sigD* gene to the *Eco*RI site within *sigD*; the vector sequences are derived from the integrational plasmid pJM102. Because this plasmid could not be efficiently cured out of integrant strains, an additional 500-bp *Hin*dIII-*Sal*I fragment from pJH6-2 (9) was subcloned into pJW9, resulting in plasmid pJW14. pJW14 was integrated as a double crossover into LMB1, LMB226, and LMB228 as described above. Double crossovers were identified by PCR using primers 5′sigdel (5′-GCATGC TGCATATTCG-3′) and OJWS3′ (5′-CAGCGCGTCCAAAGCA-3′). The deletion yields a product of 73 bp, whereas the wild-type product is 98 bp. Plasmid sequences were cured as described above. For all deletion strains, DNA surrounding the deletion was amplified by PCR and sequenced to ensure that no extraneous mutations had occurred.

For construction of *hag-lacZ* reporter strains, a lysate from *B. subtilis* HB4187

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

Strain	Genotype	Source or derivation (reference)[a]
LMB1	*trpC2*	E. Ferrari, I168[b]
LMB10	*trpC2 sigD*::pLM5 (Cm^r)	M. J. Chamberlin, CB100[b] (6)
LMB214	*trpC2 fla/che* $P_A\Delta$	Transform [LMB1: pWE4-int, Cm^r][c] (6; this study)
LMB216	*trpC2 fla/che* $P_A\Delta$ *flgM*::mini-Tn*10* (Sp^r)	Transform [LMB214: LMB213 (6), Sp^r] (6; this study)
LMB226	*trpC2 fla/che* $P_A\Delta$ $P_{D-3}\Delta$	Transform [LMB1: pSS7-1, Cm^r][c] (this study)
LMB228	*trpC2 fla/che* $P_A\Delta$ $P_{D-3}\Delta$ *flgM*::mini-Tn*10* (Sp^r)	Transform [LMB226: LMB213 (6), Sp^r] (this study)
LMB232	*trpC2* $P_{sigD}\Delta$	Transform [LMB1: pJW14, Cm^r][c] (this study)
LMB233	*trpC2 fla/che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$	Transform [LMB226: pJW14, Cm^r][c] (this study)
LMB234	*trpC2 fla/che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$ *flgM*::mini-Tn*10* (Sp^r)	Transform [LMB228: pJW14, Cm^r][c] (this study)
LMB241	*trpC2 sigD*::pLM5(Cm^r) *flgM*::mini-Tn*10* (Sp^r)	Transform [LMB10: LMB213 (6)] (this study)
LMB243	*trpC2* $P_{sigD}\Delta$ SPβc2Δ2ϕ[*hag-cat-lacZ*], Neo^r	Transduce [LMB232: HB4187 (J. Helmann), Neo^r][d] (this study)
LMB244	*trpC2* SPβc2Δ2ϕ[*hag-cat-lacZ*], Neo^r	Transduce [LMB1: HB4187 (J. Helmann), Neo^r][d] (this study)
LMB247	*trpC2 sigD*::pLM5(Cm^r) SPβc2Δ2ϕ[*hag-cat-lacZ*], Neo^r	Transduce [LMB10: HB4187 (J. Helmann), Neo^r][d] (this study)

[a] Transformation of [recipient strain: with chromosomal DNA from this strain, selecting for this resistance].
[b] Previous name of strain.
[c] This strain was subsequently cured of the chloramphenicol resistance marker.
[d] Transduction of [recipient strain: with transducing lysate from this strain, selecting for this resistance].

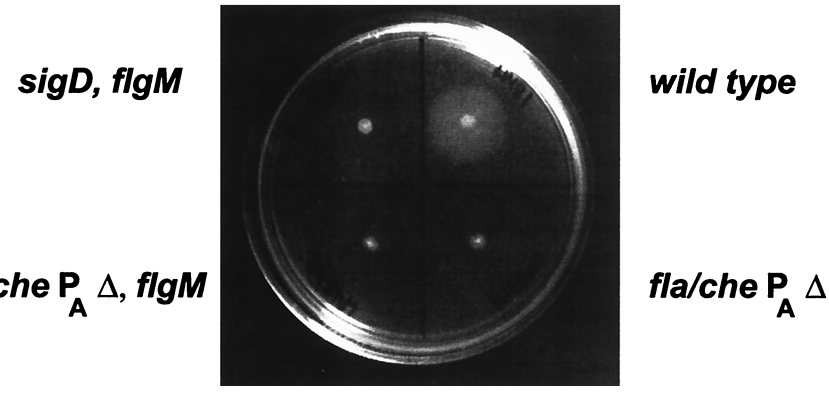

FIG. 2. Swarm assay of LMB216. Cells of the indicated genotype were inoculated onto PAB–0.4% agar plates and grown at 37°C overnight. LMB1, the wild-type strain, has swarmed from the point of inoculation, which indicates that this strain is motile. LMB241 (*sigD*::pLM5 *flgM*::mini-Tn*10*), the negative control strain, exhibits growth from the position of inoculation, but does not swarm. LMB214 (*fla/che* $P_A\Delta$) and LMB216 (*fla/che* $P_A\Delta$ *flgM*::mini-Tn*10*) also do not swarm, indicating that they are nonmotile. This indicates that in LMB216, activation of σ^D by the removal of FlgM does not restore motility.

(SPβc2Δ2ϕ[*hag-cat-lacZ*], Neo^r) was generated. HB4187 was grown overnight on an LB-neomycin plate; a colony was then inoculated into Difco Antibiotic Medium No. 3 (i.e., Penassay broth) and grown to light turbidity at 37°C with aeration. To generate SPβ phage lysate, the culture was transferred to 50°C and incubated for 90 min with aeration. Cellular debris was removed by centrifugation at 8,000 rpm in an SS34 rotor for 10 min. After transfer to a fresh tube, a drop of chloroform was added to the lysate, which was stored at 4°C. Strains LMB243 and LMB244 were generated by transducing strains LMB232 and LMB1, respectively, with this lysate. LMB1 and LMB232 were grown to mid-log phase in Difco Antibiotic Medium No. 3 (Penassay broth [PAB]) at 37°C with aeration. After addition of an equal volume of lysate, this incubation was continued for an additional 20 min. Cells were collected by centrifugation and then washed with 5 ml of 1× SC (0.15 M NaCl, 0.01 M sodium citrate; pH 7.0). Cells were then plated onto LB-neomycin to select for transductants that carried the *hag-lacZ* reporter.

RNA isolation and primer extension. Cells were grown in complex sporulation medium (2× SG) at 37°C with aeration. At T_0 and $T_{0.5}$, 25 to 50 ml of culture was transferred to Falcon conical centrifuge tubes, pelleted, and snap frozen in dry ice-ethanol. Cells were lysed by 3-min incubation in disruption buffer (30 mM Tris, pH 8; 50 mM EDTA; 100 mM NaCl; 1 mg of lysozyme per ml). This was followed by incubation with 50 U of RQ1 DNase (Promega) and 0.5 mg of proteinase K per ml. Samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1) until the interface cleared and then precipitated with ethanol and 0.3 M sodium acetate. RNA samples were examined by formaldehyde agarose gel electrophoresis, performed according to standard procedures (28), to ensure that they were intact.

Primer extensions were conducted essentially as described previously (23). For detection of *fla/che* P_A and P_{D-3} transcripts, primer OWE3 (5′-AATATCCGC TCGTCTCAAGGCAT-3′) was used, yielding extension products of 123 and 259 bases, respectively. For detection of P_{sigD} transcripts, primer OJSPE2 (5′-GCA CTGATTTCGGCAGTCCGACAG-3′) was used, yielding an extension product of 164 bases. For control *rpsB* reactions, primer RPSBE (5′-GTGACCGAAGT GAACAGG-3′) was used, also yielding an extension product of 164 bases. The following modifications were made to the protocol: unincorporated label was removed from primers using a NICK-Column (Pharmacia); prior to the annealing reaction, 0.6 pmol of labeled primer was ethanol precipitated with 50 μg of sample RNA and then centrifuged for ≥15 min in a microcentrifuge at 4°C; and air-dried pellets were resuspended in 8 μl of 1 mM vanadyl-ribonucleoside complex (VRC) for annealing. Primer extension products were resolved alongside sequencing reactions of pWE1 (6) for *fla/che* P_A and P_{D-3} and of pLM112 (9) for P_{sigD}.

Autoradiography was carried out a −80°C with intensifying screens for 5 to 15 days. Exposed X-ray films were scanned with a UMAX scanner into Adobe Photoshop 4.0.

Western blots. Cells were grown in 2× SG to $t_{0.5}$. Then, 10 to 20 ml of culture was collected by centrifugation and washed in ice-cold STE (150 mM NaCl, 10 mM Tris-Cl, 100 mM EDTA). Cells were lysed by sonication as described previously (18), and cellular debris was removed from the protein extracts by centrifugation. For detection of σ^D, 50 μg of protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel and then transferred to nitrocellulose. The filter was stained with Ponceau-S (Sigma) to ensure equal loading and transfer of protein samples across all lanes. The filter was blocked with TBST (10 mM Tris, pH 7.5; 150 mM NaCl; 0.1% Triton X-100) plus 5% powdered skim milk for 2 h at room temperature, incubated with 1:1,000 anti-σ^D polyclonal antibody 2855 (9) for 2 h at room temperature, washed four times for 10 min each time in TBST, incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:2,000 in TBST for 1 h, and washed four times for 10 min each time in TBST. σ^D protein was visualized using BCIP (5-bromo-4-chloro-3-indolylphosphate)–nitroblue tetrazolium substrate (Sigma) in distilled, deionized water.

For detection of flagellin, the procedure was as described above except that 10 μg of protein was resolved on a 10% PAGE mini-gel. The anti-flagellin polyclonal antibody (25) was used at a 1:2,000 dilution. In addition, an anti-σ^A antibody (9) was used simultaneously with the flagellin antibody at a 1:2,000 dilution. The result obtained with these two antibodies used together was as expected from pilot experiments in which the flagellin and σ^A antibodies were used individually (i.e., there was no extraneous cross-reactivity observed when the antibodies were used simultaneously).

Swarm assay. A swarm assay was conducted according to the method of Fein and Rogers (7). Cells were patched from a fresh overnight LB plate to a swarm assay plate (Difco Antibiotic Medium No. 3 plus 0.4% Bacto-agar) and incubated in a humidified chamber at 37°C overnight.

β-Galactosidase assay. To assay expression from the *hag-lacZ* transcriptional fusion, cells were grown in 2× SG medium at 37°C with aeration. Samples were removed every 18 min and stored on ice. β-Galactosidase assays were performed essentially as described previously (6). Assays were performed in duplicate or triplicate samples, and values were averaged.

RESULTS

***fla/che* expression initiated from P_{D-3} is insufficient to promote motility and does not appear to function in positive-feedback regulation of *sigD* expression.** Previous work demonstrated that σ^D-dependent gene expression and motility were abolished in a *fla/che* $P_A\Delta$ mutant strain (6). Concurrent deletion of *fla/che* P_A and *flgM* resulted in restoration of the subset of σ^D-dependent activities that were examined (6), but the question of whether disruption of *flgM* also restores motility in a *fla/che* $P_A\Delta$ strain background was not addressed. However, the proposed model (6) predicts that motility would be exhibited by the *fla/che* $P_A\Delta$ *flgM* mutant strain; basal levels of σ^D would be activated in the absence of FlgM activity, resulting in expression of the *fla/che* operon from P_{D-3}. Consequently, the HBB genes would be expressed concurrently with the σ^D-dependent motility genes, allowing assembly of functional flagella.

To test this prediction, we examined the *fla/che* $P_A\Delta$, *flgM*::mini-Tn*10* strain (LMB216) for motility. Figure 2 shows the results of a swarm plate assay. In this assay, cells were inoculated onto semisolid agar plates (7); cells that were motile swarm outward from the point of inoculation, making a halo of growth, whereas nonmotile cells were restricted to the point of inoculation. The *fla/che* $P_A\Delta$ *flgM*::mini-Tn*10* strain (LMB216) fails to swarm, indicating that it is not motile. Examination of live cells by light microscopy confirmed that LMB216 is nonmotile (data not shown).

In order to gain insight into this surprising result, we exam-

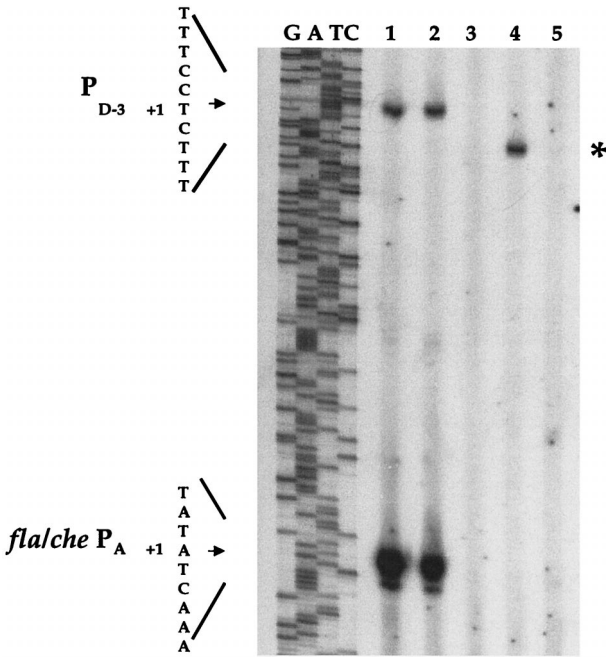

FIG. 3. Primer extension analysis of *fla*/*che* P_A and P_{D-3}. A 50 μg portion of total RNA was isolated at T_0 and subjected to primer extension analysis using primer OWE-3. In parallel, OWE-3 was utilized in a dideoxy sequencing reaction on plasmid pWE-1, which includes the *fla*/*che* promoter region and *flgB*. Lanes of the sequencing reaction are indicated as G, A, T, and C. Lane 1, LMB1 (wild type); lane 2, LMB232 ($P_{sigD}\Delta$); lane 3, LMB214 (*fla*/*che* $P_A\Delta$); lane 4, LMB216 (*fla*/*che* $P_A\Delta$ *flgM*::mini-Tn*10*); lane 5, LMB234 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$ *flgM*::mini-Tn*10*). The +1 position for P_{D-3} and *fla*/*che* P_A is indicated to the left. The sequence indicated corresponds to the template strand (i.e., is complementary to the sequence indicated in Fig. 1B). The asterisk indicates the position of the truncated P_{D-3}-specific primer extension product in LMB216 that results from internal deletion of sequences corresponding to the *fla*/*che* P_A. The result in lane 1 indicates that expression from *fla*/*che* P_A greatly exceeds expression from P_{D-3} in the wild-type strain at the end of logarithmic growth. This is the time of maximal σ^D activity (25), suggesting that activation of P_{D-3} is not serving a positive autoregulatory role in the induction of σ^D activity. Lane 3 shows that in the *fla*/*che* $P_A\Delta$ strain, primer extension products corresponding to both the *fla*/*che* P_A and the P_{D-3} transcripts are absent, indicating that expression from *fla*/*che* P_A is required for σ^D-dependent transcription at P_{D-3}. Lane 4 shows that disruption of *flgM*, in a *fla*/*che* P_A background, results in activation of P_{D-3}. However, Fig. 1 demonstrates that this expression of the *fla*/*che* operon from P_{D-3} is insufficient to promote motility. Lane 2 shows that a $P_{sigD}\Delta$ strain resembles the wild type with regard to expression of the *fla*/*che* operon. Lane 5 shows the absence of *fla*/*che* P_A- and P_{D-3}-specific primer extension products in a *fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$ *flgM*::mini-Tn*10* strain (LMB234). In control primer extension reactions utilizing a primer specific for the ribosomal *rpsB* gene, the LMB216 (lane 3), LMB234 (lane 5), and wild-type (lane 1) samples yielded comparable results (data not shown), indicating that the lack of signal observed in lanes 3 and 5 is a direct result of the promoter deletions.

ined RNA from the LMB216 strain for evidence of transcription from P_{D-3}. Figure 3 (lane 4, asterisk) shows the presence of a primer extension product that corresponds to the P_{D-3}-specific transcript. This P_{D-3}-specific product is shorter than the P_{D-3}-specific product of the wild-type strain (lane 1); the shortened length of the P_{D-3}-specific product in LMB216 results from the deletion of *fla*/*che* P_A sequences, which lie downstream of P_{D-3} (Fig. 1). The level of P_{D-3} product appears to be similar for LMB216 and the wild-type strain. However, overall *fla*/*che* transcription is greatly reduced in LMB216 compared to the wild type since LMB216 lacks the *fla*/*che* P_A-specific product, which comprises the vast majority of *fla*/*che* primer extension products in the wild-type sample (Fig. 3, lane 1). The primer extension results indicate that P_{D-3}-mediated transcription of the *fla*/*che* operon is induced in LMB216. However, this expression of *fla*/*che* genes is reduced relative to the wild type.

Figure 4A (lane 5) indicates that σ^D is present in the *fla*/*che* $P_A\Delta$ *flgM*::mini-Tn*10* strain (LMB216). However, σ^D expression is reduced in LMB216 relative to σ^D expression in the wild-type strain (Fig. 4A, lane 1). Finally, LMB216 appears to express σ^D-dependent motility genes. Figure 4B (lane 5) indicates that flagellin protein is expressed in LMB216, albeit at reduced levels compared to the wild type (lane 1). Altogether, our analysis of LMB216 indicates that the *fla*/*che* operon is expressed in the *fla*/*che* $P_A\Delta$ *flgM*::mini-Tn*10* mutant back-

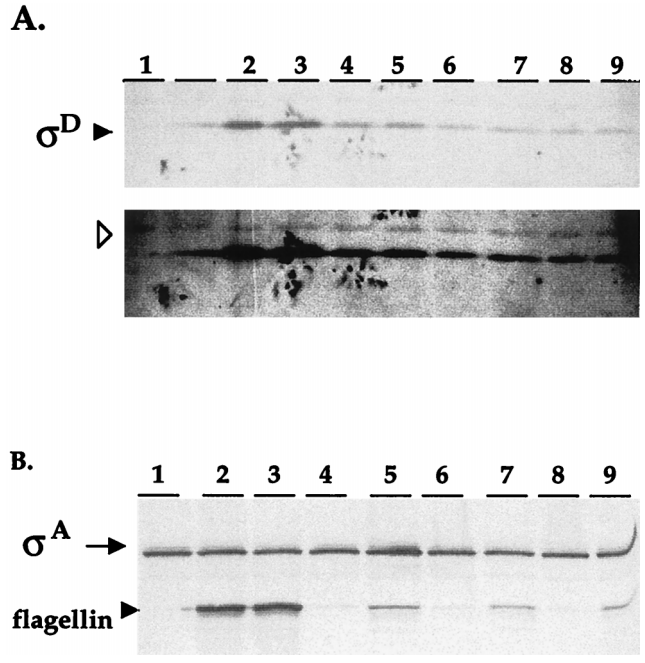

FIG. 4. (A) Western blot analysis of σ^D. (Upper panel) A total of 50 μg of protein, isolated from cultures at $T_{0.5}$, was fractionated by SDS-PAGE and probed with antibodies raised against σ^D. The blot was stained with Ponceau-S, prior to antibody incubation, to ensure equivalent loading across lanes. Lane 1, LMB10 (*sigD*::pLM5); lane 2, LMB1 (wild type); lane 3, LMB232 ($P_{sigD}\Delta$); lane 4, LMB214 (*fla*/*che* $P_A\Delta$); lane 5, LMB216 (*fla*/*che* $P_A\Delta$ *flgM*::mini-Tn*10*); lane 6, LMB226 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$); lane 7, LMB228 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ *flgM*::mini-Tn*10*); lane 8, LMB233 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$); lane 9, LMB234 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$ *flgM*::mini-Tn*10*). (Lower panel) Overexposing the same blot allows visualization of a cross-reacting band (arrowhead), which indicates uniform loading of samples. The lane adjacent to lane 1 also contains protein extract from the *sigD* null strain; spillover from lane 2 is evident in this lane. Comparison of lanes 1 and 3 shows that deletion of *fla*/*che* P_A results in a reduction in the σ^D protein level. Comparison of lanes 3 and 4 indicates that disruption of *flgM*, in a *fla*/*che* $P_A\Delta$ background, does not result in increased σ^D levels, even though transcription from P_{D-3} is activated in this strain background. Lanes 8 and 9 show that σ^D is detected in strains that lack *fla*/*che* P_A, P_{D-3}, and P_{sigD}, indicating that an additional promoter(s) contributes to *sigD* gene expression. A comparison of lanes 8 and 9 with lanes 6 and 7 reveals that σ^D detected in strains that lack all three known promoters is comparable to σ^D detected in isogenic strains that lack *fla*/*che* P_A and P_{D-3} but retain P_{sigD}; this indicates that P_{sigD} activity does not contribute significantly to the σ^D that is observed. Lane 2 indicates that σ^D is expressed at apparently wild-type levels in a strain that lacks P_{sigD} but retains the other promoters. (B) Western blot analysis of flagellin protein. A total of 10 μg of protein, isolated from cultures at $T_{0.5}$, was fractionated by SDS-PAGE and probed with antibodies specific for flagellin and for σ^A as a loading control. Lane 1, LMB10 (*sigD*::pLM5, null mutant); lane 2, LMB1 (wild type); lane 3, LMB232 ($P_{sigD}\Delta$); lane 4, LMB214 (*fla*/*che* $P_A\Delta$); lane 5, LMB216 (*fla*/*che* $P_A\Delta$ *flgM*::mini-Tn*10*); lane 6, LMB226 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$); lane 7, LMB228 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ *flgM*::mini-Tn*10*); lane 8, LMB233 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$); lane 9, LMB234 (*fla*/*che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$ *flgM*::mini-Tn*10*). In a *fla*/*che* $P_A\Delta$ strain, flagellin expression is diminished (lane 3). However, disruption of *flgM* in the *fla*/*che* $P_A\Delta$ strain background results in observable flagellin expression (lane 4) because σ^D-dependent gene expression is activated. This expression of flagellin remains reduced relative to wild-type expression (compare lanes 4 and 1). Lane 2 shows that a $P_{sigD}\Delta$ strain exhibits essentially wild-type expression of flagellin protein, indicating that P_{sigD} is not required for normal expression of this motility gene.

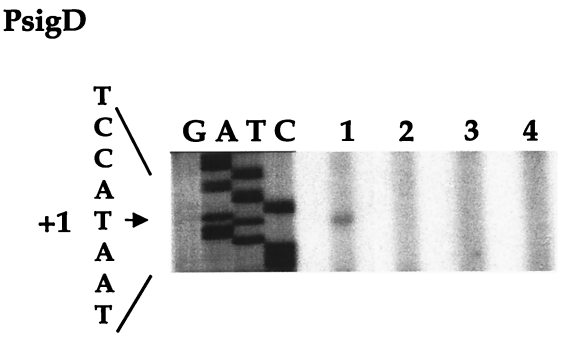

FIG. 5. Primer extension analysis of P_{sigD}. A total of 50 μg of total RNA was isolated at T_0 and subjected to primer extension analysis using primer OSPE2. In parallel, OSPE2 was utilized in a dideoxy sequencing reaction on plasmid pLM112, which contains the P_{sigD} promoter region and the 5′ half of the *sigD* gene. Lanes of the sequencing reaction are indicated as G, A, T, and C. Lane 1, LMB1 (wild type); lane 2, LMB232 ($P_{sigD}\Delta$); lane 3, LMB233 (*fla/che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$); lane 4, LMB234 (*fla/che* $P_A\Delta$ $P_{D-3}\Delta$ $P_{sigD}\Delta$ *flgM*::mini-Tn*10*). The +1 position for P_{sigD} is indicated to the left. The sequence indicated corresponds to the template strand (i.e., it is complementary to the sequence indicated in Fig. 1). Lane 1 shows the primer extension product corresponding to the weakly expressed transcript that initiates at P_{sigD}. Lanes 2 to 4 show that this product is absent in $P_{sigD}\Delta$ strains.

ground at reduced levels relative to wild-type expression. σ^D and σ^D-dependent motility genes are also expressed at reduced levels, and the strain is nonmotile. This indicates that *fla/che* expression mediated by P_{D-3} is insufficient to support motility.

In addition, our data enable us to make inferences about the role of P_{D-3} in the autoregulatory control of *sigD* expression. The primer extension data indicate that transcription from P_{D-3} is essentially abolished in LMB214, the *fla/che* $P_A\Delta$ strain (Fig. 3, lane 3). The *fla/che* $P_A\Delta$ *flgM* strain (LMB216) exhibits a severalfold induction of P_{D-3} transcript relative to LMB214 (Fig. 3, lane 4). However, there is no commensurate increase in σ^D levels in LMB216 relative to LMB214 (Fig. 4A, compare lanes 4 and 5). This indicates that the P_{D-3} transcript does not contribute significantly to σ^D protein levels, suggesting that P_{D-3} is not part of an autoregulatory feedback mechanism for inducing *sigD* expression.

Simultaneous deletion of *fla/che* P_A, P_{D-3}, and P_{sigD} suggests that other promoters contribute to expression of *sigD*. The *fla/che* $P_A\Delta$ strain (LMB214) exhibits expression of σ^D in the absence of transcription from *fla/che* P_A and P_{D-3} (Fig. 3, lane 3; Fig. 4A, lane 3). We sought to determine whether P_{sigD} could be responsible for this σ^D expression. We constructed strain LMB233, which bears chromosomal deletions of *fla/che* P_A, P_{D-3}, and P_{sigD}. In addition, we constructed LMB234, which is isogenic to LMB233 but which also bears a disruption of the *flgM* gene. We confirmed that LMB233 and LMB234 lack the P_{sigD}-specific product primer extension product that is evident for the wild-type strain (Fig. 5). Additionally, Fig. 3 (lane 5) shows that primer extension products specific to the *fla/che* P_A and P_{D-3} transcripts are absent in LMB234, a finding consistent with the chromosomal deletion of those promoters in this strain; a control primer extension utilizing a primer for the ribosomal *rpsB* gene yielded comparable results for LMB234 and the wild-type strain, indicating that the lack of primer extension products for *fla/che* P_A, P_{D-3}, and P_{sigD} results directly from deletion of these promoters in LMB234.

Surprisingly, we were able to detect σ^D in protein extracts of LMB233 and LMB234 (Fig. 4, lanes 8 and 9). This indicates that an additional promoter(s) must contribute to the expression of *sigD*. Moreover, Fig. 4A indicates that strains LMB233 and LMB234 exhibit σ^D levels comparable to those exhibited by strains LMB226 and LMB228. These strains are isogenic to LMB233 and LMB234, respectively, but retain a wild-type copy of P_{sigD}. Two inferences can be drawn from the comparison of these strains. First, P_{sigD} does not contribute significantly to the pool of σ^D protein that is expressed in the absence of *fla/che* P_A and P_{D-3} activity. Second, an additional promoter(s) appears to account for the bulk of the σ^D expression in the *fla/che* $P_A\Delta$ $P_{D-3}\Delta$ strain background.

P_{sigD} is not essential for σ^D expression or for motility functions. It has been suggested that P_{sigD} is required for normal expression of the *fla/che* operon (1). We explored this possibility by constructing strain LMB232, which carries a chromosomal deletion of P_{sigD}. We confirmed that the primer extension product specific to P_{sigD} is absent in this strain (Fig. 5, lane 4). Primer extension products specific to the *fla/che* P_A and P_{D-3} transcripts, however, are detected at levels comparable to those found in the wild-type strain (Fig. 4, lane 2). This suggests that expression of the *fla/che* operon is normal in the absence of P_{sigD}.

We next examined σ^D protein levels and σ^D activity. On a Western blot, LMB232 exhibits σ^D protein at a level roughly comparable to that found in the wild-type strain (Fig. 3B, lane 3). σ^D activity was assessed in two ways. First, we examined flagellin levels in protein lysates isolated from LMB232 at $T_{0.5}$ and found that flagellin expression is comparable to expression in the wild-type strain (Fig. 3A, lanes 2 and 3). Second, to assess σ^D activity throughout growth, we examined the expression of a *hag-cat-lacZ* transcriptional fusion in strain LMB243. This strain bears the chromosomal deletion of P_{sigD}, as well as a reporter construct consisting of the strong, σ^D-dependent promoter of the flagellin (*hag*) gene driving expression of β-galactosidase. Figure 6A shows that the profile of *hag-lacZ*-dependent β-galactosidase activity in the LMB243 ($P_{sigD}\Delta$) background is similar to the activity profile exhibited by a wild-type strain bearing the same reporter fusion. This indicates that σ^D activity, throughout growth, is normal in the $P_{sigD}\Delta$ mutant. In addition, the $P_{sigD}\Delta$ strain is motile (Fig. 6B). Altogether, our data indicate that P_{sigD} is not required for *fla/che* expression, σ^D expression, σ^D-dependent motility gene expression, or motility.

DISCUSSION

Our results allow us to draw several inferences about the relative roles of *fla/che* P_A, P_{D-3}, and P_{sigD} in regulating expression of the *fla/che* genes and in controlling motility. A number of the salient features of our findings are summarized in Fig. 7.

fla/che P_A appears to be essential for *fla/che* expression and for motility. Moreover, since expression of σ^D and σ^D-dependent motility genes is reduced in *fla/che* P_A deletion strains, *fla/che* P_A activity appears to be required for normal expression of these genes, as well. In wild-type cells, the primer extension data indicate that the majority of *fla/che* transcripts originate from *fla/che* P_A; P_{D-3} transcripts comprise only a minor fraction of the *fla/che* transcripts. Moreover, analysis of the *fla/che* $P_A\Delta$ mutant indicates that *fla/che* P_A activity is required for expression of the P_{D-3}-specific transcript, as well as for the *fla/che* P_A-specific transcript. σ^D protein is evident in a mutant strain that lacks the *fla/che* P_A, but this σ^D is incapable of activating transcription from P_{D-3}. This inactivity of σ^D is probably a consequence of the lack of HBB gene expression in the absence of *fla/che* P_A activity, which precludes the inactivation of FlgM. P_{D-3}-mediated expression of *fla/che* genes can be achieved in a *fla/che* $P_A\Delta$ strain only if the *flgM* gene is also disrupted. In this context, in which *fla/che* gene expression is

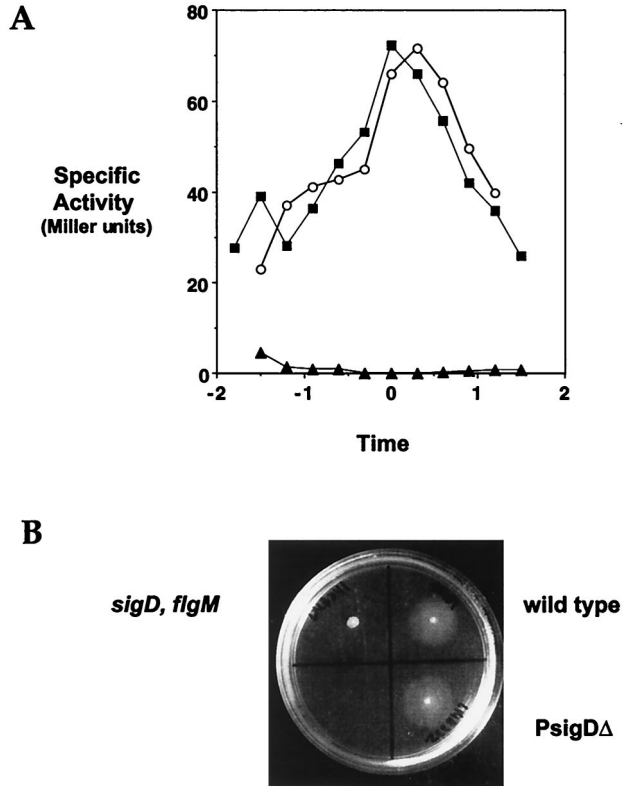

FIG. 6. (A) *hag-lacZ* expression in the $P_{sigD}\Delta$ strain. β-Galactosidase activity was monitored in strains bearing *hag-lacZ* reporter constructs throughout growth from approximately an optical density at 600 nm of 0.1. The *x* axis represents the time in culture, where 0 h represents the end of logarithmic growth. Symbols: ○, LMB244 (wild type); ■, LMB243 ($P_{sigD}\Delta$); ▲, LMB247 (*sigD*::pLM5). The $P_{sigD}\Delta$ and wild-type strains exhibit comparable patterns of *hag-lacZ* expression throughout growth. This suggests that P_{sigD} is not required for regulation of σ^D expression or activity. (B) Swarm assay of LMB232 ($P_{sigD}\Delta$). Cells of the indicated genotype were inoculated onto PAB–0.4% agar plates and grown at 37°C overnight. LMB232 swarms, like the wild-type strain, indicating that P_{sigD} is not required for motility. LMB241 (*sigD*::pLM5 *flgM*::mini-Tn*10*), the nonmotile negative control strain, does not swarm.

mediated entirely through P_{D-3}, the overall level of *fla/che* expression is substantially lower than is observed when *fla/che* P_A is active. Primer extension and Western blot data indicate that the expression of HBB (i.e., *flgB*) transcript and σ^D protein are both reduced relative to the wild type in the *fla/che* $P_A\Delta$ *flgM* double mutant strain. Moreover, this strain background exhibits a reduction of σ^D-dependent motility gene expression; Western blot data indicates that flagellin protein is diminished relative to wild-type levels of expression. Finally, the strain that lacks *fla/che* P_A and *flgM* is nonmotile.

A possible explanation for this lack of motility is that expression of motility genes is reduced below some critical threshold level. As stated above, *fla/che* expression, which is solely dependent upon P_{D-3} in this context, is greatly reduced relative to the wild type. Moreover, σ^D protein is also reduced relative to the wild type. In addition, this reduction in σ^D protein is accompanied by a reduced level of flagellin expression; since it has previously been shown that σ^D-dependent motility genes are coordinately regulated in response to σ^D activity levels (3), we presume that the expression of other σ^D-dependent motility genes is also reduced. Thus, in the *fla/che* $P_A\Delta$ *flgM*::mini-Tn*10* strain, the HBB components, *sigD*, and the σ^D-dependent motility genes are all simultaneously expressed at significantly reduced levels. The process of flagellar formation is thought to be highly conserved among *B. subtilis* and the enteric bacteria, i.e., *Salmonella* spp. and *E. coli*. In these enteric bacteria, analysis of stoichiometric ratios of HBB components has revealed that as many as 26 subunits of some components are required in each HBB structure, whereas only 5 or fewer subunits of other components are required (13). Moreover, the assembly process has been found to occur in a stepwise manner and is stalled at particular junctures in mutant backgrounds where structural components are lacking (13, 29, 30). Taking this into account, we think it is possible that the simultaneous and dramatic reduction in expression of the *fla/che* operon, *sigD*, and σ^D-dependent motility genes could give rise to a situation where one or more of the flagellar components is limiting for assembly. This may underlie the lack of motility that we observe in the strain that lacks *fla/che* P_A and FlgM activities.

An alternative hypothesis to explain this lack of motility is rooted in observations of *fla/che* expression in the mutant strains that we have examined. In the strain that lacks *fla/che* P_A activity, P_{D-3} is apparently not transcribed, and σ^D protein

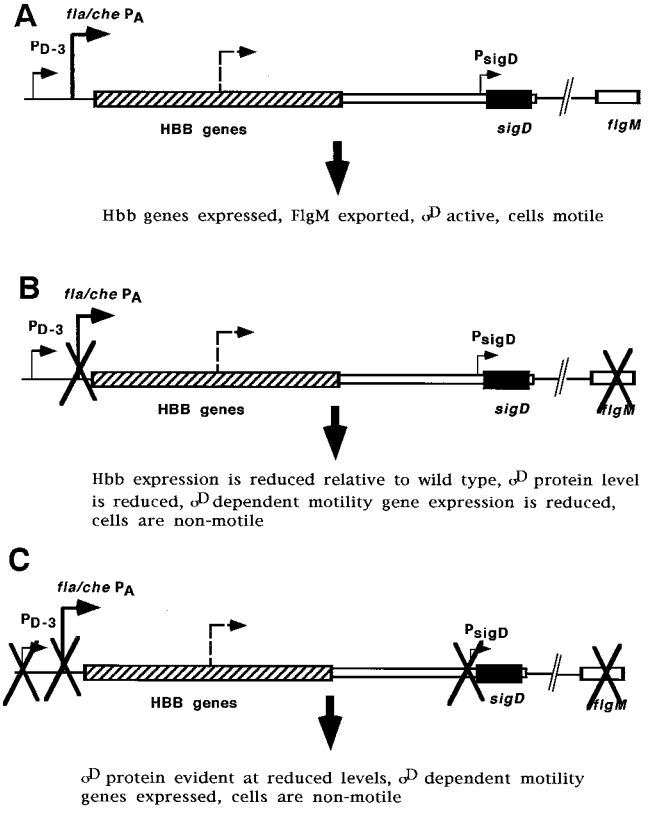

FIG. 7. Schematic representation of the *fla/che* operon and the promoters (arrows) that function in *fla/che* gene expression. This size of the arrow is proportional to the relative contribution of the designated promoter in gene expression. The dotted arrow represents the previously undescribed promoter(s), which has not yet been localized within the operon. (A) In a wild-type cell, *fla/che* P_A makes the predominant contribution to *fla/che* expression. P_{D-3} and P_{sigD} play minor roles. P_{D-3} is activated because *fla/che* P_A-dependent expression of the HBB genes allow efficient export of FlgM, with consequent activation of σ^D. (B) In the absence of *fla/che* P_A activity, *sigD* is expressed, albeit at reduced levels, from the undefined promoter (dotted line) and P_{sigD}. The resulting σ^D is inactive unless *flgM* is also disrupted. *flgM* disruption allows activation of σ^D, with consequent activation of P_{D-3} transcription, and σ^D-dependent gene expression. σ^D-dependent gene expression is reduced relative to wild-type levels, and cells are nonmotile (see Discussion). (C) σ^D protein is observed, at reduced levels, in strains that bear deletions of all three known promoters (*fla/che* P_A, P_{D-3}, and P_{sigD}), indicating that another promoter(s) contributes to *sigD* expression.

is reduced relative to the wild type. In the strain that lacks both *fla/che* P_A and FlgM activities, P_{D-3} transcription is significantly induced, but σ^D protein levels remain unchanged. There are two possible explanations for this apparent inability of the P_{D-3} transcript to contribute to *sigD* gene expression. The first is that, by comparison with the other relevant promoters, P_{D-3} makes such a slight contribution to *sigD* expression that its effect is not sufficient to be detected by Western blot. The second hypothesis is that the transcript that originates at P_{D-3} terminates upstream of the *sigD* gene. This hypothesis could explain the observed lack of motility if the P_{D-3} transcript terminated prior to completing transcription of all of the HBB component genes. In this case, the lack of one or more HBB gene products would stall assembly of the flagella. Such a hypothesis would require that the P_{D-3} transcript terminate earlier in the operon than the *fla/che* P_A transcript in order to account for the observation that *fla/che* P_A is essential for motility. We have examined the *fla/che* operon sequence for clues as to a mechanism by which such a P_{D-3}-specific termination event might occur. The P_{D-3} transcript does not appear to contain an open reading that might be subject to attenuation. Cursory examination of the 133-bp region between P_{D-3} and *fla/che* P_A (L.M.-M., unpublished observation) suggested that it might contain a site(s) for mediating rho-dependent termination (12). However, we have generated a strain isogenic to LMB216 that bears a disruption in *rho* (27) and find that motility is not restored (unpublished data). This suggests that *rho*-dependent termination of the P_{D-3} transcript in the proximal region of the operon is not the basis for the lack of motility in LMB216. A second possibility is that the transcript that initiates from *fla/che* P_A is specifically subject to some antitermination mechanism. Our cursory analysis of the *fla/che* operon sequence has not revealed any obvious intrinsic terminators. Moreover, we have not identified any potential target sites for known antitermination mechanisms.

Our results indicate the central role of the *fla/che* P_A in regulating expression of the *fla/che* operon and of the *sigD* gene. However, our results indicate that the regulatory roles of P_{D-3} and P_{sigD} are quite minor. First, as discussed previously, primer extension data indicates that P_{D-3} makes only a small contribution to *fla/che* gene expression in wild-type cells. Moreover, the RNA samples utilized for our primer extension experiments were isolated at the time points when σ^D expression and activity are maximal. This suggests that P_{D-3} activation is not the primary factor responsible for the induction of *sigD* expression and σ^D activity. Other data are also consistent with this notion that P_{D-3} activation does not have a role in the positive autoregulation of *sigD* expression. Specifically, as described above, induction of transcription from P_{D-3}, in a *fla/che* $P_A\Delta$ strain background, does not result in a commensurate increase in σ^D levels. Together, these data suggest that P_{D-3} does not contribute significantly to *sigD* expression, either in the presence or in the absence of *fla/che* P_A activity.

Our results also indicate that P_{sigD} is not involved in the regulation of *fla/che* expression. Whereas others (1) have concluded that deletion of P_{sigD} results in delayed activation, as well as reduced levels, of *fla/che* expression, our results indicate that *fla/che* expression and σ^D activation are normal in $P_{sigD}\Delta$ mutant strains. One factor that could account for the difference between these studies is the nature of the deletion strains that were employed. The earlier study utilized chromosomal insertions of plasmids carrying variants of a *sigD-lacZ* reporter (with or without P_{sigD}); our study employed a deletion of P_{sigD} in its normal chromosomal context. Aside from being expendable for *fla/che* regulation, our data indicate that P_{sigD} activity is not required for the σ^D expression that is observed in *fla/che* $P_A\Delta$ strains. Further, σ^D levels are comparable in isogenic strains that either carry deletions of *fla/che* P_A and P_{D-3} or that carry deletions of all three promoters. This indicates that P_{sigD} activity makes very little contribution to the pool of σ^D that is observed in strains lacking *fla/che* P_A and P_{D-3} activity. Altogether, our results indicate that P_{sigD} is not required for motility and that P_{sigD} contributes negligibly to overall σ^D levels in cells grown in rich medium under our culture conditions. Recently, it has been suggested that the primary role of P_{sigD} in vivo may be to allow expression of σ^D-dependent functions unrelated to motility (e.g., autolysin genes) under conditions in which it is undesirable to induce motility gene expression (31).

Finally, our results indicate that an additional, previously undescribed promoter(s) contributes to *sigD* expression, since we detect σ^D protein and activity in strains where the known promoters have all been deleted. We postulate that this promoter(s) exhibits σ^A-dependent activity, since it does not appear to be regulated by FlgM. It seems likely that this putative σ^A-dependent promoter(s) lies upstream of P_{sigD}, since the -10 region of P_{sigD} is 30 bases upstream of the initiating codon and no other promoter consensus sequences are found within this region. We have conducted a sequence pattern search of the *fla/che* operon for sequence elements closely related to the $E\sigma^A$ promoter consensus. We have identified at least two potential candidate promoters in intergenic regions internal to the operon that could contribute to expression of *sigD*; however, it should be noted that P_{sigD} is not within an intergenic region. It will be of interest to test the two aforementioned candidates, as well as other sequences in the 26-kb operon that bear close relationship to the $E\sigma^A$ promoter consensus, to assess whether they exhibit promoter activity in vitro and in vivo. The functional promoter(s) can then be tested to determine whether it is required for the *sigD* expression that we have observed in *fla/che* $P_A\Delta$ strains. Finally, it would be of interest to determine the relative contribution of this promoter(s) to overall expression of *sigD* and to define the conditions under which the promoter(s) is required in vivo.

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