

# Overproduction and Characterization of the *Bacillus subtilis* Anti-sigma Factor FlgM\*

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**FlgM is an anti-sigma factor of the flagellar-specific sigma ( $\sigma$ ) subunit of RNA polymerase in *Bacillus subtilis*, and it is responsible of the coupling of late flagellar gene expression to the completion of the hook-basal body structure. We have overproduced the protein in soluble form and characterized it. FlgM forms dimers as shown by gel exclusion chromatography and native polyacrylamide gel electrophoresis and interacts *in vitro* with the cognate  $\sigma^D$  factor. The FlgM- $\sigma^D$  complex is a stable heterodimer as demonstrated by gel exclusion chromatography, chemical cross-linking, native polyacrylamide gel electrophoresis, and isoelectric focusing.  $\sigma^D$  belongs to the group of sigma factors able to bind to the promoter sequence even in the absence of core RNA polymerase. The FlgM- $\sigma^D$  complex gave a shift in a DNA mobility shift assay with a probe containing a  $\sigma^D$ -dependent promoter sequence. Limited proteolysis studies indicate the presence of two structural motifs, corresponding to the N- and C-terminal regions, respectively.**

In bacteria genes are regulated mainly at the transcriptional level. In addition to the *cis*-acting promoter sequences, a large number of activators and repressors modulate initiation of transcription. Furthermore initiation depends on alternative sigma factors that direct the RNA polymerase to specific promoter sequences. The activity of sigma factors may itself be regulated, either by stability as is the case for the *Escherichia coli* stationary phase  $\sigma^S$  (1) or by interaction with anti-sigma factors (2). Anti-sigma factors have been characterized for  $\sigma^B$  and  $\sigma^F$  of *B. subtilis* (3–5) and for the flagellar dedicated sigma of *Salmonella* (6, 7) and  $\sigma^{70}$  of *E. coli* upon infection with the bacteriophage T4 (8). Anti-sigma factors have also been described in *Pseudomonas aeruginosa* and *Myxococcus xanthus* (9–11). The activity of the anti-sigma factors is exercised by specific protein-protein interaction, even though the details of the molecular mechanisms involved are still largely to be elucidated (12–15).

FlgM was first identified in *Salmonella* as a negative regulator of the flagellar-specific sigma factor  $\sigma^{28}$ , also called FliA

(6, 7), and later shown to interact specifically with the cognate sigma factor, forming a stable complex (15). FlgM has also been described in *B. subtilis*, where it appears to accomplish a similar regulatory function, coupling the early and late flagellar gene expression (16–18). The action of FlgM is thought to occur by specific interaction with the late flagellar sigma factor  $\sigma^D$ .

The *B. subtilis* FlgM has now been overproduced in *Escherichia coli*, purified, and shown to form a complex with purified  $\sigma^D$  protein. Limited proteolysis studies suggest that FlgM contains a relatively stable N-terminal domain.

## EXPERIMENTAL PROCEDURES

**Overexpression and Purification of FlgM**—The *flgM* gene (264 base pairs) was obtained by polymerase chain reaction amplification of *Bacillus subtilis* chromosomal DNA, using primers (forward, 5'-CGGAATTCATATGAAAATCAATCAATTTGG-3' and backward 5'-CCCGATCCTTATTGCTTTTATAAAAAATTAATC-3') with a *NdeI* and *BamHI* site, respectively; after purification and restriction, this *NdeI*-*BamHI* fragment was cloned into the expression vector pET12a (19). The resulting plasmid, pBG9, allows high level overproduction of FlgM in *E. coli* BL21 (DE3) (19).

To purify FlgM, BL21 (DE3) (pBG9) cultures were grown at 37 °C with shaking in 150 ml of Luria broth medium supplemented with 1% (w/v) glucose and 100  $\mu$ g/ml ampicillin. At  $A_{600}$  of 3–4, the cells were collected by centrifugation and resuspended in 1 liter of Luria broth medium with 100  $\mu$ g/ml ampicillin; isopropyl- $\beta$ -D-thiogalactopyranoside was added to 0.1 mM, and growth was continued for 3 h at 21 °C, until an  $A_{600}$  of 0.7. The cells were recovered by centrifugation and resuspended in 100 ml of A buffer (20 mM  $\text{NaH}_2\text{PO}_4$ , 29.6 mM  $\text{Na}_2\text{HPO}_4$ , 50 mM NaCl, 0.1 mM EDTA, pH 8.0, 10% (v/v) glycerol). Cells were lysed by sonication, and the lysate was centrifuged to remove cell debris and inclusion bodies. The supernatant, containing the soluble fraction of the protein, was loaded on a FPLC<sup>1</sup> (Pharmacia) S-Sepharose 26/10 column, pre-equilibrated with A buffer. The FlgM protein was purified by elution with a 0–0.5 M gradient of NaCl in A buffer; 19 mg of FlgM were recovered, eluting with 0.3 M NaCl. The protein was further purified by gel exclusion chromatography on an FPLC Sephacryl S-100 column in B buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , 1 mM EDTA, pH 8.0, 10% (v/v) glycerol).

**Overexpression and Purification of  $\sigma^B$ – $\sigma^B$**  was overexpressed by using a pT7–5 derivative, kindly supplied by W. G. Haldenwang. For purification we followed the procedure described by Huang *et al.* (20) for  $\sigma^W$ . The last steps (heparin-Sepharose and Superdex) were omitted and substituted by a single HiTrap Q column (Amersham Pharmacia Biotech).

**Physical Characterization of FlgM**—The molecular mass of FlgM and its fragments were determined by matrix-assisted laser desorption and ionization time-of-flight mass spectroscopy using a matrix of sinapinic acid or of  $\alpha$ -cyano-4-hydroxycinnamic acid. The N-terminal amino acid sequences were determined by automated Edman degradation using an Applied Biosystem 477A liquid pulse protein sequencer.

**FlgM- $\sigma^D$  Binding Assay**—Purified proteins FlgM and  $\sigma^D$  were incubated together at equimolar ratio at 0 °C for 1 h, and the mixture was

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<sup>1</sup> The abbreviations used are: FPLC, fast protein liquid chromatography; EGS, ethylene glycol-bis(succinic acid *N*-hydroxysuccinimide ester); DSP, dithio-bis(succinimidyl propionate); HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

fractionated by chromatography on an FPLC Superose HR 12 gel exclusion column in buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol. Fractions collected were analyzed by 15% SDS-PAGE.

**Chemical Cross-linking**—Purified proteins were cross-linked in a 50  $\mu$ l reaction mixture containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 10% glycerol by adding ethylene glycol-bis(succinic acid *N*-hydroxysuccinimide ester) (EGS, Sigma) or dithio-bis(succinimidyl propionate) (DSP, Sigma) to a final concentration of 1 mg/ml. EGS and DSP were predissolved in dimethyl sulfoxide at 10 mg/ml. After incubation at 0 °C for 2 h, L-lysine was added to a final concentration of 20 mM. Cross-linked products were separated and analyzed by SDS-PAGE.

**Isoelectric Focusing**—Isoelectric focusing was performed in non-denaturing conditions, using horizontal electrophoresis apparatus with 1 M NaOH and 1 M H<sub>3</sub>PO<sub>4</sub> as electrode solutions. The samples (5–20  $\mu$ l) were applied to a 1-mm-thick gel using isoelectric focusing applicator (Pharmacia). After focusing, proteins were visualized by Coomassie Blue staining.

**Gel Mobility Shift Analysis**—DNA binding reactions (10  $\mu$ l) contained about 0.5 pmol of 5'-end-labeled DNA fragment and purified proteins (as indicated) incubated in binding buffer (40 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 50 mM NaCl, 1 mM EDTA, pH 8.0, 20% glycerol, 5 ng/ $\mu$ l unspecific plasmid DNA) at 37 °C for 20 min prior to separation by native 5% PAGE at room temperature. To label the DNA, the PD-6 promoter containing fragment (300 base pairs) was amplified by polymerase chain reaction using unphosphorylated primers (5'-GATCAAGTGAAGCTTGGAAAT-T-3' and 5'-AATATTGTGGTAAATTCAT-3'), labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase and purified by 5% PAGE.

To analyze the protein components associated with the shifted DNA band, the corresponding portion of the gel was cut out, rerun in 15% SDS-PAGE, and transferred to a nitrocellulose filter. The filter was probed with anti-FlgM polyclonal antibodies, followed by a secondary antibody conjugated with horseradish peroxidase. The peroxidase activity was visualized with an enhanced chemiluminescence kit (ECL, Amersham Corp.).

**Limited Proteolytic Digestion**—FlgM (2.5 mg/ml) was incubated at 21 °C for 30 min in reaction containing 20 mM HEPES, pH 7.5, 50 mM KCl, 10 mM MgSO<sub>4</sub>, and 0.01 mg/ml subtilisin. Reactions were stopped by adding 10 mM phenylmethylsulfonyl fluoride and visualized by 20% SDS-PAGE. Samples were blotted to polyvinylidene difluoride membrane and analyzed by automated amino acid sequencing. In a second experiment the products of limited proteolysis were separated by reverse phase HPLC (Jasco on an Aquapore RP-300 C 8 column (25  $\times$  0.46 cm). The solvent system used was either 0.1% (v/v) trifluoroacetic acid in H<sub>2</sub>O (system A) or 0.075% trifluoroacetic acid in CH<sub>3</sub>CN (system B). The elution was with a linear gradient from 0 to 50% system B in system A, in 70 min at a flow rate of 0.7 ml min<sup>-1</sup>. The elution of peptides was monitored at 220 nm. Aliquots were analyzed by matrix-assisted laser desorption and ionization time-of-flight mass spectroscopy and Edman degradation.

## RESULTS

**Overproduction and Purification of FlgM**—The *B. subtilis* FlgM is a 9.8-kDa protein (16). The sequence coding for FlgM was obtained by polymerase chain reaction amplification of *B. subtilis* chromosomal DNA, using primers with a *Nde*I and *Bam*HI site, respectively; after purification and restriction, *flgM* was cloned in the expression vector pET12a. The coding sequence was followed by the original stop codon, and the expected product was only FlgM, without fusion to any resident sequence. The resultant plasmid, pBG9, was transformed into *E. coli* BL21 (DE3). Following induction with isopropyl- $\beta$ -D-thiogalactopyranoside expression of FlgM was obtained at high level, but in the insoluble protein fraction. After several attempts we found conditions at which the majority of FlgM was in the soluble fraction. The volume of the culture was 1 liter in a 5-liter volume flask, the incubation temperature was 21 °C, and the concentration of the isopropyl- $\beta$ -D-thiogalactopyranoside inducer 0.1 mM. The soluble fraction was purified by S-Sepharose chromatography, followed by a gel exclusion column of Sephacryl S-100. After this step, the protein was estimated to be at least 95% pure by SDS-PAGE and Coomassie staining (Fig. 1). The final yield was 19 mg of FlgM/liter of culture of *E.*

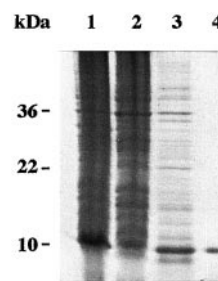


FIG. 1. Overexpression of *B. subtilis* FlgM in *E. coli*. Proteins were analyzed by 15% SDS-PAGE. Lane 1, total proteins from *E. coli* BL21/DE3 (pBG9) not induced. Lanes 2 and 3, pellet and supernatant, respectively, of a culture induced with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside at 21 °C for 3 h. Lane 4, purified FlgM, after FPLC Sephacryl S-100. The positions of the molecular mass standards are shown to the left of the figure.

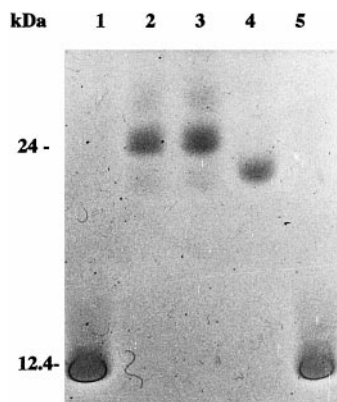


FIG. 2. Mobility of native FlgM in nondenaturing polyacrylamide gradient (from 2 to 20%, FASTA system). Standard proteins used were cytochrome *c* ( $M_r = 12,400$ ; lanes 1 and 5) and trypsinogen ( $M_r = 24,000$ ; lanes 2 and 3) both from Pharmacia. Lane 4, purified FlgM.

*coli* grown to an A<sub>600</sub> of 0.7.

**Characterization of FlgM**—N-terminal determination by Edman degradation of an aliquot of the purified protein gave the sequence MKINQFG corresponding to the first seven amino acids of FlgM as deduced from the nucleotide sequence (16). The mass, measured by mass spectrometry was 9,931 Da, in agreement with the estimated mass of 9,862 Da. We used gel exclusion chromatography to determine whether FlgM is a dimer in solution. The elution volume on a Sephacryl S-100 column corresponds to a molecular mass of about 21 kDa, suggesting that FlgM is a dimer. This was further confirmed by PAGE under nondenaturing conditions (Fig. 2). The dimer nature of FlgM is also corroborated by results of chemical cross-linking experiments. Incubation of FlgM with cross-linker generated a faster migrating species, probably due to internal cross-linking, and a band of approximately 20 kDa, again suggestive of a homodimer (Fig. 3A, lane 3). At least one other anti-sigma factor, SpoIIAB of *B. subtilis*, has been reported to be present as a dimer (4, 21). We have no knowledge of the possible oligomerization of FlgM of *Salmonella*.

**FlgM Forms a Complex with  $\sigma^D$** —FlgM is an anti-sigma factor specific for  $\sigma^D$  as shown by *in vitro* and *in vivo* experiments (17).<sup>2</sup> To investigate the interaction of the two proteins, we purified  $\sigma^D$  according to Chen and Helmann (23). Upon mixing and incubation at 0 °C for 1 h, the two proteins formed a complex that could be resolved by gel exclusion chromatography on a Superose 12HR column. The complex was stable even in 0.6 M NaCl, suggesting a participation of hydrophobic

<sup>2</sup> D. Barilla and M. Chamberlin, personal communication.

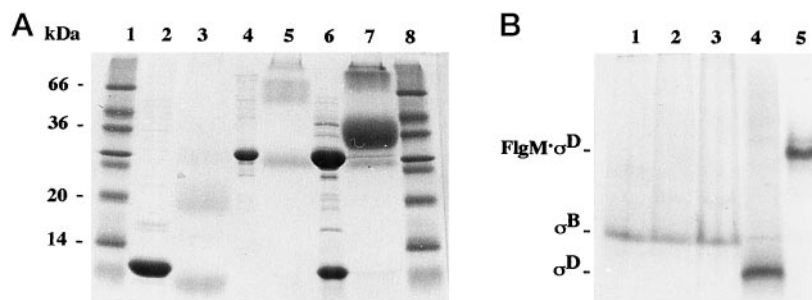


FIG. 3. **The FlgM- $\sigma^D$  complex is a specific heterodimer.** *A*, 15% SDS-PAGE analysis of the DSP cross-linked FlgM- $\sigma^D$  complex. *Lanes 1 and 8*, molecular mass standards (SIGMA); *lane 2*, purified FlgM; *lane 3*, FlgM treated with DSP; *lane 4*, purified  $\sigma^D$ ; *lane 5*,  $\sigma^D$  treated with DSP; *lane 6*, FlgM- $\sigma^D$  complex not treated with DSP; *lane 7*, the complex after DSP cross-linking. *B*, specificity of FlgM- $\sigma^D$  complex. Native 8% PAGE of samples incubated at 0 °C for 1 h. *Lane 1*,  $\sigma^B$  (250 pmol); *lane 2*,  $\sigma^B$  (125 pmol) + FlgM (250 pmol); *lane 3*,  $\sigma^B$  (125 pmol) + FlgM (625 pmol); *lane 4*,  $\sigma^B$  (118 pmol); *lane 5*,  $\sigma^B$  (59 pmol) + FlgM (250 pmol).

interaction between the two polypeptide chains in the complex.

The purified complex was treated with the homobifunctional cross-linking agents DSP and EGS. Analysis of the DSP-treated product by SDS-PAGE showed a single band of approximately 40 kDa (Fig. 3A). Similar results were obtained upon treatment with EGS (data not shown). No bands corresponding to the two proteins, FlgM and  $\sigma^D$ , were observed, suggesting that the complex is very stable and long lived, because it is not in equilibrium with the monomers. The same cross-linked product was obtained by treating the two proteins, immediately upon mixing. FlgM and  $\sigma^D$  treated separately with DSP did not produce a band of the same mobility as that obtained when the mixture was treated (Fig. 3A, lanes 3 and 5).

The complex appears to be an heterodimer by the mass of the cross-linked complex estimated by SDS-PAGE and electrophoresis of purified complex under nondenaturing gel gradient electrophoresis. In addition the mass of the EGS cross-linked complex was measured by mass spectrometry, and the observed value of 43,399 Da is in good agreement with the theoretical value of 39,311 Da (9, 862 Da of FlgM + 29, 449 Da of  $\sigma^D$ ), considering the presence of an unknown number of EGS molecules. The same type of heterodimeric structure has been reported for the *Salmonella* FlgM- $\sigma^{28}$  complex (15). The *B. subtilis* purified complex, not treated with the cross-linking agent, had a pI of about 6.8 as determined experimentally by isoelectric focusing; distinct from the observed pI of  $\sigma^D$  (5.9) and the deduced pI of 9.9 of FlgM (data not shown).

The complex gave a single band in PAGE under nondenaturing conditions (Fig. 3B, lane 5). Using antibodies anti- $\sigma^D$  to detect free  $\sigma^D$  in Western blotting, in nondenaturing conditions, we estimated that the apparent dissociation constant for the FlgM- $\sigma^D$  complex is  $10^{-6}$  M or less (data not shown).

To evaluate the specificity of the complex formation, we performed similar experiments with FlgM and a different sigma factor of *B. subtilis*,  $\sigma^B$ , which is known to interact with the cognate anti-sigma, RsbW (3). We failed to observe any interaction between the two proteins (Fig. 3B, lanes 2 and 3). (In the electrophoretic system used, FlgM is not visible, because it moves out of the gel.)

**The FlgM- $\sigma^D$  Complex Binds DNA**— $\sigma^D$  belongs to the group of sigma factors devoid of region 1, responsible for masking the rest of the molecule. Thus these factors can bind to the promoter sequence even in absence of core RNA polymerase (23).

FlgM inhibits initiation of transcription from  $\sigma^D$ -dependent promoters *in vitro*.<sup>2</sup> Unexpectedly the FlgM- $\sigma^D$  complex gave a supershift in a gel retardation assay with a probe containing the flagellin,  $\sigma^D$ -dependent promoter PD-6 (22). Not only was the probe retarded more than observed with only  $\sigma^D$ , but the amount of probe bound to the complex was much higher. The experiment was performed in the presence of cold unspecific

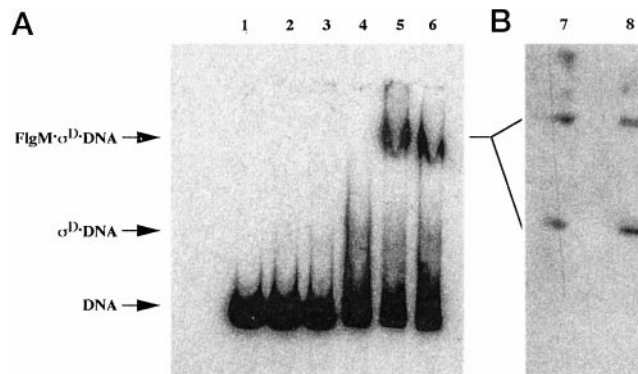


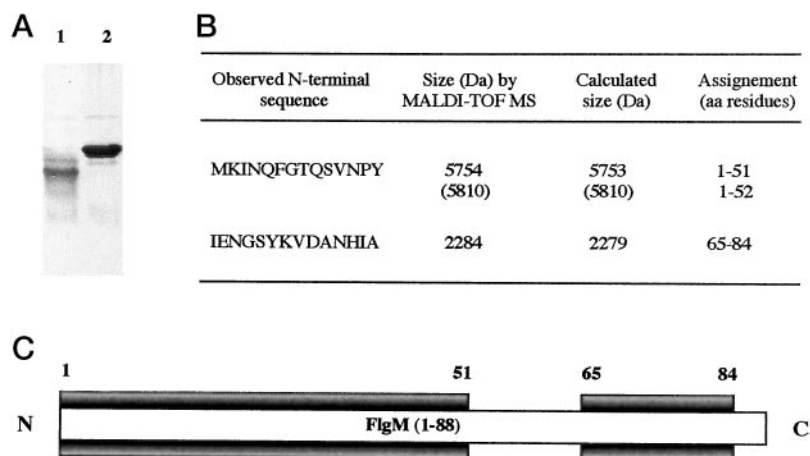
FIG. 4. **The FlgM- $\sigma^D$  complex binds DNA.** *A*,  $^{32}$ P-labeled PD-6 promoter fragment (0.5 pmol) was incubated for 20 min at 37 °C with increasing concentrations of  $\sigma^D$  protein (*lanes 2–4* contained 0.5, 1.0, and 5.0 pmol/ $\mu$ l, respectively). The sample applied to *lane 5* was incubated with 5 pmol/ $\mu$ l of  $\sigma^D$  for 15 min at 37 °C, and then 5 pmol/ $\mu$ l of FlgM were added for the remaining 5 min of incubation. *Lane 6*, incubation for 20 min with 5 pmol/ $\mu$ l of purified FlgM- $\sigma^D$  complex. Free DNA and complexes were separated by 4% native PAGE. *Lane 1* shows a probe alone control. *B*, SDS-PAGE 15% and Western blot analysis of the proteins present in the shifted DNA band (*lane 7*). *Lane 8*, standard of FlgM- $\sigma^D$  complex (0.1  $\mu$ g).

plasmid DNA. To show that the supershift was indeed due to the complex, a gel slice corresponding to the shifted band was subjected to SDS-PAGE and analyzed by Western immunoblotting with anti-FlgM antibodies. These polyclonal antibodies cross-react with  $\sigma^D$  and allowed the detection of the presence of both proteins in the shifted band (Fig. 4). The same shift was observed when the probe was first incubated with  $\sigma^D$  and an equimolar amount of FlgM added 5 min prior to the electrophoresis (Fig. 4, lane 5). In our tests,  $\sigma^D$  alone gave a weak shift of the probe (Fig. 4, lane 4), and this is probably due to incomplete renaturation of the protein solubilized by guanidine hydrochloride. The higher amount of probe retained in the presence of FlgM suggests that the anti-sigma factor may facilitate the renaturation of  $\sigma^D$ .

**Partial Proteolysis of FlgM**—To gain information on the structural organization of FlgM, we performed experiments of limited proteolysis. Partial digestion with subtilisin gave consistently one major product that in PAGE had a mobility of approximately 5 kDa (Fig. 5A). The same electrophoretic pattern was obtained following incubation at 21 °C for at least one h (data not shown). Edman degradation of the material transferred to polyvinylidene difluoride membranes, indicated the presence of the N terminus of FlgM. In a parallel experiment the products of partial proteolysis were subjected to separation by HPLC. Only one peak was observed, but two N-terminal sequences were obtained in approximately equimolar amounts:



**FIG. 5. Partial proteolysis of FlgM.** A, SDS-PAGE 20% analysis of subtilisin (lane 1) digestion of FlgM. The reaction contained 2.5 mg/ml of FlgM and 0.01 mg/ml of subtilisin. Incubation was at 21 °C for 30 min. Lane 2, untreated FlgM. B, characterization of FlgM proteolytic fragments, after reverse phase HPLC purification. C, structure of FlgM. The open bar represents the 88-amino acid FlgM peptide. The shaded boxes highlight the two structural motifs (residues 1–51 and 65–84), as determined by subtilisin limited proteolysis.



MKINQFGTQSVNPY and IENGSYKVDANHIA (Fig. 5B). The first sequence corresponds to the N terminus of FlgM, whereas the second one corresponds to residues 65–78 of the protein. Various attempts to further purify the two components were unsuccessful. The same fraction obtained by HPLC was analyzed by mass spectrometry and gave the values of 5754 and 2284 Da, respectively (Fig. 5B). The  $M_r$  of 5754 is in good agreement with the calculated value (5753 Da) of a peptide consisting of the first 51 residues of FlgM; the smaller peptide of  $M_r$  2284, should correspond to amino acids Ile<sup>65</sup> to Phe<sup>84</sup> (calculated mass, 2279 Da) toward the C terminus of the protein. A minor component of  $M_r$  5810 was also observed as a shoulder of the main peak of  $M_r$  5754; we infer that the proteolytic cleavage can occur either upstream or downstream of Gly<sup>52</sup>.

The results of the partial proteolysis are summarized in the Fig. 5C. We conclude that FlgM is organized in two regions: the N-terminal part (residues 1–51) and the C-terminal portion (residues 65–84), which appear structured and resistant to proteolytic cleavage. The two structural motifs are presumably connected by a loop, easily accessible to cleavage.

#### DISCUSSION

We have overexpressed and obtained in soluble form the anti-sigma factor FlgM of *B. subtilis*, the main regulator of  $\sigma^D$  activity. The protein is a dimer in solution, as determined by gel exclusion chromatography and PAGE electrophoresis under nondenaturing conditions. The anti-sigma factor FlgM forms a stable and long lived complex with the cognate sigma factor  $\sigma^D$ . Highly stable complexes have been reported for two other *B. subtilis* sigma-anti-sigma couples, SpoIIAB- $\sigma^F$  and RsbW- $\sigma^B$  (24, 25).

FlgM- $\sigma^D$  complex still binds to the promoter sequence, but the fact that FlgM does not interfere with the interaction of  $\sigma^D$  at the promoter site may have a marginal physiological significance, as already noted for the DNA binding by the same sigma factor (23). Therefore, if, as it seems plausible, *in vivo* the promoter recognition is performed by the holoenzyme, the specific anti-transcriptional activity of FlgM could be exerted in three ways: (i) sequestering of free  $\sigma^D$  molecules in the FlgM- $\sigma^D$  complex, (ii) removing  $\sigma^D$  from the holoenzyme, and (iii) hampering a productive interaction between the holoenzyme and the promoter.

In the case of bacteriophage T<sub>4</sub>, the anti-sigma AsiA forms a tight complex with the  $\sigma^{70}$  subunit (8, 26). Nevertheless a detailed analysis of its mechanism of action suggests that AsiA interacts with the holoenzyme, thereby modifying the interaction between the enzyme and promoter sequence and not simply dissociating the sigma factor from the core (27–29). Re-

cently it has been shown that the *Salmonella* FlgM, in addition to sequestering the free sigma factor, binds to the holoenzyme and increases the rate of dissociation of sigma from the core (30).

The anti-sigma factors themselves are regulated. In the case of SpoIIAB and RsbW, the sigma factor is set free to accomplish transcription by the action of an anti-anti-sigma factor (SpoIIAA and RsbV, respectively), which under certain conditions forms a complex with the anti-sigma protein (25, 31–33).

As for FlgM we do not yet know the mechanism regulating its activity. A clue derives from work on the *Salmonella* homologue of FlgM that has been shown to be secreted into the medium by the flagellar secretion system (34, 35). When the flagellar hook-basal body complex is assembled, the devoted class III secretory apparatus export FlgM from the cell, thereby relieving the inhibition exerted by the anti-sigma factor. In solution the free *Salmonella* FlgM is mostly unfolded, which may facilitate its secretion; it forms a stable complex with  $\sigma^{28}$ , but upon binding, the C-terminal half of FlgM becomes structured, with the N terminus still unfolded and thus potentially competent for secretion (15). The present results from partial proteolysis indicate a different organization of the *B. subtilis* FlgM polypeptide. The N-terminal region is structured, at least as deduced from partial proteolysis and not unfolded as suggested for the *Salmonella* FlgM from NMR studies. The structural differences may hint at different mechanisms in the way to regulate the anti-sigma factor activity in the cell.

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## Overproduction and Characterization of the *Bacillus subtilis* Anti-sigma Factor FlgM

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