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Protoplasting, regeneration and fusion of lactobacilli

Michael Jantschke

To the Graduate Council:

I am submitting herewith a thesis written by Michael Jantschke entitled "Protoplasting, regeneration and fusion of lactobacilli." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

P.M. Davidson, Major Professor

We have read this thesis and recommend its acceptance:

S.L. Melton, G.L. Christen, H.O. Jaynes

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Date Aug. 5, 1986

PROTOPLASTING, REGENERATION AND FUSION OF LACTOBACILLI

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Michael Jantschke

August 1986

DEDICATION

With love, I dedicate this thesis to my wife Esmeralda.

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ABSTRACT

Cells of two Lactobacillus strains were protoplasted by treatment with mutanolysin or combinations of mutanolysin and lysozyme for various incubation times. Almost all protoplastization treatments tested were suitable for reducing the number of osmotically stable cells two log cycles. Protoplasts were successfully regenerated on a complex medium containing $MgCl_2$, $CaCl_2$, gelatin, raffinose and bovine serum albumin (BSA). Maximal regeneration frequencies ranged from 6 to 10 % for L. casei subsp. rhamnosus and L. lactis, respectively. The use of agar overlays did not affect the regeneration ability of either strain. Treatment of protoplasts of both strains with 40 % polyethylene glycol (PEG) resulted in a regeneration frequency decrease of several hundredfold. Using the different resistance levels of both strains to the antibiotics kanamycin and penicillin as selected genetic markers, attempts to fuse protoplasts of these lactobacilli in the presence of 40 % PEG were not successful.

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CHAPTER I

INTRODUCTION

In recent years, the advances and applications of genetic engineering have aroused a great deal of interest in the food industry. The potential for applications in the field of fermentation processes seems to be especially promising.

Species of the genus Lactobacillus have been used in food fermentation for many years. However, knowledge of genetic transfer systems for this genus is limited. Among other things, difficulties in production and regeneration of Lactobacillus protoplasts have undermined progress (Lee-Wickner and Chassy, 1984, 1985).

One genetic engineering technique potentially applicable to strain improvement of lactobacilli is protoplast fusion. This method encompasses combining entire genomes of different cells via a process which involves the localized fusion of the cell membranes of two cells and subsequent genetic recombination. Thus, enzyme systems for metabolic functions as well as mechanisms of phage resistance could be transferred within and among species leading to strains with novel and/or improved properties.

There were several objectives developed for this research. First, was to test the applicability of the

protoplast formation and regeneration method of Lee-Wickner and Chassy (1984) on the lactobacilli used in this study and to evaluate the effect of the agar overlay method of Fodor et al. (1975) on regeneration. Second, was to select usable and reliable genetic markers for the detection of fused cells. The last objective was to ascertain the effect of the fusogenic agent polyethylene glycol (PEG) on the regeneration ability of the Lactobacillus strains used in this study and to attempt protoplast fusion of these strains by employing methods developed for other microorganisms.

CHAPTER II

REVIEW OF THE LITERATURE

1. The Genus *Lactobacillus*

Occurrence, Classification and Physiology

The genus *Lactobacillus* belongs to the family *Lactobacillaceae*, and together with the genera *Streptococcus*, *Pediococcus*, and *Leuconostoc* makes up a group of microorganisms commonly referred to as "lactic acid bacteria". Lactobacilli are ubiquitous in nature. Because of their complex nutritional requirements for protein breakdown products, vitamins, inorganic salts, purines and pyrimidines, fatty acids and fatty acid esters and their mode of energy-generating metabolism, members of this genus do not exist in soil and water but are easily isolated from man and animals, plants, and various food products (Lorenz et al., 1983). In animals including humans, lactobacilli are found in the oral cavity, the vagina and the intestinal tract (Sharpe, 1981).

Lactobacilli are gram-positive, catalase-negative, rod-shaped bacteria that have a tendency to form chains. They do not produce spores and the presence of motility is unusual. Growth of lactobacilli is enhanced by microaerophilic to anaerobic conditions in the presence of 5 - 10 % CO_2. Colonies of lactobacilli growing on the surface of agar may be classified as rough (R) or smooth

(S). Colonial morphology does not seem to be genetically controlled and can be induced by the composition of the growth medium and environmental conditions (McDonald and Frazier, 1951; Rogosa and Mitchell, 1949). However, Klaenhammer and Kleeman (1981) suggested that cells within a single population may be genetically predisposed to colonial morphologies. Lactobacilli are heat sensitive and usually destroyed by exposure to 60 - 65C for 30 minutes (Topley and Wilson, 1983). In contrast, high resistance to acid is characteristic for the genus. For example, certain strains of L. plantarum and L. casei species can grow in wine at a pH as low as 3.5 (Wibowo et al., 1985). The type species is Lactobacillus delbruecki.

Traditionally, members of the genus Lactobacillus have been subdivided into three groups, Thermobacterium, Streptobacterium and Betabacterium, primarily on the basis of their nutritional and biochemical properties (Orla-Jensen, 1919; Rogosa and Sharpe, 1959). Included in the subgenus Thermobacterium are Lactobacillus lactis and Lactobacillus bulgaricus. All members of the group are homofermentative and grow best at 37 - 45C (Rogosa and Sharpe, 1959; Topley and Wilson, 1983). Typical members of the subgenus Streptobacterium are Lactobacillus casei and Lactobacillus plantarum. They are also homofermentative (for hexoses) but their optimal growth temperature is around 30C. The streptobacteria can be distinguished from

the thermobacteria by their ability to grow at 15C, to ferment ribose and to produce CO_2 from gluconate (Sharpe, 1981). In contrast to the first two groups, members of the Betabacterium group are heterofermentative. The absence of the enzyme aldolase, a growth requirement for thiamine, the production of mannitol and the generation of CO_2 from glucose are used as criteria to distinguish the betabacteria from the homofermentative lactobacilli (Sharpe, 1981).

The most common characteristics used to differentiate species of the genus Lactobacillus are their abilities to ferment various sugars and their vitamin requirements. A newer approach to differentiation has been the use of serological analysis of cell wall antigens (London, 1976; Sharpe, 1981; Topley and Wilson, 1983). To date, seven group antigens (A-G) have been identified, four of which are species-specific. Many species are not covered by this classification, probably because they are antigenically heterogeneous. Chemically the identified antigens are glycerol teichoic acid, ribitol teichoic acid and polysaccharides. They are located in the cell wall with the exception of glycerol teichoic acid which is also found in the plasma membrane (Schleifer and Kandler, 1972; Sharpe, 1981).

Thus far, the classification methods discussed were based on phenotypic traits of microorganisms. In recent

years, the interest has shifted towards genotypical analysis of lactobacilli through determination of guanosine plus cytosine (G+C) base composition and deoxyribonucleic acid (DNA) hybridization studies (London, 1976; Sharpe, 1981). Generally the genus is divided into three G+C groups, ranging from 33 to 50 mole % (Sriranganathan et al, 1985). DNA-DNA or DNA-RNA (ribonucleic acid) hybridization procedures indicate the extent of genome homology between different bacteria and make it possible to define a species based on DNA homology. In many cases these genetic classification studies on lactobacilli have confirmed the phenotypic classification, but in some instances incongruous results have been obtained (Sriragganathan et al., 1985).

Physiologically, lactobacilli are characterized by their comparatively weak proteolytic activity and strong dependence on fermentable carbohydrates for growth. The occurrence of cell wall- and membrane-bound proteinases and peptidases in lactobacilli was reviewed by Law and Kolstad (1983). These enzymes are part of a cytological system to hydrolyze proteins and peptides to free amino acids (Thomas, 1985). Lactobacilli possess neither a functional tricarboxylic acid cycle nor a heme-linked electron transport system. The principle pathways of carbohydrate metabolism have been reviewed by Kandler (1983), Lorenz (1983), and Thomas (1985). Energy equivalents are

primarily derived through substrate-level phosphorylation during sugar fermentation, although some species are able to use citrate (Campbell and Gunsalus, 1944) and malate (Caspritz and Radler, 1983; Schuetz and Radler, 1973) as sole energy sources in the absence of carbohydrates. Members of the subgenus Thermobacterium ferment hexoses via glycolysis. Glucose is phosphorylated to fructose-1,6-diphosphate which in turn is split by an aldolase into two triose phosphate moieties. The triose phosphate then is converted to lactate with generation of energy. Because thermobacteria convert glucose to more than 85 % lactic acid solely via glycolysis, they are referred to as "obligate homofermenters" (London, 1976; Stamer, 1979). The betabacteria ferment hexoses differently. Lacking the enzyme aldolase, glucose is phosphorylated and converted to 6-phospho-gluconate. Through various steps this compound is transformed to triose phosphate, acetyl phosphate, and CO_2, the last step being catalyzed by the enzyme phosphoketolase. The triose phosphate is converted to lactate whereas acetyl phosphate yields acetate, ethanol, hydrogen peroxide, or mannitol depending on the oxidation-reduction potential of the system and the presence of hydrogen acceptors. Since only about 50 % of the glucose is transformed to lactic acid, the betabacteria are called "obligate heterofermenters". Another difference between the betabacteria and the

thermobacteria is that members of the latter group are not able to ferment pentoses since they lack the necessary enzyme systems. In the Betabacterium group, pentoses are transported through the membrane by specific permeases and converted to xylulose-5-phosphate by inducible enzymes. The xylulose phosphate is then fed into the phosphoketolase pathway. Streptobacteria, which are classified as "facultative homofermenters", possess aldolase and all enzymes of the phosphoketolase pathway. While the latter system is used primarily for pentose metabolism, hexoses are preferably fermented via glycolysis.

The disaccharide lactose is transported through the cell membrane with the help of a permease and then split into glucose and galactose by the enzyme β-galactosidase (Premi et al., 1972). The so-called Leloir pathway is used by the lactobacilli in converting galactose to glucose-6-phosphate. The glucose moiety of the lactose is phosphorylated and both molecules of glucose-6-phosphate are fed into the Embden-Meyerhoff pathway. A common lactose fermentation system of streptococci, the phosphoenol-pyruvate-dependent phosphotransferase system (PEP:PTS), is present only in a few lactobacilli such as L. casei and L. bulgaricus (Hickey et al., 1986; Jimeno et al., 1984; Lee et al., 1982).

Public Health Aspects

In recent years the notion that the ingestion of milk products fermented by lactobacilli may improve intestinal health and prevent disease has become quite popular. This notion refers primarily to the inhibition of pathogenic gram-negative bacteria and other noxious microorganisms by lactobacilli through production of lactic acid and hydrogen peroxide, the maintenance of a low pH and redox potential as well as deconjugation of bile in the intestines (Lorenz et al., 1983; Sandine, 1979). Furthermore, various members of the genus are known to produce antibiotics (Sandine, 1979). In vitro, antibiotics produced by L. acidophilus (acidophilin, lactocidin, acidolin), L. plantarum (lactolin), L. brevis (lactobrevin), and L. bulgaricus (bulgarican) have been shown to be inhibitory to species of Salmonella, Shigella, Staphylococcus, and other pathogens (Kilara and Treki, 1984; Lorenz et al., 1983; Sandine, 1979). Several studies have shown an anticarcinogenic and anticholesterolemic effect of L. acidophilus and L. bulgaricus, suggesting a reduced risk of colon cancer and coronary heart disease (Gilliland et al., 1985; Kilara and Treki, 1984). Considerable controversy has arisen concerning whether lactobacilli ingested through food are able to colonize the gastrointestinal tract. Although some of these microorganisms have been shown to exist in the stomach and small intestines in appreciable numbers

(Sharpe, 1981), at present this question cannot be answered conclusively. In general, the notion exists that lactobacilli in fermented dairy products act as a general nutritional therapeutic agent and are important in the maintenance of intestinal health (Lorenz et al., 1983).

The non-pathogenicity of lactobacilli has been established over the centuries. However, several strains of L. casei, L. casei subsp. rhamnosus, and L. acidophilus have been implicated with disease conditions such as subacute bacterial endocarditis, septicemia, abscesses, and dental caries (London, 1976; Sharpe et al., 1973). Histamine producing strains of Lactobacillus buchneri and L. brevis have been isolated from Swiss cheese and delicatessen salads, respectively. The L. buchneri strain was implicated in a food poisoning outbreak in New Hampshire (Sumner et al., 1985).

Use in Food Fermentations

Numerous species of this genus play a vital role in various food fermentations. The production of fermented milk products is of great importance to the dairy industry. To attain greater control over the fermentation process, lactic starter cultures of known composition and characteristics are used. Their main functions are lactic acid and flavor production. Examples of fermented dairy products utilizing lactobacilli, among other

microorganisms, are yogurt (L. bulgaricus), acidophilus milk (L. acidophilus), yakult (L. casei), kefir (L. caucasicus), koumiss (L. bulgaricus), Bulgarian buttermilk (L. bulgaricus), and Swiss cheese, Parmesan, Romano, and Mozzarella (L. bulgaricus, L. helveticus). In the production of cheddar cheese, lactobacilli are not used as starter cultures but L. plantarum and L. casei are usually present due to post-pasteurization contamination and contribute to ripening (Thomas, 1985).

Another area of the food industry in which the genus Lactobacillus is of considerable importance is the fermentation of vegetables, including cabbage, cucumbers, olives, and soybeans. While lactic acid bacteria, in general, and lactobacilli, in particular are difficult to isolate from living plant tissue they increase in number during harvesting and processing of certain vegetables (Sharpe and Pettipher, 1983). The sequential growth of lactic acid bacteria with subsequent predominance of L. plantarum and L. brevis is of paramount importance to sauerkraut and pickle production (Sharpe and Pettipher, 1983). The same is true for the conversion of moist forage to silage (Silley and Damoglou, 1985). Strains of the species L. delbruecki play a role in the manufacture of soy sauce (Kilara and Treki, 1984) and a combination of L. acidophilus and L. bulgaricus has been used to ferment soybean milk to soy yogurt (Sharpe, 1981).

In the United States, most fermented meat products are consumed in the form of cured ham and dry and semi-dry sausages. At present, most processors of fermented sausages use starter cultures comprising pediococci and homfermentative lactobacilli to yield products with consistent flavor, texture and shelf stability. In addition, the food poisoning microorganism S. aureus is inhibited by the low pH produced by the starter culture in fermented meats (Bacus, 1984).

Food Spoilage

Although lactobacilli are present in a variety of food products some strains are known to cause spoilage. Types of spoilage of dairy products include ropiness and slime in fluid milk, off-flavors in Edam and Gouda cheeses, excessive CO_2 during cheese ripening, discolorations in cheese, and slime formation in cheese brine (Sharpe and Pettipher, 1983). Spoilage of vegetable and fruit products by lactobacilli usually occurs when the growth sequence of the lactic flora is disturbed, leading to the predominance of undesirable organisms. Examples are red discoloration of sauerkraut, bloater formation of pickles, and off-flavor production due to diacetyl in fruit juices (Sharpe and Pettipher, 1983).

With the advent of vacuum packaging of meats and meat products, the significance of lactobacilli as spoilage

bacteria has drastically increased. Whereas they are insignificant in the spoilage of aerobically stored meat, lactobacilli become the dominant flora of vacuum packaged meat products. The increased concentration of CO_2 in the package is the single most important factor which facilitates their growth and suppresses that of aerobes. Common types of spoilage are souring, greening, slime production, gas formation, and off-flavors (Sharpe and Pettipher, 1983).

Genetics

Despite the industrial importance of the genus Lactobacillus, very little is known about the genetics of these bacteria. Difficulties in culturing these microorganisms in the laboratory and the absence of usable gene transfer systems have hampered progress considerably. Knowledge of DNA replication, plasmid maintenance, and gene expression is particularly limited (Lee-Wickner and Chassy, 1985).

Since 1976 it has become apparent that plasmids do occur in lactobacilli (Chassy et al., 1976). For example, L. helveticus subsp. jogurti contains a 13 Kb plasmid which codes for lactic acid production and fermentation of N-acetyl-D-glucosamine (Smiley et al., 1978). In L. casei, the lactose metabolism genes for the PEP:PT system are plasmid-coded (Chassy et al., 1978; Lee et al., 1982). In

the last six years, the existence of plasmids has been reported in various strains of Lactobacillus including: reuteri, helveticus, acidophilus, casei, fermentum, bulgaricus, and plantarum (Ishiwa and Iwata, 1980; Klaenhammer, 1984; Lee-Wickner and Chassy, 1985; Morelli et al., 1983; Nes, 1984; West and Warner, 1985). Most of these plasmids are cryptic. Nevertheless, in addition to lactose metabolism, resistance to the antibiotics tetracycline, erythromycin, and chloramphenicol has been linked to extrachromosomal elements (Ishiwa and Iwata, 1980; Morelli et al., 1983).

Reports of gene transfer systems for the genus Lactobacillus have been rather scarce. Conjugation, once believed to be restricted to gram-negative microorganisms has been shown to exist in gram-positive bacteria such as streptococci, S. aureus, and Bacillus subtilis (Gibson et al., 1979; Vescovo et al., 1983). It is possible to conjugally transfer certain transmissible plasmids from Streptococcus strains to various lactobacilli with frequencies of transmission ranging from 10^{-5} to 10^{-9} (Gibson et al., 1979; Vescovo et al., 1983; West and Warner, 1985).

The transfer of parts of a bacterial genome to a bacterial host via a phage vector is called transduction. Although lytic phages are rare in lactobacilli, lysogeny seems to be widespread among members of this genus

Sharpe, 1981; Yokokura et al., 1974). These temperate phages can be used as transduction vehicles. Tohyama and co-workers (1971) transduced auxotrophic strains of Lactobacillus salivarius to the prototrophic form at a frequency of 10^{-7} to 10^{-8}.

The gene transfer system of choice for recombinant DNA techniques is transformation. Transformation entails the uptake of free DNA or plasmids by bacterial cells or protoplasts. Recently the establishment of a transformation system for lactobacilli has become reality (Barach, 1985; Finer and Klaenhammer, personal communication, 1986). To apply such a system to genetic engineering, suitable plasmid cloning vectors which are stable in their respective hosts and for which detailed restriction maps are available have to be developed (Lee-Wickner and Chassy, 1985; West and Warner, 1985). Shimizu-Kadota and Kudo (1984) took a somewhat different approach by encapsulating viral DNA in liposomes with subsequent transformation of L. casei.

2. Protoplast Formation and Cell Wall Regeneration

Bacterial cytoplasm surrounded by an intact membrane which is devoid of a cell wall is referred to as a protoplast. The cell wall is enzymatically removed and the resulting osmotically fragile protoplasts are stabilized by hypertonic media containing high concentrations of

raffinose, sucrose, lactose, or other osmotically active compounds. The production of protoplasts and subsequent regeneration of the cell wall are crucial for the efficacy of gene transfer systems such as transformation and protoplast fusion.

Cell Wall Structure of Lactobacillus

The main constituent of the gram-positive cell wall is peptidoglycan, comprising 30 - 70 % of the total cell wall weight. In addition to other functions, the serological characteristics as well as phage adsorption loci are determined by this structure. In an excellent review Schleifer and Kandler (1972) described the basic wall architecture of gram-positive bacteria including lactobacilli. Peptidoglycan is a heteropolymer of glycan strands cross-linked through short peptides, forming a three-dimensional, multi-layered network, ranging in thickness from 20 - 80 nm. The glycan moiety consists of N-acetylglucosamine and N-acetylmuramic acid residues which are connected by β-1,4-glycosidic bonds, averaging about 10 to 65 disaccharide units. The peptide subunit is comprised of alternating L- and D-amino acids with amino terminal L-alanine binding to the muramic acid carboxyl group. The peptide moieties are linked by either interpeptide bridges or by direct bonds of diamino acids in the third position of one peptide subunit with the carboxyl terminus of

another subunit. Lactobacillus subgenera differ in their peptide subunit linkage. The amino acid sequence of the interpeptide bridges of thermo- and streptobacteria is of the L-lysine-D-aspartate type. The betabacteria are more heterogeneous, although the L-lys-D-asp sequence predominates.

Enzymatically attached to this peptidoglycan foundation are teichoic acids, polysaccharides and proteins (Reusch, 1984). It has been shown that peptidoglycan is cross-linked with polysaccharides and teichoic acids through a phosphodiester bond involving carbon #6 of the muramic acid residue (Araki et al., 1972). Teichoic acids are water-soluble polymers made up of a sugar moiety, D-alanine, glycerol (glycerol teichoic acid) or ribitol phosphate (ribitol teichoic acid). A special class of teichoic acids, lipoteichoic acid, are molecules that contain a polymer chain covalently bound to a lipid moiety which is anchored in the plasma membrane. In addition to surface antigen functions, lipoteichoic acids and other lipopolymers may be transiently involved in cell wall polymer biosynthesis and assembly (Reusch, 1984). Schall and colleagues (1981) suggested an intricate association of membrane and wall in gram-positive bacteria, possibly by covalent bonds. Proteins and lipoteichoic acids may constitute bridging structures which link the membrane with the peptidoglycan network.

Muralytic Enzymes and Protoplast Formation

Muralytic enzymes degrade bacterial cell walls, a property which is extensively used to lyse cells for membrane preparations and DNA isolations as well as to prepare protoplasts. Based on their mode of action these enzymes can be assigned to three classes (Yokogawa et al. 1975). Glycosidases, the most ubiquitous group, attack the glycan residue. Endopeptidases split linkages within the peptide subunit and amidases hydrolyze the bonds between glycan and peptide moieties.

Lysozyme, an endo-N-acetyl-muramidase isolated from egg white, hydrolyzes the β-1,4 bond between N-acetylglucosamine and N-acetylmuramic acid of the glycan polymer. Variability among the gram-positive microorganisms in respect to lysozyme resistance is attributed to cell wall structure and chemistry. There seems to be a positive correlation between a high degree of peptide cross-linkage, free amino groups in peptide subunits, attachment of teichoic acids to the peptidoglycan backbone and increased lysozyme resistance (Araki et al., 1972). For example, lysozyme resistance in Micrococcus lysodiekticus is linked to the presence of O-acetyl groups and N-nonsubstituted muramic acid residues with free amino groups (Araki et al., 1972; Brumfitt et al., 1958). Araki and co-workers (1972) showed that the number of N-nonsubstituted glucosamine residues in glycan also

determined the extent of lysozyme resistance in Bacillus cereus. In addition to inherent properties of the bacterium, phase of growth also affects the lytic activity of the enzyme. Neujahr et al. (1973) reported greatest sensitivity of L. fermenti during the exponential phase, but Chassy and Guiffrida (1980) found stationary phase cells of L. casei to be more susceptible.

In recent years mutanolysin, a lytic enzyme preparation obtained from Streptomyces globisporus has been shown to have activity against gram-positive bacteria such as streptococci, B. subtilis, L. acidophilus, and L. casei (Kondo and McKay, 1982; Tomochika et al., 1982; Yokogawa et al., 1975). Mutanolysin exists in two molecular forms, M1 and M2, with molecular weights of 22,000 and 11,000, respectively (Yokogawa et al., 1975). The original mutanolysin preparation from Yokogawa and colleagues possessed proteolytic activity which made it unsuitable for protoplast production. Subsequently, Siegel et al. (1981) developed a method to eliminate all proteolytic activity and today mutanolysin is commercially available in a purified form.

The knowledge of the exact mechanism of protoplasting is still rudimentary. Barker and Thorne (1970) investigated the protoplast formation of L. casei. These researchers discovered that upon incubation with trypsin and lysozyme the plasma membrane separates from the cell

wall and the protoplast extrudes through an opening in the ruptured cell wall. At that point the protoplasts are not larger than the intact bacterial cells due to high density of the cytoplasm. As time progresses, the size of the protoplast increases as a result of swelling leading to a reduction of cytoplasmic density. Lee-Wickner and Chassy (1984) observed partially emptied rodlike sacs and small protoplasts during protoplast formation of L. casei strains but were not able to detect these structures in electron micrographs. Miller et al. (1967) recognized two intermediate forms in the production of B. subtilis protoplasts: an osmotically sensitive rod-shaped form and a sphere-shaped form which is osmotically sensitive and retains vestigial cell wall material.

Cell Wall Regeneration

Successful cell wall regeneration has been reported for a host of microorganisms such as Bacillus (Akamatsu and Sekiguchi, 1984; Fodor et al., 1975), Clostridium (Jones et al., 1985; Stal and Blaschek, 1985), Streptococcus (Okamoto et al., 1983), Staphylococcus (Goetz et al., 1981; Stahl and Pattee, 1983), Streptomyces (Baltz and Matsushima, 1981), and Lactobacillus (Finer and Klaenhammer, personal communication, 1986; Lee-Wickner and Chassy, 1984; Vescovo et al., 1984). Regeneration is usually carried out on a complex medium the composition of which is dependent upon

the organism studied. In general, plasma expanders such as gelatin, bovine serum albumin (BSA), dextran, and polyvinyl pyrrolidone as well as $MgCl_2$ and $CaCl_2$ are crucial for the reversion of protoplasts to the bacillary form. In L. casei, BSA is an absolute requirement for regeneration and cannot be substituted by other plasma expanders (Lee-Wickner and Chassy, 1984). High concentrations of agar (1 - 3 %) are required for sufficient regeneration (Landman et al., 1968). Landman and Forman (1969) advanced the proposal that "...a physically solid environment in contact with the cell surface triggers derepression of previously repressed wall precursor biosynthesis." Other factors which can affect the regeneration frequency are the genetic background of each strain (Akamatsu and Sekiguchi, 1981), the choice of osmotic stabilizer (Lee-Wickner and Chassy, 1984), the addition of cell wall fragments and autoclaved cells to regeneration media (Landman and Forman, 1969), the choice of temperatures during cell growth, protoplast formation and regeneration (Baltz and Matsushima, 1981; Gabor and Hotchkiss, 1979), and the use of soft agar overlays as well as incorporation of BSA in hypertonic dilution buffers (Akamatsu and Sekiguchi, 1984; Stal and Blaschek, 1985). Successful regeneration seems to be dependent on the preservation of the enzymatic activity of the wall biosynthetic system, the physical and chemical environment of the protoplast, and the presence of vestiges

of cell wall attached to the protoplast surface (Landman et al., 1968; Miller et al., 1967).

For most microorganisms very little is known about the actual processes taking place during the reversion of protoplasts to the walled form. Landman and Forman (1969) reported a succession of three steps in the production of osmotically resistant revertents from protoplasts of B. subtilis. In the initial stages of regeneration, protein and RNA biosynthesis is maximal whereas synthesis of cell wall products occurs at later stages. In L. casei, upon reversion to the bacillary form, polyisoprenol intermediates of cell wall biosynthesis are found in the plasma membrane of the protoplast, giving the cell the potential to produce wall components over the whole surface (Barker and Thorne, 1970).

In recent years, it has been demonstrated that protoplasting and regeneration of bacterial cells can lead to loss of plasmids. Vescovo and co-workers (1984) reported the curing of a plasmid in L. reuteri at a frequency of 92%. Novick et al. (1980) observed the elimination of certain plasmids in S. aureus at high frequency. These researchers postulated that plasmid curing occurs during protoplast divisions which take place before cell wall regeneration is completed. This seemed to be caused by a disruption of the plasmid division-partition process, the proper functioning of which is dependent on an intact cell wall.

3. Fusion of Bacterial Protoplasts

Protoplast fusion has recently become an important genetic tool, especially for species in which other gene transfer systems have not been developed. It has been used for genetic recombination studies (Baltz and Matsushima, 1981; Gabor and Hotchkiss, 1979; Hotchkiss and Gabor, 1980), chromosome mapping (Gabor and Hotchkiss, 1983; Stahl and Pattee, 1983), and transfer of plasmids (Dancer, 1980; Goetz et al., 1981; Smith, 1985). This method encompasses localized membrane fusion of two or more protoplasts with subsequent genetic reassortment and stabilization, giving rise to cells that contain genetic material from different parental cells. The feasibility of this technique has been demonstrated for a wide array of organisms and cells, e.g. plants (Kao and Michayluk, 1974; Zimmermann and Scheurich, 1981), hen erythrocytes (Ahkong et al., 1975), human fibroblasts (Pontecorvo et al., 1977), Basidiomycetes (Toyomasu et al., 1986), yeasts (Panchel et al., 1984; Sakai et al., 1986), Streptomyces (Baltz, 1978; Godfrey et al., 1978; Hopwood and Wright, 1978), Brevibacterium (Kaneko and Sakaguchi, 1979; Karasawa et al., 1986), Staphylococcus (Dancer, 1980; Goetz et al, 1981; Stahl and Pattee, 1983), Bacillus (Fodor and Alfoldi, 1976; Schaeffer et al, 1976), Clostridium (Jones et al, 1985), and Streptococcus (Okamoto et al., 1983; Smith, 1985). Theoretically any two biological membranes can be fused but

in reality the "genetic relatedness" of the parental cells limits the stability and viability of the fusion products. Fusion of protoplasts usually occurs intragenerically but exceptions have been documented in the literature (Ahkong et al., 1975; Dancer, 1980; Vasil and Giles, 1975).

Protoplast fusion can be induced by inactivated viruses, electric fields, and fusogenic chemicals such as calcium ions and polyethylene glycol (PEG). Among bacterial geneticists, the fusogenic agent of choice is PEG, although the efficacy of Ca^{2+} and electrofusion has been demonstrated (Fodor and Alfoeldi, 1976; Kuta et al, 1985). Polyethylene glycol is the generic name for ethylene oxide polyethers, ranging in molecular weight from 200 to 20,000. Their primary industrial use is in the production of detergents and as bases for pharmaceuticals and cosmetics (Cox, 1978). The exact mode of action of PEG during protoplast fusion is not well understood. Apparently polymer bridging between cells as well as changes in membrane surface potential cause protoplasts to aggregate (Maggio et al., 1976). Ahkong and co-workers (1975) suggested a succession of two events during membrane fusion. First, perturbation of the bilayer structure of membrane lipids increases the fluidity of the lipid region resulting in aggregation of intramembranous proteins. Subsequently, protein-free lipid bilayer areas of adjacent membranes can interact and form bridges during the

resealing process. The perturbation of the membrane is believed to be caused by the temporary breakdown of the surface potential (Maggio et al., 1976; Zimmermann and Scheurich, 1981). Polyethylene glycol has been shown to decrease the surface potential of lipid bilayers by several hundred millivolts thus diminishing the electrostatic field which exists perpendicular to the membrane (Maggio et al., 1976). Frehel and colleagues (1979) as well as Sanchez-Rivas and Garro (1979) ascertained that the fusion of B. subtilis protoplasts involves a PEG-dependent membrane activation step and an energy consuming fusion step. Fusion appears to occur during or shortly after PEG treatment (Pontecorvo et al., 1977).

Following fusion, genetic interactions can take place because two or more copies of homologous DNA reside in the same cytoplasm for a period of time. Recombination occurs between chromosomes as well as between plasmids (Bedbrook and Ausubel, 1976). The time pattern of genetic recombination is of variable character. Hotchkiss and Gabor (1980) found that the post-fusion progeny of B. subtilis consisted of a minor fraction of stable recombinants and a majority of biparentals. The biparentals were mainly diploid cells which harbor both parental genomes but phenotypically express only one. This transient form segregated into diploid progeny, parental segregants, and late-appearing recombinants. The diploid

state lasted up to 100 cell generations and seemed to be the principle product of intertype protoplast fusion (Gabor and Hotchkiss, 1983). Similar results were obtained by Jones and colleagues (1985) for Clostridium acetobutylicum.

Practically, protoplast fusion entails mixing of parental protoplasts in the presence of PEG for various periods of time with subsequent diluting and plating on regeneration media. Goetz et al. (1981) found that addition of $CaCl_2$ to the fusion mixture increases chromosomal recombination in staphylococci but has no effect on the transfer of plasmids. Baltz and Matsushima (1981) postulated a connection between cell growth temperature and recombination frequency of Streptomyces. The optimal PEG concentration for most species seems to be close to 40 %, the polymer size being relatively unimportant (Gabor and Hotchkiss, 1979). Goetz and colleagues (1981) could not detect any relationship between exposure time of the mixed protoplasts to PEG and the rate of recombination over a 1- to 5-minute period, but prolonged exposure to PEG affects regeneration adversely (Gabor and Hotchkiss, 1979). Reverting fused protoplasts on crowded plates or on selective media exerts physiological stress on the protoplasts which results in lower or disproportionate numbers of recombinants (Gabor and Hotchkiss, 1983; Schaeffer et al., 1976). Recombination frequencies seem to be unrelated to the rate

of regeneration in B. subtilis. Fusion occurs at high frequency and appears to be adequate to produce substantially more recombinants than are detected (Gabor and Hotchkiss, 1979; Sanchez-Rivas and Garro, 1979). However, Baltz and Matsushima (1981) postulated a definite relationship between cell regeneration, fusion, and genetic recombination for Streptomyces.

CHAPTER III

MATERIALS AND METHODS

1. Test Organisms

Lactobacillus casei subsp. rhamnosus 21052 and Lactobacillus lactis 21051 were obtained from the American Type Culture Collection, Rockville, MD. Both strains were highly resistant to a variety of antibiotics (Green Cross Co., 1967). The cultures were maintained at 4C by monthly transfer in litmus milk (BBL; Cockeysville, MD) over solid $CaCO_3$ (Fisher Scientific Products; Fair Lawn, NJ) fortified with 0.5 % glucose (BBL) (Lee-Wickner and Chassy, 1984).

2. Media and Buffers

Lactobacillus Carrying Medium

Lactobacillus carrying medium (LCM) was prepared according to Efthymiou and Hansen (1962)(Appendix). Addition of 1.5 % agar (BBL) yielded LCM agar medium. The LCM broth and agar were sterilized at 121C for 15 min. LCM containing 0.1 % and 1 % glucose was prepared by adding appropriate amounts of a filter-sterilized 50 % glucose solution. Poured LCM agar plates were stored at 4C after autoclaving.

Regeneration Medium

Regeneration medium (RM) was prepared according to Lee-Wickner and Chassy (1984). It consisted of LCM base without Tween 80 (polyoxyethylene sorbitan monooleate) supplemented with 1 % glucose, 0.5 % bovine serum albumin (United States Biochemical Corp.; Cleveland, OH), 25 mM $MgCl_2$ (Aldrich Chemical Co.; Milwaukee, WI), 25 mM $CaCl_2$ (Sigma; St. Louis, Mo), 2.5 % gelatin (Difco; Detroit, MI), 0.3 M D-raffinose pentahydrate (US Biochemical), and 1.5 % agar. A 2.5 % bovine serum albumin (BSA Fraction V) stock solution was heat treated at 56C for 30 min and sterilized using a 0.45 um membrane filter. The 3 M stock solutions of anhydrous $CaCl_2$ and $MgCl_2$ were sterilized using a 0.22 um or 0.2 um membrane filter.

Upon storage of RM agar plates at 4C some of the D-raffinose recrystallized due to the high concentration used. Therefore, RM was kept not longer than three weeks in the cooler after autoclaving.

Protoplast Formation Buffer

Protoplast formation buffer (PB) consisted of 20 mM HEPES (N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid) (Sigma), pH 7.0, 0.5 % gelatin, and 0.3 M D-raffinose pentahydrate. As for LCM, the pH of PB was adjusted with 1 N NaOH employing a Fisher Accumet pH Meter Model 600. After autoclaving at 121C for 15 min, 1 mM $MgCl_2$ was added from a 3 M stock solution (Lee-Wickner and Chassy, 1984).

Muralytic Enzymes

Lyophilized mutanolysin (Sigma) was reconstituted according to manufacturer's instructions and sterilized using a 0.2 um or 0.22 um membrane filter. Aliquots of 0.5 ml were stored frozen over dessicant at -20C. Since loss of activity of this enzyme preparation was observed after three months, storage was limited to two months. For each experiment, frozen aliquots were thawed out at room temperature and diluted with PB to appropiate concentrations.

Solid lysozyme (grade I; Sigma) was weighed prior to experiments, dissolved in PB and sterilized using a 0.45 um membrane filter.

3. Growth of Test Cultures

Both strains were subcultured twice before each experiment (Lee-Wickner and Chassy, 1984). A 0.1 ml inoculum of L. casei subsp. rhamnosus was transferred from the maintenance medium to 10 ml of LCM-0.1% glucose and incubated at 37C for 16 h. Following incubation, 0.05 ml of this culture was transferred to 10 ml of LCM-0.1% glucose and grown at 30C for 16 h.

A litmus milk inoculum of L. lactis was subcultured into MRS broth (BBL) and incubated at 37C for 14 - 16 h. This culture then was transferred to LCM-0.1% glucose and grown again at 37C for 16 h. Inocula sizes and broth volumes were the same as for L. casei subsp. rhamnosus.

4. Growth Curves

Broth cultures of both strains were grown as described previously. A portion of the culture (0.04 ml) was transferred into 8 ml LCM in 13x100 mm cuvettes (Fisher). The change in absorbance due to the growth of L. lactis (37C) and L. casei subsp. rhamnosus (30C) over a period of 16 h was monitored with a Bausch & Lomb (Rochester, NY) Spectronic 20 spectrophotometer at a wavelength of 650 nm.

5. Genetic Marker Selection

LCM Evaluation

Cells of both strains were grown as described previously, washed twice by centrifugation at 9700xg for 10 min at 4C, and the pellets suspended in 10 ml sterile distilled water. Portions of the suspensions were removed, diluted in sterile distilled water and spread plated on LCM from which glucose, yeast extract, citrate, or combinations thereof had been omitted. Colonies were counted after 5 d of incubation at 37C in a Gas-Pak Anaerobic System.

Antibiotic Resistance

Centrifuged cell suspensions from both strains were prepared as outlined in LCM Evaluation. Appropriate dilutions in sterile distilled water were spread plated on LCM supplemented with 200 ug/ml or 300 ug/ml kanamycin

sulfate (Sigma) and incubated for 3 d at 37C in a Gas-Pak Anaerobic System. Additionally, dilutions were spread plated on LCM supplemented with 30, 40, 50, or 100 units/ml penicillin-G potassium salt (Sigma) and incubated under the same conditions. LCM supplemented with antibiotics was prepared by addition of appropriate aliquots of filter-sterilized aqueous stock solutions of kanamycin sulfate (50 mg/ml) or penicillin-G (15900 units/ml) to LCM after autoclaving. The stock solutions were stored at 4C for up to one week to avoid loss of activity upon storage.

6. Preparation of Protoplasts

The procedure of Lee-Wickner and Chassy (1984) was used with slight modifications. Previously grown cultures were harvested by centrifugation at 9700xg for 10 min at 4C using a refrigerated IEC centrifuge (Needham Heights, MA). The supernatants were decanted, the cells washed in 10 ml cold PB and centrifuged at 9700xg for 10 min at 4C. After decanting the supernatants, each pellet was suspended in 5 ml cold PB and chilled in ice. Aliquots of L. lactis suspension (2 ml) were mixed with the same volumes of PB containing mutanolysin yielding final enzyme concentrations of 10 ug/ml and 20 ug/ml. The mixtures were incubated for 20, 30, and 40 min at 37C with occasional agitation. L. casei suspension aliquots were treated similarly using enzyme concentration combinations of 10 ug/ml mutanolysin +

300 ug/ml lysozyme and 25 ug/ml mutanolysin + 300 ug/ml lysozyme. Incubation was conducted at 37C for 30, 60, and 90 min with occasional agitation.

After each time period portions of the mixtures were removed, diluted in sterile distilled water and spread plated on LCM-1% glucose agar. Colonies were counted after 48 h of incubation at 37C in a Gas-Pak Anaerobic System (BBL). The counts reflected the number of osmotically resistant cells and indicated the extent of the formation of protoplasts. Additionally, portions of the enzyme-protoplast mixtures were examined microscopically under a phase-contrast microscope (Bausch & Lomb) for remaining unprotoplasted cells.

7. Regeneration of Protoplasts

Protoplasts were prepared as outlined previously. After incubation with muralytic enzymes for the stated periods of time, portions of the mixtures were removed, diluted in PB and spread plated on RM. Plates were incubated at 30C in a Gas-Pak Anaerobic System. Counting of colonies was conducted after 5 and 6 d for L. lactis and L. casei, respectively.

In another series of experiments, dilutions were plated using the soft agar overlay method of Akamatzu and Sekiguchi (1984). A 0.1 ml portion of the diluted protoplast suspension was spread on the surface of RM

followed by an overlaying of 4 ml of RM containing 1 % agar. Incubation conditions were identical to the spread plating technique.

The regeneration frequency (RF) was computed for each strain using the following formula (Lee-Wickner and Chassy, 1984):

$$RF(\%) = \frac{(\text{CFU/ml on RM}) - (\text{CFU/ml on LCM})}{\text{initial CFU/ml}} \times 100$$

8. Fusion of Protoplasts

Both strains were protoplasted as was established in earlier experiments. Suspensions of protoplasted cells (8 ml) were mixed, centrifuged at 4300xg for 10 min at 4C, and the pellet suspended in 0.4 ml PB. To this suspension 3.6 ml of an autoclaved 40 % polyethylene glycol 8000 (PEG) (Fisher) solution in PB was added and the mixture incubated for 2 min at room temperature. Dilutions in PB were prepared, spread plated on RM, and incubated at 30C for 7 d in a Gas-Pak Anaerobic System. As a control, the above procedure was applied to L. casei and L. lactis protoplasts separately. Colonies were counted to ascertain the effect of PEG on the regeneration frequency of both strains. Plates with sufficient growth were replica-plated to LCM supplemented with 300 ug/ml kanamycin sulfate and 100 units/ml penicillin-G potassium salt with the help of RepliPlate colony transfer pads (FMC Corp.: Rockland,

Maine). After 3 d of incubation at 37C in a Gas-Pak Anaerobic System, plates were screened for the presence of colonies.

9. Statistical Analysis

Optimization procedures for protoplasting and regeneration of both bacterial strains were analyzed by Analysis of Variance (ANOVA). A 3x3 factorial arrangement of treatment means in a Randomized Complete Block design was used. Any significant differences ($P<.05$) detected among the treatments in the ANOVA were separated by Duncan's Multiple-Range Test.

CHAPTER IV

IV. RESULTS AND DISCUSSION

1. Growth of *L. lactis* 21051 and *L. casei* subsp. *rhamnosus* 21052

Attempts to transfer *L. lactis* from the litmus milk medium to LCM were not successful due to insufficient growth. Therefore, litmus milk cultures of this strain were subcultured into MRS broth (DeMan et al., 1960), yielding satisfactory growth. Aliquots of a *L. lactis* culture in MRS and a *L. casei* culture in LCM were then transferred to LCM broth and grown for 16 h. The change in absorbance was monitored spectrophotometrically at 2 h intervals (Fig. 1). The objective of this experiment was to ascertain the point in time when both strains reached the stationary phase.

The growth patterns of the two *Lactobacillus* species differed slightly (Fig. 1). *Lactobacillus casei* had a lag time of 3 h and reached the stationary phase after 15 h. In comparison, the lag time of 6 h for *L. lactis* was rather long, but the stationary phase started after only 11 h. These results are in agreement with growth studies conducted for various lactobacilli in LCM (Chassy and Giuffrida, 1980). These researchers also reported that lactobacilli harvested at the stationary phase were more susceptible to the action of lysozyme than log phase cells.

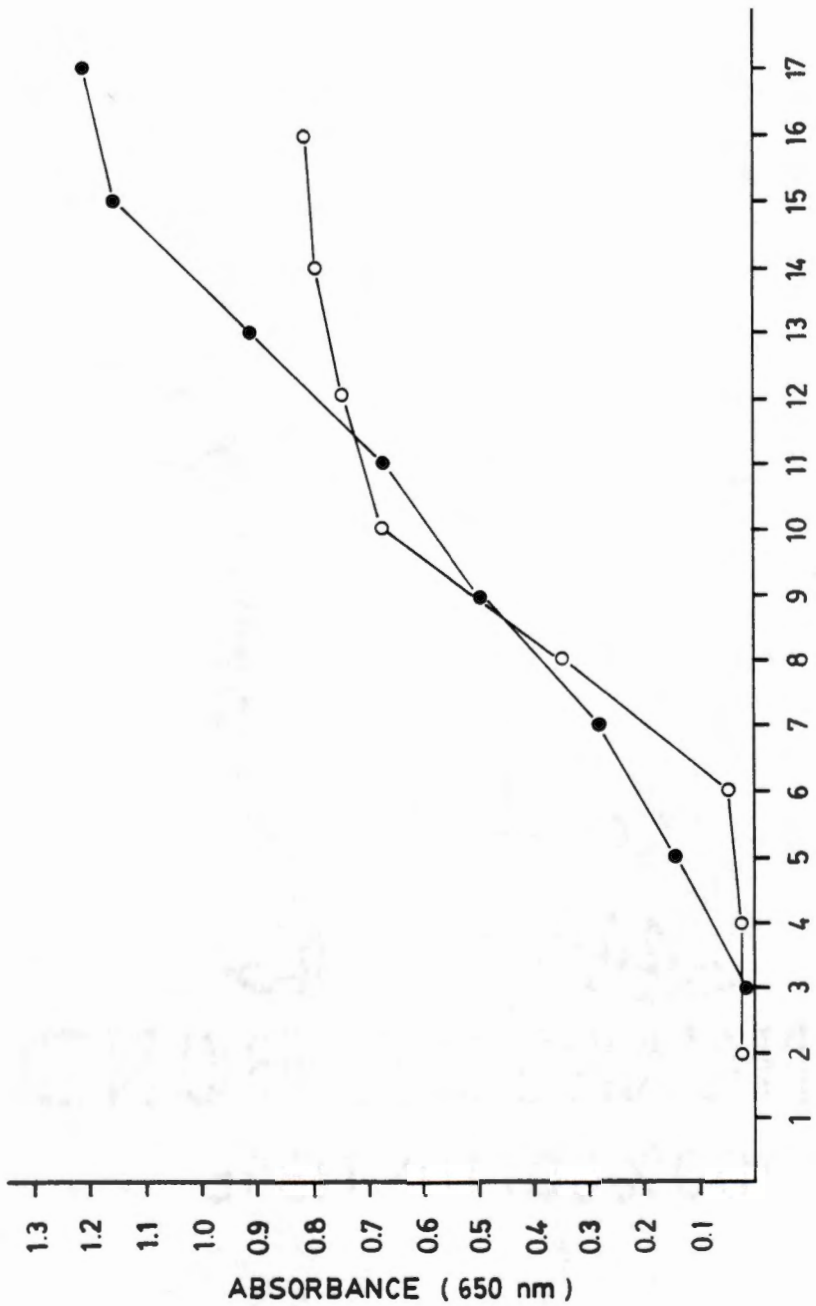

Fig. 1. The growth curves of two Lactobacillus strains in LCM broth, where each point represents the mean of two replicates. (●—●) L. casei at 30C; (o—o) L. lactis at 37C.

Later, Lee-Wickner and Chassy (1984) used 16 h old cultures of various L. casei strains for successful protoplastization. Since both strains used in this study reached stationary growth phase in 16 h, this time was used for subsequent experiments.

2. Selection of Genetic Markers

In order to detect recombinant cells after protoplast fusion, the parental cells had to possess distinct genetic properties, so-called genetic markers. Such genetic markers could be sugar fermentation characteristics as well as resistance to antibiotics.

LCM Evaluation

The objectives of this experiment, in which both strains were plated on LCM from which various nutrient components had been omitted, were twofold. Firstly, was to determine the metabolic utilization patterns of both strains. Secondly, was to assess the suitability of LCM as a selection medium by using the distinct sugar fermentation patterns of both strains as genetic markers. An extensive literature search revealed that two components of LCM, yeast extract and citrate, had the potential to interfere with the sugar fermentation markers. Table 1 shows the effect of the omission of ammonium citrate and yeast extract on the recovery of both strains used in this study.

Table 1. Effect of the Omission of LCM Components on the Recovery of L. casei subsp. rhamnosus 21052 and L. lactis 21051 after Incubation for 5 d at 37C.

Omitted Component	Replication I		Replication II	
	L. lactis	L. casei	L. lactis	L. casei
	$\log_{10}$ CFU/ml			
None	7.23	7.75	7.26	8.49
YE[a]	7.15	7.76	7.30	8.53
Cit[b]	7.11	7.76	7.36	8.46
YE+Cit	7.23	7.82	7.30	8.51
YE+Glu[c]	6.18	6.38	6.11	7.48
Cit+Glu	6.93	7.36	6.78	7.52
YE+Cit+Glu	5.78	7.46	6.43	7.18

[a]Yeast extract.

[b]Ammonium citrate.

[c]Glucose.

As long as glucose was inclued in the medium, the omission of yeast extract, citrate, or both did not affect growth of either strain. Other researchers have found that addition of these compounds to media for lactobacilli resulted in improved growth (Campbell and Gunsalus, 1944; Evans and Niven, 1951; DeMan et al., 1960). Apparently that was not true for the strains used in the present study. In the absence of glucose and yeast extract or ammonium citrate, the recovery of both strains was decreased by about one log cycle. This result could be explained by the fact that yeast extract contains unspecified glycogen breakdown products and trehalose as residual carbohydrates (Bridson, 1978). According to Bergey's Manual of Determinative Bacteriology (1977), both species used in this study were able to utilize trehalose as a carbon source. Furthermore, Campbell and Gunsalus (1944) found that a number of homofermentative streptococci and lactobacilli, including some L. casei and L. lactis strains, use citric acid as an energy source for growth in the absence of fermentable carbohydrates. Surprisingly, the omission of yeast extract, citrate, and glucose did not reduce the cell numbers of either strain by more than 90 %. Lactobacilli normally grow poorly on protein media without carbohydrates (Topley and Wilson, 1983). While the number of colony forming units (CFU) was high, the colonies on the plates with all three components omitted did not reach normal size

and usually remained small. No logical explanation for these findings can be put forward at the present time. Nevertheless, the results obtained in this experiment did show that sugar fermentation markers could not be used in conjunction with LCM as the basic medium. Since synthetic growth media for lactobacilli such as the one developed by Ledesma et al. (1977) are prohibitively laborious to prepare for routine experiments, the intention to use sugar fermentation markers had to be abandoned.

Antibiotic Resistance

Lactobacillus lactis 21051 and L. casei subsp. rhamnosus 21052 are both highly resistant to a host of antibiotics (Green Cross Co., 1967). Preliminary experiments indicated that only two antibiotic markers were amenable for further investigation, either due to unreliability of most markers or the extent of resistance. The objective of this set of experiments was to determine the suitability of penicillin and kanamycin resistance as reliable genetic markers.

There was a large difference in resistance between strains to kanamycin sulfate (Table 2). The results clearly showed that L. casei was virtually nonresistant to the antibiotic at the concentrations employed. This was in accordance with the kanamycin resistance of this strain specified in British Patent 1190386 (Green Cross Co.,

Table 2. Resistance of L. lactis 21051 and L. casei subsp. rhamnosus 21052 to Kanamycin Sulfate on LCM after Incubation for 3 d at 37C.

Rep	Concentration (ug/ml)	L. casei[a]	L. lactis[b]
		------ CFU/ml ------	
I	200	10^2	6.2×10^5
	300	<10	3.8×10^5
II	200	<10	6.9×10^5
	300	<10	6.0×10^5

[a]Initial CFU/ml: 4.9×10^8.

[b]Initial CFU/ml: 4.6×10^7.

1967). In contrast, L. lactis proved to be resistant to kanamycin at 200 and 300 ug/ml. However, the decrease in cell number of approximately two log cycles was fairly large. A review of British Patent 1190386 (Green Cross Co., 1967) confirmed that the strain should have resistance to the level of antibiotic tested but did not allow any specific comparisons concerning numbers of resistant cells because the patent simply indicated the presence or absence of growth. Nevertheless, the results did demonstrate a definite difference in resistance to kanamycin between the two Lactobacillus strains.

The difference in resistance of L. lactis and L. casei to penicillin-G was also unequivocal. Exposure of L. lactis to the antibiotic caused a reduction in viable cells to below the detection limit of the test system (Table 3). Conversely, L. casei exhibited appreciable resistance, although the decrease in viable cell numbers was quite severe. Again, these results agreed with British Patent 1190386 (Green Cross Co., 1967) without allowing any comparisons of resistant cell numbers in a given population.

In conclusion, a marked difference in the resistances of both strains to kanamycin sulfate and penicillin-G potassium salt was detected. This substantiated the suitability of these genetic properties as selection markers which would allow the detection of fused cells.

Table 3. Resistance of L. lactis 21051 and L. casei subsp. rhamnosus 21052 to Penicillin-G Potassium Salt on LCM after Incubation for 3 d at 37C.

Rep	Concentration (units/ml)	L. lactis	L. casei
		------ CFU/ml ------	
I	50	<10[a]	5.1 x 10^{4}[b]
	100	<10	2.3 x 10^{4}
II	50	<10[c]	6.0 x 10^{4}[d]
	100	<10	3.4 x 10^{4}

[a] Initial CFU/ml: 2.5 x 10^{8}.

[b] Initial CFU/ml: 6.5 x 10^{7}.

[c] Initial CFU/ml: 3.4 x 10^{8}.

[d] Initial CFU/ml: 8.2 x 10^{7}.

3. Protoplast Formation and Regeneration

Protoplast Formation

The starting point for this set of experiments was the paper by Lee-Wickner and Chassy (1984) which was the first published report of protoplastization and cell wall regeneration at high frequency for the genus Lactobacillus. The objective was to achieve a reduction in osmotically stable cells of at least 99 % by using various mutanolysin and lysozyme concentrations and incubation times. Protoplast formation conditions which enabled Lee-Wickner and Chassy to regenerate the cell walls of several L. casei strains at appreciable frequencies were used as guidelines. It was found that all protoplastization conditions tested were suitable for reducing the number of osmotically resistant L. casei subsp. rhamnosus cells at least two log cycles (Table 4). When incubation times of less than 30 min were used, protoplast formation was insufficient (data not shown). Combinations with mutanolysin at 25 ug/ml generally produced less osmotically stable cells than at 10 ug/ml (Table 4). While protoplasts of L. casei could only be obtained by the combined action of mutanolysin and lysozyme, mutanolysin alone was sufficient for protoplastization of L. lactis (Table 5). The time required with the muralytic enzyme was also lower than for L. casei. This supported findings by several researchers that some lactobacilli can be protoplasted by the action of

Table 4. Protoplast Formation of L. casei subsp. rhamnosus 21052.

Enzyme Concentration[a]	Time (min)	Log_{10} CFU/ml[b]	% PF[c]
10 + 300	30	5.06	99.86
	60	3.71	99.99
	90	3.57	>99.99
25 + 300	30	4.51	99.96
	60	3.55	>99.99
	90	2.00	>99.99

[a] ug/ml mutanolysin + ug/ml lysozyme.

[b] Average counts (two replications) on LCM after incubation for 48 h at 37C.

[c] % Protoplast Formation (PF) based on initial log_{10} CFU/ml of 7.89.

Table 5. Protoplast Formation of L. lactis 21051.

Enzyme Concentration[a]	Time (min)	Log_{10} CFU/ml[b]	% PF[c]
10	20	5.94	98.79
	30	5.33	99.69
	40	4.52	99.95
20	20	5.72	99.28
	30	4.27	99.97
	40	3.55	>99.99

[a]Mutanolysin in ug/ml.

[b]Average counts (two replications) on LCM after incubation for 48 h at 37C.

[c]% Protoplast Formation (PF) based on initial Log_{10} CFU/ml of 7.86.

mutanolysin without the presence of lysozyme (Lee-Wickner and Chassy, 1984; Shimizu-Kadota and Kudo, 1984; Tomochika et al., 1982). Table 5 also shows that, except for incubation with 10 ug/ml mutanolysin for 20 min, all treatments yielded >99 % reductions of osmotically resistant cells. However, in preliminary experiments 10 ug/ml mutanolysin was sufficient to produce protoplasts at a rate of >99 % within 20 min. Apparently 20 min was the incubation time threshhold for the enzyme concentrations used and therefore seemed less reliable than longer incubation periods. A graphical representation of the effect of time and concentration on protoplast formation clearly showed that it commenced more rapidly with both strains at higher mutanolysin concentrations (Fig. 2).

The formation of protoplasts of both strains was regularly verified with a phase-contrast microscope using the oil immersion objective. After 20 - 30 min the protoplasts became visible as small dark spheres, easily distinguishable from the larger rod-shaped cells which were rarely present. As time progressed, the dark spheres often gave rise to greyish, semi-translucent protoplasts, indicating a change in protoplasm density. These findings were in agreement with observations made by Barker and Thorne (1970) and Lee-Wickner and Chassy (1984) for L. casei.

The plating of muralytic enzyme-cell mixtures on LCM

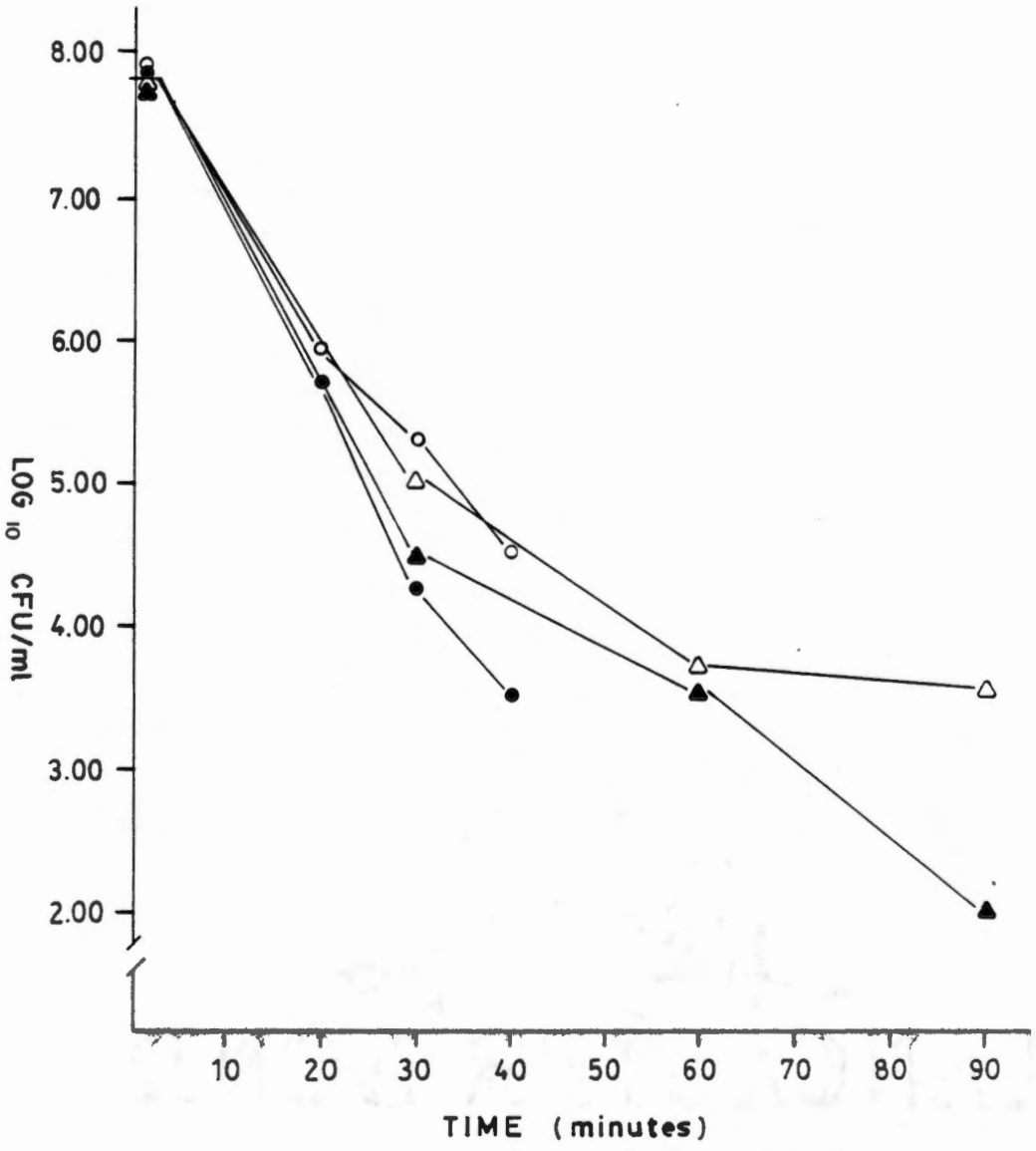

Fig. 2. Reduction of the number of osmotically stable cells as a function of the time of incubation with muralytic enzymes. L. lactis: (o—o) 10 ug/ml mutanolysin, (●—●) 20 ug/ml mutanolysin; L. casei: (△—△) 10 ug/ml mutanolysin + 300 ug/ml lysozyme, (▲—▲) 25 ug/ml mutanolysin + 300 ug/ml lysozyme.

without an osmotic stabilizer and dark-phase microscopy indicated that the cell wall loss was severe enough for the bacterium to lose its inherent shape and that the resulting "protoplasmic units" were osmotically sensitive. The magnitude of cell wall reduction could not be estimated by these methods. However, Lee-Wickner and Chassy (1984) examined L. casei protoplasts using gas chromatography and found that less than 5 % of the cell wall remained after treatment with muralytic enzymes.

Without determining the precise extent of cell wall removal, the present study did show that the number of osmotically stable cells could be decreased more than 99 % by almost all protoplastization treatments tested.

Cell Wall Regeneration

A study was then carried out in an attempt to regenerate the protoplasts and to determine how the regeneration treatments affected regeneration frequencies (RFs). The recovery and RF of L. casei subsp. rhamnosus protoplasts on RM is shown in Table 6. The pronounced decrease in regeneration capability for incubation times exceeding 30 min was unexpected. Prolonged exposure to muralytic enzymes apparently decreased the RF to nearly zero. This trend was apparent for both enzyme concentrations used (Fig. 3). In preliminary experiments, incubation periods of less than 30 min did not yield

Table 6. Regeneration of L. casei subsp. rhamnosus 21052 on RM after Incubation for 6 d at 30C.

Enzyme Concentration[a]	Time (min)	Log_{10} CFU/ml[b]	% RF[c]
10 + 300	30	6.74	6.9[d]
	60	5.70	0.6[f]
	90	5.34	0.4[f]
25 + 300	30	6.53	4.3[e]
	60	4.95	0.1[f]
	90	4.80	0.1[f]

[a] ug/ml mutanolysin + ug/ml lysozyme.

[b] Average of two replications.

[c] $\%RF = \frac{(\text{CFU/ml on RM}) - (\text{CFU/ml on LCM})}{\text{initial CFU/ml}} \times 100.$

Regeneration Frequency (RF) based on initial log_{10} CFU/ml of 7.89.

[d,e,f] Means with the same superscript are not significantly different ($p<0.05$).

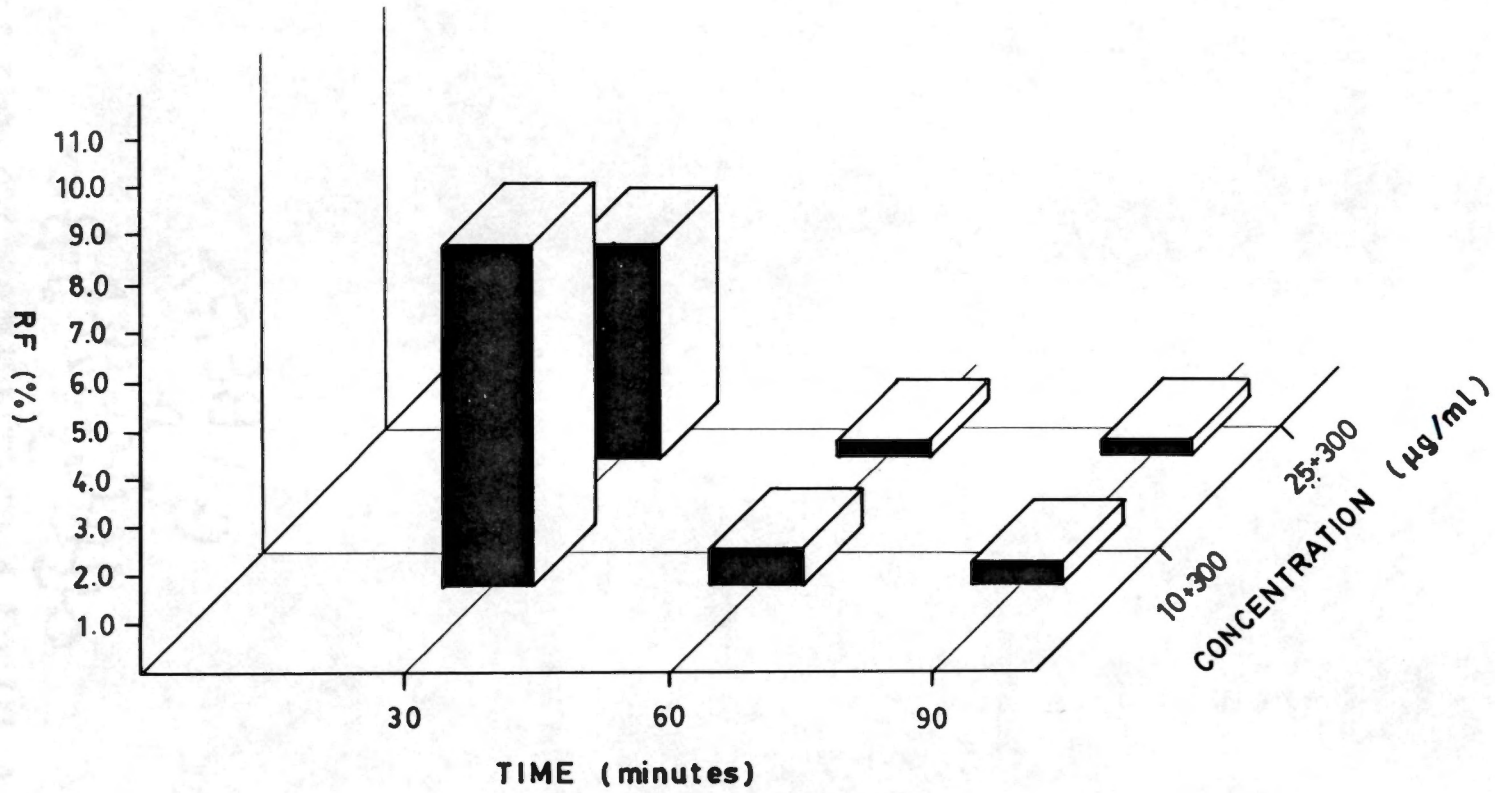

Fig. 3. The regeneration frequency (RF) of L. casei subsp. rhamnosus 21052 as a function of incubation time and muralytic enzyme concentration (mutanolysin + lysozyme).

sufficient formation of protoplasts of this strain. Therefore, successful regeneration of this strain was limited to a narrow range in time with the concentrations used. For that reason, while a difference in regeneration frequency between 10 and 25 ug/ml mutanolysin might have been statistically significant, it was of lesser importance than the effect of time on the regeneration ability of L. casei.

Similar results were obtained for L. lactis (Table 7). Incubation with 10 ug/ml mutanolysin for 20 min resulted in maximal wall regeneration; however, the extent of protoplast formation under these conditions was below the target level of 99 % (Table 5). Again, appreciable regeneration was limited to a relatively narrow range in time with the concentrations used (Fig. 4). Furthermore, regeneration of L. lactis was more reproducible than for L. casei. Likely factors to account for this discrepancy were the utilization of mono- vs. dienzymatic systems to protoplast cells of L. lactis and L. casei, respectively, as well as inherent differences in cell wall structure and composition of both strains. Additionally, lysozyme has been shown to break up chains of bacteria thus contributing to greater variability of the apparent number of colony forming units.

A comparative look at the reduction of osmotically stable cells and the corresponding RF's suggested that

Table 7. Regeneration of L. lactis 21051 on RM after Incubation for 5 d at 30C.

Enzyme Concentration[a]	Time (min)	Log_{10} CFU/ml[b]	% RF[c]
10	20	6.92	10.1[d]
	30	6.72	6.8[e]
	40	5.20	0.2[g]
20	20	6.45	3.1[f]
	30	5.69	0.6[g]
	40	5.36	0.3[g]

[a] ug/ml mutanolysin.

[b] Average of two replications.

[c] $\%RF = \frac{(\text{CFU/ml on RM}) - (\text{CFU/ml on LCM})}{\text{initial CFU/ml}} \times 100.$

Regeneration frequency (RF) based on initial log_{10} CFU/ml of 7.86.

[d,e,f,g] Means with the same superscript are not significantly different ($p<0.05$).

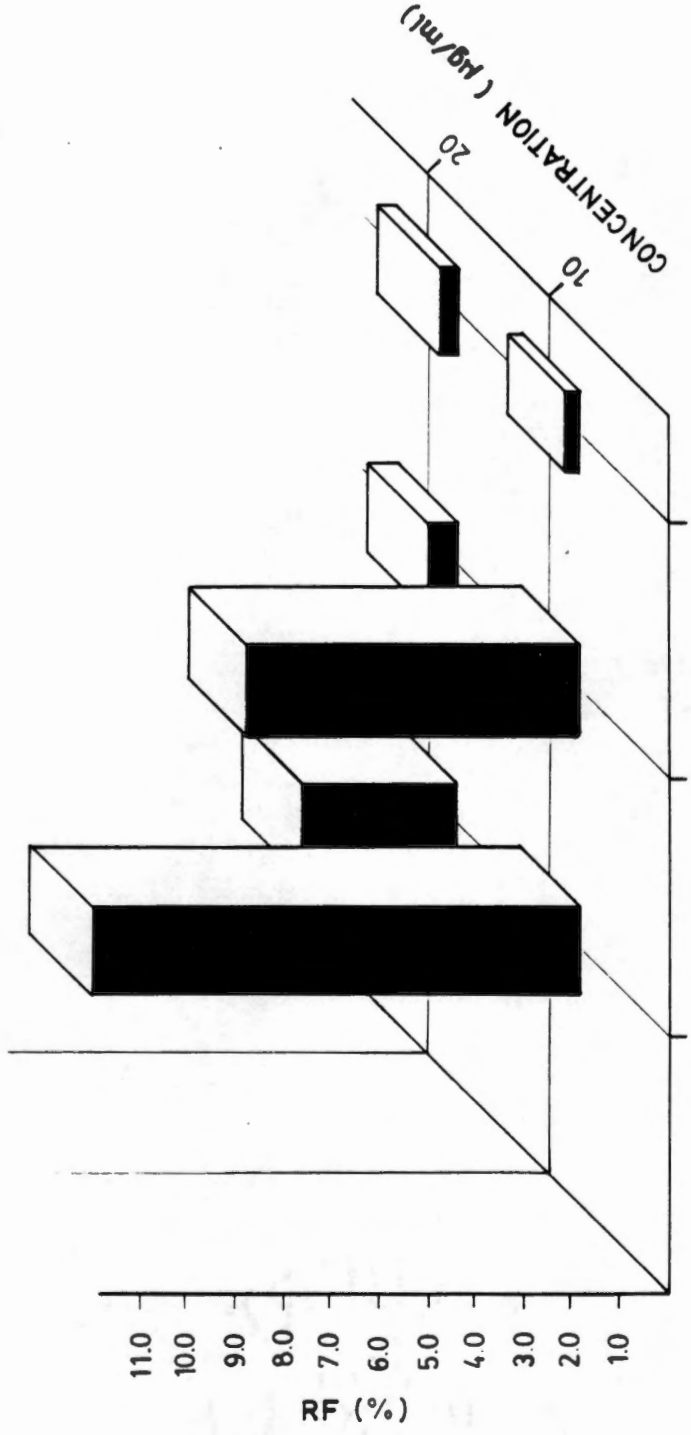

Fig. 4. The regeneration frequency (RF) of L. lactis 21051 as a function of incubation time and mutanolysin concentration.

excessive cell wall removal was counterproductive to successful regeneration (Tables 4 - 7). A possible explanation was provided by the hypothesis that the presence of residual wall primer and the preservation of the enzymatic activity of the wall biosynthetic system during protoplastization was crucial for regeneration (Miller et al., 1967; Lee-Wickner and Chassy, 1984).

Regeneration frequencies determined for both species used in this study were lower than those reported by Lee-Wickner and Chassy (1984). These researchers achieved RF's of 10 - 40 % for several L. casei strains. The genetic background as well as the different requirements of the individual strains for optimal media probably accounted for the varying RF's (Akamatsu and Sekiguchi, 1981; Lee-Wickner and Chassy, 1984). Two other research groups protoplasted and successfully regenerated lactobacilli. Vescovo et al. (1984) achieved RF's of 0.002 % and 0.2 % for L. reuteri strains and Finer and Klaenhammer (personal communication, 1986) regenerated L. bulgaricus protoplasts at frequencies of approximately 1 %. Unfortunately it was not clear how these researchers calculated the RF's, making comparisons of their results with those attained by this study impossible. In contrast to genera such as Bacillus (10 - 90 %), Clostridium (80 %), and Streptomyces (100 %), RF's for lactobacilli seemed very low (Akamatsu and Sekiguchi, 1984; Jones et al., 1985; Baltz and Matsushima,

1981). It could be envisioned that the lower growth rate and greater fragility of Lactobacillus protoplasts had an effect (Lee-Wickner and Chassy, 1984). Also, the lack of knowledge of all regeneration requirements of this genus could have been responsible for the comparatively low RF's.

Spread Plating Versus Overlay Technique

Protoplasts of both strains were prepared as previously outlined. Aliquots of dilutions in PB were either spread on RM or spread and subsequently overlayed with 4 ml RM. The objective of this experiment was to determine if the two plating methods differed in their effect on the RF of both strains. Tables 8 and 9 show that the overlay method did not affect the regeneration ability of L. casei or L. lactis. This was in sharp contrast to results of other research groups. Stal and Blaschek (1985) reported a tenfold RF reduction of Clostridium perfringens by using the overlay technique. In contrast, regeneration of various Bacillus species on semi-synthetic media was improved 40- to 10,000-fold by this method (Akamatsu and Sekiguchi, 1984). The reason why the overlay method produced such varied results is not easily understood. Shirahama and colleagues (1981), working with Streptomycetes, found that protoplasts were very heat sensitive. Heat damage might be a possibility to explain the RF reduction with C. perfringens. An adverse effect of

Table 8. Effect of Two Plating Methods on the RF of L. lactis 21051.

Rep	Enzyme Concentration[a]	Spread plate Method	Overlay Method
		------ % RF[b] ------	
I	10	4.9	5.1
	20	1.0	1.3
II	10	5.7	7.2
	20	1.3	1.2

[a] ug/ml mutanolysin.

[b] $\%RF = \frac{(CFU/ml \text{ on RM}) - (CFU/ml \text{ on LCM})}{\text{initial CFU/ml}} \times 100$

Table 9. Effect of Two Plating Methods on the RF of L. casei subsp. rhamnosus 21052.

Rep	Enzyme Concentration[a]	Spread plate Method	Overlay Method
		% RF[b]	
I	10 + 300	4.0	3.7
	25 + 300	1.0	2.0
II	10 + 300	2.9	3.4
	25 + 300	2.3	1.0

[a] ug/ml mutanolysin + ug/ml lysozyme.

[b] $\%RF = \frac{(CFU/ml \text{ on } RM) - (CFU/ml \text{ on } LCM)}{\text{initial } CFU/ml} \times 100$

the overlay agar temperature of 45C on the regeneration ability of the two Lactobacillus strains could not be inferred from the data.

The agar overlay technique was first employed by Fodor et al. (1975) to enhance the poor reversion of B. megaterium protoplasts to the bacillary form. Although these researchers did not elaborate on the exact mechanism of improving regeneration by embedding protoplasts in agar, it was assumed that the more solid environment favored cell wall regeneration. Landman and Forman (1969) postulated that a physically solid environment in contact with the protoplast surface was essential for successful regeneration. Not enough knowledge of how agar overlays affect the regeneration of protoplasts is available. Furthermore, the advantage of this technique has only been established for a limited number of genera (Akamatsu and Sekiguchi, 1984).

4. Attempted Protoplast Fusion of L. lactis 21051 and L. casei subsp. rhamnosus 21052

Effect of PEG on the Regeneration Frequency

The objective of this experiment was to determine the effect of PEG on the regeneration ability of protoplasts of both strains. In the presence of PEG a drastic reduction in the regeneration frequencies occurred (Table 10). The magnitude of reduction ranged from approximately 180-fold

Table 10. Effect of Polyethylene Glycol (PEG) on the Regeneration Capacity of L. lactis 21051 and L. casei subsp. rhamnosus 21052.

Rep	L. casei[a] 0 % PEG	L. casei 40 % PEG	L. lactis 0 % PEG	L. lactis 40 % PEG
I	3.62[b]	0.02	5.18	0.03
II	5.00	<0.01	5.48	<0.01

[a]Molecular weight of PEG, 7000-9000.

[b]Numbers in table are regeneration frequencies expressed as percentages.

to over 500-fold for both strains. Several researchers have reported on the inability of many protoplasts to recover after PEG treatment. Gabor and Hotchkiss (1979) observed a tenfold decline in the regeneration frequency of B. subtilis. Baltz and Matsushima (1981), working with Streptomyces fradiae, detected a sixfold reduction in colony formation after treatment with 30 % PEG. A 68 % reduction in viable counts of several Streptomyces species was reported by Godfrey et al. (1978). To date, two possible explanations to account for the observed decline in RF have been put forward in the literature. Baltz and Matsushima (1981) postulated that decreasing regeneration was solely the result of protoplast aggregation to form multicellular units. However, Gabor and Hotchkiss (1979) found that aggregation of B. subtilis protoplasts was reversible. Furthermore, the multicellular complexes tended to be small in number of protoplasts. A more likely explanation for the decrease of the RF is the interaction of PEG with the microbial membrane, affecting the viability of protoplasts adversely (Kuta et al., 1985). Clearly, further basic research is needed to elucidate the effect of PEG on biomembranes.

Why the RF of the two Lactobacillus strains used in this study decreased several hundredfold after PEG treatment was not easy to explain. Inspection of the PEG-treated protoplasts by light microscope revealed

extensive clumping. However, protoplast aggregation alone could not account for the magnitude of the RF decline. More than likely the PEG at the 40 % level exerted a toxic effect on the protoplasts. Possibly the preservation of the cell wall biosynthetic system was impaired. Since Lactobacillus protoplasts, even in the absence of PEG, were difficult to regenerate, treatment with PEG could have amplified inherent problems with successful cell wall regeneration of this genus. Lee-Wickner and Chassy (1984) suggested that regeneration of lactobacilli might proceed in a more complex manner than observed for other bacterial genera. Clearly then, the greater complexity of the regeneration process would have made the protoplasts more susceptible to any adverse effect PEG might have. More research is needed to investigate whether the protoplast regeneration of lactobacilli is more severely inhibited by PEG than detected for other bacteria.

Attempted Protoplast Fusion

The objective of this experiment was to determine if protoplasts of these strains could be fused under the given experimental conditions. Table 11 shows the occurrence of prototrophic cells which were able to grow in the presence of 300 ug/ml kanamycin sulfate and 100 units/ml penicillin-G potassium salt. Since prototrophs derived from separated L. casei subsp. rhamnosus and L. lactis

Table 11. Occurrence of Antibiotic Resistant Prototrophs after PEG-induced Fusion of L. casei subsp. rhamnosus 21052 and L. lactis 21051 Protoplasts.

Rep	CI[a] CFU/ml[d]	CI[a] Freq.[e]	CII[b] CFU/ml	CII[b] Freq.	T[c] CFU/ml	T[c] Freq.
I	$1.0x10^1$	0.036	$1.0x10^1$	1.0	$1.0x10^1$	0.07
II	$1.3x10^2$	4.3	$5.0x10^1$	2.6	$2.3x10^2$	2.3

[a]Control group I: Fusion performed with L. casei subsp. rhamnosus 21052 protoplasts.

[b]Control group II: Fusion performed with L. lactis 21051 protoplasts.

[c]Treatment group: Fusion performed with protoplasts of both strains.

[d]Number of prototrophs growing in the presence of 300 ug/ml kanamycin and 100 units/ml penicillin-G on LCM after incubation for 3 d at 37C.

[e]Frequency of prototrophs ($x10^{-7}$) per total input protoplasts.

protoplasts could not have been generated by interspecific fusion events, the only logical explanation for their presence was spontaneous mutation of the respective resistance genes. The observed frequencies corresponded well to the mutation rate range of 3.0×10^{-6} to 1.0×10^{-10} for antimicrobial resistance genes (Kiser et al., 1969). When protoplasts of both Lactobacillus strains were mixed in the presence of PEG, the number of prototrophs did not increase (Table 11). In fact, no difference of the treatment group (T) and the two control groups (CI,CII) could be detected. If any interspecific fusion events took place in the treatment group they could not be discerned against the spontaneous mutation background noise. The low regeneration frequency after PEG treatment made the detection of prototrophs extremely difficult. Since the fusion event itself usually occurs at a low frequency, the combined effect of these two factors limited the chances to detect fused cells in appreciable numbers. Furthermore, Baltz and Matsushima (1981) showed for Streptomyces that protoplasts which regenerated poorly did not fuse efficiently and yielded low frequencies of genetic recombination.

The absence of detectable prototrophs did not preclude the existence of fusion. Hotchkiss and Gabor (1980) postulated that the principle fusion products of B. subtilis protoplasts were diploid cells which harbored both

parental genomes but phenotypically expressed only one. Those cells would not have been able to grow upon transfer from RM to LCM supplemented with kanamycin and penicillin. Another possibility would have been the successful completion of the fusion step without subsequent genetic recombination. That could have been due to a hypothetical restriction enzyme system present in the parental protoplasts, leading to the degradation of foreign DNA. Another question was whether kanamycin and penicillin resistance were plasmid- or chromosome-coded. This was not known for lactobacilli. Several researchers have demonstrated that the transfer of plasmids occurred with greater efficiency than did the recombination of chromosomal markers (Dancer, 1980; Goetz et al., 1981).

The present study did not produce experimental evidence to substantiate any of these hypotheses. Therefore, it was concluded that protoplast fusion of the two Lactobacillus strains used in this study could not be achieved under the given conditions.

CHAPTER V

CONCLUSIONS

Lactobacillus casei subsp. rhamnosus 21052 and L. lactis 21051 cells were successully protoplasted and regenerated using the procedure of Lee-Wickner and Chassy (1984). Differences in regeneration frequencies between strains were most likely due to inherent genetic differences and requirements for optimal conditions of protoplast preparation and regeneration. Efficient regeneration could only be achieved under optimal conditions; deviations from these conditions resulted in failure of the protoplasts to regenerate. Whether this was solely caused by experimental factors or whether the genetic material of the microorganisms played a role could not be ascertained.

Treatment with polyethylene glycol (PEG) reduced the regeneration frequency of both strains several hundredfold. This result suggests that protoplasts of lactobacilli may be more fragile and more susceptible to any adverse effects of PEG than protoplasts of other bacterial genera.

Attempts to fuse protoplasts of the two strains used in this study were not successful. The low regeneration frequency after PEG treatment drastically reduced chances to detect kanamycin- and penicillin-resistant prototrophs which potentially could have been produced by fusion.

This study clearly indicates that further research is needed in order to make progress in the field of protoplast fusion of lactobacilli. Firstly, regeneration media of more defined composition have to be developed in order to use nutritional and fermentation properties of lactobacilli as reliable genetic markers. The sole dependence on antibiotic markers is not to be recommended for food-related research. Secondly, more auxotrophic mutants of Lactobacillus strains with a variety of genetic markers have to be generated. Finally, research to elucidate the effect of PEG on the cell membrane of lactobacilli is essential. Feasible alternatives to the use of PEG may have to be considered in the future.

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APPENDIX

COMPOSITION OF LCM[a] (1000 ml)

Trypticase	10 g
Yeast extract	5 g
Tryptose	3 g
K_2HPO_4	3 g
KH_2PO_4	3 g
Ammonium citrate	2 g
Tween 80	1 g
Sodium acetate	1 g
L(+)-cysteine hydrochloride	0.2g
salt solution	5 ml

pH adjusted to 6.8 with 1 N NaOH

Salt solution: 11.5 g $MgSO_4*7H_2O$, 1.68 g $FeSO_4*7H_2O$, 2.4 g $MnSO_4*2H_2O$ in H_2O to 100 ml

[a]Efthymiou and Hansen (1962).

VITA

Michael Jantschke was born on May 15, 1958 in Weilburg, Federal Republic of Germany (FRG). Upon graduating from Gesamtschule Kirchhain, FRG, in June 1977, he joined the German army for compulsory military service. In October 1978 he entered the Philipps-Universitat, Marburg, FRG, and in April 1981 he received the Vordiplom in Biology. The following September he participated in an ISEP exchange program with the University of Tennessee, Knoxville. He re-entered the Graduate School of the University of Tennessee, Knoxville, in September 1983, and began work towards a Master of Science degree in Food Technology and Science.