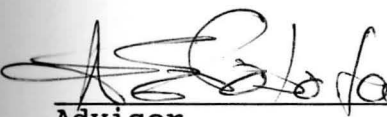FACTORS THAT AFFECT TRANSFORMATION OF

ESCHERICHIA COLI WITH PLASMID DNA

by

Karen S. Keslar

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

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ABSTRACT

FACTORS THAT AFFECT TRANSFORMATION OF

ESCHERICHIA COLI WITH PLASMID DNA

Karen S. Keslar

Master of Science

Youngstown State University, 1990

Plasmid transformation of *Escherichia coli* is an essential step in many cloning experiments, yet the process is very inefficient and the action of calcium is not clear. Transformation takes place in three stages: 1) binding of DNA to the outer membrane, 2) uptake of DNA into the cell, and 3) establishment of the plasmid as a stable replicon. This study focuses on factors which influence uptake and establishment of plasmids within *E. coli*. Glucose, multiple hot/cold cycles, and EDTA were all tested for their effects on DNA uptake. It was observed that treatments which decrease the viability of a transformation mixture increase the proportion but not the net number of transformed cells. Treatments which increase viability decrease the proportion of transformed cells. The requirement for calcium in establishment of DNA was investigated by using various chelating agents to remove calcium from the post-transformation growth medium. Removal

of calcium did not affect viability of the cells, however, transformation efficiency was decreased. The results suggest that uptake of DNA cannot be improved without drastically reducing viability and that calcium may have a role in establishing of plasmids within *E. coli*.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS (CONT'D)

LIST OF FIGURES

LIST OF FIGURES (CONT'D)

CHAPTER I

INTRODUCTION

Transformation is the process by which a bacterium absorbs pure deoxyribonucleic acid (DNA) from its surroundings and functionally integrates the exogenous DNA into its own genetic material (Watson *et al.*, 1987). The phenomenon was first observed by Frederick Griffith in 1928. He noted that nonvirulent strains of *Streptococcus pneumoniae* became virulent when mixed with their heat-killed pathogenic counterparts. Subsequent research showed that the genetic components of the heat-killed cells remain undamaged and can escape from the heat-killed cells, enter the living cells, and undergo genetic recombination with the chromosome of the recipient cell (Watson *et al.*, 1987). This discovery eventually led to the experiments that proved DNA to be the genetic material (Avery *et al.*, 1944).

Transformation occurs naturally in many species of bacteria and is a major means of genetic exchange. The process can be divided into three major steps: 1) development of competence (the ability to take up DNA), 2) binding of DNA, and 3) uptake and integration of DNA into the chromosome of the recipient cell (reviewed in Smith *et al.*, 1981).

Among transformable gram-positive species of bacteria, the ones that have been studied the most are *Streptococcus pneumoniae, Streptococcus sanguis, and Bacillus subtilis* (Smith *et al.*, 1981). The cells become competent naturally during growth, often when a critical cell concentration is reached. As competence develops, receptor proteins, DNA-binding proteins, and nucleases appear in the cell membrane. These proteins serve to bind the DNA and process it into single-stranded fragments which are taken up into the cell and integrated into the chromosome through a recombination event (Smith *et al.*, 1981).

In transformable gram-negative bacteria, *Haemophilus* and *Neisseria* species are the best known (Smith *et al.*, 1981). Competence development occurs naturally, as in gram-positive organisms; however, the exact conditions required vary between species. The uptake system in gram-negative bacteria is very selective for homologous DNA (Smith *et al.*, 1981). Although foreign DNA can bind to the cells, only DNA from the same or a closely related species will enter the cells. In contrast to gram-positive cells, the DNA is taken up as a double-stranded entity. Once it is inside the cell, one strand becomes incorporated into the host chromosome, while the other donor strand and the displaced host DNA are degraded and released into the medium (Smith *et al.*, 1981).

Transformation is an important process; nevertheless, many species of bacteria have not developed mechanisms by

which they can take up foreign DNA. These organisms can only be transformed artificially by treating the cells to make them permeable to DNA. Nearly all of the artificial transformation techniques have been developed because of their usefulness in genetic engineering and gene analysis (Smith *et al.*, 1981).

The gram-negative rod, *Escherichia coli*, is one of the best known examples of a bacterium with no natural capacity for transformation. Several different methods have been developed to introduce DNA into *E. coli*. These techniques were originally used to transfect *E. coli* with bacteriophage DNA in order to study phage genetics. Transfection refers to infection of a host cell by viral nucleic acid which is devoid of its coat (Benzinger, 1978).

One effective method for transfecting *E. coli* is to produce spheroplasts by treating the cells with lysozyme and EDTA. EDTA removes the lipopolysaccharide (LPS) from the outer membrane and exposes the peptidoglycan layer to lysozyme digestion (Benzinger, 1978). The resulting spheroplasts are permeable to phage DNA. This method is very efficient; that is, a large number of the spheroplasts become transfected (Benzinger, 1978). Spheroplasts, however, are osmotically fragile and the viability after transfection is less than one percent of the initial cell count (Benzinger, 1978). Spheroplasts also lose a few of the physiological activities of intact cells, which could make them undesirable for some applications (Benzinger, 1978).

Another method that has been used to obtain transfection of *E. coli* is to add DNA to the cells in the presence of intact "helper" phage. Helper phage are bacterial viruses that are mutated in an essential gene and thus have low plating efficiency for the host cells (Benzinger, 1978). Injection of the helper phage DNA and its presence in the cell are required for the incorporation of free DNA (Mandel and Higa, 1970). The transfecting DNA carries the gene that is missing in the helper phage, so the two DNA molecules must interact (usually by recombination) to form a plaque (Benzinger, 1978).

In one instance, the helper phage technique was used to transform a galactose-negative strain of *E. coli* to galactose-positive (Kaiser and Hogness, 1960). The transfecting DNA had been extracted from the transducing phage λ*dg*. Lambda *dg* is formed when the λ prophage is improperly excised from the *E. coli* chromosome. The locus for λ integration is closely linked to the genes controlling galactose metabolism (Lederberg and Lederberg, 1953), so lambda *dg* can "lift" (transduce) the galactose genes from the bacterium in which it is produced to the bacterium it infects (Morse, Lederberg, and Lederberg, 1956). In exchange, it loses some of the phage genes necessary for lysogeny. In order for lambda *dg* to form a lysogen, wild-type helper phage must be present to complement the missing region (Kaiser and Hogness, 1960). When galactose-negative cells are transfected with lambda dg

DNA in the presence of wild-type helper phage, the galactose genes are restored to the cells by the transfecting DNA and the cells are transformed to galactose-positive. This technique for transforming *E. coli* has limited usefulness because only the genes close to the λ attachment site are transduced.

Although little or no entry of transfecting DNA is observed in the absence of helper phage, the role of the helper phage in promoting DNA uptake was not known at that time (Mandel, 1967; Kaiser and Hogness, 1960). It had been found that when cells of *E. coli* were superinfected with helper phage they became sensitive to the antibiotic actinomycin due to an increase in the permeability of the cell wall (Mandel, 1967). However, it had not been determined whether or not the permeability change was responsible for DNA uptake (Mandel, 1967).

This question of the role of the helper phage led Mandel and Higa (1970) to investigate the effects of monovalent and divalent cations on *E. coli* cell wall permeability and its correlation with DNA uptake. As a result, they discovered that DNA from phages lambda and P2 could infect *E. coli* without the use of helper phage as long as calcium ions were present.

The method developed by Mandel and Higa twenty years ago is still used today with only slight modifications. It involves diluting an overnight bacterial culture 1:500 into

fresh medium and growing the cells at 37°C to a density of 1×10^9 cells/mL. The cells are then chilled, washed, and resuspended in half of the original culture volume of $CaCl_2$. They are stored on ice for twenty minutes, then washed and resuspended in one-tenth the original culture volume of cold $CaCl_2$. DNA in a volume of 0.1 mL is added to 0.2 mL samples of competent cells, chilled for 15 minutes, then incubated for 20 minutes at 37°C. At the end of the incubation, dilutions of the mixture are plated onto appropriate indicators. Using this method, 10^5 to 10^6 plaques are produced per microgram of DNA (Mandel and Higa, 1970).

The calcium chloride method for transfecting *E. coli* was more convenient than the spheroplast or helper phage preparations (Taketo and Kuno, 1974) and had the added advantage of allowing the cells to retain full viability (Benzinger, 1978). The method was soon applied to transfection with different types of phage DNA (Benzinger, 1978), but early attempts to transform cells with *E. coli* DNA met with failure (Mandel and Higa, 1970). It was not until two years later that Cohen, Chang, and Hsu (1972) successfully transformed *E. coli* to antibiotic resistance with purified plasmid DNA. The following year, Wackernagel (1973) and Cosloy and Oishi (1973) demonstrated transformation with linear chromosomal DNA.

The observation of Mandel and Higa that cells of *E. coli* could take up DNA in the presence of calcium ions proved to be a major development in molecular biology. The discovery in

the early 1970s of plasmid vectors that can replicate independently of the host chromosome, restriction endonucleases that cut DNA at specific sites, and a simple method to introduce DNA into *E. coli* gave biologists powerful tools for studying DNA. In the twenty years since it was developed, the calcium method for producing competent cells has become a cornerstone in recombinant DNA technology (Smith *et al.*, 1981).

Although the calcium method of Mandel and Higa is simple to use and has a wide variety of applications, the efficiency (the number of transformed cells produced per microgram of DNA) is relatively low and the role of calcium in promoting competence is poorly understood. For this reason, the method has been extensively studied since its introduction and, as a result, many of the conditions that influence transformation efficiency in *E. coli* have been worked out in detail.

First of all, the growth conditions of the cells are very important in transformation. Calcium-treated *E. coli* are most efficiently transformed when the culture is in the early log phase of growth (Humphreys *et al.*, 1978; Saunders *et al.*, 1987), with densities of 10^7-10^8 cells/mL giving the best results (Hanahan, 1983; Maniatus *et al.*, 1982).

The medium in which the cells are grown does not seem to affect transformation efficiency. A comparison of cells grown in various rich media (nutrient broth, tryptone and yeast extract, Casamino acids and yeast extract) shows no

significant difference in transformation efficiencies (Hanahan, 1983). Similarly, cells grown in nutrient broth, minimal media, and peptone meat-infusion are equally competent for transformation (Taketo, 1972). The presence of magnesium ions in the medium, however, does have an effect on transformation. When Mg^{2+} is limiting, transformation is inhibited (Jones et al., 1981), whereas the presence of 10-20 mM concentrations of the ion enhance transformation (Hanahan, 1983).

The second important factor in the transformation process is the requirement for calcium ions in the transformation mixture. E. coli cannot be transformed in the absence of divalent cations (Humphreys et al., 1978). Although calcium ions are the most effective at promoting transformation, studies with other divalent cations show that strontium, barium, or magnesium ions will also produce transformed cells, albeit at a much lower frequency (Humphreys et al., 1978; Weston et al., 1981). When monovalent or trivalent cations are substituted for calcium, no transformed cells are found (Humphreys et al., 1978).

Several ionic substances are able to enhance transformation efficiency when combined with calcium. These include Mg^{2+} (Taketo and Kuno, 1974), Mn^{2+} (Enea et al., 1975), Rb^+ and dimethyl sulfoxide (Kushner, 1978), and dithiothreitol and hexamine cobalt(III) chloride (Hanahan, 1983). Douglas Hanahan (1983) has outlined a set of conditions using these

substances in which transformation efficiencies can be improved one hundred to one thousand times over that obtained with calcium alone.

A third factor that can affect transformation is the nature of the incoming DNA. The number of transformed cells increases linearly with increasing amounts of added DNA until the system becomes saturated (Humphreys *et al.*, 1978; Weston *et al.*, 1981; Hanahan, 1983). At that point, addition of more DNA will not increase transformation efficiency (Humphreys *et al.*, 1978; Weston *et al.*, 1981). There is no requirement for specific sequences in the transforming DNA (Hanahan, 1983). The size of the plasmid, however, is important. Supercoiled plasmids transform better than their relaxed counterparts, and the probability that a plasmid will produce a transformed cell decreases with increasing plasmid size (Hanahan, 1983).

Temperature is another important parameter in transformation of *E. coli*. It is essential that the cells be kept cold (0-4°C) throughout the procedure (Mandel and Higa, 1970; Maniatus *et al.*, 1982). After the DNA is added to the cells, the mixture is subjected to a heat shock of 42°C for two minutes. It is thought that the DNA enters the cells during the heat shock (van Die *et al.*, 1983). An investigation of the temperature changes required during transformation shows that DNA enters the cells when a critical temperature range between 18-32°C is passed (van Die *et al.*, 1983). Because this is also the temperature at which membrane

lipids in *E. coli* undergo a phase transition from solid to liquid, it has been suggested that "melting" of the membrane lipids exposes channels that allow DNA entry (van Die *et al.*, 1983; Humphreys *et al.*, 1978).

In addition to revealing some of the conditions that influence transformation efficiency, studies have shown that the process of transformation in *E. coli* can be divided into three stages: adsorption of DNA, uptake across the cell membrane, and establishment of the DNA as a stable replicating element within the cell. The first two stages can be distinguished by their differing requirements for ions and temperature, as well as the susceptibility of the DNA to DNase in the transformation mixture (Humphreys *et al.*, 1978).

In stage I, DNA binds to the surface of the cell at 4°C. Calcium ions facilitate this association of DNA with the cell. When binding of radiolabelled DNA in the presence of different ions is compared, the following order of effectiveness is found: $Ca^{2+} \gg Ba^{2+} > Sr^{2+} > Mg^{2+}$ (Weston *et al.*, 1981). The efficiencies of these ions in promoting DNA binding correspond to their abilities to produce transformed cells (Weston *et al.*, 1981; Humphreys *et al.*, 1978). If DNase is added to the transformation mixture at any time during stage I, the number of transformed cells is reduced to 1% of the control without DNase (Humphreys *et al.*, 1978; Weston *et al.*, 1981). This indicates that the DNA is attached to the outside of the cell at this time (Humphreys *et al.*, 1978).

Stage II, the uptake of DNA, occurs during the heat shock step at 42°C (Humphreys *et al.*, 1978; van Die *et al.*, 1983; Weston *et al.*, 1981). Although divalent cations must be present for DNA binding to occur, they are not necessary for entry of the nucleic acid into the cell (Humphreys *et al.*, 1978). After adsorption of the DNA, the cells can be washed before stage II and no further addition of calcium or DNA is necessary for transformation to occur (Humphreys *et al.*, 1978). If DNase is added after stage II, the number of transformants is the same as the control that lacks DNase (Humphreys *et al.*, 1978; Weston *et al.*, 1981). Resistance to DNase suggests that the DNA has been taken into the cells (or at least across the outer membrane) during the heat shock (Humphreys *et al.*, 1978; Weston *et al.*, 1981).

In stage III, the transforming DNA is established as a replicating element within the cell and expression of plasmid-encoded genes begins (Humphreys *et al.*, 1978; Hanahan, 1983). This stage takes place during incubation of the cells at 37°C. Very little is known about the mechanism of establishment or the effect this stage may have in determining competence (Hanahan, 1983).

The reason for the inefficiency of calcium-mediated transformation remains a mystery. Several investigators have demonstrated that much more DNA is bound to the outside of a cell than is actually taken up (Weston *et al.*, 1981; Humphreys *et al.*, 1978). For example, binding studies of radiolabelled

DNA indicate that 80-100% of the DNA in a transformation mixture becomes cell-associated. After three washes with saline, however, only 10% of this DNA remains tightly bound to the cells, and less than 1% of the loosely bound DNA becomes DNase-resistant after the heat shock (Weston *et al.*, 1981). There is a linear relationship between the amount of DNA added to a transformation mixture and the amount that becomes tightly bound to cells. Moreover, the ability of cells to bind DNA does not become saturated with addition of increasing quantities of DNA (Weston *et al.*, 1981).

In contrast, the capacity to internalize DNA, indicated by the level of DNase-resistant binding, and the production of transformed cells both show saturation (Weston *et al.*, 1981). These data suggest that all cells in a population can bind DNA readily, but only a small minority can transport intact plasmid molecules to the interior of the cell and establish them as stable replicating elements (Weston *et al.*, 1981). In other words, either uptake or establishment of DNA is the limiting step in transformation. It is not known whether all calcium-treated *E. coli* can take up DNA, or only those cells that are destined to become transformed (Weston *et al.*, 1981). It is difficult to directly determine whether competence for transformation depends on uptake or on establishment of DNA because the proportion of competent cells in a population is so low and attempts to isolate the subpopulation of competent

E. coli cells have all failed (Humphreys *et al.*, 1978; Weston *et al.*, 1981).

Besides the problem of the low efficiency of the calcium method, the molecular basis for the binding, uptake, and establishment of plasmid DNA and the need for calcium ions in these processes is poorly understood. Calcium is known to be involved in a multitude of biological processes in eucaryotic cells. These include muscle contraction, neurotransmitter secretion, allosteric modification of enzymes, and intercellular signalling (Alberts, *et al.*, 1983). Many mechanisms have been proposed by which calcium may induce competence in *E. coli*. These can be grouped into three major categories: 1) stabilization of ionic interactions that facilitate DNA binding, 2) modification of membrane proteins to allow DNA uptake, and 3) reorganization of membrane lipids to increase permeability to DNA.

The first possible role of calcium in transformation is to foster the association of DNA with the cell surface. The interaction of a cell with exogenous DNA is basically the interaction of two complex polyanions: the DNA with its phosphate backbone, and the cell with its surface of phospholipid and lipopolysaccharide (Hanahan, 1983). The means by which calcium facilitates this unlikely association of two phosphate-rich structures is not certain (Hanahan, 1983). Divalent cations are known to shield phosphates and form stable coordination complexes with them (Sillen, 1964).

This could improve the interaction of DNA with the membrane. Calcium may also act as a chelating agent to remove molecules at the cell surface that block DNA entry, to neutralize charges that prevent DNA binding, or to bind the DNA and precipitate it onto the cell (Smith *et al.*, 1981).

It has been shown that calcium is more effective than other divalent cations at enhancing both DNA binding and the production of transformed cells (Weston *et al.*, 1981). This high degree of specificity for calcium suggests that its role in transformation of *E. coli* is more complex than merely the stabilization of ionic interactions (Humphreys *et al.*, 1978).

Cellular proteins are a second possible target for calcium action during transformation. Calcium has well-defined regulatory effects in many cell types. These effects are related to structural modifications in proteins (Alberts *et al.*, 1983). To induce transformation, calcium may affect the conformation of a membrane protein to permit DNA entry (Oishi and Irbe, 1977). When membranes of *E. coli* are treated with trypsin little DNA binding occurs, and treatment of whole cells with trypsin or pronase reduces transformation frequency (Weston *et al.*, 1981). This suggests that the protein portion of the outer membrane is essential for DNA binding (Weston *et al.*, 1981).

The third probable role of calcium in transformation is to reorient membrane lipids to increase the permeability to DNA. The membrane is normally in a fluid state in which lipid

molecules are free to rotate and diffuse. As the temperature is lowered, the membrane changes to a rigid gel state in which the lipids become closely packed and behave as a solid (Alberts *et al.*, 1983; Hanahan, 1983). DNA uptake has been found to occur when cells pass through a critical temperature range from 18°C to 32°C (van Die *et al.*, 1983). This is the same temperature at which the membrane lipids change phase (van Die *et al.*, 1983; Nichol *et al.*, 1980). The discontinuities which occur in lipid packing at the edges of solid and fluid areas of the membrane (Verkleij, 1975) could function as sites for DNA entry during transformation (van Die *et al.*, 1983). Calcium can enhance the phase transition of phosphatidylglycerol (Verkleij *et al.*, 1974) and lipopolysaccharide (van Alphen *et al.*, 1980), as well as inducing non-bilayer structures in the total lipids of *E. coli* (Burnell *et al.*, 1980; van Die *et al.*, 1983). Thus, the combination of calcium and low temperatures may produce phase transitions that compromise the integrity of the outer membrane and allow DNA to enter.

Calcium can also affect the lipopolysaccharide portion of the outer membrane. The *E. coli* cell envelope contains several hundred zones of adhesion, where the cytoplasmic and outer lipid bilayers are fused through holes in the peptidoglycan (Bayer, 1968; Mühlradt *et al.*, 1973). Complexes of LPS and membrane protein are organized around the zones of adhesion and seem to serve gate-keeping functions by

restricting the passage of molecules both into and out of the cell (Lieve, 1974; Osborn and Wu, 1980). LPS is partially held in place by binding to divalent cations (Lieve, 1974). Addition of excess calcium can shift the equilibrium of these interactions and reorganize the LPS, perhaps to orient it away from the channels it protects (Lieve, 1974; Hanahan, 1983). Moreover, preincubation in high concentrations of calcium can cause 35-45% of the LPS to be released from the outer membrane in some strains of *E. coli* (Lieve, 1974). Exposure of membrane channes by rearrangement of the LPS could provide a means for DNA to enter the cells.

It is obvious that calcium can have a multitude of effects on *E. coli*. Although the exact mechanism of calcium-mediated transformation is not known, it is most likely a combination of several of the previously mentioned possibilities. More research will have to be done to conclusively determine the means by which calcium ions promote transformation.

It has been shown that the limiting step in transformation is either uptake or establishment of the DNA (Weston *et al.*, 1981; Humphreys *et al.*, 1978), and that the mechanisms of these two processes are poorly understood. If factors could be found that affect the uptake and/or establishment of plasmids within *E. coli*, it should be possible to vary these factors to improve the efficiency of transformation. The purpose of this study was to further

characterize the transformation process by examining the effects of glucose, multiple heat shocks, and chelating agents on uptake and establishment of transforming DNA.

CHAPTER II

MATERIALS AND METHODS

Bacteria

E. coli K-12 strain RR1 (ATC 31343) was obtained from the American Type Culture Collection. It is a rec$^+$ derivative of *E. coli* HB101 with the genotype: F-, *hsdS20*(r^-_b, m^-_b), *ara-14, proA2, lacY1, galK2, rpsL20*(Sm^r), *xyl-5, mtl-1, supE44*, λ^- (Maniatus *et al.*, 1982). The cells were grown in LB medium (Difco) as described in Maniatus *et al.* (1982). For long term storage, 8.5 mL of an overnight culture was added to a sterile test tube containing 1.5 mL of sterile glycerol, mixed thoroughly, and frozen at -20°C (Maniatus *et al.*, 1982).

DNA

Plasmid pBR322 (4.4 kb) was first described by Bolivar *et al* (1977). It has the genetic markers Ap^r and Tc^r, and unique restriction sites for PstI, SalI, EcoRI, HindIII, and BamHI. The plasmid was obtained from the American Type Culture Collection in *E. coli* K-12 RR1 (ATC 37017).

Chemicals

Unless otherwise noted, all chemicals were from Sigma.

Purification of DNA

Plasmid DNA was extracted from the cells by the boiling lysis method of Maniatus *et al.* (1982). The bacterial cells from a 500 mL overnight culture were harvested by centrifuging at 4,000 x g for 10 minutes at 4°C. The pellet was washed in 100 mL of ice-cold STE buffer (0.1 M NaCl, 10 mM Tris-Cl [pH 7.8], 1 mM EDTA) and resuspended in 10 mL of STET (0.1 M NaCl, 10 mM Tris-Cl [pH 8.0], 0.1 mM EDTA, 0.5% Triton X-100).

The cells were transferred to a 50 mL Erlenmeyer flask and 1 mL of freshly prepared lysozyme solution (20 mg/mL in 10 mM Tris-Cl, pH 8.0) was added. Using a clamp, the flask was held over the flame of a bunsen burner with constant shaking until the liquid just started to boil, then immediately immersed in a 1 liter beaker of boiling water for 40 seconds. The flask was cooled by placing it in an ice-cold water bath for 5 minutes. The contents were then transferred to an ultracentrifuge tube and centrifuged at 17,000 x g for 15 minutes at 4°C.

The supernatant containing the plasmid was poured into a fresh tube and 0.1 volume of 2.5 M sodium acetate (pH 5.2) and 2 volumes of ice-cold 95% ethanol were added. The solution was kept at -20°C overnight to allow the DNA to precipitate. The next day, the sample was centrifuged at 12,000 x g for 10 minutes at 0°C and the supernatant discarded. The pellet was thoroughly dried and resuspended in 8 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNase-free RNase was added to a

final concentration of 10 μg/mL and the solution kept at room temperature for one hour. The DNA was again precipitated with ethanol and resuspended in 2 mL TE buffer.

To remove contaminating protein, an equal volume of phenol was added to the DNA solution and mixed to form an emulsion. The sample was centrifuged for 2 minutes in a microcentrifuge and the aqueous phase transferred to a fresh tube. The extraction was repeated with a 25:24:1 mixture of phenol/chloroform/isoamyl alcohol, then with 24:1 chloroform/isoamyl alcohol. After precipitating with ethanol, the DNA was resuspended in 1 mL of TE buffer. A portion of the DNA was diluted with TE buffer for use in transformation. Aliquots were stored at -20°C and used only once.

Quantification of DNA

A. Electrophoresis

Plasmid samples in buffer were loaded onto a 0.8% agarose gel and electrophoresed at 50-55 milliamps for 45 minutes. The electrophoresis buffer was 40 mM Tris acetate, 5 mM sodium acetate, 1 mM EDTA, pH 7.8. The loading buffer (10x) was 0.1% bromophenol blue, 50% glycerol, 1% SDS. Following electrophoresis, the gel was stained for 20 minutes in 1 μg/mL ethidium bromide, followed by 10 minutes destaining in water. The gel was photographed using Polaroid Type 667 film at f/8 for 30 seconds. The DNA concentration was estimated by comparing the intensity of the bands to standards (Hackett et al., 1988).

B. Spectrophotometry

Five μL of the purified plasmid was diluted to 1 mL with TE buffer and scanned from 200-300 nm with a Beckman DU-50® spectrophotometer. A cuvette containing TE only was used as a blank.

Transformation of *E. coli*

The transformation procedure was that of Maniatus *et al.* (1982). To prepare competent cells, 0.5 mL of an overnight culture was diluted into 50 mL of fresh LB broth. The cells were grown with vigorous shaking at 37°C to an OD_{600} of 0.08 (5 X 10^7 cells/mL), determined by a Bausch and Lomb Spectronic 20® spectrophotometer. The culture was transferred to a sterile centrifuge tube and chilled on ice for 10 minutes, then centrifuged at 4,000 x g for 5 minutes at 4°C. The supernatant was discarded and the cells gently resuspended in 25 mL of ice-cold, sterile transformation buffer (50 mM $CaCl_2$ and 10 mM Tris, pH 8.0). The tube was placed in an ice bath for 15 minutes, then centrifuged as above. The supernatant was discarded and the cells gently resuspended in 3.3 mL of transformation buffer (TFB). Aliquots of 0.2 mL were dispensed into prechilled microcentrifuge tubes and stored overnight (20-22 hours) at 4°C.

To transform the cells, 50 μL of TE buffer containing 40 ng of dissolved DNA was added to the 0.2 mL sample of cells and mixed gently with the pipet tip. The cells were stored on ice for 30 minutes, then transferred to a 42°C water bath for

2 minutes. One mL of prewarmed broth was added to each tube, followed by incubation at 37°C for one hour. Serial dilutions in LB media were prepared and 0.1 mL aliquots were spread on agar plates. Dilutions of 10^{-5} and 10^{-6} were plated on LB to count viable cells. The original sample and 1:10 dilution were plated on LB containing 50 μg/mL ampicillin to count transformed cells.

Effect of multiple heat shocks on transformation

Competent cells were prepared and the DNA added as described above. Samples were exposed to one, two, or three heat shocks at 42°C for two minutes, then diluted with LB broth and incubated for one hour at 37°C before plating. Between heat shocks, samples were returned to 0°C for fifteen minutes.

Effect of glucose on transformation

Competent cells were prepared and transformed as previously described using transformation buffer that contained either 0.5% or 1.0% glucose in addition to the calcium chloride and tris.

Effect of EDTA (ethylenediaminetetraacetic acid) on membrane permeability

A. EDTA treatment

The normal transformation procedure was followed for calcium treatment and addition of the DNA. After the cells had been on ice for 30 minutes, they were centrifuged for 20

seconds in a microcentrifuge at 4°C. The supernatant was discarded and the cells to be treated were resuspended in 100 μL of sterile, ice-cold 0.1 M Tris, pH 8.0, followed by 100 μL of EDTA. The control cells were resuspended in 200 μL of TFB. All tubes were transferred to a 42°C water bath for 2 minutes. One mL of broth was added to each tube and they were incubated at 37°C for one hour before plating.

B. EDTA pretreatment

An overnight culture was diluted and grown to an OD_{600} of 0.08 as in the normal transformation procedure. Ten mL of the culture was transferred to each of six centrifuge tubes and stored on ice for 10 minutes. Five of the tubes were centrifuged at 4,000 x g for 5 minutes at 4°C. The supernatants were discarded and the pellets resuspended as follows:

1) 9 mL of 5 mM EDTA and 1 mL Tris   (5 mM EDTA)

2) 1 mL of 5 mM EDTA and 9 mL Tris   (0.5 mM EDTA)

3) 0.1 mL of 5 mM EDTA and 9.9 mL Tris   (0.05 mM EDTA)

4) 10 mL Tris   (Tris control)

5) 10 mL LB medium   (0 EDTA control)

The sixth tube was the normal transformation control and was not subjected to any pretreatment. After storing the treated cells on ice for 2 minutes, all tubes were centrifuged as before. The pellets were resuspended in 5 mL TFB and kept on ice for 15 minutes. The cells were centrifuged again and resuspended in 0.66 mL TFB. Aliquots of 0.2 mL were dispensed

into chilled tubes, stored overnight, and transformed the usual way.

Effect of EDTA on cell recovery

Competent cells were prepared and transformed as usual. After the heat shock, 100 μL of 0.05 mM, 0.5 mM, or 5 mM EDTA and 0.9 mL of LB media were added to the treated cells. 100 μL of TFB and 0.9 mL of LB were added to the control cells.

Effect of other chelating agents on cell recovery

Competent cells were prepared and transformed as usual. After the heat shock, 1 mL of LB media containing either 1 mM EDTA, 1 mM EGTA (ethyleneglycol-bis-[ß-aminoethyl ether] N,N, N',N',tetraacetic acid), 1 mM phytic acid, or 1% wheat bran extract was added to the cells. The cells were incubated at 37°C for one hour and plated as usual.

Preparation of wheat bran extract

Twelve grams of Golden Harvest™ Miller's Bran® (from General Nutrition Center) was homogenized with 50 mL of 5% acetic acid in a Waring Blendor. After centrifuging at 5,000 x g for 5 minutes, the supernatant was transferred to a weigh boat and dried under a hood for 2 days. The dried extract was added to LB medium to a concentration of 1% and sterilized by filtration.

CHAPTER III

RESULTS

Quantification of DNA

The absorbance of the purified plasmid sample was measured at 260 and 280 nm. The ratio of OD_{260}/OD_{280} was 1.8, indicating that the DNA was acceptably free from contaminating RNA, protein, or phenol (Maniatus et al., 1982).

The concentration of the DNA sample was estimated from the intensity of the bands it produced following electrophoresis on a 0.8% agarose gel. The gel is shown in figure 1. The wells, from left to right, contained 10 μL of a 1:5 dilution of the DNA, 5 μL of the 1:5 dilution, 10 μL of a 1:10 dilution of the DNA, and 5 μL of the 1:10 dilution. The three bands nearest the top of the gel are three different conformations of the plasmid, which have distinct mobilities in agarose gels. The lowest of the three bands contains closed circular plasmids, the middle band is linear duplex plasmids, and the top band consists of nicked circular plasmids (Ausubel et al., 1987). The more diffuse, bottom band is digested RNA. Comparison to a known standard (Hackett et al., 1988) suggested a DNA concentration of 60 μg/mL, or 3,000 ng/50 μL.

To determine the optimal concentration of plasmid for transforming *E. coli* RR1, a dose-response experiment was performed using pBR322 purchased from Sigma as a standard. From the graph in figure 2 it can be seen that the maximum transformation efficiency was achieved with 30 ng of DNA in 50 μL of TE buffer. This suggests that the extracted DNA should be diluted 1:100.

Various dilutions of the extracted DNA were prepared and used for transformation. The results are found in figures 3 and 4. In two separate trials, a 1:75 dilution of the plasmid sample produced the most transformed cells. The DNA concentration in that dilution is 40 ng/50 μL. That concentration of DNA was used for all subsequent transformation experiments.

Effect of multiple heat shocks on transformation

In order to characterize the effects of temperature changes on transformation, cells were exposed to 1, 2, or 3 heat shocks at 42°C. Figure 5 shows that the control sample (exposed to one heat shock) had 9×10^7 viable cells/mL. The samples that were exposed to additional heat shocks each showed identical viability of 1×10^7 cells/mL.

The transformation efficiency of the control sample (expressed as transformed cells per μg of DNA) was 8.7×10^4 (figure 6). The transformation efficiency after two heat shocks dropped to 4.4×10^4 transformed cells/μg. After three

heat shocks, the efficiency was 7.2 x 10^4 transformed cells/μg.

Effect of glucose on transformation

Competent cells were prepared using transformation buffer that contained either 0.5% or 1.0% glucose. Data shown are the averages of three separate trials. When 0.5% glucose was used, the viability decreased from 5.3 x 10^7 cells/mL in the control to 3 x 10^7 cells/mL with glucose (figure 7). Transformation efficiency, on the other hand, was slightly increased from 7.8 x 10^4 cells/μg for the control to 8.6 x 10^4 cells/μg with glucose (figure 8).

When the glucose concentration was increased to 1%, the opposite effect occurred (figures 9 and 10). There was a minimal change in viability with glucose (4.5 x 10^7 cells/mL) as compared to the control (4.0 x 10^7 cells/mL). Transformation efficiency, however, was reduced from 7 x 10^4 cells/μg (control) to 3.7 x 10^4 cells/μg (glucose).

Effect of EDTA on membrane permeability

A. EDTA treatment

In order to determine whether EDTA can increase the permeability of competent *E. coli* to transforming DNA, cells were exposed to concentrations of 0.05 mM, 0.5 mM, or 5.0 mM EDTA during the heat shock. The control sample had 2.3 x 10^7 viable cells/mL (figure 11, average of 5 trials) and the transformation efficiency was 1.6 x 10^4 cells/μg DNA (figure

12, average of 2 trials). EDTA treatment increased the viability of the cells, but reduced the transformation efficiency at all concentrations as compared to control values.

A control transformation experiment was performed at the same time as the EDTA treatment to determine if centrifuging the cells after addition of the DNA would affect transformation. The viability of the cells was not changed (data not shown), however, the transformation efficiency was greatly reduced. The normal transformation procedure produced 5×10^5 transformed cells/μg of DNA, while the EDTA control showed only 8×10^3 cells/μg.

B.   EDTA Pretreatment

In order to eliminate the centrifugation step before the heat shock, the cells were exposed to EDTA prior to calcium treatment. Two controls were used in this experiment. One control was subjected to the standard transformation procedure and the other (0 EDTA control) was taken through the treatment procedure with LB broth as a substitute for EDTA.

As seen in figure 13, the two controls showed similar viability: 6.5×10^7 cells/mL for the normal transformation control and 5.8×10^7 cells/mL for the treatment control (average of 3 trials). The viability was reduced to 2.4×10^7 cells/mL with 0.05 mM EDTA, 5.7×10^6 cells/mL with 0.5 mM EDTA, and 3.3×10^4 cells/mL with 5 mM EDTA.

The transformation efficiency of the 0 EDTA control (6.3 x 10^4 cells/μg of DNA) was somewhat higher than that of the normal transformation sample (4.7 x 10^4 cells/μg), figure 14 (average of three trials). The cells treated with 0.05 mM EDTA gave the highest transformation efficiency of 7.9 x 10^4 cells/μg, even though the viability of this sample was much lower than that of either control. The other two EDTA-treated groups had very low transformation efficiencies: 5.2 x 10^2 cells/μg with 0.5 mM EDTA and 1.0 x 10^2 cells/μg with 5 mM EDTA.

Effect of EDTA on cell recovery

The ability of EDTA to protect cells by chelating calcium ions was tested by adding various concentrations of EDTA to the growth medium after transformation was completed. The control had 1 x 10^8 viable cells/mL (figure 15, average of two trials). Addition of EDTA reduced viability to less than 8 x 10^7 cells/mL. The transformation efficiency of the control was 6.3 x 10^5 cells/μg (figure 16, average of two trials). Addition of 0.05 EDTA reduced the transformation efficiency to 5.9 x 10^5 cells/μg and 0.5 mM EDTA decreased transformation to 3.5 x 10^5 cells/μg. Five millimolar EDTA increased the transformation efficiency to 6.7 x 10^5 cells/μg.

Effect of other chelating agents on cell recovery

The experiment described above using EDTA was repeated with three other chelating agents: EGTA, phytic acid, and

wheat bran extract. Data shown are the averages of three separate trials.

After transformation, cells were incubated in LB broth containing either 1 mM EDTA, 1 mM EGTA, 1 mM phytic acid, or 1% wheat bran extract. The viability of the control was 4.6 x 10^7 cells/mL (figure 17). The viability was not changed by incubation in EGTA (4.7 x 10^7 cells/mL) or phytic acid (4.3 x 10^7 cells/mL). EDTA, however, reduced viability to 3.9 x 10^7 cells/mL and wheat bran extract reduced it to 1.4 x 10^7 cells/mL. The control transformation efficiency was 1.2 x 10^5 cells/µg of DNA (figure 18). Phytic acid did not change the transformation efficiency (1.0 x 10^5 cells/µg). EDTA and EGTA caused a small decrease to 8.7 and 8.5 x 10^4 cells/µg, respectively. Wheat bran extract lowered transformation efficiency to 4.1 x 10^4 cells/mL.

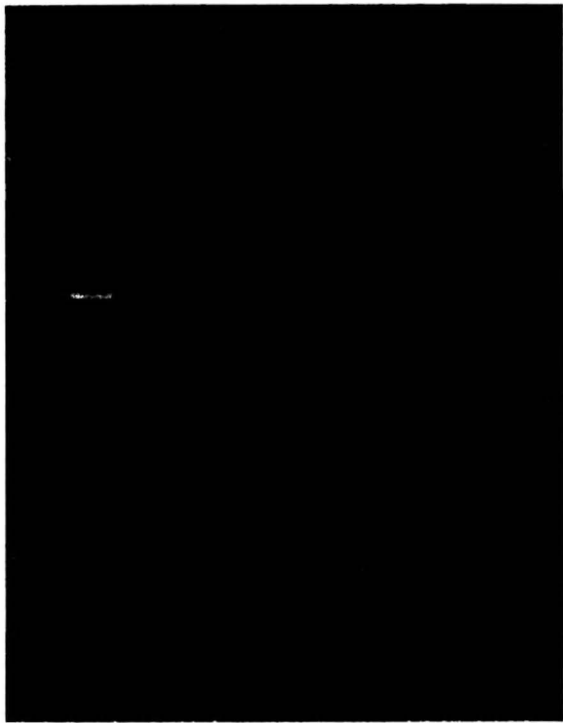Figure 1   Agarose gel electophoresis of plasmid pBR322

Figure 2   Dose-response curve with
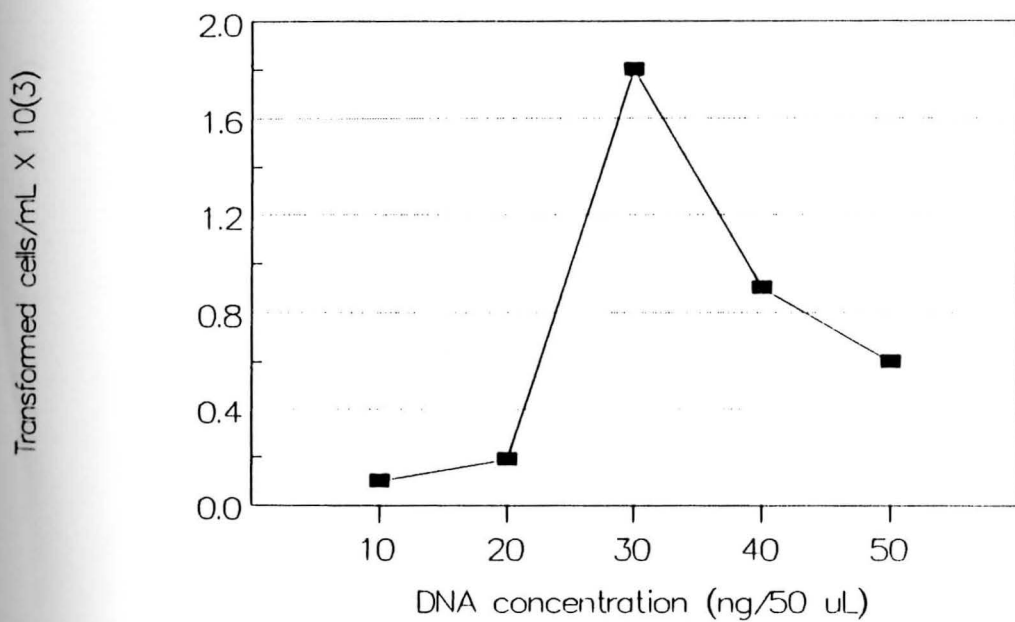## plasmid pBR322 purchased from Sigma

Figure 3   Dose–response curve with
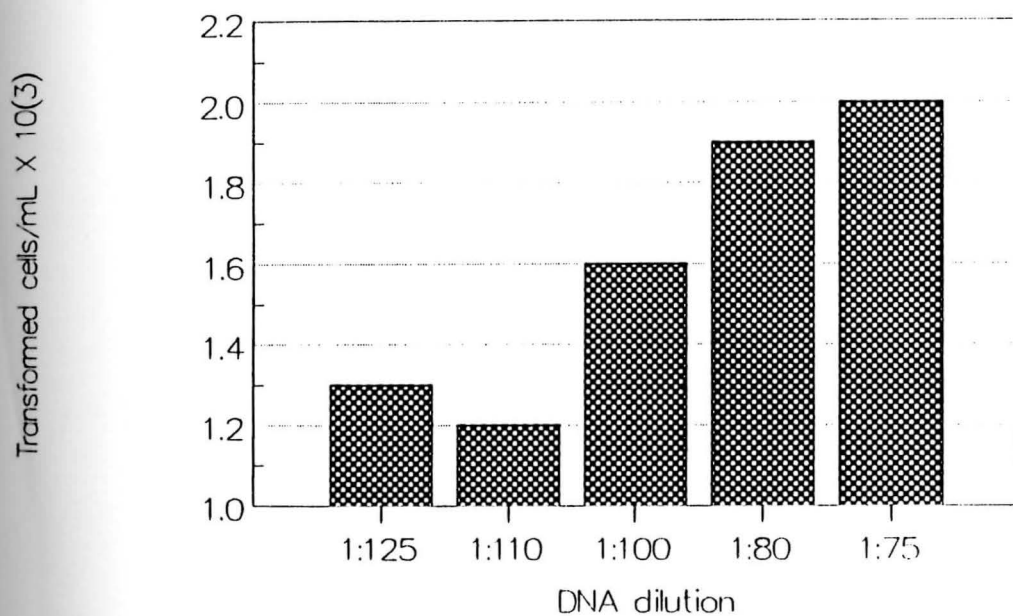
pBR322 extracted from E. coli RR1

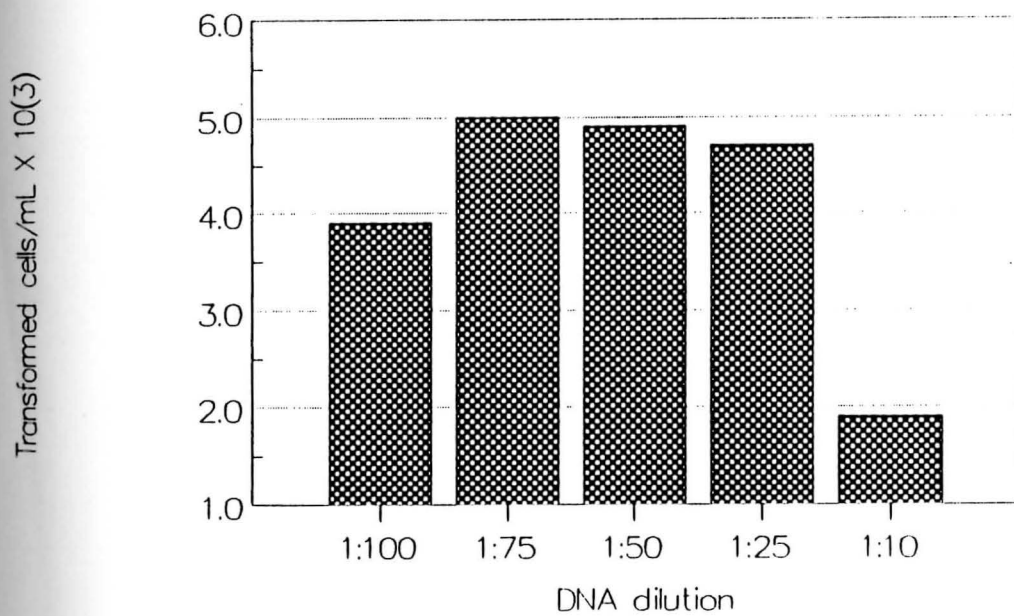Figure 4   Dose−response curve with pBR322 extracted from E. coli RR1

Figure 5 Viability after exposure

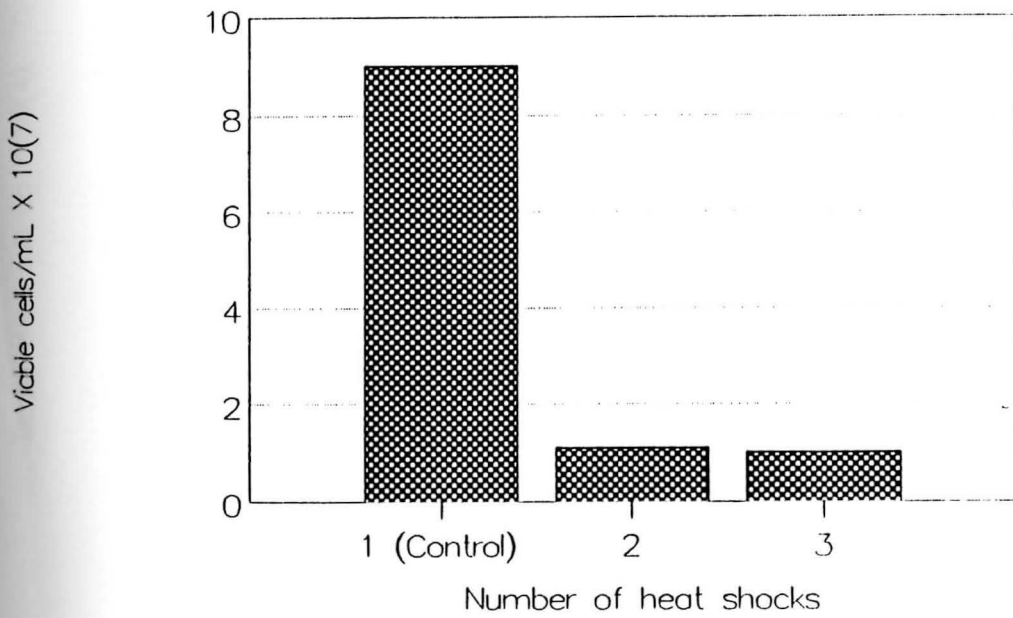to multiple heat shocks

Number of heat shocks

Figure 6   Transformation efficiency

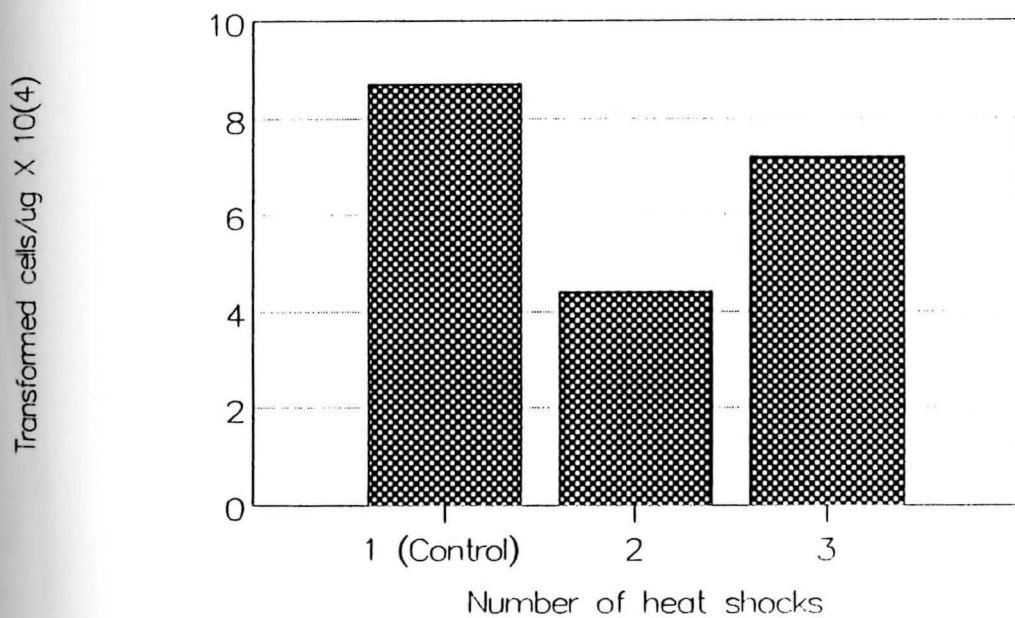after exposure to multiple heat shocks

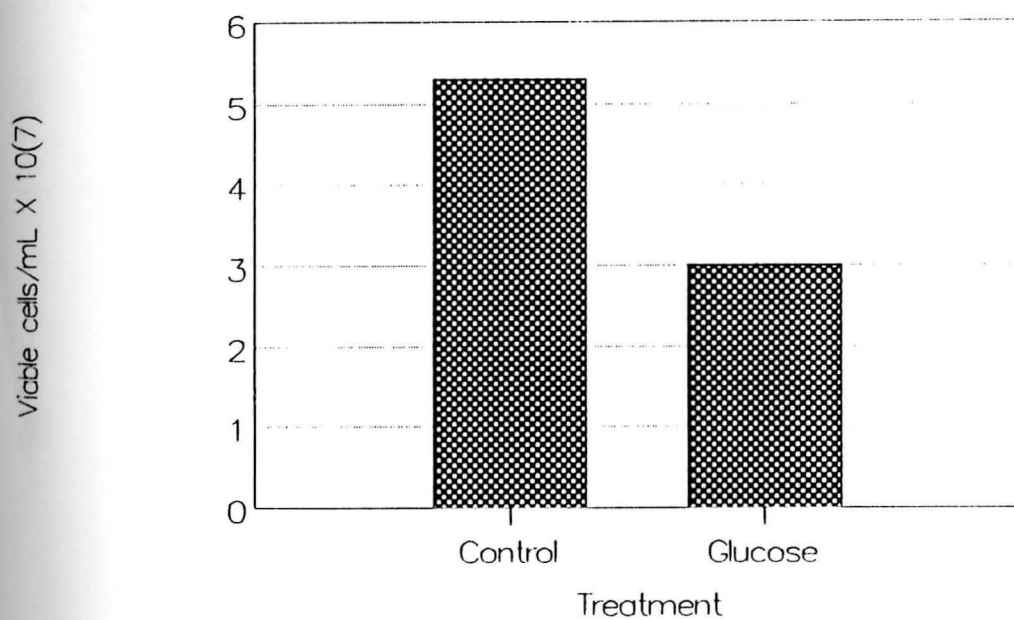Figure 7   Effect of 0.5%

glucose on viability

Figure 8   Effect of 0.5% glucose
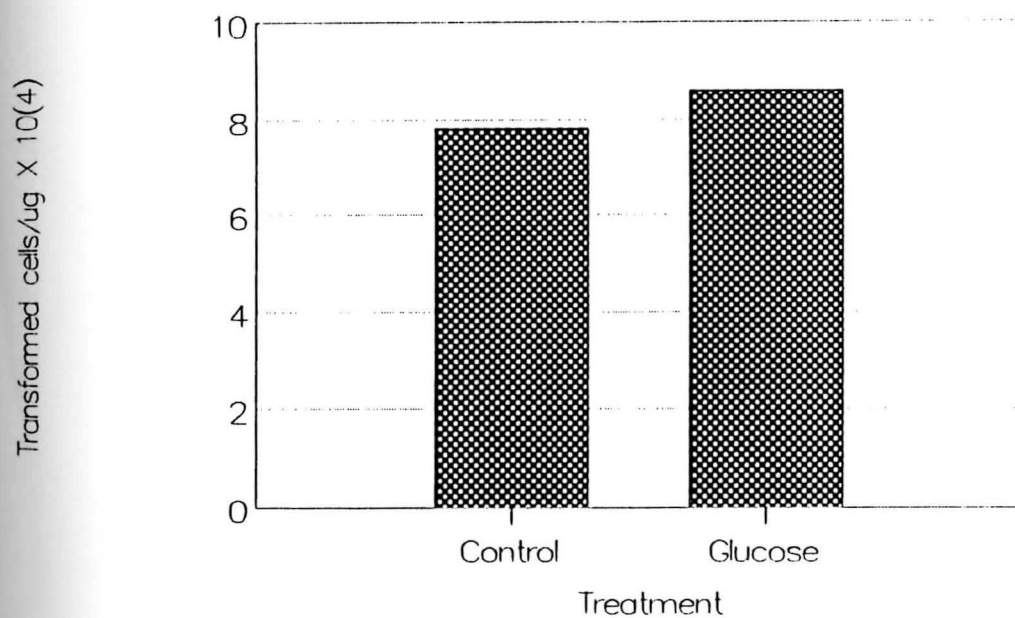
on transformation efficiency

Figure 9   Effect of 1.0%

glucose on viability

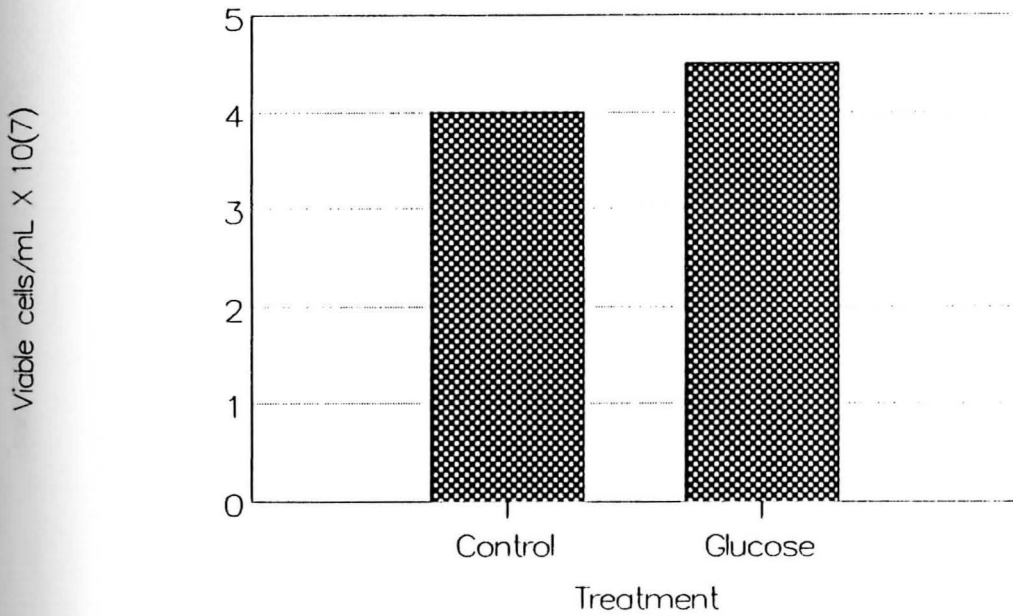Figure 10   Effect of 1.0% glucose

on transformation efficiency

Figure 11   Effect of EDTA

treatment on viability

Figure 12   Effect of EDTA treatment on transformation efficiency
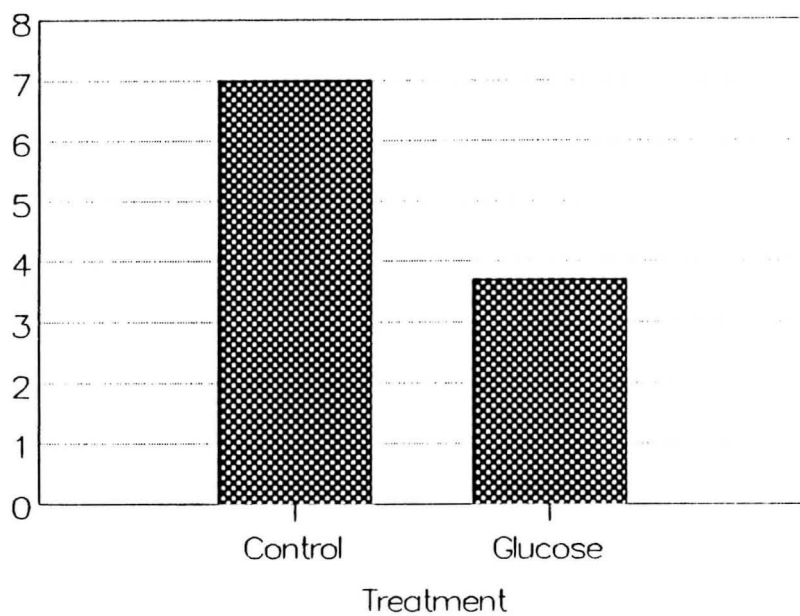
Figure 13 Effect of EDTA
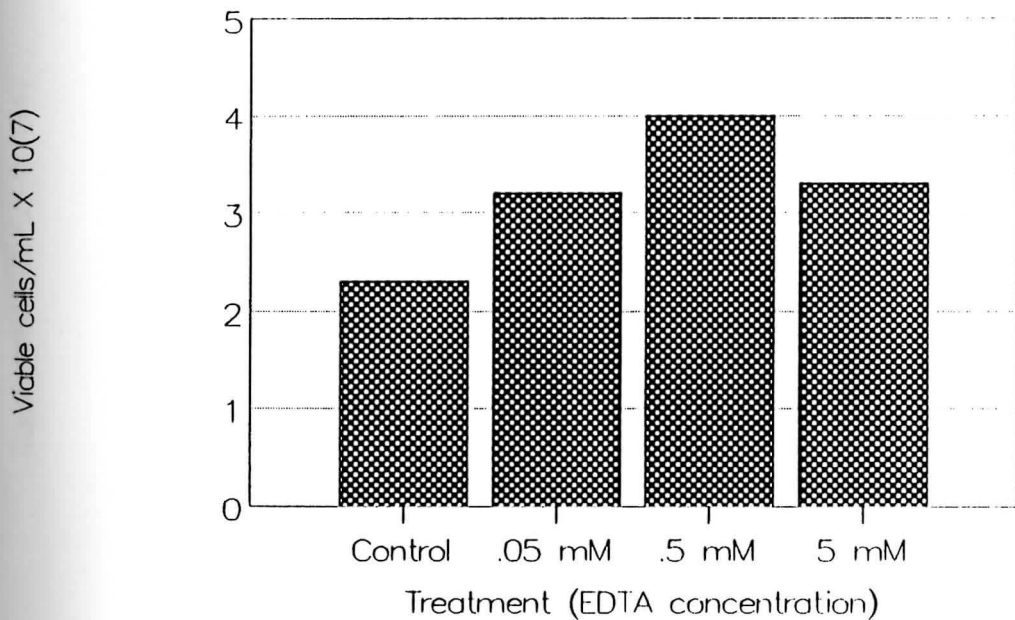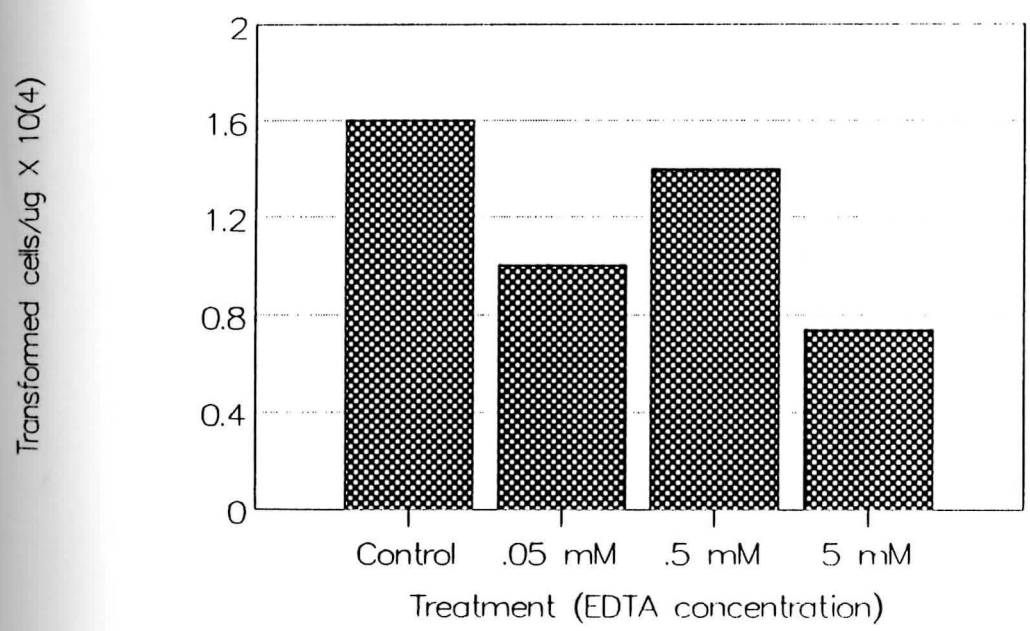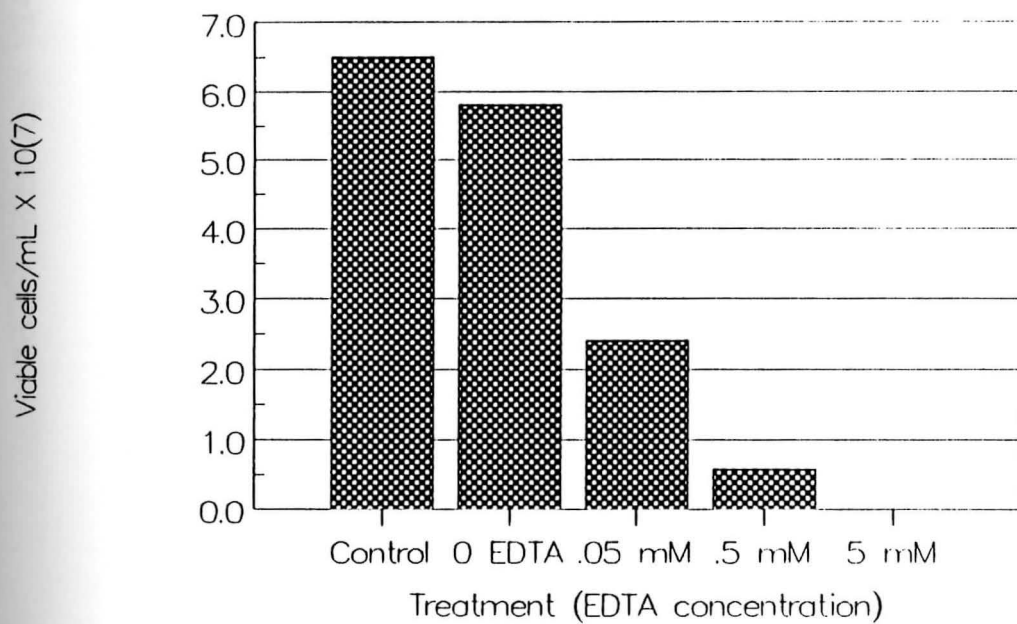
pretreatment on viability

Figure 14   Effect of EDTA pretreatment
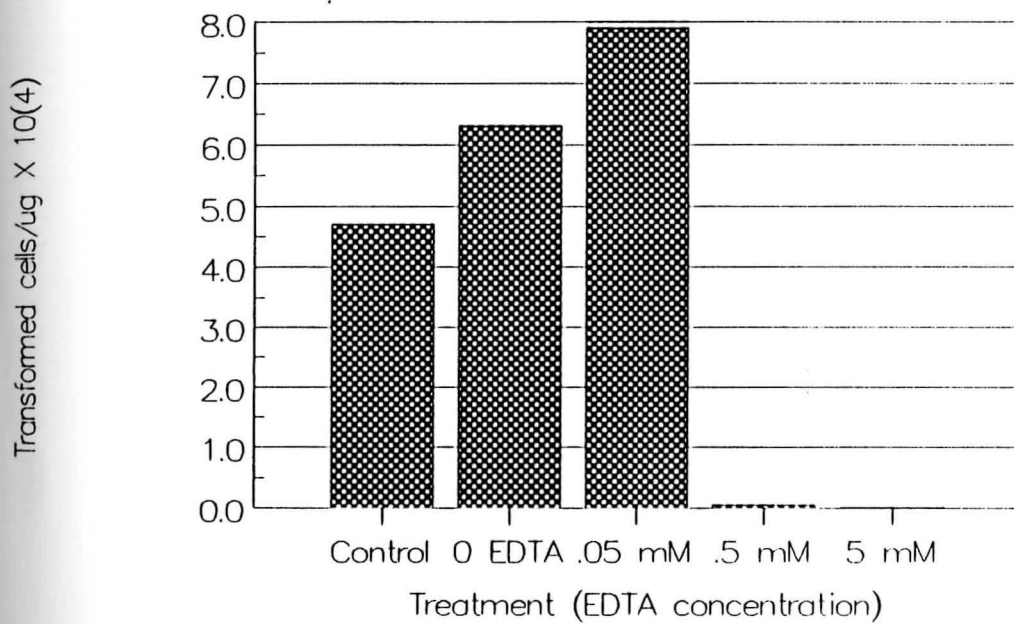
on   transformation efficiency

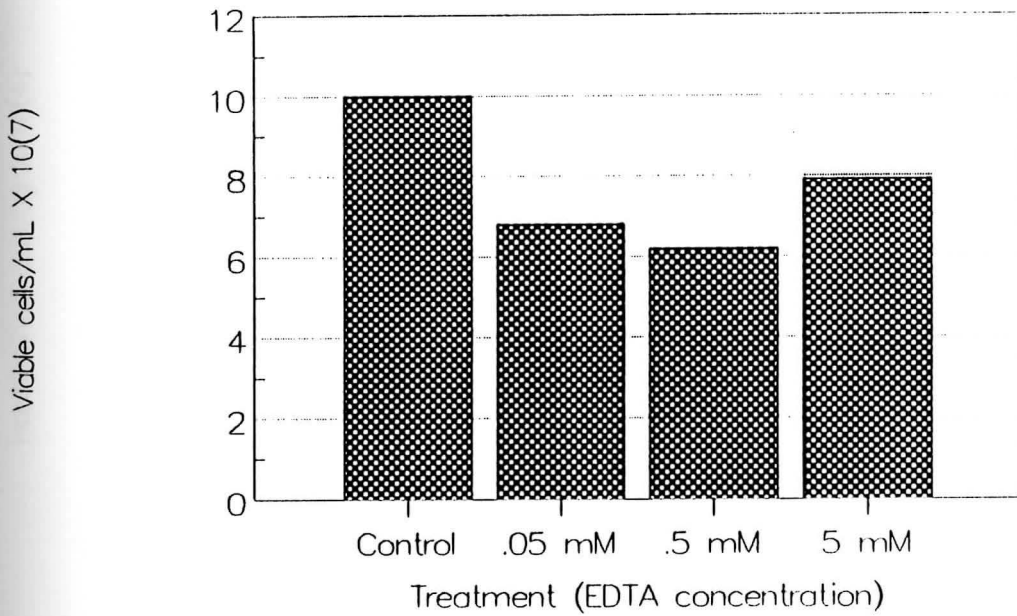Figure 15   Effect of EDTA in the
growth medium on viability

Figure 16   Effect of EDTA in the growth

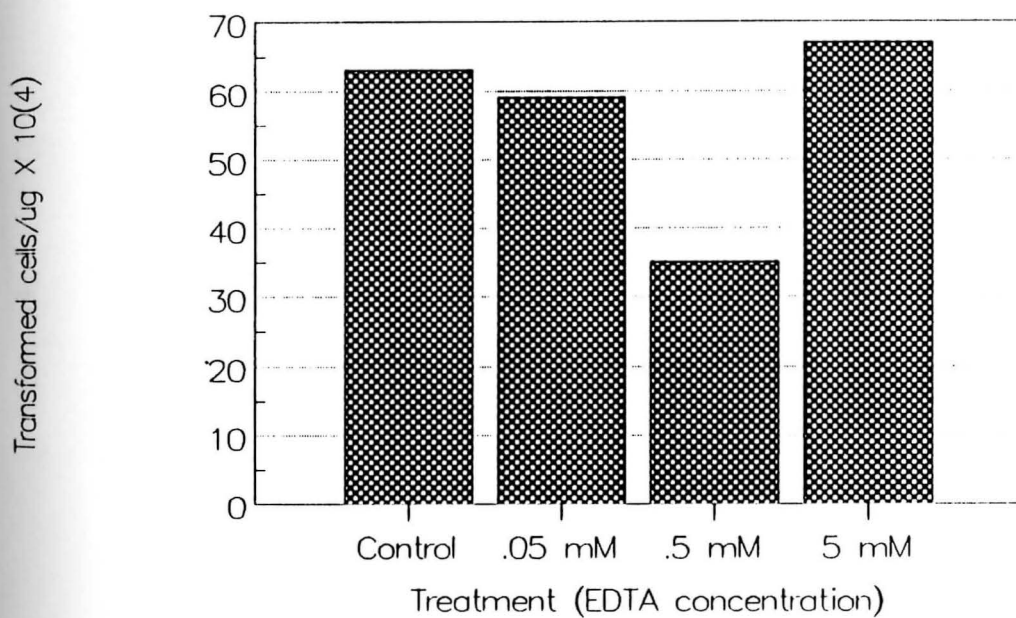medium on transformation efficiency

Figure 17   Effect of various chelating
agents in growth medium on viability
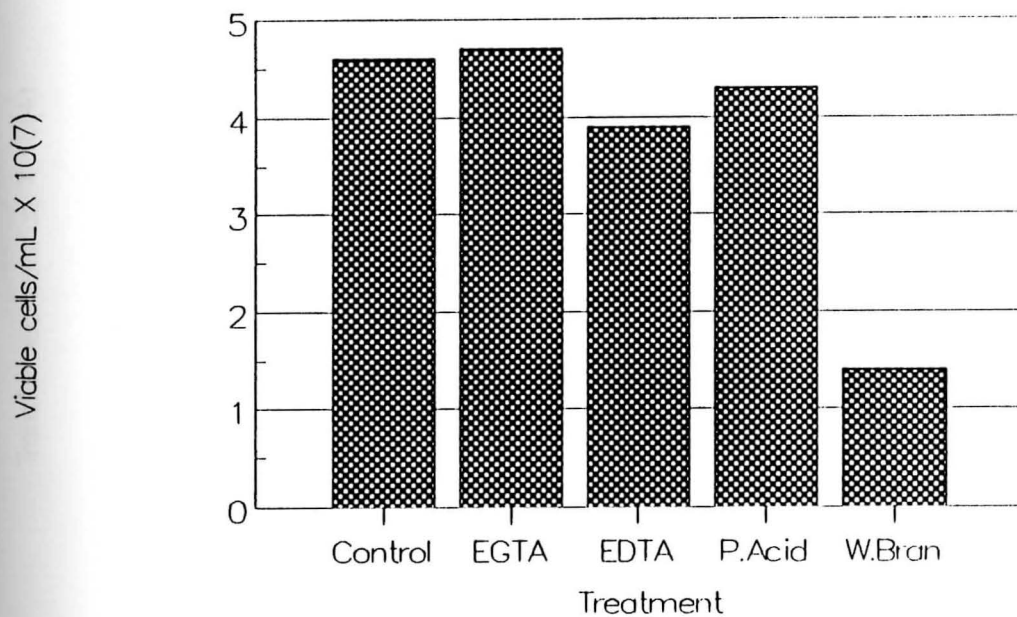
Figure 18 Effect of various chelating agents on transformation efficiency
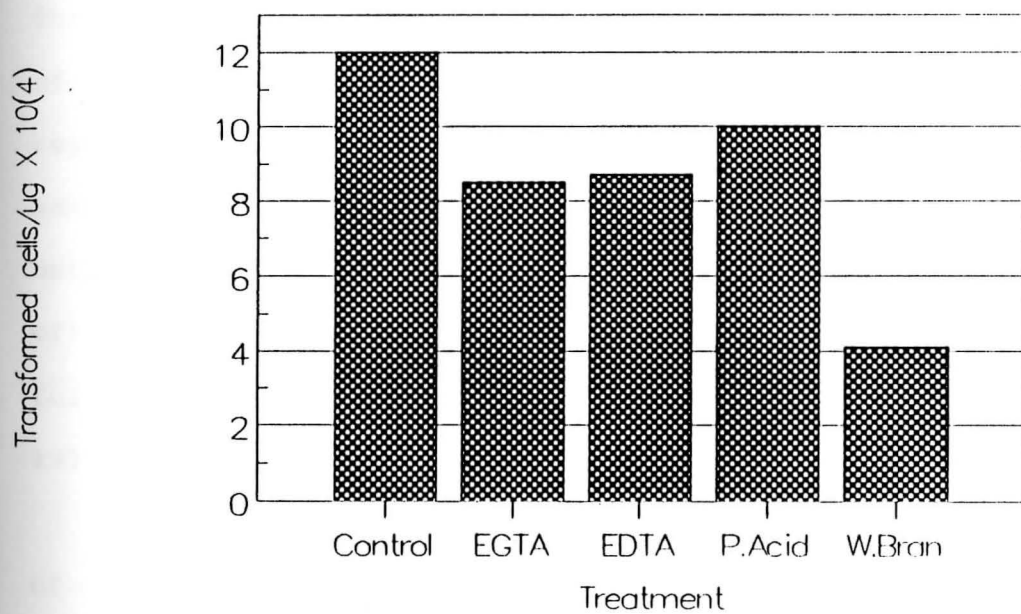
CHAPTER IV

DISCUSSION

Plasmid transformation of *Escherichia coli* is an essential step in many cloning experiments. The calcium method developed by Mandel and Higa (1970) provides a simple and inexpensive way to introduce recombinant DNA molecules from a variety of sources into *E. coli*. In spite of its ease and wide range of applications, the method is very inefficient. The number of transformed cells produced is never more than 1% of the viable cells in a sample (Weston *et al.*, 1981; Humphreys *et al.*, 1978). Because it is desirable to obtain the maximum possible number of transformed cells, the calcium method has been studied in detail in attempts to increase its efficiency. Nevertheless, there have only been a few successful improvements on the original method (Dagert and Ehrlich, 1979; Kushner, 1978).

Although the efficiency remains low and the exact action of calcium on the cells is not understood, the stages of the process of transformation in *E. coli* have been determined (Humphreys *et al.*, 1978; Weston *et al.*, 1981). In stage I, DNA binds to the surface of the cells at 0°C. During stage II, DNA is transferred into the cells at 42°C in a step that may or may not require energy (Humphreys *et al.*, 1978; van Die

49

et al., 1983). Stage III involves establishment of the DNA as a replicating unit within the cell at 37°C (Hanahan, 1983; Humphreys *et al.*, 1978; Weston *et al.*, 1981). Calcium may have an important role in each of the three stages of the process. It has been shown that calcium promotes DNA binding to the surface of the cells in stage one (Weston *et al.*, 1981); and in stage two, calcium ions are thought to affect the conformation of membrane lipids to allow DNA entry (Hanahan, 1983; van Die *et al.*, 1983). The role of calcium in establishment of the plasmid, if any, is not known at this time.

Studies with radiolabelled DNA show that cells of *E. coli* bind DNA readily and that the binding capacity of the cells does not become saturated with increasing amounts of DNA (Weston *et al.*, 1981). This indicates that DNA binding is not the limiting step in transformation (Weston *et al.*, 1981). Therefore, the low transformation efficiency of calcium-treated *E. coli* must be due to either the uptake or establishment steps. It is not known whether DNA is taken up only into transformable cells, or whether all cells can take up DNA but only a small proportion of these cells are capable of establishing the plasmid as a replicon (Weston *et al.*, 1981). For this reason, it is difficult to analyze uptake separately from establishment (Weston *et al.*, 1981).

If factors could be found that affect the uptake and/or establishment of plasmids within *E. coli*, it may be possible

to vary these factors to improve the efficiency of transformation. In this study, three different factors were analyzed for their ability to affect transformation efficiency: the number of heat shocks to which cells were exposed, the composition of the transformation buffer, and the presence of chelating agents, both during and after transformation.

The plasmid DNA used in the transformation experiments was extracted from a culture of *E. coli* RR1. To determine the optimal concentration of plasmid for transforming *E. coli*, dose-response experiments were performed using the extracted DNA and pBR322 purchased from Sigma. Comparison of the two DNA samples showed that 30 ng of Sigma DNA produced the highest number of transformed cells, whereas 40 ng of the extracted DNA gave the best transformation efficiency. The discrepancy is most likely due to differences in the purity of the two samples. The extracted DNA may be contaminated with fragments of chromosomal DNA that would make it less efficient in transforming cells than commercially available pBR322 DNA.

The first series of experiments were designed to examine the effects of multiple heat shocks on transformation efficiency. Uptake of DNA is thought to occur during the heat shock when the membrane lipids undergo a phase transition from solid to liquid (Weston et al., 1981; van Die et al., 1983). Discontinuities which occur in membrane lipid packing at the edges of solid and fluid domains could function as sites for DNA entry (Verkleij, 1975; van Die et al., 1983).

It would appear that if DNA is only taken up into a small number of cells during the transition from 0°C to 42°C, then repetition of the heat shock should enable some of the remaining cells to take up DNA and thereby increase transformation efficiency. To test this hypothesis, multiple heat shocks were administered to the cells. The viability of the samples after two heat shocks was reduced almost ten-fold and there was also a decrease in the transformation efficiency. It has been reported that leakage of ß-lactamase occurs when cells are exposed to a heat shock in the presence of divalent cations (van Die et al., 1983). This indicates that severe damage is inflicted on the outer membrane during the heat shock. Destruction of the outer membrane would explain the severe reduction in viability that was observed in these experiments after two or more heat shocks.

A comparison of the transformation efficiency and viability shows that both did not decrease at the same rate. A higher proportion of the cells that survived the second and third heat shocks were competent for transformation. This can be seen more clearly if transformation frequency is expressed as transformed cells per viable cell. One heat shock results in 3×10^{-5} transformants/viable cell, two heat shocks produces 1.2×10^{-4}, and three heat shocks 2.3×10^{-4}. Thus, the fraction of viable cells that become transformed increases with each heat shock. This would indicate that the non-competent cells are dying more rapidly than the competent

cells in response to multiple heat shocks. It has been has suggested that competent cells can receive outer membrane damage without losing viability (van Die et al., 1983). Therefore, it seems that exposing transformation mixtures to multiple heat shocks selectively increases the portion of the cells that is resistant to membrane damage and competent for transformation.

Although the multiple heat shocks increase the proportion of competent cells in terms of the total viable population, the net number of competent cells decreases. Thus, the use of multiple heat shocks has no inherent advantage over the use of one heat shock as a technique for producing transformed cells. However, the observation that there is an increase in the proportion of competent cells as the viability decreases may provide some insight into the mechanism of the transformation process.

A second factor that could affect uptake and establishment of plasmids within E. coli is the composition of the transformation buffer. Humphreys et al. (1978) have stated that transformation frequencies may be increased up to five-fold if glucose or sucrose is added to the transformation mixture. Specifically, the presence of 0.5% glucose in the transformation mixture gives a three-fold increase in transformation frequency (Humphreys et al., 1978).

In this study, addition of 0.5% glucose to the transformation buffer resulted in a 50% decrease in the viability

of the sample and a small increase in transformation efficien-
cy. This increase was considerably lower than the transfor-
mation efficiency reported by Humphreys *et al.* (1978).
However, the pattern of transformation was similar to that
observed for the multiple heat shocks. Addition of 0.5%
glucose, like the multiple heat shocks, seems to favor the
survival of those cells that are competent for transformation.

The experiment was also performed using 1.0% glucose in
the transformation buffer. The effect of this concentration
of glucose was opposite to that of the 0.5% glucose. The
number of viable cells was increased slightly with glucose,
while the number of transformed cells was reduced by almost
one-half. This observation is the reverse of the two just
discussed. A treatment that increased the number of viable
cells resulted in a decrease in the proportion of transformed
cells. These results suggest a pattern: treatments that
decrease viability increase the proportion of transformable
cells and treatments that increase viability decrease the
proportion of transformable cells.

A third factor that could affect transformation effi-
ciency in *E. coli* is the presence of chelating agents during
the transformation process. Chelating agents may be examined
for their effects on uptake or establishment of plasmid
molecules by adding them at the appropriate step of the
transformation procedure.

In the first series of experiments, the chelating agent EDTA was tested for its ability to increase the permeability of *E. coli* to transforming DNA. One of the proposed mechanisms for DNA uptake during transformation suggests that calcium ions reorient the LPS molecules in the outer membrane to expose channels for DNA entry (Hanahan, 1983; Humphreys et al., 1978). EDTA has been shown to cause an increase in the permeability of *E. coli* by releasing LPS from the outer membrane (Lieve, 1974). A method was developed to combine EDTA treatment with the transformation procedure to determine whether EDTA could enhance the action of calcium and allow DNA to enter more of the cells.

Originally, cells were centrifuged before the heat shock and resuspended in 0.05 mM, 0.5 mM or 5.0 mM EDTA. Controls were resuspended in transformation buffer. For all concentrations of EDTA, the viability of the treated cells was increased as compared to the control and the relative number of transformed cells decreased. This observation is similar to the pattern that was observed with 1% glucose in which an increase in viability was accompanied by a decrease in the proportion of transformable cells.

Incubating cells in high concentrations of calcium ions to induce competence subjects them to an unnatural stress that can reduce their viability (Hara et al., 1988; Saunders et al., 1987). The treated cells have had the calcium in the transformation buffer removed and replaced by EDTA for a short

time before being diluted into growth medium. Based on the observations of Hara *et al.* (1988), it appears that reducing the time that the cells are exposed to calcium ions and using EDTA to further decrease the concentration of calcium ions diminishes the stress on the cells and improves the viability of the sample.

Treatment with EDTA clearly increases the viability of cells and reduces the transformation efficiency. However, it was found that the centrifugation procedure alone resulted in a decrease in transformation, possibly by removing DNA from the cells. To avoid this problem, the cells were exposed to EDTA prior to calcium treatment. In each experiment, two controls were used to ensure that the procedure itself was not affecting either transformation efficiency or viability. One control was subjected to the standard transformation procedure and the other was taken through the treatment procedure with LB broth as a substitute for EDTA. Both controls showed similar viability. The transformation frequency of the treatment control was somewhat higher than that of the standard transformation control, so the procedure was not adversely affecting the experiment.

In this experiment viability was progressively reduced with increasing concentrations of EDTA. The loss of LPS from the outer membrane in the presence of EDTA is reversible (Lieve, 1974). Normally, cells are diluted into media and incubated after EDTA treatment. As the cells grow, the

permeability barrier is rapidly restored (Lieve, 1974). Here the cells were treated with EDTA, then chilled to 4°C and stored overnight in a calcium solution. Under these conditions, growth of the culture is retarded and repair of the LPS fraction of the membrane probably does not occur. Since calcium also has the ability to reorganize LPS (Hanahan, 1983), further disruption of outer membrane integrity can develop. The combination of EDTA treatment and exposure to high calcium concentrations could cause irreparable outer membrane damage that results in a significant loss in viability.

The transformation efficiencies of the 0.5 mM and 5.0 mM EDTA samples were very low because of the extreme loss of viability in these two cultures. The 0.05 mM EDTA sample had a very high transformation efficiency although its viability was lower than the controls. This observation is similar to the pattern observed with the other treatments; an induced decrease in viability results in an increase in the proportion of transformable cells.

One additional attempt was made to increase the transformation frequency. Following the heat shock, transformation mixtures are usually diluted with 1 mL of LB broth and incubated at 37°C for one hour before plating onto selective media (Maniatus et al., 1982). The purpose of this growth period is to allow for plasmid replication and expression of the plasmid-encoded antibiotic resistance genes (Maniatus et al., 1982). The processes involved in the establishment of a

plasmid within a cell, as well as the requirement for calcium ions during this stage, are not known (Hanahan, 1983). Although calcium is necessary for DNA binding and uptake, the high concentration of calcium ions present in the growth medium after transformation may actually impede plasmid establishment and cell recovery. Therefore, a series of experiments was performed in an attempt to protect the cells from the effects of the high calcium concentration.

It has been reported that incorporation of a chelating agent into the post-transformation growth medium to bind excess calcium ions improves transformation efficiency up to nine-fold (Hara *et al.*, 1988). The effect of EDTA on cell recovery was tested by adding 0.05 mM, 0.5 mM, or 5.0 mM solutions of EDTA to the LB broth after transformation. The viability of each EDTA-treated sample was lower than that of the control. The transformation frequency was practically unchanged by 0.05 mM and 5.0 mM EDTA, but notably reduced with 0.5 mM EDTA.

Thus, addition of EDTA to the recovery medium does not increase the transformation efficiency or the viability of calcium-treated *Escherichia coli*. Prolonged exposure to EDTA can be harmful to cells (Lieve, 1974), so the experiment was repeated using other chelating agents in addition to EDTA. The other chelating agents were EGTA, phytic acid, and a wheat bran extract. EGTA has a high affinity for calcium ions (Taketo and Kuno, 1974) and phytic acid is a chelating agent

found in wheat bran. Hara *et al.* (1988) have obtained a four-fold increase in transformation efficiency by incorporating phytic acid into the post-transformation growth medium, and a nine-fold increase by using a wheat bran extract that contains phytic acid.

In this study, phytic acid was found to have little effect on either transformation efficiency or viability, and wheat bran extract significantly decreased both. Some component of the wheat bran extract besides the phytic acid is obviously detrimental to cell growth after transformation since phytic acid alone has no effect. When EGTA was added to the medium, the transformation efficiency decreased slightly although the viability remained unchanged. EDTA reduced the viability and the transformation efficiency somewhat as it had done in the previous experiment.

Since neither phytic acid nor EGTA changed the viability from the control values, it can be concluded that the presence of calcium ions in the recovery medium has no effect on the growth of cells after transformation. The loss of viability seen in the EDTA sample could be due to the harmful effects of EDTA rather than the removal of calcium.

Although phytic acid did not affect transformation efficiency, EGTA and EDTA both reduced it. The decreased transformation efficiency of the EDTA sample could result from the reduced viability in that culture or from the action of EDTA on the cells. The EGTA-treated cells, however, had

reduced transformation efficiency with no loss in viability. This suggests that although calcium does not appear to affect growth or recovery of the cells, it may have a role in establishing plasmids within them.

In summary, two observations can be made concerning transformation in *E. coli*. First of all, there appears to be an inverse relationship between viability and transformation frequency. Treatments that decrease the viability of a sample, such as multiple heat shocks and 0.5% glucose, increase the proportion of transformed cells. On the other hand, factors which improve viability, such as 1.0% glucose and EDTA treatment before the heat shock, decrease the proportion of transformed cells. It seems that transfer of DNA into *E. coli* cannot occur unless the cells are stressed in some way. The standard transformation procedure subjects rapidly dividing cells to high calcium concentrations and temperature extremes, both of which cause outer membrane damage and a small loss in viability (van Die *et al.*, 1983; Humphreys *et al.*, 1978; Saunders *et al.*, 1987). Treatments that minimize or counteract this outer membrane damage will improve the viability of the sample; however, uptake of DNA will be reduced. Conversely, treatments that increase the degree of membrane damage will kill many of the cells, yet allow DNA to enter more of those that survive.

The second observation that can be made relates to the presence of calcium in the post-transformation growth medium.

Recovery and growth of the cells are not affected when the calcium is removed from the medium by chelating agents; however, the transformation efficiency is reduced. These results suggest that calcium may have a role in establishment or expression of plasmid genes within *E. coli*.

Whether or not a particular cell becomes transformed may be entirely due to chance. The extent of membrane damage probably varies from cell to cell. The cells that become transformed are those that receive enough damage to allow DNA entry but not enough to destroy the cell. Another alternative is that all calcium-treated *E. coli* can take up DNA; however, a large number of the cells are inviable due to the harsh conditions of the calcium treatment and only a small proportion of the survivors are capable of establishing or expressing the DNA (Sabelnikov and Domaradsky, 1981). It has been suggested that competent cells possess distinct properties that enable them to withstand membrane damage without losing viability (van Die *et al.*, 1983). If this is true, it would seem that competence in *E. coli* depends on the ability of a cell to establish DNA, rather than to bind it to the cell surface.

In conclusion, it is very likely that DNA uptake during the transformation procedure cannot be improved. Efforts to increase the permeability of *E. coli* to exogenous DNA reduce viability so much that the net number of transformed cells is always very low. As little is known about what happens after

uptake of DNA, future studies of transformation in *E. coli* should focus on the nature of the incoming DNA and the events surrounding its establishment.

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