NORTHERN ILLINOIS UNIVERSITY

Research Toward the Development of a Protocol To Electrotransform *Bacillus megaterium* Through Exploration of Parameters and Variables that have Been Used to Successfully Electrotransform Other Gram-Positive Bacteria

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Abstract

This project was proposed to develop a protocol to electrotransform Bacillus megaterium (B. megaterium) through investigation of techniques that have been used to electrotransform other species of gram-positive bacteria. Electrotransformation is a process by which exogenous DNA can be introduced into a recipient cell through the application of an electric pulse. It was decided that successful protocols be studied and applied as closely as possible to B. megaterium. Specific areas of inquiry included; finding the machine settings on the apparatus needed to achieve electric pulse times that have been found to be optimum in successful protocols, constructing a table of applied voltage vs incidence of cell death in the range of applied voltages used in other protocols, and investigating the effect of adding various substances, such as glycine and polyethelene glycol, to the growth medium or the electrotransformation buffer to assess whether damage to, or reconfiguration of the cell wall and capsule could be induced. It was found that similar, but not identical, voltages and pulse times as those utilized in the protocols studied could be achieved using the available apparatus. Surprisingly, applying similar pulse times and voltages to B. megaterium did not result in any significant or even measurable level of cell death -- a condition thought to be critical to successful electrotransformation. The most promising procedure proved to be the addition of glycine to the growth medium. Significant morphological changes were observed after growing a culture in varying concentrations of glycine to a stage that showed a 75-90% reduction in optical density when compared to a control grown without glycine. In addition, when an electric pulse was applied to these cells they appeared to have been damaged. In spite of this progress, when the procedure was followed using plasmid DNA as the donor, it yielded no transformants. There is some question as to the suitability of the plasmid that was used, (pHV 33) as an electrotransformation vector, adding complexity to the problem. In addition to the work described above, two plasmid DNA extractions using cesium chloride gradients were performed and another plasmid, the 8.1kb plasmid of B. megaterium was isolated using electroelution.

Introduction

Bacillus megaterium (B. megaterium) is a non-pathogenic, gram-positive soil bacteria whose properties make it well suited for genetic research. B. megaterium is a spore-forming Bacilli that germinates synchronously and efficiently. The development of genetic studies in B. megaterium has revealed that it is not closely related to other species of Bacillus, hybridizing only 8% with B. subtilus, and varying in %G+C content with 39% compared to 35% in the B. cereus, and 44% in the B. subtilus, groups (25). Genetic analyses performed in Dr. Patricia Vary's lab have isolated and characterized over 550 mutants including: auxotrophic, antibiotic resistant, and, temperature sensitive germination mutants. B. megaterium strains usually contain several resident plasmids. The plasmid sizes and compositions have been studied, and significantly, a plasmidless strain, PV361 was isolated (24). Strain has allowed PV361 B. megaterium to be used as a cloning host for the expression of genetically engineered products in addition to its use as an industrial producer of many compounds, including; synthetic penicillin, vitamin B12, and, amylases. The value of B. megaterium as a cloning host is enhanced by it's lack of pathogenicity, endotoxins, and, alkaline proteases which interfere with cloning by cutting up donor DNA before it can enter the recipient cell (25).

B. megaterium is the primary organism used for research in

Dr. Vary's lab, and developing a protocol to electrotransform the bacterium was the focus of this project. Electroporation is a phenomenon in which the membrane of a cell wall exposed to high-intensity electric field pulses can be temporarily destabilized in specific regions of the cell. During the destabilization periods, the cell membrane is highly permeable to exogenous molecules present in the surrounding media. Hence, electroporation can be regarded as a massive microinjection technique that can be used to inject a single cell or millions of cells with specific components in culture medium (4). The objective, or desired specific component in this case, is the introduction of plasmid DNA into the cell with the goal of creating specific mutants to be used in the investigation of the genetic make-up of and phenotypic expression of B. megaterium. Because the mechanism by which bacteria are transformed by electroporation is not well understood, it has been suggested by Dr. Annick Mercenier that electrotransformation is a more accurate term (15). This usage has been adopted in Dr. Vary's lab.

To date, there are two known methods of transforming B. megaterium: the protoplast/PEG method (27), a chemically mediated method involving the removal of the bacterial cell wall using lysozyme to form protoplasts, and then exposing them to the cell fusigen, polyethylene glycol (PEG) in the presence of exogenous DNA, and the more recently developed biolistic method of shooting DNA into the cell using a biolistic propulsion

apparatus (21). Both of these methods yield approximately 10^4 transformants/ug DNA. Electrotransformation, if successful, could offer a faster, simpler method of transformation without the necessity of outgrowth and replica plating steps while giving a higher yield of transformants.

Work done prior to the start of this project focused on optimizing the PEG/protoplast transformation of B. megaterium, by testing the viability of substituting simple regeneration media for the rich and easily contaminated RHAF media required for this procedure (7,26). Experiments were also performed to test the effectiveness of including selective concentrations of antibiotic to the outgrowth media.

Other areas of study for 370H and 495H classes included: reviewing and continuing work done previously testing the effectiveness of certain buffers, electric field strengths, and pulse times for electrotransformation (3,14,24). Finally, an electrotransformation experiment (20) using the Escherichia coli (E. coli) strain DH5alpha was performed to eliminate the possibility that our electroporation apparatus was unreliable. This experiment was successful yielding 10^6 transformants/ug DNA (8).

A research plan was formulated for this capstone project that centered on three factors that are fairly constant in protocols that have proven successful for the electrotransformation of gram-positive bacteria:

1. There is a range of applied electric field strengths and

pulse times that is similar for many of the gram-positive species that have been electrotransformed (1,5,11,19).

2. Most of the available literature states that there is a relationship between the number of cells killed by the applied electric field and the number of cells transformed. The optimum percentage of cells killed varies from 46-99% depending on the species (5,11,19).
3. Several gram-positive species previously recalcitrant to electrotransformation were successfully transformed when certain substances known to interfere with the formation of the cell wall were added to the growth media of cells prepared for transformation. The additions to growth media noted included: antibiotics, nutritional requirements such as glycine, and chemicals such as the cell wall fusigen, PEG (6,10,23).

Using the factors listed above as a starting point, it was decided that data for the construction of a kV vs kill rate curve should be collected and several protocols selected and modified for use on B. megaterium (1,6,10,13,18,20,23). In addition to the conditions and procedures listed above, plans were made to investigate suggestions made by Dr. Vary and Dr. Mercenier (13,22) that the cells be:

1. passed through glass wool prior to applying the electrical pulse to see if removing some of the peptidoglycan capsule facilitated transformation

2. exposed to multiple electrical pulses
3. grown with inhibiting concentrations of glycine and data collected to plot a growth curve.

Finally, plasmid DNA was obtained for experimental use by cesium chloride plasmid preparation using two methods (2,12), and another plasmid, the 8.1kb plasmid, pPY105, of B. megaterium strain PV203 was isolated by electroelution from agarose gel fragments.

Materials and Methods

Strains and culture conditions. The recipient strain of B. megaterium used for all experiments was PV361. PV361 was derived from the parent strain QM B1551 (ATCC 12778), and is a prototroph 7p- genotype (24). Two plasmids, pHT3101 and pHV33, were extracted by alkaline plasmid prep procedure (2) and cesium chloride plasmid preparation (10). Plasmid pHT3101 was given to the lab by George Rapaport of the Pasteur Institute and confers erythromycin resistance in B. megaterium. For this experiment pHT3101 was extracted from the E. coli strain JM83. Plasmid pHV33 is a derivative of pHV14 which was derived from a hybrid of pBR322 and pC194 (15). pHV33 confers chloramphenicol resistance in B. megaterium and was extracted from the E. coli strain DH5alpha.

Pulse times and electric field strength determination. Pulse times and electric field strengths close to those used in the protocols that were being adapted (1,8,11,19,) were calculated using the equation:

$$T = \frac{C(R1)(R2)}{R1+R2}$$

Where C = capacitance in Farads, R1 = the variable resistance setting on the apparatus, R2 = the internal resistance of the cuvette (the chamber the cells are placed in to receive the electric pulse), and T = pulse duration in sec. Since the buffer used influences the magnitude of the chamber resistance, individual buffers were pulsed at a fixed resistance setting to determine the pulse time under those conditions. This data allowed the chamber resistance to be calculated by rearranging the equation to solve for R2 (5). Knowing R2 permits the calculation of various pulse times keeping capacitance constant and varying the resistance setting on the apparatus.

Establishing a KV vs killing curve. Frozen, competent cells prepared by Maya Blakely were used for these experiments (3). A 40 uL sample of cells was placed in a chilled cuvette and pulsed at 10, 15, 20, and, 25 KV/cm. The resistance setting was 186 ohms, and capacitance 50 uF, giving a pulse time of approximately 9.0 ms. Trials were also run using freshly grown and frozen cells prepared by the method described in Protocol 3.

Addition of PEG to Electrotransformation Buffer and Passing Cells Through Glass Wool (8,13) Subsequent trials involved resuspending the cells in 1.0 ml of PEG 4000 (30% in TE buffer), and 1.0 ml ddH2O and then passing the cells through a Sweeney filter filled with glass wool. Cultures were also exposed to multiple pulses to see if the incidence of cell death increased.

Development of growth curves for cells grown in inhibiting concentrations of glycine. Cells were prepared according to Protocol 3. (6). During the growth period 4.0 ml aliquots were removed from the control and the four flasks containing PV 361 growing with various concentrations of glycine once an hour, and the optical density (OD) at 660 nm measured. Data was collected for cells grown in SNB broth plus glycine, and minimal (MC) broth plus glycine. A graph of optical density vs time was plotted for PV 361 grown in SNB with various concentrations of glycine (Fig. 1).

Examination of treated cells to check for morphological changes. Samples of experimental and control cultures were periodically examined under a phase contrast microscope to check for possible morphological changes in the cells caused by the presence glycine in the growth media. Cells were also observed after the administration of single and multiple electrical pulses to cells prepared according to Protocol 3.

Extraction of plasmid DNA. Plasmid DNA was extracted by two methods (Protocol 1, 2). The extractions were followed by separation of plasmid DNA from chromosomal DNA by cesium chloride gradients containing 600 ug/ml ethidium bromide at a density of 1.56. The prep was placed in a 38 ml tube and run in a tv865 ultra-centrifuge overnight. Plasmid DNA was recovered by extracting the lower band in the cesium chloride gradient--visible when illuminated by UV light--with a 10.0 ml syringe. The DNA was then pipetted into dialysis tubing and dialyzed in dilute buffer (2.5 M NaCl, 0.5 M Tris base--pH 7.5, 0.05 M Na2EDTA--50X concentration, use 20 ml/liter ddH2O) for four hours, changing the buffer every hour.

Isolation of DNA from agarose gel by electroelution. Agarose gel fragments containing a mixture of two native B. megaterium plasmids, (extracted from strain PV 203 by David Stevenson) the 8.1 and 70 kb, was isolated by electroelution using an electroelution box (International Biotechnologies Inc.). The fragments containing the DNA bands were loaded into wells in the apparatus that are just covered with degassed TAE buffer. A 3.0 M sodium acetate solution containing 0.1% volume loading dye was then loaded in the collection tubes. The apparatus was then operated for one hour at 125 volts. The DNA was then removed from the collection tube with a pipetteman and reprecipitated with ethanol.

Agarose gel electrophoresis and restriction endonuclease digestion of DNA. Plasmid DNA extracted as described in the procedures above was digested by restriction endonuclease and analyzed by agarose gel electrophoresis. The plasmids, pHT3101 and pHV33 were digested with HindIII, BamHI, and EcoRI (Gibco BRL Life Technologies) in total volumes of 20 ul (15.5 ul H2O, 2.0 ul buffer, 2.0 ul DNA, and, 0.5 ul restriction enzyme). The tubes were then placed in a 37°C incubator for two hours. After 4.0 ul of loading dye was added, the digest was loaded into a 20 ml, 0.7% agarose (in TAE buffer) gel which was run at 45 volts for approximately one hour. The gel was removed from the box and stained with ethidium bromide for 10 minutes. The gel was then illuminated with UV light from a transilluminator so that the gel could be photographed (Figure 2). This analysis was performed to confirm the identity of the plasmids. Analysis of the 8.1 kb plasmid, pPY105, was performed in a similar manner, and was performed to begin a restriction map of the plasmid. Plasmid pPY105 was cut with HindIII, BamHI, Xba, to determine which enzymes were single cutters of the plasmid. Preliminary work with the 8.1 kb plasmid was performed by Lisa Manning (14), using a 16.5 kb recombinant plasmid, pDS10 containing a 7.7 kb fragment of pPY105 cloned by David Stevenson.

Protocol 1

Alkaline Plasmid Prep

Solution I
Lysozyme solution: 2ug/ml lysozyme, 50mM glucose, 10mMEDTA, 25mM Tris (pH 8). Glucose, EDTA, tris may be stored as sterile solutions in frig. Lysozyme should be added fresh only after cells are resuspended.
Solution II
0.2 N NaOH, 1%SDS. Store at room temperature one week only.
Solution III
3.0 M Na acetate (pH 4.8). Prepare by dissolving three moles of Na acetate in 400 ml distilled water, adjust pH with glacial acetic acid -- may require up to 300 ml. Store at room temperature.
Inoculate culture (500 ml) using appropriate antibiotic. Grow at 37°C until OD_{600} is 0.6 then add chloramphenicol (25 ug/ml). Continue shaking at 37°C overnight.

1. Place culture on ice for 15 min. Harvest cells in 250 ml bottles by centrifugation at 8k rpm for 10 min.
2. Resuspend pellet in 25 ml/L of culture in solution I then add appropriate amount of lysozyme. Mix well then incubate on ice for 30 min.
3. Add 50 ml of solution II and mix gently. Incubate on ice 5 min. (Suspension should become clear and viscous).
4. Add 38 ml of solution III and mix gently, but thoroughly (a clot of chromosomal DNA should form). Incubate on ice 60 min.
5. Spin at 8 k rpm for 20 min. Transfer supernatant to another 250 ml centrifuge bottle. IF ANY OF THE PELLET COMES LOOSE REMOVE IT FROM NEW BOTTLE.
6. Add 125 ml of 100% ETOH to precipitate DNA. Place in -80°C freezer for 30 min. or dry ice ethanol bath. Spin at 8K rpm for 15 min. then discard supernatant.
7. Resuspend pellet in a total of 12.5 ml 0.1 M NaAc, 0.05 M tris (pH 8.0). Add 25ml 100% ETOH and place in -80°C freezer or dry ice ethanol bath for 30 min. Spin at 8k rpm for 15 min., discard supernatant. Air dry tubes upside down for about 30 min.

Either proceed to Cesium chloride gradients or follow the protocol that requires RNase and PEG precipitation. CsCl gradients contain 600 ug/ml ethidium bromide at a density of 1.56. They can be run in either vertical rotor depending on the volume of the prep. The tv865 holds 6.0 ml tubes and is run at 50k rpm for 16 hours or overnight. The tv850 holds 38 ml tubes and is run at 42k rpm for 16 hours or overnight.

Protocol 2.

Cesium Chloride Plasmid Preparation

1. Grow 100 ml cells in Spizizin salts + 4.0 g yeast extract per liter. Inoculate 1 loopful/100 ml media and leave at 30°C overnight. Place in 30°C shaker in morning at 300 rpm. Harvest cells when they reach O.D.660 = 0.9-1.0.
2. Spin at 7,000 rpm for 5 min. and wash twice with 50 ml 1XTES.
3. Resuspend in 4 ml of lysozyme buffer and transfer to 50 ml Sepcor tubes.
4. Add 8 mg lysozyme (= 2mg/ml) to solution.
5. Incubate at 37°C for 15-20 min. Check for protoplasting.
6. Add 16 ml freshly made SDS buffer. Mix gently. Incubate at 37°C for 30 min.
7. Add 5 ml NaCl, mix gently, leave on ice overnight.
8. Spin at 10,000 rpm at 0°C for 30 min. in Sepcor tubes in SS34.
9. Place supernatant in cold tube, add 5 ml of 50% PEG. (Type 6,000; 50% in TES) mix gently.
10. Leave on ice 3-5 hr.
11. Spin at 10,000 rpm for 10 min. at 10°C in Sepcor tube.
12. Dissolve pellet gently in 2 ml TES. Add RNase (pancreatic, cooked) to 100 mg/ml (20ug of 10 mg/ml stock in -20°C freezer). Do not have to dissolve pellet completely before adding RNase.
13. Incubate at 65°C for 30 min. Can store in refrigerator overnight.
14. Bring to 4.0 ml with TES.
15. Add CsCl at 1.0g/ml of above solution, then weigh 1.0 ml at a time by taking 1.0 ml out, tare balance to 0, put 1.0 ml back in, should weigh 1.55, not more than 1.56. Adjust by adding more CsCl or TES. Then add up to 600 ug/ml ethidium bromide from 10 mg/ml stock. Check density again and adjust if needed.
16. Load tubes for gradients. Weigh tubes to within 0.01 g, adjust with stock of CsCl in TES at correct density. Add oil to fill tube. Cap.
17. After spinning for gradients and removing the plasmid DNA, dialyze 4X against 100X volume dialysis buffer at 4°C.

Reagents for Cesium Chloride Preparation

TES

20 mM Tris pH 7.5
5 mM EDTA
100 mM NaCl

Lysozyme mix

30 mM Tris pH 8.0
50 mM EDTA
25% glucose
500ug/ml-2 mg/ml lysozyme added separately

SDS solution

2.0 ml 10% SDS in TES
2.0 ml 500 mM EDTA pH 8.0
12 ml TES pH 8.0
(final yield 1% SDS in 50 mM EDTA and TES

RNase

10 mg/ml in H2O heated 80°C for 15 minutes. Store in -20°C freezer.

Cesium Chloride Stock Solution

1.0 mg/ml in TES adjusted to 1.55 g/ml by weighing 1.0 ml of solution at room temperature.

Ethidium Bromide

10 mg/ml in H2O in dark bottle, room temperature.

50X Dialysis Buffer

2.5 M NaCl
0.5 M Tris Base (pH 7.5)
0.05 M Na2EDTA
Use 20 ml/Liter for final concentration

Protocol 3.

Optimized Electroporation Protocol, modified for B. megaterium from Dunny, et al. (6).

1. Grow cells for 12-15 hours in SNB (or MC) medium plus glycine at various concentrations.

Use a glycine concentration that gives a 70-90% reduction in the A660 of the culture, as compared with a control grown for the same period of time in the absence of glycine. Because the extent to which the growth of a given strain is inhibited by glycine is somewhat variable, it is necessary to prepare overnight cultures at several different concentrations to ensure that a culture showing the optimal growth inhibition will be obtained. Useful ranges of glycine concentrations for selected strains are from 1.0-6.0%.

2. Dilute the culture from step 1. into fresh medium (containing the same or slightly higher concentration of glycine to bring the OD_{660} to 0.05-0.08. Incubate for 60 min. (90 min. for slowly growing strains) at 30°C.
3. Chill the culture on ice, harvest the cells by centrifugation, and wash the cells in 1/3 volume of chilled electroporation solution (0.625 M sucrose-1.0 mM MgCl2, adjusted to pH 4.0 with 1 N HCl).
4. Harvest cells from the wash and suspend them in 1/30 to 1/100 of the original volume of electroporation solution. Incubate on ice 30-60 min. (Cells may be frozen in a dry ice-ethanol bath and stored for at least one year at -70°C at this point and thawed in an ice water bath just before use).
5. Add cells from Step 4. to the electroporation cuvette. Add DNA (less than 10 ul in H2O or low salt buffer). About 300 ng of DNA should ensure a successful transformation; the use of more than about 1-1.5 ug of plasmid DNA appears to be unnecessary.
6. Electroporate immediately with the 25 uF setting on the electroporation apparatus. Field strength should be 8,750-10,000 V/cm.
7. Place cells on ice for 1-2 min. and then dilute into 2 volumes of SNB media plus inducing concentrations of antibiotics, if using an inducible4 resistance gene as a selective marker. Incubate for 90-120 min. at 30°C.
8. Spread the cells on selective SNB agar plates.

Results

KV vs Kill Rate Curve

Trial 1.

Conditions: PV 361, 10^9-10^{10} cells to start, buffer, 10% glycerol
Observed pulse times: approximately 9.0 ms.

Trial 2.

Conditions: PV 361, 10^9-10^{10} cells to start,
Cells passed through a Sweeney filter filled with glass wool. Buffer, sterile H2O.
Observed pulse times: approximately 6.0 ms.

Trial 3.

Conditions: PV 361, 10^9-10^{10} cells to start, buffer,
30% PEG 4000 in TE.
Observed pulse times: approximately 9.0 ms.

Trials 4 & 5.

Conditions: PV 361, 10^9-10^{10} cells to start, cells passed through glass wool. Buffer, 30% PEG 4000 in TE.
Observed pulse times: approximately 8.0 ms.

Trial 6 & 7.

Conditions: PV 361 prepared by method described in Protocol 3.
10^6 cells to start. Electroporation buffer.
Observed pulse times: approximately 9.0 ms

Trial 8 & 9.

Conditions: PV 361 prepared by method described in Protocol 3. and frozen in dry ice/ethanol bath, defrosted on ice and resuspended in 30% PEG 3000 in TE. Three pulses administered at settings listed below.
Trial 9, half of cells pulsed in PEG, half in ETfn solution.
Observed pulse times: approximately 8.0 ms, 3 pulses

Machine settings for all trials:
Resistance: 186 ohms
Capacitance: 50 uF
Voltage: 10, 15, 20, 25 kV

For all trials described above, there was no significant difference in survival rates between controls and cells exposed to electric fields. For trials where a 25 kV pulse was administered, there was arcing across the cuvette vaporizing all cells.

Growth Curve Data

Time vs Optical Density (OD_{660})

Time Hour	Control (SNB)	0.5 (SNB)	Glycine Concentration (%) 0.75 (SNB)	1.0 (SNB)	Control (MC)	0.25 (MC)	0.4 (MC)
1	.03	.01	.02	.01	.01	.01	.02
2	.08	.04	.02	.01	.02	.01	.02
3	.32	.09	.03	.01	.03	.01	.02
4	.9	.19	.03	.01	.11	.02	.02
5	2.2	.2	.03	.02	.18	.02	.02
6	3.4	.4*	.03**	.04	.5	.04***	.02

*Cells prepped according to Protocol 3.
**After 22 hrs. this culture had an OD_{660} = 0.5
***Cells prepped as above.

Observations of the cells listed above using a phase contrast microscope before exposure to electric field: Control cells grown with both SNB and MC media appeared as whole healthy vegetative B. megaterium cells.
Cells grown with various concentrations of glycine:

SNB + glycine, cell appeared to have less interior structure and were puffy. There was greatly reduced levels of PHB, (poly-beta-hydroxy-butyrate) probably the major energy source for the cell during sporulation.

MC + glycine, some dead cells were observed, others were puffy, and many appeared to be empty. Some cells had been broken open when the coverslip was placed on the slide. Reduced PHB levels were noted. After 22 hours of growth, there were very few spores, compared with the control, which was nearly 100% spores.

All samples were examined for evidence of clumping after exposure to the electric pulse. The best example were cells grown in SNB + 0.5% glycine, where extensive clumping was observed after exposure to three pulses of approximately 9.0 ms under conditions described in kV vs kill rate section.

Restriction Endonuclease Digestions and Electrophoresis Gels of Plasmid DNA Extracts

Figure 1. (pg. 18) Shows the two bands of the 8.1 kb plasmid, pPY105, which were then cut out and electroeluted.

Figure 2. (pg. 19) shows the two bands of pPY105 after electroelution, lane 2. is the single plasmid, lane 4 shows the tetrameric form higher in the lane.

Figure 3. (pg.20) show the bands that resulted when pPY105 was digested with several restriction enzymes, most of them single cutters.

FIGURE - 1

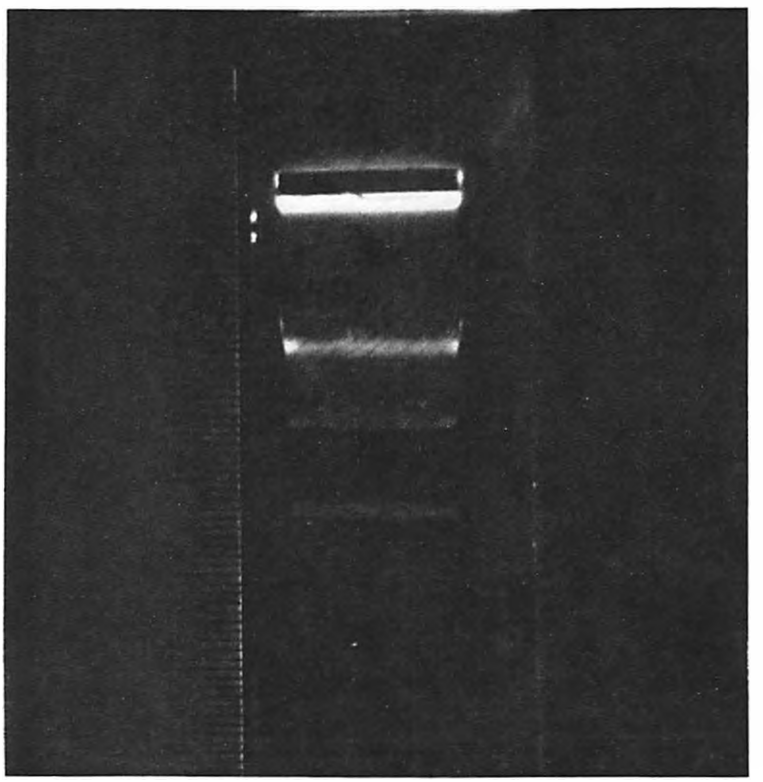


FIGURE - 2

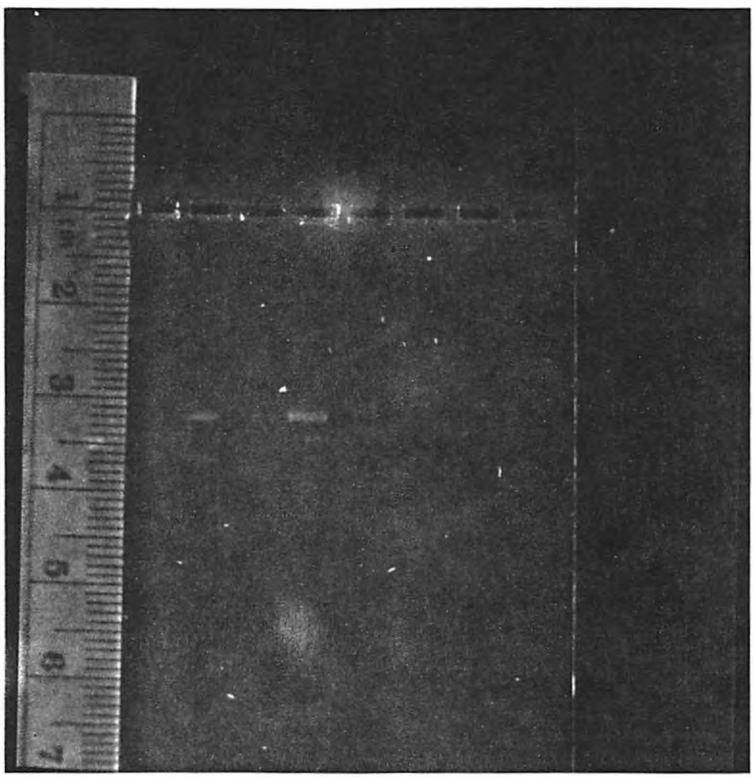
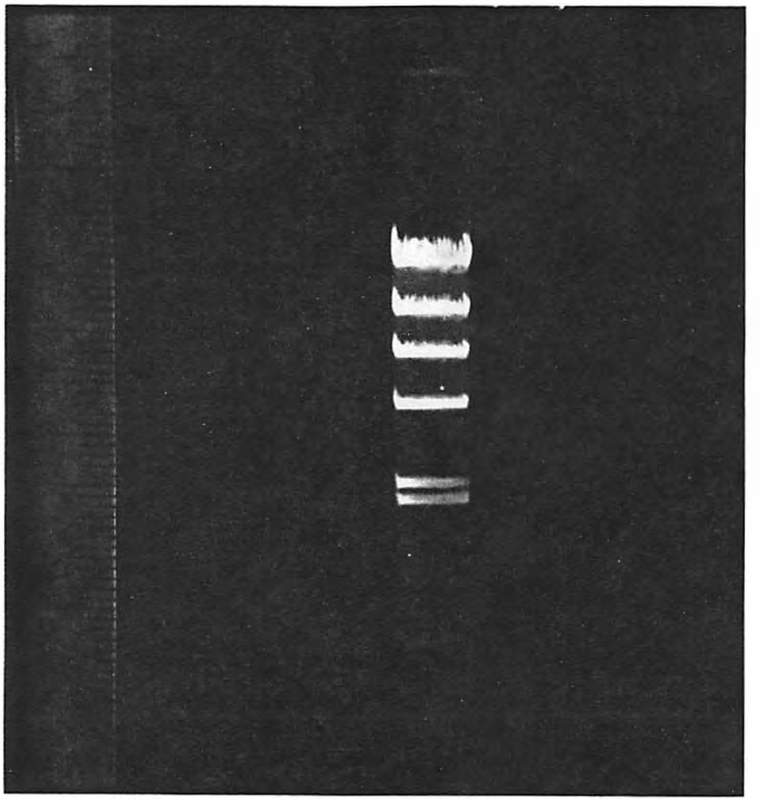
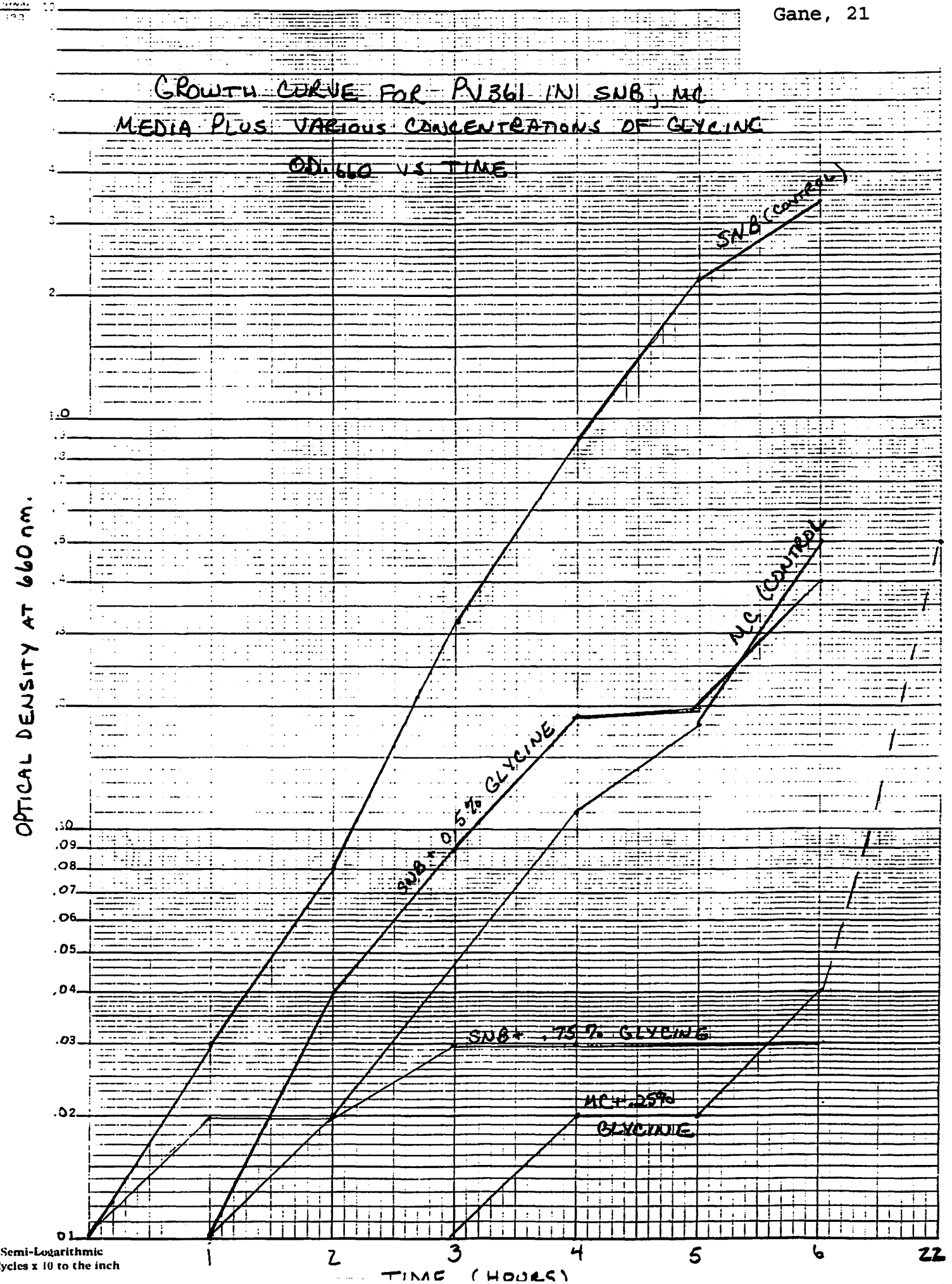


FIGURE - 3



GROWTH CURVE FOR PV361 IN SNB, MC MEDIA PLUS VARIOUS CONCENTRATIONS OF GLYCINE

OD 660 VS TIME



Semi-Logarithmic
3 Cycles x 10 to the inch

Discussion

A great deal of experimentation has been done in Dr. Vary's laboratory to investigate possible methods of electrotransforming B. megaterium. Initially, it appeared that the conditions necessary for success would be simply to find the correct buffer and growing conditions, as was the case for many species (1,13,20). Work done over the past four years has been, in large, focussed on finding the correct; buffer, electric field strength, and pulse time that would lead to the development of a protocol for the electrotransformation of B. megaterium (3,8,16,26). When these avenues proved unproductive it was decided that the problem should be approached from a slightly different perspective.

Studies with various species shown to be resistant a basic method of electrotransformation: cell culture growth to the appropriate phase, several washes and resuspension in a small volume of high or low ionic strength buffer, addition of an optimum amount of DNA, and the administration of an electric pulse of particular field strength (5), have shown that there are further procedures that can lead to successful protocols. In addition, it was observed that successful protocols shared certain commonalties beyond those conditions listed above. First, it was observed that the administration of a pulse leading to transformation usually killed between 46 and 99% of the exposed cells compared to an unpulsed control (1,13,18). This

phenomenon is linked to the success and the rate of electrotransformation occurring at different field strengths.

In an effort to define the electric field strength that would create similar conditions for B. megaterium, experiments were performed to collect data for a kV vs kill rate curve (pg. 15). It was decided that the majority of these trials be run without adding DNA since the primary objective was not to transform, but to see if any cells were killed by the exposure to the electric field. Some tests involved plating the pulsed cells and control cells to non-selective medium and counting survivors after 24 hours, while for other tests the cells were exposed to single and/or multiple pulses and then observed under the phase contrast microscope for any changes in morphology.

The results of nine trials performed under varying conditions to assess the amount of killing caused by the administration of an electrical pulse to vegetative, mid-log phase, (results pg. 15) B. megaterium cells showed that no discernible number of cells were killed. This result was unexpected and may be due in part to the limitations of the electroporation apparatus (a pulse higher than 2.0 kV caused arcing in all cases, and it was not possible to program multiple timed pulses). An investigation of the available literature suggests that Hamilton and Sale (7,16) were able to kill vegetative B. megaterium cells using a special apparatus designed to show that cell death by exposure to an electric field was not due to heat generated by the pulse, but rather, as a result of

leakage of the intercellular contents.

It is believed that the difficulty in killing the bacteria as discussed above is a result of the physiology of the B. megaterium cell wall and capsule. This led to a series of experiments designed to weaken or compromise the integrity of the cell wall and capsule. The first experiment involved passing an aliquot of prepared cells through a Sweeney filter filled with sterile glass wool, which was intended to remove some of the polysaccharide capsule. This procedure did not increase the incidence of cell death by administration of an electric pulse.

Other methods, such as; resuspending the cells in a solution of 30% PEG 4000 in TE buffer, and applying multiple pulses were also unsuccessful in causing cell death. This was also a somewhat surprising result for if pores were being formed by the administration of the electric pulse, other experiments suggest that the addition of PEG to the cell culture before pulsing causes the pore size to be enlarged (10), however, the study supporting this assertion was done with yeast and the findings may not extend to bacterial species.

Another series of tests involved growing B. megaterium cells (PV361) in media containing inhibitory concentrations of glycine (Protocol 3). Glycine inhibits cell growth in B. megaterium by preventing the formation of cross-links in the peptidoglycan of the bacterial cell wall. The mechanism of inhibition would also suggest a weakening of the cell wall. In fact, morphological changes were observed in cells grown in glycine, including: cells

with decreased levels of PHB's--which appear as bright spots inside the cells when observed under the microscope, cells that appeared "puffy" when compared with control cells grown without glycine, and cells that appeared empty or were so weakened that they broke open when the coverslip was placed on the slide (pg. 16). A growth curve was plotted to assess the amount of inhibition compared to cells grown without glycine (pg. 21). The flat area of the graph between hours 4 and 5 for SNB + 0.5% glycine is probably due to a change in temperature which induced a short lag phase. However, it is clear that glycine does slow growth in B. megaterium and delay the onset of sporulation. Unfortunately, neither an increase in kill rate, or, successful transformation were observed. The most dramatic damage to B. megaterium cells was observed when cells grown in minimal (MC) media were exposed to multiple electric pulses. However, the cell growth was so inhibited by glycine, that it was difficult to obtain enough cells to experiment with and a trial using plasmid DNA gave no transformants.

A possible explanation for the puzzling results described above may be found in information that has recently been noted in a publication. Experimenters found that plasmid pHV33 was not able to be transformed into Bacillus thuringiensis, while several other plasmids were very effective (20). This may indicate a problem with the transforming plasmid. This is a problem that has been encountered in other species, in fact, the lab that developed Protocol 3 also developed a new cloning vector to use

in their experiments with species that had proven difficult to electrotransform (6). This evidence suggests that while a plasmid may be effective in transforming a species using the PEG/protoplast method of transformation, it does not mean that the same plasmid will be effective for electrotransformation.

Plasmid DNA was first extracted using an alkaline plasmid prep (Protocol 1), and purified by cesium chloride gradients. The optical density of the DNA after dialysis was read on a Perkin-Elmer Lambda 1 Spectrometer. The OD_{260}/OD_{280} was less than 1.6, indicating protein contamination, so, addition cleaning steps were performed. During the cleaning process the plasmids were nicked when a mixture of phenol and chloroform was used that contained phenol which had not been redistilled. A second plasmid prep was performed (Protocol 2) and a gel run to confirm the identity and to estimate the density of the DNA (data not shown).

The final work done on this project involved isolating the 8.1 kb plasmid of B. megaterium, pPY105 by electroelution, and performing restriction enzyme digests to confirm which restriction enzymes are single cutters of this plasmid. The mixed DNA was run on an agarose loading gel (Figure 1) and the two lower bands cut out and electroeluted. It is thought that the plasmid runs in two bands because some of the DNA is found in the form of a tetramer, or four plasmids connected together as. It can be seen in Figure 2 that one band remains higher in the lane as expected because larger fragments of DNA move through the

gel more slowly.

The isolated DNA was digested with HindIII, BamHI, and, XbaI (Figure 3). All of these enzymes appear to be single cutters of pPY105. These experiments are of interest because plasmid pPY105 has potential for development as an effective vector for electrotransformation (22).

In conclusion, a series of experiments have been performed in an effort to develop a protocol for the electrotransformation of B. megaterium. While some progress has been made as far as causing damage to or weakening the cell wall--believed to be the primary obstacle to the process--the goal of developing and optimizing a protocol has not been fulfilled. In spite of the limited results, the project has proven to be productive from the standpoint of learning new techniques and becoming familiar with other work being done in the area of electrotransformation, and the various genetic research that is associated with transformation. In addition, the techniques involved with restriction mapping and DNA isolation will be very useful in future work.

Creating specific mutants in B. megaterium reliably and quickly is an important component of the genetic research being done in Dr. Vary's lab. For this reason, it is likely that Dr. Vary and others will continue to search for a successful protocol for the electrotransformation of B. megaterium. There is still a great deal of ground to be covered, but the work that has been done has pin-pointed some problem areas; the resistance of

B. megaterium to an applied electric field, the possibility that another plasmid may be needed to electrotransform B. megaterium, and, that, although the cell wall growth of B. megaterium can be inhibited by the addition of glycine to the growth media, some other factor(s) is needed to transform this bacteria

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