THE DEVELOPMENT OF *PEDIOCOCCUS* SPECIES AS STARTERS
FOR MOZZARELLA CHEESE

by

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ABSTRACT

The Development of *Pediococcus* Species as Starters for Mozzarella Cheese

by

Shelby L. Caldwell, Doctor of Philosophy
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Bacteriophage infection of *Streptococcus thermophilus* is a growing concern in the mozzarella cheese industry. One method to control this problem may be to replace *S. thermophilus* with a starter coccus from a different genus of lactic acid bacteria. This work evaluated the possibility of using genetically modified *Pediococcus* spp. for this approach. Electroporation was used to introduce genes for lactose utilization from *Lactococcus lactis* into strains of *P. acidilactici* and *P. pentosaceus*. The resulting lactose-positive transformants, *P. acidilactici* SAL and *P. pentosaceus* SPL-2, rapidly reduced the pH of lactose broth, accumulated [14C]lactose at a rate higher than a lactococcal control, and showed relatively high phospho-β-galactosidase activity.

When paired with *Lactobacillus helveticus* LH100 in 9% reconstituted skim milk, *P. acidilactici* SAL and *P. pentosaceus* SPL-2 demonstrated synergistic growth with LH100. Milk fermented with *Pediococcus*-LH100 starter pairs also contained significantly less free galactose than milk fermented with a control starter blend of LH100 and *S. thermophilus* TA061. Mozzarella cheese made with lactose-positive *Pediococcus*-LH100 blends was compositionally similar to cheese made with the control starter blend, but production required 60-90 additional minutes.
In an attempt to decrease the time required to produce mozzarella, *Pediococcus* spp. were transformed with lactococcal genes for an extracellular serine proteinase or an oligopeptide transport system. Constructs which expressed each system were obtained, but these strains did not display improvement in the ability to clot 9% reconstituted skim milk.

Studies to screen *P. acidilactici* and *P. pentosaceus* for lysogeny detected temperate bacteriophage in three strains of *P. acidilactici*. Morphological characterization of these new phages demonstrated that they had small isometric heads with non-contractile tails and thus belonged to the B1 group of the family *Siphoviridae*. Further characterization based on DNA-DNA homology and protein profiles suggested that the *P. acidilactici* phages can be separated into at least two different species.

As a whole, the results reported here suggest that due to their slower growth in milk, *P. acidilactici* SAL and *P. pentosaceus* SPL-2 cannot be used as direct replacements for *S. thermophilus* but may be suited for use as adjuncts to the traditional *S. thermophilus/Lactobacillus* sp. starter blend.
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Shelby L. Caldwell
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MOZZARELLA CHEESE PRODUCTION

Mozzarella is a soft, pulled curd variety of Italian cheese. This classification is based on a manufacturing step wherein the curd is given a high temperature (82°C) stretch to generate characteristic body and texture. Bacterial starter cultures used in the production of mozzarella cheese involve a combination of *Streptococcus thermophilus* and either *Lactobacillus delbrueckii* subsp. *bulgaricus* or *Lactobacillus helveticus* (44, 56). Microbiological analysis of milk fermentations that include *S. thermophilus* and *Lactobacillus* spp. has demonstrated that these bacteria undergo synergistic growth and lactate production in milk. *Streptococcus thermophilus* is the primary acid producer in the pair, initially decreasing the pH rapidly to a level that favors the more acid tolerant *Lactobacillus* (22, 44). The exocellular proteinase of the *Lactobacillus* sp. hydrolyzes caseins, releasing peptides and free amino acids into the medium which stimulate the growth of the less proteolytic *S. thermophilus* (22, 49).

In the United States, production of natural cheese has historically been dominated by Cheddar varieties. Over the past 25 years, however, dramatic growth in the domestic pizza industry has increased the demand for mozzarella cheese to a point where annual production is essentially equal to that of Cheddar cheese (38, 44, 56). Increases in mozzarella production have not been accompanied by concomitant expansion in the number of production facilities, so the rise in mozzarella output has largely occurred at the expense of existing production facilities and schedules (27, 56). As the amount of milk processed each day increases, time available for the vat sanitation and cleaning of equipment between batches decreases. Similar conditions in the Cheddar cheese industry 40 years ago demonstrated that less efficient plant sanitation created greater opportunities.
for bacteriophage to emerge and proliferate. Consequently, tighter production schedules in mozzarella plants have served to create a bacteriophage crisis reminiscent of that seen in Cheddar cheese facilities (44, 56).

At present, it is the streptococci in the thermophilic starter blend that are generally the most susceptible to phage attack (44, 56). Unlike Cheddar vats, few complete failures of the mozzarella fermentation have been noted, probably due to the continued acid production by lactobacilli. Phage attack on *S. thermophilus*, however, causes a dramatic decrease in the rate of acid production which slows the entire cheese making process. Because the incidence of phage attack has increased, interruptions in the production schedule have become an important economic concern to mozzarella producers (5).

**NATURAL PHAGE RESISTANCE MECHANISMS**

Although research on the bacteriophage which infect *S. thermophilus* has intensified in recent years, information regarding phage resistance mechanisms in this and other thermophilic lactic acid bacteria remains limited (40, 44). Therefore, in an effort to curb the phage problem in mozzarella plants, companies have implemented many of the phage control measures originally developed to protect the mesophilic species, *Lactococcus lactis*, used in Cheddar cheese manufacture (56). These strategies include strain rotation with phage unrelated starters and selection of bacteriophage insensitive mutants (BIM) (27, 28, 56).

Natural lactococcal cellular defenses against bacteriophage can be classified into three main categories: interference with phage adsorption, restriction and modification, and abortive infection mechanisms. Each of these mechanisms targets a different stage in the phage infection cycle and when combined may have a synergistic effect on phage resistance (1).

The adsorption of a phage particle to the bacterial host is the first step in the
infection cycle. This process has been reported to occur in two stages (1, 19). The first stage is reversible and believed to involve a carbohydrate fraction of the bacterial peptidoglycan. The second stage involves irreversible attachment with a specific membrane protein (19). The *L. lactis* subsp. *lactis* receptor protein for the prolate phage c2 was cloned and characterized by Geller et al. (18). Designated phage infection protein (Pip), this protein has been shown to be essential for c2 infection of *L. lactis*, but it does not function in the adsorption of small isometric phages (1).

Resistance mechanisms that interfere with phage adsorption often involve changes in the cell surface receptors (19). For instance, Geller et al. (18) reported that by mutating the Pip protein in *L. lactis* LM2301, they obtained a construct that was completely insensitive to phage c2. Although this modification was engineered, other alterations in cell surface components occur spontaneously and are the basis for the isolation of the bacteriophage resistant mutants mentioned above (1).

A subset of adsorption inhibition mechanisms is referred to as DNA injection prevention. In these cases, the phages appear to adsorb normally to the cell surface, but their DNA does not enter the host cell. It has been proposed that such mechanisms may allow the nonspecific reversible adsorption, but interfere with the irreversible attachment which is necessary for DNA injection (19).

Restriction and Modification (R/M) defense mechanisms are common among the lactic acid bacteria (8). These systems are comprised of a sequence specific restriction endonuclease that cleaves foreign (e.g., phage) DNA, coupled with a DNA methylase that modifies the host DNA in a manner that protects it from the endonuclease (11). Unfortunately, these systems are not foolproof, and phage DNA which survives restriction may become modified and thus resistant to the R/M system (19).

Abortive infection (Abi) mechanisms are a diverse group of defenses that act after DNA injection, do not involve R/M, and generally result in the death of the host cell (1,
19, 52). The death of the cell traps the developing phage and prevents its proliferation in the environment (1). Although the exact mode of action in the majority of these systems remains undefined, studies have suggested that some may interfere with DNA replication (AbiA), degrade phage mRNA (AbiB), or inhibit phage protein production (AbiC) (1, 19). An advantage of Abi systems over R/M is that bacteriophage which escape the abortive infection response remain susceptible to the mechanism in any subsequent infections.

As mentioned above, the different types of phage resistance systems can be combined, either naturally or by design, to confer an increased level of phage resistance. An example of a naturally occurring complementation of phage defense systems is the resistance plasmid pTR2030, which carries the genes for the R/M system Lla1 and for the abortive infection system, AbiA (19). In 1986, Jarvis and Klaenhammer tested the effectiveness of pTR2030-encoded bacteriophage resistance in strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. * cremoris* (24). Those authors reported that pTR2030 conferred complete resistance against all small isometric-headed phages tested. Over time, however, small isometric phages which can overcome these resistance systems and proliferate in strains carrying plasmid pTR2030 have emerged (28).

**BACTERIOPHAGE EVOLUTION**

The effectiveness of bacterial phage resistance mechanisms is limited by the ability of the bacteriophage to mutate and overcome the host’s defenses. This evolution may involve a change in morphology, expression of proteins that inhibit the host’s endonuclease, or modification of the phage DNA (11, 28). These changes are often accomplished through genetic exchange of DNA modules with the bacterial host or other phages (1, 5). Such exchanges have resulted in the development of at least two phages that are resistant to the R/M system encoded by pTR2030 (28). The first resistant phage,
5

qmk202.50 or phage 50, was shown to have acquired a functional portion of the methylase component of the Llal R/M system (20), which rendered the phage resistant to the endonuclease. The second Llal resistant phage also obtained a portion of the methylase, but it is unknown whether this phage, which appeared after phage 50, received the gene from the host or from phage 50 (28).

Even more extensive phage adaptation was described by Moineau et al. (39), who reported the evolution of φul36 to φul37 by cassette exchange with the host chromosome. These acquisitions provided the phages with a new baseplate, a different origin of replication, and resistance to AbiC.

NOVEL BACTERIOPHAGE DEFENSE STRATEGIES

Several strategies have recently been proposed to increase the length of time that a phage defense system remains effective. These schemes include the phage defense rotation strategy, a triggered suicide response, phage-encoded resistance, and the use of antisense RNA (1).

The phage defense rotation strategy employs several derivatives of a single bacterial strain (L. lactis NCK203), which contain plasmids encoding various combinations of R/M and Abi systems (13, 53). These derivatives are used in a planned rotation scheme which challenges the phage with a succession of different defense mechanisms. When the same starter strain is used throughout the rotation, the diversity of bacteriophage in the plant environment should be decreased and the consistency of the product increased (13).

In the next scheme, triggered suicide response, a phage-induced promoter from φ31, is used to activate a bacterial suicide system (12). The result resembles an Abi response in that expression of the system leads to the death of the host cell before the phage progeny are released. Unfortunately, this system is based on a phage specific
promoter that is induced only by phages closely related to \( \phi 31 \).

Phage-encoded resistance (Per) also exploits a phage genetic element (19). In this system, a phage origin of replication is cloned into a high copy number plasmid, then transferred into a bacterial host. Phage infection of the host triggers replication of the plasmid, which competes for the phage replication factors and results in a decrease in phage DNA replication (19).

A fourth strategy uses a highly conserved phage gene cloned in reverse orientation to produce an antisense RNA (19). It is assumed that the antisense RNA and the corresponding phage mRNA form an unstable duplex, which is rapidly degraded. As a consequence, less phage protein and fewer phage progeny are produced (19). Although this strategy is intriguing, attempts to implement it have had limited success in reducing phage proliferation (1).

**BACTERIOPHAGE RESISTANCE IN**

**STREPTOCOCCUS THERMOPHILUS**

As mentioned above, information about naturally occurring phage resistance mechanisms in *S. thermophilus* remains limited. Four chromosomally encoded R/M systems have been identified in this species, but none have been cloned or sequenced (40). In addition, there has been one report describing the expression of a lactococcal resistance mechanism by a strain of *S. thermophilus*. Moineau et al. (40) showed expression of the *L. lactis* LlaII R/M system in *S. thermophilus* SMQ-119 decreased the efficiency of plaquing to \( 10^5 - 10^8 \), depending on the infecting phage. The barrier to industrial application of this approach, however, is the lack of a food-grade cloning vector that will replicate in *S. thermophilus* (40).
NEED FOR STRAIN DIVERSITY

Bacteriophage often exhibit narrow host ranges and so are able to infect only bacteria that are closely related to the strains from which they were originally isolated (57). However, even closely related bacteriophage can have slightly different specificities, and host ranges tend to overlap. Overlaps in phage host ranges are problematic to industry because very few strains have completely different phage types (27). In an effort to overcome this limitation, researchers at Oregon State University used molecular probes to screen environmental samples for new strains of \textit{L. lactis}. The isolation of new strains may provide a source of genetic diversity which will confer some protection against phage attack. This hypothesis was substantiated by the successful use of four of the newly isolated strains in cheesemaking trials (8).

An alternative approach for increasing strain diversity may be to develop new starter cultures from genera or species of lactic acid bacteria that are not currently used in dairy fermentations. Oberg and Broadbent (44) suggested lactose-positive (Lac\textsuperscript{+}) pediococci may be good candidates for this approach.

\textit{PEDIOCOCCUS SPECIES}

Pediococci are widely used in meat and vegetable fermentations, but their inability to rapidly ferment lactose has historically precluded them from consideration as starters in milk fermentations (44). However, lactose-negative pediococci have been used as adjuncts to accelerate ripening in Cheddar cheese (2, 3) and to improve the quality of Cheddar and mozzarella cheese (2, 50). At least two studies have shown low fat Cheddar cheese made with pediococci had a sharp Cheddar flavor and improved body and texture compared to cheese made without an adjunct or with \textit{Micrococcus} (2, 3). Those authors concluded that the improved flavor was a result of the production of sulfur compounds by
pediococci. Tzanetakis and Litopoulou-Tzanetaki (60) found strong leucine aminopeptidase and valine aminopeptidase activity in several *Pediococcus pentosaceus* strains and speculated that these activities could play a role in cheese ripening. These studies indicate pediococci would have good potential as dairy starters if the ability to utilize lactose could be introduced into these bacteria.

**LACTOSE METABOLISM**

The ability of a microorganism to metabolize lactose is dependent on enzymes which transport the molecule into the cell and cleave it into its component sugars. The lactose transport systems used by lactic acid bacteria can be divided into two major classes: phosphotransferase systems and permeases (7).

Many lactic acid bacteria use a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) to transport lactose or other carbohydrates across the cell membrane (36). The PTS system is comprised of four main proteins that are highly conserved across bacterial genera (36, 46). Two of the proteins, Enzyme I and the histidine-containing or heat-stable protein (HPr), are constitutively expressed, non-sugar specific enzymes. The other two proteins, Enzyme IIA and Enzyme IIBC, are carbohydrate specific enzymes whose genes are often encoded by a sugar-inducible operon. Lactose translocation by PTS results in the conversion of PEP to pyruvate with the simultaneous transport and phosphorylation of lactose (36, 46). The phosphorylated disaccharide, lactose-6-phosphate, is then cleaved into glucose and galactose-6-phosphate by phospho-β-galactosidase. These two monosaccharides undergo further catabolism via the glycolytic and tagatose-6-phosphate pathways, respectively. PTS activity has been detected in pediococci, but lactose-negative strains apparently do not contain the genetic information needed to produce the lactose specific proteins Enzyme IIA\textsuperscript{lac} and Enzyme IIBC\textsuperscript{lac} (51).
One lactic acid bacterium with the ability to utilize lactose via PTS is *L. lactis* (36), and the genes encoding this ability are plasmid-coded (17, 36). Genetic analysis of the *L. lactis* 712 lac plasmid has demonstrated that it carries genes for lactose specific proteins of the PTS system (*lacE* and *lacF*), phospho-β-galactosidase (*lacG*), and tagatose-6-phosphate enzymes (*lacA-lacD*) within a single operon (9). Other studies have shown the lactose plasmid of *L. lactis* C2, a close relative of 712, undergoes spontaneous deletions at low frequency to generate a 35 kb lactose plasmid which can be moved by transduction into other lactococci (17, 36).

Two types of permease systems have been identified in lactic acid bacteria (10). In both systems, lactose is transported into the cell without modification, then cleaved into glucose and galactose by β-galactosidase (*LacZ*). The glucose is phosphorylated by a hexokinase and enters the glycolytic system, while the galactose either enters the Leloir pathway or is excreted from the cell as part of an antiport system (10).

The first type of permease resembles the LacY system of *Escherichia coli* and is found in the atypical strain of *L. lactis*, NCDO 2054 (10). This system uses proton-coupled translocation to transport the sugar into the cell. It should be noted, however, that this system has a much higher affinity for galactose than for lactose and thus may actually be a galactose transporter (10, 45).

The second permease was first described in *S. thermophilus* and is mediated by the transport protein LacS (10, 45). This system can operate as either a proton symport or as a lactose-galactose antiport, depending on the intracellular galactose concentration (10). Therefore, in strains which do not metabolize galactose, such as *S. thermophilus* or *Lactobacillus bulgaricus*, LacS functions as an antiport, while in the galactose-positive strains of *Leuconostoc lactis* and *Lactobacillus helveticus* it operates as a proton symport (10).
GALACTOSE METABOLISM

Interest in galactose utilization by mozzarella starters has grown in recent years because residual galactose has been linked to excessive browning during cheese baking (44). Excessive browning decreases the value of the cheese because it makes the product (i.e. pizza) less desirable to the consumer. Unfortunately, nearly all strains of S. thermophilus are unable to ferment the galactose moiety of lactose, and it is excreted back into the cheese as part of the LacS-mediated antiport system (10, 23). As a result, galactose accumulates in the cheese and contributes to nonenzymatic browning reactions that occur during baking. Research has shown that use of galactose-positive mozzarella cheese starters such as Lactobacillus helveticus decrease the level of residual galactose and reduce the degree of nonenzymatic browning (22, 42). Most pediococci are galactose positive (15), and these bacteria have been used to decrease browning in mozzarella cheese (50).

GROWTH IN MILK

All lactic acid bacteria require one or more essential amino acids (30). Because concentrations of free amino acids in milk are limited, rapid growth in that medium requires enzymes for lactose and casein utilization (55). Studies in L. lactis have shown that an extracellular proteinase, an oligopeptide transport system, and one or more intracellular peptidases are required for cells to efficiently obtain amino nitrogen from casein (33, 43, 62).

EXTRACELLULAR PROTEINASE

The extracellular proteinase in L. lactis is a membrane-anchored enzyme whose production requires the expression of two genes, prtP and prtM (29, 30). The product of
prtP is an inactive preproproteinase, which is translocated across the membrane then activated by the removal of 154 N-terminal amino acids (29). The exact mechanism of activation is unknown, but it requires a membrane bound lipoprotein encoded by prtM (29, 30).

The degradation of casein by the extracellular proteinase yields small- to medium-sized oligopeptides (33). For example, studies with the purified proteinase demonstrated that cleavage of β-casein by PrtP releases peptides which vary in size from 4-30 amino acid residues with approximately 17% of the fragments under nine residues in length (25, 34). A similar study performed in vivo found similar patterns of protein degradation but failed to detect the release of free amino acids or peptides containing less than five residues (31).

**OLIGOPEPTIDE TRANSPORT SYSTEM**

Once formed, small oligopeptides are taken up by the oligopeptide transport (Opp) system, a member of the ATP-binding cassette family of uptake systems. These systems are comprised of a substrate-binding protein (OppA), two hydrophobic membrane-spanning proteins (OppB and OppC), and two ATP-binding proteins (OppD and OppF) (30, 59). The lactococcal Opp system is capable of transporting peptides of between 4 and at least 10 amino acids (31, 34, 59). Studies have indicated that Opp allows *L. lactis* to obtain all amino acids necessary for growth from the small oligopeptides obtained via proteinase-catalyzed casein degradation. Thus lactococci apparently do not require extracellular peptidase activity to supply their need for free amino acids (32).

Genes for the extracellular proteinase and the Opp system in *L. lactis* C2 are located on the same plasmid as the lactose operon (62). It is therefore conceivable that transfer and expression of this plasmid could provide all of the systems essential for rapid growth in milk to organisms that cannot grow in milk because they are unable to utilize
lactose and casein.

PEPTIDASES

The peptides transported by the oligopeptide transport system are further hydrolyzed by intracellular peptidases (31, 43). Peptidase activity has been identified in several genera of lactic acid bacteria including *Lactococcus*, *Lactobacillus*, and *Pediococcus* (48, 60).

The enzymes are best characterized in *L. lactis*, where two classes of peptidases have been identified, aminopeptidases and endopeptidases (48). Aminopeptidases, such as PepX and PepN, cleave amino acids or di-peptides from the amino-terminal end of the peptide. The endopeptidases, which include PepO, hydrolyze bonds within the peptide chain. A third class, carboxypeptidases, has been reported in lactobacilli but not in lactococci (16, 48).

The importance of these enzymes to the growth of lactococci in milk was demonstrated by the creation of deletion derivatives that were deficient in the expression of five peptidases (PepX, PepT, PepO, PepC, and PepN) (31). When transferred into milk, these mutants exhibited growth rates ten times slower than the wild-type strain.

GENETIC ENGINEERING OF LACTIC ACID BACTERIA

Advances in gene transfer technology have made possible the transformation of many species previously found to be nontransformable (61). The methods most commonly used to effect gene transfer include conjugation, transduction, and electroporation.

Conjugation is a natural gene transfer process that has been widely used to genetically modify lactococci (16). The process involves transfer of a mobilized genetic element from one cell to another and requires cell-to-cell contact (54).
One drawback to the use of conjugation for development of lactic acid bacteria is that recipients typically contain plasmid DNA that encodes resistance to clinically important antibiotics such as erythromycin or chloramphenicol (4). These genes are necessary for transconjugant selection but their presence is a concern in bacteria that will be used in human food because of the potential to spread antibiotic resistance genes among pathogenic organisms (4).

Transduction involves the transfer of DNA between bacteria by way of a defective bacteriophage. The transducing phage carries a portion of the host DNA in its capsule which is injected into the next potential host bacterium as part of the infection cycle. An example of natural gene transfer by transduction was described by McKay et al. (37). Those authors demonstrated that the ability to ferment lactose could be transferred into lactose-negative derivatives of \textit{L. lactis} C2 by treatment with phage lysates obtained from the parental strain. Later studies indicated that the transducing phage in that study had packaged deletion derivatives of the \textit{L. lactis} lactose plasmid into their capsules, and it was these plasmids that conferred the lactose fermenting ability to the recipient (14, 17).

Electroporation is a transformation procedure that employs high-strength electrical fields to induce transient pores in cell membranes. Transformation occurs by the diffusion of extracellular DNA into these permeabilized cells (6, 35). This technique has an advantage over other recombinant DNA methods, such as conjugation, in not requiring antibiotic resistance markers for the selection of transformants (4). Furthermore, unlike protoplast transformation techniques which require extensive strain specific optimization, one electroporation protocol can often be used to transform several strains (47).

Transformation of \textit{Pediococcus acidilactici} has been reported but frequencies were low (26, 41). The best electroporation frequency, 61.5 transformants/µg DNA, was obtained with the 4.4 kb plasmid pGK12 (41). Maximum reported transformation frequencies in electroporated bacteria range from $10^3$-10$^7$ transformants/µg DNA for
Gram-positive organisms (21, 35) to greater than $10^9$ transformants/µg DNA in Gram-negative strains (6). It has been suggested that the lower transformation frequencies in Gram-positive bacteria can be attributed to the relatively thick cell wall acting as a barrier between DNA molecules and the permeabilized membrane (21, 47). This hypothesis is supported by studies that show increased transformation frequencies with cells that are treated with lysozyme (47) or are grown in media that contained substances, such as glycine (21) or DL-threonine (58), which inhibit cell wall synthesis (21).

The objective of this study was to use genetic manipulation of *Pediococcus* spp. to create alternative dairy starter cultures that might alleviate the bacteriophage problem in the mozzarella cheese industry by increasing the diversity of strains available for strain rotation programs. Specifically, this study sought to:

1. Determine whether Lac⁺ pediococci can be obtained by transformation with native *Lactococcus lactis* plasmid DNA.

2. Determine whether pediococci can express lactococcal proteinase or oligopeptide transport enzyme systems.

3. Investigate the suitability of genetically improved *Pediococcus* species as starter cultures for mozzarella cheese.

**REFERENCES**


CHAPTER II
DEVELOPMENT AND CHARACTERIZATION OF LACTOSE-POSITIVE
_PEDIOCOCUS_ SPECIES FOR MILK FERMENTATION

ABSTRACT

Bacteriophages against _Streptococcus thermophilus_ are a growing problem in the Italian cheese industry. One possible control method involves replacing _S. thermophilus_ in mozzarella starter blends with lactic acid bacteria from a different genus or species. In this study, we evaluated lactose-positive pediococci for this application. Because we could not identify any commercially available pediococci with fast acid-producing ability in milk, we transformed _Pediococcus pentosaceus_ ATCC 25744, _P. pentosaceus_ ATCC 25745, and _P. acidilactici_. ATCC 12697 by electroporation with pPN-1, a 35 kilobase _Lactococcus lactis_ lactose plasmid. Transformants of _P. pentosaceus_ ATCC 25745 and _P. acidilactici_. ATCC 12697 were then used to examine lactose-positive pediococci for properties related to milk fermentation. Both transformants rapidly produced acid and efficiently retained pPN-1 in lactose broth, and neither bacterium was attacked by bacteriophages in whey collected from commercial cheese facilities. Paired starter combinations of _Pediococcus_ spp. and _Lactobacillus helveticus_ LH100 exhibited synergistic pH reduction in milk and small-scale cheese trials showed these cultures could be used to manufacture part-skim mozzarella cheese. Results demonstrate lactose-positive pediococci have potential as replacement cocci for _S. thermophilus_ in Italian starter blends and may facilitate development of new strain rotation schemes to combat _S. thermophilus_ bacteriophage problems in mozzarella cheese plants.

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INTRODUCTION

Starter cultures for the manufacture of Italian cheeses such as mozzarella typically contain *Streptococcus thermophilus* and *Lactobacillus helveticus* or *L. delbruekii* subsp. *bulgaricus* (27). Explosive growth in mozzarella cheese production over the past 20 years has led to an increased incidence of bacteriophage attack on *S. thermophilus* (11, 33). Bacteriophages of starter lactobacilli appear much less frequently (27). One method to control bacteriophage problems in mozzarella plants may be to expand the number of phage-unrelated starter cocci available for strain rotation (13, 33). An approach may be to replace *S. thermophilus* in Italian starter blends with suitable lactic cocci from a different genus or species.

Pediococci are homofermentative lactic acid bacteria which, from an industrial perspective, include species primarily important for meat and vegetable fermentations (9). These bacteria sometimes dominate populations of nonstarter lactic acid bacteria (NSLAB) in ripened cheese (4), and some strains are used as adjunct cultures to improve attributes of Cheddar and mozzarella cheese (5, 29). Unfortunately, pediococci typically are unable to ferment lactose (9), which clearly restricts their application in milk fermentations. Reports which indicate nonstarter- and adjunct *Pediococcus* spp. impart desirable attributes to cheese (4, 5, 29) suggest pediococci might be good dairy starter bacteria if they possessed the ability to utilize lactose. As an example, lactose-positive (Lac+) *P. acidilactici* and *P. pentosaceus* may be suitable replacement cocci for *S. thermophilus* in Italian starter blends because these bacteria grow at 45°C, and each has a long history of safe consumption in human food (9, 27). The development of gene transfer systems for pediococci in recent years (2, 12) provides new opportunities to investigate applications for pediococci in milk fermentation. Researchers in Japan, for instance, have reported Lac+ transfer by conjugation from *Lactococcus lactis* to *P. acidilactici* (26).
This study constructed Lac⁺ *P. acidilactici* and *P. pentosaceus* strains by transformation with a naturally occurring 35 kilobase (kb) *Lactococcus lactis* lactose plasmid, pPN-1. Lactose-positive transformants were investigated for stability of the Lac⁺ phenotype, the ability to acidify milk, and other important dairy starter properties. Results indicated Lac⁺ *Pediococcus* spp. have potential as replacement cocci for *S. thermophilus* in Italian starter blends.

**MATERIALS AND METHODS**

**Bacterial cultures.** Bacteria and plasmids used in the study are listed in Table 2-1. Cultures were stored at 4°C and maintained by biweekly transfer. Pediococci and *L. helveticus* LH100 were grown at 37°C in MRS broth (7), which contained 2.0% glucose or lactose as the carbohydrate source (MRS-G or MRS-L). Lactococci were propagated at 30°C in M17 broth (32), which contained 0.5% glucose or lactose (M17-G or M17-L) as the carbohydrate source, and *S. thermophilus* TA061 was grown in M17-L at 37°C.

**Plasmids.** Plasmid DNA was isolated by the method of Anderson and McKay (3) and when necessary, purified by CsCl₂-ethidium bromide density gradient centrifugation (20). The presence of plasmids in cell lysates was established by electrophoresis in 0.6% agarose gels at 1.5 V/cm for 14 h with Life Technologies, Inc. (Gaithersburg, Md.) supercoiled DNA ladder size standards included in the gel. Restriction endonuclease mapping of pPN-1 was performed as described by Maniatis et al. (20).

**Electroporation of pediococci.** Electrottransformation of *Pediococcus* spp. with pGK12 and pPN-1 was performed with a Bio-Rad (Richmond, Calif.) GenePulser apparatus. Competent cells were prepared by overnight growth in MRS-G with 0.5 M sorbitol; then, a 1.5% inoculation was made into 800 ml MRS-G that contained 0.5 M
### TABLE 2-1. Bacteria and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterium or plasmid</th>
<th>Relevant Phenotype</th>
<th>Description (source or reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pediococcus</em> spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> ATCC 12697</td>
<td>Lac&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Wild-type (American Type Culture Collection, Rockville, Md.)</td>
</tr>
<tr>
<td><em>P. acidilactici</em> SAL</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ATCC 12697 transformed with pPN-1 (this study)</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> ATCC 25744</td>
<td>Lac&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Wild-type (American Type Culture Collection)</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> SPL-1</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ATCC 25744 transformed with pPN-1 (this study)</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> ATCC 25745</td>
<td>Lac&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Wild-type (American Type Culture Collection)</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> SPL-2</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>ATCC 25745 transformed with pPN-1 (this study)</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM2302</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Plasmid-cured derivative of strain C2 (23)</td>
</tr>
<tr>
<td>PN-1</td>
<td>Lac&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Transductant of strain LM2302 constructed as described by McKay et al. (24; this study)</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em> LH100</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Mozzarella cheese starter (Marschall Products, Madison, Wis.)</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> TA061</td>
<td>Lac&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Mozzarella cheese starter (Marschall Products)</td>
</tr>
<tr>
<td>pGK12</td>
<td>Em&lt;sup&gt;r&lt;/sup&gt;Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>4.4 kb <em>Lactococcus lactis</em> cloning vector (15)</td>
</tr>
<tr>
<td>pPN-1</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;</td>
<td>35 kb deletion derivative of the <em>Lactococcus lactis</em> C2 lactose plasmid (this study; 21).</td>
</tr>
</tbody>
</table>

Abbreviations: Lac<sup>+</sup>, able to ferment lactose; Em<sup>r</sup>, erythromycin-resistance; Cm<sup>r</sup>, chloramphenicol resistance; kb, kilobase.
sorbitol, 3% glycine, and 40 mM D/L-threonine. The bacteria were incubated for 2 to 4 h ($A_{600} = 0.4-0.6$) at 37°C then collected and washed twice in 25 ml of 0.5 M sorbitol, 10% glycerol solution. After the washes, cells were suspended in 1 ml electroporation buffer (0.5 M sorbitol, 1 mM $K_2HPO_4$, 1 mM $MgCl_2$). Eighty microliters of the suspension was mixed with 4 µl pGK12 (0.15 µg/µl) or pPN-1 (0.11 µg/µl), and the mixture was transferred to a 0.1 cm electrode-gap electroporation cuvette. The machine parameters were set at 200 Ω resistance and 25 µF capacitance, with a field strength of 1.8 kV (18 kV/cm).

Immediately after the electric pulse, 2 ml of recovery medium (MRS plus 0.5 M sorbitol, 20 mM $MgCl_2$, and 2 mM $CaCl_2$) was added. Then the cultures were kept on ice for approximately 5 min. The cells were allowed to recover for 2 h at 37°C then plated and incubated 2 to 5 days at 37°C. Cells which had been transformed with pPN-1 were identified by their ability to acidify bromcresol purple-lactose indicator agar (BCP-L; 23) which contained 0.5 M sorbitol. Transformants with pGK12 were selected on M17-G agar that contained 0.5 M sorbitol and 5 µg per ml of erythromycin. Transformation frequencies are expressed as the number of erythromycin-resistant (Em') CFU per µg pGK12 and values reported represent the mean from at least two separate experiments.

**Plasmid stability.** The stability of pPN-1 in *Pediococcus* spp. transformants was inferred by the percentage of Lac+ cells which remained in the population after successive transfer in MRS-L or MRS-G at 37°C (30). Transfers were performed at 12-h intervals over a 5-day period and the fraction of Lac+ pediococci at each interval was determined by plate count on BCP-L agar.

**Milk fermentation.** The ability to metabolize lactose was demonstrated by pH reduction on BCP-L agar or by exponential growth in MRS-L or M17-L broth. The time required for a 1% inoculum from a fresh overnight culture to coagulate 10 ml of
9% reconstituted skim milk (RSM) at 37°C was determined by visual examination at selected time intervals, based on preliminary experiments which established approximate clot times for each strain under study. Reduction in pH of 9% RSM or MRS-L broth by 1- or 2% inoculum from a fresh 4 h (>10^6 CFU/ml) culture was measured with a Beckman Instruments, Inc. (Fullerton, Calif.) model pH140 pH meter.

The ability of *P. acidilactici* SAL, *P. pentosaceus* SPL-1, and *P. pentosaceus* SPL-2 to utilize casein (22) was investigated by 2% inoculation into 10 ml tubes of RSM, and RSM fortified with 0.25% of the casein hydrosylate NZ amine (ICN Biomedicals, Inc., Aurora, Ohio) or casamino acids (Difco Laboratories, Detroit, Mich.). Milks were incubated at 37°C; then, pH and milk coagulation time were measured as described above. Cell numbers at 0- and 24 h were determined by plate count on MRS-L agar.

**Bacteriocin production.** The agar overlay assay described by Steele and McKay (31) was used to ensure pediococci did not produce compounds that would inhibit growth of *L. helveticus* LH100. Each test included *L. helveticus* LH100 and the nisin-producing bacterium *Lactococcus lactis* 11454 (31) as the negative and positive control, respectively.

**Phage sensitivity assays.** The susceptibility of *Pediococcus* spp. transformants to bacteriophages in North American dairy production facilities was evaluated by Marschall Products/Rhône-Poulenc (Madison, Wisc.) with BCP acid-inhibition tests (18) in milk fortified with MRS-L (3:1). The bacteria were tested for susceptibility to bacteriophages in whey samples collected over a 1-month period from cheese plants that used Marschall *Lactococcus lactis, S. thermophilus,* and *Lactobacillus* spp. starter cultures.

**Mozzarella cheese manufacture.** Part-skim mozzarella cheese was produced in 10-kg experimental vats essentially as described by Merrill et al. (25). Experimental
cheeses were prepared using 2% total inoculations of *P. acidilactici* SAL or *P. pentosaceus* SPL-2 with *L. helveticus* LH100 (1:1). Control cheeses were made with 1% total inoculations of *S. thermophilus* TA061 and *L. helveticus* LH100 (1:1). Cheese fat and moisture levels were measured by the modified Babcock and vacuum oven methods as described by Kosikowski (17).

**RESULTS**

**Screening wild-type pediococci for Lac⁺.** Sixteen strains of *P. acidilactici*, six *P. pentosaceus*, one *P. dextrinicus*, two *P. inopinatus*, and one undefined *Pediococcus* spp. were obtained from the American Type Culture Collection (ATCC; Rockville, Md.), the Belgian Coordinated Collections of Microorganisms (Gent, Belgium), or this laboratory and were tested for the ability to ferment lactose. Only one strain, *P. acidilactici* ATCC 31282, notably acidified BCP-L agar after 24 h at 37°C. Fatty acid analysis of this bacterium, however, revealed it was actually an *Enterococcus faecalis*. This result prompted us to subject pediococci selected for electrotransformation experiments to extensive microbiological characterization (Table 2-2).

**Isolation and characterization of pPN-1.** Plasmid pPN-1 was obtained from *Lactococcus lactis* C2 by transduction to the plasmid-free bacterium *Lactococcus lactis* LM2302 (24). Lysates of one transductant, *Lactococcus lactis* PN-1, were analyzed by agarose gel electrophoresis and were found to contain a single plasmid, designated pPN-1. Restriction endonuclease digests of pPN-1 with *BglII* and *KpnI* indicated the plasmid was approximately 35 kb in length. The size difference between pPN-1 and the 56 kb C2 lactose (*lac*) plasmid (21) suggested 20 kb of the original molecule had been deleted from pPN-1. Temperate bacteriophages responsible for *lac* plasmid transduction can accommodate only 35-36 kb of DNA; thus, *lac* plasmid deletion derivatives are consistent and natural occurrences in Lac⁺ transductants (10, 21).
### TABLE 2-2. Microbiological characterization of wild-type *Pediococcus* strains used in the study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ATCC 12697</th>
<th>ATCC 25744</th>
<th>ATCC 25745</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate enantiomers produced</td>
<td>D/L</td>
<td>D/L</td>
<td>D/L</td>
</tr>
<tr>
<td>Fatty acid analysis(^a)</td>
<td>inconclusive</td>
<td><em>P. pentosaceus</em></td>
<td><em>P. pentosaceus</em></td>
</tr>
<tr>
<td>Carbohydrate utilization(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-acetylglucosamine</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>amygdalin</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>arbutin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>celllobiose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>esculin</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>galactose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>β-gentiobiose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-glucose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>D-mannose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>melibiose</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>raffinose</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>ribose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>sucrose</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>salicin</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-tagatose</td>
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<td>+++</td>
</tr>
<tr>
<td>trehalose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>D-xylose</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**API identification\(^c\) \(P. acidilactici\)**

**API identification\(^c\) \(P. pentosaceus\)**

**API identification\(^c\) \(P. pentosaceus\)**

\(^a\)Service provided by Analytical Services, Inc. (Essex Jct., Vt., USA), similarity index (SI) values \(\geq 0.400\) indicate a good match in fatty acid profile while values \(\leq 0.250\) indicate poor match. SI for ATCC 12697 < 0.100; ATCC 25744 = 0.686; ATCC 25745 = 0.606.

\(^b\)Acid production from carbohydrate after 24 h at 37°C in API 50 CH test kit prepared for lactic acid bacteria (-, not fermented; +++, rapidly fermented).

\(^c\)Service provided by bioMérieux Vitek, Inc. (Hazelwood, Mo.) technical service department.
A restriction endonuclease map of pPN-1 was constructed and compared to the 56 kb lac plasmid of *Lactococcus lactis* 712 (10, 16), the strain from which C2 was originally derived (6). The comparison showed the lac operon on pPN-1 was intact and indicated the plasmid had undergone two distinct deletion events (Figure 2-1). A 14-kb deletion was mapped to the region between lac and the origin of replication, and a separate 6-kb deletion had removed most of the DNA required for expression of the lactococcal cell wall proteinase.

**Transformation of *Pediococcus* spp.** Electroporation with pGK12 was performed to evaluate the transformation efficiency of pediococci used in this study. Em' *P. acidilactici* ATCC 12697, *P. pentosaceus* ATCC 25744, and *P. pentosaceus* ATCC 25745 transformants were obtained at frequencies of 4.6 x 10^3, 3.4 x 10^3, and 1.8 x 10^3, respectively. The procedure was then used to transform wild-type strains with intact pPN-1, and Lac+ transformants isolated from each strain were examined for pPN-1 uptake. As shown in Figures 2-2 and 2-3, lysates of Lac+ *P. acidilactici* ATCC 12697 and *P. pentosaceus* ATCC 25745 transformants, designated *P. acidilactici* SAL and *P. pentosaceus* SPL-2, contained a new plasmid molecule which comigrated through 0.6% agarose gels with CsCl_2-purified pPN-1. Figure 2-3 also showed *P. pentosaceus* ATCC 25745 contained a native plasmid slightly smaller than pPN-1, and this molecule was retained in Lac+ transformants.

Agarose gel electrophoresis did not detect pPN-1 in Lac+ transformants of *P. pentosaceus* ATCC 25744 (designated *P. pentosaceus* SPL-1), but extensive microbiological characterization of this bacterium (tests listed in Table 2-2) showed it differed from the parental strain only by the ability to rapidly utilize lactose. Detailed microbiological characterization of *P. acidilactici* SAL and *P. pentosaceus* SPL-2 also confirmed their identity, and these two bacteria were selected as species representatives for subsequent studies to characterize genetically derived Lac+ pediococci.
FIG 2-1. Restriction endonuclease and deletion map of the 35 kilobase (kb) *Lactococcus lactis* plasmid pPN-1. Diagonal lines identify regions of the 56 kb lactococcal plasmid pLP712 that have been deleted in pPN-1. Abbreviations: B, *BgIII*; E, *BstEII*; K, *KpnI*; L, *BglII*; P, *PstI*; S, *Sall*; U, *Stul*; *lac*, lactose operon; *rep*, origin of replication; *prt*, genes for extracellular proteinase. Map of pLP712 adapted from Gasson et al. (10) and Kok and Venema (16).
FIG 2-2. Agarose gel electrophoresis of plasmid DNA isolated from *Pediococcus acidilactici* lactose-positive (Lac⁺) transformant and wild-type strain. Samples shown included a supercoiled DNA standard (lane A), wild-type *P. acidilactici* ATCC 12697 (lane B), a Lac⁺ transformant of that strain, *P. acidilactici* SAL (lanes C and D), and CsCl₂-purified pPN-1 (lane E).
FIG 2-3. Agarose gel electrophoresis of plasmid DNA isolated from *Pediococcus pentosaceus* lactose-positive (Lac⁺) transformant and wild-type strain. The gel shown contained supercoiled DNA standard (lane A), CsCl₂-purified pPN-1 (lane B), wild-type *P. pentosaceus* ATCC 25745 (lane C), and a Lac⁺ transformant of that bacterium, *P. pentosaceus* SPL-2 (lanes D and E).
Expression of Lac⁺ in transformants. Lac⁺ expression in *P. acidilactici* SAL and *P. pentosaceus* SPL-2 was evaluated in MRS-L. Both transformants grew much better than respective wild-type strains in MRS-L broth (Figure 2-4A), especially SAL. As expected, improved growth by Lac⁺ transformants in MRS-L was accompanied by a concomitant pH reduction (Figure 2-4B).

**Lac⁺ stability.** After nine sequential transfers (> 175 generations) in MRS-L broth, approximately 95% of *P. acidilactici* SAL CFU remained Lac⁺ (Figure 2-5). In contrast, growth for a similar period in MRS-G reduced the Lac⁺ population to less than 20%. Lac⁺ was slightly less stable in *P. pentosaceus* SPL-2. About 90% of the population remained Lac⁺ after nine transfers (> 175 generations) in MRS-L, while fewer than 2% retained this phenotype after comparable growth in MRS-G.

**Bacteriocin production.** Because mozzarella starter blends frequently include *L. helveticus* (27), Lac⁺ transformants were tested for production of compounds which inhibited growth of *L. helveticus* LH100. Agar overlay tests indicated *L. helveticus* LH100 was not inhibited by any of the *Pediococcus* spp. listed in Table 2-1.

**Ability to clot and reduce the pH of 9% RSM.** *P. acidilactici* SAL and *P. pentosaceus* SPL-2 each failed to coagulate RSM within 48 h at 37°C. Interestingly, RSM inoculated with *L. helveticus* LH100 alone coagulated within 18 h, but milk which contained 1:1 combinations (1% total inoculum) of SAL or SPL-2 with LH100 had significantly (*P* < 0.016; Table A-1) reduced clot times when compared to LH100 alone. Coagulation times for strain combinations were 6.75 h for LH100 with SAL, and 6.25 h for LH100 plus SPL-2.

In similar fashion, Lac⁺ transformants alone showed weak ability to acidify RSM, but 1:1 combinations (1% total inoculum) with LH100 produced final milk pH values notably lower than those obtained with SAL, SPL-2, or LH100 pure cultures (Figure 2-6). The rate of pH decrease in 9% RSM inoculated with 1%
FIG. 2-4. Growth in (A) and pH reduction of (B) MRS-L by lactose-positive (Lac⁺) *Pediococcus* spp. transformants and wild-type strains. Open symbols represent wild-type *Pediococcus acidilactici* ATCC 12697 (○) and *Pediococcus pentosaceus* ATCC 25745 (□); closed symbols denote the respective Lac⁺ transformants *Pediococcus acidilactici* SAL and *Pediococcus pentosaceus* SPL-2.
FIG. 2-5. Percentage of lactose-positive *Pediococcus acidilactici* SAL (°, ♦) and *Pediococcus pentosaceus* SPL-2 (□, ■) after serial transfer at 37°C in MRS broth that contained lactose (°, □) or glucose (♦, ■) as the carbohydrate source.
FIG. 2-6. pH reduction of 9% reconstituted skim milk (RSM) by various bacteria. Graph A shows pH changes in RSM inoculated with 1% *Pediococcus acidilactici* SAL (♦), 1% *Lactobacillus helveticus* LH100 (□), 0.5% SAL plus 0.5% LH100 (O), 1% SAL plus 1% LH100 (△), and 0.5% *Streptococcus thermophilus* TA061 plus 0.5% LH100 (◊). Graph B shows similar data for RSM inoculated with 1% *Pediococcus pentosaceus* SPL-2 (♣), 1% *Lactobacillus helveticus* LH100 (□), 0.5% SPL-2 plus 0.5% LH100 (O), 1% SPL-2 plus 1% LH100 (△), and 0.5% *Streptococcus thermophilus* TA061 plus 0.5% LH100 (◊).
Pediococcus/LH100 strain combinations was slower than that noted with the positive control, a 1% S. thermophilus TA061/LH100 blend (1:1). These differences were substantially reduced, however, when higher numbers of pediococci and lactobacilli (1% versus 0.5% each) were added to the milk (Fig. 2-6).

Pediococci require most amino acids for growth (9), and Table 2-3 shows the inability of Lac⁺ transformants to rapidly coagulate and acidify RSM was likely due to ineffective casein utilization by these bacteria. Addition of enzymatically or acid hydrolyzed casein to RSM stimulated growth of all Lac⁺ pediococci in milk and reduced pH and clot times. The effect was less pronounced in P. pentosaceus strains, which indicated these bacteria are at least weakly able to degrade casein. Studies are in progress to further investigate casein utilization by pediococci.

**Bacteriophage sensitivity.** P. acidilactici SAL or P. pentosaceus SPL-2 were tested for susceptibility to bacteriophages in 835 separate whey samples collected from North American cheese producers. At least 440 of these samples contained more than 10⁵ PFU per ml (11), but bacteriophages able to attack SAL or SPL-2 were not detected.

**Cheese production.** Moisture and fat levels of part-skim mozzarella manufactured with Lac⁺ Pediococcus/L. helveticus LH100 paired starters were similar to that of control cheese made with S. thermophilus TA061/LH100 (see Table 6-2). The time required to produce mozzarella (curd cut to stretching) with SPL-2 or SAL starter blends, however, averaged 60 and 90 min longer than the control (3.0 h).

**DISCUSSION**

The objective of this study was to develop alternative starter cocci with potential to replace S. thermophilus in Italian starter blends. P. acidilactici and P. pentosaceus appear to be good candidates for this application because each grows well at 45°C, is
### TABLE 2-3. Growth of *Pediococcus* transformants in 9% RSM with and without NZ amine or casamino acids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>CFU per ml</th>
<th>pH</th>
<th>Clot Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
<td>24 h</td>
<td>0 h</td>
</tr>
<tr>
<td><em>P. acidilactici</em></td>
<td>RSM</td>
<td>8.7 x 10⁷</td>
<td>9.0 x 10⁷</td>
<td>6.45</td>
</tr>
<tr>
<td>SAL</td>
<td>NZ amine</td>
<td>7.5 x 10⁷</td>
<td>9.2 x 10⁷</td>
<td>6.51</td>
</tr>
<tr>
<td>casamino acids</td>
<td>6.3 x 10⁷</td>
<td>8.0 x 10⁷</td>
<td>6.52</td>
<td>5.10</td>
</tr>
<tr>
<td><em>P. pentosaceus</em></td>
<td>RSM</td>
<td>7.6 x 10⁷</td>
<td>5.1 x 10⁸</td>
<td>6.47</td>
</tr>
<tr>
<td>SPL-1</td>
<td>NZ amine</td>
<td>8.7 x 10⁷</td>
<td>8.4 x 10⁸</td>
<td>6.50</td>
</tr>
<tr>
<td>casamino acids</td>
<td>6.9 x 10⁷</td>
<td>6.6 x 10⁸</td>
<td>6.49</td>
<td>5.18</td>
</tr>
<tr>
<td><em>P. pentosaceus</em></td>
<td>RSM</td>
<td>6.5 x 10⁷</td>
<td>3.5 x 10⁸</td>
<td>6.49</td>
</tr>
<tr>
<td>SPL-2</td>
<td>NZ amine</td>
<td>5.7 x 10⁷</td>
<td>7.3 x 10⁸</td>
<td>6.52</td>
</tr>
<tr>
<td>casamino acids</td>
<td>6.3 x 10⁷</td>
<td>6.0 x 10⁸</td>
<td>6.49</td>
<td>5.16</td>
</tr>
</tbody>
</table>

*RSM, 9% RSM; NZ amine, 9% RSM fortified with 0.25% of NZ amine; casamino acids, 9% RSM fortified with 0.25% casamino acids*
homofermentative, and has a long history of safe consumption in food (9, 27).
Unfortunately, dairy starter cultures must be able to rapidly ferment lactose, and wild-type pediococci typically lack this attribute (9).

Many lactococci, including *Lactococcus lactis* C2, utilize lactose via a plasmid-coded phosphoenolpyruvate-dependent phosphotransferase system (PTS; 8, 21). DNA sequence analysis of the *lac* operon has shown it includes genes for the lactose-specific PTS enzymes Enzyme IIA\textsuperscript{lac} (LacF) and Enzyme IIB\textsuperscript{lac} (LacE), phospho-β-galactosidase (LacG), and enzymes of the tagatose-6-phosphate pathway (LacA-D; 8). Complementation between lactose-specific PTS enzymes from one bacterium and nonspecific enzymes (Enzyme I and HPr) from another is well documented (21). This study used complementation between pPN-1-encoded LacE and LacF enzymes and non-sugar specific PTS enzymes from *Pediococcus* spp. (1, 8, 19, 21) to produce a functional lactose transport system in *P. acidilactici* ATCC 12697, *P. pentosaceus* ATCC 25744 and *P. pentosaceus* ATCC 24745. The Lac\textsuperscript{+} phenotype was unstable in glucose broth, but pPN-1 stability should not be a problem in commercial settings because dairy starter cultures are grown and maintained in milk or whey-based media (11).

In *Lactococcus lactis*, rapid growth and acid production in milk require genes for lactose and casein utilization. The ability to efficiently convert casein into amino acids to support growth requires the extracellular proteinase and an oligopeptide transport system (14). Since Lac\textsuperscript{+} pediococci constructed in this study required free amino acids to rapidly acidify and coagulate RSM, these bacteria apparently lack one or both enzyme systems needed for efficient casein utilization. Because of their slower activity, Lac\textsuperscript{+} pediococci developed in this study may, at present, be most valuable as adjuncts in the traditional *S. thermophilus/Lactobacillus* sp. starter blend, where they could provide greater protection against problems caused by *S. thermophilus* bacteriophage attack. Research is underway to determine whether or not lactococcal genes for the
extracellular proteinase and/or the oligopeptide transport system can be used to improve the ability of pediococci to utilize casein. Even a modest increase should facilitate use of these bacteria as direct substitutes for *S. thermophilus* because the latter species also is typified by relatively weak proteolytic activity (28).

In Italian starter blends, casein hydrolysis by the *Lactobacillus* sp. generates peptides and free amino acids required for maximal growth of *S. thermophilus* (28). This symbiotic growth between starter cocci and rods is a characteristic and desirable property of Italian starter blends because it provides a synergistic increase in lactate production (27). Significantly, synergistic pH reduction was also noted in RSM which contained Lac⁺ pediococci and *L. helveticus* LH100 (Fig. 2-5). Although the rate of pH decrease was slower than that of RSM inoculated with *S. thermophilus* TA061 and LH100, small-scale cheese make experiments showed Lac⁺ *Pediococcus* spp. can be used to manufacture part-skim mozzarella cheese. As a whole, these data show Lac⁺ pediococci have clear potential as replacement cocci for *S. thermophilus* in Italian starter blends, and this application may facilitate development of new strain rotation schemes to combat *S. thermophilus* bacteriophage problems in mozzarella cheese plants.

REFERENCES


11. **Gillies, K.** Personal communication.


Chapter III

LACTOSE AND GALACTOSE UPTAKE BY GENETICALLY ENGINEERED PEDIOCoccus SPECIES

Abstract The ability to utilize lactose is requisite for lactic acid bacteria used as starters in the dairy industry. Modern genetic recombination techniques have facilitated the introduction of the lactose-positive phenotype into bacteria such as Pediococcus species, which traditionally have not been used as dairy starters. This study investigated lactose and galactose uptake along with phospho-β-galactosidase activity in pediococci which had been transformed with a Lactococcus lactis lactose plasmid. Lactose-positive transformants, Pediococcus acidilactici SAL and Pediococcus pentosaceus SPL-2, demonstrated an ability to accumulate [14C]lactose at a rate greater than the Lactococcus lactis control. Phospho-β-galactosidase activity was also higher in transformants versus Lactococcus lactis. Studies of [3H]galactose uptake suggested that a wild-type galactose transport system and the introduced lactose phosphotransferase system both functioned in galactose uptake by Pediococcus spp. transformants. Significantly lower levels of free galactose were detected in milk fermented with Lactobacillus helveticus LH100 and SAL or SPL-2 than in milk fermented with a LH100 plus Streptococcus thermophilus TA061 control starter blend.

Introduction

Lactic acid bacteria are a group of gram-positive, non-sporeforming bacteria that produce lactate as the main product of carbohydrate metabolism (Axelsson 1993). This group includes several genera (e.g., Lactococcus, Lactobacillus, Leuconostoc, Pedicoccus, and Streptococcus) that are important in meat, vegetable, and dairy fermentations. In the dairy industry, fermentation of milk to produce cheese, yogurt, or buttermilk requires lactic acid bacteria that possess the ability to metabolize the milk sugar lactose. This attribute is dependent upon enzymes which transport lactose across the cell membrane and cleave it into its component sugars (De Vos and Vaughan 1994).

Many lactic acid bacteria transport lactose and other carbohydrates across the cell membrane by a phosphoenolpyruvate-dependent phosphotransferase system (PTS). In these strains, sugar translocation and phosphorylation are coupled, and then the phosphorylated lactose is cleaved by phospho-β-galactosidase (P-β-Gal) (EC 3.2.1.85) into glucose and galactose-6-phosphate. These monosaccharides are further metabolized through the glycolytic and tagatose-6-phosphate pathways, respectively (De Vos and Vaughan 1994; McKay 1982). In Lactococcus lactis, the genes necessary for lactose fermentation are encoded within an 8 kilobase pair lac operon which is frequently located on plasmid DNA. These genes encode the lactose-specific PTS enzymes (LacE-F), P-β-Gal (LacG), as well as enzymes for the tagatose-6-phosphate pathway (LacA-D) (Gasson et al. 1987; De Vos et al. 1990; De Vos and Vaughan 1994; McKay 1982).

Most strains of Streptococcus thermophilus, the traditional coccus in Italian cheese starter blends, metabolize the glucose moiety of lactose and export the galactose moiety into the medium via an antiport system for lactose uptake (Hutkins and Ponne 1991; Poolman 1993). Residual galactose in cheese can promote Maillard browning during high-temperature cooking of cheese on pizza, and this reaction can be problematic because excess browning makes the product less appealing to consumers (Matzdorf et al. 1994).
Thus, the capability to metabolize galactose is a useful characteristic in dairy starter cultures, particularly those involved in Mozzarella cheese production (Oberg and Broadbent 1993).

Pediococci typically metabolize galactose (Garvie 1986), but their inability to rapidly ferment lactose has historically precluded them from consideration as starters in dairy fermentations (Oberg and Broadbent 1993). To overcome this limitation, our laboratory transformed strains of *Pediococcus acidilactici* and *Pediococcus pentosaceus* with a naturally occurring lactococcal lactose plasmid, pPN-1, and showed that transformants were able to rapidly acidify lactose broth (Caldwell et al. 1996). In this study, [*14C]lactose and [*3H]galactose uptake, P-β-Gal and β-galactosidase (β-Gal; EC 3.2.1.23) activities, lactose plasmid copy number, and levels of residual galactose in milk were examined to further investigate the utilization of lactose and galactose in the transformed *Pediococcus* spp.

**Materials and methods**

**Bacterial cultures**

Bacteria used in the study are listed in Table 3-1. Cultures were stored at 4°C and maintained by biweekly transfer. Pediococci and *Lactobacillus helveticus* LH100 were grown at 37°C in MRS broth (De Man et al. 1960) which contained 2.0% glucose or lactose as the carbohydrate source (MRS-G or MRS-L). *L. lactis* PN-1 was propagated at 30°C in M17 broth (Terzaghi and Sandine 1975) which contained 0.5% lactose (M17-L) as the carbohydrate source, and *Streptococcus thermophilus* TA061 was grown in M17-L at 37°C.

**Carbohydrate uptake**

Lactose uptake assays were performed using a modified version of the procedure described by Hutkins and Ponne (1991). Overnight cultures were harvested by...
Table 3-1  Bacteria and plasmid DNA used in this study

<table>
<thead>
<tr>
<th>Bacterium or plasmid</th>
<th>Relevant Phenotype</th>
<th>Description (source or reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pediococcus</em> spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. acidilactici</em> ATCC 12697</td>
<td>Lac⁺, Gal⁺</td>
<td>Wild-type (American Type Culture Collection, Rockville, Md.)</td>
</tr>
<tr>
<td><em>P. acidilactici</em> SAL</td>
<td>Lac⁺, Gal⁺</td>
<td>ATCC 12697 transformed with pPN-1 (Caldwell et al. 1996)</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> ATCC 25745</td>
<td>Lac⁻, Gal⁺</td>
<td>Wild-type (American Type Culture Collection)</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> SPL-2</td>
<td>Lac⁺, Gal⁺</td>
<td>ATCC 25745 transformed with pPN-1 (Caldwell et al. 1996)</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. lactis</em> LM0230</td>
<td>Lac⁻</td>
<td>Plasmid-cured derivative of strain C2 (McKay et al. 1972)</td>
</tr>
<tr>
<td><em>L. lactis</em> PN-1</td>
<td>Lac⁺</td>
<td>A derivative of strain LM2302 that contains pPN-1 (Caldwell et al. 1996)</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em> LH100</td>
<td>Lac⁺, Gal⁺</td>
<td>Mozzarella cheese starter (Marschall Products/ Rhône-Poulenc, Madison, Wis.)</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> TA061</td>
<td>Lac⁺, Gal⁺</td>
<td>Mozzarella cheese starter (Marschall Products/ Rhône-Poulenc, Madison, Wis.)</td>
</tr>
<tr>
<td>Plasmid pPN-1</td>
<td>Lac⁺</td>
<td>35 kb deletion derivative of the <em>L. lactis</em> C2 lactose plasmid (Caldwell et al. 1996).</td>
</tr>
</tbody>
</table>

Abbreviations:  Lac⁺, able to ferment lactose; Lac⁻, unable to ferment lactose; Gal⁺, able to ferment galactose; Gal⁻, unable to ferment galactose; kb, kilobase.
centrifugation and washed twice in citrate phosphate buffer (pH 6.5). After the second wash, cell density was adjusted to A_{600} 1.0-1.2 in wash buffer. Cells were warmed for 5 min at 35°C prior to addition of 1 mM [^{14}C]lactose (1.4 x 10^4 CPM/mM). Samples of 1 ml were taken at timed intervals and the cells were separated from the buffer by centrifugation through silicon oil as described by Nannen and Hutkins (1991).

Radioactivity of each fraction (pellet and supernatant) was determined with a Beckman LS 3801 or LS 6500 scintillation counter (Beckman Instruments, Fullerton, Calif.). The isotope counts were then used to calculate the nmoles of carbohydrate taken into the cells based on a dry weight of 0.35 mg/ml OD^1 and intracellular volume of 3.80 µl/mg (dry weight) for _Pediococcus_ cells (Christensen and Hutkins 1992) or 2.34 µl/mg (dry weight) for _L. lactis_ cells (Nannen and Hutkins 1991).

Galactose uptake was investigated as described above using 1 mM [^3H]galactose (2.0 x 10^4 CPM/mM) instead of [^{14}C]lactose and cells which had been grown overnight in MRS containing galactose as the sole carbohydrate. [^3H]galactose uptake studies were also performed with 20 mM non-radiolabeled lactose or galactose added as a competitive substrate (Martin and Russell 1987). In these experiments, non-radiolabeled substrates were added just prior to the addition of the radiolabeled galactose. All carbohydrate uptake experiments were replicated at least one time.

Enzyme assays

β-Galactosidase and P-β-Gal activities were measured spectrophotometrically as described previously (Citti et al. 1965; Inamine et al. 1986). Cells were grown overnight, harvested by centrifugation, washed twice in 100 mM Tris buffer (pH 7.0), then suspended in the same buffer to an A_{600} of approximately 20.0 (generally about 1/20 of the original volume). A 1-ml sample of the cell suspension was transferred to a 1.5-ml microcentrifuge tube, and the cells were disrupted with glass beads. Cell debris and glass beads were
beads were removed by centrifugation, and the protein levels in the cell-free extracts were
determined as described by Bradford (1976).

Assays for β-Gal and P-β-Gal were conducted at room temperature (22°C) and
initiated by the addition of 10-50 µl of cell-free extract to 0.5 ml Tris buffer (20 mM, pH
6.5), which contained 1 mM o-nitrophenyl-β-D-galactopyranoside (ONPG) or o-
nitrophenyl-β-D-galactopyranoside-6-phosphate (ONPG-6P), respectively. Release of
o-nitrophenol (ONP) was followed at 420 nm on a Beckman DU-64 spectrophotometer for
5 min with readings at 15-sec intervals. The nmoles ONP released were calculated as
described by Inamine et al. (1986) using an extinction coefficient of 0.001 optical density
units per 0.214 nmole ONP. All of the enzyme assays were performed in duplicate on two
separate occasions.

Determination of pPN-1 copy number

An estimate of the copy number of pPN-1 in *L. lactis* PN-1, *P. acidilactici* SAL and
*P. pentosaceus* SPL-2 was obtained using a quantitative polymerase chain reaction (PCR)
method. The procedure for the experiment was developed using previously published
guidelines (Coen 1992; Diaco 1995). Template DNA was isolated using a method
developed by C. A. Batt (personal communication). Cells (250 µl) from an overnight
culture were collected by centrifugation, washed once in 100 µl of phosphate-buffered
saline (PBS) (140 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4), and
suspended in 10 µl 10X PCR Buffer II (Perkin-Elmer Corp., Norwalk, Conn.), 85 µl
sterile distilled H₂O, and 4 µl freshly prepared chicken egg-white lysozyme (10 mg/ml)
(Sigma Chemical Co., St. Louis, Mo.). After a 15-min incubation at 37°C, 1 µl proteinase
K (50 mg/ml) (Sigma Chemical Co.) was added and the suspension was incubated at 50-
55°C for 1 h, then boiled for 10 min to inactivate cellular enzymes. Cell debris was
removed by centrifugation and the template DNA was stored at -20°C until needed.
Oligonucleotide PCR primers (Table 3-2) were designed using published lacE and L-(-)-lactate dehydrogenase (ldhL) gene sequences (De Vos et al. 1990; Garmyn et al. 1995; Llanos et al. 1992); these primers were then obtained through Life Technologies Inc. (Gaithersburg, Md.). The first set of primers (lacEF and lacER) amplified a 352-bp fragment of the lactococcal lacE gene encoded on pPN-1. The second set of primers (LldhF and LldhR or PldhF and PldhR) was included as an internal control (Diaco 1995) and was designed to amplify a 195-bp fragment of the ldhL gene, which is present on the chromosome in a single copy (Garmyn et al. 1995; Llanos et al. 1992).

The PCR reaction consisted of 0.2 µg DNA, 4 U FisherBiotech Taq DNA polymerase (Fisher Scientific, Pittsburgh, Pa.), 1 X Fisher Biotech assay buffer B (10 mM Tris (pH 8.3), 50 mM KCl), 2.5 mM MgCl₂, deoxyribonucleotide triphosphates (100 µM each), lacEF and lacER (1 mM each for all templates), LldhF and LldhR (1 µM each for L. lactis templates) or PldhF and PldhR (1 µM each for Pediococcus spp. templates), and sterile distilled H₂O to a final volume of 50 µl. PCR was performed with a Perkin Elmer DNA thermal cycler 480 (Perkin-Elmer Corp.) programmed for 25 cycles of 96°C for 15 s, 57°C for 30 s, and 72°C for 1 min 30 s.

The amplified fragments were separated by electrophoresis in 1.5% agarose gels at 8.5 V/cm for 1.5 h. Gels were stained with ethidium bromide (5 µg/ml) and the density of each DNA band was determined using an Alphalmager 2000 documentation and analysis system (Alpha Inotech Corp., San Leandro, Calif.). The copy number of pPN-1 in each strain was estimated by comparing the relative density reading of the lacE fragment to that of the ldhL gene fragment. The lactose-negative strains L. lactis LM0230, P. acidilactici ATCC 12697, and P. pentosaceus ATCC 25745 were included in PCR experiments to establish primer specificity.
Table 3-2 Oligonucleotide primers used for quantitative polymerase chain reaction

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. lactis</em> lacE</td>
<td>lacEF</td>
<td>TGAACTCATTCTCTGCaACC</td>
</tr>
<tr>
<td></td>
<td>lacER</td>
<td>AAGCCACTGTACCTCaACC</td>
</tr>
<tr>
<td><em>L. lactis</em> ldhL</td>
<td>LdhF</td>
<td>CTTCCCTGTCGTGCTAACC</td>
</tr>
<tr>
<td></td>
<td>LdhR</td>
<td>TCTAGGTCACCCTGTCCACC</td>
</tr>
<tr>
<td><em>P. acidilactici</em> ldhL</td>
<td>PldhF</td>
<td>CTTCCCTGTCGTGCTAACC</td>
</tr>
<tr>
<td></td>
<td>PldhR</td>
<td>TCACTGTCACCATGTCCACC</td>
</tr>
</tbody>
</table>

Residual galactose

The concentration of free galactose in 9% reconstituted skim milk (RSM) fermented with single strain cultures or starter blends was determined using Boehringer Mannheim lactose/D-galactose test kits (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The individual cultures used in these experiments were *Lactobacillus helveticus* LH100, *S. thermophilus* TA061, *P. acidilactici* SAL, or *P. pentosaceus* SPL-2. The blends contained LH100 in combination (1:1) with each coccus. For each single strain or culture blend, 0.2 ml of an overnight culture was inoculated into 10 ml RSM (initial cell count = 3 to 6 x 10⁷ CFU per ml) and incubated for 24 h at 37°C. Because *P. acidilactici* SAL and *P. pentosaceus* SPL-2 are proteolytic-deficient (Caldwell et al. 1996), RSM for single-strain studies of these bacteria was supplemented with 0.25% of the casein hydrolysate NZ amine (ICN Biomedicals, Inc., Aurora, Ohio).

Results

Lactose uptake and galactosidase activities

No lactose uptake or P-β-Gal activity was detected in the wild-type parental strains, *P. acidilactici* ATCC 12697 or *P. pentosaceus* ATCC 25745. In contrast, *P. acidilactici* SAL accumulated [¹⁴C]lactose at a rate that was similar to the *L. lactis* control
strain, PN-1, while [¹⁴C]lactose accumulation in *P. pentosaceus* SPL-2 was nearly threefold higher than SAL or PN-1 (Figure 3-1). In addition, P-β-Gal activity in both *P. acidilactici* SAL and *P. pentosaceus* SPL-2 was nearly double that detected in *L. lactis* PN-1 (Figure 3-2).

Assays using ONPG as a substrate detected low levels of β-Gal activity in all of the strains used in this study. *P. pentosaceus* SPL-2 showed the highest level of β-Gal activity (16.1 nmoles ONP released/mg protein/min), while β-Gal activities for *L. lactis* PN-1, *P. pentosaceus* ATCC 25745, *P. acidilactici* ATCC 12697, and *P. acidilactici* SAL were 0.1, 1.9, 0.3, and 1.5 nmoles ONP released/mg protein/min, respectively.

**Copy Number of pPN-1**

Quantitative PCR reactions yielded amplicons of the expected sizes for *lacE* and *ldhL* fragments, and the specificity of each primer pair was established by the presence of a *ldhL* fragment and the absence of a *lacE* band in lactose-negative control strains (*L. lactis* LM0230, *P. acidilactici* ATCC 12697, and *P. pentosaceus* ATCC 25745) (Figure A-1). Comparisons between the band intensities of the *lacE* and the *ldhL* amplicons by densitometry gave *lacE*: *ldhL* ratios of 1.2:1 for *L. lactis* PN-1, 1.5:1 for *P. acidilactici* SAL, and 1.0:1 for *P. pentosaceus* SPL-2, which indicated that each strain contained essentially one copy of pPN-1 per cell.

**Galactose uptake**

Transformation of *P. acidilactici* ATCC 12697 and *P. pentosaceus* ATCC 25745 with the lactococcal lac operon encoded by pPN-1 also influenced galactose uptake in these bacteria. The amount of galactose that was accumulated by these transformants under non-competitive conditions was approximately threefold higher than that of the respective parental strains (Figure 3-3). When 20 mM lactose was included in the assay, substrate
Fig. 3-1 [\(^{14}\)C]Lactose uptake by *Pediococcus acidilactici* ATCC 12697 (▲), *Pediococcus acidilactici* SAL (●), *Pediococcus pentosaceus* ATCC 25745 (□), *Pediococcus pentosaceus* SPL-2 (○), and *L. lactis* PN-1 (+). Data points were taken from a representative experiment.
Fig. 3-2 Phospho-β-galactosidase activity in *Pediococcus acidilactici* ATCC 12697 (▲), *Pediococcus acidilactici* SAL (●), *Pediococcus pentosaceus* ATCC 25745 (□), *Pediococcus pentosaceus* SPL-2 (○), and *L. lactis* PN-1 (+). Data points were taken from a representative experiment.
Fig. 3-3 [³H]Galactose uptake by bacteria used in the study. Graph A shows galactose uptake by *Pediococcus acidilactici* ATCC 12697 incubated with [³H]galactose only (◇), plus lactose (○), plus non-radiolabeled galactose (△); *Pediococcus acidilactici* SAL incubated with [³H]galactose only (■), plus lactose (●), plus non-radiolabeled galactose (▲). Graph B shows galactose uptake by *Pediococcus pentosaceus* ATCC 25745 incubated with [³H]galactose only (◇), plus lactose (○), plus non-radiolabeled galactose (△); *Pediococcus pentosaceus* SPL-2 incubated with [³H]galactose only (■), plus lactose (●), plus non-radiolabeled galactose (▲). Data points for graphs a and b were taken from representative experiments.
competition reduced the level of galactose uptake in *P. acidilactici* SAL to that of its wild-type strain, *P. acidilactici* ATCC 12697 (Figure 3-3A). In *P. pentosaceus* SPL-2, however, competition from lactose resulted in a level of galactose accumulation that was intermediate to the non-competitive and parental levels (Figure 3-3B). As expected, addition of 20 mM non-radiolabeled galactose to saturate the galactose uptake system virtually eliminated [3H]galactose accumulation in all strains.

Residual galactose in milk

Levels of residual galactose were significantly lower (*P* < 0.01; Table A-2) in RSM fermented with *Lactobacillus helveticus* LH100 and *P. acidilactici* SAL or *P. pentosaceus* SPL-2 starter blends than in RSM fermented with a traditional Mozzarella starter pair (*Lactobacillus helveticus* LH100 and *S. thermophilus* TA061). Levels of residual galactose were also lower in RSM fermented with single-strain pediococci versus *S. thermophilus*, but this result may be due to less lactose utilization since the final pH of the milk differed by nearly one pH unit (Table 3-3).

Discussion

This study showed *Pediococcus* spp. that had been transformed with the lactococcal lactose plasmid pPN-1 were able to transport [14C]lactose at rates that were equal to or greater than the lactococcal control (Figure 3-1). Furthermore, enzyme assays demonstrated that the transformants possessed a level of P-β-Gal activity that was 2-fold greater than the *L. lactis* control (Figure 3-2). Quantitative PCR showed that increased P-β-Gal activity (or rate of lactose uptake) in Lac+ pediococci was not due to a gene dosage effect. However, these experiments did not address possible differences in the transcriptional or translational efficiency of pPN-1 coded genes by pediococci and *L. lactis* PN-1.
Table 3-3 Galactose concentrations in 9% RSM after 24 h incubation at 37°C.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Final pH</th>
<th>% Galactose (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninoculated 9% RSM</td>
<td>6.44</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>uninoculated 9% RSM + 0.25% NZ amine</td>
<td>6.49</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td><em>S. thermophilus</em> TA061</td>
<td>4.34</td>
<td>0.62 ± 0.04</td>
</tr>
<tr>
<td><em>P. acidilactici</em> SAL</td>
<td>5.12</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> SPL-2</td>
<td>5.18</td>
<td>0.05 ± 0.05</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em> LH100</td>
<td>4.20</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>LH100 with TA061</td>
<td>4.03</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td>LH100 with SAL</td>
<td>4.12</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>LH100 with SPL-2</td>
<td>4.00</td>
<td>0.46 ± 0.07</td>
</tr>
</tbody>
</table>

Abbreviation: RSM, reconstituted skim milk.

\(^a\)Values reported are the means of three replications ± the standard deviations

Lactose transport ability and P-β-Gal activity in *P. acidilactici* SAL and *P. pentosaceus* SPL-2 were expected in bacteria which had acquired genes for a lactose PTS. It was interesting to note, however, that even though pPN-1 does not encode β-Gal, *P. pentosaceus* SPL-2 exhibited eightfold higher β-Gal activity than its parental strain, *P. pentosaceus* ATCC 25745. This suggested that growth in lactose may have induced a native β-Gal in the transformant. That hypothesis is supported by the work of Bhowmik and Marth (1990), who reported lactose-inducible β-Gal activity in several *Pediococcus* strains, including *P. pentosaceus* ATCC 25745.

*Pediococcus* spp. that contained pPN-1 showed a 3-fold increase in galactose uptake over wild-type cells (Figure 3-3). The lactose PTS has been implicated as a mechanism of galactose transport in lactococci (De Vos and Vaughan 1994), and it
appeared to provide a similar function in Lac⁺ pediococci. This activity was most clearly evident in *P. acidilactici* SAL, where the addition of nonradioactive lactose reduced the rate of [³H]galactose uptake to the wild-type level (*P. acidilactici* ATCC 12697) (Figure 3-3). In *P. pentosaceus* SPL-2, however, the interaction between the lactose PTS and the native galactose uptake system was not as clear. The reduction in galactose uptake during substrate competition with lactose in SPL-2 suggested that the lactose PTS was also involved in galactose accumulation in this strain, but since this level was still above that of the parental strain, other factors may also be involved. One possible explanation is that lactose or one of its products acted as an inducer of the native galactose transport system in *P. pentosaceus* SPL-2. Nevertheless, [³H]galactose transport studies showed that in the absence of lactose, Lac⁺ transformants were able to take up galactose through both native and pPN-1-encoded transport systems, and that native enzymes for galactose uptake remained active in these cells even when lactose was present.

Because lactose uptake in *S. thermophilus* occurs by a lactose-galactose antiport through the LacS protein (De Vos and Vaughan 1994; Hutkins and Ponne 1991), galactose accumulates in the growth medium and may contribute to Maillard browning in milk products fermented with that bacterium (Matzdorf et al. 1994). In this study, milk fermented with *Lactobacillus helveticus* LH100 and *Pediococcus* spp. starter pairs contained significantly lower levels of residual galactose than the *Lactobacillus helveticus* LH100 and *S. thermophilus* TA061 control, despite similar final pH values. Since galactose accumulates in RSM fermented with LH100, these data indicated that lactose utilization by Lac⁺ pediococci involved co-metabolism of the glucose and galactose moieties. This hypothesis is supported by the fact that pPN-1 encoded genes for the lactose-specific PTS enzymes and P-β-Gal, whose expression was demonstrated in Lac⁺ pediococci (Fig. 3-1 and 3-2), are co-transcribed with genes for galactose metabolism via the tagatose-6-phosphate pathway (De Vos and Vaughan 1994). If Lac⁺ pediococci were
unable to express native or pPN-1 encoded tagatose enzymes, lactose PTS and P-β-Gal activity would result in the accumulation of toxic levels of galactose-6-phosphate. Since the bacteria grow well on lactose, and galactose does not accumulate in the growth medium, Lac⁺ pediococci must have the ability to metabolize galactose-6-phosphate.

References


Terzaghi BE, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophages. Appl Microbiol 29:807-813
CHAPTER IV

CASEINOLYTIC ACTIVITY IN PEDIOCOCUS SPECIES

ABSTRACT

Pediococcus spp. are lactic acid bacteria that are often used in meat and vegetable fermentations but have a limited ability to grow in milk. Previous work by this laboratory indicated that an inability to efficiently obtain amino nitrogen from casein may contribute to this limitation. Consequently, this study investigated caseinolytic ability in Pediococcus spp. Results indicated that the inability of the pediococci to derive nitrogen from milk proteins was not due to a deficiency in aminopeptidase activity, since general aminopeptidase activity in both \textit{P. pentosaceus} ATCC 25745 and \textit{P. acidilactici} ATCC 12697 was as high as or higher than that of \textit{Lactococcus lactis} C2. Assays for proteinase or oligopeptide transport activity in pediococci indicated that they were deficient in these activities. Attempts were therefore made to increase the caseinolytic ability of \textit{P. acidilactici} and \textit{P. pentosaceus} strains by transformation with \textit{Lactococcus lactis} genes for the extracellular proteinase (\textit{prtP/prtM}) and the oligopeptide transport system (\textit{oppA-D}). Low-level proteinase activity was detected in a derivative of \textit{P. acidilactici} ATCC 12697 which contained the proteinase-coding plasmid pGKV552, but the ability of this strain to grow in milk was not improved. Introduction of the pVS8-encoded oligopeptide transport system into \textit{P. pentosaceus} ATCC 25745 and SPL-2 allowed these strains to obtain histidine from the $\alpha_{s1}$-casein-(1-8)-peptide, which confirmed the expression of the oligopeptide transport system. Nonetheless, pVS8 constructs remained unable to rapidly clot 9% reconstituted skim milk. These data showed that transformation with the lactococcal genes for either the extracellular serine proteinase or the oligopeptide transport system alone was insufficient to increase the ability of \textit{Pediococcus} spp. to grow in milk.
INTRODUCTION

Lactic acid bacteria (LAB) are a fastidious group of microorganisms that are generally auxotrophic for one or more amino acids (2, 16). Although many species of LAB are used in dairy fermentations, milk is not an ideal substrate for amino acid auxotrophs because of its low level of free amino acids. Studies in *Lactococcus lactis* have demonstrated that rapid growth in milk requires the ability to obtain amino nitrogen from casein. Several enzymes are involved in the release of free amino acids from milk caseins, including a cell-wall bound extracellular serine proteinase, an oligopeptide transport system, and a variety of intracellular peptidases (18, 22).

*Pediococcus* spp. are LAB that are commonly associated with meat and vegetable fermentations and have been identified as non-starter organisms in dairy products (3). Previous work in this laboratory sought to improve the growth of *P. acidilactici* and *P. pentosaceus* in milk by transformation with lactococcal genes for lactose utilization (5). Characterization of the lactose-positive transformants revealed that they were still unable to rapidly grow in milk. Because this deficiency could be corrected by the addition of hydrolyzed casein to 9% reconstituted skim milk (RSM), we hypothesized that these strains were limited by their ability to obtain free amino acids from casein.

This study was initiated to investigate and make an attempt to increase the caseinolytic ability of *Pediococcus* spp. The pediococci were screened for native aminopeptidase, extracellular proteinase, and oligopeptide transport activity. Then, strains of *P.acidilactici* and *P. pentosaceus* were transformed with plasmids encoding the lactococcal genes for either the extracellular proteinase or the oligopeptide transport system, and transformants were assayed for the ability to clot 9% RSM.
MATERIALS AND METHODS

Bacterial cultures. Bacteria and plasmids used in this study are listed in Table 4-1. Cultures were stored at 4°C and maintained by biweekly transfer. Pediococci and *Lactobacillus helveticus* strains were grown at 37°C in MRS broth (6), which contained 2.0% glucose or lactose as the carbohydrate source (MRS-G or MRS-L). Lactococci were propagated at 30°C in M17 broth (24), which contained 0.5% glucose or lactose (M17-G or M17-L) as the carbohydrate source, and *Streptococcus thermophilus* TA061 was grown in M17-L at 37°C.

Plasmids. Plasmid DNA was isolated by the method of Anderson and McKay (1) and when necessary, purified by CsCl₂-ethidium bromide density gradient centrifugation (20). The presence of plasmids in cell lysates was established by electrophoresis in 0.6% agarose gels at 5.7 V/cm for 4 h with Life Technologies, Inc. (Gaithersburg, Md.) supercoiled DNA ladder size standard included in the gel.

Aminopeptidase activity in pediococci. Aminopeptidase activity was determined using the colorimetric method of Dias and Weimer (9), except that the cell free extracts were prepared from an initial culture volume of 20 ml, and the final protein concentration of the extract was diluted to 100 µg protein/ml before initiation of the assay. The substrates used in the assay were L-alanine-p-nitroanilide, L-γ-glutamyl-p-nitroanilide, glycine-p-nitroanilide, L-leucine-p-nitroanilide, and L-methionine-p-nitroanilide (Sigma Chemical Co., St. Louis, Mo.). The L-alanine-p-nitroanilide stock solution (10 mM) was prepared directly in 10 ml sterile 0.05 M sodium phosphate buffer (pH 7.2). The remaining substrates were dissolved in 0.5 ml N,N-dimethylformamide prior to dilution to 10 ml with the sodium phosphate buffer. Stock solutions were divided into 1-ml aliquots and stored at -20°C until needed. *Lactobacillus helveticus* CNRZ32 and *L. lactis* C2 were included in each experiment as positive controls. The aminopeptidase activity values reported represent the mean of at least two separate experiments, each
Table 4-1. Bacteria and plasmids used to study caseinolytic activity in *Pediococcus* spp.

<table>
<thead>
<tr>
<th>Bacterium or plasmid</th>
<th>Relevant phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pediococcus acidilactici</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 12697</td>
<td>Lac' Prt' Opp'</td>
<td>American Type Culture Collection (ATCC), Rockville, Md.</td>
</tr>
<tr>
<td>12697:pGKV552</td>
<td>Lac'</td>
<td><em>P. acidilactici</em> ATCC 12697 transformed with pGKV552</td>
</tr>
<tr>
<td>12697:pVS8</td>
<td>Lac'</td>
<td><em>P. acidilactici</em> ATCC 12697 transformed with pVS8</td>
</tr>
<tr>
<td>SAL</td>
<td>Lac' Prt' Opp'</td>
<td><em>P. acidilactici</em> SAL transformed with pGKV552</td>
</tr>
<tr>
<td>SAL:pGKV552</td>
<td>Lac'</td>
<td><em>P. acidilactici</em> SAL transformed with pVS8</td>
</tr>
<tr>
<td>SAL:pNZ521</td>
<td>Lac'</td>
<td><em>P. acidilactici</em> SAL transformed with pVS8</td>
</tr>
<tr>
<td>SAL:pPS12</td>
<td>Lac'</td>
<td><em>P. acidilactici</em> SAL transformed with pPS12</td>
</tr>
<tr>
<td>SAL:pVS8</td>
<td>Lac'</td>
<td><em>P. acidilactici</em> SAL transformed with pVS8</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25745</td>
<td>Lac' Prt' Opp'</td>
<td>ATCC</td>
</tr>
<tr>
<td>25745:pGKV552</td>
<td>Lac'</td>
<td><em>P. pentosaceus</em> ATCC 25745 transformed with pGKV552</td>
</tr>
<tr>
<td>25745:pVS8</td>
<td>Lac'</td>
<td><em>P. pentosaceus</em> ATCC 25745 transformed with pVS8</td>
</tr>
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<td>SPL-2</td>
<td>Lac' Prt' Opp'</td>
<td>(5)</td>
</tr>
<tr>
<td>SPL-2:pGKV552</td>
<td>Lac'</td>
<td><em>P. pentosaceus</em> SPL-2 transformed with pGKV552</td>
</tr>
<tr>
<td>SPL-2:pVS8</td>
<td>Lac'</td>
<td><em>P. pentosaceus</em> SPL-2 transformed with pVS8</td>
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<tr>
<td><em>Lactococcus lactis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM2301</td>
<td>Lac' Prt' Opp'</td>
<td>(26)</td>
</tr>
<tr>
<td>C2</td>
<td>Lac' Prt' Opp'</td>
<td>(27)</td>
</tr>
<tr>
<td>LM2301:pGKV552</td>
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<td><em>L. lactis</em> LM2301 transformed with pGKV552</td>
</tr>
<tr>
<td>LM2301:pNZ521</td>
<td>Lac' Prt' Opp'</td>
<td><em>L. lactis</em> LM2301 transformed with pNZ521</td>
</tr>
<tr>
<td>LM2301:pPS12</td>
<td>Lac' Prt' Opp'</td>
<td><em>L. lactis</em> LM2301 transformed with pPS12</td>
</tr>
<tr>
<td>LM2301:pVS8</td>
<td>Lac' Prt' Opp'</td>
<td><em>L. lactis</em> LM2301 transformed with pVS8</td>
</tr>
<tr>
<td><em>Lactobacillus helveticus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH100</td>
<td>Lac'</td>
<td>Rhodia Inc., Madison, Wis.</td>
</tr>
<tr>
<td>CNRZ32</td>
<td>Lac' AP'</td>
<td>(9)</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA061</td>
<td>Lac'</td>
<td>Rhodia Inc.</td>
</tr>
<tr>
<td>Plasmids</td>
<td>Prt' Em'</td>
<td></td>
</tr>
<tr>
<td>pGKV552</td>
<td>Prt' Cm'</td>
<td>(11)</td>
</tr>
<tr>
<td>pNZ521</td>
<td>Prt' Em'</td>
<td>(8)</td>
</tr>
<tr>
<td>pPS12</td>
<td>Prt' Em'</td>
<td>(15)</td>
</tr>
<tr>
<td>pVS8</td>
<td>Opp' Cm'</td>
<td>(25)</td>
</tr>
<tr>
<td>pGK12</td>
<td>Em' Cm'</td>
<td>(17)</td>
</tr>
</tbody>
</table>

Abbreviations: Lac', able to ferment lactose; Prt', extracellular proteinase activity; Opp', oligopeptide transport activity; AP', aminopeptidase activity; Em', erythromycin-resistance; Cm', chloramphenicol resistance.
performed in duplicate. Statistical comparisons between mean values from different strains were performed using two-sample t tests (19).

**Transformation.** Electrotransformation of *Pediococcus* spp. was performed as described previously (5), except that the washed cells were suspended in 1 ml SG (0.5 M sorbitol, 10% glycerol) instead of electroporation buffer, and the volume of the cell suspension used in the procedure was decreased to 45 µl. The selective medium used in the experiments was MRS agar that contained 0.5 M sorbitol and 5 µg/ml erythromycin or 10 µg/ml chloramphenicol. The competence of the treated cells was checked by performing an electrotransformation with the 4.4-kb erythromycin resistance plasmid pGK12, and the uptake of the plasmid DNA by antibiotic resistant isolates was confirmed by agarose gel electrophoresis (5, 20).

Electroporation of *L. lactis* strains was performed as described above except the competent cells were prepared by the method of Holo and Nes (13), and the selective medium was M17-G agar that contained 5 µg/ml erythromycin or 10 µg/ml chloramphenicol.

**Oligopeptide transport activity.** *L. lactis* strains were grown in the defined SA medium of Jensen and Hammer (14). The pediococci were grown in the chemically defined media (CDM) described by Herawati and Ishizaki (12) but modified by the addition of 100 µl of 10% mevalonic acid lactone per liter of medium. Histidine is an essential amino acid for lactococci and pediococci, and both CDM were prepared without the addition of histidine. The media were sterilized by filtration through 0.45 micron bottle top filters (Corning Inc., Acton, Mass.). Cells for the assays were collected from 10-ml overnight cultures grown in MRS (*Pediococcus* spp.) or M17 (lactococci), washed once with 0.85% NaCl, and suspended in 10 ml of saline before being used to inoculate (1%) the appropriate CDM.
The initial screening experiments followed the growth of *P. acidilactici* ATCC 12697 and *P. pentosaceus* ATCC 25745 in CDM, which either lacked histidine or contained histidine (1 µM final conc.) in one of three forms: as a free amino acid, as α₁-casein (f1-8; R-P-K-H-P-I-K-H), or as a synthetic pentapeptide, E-G-I-H-V. Later experiments included *P. acidilactici* ATCC 12697, *P. acidilactici* SAL, *P. pentosaceus* ATCC 25745, *P. pentosaceus* SPL-2, *L. lactis* LM2301, and derivatives of these strains which contained pVS8 (Table 4-1). After inoculation, the absorbance at A₆₀₀ was recorded and then the cultures were incubated overnight at 30°C (*L. lactis*) or 37°C (*P. pentosaceus* and *P. acidilactici*). A second A₆₀₀ reading was taken after the overnight incubation, and a significant (α = 0.05) increase in A₆₀₀ was used as an indication that the strains had been able to obtain the histidine required for growth. The significance of the increase in absorbance was determined by a two-sample *t* test comparison between the mean increase in A₆₀₀ in CDM without histidine to the mean increase in A₆₀₀ in each broth containing histidine (21).

**Proteinase activity.** Wild-type *Pediococcus* spp. and derivatives that were transformed with pGKV552, pNZ521, or pPS12 (Table 4-1) were assayed for the ability to hydrolyze α₁-casein-(f1-23) as described previously (4, 10). Overnight cultures that had been washed once with one volume (10 ml) 0.85% NaCl were used to inoculate (1%) 10 ml modified citrated milk (27) that had been prepared double strength then diluted to single strength with CDM that contained 0.1 µM free histidine. The citrated milk cultures were incubated overnight at 30°C (*L. lactis*, *P. pentosaceus*, and *P. acidilactici*) or 37°C (*P. pentosaceus* and *P. acidilactici*), then collected by centrifugation and washed twice with 50 mM Na₂PO₄ (pH 6.8). The concentration of the washed cells was adjusted to A₀₆₀₀ = 0.7 with buffer, and then the cells were collected by centrifugation, suspended in 1/10 volume of wash buffer, and assayed for proteinase activity toward α₁-casein-(f1-23). The samples were incubated with the substrate for 3 h, then analyzed by HPLC as
described previously (4). Derivatives of *L. lactis* LM2301 containing these plasmids and the proteinase-negative parental strain were used in each assay as positive and negative controls, respectively.

**Milk clotting ability.** *P. acidilactici* 12697:pGKV552 and *P. pentosaceus* 25745:pVS8 were inoculated into 9% RSM containing 0.5% glucose and *P. pentosaceus* SPL-2:pVS8 was inoculated into 9% RSM. Each strain was inoculated into the milk as a single strain culture or in combination with *Lactobacillus helveticus* LH100. The samples were incubated at 37°C, and the time required to clot the milk was recorded. RSM inoculated with *Streptococcus thermophilus* TA061 or the caseinolytic-negative *Pediococcus* parental strains alone or in combination with *Lactobacillus helveticus* LH100 was included as fast- or slow-milk clotting controls, respectively.

**RESULTS AND DISCUSSION**

**Aminopeptidase activity.** Aminopeptidase activity (9, 18) was detected in *P. acidilactici* ATCC 12697, *P. pentosaceus* ATCC 25745, *L. lactis* C2, and *Lactobacillus helveticus* CNRZ32. As shown in Figure 4-1, the activity detected in *P. pentosaceus* ATCC 25745 was approximately the same as that found in the *L. lactis* C2 control (*P* > 0.19 except with L-alanine-p-nitroanilide where *P* < 0.003). Although this activity level was significantly (*P* < 0.002) lower than that of *Lactobacillus helveticus* CNRZ32, it does not limit the ability of *L. lactis* C2 to grow rapidly in milk (27). The aminopeptidase activity in *P. acidilactici* ATCC 12697 also approximated that of *L. lactis* C2 (*P* > 0.054) except when L-methionine-p-nitroanilide was used as a substrate (*P* < 0.014). From these data, we conclude that the inability of *Pediococcus* spp. to grow in milk is not due to inadequate aminopeptidase activity.

**Oligopeptide transport.** Significant (*P* < 0.05) growth in CDM that contained histidine in the form of α₅-casein-(f1-8) was noted for *P. pentosaceus* 25745:pVS8 and
*P. pentosaceus* SPL-2:pVS8, but not for either of their parental strains (Table 4-2). Although not statistically significant (*P* > 0.06), the increase in absorbance demonstrated by the *P. pentosaceus* SPL-2 parental culture suggested that this strain may have some native ability to transport small peptides. The capability to transport small peptides may explain the ability of SPL-2 to clot milk in 48 h (see Table 2-3). This hypothesis is supported by the lack of an increase in absorbance in the *P. acidilactici* parental cultures accompanied by the inability of these strains to clot milk in under 120 h.

**Extracellular proteinase activity.** Low levels of α₁-casein-(f1-23) breakdown products were detected in the reaction mixtures that contained *P. acidilactici* 12697:pGKV552 (Figure 4-2A). Although these levels were much lower than those detected in the *L. lactis* control (Figure 4-2B), the pattern of peptide cleavage was similar, suggesting that the lactococcal proteinase specificity was retained in the *Pediococcus* spp. transformant. Furthermore, because the strains used in this study had been shown to lack oligopeptide transport capability (Table 4-2), the reduced level of breakdown products detected in the *P. acidilactici* 12697:pGKV552 samples was attributed to a lower level of proteinase activity rather than to a loss of peptides due to cellular metabolism during the 3-h incubation period. No proteinase activity was detected in any of the other *Pediococcus* strains tested (Table 4-1).

**Utility of constructs.** Neither the proteinase activity demonstrated by *P. acidilactici* 12697:pGKV552 nor the oligopeptide transport activity demonstrated by *P. pentosaceus* 25745:pVS8 and SPL-2:pVS8 was sufficient to decrease the time required to clot 9% RSM (Table 4-3). These results prompted attempts to create transformants that contained genes for the extracellular proteinase and an oligopeptide transport. Experiments to introduce pVS8 into *P. acidilactici* 12697:pGKV552 were unsuccessful despite a transformation frequency of 2.5 x 10⁵ transformants/µg pGK12 in the positive
Table 4-2 Increase in $A_{600}$ after overnight growth in CDM with or without added histidine

<table>
<thead>
<tr>
<th>Strain</th>
<th>No added His</th>
<th>Free amino acid</th>
<th>pentapeptide (EGIHV)</th>
<th>$\alpha_1$-CN-(f1-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. lactis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM2301</td>
<td>ND</td>
<td>0.95 ± 0.11</td>
<td>ND</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>LM2301:pVS8</td>
<td>ND</td>
<td>0.87 ± 0.02</td>
<td>ND</td>
<td>0.59 ± 0.18</td>
</tr>
<tr>
<td><em>P. acidilactici</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 12697</td>
<td>ND</td>
<td>0.55 ± 0.03</td>
<td>ND</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>12697:pVS8</td>
<td>ND</td>
<td>0.55 ± 0.01</td>
<td>ND</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>SAL</td>
<td>0.05 ± 0.03</td>
<td>1.29 ± 0.58</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>SAL:pVS8</td>
<td>0.05 ± 0.01</td>
<td>1.42 ± 0.39</td>
<td>0.04 ± 0.03</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td><em>P. pentosaceus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 25745</td>
<td>ND</td>
<td>0.59 ± 0.19</td>
<td>ND</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>25745:pVS8</td>
<td>ND</td>
<td>0.68 ± 0.08</td>
<td>ND</td>
<td>0.44 ± 0.04$^a$</td>
</tr>
<tr>
<td>SPL-2</td>
<td>0.07 ± 0.01</td>
<td>0.70 ± 0.14</td>
<td>0.58 ± 0.08</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>SPL-2:pVS8</td>
<td>0.06 ± 0.02</td>
<td>0.72 ± 0.16</td>
<td>0.47 ± 0.1</td>
<td>0.24 ± 0.1$^a$</td>
</tr>
</tbody>
</table>

$^a$Significant at $\alpha = 0.05$ (See Table A-3)
Abbreviation: ND, Not determined

control. The cause of this failure was most likely plasmid incompatibility between pGKV552, which is a pWVO1 derivative, and pVS8, which contains the origin of replication from pSH71 (7, 11, 25). Plasmids pWVO1 and pSH71 are highly similar, differing in only a few nucleotides and by the presence of a direct repeat on pWVO1 (7). Thus the origins of replication for the two plasmids are almost identical and may not be recognized as different during plasmid DNA replication. This would result in the random replication and/or segregation of only one of the two plasmids (19). Consequently, only one of the two plasmids, pGKV552 or pVS8, can be stably maintained within a single cell. Therefore, further study is required to fully evaluate the effect of paired proteinase and oligopeptide transport activities on the ability of pediococci to grow in milk.

The results of this study confirmed our hypothesis that a deficiency in caseinolytic activity was at least partly responsible for the inability of *Pediococcus* spp. to grow rapidly in milk. It has been shown that *L. lactis* requires lactose metabolism, proteinase
FIG. 4-2. HPLC chromatograms showing cleavage products obtained by incubating the \( \alpha_{s1}\)-casein-(f1-23) with whole cells for 3 h at 30°C. Panel A: Black line, *Pediococcus acidilactici* ATCC 12697; Grey line, *Pediococcus acidilactici* 12697:pGKV552. Panel B: Black line, *Lactococcus lactis* LM2301; Grey line *Lactococcus lactis* LM2301:pGKV552. Peaks that were identified in the chromatogram include: 1, \( \alpha_{s1}\)-casein-(f1-9); 2, \( \alpha_{s1}\)-casein-(f1-13); 3, \( \alpha_{s1}\)-casein-(f1-16); 4, \( \alpha_{s1}\)-casein-(f14-23); 5, \( \alpha_{s1}\)-casein-(f1-23) (uncleaved substrate).
Table 4-3. Hours required to clot 9% RSM using single strain cultures or mixed cultures containing *Lactobacillus helveticus* LH100 and either *Streptococcus thermophilus* TA061 or a *Pediococcus* strain

<table>
<thead>
<tr>
<th>Strain</th>
<th>Single strain culture</th>
<th>Combined culture with LH100</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acidilactici</em> ATCC 12697</td>
<td>&gt; 24</td>
<td>7</td>
</tr>
<tr>
<td><em>P. acidilactici</em> 12697:pGKV552</td>
<td>&gt; 24</td>
<td>7</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> ATCC 25745</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> 25745:pVS8</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> SPL-2</td>
<td>&gt; 24</td>
<td>13.5</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> SPL-2:pVS8</td>
<td>&gt; 24</td>
<td>13.5</td>
</tr>
<tr>
<td><em>Streptococcus thermophilus</em> TA061</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*a* Note: 0.5% glucose was added to cultures containing *P. acidilactici* ATCC 12697 or 12697:pGKV552 and *P. pentosaceus* ATCC 25725 and 25745:pVS8 to compensate for their inability to metabolize lactose.

activity, and oligopeptide transport to demonstrate rapid milk clotting ability (23), and it is possible that pediococci have similar needs. Earlier work in this laboratory demonstrated the expression of the lactococcal lactose PTS system in pediococci (5). This study showed that *P. pentosaceus* strains not only have some native ability to transport small peptides, but can also express the lactococcal oligopeptide transport system (Table 4-2).

Unfortunately, inadequate expression of the lactococcal extracellular serine proteinase in transformed *Pediococcus* spp. prevented a full exploration of their caseinolytic requirements. Future work in this area could involve the isolation of *Pediococcus* spp. with native proteinase activity or the replacement of the lactococcal extracellular proteinase genes with proteinase genes from another bacterial genus.

REFERENCES


CHAPTER V

INDUCTION AND CHARACTERIZATION OF *Pediococcus acidilactici* TEMPERATE BACTERIOPHAGE

ABSTRACT

Mitomycin C was used to induce temperate bacteriophage from three strains of *Pediococcus acidilactici*. The new bacteriophage, designated pa97, pa40, and pa42, were characterized based on morphology, DNA homology, and major protein profiles. Morphological attributes (small, isometric heads with non-contractile tails) place these bacteriophages within the B1 group of the family *Siphoviridae*. Restriction endonuclease digests indicated that the bacteriophage genomes were linear molecules without cohesive ends, and between 33 and 37 kilobases in length. All three bacteriophages possessed one major protein with an estimated mass of 30 to 35 kilodaltons. Bacteriophage pa42 also contained a second major protein of approximately 47 kilodaltons. DNA-DNA hybridization showed bacteriophages pa40 and pa42 were homologous to each other, but not to pa97, suggesting that *Pediococcus acidilactici* bacteriophage fall into at least two different species.

INTRODUCTION

Bacteriophage infection of the starter culture is the leading cause of slow acid production in dairy fermentations (8). The negative economic impact of bacteriophage attack has prompted extensive research on the bacteriophage and bacteriophage resistance of several industrially important lactic acid bacteria, including *Lactococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus* spp. (for reviews, see 2, 10). One of the

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1 Coauthored by S. L. Caldwell, D. J. McMahon, C. J. Oberg, and J. R. Broadbent.
strategies that has been developed to inhibit bacteriophage proliferation involves the rotation of phage-unrelated strains (2). This scheme inhibits the proliferation of bacteriophage by limiting the time a particular host phage-type is susceptible to infection. The effectiveness of these programs, however, is restricted by the relatively small number of strains with different bacteriophage sensitivities (13).

In an effort to address this limitation, our laboratory has studied the possibility of using genetically engineered *Pediococcus* spp. to replace *S. thermophilus* in the mozzarella cheese fermentation (5), and thus increase the number of phage-unrelated bacteria available for strain rotation programs. Results from that study showed the lactose-positive constructs, *P. acidilactici* SAL and *P. pentosaceus* SPL-2, were not sensitive to any bacteriophages currently found in whey from commercial cheese plants (5). While those data showed that *Pediococcus* spp. bacteriophages do not currently exist in commercial cheese plants, the introduction of lysogenic pediococci may lead to the emergence of lytic bacteriophage against these species (14). No lytic or temperate *Pediococcus* spp. bacteriophages have been described to date (9), but lysogeny is common in the lactic acid bacteria (14). In this study, mitomycin C was used to screen strains of *P. acidilactici* and *P. pentosaceus* for evidence of lysogeny. Temperate bacteriophages were detected in three of eight strains of *P. acidilactici*, but not in any of five *P. pentosaceus* strains tested. This report describes major characteristics of the newly discovered *P. acidilactici* bacteriophages.

**MATERIALS AND METHODS**

**Bacterial cultures.** The bacteria and bacteriophages used in this study are listed in Table 5-1. *Pediococcus* spp. were grown at 37°C in MRS broth (7) which contained 2.0% glucose as the carbohydrate source. *Lactococcus lactis* C2 was propagated at 30°C in M17 broth (19) which contained lactose as the carbohydrate source.
Table 5-1. Bacteria and bacteriophage used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactococcus lactis</em> C2</td>
<td>18</td>
</tr>
<tr>
<td><em>Pediococcus acidilactici</em></td>
<td></td>
</tr>
<tr>
<td>ATCC 8041</td>
<td>American Type Culture Collection (ATCC), Rockville, Md</td>
</tr>
<tr>
<td>ATCC 8042</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 12697</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 25740</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 25741</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 25742</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 25743</td>
<td>ATCC</td>
</tr>
<tr>
<td>LMG 11384</td>
<td>Belgian Coordinated Collections of Microorganisms (BCCM), Gent, Belgium</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em></td>
<td></td>
</tr>
<tr>
<td>ATCC 25744</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 25745</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 33316</td>
<td>ATCC</td>
</tr>
<tr>
<td>FBB-61</td>
<td>This laboratory</td>
</tr>
<tr>
<td>LMG 11487</td>
<td>BCCM</td>
</tr>
<tr>
<td>Bacteriophage:</td>
<td></td>
</tr>
<tr>
<td>pa97</td>
<td>Temperate bacteriophage induced from <em>P. acidilactici</em> ATCC 12697 (this study).</td>
</tr>
<tr>
<td>pa40</td>
<td>Temperate bacteriophage induced from <em>P. acidilactici</em> ATCC 25740 (this study).</td>
</tr>
<tr>
<td>pa42</td>
<td>Temperate bacteriophage induced from <em>P. acidilactici</em> ATCC 25742 (this study).</td>
</tr>
</tbody>
</table>
All cultures were stored at 4°C and maintained by biweekly transfer.

**Prophage induction.** Eight strains of *P. acidilactici* and five strains of *P. pentosaceus* were screened for the presence of prophages by the mitomycin C (0.5 µg/ml) induction method of Cuesta et al. (6). *Lactococcus lactis* C2 was included in these induction experiments as a positive control (18). Cell lysis was monitored spectrophotometrically at A₆₀₀ and putative bacteriophages were concentrated from cell lysates by the polyethylene glycol precipitation method for the isolation of bacteriophages without indicator strains (6). Purified bacteriophages were suspended in phage storage medium (SM; 20 mM Tris·HCl, 100 mM NaCl, 10 mM MgSO₄, 10 mM Ca(NO₃)₂; pH 7.25) and stored at 4°C (6).

**Screening for indicator strains.** Each of the 13 *Pediococcus* strains used in this study was swabbed onto MRS agar plates to form a lawn of cells (19). The inoculum was allowed to dry and 10 µl of supernatant from each mitomycin C-induced culture was spotted onto the agar surface. The plates were incubated overnight at 37°C and checked for zones of inhibition. Ten microliters of MRS broth that contained 0.5 µg/ml mitomycin C was also spotted onto each plate as a negative control. Lysates which produced a zone of inhibition were assayed for plaque forming particles by the method of Terzaghi and Sandine (19).

**Electron microscopy.** Concentrated suspensions of *P. acidilactici* ATCC 25740 and ATCC 25742 bacteriophage preparations were diluted 1:100 with SM and adsorbed to Formvar coated 600H copper mesh grids (EMS, Fort Washington, Pa.). The *P. acidilactici* ATCC 12697 bacteriophage concentrate was adsorbed to the grids without dilution. Negative staining of the bacteriophage particles was then performed by standard methods (4) using 12 mM uranyl oxalate, pH 6.8. The particles were examined at 80 kV on a Zeiss 902 CEM electron microscope (Zeiss, Thornwood, N. Y.), and the images
were recorded at a magnification of 140,000X on Kodak SO 163 film (Eastman Kodak Co., Rochester, N.Y.).

**Protein profiles.** Bacteriophage proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Mata et al. (17) with minor modifications. The protein concentration of the purified bacteriophage samples was determined using the Pierce bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, Ill.). Individual bacteriophage samples containing 10 µg protein were transferred to a microcentrifuge tube containing 0.06 M Tris hydrochloride, pH 6.8, 5% β-mercaptoethanol, and 2% sodium dodecyl sulfate and boiled for 10 min. The boiled protein samples were loaded into a SDS-PAGE gel (4% stacking, 12% separating) and then electrophoresis was performed by standard methods (15), and proteins were stained with coomassie blue. Mass estimates for *P. acidilactici* bacteriophage proteins represent the average from three separate phage preparations and SDS-PAGE gels.

**Isolation of bacteriophage DNA.** Bacteriophage DNA was collected essentially as described by Maniatis et al. (16). A reaction mixture that contained 400 µl of the polyethylene glycol-purified bacteriophage suspension, 20 mM EDTA (pH 8.0), 50 µg/ml proteinase K, and 0.5% SDS was incubated at 65°C for 1 h. Three extractions were then performed: first with phenol equilibrated with TE (10 mM Tris, pH 8.0; 1 mM EDTA), then phenol:chloroform:isoamyl alcohol (25:24:1), and finally chloroform:isoamyl alcohol (24:1). The aqueous phase containing bacteriophage DNA was transferred to a Centricon-30 concentrator (Amicon Inc., Beverly, Mass.) and washed three times with TE. The purified DNA was stored at -20°C until needed.

**DNA analysis.** Restriction endonuclease digests and mapping of bacteriophage DNA were performed as described by Maniatis et al. (16). The method of Boyce et al. (3) was used to determine whether or not the bacteriophage DNA had cohesive ends.
Fragment sizes in each digest were determined, and the mean total from duplicate experiments with 10 different restriction endonucleases (New England Biolabs, Inc., Beverly, Mass.) was used to estimate bacteriophage genome size (Table 5-2). To investigate DNA homology among *Pediococcus* bacteriophages, DNA-DNA hybridizations were performed using probes made from each bacteriophage genome by random digoxigenin-11-dUTP (DIG) labeling of a *Hind*III digest of purified bacteriophage DNA (Genius System, Boehringer Mannheim, Indianapolis, Ind.). Transfer of unlabeled *Hind*III digested bacteriophage DNA to positively charged nylon membranes (Boehringer Mannheim), DNA-DNA hybridization in NaCl/Nacitrate solution, and chemiluminescent detection was performed as directed by the DIG kit supplier.

**RESULTS AND DISCUSSION**

**Induction of temperate bacteriophage.** Of the 13 *Pediococcus* spp. strains tested, only *P. acidilactici* ATCC 25740 and *P. acidilactici* ATCC 25742 showed a decrease in A$_{600}$ that began 1.5 to 2 h after the addition of mitomycin C. In addition, growth of a third strain, *P. acidilactici* ATCC 12697, slowed at the same time point where strains ATCC 25740 and ATCC 25742 began to lyse (Figure A-2).

Filtered supernatants from these three strains were then spotted onto lawns of the thirteen pediococci used in this study. A small (1 cm in diameter) zone of inhibition was observed when the supernatant from *P. acidilactici* ATCC 25742 was spotted onto a plate inoculated with *P. acidilactici* LMG 11384, but plaque assays did not detect a lytic bacteriophage. These results are similar to a phenomenon previously described by Jarvis (12) and may indicate that the supernatant contained either a bacteriophage lysin or a bacteriocin. Furthermore, our inability to identify indicator strains for the new
bacteriophage, while inconvenient, is not uncommon among the temperate bacteriophages of lactic acid bacteria (18).

**Morphology.** Electron microscopy confirmed that the supernatants of *P. acidilactici* ATCC 12697, ATCC 25740, and ATCC 25742 contained bacteriophage, designated as pa97, pa40, and pa42, respectively, with small isometric heads and non-contractile tails (Figure 5-1). These morphological characteristics place them within the B1 group of the family *Siphoviridae*, the family that includes most of the bacteriophages described in the lactic acid bacteria (1, 12). The *P. acidilactici* bacteriophage, although structurally similar, differed slightly in head diameter (63, 67, and 52 nm, respectively, for bacteriophages pa97, pa40, and pa42) and more substantively in tail length (300, 185, and 138 nm for bacteriophages pa97, pa40, and pa42, respectively).

**Protein profiles.** The protein profiles for the three *P. acidilactici* bacteriophages, pa40, pa42, and pa97, are shown in Figure 5-2. Each bacteriophage contained one major protein whose estimated size was between 33 and 35 kDa. In addition, pa42 contained a second major protein of about 47 kDa.

Analysis of the protein profiles from the three *P. acidilactici* bacteriophages showed that each bacteriophage contained at least three other detectable proteins. Bacteriophages pa40 and pa42 each contained a similar pair of minor proteins whose estimated masses were between 28 and 30 kDa. No size similarities were noted between the minor proteins of bacteriophage pa97 and those of bacteriophages pa40 and pa42. Estimated mass values for the other minor bacteriophage proteins were 51 kDa for pa40, 75-79 kDa for pa42, and 55-60, 36-40, and 31 kDa for the three minor proteins of pa97. Bacteriophage pa40 contained one additional protein, with an estimated mass of between 48 and 50 kDa, whose abundance was intermediate to the major and minor proteins.

**Restriction digests.** Agarose gel electrophoresis of heated and unheated restriction endonuclease digests of bacteriophage DNA indicated that all three genomes
FIG. 5-1. Electron micrographs of *Pediococcus acidilactici* bacteriophage. The bacteriophage present in each panel was A) pa97; B) pa40; and C) pa42. Bar = 50 nm.
FIG. 5-2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of bacteriophage proteins. Samples present in each lane include A) protein molecular weight size standard; B) pa40; C) pa42; and D) pa97.
fragments indicated that the size of each genome was approximately 37, 36, and 34 kilobasepairs (kb) for pa97, pa40, and pa42, respectively. Restriction digests with different enzymes also indicated that the bacteriophages were dissimilar at the nucleotide sequence level (Table 5-2) and this possibility was further supported by the construction of a simple restriction endonuclease map for each genome (Figure 5-3).

**DNA homology.** DNA-DNA hybridization of a DIG-labeled *HindIII* digest of bacteriophage pa42 to *HindIII*-cut pa40 and pa97 DNA showed that the probe hybridized to eight of nine pa40 fragments (only a 1.6-kb fragment did not hybridize), and to a 4.4-kb *HindIII* fragment of pa97 (Figure 5-4). Similarly, DNA-DNA hybridization of a pa40 DNA probe to *HindIII*-cut pa42 and pa97 genomes showed hybridization to the 4.4-kb pa97 fragment and to eight of nine pa42 fragments (a 1.5-kb band did not hybridize) (Figure A-3). Finally, hybridization of pa97 to pa40 and pa42 DNA showed homology between pa97 and the latter bacteriophages was limited to an 11.3-and a 10.4-kb *HindIII* fragment in pa40 and pa42, respectively (Fig. A-3).

In summary, this report provides the first description of three bacteriophages for the genus *Pediococcus*. Morphological characteristics, protein profiles, and DNA-DNA hybridization results showed that *P. acidilactici* bacteriophages pa40 and pa42 are closely related, but not identical. In contrast, bacteriophage pa97 exhibited little DNA homology with pa40 or pa42, and also had a distinct tail length morphology and protein profile. From these data we conclude that *P. acidilactici* bacteriophages fall into at least two different species.

Lysogeny is common among dairy starter cultures and may contribute toward the emergence of new phages in the dairy industry (11). The presence of prophage in *P. acidilactici* should not preclude their application as milk starters, but it may accelerate the emergence of lytic bacteriophages against this species in dairy plants. Thus,
Table 5-2. Number of cleavage sites demonstrated by restriction endonuclease digests of bacteriophage DNA

<table>
<thead>
<tr>
<th>Endonuclease</th>
<th>pa40</th>
<th>pa42</th>
<th>pa97</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvaI</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>BamHI</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>BssHII</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BstEII</td>
<td>8</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>HindIII</td>
<td>9</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>KpnI</td>
<td>6</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>SacI</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>SacII</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>XbaI</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>XmaI</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

FIG. 5-3. Restriction endonuclease maps of the *Pediococcus acidilactici* bacteriophage DNA.
FIG. 5-4. Hybridization of a *Hind* III-restricted bacteriophage pa42 probe to *Hind* III-cut genomic DNA from phages pa40, pa42, and pa97. The panel on the right shows the X-ray film obtained after DNA-DNA hybridization of the digoxigenin-11-dUTP-labeled probe to a blot made from the agarose gel shown on the left. The DNA samples present in each lane were A) Hi-Lo DNA size standard (Minnesota Molecular, Minneapolis, Minn.); B) pa97; C) pa40; D) pa42; E) undigested pa97 DNA; F) undigested pa40 DNA; and G) undigested pa42 DNA.
continued phage testing will be necessary to avoid the development of a bacteriophage problem in cheese plants using *P. acidilactici* in their starter blends.

REFERENCES


CHAPTER VI
SUMMARY AND CONCLUSIONS

The modern dairy industry relies on bacterial starter cultures to consistently produce high-quality products. To maintain desired characteristics, manufacturers have embraced the use of defined strain starter systems. Unfortunately, the long-term use of a limited number of strains can facilitate the emergence of bacteriophage, which cause slowed or failed fermentations. This problem became chronic in Cheddar cheese facilities over 40 years ago, and it continues to fuel intensive basic and applied research on bacteriophage defense mechanisms in the Cheddar starter bacterium, *Lactococcus lactis*. Unfortunately, a parallel phage problem has been growing in recent years in the Italian-type cheese industry. U.S. production of these varieties, especially Mozzarella, has exploded from 394 million lbs in 1970 to over 2.4 billion lbs in 1998. Increased manufacture of these varieties has been followed by a concomitant rise in bacteriophage problems that have focused new attention toward bacteriophage that attack thermophilic starters.

The thermophilic starter blends used in the manufacture of Italian-type cheeses usually consist of a *Streptococcus thermophilus* paired with a *Lactobacillus* species. These two bacteria exhibit a synergistic growth pattern in milk, which stimulates acid production. *S. thermophilus* is the primary acid producer in the pair, rapidly decreasing the pH to a level that favors the more acid-tolerant *Lactobacillus* sp. (5). The exocellular proteinase of the *Lactobacillus* sp. hydrolyzes milk protein, releasing small peptides and free amino acids into the medium where they fuel growth of the less proteolytic *S.*

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1 Portions of this chapter were previously printed in the following article and are reprinted with permission (Appendix B): Caldwell, S., D. J. McMahon, C. J. Oberg, and J. R. Broadbent. 1998. An alternative approach to phage control in Italian-type Cheese. Euro. Dairy Mag. 4:37-40.
thermophilus. Although bacteriophages against both cultures have been isolated from Mozzarella cheese plants, *S. thermophilus* is the most common target and because it is the major acid producer, the consequences of phage attack are more severe (5).

Because information concerning bacteriophage and bacteriophage resistance in *S. thermophilus* remains limited, U.S. producers of Italian-type cheese have implemented many of the bacteriophage control methods originally developed for Cheddar cheese starters. These techniques include strain rotation and the isolation of bacteriophage-resistant mutants by superinfection (5). Our laboratory has explored an alternative approach to phage control that involves the introduction of a new thermophilic starter from a different genus of lactic acid bacteria.

*Pediococcus* spp. are homofermentative lactic acid bacteria that are used as starter cultures in fermented meat and vegetable foods. Although not traditionally used in milk fermentations, these organisms are sometimes found as non-starter bacteria in ripened cheeses. Because of their long history in food and ability to grow at higher temperatures, it was our hypothesis that pediococci might be suitable replacements for *S. thermophilus* in thermophilic starter blends. Unfortunately, pediococci typically lack the enzyme system that is necessary for rapid utilization of lactose (2).

In an effort to overcome this limitation, we introduced a native, 35-kilobase pair *Lactococcus lactis* lactose plasmid into *P. acidilactici* ATCC 12697 and *P. pentosaceus* ATCC 25745. The wild-type parental strains were unable to transport lactose and had no detectable phospho-β-galactosidase (P-β-Gal) activity, but the new strains, *P. acidilactici* SAL and *P. pentosaceus* SPL-2, contained a level of P-β-Gal activity that was twofold greater than the *L. lactis* control (Figure 3-2). These strains also acquired the ability to produce acid from lactose (Figure 2-3) and displayed relatively high rates of lactose transport (Figure 3-1).
During growth on lactose, strains of *S. thermophilus* characteristically metabolize only the glucose moiety of the disaccharide and export the galactose residue into the medium via an antiport system for lactose uptake (3). This mechanism for lactose catabolism can be problematic because residual galactose in cheese promotes Maillard browning during high-temperature cooking of pizza, and excess browning makes the product less appealing to consumers. Thus, the capability to metabolize galactose has become a desirable characteristic in starter cultures for Mozzarella cheese production (5).

Most pediococci are able to ferment galactose, and the lactococcal lactose plasmid that was introduced into *P. acidilactici* and *P. pentosaceus* also includes genes for galactose catabolism through the tagatose-6-phosphate pathway (1). Galactose uptake studies showed that in the absence of lactose, *P. acidilactici* SAL and *P. pentosaceus* SPL-2 accumulated approximately threefold more galactose than did the respective parental strains (Figure 3-3). Our investigation indicated that SAL and SPL-2 were able to transport galactose through the lactococcal lactose transport system and the native (wild-type) galactose uptake system, and it also showed that the native enzymes remained active even when lactose was present. More importantly, 9% reconstituted skim milk (RSM) fermented with *Lactobacillus helveticus* LH100 and *P. acidilactici* SAL or *P. pentosaceus* SPL-2 starter pairs contained a significantly lower level of residual galactose (*P* < 0.01) than RSM fermented with a traditional mozzarella starter pair (Tables 3-3 and A-2). These observations suggest that *Pediococcus* strains may be effective cultures for the control of nonenzymatic browning in mozzarella cheese.

As mentioned above, symbiotic growth between starter cocci and rods is a desirable property of thermophilic starter blends because it accelerates lactic acid production. To determine if similar interactions might occur between pediococci and lactobacilli, SAL and SPL-2 were grown in 9% RSM alone and in combination with a commercial Mozzarella starter, *Lactobacillus helveticus* LH100. Because they are
deficient in the ability to utilize milk proteins (Table 2-3, Chapter IV), neither *Pediococcus* spp. was able to coagulate 9% RSM within 48 h at 37°C; however, *Lactobacillus helveticus* LH100 alone gave a clot time of 18 h. Nonetheless, when LH100 and SAL or SPL-2 were combined 1:1 (1% total inoculum), milk clot time was reduced to 6.75 and 6.25 h, respectively (see Chapter II), and pH reduction curves indicated synergistic lactate production was occurring (Figure 2-5). Synergism between pediococci and *Lactobacillus helveticus* LH100 was also supported by plate count data. Milk inoculated with SAL, SPL-2, or *Lactobacillus helveticus* LH100 single strains always contained lower final cell populations, after 24 h at 37°C, than SAL- or SPL-2-*Lactobacillus* paired starters (Table 6-1).

Experimental-scale part-skim Mozzarella cheese was manufactured using *Lactobacillus helveticus* LH100 and *P. acidilactici* SAL or *P. pentosaceus* SPL-2 paired starters (1:1, 2% total inoculation) and a commercial starter pair (*S. thermophilus* TA061 with *Lactobacillus helveticus* LH100 in 1:1 ration, 1% total inoculation). As shown in Table 6-2, cheese fat and moisture levels in Mozzarella made with *Pediococcus-* *Lactobacillus helveticus* paired starters were similar to those of the control cheese. The time required to produce Mozzarella (curd cut to stretching) with SPL-2 or SAL starter blends, however, averaged 60 and 90 min longer than the control. Those longer make times prompted an examination of the caseinolytic ability of the *Pediococcus* spp. used in this study.

Results from experiments that screened wild-type *Pediococcus* spp. for aminopeptidase, extracellular proteinase, and oligopeptide transport system activity indicated that *P. acidilactici* ATCC 12697 and *P. pentosaceus* ATCC 25745 exhibited general aminopeptidase activity (Figure 4-1), but lacked one or both of the latter systems. Attempts were then made to improve the ability of *Pediococcus* spp. to grow in milk by introducing the lactococcal genes for either the extracellular proteinase or the oligopeptide
Table 6-1. Synergistic increase in cell numbers\textsuperscript{a} in combined *Lactobacillus-Pediococcus* cultures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Single culture</th>
<th>Combined culture</th>
<th>Increase in cell numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus helveticus</em> LH100</td>
<td>$3.49 \times 10^8$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pediococcus acidilactici</em> SAL</td>
<td>$5.06 \times 10^7$</td>
<td>$6.88 \times 10^8$</td>
<td>$2.75 \times 10^6$</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em> SPL-2</td>
<td>$2.21 \times 10^8$</td>
<td>$7.59 \times 10^8$</td>
<td>$1.82 \times 10^8$</td>
</tr>
</tbody>
</table>

\textsuperscript{a}CFU/ml

transport system into those strains and their Lac\textsuperscript{+} transformants, *P. acidilactici* SAL, and *P. pentosaceus* SPL-2; however, these experiments proved unsuccessful.

Since the primary objective of our work was to develop an alternative starter coccus that could be used to thwart bacteriophage proliferation in Mozzarella cheese plants, it was important to screen *P. acidilactici* SAL and *P. pentosaceus* SPL-2 for sensitivity to bacteriophages that were already present in the industry. In collaboration with Rhodia, Inc. (Madison, Wis.), *P. acidilactici* SAL and *P. pentosaceus* SPL-2 were tested for susceptibility to bacteriophages in 835 separate whey samples collected from North American cheese producers. More than half of the whey samples contained greater than $10^5$ plaque forming units per ml, but none of these bacteriophages was able to attack SAL or SPL-2.

Although bacteriophage which attack *Pediococcus* spp. had not been previously reported, the possibility for the development of lytic phages from temperate strains made the study of lysogeny in pediococci advisable (4). Therefore, mitomycin C was used to screen strains of *P. acidilactici* and *P. pentosaceus* for lysogeny. Temperate bacteriophage were induced from three strains of *P. acidilactici*. These phages
Table 6-2. Composition of Mozzarella cheese made with *Lactobacillus helveticus* LH100 paired with *Streptococcus thermophilus* TAO061, *Pediococcus pentosaceus* SPL-2, or *Pediococcus acidilactici* SAL

<table>
<thead>
<tr>
<th>Cheese content</th>
<th><em>S. thermophilus</em> TAO061</th>
<th><em>P. pentosaceus</em> SPL-2</th>
<th><em>P. acidilactici</em> SAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Moisture</td>
<td>51.0 ± 1.7</td>
<td>46.6 ± 0.14</td>
<td>50.8 ± 3.0</td>
</tr>
<tr>
<td>% Fat</td>
<td>18.8 ± 0.90</td>
<td>22.1 ± 4.1</td>
<td>18.8 ± 1.8</td>
</tr>
<tr>
<td>% Protein</td>
<td>25.9 ± 1.7</td>
<td>27.0 ± 1.2</td>
<td>25.1 ± 0.85</td>
</tr>
<tr>
<td>% Ash</td>
<td>2.6 ± 0.36</td>
<td>2.7 ± 0.24</td>
<td>2.9 ± 0.82</td>
</tr>
<tr>
<td>pH</td>
<td>5.23</td>
<td>5.32</td>
<td>5.32</td>
</tr>
</tbody>
</table>

demonstrated morphological characteristics which placed them in the B1 group of the family *Siphoviridae*. Furthermore, DNA-DNA homology studies suggest that the phages may represent at least two different species of *P. acidilactici* bacteriophage.

As a whole, these results addressed the specific objectives of the study as follows:

Objective 1: Determine whether Lac⁺ pediococci can be obtained by transformation with native *Lactococcus lactis* plasmid DNA.

Data presented in Chapters II and III showed that *Pediococcus* strains with the ability to metabolize lactose could be developed through transformation of *P. acidilactici* ATCC 12697 and *P. pentosaceus* ATCC 25745 by electroporation using pPN-1, a 35-kb deletion derivative of the lactose plasmid from *L. lactis* C2. Furthermore, greater than 85% of the transformants, *P. acidilactici* SAL and *P. pentosaceus* SPL-2, remained Lac⁺ after nine sequential transfers in MRS containing lactose as the sole carbohydrate (Figure 2-5).
Objective 2: Determine whether pediococci can express lactococcal proteinase or oligopeptide transport enzyme systems.

The data described in Chapter IV showed that *P. acidilactici* ATCC 12697 could express low levels of proteinase activity (Figure 4-2) when transformed with the proteinase plasmid pGKV552 and that *P. pentosaceus* strains demonstrated oligopeptide transport activity (Table 4-2) when the pVS8-encoded genes for this system were introduced. The expression of the individual systems, however, was not sufficient to decrease the amount of time that these strains required to clot 9% RSM (Table 4-3).

Objective 3: Investigate the suitability of genetically improved *Pediococcus* species as starter cultures for Mozzarella cheese.

The inability of *Pediococcus* spp. to efficiently utilize casein (Chapter IV) currently precludes their application as direct replacement cocci for *S. thermophilus*. The expression of lactococcal enzymes for lactose and galactose utilization (Chapters II and III), however, makes these strains attractive adjunct cultures for the manufacture of Mozzarella cheese. As adjuncts, Lac⁺ pediococci have two potential benefits. They may serve to continue acid production in the event of a bacteriophage attack on the *S. thermophilus* starter and thus complete the fermentation, or they may alleviate Maillard browning of the cheese by reducing the level of free galactose in the curd.

References


APPENDICES
APPENDIX A
DATA NOT INCLUDED IN TEXT
Table A-1. Statistical analysis of differences in milk clotting times

<table>
<thead>
<tr>
<th>Culture</th>
<th>Clot time (h)(^a)</th>
<th>T(^b)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus helveticus</em> LH100</td>
<td>17.9 ± 0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH100 + <em>P. acililactici</em> SAL</td>
<td>6.75 ± 0.35</td>
<td>39.8</td>
<td>0.016</td>
</tr>
<tr>
<td>LH100 + <em>P. pentosaceus</em> SPL-2</td>
<td>6.25 ± 0.35</td>
<td>41.6</td>
<td>0.015</td>
</tr>
</tbody>
</table>

\(^a\)Values reported are the means ± the standard deviations

\(^b\)The hypothesis tested is that the clot time of the combined culture is equal to the clot time of *Lactobacillus helveticus* LH100 alone

Table A-2. Statistical values for the comparison of residual galactose in milk

<table>
<thead>
<tr>
<th>Culture</th>
<th>T(^a)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus helveticus</em> LH100</td>
<td>4.76</td>
<td>0.018</td>
</tr>
<tr>
<td>LH100 + <em>Pediococcus acililactici</em> SAL</td>
<td>5.87</td>
<td>0.010</td>
</tr>
<tr>
<td>LH100 + <em>P. pentosaceus</em> SPL-2</td>
<td>7.21</td>
<td>0.006</td>
</tr>
</tbody>
</table>

\(^a\)All T-tests test the hypothesis that the mean % galactose (Table 4-) equals the mean % galactose found in the milk fermented with the control culture LH100 + *Streptococcus thermophilus* TA061
Table A-3. Statistical analysis of the data presented in Table 4-2.

<table>
<thead>
<tr>
<th>Culture (a)</th>
<th>Hypothesis (b)</th>
<th>T =</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactococcus lactis</em></td>
<td>( \mu = \mu_{\text{LM2301-C}} )</td>
<td>4.23</td>
<td>0.15</td>
</tr>
<tr>
<td>LM2301:pVS8-C</td>
<td>( \mu = \mu_{\text{ATCC 12697-C}} )</td>
<td>8.22</td>
<td>0.077</td>
</tr>
<tr>
<td><em>Pediococcus acidilactici</em></td>
<td>( \mu = \mu_{\text{SAL-N}} )</td>
<td>0.10</td>
<td>0.93</td>
</tr>
<tr>
<td>12697:pVS8-C</td>
<td>( \mu = \mu_{\text{SAL-N}} )</td>
<td>0.20</td>
<td>0.85</td>
</tr>
<tr>
<td>SAL-P</td>
<td>( \mu = \mu_{\text{SAL:pVS8-N}} )</td>
<td>0.50</td>
<td>0.70</td>
</tr>
<tr>
<td>SAL-C</td>
<td>( \mu = \mu_{\text{SAL:pVS8-N}} )</td>
<td>1.51</td>
<td>0.18</td>
</tr>
<tr>
<td>SAL:pVS8-C</td>
<td>( \mu = \mu_{\text{SAL-C}} )</td>
<td>0.83</td>
<td>0.45</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em></td>
<td>( \mu = \mu_{\text{ATCC 25745-C}} )</td>
<td>14.57</td>
<td>0.044</td>
</tr>
<tr>
<td>25745:pVS8-C</td>
<td>( \mu = \mu_{\text{SPL-2-N}} )</td>
<td>9.40</td>
<td>0.067</td>
</tr>
<tr>
<td>SPL-2-P</td>
<td>( \mu = \mu_{\text{SPL-2-N}} )</td>
<td>0.50</td>
<td>0.64</td>
</tr>
<tr>
<td>SPL-2-C</td>
<td>( \mu = \mu_{\text{SPL-2:pVS8-N}} )</td>
<td>5.86</td>
<td>0.11</td>
</tr>
<tr>
<td>SPL-2:pVS8-P</td>
<td>( \mu = \mu_{\text{SPL-2-C}} )</td>
<td>4.14</td>
<td>0.014</td>
</tr>
<tr>
<td>SPL-2:pVS8-C</td>
<td>( \mu = \mu_{\text{SPL-2-C}} )</td>
<td>4.00</td>
<td>0.016</td>
</tr>
</tbody>
</table>

(a) -N= grown in chemically defined media (CDM) without added histidine; -P, grown in CDM with a pentapeptide containing histidine added; -C, grown in CDM with \( \alpha_\text{T-casein} \)-(1-8) fragment added.

(b) All tests compare the mean increase in \( A_{600} \) after 24h growth in CDM (Table 4-2).
Fig A-1. Agarose gel electrophoresis of amplicons obtained by quantitative polymerase chain reactions. Samples shown included Hi-Lo DNA size standard (Minnesota Molecular, Minneapolis, Minn.) (lane A); amplicons from *L. lactis* LM2301 template DNA (lane B); amplicons from *L. lactis* PN-1 template DNA (lane C); amplicons from *P. acidilactici* ATCC 12697 template DNA (lane D); amplicons from *P. acidilactici* SAL template DNA (lane E); amplicons from *P. pentosaceus* ATCC 25745 template DNA (lane F); amplicons from *P. pentosaceus* SPL-2 template DNA (lane G); negative control (no template DNA) (lane H).
FIG. A-2. Induction curves of *Pediococcus acidilactici* strains with (closed symbols) or without (open symbols) 0.5 µg/ml mitomycin C. *Pediococcus acidilactici* ATCC 25740 (■, □); ATCC 25742 (▲, △); ATCC 12697 (●, ○).
FIG. A-3. Hybridization of *Hind* III-restricted bacteriophage probes to *Hind* III-cut genomic DNA. The panel on the left shows the X-ray film obtained after DNA-DNA hybridization of the digoxigenin-11-dUTP-labeled pa40 probe to a blot made from the agarose gel shown in Fig. 5-4. The panel on the right shows the X-ray film obtained after DNA-DNA hybridization of the digoxigenin-11-dUTP-labeled pa97 probe to the same blot. The DNA samples present in each lane were A) Hi-Lo DNA size standard (Minnesota Molecular, Minneapolis, Minn.); B) pa97; C) pa40; D) pa42; E) undigested pa97 DNA; F) undigested pa40 DNA; and G) undigested pa42 DNA.
APPENDIX B

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<th>Year</th>
<th>Institution</th>
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<td>Major: Microbiology</td>
<td>1993</td>
<td>Weber State University</td>
</tr>
<tr>
<td></td>
<td>Minor: Chemistry</td>
<td></td>
<td>Ogden, Utah 84408</td>
</tr>
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<td>Nutrition and Food Sciences</td>
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<td></td>
<td>GPA: 3.92</td>
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</table>

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