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JERREY W. COOPER

ENTITLED "TIME OF PROTEINASE ACTION IN MAIZE SEED"

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

DEGREE OF BACHELOR OF SCIENCE

IN BIOCHEMISTRY

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Instructor in Charge

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MAPPING OF CHEMOTAXIS GENES IN BACILLUS SUBTILIS

BY

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I wish to express my thanks to George Ordal for giving me the opportunity to participate in a fantastic educational process. I'd also like to thank Jeff Ahlgren, Tony Burgess-Cassier, Danny Goldman, Dave Kettleton, Jaffer Ullah, and Dennis Werner for all your time, your thoughts and advice, and all your help to make the lab such an educational and enjoyable experience.
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1. INTRODUCTION

Chemotaxis is the ancient process by which bacteria swim toward concentrations of attractant and away from concentrations of repellents (13,8). Un-stirred, bacteria swim and tumble normally with random tumbling occurring in wild type Bacillus subtilis every 1.5 to 2.5 seconds (I.O. Nettleton, unpublished results). Upon addition of attractant (e.g. many amino acids and sugars) the bacteria swim a longer length of time (11). Upon adding repellent (e.g. uncouplers of oxidative phosphorylation) the bacteria tumble more frequently (12). The bacteria respond chemotactically to a temporal gradient of attractant or repellent, as opposed to spatial concentration changes (1).

The hypothesized mechanism for these events is as follows: the attractant binds to a membrane receptor (2). This receptor-attractant complex binds to a methyl-accepting chemotaxis protein (MCP). The MCP is then methylated with Methyl Transferase (MTase), the methyl group being donated by S-adenosylmethionine (SAM) (10,4). The methylation somehow affects a change in rotation of the flagella. Normally the flagella tumbles rotate counterclockwise (viewed from flagella to cell) for swimming and rotate clockwise when briefly tumbling (7). The flagella are right hand protein helices which when rotating counterclockwise pull together and propel the bacterium through solution. Clockwise rotation throws the flagella apart at random causing tumbling (7).

For illustration (Figure 1), in a neutral solution bacteria will swim and tumble normally (4). Upon addition of attractant
the bacteria will swim smoothly (1). Adaptation to the new level of attractant occurs at (C) as there are no more MCPs being methylated, so the bacteria return to normal swimming and tumbling. At (D) attractant is diluted away and tumbling occurs. At (E) the bacteria have again readapted to the new level of attractant or methylation level. Diluting attractant is analogous to increasing repellent, producing the same effect of increased tumbling.

In nature a tumble will randomly orient a bacterium. If a gradient of attractant or repellent is not sensed through a chemoreceptor, it will continue swimming and tumbling. If it senses an attractant gradient, the bacterium will swim smoothly. If the direction is toward the attractant (up the gradient), smooth swimming will continue to propel the bacterium to the attractant. If it finds itself swimming away from the attractant, the methylation levels will decrease causing tumbling. This serves to reorient the bacterium and bias it toward the higher concentration of attractant. This all serves to maintain the bacterium in a favorable environment.

Mutants of *Bacillus subtilis* have been isolated which show differences in chemotaxis. Capillary assays (3), done by inserting an attractant filled capillary tube into a pond of bacteria and counting the bacteria which chemotax positively, entering the tube, versus a control of no attractant, can be used to characterize these mutants. Another method is by microscopic observation of tumbling frequency. The method used in the following experiments to characterize chemotactic mutants
Figure 1

[Diagram showing a graph with X-axis labeled as 'TIME' and Y-axis labeled with levels of attractant and [CH₃-MCP].

- ST: Normal swimming and tumbling
- SS: Smooth swimming
- TT: Tumbling

Legend:
is swarm plating (1,9) due to its ease and clarity.

In this method, a metabolisable attractant (mannitol) was used. Petri plates containing mannitol and agar supplemented with necessary nutrients were solidified. Bacteria are spotted in the plate and incubated. As the mannitol is metabolised, a gradient is formed from the center increasing outward. Normal *Bacillus subtilis* forms a ring with a clear middle as it swarms outward. Different mutants produce different phenotypic swarms, including non-swarmers, fuzzy rings, no rings, etc.

Using some of these mutants with chemotactic deficiencies I proposed to map some of the genes affecting these behaviors on the *Bacillus subtilis* chromosome (Figure 2) (10). Before their products and biochemical functions can be explored, we must be able to locate the genes. Mapping can be used to locate these genes and group genetically similar mutants. This is an important step to the elucidation of the biochemical reactions involved in chemotaxis in *Bacillus subtilis*.

The mapping procedure used was phage transduction using P22 bacteriophage (15). P22 is a T4 phage (50-100 generations (16)) which attaches to the cell wall of the bacteria and slides up to the basal body at the cell membrane (16). Here it injects its DNA into *B. subtilis* producing a virulent infection (17). Some of the phage contain 1 to 8 percent of *B. subtilis* chromosomal DNA (17) from previous infection on another strain and act as donors. As this DNA enters the recipient cells it recombines with their chromosome, providing a wild-type or mutant recombinant depending on the donor.
This is the active transcription.

Since the phase attach to the cell wall, they can interfere with chemotaxis. This can be circumvented by using appropriate controls to determine whether transfection of phase interference is causing the observed chemotactic behavior. But to facilitate further experiments it was suggested (S.R. Oral, personal communication) anti-FBS1 antibody be made to bind any free FBS1 after infection to prevent further attachment and scission of non-motile mutants.

This is being accomplished by purifying the phase by density centrifugation (method by R. Burgess-Cassler) and dialysis. This phase was then emulsified with Freund's adjuvant and injected into a rabbit to induce antibody synthesis.
**Lil Aar for phase titers**

1.2 Aar
1 Lactotryptone
0.5 NaCl
0.5 Yeast Extract

**Tryptone Soft top aar for phase titers**

0.5 Aar
1 Lactotryptone
0.8 NaCl

Glucose added after a tooclaves

**Selection plates for amino acids**

1.5 Aar
10 ml KHPO4
1 ml MgCl2
1 ml (NH4)2SO4 (for work with strain 1A11 substitute 0.5 ml HCl 1.5 ml and 0.2 ml Asn)
0.5 Glucose
0.1 ml amino acids for selection

**Minimal plates are selection plates without amino acid supplements**

**Lil Aar for cloning plates**

1.5 Aar
1 Lactotryptone
0.5 NaCl

**Swarm plates 1**

0.35 Aar
10 ml KHPO4
1 ml MgCl2
1 ml (NH4)2SO4
0.1 ml Mannitol
0.01 ml per plateThr (no aar)
0.1 ml amino acids for maintaining selection
Antimicrobial broth

1. 0.9% NaCl
2. 0.4% glucose
3. 0.4% yeast extract
4. 0.05% sodium citrate
5. 0.02% C<sub>6</sub>H<sub>12</sub>O<sub>7</sub>

Preparation of the broth:

1. Antibiotic selection
2. Autoclaving of the broth
3. Preparation of Blood Agar Base Plates (BAPB)
4. Inoculate and pour plates

All chemicals were obtained from commercial sources.
**Macillus ligniformis**

**Macillus garrulus:**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Swarm Plate Phenotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>Lug</td>
<td>Che+ Leu- Hiz- Trp+</td>
</tr>
<tr>
<td>1111</td>
<td>lug</td>
<td>Che- Leu- Hiz- Trp+</td>
</tr>
<tr>
<td>1094</td>
<td>Su</td>
<td>Che- Leu- Hiz- Trp+</td>
</tr>
<tr>
<td>1124</td>
<td>s</td>
<td>Che- Leu- Hiz- Trp+</td>
</tr>
<tr>
<td>1118</td>
<td>na</td>
<td>Che- Leu- Hiz- Trp+</td>
</tr>
<tr>
<td>1125</td>
<td>ng</td>
<td>Che- Leu- Hiz- Trp+</td>
</tr>
<tr>
<td>1111</td>
<td>Lug</td>
<td>Che+ His+ Phe+</td>
</tr>
<tr>
<td>1008</td>
<td>Lug</td>
<td>Che+ His+ Phe+</td>
</tr>
<tr>
<td>1004</td>
<td>Lug</td>
<td>Che+ Trp+ Trp+ Trp+ Hiz+ Phe+</td>
</tr>
</tbody>
</table>

From J.C. Nettleton's Collection:

| XI 10  | sf                    | Che- His+ Leu- Trp- |
| XI 24  | sf                    | Che- His+ Leu- Trp- |
| XI 42  | sf                    | Che- His+ Leu- Trp- |
| XI 47  | sf                    | Che- His+ Leu- Trp- |
| XI 50  | sf                    | Che- His+ Leu- Trp- |
| XI 20  | sf                    | Che- His+ Leu- Trp- |

Swarm Plate Phenotype Key

- Lug: Large uniform ring (Wild Type)
- Sug: Small uniform ring (slower)
- Su: Slow uniform disk
- ns: No swarming—growth only around starch
Taint dressing
Grow 2 hours 37°C/ shaking in 1 ml TBR, add 0.9 ml 20% glycerol, 30 min. 37°C/ shaking, freeze.

Titer for Phage
Grow *B. licheniformis* overnight on TBAB plates, inoculate 5 mls. of TBR, grow to 100 Klett Units 37°C/ shaking to .3 ml bacteria and 0.1 ml phage, incubate 20 min. 37°C/ shaking, add 3 ml Top agar, plate on TBR plates, dry incubate 5 hours, count plaques.

Transduction I
Grow recipient bacteria overnight in TBR, inoculate 0.1 ml. bacteria in 5 ml. FAB, grow 37°C/ shaking to 120 Klett Units, add to 1 ml bacteria MOI 3 donor phage, do control without phage, grow 20 min. 37°C/ shaking, add 5 ml. 1X spisigen salts, centrifuge 1500 rpm for 10 min., pour off supernatant, resuspend in 1X spisigen salts, spread 0.1 ml. on selection plates, dry incubate overnight.
For example, phage (201) infecting 1134 would be plated on selection plates containing histidine to select for methionine prototrophs.

Transduction II
Grow *B. licheniformis* overnight on TBAB, inoculate loopful into 2.5 ml. FAB, grow 3 hours 37°C/ shaking, add 0.5 ml PBS1 bacteriophage, sit 20 min. 37°C, add 10 ml. FAB, incubate 3 hours 37°C/ shaking, let sit overnight at 4°C, centrifuge 1500 rpm for 10 min., save supernatant phage.
and add 0.3 ml. chloroform to prevent bacterial growth in
the phage preparation.
Repeat, infecting B. subtilis 201 in place of E. licheniformis.
Transduce: same to 20 min. 37°C sitting but shake instead,
centrifuge 10 min. at 1500 rpm, resuspend pellet in 0.2 ml.
spisizen salts (1X) and plate all.

Cloning

Pick colonies from selection plates and streak out on clone
plates, dry incubate overnight 37°C.

Swarm Plating

Pick individual cloned colonies and stab into swarm plate
(approximately 1 per plate), wet incubate 37°C for 8 to 10
hours, score phenotypes.
A titer was done for phase grow of 201. This gave 2.73 \(10^{10}\) phase/ml, which is in the typical range for all the phase titers.

A test was done for cotransduction between ILV\(E\) and Leu\(E\) on 201. Transduction with phage known on 1134 (resistant PB31 (1134)), which is Leu\(E\) and ILV\(E\), found in 201 was transduced to Leu\(E\), then 50/50 = 100% cotransduction occurs.

This experiment was a test to use further cotransduction experiments on between changes in amino acid requirements and chemotaxis changes. Since PB31 takes in 8% of the L. lactis chromosome, and ILV\(E\) and Leu\(E\) have been mapped next to each other, my results are in agreement.
Summary of experiments showing percentage of cotransduction of genes for amino acid requirements and chemotaxis genes.

<table>
<thead>
<tr>
<th>Locus checked for cotransduction with che gene: Recipient Phage bacteria</th>
<th>DIF</th>
<th>HET</th>
<th>H1HA</th>
<th>H1L</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>201 1134</td>
<td>/51=8</td>
<td>21/0=3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>201 1094</td>
<td>2/59=3</td>
<td>29/52=56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>201 1117</td>
<td>16/3=42</td>
<td>0/3=0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>201 1118</td>
<td>16/3=42</td>
<td>0/3=0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>201 1125</td>
<td>0/4=0</td>
<td>0/4=0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1117 1006</td>
<td>3/49=6</td>
<td>14/54=26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1118 1008</td>
<td>7/0=12</td>
<td>3/2=49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1125 1008</td>
<td>7/1=12</td>
<td>9/0=100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1117 1A11</td>
<td>4/19=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1118 1A11</td>
<td>7/5=84</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1125 1A11</td>
<td>24/48=50</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1134</td>
<td>24/24=100</td>
<td></td>
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<td></td>
</tr>
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<td>1094</td>
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<tr>
<td>1117</td>
<td>17/18=94</td>
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</tr>
<tr>
<td>1118</td>
<td>18/18=100</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1125</td>
<td>7/12=58</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1008</td>
<td>12/19=3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A11</td>
<td>24/24=100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
IV. DISCUSSION

These summaries are the culmination of five to six separate experiments for each mapping. Difficulties were encountered in individual experiments through contamination, but this was minimized and did not occur frequently. Reversion was a problem with some strains, especially 1094, which required reisolation several times. This could account for the HisF and MetC low percentages of cotransduction found, which should theoretically be zero (no cotransduction can occur between markers this distant).

Super-infection could be an explanation for the low amount of MetC and HisA cotransduction. Since the infecting phage no longer has its own DNA, multiple infection cannot be prevented. This would enable both markers to be cotransduced, but at a low frequency. A check on this can be done by lowering the amount of phage added and observing whether the low cotransduction percentages go to zero, implying super-infection was occurring before.

Phage interference is another problem. When PBS1 attaches to flagella, it inhibits motility and therefore modifies the actual behavior. Antibody to PBS1 (later described) is being made to circumvent this.

Appropriate controls can be utilized to determine whether the actual behavior is being observed, or a modified behavior due to one or more of the above problems. These considerations have made these mapping experiments very challenging.

Summed up to minimize experimental error, this presents a
good, generalised location of several genes important in chemotaxis.

Conclusions:

1134 maps near MetC.
1094 maps near MetC.

MetC is chosen over MetB due to the lack of cotransduction of Met with HisB. These Che genes may be the same since they map closely and 1134 and 1094 have the same phenotype (Su).

1117 seems to map around HisB due to the 201-1117 results. Yet 1117-1008 mapping implies that HisA is involved. These results are less valid in light of some cotransduction occurring with MetC and HisA, which is impossible. 1117-1A11 gives some CtrA but the percentage is very low and could be due to interference. It appears the Che gene in 1117 is near HisB.

1118 maps around HisB from 201-1118 results. However, 1118-1008 supports HisA, but high CtrA cotransduction implies the gene maps closer to CtrA.

Both 1117 and 1118 have similar inconsistencies yet different phenotypes. This might otherwise have suggested a two Che gene involvement. The genotype of 1117 and 1118 may really be His A instead of HisB. This is unlikely, but would satisfy the inconsistency.

1125 offers little conclusion due to difficulties in getting it to grow, and the poor control results. The chemotaxis gene being near CtrA is a possibility.

Further experiments are needed to assure the inconsistencies. Checking the 1117 and 1118 His gene location is being done by
infecting 201 on 1117 and 1118 and looking again for cotransduct of His and Met. If a high percentage is obtained, this would imply that 1117 and 1118 are genotypically HisA and MetB. If a low percentage is observed, it follows that 1117 and 1118 are MetB or MetC and HisA, and further experiments are needed.

Also finer mapping needs to be done with a phage that incorporates a smaller chromosome percentage, and by transformation for even better resolution.
V. RESULTS AND DISCUSSION

Transduction technique II and swarm plates II were used for clearer results, less time consumption, and ease.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Recipient</th>
<th>HisB</th>
<th>MetB</th>
<th>LyrD</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>KI 10</td>
<td>0/21</td>
<td>0/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>KI 24</td>
<td>0/18</td>
<td>0/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>KI 42</td>
<td>0/12</td>
<td>0/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>KI 47</td>
<td>11/18</td>
<td>1/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>KI 31</td>
<td>0/18</td>
<td>0/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>201</td>
<td>KI 28</td>
<td>0/18</td>
<td>0/18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

KI 47 seems to map near HisB. For better statistics:

<table>
<thead>
<tr>
<th>Phase</th>
<th>Recipient</th>
<th>HisB</th>
<th>MetB</th>
<th>LyrD</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>KI 47</td>
<td>54/60</td>
<td>57/60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI 47</td>
<td>1004</td>
<td></td>
<td>0/18</td>
<td>0/18</td>
<td></td>
</tr>
<tr>
<td>KI 24</td>
<td>1004</td>
<td>0/6</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI 28</td>
<td>1004</td>
<td>0/6</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

KI 47 maps around MetB and HisB, slightly closer to MetB.

For closer mapping trying IlvA:

<table>
<thead>
<tr>
<th>Phase</th>
<th>Recipient</th>
<th>IlvA</th>
<th>LyrD</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>KI 47</td>
<td>1004</td>
<td>5/6</td>
<td>0/18</td>
<td></td>
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</tbody>
</table>

KI 47 maps near IlvA and MetB. Trying PyrD for others:

<table>
<thead>
<tr>
<th>Phase</th>
<th>Recipient</th>
<th>LyrD</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>KI 30</td>
<td>1004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI 24</td>
<td>1004</td>
<td>0/24</td>
<td>0/12</td>
</tr>
<tr>
<td>KI 42</td>
<td>1004</td>
<td>0/24</td>
<td>0/12</td>
</tr>
<tr>
<td>KI 10</td>
<td>1004</td>
<td>0/24</td>
<td>0/12</td>
</tr>
<tr>
<td>KI 28</td>
<td>1004</td>
<td>1/18</td>
<td>0/1</td>
</tr>
</tbody>
</table>
KI 26 may map near PyrI, but more statistics are needed.

There is some disagreement as to whether the Met marker is MetB or MetC. Previous work (G.W. Ordel) had shown no cotransduction of MetB and HisB in strain 1085, from which KI 47 is derived. The high cotransduction in my experiment implies the marker is MetB, assuming HisB is known positively.

Revertants of KI 47 were looked for to do 3 factor crosses (D.O. Nettleton), but none could be found. There is still some inconsistency in this area.

PBS1 (201) is being infectei on 1085 which is a Met-, HisB-, TrpF- strain to test for Met and HisB cotransduction. Since KI 47 is derived from 1085, it contains the same genetic markers. Thus if Met and HisB cotransduce in 1085, this would verify the KI 47 results demonstrating the Met marker is MetB. If not, the the original 201-KI47 experiment needs to be repeated with pure KI 47 and PBS1 (201) with antibody to check the results.

In conclusion, there are now four areas of interest where several chemotactic mutants have been mapped: MetC, HisB, MetB, and CtrA.
VI. MATERIALS AND METHODS II

PREPARING ANTIBODY TO PBSI

For the phage purification for antibody formation, 5 ml. of PBSI grown on 201 was used. The bacteria were removed by centrifuge at 1500 rpm for 10 minutes. Then differential centrifugation was employed. The gradient was prepared as follows:

2 ml. 30% Sucrose
1 ml. 1.4 CaCl₂, 10 mM Tris pH 7.5, 5 mM MgCl₂
1 ml. 1.4 CaCl₂, 10 mM Tris pH 7.5, 5 mM MgCl₂
Add 5 ml. phage and balance with H₂O
Spin at 30,000 rpm 2.5 hours

One clear band was found at the 1.4-1.6 interface (fraction 1) (assumed to be whole phage, verified by high titer (see below)).

Two bands were at the 1.4-30% sucrose interface (fraction 2) (assumed to be incomplete phage and other proteins, verified by lower titer (see below)).

Fractions 1 and 2 were collected and dialyzed separately overnight in 2 liters of glass distilled water. The fractions were then collected and lyophilized overnight.

Determination of mg/ml protein of the phage for injection was attempted using a Coomassie protein assay. There was no evidence of protein in the sample, possibly due to a lipid coat on the phage, or simply that Coomassie cannot bind to the protein for some reason.

So a titer was done:
Fraction 1 = \(1.8 \times 10^9\) phage/ml.
Fraction 2 = \(5 \times 10^7\) phage/ml.

Fraction 1 (whole phage) was used, rehydrating to get
5 \(10^7\) phage in 1.5 mls. The phage were emulsified with 1.7 ml.
Freund's adjuvant added dropwise while rapidly mixing, forming
a creamy texture. The rabbit was injected intradermally with
0.75 ml. in each hind quarter and shoulder. This will be repeated
three times, once a week, before collecting the anti-phage
antiserum.

This antibody will be added to the transduction II procedure
after centrifugation and before plating. This will bind excess
phage and prevent interference with chemotaxis. Using this
antibody should clarify results, making interpretation easier
in the future.
REFERENCES


