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JONATHAN WALTER STOCKER

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George W. Orbath
Instructor in Charge

APPROVED:

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THE STUDY OF THE METHYLATION OF
THE METHYL-ACCEPTING CHEMOTAXIS PROTEINS IN
BACILLUS SUBTILIS

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Jonathan Walter Stocker

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# Table of Contents

**PART I: IN VIVO METHYL TURNOVER EXPERIMENTS --**

- **Introduction**  
  Page 3

- **Materials and Methods**  
  Page 8

- **Results and Discussion**  
  Page 11

- **Conclusions**  
  Page 23

**PART II: SCREENING FOR METHYL-ACCEPTING CHEMATAXIS PROTEIN MUTANTS--**

- **Introduction**  
  Page 25

- **Materials and Methods**  
  Page 26

- **Results and Discussion**  
  Page 26

- **Conclusions**  
  Page 28

**PART III: SOLUBILIZATION OF THE METHYL-ACCEPTING CHEMATAXIS PROTEINS--**

- **Introduction**  
  Page 33

- **Materials and Methods**  
  Page 33

- **Results and Discussion**  
  Page 34

- **Conclusions**  
  Page 35

**Endnotes**  
Page 37
IN VIVO METHYL TURNOVER EXPERIMENTS

INTRODUCTION

The Gram positive Bacillus subtilis exhibits a "random walk" behavior when in an isotropic medium. The "random walk" is characterized by the bacterium alternating between smooth swimming and tumbling. The bacterium smooth swims by rotation of its peritrichous flagella counterclockwise in a coordinated bundle, and tumbling is achieved by rotation of the flagella clockwise which results in the bundle breaking apart. With no chemotactic stimulants present, the bacterium continually randomly orients itself after each period of tumbling. So a bacterium will swim smoothly for a short period of time, tumble, become randomly reoriented, and swim smoothly again. This series of events then repeats itself.

Chemotaxis is the process by which bacteria travel to higher concentrations of attractant or lower concentrations of repellent. The bacteria achieve this movement by swimming longer when moving in a favorable direction (either up an attractant concentration gradient or down a repellant concentration gradient) and by swimming for a shorter period of time when headed in an unfavorable direction. In this way the bacterium no longer simply moves randomly but actually begins to move toward a higher concentration of attractant or away from a higher concentration of repellent.

The bacterium is also observed to go through a period of adaptation which ends in the bacterium returning to its normal pattern of smooth swimming interspersed with periods of tumbling.
(i.e., 'random walk'). The amount of time it takes for the bacterium to adapt is dependent on the concentration of attractant or repellent to which the bacterium has been exposed.\textsuperscript{3,4} For example, at 20\% Receptor Occupancy of aspartate at 25\(^\circ\)C, it takes about 10 seconds after the initial addition of aspartate for B. subtilis to return to its random walk behavior.

A goal of the Ordal laboratories is to elucidate the molecular mechanism by which this relatively simple sensory system operates. One important finding is that a group of closely related membrane proteins, collectively called the methyl-accepting chemotaxis proteins (MCPs), undergo methylation upon addition of attractant to Escherichia coli.\textsuperscript{5,6} This sensory system in the bacterium Escherichia coli has been extensively studied, and it has been found that certain specific mutants in these methyl-accepting chemotaxis proteins do not adapt to stimulation by attractants.\textsuperscript{7,8,9} This provides strong evidence that these MCPs are intricately involved in the molecular mechanism by which these bacteria chemotax.

Studies done on the bacterium Bacillus subtilis, which is thought to have at least some similarities to E. coli, indicate that this bacterium also has MCPs which could be intricately involved in its sensory system for chemotaxis. The existence of at least three individual methyl-accepting chemotaxis proteins have been postulated. In general, it has been noted that addition of attractants to B. subtilis causes little or no net demethylation of the MCPs.\textsuperscript{10} This is the exact opposite response of what is observed in E. coli. Upon addition of the attractant aspartate, it has also been observed that there is an internal shifting of methyl groups.\textsuperscript{11} The
uppermost MCP (as visualized on a PAGE-SDS gel) loses most of its methyl groups while the second and third MCPs (bands) become more highly methylated. While this phenomenon is not observed for some of the other attractants, it was thought that this provides some evidence for the importance of MCPs in the chemotaxis system.

Along with the change in MCPs, it was found that B. subtilis produces a large amount of methanol upon initial addition of attractant. It was found that there is a burst of methanol evolution which is followed by a somewhat gradual decrease of methanol evolution. Methanol evolution does not reach baseline levels until after the cells have adapted behaviorally.\textsuperscript{12} It is, therefore, suspected that methanol evolution is also closely linked with the chemotactic mechanism.

Another very important finding was that a methyltransferase and methylesterase enzyme are involved in adding and removing methyl groups from the MCPs, respectively. Methyltransferase has been shown to transfer methyl groups from S-adenosyl methionine to the MCP glutamate residues as methyl esters.\textsuperscript{13,14} A methylesterase enzyme has been purified which removes methyl groups from MCPs and releases them as methanol.\textsuperscript{15,16} The methyl linkages are methyl ester linkages and are therefore alkali labile in vitro. A methyltransferase mutant in Bacillus subtilis was isolated and was found to be a chemotactic mutant also. Since this mutant, which was named the 1100 strain, has no (or very little) methyl transferase, no transfer of methyl groups either on or off of the MCPs is possible; therefore, no methyl groups can be shuttled through the MCPs. An important link between the methyl groups
associated with the MCPs and the methyl groups evolved as methanol was found when it was discovered that the 1100 mutant was also mutant in methanol evolution.\textsuperscript{17} This evidence seems to suggest that all methyl groups which are evolved as methanol from the bacterium must at one time or another be shuttled through the MCPs. Since there is a strong link between the chemotactic behavioral response and methanol evolution, there would also seem to be a strong link between the chemotactic behavioral response and the shuttling of methyl groups through the MCPs.

A hypothetical scheme for the mechanism by which the chemotaxis sensory system works has been proposed. It is suggested that there are receptor sites on the outside of the cell (membrane bound proteins).\textsuperscript{18} Upon binding of an attractant to these receptors, the enzymatic activity of methyltransferase and methylesterase is increased at the MCP methyl sites. This increase in activity could also be used to explain the sudden burst of methanol production seen upon addition of attractants.

A recent development in the research has led to the proposal of an intermediate in the chemotactic system.\textsuperscript{12} Thaelke, Bedale, Nettleton, and Ordal have suggested that there is an intermediate receptor of the methyl groups which come off of the MCPs. This intermediate serves as a receptor for the methyl groups which are being removed from the MCPs by methylesterase. The intermediate then serves as a source of methyl groups for a yet undiscovered enzyme which produces methanol.

Due to the great interest in what is actually happening to the methyl groups at the MCPs, a new 'turnover' experiment was
developed. This new experimental procedure is described in this paper. Results of a wide range of 'turnover' experiments are discussed as well as the possible implications of these findings on the current model for the chemotactic system in B. subtilis.
MATERIALS AND METHODS

*Strains* -- 011085 is a chemotactically wild strain of B. subtilis, which is trp F7, his H2, met C19. 1088, M127, and M141 are all transposon generated mutants.

*Chemicals* -- L-(methyl-\(^3\)H)Methionine (15 Ci/mmol) were obtained from Amersham Corp., New England Nuclear, or ICN. L-Aspartic acid potassium salt was purchased from Sigma. Electrophoresis reagents were all electrophoresis grade. All other chemicals were reagent grade.

*Solutions and Media* -- Tryptone broth (Tbr) is 1% tryptone, 0.5% NaCl. Luria broth (Lbr) is 1% tryptone, 0.5% yeast extract. Chemotaxis buffer (CB) is as described in reference 20. Protoplast buffer (PB) is as described in 1 with addition of 0.1 mg of chloramphenicol/ml.

'Turnover' methylation determination -- The finalized version of this turnover experiment consisted of always allowing the bacteria to be in the presence of \(^3\)H-methionine for only 20 seconds. The basic experiment consisted of the following steps. The B. subtilis was grown from spores in Tbr overnight at 37\(^0\)C. The cells were then diluted 1:50 into Lbr, grown to 180-185 Klett units, and harvested by centrifugation into pellets. The cells were washed in CB twice by resuspending followed by pelleting. A final wash was carried out in PB. The cells were then pelleted a final time (extra time was allowed for centrifugation in PB since PB is quite viscus). Finally, the cells were resuspended in PB which contained 1 mg/ml lysozyme at an \(A_{600}\) of 0.9. Each flask will contain a final volume of 2.5 ml of solution in which the cells are suspended. These cells were then
incubated in a slow shaking water bath which was held at 37°C for 25-30 minutes. (This incubation time allows the formation of protoplasts.) The flasks containing the cells were then quickly dipped in cool water and placed on a shaker which was in air (at room temperature--about 25°C). The cells were allowed to equilibrate to the new temperature (room temperature) for five minutes.

1. Basic 'turnover' experiment -- Once the B. subtilis cells were prepared in the above manner, 25 ul of ³H-methionine were added to the cells. After 15 seconds, 250 ul of effector was added to the cells. The cells were then 'killed' in a dry ice-acetone bath five seconds after the addition of the effector. (Note: The final volume in which the cells are suspended is 2.5 ml.)

2. Time-course 'turnover' experiment -- The constant factor in these time-course experiments was that the cells were always only exposed for 20 seconds to the ³H-methionine. The addition of effector could then be varied greatly and, thereby, the turnover rate at different times after addition of effector could be monitored. For example, a frequently used time-course experiment consisted of four different time points.

-15 sec: ³H-methionine addition, 15 seconds, effector addition, 5 seconds, cells 'killed' (20 seconds of total methylation time.)

0 sec: ³H-methionine and effector added simultaneously, 20 seconds, cells 'killed' (20 seconds of total methylation time).
15 sec: Effector addition, 15 seconds, $^3$H-methionine addition, 20 seconds, cells 'killed' (20 seconds of total methylation time).

30 sec: Effector addition, 30 seconds, $^3$H-methionine addition, 20 seconds, cells 'killed' (20 seconds of total methylation time).

Note that the number associated with each time point indicates the amount of time that has elapsed between the addition of effector and the addition of the $^3$H-methionine.

Polyacrylamide Gel Electrophoresis -- Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli. $^3$ Each of the reaction mixtures, which contained the protoplasts, were slowly thawed and kept at below 4°C. The protoplasts were pelleted by centrifugation and any excess PB was removed with a Kimwipe. The samples were solubilized by boiling several minutes in 100 ul of 2× Laemmli sample buffer and then applied to sodium dodecyl sulfate-polyacrylamide gels (10% acrylamide and 0.125% bisacrylamide). $^{21}$ Gels were run at 25 milliamperes and were then prepared for fluorography by the method of Laskey and Mills. $^{22}$
RESULTS AND DISCUSSION

The 'turnover' experiment was designed to study the effect of the addition of attractant/repellent on the actual on/off rate of $^3$H-methyl groups at the MCPs. The experiments were designed to allow a minimal methylation time so that 'background' (normal) methylation of MCPs would be kept at a minimum. The general format of these experiments was to consist of exposing the bacteria for a relatively short period of time to $^3$H-methionine. This short methylation time was devised to allow the $^3$H-methionine to enter the cell and be metabolized into $^3$H-SAM but not allow enough time for a large amount of incorporation of $^3$H-methyl groups into the MCPs. At the point at which a $^3$H-SAM pool would be created within the cell but the MCPs had no or very few $^3$H-methyl groups incorporated onto them, the bacteria could be stimulated by addition of an attractant or repellent. By adding attractant/repellent and then quickly 'killing' (freezing in dry ice-acetone bath) cells, the immediate effects of the turnover of methyl groups on the MCPs could be monitored. These samples could then be worked up in the normal procedure and run on an SDS-PAGE gel.

The initial experiments run in this fashion showed promising results. It was seen, that there was an overall increase in the amount of $^3$H-methyl groups incorporated in the MCPs of cells which had been treated with aspartate. This overall increase indicated that the aspartate had caused an increased rate of turnover of methyl groups on the MCPs. According to the current model, this means an increased activity of methyltransferase and methylesterase. Since the pool from which new methyl groups were
methylesterase. Since the pool from which new methyl groups were being placed on the MCPs was radioactive, an increased turnover rate which would cause an increase in uptake and attachment of new methyl groups on the MCPs would, therefore, cause a net increase of $^3$H-methyl groups incorporated in MCPs.

The established procedure for methylation experiments had involved the stimulation of cells by potassium aspartate concentrations which afforded 70%-90% receptor occupancy of the aspartate receptors on the B. Subtilis bacteria. While this concentration range was found useful in studying the overall effect of aspartate on the MCPs (especially the demethylation of the H1 band with concurrent stronger methylation of H2 and H3), these relatively high receptor occupancy levels seemed to obscure the results of these new turnover experiments. Since in these turnover experiments, the focus of the study was to observe the net change in methylation of the MCPs, any 'internal' changes between the relative methylation of different bands within the MCP region simply obscured the results being sought. This 'internal' shifting of $^3$H-methyl groups from one band to another in the MCP region made it much more difficult to determine net changes in the entire MCPs. These observations led to the testing of lower concentrations of potassium aspartate (i.e. lower % receptor occupancies) which would not produce such drastic 'internal' changes (also referred to as 'stripping') within the bands making up the MCP region. It was found that at 20% receptor occupancy concentrations of aspartate ($2.5 \times 10^{-3}$ M potassium aspartate) much less 'internal' changes within the bands of the MCPs was seen but that there still
was a noticeable net increase of turnover of \(^3\)H-methyl groups in cells treated with this lower aspartate concentration when compared to unstimulated cells (control). It was, therefore, determined that the 20% receptor occupancy levels of aspartate (2.5 x 10\(^{-3}\) M) would be the optimum concentration to use.

The actual methylation time (amount of time that cells are exposed to the \(^3\)H-methionine) was determined by a set of time-course experiments. For these turnover experiments, an optimum time of methylation was needed which would allow the \(^3\)H-methionine to get into the cell and form a \(^3\)H-SAM pool in the cell but not allow enough time for much incorporation of these \(^3\)H-methyl groups into the MCPs. Initially, experiments using 30, 60, 90, and 120 second methylation times were executed. It was found that the 30 second methylation time gave the largest quantitative difference between the unstimulated (control cells which have only buffer-0.1M KCl added to them as effector) cells and stimulated (aspartate added) cells (See Fig. 1). The stimulated cells showed a relatively large amount of \(^3\)H-methyl group incorporation in their MCPs while the unstimulated (control) cells showed very little incorporation of \(^3\)H-methyl groups in their MCPs. These were exactly the type of optimum conditions that were wanted. The small amount of incorporation of \(^3\)H-methyl groups in the control cells indicated that the 30 second methylation time was short enough not to allow very much of the \(^3\)H-methyl groups to be incorporated into the MCPs, but the relatively large increase in \(^3\)H-methyl groups present in the MCPs of stimulated cells indicated that the 30 seconds had allowed enough time to establish a pool of \(^3\)H-SAM in the cell. Later
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Fig. 1: 1- 25 sec methyl.-KCl-5 sec-kill; 2 - 25 sec methyl.-Asp-5 sec-kill; 3 - 40 sec methyl.-KCl-5 sec-kill; 4 - 40 sec methyl.-Asp-5 sec-kill; 5 - 55 sec methyl.-KCl-5 sec-kill; 6 - 55 sec methyl.-Asp-5 sec-kill; 7 - 70 sec methyl.-KCl-5 sec-kill; 8 - 70 sec methyl.-Asp-5 sec-kill.

Fig. 2: 20 second methylation time for all samples/5 second effector time. Effectors: 1 - KCl (control); 2 - Aspartate; 3 - Histidine; 4 - Asparagine; 5 - Serine.
experiments showed that even a 20 second methylation time produced the same if not greater difference between the MCPs of the stimulated and unstimulated cells.

Since all of the experiments that had been conducted so far had used aspartate as the attractant, it was decided that other known chemotactic attractants (based on behavioral studies) should be tested using this new turnover experiment. Based on behavioral studies, it had been found that histidine, asparagine, and serine are also chemotactic attractants. At 70% receptor occupancy levels of these three other attractants a noticeable increase in turnover was observed (See Fig. 2). It was especially evident in the cells treated with asparagine and serine that an increased turnover rate of $^3$H-methyl groups had been effected. The cells treated with histidine showed some increase in turnover but not nearly as dramatic a change as aspartate, asparagine, or serine. All four of these amino acids have been identified through behavioral studies as chemotactic attractants and they all cause an increase in turnover of $^3$H-methyl groups at the MCPs. This provides strong evidence which links this increase in the rate of turnover to the chemotactic system.

Another interesting finding involved the length of time which the increased turnover rate seemed to last after initial stimulation. A time course turnover experiment was done with cells that were being treated with 20% R.O. aspartate concentrations. The time course experiments always allowed the cells to be in contact with the $^3$H-methionine for only 20 seconds and varied the time at which the attractant or repellent was added. The interesting result
observed was that the increased turnover rate seemed to last for a longer period of time than was expected. For example, cells treated with 20% R.O. aspartate concentrations showed that the increased turnover rate did not slow down even 30 to 40 seconds after the initial addition of aspartate (See Fig. 3). This was somewhat surprising in that it only takes the cells about 10 seconds to adapt to 20% R.O. levels of aspartate. This result indicates that the rate of turnover of methyl groups at the MCPs is not unequivocally and directly linked to the behavioral response. That is to say that there appears to be some type of 'slack' between the behavioral response and the rate of methyl group turnover at the MCPs. It is already known that the increased rate of turnover does eventually return to prestimulus levels. Thoelke has completed experiments which indicate that the prestimulus turnover rate had been achieved at between three and five minutes after the addition of 20% R.O. level aspartate concentrations. Further experiments will need to be done in order to exactly pinpoint the time at which the turnover rate returns to baseline-prestimulus levels.

This slight discrepancy between the amount of time it takes for adaptation versus the amount of time it takes for the rate of turnover to return to prestimulus levels could be explained by the proposed methyl accepting intermediate. It could be this intermediate which is 'taking up the slack'. That is to say that this intermediate might be accepting the seemingly extra methyl groups which are being shuttled through the MCPs but are not necessarily being released as methanol.

Turnover experiments which were done using different
Fig. 3: 20 second methylation time for all samples/varying effector times. Effector (effector time): 1 - KCl (5 sec); 2 - Asp (5 sec); 3 - Asp (20 sec); 4 - Asp (35 sec); 5 - Asp (50 sec). All aspartate concentrations yield 20% R.O.
concentrations of aspartate (and, consequently, different % receptor
occupancy (R.O.) levels) have also yielded interesting results. It
appears that, when cells treated with 20% R.O. aspartate levels (2.5
x 10^{-3} M) are compared with those treated with 70% R.O. aspartate
levels (9.0 x 10^{-2} M), very little difference can actually be seen on
the SDS-PAGE gel. It might be expected that since the 70% R.O. level
of aspartate causes a much longer period of smooth swimming that
a greater amount of turnover might also be seen at the MCPs when
compared with 20% R.O. levels. However, a very important point
must be made at this time. A possible inherent limitation of the
turnover experiment and the current form in which it is executed is
that the actual quantity of turnover which is occurring at the MCPs
cannot be measured. It is postulated that only certain of the methyl
positions on the MCPs are readily labile. Even though the increased
rate of turnover lasts for at least 30 seconds after stimulation with
70% R.O., cold chase time course experiments have indicated that
certain of the methyl groups do not immediately leave the MCPs
upon addition of aspartate. This indicates that these few methyl
groups are not as readily labile to turnover as the rest of the methyl
groups attached to the MCPs. When a SDS-PAGE gel is
audioradiographed, the ^3H-methyl groups which are visualized are
only those that were attached to the MCPs at the time at which the
cells were 'killed' (placed in a dry ice-acetone bath). This experiment
can in no way indicate how many ^3H-methyl groups had already
'passed through' (been attached to the MCPs by methyltransferase
and then removed by methylesterase) the MCPs. Consequently, this
turnover experiment can indicate if the rate of turnover has
increased when compared to baseline levels of unstimulated cells, but there is a limitation of not necessarily being able to distinguish between different increases in the rate of turnover. At this time, a true comparison of the rates of turnover at the MCPs of cells treated with differing %R.O. is inconclusive.

Although aspartate has been the attractant usually used when studying the effects on MCPs in the Ordal laboratory, it could also prove useful to use these other attractants to study the turnover at the MCPs. As mentioned before aspartate, besides causing increased turnover, causes the internal shifting of $^3$H-methyl groups from the H1 band to the H2 and H3 bands. This shifting is an effect which tends to confuse the results of turnover experiments since it is very hard to compare the net changes in the MCPs when there is also internal shifting occurring. Asparagine and serine, however, are found not to produce this internal shifting effect of the $^3$H-methyl groups and, therefore, may give clearer results than aspartate. Since asparagine and serine do not cause this internal shifting, it is also possible to use much higher % receptor occupancy levels and still obtain clear results (This can be observed on the gels run with samples treated with 70% levels of asparagine and serine.) Histidine, while not causing nearly as much internal shifting of $^3$H-methyl has been observed to cause a slight shifting in the MCP bands.

The next logical step in the testing of the rate of turnover was to observe how repellents would effect the rate of turnover of methyl groups at the MCPs. Unstimulated cells were compared with those treated with chlorpromazine (a known chemotactic repellent).
Fig. 4: 20 second methylation time for all samples. Effector (effector time): 1 - KCl (5 sec); 2 - Chlorpromazine (5 sec); 3 - Chlorp. (20 sec); 4 - Chlorp. (35 sec); 5 - Chlorp. (50 sec). Chlorpromazine at 70% R.O. crossover concentration.

Fig. 5: 20 second methylation time for all samples/5 second effector time. 1 - 1085-KCl; 2 - 1085-Asp; 3 - 1088-KCl; 4 - 1088-Asp; 5 - M127-KCl; 6 - M127-Asp; 7 - M141-KCl; 8 - M141-Asp
It was found that immediately after the addition of chlorpromazine, the rate of turnover of methyl groups is actually depressed. Observations showed that the chlorpromazine treated cells showed a slower rate of methylation as compared to unstimulated cells (or those treated with 0.1M KCl--which is known to be chemotactically inactive) (See Fig. 4). This observation was quite interesting and exciting. Earlier methanol evolution experiments had found that, while there is an initial burst of methanol evolution which immediately follows the addition of a repellent, methanol evolution after this small burst declines below normal baseline production. The fact that the data also shows that repellents cause an initial slow-down of the turnover of methyl groups at the MCPs provides a further link between methanol evolution and turnover at the MCPs. It might be expected that, if methanol evolution drops below normal baseline levels after stimulation by a repellent, the initial source of these methyl groups, which is hypothesized to be the MCPs, would also show a decrease in methyl groups. In this case, the turnover of methyl groups at the MCPs is reduced and according to the current theory, this could cause a decrease in the amount of methanol evolved. It is also noteworthy to point out that the turnover at the MCPs is also maintaining its close link to the behavioral response of the bacterium B. subtilis. The attractant can be thought of as giving a positive behavioral response of smooth swimming while a repellent can be thought of as giving a negative behavioral response of tumbling. In the same manner, an attractant can be thought of as giving a positive turnover response of increasing the turnover of methyl groups at the MCPs while the repellent might be thought of
as giving a negative turnover response of a decrease in the rate of turnover of methyl groups.

Further studies done on the effect of the addition of repellent (chlorpromazine) to B. subtilis, indicated that while the addition of repellent gives an initial suppression of turnover at the MCPs, this effect does not appear to last long. It was shown that within 20 to 30 seconds after the addition of chlorpromazine (at 70% receptor occupancy crossover levels -- when compared with aspartate) little if any suppression of the rate of turnover of methyl groups at the MCPs can still be observed.\(^{12}\) This result does not completely correspond with the methanol evolution experiments in that it takes a relatively longer period of time for the amount of methanol evolution to return to baseline levels.

This new turnover experiment could also be used as a new way in which to screen chemotaxis mutants. It would be very interesting to learn if any of the observed chemotaxis mutants also do not show increase \(^3\)H-methyl group turnover when stimulated by an attractant. Such a finding would provide another piece of evidence linking the chemotaxis system to this observed turnover of \(^3\)H-methyl groups.

The strains 1088, 1082, M141, and M127 are all chemotaxis mutants (behaviorally determined) and have been characterized by Chingwen Ying as methanol evolution mutants also.\(^{26}\) That is to say that these mutants do not show the normal evolution of methanol upon stimulation by aspartate. It was thought that a turnover study of these mutants may prove interesting since there could be a link in the chemotaxis system between the methanol evolution and methyl
group turnover. A turnover experiment showed that 1088 and 1082 show quite normal turnover response to stimulation by aspartate (See Fig. 5). While M141 and M127 do not seem to show quite as much increase in turnover, it was observed that these two mutants do show increased turnover when stimulated by aspartate (See Fig. 5). While these mutant strains were not found to be totally lacking the turnover response, this type of turnover experiment may serve as a useful screening technique for mutants.

CONCLUSIONS

One of the most interesting and exciting findings was that the rate of turnover of methyl groups at the methyl-accepting chemotaxis proteins is increased by the presence of certain known chemotactic attractants. Possibly just as interesting was the finding that in the presence of a known chemotactic repellent the rate of methyl group turnover appeared to decrease below normal baseline levels of turnover. Both of these results provide strong evidence for a model which includes the methyl-accepting chemotaxis proteins as an intrinsic part of the chemotactic sensory system in Bacillus subtilis.

However, it was also found that the turnover at the MCPs is not linked 100% directly to methanol evolution of the cell. Certain experiments showed that the increased rate of methyl group turnover seemed to last for a longer period of time than might be predicted if the methyl groups released from the MCPs were directly evolved as methanol.

In terms of the model for the chemotaxis system in B. subtilis,
these results show that the MCP could be the proteins through which all of the methyl groups must cycle before either moving on to the formation of methanol or possibly to an intermediate. The rate of turnover of methyl groups at the MCPs is part of the cell's response to stimulation by attractant and the control of this rate may also be closely linked with how the cell actually adapts to the chemotactic stimulation. The results that show that the turnover rate of methyl groups at the MCPs was still relatively high even when methanol evolution had returned to near baseline levels also provides very strong support for the recently proposed intermediate which could accept methyl groups before they were evolved as methanol.

Further experiments will need to be completed in order to actually determine when the rate of methyl group turnover returns to baseline levels. Experiments dealing with the stimulation of cells by mixtures of both attractants and repellents should also be carried out in order to see what effect, if any, there is on the rate of turnover. These types of turnover experiments could also serve as a useful tool for screening mutants.
SCREENING FOR METHYL-ACCEPTING CHEMOTACTIC PROTEIN MUTANTS

INTRODUCTION

As has already been mentioned, there is evidence which shows that the methyl-accepting chemotactic proteins (MCPs) could play an integral role in the chemotactic sensory system of B. subtilis. Specific mutants in the MCPs of Escherichia coli have been found to lack a normal chemotactic response. These isolated MCP mutants of E. coli have also proved quite useful in the study of how the proteins are involved in the chemotactic system. A very common path to pursue when studying and characterizing a protein is trying to find a specific mutant in the protein in question. The Ordal Laboratories have generated several mutants of Bacillus subtilis. Initial screening has shown that some of these mutants are chemotactic mutants. One set of these mutants was produced through transposon insertion. If a MCP mutant can be found, the location of the transposon insertion can be mapped yielding information about the location of the MCP gene.

One method for further screening of the mutants which were already determined to be chemotactic mutants is to test for the presence of MCPs and observe whether the MCPs have a 'normal' response to the addition of an attractant. A relatively well established procedure for the methylation of MCPs with $^3$H-methyl groups was used to first establish the existence of MCPs which can be methylated. Secondly, this screening technique was used to determine whether the MCPs responded normally to stimulation by
attractant.

**MATERIALS AND METHODS**

*Strains* -- Of 1085 is a chemotactically wild strain of B. subtilis, which is trp F7, his H2, and met C.\(^1\)\(^9\) The strains 1800, 1801, 1803, 1815, 1816, 1817, 1818, 1819, 1820, 1821, 1822, 1823, 1824, 1829, 1830, 1831, 1832, 1833, 1834, 1836 were all generated by transposon insertion.

*Chemicals* -- Same as in Part I.

*Solutions and Media* -- Same as in Part I.

*In Vivo Methylation Assay* -- In vivo methylation was performed as described by Ullah and Ordal.\(^1\)\(^9\) B. subtilis were grown to 180-185 KU, harvested, washed twice with CB, and washed once with PB. Cells were resuspended in PB (containing 1 mg/ml lysozyme) at an \(A_{600}\) of 0.9 for methylation. Cells were methylated for five minutes, at which time effector was added and the cells were incubated for one more minutes. The reaction was terminated by freezing the flasks in a dry ice-acetone bath.

**RESULTS AND DISCUSSION**

The results from a large amount of mutants screened proved quite interesting. While lack of methylation of the MCPs did not prove that the particular strain was an MCP mutant, it did provide a method for screening and identifying possible MCP mutants. For example, no methylation of the MCPs could also indicate that the particular strain is mutant in methyltransferase of methylesterase or possibly another component related to the methylation
mechanism. However, any mutant that showed no methylation or an abnormal methylation response to aspartate, could prove very interesting to the study of the chemotactic system.

The strains 1801, 1803, 1816, 1817, 1818, 1819, 1821, 1822, 1823, 1824, 1829, 1831, and 1833 all showed relatively normal methylation patterns and normal methylation responses to aspartate (See Figures 6, 7, 8). While there were some small difference in some of the methylation patterns when compared to the control 1085 MCP methylation pattern. These slight difference did not warrant further investigation at this time. For example, 1821, 1822, 1823, 1824, and 1825 all did not seem to show the normal increase in H2 and H3 upon addition of aspartate even though H1 was quite demethylated. These slight differences did not show that there was actually any mutation in the MCP proteins themselves.

The strain 1800 while showing a relatively normal MCP methylation profile did show an abnormally large amount of demethylation upon stimulation by attractant (See Fig. 9). The strains 1815 and 1835 showed a very weak amount of methylation in both unstimulated cells and cells stimulated with aspartate (See Fig. 9). This type of weak methylation could simply be do to a abnormally small amount of methylesterase or transferase or a slight mutation in either of these enzymes.

The 1820 strain showed very interesting results upon attempted methylation of its MCP region. There was only very faint methylation of the MCP region (See Fig. 10). This could indicate possible inactivity or problems with the methylesterase or
methyltransferase enzymes. However, these results could also indicate that only a very small percentage of 'operational' MCPs are present in the membrane of this B. subtilis cell. This might mean that this is a leaky MCP mutant. Further in vitro experimentation should be carried out in order to further study this mutant. For example, the 1820 strain could be separated into into its membrane and crude supernatant fractions. Each fraction could then be mixed with the 1085 complementary fraction and, thereby, further information about whether the 1820 strain is mutant in its membranes or its supernatant.

The strains 1830, 1832, 1834, and 1836 all showed no methylation of in the MCP region (See Fig. 11). This indicates that these are very interesting strains and are possible candidates for MCP mutants. Again, in vitro studies need to be carried out on these strains to learn if the mutation has affected a product in the membranes or the crude supernatant. For example, if 1830 membranes is mixed with 1085 crude supernatant and the reaction mixture is allowed to methylate in vitro, MCP methylation could possible be seen and this would indicate that 1830 contains something in its crude supernatant which is mutant and not functioning properly.

CONCLUSIONS

The methylation experiment does serve as a good first step toward screening these known chemotaxis mutants. While some of the mutant strains did not look that interesting in terms of an unusual methylation pattern in the MCP region, 1815, 1820, 1830,
1832, 1834, and 1836 all yielded little or no methylation in the MCP region. These strains are candidates for further study (in fact, some of these strain are already being studied using an in vitro system). The next logical screening procedure for MCP mutation would be to try and methylate the membranes of each of these strains with the crude supernatant of 1085 (wild type) in an in vitro system. Those mutants that still showed no methylation would be very strong candidates for MCP mutants.
Fig. 6: 5 minutes methylation time/1 minute effector time
K-KCl and A-Asp as effectors. 1 - 1085; 2 - 1801; 3 - 1803; 4 - 1816; 5 - 1817.

Fig. 7: 5 minutes methylation time/1 minute effector time
K-KCl and A-Asp as effectors. 1 - 1818; 2 - 1819; 3 - 1821; 4 - 1822; 5 - 1823.
Fig. 8: 5 minutes methylation time/1 minute effector time
K=KCl and A=Asp as effectors. 1 - 1824; 2 - 1829; 3 - 1831; 4 - 1833.

Fig. 9: 5 minute methylation time/1 minute effector time
K=KCl and A=Asp as effector in the lane. 1 - 1085; 2 - 1800; 3 - 1815; 4 - 1835.
Fig. 10: 5 minute methylation time/1 minute effector time
K=KCl and A=Asp as effectors. 1 - 1085; 2 - 1820

Fig. 11: 5 minutes methylation time/1 minute effector time
K=KCl and A=Asp as effectors. 1 - 1085; 2 - 1830; 3 - 1832; 4 - 1834; 5 - 1836.
INTRODUCTION

The great interest in the methyl-accepting chemotactic proteins (MCPs) with respect to the chemotactic sensory system has also led to interest in the development of an in vitro methylation system. As is common in biochemical research, it is often important to develop an in vitro system so that the many different parts of a particular system can be studied. An in vitro methylation system for the MCP region has been developed which consists of a mixture of membranes and the crude supernatant. One step toward obtaining a less complex in vitro system and also a step towards the purification of the methyl-accepting chemotaxis proteins is the solubilization of these membrane bound proteins.

MATERIALS AND METHODS

Strains -- The wild-type strain of B. subtilis was 01 1085 which is trp F7, his H2, Met C. Mutant strain 01 1100 was derived from 01 1085 by mutagenesis with ethyl methane sulfonate and is a methyltransferase II mutant.

Chemicals -- Same as in Part I.

Solutions and Media -- Same as in Part I and methyltransferase buffer was 10 mM potassium phosphate (pH 7), 1 mM magnesium chloride, 0.1 mM EDTA, 1 mM B-mercaptoethanol.

Solubilization and in vitro methylation of MCPs --

Solubilization -- Membranes were prepared from a lysate of
the bacterium. The membranes were washed with FPB. The membranes were then resuspended in FPB which contained 1% Triton X-100. This suspension was allowed to incubate on ice for three hours. The debris was spun down at 40 K for four hours. The supernatant was removed and filtered through a 0.45 μm millipore filter to remove any membrane contamination.

**In Vitro Methylation Assay** -- In vitro methylation assays consisted of 5 μL of bacterial membrane (about 75 μg of protein), 2 μL of 0.1M MgCl₂, 10 μL of 80% glycerol, 5 μL of effector, and 25 μL of 1085 crude supernatant. The methylation was initiated by the addition of 5 μL of S-adenosyl(methyl-³H)-methionine. Reactions were terminated by addition of 25 μL of 2× Laemmli sample buffer.

**RESULTS AND DISCUSSION**

It was found that the in vitro methylation of these solubilized methyl-accepting chemotaxis proteins did not occur nearly as well as in vitro methylation of membranes. The product of the solubilization procedure also responded to aspartate by showing a large increase in the methylation of the H3 band but a very small increase in the methylation of bands H1 and H2 (See Fig. 12). Another difficulty found in this solubilization procedure was that it was very difficult to obtain two preparations of solubilized MCPs that contained the same percent of proteins or that methylated in the same fashion. Even with all these difficulties, it was found that solubilized MCPs can be methylated by this in vitro system.
CONCLUSIONS

It is evident that this in vitro system can cause methylation of the solubilized MCPs and that this methylation process also responds to aspartate. It was found that aspartate increases the net methylation of the solubilized MCPs. However, the methylation profile of these solubilized MCPs is weak and not the same as the in vitro methylation profile of membranes. Further investigation needs to be done in order to develop an even better in vitro methylation system. This set of experiments does show, however, that the MCPs can be solubilized and that this solubilization procedure may be an important step in the purification of the methyl-accepting chemotaxis proteins (MCPs).
Fig. 12: In vitro methylation for 3 hours. K-KCl and A-Asp as effectors in reaction mixture.
1 - 1100 membrane preparation
2 - 1100 solubilized MCP preparation
(17) Ordal, G., unpublished data.
(25) Thoelke, M. S., and Ordal, G. W., unpublished data.
(26) Ying, C., and Ordal, G. W., unpublished data.