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Isolation and characterization of plasmid DNA from *Propionibacterium*

Rehberger, Thomas G., Ph.D.

Iowa State University, 1989

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Isolation and characterization of plasmid DNA from *Propionibacterium*

by

Thomas G. Rehberger

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major: Food Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1989

TABLE OF CONTENTS

INTRODUCTION

Propionibacteria

Bacteria of the genus Propionibacterium are gram-positive, pleomorphic rods which are anaerobic to aerotolerant, nonmotile and nonsporeforming (20). The genus Propionibacterium contains two groups of organisms, the classical or dairy propionibacteria and the acnes group or cutaneous propionibacteria. These two groups of organisms both produce large amounts of propionic and acetic acids as end products of metabolism. The acnes group strains were formerly members of the genus Corynebacterium whereas the dairy propionibacteria are similar to the organisms originally isolated by von Freudenreich and Orla-Jensen (73).

Four species of the classical or dairy propionibacteria are recognized: P. freudenreichii, P. jensenii, P. thoenii, and P. acidipropionici (20). Organisms belonging to these species are found in dairy products and in natural fermentations such as silage and olives. Their natural habitat is the digestive tract of ruminants. They have also been found in soil.

The four species of the cutaneous propionibacteria are P. acnes, P. avidium, P. granulosum, and P. lymphophilum (20). Organisms belonging to these species are found in

abscesses, subgingival plaque, vaginal secretions and on human skin.

Industrial applications

The classical or dairy propionibacteria are an industrially important group of microorganisms that have been used for the production of propionic acid, vitamin B_{12}, and Swiss cheese.

The most common application of the propionibacteria in industrial fermentations is their use by the dairy industry as starter cultures for the manufacture of Swiss-type cheeses (65). In 1986, over 225 million pounds of Swiss cheese were produced in the United States (3). As dairy starter cultures, the propionibacteria ferment the lactic acid generated by the lactic starter cultures to produce the characteristic eyes and contribute to the development of the typical flavor of Swiss cheese (6, 44, 45, 46, 47, 70). The role of the propionibacteria in Swiss cheese manufacture and the microbiology and flavor development of Swiss cheese have been reviewed by Reinbold (66) and Langsrud and Reinbold (44, 45, 46, 47).

In addition to their beneficial role in Swiss cheese production, the propionibacteria have also been implicated in several defects of Swiss cheese (35, 36, 65). The most notable of these is the split defect which is characterized

by the formation of cracks and fissures in the cheese body (65). The split defect represents an important economic problem to the Swiss cheese producer because cheese with this defect is unfit for direct sale to the consumer in presliced and retail-sized packages.

Numerous researchers have investigated the split defect of Swiss cheese and have identified several properties of the propionibacteria that may be important in the formation of this defect. Park et al. (59) hypothesized that splitting was the result of bacterial growth during cold room incubation. They reported that Swiss cheese produced with a Propionibacterium strain that grew well at low temperatures (7.2°C) showed a higher tendency to split than Swiss cheese produced with a strain unable to grow at low temperatures. Park et al. (60) also examined the ability of propionibacteria isolated from Swiss cheese to grow at low temperatures. Many of the strains isolated were able to grow at temperatures used for cold room curing of Swiss cheese (7.2°C) and some grew at temperatures as low as 2.8°C.

Hettinga et al. (35) confirmed the hypothesis of Park et al. (59). Strains of propionibacteria that grew well at low temperatures had a greater disposition than those that grew slowly at low temperatures to produce cheese with splits. Carbon dioxide production measured during the warm-room curing (21°C) of Swiss cheese made with low-temperature-

growing strains was nearly twice that of cheese made with strains unable to grow at low temperatures.

The metabolic characteristics of the low-temperature-growing strains were examined by Hettinga and Reinbold (36). Lactate and malate dehydrogenase enzymes isolated from low-temperature-growing strains had greater activity at lower temperatures and low pH. These enzymes play a vital role in carbon dioxide, acetate and propionate production. Strains which have greater enzyme activity at low temperature are capable of carbon dioxide production and increase the possibility for Swiss cheese to split.

The fatty acid composition of three low-temperature-growing strains and three strains which did not grow at low temperature were studied at 10 and 32°C by Hofherr et al. (39). They concluded that the change in fatty acid composition with a change in temperature was limited, and that strains able to grow at low temperatures possess a fatty acid composition consistent with this ability.

Brendehaug and Langsrud (13) investigated amino acid metabolism in resting cell suspensions of propionibacteria to determine if amino acids could serve as substrates for carbon dioxide production. Large differences were detected in the ability to degrade amino acids among the four strains examined. Anaerobic conditions increased amino acid catabolism for two strains, decreased catabolism in one

strain, and had no effect on another strain. The addition of lactate delayed amino acid degradation in one strain and did not affect degradation in another strain. The amount of carbon dioxide produced in the mixture of amino acids and lactate was less than the sum produced individually in the former strain and equal to the sum produced individually in the latter strain. They concluded that amino acids are possible substrates for carbon dioxide production which may contribute to the split defect of Swiss cheese. Whether or not the strain utilizes amino acids and lactate simultaneously may influence carbon dioxide production and the incidence of split defects.

Crow (18) also studied the influence of lactate on amino acid metabolism. Only aspartate was metabolized during lactate fermentation by P. freudenreichii ATCC 9614. Alanine fermentation occurred only after lactate exhaustion and then only at a slow rate. The specific activity of alanine dehydrogenase increased more than 10-fold after lactate exhaustion.

Aspartate metabolism and its possible influence on the production of carbon dioxide by the propionibacteria were studied by Crow (17). The conversion of aspartate to fumarate and ammonia by the enzyme aspartase followed by the reduction of fumarate to succinate occurred in all strains studied. Aspartase activity increased during and after

lactate fermentation. In complex media, similar to a Swiss cheese environment, aspartate was metabolized to succinate and ammonia during lactate fermentation. As an intermediate of this reaction, fumarate, which is a terminal oxidant, was generated. Crow found that in order to maintain the oxidation-reduction balance during aspartate metabolism, more lactate was fermented to acetate and carbon dioxide than was fermented to propionate. He concluded that aspartate is the only amino acid likely to be metabolized by propionibacteria in Swiss cheese. Carbon dioxide production was enhanced as a consequence of aspartase activity, and this may play a role in the split defect of Swiss cheese.

The fact that propionibacteria can contribute to the split defect of Swiss cheese has clearly been established. The majority of research to date has focused on determining the metabolic activities of the propionibacteria that may be responsible for split formation. To date, only a single report has been published in which cultures were manipulated to reduce the incidence of this defect. Hofherr and Glatz (38) used N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis to produce cold-sensitive mutants of strains of propionibacteria. The cold-sensitive mutants had growth rates nearly equal to that of the parent strain at 32°C but had much lower growth rates than the parent at 14°C. The use of mutants incapable of growth and carbon dioxide production

at low temperatures may reduce the incidence of split formation in Swiss cheese.

Vitamin B_{12} is an important coenzyme that enables cobalamine enzymes to catalyze rearrangement and methylation reactions (72). Although required by animals and humans, the vitamin is synthesized exclusively by microorganisms. Animal needs for this vitamin are met by food intake or by absorption of the vitamin produced by the animal's intestinal microorganisms. Humans, which are unable to absorb vitamin B_{12} produced in the large intestine, are dependent on food intake for this vitamin (19).

The commercial production of vitamin B_{12} is currently carried out by microbial fermentation. The processes have been described by Perlman (62) and Florent and Ninet (28). Propionibacterium and Pseudomonas strains have been used for this process. Reported yields with strains of P. freudenreichii and P. shermanii have ranged from 15 to 23 mg/ml. Recently, Pseudomonas strains have been developed with yields of 60 mg/ml of the vitamin (19).

Possible applications of the propionibacteria in utilizing waste-products for vitamin B_{12} production have been described by a number of researchers. Beszedits (5) described the incorporation of P. freudenreichii with cobalt into activated sludge to increase the vitamin B_{12} content of sludge and enhance its use as an animal feed supplement.

Fermentation by P. freudenreichii increased the vitamin B_{12} content of sewage sludge 70-fold and when dairy waste was added to sewage sludge a 140-fold increase was observed.

The distillation residue produced in the industrial processing of limes was also investigated as a possible substrate for vitamin B_{12} production (61). Fermentation of residue supplemented with corn steep liquor and pH-adjusted with sodium hydroxide gave the highest yields. Under anaerobic conditions, the addition of 5,6-dimethylbenzimidazole had a positive effect on vitamin B_{12} synthesis.

Soybean wastes were also examined as possible substrates for vitamin B_{12} production (76). Soybean curd whey served as a better substrate for vitamin production than did waste waters from soystarch or soymilk production. Fermentation of soybean curd whey under optimum conditions produced 889 micrograms of vitamin B_{12} per gram of dry cells.

Propionic acid, which is a primary metabolite of the propionibacteria, has a variety of industrial uses including the production of cellulose plastics, herbicides, and perfumes (63). Propionic acid is also an important mold inhibitor and can be used as a food and feed preservative (10, 29, 78). Several commerical products either produced by fermentation with propionibacteria or containing propionibacteria are currently being used in the food and

feed industry as mycostats (1, 11).

The production of propionic acid by fermentation results in acid concentrations of 2-3% in 7-10 days (63). The processes for fermentation, product recovery, and purification of propionic acid have been reviewed by Playne (63). Currently, fermentation by the propionibacteria to produce propionic acid is not an economically competitive process. Commercial production of propionic acid is by chemical synthesis from petrochemicals. However, research on fermentation processes and methods to stimulate propionic acid production by the propionibacteria have continued.

Anderson et al. (2) described inhibitory effects of autoclaved whey-based media used for the production of propionic acid by P. shermanii. Increasing whey concentrations decreased propionic acid production in autoclaved media but not in pasteurized media. The inhibitory effect of autoclaved media could be reduced by the addition of yeast extract. This same group of researchers reported that propionic acid could be produced in autoclave-sterilized whey by growing P. shermanii in mixed culture with Lactobacillus casei (8). In medium containing 12% autoclaved whey solids and 1% yeast extract, no acid production was detected if P. shermanii was grown alone. However, when L. casei and P. shermanii were grown together in this same medium, the final concentration of propionic acid was 3%.

Use of a pH-controlled fermenter with the mixed culture increased propionic acid production. Propionic acid production was also examined in ultra-high-temperature (UHT) sterilized whey (9). Propionibacterium shermanii grown with pH control in UHT sterilized medium containing 12% whey solids and 1% yeast extract produced 1.9% propionic acid. Mixed cultures of L. casei and P. shermanii produced greater than 3% propionic acid under identical conditions. Increasing the whey solids content to 18% increased the final propionic acid concentration to 6.5%.

Continuous fermentation processes for the production of propionic acid have been described. Boyaval and Corre (12) described a continuous culture system which utilized ultrafiltration for cell recycling. Schutz et al. (69) described a continuous system that used P. freudenreichii with either Streptococcus lactis or Lactobacillus acidophilus. Measurement of the carbon dioxide formed was used to control the addition of fresh medium. Continued research on propionic acid production may provide the technical advances necessary to make this fermentation more economically competitive.

Potential applications

Other applications of the propionibacteria have been suggested, including their use as a probiotic for animal

health and nutrition and as a human dietary adjunct for use in the prophylaxis and treatment of gastrointestinal disorders.

The potential use of propionibacteria as a probiotic for animals was investigated by Mantere-Alhonen (49). A probiotic is a viable organism introduced through food and feed supplements for nutritional and therapeutic benefits to the consumer (27). The addition of propionibacteria to fodder increased growth rates and stabilized the intestinal microflora of swine. Higher numbers of propionibacteria were isolated from the intestinal contents of groups fed the probiotic compared to control groups that were not fed the probiotic. However, no adhesion of the propionibacteria could be demonstrated in any part of the intestinal tract.

Vuorinen and Mantere-Alhonen (74) studied the trace element content of Propionibacterium cells in an attempt to explain the beneficial effects observed when adding propionibacteria to swine fodder. Atomic-absorption spectrophotometry identified several trace elements: magnesium, iron, calcium, manganese, zinc, copper, cobalt, nickel, chromium, cadmium, and selenium. Compared to the concentration in commercial swine fodder the concentration of trace elements in propionibacteria was 20-fold higher for manganese, five-fold higher for cobalt, and two-fold higher for magnesium. The remaining trace elements were not

noticeably different in the two preparations. The authors concluded that the higher amount and different biological form of trace elements may explain the favorable effects observed on feeding propionibacteria to animals.

Mantere-Alhonen (50) also studied the survival of propionibacteria during *in vitro* gastric digestion. Simulated gastric digestion consisting of incubation of cells with 0.15 N hydrochloric acid, 2.1% rennet and 1% pepsin with constant shaking at 37°C for one to six hours did not affect the viability of *P. freudenreichii*. It was concluded that propionibacteria are not influenced by strong gastric digestion and therefore can function in microbe-microbe interactions in the intestinal tract.

The use of propionibacteria in the production of fermented milk products which have potential use as human dietary adjuncts has also been described. Kornyeva (43) reported the establishment of propionic acid bacteria in the intestine of infants given propioni-acidophilus milk. Infants with various intestinal infections were fed acidophilus milk made with *P. shermanii*. After seven days of treatment, 87.5% of the 92 infants were excreting *P. shermanii*. When a group of healthy infants was given the propioni-acidophilus milk, 92% were found to excrete the organism after seven days. The fecal concentration of *P. shermanii* for both groups increased 100-fold during 14 days

of treatment.

Nabukhotnyi et al. (55) described the use of propioni-acidophilic formulas in the treatment of acute gastrointestinal diseases of infants. The fermented milk products 'Malyutka' and 'Malysh' were modified by adding a P. shermanii culture. The optimal products were obtained by adding 5% of a three-day culture of P. shermanii and 1-2% of a Lactobacillus acidophilus culture. The products were stable for three days when stored at 4-8°C. Formulas containing the propionibacteria proved more efficacious than the original 'Malyutka' and 'Malysh' formulas in treating infants with gastrointestinal disease.

Sidorchuk and Bondarenko (71) selected a stable mutant of P. shermanii which was multiply resistant to antibiotics, displayed a broad spectrum of antagonistic effects, and synthesized high levels of vitamins B_1, B_2, and B_{12}. A sour milk product, propioni-acidophilic milk (PAM), was made with this mutant and used in the treatment of infants with bacterial intestinal infections. The administration of PAM for 5-12 days restored the normal microflora in the intestine.

Elvit is another fermented milk product made with propionibacteria in combination with L. acidophilus (51). Mantere-Alhonen and Makinen (51) recently developed a similar new sour milk product. The optimal time-temperature

combinations for fermentation and the balance of inocula were determined to obtain a product of acceptable flavor, acidity and consistency. The best product was obtained by inoculating with 3% P. freudenreichii and 1% L. acidophilus followed by incubation at 35°C for 20-22 hours. The fermented product contained active enzymes and inhibitory activity against Escherichia coli.

Plasmids

Many bacteria contain extrachromosomal, autonomously replicating DNA molecules called plasmids. Most procaryotic plasmids are covalently closed circular DNA molecules; however, linear plasmid DNA molecules have been isolated from Streptomyces (33). In general, plasmids are considered nonessential since their presence is not normally required for the survival of the cell. Plasmids range in size from less than one megadalton to greater than 300 megadaltons and may be maintained in the cell as a single copy or in multiple copies.

A great diversity of plasmid-encoded phenotypes has been described, including antibiotic resistance, heavy-metal resistance, bacteriocin production, enterotoxin production, carbohydrate fermentation, degradation of aromatic compounds, host-controlled restriction and modification systems, cell

aggregation and many other metabolic functions. In addition to phenotypically-defined plasmids, most bacterial species contain a large number of plasmids that confer no known phenotype. These plasmids are called cryptic.

Importance in dairy starter cultures

In recent years, genetic studies of the lactic acid bacteria have established that numerous metabolic characteristics are encoded by plasmid DNA. Plasmids have been found to be associated with carbohydrate fermentation, proteolytic activity, bacteriocin production, citrate utilization, restriction and modification systems, phage adsorption, antibiotic resistance, cell aggregation, and other metabolic properties. McKay (52), Davies and Gasson (22) and Sandine (68) have reviewed the genetics of the lactic acid bacteria.

The instability of metabolic properties, such as lactose metabolism and proteinase activity in the Lactococcus group, has long been known (52). Lactococci are dependent on these functions for rapid growth and acid production. Plasmid association of these functions explains their instability and establishes the critical role of plasmids in strain performance for dairy fermentations.

Only recently have plasmids been reported in the propionibacteria (56, 58). Panon (58) detected three

different plasmid profiles from twenty strains found to contain plasmids. Plasmid DNA from a P. acidipropionici strain was digested with several restriction enzymes and estimated to be 4.33 Mdal in size. Naud et al. (56) detected extrachromosomal DNA molecules in a stable morphological variant of a P. freudenreichii strain but not in the parent. The appearance of DNA molecules, tentatively called plasmids, was accompanied by other metabolic changes. Morphological reversion from round to rod shape occurred in medium in which glucose was absent or was replaced by lactate. Revertant cells lacked plasmids and had fermentation and resistance profiles very similar to the parent strain.

Plasmid DNA isolation

The purpose of this section of the introduction is to provide the reader with an overview of the techniques and strategies used for the isolation of plasmids. Since an overwhelming number of techniques has been published this will, of necessity, not be an all-inclusive review. Instead, some of the basic techniques which have been employed in the majority of plasmid isolation methods will be presented. Basic principles of plasmid DNA isolation can be found in several manuals of methods for microbiology and recombinant DNA techniques (16, 23, 48).

A wide variety of plasmid DNA isolation methods has been

reported for both Gram-negative and Gram-positive organisms. These methods employ a variety of procedures for cell lysis and subsequent plasmid DNA purification. Although the initial steps of cell lysis can differ greatly between Gram-positive and Gram-negative microorganisms, many of the same purification strategies are used once the plasmid DNA has been released from the cell.

The first step in any successful plasmid DNA isolation procedure is the effective lysis of the host cells. Differences between the cell walls of Gram-positive and Gram-negative bacteria dictate the methods used for lysis. Gram-positive organisms contain a larger layer of peptidoglycan than Gram-negative organisms and in general can be more resistant to cell lysis. Therefore, they may require additional treatments or more severe lysis conditions.

Enzyme treatment of the cells is one of the most common procedures used to disrupt bacterial cell walls. Enzymes such as lysozyme, lysostaphin, mutanolysin and achromopeptidase as well as combinations of these enzymes have been employed in bacterial lysis procedures. These enzymes cleave specific bonds within the cell wall peptidoglycan, removing the cell walls to produce spheroplasts or protoplasts. Lytic enzyme treatment in the presence of buffered hypertonic medium maintains the integrity of the cytoplasmic membrane during enzymatic

treatment. Several workers have reported that treatment of cells with lytic enzymes in the presence of unbuffered hypertonic medium increased the efficiency of lysis and plasmid DNA yields (53, 57).

Proteolytic enzymes such as pronase E and proteinase K have also been used, usually in conjunction with lytic enzymes, to increase the efficiency of lysis. In addition to aiding in the breakdown of the cell wall material, nonspecific proteases such as pronase may be helpful in controlling endogenous nucleases.

The susceptibility of bacterial strains to lysis by bacteriolytic enzymes can often be increased by one of several pretreatments. Many Gram-positive organisms are recalcitrant to bacteriolytic enzymes. Treatments such as growth in modified medium and washing of the cells prior to enzymatic digestion may increase the susceptibility of these strains to bacteriolytic enzymes. Supplementation of the growth medium with D,L-threonine, glycine or penicillin has been reported to weaken the cell wall structure (14, 16, 31). Glycine is incorporated into the peptidoglycan in place of the D- and L-alanine of the tetrapeptide (31). Since glycine precursors are poor substrates in the transpeptidation reaction, the peptidoglycan is not efficiently cross-linked. D,L-threonine also inhibits cell wall cross-linking by a mechanism that is not exactly known. The addition of

penicillin to the growth medium inhibits the transpeptidase enzyme by forming a covalent bond with a serine residue in the active site of the enzyme (75).

Washing the cells before digestion with a bacteriolytic enzyme has also been shown to increase the susceptibility of the cells to enzyme digestion. Heath et al. (34) recently reported a simple and generally applicable procedure for releasing DNA from a variety of Gram-positive and Gram-negative bacterial cells. Treatment of the cells with acetone before digestion with lysozyme made the cells susceptible to lysis by sodium dodecyl sulfate. Washing the cells with 3% sodium chloride prior to enzyme digestion may also increase the cells' susceptibility to lytic enzymes by removing cell-associated exopolymers (67).

Another technique that can be used to increase the lysozyme susceptibility of bacterial cells is acetylation of the cell wall muramic acid residues with acetic anhydride (26). Although this procedure has been shown to be effective with Streptococcus species it is tedious and has not been used routinely in plasmid DNA isolaton procedures.

At the completion of any pretreatment and lytic enzyme digestion, the bacterial cells should be susceptible to lysis by detergents. Ionic detergents such as sodium dodecyl sulfate (SDS) and sodium N-lauryl sarcosinate or the nonionic detergent Triton X-100 are often used to lyse bacterial

cells. Alkaline detergent extraction is also effective in lysing bacterial cells and simultaneously irreversibly denaturing the chromosomal DNA (7). Boiling the cells has also been used for lysis and will provide some degree of purification by denaturation of the chromosomal DNA (40).

Effective lysis of the bacterial cells releases the cytoplasmic macromolecules, including any resident plasmids, into the hypertonic buffer. In addition, nonlysed cells and cell wall debris are also present. Purification procedures can be used to remove these contaminating materials. Generally, minimum purification is necessary for the simple detection of resident plasmids by rapid screening techniques and agarose gel electrophoresis (54). Eckhardt (25) has developed a method in which the bacteria are lysed directly in the slots of an agarose gel. Lysozyme-treated cells in the slots of a gel were lysed by the addition of SDS. The slots were sealed and the samples separated by electrophoresis. No purification steps were necessary to detect plasmids.

Often plasmids cannot be adequately resolved or detected in a complex lysate mixture without additional purification. In addition, many subsequent techniques may require a more purified DNA sample. Purification techniques serve to enrich the covalently closed circular (CCC) plasmid DNA content of the lysate. This can be accomplished by removing or reducing

the content of proteins, RNA, chromosomal DNA and the open circular (OC) form of plasmids.

Proteins are known to interfere with many of the enzymes reactions utilized in recombinant DNA techniques. Removal of contaminating proteins is usually accomplished by extraction with phenol and/or chloroform. Phenol denatures proteins and selectively removes denatured DNA from aqueous solutions. Chloroform, which is usually used as a mixture with isoamyl alcohol (24:1, v/v), also removes proteins and any residual phenol from the aqueous phase. Deproteinization with the two organic solvents is more efficient than deproteinization with one solvent. Phenol extraction is most often used as a final purification step prior to concentration of the DNA.

Contaminating RNA can obscure the detection of low molecular weight plasmids and may interfere with enzymes utilized in genetic analysis and cloning. The most common technique used to remove RNA is treatment with RNase. Prior to the use for this treatment the RNase preparation is freed of DNase activity by heating it at 100°C for 15 minutes (48).

Several techniques have been developed to reduce the chromosomal and OC DNA present in a crude lysate. Many of the techniques developed rely on the nature of the CCC configuration and other properties unique to plasmid DNA.

Separation of CCC plasmid DNA from the OC form and chromosomal DNA can be accomplished by ethidium bromide

density gradient centrifugation (48). Ethidium bromide intercalates into DNA, decreasing its density. The CCC form of plasmids binds less of the intercalating dye and thus has a greater density than open circular or linear chromosomal DNA which bind more of the dye (4). In the presence of a cesium chloride (CsCl) gradient, the CCC plasmid molecules can be separated from linear chromosomal and OC DNA. Clewell and Helinski (15) used a gentle method of nonionic detergent lysis followed by low-speed centrifugation to remove most of the chromosomal DNA from the lysate. The plasmid DNA remained in the supernatant fraction, which is called the cleared lysate, and was further purified by CsCl-ethidium bromide density gradient centrifugation.

Guerry et al. (30) and Dunny et al. (24) modified the procedure of Hirt (37) for the selective precipitation of high molecular weight chromosomal DNA. The precipitation was accomplished by the addition of SDS in the presence of a high concentration of salt. The technique did not rely on the CCC form of the plasmid and thus the OC forms of plasmids also were isolated. Although the method is presumably based on selective precipitation according to size, the procedure was reported to be applicable for plasmids over a wide range of sizes (5-65 Mdal).

Currier and Nester (21) described a method for isolating CCC plasmid DNA which involved shearing and alkaline

denaturation of the chromosomal DNA. At a pH range of 12.0 to 12.5, linear DNA was selectively denatured whereas CCC plasmid DNA was unaffected. Shearing lysates to reduce viscosity was the most critical step in the successful alkaline denaturation. Neutralization and phenol extraction following alkaline denaturation removed the denatured linear chromosomal DNA.

The resistance of CCC plasmid molecules to denaturation by heat has also been exploited in developing procedures to remove contaminating chromosomal and OC DNA. Holmes and Quigley (40) and Ranhand (64) described boiling methods which reduced the amount of chromosomal and and OC DNA. In the method described by Ranhand, sheared cell lysates were boiled for 15 minutes and quenched on ice. Both large and small plasmids were detected using this technique. In the Holmes and Quigley protocol, cells briefly treated with lysozyme were boiled for 40 seconds. Only small plasmids were detected using this procedure.

Zasloff et al. (77) described a specialized, selective extraction procedure based on differential partitioning of the CCC plasmid DNA and noncovalently closed DNA extracted with phenol at acid pH and low ionic strength. Plasmid DNA in the CCC form remained in the aqueous phase while all other DNA forms were removed into the organic phase. Three extractions were required to remove 99% of the contaminating

DNA.

The successful removal or reduction of the major contaminating macromolecules enriches the sample for CCC plasmid DNA. The concentration of plasmid DNA in the sample is often too low for further experimentation so a precipitation is used to concentrate the plasmid DNA. Precipitation with ethanol and isopropanol are the most commonly used methods (48). The addition of two volumes of ethanol or one volume of isopropanol to a given volume of a DNA solution containing the proper concentration of monovalent cations, often followed by incubation at low temperature (-20°C), results in the precipitation of DNA. Polyethylene glycol at a final concentration of 10% has also been reported to quantitatively precipitate DNA (41). At the completion of precipitation, the DNA is redissolved in an appropriate buffer at the desired concentration.

Many excellent plasmid isolation techniques have been described which utilize a combination of the purification techniques described to remove contaminating macromolecules. Hansen and Olsen (32) developed a protocol which incorporated alkaline denaturation, sodium chloride-SDS precipitation and polyethylene glycol concentration to isolate large plasmids from a number of Gram-negative organisms. Birnboim and Doly (7) described a rapid procedure which employed alkaline denaturation followed by neutralization and precipitation of

contaminating materials with a high concentration of sodium acetate in the presence of SDS. Kado and Liu (42) described a plasmid screening procedure in which cells were lysed by alkaline detergent treatment. The lysate was then heated to denature linear chromosomal DNA and extracted with phenol-chloroform to remove proteins and the denatured DNA.

The techniques described in the preceding paragraphs are some of the basic methods used in the purification and concentration of plasmid DNA. Traditional techniques, such as phenol extraction and ethanol precipitation, are often tedious and recoveries are variable and sometimes low (21). Alternate purification protocols developed for use in an array of commercial products are now available to minimize the number of handling steps and maximize DNA recovery. These purification products are not used in routine, rapid detection and screening methods but are a faster, more efficient, and less expensive way to obtain preparative amounts of highly purified DNA. Some of the most popular products utilize ion-exchange or gel-filtration chromatographic techniques (23). Success with any of these products is only possible in combination with methods for effective cell lysis.

Explanation of Dissertation Format

The varied industrial applications and potential uses of the propionibacteria in agricultural biotechnology make these organisms an attractive group for genetic studies. Genetic research will provide important information that could be applied to other dairy starter cultures, improve strains, and develop beneficial alternate applications of these organisms for human and animal use. Improved strains for industrial applications could make these fermentations more economically competitive and increase the quality of current products. The incorporation of recombinant DNA techniques in strain improvement programs should make it possible to engineer strains for specific applications.

A basic understanding of the native plasmids in propionibacteria is essential for the future application of recombinant DNA techniques. A prerequisite in this regard is a plasmid isolation procedure.

The goals of this research were to determine if plasmids were present in the dairy propionibacteria, to explore the relationships among the native plasmids, and to determine the functional properties the plasmids encode. The manuscripts presented here are the results of research efforts directed at attaining these goals. Manuscript I describes the isolation procedure and initial results of plasmid screening

studies. The characterization of several Propionibacterium plasmids by restriction endonuclease analyses, DNA-DNA hybridizations and curing studies is reported in Manuscript II. Manuscript III describes genetic and enzyme analyses of plasmid-associated lactose utilization in a P. freudenreichii strain. The manuscripts are written in the form of scientific journal articles, acceptable as an alternate dissertation format. They will be submitted for publication in appropriate scientific journals. All research included in these manuscripts was completed by this author under the supervision of Bonita A. Glatz.

MANUSCRIPT I.

ISOLATION AND PARTIAL CHARACTERIZATIOM OF PLASMID DNA FROM *Propionibacterium*

Isolation and partial characterization of plasmid DNA from *Propionibacterium*

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ABSTRACT

A simple and rapid method was developed for the isolation of plasmid DNA from propionibacteria. Effective cellular lysis was achieved in all species when cells were treated with a high concentration of lysozyme. With this method, 119 strains representing all species of the classical or dairy propionibacteria were lysed and screened for the presence of plasmid DNA. Twenty strains were found to contain plasmids that ranged in size from 4.4 to greater than 119 Mdal. Additional purification by acid phenol extraction was required to remove contaminating chromosomal and open circular DNA and to obtain complete endonuclease digestions. Restriction endonuclease analysis revealed that eleven strains contained an identical 4.4 Mdal plasmid. Two other strains contained an identical 6.3 Mdal plasmid and one of these strains contained a multimeric form of this plasmid.

INTRODUCTION

Bacteria of the genus Propionibacterium are gram-positive, pleomorphic rods which are anaerobic to aerotolerant, nonmotile and nonsporeforming (7). The classical or dairy propionibacteria have been used industrially in the production of propionic acid, vitamin B_{12} and Swiss cheese. As dairy starter cultures the propionibacteria are responsible for the development of the characteristic flavor and appearance of Swiss cheese (2, 20, 32). They also have been implicated in the development of the split defect of Swiss cheese, which is characterized by the formation of cracks and fissures in the cheese body (15, 16, 28). Although the metabolic properties and nutritional requirements of the propionibacteria have been studied extensively (12, 13, 14), little information is available about the genetics of these organisms.

In recent years, genetic studies of the lactic acid bacteria have established the role plasmids play in carrying genes important for industrial fermentations (23, 31). Only recently have plasmids been reported in the propionibacteria (26, 27); to date, no information is available on the functional properties of these plasmids. A basic understanding of the native plasmids in the propionibacteria is essential for the future application of recombinant DNA

techniques in strain improvement programs. A prerequisite for understanding the functional role of plasmids is the development of a reliable procedure for plasmid DNA isolation. This report describes a simple procedure for the isolation of plasmid DNA from propionibacteria and the partial characterization of the isolated plasmids by means of restriction endonuclease cleavage.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All *Propionibacterium* strains were obtained from the Department of Food Technology culture collection, and were grown in sodium lactate broth (NLB) at 32°C (17). *Agrobacterium tumefaciens* A277 was propagated in nutrient broth (Dico Laboratories, Detroit, MI) at 28°C. *Escherichia coli* V517 was propagated in Luria broth (22) at 37°C. All cultures were stored at -70°C in their respective broths supplemented with 10% glycerol. Plasmids isolated from *A. tumefaciens* A277 and *E. coli* V517 served as mobility reference molecules in agarose gel electrophoresis analysis (21, 30). Table 1 lists representative strains of the four species of the classical propionibacteria used in this study.

Lysis Procedures for Plasmid DNA Isolation

Only the final procedures are described here. All solutions were made weekly except for lysozyme and pronase which were made fresh daily. Mutanolysin, lysozyme and pronase were obtained from Sigma Chemical Co., St. Louis, MO. Achromopeptidase was a gift from Dr. P. Pattee,

Table 1. Propionibacteria strains

ISU Number	Species and Strain	Plasmid content Number	Size(Mdal)
	P. acidipropionici		
P3	E14	1	4.4
P5	E214	1	4.4
P58	ATCC 4875	1	4.4
P88	22	1	4.4
P108	ATCC 14072	1	4.4
	P. freudenreichii		
P7	52	none	
P22	123	2	12.5, >119
P77	PS49	1	4.4
P80	PS18	1	4.4
P83	SAUER	1	4.4
P84	10	2	4.4, >119
P93	91	1	25
P97	8262	1	22
P101	ATCC 9614	2	12.5, >119
P103	5932	1	30
P113	F32	1	30
	P. jensenii		
P38	13	2	4.4, 35
P54	E.1.1	1	6.3
P63	PJ54	2	6.3, 19
P81	PP798	1	4.4
	P. thoenii		
P114	114	1	5.0

Department of Microbiology, Iowa State University. Chemicals were obtained from Fisher Scientific, Fair Lawn, NJ., or Sigma.

Propionibacterium strains grown to mid-log phase in 10 ml of NLB at 32°C provided a 1% inoculum for cultures from which plasmids were to be extracted. Cultures for plasmid isolation were incubated at 32°C until they reached late-log phase (generally, 24-48 h). Cells were harvested by centrifugation and washed once with a one-tenth volume of 3% NaCl in 50 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0.

Rapid microscale screening procedure

The washed cell pellet from a 10 ml culture was resuspended in 1 ml of 15% (w/v) sucrose in 50 mM Tris-HCl, 50 mM Na_2EDTA, pH 8.0, and transferred to a 1.5 ml microcentrifuge tube. Resuspended cells were used immediately or stored at -20°C for later use. The cells were pelleted by centrifugation (10,000 x g, 5 min) and resuspended to a final volume of 240 ul with a buffer that contained 15% sucrose in 50 mM Tris-HCl, 50 mM Na_2EDTA, pH 8.0. Lysozyme (100 mg/ml in 50 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0) was added to a final concentration of 20 mg/ml. The sample was thoroughly mixed with a vortex mixer and incubated at 32°C for 1 h. Pronase E (10 mg/ml in 50 mM Tris-HCl, 5 mM Na_2EDTA, 50 mM NaCl, pH 8.0), which had been

preincubated for 1 h at 37°C, was added to a final concentration of 1 mg/ml. The mixture was gently agitated by several inversions of the tube and incubated for 1 h at 32°C. Then 111 µl of 0.25 M Na_2EDTA at pH 8.0 were added and mixed gently by inversions. After a 15 min incubation on ice, 111 µl of 20% (w/v) sodium dodecyl sulfate (SDS) in 50 mM Tris-HCl, 25 mM Na_2EDTA, pH 8.0, were added while the tube was repeatedly tapped and inverted. The tube was returned to ice for 15 min; 70 µl of 5 M potassium acetate were then added. After thorough mixing, the tube was held on ice for 30 min. Cell debris was pelleted by centrifugation at 14,000 x g for 12 min. One ml of cold (-20°C) 95% ethanol was added to the supernatant fluid and mixed by inversion. The sample was incubated at -20°C for 1 h, after which the precipitated plasmid DNA was collected by centrifugation at 14,000 x g for 15 min. The resulting crude DNA pellet was thoroughly dried, dissolved in 75 µl of 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.5, and analyzed by agarose gel electrophoresis as described below.

Mobility reference plasmids were isolated from *E. coli* V517 by the method of Birnboim and Doly (3) and from *A. tumefaciens* A277 by the method of Casse *et al.* (4).

Preparative plasmid DNA isolation

The washed cell pellet from a 1-liter culture was resuspended in approximately 50 ml of 15% sucrose in 50 mM Tris-HCl, 50 mM Na_2EDTA, pH 8.0. To improve the reproducibility of the lysis procedure the optical density (OD) at 600 nm of the cell suspension was adjusted to 20 (i.e., 1:100 dilution of the cells were at OD=0.2) by adjustment of the buffer volume. Cells from 50 ml of this suspension were harvested by centrifugation at 10,000 x g for 10 min and resuspended to a final volume of 24 ml. Cell lysis was achieved as in the microscale method described above; the volumes of all reagents were increased 10-fold. Following the addition of 20% SDS and incubation on ice for 15 min, 5 M NaCl was added by gentle mixing to a final concentration of 1 M (10). The lysate was stored on ice overnight, after which the precipitated cell debris, NaCl and SDS were removed by centrifugation at 15,000 x g for 30 min. The plasmid DNA present in the supernatant fluid was precipitated with two volumes of cold (-20°C) 95% ethanol at -20°C for 1 h and collected by centrifugation at 10,000 x g for 15 min. The DNA pellet was dried, dissolved in 7.5 ml of 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.5, and loaded onto a preparative agarose gel or further purified as described below.

Plasmid DNA Purification

Preparative plasmid DNA samples were purified of contaminating proteins, chromosomal and open circular (OC) DNA by acid phenol extraction (34).

Acid phenol extraction

The dissolved plasmid DNA sample (7.5 ml) was extracted with an equal volume of buffer-saturated redistilled phenol (22). After centrifugation, (10,000 x g, 5 min) the aqueous phase was transferred to a clean centrifuge tube and extracted with an equal volume of chloroform-isoamylalcohol (24:1). The phases were separated by centrifugation at 10,000 x g for 5 min, the aqueous phase was removed, and two volumes of cold (-20°C) 95% ethanol were added. The sample was held at -20°C for 1 h, after which the precipitated DNA was collected by centrifugation (12,000 x g, 15 min). The DNA pellet was thoroughly dried and resuspended in 4.5 ml of 10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.5. The dissolved DNA sample was transferred to a clean centrifuge tube and 500 ul of DNase-free RNase (1mg/ml in 50 mM sodium acetate, pH 5.0) were added. The sample was incubated at 37°C for 1 h, after which a one-tenth volume of 3 M sodium acetate was added and the DNA was again precipitated by the addition of ethanol. The plasmid DNA pellet was dissolved in 5 ml of 50 mM sodium

acetate, 75 mM NaCl, pH 4.0, and extracted at 0°C with an equal volume of acid phenol (redistilled phenol equilibrated with 50 mM sodium acetate, pH 4.0). Usually two extractions were necessary to remove all contaminating chromosomal and OC DNA. Upon completion of the acid phenol extractions, 1 M Tris-HCl, pH 8.5, was added to a final concentration of 50 mM. Residual phenol was removed by chloroform extraction as described above. The remaining covalently closed circular (CCC) plasmid DNA in the aqueous phase was precipitated by the addition of cold (-20°C) ethanol and dissolved in 1 ml of 10 mM Tris-HCl, 1mM Na_2EDTA, pH 7.5.

Alternative plasmid DNA purification methods

In some experiments, alternative DNA purification methods were used. Cell lysis was achieved as described for preparative scale volumes. Lysates were then purified by the alkaline denaturation method of Currier and Nester (8) or by heat treatment (15 min, 100°C) as described by Ranhand (29). A modification of the Currier and Nester (8) alkaline denaturation method that omitted the phenol extraction step was also used.

Agarose Gel Electrophoresis

Plasmid DNA samples were subjected to electrophoresis in horizontal gels of 0.6 or 0.7% agarose dissolved in Tris-borate buffer at pH 8.1 (25). The dissolved plasmid DNA samples (75 µl) were each mixed with 25 µl of tracking dye and divided into two 50 ul subsamples. One subsample was loaded onto a 0.6% agarose gel for the detection of large plasmids, while the other subsample was loaded onto a 0.7% agarose gel for the detection of small plasmids. Electrophoresis was carried out at 55-70 volts until the tracking dye front neared the bottom of the gel for the detection of small plasmids, and at 55-70 volts for 20-24 h for the detection of large plasmids. To detect plasmids that may have been masked by the chromosomal DNA band, gels were stained in a solution of ethidium bromide (0.5 µg/ml) for 1 h, returned to the electrophoresis apparatus, and the voltage was reapplied for 2 h (1). Gels were then examined to identify any previously hidden plasmids.

Gels were stained in ethidium bromide (0.5 µg/ml in distilled water), observed on a UV transilluminator (Foto 300, Fotodyne Inc., New Berlin, WI), and photographed through 23A and 2B Wratten filters with a Polaroid MP4 camera (film type 55).

Preparative gels were loaded with 7.5 ml of dissolved

DNA from the preparative scale procedure. Electrophoresis was carried out as described above. The plasmid DNA band of interest was located in the stained gel, and a slice of agarose containing the band was cut out. Plasmid DNA was recovered from the agarose slice by electroelution into dialysis bags (22) and purified by phenol-chloroform extraction (22).

Restriction Endonuclease Digestions

Restriction endonuclease digestions were performed according to the manufacturer's directions (Boehringer Mannheim Biochemicals, Indianapolis, IN) and the method of Maniatis et al. (22). Digests were stopped by heating the samples for 10 min at 65°C and then cooling them on ice. Restriction fragments were separated on horizontal 0.8-1.0% agarose gels run in Tris-borate buffer for 12 to 14 h at 55 V. Lambda DNA digested with Pst I, Hind III or EcoR I served as mobility reference standards.

RESULTS

Lysis and Plasmid Isolation

A group of strains, composed of two representatives of each of the classical species of propionibacteria, was chosen for initial plasmid screening experiments. Initial attempts to detect plasmids in these strains by using several published plasmid DNA isolation methods and modifications of these methods were unsuccessful (5, 6, 9, 11, 18, 24, 33). None of the methods reliably isolated plasmid or chromosomal DNA from all Propionibacterium species. However, when a high concentration of lysozyme (20 mg/ml) followed by treatment with pronase was used, plasmids were detected in two strains and chromosomal DNA was detected in all strains. The critical step in the successful isolation of plasmid DNA appeared to be effective cell lysis achieved with the high lysozyme concentration.

Further experiments were conducted to select the best concentration (5, 10, 20 and 40 mg/ml), time (15, 30, 45, 60, and 120 min) and temperature (0°C, 32°C and 37°C) of lysozyme treatment. Plasmids could be isolated consistently from all species when cells were exposed to 20 mg/ml lysozyme for 1 h at 32°C. Lower concentrations, incubation times or temperatures reduced the efficiency of lysis of

strains of P. thoenii and P. jensenii.

Other lytic enzymes were also tested for their ability to lyse Propionibacterium cells. Mutanolysin at 50, 100 and 200 ug/ml and achromopeptidase at 300 and 3000 units/ml both with and without pronase treatment at 1 mg/ml for 1 h, either did not lyse all species of propionibacteria or were no more effective than lysozyme. Pronase treatment at a concentration of 1 mg/ml for 1 h following lysozyme treatment greatly improved plasmid DNA yields with most strains. Other critical factors that improved plasmid DNA yields were the addition of EDTA and the SDS concentration. Without the addition of EDTA prior to cell lysis, the DNA of most strains exhibited excessive smearing in the agarose gels. The detergent SDS was found to be more effective than Triton X-100 at 0.5 or 1% or Sarkosyl at 2 or 4%. A final concentration of 4% SDS significantly increased the DNA yields from most strains.

Plasmid DNA Purification

Purification of the isolated plasmid DNA from contaminating proteins and from chromosomal and open circular DNA was attempted with heat treatment, alkaline denaturation, alkaline denaturation followed by phenol extraction, and acid phenol extraction. The effects of

these procedures on the isolation of plasmids found in strains P54 and P113 are shown in Figure 1. Heat treatment following cell lysis reduced the amount of chromosomal DNA but produced a distorted plasmid DNA band (lane A). Alkaline denaturation did not reduce the chromosomal DNA content and produced irreversibly denatured forms of the plasmids which migrated just ahead of the CCC form (lanes B and D) (3). Denaturation conditions and the effects of alkaline denaturation varied from strain to strain and once optimized for a given strain were not always reproducible in the microscale procedure. Alkaline denaturation followed by renaturation and phenol extraction also failed to remove contaminating DNA (lane E). Purification by acid phenol extraction, however, significantly reduced the contaminating chromosomal and open circular DNA (lanes C and F). Optimal extraction conditions were found to be identical to those reported by Zasloff et al. (34), i.e., 75 mM NaCl, pH 4.0. Ether extraction was not used following phenol extraction because it reduced the yield of larger plasmids.

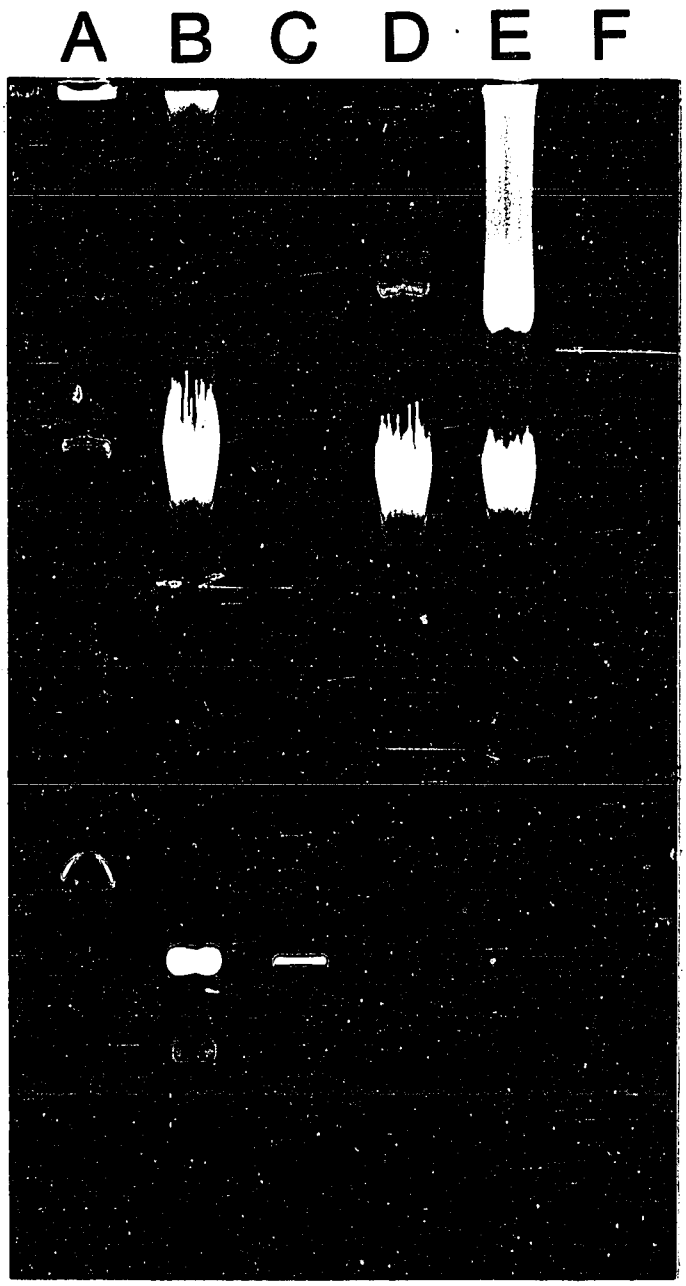
Plasmid Screening

A total of 119 strains of propionibacteria were screened for plasmid DNA by using the rapid microscale procedure. Typical gel patterns of plasmids isolated from 10 strains of propionibacteria are shown in Figure 2. Bands

Figure 1. Purification of plasmid DNA from strains P54 and P113

Lane A, heat treatment of DNA from strain P54; lanes B and D, alkaline denaturation of DNA from strains P54 and P113; lane E, alkaline denaturation followed by phenol extraction of DNA from strain P113; lanes C and F, acid phenol extraction of DNA from strain P54 and P113.

All samples loaded onto the gel were adjusted to represent an equal volume of cells.


A
B
C
D
E
F

in agarose gels corresponding to different DNA conformations of the same plasmid were identified by the removal of linear and OC forms with acid phenol extraction. Potential OC forms were initially identified by comparing their migration to the known set of OC forms produced in the mobility reference plasmids (21).

Twenty strains were found to contain plasmids that ranged in size from 4.4 to greater than 119 Mdal (Table 1). Most strains contained a single plasmid and no strain was found to contain more than two distinct plasmids. Nine strains were found to contain a single 4.4 Mdal plasmid and two strains, P38 and P84, contained the 4.4 Mdal plasmid in addition to a larger plasmid. Two P. jensenii strains, P54 and P63, each contained a 6.3 Mdal plasmid. However, P63 also contained a 19 Mdal plasmid. The plasmid profiles of two pairs of P. freudenreichii, strains P22 and P101 as well as P103 and P113, were identical. Figure 3 is a closeup photograph of a gel that contains the large Propionibacterium plasmids. The largest plasmids isolated to date were from P. freudenreichii strains P22, P84 and P101. These plasmids were significantly larger than 119 Mdal.

Figure 2. Agarose gel electrophoresis of plasmid DNA from Propionibacterium strains

Lanes C thru L contain DNA isolated by using the microscale procedure from Propionibacterium strains. The identities of the plasmid bands in each lane are listed from the wells down. Lane C, 4.4-Mdal OC, 4.4-Mdal linear, 4.4-Mdal CCC from P3; lane D, 4.4-Mdal CCC from P5; lane E, no plasmids from P7; lane F, 12.5-Mdal CCC from P22; lane G, 35-Mdal CCC, 4.4-Mdal OC, 4.4-Mdal CCC from P38; lane H, 6.3-Mdal OC, 6.3-Mdal CCC from P54; lane I, 4.4-Mdal OC, 4.4-Mdal linear, 4.4-Mdal CCC from P58; lane J, 19-Mdal multimer of the 6.3-Mdal plasmid, 6.3-Mdal OC, 6.3-Mdal CCC from P63; lane K, 25-Mdal CCC from P93; lane L, 30-Mdal CCC from P113. A. tumefaciens A277 (lanes B and M) and E. coli V517 (lane A) mobility reference plasmids with molecular masses indicated in Mdal. Chr. indicates chromosomal DNA fragments.

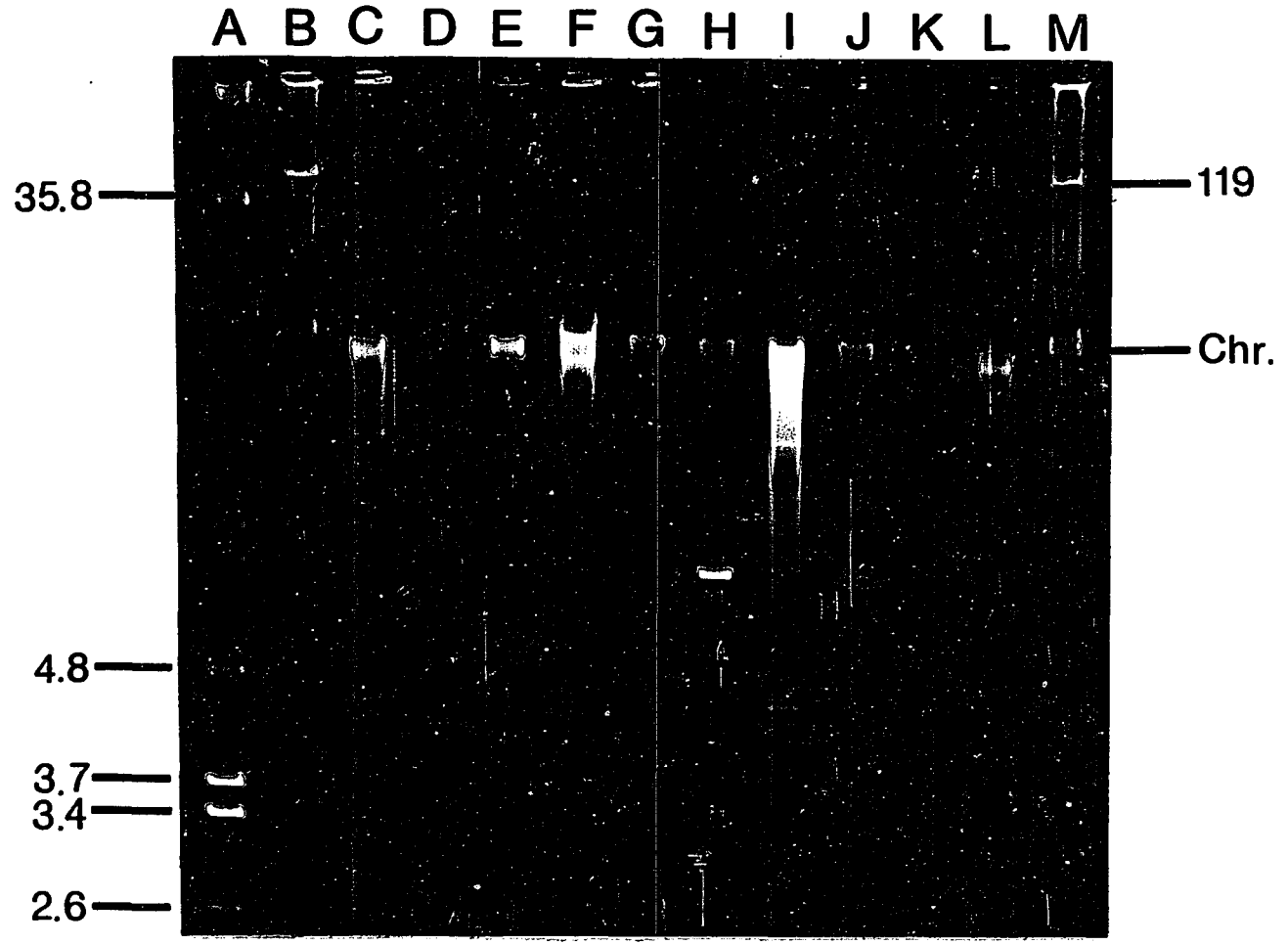
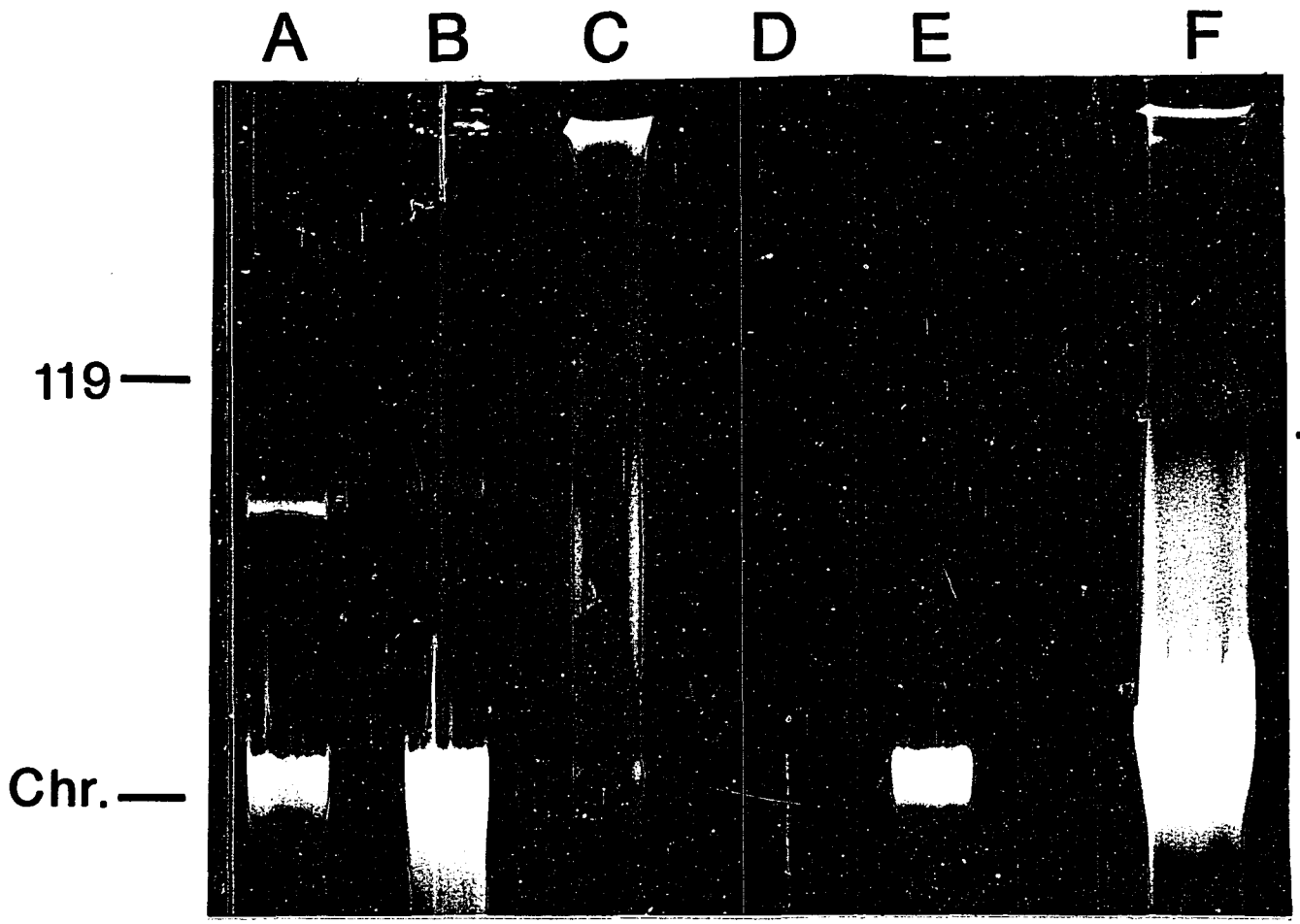

A B C D E F G H I J K L M
35.8
4.8
3.7
3.4
2.6
119
Chr.

Figure 3. Closeup photograph of an agarose gel showing the large plasmids from *Propionibacterium* strains

Lane A, P93; lane B, P113; lane E, P103; lane F, P22. *A. tumefaciens* A277 (lane C) and *E. coli* V517 (lane D) mobility reference plasmids have molecular masses indicated in Mdal. Chr. indicates chromosomal DNA fragments.


A
B
C
D
E
F
119
Chr.
35.8

Restriction Endonuclease Analysis

Plasmids of the same molecular weight from different strains were purified and digested with restriction enzymes to determine if they were, in fact, identical.

The 4.4 Mdal plasmids from strains P5, P58 and P108 were purified by acid phenol extraction and digested with restriction endonucleases BamH I, EcoR I and Sac I (Figure 4). All three plasmids produced identical DNA digestion patterns for all enzymes tested, which indicates that these plasmids are identical. Further analysis of the 4.4 Mdal plasmids in the remaining strains indicated that all strains contained this same plasmid. The 4.4 Mdal plasmid contained a single site for BamH I and two sites for EcoR I and Sac I.

Plasmid DNA from strains P54 and P63 was isolated by using the preparative scale procedure and was purified by electroelution. Restriction endonuclease digestions of the 6.3 and 19 Mdal plasmids in strain P63 and the 6.3 Mdal plasmid in P54 produced identical DNA digestion fragments (Figure 5). Therefore, the 19 Mdal was identified as a multimeric form of the small 6.3 Mdal plasmid. Partial digestion fragments were evident; these probably resulted from the presence of impurities in the DNA samples. Complete digestion was observed if the DNA was purified by acid phenol extraction prior to electroelution. The 6.3

Figure 4. Restriction endonuclease analysis of the 4.4 Mdal plasmids in strain P5, P58 and P108

Lanes A-C, undigested plasmids from P5, P58 and P108; lane D-F, BamH I digests of above plasmids; lanes G-I, EcoR I digests of above plasmids; lanes J-L, Sac I digests of above plasmids; lane M, Lambda DNA digested with Pst I. Fragment sizes are indicated in kilobases (kb).

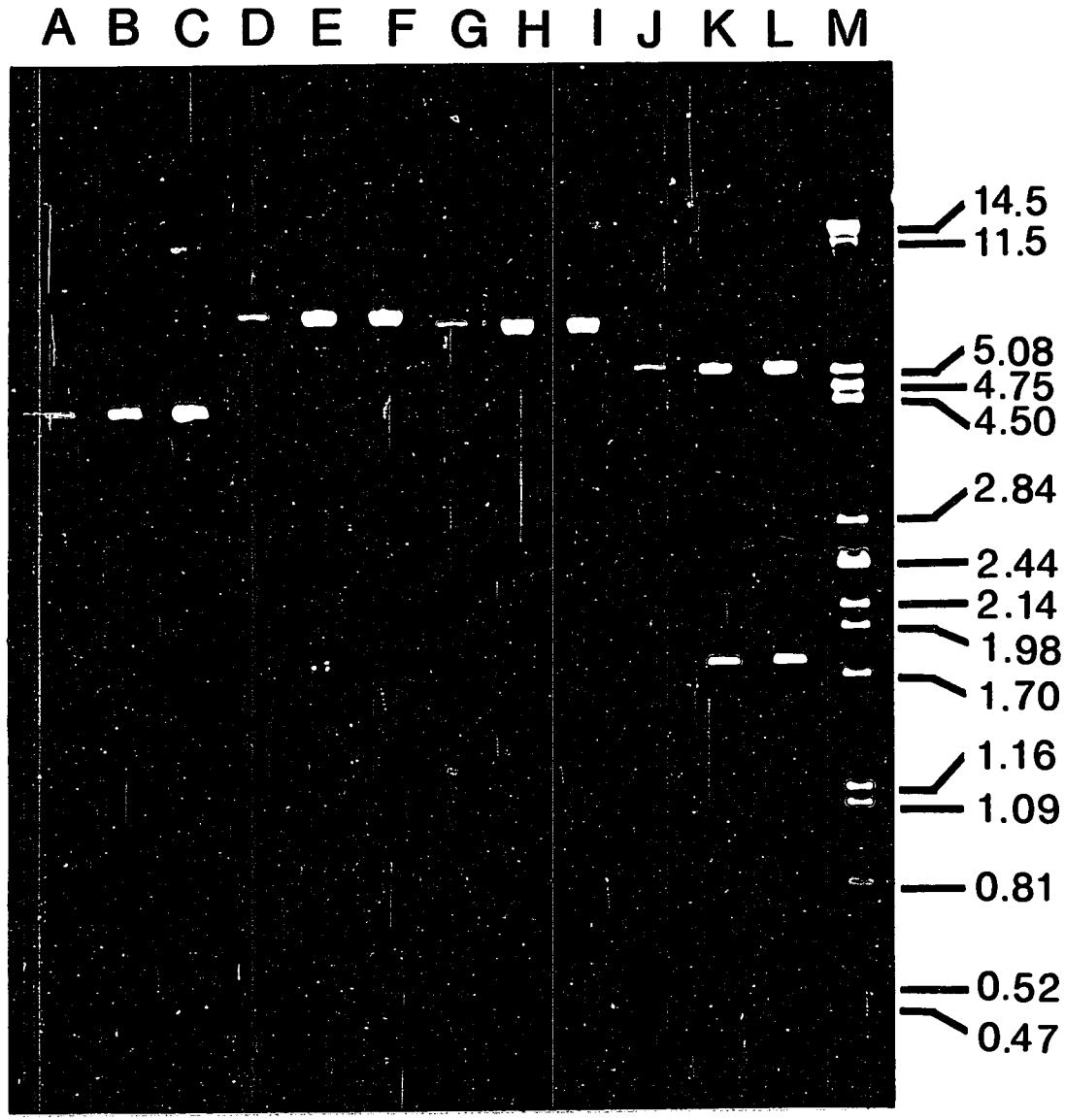
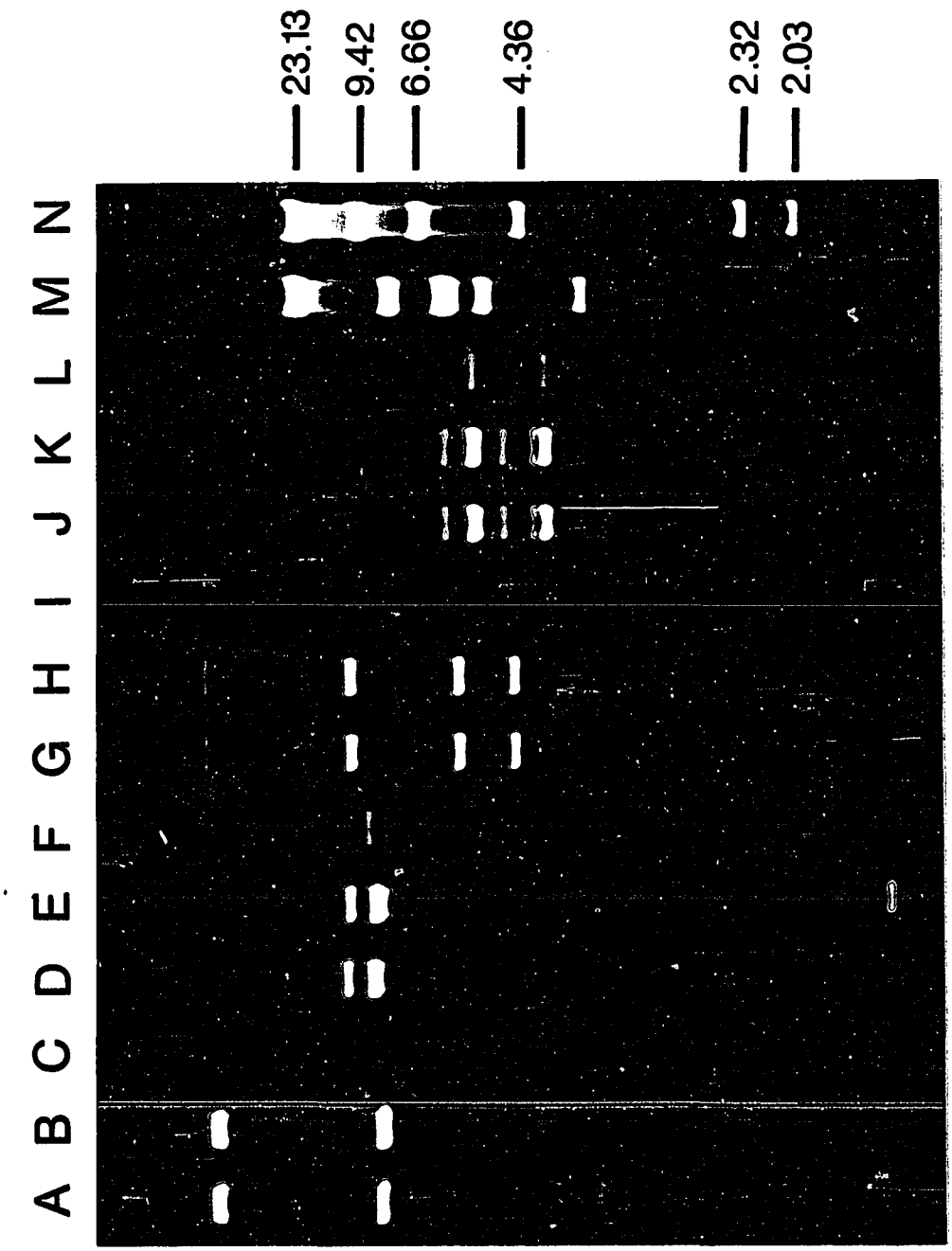

A B C D E F G H I J K L M
14.5
11.5
5.08
4.75
4.50
2.84
2.44
2.14
1.98
1.70
1.16
1.09
0.81
0.52
0.47

Figure 5. Restriction endonuclease analysis of plasmids from P54 and P63

Lane A, undigested 6.3 Mdal plasmid from P54; lane B, undigested 6.3 Mdal plasmid from P63; lane C, undigested 19 Mdal plasmid from P63; lanes D-F, EcoR I digests of above plasmids; lanes G-I, Xho I digests of above plasmids; lanes J-L, EcoR I-Xho I digests of above plasmids; lane M, lambda DNA digested with EcoR I; lane N, lambda DNA digested with Hind III. Fragment sizes are indicated in kilobases (kb).


23.13
9.42
6.66
4.36
2.32
2.03
A B C D E F G H I J K L M N

Mdal plasmid was found to contain two EcoR I and Xho I sites.

DISCUSSION

The protocol described here was designed for the reliable isolation and detection of both large and small plasmids from all species of the classical propionibacteria. It is a relatively rapid procedure that requires about 4 to 5 h to obtain samples for agarose gel electrophoresis, and requires only a small amount of cells (less than 10 ml of culture) for the detection of all plasmids. Treatment of cells with a high concentration of lysozyme for 1 h was the critical step in obtaining adequate lysis for plasmid DNA isolation. Although such intense lysis conditions have been reported to be detrimental to plasmid isolation in some lactobacilli (19), reduced lysozyme concentrations, incubation times, and incubation temperatures did not work effectively. In addition, if lysozyme treatment was extended to 2 h plasmid isolation was not adversely affected.

Treatment with pronase was found to increase plasmid yields in most strains, especially those of P. jensenii and P. thoenii that were most insensitive to lysozyme. The addition of EDTA and the use of 4% SDS were also found to increase plasmid yields. It is possible that the use of these reagents eliminated endogenous nuclease activity. If they were not used, excessive smearing of the DNA of most

strains was observed in agarose gels.

Complete purification of isolated plasmid DNA was only possible with acid phenol extraction, which removed all contaminating chromosomal and OC DNA after two extractions. Purified plasmid DNA produced with this method could be used to identify the various conformational forms of plasmids that appeared in agarose gels. Complete digestion by restriction endonucleases was also obtained with this purified DNA.

Twenty strains out of 119 strains screened to date have been found to carry plasmids, which range in size from 4.4 to greater than 119 Mdal. The distribution of plasmids is interesting and may be species-related. The P. freudenreichii strains contained the greatest diversity of plasmid profiles while P. acidipropionici strains contained only the 4.4 Mdal plasmid. The single 4.4 Mdal plasmid was observed in three of the four species and 45% of the plasmid-carrying strains.

Restriction endonuclease analysis revealed that the 4.4 Mdal plasmids present in 11 strains were identical. The 6.3 Mdal plasmids present in two strains were identical and a multimeric form of this plasmid was also present. The similar size plasmids in other pairs of strains (P22 and P101 and P103 and P113) have not been analyzed by restriction endonuclease digestions to determine if they are

also identical to each other.

Recently, Panon reported the isolation of three different plasmid profile patterns (designated group 1, 2 and 3) from 20 strains of propionibacteria (27). Panon did not distinguish different plasmid conformations and did not present the molecular sizes of these plasmids. However, the OC and CCC forms of the 4.4 Mdal plasmid that we have described closely resemble the plasmid profile of Panon's group 1. Additionally, the OC and CCC forms of the 6.3 Mdal plasmid resemble the plasmid profile of Panon's group 2. We have found no strains with plasmid profiles similar to those of Panon's group 3.

Further evidence that the 4.4 Mdal plasmid is identical to the plasmids of Panon's group 1 comes from restriction endonuclease analysis. The fragments produced by digestion with BamH I and Sac I as reported by Panon (27) appear to be the BamH I and Sac I digestion fragments of the 4.4 Mdal plasmid. Panon however, reported the molecular mass to be 6.5 kb as determined by a single EcoR I fragment (27). Our results indicate that the 4.4 Mdal (6.7 kb) plasmid has two EcoR I sites that produce fragments of 6.35 and 0.25 kb. It is possible that Panon did not detect the smaller EcoR I fragment.

The results reported here and by Panon (27) establish the similarity of Propionibacterium plasmid profiles from

strains isolated from diverse geographical locations. They also point to the wide distribution of the 4.4 Mdal plasmid, which was found in 11 of 20 plasmid-carrying strains in this study and in 16 of 20 strains reported by Panon. The role these plasmids may play in the metabolic processes of their host strains has yet to be established and remains an important area for future investigations. It is hoped that the plasmid isolation and detection method described in this study will be a useful tool for these and other genetic studies of the propionibacteria.

ACKNOWLEDGMENTS

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

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MANUSCRIPT II.

CHARACTERIZATION OF *Propionibacterium* PLASMIDS

Characterization of *Propionibacterium* Plasmids

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ABSTRACT

Plasmid DNA from 15 strains of Propionibacterium was characterized by using restriction endonuclease analyses, DNA-DNA hybridizations and curing experiments. Restriction endonuclease analysis identified seven distinct plasmids (pRG01 through pRG07). Detailed restriction maps were constructed for four of these plasmids. DNA-DNA hybridization analysis revealed that plasmids pRG01 and pRG02 shared extensive sequence homology and both were homologous to pRG07 and to identical sequences of pRG05. Plasmids pRG04 and pRG06 did not share any significant sequence homology with any of the other plasmids. Plasmid pRG03 had partial sequence homology only with pRG07. Curing of plasmids pRG01, pRG02 and pRG05 was achieved by treatment with acriflavin, but failed to identify any plasmid-encoded bacteriocin production, carbohydrate fermentation or antibiotic resistance. However, physical evidence was obtained that tentatively linked the clumping phenotype of P. jensenii strain P38 to plasmid pRG05.

INTRODUCTION

The propionibacteria are important organisms used in several industrial fermentations. Their metabolic properties and nutritional requirements have been studied extensively (11, 12, 13). The most common application of the propionibacteria in industrial fermentations is their use by the dairy industry as starter cultures for the manufacture of Swiss-type cheeses. As dairy starter cultures, the propionibacteria ferment the lactic acid generated by the lactic starter cultures to produce the characteristic eyes and contribute to the development of the typical flavor of Swiss cheese (3, 19, 20). They also have been implicated in several defects of Swiss cheese (14, 15, 21, 28).

Several other industrial applications of the propionibacteria have been described including their use as a probiotic (25, 26), as an inoculant for silage and grain (9, 34) and in the production of vitamin B_{12} and propionic acid (29, 30). Propionibacteria have been used in the industrial production of vitamin B_{12}, but the recent development of Pseudomonas strains with increased yields of the vitamin may make their use in this regard obsolete (6). Fermentation by propionibacteria to produce propionic acid is presently not an economically competitive process. Propionic acid has a variety of industrial uses including the production of

cellulose plastics, herbicides and perfumes (30). Propionic acid is also an important mold inhibitor and is used commercially as a food and feed preservative (30). Advances in fermentation technology and product separation along with the development of genetically engineered strains may supply the necessary increase in performance to make these fermentations more economically competitive and may also improve the quality of current products.

The need for improved strains for industrial fermentations has prompted genetic studies of these organisms. A basic understanding of the native plasmids in the propionibacteria is essential for the future application of recombinant DNA techniques in strain improvement programs. Recently, a lysis procedure for plasmid DNA isolation from the propionibacteria was developed in our laboratory (31). To date, twenty strains have been found to contain plasmids that range in size from 4.4 to greater than 119 Mdal. Initial characterization by treatment with restriction endonucleases revealed that 11 strains contained an identical 4.4 Mdal plasmid and two other strains contained an identical 6.3 Mdal plasmid. The role of these plasmids in the growth and metabolism of the propionibacteria has yet to be identified.

Work on the characterization of Propionibacterium plasmids has continued. We report here the construction of

restriction maps of four plasmids as well as the identification of a plasmid phenotype from plasmid curing experiments. Additionally, the relationships among Propionibacterium plasmids were examined by using DNA-DNA hybridizations.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All Propionibacterium strains were obtained from the Department of Food Technology culture collection, and routinely grown in sodium lactate broth (NLB) at 32°C (16). Escherichia coli strains were propagated at 37°C in Luria broth (LB)(24). Solid media (NLA and LBA) contained 1.5% agar. All cultures were permanently stored at -70°C in their respective broths supplemented with 10% glycerol. Table 1 lists the Propionibacterium strains used in this study.

Plasmid DNA Isolation and Purification

Plasmid DNA was isolated from Propionibacterium strains by using the microscale screening procedure or the preparative scale isolation method previously described (31). Plasmid DNA was purified by acid phenol extraction (35) or by cesium chloride (CsCl)-ethidium bromide density gradient centrifugation (24). 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-HCl, 1 mM Na_2EDTA, pH 7.5, by using a Centricon-30 microconcentrator according to the manufacturer's

Table 1. *Propionibacterium* strains

ISU Number	Species and Strain	Plasmid content Size(Mdal)	Name
	P. acidipropionici		
P3	E14	4.4	pRG01
P5	E214	4.4	pRG01
P58	ATCC 4875	4.4	pRG01
P88	22	4.4	pRG01
P108	ATCC 14072	4.4	pRG01
	P. freudenreichii		
P77	PS49	4.4	pRG01
P80	PS18	4.4	pRG01
P83	SAUER	4.4	pRG01
P93	91	6.3, 25	pRG07, pRG03
P103	5932	5.6, 30	pRG06, pRG04
P113	F32	5.6, 30	pRG06, pRG04
	P. jensenii		
P38	13	4.4, 35	pRG01, pRG05
P54	E.1.1	6.3	pRG02
P63	PJ54	6.3, 19	pRG02, multimer pRG02
P81	PP798	4.4	pRG01

instructions (Amicon Corp. Danvers, MA).

Plasmid DNA was isolated from E. coli by using the rapid screening procedure of Birnboim and Doly (4). Large scale isolation of plasmid DNA was performed by the cleared lysate method of Clewell and Helinski (5) and the DNA was purified by CsCl-ethidium bromide density gradient centrifugation.

Restriction Endonuclease Analysis and Agarose Gel Electrophoresis

Restriction endonuclease digestions were performed according to manufacturer's directions (Boehringer Mannheim Biochemicals, Indianapolis, IN), and to methods described by Maniatis et al (24). Restriction fragments were separated in horizontal 0.9% agarose gels run in Tris-borate buffer for 12 to 14 h at 55 V. Lambda DNA digested with Pst I and a commercial 123 bp DNA ladder (Bethesda Research Laboratories Inc. (BRL), Gathersburg, MD) served as DNA standards for molecular weight determinations.

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) (27).

Preparative agarose gels (0.7%) were loaded with 2.0 ml

of purified plasmid DNA. Electrophoresis was carried out as described above. The plasmid DNA band of interest was located in the stained gel, cut out, and electroeluted from the agarose slice in an Elutrap chamber according to the manufacturer's instructions (Schleicher and Schuell Inc., Keene, NH).

Nick Translation

Biotinylated plasmid DNA probes were made by using a nick translation kit and biotin-11-dUTP as described by the manufacturer (BRL). After nick translation, unincorporated biotin-11-dUTP was removed with a Centricon-30 microconcentrator according to manufacturer's instructions (Amicon). When more than one plasmid was present in a strain the plasmid to be labeled was first separated on a preparative agarose gel, excised and electroeluted.

DNA-DNA Hybridization and Detection

Restriction fragments of plasmid DNA were transferred to nitrocellulose sheets (Trans-blot nitrocellulose membranes, Bio-Rad Laboratories, Richmond, CA) by using the Southern blotting technique (32). Prehybridization and hybridization buffers (10ml/100cm^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's reagent and 25 mM sodium phosphate (22). Filters were subjected to prehybridization treatment in a sealed bag at 42C with constant agitation for at least 4 h. Biotinylated probe DNA was denatured at 95C for 10 min. and cooled in an ice bath. The denatured probe was added to the hybridization buffer at a concentration of 100-200 ng/ml. The filter and hybridization buffer were sealed in a bag and incubated at 42C for 24-36 h. Posthybridization washes, filter blocking, and detection of homologous sequences were performed as described by the manufacturer of the biotinylated DNA detection system (BRL).

Cloning in *E. coli*

Plasmid pRG04, the 30 Mdal plasmid from strain P103 or P113, was isolated on a preparative agarose gel, excised and purified. Purified pRG04 insert DNA and pUC19 vector DNA were digested with *Eco*R I or *Pst* I, extracted once with an equal volume of phenol and once with chloroform and then ethanol-precipitated. Cleaved vector and insert DNA were mixed in a 1:3 ratio (pmoles ends/pmoles ends) at a final DNA concentration of 5-10 µg/ml. Ligation was performed with 1 unit of T4 DNA ligase in a final volume of 50 µl at 14°C for 16 h (24). The ligated DNA sample was used to transform

competent cells of E. coli DH5 as described by Hanahan (10). Transformants were selected on LBA containing 50 µg/ml ampicillin and 40 µg/ml 5-bromo-4-chloro-3-indoyl-B-D galactoside (X-gal) (24).

Plasmid Curing

Propionibacterium strains grown to mid-log phase in 10 ml of NLB at 32°C provided inocula for curing trails. Cultures were inoculated at 1% into tubes of NLB that contained either 5 to 50 µg/ml acriflavin, 10 to 100 µg/ml acridine orange or 1 to 25 µg/ml ethidium bromide and incubated at 32°C for 48 h. For each curing agent and bacterial strain, the highest concentration that allowed growth was noted, and 0.1 ml of the culture in that medium was transferred to 10 ml of fresh medium and reincubated. After at least seven successive transfers, cultures were diluted and plated onto NLA. After incubation for one week at 32°C individual colonies were transferred to NLB and incubated for 24-36 h at 32°C. These cultures were then inoculated at 1% into 10 ml of NLB and incubated at 32°C for 36-42 h, to provide the cultures to be screened for plasmid DNA. Cured derivatives were identified by comparing the plasmid profile of the parent strain to the profile of cultures exposed to the curing agent.

Screening of Plasmid-Cured Derivatives

Propionibacterium strains P5, P38, P54, P63 and their cured derivatives were grown to mid-log phase in 10 ml of NLB and inoculated onto plates for detection of bacteriocin production, carbohydrate fermentation and antibiotic resistance as described below.

Bacteriocin production

Cultures were inoculated as spots onto NLA plates, incubated 10 days at 32°C in a GasPak jar (BBL Microbiology Systems, Cockeysville, MD) flushed with CO_2 and screened for bacteriocin production by the deferred antagonism method (18). Soft agar (5 ml, 0.7%) was inoculated with 0.1 ml of an overnight culture of an indicator organism and poured on the inverted surface of the plate. Cultures used as indicators included: Aeromonas hydrophila ATCC 7965, Bacillus cereus USDA 201, Apiotrichum curvatum ATCC 20509, E. coli H10407, Lactobacillus bulgaricus AR2, Leuconostoc cremoris, Listeria monocytogenes strains V7 and Scott A, P. acidipropionici strains P42 and P68, P. freudenreichii strains P1 and P104, P. jensenii strains P9 and P69, P. thoenii strains P4 and P15, Pseudomonas aeruginosa, Pseudomonas fluorescens 949, Salmonella typhimurium, Staphylococcus aureus Z88, Streptococcus cremoris B1,

Streptococcus diacetylactis 18-16, Streptococcus lactis C2, Streptococcus thermophilus AC2, and Yersinia enterocolitica ATCC 23715. Cured derivatives of Propionibacterium strains P5, P38, P54, and P63 were also used as indicators. Bacteriocin assay plates were incubated at 32°C for 12-24 h and examined for zones of growth inhibition.

Carbohydrate fermentations

Cultures were inoculated as spots onto bromocresol purple basal medium plates (1% trypticase, 1% yeast extract, 1.5% agar, 0.025% K_2HPO_4, 0.02% bromocresol purple, pH 7.2) (23) supplemented with the following carbohydrates at 1% final concentration: adonitol, arabinose, cellobiose, erythritol, fructose, galactose, glucose, glycerol, lactate, lactose, maltose, mannitol, mannose, raffinose, rhamnose, ribose, sorbitol, starch, sucrose, trehalose, and xylose. Plates were incubated at 32°C for one week and examined for acid production as evidenced by a color change from purple to yellow. Carbohydrates were obtained from Aldrich Chemical Co., Milwaukee, WI, or Fisher Scientific Co., Fair Lawn, NJ.

Antibiotic Resistance

Susceptibility testing of Propionibacterium cultures was performed by the agar dilution technique (2). Cultures were inoculated as spots onto NLA plates containing various concentrations of the following antibiotics: ampicillin,

bacitracin, chloramphenicol, cephalothin, cloxacillin, erythromycin, fusidic acid, gentamycin, kanamycin, lincomycin, methicillin, naladixic acid, neomycin, novobiocin, oxacillin, penicillin, rifampicin, streptomycin, tetracycline, trimethoprim, and vancomycin. All antibiotics were obtained from Sigma Chemical Co., St. Louis, MO. After incubation for 7-10 days at 32°C the presence or absence of growth at the various concentrations of antibiotics was recorded. The lowest concentration of each antibiotic that completely inhibited the growth of a strain was recorded as the minimum inhibitory concentration (MIC) for that strain.

Clumping

Propionibacterium jensenii strain P38 and its cured derivatives were observed for clumping as follows. A mid-log phase culture was harvested, resuspended in one-tenth volume of fresh NLB, and inoculated (100 ul) into 10 ml of fresh NLB. This culture was incubated at 32°C and observed over a 24 h period for the presence of visible cell aggregates.

Induction of the clumping phenotype was tested by using the method of Dunny et al. (7). A mid-log phase culture was harvested by centrifugation, the supernatant fraction was filtered through a 0.2 um filter, and a portion was added to a nonclumping test culture in an assay mixture that consisted of 5 ml filtrate, 4.9 ml fresh NLB and 0.1 ml harvested cell

suspension. In some assays the fresh NLB was eliminated. The mixture was incubated at 32°C and observed for cell clumping.

RESULTS

Restriction Endonuclease Analysis

Restriction endonuclease analysis of plasmid DNA from 15 Propionibacterium strains identified seven distinct plasmids. Their names, sizes and strains in which they were found are shown in Table 1. Like plasmids pRG01 and pRG02, which were previously found to reside in several different strains (31), plasmids pRG04 and pRG06 were also found in more than one strain. Plasmids pRG06 and pRG07 had been previously undetected and were found in strains thought to contain a single plasmid.

The numbers of restriction endonuclease recognition sites in plasmids pRG01 through pRG05 are shown in Table 2. Enzymes (Hae III and Msp I) that recognized sequences of four bases composed of nonalternating guanine and cytosine residues had a large number of cleavage sites in all plasmids. In contrast, Cfo I which recognizes a four-base site composed of alternating guanine and cytosine residues had no cleavage sites in any of the plasmids.

Restriction endonuclease cleavage maps of plasmids pRG01, pRG02 and pRG03 were constructed by using results from single and multiple enzyme digestions (Figures 1-3). Comparison of the pRG01 and pRG02 restriction maps identified

Table 2. Restriction endonuclease cleavage sites in *Propionibacterium* plasmids

Enzyme	Recognition Site	Plasmid pRGO1	pRGO2	pRGO3	pRGO4	pRGO5
Apa I	GGGCC*C	6	11	4	11	15
BamH I	G*GATCC	1	3	1	13	13
Bgl II	A*GATCT	0	0	2	7	0
Cfo I	GCG*C	0	0	0	0	0
EcoR I	G*AATTC	2	2	0	2	5
Hae III	GG*CC	>50	>50	>75	>75	>75
Hind III	A*AGCTT	0	0	2	0	0
Hpa I	GTT*AAC	1	1	1	0	0
Kpn I	GGTAC*C	0	1	5	0	7
Msp I	C*CGG	>50	>50	>75	>75	>75
Nhe I	G*CTAGC	2	2	2	0	0
Pst I	CTGCA*G	0	2	0	10	13
Pvu II	CAG*CTG	0	0	10	12	12
Sac I	GAGCT*C	2	3	16	13	14
Sal I	G*TCGAC	1	4	3	22	18
Sph I	GCATG*C	1	1	0	14	16
Xho I	C*TCGAG	1	2	0	8	12
Xba I	T*CTAGA	0	0	0	0	0

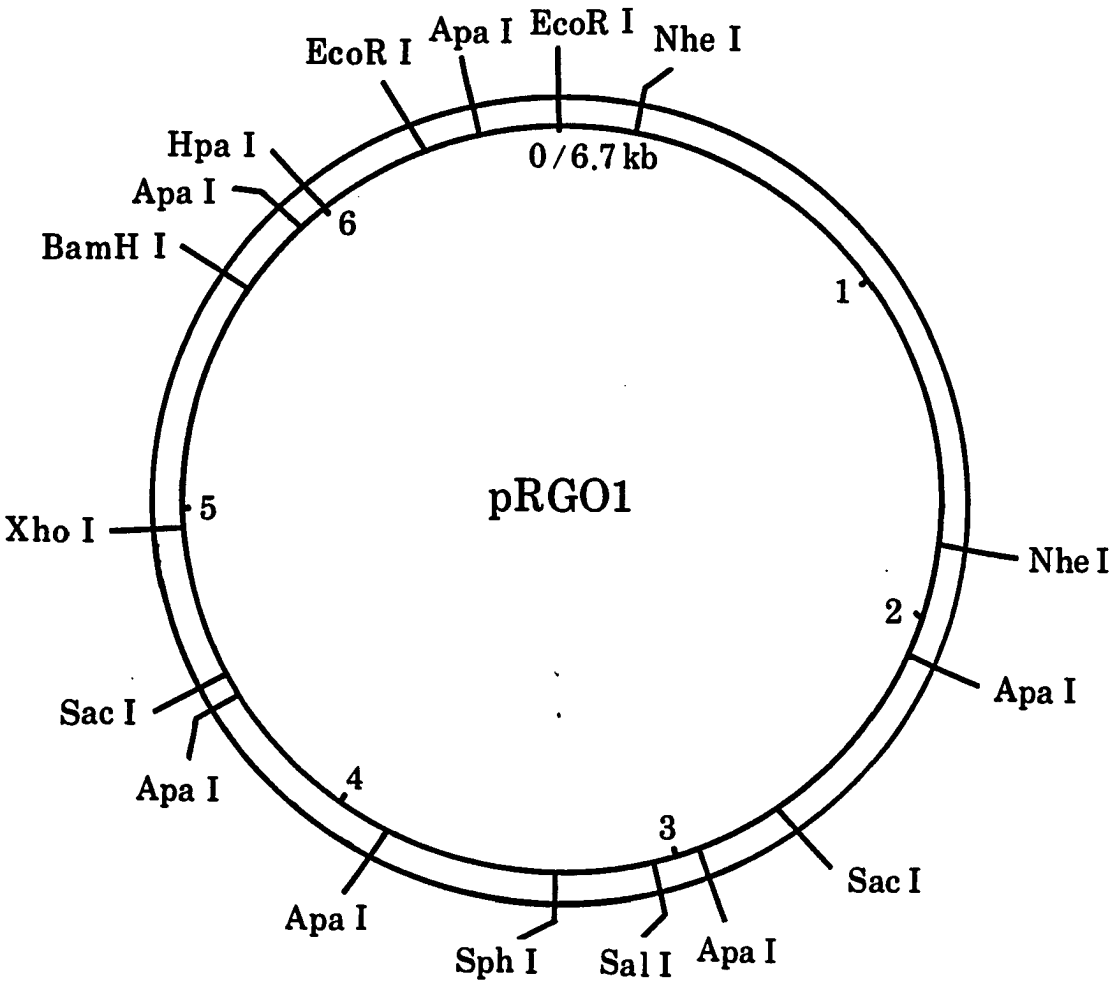


Figure 1. Restriction endonuclease cleavage map of plasmid pRG01

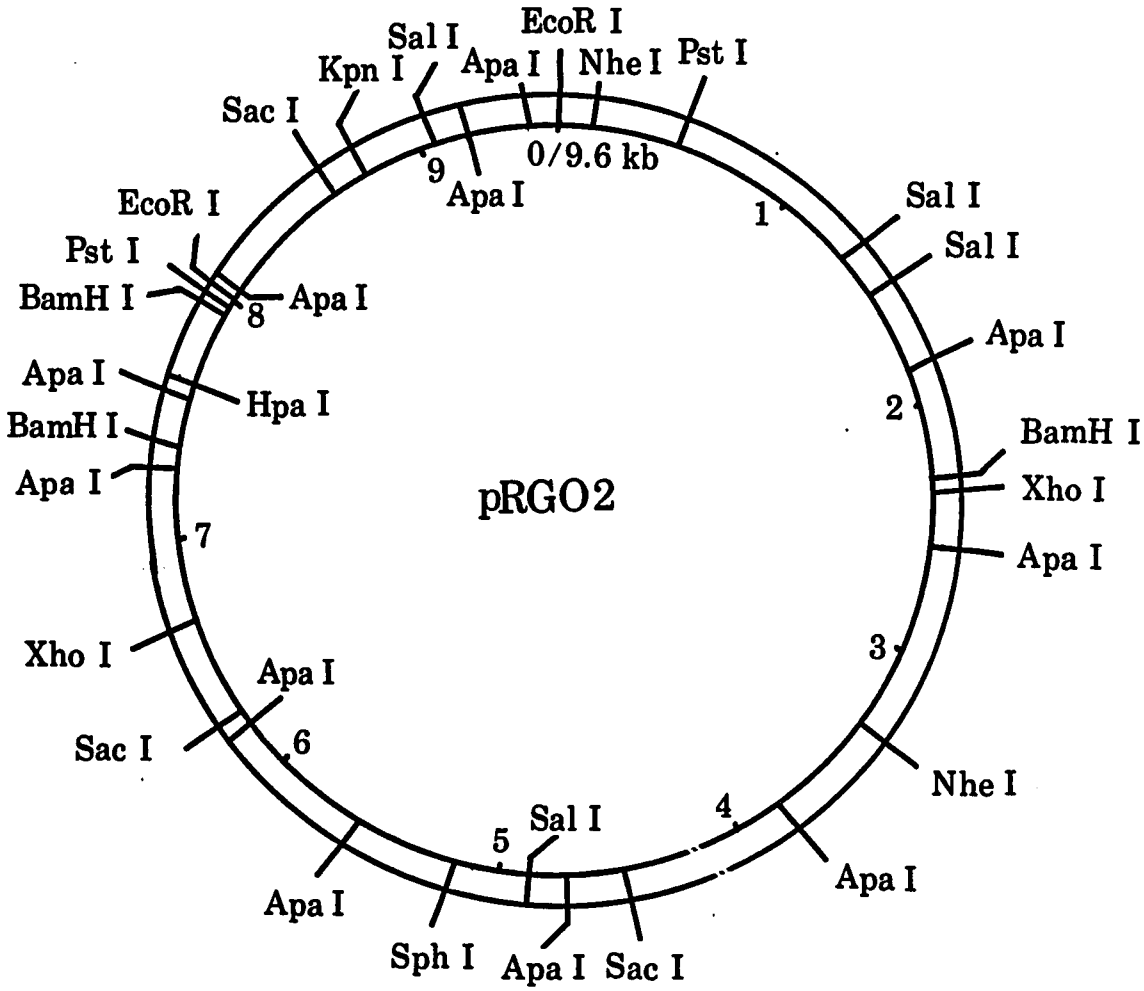


Figure 2. Restriction endonuclease cleavage map of plasmid pRG02

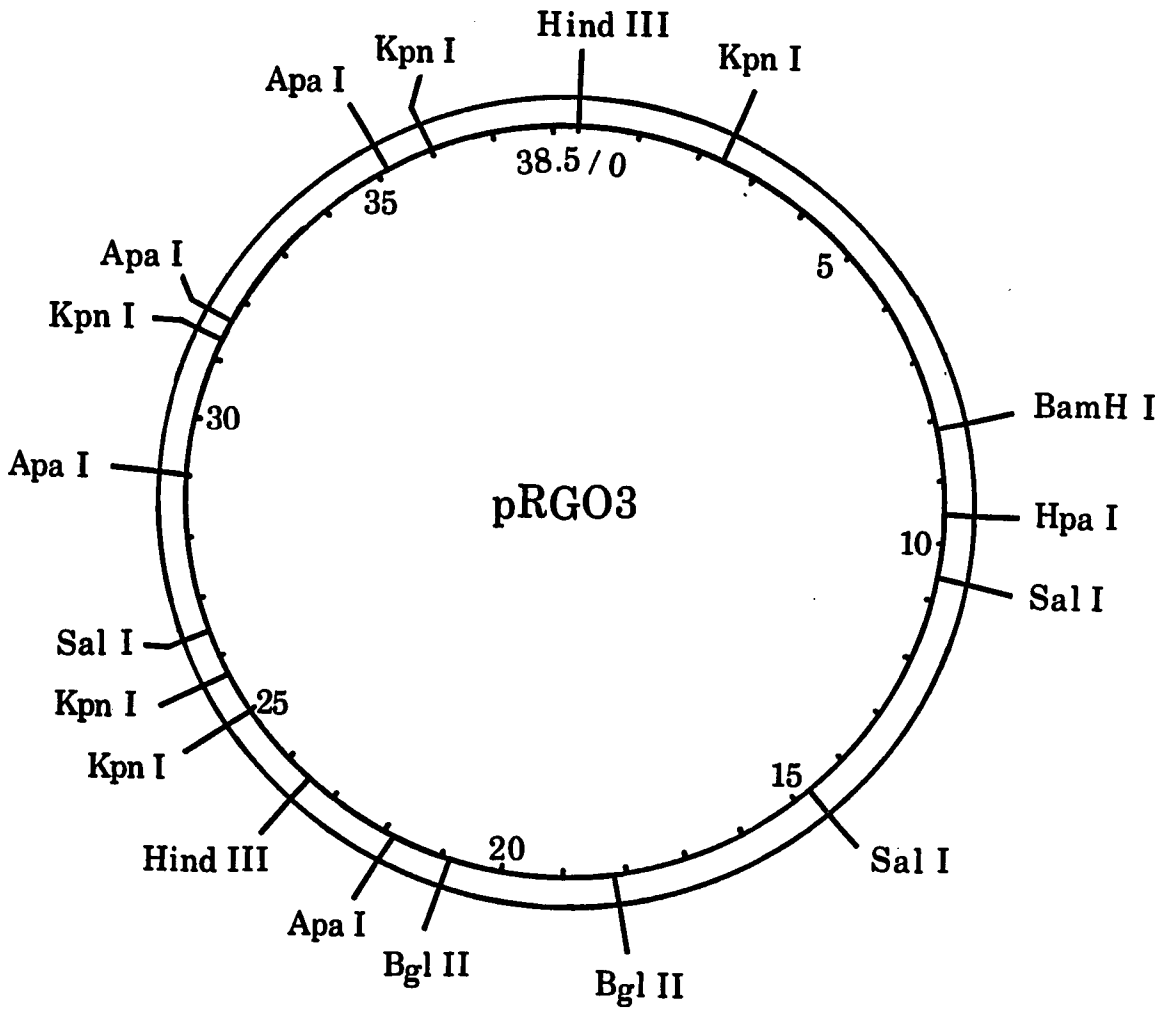


Figure 3. Restriction endonuclease cleavage map of plasmid pRG03

an identical order of enzyme recognition sites and fragment sizes (Apa I-Sac I-Apa I-Sal I-Sph I-Apa I-Apa I-Sac I-Xho I) conserved between these two plasmids. The order of recognition sites on plasmid pRG03 was not similar to those of plasmids pRG01 or pRG02. The positions of 10 Pvu II sites and 16 Sac I sites are not indicated on the restriction map of pRG03. Most of the Pvu II and Sac I sites were clustered in areas where they were uninterrupted by other enzyme recognition sites. Therefore, these sites could not be unambiguously placed on the restriction map with the techniques used here.

Complete restriction endonuclease mapping of plasmid pRG04 by single and multiple digestions was unsuccessful because only one enzyme had less than seven recognition sites. Therefore, to complete the restriction map of pRG04, EcoR I and Pst I fragments were ligated into their respective sites in the plasmid vector pUC19 and transformed into competent E. coli cells. Recombinants that contained 6 of the 10 Pst I fragments and both EcoR I fragments of pRG03 were identified. Restriction maps of these recombinant plasmids were constructed and used with results from single and double digestions of the whole plasmid to assemble a partial restriction map of plasmid pRG04 (Figure 4). Four EcoR I sites and three Pst I could not be placed unambiguously on the restriction map. Comparison of the

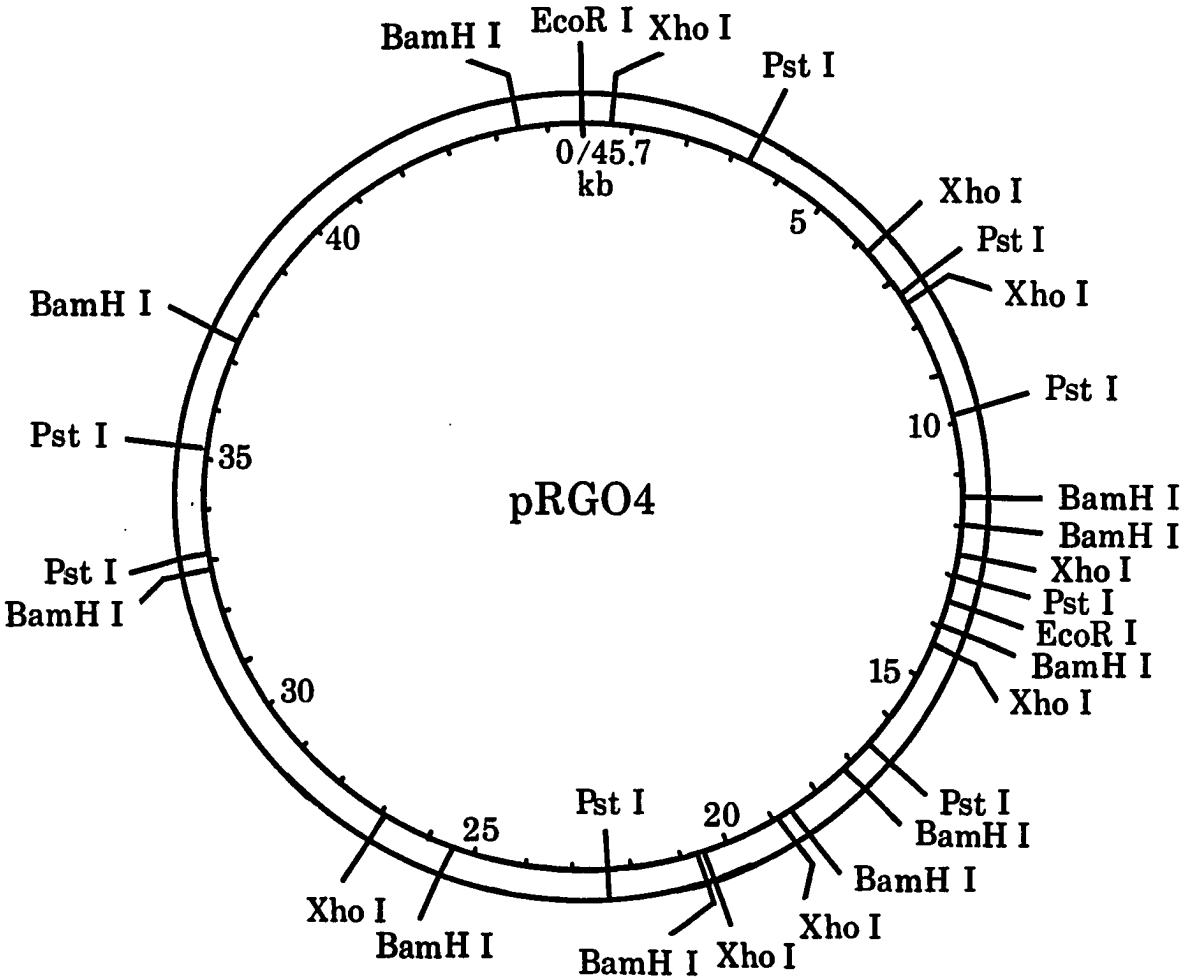


Figure 4. Restriction endonuclease cleavage map of plasmid pRG04

restriction map of pRG04 to maps of pRG01, pRG02 and pRG03 did not identify any regions where the order of the enzyme cleavage sites or fragment sizes was conserved.

DNA-DNA Hybridizations

To examine further the relationship among the Propionibacterium plasmids, total plasmid DNA was isolated from a representative group of strains and digested with restriction enzymes. Digestion with Sac I of plasmid pRG01 from strains P5, P58 and P108 produced two fragments of 4.95 and 1.8 kb (Figures 5-8 I, lanes A-C). Digestion with Pst I of plasmid pRG02 from strains P54 and P63 produced two fragments of 7.42 and 2.18 kb (Figures 5-8 I, lanes D and E). Digestion with Kpn I of total plasmid DNA from strain P93 produced six fragments: five fragments from pRG03 (23.5, 6.0, 4.7, 3.52, and 0.78 kb; a 6.78 kb partial digestion fragment was also visible in many of the gels) and a single 9.6 kb fragment from pRG07 (undigested forms of this plasmid were also visible in many of the gels) (Figures 5-8 I, lane G). Digestion with Xho I of total plasmid DNA from strains P103 and P113 produced 10 fragments: eight from pRG04 (20.0, 6.4, 5.6, 5.15, 4.3, 1.85, 1.6 and 1.1 kb) and two from pRG06 (4.65 and 1.15 kb) (Figures 5-8 I, lanes H and I). Digestion with BamH I of total plasmid DNA from strain P38 produced 15

Figure 5. Hybridization of biotin-labeled pRG01 DNA to restriction fragments of plasmids isolated from Propionibacterium strains. Panel I, agarose gel electrophoresis of restriction digests of plasmids from Propionibacterium strains. Panel II, corresponding hybridization of the pRG01 probe to a nitrocellulose filter containing restriction fragments of plasmid DNA shown in panel I. Lanes A-C, Sac I digest of plasmid pRG01 isolated from strains P5, P58, and P108 respectively; lanes D and E, Pst I digest of plasmid pRG02 isolated from strains P54 and P63 respectively; lane F, Hind III digest of biotin-labeled lambda DNA; lane G, Kpn I digest of plasmids pRG03 and pRG07 isolated from strain P93; lanes H and I, Xho I digest of plasmids pRG04 and pRG06 isolated from strains P103 and P113 respectively; lane J, BamH I digest of plasmids pRG01 and pRG05 isolated from strain P38; lane K, Pst I digest of lambda DNA

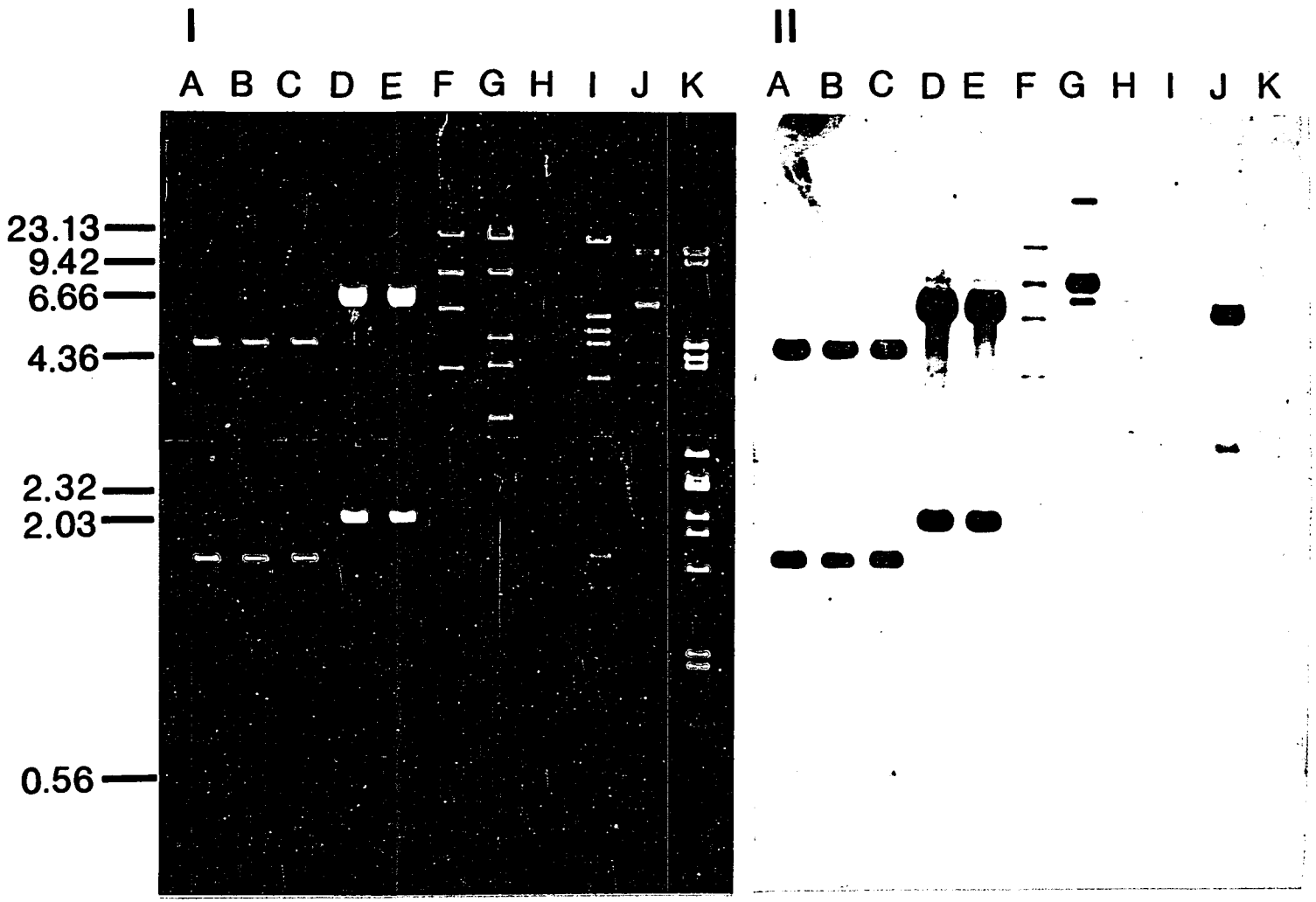

I
A B C D E F G H I J K
23.13
9.42
6.66
4.36
2.32
2.03
0.56
II
A B C D E F G H I J K

Figure 6. Hybridization of biotin-labeled pRG02 DNA to restriction fragments of plasmids isolated from Propionibacterium strains. Panel I, agarose gel electrophoresis of restriction digests of plasmids from Propionibacterium strains. Panel II, corresponding hybridization of the pRG02 probe to a nitrocellulose filter containing restriction fragments of plasmid DNA shown in panel I. Lanes A-C, Sac I digest of plasmid pRG01 isolated from strains P5, P58, and P108 respectively; lanes D and E, Pst I digest of plasmid pRG02 isolated from strains P54 and P63 respectively; lane F, Hind III digest of biotin-labeled lambda DNA; lane G, Kpn I digest of plasmids pRG03 and pRG07 isolated from strain P93; lanes H and I, Xho I digest of plasmids pRG04 and pRG06 isolated from strains P103 and P113 respectively; lane J, BamH I digest of plasmids pRG01 and pRG05 isolated from strain P38; lane K, Pst I digest of lambda DNA

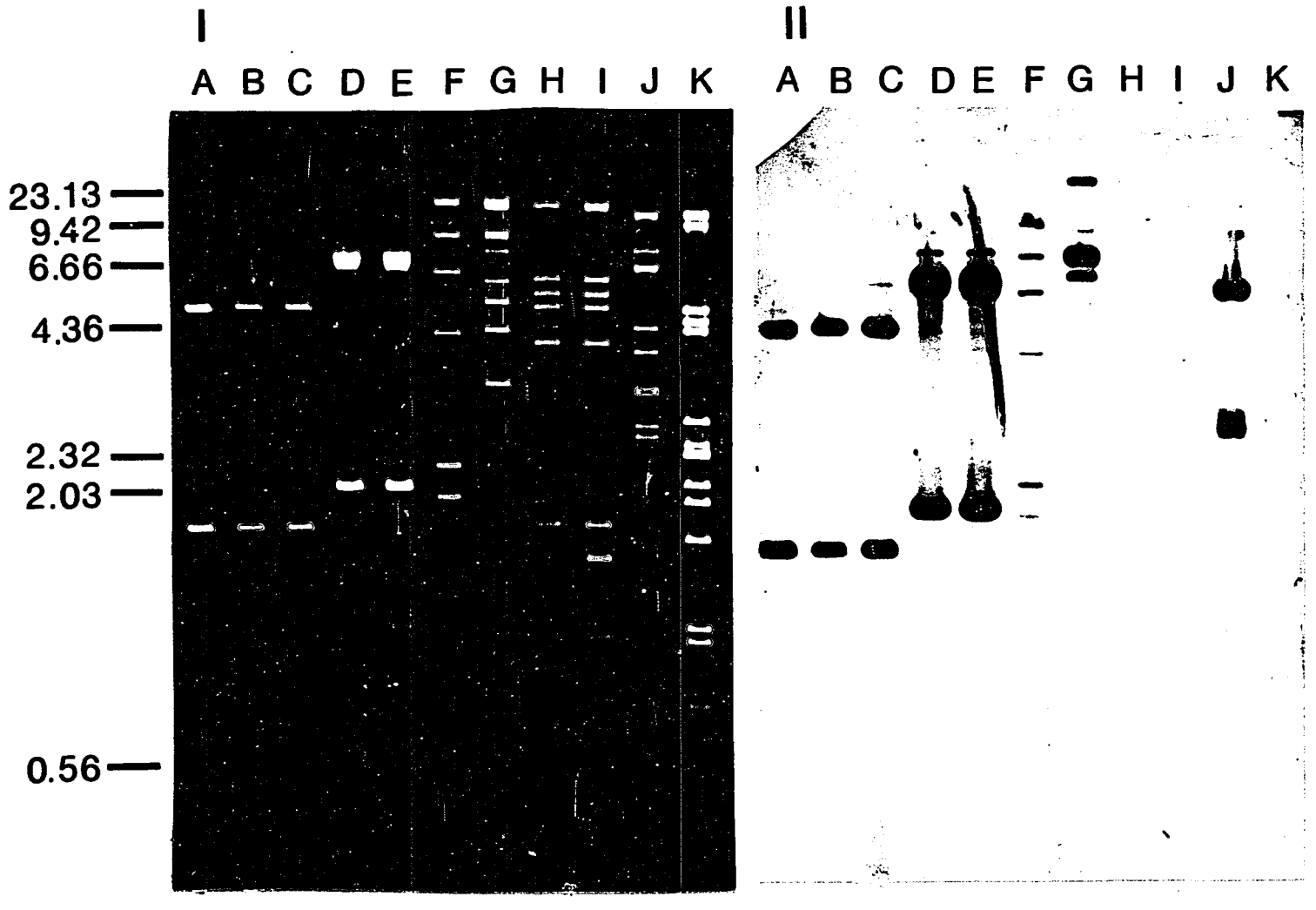

I
A B C D E F G H I J K
23.13
9.42
6.66
4.36
2.32
2.03
0.56
II
A B C D E F G H I J K

Figure 7. Hybridization of biotin-labeled pRG03 DNA to restriction fragments of plasmids isolated from Propionibacterium strains. Panel I, agarose gel electrophoresis of restriction digests of plasmids from Propionibacterium strains. Panel II, corresponding hybridization of the pRG03 probe to a nitrocellulose filter containing restriction fragments of plasmid DNA shown in panel I. Lanes A-C, Sac I digest of plasmid pRG01 isolated from strains P5, P58, and P108 respectively; lanes D and E, Pst I digest of plasmid pRG02 isolated from strains P54 and P63 respectively; lane F, Hind III digest of biotin-labeled lambda DNA; lane G, Kpn I digest of plasmids pRG03 and pRG07 isolated from strain P93; lanes H and I, Xho I digest of plasmids pRG04 and pRG06 isolated from strains P103 and P113 respectively; lane J, BamH I digest of plasmids pRG01 and pRG05 isolated from strain P38; lane K, Pst I digest of lambda DNA

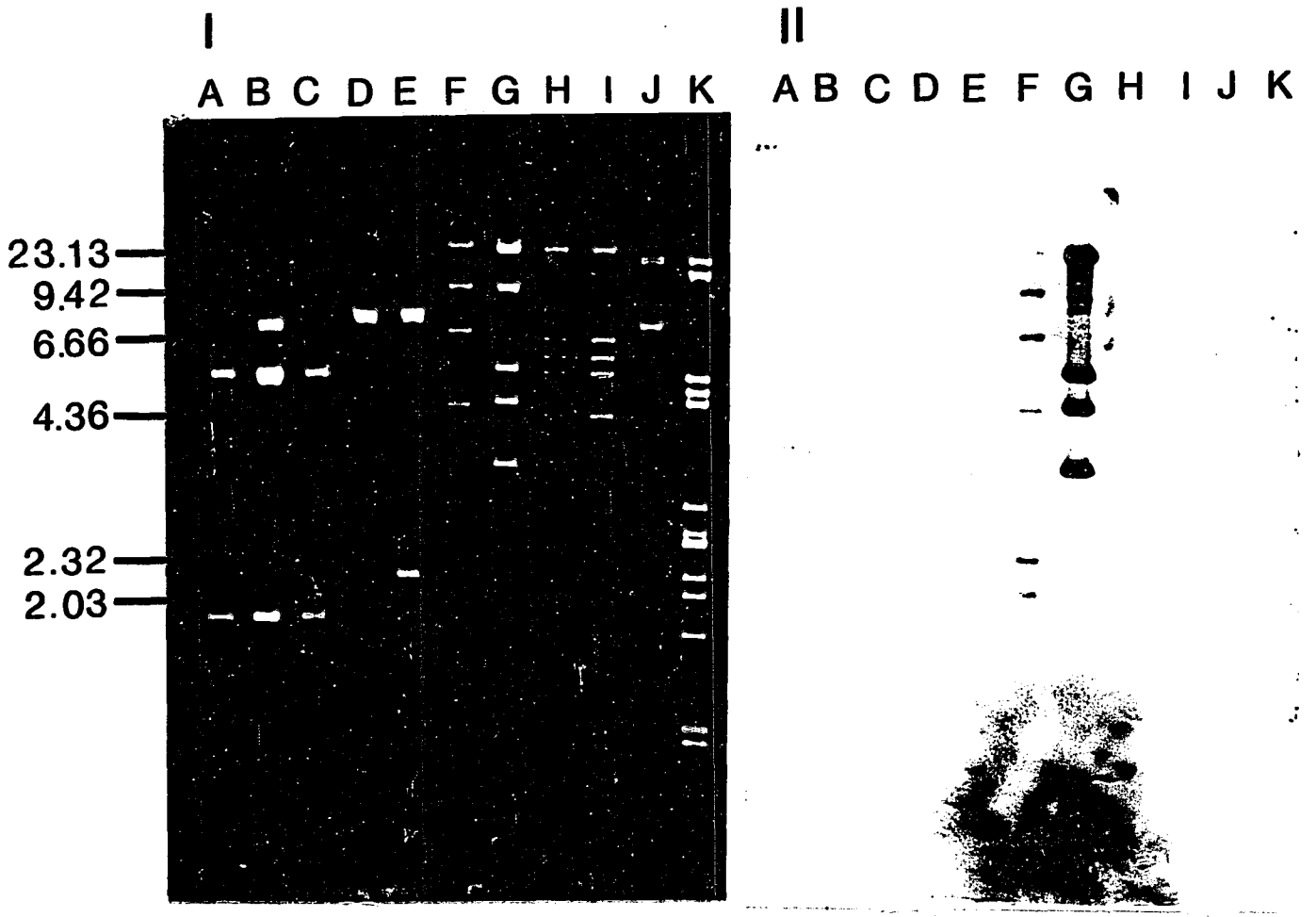
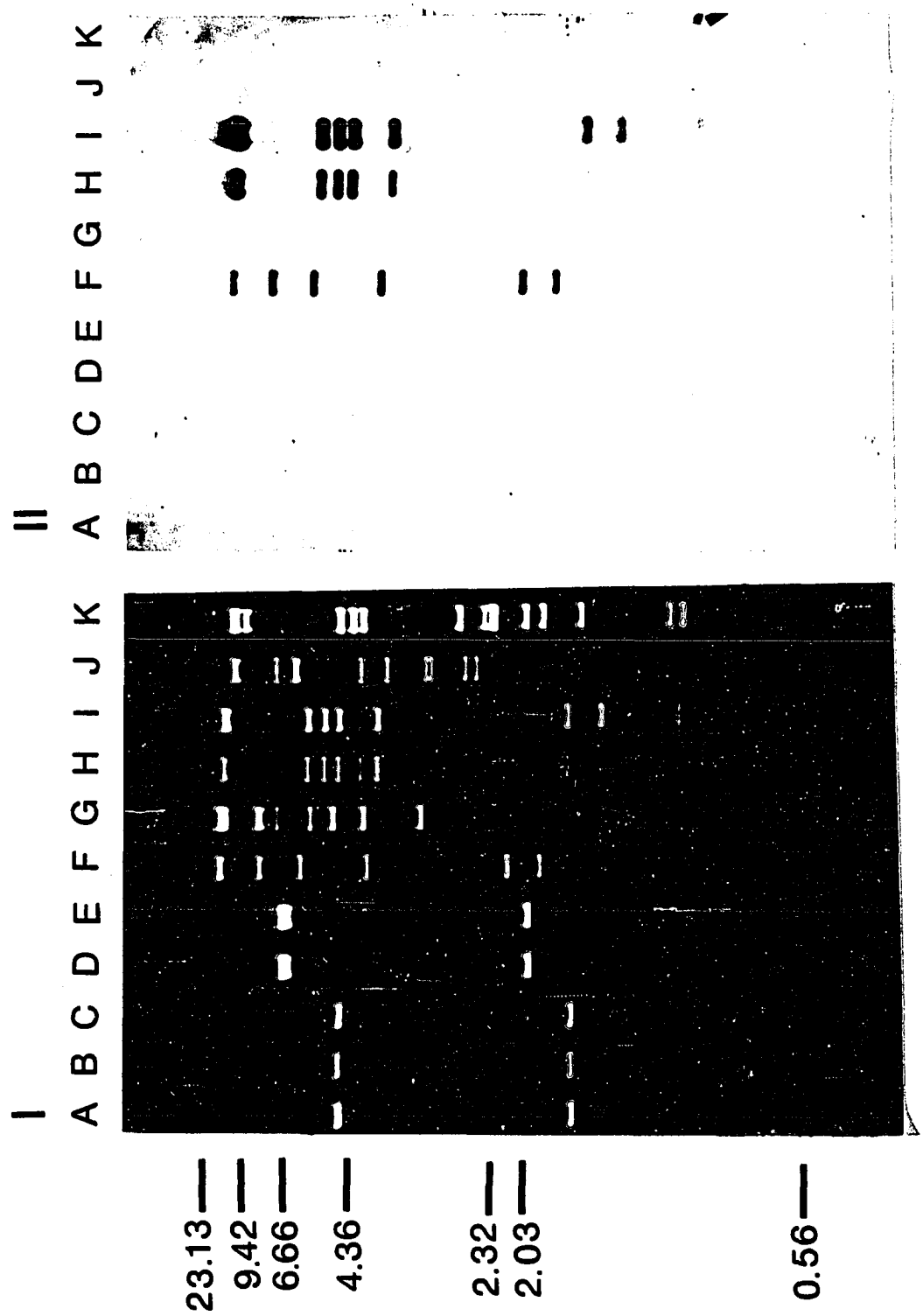

I
A B C D E F G H I J K
23.13
9.42
6.66
4.36
2.32
2.03
II
A B C D E F G H I J K

Figure 8. Hybridization of biotin-labeled pRG04 DNA to restriction fragments of plasmids isolated from Propionibacterium strains. Panel I, agarose gel electrophoresis of restriction digests of plasmids from Propionibacterium strains. Panel II, corresponding hybridization of the pRG04 probe to a nitrocellulose filter containing restriction fragments of plasmid DNA shown in panel I. Lanes A-C, Sac I digest of plasmid pRG01 isolated from strains P5, P58, and P108 respectively; lanes D and E, Pst I digest of plasmid pRG02 isolated from strains P54 and P63 respectively; lane F, Hind III digest of biotin-labeled lambda DNA; lane G, Kpn I digest of plasmids pRG03 and pRG07 isolated from strain P93; lanes H and I, Xho I digest of plasmids pRG04 and pRG06 isolated from strains P103 and P113 respectively; lane J, BamH I digest of plasmids pRG01 and pRG05 isolated from strain P38; lane K, Pst I digest of lambda DNA


II
A B C D E F G H I J K
I
A B C D E F G H I J K
23.13
9.42
6.66
4.36
2.32
2.03
0.56

fragments: 14 from pRG05 (14.7, 14.5, 7.5, 4.5, 3.8, 3.15, 3.1, 2.75, 2.55, 1.61, 1.48, 0.98, 0.75 and 0.72 kb; the two smallest fragments are only partially visible on many of the gels) and one 6.75 kb fragment from pRG01 (Figures 5-8 I, lane J).

After separation by electrophoresis, fragments were transferred by capillary blotting to a nitrocellulose membrane filter and hybridized to biotin-labeled plasmid DNA probes prepared from plasmids pRG01, pRG02, pRG03 and pRG04. The results of the hybridizations are shown in Figures 5-8.

The probe prepared from plasmid pRG01 (Figure 5 II) strongly hybridized to the positive controls: both Sac I fragments of pRG01 from strains P5, P58 and P108 (lanes A-C) and the single Bam HI fragment of pRG01 from strain P38 (lane J). The probe also strongly hybridized to both Pst I fragments of plasmid pRG02 from strains P54 and P63 (lanes D and E) and the single Kpn I fragment of plasmid pRG07 from strain P93 (lane G). The other hybridization signals detected in lane G correspond to undigested forms of pRG07. Weak signals detected in lanes A through E correspond to partially digested forms of plasmids pRG01 and pRG02. Weaker hybridization occurred to the 3.15 and 3.1 kb Xho I fragments of pRG05 (lane J). Very faint hybridization was observed to various fragments of pRG04 (lanes H and I), pRG03 (lane G) and pRG05 (lane J).

The hybridization results with the pRG02 probe are shown in Figure 6 II. The probe strongly hybridized to the Pst I-digested pRG02 positive control fragments (lanes D and E), and to all fragments of pRG01 (lanes A-C and J). The weaker hybridization signals detected in lanes C, D and E correspond to partially digested forms of the resident plasmids. Strong hybridization also occurred to the Kpn I fragment and undigested forms of pRG07 (lane G), as well as to the 3.15 and 3.1 kb Xho I fragments of pRG05 from strain P38 (lane J). Very faint hybridization was detected to the Kpn I fragments of pRG03 (lane G). No hybridization was seen to any fragments of pRG04 from strains P103 and P113 (lanes H and I).

Hybridization results with plasmid pRG03 as probe DNA are shown in Figure 7 II. Strong hybridization was detected to the Kpn I-digested pRG03 positive control fragments (lane G). Weak hybridization was detected to the single 9.6 kb Kpn I fragment of pRG07 (lane G). No hybridization was detected to any other plasmid fragments.

Hybridization results with the pRG04 probe are shown in Figure 8 II. Strong hybridization was detected to the Xho I positive control fragments of pRG04 (lanes H and I). Very weak hybridization was detected to the 4.65 kb Xho I fragment of pRG06 (lanes H and I), and to various fragments of pRG03 and pRG05 (lanes G and J respectively).

Plasmid Curing

Curing studies were initiated to determine the functional properties of the Propionibacterium plasmids. Treatment of strains P5, P38, P54 and P63 with acriflavin produced plasmid-cured derivatives. When strains P54 and P63 were grown in the presence of 20 ug/ml acriflavin, 20% of the colonies screened were cured of plasmid pRG02. When strain P5 was grown in the presence of 15 ug/ml acriflavin, 15% of the colonies screened were cured of plasmid pRG01. After growth of strain P38 with 25 ug/ml acriflavin, 80% of the colonies screened were cured of both plasmids pRG01 and pRG05. Derivatives of strain P38 that had been cured of only one of these plasmids were not obtained. Growth with acridine orange or ethidium bromide produced cured derivatives only for strains P5 and P38, and only at a frequency of 1-2%.

To determine the phenotypic roles of plasmid pRG01, pRG02 and pRG05, cured derivatives and their parent strains were screened for bacteriocin production, carbohydrate fermentations, antibiotic resistances and clumping ability. No inhibition of the indicator cultures by strains P5, P38, P54, P63, or by their cured derivatives was detected in the bacteriocin production assay. The fermentation patterns for 21 carbohydrates did not differ between parent strains and

their cured derivatives. No detectable differences in the MIC of 21 antibiotics were observed between parent strains and their plasmid-cured derivatives. Therefore, no evidence was found to indicate plasmid association for bacteriocin production, carbohydrate fermentation, or antibiotic resistance in these four strains.

Strain P38 was observed to form cell aggregates or clumps when inoculated into fresh broth. Cured derivatives of strain P38 did not exhibit the clumping phenomenon. These results suggested that the clumping phenotype was plasmid-associated in strain P38. However, it could not be specifically linked to plasmid pRG01 or pRG05 because all cured derivatives were missing both plasmids. The fact that none of the 10 other strains that contain plasmid pRG01 exhibit clumping suggests that this phenotype is associated with plasmid pRG05.

Clumping induction assays were conducted to determine if the clumping phenomenon is mediated by soluble extracellular substances produced by strain P38. Cell-free supernates of strain P38 were added to growing cultures of the cured derivatives of strain P38 and of 10 other nonclumping propionibacteria strains. Clumping was not observed in any of the strains tested.

DISCUSSION

We describe here the use of restriction endonuclease analyses, DNA-DNA hybridizations and curing studies to characterize several Propionibacterium plasmids. Seven distinct plasmids have been identified to date by restriction endonuclease analysis. At least four additional unique plasmids have been isolated: a 5.0 Mdal plasmid in strain P114; a 22 Mdal plasmid in strain P97; a 12.5 Mdal plasmid in strains P22 and P101; and a plasmid greater than 200 Mdal present in strains P22, P84, P101 (data not shown). To date, these plasmids have not been characterized by restriction analysis, and therefore it is not known whether the plasmids of similar size present in different strains are identical. We will continue to characterize these plasmids to define their relationship to other Propionibacterium plasmids and to determine their functional properties.

Similarities in the distribution of restriction enzyme recognition sites in the Propionibacterium plasmids indicate that although these plasmids are relatively heterogenous in structure, some compositional homogeneity exists. The classical propionibacteria have been reported to have DNA base compositions in the range of 65 to 68% guanine + cytosine (GC)(17). Plasmids from propionibacteria also appear to have a high GC content. Restriction enzymes whose

recognition sites were composed largely of nonalternating guanine and cytosine residues cut the plasmids much more frequently than did enzymes with a low GC content in their recognition sites. This fact should help to predict the activity of other restriction enzymes in future studies.

Comparison of restriction maps allows the identification of conserved regions between plasmids pRG01 and pRG02. These results provide useful information about the molecular relationship among Propionibacterium plasmids and will assist in the construction of future Propionibacterium cloning vectors.

The relationship among Propionibacterium plasmids was further examined by DNA-DNA hybridizations. Extensive sequence homology exists among some of the plasmids. Plasmids pRG01 and pRG02 share extensive sequence homology and are both homologous to pRG07 and to the same fragments of pRG05. The relationship between the regions in pRG05 and pRG07 that are homologous to pRG01 and pRG02 is currently unknown. Plasmids pRG04 and pRG06 do not share significant sequence homology with any other of the known Propionibacterium plasmids. Plasmid pRG03 shares partial sequence homology only with pRG07, which is carried by the same strain. These hybridization data are useful in defining plasmid homology groups and in identifying possible plasmid incompatibilities that could interfere in future gene

transfer experiments (33).

Cured derivatives of plasmid-carrying strains were isolated to begin to characterize the functional properties of Propionibacterium plasmids. No traits associated with bacteriocin production, carbohydrate fermentation, or antibiotic resistance were linked to plasmids pRG01, pRG02 or pRG05. However, physical evidence was obtained to link the clumping phenotype in strain P38 to plasmid DNA, probably to plasmid pRG05.

Plasmid-associated cell aggregation or clumping has been well documented in Streptococcus faecalis and Streptococcus lactis (1,7). In both cases this phenomenon was observed to play a role in their native conjugal DNA transfer systems, even though the systems are quite different. Experiments with S. faecalis identified a sex pheromone produced by the recipient which induced certain donor strains to aggregate. Donor cells contained the genes responsible for aggregation which were expressed only after induction by the proper recipient's sex pheromone (8). The S. lactis clumping phenomenon is not pheromone-dependent and has been observed in some transconjugants from conjugal matings with strain ML3 (1). This strain contains the genes for clumping on a low copy number plasmid. Transconjugants that clump contain the genes on a cointegrate replicon that is maintained at a higher copy number (1).

The clumping phenotype in Propionibacterium strain P38 appears to be a different phenomenon. Strain P38 clumps without induction or gene transfer from another strain. The clumping phenomenon does not appear to be dependent on soluble extracellular substances. Work is in progress to determine if this plasmid contains the genes for conjugal DNA transfer or if the clumping phenomenon may play a role in a yet undiscovered native conjugal DNA transfer system.

ACKNOWLEDGMENTS

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

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MANUSCRIPT III.

PLASMID-ASSOCIATED LACTOSE FERMENTATION IN *Propionibacterium freudenreichii* STRAIN P93

Plasmid-associated lactose fermentation in *Propionibacterium freudenreichii* strain P93

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ABSTRACT

Propionibacterium freudenreichii P93 was previously found to contain two plasmids, pRG07 and pRG03, of 6.3 and 25 megadaltons (Mdal), respectively. Physical evidence was obtained linking lactose fermentation and the ability of the cells to pellet after centrifugation to plasmid DNA in this strain. Cured derivative strains produced by acriflavin treatment, which had lost the ability to ferment lactose and did not form pellets after centrifugation, were found to be cured of plasmid pRG03 or of both resident plasmids. The parental strain and strains which were cured of plasmid pRG07 only, fermented lactose and formed a pellet after centrifugation. Thus, lactose fermentation and the pelleting phenotype appear to be associated with plasmid pRG03. Evidence from Beta-galactosidase assays of whole cells, toluene-acetone-treated cells, and cell-free extracts of strain P93 indicated that plasmid pRG03 codes for a constitutive Beta-galactosidase enzyme. Maximum enzyme activity was observed in late-log cells assayed at 50°C in pH 7.5 buffer. An *E. coli lacZ'* probe did not hybridize to restriction digests of plasmid pRG03.

INTRODUCTION

The propionibacteria are a diverse group of microorganisms capable of producing large amounts of propionic acid from a wide variety of carbohydrates. The most common industrial application of the classical propionibacteria is their use as dairy starter cultures in the manufacture of Swiss-type cheeses (15, 16, 17). Other applications of the propionibacteria have been described including their use in fermented dairy-based drinks (23), as a probiotic (22) and in the industrial production of vitamin B_{12} (10, 28) and propionic acid (29).

Recently, studies in our laboratory have examined the distribution of plasmids in the dairy propionibacteria (31). To date, 20 of 119 strains screened have been found to contain plasmids. Initial characterization of the resident plasmids by using restriction endonucleases has identified 11 strains that contain a 6.7 kb plasmid, pRG01; two strains that contain a 9.6 kb plasmid, pRG02; and two strains that contain both a 46 kb plasmid, pRG04, and a 8.6 kb plasmid, pRG06. Three plasmids were carried by single strains: pRG07 (9.6 kb) and pRG03 (38.5 kb) in strain P93, and pRG05 (54 kb) in strain P38.

Hybridization studies have revealed that plasmids pRG01 and pRG02 share extensive regions of homology and both are

homologous to two other plasmids, pRG05 and pRG07 (31). Plasmids pRG03, pRG04 and pRG06 do not share significant regions of homology with any other of the known Propionibacterium plasmids. Plasmid curing studies have provided preliminary physical evidence linking the clumping phenotype of P. jensenii strain P38 to a 55 kb plasmid, pRG05. The role the remaining plasmids may play in the metabolic processes of their host strains has yet to be established.

Plasmid-associated lactose utilization has been well documented in the lactic acid bacteria (2, 4, 11, 14, 25, 26, 32). In this report we present physical evidence linking lactose utilization and a cell pelleting phenomenon to plasmid DNA in P. freudenreichii strain P93. In addition, we have characterized the plasmid-encoded lactose hydrolyzing enzyme system.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All cultures were obtained from the Department of Food Technology culture collection. Propionibacteria were routinely propagated in sodium lactate broth (NLB) or on sodium lactate agar (NLA) at 32°C (13). Escherichia coli was propagated in Luria broth at 37°C (21). Table 1 lists the bacterial strains used in this study.

Plasmid DNA Isolation

Plasmid DNA was isolated from Propionibacterium strains by using the microscale screening procedure or preparative scale isolation method previously described (30). Escherichia coli plasmid DNA was isolated by the method of Birnboim and Doly (3) or the cleared lysate method of Clewell and Helinski (7) and purified by cesium chloride-ethidium bromide density gradient centrifugation (21).

Table 1. Bacterial strains used in the current study

ISU Number	Species and Phenotype	Plasmid content Size (Mdal)	Name
	P. freudenreichii		
P93	Lac^+, Pel^+[a]	6.3, 25	pRG07, pRG03
P39308	Lac^+, Pel^-	6.3	pRG07
P39310	Lac^-, Pel^+	25	pRG03
P39317	Lac^-, Pel^-	none	
P39337	Lac^-, Pel^-	none	
P39341	Lac^-, Pel^-	none	
	Escherichia coli JM109		
E50	Lac^+	1.86	pUC19

[a]Lac=ability to ferment lactose to produce acid. Pel=ability to form a firm pellet after centrifugation of a culture.

Restriction Endonuclease Digests and Agarose Gel Electrophoresis

Restriction endonuclease digestions were performed according to the manufacturer's directions (Boehringer Mannheim Inc., Indianapolis, IN) and to methods described by Maniatis et al. (21). Restriction fragments and plasmid DNA samples were subjected to electrophoresis in horizontal gels of 0.7 to 0.8% agarose dissolved in Tris-borate buffer at pH 8.1 (27). Electrophoresis was carried out at 55-70 volts for 12-14 h. 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 filters with a Polaroid MP4 camera (film type 55).

Preparative agarose gels (0.8%) were loaded with 2.0 ml of digested plasmid DNA. Electrophoresis was carried out as described above. The DNA fragment of interest was located in the stained gel, cut out, and electroeluted from the agarose slice in an Elutrap chamber according to the manufacturer's instructions (Schleicher and Schuell, Inc., Keene, NH).

Plasmid Curing and Carbohydrate Screening

Propionibacterium freudenreichii strain P93 was grown to mid-log phase and transferred (1%) to NLB that contained 5 to 50 µg/ml acriflavin, 10 to 100 µg/ml acridine orange or 1 to 25 µg/ml ethidium bromide for curing trials. After incubation for 48 h, 0.2 ml of the culture that contained the highest concentration of each curing agent that allowed growth was transferred to 10 ml of fresh broth supplemented with the appropriate concentration of the curing agent, and incubated again. After at least seven successive transfers, cultures were diluted and plated onto NLA. Individual colonies were transferred to NLB, grown to mid-log phase and transferred (1%) to fresh NLB for plasmid DNA screening. Cured derivatives were identified by comparing the plasmid DNA profile of the parent strain to plasmid profiles of cultures exposed to the curing agents.

Strain P93 and the isolated cured derivatives were replicated onto bromocresol purple basal plates (1% trypticase, 1% yeast extract, 1.5% agar, 0.025% K_2HPO_4, 0.02% bromocresol purple, pH 7.2) (20) supplemented with the following carbohydrates at 1% final concentration: adonitol, arabinose, cellobiose, erythritol, fructose, galactose, glucose, glycerol, lactate, lactose, maltose, mannitol, mannose, raffinose, rhamnose, ribose, sorbitol, starch,

sucrose, trehalose, and xylose. Plates were incubated at 32°C for one week and examined for acid production as evidenced by a color change from purple to yellow.

Cell-Free Extracts

Cells from 4 l of a 25 h culture were harvested by centrifugation (10,000 x g, 10 min), washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and resuspended to a final volume of 40 ml. The cell suspension was disrupted by passing it through a chilled French pressure cell maintained at 18,000 lb/in^2. Three passes were necessary to obtain adequate cell disruption. Cell debris was removed from the disrupted cell suspension by centrifugation at 27,000 x g for 20 min. The resulting cell-free extract was used for enzyme assays. Protein concentrations were determined by the Lowry procedure with a protein assay kit (Sigma Chemical Co., St. Louis, MO) for cell-free extracts. Dry cell weights were converted to protein concentrations by using the conversion factor of 55% protein per dry cell weight (1).

Enzyme Assays

Cultures for enzyme assays were grown in basal broth (1% trypticase, 1% yeast extract and 0.25% K_2HPO_4, pH 7.2)

supplemented with 1% sodium lactate. Cultures were incubated for 24 to 28 h at 32°C, harvested by centrifugation, washed twice and resuspended to one-tenth the original volume in 50 mM sodium phosphate buffer, pH 7.5. Toluene-acetone-treated cells were prepared as described by Citti et al. (6). All assays were performed in duplicate on two independent cultures.

Beta-galactosidase (B-gal) activity was measured by determining hydrolysis of the chromogenic substrate o-nitrophenyl-B-D-galactopyranoside (ONPG) (19). The assay mixture contained 0.4 ml of whole cells, toluene-acetone-treated cells or cell-free extracts and 1.6 ml of 5 mM ONPG in 50 mM sodium phosphate, 0.4 mM $MnCl_2$, pH 7.5. The mixture was incubated at 50°C for 15 min during which time the rate of hydrolysis of ONPG remained linear. The reaction was stopped by the addition of 2.0 ml cold 0.5 M sodium carbonate. Cells from two 1.5 ml portions of each assay tube were removed by centrifugation for 3 min in a microcentrifuge. The absorbance of the supernatant fraction was measured at 420 nm and converted to a molar concentration of o-nitrophenyl by using a standard curve for o-nitrophenyl. Dry weights were determined from a standard curve for each culture that related the optical density at 600 nm of the culture suspension to the culture dry weight. Enzyme activity was defined as nmoles of o-nitrophenyl liberated

from ONPG per mg of cell dry weight (or per mg of protein) per minute.

Phospho-B-galactosidase (P-B-gal) activity was measured by following the hydrolysis of o-nitrophenyl-B-D-galactopyranoside 6-phosphate (ONPG-6-P). The assay mixture contained 125 µl of whole cells, toluene-acetone-treated cells or cell-free extracts and 500 µl of 10 mM ONPG-6-P in 50 mM sodium phosphate, 0.4 mM $MnCl_2$, pH 7.5. The mixture was incubated at 50°C for 15 min at which time 625 µl of cold 0.5 M sodium carbonate were added to stop the reaction. All other procedures were identical to those of the B-galactosidase assay. Specific enzyme activity was defined as the number of nmoles of o-nitrophenyl liberated from ONPG-6-P per mg of cell dry weight (or per mg of protein) per minute.

Induction Experiments

Cultures to be used for B-gal induction experiments were grown to mid-log phase in basal broth supplemented with 1% sodium lactate. Two-ml portions of these cultures were used to inoculate flasks containing 200 ml of basal broth supplemented with potential inducing agents and carbohydrates at the desired concentrations. Cultures were incubated at 32°C for 28 h, harvested by centrifugation, and assayed for B-gal activity.

DNA-DNA Hybridizations

Purified pUC19 plasmid DNA was digested with Hae II and the fragments were separated on a preparative agarose gel. The 445-bp fragment containing the lacZ' gene was excised, purified and self-ligated prior to nick translation. The lacZ' probe was biotin-labeled with a nick translation kit and biotin-11-dUTP as described by the manufacturer (Bethesda Research Labs Inc. (BRL), Gathersburg, MD). After nick translation, unincorporated biotin-11-dUTP was removed by using a Centricon-30 microconcentrator according to the manufacturer's instructions (Amicon Inc., Danvers, MA).

Restriction fragments of the lactose plasmid, pRG03, were transferred to nitrocellulose sheets (Transblot nitrocellulose, Bio Rad Laboratories, Richmond, CA) by using the Southern blotting technique (33). Prehybridization and hybridization buffers (10ml/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's reagent and 25 mM sodium phosphate (18). Filters were subjected to prehybridization treatment in a sealed bag at 42°C with constant agitation for at least 4 h. The biotinylated probe DNA was denatured at 95°C for 10 min., cooled in an ice bath and added to the hybridization buffer at a concentration of 100-200 ng/ml. The filter and hybridization buffer were

sealed in a bag and incubated at 42°C for 24-36 h. Posthybridization washes, filter blocking, and detection of homologous sequences were performed as described by the manufacturer of the biotinylated DNA detection system (BRL).

Hybridization at low stringency was performed by reducing the formamide concentration in the hybridization buffer to 35% and the temperature of hybridization to 37°C (35). In addition, the temperature of the high temperature posthybridization wash was reduced to 35°C.

RESULTS

Plasmid Linkage of Lactose Utilization

Previous work had demonstrated that P. freudenreichii strain P93 contained two plasmids, pRG03 and pRG07, of molecular mass 25 and 6.3 Mdal respectively (31). Although these plasmids had been characterized by restriction endonuclease mapping and their relationship to other native plasmids had been examined by DNA-DNA hybridization studies, no information was available on their phenotypic role.

To begin to understand the role of plasmids pRG03 and pRG07 in strain P93, derivatives cured of their plasmids were sought. Acriflavin, ethidium bromide and acridine orange were used to treat cultures of P93. Cured derivatives were isolated following treatment only with acriflavin or ethidium bromide. After growth in the presence of 20 ug/ml acriflavin, 15% of the colonies screened for plasmid DNA were identified as cured derivatives. Of these, 50% were cured of both plasmids, 33% were cured of plasmid pRG07 only and 17% were cured of pRG03 only. After growth with 10 ug/ml ethidium bromide, only 5% of the colonies screened were found to be cured of one or both plasmids. Figure 1 shows the results of agarose gel electrophoresis of DNA isolated from representative cured derivatives.

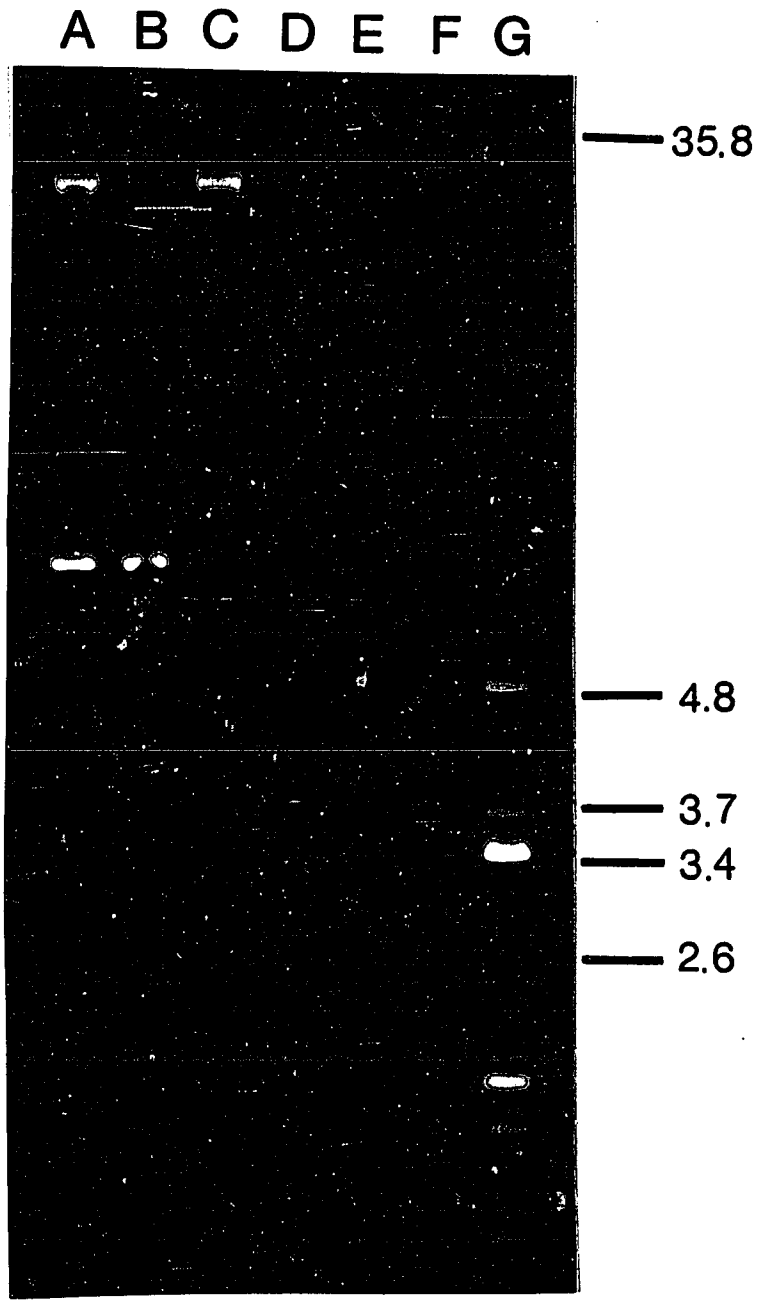


Figure 1. Plasmid DNA profiles of P. freudenreichii P93 and cured derivative strains. Lane A, P93 (25, 6.3 Mdal); lane B, P39308 (6.3 Mdal); lane C, P39310 (25 Mdal); lane D, P39317; lane E, P39337; lane F, P39341; lane G, E. coli V517, molecular mass (Mdal) of plasmids are shown. Positions of the open circular form of the 25 Mdal plasmid (OC) and

The phenotypic properties of the parent strain and of all cured derivatives (12 were tested) were compared in fermentation tests on 21 carbohydrates. The parent strain fermented lactose (lac+) but eight of the cured derivatives did not ferment lactose (lac-). Of these eight two were cured of plasmid pRG03 and six were cured of both plasmid pRG03 and plasmid pRG07. All lac+ cured derivatives contained plasmid pRG03. These results provided physical evidence linking plasmid pRG03 to lactose fermentation in strain P93. No phenotype has been found to be associated with plasmid pRG07.

Plasmid Linkage of Cell Pelleting

Large-scale cultures of cured derivatives P39308, P39310, P39337 and P39341 and of the parent strain were grown to reconfirm their plasmid content and to provide cells for enzyme studies. The cells were harvested from 500-ml cultures by centrifugation at 14,000 x g for 20 min. These were standard centrifugation conditions to produce a solid pellet of cells. However, centrifugation of strains P39308, P39337 and P39341 under these conditions produced an extremely loose pellet which was decanted with the supernate. Pellets of the parent strain and of P39310, which contained plasmid pRG03, appeared normal. Further examination of the

remaining cured derivatives indicated an association between plasmid pRG03 and the firm, coherent pellet phenotype. All derivatives cured of pRG03 produced loose pellets after centrifugation. All derivatives that contained pRG03 produced normal pellets. There was no evidence of clumping in strain P93 or its cured derivatives. The nature of the pelleting phenomenon associated with plasmid pRG03 merits further investigation.

Enzyme Activities

To characterize the lactose hydrolyzing system encoded by plasmid pRG03, beta-galactosidase (B-gal) and phospho-beta-galactosidase (P-B-gal) assays were performed on strain P93 and cured derivative P39310. Untreated and toluene-acetone-treated whole cells were first used to determine the optimal B-gal assay conditions.

Assay temperature was varied between 25 and 70°C. For all samples, enzyme activity increased with increasing temperature to a maximum at 50°C (data not shown). The B-gal activity of toluene-acetone-treated cells was much lower than that of untreated cells for both strains.

When assay pH was varied from pH 5.5 to pH 9.0, maximum enzyme activity of untreated and treated cells of both strains was obtained at pH 7.5. Treated cells again

exhibited significantly lower enzyme activity.

Enzyme activity was assayed at 50°C and pH 7.5 in cultures of P93 and P39310 at all stages of growth from 12 to 72 h. Cells of both strains in the late-log phase (25 h) had the highest B-gal activity.

Late-log phase cultures of all cured derivatives were assayed for B-gal activity under optimal conditions. Derivatives cured of plasmid pRG03 had no detectable B-gal activity, whereas derivatives containing plasmid pRG03 had B-gal activity equal to that of the parent strain.

A reduction in enzyme activity following solvent treatment has been reported to be initial evidence of an alternate pathway of lactose metabolism (24). To investigate if this was also the case with strain P93, cells of strains P93 and P39310 were assayed for P-B-gal activity with the substrate ONPG-6-P. In addition, the effects of phosphoenolpyruvate and sodium fluoride on beta-galactosidase of both strains were examined.

Enzyme activities of untreated cells, toluene-acetone-treated cells and cell-free extracts of strain P93 under various conditions are shown in Table 2. The B-gal activity was greatly reduced in toluene-acetone-treated cells and cell-free extracts. The addition of phosphoenolpyruvate to these preparations did not recover the enzyme activity. Sodium fluoride did not inhibit the enzyme activity of any

Table 2. Beta-Galactosidase and phospho-beta-galactosidase activity of P. freudenreichii P93

Culture Treatment	Specific enzyme activity[a]			
	ONPG	ONPG+PEP	ONPG+NaF	ONPG-6-PO_4
None (whole cells)	75.6	83.5	82.7	20.9
Toluene-acetone	8.5	10.0	12.9	7.5
Cell-free extract	3.1	3.3	3.3	2.5

[a]Cells were harvested after 25 h of incubation and assayed in sodium phosphate buffer, pH 7.5, at 50°C for 15 min. Units are expressed as nmoles/min/mg protein. Values are averages of two trials.

cell preparation. Similar results were observed for strain P39310. These results suggest that the pathway of lactose metabolism encoded by plasmid pRG03 is not the phosphoenolpyruvate-dependent phosphotransferase system.

Some P-B-gal activity was detected in untreated cells, at a much lower level than B-gal activity. In toluene-acetone-treated cells and cell-free extracts, P-B-gal activity was nearly equal to the remaining B-gal activity. It is currently not known if a true P-B-gal enzyme is present or if the hydrolysis of ONPG-6-P in the assay employed here is due to a lack of substrate specificity of beta-galactosidase in P93.

Induction Studies

No induction of B-gal activity by various beta-D-galactosides was seen in strains P93 and P39310 (Table 3). Enzyme activity of cells grown in the presence of the inducers and repressors examined was not significantly different from enzyme activity of the control cells grown without a carbohydrate source. The addition of lactose to the growth medium slightly reduced B-gal activity.

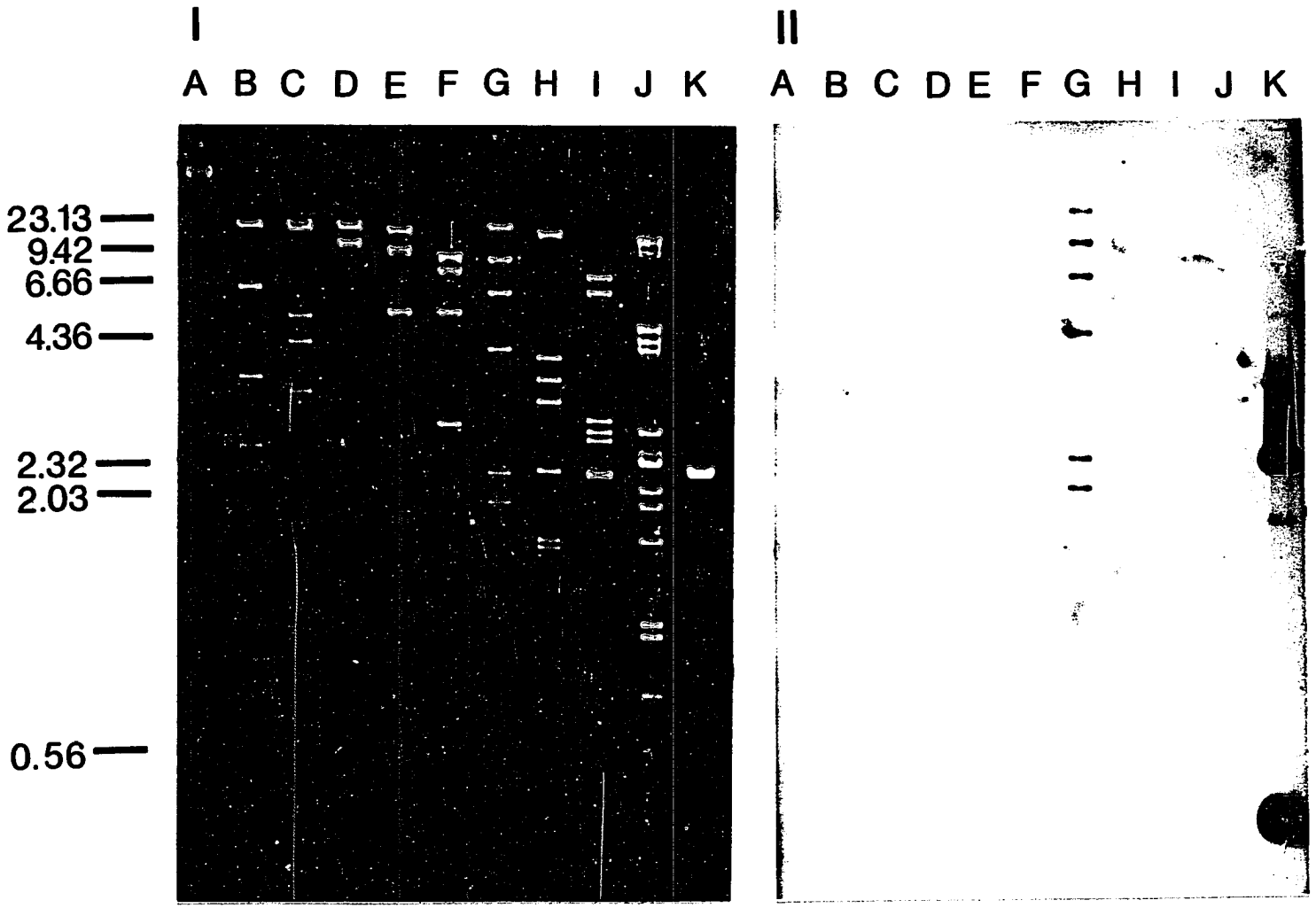
Table 3. Effect of various compounds on beta-galactosidase activity in P. freudenreichii P93 and P39310

Addition to basal medium	Beta-Galactosidase Activity[a]	
	P93	P39310
None	37.1	38.2
Sodium lactate (1%)	37.7	36.3
Lactose (0.2%)	29.9	31.7
Lactose (0.5%)	30.7	29.3
IPTG (1 mM)	34.8	33.5
IPTG (1 mM) + sodium lactate (1%)	36.7	37.6
Glucose (0.2%)	33.1	35.5
Galactose (0.2%)	31.5	33.8
Glucose (0.1%) + galactose (0.1%)	37.0	35.5

[a]Cells were grown in basal medium with the listed additives for 28 h and assayed in sodium phosphate buffer, pH 7.5 at 50°C for 15 min. Units expressed as nmoles/min/mg dry weight. Values are averages of two trials.

Hybridization Analysis

Results of enzyme assays indicated that the pathway of lactose metabolism in strain P93 may be similar to the system found in E. coli. Therefore, DNA-DNA hybridizations were conducted to determine if an E. coli lacZ' probe was homologous to any portion of the lactose plasmid pRG03. No detectable homology was observed between the lacZ' probe and restriction fragments of pRG03 at both high (data not shown) and low stringency (Figure 2).


I
A B C D E F G H I J K
23.13
9.42
6.66
4.36
2.32
2.03
0.56
II
A B C D E F G H I J K

DISCUSSION

In this report, physical evidence was presented to link lactose fermentation and a cell pelleting phenotype to a 25 Mdal plasmid, pRG03, in P. freudenreichii strain P93.

Plasmid-associated lactose fermentation has been well documented in many bacteria, including the lactic acid bacteria used as starter cultures in the dairy industry (2, 4, 5, 8, 11, 14, 25, 26, 32). Lactose plasmids found in Streptococcus lactis and S. cremoris have been found to encode components of the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), the enzyme phospho-beta-galactosidase, and enzymes of the tagatose phosphate pathway (2, 9). Most lactose plasmids reported in the lactic acid bacteria encode genes for the PEP-PTS pathway, but evidence of plasmid association of the lactose permease-B-gal system has also been reported (4).

In the present study, enzyme assays of whole cells, toluene-acetone-treated cells, and cell-free extracts of strain P93 and its cured derivatives indicated that plasmid pRG03 codes for a beta-galactosidase. Treatment of strain P93 with toluene-acetone greatly reduced B-gal activity, which suggested that this strain possessed a PEP-dependent PTS pathway for beta-galactosides. However, in other microorganisms with this pathway, the addition of

phosphoenolpyruvate to solvent-treated cells recovers the B-gal activity. This did not occur in strain P93.

Only low levels of P-B-gal activity were measured in whole cells, toluene-acetone-treated cells, and cell-free extracts of P93. In addition, sodium fluoride, which is known to inhibit the PEP-PTS pathway, had no effect on the B-gal activity of whole cells of strain P93. These results suggest that the loss of B-gal activity in solvent-treated cells of strain P93 cannot be attributed to disruption of a PEP-PTS pathway as described in Staphylococcus aureus and S. lactis (24). The reduction in B-gal activity after solvent treatment may be due to sensitivity of the beta-galactosidase to toluene or acetone. The low enzyme activity in cell-free extracts indicates that beta-galactosidase in P93 is sensitive to cell disruption. It may be membrane-bound in this strain.

It is presently not known whether the low P-B-gal activity detected in P93 is due to the presence of a true phospho-beta-galactosidase or to the lack of substrate specificity of the beta-galactosidase. Alternatively, the measured hydrolysis of ONPG-6-P could be due to conversion of ONPG-6-P to ONPG by the action of an endogenous phosphatase as described in S. thermophilus (34).

In a previous study of beta-galactosidase of P. shermanii, Hartley and Vedamuthu reported that five strains

showed enhanced B-gal activity and five other strains showed reduced B-gal activity after toluene-acetone treatment (12). The optimal temperature and pH for B-gal activity, 52°C and 7.5 respectively, were identical for representatives of these two groups of strains, and B-gal activity was constitutive. In the present study, the optimal temperature and pH for B-gal activity in strain P93 and the constitutive nature of this enzyme agree with the results of Hartley and Vedamuthu.

The solvent-sensitive strain P7 studied by Hartley and Vedamuthu (12) does not contain any detectable plasmids (30). Therefore, the beta-galactosidase of this strain appears to be encoded on the chromosome. The properties examined to date of this enzyme and of the enzyme in P93 are very similar. It is possible that the enzymes in these strains, one chromosomal and one plasmid-encoded, may be identical. Hybridization experiments will be necessary to confirm this possibility. In contrast, evidence from hybridization studies revealed that the beta-galactosidase alpha peptide of E. coli is not related to the beta-galactosidase of P93.

Plasmid-mediated lactose fermentation is not a common trait among the propionibacteria, as it is in L. casei, S. lactis and S. cremoris strains. Examination of over 119 strains of propionibacteria has identified only a single plasmid in a single strain to be associated with lactose fermentation. The pelleting phenomenon is also unique. At

present it is unknown if the pelleting phenotype is directly related to lactose fermentation or plays another role in the host strain. Currently, work is under way to provide genetic evidence for these plasmid-mediated functions as well as to clone the B-gal gene of pRG03 for genetic analysis. These and other studies should provide basic information on the expression and regulation of the B-gal gene in P. freudenreichii and on the possible origin of the lactose plasmid.

ACKNOWLEDGMENTS

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

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SUMMARY

The importance of plasmids in dairy starter cultures has been well established. Plasmids have been found to be associated with metabolic properties vital for successful dairy fermentations. The purpose of this study was to determine if plasmids were present in the dairy propionibacteria and to understand what role they may play in the growth and metabolic activities of these organisms.

A simple and rapid method was developed for the isolation of plasmid DNA from propionibacteria. Effective cellular lysis was achieved in all species when cells were treated with a high concentration of lysozyme. Treatment with pronase was found to increase plasmid yields in most strains, especially those of P. jensenii and P. thoenii that were most insensitive to lysozyme. The addition of EDTA and the use of 4% SDS during the lysis procedure were also found to increase plasmid yields. Following lysis, additional purification by acid phenol extraction was required to remove contaminating chromosomal and open circular DNA and to obtain complete endonuclease digestions.

Twenty strains out of 119 screened to date have been found to carry plasmids, which range in size from 4.4 to greater than 119 Mdal. Twelve strains contained a single plasmid and no strain contained more than two distinct

plasmids. Ten plasmids of different molecular mass were detected on agarose gels. Eleven strains were found to contain a 4.4 Mdal plasmid; two of these strains contained an additional plasmid, one greater than 119 Mdal and the other of 35 Mdal. Three strains were found to contain a 6.3 Mdal plasmid; two of these strains contained an additional plasmid, one of 19 Mdal and the other of 25 Mdal. Two strains contained 5.6 and 30 Mdal plasmids, and two other strains contained 12.5 Mdal plasmids and plasmids greater than 119 Mdal.

Plasmids of the same molecular mass from different strains were digested with restriction endonucleases to determine if they were, in fact, identical. Restriction endonuclease analysis revealed that the same 4.4 Mdal plasmid was present in eleven strains (pRG01). The 6.3 Mdal plasmids present in three strains were not identical. Plasmid pRG02 was present in two strains, and pRG07 was present in one strain. The 5.6 and 30 Mdal plasmids present in two strains were identical, and were named plasmids pRG06 and pRG04, respectively. The similar size plasmids (12.5 and those larger than 119 Mdal) in other pairs of strains have not been analyzed by restriction endonuclease digestion to determine if they are also identical to each other. Detailed restriction maps were constructed for plasmids pRG01, pRG02, pRG03, and pRG04.

The relationship among Propionibacterium plasmids was further examined by DNA-DNA hybridizations. Extensive sequence homology exists among some of the plasmids. Plasmids pRG01 and pRG02 share extensive sequence homology and are both homologous to pRG07 and to the same fragments of pRG05. Plasmids pRG04 and pRG06 do not share significant sequence homology with any other of the known Propionibacterium plasmids. Plasmid pRG03 shares minor sequence homology only with pRG07.

Cured derivatives of plasmid carrying-strains were isolated to characterize the functional properties of Propionibacterium plasmids. No phenotypic traits associated with bacteriocin production, carbohydrate fermentation, or antibiotic resistance were linked to plasmids pRG01, pRG02 or pRG05. However, physical evidence was obtained linking the clumping phenotype in strain P38 to plasmid DNA, possibly to plasmid pRG05. In addition, physical evidence was obtained linking lactose fermentation and a cell pelleting phenotype to the 25 Mdal plasmid, pRG03, in strain P93. The remaining plasmids remain cryptic.

Enzyme assays were used to characterize the lactose hydrolyzing system of strain P93 encoded by plasmid pRG03. Evidence from B-gal assays of whole cells, toluene-acetone-treated cells, and cell-free extracts of strain P93 and of its cured derivatives indicated that plasmid pRG03 codes for

a constitutive beta-galactosidase enzyme. Evidence from hybridization studies revealed that the beta-galactosidase alpha peptide of E. coli is not related to the beta-galactosidase of strain P93.

The characterization of plasmids in Propionibacterium should continue. The roles these plasmids may play in the metabolic processes of their host strains have yet to be established for a majority of the plasmids and this remains an important area for future investigations. Currently, work is underway to provide genetic evidence for these plasmid-mediated functions as well as to clone the beta-galactosidase gene of pRG03 for genetic analysis. These and other studies should provide basic information on the expression and regulation of plasmid-encoded genes in Propionibacterium. Research efforts also need to be directed at utilizing the native plasmids as cloning vectors. With continued research, the application of recombinant DNA techniques for strain improvement and genetic analyses of the propionibacteria will be a reality.

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ACKNOWLEDGMENTS

I would like to express my sincere appreciation to Dr. Bonita A. Glatz for her guidance and patience throughout the many years. She provided an environment which allowed me to work, explore, and learn with few constraints.

I would like to thank Dr. Peter Pattee, Dr. Earl Hammond and Dr. Paul Hartman for serving on my committee. Their helpful discussions and advice were greatly appreciated. Special thanks to Clark Ford for joining my committee on very short notice.

Thanks are expressed to the past and present members of the Propionibacteria Research Group (PRG): LeAnn Baehman, Kathleen Anderson, Dale Grinstead, and Steve Woskow. Their discussions, criticisms and ideas were an important and cherished part of my graduate education. Thanks also to other members of the lab group, Wan Soo Park and Anand Rao for their companionship, enthusiasm, and assistance. Anand's willingness to lend a helping hand was greatly appreciated.

I would like to express my appreciation to my parents and father-in-law, who in addition to willingly providing financial support, offered the encouragement that made this work possible. Finally, I would like to thank my wife Carolyn, without whom none of this would have been possible. Her love, support and sacrifice have been a constant for our family.

APPENDIX

Table A1. Minimum inhibitory concentrations of selected antibitotics for *Propionibacterium* strains in µg/ml

ANTIBIOTIC	STRAIN							
	P1	P2	P3	P4	P5	P6	P7	P8
Chloramphenicol	5.0	5.0	5.0	1.0	5.0	10.0	5.0	5.0
Cloxacillin	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Erythromycin	5.0	5.0	5.0	2.5	2.5	2.5	1.0	2.5
Fusidic Acid	0.1	1.0	1.0	1.0	1.0	0.25	0.1	1.0
Gentamycin	50.0	250.0	125.0	50.0	350.0	125.0	50.0	250.0
Kanamycin	250.0	250.0	350.0	25.0	250.0	500.0	250.0	500.0
Methicillin	75.0	250.0	100.0	250.0	250.0	5.0	50.0	100.0
Nafcillin	5.0	5.0	5.0	5.0	5.0	0.25	0.05	5.0
Neomycin	125.0	25.0	125.0	25.0	125.0	50.0	50.0	125.0
Novobiocin	0.05	1.0	5.0	0.25	5.0	0.05	0.05	1.0
Oxacillin	10.0	10.0	10.0	10.0	10.0	0.5	0.5	10.0
Penicillin	1.0	5.0	5.0	0.25	2.5	10.0	0.25	5.0
Rifampicin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.0
Streptomycin	250.0	25.0	100.0	10.0	250.0	100.0	100.0	250.0
Tetracycline	10.0	5.0	5.0	5.0	10.0	50.0	5.0	10.0
Vancomycin	1.0	0.5	1.0	0.5	1.0	5.0	1.0	1.0

Table A1 (Continued)

ANTIBIOTIC	CULTURE							
	P9	P10	P11	P12	P13	P14	P15	P16
Chloramphenicol	1.0	1.0	5.0	5.0	5.0	5.0	5.0	5.0
Cloxacillin	10.0	10.0	10.0	10.0	5.0	10.0	10.0	10.0
Erythromycin	2.5	25.0	2.5	5.0	5.0	10.0	5.0	10.0
Fusidic Acid	1.0	1.0	1.0	0.1	0.1	0.1	1.0	1.0
Gentamycin	250.0	250.0	125.0	250.0	125.0	250.0	25.0	250.0
Kanamycin	125.0	125.0	350.0	350.0	250.0	500.0	25.0	500.0
Methicillin	75.0	250.0	75.0	100.0	10.0	50.0	250.0	75.0
Nafcillin	5.0	5.0	5.0	5.0	0.5	1.0	5.0	5.0
Neomycin	25.0	50.0	125.0	125.0	125.0	125.0	125.0	125.0
Novobiocin	1.0	1.0	0.25	0.05	0.05	0.05	0.75	0.5
Oxacillin	10.0	10.0	10.0	10.0	2.5	5.0	10.0	10.0
Penicillin	5.0	1.0	1.0	1.0	0.25	1.0	1.0	1.0
Rifampicin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	5.0
Streptomycin	25.0	50.0	100.0	250.0	250.0	1000.0	1000.0	250.0
Tetracycline	5.0	5.0	10.0	5.0	10.0	10.0	5.0	5.0
Vancomycin	0.5	0.5	1.0	1.0	1.0	1.0	1.0	5.0

Table A1 (Continued)

ANTIBIOTIC	CULTURE							
	P17	P18	P19	P20	P21	P22	P23	P24
Chloramphenicol	1.0	5.0	5.0	5.0	0.5	5.0	5.0	5.0
Cloxacillin	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Erythromycin	25.0	10.0	2.5	2.5	25.0	2.5	1.0	2.5
Fusidic Acid	0.75	0.75	0.1	1.00	0.75	0.1	0.1	0.1
Gentamycin	250.0	50.0	125.0	125.0	250.0	125.0	250.0	125.0
Kanamycin	250.0	250.0	50.0	250.0	250.0	350.0	350.0	250.0
Methicillin	250.0	100.0	75.0	250.0	250.0	20.0	10.0	100.0
Nafcillin	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Neomycin	125.0	125.0	50.0	50.0	50.0	125.0	125.0	125.0
Novobiocin	0.5	0.25	0.05	1.0	0.50	0.05	0.05	0.1
Oxacillin	10.0	10.0	5.0	10.0	10.0	10.0	10.0	10.0
Penicillin	1.0	2.5	1.0	5.0	1.0	0.5	0.5	1.0
Rifampicin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Streptomycin	50.0	1000.0	50.0	50.0	50.0	100.0	1000.0	50.0
Tetracycline	5.0	10.0	5.0	10.0	5.0	5.0	5.0	10.0
Vancomycin	1.0	5.0	1.0	0.5	0.5	1.0	1.0	1.0

Table A1 (Continued)

ANTIBIOTIC	CULTURE							
	P25	P26	P30	P38	P54	P63	P93	P113
Chloramphenicol	1.0	1.0	5.0	5.0	1.0	5.0	5.0	5.0
Cloxacillin	10.0	10.0	10.0	10.0	5.0	10.0	10.0	10.0
Erythromycin	2.5	2.5	2.5	2.5	5.0	5.0	10.0	10.0
Fusidic Acid	1.0	1.0	1.0	1.0	1.0	1.0	0.5	0.1
Gentamycin	250.0	250.0	250.0	250.0	250.0	250.0	125.0	250.0
Kanamycin	250.0	250.0	125.0	250.0	125.0	125.0	250.0	250.0
Methicillin	250.0	250.0	250.0	250.0	250.0	500.0	100.0	75.0
Nafcillin	5.0	5.0	5.0	5.0	1.0	1.0	5.0	0.5
Neomycin	50.0	50.0	125.0	50.0	50.0	125.0	125.0	125.0
Novobiocin	1.0	0.5	0.5	1.0	0.5	0.5	1.0	0.1
Oxacillin	10.0	10.0	10.0	10.0	10.0	10.0	10.0	2.5
Penicillin	2.5	2.5	1.0	10.0	5.0	5.0	5.0	10.0
Rifampicin	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Streptomycin	50.0	50.0	25.0	25.0	50.0	100.0	100.0	100.0
Tetracycline	5.0	10.0	10.0	10.0	5.0	10.0	5.0	5.0
Vancomycin	1.0	1.0	1.0	1.0	1.0	1.0	5.0	1.0