JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 701–704 Vol. 178, No. 3
0021-9193/96/$04.00+0
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Sequencing, Distribution, and Inactivation of the Dipeptidase A Gene (*pepDA*) from *Lactobacillus helveticus* CNRZ32

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Received 28 August 1995/Accepted 13 November 1995

Previously, the gene for a general dipeptidase (*pepDA*) was isolated from a gene bank of *Lactobacillus helveticus* CNRZ32. The *pepDA* gene consists of a 1,422-bp open reading frame which could encode a polypeptide of 53.5 kDa. No significant identity was found between the deduced amino acid sequence of the *pepDA* product and the sequence for other polypeptides reported in GenBank. Southern hybridization studies with a *pepDA* probe indicated that the nucleotide sequence for *pepDA* is not well conserved among a variety of lactic acid bacteria. Growth studies indicated that a *pepDA* deletion had no detectable effect on growth rate or acid production by *L. helveticus* CNRZ32 in milk. Furthermore, no difference in total cellular dipeptidase activity was detected between the mutant and wild-type strains during logarithmic growth in MRS medium.

Lactic acid bacteria (LAB) are a heterogeneous family of bacteria having utility in the manufacture of fermented dairy products. LAB are unable to synthesize a number of amino acids (22) and thus must obtain these from the milk. However, the level of free amino acids and small peptides in milk is not sufficient to support growth of LAB to high cell densities (22). Therefore, these organisms utilize a complex proteolytic system to obtain essential amino acids from casein, the primary protein present in milk (22). This hydrolysis of casein is also important in cheese ripening, because the casein degradation products produced by LAB contribute to cheese flavor development (26).

To initiate the characterization of the proteolytic system of *Lactobacillus helveticus* CNRZ32, a gene bank of this organism was constructed and screened for peptidase activities (20). Six peptidase genes were identified, and many of these enzymes have been characterized further on the molecular level (8, 12, 13, 29). Currently, inactivation of individual peptidases is being used to determine the physiologic role of each peptidase as well as the role that each plays in cheese flavor development. This study describes the characterization and inactivation of the gene for one of the broad-specificity dipeptidases, designated dipeptidase A (PepDA; previously designated DPI) of *L. helveticus* CNRZ32.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The bacteria and plasmids used in this study are listed in Table 1. All cultures were maintained at −80°C in 6.9% nonfat dry milk and 10% glycerol. *Escherichia coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was grown in LB (23) medium. *E. coli* CM89 (19) was grown in LB medium supplemented with 0.3 mM thymine and 0.05 mM thiamine. All *E. coli* cultures were incubated at 37°C with aeration. *L. helveticus* was routinely grown in MRS (Difco Laboratories, Detroit, Mich.) and incubated without shaking at 42°C. When antibiotics were included to select for plasmid constructs, the concentrations used were as follows: pUC19, 60 μg of ampicillin (Ap) per ml; pSA3 in *E. coli*, 1.0 mg of erythromycin (Em) per ml; and pSA3 in *L. helveticus*, 4 μg of erythromycin Em per ml. All antibiotics were purchased from Sigma Chemical Company (St. Louis, Mo.). For experiments with α-complementation, isopropyl-β-thiogalactopyranoside (IPTG) (Promega Corporation, Madison, Wis.) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; Gibco-BRL, Gaithersburg, Md.) were incorporated into agar medium at concentrations of 0.12 g and 20 mg per liter, respectively.

Molecular cloning and enzymatic screening. Recombinant DNA and plasmid isolation techniques were essentially those described by Sambrook et al. (23). Klenow fragment, T4 DNA ligase, and restriction endonucleases were used as recommended by the manufacturer (Gibco-BRL). *E. coli* transformation was performed with a Gene Pulser following the instructions recommended by the manufacturer (Bio-Rad Laboratories). Transformation of *L. helveticus* was performed as described previously (6).

Plasmid-encoded PepDA activity in *E. coli* was confirmed as described by Nowakowski et al. (20). Met-Ala (Sigma) was used as the substrate.

Cell extract preparation. Cell extracts were prepared essentially as described by Bhowmik and Marth (5). However, a Red Devil 5410 paint mixer was used rather than a Braun homogenizer.

Quantitative dipeptidase assays. The dipeptide-hydrolyzing activity of cell extracts was measured by the Cd-ninhydrin method essentially as described by Doi et al (11). Assays were performed at pH 5.5 in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid. Four time points over 17 min were measured to ensure measurement of initial velocity.

Southern hybridizations. Chromosomal DNA isolation, the Southern hybridization procedure, and sources for bacteria used in distribution studies were described previously (12). Probe synthesis was performed as described in the Genius kit (Boehringer Mannheim, Indianapolis, Ind.), with a 0.65-kb *Kpn*I-*Hpa*I internal *pepDA* fragment. Hybridization was carried out at 42°C as described by Boehringer Mannheim. Low and high stringencies were achieved by incorporating 10 or 50% formamide, respectively, into the prehybridization and hybridization steps.

RNA isolation and Northern (RNA blot) hybridization. Total RNA was isolated from *L. helveticus* by pelleting 1.5 ml of cells and washing them twice in double-distilled water. Cells were resuspended in 130 μl of 0.5 M sucrose–10 mM EDTA (pH 7.5). Next, 10 μl of 10-mg/ml lysozyme and 10 U of mutanolysin were added, and cells were incubated at 37°C for 10 min. Cells were lysed by the addition of 150 μl of 2% sodium dodecyl sulfate (SDS)–10 mM EDTA (pH 7.5), and purification of total RNA was completed as described by Liu et al (18).

For Northern hybridizations, total RNA was separated in a 1.0% agarose gel and blotted onto a MagnaGraph nylon membrane (Micron Separations, Inc., Westborough, Mass.) as described by Trnovsky (25) with a Trans-Blot SD (Bio-Rad Laboratories). Hybridization and detection were performed as described by Boehringer Mannheim with the *pepDA* probe described above. RNA size standards (9.5 to 0.36 kb; Promega Corporation, Madison, Wis.) stained by the method of Gründemann and Koepsell (15) were used for size determinations.

DNA sequencing. Nested sets of deletions were generated by using the Erase-a-Base Kit (Promega). Plasmid DNA was purified by the modified mini-alkaline lysis/polyethylene glycol precipitation procedure of Applied Biosystems (Foster City, Calif.). Sequence determination was performed by the University of Wisconsin-Madison Biotechnology Center with an automated fluorescence sequencer. Sequence analysis was performed with the GCG fragment assembly program (Genetics Computer Group, Madison, Wis.). Amino acid sequence comparisons were performed with BLAST (1). Motifs were searched for in the deduced amino acid sequence by using PROSITE (2).

Construction of a *pepDA* mutant of *L. helveticus* CNRZ32. A deletion was

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
DH5α	F$^-$ φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *hsdR17* (r_k^- m_k^+) *deoR recA1 endA1 supE44* λ$^-$ *thi-1 gyrA96 relA1*	BRL[a]
CM89	*leu-9* Δ (*pro-lac*) *met thyA pepN102 pepA11 pepQ10 pepB1*	19
Lactobacillus helveticus		
CNRZ32	Parental strain	Stock
JLS222	Em^s PepDA$^-$ derivative of CNRZ32	This study
Plasmids		
pSUW10	Em^r PepDA$^+$; pJDC9 with a 5.8-kb CNRZ32 chromosomal fragment	20
pUC19	Ap^r *lacZ*	28
pSA3	Em^r	9
pSUW96	Ap^r PepDA$^+$; pUC19 with a 2.3-kb CNRZ32 chromosomal fragment	This study
pSUW98	Em^r PepDA$^-$; pSA3 with the internally deleted pepDA	This study

[a] BRL, Bethesda Research Laboratories.

made in *pepDA* by removing the internal 0.25-kb *Bcl*I-*Hpa*I fragment, and an integration vector was constructed by introducing the deleted *pepDA* gene into pSA3. This deletion inactivated the enzyme, as determined by qualitative enzyme assays (data not shown). The resulting construct, designated pSUW98, was used to construct a *pepDA*-negative derivative of *L. helveticus* CNRZ32 as described previously (4).

Growth of strains in milk. The characterization of growth rate and acid production of *L. helveticus* JLS222 was performed at 42°C as previously described (29). All growth studies were performed in duplicate.

Nucleotide sequence accession number. The sequence for the 2.3-kb insert of pSUW96 has been submitted to GenBank and assigned accession number U34257.

RESULTS

Subcloning of *pepDA*. Previously (20), the *L. helveticus* CNRZ32 *pepDA* gene had been isolated on a 12.7-kb plasmid designated pSUW10. Tn*5* mutagenesis (10) further localized the gene to a 2.3-kb *Sma*I-*Stu*I fragment of pSUW10 (data not shown). This fragment was successfully cloned into pUC19 in both orientations. Transformants containing either of these two constructs exhibited PepDA activity. One construct, designated pSUW96, was arbitrarily chosen for further study. The PepDA-encoding insert along with relevant restriction sites is shown in Fig. 1.

Sequencing of *pepDA* of *L. helveticus* CNRZ32. By sequencing the entire 2.3-kb insert of pSUW96, a 1,422-bp open reading frame (ORF) was identified and designated *pepDA* (Fig. 2). The ORF could encode a polypeptide consisting of 474 amino acids, with a deduced mass of 53.5 kDa. Preceding the ORF was a putative ribosome-binding site (AAGGAA; nucleotides −12 to −7) which resembles the consensus ribosome-binding site of *L. helveticus* (21). Following *pepDA* is a putative rho-independent transcriptional terminator (TAAGAGCTTGA GA [nucleotides 1446 to 1459] and TCTCAAGCTCTTA [nucleotides 1464 to 1476]) with a Δ*G* of −19.8 kcal (24). BLAST did not identify any other sequences in GenBank (7) with significant identity ($P < 0.05$) to the deduced amino acid sequence of *pepDA*, and no motifs defined in PROSITE were identified. A hydrophilicity plot (17) of the deduced amino acid sequence did not reveal a hydrophobic region at the amino terminus that might function as a signal peptide or membrane anchor. Therefore, it seems likely that PepDA is an intracellular enzyme.

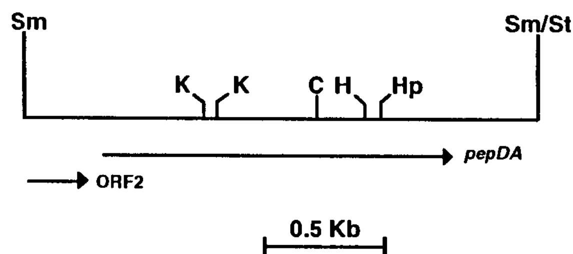

FIG. 1. Restriction endonuclease map of the subcloned 2.3-kb *Sma*I-*Stu*I fragment of pSUW10. Arrows represent the limits of *pepDA* and ORF2, with the arrow indicating the direction of transcription. Abbreviations for restriction endonucleases: Sm, *Sma*I; Sm/St, *Sma*I/*Stu*I hybrid site; K, *Kpn*I; C, *Bcl*I; H, *Hin*dIII; Hp, *Hpa*I.

A second ORF (ORF2) was found 5′ of *pepDA* (Fig. 1 and 2). The 87-amino-acid polypeptide deduced from ORF2 contains the conserved sequence motif for the XylS/AraC family of prokaryotic transcriptional regulators (14). No rho-independent transcriptional terminator was found between ORF2 and *pepDA*. However, Northern hybridizations with a *pepDA* probe and total RNA extracted from logarithmically growing *L. helveticus* cells in MRS have indicated that *pepDA* is transcribed monocistronically under these conditions, as a 1.5-kb transcript was detected (data not shown). More studies are needed to determine whether the protein encoded by ORF2 plays a role in *pepDA* regulation.

Distribution of *pepDA* in LAB. A nucleic acid probe was synthesized from an internal portion of *pepDA*. When low-stringency (10% formamide and 42°C) conditions were employed, hybridization with all *L. helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* strains tested was detected (Fig. 3), although hybridization with *L. delbrueckii* subsp. *bulgaricus* JLS110 was weak (Fig. 3, lane D). No hybridization was detected with any *Lactobacillus casei*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, or *Streptococcus thermophilus* strains tested (data not shown). Under high-stringency conditions (50% formamide), hybridization only with the *L. helveticus* strains and *L. delbrueckii* subsp. *bulgaricus* JLS160 was detected (data not shown).

Characterization of *L. helveticus* JLS222. To determine the effect of a *pepDA* mutation on the growth of *L. helveticus* CNRZ32 in milk, growth studies were conducted with the wild-type strain and JLS222 (PepDA$^-$ derivative of (NRZ32). The two strains grew in and acidified milk at the same rate (data not shown).

Ninhydrin assays were performed on wild-type and mutant cell extracts to determine if any difference in dipeptide cleaving activity could be detected between the two cultures. Activity was tested with the dipeptide substrates Leu-Leu, Phe-Met, Arg-Leu, Lys-Phe, Leu-Arg, and Met-Glu, all of which had been identified as substrates for *pepDA* previously in cell ex-

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                                                       CCCAATT  -337
                                                       P  N  C
GCAACATTGTGGAGCTTTGCAACAGGTTAGGTGTCTCACGTAGTTTTTATATTCATTAT  -277
 N  I  V  E  L  C  N  R  L  G  V  S  R  S  F  L  Y  S  L  F
TTAGAGAAAATACTAATATTTCACCGCAAAAATATCTGATGCAGCTGCGCATGGAAGCAG  -217
 R  E  N  T  N  I  S  P  Q  K  Y  L  M  Q  L  R  M  E  A  A
CCAAAAAAGAATTGCAAAATACCACATCCAACTTAAAAGAAATCGCCCACAAAGTTGGCT  -157
 K  K  E  L  Q  N  T  T  S  N  L  K  E  I  A  H  K  V  G  Y
ATGGTGACGAATTTACTTTTTCAAAAGCATTTAAGCGTTACAGTGGTGTTAGCCCCAATG   -97
 G  D  E  F  T  F  S  K  A  F  K  R  Y  S  G  V  S  P  N  V
TATTTCGTAGAAATTAATTATATTCTTTGCTTAACTCAGGCTTCTTTGCATACAATATAT   -37
 F  R  R  N  *
AGTGTATAACTTATTAAAACAGTAAAGGAATGATTCAATGAAACAAACAGAATGTACTAC    23
                                       M  K  Q  T  E  C  T  T   8
TATCTTAGTAGGTAAAAAAGCAAGTATTGACGGCTCAACTATGATCGCCCGTAGTGAAGA    83
 I  L  V  G  K  K  A  S  I  D  G  S  T  M  I  A  R  S  E  D  28
TGGTGGCCGTGTAATTATCCCTGAAGGCTTTAAAGTAGTTAATCCAGAAGACCAACCTAA   143
 G  G  R  V  I  I  P  E  G  F  K  V  V  N  P  E  D  Q  P  K  48
GCACTACACTAGCGTTATCAGCAAGCAAAAGATCGATGATGAAGATTTAGCTGAAACTCC   203
 H  Y  T  S  V  I  S  K  Q  K  I  D  D  E  D  L  A  E  T  P  68
ACTTCGTTATACTAGTGCTCCAGATGTTTCTGGTAAAAATGGTATTTGGGGCGCTGCCGG   263
 L  R  Y  T  S  A  P  D  V  S  G  K  N  G  I  W  G  A  A  G  88
TATCAACGCTGATAACGTGGCAATGACCGCTACTGAAACTATTACCACTAATTCACGCAT   323
 I  N  A  D  N  V  A  M  T  A  T  E  T  I  T  T  N  S  R  I 108
CCAAGGCGTTGATCCTATTCTTGATCCAAGCGAAGGTGGTCTTGGGGAAGAAGACTTTGT   383
 Q  G  V  D  P  I  L  D  P  S  E  G  G  L  G  E  E  D  F  V 128
TACTTTAACTCTTCCATATCTTCACTCTGCTTTCGATGGCGTTAAGCGTGTCGGCTACCT   443
 T  L  T  L  P  Y  L  H  S  A  F  D  G  V  K  R  V  G  Y  L 148
TGTTGAAAAATATGGTACCTACGAAATGAACGGTATGGCCTTCTCAGATAAAGACAATAT   503
 V  E  K  Y  G  T  Y  E  M  N  G  M  A  F  S  D  K  D  N  I 168
CTGGTACCTTGAAACTATTGGTGGTCACCACTGGATTGCACGCCGCATCCCTGATGATGC   563
 W  Y  L  E  T  I  G  G  H  H  W  I  A  R  R  I  P  D  D  A 188
CTACGTTATCGCACCAAACCGTTTAAACATCGATACTTTCGACTTCGATGACAGCGAAAA   623
 Y  V  I  A  P  N  R  L  N  I  D  T  F  D  F  D  D  S  E  N 208
CTTCGCTACAGCTAGCGACTTAAAGGATTTAATCGATGAATATCATTTAAATCCAGACCG   683
 F  A  T  A  S  D  L  K  D  L  I  D  E  Y  H  L  N  P  D  R 228
TGAAGGTTACAATATGCGTCACATCTTTGGTTCTTCAACTATCAAAGATGCACACTACAA   743
 E  G  Y  N  M  R  H  I  F  G  S  S  T  I  K  D  A  H  Y  N 248
CAACCCACGTGCTTGGTACATTCACAACTACTTTGATCCCGACTTTGGCGGCACTCCAGC   803
 N  P  R  A  W  Y  I  H  N  Y  F  D  P  D  F  G  G  T  P  A 268
AGATCAAGATCAACCTTTCATTTGCCGTGCTAACCGCTTAATCTCAATCGAAGATATTAA   863
 D  Q  D  Q  P  F  I  C  R  A  N  R  L  I  S  I  E  D  I  K 288
GTGGGCTGAAAGCTCACACTACCAAGACACCCCTTACGATGCATACGGTGATCAAGGTAC   923
 W  A  E  S  S  H  Y  Q  D  T  P  Y  D  A  Y  G  D  Q  G  T 308
TCCTGAACAAAAGAAGACCTTCCGTCCAATCGGAATTAACAGAAACTTTGAAACCCACAT   983
 P  E  Q  K  K  T  F  R  P  I  G  I  N  R  N  F  E  T  H  I 328
TTTACAAATCAGAAATGACGTTCCTGCAGAAATTGCGGGTGTCCAATGGTTAGCATTTGG  1043
 L  Q  I  R  N  D  V  P  A  E  I  A  G  V  Q  W  L  A  F  G 348
TCCTAACACCTTCAACTCAATGTTGCCATTCTACACCAACGTCACAACCACTCCAGAAGC  1103
 P  N  T  F  N  S  M  L  P  F  Y  T  N  V  T  T  T  P  E  A 368
TTGGCAAACCACTCCTAAGTTTAACTTGAACAAGATTTTCTGGTTAAACAAGTTAACTGC  1163
 W  Q  T  T  P  K  F  N  L  N  K  I  F  W  L  N  K  L  T  A 388
TCAACTTGGCGACACCAACTACCGTGTATATGGCGAACTTGAAGATGCCTTTGAACAAAA  1223
 Q  L  G  D  T  N  Y  R  V  Y  G  E  L  E  D  A  F  E  Q  K 408
GAGTTTAGCGCAATGCCACAAGATTCAACATGAAACTGATAAAGAAGTTAAGAATCTTTC  1283
 S  L  A  Q  C  H  K  I  Q  H  E  T  D  K  E  V  K  N  L  S 428
AGGCAAGGAATTGCAAGATAAATTAATTGCAGCTAACCAAAAGATGTCTGACACTGTTTA  1343
 G  K  E  L  Q  D  K  L  I  A  A  N  Q  K  M  S  D  T  V  Y 448
CAACAATACTGTCGAACTTTTGGGTCAAATGGTTGACGAAGGTCACGGCTTAATGACTTT  1403
 N  N  T  V  E  L  L  G  Q  M  V  D  E  G  H  G  L  M  T  L 468
GAAGTACGACTTGCTCGACTAATAATTCAAATATAAAAAGCAATAAGAGCTTGAGAATCA  1463
 K  Y  D  L  L  D  *  *                                     474
TCTCAAGCTCTTATTTTTTACTCAAATAATGGAATTGCATCGGCAAGTGTTTTGACTACA  1523
GTCACCTTTTTGGTTATTCCTTCTGACGGCATCACTTGGTCTGGAACAAGTACCACATCA  1583
ATTCCTGCATTTAATCCTGATTCCACTCCATTTGGTGCATCTTCAAAAATCACTGCTTCA  1643
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FIG. 2. Nucleotide sequence of *pepDA* (nucleotides 1 to 1422) and 3′ end of ORF2 (nucleotides −343 to −83). The predicted amino acid sequence is shown in the single-letter code. The putative Shine-Dalgarno sequence (nucleotides −13 to −8) and rho-independent transcriptional terminators (nucleotides 1447 to 1459 and 1464 to 1476) are underlined.

tracts of *E. coli* CM89(pSUW96). However, no differences between mutant and wild-type cell extracts were detectable.

DISCUSSION

L. helveticus CNRZ32 is a strain of LAB which has shown promise as a starter adjunct in the manufacture of aged cheeses (3). While the proteolytic activity of the adjunct within the cheese matrix is thought to play a significant role in flavor development in these cheeses, the role of individual components of the proteolytic system has not been studied.

This study focused on a broad-specificity dipeptidase which had been previously cloned from *L. helveticus* CNRZ32 (20). The *pepDA* gene was further subcloned in both possible orientations with respect to the *lacZ* promoter in pUC19. Since transformants containing either of these constructs exhibited PepDA activity, this suggests that the entire *pepDA* gene, including transcriptional promoters, has been subcloned.

Nucleotide sequencing of the 2.3-kb insert from pSUW96 revealed a 1,422-bp ORF which could encode a putative 53.5-kDa polypeptide. Analysis of the deduced amino acid sequence

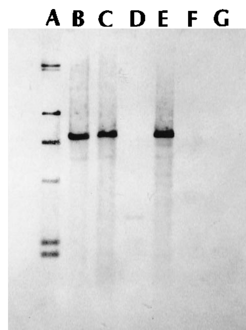

FIG. 3. Low-stringency (10% formamide, 42°C) Southern hybridization of *Eco*RI-digested total genomic DNA samples of *Lactobacillus* spp., using a probe synthesized from a 0.65-kb *Kpn*I-*Hpa*I fragment of the *L. helveticus* CNRZ32 *pepDA* gene. Lanes: A, *Hin*dIII-cleaved lambda DNA (molecular size standards); B, *L. helveticus* CNRZ32; C, *L. helveticus* ATCC 10797; D, *L. delbrueckii* subsp. *bulgaricus* JLS110; E, *L. delbrueckii* subsp. *bulgaricus* JLS160; F, *L. casei* JLS20; G, *L. casei* subsp. *pseudoplantarum* ATCC 25598.

revealed no significant identities to the sequences of any other known proteins. However, to the best of our knowledge, the sequences of only two broad-specificity bacterial dipeptidases, the products of *pepD* from *E. coli* K-12 (16) and *pepV* from *L. delbrueckii* subsp. *lactis* DSM7290 (27), are known. It seems likely that the absence of sequences related to *pepDA* is the result of limited knowledge of broad-specificity bacterial dipeptidases.

Southern hybridizations were performed to study the distribution of *pepDA* among a variety of LAB. Hybridization was detected only with chromosomal DNA from *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* strains. Therefore, either PepDA is not present in most LAB, or the homology between *pepDA* and its homologs in other LAB is too low to detect by this technique. Similar studies have been performed with the peptidase genes *pepXP*, *pepN*, *pepC*, and *pepPN* of *L. helveticus* CNRZ32 (8, 12, 13, 29). The distribution studies for these peptidases indicate that *pepDA* is the least conserved of the five genes among the LAB strains tested.

To determine whether *pepDA* plays a significant role during the growth of *L. helveticus* CNRZ32 in milk, the gene encoding this peptidase was inactivated. No differences in final cell densities or rate of acid production were detected between the wild-type and mutant strains. Thus, PepDA is not necessary for the liberation of essential amino acids from casein. The construction and characterization of single and multiple peptidases mutants of *L. helveticus* CNRZ32 should clarify the physiological role of individual peptidases in casein hydrolysis. Currently, the only known components of the proteolytic system of LAB which are essential for growth in milk are the proteinase and the oligopeptide transport system of *Lactococcus lactis* (22).

In addition, no difference was detected in cellular dipeptidase activity during logarithmic growth between the wild type and JLS222 with a variety of substrates known to be cleaved by PepDA. Therefore, it appears that other, more active peptidases from *L. helveticus* CNRZ32 have overlapping specificities with PepDA. Further investigations will be required to determine the number of dipeptidases present in *L. helveticus* CNRZ32 and their physiological roles.

ACKNOWLEDGMENTS

This project was supported in part by the United States Department of Agriculture National Research Initiative, the College of Agricultural and Life Science at the University of Wisconsin-Madison, and the

Center for Dairy Research through funding from the National Dairy Promotion and Research Board.

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