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Characterization of the *cysK2-ctl1-cysE2* gene cluster involved in sulfur metabolism in *Lactobacillus casei*

Biljana Bogicevic [a,b,*], Stefan Irmler [a], Reto Portmann [a], Leo Meile [b], Hélène Berthoud [a]

[a] Agroscope Liebefeld Posieux Research Station ALP, Bern, Switzerland
[b] Laboratory of Food Biotechnology, Institute of Food Science and Nutrition, ETH Zürich, Switzerland

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ABSTRACT

The up- and downstream regions of *ctl1* and *ctl2* that encode a cystathionine lyase were analyzed in various *Lactobacillus casei* strains. *ctl1* and *ctl2* were found to be part of a gene cluster encoding two other open reading frames. One of the two open reading frames precedes *ctl1* and encodes a putative cysteine synthase. The other open reading frame lies downstream of *ctl1* and encodes a putative serine acetyltransferase. The gene cluster is not present in the publicly available genome sequences of *L. casei* ATCC 334, BL23 and Zhang. Apparently, the gene cluster was acquired by a horizontal gene transfer event and can also be found in other lactic acid bacteria such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. RT-PCR was used to analyze the expression of the gene cluster. Additionally, an mass spectrometry-based selected reaction monitoring method was developed for quantifying Ctl1 in a cell-free extract of lactic acid bacteria. The gene cluster *cysK2-ctl1-cysE2* was expressed as single transcript, and expression was down-regulated by cysteine. In addition, cystathionine lyase activity present in cell-free extracts disappeared when *L. casei* was grown in the presence of cysteine. Whereas the transcript and the gene product of *ctl1* protein were found in all studied *ctl1*$^+$ *L. casei* strains, only the transcript but not the protein or cystathionine lyase activity was detected in *L. helveticus* FAM2888, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and *S. thermophilus* FAM17014, which actually possess a homolog of the *cysK2-ctl1-cysE2* gene cluster.

1. Introduction

While cheese ripens, non-starter lactobacilli start to grow and often dominate the microflora at the end of the ripening process. A predominant species found in many cheese varieties is *Lactobacillus casei* (Beresford and Williams, 2004). During sulfur metabolism, most bacteria synthesize cysteine de novo by taking up inorganic sulfate, reducing it to sulfide and incorporating it into O-acetyl-L-serine (OAS) (Kredich, 1996). However, the *L. casei* ATCC 334 genome data show that this bacterium lacks homologs of the enzymes necessary for sulfur assimilation (Makarova et al., 2006). Instead, several amino acid uptake systems are present, indicating that the sulfur-containing amino acids are taken up from the environment.

Proteolysis is one of the main biochemical events that occurs when cheese ripens. Thereby, peptides and amino acids are released from the caseins. Since methionine is present in the caseins at a higher concentration than cysteine, methionine can be assumed to be the main sulfur source for *L. casei* in cheese.

In enteric bacteria such as *Escherichia coli*, cysteine biosynthesis involves two steps. First, L-serine-O-acetyltransferase (SAT; EC 2.3.1.30), which is encoded by *cysE*, catalyzes the formation of O-acetyl-L-serine (OAS) from serine and acetyl-CoA. Then O-acetyl-L-serine sulfhydrylase (OASS, EC 2.5.1.47), also often called cysteine synthase, forms cysteine from OAS and sulfide. With regard to the cysteine synthase, two isoenzymes, called OASS-A and OASS-B, which are encoded by *cysK* and *cysM*, respectively, are found. Both isoenzymes show approximately 40% identity in the amino acid sequence and can be differentiated enzymatically since OASS-B, in contrast to OASS-A, can use thiosulfate instead of sulfide to give cysteine thiosulfonate, which is subsequently reduced to cysteine (Zhao et al., 2006). A homolog of *cysK* is present in the genome of *L. casei* ATCC 334 (Makarova et al., 2006).

An alternative cysteine biosynthesis pathway is the reverse transsulfuration pathway that converts homocysteine, derived from methionine degradation, via cystathionine to cysteine. The pathway involving the action of cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CGL; EC 4.4.1.1) has rarely been reported in bacteria. The pathway's existence has been demonstrated in Gram-negative *Klebsiella pneumonia* (Seilflein and Lawrence, 2006), Gram-positive *Bacillus subtilis* (Hullo et al., 2007) and *Mycobacterium tuberculosis* (Wheeler et al., 2005).

* Corresponding author at: Agroscope Liebefeld Posieux Research Station ALP, Schwarzenburgstrasse 161, Bern, Switzerland. Tel.: +41 31 323 81 55; fax: +41 31 323 82 27.
E-mail address: biljana.bogicevic@ilw.agrl.ethz.ch (B. Bogicevic).

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Table 1
Presence of the *cbs-cblB(cglB)-cysE* gene cluster in various lactic acid bacteria.

Bacterium	GenBank accession no: gene region	Original annotations of the genes	Reference
S. thermophilus			
LMG 18311	CP000023: 777824..780464	*cysM2-metB2-cysE2*	Bolotin et al. (2004)
LMD-9	CP000419: 817570..820210	*STER_0885-STER_0886-STER_0887*	Makarova et al. (2006)
CNRZ1066	CP000024: 784239..786762	*cysM2-metB2-cysE2*	Bolotin et al. (2004)
ND03	CP002340: 794899..797539	*STND_0833-STND_0834-STND_0835*	Sun et al. (2011)
L. delbrueckii subsp. Bulgaricus			
ATCC BAA-365	CP000412: 1147217..1150036	*LBUL_1235-LBUL_1236-LBUL_1237*[a]	Makarova et al. (2006)
ATCC 11842	CR954253: 1127887..1130302	*Ldb1325-Ldb1326-Ldb1327*[a]	van de Guchte et al. (2006)
ND02	CP002341: 1305636..1308276	*LDBND_1271-LDBND1272-ORF(1307695..1208276)*[b]	GenBank
L. helveticus			
DPC 4571	CP000517: 1873812..1876452	*lhv_1905-lhv_1904-lhv_1903*	Callanan et al. (2008)
CNRZ32	DQ826147: 1..2641	*cysK-cbl-cysE*	Smeianov et al. (2007)
DSM 20075	ACLM01000165: 653..3293	*cysK2-metC-cysE*	GenBank
L. rhamnosus			
Lc 705	FM179324: 34306..36946	*cysK-cgl-cysE*	Kankainen et al. (2009)
LMS2-1	ACIZ01000037: 466..3078	*cysM- metB-1-HMPREF0539_0807*	GenBank
L. casei			
FAM18101	JF272516; EU340836; JF272515	*cysK2-ctl1-cysE2::ISLca2*	This study, Irmler et al. (2009)
FAM18108	JF495406; EU340837; JF495407	*cysK3-ctl2-cysE3*	This study, Irmler et al. (2009)
FAM18149	JF272516; EU340836; JF272514	*cysK2-ctl1-cysE2*	This study, Irmler et al. (2009)

[a] *LBUL_1237* and *Ldb1327* carry insertion sequences.
[b] Region 1307695…1208276 comprises an uncharacterized open reading frame encoding a putative serine acetyltransferase.

CGL is a key enzyme in reverse transsulfuration and catalyzes the breakdown of cystathionine to cysteine, α-ketobutyrate and ammonia. Recently, we found that several *L. casei* strains isolated from cheese and milk possess a gene that encodes a CGL (Irmler et al., 2009). We found two variants of the gene that had 81% nucleotide sequence identity and named them *ctl1* and *clt2*. Neither variant is present in the *L. casei* strains ATCC 334, BL23 and Zhang. *Ctl1* shows 99% nucleotide sequence identity to *metB2* of *Streptococcus thermophilus* CNRZ1066 (Bolotin et al., 2004). In *S. thermophilus*, CNRZ1066 m*etB2* is part of a gene cluster containing two more open reading frames named *cysM2* and *cysE2* that putatively encode an OASS and a serine acetyltransferase, respectively. The gene cluster *cysM2-metB2-cysE2* is found under different names in various lactic acid bacteria (Table 1) and shows striking sequence conservation. Based on comparative sequence analysis, Liu et al. (2008) suggested that the first open reading frame of this gene cluster (often annotated as *cysM* or *cysK*) could also encode a cystathionine β-synthase. Thus, the authors proposed annotating the cluster as *cbs-cblB(cglB)-cysE* (Liu et al., 2009). However, the functional role of the *cysM*/*cysK*/*cbs* and *cysE* gene products remains to be determined.

Table 2
Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
L. casei strains		
ATCC 334	Genome sequenced strain, *ctl1* and *ctl2* negative	American Type Culture Collection
FAM18101	*ctl1*$^+$	Agroscope Culture Collection
FAM18105	*ctl1*$^+$	Agroscope Culture Collection
FAM18108	*ctl2*$^+$	Agroscope Culture Collection
FAM18124	*ctl2*$^+$	Agroscope Culture Collection
FAM18125	*ctl1*$^+$	Agroscope Culture Collection
FAM18129	*ctl2*$^+$	Agroscope Culture Collection
FAM18133	*ctl1*$^+$	Agroscope Culture Collection
FAM18144	*ctl1*$^+$	Agroscope Culture Collection
FAM18145	*ctl1*$^+$	Agroscope Culture Collection
FAM18149	*ctl1*$^+$	Agroscope Culture Collection
FAM18168	*ctl1*$^+$	Agroscope Culture Collection
FAM18172	*ctl2*$^+$	Agroscope Culture Collection
FAM8407	*ctl2*$^+$	Agroscope Culture Collection
L. helveticus FAM2888	*ctl1* homolog	Agroscope Culture Collection
L. delbrueckii subsp. *bulgaricus* ATCC 11842	Genome sequenced strain, *ctl1* homolog (=*Ldb1326*)	American Type Culture Collection
S. thermophilus FAM17014	*ctl1* homolog	Agroscope Culture Collection
Plasmids		
pEX5-CT/TOPO	Amp^R	Invitrogen
pEX5-CT/*cysK2*	pEX5-CT/TOPO carrying *cysK2* from FAM18149, Amp^R	This study
pEX5-CT/*cysE2*	pEX5-CT/TOPO carrying *cysE2* from FAM18149, Amp^R	This study
pEX5-CT/cysK3	pEX5-CT/TOPO carrying *cysK3* from FAM18108, Amp^R	This study
pEX5-CT/cysE3	pEX5-CT/TOPO carrying *cysE3* from FAM18108, Amp^R	This study
pGEM-T Easy	Amp^R	Promega
pGEM-T/*cysE2::ISLca2*[b]	pGEM-T Easy carrying *cysE2* from FAM18101, Amp^R	This study
pET/*ctl1*	pET Sumo carrying *ctl1* from FAM18168, Kan^R	(Irmler et al., 2009)

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With regard to *ctl2*, the gene shows 99% nucleotide identity to *metB-1* from *Lactobacillus rhamnosus* LMS2-1, whose genome was recently published as a draft sequence. *MetB-1* seems also to be organized in a gene cluster (Table 1).

In the study reported here, we first studied the *ctl1* and *ctl2* flanking regions in various *L. casei* strains. Furthermore, we investigated the gene expression of the *cysK2-ctl1-cysE2* gene cluster. Finally, we studied the influence of cysteine and methionine on gene expression.

2. Materials and methods

2.1. Bacteria and culture conditions

The strains used in this study are listed in Table 2. The *L. casei* strains were propagated at 30 °C in MRS broth (de Man et al., 1960) or in a chemical defined medium (CDM) that was prepared as described by Christensen and Steele (2003). *L. helveticus* FAM2888 and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 were grown in MRS broth at 37 °C while *S. thermophilus* FAM17014 was grown in M17 medium (Terzaghi and Sandine, 1975) supplemented with 0.5% glucose at 37 °C. *E. coli* strains were grown in LB (Sambrook et al., 1989) supplemented, if necessary, with ampicillin (100 μg/mL).

2.2. Genomic DNA extraction and PCRs

Cells from a 1 mL overnight culture were collected by centrifugation. First, they were treated with 0.05 M NaOH for 15 min at room temperature and then with TES buffer (0.1 M Tris–HCl [pH 8.0], 10 mM EDTA, 25% sucrose) supplemented with lysozyme (1 mg/mL) for 1 h at 37 °C. Genomic DNA was finally extracted with robot extraction (BioRobot EZ1, EZ1 DNA Tissue kit; Qiagen).

Genomic DNA was screened for the presence of *cysK2*, *cysK3*, *cysE2* and *cysE3* with a 35-cycle PCR using the primers listed in Table 3. PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems, Rotkreuz, Switzerland) and the following cycle conditions: 30 s at 48 °C (cysM_Lrham/cysM_Lrham_R) at 52 °C, (cysE2_F/cysE2_R) or at 55 °C (cysE_Lrham/cysE_Lrham_R) and 30 s at 72 °C. PCR products obtained with cysM_Lrham/cysM_Lrham_R were restricted with *Rsa*I. PCR products and restricted DNA were separated by electrophoresis in 1.5% agarose gels and visualized with GelRed staining.

2.3. Construction of plasmids and sequencing

DNA fragments containing *cysK2 and cysE2,* were amplified from genomic DNA of *L. casei* FAM18149, while *cysK3* and *cysE3* were amplified from genomic DNA of *L. casei* FAM18108 (Table 3). PCR products were cloned into the pEX5-CT/TOPO (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The plasmids were named pEX5-CT/*cysE2*, pEX5-CT/*cysK2*, pEX5-CT/*cysE3* and pEX5-CT/*cysK3* and were maintained in *E. coli* BL21 (DE3).

The PCR product obtained from genomic DNA of *L. casei* FAM18101 with the *cysE2* specific primer pair was cloned into pGEM-T Easy (Promega, Madison, USA) according to the manufacturer's conditions. The plasmid was named pGEM-T/*cysE2::ISLca2* and maintained in *E. coli* DH5α.

Inserts in the plasmids were sequenced using the BigDye Terminator Cycle sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

2.4. RNA isolation and preparation of cell-free extracts

Bacteria were grown in either 50 mL medium MRS or CDM containing 0.08 mg/mL of L-methionine and various concentrations of L-cysteine (none, 800 ng/mL and 8 μg/mL) for 16 h at 30 °C. An aliquot of 2 mL was withdrawn from the cell culture and immediately used for RNA isolation. The remaining 48 mL of cell culture was used to prepare cell-free extract.

To isolate the RNA, cells were harvested by centrifugation (17950×*g*, 10 min, 4 °C). Total RNA was isolated with the Ribopure™-Bacteria kit (Ambion, Applied Biosystems, USA) followed by DNase I treatment according to the manufacturer's instructions. RNA concentration, RNA ratio (23S:16S), and RNA integrity number were determined with the Agilent 2100 BioAnalyser (Agilent Technologies, Palo Alto, CA) using RNA 6000 NANO chips according to the manufacturer's instructions.

To prepare cell-free extract, cells were harvested by centrifugation (3000×*g*, 10 min, 20 °C) and washed twice with 20 mM sodium phosphate (pH 7.4). The cells were then suspended in 500 μL of 20 mM sodium phosphate (pH 7.4), and disrupted with glass beads (212–300 μm) by violent agitation in a Mini-Beadbeater-8™ (Biospec Products, Inc.). Cell debris was removed by centrifugation. Soluble protein concentration in the supernatant was determined with bicinchoninic acid (Smith et al., 1985) and bovine serum albumin as the standard.

2.5. Tagged RT-PCR

The RT-PCR method described by Aguena and Spira (2003) was used. Total RNA (500 ng) was first incubated with either 2 pmol of tag-cysE2 primer or 2 pmol of tag-ctl1 primer and 10 pmol of dNTPs at 65 °C for 5 min and then chilled on ice. For the RT reaction, a mixture

Table 3
Oligonucleotides used for amplification, real-time PCR, and cDNA synthesis.

Primer	Sequence (5′ to 3′) [a]	Reference
Amplification of cysK2 and cysK3; cloning of cysK3		
cysM_Lrham	ATGCTTATTCAATCCGTTTC	This study
cysM_Lrham_R	GTTGTAAATGTTTTCACTCATG	
cloning of cysK2		
cysK_Ldel	ATGCTTATTCATTCCATTTCAG	This study
cysK_Ldel_R	ATTATAAATATTTTCACTCATGTAAC	
Amplification and cloning of cysE2		
cysE2_F	ATGAGCTTAAGAGGAGCAAGA	This study
cysE2_R	ATTTTTAGTGGTTGACGTGTTT	
Amplification and cloning of cysE3		
cysE_Lrham	ATGTTTCAAACAGCAGGGTCCAT	This study
cysE_Lrham_R	AATCGGATGTGGGGCAATC	
Analysis of genetic organization		
cysK_Ldel	ATGCTTATTCATTCCATTTCAG	This study
1236LbdelbulgR2	TCACGCTTGAGAATGGACCTT	(Irmler et al., 2009)
Tagged RT-primer		
tag-ctl1	agtggtacacgcagagtacttCCGGCGGAGAACATT	This study
tag-cysE2	agtggtacacgcagagtacttTGACTTTAGCGGGTAACTTG	
Transcript analysis		
cysK2_584	CGACTCAAACCATCGTTGT	This study
tag (anchored reverse primer)	agtggtacacgcagagtactt	(Aguena and Spira, 2003)
Real time-PCR for ctl1		
Q-ctl1_F	GCACTGGAAAGCTTGATCGAA	This study
Q-ctl1_R	ACCGAATGTCACGTGGAATTG	
Q-ctl1_FAM	CGGCCTTGATGACCCACGGC	
Real time-PCR for cysE2		
Q-cysE2_F	ATTGCCGGGTTCCTAAGTCA	This study
Q-cysE2_R	GCTTCCGGCGAAAATTGAA	
Q-cysE2_FAM	CATGCCGCCAAAGTAACCGGGA	

[a] Lowercase letters show tag-sequence.

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of 1x first-strand buffer (50 mM Tris–HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$], 10 mM dithiothreitol and 200 U of M-MLV reverse transcriptase (Invitrogen) was added to the final volume of 20 μL. The samples were incubated first at 25 °C for 10 min, then at 37 °C for 50 min and finally at 70 °C for 15 min.

One-twentieth of the final cDNA product (1 μL) was used for the PCR reactions. First, PCR with the primer pair Q_ctl1_F/tag was carried out with the cDNA obtained with tag-ctl1 primer. Then, PCR with the primer pair cysK2_584/tag was performed with the cDNA obtained with the tag-cysE2 primer. Both PCR amplifications were performed for 35 cycles, with each cycle consisting of denaturation for 20 s at 95 °C, annealing for 30 s at 52 °C and elongation for 60 s at 72 °C. Water and reactions performed without reverse transcriptase served as negative controls. The PCR products were analyzed on the Agilent 2100 BioAnalyser (Agilent Technologies) using DNA 7500 chips according to the manufacturer's instructions.

2.6. Real-time RT-PCR

Total RNA (500 ng) was first incubated with 50 ng of random hexamers (Invitrogen) and 10 pmol of dNTPs at 65 °C for 5 min and then chilled on ice. For the RT reaction, a mixture of 1x first-strand buffer (50 mM Tris–HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$], 10 mM dithiothreitol and 200 U of M-MLV reverse transcriptase (Invitrogen) was added to the final volume of 20 μL. The samples were incubated first at 25 °C for 10 min, then at 37 °C for 50 min and finally at 70 °C for 15 min.

Real-time PCR was carried out using the Rotor-Gene RG 3000-A machine (Corbett Research, Sydney, Australia). To quantify the *ctl1* transcript, the PCR reaction mixture (12 μL) contained 1x TaqMan Universal PCR Master Mix (Invitrogen), 50 nM of Q-ctl1_F (forward primer), 50 nM of Q-ctl1_R (reverse primer), 150 nM of Q-ctl1_FAM (TaqMan probe) and 1 μL of cDNA. To quantify the *cysE2* transcript, the PCR reaction mixture (12 μL) contained 1x TaqMan Universal PCR Master Mix (Invitrogen), 50 nM of Q-cysE2_F (forward primer), 300 nM of Q-cysE2_R (reverse primer), 200 nM of Q-cysE2_FAM (TaqMan probe) and 1 μL of cDNA. The sequences of the primers and probes are described in Table 3. Water and total RNA were used as negative controls. The cycling conditions were as follows: 10 min at 95 °C followed by 40 cycles consisting of 15 s at 95 °C and 60 s at 60 °C. All PCR reactions were done in triplicate.

To quantify the transcripts, a standard curve with serial dilutions of the plasmids pET/*ctl1* and pEX5-CT/*cysE2* was used. Data were normalized to RNA concentration.

2.7. Cystathionine lyase assay

Cystathionine lyase (CL) activity was assayed in cell-free extracts by measuring the formation of free thiol groups with spontaneous disulfide interchange with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) similar to the method described by Uren (1987). The reaction buffer (200 μL) contained 50 mM sodium phosphate (pH 5.5), 200 μM DTNB, 20 μM pyridoxal-5′-phosphate, 2 mM cystathionine and cell-free extract. The reaction was initiated by adding the cell-free extract and was carried out at 37 °C. The optical density at 412 nm was recorded at 5 min intervals for 90 min.

2.8. Quantification of Ctl1 in cell-free extracts with LC-SRM

The concentration of Ctl1 was determined with selected-reaction monitoring (SRM) based on heavy-isotope labeled peptide Ctl1_(V149-K157)* that served as the internal standard. This peptide contained a stable-isotope labeled C-terminal lysine (K-^{13}C6; ^{15}N2) and was obtained from Thermo Fischer Scientific (Ulm, Germany).

The total protein (20 μg) of the cell-free extracts was digested overnight at 37 °C in 50 μL of 10 mM ammonium bicarbonate pH 8.0 containing trypsin (4 ng/μL). One-fifth of the tryptic digest was coinjected with 3 pmol of the reference peptide Ctl1_(V149-K157)*.

Peptides were separated on a Rheos 2200 HPLC (Flux Instruments, Switzerland) equipped with an XTerra MS C18 column (3.5 μm, 1.0 mm i.d. × 150 mm, Waters). Chromatography was conducted at a flow rate of 80 μL min^{-1} with a gradient from 5% to 80% B in 30 min. Solvent A was water, and solvent B was acetonitrile; both contained 0.1% formic acid. The column temperature was maintained at 25 °C.

The HPLC eluent was introduced into a LTQ linear ion trap mass spectrometer (Thermo Scientific, Switzerland) 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. The SRM transitions (in m/z) 444.5 (±1) → 687.8 (±1) for the native and 448.5 (±1) → 695.7 (±1) for the reference peptide were monitored to quantify Ctl1. Additionally, the SRM transition 821.5 (±1) → 943.1 (±1) for the peptide TLDLGEAGDNVGVLLR and the SRM transition 836.9 (±1) → 944.1 (±1) for the peptide QLDEGIAGDNVGVLLR were monitored. The former peptide is part of elongation factor Tu (EF-Tu) from *L. casei* (GenBank accession number YP_806555), *L. helveticus* (ZP_05752017) and *L. delbrueckii* subsp. *bulgaricus* (YP_618840), and the latter originates from the EF-Tu of *S. thermophilus* (YP_140901). Each sample was analyzed three times, and two independent biological repetitions were performed.

The peak areas for the native and internal standard were determined with LCquan software (ThermoFisher Scientific). The ratio of the light/heavy transitions was multiplied by 3 pmol (amount of reference peptide) and divided by the amount of total protein for normalization.

2.9. Nucleotide sequence accession numbers

The nucleotide sequence of *cysK2*, *cysK3*, *cysE2*, *cysE2::ISLca2* and *cysE3* were deposited in the GenBank. The accession numbers are listed in Table 1.

3. Results

3.1. Detection of cysK2 and cysK3

Ctl1 and *ctl2* positive *L. casei* strains (Table 2), *L. helveticus* FAM2888 and *S. thermophilus* FAM17014 were screened for the presence of the *cysM/cysK* homolog of the *cbs-cblB(cglB)-cysE* gene cluster with PCR using the primer pair cysM_Lrham/cysM_Lrham_R. The primer sequences were deduced from *cysM* from *L. rhamnosus* LMS2-1 and should be able to anneal the 5′- and 3′-end of the *cysM/cysK* homologs listed in Table 1. Genomic DNA from *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and from *L. casei* ATCC 334 served as the positive and negative controls, respectively. From all strains except ATCC 334, an amplicon of approximately 900 bp was obtained (data not shown).

Restriction with *Rsa*I clarified whether the amplicon was identical in all strains. The restriction pattern of the strains possessing *ctl1* or the *ctl1* homolog revealed bands of approximately 85, 270 and 570 bp (Fig. 1). Strains possessing *ctl2* showed two bands of approximately 140 and 750 bp and a weak band of approximately 900 bp. Based on the different restriction pattern, the amplified genes were named *cysK2* and *cysK3*.

The genetic organization of *cysK2* and *ctl1* was analyzed with PCR with the primer pair cysK_Ldel/1236LbdelbulgR2 in FAM18149 and FAM18168. *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 was used as the positive control. From all strains, a PCR product of approximately 1900 bp was obtained showing that *cysK2* is located upstream of *ctl1* (data not shown).

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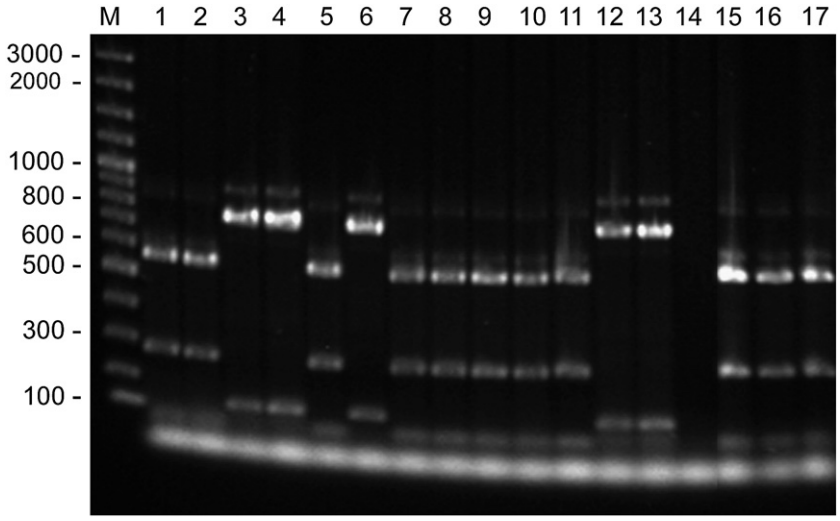

Fig. 1. Separation of the *Rsa*I restricted PCR products obtained from various lactic acid bacteria (Table 2) using the primer pair cysM_Lrham/cysM_Lrham_R with agarose gel electrophoresis. Lane M, DNA ladder, lane 1, FAM18101; lane 2, FAM18105; lane 3, FAM18108; lane 4, FAM18124; lane 5, FAM18125; lane 6, FAM18129; lane 7, FAM18133; lane 8, FAM18144; lane 9, FAM18145; lane 10, FAM18149; lane 11, FAM18168; lane 12, FAM18172; lane 13, FAM8407; lane 14, ATCC 334; lane 15, FAM2888; lane 16, ATCC 11842; lane 17, FAM17014. ATCC 334 was used as a negative control. The molecular weights of the DNA ladder are shown in bp on the left.

3.2. Detection of *cysE2*

Furthermore, the strains were analyzed for the presence of a *cysE2* homolog. The primer pair cysE2_F/cysE2_R was designed to anneal to the 5′- and 3′-end of the *cysE2* gene. From all *ctl1*$^{+}$ *L. casei* strains, *L. helveticus* FAM2888, *L. delbrueckii* ATCC 11842 and *S. thermophilus* FAM17014, a PCR product was amplified (Fig. 2A). No amplicons were found in ATCC 334 and the strains possessing *ctl2*.

The amplicon was approximately 580 bp except in FAM18101 and FAM18105, which yielded an amplicon of approximately 2500 bp. The PCR product from ATCC 11842 was approximately 800 bp, which is explained by the presence of a 179-bp insertion sequence (Liu et al.,

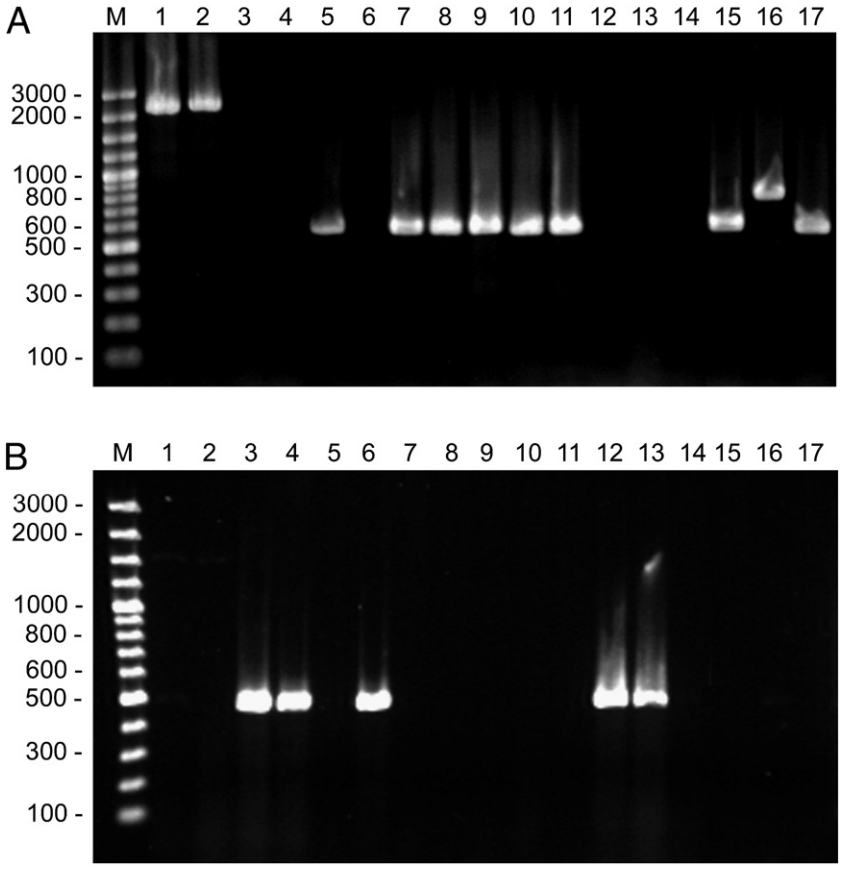

Fig. 2. Detection of *cysE2* and *cysE3* in various lactic acid bacteria. PCR products obtained by using primer pair cysE2_F/cysE2_R (A) and cysE_Lrham/cysE_Lrham_R (B) were separated with agarose gel electrophoresis. Lane M, DNA ladder, lane 1, FAM18101; lane 2, FAM18105; lane 3, FAM18108; lane 4, FAM18124; lane 5, FAM18125; lane 6, FAM18129; lane 7, FAM18133; lane 8, FAM18144; lane 9, FAM18145; lane 10, FAM18149; lane 11, FAM18168; lane 12, FAM18172; lane 13, FAM8407; lane 14, ATCC 334, lane 15, FAM2888; lane 16, ATCC 11842; lane 17, FAM17014. ATCC 334 was used as a negative control. The molecular weights of the DNA ladder are shown in bp on the left.

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2009). The amplified 580 bp gene in the *L. casei* strains was named *cysE2*.

3.3. Detection of cysE3

The primer pair used to detect *cysE2* cannot anneal to the *cysE* homolog *HMPREF0539_0807* from *L. rhamnosus* LMS2-1. Hence, the primer pair cysE_Lrham/cysE_Lrham_R was designed to anneal the 5′- and 3′-end of this gene. Again, a PCR reaction with the genomic DNA of all strains was performed. This time, an amplicon of approximately 500 bp was obtained from the *ctl2*$^+$ strains but not from the strains possessing either *ctl1* or a *ctl1* homolog and not from ATCC 334 (Fig. 2B). The amplified gene was named *cysE3*.

3.4. Sequence analysis of PCR products

The amplified genes *cysK2* and *cysE2* from FAM18149 and *cysK3* and *cysE3* from FAM18108 were cloned and sequenced. In addition, the nucleotide sequence of the 2500 bp amplicon from FAM18101 was determined. A multiple-nucleotide sequence alignment of the *cysK2* sequence revealed 99% of the nucleotide sequence identity to the first coding sequence of the *cbs-cblB(cglB)-cysE* gene cluster from *S. thermophilus*, *L. bulgaricus*, *L. helveticus* and *L. rhamnosus* Lc 705 and 76% identity to *cysM* from *L. rhamnosus* LMS2-1 and *cysK3* from *L. casei* FAM18108.

Similar results were observed for the *cysE2* gene. The nucleotide sequence of *cysE2* from *L. casei* showed 99% identity to *cysE* from *L. rhamnosus* Lc 705, 98% identity to the *lhv_1903* gene from *L. helveticus* DPC 4571 and *cysE* from *L. helveticus* CNRZ32, 97% to *cysE2* from *S. thermophilus* CNRZ1066 and *S. thermophilus* LMG 18311 and 70% identity to *HMPREF0539_0807* from *L. rhamnosus* LMS2-1 and *cysE3* from *L. casei* FAM18108.

Sequencing of *cysE2* from *L. casei* FAM18101 revealed at position 366 bp a 1569 bp insertion sequence. A search of the IS finder database (http://www-is.biotoul.fr/) showed that the insertion sequence was identical to *ISLca2* from *L. casei* BL23. In FAM18101, the insertion sequence is characterized by 6 bp direct repeats, by 7 bp inverted repeats (5′-GTGGCTG-3′) and by a coding sequence that shows high similarity to IS5 family transposases. The insertion probably leads to a non-functional, truncated CysE protein.

3.5. Transcript analysis

To evaluate if the gene cluster *cysK2-ctl1-cysE2* is a functional transcriptional unit, a tagged RT-PCR was performed. Therefore, RNA isolated from overnight cultures of *L. casei* FAM18101, FAM18149, and ATCC 334 was used in two different PCR reactions. First, a RT-PCR reaction was performed with the tag-ctl1 primer for cDNA synthesis and the cysK2_584/tag primer pair (Fig. 3A) for the amplification. From FAM18101 and FAM18149 but not from ATCC 334, an amplicon of approximately 630 bp was obtained (Fig. 3B). Second, the tag-cysE2 primer was used for cDNA synthesis, and the PCR reaction was performed with the Q-ctl1_F/tag primer pair (Table 2). In this case, a PCR product of approximately 700 bp was obtained only from FAM18149. Since the annealing site for tag-cysE2 is shifted more than 1500 bp downstream in FAM18101, no RT-PCR product was obtained. No PCR products were obtained when reverse transcriptase was omitted from the reactions showing that the RNA samples were free of DNA contamination.

3.6. Regulation of the gene expression of the cysK2-ctl1-cysE2

CL activity was measured in cell-free extracts of *L. casei* FAM18149 that were grown in chemically defined medium supplemented with various concentrations of L-cysteine. The activity dramatically decreased when more than 8 μg/mL of L-cysteine was present in the medium (Fig. 4A). To test if gene expression was down-regulated, transcripts of *ctl1* and *cysE2* were quantified with real-time PCR. The decrease in CL activity correlated with the decrease of *ctl1* and *cysE2*

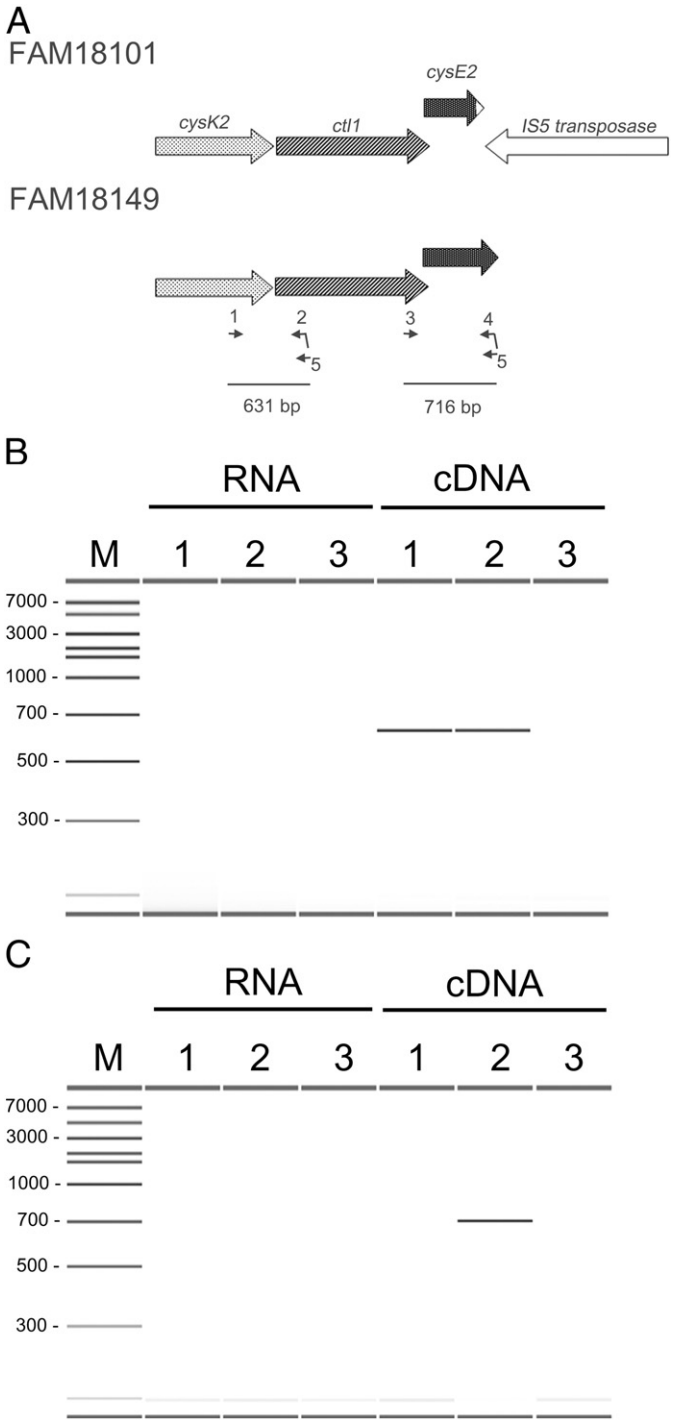

Fig. 3. Transcription analysis of the *cysK2-ctl1-cysE2* gene cluster with tagged RT-PCR. A. Schematic representation of the *cysK2-ctl1-cysE2* gene cluster from FAM18101 and FAM18149. The coding sequences are illustrated with large arrows. The white tip of the arrowhead of FAM18101 represents the codons originating from the insertion sequence. Small numbered arrows indicate the annealing regions of the primers used for the RT-PCR experiments. The primers were as follows: 1, cysK2_584; 2, tag-ctl1; 3, Q-ctl1_F; 4, tag-cysE2; 5, tag. B. PCR product of approximately 630 bp was obtained using the primers 1 and 5 with cDNA synthesized using tagged primer 2. C. PCR product of approximately 700 bp was obtained using primers 3 and 5 with cDNA synthesized using tagged primer 4. Lane M, DNA ladder; lane 1, FAM18101; lane 2, FAM18149; lane 3, ATCC 334. Molecular weights of the DNA ladder are shown in bp on the left. The PCR products were separated on the Agilent 2100 Bioanalyzer using DNA 7500 chips.

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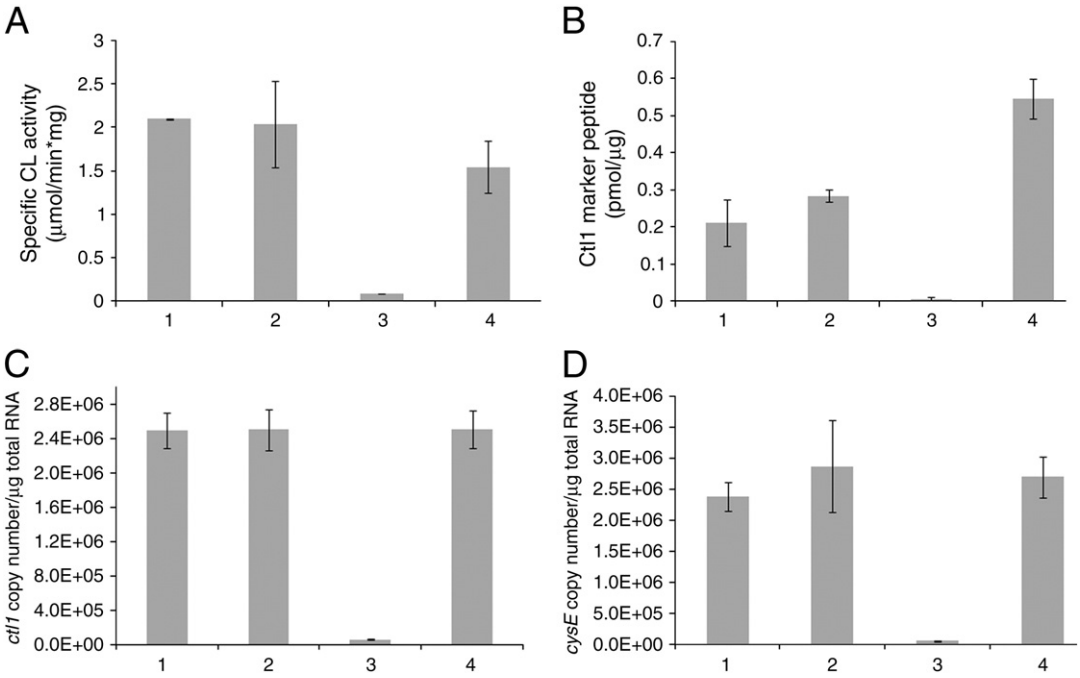

Fig. 4. Influence of cysteine on the expression of *ctl1*, *cysE2* and cystathionine lyase activity from *L. casei* FAM18149. The strain was grown overnight in MRS (4) or in CDM with various cysteine concentrations: no cysteine (1), 0.8 µg/mL L-cysteine (2), 8 µg/mL L-cysteine (3). A. illustrates the specific cystathionine lyase activity found in cell-free extracts. An LC-SRM method with a stable isotope-labeled peptide standard was used to quantify a marker peptide of the Ctl1 protein (B.). The amount of transcript for *ctl1* (C.) and for *cysE*2 (D.) was determined with real-time RT-PCR.

transcripts when the cells were grown in the presence of 8 µg/mL of L-cysteine (Fig. 4C and D). When the experiments were performed with strains grown in CDM with methionine as the sole sulfur source, no influence on gene expression or CL activity was found (data not shown).

Surprisingly, we did not detect CL activity in cell-free extracts from *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. helveticus* FAM2888 and *S. thermophilus* FAM17014 although we found transcripts of the *ctl1* homolog in all three strains with quantitative RT-PCR showing that the genes were expressed (Table 4).

3.7. Quantification of Ctl1 in cell-free extracts by mass spectrometry

A selected reaction monitoring (SRM) method was used to quantify the Ctl1 protein in cell-free extracts. First, recombinant Ctl1 was trypsinized and analyzed with LC-MS/MS. The doubly positive charged peptide Ctl1_(V149-K157) showed good signal intensity with low noise. The most intensive fragment ion with m/z 687.8 was chosen for quantification. The isotopically-labeled version of this peptide, Ctl1_(V149-K157)*, was used as the internal standard to quantify Ctl1 in cell-free extracts of different bacterial strains. The level of Ctl1 protein dramatically decreased when the strain *L. casei* FAM18149 was grown in CDM supplemented with 8 µg/mL of L-cysteine (Fig. 4B). This method was used to quantify the Ctl1_(V149-K157) peptide in cell-free extracts of different *L. casei* strains and in cell-free extracts of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. helveticus* FAM2888 and *S. thermophilus* FAM17014. The marker peptide of the Ctl1 protein was detected and quantified in the studied *L. casei* strains except in ATCC 334 (Table 3). The Ctl1_(V149-K157) marker peptide was not found in ATCC 11842 or in FAM2888 or in FAM17014 although the transcripts encoding the protein were present (Table 3). To make sure that this observation did not result from an incomplete or inhibited tryptic digest, a marker peptide for the elongation factor Tu was also monitored. In all samples, equal amounts of these peptides were detected, confirming that the tryptic digest worked properly.

4. Discussion

Usually, bacteria reduce sulfate to sulfide that is then incorporated into cysteine. To synthesize methionine, cysteine is converted to methionine. *L. casei* is a species found in many types of cheese (Beresford and Williams, 2004). The genome data of *L. casei* ATCC 334, *L. casei* BL23 and *L. casei* Zhang are publicly available. Exploitation of the genome data suggests that these strains lack the genes for sulfate utilization. Instead, these strains probably take up sulfur-containing

Table 4
PCR-based presence of the *ctl1* gene, transcription and protein level of *ctl1* and cystathionine lyase (CL) activity of cell-free extract in various lactic acid bacteria.

Strain	Presence of gene	*ctl1* transcript (copy number/µg total RNA)	Ctl1_(V149-K157)[a] (pmol/µg total protein)	CL activity
L. casei FAM18101	+	1.3E + 06	0.34	+
L. casei FAM18149	+	9.7E + 05	0.64	+
L. casei FAM18168	+	8.9E + 05	0.76	+
L. casei ATCC 334	−	n. d. [b]	n. d.[b]	−
L. bulgaricus ATCC 11842	+	6.5E + 04	n. d.[b]	−
S .thermophilus FAM17014	+	7.5E + 02	n. d.[b]	−
L. helveticus FAM2888	+	4.5E + 04	n. d.[b]	−

[a] Amino acid sequence: VTDIAAVAK.
[b] Not detected.

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peptides and amino acids from the environment, e.g., released by casein proteolysis in cheese during ripening. Since methionine is present in the caseins in larger amounts than cysteine, methionine is probably the main source for sulfur in cheese, and bacteria possessing the genes required to convert methionine to cysteine have an advantage.

We have previously identified a strain-specific cystathionine lyase in various *L. casei* strains (Irmler et al., 2009). Two different genetic variants named *ctl1* and *ctl2* were characterized, and the presence of these genes correlated with the ability to grow in a chemically defined medium that contained only L-methionine as the sole sulfur source. This implies that *ctl1* (and *ctl2*) enables the strains to convert methionine to cysteine. In the report here, we investigated the up- and downstream regions of the *ctl1* and *ctl2* gene.

An open reading frame (ORF) encoding a putative cysteine synthase (*cysK*) was detected upstream, and an ORF encoding a serine O-acetyltransferase (*cysE*) was detected downstream. Strains possessing *ctl1* have the *cysK2-ctl1-cysE2* gene cluster, and strains with *ctl2* have the *cysK3-ctl2-cysE3* gene cluster. The organization resembled the *cbs-cblB(cglG)-cysE* gene cluster found in *L. delbrueckii* subsp. *bulgaricus, L. helveticus* and *S. thermophilus* (Liu et al., 2009). In *L. delbrueckii* subsp. *bulgaricus*, the *cysE* gene is probably inactive due to a small insertion sequence. We found that *L. casei* FAM18101 and FAM18105 also carry an insertion sequence in the *cysE2* gene that apparently renders the gene inactive. Both strains grew in CDM containing methionine as the sole sulfur source (Irmler et al., 2009), indicating that a functional *cysE2* is not required for cysteine biosynthesis. Apparently, a second gene is present that takes over *cysE2*'s functional role.

The gene cluster *cysK2-ctl1-cysE2* is present but differently annotated in various species of LAB. First, Bolotin et al. (2004) presented the hypothesis that the *cysM2-metC-cysE2* gene cluster in *S. thermophilus* CNRZ1066 and *S. thermophilus* LMG 18311 was transferred from *L. delbrueckii* subsp. *bulgaricus* through a horizontal gene transfer event. Recently, Liu et al. (2009) further supported this hypothesis by analyzing the gene cluster that was renamed *cbs-cblB (cglB)-cysE* of *L. delbrueckii* subsp. *bulgaricus, S. thermophilus* and *L. helveticus* using bioinformatics tools. We used genomic sequencing to obtain nucleotide sequences upstream from the gene cluster in *L. casei* FAM18149 and FAM18168 and found an ORF encoding a putative transposase (data not shown). Apparently, the gene cluster in the *L. casei* strains was also obtained through a horizontal gene transfer event and may explain why the gene cluster is not present in *L. casei* ATCC 334, BL23 or Zhang.

The *cysK2-ctl1-cysE2* was expressed as one transcript in *L. casei* FAM18149 and FAM18101 (Fig. 3), indicating that the gene cluster forms an operon. The expression was down-regulated when L-cysteine (8 μg/mL) was added to the medium (Fig. 4). In addition, CL activity was no longer detected, which supports the hypothesis that the cystathionine lyase activity present in cell-free extracts of *ctl1*$^+$ positive *L. casei* strains results from the Ctl1 protein (Irmler et al., 2009). Since the expression is negatively affected by cysteine, we think that the gene cluster plays a role in cysteine biosynthesis. A similar regulation was reported for the *metC-cysK* operon in *Lactococcus lactis*, which is involved in cysteine biosynthesis and whose expression is down-regulated by cysteine (Fernandez et al., 2002).

No cystathionine lyase activity and no marker peptide from the gene product of the *clt1* homolog were found in *S. thermophilus* FAM17014, *L. helveticus* FAM2888 and *L. bulgaricus* ATCC 11842 although we detected the transcripts. We assume that in these species transcription and translation do not occur together and a post-transcriptional regulation mechanism is present. A similar observation was reported for *patC* encoding a cystathionine β-lyase in *L. delbrueckii* subsp. *bulgaricus*. Several strains possessing the *patC* gene did not exhibit cystathionine lyase activity although transcripts of the *patC* gene were present (Aubel et al., 2002).

A recent proteomics study of *S. thermophilus* LMG18311 showed that the genes of the *cysM2-metB2-cysE2* gene cluster were fully induced during the late stage of growth in milk (Herve-Jimenez et al., 2008). This report emphasized the importance of cysteine and methionine biosynthesis in *S. thermophilus* growing in a dairy environment. Cheese mainly consists of caseins. Cysteine is present only in the α_{SN2} and κ-casein that are slightly hydrolyzed during cheese ripening (Sousa et al., 2001). Apparently, the gene cluster is involved in cysteine biosynthesis from methionine, and the transfer of the gene cluster between various species of lactic acid bacteria isolated from cheese and milk may be the result of limiting cysteine availability.

Lactic acid bacteria are widely used in the food industry. Genome sequencing and exploiting these data with bioinformatics are powerful tools for predicting metabolic pathways and identifying the genes involved in these pathways. The tools, however, cannot predict metabolite (e.g., cysteine)-regulated gene expression or a post-transcriptional mechanism. To select strains for specific applications, e.g., use as flavor-forming adjuncts in cheese making, studying the transcription and translation of genes of interest and determining the enzymatic properties of the gene products will still be essential.

References

Aguena, M., Spira, B., 2003. RT-PCR of long prokaryotic operon transcripts without DNase treatment. Journal of Microbiological Methods 55, 419–423.

Aubel, D., Germond, J.E., Gilbert, C., Atlan, D., 2002. Isolation of the *patC* gene encoding the cystathionine ß-lyase of *Lactobacillus delbrueckii* subsp *bulgaricus* and molecular analysis of inter-strain variability in enzyme biosynthesis. Microbiology 148, 2029–2036.

Beresford, T., Williams, A., 2004. The microbiology of cheese ripening. In: Fox, P.F., McSweeney, P.L.H., Cogan, T.M., Guinee, T.P. (Eds.), Cheese: chemistry, physics and microbiology. Elsevier Academic Press, pp. 287–317.

Bolotin, A., Quinquis, B., Renault, P., Sorokin, A., Ehrlich, S.D., Kulakauskas, S., Lapidus, A., Goltsman, E., Mazur, M., Pusch, G.D., Fonstein, M., Overbeek, R., Kyprides, N., Purnelle, B., Prozzi, D., Ngui, K., Masuy, D., Hancy, F., Burteau, S., Boutry, M., Delcour, J., Goffeau, A., Hols, P., 2004. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. Nature Biotechnology 22, 1554–1558.

Callanan, M., Kaleta, P., O'Callaghan, J., O'Sullivan, O., Jordan, K., McAuliffe, O., Sangrador-Vegas, A., Slattery, L., Fitzgerald, G.F., Beresford, T., Ross, R.P., 2008. Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. Journal of Bacteriology 190, 727–735.

Christensen, J.E., Steele, J.L., 2003. Impaired growth rates in milk of *Lactobacillus helveticus* peptidase mutants can be overcome by use of amino acids supplements. Journal of Bacteriology 185, 3297–3306.

de Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. Journal of Applied Bacteriology 131, 82–91.

Fernandez, M., Kleerebezem, M., Kuipers, O.P., Siezen, R.J., Kranenburg, R., 2002. Regulation of the *metC-cysK* operon, involved in sulfur metabolism in *Lactococcus lactis*. Journal of Bacteriology 184, 82–90.

Herve-Jimenez, L., Guillouard, I., Guedon, E., Gautier, C., Boudebbouze, S., Hols, P., Monnet, V., Rul, F., Maguin, E., 2008. Physiology of *Streptococcus thermophilus* during the late stage of milk fermentation with special regard to sulfur amino-acid metabolism. Proteomics 8, 4273–4286.

Hullo, M.F., Auger, S., Soutourina, O., Barzu, O., Yvon, M., Danchin, A., Martin-Verstraete, I., 2007. Conversion of methionine to cysteine in *Bacillus subtilis* and its regulation. Journal of Bacteriology 189, 187–197.

Irmler, S., Schaefer, H., Beisert, B., Rauhut, D., Berthoud, H., 2009. Identification and characterization of a strain-dependent cystathionine ß/g-lyase in *Lactobacillus casei* potentially involved in cysteine biosynthesis. FEMS Microbiology Letters 295, 67–76.

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A.P.A., Lebeer, S., De Keersmaecker, S.C.J., Vanderleyden, J., Hamalainen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K.T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjarvi, T., Auvinen, P., de Vos, W.M., 2009. Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. Proceedings of the National Academy of Sciences of the United States of America 106, 17193–17198.

Kredich, N.M., 1996. Biosynthesis of cysteine. In *Escherichia coli and Salmonella*. In: Neidhardt, F.C., Curtiss, I.R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., Umbarger, H.E. (Eds.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C, pp. 514–527.

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Liu, M., Nauta, A., Francke, C., Siezen, R.J., 2008. Comparative genomics of enzymes in flavor-forming pathways from amino acids in lactic acid bacteria. Applied and Environmental Microbiology 74, 4590–4600.

Liu, M., Siezen, R.J., Nauta, A., 2009. In silico prediction of horizontal gene transfer events in *Lactobacillus bulgaricus* and *Streptococcus thermophilus* reveals proto-cooperation in yogurt manufacturing. Applied and Environmental Microbiology 75, 4120–4129.

Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. Proceedings of the National Academy of Sciences of the United States of America 103, 15611–15616.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.

Seilflein, T.A., Lawrence, J.G., 2006. Two transsulfurylation pathways in *Klebsiella pneumoniae*. Journal of Bacteriology 188, 5762–5774.

Smeianov, V.V., Wechter, P., Broadbent, J.R., Hughes, J.E., Rodriguez, B.T., Christensen, T.K., Ardo, Y., Steele, J.L., 2007. Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. Applied and Environmental Microbiology 73, 2661–2672.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150, 76–85.

Sousa, M.J., Ardö, Y., McSweeney, P.L.H., 2001. Advances in the study of proteolysis during cheese ripening. International Dairy Journal 11, 327–345.

Sun, Z., Chen, X., Wang, J., Zhao, W., Shao, Y., Wu, L., Zhou, Z., Sun, T., Wang, L., Meng, H., Zhang, H., Chen, W., 2011. Complete genome sequence of *Streptococcus thermophilus* strain ND03. Journal of Bacteriology 193, 793–794.

Terzaghi, B.E., Sandine, W.E., 1975. Improved medium for lactic streptococci and their bacteriophages. Applied Microbiology 29, 807–813.

Uren, J.R., 1987. Cystathionine ß-lyase from *Escherichia coli*. Methods in Enzymology 143, 483–486.

van de Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., Robert, C., Oztas, S., Mangenot, S., Couloux, A., Loux, V., Dervyn, R., Bossy, R., Bolotin, A., Batto, J.M., Walunas, T., Gibrat, J.F., Bessieres, P., Weissenbach, J., Ehrlich, S.D., Maguin, E., 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. Proceedings of the National Academy of Sciences of the United States of America 103, 9274–9279.

Wheeler, P.R., Coldham, N.G., Keating, L., Gordon, S.V., Wooff, E.E., Parish, T., Hewinson, R.G., 2005. Functional demonstration of reverse transsulfuration in *Mycobacterium tuberculosis* complex reveals that methionine is the preferred sulfur source for pathogenic mycobacteria. Journal of Biological Chemistry 280, 8069–8078.

Zhao, C., Kumada, Y., Imanaka, H., Imamura, K., Nakanishi, K., 2006. Cloning, overexpression, purification, and characterization of O-acetylserine sulfhydrylase-B from *Escherichia coli*. Protein Expression and Purification 47, 607–613.

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