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Development and Characterization of Lactose-Positive *Pediococcus* Species for Milk Fermentation†

SHELBY L. CALDWELL,[1] DONALD J. MCMAHON,[1] CRAIG J. OBERG,[2] AND JEFFERY R. BROADBENT[1]*

Western Center for Dairy Proteins Research and Technology, Department of Nutrition and Food Sciences, Utah State University, Logan, Utah 84322-8700,[1]† and Department of Microbiology, Weber State University, Ogden, Utah 84408-2506[2]

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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 *Pediococcus acidilactici* ATCC 12697 by electroporation with pPN-1, a 35-kb *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 that these cultures could be used to manufacture part-skim mozzarella cheese. Results demonstrate that lactose-positive pediococci have potential as replacement cocci for *S. thermophilus* in Italian cheese starter blends and may facilitate development of new strain rotation schemes to combat *S. thermophilus* bacteriophage problems in mozzarella cheese plants.

Starter cultures for the manufacture of Italian cheeses such as mozzarella typically contain *Streptococcus thermophilus* and *Lactobacillus helveticus* or *Lactobacillus delbrueckii* 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). One approach may be to replace *S. thermophilus* in Italian cheese 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 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 indicating that nonstarter and adjunct *Pediococcus* spp. impart desirable attributes to cheese (4, 5, 29) suggest that pediococci might be good dairy starter bacteria if they possess the ability to utilize lactose. As an example, lactose-positive (Lac^+) *Pediococcus acidilactici* and *Pediococcus 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-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 that Lac^+ *Pediococcus* spp. have potential as replacement cocci for *S. thermophilus* in Italian starter blends.

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MATERIALS AND METHODS

Bacterial cultures. The bacteria and plasmids used in the study are listed in Table 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 (7) which contained 2.0% glucose or lactose as the carbohydrate source (MRS-G and MRS-L, respectively). Lactococci were propagated at 30°C in M17 broth (32), which contained 0.5% glucose or lactose (M17-G and M17-L, respectively) 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 $CsCl_2$-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 supercoiled DNA ladder size standards from Life Technologies, Inc. (Gaithersburg, Md.), included in the gel. Restriction endonuclease mapping of pPN-1 was performed as described by Maniatis et al. (20).

Electroporation of pediococci. Electrotransformation of *Pediococcus* spp. with pGK12 and pPN-1 was performed with a Bio-Rad (Richmond, Calif.) Gene-Pulser apparatus. Competent cells were prepared by overnight growth in MRS-G with 0.5 M sorbitol, and then a 1.5% inoculation was made into 800 ml of MRS-G that contained 0.5 M sorbitol, 3% glycine, and 40 mM DL-threonine. The bacteria were incubated for 2 to 4 h (A_{600}, 0.4 to 0.6) at 37°C and then collected and

* Corresponding author. Phone: (801) 797-2113. Fax: (801) 797-2379.

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TABLE 1. Bacteria and plasmids used in this study

Bacterium or plasmid	Relevant phenotype[a]	Description (source or reference)
Pediococcus spp.		
P. acidilactici ATCC 12697	Lac^-	Wild type (ATCC)
P. acidilactici SAL	Lac^+	ATCC 12697 transformed with pPN-1 (this study)
P. pentosaceus ATCC 25744	Lac^-	Wild type (ATCC)
P. pentosaceus SPL-1	Lac^+	ATCC 25744 transformed with pPN-1 (this study)
P. pentosaceus ATCC 25745	Lac^-	Wild type (ATCC)
P. pentosaceus SPL-2	Lac^+	ATCC 25745 transformed with pPN-1 (this study)
Lactococcus lactis		
LM2302	Lac^-	Plasmid-cured derivative of strain C2 (23)
PN-1	Lac^+	Transductant of strain LM2302 constructed as described by McKay et al. (reference 24 and this study)
Lactobacillus helveticus LH100	Lac^+	Mozzarella cheese starter (Marschall Products/Rhône-Poulenc)
Streptococcus thermophilus TA061	Lac^+	Mozzarella cheese starter (Marschall Products/Rhône-Poulenc)
Plasmids		
pGK12	Em^r Cm^r	4.4-kb *Lactococcus lactis* cloning vector (15)
pPN-1	Lac^+	35-kb deletion derivative of the *Lactococcus lactis* C2 lactose plasmid (this study and reference 21)

[a] Abbreviations: Lac^+, able to ferment lactose; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant.

washed twice in 25 ml of a 0.5 M sorbitol–10% glycerol solution. After the washes, the cells were suspended in 1 ml of electroporation buffer (0.5 M sorbitol, 1 mM K_2HPO_4, 1 mM $MgCl_2$, pH 7.0). Eighty microliters of the suspension was mixed with 4 μl of 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. The cultures were then kept on ice for approximately 5 min. The cells were allowed to recover for 2 h at 37°C and then plated and incubated for 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) which contained 0.5 M sorbitol. Transformants with pGK12 were selected on M17-G agar that contained 0.5 M sorbitol and 5 μg of erythromycin per ml. Transformation frequencies are expressed as the number of erythromycin-resistant CFU per microgram of 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 from 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 on the basis of preliminary experiments which established approximate clot times for each strain under study. Reduction in pH of 9% RSM or MRS-L broth by a 1 or 2% inoculum from a fresh 4-h ($>10^6$-CFU/ml) culture was measured with a Beckman Instruments (Fullerton, Calif.) model pHI40 pH meter.

The ability of *P. acidilactici* SAL, *P. pentosaceus* SPL-1, and *P. pentosaceus* SPL-2 to utilize casein (22) was investigated by introduction of a 2% inoculum of these strains 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.). The milk samples were incubated at 37°C, and 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 that pediococci did not produce compounds that would inhibit the growth of *Lactobacillus helveticus* LH100. Each test included *Lactobacillus helveticus* LH100 and the nisin-producing bacterium *Lactococcus lactis* 11454 (31) as the negative and positive controls, respectively.

Phage sensitivity assays. The susceptibility of *Pediococcus* sp. transformants to bacteriophages in North American dairy production facilities was evaluated by Marschall Products/Rhône-Poulenc (Madison, Wis.) 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* sp. 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 with 2% total inoculations of *P. acidilactici* SAL or *P. pentosaceus* SPL-2 with *Lactobacillus helveticus* LH100 (1:1). Control cheeses were made with 1% total inoculations of *S. thermophilus* TA061 and *Lactobacillus 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 of *P. pentosaceus*, one of *Pediococcus dextrinicus*, two of *Pediococcus inopinatus*, and one undefined *Pediococcus* sp. were obtained from the American Type Culture Collection (ATCC; Rockville, Md.), the Belgian Coordinated Collections of Microorganisms (Ghent, 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 that it was actually *Enterococcus faecalis*. This result prompted us to subject pediococci selected for electrotransformation experiments to extensive microbiological characterization (Table 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 found to contain a single plasmid, designated pPN-1. Restriction endonuclease digests of pPN-1 with *Bgl*II and *Kpn*I indicated that the plasmid was approximately 35 kb in length. The size difference between pPN-1 and the 56-kb C2 lactose (*lac*) plasmid (21) suggested that 20 kb of the original molecule had been deleted from pPN-1. Temperate bacteriophages responsible for *lac* plasmid transduction can accommodate only 35 to 36 kb of DNA; thus, *lac* plasmid deletion derivatives are consistent and natural occurrences in Lac^+ transductants (10, 21).

A restriction endonuclease map of pPN-1 was constructed and compared with that of the 56-kb *lac* plasmid of *Lactococcus lactis* 712 (10, 16), the strain from which C2 was originally derived (6). The comparison showed that the *lac* operon on pPN-1 was intact and indicated that the plasmid had undergone two distinct deletion events (Fig. 1). A 14-kb deletion was mapped to the region between *lac* and the origin of replication,

TABLE 2. Microbiological characterization of wild-type *Pediococcus* strains used in this study

Characteristic	ATCC 12697	ATCC 25744	ATCC 25745
Lactate enantiomers produced	D and L	D and L	D and L
Fatty acid analysis[a]	Inconclusive	*P. pentosaceus*	*P. pentosaceus*
Carbohydrate utilization[b]			
N-Acetylglucosamine	++	++	++
Amygdalin	−	+	++
L-Arabinose	+++	+++	+++
Arbutin	+	+	+
Cellobiose	+++	+++	+++
Esculin	+++	+++	+++
D-Fructose	+++	+++	+++
Galactose	+++	+++	+++
β-Gentiobiose	+	+	+
D-Glucose	+++	+++	+++
D-Mannose	+++	+++	+++
Melibiose	−	+++	+++
Raffinose	−	+++	+
Ribose	+++	+++	+++
Sucrose	−	+++	+++
Salicin	+	+	+
D-Tagatose	+++	+++	+++
Trehalose	+++	+++	+++
D-Xylose	+++	++	+++
API identification[c]	*P. acidilactici*	*P. pentosaceus*	*P. pentosaceus*

[a] Service provided by Analytical Services, Inc. (Essex Junction, Vt.). Similarity index values of ≥0.400 indicate a good match in fatty acid profile while values of ≤0.250 indicate a poor match. Similarity index values: 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 carbohydrate test kit prepared for lactic acid bacteria (values range from not fermented [−] to rapidly fermented [+++]).

[c] Service provided by the bioMérieux Vitek, Inc. (Hazelwood, Mo.), technical service department.

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^r *P. acidilactici* ATCC 12697, *P. pentosaceus* ATCC 25744, and *P. pentosaceus* ATCC 25745 transformants were obtained at frequencies of 4.6×10^3, 3.4×10^3, and 1.8×10^3, 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 Fig. 2, 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 2b also shows that *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 (by tests listed in Table 2) showed that 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 identities, and these two bacteria were selected as species representatives for subsequent studies to characterize genetically derived Lac^+ pediococci.

Expression of Lac^+ in transformants. Lac^+ expression in *P. acidilactici* SAL and *P. pentosaceus* SPL-2 in MRS-L was evaluated. Both transformants, especially SAL, grew better than the respective wild-type strains in MRS-L broth (Fig. 3a). As expected, improved growth by Lac^+ transformants in MRS-L was accompanied by a reduction in pH (Fig. 3b).

Lac^+ stability. After nine sequential transfers (>175 generations) in MRS-L broth, approximately 95% of *P. acidilactici* SAL CFU remained Lac^+ (Fig. 4). 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 *Lactobacillus helveticus* (27), Lac^+ transformants were tested for production of compounds which inhibited growth of *Lactobacillus helveticus* LH100. Agar overlay tests indicated that *Lactobacillus helveticus* LH100 was not inhibited by any of the *Pediococcus* spp. listed in Table 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 *Lac-*

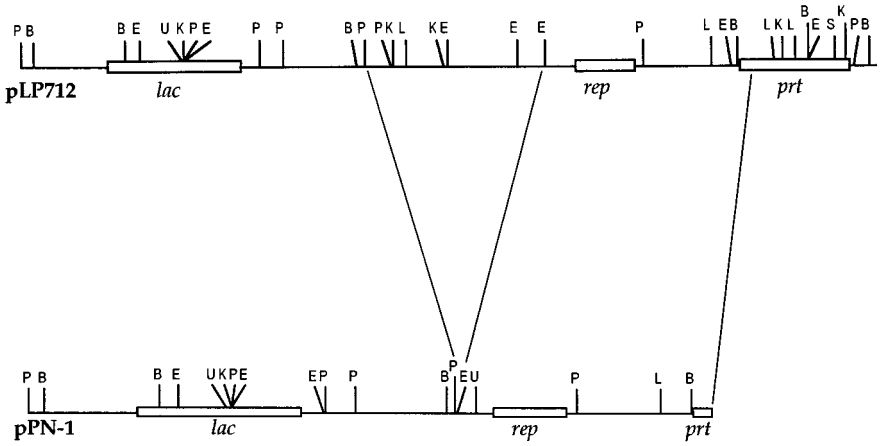

FIG. 1. Restriction endonuclease and deletion map of the 35-kb *Lactococcus lactis* plasmid pPN-1. Diagonal lines identify regions of the 56-kb lactococcal plasmid pLP712 that have been deleted in pPN-1. Abbreviations: B, *Bgl*II; E, *Bst*EII; K, *Kpn*I; L, *Bgl*I; P, *Pst*I; S, *Sal*I; U, *Stu*I; *lac*, lactose operon; *rep*, origin of replication; *prt*, genes for extracellular proteinase. The map of pLP712 is adapted from articles by Gasson et al. (10) and Kok and Venema (16).

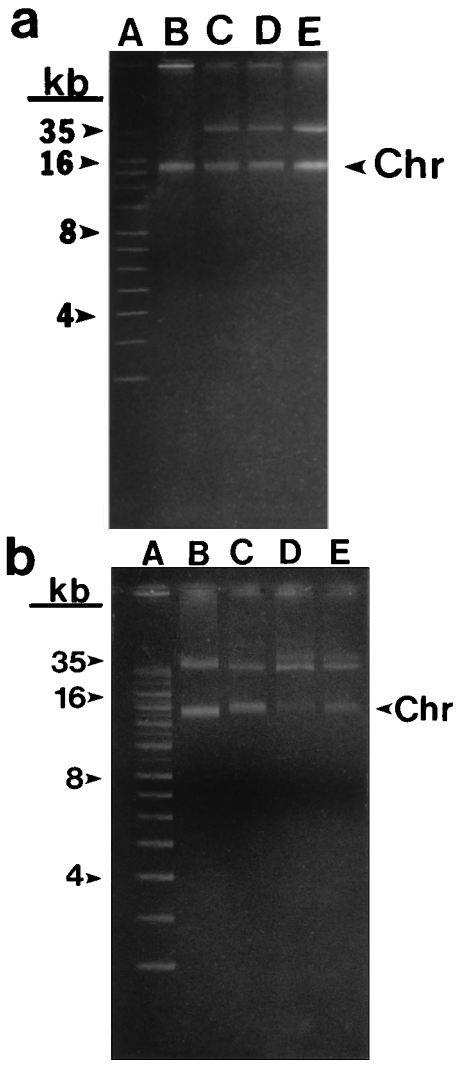

FIG. 2. Agarose gel electrophoresis of plasmid DNA isolated from *Pediococcus* sp. lactose-positive (Lac^+) transformants and wild-type strains. (a) Lanes: A, supercoiled DNA standard; B, wild-type *P. acidilactici* ATCC 12697; C and D, Lac^+ transformants of strain ATCC 12697, namely, *P. acidilactici* SAL; E, $CsCl_2$-purified pPN-1. (b) Lanes: A, supercoiled DNA standard; B, $CsCl_2$-purified pPN-1; C, wild-type *P. pentosaceus* ATCC 25745; D and E, Lac^+ transformants of *P. pentosaceus* ATCC 25745, namely, *P. pentosaceus* SPL-2.

tobacillus 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 ($P < 0.003$) reduced clot times when compared with those of 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 a 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 (Fig. 5). The rate of pH decrease in 9% RSM inoculated with 1% *Pediococcus* sp.–LH100 strain combinations was lower 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% rather than 0.5% each) were added to the milk (Fig. 5).

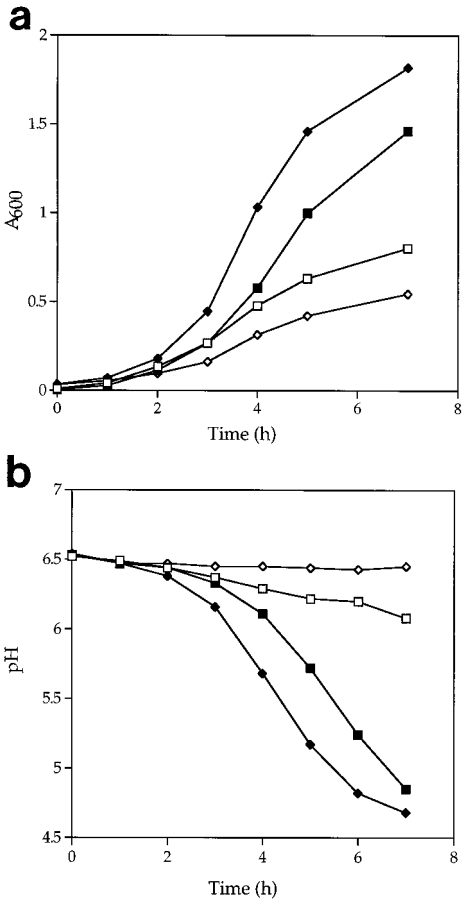

FIG. 3. Growth in (a) and pH reduction of (b) MRS-L by Lac^+ *Pediococcus* sp. transformants (closed symbols) and wild-type strains (open symbols). Symbols: ◇, *P. acidilactici* ATCC 12697; □, *P. pentosaceus* ATCC 25745; ◆, *P. acidilactici* SAL; ■, *P. pentosaceus* SPL-2.

Pediococci require most amino acids for growth (9), and Table 3 shows that the inability of Lac^+ transformants to rapidly coagulate and acidify RSM was likely due to ineffective casein utilization by these bacteria. The addition of enzymat-

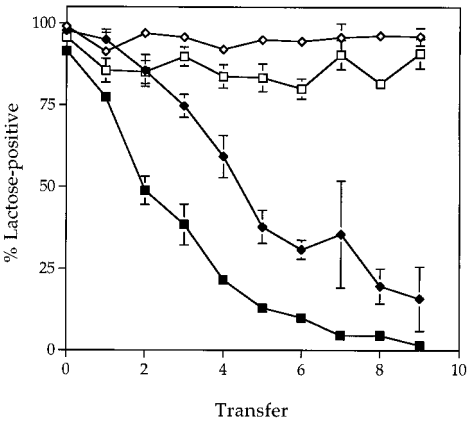

FIG. 4. Percentage of lactose-positive *P. acidilactici* SAL (◇, ◆) and *P. pentosaceus* SPL-2 (□, ■) after serial transfer at 37°C in MRS-L (◇, □) or MRS-G (◆, ■).

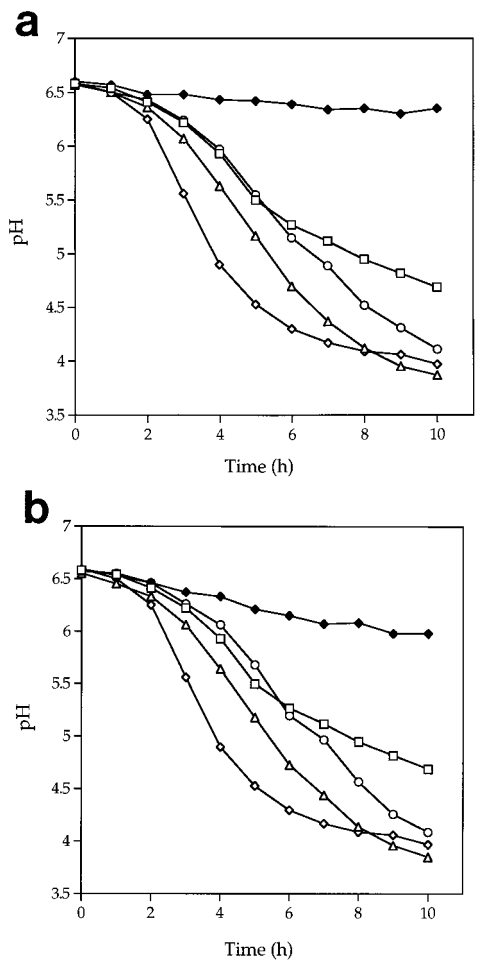

FIG. 5. pH reduction of 9% RSM by various bacteria. (a) pH changes in RSM inoculated with 1% *P. acidilactici* SAL (◆), 1% *Lactobacillus helveticus* LH100 (□), 0.5% SAL plus 0.5% LH100 (○), 1% SAL plus 1% LH100 (△), and 0.5% *S. thermophilus* TA061 plus 0.5% LH100 (◇); (b) pH changes in RSM inoculated with 1% *P. pentosaceus* SPL-2 (◆), 1% *Lactobacillus helveticus* LH100 (□), 0.5% SPL-2 plus 0.5% LH100 (○), 1% SPL-2 plus 1% LH100 (△), and 0.5% *S. thermophilus* TA061 plus 0.5% LH100 (◇).

ically 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 that 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 and *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 10^5 PFU/ml (11), but bacteriophages able to attack SAL or SPL-2 were not detected.

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

DISCUSSION

The objective of this study was to develop alternative starter cocci with the 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 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 that it includes genes for the lactose-specific PTS enzymes enzyme IIA^{lac} (LacF) and enzyme IIBC^{lac} (LacE), phospho-β-galactosidase (LacG), and enzymes of the tagatose-6-phosphate pathway (LacA to LacD) (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$^+$ 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

TABLE 3. Growth of *Pediococcus* transformants in 9% RSM with and without NZ amine or Casamino Acids

Strain	Medium[a]	CFU/ml		pH		Clotting time (h)
		0 h	24 h	0 h	24 h	
P. acidilactici SAL	RSM	8.7×10^7	9.0×10^7	6.45	5.72	>120
	NZ amine	7.5×10^7	9.2×10^8	6.51	4.95	<24
	Casamino Acids	6.3×10^7	8.0×10^8	6.52	5.10	<24
P. pentosaceus SPL-1	RSM	7.6×10^7	5.1×10^8	6.47	5.34	48
	NZ amine	8.7×10^7	8.4×10^8	6.50	5.16	<24
	Casamino Acids	6.9×10^7	6.6×10^8	6.49	5.18	<24
P. pentosaceus SPL-2	RSM	6.5×10^7	3.5×10^8	6.49	5.37	48
	NZ amine	5.7×10^7	7.3×10^8	6.52	5.10	<24
	Casamino Acids	6.3×10^7	6.0×10^8	6.49	5.16	<24

[a] RSM, 9% RSM; NZ amine, 9% RSM fortified with 0.25% NZ amine; Casamino Acids, 9% RSM fortified with 0.25% Casamino Acids.

growth requires the extracellular proteinase and an oligopeptide transport system (14). Since Lac^+ 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^+ 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 under way to determine whether 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 the 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 *Lactobacillus helveticus* LH100 (Fig. 5). Although the rate of pH decrease for this RSM was lower than that for RSM inoculated with *S. thermophilus* TA061 and LH100, small-scale cheese-making experiments showed that Lac^+ *Pediococcus* spp. can be used to manufacture part-skim mozzarella cheese. As a whole, these data show that Lac^+ pediococci have clear potential as replacement cocci for *S. thermophilus* in Italian starter blends, and this application may facilitate the development of new strain rotation schemes to combat *S. thermophilus* bacteriophage problems in mozzarella cheese plants.

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