NORTHERN ILLINOIS UNIVERSITY

An Investigation of Bacterial Transformation Techniques

A Thesis submitted to the

University Honors Program

In Partial Fulfillment of the

Requirements of the Baccalaureate Degree

With University (Upper Division) Honors

Department of Biology

by

Kimberly M. Moldovan

DeKalb, Illinois

May 9, 1992

HONORS THESIS ABSTRACTS
THESIS SUBMISSION FORM

AUTHOR: KIMBERLY MICHELLE MOLDOVAN

THESIS TITLE: An Investigation of Bacterial Transformation Techniques

ADVISOR: Dr. Patricia Vary ADVISOR'S DEPT: BIOLOGY

DISCIPLINE: Completion of B.S. degree in Biology YEAR: 1992

HONORS PROGRAM: University Honors Program

NAME OF COLLEGE: Liberal Arts and Sciences

PAGE LENGTH: 24 BIBLIOGRAPHY (YES OR NO): YES ILLUSTRATED (YES OR NO): YES

PUBLISHED (YES OR NO): NO IF YES, LIST PUBLICATION:

COPIES AVAILABLE (HARD COPY, MICROFILM, DISKETTE):

SUBJECT HEADINGS: (Choose 5 key words or phrases by which a reader could find your thesis)

Electroporation CsCl plasmid preparation

Bacillus megaterium Bacterial Genetic Engineering

Protoplast transformation

ABSTRACT (100-200 WORDS): This thesis contain information regarding two possible means of causing B. megaterium to take into its own genome, exogenous DNA. The whole goal of the transformation process, be it via electroporation or protoplast transformation, is to get the cell to not only accept the foreign DNA, but to express the proteins the exogenous genes code for. The electroporation process consists of subjecting the bacterial cells to various strengths of electric shock in order to cause pore formation in the cell wall and subsequent entrance of the exogenous DNA of interest. The disruption of the cell wall must not be too great that it is unable to synthesize new cell wall components and express the new genetic material. Protoplast transformation involves the complete removal of the bacterial cell wall, membrane fusion, membrane separation, cell wall regeneration, and finally, expression of the new genetic matter. The former process would appear to be more efficient and successful, but per my research of one year and a half, it seems the exact opposite it true. This thesis investigated the parameters of both transformation processes and suggests future studies in this relatively new and exciting field of Microbial genetics. Included in the work are the parameters explored and several suggestions for further research. One of the more radical suggestions made about future studies is the concept of a fusion of techniques from both processes to increase transformation efficiencies. The concept is one of creating the cell wall-less protoplasts and add exogenous DNA, but then subject this to the electric shock.

For Office Use:
THESIS NO:

Approved: Patricia S. Yorey

Department of: Biological Sciences

Date: May 7, 1992

INTRODUCTION

Transformation of bacteria has been a cornerstone of bacterial genetic investigations and engineering for several years now. The transformation process consists of the creation of a competent recipient cell-type that is capable of accepting exogenous DNA, and in turn, expressing this extraneous DNA as a part of its own genome. As one might imagine, this is not always an easy process to accomplish. As a result, new techniques have been developed to try to improve the level of efficiency of this process. The main focus of my ongoing year and a half of research has been the improvement of the techniques used in two forms of the transformation process. The first technique that was investigated in depth was that of the process of electroporation. With this process, the basic idea is to subject the bacterial recipient to an electric current in order to cause the formation of pores in the cell wall. As a result of this traumatic experience, it is our hope that the cell will respond by taking in the exogenous DNA offered, regenerate the missing cell wall fragments, and begin expressing the new DNA's protein products. The alternate form of transformation that was investigated is termed protoplast transformation and is a much more well known process in comparison to the electroporation technique. This alternate process consists of first the removal of the bacterial cell walls (protoplast formation), the addition of exogenous DNA to the suspension, protoplast fusion and subsequent separation, regeneration of the cell walls, and finally, the expression of the new DNA's proteins. It is easy to see that this technique takes quite a bit of skill to accomplish at high levels of efficiency. With this, I will begin the discussion of:

I. Electroporation experiments conducted using DNA samples as well as a time and material saving fluorescent dye in order to begin to narrow down the parameters of what is to be a successful protocol for the process.

II. Protoplast transformation experiments done to improve the process's efficiency and to produce a protocol that will uniformly deliver a high yield of transformants recovered per micro liter (microL) of DNA added to the sample. The improved yield from both of these processes will not only correspond to the needs of my particular lab but the microbial genetic community at large as well. As will be seen during the course of this thesis, the experiments conducted on the electroporation front were less successful than those in the protoplast area. This is not a result of lack of experimentation, but may have been a factor of having to sort through and select for the correct set of variables that are necessary in conjunction with each other to result in the desired increased efficiencies. The protoplast transformation experiments were found to be resulting in efficiencies that were as high as any results that have been observed in our laboratory. This was exactly the result desired and consistency was now a key factor in the process to improve the general usefulness of the process in everyday laboratory use.

MATERIALS & METHODS

For the electoporation experiments, the following was used:

source stock of pV 361 (Bacillus megaterium)

Plasmid DNA (pTV 1) isolated from B. subtilis via a CsCl gradient

LBG broth: per Quinn and Dancer, 10g tryptone, 5g yeast extract, 10g NaCl, and 10mL of 10% w/v glucose solution added after autoclaving

10mM HEPES adjusted to pH 7 with concentrated NaOH

Double distilled, autoclaved H_2O

SNB plates with 5 microgram/mL concentration of chloramphenicol

LBG plates with 5 microgram/mL concentration of chloramphenicol

BHI plates with 5 microgram/mL concentration of chloramphenicol

Pipettemen (20, 200, and 1000 microliter sizes)

1mm and 2mm size cuvettes (microchambers)

ice bath

FITC-Dextran dye (concentration 4mg/mL)

fluorescent microscope (courtesy of Dr. Briles)

Dextranase (at concentrations of 10u/mL and 2u/mL)

B. subtilis stock strain 168

Sorvall refrigerated centrifuge (GSA rotor and SS-34 rotor)

E. coli stock strain DH10B (courtesy of Dr. Joel Stafstrom)

10% v/v glycerol

pUC 19 DNA

SOC recovery media: 98mL of SOB plus 1mL sterile 2M Mg^{++} stock and 1mL 2M glucose

SOB media: 20g bacto-tryptone, 5g of bacto-yeast extract, 0.584g of NaCl, 0.186g of KCl, and distilled or deionized H_2O to 1L.

2M Mg^{++} stock: 0.2033g of $MgCl_2$-$6H_2O$, 0.246g of $MgSO_4$-$7H_2O$, and distilled H_2O to 100mL; autoclaved.

2M glucose stock solution: 36.04g dextrose and distilled H_2O to 100mL; filter sterilized

LB plates with a concentration of 60 micrograms/mL of ampicillin

For the protoplast transformation experiments, the following was used:

RHAF: Broth and plates (VonTersch and Carlton 1981 J. Bacteriol. 155:866) Soln A= 5g yeast extract, 5g tryptone, 500mL ddH_2O (Plates: 10g Agar) Soln B= 12g Trisma base, 2g glucose, 68.46g sucrose, 0.14g KH_2PO_4, 10mL HAF salts, 470mL ddH_2O Soln B is titrated to pH 7.5 with concentrated HCl; autoclave, combine A and B, then add 10mL of 2M MgCl-$6H_2O$

HAF Protoplasting buffer (Fodor et al., 1975 J. Bacteriol. 121:390):
12g Tris base, 68.46g sucrose, 10mL HAF salts, 970mL ddH_2O
Titrated to pH 7.5, autoclave and add 10mL of 2M $MgCl_2$

HAF salts (100X): Added in order 0.35g KCl, 0.58g NaCl, 1.3g Na_2SO_4, 10g NH_4Cl, 100ml ddH_2O mixed and stored at room temp.

2M MgCl stock solution: 40.6g $MgCl\text{-}6H_2O$ and 100mL ddH_2O
mixed, autoclaved and stored at room temp.

SNB media (Shay and Vary 1978, BBA 538:284-292): A. in 2L flask combine 8mL SNB salts, 8g nutrient broth, 900mL ddH_2O, 15g agar for plates B. combine in milk dilution bottle 10mL 0.1M $CaCl_2\text{-}2H_2O$, 10mL 10% w/v glucose (1g), 80mL of ddH_2O Autoclave A and B separately, then combine

SNB Salts (sterilize,refrigerate): 50mL of 1mM $FeSO_4$, 100mL of 10mM $MnCl_2$, 200mL of 25% w/v KCl, and 50mL of 1M $MgSO_4\text{-}7H_2O$

Lysozyme stock: 5mL HAF plus 10mg lysozyme divided into 10 aquilots of 0.5mL, stored in -20°C freezer

50mL and 15mL disposable plastic centrifuge tubes

Stock solution of 30% PEG in HAF

RHAF plates with 1microgram/mL concentration of erythromycin

15% Sucrose Nutrient Agar plates

SNB plates with 5microgram/mL concentration of erythromycin

RHAF plates with 2microgram/mL concentration of chloramphenicol

SNB plates with 5microgram/mL concentration of chloramphenicol

Stock pHV 33 plasmid DNA (CsCl prepared)

Stock pHT 3101 plasmid DNA (CsCl prepared)

1% and 0.8% agarose solutions (in TE)

4'x5' Royal Pan film

Kodak D 11 developer

Rapid Fix

EcoR1 cut lambda phage

E. coli V 517 uncut plasmid standard

loading dye

Solution I- lysozyme solution: 2mg/mL lysozyme, 50mM glucose, 10mM EDTA, 25mM Tris-HCl (pH 8). Prepared fresh daily from crystalline lysozyme and a stock solution of the other components. Stored at $0^{o}C$ or sterile at $4^{o}C$

Solution II- Alkaline SDS solution: 0.2 N NaOH, 1% SDS. Stored at room temp.

Solution III- High salt solution: "3M" Na acetate (pH 4.8). Prepare by dissolving 3 moles of Na acetate in 400mL of water, adjusting pH to 4.8 with glacial acetic acid, and adjusting volume to 1L. Store at room temp.

10mg/mL RNAse A

TE: 10mM Tris, 1mM EDTA at pH 7.5

EtBr at concentration of 10mg/mL

Sorvall vertical rotor ultracentrifuge

Sorvall tv850 rotor run 16hrs to overnight

BTX 600 electro cell manipulator

The following are the parameters used with the electroporation experiments:

In all cases, High voltage mode on the BTX 600 was used, the capacitance was 50 microFarads, all pV 361 B. megaterium cell samples plus pTV 1 DNA were tested in 80 microL amounts.

	cells	OD 660	fld st	R	pulse t	peak V	buffer	DNA
1.	pV361	0.2	1 kV/cm	72 ohms	3.09msec	0.2kV	10%glycerol	pTV 1

	cells	OD 660	fld st	R	pulse t	peak V	buffer	DNA	
2.	pV361	0.2	1kV/cm	129ohms	5.48msec	0.2kV	10% glycerol	pTV1	
3.	"	"	"	186ohms	7.88msec	"	"	"	
4.	"	"	6kV/cm	72ohms	2.95msec	0.60kV	"	"	
5.	"	"	"	129ohms	5.37msec	0.62kV	"	"	
6.	"	"	"	186ohms	7.74msec	"	"	"	
7.	"	"	12kV/cm	72ohms	2.94msec	1.15kV	"	"	
8.	"	"	"	129ohms	5.40msec	1.17kV	"	"	
9.	"	"	"	186ohms	7.70msec	1.18kV	"	"	
10.	"	"	6kV/cm	129ohms	4.94msec	0.62kV	"	"	no ice
11.	pV361	0.2	1kV/cm	129ohms	4.84msec	0.19kV	10%glycerol in HEPES	pTV 1	
12.	"	"	"	186ohms	6.73msec	"	"	"	
13.	"	"	"	246ohms	9.03msec	"	"	"	
14.	"	"	6kV/cm	129ohms	4.90msec	0.61kV	"	"	
15.	"	"	"	186ohms	6.72msec	0.62kV	"	"	
16.	"	"	"	246ohms	8.43msec	"	"	"	
17.	"	"	12kV/cm	129ohms	4.49msec	1.15kV	"	"	
18.	"	"	"	186ohms	5.06msec	1.17kV	"	"	
19.	"	"	"	246ohms	7,53msec	"	"	"	
20.	"	"	6kV/cm	186ohms	6.78msec	0.62kV	"	"	no ice

	cells	OD 660	fld st	R	pulse t	peak V	buffer	DNA	
21.	pV361	0.4	1kV/cm	72ohms	106.1msec	-0.6kV	10% glycerol	pTV 1	
22.	"	"	"	129ohms	5.38msec	0.119kV	"	"	
23.	"	"	"	186ohms	7.79msec	0.19kV	"	"	
24.	"	"	6kV/cm	72ohms	3.06msec	0.58kV	"	"	
25.	"	"	"	129ohms	5.29msec	0.60kV	"	"	
26.	"	"	"	186ohms	7.65msec	"	"	"	
27.	"	"	12kV/cm	72ohms	3.00msec	1.13kV	"	"	
28.	"	"	"	129ohms	5.22msec	1.16kV	"	"	
29.	"	"	"	186ohms	7.52msec	1.17kV	"	"	
30,	"	"	6kV/cm	129ohms	5.29msec	0.60kV	"	"	no ice
31.	pV361	0.4	1kV/cm	129ohms	4.90msec	0.20kV	10%glycerol in HEPES	pTV 1	
32.	"	"	"	186ohms	6.62msec	"	"	"	
33.	"	"	"	246ohms	8.20msec	"	"	"	
34.	"	"	6kV/cm	129ohms	4.74msec	0.60kV	"	"	
35.	"	"	"	186ohms	6.56msec	0.61kV	"	"	
36.	"	"	"	246ohms	8.21msec	0.62kV	"	"	
37.	"	"	12kV/cm	129ohms	4.74msec	1.15kV	"	"	
38.	"	"	"	186ohms	6.21msec	1.17kV	"	"	
39.	"	"	"	246ohms	7.44msec	1.18kV	"	"	
40.	"	"	6kV/cm	186ohms	6.32msec	0.62kV	"	"	no ice
41.	pV361	0.6	1kV/cm	72ohms	3.02msec	0.19kV	10%glycerol	pTV 1	
	"	"	"	129ohms	5.28msec	"	"	"	
43.	"	"	"	186ohms	7.47msec	"	"	"	

	cells	OD 660	fld st	R	pulse t	peak V	buffer	DNA	
44.	pV361	0.6	6kV/cm	72ohms	3.02msec	0.60kV	10%glycerol	pIV 1	
45.	"	"	"	129ohms	5.25msec	0.61kV	"	"	
46.	"	"	"	186ohms	7.45msec	"	"	"	
47.	"	"	12kV/cm	72ohms	3.00msec	1.14kV	"	"	
48.	"	"	"	129ohms	5.19msec	1.16kV	"	"	
49.	"	"	"	186ohms	7.36msec	1.18kV	"	"	
50.	"	"	6kV/cm	129ohms	5.15msec	0.62kV	"	"	no ice
51.	pV361	0.6	1kV/cm	129ohms	4.81msec	0.19kV	10%glycerol in HEPES	pIV 1	
52.	"	"	"	186ohms	6.50msec	"	"	"	
53.	"	"	"	246ohms	8.16msec	"	"	"	
54.	"	"	6kV/cm	129ohms	4.75msec	0.60kV	"	"	
55.	"	"	"	186ohms	6.37msec	0.61kV	"	"	
56.	"	"	"	246ohms	8.09msec	"	"	"	
57.	"	"	12kV/cm	129ohms	4.56msec	1.15kV	"	"	
58.	"	"	"	186ohms	destroyed by arcing		"	"	
59.	"	"	"	246ohms	5.93msec	1.16kV	"	"	
60.	"	"	6kV/cm	186ohms	6.67msec	0.61kV	"	"	no ice

ELECTROPORATION WITH FLUORESCEIN ISOTHIOCYANATE-DEXTRAN DYE

(FITC-DEXTRAN)

	cells	OD 600	fld st	R	pulse t	peak V	washed	dex'ase?
1.	Bs 168	0.6	6.25kV/cm	720ohms	4.82msec	1.19kV	in PEB	no
2.	"	"	"	"	not electroporated		"	"

	cells	OD 600	fld st	R	pulse t	peak V	washed	dex'ase	DYE?
3.	Bs 168	0.6	6.25kV/cm	720ohms	5.4msec	not zapped	in PEB	no	6microL FITC
4.	"	"	"	"	"	"	"	"	12microL FITC
5.	"	"	"	"	"	"	"	"	24microL FITC
6.	"	"	"	"	"	"	"	yes 10u/mL and 16u/mL dextranase added	48microL FITC
samples=50microL		OD 550							
7.	DH10B	0.8	13.0kV/cm	129ohms	3.02msec	1.21kV	in 10% glycerol	no	24microL FITC
8.	"	"	"	"	not zapped		"	no	"

Electroporation with FITC DYE was discontinued at this point because of its lack of significant saving of time or materials.

PROTOPLAST TRANSFORMATIONS

In order to conduct the protoplast transformations, it was necessary that I learn how to CsCl prepare large amounts of DNA (pHV 33 and pHT 3101) and learn how to run these plasmids out on agarose gels to make sure I have obtained the correct DNA material for the transformations. A brief summary of the CsCl preparation process is as follows: (Based on Birnborn and Doly, Nucleic Acids Res. 7, 1513, 1979)

Grow cells overnight (E. coli containing pHV 33 plasmid) in LB, shaking at 37°C. Overnight culture was 5mL with Amp at 25 microgram/mL. 2L of culture were innoculated with the E. coli and ampicillin and grown overnight.

1. Culture placed on ice for 15 min. Harvest cells in 250 mL bottles at 8k for 10 min.

2. Resuspend pellet in 12.5mL (for each 500mL of culture) in Solution I (I combined some of the bottles at this point) then add appropriate amount of lysozyme. Mix well and incubate on ice for 30 min.

3. Add 25 mL (all directions per 500mL of culture) of Solution II and mix gently. Incubate on ice 5 min. (Suspension should become clear and slightly viscious).

4. Add 19mL of Solution III and mix gently, but thoroughly. (a clot of chromosomal DNA should form) Incubate on wet ice for 60 min.

5. Centrifuge at 8k for 20 min. Transfer supernatant to a different 250mL centrifuge bottle (if pellet is not tight, remove bits of pellet that are decanted into fresh bottle with the supernatant).

6. Add 125mL of 100% EtOH and precipitate in -80^{o}C freezer for 30 min. Spin at 8k for 15 min and discard supernatant. Turn bottles over on paper towels for 10-15 min as a drying step.

7. Resuspend pellet in a total of 12.5mL of TE. Add 25mL of 100% EtOH and precipitate in -80^{o}C freezer for 30 min. Spin at 8k for 15 min and discard supernatant. Drying step is necessary at this point as well.

Step 8 is followed for myself because of the need to rid my sample of RNA. Add 19 microL of 10mg/mL RNAse A (Note: when preparing RNAse solution, heat at 100^{o}C for 5 min to destroy DNAse, in 50 mM NaAc pH 4.8) Incubate in 37^{o}C water bath for 15 min.

9. Resuspend in 7mL of TE.

10. Add CsCl (approximately 1g/mL) to a density of 1.56-1.57. Check the density by weighing 1mL of the solution (Remove 1mL, tare the balance, and add it back). It should weigh 1.56-1.57g.

11. Add Ethidium Bromide from a 10mg/mL stock to yield a final concentration of 600 microgram/mL. CAUTION EB IS A STRONG MUTAGEN: GLOVES MUST BE WORN.

12. Recheck the density and add CsCl if necessary to bring density to 1.56-1.57.

13. Balance the ultracentrifuge tubes by adding CsCl solution at a density of 1.56 if necessary. Use light mineral oil to fill the tubes. All CsCl gradients should be run in full tubes no matter which rotor is used.

The gradients can be run in either Sorvall vertical rotor depending on the volume of the preparation. The tv865 holds 6mL tubes and is run at 50k for 16 h to overnight. The tv850 holds 38mL tubes and is run at 42k for 16 h or overnight.

The preceding preparation is an E. coli alkaline plasmid preparation and should only be followed as such.

The general procedure followed for every protoplast transformation is as follows (Modification of VonTersch and Carlton, J. Bact. 1983 155):

Streak recipient (pV361) on SNB, Incubate at 25°C overnight
Innoculate heavily 2 prewarmed RHAF broths
(50mL in 500mL flasks)

↓ 37°C shaking to OD_{660}= 0.6-0.8 (3-5 h)

Pour into 2 plastic conical screw cap 50mL centrifuge tubes

3000 rpm, 10 min, room temp.

Resuspend both tubes in total of 4.5mL HAF, vortex, combine

se all plastic from his point on.....

Add 0.5mL HAF with 2mg/mL lysozyme (from frozen stock)
Invert several times

37°C 100 rpm 12-15 min
Check in microscope for 90-100% protoplasting

1500 rpm 10 min room temp.

Respend by inverting after adding 5mL of 37°C RHAF

1500 rpm 10 min room temp.

Resuspend by inverting after adding 2mL of 37°C RHAF

→

12.

Tube: 1 2 3 4

Cells: 0.5 mL each tube (in disposable 15mL plastic tubes)

DNA: 0 1-5 1-5 1-5 micrograms

0=control (Add DNA--different types or amts)

Mix gently (also drop DNA onto RHAF to check for sterility)

IMMEDIATELY ADD 1.5mL of 30% PEG in HAF and invert several times

Incubate at room temp. for 4 min, no more (PEG toxic)

Add 5mL of 37°C RHAF, invert again **

1500 rpm 10 min room temp.

Add 0.5mL RHAF to each pellet and gently resuspend by inverting

Plate 10^{0}-10^{-3} (dilute in HAF) on RHAF plates (no antibiotic) for transformants
Incubate at 30°C for 12-19 hrs
Replica plate to SNB + selective antibiotic (chloramphenicol for pHV 33 and erythromycin for pHT 3101)
Incubate 30°C for 12-24 hrs and count transformants

** may stop at this point and dilution plate from this point, spin step may be then be used to get the 10^{-1} dilution

---

Agarose gels were prepared at 1%and 0.8% in TE. These gels are poured, solidified and placed in the electrophoresis chamber for 10 min. At that time, TB buffer (1X) is added until the solution just covers the wells. Depending on the specific concentration of the DNA to be run determines the extent to which the sample must be diluted, but 1 microL of loading dye is a common factor. The Wells of the gel are loaded using a pipetteman and the gel can be run at 40 volts for 1 and 1/2 h to 2 hours. When the DNA has been run the length of the gel (mini gel in my investigation) it is subjected to 25 microL of 10mg/mL EtBr. This treatment is for 10 minutes and then it is washed with buffer. If a picture is to be taken, the gel is put on the UV box and the extremely light sensitive film is loaded in the camera in complete darkness. The film is exposed to the UV light for 30sec to 1 min and then placed in the developer for 5 minutes. Then it is transferred to the rapid fix for an additional 3 min. The film may now be subjected

to light and must be rinsed under running water for 10-15 minutes. After this, the film is hung up to air dry and may be left as is or may be turned into prints of the gels.

Results

In the electroporation trials done with DNA present (pTV 1), the procedure for trials numbered 1-60 were conducted in the following manner:

One of the 1mL aquilots of washed pV361 samples was removed from the -70°C freezer and with the pTV1 plasmid DNA, allowed to thaw on ice. Next, I found it necessary to take 800 microL of the cell sample and add 20 microL of the pTV1 DNA (0.425 micrograms/lambda) and place the mixture in a separate eppendorf tube. Before each trial, the mixture was allowed to sit for 1-2 min. before 80 microL was pipetted out and placed in the 1mm microchamber. Variables such as field strengths (fld st), buffers, resistances (R), presence of a post electric shock ice bath were conducted and the results duly noted. Generally, after the delivery of the electric shock, the cuvette was carefully placed back into an ice bath and allowed to sit there for 10 min. undisturbed. At the end of that interval, 1mL of LBG broth is pipetted into the cuvette and then carefully and extremely slowly pipetted out again with the electroporated cells incorporated. This was placed in a 15mL disposable plastic tube and the labelled tube was placed in the 37°C incubator with shaking for 1-2 hours. The shaking was for aeration purposes and was not very vigorous in nature. This was considered the outgrowth period and soonafter, each sample was plated on the differential SNB, BHI, and LBG plates all of which contained 5 micrograms/mL concentrations of chloramphenicol. Two plates of each media were plated for each trial in amounts of 0.1mL and 0.2mL. It should also be noted that controls that were created differed from the test samples in that they were subjected to exactly the same conditions as the tests except the electric shock itself. The plates were incubated for anywhere from

1-4 days and the results were noted at that time. The results of trials 1-60 were negative in nature. This was not to say that nothing was gained from the experiments, though. The negative results of these trials was used in order to narrow down the myriad of variables that may effect the efficiency of this transformation process. The variables that were tried at the time were later ruled out as possible areas of great importance and left room for new variable to be explored.

The results from the FITC-Dextran dye experiments were not encouraging in the slightest:

1. Fluorescence observed everywhere because at the time I did not know that the sample had to be washed in the proper buffer (PEB) several times in order to remove the background fluorescence from the true fluorescence.
2. This was the control for trial 1 and there was no clear difference in flourescence levels between the two trials. This was a very inconclusive pair of trials.

3.-6. Trials were set up as a test of what might be the necessary amount of the concentrated dye that would give the best resolution. It was also suggested by a BTX technical support advisor (personal contact 1991) that a dextranase might be helpful in reducing extraneous fluorescence in my samples. The 48 microL sample of FITC was used to test the dextranase at the concentrations noted on page 9 of this analysis. What happened is that the fluorescence was indeed reduced, but not to a readily distinguishable level that could be used as conclusive scientific evidence.

7.-8. These trials were conducted with E. coli (DH10B) in order to get a fool-proof positive transformation result from the electroporation process. Unfortunately, this was not the case and with the lack of success with the dye, I was forced to go back to the brute force method of electroporation at the request of my advising professor.

The results for the CsCl preparation were extremely favorable when first noted. The process, which is as extensive as delineated on 9-11 of this thesis, was followed as described and took a total of almost a week and a half to complete.

Ultracentrafuge specifics:

Rotor temperature= 20°C

Temp. limit= 25-30°C

Time set at infinity in order to complete what was to be a 16 h run

Rotor speed= 42k

Rotor used= tv850

After the gradients were run, they were cut as to separate the plasmid band of DNA from the excess protein and chromosomal bands of DNA. This was done with an automated machine in Dr. Hudspeth's lab with the kind help of VJ. Once the plasmid DNA was obtained, it was extracted 4 times with N-butanol in order to separate out the EtBr from the DNA. The DNA was subsequently dialyzed against the following buffer: (use 20 mL/L ddH_2O) 2.5M NaCl, 0.5M Tris pH 7.5, and 0.05M EDTA. This was changed 4 times in a 24 h period in 1L increments. The final plasmid DNA (pHV 33) was concentrated down to a 1mL sample that after a spectrophotometric analysis was done, was calculated to contain 664 micrograms/mL. This was a very good yield for a first try at the process. This DNA was shared with everyone in the lab and it was later discovered that an impurity in the DNA vial may have been spores that have subsequently germinated and are currently destroying the DNA that was so hard to obtain in the first place. This is why I was forced to begin doing experiments with another colleague's CsCl prepared pHT 3101 DNA. Protoplast transformation results, in general, improved tremendously when I began to use the pHT3101 versus my own pHV 33 DNA.

The process of protoplast transformation that was conducted is represented on pages 11 and 12 of this thesis and were followed as directed except where the **marked procedure was altered. The results were positive and are as follows:

Transformation Experiment 1:

Plate #	DNA	Dil.	Regener	Select	cfu a	extra b	plates c
1	0	10^{-1}	RHAF	SNB+cm5	0	0	0
2	0	10^{-2}	"	"	0	0	
3	0	10^{-3}	"	"	0	0	
4	0	10^{-4}	"	"	0	0	
5	2 microL pHV33	10^{-1}	"	"	0	0	0
6	"	10^{-2}	"	"	2	0	
7	"	10^{-3}	"	"	0	0	
8	"	10^{-4}	"	"	0	0	
9	5 microL pHV33	10^{-1}	"	"	0	0	0
10	"	10^{-2}	"	"	3	0	
11	"	10^{-3}	"	"	0	0	
12	"	10^{-4}	"	"	0	0	
13	10 miroL pHV33	10^{-1}	"	"	1	0	0
14	"	10^{-2}	"	"	2	1	
15	"	10^{-3}	"	"	2	0	
16	"	10^{-4}	"	"	0	0	

ontrol plus junk from the bottom of tube was replica plated and 4-5 cfu's were found.

It was not wholly unexpected that the very first protoplast transformation would not be a great success, but these results are rather discouraging. As my technique and skill improved, so did the results as will be shown shortly.

Transformation Experiment 2:

This experiment appeared to be immensely more successful at the regeneration stage than the first experiment ever did. This was a big disappointment when I discovered that possibly during the replica plating process, massive contamination had occurred. Plates that originally had characteristic B. megaterium colony morphology growth were found to subsequently have extremely mucoid growth on the master plates as well as the replicated ones. I was able to identify some transformants that were B. meg. but the growth was so obscured by the mucoid growth that a statistically accurate count could not be made. This will be further explored in the Discussion section.

Transformation Experiment 3:

Plate #	DNA	Dil.	Regener	Select	cfu a	plates b
1	0 microL	10^{-1}	RHAF	SNB+cm5	background growth on both	
2	"	10^{-2}	"	"		"
3	"	10^{-3}	"	"		"
4	"	10^{-4}	"	"		"
5	"	10^{-5}	"	"		"
6	2 microL pHV33	10^{-1}	"	"	21+bg	confluent cfu and bg
7	"	10^{-2}	"	"	57+bg	17+bg
8	"	10^{-3}	"	"	background growth only (bg)	
9	"	10^{-4}	"	"		"
10	"	10^{-5}	"	"		"
11	5 microL pHV33	10^{-1}	"	"	202+bg	55+bg
12	"	10^{-2}	"	"	44+bg	7+bg
13	"	10^{-3}	"	"	background growth only	

Transformation Experiment 3 (cont):

Plate #	DNA	Dil.	Regener	Select	cfu a	plates b
14	5 microL pHV33	10^{-4}	RHAF	SNB+ cm5	background growth only (bg)	
15	"	10^{-5}	"	"	"	

DNA control had contamination of RHAF and residual on SNB+cm5 but not enough to conclude that most if not all of the colonies were transformants. The technique seemed to improve, but the numbers of cfu's were still not accurate enough to be statistically sound.

Transformation Experiment 4:

Plate #	DNA	Dil.	Regener	Select	cfu alpha	plates beta	made ankh
1	0 microL	10^{-1}	♂ RHAF	SNB+ery5	lyellow c.		
			♑ 15% SNA	SNB+ ery5		low bg	
			♀ RHAF+ cm2	no			TNTC
2	"	10^{-2}	RHAF	SNB+ery5	0		
			15% SNA	SNB+ery5		low bg	
			RHAF+cm2	no			TNTC
3	"	10^{-3}	RHAF	SNB+ ery5	0		
			15% SNA	SNB+ery5		low bg	
			RHAF+cm2	no			426
4	5 microL pHV33	10^{-1}	RHAF+cm2	no			TNTC
			15%SNA	SNB+cm5	low level meshlike bg		
5	"	10^{-2}	RHAF+cm2	no			TNTC
			15% SNA	SNB+cm5	low level bg		

Transformation Experiment 4 (cont.)

Plate #	DNA	Dil.	Regener	Select	cfu alpha	plates beta	made ankh
6	5 microL pHV 33	10^{-2}	RHAF+cm2	no			TNTC
			15% SNA	SNB+cm5	low level meshlike bg		
7	2 microL pHT3101	10^{-1}	RHAF	SNB+ery5	TNTC		
8	"	10^{-2}	"	"	223		
9	"	10^{-3}	"	"	56		
10	5 microL pHT3101	10-1	"	"	TNTC		
11	"	10^{-2}	"	"	334		
12	"	10^{-3}	"	"	55		

controls created for each type of media used was tested and replicated with negative growth in all cases.

Calculation of transformation efficiency:

[pHT 3101] = 0.8 mg/mL

Using trial 9: 56 cfu at 10^{-3}

$56 \times \frac{1}{10^{-3}} = 5.6 \times 10^4$ cfu/mL

using 2.0 mL of pHT3101 = 1.6 mg DNA used

cell / mg DNA

$\frac{5.6 \times 10^4}{1.6 \text{ mg}} = 3.5 \times 10^4$ Trnsf / mg DNA

Transformation Experiment 5:

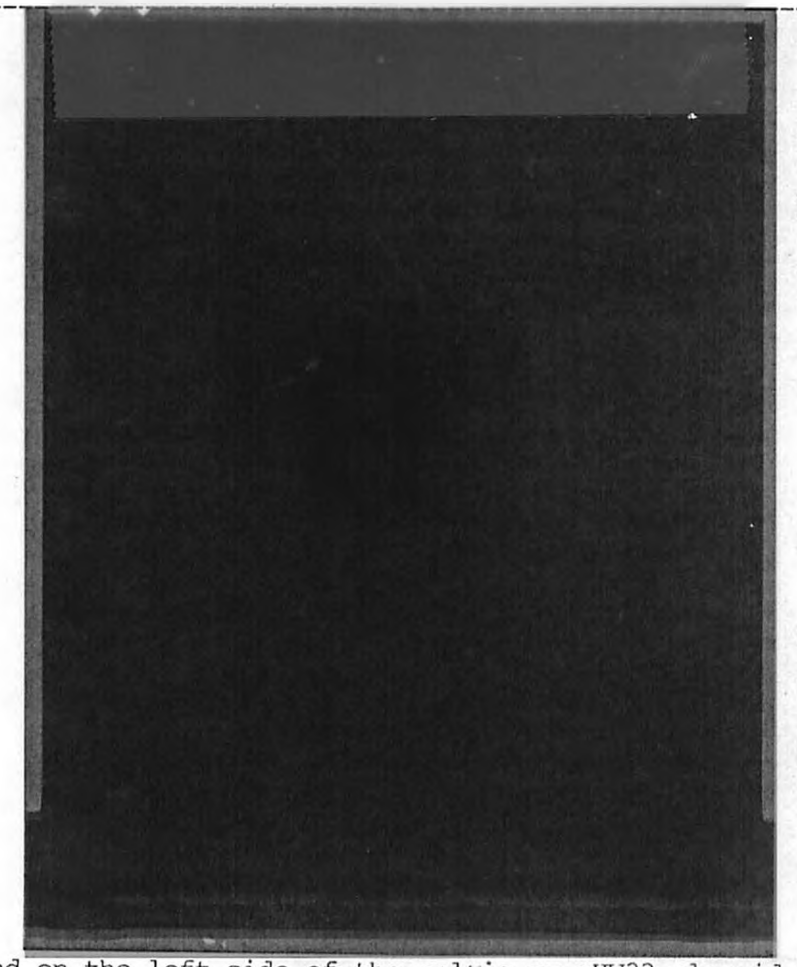
Plate #	DNA	Dil.	Regener	Select	cfu Ar	plates B	made Cr
1	0 microL	10^{-1}	RHAF	SNB+ery5	0		
			RHAF+eryl	no		0	
			15%SNA	SNB+ery5			0
2	"	10-2	RHAF	SNB+ ery5	0		
			RHAF+eryl	no		0	

Transformation Experiment 5 (cont.):

Plate #	DNA	Dil.	Regener	Select	cfu plates made Ar	B	Cr
2	0 microL	10^{-2}	15% SNA	SNB+ery5			0
3	"	10^{-3}	RHAF	SNB+ery5	0		
			RHAF+eryl	no		0	
			15% SNA	SNB+ery5			0
4	1 microL pHT 3101	10^{-1}	RHAF	SNB+ery5	6+ bg		
			RHAF+eryl	no		9+yellow cont.	
			15% SNA	SNB+ery5			0
5	"	10^{-2}	RHAF	SNB+ery5	21		
			RHAF+eryl	no		65	
			15% SNA	SNB+ery5			TNTC
6	"	10^{-3}	RHAF	SNB+ery5	0		
			RHAF+eryl	no		0	
			15% SNA	SNB+ery5			467
7	1.5 microL pHT3101	10^{-1}	RHAF	SNB+ery5	TNTC≅ 500		
			RHAF+eryl	no		0	
			15% SNA	SNB+ery5			21+ yellow cont.
8	"	10^{-2}	RHAF	SNB+ery5	475		
			RHAF+eryl	no		1	
			15% SNA	SNB+ery5			0
9	"	10^{-3}	RHAF	SNB+ery5	12		
			RHAF+eryl	no		0	
			15% SNB	SNB+ery5			0
10	2 microL pHT3101	10-1	RHAF	SNB+ery5	TNTC≅ 600		

Transformation Experiment 5 (cont.):

Plate #	DNA	Dil.	Regener	Select	cfu Ar	plates B	made Cr
10	2 microL pHT3101	10^{-1}	RHAF+eryl	no		4	
			15% SNA	SNB+ery5			0
11	"	10^{-2}	RHAF	SNB+ery5	30		
			RHAF+eryl	no		0	
			15% SNA	SNB+ery5			0
12	"	10^{-3}	RHAF	SNB+ery5	1		
			RHAF+eryl	no		0	
			15% SNA	SNB+ery5			0



Electrophoretic Gel Analysis of Plasmid DNA

The lowest band on the left side of the gel is my pHV33 plasmid which is 7.3kb.

The center band is E. coli v517 which is a plasmid standard.

The lowest band on the right side is the pHT3101 plasmid at 6.4kb.

Discussion and Conclusions

This past semester has been one of a great deal of learning that had to be accomplished before I could expect any success in my transformation experiments. I have learned most of the important parameters associated with electroporation, how to run a CsCl DNA gradient, how to efficiently conduct protoplast transformation experiments, and how to run and analyze electrophoretic gels and their photos. Some important things that I have also encountered these past weeks is that nothing is impervious to contamination, even if I am extremely careful. There is also the notion that the frozen stock lysozyme I made takes approximately 16 min to completely protoplast while fresh lysozyme takes 12 min. What was discovered was that freezing and thawing is capable of reducing lysing power by approx. 30% per freezing and thawing event. As far as protoplast transformation goes, some of the keys is to never pour off the gelatinous pellet found after the lysozyme spin (this may be the competent cells), always resuspend as gentle as possible when it comes to the protoplasts, and when the protocol says 4 min with the PEG it means 4 minutes and no more.

As far as electroporation goes, I am still very interested in getting this process to work because it has not been satisfactorily done in B. megaterium. If it is possible, I would like to think that I could be the one to work out the optimal conditions since I have spent almost 1 year on the project so far. The problem lies in the fact that there are so many variables that may be preventing the uptake of exogenous DNA that its hard to eliminate any variable for sure. It is entirely possible that it may be a combination of variables, but we can't know this for sure until more research can be done. If I continue on at this university for graduate school, I intend to remain in Dr. Vary's laboratory and devote most, if not all of my research to transformation factors, with electroporation as a special area of focus.

I truly believe that when I was using the FITC-Dextran dye, that it may not be my fault entirely for its failure to be useful. At the peak of my frustration with the product I did call the BTX tecnical support analyst. I was very shocked and dismayed that the person I talked to did not even know the correct concentration of the dye that should be used. This is the same person who called up Dr. Vary to tell her how wonderful this product is and that its something that would be very helpful. It only became my worst nightmare and an almost complete waste of time. The one good learning experience that did come out of this mess is that I learned the concept of fluorescent microscopy. This will definitely be an asset in the future.

The problems that I encountered in protoplast transformation with contamination seemed to be solved when the filters in the lab's air system were changed and I began to do my replica plating in the less crowded area of the lab where people would not walk by and inadvertently contaminate my experiment. I have also implicated the replicating velvets as a source of contamination which has been taken care of with better washing techniques, longer autoclaving times, better penetration of steam through paper bag containers, and the purchase of new velvets. Since these velvet improvements, I have had little or no contamination problems.

Last semester I set myself a goal of getting positive data. With the completion of this Capstone project, that goal has been reached, and at an extremely satisfactory level. I am not usually pleased with that amount of work that I accomplish in a semester's time, but this time, I feel I have accomplished a lot. The number of experiments may seem low, but the amount of time spent in preparation to do the experiments took the most time to learn and then perform accurately. I consider this semester one of the best and most challenging learning experiences I have ever had to face during my college career.

Future Goals

This seems to be the hardest part of the thesis to write because I really do not know what is going to happen with my educational career in the coming semester. My goal is to find some way to afford to stay going to school at N.I.U., to become an important member of the Graduate School, and to continue my investigations into bacterial genetic engineering. I feel this is one of the major areas of research in the future and it never hurts to train for a career that will be in demand in such a harsh job market.

As far as future projects are concerned, I think my research should go in the direction of trying to narrow down more of the electroporation parameters. Perhaps it is even possible to create protoplasts and electroporate them, or add lysozyme to the sample in the cuvette to weaken the cell walls right before the shock is administered. The protoplast transformation experiments seem to be working very well and the only complaint that could be investigated in the future might be the development of a regeneration media that is not as susceptible to contamination as the RHAF tends to be. I have tried 15% Sucrose Nutrient agar, but I feel that this did not work as well as expected. Perhaps there should be a higher concentration of sucrose, or perhaps another C source is what is needed. This is a fascinating project to work with because it turns out to be a lot more like and art than pure science. I like this aspect of my research and I wouldn't trade doing this kind of work for anything.

REFERENCES

BELLIVEAU, BRIAN H. and JACK T. TREVORS. 1989. Transformation of Bacillus cereus vegetative cells by electroporation. Applied and Environmental Microbiology, 55(6):1649-1652.

BIRNBORN and DOLY. 1979, Alkaline plasmid prep for E. coli . Nucleic Acids Res. 7, 1513.

VARY, P.S. and Y-P TAO. 1988. Development of genetic methods in Bacillus megaterium. Genetics and Biotechnology of Bacilli, 2:403-407.

VONTERSCH, and CARLTON. 1983. Transformation of B. megaterium. J. Bact. 155.