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Improved production of propionic acid by strains of Propionibacterium

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Improved production of propionic acid by strains of *Propionibacterium*

by

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GENERAL INTRODUCTION

Strains of the genus *Propionibacterium* are industrially important microorganisms used in the production of Swiss-type cheeses. The products from the metabolism of lactate are responsible for the characteristic eyes and contribute to the flavor, texture, aroma, and appearance of Swiss cheese. Industrially, propionibacteria are also used as silage inocula, as probiotics, and for the production of vitamin B_{12} and propionic acid.

As a preservative, propionic acid extends the shelf-life of food and agricultural products by inhibiting molds and some bacteria. Although preservatives derived from propionibacteria are available, most propionic acid used by the food and agricultural industries is produced by chemical synthesis. If higher yields could be obtained, production by fermentation may become economically competitive and may offer several advantages to chemical synthesis.

Increases in propionic acid production by propionibacteria will be accomplished by improving both the strains and the fermentation methods employed.

Batch-type fermentations are most often used to produce propionic acid. Recently, continuous, semi-continuous, cell immobilization, and cell-recycle systems have been advocated for propionibacteria fermentations to increase the yield of propionic acid. The major factor that limits the production of propionic acid during fermentation is end-product inhibition by the acid. A propionic acid-tolerant variant of a strain of *P. acidipropionici* was sought in this study and a method for semi-continuous fermentation with this strain was developed that improved the yield of propionic acid.

The initial goal of this study was to develop a transposon delivery system in strains of *Propionibacterium*. In order to achieve this goal and to apply genetic engineering techniques to improve propionic acid yield in these organisms, a reliable and efficient method for introducing DNA into propionibacteria is essential. Polyethylene glycol-induced transformation of protoplasts is an effective method for introducing foreign DNA into gram-positive bacteria. Other methods, including electroporation and conjugation, may also effect gene transfer in strains of *Propionibacterium*. In this study a more efficient method for removing the cell wall to form protoplasts was developed. Protoplasts produced by this method, as well as whole cells, were used in gene transfer experiments.

Explanation of Thesis/Dissertation Format

The thesis follows the alternative format and is divided into four parts (papers). Each contains Introduction, Materials and Methods, Results, Discussion, and References sections. Each section is written to conform to the specifications outlined by the American Society for Microbiology for papers submitted to Applied and Environmental Microbiology. Section I was published in Applied and Environmental Microbiology 57:2821-2828 (1991). Literature review and general summary sections are also included with references cited in those sections following the general summary. The candidate conducted the research described in each paper, or directed the efforts of undergraduate research assistants as noted.

LITERATURE REVIEW

Characteristics and Metabolism of Propionibacteria

The genus *Propionibacterium* consists of two principal groups of organisms, the "classical" propionibacteria and the acnes group. This review will focus on current knowledge of the classical group of propionibacteria. The species *P. freudenreichii*, *P. thoenii*, *P. jensenii*, and *P. acidipropionici* are described as the classical or dairy propionibacteria (21). These strains of propionibacteria have been isolated from raw milk, Swiss cheese and other dairy products, and some strains are used extensively as dairy starter cultures (35, 46, 47, 48, 49).

The propionibacteria are gram-positive, catalase-positive, nonsporeforming, nonmotile, pleomorphic rods, which are anaerobic to aerotolerant (21). They are unique in that they are capable of producing propionic acid, acetic acid, and carbon dioxide from the metabolism of lactate (35, 63, 77).

Data comparing sequences from reverse transcriptase sequences of 16S ribosomal RNA have shown that the genus represents a well defined taxon (13). *Propionibacterium jensenii*, *P. thoenii* and *P. acidipropionici* form a cluster well separated from *P. freudenreichii*. These species also differ from *P. freudenreichii* in the type of diaminopimelic acid in their peptidoglycan, and in their fermentation of sucrose and maltose.

The propionibacteria are metabolically complex organisms that are capable of fermenting substrates by a number of different pathways. A number of excellent reviews have been written (35, 36, 63, 77) and the reader is referred to these for detailed descriptions of the metabolism of these organisms. Briefly, the propionibacteria utilize the succinate-propionate pathway in which lactate is

converted to propionate via pyruvate and succinate (77). The initial steps in the reaction involve the conversion of lactate to pyruvate. Oxaloacetate is then formed in a transcarboxylation reaction and further reduced to succinate by a number of enzymes. Succinyl-CoA is then formed in a CoA transferase reaction and is rearranged to (R)-methylmalonyl-CoA which is converted to the (S)-enantiomer by a racemase enzyme. Transcarboxylation of (S)-methylmalonyl-CoA yields propionyl-CoA and CoA transfer to succinate yields propionate. The overall metabolism of 1.5 moles of glucose or 3 moles of lactate theoretically yields 2 moles of propionate, 1 mole of acetate, and 1 mole of carbon dioxide (77).

A number of other reactions occur in the propionibacteria that may be important in the overall ratios of products formed. A transphosphorylase reaction in which oxaloacetate and pyrophosphate are produced from phosphoenolpyruvate, carbon dioxide, and inorganic phosphate may influence the overall product ratios (77). This reaction is catalyzed by PEP carboxy-transphosphorylase and is unique in that carbon dioxide is fixed and the function of ATP is replaced by inorganic pyrophosphate. This reaction occurs when high carbon dioxide fixation is essential to replace a C_4 dicarboxylic acid removed from the cycle, as when succinate accumulates as an end product. When this occurs methylmalonyl-CoA is not regenerated fast enough to replenish the supply of oxaloacetate because the decarboxylation of succinate to propionate is a slow reaction in the propionibacteria (77). Therefore, for the cycle to continue, oxaloacetate must be regenerated by carbon dioxide fixation, thus altering the molar product ratios (18, 19, 77).

Citric acid cycle intermediates have been detected in propionibacteria and under aerobic conditions, metabolism is believed to occur via the citrate cycle (18,

19, 77). Wood (77) has shown that the citrate cycle in conjunction with the transcarboxylase reaction accounts for some of the atypical fermentation patterns observed. Metabolism via the citrate cycle causes shunting of acetyl-CoA from the production of acetate into the citrate pathway. Crow (18, 19) has shown that strains of propionibacteria used in Swiss cheese making were capable of metabolizing aspartate via the citric acid cycle such that 3 moles of aspartate and 1 mole of propionate were converted to 3 moles of succinate, 3 moles of ammonia, 1 mole of acetate, and 1 mole of carbon dioxide. Thus, as a consequence of aspartate metabolism, more lactate is fermented to acetate and carbon dioxide than to propionate.

Crow (19) suggested that aspartase and carboxytransphosphorylase activity explain the presence of succinate in Swiss cheese and the reason that the ratio of propionate to acetate is frequently less than 2:1 in cheese samples. Citric acid cycle intermediates and enzymes have been shown to be present in propionibacteria (77). It appears likely that, as Crow suggests (19), the citrate, transcarboxylase, transphosphorylase, and possibly the glyoxylate pathways operate during lactate fermentation and affect the product ratios.

Polysaccharide production from the metabolism of both glucose and lactose has also been observed in the propionibacteria (20). These polysaccharides are principally polymers of methylpentoses with some glucose and galactose. The production of polysaccharides alters the propionate:acetate product ratio by increasing the formation of acetate and carbon dioxide without any associated propionate production.

Another important trait of propionibacteria is the ability to produce high cell yields from the fermentation of glucose (77). This may have different implications

depending on the fermentation product desired. If biomass or a biomass-related product effluent is desired, high cell yields should mean reduced production costs. If a soluble metabolite is desired, high biomass production could mean that too little substrate is being converted to desired product, and higher downstream processing costs will be needed to remove cells.

Industrial Uses of Propionibacteria

In the manufacture of Swiss-type cheeses, the propionibacteria are primarily used to form the characteristic eyes (holes) but also contribute to the flavor, texture, and shelf life of these cheeses (35, 36, 46, 47, 48, 49, 54). Initially, in Swiss cheese making, lactose is fermented to lactic acid by strains of *Lactobacillus* and *Streptococcus*. During curing, the eyes are produced by the production of carbon dioxide from the metabolism of lactate by propionibacteria. The organic acids produced by propionibacteria are inhibitory to molds, yeasts, and some bacteria and thus improve the shelf life of the final product (30, 34, 35, 40, 54, 63, 69). The characteristic flavor of Swiss cheese is due, in part, to the production of amino acids, metabolic pathway intermediates, and short-chain fatty acids by the propionibacteria (36, 46, 47, 48, 49, 50, 51).

Propionibacteria may also contribute to some of the defects associated with Swiss cheese production. Splits often occur in the cold room and frequently are referred to as a "second fermentation" (18, 19). Brendehaug and Langsrud (7) have suggested that the split defect is a result of carbon dioxide being produced from decarboxylation of amino acids by the propionibacteria in the cold room.

It has been suggested that propionibacteria be used to preserve high-moisture corn (24) and silage (34). Propionic acid inhibits the growth of molds and

yeasts. The preservation of food crops especially important in countries located in tropical or subtropical areas where the costs of drying are high (24). However, the slow growth of propionibacteria and their poor ability to compete with other bacteria have limited their inclusion in commercial silage inoculants.

Propionibacteria are also used industrially as a probiotic (55, 56), in the production of vitamin B_{12} (32, 78), and for the production of food preservatives (5, 74). Currently three products of propionibacteria fermentation are sold commercially. Microgard, a fermented milk product produced by Wesman Foods in Beaverton, OR, is used in the cheese industry to increase the shelf life of cottage cheese. CAPARVE, a food preservative produced by PTX Food Corp. of Elmsford, NY, is marketed as a "natural mold inhibitor." Upgrade, produced by Microlife Techniques of Sarasota, FL, is also used as a preservative. These products are made by fermentation of natural materials such as milk and whey and are sold as "value-added" products. Food preserved with these products can still be marketed as a natural product.

Propionic Acid Production

As a preservative, propionic acid extends the shelf life of food products by inhibiting molds, yeasts, and some bacteria (24, 30, 40, 69). Although preservatives derived from propionibacteria fermentations are available, most propionic acid is produced by chemical synthesis. An economic analysis comparing the cost of producing propionic acid by fermentation to the current market price of $1.52 per kilogram propionic acid is shown in Appendix A. Even when an inexpensive substrate (corn steep liquor) is used, the cost of producing a propionic acid product is $7.78 per kilogram propionic acid.

If higher yields of propionic acid could be obtained, production by fermentations may become economically competitive and may offer several advantages to chemical synthesis. These advantages include: possible bacteriocin production that can increase the spectrum of antimicrobial activity; the ability to label the product as a "natural preservative" and thus allow its use in the natural foods market; and the opportunity to use food processing wastes as fermentation substrates, thus lowering production costs while accomplishing waste reduction.

Several processes have been patented for producing propionic acid by fermentation (for review see reference 63.) Batch methods using a variety of substrates typically produce 1-3% propionic acid in 7 to 14 days (5, 14, 25, 63). The slow growth rate of propionibacteria and inhibitory effect of propionic acid on the growth rate limit the usefulness of these bacteria in commercial processes (58, 60). To overcome these problems, other processes, including fed-batch (75), cell immobilization (9, 10, 11, 14), continuous (9, 14), continuous cell recycle (4, 6, 9), semi-continuous (25, 75), and multi-stage processes (63) have been used. This review will focus on the most current research because reviews of early work in this area are available (63).

Clausen and Gaddy (14) compared the production of organic acids from hydrolyzed corn stover in a continuous stirred tank reactor (CSTR) and a immobilized cell reactor (ICR), and developed a process for converting corn stover to organic acids. The process involved acid hydrolysis of corn stover to produce two sugar streams, one containing xylose and the other glucose. A strain of *Propionibacterium acidipropionici* capable of fermenting both sugars at low pH was used.

The continuous fermentation in the CSTR was studied over a retention time

of 5 to 96 hours (14). More than 90% conversion of glucose and 86% conversion of xylose were obtained in 72 hours in a CSTR. The maximum productivity for both sugar substrates occurred at a 72-hour retention time. Organic acid production levels of 3.74 g/l propionic acid and 2.8 g/l acetic acid were reached with an initial sugar concentration of 30 g/l.

To immobilize cells, Clausen and Gaddy (14) coated Raschig rings with a 20% gelatin and 1.5% agar solution. A seed culture was pumped through the column and incubated for 48 hours to establish a film of microorganisms cross-linked to the rings. Following the acclimation period of 48 hours, the ICR was operated with a 28-hour retention time. At this rate, 92% of glucose and 75% of xylose were converted to product and 20 g/l of organic acids were produced. This represented a major improvement in the productivity (in grams per liter per hour) of organic acids. This method also reduced the cost of downstream processing.

Economic analysis by Clausen and Gaddy (14) showed that cost of the recovered acid from corn stover would be $.366/kg. Based on 1984 market values for acids, this could allow a profit to be made from the production of acids by fermentation. Further benefit could be realized from reduced waste disposal costs.

Champagne *et al.* (11) used cells of *Propionibacterium shermanii* immobilized in calcium alginate beads in batch fermentations to produce propionic acid from acid whey. The advantage to using immobilized cells is that they can be easily recovered and used to re-inoculate fresh medium. A high-density inoculum was used to decrease the fermentation time. The effects of agitation, temperature, pH, type of neutralizer, and re-utilization of immobilized cells on acid production were examined. Optimum propionic acid production was achieved with whey that contained 1 to 2% lactate, neutralized to pH 7.0 with $Ca(OH)_2$. By agitating the

beads to remove end-products that tend to accumulate at the center of the beads, faster fermentation was achieved but the propionic acid to acetic acid ratio was reduced. Beads containing immobilized propionibacteria were used for ten consecutive fermentations without contamination.

Cavin *et al.* (10) investigated the use of a continuous reactor with propionibacteria trapped in calcium alginate beads for producing cheese flavors. The continuous process was maintained for 20 days. Once the fermentation was established, more organic acids could be produced in 30 minutes than could be produced by batch fermentation in 96 hours. Immobilizing the cells in beads allowed the maintenance of high levels of cells, thus improving the performance of continuous fermentations.

Continuous fermentation with cell recycle has been used to reduce the propionic acid concentration in the reactor and to increase the concentration of viable cells. One of the limitations to continuous processes is that low organic acid production results from low cell densities in the reactors. By using cell recycle, high cell densities are obtained, resulting in accelerated fermentations and higher organic acid productivity.

Carrondo *et al.* (9) compared the production of propionic acid by batch fermentation with three different types of continuous reactor types: CSTR, immobilized-cell columnar reactor, and CSTR with ultrafiltration cell-recycle. Higher cell densities were maintained in the immobilized cell reactor and the CSTR with cell recycle. As a consequence of this, higher organic acid productivity was maintained in both reactors than in the other types of reactors. The immobilized cell reactor performed the best in these comparisons, producing 100% of the maximum theoretical yield of propionic acid. However, immobilized cell reactors

were not without their problems. Carbon dioxide produced during fermentation reduced the useful reactor volume and limited cell access to fresh nutrients.

Other authors have reported successful use of continuous fermentation with cell recycle (4, 6, 32). In at least one case, two products could be sold: the effluent containing organic acids and a concentrated culture that could be used as a dairy starter culture (4). The disadvantage of these systems is the extra cost and high maintenance associated with the equipment used. If enough of the fermentation product can be sold, the extra cost may be recovered.

Production of Other Compounds

Most efforts to produce commercial products by fermentation focus on the production of a single compound. But for organisms as metabolically versatile as the propionibacteria, it may be advantageous to isolate multiple products from a single fermentation. The propionibacteria produce a number of metabolic end products of commercial value. These include: vitamin B_{12} (32, 78), amino acids (7, 19, 50, 51), and carbohydrates (20, 36, 76).

Vitamin B_{12} is an essential nutrient for humans, needed for the proper development of nerve tissue and red blood cells (32). Unlike propionic acid, vitamin B_{12} is produced by fermentation since chemical synthesis of the vitamin is difficult (32). Production of vitamin B_{12} by propionibacteria is a cell-associated process; therefore, vitamin B_{12} yields are correlated with cell yields. Batch processes yield approximately 6 to 8 g dry cells per liter and 2 mg per liter of vitamin B_{12} (32, 36, 78).

Hatanaka *et al.* (32) applied a cultivation system with a hollow-fiber module to remove propionic acid and acetic acid that inhibit growth during the

fermentation. The result was an increase in dry cell and vitamin concentrations of 35-fold (227 g/l) and 24-fold (52 mg/l), respectively. Though the yield of vitamin B_{12} from propionibacteria was improved by this method, other industrial strains such as *Butyribacterium methylotrophicum* produce higher amounts (92.5 mg/l) (32).

The propionibacteria are able to synthesize other organic acids and amino acids (7, 19, 50, 51). The dicarboxylic acid succinate and the amino acid proline may be produced in high enough concentrations to be considered for commercial production (41). Succinate is usually found in only trace amounts as a product of lactate fermentation (9, 41). However, appreciable amounts of succinate can be formed from glucose, glycerol, or aspartate fermentation (9). Proline is produced in appreciable amounts during ripening of Swiss-type cheese. Production of proline in Swiss cheese by propionibacteria is believed to occur through both hydrolysis of casein and through biosynthesis.

The formation of complex carbohydrates has been reported for propionibacteria when grown on glucose-based medium or medium containing a high lactose concentration (35, 76). Crow (20) suggested that low carbon recovery and low propionate to acetate ratios during fermentation by *P. freudenreichii* subsp. *shermanii* ATCC 9614 were caused by the production of a polysaccharide containing methylpentose (major component), glucose, and galactose. Stjernholm (76) identified the carbohydrate produced by three species of propionibacteria as 1-0-α-D-glucopyranosyl-α-D-glucopyranoside, commonly known as trehalose. In the food industry, trehalose is being used as a cryoprotectant to preserve foods during freeze drying or quick freezing (67). Other carbohydrates produced by propionibacteria (20, 35) may be effective stabilizers, thickeners, or gums for use

as food additives.

These few examples of other products that can be derived from propionibacteria fermentation illustrate the diversity of these organisms. The challenge for the industrial microbiologist or engineer will be in developing the downstream processing needed to recover the various products.

Methods of Culture Improvement

Maximizing the production of products from propionibacteria fermentations will require optimization of both the fermentation process and proper selection or manipulation of the strains used. Traditionally, cultures have been selected for fermentations by screening natural isolates for hyperproducing strains or selecting mutants capable of hyperproduction of product under specific conditions (64). Recently, with the proliferation of genetic tools available, the possibility of using genetic engineering to alter metabolic pathways or even create new pathways exists (42).

Overproduction of products has been a target in genetic engineering of industrial strains (64). Various techniques have been developed for overproduction of cloned gene products such as uses of runaway-replication plasmids, efficient expression vectors, and on-off regulation of gene expression (41). However, before these techniques can be applied to propionibacteria, more information on the basic biology of the propionibacteria and development of gene transfer techniques will be necessary.

Genetics of Propionibacteria

Relatively little is known about the genetics of the propionibacteria. Methods for isolation of DNA from propionibacteria have been developed (66) and 15 strains were screened for the presence of plasmid DNA (65). Seven distinct plasmids were isolated that ranged in size from 4.4 megadalton (MDa) to greater than 200 MDa. Restriction mapping and DNA hybridization revealed sequence homology among many of the plasmids found in propionibacteria. Only two of the seven plasmids isolated had no homology with any other *Propionibacterium* plasmids.

Derivatives cured of plasmids pRGO1, pRG02, pRG03, and pRG05 were screened for resistance to 18 antibiotics and antimicrobials, bacteriocin production, and the fermentation of 15 carbohydrates. Only two phenotypes, cell clumping and lactose utilization, were tentatively linked to plasmids pRG05 isolated from *P. jensenii* P38 and pRG03 from *P. freudenreichii* P93, respectively (66). Attempts to clone the lactose utilization genes into *E. coli* were unsuccessful.

Naud *et al.* (59) were able to induce production of spherical cells of *Propionibacterium freudenreichii* by growing cells under growth-limiting conditions and then plating on medium containing chloramphenicol or erythromycin. This treatment produced a change in morphology, a simultaneous resistance to antibiotics, a change in fermentation patterns, and the appearance of at least one new enzyme. Also, three plasmids not present in the parental strains were found in cell extracts of round cells. No information was presented to confirm that round cells were propionibacteria and not contaminants.

Recently, the structural genes for methylmalonyl-CoA mutase were successfully cloned from *Propionibacterium shermanii* into *E. coli* (57). This

enzyme catalyzes the structural rearrangement of succinyl-CoA into (2R)-methylmalonyl-CoA as part of the propionic acid fermentation. Oligonucleotide probes derived from purified peptide fragments were used to screen a *P. shermanii* library consisting of 1.0 to 1.8 kb fragments in plasmid pUC13. Positive transformants were identified, subcloned into plasmids M13 mp 18 and M13 mp 19, and sequenced. A two-gene sequence was identified. Nucleotide sequence analysis of a 4.5-kb piece of DNA showed that the region upstream of the first gene did not contain consensus promoter sequences found in other procaryotes. However, an *E. coli*-like potential ribosome-binding site was present before the start of each gene, and when the genes were placed on a plasmid behind a strong *E. coli* promoter both mutase genes were expressed in *E. coli*. The two genes were found to lie very close together with the last G of the C-terminal lysine of the *mutA* gene being the first base of the GTG initiation codon of the *mutB* gene. A region of inverted symmetry, which is a potential transcription termination structure, was present 20 bp downstream of the termination codon of the second gene. These results suggest that the genes are probably transcribed as an operon.

More information is needed on the basic genetics of these organisms, especially the mechanism of plasmid replication and gene expression. The small cryptic plasmids present in strains of *Propionibacterium* will be useful in the construction of cloning vectors. However, nothing is known about the mode of replication of these plasmids or the compatibility of these plasmids in other bacteria. Also, information on how genes are expressed in propionibacteria is needed. Isolation and genetic analysis of promoters and the origin of replication from propionibacteria plasmids will provide valuable information and also form the basis for cloning vectors. Also needed: an examination of restriction-modification

systems, the physical and genetic map of the chromosome, and the structure and control of genes.

Gene Transfer Methods

Transfer of genetic information in gram-positive bacteria can occur by at least four mechanisms: transformation, conjugation, transduction, and protoplast fusion. These gene transfer methods are essential for genetic studies and application of genetic engineering for strain construction.

Transformation is defined as the process by which a cell takes naked DNA from the surrounding medium and expresses the newly acquired genes (43). Some gram-positive bacteria are able to undergo "natural transformation," the most well characterized of which include *Bacillus subtilis*, *Streptococcus sanguis*, and *Streptococcus pneumoniae* (73). However, most gram-positive bacteria do not possess natural transformation systems, and therefore artificial techniques that allow direct cloning of foreign genes into these organisms have been developed.

One method commonly employed in gene cloning is polyethylene glycol-induced transformation of protoplasts (cells with the cell wall removed). Protoplast transformation was first described in *Streptomyces* (3) and yeast (38) and then extended to *B. subtilis* by Chang and Cohen (12). Most of the published protoplast transformation procedures in bacteria are modified versions of the original method of Chang and Cohen. The general method of protoplast transformation is as follows: cells are grown in an appropriate medium, harvested by centrifugation, washed, and resuspended in buffer containing an osmotic stabilizer (usually a carbohydrate or amino acid). Protoplasts are formed by adding muralytic enzymes to the cell suspension and incubating for the appropriate time. Once protoplasts

are formed, the DNA is added to the suspension followed immediately by addition of polyethylene glycol. After an incubation period, the protoplasts are washed, resuspended in broth to allow for expression of the cloned genes, and then plated onto selective media supplemented with an osmotic stabilizer.

More recently, a procedure termed electroporation has been successfully applied to gram-positive bacteria. Electroporation is now an established procedure for transforming a variety of cell types (8, 22, 53, 71). Electroporation involves polarization of the cell membrane by the application of an electric pulse, resulting in the formation of transient pores in the cell membranes (8, 71). The resultant pores are large enough for macromolecules such as DNA to pass through and, as long as the electric field does not exceed a critical limit, the permeability is reversible. Unlike protoplast transformation, electroporation requires the purchase of expensive electroporators.

Conjugation is a mechanism of gene transfer that occurs when a donor and recipient cell are in close physical contact (43). In gram-positive bacteria conjugation can be performed in broth (15), by forced physical contact on membrane filters (17, 70), or by plating cells on an agar medium (43). Gene transfer from the donor to the recipient strain is usually mediated by a conjugative plasmid or transposon that possesses transfer capabilities.

The conjugative transposons Tn*916,* first identified in *Enterococcus faecalis* DS16 (26, 28), and Tn*919* from *Streptococcus sanguis* FC1 (23) have been useful tools for genomic analyses of gram-positive bacteria (16, 15, 17, 26, 37). These transposons possess a number of similar characteristics that have been exploited for both genome mapping and targeting genes for cloning in *E. coli (29).* Both transposons encode tetracycline resistance, are similar in size (15 and 16.5 kb for

Tn*916* and Tn*919*, respectively), are capable of conjugative transfer at frequencies ranging from 10^{-5} to 10^{-8}, and can randomly insert into either the chromosome or plasmids of host cells. Both transposons have been transferred to a number of gram-positive bacteria by filter mating. A high-frequency conjugal delivery system for Tn*919* has been developed in *Lactococcus lactis* (37) and for Tn*916* in *Enterococcus faecalis* (28)

Transfer of conjugal plasmids can also be used to facilitate genetic analysis and as an alternative gene transfer method in strains in which other gene transfer systems are either inefficient or don't exist (15, 17, 29, 31, 33, 45, 52, 61, 70, 72, 79). Plasmids pAMβ1 (15, 17) and pIP501 (39) are capable of conjugal transfer into a wide range of hosts and have been used extensively in genetic studies of gram-positive bacteria (2, 15, 27, 31, 33, 43, 45, 61, 70, 72, 79). Plasmid pAMβ1 isolated from *E. faecalis* DS5 is a 26.5 kb-plasmid expressing MLS (macrolide-lincosamide-streptogramin B) antibiotic resistance. Plasmid pIP501 first isolated from *Streptococcus agalactiae* is a 30.2-kb plasmid that encodes resistance to both MLS antibiotics and chloramphenicol. Both plasmids have been characterized at the molecular level and have been used to develop gene cloning and transposon delivery systems (26, 44).

Certain bacteriophages are able to transfer host genes between bacterial cells (43). Transduction is not a method of gene transfer commonly employed in gene cloning experiments but has been used for mapping the genome of bacteria and strain construction.

Protoplast fusion of two phenotypically distinct strains can cause recombination of genetic information to occur (43). In this procedure, protoplasts of two strains are formed and caused to fuse by addition of polyethylene glycol.

Regeneration of the cell wall results in whole cells that may form recombinants containing genetic information from both strains. As with transduction, protoplast fusion has had more application in genome mapping than in gene cloning.

Gene Transfer in Propionibacteria

A procedure for the production and regeneration of protoplasts was first developed by Baehman and Glatz (1). Baehman and Glatz (1) used lysozyme (20 mg/ml final concentration) to remove the cell wall from *P. freudenreichii* strain P104. Optimum protoplast formation was achieved by using logarithmic-phase cells suspended in Tris-HCl buffer containing 0.5 M sucrose and 10 mM $MgCl_2$. Greater than 99% of the cells were converted to protoplasts. Plating by the overlay method onto regeneration medium containing 0.5 M sucrose and 2.5% gelatin resulted in regeneration frequencies of 10 to 30%. Attempts to transform protoplasts with plasmid DNA by the method of Chang and Cohen (12) were unsuccessful.

Other groups have since reported formation and regeneration by similar methods (68). Protoplasts of *P. shermanii* ATCC 9614 were used to examine cell wall, membrane, and intracellular fractions for peptidase activity (68). Protoplasts were produced by pre-conditioning cells in a 20% (wt/vol) sucrose solution incubated for 2 hour at 4°C with shaking. Pre-conditioned cells were resuspended in buffer and treated for 90 min with lysozyme.

Pai (62) later examined other factors that affect protoplast production. By incubating the cells in an anaerobic environment and washing the cells after harvesting and after lysozyme treatment, consistently higher regeneration frequencies were obtained. Several factors were investigated to develop a

procedure for polyethylene glycol (PEG)-induced transformation of protoplasts of strain P104. The amount of DNA added to the protoplast suspension ranged between 1 and 12.5 μg. The concentration of PEG varied from 40 to 60%. The incubation time for the protoplast-PEG-DNA reaction mixture ranged between 4 and 90 min. The length of the incubation time for expression of plasmid DNA in transformants was 20 hours.

Erythromycin-resistant putative transformant colonies were obtained in transformation with plasmid pE194, a small *Staphylococcus aureus* plasmid that codes for erythromycin resistance (62). However, no free plasmid DNA was observed when total DNA extracts from these colonies were examined by agarose gel electrophoresis. Purified DNA from 19 putative transformants was transferred to a nitrocellulose membrane in a slot blot apparatus and hybridized with a biotinylated pE194 probe. After hybridization, the DNA from eight cultures gave a positive color reaction, while the DNA from strain P104 gave no reaction.

Pai speculated that plasmid DNA may have integrated into the chromosome. To test this hypothesis, chromosomal DNA from eight putative transformants was digested with a restriction enzyme that cut pE194 at one site. The digested DNA was transferred to nitrocellulose and probed with biotinylated pE194. The DNA from two cultures hybridized with the pE194 probe; no hybridization with P104 DNA was observed. One culture produced two bands, indicating possible integration of plasmid pE194 into the chromosome. Only one band was observed for the other culture, suggesting that the plasmid had not integrated into the chromosome. However, definitive proof that the erythromycin-resistant colonies were true transformants was not obtained by Pai since the hybridization produced only faint bands that were not even dark enough to be

photographed.

Luchansky *et al.* (53) transformed cells of *Propionibacterium jensenii* strain B-77 by electro-transformation. However, the transformation frequencies reported were too low to be useful for gene cloning. Successful gene transfer into propionibacteria by conjugation or protoplast fusion has not been reported.

SECTION I

PROPIONIC ACID PRODUCTION BY A PROPIONIC ACID-TOLERANT STRAIN OF *PROPIONIBACTERIUM ACIDIPROPIONICI* IN BATCH AND SEMICONTINUOUS FERMENTATION

Propionic acid production by a propionic acid-tolerant strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation†

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ABSTRACT

A propionic acid-tolerant derivative of *Propionibacterium acidipropionici* strain P9 was obtained by serially transferring strain P9 through broth that contained increasing amounts of propionic acid. After one year of repeated transfers, a strain (designated P200910) capable of growth at higher propionic acid concentrations than P9 was obtained. An increase in the proportion of cellular straight chain fatty acids and uncoupling of propionic acid production and growth were observed for strain P200910. Growth rate, sugar utilization, and acid production were monitored during batch and semicontinuous fermentation of semidefined medium and during batch fermentation of whey permeate for both strain P200910 and strain P9. The highest propionic acid concentration (47 g/l) was produced by P200910 in a semicontinuous fermentation. Strain P200910 produced a higher ratio of propionic to acetic acid, utilized sugar more efficiently, and produced more propionic acid per gram of biomass than did its parent in all fermentations.

INTRODUCTION

Strains of the genus *Propionibacterium* are used in several industrial processes because of their ability to convert lactate and carbohydrates to propionic acid, acetic acid, and carbon dioxide. The metabolism of glucose by propionibacteria theoretically yields 2 moles of propionate, 1 mole of acetate, and 1 mole of carbon dioxide from 1.5 moles of glucose (29, 41). Propionibacteria are primarily used by the dairy industry for the production of Swiss-type cheeses. The products from the metabolism of lactate are responsible for the characteristic eyes and contribute to the flavor, texture, and shelf life of Swiss cheese (16). Though they are used mainly in cheese production, propionibacteria are also used industrially as a silage inoculum (12), as a probiotic (24, 25), and for production of vitamin B_{12} (14, 29, 42) and propionic acid (29).

As a preservative, propionic acid extends the shelf life of food products by inhibiting molds and some bacteria (15, 20, 22). Although preservatives derived from propionibacteria fermentations are available, most propionic acid used by the food industry is produced by chemical synthesis (29). If higher yields of propionic acid could be obtained, production by fermentation may become economically competitive and may offer several advantages to chemical synthesis. These advantages include: bacteriocin production (17) that can increase the spectrum of antimicrobial activity, the ability to label the product as a "natural preservative," and an opportunity to use food-processing wastes as fermentation substrate, thus lowering disposal costs.

Several processes have been patented for producing propionic acid by fermentation (29). Batch methods using a variety of substrates typically produce 1-

3% propionic acid in 7 to 14 days (2, 3, 4, 29). Other processes, including fed batch (16), cell immobilization (6, 7), continuous (2, 4, 6, 8, 29), semicontinuous (13), and multistage processes (29) have been used to improve the yield of propionic acid. Despite these efforts, the maximum reported yield of propionic acid obtained by fermentation is still too low to be economically competitive with chemical synthesis.

The major factor that limits the production of propionic acid during fermentation is end-product inhibition by the acid (22). To overcome the inhibitory effect of propionic acid, continuous processes combined with cell recycling have been employed to remove metabolic end products (27, 28). Though these processes were able to increase the yield, the production of propionic acid was not large enough to offset the higher costs of continuous processes. Mutant strains resistant to end products have been used to increase the production of ethanol from *Clostridium thermocellum* (35), butanol from *Clostridium acetobutylicum* (23), and ethanol from yeasts (21).

To improve the yield of propionic acid, we have developed a propionic acid-tolerant mutant, designated P200910, by using a simple enrichment technique. When used in a semi-continuous fermentation, P200910 produced greater amounts of propionic acid than did the parental strain. We report here the characterization of P200910 in batch fermentation and the development of a semicontinuous process. Also, the physiological changes that occur in the mutant strain and their contribution to acid tolerance are discussed.

MATERIALS AND METHODS

Strains and culture maintenance. *Propionibacterium acidipropionici* strain P9 was obtained from the culture collection of the Department of Food Science and Human Nutrition, Iowa State University. Strain P9 and its propionic acid-tolerant derivative P200910 were grown in sodium lactate broth (NLB) at 32°C (19). Working cultures were maintained on sodium lactate agar (NLA) and stored at 4°C. All cultures were permanently stored at -70°C in NLB supplemented with 10% glycerol. Fermentation broth (FB) consisted of 0.6% yeast extract, 0.3% trypticase, and 3% glucose at pH 7.0. Glucose was sterilized separately in the autoclave and added aseptically to the fermentation broth before the start of fermentation.

Culture conditions. Primary cultures were prepared by inoculating 10 ml of NLB with isolated colonies from an NLA plate and incubating at 32°C for 24-36 h. For small-scale cultures, a 1% inoculum of this culture was transferred into 10 ml of the appropriate fresh medium and incubated at 32°C. For 500-ml fermentation experiments, a 1% inoculum of the primary culture was transferred to 25 ml of FB in a 100-ml Erienmeyer flask. The culture was incubated at 32°C and harvested while in exponential phase (OD_{550} of 0.8). All seed cultures were incubated without agitation. The entire contents of the flask were used to inoculate the fermenter.

Growth rate measurements. Small-scale (10 ml) cultures of P9 and P200910 were grown in the desired medium, incubated at 32°C, and observed for changes in optical density at 550 nm (OD_{550}) with a Spectronic 21 spectrophotometer (Milton Roy, Rochester, NY). Specific growth rates were determined by plotting the natural log (ln) of the OD_{550} vs. time. Regression analysis was performed on the values taken from the linear portion of the curve,

and the specific growth rate was calculated from the slope of the least-squares regression line. Analysis of variance was performed on data obtained from replicate trials.

Fatty acid analysis. One-liter cultures of strains P9 and P200910 were grown in NLB for 48 h at 32°C to an OD_{550} of 1.0. The cells were removed by centrifugation and washed 3 times with 0.01 M phosphate buffer (pH 7.0). Fatty acid methyl esters (FAMES) of cellular lipids were prepared by the method of Baer *et al.* (1) with the following modifications. The methanolic-base reagent was prepared by mixing 170 ml sodium methoxide (Fluka Chemie AG, Buchs, Switzerland) with 750 ml anhydrous methanol. A 0.5-g sample of wet-packed cells, 1 ml benzene, and 1 ml methanolic-base reagent were added to a screw-cap test tube (18 x 150 mm). The tube was sealed and heated in a water bath at 80°C for 20 min. The sample was cooled to room temperature before 3 ml water and 3 ml diethyl ether were added. The contents of the tube were mixed, the lower aqueous phase was removed, and the upper benzene-ether layer was washed twice with 2 ml water. Residual water was removed by drying over sodium sulfate crystals. The solvent layer, containing the FAMES, was transferred to 1-dram vials and stored under nitrogen at -20 °C.

Immediately before analysis, the remaining solvent was removed by evaporation under a gentle stream of nitrogen. The FAMES were resuspended in 50 µl hexane and a 1-µl sample was injected into a Varian 3700 gas chromatograph (Varian Aerograph, Palo Alto, CA) equipped with a flame ionization detector and a fused silica capillary column (15 m x 0.32 mm) coated with SP-2330. The column temperature was programmed for 100°C to 230°C at 4°C/min. The injection and detector temperatures were set at 230°C. An integrator (model

3396A Hewlett-Packard, Arondale, PA) was used to analyze data and plot chromatograms. The FAMES were identified by comparing the retention times with FAME standards (Analabs, Foxboro Co., North Haven, CT). The peak heights were measured, and the percentage of each peak in the sample was calculated from the ratio of the individual peak height to the sum of the heights of all detected peaks.

Batch fermentation. Batch fermentations (500-ml working volumes) were performed in a model C30 bench-top fermenter (New Brunswick Scientific Co., New Brunswick, NJ) with accessory pH controller (model pH-40, New Brunswick) or a Multigen bench-top fermenter (New Brunswick) equipped with a model TTT2 automatic titrator (Radiometer, Copenhagen, Denmark) for pH control. The vessel with 450 ml of FB was sterilized in the autoclave for 20 min, and 50 ml of a sterile 30% glucose solution was added aseptically. Temperature was controlled at 32°C, agitation rate was 200 rpm, and pH was maintained at 7.0 by addition of 2 N NaOH.

To determine if acid production and cell growth decreased during batch fermentation because of product inhibition or nutrient depletion, spent broth was obtained from a batch fermentation, centrifuged to remove cells, and sterilized by filtration through a 0.22-μm filter. A 9-ml sample was placed in screw-cap test tubes, and 1 ml of filter-sterilized 10X FB was added to each tube. A 2% inoculum of an active 48-h culture of either P9 or P20091 was added and the tubes were incubated at 32°C. Culture growth over time was monitored at OD_{550}. Control tubes contained FB and spent broth with no added nutrients.

Semicontinuous fermentation (SCF). Initial fermentation conditions were the same as described for batch fermentation. After the initial 48 h of

fermentation, 50 ml of 10X FB and 25 ml of a 60% glucose solution were added while an equal volume of spent FB was removed from the fermenter. This procedure was repeated every 24 to 36 h until the measured amount of propionic acid in the fermentation broth did not increase.

Fermentation of whey permeate. Sweet whey was obtained from Swiss cheese production in the Food Science and Human Nutrition Department, Iowa State University. The whey was filtered through an Amicon DC10L ultrafiltration unit containing a H5MP01-43 0.1-μm hollow-fiber filter cartridge (Amicon Div., W.R. Grace and Co., Danvers, MA) and filtered a second time through a S10Y10 spiral-wound ultrafiltration cartridge with a 10,000 MW cutoff (Amicon). Whey permeate was adjusted to pH 7.0 by addition of NaOH, filter-sterilized, and stored at 4°C. Batch fermentations of whey permeate in 9-l working volumes were performed in a model NLF 22 fermenter (Bioengineering Corp., Wald, Switzerland). The culture was grown in the fermenter at 32°C, pH 7.0, and agitated at 200 rpm. Temperature, pH, and agitation were monitored and controlled by computer (Nomad System Inc., San Jose, CA).

Analysis of products. Glucose, lactose, propionic acid, and acetic acid were separated by high-performance liquid chromatography with a Waters model 501 pump (Waters, Div. of Millipore, Milford, MA) and a HPX-87H column (Bio-Rad, Richmond, CA) operated at 65°C, with 0.012 N H_2S0_4 (pH 2.0) as the mobile phase at 0.8 ml/min flow rate. Peaks were detected with a Waters differential refractometer (model R401). Samples for analysis were centrifuged to remove cells, filtered through 0.22-μm filter, and stored at -70°C before analysis. A Maxima 820 software program (Waters) was used to analyze the data and plot the chromatograms. The product concentration was calculated by comparing the peak

areas with those of external standards (Aldrich Chemical Co., Milwaukee, WI).

Biomass was determined from a standard curve of optical density vs. dry weight. Dry weights for the standard curve were obtained by filtering aliquots of culture through prerinsed, dried, and weighed 0.22-μm filters, rinsing with 0.1 M phosphate buffer (pH 7.0), and drying filters in a microwave oven (Tappon/O'Keefe & Merrit, Chicago, IL). The standard curve was plotted from the mean values of two determinations, and biomass was obtained from the least-squares regression line.

All fermentations were performed at least twice. Results are the averages of these replicate trials.

RESULTS

Development of a propionic acid-tolerant strain. The propionic acid-tolerant strain was derived from strain P9 by a modification of the serial dilution method of Lin and Blaschek (23). A 1% inoculum of a primary culture of P9 was transferred to a series of tubes with 10 ml NLB that contained 0.5 to 5% propionic acid. The tubes were incubated at 32°C for 24 h, and the change in OD_{550} was monitored. Cells from the broth with the highest propionic acid concentration that showed growth were repeatedly transferred into fresh medium containing that concentration of propionic acid. Once the growth rate of the tolerant strain reached approximately 80% of that of the unchallenged parental strain, the tolerant strain was transferred into broth containing a slightly greater amount of propionic acid, and the process was repeated.

After one year of such repeated transfers, a strain designated P200910 was obtained that was able to grow at higher concentrations of propionic acid than P9. Electron micrographs of strain P200910 showed no differences in morphology from that of the parent strain (data not shown). Strain P200910 is identical to the parental strain in Gram reaction, fermentation of sucrose, maltose and mannitol, reduction of nitrate, and pigment production.

Growth characteristics of P200910. A plot of the specific growth rates for P200910 and P9 in NLB that contained different concentrations of propionic acid is shown in Fig. 1. Analysis of variance showed significant differences ($P<0.05$) between the growth rates of P9 and P200910 at propionic acid concentrations between 1 and 7%. No statistically significant differences were observed for growth rates of the two strains at 0% or 8% propionic acid. However, P200910

had a slightly faster specific growth rate at 8% propionic acid (0.047 vs. 0.033) and a slightly slower specific growth rate (0.185 vs. 0.199) at 0% propionic acid. In NLB, strain P200910 had a longer lag time and grew to a lesser final cell density than did P9, even after several transfers.

The stability of strain P200910's tolerance to propionic acid was determined by serially transferring this strain 10 times in NLB or in NLB with 0.5% propionic acid and then inoculating these cultures into NLB with 2% propionic acid. No difference in growth was observed if inocula were serially transferred with or without propionic acid.

Fatty acid analysis. Fatty acid analysis was performed to characterize any physiological changes associated with propionic acid tolerance. Typical chromatograms are presented in Fig. 2. Table 1 lists the relative amounts of the 12 major peaks that represent greater than 90% of the total peaks detected. Eight of the peaks have been identified by comparing their retention times with those of known FAME standards. These peak identifications agreed with previously published fatty acid profiles of propionibacteria (18). The predominant fatty acids were 15- and 17-carbon iso- and anteiso-branched-chain and straight-chain fatty acids. Hofherr *et al.* (18) also reported the presence of hydrocarbon peaks tentatively identified as branched 19- and 21-carbon species. In this work, peaks 10 and 12 have retention times corresponding to 19:0 and 20:0 straight-chain fatty acids. Four peaks could not be identified. The relative amounts of the predominant fatty acids were different for strains P9 and P200910. Strain P200910 had less branched-chain and more normal-chain fatty acids.

Fig 1. Specific growth rates of P9 (□) and P200910 (■) in NLB that contained various amounts of propionic acid. Error bars represent standard deviations for four replicate triais. Specific growth rates were determined by plotting the natural log (ln) of the OD_{550} vs. time. Regression analysis was performed on the values taken from the linear portion of the curve, and the specific growth rate was calculated from the slope of the least-squares regression line.

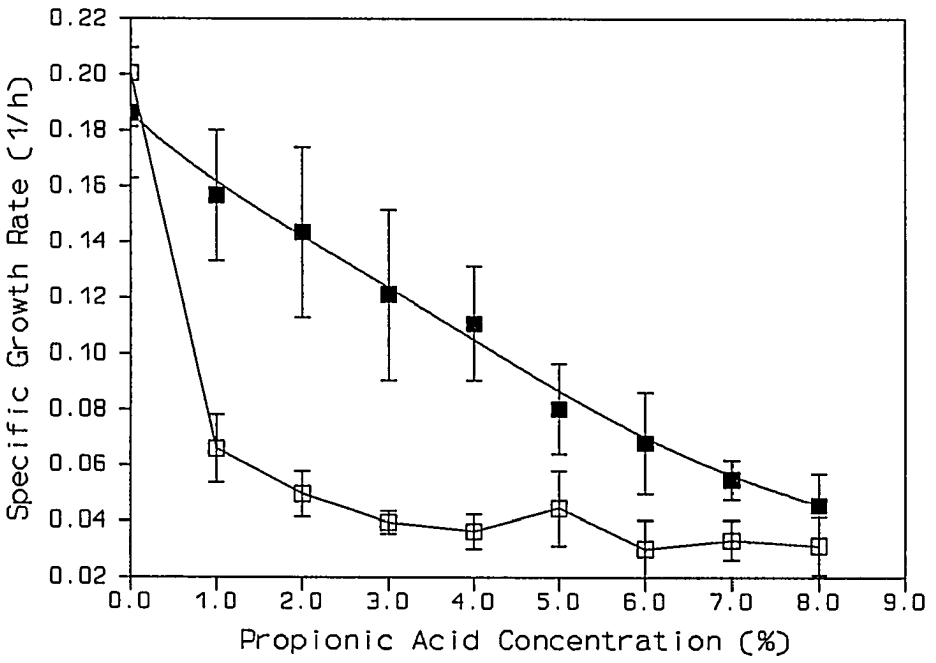
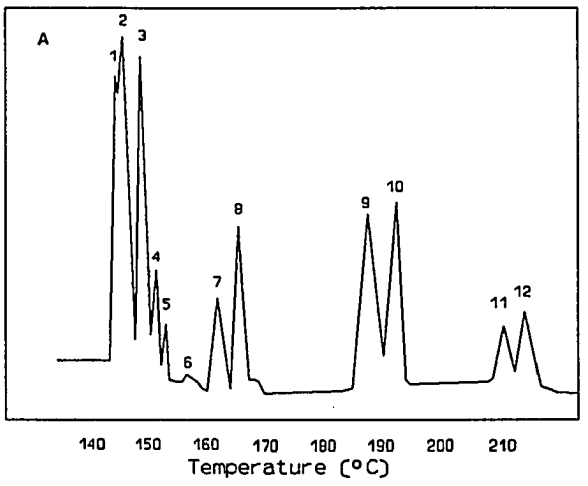

Specific Growth Rate (1/h)
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
Propionic Acid Concentration (%)

Fig. 2. Typical gas chromatograms of fatty acid metyl esters prepared from strains P200910 (A) and P9 (B).


A
1
2
3
4
5
6
7
8
9
10
11
12
140
150
160
170
180
190
200
210
Temperature (°C)

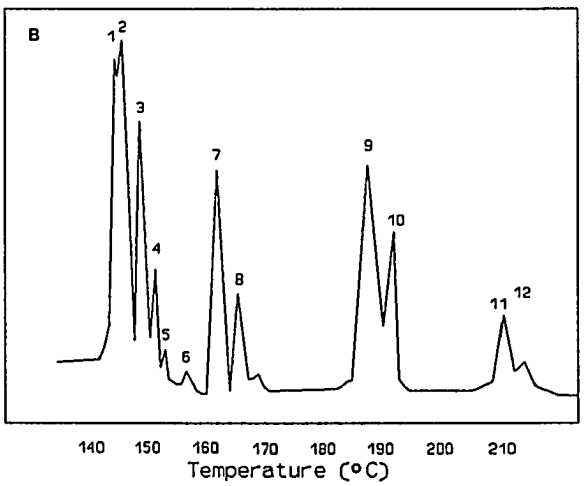

B
1
2
3
4
5
6
7
8
9
10
11
12
140
150
160
170
180
190
200
210
Temperature (°C)

Table 1. The identity and relative amounts of the major fatty acids in cellular lipids of strains P9 and P200910, as determined by gas chromatography.

		% Fatty Acids Present	
Peak #	Identification[a]	P9	P200910
1	i15:0	16.9	15.4
2	a15:0	18.3	17.5
3	n15:0	13.6	16.6
4	HC	6.4	6.1
5	HC	2.2	3.5
6	i17:0	0.9	0.9
7	a17:0	11.4	4.8
8	n17:0	5.0	8.3
9	HC	11.4	9.2
10	HC[b]	8.2	9.6
11	HC	4.1	3.5
12	HC[c]	1.4	4.4

[a] n = normal; a = anteiso; i = iso; HC = hydrocarbon.
[b] Unidentified hydrocarbon, retention time corresponds to 19:0 straight-chain fatty acids.
[c] Unidentified hydrocarbon, retention time corresponds to 20:0 straight-chain fatty acids.

Batch fermentation. Data from typical batch fermentations for strains P9 and P200910 are shown in Fig. 3, and selected parameters are tabulated in Table 2. The strains had similar lag times (12 h). Growth rate during exponential phase of P200910 (0.84 g/liter per h) was faster than that of P9 (0.45 g/liter per h), but strain P9 remained in exponential phase longer (36 h) and reached a larger final biomass. Strain P9 entered stationary phase at 48 h; strain P200910 at 28 h.

After 60 h of fermentation, P200910 had produced more propionic acid than had P9. Propionic acid produced per gram of biomass was much greater for P200910 (0.91 vs. 0.50). Strain P200910 produced propionic acid faster during exponential phase, and continued production after reaching stationary phase. Propionic acid production was fastest between 20 and 38 h for both strains. Final acetic acid concentration and rate of acetic acid production were similar for both strains but acetic acid produced per gram biomass was greater for strain P200910 than for P9 (0.21 vs. 0.13). Strain P200910 converted glucose to propionic acid somewhat more efficiently than did P9, but both strains produced less acid than the theoretical maximum. Propionic acid to acetic acid (PA:AA) ratios for both strains were higher than the theoretical ratio of 2:1.

Strain P200910 exhibited biomass and propionic acid production patterns typical of nongrowth-associated product formation, whereas P9 followed typical growth-associated product formation patterns (31). Fastest growth of P200910 occurred earlier than the period for maximum propionic acid production or maximum glucose utilization (Fig. 4). In contrast, periods of maximum growth and glucose utilization coincided for strain P9.

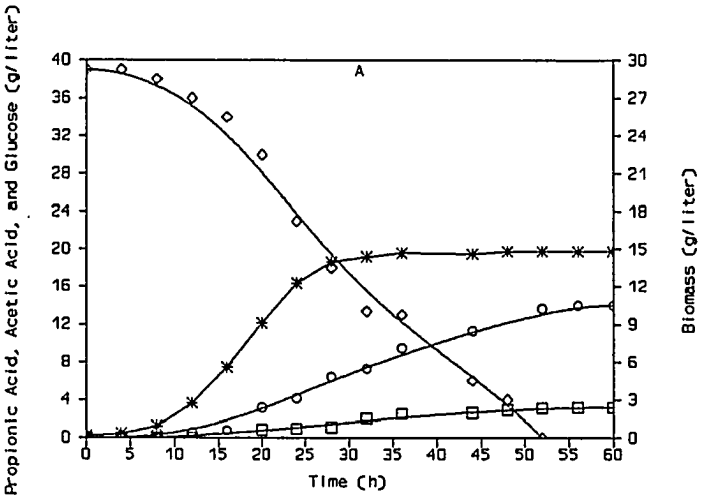
To determine whether acid and biomass production in batch fermentations eventually decreased because of product inhibition or nutrient depletion, the growth

rates of strains P9 and P200910 in FB were compared in spent fermentation broth that contained 0.6% propionic acid (SB) and in spent broth supplemented with fresh fermentation broth (SBFB) (Table 3). Both strains showed little growth in SB. Growth rates of P9 and P200910 grown in supplemented SB were 76% and 79%, respectively, of their growth rates in FB. Nutrient depletion and product inhibition may both play a role in growth limitation during batch fermentations, though it seems that nutrient depletion has a greater effect.

Whey permeate fermentation. Performance of P9 and P200910 in batch fermentation of a natural substrate, cheese whey permeate, was evaluated (Fig. 5 and Table 2). Growth and acid production were much slower than in batch fermentations in defined medium. Even after 8 days of fermentation, only 44% and 36% of the lactose had been used by P200910 and P9, respectively. Although total biomass and organic acid concentrations were lower in whey permeate than in semidefined medium, the yields of propionic and acetic acid per gram sugar utilized were greater in whey permeate fermentations.

Strain P200910 grew to a much lower biomass concentration than did P9, but it produced more propionic acid. Its production of propionic acid per gram biomass was much greater than that of strain P9 (5.4 vs. 1.1). Much more lactose was converted to propionic acid than to biomass or acetic acid by P200910. The propionic:acetic acid ratio was about double that of strain P9.

Fig. 3. Growth, glucose utilization, and acid production by strains P200910 (A) and P9 (B) in semidefined medium in a batch fermentation process. Symbols: *, biomass; ◊, glucose; O, propionic acid; □, acetic acid. Results are the averages of three replicate trials.


A
Propionic Acid, Acetic Acid, and Glucose (g/liter)
Biomass (g/liter)
Time (h)
0 4 8 12 16 20 24 28 32 36 40
0 3 6 9 12 15 18 21 24 27 30
0 5 10 15 20 25 30 35 40 45 50 55 60

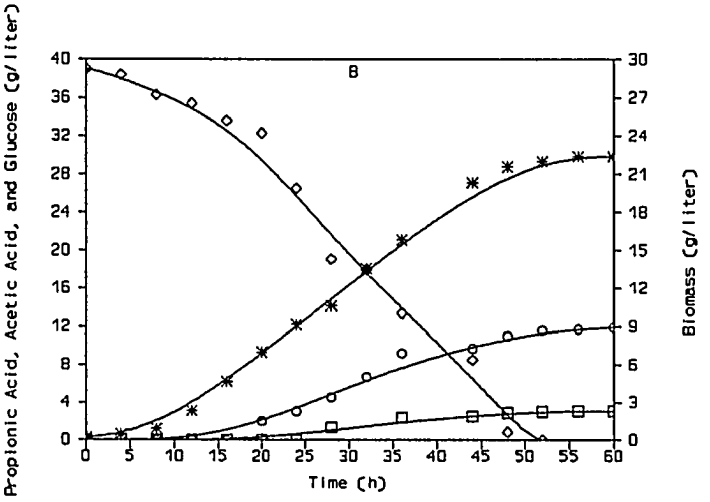
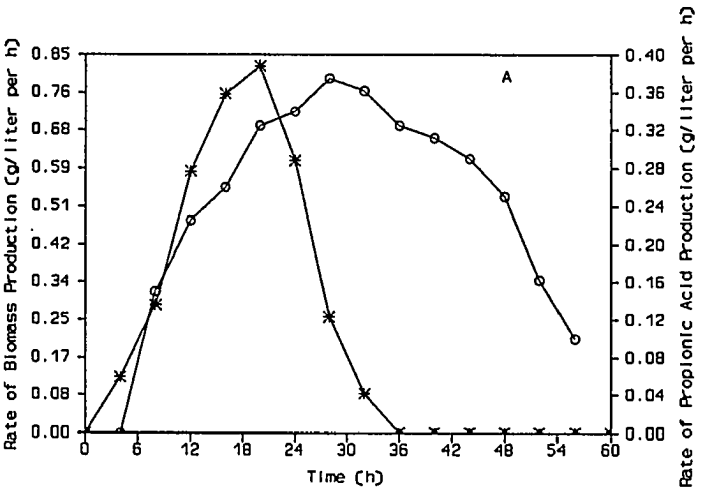

B
Propionic Acid, Acetic Acid, and Glucose (g/liter)
Biomass (g/liter)
Time (h)
0 4 8 12 16 20 24 28 32 36 40
0 3 6 9 12 15 18 21 24 27 30
0 5 10 15 20 25 30 35 40 45 50 55 60

Table 2. Characteristics of fermentations with strains P9 and P20010.

Strain	Medium[a]	Process[b]	X_{max}[c] (g/liter)	Final acid concn[d] (g/liter)		Volumetric productivity[e] (g/liter·h)		$Y_{p/s}$[f] (g/g)			Yield[g] (% theoretical maximum)		Ratio[h] PA:AA
				PA	AA	PA	AA	X	PA	AA	PA	AA	
P9	FB	Batch	23	11.6	3.0	0.33	0.07	0.59	0.30	0.08	54	36	3.0
P200910	FB	Batch	15	13.6	3.1	0.39	0.07	0.34	0.35	0.08	64	36	3.5
P9	WP	Batch	7.8	8.8	3.4	0.044	0.002	0.37	0.55	0.21	-	-	2.1
P200910	WP	Batch	1.7	9.2	1.9	0.048	0.007	0.13	0.70	0.14	-	-	3.9
P9	FB	SCF	78	32	9.1	0.26	0.10	0.74	0.31	0.09	56	41	2.9
P200910	FB	SCF	58	47	10.5	0.37	0.12	0.55	0.45	0.10	82	45	3.6

[a] FB, fermentation broth; WP, whey permeate.
[b] Batch = 60-h fermention in FB or 8-d fermentation in WP, SCF = semicontinuous fermentation for 8 d.
[c] X_{max}, maximum biomass production.
[d] PA, propionic acid; AA, acetic acid.
[e] Maximum rate of production calculated from the linear portion of the best fit curve from Figs. 3, 5, 6.
[f] Yield coefficient for product on carbon substrate, g of product produced per g sugar utilized.
[g] Calculated as the percent of the theoretical maximum yield of 55 g propionic acid and 22 g acetic acid per 100 g glucose (41).
[h] Molar ratios of propionic and acetic acid produced.

Fig. 4. Rates of biomass (*) and propionic acid (O) production by strains P200910 (A) and P9 (B) in semidefined medium in batch fermentation process. Rates were calculated from the averaged results from three trials.


A
Rate of Biomass Production (g/liter per h)
Rate of Propionic Acid Production (g/liter per h)
Time (h)
0.85
0.76
0.68
0.59
0.51
0.42
0.34
0.25
0.17
0.08
0.00
0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
0
6
12
18
24
30
36
42
48
54
60

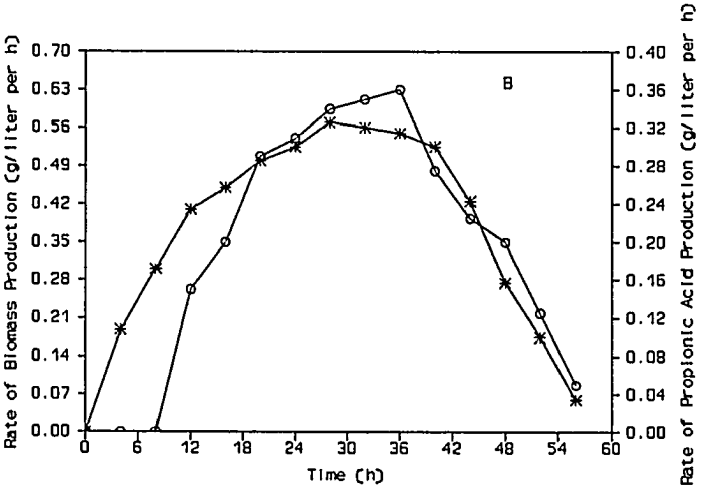

B
Rate of Biomass Production (g/liter per h)
Rate of Propionic Acid Production (g/liter per h)
Time (h)
0.70
0.63
0.56
0.49
0.42
0.35
0.28
0.21
0.14
0.07
0.00
0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
0
6
12
18
24
30
36
42
48
54
60

Table 3. Specific growth rates of P9 and P200910 grown for 24 h in spent fermentation broth or a mixture of spent and fresh fermentation broth.

	Specific Growth Rate (h^{-1})[a]		
Strain	FB[b]	SB[c]	SBFB[d]
P9	0.21	0.05	0.16
P200910	0.14	0.04	0.11

[a] Specific growth were determined by plotting the natural log (ln) of the OD_{550} vs. time. Regression analysis was performed on the values taken from the linear portion of the curve, and the specific growth rate was calculated from the slope of the least-squares regression line.
[b] Fermentation broth.
[c] Spent fermentation broth, obtained from a batch fermentation experiment after 96 h of cultivation, containing 0.6% propionic acid and no detectable glucose.
[d] Spent fermentation broth mixed with 10X concentrated fresh fermentation broth at 9:1 vol:vol.
Values given are the averages from two separate experiments.

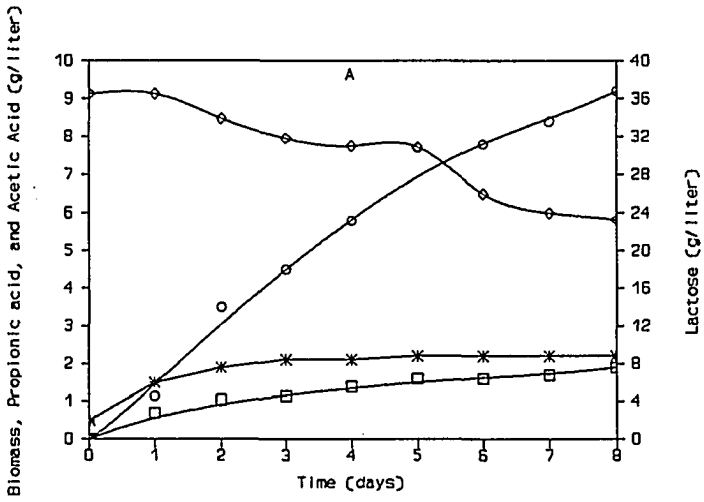
Semicontinuous fermentation. Strains P9 and P200910 were cultivated in a semi-continuous fermentation (SCF) in which fresh fermentation broth was added to the vessel at regular intervals, at which times an equal volume of spent broth was removed. Data are presented in Fig. 6 and Table 2. Much greater final concentrations of biomass and organic acids were obtained in semicontinuous than in batch fermentations. These were achieved by extending growth and acid production over a longer time and by supplying more sugar as the fermentation progressed. Yields of biomass from sugar used were greater in semicontinuous than in batch fermentation. Yields of acids from sugar used were approximately

the same as in batch fermentations, with propionic acid production being favored in semicontinuous fermentation by strain P200910.

As was seen in batch fermentations, P200910 produced less biomass, but more propionic acid than strain P9. Growth rates of both strains were rapid through 5 days of incubation, at which time the propionic acid concentration was 26 g/liter for P9 and 38 g/liter for P200910. The amount of propionic acid produced per gram biomass was greater for P200910 (0.81 g/g), than for P9 (0.41 g/g). Rates of propionic acid production decreased for both strains after 4 days of incubation.

Early in the fermentation (days 2 to 4), P9 was able to utilize added glucose within 36 h. After day 4, P9 required at least 48 h to exhaust the added glucose. After day 6, complete glucose utilization did not occur. In contrast, strain P200910 continued to use glucose at a high rate throughout the fermentation. Figure 7 shows the fermentation profile for P200910 in the 36-h period following the first and sixth nutrient addition. After the first nutrient addition, glucose was exhausted by 24 h, and biomass and organic acid concentrations increased through 36 h. After the sixth nutrient addition glucose again was exhausted by 24 h, but biomass and organic acid concentrations showed little or no change.

Fig. 5. Growth, lactose utilization, and acid production by strains P200910 (A) and P9 (B) in whey permeate in a batch fermentation process. Symbols: *, biomass; ◊, lactose; O, propionic acid; □, acetic acid. Results are the averages of two replicate trials.


A
Biomass, Propionic acid, and Acetic Acid (g/liter)
Lactose (g/liter)
Time (days)
0
1
2
3
4
5
6
7
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9
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12
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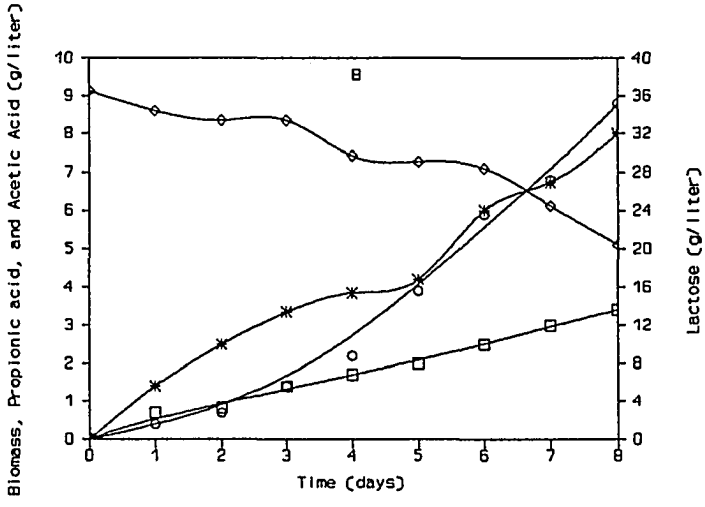
32
36
40


B
Biomass, Propionic acid, and Acetic Acid (g/liter)
Lactose (g/liter)
Time (days)
0
1
2
3
4
5
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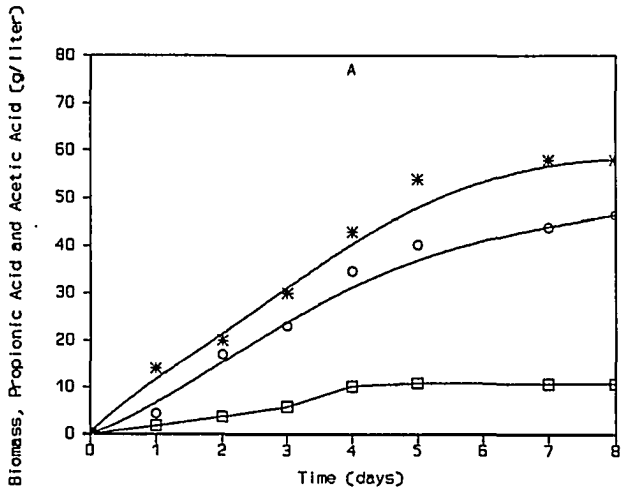
8
9
10
12
16
20
24
28
32
36
40

Fig. 6. Growth and acid production by strains P200910 (A) and P9 (B) in semidefined medium in a semicontinuous fermentation process. Symbols: *, biomass; O, propionic acid; □, acetic acid. Results are the averages of three replicate trials.


A
Biomass, Propionic Acid and Acetic Acid (g/liter)
Time (days)
0
10
20
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40
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70
80
0
1
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4
5
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7
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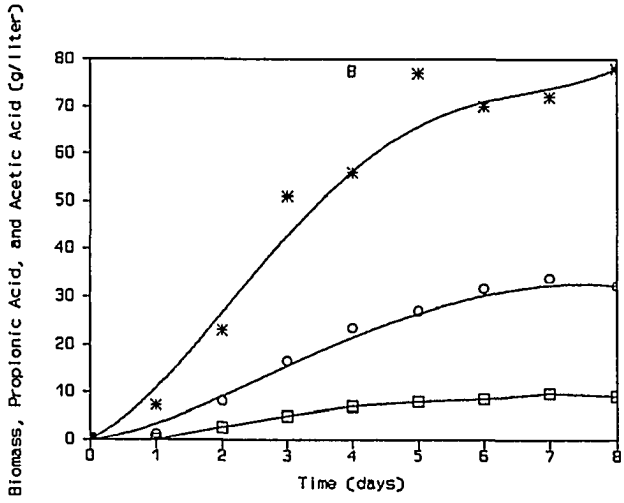
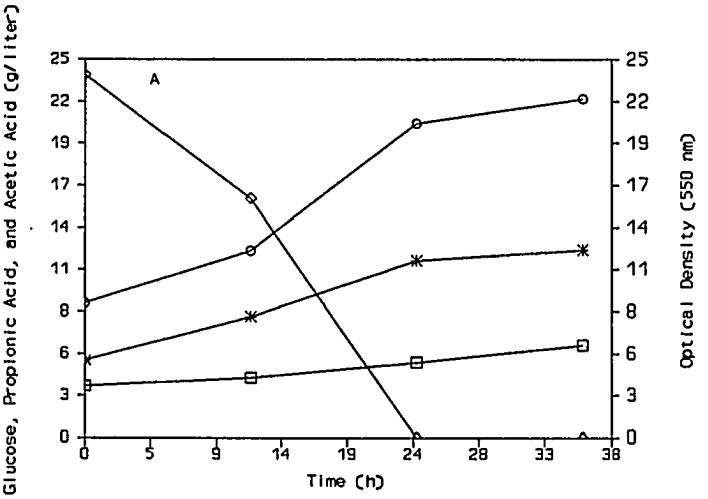
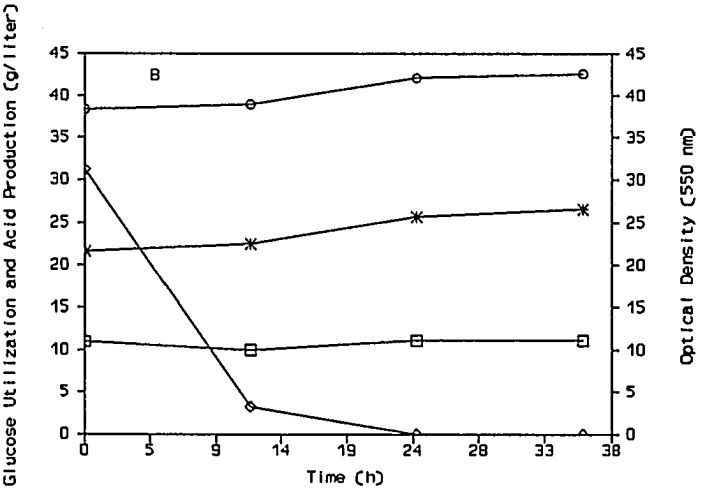

B
Biomass, Propionic Acid, and Acetic Acid (g/liter)
Time (days)
0
10
20
30
40
50
60
70
80
0
1
2
3
4
5
6
7
8

Fig. 7. Growth, glucose utilization, and acid production by strain P200910 during the 36-h periods following the first (A) and sixth (B) nutrient additions during semicontinuous fermentation. Samples were analyzed at 12, 24, and 36 h after nutrient addition. Symbols: * optical density; ◊, glucose; O, propionic acid; □, acetic acid.


A
Glucose, Propionic Acid, and Acetic Acid (g/liter)
Optical Density (550 nm)
Time (h)
0
3
6
8
11
14
17
19
22
25
0
5
9
14
19
24
28
33
38


B
Glucose Utilization and Acid Production (g/liter)
Optical Density (550 nm)
Time (h)
0
5
10
15
20
25
30
35
40
45
0
5
9
14
19
24
28
33
38

DISCUSSION

This study was undertaken to develop a strain of *Propionibacterium* able to produce greater amounts of propionic acid. The commercial use of fermentation processes for the production of organic acids is limited by low yields and high recovery costs. This limitation could be overcome if higher yields of acids could be obtained. The propionic acid-tolerant mutant, P200910, produced more propionic acid even though it grew to a lesser biomass than did the parental strain.

Continued product synthesis during fermentation is often inhibited by the end products produced during the fermentation (27, 28, 31). Strains of bacteria and yeasts tolerant to fermentation end products have been successfully used to increase production of ethanol and butanol (21, 23, 35, 36). Lin and Blaschek (23) used a serial dilution method to increase the butanol tolerance of *C. acetobutylicum.* The resultant strain was able to produce greater amounts of butanol. We adapted this procedure to develop propionic acid tolerance in a strain of *Propionibacterium.* This tolerance seems to be a stable trait maintained in cultures grown in medium without propionic acid.

Changes in lipid composition occur in bacteria to assure survival under adverse conditions such as changes in temperature, starvation, exposure to irradiation, and the presence of organic acids and solvents (26). Warth (39) has shown that propionic acid-resistant yeast strains are less permeable to propionic acid than are sensitive strains. Baer *et al.* (1) showed that a butanol-tolerant mutant of *Clostridium acetobutylicum* responded to the presence of butanol by increasing the percentage of 16:0 and 18:0 fatty acids and decreasing the percentage of 16:1 and 18:1 fatty acids in its membrane lipids.

Normally, propionibacteria contain predominantly branched-chain 15:0, 16:0, 17:0 fatty acids and no unsaturated fatty acids (18). Gas chromatographic analysis showed greater amounts of straight-chain fatty acids in P200910 than in P9. The physiochemical effect of a methyl branch or a cis-double bond in a fatty acid instead of a saturated straight chain is to decrease the melting point and increase the surface area of the membrane that contains the lipid (11). Therefore, lesser amounts of branched-chain fatty acids and greater amounts of straight-chain fatty acids would likely result in a less fluid, less permeable membrane. This seems to be the situation with strain P200910. The significance of this change in cell lipids is unknown at this time.

Reductions in the yield of cells grown in the presence of preservative have been reported (30, 37, 38, 40). We observed that P200910 had smaller biomass yield than its parent, yet it continued to utilize glucose in the presence of high propionic acid concentrations. Perhaps this acid-tolerant strain may be expending energy to rid itself of excess acid.

Strain P200910 was superior to the parental strain P9 in several respects. Its yield of propionic acid from substrate, production of acid per gram biomass, propionic to acetic acid ratio, and percentage of theoretical maximum yield attained were greater than the corresponding values for P9 for all fermentation methods used. The major reason for the differences in these parameters is the shift in P200910 to greater production of propionic acid at the expense of acetic acid and biomass. Also, this tolerant strain was able to continue acid synthesis as high acid concentrations accumulated in the medium during fermentation.

Batch fermentations of semidefined medium showed that acid production by strain P200910 had seemingly shifted from a growth-associated to a nongrowth-

associated phenomenon. This shift may be advantageous to fermentation productivity as incoming glucose can be converted principally to organic acids. A decrease in the amount of biomass produced may reduce downstream processing costs during large-scale production by reducing the time and energy required for removing biomass.

Whey fermented by propionibacteria is considered to be a natural preservative and is produced commercially for use in bakery products (2, 4, 7, 33). Whey permeate is a cheap, readily available substrate for the production of propionic acid, but fermentations generally require 10 to 14 days to complete and produce 2 to 11 g/liter acid (2, 4, 7, 29, 33).

We observed that strain P200910 produced more propionic acid from whey permeate than did its parent, but fermentation time was too long to make this an economically feasible process. After 8 days of fermentation, less than half of the total lactose had been used. Strains able to utilize lactose at higher rates are needed. It may be possible to increase propionic acid tolerance in such a strain or to select for faster-growing variants of strain P200910.

Several fed-batch, semicontinuous, and continuous propionic acid fermentation processes have been patented (29). These yield 20 to 30 g/l propionic acid and require 5 to 14 days to complete (3, 29). Recent work on propionic acid production has focused on using continuous methods to increase the product yields during fermentation. Clausen and Gaddy (8) were able to produce 20 g/liter propionic acid in an immobilized cell reactor with a plug flow tubular column. Carrondo *et al.* (5) compared the performance of three types of continuous reactors and reported that a continuous stirred-tank reactor with ultrafiltration cell recycle was most efficient, with propionic acid concentrations at 18

g/liter and maximum volumetric productivity of 2.2 g/liter/h. Blanc and Goma (2) and Boyaval and Corre (4) also reported efficient continuous culture-cell recycle systems that produced 17 and 25 g/liter propionic acid at maximum volumetric productivities of 5.0 and 14.3 g/liter/h, respectively. The maximum volumetric productivities of propionic acid obtained in this study were similar to those reported for batch fermentations and for continuous culture systems without cell recycle (3, 5), but were less than values reported for continuous systems with cell recycle (2, 4, 5).

In this work, we have obtained a propionic acid concentration of 47 g/liter with strain P200910 grown in semidefined medium in a semicontinuous fermentation. This level is 40 to 50% higher than those previously reported in the literature (2, 3, 4, 5, 6, 7, 8, 13, 29, 34,), but is less than is theoretically possible.

In practice, yields of propionic and acetic acids and their molar ratios often deviate from theoretical values derived from known pathways of glucose metabolism. This study was no different in this regard. Values obtained were typical of propionibacteria fermentations and likely were affected by the production of succinate (9, 32) and an extracellular polysaccharide (10). During fermentations, we observed both the accumulation of succinate in the medium (data not shown) and an increase in viscosity.

The semicontinuous process used in this study shows promise as a means to produce propionic acid with P200910. It was run for 8 days but could be performed in a shorter period. Nutrients were added every 24 to 36 h, but P200910 exhausted the glucose within 12 to 16 h. A commercial process might be run by initially operating the fermenter in a fed-batch mode until the maximum propionic acid concentration was achieved. From that point, spent medium could

be removed as fresh nutrients were added. The addition of nutrients could be triggered by the glucose level reaching a predetermined lower limit. This process could yield a continuous supply of broth with higher propionic acid concentrations than have been reported previously.

ACKNOWLEDGMENTS

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SECTION II

PRODUCTION AND TRANSFORMATION OF PROTOPLASTS OF *PROPIONIBACTERIUM*

64B

Production and transformation of protoplasts of *Propionibacterium*

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ABSTRACT

An improved method for production of protoplasts of *Propionibacterium freudenreichii* strain P104 is reported. Protoplasts were prepared from cells grown in sodium lactate broth plus 1% threonine and treating the cells with a combination of lysozyme and chymotrypsin. The protoplasts were osmotically sensitive and were able to regenerate on hypertonic medium to walled cells. Polyethylene glycol-induced transformation of protoplasts of strain P104 was attempted. The protoplasts seemingly were able to undergo transformation at low frequency but autonomous plasmid DNA was not detected by agarose gel electrophoresis. Intracellular nuclease activity was detected in strain P104 and may explain the lack of success in transforming this strain.

INTRODUCTION

Strains of the genus *Propionibacterium* are industrially important microorganisms used in the production of Swiss-type cheeses (5, 8), silage inocula (6), probiotics (19), vitamin B_{12} and propionic acid (24).

To apply genetic engineering techniques to these industrially important organisms, a reliable and efficient method for introducing DNA is essential. Luchansky *et al.* (17) transformed cells of *Propionibacterium jensenii* strain B-77 by electrotransformation. However, the transformation frequencies reported were too low to be useful for gene cloning. Ziernstein and Rehberger (31) recently reported electroporation-mediated transformation of three strains of *Propionibacterium* using the *Staphylococcus aureus* plasmid pC194. However, no autonomous plasmid DNA was observed in the transformants. A comparison of hybridization patterns of digested chromosomal DNA from transformants and from the parent with a pC194 probe showed that the plasmid had integrated into the host genome.

Polyethylene glycol (PEG)-induced transformation of protoplasts is an effective method for introducing foreign DNA into gram-positive bacteria (4, 11, 12, 15, 20, 21, 26, 27, 28, 29, 30). Fusion of bacterial protoplasts has also been useful for promoting genetic exchange between microorganisms (4, 7). Both methods of gene transfer are dependent on the successful production and regeneration of protoplasts.

Though procedures for the production and regeneration of protoplasts in classical propionibacteria have been developed, successful protoplast transformation has not been accomplished. Baehman and Glatz (1) were the first to report the production and regeneration of protoplasts from *Propionibacterium*

freudenreichii. Pai (22) extended the procedure to other species of *Propionibacterium.* Though these authors were able to show the conversion of rod-shaped cells to spherical forms, they were unable to produce protoplasts that burst when suspended in buffer without osmotic stabilizer (1). Woskow and Kondo (30) showed that protoplast transformation of *Lactococcus iactis* would not occur in cells that remained osmotically stable after muralytic enzyme treatment. The inability to produce *Propionibacterium* protoplasts that lyse when suspended in buffer without an osmotic stabilizer may be responsible for lack of successful transformation.

In this study we report the development of an improved method for the production of protoplasts in a classical *Propionibacterium* strain. By growing cells in broth containing threonine, and treating these cells with a combination of lysozyme and chymotrypsin, we were able to produce and regenerate osmotically sensitive cells of *P. freudenreichii.* Transformation of protoplasts with plasmid DNA produced antibiotic-resistant colonies that may contain transforming DNA integrated into the host chromosome.

MATERIALS AND METHODS

Bacterial strains and media. *Propionibacterium freudenreichii* P104 was obtained from the culture collection of the Department of Food Science and Human Nutrition, Iowa State University. It was grown at 32°C in either sodium lactate broth (NLB) (1) or sodium lactate broth supplemented with 1% threonine (NLBT). Working cultures were maintained at 4°C on sodium lactate agar (NLA, NLB plus 1.5% agar).

Staphylococcus aureus strain ISP1390, containing plasmid pE194 (9) which confers erythromycin-inducible resistance (Em^R) to macrolide, lincosamide, and streptogramin (MLS) antibiotics, and *S. aureus* ISP1386, containing plasmid pC194 (10) which confers chloramphenicol resistance (Cm^R), were provided by Dr. P. A. Pattee, Department of Microbiology, Iowa State University, Ames. Cultures of *S. aureus* were grown at 37°C in trypticase soy broth (TSB, BBL Microbiology Systems, Cockeysville, MD) or trypticase soy agar (TSA, BBL), supplemented with either 5 μg/ml erythromycin (Em) or 5 μg/ml chloramphenicol (Cm). Working cultures were maintained on TSA at 4°C and transferred biweekly. All cultures were permanently stored at -70°C in their respective media supplemented with 10% glycerol.

Regeneration medium (RM) was prepared as described by Baehman and Glatz (1), and consisted of NLA plus 0.5 M sucrose and 2.5% gelatin. Soft agar overlays contained 0.5% agar in the RM base medium. All media were sterilized in an autoclave at 121°C for 15 min and tempered in a water bath.

Buffers and reagents. Protoplast buffer (PB) consisted of 0.5 M sucrose, 100 mM Tris-hydrochloride, and 10 mM $MgCl_2$, adjusted to pH 7.0. For some

applications sucrose was omitted from PB. Buffers were sterilized at 121°C for 15 min and immediately cooled to room temperature in a water bath. Lysozyme (Sigma Chemical Co., St. Louis, MO), chymotrypsin (Boehringer Mannheim, Indianapolis, IN), pronase E (Sigma), achromopeptidase (Sigma), and trypsin (Boehringer Mannheim) were dissolved in PB or PB without sucrose at 20 mg/ml final concentration and sterilized by passage through a 0.22-μm membrane filter. Pronase was incubated for 1 h at 37°C prior to use. Lysostaphin (Sigma) was dissolved in PB without sucrose at 1 mg/ml final concentration and sterilized by filtration.

DNA Isolation. Rapid microscale plasmid isolation from propionibacteria was performed by the method of Rehberger (25). Preparative-scale isolation of DNA from strain P104 was performed as described by Rehberger (25) except that 500 μl of diethyl pyrocarbonate (Sigma) were added prior to pronase digestion. Plasmid DNA was separated by electrophoresis of DNA samples through a 0.6% horizontal agarose gel in TBE buffer (0.089 M Tris hydrochloride, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) for 6 to 12 h at 50 V.

Gels were stained in ethidium bromide (0.5 μg/ml in distilled water), observed on a UV transilluminator (Foto UV 300; Fotodyne Inc., New Berlin, WI), and photographed through 23A and 2B Wratten gel filters with a Polaroid MP4 camera (film type 55). Large-scale isolation of plasmid DNA from *S. aureus* was performed by the method of Pattee (23), and the DNA was purified by CsCl-ethidium bromide density gradient centrifugation. Gradient-purified plasmid DNA samples were extracted with isopropanol saturated with 5 M NaCl to remove the ethidium bromide and desalted and concentrated in 10 mM Tris hydrochloride-1 mM disodium EDTA (pH 7.5) by using a Centricon-30 microconcentrator according

to the instructions of the manufacturer (Amicon Corp., Danvers, MA).

Nuclease activity assay. A 1-liter culture of P104 was incubated at 32°C for 36 h, harvested by centrifugation at 12,000 x *g* for 10 min, and washed twice in 0.01 M phosphate buffer (pH 7.0). Wet packed cells were resuspended in 10 ml of cold 0.01 M phosphate buffer (pH 7.0) and held on ice. Crude cell extracts were prepared by five passages through a French press (SLM Instruments, Inc., Urbana, IL). Cell debris was removed from the mixture by centrifugation at 12,000 x *g* for 15 min followed by passage of the supernatant through a 0.22-μm filter.

A 50-μl volume of cell-free extract was placed in 1.5-ml tubes and 1 μg of plasmid DNA was added. The mixture was incubated for either 1 h or 2 h at 37°C. The reaction was stopped by addition of an equal volume of phenol:chloroform (Amresco, Solon, OH) followed by centrifugation at 12,000 x *g* for 15 min. The DNA in the upper aqueous layer was precipitated with ethanol and the precipitate was resuspended in 20 μl of 10 mM Tris hydrochloride-1 mM disodium EDTA (pH 7.5). A 1-μl volume of RNase (Boehringer Mannheim) was added and the mixture incubated for 1 h at 37°C. The DNA was separated by electrophoresis as described above.

Nick translation, DNA hybridization, and detection. Biotinylated plasmid DNA probes were made by using a nick translation kit and biotin-11-dUTP as described by the manufacturer (Bethesda Research Laboratories Inc., Gaithersburg, MD). A Centricon-30 microconcentrator was used to remove unincorporated biotin 11-dUTP after nick translation according to the instructions of the manufacturer.

Total cell DNA was transferred to nitrocellulose sheets (Trans-blot nitrocellulose membranes; Bio-Rad Laboratories, Richmond, CA) by using the

Southern blotting technique (18). Prehybridization and hybridization buffers (10 ml/100 cm^2 of filter) consisted of 45% deionized formamide, 350 μg/ml denatured salmon sperm DNA, 0.15 M NaCl, 15 mM sodium citrate, 5X Denhardt reagent, and 25 mM sodium phosphate. Filters were subjected to the prehybridization treatment in a sealed bag at 42°C with constant agitation for at least 4 h. Biotinylated probe DNA was denatured at 95°C for 10 min and cooled in an ice bath. The denatured probe was added to the hybridization buffer at a concentration of 100 to 200 ng/ml. The filter and hybridization solution were sealed in a bag and incubated at 42°C for 24 to 36 h. Posthybridization washes, filter blocking, and detection of homologous sequences were performed as described by the manufacturer of the biotinylated DNA detection system (Bethesda Research Laboratories).

Production and regeneration of protoplasts. The final procedure for the formation of protoplasts of *P. freudenreichii* was performed as described previously (1) with the following modifications in the growth medium and the enzymes used to remove the cell wall. Isolated colonies from an NLA plate were inoculated into 10 ml of NLB and incubated at 32°C for approximately 24 h. This culture was inoculated at 2% (vol/vol) into 30 ml of NLBT. The NLBT culture was incubated for 24 to 36 h at 32°C to a final cell density of approximately 10^9 colony-forming units/ml. The cells were removed by centrifugation at 7,600 x *g* for 5 min, washed in cold (4°C) deionized water, and suspended in 9 ml PB. A sample of the cell suspension was withdrawn, diluted in sterile water, and spread on NLA plates to determine the initial colony-forming units (CFU). A 1-ml aliquot of lysozyme in PB was added to 9 ml of cells to give a 2 mg/ml lysozyme concentration. The cell suspension was incubated at 37°C for 15 min, at which time 1 ml of chymotrypsin

in PB was added (2.0 mg/ml final chymotrypsin concentration). After incubation at 37°C for another 30 min, the cells were sedimented by centrifugation at 2000 x *g* for 10 min, washed in 5 ml PB, and suspended in 9 ml PB.

Osmotically sensitive cells were enumerated by diluting aliquots of the cell suspension in sterile water and spread-plating 0.1-ml samples onto NLA. Initial cells and protoplasts were enumerated as described by Baehman and Glatz (1). The NLA plates were counted after 7 days, and the RM plates counted after 30 days of incubation. Percent protoplast regeneration was calculated as: [(CFU on RM - CFU on NLA after enzyme treatment) ÷ (initial CFU on NLA - CFU on NLA after enzyme treatment)] x 100 = % regeneration. For comparison with previously published work, the equation of Lee-Wickner and Chassy (14) was also used for calculating regeneration frequencies: [(CFU on RM - CFU on NLA after enzyme treatment) ÷ initial CFU on NLA] X 100= % regeneration.

Transformation. A 0.5-ml portion of a protoplast suspension was placed in a 50-ml centrifuge tube. Plasmid DNA (1 μg in 10 mM Tris hydrochloride, 1 mM disodium EDTA, pH 7.5) suspended in an equal volume of 2X PB was added, followed immediately by 1.5 ml of a 30% polyethylene glycol 6000 (PEG, Sigma) solution in PB. Controls received no DNA. After gentle shaking, the mixture was kept at room temperature for 5 min at which time 5 ml PB were added. Protoplasts were recovered by centrifugation at 4,500 x *g* and were resuspended in 1 ml NLB supplemented with 0.5 M sucrose, 5 mM $MgCl_2$, and 1 μg/ml Em. After incubation at room temperature for 2 h, a 0.2-ml aliquot was added to 5 ml soft RM containing 10 μg/ml Em, and overlaid onto RM containing 10 μg/ml Em. The plates were incubated anaerobically at 32°C for 21-30 days.

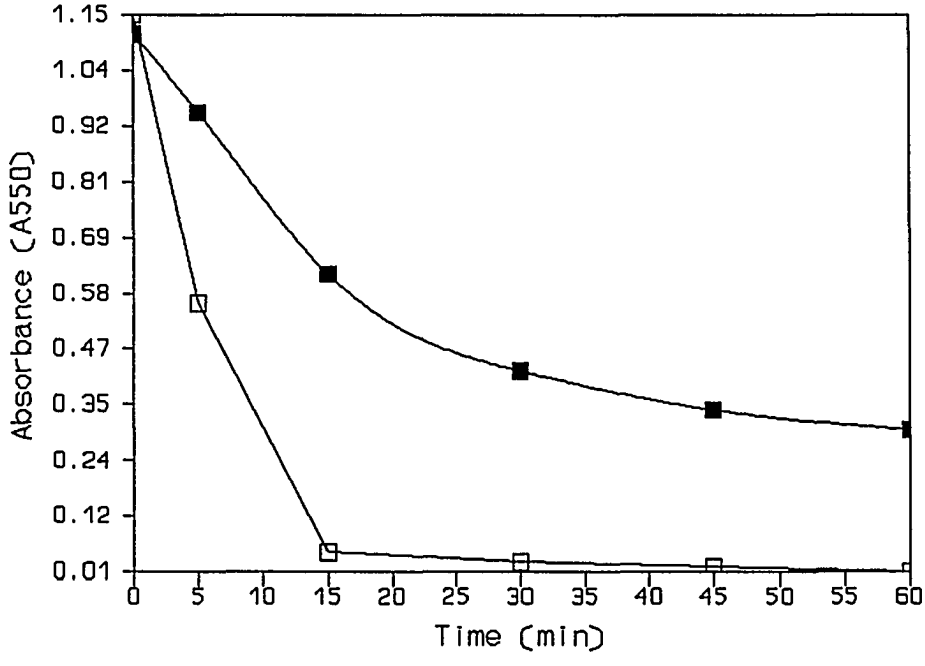
RESULTS

Lysis of P104 cells grown in NLB or NLBT. Strain P104 was incubated in NLB or NLBT and prepared for protoplast formation as described in Materials and Methods, except that cells were suspended in 9 ml PB without sucrose. A 1-ml portion of lysozyme was added (2 mg/ml final concentration) and the mixture was incubated for the desired time. To circumvent the lengthy plate count method to count protoplasts, cell lysis upon exposure to SDS was taken as the indication of lysozyme activity. A 2-ml sample of the lysozyme-cell mixture was added to 2 ml of a 4% SDS solution in deionized water. The suspension was mixed and the A_{550} measured after 2 min at room temperature.

Growth in NLBT resulted in greater sensitivity to cell wall digestion by lysozyme than cells grown in NLB (Fig. 1). Almost complete reduction in absorbance of NLBT-grown cells was seen after 15 min exposure to lysozyme followed by SDS treatment, compared to a 44% reduction in absorbance of NLB-grown cells after the same treatment. Even after 60 min incubation with lysozyme, only a 69% reduction in absorbance of cells grown in NLB was seen.

Activity of different lytic enzymes on P104. Cultures were grown in NLBT, suspended in PB without sucrose, and incubated at 37°C with lysozyme, achromopeptidase, lysostaphin, lysozyme and chymotrypsin, or lysozyme and achromopeptidase. Lysostaphin was used at 50 μg/ml final concentration and all other enzymes were added at 2 mg/ml final concentrations. Protoplast formation was again determined by observing the decrease in absorbance when cells were mixed with SDS. Results are shown in Fig. 2.

Fig. 1. Effect of time of lysozyme treatment on lysis of cells of strain P104 grown in NLB (■) or NLB with 1% threonine (□). Absorbance of each sample was measured 2 min after adding 2% SDS. Results are from averages of duplicate trials.


1.15
1.04
0.92
0.81
0.69
0.58
0.47
0.35
0.24
0.12
0.01
Absorbance (A550)
0
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)

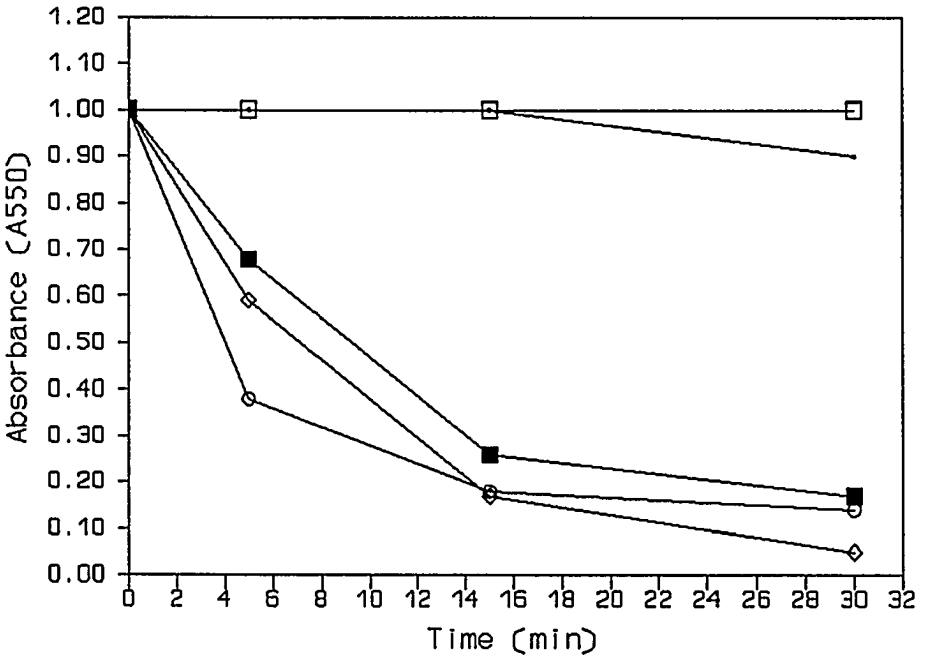
No decrease in absorbance was observed for cell suspensions treated with chymotrypsin or lysostaphin. Achromopeptidase showed some activity, but only a 10% reduction in absorbance was seen after 30-min treatment with the enzyme. Incubation with lysozyme alone or with lysozyme in combination with other enzymes was effective; after 30-min treatments, 86% to 95% reductions in absorbance of cell suspensions were observed upon exposure to SDS.

Incubation of the cell-enzyme mixtures was continued for another 12 h at which time 2-ml samples were removed into 2 ml water rather than into an SDS solution, and the A_{550} measured. At this time no decrease in absorbance was observed for cells incubated with lysozyme, lysostaphin, chymotrypsin, achromopeptidase, or lysozyme/achromopeptidase. However, the absorbance of lysozyme/chymotrypsin-treated cells decreased by 30% upon dilution with water.

Production of osmotically sensitive cells. To test for the generation of osmotically sensitive cells (i.e. cells that lyse upon suspension in hypotonic medium) upon exposure to lysozyme and proteolytic enzymes, strain P104 was grown in NLBT or NLB, suspended in PB without sucrose, and incubated at 37°C with lysozyme, chymotrypsin, pronase, and trypsin in various combinations. Production of osmotically sensitive cells was determined by observing the decrease in absorbance of cells mixed with water. Results are presented in Fig. 3.

No decrease in absorbance was observed when cultures were exposed to proteolytic enzymes before they were mixed with water. No lysis of NLB-grown cells was seen after lysozyme treatment; only prolonged exposure to lysozyme resulted in some lysis of NLBT-grown cells upon mixing. However, incubation of cells with enzyme combinations resulted in significant lysis. Cells grown in NLBT showed greater losses of absorbance after dilution with distilled water, at shorter

Fig. 2. Effect of time of exposure to various enzymes on lysis of cells of strain P104 grown in NLBT. Absorbance of each sample was measured 2 min after adding 2% SDS. Enzymes: □, lysostaphin or chymotrypsin; ◊, lysozyme; :, achromopeptidase; ○, lysozyme plus achromopeptidase; ■, lysozyme plus chymotrypsin. Results are from duplicate trials.

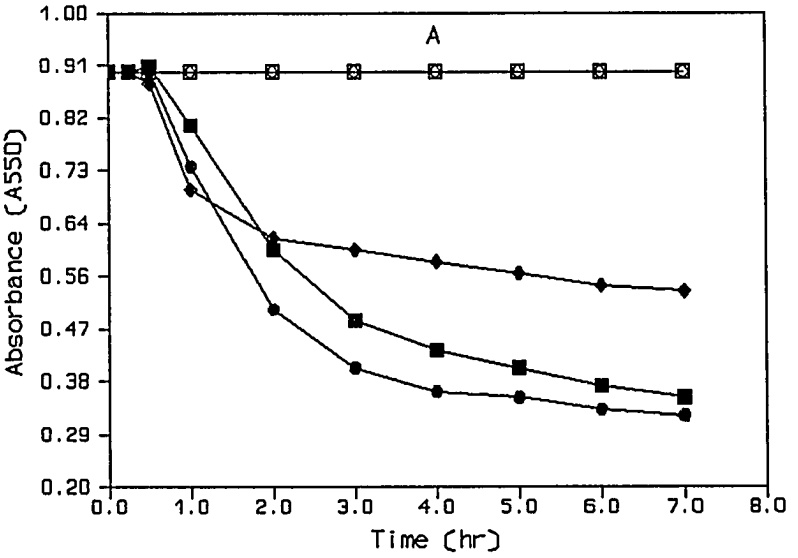

1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Absorbance (A550)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (min)

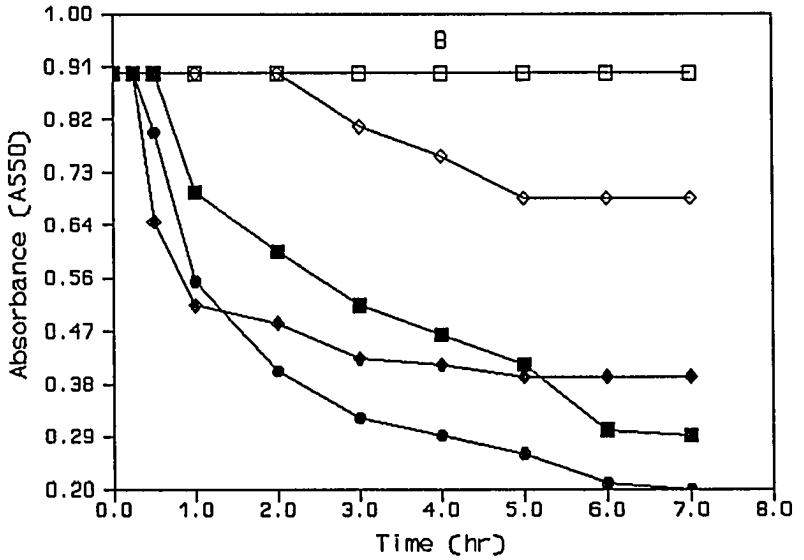
enzyme exposure times, than did cells grown in NLB.

Production and regeneration of protoplasts. Growth in NLBT and exposure to lysozyme plus a proteolytic enzyme yielded a high percentage of osmotically sensitive cells. The ability of these osmotically sensitive cells to regenerate to walled cells was examined next. Strain P104 was grown in either NLB or NLBT. The NLBT-grown cells were treated with lysozyme/chymotrypsin as described in Materials and Methods. The NLB-grown cells were incubated for 45 min with 2 mg/ml lysozyme. A comparison of these two treatments for protoplast formation and regeneration efficiencies is presented in Table 2.

Treatment of NLBT-grown cells with lysozyme/chymotrypsin resulted in conversion of 99.1% of the initial cells to protoplasts. Of those protoplasts, only 3.4% regenerated to walled cells when plated on RM. A much lower percentage (12.7%) of NLB-grown cells was converted to protoplasts after lysozyme treatment, but a higher percent regeneration (28%) of these protoplasts was observed. When the equation of Lee-Wickner and Chassy (14) was used to calculate regeneration frequencies, values for NLB-grown cells and NLBT-grown cells were essentially the same (3.6% and 3.4%, respectively). Regenerant colonies appeared in 21 days for both treatments.

Fig 3. Effect of time of exposure to various enzyme treatments on lysis of cells of strain P104 after dilution with distilled water. Strain P104 was grown in NLB (A) or NLBT (B) and incubated with: □, pronase, chymotrypsin or trypsin; ◊, lysozyme; ■, lysozyme plus chymotrypsin; ♦, lysozyme plus pronase; ●, lysozyme plus trypsin. When enzyme combinations were used, proteolytic enzymes were added 15 min after addition of lysozyme. Results are the averages of duplicate trials.


A
1.00
0.91
0.82
0.73
0.64
0.56
0.47
0.38
0.29
0.20
Absorbance (A550)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Time (hr)


B
1.00
0.91
0.82
0.73
0.64
0.56
0.47
0.38
0.29
0.20
Absorbance (A550)
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Time (hr)

Transformation. Protoplasts produced from cells grown in NLBT and treated with lysozyme and chymotrypsin were transformed with 1 μg of plasmid pE194 DNA as described in Materials and Methods. Erythromycin-resistant colonies began to appear after 21 days of incubation and continued to appear through 27 days. A total of 60 Em^R-colonies appeared on the experimental plates; control plates (no added DNA) contained 15 spontaneous Em^R colonies. All colonies showed resistance to 1 mg/ml erythromycin, whereas the parental strain was sensitive to 1 ug/ml erythromycin.

The presumptive transformants were examined for the presence of covalently closed circular (CCC) DNA. When DNA preparations isolated by the microscale procedure were subjected to gel electrophoresis, faint bands that comigrated with plasmid pE194 seemed to be present in some of the transformants (Fig. 4). A few such transformants were grown in 1-liter cultures, and DNA was isolated by the preparative scale procedure. After cesium chloride-ethidium bromide density gradient centrifugation, only single bands that were identified as chromosomal DNA were observed. Because plasmid DNA could not be isolated from the putative transformants, it was postulated that the donor plasmid may have integrated into the recipient chromosome.

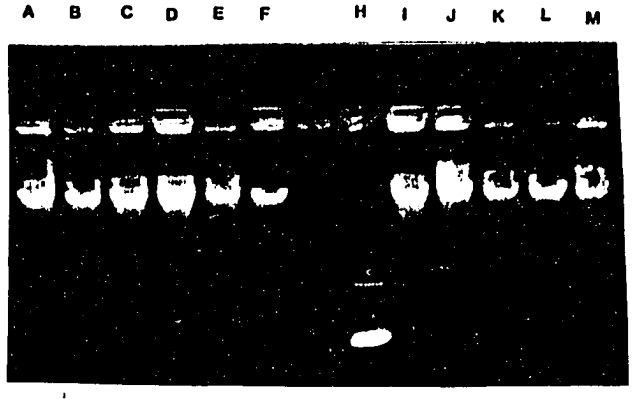
The DNA from 10 putative transformants was isolated, seperated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with a biotinylated pE194 probe. Faint bands migrated at the same rate as did pE194 were observed in the gel for the transformants (Fig. 4). However, the pE194 probe did not hybridize to these bands. Instead, the probe hybridized to fragmented chromosomal DNA. No hybridization with chromosomal DNA from P104 was seen. These results suggest that part or all of plasmid pE194 may have

integrated into the recipient chromosome.

To investigate the reasons for the low frequency of transformation and the inability to isolate CCC DNA from transformants, cell-free extracts of P104 were mixed with plasmids pE194 and pC194, incubated at 37°C, and subjected to agarose gel electrophoresis to observe possible effects of nuclease activity on isolated DNA. Resuts are illustrated in Fig. 5. Much of the CCC DNA disappeared after 1 h of incubation with cell-free extract, and complete loss of CCC DNA occurred after 2 h of incubation. After 2 h of incubation with cell-free extract, plasmid pC194 and plasmid pE194 were converted to the linear form.

Fig 4. Hybridization of biotin-labelled pE194 DNA to DNA isolated from Em^R transformants of strain P104. (I) Agarose gel electrophoresis of DNA. (II) Nitrocellulose filter to which the plasmid DNA shown in panel I had been transferred, and hybridized with labelled pE194 probe DNA. Lanes: A-E and I-M, undigested DNA from different P104 transformants; F, DNA from *P. freudenreichii* strain P104; H, pE194 DNA

I



II

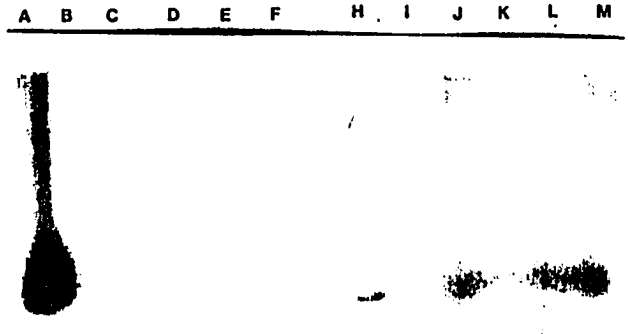
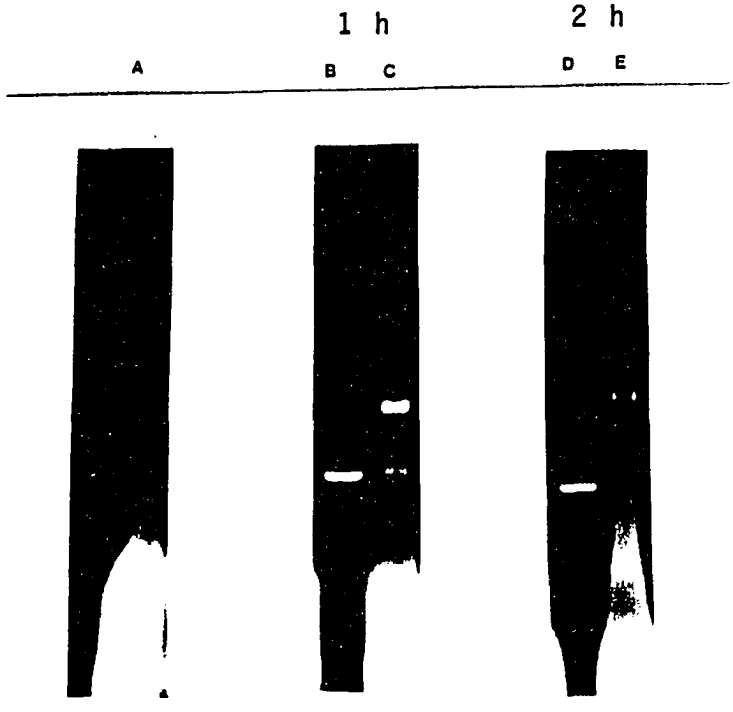


Fig 5. Agarose gel electrophoresis of plasmid pE194 DNA incubated for 1 or 2 h at 37°C with and without cell-free extract (CFE) of P104. Lanes: A, P104 CFE alone; B, pE194; C, P104 CFE plus pE194; D, pE194; E, P104 CFE plus pE194.


1 h
2 h
A
B
C
D
E

DISCUSSION

Polyethylene glycol-induced transformation of protoplasts is an effective method for incorporating foreign DNA into bacteria. Though the production of protoplasts of *P. freudenreichii* has been reported (1), transformation of these protoplasts was unsuccessful. We attempted to develop a method to produce osmotically sensitive cells (cells that burst when resuspended in hypotonic buffers), because others have demonstrated that their production was essential for successful protoplast transformation (30). By growing strain P104 in NLB plus 1% threonine and treating these cells with a combination of lysozyme and proteolytic enzyme, were able to produce osmotically sensitive cells. Production of osmotically sensitive cells able to regenerate to walled cells should facilitate the development of protoplast transformation in these organisms.

The ability to produce protoplasts can be affected by the culture's growth medium. The addition of penicillin (11), glycine (27, 28), and threonine (3) to growth media has been used to enhance cell wall removal by lysozyme. In this work, growth in NLB containing 1% threonine increased sensitivity of *P. freudenreichii* P104 to lysozyme treatment. This enhancement of lysozyme sensitivity is unique to strains, like P104, that are already sensitive to lysozyme. Strains that were recalcitrant to lysozyme treatment were still recalcitrant after growth in NLBT (data not shown). The activity of threonine could be due to its replacement of cell wall amino acids (3, 5)

All three proteolytic enzymes tested improved the activity of lysozyme against *P. freudenreichii* P104. It is unknown why proteolytic enzymes enhance protoplast production. Proteolytic activity is known to stimulate lytic activity of

various enzymes, possibly by clearing away cell debris and thus allowing greater access to the peptidoglycan layer (30).

We decided to use the lysozyme/chymotrypsin combination even though treatment of P104 with this combination of enzymes did not produce osmotically sensitive cells as rapidly as did the other enzyme combinations. Woskow and Kondo (30) studied the same enzyme combinations on *Lactococcus lactis*, and showed that the lysozyme/chymotrypsin combination resulted in higher transformation frequencies even though it was not as effective at producing osmotically sensitive cells. Also, though the production of osmotically sensitive cells is required for transformation, optimal transformation frequencies usually are obtained when there is limited cell wall digestion (4, 27, 30). Incubation of cells with lysozyme/pronase or lysozyme/trypsin might cause overdigestion of the cell wall, resulting in protoplasts that are no longer viable and/or are unable to regenerate cell wall (2).

Cells grown in NLBT were more sensitive to lysozyme, but regenerated at a lower frequency than did NLB-grown cells. Lee-Wickner and Chassy (14) showed that three factors are involved in successful regeneration: the presence of residual primer, the preservation of enzymatic activity of the wall biosynthetic system, and the ability of the strain to develop in a rich hypertonic medium. In the present case, the lower regeneration frequencies could be due to either the lack of primer or the digestion of cell wall biosynthetic proteins (2). Alternatively, the use of proteolytic enzymes may permanently damage membrane proteins vital for cell integrity. Though these regeneration frequencies are low, they are still high enough to achieve transformation; transformation of bacterial protoplasts with less than 1% regeneration has been reported (7, 11, 12).

We calculated regeneration frequencies based on the conversion of protoplasts to walled cells irrespective of the initial numbers of cells present. A lower regeneration frequency is calculated by using the equation of Lee-Wickner and Chassy (14), which uses the initial number of cells. If the production of protoplasts is low, this equation can yield a low value for regeneration frequency. We believe the equation used in this study better accounts for the actual number of protoplasts regenerated.

Baehman and Glatz (1) reported regeneration frequencies between 10 and 30%, calculated by using the Lee-Wickner and Chassey equation. In the current study, we were only able to achieve 3 to 4% regeneration as calculated by the same method. The difference may be due to the number of protoplasts formed. Baehman and Glatz (1) used higher concentrations of lysozyme (20 mg/ml) and were able to convert 99% of the initial cells to protoplasts. In this study, only 12.7% of cells grown in NLB and treated with 2 mg/ml lysozyme were converted to protoplasts.

A number of factors are involved in the successful transformation of bacterial cells. The DNA must be able to cross the cell wall and membrane, and be maintained and expressed once inside the cell. The method developed in this study has been used to produce protoplasts for preliminary transformation trials. The protoplasts seemingly were able to undergo PEG-induced transformation, albeit at low transformation frequency, and it seems that the transforming DNA may have integrated into the chromosome. Chromosomal integration of transforming plasmid DNA has been shown to occur in gram-positive bacteria (4, 13, 16). Zirnstein and Rehberger (31) transformed three strains of propionibacteria with the *S. aureus* plasmid pC194. Transformation frequencies of $1.1X10^2$ were

obtained. However, plasmid DNA was not observed in any of the transformants. Integration of pC194 plasmid DNA into the host chromosome was verified by comparing hybridization signals detected between parental and transformant DNA. In other organisms integration of plasmids has been exploited in a manner similar to that used for transposons to examine chromosome organization (13).

The presence of a restriction/modification system may also be responsible for low transformation frequencies and forced integration of plasmid DNA. The presence of intracellular nuclease activity in strain P104 is indicative of a restriction/modification system. A number of different strategies have been employed to circumvent the presence of such systems in recipient strains used for transformation. If low-frequency transformation can be accomplished, DNA for future transformation experiments can be isolated from the recipient strain; these experiments usually show increased transformation frequencies (11, 12, 13, 16, 25). Other methods to avoid nuclease activity include entrapping DNA in liposomes (23) and using strains that have lost restriction/modification capability (20). Even though we can produce protoplasts permeable to DNA, successful transformation of *P. freudenreichii* may not be possible until strains free of nuclease activity are available.

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SECTION III

CONJUGATION IN *PROPIONIBACTERIUM*

Conjugation in *Propionibacterium*

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Iowa State University

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ABSTRACT

Conjugal transfer of plasmid DNA into strains of propionibacteria has not been reported previously. A spontaneous streptomycin-resistant derivative (designated P23801) of *Propionibacterium thoenii* strain P38 was obtained by repeated transfers in broth containing streptomycin and streaking onto streptomycin gradient plates. Conjugal transfer between the streptomycin resistant strain and two *Enterococcus faecalis* donor strains, one carrying plasmid pAMβ1 (erythromycin resistance) and the other carrying plasmid pIP501 (erythromycin and chloramphenicol resistance), was attempted by using the filter mating technique. Both matings yielded putative transconjugants, but agarose gel electrophoresis of total cellular DNA did not reveal the presence of autonomous plasmids. Transfer of the conjugal transposons Tn*919* from *Lactococcus lactis* and Tn*916* from *Enterococcus faecalis* to *Propionibacterium jensenii* strain P22501 (erythromycin-resistant, tetracycline-sensitive) was also attempted. No transconjugants were obtained in these filter matings.

INTRODUCTION

Strains of the genus *Propionibacterium* are used in production of Swiss-type cheeses (3) and in other industrial processes (16). Despite their industrial importance very little is known about the genetics of these organisms.

Transposons Tn*916*, first identified in *Enterococcus faecalis* DS16 (9, 10), and Tn*919* from *Streptococcus sanguis* FC1 (8) have been useful tools for genomic analyses of gram-positive bacteria (4, 5, 7, 9, 17, 21). These transposons possess characteristics that have been exploited for genome mapping and to target genes for cloning in *Escherichia coli (11).* Both transposons encode tetracycline resistance, are similar in size (15 and 17 kb for Tn*916* and Tn*919* respectively), are capable of conjugative transfer at frequencies ranging from 10^{-5} to 10^{-8}, and can randomly insert into either the chromosome or plasmids of host cells. Both transposons have been transferred to a number of gram-positive bacteria by filter mating; a high-frequency conjugal delivery system for Tn*919* has been developed in *Lactococcus lactis* (17), and for Tn*916* in *E. faecalis* (10).

Transfer of conjugal plasmids can also be used to facilitate genetic analysis and as an alternative gene transfer method in strains in which other gene transfer systems are either inefficient or do not exist (5, 6, 11, 13, 14, 24, 25, 27, 30, 31, 34). Plasmids pAMβ1 (5, 6) and pIP501 (20) are capable of conjugal transfer into a wide range of hosts and have been used extensively in genetic studies of gram-positive bacteria (1, 6, 12, 13, 14, 22, 24, 27, 30, 31, 34). Plasmid pAMβ1, isolated from *E. faecalis* DS5, is a 26.5-kb plasmid expressing MLS (macrolide-lincosamide-streptogramin B) antibiotic resistance. Plasmid pIP501, first isolated from *Streptococcus agalactiae*, is a 30.2-kb plasmid that encodes resistance to

both MLS antibiotics and chloramphenicol. Both plasmids have been characterized at the molecular level and have been used to develop gene cloning and transposon delivery systems (9, 23).

To apply genetic engineering techniques to the propionibacteria, gene transfer systems similar to those described for other gram-positive bacteria must be developed (6, 8, 15, 22, 25). In this study, we report attempts to transfer plasmids pAMβ1 and pIP501 to strains of *Propionibacterium*. Also, transfer of the conjugal transposons Tn*916* and Tn*919* into propionibacteria was attempted.

MATERIALS AND METHODS

Bacterial strains, media and reagents. The bacterial strains and plasmids used in this study are shown in Table 1. *E. faecalis* was grown at 37°C in Brain Heart Infusion medium (BHI, Difco Laboratories, Detroit, MI). *Lactococcus lactis* was grown in M17G (31) at 32°C. All propionibacteria were grown in sodium lactate broth (NLB) at 32°C (19). Solid medium (sodium lactate agar, NLA) contained 1.5% agar and was incubated at 32°C in an anaerobic atmosphere produced by the Gas-Pak system (BBL Microbiology Systems, Cockeysville, MD) . When present as selective agents in growth medium, antibiotics were used at the following concentrations: erythromycin (Em), 10 μg/ml; chloramphenicol (Cm), 5 μg/ml; streptomycin sulfate (Str), 1200 μg/ml; tetracycline (Tc), 10 μg/ml. All antibiotics were obtained from Sigma Chemical Co., St. Louis, MO. All strains were permanently stored at -70°C in their respective broths supplemented with 10% glycerol.

Antibiotic susceptibility and MIC testing. Initially, susceptibility testing was performed by spot plating 0.02-ml amounts of broth cultures onto NLA containing various concentrations of the appropriate antibiotic. Plates were incubated anaerobically in a Gas-Pak system for 2 to 7 days at 32°C. Growth at the various concentrations was recorded and compared to growth on NLA. The MIC was taken as the lowest antibiotic concentration that suppressed the growth of the cultures.

Prior to conjugation experiments, all cultures used were tested for growth on antibiotic-containing solid media. Serial dilutions of each culture were spread-plated onto NLA containing the MIC of the appropriate antibiotic. Plates were

Table 1. Bacterial strains and plasmids used in this study.

Strain[a]	Source	Relevant genotype	Relevant phenotype	Remarks
P. thoenii P38	A		Em^s Str^s Cm^s	
P. jensenii P22501	A		Em^r Tc^s	Spontaneous Em^r derivative of *P. jensenii* P25 (28)
P. thoenii P23801	A		Str^r Em^s Cm^s	Spontaneous Str^r derivative of *P. thoenii* P38 (this study)
L. lactis CH919	B	[Chr::Tn*919*]	Tc^r Str^s	(17)
E. faecalis CG110	C	[Chr::Tn*916*]	Tc^r Str^s	(9, 10)
E. faecalis JH2-2 (pAMβ1)	C	(pAMβ1)	Em^r Str^s	(4)
E. faecalis JH2-2 (pIP501)	C	(pIP501)	Em^r Cm^r Str^s	(30)

[a] Sources of strains: A, Department of Food Science and Human Nutrition, Iowa State University; B, G. F. Fitzgerald, Department of Food Microbiology, University College, Cork, Ireland; C, P. A. Pattee, Department of Microbiology, Iowa State University.

incubated for 7 days at 32°C both aerobically and anaerobically. Plate counts on NLA plus antibiotic were compared to those on NLA.

Development of antibiotic resistance in propionibacteria. Spontaneous streptomycin-resistant mutants of strain P38 were derived by repeated transfer into broth containing Streptomycin. Initially, a 1% inoculum of strain P38 was made into 10 ml of NLB plus 10 μg/ml of Streptomycin. Cultures were incubated 24 at 32°C, and repeated transfers (1% inoculum) into fresh media were made. Once the growth rate of the strain in antibiotic medium was roughly equivalent (determined by visual inspection) to that in NLB, the antibiotic-tolerant strain was transferred to antibiotic gradient plates as described by Carlton and Brown (2). Cells were removed with a sterile loop from the end of a single cross-streak at the high antibiotic concentration and restreaked onto NLA plus antibiotic. Isolated colonies from the streak plate were transferred into NLB containing a slightly higher amount of antibiotic and the process was repeated.

Conjugation. Filter matings were performed as described (8) with the following modifications. Log-phase broth cultures of donor and recipient strains were mixed at various ratios and filtered through 0.22-μm Gellman nitrocellulose filters. The filters were washed with 3 volumes of sterile deionized water and aseptically placed onto NLA, cell side up. After at least 2 days of anaerobic incubation at 32°C, the filters were aseptically removed into 1 ml of 0.01 M phosphate buffer (pH 7.0) and vortexed to remove cells. A 0.1-ml sample was plated onto NLA containing the appropriate selective antibiotics at the concentrations described above. After 7 to 10 days of incubation at 32°C, the plates were scored for the appearance of antibiotic-resistant colonies. Donor and recipient populations also were enumerated by plating onto NLA and incubating 7

days at 32°C. Conjugation frequencies were expressed as the number of Em^r Str^r (pAMβ1 transfer), or Em^r Cm^r Str^r (pIP501 transfer) colonies divided by the number of recipient cells in the mating mixture. Controls consisted of donor and recipient cells plated alone onto NLA containing the appropriate antibiotics.

For broth matings, cells were resuspended in NLB at 1:1, 1:5, and 1:10 donor:recipient ratios and incubated in NLB at 32°C for 24 h. A 0.1-ml sample was then plated onto NLA containing the appropriate selective antibiotics.

Plasmid DNA isolation and purification. Plasmid DNA was isolated, purified, desalted, and concentrated from *Propionibacterium* strains by using the procedure of Rehberger (28). Total cellular DNA was separated by electrophoresis through a 0.6% horizontal agarose gel in TBE buffer (0.089 M Tris hydrochloride, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) for 8 to 12 h at 50 V. Gels were stained in ethidium bromide (0.5 μg/ml in distilled water), observed on a U.V. transilluminator (Foto UV 300; Fotodyne Inc., New Berlin, WI), and photographed through 23A and 2B Wratten gel filters with a polaroid MP4 camera (film type 55).

RESULTS

Strain selection. *Propionibacterium* strains P38 and P22501 used in mating experiments were selected based on the availability of small-scale procedures for the isolation of plasmid DNA. Also, strain P38 exhibits a clumping phenomenon (29) similar to that which has been associated with high-frequency conjugation in other bacteria (33).

Development of antibiotic resistance in strain P38. After repeated transfers, a Str^r derivative of parental strain P38, designated P23801, was obtained. Strain P23801 showed no differences from its parent in morphology, gram reaction, reduction of nitrate, and pigment production. The MIC of Streptomycin was 1400 μg/ml for strain P23801 compared to 100 μg/ml for P38. In contrast, the donor strains used in the conjugation experiments were sensitive to Streptomycin at 250 μg/ml.

Mating experiments. Transfer of Tn*919* from *L. lactis* CH919 to *P. jensenii* P22501 and transfer of Tn*916* from *E. faecalis* CG110 to strain P22501 were attempted by filter matings. Log-phase cultures were adjusted to OD_{660} of 0.7 by diluting with fresh sterile medium. Conjugations were performed using donor:recepient ratios of 1:1, 1:2, 1:5, 1:10, and 1:20. Duplicate attempts were made at each ratio but no transconjugants appeared on any of the plates from these matings.

Conjugal transfer of plasmids pAMB1 and pIP501 was attempted by using a 1:10 donor:recipient ratio. Filter matings between P23801 and JH2-2 (pAMB1) resulted in the appearance of Em^r Str^r colonies at all ratios (Table 2). No propionibacteria appeared on the control plates. Fifty putative transconjugants

were picked and streaked onto fresh NLA containing Em (10μg/ml) and Str (1000 μg/ml). The growth rate on solid media containing antibiotics was equivalent to the growth of the parental strain on solid media without added antibiotic. All Em^r Str^r colonies were able to grow in media containing greater than 100 μg/ml Em while the parental strain was inhibited by 1 μg/ml Em. However, no autonomous plasmids were detected in any of the Em^r Str^r strains.

Further filter matings were performed between donor strain *E. faecalis* JH2-2 that contained the double antibiotic resistance plasmid pIP501 and the recipient strain P23801. Colonies (total of 39) showing resistance to all three antibiotics appeared after 9 days of incubation. No propionibacteria appeared on the control plates. The growth rate of these colonies on NLA or in NLB plus Em and Cm was slower than that of the parental strain.

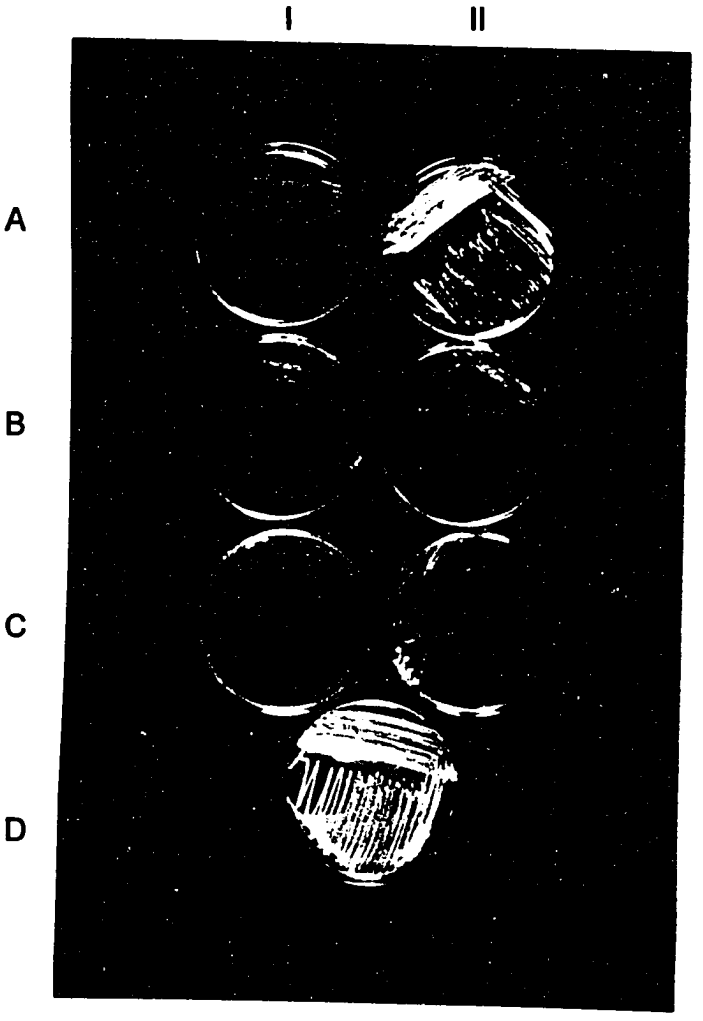
Antibiotic-resistant colonies and the parental antibiotic-sensitive strain were streaked onto NLA plates containing 5 μg/ml each of Em alone, Cm alone, or Em and Cm, and also onto control plates (Fig 1). No growth of the parental strain occurred on any of the plates containing antibiotics. Growth of putative transconjugants on medium containing Em was equivalent to that on medium without antibiotics, but was slower on media containing Cm alone or Cm plus Em.

Table 2. Frequency of transfer of antibiotic resistance from donor strains to P22501 or P23801 in filter mating experiments.

Donor	Recipient	Selected Phenotype	Transfer frequency (per recipient)[a]
CG110 (Tn*916*)	P22501	Tc^r Em^r	$<4.0 \times 10^{-8}$
CH919 (Tn*919*)	P22501	Tc^r Em^r	$<6.0 \times 10^{-8}$
JH-2 (pAMβ1)	P23801	Em^r Str^r	1.2×10^{-7}
JH-2 (pIP501)	P23801	Em^r Cm^r Str^r	9.75×10^{-8}

[a] Transfer frequency is the number of antibiotic-resistant colonies per recipient. For filter matings with CG110 and CH919, total recepient cells were 4.0×10^8 and 6.0×10^8 CFU respectively.

Fig 1. Growth of P23801 (I) and putative P23801 pIP501 transconjugants carrying pIP501 DNA (II) on NLA and NLA plus antibiotics after 7 days of incubation at 32°C. Row: A, NLA plus 10 ug/ml Em; B, NLA plus 5 ug/ml Cm; C, NLA plus 10 ug/ml EM and 5 ug/ml Cm; D, NLA.


I
II
A
B
C
D

DISCUSSION

The transposons Tn*916* and Tn*919* have unique properties that have made them useful in genetic analysis of gram-positive bacteria. First, they are capable of conjugal transfer in the absence of plasmid DNA at frequencies of 10^{-8} to 10^{-5} per recipient cell in a wide range of gram-positive bacteria (6, 7, 17, 21). Second, the excision of the transposons is precise in that the function of insertionally inactivated genes is restored after the excision event (12, 17). Third, both transposons have been cloned in *E. coli* in which growth in the absence of tetracycline resulted in high-frequency precise excision (12, 17). These characteristics have been exploited by Gawron-Burke and Clewell (11) who developed a system for cloning insertionally inactivated genes from the chromosome of streptococci into *E. coli*.

In our laboratory, interest in transposons concerns their use as a tool in advancing genetic studies in the industrially important propionibacteria. Matings between propionibacteria and strains containing Tn*916* and Tn*919* were performed, but no antibiotic-resistant transconjugants appeared. It's possible that congugation occurs at such a low frequency that more matings would have to be performed to detect it. A higher probability of success may be achieved by using strains of lactococci that are capable of conjugative transfer at high frequencies (1.25×10^{-4} per recipient) (17). Unfortunately, we were unable to obtain these strains.

In this study we attempted to conjugally transfer the broad host range plasmids pIP501 and pAMβ1 into propionibacteria. These plasmids have been shown to transfer to a wide range of gram-positive bacteria and derivatives of these plasmids are used extensively as cloning vectors (1, 4, 5, 6, 12, 13, 14, 24,

25, 27, 30, 31, 34). Our interest in these plasmids is to develop a conjugation system similar to that of other gram-positive bacteria (6, 15, 22) and to develop conjugative mobilization as an alternative vector delivery system (31) for propionibacteria. Filter matings with *E. faecalis* containing plasmid pAMβ1 resulted in the appearance of colonies 100 times more resistant to erythromycin than was the parental strain. However, no autonomous plasmids were detected in any of the antibiotic-resistant strains. Possibly, either all or part of the plasmid integrated into the chromosome of the recipient strains. Alternatively, an inducible Em^r gene may be present in strains of propionibacteria, and was expressed under conditions used in this study. The possibility of plasmid integration needs to be xplored by probing putative transconjugants with labelled pAMβ1 DNA.

The doubly marked plasmid pIP501 was used in further filter mating experiments to avoid the possibility that the appearance of the single trait during matings was the result of spontaneous mutation. Following filter matings, the appearance of Em^r Cm^r colonies required greater than 7 days of incubation. This is a considerably longer period of time than that required for colony appearance of the parental strain on NLA. As was the case with pAMβ1, no autonomous plasmid was detected in any of the Em^r Cm^r colonies. When Em^r Cm^r colonies were streaked onto fresh NLA containing antibiotics, only the colonies streaked onto plates with Em alone showed growth equivalent to that on medium without antibiotics. If part or all of the plasmid integrated into the host chromosome, then the chloramphenicol acetyltransferase gene either did not integrate or integrated but did not fully express. Alternatively, induction of an Em^r resistance gene rather than plasmid transfer in the recipient occurred.

The work presented in this paper does not represent an exhaustive study of

the development of conjugation in the propionibacteria. It is interesting to note that when attempts were made to introduce plasmid DNA by transformation, putative recipients that were antibiotic-resistant but did not carry independent plasmid DNA were also obtained (data not shown).

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SECTION IV

ELECTROPORATION OF *PROPIONIBACTERIUM*

Electroporation of *Propionibacterium*

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ABSTRACT

In attempts to develop an electroporation method for transformation of *Propionibacterium* cells, we tested four different strains as recipients, 13 different buffers, and three different plasmids as vectors. Antibiotic-resistant colonies appeared on experimental plates in some experiments. However, when the putative transformants were examined for the presence of autonomous plasmid DNA, none was detected. Possible reasons for lack of success are discussed.

INTRODUCTION

Strains of the genus *Propionibacterium* are used in production of Swiss-type cheeses (10) and other products. Despite their industrial importance, little is known about the genetics of these organisms and only two reports of gene transfer exist (16, 29).

Electroporation is an established procedure for transforming a variety of cell types (1, 4, 16, 25). According to one theory, in electroporation transient pores open in the cell membrane as an electric pulse causes polarization of the membrane (24). The resultant pores are large enough for macromolecules such as DNA to pass through and, as long as the electric field does not exceed a critical limit, this permeability is reversible.

Electroporation-induced transformation has been used successfully to transform plant (14), animal (19), and both gram-positive (1, 2, 4, 6, 16, 17, 20, 28) and gram-negative bacteria (5, 8, 13, 25). Recently, Zirnstein and Rehberger (29) successfully introduced the *Staphylococcus aureus* plasmid pC194 into several *Propionibacterium* strains. This report describes attempts at electroporation of propionibacteria.

MATERIALS AND METHODS

Bacterial strains, culture conditions and plasmids. The bacterial strains and the plasmids used in this work are listed in Table 1. Propionibacteria were grown in sodium lactate broth (NLB) (11) at 32°C. Working cultures were maintained on sodium lactate agar (NLA, NLB plus 1.5% agar) at 4°C. Cultures of *Staphylococcus aureus* were grown in trypticase soy broth (TSB, BBL Microbiology Systems, Cockeysville, MD) or trypticase soy agar (TSA, BBL) that contained 5 μg/ml chloramphenicol (Cm) or erythromycin (Em) at 37°C. Working cultures were maintained on TSA at 4°C and transferred biweekly. Cultures of *E. coli* were grown aerobically in Luria broth (14) at 37°C. All cultures were permanently stored at -70°C in their respective media supplemented with 10% glycerol.

Plasmid DNA Isolation. Rapid microscale plasmid isolation from propionibacteria was performed by the method of Rehberger (21). Plasmid DNA was separated by electrophoresis of DNA samples through a 0.6% horizontal agarose gel in TBE buffer (0.089 M Tris hydrochloride, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) for 6 to 12 h at 50 V. Gels were stained in ethidium bromide (0.5 μg/ml in distilled water), observed on a UV transilluminator (Foto UV 300; Fotodyne Inc., New Berlin, WI), and photographed through 23A and 2B Wratten gel filters with a Polaroid MP4 camera (film type 55) (22).

Large-scale isolation of plasmid DNA from *S. aureus* was performed by the method of Pattee (18). The DNA was purified by CsCl-ethidium bromide density gradient centrifugation. The purified plasmid DNA samples were extracted with isopropanol saturated with 5 M NaCl to remove the ethidium bromide, desalted and concentrated in 10 mM Tris hydrochloride-1 mM disodium EDTA (pH 7.5) by using a

Table 1. Bacterial strains and plasmids.

Strain	Source[a]	Plasmid	Relevant phenotype[b]	Remarks/ Reference
P. jensenii P36301	A			Plasmid-free derivative of *P. jensenii* P63
P. freudenreichii P104 (ATCC6207)	A			
P. freudenreichii P22	A			
P. freudenreichii P7	A			
P. jensenii P53	A			
S. aureus 1390	B	pE194	Em^r	
S. aureus ISP 1386	B	pC194	Cm^r	(12)
S. aureus ISP1768	B	pTV32(ts)	Em^r Cm^r	(25)
S. aureus ISP 1869	B	pTV1(ts)	Em^r Cm^s	(25)
E. coli BHB2600	C	pGKV210	Em^r	(15)
E. coli (BHB2602)	C	pNZ12	Cm^r Km^r	(6)

[a] Sources of strains; A, Department of Food Science and Human Nutrition, Iowa State University; B, P. A. Pattee, Department of Microbiology, Iowa State University; C, W. M. de Voss, Netherlands Institute for Dairy Research (NIZO), Ede, Netherlands.
[b] Relevant phenotype: Cm, chloramphenicol resistance; Em, erythromycin resistance; Km, kanamycin resistance.

Centricon-30 microconcentrator according to the instructions of the manufacturer (Amicon Corp., Danvers, MA).

Large-scale isolation of plasmid DNA from *E. coli* was performed by the cleared lysate method of Clewell and Helinski (7) and the DNA was purified by CsCl-ethidium bromide density gradient centrifugation.

Electroporation buffers. The following buffers were used: deionized water (dH_2O); EB (10% glycerol in deionized H_20, pH 7.0); HEB (272 mM sucrose, 1 mM $MgCl_2$, 7 mM HEPES buffer, pH 7.0); HB (1 mM $MgCl_2$, 7 mM HEPES buffer, pH 7.0); PEB (272 mM sucrose, 1 mM $MgCl_2$, 7 mM potassium phosphate, pH 7.0); PB (1 mM $MgCl_2$, 7 mM HEPES buffer, pH 7.0); GP (0.2 M potassium phosphate, 1 mM $MgCl_2$, pH 7.0); PEG (30% polyethylene glycol in dH_20, pH 7.0); PM (7 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.0); PPM (30% polyethlylene glycol, 7 mM potassium phosphate, 1 mM mercaptoethanol, pH 7.0). For some experiments, PEB and HEB were used at greater than 1X concentration.

Electroporation protocol. Electroporation was performed according to the procedure of McIntyre and Harlander (17) with modifications. A 1-ml sample of a 36-h culture of propionibacteria was inoculated into 50 ml of NLB and grown to an optical density at 600 nm of 0.8. Cells were removed by centrifugation (6000 X *g*, 10 min, 4°C), washed twice with 10 ml of cold sterile deionized water and suspended in 1 ml of buffer to approximately 5 x 10^{10} CFU/ml final concentration. A 200-µl amount of cell suspension was placed in a 1.5-ml microcentrifuge tube and frozen at -70°C.

Cells were thawed and held on ice immediately prior to electroporation. Plasmid DNA (1 to 10 µg) was added to the cell suspension. The DNA and cells were mixed thoroughly and transferred to a semi-micro disposable cuvette (VWR Scientific Inc., Chicago, IL). In later experiments, the Flat-Pack electrode system

(BTX Inc., San Diego, CA) was used. The cell-DNA suspension was exposed to a high-voltage electric pulse with the BTX Transfector 100 equipped with the BTX Power-Plus system as described below.

For most experiments, the electric pulse was provided by a BTX transfector 100 equipped with a Power-Plus system capable of generating field strengths up to 25 kV/cm. The electrode head assembly consisted of either a 0.5-mm electrode inserted into a semi-micro disposable cuvette or the BTX Flat-Pack system. Voltages of 200, 400, 600, 800, 900, 1200, 1500, and 2000 at a pulse duration of 5 or 10 ms were selected. Analysis of the pulse time, actual field strength, peak voltage or pulse decay time is not possible with the BTX Transfector 100.

For some experiments (see table 1) the Bio-Rad Gene Pulser apparatus equipped with a Bio-Rad pulse controller (Bio-Rad Laboratories, Richmond, CA) was used according to the instructions of the manufacturer. Cell suspensions were prepared as described above and a 0.8-ml sample was placed in a chilled Gene Pulser cuvette containing two electrodes separated by 2 mm. Cells were exposed to a single pulse (peak voltage 2.5 kV; capacitance, 25 μF) that generated a peak field strength of 12.5 kV/cm, and treated as described above.

Following electroporation cells were held on ice 10 min, diluted in NLB containing 1 μg/ml of the appropriate antibiotic and incubated at 32°C for 2 h before plating. Plasmid pNZ12 and pC194 transformants were selected on NLA containing 5 μg/ml Cm. Plasmid pGKV210 transformants were selected on NLA containing 10 μg/ml Em. Plasmid pTV1 transformants were selected by the double soft agar induction-selection system overlay method of Youngman (27). In this method, porated cells were mixed with soft agar (NLA plus 0.7% agar) containing Em (1 μg/ml) and Cm (1 μg/ml) and overlayed onto an antibiotic-free NLA plate. After 6 h of incubation

at room temperature, a second agar overlay containing Em (40 μg/ml) and Cm (200 μg/ml) was applied. After plates solidified, they were incubated anaerobically at 32°C.

Suspensions of cells without added DNA served as controls. Putative transformants were subjected to agarose gel electrophoresis to confirm the presence of plasmid DNA. Protoplasts were prepared by the method of Baehman and Glatz (3).

RESULTS

Electroporation. Table 2 shows the different combinations of strains, buffers and plasmid DNA preparation used. Initial electroporation trials were performed using strain P36301, in EB buffer exposed to 500, 600, 700, 800, and 900 V and plated onto NLA to determine survivability. A 100% survival rate was observed for cells subjected to 500, 600, 700, and 800 V. At 900 V arcing occurred and only 25% of the cells survived at this voltage.

The only putative transformants of strain P36301 appeared when 600 V was applied to a cell suspension in 1.5X HEB with pTV32(ts) as donor DNA. Seven colonies arose on NLA plates containing Cm and Em. These colonies were replated onto fresh selective media and isolated colonies were picked into NLB containing Em and Cm. Growth of the putative transformants was slower than that of the parental strain in either NLA or NLB. Agarose gel electrophoresis of the putative transformants did not reveal the presence of autonomous plasmid DNA.

Electroporation of whole cells of strain P104 was tried once at each enzyme-buffer combination. Seven Em^r colonies grew on plates containing cells porated at 1500 V with plasmid pGKV210; no colonies were present on control plates. Agarose gel electrophoresis did not show the presence of autonomous plasmid DNA. Three Cm^r colonies grew on plates containing cells that had been subjected to 1500 V with plasmid pC194; no colonies grew on control plates. Again, no autonomous plasmid DNA was detected by agarose gel electrophoresis.

Two attempts at transferring plasmid pC194 into strain P22 were made. No transformants were detected in either trial. Electroporation of strain P7 was attempted once with each buffer (PB, PM, and PPM). No transformants were detected.

Likewise, no transformants were detected in two attempts to transform P104 and P36301 protoplasts.

Table 2. A summary of electroporation trials with *Propionibacterium* strains.

Strain	Buffer	Plasmid[a]
P36301	dH20	pNZ12, pTV1(ts)
P36301	EB	pNZ12, pTV1(ts), pTV32(ts)
P36301	GP	pTV1(ts)
P36301 protoplasts	GP	pTV1(ts)
P36301	HEB (no sucrose)	pNZ12, pTV1(ts)
P36301	1X, 1.5X, 2X HEB	pNZ12, pTV1(ts)
P36301[b]	1X, 1.5X, 2X HEB	pNZ12, pTV1(ts)
P7	PB, PM, PPM	pC194
P22	HEB	pC194
P104	EB, PB, GP	pC194, pNZ12, pTV32(ts), pE194, pGKV210
P104	PB, PM, PPM	pC194
P104 protoplasts	HEB	pC194
P104	HEB	pC194, pNZ12, pTV32(ts)

[a] Each trial was performed with single plasmids.
[b] Voltage applied with a Bio-Rad Gene Pulsar system. All other trials were performed using the BTX Transfector 100.

DISCUSSION

Transformation of gram-positive bacteria is often a laborious and difficult task (6, 23). Prior to the use of electroporation, most transformation procedures for gram-positive bacteria involved the formation and subsequent regeneration of protoplasts. Electroporation of vegetative cells has simplified procedures used to transform gram-positive bacteria. Also, electroporation has been effective for transforming bacteria that were recalcitrant to other methods (16).

Two reports of gene transfer in the propionibacteria have appeared in the literature. Zirnstein and Rehberger (29) introduced plasmid pC194 into four strains of *Propionibacterium* by electroporation. The most efficient transformation of *P. freudenreichii* P7 was achieved with mid-log cells in a buffer containing 30% PEG (M.W. 10,000) in distilled H_2O with either of two field strength and pulse duration combinations, 5.4 kV/cm with a 5 ms pulse or 37.8 kV/cm with a pulse of approximately 40 μsec. About 1.1×10^2 transformants per μg of DNA were obtained, but no autonomous plasmid appeared in any of the transformants. Integration of pC194 into the *Propionibacterium* chromosome was verified by comparing hybridization signals detected between parental and transformant DNA digested with restriction enzymes.

Luchansky *et al* (16) electroporated cells of *Propionibacterium jensenii* B-77 by using the Bio-Rad Gene Pulsar system. They obtained Cm-resistant transformants at a frequency of 3.2×10^1 per μg pGK12 plasmid DNA. However, the results of agarose gel electrophoresis of whole cell DNA were not presented in their paper, and attempts by us and others (Dr. T. Rehberger, personal communication) to duplicate their procedure have been unsuccessful.

We attempted electroporation using conditions similar to those of Zirnstein and Rehberger (29) but were unsuccessful. Only one attempt was made with strain P7. It is possible that we did not allow a long enough incubation period for transformants to appear on plates; Zirnstein and Rehberger (29) observed transformants after 14 days of incubation (Dr. T. G. Rehberger, personal communication).

Various biological and physical factors influence the outcome of electroporation. Among the most important parameters for bacteria are the field strength, pulse duration, and shape of the pulse (5, 24). Variations in the cell growth phase and cell concentration, medium for washing and suspending cells, concentration of purified plasmid DNA, and ionic strength of the buffers also seem to influence the success of electroporation (5, 8, 13, 17, 24).

The BTX Transfector 100 consists of a bank of capacitors that are charged to a peak voltage. An exponential pulse is generated by the discharge of the capacitors through the cell suspension to generate the required electrical field. It's generally believed that because of their smaller size, bacteria require a much larger electric field to induce poration than do mammalian or plant cells (5). At the time this study was undertaken, the BTX Transfector 100 was capable of delivering higher field strengths (17 Kv/cm) than other commercially available instruments.

The pulse length is largely determined by the resistance of the suspending medium. When buffers of low ionic strength (high resistance) are used, the pulse length is determined by the setting on the instrument. High ionic strength (low resistance) buffers produce shorter pulse times and in our hands resulted in arcing. Decreasing the pulse length increases the amplitude of the field required to transform cells maximally (5, 8). Experience with the BTX system shows that optimal transformation is achieved by using low ionic strength buffers (17). The buffers used

in this study varied in conductivity from low (dH_2O) to high (PEB and HEB) and many contained ingredients chosen to minimize osmotic shock (sucrose, glycerol, PEG). Mercaptoethanol was incorporated into buffers to minimize oxidation that occurs during electroporation. All the buffers used in this study are those reported in the literature to be suitable for electroporation (5, 8, 13, 16, 20, 25).

Generally, the transformation frequency goes up as the concentration of cells is increased (8, 17). High cell densities recommended by the manufacturer were used in this study. No attempts were made to use dilute cultures. It's probable that electro-transformation of highly concentrated cells may not be successful for all bacteria and attempts should be made with dilute cell suspensions.

Electroporation of most bacteria is optimal when cultures are harvested in mid- to late-log phase (1, 5, 6, 8, 13, 16, 17, 20, 24, 25, 26). We used only one set of culture conditions to produce cells for electroporation. Cultures typically took between 24 to 36 hr to reach an OD of 0.6 to 0.8, and were considered to be in mid-to late-log phase at this point.

Some authors have reported increased electro-transformation when cells were treated with lysozyme (19, 24, 26). To render cells more accessible to transforming DNA we used the method of Baehman and Glatz (3) to form protoplasts. This method involves the use of high concentrations of lysozyme which may remove more of the cell wall than is necessary or may interfere with the electric field. Further work should be done using cells treated with less enzyme for shorter times.

Conflicting reports exist on the relationship between the frequency of transformation by electroporation and cell killing (5, 6, 13). Some authors claim that optimal transformation is achieved at voltages that kill a large percentage of cells (5, 8, 13) while others claim optimal frequencies when a low percentage or no cells

are killed (9, 28). Because of these conflicting reports, we decided to perform our experiments using voltages that result in no kill or low kill (200 V), 50% kill (500 V), 75% kill (1000 V), and > 90% kill (>1200 V) (Dr. T. Rehberger, personal communication).

A number of possible reasons for the inability to obtain transformants exist: the cells may not be permeable to plasmid DNA; cells may be permeable to DNA but nucleases degrade the plasmid DNA upon entry into the cell; the bacteria may possess an effective restriction/modification system that degrades newly introduced unmodified DNA; plasmids used in this study do not replicate in propionibacteria; appropriate conditions for electroporation were not used.

Electroporation of propionibacteria should be applicable to introduce DNA into propionibacteria. Further research needs to be performed, however, to gain an understanding of the basic biology of the propionibacteria, especially the existence of possible restriction/modification systems, to allow successful application of electro-transformation in these organisms.

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GENERAL SUMMARY

A propionic acid-tolerant derivative of *Propionibacterium acidipropionici* strain P9 was obtained by serially transferring strain P9 through broth that contained increasing amounts of propionic acid. After one year of repeated transfers, a strain (designated P200910) capable of growth at higher propionic acid concentrations was obtained. Also, it was observed that strain P200910 responded to growth in propionic acid by altering the proportion of straight-chain fatty acids in cellular lipids and by uncoupling acid production and growth. Growth rate, sugar utilization, and acid production were monitored during batch and semi-continuous fermentation of semi-defined medium and during batch fermentation of whey permeate for both strain P200910 and strain P9. The highest propionic acid concentration (47 g/l) was produced by P200910 in a semi-continuous fermentation. Strain P200910 produced a higher ratio of propionic acid to acetic acid, utilized sugar more efficiently, and produced more propionic acid per gram of biomass than did its parent in all fermentations.

A new method for producing protoplasts of *Propionibacterium fruedenreichii* was developed. The improved procedure involves growing cells in broth containing 1% threonine and treating cells with a combination of lysozyme and chymotrypsin. This procedure produces osmotically fragile cells (cells that burst when suspended in hypotonic buffer) more rapidly, and uses lower lysozyme concentrations than previously reported procedures.

Gene transfer of transposons and plasmid DNA was attempted by various methods: polyethylene glycol-induced protoplast transformation, conjugation, and electroporation. Antibiotic-resistant colonies would often appear as putative

recombinants during these experiments, but no autonomous piasmid DNA was ever detected by agarose gel electrophoresis of these cells. Conclusive evidence of gene transfer was not obtained, however, it is possible that integration of plasmid DNA into the host chromosome is took place. Nuclease activity was detected in at least one strain of *Propionibacterium* and may be responsible for the lack of successful gene transfer or the disappearance of autonomous plasmids.

Resistance to high levels of antibiotics, especially erythromycin, was observed during transformation and conjugation experiments. Most of the plasmids used in this study contained genes coding for erythromycin resistance; chromosomal integration of these genes may have occurred.

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APPENDIX A

Cost of propionic acid produced by chemical synthesis.

$1.52 per kg for a liquid product packaged in 55 gallon drums.

Cost of propionic acid produced by fermentation (1500 l).

Corn Steep Liquor	$200
Labor, Shipping, 55 gal Drum	$150
Total Cost	$350

At 3% yield - 45 kg of propionic acid would be produced in 1500 l.

Total Cost =$7.78 per kg

Note, this does not include any downstream processing

Cost of commercial mold inhibitors for agricultural use.

Liquid products cost $0.77 to $2.20 per kg. These products contain from 50 to 98% propionic acid and are applied at 0.5 to 1.0 kg per ton of feed.

Dry products cost $1.32 to $4.40 per kg. These products contain approximately 50% propionic acid and are applied as described above.