ELECTROPORATION MEDIATED INSERTION OF pC194 INTO THE *PROPIONIBACTERIUM* GENOME

By

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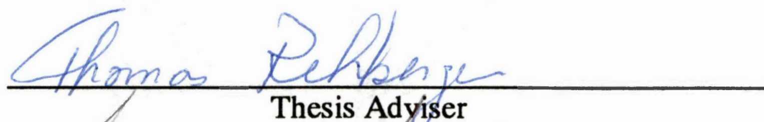

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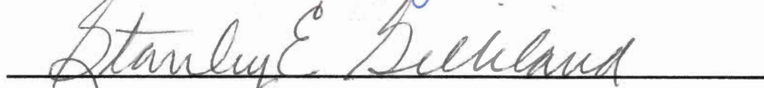

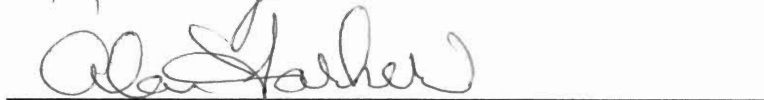

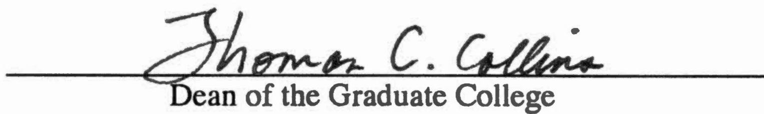

Dean of the Graduate College

ACKNOWLEDGEMENTS

This body of work is dedicated to the memory of Mr. Clovis H. Zirnstein and Mrs. W. Marie Zirnstein, my parents. I know they wanted to see me to the end of this project and I hope they somehow know it is finally finished. May they sincerely rest in peace.

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CHAPTER I

INTRODUCTION

Bacteria of the genus *Propionibacterium* are Gram-positive, pleomorphic rods that tend to clump into "Chinese character" arrangements under certain conditions. They are anaerobic to aerotolerant and are generally catalase-positive. Members of this genus can be divided into two groups of organisms that differ in their natural habitats. One group, primarily found on human skin, is referred to as the "acnes group" of propionibacteria. The second group is often referred to as the "classical propionibacteria" or the "dairy propionibacteria". Members of this second group of bacteria are found in cheese and dairy products, silage, fermenting olives and other natural habitats. Fermentation products of these bacteria include large amounts of propionic and acetic acids.

Due to the commercial importance of products produced by propionibacteria, it is desirable to know more about the genetics of these bacteria for future strain improvement. Only one group of researchers to date has reported being able to generate transformants of a *Propionibacterium* strain, an important first step to future genetic work with these bacteria. Unfortunately, workers in other laboratories have not been able to generate transformants using the published method or any other method, including protoplast transformation. Since the initial transformants were reported to have been produced by the use of a commercially available electroporation apparatus, we decided further work should include this method of molecular transfer.

The purpose of this investigation was to develop a method to generate transformants of propionibacteria that was repeatable, that used electroporation as the means of genetic transfer, and that would increase the transformation efficiency over that reported previously. The major components of the electroporation protocol that were to be studied in detail were; (1. optimium strain of *Propionibacterium* to electroporate, (2. electroporation buffer, (3. electrical field strength, (4. electrical pulse duration. A thorough study of plasmids that would replicate efficiently in strains of *Propionibacterium* was intended but was pre-empted by work related to the discovery that pC194 can insert into the *Propionibacterium* genome.

CHAPTER II

REVIEW OF LITERATURE

Gene Transfer in Propionibacteria

The development of gene transfer methods for genetic analysis and strain improvement in *Propionibacterium* has progressed slowly and few positive results have been reported. To date, only electroporation has been used successfully to transfer plasmid pGK12 into *Propionibacterium jensenii* (27). Low transformation efficiencies were obtained, about 3.2×10^1 transformants/μg of plasmid DNA, by electroporation with a field strength of 6.25 kV/cm and a capacitance of 25 μFarads (the actual pulse duration was not published, it is assumed to be between 3 and 20 milliseconds (msec) in length). Plasmid pGK12 was found to replicate autonomously in transformants. Other gene transfer methods such as naturally competent transformation, competence-induced transformation, protoplast transformation, conjugation, transfection, and transduction have been examined. Nevertheless, no reports of successful gene transfer in propionibacteria using these other methods have appeared in the literature.

Transformation occurs when exogenous DNA is taken up by a recipient cell, sphaeroplast, or protoplast and incorporated into either the chromosome or a plasmid by homologous recombination. Alternatively, the exogenous DNA may replicate as an autonomous plasmid. The ability of cells to take up DNA is called competence. The natural competence of certain bacteria is generally transient, occurring only during certain growth phases (usually towards the end of log phase) or after shifts in the nutritional status of the medium. This type of competence is associated with the

induction of the synthesis of various competence-specific proteins that may or may not be diffusible (communicable to other cells of its kind) (49). In some instances competence can be induced in cells that cannot undergo transformation under natural conditions. Whole cells of *Escherichia coli* and other Gram-negative bacteria can be made competent with high (millimolar) concentrations of $CaCl_2$, sometimes accompanied by heat shock. Their sphaeroplasts can be made competent by low concentrations of divalent cations (49). Dimethyl sulfoxide (DMSO), potassium 2-N-morpholinoethane sulfonate, RbCl, $MnCl_2$, hexamine cobalt (III) chloride, glycerol, and polyethylene glycol (PEG) are all chemicals normally found in competent cell preparation methods for *E. coli* (26). Gram-positive cells, which are often more difficult to transform, can first be treated with lysozyme to produce protoplasts and then mixed with polyethylene glycol in the presence of the transforming DNA. Transformed protoplasts can then be regenerated into viable cells (23, 49). Although a procedure for the production and regeneration of protoplasts of *Propionibacterium freudenreichii* has been developed, thus far, it appears that propionibacteria are recalcitrant to transformation by this method (3).

To date, conjugation has not been reported as a gene transfer mechanism in propionibacteria. Bacterial conjugation refers to a donor cell transferring DNA to a recipient cell while the cells are in physical contact. The donor characteristic is usually conferred to a recipient by the intracellular presence of a conjugative plasmid, although in some cases a transposon may mediate the conjugation (49). Conjugal transfer has been demonstrated in some group N streptococci in which lactose plasmids are transferred between strains (49, and references therein). Steele *et al.* (53) reported a Lac^+ plasmid, pKB32, that was conjugally transferred by cointegrate formation with plasmid pJS88 in *Lactococcus lactis* subsp. *lactis* 11007. The pKB32:pJS88 cointegrate formation and resolution by *rec*- independent mechanisms suggested to them the involvement of a transposable element.

Transformation of a bacteria by viral genomic DNA is termed transfection. Transduction, in contrast, occurs when a phage replicates in a donor cell and in the process some of its progeny virions encapsidate donor DNA (either chromosomal or plasmid). These progeny virions can then adhere to new host cells and transfer the donor DNA to the new cells (49). Raya *et al.* (44) demonstrated that phage ϕadh mediates plasmid transduction in *Lactobacillus acidophilus* ADH. The highest frequencies of plasmid transduction were observed for the small plasmids pC194 and pGK12, which were transferred as whole plasmids with no deletions or rearrangements (44). Unfortunately, no such system has ever been reported for the dairy propionibacteria.

The nearly complete lack of gene transfer systems for propionibacteria, except for the one reported case of transformation by electroporation (27), led us to expand on the initial success of the electroporation method in this study.

Electroporation as a Gene Transfer Method

Electroporation utilizes the fact that the cell membrane can act as an electrical capacitor that is unable to pass current, except through ion channels. Membranes subjected to high-voltage electrical fields temporarily break down, with the subsequent formation of pores in the cell membrane that are large enough to allow macromolecules (and smaller molecules such as ATP) to enter or leave the cell. The reclosing of the membrane is a natural decay process that can be delayed by keeping the temperature near 0° C. It is thought that the physical, rather than the biochemical, nature of electroporation is most likely to account for its wide applicability (42).

Flourescent Dye Studies of Membranes During Electroporation

Sowers and Lieber (52), in a study of the efflux of soluble, flourescent-tagged molecules out of erythrocyte ghosts loaded with flouresceinated dextran, found that an electrical pulse-induced loss of flourescence labels occurs in intervals. Large, immediate losses of the flourescent-tagged dextran molecules were seen over a very short time interval, followed by little or no loss in a longer time interval following the first, short interval. This was taken as evidence that the electropores created in the membranes of the erythrocyte ghosts open to some peak radius for a set amount of time and then quickly reseal. In addition, during the electroporation, a cloud of flourescence appeared *only* outside the hemispheres of the erythrocyte ghosts closest to the negative electrode. This indicated that more, larger, or longer-lived pores were produced in that hemisphere of the ghost. However, in a similar study by Mehrle *et al.* (35) of the permeability properties of the plasmalemma of oat mesophyll protoplasts, the application of a high intensity, short duration DC field pulse (1-2 kV/cm;20 μs duration) resulted in an immediate, distinct increase in flourescence which always started from that part of the protoplast directed towards the anode (positive electrode). It was first thought the discrepancy in the polarity may be due to experimental differences and differences in the membrane properties (52). However, a more careful analysis of the problem revealed that if the tracer molecule is inside the cell (52) then it can exit only through electropores in the hemisphere facing the negative pole; if the tracer molecule is outside the cell (35) then it can enter the cell only through pores facing the positive pole (12). The best hypothesis to date to explain this phenomenon of molecular motion is electroosmosis (12 and references therein).

Electroosmosis (electroendosmosis) is the movement of a charged fluid, relative to a fixed medium carrying the opposite charge, under the influence of an electrical

gradient. In the case of a negatively charged cell membrane, the charge on the membrane surface leads to redistribution of the ions in the solution, forming a layer of positive ions near the negatively charged surface of the membrane. The electrical field causes motion of the positive ions accumulated about the negatively charged cell membrane (which can be considered to have a fixed position in relation to the ions in solution), creating hydrodynamic flow. For biological membrane surfaces which are negatively charged, the hydrodynamic flow is directed towards the negative electrode and away from the positive electrode. The net ion movement causes a hydrodynamic flow of *all* molecules which are nearby in solution, with the flow even being strong enough to carry negatively charged molecules away from the attraction of the positively charged anode (12, 49). It is this mechanism which is now believed to be responsible for the successful movement of charged molecules (such as DNA and RNA) through the pores (created by electrically-induced membrane breakdown) and into the cell during an electroporation pulse. Diffusion and electrophoresis seem to be ruled out or reduced in importance as possible mechanisms for molecular transfer during electroporation due to their slow velocities of transfer and other reasons (12).

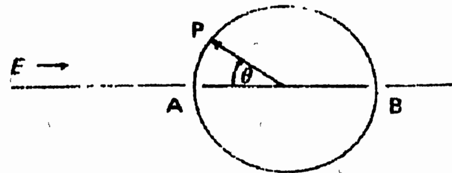
Membrane Permeabilization Relates Mathematically to Cell Size and Shape

The factors involved in permeabilization of a biological membrane can be expressed in mathematical terms. A spherical, membrane-bound particle of radius r (which can be either a cell or an intracellular organelle) will be exposed to a voltage difference V across the membrane at any point P if placed in a plane electric field of strength E as given by the following equation:

Equation 1 $$V = C\,r\,E\,(\cos\theta)$$

where C is a constant and θ is the angle made by point P relative to the direction of the field (Figure 1):

Figure 1



Vmax, the maximum voltage difference, will develop across the membrane when the cosine of θ = (+) 1 or (-) 1, i.e. the points on the sphere which are closest to the anode and the cathode (points A and B in Figure 1). This is in good agreement with the observation that pores have a tendency to form more readily at these points (12). The constant C is determined by the radius of the cell, the thickness of the membrane, and the relative electrical conductance of the membrane and the fluids within and outside the particle. This equation can be simplified. If the conductance of the membrane is much smaller than that of the internal and external fluid, and the thickness of the membrane is much less than the radius of the particle, then the value of C approaches a limit of 1.5 with the following result:

Equation 2 $$V_{max} = 1.5\, r\, E$$

Equation 2 makes it apparent that the value of Vmax for a given electrical field is determined by the radius of the particle (24). This implies that small bacteria such as propionibacteria would require electroporation field strengths greater than those for larger bacteria or eukaryotic cells in order to bring Vmax up to the threshhold level

necessary for pore formation in the membrane. It also implies that all of the cells in a culture need to be of a uniform size and shape to electroporate uniformly and at a high frequency (24).

Rate of Cell Lysis During Electroporation
Can Be Expressed Empirically

Several researchers have noted that high yields of transformants are usually achieved with electric field strength-pulse duration conditions which result in lysis of some of the cells (9, 13, 21, 43). This knowledge can be helpful in establishing successful electrical operating parameters by constructing a lysis/viability curve: percentage of cell survival versus field strength. Above a critical field strength, lysis can begin quite suddenly. If the intensity of the field during electroporation is excessive, cells may lyse due to failure of the membrane pores to reseal or osmotic swelling which leads to excess tension in the membrane. The relative rate of lysing can be expressed by the following empirical relationship (21):

Equation 3 $$L = 1 - (t/t_c)\exp[-(E - E_c)/K]$$

where L is the relative rate of lysing, t is the treatment time (the product of the pulse width times the number of pulses, in microseconds), t_c is the threshhold value for the treatment time in microseconds, E is the applied electric field strength in kilivolts per centimeter, E_c is the threshold value for the field strength in killivolts per centimeter, and K is a model constant. The species dependent constants are t_c, E_c, and K. Typical values for bacteria are: $t_c = 35\ \mu$sec, $E_c = 6$ kV/cm, K = 6.3 cm/kV (21). While constants for the above equation are generally not determined for their particular bacteria by most

researchers, it does serve to show how the major variables involved in cell lysis by electroporation are related to one another.

Applications of Electroporation to Bacterial Transformation

Bacteria Successfully Transformed by Electroporation

Numerous types of bacteria have been transformed by electroporation. Bringel *et al.* (8) compiled a long list of Gram-positive bacteria that have been reported to be transformable by electroporation; the list is reproduced below in Table I:

TABLE I

GRAM-POSITIVE BACTERIA TRANSFORMED BY ELECTROPORATION

Species	Reference
Bacillus amyloliquefaciens	Vehmaanpera (1989) FEMS Microbiol Lett 61:165-170
B. brevis	Takagi et al. (1989) Agric Biol Chem Tokyo 53:3099
B. cereus[a]	Shivarova et al. (1983) Z Allg Mikrobiol 23:593-599
	Belliveau and Trevors (1989) Appl Environ Microbiol 55:1649-1652
B. circulans	Lian-Ying and Wong (1984) Chin J Antibiot 9:450-454
B. sphaericus	Taylor and Burke (1990) FEMS Microbiol Lett 66:125-127
B. thuringiensis	Mahillon et al. (1989) FEMS Microbiol Lett 60:205-210
	Masson et al. (1989) FEMS Microbiol Lett 60:273-278

TABLE I (Continued)

Species	Reference
Brevibacterium lactofermentum	Haynes and Britz (1989) FEMS Microbiol Lett 61:329-333
Enterococcus faecalis[a]	Fiedler and Wirth (1988) Anal Biochem 170:38-44
	Luchansky et al. (1988) Mol Microbiol 5:637-646
Clostridium acetobutylicum	Oultram et al. (1988) FEMS Microbiol Lett 56:83-88
C. perfringens	Allen and Blaschek (1988) Appl Environ Microbiol 54:2322-2324
Corynebacterium glutamicum[a]	Wolf et al. (1989) Appl Microbiol Biotechnol 30:293-289
Lactobacillus acidophilus	Luchansky et al. (1988) Mol Microbiol 5:637-646
L. casei	Chassy and Flickinger (1987) FEMS Microbiol Lett 44:173-177
L. fermentum	Luchansky et al. (1988) Mol Microbiol 5:637-646
L. plantarum	Aukrust and Nes (1988) FEMS Microbiol Lett 52:127-131
Leuconostoc dextranicum	Luchansky et al. (1988) Mol Microbiol 5:637-646
L. lactis	Luchansky et al. (1988) Mol Microbiol 5:637-646
L. paramesenteroides	David et al. (1989) Appl Environ Microbiol 55:1483-1489
L. innocua	Luchansky et al. (1988) Mol Microbiol 5:637-646
Listeria monocytogenes	Luchansky et al. (1988) Mol Microbiol 5:637-646
Pediococcus acidilactici	Luchansky et al. (1988) Mol Microbiol 5:637-646
Propionibacterium jensenii	Luchansky et al. (1988) Mol Microbiol 5:637-646
Staphylococcus	Augustin and Gotz (1990) FEMS Microbiol Lett 66:203-208
Streptococcus cremoris	van der Lelie et al. (1988) Appl Environ Microbiol 54:865-871

TABLE I (Continued)

Species	Reference
S. lactis[a]	Harlander (1987) In: Ferretti and Curtiss III, Streptococcal genetics, 229-233, American Society for Microbiology, Washington, DC Powell et al. (1988) Appl Environ Microbiol 54:655-660
S. pyogenes	Suvorov et al. (1988) FEMS Microbiol Lett 56:95-99
S. sanguis Challis	Somkuti and Steinberg (1989) Curr Microbiol 19:91-95
S. thermophilus	Somkuti and Steinberg (1988) Biochimie 70:579-585
Streptomyces lividans[a]	MacNeil (1987) FEMS Microbiol Lett 42:239-244

[a] When protoplasts or lysozyme-treated cells were electroporated

While the above list is not entirely complete, it does demonstrate the rapid increase in the use of electroporation for the transformation of Gram-positive bacteria within the last three to four years. Shivarova *et al.* (47) were the first researchers to use electroporation with Gram-positive bacteria. *Bacillus cereus* protoplasts were given three 5 μs pulses of 14 kV/cm, increasing the transformation frequency over the older PEG-induced protoplast transformation method. The earliest report encountered of electroporation of *intact* bacterial cells was by Harlander (18). She was able to transform intact cells of *Streptococcus lactis* to an efficiency of about 10^4 transformants/μg of DNA. While improving the protocols for the electroporation of Gram-positive bacteria is gradually increasing transformation frequencies for these bacteria it will probably be some time before a majority of strains will be able to match the high transformation

efficiencies, consistently 10^9 to 10^{10} transformants/ug of DNA, seen for *Escherichia coli* strains (14). Other Gram-negative bacteria reported to have been successfully electroporated are *Salmonella typhimurium* (39), *S. typhi* (39), *Vibrio cholerae* (31), *Pseudomonas aeruginosa* (13, 16), *P. putida* (17), *Campylobacter jejuni* (37), *Bacteriodes ruminicola* (55), *B. uniformis* (55), *Bordetella pertussis* (59) and, *B. parapertussis* (59).

Factors that Affect Transformation by Electroporation

Effect of Field Strength

Many researchers have found that as the field strength (kV/cm) of the electroporating pulse was increased the transformants/μg of plasmid DNA and the percentage of cells killed also increased (9, 13, 43). On the other hand, an increase in field strength may lead to a decrease in transformation efficiency (so long as the pulse length is held constant) due to excessive cell death (30, 32, 51). Some researchers suggest that a point on the survival curve (variation of viable cells with increasing field strength) where a significant amount of kill is noted may be a good point to expect efficient electroporation (10, 29). In contrast, separate studies of *Bordetella pertussis* (59) and *Campylobacter jejuni* (37) showed no statistically significant reduction in cell viability even at the highest field strengths applied, which were 25.5 kV/cm and 12 kV/cm respectively. This suggested that, for these strains at least, lethality is not necessarily linked to efficient transformation. It should also be noted that the field strength necessary for maximal transformation varies from strain to strain (10).

Occasionally, an apparent increase will be seen in the numbers of surviving colony forming units with increasing field strength. This was observed with *Lactobacillus casei* which grows in chains of 10 to 20 cells, and with *Lactobacillus*

plantarum. It may be that the apparently higher numbers resulted from electroporation-induced separation of cells rather than a net increase in the number of viable cells (8, 10).

Effect of Pulse Duration

Pulse duration is another key element in the efficient electroporation of bacteria. Intact cells of *Lactococcus lactis* subsp. *lactis* were electroporated at an electric field strength of 17 kV/cm and a pulse duration of 5 ms to obtain the highest transformation rate with a BTX Transfector 100 electroporation unit (33). Other investigators, using a Bio-Rad Gene-Pulser apparatus with a Hewlett Packard Co. digitizing oscilloscope to measure the pulse duration, found that a pulse length of from 3.5 to 5.0 ms worked well within a range of field strengths from 3.75 to 6.25 kV/cm (43). Still another group using the Gene-Pulser found that the optimal transforming conditions for *Bacillus thuringiensis* was a single pulse of 4.7 ms at a field intensity of between 8.75 and 10 kV/cm (32). Intact cells of *Corynebacterium glutamicum* were found to transform most efficiently with an extremely short pulse duration of 450-500 μs at a high field strength of 35-40 kV/cm (57).

In some cases research has revealed a compensatory relationship between the optimal pulse duration and the field strength. The relationship between these two factors is considered to be inverse (5, 14, 37, 41). This can lead to a region of pulse length-field strength settings with similar abilities to transform.

Effect of Multiple Pulses

While some researchers report increased transformation efficiency using multiple pulses (5, 17) others find no increase in transformation efficiency (59) and some even see a decrease in transformation efficiency (54). It was apparent that in some cases the

transformation efficiency only dropped with multiple pulses because the sample was not cooled between pulses, resulting in temperatures high enough for cell death (17, 43).

Shape of the Pulse Waveform

Two types of waveforms are commonly used for electroporation: square and exponential decay. Of the two, the exponential decay waveform is used most often. A square wave is produced by rapidly increasing the voltage to the desired amplitude, holding the specified voltage for a controlled time (pulse width), and quickly dropping the voltage to zero, giving the pulse its square shape. Exponential decay waveforms, on the other hand, are generated when the charge from a capacitor is directed to a sample placed between two electrodes; the voltage across the electrodes is raised rapidly to a peak voltage (known as the initial voltage, V_O), and declines immediately in a smooth curve downwards (exponential decay). The pulse length is defined as the time (in seconds) it takes for the voltage to decline to 1/e, or approximately 37% of the peak value of the voltage. The voltage gradient between the electrodes, i.e. the electric field (E) is described by:

Equation 4 $$E=V/d$$

where E is measured in kV/cm, V is the voltage in kV, and d is the distance between the electrodes in cm. The time constant is defined as:

Equation 5 $$\tau=RC$$

where τ is measured in seconds, R is the resistance in the system in ohms (Ω), and C is the capacitance in farads (F). Larger capacitors require longer times to discharge through a given resistance and any given capacitor will discharge more slowly through a higher

resistance. The time constant (or pulse duration) can be adjusted by changing the size of the capacitor in the circuit, by changing the resistance across the sample chamber, or (if available with the given equipment) by selecting a different size of parallel resistor (40).

Although the bulk of experimentation with electroporation up to this point has been performed with exponential decay equipment, there are at least two reports in the literature that indicate higher numbers of transformants were obtained with apparatus generating a square pulse (8, 15).

Electroporation Buffers

The ionic strength of the buffer used to suspend cells during electroporation affects the pulse length and the transformation efficiency. Using a BTX Transfector 100 adjusted for a theoretical pulse length of 5 ms, a field strength of 17 kV/cm, and a *Lactobacillus lactis* subsp. *lactis* cell suspension, buffers of different ionic strength were compared. The actual pulse length was found to vary from 0.60 msec with a highly conductive buffer (0.5 M sucrose, 1 mM $MgCl_2$, 7 mM K_2HPO_4-KH_2PO_4, pH 7.4) to 5.53 msec with a suspending solution of low electrical conductivity, like double distilled H_2O (ddH_2O). Transformation efficiency had a tendency to decrease as the voltage was increased with the highly conductive buffers but the low conductivity of ddH_2O allowed for a large increase in electroporation efficiency (33). In contrast, a study by Brigidi *et al.* (7) showed an increase in transformation efficiency with increasing buffer strength up to 1 x concentration using HEB (272 mM sucrose, 1 mM $MgCl_2$, 7 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), pH 7.4) and PEB (272 mM sucrose, 1 mM $MgCl_2$, 7 mM potassium phosphate, pH 7.4) to electroporate *Bacillus subtilis* cells. Past 1 x concentration of PEB and HEB there was a significant decrease in transformation efficiency. Oddly, at the 1 x concentration it was the more conductive PEB buffer that gave higher transformation efficiencies over HEB (7). Bonamy *et al.* (5)

were able to achieve high transformation efficiencies (more than 10^7 transformants/μg DNA) with corynebacteria strains using 10% glycerol as the electroporation medium. Dower *et al.* (14) were able to transform *E. coli* up to 10^{10} transformants/μg DNA with the same medium. Liebl *et al.*(25) found 10% glycerol gave three times the number of transformants compared to 10% sucrose when used to wash and electroporate *Corynebacterium glutamicum* cells. Haynes *et al.* (19) had success with a slightly higher level of glycerol, 15%. Chassy and Flickinger (10) used a HEPES buffer (7 mM HEPES, 272 mM sucrose, 1 mM $MgCl_2$, pH 7.4) to achieve electroporation in the 10^4 range of efficiency with cells of *Lactobacillus casei*. Cells of *Rhodococcus fascians* were transformed to an efficiency of 10^3-10^4 CFU/μg of DNA using water as the electroporation medium but a 3- to 10-fold increase in transformation efficiency was seen with the use of a 30% solution of polyethylene glycol with a molecular weight of 1000 (PEG 1000) (11). An electroporation medium of 25% (w/v) PEG (MW 6,000) was found to significantly increase the transformation, up to 10-fold, of *Lactobacillus casei* IAM1045 with plasmid pAMβ1 - 1 DNA (38). Mahillon *et al.* (30) found that omitting PEG from the electroporation suspension decreased the transformation frequency of all *Bacillus thuringiensis* strains tested, except strain HD12 which transformed 100 times more efficiently in water. In a comparison of PEG having different molecular weights (PEG 400, PEG 1000, PEG 6000, and PEG 20,000), PEG 1000 performed best with strains of *Bacillus thuringiensis* (30). Solioz and Waser (50), on the other hand, found sucrose, polyethylene glycol, and glycerol had no marked effect on the transformation efficiency of *Enterococcus hirae* electroporated with plasmid DNA.

Effect of Monovalent and Divalent Cations in the Electroporation Medium. Addition of 1 mM $MgCl_2$ to the electroporation buffer led to significant improvements in transformation efficiencies when electroporating *Pseudomonas aeruginosa* with plasmid DNA (13). Traces (0.1 mM) of $MgCl_2$, $CaCl_2$, and EDTA lowered the transformation

efficiency 10- to 100-fold for *Enterococcus hirae*. NaCl and K_2SO_4 were only slightly inhibitory at those concentrations (50). Decreased transformation frequencies were also seen when low levels of Mg^{+2}, Ca^{+2}, and Mn^{+2} were included in the electroporation buffer of *Lactobacillus casei* (38).

Effect of Buffer pH. When electroporating cell suspensions of *Lactobacillus casei* IAM1045 (38), changing the pH over a range of 6 to 8 with 8 mM sodium phosphate buffer and from pH 8 to 9 with 8 mM Tris-HCl buffer resulted in a moderate increase in transformation frequency with increasing pH. Very little attention, if any, has been paid to this factor by most researchers.

Effect of DNA Concentration During Electroporation

In some cases, as the plasmid DNA concentration during electroporation increases the total number of transformants will increase (30, 33), but in other cases the number of transformants will level off at high DNA concentrations (33). Interestingly enough, some strains of bacteria with certain plasmids show that the number of transformants recovered are not directly proportional to the DNA concentration and may even decrease as the amount of DNA is increased (43).

Effect of Cell Concentration During Electroporation

It is generally observed that an increase in the density of the cells being electroporated results in a subsequent increase in the transformation efficiency (5, 14, 25). On the other hand, if the concentration of the cells is so great as to increase the ionic strength of the suspension, then excessive heating of the cell suspension, a higher incidence of arcing, and a decrease in the transformation efficiency can occur (5, 25). It is also sometimes observed that the yield of transformants will increase with increasing

cell concentration up to a certain point and then level off, suggesting that the DNA level may be a limiting factor in some cases (5).

Type of Plasmid

The type of plasmid used in an electroporation experiment will affect the ability to recover transformants. The plasmid that transformed *Lactobacillus plantarum* CCM 1904 the best was pGK12, which is based on a lactococcal replicon, pWV01. Good results were also obtained with plasmids pC194 and pNZ12, but plasmid pULP8 based on a *Lactobacillus plantarum* replicon did not transform *Lactobacillus plantarum* as efficiently as the above heterologous plasmids. This may be due to the fact that pULP8 plasmid DNA used in this experiment was amplified in *E. coli* JM103, and an unknown restriction-modification system may be lowering the transformation efficiency in this case. All of the plasmids tested were based on replicons replicating by way of single stranded intermediates (8). Silke *et al.* (48) used the broad host range plasmid vectors pNZ12 and ΔpAMβ1 to perform their initial electroporation experiments. Transformation efficiencies were as high as 4 x 10^3 for pNZ12 but only 2 x 10^2 for ΔpAMβ1. It is generally a good idea to electroporate using broad host range vectors known to function in species related to the target strain when conjugation, conjugative mobilization, or the existence of a native plasmid with a suitable marker is not available as an alternative. Traits prerequisite for a plasmid to be considered a good vector are (1. small size, (2. presence in high copy number, (3. suitable restriction sites, and (4. a proven antibiotic resistance marker (36). pC194 is such a vector and has been widely used in electroporation experiments (4, 6, 7, 8, 27, 28, 30, 32, 56). Over 5 x 10^6 transformants/μg of pC194 DNA were obtained with *Bacillus thuringiensis* cells (32). In a different study, seven strains of *Bacillus thuringiensis* were transformed by electroporation with eight different plasmids. Plasmid pC194 accounted for the greatest

number of transformants for four of the strains in the study. The transformation efficiency was less than four times lower for pC194 compared to the efficiencies of the other seven plasmids when electroporated with the remaining three strains of *Bacillus thuringiensis* (6). Similar results were seen by Mahillon *et al.* (30) with pC194 consistently yielding higher transformation efficiencies than three other plasmids (including pE194) when tested with 21 strains of *Bacillus thuringiensis.*

<u>Plasmid Size May Affect Transformation</u>. The size of the plasmid DNA used in an electroporation experiment may or may not affect transformation efficiency. Because of its smaller mass, plasmid pVA736 (7.6 kb in size) was taken up more efficiently than pAMβ1 (26.5 kb) in *Streptococcus sanguis* Challis (51). However, the transformation frequency of lysozyme-treated *Streptococcus lactis* LM0230 electroporated with plasmids pLS1 (4.4 kb), pMU1328 (7.4 kb), and pAMβ1 (26.5 kb) showed no relationship between plasmid size and transformation efficiency (43). Similarly, *Bacillus cereus* 569 UM20-1 cells were electroporated with the chloramphenicol resistance plasmid pC194 (2.8 kb) and with a 200 kb mercury-resistant plasmid from *Bacillus cereus* strain 5 with comparable frequencies (4).

<u>Effect of Plasmid Form on Transformation</u>. The effect of the plasmid form on transformation efficiency has been studied by several groups of researchers. In one study, *Staphylococcus epidermidis* was electroporated with equal amounts of either supercoiled (ccc), *Hind*III-digested (linear), or religated (open circular) pC194 DNA. The supercoiled pC194 gave the highest transformation efficiency (1.2 x 10^5), followed by re-ligated pC194 (2.0 x 10^4). Linear pC194 yielded only 85 transformants/μg DNA, indicating the circular forms of pC194 greatly increased the transformation ability of the DNA (2). Park and Stewart (41) had similar findings with *Listeria monocytogenes.* They incubated pCK1 ccc plasmid DNA with various concentrations of DNA

topoisomerase I, with a resultant decrease in the transformation efficiency of the most relaxed DNA to a level of only 40% that of the native supercoiled form. In addition, restricted and religated plasmid DNA gave only 22% of the transformants seen with supercoiled pCK1. If, however, the ligated DNA was given limited treatment with DNA gyrase to introduce negative supercoils into the DNA template, a two-fold stimulation in transformation efficiency could be obtained over restricted and religated plasmid DNA. Plasmid molecules given a superhelicity above that of the native plasmid transformed less efficiently, while linearized DNA gave no transformants.

Restriction-Modification Systems

Transformation by electroporation appears most likely to be achieved if attention is paid to potential problems arising from restriction systems in the bacteria to be transformed. Allen and Blaschek (1), in an attempt to transform two strains of *Clostridium perfringens* that would not otherwise transform with pAK201 DNA , methylated the adenosine and cytosine bases in the transforming DNA by passage through *E. coli* DH5-alpha. The pAK201 DNA was confirmed to be protected by methylation by the inability of *Mbo* I to digest pAK201. The attempt failed, however, because crude lysates from the non-transformable strains were still able to degrade the modified pAK201 DNA. Restriction barriers have also been reported between *Corynebacterium glutamicum, Brevibacterium lactofermentum*, and *E. coli.* Although *C. glutamicum* and *B. lactofermentum* are related to the extent both of them can use the origins of replication of pUL340 and pCSL17, the barrier to transformation is as great between these two species as that seen for DNA derived from *E. coli* (20, 58). One approach to overcome restriction-modification systems is to use mutation with nitrosoguanidin (NTG) to isolate restriction deficient mutants which could be efficiently transformed with foreign DNA (25). The use of restriction minus mutants in the

electroporation of *Salmonella* species resulted in 10^2 increases in transformants over that of restriction positive strains (39). In addition to restriction-modification systems, many bacteria produce extracellular nucleases that can degrade the transforming DNA before it enters the cell. If foreign DNA should gain access to the inside of the cell it still faces the possibility of being degraded before a functional protein can be produced. Consequently, decreased numbers of transformants may be recovered.

Physiological State of the Cells May Affect Their Ability to be Transformed

While many factors are influencial in transforming bacteria during electroporation, one of the most obvious factors is the growth phase of the bacteria. Cells in the stationary phase were reported to give a marked increase in transformation efficiency at high cell concentrations (5×10^{10}) for *Lactococcus lactis* subsp. *lactis* (33). In contrast, other researchers studying the same organism found cells from the early-exponential phase to give a higher number of transformants under their experimental conditions (43). High transformation efficiency was found with cells of *E. coli* in the mid-log phase (14). And, to complete the picture, culture age did not appear to be a significant factor in the electroporation of *Streptococcus sanguis* Challis or *Bacillus thuringiensis* cells (30, 51).

Dilution, Expression Time, and Recovery Medium after Electroporation

McIntyre and Harlander found an expression period of 2 hours in nonselective media after electroporation gave consistently better results than no expression period while studying *Lactococcus lactis* subsp. *lactis*. In addition, if the electroporated sample was diluted 1:100 after electroporation the number of survivors increased 3 to 4 times the

initial value with a comparatively much larger (30 to 50 times) increase in transformant numbers over the initial values (33). Haynes and Britz (20) found similar phenomena when electroporating *Corynebacterium glutamicum cells.* Dilution of the *C. glutamicum* cells in distilled water immediately after being electrically pulsed but before decimal dilution in buffer resulted in substantially fewer transformants compared to immediate dilution in buffer after the pulse. This was taken as evidence that transformants arising after electroporation were osmotically and/or electrochemically fragile. The number of transformants increased five-fold after a recovery period, and transfer to a high ionic strength, osmotically-protective medium also increased the number of transformants 10- to 10^3-fold. The fragility was eliminated after a recovery period of up to one hour (20). In contrast, after electroporation of *Bacteroides ruminicola,* no significant difference in transformant recovery occurred when the cells were incubated 0 to 4 hours in antibiotic free medium before plating onto selective medium (55). The best results with *Bacillus amyloliquefaciens* occurred when the cells were immediately transferred into expression medium and diluted 1:10, followed by 1 to 1.5 hours incubation to allow phenotypic expression of their plasmid DNA (56).

Sample Temperature Before Electroporation

Cells of *Bacillus amyloliquefaciens* held at 0° C for 20 to 30 minutes before pulsing were found to give optimal transformation efficiencies (56). Cells of *E. coli* behaved in a similar manner, yielding 2 to 10 times the number of transfectants when precooled to 0° C as opposed to electroporating at room temperature (54). On the other hand, cells of *Corynebacterium glutamicum* demonstrated increased transformant yields with increased prepulse temperatures up to 30° C (57).

Other Miscellaneous Parameters to Investigate for Optimum Transformation

Growth in certain media or treatment of cells with various substances before electroporation often results in improved recovery of transformants. Some of the growth media additives investigated by various researchers were Tween 80 (19), glycine (19, 20, 22, 33), isonicotinic acid hydrazide (20), DL-threonine (33), and penicillin (41). Growth in defined media has been reported to increase electroporation efficiency also (34). Pre-treatment of cells with lysostaphin (46), lysozyme (57), and freeze-thaw cycles (16, 57) also aided in transformation with some strains.

Future Prospects for Gene Transfer in Propionibacteria

While there is one report in the literature of *Propionibacterium jensenii* having been transformed by electroporation with plasmid pGK12 DNA at low transformation efficiencies (27), to date, no other researchers have reported being able to transform any species of the genus *Propionibacterium* using the previously published electroporation protocol or by any other method. While this is discouraging at first, it must be realized that an extremely large number of variables are able to influence the outcome of an electroporation experiment. The first considerations are to determine the best strain of propionibacteria to use, the field strength to use and the pulse duration that gives the best transformation efficiencies. The type of transforming DNA is important also, and initial experiments should include the same DNA or DNA with characteristics similar to that which has already been reported as successful. Screening of cultures should be performed to determine whether or not they degrade the transforming DNA with nuclease enzymes. These and many other factors were considered in the present study, with some success. While electroporation is one approach, another that would be worth investigating would be conjugation.

It has been reported that *E. coli* S17-1, which carries a derivative of the RP4 mobilization plasmid in its chromosome, could successfully transfer pECM1, an *E. coli-Corynebacterium glutamicum* shuttle vector to *C. glutamicum* by conjugation (45). The pECM1 shuttle vector only worked well with strains of bacteria that were closely related physiologically to *C. glutamicum*. However, shuttle vectors based specifically on a *Brevibacterium linens* replicon were successfully mobilized from *E. coli* S17-1 to *B. linens* using the same protocol. Perhaps a similar system can be developed in the future that would allow for the conjugal transfer of genetic material to propionibacteria.

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CHAPTER III

ELECTROPORATION MEDIATED GENE TRANSFER OF pC194 INTO THE *PROPIONIBACTERIUM* GENOME

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ABSTRACT

A vital step in further studies of the genetics of the genus *Propionibacterium* is the development of a reliable DNA transfer system. Using electroporation, plasmid pC194 from *Staphylococcus aureus* was successfully introduced into several strains of *Propionibacterium*. The plasmid contains a gene coding for chloramphenicol resistance. *Propionibacterium freudenreichii* P7 was chosen to determine the optimal conditions for electroporation. Parameters found to influence the electroporation-induced transformation frequency were field strength, pulse duration, and electroporation buffer. The most efficient transformation of strain P7 was achieved with cells suspended in ddH_2O given an electric pulse with a field strength of 5.4 kV/cm and a pulse duration of 5 msec. Use of these conditions yielded 1.5×10^2 transformants per μg of DNA. To date, three other strains have been successfully transformed with pC194 using these conditions. Isolation of plasmid DNA from putative transformants and subsequent agarose gel electrophoresis failed to confirm the presence of an autonomous plasmid. Southern hybridizations indicated that pC194 was homologous to the chromosomes of the parent strains of *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii*, which were sensitive to chloramphenicol. Integration of pC194 into the *Propionibacterium* chromosome was verified by comparing hybridization signals detected between parental and transformant DNA digested with *Hin*f I, *Eco*R I, and *Hin*d III.

INTRODUCTION

The genus *Propionibacterium* includes four species isolated from cheese and dairy products. These are often referred to as the "dairy" or "classical" propionibacteria. Morphologically, these strains are Gram-positive, nonmotile, nonsporing, pleomorphic rods, often diptheroid or club-shaped and may be found as single cells, pairs, or short chains. Some species produce extracellular slime (3). Propionibacteria are used industrially as starter cultures in Swiss cheese and fermented dairy product-based drinks, in the production of vitamin B_{12}, and in the production of propionic acid (15). High yields of B_{12} have been obtained by *Propionibacterium freudenreichii* and *Propionibacterium shermanii*; the crude B_{12} product is used as a feed additive while further purification yields a product suitable for human use (2).

The development of dependable gene transfer systems and cloning vectors for propionibacteria will allow for future applications of recombinant DNA techniques in strain improvement programs. To date, the only published report of successful gene transfer in propionibacteria has been by electroporation-mediated transformation of *Propionibacterium jensenii* with the plasmid pGK12 (11). In this study we report the transformation by electroporation of *Propionibacterium freudenreichii,* and *Propionibacterium acidipropionici* with pC194, a 2.9 kb, broad host range plasmid from *Staphylococcus aureus* coding for chloramphenicol resistance (8). Response surface regression analysis was performed to determine a region of optimum field strengths and time constants for electroporation. Restriction endonuclease digestion and Southern hybridization analysis were used to demonstrate insertion of pC194 into the genomes of the tested strains and the existence of native *Propionibacterium* homology with pC194.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All strains of *Propionibacterium* were obtained from the culture collection of the Department of Animal Science and were routinely grown in sodium lactate broth (NLB) at 32°C (7). Strains of *Propionibacterium* grown to mid-log phase in 10 ml of NLB at 32°C provided a 1% inoculum for cultures from which plasmid or chromosomal DNA was to be extracted. Cultures of *Propionibacterium* for DNA isolation were incubated at 32°C until they reached late-log phase (generally, 24-48 hours). Solid media (NLA) contained 1.5% agar. Transformants were selected on NLA containing 20 µg of chloramphenicol per ml and were transferred to NLB containing 5 to 20 µg/ml of chloramphenicol for propagation before harvest and storage. Putative transformants were challenged with 25 to 1000 µg/ml of chloramphenicol in NLB to examine their relative resistance to chloramphenicol. Table 1 lists the strains of *Propionibacterium* used in this study.

Staphylococcus aureus ISP 1386 containing the plasmid pC194 was propagated at 37°C in trypticase soy broth (TSB) containing 5 µg/ml of chloramphenicol. One percent inoculums from 24 hour cultures of *S. aureus* ISP 1386 grown in TSB containing chloramphenicol were used to inoculate brain-heart infusion (BHI) broth cultures containing 5 µg/ml of chloramphenicol. Cultures of *S. aureus* ISP 1386 in BHI broth containing 5 µg/ml of chloramphenicol were grown at 37°C to an absorbance of 1.5 at 600 nanometers (A_{600}) before harvest and subsequent plasmid isolation procedures. All cultures were stored at -70°C in their respective broth medium containing 10% glycerol.

Table 1. *Propionibacterium* strains used in this study

Genus and species	Strain	Plasmid Content
Propionibacterium acidipropionici	P3	pRGO1
Propionibacterium acidipropionici	P5-3	Cured derivative of strain P5
Propionibacterium acidipropionici	P9	No plasmid
Propionibacterium freudenreichii	P7	No plasmid
Propionibacterium freudenreichii	P93-37	Cured derivative of strain P93
Propionibacterium freudenreichii	P104	No plasmid
Propionibacterium jensenii	P38	pRGO1, pRGO5

DNA Isolation and Purification

Plasmid DNA was isolated from *S. aureus* using the preparative scale method of Jones and Pattee (9). Large-scale isolation of plasmid DNA from *E. coli* was performed by the cleared-lysate method (12).

Putative *Propionibacterium* transformants were screened for plasmid DNA using the microscale screening procedure previously described (14). A preparative scale plasmid DNA isolation procedure was used to confirm the absence of plasmids in transformant strains (14). Chromosomal DNA from *Propionibacterium* for hybridization analysis was isolated from parent and transformant strains using a modification of the preparative plasmid isolation procedure. Briefly, the procedure was as follows; cells from 50 ml of a concentrated propionibacteria cell suspension previously adjusted to an A_{600} of 20 (i.e. a 1:100 dilution of the concentrated cells gave an absorbance reading of 0.2 at 600 nanometers) in NLB were harvested by centrifugation at 12,000 x g for 10 minutes. Parent and putative transformant cells of strain P7 were an exception and required 25 minutes of centrifugation. The cell pellet was resuspended to a final volume of 25 ml of a solution containing 15% sucrose, 50 mM Tris-HCl and 50 mM Na_2EDTA at pH 8.0. Lysozyme solution (100 mg/ml of lysozyme in a solution of 50 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0) was added to a final concentration of 20 mg/ml. The samples were vigorously mixed and then incubated 1 hour at 32° C. Pronase E (10 mg/ml in a solution containing 50 mM Tris-HCl, 5 mM Na_2EDTA and 50 mM NaCl at pH 8.0, preincubated for 1 hour at 37 ^{o} C) was added to a final concentration of 1 mg/ml. The mixture was gently mixed and incubated for 1 hour at 32° C. Following incubation, 11.5 ml of 0.25 M Na_2EDTA at pH 8.0 were added with gentle mixing, and the tube containing the mixture was held for 15 minutes, nearly submerged, in a container of crushed ice (on ice). This was followed by the addition of 11.5 ml of 20% (w/v) sodium dodecyl sulfate (SDS) in 50 mM Tris-HCL, 25 mM Na_2EDTA, pH 8.0, with inversion

and gentle mixing of the mixture. This mixture was chilled on ice for 15 min. Following incubation, 7.5 ml of 5.0 M NaCl was added with gentle mixing. This was followed by the addition of 70 ml of phenol saturated with 3% NaCl, 0.1 M Tris-free base (pH ≅ 8.0). The mixture was shaken vigorously and held for a period of 5 minutes at room temperature. To ensure phase separation, 20 ml of chloroform was added followed by vigorous shaking. The phases were separated by centrifugation at 12,000 x g for 15 min, the aqueous phase was removed and extracted with 75 ml of chloroform:isoamyl alcohol (24:1), followed by centrifugation at 12,000 x g for 15 min. After centrifugation, the aqueous phase was removed and two volumes of 95% ethanol (≅ 160 ml) were added. The sample was gently mixed and stored overnight at - 20° C. The precipitated DNA was harvested by centrifugation at 12,000 x g for 15 min. The supernatant was discarded and the DNA pellet was thoroughly dried before the addition of 14 ml of a solution of 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.5.

All preparative scale DNA samples were purified by CsCl-ethidium bromide density gradient centrifugation (12). The purified DNA was extracted with isopropanol saturated with 5 M NaCl to remove the ethidium bromide. It was desalted and concentrated by using a Centricon-30 microconcentrator (Amicon Corp., Danvers, MA.).

Electroporation Procedure

Plasmid-free strains of propionibacteria were grown to the appropriate growth phase in NLB. The cells were harvested (12,000 x g for 10 minutes) and washed three times in aliquots of electroporation buffer equal to 1/20th the volume of the broth culture before harvest (the buffers examined are listed in Table 2). An exception to this was strain P7 which, due to the production of extracellular slime, required 25 minutes of centrifugation to form a pellet firm enough to allow complete recovery. The washed cell pellet was resuspended to 1/20th the original volume in the same buffer to be used for

Table 2. Electroporation Buffers

Buffer Number	Composition	Reference
1.)	10% glycerol, 0.2 mM K_2HPO_4, pH 7.5.	Calvin and Hanawalt [10]
2.)	15% glycerol, 0.2 mM K_2HPO_4, pH 7.5	Calvin and Hanawalt [10]
3.)	0.2 mM K_2HPO_4, pH 7.5.	Calvin and Hanawalt [10]
4.)	272 mM sucrose, 1 mM $MgCl_2$, 7 mM Hepes, pH 7.3.	Luchansky *et al.* [5]
5.)	272 mM sucrose, 1 mM $MgCl_2$, 7 mM K_2HPO_4-KH_2PO_4, pH 7.4.	Luchansky *et al.* [5]
6.)	270 mM sucrose, 1 mM $MgCl_2$, 5 mM Na_2HPO_4, pH 7.4.	Allen and Blaschek [11]
7.)	0.5 M sucrose, 1 mM $MgCl_2$, 7 mM K_2HPO_4-KH_2PO_4, pH 7.4.	Powell *et al.* [12]
8.)	Milli-Q(R) deionized H_2O (18 MΩ·cm at 25° C).	McIntyre *et al.* [13]
9.) through 14.):	30% (w/v) Polyethylene glycol (PEG, Sigma Chemical Co., St. Louis, MO) of the following molecular weights in Milli-Q(R) H_2O; 9.) 600, 10.) 1000, 11.) 1450, 12.) 3350, 13.) 8000, 14.) 10,000	Mahillon *et al.* [14]
15.)	$T_{10}E_1$ (10 mM Tris-HCL, 1 mM Na_2EDTA, pH 7.5)	*a*

a This buffer was used on a limited basis. Although it allowed for the recovery of transformants at low field strengths it caused excessive arcing at moderate field strengths.

electroporation and stored on ice for subsequent use. Cells prepared for electoporation were held on ice for no more than one hour. Cells of *Propionibacterium* strains that were to be frozen and thawed for later use in electroporation experiments were harvested and then washed five times in 1/10th the original culture volume of ddH_2O (Milli-Q(R) deionized H_2O, Millipore Corp., Bedford, MA). The washed pellet was resuspended to 1/20th the original volume in NLB containing 10% glycerol. One ml aliquots of the cell suspension were placed in microcentrifuge tubes, quick-frozen by submersion in liquid nitrogen, and stored at -70°C. Frozen cultures were thawed on ice, washed three times in the same volume of the appropriate buffer and stored on ice prior to electroporation. One μg of pC194 plasmid DNA (in 4 to 10 μl $T_{10}E_1$, pH 7.5) and 75 to 81 μl of concentrated cells (for a total volume of 85 μl) were added to a microcentrifuge tube (that previously had been cooled on ice) and were gently mixed. The bacteria-DNA mixture was transferred to a BTX Flatpack electroporation chamber (85 μl capacity) (BTX, Inc., San Diego, Calif.) previously cooled by storage in a sterile, custom-made, aluminum rack which was kept on ice. The loaded chamber was then inserted into the isolation chamber and the cell-DNA mixture was electroporated with a single pulse of the desired field strength and duration. The majority of the work was performed with a BTX Transfector 100 with a BTX Power Plus unit for theoretical field strengths that varied from 0.89 to 43.39 kV/cm and theoretical pulse durations from approximately 100 μsec to 1 second. Due to the nuclease activity of the strains of propionibacteria studied, the BTX Transfector 100 unit was pre-charged (not recommended by the manufacturer) before the sample was inserted and electroporated, reducing the amount of time the DNA was exposed to the cell suspension and maintaining the sample temperature. Later work was performed with the BTX ECM 600 using 1 mm gap, 85 μl capacity cuvettes. The BTX ECM 600 allows for greater control over the pulse duration at extremely high field strengths and also charges rapidly, which eliminated the need to pre-charge the

electroporation unit before inserting the cuvette. The 1 mm cuvettes allow for more convenient and thorough sample chilling prior to electroporation.

Immediately after the pulse was applied the cells were quickly flushed from the Flatpack chamber (or 1 mm cuvette) with 600 μl of cold NLB to a test tube containing 10 ml of cold NLB (approximately a 133 to 1 dilution of the electroporated cells). The cells were then held at least 10 minutes on ice, followed by 13 hours of incubation at 32°C. The cells were harvested (6,000 x g for 30 minutes), resuspended to 0.3 ml in NLB, and plated on three plates (100 μl/plate) of NLA containing 20 μg/ml of chloramphenicol. Plates were incubated in an anaerobic system (BBL(R) Gas Pack *Plus;* BBL Microbiology Systems, Cockeysville, MD) for 25 to 35 days at 32°C before colonies were large enough to count. Controls in which either the electric pulse or the plasmid DNA was omitted were included. Numbers of colonies arising on plates for the controls were considered to be a representative background level of spontaneous mutants and thus were subtracted from the numbers of transformants on the plates from the treatments.

Parameters examined in the course of this study were electrical field strength, pulse duration, electroporation buffer, strains of *Propionibacterium*, and the growth phase of the cultures used. In addition, the use of fresh cells compared to frozen cells was examined as was the effect of varying the amount of pC194 plasmid DNA added during electroporation. Transformation efficiency was defined as the number of Cm^r transformants (previously adjusted for the occurrence of spontaneous Cm^r mutants) recovered per 1 μg of pC194 transforming DNA. Transformation frequency was defined as the number of Cm^r transformants/μg of pC194 transforming DNA /10^9 colony forming units pulsed during electroporation.

Genomic analysis of pC194 transformants

Chromosomal DNA isolated from parent and transformant strains was digested with *Eco*R I, *Hin*d III, and *Hin*f I and separated on 0.9% agarose gels (50 volts for 13 hours; 1.0 x : 0.045 M Tris base, 0.045 M boric acid, 0.001 M EDTA, pH 8.0 electrophoresis buffer (TBE)). DNA fragments were transferred to nitrocellulose filters by the method of Southern (16). The pC194 probe was made by using a nick translation kit with biotin-7-dATP according to the manufacturer's instructions (BRL, Gaithersburg, MD). Plasmid DNA to be labeled was separated on a preparative agarose gel, excised, and electroeluted from the gel slice using an Elutrap chamber according to the instructions of the manufacturer (Schleicher & Schuell, Inc., Keene, N.H.). DNA fragments with homology to pC194 were detected using the BRL BluGENE™ DNA detection system. Biotinylated λ DNA digested with *Hin*d III served as a DNA standard for molecular weight determinations (BRL, Gaithersburg, MD).

Sonication of Cells

Concentrated, mid-log phase cell suspensions of strain P9, either frozen in electroporation Buffer 3 at -20°C or freshly harvested and resuspended in electroporation buffer 6, were examined for a variation in numbers of colony forming units after the application of increasing amounts of sonication. Three milliliters of the cell suspension was added to a sterile, 4.5 ml Vanguard Cryos™ tube (Vanguard International, Neptune, N.J.) and held on ice during the sonication procedure. A Fischer(R) Sonic Dismembrator model 150 (Artek Systems Corp., Farmingdale, N.Y.) adjusted to a dial setting of 60 and fitted with a micro-tip was used to apply 20 second bursts of sonication to the cell samples. The sonicator tip was cooled in sterile ddH_2O after each 20 second burst. Portions of the cell suspension (100 μl) were removed after each 20 second burst,

serially diluted, and plated on NLA. Plates were incubated for one week at 32°C before enumeration.

Nuclease Testing

Samples of DNA were mixed with 450 μl of concentrated cell suspensions of three strains of propionibacteria, P5-3, P7, and P9 to detect the presence of extracellular nucleases. Cells from 24 hour cultures were harvested (12,000 x g, 10 minutes), washed twice in 1/20th the original culture volume with electroporation buffer 7, and resuspended to 1/20th the original volume in electroporation Buffer 7. One μg of DNA to be tested was added to each concentrated cell suspension and allowed to incubate on ice for 0, 5, 10, or 30 minutes depending on the trial. DNA samples included λ, pUC19, and pRGO1. Following incubation, cells were pelleted by centrifugation (4 minutes at 12,000 x g) and the supernatant containing the DNA was removed. To precipitate the DNA left in the supernatant, 1/10th (final) volume of 3 M sodium acetate (NaAc) was added followed by two volumes of cold ethanol and incubation at -20°C overnight. Precipitated DNA was harvested by centrifugation, dried, and resuspended in 40 μl of $T_{10}E_1$ before the addition of 10 μl of tracking dye. DNA was separated in a 0.7% agarose gel (50 volts for 8 hours, 1.0 x TBE electrophoresis buffer). The gels were stained in ethidium bromide solution (0.5 μg/ml in distilled H_2O) for 45 minutes and destained in distilled H_2O for the same amount of time before observation on a UV transilluminator. Controls consisted of 1 μg of test DNA with no exposure to the test reagents (except for the $T_{10}E_1$ and tracking dye used to load the sample) and 1 μg of test DNA exposed to the test reagents for 5 minutes before agarose gel electrophoresis.

Statistical Analysis

In the analysis of the first comprehensive field strength-time constant study, duplicate responses from separate days were collected for each randomized electroporation treatment and analyzed using the response surface regression analysis (RSREG) procedure of SAS (SAS Institute Inc, Cary, N.C.). The second comprehensive study of field strengths and time constants was analyzed using the general linear model (GLM) and regression (REG) procedures of SAS .

The One-Way Analysis of Variance (ANOVA) and Two-Sample Analysis (Student's t) procedures of StatGraphics Ver. 2 (STSC Inc., Rockville, MD) were used to test for significant differences (95% level) in transformation efficiency between the growth phases of P7 and between fresh and frozen P7 cells respectively. Significant differences between the transformation frequencies of strain P7 growth phases were also analyzed by the ANOVA procedure. All ANOVA procedures were followed by LSD, Tukey HSD, and Scheffe multiple range analysis (95% level).

RESULTS

Preliminary Electroporation Experiments: Effects of Buffer Composition, Field Strength and Strain on Survival and Transformation Efficiency

Preliminary transformation experiments were performed with strains P5-3, P7, P9, P93-37 and P104 at field strengths of 5.36, 12.5, and 16.07 kV/cm. The BTX T100 was used with Flatpacks and the time constant was set at 5 msec for all treatments. The second set of preliminary transformation experiments was conducted with the same four strains by using the BTX T100 with the Power Plus module and Flatpacks. The field strengths tested were 24.1, 33.75, and 43.39 kV/cm with pulse lengths controlled by the conductivity of the cell suspension in buffer. All preliminary electroporations were

performed with 1 μg of pC194 using cells harvested from the mid-log growth phase. The buffers examined were numbers 1, 3, 4, 6, 7, 8, and 9 through 15 (Table 2).

A summary of the highest single-trial transformation efficiencies obtained for the five strains is presented in Table 3. The use of other electroporation conditions often resulted in transformants, but not to the degree noted in Table 3. Transformation results varied widely from strain to strain, with strain P7 producing more consistent high numbers of transformants under several different sets of electroporation conditions than any other strain. Figure 1 illustrates the effect of field strength on survival and transformation efficiency of strain P7. The results indicated that transformation efficiency decreased with a decrease in survival beyond a field strength of approximately 4 kV/cm. Transformation efficiencies were much higher for buffer 8 than buffer 6.

Examination of the effect of high field strengths on transformation efficiency are shown in Table 4. Buffer 14, which was 30% (w/v) polyethylene glycol in ddH_2O, gave the best results for the high field strength conditions. Buffer 14 was the most viscous and waxy of the PEG buffers examined and aided in reducing arcing events and loss of sample from the Flatpack. Once again, buffers of low conductivity such as Buffer 8 and Buffer 14 gave higher transformation efficiencies.

Occasionally, a slight increase in the number of transformants recovered appeared at or past the field strength where arcing occurred (approximately 13 to 16 kV/cm when using a 5 msec theoretical pulse duration, depending on the buffer and strain). This increase never approached the optimum levels of transformants recovered at lower field strengths. This is felt to have occurred primarily due to the uneven application of the electrical field to the cells, which is supported by survival curve data showing simultaneous small increases in the numbers of surviving colony forming units at field strengths that allow arcing. A small increase in transformants at field strengths and time constants that result in arcing should not be construed as meaning these would be good settings for future electroporation experiments. In general, buffers with a higher

Table 3. Electroporation conditions for transformation of *Propionibacterium* strains

Strain	Transformation efficiency [a,d]	Conditions
P7	a.) 1.5×10^2	Buffer 8, 5.36 kV/cm, 5 msec pulse.
	b.) 1.4×10^2	Buffer 14, 3.57 kV/cm, 50 msec pulse.
	c.) 1.4×10^2	Buffer 14, 43.40 kV/cm, 40 to 160 μsec pulse [c].
P5-3	1.0×10^2	Buffer 10, 24.1 kV/cm, 40 to 160 μsec pulse [c].
P104	1.0×10^0	Buffer #4, 24.1 kV/cm, 40 to 160 μsec pulse [c].
P9	0	No results.
P93-37	1.3×10^1 [b]	Buffer #4, 33.75 kV/cm, 40 to 160 μsec pulse [c].

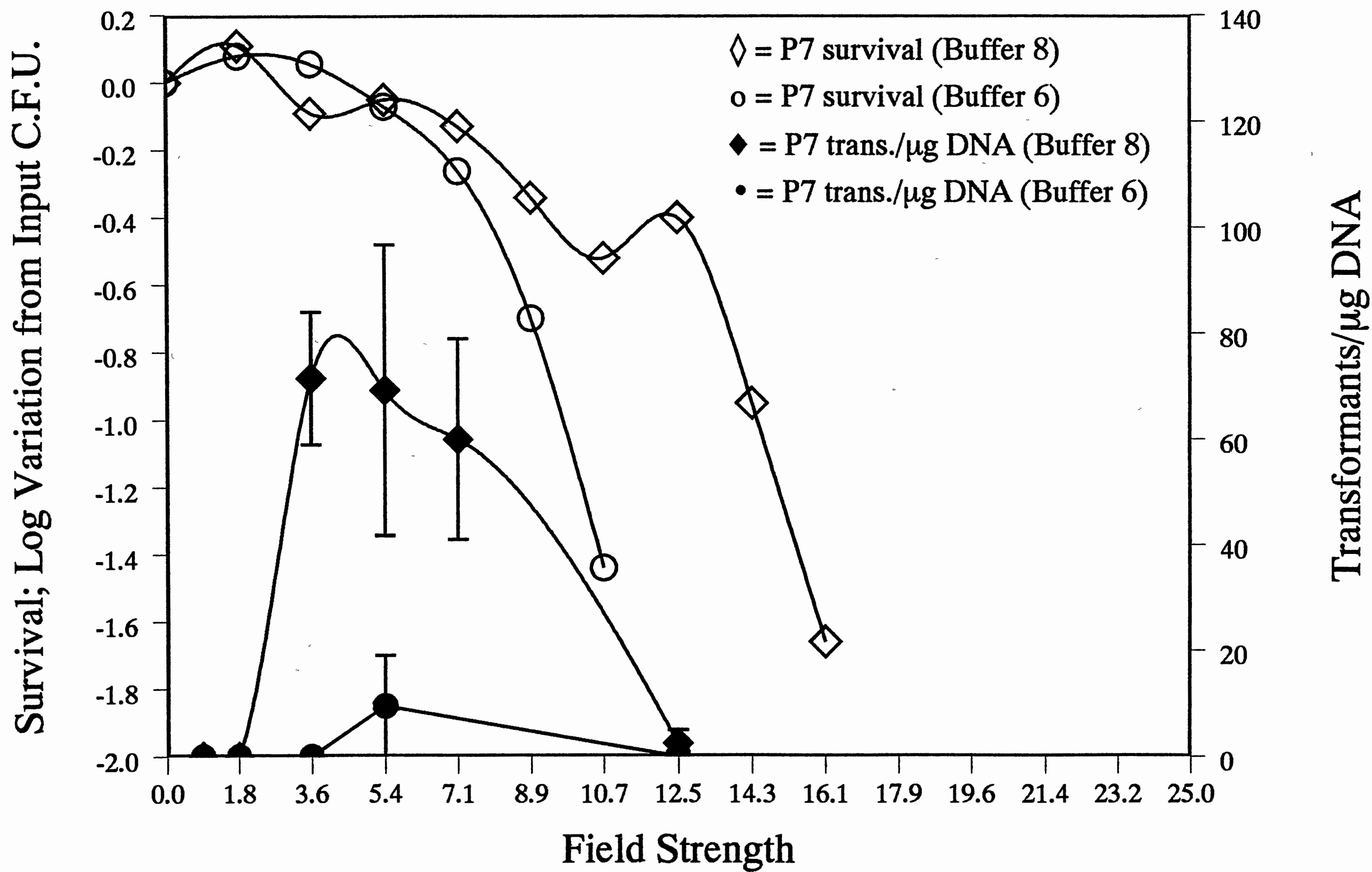
[a] Transformants per μg of pC194 plasmid DNA.

[b] Only phenotypic evidence is available for this strain presently.

[c] High field strengths using the BTX Power Plus unit resulted in very short pulse durations estimated to be in this range.

[d] The single highest efficiency from at least two replications per treatment.

Figure 1. Survival compared to transformation efficiency with increasing field strength and different buffers using a BTX Transfector 100 and Flatpacks. Each electroporation was performed with 1 µg of pC194 plasmid DNA mixed with a 1/20th volume concentrated cell suspension of strain P7 mid-log cells, except for treatments establishing the survival curves which did not include DNA. Theoretical field strengths examined for the survival curves were 0, 1.79, 3.57, 5.36, 7.14, 8.93, 10.71, 12.50, 14.28, and 16.07 kV/cm. The theoretical time constant for all treatments was 5 msec. Data was not collected past the point of severe arcing. Standard error bars were included for transformation efficiencies except at points where only one trial was performed or where arcing occurred frequently and only one data point was obtained. The initial population (average of two replications) in Buffer 6 was 2.0×10^{10} c.f.u./ml and in Buffer 8 it was 1.1×10^{10} c.f.u./ml.



Survival; Log Variation from Input C.F.U.
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
◊ = P7 survival (Buffer 8)
o = P7 survival (Buffer 6)
♦ = P7 trans./µg DNA (Buffer 8)
• = P7 trans./µg DNA (Buffer 6)
140
120
100
80
60
40
20
0
Transformants/µg DNA
0.0
1.8
3.6
5.4
7.1
8.9
10.7
12.5
14.3
16.1
17.9
19.6
21.4
23.2
25.0
Field Strength

Table 4. Effect of buffer and very high field strengths on transformation of *Propionibacterium freudenreichii* strain P7 by electroporation

	Buffers		
Field Strength (kV/cm) [a]	Milli-Q H_2O (Buffer #8)	30% PEG 10,000 in dH_2O (Buffer #14)	5 mM Na_2HPO_4 Buffer (Buffer #6)
24.10	33	111	0
33.75	45	80	14.5
43.40	31	138 [b]	5 [b]

[a] Concentrated suspensions of mid-log phase cells of strain P7 electroporated with 1 μg of pC194. The electroporation equipment used was a BTX T100 with a BTX Power Plus unit and 0.56 mm gap Flatpack electrodes. Field strengths above 16.07 kV/cm (achieved with the use of a Power Plus module) have a pulse length (controlled by the buffer conductivity) of approximately 40 to 160 μsec.

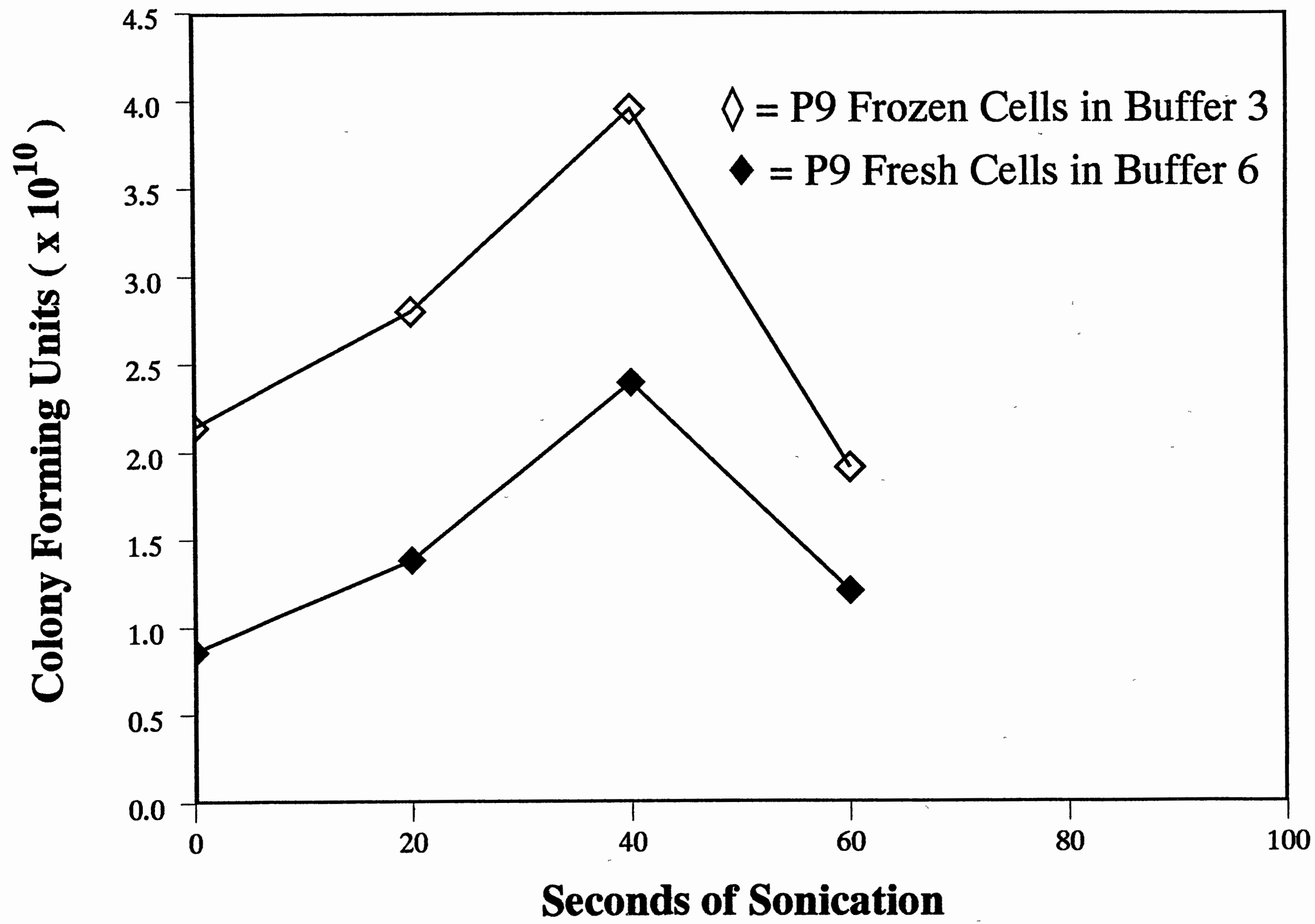
[b] These treatments are single reps., due to arcing. All other values in the table are the means of two replications.

ionic strength, such as buffer 6, yielded lower numbers of transformants and arced at lower field strengths than buffers of lower ionic strength such as buffer 8.

Effect of voltage and buffer on survival. Survival curves were determined using the first eight buffers listed in Table 2 and *Propionibacterium* strains P5-3, P7, and P9 to observe the effect of electroporation on cell survival. Survival curves of strain P7 electroporated in buffer 6 and in buffer 8 are shown in Figure 1. In the 1 to 4 kV/cm range the number of surviving colony forming units actually increased over the number of input colony forming units, most likely the result of disruption of cell clumps. Further evidence that this cell clump disruption occurs was provided by the influence of sonication on numbers of c.f.u. (Figure 2). An increase in surviving colony forming units occurred more frequently and reached higher levels when cells were electroporated in the highly resistive buffers (such as Buffer 8) rather than highly conductive buffers (such as Buffer 6). The use of high ionic strength buffers resulted in more rapid declines in the number of surviving colony forming units past approximately 5 kV/cm field strength.

The survival curve for strain P5-3 in buffer 8 (data not shown) was similar to the curve developed for strain P7 in buffer 6. Both of these curves indicated that arcing occurred at a lower field strength and that survivors decreased more rapidly than the survival curve for strain P7 in buffer 8. One possibility is that the differences in survival curves and transformation efficiencies between P5-3 and P7 originate with the extracellular slime produced by P7. Survival curves for strain P9 were similar to those for P7 (data not shown) but no pC194-P9 transformants could be recovered, which indicates some factor more important than survival was affecting the ability of strain P9 to be transformed.

Figure 2. Mid-log, concentrated cell suspensions of *Propionibacterium* strain P9 exposed to 0, 20, 40, and 60 seconds of sonication in 20 second bursts. Values represent one replication and trials using different buffers were performed on separate days.



4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Colony Forming Units (x 10^10)
◊ = P9 Frozen Cells in Buffer 3
♦ = P9 Fresh Cells in Buffer 6
0
20
40
60
80
100
Seconds of Sonication

Nuclease Activity

Preliminary studies were conducted to determine if strains of propionibacteria possessed nuclease activity that would be a barrier to successful transformation. Concentrated cell suspensions of strains P5-3, P7, and P9, were mixed with DNA and incubated for 0, 5, 10, or 30 minutes to detect the presence of extracellular nucleases. All strains exhibited nuclease activity when their whole cells were exposed to pUC19 DNA isolated from *E. coli* (Figure 3). When λ DNA was added to the cell suspensions downward streaking of the DNA was observed for all strains (Figure 4). This was presumably caused by extracellular nucleases. Plasmid pRGO1, the only DNA examined that was isolated from propionibacteria (14), was also degraded by all strains (Figure 4). In addition, incubation of pRG01 with strains P7 and P9 resulted in a steady increase in linear pRGO1 DNA at the expense of ccc DNA . This may provide initial evidence for the existence of an endonuclease enzyme produced by these two strains. Strain P5-3 also increased linear pRGO1 at the expense of the ccc form of the plasmid when compared to the control DNA . However, the presence of other nuclease activity that rapidly degraded pRG01 may have masked the endonuclease activity. Strain P5-3 had more nuclease activity than strain P9 or P7. Strain P7 appeared to have the least amount of nuclease activity of the strains tested. The presence of nuclease enzymes in propionibacteria strains dictated that steps to control this activity be included in the in the electroporation protocol. To minimize the effect of nucleases, cells were chilled at all times and the exposure of DNA to the cell suspension was kept to a minimum prior to and during electroporation.

Optimization of Electroporation Conditions

Preliminary electroporations of strain P7 with pC194 produced over 10^2 transformants at a field strength of 5.36 kV/cm and pulse duration of 5 msec. In

Figure 3. Agarose gel (0.7%) demonstrating exonuclease, restriction endonuclease, and possible ligase enzyme activities of propionibacteria used in this study on pUC19 plasmid DNA from *E. coli*. Concentrated, whole cells in electroporation buffer #7 were exposed to 1 μg of pUC19 for 0 and 5 minutes. Lanes: A, 1 μg pUC19 control DNA with no exposure to the test reagents; B, 1 μg pUC19 control DNA with 5 minutes of exposure to the test reagents; C and D, strain P5-3, 0 and 5 min. exposure; E and F, strain P7, 0 and 5 min. exposure; G and H, strain P9, 0 and 5 min. exposure. Strain P5-3 shows the most streaking indicative of exonuclease activity. All strains exhibit what appears to be an extra linear band that indicates the possible existence of an endonuclease enzyme, and all exhibit some degree of ability to produce a pUC19 multimeric ladder.

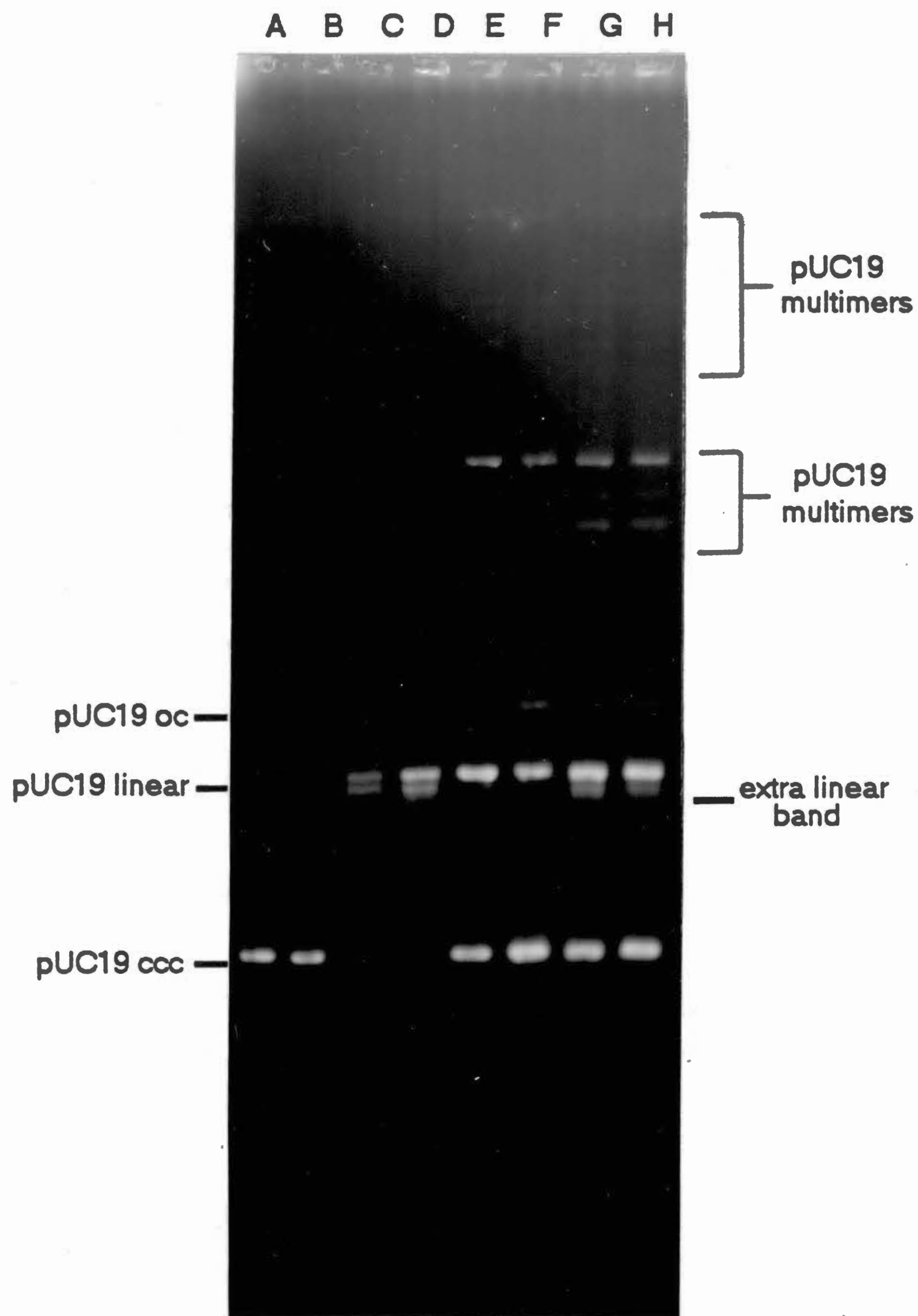
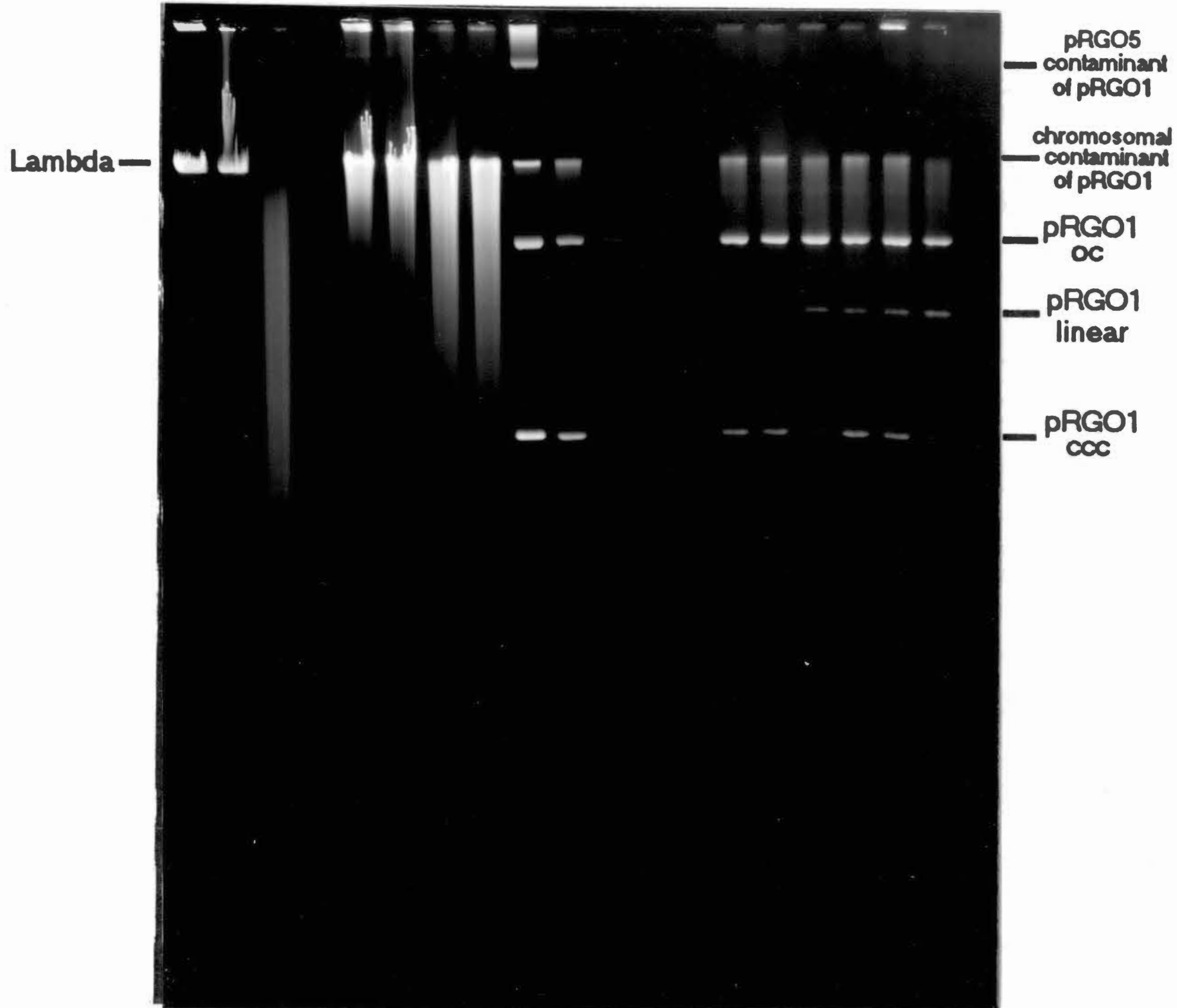

A B C D E F G H
pUC19 multimers
pUC19 multimers
pUC19 oc
pUC19 linear
extra linear band
pUC19 ccc

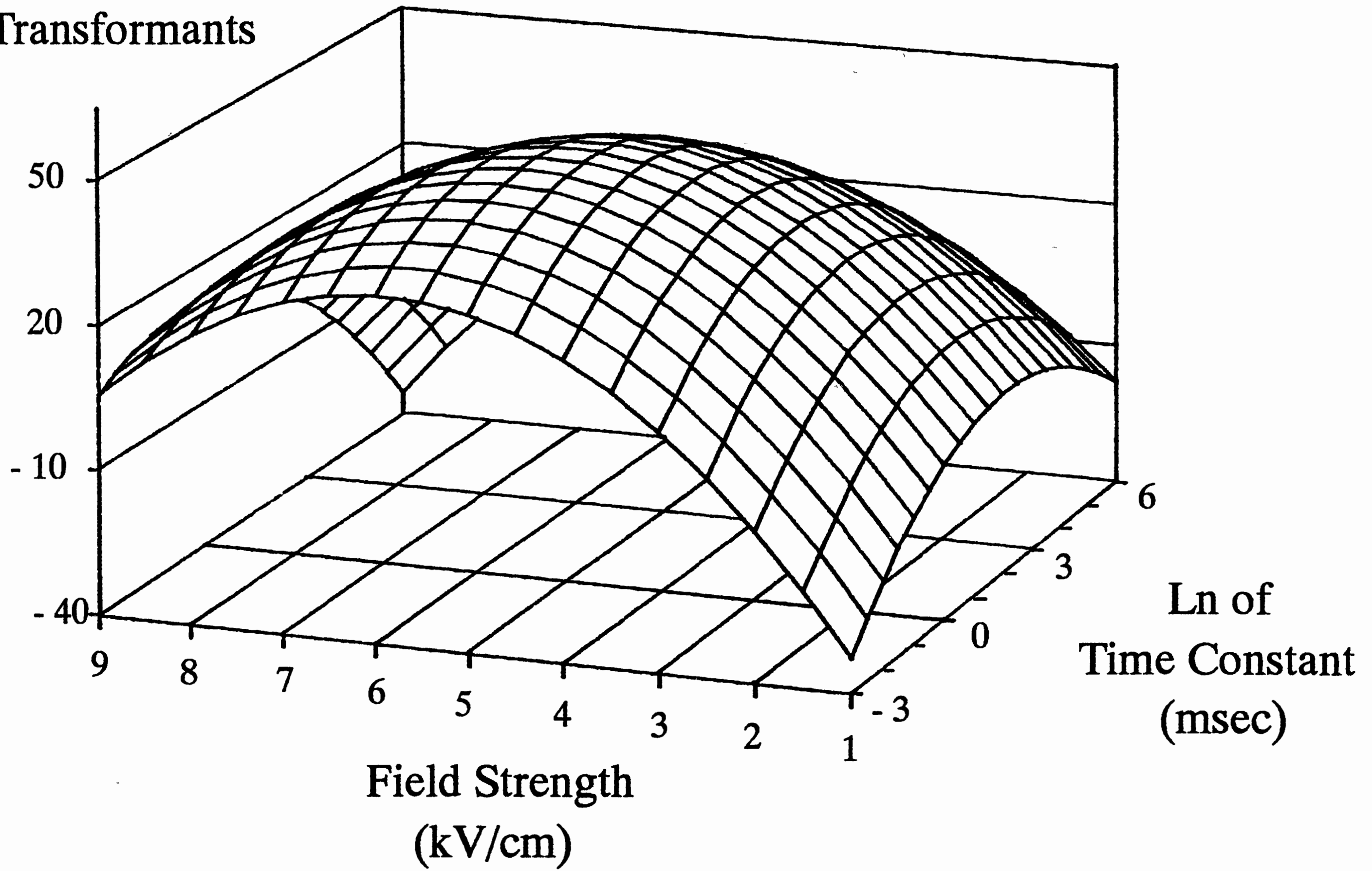
Figure 4. Agarose gel (0.7%) demonstrating exonuclease and endonuclease activities of propionibacteria when exposed to λ and pRGO1 DNA. Concentrated, whole cells in electroporation buffer #7 were exposed to 1 μg of λ DNA for 0 and 5 minutes and to 1 μg of pRGO1 plasmid DNA from propionibacteria for 0, 5, and 10 minutes. Lanes: A, 1 μg of λ control DNA with no exposure to the test reagents; B, 1 μg of λ control DNA with 5 minutes of exposure to the test reagents; C and D, strain P5-3, 0 and 5 min. exposure to λ; E and F, strain P7, 0 and 5 min. exposure to λ; G and H, strain P9, 0 and 5 min. exposure to λ; I, 1 μg of pRGO1 control DNA with no exposure to the test reagents; J, 1 μg of pRGO1 control DNA with 5 minutes of exposure to the test reagents; K through M, strain P5-3, 0, 5, and 10 min. exposure to pRGO1; N through P, strain P7, 0, 5, and 10 min. exposure to pRGO1; Q through S, strain P9, 0, 5, and 10 min. exposure to pRGO1. A different source of pRGO1 had to be used to be able to include the control in lane I. The uppermost band in lane I is plasmid pRGO5 which was present in that sample of pRGO1 DNA. All other pRGO1 lanes used DNA from the same source. Also present in the pRGO1 samples was some residual chromosomal DNA which migrated to about the same level as λ in the gel. Strain P5-3 demonstrates a strong ability to degrade both λ and pRGO1 DNA. Strains P7 and P9 demonstrate less degradative downward streaking but also demonstrate a steady increase in linear DNA at the expense of ccc DNA over time, likely due to the presence of an endonuclease enzyme.


A B C D E F G H I J K L M N O P Q R S
Lambda
pRGO5 contaminant of pRGO1
chromosomal contaminant of pRGO1
pRGO1 oc
pRGO1 linear
pRGO1 ccc

addition to being the strain with the highest observed transformation frequency in preliminary studies, strain P7 also was found to have the least nuclease activity of the strains examined. Therefore, strain P7 was chosen to determine the effect of field strength, pulse duration, growth phase of cells, DNA concentration and frozen storage of cells on electroporation efficiency.

Electrical Parameters. Two comprehensive studies were performed to further clarify the field strength-pulse duration combinations for the optimal transformation efficiency. In the first comprehensive study, strain P7 was electroporated at low field strengths (1.79, 3.57, 5.36, and 7.14 kV/cm) over a range of theoretical time constants (100 µs, 500 µs, 1 ms, 5 ms, 10 ms, 50 ms, 100 ms, 500 ms, and 1 second) using a BTX Transfector 100 with Flatpack chambers. A second comprehensive study was performed to determine the effects of high field strengths on transformation efficiency. Strain P7 was electroporated at various field strengths (9.0, 13.5, 18.9, and 24.3 kV/cm) with a range of timing resistors (13, 48, 186, and 720 ohms) in parallel with the output that resulted in pulse durations of approximately 0.61, 2.0, 5.2, and 9.1 msec using a BTX ECM 600 electroporation unit and BTX 1 mm gap electrodes. The capacitance was fixed at 50 µF. Cells of strain P7 from the mid-log growth phase resuspended in ddH_2O with 1 µg of pC194 were used in both of the studies. Figure 5 illustrates a contour plot of the number of transformants obtained from the results of the low field strength study analyzed using a response surface technique. The influences of field strength, time constant, and the field strength-time constant interactions were all found to be statistically significant. No significant difference was found between replications performed on separate days. The plot defined the range of optimum field strengths from 3.6 to 7.1 kV/cm and optimum time constants from 0.17 to 27.1 msec. The fitted equation for the regression model is $\hat{y}$ = intercept + f + $(f)^2$ + ln t + $(\ln t)^2$ + f(ln t), where f is the field strength and t is the time constant in msec (ln refers to the natural logarithm, base 2.71828). Parameter estimates for the equation are; intercept =

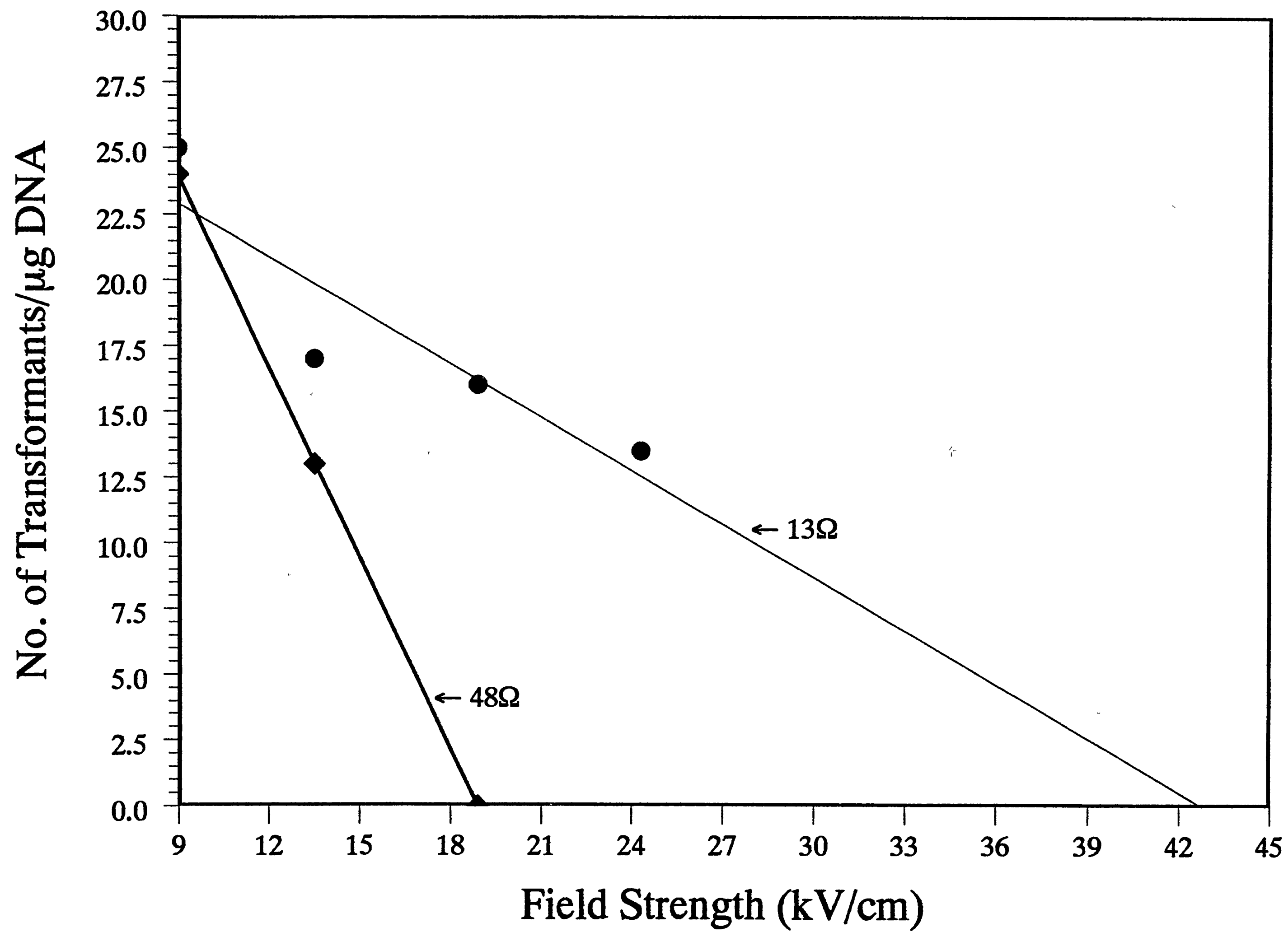
Figure 5. Surface response of transformants produced by electroporation of 1 μg of pC194 plasmid DNA in $T_{10}E_1$ (pH 7.5) with mid-log, *P. freudenreichii* strain P7 cells resuspended to 1/20th volume in ddH_2O. The plot was developed over a range of theoretical field strengths from 1.79 to 7.14 kV/cm and a range of theoretical time constants from 100 μs to 1 second. Two replications on separate days were analyzed at all combinations of field strengths and time constants. Treatments performed on any one day were randomized. The equipment used was a BTX Transfector 100 unit with 0.56 mm Flatpack electrodes.


Predicted Number
of Transformants
50
20
- 10
- 40
9
8
7
6
5
4
3
2
1
Field Strength
(kV/cm)
6
3
0
- 3
Ln of
Time Constant
(msec)

- 34.690261, f = 29.414765, ln t = 5.728464, $(f)^2$ = -2.696210, $(\ln t)^2$ = -1.109102, and f(ln t) = -0.771716. The approximate center of the region for optimal transformation efficiency is a field strength setting of 5.4 kV/cm with a time constant of 2.5 msec.

The results of the second comprehensive transformation study for optimizing the field strength and pulse duration are shown in Figure 6. Linear regression was performed on the means of two replications to determine the equation of the line for the data sets at each resistance setting, 13, 48, 186, and 720 ohms (actual time constants at these resistance settings were approximately 0.61, 2.0, 5.2, and 9.1 msec). Significant linearity was found only for the resistance settings of 13 and 48 ohms. The data collected at 186 and 720 ohms showed that, at the field strengths tested, the number of transformants dropped to zero so quickly a thorough analysis could not be conducted and significant results would not be expected. The regression equations and r values for the means of two replications were; 13 ohms, y = (-6.78)(x) + 29.02, r = -0.904 and 48 ohms, y = (-24.24)(x) + (45.78), r = -0.999. The actual time constants had a tendency to decrease slightly as the field strength was increased and this phenomenon became more pronounced as the resistance was increased. The theoretical field strengths tested were 9, 13.5, 18.9, and 24.3 kV/cm. The number of transformants decreased to zero within this tested range of field strengths, except when the resistance setting was 13 ohms. By examining an extension of the regression line past 24.3 kV/cm, the short pulse duration at 13 ohms of resistance (only 0.61 msec) theoretically would allow a field strength of 43 kV/cm to be applied before transformants would cease to be observed. Worth noting is that the highest number of transformants, roughly 23 at 9 kV/cm in Figure 6 occurred when the pulse duration was 2 msec. The optimal transformation conditions described in the surface response of Figure 5 were also near 2 msec. In addition, using the equation of the contour plot in Figure 5, roughly 7 transformants would be predicted for a field strength of 9.0 kV/cm and a 5 msec pulse duration. Considering the data sets for the two comprehensive transformation studies described in Figures 5 and 6 were generated using

Figure 6. Linear regression analysis demonstrating the negative effect of increasing field strength on the number of P7 transformants recovered after electroporation with 1 μg of pC194 DNA. The electroporation unit used was a BTX ECM 600 with 1 mm gap, 85 μl capacity, cuvette electrodes. Two resistors in parallel with the electrical discharge were used to control the pulse length. Resistors of 13 and 48 ohms produced pulse lengths of approximately 0.61 and 2.0 msec. Two replications on separate days were analyzed at all combinations of field strengths and resistance settings. Treatments performed on any one day were randomized.



No. of Transformants/µg DNA
30.0
27.5
25.0
22.5
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
9
12
15
18
21
24
27
30
33
36
39
42
45
Field Strength (kV/cm)
← 13Ω
← 48Ω

different equipment, there appears to be good agreement between them concerning the optimum time constant and the fact that the number of transformants recovered will decline to zero at some point past a peak field strength near 5 or 6 kV/cm. These two comprehensive studies also confirmed the results of the first preliminary study of strain P7 electroporated in ddH_2O with a 5 msec time constant (Figure 1).

Growth Phase. Samples were removed at early-log, mid-log, and late-log phases of growth (A_{620} of 0.52, 0.90, and 1.35 respectively) from a static culture of strain P7 to determine the effect of growth phase on transformation efficiency. Samples for each growth phase were harvested and electroporated with 1 μg of pC194 at a field strength of 3.75 kV/cm and a pulse duration of 5.9 msec (capacitance setting of 400 μF and a resistance setting of 13 ohms). No statistically significant differences were found among the transformation efficiencies of early-log, mid-log, and late-log phase cells. Although not statistically significant, higher transformation efficiencies were noted for cells from the early-log growth phase. Table 5 illustrates the transformation frequencies at each growth phase. When transformation frequencies for the same experiment were compared, which tends to compensate for the lower concentration of cells of strain P7 at the earlier growth phases, the analysis of variance of the results still indicated no significant differences between the growth phases. Further tests with (unprotected) LSD, Tukey HSD, and Scheffe multiple range analyses all indicated a significant difference did exist between the transformation frequencies of the early and late-log growth phase cells of strain P7 ($P < 0.05$). The transformation frequency of cells from the mid-log growth phase was not found to be significantly different than the transformation frequencies of early or late-log growth phase cells. This indicates a small advantage may be gained by using early-log growth phase cells of strain P7.

DNA Concentration. The effect of DNA concentration on transformation efficiency was examined using cells of strain P7 from the mid-log growth phase electroporated with various concentrations (0.000001, 0.001, 0.1, 1.0, 2.0 μg) of pC194.

Table 5. Transformation frequency at early, mid, and late-log growth phases of strain P7 electroporated with pC194 plasmid DNA

Growth Phase	Absorbance at 620 nm	C.F.U./ml	Transformants/10^9 C.F.U.
Early-log	0.52	2.4×10^9	10.0
Mid-log	0.90	3.9×10^9	7.3
Late-log	1.35	7.6×10^9	4.6

- Cells were concentrated to 1/20th volume in ddH_2O and 1 μg of pC194 was added before pulsing at a theoretical field strength of 3.57 kV/cm (measured field strength of 3.34 kV/cm) with a measured pulse duration of 5.9 msec (a capacitance setting of 400 μF and a resistance setting of 13 ohms were used to control the pulse length). The experiment was performed with a BTX ECM 600 unit using BTX electrode cuvettes (1 mm gap, part no. 610). Results were calculated from two trials on separate days.

Cells were electroporated at a field strength of 3.57 kV/cm and a pulse duration of 5.9 msec. The results indicate as the amount of pC194 DNA added to the cells increased, the transformation efficiency decreased by nearly seven orders of magnitude in a nearly linear fashion (Figure 7). It appeared that sufficient plasmid DNA was present, when just 1 picogram of pC194 was added to the cell suspension, to enter all of the receptive cells during the electroporation pulse and produce Cm^r colonies.

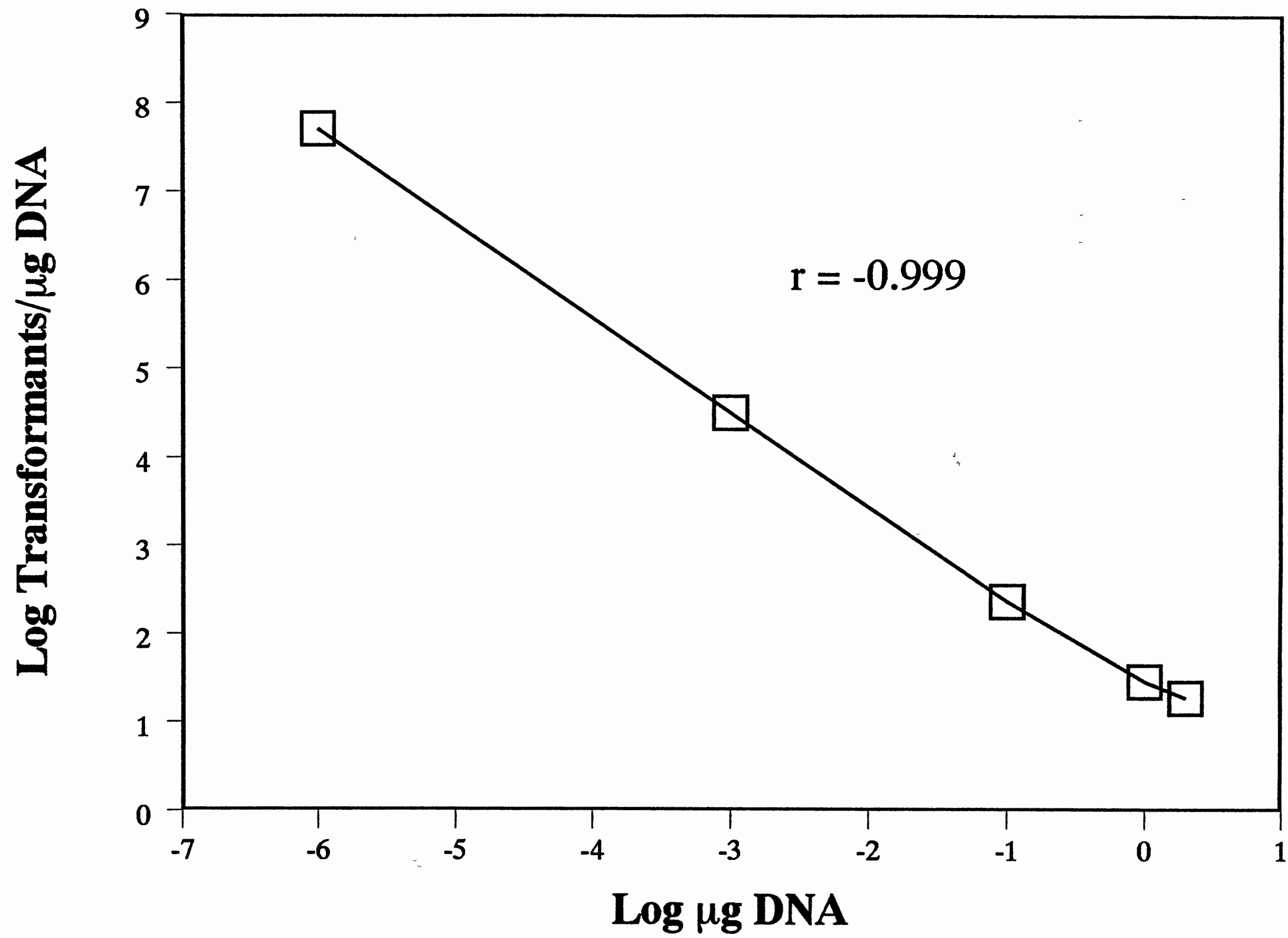
Fresh Cells Compared to Frozen Cells. The effect of frozen storage (-70°C) of cells on electroporation efficiency was compared by using freshly prepared and thawed cells of strain P7 from the mid-log growth phase. No statistically significant difference was found between the transformation efficiencies of freshly prepared cells and cells that had been frozen (data not shown). The use of frozen cells would eliminate the tedious process of growing, harvesting and washing the cells several times before each electroporation trial.

Analysis of Transformants Obtained With pC194

Isolation of plasmid DNA from putative transformants and subsequent agarose gel electrophoresis failed to confirm the presence of an autonomous plasmid in all strains transformed. Preparative scale DNA isolation from putative transformants failed to identify plasmid DNA in the CsCl density gradients. DNA samples of putative transformants from CsCl gradients were analyzed by DNA hybridization using pC194 as the probe in a slot blot apparatus. Hybridization signals were detected in DNA from all transformants. Unexpectedly, DNA from all propionibacteria strains used as recipients also produced a hybridization signal.

In order to confirm the presence of pC194 in the transformants and further characterize the regions of pC194 homology in the strains of propionibacteria, restriction endonuclease digests of parent and putative transformants were analyzed by Southern

Figure 7. Transformation efficiency compared to the amount of pC194 plasmid DNA added to strain P7 mid-log cells. The amounts of transforming DNA used were 1 picogram, 1 nanogram, 0.1 µg, 1 µg, and 2 µg. The theoretical field strength was 3.57 kV/cm (measured field strength was 3.34 kV). The pulse duration was controlled by a capacitance setting of 400 µF and a resistance setting of 13 ohms for a measured duration of 5.9 msec for the pulse. A BTX ECM 600 unit and 1mm gap, cuvette electrodes were used for two replications of each treatment. Duplicates were performed on separate days.



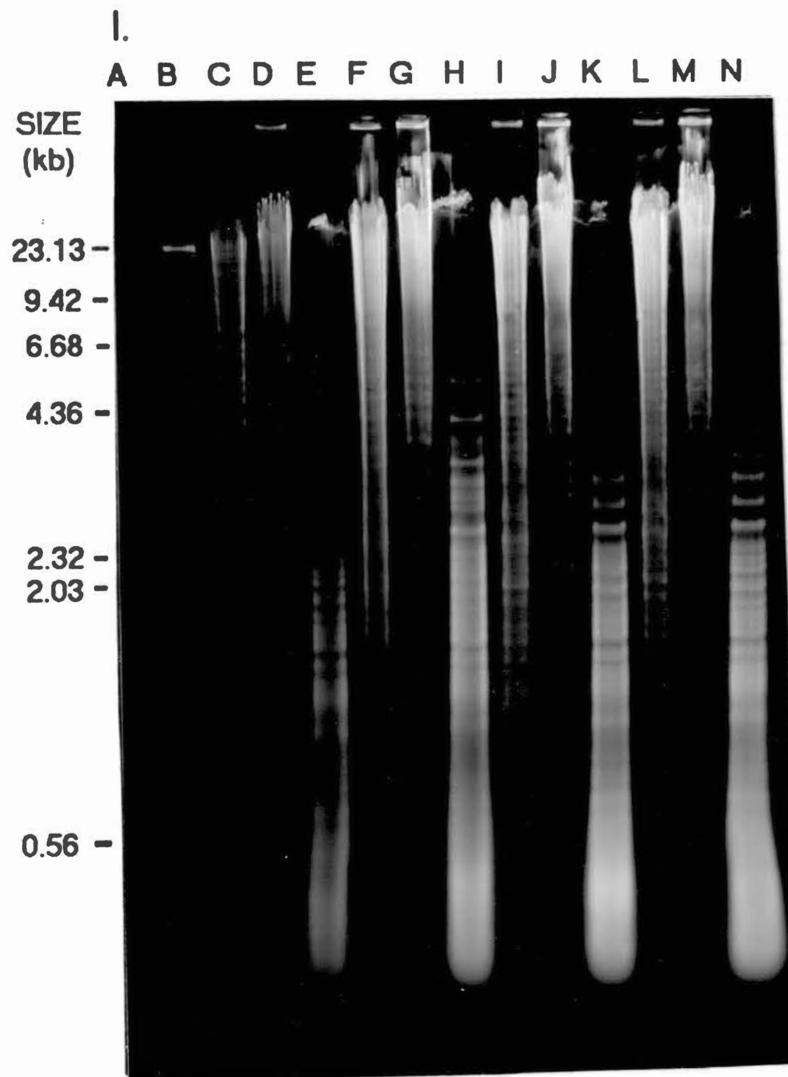
Log Transformants/µg DNA
9
8
7
6
5
4
3
2
1
0
r = -0.999
-7
-6
-5
-4
-3
-2
-1
0
1
Log µg DNA

hybridization with pC194 as the probe. Chromosomal DNAs were digested with *Eco* R I, which has no restriction site on pC194; *Hin*d III, which has one restriction site on pC194; and *Hin*f I, which has 3 sites on pC194. The results are presented in Figures 8, 9 and 10. Hybridization bands were detected between pC194 and chromosomal DNA of P7 (Fig. 8, lanes C, D, and E), P104 (Fig. 9, lanes C, D, and E), P5-3 (Fig. 9, lanes I, J, and K; Fig. 10, lanes C and D), and P9 (Fig. 9, lanes L, M, and N). Transformants retained some of the native bands of homology and, in addition, exhibited extra bands of homology, suggesting that pC194 had integrated into the chromosome. The sizes, in kb, of restriction fragments that hybridized with the pC194 probe DNA are listed in Table 6. Also listed in Table 6 are the sizes of the restriction fragments to be expected when the transforming pC194 DNA is cut with the same enzymes.

Two transformants of strain P7 exhibiting extra hybridization bands are shown in Figure 8, lanes F through K. A typical colony that proved not to be a transformant can be seen in lanes L, M, and N. The *Eco*R I digests of the P7 transformants (lanes F and I) showed hybridization signals identical to the parent (lane C) and the presence of additional signals. Both transformants had a band of hybridization nearly corresponding to the linear form of pC194. Transformant P3007/11 had an additional two bands. Integration of pC194 into the chromosome by homolgous recombination should produce a single additional band since Eco R I has no site on pC194.

The *Hin*d III digest of strain P7 transformants are shown in lane G and J of Figure 8. Both transformants produced a band of roughly 2.96 kb in size that could be either native homology or linear pC194. Transformant P3007/11 had two additional bands that were 4.8 and 2.6 kb. These two bands may represent restriction fragments composed of pC194:P3007/11 chromosomal DNA. The 10.6 kb fragment in the same lane may be a partial digest fragment of the 4.8, 3.0, and 2.6 kb fragments. The absence of hybridization bands corresponding to junction fragments may be due to comigration with the existing bands or because they were too small to be detected.

Figure 8. Hybridization of biotin-labeled pC194 DNA to restriction fragments of parent and transformant chromosomal DNA from *Propionibacterium* strain P7. (I.) Agarose gel electrophoresis of restriction digests of chromosomal DNA from *Propionibacterium* strains. Lanes: A, pC194 plasmid DNA control; B, *Hin*d III digest of biotin-labeled lambda DNA; C through E, *Eco*R I, *Hin*d III, and *Hin*f I digests of P7 parent chromosomal DNA; F through H, *Eco*R I, *Hin*d III, and *Hin*f I digests of P7 transformant P3007/11 DNA; I through K, *Eco*R I, *Hin*d III, and *Hin*f I digests of P7 transformant P3007/15 DNA; L through M, *Eco*R I, *Hin*d III, and *Hin*f I digests of a P7 Cm^R strain (P3007/28) which is not a transformant. (II.) Hybridization of the pC194 probe to a nitrocellulose filter containing restriction fragments of chromosomal DNA shown in panel I.


I.
A B C D E F G H I J K L M N
SIZE (kb)
23.13
9.42
6.68
4.36
2.32
2.03
0.56

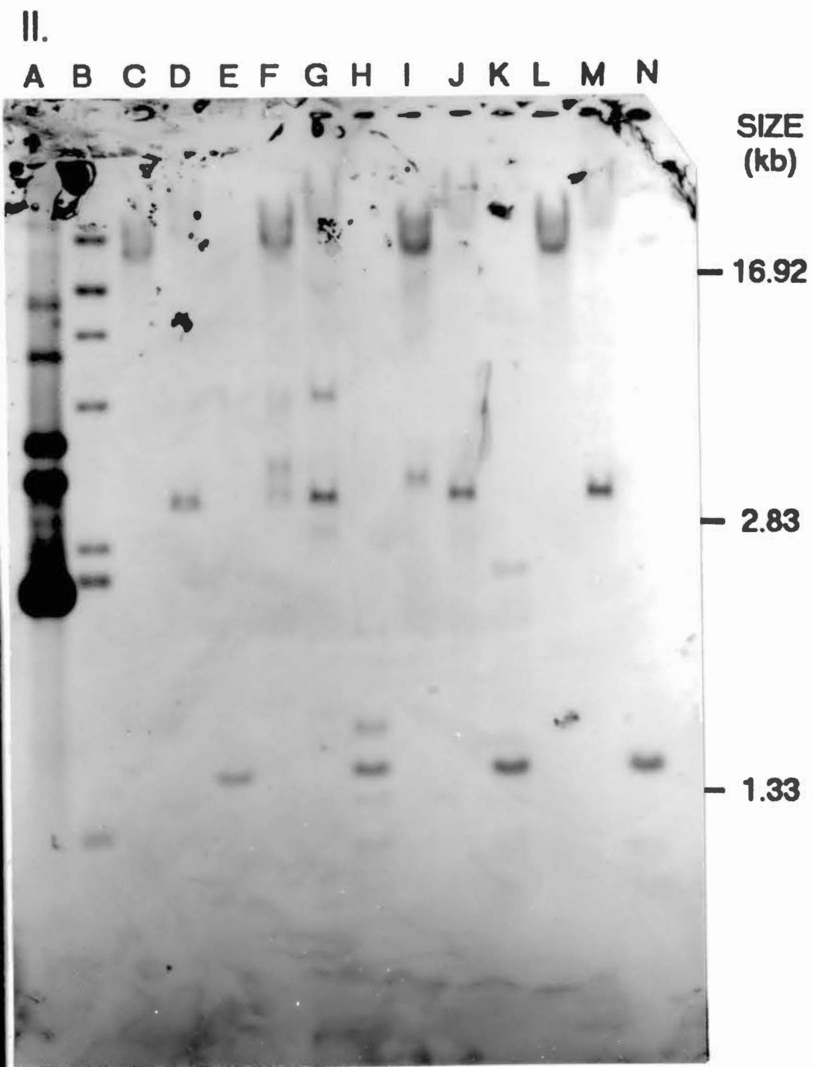
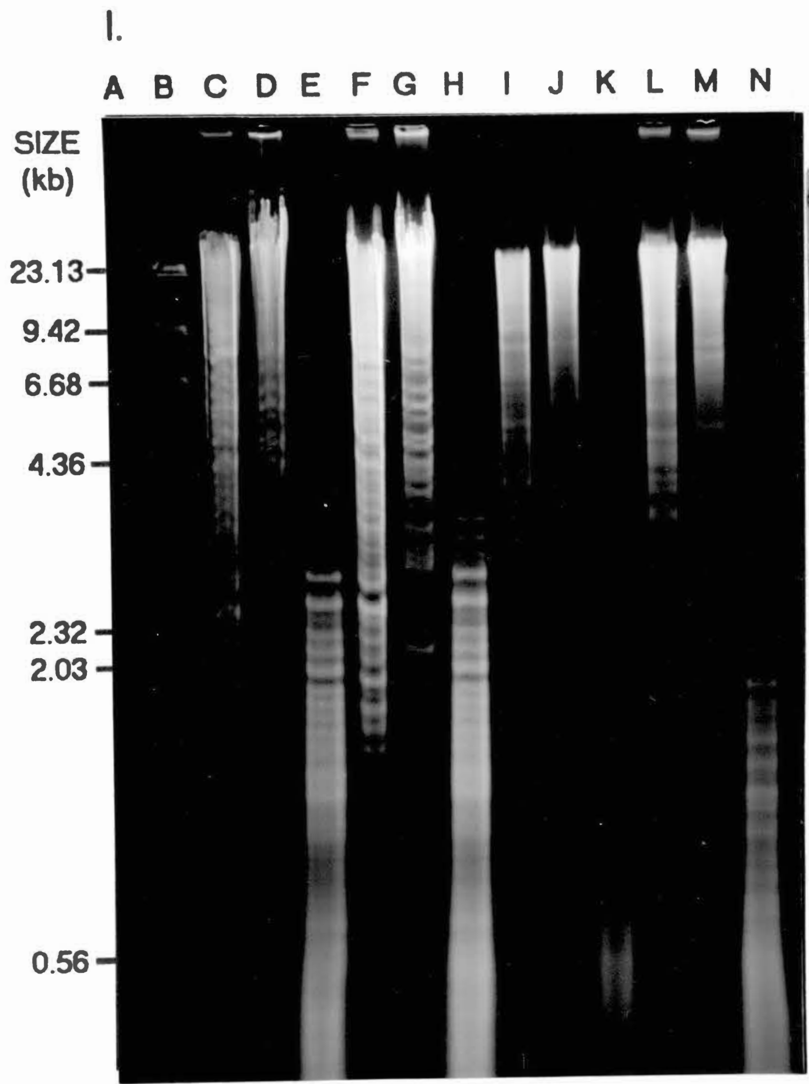

II.
A B C D E F G H I J K L M N
SIZE (kb)
16.92
2.83
1.33

Figure 9. Hybridization of biotin-labeled pC194 DNA to restriction fragments of parent and transformant chromosomal DNA from *Propionibacterium* strains. (I.) Agarose gel electrophoresis of restriction digests of chromosomal DNA from *Propionibacterium* strains. Lanes: A, pC194 plasmid DNA control; B, *Hin*d III digest of biotin-labeled lambda DNA; C through E, *Eco*R I, *Hin*d III, and *Hin*f I digests of P104 parent chromosomal DNA; F through H, *Eco*R I, *Hin*d III, and *Hin*f I digests of P104 transformant P3104/1 chromosomal DNA; I through K, *Eco*R I, *Hin*d III, and *Hin*f I digests of P5-3 parent chromosomal DNA; L through N, *Eco*R I, *Hin*d III, and *Hin*f I digests of P9 parent chromosomal DNA. (II.) Hybridization of the pC194 probe to a nitrocellulose filter containing restriction fragments of chromosomal DNA shown in panel I.


I.
A B C D E F G H I J K L M N
SIZE (kb)
23.13
9.42
6.68
4.36
2.32
2.03
0.56

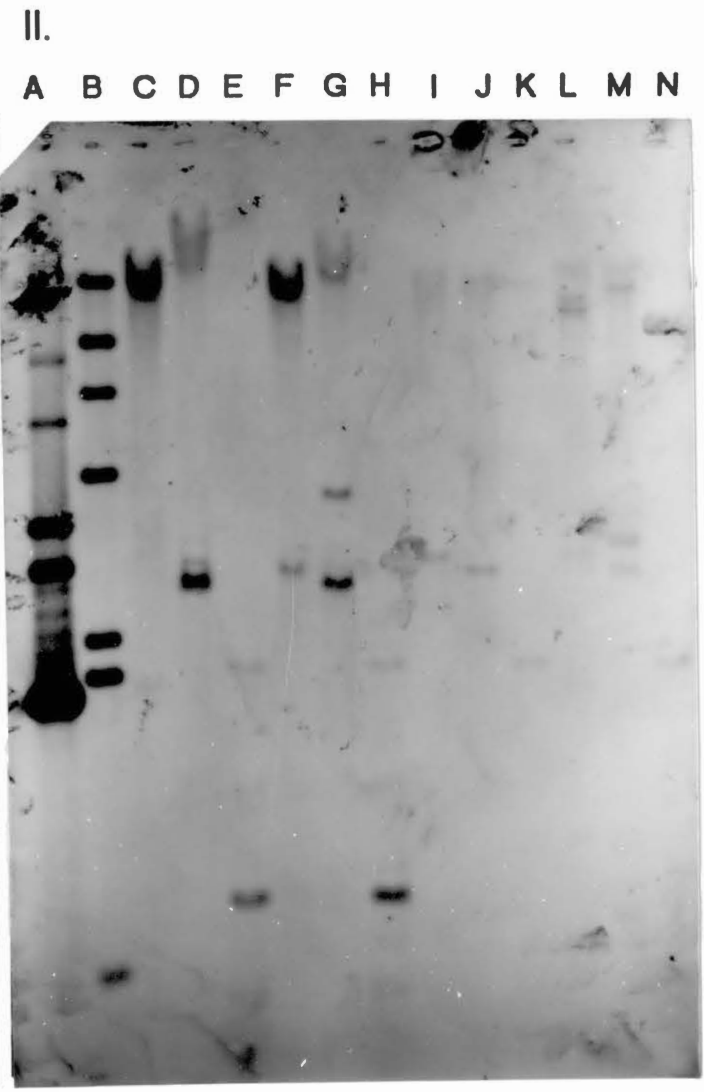
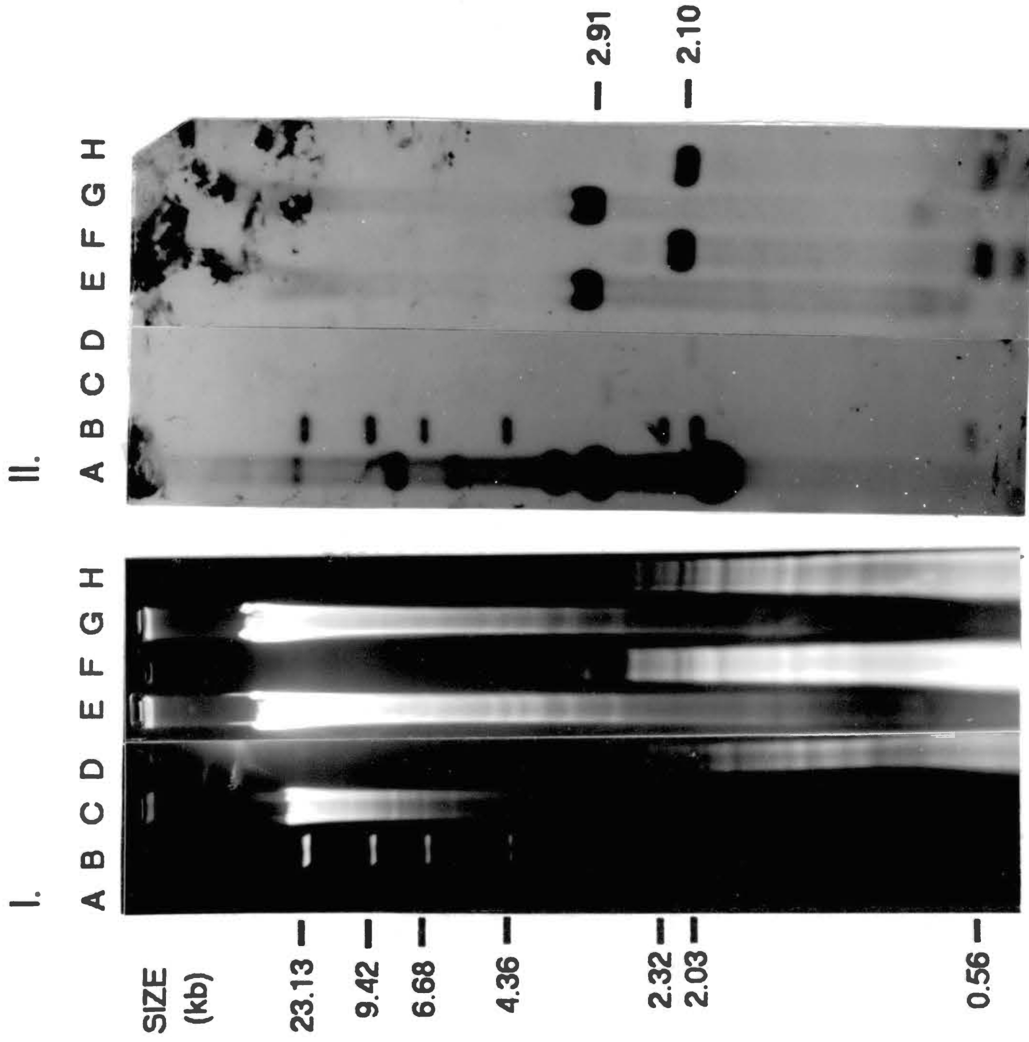

II.
A B C D E F G H I J K L M N

Figure 10. Hybridization of biotin-labeled pC194 DNA to restriction fragments of parent and transformant chromosomal DNA from *Propionibacterium* strain P5-3. (I.) Agarose gel electrophoresis of restriction digests of chromosomal DNA from *Propionibacterium* strain P5-3. Lanes; A, pC194 plasmid DNA control; B, *Hind* III digest of biotin-labeled lambda DNA; C and D, *Hind* III, and *Hinf* I digests of P5-3 parent chromosomal DNA; E and F, *Hind* III, and *Hinf* I digests of P5-3 transformant P3005-3/62 chromosomal DNA; G and H, *Hind* III, and *Hinf* I digests of P5-3 transformant P3005-3/63 chromosomal DNA. (II.) Hybridization of the pC194 probe to a nitrocellulose filter containing restriction fragments of chromosomal DNA shown in panel I. All of the lanes shown are from the same gel and its corresponding Southern blot filter.



I.
A B C D E F G H
II.
A B C D E F G H
2.91
2.10
SIZE
(kb)
23.13
9.42
6.68
4.36
2.32
2.03
0.56

Table 6. *Propionibacterium* parent and transformant restriction fragments hybridized with pC194 probe DNA

Species and strain	Transformant Code #	Size (kb) of restriction fragments		
		*Eco*R I	*Hin*d III	*Hin*f I
P. acidipropionici				
P5-3 (Parent)		3.195	2.908	2.101
P5-3	P3005-3/62	[a]	3.052 0.978 0.578	2.114 0.414 0.133
P5-3	P3005-3/63	[a]	3.028 1.013	2.101 0.380 0.125
P9 (Parent)		24.062 16.810 13.793 3.168 1.977 1.690	26.359 20.312 3.367 2.981 1.305	2.144
P. freudenreichii				
P7 (Parent)		16.916	25.477 2.833	1.333
P7	P3007/11	23.056 4.534 3.455 2.998	40.242 10.614 4.760 2.969 2.559	1.808 1.565 1.377 1.108 0.585
P7	P3007/15	20.975 3.173	37.801 2.969	2.178 1.377
P104 (Parent)		18.080 1.998	29.008 3.455 3.140 2.855	2.130 1.432 0.333

Table 6. (Continued from the previous page)

Species and strain	Transformant Code #	Size (kb) of restriction fragments		
		*Eco*R I	*Hin*d III	*Hin*f I
P104	P3104/1	17.425 3.033	24.062 4.101 3.367 3.086 2.855	2.144 1.746 1.447 0.463
pC194 plasmid DNA		No sites	One site 2.910	Three sites 2.064 0.472 0.374

[a] P5-3 transformants were never analyzed using *Eco*R I for digestion of chromosomal DNA samples.

The *Hinf* I digests of strain P7 transformants are shown in lane H and K (Figure 8). Both transformants had a hybridization signal corresponding to the band in the parent strain (lane E). Transformant P3007/11 produced several additional fragments. The exact nature of these fragments is unclear. The hybridization bands detected may be junction fragments or partial digest fragments or a combination of these two possibilities. Transformant P3007/15 (lane K) produced a fragment 2.18 kb in size in addition to a 1.377 kb band possibly the same as the native homology band to pC194. No other small bands were present.

The additional hybridization bands detected in P7 transformants are evidence for chromosomal integration of pC914 but do not suggest any simple integration model such as Campbell-like integration (10). Integration followed by rearrangements could explain the hybridization signals detected.

Restriction digests of P104 parent and transformant DNA can be seen in Figure 9, lanes C through H. The *Eco*R I digests of the P104 transformant (lane F) showed two bands of hybridization. The 17.4 kb signal may be identical to the largest band of hybridization detected in the parent (lane C). The additional signal (3.0 kb) is nearly equivalent to the linear form of pC194 and was larger than the faint hybridization band detected in the parent (1.9 kb) in lane C. The *Hin*d III digest of P3104/1 in lane G contained one extra band besides the native homology bands seen for the parent strain (lane D). This extra band is 4.1 kb which may be a junction fragment of pC194:P104 chromosomal DNA. The bands of hybridization detected in the *Hinf* I digest of P3104/1 (lane H) differed from the native bands of homology. The transformant had one additional band of homology of 1.75 kb and a band of 0.46 kb which may represent a junction fragment. There is a band in both the parent and transformant *Hinf* I digests (lanes E and H) that is about 2.1 kb and is believed to be the 2.064 kb band expected from a *Hinf* I digest of pC194.

Two transformants of strain P5-3 are shown in Figure 10, lanes E, F, G, and H. Both P5-3 transformants had an intense band of homology of approximately 3.05 kb in size when their chromosomal DNA was digested with *Hin*d III (lanes E and G). In addition, transformant P3005-3/62 had two additional bands of homology at 0.98 and 0.58 kb (lane E) and transformant P3005-3/63 had one additional band at 1.0 kb (lane G). The absence of a second additional band in lane G may be due to comigration of the band with another band, or the band was too small or indistinct to be detected. Chromosomal DNA of the P5-3 parent gave a hybridization signal at approximately 2.91 kb when cut with *Hin*d III (lane C) which is identical in size to the linear form of pC194. These results are consistent with the integration of pC194 into the chromosome by a Campbell-like mechanism with subsequent amplification of the pC194 fragment. Two (or more) linear copies of pC194 separated by a relatively small direct repeat of the homologous chromosomal DNA fragment digested with *Hin*d III would account for the presence of the intense 3.05 kb band in lanes E and G of Fig. 10.

Lanes F and H of Figure 10 show the results of digesting P3005-3/62 and P3005-3/63 chromosomal DNA with the restriction enzyme *Hin*f I. Both transformants had a intense band at 2.1 kb, the expected size of the largest *Hin*f I fragment of pC194, and two additional bands of homology. The smaller bands may represent junction fragments of pC194 and the native homologous fragment. These results would be consistent with integration of pC194 followed by amplification and subsequent rearrangement.

All transformants were screened for characteristic biochemical and fermentation patterns to confirm the identity of each isolate. Transformants obtained from P7 and P5-3 were identical to their respective parent strains. However, the single transformant of strain P104, designated P3104/1, lost its ability to reduce nitrate to nitrite. Restriction fragment patterns of chromosomal DNA from strain P104 and P3104/1 were identical. This suggests that pC194 inserted into the chromosome of strainP104 at or near the genes for nitrate reduction causing these genes to be inactivated.

DISCUSSION

Conditions for successful transformation of propionibacteria using electroporation were established. Three of the five strains examined were transformed with pC194. A fourth strain, P93-37, is believed to have been transformed due to its alteration in phenotype to Cm^r, but this has not yet been confirmed by Southern blot analysis. It is interesting to note that *Propionibacterium freudenreichii* P7, which produces extracellular slime, had the highest efficiency of transformation (1.49 x 10^2 transformants per ug of DNA).

Several parameters were examined and found to influence the efficiency of transformation of strain P7. Field strength, pulse duration, electroporation buffer and DNA concentration all had a significant effect on transformation efficiencies. The growth phase of the cells and electroporation with fresh or frozen cells did not significantly affect transformation efficiencies. Both ddH_2O and 30% PEG (M.W. 10,000) in ddH_2O work well for transforming P7, even in the two extremes of field strength we have found to be successful.

A number of investigators have indicated that field strength and pulse duration are the most important parameters in developing and optimizing bacterial transformation systems. Optimal electric field parameters have generally been reported as the single pulse duration and field strength combination that resulted in the highest transformation efficiency. Since pulse duration and field strength were expected to have a significant interaction, response surface analysis was used to determine the optimum electrical parameters for transformation. The results indicated that strain P7 transforms most efficiently between field strengths of 3.6 to 7.1 kV/cm and time constants from 0.17 to 27.1 msec. To our knowledge, no other reports have been published using this statistical technique to optimize electrical parameters for transformation efficiency. The advantage

of using this technique is that it accurately predicts the optimal conditions for transformation without running all combinations of pulse duration and field strength settings. Thus, optimal electrical settings can be determined more rapidly with less dependence on a single pulse duration-field strength setting for optimal results. In addition, since field strength and pulse duration have a compensatory effect, the region of optimal electric parameters predicted by the response surface are broader and a more realistic view of the optimal pulse duration-field strength settings. Although this technique is useful to predict optimal conditions, care must be taken not to rely on extrapolations beyond the experimental parameters since these predictions are untrustworthy.

A pulse duration of 5 msec worked well when the field strength was low, near 5 kV/cm. However, at extremely high field strengths (near 40 kV/cm) it was necessary to lower the pulse duration to around 100 μsec to obtain transformation. Transformation at field strengths near 40 kV/cm may be due to the more square shape of the electrical pulse when the Power Plus module was used. A square waveform was reported to have produced about one log more transformants per μg of pUC18 plasmid DNA in one study (13) and from 0.1 to 0.6 logs more transformants per μg of pC194 plasmid DNA in a second study (1). However, Wolf *et al.* (17) reported efficient transformation of intact cells of *Corynebacterium glutamicum* using a custom-made electroporation apparatus set at field strengths of 35-40 kV/cm and time constants of 450-500 μsec which produced exponentially decaying pulses. Accurate control of the shape of the electrical pulse and its duration might prove useful for increasing the transformation efficiency of pC194 with propionibacteria. No transformants were noted below a threshold field strength of about 1.7 kV/cm when propionibacteria were electroporated in ddH_2O. This threshold phenomenon was also noted by earlier researchers (5).

The optimum electroporation conditions established for strain P7 have not been successful in achieving genetic transformation of all strains of propionibacteria tested.

Only one transformant was recovered from strain P104. No transformants were recovered from strain P9. The difficulty in transforming strains P9 and P104 with pC194 DNA may be due to a number of reasons including the presence of DNA restriction systems, extracellular nucleases, structure of the cell wall, cell aggregation and strain specific factors affecting integration and plasmid maintenance. All propionibacteria strains tested exhibited extracellular nuclease activity that has been shown to degrade the transforming DNA. Strain P9 is also recalcitrant to standard lysis procedures, so it is conceivable that transforming DNA entering the cell would have to breach a formidable barrier. Cells of strain P9 have also been shown to form aggregates that could reduce the availability of the transient pores formed during electroporation. Indications are that different strains will require modifications of the electroporation procedure for successful transformation.

Putative transformants were screened for plasmid DNA to confirm the presence of pC194 in the electroporation-induced transformants. Plasmid DNA was not detected in any of the transformants using a micro-scale or preparative scale lysis procedure. These results indicate that pC194 integrated into the chromosome of propionibacteria recipients. This conclusion is based on the following evidence; no autonomous plasmids were detected in any of the transformants, homology was detected between pC194 and the chromosome of all strains of propionibacteria tested, Southern hybridization of undigested chromosomal DNA of transformants showed a single band of DNA homology that comigrated only with the undigested chromosomal DNA (data not shown), and additional bands of homology were detected in digests of transformants. The mechanism of integration of pC194 into the propionibacteria recipients remains to be elucidated fully but some preliminary assumptions about the mechanism can be made.

Integration of pC194 into the *Propionibacterium* chromosome may have occurred by a Campbell-like integration using the sequence homology between the plasmid and the chromosome. Southern hybridization analysis of restriction digests of chromosomal

DNA from transformants identified a fragment in all transformants that was roughly equivalent to linear pC194 in *Hin*d III digests. This can be explained by integration into the chromosome followed by amplification of pC194. A Campbell-like mechanism of integration has been proposed to lead to duplication of the homologous chromosomal insert and may create a substrate for amplification and subsequent rearrangements (10, 18). This type of integration has been well documented in *B. subtilus* and *Lactococcus lactis*. The hybridization results obtained by integration of pC194 into the chromosome of P5-3 appears to be very similar to that of *E. coli* plasmid pHV60 when inserted into the chromosome of *L. lactis* subsp. *lactis* MG1363 as reported by Leenhouts *et al.* (10). Digests of MG1363 transformant DNA cut with restriction enzymes that have unique restriction sites in pHV60 showed one intense signal corresponding to the linear form of pHV60 and two additional bands. The two additional bands were thought to be junction fragments of plasmid:chromosomal DNA. They also detected homology between pHV60 and the chromosome of strain MG1363. Integration of pHV60 was proposed to occur by a Campbell-like mechanism and subsequent amplification. We believe this to be the most likely mechanism of pC194 integration into the P5-3 chromosome at this time. Further work will be necessary to confirm this mechanism of integration.

Southern hybridization analysis of two transformants from strain P7 does not provide a straightforward interpretation of possible mechanisms for the integration of pC194 into the chromosome. Digestion of transformant chromosomal DNA with a restriction enzyme which has no sites in pC194 would produce a single hybridization band expected to be larger than the native-homology band. However when *Eco* R I, which has no sites in pC194, was used to digest P7 transformant DNA , hybridization signals were detected at the position of linear pC194 in both transformants. In addition, transformant P3007/11 had two additional hybridization bands that were also smaller than the bands detected in the parent strain. These hybridization bands could be the result of rearrangements in the amplified structure or integration at homolgous regions

undetected in the parent strain. Alternatively, integration of pC194 at multiple, secondary sites may also explain the presence of additional bands. The *Hin*d III and *Hin*f I digests of transformant P3007/11 also produced additional bands not predicted by a single Campbell integration. Although these bands can be interpreted as amplified segments of pC194, junction fragments and partial digestion products, similar results could be explained by rearrangements of the amplified structure of pC194. Likewise, the absence of additional bands in the *Hin*d III and *Hin*f I digest of transformant P3007/15 may also be due to rearrangements. In this case the resulting fragments may have been too small to be detected clearly. Hybridization analysis of the P104 transformant also produced bands that may reflect rearrangements or other complex mechanisms of integration.

Insertion of pC194 into a short sequence of DNA outside of the native bands of homology would be a second way to account for the hybridization bands seen in some of the transformants. A recombination model involving a conservative, reciprocal strand exchange between a recombination site on pE194 and a short, homologous site in the chromosome of *Bacillus subtilis* has been proposed (4). The origin of replication of pE194, a *Staphylococcus aureus* plasmid related to pC194, contains a GC-rich dyad symmetry element. Five of seven pE194-integrated strains of *B. subtilis* analyzed had been produced by recombination at different locations within this 70-base-pair interval of pE194. Recombination had occurred between regions of short nucleotide homology (6 to 14 base pairs) and the integrated pE194 molecule was bounded by direct repeats of the short homology. It is possible that pC194 may have integrated into a short region of homology in the *Propionibacterium* chromosome that may or may not have been part of the hybridization signals detected. This mode of insertion could have occurred even if the *Propionibacterium* strains we used did not have a system of major homology-dependent recombination, such as the RecE recombination system of *Bacillus subtilis* and *E. coli* (4). Since pC194 replicates by way of a single-stranded DNA intermediate by

what is believed to be a rolling circle replication mechanism, the production of ssDNA greatly increases the recombination capacity both by homologous and illegitimate recombination (6).

The insertion of pC194 into the chromosome of strain P104 appears to have eliminated the ability of this strain to reduce nitrate to nitrite. If pC194 insertion actually caused this loss, this would predict that the nitrate reductase gene must lie somewhere near the site of insertion of pC194. This would allow us to clone this gene for further analysis and would clearly demonstrate the feasibility of using an integration strategy for genetic analysis of propionibacteria chromosomal DNA.

The native *Propionibacterium* bands of homology to pC194 seem to migrate a distance that would be expected of a nearly full complement of pC194 plasmid DNA. However, this may be purely coincidental. If it were the case, however, then a nearly complete copy of pC194 already exists in the chromosome of the *Propionibacterium* strains tested. Since all strains tested are sensitive to chloramphenicol, it would be expected that the inserted chloramphenicol acetyl transferase (CAT) gene is defective. All strains examined appear to have unique hybridization profiles when probed with pC194. However, two hybridization fragments appear to be conserved, a 2.9 kb *Hin*d III fragment and a 2.1 kb *Hin*f I fragment. It is interesting to note that the 2.9 and 2.1 kb size for these fragments are equivalent to the linear form of pC194 produced by *Hin*d III digestion and the largest fragment produced by *Hin*f I digestion respectively. This may also be a clue as to why one of the *Propionibacterium* strains examined by Naud *et al.* (13) was capable of gaining resistance to chloramphenicol when placed under various environmental stresses.

The first reported result of successful electroporation in propionibacteria was by Luchansky *et al.* (11). They reported the transformation of a single strain of *Propionibacterium jensenii* with plasmid pGK12. This plasmid contains the Cm^r gene of pC194 but not the origin of replication of pC194. They reported the identification of an

autonomous plasmid in the transformants they isolated. It could be that this *Propionibacterium jensenii* strain had no native homology with the portion of pC194 in pGK12. Alternatively, this strain may have no homology with pC194. To date, all strains of propionibacteria examined in our lab contain native homology with pC194.

Electroporation is a well established method for transformation of Gram-positive and Gram-negative bacteria. Other methods of gene transfer, such as protoplast transformation, have not been successful to date in propionibacteria. Electroporation has many advantages over other gene transfer methods. The actual procedure takes only a few minutes to perform once the cells are prepared. Frozen cells may be used in lieu of fresh cells to further simplify the procedure. In the future, as the electroporation protocol improves, it is hoped that this technique will be useful for genetic analysis and improvement of propionibacteria.

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CHAPTER IV

SUMMARY AND CONCLUSIONS

A repeatable method for the transformation of dairy propionibacteria has been developed that uses electroporation as the means of molecular transfer. Three strains of propionibacteria have been proven by Southern blot analysis to be transformed by the plasmid pC194. A fourth strain has gained resistance to chloramphenicol and is also believed to have been transformed by pC194. The pC194 plasmid did not replicate as an autonomous plasmid in the strains of *Propionibacterium* tested. Instead, the transforming plasmid integrated into the *Propionibacterium* genome. This integration was most likely into the chromosome, but there is a remote possibility that it may have integrated into a large plasmid of similar size to the chromosomal DNA.

Transformation occurred with greater efficiency near a field strength of 5.4 kV/cm with a 5 ms pulse duration and at a field strength near 40 kV/cm with about a 100 to 160 μsec pulse duration. The highest transformation efficiency to date was with cells of strain P7 from the mid-log phase in ddH_2O as the electroporation buffer, a 5.4 kV/cm field strength, and a 5 msec pulse duration for a yield of 1.5×10^2 transformants/μg of pC194 plasmid DNA. Early indications are that cells that had been frozen can be used instead of fresh cells with no significant decrease in transformation efficiency. Also, limited testing indicated a small advantage may be gained by using cells from the early-log phase of growth instead of mid-log phase cells in the electroporations. The highest transformation efficiencies were achieved with picogram levels of pC194 DNA. This indicated some sort of limiting factor was involved besides the amount of transforming

DNA added during the electroporation experiment. Factors that could limit the number of transformants obtained could be the number of electropores produced in the cells that would allow the passage of transforming DNA, the number of cells in the population competent for pore formation and/or integration of the plasmid, or the number of molecules of pC194 plasmid DNA in a form suitable for integration (possibly some ssDNA intermediate). Transformation may also have varied in efficiency from strain to strain due to variations in nuclease enzyme activity and the rate at which cells of a particular strain are killed by the transforming pulse, not to mention differences in their cell walls or membranes. The ability of a strain to produce slime may be another factor involved in efficient electroporation of propionibacteria. *Propionibacterium freudenreichii* strain P104 yielded only one transformant, that was highly resistant to chloramphenicol. It appears at this time that the entry of the pC194 plasmid molecule into the chromosome of this transformant caused insertional inactivation of a gene in the metabolic pathway that reduces nitrate to nitrite.

Future work should include trying to improve the electroporation procedure. Data related to the ability of lysozyme to produce protoplasts of the four *Propionibacterium* strains used in this study has already been collected. This data should be used to determine if removal of part or all of the cell wall of these bacteria will improve their electroporation efficiencies. The location of the integration site of pC194 into the chromosome of the P104 transformant should also be determined. The DNA sequence surrounding this site should be analyzed to determine the mechanism of pC194 integration and to determine the sequence of the gene related to the loss of the ability to reduce nitrate to nitrite. The integration sites for strains P7 and P5-3 should also be determined. What appears to be amplified pC194 regions in the P5-3 chromosome should be further analyzed. This could lead to a method for inserting any desired gene into the chromosome at that site and amplifying it for the high level of expression of a desired trait. Furthermore, with the use of pulse gel electrophoresis and insertion of

pC194 into the chromosomes of these bacteria it is probable that the locations of other genes can be mapped. The enzymes responsible for the endonuclease, exonuclease, and ligase enzyme activities noted by the *Propionibacterium* strains used in this study should also be isolated for further analysis.

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APPENDIXES

APPENDIX A

DATA FROM TREATMENTS

TABLE 7

FIRST COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY;
DATA FROM THE FIRST REPLICATION

Estimated Field Strength (kV/cm)	Voltage Setting (kV)	Time (msec)								
		0.1	0.5	1	5	10	50	100	500	1000
0.89	0.05	0	0	0	0	0	0	0	0	0
1.79	0.1	0	0	0	0	0	0	0	0	0
3.57	0.2	46	70	20	59	60	31	22	11	16
5.36	0.3	28	56	14	59	45	1	13	1	0
7.14	0.4	21	48	59	41	41	0	0	0	0

- P7 mid-log cells electroporated with 1 ug of pC194 plasmid DNA in Milli-Q H_2O.
- Values represent transformants recovered from the first of two replications.

TABLE 8

FIRST COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY;
DATA FROM THE SECOND REPLICATION

Estimated Field Strength (kV/cm)	Voltage Setting (kV)	Time (msec)								
		0.1	0.5	1	5	10	50	100	500	1000
0.89	0.05	0	0	0	0	0	0	0	0	0
1.79	0.1	0	0	0	0	0	0	3	0	0
3.57	0.2	0	68	60	84	75	0	0	21	6
5.36	0.3	0	33	32	44	35	15	29	1	12
7.14	0.4	0	52	49	79	49	6	0	0	0

- P7 mid-log cells electroporated with 1 ug of pC194 plasmid DNA in Milli-Q H_2O.
- Values represent transformants recovered from the second of two replications.

TABLE 9

FIRST COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY; TRANSFORMANT MEANS

Estimated Field Strength (kV/cm)	Voltage Setting (kV)	Time (msec)								
		0.1	0.5	1	5	10	50	100	500	1000
0.89	0.05	0	0	0	0	0	0	0	0	0
1.79	0.1	0	0	0	0	0	0	1.5	0	0
3.57	0.2	23	69	40	71.5	67.5	15.5	11	16	11
5.36	0.3	14	44.5	23	51.5	40	8	21	1	6
7.14	0.4	10.5	50	54	60	45	3	0	0	0

- P7 mid-log cells electroporated with 1 ug of pC194 plasmid DNA in Milli-Q H_2O.
- Values represent the means of transformants recovered from two replications.

TABLE 10

SECOND COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY; TRANSFORMANT DATA AND ACTUAL OSCILLOSCOPE READINGS

13 Ohms Resistance

Field Strength (kV/cm)	Average Actual kV	Average Actual msec	Trial 1 Transformants	Trial 2 Transformants	Mean Transformants
9.0	0.715	0.607	34	16	25
13.5	1.10	0.612	27	7	17
18.9	1.58	0.611	23	9	16
24.3	2.05	0.608	17	10	13.5

48 Ohms Resistance

Field Strength (kV/cm)	Average Actual kV	Average Actual msec	Trial 1 Transformants	Trial 2 Transformants	Mean Transformants
9.0	0.78	2.085	32	16	24
13.5	1.23	2.035	22	4	13
18.9	1.76	1.96	0	0	0
24.3	2.25[a]	1.91[a]	0[b]	0[b]	0[b]

TABLE 10 (Continued)

186 Ohms Resistance

Field Strength (kV/cm)	Average Actual kV	Average Actual msec	Trial 1 Transformants	Trial 2 Transformants	Mean Transformants
9.0	0.815	5.485	51	13	32
13.5	1.265	4.82	0	4	2
18.9	[c]	[c]	0	0	0
24.3	[c]	[c]	0[b]	0[b]	0[b]

720 Ohms Resistance

Field Strength (kV/cm)	Average Actual kV	Average Actual msec	Trial 1 Transformants	Trial 2 Transformants	Mean Transformants
9.0	0.835	9.125	24	4	14
13.5	1.24[a]	3.23[a]	0	0	0
18.9	[c]	[c]	0[b]	0[b]	0[b]
24.3	[c]	[c]	0[b]	0[b]	0[b]

[a] One of the two trials had no oscilloscope reading, due to arcing.

[b] These values were excluded from the statistical analysis.

[c] No oscilloscope reading could be collected for either trial, due to arcing.

APPENDIX B

STATISTICAL ANALYSES

TABLE 11

ANALYSIS OF VARIANCE FOR THE FIRST COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY

Analysis of Variance Procedure

Dependent Variable Y

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	36	40040.50000000	1112.23611111	7.16	0 0001
Error	35	5440.37500000	155.43928571		
Corrected Total	71	45480 87500000			

R-Square	C.V.	Root MSE	Y Mean
0.880381	59 25162	12.46752925	21 04166667

Source	DF	Anova SS	Mean Square	F Value	Pr > F
REP	1	1.12500000	1.12500000	0 01	0.9327
V	3	12230.70833333	4076.90277778	26 23	0 0001
T	8	19034 50000000	2379.31250000	15 31	0 0001
V*T	24	8774.16666667	365.59027778	2 35	0.0104

TABLE 12

ANALYSIS OF VARIANCE AND PARAMETER ESTIMATES OF THE REGRESSION MODEL FOR THE FIRST COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	5	21807.83272	4361.56654	12.160	0.0001
Error	66	23673.04228	358.68246		
C Total	71	45480.87500			

Root MSE	18 93891	R-square	0.4795
Dep Mean	21 04167	Adj R-sq	0 4401
C V	90 00672		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	T for H0: Parameter=0	Prob > |T|
INTERCEP	1	-34.690261	13.27927892	-2.612	0 0111
V	1	29.414765	6.41887941	4.583	0 0001
LOGT	1	5.728464	2.29635602	2 495	0 0151
V2	1	-2.696210	0.70001656	-3.852	0 0003
LOGT2	1	-1.109102	0.28434511	-3 901	0 0002
VLOGT	1	-0.771716	0.37498629	-2 058	0 0435

TABLE 13

SAS ANALYSIS AT 13 OHMS OF RESISTANCE FOR THE SECOND COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY

GLM RUN WITH LINEAR ONLY TERM IN THE MODEL, TRIAL*VOLT OUT

OHMS=13

General Linear Models Procedure

Dependent Variable COLONIES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	556 48156682	278 24078341	18 21	0 0051
Error	5	76 39343318	15 27868664		
Corrected Total	7	632 87500000			

R-Square	C V	Root MSE	COLONIES Mean
0 879291	21 86739	3 90879606	17 87500000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRIAL	1	435 12500000	435 12500000	28 48	0 0031
VOLTAGE	1	121 35656682	121 35656682	7 94	0 0372

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRIAL	1	435 12500000	435 12500000	28 48	0 0031
VOLTAGE	1	121 35656682	121 35656682	7 94	0 0372

Parameter		Estimate	T for H0: Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT		21 64343318 B	4 91	0 0044	4 41059355
TRIAL	1	14 75000000 B	5 34	0 0031	2 76393620
	2	0 00000000 B			
VOLTAGE		-6 78443420	-2 82	0 0372	2 40727112

NOTE The X'X matrix has been found to be singular and a generalized inverse was used to solve the normal equations Estimates followed by the letter 'B' are biased, and are not unique estimators of the parameters

TABLE 14

SAS ANALYSIS AT 48 OHMS OF RESISTANCE FOR THE SECOND COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY

GLM RUN WITH LINEAR ONLY TERM IN THE MODEL, TRIAL*VOLT OUT

---------- OHMS=48 ----------

General Linear Models Procedure

Dependent Variable COLONIES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	769 98901099	384 99450549	11 86	0 0376
Error	3	97 34432234	32 44810745		
Corrected Total	5	867 33333333			

R-Square	C V	Root MSE	COLONIES Mean
0 887766	46 18641	5 69632403	12 33333333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRIAL	1	192 66666667	192 66666667	5 94	0 0928
VOLTAGE	1	577 32234432	577 32234432	17 79	0 0244

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRIAL	1	192 66666667	192 66666667	5 94	0 0928
VOLTAGE	1	577 32234432	577 32234432	17 79	0 0244

Parameter		Estimate	T for H0 Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT		40 11355311 B	4 67	0 0185	8 58438539
TRIAL	1	11 33333333 B	2 44	0 0928	4 65102909
	2	0 00000000 B			
VOLTAGE		-24 23687424	-4 22	0 0244	5 74595356

NOTE The X'X matrix has been found to be singular and a generalized inverse was used to solve the normal equations Estimates followed by the letter 'B' are biased, and are not unique estimators of the parameters

TABLE 15

SAS ANALYSIS AT 186 OHMS OF RESISTANCE FOR THE SECOND COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY

GLM RUN WITH LINEAR ONLY TERM IN THE MODEL, TRIAL*VOLT OUT

-- OHMS=186 --

General Linear Models Procedure

Dependent Variable COLONIES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	1160 41758242	580 20879121	2 04	0 2763
Error	3	854 91575092	284 97191697		
Corrected Total	5	2015 33333333			

R-Square	C V	Root MSE	COLONIES Mean
0 575794	148 9510	16 88111125	11 33333333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRIAL	1	192 66666667	192 66666667	0 68	0 4712
VOLTAGE	1	967 75091575	967 75091575	3 40	0 1626

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRIAL	1	192 66666667	192 66666667	0 68	0 4712
VOLTAGE	1	967 75091575	967 75091575	3 40	0 1626

Parameter		Estimate	T for H0. Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT		48 97069597 B	1 92	0 1499	25 43990898
TRIAL	1	11 33333333 B	0 82	0 4712	13 78336962
	2	0 00000000 B			
VOLTAGE		-31 37973138	-1 84	0 1626	17 02818885

NOTE The X'X matrix has been found to be singular and a generalized inverse was used to solve the normal equations Estimates followed by the letter 'B' are biased, and are not unique estimators of the parameters

TABLE 16

SAS ANALYSIS AT 720 OHMS OF RESISTANCE FOR THE SECOND COMPREHENSIVE FIELD STRENGTH-TIME CONSTANT STUDY

GLM RUN WITH LINEAR ONLY TERM IN THE MODEL, TRIAL*VOLT OUT

-- OHMS=720 --

General Linear Models Procedure

Dependent Variable COLONIES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	296 00000000	148 00000000	1 48	0 5025
Error	1	100 00000000	100 00000000		
Corrected Total	3	396 00000000			

R-Square	C V	Root MSE	COLONIES Mean
0 747475	142 8571	10 00000000	7 00000000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
TRIAL	1	100 00000000	100 00000000	1 00	0 5000
VOLTAGE	1	196 00000000	196 00000000	1 96	0 3949

Source	DF	Type III SS	Mean Square	F Value	Pr > F
TRIAL	1	100 00000000	100 00000000	1 00	0 5000
VOLTAGE	1	196 00000000	196 00000000	1 96	0 3949

Parameter		Estimate	T for H0. Parameter=0	Pr > |T|	Std Error of Estimate
INTERCEPT		37 00000000 B	1 42	0 3897	25 98076211
TRIAL	1	10 00000000 B	1 00	0 5000	10 00000000
	2	0 00000000 B			
VOLTAGE		-31 11111111	-1 40	0 3949	22 22222222

NOTE The X'X matrix has been found to be singular and a generalized inverse was used to solve the normal equations Estimates followed by the letter 'B' are biased, and are not unique estimators of the parameters

VITA

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