Overexpression of Peptidases in *Lactococcus* and Evaluation of Their Release from Leaky Cells

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ABSTRACT

Walker and Klaenhammer (2001) developed a novel expression system in *Lactococcus lactis* that facilitated the release of β -galactosidase (117 kDa monomer) without the need for secretion or export signals. The system is based on the controlled expression of integrated prophage holin and lysin cassettes via a lactococcal bacteriophage ϕ 31 transcriptional activator (Tac31A) that resides on a high-copy plasmid. Approximately 85% of β -galactosidase activity was detected in the supernatant of leaky lactococci without evidence of hindered growth, cell lysis, or membrane damage. The objective of this study was to determine if intracellular peptidases were externalized from leaky lactococci. Five *L. lactis* peptidases (PepA, PepC, PepN, PepO and PepXP) and two *Lactobacillus helveticus* peptidases (PepN and PepO) were cloned and overexpressed on two high-copy vectors. The lactococcal peptidases were also cloned into the high-copy vector that contained the Tac31A transcriptional activator to determine if they were externalized from the leaky prophage-containing *L. lactis* subsp. *lactis* strain NCK203. Two of the lactococcal peptidases (PepA and PepO) required an additional strong promoter (*Lactobacillus paracasei* P144) and optimized assay conditions to detect enzyme activity. Results showed different levels of enzymatic overexpression associated with the cellular fraction (2 to 250-fold increases in activity) and negligible amounts of activity present within the supernatant fraction (0 to 6% of total peptidase activity). The lactococcal phage-based protein release mechanism did not facilitate the externalization of the lactococcal peptidases investigated in this study. (**Key words:** Leaky *Lactococcus*, peptidase)

Abbreviation key: Cm = chloramphenicol, Em = erythromycin, LAB = lactic acid bacteria, MCA = methylcoumarin, Pep = peptidase, pNA = para-nitroanilide.

INTRODUCTION

Lactic acid bacteria (LAB) are responsible for flavor and texture development of milk-based fermentation products. Of particular importance in milk fermentation is the proteolysis of casein. The LAB species have several amino acid auxotrophies, and possess proteolytic systems that enable them to acquire amino acids from casein by the use of various enzymes and transport systems. Because of their importance in cheese manufacture, the enzymatic activities of various *Lactobacillus* and *Lactococcus* strains have been studied with the objective of producing a superior final product in a shorter amount of time. The lactococcal proteolytic system consists of an extracellular, cell wall-associated proteinase, several oligopeptide and amino acid transport systems, and over ten different intracellular peptidases (Christensen et al., 1999; Kunji et al., 1996). This system is essential for their rapid growth in milk where the amount of small peptides and free amino acids is low. In addition, this process contributes to the final product by producing the precursors to volatile aromas and flavors.

Starter culture bacteria inoculated at the start of cheese making increase 100-fold by the end of the process (5-6 hrs.) and decrease during the early stages of ripening. The rate of starter culture decline is dependent on lactose content, salt concentration, autolytic behavior, redox potentials and ripening temperatures (Farkye, 1999). The onset of cell death and subsequent lysis is very important to the cheese making process, because as intracellular enzymes are released, native milk peptides are converted into amino acids and flavor compounds. Several studies have shown that the reduction of bitterness and overall acceptable flavor development in cheese correlates to the release of these intracellular enzymes and their subsequent hydrolysis of peptides (Baankreis, 1992; Meijer et al., 1998; Prost and Chamba, 1994; Tan et al., 1993). Autolysis and intracellular enzyme release may be rate-limiting steps in the ripening process. A slow onset of lysis can cause cheese manufacturers valuable time and money. The ripening of different varieties of cheese can take as little as 4 wk to as long as 4 yr. El Soda (1997) calculated that

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ripening time for Cheddar cheese adds an additional 1.5 to 3 cents per month per pound to the cost of the final product. This makes ripening the most expensive step in the cheese making process. If ripening could be accelerated and controlled, it could improve both the efficiency and quality of the process.

Several approaches have been proposed to accelerate cheese ripening by controlling the lysis onset time using various phage- and autolysin-based mechanisms. Buist et al. (1997) induced *acmA* expression in *Lactococcus lactis* subsp. *cremoris* LL302 by using mitomycin and the phage *r1t* promoter region. They reported an increase in *acmA* expression 4 h after induction, which correlated with lysis and release of intracellular proteins. This study also reported that once *AcmA* was released from the cell, it acted extracellularly on non-*AcmA* producing strains. This extracellular behavior is similar to the widespread lysis produced by released bacteriophage lysins. De Ruyter et al. (1997) combined the holin and lysin genes (*lytA* and *lytH*) of the lactococcal bacteriophage ϕ US3 with the nisin-inducible expression system to control the lysis of *L. lactis* starter cultures by using subinhibitory amounts of the lantibiotic nisin. This system provided mechanisms for both complete and partial lysis. When holin and lysin are expressed together, the complete lysis of all existing cultures occurred as a result of lysin being released into the supernatant. Other published methods of decreasing the lysis onset time also utilized phage-lysis machinery. Fiertag and McKay (1987) used chemical mutagenesis techniques to isolate temperature sensitive *L. lactis* C2 strains that lysed after 1 h of incubation at typical cheese cooking temperatures (38–40°C). This strain harbors a resident prophage, which likely became sensitive to higher temperatures as a result of chemical mutagenesis. A shift in temperature induced the mutagenized phage into its lytic cycle leading to cell lysis. Another method controlling the onset of lysis involved overexpression of the *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H lysin in *Escherichia coli*. Vasala et al. (1995) suggested that this lysin enzyme could be expressed in *E. coli*, purified, and then used as a food additive for the cheese making process. The majority of accelerated ripening models are based on control of the level and onset of cell lysis and all facilitate an early onset of cell death, which can lower cheese quality. Crow et al. stated that a balance between lysed and intact cells is important in order to create a final balanced cheese flavor and texture.

A novel enzyme delivery system created by Walker and Klaenhammer (2001) has been shown to externalize β -galactosidase (β -gal) from *L. lactis* cells without the need for export signals and without effecting cell growth. The system is based on the controlled ex-

pression of a holin and lysin cassette integrated in the host chromosome. The Tac31A transcriptional activator from a lytic lactococcal bacteriophage ϕ 31, encoded on a high copy vector (pTRK391), activates low-level expression of the holin and lysin genes. This low level of expression results in the externalization of the large (117 kDa monomer) β -gal protein independently of cell lysis, membrane damage or reduced growth rates. The objective of this study was to determine whether leaky lactococci released intracellular peptidases into the supernatant. Leaky lactococcal starter cultures that express proteolytic enzymes and efficiently release them into the cheese matrix could potentially be used to accelerate the ripening process.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The strains and plasmids used in this study are listed in Table 1. *L. lactis* cells were grown at 30°C in M17 medium (Difco, Detroit, Mich.) (Terzaghi and Sandine, 1975) supplemented with 0.5% (wt/vol) glucose (GM17). Erythromycin (**Em**) and chloramphenicol (**Cm**) were used as selective agents for lactococci at a concentration of 5 μ g/ml when needed. *E. coli* XL1-Blue strains were grown in Luria-Bertani medium (Difco) with shaking or on Brain Heart Infusion medium (BHI, Difco) supplemented with 1.5% agar. Em (200 μ g/ml) and Cm (5 μ g/ml) were used for selection of *E. coli* as appropriate.

DNA Isolation and PCR Methods

Genomic DNA from *Lactobacillus helveticus* CNRZ32 and *L. lactis* MG1363 was isolated as described by Walker and Klaenhammer (1994). Small-scale *E. coli* plasmid preparations for screening transformants were performed using alkaline sodium dodecyl sulfate lysis (Sambrook et al., 1989). Small-scale *E. coli* plasmid preparations used for transformation of lactococci were performed using the QIAprep Spin Miniprep kit according to the manufacturer's specifications (Qiagen, Valencia, Calif.). Small-scale isolation of lactococcal plasmid DNA was carried out as described by O'Sullivan and Klaenhammer (1993) except that ethidium bromide was not added prior to the phenol/chloroform extraction step. Standard procedures were used for all DNA manipulations described in this study (Sambrook et al., 1989). Restriction enzymes and T4 DNA ligase were obtained from Roche Molecular Biochemicals (Indianapolis, Ind.) and New England Biolabs, Inc. (Beverly, Mass.), respectively. DNA was purified using the QIAquick PCR purification kit (Qiagen) prior to use in cloning reactions. All PCR reactions were performed using standard procedures (White, 1997) and the Ex-

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Description ^a	Source and/or reference
Bacterial strains		
<i>Lb. helveticus</i> CNRZ32	Cheese isolate	Steele ^b Bhowmik et al. (1993)
<i>L. lactis</i> subsp. <i>cremoris</i> MG1363	Plasmid-free derivative of NCDO712	Gasson (1983)
<i>L. lactis</i> subsp. <i>lactis</i> NCK203	KP1 derivative	Hill et al. (1989)
<i>E. coli</i> XLI-Blue	<i>E. coli</i> cloning host	Gibco-BRL ^c
Plasmids		
pTRKH2	Em ^r ; high-copy number cloning vector	O'Sullivan and Klaenhammer (1993)
pNZ123	Cm ^r ; high-copy number cloning vector	de Vos (1987)
pTRK391	Em ^r ; P _{15A10} :: <i>lacZ.st</i> on pTRKH2	Walker and Klaenhammer (2001)
pTRK619	Em ^r ; P _{15A102X} :: <i>pepXP</i> on pTRKH2	Walker and Klaenhammer (2001)
pTRK568	Em ^r ; <i>Lb. acidophilus</i> P6 on pTRKH2	Djordjevic et al. (1997)
pTRK528	Em ^r ; <i>LafA</i> translational fusion vector	Allison and Klaenhammer (1996)
pTRK701	Em ^r ; <i>Lb. helveticus pepN</i> on pTRKH2	This study
pTRK702	Cm ^r ; <i>Lb. helveticus pepN</i> on pNZ123	This study
pTRK703	Em ^r ; <i>L. lactis pepN</i> on pTRKH2	This study
pTRK704	Cm ^r ; <i>L. lactis pepXP</i> on pNZ123	This study
pTRK705	Cm ^r ; <i>L. lactis pepC</i> on pNZ123	This study
pTRK706	Cm ^r ; <i>Lb. helveticus pepO</i> on pNZ123	This study
pTRK707	Em ^r ; P _{15A10} on pTRKH2	This study
pTRK708	Em ^r ; <i>L. lactis pepC</i> on pTRK707	This study
pTRK709	Em ^r ; <i>L. lactis pepN</i> on pTRK707	This study
pTRK710	Em ^r ; P _{15A10} on pTRK568	This study
pTRK711	Em ^r ; P _{15A10} on pTRK528	This study
pTRK712	Em ^r ; <i>L. lactis pepA</i> on pTRK711	This study
pTRK713	Em ^r ; <i>L. lactis pepO</i> on pTRK711	This study
pTRT001	Em ^r ; <i>L. lactis pepXP</i> on pTRKH2	This study
pTRT002	Cm ^r ; <i>L. lactis pepA</i> on pNZ123	This study
pTRT003	Cm ^r ; <i>L. lactis pepO</i> on pNZ123	This study
pTRT004	Em ^r ; <i>L. lactis pepA</i> on pTRK707	This study
pTRT005	Em ^r ; <i>L. lactis pepO</i> on pTRK707	This study
pTRT006	Em ^r ; <i>L. lactis pepA</i> on pTRK710	This study
pTRT007	Em ^r ; <i>L. lactis pepO</i> on pTRK710	This study

^aAbbreviations: Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance.

^bJames Steele, Dept. of Food Science, University of Wisconsin-Madison.

^cGibco-BRL, Grand Island, N.Y.

pand High Fidelity PCR system (Roche Molecular Biochemicals). Synthesized oligonucleotides were obtained from Integrated DNA Technologies (Coralville, Iowa). When blunt-end inserts were required, *Pwo* DNA polymerase (Roche Molecular Biochemicals) was used.

Plasmid Constructions

The plasmids constructed in this study are listed in Table 1. A 3.8 kb insert carrying the *L. helveticus pepN* gene (Christensen et al., 1995) was obtained from pSUW32 by digestion with *Xba*I and then ligated into pTRKH2 (O'Sullivan and Klaenhammer, 1993) and pNZ123 (de Vos, 1987) to produce pTRK701 and pTRK702 respectively. The other peptidase (**PEP**) genes were amplified from *L. helveticus* CNRZ32 and *L. lactis* MG1363 with their own transcription and translation start sites, with the exception of the *L. lactis pepO* where the translation initiation region originates in the oligopeptide transport system operon (Mierau et

al., 1993). The primers used to amplify the *Lactobacillus* and *Lactococcus* peptidases are listed in Table 2. To construct vectors that encode both Tac31A of the lytic bacteriophage ϕ 31 and the peptidase genes, the 3.0 kb fragment containing *lacZ.st* on pTRK391 was removed by digestion with *Pst*I/*Sal*I. T4 DNA polymerase (Roche Molecular Biochemicals) was used to create blunt ends, followed by ligation and transformation into *E. coli* XL1-Blue to create the vector pTRK707. All five of the lactococcal peptidases were then amplified using *Pwo* DNA polymerase and the primers described in Table 2 to create fragments with blunt ends. The PCR products were purified prior to ligation with pTRK707, which had been digested with *Eco*RV (See Figure 1 for a diagram of this construct). The genes for PepA, PepC, PepN, and PepO were cloned into pTRK707 to construct pTRT004, pTRK708, pTRK709, and pTRT005, respectively. Figure 2 depicts the construction of pTRK711 and its derivatives. The 0.8 kb insert carrying the phage promoter P_{15A10} was obtained from pTRK391 by diges-

Table 2. Primer sets used for the construction of peptidase overexpression vectors.

Gene	Primer set	Enzyme sites	Size	Reference ^a
<i>pepN^c</i>	F-CGAGATCTGCGGACGTTTCGGATGAGCCTAATTA R-GCGAGCTCGCGGAAGTCAAGCTCTTCAAGCACTT	<i>Bgl</i> II/ <i>Sac</i> I	3.0 kb	Tan et al., (1992)
<i>pepXP</i>	F-CGCTGCAGACGTTAGCAACCGCACCATTACC R-GCGGATCCAAATTGAGCATGAGGTTCTCGCC	<i>Pst</i> I/ <i>Bam</i> HI	2.8 kb	Nardi et al., (1991)
<i>pepXP</i>	F-GCCTCGAGGAGTAGGGCAGTTCCAACAA R-GCCTCGAGATGAGGTTCTCGCCATAGA	<i>Xho</i> I/ <i>Xho</i> I	2.8 kb	Nardi et al., (1991)
<i>pepC</i>	F-GCAGTACTACCGTGGCTTTTAGAAGCA R-GCGAGCTCTCGTATTCCAGACTCTGTCC	<i>Sca</i> I/ <i>Sac</i> I	1.7 kb	Chapot et al., (1993)
<i>pepA</i>	F-CGGAATTCACCATGATAAGCTGCGTGAG R-CGGAGCTCGTGATATAGCTGCTGGTTGG	<i>Eco</i> RI/ <i>Sac</i> I	1.4 kb	l'Anson et al., (1995)
	F-CGCCTGCAGTTTTTCAGTATCCCGGAGGAG R-CGCCTCGAGGTGATATAGCTGCTGGTTGG	<i>Pst</i> I/ <i>Xho</i> I	1.2 kb	
	F-GCGCTCTAGACTGACAGATTTGTCA R-GCGGTCGACGTGATATAGCTGCTGGTTGG	<i>Xba</i> I/ <i>Sal</i> I	1.2 kb	
<i>pepO^b</i>	F-CGAAGCTTGGCTTTTCGACTATCGCAGAT R-CGCTCGAGAACTTGCCAATGACCCGTGA	<i>Hind</i> III/ <i>Xho</i> I	2.3 kb	Chen and Steele, (1998)
<i>pepO</i>	F-GCTCTAGACAGGAGCAAATGACTGGGA R-GCCTCGAGGTATCCTAACCGTGCTAGC	<i>Xba</i> I/ <i>Xho</i> I	2.5 kb	Mierau et al. (1993)
	F-GCGAGCTCCAGGAGCAAATGACTGGGA R-GCCTCGAGGTATCCTAACCGTGCTAGC	<i>Sac</i> I/ <i>Xho</i> I	2.5 kb	
	F-GCTCTAGACAGGAGCAAATGACTGGGA R-GCCTCGAGGTATCCTAACCGTGCT	<i>Xba</i> I/ <i>Xho</i> I	2.5 kb	

Restriction sites are underlined.

Abbreviations: F, forward primer; R, reverse primer.

^aGenetic sequence reference.

^bPeptidase amplified from *Lb. helveticus* CNRZ32 genome; all other peptidase were amplified from *L. lactis* MG1363.

tion with *Sma*I and ligated into the same site downstream of P144 in pTRK528 (Allison and Klaenhammer, 1996) to construct pTRK711. The *L. lactis* PepA and PepO coding regions were amplified to contain their ribosomal binding sites, without a native promoter, using the primers listed in Table 2. The peptidase fragments were cloned behind P144 (Djordjevic et al., 1997) in pTRK711.

Bacterial Transformations

Ligations were transformed into RbCl-competent *E. coli* XL1-Blue strains. RbCl-competent *E. coli* cells were prepared and transformed using the heat-shock method of transformation described for CaCl₂-competent cells (Sambrook et al., 1989). After screening *E. coli* transformants for the proper insert size and orientation, plasmid DNA was isolated and electroporated into competent *L. lactis* NCK203 cells using a modification of the procedure described by Holo and Nes (1989). A culture of *L. lactis* was inoculated (1%) into GM17 and grown until an OD₆₀₀ of 0.6 was reached. The cells were then washed three times with ice-cold electroporation buffer (0.5 M sucrose, 10% (vol/vol) glycerol) and resuspended in 1/10 of the original volume of this same buffer. Electroporations were carried out as described previously (Walker and Klaenhammer, 1998).

Enzyme Assays

L. lactis cells (1 ml) were harvested at an OD₆₀₀ of 0.6. The supernatant was harvested and the pelleted cells were washed once with 20 mM Tris-HCl (pH 6.5) assay buffer. The washed cells were then resuspended in 1 ml of this same buffer. For chromogenic substrates, the following procedure was adapted from Mierau et al. (1996) for the measurement of enzyme activity. Permeation of the membrane was accomplished by adding 5 µl of toluene to 1 ml of resuspended cells. Incubation of this mixture was done at 37°C for 30 min to facilitate toluene evaporation. Para-nitroanilide (pNA) substrates (Sigma) were used at 1 mg/ml concentrations. Individual molar concentrations of each substrate, suspended in 20 mM Tris-HCl (pH 6.5), were 0.54 mM L-lysine-pNA for PepN and PepC, 0.76 mM L-glutamic acid-γ-pNA for PepA, 0.50 mM gly-pro-pNA for PepXP, and 1.69 mM N-Benzoyl-val-gly-arg-pNA, 1.47 mM N-Benzoyl-pro-phe-arg-pNA, and 1.47 mM N-Benzoyl-phe-val-arg-pNA for PepO. The tubes were incubated in a 37°C water bath after the addition of 300 µl of the appropriate substrate to 1 ml samples of cell or supernatant fractions. The reactions were stopped after 10 min with 500 µl of 30% (vol/vol) acetic acid. Cellular debris was removed by centrifugation and spectrophotometric readings were taken at 410 nm. Enzyme activity

Figure 1. Construction of vectors encoding Tac31A and the peptidase genes. The *lacZ.st* gene of pTRK391 (Walker and Klaenhammer, 2001) was excised to create pTRK707. Plasmid pTRK707 was digested with *EcoRV* and ligated to the peptidase fragments amplified with *Pwo* polymerase. Orientation was determined through restriction enzyme digestion patterns. MCS, multiple cloning site.

is presented as the change in absorbance (410 nm) per minute.

For fluorogenic substrate assays, cell and supernatant fractions were placed on ice for 10 min prior to bead-beating. Chilled cell fractions were added to tubes that contained silica beads (106 micron diameter) and exposed to three 1-min cycles in a Mini Bead Beater (Biospec Products, Bartlesville, Okla.). The tubes were placed on ice for 1 min between each beating cycle. Cellular debris and beads were removed by centrifuga-

tion. Each 50 μ l sample of cell extract or supernatant was placed in a 400 μ l well within a black, Nunc Maxisorp 96-well microtiter plate (Nalge Nunc International, Rochester, NY). The substrates used were L-arginine-7-amido-4-methylcoumarin (MCA) for PepN and PepC, L-glutamic acid- β -MCA, L-aspartic acid- γ -MCA, and N-glutaryl-L-phenylalanine-MCA for PepA, gly-pro-MCA for PepXP, and N-Benzoyl-phe-val-arg-MCA and N-Benzoyl-arg-MCA for PepO (all MCA substrates were obtained from Sigma). The substrates were

Figure 2. Construction of PepO and PepA expression vectors with the P144 promoter. Plasmid pTRK391 was digested with *Sma*I to isolate P_{15A10} and the fragment was ligated to *Sma*I digested pTRK528 vector (Allison and Klaenhammer, 1996) to create pTRK711. The promoterless *pepA* and *pepO* genes were amplified by PCR and cloned downstream of P144 on pTRK711 as *Xba*I/*Sma*I (PepA) or *Xba*I/*Xho*I (PepO) fragments.

initially dissolved in dimethyl sulfoxide, diluted Tris-HCl (pH 6.5) and added to a final assay concentration of 0.2 mM. The substrates were added immediately before placing the microtiter plate into the CytoFluor® II fluorescence multi-well plate reader (PerSeptive Biosystems, Inc., Framingham, MA). The reader collected

data every 2 min at room temperature for a total of 30 minutes. Three readings per well were taken during each scan and the average of these three values was recorded. A gain value of 50 was used with excitation and emission wavelengths of 360 nm and 460 nm, respectively. Standard curves were used to calculate sub-

strate hydrolysis rates (nmol/min) and percentages were determined with the formula (nmol/min) supernatant/((nmol/min) supernatant + (nmol/min) cell extract) $\times$ 100. It should be noted that as a cysteine aminopeptidase, PepC required 5 mM dithiothreitol to achieve reduced conditions. In addition, since both PepN and PepC are general aminopeptidases and hydrolyze the same type of substrates, it was necessary to also add 1 mM EDTA to the PepC reaction wells in order to chelate the metal cofactors required by PepN. This assured that only PepC activity was being detected. Finally, the two-component PepO procedure was performed as stated in Lian et al. (1996) using Succinyl-arg-pro-phe-his-leu-leu-val-tyr-MCA as a substrate and 50 mM ammonium acetate (pH 7.0) as a buffer. Briefly, the sample and substrate were incubated at 37°C for the desired time and stopped by adding the PepO enzyme inhibitor, phosphoramidon, to a final concentration of 5 μ M. The PepO activity produces two smaller PepM sensitive peptides. The addition of 4 μ g of commercially prepared PepM (Sigma) for a further 60 min liberates the MCA for fluorescent detection. The amount of MCA released by PepM is proportional to the amount of original substrate hydrolyzed by PepO. Fluorescence was measured using the CytoFluor® II plate reader and hydrolysis rates and percentages were calculated as stated above.

RESULTS

Construction of Vectors for the Overexpression of Peptidases

A series of vectors were constructed to facilitate the overexpression of several *Lactococcus* (PepA, PepC, PepN, PepO, and PepXP) and *Lactobacillus* (PepN and PepO) peptidases. With the exception of the *L. helveticus* *pepN* gene, the peptidase genes were amplified by PCR from *L. lactis* subsp. *cremoris* MG1363 or *L. helveticus* CNRZ32 genomic DNA and directionally cloned into high-copy vectors pTRKH2 (O'Sullivan and Klaenhammer, 1993) and pNZ123 (de Vos, 1987). The *L. helveticus* *pepN* gene was subcloned as a 3.8 kb *Xba*I fragment from pSUW32 (Christensen et al., 1995) into both high-copy vectors.

Detection of Peptidase Activity Using *p*-Nitroanilide Substrates

Para-nitroanilide (pNA) assays were performed on *L. lactis* NCK203 and its derivatives transformed with the plasmids encoding the peptidase genes. Enzymatic activities were detected only for *Lactococcus* PepN and

Figure 3. PepN activities detected by chomogenic pNA substrate assays in the cells and supernatant of *L. lactis* NCK203 wildtype and pTRK701 and pTRK702 transformants. Plasmids pTRK701 and pTRK702 encode the *L. helveticus* PepN on pTRKH2 and pNZ123, respectively.

PepXP and *Lactobacillus* PepN activities using pNA substrates. PepA, PepO and PepC were not detected by pNA assays in wildtype NCK203 or transformants with the plasmid-encoded *pep* genes under any conditions (data not shown).

L. lactis NCK203 wild-type PepN activity was low. Expression of the *L. helveticus* PepN from either high-copy vector caused an increase in cell-associated activity (Figure 3), but the levels were highly variable using pNA substrates. In contrast, PepXP activity was found to be consistently high in wild type *L. lactis* NCK203 cells (Figure 4). PepXP activity was not increased sub-

Figure 4. PepXP activities detected by chomogenic pNA substrate assays in the cells and supernatant of *L. lactis* NCK203 wildtype and pTRT001 and pTRK704 transformants. Plasmids pTRT001 and pTRK704 encode the *L. lactis* PepXP on pTRKH2 and pNZ123, respectively.

Figure 5. PepN activities detected by fluorogenic MCA substrate assays in the cells and supernatant of *L. lactis* NCK203 wildtype and pTRK701 and pTRK702 transformants. Plasmids pTRK701 and pTRK702 encode the *L. helveticus* PepN on pTRKH2 and pNZ123, respectively.

stantially over the wildtype levels when cloned into the high-copy vector pTRKH2 but PepXP expressed from pNZ123 produced significantly more activity than *L. lactis* NCK203 cells (Figure 4).

Detection of Peptidase Activity Using 7-Amino-4-Methylcoumarin Substrates

The assays performed with the pNA substrates were repeated with 7-amino-4-methylcoumarin (MCA) substrates because of the inability of the pNA assays to detect the PepC, Pep O and PepA activity and the variability in the levels of peptidase activity detected with pNA substrates. The MCA peptidase substrates, which generate a fluorescent product after enzymatic hydrolysis provided a considerable advantage due to the absence of background interference. The results obtained with the MCA substrates revealed significant differences in PepN activity between strains harboring *pepN* on pNZ123 and pTRKH2-based vectors. PepN activity associated with cells harboring the pTRKH2 construct was significantly higher compared to both the wildtype and the pNZ123-based construct (compare Figure 3 and 5). Native PepXP levels remained high in *L. lactis* NCK203 and the pNZ123:*pepXP* construct (pTRK704) produced higher activity levels than the wildtype or when the *pepXP* was encoded on pTRKH2 (Figure 6).

Detection of Peptidase Externalization Using 7-Amino-4-Methylcoumarin Substrates

To assess externalization of the peptidases from leaky lactococcal cells, *pepA*, *pepC*, *pepN*, and *pepO* were

Figure 6. PepXP activities detected by fluorogenic MCA substrate assays in the cells and supernatant of *L. lactis* NCK203 wildtype and pTRT001 and pTRK704 transformants. Plasmids pTRT001 and pTRK704 encode the *L. lactis* PepXP on pTRKH2 and pNZ123, respectively.

cloned into pTRK707 (Figure 1), a derivative of pTRK391 that encodes Tac31A (O'Sullivan et al., 1996). A plasmid encoding both Tac31A and PepXP was previously constructed in our laboratory (Walker and Klaenhammer, 2001). Lactococcal strains harboring these plasmids were subjected to MCA assays to detect peptidase activity in the cells and supernatants (Figure 7). Introduction of the *pepN* and *pepC* genes on pTRK707 resulted in a significant increase in the activity of these enzymes over background levels. Native

Figure 7. Peptidase activities in the cells and supernatants of *L. lactis* NCK203 wildtype and with vectors encoding Tac31A and lactococcal peptidase genes. Enzyme activity associated with cell (thatched bars) and the supernatant (solid bars) fractions was detected by fluorogenic MCA substrate assays.

Table 3. Percentage of peptidase activity found in the supernatant fraction of wild type *L. lactis* NCK203 and strains harboring leaky, overexpression vectors.

Peptidase	Vector present	% Activity in supernatant
Aminopeptidase A	— ^a	0.00 %
	pTRK712	0.00 %
Aminopeptidase C	—	0.00 %
	pTRK708	5.72 %
Aminopeptidase N	—	0.00 %
	pTRK709	1.24 %
Endopeptidase O	—	1.87 %
	pTRK713	3.12 %
X-prolyl-dipeptidyl aminopeptidase	—	3.76 %
	pTRK619	4.62 %

^a—, wild type *L. lactis* NCK203, no vector constructs present.

PepXP activity was again not significantly effected by the presence of extra copies of *pepXP* on a pTRKH2 derivative. However, the presence of Tac31A on these constructs, which resulted in the externalization of the β -gal enzyme, did not result in detectable levels of PepN, PepXP or PepC in the supernatants. Although the peptidases were significantly expressed, less than 6% of their total activity was found in supernatant fractions (Table 3). PepA and PepO activities remained below detection limits and it could not be determined whether or not they were externalized.

Construction of Vectors for the Overexpression and Externalization of PepO and PepA Using Alternative Strong Promoters

No measurable enzyme activity was detected during preliminary peptidase assays using either pNA or MCA substrates on strains harboring vectors constructed to overexpress PepA or PepO. Therefore, new high-copy vectors were created to place a strong promoter upstream of each of the two *pepA* and *pepO* genes. Initially, transcriptional fusions were made with the *Lactobacillus acidophilus* P6 promoter (Djordjevic et al., 1997) and the *pepA* and *pepO* genes. These vectors were stable in *E. coli* XLI-Blue but deletions occurred after transformation into *L. lactis* NCK203 (data not shown). Transcriptional fusions were also made to the *pepA* and *pepO* genes with the *Lactobacillus paracasei* P144 promoter (Djordjevic et al., 1994) using the cloning strategies illustrated in Figure 2. In contrast to the plasmid encoding the P6 promoter, plasmids pTRK712 and pTRK713 appeared to be stable both in *E. coli* and *L. lactis*. These vectors also encoded Tac31A in order to induce leaky behavior when introduced into lactococcal cells.

Figure 8. PepO activities in the cells and supernatants of *L. lactis* NCK203 wildtype and NCK203 with pTRK713. Plasmid pTRK713 encodes the *L. lactis pepO* gene under the control of the strong *Lactobacillus* promoter P144 on a high-copy vector containing Tac31A. Enzyme activity associated with cell and the supernatant fractions was determined by fluorogenic MCA substrate assays as described by Lian et al. (1996).

Detection of PepO and PepA Externalization Using a Modified Assay Procedure with 7-Amido-4-Methylcoumarin Substrates

PepO activity could not be detected using the short N-Benzoyl-phe-val-arg-pNA or N-Benzoyl-arg-MCA substrates. However, using the Succinyl-X₈-MCA substrate described by Lian et al. (1996), activity was detected in cell free extracts of *L. lactis* NCK203 and doubled by introducing *pepO* behind P144 on pTRK713 (Figure 8). PepA also required modified assay conditions to detect activity when expressed behind the P144 promoter. The PepA assay employed the N-glutaryl-L-phe-MCA substrate for an extended incubation time (1 h), at an elevated incubation temperature (55°C), and with concentrated cell extracts (100-fold). Wildtype levels of PepA activity were determined and a 2-fold increase in activity were detected in cells when *pepA* was expressed behind P144 on pTRK707 (Figure 9). The presence of Tac31A on the same plasmid did not result in the externalization of either PepA or PepO activities (Table 3).

The overall amount of PepA activity detected was three to four logs less than the amounts produced by the other four peptidases. To explore if PepA enzymatic activity was affected by pH, reactions were carried out at six different pH levels between 6.0 and 8.5 and at various pH levels after a 15 h incubation period at 55°C (Table 4). The activity was also assayed after 10 and 60 min of incubation, but these values were very low and showed no discernable difference among the differ-

Figure 9. PepA activities in the cells and supernatants of *L. lactis* NCK203 wildtype and NCK203 with pTRK712. Plasmid pTRK712 encodes the *L. lactis* *pepA* gene under the control of the strong *Lactobacillus* promoter P144 on a high-copy vector containing Tac31A. Detection of enzyme activity associated with the cell and supernatant fractions was done by fluorogenic MCA substrate assays under modified conditions (see text for details).

ent pH levels (data not shown). The data in Table 4 indicate that PepA is most active at pH 7.0, and extended periods of time are required to detect a significant level of activity. After 15 h of incubation, no PepA activity was detected in the supernatant.

DISCUSSION

This paper describes the cloning and overexpression of several *Lactococcus lactis* peptidases (PepA, PepC, PepN, PepO and PepXP) and *Lactobacillus helveticus* peptidases (PepN and PepO) on two different high-copy vectors (pTRKH2 and pNZ123). It also describes the cloning of the lactococcal peptidases into pTRK707 (pTRKH2:P_{15A10}), the vector responsible for producing a leaky phenotype, to determine if they could be exter-

nalized from the prophage-containing *L. lactis* subsp. *lactis* NCK203. The results showed that the level of peptidase overexpression varied considerably among the different enzymes investigated and that none were externalized at significant levels by leaky lactococci.

The PepN activity levels detected in *Lactococcus* in this study are similar to the results of Christensen et al. (1995) and Van Alen-Boerrigter et al. (1991). Christensen et al. (1995) reported a 180-fold increase in activity when the *L. helveticus* CNRZ32 PepN was expressed from a high-copy vector (pIL253) in *L. lactis* subsp. *cremoris* LM0230. Van Alen-Boerrigter et al. (1991) found a 20-fold increase in activity when the *L. lactis* MG1363 PepN was cloned into pIL253 and transformed into *L. lactis* subsp. *cremoris* MG1363. We observed a 40-fold increase in activity with the pIL253-based pTRK701. In addition, the MCA assays revealed significantly higher levels of activity when *pepN* was encoded on pTRKH2 than the pNZ123-based pTRK702. In contrast to PepN, the *pepXP* gene on pTRKH2 failed to increase PepXP levels above the high wildtype levels, but *pepXP* on pNZ123 increased PepXP activity by a factor of seven. Previous attempts to overexpress PepXP on pTRKH2 were unsuccessful (Walker and Klaenhammer, 2001). Two additional studies have attempted to overexpress *L. helveticus* *pepXP* genes with varying results. Yuksel and Steele (1996) cloned the CNRZ32 *pepXP* gene into both a low-copy (pGK12; Kok et al., 1984) and a high-copy vector (pIL253). None of the constructs increased PepXP activity in *L. lactis* LM0230. However, when the pGK12 based-vector was placed back in CNRZ32, a 4-fold increase in activity was observed. In contrast, Luoma et al. (2001) found a 5-fold increase in activity when the *L. helveticus* 53/7 PepXP was cloned into a low-copy pGK12 derivative and placed in *L. lactis* MG1363. The significance of the different reported peptidase activities cannot be resolved in the absence of mRNA analysis and a direct

Table 4. Level of PepA activity detected in strains of *L. lactis* NCK203 after overnight (15 h) incubation at 55°C under variable pH conditions.^{ab}

Vector	Fraction	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.5
—	C	0.00	0.00	0.39	0.00	0.00	0.00
— ^c	C	1.11	1.84	2.78	1.96	1.95	1.28
—	S	0.00	0.00	0.00	0.00	0.00	0.00
pTRK712	C	0.00	0.00	0.92	0.00	0.00	0.00
pTRK712 ^c	C	2.32	2.65	4.09	3.29	3.19	1.90
pTRK712	S	0.00	0.00	0.00	0.00	0.00	0.00

^aOvernight incubation was done using 0.2 mM N-glutaryl-L-phe-MCA in a covered 96-well microtiter plate. Data shown represent the nanomoles of substrate hydrolyzed after 15 h of incubation.

^bAbbreviations: —, wild type *L. lactis* NCK203, contains no overexpression vector; C, cell extract; S, supernatant.

^c100-fold concentrations were made by harvesting 50 ml of cells by centrifugation and resuspension in 0.5 ml of the appropriate buffer.

comparison of the copy number of each vector encoding the different *pep* genes.

There are less data available on the overexpression of the other three peptidases investigated in this study. Only recently has data been published on the cloning and expression of a *L. helveticus pepC* in *L. lactis* MG1363. Luoma et al. (2001) reported a doubling of PepC activity when cloned on the low-copy vector pGK12. In this study, the *L. lactis pepC* gene was cloned into the high-copy vector pTRKH2 along with Tac31A in an attempt to determine whether PepC was externalized by the Tac31A-induced leaky state. Unexpectedly, the *L. lactis pepC* expressed on a high-copy vector produced approximately the same increase in activity as the low-copy pGK12 *pepC* construct.

To our knowledge, this study describes the first report of the overexpression of the *Lactococcus* PepO and PepA in *L. lactis*. All previous studies have utilized concentration and purification methods to detect activity. Cloning of these two enzymes required additional expression signals and tailored assay conditions in order to detect activity. In addition to cloning a strong promoter (P144) in front of *pepO*, the two-component assay procedure described by Lian et al. (1996) was successfully adopted for measuring PepO activity in cell free extracts. Overexpression in pTRK528:P_{15A10}:*pepO* produced twice as much PepO activity as the wild type strain. However, it should be noted that after 15 min, a plateau effect occurred even when the PepO assays were performed with excess substrate (data not shown). This effect may be linked to the fact that hydrolysis of the MCA substrate by PepO produces two small non-fluorescent oligopeptides (6 and 4 molecules in length). It has been shown that lactococcal endopeptidases have the ability to hydrolyze a variety of different peptide bonds within a variety of different-sized oligopeptides (Baankreis et al., 1995; Lian et al., 1996; Tan et al., 1991). Because it lacks active site specificity, PepO has the ability to hydrolyze the newly released N-terminal peptide instead of the original, intact MCA peptide substrate. If the peptidase cleaves these smaller N-terminal peptides, which do not have a fluorescent MCA molecule attached to them, the accurate measurement of hydrolysis becomes compromised. Therefore, even though the results displayed a plateauing of activity, suggesting substrate exhaustion, it is possible that PepO was interacting and cleaving peptides that do not possess detectable fluorescent markers.

Of the peptidases investigated, PepA activity proved the most difficult to detect. A study by Exterkate and de Veer (1987) tested over 30 different substrates with a lactococcal PepA to determine its hydrolytic activity towards various peptides and synthetic substrates. Specificity was found towards peptides possessing N-

terminal L- α -glutamyl or L- α -aspartyl groups with free γ -carboxyl groups. It was believed that the structure of these alpha amino groups reduced steric hindrance and allowed for proper alignment of the substrate in the active site. In the study by Exterkate and de Veer (1987), only nine out of the 30 substrates tested fit these requirements, and only one was still commercially available as a MCA derivative, N-glutaryl-L-phe-MCA. After several rounds of assay optimization, a measurable amount of activity was attained when a 100-fold cell extract concentration was incubated at pH 7.0 and 55°C for 1 h. However, even with the assay modifications and determination of optional pH conditions for PepA, the level of PepA activity detected was significantly lower than that of the other peptidases.

After successful expression and detection of these peptidases was accomplished, their externalization was investigated. Walker and Klaenhammer (2001) had shown previously that leaky lactococci externalized 85% of the intracellular enzyme β -galactosidase. β -gal is a 117 kDa monomer and is thought to be functional only as a dimer or tetramer suggesting the leaky system could potentially export molecules up to 500 kDa in size without the need for secretion or export signals. Young and Blasi (1995) discussed the apparent lack of specificity that holin proteins have shown in the past towards different heterologous lysin proteins. If the holin proteins supposedly facilitated the non-specific externalization of lysins, then it was plausible that peptidases would leak via this system. In the *E. coli* λ bacteriophage lysis model described by Young and Blasi (1995), it is believed that holin proteins, which are produced during the late expression phase of the phage lytic cycle, associate with the membrane just prior to the release of progeny phage. Once oligomerized across the cell membrane, a conformational change occurs allowing the lysin proteins to cross the membrane where they begin cleaving the peptidoglycan layer and ultimately cause cell lysis. The leaky state may occupy a small window between compromised cell membrane integrity and complete cell lysis. Since all of the peptidases under investigation in this study range between 30-100 kDa (as monomers) in size, it was projected that they might also be released by cells in a leaky condition. The fact that the peptidases were not released into the supernatant of the leaky cells suggests that protein size is not the determining factor for externalization.

In conclusion, over the course of this study, vectors were constructed with several *Lactococcus* and *Lactobacillus* peptidases. Two different enzymatic assay techniques were employed for the detection of peptidase activity both in the cells and supernatants. It was established that lactococcal peptidases do not leak out of leaky lactococci under the conditions investigated in

this study. The mechanism responsible for the leaky state remains unknown. Efforts are underway to control the leaky versus lysis condition, thereby defining avenues to deliver peptidases and other useful proteins (i.e. vaccines, bioactive peptides, etc.) out of the cell without compromising membrane integrity and overall cell viability.

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