RESEARCH LETTER

Identification and characterization of a strain-dependent cystathionine β/γ-lyase in *Lactobacillus casei* potentially involved in cysteine biosynthesis

Stefan Irmler[1], Heike Schäfer[1], Beata Beisert[2], Doris Rauhut[2] & Hélène Berthoud[1]

[1]Agroscope Liebefeld-Posieux Research Station ALP, Bern, Switzerland; and [2]Geisenheim Research Centre, Geisenheim, Germany

Correspondence: Stefan Irmler, Agroscope Liebefeld-Posieux Research Station ALP, Schwarzenburgstrasse 161, CH-3003 Bern, Switzerland. Tel.: +41 31 323 81 55; fax: +41 31 323 82 27; e-mail: stefan.irmler@alp.admin.ch

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Abstract

The *trans*-sulfuration pathways allow the interconversion of cysteine and methionine with the intermediary formation of cystathionine and homocysteine. The genome database of *Lactobacillus casei* ATCC 334 provides evidence that this species cannot synthesize cysteine from methionine via the *trans*-sulfuration pathway. However, several *L. casei* strains use methionine as the sole sulfur source, which implies that these strains can convert methionine to cysteine. Cystathionine synthases and lyases play a crucial role in the *trans*-sulfuration pathway. By applying proteomic techniques, we have identified a protein in cell-free extracts of *L. casei*, which showed high homology to a gene product encoded in the genome of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus* and *Lactobacillus helveticus* but not in the genome of *L. casei* ATCC 334. The presence of the gene was only found in strains able to grow on methionine as the sole sulfur source. Moreover, two gene variants were identified. Both gene variants were cloned and expressed heterologously in *Escherichia coli*. The recombinant enzymes exhibited cystathionine lyase activity *in vitro* and also cleaved cysteine, homocysteine and methionine releasing volatile sulfur compounds.

Introduction

The flavor of cheese is primarily determined by the degradation pathways of lactose, citric acid, amino acids and fat (McSweeney, 2004). Among the numerous flavor compounds, the volatile sulfur compounds (VSCs) methanethiol, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), hydrogen sulfide and methional have low-perception threshold values. They are found in many cheese types and are important flavor components (Walker, 1959; Grill *et al.*, 1966; Urbach, 1995; Curioni & Bosset, 2002). Methionine and cysteine are regarded as the source for the bacterial formation of methanethiol, methional and hydrogen sulfide. DMDS and DMTS can be produced by the oxidation of methanethiol (Chin & Lindsay, 1994). Because methionine is present in the caseins at a higher concentration than cysteine, VSCs in cheese presumably originate from methionine. Methionine also plays a role in a variety of other cellular functions. It is not only the universal initiator of protein synthesis but is also involved in the cycle of active methyl groups, polyamine biosynthesis and in the *trans*-sulfuration pathway. The addition of mutants with selectively enhanced enzymatic activities in amino acid metabolism in the form of adjunct cultures during the process of cheese making is a possible way to enhance flavor development and ripening. A greater understanding of the pathways and enzymes involved in sulfur metabolism will facilitate the identification of strains that contribute to the production of VSCs.

Regarding the conversion of methionine to VSCs in *Lactococcus lactis* two enzymatic pathways have been identified. A pathway is initiated by transamination of methionine to 4-methylthio-2-oxobutyric acid, which is then further degraded to methanethiol and methional (Gao *et al.*, 1998; Rijnen *et al.*, 2003). The other pathway involves the deamination and demethylthiolation of methionine by lyases such as cystathionine β-lyase (CBL, EC 4.4.1.8) and cystathionine γ-lyase (CGL, EC 4.4.1.1) (Alting *et al.*, 1995; Bruinenberg *et al.*, 1997; Fernandez *et al.*, 2000; Martinez-Cuesta *et al.*, 2006).

Lactococcus lactis does not survive the scalding temperatures of 50–55 °C used for the manufacture of several Swiss cheese varieties such as Gruyère or Emmental and therefore

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cannot be used as adjunct culture in hard-cooked cheeses. Instead *Lactobacillus casei*, which is found in high numbers in many hard cheeses and semi-hard cheeses (Beresford & Williams, 2004) survive the high cooking temperatures and therefore could be used as adjuncts.

Our interest in the methionine catabolic pathway in *L. casei* was stimulated when we found that several *L. casei* strains isolated from Gruyère cheese were capable of producing DMDS in the absence of an amino acceptor (Irmler *et al.*, 2006), indicating that the degradation does not necessitate the action of aminotransferases. These strains exhibited considerable cystathionine lyase activity and were able to use methionine as the sole sulfur source for growth (Irmler *et al.*, 2008). These findings indicated that cystathionine lyases may play a role in the production of VSCs and are involved in the conversion of methionine to cysteine. The genome sequence of *L. casei* ATCC 334 was used to clone two genes encoding cystathionine lyases from the strain FAM18168. However, the biochemical properties of both enzymes did not explain the observed cystathionine lyase activity of FAM18168, indicating that a third gene encoding a cystathionine lyase might be present.

In this work, we describe the identification a cystathionine lyase in several *L. casei* strains, which is not present in *L. casei* ATCC 334. Two variants of the cystathionine lyase-encoding gene are present, and we cloned both variants and investigated the enzymatic properties of the recombinant proteins.

Materials and methods

Strains and growth conditions

Lactobacillus casei strains from the Agroscope Liebefeld-Posieux culture collection were used in this study and cultured anaerobically in 10 mL of MRS broth (de Man *et al.*, 1960) at 37 °C.

Chemicals were purchased from Merck (Dr Grogg Chemie AG, Stettlen, Switzerland) and Sigma-Aldrich (Buchs, Switzerland).

Gel electrophoresis and LC-electrospray ionization (ESI)-MS/MS

To detect peptides of cystathionine lyases by mass spectroscopy (MS), 25 μg of the crude protein extracts obtained from *L. casei* FAM6161, FAM8407, FAM17407, FAM18099, FAM18108, FAM18124 and FAM18168 were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were visualized with colloidal Coomassie staining. The region with a molecular weight between 40 and 50 kDa was excised from the gel of all strains. The gel piece from each strain was then cut into four pieces of equal size and subjected to in gel tryptic digest (Schaefer *et al.*, 2003). Peptides were extracted with 80% acetonitrile and 0.1% formic acid.

The peptides were separated on a ZIC-HILIC column (1.0 mm ID × 15 cm length, 5 μm, 200 Å, SeQuant GmbH, Haltern, Germany) using the Rheos 2200 HPLC (Flux Instruments, Basel, Switzerland). The temperature of the column was maintained at 30 °C. The following solvent system was used for the analysis: solvent A, 0.1% formic acid in water and solvent B, 0.1% formic acid in acetonitrile. The flow was set to 80 μL min^{-1} and separation was performed with a linear gradient of 80% B to 20% B over 28 min.

The HPLC eluent was introduced into a Finnigan LTQ ion trap mass spectrometer (San Jose, CA) using an ESI interface. The ESI conditions were as follows: source voltage 4.0 kV, capillary voltage 20 V, tube lens 200 V, capillary temperature 250 °C and sheath gas flow 25 arbitrary units. The ion trap mass spectrometer was operated in positive ion mode scanning from *m/z* 300 to *m/z* 2000. A full MS spectrum was followed by four MS/MS spectra of the most intensive ions (dynamic exclusion time of 1 min).

Database analysis

All MS/MS spectra were used to search a lactic acid bacteria subdatabase of the NCBI protein sequence database (http://www.ncbi.nlm.nih.gov/, November 2006 release) with the BIOWORKS search program (Thermo Scientific, San Jose). For positive protein identification two unique peptides had to be allocated and more than half of the possible ions of the *b*- and *y*-series had to be assigned in the related MS/MS spectra for peptide identification. For additional protein characterization the MODIRO software (Protagen AG, Dortmund, Germany) was used.

Species-specific PCR and DNA sequencing

To test a contamination of the *L. casei* strains with *Lactobacillus delbrueckii*, *Streptococcus thermophilus* and *Lactobacillus helveticus*, genomic DNA was extracted using the EZ1 DNA tissue Kit (Qiagen) and PCR amplifications with species-specific primers were performed (Table 1). Genomic DNA preparations from *L. casei* ATCC 334, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, ALP starter culture MK 401 and *L. helveticus* ATCC 15009 served as positive controls.

To confirm the identity of the *L. casei* strains, an 803-bp portion of the *tuf* gene (encoding the elongation factor Tu) was amplified using the primer pair Keu1 and Keu2 (Table 1). The purified PCR products were sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) and analyzed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The nucleotide (excluding primers) and the translated amino acid sequences were used to perform BLAST searches against the GenBank database.

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Table 1. PCR primers used in this study

Primers	Sequences (5′–3′)	Primer target	Anneal sites	Amplicon size (bp)	References
L. casei-group specific*				733	Kwon *et al.* (2004)
IDL11F	TGGTCGGCAGAGTAACTGTTGTCG	16S rRNA gene	479–502		
IDL03R	CCACCTTCCTCCGGTTTGTCA	16S rRNA gene	1191–1211		
L. delbrueckii specific				185	Kwon *et al.* (2004)
IDL31F	TGGTCGGCAGAGTAACTGTTGTCG	16S rRNA gene	1015–1039		
IDL03R	CCACCTTCCTCCGGTTTGTCA	16S rRNA gene	1180–1200		
Streptococcus specific				197	Picard *et al.* (2004)
Str1	GTACAGTTGCTTCAGGACGTATC	*tuf*	683–705		
Str2	ACGTTCGATTTCATCACGTTG	*tuf*	859–879		
L. helveticus specific				858	Pillonel *et al.* (2005)
HE1	AGCAGATCGCATGATCAGCT	16S rRNA gene	199–218		
HE2	CCGAAGGAACNCCTAATCTCTTA	16S rRNA gene	1033–1056		
tuf amplification				803	Chavagnat *et al.* (2002)
Keu1	AAYATGATIACIGGIGCIGCICARATGGA	*tuf*	274–302		
Keu2	AYRTTITCICCIGGCATIACCAT	*tuf*	1054–1076		
LBUL_1236/ctl amplification				1185	This study
1236LbdelbulgFNhe	GATATAAGGCTAGCATGGAATTTGA TACAAAATTAATTCATG	*LBUL_1236/ctl*	1–28		
1236LbdelbulgR2	TCACGCTTGAGAATGGACCTT	*LBUL_1236/ctl*	1165–1185		

*The *L. casei* group includes *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*.

Cloning, expression and purification of His-tagged proteins

The gene *ctl1 and ctl2* were amplified from genomic DNA of *L. casei* FAM18168 and FAM18108, respectively, by PCR with the primer set 1236LbdelbulgFNhe/1236LbdelbulgR2 (Table 1). Following amplification, the PCR products were cloned into the pET SUMO vector (Champion™ pET SUMO Protein Expression System, Invitrogen), juxtaposing the fragment downstream of the coding sequence for the His-tag and the SUMO protein. To remove the region encoding the SUMO fusion protein, the plasmid was purified with the QIAprep Spin Miniprep Kit (Qiagen) and then digested with NheI. The DNA digest was then separated by agarose gel electrophoresis and the DNA fragment of *c.* 6.4 kbp was purified from the gel and treated again with ligase. Thereby a region encoding 21 amino acids including the polyhistidine region was added in frame directly upstream of the PCR product. The DNA sequences and research for DNA similarity were determined as described above.

The plasmids containing *ctl1* and *ctl2* were expressed in *Escherichia coli* BL21 (DE3) and the recombinant proteins were purified by Ni^{2+} affinity chromatography as described previously (Irmler *et al.*, 2008). Bound proteins were eluted with 20 mM sodium phosphate (pH 7.4), 500 mM NaCl, 350 mM imidazole. Imidazole was immediately removed by applying the eluate to NAP columns (GE HealthCare, Uppsala, Sweden), which had been equilibrated with 20 mM sodium phosphate (pH 7.4) and 150 mM NaCl. The protein concentration was determined using the Bio-Rad Protein Assay reagent and bovine serum albumin as standard; the purity of the eluted protein fraction was verified by SDS-PAGE.

Enzyme assays

Enzyme activity toward various substrates was measured by determination of thiol formation with 5,5′-dithiobis (2-nitrobenzoic acid) as described by Uren (1987) and determination of keto acid production with 3-methyl-2-benzothiazolinone hydrazone as described by Esaki & Soda (1987), using either sodium pyruvate or α-ketobutyrate as a standard. The standard reaction mixture contained 50 mM sodium phosphate (pH 5.5 or 6.8), 5 μM pyridoxal-5′-phosphate and various concentrations of substrate. Reactions were performed at 37 °C. The Hanes–Woolf transformation ($S\,V^{-1}$ vs. S^{-1}), where V is the formation rate of either thiols or keto acids (nmol min^{-1} mg^{-1} protein) and S is the concentration (mM) of each substrate, was employed to calculate K_m and V_{max} values.

Keto acids were identified by HPLC. Therefore, samples were separated on a 300 × 7.8 mm ion exchange column HPX-87H Aminex (Bio-Rad) protected with a cation H^+ Microguard cartridge (Bio-Rad) and with 3.8 mM H_2SO_4 as mobile phase. The operating conditions were as follows: flow rate of 0.6 mL min^{-1}, column temperature of 65 °C and detection at 210 nm. Chromatographic identification of

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target keto acids was performed by comparing retention times to those of pure standards.

Sample preparation and analysis of VSCs were conducted as described previously (Rauhut *et al.*, 2005; Irmler *et al.*, 2008). Briefly, VSCs were analyzed on a gas chromatograph (6890, Agilent Technologies) equipped with a Headspace Sampler (HSS, Multi-Purpose-Sampler, MPS 2; Gerstel), a cooled injection system (CIS-4, Gerstel) and a pulsed-flame photometric detector (PFPD Model 5380, O.I. Analytical, TX). Enzymatic assays were performed at a 1-mL scale in sealed 10-mL vials (which were flushed with Argon) and stopped by heating to 95 °C for 5 min. After preheating the sample at 60 °C for 45 min, 1 mL of the headspace was injected into the cooled injection system. The liner of the injector was packed with deactivated glass wool (Siltek CIS4 & PTV Inlet Liner w/wool, Restek, Germany) and cooled to − 100 °C. Sample components were vaporized with the following temperature program: heated to 40 °C at a rate of 12 °C s^{-1}, held for 1 min, then heated to 180 °C with a final hold time of 8 min. The analytes were transferred to the column in the split mode (10 : 1) and separated on a SPB-1 sulfur capillary column (30 m × 0.32 mm ID, 4-μm film thickness, Supelco) with helium as carrier gas. The oven temperature program was as follows: 29 °C held for 7 min, heated to 180 °C at a rate of 10 °C min^{-1} and held for 10.5 min. Chromatographic identification of target sulfur compounds was performed by comparing retention times to those of pure standards.

Nucleotide sequence accession number

The sequences of *ctl1* and *ctl2* were deposited in GenBank under the accession numbers EU340836 and EU340837.

Results

Identification of a *L. casei* cystathionine lyase by MS

Data analysis of the MS/MS spectra revealed that the *L. casei* strains FAM8407, FAM18108, FAM18124 and FAM18168 contained peptides that could unambiguously be assigned to the gene product of *LBUL_1236* (GenBank YP_813202) from *L. delbrueckii* ssp. *bulgaricus* ATCC BAA-365 (Table 2), which is annotated as a protein with possible CBL/CGL activity. None of the peptides were found in the protein samples obtained from the strains FAM6161, FAM17407 and FAM18099.

Additionally, we found that the peptides could be assigned to a putative CBL of *S. thermophilus* LMG19311, CNRZ1066 and LMD-9 (GenBank YP_139336, YP_141252 and YP_820317) and CBL of *L. helveticus* CNRZ32 and DPC 4571 (GenBank ABH11638 and ABX27743).

By searching the MS/MS datasets for unknown mass shifts with the MODIRO software and the amino acid sequence of LBUL_1236 as target sequence, the peptide VTDIAAVAK was identified in all four *L. casei* strains. Additionally, we found that one peptide from FAM8407 and FAM18108 and

Table 2. Peptides identified in several *Lactobacillus casei* strains by LC-ESI-MS/MS analyses

Peptides from LBUL_1236	*L. casei* FAM8407	*L. casei* FAM18108	*L. casei* FAM18124	*L. casei* FAM18168
MEFDTK			+	+
LIHGGISEDK	+	+	+	+
ATGAVSVPIYMASTFHQQK				+
IGENQYEYSR	+	+	+	+
FGMTFTVVDTR			+	+
DLAAVEEAITPNTK				+
AIYLE TPTNPLLR			+	+
VTDIVAVAK	+*	+*	+*	+*
SHQILSIIDNTFSSPYVQR				+
PLEQGVDIVLHSASK			+†	+
YLGGHSDVIAGLVVTK	+	+	+	+
IGYLQNAIGGILAPQESWLLQR	+		+	+
AHLANAEAVFNYLSNQPLVSK				+
IYYPGDPNNPDYEVAK	+	+	+	+
QMHGFGAMISFELQDGLDPK	+‡	+‡	+‡	+
QFVEQLHVITLAESLGALESLIEIPALMTHGSIPR				+
LSVGVEDQK	+	+	+	+
DLLADLER	+	+	+	+
GFNELKR				+

*Sequence found was VTDIAAVAK.

†Sequence found was PLDLGVDIVLHSASK.

‡Sequence found was QMHGFGAMISFELQAGLDPK.

Amino acid substitutions that were identified with the help of the MODIRO software are underlined.

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two peptides from FAM18124 carried amino acid substitutions in comparison with FAM18168 (Table 2).

Species-specific PCR and sequencing of the *tuf* gene

To test the *L. casei* strains for a contamination with *L. delbrueckii*, *S. thermophilus* or *L. helveticus*, we performed PCR amplifications with primer pairs that have previously been described to detect specifically the species mentioned above (Table 1). Only the primer pair IDL11F/IDL03R, which is specific for the *L. casei* group amplified a DNA fragment of *c.* 750 bp (expected 733 bp). No amplification products were observed with all other primer pairs (data not shown). As the primer IDL11F does not differentiate *L. casei* from *Lactobacillus rhamnosus* and *Lactobacillus paracasei*, we amplified a portion of the *tuf* gene. Sequence analysis revealed 99–100% identity to the *tuf* gene of *L. casei* ATCC 334 and *L. casei* BL23 (data not shown).

Distribution and variation of *ctl* in *L. casei* strains

The sequence of *LBUL_1236* was used to design the primer pair 1236LbdelbulgFNhe and 1236LbdelbulgR2 which amplifies the protein-coding region (Fig. 1a). First, we applied the primer pair in a PCR reaction to study whether the gene is present in different *L. casei* strains and found that a product of *c.* 1200 bp was amplified in some but not all *L. casei* strains (Fig. 1b). The amplified gene product was also incubated with the restriction enzyme RsaI, which would yield two fragments of *c.* 890 and 310 bp, if the gene was identical to that of *L. delbrueckii* ATCC BAA-365. We observed that not all *L. casei* strains carrying the gene yielded the expected DNA fragments, but instead revealed fragments of *c.* 600 and 520 bp (Fig. 1c).

The genes from FAM8407, FAM18108, FAM18124 and FAM18168 were sequenced. It was found that the nucleotide sequences of the first three strains were identical but showed only 80% similarity to the gene of FAM18168. Therefore, the gene variant from FAM18168 and from FAM18108 have been named *ctl1* and *ctl2*, respectively. An alignment of the deduced amino acid sequence of Ctl1 showed 91% identity to Ctl2. Additionally, a comparison of Ctl1 with proteins that have been shown to degrade cystathionine in other lactic acid bacteria revealed 58% identity to MetC of *L. lactis* (GenBank AF170901), 45% to MetB of *L. casei* (GenBank ABR68291) and 6% to MalY of *L. casei* (GenBank ABR68292), PatC of

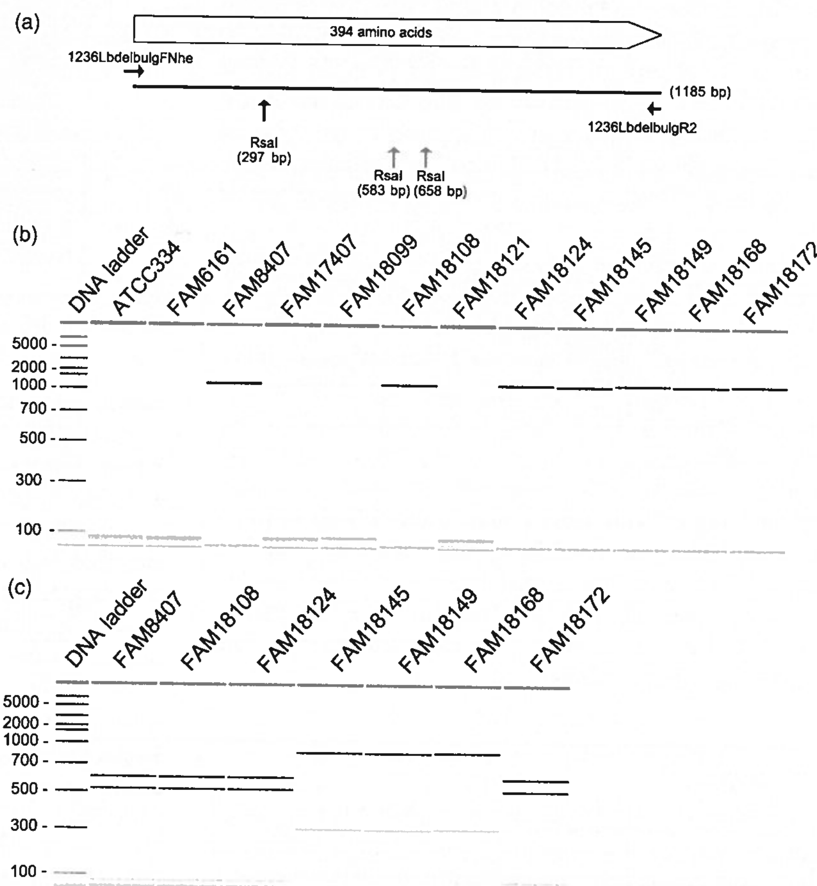

Fig. 1. (a) Schematic representation of the *ctl* gene, the primer set 1236LbdelbulgNhe/1236LbdelbulgR2 and the restriction sites of RsaI for *ctl1* (black arrow) and *ctl2* (gray arrows). (b) PCR products obtained with the primer set 1236LbdelbulgNhe/1236LbdelbulgR2 from several *Lactobacillus casei* strains. (c) Restriction products obtained after treatment with RsaI. DNA fragments were separated on the Agilent Technologies 2100 Bioanalyser and are illustrated in a gel-like view. Numbers on the left designate the size in base pairs.

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L. delbrueckii (GenBank AF423071) and YtjE of *L. lactis* (GenBank NP_268073).

Enzyme characterization of recombinant Ctl1 and Ctl2

To facilitate protein purification, the *ctl1* and *ctl2* genes were cloned in frame behind a six-histidine-coding region. Gene expression in *E. coli* produced soluble recombinant proteins, which were purified under native conditions by affinity chromatography. Denaturating SDS-PAGE showed that the recombinant Ctl1 and Ctl2 had a size of *c.* 43 kDa (predicted 45.2 kDa) and 41 kDa (predicted 45.1 kDa), respectively, and that the purified proteins had a high degree of purity (Fig. 2).

The recombinant Ctl1 and Ctl2 showed high activity toward the degradation of L-cystathionine at pH 5.5. At pH 6.8 only Ctl1 still exhibited high cystathionine lyase activity whereas cystathionine lyase activity of Ctl2 was strongly reduced (Table 3). No cystathionine lyase activity was detected at pH 9.0. In the presence of 4% NaCl the reaction rate of both enzymes was decreased to *c.* 40% of the original rate at pH 5.5. On the contrary, NaCl considerably enhanced the reaction rate at pH 6.8. Both enzymes did not lose activity after incubation at 42 °C for 30 min, but activity was lost at 57 °C.

Furthermore, we found that both enzymes degraded L-cysteine, DL-homocysteine and L-methionine at pH 5.5. No activity toward the three substrates was detected at pH 6.8 or 9.0. By comparing Ctl1 with Ctl2, it was found that the former showed higher V_{max} values toward cysteine and homocysteine (Table 3). No significant differences were found in the kinetic parameters concerning the release of thiols from methionine.

HPLC analysis showed that Ctl1 and Ctl2 released pyruvate and α-ketobutyrate from cystathionine (Fig. 3a). It was also observed that the reaction vials emitted a rotten egg odor and GC-PFPD confirmed that hydrogen sulfide was produced (Fig. 3b). Additionally, the enzymes also formed hydrogen sulfide from homocysteine and cysteine and produced α-ketobutyrate and pyruvate, respectively (data not shown). Using methionine as substrate, methanethiol, DMDS and DMTS were detected by GC-PFPD (Fig. 3c). The signal intensities of these VSCs were > 25-fold higher than those from methionine incubated without enzyme preparations (Fig. 3d). Furthermore, HPLC analysis revealed that the enzymes formed α-ketobutyrate from methionine (data not shown).

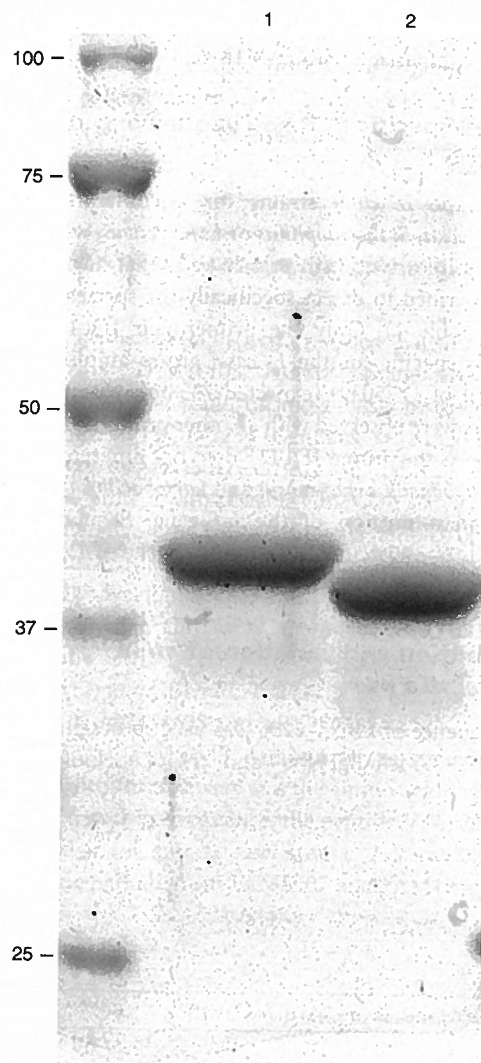

Fig. 2. SDS-PAGE analysis of the purified recombinant Ctl1 (lane 1) and Ctl2 (lane 2). The molecular mass markers are indicated in kDa.

Discussion

In this study proteomic techniques enabled us to discover peptides of LBUL_1236 in several *L. casei* strains. Additionally, we identified by in-depth analysis of the LC-MS/MS spectra peptides that carried amino acid substitutions compared with LBUL_1236. Homologs of the LBUL_1236-encoding gene can be found by BLAST searches in the genome of *S. thermophilus* and *L. helveticus* but not of *L. casei* ATCC 334 or BL23. The study strains have previously been identified as *L. casei* by 16S rRNA gene sequencing (Irmler *et al.*, 2006). Despite its importance in the food industry, the taxonomic status of *L. casei* is still a matter of debate because studies on the molecular level show that the majority of *L. casei* strains are more related to *L. casei* ATCC 334 than to the official type strain ATCC 393 (Dellaglio *et al.*, 2002). To clarify the identity of the study strains, we used partial *tuf* gene sequences as a phylogenetic marker (Chavagnat *et al.*, 2002), which showed highest identity to *L. casei* ATCC 334 and *L. casei* BL23. Thus, the study strains are designated as *L. casei* in this work.

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Table 3. Kinetic parameters of the recombinant Ctl1 and Ctl2. Data were determined at 37 °C and are means ± SD from three experiments

	Ctl1		Ctl2	
Substrate	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})
L-Cystathionine*	0.37 ± 0.06	829 ± 145	0.41 ± 0.07†	22 ± 3†
L-Cystathionine‡	0.35 ± 0.02	1572 ± 179	0.33 ± 0.01	1305 ± 115
L-Methionine‡	32.8 ± 2.1	3.4 ± 0.3	34.2 ± 5.7	4.2 ± 0.7
L-Cysteine‡	1.0 ± 0.2	184 ± 8	0.3 ± 0.2	39 ± 2
DL-Homocysteine‡	1.4 ± 0.6	85 ± 28	1.0 ± 0.7	40 ± 11

*Enzymatic assay performed with sodium phosphate buffer (pH 6.8).
†Enzyme activity was detectable after 60 min of incubation.
‡Enzymatic assay performed with sodium phosphate buffer (pH 5.5).

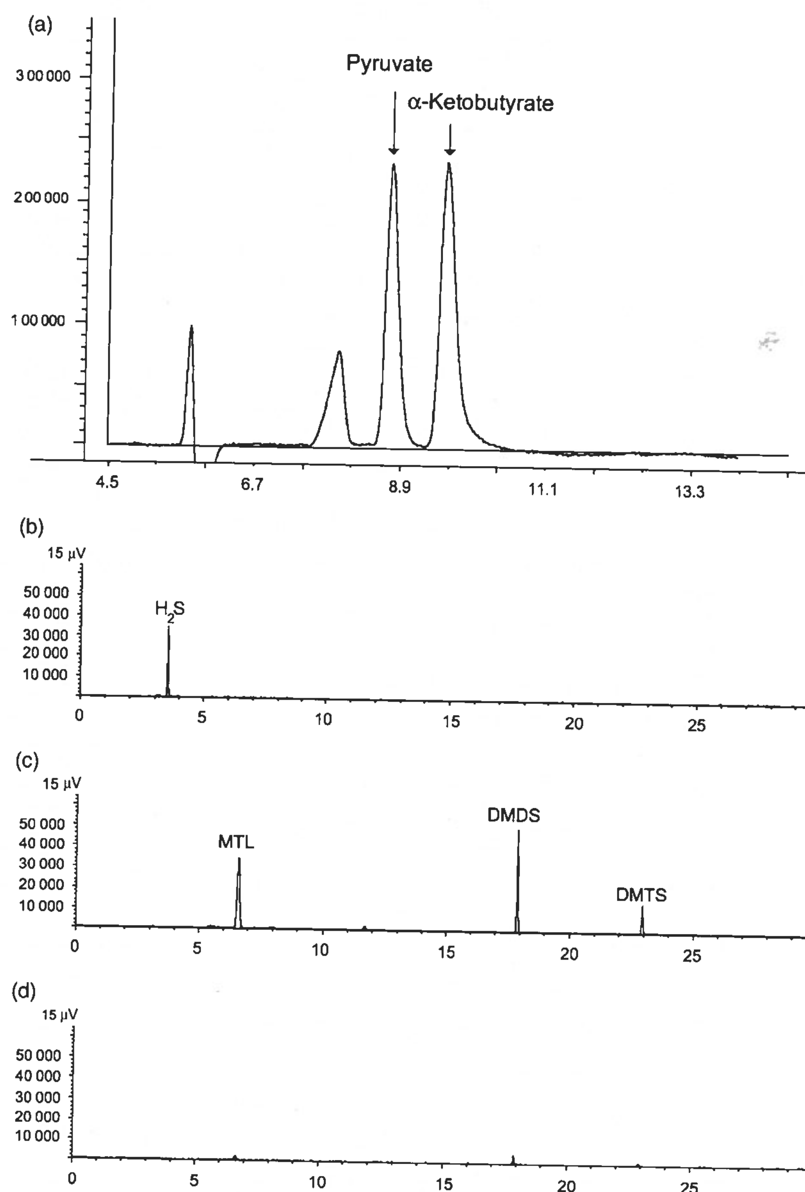

Fig. 3. HPLC (a) and GC-PFPD (b, c) analysis of the products formed by incubating the recombinant Ctl2 at 37 °C with 2 mM L-cystathionine (a and b) for 1 h and 10 mM L-methionine (c) for 4 h. Similar results were obtained with Ctl1 (data not shown). (d) Illustrates the GC-PFPD chromatogram obtained from L-methionine incubated without enzyme.

The occurrence of a protein that is nearly similar to proteins found in other species, could also suggest that the *L. casei* isolates presented in this study were not pure. Because the study strains except ATCC 334 were isolated on facultatively heterofermentative agar plates, which is selective for facultatively heterofermentative lactobacilli (Isolini

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et al., 1990), it was unlikely that the strains were contaminated with *L. delbrueckii*, *S. thermophilus* or *L. helveticus*. For an additional confirmation we applied sensitive species-specific PCR methods but did not detect a contamination and therefore concluded that a *LBUL_1236* homolog is present in several *L. casei* strains.

Primers were designed on the basis of the *LBUL_1236* and an amplicon was only obtained from *L. casei* strains that were found to exhibit cystathionine lyase activity at pH 5.5 and were able to grow in media with methionine as the sole sulfur source (Irmler *et al.*, 2008). This observation implied that the PCR product contained a gene related to the *trans*-sulfuration pathway. DNA sequencing confirmed that the amplicon encoded a putative cystathionine lyase and that, depending on the strain, two gene variants, were present. It is very interesting that the gene has a high degree of homology in at least four species usually found in milk products. This implies that the gene may be beneficial to bacteria that are adapted to a dairy environment. The published genomes of *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus* and *L. helveticus* (Bolotin *et al.*, 2004; Makarova *et al.*, 2006; Callanan *et al.*, 2008) show that upstream of the *ctl1/2* homolog is an ORF that codes for a cysteine synthase. Whereas Bolotin *et al.* (2004) postulated that this region plays a role in methionine biosynthesis we think it is rather involved in the synthesis of cysteine, a rare amino acid in cheese. This hypothesis is supported by sequence alignments, which suggest that these cysteine synthases may exhibit cystathionine β-synthase activity (Liu *et al.*, 2008). Currently, we are investigating if a cysteine synthase-encoding gene is also present upstream of *ctl1* or *ctl2*. Furthermore, we are about to investigate if the disruption of *ctl1* or *ctl2* leads to cysteine auxotroph strains or if strains are able to overcome cysteine auxotrophy after a transformation with *ctl1* or *ctl2*.

Up to now, only a few CBL/CGL enzymes have been characterized experimentally in lactic acid bacteria and analysis of the gene sequences is not sufficient to predict substrate and reaction specificity (Liu *et al.*, 2008). To our knowledge, the enzymatic activities of the Ctl homologs in *L. delbrueckii*, *S. thermophilus* and *L. helveticus* have not been described yet. Lee *et al.* (2007) demonstrated that a CBL overexpression variant of *L. helveticus* produced more VSCs, but the authors did not describe the enzymatic activities of the isolated protein. Our results show that the recombinant Ctl1 and Ctl2 degraded the four sulfur-containing substrates methionine, cystathionine, cysteine and homocysteine. Under the conditions used, the Ctl1 and Ctl2 exhibited higher affinity for cystathionine than for the other amino acids (Table 3) and, probably, they function primarily as cystathionine lyases within the cell.

The degradation of homocysteine and cysteine to keto acids and hydrogen sulfide already indicated that the enzyme catalyzes α,β- and α,γ-elimination reactions. The identification of pyruvate and α-ketobutyrate as degradation products of cystathionine confirmed further that the enzymes are cystathionine β,γ-lyases. This dual catalytic activity on the same substrate has also been reported for the MetC of *L. lactis* ssp. *cremoris* (Alting *et al.*, 1995; Dobric *et al.*, 2000). The release of hydrogen sulfide demonstrated that cystathionine is broken down in two steps. After the formation of pyruvate, α-ketobutyrate, cysteine, homocysteine and probably ammonia, the two sulfur-containing amino acids are further broken down to pyruvate, α-ketobutyrate and hydrogen sulfide.

Methanethiol, DMDS and DMTS, which are key flavors in several cheese types were detected in the headspace of the reaction vials with methionine. It has been reported that methanethiol can also be generated chemically from methionine in the presence of pyridoxal-5′-phosphate (Wolle *et al.*, 2006). We also observed the formation of methanethiol from methionine in the absence of enzyme, but the amounts were considerably lower showing that Ctl1 and Ctl2 catalyze the production of methanethiol. It has been shown that methanethiol can also be produced by the treatment of methionine or casein with hydrogen sulfide (Manning, 1979). Thus, the formation of both sulfur compounds in cheese are probably strongly related and it is worthwhile studying the production of both compounds together in bacteria and in cheese. Up to now, three enzymes releasing VCSs have been identified in *L. casei*. The presence of more than one cystathionine lyase gene and the possibility of chemical formation of methanethiol might explain why *cbl* or *metC* knock-out mutants are still capable of producing VSCs that were observed in *L. lactis* and *L. helveticus* (Fernandez *et al.*, 2000; Lee *et al.*, 2007).

The ability of Ctl1 and Ctl2 to release VSCs from several substrates at a pH usually found in cheese and in the presence of elevated salt concentrations indicate that the enzyme may have a significant role in the development in cheese flavor. In comparison, MetB (LSEI_0600) of *L. casei* favored a biosynthetic acitivity at neutral and acidic pH and was strongly inhibited by salt. MalY (LSEI_0894) only showed α,β-elimination reactions and did not degrade methionine (Irmler *et al.*, 2008). Thus, we think that *L. casei* strains especially overexpressing *ctl1* or *ctl2* have the potential to be used as flavor-intensifying adjunct cultures for cheese making.

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