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Nucleotide Sequence and Characterization of a *Bacillus subtilis* Gene Encoding a Flagellar Switch Protein

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The nucleotide sequence of the *Bacillus subtilis fliM* gene has been determined. This gene encodes a 38-kDa protein that is homologous to the FliM flagellar switch proteins of *Escherichia coli* and *Salmonella typhimurium*. Expression of this gene in Che$^+$ cells of *E. coli* and *B. subtilis* interferes with normal chemotaxis. The nature of the chemotaxis defect is dependent upon the host used. In *B. subtilis*, overproduction of FliM generates mostly nonmotile cells. Those cells that are motile switch less frequently. Expression of *B. subtilis* FliM in *E. coli* also generates nonmotile cells. However, those cells that are motile have a tumble bias. The *B. subtilis fliM* gene cannot complement an *E. coli fliM* mutant. A frameshift mutation was constructed in the *fliM* gene, and the mutation was transferred onto the *B. subtilis* chromosome. The mutant has a Fla$^-$ phenotype. This phenotype is consistent with the hypothesis that the FliM protein encodes a component of the flagellar switch in *B. subtilis*. Additional characterization of the *fliM* mutant suggests that the *hag* and *mot* loci are not expressed. These loci are regulated by the SigD form of RNA polymerase. We also did not observe any methyl-accepting chemotaxis proteins in an in vivo methylation experiment. The expression of these proteins is also dependent upon SigD. It is possible that a functional basal body-hook complex may be required for the expression of SigD-regulated chemotaxis and motility genes.

The control of the direction of flagellar rotation is crucial in allowing a bacterial cell to respond to gradients of attractants and repellents. Counterclockwise rotation of flagella promotes the organized formation of the flagellar bundle and results in smooth swimming. Reversal of the flagellar motor so that it rotates in a clockwise direction results in a disruption of the bundle. During this time, the bacterium tumbles until the motor is reversed again. Tumbling behavior serves to reorient the bacterium for the next period of smooth swimming in a random direction. The frequency of tumbling increases when the bacterium is heading in an unfavorable direction and decreases when the bacterium is heading in a favorable direction.

The molecular mechanism behind chemotaxis has been the focus of intense study in the last decade (reviewed in references 5 and 19; see reference 10 for a nomenclature change). Much of the information has been generated from experiments performed on *Escherichia coli* and *Salmonella typhimurium*, and a model has been proposed (2). The separate components involved in chemotaxis, namely, the methyl-accepting chemotaxis proteins (MCPs), the six Che proteins, and the structural components of the flagella (which include the basal body, the hook, and the filament), have been studied genetically and biochemically. In contrast, the biochemical characterization of the switch complex has been hindered by the lack of a suitable purification procedure that allows the proteins to be retained in a complex. Some new features on the cytoplasmic face of the basal body have been recently identified in electron micrographs (4). These structures appear to be associated with the M ring (a component of the basal body) and are also observed in strains lacking the MotA or MotB protein. Direct confirmation that these novel structures contain the switch proteins is not possible because switch mutants do not possess basal bodies (31).

The presence of a switch complex has been inferred from genetic analysis of switch mutants. In *E. coli* and *S. typhimurium*, three proteins that are thought to form part of the switch complex have been identified. They are called FliG, FliM, and FliN. The proteins appear to be required very early in basal body formation, shortly after the M ring has been inserted into the membrane (11). A null mutation in any switch protein gene confers a Fla$^-$ phenotype. Other mutations that confer defects in chemotaxis or that generate paralyzed flagella have also been mapped to these genes (38).

The three genes encoding the switch proteins have been cloned from both *E. coli* and *S. typhimurium*. The proteins are extremely similar to their respective counterparts (12, 15, 21). FliM interacts with the other two switch proteins and with MotB, CheY, and CheZ (38). Experiments with *S. typhimurium* have identified two regions in the FliM protein that can suppress the swimming bias of CheY mutant strains (20).

We have reported previously that the mechanism of chemotaxis in *Bacillus subtilis* is different in some respects from that observed in *E. coli* and *S. typhimurium*; the differences appear to be in the role that methyl transfer plays in excitation and adaptation (33–35). To characterize these differences further, we have been isolating and studying chemotaxis mutants (28, 29). We have identified several loci that appear to affect chemotaxis and motility and have cloned a large chemotaxis operon (28, 41).

All chemotaxis and flagellum genes expressed late in *E. coli* and *S. typhimurium* are regulated by SigF, a minor sigma factor that is the product of the *fliA* gene (16, 27). The SigF protein of *E. coli* is homologous to *B. subtilis* SigD (8). There are some chemotaxis and motility loci in *B. subtilis* that appear to be regulated by SigD. These include the *hag* locus, which encodes flagellin (26), and the *mot* locus (40). In addition, MCPs were not observed in a *sigD* mutant (24).

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Comment or reference
E. coli		
TG1	M13 cloning host	Amersham
K38	Contains pGP1-2	S. Tabor (32)
YK410	Che$^+$	R. Macnab (14)
RP4187	*fliM75*(Am)	R. Macnab (30)
B. subtilis		
OI1318	*his leu*	Che$^+$
OI2273	Tn*917 recE4*	Che$^+$; R. Yasbin
OI2282	Tn*917lacZΩ1831*	*cheX* mutant (40)
OI2283	Tn*917lacZΩ1833*	*mot* mutant (40)
OI2299	Tn*917lacZΩ1846*	*hag* mutant (40)
OI2553[a]	*his leu fliM1*	4-bp insertion in *fliM*; this work
OI2554[a]	*his leu recE4* Tn*917*	Che$^+$ *recE4*; this work
OI2555[a]	*his leu fliM1 recE4* Tn*917*	This work

[a] Isogenic to OI1318.

Other loci are transcribed by a major vegetative SigA-containing RNA polymerase. These include the chemotaxis operon discussed above and the *cheX* locus (40). The regulation of the *cheR* locus has not been determined, but normal levels of CheR were present in a *sigD* mutant (24). In *S. typhimurium*, the *cheR* gene is part of a class III transcript (16), so its transcription is dependent upon FliA (SigF). In *B. subtilis*, the transcription of the *cheR* gene is not dependent upon SigD (the SigF homolog). These data suggest that there are some differences in the regulation of at least some chemotaxis genes in *B. subtilis*.

We report here the nucleotide sequence of a *B. subtilis* gene that encodes a protein that is homologous to the FliM protein of *E. coli* and *S. typhimurium*. We inactivated the gene and generated a Fla$^-$ mutant phenotype consistent with the hypothesis that the protein forms part of the switch complex in *B. subtilis*. Interestingly, we found that the isolated mutant does not express genes from the SigD-regulated *hag* and *mot* loci. In addition, no MCPs were observed in the mutant. We suggest that an intact basal body may be required for the induction of *sigD* at the end of exponential growth.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the *E. coli* and *B. subtilis* strains used.

Plasmids. Plasmids pAZ203 and pGO103 have been described before (41). Plasmid pAZ203 contains a 5-kb *Pst*I fragment of *B. subtilis* DNA from the *che* operon. This DNA overlaps the 7.7- and 4.0-kb *Eco*RI fragments. Plasmid pGO103 contains the 4.0-kb *Eco*RI fragment from the *che* operon. A 1.46-kb *Dra*I fragment was isolated from pAZ203. This fragment carries the entire *fliM* gene. The location of this *Dra*I fragment in the *che* operon and the location of the open reading frames (ORFs) reported in this paper are shown in Fig. 1.

Complementation experiments in *E. coli* and *B. subtilis* were performed with derivatives of plasmid pSI-1 (39). This plasmid confers Cm^r and is a *B. subtilis-E. coli* shuttle expression vector. Expression from the cloned insert in this vector is under the control of the spac promoter. It can be induced by the addition of isopropylthiogalactopyranoside (IPTG; Sigma). The expression vector used to visualize the FliM protein was a derivative of plasmid pT7-5 (32). Gene conversion in *B. subtilis* was performed with plasmid pUB18 (36a). This plasmid confers Kn^r and was used as the vector when the *fliM* gene was mutagenized. Other plasmids are described in the text.

DNA sequence and analysis. *B. subtilis* DNA fragments from pAZ203 and pGO103 were subcloned into M13 phages M13mp18 and M13mp19 to generate specific clones. Other templates were derived from exonuclease III-generated deletions of the 4.0-kb *Eco*RI fragment in M13mp18 (9). Sequencing was performed with a Sequenase sequencing kit (US Biochemicals) and [^{35}S]dATP (Amersham). Electrophoresis was performed on polyacrylamide gradient gels (1). Analyses of DNA sequences and alignments of proteins were performed with computer software from DNASTAR, Madison, Wis. Hydropathy profiles were generated with the program AMPHI (University of Illinois Biotechnology Center). The algorithms used were those of Kyte and Doolittle (17).

Transformation. Competent cells of *E. coli* were prepared by an RbCl procedure (7). Competent *B. subtilis* cells were prepared as described previously (37). The *recE4* mutation was introduced into OI1318 and OI2553 by congression with chromosomal DNA from OI2273 and selection for resistance to the macrolide-lincosamide-streptogramin S-type antibiotics (1 μg of erythromycin and 25 μg of lincomycin per ml). The transposon insertion in OI2273 does not, by itself, confer a Che$^-$ phenotype. Transformants were screened for sensitivity to 0.05 μg of mitomycin C per ml.

Expression experiments. The procedure of Tabor and Richardson (32) was used. Plasmid pT7-5 derivatives were introduced into competent *E. coli* K38(pGP1-2) cells. Plasmid-encoded proteins were expressed after incubation of transformants at 42°C in the presence of rifampin, and L-[^{35}S]methionine was added. Cells were solubilized and electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels. The labeled proteins were visualized by autoradiography.

Other procedures. Standard techniques were used for plasmid isolation, fragment purification by electroelution, and ligation (22). The 3′-recessed ends of DNA generated after digestion with *Eco*RI and *Sal*I were filled in with Sequenase enzyme in the presence of 20 μM deoxynucleotide triphosphates. Chromosomal DNA was prepared as described by Marmur (23). Southern blots were performed on nitrocellulose. Radioactive probes were generated by random primer labeling with a kit from Bethesda Research Laboratories and [^{32}P]dCTP from ICN.

Swarm plates. *E. coli* and *B. subtilis* transformants were spotted onto tryptone-containing swarm plates (28) containing IPTG (10^{-6} to 10^{-3} M) and chloramphenicol (8 μg/ml for *E. coli* and 5 μg/ml for *B. subtilis*). Swarm plates were incubated at 37°C, and the swarm diameter was measured periodically. *E. coli* cells were spotted onto swarm plates and incubated through the day. Some *B. subtilis* transformants grew more slowly because of the presence of the *recE4* mutation. They were spotted onto swarm plates, incubated overnight at 30°C, and transferred to 37°C to measure the swarm rate.

Motility. Cultures were grown in LB media (25) with and without IPTG. Cells were examined under a microscope, and their behavior was monitored by recording onto VHS cassette tapes with a Panasonic BL200 camera attached to a Zeiss phase-contrast microscope.

In vivo methylation. In vivo methylation was performed as described earlier (6).

Nucleotide sequence accession number. The nucleotide

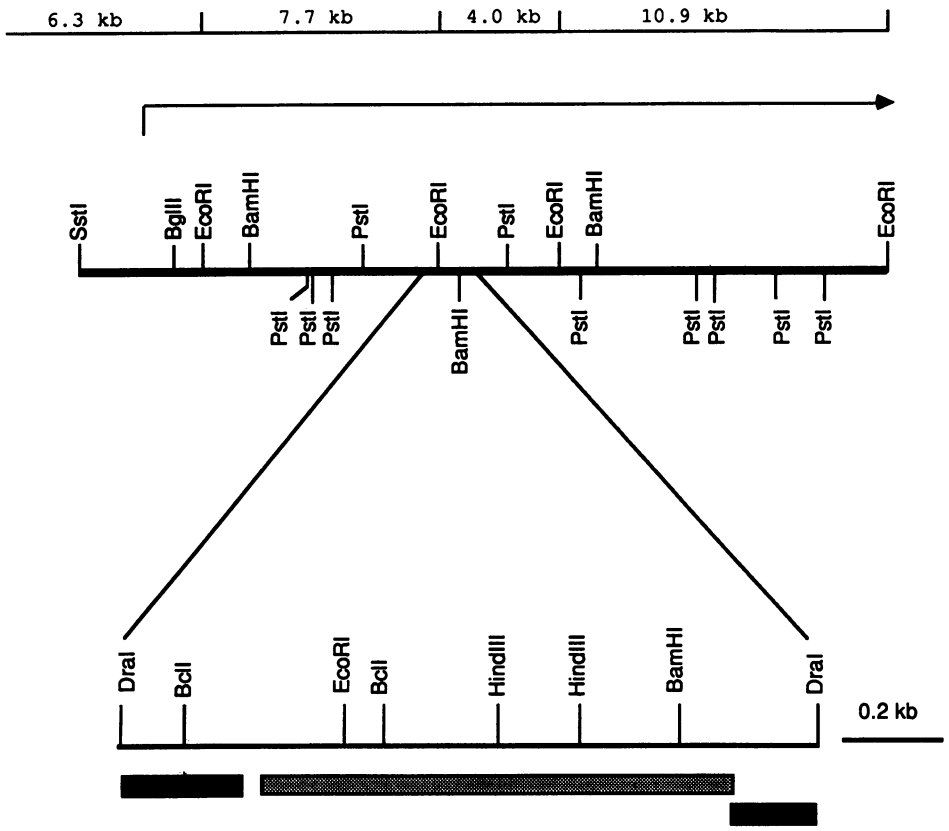

FIG. 1. Localization of the *fliM* gene within the chemotaxis operon. A restriction map of the chemotaxis operon is shown. The sizes of the *Eco*RI restriction fragments are shown above the restriction map. The arrow corresponds to the direction and extent of transcription of this operon (41). Below the restriction map is shown the 1.46-kb *Dra*I fragment that contains the entire *fliM* gene (hatched area). The restriction map of this fragment is drawn on a different scale. The black rectangles represent ORFs upstream and downstream of *fliM*. The downstream ORF is the *cheD* gene (unpublished data).

sequence data reported in this paper have been submitted to GenBank and assigned the accession number M37691.

RESULTS

Nucleotide sequence. A 1.46-kb *Dra*I fragment (Fig. 1) was isolated from pAZ203, and its nucleotide sequence was determined. Analysis of the DNA sequence revealed the presence of one complete ORF and two partial ORFs on the *Dra*I fragment. The orientation of these ORFs agrees with the known direction of transcription through this region of DNA (41). The complete ORF has the capacity to encode a polypeptide with an M_r of 37,500. The nucleotide sequence and deduced polypeptide sequence of this ORF are shown in Fig. 2. The ORF has a potential ribosome-binding site (RBS) located 10 bp upstream from an AUG initiation codon. The calculated free energy of interaction of this RBS with 16S rRNA is -21 kcal $\cdot$ mol^{-1} (ca. -88 kJ $\cdot$ mol^{-1}) (36). The deduced amino acid sequences of the partial ORFs immediately upstream and downstream of this complete ORF are also shown in Fig. 2. The downstream ORF probably corresponds to the *cheD* gene (unpublished data). The initiation codon for *cheD* occurs within the 3′ end of the complete ORF, and it is preceded by a potential RBS.

Homology to *E. coli* and *S. typhimurium* FliM. A computer search of the NBRF protein data base with the deduced polypeptide encoded by the complete ORF revealed substantial homology to the *E. coli* and *S. typhimurium* FliM proteins (Fig. 3). The *E. coli* and *S. typhimurium* FliM proteins share 95.5% identity in amino acid sequence (12) (Fig. 3). In contrast, *B. subtilis* FliM shares only 29.1% identity to *E. coli* FliM and 27.9% identity to *S. typhimurium* FliM. There appear to be a few regions that are more well conserved than others. The most striking of these regions is the sequence LSQ-EIDALL close to the amino termini of the three proteins. The sequence is identical in all three FliM proteins. The amino acid residue between the sequences LSQ and EIDALL does not appear to be conserved. In *E. coli* and *S. typhimurium*, this amino acid is an alanine (nonpolar). The corresponding amino acid in *B. subtilis* is an asparagine (polar, uncharged).

Hydropathy profile of FliM. The *E. coli* and *S. typhimurium* FliM proteins have been shown to associate with the inner cell membrane but are not integral membrane proteins (3, 12). The hydropathy profile of *B. subtilis* FliM is essentially similar to that of *S. typhimurium* (Fig. 4). The NH_2-terminal portions of both proteins (amino acids 1 to 80) have a similar profile. The COOH-terminal portions of both proteins (amino acids 230 to 330) are also similar. However, there are two regions in which small differences in hydropathy are apparent. These regions are between amino acids 80 and 160 and between amino acids 190 and 230. The *S. typhimurium* FliM protein is membrane associated. It is likely that the *B. subtilis* FliM protein is also membrane associated. The fact that the two proteins differ slightly in relative hydropathy suggests that they may interact differ-

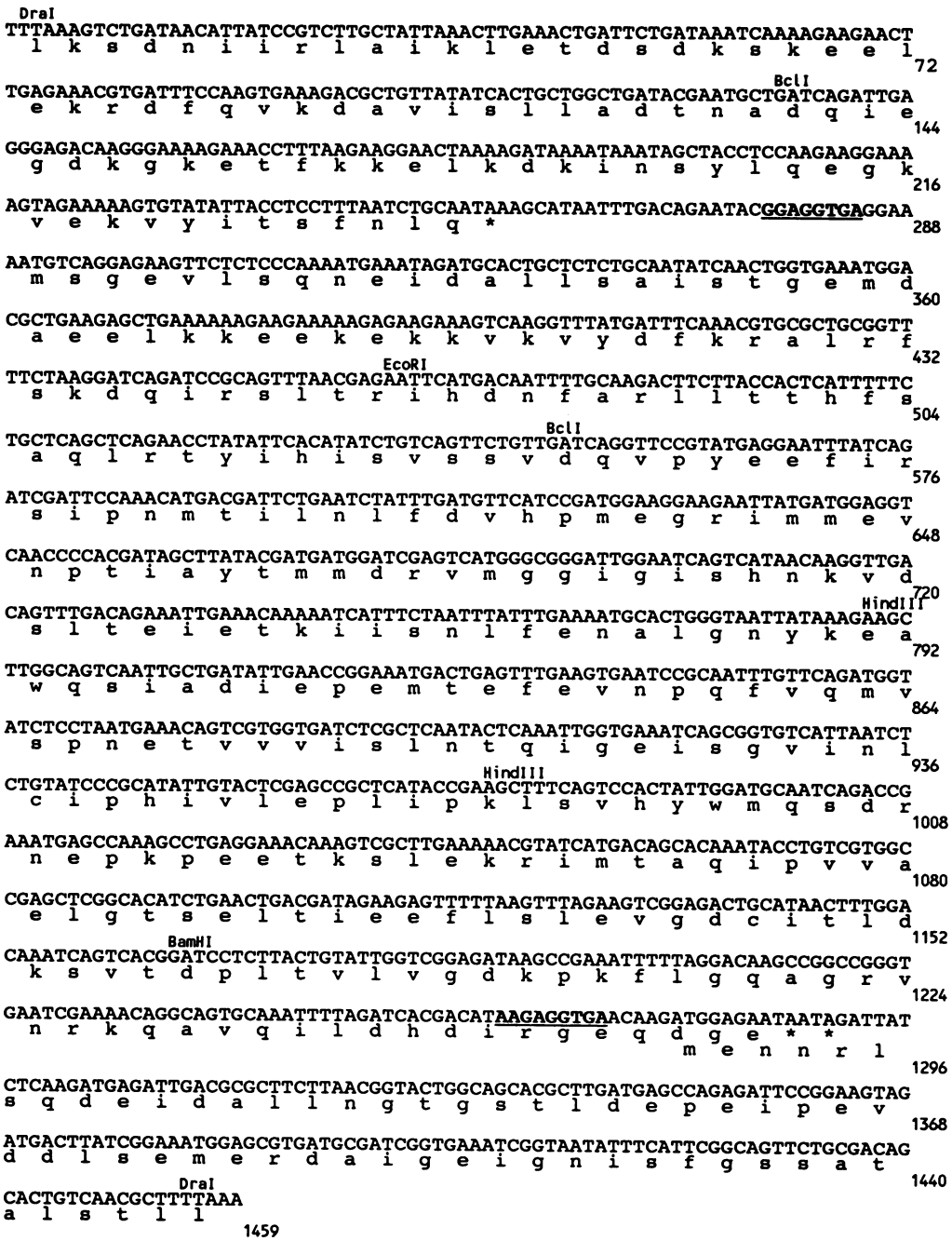

FIG. 2. Nucleotide sequence of the 1.46-kb *Dra*I fragment. The amino acid sequences of ORFs present within this fragment are shown below the nucleotide sequence. The first ORF terminates at a UAA codon at position 254. The ORF corresponding to *fliM* begins at position 290 and terminates at position 1286. The putative initiation codon for *cheD* is at position 1278. Potential RBS sequences are in boldface type and underlined. Restriction sites are also shown.

ently with the cell membrane or with other proteins. It is also possible that the differences reflect slightly different internal interactions that may affect the tertiary structure of the proteins.

Expression of FliM. The *Dra*I fragment was subcloned into the *Sma*I site of plasmid pT7-5 in both orientations. The derivative plasmids were termed pAZ244 and pAZ245. Plasmid pAZ245 should express FliM. Both plasmids and pT7-5 were introduced into *E. coli* K38, and the plasmid-encoded proteins were expressed. A 38-kDa polypeptide was expressed from pAZ245 (Fig. 5). No proteins of this size were observed to be expressed from pAZ244 or pT7-5. The size of the 38-kDa polypeptide agrees very well with the size of the FliM protein predicted from the DNA sequence.

Mutagenesis of the *fliM* gene. The *fliM* gene is within a large chemotaxis operon that contains at least 14 genes and possibly spans 25 kb (41). A null mutation was created by generating a 4-bp insertion within the *fliM* gene. The 3′ overhangs of the *Eco*RI site within the *fliM* gene were filled in, and the plasmid-borne mutation was transferred onto the

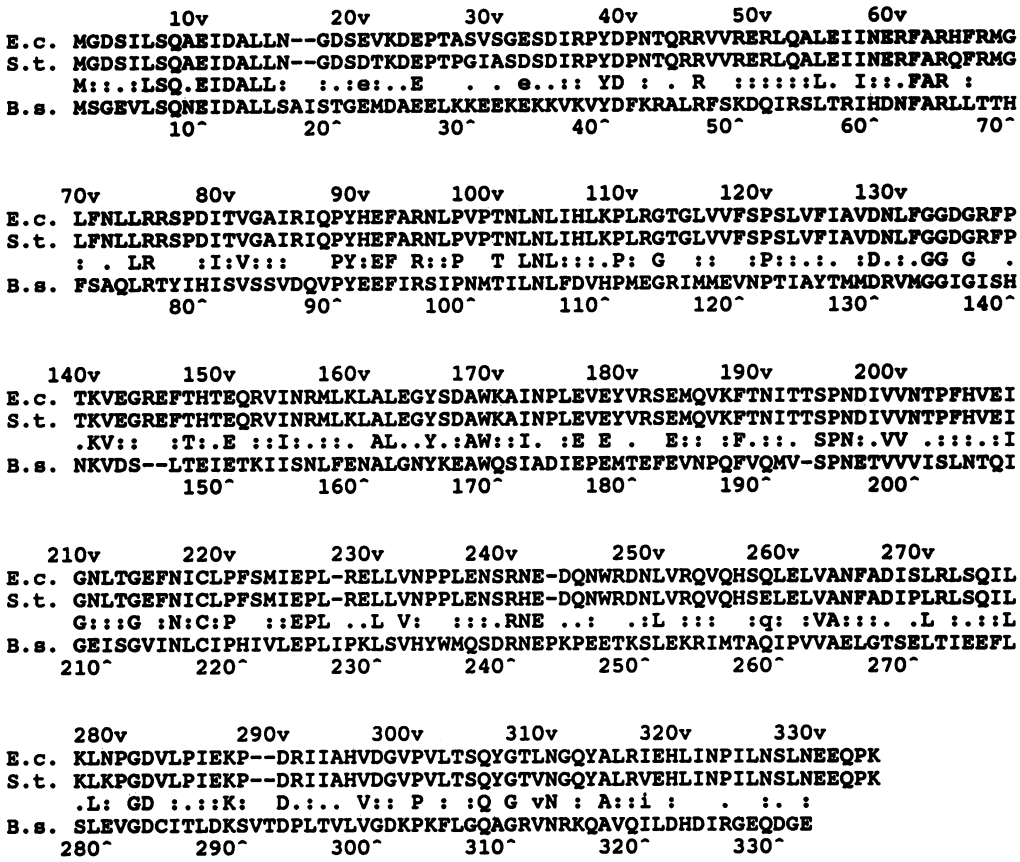

FIG. 3. Amino acid alignment of FliM from *E. coli* (E.c.), *S. typhimurium* (S.t.), and *B. subtilis* (B.s.). Alignment was performed with the AALIGN program on DNASTAR computer software. The standard protein similarity matrix was used to score the alignment (18). Amino acids that appear to be positively related in the standard protein similarity matrix are shown with a colon, and those with a zero-value relationship are shown with a period. Amino acids that appear to be negatively related are shown with a blank. Matching amino acids are indicated. If the amino acid is in uppercase type, the amino acid is present in all three proteins; if it is in lowercase type, the amino acid of *B. subtilis* FliM is identical to an amino acid of either *E. coli* or *S. typhimurium* FliM. v and ^ immediately after a number identify the position of the specific amino acid indicated.

chromosome of OI1318 by gene conversion. Mutants were identified by their defective swarm phenotype on tryptone-containing swarm plates. One representative mutant was designated OI2553.

To confirm that OI2553 had acquired the desired mutation and was not a spontaneous mutant, we performed Southern blotting. Chromosomal DNA was prepared from the strain and probed with labeled pAZ203. The *fliM* gene spans *Eco*RI fragments of 7.7 and 4.0 kb. Destruction of the *Eco*RI site present within the *fliM* gene would be expected to generate a single *Eco*RI fragment of 11.7 kb (7.7 + 4.0) that could hybridize to pAZ203. This result was observed in OI2553 (Fig. 6). Microscopic observation of OI2553 revealed that the cells were nonmotile. Furthermore, when the cells were incubated with antiflagellar antibody, no agglutination was observed under conditions in which the Che^+ parent did agglutinate. This result suggests that flagella are absent from OI2553. The same phenotype is also observed in *E. coli* and *S. typhimurium* FliM null mutants.

Complementation of the *fliM* mutant. The 1.46-kb *Dra*I fragment containing the *fliM* gene was subcloned into the blunt-ended *Sal*I site of pSI-1. Two plasmids that differed in the orientation of insertion of the *Dra*I fragment into the vector were recovered. They were designated pAZ273 and pAZ274. Expression of *fliM* is under the control of the inducible spac promoter in plasmid pAZ273. Plasmid pAZ274 contains the *Dra*I fragment in the opposite orientation and is unable to express *fliM*.

Both plasmids and the parent vector pSI-1 were introduced into OI2555, a *recE4* derivative of OI2553. Transformants were scored for motility in the presence of different concentrations of IPTG. Only plasmid pAZ273 was observed to complement the *fliM* mutant. Microscopic examination revealed that, even in the absence of any exogenous IPTG, approximately 50% of the cells were motile because of residual expression from the spac promoter, which was not fully repressed. Most of the cells appeared to swim smoothly, implying a counterclockwise bias in the direction of flagellar rotation. Transformants grown in the presence of 10 μM IPTG appeared to swim faster than did transformants grown in the absence of IPTG. Approximately 70% of the cells were motile, and a swimming bias was still apparent. In contrast, growth in 100 μM IPTG inhibited the motility of transformants. The transformants were mostly nonmotile. The motile cells swam very slowly and only occasionally tumbled.

The motility observed in OI2555 transformants carrying pAZ273 did not, however, restore the swarm phenotype back to the wild type. The swarm phenotype had a speckled appearance on swarm plates containing 1, 10, and 100 μM

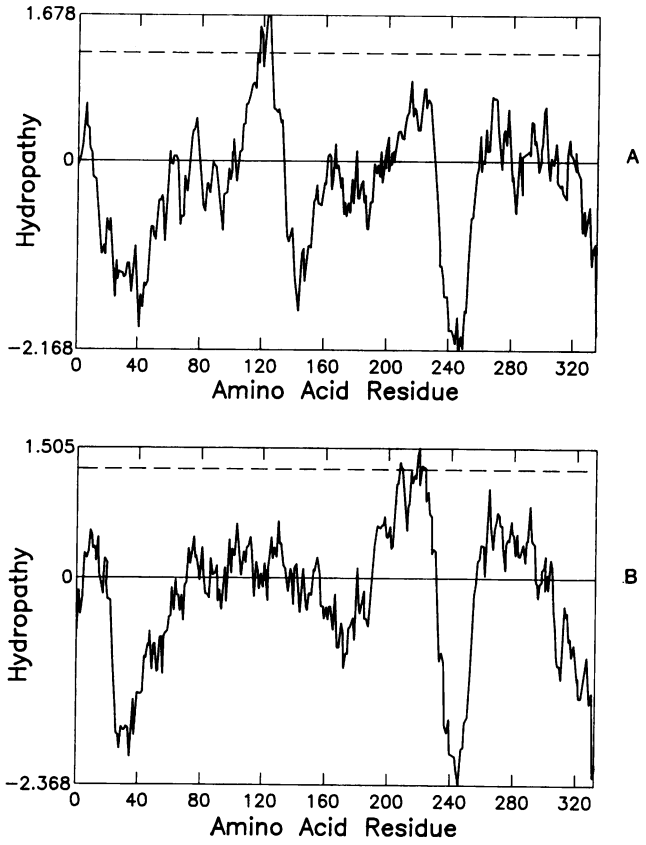

FIG. 4. Hydropathy profiles of FliM from *S. typhimurium* (A) and *B. subtilis* (B). The algorithm of Kyte and Doolittle (17) was used with a window span of 19. The hydropathy values are shown on the vertical axis. The amino acid residue number is plotted on the horizontal axis. The plot for *S. typhimurium* is reproduced from the data of Kihara et al. (12) and is shown with permission from R. Macnab.

IPTG. The data suggest that even though plasmid pAZ273 appears to restore motility to OI2555, there is still a defect in chemotaxis, probably in the control of switching of the flagellar motor. Overproduction of FliM leads to a defective swarm phenotype (see below), and it is possible that even in the absence of IPTG too much FliM protein is made.

Effect of overproduction of FliM in *B. subtilis*. Plasmids pAZ273, pAZ274, and pSI-1 were introduced into OI2554.

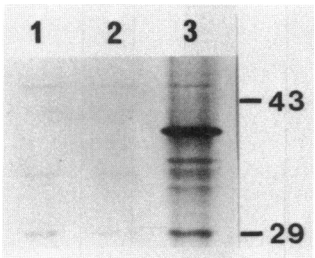

FIG. 5. Expression of *B. subtilis* FliM from a T7 phage promoter in *E. coli* K38. An autoradiograph of the protein expression pattern from three plasmids is shown. Lanes: 1, *E. coli* K38 cells containing pT7-5; 2, transformants containing pAZ244; 3, transformants containing pAZ245. The molecular masses in kilodaltons of two protein standards are indicated.

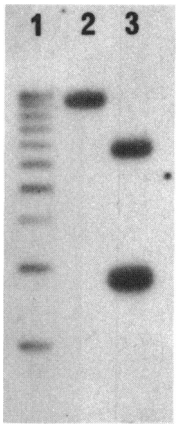

FIG. 6. Mutagenesis of the *B. subtilis fliM* gene. A Southern blot is shown. *Eco*RI-digested chromosomal DNAs from OI1318 (lane 3) and OI2553 (lane 2) were hybridized to labeled pAZ203. The 1-kb ladder from Bethesda Research Laboratories (lane 1) was used as a size marker; it was visualized by the addition of a labeled 1-kb DNA ladder to the blot.

Transformants were spotted onto tryptone swarm plates containing different concentrations of IPTG to determine the effect of overexpression of *fliM* on wild-type cells. Overexpression of *fliM* led to a reduction in the swarm rate (Fig. 7). The effect was noticed at 100 μM IPTG, and no significant inhibition occurred in the presence of 1 or 10 μM IPTG. The reduction in the swarm rate coincided with a change in the swarm morphology. At 100 μM and 1 mM IPTG, the swarm phenotype of OI2554 containing pAZ273 had a speckled appearance. This phenotype signifies that most cells have a motility defect. Occasionally, a cell acquires the ability to move through the agar, but most descendents are nonmotile.

To characterize the inhibitory effect of overproduction of FliM in OI2554 containing pAZ273 in more detail, we grew this culture in LB medium containing 100 μM IPTG. Microscopic examination of the cells revealed that overproduction of FliM caused a partially filamentous phenotype during exponential growth. The filaments became shorter as the culture reached the late logarithmic stage of growth, but the cells remained nonmotile. A small proportion (15 to 20%) of the culture was motile. Both smooth swimming and tumbling behavior were observed, but the switching frequency was reduced as compared with that of the same culture grown in the absence of IPTG. The addition of 100 μM IPTG to a mid-logarithmic culture generated an intermediate phenotype. There were fewer nonmotile filaments and a greater number of motile cells. The switching frequency was lower than that observed in the absence of IPTG.

Antibody directed against flagellin was added to samples of each culture when each was maximally motile. Cells grown in the absence of IPTG immediately agglutinated and formed large clumps, indicating that flagellin was present on the cell surface, as expected. The addition of antibody to cells grown in 100 μM IPTG did not result in significant agglutination. This result suggests that the nonmotile majority of cells do not possess flagella. Thus, overexpression of *fliM* in *B. subtilis* affects the ability of the cells to produce the flagellar apparatus.

Expression of *B. subtilis fliM* in *E. coli*. We wished to determine whether the *B. subtilis fliM* gene could complement an *E. coli fliM* mutant. We also wished to determine the effects of overexpression of *fliM* in Che^+ cells of *E. coli*.

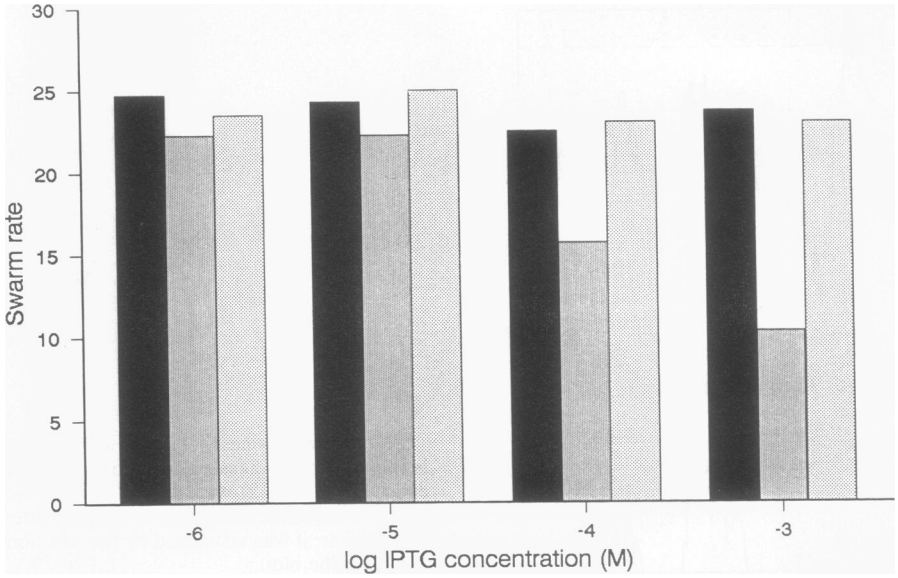

FIG. 7. Effect of overexpression of *B. subtilis fliM* on swarm rates of *B. subtilis* OI2554 Che^+ cells. The swarm rate (100 × actual swarm rate in millimeters per minute) of transformants is plotted against $\log_{10}$ IPTG molar concentrations. The leftmost bars in each set represent transformants containing pSI-1. The middle bars correspond to transformants containing pAZ273. The rightmost bars correspond to transformants containing pAZ274.

Plasmids pAZ273, pAZ274, and pSI-1 were introduced into *E. coli* YK410 and RP4187. These strains are Che^+ and *fliM*, respectively. Representative transformants were spotted onto tryptone swarm plates, and the swarm rates were measured at different concentrations of IPTG.

No complementation was observed in the *E. coli fliM* mutant at any IPTG concentration tested. This result suggests that even though the two FliM proteins are homologous, the *B. subtilis* protein cannot substitute in *E. coli*. We did observe, however, that the expression of *B. subtilis fliM* from pAZ273 conferred a Che^- phenotype upon YK410 (Fig. 8). The effect was first measured at 10 μM IPTG. Although the *B. subtilis* FliM protein cannot replace the corresponding *E. coli* protein, it can apparently interfere with normal chemotaxis.

Microscopic examination of *E. coli* YK410 cells containing pAZ273 and grown in 100 μM IPTG revealed that overproduction of FliM led to most of the cells becoming nonmotile. A few motile cells (fewer than 1%) were observed, and these appeared to have a marked tumble bias.

Effect of the *fliM* mutation on the expression of SigD-regulated proteins. We have characterized three Tn*917lacZ*

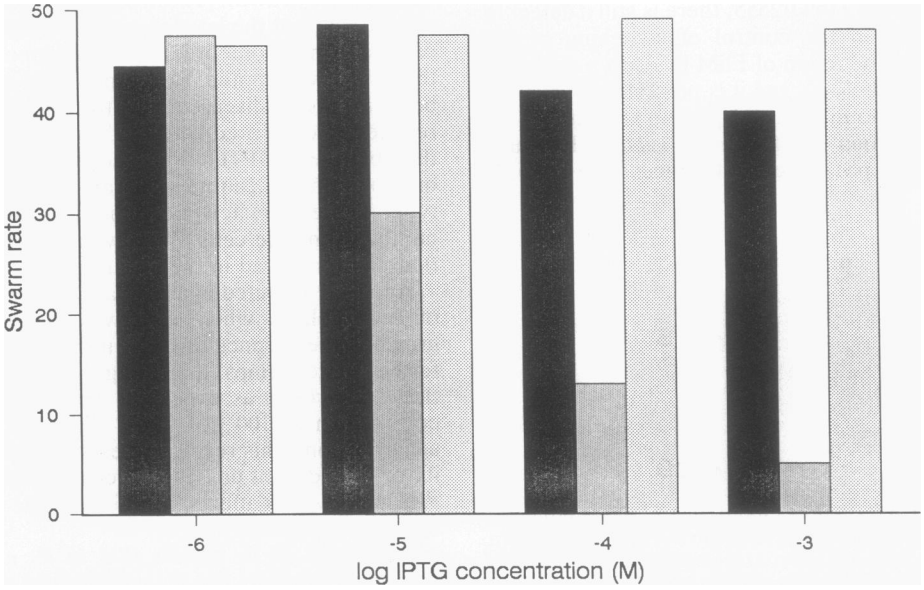

FIG. 8. Effect of expression of *B. subtilis* FliM in *E. coli* YK410 Che^+ cells. The swarm rate (100 × actual swarm rate in millimeters per minute) of transformants is plotted against $\log_{10}$ IPTG molar concentrations. The bars are as described in the legend to Fig. 7.

transposons inserted into the *hag*, *mot*, and *cheX* loci (40). The corresponding mutants can all express β-galactosidase, and expression is under the control of the promoter(s) of the mutated locus. Transcription of *hag* and *mot* is dependent upon SigD, and transcription of *cheX* is dependent upon SigA.

OI1318 and OI2553 were transformed to macrolide-lincosamide-streptogram S-type antibiotic resistance with chromosomal DNA from strains OI2299, OI2283, and OI2282. These strains contain transposon insertions in the *hag*, *mot*, and *cheX* loci, respectively. The transformants were spotted onto plates containing the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The expression of β-galactosidase from the *hag* and *mot* loci was eliminated in a *fliM* mutant. The expression of β-galactosidase from the *cheX* locus was unaffected by the mutation in *fliM*.

The presence of MCPs is also dependent upon SigD. An in vivo methylation experiment was performed with OI1318 and OI2553. No methylation was observed in the *fliM* mutant (data not shown). The data suggest that all SigD-dependent chemotaxis and motility functions are defective or absent in the presence of the *fliM* mutation.

DISCUSSION

We have sequenced an ORF from the *B. subtilis che* locus. The corresponding protein has substantial homology to the *E. coli* and *S. typhimurium* FliM proteins. Accordingly, we designate the *B. subtilis* ORF *fliM*, in keeping with a unified nomenclature, as suggested by Iino et al. (10).

The *E. coli* and *S. typhimurium* FliM proteins are almost identical. This fact has prevented the identification of specific amino acids that are involved in interactions with other proteins. The *B. subtilis* FliM protein sequence is less homologous, and only a few regions are identical. These regions are probably very important in the function of the protein.

One interesting aspect of the comparison of the protein sequences of *E. coli*, *S. typhimurium*, and *B. subtilis* is the sequence identity close to the start of all three proteins. This sequence, LSQ-EIDALL, is also observed near the amino terminus of CheD. The *cheD* gene is immediately downstream of *fliM* (Fig. 2) and probably also encodes a flagellar switch protein (1a). Closer examination of the conserved sequence suggests that this region may form an interface either with the cytoplasm or with another protein. The amino acids leucine, isoleucine, and alanine are all nonpolar and would be expected to favor a hydrophobic environment. Serine and glutamine are both polar and uncharged, whereas aspartic acid and glutamic acid are charged and polar. Alternatively, this region may represent a consensus sequence that serves to target the protein to the correct location near the membrane.

One interesting observation is that overproduction of FliM in Che^+ *B. subtilis* and Che^+ *E. coli* leads to a defective chemotaxis phenotype. The phenotypes were slightly different, depending upon the host used. Overproduction of FliM in *B. subtilis* generated mostly nonmotile cells. Smooth swimming and tumbling behavior were observed in the small proportion of cells that were motile, but the rate of switching was reduced. The nonmotile cells did not possess flagella. It appears that overproduction of FliM interferes with the normal synthesis, export, or assembly of flagellar proteins. In cases in which the flagellar apparatus was exported, the cells were motile, but the motor was unable to switch normally.

The expression of *B. subtilis* FliM in *E. coli* generated mostly nonmotile cells, but those that were motile appeared to have a tumble bias. Overproduction of *E. coli* FliM in *E. coli* affects motility by slowing the rate of swimming and reducing the frequency of tumbling (3). These different phenotypes suggest that the *B. subtilis* FliM protein must interact differently with the chemotactic machinery of *E. coli*. The failure of *B. subtilis* FliM to complement the corresponding *E. coli* mutant also suggests that the two proteins may function differently. It is known that the *S. typhimurium* and *E. coli* FliM proteins form part of the switch complex of the flagellar apparatus. The *B. subtilis* FliM protein may be sufficiently different from the enteric homologs so that it cannot generate a functional switch complex with other *E. coli* switch proteins.

Previous data suggested that the *cheI*, *cheJ*, and *cheK* complementation groups all spanned the *Eco*RI site between the 7.7- and 4.0-kb *Eco*RI fragments (29). This conclusion was based on the observations that a SPβ phage carrying either the 7.7- or the 4.0-kb *Eco*RI fragment could not complement any of these three mutants, whereas a phage carrying the 7.7- and the 4.0-kb *Eco*RI fragments could complement all three mutants. These results would suggest that the corresponding mutations all mapped to *fliM*. However, the cloned *fliM* gene on a plasmid was unable to complement a *cheI* mutant (unpublished data). Additional complementation experiments were performed with an SPβ transducing phage that carried a mutation in *fliM*. The data suggested that these three *che* loci and *fliM* mapped to different complementation groups (27a). Additional experiments showed that the *cheI* and *cheJ* mutations both mapped to the 7.7-kb *Eco*RI fragment (41). One explanation for the incorrect mapping of *cheI*, *cheJ*, and *cheK* is that the original SPβ phage that carried the 7.7-kb *Eco*RI fragment (29) may have had a deletion in the cloned chemotaxis DNA.

As no mutations were available in the *fliM* gene, a null mutant that truncated the protein close to the NH_2 terminus was constructed. It is possible that the frameshift mutation generated within the *fliM* gene could be polar for downstream genes in the operon, even though these genes are known to possess their own RBSs (unpublished data). However, the *fliM* mutant was nonflagellated, because the mutant cells became motile when the FliM protein was produced from pAZ273. The failure of this plasmid to fully complement a *B. subtilis fliM* mutant could have been due to additional defects in the expression of downstream genes as a result of the mutation generated or to the effects of subtle changes in the stoichiometry of the flagellar switch complex. *E. coli* and *S. typhimurium fliM* null mutants are also nonflagellated. The formation of the basal body-hook complex of flagella in *E. coli* and *S. typhimurium* is dependent upon FliM, and this is probably true of *B. subtilis* as well.

We observed that the presence of the *fliM* mutation in *B. subtilis* eliminated expression from the SigD-regulated *hag* and *mot* loci, as determined from Tn*917* insertions that generated transcriptional fusions to *lacZ*. There was also no in vivo methylation of the MCPs in the *fliM* mutant. MCPs are also apparently regulated by SigD (24). These observations have also been documentated in *E. coli* and *S. typhimurium* (13, 16, 27). Flagellar operons in these organisms are transcribed in a complex hierarchy that requires that all transcripts of one class be expressed before the next series of chemotaxis and motility operons is transcribed. The data reported here are the first evidence that a similar transcrip-

tional hierarchy may also exist in *B. subtilis*. The nature of the *fliM*-dependent expression of late operons is not well understood. It is unlikely that FliM itself is a transcriptional activator. One possibility is that there is a morphological switch in the flagellar basal body or switch complex. The correct organization of these structures may, in some way, promote the expression of the late operons. Additional experiments are in progress to test this hypothesis.

Because *B. subtilis* FliM was unable to complement an *E. coli* mutant, it is possible that *B. subtilis* FliM is related to *E. coli* FliM but that it has a different specific function. We consider this unlikely for the following reasons. The FliM protein of the enteric bacteria is known to interact with several other proteins. The failure of *B. subtilis* FliM to complement an *E. coli* mutant probably reflects a deficiency in one of these interactions with other *E. coli* proteins. We have also observed that the *B. subtilis* CheA and CheY homologs do not complement the corresponding *E. coli* mutants (unpublished data). Both of these proteins also interact with other proteins in a complex. The mechanism of signal transduction and flagellar rotation is probably quite similar in *B. subtilis* and *E. coli*. Nevertheless, sufficient changes have probably arisen so that the conformation of individual proteins may not be conserved. The conformation of the complete complexes, however, may be quite similar because of compensating mutations in other protein subunits.

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