JOURNAL OF BACTERIOLOGY, June 1990, p. 3435–3443
0021-9193/90/063435-09$02.00/0

Vol. 172, No. 6

# Studies of $\sigma^D$-Dependent Functions in *Bacillus subtilis*

LETICIA M. MÁRQUEZ,[1] JOHN D. HELMANN,[2] EUGENIO FERRARI,[3] HELEN M. PARKER,[4] GEORGE W. ORDAL,[4] AND MICHAEL J. CHAMBERLIN[1]*

*Department of Biochemistry, University of California, Berkeley, California 94720[1]; Department of Biological Chemistry & Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115[2]; Department of Research, Genencor, South San Francisco, California 94080[3]; and Department of Biochemistry, University of Illinois, Urbana, Illinois 61801[4]*

Received 27 November 1989/Accepted 1 March 1990

**Gene expression in *Bacillus subtilis* can be controlled by alternative forms of RNA polymerase programmed by distinct σ factors. One such factor, $\sigma^D$ ($\sigma^{28}$), is expressed during vegetative growth and has been implicated in the transcription of a regulon of genes expressed during exponential growth and the early stationary phase. We have studied several functions related to flagellar synthesis and chemotaxis in *B. subtilis* strains in which $\sigma^D$ is missing or is present at reduced levels. Previous studies showed that a null mutant, which contains a disrupted copy of the $\sigma^D$ structural gene (*sigD*), fails to synthesize flagellin and grows as long filaments. We now show that these defects are accompanied by the lack of synthesis of the methyl-accepting chemotaxis proteins and a substantial decrease in two autolysin activities implicated in cell separation. A strain containing an insertion upstream of the *sigD* gene that reduces the level of $\sigma^D$ protein grew as short chains and was flagellated but was impaired in chemotaxis and/or motility. This reduced level of $\sigma^D$ expression suggests that the *sigD* gene may be part of an operon. A strain containing an insertion downstream of the *sigD* gene expressed nearly wild-type levels of $\sigma^D$ protein but was also impaired in chemotaxis and/or motility, suggesting that genes downstream of *sigD* may also be involved in these functions. Genetic experiments demonstrate that *sigD* is allelic to the *flaB* locus, which was initially isolated as a locus affecting flagellin expression (G. F. Grant and M. I. Simon, J. Bacteriol. 99:116–124, 1969).**

The specificity of promoter recognition by bacterial RNA polymerases is determined by proteins known as σ factors that interact with the core RNA polymerase (35). These factors are now known to be involved in a variety of developmental and physiological transitions in many different bacterial species. The $\sigma^D$ factor of *Bacillus subtilis* is present and active in vegetatively growing cells and controls the coordinate expression of a regulon of genes from a distinct set of promoter sequences, designated $P^{28}$ or $P^D$ (12, 25). Disruption of the $\sigma^D$ gene produces cells that are viable but do not synthesize flagellin protein and grow as long filaments (16). Subsequent studies have revealed that the *B. subtilis* flagellin gene (*hag*) is transcribed solely from a $P^D$ promoter (25). In addition, enteric bacteria have been found to contain a counterpart to $\sigma^D$, $\sigma^F$, that transcribes certain flagellar and chemotaxis genes from promoters that bear the $P^D$ consensus (4). These results suggest that the *B. subtilis* $\sigma^D$ regulon may include genes for elements of the flagellar, motility, and chemotaxis system (15).

The filamentous phenotype of a strain lacking $\sigma^D$ suggests that $\sigma^D$ may be involved in the synthesis of one or more of the autolysins; these enzymatic activities are responsible for autolysis of the bacterial cell wall, and loss of their function results in filamentous growth (6, 10, 11, 33). In fact, the lack of flagellin expression in *B. subtilis* has often been found to correlate with a lack of autolysin expression. The autolytic enzymes of bacilli have been proposed to participate in a variety of different processes, including sporulation (11), flagellin synthesis (6, 10, 36), and DNA-mediated transformation (2, 34, 46). Additionally, *B. subtilis* mutant strains partially deficient in autolytic enzyme activity have been shown to be hyperproducers of protease and α-amylase (6, 44). However, in most of these studies it has not been clear whether the pleiotropic phenotypes observed in these strains result from a single mutation at a regulatory locus affecting all these functions or are due to indirect effects resulting from the lack of autolysin in the cell.

To gain more information about the role of the $\sigma^D$ factor in expression of specific cellular functions, we examined flagellar synthesis, motility, chemotaxis, and autolysin expression in several *B. subtilis* strains that lack $\sigma^D$ or bear reduced levels of that factor.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The *B. subtilis* strains used in this study are listed in Table 1. Plasmid subclones were grown in *Escherichia coli* HB101 [*hsdS20* ($r_B^-$ $m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* ($Sm^r$) *xyl-5 mtl-1 sup E44* $F^-$ $\lambda^-$] and TG-2 [Δ(*lac pro*) *supE thi hsdD5* F′(*traO36 proA proB lacI*$^q$) ZΔ*M15 recA* $EcoK^-$]. Integrational vectors pLM5 and pLM112 were subcloned from pJH6-2 (16), and pLM3 contains the insert from pJH1-1 (16). Restriction fragments were gel purified and cloned into the integrational plasmid vector pJM102 (16). *B. subtilis* strains were grown in Penassay broth media (PA) or on tryptose blood agar base (TBAB) (Difco Laboratories) plates unless otherwise indicated. Chloramphenicol (5 μg/ml) was added where appropriate. *E. coli* strains were grown on Luria broth (23).

**Materials.** Rabbit antisera to the *B. subtilis* $\sigma^D$ factor (17) and flagellin protein (30) were prepared previously. Antiserum to the *B. subtilis* $\sigma^A$ ($\sigma^{43}$) factor was generously provided by Roy Doi (University of California, Davis). $Na^{125}I$ and *S*-adenosyl[*methyl*$^{-3}$H] methionine (15 Ci/mmol) were purchased from Amersham Corp. $^{125}I$-labeled *Staphylococcus aureus* protein A was prepared as described by Hunter and Greenwood (20). Nitrocellulose was obtained from Schleicher & Schuell Co.

**Construction of plasmid integrants.** Intregrational vectors

* Corresponding author.

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

| Strain | Genotype | Source or derivation |
|---|---|---|
| CB1 | *trpC2* | E. Ferrari, I168[a] |
| CB100 | *sigD*::pLM5 *trpC2* CAT | Tfm(CB1:pLM5, Cm$^r$)[b]; DP-I[a] (16) |
| CB101 | *sigD*::pLM112 *trpC2* CAT | Tfm(CB1:pLM112, Cm$^r$); MP-I[a] (16) |
| CB103 | *sigD*::pLM3 *trpC2* CAT | Tfm(CB1:pLM3, Cm$^r$); this study |
| M104 | *che trpC2 his met* | T. Leighton (30) |
| OI1085 | *trpC2 his met* | G. W. Ordal (30) |
| OI1100 | *cheR trpC2 his met* | G. W. Ordal (30) |
| OI2069 | *sigD*::pLM5 *trpC2 his met* | Tfm(OI1085:CB100, Cm$^r$); this study |
| JH648 | *trpC2 phe-1 spo0B136* | T. Leighton |
| BG40 | *ura argC metC* | E. Ferrari |
| 1A6 | *ilvA1 pyrD1 thyA1 thyB1 trpC2* | BGSC[c] |
| fla-TS4 | *flaA4 hag-1 lys trpC2* | BGSC (14) |
| fla-TS2 | *flaB2 lys trpC2* | BGSC (14) |

[a] Name given to strain previously.
[b] Tfm( ), Transformation of (recipient strain: with plasmid listed, selecting for given resistance).
[c] BGSC, *Bacillus* Genetic Stock Center.

containing an internal portion of the *sigD* gene (pLM5 [16]), the 5′ half of the gene (pLM112 [16]), or the 3′ end of the gene (pLM3; see Fig. 7) were cut at a unique site and ligated to form concatamers (8). The multimeric integrational vectors were then used to transform strain CB1 to chloramphenicol resistance, giving rise to integrant strains CB100 (previously called DP-I [16]), CB101 (previously called MP-I [16]), and CB103, respectively. Genomic Southern blot analysis was performed as previously described (16) to confirm that the expected integrational event had occurred. The integrational events at the chromosomal *sigD* locus are depicted schematically in Fig. 1.

**Immunoblot analysis.** The levels of $\sigma^D$ ($\sigma^{28}$), flagellin, and $\sigma^A$ ($\sigma^{43}$) proteins in the various strains were determined by Western immunoblot analysis with iodinated protein A. The assay was determined to be linear by including a standard of increasing amounts of $\sigma^D$ holoenzyme for the $\sigma^D$ immunoblot. For the flagellin and $\sigma^A$ immunoblots, increasing amounts of CB1 extract were used as a standard. Frozen cell pellets, representing 10 to 120 ml of cell culture grown to an $A_{600}$ of 0.4 to 5.4 in 2× SG sporulation medium (37), were suspended in 0.4 ml of lysis buffer and lysed as described previously (16). Protein concentration was assayed with a Bio-Rad protein assay kit with bovine serum albumin as a standard. Samples (50 μg) of protein from each lysate were resolved by electrophoresis on 12.5% sodium dodecyl sulfate–polyacrylamide gels and electrophoretically transferred to nitrocellulose (40). The nitrocellulose was stained with Ponceau S (Sigma) to monitor transfer efficiency and placed into blocking buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Nonidet P-40, 2% nonfat dried milk, and 5 mM sodium azide) for 1 h. The primary antibody incubation contained a 1:500 dilution of $\sigma^D$ antiserum or a 1:1,000 dilution of flagellin or $\sigma^A$ antiserum in blocking buffer. The primary incubation was done at room temperature, overnight for the anti-$\sigma^D$ blot or 2 h for the anti-flagellin and anti-$\sigma^A$ blots. Nitrocellulose filters were rinsed three times for 15 min each in blocking buffer before being incubated at room temperature for 1 h in a 1:1,000 to 1:5,000 dilution of $^{125}$I-labeled protein A in blocking buffer. The blot was then rinsed three times for 30 min each in blocking buffer and dried, and the reactive bands were visualized by autoradiography on XAR film with an intensifying screen (the film was preflashed for the anti-$\sigma^D$ blot). Occasionally, increased wash times were required to remove nonspecifically bound $^{125}$I-labeled protein A. Quantitation of the reactive material was accomplished by densitometric analysis of the exposed film. Several exposures of the same blot were taken to insure that the densitometric reading from each band was within the linear range of exposure. Anti-flagellin and anti-$\sigma^A$ immunoblots were also quantitated by excising specific bands from the nitrocellulose and counting them in an LKB 1272 clinigamma counter.

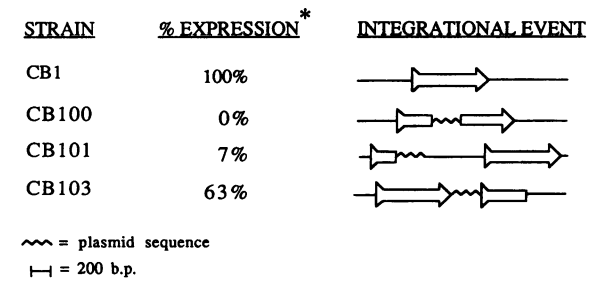


FIG. 1. Diagrammatic representation of the integrational event at the chromosome for each of the *sigD* mutant strains used in this study and the parent strain, CB1. The arrow represents an intact *sigD* open reading frame. The percent expression is the $\sigma^D$ protein level at the end of exponential growth for each strain relative to that of the parent strain.

**Swarm plates.** Chemotaxis and motility were judged by the swarm size of strains patched onto swarm plates (29). Swarm plates contained 0.35% Bacto-Agar (Difco), $4 \times 10^{-4}$% tryptone (Difco), 0.1 mM mannitol, 10 mM potassium phosphate (pH 7.0), 1 mM $(NH_4)_2SO_4$, and 0.1 mM tryptophan. Strains to be tested were patched onto swarm plates, which were placed into a container lined with wet bench paper and covered with Saran Wrap to prevent dehydration, and incubated at 37°C.

**Capillary assays.** Chemotaxis and motility were also studied by capillary assay as described by Ordal and Goldman (27). Bacteria were grown to late-exponential phase in Luria broth at 37°C, and 0.1 ml was used to inoculate 5 ml of minimal medium for overnight growth at 30°C without shaking (10 h). Minimal medium contained 50 mM potassium phosphate (pH 7.0), 1 mM $MgCl_2$, 3 mM $(NH_4)_2SO_4$, 1× sporulation salts (1,000× sporulation salts is 0.14 M $CaCl_2$, 0.01 M $MnCl_2$, 0.2 M $MgCl_2$), 0.3 mM tryptophan, and 0.02 M sorbitol. The next morning a portion of the stationary-phase culture was removed and replaced with the same volume of minimal medium such that the $A_{600}$ of the starting

culture would be 0.15. Bacteria were then grown for 2.5 h at 37°C with high aeration before the addition of 0.05 ml glycerol-lactate (1.87 g of 60% sodium lactate syrup, 1 g of glycerol brought to 20 ml with deionized, distilled water). Growth was continued for 15 min. The bacteria were harvested and washed in chemotaxis buffer. Chemotaxis buffer contained 10 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 0.05% glycerol, 5 mM lactate, 0.14 mM $CaCl_2$, and 0.3 mM $(NH_4)_2SO_4$ (27). Cells were spun for 5 min in a clinical centrifuge kept at 37°C, and the supernatant was removed. The cell pellet was then gently suspended in 5 ml of chemotaxis buffer, also kept at 37°C, and this procedure was repeated three times.

Capillary assays were performed by the method of Adler (1) with 1-μl microcapillaries and a modified pond apparatus (S. Chasalow, Master's thesis, University of California, Berkeley, 1988). Microcapillaries contained an unidentified attractant (26; this study) and were therefore soaked overnight in deionized, distilled water and rinsed four times in deionized, distilled water before being filled with buffer or attractant (1). Ponds contained a bacterial suspension at an $A_{600}$ of $5 \times 10^{-4}$ in chemotaxis buffer. Microcapillaries containing only chemotaxis buffer or 1 mM proline in chemotaxis buffer were inserted into the pond for 20 min. Ponds were maintained at 37°C and at 100% relative humidity. Bacteria accumulating within the microcapillary were expelled into 0.5 ml of T broth (1% tryptone and 0.5% NaCl). The suspension was then added to 3 ml of T agar (T broth with 1.5% agar) at 50°C, poured onto T-agar plates and incubated at 37°C overnight. The next morning bacterial colonies were counted and used as a measure of motility and chemotactic ability (1). Motility was measured as the number of bacteria that accumulated in a microcapillary containing the same buffer as the bacterial pond, whereas chemotaxis was measured as the ratio of the number of bacteria that accumulated in a microcapillary containing an attractant to the number of bacteria that accumulated in a microcapillary containing buffer.

**MCP methylation.** Methylation of the methyl-accepting chemotaxis proteins (MCPs) was determined both in vivo and in vitro by using radiolabeled methionine. In vivo methylations were done by the method of Ullah and Ordal (41). In vitro methylations of partially purified membranes were done by using crude cytoplasmic extract as the source of the methyltransferase enzyme (13). Methylated samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and prepared for fluorography (21).

**Autolysin assays.** Total autolysin activity in the various strains was determined by two methods. Endogenous activity was measured by monitoring the self-digestion of cell wall preparations, which contain most of the cell content of autolysin. These cell wall preparations will be referred to as native walls. Native walls were prepared as described by Fein and Rogers (11) from cells grown to an $A_{600}$ of 1.0 to 1.2 (mid-log phase). Cells were collected and disrupted by sonication. The supernatant fluid was removed by sedimentation, and the pellet was washed repeatedly with TL buffer (0.05 M Tris, 0.024 M LiCl [pH 7.1]). The final cell wall preparation was resuspended to an $A_{540}$ of 0.2 in reaction buffer (TL buffer plus 0.01 M $MgCl_2$). Autolysin activity was measured by incubating samples in reaction buffer at 40°C and monitoring the decrease in the $A_{540}$. A unit of enzyme was defined as the amount required to decrease the turbidity of the substrate by 0.001 optical density unit per min (11).

Total autolytic activity was also determined by partially purifying autolysin from the native walls and adding it back to cell walls that lacked the enzymatic activity. Autolysin was extracted from native walls by cellular autolysis (45). After 90% of the turbidity had been lost (approximately 2 h), the supernatant fluid containing autolysin activity was decanted after centrifugation and used in subsequent experimentation. Substrate cell walls lacking autolytic activity were prepared by heat inactivation of the endogenous autolytic activity in native walls. Native walls were suspended in TL buffer to an $A_{540}$ of 0.2, and the suspension was incubated at 100°C for 30 min (6). The substrate walls were then collected by centrifugation and suspended in reaction buffer to the appropriate optical density with the addition of the autolysin extract to be tested.

To determine the endogenous activity of two autolysins, *N*-acetylmuramyl-L-alanine amidase (amidase) and endo-β-*N*-acetylglucosaminidase (glucosaminidase), native walls were prepared in buffers shown to be optimal for the activity of each enzyme. Native walls prepared in 0.012 M sodium borate containing 0.075 M LiCl (BL buffer) at pH 8.6 were assayed for amidase activity by incubating the native walls in BL buffer at 37°C and monitoring the rate of autolysis spectrophotometrically (11). Similarly native walls prepared in 0.1 M sodium acetate buffer at pH 5.6 were assayed for glucosaminidase activity by incubating them at 37°C in sodium acetate buffer containing 0.01 M $MgCl_2$ and monitoring the decrease in turbidity (11).

**Sporulation frequency.** The sporulation frequency was determined by comparing the viable counts obtained from test strains grown in sporulation medium with and without chloroform treatment. Strains were grown in 2× SG sporulation medium (37), with the addition of 100 μg of tryptophan per ml for 24 h at 37°C. Duplicate 4-ml samples were placed into separate test tubes, and 100 μl of chloroform was added to one tube. Samples were vortexed for 30 s and the chloroform was allowed to settle before the cells were diluted and plated onto TBAB plates. The percent sporulation was defined as the ratio of chloroform-treated CFU to untreated CFU.

**Genetic techniques.** PBS1 transduction was performed as described by Hoch et al. (19). Chromosomal and plasmid transformation was performed by the method of Anagnostopoulos and Spizizen (3). Chromosomal DNA was prepared by the method of Massie and Zimm (24). Plasmid DNA amplified in *E. coli* was prepared by alkaline lysis (23) and concatermerized before transformation of *B. subtilis* (8).

## RESULTS AND DISCUSSION

**Measurement of $\sigma^D$ and flagellin protein levels in *sigD* mutant strains.** Prior studies had failed to detect significant amounts of $\sigma^D$ in either the *sigD* gene disruption strain (CB100) or the upstream integrant strain (CB101) (16). CB101 had reduced levels of $\sigma^D$ protein, averaging 7% of the wild-type level at the end of exponential growth ($t_0$), whereas CB100, as expected, had no detectable $\sigma^D$ protein (Fig. 2). Note that all of our measurements of $\sigma^D$ levels were done at $t_0$. Previous studies have shown that $\sigma^D$-dependent functions are maximally expressed at the end of logarithmic growth (25; V. L. Singer, Ph.D. thesis, University of California, Berkeley, 1987), and recent work has demonstrated that $\sigma^D$ protein levels are also maximal shortly after $t_0$ (Márquez and Chamberlin, unpublished data). Because the upstream integrant CB101 contains an intact copy of *sigD*, it appears that *cis*-acting elements more than 637 base pairs upstream of the *sigD* structural gene are required for the synthesis of wild-type levels of the $\sigma^D$ polypeptide. This result suggests that

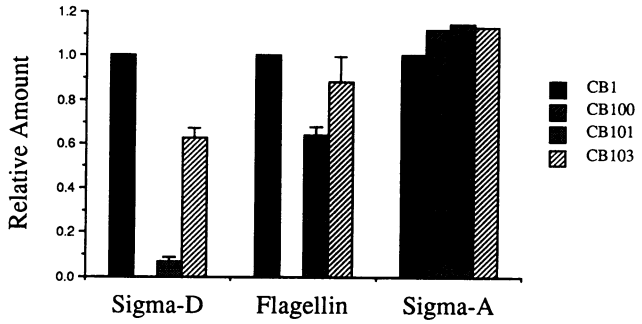


FIG. 2. Radioimmune assays of $\sigma^D$, flagellin, and $\sigma^A$ levels in mutant strains. Levels of $\sigma^D$, flagellin, and $\sigma^A$ were detected by immunochemical analysis by specific antisera and $^{125}$I-labeled protein A. Results were quantitated by autoradiography and densitometry ($\sigma^D$), or by directly counting the reactive bands (flagellin, $\sigma^A$). Values were normalized to those of the parent strain, CB1. Results given for $\sigma^D$ and flagellin are the results of three experiments (error bars are given where possible), whereas the results given for $\sigma^A$ are the result of a single experiment.

the *sigD* gene may be part of an operon of genes, a result consistent with the presence of four open reading frames upstream of the *sigD* gene (16). The downstream integrant CB103 also has somewhat reduced levels of $\sigma^D$ at $t_0$, averaging about 60% of the wild-type level. The effects of the downstream insertion in somewhat reducing $\sigma^D$ levels may reflect some decrease in mRNA stability due to the insertion. Studies of the size, stability, and steady-state levels of the $\sigma^D$ mRNA should clarify each of these effects.

Previous studies had shown that the null mutant (CB100) failed to produce flagellin, whereas the upstream integrant (CB101) produced apparently wild-type levels of this protein despite greatly reduced $\sigma^D$ levels (16). These studies, however, were not quantitative. Flagellin levels in these strains were therefore quantitatively compared by Western analysis with iodinated protein A. As an internal control, antiserum to the primary sigma factor ($\sigma^A$) was used in a parallel immunoblot (Fig. 2). Although the levels of $\sigma^D$ and flagellin varied considerably among strains, the level of $\sigma^A$ remained within 10% of the wild-type level, as expected. In the null mutant (CB100) the lack of $\sigma^D$ expression resulted in a complete lack of detectable flagellin synthesis. However, low-level synthesis of the $\sigma^D$ polypeptide resulted in only slightly decreased levels of the flagellin protein. In the upstream integrant (CB101), 7% of the wild-type level of $\sigma^D$ protein resulted in the synthesis of 64% of the wild-type flagellin, whereas 63% production of $\sigma^D$ in the downstream integrant (CB103) gave rise to 88% of wild-type flagellin synthesis (Fig. 2). The lack of a direct correlation between the level of $\sigma^D$ in the cell and the amount of flagellin protein produced suggests that wild-type $\sigma^D$ levels are in excess of those needed to efficiently transcribe the *hag* promoter (16), consistent with the observation that the *hag* $P^D$ is relatively strong (25).

**Alterations in the chemotaxis and motility of $\sigma^D$ mutant strains.** Analysis of alterations in chemotaxis and motility in $\sigma^D$ mutants is complicated by the fact that these functions depend on the presence of flagella. Hence cells lacking $\sigma^D$ protein and flagellin are not motile and therefore cannot swim up a chemical gradient. However, the upstream (CB101) and downstream (CB103) integrants synthesize $\sigma^D$ protein and express flagellin, thereby allowing the characterization of chemotaxis and motility in these strains directly. Swarm plate assays demonstrated a decrease in swarm size (55 ± 10% of the wild-type size) for both the CB101 and CB103 mutant strains (Fig. 3). This is consistent with a decrease in chemotactic ability or motility in these strains. As expected, the null mutant (CB100) showed no motility or chemotaxis by this assay.

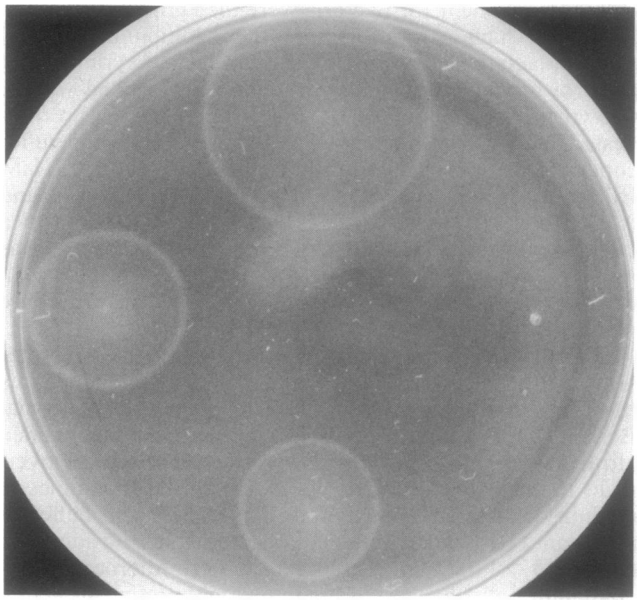

FIG. 3. Swarm plate analysis of parent and mutant strains. From the top and proceeding clockwise: strains CB1, CB100, CB101, and CB103. Strains were patched onto swarm plates and incubated at 37°C for 16 h at 100% relative humidity.

Capillary assays were utilized in an effort to differentiate between defects in chemotaxis and motility for the mutant strains. Chemotaxis in *B. subtilis* is often measured as the ratio of the number of bacteria that accumulate in a microcapillary containing an attractant to the number of bacteria that accumulate in a microcapillary containing only buffer (44). The magnitude of the buffer only or the background number is used as a direct measure of motility (1). However, the background numbers obtained for the different strains varied greatly (Table 2), and the number of cells entering the capillary did not increase linearly with time (data not shown). Conversely, the accumulation of cells in a microcapillary containing attractant (1 mM proline) was reproducible (Table 2), and the rate was constant over a period of 30 min (data not shown). Therefore we have used the number of bacteria that accumulated after a 20-min incubation as a combined measure of chemotaxis and motility in the parent and mutant strains without differentiating between the two functions. These results are consistent with those obtained by swarm plate assay; whereas the upstream and downstream integrant strains expressed only slightly reduced

TABLE 2. Accumulation of bacteria in microcapillaries after 20 min of incubation in bacterial pond

| Strain | CFU | |
|---|---|---|
| | Buffer | 1 mM proline |
| CB1 (parent) | 2.7 ± 4.2 | 1,337 ± 15.6 |
| CB100 (null mutant) | 2.0 ± 1.4 | 2 ± 1.4 |
| CB101 (upstream integrant) | 36 ± 34 | 962 ± 16.8 |
| CB103 (downstream integrant) | 12.5 ± 2.1 | 939 ± 28.0 |

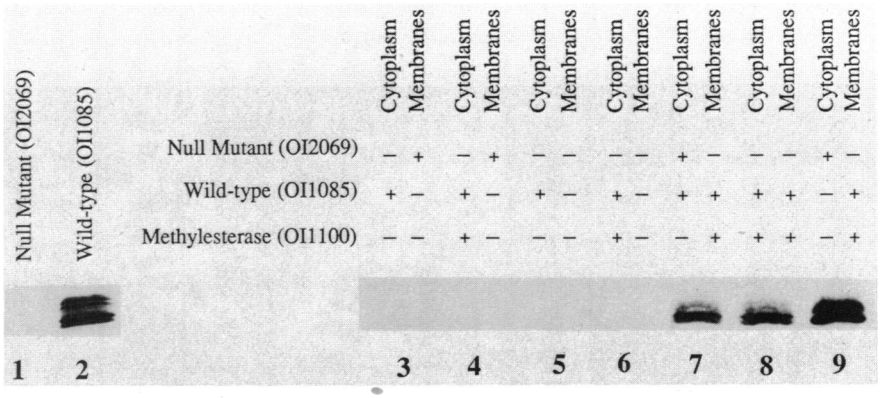


FIG. 4. In vivo and in vitro methylation of MCPs. Lanes: 1, in vivo methylation pattern obtained from a strain containing the CB100 mutation; 2, in vivo methylation pattern of the parent strain; 3 through 9, in vitro mixing experiments. Null mutant and wild-type cytoplasm and membranes were isolated and mixed as indicated.

levels of flagellin, they were slightly impaired in their motility and/or chemotaxis.

For the upstream integrant this defect in motility and chemotaxis might be related to the somewhat reduced level of flagellin in the strain or might result from the reduced level of $\sigma^D$ protein, leading directly or indirectly to lower expression of motility and chemotaxis functions. Alternatively, the defect may be the result of reduced expression of genes downstream of *sigD*, whose products may be involved in these functions. The latter possibility is supported by the fact that in the downstream integrant $\sigma^D$ and flagellin levels were nearly normal (Fig. 2) and yet a similar impairment in chemotaxis and motility was found.

Although chemotaxis cannot be studied directly in the null mutant, we can study the proteins responsible for sensing the presence of attractants and repellents in the surrounding environment, the MCPs (28). These proteins undergo methyl group transfer in response to attractants and can be easily identified as radiolabeled proteins migrating at about 70 kilodaltons on a sodium dodecyl sulfate-polyacrylamide gel after radiolabeling of cells with *S*-adenosyl[*methyl*-$^3$H]methionine (28, 39). We monitored the methylation of MCPs as a way of detecting the presence of these proteins in the cell.

Wild-type and *sigD* null mutant cells were radiolabeled, total protein was extracted, and the proteins were resolved on a denaturing gel. Whereas the characteristic pattern of radiolabeled MCPs was found in total protein isolated from the wild-type strain (Fig. 4, lane 2), no MCPs were observed in protein isolated from the null mutant (lane 1). This could be due to a lack of MCPs or a deficiency in either the methyltransferase or methylesterase activities of the mutant strain.

To determine which proteins were missing in the null mutant, in vitro complementation assays were performed to directly measure MCP levels and methyltransferase activities. Extracts of each strain were fractionated into membrane and soluble fractions. Membrane fractions from a chemotactically wild-type strain, OI1085, contain the MCPs, whereas the cytoplasm from this strain contains the methyltransferase activity. If the mutant membranes contain wild-type MCPs, incubation of mutant membranes with wild-type cytoplasm will lead to the production of 70-kilodalton radiolabeled proteins. Conversely, if the mutant cytoplasm contains active methyltransferase, the MCPs in wild-type membranes will be radiolabeled. In the former case a negative result can also be obtained if the MCPs of the null mutant are fully methylated and the wild-type cytoplasm is low in methylesterase. Then methylation by *S*-adenosyl[*methyl*-$^3$H] methionine might not be detected. If, however, cytoplasm from OI1100, a methyltransferase mutant whose cytoplasm is rich in methylesterase (G. W. Ordal and H. M. Parker, unpublished results), is included in the reaction mixture the additional methylesterase will remove unlabeled methyl groups from the MCPs, allowing the in vitro methylation to proceed normally. Wild-type cytoplasm with or without methylesterase from OI1100 cytoplasm was unable to methylate *sigD* mutant membranes (Fig. 4, lanes 3 and 4). However, null mutant cytoplasm was able to methylate wild-type and OI1100 membranes without additional methylesterase (Fig. 4, lane 9). These studies demonstrate that $\sigma^D$ is required for the synthesis of wild-type MCPs but is not required for production of the methyltransferase or methylesterase. An alternate possibility is that although the MCPs are synthesized in the null mutant they are not inserted into the cell membrane.

**Autolytic enzyme expression.** Microscopic observation revealed that the null mutant no longer grew as single, rod-shaped cells but instead grew as long, tangled filaments (Fig. 5A). Since the lack of flagellin expression has often been found to be correlated with a lack of autolysin expression and filamentous growth (6, 10, 11, 33), we monitored autolysin activity in the mutant strains. The endogenous autolytic activity of the null mutant was 33% of the activity of the parent strain (Fig. 6A). The residual activity was heat labile, as demonstrated by the lack of activity in native walls prepared from CB100 and heated to 100°C before being assayed (Fig. 6A). The upstream and downstream integrants also produced reduced levels of autolytic activity, expressing 66% of the wild-type level (Fig. 6A).

The decrease in autolytic activity observed in these strains could be due to a lack of autolysin synthesis or to an abnormal cell wall structure that is no longer recognized by the autolytic enzymes as a substrate for hydrolysis. The latter possibility was tested by incubating heat-inactivated native walls (substrate walls) from the null mutant in the presence of autolysin extracted from the parent strain. The mutant substrate walls were hydrolyzed by the wild-type autolysin extract (Fig. 6B) with kinetics similar to those obtained with wild-type cell walls as a substrate (data not shown). These results demonstrate that total autolytic activity in the *B. subtilis* cell is dependent on the synthesis of the $\sigma^D$ factor.

To determine whether the decrease in total autolytic activity was due to a decrease in the activity of amidase

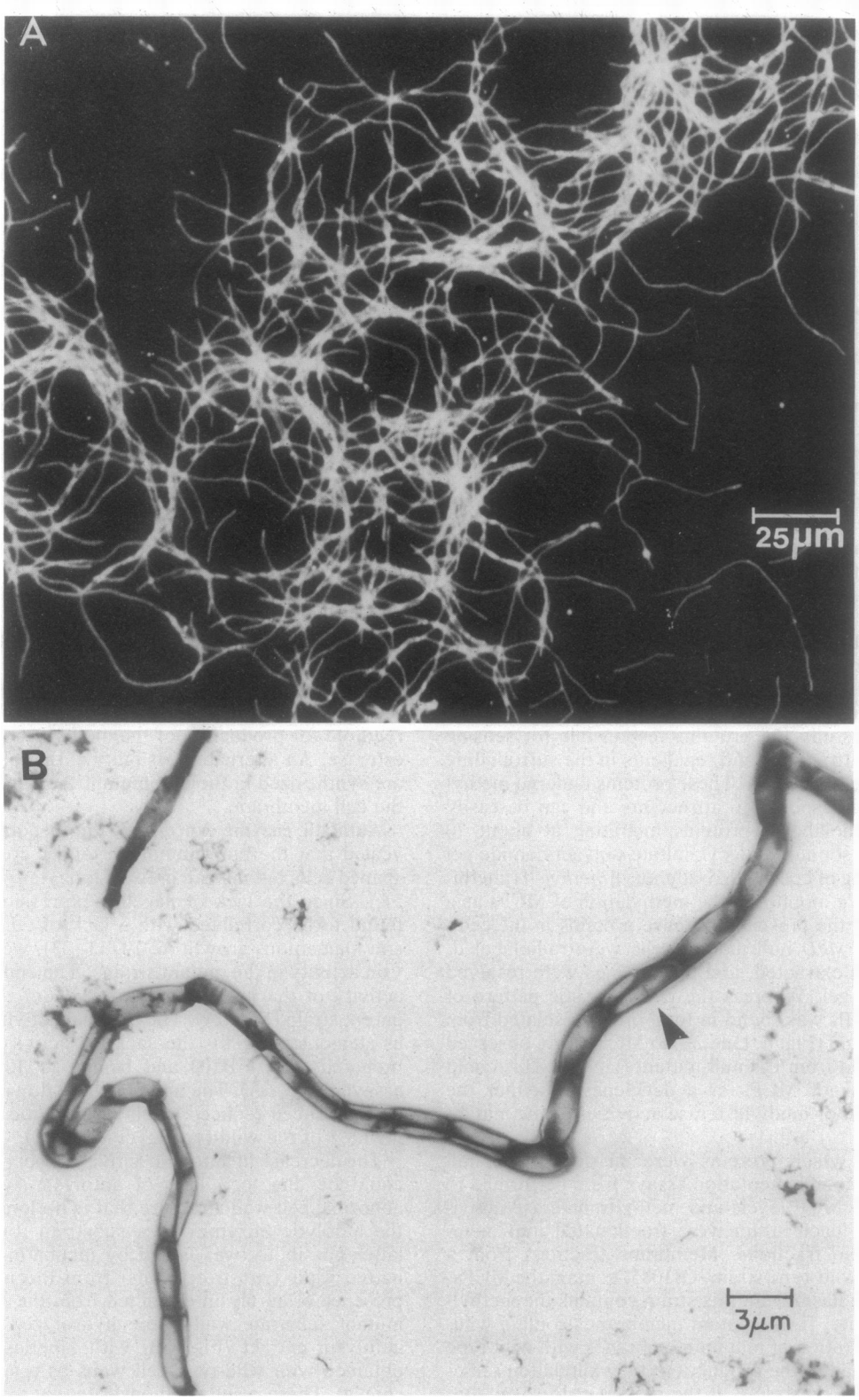


FIG. 5. Microscopic analysis of the null mutant. (A) Null mutant (CB100) cells as seen by light microscopy (×400 magnification). (B) Electron micrograph of a single filament (×1,900 magnification). A normal septum is indicated by the arrow.

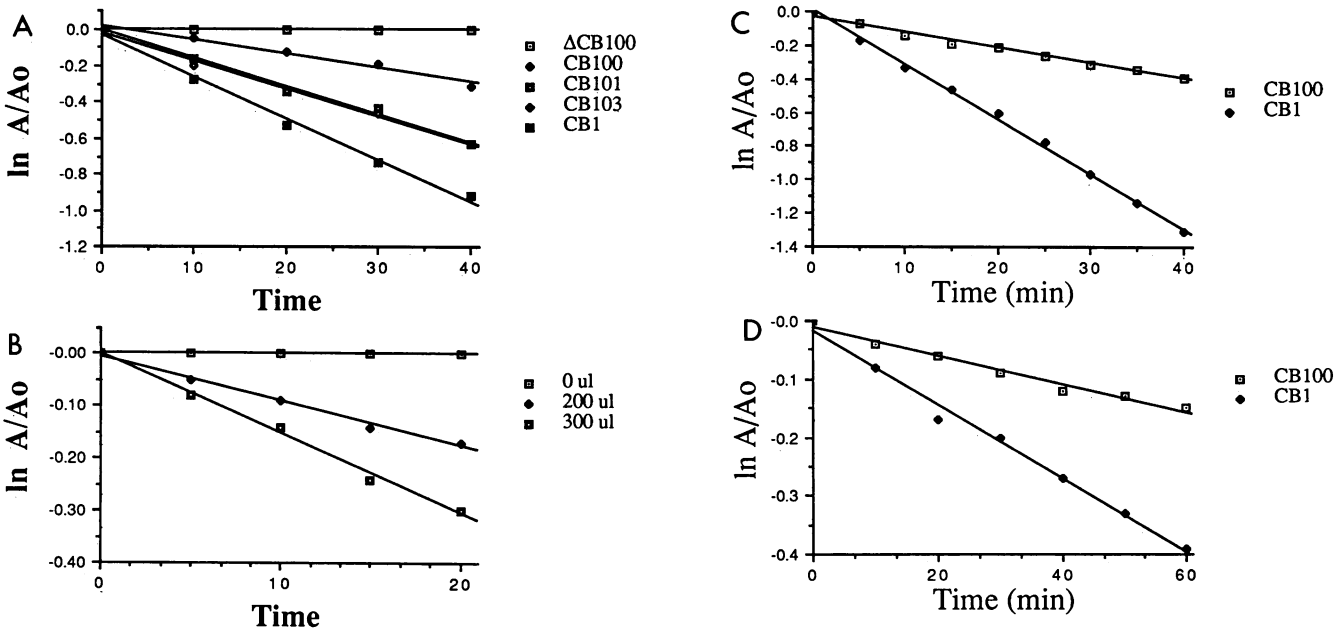


FIG. 6. Determination of autolytic enzyme levels. (A) Endogenous autolytic activity in strains CB1, CB100, CB101, and CB103 and heat-inactivated native walls of CB100 (ΔCB100): Each data point represents three to five experiments. The standard deviation for each point ranged between 2 and 15% of the value given. (B) Hydrolysis of CB100 substrate walls by wild-type autolysin (0, 200, and 300 μl of autolysin extract added). Data presented are the results of four experiments. The standard deviation for each point ranged between 1 and 3% of the value given. (C) Amidase activity assayed at pH 8.6. Each point given is the average of two experiments. (D) Glucosaminidase activity measured at pH 5.6. Each point given is the average of two experiments.

and/or glucosaminidase, native walls were prepared from the parent and null mutant strains under conditions that were optimal for each enzymatic activity. Null mutant native walls exhibited a decrease in both activities (Fig. 6C and D). Amidase activity in the null mutant was 29% of that of the parent strain, whereas glucosaminidase activity was 40% of the wild-type level; that is, there were significant levels of both autolysin activities in the null mutant. The residual levels of each activity detected in CB100 may be due to either partial expression of the amidase and/or glucosaminidase or the presence of other activities in the cells that react in these assays. Hence it cannot be determined from these data whether the effects of $\sigma^D$ on expression of the autolysins is direct, involving transcription of the structural genes by $\sigma^D$ holoenzyme, or indirect, involving transcription of a regulator of the genes or some other second-order effect. It should be noted that the amidase activity is reported to be controlled by a regulatory factor that enhances its activity by about threefold (18); failure to synthesize this factor in the null mutant would be expected to lead to the observed effect on amidase activity.

Filamentous strains containing *div* mutations have also been isolated (42). In *div* mutants, however, filamentous growth is a result of a defect in septum formation and consequently cell division. These defects are not present in the null mutant. Electron microscopic analysis of this strain clearly demonstrated normal septum formation (see arrow in Fig. 5B). Additionally, the growth kinetics of the null mutant and parent strain were identical in both rich and minimal media, indicating that cell division was proceeding at a wild-type rate in the mutant strain (data not shown). Filamentous growth in the null mutant can be entirely attributed to the lack of cell wall separation, probably as a result of the decrease in autolysin production.

**Functions not dependent on the $\sigma^D$ factor.** $\sigma^D$ protein levels are maximal at the end of logarithmic growth (Márquez and Chamberlin, unpublished data) as nutrients become limiting for the cell. At this point in development the cell becomes hypermotile (31), secretes increased amounts of degradative enzymes and antibiotics (32), becomes competent for transformation (9), and initiates a developmental program that ultimately results in the formation of the endospore (22, 32). Interestingly this list includes many of the processes in which bacterial autolysin is thought to have a role (2, 6, 10, 11, 34, 36, 44, 46). Having characterized the $\sigma^D$ mutant strains for their ability to swim and produce autolysin, we

TABLE 3. Sporulation frequency as measured by resistance to chloroform treatment

| Strain | Spores per ml[a] | Cells per ml[b] | % Sporulation[c] |
|---|---|---|---|
| JH648 (Spo⁻ control) | $4.0 \times 10^2 \pm 3.0 \times 10^2$ | $1.2 \times 10^9 \pm 0.3 \times 10^9$ | $5.0 \times 10^{-5}$ |
| CB1 (parent) | $8.0 \times 10^8 \pm 1.3 \times 10^8$ | $7.1 \times 10^8 \pm 0.3 \times 10^8$ | 100 |
| CB100 (null mutant) | $1.0 \times 10^9 \pm 0.2 \times 10^9$ | $1.2 \times 10^9 \pm 0.1 \times 10^9$ | 72 |
| CB101 (upstream integrant) | $8.5 \times 10^8 \pm 2.5 \times 10^8$ | $1.4 \times 10^9 \pm 0.2 \times 10^9$ | 71 |

[a] The number of CFU obtained after chloroform treatment.
[b] The number of CFU obtained without chloroform treatment.
[c] Ratio of the number of chloroform-treated CFU to untreated CFU.

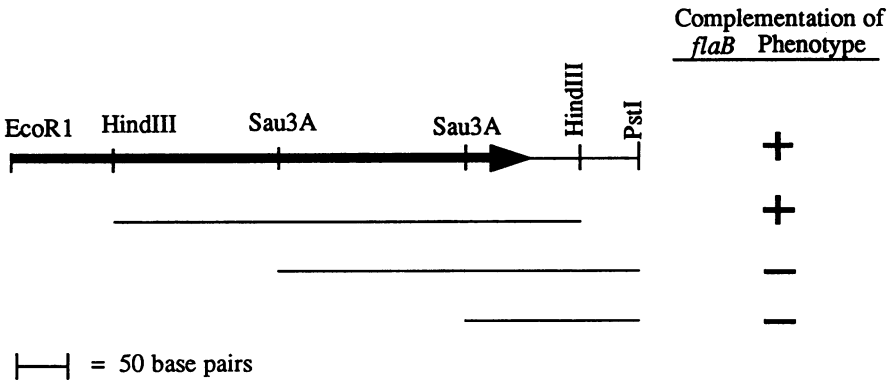


FIG. 7. Complementation of the *flaB* mutation by integration of wild-type *sigD* sequences. Diagrammatic representation of the pLM3 insert and the phenotype generated upon transformation of the *flaB* strain with integrational vectors containing the indicated fragments. The 3′ end of the *sigD* gene is represented by the bold line.

next examined these strains for their ability to sporulate, to synthesize extracellular enzymes, and to become competent for transformation.

Sporulation frequency was measured as the ratio of chloroform-resistant CFU to untreated CFU in wild-type and *sigD* mutant strains. $\sigma^D$ RNA polymerase was not required for normal endospore formation (Table 3). In addition, these strains were catabolite repressed for sporulation, secreted wild-type levels of protease, α-amylase, and antibiotics, and were not significantly impaired in their ability to develop competence (data not shown). One exception was the null mutant, which exhibited a slight but reproducible decrease in protease synthesis.

Although mutations at the *sigD* locus affect flagellin expression, chemotaxis, motility, and autolytic enzyme production, many of the phenotypes thought to be dependent on autolysin activity are normal in $\sigma^D$ mutant strains. Our results suggest that a 67% decrease in *B. subtilis* autolysin levels and the lack of $\sigma^D$ in the cell do not affect the processes of sporulation, extracellular enzyme synthesis, or the competence of the bacterium for transformation.

The *B. subtilis* $\sigma^D$ factor and the heat shock sigma factor from *E. coli*, $\sigma^{32}$, have been shown to display overlapping promoter specificity, leading to the suggestion that $\sigma^D$ might regulate the heat shock response in *B. subtilis* (7). This possibility was tested by comparing the pattern of expression of heat-inducible polypeptides in the null mutant to the pattern obtained with the parent strain after a sudden shift in temperature. The pattern was identical in both strains, although the *dnaK* protein level (5) was reduced in the null mutant 10 min after the shift in temperature (data not shown). This result suggests, that unlike $\sigma^{32}$ in *E. coli*, $\sigma^D$ is not a primary regulator of the heat shock response in *B. subtilis*.

***sigD* is allelic to *flaB*.** A PBS1 lysate prepared on the downstream integrant, CB103, was used to transduce the polyauxotrophic strain BG40 (*ura met argC*) to chloramphenicol resistance. Linkage of the chloramphenicol resistance determinant to *ura* was found, and subsequent transductions with strain 1A6 (*ilvA1 pyrD1 thyA1 thyB1 trpC2*) as the recipient strain placed the *sigD* gene between *pyrD1* and *thyA1* on the bacterial chromosome. The *flaA* (fla-TS4) and *flaB* (fla-TS2) mutations, isolated as loci affecting flagellin expression (14), have been mapped to this region of the chromosome (33). Chromosomal DNA isolated from the downstream disruption (CB103) was used to transform the *flaA* and *flaB* strains to chloramphenicol resistance. Transformant strains were grown in PA broth and characterized by light microscopy for their ability to swim and therefore express flagellin. Motility was found only for the *flaB* transformants, indicating that *sigD* is linked to the *flaB* mutation.

To determine whether the *flaB* mutation was within the *sigD* structural gene, integrational vectors containing the 5′ (pLM112) and 3′ (pLM3) end of the gene and flanking DNA were used to transform the *flaB* strain to chloramphenicol resistance. Rescue of the *fla* phenotype was only found when the 3′ end of the gene, plus an additional 88 base pairs of flanking DNA, was used to transform the *flaB* strain to chloramphenicol resistance. Subcloning of the pLM3 insert onto integrational vectors and subsequent utilization of these constructs in recombination experiments localized the *flaB* mutation within the *sigD* structural gene (Fig. 7), demonstrating that *sigD* and *flaB* are allelic.

## ACKNOWLEDGMENTS

We are grateful to Terry Leighton and Jim Hoch for providing strains and technical advice.

This research was supported by Public Health Service research grant GM12010 (to M.J.C.) and by a National Science Foundation graduate minority fellowship (to L.M.M.).

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