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Identification of Mur, an Atypical Peptidoglycan Hydrolase Derived from *Leuconostoc citreum*

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A gene encoding a protein homologous to known bacterial *N*-acetyl-muramidases has been cloned from *Leuconostoc citreum* by a PCR-based approach. The encoded protein, Mur, consists of 209 amino acid residues with a calculated molecular mass of 23,821 Da including a 31-amino-acid putative signal peptide. In contrast to most of the other known peptidoglycan hydrolases, *L. citreum* Mur protein does not contain amino acid repeats involved in cell wall binding. The purified *L. citreum* Mur protein was shown to exhibit peptidoglycan-hydrolyzing activity by renaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An active chimeric protein was constructed by fusion of *L. citreum* Mur to the C-terminal repeat-containing domain (cA) of AcmA, the major autolysin of *Lactococcus lactis*. Expression of the Mur-cA fusion protein was able to complement an *acmA* mutation in *L. lactis;* normal cell separation after cell division was restored by Mur-cA expression.

Bacteria produce one or several peptidoglycan hydrolases (PGHs), which are capable of hydrolyzing covalent bonds in the peptidoglycan of their own cell envelope (for reviews, see references 46 and 49). Some of them, named autolysins, are able to trigger cell autolysis. PGHs are located in the cell wall and are involved in various cellular functions, including cell wall expansion, cell wall turnover, or cell separation. On the basis of their cleavage site in the peptidoglycan, four types of PGHs are defined: (i) *N*-acetyl-muramidases, (ii) *N*-acetyl-glucosaminidases, (iii) *N*-acetyl-muramoyl-L-alanine amidases, and (iv) peptidases. Most of the PGHs characterized so far have a modular structural organization with two domains: a catalytic domain containing the enzyme active site and a cell wall binding domain composed of several amino acid repeats (22, 30).

Autolysis of lactic acid bacteria (LAB) used as starters for cheese manufacturing plays an important role in flavor development during ripening (for reviews, see references 13 and 18). It has been shown that lysis of *Lactococcus lactis* starter strains leads to the release of intracellular peptidases in the cheese curd, and as a result more free amino acids (which are aroma precursors) are produced and hydrophobic bitter peptides are degraded (12, 35, 52).

The PGH activities present in *L. lactis* were studied by renaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which allowed their detection after renaturation in a substrate-containing gel. Several activity bands were evidenced by this technique (9, 36, 40, 44). The major autolysin, AcmA, was characterized at the genetic level. It is an *N*-acetyl-muramidase that is required for proper cell separation after cell division (9) and is involved in autolysis observed during stationary phase after growth in liquid medium (10).

Leuconostocs are heterofermentative LAB used as cheese starters in association with lactococci. They contribute to the development of cheese organoleptic properties by metabolizing citrate to diacetyl, an important flavor compound, and to CO_2, which is responsible for eye formation in some Dutch cheeses (17). Like lactococci, leuconostocs contain a diverse pool of peptidases (23). Thus, autolysis of *Leuconostoc* starter strains could contribute to peptidolysis during cheese ripening. Recently, researchers have demonstrated several PGH activities in dairy leuconostocs (15). In order to understand and control autolysis, a first step is to identify and to characterize at the molecular level the enzymes involved in this phenomenon.

In the present study, we report the cloning, sequencing, and expression of a PGH-encoding gene, named *mur*, from *Leuconostoc citreum* 22R. The *L. citreum* Mur protein shows sequence homology to the N-terminal catalytic domain of several known bacterial muramidases. However, in contrast to these muramidases, *L. citreum* Mur is devoid of a specific cell wall binding domain. We constructed an active chimeric protein by fusion of *L. citreum* Mur and the *L. lactis* AcmA cell wall binding domain and showed that it complements an AcmA deficiency in *L. lactis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *Leuconostoc* strains and *L. lactis* strains were grown at 30°C in MRS medium (20) and M17 medium containing 0.5% glucose (M17-glu) (50), respectively. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C under vigorous shaking conditions. When required, antibiotics were added at the following concentrations, except where otherwise stated: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 10 μg/ml; and erythromycin, 5 μg/ml.

General DNA techniques, PCR, and transformation. Molecular cloning techniques were performed essentially as described previously (45). Total DNA was isolated from *L. citreum* 22R according to de los Reyes-Gavilan et al. (19) except that 10 IU of mutanolysin (Sigma Chemicals, St. Louis, Mo.) was added to TES

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
E. coli		
TG1	*supE hsdΔ5 thi* Δ(*lac proAB*) F′ [*traD36proAB*+*lacI*^{q} *lacZ*ΔM15]	25
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$ F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(tet^r)]	Stratagene
L. citreum 22R	Wild-type strain	CNRZ collection
L. mesenteroides 50M	Wild-type strain	CNRZ collection
L. lactis		
MG1363 *acmAΔ1*	Derivative of MG1363 carrying a 701-bp *Sac*I-*Spe*I deletion in *acmA*	9
NZ9000	Derivative of NZ3000; *pepN*::*nisRnisK*	O. Kuipers[a]
NCDO763	Wild-type strain	CNRZ collection
Plasmids		
pBluescript SK+ (pBSK+)	Ap^r	Stratagene
pQE30	Ap^r, expression vector, six-His tag at the N terminus of the protein	Qiagen
pCYT1	Cm^r, pNZ8010 derivative carrying a transcriptional fusion of the *nisA* promoter, the *usp45* RBS, and the *S. aureus nucA* gene	P. Langella[b]
pNZ9520	Em^r, *nisRK* genes cloned in high-copy-number cloning vector pIL253 (48)	31
pNZ9530	Em^r, *nisRK* genes cloned in low-copy-number cloning vector pIL252 (48)	31
pTIL340	Ap^r, pBSK+ carrying the 552-bp *Bam*HI-*Hin*dIII fragment encoding *L. citreum* Mur amplified with the primers LnP5 and LnP6	This study
pTIL341	Ap^r, pBSK+ carrying the 636-bp *Pst*I-*Cla*I fragment encoding *L. citreum* Mur amplified with the primers LnP7 and LnP8	This study
pTIL342	Ap^r, pBSK+ carrying the 740-bp *Cla*I-*Xho*I fragment encoding the amino acid repeats of *acmA* (cA) amplified with the primers LnP9 and LnP10	This study
pTIL343	pQE30 derivative with the 552-bp *Bam*HI-*Hin*dIII insert of pTIL340	This study
pTIL344	Cm^r, pCYT1 derivative carrying the 1,370-bp chimeric *mur-cA* gene under control of the *nisA* promoter	This study
pTIL345	Ap^r, pBSK+ carrying the 648-bp *Pst*I-*Hin*dIII fragment encoding *L. citreum* Mur amplified with the primers LnP6 and LnP7	This study
pTIL346	Cm^r, pCYT1 derivative carrying the 648-bp *Pst*I-*Hin*dIII insert of pTIL345 under control of the *nisA* promoter	This study

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buffer (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl [pH 8.0]). Plasmid DNA was isolated essentially as described previously (6); for *L. lactis,* cells were incubated in TES buffer containing 10 mg of lysozyme per ml at 37°C for 10 min before alkaline lysis. Restriction enzymes, DNA ligase, T4 DNA polymerase, and Klenow enzyme were obtained from Gibco BRL or New England Biolabs and used according to the suppliers' instructions. PCR was performed with a Perkin-Elmer Cetus (Norwalk, Conn.) thermocycler. Electroporation of *L. lactis* was performed as described before (33), and transformants were plated on M17-glu agar plates containing the required antibiotic. DNA sequencing was performed by the dideoxy-chain termination method with the Dye Terminator ABI Prism cycle sequencing kit (Perkin-Elmer). DNA sequence was determined with an automated Applied Biosystems 373 DNA sequencer (Perkin-Elmer). Protein homology searches were carried out with the Blast program (1).

Southern hybridization was performed according to standard protocol (45). Total DNA of *Leuconostoc* strains was digested with *Hin*dIII or *Eco*RI, electrophoresed in an agarose gel, and blotted onto a Hybond-N$^+$ nylon membrane. Two primers (5′-GCGCAGGCTATTTTAG-3′, 465–480 forward, and 5′-ATG CATTAGCTGCTGC-3′, 740–724 reverse) were used to amplify a 276-bp DNA fragment corresponding to the internal region of *L. citreum mur*. This fragment, labeled with [α-^{32}P]dCTP with a random primed DNA labeling kit, was used as a probe in a hybridization experiment under low-stringency conditions (20% formamide).

Cloning of the *L. citreum mur* gene. Two conserved stretches of amino acids were selected from the alignment of the N-terminal regions of the *N*-acetylmuramidases of *L. lactis* (8), *Enterococcus faecalis (5*), and *Enterococcus hirae* (13). These were used to design the degenerate primers LnP1 and LnP2 (Table 2). A PCR with these primers on *L. citreum* 22R total DNA gave rise to a single DNA fragment with the expected size. The nucleotide sequence of this 323-bp fragment was determined, and the deduced amino acid sequence revealed similarity with the N-terminal region of AcmA, the muramidase of *L. lactis*. The entire gene was cloned by reverse PCR as previously described (47) with the divergent primers LnP3 and LnP4 (Table 2), which correspond to internal sequences of the 323-bp fragment. Total DNA from *L. citreum* 22R was digested with *Hin*dIII, and the resulting fragments were self ligated and used as the template for a PCR with the divergent primers. A 3.6-kb DNA fragment was amplified and sequenced.

Expression and purification of the six-His-tagged Mur protein in *E. coli*. The expression vector pQE30 (Qiagen) was used for overproduction of the *L. citreum* Mur protein in *E. coli*. A DNA fragment encoding *L. citreum* Mur without its putative signal peptide was amplified with the primers LnP5 and LnP6 (Table 2) and fused in frame downstream of the N-terminal six-His box sequence in pQE30. The resulting plasmid (pTIL343) was used to transform *E. coli* XL1-Blue competent cells. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to the culture at an optical density at 650 nm of 0.6 to 0.8 to induce the expression of the six-His-tagged Mur protein. The culture was further incubated at 37°C for 4 h. The cells were harvested by centrifugation and broken by a freezing and thawing process followed by sonication. The inclusion bodies containing the recombinant protein were collected by centrifugation at 15,000 × *g* for 10 min at 4°C. The recombinant protein was solubilized in 8 M urea and purified on a nickel-nitriloacetic acid (Ni-NTA) spin column (Qiagen) as recommended by the supplier. Protein concentration was determined with the Coomassie protein assay kit (Pierce).

Mass spectrometry. Protein molecular mass was determined by mass spectrometry with a matrix-assisted laser desorption ionization–time of flight system (LD-TOF G 2025A; Hewlett-Packard).

TABLE 2. Oligonucleotides used in this study

Primer[a]	Sequence[b]	Restriction site	Positions[c]
LnP1	GTNATGATNGCNCARGC		Conserved region; aa 86-91[d]
LnP2(r)	GGRTCNGTNGCRTANC		Conserved region; aa 190-195[d]
LnP3(r)	CATTCCAACTCGTGCCATGA		nt 683 to 664 of the sequenced fragment
LnP4	CGGCCCAATATCAGCATGTT		nt 684 to 703 of the sequenced fragment
LnP5	CGCGGATCCACTTTTTGGTCGCAAACAC	*Bam*HI	nt 305 to 323 of the sequenced fragment
LnP6(r)	GGAAGCTTCGGAACATTTCCGTT	*Hin*dIII	nt 857 to 843 of the sequenced fragment
LnP7	GCCTGCAGCAAAAAGAAAAAGACGT	*Pst*I	nt 216 to 232 of the sequenced fragment
LnP8(r)	CCATCGATTTTTTGTGGTACATCAAAGCG	*Cla*I	nt 838 to 818 of the sequenced fragment
LnP9	CCATCGATGCTGGTACTTCTAATTCCGGT	*Cla*I	nt 652 to 675 of *acmA*[e]
LnP10(r)	CCGCTCGAGTCATCTTCAGTCTGTTTTAAAAG	*Xho*I	nt 1382 to 1359 of the 3′ end of the transcription terminator downstream *acmA*[e]

[a] (r), complementary primer.
[b] N = A or C or T or G; R = A or G. Restriction sites are underlined.
[c] aa, amino acid; nt, nucleotide.
[d] Positions refer to the *L. lactis* MG1363 AcmA sequence (9).
[e] Positions refer to the *L. lactis* IL1403 *acmA* sequence (37).

Construction of a chimeric protein between *L. citreum* Mur and the C-terminal domain of *L. lactis* AcmA and expression in *L. lactis*. A chimeric protein was constructed by fusion of *L. citreum* Mur with the C-terminal domain (cA) of *L. lactis* AcmA containing the amino acid repeats involved in cell wall binding (11). The Mur-cA chimeric protein was expressed in *L. lactis* using the nisin-inducible expression system (21) with the plasmid vector pCYT1 (P. Langella, personal communication). pCYT1 is a derivative of pNZ8010 (21), in which the *gusA* gene was replaced by a DNA fragment carrying the *usp45* ribosome binding site (RBS) (51) fused to the *Staphylococcus aureus nuc* gene (34). pCYT1 was digested with *Nsi*I and *Xho*I to remove *nuc* and to place subsequently the *mur-cA* gene fusion under control of the *nisA* promoter and to express it via the *usp45* RBS. The oligonucleotides used in the study are listed in Table 2.

The *L. citreum mur* gene was amplified by PCR (with primers LnP7 and LnP8), and the 3′ end of the *acmA* gene (*cA*) was amplified by PCR from *L. lactis* IL1403 total DNA (with primers LnP9 and LnP10). The blunted PCR products were cloned in the *Eco*RV site of pBSK+ to yield plasmids pTIL341 (for *L. citreum mur*) and pTIL342 (for *cA*). pTIL341 and pTIL342 were subsequently digested with *Pst*I and *Cla*I and with *Cla*I and *Xho*I, respectively, to recover the inserts. The 636-bp *Pst*I-*Cla*I fragment carrying the *L. citreum mur* gene plus the 740-bp *Cla*I-*Xho*I fragment carrying the 3′ end of *acmA* were mixed with pCYT1 vector digested with *Nsi*I and *Xho*I, and the mixture was ligated. Plasmid pTIL344 containing both fragments, allowing in-frame fusion of *L. citreum mur* and the 3′ end of *acmA*, was selected. It was used to transform *L. lactis* NZ9000 (kindly provided by Oscar Kuipers, Netherlands Institute for Dairy Research, (NIZO), Ede, The Netherlands), which contains the regulatory *nisRK* genes integrated in its chromosome and the *acmA*-negative mutant, *L. lactis* MG1363*acmAΔ1* (9). In the latter case, MG1363*acmAΔ1* harboring pTIL344 was then transformed with pNZ9520 or pNZ9530 plasmid (31), which carries the regulatory *nisRK* genes.

A similar construction was made with *L. citreum mur*. The *L. citreum mur* gene was amplified by PCR from total DNA from *L. citreum* 22R with the primers LnP6 and LnP7. The amplified DNA fragment was blunt ended and first cloned in the *Eco*RV site of plasmid pBSK+. The resulting plasmid, pTIL345, was subsequently digested with *Hin*dIII and *Pst*I, and the 648-bp insert was transferred into the plasmid vector pCYT1 predigested with *Nsi*I and *Hin*dIII. The resulting plasmid, pTIL346, was used to transform *L. lactis* NZ9000 or MG1363*acmAΔ1* as described above.

For induction of the *nisA* promoter, strains were grown until an optical density at 650 nm (OD_{650}) of 0.5 was reached and nisin was added at a final concentration of 2.5 ng/ml. Growth was continued for 5 h, and cells were harvested. SDS cell extracts were prepared as described below and submitted to SDS-PAGE or tested for bacteriolytic activity by renaturing SDS-PAGE.

SDS-PAGE and detection of bacteriolytic activity by renaturing SDS-PAGE. SDS-PAGE was performed as described by Laemmli (32) with 10 to 15% (wt/vol) polyacrylamide separating gels. Gels were stained with Coomassie brilliant blue R250 (Sigma).

For renaturing SDS-PAGE, autoclaved cells of *Micrococcus lysodeikticus* ATCC 4698 (0.2% [wt/vol]) (Sigma), of *Leuconostoc mesenteroides* subsp. *dextranicum* 50 M (0.4% [wt/vol]), or of *L. lactis* subsp. *lactis* NCDO763 (0.4% [wt/vol]) were incorporated into polyacrylamide gels as the substrate. Preparation of samples and detection of bacteriolytic activity were performed essentially as described previously (36, 42). For the preparation of *L. citreum* SDS cell extract, 4 ml of culture was centrifuged and the cell pellet was resuspended in 40 μl of SDS-PAGE sample buffer. The suspension was boiled at 100°C for 3 min and centrifuged at 13,000 × *g* for 10 min, and the supernatant (SDS cell extract) was loaded on the gel. The culture supernatant was concentrated 25 times with a Centriplus concentrator (Amicon, Beverly, Mass.) with a 10,000-Da molecular mass cutoff. Following electrophoresis, the gels were soaked in 250 ml of distilled water for 30 min at room temperature under gentle agitation. They were transferred to 200 ml of renaturation buffer consisting of 50 mM potassium phosphate (pH 6.5) buffer with 1% (vol/vol) Triton X-100 and incubated at 37°C for an additional 6 h with gentle shaking. The gels were then stained with 0.1% meth-

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TAAAACAGAGCATGAGGTGTTACAGCAAATATTTACTGTATTAGCAAATCGCACAATTAT      60
  K  T  E  H  E  V  L  Q  Q  I  F  T  V  L  A  N  R  T  I  I

CTGGGTGACACATCATTTGACGGGTATTTGGGCAGTTGATCAAGTTTATTTTCTTGAAAA     120
  W  V  T  H  H  L  T  G  I  W  A  V  D  Q  V  Y  F  L  E  K

                                                    -35
AGGGCAATTTACTTTGTCGGGGACACCTACTGATTTAGCAAAAAATGCAACACACTTTCA     180
  G  Q  F  T  L  S  G  T  P  T  D  L  A  K  N  A  T  H  F  Q

     -10        RBS
ACAACTATTAGCAATGGATGATTTGGGTTAAATGGCAAAAAGAAAAAGACGTGGCAAAAA     240
  Q  L  L  A  M  D  D  L  G  *  M  A  K  R  K  R  R  G  K  K      10
                                  -----> Lnmur
GAAAATATTTCGTTTGAATCTCTGGTTATGGATAAGCAGTATTTTAGTGTTGGCAATTGG     300
  K  I  F  R  L  N  L  W  L  W  I  S  S  I  L  V  L  A  I  G      30

CGCAACTTTTTGGTCGCAAACACTAATATACAACGAAGAAAATGCACAAGGACAAGACGT     360
  A  T  F  W  S  Q  T  L  I  Y  N  E  E  N  A  Q  G  Q  D  V      50
  ▲
TGTGCGTCAACAAAATATAGCTTGGATTAATCAACTAGCACCATATGCGCGATTAATGCA     420
  V  R  Q  Q  N  I  A  W  I  N  Q  L  A  P  Y  A  R  L  M  Q      70

AGAAAAGTACGGTGTGATTGCGTCAATTAGTATCGCGCAGGCCATTTTAGAGTCAGATTG     480
  E  K  Y  G  V  I  A  S  I  S  I  A  Q  A  I  L  E  S  D  W      90

GCATACTTCGACGCTATCGCCACAGTATAATAATTTATTCGGCATTAAAGCTGACGCAGG     540
  H  T  S  T  L  S  P  Q  Y  N  N  L  F  G  I  K  A  D  A  G     110

GCAAAAAGTGTTGTTTTACCAACCCAGGAGTTTCAAAATGGCGAATGGGTCACTATTCA     600
  Q  K  S  V  V  L  P  T  Q  E  F  Q  N  G  E  W  V  T  I  Q     130

AGGGCGCTTTGCAGCTTATGATTCATGGCAAGAGAGTATGAAGGCGCATGCTGAATTACT     660
  G  R  F  A  A  Y  D  S  W  Q  E  S  M  K  A  H  A  E  L  L     150

TTATCATGGCACGAGTTGGAATGCGGCCCAATATCAGCATGTTTTAGCGGCCAAAGATTA     720
  Y  H  G  T  S  W  N  A  A  Q  Y  Q  H  V  L  A  A  K  D  Y     170

TCAGGCAGCAGCTAATGCATTGACAAAAGATGGGTATGCCACTGATCCAACTTATGCAGA     780
  Q  A  A  A  N  A  L  T  K  D  G  Y  A  T  D  P  T  Y  A  E     190

AAAGCTCATCGCAATTATTCAGACGTGGCATTTGTCGCGCTTTGATGTACCACAAAAATA     840
  K  L  I  A  I  I  Q  T  W  H  L  S  R  F  D  V  P  Q  K  *     209

AAAACGGAAATGTTCCGTTTTTTTATTTT                                    869
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FIG. 1. Nucleotide sequence (nucleotides 1 to 869) and deduced amino acid sequences of the *L. citreum* DNA fragment encoding Mur (Lnmur) (accession number AF176553). The putative −10 and −35 sequences are double underlined, and the putative RBS is indicated with a dotted line. The stop codon is indicated with an asterisk, and a putative transcription terminator is underlined. The putative peptide signal cleavage site is indicated with an arrow.

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EfAutol   FIAEIATYAQPIAQANDLYASVMIAQAVVESGWGSSALSQA---PYYN-LFGIKGSYQGQ
Mur2      FIAELARCAQPIAQANDLYASVMMAQAIVESGWGASTLSKA---PNYN-LFGIKGSYNGQ
AcmA      FIESIAASAKPVADANGLYPSVMIAQAILESNWGSSQLSRA---PYYN-LFGIQGTYQGK
FlgJ      FLARLSLPARLASEQSGVPHHLILAQAALESGWGQRQILRENGEPSYN-VFGVKATASWK
Lnmur     WINQLAPYARLMQEKYGVIASISIAQAILESDWHTSTLS-----PQYNNLFGIKADAGQK
          ::  ::  *:   :  .:   : :*** :**.*    :      * ** :**::.    :

EfAutol   --TVYMDTLEYLNNKWVSKKEPFRQYPSFAESFNDNAYVLR-NTSFGNG-YYYAGTWKSN
Mur2      --SVYMDTWEYLNGKWLVKKEPFRKYPSYMESFQDNAHVLK-TTSFQAGVYYYAGAWKSN
AcmA      --SVVFKTQEYLNGKWVTKDMPFRVYPSFNQSFQDNAYVLK-TTNFGNG-PYYAKAWRAN
FlgJ      GPVTEITTTEYENGEAKKVKAKFRVYSSYLEALSDYVALLTRNPRYAAV--TTAATAE-
Lnmur     --SVVLPTQEFQNGEWVTIQGRFAAYDSWQESMKAHAELLYHGTSWNAAQYQHVLAAK-D
             . : * *: *.:    .  *  * *: :::.  . :*   . :       . : .

EfAutol   TKSYTDATACLTGR-YATDPGYAGKLNNIITTYGLTKYDTP--
Mur2      TSSYRDATAWLTGR-YATDPSYNAKLNNVITAYNLTQYDTP--
AcmA      AATYQDATAALTGR-YATDPSYGASLNRIISQYNLTRFDGA--
FlgJ      ----QGAVALQNAG-YATDPNYARKLTSMIQQLKAMSEKVS-
Lnmur     ---YQAAANALTKDGYATDPTYAEKLIAIIQTWHLSRFDVPQK
               *.   .   ***** *  .*  :*        . .
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FIG. 2. Alignment of the amino acid sequences of the Mur protein of *L. citreum* 22R (Lnmur) and the catalytic domains of AcmA of *L. lactis* MG1363, autolysin (EfAutol) of *E. faecalis*, muramidase-2 (Mur2) of *E. hirae*, and flagellar protein (FlgJ) of serovar Typhimurium. Alignment was made using the ClustalW program. Identical residues present in all the sequences are indicated with an asterisk, and similar residues are indicated with a point. Putative acidic residues present in the catalytic site are in bold letters.

ylene blue in 0.01% KOH and subsequently destained with distilled water. Bacteriolytic activity bands appeared as clear zones in the opaque background. Molecular masses were determined with standards run on the same gel.

Fluorescent in situ hybridization. Fluorescent in situ hybridization was performed as described by Amann et al. (3). Cells were treated with lysozyme, and a 16S RNA-targeted probe (EUB338) (2) labeled with fluorescein or rhodamine was used for hybridization. Photographs of cells were taken with an epifluorescence microscope (Nikon).

Nucleotide sequence accession number. The nucleotide sequence described in this paper is deposited in the EMBL/NCBI/DDBJ sequence databases under accession number AF176553.

RESULTS

Cloning and sequencing of the *mur* gene from *L. citreum* 22R. Using the PCR-based strategy described in Materials and Methods, we cloned a 3.6-kb DNA fragment from *L. citreum* 22R total DNA. Analysis of the nucleotide sequence of this fragment revealed the presence of a 630-bp open reading frame (ORF) (*mur*) showing homology with *L. lactis acmA* (Fig. 1). *L. citreum mur* is preceded by a putative RBS as well as by consensus −10 and −35 regions corresponding to a putative promoter. A potential transcription terminator is present downstream of the ORF.

L. citreum mur specifies a 209-residue polypeptide (Mur), with a calculated molecular mass of 23,821 Da. The first 31 amino acids are predicted to serve as a signal peptide (39). The protein has a predicted isoelectric point (pI) of 9.6. Cleavage of the signal peptide would yield a 178-residue mature protein with a calculated molecular mass of 20,171 Da and a predicted pI of 5.2.

Sequence homology searches revealed that *L. citreum* Mur has sequence identity with the N-terminal regions of the *L. lactis* muramidase AcmA (37%) (9), the *E. hirae* muramidase-2 (37%) (14), and the *E. faecalis* autolysin (38%) (5) (Fig. 2). A significant level of identity was also found with the C-terminal region of the flagellar protein FlgJ of *Salmonella enterica* serovar Typhimurium (37%), which possesses peptidoglycan-hydrolyzing activity (28, 38), and the *E. coli* FlgJ homolog (37%) (7) (Fig. 2). These proteins have a modular structural organization with a catalytic domain fused to a cell wall binding domain (30). Surprisingly, *L. citreum* Mur comprises the catalytic domain of these proteins but lacks a domain containing amino acid repeats. *L. citreum* Mur contains several acidic residues separated by 13 to 33 residues, which could be involved in the catalytic site of the enzyme as proposed for other muramidases (30, 38). For example, E120 and D137 are separated by 16 amino acids.

Part of an ORF (ORFB) was found upstream of *L. citreum mur* (Fig. 1), but no homology was found with sequences present in the databases. Downstream of *L. citreum mur*, another complete ORF was identified (accession number AF176554); it encodes a 750-residue polypeptide with high sequence similarity with *Staphylococcus aureus* DNA helicase PcrA (51%) (27) and the *Bacillus subtilis* homolog (55%) (41).

Distribution of the *L. citreum mur* gene. Southern hybridization was carried out with a 276-bp probe derived from the *L. citreum mur* sequence with the DNA of several *Leuconostoc* strains (16) under low-stringency conditions. One hybridization band was detected in *L. citreum* 22R and in *L. citreum* 50A

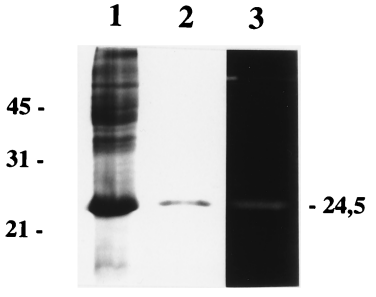

FIG. 3. SDS-PAGE and renaturing SDS-PAGE analysis of the purified six-His-tagged *L. citreum* Mur protein. Coomassie blue staining was used for lanes 1 and 2. Lane 1, whole SDS cell extract of *E. coli* XL1-Blue harboring pTIL343 induced for 4 h with isopropyl-β-D-thiogalactopyranoside; lane 2, recombinant *L. citreum* Mur purified on Ni-nitrilotriacetic acid resin; lane 3, activity of the purified recombinant protein by renaturing SDS-PAGE containing 0.2% autoclaved *M. lysodeikticus* cells. The molecular masses (in kilodaltons) of standard proteins are indicated on the left.

as well as in *L. mesenteroides* subsp. *dextranicum* 19S and *L. mesenteroides* subsp. *mesenteroides* 10L under the conditions used. Besides, a gene homologous to *L. citreum mur*, named *mur1*, was isolated from *Streptococcus thermophilus* (26), and a homologous one was identified in the complete sequence of *L. lactis* IL1403 (A. Bolotin and A. Sorokin, personal communication). All these data suggest a wide distribution of the gene, both in *Leuconostoc* spp. and in other LAB.

***L. citreum* Mur has peptidoglycan-hydrolyzing activity.** The *L. citreum* Mur protein devoid of its putative signal sequence was overproduced in *E. coli* as a six-His N-terminally tagged protein. It was purified from inclusion bodies by metal chelation affinity chromatography on a Ni-nitrilotriacetic acid spin column. The eluted fraction was analyzed by SDS-PAGE, and a single band with an apparent molecular mass of 24.5 kDa was detected after Coomassie blue staining (Fig. 3, lanes 1 and 2). The apparent molecular mass of the protein was higher than that calculated (21,569 Da). Nevertheless, the molecular mass of the purified protein determined by mass spectrometry (21,680 ± 100 Da) fits the calculated mass, thus indicating that the electrophoretic mobility of the protein may be altered by the six-His tag.

The peptidoglycan-hydrolyzing activity of the purified protein was assayed by renaturing SDS-PAGE with autoclaved cells of *M. lysodeikticus* as the substrate. Activity was observed as a clear band at a molecular mass of around 24 kDa on the opaque background after incubation in renaturation buffer (Fig. 3, lane 3), thus indicating that *L. citreum* Mur has peptidoglycan-hydrolyzing activity. Also, *L. citreum* Mur exhibits hydrolyzing activity on *L. lactis* and *L. mesenteroides* substrates (results not shown). Several activity bands can be detected in *L. citreum* 22R cell extracts by renaturing SDS-PAGE (15). In order to determine whether the activity corresponding to that of *L. citreum* Mur could be detected in *L. citreum* 22R, whole SDS cell extract and concentrated culture supernatant were tested under the same conditions as the purified *L. citreum* Mur. However, even after 2 days of incubation of the gel in renaturation buffer, no activity at the expected molecular mass could be detected (data not shown). This suggests that the quantity of enzyme produced is too low to be detected in these conditions.

A chimeric fusion protein between *L. citreum* Mur and the *L. lactis* AcmA C-terminal domain is able to complement AcmA deficiency in *L. lactis*. *L. citreum* Mur is devoid of a specific cell wall binding domain. Thus, a chimeric protein was constructed by fusion of *L. citreum* Mur with the *L. lactis* AcmA C-terminal domain (cA),which contains 3 amino acid repeats (11). The *mur-cA* chimeric gene and the *L. citreum mur* gene were then expressed in the *acmA* deletion mutant, *L. lactis* MG1363*acmAΔ1* (9), in order to investigate whether they could complement an *acmA* mutation.

For expression purposes, the nisin-inducible expression system was used (21). The *mur-cA* and *L. citreum mur* genes were cloned under the control of the *nisA* promoter in pCYT1 (Table 1). The resulting plasmids carrying the fusion *mur-cA* or *mur* gene were first transformed in *L. lactis* NZ9000, which carries the regulatory *nisRK* genes integrated in its chromosome. Production of Mur-cA fusion protein and *L. citreum* Mur after nisin induction was checked by renaturing SDS-PAGE with *M. lysodeikticus* as the substrate. Activity bands at

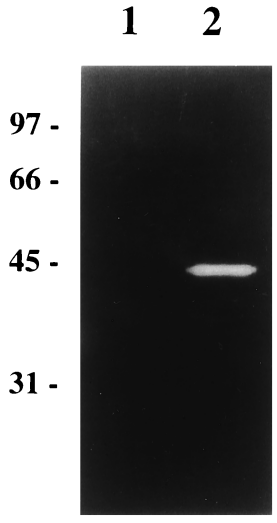

FIG. 4. Renaturing SDS-PAGE analysis of the chimeric Mur-cA protein expressed in *L. lactis* MG1363*acmAΔ1*. Lane 1, *L. lactis* MG1363*acmAΔ1* harboring plasmid pTIL344; lane 2, *L. lactis* MG1363*acmAΔ1* harboring pTIL344 and pNZ9520 carrying *nisRK* genes. SDS cell extract of each strain was prepared from a noninduced culture and loaded onto polyacrylamide gel containing 0.2% autoclaved *M. lysodeikticus* cells. The molecular masses (in kilodaltons) of standard proteins are indicated on the left of the gel.

44 and 24.5 kDa were revealed in NZ9000 harboring pTIL344 and pTIL346, respectively, which correspond to the expected molecular masses of the fusion proteins Mur-cA and *L. citreum* Mur, respectively (data not shown). This indicated that the genetic constructions were functional. The plasmids pTIL344 and pTIL346 were then transferred in *L. lactis* MG1363*acmAΔ1*. Since strain MG1363 does not contain the regulatory *nisRK* genes, plasmid pNZ9520 (Table 1) carrying *nisRK* was also introduced into MG1363*acmAΔ1* harboring pTIL344 or pTIL346.

L. lactis MG1363*acmAΔ1* is characterized by the presence of long bacterial chains and the absence of any activity band in renaturing SDS-PAGE with *M. lysodeikticus* as the substrate. However, both of these phenotypes are reversed in the MG1363*acmAΔ1* strain harboring plasmids pTIL344 and pNZ9520 even in the absence of nisin. A 44-kDa activity band was observed using either *M. lysodeiktikus* (Fig. 4, lane 2), *L. mesenteroides*, or *L. lactis* cells as substrates (data not shown). This activity was absent from MG1363*acmAΔ1* harboring pTIL344 alone (Fig. 4, lane 1). In addition, we observed that *L. lactis* MG1363*acmAΔ1* harboring plasmids pTIL344 and pNZ9520 lost its sedimentation properties and formed short chains similar to those of wild-type MG1363 (Fig. 5). These results indicate that the chimeric protein Lnmur-cA is functional and able to complement an AcmA deficiency. In contrast, MG1363*acmAΔ1* harboring plasmids pTIL346 and pNZ9520 still formed long chains even after nisin induction. It is worth noting that, although we checked that the construction was functional in strain NZ9000, *L. citreum* Mur was not detected in MG1363*acmAΔ1* harboring plasmids pTIL346 and pNZ9520, most probably due to the low expression level and a lower specific activity of *L. citreum* Mur compared to those of Mur-cA. Nevertheless, the results suggest that unlike Mur-cA,

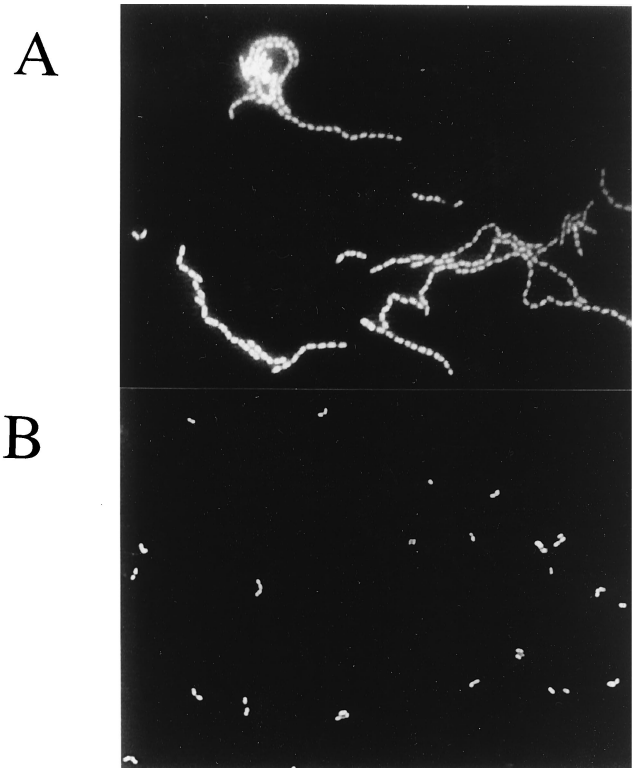

FIG. 5. Epifluorescent micrograph of *L. lactis* MG1363*acmA*Δ*1* (A) and *L. lactis* MG1363*acmA*Δ*1* harboring pTIL344 and pNZ9520 (B). Bacteria were grown in M17-glu medium and were not induced with nisin. Micrographs were taken after in situ hybridization of the lysozyme-treated bacteria with the universal probe EUB338.

L. citreum Mur alone was not able to complement the AcmA deficiency.

We were surprised to detect Mur-cA activity in the absence of nisin inducer, as well as a lack of induction after nisin addition. Previously, Kleerebezem et al. (31) also reported a significant level of transcription from the *nisA* promoter in the absence of nisin, when the *nisRK* genes were carried by the high-copy-number pNZ9520, as well as a reduced or abolished inducibility of the *nisA* promoter. They overcome this problem by the use of the low-copy-number plasmid pNZ9530 (Table 1) carrying the *nisRK* genes. In our case, even with pNZ9530 instead of pNZ9520 and regardless of the nisin concentration, nisin-inducible expression of the *mur-cA* and *L. citreum mur* genes was not obtained.

DISCUSSION

To our knowledge, this is the first report concerning the identification of a PGH gene from a *Leuconostoc* species. The *L. citreum mur* gene isolated from *L. citreum* encodes a PGH homologous to bacterial *N*-acetyl-muramidases.

In contrast to most of the other previously described bacterial muramidases, *L. citreum* Mur contains only the catalytic domain and is devoid of the cell wall binding domain that typically consists of repeated sequences (30). Very recently, a similar PGH, exhibiting 35% sequence identity with *L. citreum* Mur, was identified in the lactic acid bacterium *Streptococcus thermophilus* (26). Despite the lack of amino acid repeats, *L. citreum* Mur is endowed with peptidoglycan-hydrolyzing activity, as detected in vitro (Fig. 3) and in an overexpression system in *L. lactis* (data not shown). These results are in agreement with previous data showing that the AcmA N-terminal domain (10) or the FlgJ C-terminal domain (38) without amino acid repeats retains enzymatic activity. In addition, we constructed an active fusion protein between *L. citreum* Mur and the AcmA C-terminal domain containing amino acid repeats. The resulting chimeric protein was able to play the role of AcmA in cell separation after cell division in the *L. lactis acmA* deletion mutant, thus indicating that *L. citreum* Mur is also functional on the cell wall in vivo.

Nisin-inducible expression of the *mur-cA* fusion gene was not obtained in the MG1363*acmA*Δ*1* deletion mutant, in which *nisRK* genes required for nisin-mediated signal transduction were plasmid carried. However, since inducible expression was observed in *L. lactis* NZ9000 with the *nisRK* genes integrated into its chromosome, this suggests that the problems encountered are most probably linked to the strain used, that is, the *acmA* deletion mutant. This observation could be due to a modification of the cell surface of the mutant devoid of AcmA, which could alter the interaction of nisin with the NisK sensor protein located in the cell cytoplasmic membrane.

No activity band migrating at the molecular mass expected for *L. citreum* Mur could be revealed in *L. citreum* 22R extract or in culture supernatant by renaturing SDS-PAGE either in cell extract or in culture supernatant. This is most probably due to a low expression level of the protein since the expression consensus sequences, putative promoter, and RBS sequences were identified upstream of the ORF encoding *L. citreum* Mur.

As discussed above, *L. citreum* Mur does not contain amino acid repeats involved in cell wall attachment. In the case of the *S. thermophilus* homolog, the protein was shown to be cell associated (26). The structural similarity between these proteins leads us to suggest that the Mur protein in *L. citreum* is thus also cell associated. Nevertheless, *L. citreum* Mur does not possess the characteristics described for surface proteins, such as an LPXTG motif (24) or a region rich in Pro-Gly and Ser-Thr (43). Other means of protein association with the cell wall are (i) via membrane association, i.e., by a transmembrane segment on the protein or indirectly by protein interactions with a membrane protein or (ii) via protein interactions with a cell wall component, such as teichoic acids or lipoteichoic acids (8, 29). It is worth noting that the PGH amino acid repeats have been proposed to direct the enzyme to the cell division site (4). The absence of these repeats could allow a more homogeneous distribution of the enzyme in the cell wall.

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