Journal of Supramolecular Structure 2:360–371 (1974)

THE REGULATION OF FLAGELLAR FORMATION AND FUNCTION

M. Hilmen, M. Silverman, and M. Simon

Department of Biology, University of California, San Diego, La Jolla, California 92037

The basal structure of the flagellum controls both activity and assembly. In order to define the steps involved in these processes, genetic analysis was performed. Twenty genes were found to be required for the complete assembly and function of the organelle. FlaE controls the length of the hook, flaA is required both to maintain flagellar structure and for chemotaxis, and flaI plays a role in regulating the synthesis of the entire structure. Mutations mapping close to flaI (the cfs mutations) release flagellar synthesis from control by catabolite repression.

The basal structure was purified and isolated. On SDS acrylamide gel electrophoresis, it contained at least six distinguishable components. One major band corresponded to the hook subunit with an apparent molecular weight of 42,000 daltons. The others had apparent molecular weights of 60,000, 40,000, 28,000, 25,000, and 18,000 daltons. The genes that correspond to these polypeptides have not been identified.

In exploring the role of the mot and che genes, assays were developed for the function of individual flagellar filaments. The filaments were found to rotate and rotation could be modulated by changing their direction. Chemotaxis results from the modulation of flagellar rotation. Using the rotation assay the response of nonmotile cells to attractants and repellents was followed.

INTRODUCTION

The bacterial flagellar system poses a number of interesting questions about the mechanism of assembly of subcellular organelles. In contrast to bacteriophage, which are generally assembled and released from the cell in a terminal act, flagella are true organelles, i.e., they are continuous and integral parts of the cell. Their synthesis, assembly, number, and distribution on the cell surface are regulated so that they are appropriate to efficient flagellar function and to the physiological state of the cell. These regulatory processes must be rather precise since there is no way to discard defective organelles once they have been assembled. Furthermore, in order to function, the flagellar organelles must be coupled to the systems which energize the membrane (1) and to receptor proteins (2) that sample the extracellular environment. Therefore, in addition to tight regulation of flagellar assembly, there are a series of mechanisms that control and integrate flagellar function.

In order to understand these regulatory processes, it is necessary that we have a clear picture of the structure of the organelle. Biochemical and electron microscope (3) studies have shown that the flagella are composed of three morphologically distinct sections: a) the flagellar filament (4) accounts for over 95% of the mass of the organelle and it is made up of identical protein subunits (flagellin); b) the filament ends in a short "hook" region which is also made up of subunits – however, the subunits in the hook (5) differ from those in the filament; c) the hook (6, 7) is attached to a caplike structure, which in gram positive organisms (7) consists of two discs that are mounted on a thin tubular shaft. The discs are bound to the cell membrane and the cell wall. In gram negative organisms (7, 8) there is a more complicated structure with two pairs of discs. The lower set is bound to the cell membrane and the upper set to the cell wall.

The basal structures are intimately involved in both the regulation and function of the organelles. For example, flagellar filaments have been shown to elongate (9, 10) by the addition of subunits to the distal tip. The basal structure must be involved in transporting these subunits to the flagellar tip. The basal structures and the hook are also directly involved in the processes that convert chemical energy into motion. Finally, it would be logical to assume that the ultimate rate of initiation of flagellar synthesis depends upon the initiation and rate of assembly of the basal structure (11).

The approach that we have taken toward analyzing the role that the basal structure plays in these regulatory mechanisms involves three steps. First, the use of genetic techniques to define the number of discrete components involved in the structure and function of the organelle; second, the isolation of structural components and their correlation with the specific genes that control their synthesis; and third, we are studying the mechanisms involved in the assembly and function of these components.

Figure 1 summarizes our current understanding of these genes. Twenty specific cistrons have been defined using genetic complementation tests in E. coli. Many of these genes are homologous to those described in studies on Salmonella (12). Thus, the mot gene appears to control the conversion of energy into flagellar motion. All the mutants that are in this group are paralyzed, i.e., they have structurally intact flagella but these flagella do not function. The hag gene is the structural gene for the major protein subunit, flagellin. In E. coli K12, this is a single polypeptide with a molecular weight of approximately 53,000 daltons. The cheC, cheA, and cheB genes were defined by Armstrong and Adler (13). Mutations in these genes result in cells that are able to make flagella and are motile but do not show chemotaxis. All of the cheC mutations were found to be in the same cistron as the flaA mutations. FlaA mutations result in cells that have no discernible flagellar-related structures. Thus, we have concluded (14) that the cheC mutations are specific alleles of a gene (flaA) whose product functions both as a structural component of the flagellum and as the terminal step in the chemotactic pathway.

The genes were grouped on the chromosome by using P1 transduction and by mapping with deletions on episomes. They were further ordered into units that are cotranscribed by preparing a series of bacteriophage Mu induced mutations (14). The arrows over the group of genes indicate the genes that are cotranscribed and the direction of transcription. There are at least four operon-like units accounting for more than half of the flagellar genes.

Most of the fla mutants (mutants that produce no clearly discernbile flagellar filament structure) were examined by electron microscopy. Only two classes of mutants showed any flagellar-related structure. The hag mutants, in agreement with previous observations (15), had hook structures. The flaE mutants were found to have long curly

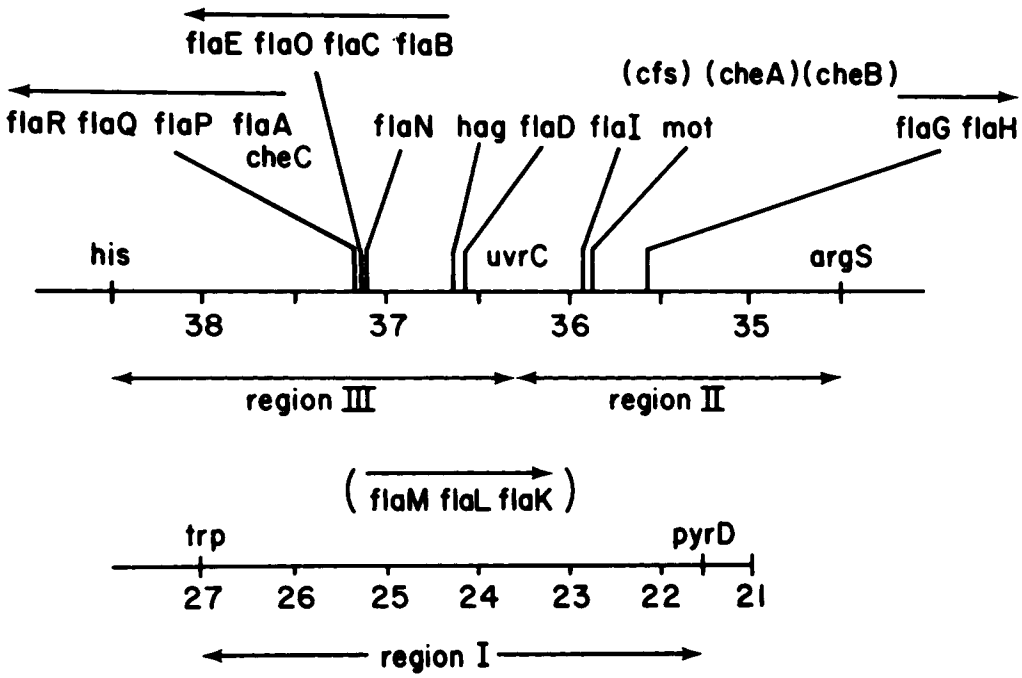

Fig. 1. The distribution of flagellar genes on the genetic map of E. coli. The arrows above groups of genes indicate organization into multicistronic transcriptional units with transcription in the direction shown by the arrow. The precise orientation and position of the genes included in parentheses is not known.

structures (16) identified as polyhooks. They were composed of one kind of protein subunit, a polypeptide with molecular weight corresponding to 42,000 daltons. The polyhooks appear to be analogous to the polytail mutants described in studies with bacteriophage λ (31). The flaE gene controls the mechanism that limits the size of the hook structure. Thus, mutations in this gene result in structures that cannot be terminated. An homologous mutation has been described (17) in Salmonella.

The cfs mutations (18) appear to describe a regulatory function. Ordinarily both the synthesis of the hook protein and the synthesis of flagellin are sensitive to catabolite repression. Thus, when the intracellular level of cyclic AMP is lowered by adding glucose to the cells or by introducing mutations in the gene that controls the synthesis of adenylcyclase (cya), flagella synthesis stops (19). The cfs mutation releases flagella synthesis from control by cyclic AMP. Thus, strains carrying the cfs mutation make flagella in the presence of catabolites, in the absence of cyclic AMP, or of the cyclic AMP binding protein (crp mutations).

We have further shown that the release from dependence on cyclic AMP requires both the cfs mutation and an intact flaI gene in cis position to the cfs mutation. These results led us to suggest that cfs defines a cyclic AMP dependent promoter region that controls the synthesis of the flaI gene product. The flaI gene product may be a regulatory protein or a flagellar structural protein which in turn activates the other flagellar operons.

Thus, while 20 specific gene loci can be shown to be involved in flagellar synthesis and function, there is only scant information concerning 5 or 6 of these genes and no

information about the role of the others. We are continuing efforts to characterize the function of these other genes – first, by isolating the structural components of the flagellum, and second, by more clearly defining how the flagellar apparatus works. In this article we will present some of our recent results in these two areas.

METHODS

Preparation of Radioactively Labeled Flagellar Hooks

E. coli strain MS1350 (14) was grown on minimal medium containing limiting amounts of sulfer. Ten μgm/ml of sodium sulfate was just sufficient to allow growth till stationary phase. Twenty-five mCi of carrier-free $^{35}SO_4$ (New England Nuclear) was added to a 500 ml culture of cells. They were grown for 10 hr or until the middle of logarithmic growth phase. The cells were harvested and intact flagella were prepared by the method of DePamphilis and Adler (7). The intact flagella were suspended in 0.1 M Tris, pH 7.8, and 5×10^{-4} M EDTA and they were disaggregated by adjusting the pH to 2.7 and incubating for 5 min at room temperature. The solution was neutralized and Triton X-100 was added to a final concentration of 0.1% and EDTA was added to a final concentration of 10^{-3} M. This entire solution was suspended on top of a gradient prepared by layering successive 1 cc samples containing 60, 53, 50, 47, 44, 41, 38, 36, and 34% sucrose followed by 10 cc of 5% sucrose. All of the sucrose solutions were made up in 0.1 M Tris, pH 7.8, 1×10^{-3} M EDTA and 0.1% Triton X-100 (TTE buffer). The gradient was centrifuged in the SW27 rotor at 24,000 rpm for 6½ hr. The gradient was dripped and 0.3 ml samples removed. Five microliters from each sample were counted.

Measurement of Antibody Precipitable Radioactivity

Five microliters of each fraction were removed and mixed with 5 μl of antihook antibody in a final volume of 0.5 ml in 0.01 M Tris, pH 7.2, and 0.1 M NaCl. The mixture was incubated for 30 min at 37°C and sufficient goat anti-rabbit gamma globulin was added to precipitate all of the rabbit antibody. The incubation was continued for 1 hr and then left overnight at 4°C. The precipitates were collected by centrifugation, dissolved in NCS (Amersham and Searle), and counted in toluene base scintillation fluid.

Velocity Sedimentation in Sucrose Gradients

Samples 19 and 21 labeled A and B, respectively, were diluted with three volumes of TTE buffer and 0.8 cc was layered onto a preformed 15–30% linear sucrose gradient. The gradient was centrifuged in an SW41 rotor at 32,000 rpm and 5°C for 180 min. 0.3 ml samples were collected and 10 μl of each sample was counted.

In order to collect the hook structures the contents of the tubes containing the major amounts of radioactive material were pooled and diluted with five volumes of TTE. The hooks were sedimented by centrifugation in the SW50 centrifuge rotor at 45,000 rpm for 2 hr.

Acrylamide gels

Acrylamide gels were prepared and run according to the methods described by Gelfand and Hayashi (20).

Bacterial Strains and Media

The strains of bacteria, the specific antiserum, and the medium have all been described previously (14, 16, 21).

RESULTS AND DISCUSSION

Isolation of Flagellar Components

By using gentle cell lysis techniques followed by ammonium sulfate precipitation and isopycnic centrifugation, it has been possible to isolate whole intact flagella from B. subtilis (6, 7) or from E. coli. (7). Furthermore by selective disaggregation the flagellar filament could be removed from intact B. subtilis flagella and the remaining hook and basal region isolated by velocity and isopycnic gradient centrifugation (5). The same approach has been used to purify the basal structures from E. coli. Figure 2 shows the results of sucrose gradient centrifugation of intact E. coli flagella that had been disaggregated by incubation at pH 2.7. The basal structures and the hook-like material were identified in two ways: a) by direct visualization in the electron microscope, and b) by precipitation with specific antihook antibody. Clearly the major portion of hook containing material is in the leading peak. Over 85% of the radioactivity in this peak is precipitable by antihook antibody. Figure 3 shows electron micrographs of material taken from the front part and the back part of the main peak. Most of the hooks in the front part of the peak appear to be intact, i.e., they have the hook region as well as all four basal discs. On the other hand, the material in the back of the peak is mostly degraded i.e., about 70% of the structures appear to contain only the hook region and lack the basal discs. These fractions could be further purified by velocity sedimentation. Figure 4 shows that the intact hook sediments in a relatively homogeneous peak with an average estimated S value of 90S–100S. The partially degraded hook has an average S value of 60S–75S. Each of these bands was collected by sedimentation at 45,000 rpm for 2 hr and then disaggregated by heating in sodium dodecyl sulfate. The individual polypeptide components were then separated by acrylamide gel electrophoresis. Figure 5 shows that the intact hook preparation resulted in five to possibly nine distinct peaks. In many gels of preparations of this sort six distinct peaks have appeared consistently in approximately the same protein ratios. They correspond to molecular weights of 60,000, 42,000, 40,000, 28,000, 25,000, and 18,000 daltons. There are some minor components which appear from time to time and the gel preparation has not clearly resolved the low molecular weight material. We can conclude, however, that the intact hook structure is composed of at least six components. The polypeptide with MW of 42,000 is the major component. It has the same molecular weight as the hook subunit protein isolated from polyhooks. Figure 5 also shows the polypeptide profile of the degraded hook material. Almost all of the peaks are missing except the major hook structural component which migrates at a velocity corresponding to a molecular weight of 42,000 daltons. Thus, we can assign at least six structural polypeptide components to the bacterial hook and basal structure. These presumably result from the fla genes previously defined. It is possible that during the purification procedure we have removed some labile auxiliary structures. The components that we find are therefore a minimum number. There may exist other proteins that are necessary for flagella function but are not necessary to maintain the

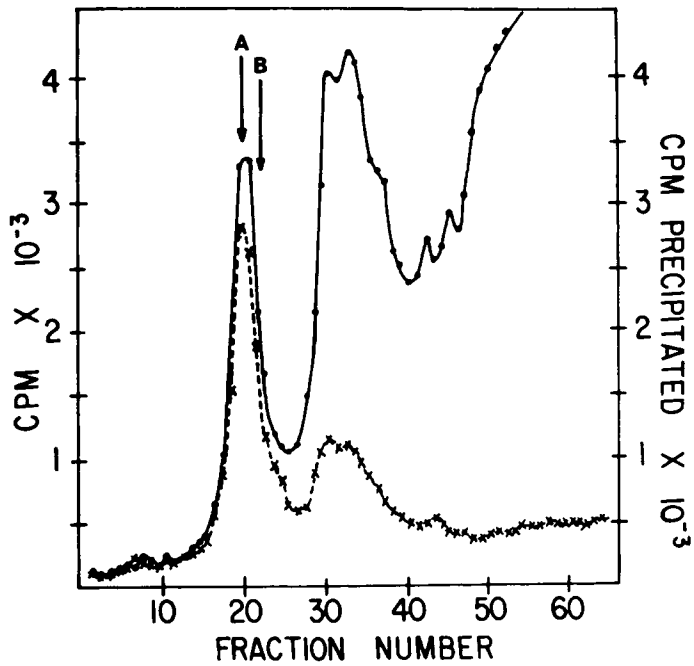

Fig. 2. The separation of intact hook structures. Intact labeled flagella were disaggregated and applied to a preformed sucrose density gradient. The arrows indicate fraction A and fraction B which were removed for subsequent study. (●) total counts, (X) counts precipitable by antihook antibody.

structural appearance of the basal region. In one experiment where intact hooks were isolated from bacteriophage Mu induced mot mutants, exactly the same six,bands were found, suggesting that none of them corresponds to the mot gene product and that the mot gene product is not necessary for the structural integrity of the basal region.

While direct isolation is useful for characterizing some flagellar components, other methods will have to be developed to show which genes these proteins correspond to and how they function.

The Mechanism of Motility and Chemotaxis

Motility and chemotaxis appear to be controlled by at least four distinct genes – mot, cheA, cheB, and cheC. The methods used to study motility and chemotaxis depend upon the behavior of the whole organism. Since this is a complex result of the activity of a number of flagella, it is difficult to analyze the results obtained in terms of the possible functions of specific genes. It would be useful to follow the motion of a single flagellum and to determine how it behaves. This cannot be done directly since the diameter of the flagellar filament is at the limit of resolution possible by light microscopy. Furthermore, in motile strains the constant motion of the organism makes analysis difficult. We therefore used two kinds of bacteria: a) flaE, hag double mutants; these cells make polyhooks which can be from 1 to 2 microns long and they are nonmotile. When dilute antipolyhook antibody is added they clump together or get tethered to the glass slide and then rotate. b) E. coli W3110 was also used. It carries a mutation in the hag gene leading to the formation of straight flagella (22). These cells are also nonmotile. They were grown on glucose

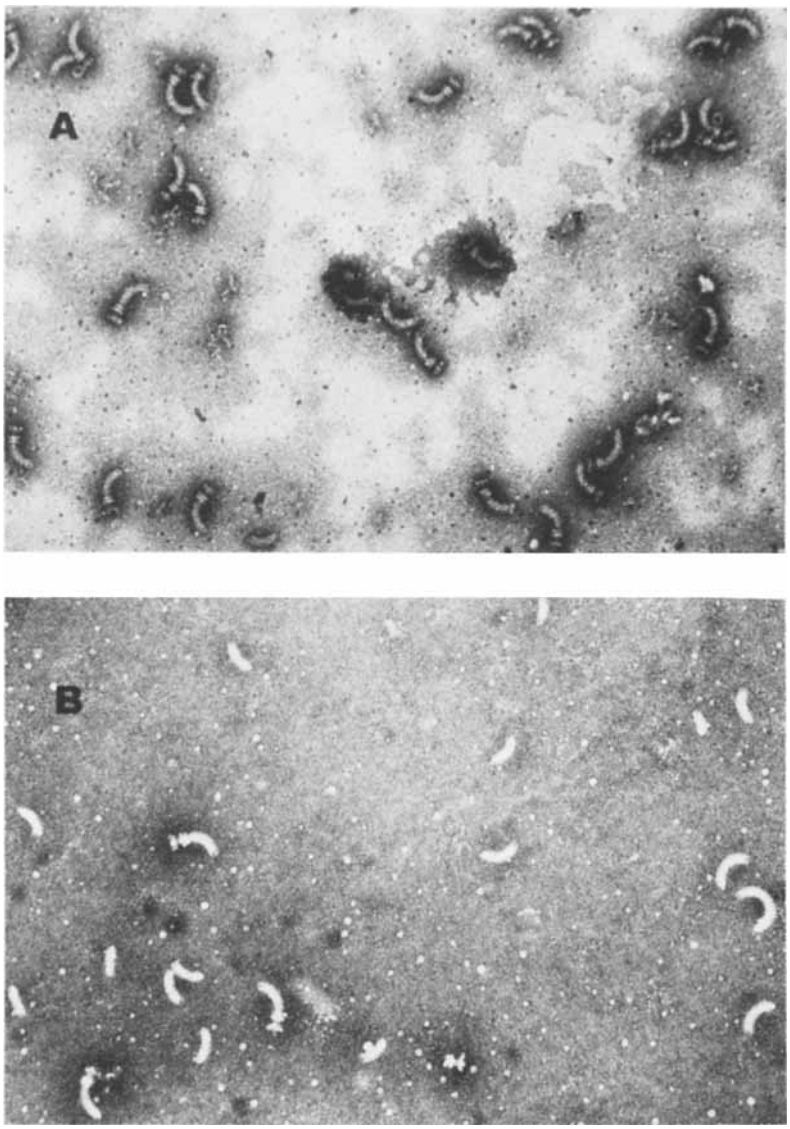

Fig. 3. Electron micrographs of fraction A and fraction B. Samples removed from the density gradient were applied to ionized formvar-carbon coated grids. They were then stained with 2% phosphotungstic acid. The diameter of the hook is 18 nm. (A) fraction A and (B) fraction B.

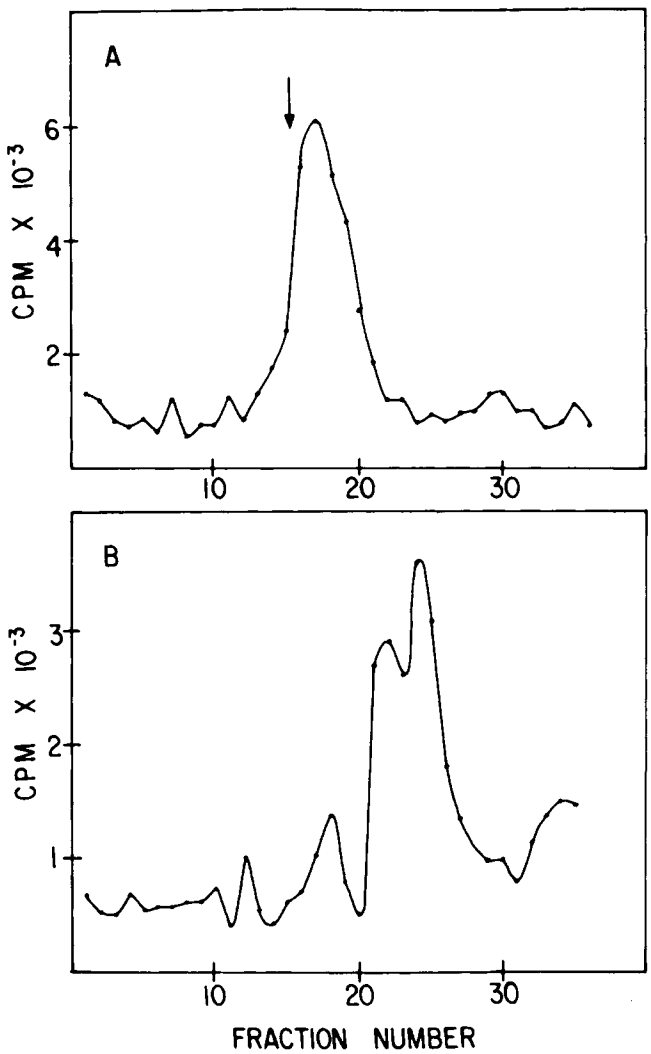

Fig. 4. Sucrose velocity gradient of intact and degraded hooks. Fractions A and B were layered onto a 15–30% sucrose gradient. Radioactive ØX174 and MS-2 phage were used as markers. The arrow indicates the position in the gradient that corresponds to 100S. (A) fraction A, (B) fraction B.

to reduce the number of flagella per cell and dilute antiflagellar antibody was added. The cells either clumped to each other or were tethered to the glass and began to rotate rapidly (Fig. 6). These tethered bacteria were used as an indirect way to follow the activity of a single filament. By observing the behavior of these bacteria and assuming that their filaments are firmly tethered and cannot move independently, we have concluded that flagella rotate (23). When they are tethered the result is rotation of the bacterial cell. The conclusion that the cell rotates is based on these observations: a) Bacteria have been found which appear to be tethered at their center and rotate in a plane. Both ends of the cell body can be followed and each end moves through a 360° circle. b) Occasional

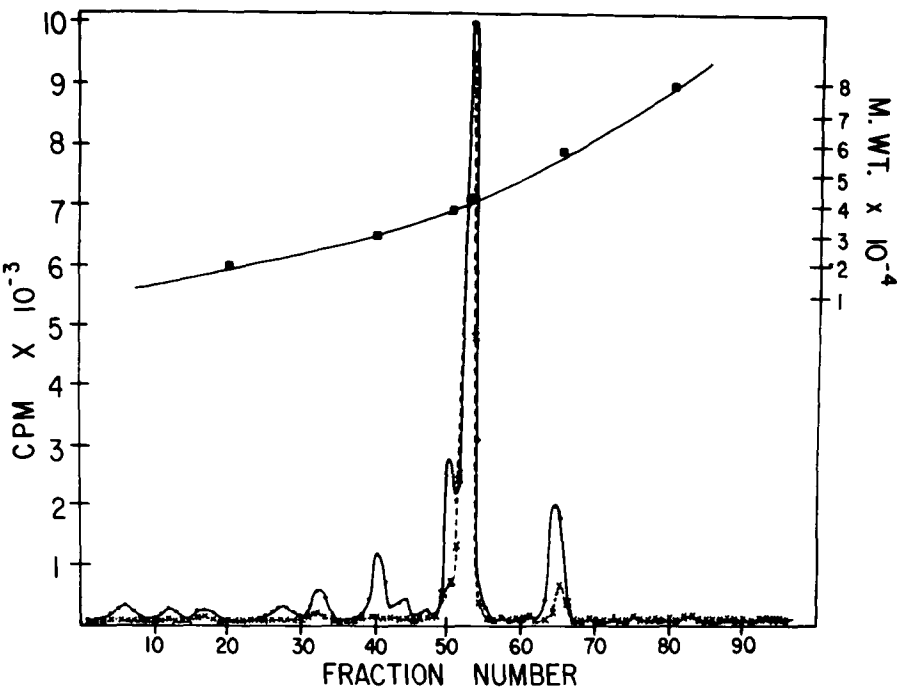

Fig. 5. Acrylamide gel electrophoresis of intact and degraded hooks. The peak samples from the sucrose velocity gradients were pooled and concentrated by centrifugation. They were then disaggregated and applied to the gel. (●) fraction A and (X) fraction B.

bacteria can be found with a discontinuity on the surface or with a piece of cell debris attached. The orientation of the marker with respect to the direction of motion of the cell remains the same through the entire revolution, indicating that the cell rotates rather than precesses around the point of attachment. In addition to seeing the cell body rotate the filament can be followed more directly in two ways: a) Small latex beads coated with antihook antibody were added. An occasional polyhook will bind as many as three latex beads and the rotation of the beads relative to each other can be followed. b) Individual latex beads coated with antiflagellar antibody were bound to flagellar filaments and observed to rotate.

These results are all best explained by a model in which the flagellum rotates. Berg and Anderson (24) have reviewed the evidence for rotation and Berg (25) has suggested specific models to describe the mechanical basis of rotation.

If the rotation of the bacterium is followed, it exhibits a variety of modes of behavior. It rotates generally counterclockwise if bound to the glass slide surface and occasionally it reverses for two or three revolutions and then proceeds to rotate counterclockwise. Occasionally the cell will stop rotating and then abruptly resume motion. We suggested (23) that the modulation of flagellar rotation could be the basis for chemotaxis. Larsen et al. (26) adopted the tethered cell assay to normal flagellar filaments and used a large variety of chemotactic mutants to show a clear correlation between the direction of rotation and chemotaxis. They found that in the presence of attractant the

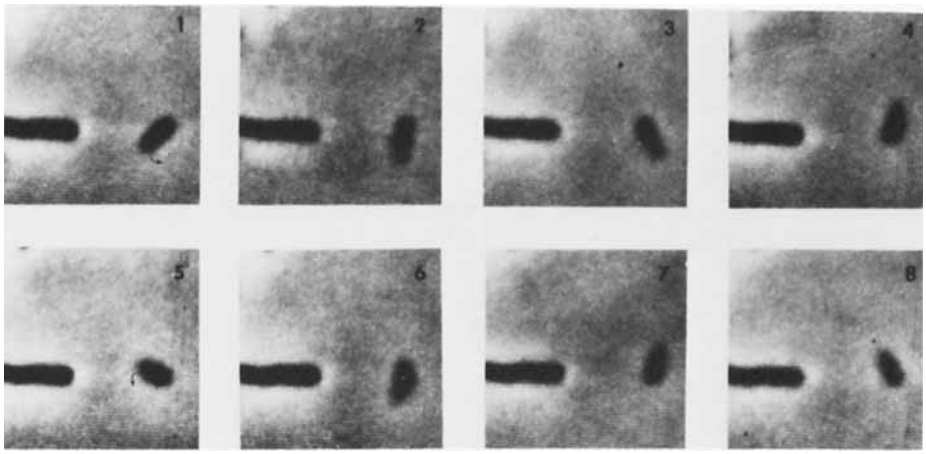

Fig. 6. The rotation of E. coli tethered to the glass slide via antipolyhook antibody, E. coli strain MS1381 was grown on minimal medium with glycerol as the carbon source. Cells were put on a microscope slide and an equal volume of antipolyhook antibody diluted to 1:200 was added. Their behavior was recorded through a Zeiss phase contrast microscope onto video tape and then transferred to film. The pictures presented represent frames taken at intervals of 1/12 of a second. The cell at the left did not move during the sequence and it provides a reference point.

cells turned predominantly in one direction, while on addition of repellent the cells reversed direction. Furthermore, temporal changes in concentration of attractant and repellent affected the direction of rotation. Increases in attractant concentration increased counterclockwise rotation and increases in repellent concentration led to clockwise rotation.

We have followed the behavior of tethered polyhook and straight mutants after the addition of attractants and repellents. A detailed description of these experiments will appear elsewhere. However, our results with these nonmotile cells can be summarized as follows: a) The predominant direction of rotation of bacteria tethered to the glass slide surface is counterclockwise. We have seen less than one bacterium in 80 that showed predominant rotation in the clockwise direction. b) The bacteria, while rotating predominantly counterclockwise will switch direction and rotate two or three and sometimes as many as twenty revolutions in the clockwise direction but always resume counterclockwise rotation. c) Upon addition of an attractant (e.g., 10^{-4} M aspartate) to bacteria tethered in either minimal medium or chemotaxis medium (27) the frequency of direction reversal drops immediately and cells rotate counterclockwise without a single reversal for 5 to 10 min, after which the frequency of reversal increases to from 5 to 25 reversals per minute. d) If a relatively high concentration of repellent (e.g., 10^{-1} M isoleucine) is added, the frequency of reversal increases immediately and cells will reverse the direction of rotation as frequently as 30 to 80 times a minute, or occasionally spin in a clockwise direction continuously for 15 to 30 seconds and then reverse direction at a high frequency (26).

Thus, the frequency of reversal of direction correlates with changes in concentration of attractant and repellent. The question that remains is how does this correlate with chemotaxis? Berg and Brown (28, 32) tracked swimming bacteria and observed that they demonstrated occasional tumbling behavior which resulted in a change of direction. They suggested that chemotaxis could result from the control of tumbling behavior. Thus, if

there is a randomly generated transient effect on flagella that results in tumbling, attractants would inhibit and repellants enhance this effect. Macnab and Koshland (29) demonstrated clearly that temporal changes in the concentration of attractants or repellents affected the frequency of tumbling, with increases in attractant concentration decreasing the frequency of changes in direction and decreases in attractant concentration increasing the frequency. Larsen et al. (26) concluded that clockwise rotation of the bacterium (and thus the clockwise rotation of the flagellum) correlated with tumbling. Our observations suggest that when a single flagellum reverses rotation it can reverse for a fraction of a turn or for a number of turns. It is not clear how such reversal would generate tumbling behavior. Perhaps partial reversals of a single flagellum are not sufficient to induce tumbling but rather the simultaneous reversal of a number of flagella in the bundle or the persistent reverse rotation of one flagellum is required. Perhaps when a flagellum reverses rotation it also "flies out" of the bundle. Taylor and Koshland (30) showed that in monotricious organisms the reversal of rotation of the single flagellum is sufficient to account for its chemotactic behavior.

While there are many details to clarify concerning the correlation between the reversal of flagellar rotation and tumbling a coarse picture has begun to emerge. However, the molecular basis for flagellar rotation and for changes in the direction of rotation remain totally obscure. The clue to these mechanisms surely lies in the nature of the mot and che gene products.

ACKNOWLEDGMENT

This work was supported by a grant from the National Science Foundation.

REFERENCES

1. Larsen, S. H., Adler, J., Gorgus, J. and Hogg, R., Proc. Nat. Acad. Sci. U.S. (in press).
2. Adler, J., Science 166:1588 (1969).
3. Abram, D., Vatter, A. E. and Koffler, H., J. Bacteriol. 95:2045 (1966).
4. Abram, D., Mitchen, J. R., Koffler, H., and Vatter, A. E., J. Bacteriol. 101:250 (1970).
5. Dimmitt, K. and Simon, M., J. Bacteriol. 105:282 (1971).
6. Dimmitt, K., and Simon, M., J. Bacteriol. 105:369 (1971).
7. DePamphilis, M., and Adler, J., J. Bacteriol. 105:376 (1971).
8. DePamphilis, M., and Adler, J., J. Bacteriol. 105:396 (1971).
9. Iino, T., J. Gen. Microbiol. 56:227 (1969).
10. Emerson, S., Tokuyasu, K., and Simon, M., Science 169:190 (1970).
11. Suzuki, H., and Iino, T., J. Mol. Biol. 81:57 (1973).
12. Yamaguchi, S., Iino, T., Horiguchi, T., and Ohta, K., J. Gen. Microbiol. 70:59 (1972).
13. Armstrong, J. B., and Adler, J., J. Bacteriol. 97:156 (1969).
14. Silverman, M., and Simon, M., J. Bacteriol. 116:114 (1973).
15. Yamaguchi, S., Iino, T., and Kuroiwa, T., J. Gen. Microbiol. 70:299 (1973).
16. Silverman, M., and Simon, M., J. Bacteriol. 112:986 (1972).
17. Patterson-Delafield, J., Martinez, R., Stocker, B. A. D., and Yamaguchi, S., Arch. Mikrobiol. 90:107 (1973).
18. Silverman, M., and Simon, M., J. Bacteriol. (in press)
19. Yokota, T., and Gots, J., J. Bacteriol. 103:513 (1970).
20. Gelfand, D., and Hayashi, J., J. Mol. Biol. 44:501 (1969).
21. Silverman, M., and Simon, M., J. Bacteriol. 117:73 (1974).
22. Vary, P. S., and Stocker, B. A. D., Genetics 73:229 (1973).
23. Silverman, M., and Simon, M., Nature 249:73 (1974).
24. Berg, H. C., and Anderson, R. A., Nature 245:380 (1973).
25. Berg, H. C., Nature 249:77 (1974).
26. Larsen, S. H., Reader, R., Kort, E., Tso, W. W., and Adler, J., Nature 249:74 (1974).
27. Adler, J., and Dahl, M. M., J. Gen. Microbiol. 18:161 (1967).
28. Berg, H. C., and Brown, D. A., Nature 239:500 (1972).
29. Macnab, R. M., and Koshland, D. E., Jr., Proc. Nat. Acad. Sci. U.S. 69:2509 (1972).
30. Taylor, B., and Koshland, D. E., Jr., Nature (in press).
31. Katsura, I. and Kühl, P. W., J. Supramolecular Structure 2:239 (1974).
32. Brown, D. A., and Berg, H. C., Proc. Nat. Acad. Sci. U. S. 71:1388 (1974).