UNIVERSITY OF ILLINOIS

April 30 19 21

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Anthony Schmitt

ENTITLED Biochemical Analysis of the Flagellar Hook-Basal Body Complex in Bacillus subtilis

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science

Instructor in Charge

APPROVED:

HEAD OF DEPARTMENT OF Biochemistry

BIOCHEMICAL ANALYSIS OF THE FLAGELLAR HOOK-BASAL BODY COMPLEX IN *BACILLUS SUBTILIS*

BY

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THESIS

for the

DEGREE OF BACHELOR OF SCIENCE

IN

LIBERAL ARTS AND SCIENCES

College of Liberal Arts and Sciences

University of Illinois

Urbana, Illinois

1991

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I. INTRODUCTION

Bacillus subtilis is a gram-positive bacterium that propels itself by rotating its flagella. When rotated counterclockwise, the flagellar filaments come together in a bundle which moves like a wave away from the cell, pushing it forward (1). Figure 1 illustrates the flagellar motion of a straight-swimming bacterium (2). When the filaments are rotated in the opposite (clockwise) sense, they come apart and the cell is randomly re-oriented (1).

A bacterium can advantageously bias its movement simply by controlling the diretion its flagella rotate. A low frequency of clockwise rotations produces a relatively straight swim. An increased frequency of clockwise rotations produces random re-orientations (tumbles) which cause abrupt changes in direction. The process whereby bacteria use these swims and tumbles to move towards chemical attractants and away from chemical repellents is called chemotaxis (3).

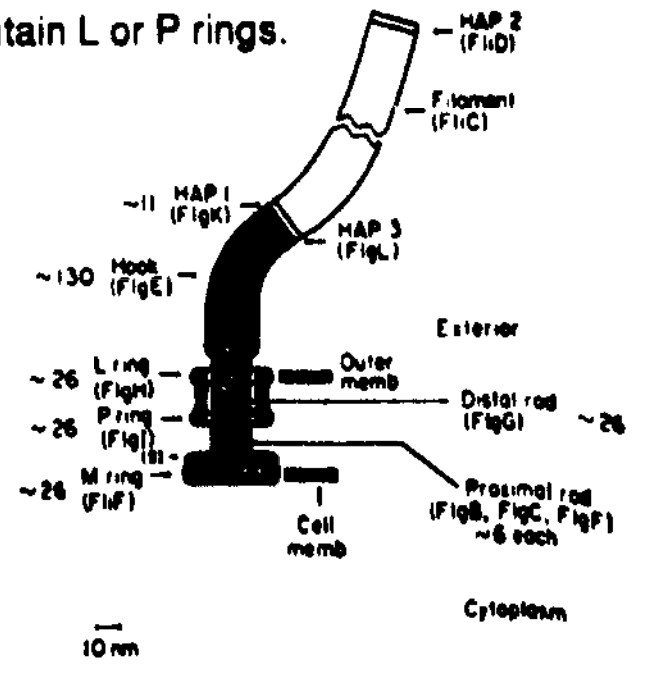
In an effort to understand chemotaxis, some attention has been given to the rotary mechanism of the flagella, particularly in *Salmonella typhimurium*. The two major components of the bacterial motor are the hook-basal body (HBB) which connects the flagella to the cell, and the

FIGURE 1 shows the forward motion produced by the counterclocwise rotation of bacterial flagellar filaments.

Taken from Berg (2), p. 78.

FIGURE 2 is a cartoon of the flagellated HBB complex of *S. typhimurium*. The structure is rooted to the outer membrane by the L and P rings. It is thought that in *B. subtilis* (which lacks an outer membrane) the complex does not contain L or P rings.

Taken from Jones (5), p. 378.



switch, which controls the direction of rotation (4). Of these, the HBB is the only one to have been isolated intact. This structure has also been directly visualized with electron microscopy. Other than the flagella itself, the HBB is the only structure involved in cell motility that can be directly visualized.

Large scale isolation of intact HBB complexes from *S. typhimurium* was performed by Robert Macnab, et. al. in 1984. Since then, most of the HBB proteins and genes from this organism have been characterized. Figure 2 sums up our current understanding of the HBB structure in *S. typhimurium* (5). As shown in the figure, the HBB is composed of a variety of rings and rods, a hook, and several hook-associated proteins (HAPs). The DNA coding each of these components has been located and sequenced. In addition, stoichiometries of the individual proteins have been determined (5).

In contrast, relatively little work has been done on the HBB complex in *B. subtilis*. Since this species lacks the outer membrane present in *S. typhimurium*, one would expect there to be fundamental differences in its basal body structure. Early studies by DePamphilis and Adler suggest that this is the case (6, 7). Their analysis by electron microscopy reveals that *B. subtilis* has just one set of rings (the M and S rings), while *Escherichia coli* and *S. typhimurium* each have an additional set of rings (the S and P rings).

No detailed analysis of the individual components of the *B. subtilis* HBB complex has been done. In 1970 Mel Simon developed a new procedure for isolating *B. subtilis* flagella with HBB complexes attached (8). He studied the thermal stability of these filaments and characterized them with electron microcopy. No biochemical analysis was done (presumably because of yield constraints). Previously mentioned studies by DePamphilis and Addler involved electron microscopic characterization of *B. subtilis* and other HBB complexes, but the individual protein components were not analyzed (6, 7).

Although none of the *B. subtilis* HBB proteins have been directly studied, some of their corresponding genes have been identified on the basis of homology with *S. typhimurium*. These are Flg B and Flg C (components of the proximal rod), Flg G (the distal rod), and Fli F (the M-ring) (9).

It is the goal of this study to characterize individual components of the *B. subtilis* HBB complex. N-terminal sequencing will allow correlation between the proteins and previously studied gene sequences. It is hoped that novel HBB protein sequences will be found so that their corresponding genes can be located and studied.

II. ISOLATION OF INTACT HBB COMPLEXES FROM *B. SUBTILIS*

A. THE MACNAB PROCEDURE

Intact HBB complexes have been successfully isolated from *S. typhimurium* by Macnab and colleagues (10). Figure 3 is an electron micrograph of a *S. typhimurium* HBB preparation. Initial attempts at purification from *B. subtilis* were based directly on the procedure used for *S. typhimurium*. This initial procedure is briefly summarized below. A lengthy and descriptive protocol is not given here because most of the steps are identical to those in the final isolation procedure. All pertinent details and observations are described fully there.

STEP 1: Cell Lysis and Solubilization of Membranes

A liter of wildtype (strain 1085) *B. subtilis* cells were grown to late-log growth phase and resuspended in a sucrose solution (0.5 M sucrose, 0.1 M Tris base, pH 8.0) by gently sending the mixture in and out of a 10 ml pipette. Lysozyme and ethylenediamine tetraacetic acid (EDTA) were added, and the mixture was put on ice in the cold room for 2.5 hours. 10 ml of 10% Triton X-100 were added and the solution was left in the cold room (4^{o} C) overnight.

STEP 2: Removal of DNA

DNase 1 was added in the presence of $MgSO_4$, and the mixture was incubated at 30^{o} C for one hour.

STEP 3: Differential Centrifugation

Unlysed cells and cell debris were removed with a low-speed spin. In some trials, the supernatant was brought to pH 11 with NaOH. In *S. typhimurium*, a major HBB contaminant is outer membrane vesicles, which are very resistant to detergents. pH 11 treatment causes dissolution of the vessicles yet leaves the flagella intact. *B. subtilis* has no outer membrane, and so the use of a pH altering step is of questionable value. The supernatant was spun at high speed to pellet the flagellin.

STEP 4: Cesium Chloride Density-Gradient Spin

The pellet was dried and washed, then subjected to a CsCl gradient spin, which produced a thick, cloudy white flagella band. The flagellin was collected and dialyzed to remove the cesium.

STEP 5: Isolation of HBB Complexes from Flagellin

The flagellin was pelleted and redissolved in an acidic (pH 2.5) solution. The acidic treatment dissociates the flagellin but not the

HBB complex, which can subsequently be pelleted and redissolved.

The procedure described above is almost identical to the one used by Macnab (10). The only differences between the two are:

1. Macnab's procedure uses *S. typhimurium* instead of *B. subtilis*.
2. Macnab only incubated for 40 minutes after the addition of EDTA before adding Triton X-100.
3. Macnab uses less lysozyme (10 μg versus 15 μg per ml).
4. Macnab relies solely on endogenous DNase to act upon addition of $MgSO_4$, whereas in the above procedure DNase is added.
5. Macnab uses polyallomer ultracentrifuge tubes; polycarbonate ones were used here.
6. Macnab uses 36 g of CsCl in 90 ml of solution, whereas 8.2 g of CsCl in 20 ml are used in this procedure. The appropriate concentration of CsCl was empirically determined for *B. subtilis* by Aamir Zuberi, who also provided much of the guidance for this study.

Samples prepared by the above procedure were analyzed with a Hitachi H-600 transmission electron microscope at the University of Illinois Center

for Electron Microscopy. Carbon coated formvar grids were used to support the samples, which were negatively stained with 2% phosphotungstic acid. Basal bodies were not visible in the samples. In the flagellated preparations (samples which had not been subjected to acid treatment), large amounts of flagellin were seen, but their ends were void of basal bodies. No differences were observed between pH 7 and pH 11 preparations.

B. MINOR MODIFICATIONS IN THE MACNAB PROCEDURE

One possible explanation for the failure is that the flagella were being sheared from the cells before lysis, leaving the basal body embedded in the cell membrane. This would account for the lack of basal bodies in the flagella preparations. The procedure was repeated, except that rather than using a 10 ml pipette to resuspend the cells, the mixture was put on ice and placed on a rotary shaker until the cells were dissolved. Electron microscopy of the resulting preparation showed that it still lacked intact HBB complexes.

Another explanation for the failure is that the detergent is not satisfactorily dissolving the membrane. The purpose of adding detergent is

to convert an organelle embedded in a membrane to one enclosed in a micelle. If the detergent fails to separate the HBB from the membrane, the isolation will fail. During the CsCl gradient spin, the HBB will be caught between a flagellar filament which is banding in the middle of the tube and the membrane, which is pelleting at the bottom. This "tug-of-war" will cause the filament to break and the HBB to be lost at the bottom of the tube. An increase in the concentration of the detergent Triton X-100 from 0.1% to 0.5% in the solubilization step was tried. Electron microscopy revealed that these samples were still lacking HBB complexes.

C. HBB ISOLATION BY A COMBINED SIMON / MACNAB PROCEDURE

Mel Simon in 1970 developed a procedure for the isolation of flagella-HBB complexes which was specific for *B. subtilis* (8). Two major differences between Simon's procedure and the ones previously described are:

1. Simon uses a different detergent (Brij-58 polyoxyethylene 20 cetyl ether rather than Triton X-100).
2. Simon does an ammonium sulphate fractionation before the CsCl gradient spin.

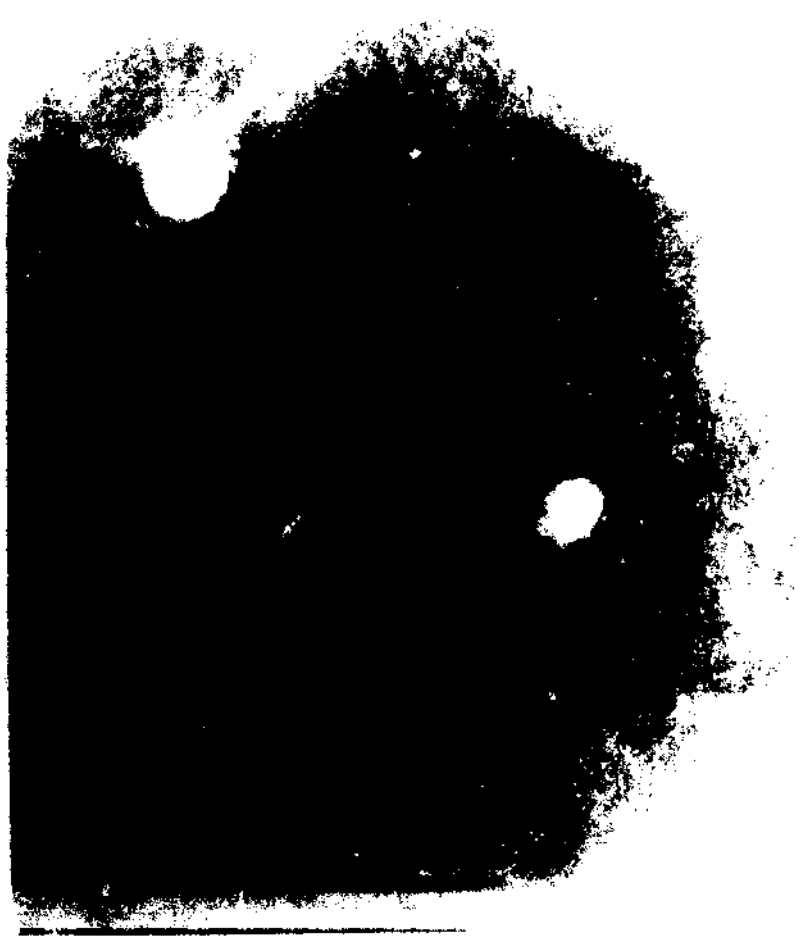
A combined Simon/Macnab procedure was tried, where the cells were lysed and solubilized according to the Simon procedure (with Brij-58), but were then subjected to differential and density-gradient centrifugations according to the Macnab procedure (thus avoiding the ammonium sulphate fractionation).

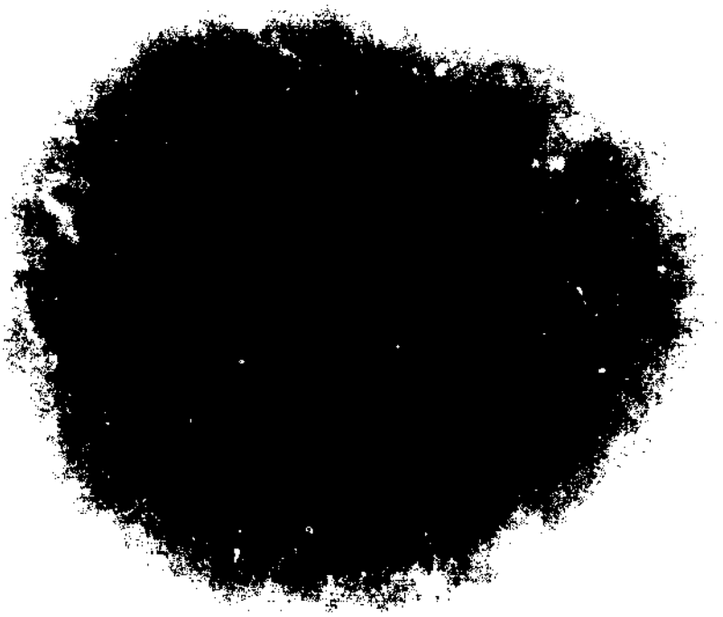
Electron microscopy of samples prepared by the combined procedure revealed that a significant portion of the flagella contained intact HBB complexes at their ends (Figures 4 and 5). HBB structures were also observed in samples where the flagellin had been removed with the acid treatment (Figures 6, 7, and 8).

The detergent type thus appears to be important in the purification of *B. subtilis* HBB complexes. Triton X-100 and Brij-58 are both non-ionic detergents containing hydrophobic chains between 10 and 15 carbons long. Triton has an aromatic ring in the nonpolar region, while Brij has a straight chain (11). Both structures are shown in Figure 9.

The combined Simon/Macnab procedure was used for all subsequent HBB preparations. The following is a detailed description of HBB isolation by this method. Appendix 1A contains a brief overall scheme for the isolation procedure, and in Appendix 1B is the actual protocol that was developed for

FIGURES 4-8 (next five pages) are electron micrographs of samples obtained using the combined Simon/Macnab procedure. Figures 4 and 5 show flagellated samples (no acidic treatment). Figures 6-8 show HBB complexes (flagellin removed). All micrographs were taken at 100,000 times magnification.





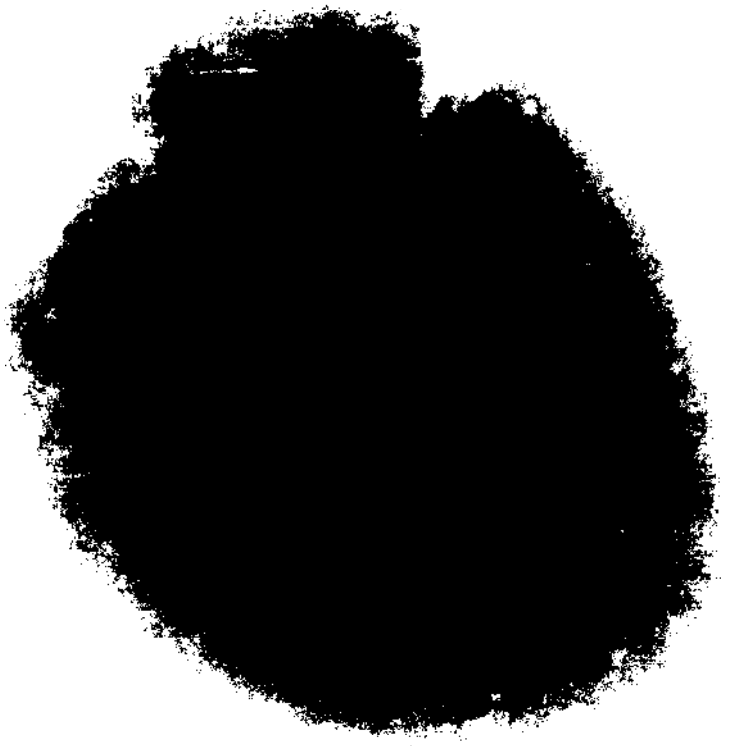


FIGURE 9 shows the structures of the two non-ionic detergents Triton X-100 and Brij-58.

Taken from Gennis (11), p. 90.

the isolation.

STEP 1: Cell Lysis and Solubilization of Membranes

A *B. subtilis* colony (strain 1085) from a tryptose blood agar base (TBAB) plate is used to innoculate 10 ml of sterile L Broth (10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl in water to 1 liter) in a sterile flask. The cell solution is incubated at 37^{o} C shaking for 14-16 hours. 5 ml of the overnight culture are added to each of two 2000 ml erlenmeyer flasks containing 500 ml of sterile L Broth. The flasks are incubated at 37^{o} C shaking until the late-log phase of cell growth (when almost all cells appear motile under the microscope, typically about 4 hours).

The cells are transferred to 250 ml centrifuge buckets and spun at 4000 g in a Beckman J-21B centrifuge for 20 minutes at 4^{o} C. To each of the cell pellets is added 8.3 ml of 0.1 M Trizma base, pH 8.0. The buckets are put in ice on a rotary shaker until the cells are dissolved, resulting in a 30 times concentrated cell suspension which is very thick and cloudy. The four fractions are combined and treated with lysozyme (1 ml of a 3.3 mg/ml solution). The lysozyme is allowed to react for 2 hours at 4^{o} C, and then the

solution is made 0.5% Brij-58 by adding 3.8 ml of 5% Brij. The mixture is stored at 4^{o} C until lysis is complete (usually overnight). After the overnight solubilization, the solution turns clear yellow and is somewhat viscous.

STEP 2: Removal of DNA

The solution is made .01 M $MgCl_2$ through the addition of 0.78 ml of 0.5 M $MgCl_2$. 0.78 mg DNase 1 is added and the mixture is incubated at 30^{o} C for one hour. The DNase treatment removes the viscosity.

STEP 3: Differential Centrifugation

Membrane debris is removed by spinning at 4000 g for 20 minutes at 4^{o} C. The supernatant is saved and spun at 70,000 g in a Beckman L5-75 ultracentrifuge for one hour at 4^{o} C. 65 ml polycarbonate metal capped tubes are used. A thick translucent membraneous pellet is produced which is colorless around the edges and has some dark brown splotches near the center. The supernatant is discarded and 20 ml of 0.1 M Tris base, 0.5% Brij-58, pH 8.0 are added. No effort is made at dissolving the pellet. The mixture is respun at 70,000 g, 4^{o} C for one hour.

STEP 4: Cesium Chloride Density-Gradient Centrifugation

To the pellet is added 18 ml of room temperature TEB (1.58 g Trizma base, 1.86 g EDTA, 5.00 g Brij-58 to one liter in water). Considerable effort is made at dissolving the pellet in the TEB. Typically, it takes about 20 minutes of inversions just to dislodge the pellet from the tube wall. The mixture can be transferred to a screw-top test tube and put in a mechanical inverter for a few hours to get the pellet completely dissolved. 8.2 g of CsCl are added all at once. (DePamphilis and Adler (6) mention that the flagellin band is much less diffuse when the CsCl is added all at once). The CsCl solution is spun in a 25 ml polycarbonate metal capped tube at 70,000 g in a Beckman L5-75 ultracentrifuge for at least 16 hours at 15^{0} C. The flagellin band is cloudy white, thick, and very dense. It usually settles about a third of the way down the tube. Some debris normally settles at the bottom of the tube, and the liquid at the very top is usually orange. The orange material is removed first, then the flagellin band is collected with a Pasteur pipette. Normally 4-5 ml of material can be collected before the band becomes too diffuse to see. The flagellin is then

dialyzed in cellulose tubing (Sigma No. D-9277, which retains proteins of molecular weight 12,000 or greater) versus TEB. Three different batches of TEB are used, at least one of which is an overnight dialysis. Once dialysis has been completed, a small amount of material is saved (flagellin preparation) and the rest is used to make HBB complexes.

STEP 5: Isolation of the Hook-Basal Body from Flagellin

Spinning the sample at 100,000 g in a Beckman TL-100 ultracentrifuge at 4^{o} C for one hour yields a thick membraneous pellet, which is subsequently dissolved in Acidic solution (0.375 g glycine, 0.5 g Brij-58, pH 2.5, in water to 100 ml). The acidic mixture is allowed to dissolve overnight, then is respun at 100,000 g, which produces a much smaller and less membraneous pellet. This pellet is dissolved in TEB (typically 50 µl).

III. BIOCHEMICAL ANALYSIS OF THE *BACILLUS SUBTILIS* HOOK-BASAL BODY COMPLEX

A. EXPECTATIONS

The proteins in the HBB will be analyzed in terms of size (by Sodium Dodecyl Sulphate Polyacrylamide Gel Elecrophoresis, or SDS-PAGE), stoichiometry (by banding intensities), and sequence (by N-terminal analysis).

1. Size

Four of the *B. subtilis* HBB genes have been identified (through homology with *S. typhimurium*) and sequenced. Their predicted sizes are given in Table 1, and their predicted amino acid sequences are given in Appendix 2. One would expect these proteins to be among those present in the HBB preparations.

Additionally, the components of the *S. typhimurium* HBB complex have been previously analyzed by SDS-PAGE. The sizes of these peptides may be similar to the ones determined for *B. subtilis*.

2. Stoichiometry

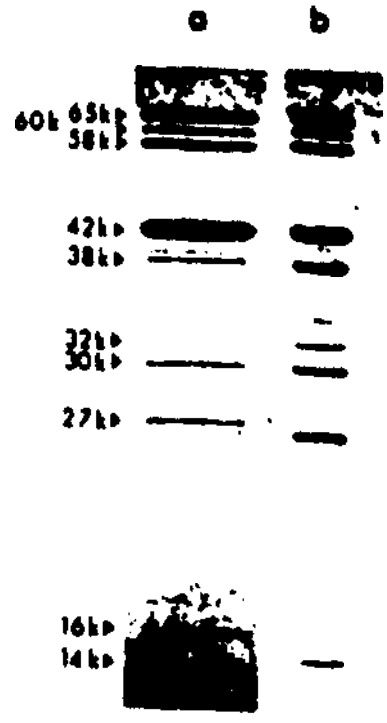
The stoichiometries of the components of the *S. typhimurium* HBB complex have been determined (5). The hook protein is by far the most abundant

TABLE 1: Predicted Sizes of *B. subtilis* HBB Proteins

Based on Known Gene Sequences

B. subtilis Gene	HBB Protein	Predicted Size
Flg B	Proximal Rod	14.4 kDa
Flg C	Proximal Rod	16.3 kDa
Flg G	Distal Rod	27.5 kDa
Fli F	M-Ring	59.3 kDa

FIGURE 10 is an SDS gel of HBB complexes purified from *S. typhimurium*. Bands are visualized with Coomassie (lane a) and silver (lane b). The most intense band is that of the hook protein, which has an apparent molecular weight of 42 kDa. Taken from Aizawa (10), p. 840.



(approximately 130 subunits per complex). The next most abundant proteins are the distal rod and the M-ring at 26 subunits each. Based on this, one would expect the most intense band on any protein gel of a HBB preparation to be that of the hook protein. This is illustrated in Figure 10, an SDS gel of a HBB preparation in *S. typhimurium* .

3. Sequence

An N-terminal amino acid sequence of any of the four known *B. subtilis* HBB proteins should be identical to the corresponding sequence in Appendix 2. Other HBB components are expected to be identifiable based on homology with known *S. typhimurium* amino acid sequences.

B. RESULTS

Purified *B. subtilis* HBB complexes were analyzed by SDS-Polyacrylamide Gel Electrophoresis. The actual protocol used (based on the method of Laemmli (12) is provided in Appendix 3. All samples were run with a polyacrylamide concentration of 12.5%. High molecular weight markers from Bethesda Res. Laboratories were loaded on all gels run. The mixture contains proteins of 200, 97.4, 68.0, 43.0, 29.0, 18.4, and 14.3 kd. Early samples (not shown) were silver stained by the method of Burk (13).

Figure 11 shows a Coomassie-stained SDS gel of a *B. subtilis* HBB preparation at each step in the isolation procedure. Lane descriptions for the gel are given in Table 2. Molecular weights were calculated relative to the molecular weight markers in lane 8. A best-fit third order polynomial curve was drawn between the data points to generate a standard curve.

As can be seen from lane 6, flagellin was obtained with little contamination. The observed molecular weight for flagellin is 35 kd.

1. Size

Based on size, the HBB preparation (lane 7) coincides moderately well with the four proteins of known gene sequences. The 14 and 17 kd bands might correspond to Flg B and Flg C, respectively. The 28 kd band could be the distal rod. There is no band with a molecular weight corresponding to that predicted for the M-ring (59.3 kd).

2. Stoichiometry

Based on stoichiometry one would not expect the 28 kd band to be the distal rod. It is the brightest and should therefore be the hook.

3. Sequence

Sequence analysis was performed on a different sample, whose Coomassie-stained SDS gel banding pattern is shown in Figure 12. As

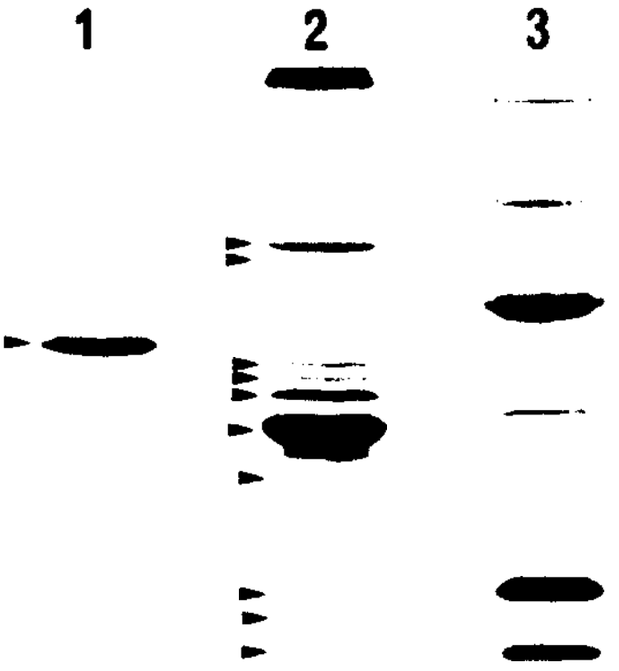
FIGURE 11 (next page) is a Coomassie stained SDS gel illustrating the isolation scheme used for *B. subtilis* HBB complexes. Lane descriptions are given in Table 2. Bands in lane 7 thought to represent actual HBB components are indicated by arrows. The apparent molecular weights of these bands, from top to bottom, are 51, 45, 32, 31, 28, 25, 18, 17, and 14 kDa. Flagellin (lane 6) has an apparent molecular weight of 35 kDa.

1 2 3 4 5 6 7 8

TABLE 2: Lane Descriptions for the Isolation Scheme Gel (Figure 11)

Lane	Description
1	5 µl whole cells
2	5 µl cells lysed with lysozyme and solubilized with Brij-58
3	5 µl solubilized cells after removal of cell debris by a low-speed spin
4	20 µl after the first high-speed spin
5	10 µl after the second high-speed spin
6	5 µl purified flagella preparation (after gradient spin and dialysis)
7	30 µl purified HBB preparation
8	5 µl BRL high molecular weight markers

FIGURE 12 (next page) is a Coomassie stained SDS gel of the *B. subtilis* HBB preparation that was analyzed by N-terminal analysis. Shown are flagellated HBBs (lane 1), HBBs (lane 2), and BRL high molecular weight markers (lane 3). The flagellin has an apparent molecular weight of 34 kDa. Bands in lane 2 thought to represent actual HBB proteins are labelled with arrows. Their apparent molecular weights are (from top to bottom) 52, 48, 33, 32, 31, 28, 26, 18, 16, and 14 kDa.


1
2
3

expected, the darkest band is at 28 kd. This band was submitted for N-terminal sequence analysis. It was hoped that the amino acid sequence would be homologous to that of the hook protein in *S. typhimurium*, which has been recorded by Homma (14). No known gene sequence in *B. subtilis* shows homology to the *S. typhimurium* hook -- thus by sequencing the hook protein, one might be able to locate and characterize the hook gene in *B. subtilis*.

Sequencing was done on protein electroblotted onto PVDF paper as described in Appendix 4. The 28 kd band was submitted to Charles Mitchell at the University of Illinois Genetic Engineering Facility. Protein analysis was done by Edman degradation, with the residues being detected by reverse-phase HPLC (15).

Results of the analysis are shown in Table 3. This sequence was compared to known *B. subtilis* basal body gene sequences, and a striking resemblance was observed upon comparison with the Flg G (distal rod) sequence (Figure 13). Thus it has been concluded that the 28 kd *B. subtilis* HBB protein is in fact the product of Flg G -- the distal rod.

Additional analysis of the HBB preparations by Charles Mitchell at the Genetic Engineering Facility revealed that the 28 kd band is actually composed of two smaller bands (Figure 14). The heavier upper band is Flg G.

TABLE 3: N-Terminal Analysis of the 28 kDa *B. subtilis* HBB Protein

Cycle	Residue	Cycle	Residue	Cycle	Residue
1	Met	5	Leu	9	Ile
2	Leu	6	Tyr	10	?
3	Arg	7	Ser	11	Gly
4	Ser	8	Gly	12	Met

FIGURE 13 gives the amino acid sequence for the *B. subtilis* distal rod protein as deduced from its gene sequence (Flg G). This sequence correlates very well with the one shown above in Table 3.

Taken from Zuberi (9).

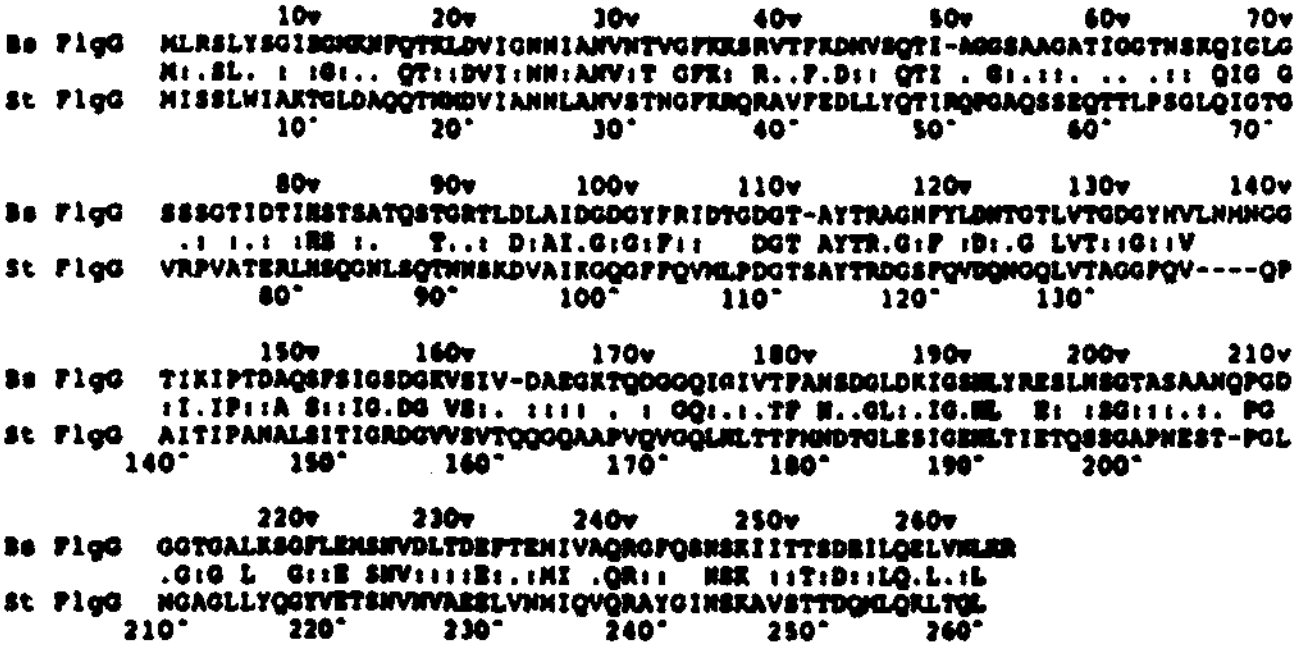


FIGURE 14 is a silver stained SDS gel of a *B. subtilis* HBB preparation run by Charles Mitchell at the University of Illinois Genetic Engineering Center. His analysis revealed that the 28 kDa band is actually composed of two proteins with apparent molecular weights of 28 kDa and 27 kDa. The HBB sample used for this analysis is the same as that used to generate the SDS gel in Figure 11.

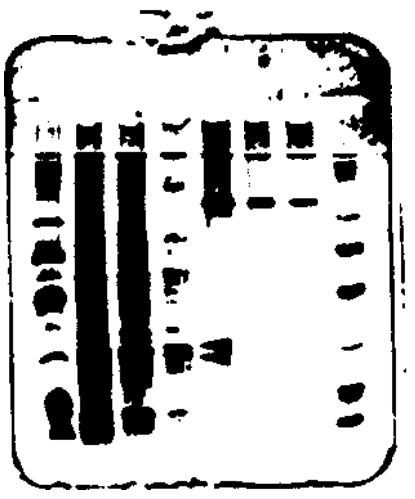


TABLE 4: N-Terminal Analysis of the 27 kDa *B. subtilis* HBB Protein

Cycle	Residue	Cycle	Residue	Cycle	Residue
1	Ala	5	Ile	9	Gln
2	Phe	6	Lys	10	Ala
3	Asn	7	Asp?	11	Asp?
4	Asp	8	Lys	12	Glu?

The lower band (27 kd) was sequenced, and the results are given in Table 4. This sequence does not correspond to any known *B. subtilis* basal body genes. Also, there is no homology to any of the *S. typhimurium* basal body or related proteins. Thus the 27 kd protein is either an HBB component with significant divergence from its *S. typhimurium* counterpart, or is a non-HBB contaminating protein. Since most known *B. subtilis* motility genes show extensive homology to those in *S. typhimurium*, it is assumed that the latter is the case.

IV. ISOLATION AND ANALYSIS OF HOOKS FROM *B. SUBTILIS*

It was initially hoped that the hook protein would be easily identifiable on an HBB protein gel. Since this turned out not to be the case, additional measures were taken in an effort to find the hook. In the original article describing *S. typhimurium* HBB isolation (10), Bob Macnab also gave a procedure for the isolation of hooks alone. Since this method is quite similar to the one for HBB isolation, only a brief description of it will be given, with emphasis on the parts that are different.

A. HOOK ISOLATION

One liter of *B. subtilis* cells are grown to the late-log phase of growth as

previously described. The cells are pelleted and resuspended in a total of 60 ml of phosphate buffer (13 mM phosphate, 150 mM NaCl, pH 7.0). The suspension is divided into 10 ml aliquots, and each aliquot is taken up and expelled four times by a 10 ml syringe with a 21-gauge needle. The fractions are combined and centrifuged at low speed to remove cell debris. The remainder of the procedure is identical to that for HBB isolation (beginning with the first ultracentrifugation).

B. ANALYSIS OF HOOK PREPARATIONS

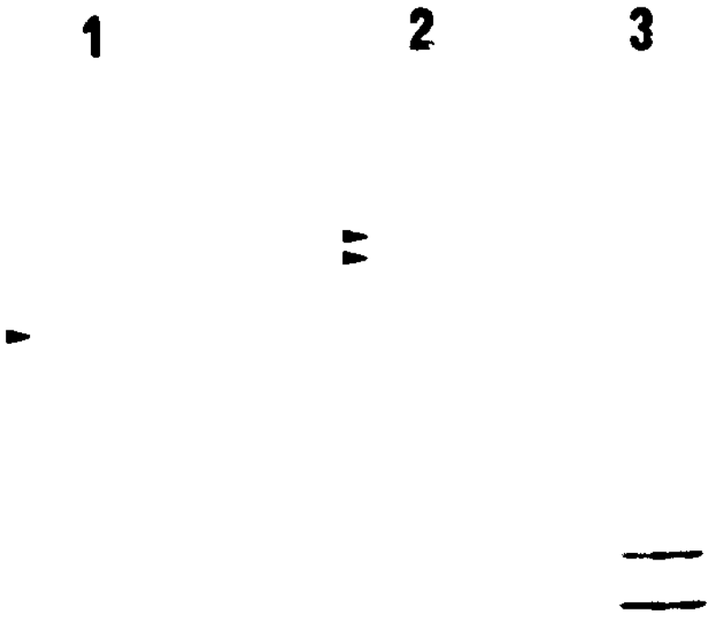
Electron micrographs taken at 30,000 times magnification of a typical hook preparation are shown in Figures 15-18. Hook structures are present in fair yield, but there is a lot of contamination from flagellin and cell debris. The ends of the hooks tend to attach themselves to the debris, so it is difficult to discern whether or not they have indeed been purified away from the basal bodies. SDS-PAGE analysis (not shown) yields a banding pattern similar to that for an HBB prep -- thus hook purification was unsuccessful.

C. ANALYSIS OF A UNIQUE HOOK PREPARATION

One attempt at hook isolation gave very different results (though the same procedure was used). SDS-PAGE analysis of this preparation (Figure 19) shows two faint bands -- 65 and 53 kd. Electron microscopy

FIGURES 15-18 (next four pages) are electron micrographs taken at 30,000 times magnification of a typical preparation obtained by following the hook isolation procedure for *B. subtilis*. SDS-PAGE analysis indicates that these samples are contaminated with basal bodies.

FIGURE 19 (next page) is a Coomassie stained SDS gel of a unique *B. subtilis* hook preparation. Unlike most of the preparations, this one appears to be uncontaminated by basal bodies. Shown are flagellin (lane 1), hook preparation (lane 2), and BRL high molecular weight markers (lane 3). The indicated flagellin band in lane 1 has an apparent molecular weight of 35 kDa. The marked bands in lane 2 have apparent molecular weights of 65 kDa (top) and 53 kDa (bottom).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2
3

FIGURES 20-22 (next three pages) are electron micrographs of the unique hook preparation which produced the gel in Figure 18. The first two micrographs were taken at 100,000 times magnification, while the third was taken at 200,000 times magnification.

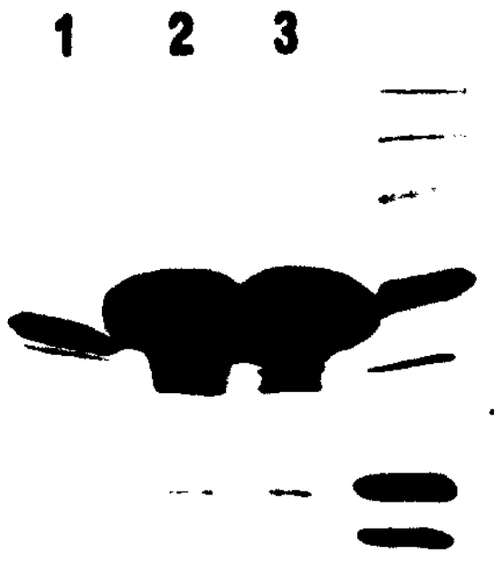
(Figures 20-22) indicates that hooks are pesent, but in low amounts. Again, it is difficult to discern whether basal bodies are attached.

The protein gel (Figure 19) was encouraging, because one would expect to see only two or three bands in a hook preparation (the hook protein and one or two HAPs). The darker (65 kd) band was submitted for N-terminal analysis, but the protein amount was too small for a sequence to be obtained.

D. pH STABILITY OF THE HOOK

The stability of the hook at pH 2.5 (the pH used to dissociate the flagellin away from the HBB) was questioned. If the pH treatment was in fact dissolving hooks as well as flagellin, this would explain the observation that the hook is not the dominant protein in an HBB preparation. It would also account for the low yields of the hook preparations. To test this hypothesis, acidic solutions of various pHs were used in place of the traditional pH 2.5 acidic solution in the hook isolation procedure. One would expect a dramatic increase in the intensity of the hook band if the pH change were to prevent its dissociation. The SDS-PAGE results are shown in Figure 23. Of the three pHs tried (2.5, 3.5, and 3.9), only pH 2.5 (lane 1) successfully dissociated flagellin. The high molecular weight (65 and 53 kd) bands did not increase in intensity as a result of the pH change. As for the lower molecular weight

FIGURE 23 (next page) is a Coomassie stained SDS gel of flagella-hook preparations treated with varying degrees of acidity: pH 2.5 (lane 1), pH 3.5 (lane 2), and pH 3.9 (lane 3). The flagellin dissociated only under the harshest conditions (pH 2.5).


1
2
3

proteins, several bands are brighter at higher pH. Thus the experiment failed to reveal the hook band.

V. PROBING HBB SAMPLES WITH ANTIBODIES

Western blotting was utilized as a tool which could possibly reveal the hook band. Antibodies versus flagellin were readily available. The flagellin that these were raised against had been purified by shearing cells in a blender, pelleting the cells, and isolating flagellin from the supernatant. It was hoped that some hooks had been sheared off with the flagellin, and that the antibodies would therefore cross-react with the hook band of an HBB preparation.

The procedure used for western blotting is found in Appendix 5. Figure 24 shows the result of a Western blot done with anti-flagellin antibodies versus 30 µl of a HBB preparation isolated in the usual manner. It is evident from the result that the anti-flagellin antibodies cross-react with several HBB components -- thus the hook cannot be identified by this means.

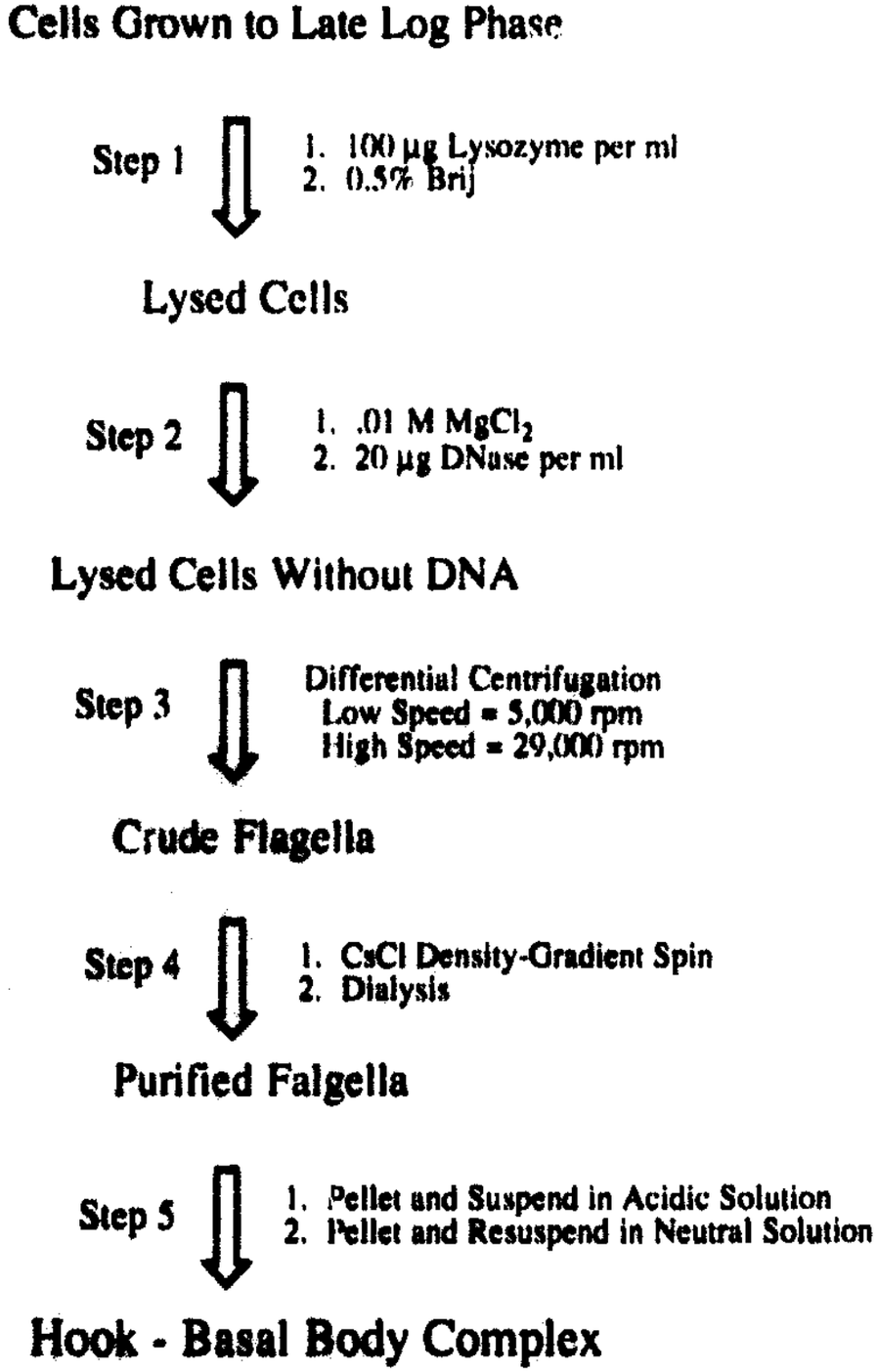
A similar experiment may be tried in the future in which antibodies versus the *S. typhimurium* hook are obtained and used against a *B. subtilis* HBB preparation. Pure hook samples are obtainable from *S. typhimurium*, because in that organism the hook gene has been cloned and over-expressed.

FIGURE 24 (next page) shows the results of a Western blot against a HBB preparation using anti-flagellin antibodies. The antibodies cross-reacted with several basal body components.

VI. CONCLUSION

The goal of this study was to isolate intact hook-basal body complexes from *B. subtilis* and to characterize their individual protein components. A procedure was developed which successfully purifies *B. subtilis* HBBs, as confirmed by electron microscopy. Apparent molecular weights of the HBB proteins were obtained through SDS-PAGE analysis. The identity of the 28 Kd band was revealed by N-terminal sequence analysis, showing it to be the product of Flg G -- the gene homologous to the one coding the distal rod in *S. typhimurium*. The hook protein is not the most abundant component of the proteins obtained by the isolation procedure. This is surprising, considering the known dominance of hook in the HBB complex of *S. typhimurium*. If hooks are being lost or destroyed in the isolation procedure, it is probably not due to the pH 2.5 treatment, since relaxation of the acidity does not produce a new dominant HBB band.

APPENDIX 1A: OVERALL SCHEME FOR BASAL BODY ISOLATION



APPENDIX 1B: PROTOCOL FOR HBB ISOLATION

STEP 0: GROW CELLS

Innoculate 10 ml L Br with a *B. Subtilis* colony. Incubate at 37° C, shaking for 14-16 hours. Add 5 ml of the cell solution to each of two 2000 ml erlenmeyer flasks containing 500 ml of autoclaved L Br. Incubate flasks at 37° C, shaking until almost all the cells are moving (3.5-4.5 hours).

STEP 1: LYSE CELLS AND SOLUBILIZE MEMBRANES

Transfer cells to 250 ml centrifuge buckets and spin at 5000 rpm; 4° C for 20 minutes. Save the pellets on ice, and to each add 8.3 ml of 0.1 M Tris, pH 8. Shake on ice until pellets are resuspended. Combine the fractions and add 1 ml of 3.32 mg/ml lysozyme. Incubate for 2 hours at 4° C, or until most of the cells convert to spheroplasts. Make the solution0.5% Brij by adding 3.8 ml 5% Brij. Store at 4°C until lysis is complete and the solution turns clear (probably overnight).

STEP 2: REMOVE DNA

Make the solution .01 M $MgCl_2$ by adding 0.78 ml of 0.5 M $MgCl_2$. Add 0.775 mg DNase to the mixture and incubate for 1 hour at 30° C so that the solution is no longer viscous.

STEP 3: DIFFERENTIAL CENTRIFUGATION

Remove cell debris by spinning at 5000 rpm; 4° C for 20 minutes. Spin the supernatant at 29,000 rpm; 4° C for 1 hour. Dissolve the pellet in 20 ml of 0.1 M Tris, 0.5% Brij, pH 8.0. Respin at 29,000 rpm; 4° C for 1 hour.

STEP 4: CESIUM CHLORIDE DENSITY-GRADIENT SPIN

Add 18 ml of room temperature TEB to the pellet and mix until dissolved (possibly an hour or more). Add 8.2 g CsCl all at once, and spin at 29,000 rpm; 15° C for at least 16 hours. Remove the thick cloudy CsCl flagella band. Dialyze versus TEB once for 2 hours, once for 3 hours, and then overnight. Save 100 µl of the purified flagella.

STEP 5: ISOLATION OF HOOK - BASAL BODY COMPLEX FROM FLAGELLA

Spin the remaining material at 50,000 rpm; 4°C for 1 hour in a TL-100 ultracentrifuge. Remove the supernatant and dissolve the pellet in 2 ml Acidic Solution. Mix at 4°C until pellet is completely dissolved (possibly several hours). Respin as 50,000 rpm; 4°C for 1 hour. Suspend in TEB buffer to achieve desired concentration (normally 50 µl).

APPENDIX 2: SEQUENCES OF KNOWN *B. SUBTILIS* BASAL BODY GENES

Source: Zuberi (9)

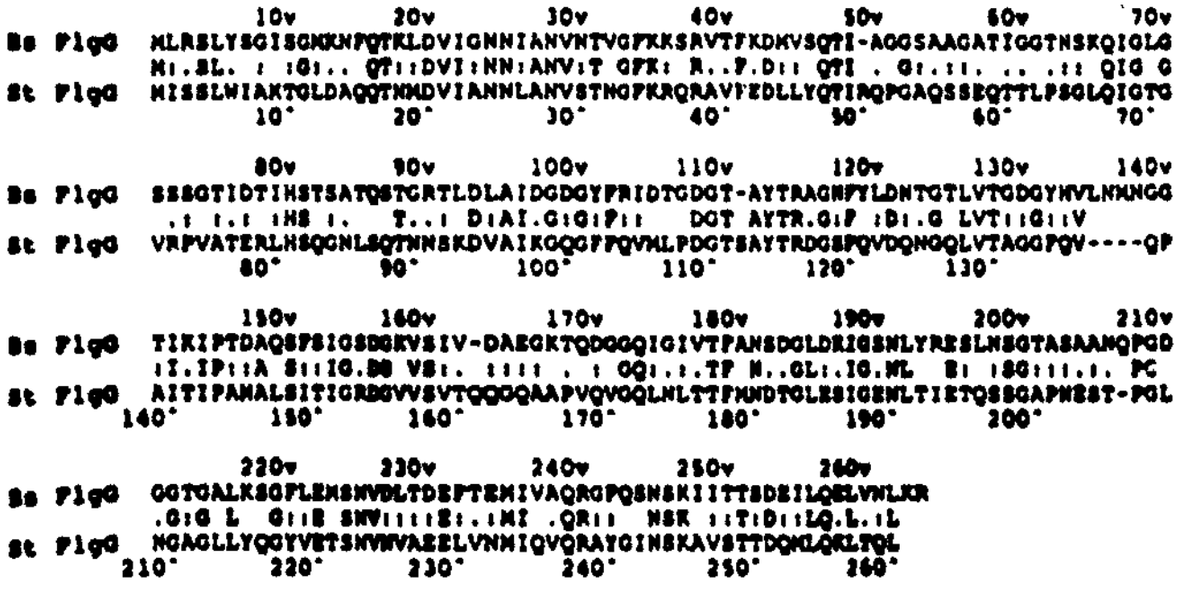
1. Flg B (Proximal Rod)

```
                10v        20v        30v        40v        50v
Bs FlgB   M-SLFSGTIQNLENALSRADIKQKVITNNIANIDTPNYKAKKVSFQNLLDQ
          M . :.:::: ::AL: . :Q.:::.NIAN DTP.Y:A:.:.F.: L.:
St FlgB   MLDRLDAALRFQQEALNLRAQRQEILAANIANADTPGYQARDIDFASELKK
                10^        20^        30^        40^        50^

                60v        70v        80v              90v
Bs FlgB   --ESSRLEAIKTDYRHVDFSDTDSNYSIVASGDTSY------QQNGNNVDV
            .:R E: .. .. :. :: :: D Y :GN.VD:
St FlgB   VMVRGREETGGVALTLTSSHHIPAQAVSSPAVDLLYRVPDQPSLDGNTVDM
              60^        70^        80^        90^       100^

             100v       110v       120v
Bs FlgB   DKEMTELAQNQINYQALVERMNGKFNSLKTVLTGGK
          D:E.T::A:N ::YQ :. :.::::::..VL GG:
St FlgB   DRERTQFAENSLKYQMGLTVLGSQLKGMMNVLQGGN
              110^       120^       130^
```

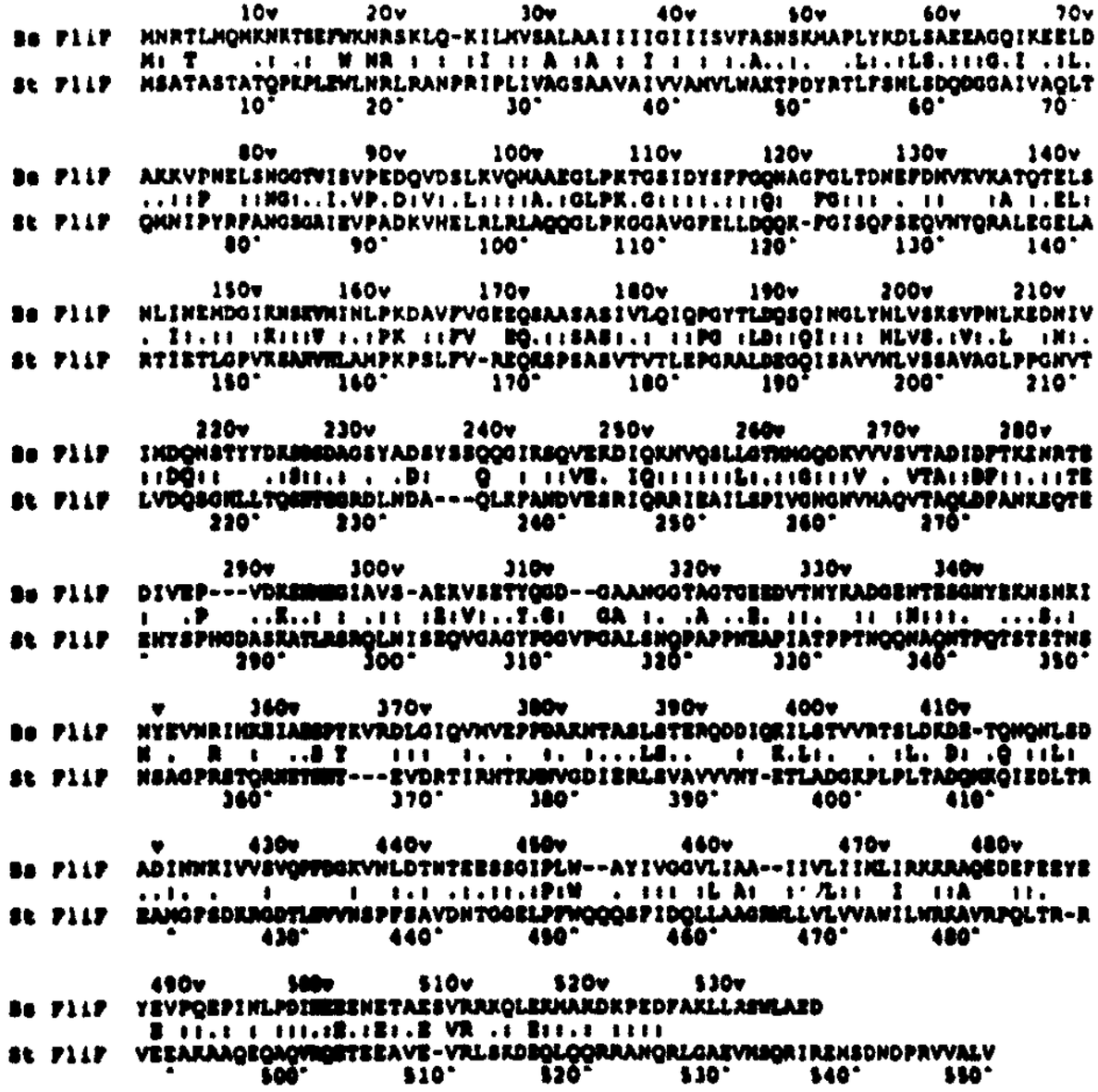
2. Flg C (Proximal Rod)

```
                10v        20v        30v        40v        50v
Bs FlgC   MTAFHSLNVSASALTAQRVRMDVVSSNLANMDTTRAKQVNGEWVPYRRKMV
          M: :: :::::SAL:AQ. R::V.:SNLAN D:. : : PYR K V
St FlgC   MALLNIFDIAGSALAAQSKRLNVAASNLANADSVTG----PDQQPYRAKQV
                10^        20^        30^              40^

               60v        70v        80v        90v       100v
Bs FlgC   SLQSKGESFSSILNSQMSGSGNAGNGVKVSKITEDDSDFNLVYDPTDPDAN
            :Q :::G:A..GVKV:.:.E.:: :LVY:P.:P A:
St FlgC   VFQ------------VDAAPGQATGGVKVASVIESQAPEKLVYEPGNPLAD
          50^                    60^        70^        80^

              110v       120v       130v       140v       150v
Bs FlgC   AEGYVQKPNVDPLKEMVDLVSSTRSYEANVTAMNATKGMLMKALEIGK
          A:GYV:.PNVD : EMV: :S::R:Y:AN:..:N:.K:M::K:L.:G:
St FlgC   ANGYVKMPNVDVVGEMVNTMSASRTYQANIEVLNTVKSMMLKTLTLGQ
            90^       100^       110^       120^       130^
```

3. Flg G (Distal Rod)



4. Fli F (M-Ring)



APPENDIX 3: PROTOCOL FOR SDS-PAGE

I. MATERIALS

Acrylamide Stock

30% Acrylamide
0.8% Bis-acrylamide

10X Reservoir Buffer

0.25 M Tris Base
1.92 M Glycine pH 8.3
1.0% SDS

Coomassie Blue Destain

90 ml Acetic Acid
450 ml Methanol
450 ml Water

Sample Application Buffer

0.125 M Tris-Hcl
4% SDS
20% Glycerol
0.002% Bromophenol Blue
Water to a final volume of 20 ml

Coosmassie Blue Stain

225 ml Methanol
45 ml Acetic Acid
1.25 g Coomassie Blue Dye
225 ml water

II. MAKING THE GELS

A. The Running Gel

	PERCENT GEL					
	5%	7.5%	10%	12.5%	15%	17.5%
Bis-Acryl. Stock (ml)	2.5	3.75	5.0	6.25	7.5	8.75
1.5M Tris HCl pH 8.8 (ml)	3.75	3.75	3.75	3.75	3.75	3.75
Water (ml)	8.5	7.25	6.0	4.75	4.5	2.25
10% APS (µl)	50	50	50	50	50	50
10% SDS (µl)	150	150	150	150	150	150
TEMED (µl)	10	10	10	10	10	10

B. The Stacking Gel (5% Acrylamide)

Bis-Acrylamide, Stock	1.25 ml
0.5M Tris HCl, pH 6.8	1.90 ml
Water	4.25 ml
10% APS	25 µl
10% SDS	75 µl
TEMED	5 µl

III. POURING THE GEL

Pour the running gel between the vertical gel plates with a Pasteur pipette. Leave about four cm on top for the stacking gel. Seal the top with water, and let stand at least 1.5 hours. The gel can be left at this stage overnight if it is covered with plastic wrap.

To add the stacking gel, first pour off the top layer of water and rinse the upper part of the gel space with more water. Residual liquid can be removed by sliding a piece of filter paper between the plates. Add the stacking gel mixture until the plates are almost filled, then insert the comb, taking care not to make any air bubbles in the gel mixture. Let the gel stand for about 1.5 hours before loading.

IV. PREPARING AND LOADING THE SAMPLES

First, prepare the sample application buffer by removing an aliquot and making it 10% 2-Mercaptoethanol. Add one volume of this mixture to each of the samples and boil for 2-5 minutes.

Once the stacking gel has polymerized, remove the bottom spacer and clear away any grease or debris that remains on the bottom of the gel. Fasten the plates to the gelbox, and add 1X Reservoir Buffer to the top and bottom gelbox compartments. Carefully remove the comb and rinse out the wells with buffer. Remove the air bubbles on the bottom of the gel with a Pasteur pipette. Load samples into the wells with a Hamilton syringe.

V. ELECTROPHORESIS

Set the power supply so that current is selected, and run at 25 mA. Once the dye front has entered the running gel, current can be increased to 30 mA, if desired. Remove any bubbles that form on the underside of the gel during the run.

VI. STAINING AND DESTAINING

Pry open the gelplates with a razor blade, and put the gel into a glass dish. Cover the gel with coomassie blue stain, wrap the dish with plastic wrap (leaving one corner open), and microwave for 2-5 minutes, or until the mixture starts to bubble. Pour off the Coomassie mix and destain. Sensitivity of Coomassie stain is 1 μg / band.

APPENDIX 4: PROTOCOL FOR ELECTROBLOTTING

I. Materials

10X CAPS Buffer

22.13 g CAPS in 1 liter H_2O. pH 11 (with NaOH).
CAPS = 3-[cyclohexylamino]-1-propanesulfonic acid.

Electroblotting Buffer (2 liters)

200 ml 10X CAPS buffer
200 ml methanol
1600 ml water

II. Procedure

Fractionate the proteins by SDS-PAGE as usual. Cut two pieces of filter to the size of the gel. Use Immobilon (PVDF membrane) for protein sequence analysis, and nitrocellulose for Western blots. Also, cut three pieces of Whatman paper to the same size.

Prepare the filters by wetting in methanol (for PVDF) or water (for nitrocellulose) a few seconds, and then soaking in electroblotting buffer. Soak the gel in electroblotting buffer for five minutes.

Assemble the sandwich. Place the "+" side of the grid in a large dish full of electroblotting buffer. Lay one of the sponges on the grid, followed by two pieces of Whatman paper, the two membrane filters, the gel, the third piece of whatman paper, and the other sponge. With a 5 ml pipette, roll any air bubbles out of the sandwich. Fasten the "-" side of the grid to the sandwich, and transfer very quickly to the

electroblotter. Run a bent spatula along the grids to release any air bubbles. Electroblot at 90V for 10 minutes.

PVDF can be stained by soaking in coosmassie stain for five minutes, then in a destain solution for about 20 minutes. Dry the filter between sheets of Whatman paper.

APPENDIX 5: PROTOCOL FOR WESTERN BLOTTING

I. Materials

Tris Solution

2.42 g Tris base
14. 62 g NaCl pH to 7.5
water to 1 liter with HCl

Tris-PM

95.8 g powdered milk per liter of Tris Solution

Tris-PM-Tween 20

Add .05% Tween 20 to Tris-PM

Keep 100 ml Tris Solution, add powedered milk to 900 ml and keep 300 ml of that. Add Tween-20 to the remaining 600 ml.

AP Buffer

100 mM Tris
100 mM NaCl pH 9.5
5 mM $MgCl_2$

Reaction Stop / Storage Buffer

20 mM Tris pH 8.0
5 mM EDTA

BCIP Stock

50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate

NBT Stock

50 mg/ml Nitro blue tetrazolium

II. Procedure

1. Fractionate proteins by SDS-PAGE, and electroblot to nitrocellulose. Place the filter face up in a tray containing 100 ml of Tris-PM. Put on a low-speed shaker in the warm room (35-37^{0} C) for 1.5 hrs.

2. Pour off the Tris-PM and rinse with water. Add 40 ml Tris-PM with antibody at 1/200 (200 µl). Shake at room temperature for 1 hour.

3. Pour off the antibody solution, and rinse with water. Add 50 ml of Tris-PM-Tween 20 and shake for 10 minutes at room temperature. Repeat this wash a total of three times.

4. Add 40 ml of Tris-PM with secondary goat anti-rabbit antibody (AP-conjugated) at 1:7500 (5.3 µl). Protect the dish from light for the remainder of the procedure. Put the dish on a shaker for 1 hour.

5. Repeat the wash cycle of step 3.

6. Make 25 ml of Color Development Solution by adding 165 µl NBT stock to 25 ml AP buffer, mixing, then adding 82.5 µl BCIP stock. Color should develop after a few minutes, and will continue for at least 4 hours.

7. When color development is satisfactory, pour off the color development solution and replace it with stop / storage buffer.

ACKNOWLEDGEMENTS

I am thankful to my thesis adviser, Dr. George Ordal for giving me the opportunity to do this work, and to Aamir Zuberi for the extensive amount of guidance he gave to me.

In addition, I wish to thank Rick Olson and the University of Illinois Center for Electron Microscopy, as well as Charles Mitchell and the University of Illinois Genetic Engineering Facility for their time and for the use of these excellent facilities.

Finally, I am grateful to the graduate students of the Ordal lab for their copious output of encouragement, constructive criticism, and opinions.

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