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PRODUCTION AND REGENERATION OF PROTOPLASTS IN PROPIONIBACTERIUM

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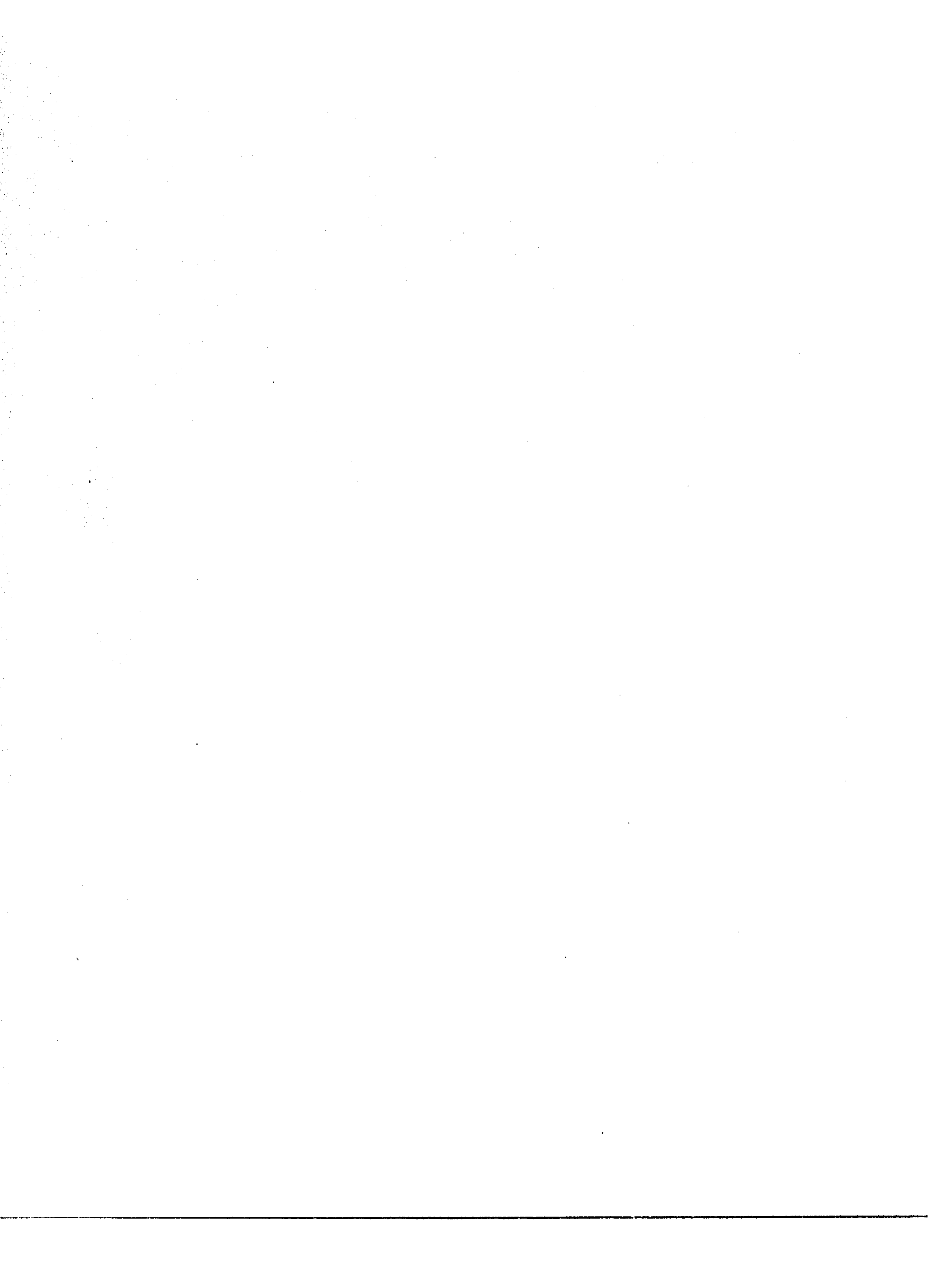

Production and regeneration of protoplasts

in *Propionibacterium*

by

LeAnn R. Baehman

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major: Food Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major Department

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For the Graduate College

Iowa State University
Ames, Iowa

1986

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INTRODUCTION

Considerable research has been devoted to understanding, controlling, and improving microbial fermentations. In the past, strain improvement relied upon techniques of mutagenesis and selection to produce organisms with altered characteristics.

Currently, more sophisticated techniques are available for the genetic manipulation of microorganisms. Instead of altering the DNA within the cell by mutagenesis, novel DNA is introduced from other sources. If the cell will accept and express the new genetic material, an organism with altered characteristics is produced. Recombinant DNA methods provide the means by which specific genetic information can be introduced into an organism. The potential for industrial strain improvement by the use of these methods is enormous. Recombinant DNA techniques require a method by which DNA that has been manipulated *in vitro* can be introduced into a host cell for expression. Techniques for the introduction of DNA into an organism therefore are essential for the alteration and improvement of industrial microorganisms.

Protoplasts of Gram-positive bacteria are produced by the careful removal of the bacterial cell wall by

enzymatic digestion. Since the cell wall provides osmotic protection for the cell, wall removal must be accomplished under hypertonic conditions to support and protect the osmotically fragile protoplasts. In the proper environment, protoplasts can resynthesize their cell wall and regenerate to normal cells. Protoplast production and regeneration techniques are essential to some genetic transfer systems. For example, protoplasts can be induced to fuse together. This fusion can result in the exchange of genetic material, and can be useful in the development of industrial microorganisms with novel characteristics. Protoplast transformation, the introduction of DNA into protoplasts, also has been reported for a variety of microorganisms.

The propionibacteria are an industrially important group of organisms. Propionibacteria are used to produce vitamin B_{12}, a nutrient essential to the diet of humans. Propionibacteria may also be used to produce propionic acid on an industrial scale, although the process currently is not employed for economic reasons. The most familiar use of propionibacteria is in the production of Swiss cheese. In 1985, over two hundred million pounds of Swiss cheese was produced in the United States (101). The propionibacteria are responsible for much of the typical

flavor of the cheese, as well as the characteristic eyes of the cheese.

The nutrition and metabolism of the propionibacteria have been studied extensively, but little research has been devoted to the genetics of these industrially important organisms. The development of genetic transfer systems in the propionibacteria would be invaluable, both to basic studies of *Propionibacterium* genetics and to the improvement of industrial strains. Methods for protoplast production and regeneration are prerequisites for some types of genetic exchange. The purpose of conducting this research was to create a method for the production of protoplasts of *Propionibacterium*, and to devise a medium that would support regeneration of the protoplasts to walled cells.

LITERATURE REVIEW

Propionibacteria

Propionibacteria are Gram-positive, nonspore-forming, facultatively anaerobic bacteria that produce large amounts of propionic acid. Individual cells are usually rod-shaped, and pleomorphic forms are quite common. The eighth edition of Bergey's Manual of Determinative Bacteriology (7) lists eight species, four of which are dairy-related (*Propionibacterium freudenreichii*, *P. theonii*, *P. acidi-propionici*, and *P. jensenii*). The other four species (*P. avidum*, *P. acnes*, *P. lymphophilum*, and *P. granulosum*) are found on the skin of humans, and in abscesses and wounds. *Propionibacterium freudenreichii* is further divided into the subspecies *freudenreichii*, *globosum*, and *shermanii*.

Characteristics

Propionibacteria were first isolated from Emmentaler cheese by von Freudenreich and Orla-Jensen, as described by Langsrud and Reinbold (53). The primary natural habitat of propionic acid bacteria is the rumen of herbivores, where the organisms ferment the lactate produced by other members of the rumen population. In addition to the fermentation of lactate, the propionibacteria ferment a wide variety of other

carbohydrates. The best growth is usually obtained under anaerobic conditions, or low oxygen tensions and elevated carbon dioxide tensions; however, the propionibacteria do contain cytochromes and catalase (15), and are capable of aerobic growth.

Malik et al. (61) evaluated the taxonomy of the genus *Propionibacterium*. Fifty-six cultures of propionibacteria were collected, and 70 characteristics were determined for each culture. Thirty-two of the characteristics were uniform for all species, the other 38 characteristics were used to sort the cultures into groups. Past attempts to classify the propionibacteria had placed considerable emphasis on carbohydrate fermentation patterns. Malik et al. noted many inconsistencies between their fermentation results and the results that had been obtained by other researchers, as well as variation of results between replications of their own experiments. They suggested that some of the *Propionibacterium* species be consolidated, and that classification of the propionibacteria be improved by the use of additional physiological, genetic, serological, and phage host-range characteristics.

Attempts to discover additional features by which the propionibacteria could be classified produced reports of

capsulation of propionibacteria (95) and slime production (82). Antibodies produced against whole cell antigens of *P. freudenreichii* and *P. shermanii* cross-reacted in serological tests, and were not useful for identification and classification. Antibodies to capsular materials could not be produced.

The growth, nutritional requirements, and metabolism of the propionibacteria have been reviewed in detail by Hettinga and Reinbold (30, 31, 32). The nutritional requirements are complex, and vary from species to species. A defined medium has been developed (30), as well as a minimal medium (104). For routine culture purposes, the propionibacteria grow well in a medium that contains yeast extract, an enzymatic digest of protein, and a fermentable carbohydrate such as lactate.

Metabolism

Much of the research on *Propionibacterium* metabolism has been conducted by Wood (107). The major fermentation products of propionibacteria are propionic acid, acetic acid, and carbon dioxide. Lactate is initially oxidized to pyruvate. Part of the pyruvate is further oxidized to acetyl-CoA and carbon dioxide, and ATP is produced in the conversion of acetyl-CoA to acetate. Propionate formation from pyruvate occurs through a complex cycle of reactions

that involve both CoA transfers and transcarboxylations to form four-carbon intermediates between pyruvate and the final accumulating end product, propionate. One molecule of acetate is produced for every two molecules of propionate generated. Doelle (17) and Moat (69) both present excellent discussions of the individual reactions involved.

In practice, the expected ratio of two parts propionate to one part acetate is not always obtained for *Propionibacterium* fermentations. Succinate may accumulate, which causes a break in the cycle of reactions from pyruvate to propionate. The yields of both propionate and ATP are reduced in this case.

In some instances, the ratio of propionate to acetate is greater than two to one, and ATP yields are also increased. Wood (106) reported that inorganic pyrophosphate can replace ATP as a source of energy in some fermentation reactions of *P. shermanii*. The utilization of inorganic pyrophosphate as a source of energy is thought to be responsible for the increased yields of ATP in propionic acid fermentations.

When propionibacteria are grown aerobically, fermentation is suppressed, and energy is obtained by oxidative phosphorylation (15). DeVries et al. (16) have

determined that cytochrome b of P. freudenreichii functions in electron transport and the generation of ATP. Nitrate can act as an electron acceptor under anaerobic conditions (43). The nitrite produced by the reduction of nitrate is toxic to propionibacteria, and will inhibit growth unless further reduced to nitrous oxide. The reduction of nitrite to nitrous oxide is considered to be a mechanism for detoxification rather than part of an energy-yielding reaction (43).

Pritchard et al. (80) studied the effects of oxygen on P. shermanii. A system of continuous culture was used for the experiments, to determine whether the organisms could be adapted to low concentrations of oxygen. At low oxygen tensions, oxygen had a deleterious effect on growth. The concentration of cytochrome b fell to 25% of the anaerobic cytochrome b level. At a higher oxygen tension, cytochrome b production increased, as did cell yield. It should be noted, however, that no dissolved oxygen could be detected in the culture, even at the higher oxygen tensions. When the flow of oxygen into the culture was increased to the point that dissolved oxygen could be detected, severe growth inhibition occurred.

Industrial applications

The propionibacteria are important to several industrial processes. They produce large quantities of propionic acid that can be utilized commercially, and can also be used for the production of vitamin B_{12}. The propionibacteria are also essential to the dairy industry, where they are used for Swiss cheese manufacture.

Although organic acids may be produced by microbial fermentation, many, including propionic acid, currently are produced mainly by chemical synthesis (79). Propionic acid is used in a variety of industrial processes. Esters of propionic acid are used in the perfume industry. Salts of propionic acid have preservative and fungicidal properties, and cellulose propionate is an important thermoplastic (79).

Fermentation by *Propionibacterium* to produce propionic acid currently is not economically competitive with chemical production methods. Research is being conducted on the fermentation process, however, and may eventually lead to the commercial production of propionic acid by propionibacteria. Playne (79) has reviewed the process in detail, as well as the current research on propionic acid fermentation. Fermentation equations have been derived for the production of propionate from a

variety of sugars (73). Such information is valuable in the prediction of product yields from a number of substrates. The antibiotic monensin, when incorporated into cattle feed, increases the proportion of propionic acid that is produced in the rumen, but it cannot be used in pure cultures of propionibacteria to increase propionate yields (42). Continuous culture fermentation methods and immobilized cell reactors have been developed for propionic acid production (11). Technical advances such as these, combined with culture improvements, may lead to industrial production of propionic acid by fermentation.

Vitamin B_{12}, or cyanocobalamin, is an essential nutrient in the human diet. A deficiency of B_{12}, or the inability to adsorb the vitamin, results in pernicious anemia. Vitamin B_{12} is produced both for human consumption and as a growth factor in animal feed. Humans are completely dependent upon food consumption as a source of B_{12} (100).

The chemical structure of vitamin B_{12} is extremely complex. Floret and Ninet (18) outline the general structure of the molecule. The vitamin has been synthesized chemically (18), but the procedure is difficult. For industrial purposes, B_{12} is produced by

microbial fermentation.

Vitamin B_{12} production processes have been outlined by Perlman (77), and, more recently, by Floret and Ninet (18). Both *Propionibacterium* and *Pseudomonas* are used for industrial B_{12} production; however, only processes and research that involve the propionibacteria will be discussed.

Propionibacterium freudenreichii and *P. shermanii* have been used to produce vitamin B_{12} industrially. The fermentation medium generally consists of glucose or molasses, buffering agents, mineral salts, and nitrogenous compounds such as yeast extracts, casein hydrolysates, or corn steep liquor. Temperature of the fermentation is 30C, and the pH is maintained at 6.5-7.0.

To obtain a high yield of vitamin B_{12} from *Propionibacterium*, the first part of the fermentation must be run under anaerobic conditions. After cell growth has been completed and almost all the sugar in the medium has been utilized, oxygen is introduced into the fermentation to create a microaerobic environment. Biosynthesis of dimethylbenzimidazole, a component of the B_{12} molecule, proceeds under the aerobic conditions, and the completed vitamin B_{12} molecule is produced.

Extraction of the vitamin is accomplished by heating

the whole fermentation broth or an aqueous suspension of the harvested cells at 80-120C for 10-30 minutes at pH 6.5-8.5. The cobalamins are converted to cyanocobalamins by treatment of the heated suspension with cyanide or thiocyanate. The cyanocobalamins are then purified by extraction with organic solvents, and then the vitamin is crystallized.

Production of vitamin B_{12} by propionibacteria was described by Leviton and Hargrove (59) in 1952. These researchers also developed a fermentation process that utilized whey for B_{12} production. Whey was inoculated with a culture of Lactobacillus casei, to ferment the lactose in the whey and produce lactate. A Propionibacterium inoculum was then added, which consumed the lactate and produced vitamin B_{12}.

Bullerman and Berry (8) used whey as a direct substrate for P. shermanii, without prior fermentation by a lactic culture. It was necessary to supplement the whey with yeast extract, and the level of the yeast extract in the medium was critical for the efficient production of B_{12}. The addition of cobalt to the whey also stimulated B_{12} production (5). Highest vitamin yields were obtained if the culture was grown anaerobically until maximum cell growth had occurred, and then was aerated (9).

The distillation residue that results from the industrial process of obtaining oil from Mexican limes has also been studied as a possible fermentation substrate for vitamin B_{12} production by propionibacteria (76). The pH of the residue was adjusted, and corn steep liquor and cobalt were added to create a substrate suitable for the production of B_{12} by P. shermanii. Highest yields of the vitamin were obtained when the culture was grown in a typical two-stage anaerobic/aerobic process; however, B_{12} was produced most efficiently under completely aerobic conditions (91).

Propionibacteria are essential to the production of Swiss cheese. They ferment the lactate produced in milk by lactic acid starter cultures to contribute much of the characteristic flavor of the cheese. In addition, carbon dioxide released by the propionibacteria causes the formation of the eyes typical of Swiss cheese.

Langsrud and Reinbold (52, 53, 54, 55) and Reinbold (85) have reviewed the microbiology and flavor development of Swiss cheese, and the role of the propionibacteria in Swiss cheese manufacture.

Starter cultures of Propionibacterium and lactic acid bacteria are added to the milk. Lactobacillus bulgaricus, L. helveticus, and Streptococcus thermophilus, either

singly or in combination, are commonly used lactic starters for Swiss cheese.

Most of the growth of propionibacteria in Swiss cheese occurs after the milk has been coagulated, and the cheese curds have been cut, cooked, and pressed into the desired forms. The unripe cheese usually is stored at 7-14C for 7-10 days, and then is moved to a warm room at 21-25C. During warm room incubation, the cheese becomes elastic and active growth of the propionibacteria occurs. Flavor development and eye formation take place as the cheese ripens. When the eyes are large enough, the cheese is moved to a cooler, and is kept at 2-5C for 2-9 months for full flavor to develop.

During warm room growth, the lactic acid produced by the lactic acid starter culture is fermented primarily to propionic and acetic acids. Carbon dioxide production causes the formation of eyes within the cheese. Proteolysis also occurs, and the content of free amino acids in the cheese increases. Some researchers consider the production of these amino acids important to cheese flavor. Proline in particular is believed to contribute to the sweetness of Swiss cheese.

Much of the research related to the propionibacteria has concerned their role in Swiss cheese ripening, and the

problems that may occur during the ripening process.

Growth of the propionibacteria in cheese may be inhibited by the presence of antibiotics in the milk used for cheese manufacture. Antibiotics are used for the treatment of mastitis in cows, and traces of the drugs can be found in the milk of cows that have undergone antibiotic therapy. Reddy et al. (81) determined the inhibition patterns of 39 antibiotic or antimicrobial agents against 39 different Propionibacterium species. The propionibacteria were particularly sensitive to penicillin G, but were resistant to most penicillin derivatives. Resistance to sulfonamides was also noted. On further examination of sulfonamide resistance in propionibacteria, Reddy et al. (83) found that resistance was due mainly to the inability of propionibacteria to transport the sulfonamide across the cell wall.

Vedamuthu et al. (102) described the inhibitory activity of whey on propionibacteria. The inhibition was destroyed by heat treatment of the whey. Further investigation of the effect by Vedamuthu, Washam, and Reinbold (103) revealed that the inhibitory factor was pseudoglobulin, one of the immune globulins of milk.

Diacetyl produces a buttery flavor in dairy products, and is an important flavor component of Swiss cheese. Lee

et al. (57) studied diacetyl production in milk by *P. shermanii*. The organisms utilized citrate present in the milk to produce diacetyl. The pH and temperature of the milk affected the amount of diacetyl produced. Production of the compound was slower at 21C than at 32 or 37C, but more was produced at the lower temperature. Four times more diacetyl was produced at pH 4 than at pH 5. The propionibacteria alone could not reduce the pH of the milk to pH 4. When a mixed culture of *Streptococcus lactis* and *P. shermanii* was inoculated into milk, the growth of the *S. lactis* culture reduced the pH of the milk to a level at which good diacetyl production could take place.

Fissures or cracks can appear in Swiss cheese during the final cold room curing of the cheese. This event is referred to as the split defect of Swiss cheese, and is an important economic problem for cheese manufacturers, since split cheese is unfit for sale in small packages or as presliced cheese.

Hammond and Reinbold (29) studied the split defect of Swiss cheese. It was thought that splits were caused by lack of cheese curd plasticity or by abnormal microbial fermentation. Cheeses were sampled and analyzed during the ripening process. No difference in the microbial flora could be detected between split and unsplit cheeses.

In addition, no obvious relationship between splitting and carbon dioxide levels in the cheese, the degree of proteolysis, or shear strength of the cheese were discernible.

The ability of the propionibacteria to grow at low temperature was investigated by Park et al. (75). Many of the propionibacteria that were isolated from Swiss cheese were able to grow at 7.2C, and some could grow at 2.8C. Park et al. (74) also studied the role of the propionibacteria in the split defect of Swiss cheese. They hypothesized that splitting was a result of bacterial growth during cold room incubation. They reported that Swiss cheese prepared with a strain of *Propionibacterium* that grew well at 7.2C, a common temperature for cold room ripening, had a greater tendency to split than did cheese made with a strain that did not grow at 7.2C.

Hettinga et al. (34) also reported that Swiss cheese produced with *Propionibacterium* strains able to grow at low temperatures showed a higher incidence of splits than cheese produced with strains unable to grow at low temperatures. Carbon dioxide production by strains that grew at low temperatures was almost twice that of strains that lacked this ability.

Hettinga and Reinbold (33) determined that the

metabolic characteristics of Propionibacterium strains that could not grow at low temperatures were different from those of strains able to grow at low temperatures. Enzymes from low-temperature strains had a greater activity at low temperatures and low pH. Such strains, when used for Swiss cheese manufacture, are capable of growth and carbon dioxide production at low temperatures, and increase the tendency of the cheese to split during cold room storage and ripening.

In an attempt to reduce the incidence of the split defect, Hofherr et al. (35) isolated mutants of P. freudenreichii that were unable to grow at low temperatures. Mutant strains grew as rapidly as the parent strains at 32C, but much more slowly than the parent strains at 14C. Other characteristics, such as carbohydrate fermentation and nitrate reduction, were identical to those of the parent strain. Hofherr et al. (36) also studied the relation of growth temperature to fatty acid composition in Propionibacterium. They concluded that strains able to grow at low temperatures may have developed a fatty acid composition consistent with this ability; however, the ability of a particular strain to adjust fatty acid composition with changes in growth temperature is limited.

Protoplasts

The envelopes of bacteria consist of a cell wall overlying a cytoplasmic membrane. The complete removal of the cell wall, in a hypertonic environment that maintains the osmotic integrity of the cell and leaves the membrane intact, produces a protoplast. The cell wall structure of Gram-negative bacteria makes complete removal of the cell wall difficult; however, partial removal is possible. Such cells are referred to as spheroplasts. Originally, it was recommended that only cells completely devoid of any cell wall be referred to as protoplasts, and that cells with residual cell wall, whether Gram-positive or Gram-negative, be referred to as spheroplasts (63). Over the years, however, this terminology has become somewhat blurred, and the term protoplast is often used to describe Gram-positive cells that have been rendered osmotically fragile by partial or complete cell wall removal. The term spheroplast is used to refer to Gram-negative cells that have had part of the cell wall removed. This review will concentrate on the literature that deals with protoplasts of Gram-positive organisms.

Salton (88), in 1952, demonstrated that isolated cell walls of Micrococcus lysodeikticus were dissolved by the enzyme lysozyme. Shortly thereafter, Weibull (105)

treated cells of Bacillus megaterium with lysozyme in a hypertonic sucrose solution. The cell wall was completely digested, and osmotically fragile spheres were produced. Weibull named these cell wall-free bodies protoplasts. Much of the subsequent research with bacterial protoplasts involved studies of cell structure, and the roles of the cell wall and membrane in cellular metabolism (64). Disintegration of protoplasts by osmotic shock provided a method for the isolation of subcellular fragments, such as cell membranes, and the preparation of cell-free extracts. The gentle conditions used in lysing the protoplasts permitted the purification of highly polymerized, biologically active DNA, as well as enzymes that had not been detected in cell-free extracts prepared by other methods (98).

In the proper environment, protoplasts can replace the cell wall, regain normal osmotic stability, and produce walled progeny. This process is referred to as regeneration. The efficiency of this process is expressed as the regeneration frequency. This value can be calculated in several different ways, but generally indicates that percentage of a protoplast population that has regenerated.

Regeneration of protoplasts to walled cell forms was

reported in 1963 by Landman and Halle (50). Protoplasts of Bacillus subtilis that were produced by lysozyme digestion of the cell wall were able to regenerate to walled, bacillary cells when incubated in hypertonic medium supplemented with 25% gelatin.

Detailed studies on protoplast formation and regeneration in Bacillus followed Landman and Halle's report. Miller, Zsigray, and Landman (67) explored factors involved in protoplast formation. The rapidity of cell wall removal by lysozyme fluctuated greatly during the growth of the cell, probably due to subtle changes in the cell wall during growth. When cellular protein synthesis was halted with chloramphenicol, the cell wall thickened, and sensitivity to lysozyme decreased. It was proposed that the bacilli pass through several stages during lysozyme treatment: rod-shaped, osmotically resistant cells; rod-shaped, osmotically fragile cells; spherical, osmotically fragile cells with some cell wall residues; and spherical protoplasts with no cell wall.

Regeneration of Bacillus subtilis protoplasts to walled, bacillary cells was studied by Landman's research group (13, 49, 51, 66). These investigations defined the sequence of events in protoplast regeneration, and factors that affect them.

Replacement of the cell wall precedes multiplication of the bacterium (51). A thin coat of wall develops and increases uniformly until a wall of normal thickness is established. Rod-shaped cells grow out of these bodies in an irregular fashion. Regrowth of the cell wall is fairly synchronous, but subsequent division of the regenerants is very asynchronous. Photomicrographic studies of the regenerating protoplasts reveal that some regenerants divide only once while others divide many times (66).

As was described earlier, regeneration of the protoplasts was accomplished by embedding them in a hypertonic medium that contained 25% gelatin. Brief incubation of the protoplasts in casein hydrolysate prior to embedding them in the gelatin medium stimulated regeneration. Melting and re-solidification of the gelatin-protoplast suspension caused delays in regeneration; however, cells were sensitive to this disruption only at certain times during regeneration (51). The addition of *B. subtilis* cell walls, or of intact autoclaved cells of *B. subtilis*, *E. coli*, pseudomonads, or yeast to the regeneration medium enhanced regeneration of the protoplasts. Protoplasts were also able to regenerate when supported on membrane filters (13). These results suggested that aggregations of excreted cell products

accumulated at the protoplast surface, and that physical immobilization of these products was necessary for cell wall regeneration.

The effects of inhibitors of cell wall, protein, and nucleic acid synthesis on regeneration of *B. subtilis* protoplasts were investigated (49). The first step of regeneration, priming, was not affected by lysozyme, penicillin, or DNA synthesis inhibitors, but was blocked by chloramphenicol, puromycin, and actinomycin D. Protein and RNA synthesis were therefore necessary to this step, but no wall mucopeptide was made. The second step was inhibited by penicillin, cycloserine, and lysozyme. Inhibitors of protein and nucleic acid synthesis caused only a slight, temporary inhibition. Mucopeptide synthesis occurred during this step, and RNA, DNA, and protein synthesis were not essential. After this phase, the newly regenerated cells were misshapen and were still osmotically sensitive. Development of final osmotic stability was blocked by chloramphenicol and actinomycin D. It was proposed that synthesis of teichoic acid was required for this final step.

In 1977, DeCastro-Costa and Landman (14) reported that an inhibitory protein controlled the regeneration of protoplasts of *B. subtilis*. The protein blocked

regeneration when the protoplast concentration exceeded 5×10^5 cells/ml. The addition of trypsin to the protoplast suspension enabled regeneration to proceed normally. Comparison of the autolysin of *B. subtilis* to the inhibitory protein suggested that the protein was indeed *B. subtilis* autolysin.

Methods for the preparation and regeneration of protoplasts of streptomycetes were also being developed during this time period. The mycelial growth characteristics of these organisms caused problems in the production of protoplasts, because the individual "cells" of the mycelia varied in age and physiological state, and therefore in sensitivity to lysozyme.

It was found that *Streptomyces* mycelia that had been grown in a medium that contained slightly inhibitory levels of glycine were much more sensitive to lysozyme than mycelia grown in the absence of added glycine (87). Okanishi, Suzuki, and Umezawa (72) reported that the optimal glycine concentration was different for each *Streptomyces* species used. Similar results were obtained by Hopwood et al. (39). During lysozyme treatment, the mycelia gradually disappeared, and spherical protoplasts were produced. Any mycelia that remained were removed by filtration, and a suspension that contained only

protoplasts was obtained. Baltz (3) fractured glycine-grown mycelia with ultrasonic treatment prior to lysozyme digestion. This method resulted in a more uniform cell suspension for protoplast production.

The growth phase of the Streptomyces culture has a great effect on the lysozyme sensitivity of the mycelia, and the phase that yields the most sensitive mycelia varies between species. Mid-logarithmic phase mycelia are required with some streptomycetes (72), and others are most sensitive to lysozyme during the transition between the logarithmic phase and the stationary phase (4).

Conditions for the efficient regeneration of the protoplasts also vary among species of Streptomyces. Okanishi, Suzuki, and Umezawa (72) studied the regeneration of two species, and noted that each required a different medium for optimum regeneration. Their R2 medium (72) has been used, with minor modifications, by many other researchers for the regeneration of protoplasts of Streptomyces species. Regeneration is also affected by physical conditions such as temperature and the method of plating of the protoplasts. In some instances, incubation at sub-optimum growth temperatures enhanced regeneration frequencies (4). Some Streptomyces protoplasts are able to regenerate on an agar surface (72), but others require

plating in soft agar overlays (4). Dehydration of the basal agar medium (about 20% water loss) on which *S. fradiae* protoplasts in soft agar overlays were plated increased regeneration frequencies by a factor of ten (4). This dehydration also served to reduce the inhibitory effect that colonies from rapidly regenerating protoplasts or nonprotoplasted cells have upon nearby protoplasts. This phenomenon of auto-inhibition (4) does not occur in all *Streptomyces* species.

Most of the early methods for bacterial protoplast formation and regeneration were developed with *Bacillus* spp. and *Streptomyces* spp. Currently, such procedures exist for many other bacteria.

Kaneko and Sakaguchi developed a method for the formation and regeneration of protoplasts of *Brevibacterium flavum* (41). These bacteria were extremely resistant to lysozyme. To overcome this difficulty, cells were grown in the presence of sub-inhibitory levels of penicillin. This produced cells that were more sensitive to lysozyme, although a lengthy incubation with the enzyme was required to form protoplasts. Regeneration was poor on an agar surface; soft agar overlays supported regeneration. Santamaria et al. (90) modified the method for use with *B. lactofermentum*.

Protoplast production and regeneration procedures have been reported for several species of *Clostridium*. Pregrowth in a medium supplemented with glycine was required for efficient production of *C. acetobutylicum* protoplasts (2). Strict anaerobiosis was necessary for successful regeneration. The addition of killed *C. acetobutylicum* cells or bovine serum albumin to the regeneration medium increased regeneration frequencies.

Minton and Morris (68) did not use glycine in the production of *Clostridium pasteurianum* protoplasts. Regeneration was inhibited by gelatin, and occurred on an agar surface.

Soft agar overlays were required for regeneration of *Clostridium tertium* protoplasts (45). When regenerant colonies were isolated and subjected to the protoplast production and regeneration procedure, a wide variation in regeneration frequencies for the individual isolates was observed. One isolate yielded protoplasts that regenerated at frequencies of nearly 100%. This ability was unstable, however, and decreased after numerous transfers, although the isolate still exhibited higher regeneration frequencies than did its parent strain.

Some species of *Corynebacterium* are resistant to the action of lysozyme, and the formation of protoplasts is

difficult. Two solutions to this problem have been reported. Katsumata et al. (44) grew C. glutamicum cells in penicillin prior to lysozyme digestion. Protoplast formation was good, but regeneration frequencies were poor. It was suspected that the use of penicillin for protoplast production caused inefficient regeneration of the cells.

Smith et al. (96) isolated lysozyme-sensitive mutants of Corynebacterium lilium. These mutant strains could be converted to protoplasts without the use of penicillin. Up to 50% of the protoplasts produced were capable of regeneration; however, this frequency varied considerably among experiments.

Ryu et al. (86) produced protoplasts from the actinomycete Micromonospora rosaria. The conversion of the mycelia to spherical protoplasts was monitored by light microscopy after suspension of the cells in a stain that contained sucrose and crystal violet. This staining method is useful for the detection of protoplasts when a phase-contrast microscope, routinely used to examine protoplasts, is unavailable.

Protoplast formation and regeneration methods have been developed for several types of lactic acid bacteria. Gasson (22) used lysozyme to produce protoplasts of

Streptococcus lactis, S. lactis var. diacetylactis, S. cremoris, S. thermophilus, and S. faecalis. The protoplasts were plated on an agar surface. Regeneration frequencies varied widely among species, and among strains of a species.

Kondo and McKay (46) produced protoplasts of S. lactis with the enzyme mutanolysin instead of lysozyme. Regeneration occurred on an agar surface (47); however, in a later study (48) it was reported that plating the cells in a soft agar overlay increased regeneration frequencies.

Okamoto, Fujita, and Irie (71) used a combination of lysozyme and bacterial alpha-amylase to produce protoplasts of S. lactis. Surface regeneration occurred at frequencies of 3-10%.

Lactobacillus casei protoplasts were produced by Lee-Wickner and Chassy (58) by treatment with a mixture of lysozyme and mutanolysin. Optimum conditions for protoplast formation and regeneration, and regeneration frequencies varied among L. casei strains. The most efficient method for protoplast production did not necessarily yield the highest regeneration frequency.

Uses of Protoplasts

Useful applications exist for protoplast formation and regeneration. Once the barrier of the cell wall has been removed, genetic manipulation of the protoplasts is possible. Fusion of protoplasts, curing of plasmid DNA, and transformation of protoplasts all depend upon successful protoplast formation and regeneration.

Protoplast fusion

The addition of polyethylene glycol (PEG) to a suspension of protoplasts causes the cells to clump together. Fusion of individual protoplasts occurs to form multicellular aggregates that are composed of two or more protoplasts within a continuous cell membrane. Thus, protoplast fusion provides a method for the exchange of entire genomes between protoplasts of different genetic backgrounds.

Fusion of mammalian cells had been described for many years prior to the first reports of bacterial protoplast fusion (37). In 1976, Fodor and Alfoldi (19) and Schaeffer et al. (92) simultaneously reported the successful fusion of protoplasts of *Bacillus*.

Fodor and Alfoldi (19) constructed two doubly auxotrophic derivatives of a strain of *Bacillus megaterium*. Each strain was converted to protoplasts,

equal volumes of the two protoplast suspensions were mixed, and a fusing agent was added. The cells were then plated in a minimal hypertonic soft agar medium to regenerate. The medium lacked at least one growth factor required by each of the parental auxotrophic strains. Thus, growth and colony formation depended on the combination of the parental genomes within the fusion bodies. Parental bacteria that had not been converted to protoplasts did not yield prototrophic colonies when exposed to fusion conditions. Lysates of the protoplasts or purified DNA from the parental bacteria did not transform either protoplasts or parental cells, and the presence of DNAse in the standard fusion procedure did not decrease the number of prototrophs obtained. These results indicated that fusion of the protoplasts, rather than transformation, had occurred. Prototrophic colonies produced by fusions were found to be mixed populations of individual cells that contained parental and recombinant genotypes. This result would be expected if fusion of the protoplasts had taken place, since fusion would produce di- or multi-nucleated cells that would segregate during or after reversion to produce colonies of bacteria with different genotypes.

Schaeffer et al. (92) reported a procedure for the

fusion of *Bacillus subtilis* that was similar to that of Fodor and Alfoldi. The most significant difference was that, in the procedure of Schaeffer et al., fused protoplasts were not immediately exposed to the selective conditions of a minimal medium. Rather, the cells were allowed to regenerate on a rich hypertonic medium, and were subsequently plated onto various selective media to determine what types of recombinants had been produced by fusion. The detailed chromosomal map that was available for *B. subtilis* allowed the researchers to gain insight into how recombinants are generated by protoplast fusion. The types of recombinants obtained, and their relative frequency of occurrence, are strongly dependent on the number and chromosomal location of the markers used for recombinant selection. In general, the more crossover events that are required to produce a given type of recombinant, the less frequently the recombinant will be observed.

Gabor and Hotchkiss (21) studied the factors that affect the regeneration and genetic recombination of *B. subtilis* protoplasts after fusion. They improved regeneration frequencies substantially, and could routinely obtain 100% regeneration of nonfused protoplasts. After fusion, 10 to 75% of the protoplasts

could be regenerated, also a significant improvement over previous methods. Modification of the regeneration medium, and the addition of bovine serum albumin to the buffer used for protoplast formation and dilution were primarily responsible for the improvements. Although regeneration frequencies had increased, genetic recombination frequencies had not. When regeneration of the fused protoplasts was deliberately reduced by experimental conditions, recombination did not decrease proportionately. These results suggested that regeneration of the recombinant-forming cells was independently determined, and was not related to the average regeneration capacity of the protoplast population. By varying the number of parental protoplasts present in a fusion population, Gabor and Hotchkiss determined that protoplast collision was not the limiting factor in determining the number of recombinants obtained from fusion.

Protoplast fusion has also been studied as a method for genetic recombination in *Streptomyces*. This genus is of great economic importance as a source of antibiotics, and techniques for the genetic manipulation and improvement of the organisms were of considerable interest.

Hopwood et al. (39) reported a method for the fusion of protoplasts of five Streptomyces species. Hopwood and Wright (38) determined optimum conditions for the fusion procedure. The concentration and molecular weight of the PEG was critical for the production of recombinants. A 50% solution of PEG of a molecular weight of 1000 yielded the best results.

Baltz (3) described a method for fusion of protoplasts of Streptomyces fradiae. A higher molecular weight of PEG was used, and the yield of recombinants was lower than the reported values for the method of Hopwood et al. Baltz and Matsushima (4) compared the fusion of S. fradiae protoplasts with PEG 1000 and PEG 6000, and found that the maximum frequencies of recombinants were obtained by the use of a 40-50% solution of PEG 1000. Both Hopwood et al. (39) and Baltz (3) noted that, with some Streptomyces species, regeneration of confluent lawns of fused protoplasts was inhibited by both rapidly regenerating protoplast colonies and by colonies from mycelial fragments that had not been converted to protoplasts by lysozyme. Baltz and Matsushima (4) were able to eliminate this inhibition by dehydration of the regeneration agar plates upon which the protoplasts, in soft agar overlays, were plated.

Techniques developed by Kaneko and Sakaguchi (41) for the fusion of Brevibacterium flavum protoplasts were the first method reported for genetic exchange in the genus. Each parent strain carried unique antibiotic-resistance and auxotrophic markers. After fusion, the protoplasts were plated on a rich hypertonic medium supplemented with antibiotics to select for recombinants. After regeneration of the recombinants had occurred, the colonies were analyzed for their auxotrophic properties.

In 1980, Gasson (22) described a method for the fusion of protoplasts of lactic streptococci. Chromosomal recombinants were isolated, and the method also proved useful for the efficient transfer of plasmid DNA.

Gotz et al. (27) utilized protoplast fusion to achieve genetic recombination and plasmid transfer in Staphylococcus. Recombination of chromosomal genes in the fused protoplasts required both PEG and calcium chloride; plasmid transfer did not require the presence of calcium chloride. Interspecies plasmid transfer was also accomplished, although lower transfer frequencies were obtained than for intraspecies plasmid transfer.

Stahl and Pattee (99) refined protoplast fusion in Staphylococcus aureus to make it suitable for use in chromosome mapping. Their method produced protoplasts at

a high efficiency, with virtually no nonprotoplasts present in the population. Fusion conditions were carefully controlled to allow for random exchange of genetic information between parents. The fusants were regenerated on a rich medium with no selective agents. After regeneration had occurred, the cells were harvested from the surface of the agar, sonicated to disperse cell aggregates, and analyzed on various selective media to determine the types and relative numbers of recombinants present. Under the conditions of random exchange of genetic material, the coinheritance of any two markers from either parent was a function of their relative positions on the parental chromosome. The authors warned that care must be taken in the use of protoplast fusion for genomic mapping. It cannot be assumed that all types of recombinants will regenerate with equal efficiency. Protoplast fusion was most useful in the detection of new linkage relationships that then could be tested by standard transformation methods.

Curing

Protoplast formation and regeneration procedures can also be used to increase the frequency of plasmid loss in bacteria. Such a loss is referred to as curing a cell of its plasmid(s). Novick et al. (70) observed that, in some

instances, up to 80% of the cells in an *S. aureus* culture lost plasmid DNA during the formation and regeneration of protoplasts. The frequency of curing varied considerably for each plasmid tested; in general, larger plasmids were more difficult to cure. Physical loss or expulsion of the plasmid DNA from the cells during protoplast formation could not be detected. Most of the regenerant colonies consisted of mixed populations of plasmid-free and plasmid-containing organisms. The authors concluded that plasmid elimination occurs during the divisions that occur within the protoplast prior to the completion of cell wall regeneration and normal cell division. The system for the partitioning of plasmid DNA during cell division is disrupted because of removal of the cell wall.

Protoplast formation and regeneration has also been reported to cause plasmid loss in *Streptomyces* (37), *Bacillus subtilis* (20), and *Streptococcus lactis* (24). Gasson (24) found the technique useful for the identification of cryptic *S. lactis* plasmids. Many lactic streptococci carry multiple plasmids. Protoplast-induced curing facilitates the isolation of strains that carry only a single plasmid or a specific plasmid complement.

Protoplast transformation

Perhaps the most useful application of protoplast production and regeneration methods is protoplast transformation. The introduction of purified DNA into bacteria is a critical component of any gene cloning system, since DNA that has been manipulated *in vitro* must eventually be introduced into a host cell for replication and expression. Removal of the cell wall barrier permits the introduction of DNA into organisms for which no known system of natural competence exists.

Bibb et al. (6) first reported the transformation of *Streptomyces* protoplasts with plasmid DNA. Protoplasts were mixed with DNA, and a solution of PEG 1000 was then added. After several minutes of incubation, the protoplast-DNA-PEG suspension was diluted, and the cells were plated on regeneration medium. No selection was present in the regeneration medium; acquisition of the plasmid by the protoplasts was tested for after regeneration was complete. A much lower PEG concentration than that used for protoplast fusion was optimum for protoplast transformation. Noting this result, Hopwood (37) suggested that protoplast fusion and protoplast transformation, although both promoted by PEG, are different processes, and that DNA uptake may not occur

simply because the DNA is trapped between fusing protoplasts.

Chang and Cohen (10) developed a method for the transformation of Bacillus subtilis protoplasts with plasmid DNA. A 30% PEG 6000 solution was used to promote DNA uptake by the protoplasts. In some experiments, regenerants were transferred to selective media to identify transformants; in others, the transformed protoplasts were plated directly onto a selective regeneration medium. Both methods yielded transformants. Covalently closed circular (CCC) plasmid DNA was transformed at a frequency of up to 4×10^7 transformants per microgram of DNA. Linear plasmid DNA was transformed at a frequency one to three orders of magnitude lower than the frequency obtained for CCC DNA.

Protoplast transformation methods have been reported for many other Gram-positive bacteria. As was the case for protoplast fusion, much of the research has been conducted with organisms of commercial interest, such as the corynebacteria and the lactic streptococci.

Santamaria et al. (90) used protoplast transformation to develop a molecular cloning system in Brevibacterium lactofermentum. The detailed study of the protoplast transformation system was reported in 1985 by Santamaria

et al. (89). The CCC forms of plasmid DNA were transformed one hundred-fold more efficiently than were linear forms. Recently, Smith et al. (96) described the introduction of an alpha-amylase gene from *Bacillus amyloliquefaciens* into *B. lactofermentum* protoplasts by transformation. The alpha-amylase gene was carried on a plasmid cloning vehicle constructed from a cryptic *B. lactofermentum* plasmid.

Molecular cloning systems have also been developed for *Corynebacterium* species. Katsumata et al. (44) reported of a method for protoplast transformation of *Corynebacterium glutamicum*. Transfection, the transformation of the protoplasts with purified bacteriophage DNA, was also described. The frequency of transfection was two orders of magnitude higher than the frequency of plasmid transformation. This difference in frequency was attributed to the fact that transfection only requires DNA uptake and expression by the protoplasts. Regeneration of the protoplasts is not required to detect transfectants. To detect transformants, however, the transformed protoplasts must be capable of regeneration and colony formation. The regeneration frequency of the *C. glutamicum* protoplasts was 3-7%, and many protoplasts that may have acquired

plasmid DNA during transformation could not regenerate and display the transformed phenotype.

Yoshihama et al. (108) described a cloning vector system for *C. glutamicum*. A plasmid vector that was capable of replication in *Bacillus subtilis* and *C. glutamicum* was constructed. This cloning system also depended upon protoplast transformation for the introduction of plasmid DNA into *C. glutamicum*.

Protoplast transformation (94, 47) and transfection (25) procedures have also been reported for the lactic streptococci. Kondo and McKay (47) used mutanolysin to form protoplasts of *Streptococcus lactis*, and transformed these with plasmid DNA from another *S. lactis* strain. A later report by Kondo and McKay (48) described a detailed study of the transformation system, and its use for molecular cloning. The CCC plasmid DNA was transformed at the highest efficiency; linear plasmid DNA, or plasmid DNA that had been cut and ligated *in vitro* were transformed at lower frequencies.

MATERIALS AND METHODS

Bacterial Strains

All *Propionibacterium* strains were obtained from the culture collection of the Food Technology Department, Iowa State University, Ames. *Propionibacterium freudenreichii* subsp. *freudenreichii* strains P1 and P104 were used for the development of protoplast production and regeneration procedures, and strain P6 was used for plasmid curing studies. *Propionibacterium* cultures were maintained on sodium lactate agar (NLA) plates or slants.

Staphylococcus aureus strain ISP1384, which contains the tetracycline resistance plasmid pT181 (40), and *S. aureus* strain ISP1390, which contains the erythromycin resistance plasmid pE194 (40), were provided by Dr. P. A. Pattee, Department of Microbiology, Iowa State University, Ames. *Staphylococcus aureus* cultures were maintained on supplemented brain heart infusion agar slants.

Streptococcus faecalis strain 760, which contains the conjugative erythromycin resistance plasmid pAMβ1 (12) was provided by Dr. P. A. Pattee, and was grown in Antibiotic Assay Medium 3 (AB3). All bacterial strains were also stored as frozen cultures at -60C.

Media

Unless otherwise noted, all microbiological media were sterilized in an autoclave at 121C and 15 psi for 15 min.

Sodium lactate broth (NLB) (35) consisted of 1.0% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), 1.0% yeast extract (Difco Laboratories, Detroit, MI), and 1.0% sodium lactate syrup (Fisher Scientific Co., Fair Lawn, NJ). Sodium lactate agar (NLA) consisted of NLB plus 2.0% Bacto-agar (Difco Laboratories).

Brain heart infusion (BHI) (Difco Laboratories) agar and GL broth were supplemented with thymine (20 ug/ml), and with adenine, guanine, cytosine, and uracil (each at 5 ug/ml) (99). GL broth contained the following, per liter: Casamino acids (Difco Laboratories), 3 g; yeast extract (Difco Laboratories), 3 g; NaCl, 5.9 g; sodium lactate syrup, 3.3 ml; and glycerol, 4 ml, pH 7.8.

Antibiotic Assay Medium #3 (AB3) contained the following, per liter: Beef extract (Difco Laboratories), 1.5 g; yeast extract (Difco Laboratories), 1.5 g; peptone (Difco Laboratories), 5 g; dextrose, 1 g; NaCl, 3.5 g; K_2HPO_4, 3.68 g; and KH_2PO_4, 1.32 g.

Regeneration (R) agar for <u>Propionibacterium</u>

protoplasts consisted of NLA plus 0.5 M sucrose, and 2.5% gelatin (Difco Laboratories). Sucrose was added to R agar as a 1.0 M stock solution. Soft regeneration agar for overlays contained 0.5% agar. The composition of the agar overlay was always identical to that of the R agar base. Regeneration agar was occasionally supplemented with soluble starch (Fisher Scientific), bovine serum albumin (Sigma Chemical Co., St. Louis, MO), casamino acids (Difco), autoclaved *Propionibacterium* cells, $MgCl_2 \cdot 6H_2O$, and $CaCl_2$. Autoclaved *Propionibacterium* cells were prepared by sterilizing an overnight NLB culture of *P. freudenreichii* strain P104 cells in an autoclave at 121C and 15 psi for 15 min. Bovine serum albumin, $MgCl_2$ and $CaCl_2$ were prepared as stock solutions, sterilized by filtration, and added to melted, cooled (50C) sterile R agar and soft agar immediately before the plates were poured. Regeneration agar base plates were poured the afternoon before they were used, and were allowed to dry at room temperature overnight.

Buffers

Protoplast buffer (PB) consisted of 0.5 M sucrose, 100 mM Tris-hydrochloride (pH 7.0), and 10 mM $MgCl_2$. The final pH of the buffer was adjusted to pH 7.0. Lysostaphin buffer (99) consisted of 50 mM Tris-HCl and

150 mM NaCl at pH 7.5. The SMM buffer consisted of 0.5 M sucrose, 10 mM $MgCl_2 \cdot 6H_2O$, and 20 mM maleate, pH 6.5. The $T_{25}E_{50}$ buffer contained 25 mM Tris-HCl and 50 mM EDTA, pH 8.0. The $T_{10}E_1$ buffer consisted of 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. The TES buffer contained 50 mM Tris-HCl, 5 mM EDTA, and 50 mM NaCl, pH 7.5.

Reagents

Lysozyme (Sigma) used for protoplast production was dissolved in PB at twice the desired final concentration, and sterilized by passage through a 0.22 um membrane filter (Gelman Instrument Co., Ann Arbor, MI). Lysostaphin (Sigma) was dissolved at 10 mg/ml in lysostaphin buffer, sterilized by filtration, and stored at -20C. Mutanolysin (Sigma) was dissolved in 0.1 M potassium phosphate buffer, pH 6.2, and was stored at -20C. Polyethylene glycol (PEG) 6000 (Sigma) was dissolved in SMM buffer, and sterilized by filtration. Distilled phenol for plasmid DNA isolation was provided by Dr. P. A. Pattee. The phenol was extracted as described by Maniatis et al. (62).

Production and Regeneration of Protoplasts

Isolated colonies of Propionibacterium from an NLA plate were inoculated into 10 ml NLB and incubated at 32C for approximately 24 hr. This culture was used to

inoculate 100 ml of NLB in a 250-ml nephelometer flask to an optical density of 0.02 at 600 nm. The culture was incubated at 32C for 16 hr, at which time it attained a final optical density of approximately 0.25. Cells were harvested by centrifugation at 5000 rpm for 10 min at 4C (Sorvall RC2-B refrigerated centrifuge, GSA rotor). The cells were then resuspended in 1 to 2 ml of PB.

A portion of the cell suspension (usually 0.5 ml) was mixed with an equal volume of lysozyme solution in a sterile 1.5-ml microcentrifuge tube. A sample of the cell suspension was immediately withdrawn, diluted in PB, and spread on NLA plates to determine the initial colony-forming units (CFU). The cells-lysozyme suspension was incubated in a water bath at 32C for 15 min, after which the cells were harvested by centrifugation at 4000 rpm for 5 min at room temperature (Beckman Microfuge 11, Beckman Instruments Inc., Palo Alto, CA). The supernatant fluid was carefully decanted, and the cells were washed once with PB if desired. The final cell pellet was gently resuspended in PB to the original volume of the cells-lysozyme mixture.

Cells were diluted in PB, and 0.1-ml samples of the appropriate dilutions were spread onto NLA plates to enumerate nonprotoplasts, and onto dried R agar plates for

regeneration of the protoplasts. All dilutions were plated in triplicate. The R agar plates were overlaid with approximately 5 ml of melted soft R agar that had been cooled to 40C. All plates were incubated at 32C in sealed plastic sleeves. The NLA plates were counted after 7-10 days; R plates were counted after 21 to 28 days. Regeneration frequency was calculated as the ratio of net regenerants (regenerants minus nonprotoplasts) per initial cell number, as follows:

[(CFU on R - CFU on NLA)/ initial CFU on NLA] X 100.

Photographs of P. freudenreichii cells and protoplasts were taken with Kodak Ecktachrome 35mm slide film. Cells and protoplasts were stained with crystal violet by the method of Ryu et al. (86).

Protoplast Transformation

Protoplasts of strain P104 were prepared as described earlier. Protoplasts were harvested by centrifugation, washed once in SMM, and resuspended in SMM to the original volume of the cells-lysozyme suspension. A sample of the protoplast suspension was removed, diluted in PB, and plated on NLA and R media. The rest of the protoplasts were used in transformation experiments.

Plasmid DNA (approximately 1 µg in $T_{10}E_1$ buffer) was mixed with an equal volume of 2X SMM buffer. A 0.1-ml

portion of the protoplast suspension was added to the DNA solution, followed immediately by the addition of 1.8 ml of a PEG 6000 solution. Concentration of the PEG 6000 solution varied from 15 to 30%, depending upon the experimental conditions. The protoplast suspension was incubated at room temperature for 2 to 30 minutes. In some experiments, immediately after addition of the PEG, the protoplasts were incubated on ice for 5 min, then transferred to a 37C water bath and incubated for 5 min with gentle swirling.

After incubation with PEG, all protoplast suspensions were diluted by the addition of 5 ml of SMM. The protoplasts were then recovered by centrifugation at 4000 rpm for 5 min at 4C (Sorvall RC2-B centrifuge; SS34 rotor). The supernatant liquid was discarded carefully, and the pellet (usually not visible) was resuspended gently in 1 ml NLB supplemented with 0.5M sucrose. The protoplasts were incubated at 32C for 2 to 24 hr for expression of plasmid resistance markers.

After incubation, 0.1-ml samples of the protoplasts were plated in regeneration medium that contained erythromycin (5 μg/ml) or tetracycline (5 μg/ml). Both the R agar base and the soft R agar overlays contained the antibiotic. Plates were incubated at 32C in plastic

sleeves. Controls that omitted either plasmid DNA, PEG, or lysozyme were incorporated into the protoplast transformation experiments.

Staphylococcus aureus Plasmid DNA Isolation

The method for isolation of *S. aureus* plasmid DNA was provided by Dr. P. A. Pattee. Cells were harvested from 100 ml of a culture grown in GL broth at 37C overnight. The cells were washed twice with 3% NaCl, and were resuspended in 10 ml $T_{25}E_{50}$ buffer plus 15% sucrose. Lysostaphin (Sigma) (0.4 ml of a 1 mg/ml solution) was added, and the suspension was incubated at 35C for 25 min. Pronase (Sigma) (1.0 ml of a 10 mg/ml solution in TES buffer) was added, and the mixture was incubated at 37C for 30 min. Pronase was freshly prepared, and was pre-incubated at 37C for 60 min to digest any nucleases present. Sodium dodecyl sulfate (4.0 ml of a 20% solution in $T_{25}E_{50}$ buffer) was added, and the tube was inverted to mix the suspension. If necessary, the suspension was held at room temperature for 5 min to allow complete lysis.

The pH of the lysate was adjusted to 12.35 by the addition of 1 M NaOH. The lysate was held at the high pH for 10 min, then was adjusted to pH 8.3 by the addition of 2 M Tris-HCl (pH 7.0). Enough 1.5 M NaCl solution was added to bring the final concentration of the lysate to 3%

NaCl.

The lysate was extracted with an equal volume of NaCl-saturated phenol, and then the phases were separated by centrifugation (10,000 rpm, Sorvall SS34 rotor, 4C). The aqueous phase was transferred to a clean centrifuge tube, and extracted with chloroform-isoamyl alcohol (24:1). The phases were again separated by centrifugation, and the aqueous phase was transferred to a clean centrifuge tube. Two volumes of cold (-20C) ethanol were added, the tube was held at -20C overnight, and the precipitated nucleic acids were collected by centrifugation (Sorvall SS34 rotor, 12,000 rpm, 15 min, 4C). The pellet was dried, and resuspended in $T_{10}E_1$ buffer.

Cesium chloride gradient centrifugation

The lysis procedure was scaled up as necessary to accommodate large volumes of cells. Precipitated DNA from 1 liter of cells was resuspended in 7 ml $T_{10}E_1$ buffer. Ethidium bromide (0.7 ml of a 5 mg/ml solution) and approximately 7.5 g cesium chloride were added to the DNA solution, and the refractive index of the solution was adjusted to 1.394 by the addition of either CsCl or $T_{10}E_1$ buffer. The solution was transferred to a Beckman polyallomer tube, and plasmid DNA was separated by

centrifugation (Beckman L-8 centrifuge with Ti 70.1 rotor, Beckman Instruments, Palo Alto, CA) at 43,500 rpm for 44 hr at 14C. Plasmid DNA was detected by fluorescence under UV light, and was collected by puncturing the centrifuge tube with an 18.5-gauge hypodermic syringe needle. Ethidium bromide was removed by repeated extraction of the plasmid DNA solution with CsCl-equilibrated butanol. Further purification and removal of the CsCl was accomplished with the use of microconcentrators (Amicon Centricon-30 microconcentrators, Amicon Products, Danvers, MA) according to the directions of the manufacturer. The concentrated DNA solution was precipitated, dried, and resuspended in a minimal volume of sterile $T_{10}E_1$ buffer, and stored at 4C. The quantity of plasmid DNA was determined by agarose gel electrophoresis by the method of Maniatis et al. (62).

Streptococcus faecalis Plasmid DNA Isolation

The method for isolation of *S. faecalis* plasmid DNA was provided by Dr. P. A. Pattee. A 1% inoculum of an overnight culture of *S. faecalis* was transferred into 100 ml of AB3, and the culture was incubated at 37C. After 4.5 hr of incubation (OD_{600}=0.25), glycine was added to the culture to a final concentration of 3%, and incubation was continued for 1 hr. The cells were harvested, and

resuspended in 3 ml of $T_{25}E_{50}$ buffer plus 25% sucrose. The cell suspension was incubated in a 37C water bath shaker with gentle shaking during the lysis procedure. One ml of lysozyme (5 mg/ml) and 50 units of mutanolysin were added, and the cell suspension was incubated for 15 min at 37C. A 1.8-ml portion of a 0.25 M EDTA solution (pH 8.0) was added, and incubation was continued for 15 min. Pronase (1 ml of a 5 mg/ml solution in TES buffer) was then added, and the cells were incubated for an additional 30 min. Sarkosyl (Sigma) (3 ml of a 2% solution) was added, and lysis proceeded for 15 min. The lysed cell suspension was then removed from the 37C shaker, and 0.1 volume of 5 M NaCl was added. The lysate was then extracted with phenol and chloroform-isoamyl alcohol, and DNA was precipitated as described earlier for *S. aureus* plasmid DNA. Precipitated DNA from the lysis of 1 to 1.5 liters of *S. faecalis* cells was resuspended and prepared for CsCl-ethidium bromide gradient centrifugation, and was recovered from the gradient, purified, and quantitated by the methods previously described for *S. aureus*.

Propionibacterium Plasmid DNA Isolation

Plasmid DNA from *Propionibacterium* strains was isolated by a method developed in this laboratory by

Thomas G. Rehberger (84).

Agarose Gel Electrophoresis

Plasmid DNA preparations were subjected to electrophoresis in a 0.7% agarose gel (IBI Ultrapure Reagent Electrophoresis Grade agarose, IBI Biotechnologies, Inc., New Haven, CT). Agarose was dissolved in a buffer that consisted of 39 mM Tris-HCl; 2.5 mM EDTA; and 89 mM boric acid (65). A tracking dye that consisted of 0.07% bromphenol blue, 7% sodium dodecyl sulfate, and 33% glycerol (65) was added to each DNA sample prior to loading of the samples onto the agarose gel. Electrophoresis was conducted on horizontal gels in a BRL Model H4 electrophoresis system (Bethesda Research Laboratories, Gaithersburg, MD) with a BioRad Model 500 power supply (BioRad Instruments, Richmond, CA). Electrophoresis was carried out at 30 volts until the tracking dye front neared the bottom of the gel. Then the gel was stained in a solution of ethidium bromide (0.5 μg/ml in water) for 30 to 60 min. The gel was viewed on a Fotodyne UV 300 transilluminator (Fotodyne Products, New Berlin, WI), and photographed through Wratten 23A and Wratten 2B gelatin filters with a Polaroid MP4 Land camera system.

Curing of Plasmids from Strain P6

A 0.5% inoculum of an overnight NLB culture of strain P6 was transferred into tubes containing 10 ml NLB and 0, 0.001, 0.005, 0.01, and 0.05 μg/ml novobiocin. The tubes were incubated at 32C for 24 hr, and 0.5% aliquots from the tubes that showed poor but detectable growth were inoculated into fresh tubes of NLB plus the appropriate levels of novobiocin. After 10 successive transfers through NLB plus novobiocin, the cultures were carried through two transfers in NLB. The final NLB cultures were diluted and plated onto NLA plates which were incubated at 32C for 48 hr. Colonies on the plates were transferred to NLA and NLA plus 50 μg/ml tetracycline by the velveteen replica-plating technique (56). Colonies that had failed to grow on the NLA plus tetracycline plates were picked from the corresponding NLA plate, and the loss of the tetracycline resistance phenotype was confirmed by again plating the cells on NLA with and without tetracycline. Plasmid DNA was isolated from representative tetracycline-sensitive colonies, and the DNA was analyzed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

Protoplast Production

The production of protoplasts of *P. freudenreichii* strain 104 was investigated by exposing the cells to lysozyme under various environmental conditions in osmotically stabilizing environments. The appearance of osmotically fragile cells during enzymatic treatment was considered an indication of cell wall digestion by lysozyme. Cells were considered to be osmotically fragile if they were unable to grow on normal (hypotonic) sodium lactate agar (NLA). In addition, the conversion of rod-shaped *Propionibacterium* cells to spherical cells could be observed by light microscopy (Figure 1). The number of osmotically fragile cells present in a cell suspension was calculated by determining the difference between the number of osmotically resistant colony-forming units (CFU) before and after lysozyme treatment.

Effect of lysozyme

Exponential-phase cells of P104 were suspended in PB and PB plus 20 mg/ml lysozyme to determine if osmotically fragile cells were produced by lysozyme. Osmotically stable CFU were determined initially, and after 15 minutes of incubation at 32C. The results are presented in Table 1.

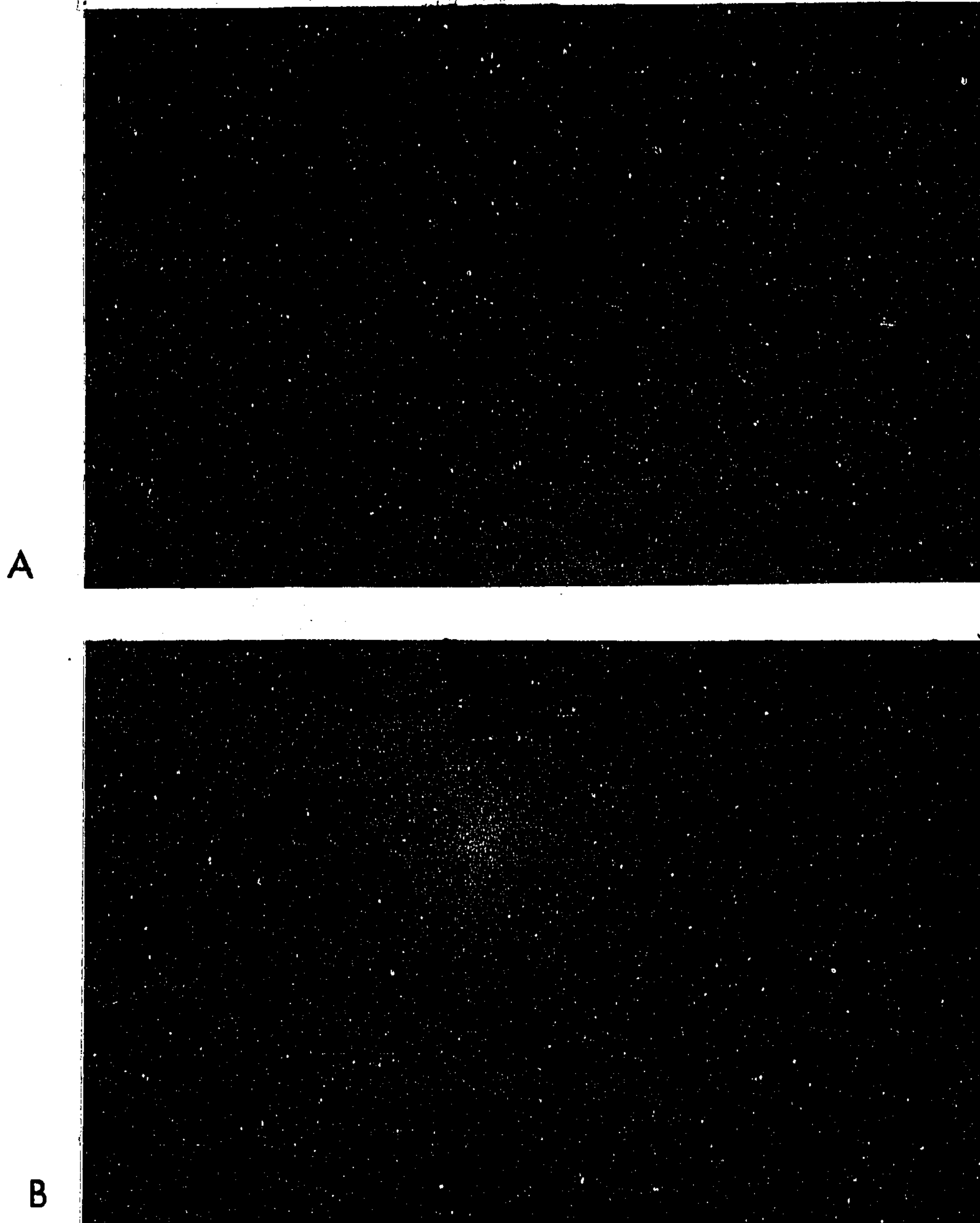

FIGURE 1. Formation of protoplasts of *Propionibacterium freudenreichii*. (A) Normal cells. (B) Cells after lysozyme treatment. For the sake of clarity, the protoplasts were diluted prior to photography.

Table 1. Effect of lysozyme on the production of protoplasts of *P. freudenreichii*

Treatment	Initial CFU	Final CFU
Lysozyme	1.2×10^9	6.2×10^5
No lysozyme	1.2×10^9	1.2×10^9

A 99.9% reduction in the count of osmotically stable cells was obtained in this experiment. Lysozyme produced osmotically fragile forms of strain P104. Factors such as buffer composition and cell growth phase were found to affect the efficiency of protoplast formation by lysozyme.

Determination of nonprotoplast CFU

The fraction of cells in the culture that was not rendered osmotically fragile (i.e., the nonprotoplast population) was determined in the above experiment by diluting the suspension in PB and plating the cells on NLA, a hypotonic medium. Nonprotoplast CFU may also be enumerated by diluting the protoplast suspension in water, and plating the cells on either hypotonic or regeneration media. When a culture was subjected to lysozyme treatment and the nonprotoplast population was enumerated by these three methods, the results presented in Table 2 were obtained.

Table 2. Comparison of nonprotoplast enumeration methods

Diluent	Plating Medium	CFU
PB	NLA	8.8×10^5
H_2O	R	8.6×10^5
H_2O	NLA	8.6×10^5

The three methods of assessing the nonprotoplast population provide similar results. Each procedure permits survival only of cells able to withstand hypotonic conditions, either within the dilution blank or in the growth medium. Dilution of the protoplast suspension in PB and plating the cells on NLA was the most convenient method, and was used to determine nonprotoplast CFU in all future experiments.

Growth phase of culture

The effect of the growth phase of the culture upon protoplast formation was determined in the following manner. A culture of strain P104 was inoculated and incubated according to the standard method for protoplast production. Samples were removed from the culture at various times, and were carried through the standard protoplast formation procedure. A previously determined growth curve for strain P104 was used to select the proper

times to withdraw samples to obtain cells in various stages of growth. Table 3 presents the results of these experiments.

Table 3. Effect of growth phase on the production of protoplasts of *P. freudenreichii*

Growth phase	Hours of incubation	% protoplasts formed
Early logarithmic	12	99.96
Mid-logarithmic	16	99.97
Late-logarithmic	20	99.95
Late-logarithmic/ early stationary	24	98.78
Stationary	36	65.25

The growth phase of the cells had a great effect upon the efficiency of production of protoplasts. Cells in the logarithmic phase of growth are easily converted to protoplasts; once the cells reached the stationary phase, they were more resistant to lysozyme digestion.

<u>Effect of inoculum preparation</u>

The culture for protoplast formation is prepared by inoculating a small volume of NLB with cells from a plate or a slant culture. After growth, this culture is used to

inoculate a larger volume of NLB that will provide the cells for protoplast production. This method of culture preparation was found to be essential for efficient protoplast formation. Cells carried in liquid media rather than on plates or slants tended to produce slime upon subsequent transfer into fresh liquid media. When these cells were used for inoculum preparation, protoplast formation was difficult, as the results presented in Table 4 indicate.

Table 4. Effect of inoculum preparation method on production of protoplasts of P. *freudenreichii*

Source of inoculum cells	% protoplasts formed
Plate or slant culture	99.9
Broth culture	83.8

Effect of magnesium chloride in protoplast buffer

Magnesium chloride ($MgCl_2$) is used in protoplast formation buffers to stabilize the protoplasts and prevent clumping of the cells. The effect of $MgCl_2$ on protoplast formation was investigated by exposing cells to lysozyme in PB containing various concentrations of this salt. The results are presented in Table 5.

Table 5. Effect of magnesium chloride concentration on production of protoplasts of *P. freudenreichii*

$MgCl_2$ concentration (mM)	% protoplast formation
0	99.96
1	99.97
10	99.97
20	99.43
50	81.57
100	81.25

Protoplast formation was equally efficient with no $MgCl_2$, or with low concentrations. However, protoplast production decreased at high $MgCl_2$ concentrations. Unusual cell morphology was observed in cell suspensions that contained 0 and 1 mM $MgCl_2$. Instead of spherical protoplasts, the cells appeared as irregular, gnarled forms that clumped together. If the concentration of $MgCl_2$ in such a cell suspension was increased to 10 mM, the cells converted to spherical protoplasts, and clumping was reduced. A $MgCl_2$ concentration of 10 mM was used in future experiments, since it supported efficient protoplast production and prevented clumping of the cells.

Effect of lysozyme concentration and time of incubation

The effect of time of incubation with lysozyme upon protoplast formation was next investigated. Suspensions of cells in PB with various concentrations of lysozyme were incubated at 32C. Samples were removed at various times, and were diluted in PB and plated onto NLA to enumerate the nonprotoplasts remaining in the cell suspension. Figure 2 displays typical results.

Most of the protoplasts are produced within the first 15 minutes of incubation with lysozyme. In each case, a plateau was reached, and further protoplast production occurred very slowly, if at all. It was decided to use a lysozyme concentration of 20 mg/ml in all future experiments, and to attempt to reduce the number of residual nonprotoplasts present in the cell suspension.

Effect of incubation temperature

Protoplast production at elevated temperatures was employed to increase lysozyme activity and to reduce the number of osmotically resistant cells present after lysozyme treatment. Protoplast production at 32C, 37C, 40C, and 45C were compared. The results are presented in Table 6.

No significant differences were observed among the numbers of osmotically stable cells remaining after

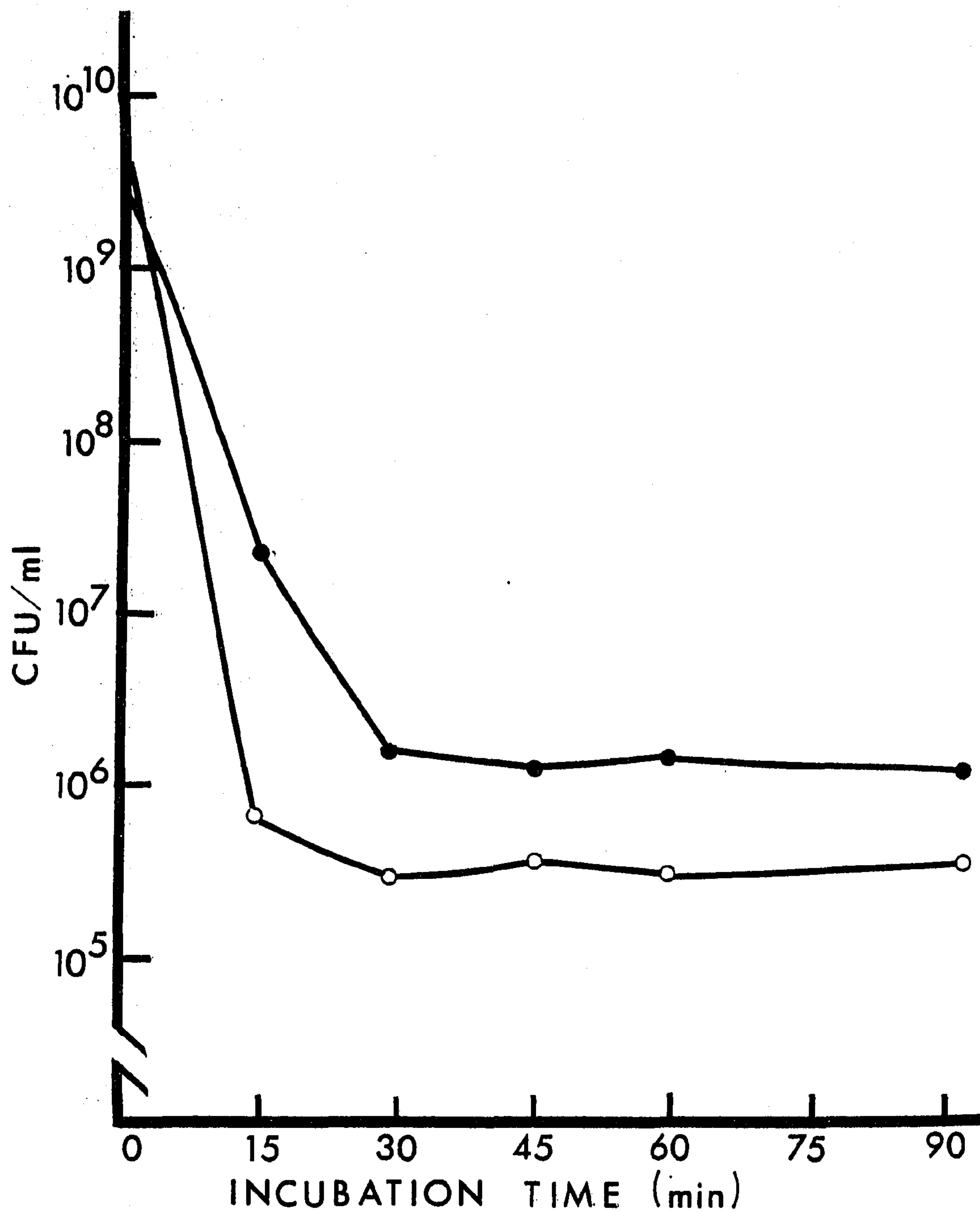

FIGURE 2. Production of osmotically fragile cells of _P. freudenreichii_. Lysozyme concentrations: 10 mg/ml (●), 20 mg/ml (○)

Table 6. Protoplast production at elevated temperatures

Temperature of lysozyme treatment	Initial CFU on NLA	Final CFU on NLA
32C	1.3×10^{10}	1.4×10^{6}
37C	1.4×10^{10}	1.6×10^{6}
40C	1.2×10^{10}	9.0×10^{5}
45C	1.1×10^{10}	2.0×10^{6}

incubation at the temperatures tested. Thus, elevated incubation temperature did not improve the efficiency of protoplast production.

Effect of glycine

Growth of microorganisms in media that contain glycine has been used by many researchers to produce cells that can be converted to protoplasts. The replacement of D-alanine residues by glycine in the peptidoglycan of the cell wall interferes with cross-linking, and a weakened cell wall is produced (28). The technique has proven particularly useful with *Streptomyces* (3, 39) and *Corynebacterium* (108). Glycine was added to the medium used to grow strain P104 cultures to make the cells more susceptible to lysozyme treatment and to reduce the number of residual nonprotoplasts. Glycine levels of 0.25 and

0.5% were found to be slightly inhibitory to growth of strain P104. Cells grown in the presence of 0.25 glycine were not converted to protoplasts more efficiently than cells grown in the absence of added glycine. Glycine levels of 0.05, 0.1, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, and 5.0% were also evaluated, but were ineffective in reducing the residual nonprotoplast population (data not shown).

Discussion of Protoplast Production

Protoplast formation of *Propionibacterium* was affected by many factors. Successful protoplast production depended upon how the cells were prepared for lysozyme treatment, as well as upon the conditions of the treatment. Harvesting the cells at the proper growth phase was critical for the formation of protoplasts. *Propionibacterium freudenreichii* cells in the exponential phase of growth were efficiently converted to protoplasts by lysozyme. Cells that had reached the stationary phase, however, were more resistant to lysozyme. Extended incubation of the stationary-phase cells in lysozyme did not increase the number of protoplasts that were produced.

A population of cells resistant to lysozyme was always present in protoplast suspensions that were prepared from exponential-phase cells of strain P104. This nonprotoplast fraction represented approximately

0.05-0.1% of the total cell population; thus, the majority of the cells were osmotically fragile. Although the nonprotoplasts represented only a minor fraction of the cell population, their presence was a matter of concern because other researchers have reported that nonprotoplasts can inhibit the regeneration of protoplasts (4, 39, 99). Prolonged incubation in lysozyme did not decrease the nonprotoplast population. Similar results have been obtained by researchers working with protoplasts of <u>Brevibacterium</u> (41), <u>Streptococcus</u> (46), and <u>Clostridium</u> (45, 68). Growth of the cells to be converted to protoplasts in a medium that contained glycine has been used to increase protoplast production efficiency in <u>Streptomyces</u> (3, 39), but this technique was ineffective with strain P104.

The resistance of a small fraction of a cell population to the action of lysozyme is probably due to the conformation of cell wall of the organisms. If lysozyme resistance was caused by unusually thick cell walls, it would be expected that protoplast formation would be slow, but would eventually occur when sufficient wall digestion had been accomplished. This was not the case with strain P104. Incubation of the cells at temperatures designed to increase the activity of

lysozyme, or prolonged incubation of the cells in lysozyme did not decrease the number of osmotically resistant cells present. Miller et al. (67), in a discussion of the lysozyme sensitivity of *Bacillus subtilis*, noted that sensitivity varied during the growth of the culture. They attributed this change in sensitivity to subtle changes in the cell wall during growth. It may be that at the time the *Propionibacterium* cells are harvested, a small fraction of the cells have wall compositions that render them resistant to the action of lysozyme.

Changes in cell wall composition may also account for the difficulty encountered in attempts to form protoplasts from cells in the stationary phase of growth. Other researchers have noted that the growth phase of the culture affects protoplast formation. It is interesting that not all organisms are most sensitive to lysozyme when in the exponential growth phase. For example, Lee-Wickner and Chassy (58) used early stationary-phase cells to produce protoplasts of *Lactobacillus casei*. Baltz (3) reported that optimum protoplast formation occurred in *Streptomyces* when the mycelia were harvested at the transition between the exponential and stationary phases.

The concentration of $MgCl_2$ in the protoplast buffer affected the physical appearance of the cells, the

efficiency of protoplast production, and clumping. At $MgCl_2$ concentrations of 1 mM, the cells were irregularly shaped and clumped together to form large aggregates. Spherical protoplasts were produced at $MgCl_2$ concentrations of 10 mM and above. Knowlton et al. (45) and Siegel et al. (93) noted that the presence of magnesium in protoplast formation buffer prevented cell clumping with Clostridium tertium and Streptococcus mutans, respectively. Indeed, most media used for protoplast production contain some magnesium salt. However, in the current research, a high level of $MgCl_2$ in PB decreased protoplast production. The increase in the ionic strength of the buffer with increasing $MgCl_2$ was probably responsible for this effect; the activity of lysozyme is inhibited at high ionic strengths.

Regeneration of Protoplasts

The ultimate test of a protoplast production method is whether or not the protoplasts are capable of regeneration to walled cells. A variety of media were evaluated for their ability to support regeneration of Propionibacterium protoplasts. None of the reported regeneration media for Streptomyces (72), Bacillus (13), or Brevibacterium (41) were suitable for regeneration of Propionibacterium protoplasts.

Regeneration was observed first in a medium composed of NLA supplemented with 0.5 M sucrose and 2.5% gelatin. Protoplasts were able to produce colonies in soft agar overlays of the regeneration medium. The regenerant colonies contained osmotically stable cells, as evidenced by their ability to grow when transferred to hypotonic media.

The first regeneration frequencies obtained were low. Only 0.1-1% of the initial cell suspension could be recovered after protoplast formation. Factors that affected protoplast regeneration were studied to optimize the procedure and increase the regeneration frequencies.

Method of plating

The first evidence of protoplast regeneration was obtained by spreading the cells on the surface of regeneration agar, and overlaying the cells with soft regeneration agar. This method was compared with simple plating of the cells on the R agar surface to determine if soft agar overlays were required for protoplast regeneration. The results are presented in Table 7.

Although limited growth occurred on the surface of R agar, regeneration frequencies obtained by this method were at least tenfold lower than when the protoplasts were allowed to regenerate in soft agar. In addition, the

Table 7. Effect of method of plating on regeneration of protoplasts of *P. freudenreichii*

	Regeneration frequency (%)		
Method of plating	Exp. 1	Exp. 2	Exp. 3
Spread plates	0.7	0.5	0.4
Soft agar overlays	18	17	11

colonies that formed on the R agar surface were very small, and did not increase beyond the pinpoint size even after incubation for 40 to 50 days. Soft agar overlays clearly provided a better environment for regeneration of strain P104 protoplasts.

Effect of sucrose concentration

The addition of sucrose to NLA provides a hypertonic environment and helps to stabilize the osmotically fragile protoplast until a cell wall can be regenerated. The sucrose concentration in both the protoplast buffer and the R medium was varied to determine the effect of sucrose concentration upon regeneration of strain P104 protoplasts. The results are presented in Table 8.

Below a sucrose concentration of 0.45 M, regeneration frequencies were very low. At sucrose

Table 8. Effect of sucrose concentration of media on regeneration of protoplasts of *P. freudenreichii*

Sucrose conc. (M) in buffer and medium	Regeneration frequency (%)	
	Experiment 1	Experiment 2
0	< 0.1	< 0.1
0.30	< 0.1	< 0.1
0.35	< 1.0	< 1.0
0.40	2.0	< 1.0
0.45	7.0	3.0
0.50	7.0	3.0
0.80	6.0	1.0

concentrations above 0.5 M, regenerants grew more slowly in R medium than they did at lower sucrose levels. Protoplast formation and regeneration media were prepared with 0.5 M sucrose for all future experiments

Effect of gelatin and other additives

Regeneration media provide a rich, hypertonic environment for protoplasts. Components such as gelatin, starch, $MgCl_2$, and $CaCl_2$ have been incorporated into regeneration media to stimulate protoplast regeneration in other microbial systems. The effects of various

supplements to R agar on protoplast regeneration in propionibacteria were investigated. Because preliminary results had demonstrated that gelatin was necessary for efficient regeneration of strain P104 protoplasts, the effect of gelatin concentration upon regeneration was studied first. The results of these experiments are presented in Table 9.

Table 9. Effect of gelatin concentration in R agar on regeneration of protoplasts of *P. freudenreichii*

Gelatin conc. (%)	Regeneration frequency			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0	< 0.1	< 0.1	< 0.1	< 0.1
0.1	1.3	--[a]	--[a]	1
0.5	1.7	1.3	0.2	--[a]
1.0	5.4	2.8	1.0	10
2.5	7.2	6.8	8.3	25
5.0	3.8	3.5	6.2	17
7.5	--[a]	2.9	5.0	--[a]

[a]Data not obtained.

Gelatin was required in R agar for regeneration of strain P104 protoplasts. Although the regeneration

frequencies obtained did not vary greatly within an experiment with differences in gelatin concentration, the trends observed were reproducible. A gelatin level of 2.5% yielded the best regeneration frequencies, and R agar was prepared with 2.5% gelatin in all subsequent experiments.

Although NLA plus 0.5 M sucrose and 2.5% gelatin supported reasonably good regeneration of protoplasts, it was possible that additional supplementation of the medium might increase regeneration frequencies. A number of supplements were added to R agar singly and in various combinations and tested for their influence on measured regeneration frequencies. Table 10 lists the supplements that were employed and the concentrations that were used. Combinations of two, three and four supplements were also tested.

Protoplasts were plated in R agar (NLA plus sucrose and gelatin) with and without the supplement that was being evaluated. The regeneration frequencies obtained in each experiment were compared. A supplement was considered to be stimulatory if its addition to R agar resulted in an increase in regeneration frequency when compared to the frequency obtained with unsupplemented R agar. A supplement was considered inhibitory if its

Table 10. Supplements added to R agar that had no effect on regeneration of *P. freudenreichii* protoplasts

Supplement	Concentrations used (%)
Bovine serum albumin	0.01, 0.1, 1.0
Casamino acids	0.01, 0.1, 1.0
Killed cells	0.1, 1.0, 5.0
Starch	1.0, 2.5, 5.0, 10.0

addition reduced regeneration frequencies. A supplement that didn't change regeneration frequencies was considered to have no effect. Combinations of supplements were evaluated by the same critera as single supplements.

None of the supplements to R agar, either singly or in any combination, were stimulatory to regeneration. Bovine serum albumin, starch, killed strain P104 cells, and casamino acids had no discernible effect on protoplast regeneration when added to R agar. The substitution of bovine serum albumin or starch for gelatin in R agar resulted in very poor protoplast regeneration.

When $MgCl_2$ and $CaCl_2$ were added to R agar, either singly or in combination, protoplast regeneration was inhibited. Table 11 presents the results of these experiments.

Table 11. Effect of magnesium chloride and calcium chloride in R agar on regeneration of protoplasts of *P. freudenreichii*

Salts added to R agar		Regeneration frequency (%)	
$MgCl_2$ (mM)	$CaCl_2$ (mM)	Exp. 1	Exp. 2
0	0	23	14
10	0	17	<1
0	1	6	<1
10	1	3	<1

The severity of inhibition of protoplast regeneration by $MgCl_2$ and $CaCl_2$ varied, but the addition of the salts always resulted in a decrease in regeneration frequencies when compared to regeneration frequencies obtained with R agar that did not contain the salts. The best medium for regeneration of strain P104 protoplasts was NLA containing 0.5 M sucrose and 2.5% gelatin, but not supplemented with $MgCl_2$ or $CaCl_2$.

Effect of extended lysozyme digestion

The effect upon regeneration efficiency of extended lysozyme treatment (20 mg/ml) of cells of strain P104 was studied. It was expected that regeneration frequencies would decrease as the time of lysozyme treatment

increased. Table 12 presents the results of these experiments.

Table 12. Effect of extended lysozyme digestion on regeneration of *P. freudenreichii* protoplasts

Minutes of lysozyme digestion	Regeneration frequency (%)	
	Experiment 1	Experiment 2
15	10	84
30	6	52
45	6	49
60	5	55
90	6	50

Incubation of the cells with lysozyme for 30 minutes decreased regeneration frequencies compared to those obtained after 15 minutes of incubation. Longer lysozyme treatments did not decrease regeneration frequencies further.

Other strains of propionibacteria

Shortly after the first evidence of successful regeneration of protoplasts of P1 had been obtained,

protoplast formation and regeneration were tested with other strains of *Propionibacterium*. Cultures were treated with 20 mg/ml lysozyme in PB at 32C for 15 minutes, and were then diluted in PB and plated onto NLA and in soft R agar overlays. The results are presented in Table 13.

Protoplast production and regeneration was accomplished with *P. freudenreichii* subsp. *shermanii* strains P70, P71, P55, P7, and P100, *P. freudenreichii* subsp. *globosum* strain P22, and *P. freudenreichii* subsp. *freudenreichii* strain P104. Regeneration frequencies were low, but were typical of the frequencies obtained at that point in the study. Regenerant colonies of strain P104 appeared 5 to 7 days earlier than did colonies of strain P1. Because of this faster regeneration, strain P104 was used in all subsequent experiments. Experiments that had been conducted with strain P1 were repeated with strain P104. Formation of protoplasts of *P. acidi-propionici* and *P. theonii* by the standard method were not successful.

Effect of centrifugation

In the early experiments on protoplast regeneration, the protoplasts were harvested by centrifugation and washed once before the cells were plated on R medium. The necessity of this step was investigated, because it was of concern that centrifugation and resuspension of the cells

Table 13. Protoplast formation and regeneration of various strains of *Propionibacterium*

Strain	Protoplast formation (%)	Regeneration frequency (%)
P. freudenreichii subsp. *freudenreichii* strain P104	99.97	4
P. freudenreichii subsp. *shermanii* strain P7	99.31	1
P. freudenreichii subsp. *shermanii* strain P55	99.92	1
P. freudenreichii subsp. *shermanii* strain P70	99.93	5
P. freudenreichii subsp. *shermanii* strain P71	99.24	<1
P. freudenreichii subsp. *shermanii* strain P100	98.23	<1
P. freudenreichii subsp. *globosum* strain P22	99.26	1
P. acidi-propionici strain P50	<10	--[a]
P. acidi-propionici strain P90	<10	--[a]
P. theonii strain P17	<10	--[a]

[a]Not applicable.

might cause protoplast lysis and a reduction in regeneration frequency. The results are presented in Table 14.

Table 14. Effect of centrifugation on regeneration of protoplasts of *P. freudenreichii*

CFU	Treatment of cells	
	Centrifugation	No centrifugation
Initial	6.3×10^9	6.3×10^9
Nonprotoplast	8.8×10^5	2.1×10^6
Regenerant	2.0×10^9	2.0×10^9

Centrifugation and washing of the protoplast suspension neither increased nor decreased regeneration frequency. This process was considered to be unnecessary and was omitted from future experiments.

Effect of gelatin in protoplast buffer

As was demonstrated earlier, efficient regeneration of protoplasts required the presence of gelatin in R agar. Lee-Wickner and Chassy (58) incorporated gelatin into the buffer used for protoplast formation. The effect of gelatin in PB upon regeneration of protoplasts of strain P104 was investigated. Regeneration frequencies obtained for protoplasts formed in PB plus gelatin were compared with the frequencies obtained for protoplasts prepared in PB with no added gelatin. Initially, it appeared that the addition of gelatin to PB would result in increased regeneration frequencies. The effect was not

reproducible, however, as indicated in Table 15.

Table 15. Effect of gelatin in protoplast buffer (PB) on regeneration of *P. freudenreichii* protoplasts

	Regeneration frequency (%)	
Experiment	Gelatin in PB	No gelatin in PB
1	41	10
2	32	9
3	7	7
4	25	33
5	30	84
6	10	2

Discussion of Protoplast Regeneration

Three of the essential requirements for regeneration of *Propionibacterium* protoplasts are the use of soft agar overlays, sufficient sucrose in the medium to provide osmotic support, and the presence of gelatin in the regeneration agar.

Soft agar overlays have been used for regeneration of *Brevibacterium* (41), *Streptococcus* (48), and *Streptomyces* (4) protoplasts. The use of a soft agar overlay was

necessary for the efficient regeneration of strain P104. Limited growth could occur on the surface of R agar, but the colonies that formed were of pinpoint size, and did not become larger, even after several extra weeks of incubation. Furthermore, the regeneration frequencies obtained for surface-plated protoplasts were at least tenfold less than the frequencies that were obtained for protoplasts plated with overlays. Kaneko and Sakaguchi (41) have reported similar results when comparing methods of plating Brevibacterium protoplasts. Apparently, the protoplasts need to be surrounded by a moist, hypertonic environment for regeneration to occur, and a soft R agar layer provides this support.

A sucrose concentration of at least 0.45 M in protoplast formation and regeneration media is necessary for survival and regeneration of strain P104 protoplasts. Reduction of the sucrose concentration below 0.45 M resulted in reduced regeneration frequencies. Increasing the sucrose level to 0.8 M did not increase frequencies, but caused slower growth of the organisms. Sucrose provides the hypertonic environment needed to stabilize the protoplasts and prevent lysis. Other researchers (22, 72, 99) have reported the use of sucrose concentrations from 0.3 M to 0.8 M in protoplast media. Sucrose

concentrations below 0.45 M apparently do not provide adequate osmotic support for strain P104 protoplasts.

The presence of gelatin in the R agar was essential for good regeneration of strain P104 protoplasts. The propionibacteria used in this study do not hydrolyze gelatin (7), so it is unlikely that gelatin serves as a source of nutrients.

Gelatin in the regeneration medium improves protoplast regeneration of other organisms (2, 45, 49), but the reason for this is unclear. Clive and Landman (13) suggested that physical contact between the protoplast and a solid gelatin barrier was responsible for the stimulatory effect of gelatin upon regeneration. Such contact was thought to keep cell wall precursors and enzymes in close proximity to the protoplast membrane and encourage resynthesis of the cell wall. Clive and Landman's results were obtained with *B. subtilis* protoplasts plated on the surface of a medium that contained 25% gelatin, but no agar.

A soft agar environment without gelatin ought to provide a physical barrier similar to that provided by high gelatin concentrations, if such a barrier were required for protoplast regeneration. Omission of the gelatin from R agar, however, resulted in very poor

regeneration of strain P104 protoplasts. Regeneration frequencies did change with differences in gelatin concentration, and although the differences were small, the trends were reproducible. Perhaps the combination of 0.5% agar and 2.5% gelatin provides the optimum physical conditions for cell wall regeneration in strain P104.

Regeneration agar was supplemented with a variety of ingredients in attempts to increase regeneration frequencies. In contrast to results obtained for *Bacillus subtilis* (13), the addition of killed *Propionibacterium* cells to R medium did not increase regeneration frequencies. Killed cells are thought to provide physical support of regenerating protoplasts in the same manner as does gelatin. The use of killed cells in regeneration media for *Clostridium* (45, 68) and *Lactobacillus* protoplasts (58) was also ineffective for increasing regeneration frequencies. Bovine serum albumin, essential to regeneration of *Lactobacillus casei* protoplasts (58), had no effect on regeneration of strain P104 protoplasts. Many protoplast regeneration media for *Streptomyces* contain casamino acids (3, 4, 72), but this additional source of nutrients did not enhance strain P104 protoplast regeneration. Starch has proven stimulatory to regeneration of *Staphylococcus aureus* protoplasts (99),

but had little or no effect on regeneration of P104 protoplasts.

Akamatsu and Sekiguchi (1) suggested that complex macromolecules such as gelatin, bovine serum albumin, and horse serum support growth and division of protoplasts during the early stages of regeneration. Plasma expanders such as polyvinyl pyrrolidone and dextran could substitute for gelatin, bovine serum albumin, and horse serum in Bacillus subtilis regeneration medium. When polyvinyl pyrrolidone and dextran were replaced with their monomeric forms (vinyl pyrrolidone and glucose, respectively), support of protoplast regeneration was not observed (1). The stimulatory effect of these compounds on protoplast regeneration may be due to their macromolecular nature. Stahl and Pattee (99) suggested that the enhancement of regeneration of S. aureus protoplasts by starch might be related to the effects of serum and gelatin with Bacillus.

If bovine serum albumin, starch, and gelatin support protoplast regeneration in a similar manner, the use of more than one compound in regeneration would probably be superfluous. This could explain why the addition of bovine serum albumin or starch to regeneration medium that already contained gelatin had no discernible effect on regeneration of Propionibacterium protoplasts. Bovine

serum albumin or starch could not substitute for gelatin in the regeneration medium. Gelatin seems to be particularly suited to the support of strain P104 protoplasts. It would be interesting to determine whether polyvinyl pyrrolidone or dextran could substitute for gelatin in the regeneration of strain P104 protoplasts. Such experiments might help elucidate the nature of gelatin's support of *Propionibacterium* protoplast regeneration.

The unique characteristics of each organism may require different macromolecules for support of protoplast regeneration. Lee-Wickner and Chassy (58) reported that bovine serum albumin was required for regeneration of *Lactobacillus* protoplasts. Neither 5% gelatin nor 3% polyvinyl pyrrolidone could substitute for the bovine serum alumin in the regeneration medium.

Salts such as $MgCl_2$ and $CaCl_2$ are required for regeneration of *Lactobacillus casei* (58) and *Streptomyces* (72), but were inhibitory to regeneration of strain P104 protoplasts. Reduction of regeneration frequencies by the use of $MgCl_2$ and $CaCl_2$ has been reported for *Clostridium acetobutylicum* (2). Magnesium chloride contributes to protoplast stability, and lack of $MgCl_2$ affected strain P104 protoplast morphology. Excess levels of the salts

may have interfered with the reactions of cell wall resynthesis, or inhibited growth of the regenerants.

Some researchers (58, 67) have speculated that the successful regeneration of protoplasts requires that some residual cell wall remain on the protoplast. These cell wall fragments are thought to act as a primer for the resynthesis of the wall during regeneration. Enzymatic digestion must thus be balanced between the need to remove sufficient cell wall to produce osmotically fragile cells and the need to avoid overdigestion that may result in poor regeneration. Extended exposure of strain P104 cells to lysozyme resulted in decreased regeneration frequencies; however, a stage was reached where further lysozyme digestion did not affect regeneration. It may be that the cells able to regenerate, even after lengthy exposure to lysozyme, possessed cell walls that were refractory to complete digestion by lysozyme. Thus, they were rendered osmotically fragile but were not stripped of their complete cell wall. Some cell wall remained to act as a primer for cell wall resynthesis and regeneration.

Gelatin is occasionally added to media used for the production and dilution of protoplasts, presumably to stabilize and protect the protoplasts (58). Gelatin was an essential component of R agar in this study, but its

role as an ingredient in protoplast buffer was difficult to establish. Initial experiments indicated that increased regeneration frequencies could be obtained if protoplasts were prepared and diluted in PB plus gelatin. When the experiments were repeated, however, it became obvious that the effect of adding gelatin to PB was not reproducible. The reasons for this inconsistency could not be determined. Irreproducible results were obtained with several gelatin concentrations. If the presence of gelatin in the diluent caused clumping of the protoplasts, one would expect consistently lower frequencies from buffer with gelatin than from buffer without gelatin. This result was not observed.

Centrifugation and washing of the protoplast suspension prior to plating the cells did not improve regeneration frequencies. Such a procedure would serve to remove residual lysozyme from regenerating protoplasts. However, dilution of the protoplasts for plating appeared to dilute the lysozyme sufficiently to allow regeneration of the protoplasts. Regeneration frequencies also were not appreciably decreased by centrifugation, which indicated that the conditions used to harvest the cells were sufficiently gentle to prevent protoplast lysis. This information is applicable to procedures in which

centrifugation and resuspension of the protoplasts is necessary, such as protoplast fusion and transformation.

Variability of regeneration frequencies occurred throughout this study. Regeneration frequencies obtained ranged from 1% to 84%. Both of these cases are extremes, and regeneration frequencies were generally between 10-30%. This variability made comparison of results between experiments difficult. Repetition of experiments indicated that trends observed within an experiment were reproducible, and this allowed the optimum concentrations of R medium constituents to be determined. Every aspect of the protoplast production and regeneration method was controlled as rigidly as possible and was repeated carefully to reduce the degree of variability. Experiments in which the preparation of the R agar was varied by altering the order in which the ingredients were added to the medium, or in which the heat treatment used to remelt the agar was varied did not reveal the factors responsible for the variability in regeneration frequencies. A large quantity of microbiological media was consumed during the course of this study. It is possible that variation between lots of media used in the preparation of R agar contributed to the variability of regeneration frequencies observed with protoplasts of

strain P104.

Variability of protoplast regeneration frequencies is by no means unique to the propionibacteria. Researchers working with protoplasts of *Lactobacillus* (58), and with *Brevibacterium* and *Corynebacterium* (96) have commented upon the variation in regeneration frequency, yet have applied their methods successfully to protoplast fusion and transformation procedures.

The procedure for formation and regeneration of *Propionibacterium* protoplasts was developed primarily with two strains of *Propionibacterium freudenreichii* subsp. *freudenreichii*. The method was successfully applied to several strains of *P. freudenreichii* subsp. *shermanii*. Other species of propionibacteria were more difficult to convert to protoplasts. The propionibacteria vary in their resistance to lysozyme, and it may be necessary to modify the conditions of lysozyme treatment when the protoplast production method is applied to different strains or species of propionibacteria. Lee-Wickner and Chassy (58) noted that different enzymatic treatments were necessary to produce protoplasts from different *Lactobacillus casei* species.

The development of colonies on R agar occurred during the last 5 to 7 days of the 3 to 4 week period required

for regeneration of *Propionibacterium* protoplasts. Asynchronous regeneration of this type has been reported by Baltz and Matsushima (4) and Hopwood et al. (39) for protoplasts of *Streptomyces*. Colonies that regenerate rapidly may inhibit the regeneration of surrounding protoplasts by the production of substances such as autolysins (14) or metabolic byproducts toxic to the protoplasts. Autoinhibition has been described for protoplasts of *Streptomyces* (4, 39) and *Staphylococcus* (99). Baltz and Matsushima (4) noted that autoinhibition did not occur in all *Streptomyces* species, and that it could be eliminated in the species in which it did occur by plating the protoplasts on a partially dehydrated regeneration agar base and overlaying the cells with soft regeneration agar. Confluent lawns of mycelia could be produced by this plating method.

Autoinhibition was minimal with *Propionibacterium*. Examination of the R agar plates during regeneration indicated that colonies produced by rapidly regenerating protoplasts did not inhibit the growth of nearby regenerants. Furthermore, when undiluted protoplast suspensions that also contained nonprotoplasts were plated, the colonies that arose from nonprotoplasts did not inhibit the development of confluent lawns of

regenerants. The strains of propionibacteria used in these experiments may not produce autoinhibitory substances. Autoinhibition of protoplast regeneration can present serious barriers to protoplast transformation and fusion procedures, because it could prevent the regeneration of recombinants. The failure to observe autoinhibition in the strains used in this study is encouraging when considering the eventual goals of using protoplast transformation and fusion for genetic exchange in the propionibacteria.

Protoplast Transformation

Attempts were made to transform protoplasts of strain P104 with plasmid DNA. Experimental procedures were based on methods that had been developed for protoplast transformation of *Streptococcus lactis* (47, 48), *Corynebacterium* (44, 108), and *Brevibacterium* (89, 90), and are summarized in Materials and Methods.

Plasmids pAMB1, pT181, and pE194 were used as transforming DNA. pAMβ1 is a 17-megadalton (Mdal) plasmid that confers resistance to erythromycin, originally isolated from *Streptococcus faecalis* (12). The plasmid was used for protoplast transformation experiments despite its relatively large size because pAMβ1 has been demonstrated to have a wide host range and has been introduced into a

variety of lactic acid bacteria by conjugation (23). pAMB1 has also been introduced into a strain of Propionibacterium by conjugation (T. Rehberger, Dept. of Food Technology, Iowa State Univ., unpublished data). The Staphylococcus aureus plasmids pT181 and pE194 are small plasmids (3.0 and 2.4 Mdal, respectively) (40). Plasmid pT181 is a tetracycline-resistance plasmid; pE194 is an erythromycin-resistance plasmid (40). Small plasmids are generally transformed at greater efficiencies than are large plasmids; therefore, these plasmids were included in protoplast transformation experiments with strain P104.

Experimental parameters such as PEG concentration, duration of incubation of the protoplasts and DNA in PEG, and the length of post-transformation incubation to allow for expression of the plasmid DNA were all varied to discover a combination of conditions that would produce transformants. The protoplast formation method was also modified in some experiments; i.e., cells were treated with lysozyme for 5 minutes, rather than 15 minutes, to encourage permeability of the cell wall to exogenous DNA but to avoid unnecessary cell wall digestion.

No antibiotic-resistant transformants were obtained with either pT181 or pE194 in any of the experiments. Over 50 attempts were made with each plasmid. Five of the

attempts to transform strain P104 protoplasts with pAMβ1 DNA produced erythromycin-resistant colonies. Transformation conditions in these experiments were as follows. Protoplasts were prepared by the standard, 15-minute lysozyme treatment. Incubation of the protoplasts with DNA and 20% PEG was for 5 to 10 minutes. Post-transformation incubation for expression of the plasmid varied from 4 to 10 hours.

No erythromycin-resistant colonies ever appeared on control plates that contained protoplasts which had been incubated without DNA. Experimental plates that contained protoplasts incubated with transforming DNA sometimes contained erythromycin-resistant colonies. Fewer than ten colonies were produced in any experiment. Repetition of the procedure did not always result in the production of erythromycin-resistant regenerants. If these colonies were transformants, the frequency of transformation was extremely low, less than 10 transformants per μg of DNA. The erythromycin resistance of the isolates was confirmed; all grew in media that contained 50 μg/ml erythromycin. Lysates of the organisms were examined by agarose gel electrophoresis to determine if the cells had acquired plasmid DNA. No plasmid DNA could be detected in any of the lysates. Large-scale lysate preparation and cesium

chloride gradient centrifugation also failed to result in the detection of plasmid DNA in the isolates.

It is possible that the erythromycin-resistant organisms were mutants. However, control plates designed to detect the presence of spontaneous resistance mutants had no colonies. It is also possible that the plasmid was successfully transformed into the protoplasts, but was present in such low copy number that it could not be detected by cesium chloride gradient centrifugation or agarose gel electrophoresis.

Alternatively, all or part of the plasmid may have been integrated into the transformant's chromosomal DNA. To determine if integration had occurred, it would be necessary to perform Southern hybridizations (97) to analyze the chromosomal DNA of the erythromycin-resistant organisms for homology with pAMβ1 DNA. If transformation of the plasmid did indeed occur, it occurred at a very low frequency, and this could explain why repetition of the transformation experiments did not always produce erythromycin-resistant organisms.

Santamaria et al. (90) encountered results similar to those described here in the development of a cloning system for Brevibacterium. When Brevibacterium protoplasts were transformed with plasmid DNA, regenerants

with the antibiotic resistance phenotype encoded by the plasmid were isolated. The absence of resistant colonies on control plates indicated that spontaneous mutation to antibiotic resistance was not occurring at an observable rate, yet no plasmid DNA could be isolated from the putative transformants. Integration of all or part of the plasmid was suspected, because the plasmid contained the transposon *Tn*5. Confirmation of the hypothesized plasmid integration was not pursued by these researchers.

Plasmid pAMβ1 is much larger than either pT181 or pE194. Smaller plasmids are generally transformed at greater efficiencies than are large plasmids, but no transformants were obtained when pT181 or pE194 were used in this study. None of the three plasmids are native to *Propionibacterium*, and it is not yet known what barriers of restriction and modification may be present in the propionibacteria. Plasmid pAMβ1 has displayed a broad host range when transferred among Gram-positive organisms by conjugation (23), and, despite its size, has been successfully transferred into *Streptococcus lactis* by protoplast transformation (48). Possibly pAMβ1 is compatible with the propionibacteria, but the other plasmids employed in this study were not.

Plasmid Curing of Strain P6

An ideal source of DNA for protoplast transformation studies with *Propionibacterium* would be plasmid or bacteriophage DNA native to the propionibacteria. Both sources would provide DNA that would not be affected by whatever restriction barriers may exist in these organisms. To date, no bacteriophage have been isolated from the dairy propionibacteria.

In a systematic study of the antibiotic-resistance patterns of the *Propionibacterium* strains in the culture collection, strain P6 grew in the presence of 50 μg/ml tetracycline (T. Rehberger, Dept. of Food Technology, Iowa State Univ., personal communication). This strain also contained several plasmid species (T. Rehberger, personal communication). A plasmid-curing regimen was employed to determine if a plasmid carrying an antibiotic-resistance marker was present in this strain.

A culture was grown in NLB plus novobiocin as described in Materials and Methods to produce cells cured of plasmid DNA. Isolates that had decreased resistance to tetracycline (minimum inhibitory concentration in NLA=1 μg/ml) were obtained from the treated population. Cleared lysates of representative isolates were examined for plasmid content by agarose gel electrophoresis.

Typical results obtained for one of the isolates are presented in Figure 3. Similar results were obtained for 23 other tetracycline-sensitive isolates.

Comparison of the plasmid content of the tetracycline-sensitive isolate and a tetracycline-resistant strain P6 control indicated that the tetracycline-sensitive isolate had lost a small plasmid. The plasmid has a size of approximately 2.8 Mdal, as calculated by comparing the mobility of its covalently closed circular form with that of the covalently closed circular forms of the eight plasmids of *Escherichia coli* strain V517 (60).

The concomitant loss of tetracycline resistance and the 2.8 Mdal plasmid provides presumptive evidence that the plasmid is responsible for tetracycline resistance in strain P6. To obtain conclusive evidence that the plasmid encodes the gene for tetracycline resistance, it would be necessary to transform a tetracycline-sensitive organism to tetracycline resistance by introducing the plasmid into the sensitive organism.

An isolate of strain P6 that contains only the 2.8 Mdal plasmid would be valuable in future experiments with the plasmid. Such a culture would provide a source for the production and isolation of large amounts of 2.8-Mdal

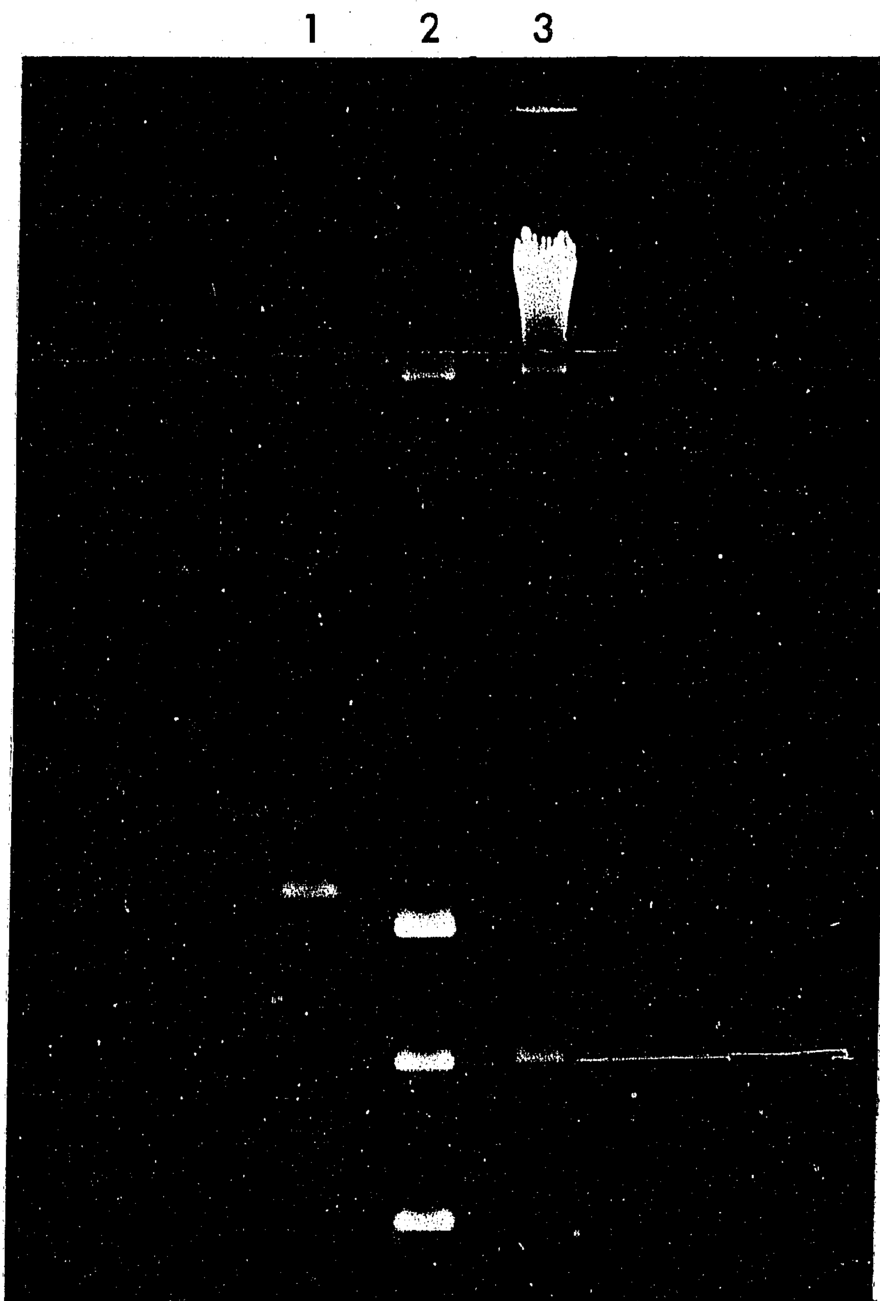

FIGURE 3. Agarose gel electrophoresis of plasmid DNA from tetracycline-resistant (lane 2) and tetracycline-sensitive (lane 3) strain P6. Lane 1 contains plasmid DNA from *Escherichia coli* V517.

plasmid DNA uncontaminated with the other plasmids of strain P6. A plasmid DNA preparation that contained all the strain P6 plasmids was isolated during this study, and several attempts were made to transform protoplasts of strain P104 with this plasmid preparation; however, no tetracycline-resistant regenerants were produced. Strain P6 was extremely resistant to protoplast formation with lysozyme, which prevented efforts to transform cured tetracycline-sensitive derivatives of strain P6 with the tetracycline plasmid. Despite these results, the small size of the plasmid and its selectable marker may make it useful in future studies of genetic exchange in the propionibacteria.

CONCLUSIONS

A procedure was developed for the production of stable protoplasts of *Propionibacterium* and for the regeneration of the protoplasts to walled cells. Protoplast formation was accomplished by exposure of the cells to lysozyme in an osmotically stabilizing environment. The growth phase of the cells, the duration of lysozyme treatment, and the composition of the protoplast buffer were all critical to the efficient production of protoplasts that would be capable of regeneration.

Incubation of mid-exponential phase cells with lysozyme produced a cell population that consisted of greater than 99.9% osmotically fragile cells. Longer exposure to lysozyme did not affect the number of residual osmotically stable cells (nonprotoplasts), but it decreased protoplast regeneration frequencies. Lysozyme treatment at elevated temperatures was ineffective in reducing the nonprotoplast population, as was growth of the cells in medium supplemented with glycine prior to lysozyme treatment. The presence of 10 mM $MgCl_2$ in protoplast formation buffer was necessary to prevent clumping of the protoplasts, but higher $MgCl_2$ levels inhibited protoplast formation.

Regeneration of the protoplasts to walled cells took 21 to 28 days. Because of this lengthy regeneration time, extreme attention to aseptic techniques was necessary throughout the entire protoplast production and regeneration procedure. The use of soft regeneration agar overlays was required for efficient regeneration of the protoplasts, as was the presence of gelatin in the regeneration medium. Salts such as $MgCl_2$ and $CaCl_2$ were inhibitory to regeneration. Regeneration frequencies were generally in the range of 10 to 30%, although frequencies varied considerably throughout the study. Occasionally frequencies as high as 75 to 85% were obtained.

The method for production and regeneration of protoplasts of *Propionibacterium* was developed with *P. freudenreichii* subsp. *freudenreichii*. It was successfully applied to several other strains of *Propionibacterium*. It is expected that modifications of the method, particularly those procedures that pertain to protoplast formation, will need to be made to adapt it for use with other species of propionibacteria.

Attempts to transform *Propionibacterium* protoplasts with plasmid DNA from other Gram-positive organisms were not successful. A wide variety of transformation conditions were investigated. Several of the attempts to

transform protoplasts with pAMβ1 yielded putative erythromycin-resistant regenerants. However, examination of cleared lysates from these organisms by agarose gel electrophoresis did not reveal the presence of plasmid DNA. The organisms may have been spontaneous erythromycin-resistant mutants, or may have been true transformants with a low copy number plasmid, or all or part of the plasmid was integrated into the *Propionibacterium* chromosome.

Elimination of a small plasmid from *P. freudenreichii* strain P6 resulted in the loss of tetracycline resistance, which suggests that tetracycline resistance in this strain is plasmid-mediated. This plasmid may be useful in studies of genetic exchange in the propionibacteria because it might be a source of DNA less affected than DNA from other organisms by whatever restriction barriers are present in the genus.

The method developed for protoplast production and regeneration should provide the basis for further research on genetic exchange in the propionibacteria. The procedure should be applicable to studies of protoplast fusion and protoplast transformation. The ability to introduce DNA into cells by these methods will be useful, both to studies of *Propionibacterium* genetics and to the

development of improved strains of this industrially important group of microorganisms.

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ACKNOWLEDGEMENTS

I wish to thank Dr. Bonita A. Glatz, my major professor, for her guidance during the course of my graduate career. I also thank the other members of my committee, particularly Dr. P. A. Pattee for providing bacterial strains, advice, laboratory procedures, and redistilled phenol.

I also wish to thank my fellow Prop Research Group members for their suggestions, ideas, and criticisms. Tom Rehberger deserves special thanks for allowing me to use the methods for *Propionibacterium* lysis that he developed, and for all his assistance during this project.

Finally, I thank my husband, Dr. Timothy A. Bender, for his extraordinary patience while I was completing my research, and for his technical expertise in preparing the dissertation.

This study was supported in part by a grant from the National Dairy Promotion and Research Board.