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"Did *Bacillus megaterium* pick up plasmid virulence genes?"

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By Sharin M. Gabl

DeKalb, Illinois

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Student name: Sharin M. Gabl

Approved by: _____

Department of: Biological Sciences

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HONORS THESIS ABSTRACT

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ABSTRACT

Plasmids are known to carry genes that allow bacteria to survive in different environments. Virulence genes that cause bacteria to be pathogenic are also found on plasmids. *Bacillus megaterium* is a non-pathogenic, spore-forming bacteria that is found in soil, but recently a strain of *B. megaterium* was reported to cause a mild case of diarrhea in an infant. This appears to be the first case of infection caused by *B. megaterium* (CH1). *Bacillus cereus* is a related species and a known gastroenteric pathogen. Research has shown that the pathogenicity in some strains of *B. cereus* is caused by either an operon containing four genes, *hblA/B/C/D*, or by a single gene, *bceT*. The pathogenic strain of *B. megaterium*, (CH1), was tested by PCR and hybridization, to see if it picked up either of these two factors that cause pathogenicity in *B. cereus* by plasmid exchange. PCR products were obtained in CH1 for the genes *hblA* and *bceT* using specific primers suggesting that such genes are present in the *B. megaterium* (CH1) strain. However, a hybridization experiment using *bceT* product as a probe for CH1 failed to show a signal.

INTRODUCTION

Bacillus megaterium QM B1551 is a Gram-positive, rod-shaped, aerobic spore-forming bacterium that contains seven indigenous plasmids (extra-chromosomal DNA) (Kieselburg *et al.*, 1984). Plasmids are known to carry genes that allow bacteria to survive in different environments and also carry virulence genes that cause bacteria to be pathogenic. *B. megaterium* has never been characterized as a pathogenic bacteria. It is best known as a non-pathogenic soil bacterium that can be found in environments such as rice paddies, dried food, seawater, sediments, fish, normal flora and bee honey (Vary, 1994).

B. cereus is a *Bacillus* species that is known to be pathogenic. It is reported that almost fifty percent of *B. cereus* strains are pathogenic (Lindstrom & Mantynen, 1998). These pathogenic strains cause enteric and emetic disease, enteric disease being the type that causes diarrhea and emetic disease causing vomiting. It has been determined that the emetic syndrome is primarily caused by strains found in fried rice dishes with a one to six hour incubation period. The enteric disease, in contrast, is caused by bacteria found in meat, vegetables, desserts, sauces, and soups with a ten to twelve hour incubation period (DeBuono *et al*, 1988). The research that is being addressed in this paper focuses on the enteric component(s).

There are conflicting theories about the cause of pathogenicity. One report indicates that the *bceT* gene codes for a protein that causes *B. cereus* to be pathogenic (Agata *et al.*, 1995). This report goes on to describe how researchers

cloned the gene into a non-pathogenic strain of *E. coli*, where it subsequently produced all of the characteristics of the enterotoxin. Ryan, *et al.*, (1997), reported that another toxin is produced by an operon composed of three or four genes. This Hemolysin BL operon is composed of B, L1 and L2 protein components produced by the *hblA*, *hblC*, and *hblD* genes, respectively. A fourth gene, *hblB*, has some homology with *hblA* and appears to be part of the mechanism creating the toxin, but researchers have not as yet characterized the gene (figure A) (Granum & Lund, 1997). Later, Lund and Granum, (1999) found that the Hbl operon also codes for a non-hemolytic enterotoxin (NHE). The only difference in the protein of the non-hemolytic strain and the hemolytic strain (HBL) is the size of the components. The size of the B, or binding component, is much greater in the NHE strain of *B. cereus* than the HBL strain (Table 1) (Granum & Lund, 1997).

Detection of the genes can be done by two methods: PCR and hybridization. PCR is a method in which specific genes can be identified and amplified by using small, specific, oligonucleotides, base-pairing in opposite directions with DNA and then synthesizing the DNA sequences in between. In the hybridization technique, a specific piece of DNA (i.e., a part of a gene sequence), is chemically labeled and subsequently exposed to the another DNA strand. The smaller, specific piece of DNA will then bind to DNA sequence that is similar and the label of only the bound DNA is then detected by x-ray film.

The CH1 strain was confirmed, in our laboratory, as a strain of *B. megaterium* through observation of the morphology of cells and colonies and through bacteria phage sensitivity specific for *B. megaterium*. Plasmid analysis of this strain showed five to seven plasmids ranging from 2 – 7 kb in size. In this study, the CH1 strain was further characterized to identify the gene causing the enteric disease. Since *B. cereus* and *B. megaterium* are known to exchange plasmids (Koehler and Thorne, 1987), we hypothesized that strain CH1 had acquired the enteric genes from *B. cereus* through plasmids. Therefore, the CH1 strain may contain the genes, which might be detected by specific PCR and/or hybridization techniques.

MATERIALS AND METHODS

Bacterial strains used and their culture conditions. The *B. cereus* strain ATCC 14579 is known to have the *hbl* genes. (Correspondence between Professor Anne-Brit Kolsto and Patricia Vary). This strain was obtained from the laboratory of Dr. Anne-Brit Kolsto of the Biotechnology Centre of Oslo and Institute of Pharmacy at the University of Oslo. The CH1 strain of *B. megaterium* was obtained from Judith E. Epstein, M.D. and Edward O'Rourke, M.D., both of Children's Hospital at Harvard Medical School. *B. cereus* 14579 and *B. megaterium* CH1 were both grown overnight at 27° C on SNB media.

B. cereus F837/76 is known to contain the *bceT* gene (Schoeni & Wong. 1999). This strain was obtained from the laboratory of Dr. A. C. Wong, of the Food Research Institute, Department of Food Microbiology and Toxicology at the University of Wisconsin, Madison. *B. cereus* F837/76 was grown overnight at 27° C on BHIG media.

Test for hemolytic activity. In order to test for hemolytic capability of CH1, the strain was streaked on to a sheep blood agar plate and incubated overnight at 30° C. Observations for lysis were made the following morning.

Test for antibiotic resistance. In order to test for CH1 resistance to antibiotics, the strain was streaked on to separate SNB plates that contained Km, Tet, Em, Cm and Neo, respectively, and incubated overnight at 30° C. Observations were made the following morning.

DNA isolation. Overnight grown cells were used to heavily inoculate 200 ml of broth (BHIG media for *B. cereus* F837/76 and SNB media for *B. cereus* 14579 and *B. megaterium* CH1). The cultures were grown at 37° C, while shaking at 250 rpm in the incubator until an O.D. reading of > 1.0 was achieved. Total genomic DNA was isolated using a modified method developed by Bovre & Szybalski (1971). The 200 ml culture was split into two (2) 250 ml tubes and cells were pelleted by spinning at 5000 g for 5 minutes. Each pellet was then washed with 100 ml SET and spun again at 5000 g for 5 minutes. The supernatant was discarded and 13.5 ml of SET was added to each tube. The mixture was vortexed until the pellet dissolved completely. Lysozyme solution, 1 ml, (20 mg lysozyme per 1 ml of SET) was added along with 400 ul of 50mg/ml RNase. Tubes were incubated at 37° C, with slow shaking for 30 minutes. Then lytic solution, 30 ml, (20% SDS in water) was added, and the solution was incubated an additional 5 minutes at 37° C. The tubes were then spun at 4000 g for 5 minutes and the supernatant was poured into new tubes. Approximately 90 ml, or 2X total volume, of 100% EtOH was added to supernatant and mixed well. The tubes were then spun at 16000 g for 20-25 minutes. The supernatant was poured off and the pellet was dried and dissolved in 3 ml of 1X TE. The DNA was transferred to Eppendorf tubes for storage at 4° C until needed.

Cleaning and concentration of DNA. To clean and concentrate the DNA, 10 ul of 50 mg/ml RNase was added for 750 ul of DNA in 1X TE and incubated at 37° C for 30 minutes. Phenol was then added, 600 ul, and the mixture was vortexed 15

seconds, then spun 3 minutes. The supernatant was then transferred to a new tube. (If a thick interface was seen, the phenol procedure was repeated.) Then, an equal volume of phenol: chloroform (1:1) was added and vortexed for 15 – 30 seconds and then spun for 3 minutes. The supernatant was transferred to a new tube. An equal volume of chloroform: isoamyl alcohol (24:1) was then added and vortexed 15 – 30 seconds and spun for 3 minutes. The supernatant was then transferred to a new tube. NaAcetate (pH 5.2) was added, in the amount of 1/10 volume of the supernatant. In addition, 2X volume of 100% EtOH was added and the solution was mixed. DNA thread was visible. (If DNA had not been visible, tube would have been kept at –80° C for minimum of 30 minutes before proceeding on to next step.) The tube was spun for 20 minutes and the pellet was then washed twice by adding 1 ml 70% EtOH and spinning for 3 minutes each time. The pellet was dried and re-suspended in 30 ul 1X TE.

Polymerase Chain Reaction. Custom primers were prepared in the Biology Department Core Facility using the known sequences from GenBank for all genes using PC Gene and Oligo Calculator Program. Primers were diluted to 100 ng/ul for use in PCR reactions. PCR reaction volume was 100 ul.

Sample reaction:

10X buffer	10.0 ul
$MgCl_2$ (25 mM)	8.0 ul
dNTP's (200 uM each)	6.0 ul
Template DNA (variable)	2.0 ul
Sterile, deionized water	69.5 ul
Primer A (200 ng)	2.0 ul
Primer B (200 ng)	2.0 ul
Taq DNA Polymerase	2.5 units
	.....
	100.0 ul

Variations in the amount of template DNA and primers were made throughout the experiments to obtain PCR product. An amount of 20 ul mineral oil was placed on top of each reaction mixture to prevent evaporation of water. Tube(s) were placed in a thermocycler, DNA was denatured at 95° C for 10 minutes, then 30 cycles of 95° C for 1 minute, annealing temperature (varied with each set of primers) for 2 minutes, 72° C for 1 minute. After 30 cycles were completed, final extension was done at 72° C for 7 minutes.

Preparation of Probe. The probe was prepared according to instructions provided by NEBlot Phototope® Kit. An 80 ul PCR product from custom *bceT* primers was used to make the probe. Amounts of 200 ul 1X TE and 300 ul chloroform were added to the PCR product. This mixture was then vortexed for 15 seconds and spun for 3 minutes. The supernatant was transferred to a new tube and 1/10 volume of sodium acetate (pH 5.2), was added along with 2 ul glycogen and 1 ml 100% EtOH. The tube was mixed well (not vortexed), and then kept at –80° C for 30 minutes. After spinning for 25 minutes the supernatant was discarded and the pellet was washed once with 1 ml 70% EtOH. The pellet was

then dried and dissolved in 20 ul sterile, deionized, water. The DNA was denatured in boiling water for 5 minutes and then quickly put on ice for 5 minutes. Labeling mix (5X), 10 ul, 5 ul of dNTP mix, 1 ul DNA Pol I (Large Klenow Fragment) were all added to the DNA. This mixture was incubated at 37° C overnight. An amount of 5 ul of 0.2M EDTA pH 8.0 was then added to the mixture to terminate the reaction. Added 5 ul of 4M LiCl and 150 ul of 100% EtOH and kept at –80° C for 30 minutes. The tube was then spun for 25 minutes at 4° C. The pellet was washed with 70% EtOH twice and dried. Once dry the pellet was resuspended in 200 ul 1X TE and stored in –20° C until needed.

Hybridization. In order to find the fragment containing the *bceT* gene homolog a hybridization experiment was carried out using CH1 and the positive control, *B. cereus* F837/76 DNA strains. DNA from both strains was restricted with *EcoRI*, *BamHI*, and *PstI* (figure B). In addition, PV361, a derivative of QM B1551, with no plasmids, was used as a negative control since it did not produce any PCR product. The restricted DNA along with the lambda marker was loaded into 0.7% agarose gel and run overnight at 20V. Southern Hybridization was carried out according to protocol provided by NEBlot Phototope® Kit. The DNA was depurinated by soaking the gel twice, with slow agitation in 0.25M HCl for 15 minutes. After the gel was soaked in HCl, it was washed in water for 15 minutes to remove any excess acid. Then the DNA was denatured by soaking the gel, again, in denaturation solution for 30 minutes. Following denaturation, the DNA was neutralized by twice soaking the gel in neutralization solution for 15 minutes

each time. A piece of nylon membrane, the same size as the gel, was secured and wet in 2X SSC solution. An apparatus for transferring DNA by capillary blotting method was set up, placing membrane on top of gel (figure C). A solution of 10X SSC was poured into the apparatus and it was covered to prevent evaporation. Weight was added to the top of the apparatus to increase capillary action. The apparatus was left overnight. The membrane was then removed and placed in the UV crosslinker for 1 minute to fix DNA on to membrane.

The membrane was then floated in 6X SSC for 2 minutes and then placed on oversized screen piece, rolled slightly and placed into glass tube with DNA side of membrane facing inside of tube. Pre-hybridization solution containing denatured salmon sperm DNA was placed into the tube and the tube was then placed in to the hybridization oven, constantly rotated and kept at 55° C for 1 hour. During last 10 minutes of this incubation period, the probe that was previously prepared was boiled for 5 minutes to denature. The entire volume of 200 ul of the probe was added to the glass tube containing the membrane and the pre-hybridization solution. The tube was then kept in the hybridization oven overnight, rotating, at 55° C.

The hybridization solution was then removed and 20 ml of 2X SSC, 0.1% SDS solution was placed into the tube for 5 minutes two times. Then, 20 ml 0.1X SSC, 0.1% SDS solution was added to tube and the tube was rotated in hybridization oven for 15 minutes at room temperature two times. Then the solution was removed and 20 ml of blocking solution was added to the tube and

incubated at room temperature. This solution was discarded and 10 ml of blocking solution containing 10 ul streptavidin was added to the tube, and incubated while rotating for 5 minutes at room temperature. After discarding the old solution, 20 ml of Wash solution I was added and the tube was incubated for 5 minutes at room temperature, then repeated. The last solution was replaced by 10 ml of blocking solution containing 10 ul of biotinylated alkaline phosphatase. The tube was incubated for 5 minutes at room temperature. This solution was then discarded and 20 ml of Wash solution II was added and the tube was incubated for 5 minutes at room temperature two times. A mixture was made containing stock solution of Lumigen – PPD (25X) buffer, 40 ul, 950 ul sterile, deionized water and finally, 10 ul of Lumigen – PPD. The membrane was taken from the tube and placed into a Ziploc bag. The diluted Lumigen – PPD was added to the Ziploc bag and the bag was sealed. Using squeegee motions for 5 minutes, solution was moved over membrane so that the solution came into contact with entire membrane. The membrane was then removed from bag and wrapped in Saran Wrap. Then the membrane was to exposed to x-ray film for 10 minutes and the film was developed.

RESULTS

The CH1 strain was tested for hemolytic activity along with *B. cereus* 14579 as a positive control. The *B. cereus* strain showed slight evidence of hemolytic activity. There was a 1 – 2 mm area around the culture that appeared clear. However, the CH1 strain did not show any hemolytic activity. CH1 did not show resistance to any of the antibiotics it was exposed to, except for neomycin. There was minimal growth of CH1 on the neomycin SNB plate.

PCR testing was begun on the *B. cereus* strains 14579 and F837/76 obtained as positive controls and on the *B. megaterium* CH1 strain. All three strains were tested for the *bceT* gene, 14579 and CH1 was tested for the *hblC/D* genes, and CH1 was tested for the *hblA* gene.

A) Testing for *bceT*

PCR products were obtained using *B. cereus* 14579 and custom primers for the *bceT* gene. The expected 2.8 kb product was observed in 2 lanes of the gel in addition to several smaller bands (Figure D). The successful reaction volumes of 100 ul consisted of the following:

10X Buffer	10.0 ul	10.0 ul
$MgCl_2$	8.0 ul	8.0 ul
dNTP's	6.0 ul	6.0 ul
Template DNA	1.0 ul	5.0 ul
Sterile, deionized water	70.5 ul	66.0 ul
2k081 primer	2.0 ul	2.0 ul
2k082 primer	2.0 ul	2.0 ul
Taq DNA Polymerase	0.5 ul	0.5 ul
	.....	.....
	100.0 ul	100.0 ul

The annealing temperature used for this experiment was 56° C.

A PCR experiment using CH1 and the custom primers for the *bceT* gene was successful in obtaining the expected product of 2.8 kb (figure E). A different DNA polymerase, Vent DNA Polymerase, was used for this experiment. The reaction volume of 100.0 ul consisted of the following:

Buffer	10.0 ul
dNTP's	6.0 ul
Template DNA	2.0 ul
2k081 primer	2.0 ul
2k082 primer	2.0 ul
Sterile, deionized water	77.0 ul
Vent DNA Polymerase	1.0 ul
	.....
	100.0 ul

The annealing temperature used for this experiment was 55° C. This product was then used in preparation of the probe for hybridization.

Four other PCR experiments were conducted using custom primers for the *bceT* gene and *B. cereus* F837/76, the positive control for the *bceT* gene. There was no product observed in these experiments.

B) Testing for *hblC/D*

A PCR experiment using the positive control *B. cereus* 14579 and custom primers for the *hblC/D* genes was successful in obtaining the expected product of 3.7 kb (figure F). The successful reaction volume consisted of the following:

10X Buffer	10.0 ul
$MgCl_2$	8.0 ul
dNTP's	6.0 ul
Template DNA	5.0 ul
Sterile, deionized water	60.5 ul
2k238 primer	5.0 ul
2k239 primer	5.0 ul
Taq DNA Polymerase	0.5 ul
	.....
	100.0 ul

The annealing temperature used was 49° C.

There were two unsuccessful attempts to duplicate the above mentioned results therefore, a subsequent PCR experiment was conducted using the 3.7 kb product of the above-mentioned experiment as the template DNA. This experiment was done to increase the volume of the product previously obtained for use as a probe for hybridization. Because the template being used was a previous PCR product, the volume of the primers was changed to 3.0 ul, the template DNA was 10.0ul, the Taq DNA Polymerase was 1.0 ul and the water volume was 67.0 ul. A 3.7 kb product was observed, however, there were at lease 3 smaller bands also observed (Figure G).

One unsuccessful PCR experiment was conducted using custom primers for the *hblC/D* gene and the CH1 strain of *B. megaterium*. Template DNA volumes of 3.0, 5.0 and 10.0 ul were attempted using 5.0 ul of each of the primers.

C) Testing for *hblA*

Since no product was obtained from primers for *hblC/D*, another PCR experiment using CH1 and custom primers for the *hblA* gene was done and proved successful in obtaining the expected product of 1.9 kb (figure H). The reaction volume of 100.0 ul consisted of the following:

Buffer	10.0 ul
dNTP's	6.0 ul
Template DNA	2.0 ul
hblA #1 primer	2.0 ul
hblA #2 primer	2.0 ul
Sterile, deionized water	77.0 ul
Vent DNA Polymerase	1.0 ul
	100.0 ul

The annealing temperature used for this experiment was 45° C.

PCR Experiments

Template DNA	**bceT primers** 2.8 kb expected	**hblA primers** 1.9 kb expected	**hblC/D primers** 3.7 kb expected
F837/76	no product	not tested	not tested
14579	2.8 kb product	not tested	3.7 kb product
CH1	2.8 kb product	1.9 kb product	no product

The probe for hybridization was prepared using the *bceT* 2.8 kb product from the PCR experiment using CH1 template DNA.

CH1 DNA and *B. cereus* F837/76 DNA were both restricted with *EcoRI, BamHI,* and *PstI*. According to the map of the *bceT* gene (figure B), these enzymes are known to produce fragments in the genes of the following sizes:

EcoRI (5 fragments)	0.7 kb, 1.3 kb, 1.8 kb, 2.0 kb, 2.6 kb
BamHI (1 fragment)	1.5 kb
PstI (1 fragment)	2.3 kb

The hybridization experiment using CH1 DNA and *B. cereus* F837/76 DNA with the CH1 PCR product failed to show a signal.

DISCUSSION AND CONCLUSIONS

The purpose of this experiment was to try to find out whether the CH1 strain of *B. megaterium* had any of the known genes that cause pathogenicity in *B. cereus*. These genes include the genes of the *HBL* operon and the *bceT* gene. Due to time constraints, the total DNA was tested instead of separately testing the plasmid DNA and the genomic DNA.

PCR experiments were conducted using specific primers that were designed from the known sequences of these genes. PCR products were obtained from experiments using the CH1 DNA for the *bceT* gene and the *hblA* gene. This suggests that the possibility exists that these two genes reside on the CH1 strain of *B. megaterium*. However, because of the failure of the hybridization experiment, this was not confirmed. It's possible that the concentration of the probe was not high enough to detect homology with the DNA since the positive control did not work. However, it appears that the techniques used for this experiment were performed correctly as the pre-biotinylated lambda marker was evident in the x-ray film.

In the course of this research, a PCR experiment was also done with *B. cereus* 14579 and the custom primers for the *bceT* gene. This experiment showed that the possibility exists that this gene is also on 14579 which was not previously known.

Table 1

Characteristics of the three enterotoxins from *B. cereus*

pceT

	Enterotoxin HBL	Enterotoxin NHE	Enterotoxin T
Number of components	3	3	1
Size of active component(s)			
L_2	46 kDa	45 kDa	41 kDa
L_1	38 kDa	39 kDa	
B	37 kDa	105 kDa	
Haemolytic	Yes	No	No
Toxicity in cell tests	80 ng	70 ng	Not determined
Shown to be involved in food poisoning	Yes	Yes	No
Cloned and sequenced	Yes	No	Yes

Figure A

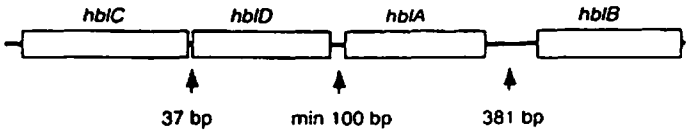


Fig. 1. The map of the *hbl* operon [19,20]. The operon includes the three known proteins of the HBL: L_2 protein encoded by *hblC*, L_1 protein by *hblD*, and B protein by *hblA* and the B' protein by *hblLB*, with 73% identity to the B protein in the first 158 amino acids [5].

Figure B

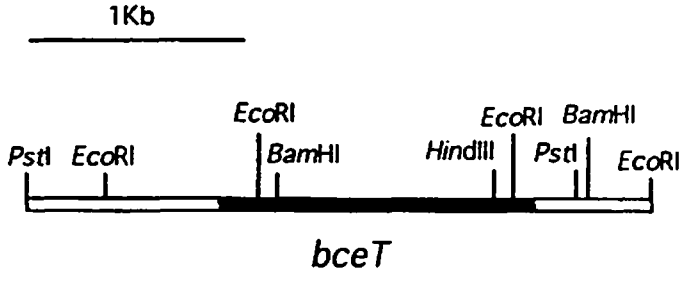
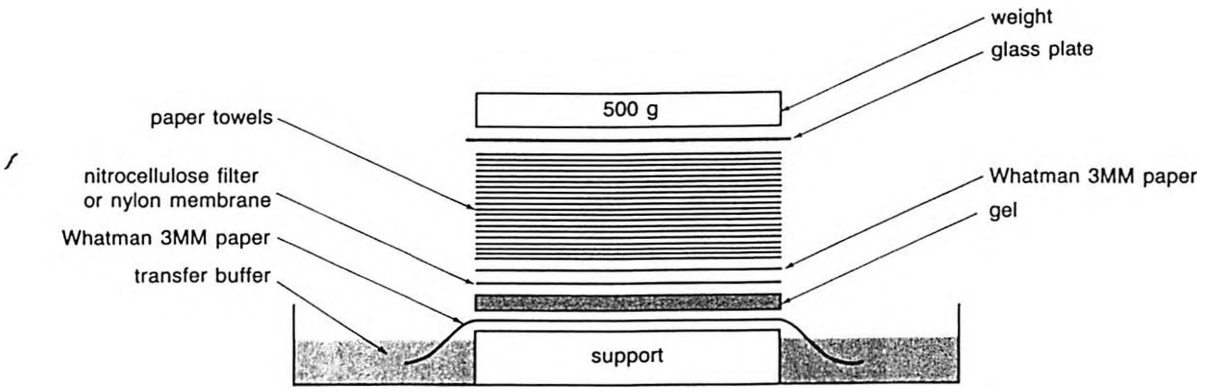


Fig. 1. Restriction map and sequencing strategy for the *bceT* gene. The 2·9 kb *Pst*I–*Eco*RI fragment from *B. cereus* B-4ac is on plasmid pAGA118.

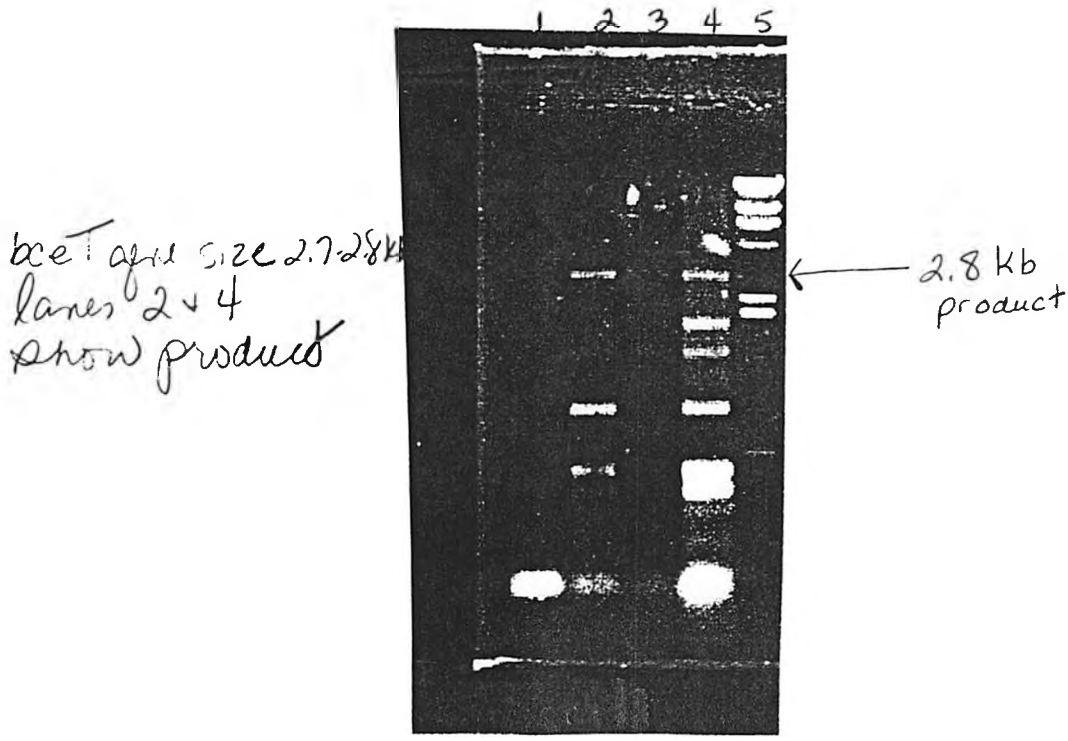
Figure C



Southern hybridization – Capillary blot set-up

Figure D

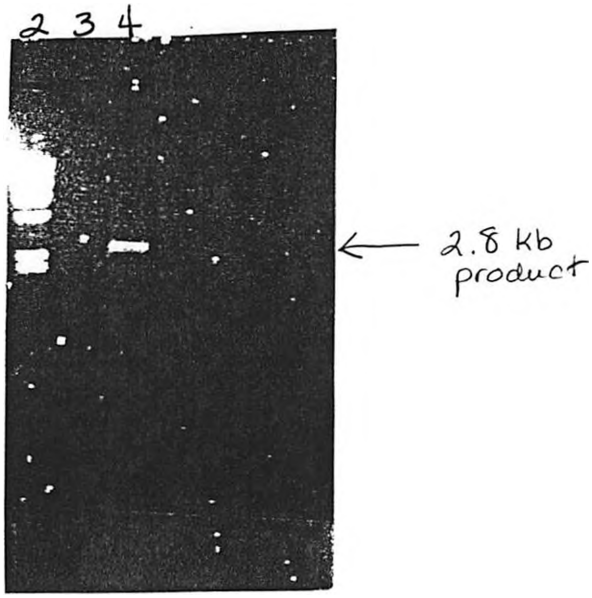
Bacillus cereus 14579 with bceT primers



Results in lanes 1 – 4
Lane 1 = negative control (no template used)
Lanes 2 and 4 show 2.8 kb product
Lane 3 = no product obtained
Lane 5 is standard = lambda cut with HindIII

Figure E

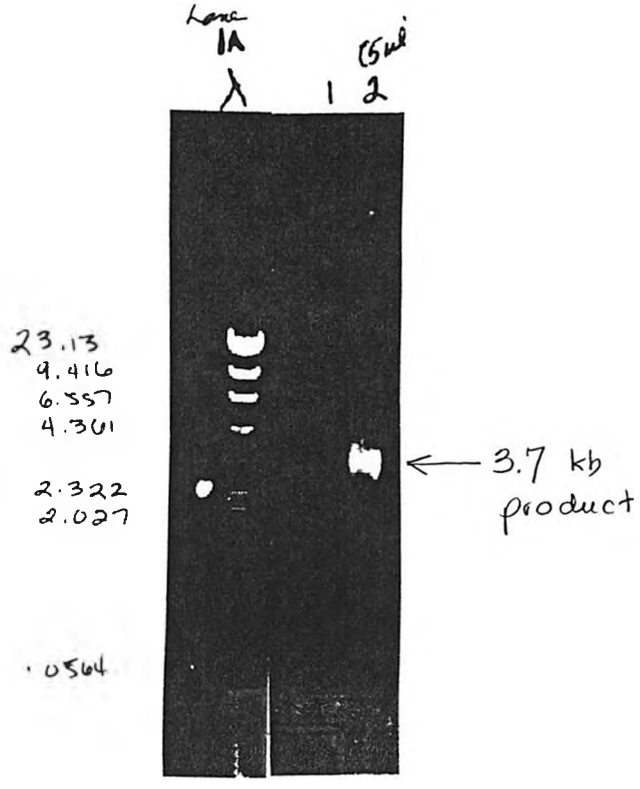
Bacillus megaterium CH1 with bceT primers



Results in Lane 4
2.8 kb product in lane 4
Lane 2 is standard = lambda cut with HindIII

Figure F

Bacillus cereus 14579 with hblC/D primers



Results in lanes 1 – 2
Lane 1 = negative control (no template used)
Lane 2 shows 3.7 kb product
Lane 1A is standard = lambda cut with HindIII

Figure G

3.7 kb product from previous PCR product

Product in lane 1, standard in lane 2
(Standard = lambda cut with HindIII)

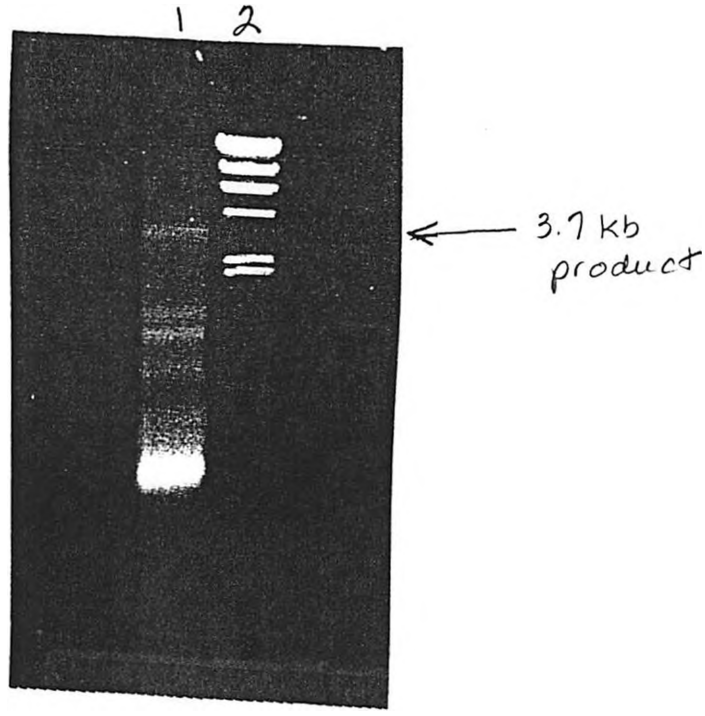
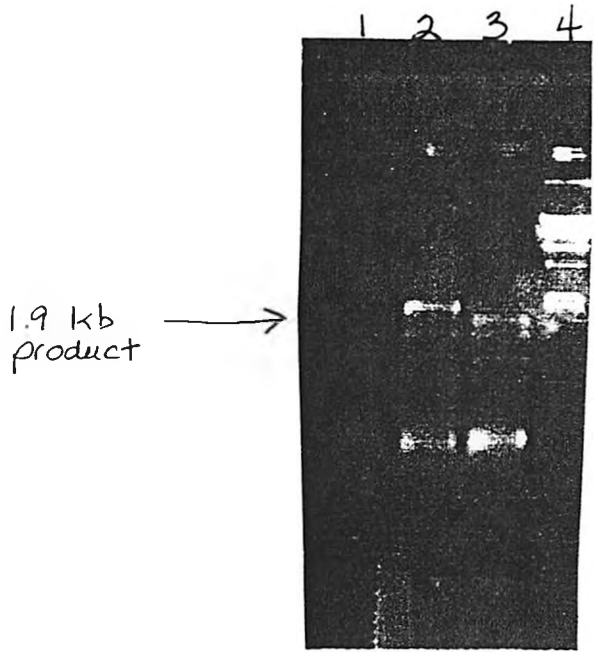


Figure H

Bacillus megaterium CH1 with hblA primers



Results in lanes 1 – 3
Lane 1 = negative control (no template used)
Lanes 2 and 3 show 1.9 kb product
Lane 4 is standard = lambda cut with HindIII

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