Isolation of Genetic Second-Site Suppressor Mutations of the *Mot* genes in Escherichia coli to Determine the Function of the Mot proteins

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ABSTRACT

Bacteria swim through their environment, but not that much is known about the "motor" which propels them. In Escherichia coli, a bacteria found in human intestines, six to eight flagella(4) move the cell around so that it can travel to "favorable environments." Both MotA and MotB, which are found in the cytoplasmic membrane(2,11), form integral parts of this "motor," but in E. coli, it is not known what other proteins work in the motor. The motor is important because it transforms the electrochemical potential energy of H^+ ions into a physical force on the molecular level. My research has revealed that there is a protein that interacts with MotA.

INTRODUCTION

Bacteria are small organisms able to swim through their environment using a structure called the flagellum. A flagellar filament, which is a polymer of the protein flagellin (4), looks like a corkscrew in three dimensions. E. coli has six to eight different flagella per cell(4). These flagella rotate(8), pushing the bacteria along with a helical propeller. This type of movement is unique in nature since it involves the use of true rotary motion (movement in a circle) to propel the cell (8). Some kind of motor must turn these flagella in order for the cell to swim. It is thought that two proteins, MotA and MotB (Mot for motility), form this motor that turns the flagella.(4,5) When these two proteins are present in the cell the flagella turn, but when they are absent the flagella do not move(5). In Mot^- cells (cells that do not have functional copies of the Mot proteins) the flagella are paralyzed but are not jammed(3). In the laboratory, I am performing genetic experiments on E. coli to determine what other flagellar proteins interact with these Mot proteins to turn the flagella.

BACKGROUND

THE FLAGELLA

Escherichia coli has developed a complex mechanical apparatus, called the flagellum, in order to propel itself. The flagellar fillament of E. coli resembles a corkscrew. Unlike the flagella of eukaryotic cells, the flagellum of E. coli propels the cell by true rotary motion(4). A cell tethered to a glass microscope slide by an antibody connected to one of its flagella will spin like a propeller, not whip back and forth. Structurally, there are three main sections to the flagella: the filament, the hook, and the basal body (see Figure 1). Synthesis of a flagellum requires over thirty seven different proteins, most of these being in the basal body, in the adjacent membranes, or having a catalytic or regulatory function(4).

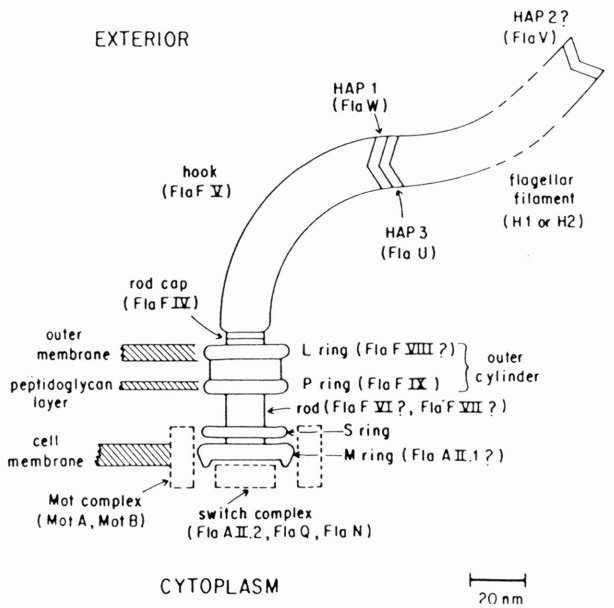


Figure 1: The Flagellum

Source: 4

The filament is the longest section of the flagellum. It can range from 5 to 10 micrometers, in length but has a constant diameter of 20 nanometers(4). It is made up of a single protein, flagellin, repeated over and over(4). When flagellin polymerizes into the filament, it forms a helix(4), and it is this "corkscrew" that twists its way through the environment. The hook, which is also composed of a repeating subunit, forms the connection between the filament and the basal body(4). The hook works as a "flexible coupling, or universal joint"(4) between the cell and the filament. Finally, the basal body acts as an anchor for the filament, extending from the outside of the cell through the cell's membranes to the cytoplasm(4). The basal body passes through the outer membrane, the periplasm and peptidoglycan layer and through the cell's inner membrane, passing from the interior of the cell through all of its membranes(4). The basal body is made up of the rod and several rings(4). The motor complex is thought to be attached at the bottom of the basal body(4,5). The motor complex has never been copurified with the basal body, however.

SWIMMING

Some bacteria swim through their environment using flagella. E. coli has six to eight flagella per cell(4). The flagella are able to turn in both directions: clockwise and counterclockwise(5). When the flagella turn in these two different directions, they generate two different modes of swimming: running and tumbling(1,5). The cell switches between these two modes as it swims. A cell will run, then tumble, run, then tumble, and so on. Now let us examine running and tumbling more closely.

Running

A run for a cell is a straight burst of forward movement. A run occurs when the flagella of the cell turn in a counterclockwise direction(1). At this time , all the flagella of the cell associate as a bundle and push the cell(5) forward (see Figure 2).

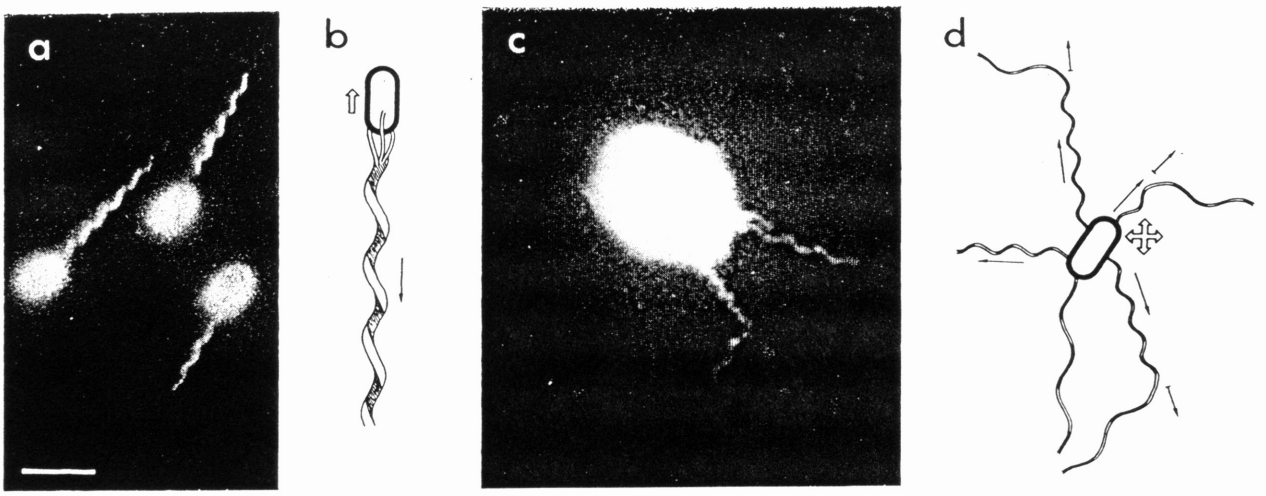


Figure 2: Running and Tumbling

Source: 5

Figure 2 shows actual swimming cells in the running and tumbling states. Part A shows swimming cells in a run with the flagellar filaments associated in a bundle, pushing the cell forward. The filaments sre visualized by high-intensity dark-field microscopy(5). Part B shows a schematic of a cell in a run: the flagella coalesce in a bundle behind the cell, pushing the cell forward. In this figure, in part A and B, the flagella associate together and push the cell in a forward direction. The run lengths for a cell follow an Poisson (exponential) distribution with a mean duration of one to two seconds(5); the runs average 43 micrometers in length(5). The average speed is 25 micrometers per second(5).

Tumbling

The opposite of running is tumbling. Tumbling occurs when the flagella of the cell turn in the clockwise direction(5). When this switch occurs, the flagellar bundle flies apart(5). Now, the cell is no longer able to travel forward because all of its flagella are pulling it in different directions (see Figure 2). Part C and D of Figure 2 show a cell tumbling with its flagella all pointing in different directions. Since none of the flagella are working together, the cell is being pulled in these different random directions and cannot move in any single direction. The cell will "tumble" through its environment, randomly orienting itself. Now when the cell starts a new run, it will be pointed in a new direction determined by its tumbling(5).

It is this run--tumble type of swimming that is most beneficial to the cell. A cell that only tumbled would never go anywhere. It would stay in one spot, waiting for what diffusion would bring its way. Because of rotational diffusion of the cell, a cell that only ran would always swim in circles, and it would never travel substantial distances, either. The run--tumble type of swimming helps the cell since it can be modulated to produce chemotaxis.

CHEMOTAXIS

Bacteria must be able to interact with and adapt to their environment. Otherwise, they will not be able to survive or compete. Chemotaxis is an ability of a bacteria to receive information about its environment, interpret that data, and react to it(5). Bacteria have receptors for oxygen, light, acidity(pH), temperature, and both organic and inorganic

attractants and repellents(5). Through the use of these receptors, bacteria can decide if the environment they are in is favorable, and if they should linger. Bacteria travel toward attractants and away from repellents, but they do not just respond to their presence(5). E. coli responds to an *increasing concentration* of attractants or a *decreasing concentration* of repellents (see Figure 3).

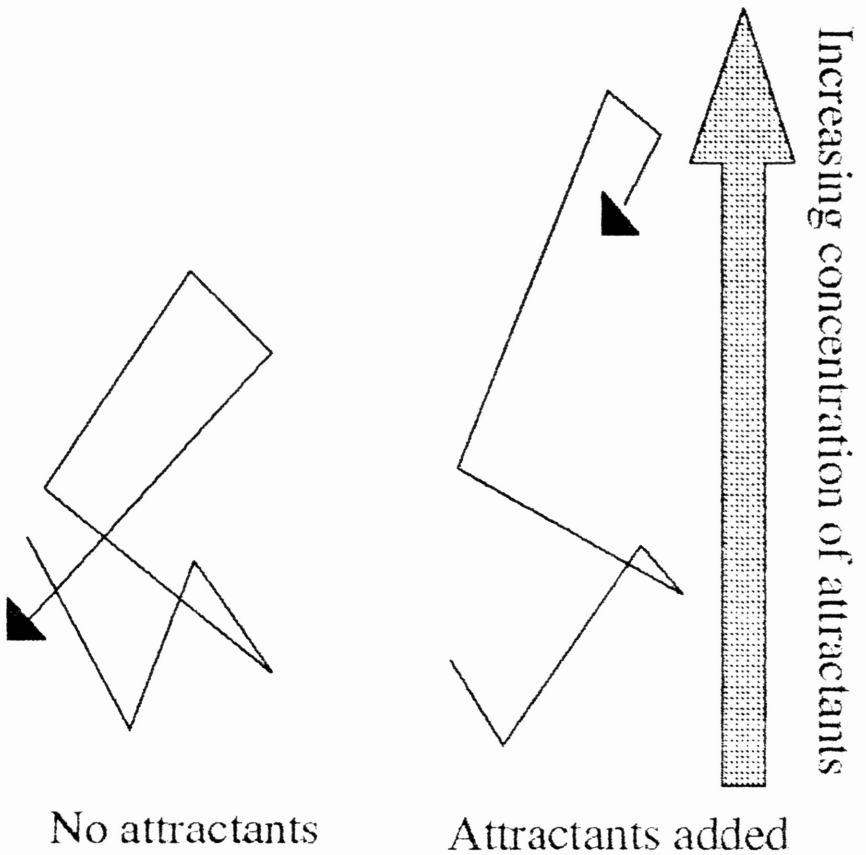


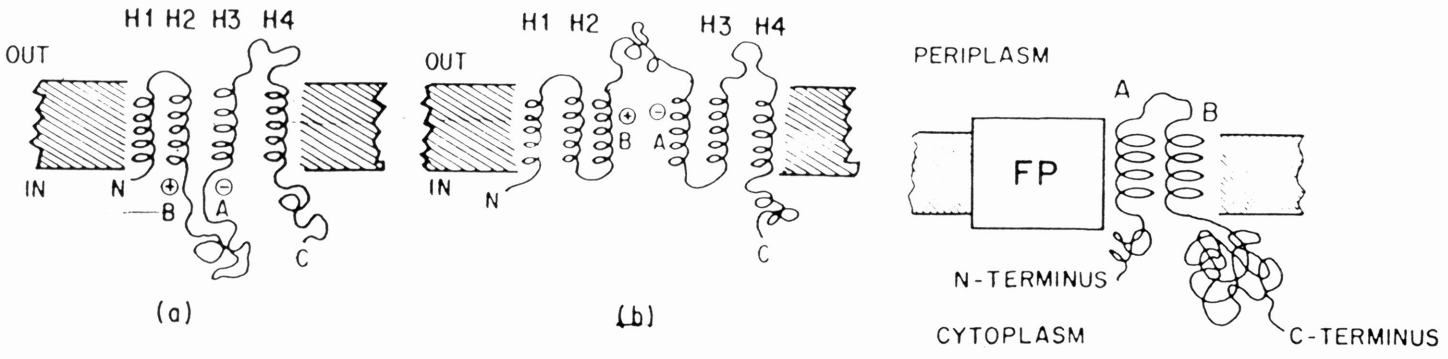
FIGURE 3: Chemotaxis
Source: 5 Adapted by D. Graybeal

As a cell moves through its environment, the runs and tumbles describe a three-dimensional random walk(1). When the cell finds an increasing concentration gradient for an attractant, it extends runs in the direction of the gradient, suppressing the tendency to tumble(1,5) and biasing the random walk in the general direction of the gradient. When the cell tumbles, it still points itself in a random direction, even if it is in a direction directly opposite of the gradient (see Figure 3). The purpose of chemotaxis is to take the cell to a more favorable environment. The cell does not accomplish chemotaxis in the way we would design as most efficient for a larger organism, but the bacterium is constrained by its small size and the consequent inability to swim in straight lines (because of rotational diffusion) to use the strategy it does.

THE MOT PROTEINS

GENERAL PROPERTIES

The Mot proteins are essential for flagellar function. If they are absent from a cell or do not work properly, the flagella do not rotate(3,4,5). The flagella are not jammed, however and can be turned by an outside force(3). MotB, and especially MotA, are integral membrane proteins, penetrating the inner cell membrane to some extent (see Figure 4). The two proteins associate peripherally around the bottom of the basal body(4,5). It is at this location that the two proteins must act to turn the flagella, probably by channeling H^+ ions to the base of the flagellum(6,12). Now, we will look at each protein individually.



MotA MotB

FIGURE 4: Possible Models for MotA and MotB

Sources: 2 and 11

MOTA

The MotA protein is an integral membrane protein having a molecular weight of 31,974(2) and containing 295 amino acids(2). The *motA* gene sequence is known and has an open reading frame of 885 nucleotides(2). The protein probably has four or six alpha helices, spanning the inner membrane twice(2). There are two areas of unbalanced electrostatic charge, one negative, one positive(2), within the protein. These might be present to provide a channel for H^+ ions through the hydrophobic membrane(12). Each cell contains about "600, plus or minus 250(12)" copies of the MotA protein. When the MotA protein is overproduced(12), the membrane is partially "short-circuited." This means that the MotA protein can act as an ionophore (channel for ions) (D. Blair, personal communication).

MOTB

The MotB protein is an integral or peripheral membrane protein having a molecular weight of 39,000(11) and containing 308 amino acids(11). The *motB* gene has 924 nucleotides in an open reading frame(11). The protein probably has one alpha helix spanning the inner membrane, since there is only one hydrophobic region(11) in the protein. The MotB protein is thought to work with several other proteins in Salmonella typhimurium(13), and it probably works with some of the same proteins in E. coli. When the MotB protein is overproduced, only a small fraction of it goes into the membrane (site limited incorporation, 11). Most of the overproduced MotB protein is found in the cytoplasm(11). The MotB protein is probably associated with the basal body(4,5).

THE PROJECT

OBJECTIVE

The MotA and MotB proteins are known to be integral parts of the flagellar motor. When a *mot*$^-$ cell is transformed with a plasmid containing the wild type *mot* genes, the flagella start to rotate again(5). How the motor works is not known however. Through some mechanism, these proteins transform the electrochemical potential energy of the H^+ ions across the cytoplasmic membrane into a physical force. I am performing genetic experiments on E.coli to determine what other proteins work with MotA and MotB in the motor.

First, I mutated a strain of E. coli that is deleted for a segment of the chromosome containing the *motA* and *motB* genes to create point mutations in a flagellar gene that codes for a protein which interacts with one of the Mot proteins. Then, I reintroduced mutated *mot* genes, each containing a different *motA* or *motB* point missense mutation, into the cell, using a lysogenic bacteriophage (virus). In cells where the mutated flagellar protein can function with the mutated Mot protein, motility is restored. These possible "suppressor" strains will be screened to ascertain that the mutation is in a second site on the genome, and is not simply a reversion of the *mot* mutation on the phage. Once I have established this, I can map the position of the second-site mutation by transducing with the phage P1 from strains containing transposable elements genetically linked to the different flagellar gene regions and looking at the frequency of co-transduction of the transposable element and the wild-type form of the gene containing the suppressor mutation. (Recombination of the wild-type gene into the genome will make the cell non-motile.) The identity of this gene can be determined through fine-scale genetic mapping. Once I have identified the suppressor gene, I will know what protein it codes for and have evidence that this protein interacts with a Mot protein.

MUTAGENESIS WITH EMS

Ethylmethyl sulfonate was chosen since it produces random point mutations. This procedure comes from Miller (7). Cells of BL104, a deletion mutant of E. coli (deficient for both *motA* and *motB*) were grown overnight in L broth. The cells were spun down and washed twice in minimal media A. They were resuspended in half the original volume of MMA containing 0.2M Tris, pH 7.5. Then I added 0.03 ml EMS to 2 ml of this suspension and mixed thoroughly. Cells were aerated at 37^{o}C for one and a half hours., then washed twice. Afterwards, the cells were diluted 10X and grown up in L (nutrient) broth overnight. The next day, the cells were spun down and resuspended in a half volume of 10mM $MgSO_4$. Ten microliters of this suspension was plated in a trough in a TB swarm plate (a tryptone plate with only 3 gms. agar instead of 15 gms/ liter of solution) to be sure there were no motile contaminants. There were none. The killing percentage was 95.2%.

SELECTION FOR SUPPRESSOR STRAINS

One hundred microliters of mutagenized BL104 was added to 5 mL of L broth and aerated at 37^{o}C overnight. The next day, the culture was diluted 10X and grown for two hours at 37^{o}C to produce mid-log cells. Afterwards, cells and phage (a recombinant lambda virus containing either a *motA* or a *motB* mutation) were mixed and incubated at 30^{o}C for 30 minutes. Cells and phage were mixed in ratios of 1:1, 10:1, and 100:1. After incubation, each cell/phage mixture was plated on a TB swarm plate(see Figure 5). Figure 5 shows a

TB swarm plate with the cell/phage mixture plated out in a trough down the middle of the plate. Any motile cells will swim out of the trough produing a swarm ring. The plates were incubated at 30°C for 24 to 48 hours. When motile cells swam out of the trough in swarm rings (see Figure 5), they were isolated and plated onto Na-citrate L plates. Next these cells were screened for lambda lysogeny and motility.

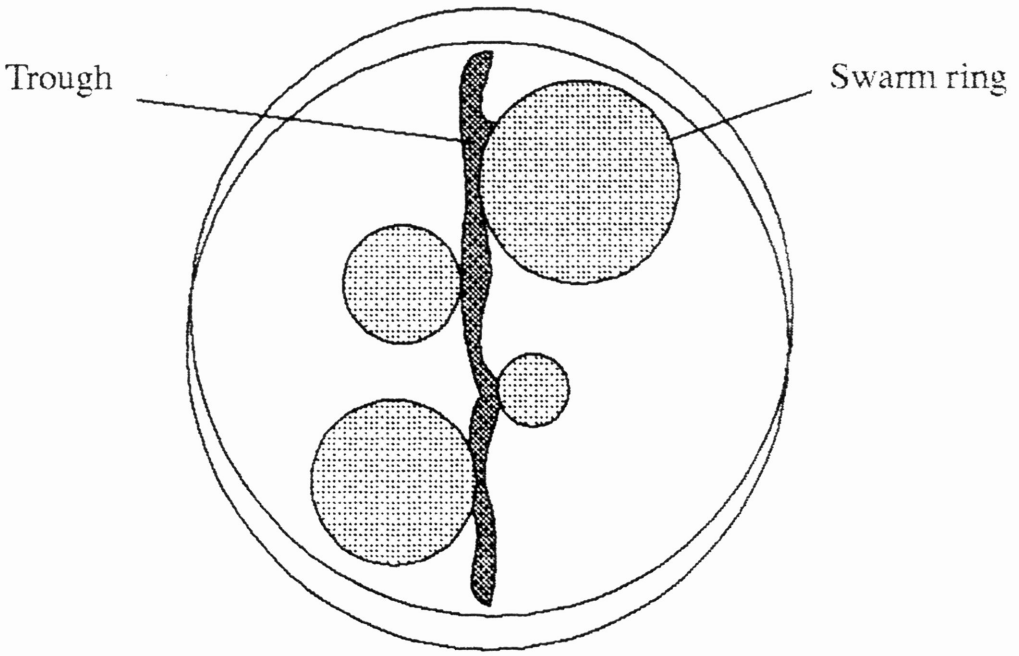


FIGURE 5: The Trough in a Swarm Plate

Adapted by D. Graybeal

CONFIRMATION OF SECOND SITE MUTATION

Lysogenic strains that were motile were grown overnight at 30°C. Then, they were shocked at 42°C for 20 minutes to induce the phage to start the lytic cycle. Cured cells (cells that no longer have the phage but have not died) were collected as well as the lysate of phage produced from each strain. Phage from this lysate were then lysogenized into BL104 to check if the *mot* mutation on the phage had reverted. Reverted phage would produce motile cells. Then the cured cells were lysogenized with their original phage and also with the wild-type recombinant phage (called lambda *che22*). The wild-type phage was used to see if the second-site mutation was allele-specific for the mutation.

P1 LYSATES OF LINKED TRANSPOSABLE ELEMENTS

Seven strains of E. coli with linked transposable elements in their genomes were obtained(10). These strains were picked because they had transposable elements flanking the FlaI, FlaII, and FlaIII regions. These regions contain all of the known genes involved in flagellar synthesis. These strains were:

Strain	Insertion location		Antibiotic resistance
zcc-282/Tn10	Tn10	22.25	Tetracycline
PutP5/Tn5	Tn5	24.75	Kanamycin
zce-726/Tn10	Tn10	24.25	Tetracycline
eda-51/Tn10	Tn10	40.75	Tetracycline
uvrC279/Tn10	Tn10	42.25	Tetracycline
zed3069/Tn10	Tn10	43.00	Tetracycline
zee3129/Tn10	Tn10	44.25	Tetracycline.

The Fla regions are located at 22, 41, and 43 minutes, respectively for the FlaI, FlaII, and FlaIII regions.

The procedure for P1 lysis comes from Silhavy (9). Cells were grown in overnight culture in L broth. The next day, they were diluted 100X and grown in L broth with .2% glucose and 10mM $CaCl_2$ for 2 hours at 37^oC. Then 0.1 ml of P1 lysate (approx. 10^8 phage) was added, and cultures were grown at 37^oC for 2 to 3 hours or until they lysed. Then, 0.1 ml of chloroform was added to kill all the remaining cells. The lysate was spun down, the supernatant collected, and the lysates titered. All titers were approx 1×10^8 phage/ ml.

P1 TRANSDUCTION AND MAPPING

Motile, lysogenic strains that have confirmed second-site suppressor mutations were grown up overnight in L broth at 30^oC. The next day, $CaCl_2$ was added to a final concentration of 10 mM. Then, to 0.9 ml of cells, 0.1 ml of phage was added and incubated for 20 minutes at 30^oC. Afterwards, 0.1 ml of Na-citrate was added.

Then 0.1 ml of suspension was plated out on Na-citrate, tetracycline L plates or Na-citrate, kanamycin L plates. Plates were incubated at 30^oC overnight , and then all colonies that appeared were tested for their motility on tetracycline TB swarm plates or kanamycin TB swarm plates. The procedure for P1 transduction comes from
Silhavy (9).

If the transposon is linked to the suppressor gene both the antibiotic resistance and the wild-type gene (of our second site suppressor mutation) will recombine onto the chromosome in a certain percentage of these transduced cells. Since the wild-type protein will not function with the mutated Mot protein, these cells will become non-motile. All the other transduced cells should remain motile. The frequency of transduction from motile to non-motile will be a measured of the map distance between the Tn10 (or Tn5) and the

suppressor mutation and the identity of a gene whose product interacts with the Mot proteins.

RESULTS

In the lab we have a number of *motA* mutations and *motB* mutations carried on lambda phage. I worked with the *motA* mutations numbered *motA1, motA6* and *motA10*. I also worked with the *motB* mutations *motB2, motB3, motB8, motB13*, *motB16* and *motB17*. I used these phage to look for second-site suppressor mutations in strain BL104. The phage lambda *motB2* never checked out with the correct phenotype for a *motB* mutation, so I stopped selecting with this phage. When I selected for motility using the *motB3* phage, I never found any motile cells, not even revertents of the mutation on the phage. Because of this I stopped using *motB3*. With *motB13* and *motB17*, I found motile cells, but these cells were always either non-lysogens or were non-motile lysogens when screened later. Right now I have strains lysogenic for lambda *motB8, motB16, motA6* and *motA10* which are motile. I have not screened these strain, however, and they could simply be revertents of the mutation on the phage.

I have had the most success with lambda *motA1*. I have found eight lysogens of lambda *motA1* which are also motile. I cured these strains of the phage and collected both the phage and the cured cells. With the phage I produced from these strains I reinfected strain BL104; all of these lysogens were non-motile, as expected, because the second-site mutation is not present to suppress the motA1 mutation. I then reinfected the cured cells (without phage) with either lambda *motA1* or lambda *che 22* (*motA*$^{+}$). All of these lysogens were motile. The second-site mutation was not allele-specific to the *motA1* mutation because it allowed motility with lambda *che22*.

I am now in the process of performing the P1 transduction that will map the location of the second-site mutation for *motA1*. I do not as yet have any data for this mapping and cannot yet assign an identity to this second-site mutation.

CONCLUSIONS

The motor project has been an interesting project to work on. We have a second-site mutation that suppresses the *motA1* mutation. As yet, however, we cannot assign the suppressor mutation to a specific gene. The evidence proves, though, that MotA interacts with another protein in its function in the motor. Since we do not know the identity of this protein, we cannot speculate on the role of MotA or the mechanism of the motor. The eight strains that have a second-site mutation might have all of their mutations in the same gene. Only genetic mapping will tell us if we have mutations in more than one gene. Also, we may have suppressor mutations for *motB8, motB16, motA6* and *motA10*, but these strains need to go through further screening. The motor has been a hard project to work on. It has been tricky at times, even frustrating when things did not work out right. But now, with the tools at hand, in the near future we will know the identity of the proteins that interact with the Mot proteins and can begin to formulate a mechanism for the motor.

ACKNOWLEDGEMENT

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