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# The *Bacillus subtilis* $\sigma^D$-Dependent Operon Encoding the Flagellar Proteins FliD, FliS, and FliT

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**During a genetic screen to identify metalloregulated loci in *Bacillus subtilis*, we isolated a Tn*917-lacZ* insertion in the second gene of an operon downstream of the flagellin (*hag*) gene. Sequence analysis indicates that this gene encodes a homolog of the enteric flagellar filament cap protein FliD. The *fliD* gene is followed by homologs of the *fliS* and *fliT* genes. Transcription of the *fliD-lacZ* fusion is $\sigma^D$ dependent, with peak expression at the end of logarithmic-phase growth. Like other $\sigma^D$-dependent genes, expression of *fliD-lacZ* is greatly reduced by mutations in genes essential for assembly and function of the basal body and hook complex (class II functions). These results suggest that *B. subtilis* flagellar genes are organized in a hierarchy of gene expression similar to that found in enteric bacteria with *hag* and *fliD* as class III genes. Expression from the *fliD* operon promoter, but not the *hag* promoter, is repressed by iron, which suggests that the target of metalloregulation is the promoter rather than the $\sigma^D$ protein.**

A large regulon of fifty or more genes is required for bacterial motility and chemotaxis (10, 22, 31). In *Escherichia coli* and *Salmonella typhimurium*, flagellar genes are expressed in a complex hierarchy (20). The master regulators, encoded by the *flhDC* loci, are expressed from a $\sigma^{70}$-dependent promoter under control of the cyclic AMP-cyclic AMP receptor protein complex. The FlhD and FlhC proteins activate expression of class II genes required for assembly of the flagellar basal body and hook (4, 20). One of the class II genes, *fliA*, encodes an alternative $\sigma$ factor which activates expression of class III genes encoding flagellin, the hook-associated proteins, the motor proteins, and the methyl-accepting chemotaxis proteins (2, 30).

In recent years many of the flagellar and chemotaxis genes of *Bacillus subtilis* have been cloned and sequenced (31). These genes are subject to complex regulation and also appear to be arranged in a hierarchy of interdependent gene expression (6, 40, 41). Many of the genes required for synthesis of the hook and basal body (the equivalent of the enteric class II functions) are present in a large operon designated the *flaA* locus (1, 31). One of the last genes in the *flaA* operon encodes the alternative $\sigma$ factor, $\sigma^D$, which is absolutely required for expression of flagellin, motor proteins, and methyl-accepting chemotaxis proteins and contributes to the expression of autolysins (10, 11, 19, 23, 28, 29). Genetic analyses indicate that $\sigma^D$-dependent promoters are often clustered. At least three other $\sigma^D$-dependent loci are present near the flagellin gene, *hag* (28). These are defined by the $\sigma^D$-dependent promoters, $P_{D-7}$, $P_{D-1}$, and $P_{D-8}$ (8, 31).

In the course of a screen to identify metalloregulated genes in *B. subtilis*, we isolated a transposon insertion downstream of the $P_{D-8}$ promoter (28) which lies immediately downstream of *hag* (28). We report the DNA sequence of this operon which encodes homologs of the *E. coli* FliD, FliS, and FliT proteins (17). Transcription of the *fliD* operon depends on the $\sigma^D$ regulatory protein and is reduced in mutant strains defective for flagellar assembly and function (class II functions). The $P_{D-8}$ promoter is repressible by iron, but the mechanism and significance of this effect are unclear. Iron regulation of motility functions has been previously reported for *Vibrio parahaemolyticus* (24). In addition, iron and several other metals induce transcription of the *E. coli* flagellin gene (9), and it has been suggested that the iron uptake repressor (Fur protein) interacts with the promoter region of the master operon of the flagellar hierarchy, *flhDC* (36).

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** All *B. subtilis* strains and plasmids used in this study are listed in Tables 1 and 2. Strain HB1058 was produced by transformation of CU1065 competent cells with chromosomal *B. subtilis* DNA containing random insertions of transposon Tn*917-lacZ* (21). Strain HB1002 was produced by transformation of CU1065 with chromosomal DNA from strain CB125 containing a translational fusion between flagellin and *lacZ* (a gift from D. Mirel and M. Chamberlin). This translational fusion links codon 71 of *hag* (flagellin) to the *lacZ* gene and is present in the integrational plasmid pDM633D (27). Strain IS876 contains a kanamycin cassette disrupting the *sinR* (*flaD*) gene and was obtained from I. Smith.

**Media and growth conditions.** Iron-deficient minimal medium was prepared as described elsewhere (7) and contained the indicated metal ions. Minimal media and plates were prepared with high-purity (Milli-Q) water to control the level of adventitious iron contamination. *E. coli* strains were grown on Luria-Bertani plates and 2× YT broth (34) with ampicillin (200 μg/ml) as appropriate. *B. subtilis* strains containing integrated Tn*917* were selected with erythromycin (1 μg/ml) and lincomycin (25 μg/ml) ($MLS^r$). Chloramphenicol (5 μg/ml) or kanamycin (10 μg/ml) was added as indicated.

**DNA manipulations and sequencing.** Isolation of *B. subtilis* chromosomal DNA and transformation were as previously described (13). For several experiments, a modified one-step transformation procedure was used (18). Restriction endonucleases and DNA ligase were from New England Biolabs and were used in accord with the manufacturer's instructions. Single-stranded DNA was isolated by infection of JM101 cells containing pLC5810, pLC5820, or pLC5830 with M13K07

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TABLE 1. Bacterial strains

| Strain | Characteristics | Source or reference |
|---|---|---|
| *B. subtilis* | | |
| CU1065 | W168 *trpC2 att*SPβ | 38 |
| CB100 | *trpC2 sigD*::pLM5 ($Cm^r$) | 23 |
| HB1002 | CU1065 *fliC-lacZ* ($MLS^r$) | This work |
| HB1058 | CU1065 *fliD*::Tn*917-lacZ* ($MLS^r$) | This work |
| HB2058 | CU1065 *fliD*::Tn*917-lacZ*::pTV21Δ2 ($Cm^r$) | This work |
| HB3058 | CU1065 *fliD*::Tn*917-lacZ*::pLC5810 ($MLS^r$ $Cm^r$) | This work |
| HB3158 | CU1065 *fliD*::Tn*917-lacZ*::pLC5820 ($MLS^r$ $Cm^r$) | This work |
| HB3519 | CU1065 *fliS*::pLC5809 ($Cm^r$) | This work |
| HB3516 | CU1065 ORF189::pLC5806 ($Cm^r$) | This work |
| OI2537 | *fliM*::*cat* ($Cm^r$) | 40 |
| OI2826 | *fliP*::*cat* ($Cm^r$) | 5 |
| IS876 | *sinR*::*kan* ($Km^r$) | I. Smith |
| HB1057 | HB1058 *fliM*::*cat* ($MLS^r$ $Cm^r$) | This work |
| HB1068 | HB1058 *fliP*::*cat* ($MLS^r$ $Cm^r$) | This work |
| HB1078 | HB1058 *sin*::*kan* ($MLS^r$ $Km^r$) | This work |
| HB1052 | HB1002 *fliM*::*cat* ($MLS^r$ $Cm^r$) | This work |
| HB1062 | HB1002 *fliP*::*cat* ($MLS^r$ $Cm^r$) | This work |
| HB1076 | HB1002 *sinR*::*kan* ($MLS^r$ $Km^r$) | This work |
| HB1001 | CB100 *fliD*::Tn*917-lacZ* ($MLS^r$ $Cm^r$) | This work |
| *E. coli* | | |
| JM101 | *supE hsdD5 thi*Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*$^q$ *lacZ*ΔM15) | 7 |
| $JM2r^-$ | *mcrAB hsdR hsdM*$^+$ *recA1* Δ(*lac proAB*) *thi gyrA96 relA1 srl*::Tn*10* F′ (*proAB lacZ*ΔM15) | 7 |

helper phage. Double-stranded plasmid DNA was prepared by alkaline lysis and polyethylene glycol precipitation as previously described (34). Sequencing reactions with single- and double-stranded templates were performed with Sequenase version 2.0 (United States Biochemicals, Cleveland, Ohio) and the Sequenase kit according to the manufacturer's directions. The M13−40 primer, reverse primer, a synthetic primer corresponding to the left end of transposon Tn*917* (5′TTTCTTA TCGATACAAATTC3′), and primers complementary to the T7 and T3 RNA polymerase recognition sequences were used to prime DNA synthesis. DNA sequence was determined by a combination of random and directed cloning of DNA fragments between the two regions of known sequence.

**Plasmid rescue experiments.** To recover DNA linked to the Tn*917* insertion in strain HB1058, linearized plasmid pTV21Δ2 (39) was introduced by homologous recombination to generate HB2058. This plasmid contains an ampicillin resistance gene and a ColE1 origin of replication. Chromosomal DNA from strain HB2058 was isolated and digested with restriction endonuclease *Hin*dIII or *Eco*RI to isolate upstream DNA or with *Bam*HI to recover downstream-linked DNA. Each restriction digest was treated with T4 DNA ligase under dilute conditions and used to transform *E. coli* $Jm2r^-$ (38) to ampicillin resistance.

**β-Galactosidase assays.** Samples (1 ml) were removed from each culture, centrifuged for 5 min, and stored at −20°C for at least 30 min. Samples were assayed for β-galactosidase levels by the method of Miller (26) at room temperature (approximately 21°C). Frozen cells were resuspended in working buffer (Z buffer with 400 μM dithiothreitol) and stored on ice. Samples of each cell suspension were diluted to a final volume of 1 ml with working buffer and partially lysed by incubation for 5 min at 37°C with 100 μg of lysozyme per ml. All assays were performed on duplicate samples, and the values were averaged.

**Computer analysis.** Sequence comparisons and data base searches were performed with the Genetics Computer Group software package. The protein sequence alignments of Fig. 3 and 4 were generated with the GAP program.

**Nucleotide sequence accession number.** The DNA sequence as illustrated in Fig. 2 has been deposited in the GenBank and EMBL data bases under accession numbers U07822 and Z31376, respectively.

TABLE 2. Plasmids

| Plasmid | Characteristics | Source or reference |
|---|---|---|
| pTV21Δ2 | $Amp^r$ and pBR322 replicon; $Cm^r$ and pE194 replicon | 39 |
| pGEM3zf(+)-cat-1 | $Amp^r$ $Cm^r$; pBR322 replicon | 39 |
| pBluescript SK(+) | $Amp^r$; pBR322 replicon | Stratagene |
| pLC58-RI | Plasmid rescue from *Eco*RI-digested HB2058 DNA | This work |
| pLC58-H3 | Plasmid rescue from *Hin*dIII-digested HB2058 DNA | This work |
| pLC5810 | pGEM-3zf(+)-cat-1 with *Eco*RI-*Bam*HI fragment from pLC58-RI | This work |
| pLC5820 | pGEM-3zf(+)-cat-1 with *Hin*dIII-*Bam*HI fragment from pLC58-H3 | This work |
| pLPJ58Bm | Plasmid rescue from *Bam*HI-digested HB2058 DNA | This work |
| pLC5890 | pBluescript SK(+) with *Pst*I-*Pst*I fragment from pLPJ58Bm | This work |
| pLC5895 | pBluescript SK(+) with *Sna*BI-*Spe*I fragment from pLPJ58Bm | This work |
| pLC5809 | pGEM-3zf(+)-cat-1 with a *Sma*I-*Sph*I fragment internal to *fliS* | This work |
| pLC5806 | pGEM-3zf(+)-cat-1 with a *Pst*I-*Bam*HI fragment containing internal sequence of ORF189 | This work |

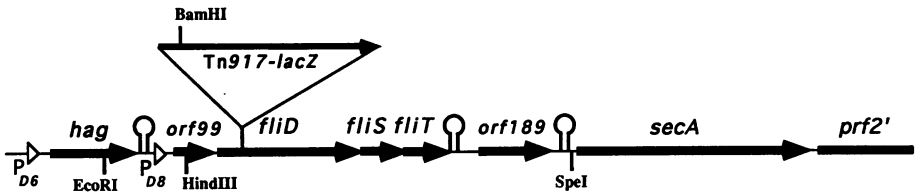


FIG. 1. Gene organization in the vicinity of the *fliD* operon (310°). $P_{D-6}$ and $P_{D-8}$ are the two *sigD*-dependent promoters (indicated by open triangles), and putative rho-independent terminators are indicated by the stem-loop structures. The DNA sequence between the indicated *Eco*RI and *Spe*I sites is shown in Fig. 2.

## RESULTS

**Isolation of the *mrgB-lacZ* (*fliD-lacZ*) transcriptional fusion.** We have transformed a wild-type *B. subtilis* strain (CU1065) with a chromosomal DNA library containing random Tn*917-lacZ* fusions (21). The resulting insertion library was screened for strains that demonstrated visibly enhanced production of β-galactosidase on plates containing the iron chelator ethylenediamine di(*o*-phenylacetic acid) (EDDA) (7). One such strain, *B. subtilis* HB1058, contained a candidate metalloregulated gene fusion (*mrgB-lacZ*) as described in more detail below. Sequence analysis demonstrated that *mrgB* is transcribed from a previously characterized $\sigma^D$-dependent promoter, $P_{D-8}$, and is the *B. subtilis fliD* gene. In this study we describe the isolation, sequencing, and regulation of the *fliD* operon.

**Identification of the promoter directing *fliD-lacZ* transcription.** To identify the *mrgB* gene and its promoter region, DNA linked to the inserted Tn*917-lacZ* transposon was isolated and sequenced as follows. Plasmid pTV21Δ2 (39) was integrated by homologous recombination at the site of transposon insertion to generate strain HB2058. Purified chromosomal DNA from strain HB2058 was digested with restriction endonucleases and recircularized to generate plasmid molecules containing upstream or downstream flanking DNA. These molecules were recovered by transformation into *E. coli* JM2r$^-$ with selection for ampicillin resistance.

To define the promoter region for expression of the *mrgB* gene, DNA upstream of the transposon insertion was cloned into the integrational plasmid vector, pGEM-3zf(+)-cat (39). Transformation of strain HB1058 with one such plasmid, pLC5810 (Table 2), generated strain HB3058. This strain still expressed β-galactosidase, and β-galactosidase levels were still affected by metal ions, though not in precisely the same way as in the parent strain HB1058 (see below). This suggested that the metal-responsive promoter for *mrgB* transcription was located downstream of the *Eco*RI site (Fig. 1). In contrast, transformation of HB1058 with a plasmid containing less upstream DNA, pLC5820 (Table 2), eliminated expression of β-galactosidase. These results localized the putative metal-regulated promoter to a 471-bp *Eco*RI-to-*Hin*dIII fragment. Sequence analysis of this DNA indicated that this fragment contained the intergenic region downstream of the flagellin gene and suggested that the *mrgB* promoter was $P_{D-8}$ (28). This conclusion was strengthened by the finding that *mrgB-lacZ* expression was eliminated in a *sigD* mutant strain (Table 3).

**Sequence analysis of the *fliD* operon.** To determine the complete DNA sequence of this $\sigma^D$-dependent operon, we isolated 8 kb of downstream DNA by plasmid rescue as described in Materials and Methods. DNA sequence analysis of this downstream region revealed overlap with a region of *B. subtilis* DNA which encodes a *secA* homolog (33) and protein release factor 2 (32). The genetic organization of this region of the chromosome is presented in Fig. 1.

We determined the sequence of the *fliD* operon including 2.7 kb of new sequence between the start of the *fliD* open reading frame and the DNA fragment containing the *secA* gene (Fig. 2). This analysis suggests that the $P_{D-8}$ promoter controls an operon of four genes designated ORF99, *fliD*, *fliS*, and *fliT*. The ORF99 reading frame is similar to that proposed previously for this region (28), except that we hypothesize that translation initiates with the indicated TTG codon rather than with a downstream ATG. The predicted protein product of ORF99 is apparently unrelated to proteins in the GenBank data base.

The second open reading frame encodes a homolog of the enterobacterial hook-associated protein 2, FliD (Fig. 3). The Tn*917-lacZ* insertion is after the 87th codon of the *fliD* gene, indicating, as suggested above, that *mrgB* is *fliD*. FliD functions in *E. coli* as a filament cap which forms the distal end of the flagellum (15). As expected for an insertion in an essential flagellar gene, strain HB1058 and its derivatives are Fla$^-$ (see below).

Downstream of *fliD* are two small reading frames with significant similarity to the *E. coli fliS* and *fliT* genes (Fig. 2 and 4). These encode essential flagellar proteins in *E. coli* but are not thought to be part of the final flagellar structure (17). It has been speculated that FliS or FliT might be regulatory rather than structural proteins, since mutations in the *fliD* operon led to an overexpression of other late flagellar genes (17, 20).

Following the *fliT* structural gene is a 560-bp intergenic region. This region includes a possible rho-independent terminator (Fig. 2) which may define the end of the *fliD* operon. The next open reading frame, encoding a 189-amino-acid polypeptide designated ORF189, initiates approximately 400 bp downstream of this proposed terminator. The carboxyl-terminal 117

TABLE 3. Expression of β-galactosidase in *B. subtilis* strains

| Gene fusion and strain | Relevant genotype | β-Gal U[a] (% control) |
|---|---|---|
| *fliD-lacZ* | | |
| HB1058 | *fliD-lacZ* | 100 |
| HB3058 | *fliD-lacZ*::pLC5810 | 200 |
| HB1001 | *fliD-lacZ sigD* | 0 |
| HB1057 | *fliD-lacZ fliM::cat* | 0 |
| HB1068 | *fliD-lacZ fliP::cat* | 0 |
| HB1078 | *fliD-lacZ sin::kan* | 13.6 |
| *hag-lacZ* | | |
| HB1002 | *hag-lacZ* | 100 |
| HB1052 | *hag-lacZ fliM::cat* | 0.67 |
| HB1062 | *hag-lacZ fliP::cat* | 0.42 |
| HB1072 | *hag-lacZ sin::kan* | 20.5 |

[a] Maximal activity measured during late logarithmic phase relative to that of the control strain (first line in each set); 100% activity is 37 Miller units for *fliD-lacZ* and 1,270 Miller units for *hag-lacZ*. β-Gal, β-galactosidase.

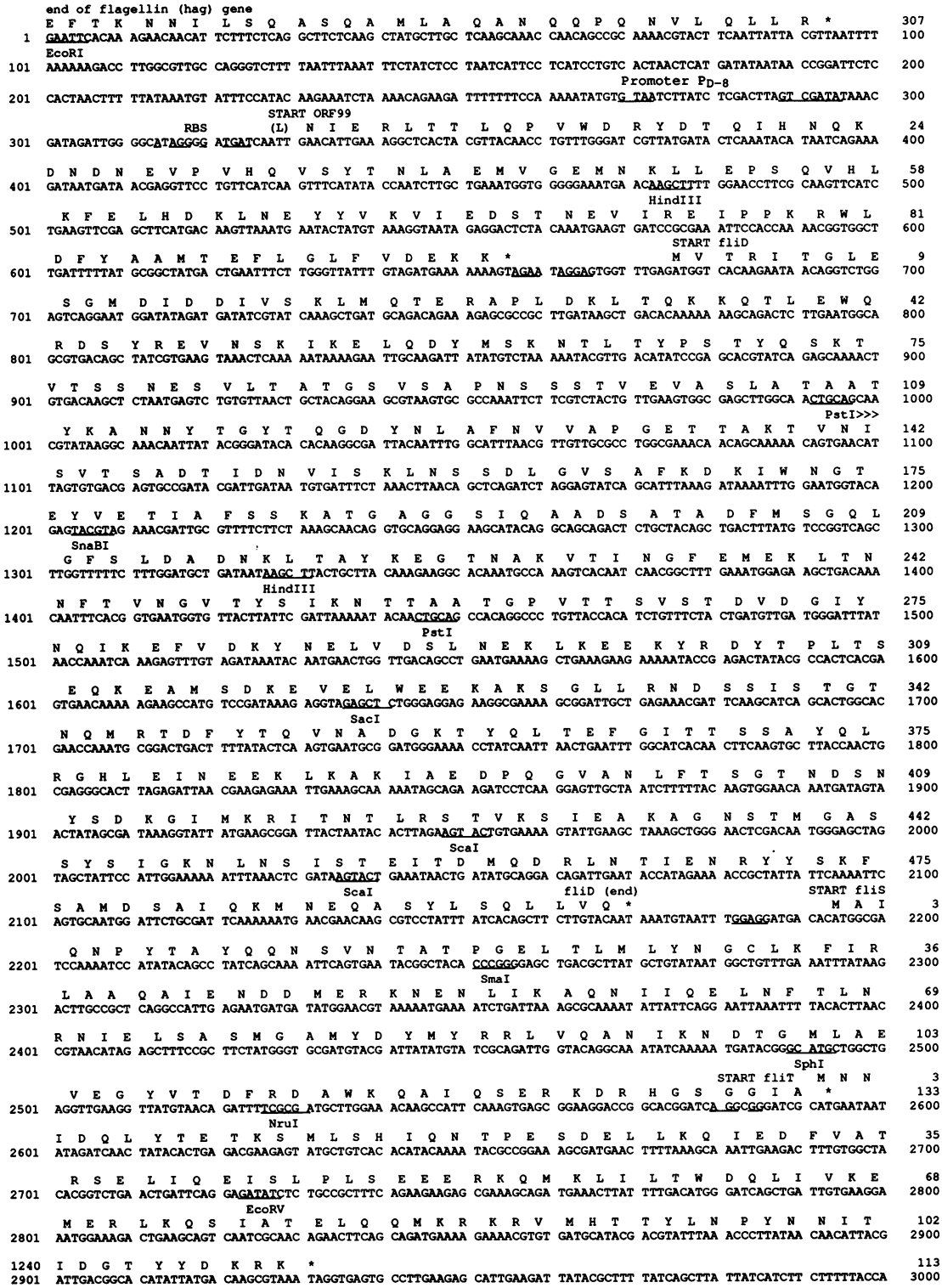


FIG. 2. Complete nucleotide sequence of the 4.2-kb *Eco*RI-to-*Spe*I fragment containing the *fliD* operon. Promoter $P_{D-8}$ and the putative ribosome-binding sites (RBS) for the ORF99 and *fliD* genes are underlined. Arrows indicate regions of dyad symmetry which, when indicated, are hypothesized to function as rho-independent terminators. Deduced amino acid sequences are shown on top of the corresponding coding strand. The region of DNA not previously sequenced is bracketed by arrowheads (>>> and <<<) and extends from the *Pst*I site at position 992 to the *Cfr*131 site at position 3708.

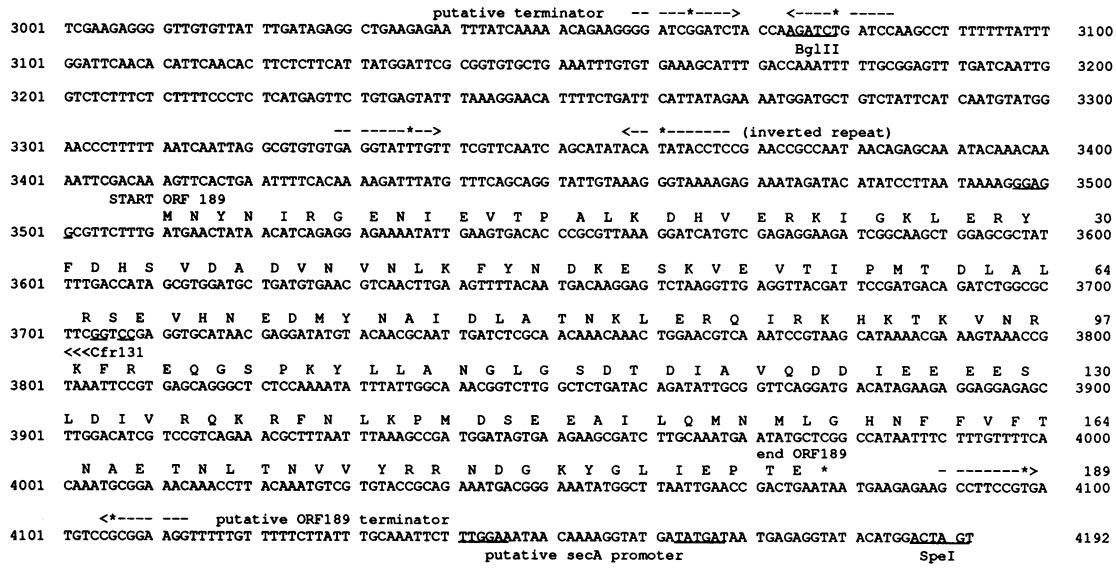


FIG. 2—*Continued.*

amino acids of ORF189 are identical to ORF1 in the sequence of Sadaie et al. (33). The amino-terminal half of ORF189 is highly related to a family of small proteins frequently encoded downstream of *rpoN* genes (Fig. 4C). It is thought that these genes might play a role in modulating $\sigma^{54}$-dependent promoter activity (25), but the mechanism of this effect is unclear. This open reading frame is followed by a putative rho-independent terminator and the proposed promoter for the *secA* gene (33).

**Genetic analysis of *fliD*, *fliS*, and ORF189 mutants.** We measured motility in the *fliD*::Tn*917-lacZ* fusion strain HB1058 and found that this strain was completely nonmotile by swarm plate and light microscopy assays (data not shown). In addition, we were unable to raise a PBS1 phage-transducing lysate of strain HB1058, which is consistent with the requirement of a flagellum for PBS1 phage infection. Electron microscopy confirmed the absence of flagella in strain HB1058 (data not shown). Integration of a plasmid into the *fliS* gene (strain HB3519) also led to a Fla$^-$ phenotype, consistent with the fact that FliS and FliT are essential flagellar proteins in *E. coli*. In contrast, integration of a plasmid into ORF189 did not impair motility (strain HB3516).

**Regulation of the *fliD* operon by other flagellar genes.** As predicted for a $\sigma^D$-dependent operon, *fliD-lacZ* transcription is eliminated in a *sigD* mutant (Table 3). This suggests that $P_{D-8}$ is responsible for *fliD* expression but does not rule out contributions from the upstream flagellin promoter, $P_{D-6}$. Like other $\sigma^D$-dependent genes, *fliD-lacZ* expression is regulated by growth phase (see below). Transcription is induced at the end of logarithmic phase and declines upon entry into stationary phase. Therefore, we analyzed $P_{D-8}$-dependent gene expression at the end of logarithmic growth and during early stationary phase. The growth phase dependence of gene expression probably reflects the regulation of *sigD* gene expression (19) and the consequent modulation of $\sigma^D$ protein levels.

Expression of at least some $\sigma^D$-dependent genes is also affected by mutations in other flagellar loci or in the *sinR* gene. We have tested the influence of *fliM*::*cat* and *fliP*::*cat* insertions on the maximal expression of both *fliD-lacZ* and *hag-lacZ* (Table 3). The *fliM* and *fliP* genes encode a component of the flagellar switch and a protein required for early stages of flagellar assembly, respectively, and have previously been

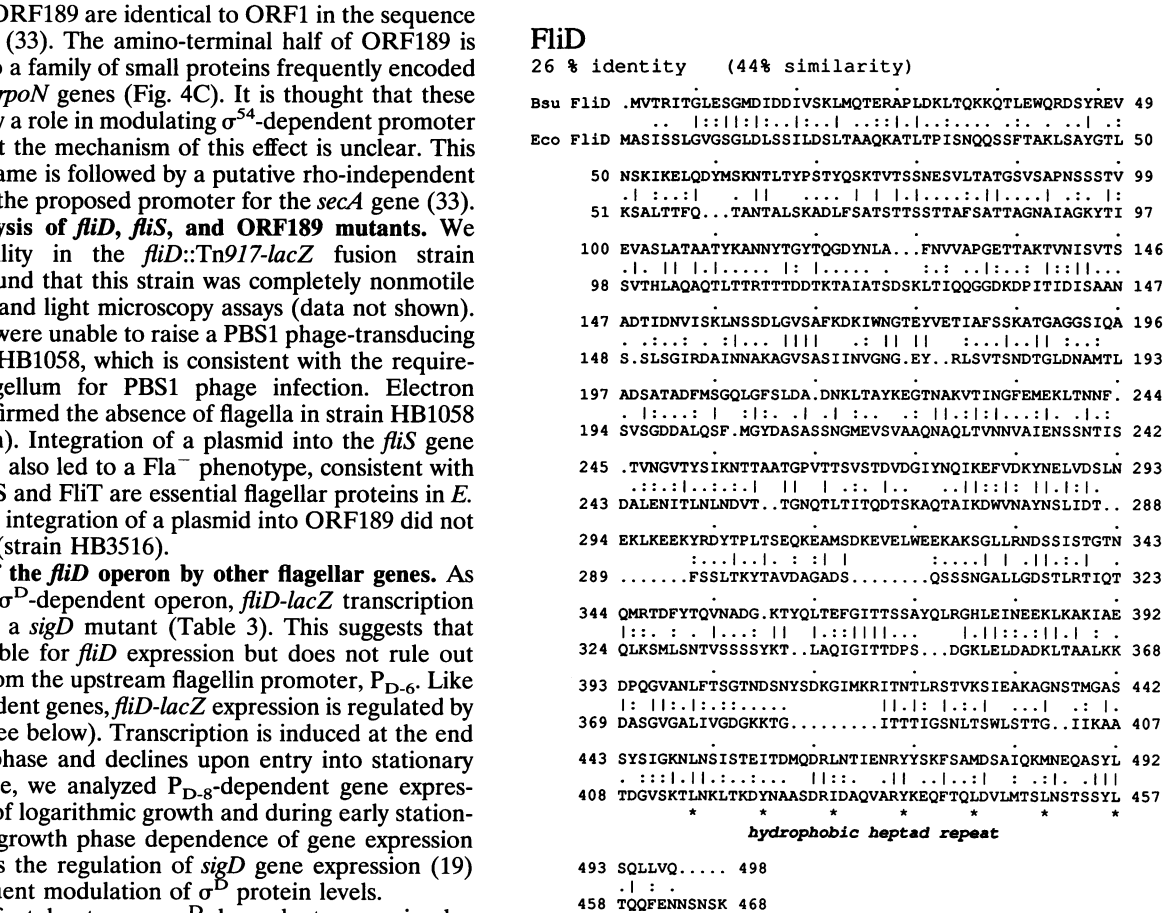


FIG. 3. Comparison of the deduced amino acid sequence of the *B. subtilis* FliD protein to the *E. coli* FliD protein. Identical residues are indicated by the vertical lines, and conservatively substituted residues are indicated by one or two dots as assigned by the Genetics Computer Group GAP program. Asterisks indicate the conserved hydrophobic heptad repeat proposed to be an important structural element for the *S. typhimurium* FliD protein (14).

## A. FliS

32% identity (55% similarity)

```
Bsu FliS MAIQNPYTAYQQNSVN....TATPGELTLMLYNGCLKFIRLAAQAIENDD 46
Eco FliS MYAAKGTQAYAQIGVESAVMSASQQQLVTMLFDGVLSALVRASLFMQDNN 50

      47 MERKNENLIKAQNIIQE...LNFTLNRNIELSASMGAMYDYMYRRLVQAN 93
      51 QQGKGVSLSKAINIIENGLRVSLDEESKDELTQNLIALYSYMVRRLLQAN 100

      94 IKNDTGMLAEVEGYVTDFRDAWKQAIQSERKDRHGSGGIA 133
     101 LRNDVSAVEEVEALMRNIADAWKESLLSPSLIQDPV.... 136
```

## B. FliT

22% identity (45% similarity)

```
Bsu FliT MNNIDQLY......TETKSMLSHIQNTPESDELLKQIEDFVATRSELIQ. 43
Eco FliT MNHAPHLYFAWQQLVEKSQLMLRLATEEQWDELIASEMAYVNAVQEIAHL 50

      44 ....EISLPLSEEERKQMKLILTWDQLIVKEMERLKQSIATELQQMKRKR 89
      51 TEEVDPSTTMQEQLRPMLRLILDNE....SKVKQLLQIRMDELAKLVGQS 96

      90 VMHTTYLNPYNNITIDGTYYDKRK.... 113
      97 SVQKSVLSAYGD...QGGFVLAPQDNLF 121
```

## C. Orf189 vs. KpnOrf95

37% identity (61% similarity)

```
BsuOrf189 1 MNYNIRGENIEVTPALKDHVERKIGKLERYFDHSVDADVNVNLKFYNDKE 50
KpnOrf95  1 MQLNITGHNVEITPAMRDFVTAKFSKLEQFFDRI..NQVYIVLKV..EKV 46

         51 SKV.EVTIPMTDLALRSEVHNEDMYNAIDLATNKLERQIRKHKTKVNRKF 99
         47 TQIADANLHVNGGEIHASAEGQDMYAAIDGLIDKLARQLTKHKDKLKQH. 95
```

FIG. 4. Comparison of the deduced amino acid sequences of the *B. subtilis* FliS (A), FliT (B), and ORF189 (C) proteins to the homologs from *E. coli* (Eco) or *Klebsiella pneumoniae* (Kpn). Alignments were optimized with the Genetics Computer Group GAP program as described for Fig. 3.

shown to be required for high-level *hag-lacZ* expression (31, 40). We find that either of these nonpolar insertions prevents transcription of both *fliD-lacZ* and *hag-lacZ*. These results suggest that like *hag* (6, 31, 41), the *fliD* gene is at level III of the *B. subtilis* flagellar hierarchy. In contrast, the presence of a *sinR* mutation reduced but did not eliminate transcription of *hag-lacZ* and *fliD-lacZ*. (Table 3).

**Regulation of the *fliD* operon by metal ions.** Since the *fliD-lacZ* gene fusion was originally detected during a genetic screen for iron starvation-regulated genes, we tested the effects of metal ions on gene expression. For these studies, we have used three different strains, as illustrated in Fig. 5. Strain HB1058 contains the original Tn*917-lacZ* transposon insertion in the *fliD* gene. Strain HB3058 is derived from strain HB1058 by insertion of plasmid pLC5810 and selection for chloramphenicol resistance. This strain expresses *fliD-lacZ* from the $P_{D-8}$ promoter element, but the flagellin gene and its associated promoter element are now several kilobases removed. Finally, we have used strain HB1002 (containing a translational *hag-lacZ* fusion [27]) to test whether a different $\sigma^D$-dependent gene might also be affected by metal ions.

Assays of β-galactosidase production as a function of growth phase and iron concentration for these three strains are shown in Fig. 6. In the case of strain HB1058, iron starvation does not alter the induction of β-galactosidase, but the persistence of β-galactosidase activity in stationary-phase cells is enhanced about 10-fold (Fig. 6A). This may explain why this strain was originally recovered during our screens and could reflect either decreased turnover or increased synthesis of β-galactosidase during stationary phase. We measured the effect of various metal ions on the level of β-galactosidase in strain HB1058 at 24 h of incubation (Fig. 7), a time when the effect of iron supplementation is at a maximum (Fig. 6A). These data demonstrate that supplementation with micromolar levels of Mn(II) and Fe(III) [but not Ca(II), Zn(II), or Co(II) (data not shown)] all decreased the stationary-phase level of β-galactosidase. Addition of Cu(II) also repressed β-galactosidase, though at significantly higher concentrations than for Mn(II) or Fe(III). The origins of these effects are not yet understood, but since the vast majority of the *lacZ* transcriptional fusions we have characterized are not repressible by addition of metal ions, we favor the hypothesis that this repression indicates metalloregulation of gene expression rather than a decrease in β-galactosidase stability. Consistent with this idea, we note that the levels of iron or manganese which reduced the stationary-phase level of β-galactosidase in strain HB1058 are the same (1 to 10 μM) as the levels required for the metalloregulation of other genes (7).

Surprisingly, when *fliD-lacZ* regulation in strain HB3058 (Fig. 6B) was compared with that in strain HB1058 (Fig. 6A), several differences were noted. First, although the temporal

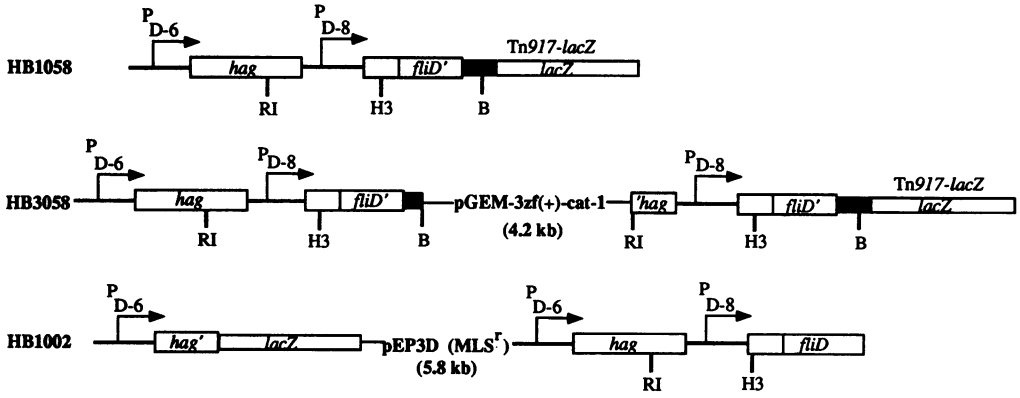


FIG. 5. Organization of chromosomal DNA in strains HB1058, HB3058, and HB1002. The positions of the *sigD*-dependent promoters $P_{D-6}$ and $P_{D-8}$ are indicated by arrows. RI, H3, and B refer to *Eco*RI, *Hin*dIII, and *Bam*HI restriction sites, respectively. Strains HB1058 and HB3058 both contain Tn*917-lacZ* transcriptional fusions to the truncated *fliD* gene but differ by the presence of plasmid pLC5810 integrated via a Campbell insertion. This leads to a duplication of the *Eco*RI-to-*Bam*HI fragment containing the $P_{D-8}$ promoter. Strain HB1002 contains a translational fusion between the *hag* gene and *lacZ*.

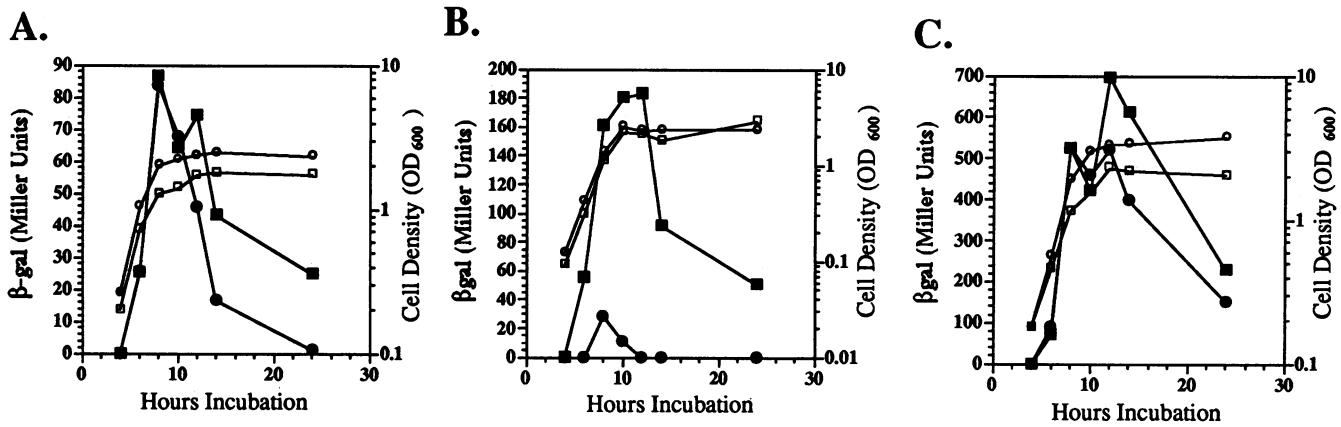


FIG. 6. Effect of Fe(III) on the expression of β-galactosidase (βgal) in strains HB1058, HB3058, and HB1002. Cells were grown in minimal medium lacking added iron (squares) or containing 5 μM Fe(III) (circles), and both cell growth (open symbols, right axis) and gene expression (closed symbols, left axis) were monitored versus time. $OD_{600}$, optical density at 600 nm.

regulation of *fliD-lacZ* expression was the same in the two strains, the level of expression in iron-deficient minimal medium was reproducibly increased about twofold by integration of plasmid pLC5810 (fold increase of 2.2 ± 0.53 [mean ± standard deviation] in 14 experiments). The second significant difference between strain HB1058 and strain HB3058 was the response to metal ions. Expression of β-galactosidase in strain HB3058 was no longer reduced by Mn(II) or Cu(II) addition, but the repression by Fe(III) was much more dramatic. When separated from the upstream flagellin gene by plasmid integration, the $P_{D-8}$ promoter was clearly iron repressible. However, not all $\sigma^D$-dependent promoters were affected by iron, since no significant effect was seen in strain HB1002 expressing a *hag-lacZ* fusion protein (Fig. 6C). The complexity of the metalloregulation we have observed is surprising and suggests that there are several independent metal-sensing systems influencing gene expression in *B. subtilis*.

## DISCUSSION

On the basis of its apparent metalloregulation, we have identified a transcriptional fusion in an essential flagellar operon. The transposition event that generated the *mrgB-lacZ* fusion disrupts the *fliD* gene in *B. subtilis* and will therefore be referred to as *fliD-lacZ*. Measurements of β-galactosidase demonstrate that promoter $P_{D-8}$ is functional in vivo and is dependent on the presence of the alternative sigma factor, $\sigma^D$, as predicted from previous characterization (8, 28, 35).

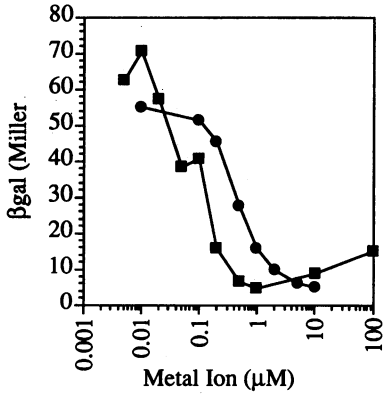


FIG. 7. Effects of Fe(III) or Mn(II) supplementation on the level of β-galactosidase (βgal) in strain HB1058 during stationary phase (24 h postinoculation). Cells were grown in minimal medium (7) containing the indicated concentration of $FeCl_3$ (●) or $MnCl_2$ (■). For the Fe(III) titration, the minimal medium contained 80 nM Mn(II) to allow for good growth.

**The *fliD* operon.** Sequence data suggest that the $P_{D-8}$ promoter element controls expression of an operon of four genes expressing homologs of the enterobacterial FliD, FliS, and FliT proteins and a predicted protein of 99 amino acids (ORF99). The role of the ORF99 gene product is unknown. Insertions in this operon led to a $Fla^-$ phenotype, suggesting that one or more of these genes are essential for flagellar structure or function. By analogy with the enterobacterial system, we hypothesize that FliD, FliS, and FliT are all essential flagellar proteins. *S. typhimurium fliD* mutants fail to make hook-associated protein 2. Flagellin is still synthesized in these mutant strains but fails to assemble onto the basal body-hook structure and accumulates to a high level in the medium (15, 16). In contrast, we do not detect obvious accumulation of the *B. subtilis* flagellin in the spent growth medium of strain HB1058 by either Coomassie blue staining or immunoblotting of sodium dodecyl sulfate-polyacrylamide protein gels (data not shown).

Following the *fliD* operon, there is an intergenic region and a gene (ORF189) whose product is homologous to a family of small proteins postulated to play a role in regulating $\sigma^{54}$ function in gram-negative organisms (25). Insertional disruption of the ORF189 reading frame did not impair motility. We speculate that transcription of the *fliD* operon terminates in the intergenic region between FliT and ORF189. The ORF189 reading frame is followed by the *B. subtilis secA* and *prf2* genes sequenced previously (32, 33).

**Regulation of the *fliD* operon.** The $\sigma^D$ regulon includes genes involved in flagellar synthesis, motility, and autolysis (10). In all cases studied, the expression of these genes is primarily regulated by the availability of the $\sigma^D$ protein (19, 23, 31). Levels of $\sigma^D$ protein reach a peak of about 200 molecules per cell at the end of logarithmic-phase growth (12). Transcriptional studies of a *sigD-lacZ* fusion are consistent with the hypothesis that this induction is largely transcriptional in origin (19). Proteolytic turnover of the $\sigma^D$ polypeptide probably accounts for the shutdown of $\sigma^D$-dependent transcription observed in stationary-phase cells (23). We have observed that

the *fliD* operon, like the *hag* gene, is completely dependent on the $\sigma^D$ for expression and is temporally regulated with a peak in expression at the end of logarithmic growth.

We have shown that mutations in either of two structural genes of the *flaA* operon block expression of the *hag* gene and the *fliD* operon, consistent with the presence of a hierarchy of flagellar gene expression in *B. subtilis*, as noted previously (6, 31, 40, 41). In addition, we find that expression of both the *fliD* and the *hag* genes is reduced but not eliminated by a mutation in the *sinR* gene (3, 37). Both the *hag-lacZ* and *fliD-lacZ* fusions display a fivefold or greater decrease in peak levels of expression in the presence of the *sinR* mutation. Both *hag* and *fliD* are completely dependent on *sigD* for expression, suggesting that there is active $\sigma^D$ in the *sinR* mutant strain. This is despite the fact that *sigD-lacZ* transcription is severely reduced by a *sin* mutation (19). It has previously been shown that even very low levels of $\sigma^D$ protein can support near-normal levels of flagellin synthesis (23), consistent with the idea that a *sin* mutation reduces but does not eliminate expression of *sigD*.

Our data reveal that insertion of a plasmid between the flagellin (*hag*) gene and the *fliD* operon reproducibly increase the level of expression of the *fliD-lacZ* fusion about twofold in iron-deficient minimal medium (Fig. 6B versus 6A). If readthrough transcription from the highly expressed $P_{D-6}$ promoter was contributing to *fliD-lacZ* expression, we would have expected the opposite result. One possibility is that active transcription of the *hag* gene reduces the local negative superhelical density in the vicinity of the *fliD* promoter and thereby decreases the rate of initiation. Transcription from $\sigma^D$-dependent promoters in vitro is known to be greatly stimulated by negative supercoiling of the template DNA (10a). Further experiments are needed to test this hypothesis.

**Metalloregulation of the *fliD* operon.** Superimposed on the factors that affect overall levels of $\sigma^D$ activity, there are likely to be promoter-specific regulatory controls. In this study, we see evidence that expression from $P_{D-8}$, but not $P_{D-6}$, is regulated by levels of iron in the medium. This repression is most striking when the $P_{D-8}$ promoter is separated from the upstream *hag* gene by plasmid integration (Fig. 6B). Curiously, the repressive effect of Mn(II) [and Cu(II) (data not shown)] we noted in strain HB1058 (Fig. 7) was lost when a plasmid was inserted upstream of $P_{D-8}$. This suggests that the repression elicited by these ions operates through a different mechanism or requires different *cis*-acting sites than the iron-mediated repression. This is consistent with our previous suggestion that iron and manganese metalloregulation require separate regulatory proteins, since some genes are regulated exclusively by iron and others are regulated exclusively by manganese (7).

We have previously described an iron-regulated gene from *B. subtilis* designated *mrgC* which requires an iron box sequence similar to those recognized by the *E. coli* ferric uptake repressor (Fur) protein for efficient metalloregulation (7). Similar iron box sequences upstream of the *B. subtilis* ferric hydroxamate uptake (*fhu*) locus have been described, and they function in *E. coli* to bind Fur protein (36). There are two possible binding sites for a Fur-like protein downstream of the $P_{D-8}$ promoter and within the coding sequence for the ORF99 protein (13 of 19 and 14 of 19 matches to the iron box consensus). The role of these sequences in the iron effects we have observed, if any, is not yet clear.

We do not understand the significance of the observed metalloregulation of the *fliD* operon. We note, however, that regulation of flagellar gene expression by iron has been reported for both *E. coli* (9) and *V. parahaemolyticus* (24). Therefore, we hypothesize that the availability of iron and other metal ion nutrients serves to modulate the growth-phase-dependent activation of the motility and chemotaxis systems of *B. subtilis*.

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