Characterization of AcmB, an N-acetylglucosaminidase autolysin from Lactococcus lactis

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A gene encoding a putative peptidoglycan hydrolase, named acmB, which is a paralogue of the major autolysin acmA gene, was identified in the Lactococcus lactis genome sequence. The acmB gene is transcribed in L. lactis MG1363 and its expression is modulated during cellular growth. The encoded AcmB protein has a modular structure with three domains: an N-terminal domain, especially rich in Ser, Thr, Pro and Asn residues, resembling a cell-wall-associated domain; a central domain homologous to the Enterococcus hirae muramidase catalytic domain; and a C-terminal domain of unknown function. A recombinant AcmB derivative, devoid of its N-terminal domain, was expressed in Escherichia coli. It exhibited hydrolysing activity on the peptidoglycan of several Gram-positive bacteria, including L. lactis. Though showing sequence similarity with enterococcal muramidase, AcmB has N-acetylglucosaminidase specificity. The acmB gene was inactivated in order to evaluate the role of the enzyme. AcmB does not appear to be involved in cell separation but contributes to cellular autolysis.

INTRODUCTION

Peptidoglycan, a polymer of amino sugars cross-linked by short peptides, is the major component of Gram-positive bacterial cell walls and ensures cell wall integrity and rigidity. Bacteria synthesize peptidoglycan hydrolases capable of hydrolysing their own peptidoglycan (Shockman & Hölting, 1994). Although threatening cell integrity, peptidoglycan hydrolases are synthesized during cellular growth. They are involved in a number of cellular functions which require cell wall remodelling, such as cell separation after division, cell wall turnover and cell wall expansion (Smith et al., 2000). According to the chemical bond cleaved inside the peptidoglycan molecule, four different specificities are defined: N-acetylmuramidase, N-acetylglucosaminidase, N-acetylglucosaminyl-L-alanine amidase and endopeptidase.

Lactococcus lactis is a lactic acid bacterium widely used in starters for cheese making. During cheese ripening, the bacterial intracellular enzyme content contributes to the development of organoleptic properties (Chapot-Chartier, 1996; Crow et al., 1995). The cell envelope appears as a physical barrier for the bacterial enzymes to reach the extracellular substrates. Bacterial autolysis was previously shown to enhance the contribution of enzymes to cheese flavour formation. Especially, autolysis leads to the release of the intracellular peptidases into the cheese curd, and as a result more free amino acids, which are aroma precursors, are produced and hydrophobic bitter peptides are degraded (Chapot-Chartier et al., 1994; Wilkinson et al., 1994). Besides this traditional use in dairy fermentation, L. lactis has been proposed as a vaccine vehicle or delivery vector for use in human medicine (Drouault et al., 1999; Wells et al., 1996). For these applications, L. lactis autolysis in the gastro-intestinal tract is also a critical parameter to obtain an optimal response. In order to get insight into the autolysis mechanism and to control it, it is necessary to identify and characterize the peptidoglycan hydrolases involved.

Up to now, only the major autolysin, named AcmA, has been characterized at the molecular level in L. lactis (Buist et al., 1995). Like most bacterial peptidoglycan hydrolases, AcmA has a modular structure with two domains. The N-terminal domain exhibits sequence similarity with the catalytic domain of Enterococcus hirae muramidase Mur-2, and the C-terminal one contains three amino acid repeats.
involved in cell wall binding (Buist, 1997). As regards its enzymic specificity, AcmA was recently shown to be an N-acetylglucosaminidase (Steen et al., 2001), rather than an N-acetylmuramidase as predicted by sequence similarity. AcmA is required for proper separation of cells after cell division and is involved in cellular autolysis in stationary phase in synthetic culture medium (Buist et al., 1997). However, other factors than AcmA contribute to determining the autolysis rate when cells are in the cheese matrix environment (Pillidge et al., 1998). This prompted us to search for new peptidoglycan hydrolases and evaluate their contribution to cellular autolysis in L. lactis.

In the genome sequence of L. lactis subsp. lactis IL1403 (Bolotin et al., 2001), we could identify four putative peptidoglycan hydrolases, in addition to AcmA, on the basis of a sequence similarity search. In this study, we have investigated the functionality, hydrolytic bond specificity and the role of one of these peptidoglycan hydrolases, named AcmB.

**METHODS**

**Bacterial strains and plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli strains were grown at 37°C in Luria–Bertani (LB) medium with shaking. L. lactis strains were grown in M17 medium (Difco) supplemented with 0.5% (w/v) glucose at 30°C. Growth was monitored by measuring OD650 with a spectrophotometer (UVicon 931, Biotek Kontron). The following antibiotics were added to the growth medium with shaking.

- E. coli: ampicillin (100 μg ml⁻¹ for E. coli), chloramphenicol (5 μg ml⁻¹ for L. lactis; 20 μg ml⁻¹ for E. coli) and ampicillin (100 μg ml⁻¹ for E. coli).

**General recombinant DNA techniques.** Molecular cloning techniques were performed using standard procedures (Sambrook et al., 1989). Restriction enzymes (Eurogentec or Roche Molecular Biochemicals), T4 DNA ligase (Epipcentre), Taq DNA polymerase (Appligene Oncor) and calf intestinal alkaline phosphatase (New England Biolabs) were used according to the suppliers’ recommendations. The oligonucleotides were purchased from Invitrogen. L. lactis DNA was isolated as described previously (Posspich & Neumann, 1995). PCR amplifications were carried out in a GeneAmp PCR System 2400 (Perkin Elmer). pGEMT easy vector (Promega Corporation) was used to clone PCR products in E. coli. DNA sequences were determined on an Applied Biosystems 370A automated DNA sequencer using the ABI PRISM Dye Terminator Cycle Sequencing Kit and the Dye Primer Cycle Sequencing Kit (Perkin Elmer). DNA and protein sequences were assembled and analysed with the Genetic Computer Group sequence analysis package (GCC, Madison, WI, USA) and the tools available on the Expasy (Expert Protein Analysis System) Biology Server of the Swiss Institute of Bioinformatics (SIB) (http://www.expasy.org). Electro- poration of L. lactis was performed as described by Holo & Nes (1995) and transformants were plated on M17 glucose agar plates containing the required antibiotic.

**Cloning of the acmB gene from L. lactis MG1363.** With the primers AU4 (5'-GATTATCTTTATCTGCTACAC-3' and AU11 (5'-CAACTGCAGACAAATTCC-3') selected from the IL1403 sequence genome (Bolotin et al., 2001), a 1.6 kb DNA fragment was amplified by PCR from L. lactis subsp. cremoris MG1363 total DNA. This fragment contained only part of the MG1363 acmB gene. PCR cloning of the 5'-end failed, although several pairs of primers were tested. The sequence of the 1077 bp region upstream of the 1674 bp

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype/phenotype</th>
<th>Source/reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli TG1</td>
<td>supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB+ lacF' lacZAM15]</td>
<td>Gibson (1984)</td>
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<tr>
<td>E. coli TG1repA+</td>
<td>TG1 derivative with repA gene integrated into the chromosome, allowing replication of L. lactis plasmids</td>
<td>P. Renault*</td>
</tr>
<tr>
<td>E. coli M15(pREP4)</td>
<td>Derivative of E. coli K-12, containing pREP4 plasmid ensuring the production of high levels of lac repressor protein; Kan’</td>
<td>Qiagen</td>
</tr>
<tr>
<td>L. lactis IL1403</td>
<td>Plasmid-free strain</td>
<td>Chopin et al. (1984)</td>
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<tr>
<td>L. lactis MG1363</td>
<td>Plasmid-free strain</td>
<td>Gasson (1983)</td>
</tr>
<tr>
<td>L. lactis MG1363acmA1</td>
<td>Derivative of MG1363 carrying a 701 bp deletion in acmA</td>
<td>Buist et al. (1995)</td>
</tr>
<tr>
<td>B. subtilis HR</td>
<td>Derivative of B. subtilis 168, trpC2</td>
<td>S. J. Foster†</td>
</tr>
<tr>
<td>L. lactis MG1363acmB</td>
<td>Derivative of MG1363 carrying a 414 bp deletion in acmB and a Cm’ cassette inserted; Cm’</td>
<td>This study</td>
</tr>
<tr>
<td>L. lactis MG1363acmA1acmB</td>
<td>Derivative of MG1363 carrying deletions in both acmA and acmB; Cm’</td>
<td>This study</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pQE60</td>
<td>Expression vector for C-terminal hexa-His-tag fusion; Ap’</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pG’+host9</td>
<td>Thermosensitive plasmid, for gene replacement by double-crossover integration; Ery’</td>
<td>Biswas et al. (1993)</td>
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<tr>
<td>pTIL328</td>
<td>pG’+host9 with 414 bp deleted acmB gene and a Cm’ cassette inserted</td>
<td>This study</td>
</tr>
<tr>
<td>pGEMT-easy</td>
<td>Cloning vector for PCR products; Ap’</td>
<td>Promega</td>
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†University of Sheffield, UK.
amplified region was obtained from a long-range 10 kb DNA fragment amplified by Multiplex Long accurate PCR from MG1363 total DNA (Boilot et al., 2002). This 10 kb fragment was amplified with two primers derived from the IL1403 sequence: mc91.pri (5'-ATCCGTACGCGCAGTTG-3') and mb95.pri (5'-GGTA- TGGTAACTACCCAGTGTC-3'), corresponding to metK and fbaA MG1363 sequencing tags. Finally, we obtained the sequence of a 2751 bp DNA fragment encompassing the acmB gene, which was located in the central region of the fragment.

Inactivation of acmB by double crossing-over integration. A 2-4 kb fragment encompassing the acmB gene was amplified by PCR with the two primers: AU57 (5'-TCCCCGGCGAATGCGGACCAATGGTGG-3'; Smal restriction site underlined) and AU58 (5'-GGGTTACCTTTTCATCGGAAGGTTGACGTTG-3'; KpnI site underlined) with MG1363 DNA as template. The PCR product was cloned into the pGEMT-easy vector. The insert was recovered with Smal and KpnI digestion and then ligated in the corresponding sites of pGEMH9 host plasmid. The ligation mixture was used to transform E. coli TG1repA competent cells. In the resulting recombinant plasmid, an internal 414 bp fragment of the acmB gene was deleted by digestion with EcoRI and PstI. A chloramphenicol-resistance cassette with a size of 0-9 kb (Trieu-Cuot et al., 1992) was then ligated into the PstI and EcoRI sites. The final plasmid pTIL328 was electroporated into L. lactis MG1363 and MG1363acmA1. Integration of pTIL328 into the chromosome and subsequent excision was achieved according to the previously developed protocol (Biswas et al., 1993). Mutant strains were screened first on their resistance to chloramphenicol and second on the size of the fragment amplified by PCR with primers AU57 and AU58. The presence of a correct insertional event was further verified by Southern blotting. When the properties of the acmB mutants were analysed, chloramphenicol was omitted from the culture.

Northern blotting. Total RNA fractions were extracted from L. lactis MG1363 grown at 30°C as previously described (Anba et al., 1995), at different stages of growth, corresponding to OD650 0-1, 0-5, 1-0, 1-5, 2-0, 2-5 and 3-0. Northern blot hybridization was performed according to a standard protocol (Sambrook et al., 1989). Twenty micrograms of total RNA were electrophoresed in a 1% (w/v) agarose gel (Seakem GTG Agarose, BMA Bioproducts), transferred to Hybond-N+ nylon membrane (Amer sham Pharmacia Biotech) and fixed by heat treatment (80°C, 2 h). The membrane was probed with a 1-5 kb DNA fragment corresponding to the entire acmB gene, and labelled with [32P]-PcITP with the Random Primed DNA Labelling Kit (Roche Molecular Biochemicals). Hybridization was performed under high-stringency conditions (50% formamide). The membrane was then dehybridized by immersion in boiling 1 x SSC/0-2% SDS and subsequent incubation for 1 h at room temperature. It was rehybridized with a 165 ssDNA specific probe obtained by PCR amplification as described by Cibik et al. (2000). Radioactivity was quantified with a PhosphorImager (Molecular Dynamics) with the ImageQuant program. The relative amounts of acmB transcript were standardized by hybridization with a L. lactis 165 ssDNA specific probe.

Expression and purification of His-tagged proteins in E. coli. The proteins were overexpressed in E. coli M15(pREP4) as C-terminal hexa-His-tagged proteins, with the expression vector pQE60 (Qiagen). Two truncated derivatives of the acmB gene encoding polypeptides corresponding to the AcmA catalytic domain alone, and to the catalytic domain plus the C-terminal domain of AcmA, were amplified by PCR from L. lactis MG1363 total DNA. The DNA fragments were amplified respectively with the primers AU65 (5’-CATGCCATGTTATATGGGCGGCTTATT-3’; NcoI restriction site underlined) and AU68 (5’-GAAGATCTACTGCGGACATATT-CTATAAAA-3’; BglII restriction site underlined), and AU65 and AU70 (5’-GAAGATCTTGGTGAATTGATAGTTCG-3’; BglII restriction site underlined). PCR fragments were digested by Ncol and BglII introduced with the primers and cloned in-frame upstream of the hexa-His box sequence in the pQE60 vector, precinct by the same enzymes. E. coli M15(pREP4) competent cells were transformed with the resulting plasmids. IPTG was added at a final concentration of 1 mM to the culture at an OD650 of 0-5 to induce the expression of the hexa-His-tagged proteins. Bacteria were grown at 37°C until IPTG addition and were then transferred at 28°C during the expression time (4 h) to avoid the formation of inclusion bodies. The cells were harvested by centrifugation and broken by one passage at a pressure of 1600 bar with a Constant Cell Disruption System (Constant System, Warwickshire, UK). The soluble fraction containing the recombinant protein was collected by centrifugation at 15 000 g for 15 min at 4°C. The hexa-His-tagged proteins were purified by affinity chromatography on Ni2+-nitrilotriacetic acid (Ni-NTA) spin columns (Qiagen), according to the manufacturer’s instructions.

Preparation of cell wall peptidoglycan. Peptidoglycan from Bacillus subtilis HR vegetative cells was prepared as described previously (Arthi et al., 1999). Peptidoglycan from L. lactis MG1363 was prepared from an exponentially growing culture (OD650 0-7) according to the protocol of Mainardi et al. (2000). Briefly, cells were boiled in 4% (w/v) SDS for 30 min. Cell walls were recovered by centrifugation for 90 min at 140 000 g and washed three times with water to eliminate SDS. To remove proteins, the cell wall pellet was treated with Pronase (200 μg ml−1) for 16 h at 37°C, then by trypsin (200 μg ml−1) for 16 h at 37°C. The pellet containing peptidoglycan was washed twice with water and stored at −20°C.

SDS-PAGE and renaturing SDS-PAGE. SDS-PAGE was performed as described by Laemmli (1970) with 15% (w/v) polyacrylamide separating gels. Renaturing SDS-PAGE was performed as previously described (Lepeuple et al., 1998). The polyacrylamide gels contained 0-2% (w/v) Microcococcus luteus ATCC 4698 (Sigma) or 0-4% (w/v) L. lactis autotolaved cells, or 0-16% (w/v) MG1363 peptidoglycan as enzyme substrate. Gels were washed for 1 h in deionized H2O at room temperature and then incubated in buffer containing 1% (w/v) Triton X-100, overnight at 37°C. The following incubation buffers were used: 25 mM sodium citrate/50 mM sodium phosphate at pH 3-0, 4-0 or 5-0; 50 mM MES at pH 6-0; and 50 mM Tris/HCl at pH 7-0 and 8-0. The gels were subsequently washed for 1 h in deionized H2O and when required, with 10 mM Tris/HCl, pH 7-0, containing 0-1% SDS to remove precipitated proteins from the gels. The gels were then stained with 0-1% (w/v) methylene blue in 0-01% (w/v) KOH for 2 h at room temperature and destained in deionized H2O. Gel images were generated with a Duoscan T1200 scanner (Agfa-Gevaert) customized for proper gel handling and controlled by the Agfa Photolook 3.0 software.

Determination of hydrolytic bond specificity. The hydrolytic bond specificity of AcmA was analysed using peptidoglycan from B. subtilis HR vegetative cells as substrate. Peptidoglycan (5 mg) was incubated overnight at 37°C, with 500 μg purified hexa-His-tagged recombinant AcmA[C+Z]-His in a final volume of 500 μl buffer (25 mM sodium citrate/50 mM sodium phosphate, pH 4-0). Samples were boiled for 3 min to stop the reaction. Insoluble material was removed by centrifugation at 14 000 g. Half of the soluble muropeptide fraction was further digested with Cellosyl (250 μg ml−1). The soluble muropeptides obtained after digestion with AcmA[C+Z]-His or AcmA[C+Z]-His plus Cellosyl were reduced with sodium borohydride (8 mg ml−1) as described previously (Arthi et al., 1999). The reduced muropeptides were separated by RP-HPLC with a Biocad Sprint system (Perkin Elmer) equipped with a variable-wavelength double detector and an automatic collector (Advance) using a Hypersyl PEP100 C18 column (250 × 4-6 mm, 5 μm, 100 Å) (Thermo Finnigan). Elution was carried out at a flow rate of 1 ml min−1 for 10 min with 100% solvent A (TFA/H2O 1:15:1000, v/v) and subsequently with a 120 min linear gradient (0 to 20%) of solvent B.
membranes. Bacterial suspension (1 ml) was incubated with 30 μl cationic surfactant used to permeabilize bacterial cytoplasmic membrane. Treatment with cetyltrimethylammonium bromide (CTAB) (Sigma), a whole population of cells was estimated by PI-induced fluorescence after washing from the steel plate and was allowed to air dry. The peptides were desorbed and ionized by a N2 laser in the positive- and/or negative-ion mode. Each mass spectrum was a mean of 200 scans. For all experiments, the accelerating potential was held at 20 kV and laser power was set to the minimum level necessary to generate a reasonable signal. An external mass calibration standard containing bradykinin fragment 1-5, angiotensin I, neurotensin and melittin was employed for all the analyses.

**Autolysis in buffer solution.** *L. lactis* strains were grown in M17 medium to mid-exponential phase (OD650 1.0). Cells were harvested by centrifugation at 5000 g for 15 min at 4 °C and washed once with sterile deionized water. They were resuspended in 50 mM potassium phosphate buffer pH 7.0 at OD650 0.8 and incubated at 30 °C. Autolysis was monitored by measuring the OD650 of the cell suspension. The extent of autolysis was expressed as the percentage decrease in OD650. The intracellular X-prolyldipeptidyl aminopeptidase (PepX) was chosen as a marker for cell autolysis (Lepeuple *et al.*, 1998). Its release into the culture supernatant was monitored by measuring enzymic activity with Ala-Pro-p-nitroanilide (Bachem) chromogenic substrate. Enzymic activity was expressed in katalas (1 katal is the quantity of enzyme releasing 1 mol p-nitroaniline s⁻¹) per ml of culture.

**Determination of the proportion of cells with a damaged membrane.** The membrane integrity of the cells incubated in buffer solution as described above was investigated by the fluorescence labelling procedure described by Niven & Mulholland (1998). The fraction of permeable cells in the whole *L. lactis* cell population was estimated by labelling with propidium iodide (PI), a fluorescent dye, by centrifugation at 5000 g for 5 min and emission wavelength of 600 nm with a Kontron fluorimeter (Perseptive Biosystem, Framingham, USA). The precision in mass determination was 0.5% by mass. Tof mass spectrometry using a Voyager DE STR mass spectrometer (Applied Biosystems, Framingham, USA) was performed. The samples were prepared for MALDI-TOF mass spectrometry using a Voyager DE STR mass spectrometer as described above. The mass spectrum was acquired with a pulsed nitrogen laser operating at 355 nm in the positive- and/or negative-ion mode. Each mass spectrum was a mean of 200 scans. For all experiments, the accelerating potential was held at 20 kV and laser power was set to the minimum level necessary to generate a reasonable signal. An external mass calibration standard containing bradykinin fragment 1-5, angiotensin I, neurotensin and melittin was employed for all the analyses. A mass of 200 scans. For all experiments, the accelerating potential was held at 20 kV and laser power was set to the minimum level necessary to generate a reasonable signal. An external mass calibration standard containing bradykinin fragment 1-5, angiotensin I, neurotensin and melittin was employed for all the analyses.

**Acmb, a peptidoglycan hydrolase with an uncommon three-domain modular structure**

The *acmB* gene encodes a 538-residue protein with calculated molecular mass of 57.1 kDa and pl of 4.72. The first 33 N-terminal residues of Acmb possess the characteristics of a signal peptide (Nielsen *et al.*, 1997). They could also constitute an N-terminal signal anchor sequence since a transmembrane helix is predicted in positions 14–33 (Tusnady & Simon, 2001). According to protein sequence similarities, the Acmb protein has a three-domain modular organization (Fig. 1A).

The central domain (residues 212–390) of Acmb exhibits sequence similarity with the catalytic domain of Ent. hirae muramidase-2 (39–9% identity) (Chu *et al.*, 1992) and numerous related proteins present in the databases. In particular, sequence similarity was found with peptidoglycan hydrolases from lactic acid bacteria – *L. lactis* major autolysin AcmA (41–5%) (Buist *et al.*, 1995), Streptococcus thermophilus Mur1 (44–5%) (Husson-Kao *et al.*, 2000), Leuconostoc citreum Mur (38%) (Cibik *et al.*, 2001) – and with two other *L. lactis* paralogue proteins (Bolotin *et al.*, 2001) (Fig. 2). The catalytically important acidic residues identified in the enterococcal muramidase family (Joris *et al.*, 1992) are conserved in *L. lactis* Acmb (Glu at position 299 and Asp at position 319).

The C-terminal domain (residues 390–538) shares sequence similarity with several cell-wall-bound proteins such as the putative transfer protein TraG of *Staphylococcus aureus*.

**RESULTS**

**Cloning of acmB from *L. lactis* subsp. cremoris MG1363**

From the sequence similarity search, a gene named *acmB*, encoding a putative peptidoglycan hydrolase, was identified in the complete genome sequence of *L. lactis* subsp. *lactis* IL1403 (Bolotin *et al.*, 2001). Since the IL1403 genome contains several inducible prophages, and the induction can cause cellular lysis (Chopin *et al.*, 2001), we chose to study *acmB* from *L. lactis* subsp. cremoris MG1363, a strain cured of its inducible prophage (Gasson, 1983). The homologous *acmB* gene from MG1363 total DNA was first cloned. The nucleotide sequence of a 2751 bp DNA fragment encompassing *acmB* was determined (accession number AJ414526). *acmB* contains 1616 bp and is preceded by a putative ribosome-binding site. Two putative promoters as identified by −35 and −10 boxes are present upstream of the start codon and a putative transcription terminator is present downstream of the stop codon.

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**Fig. 1.** Schematic representation of the domain structure of Acmb (A) and the His-tagged Acmb derivatives (B). SP, signal peptide; [A], putative cell-wall-associated domain; [C], putative catalytic domain; [Z], C-terminal domain containing a putative zinc-binding motif. Black boxes indicate the hexa-His tag added at the C-terminus of Acmb derivatives expressed in *E. coli*.
involved in conjugative transfer of plasmids (Morton et al., 1993), the immunogenic secreted protein (Isp) of group A streptococci (McIver et al., 1996) and the major secreted protein Usp45 with unknown function (van Asseldonk et al., 1990). The precise role of this domain in the proteins is not known. Interestingly, the AcmB C-terminal domain contains a 33-amino-acid sequence Tyr-X-His-X 7-Tyr-X13-Gly-X7-His resembling a zinc-binding motif (Ramadurai et al., 1999).

The N-terminal domain (residues 34–211) has a remarkably high content of the amino acids Ser (29 %), Thr (15 %), Asn (10 %) and Pro (8 %). This characteristic is encountered in protein domains associated with the cell wall in Gram-positive bacteria (Fischetti et al., 1991). A very similar Ser-, Thr-, Gly-, Pro-rich domain was previously described in the cell-bound fructosyltransferase of Streptococcus salivarius (Rathsam et al., 1993) and this domain was shown to play a role in cell surface attachment of the protein (Rathsam & Jacques, 1998).

A putative protein similar to AcmB and composed of three homologous domains with 39 % sequence similarity was found only in the Staph. aureus genome sequence (Kuroda et al., 2001).

acmB expression is regulated during cellular growth

Transcriptional analysis of acmB during cellular growth was studied by Northern blot analysis. Total RNA was isolated from cells harvested at different stages of growth at 30˚C in M17 medium. Using an acmB probe, we detected a single 1.6 kb transcript (Fig. 3A). This size corresponds to a monocistronic organization of acmB. Quantitative mRNA analysis revealed that the relative abundance of the transcript varied during growth (Fig. 3B). Early-exponential-phase cells (OD650 0.1) contained a substantial amount of acmB mRNA, which rapidly declined to become

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**Fig. 2.** Alignment of amino acid sequences of AcmA, AcmB, AcmC and AcmD of L. lactis, muramidase-2 of Ent. hirae (Mur2), Mur1 of Strep. thermophilus and Mur of Ln. citreum. Alignment was done with the ClustalW program. (*), identical amino acids in the seven proteins; (:), similar amino acids. Glu and Asp residues conserved in the active centre of the enzymes are shaded.

**Fig. 3.** Northern blot analysis of acmB expression during cellular growth. Total RNA was prepared from L. lactis MG1363 cells, grown in M17 medium, at different times of the culture and analysed by Northern blotting. (A) Autoradiogram of the membrane hybridized successively with radioactive acmB and 16S rRNA probes. (B) Quantitative analysis of the acmB transcript during cellular growth. Growth was followed by OD650 measurement. Hybridization signals were quantified with a PhosphorImager. The relative amount of acmB transcript (■) is expressed with respect to the highest level of transcript (measured at OD650 0-1). The relative amount of acmB transcript was standardized by hybridization with a L. lactis 16S rRNA specific probe.
3.5 times less abundant at an OD\textsubscript{650} of 0.5. Then, the amount of acmB mRNA increased slightly during the exponential growth phase, followed by a slow decline until stationary phase. These results indicate that acmB is transcribed in \textit{L. lactis} MG1363 and its transcription is regulated during cell growth.

**AcmB has peptidoglycan-hydrolysing activity**

No activity band which could correspond to AcmB was detected by renaturing SDS-PAGE with \textit{M. luteus} or \textit{L. lactis} cells as substrate, in cell extracts from \textit{L. lactis} MG1363 or from the acmA mutant, although this latter strain lacks the activity bands of AcmA and its degradation products (data not shown). Therefore, in order to examine AcmB peptidoglycan-hydrolysing activity, the enzyme was over-produced in \textit{E. coli}. The full-length protein without its putative signal sequence and with a C-terminal hexa-His tag could not be produced in \textit{E. coli} despite several attempts with different expression vectors and recipient strains. Conversely, two truncated derivatives of AcmB were obtained in large amounts with the pQE60 expression system. These derivatives correspond to the catalytic domain plus the C-terminal domain of AcmB (AcmB [C+Z]-His) and to the catalytic domain of AcmB alone (AcmB[C]-His), which were expressed without signal peptide and with a C-terminal hexa-His tag (Fig. 1B).

The His-tagged derivatives of AcmB were purified by nickel affinity chromatography under native conditions (Fig. 4A). Their peptidoglycan-hydrolysing activity was examined by renaturing SDS-PAGE with \textit{M. luteus} autoclaved cells as substrate (Fig. 4B). The two proteins gave a clear hydrolysis band with incubation buffer at pH 4-0. The detection of AcmB[C]-His activity indicates that the [C] domain retains peptidoglycan hydrolase activity and confirms its assignment as the catalytic domain. In addition, these results indicate that cell wall binding is not an absolute requirement for enzyme activity. It is worth noting that a rather high amount of the purified His-tagged proteins was loaded on the gel for activity to be detected. Thus, the absence of detection of AcmB activity in \textit{L. lactis} cellular extracts results most probably from too low an amount of protein expressed in the cells.

The activity of AcmB[C+Z]-His was also examined by renaturing SDS-PAGE on \textit{L. lactis} cell walls with incubation buffer at pH 4-0. A clear hydrolysis band was observed (data not shown). Since the N-terminal [A] domain is most probably not involved in specific recognition of the cell wall, we can conclude that AcmB is an autolysin.

The influence of the incubation buffer pH on activity detection was tested on \textit{M. luteus} cell substrate. AcmB [C+Z]-His activity was detected at pH 4-0 and 5-0 but not at pH 6-0 and higher, nor at pH 3-0. AcmB[C+Z]-His has a low pl (5-03) and interestingly, its activity was detected only at acidic pH in the renaturing electrophoresis test. This result suggests that interaction with the negatively charged cell walls is favoured by positive or neutral charge of the protein.

**AcmB is an N-acetylmuramidase**

The purified recombinant N-terminal-truncated AcmB (AcmB[C+Z]-His) was found by renaturing SDS-PAGE to hydrolyse \textit{B. subtilis} vegetative cell walls (data not shown). Therefore, the hydrolytic bond specificity of AcmB was investigated on \textit{B. subtilis} vegetative peptidoglycan, whose fine structure has been previously studied in detail (Atrih et al., 1999).

Peptidoglycan extracted from \textit{B. subtilis} vegetative cells was incubated with purified recombinant AcmB[C+Z]-His overnight at 37 °C in phosphate/citrate buffer at pH 4-0. A slight reduction of OD\textsubscript{550} was observed (less than 5%). The soluble fraction was recovered and analysed by RP-HPLC (Fig. 5A). Peaks 1 and 2 were products of AcmB digestion since they were absent from the control consisting of cell walls incubated without enzyme (data not shown). When analysed by MALDI-TOF mass spectrometry, they gave molecular ions with \textit{m/z} values of 892-36 and 1814-70 respectively. According to the data of Atrih et al. (1999), these \textit{m/z} values correspond to peak 1 containing the disaccharide tripeptide muropeptide, and peak 2 containing...
Properties of an *acmB* mutant

To investigate the role of AcmB, a single *acmB* mutant and a double *acmA acmB* mutant were constructed by gene replacement with the pGp*+* host-9 thermosensitive vector in the MG1363 strain. The chromosomal copy of *acmB* was replaced by a mutant copy obtained by deletion of a 414 bp fragment inside the region encoding the catalytic domain of AcmB, followed by insertion of a chloramphenicol-resistance cassette.

The *acmB* mutant was not impaired in cellular growth nor in cell separation compared to the wild-type strain. No change in the cellular morphology of the mutants was observed by phase-contrast microscopy. The double *acmA acmB* mutant was not affected in its growth rate and formed long chains of cells compared to MG1363, as previously described for the *acmA* single mutant (Buist et al., 1995).

Autolysis of the *acmB* mutant was measured in 50 mM potassium phosphate buffer solution at pH 7-0, and compared to that of the wild-type MG1363. As shown in Fig. 7, the *acmB* mutant autolysed at a slightly but significantly lower rate than the wild-type strain. Also the final extent of autolysis was reduced in the mutant strain (61%) compared with the wild-type strain (78%). The OD<sub>650</sub> decrease was accompanied by a concomitant release of intracellular peptidase PepX into the culture supernatant (Fig. 7), which confirms that the OD<sub>650</sub> decrease reflected cellular autolysis and release of intracellular content. The *acmB* mutant released a lower amount of PepX activity than the wild-type MG1363, confirming the differences revealed by optical density measurement.

The membrane integrity of the cells incubated in buffer solution was investigated by labelling with PI. PI is a fluorescent marker for double-stranded DNA, which cannot cross an intact cell membrane and which thus selectively labels cells with a damaged membrane. For both

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**Table 2.** Calculated and observed *m/z* values for protonated, sodiated or deprotonated molecular ions of muropeptides obtained after hydrolysis of *B. subtilis* peptidoglycan by AcmB (A) or by AcmB followed by Cellosyl (B) and purification by RP-HPLC

<table>
<thead>
<tr>
<th>Peak</th>
<th>Ion</th>
<th><em>m/z</em></th>
<th>Δ<em>m</em> (Da)&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Muropeptide identification&lt;sup&gt;†&lt;/sup&gt;</th>
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<tr>
<td></td>
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<tr>
<td>Peak 1 (A)</td>
<td>(M+Na)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>892-12</td>
<td>893-36</td>
<td>-1-24</td>
</tr>
<tr>
<td>Peak 2 (A)</td>
<td>(M+Na)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1814-70</td>
<td>1816-76</td>
<td>-2-06</td>
</tr>
<tr>
<td>Peak 3 (B)</td>
<td>(M+Na)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>689-11</td>
<td>893-76</td>
<td>-204-65</td>
</tr>
<tr>
<td>Peak 4 (B)</td>
<td>(M+H)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1386-66</td>
<td>1794-80</td>
<td>-408-14</td>
</tr>
<tr>
<td>Peak 5 (B)</td>
<td>(M+Na)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1408-65</td>
<td>1816-76</td>
<td>-408-11</td>
</tr>
<tr>
<td></td>
<td>(M–H)&lt;sup&gt;−&lt;/sup&gt;</td>
<td>1611-96</td>
<td>1816-76</td>
<td>-204-80</td>
</tr>
<tr>
<td></td>
<td>(M–H)&lt;sup&gt;−&lt;/sup&gt;</td>
<td>1588-04</td>
<td>1792-76</td>
<td>-204-76</td>
</tr>
</tbody>
</table>

<sup>+</sup>Δ*m*, difference between the calculated *m/z* and the observed *m/z*.  
<sup>†</sup>ds, disaccharide (MurNAc-GlcNAc); di, dipeptide; tri, tripeptide; tetra, tetrapeptide.
the wild-type and acmB mutant strains, the percentage of labelled cells at the beginning of incubation in the buffer solution at pH 7.0 was close to zero. After 6 h incubation, this percentage reached 36% and 30% for wild-type MG1363 and the acmB mutant, respectively. After 24 h incubation, 100% of the MG1363 cells were labelled, whereas only 78% of the acmB mutant cells were labelled. Similar results were obtained in three independent experiments (data not shown). As a conclusion, acmB inactivation leads to a slower rate of membrane integrity loss under starvation conditions in buffer solution.

For the double MG1363 acmA acmB mutant, no difference in autolytic properties in buffer solution was observed compared with the single MG1363 acmA mutant. Both of them exhibited a lower autolysis rate and lower autolysis extent compared with the wild-type strain MG1363 (Fig. 7). Thus, AcmB does not appear to be responsible for the residual autolytic activity detected in the MG1363 acmA mutant. The fact that acmB inactivation leads to a reduction of the autolysis rate and extent in wild-type MG1363, but not in the acmA mutant, suggests that AcmB could act only in concert with AcmA and that AcmA could potentiate the hydrolytic action of AcmB.

**DISCUSSION**

In this work, we have characterized at the molecular level a second peptidoglycan hydrolase of *L. lactis* named AcmB. The acmB gene was identified on the basis of sequence similarity in the complete genome sequence of *L. lactis* IL1403. Our results show that acmB is expressed during growth in *L. lactis* MG1363 and that it specifies an active autolysin with N-acetylmuramidase specificity. Like most of the previously described bacterial peptidoglycan...

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**Fig. 6.** Structure of the muropeptides from *B. subtilis* peptidoglycan obtained after AcmB digestion (1 and 2) or AcmB and Cellosyl digestion (3, 4 and 5). Numbers refer to the peaks on the chromatograms presented in Fig. 5.

**Fig. 7.** Autolysis of *L. lactis* MG1363 and its derivative mutants in 50 mM potassium phosphate, pH 7.0, at 30°C. Cells were grown in M17 medium up to an OD<sub>650</sub> of 1.0, washed and resuspended in the buffer at an OD<sub>650</sub> of 0.8. Lysis of wild-type MG1363 (●), acmB mutant (▲), acmA mutant (○) and acmA acmB mutant (△) was monitored by measuring the OD<sub>650</sub> decrease of the cell suspension (continuous lines) and the PepX activity released into the buffer (dashed lines). PepX activity was measured with Ala-Pro-p-nitroanilide as substrate. Similar results were obtained in three independent experiments.
hydrolases, AcmB protein has a modular structure. It has an uncommon structure with three domains.

AcmB exhibits sequence similarity with the Ent. hirae muramidase-2 catalytic domain (Joris et al., 1992). However, like the major L. lactis autolysin AcmA (Steen et al., 2001) and B. subtilis peptidoglycan hydrolase LytG (Horsburgh, 2001), which are also homologous to the Ent. hirae muramidase-2, AcmB exhibits N-acetylmuramidase hydrolytic specificity. Thus, it appears that the enterococcal muramidase family defined by Smith et al. (2000) on the basis of sequence similarity contains glycosidases with the two types of specificities: muramidase or glucosaminidase. Hitherto, one muramidase, Mur-2 of Ent. hirae, and three glucosaminidases, LytG, AcmA and AcmB, had been identified, whereas the hydrolytic specificity of the other homologous enzymes was not determined experimentally.

B. subtilis LytG was proposed to have exoglucosaminidase specificity (Horsburgh, 2001). The fact that AcmB leads to only limited hydrolysis of B. subtilis peptidoglycan (less than 5% optical density decrease), and that in these conditions only small muropeptides were released, suggests that AcmB could also be an exoglucosaminidase.

AcmB is devoid of sequence repeats termed LysM domains (Bateman & Bycroft, 2000) previously described as cell-wall-targeting and peptidoglycan-binding sequences. In contrast, three such LysM repeats are found in L. lactis AcmA major autolysin. Also, no LPXTG motif, enabling covalent binding to peptidoglycan (Fischetti et al., 1990), could be found close to the C-terminus. However, AcmB contains a domain with a high content of Ser, Thr, Asn and Pro residues, which could be involved in cell wall interaction. Such a domain was previously described in Strep. salivarius fructosyltransferase, and was shown to be required in combination with a C-terminal transmembrane anchor, for cell wall attachment of the enzyme (Rathsam et al., 1993). By analogy, in AcmB, this domain plus the N-terminal putative transmembrane sequence could be involved in cell wall attachment of the protein. Like other Pro-, Ser-, Thr-rich domains (Fischetti et al., 1991), the AcmB N-terminal domain is likely to have an extended conformation, and could serve as a cell wall spacer allowing exposure of the catalytic domain of AcmB at the cellular surface. Another remarkable feature of this domain is the presence of several consensus sequences (Asn-X-Ser/Thr) for N-glycosylation and the high amount of Ser and Thr residues, which could be sites for O-glycosylation. Glycosylation of the protein could protect it from proteolytic degradation in the cell wall (Moens & Vanderleyden, 1997).

Recent studies provided evidence that actively metabolizing B. subtilis cells secrete protons, which bind to anionic sites in the cell wall (Calamita et al., 2001). It was proposed that acidification of the cell wall during growth may be one means for autolysin inhibition (Kemper et al., 1993). We can speculate that in contrast AcmB, which has a low pI (4.65) and is active at acidic pH, may be active during cellular growth.

In this study, we have shown that AcmB contributes to cellular autolysis but to a lesser extent than AcmA. In addition, AcmB action appears to be potentiated by that of AcmA. This feature is consistent with AcmB being an exoenzyme. It has been demonstrated previously that the amino acid repeats present in Staph. aureus Atl autolysin direct the enzyme to cell division sites (Baba & Schneewind, 1998). AcmB is devoid of such repeats and thus could have a uniform localization around the cell surface. In contrast to AcmA, which plays a role in cell separation, AcmB could be more likely involved in other cellular processes such as cell enlargement and insertion of new peptidoglycan units. The fact that inactivation of AcmB did not impair cellular growth does not exclude such a role for this protein. Indeed, functional redundancy was previously observed among B. subtilis autolysins (Smith et al., 2000). Thus, other L. lactis peptidoglycan hydrolases could compensate for its absence. Besides AcmA and AcmB, at least three other putative peptidoglycan hydrolases are present in the L. lactis IL1403 genome sequence.

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