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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

CysK from *Lactobacillus casei* encodes a protein with *O*-acetylserine sulfhydrylase and cysteine desulfurization activity

Biljana Bogicevic · Hélène Berthoud · Reto Portmann · Leo Meile · Stefan Irmler

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Abstract A gene encoding an *O*-acetyl-L-serine sulfhydrylase (c*ysK*) was cloned from *Lactobacillus casei* FAM18110 and expressed in *Escherichia coli*. The purified recombinant enzyme synthesized cysteine from sulfide and *O*-acetyl-L-serine at pH 5.5 and pH 7.4. At pH 7.4, the apparent K_M for *O*-acetyl-L-serine (OAS) and sulfide were 0.6 and 6.7 mM, respectively. Furthermore, the enzyme showed cysteine desulfurization activity in the presence of dithiothreitol at pH 7.5, but not at pH 5.5. The apparent K_M for L-cysteine was 0.7 mM. The synthesis of cystathionine from homocysteine and serine or OAS was not observed. When expressed in a *cysMK* mutant of *Escherichia coli*, the cloned gene complemented the cysteine auxotrophy of the mutant. These findings suggested that the gene product is mainly involved in cysteine biosynthesis in *L. casei*. Quantitative real-time PCR and a mass spectrometric assay based on selected reaction monitoring demonstrated that *L. casei* FAM18110 is constitutively overexpressing *cysK*.

Keywords *Lactobacillus casei* · Cysteine · Hydrogen sulfide · *O*-acetylserine sulfhydrylase · Desulfurization

B. Bogicevic · H. Berthoud · R. Portmann · S. Irmler (✉)
Agroscope Liebefeld-Posieux Research Station ALP,
Bern, Switzerland
e-mail: stefan.irmler@alp.admin.ch

B. Bogicevic · L. Meile
ETH Zurich, Institute of Food, Nutrition and Health (IFNH),
Laboratory of Food Biotechnology,
Zürich, Switzerland

Introduction

Volatile sulfur compounds (VSCs) such as methanethiol and hydrogen sulfide are odor-active compounds present in several cheese types. Because of their low odor threshold, these compounds contribute significantly to the aroma and sensorial properties of cheese. The formation of VSCs results from the metabolism of methionine and cysteine by bacteria present in the cheese matrix. On this account, the study of sulfur metabolism in bacteria associated with cheese (e.g., of the genera *Brevibacterium*, *Corynebacterium*, *Lactococcus*, and *Lactobacillus* spp.) is of great interest (Bonnarme et al. 2001; Bustos et al. 2011; Landaud et al. 2008; Weimer et al. 1999).

Two major pathways for cysteine biosynthesis in microorganisms have been described (Guedon and Martin-Verstraete 2007). One is the reverse transsulfuration pathway that converts homocysteine, a product of methionine degradation, to cysteine. In this pathway, cystathionine β-synthase (CBS) catalyzes the formation of cystathionine from homocysteine and serine. The cystathionine is then degraded by cystathionine γ-lyase (CGL) to cysteine, α-ketobutyrate, and ammonia. Whereas CGL activity was reported in several strains of lactic acid bacteria (Bruinenberg et al. 1997; de Angelis et al. 2002; Dobric et al. 2000; Irmler et al. 2009; Knoll et al. 2010; Smacchi and Gobbetti 1998), the presence of CBS activity has not been shown yet. Interestingly, it was shown that CGLs of lactic acid bacteria not only cleave cystathionine, but also catalyze the breakdown of methionine and cysteine to yield methanethiol and hydrogen sulfide, respectively.

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The other pathway is the thiolation pathway which converts serine to cysteine. It involves the action of serine acetyltransferase (SAT), which catalyzes the acetylation of serine and *O*-acetylserine sulfhydrylase (OASS, also known as cysteine synthase), which, in turn, catalyzes the replacement of the acetate group by inorganic sulfide. To our knowledge, SAT activity has not been demonstrated in lactic acid bacteria and OASS activity was only reported for *Lactococcus lactis* (Fernandez et al. 2000).

In *Enterobacteriaceae*, there are two isoenzymes of OASS called CysK (also known as OASS-A) and CysM (also known as OASS-B). Both enzymes catalyze the insertion of sulfide into *O*-acetyl-L-serine to form cysteine and acetate (Kredich 1996). The amino acid sequences of CysK and CysM show 43% identity. In contrast to CysK, CysM can also use thiosulfate instead of sulfide to synthesize *S*-sulfo-L-cysteine (Zhao et al. 2006). Furthermore, it has been proposed that CysM is preferentially used during growth in anaerobic conditions, since CysK is predominantly expressed in higher amounts under aerobic conditions (Kredich 1996).

Many enzymes catalyze forward and backward reactions. And, in fact, it has been shown that CysK and CysM from *Escherichia coli* not only catalyze the synthesis of cysteine, but also have desulfurization activity (Awano et al. 2005; Flint et al. 1996). In a similar way, the formation of hydrogen sulfide from cysteine was also reported for OASS from *Fusobacterium nucleatum* and *Aeropyrum pernix* (Fukamachi et al. 2002; Mino and Ishikawa 2003).

The biosynthesis and degradation of cysteine by OASS has not been extensively studied in lactic acid bacteria. Genome data analyses show that a *cysK* homolog is present in several species of lactobacilli and lactococci (Liu et al. 2008). It has been reported that *cysK* of the *metC-cysK* operon in *L. lactis* encodes a protein with cysteine synthase activity, but enzymatic data were not presented (Fernandez et al. 2002). Moreover, it was proposed that the lactococcal *cysK* gene product may also encode a CBS (Sperandio et al. 2005). This hypothesis is based on several observations. First, the gene forms an operon with *metC* encoding a cystathionine lyase (Fernandez et al. 2000). Second, expression of the operon is downregulated by cysteine (Fernandez et al. 2002). Furthermore, inactivation of *cysK* results in cysteine auxotrophy in the presence of methionine (Sperandio et al. 2005). Finally, cystathionine synthesis activity was observed for *cysK* homologs from *Bacillus subtilis* and *A. pernix* (Hullo et al. 2007; Mino and Ishikawa 2003).

Our interest in the pathway for biosynthesis of cysteine arose from the observation that several *Lactobacillus casei* strains isolated from Gruyère cheese and raw milk grew in a chemically defined medium containing methionine as the sole sulfur source (Irmler et al. 2008). This indicated that cysteine biosynthesis occurred probably by reverse transsulfuration. In fact, it was found that these strains possess a gene encoding a CGL (Irmler et al. 2009), but CBS activity has not been detected yet. The hypothesis that the gene product of *cysK* from *L. casei* may exhibit CBS activity remains to be clarified.

This paper reports the cloning of a *cysK* homolog of *L. casei*. To address the question of cysteine biosynthesis in lactobacilli, the biochemical properties of the gene product and the expression in various *L. casei* genotypes were studied. Finally, the role of CysK in the formation of cysteine and hydrogen sulfide is discussed.

Materials and methods

Bacterial strains, plasmids, and primers

The strains, plasmids, and primers used in this study are listed in Table 1. *L. casei* strains were grown at 30°C in MRS broth (de Man et al. 1960). Chemically defined media (CDM) for lactobacilli was prepared as described by Christensen and Steele (2003). *E. coli* strains were grown at 37°C in LB broth (Sambrook et al. 1989) with, when necessary, kanamycin (50 μg mL^{-1}). Solid media contained 1.5% agar.

Isolation of RNA and DNA

Genomic DNA from *L. casei* strains was extracted from 1 mL of overnight culture by robot extraction (BioRobot EZ1, EZ1 DNA Tissue kit; Qiagen) after cells had been first treated with 0.05 N NaOH for 15 min at room temperature, followed by 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.

Isolation of *E. coli* plasmid DNA was performed with the QIAprep Spin Miniprep Kit (Qiagen).

To isolate the RNA from *L. casei*, cells from 2 mL culture grown in MRS at 37°C for 16 h were harvested by centrifugation (17,950×*g*, 10 min, RT). 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.

Nucleic acid hybridization analysis

Genomic DNA (800 ng) was digested with restriction enzymes, separated in a 0.8% agarose gel, transferred to a nylon membrane by vacuum transfer and then hybridized with a digoxigenin (DIG)-labeled *cysK* probe. The DIG-

Table 1 Strains, plasmids, primers, and peptides used in this study

Strain	Genotype or relevant properties	Source or reference
E. coli		
BL21 (DE3)	F$^-$ *ompT hsdS*B (r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
NK3	Δ*trpE5 leu-6 thi hsdR hsdM*$^+$ *cysK cysM*	Hulanicka et al. (1986)
L. casei		
FAM18099	Δ*malY1*	Gruyère, Agroscope Culture Collection
FAM18110, FAM18121		Gruyère, Agroscope Culture Collection
FAM18149, FAM18168	*ctl1*$^+$	Raw milk, Agroscope Culture Collection
FAM18172	*ctl2*$^+$	Raw milk, Agroscope Culture Collection
ATCC 334		American Type Culture Collection
Plasmid		
pET SUMO	*E. coli* cloning vector, kan^r	Invitrogen
pET SUMO/CAT	Control vector expressing a His-tagged SUMO-CAT fusion protein, kan^r	Invitrogen
pET SUMO/cysK	pET SUMO containing *cysK* from *L. casei* FAM18110	This study
pET/cysK	pET SUMO/cysK lacking the part encoding SUMO	This study
pET/ctl1	Expression plasmid encoding His-tagged Ctl1	Irmler et al. (2009)
pET/malY	Expression plasmid encoding His-tagged MalY	Irmler et al. (2008)
pET/metB	Expression plasmid encoding His-tagged MetB	Irmler et al. (2008)
Primer	Sequence (5′ to 3′)	Used for
cysK_NheI [a]	GATATAAGGCTAGCATGGTGACAGCAGCAGAT	Cloning of *cysK*
cysK_R	TTAATCTTCGAACTTAAACAAGTCCGTTG	Cloning of *cysK*
cysKI_F	CCGGCGGTTCTGTCAAAG	Real-time PCR
cysKI_R	CCCTTGTATTCGGCATCTTCA	Real-time PCR
cysKI_FAM	FAM-CCGAATTGCCTTGGCCATG-BHQ1	Real-time PCR, fluorogenic hybridization probe
cysK_DIG_F	ACCCCAGACGCCTTCGTT	Preparation of hybridization probe
cysK_DIG_R	TCTTGCCTTTGCCAAGTTTCTT	Preparation of hybridization probe
tag-cysK	agtggtacacgcagagtacttCCCTTGTATTCGGC	Tagged RT-PCR
tag (anchored reverse primer)	agtggtacacgcagagtactt	Tagged RT-PCR
cysE_F_Tr	CCGAAATGGATGTCCGCG	Tagged RT-PCR
Peptide	Sequence	
CysK(S165-R189)*	SFDGGTPDAFVAGVGTGGTLTGVGR (Containing U-[^{13}C6], [^{15}N4]-arginine)	Internal standard for SRM

[a] Contains a *Nhe*I restriction site (underlined)

dUTP-labeled probe was prepared from the genomic DNA of *L. casei* ATCC 334 with the primers cysK_DIG_F and cysK_DIG_R (Table 1) and the PCR DIG Probe Synthesis Kit (Roche, Switzerland). Hybridization was performed overnight in 5× SSC, 0.02% SDS, 0.1% *N*-lauroylsarcosine and 1% blocking reagent (Roche) with the DIG-labeled probe at 68°C. Afterwards, membrane was washed two times in 2× SSC and 0.1% SDS at room temperature for 5 min followed by two washes in 0.2× SSC and 0.1% SDS at room temperature for 15 min. Hybridization signals were visualized using alkaline phosphatase-coupled anti-digoxigenin IgG (Roche) and NBT/BCIP stock solution (Roche) according to the manufacturer's instructions.

Tagged RT-PCR

A slightly modified RT-PCR method as described by Aguena and Spira (2003) was used. The total RNA was denatured by incubation at 65°C for 5 min and rapidly cooled on ice. Single-stranded cDNA was synthesized from 500 ng of total RNA using the M-MLV reverse transcrip-

tase (Invitrogen) and 2 pmol of tagged tag-cysK primer under the following conditions: 53°C for 50 min, 70°C for 15 min. One twentieth of the final cDNA product (1 μL) was used for the PCR reaction, which was carried out with the primer pair cysE_F1_Tr/tag. PCR amplification was 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 1000 chips according to the manufacturer's instructions.

Construction of plasmids

Based on the nucleotide sequence of *LSEI_0480*, the primer pair cysK_NheI/cysK_R was designed and used to amplify *cysK* from genomic DNA of *L. casei* FAM18110 by PCR (Table 1). The PCR product was cloned into the pET SUMO expression vector (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. A plasmid containing *cysK* in the proper orientation was selected and called pET SUMO/*cysK*. The primer cysK_NheI contained a *Nhe*I restriction site to remove the part encoding SUMO (~0.3 kb) from the plasmid. Therefore, the plasmid pET SUMO/*cysK* was digested with *Nhe*I, yielding a 6.3-kb fragment and a 0.3-kb fragment. The larger fragment was isolated from an agarose gel with the JETSORB Gel Extraction Kit (Genomed, Germany) and ligated, obtaining the expression plasmid pET/*cysK*. Restriction, ligation, and calcium-chloride-mediated transformation of *E. coli* cells were performed using standard procedures (Sambrook et al. 1989).

Expression and purification of His-tagged proteins

E. coli BL21 (DE3) transformed with pET/*cysK* was grown in 100 mL LB broth containing kanamycin (50 μg mL^{-1}) at 37°C on a shaker. When the optical density at 600 nm reached 0.5, expression of recombinant c*ysK* was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (final concentration) and by lowering the incubation temperature to 27°C. After 4 h incubation on a shaker, the bacterial cells were harvested by centrifugation, washed twice with 20 mM sodium phosphate (pH 7.4), and frozen at −20°C.

To purify the recombinant protein, cells were thawed and suspended in 500 μL binding buffer (20 mM sodium phosphate [pH 7.4], 150 mM NaCl, 20 mM imidazole). After adding approximately 0.4 g of glass beads (212–300 μm), the cells were disrupted by violent agitation in a Mini-Beadbeater-8 (Biospec Products, Inc., OK, USA). The extract was cleared by centrifugation at 17,900×*g* for 15 min at 4°C and then applied to a 1 mL HiTrap Chelating HP column (GE HealthCare, Uppsala, Sweden), which had been loaded with Ni^{2+} and equilibrated with binding buffer. The column was extensively washed with binding buffer, and bound proteins were eluted with 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, and 500 mM imidazole.

Imidazole was immediately removed by applying the eluent to NAP-5 columns (GE HealthCare, Uppsala, Sweden), which had been equilibrated with 20 mM Tris–HCl (pH 7.4) and 150 mM NaCl. The eluted protein fraction was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by colloidal Coomassie staining.

Purified His-tagged MalY, MetB, Ctl1, and SUMO/CAT fusion protein were produced and purified as described previously (Irmler et al. 2009; Irmler et al. 2008).

Enzyme assays

To determine OAS sulfhydrylase activity, the nitrous acid method described by Kredich and Tomkins (1966) was used. To determine the K_M for OAS, assays with various concentrations of OAS (0.1–12.5 mM) with a fixed concentration of sodium sulfide (3 mM) were performed. For the K_M of sulfide, various concentrations of sodium sulfide (0.2–10 mM) with a fixed concentration of OAS (5 mM) were used. A calibration curve was prepared with different concentrations of L-cysteine.

To study *S*-sulfocysteine synthesis, the acid ninhydrin reagent described by Gaitonde (1967) was used; it forms a pink color when incubated with *S*-sulfocysteine. Enzyme reactions containing sulfide and a solution of 1 mM *S*-sulfo-L-cysteine (Sigma-Aldrich, Switzerland) served as positive controls.

To detect the release of hydrogen sulfide from cysteine, two methods were used: assay I: precipitation with lead ions as described by Zdych et al. (1995), assay II: formation of methylene blue as described by Siegel (1965). For assay I, cell-free extract (CFE) or purified protein was separated by non-denaturing electrophoresis in an 8% polyacrylamide gel. After electrophoresis, the gel was incubated in activity stain buffer (100 mM Tris–HCl [pH 7.5], 10 mM L-cysteine, 0.5 mM $Pb(NO_3)_2$ and 4 μM pyridoxal-5′-phosphate) at 37°C until a dark brown to black precipitate had formed. To detect activity at low pH, the gel was first incubated two times for 15 min in 100 mM 2-morpholinoethanesulfonate (pH 5.5) and finally incubated in the activity stain buffer in which Tris had been replaced by 2-morpholinoethanesulfonate (pH 5.5). For assay II, recombinant CysK was incubated in 0.2-mL reactions containing 50 mM Tris–HCl (pH 7.5) or 2-morpholinoethanesulfonic (pH 5.5), 20 μM pyridoxal-5′-

phosphate and various concentrations of L-cysteine or DL-homocysteine at 37°C for 10 min. Reactions were carried out in the absence and presence of 2.5 mM dithiothreitol (DTT). The reaction was initiated by the addition of 10 μL of enzyme and terminated after 10 min incubation at 37°C by the successive addition of 20 μL of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride dissolved in 7.2 M HCl and 20 μL of 30 mM $FeCl_3$ dissolved in 1.2 M HCl. After 30 min at room temperature, methylene blue formation was measured at 670 nm.

K_M and V_{max} for the desulfurization activity were determined with assay II. The values for cysteine were determined with a fixed concentration of DTT (2.5 mM) and variable concentrations of cysteine (0.125–5 mM); the values for DTT were determined with a fixed concentration of cysteine (1 mM) and variable concentrations of DTT (0.1–10 mM). The amount of released hydrogen sulfide was calculated using the molar extinction coefficient of 28.5×10^3 M^{-1} cm^{-1}.

K_M and V_{max} values were determined using the Hanes–Woolf transformation (S V^{-1} vs. S), where V is the formation rate of cysteine or sulfide and S is the concentration of the substrate.

To study cystathionine synthesis, enzymatic reactions were carried out in 500 μL containing 200 mM sodium phosphate (pH 7.4), 0.2 mM pyridoxal-5′-phosphate, 10 mM OAS or 10 mM L-serine, 20 mM DL-homocysteine, and recombinant CysK at 37°C for 1 h. Solutions containing L-cystathionine were used as standard. Cystathionine was detected by the method described by Kashiwamata and Greenberg (1970).

Complementation of *E. coli* NK3

A cysteine-auxotroph, *E.coli* NK3, was transformed with pET/*cysK* or with pET SUMO/CAT as a control. For genetic complementation of the cysteine requirement, the transformed *E. coli* was cultured on M9 agar medium (Sambrook et al. 1989) supplemented with 0.02% leucine, 0.02% tryptophan, 0.01% thiamine, 1 mM IPTG and kanamycin (50 μg/mL) at 37°C for 2 days.

Real-time RT-PCR

Single-stranded cDNA was synthesized from 500 ng of total RNA using the M-MLV reverse transcriptase (Invitrogen) and 50 ng of random hexamer primer (Invitrogen) under the following conditions: 25°C for 10 min, 37°C for 50 min, 70°C for 15 min. Exactly 1.0 μL of the cDNA was used as template for PCR amplification.

Real-time PCR was carried out using the Rotor-Gene RG 3000-A machine (Corbett Research, Sydney, Australia). The PCR reaction mixture (12 μL) contained 6 μL 2× TaqMan Universal PCR master mix (Invitrogen), 0.3 μM of cysKI_F (forward primer), 0.9 μM of cysKI_R (reverse primer), 0.15 μM of cysKI_FAM (TaqMan probe), and 1 μL of cDNA. The sequences of the primers and probes are described in Table 1. Water and total RNA were used as controls. 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 triplicates with cDNA synthesized from two independent RNA samples.

The crossing threshold cycle (C_T) is the cycle at which there is a significant increase in fluorescence above the background and was determined with the Rotor-Gene Real-Time Analysis Software 6.0. The C_T is inversely proportional to the logarithm of the initial number of template molecules. The reaction efficiency was calculated from the slopes of a calibration curve, which was constructed with serial dilutions of pET/*cysK*. Relative quantification was performed with the comparative C_T method (Applied Biosystems 2001).

Preparation of cell-free extracts from *L. casei*

Cells from 50 mL cultures grown overnight were harvested by centrifugation and washed twice with 20 mM sodium phosphate (pH 7.4). Finally, cells were suspended in 500 μL of 20 mM sodium phosphate (pH 7.4) and disrupted by violent agitation in a Mini-Beadbeater-8 (Biospec Products, Inc.) with the aid of glass beads (212–300 μm). The extract was cleared by centrifugation (17,900×*g*, 15 min, 4°C), and protein concentration was determined by Bio-Rad protein assay according to the manufacturer's instructions. Bovine serum albumin was used to obtain a standard curve.

Quantitation of CysK by mass spectrometry

CysK concentration was determined by selected-reaction monitoring (SRM), which is a sensitive and specific mass spectrometry assay. The isotope-labeled reference peptide CysK(S165-R189)*, which contained a stable-isotope-labeled C-terminal arginine, was obtained from Thermo Fisher Scientific (Ulm, Germany) and served as an internal standard (Table 1).

All protein (20 μg) was digested in 50 μL of 10 mM ammonium bicarbonate containing trypsin (4 ng μL^{-1}) overnight at 37°C. The tryptic digest (10 μL) was then coinjected with 2 μL of the reference peptide CysK(S165-R189)* corresponding to 3 pmol.

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) 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 10%

water/90% acetonitrile, both containing 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*) 766.6 (±1)→754.2 (±1) for the native and 769.6 (±1)→757.4 (±1) for the internal standard peptide were monitored for the quantification of CysK. Additionally, the SRM transition 821.5 (±1)→943.1 (±1) for the elongation factor Tu (EFTu) was monitored for normalization. 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 the LCquan software (ThermoFisher Scientific). The ratio of the light/heavy transitions was calculated and divided by the peak area of EFTu for normalization.

Accession numbers

Sequence data from this article can be found in the NCBI database under the accession number HQ537477.

Results

Presence of *cysK* in various *L. casei* strains

The gene *LSEI_0480* of *L. casei* ATCC 334 (Genbank accession no. CP000423: 497885.498814) putatively encodes an *O*-acetylserine sulfhydrylase. The presence and copy number of *LSEI_0480*, which is called *cysK* in this report, was analyzed in seven *L. casei* strains (Table 1) by Southern blot analysis. Restriction of genomic DNA was performed with *Eco*RI, *Hin*dIII and *Bam*HI, respectively. When hybridized with a specific 352-bp *cysK* probe, a single band appeared in each digest (Fig. 1). Remarkably, the size of the hybridizing band varied between the strains, and the differences were highest in the *Eco*RI digests. In the *Eco*RI, *Bam*HI, and *Hin*dIII digests of ATCC 334 (Fig. 1, lane 7), the size of the band had an apparent size of 8.3, 6.7, and 1.6 kb, respectively. The experimental data matched quite well with those calculated from the genomic data (7.6, 6.5, and 1.8 kb).

Transcript analysis

The genomic sequence of *L. casei* ATCC 334 shows that upstream of *cysK* (*LSEI_0480*) lies another open reading frame (*LSEI_0479*), which putatively encodes a homoserine succinyltransferase. The two genes are separated by 33 bp of non-coding sequence, and the vicinity implies that they form a transcriptional unit. To analyze if these two genes are transcribed together, a tagged RT-PCR was performed with RNA isolated from *L. casei* ATCC 334 and *L. casei* FAM18110. A PCR product of approximately 570 bp was amplified in both RNA samples (Fig. 2b). No PCR product was obtained when the reverse transcriptase was omitted from the reaction.

Purification of recombinant CysK

A PCR product of approximately 960 bp was amplified with the primer pair cysK_NheI / cysK_R from genomic DNA of *L. casei* FAM18110. The PCR product named *cysK* was cloned into pET SUMO expression vector and sequenced. The deduced amino acid sequence showed the following sequence similarities: 100% with LSEI_0480 from *L. casei* ATCC 334, 49% with CysK and 40% with CysM from *E. coli*, 60% with CysK and 42% with YhrA from *B. subtilis*, 62% with CysK from *L. lactis* subsp. *cremoris*, and 23% with CysK from *A. pernix* (Fernandez et al. 2002; Hullo et al. 2007; Kredich and Tomkins 1966; Mino and Ishikawa 2003; Zhao et al. 2006). All these enzymes except LSEI_0480 have been biochemically demonstrated to exhibit OASS activity.

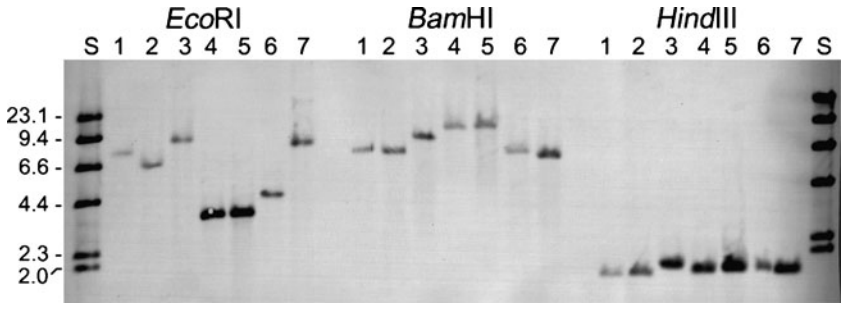

Fig. 1 Southern hybridization of digested genomic DNA from *L. casei* FAM18099 (*1*), FAM18110 (*2*), FAM18121 (*3*), FAM18149 (*4*), FAM18168 (*5*), FAM18172 (*6*), and ATCC 334 (*7*). DNA was digested with *Eco*RI, *Bam*HI, or *Hin*dIII, and a 352-bp fragment of *cysK* was used as probe. The DNA sizes of the standard (S) are indicated on the left-hand-side in kilobases

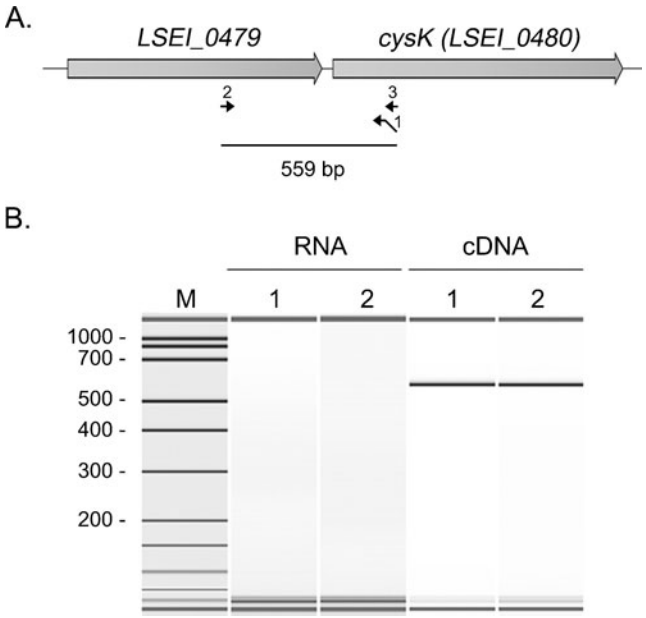

Fig. 2 Transcription analysis of *cysE–cysK* gene cluster by tagged RT-PCR. **a** Schematic representation of the *LSEI_0479-LSEI_0480* gene cluster from *L. casei* ATCC 334. *Large arrows* illustrate the coding sequences. *Small numbered arrows* indicate the annealing regions of the primers used for the tagged RT-PCR experiments. The primers are as follows: *1*, tag-cysK; *2*, cysE_F_Tr; *3*, tag. **b** A PCR product of approximately 570 bp was obtained using primers 1 and 3 with cDNA synthesized using tagged primer 2. *Lane M*, DNA ladder; *lane 1*, FAM 18110; *lane 2*, ATCC 334. Molecular weights of the DNA ladder are shown in base pairs on the left. The PCR products were separated on the Agilent 2100 BioAnalyser using the Agilent DNA 1000 chip reagent kit

With the expression vector called pET SUMO/*cysK*, a fusion protein containing a 6× His-tag, SUMO, and CysK could be produced in *E. coli* BL23 (data not shown). To rule out that SUMO influences the enzymatic activity, the nucleotide region encoding SUMO was removed as described in the M&M section. The resulting expression plasmid named pET/*cysK* encoded CysK fused to a 21-amino-acid peptide containing the 6× His-tag. Heterologous expression in *E. coli* BL21 (DE3) yielded a soluble recombinant protein that could be purified by nickel affinity chromatography. A single protein band was observed by SDS-PAGE and non-denaturing PAGE (Fig. 4, lane 9). Under denaturing conditions, the recombinant protein had an apparent molecular weight of 32 kDa, which is slightly lower than calculated (34.9 kDa).

Sulfhydrylation activity of recombinant CysK

For the sulfhydrylation reaction, purified CysK was incubated with OAS and sodium sulfide at pH 7.4 and 5.5. Furthermore, OAS was replaced by serine, phosphoserine, or *O*-succinyl-L-homoserine. Further controls were enzyme assays in which either OAS or sulfide was omitted. By using the nitrous acid assay or the acid ninhydrin reagent, we only observed synthesis of a product, when CysK was incubated with OAS and sulfide. Thin-layer chromatography confirmed that the reaction product was cysteine (data not shown). The synthesis of cysteine was observed at neutral and acidic pH. For the *S*-sulfocysteine synthetic reaction, sulfide was replaced with 3 and 20 mM thiosulfate, but no color formation was observed using the acid ninhydrin reagent. The specific and highly sensitive nitrous acid assay was used to determine the K_M and V_{max} for OAS and sodium sulfide (Table 2). Additionally, the OAS sulfhydrylation assay was performed in the presence of 4% NaCl. No effect on enzymatic activity was observed (data not shown).

To confirm that *cysK* is a functional OASS in vivo, the plasmid pET/*cysK* was transformed into the cysteine-auxotroph mutant *E. coli* NK3. NK3 transformed with pET SUMO/CAT was used as a negative control. It was observed that NK3 (pET/*cysK*) grew in minimal medium M9, whereas NK3 (pET SUMO/CAT) could not grow (Fig. 3).

Desulfurization activity of recombinant CysK

To study the cysteine desulfurization activity, purified CysK was electrophoresed under non-denaturing conditions together with purified recombinant MetB, Ctl1, and MalY from *L. casei*, proteins known to cleave cysteine (Irmler et al. 2009; Irmler et al. 2008). Purified SUMO/CAT fusion protein was used as a negative control. In addition, cell-free extract (CFE) proteins of various *L. casei* strains were electrophoresed. The activity staining was performed at pH 7.5 and pH 5.5 (Fig. 4a, c). At pH 7.5, a black precipitate formed at the site of MetB, CysK, and MalY (Fig. 4a, lanes 6, 9, and 10). In the separated CFE proteins from the *L. casei* strains, two bands became visible at pH 7.5. This was not the case for FAM18099, where only the band with the apparent lower molecular mass was observed (Fig. 4a, lane 5). At pH 5.5, a black band

Table 2 Kinetic parameters of OAS sulfhydration and cysteine desulfurization reaction of the recombinant CysK from *L. casei* (values were determined at pH 7.4 and 37°C and are means±S.D. from three independent experiments)

Activity	Substrate	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})
OAS sulfhydrylation	*O*-acetyl-L-serine	0.6±0.1	89±19
	Na_2S	6.7±0.3	131±6
Desulfurization	L-cysteine	0.7±0.2	0.027±0.008
	DTT	0.7±0.1	0.037±0.017

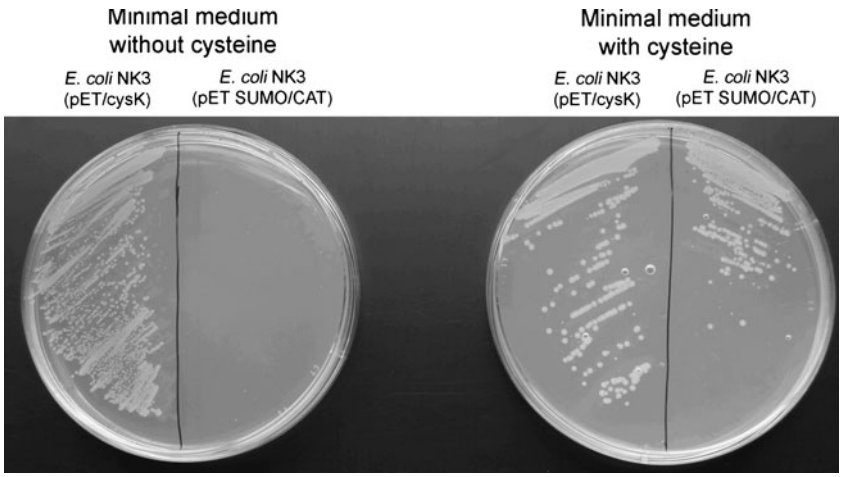

Fig. 3 Complementation of the cysteine auxotrophy of *E. coli* NK3 by *cysK* from *L. casei* FAM18110. *E. coli* NK3 was transformed either with the pET SUMO/CAT plasmid (negative control) or with pET/*cysK*. Transformants were streaked onto an M9 minimal agar plate supplemented with leucine, tryptophan, IPTG, and kanamycin in the presence or absence of 0.02% L-cysteine and incubated for 2 days at 37°C

appeared at the sites of Ctl1 and MalY (Fig. 4c, lanes 7 and 10), and in the CFE proteins, only the band with the apparent higher molecular mass appeared. Again, this band was not present in FAM18099.

To determine K_M and V_{max} for the cysteine desulfurization activity, formation of hydrogen sulfide by CysK was studied with the methylene blue assay (Table 2). In this assay, production of hydrogen sulfide was only observed in the presence of DTT and at pH 7.5. No cysteine desulfurization activity was observed at pH 5.5, confirming the results of the native gel electrophoresis. No production of hydrogen sulfide was observed when cysteine was replaced with homocysteine.

Expression of *cysK* in various *L. casei* strains

The levels of *cysK* mRNA were determined by real-time RT-PCR. Total RNA from cultures of the seven *L. casei* strains grown under the same conditions was analyzed. The RNA gave clear capillary electrophoretic patterns with prominent 23S and 16S ribosomal RNA bands having a 16S/23S ratio between 1.2 and 2.0 (data not shown). The RNA Integrity Number (RIN) was between 8.9 and 10, confirming that RNA samples were intact (Schroeder et al. 2006).

CysK transcripts were detected in all strains, and C_T values ranged from 15 to 20 (Table 3). The highest expression level was determined in *L. casei* FAM18110 with an average C_T value of 15, whereas the lowest expression was determined in *L. casei* ATCC 334 with a C_T value of 19.4. This means that *cysK* in FAM18110 is approximately 11-fold more expressed than in ATCC 334. The other five strains showed only slightly higher levels of *cysK* expression.

The amount of CysK protein was determined with selected reaction monitoring (SRM). In preliminary studies, tryptic digests of CFE proteins were analyzed by mass spectrometry. Thereby, the tryptic peptide SFDGGTPDAF VAGVGTGGTLTGVGR from CysK was readily detected,

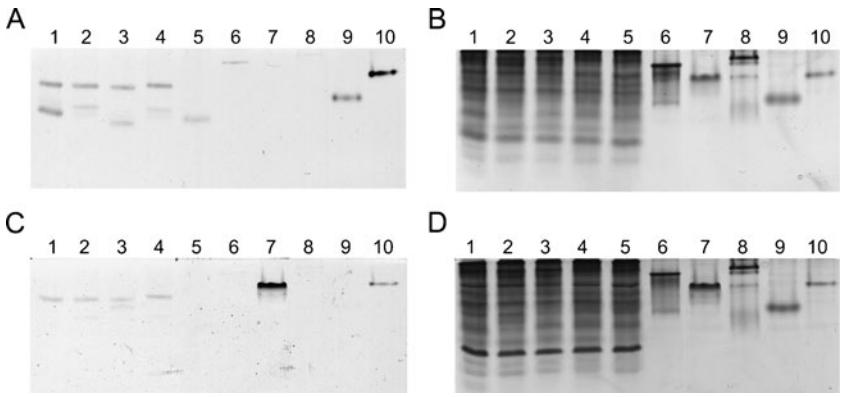

Fig. 4 Detection of cysteine desulfurization activity in native gels. Proteins were separated under non-denaturing conditions followed by activity staining with L-cysteine as substrate at pH 7.5 (**a**) and at pH 5.5 (**c**). Subsequently, proteins were visualized by Coomassie Blue G staining (B, D). *Lane 1*, CFE of *L. casei* FAM18110; *lane 2*, CFE of *L. casei* FAM18145; *lane 3*, CFE of *L. casei* FAM18168; *lane 4*, CFE of *L. casei* FAM18108; *lane 5*, CFE of *L. casei* FAM18099; *lane 6*, recombinant MetB; *lane 7*, recombinant Ctl1; *lane 8*, recombinant SUMO-CAT; *lane 9*, recombinant CysK; *lane 10*, recombinant MalY. Protein amount was 20 μg of CFE and 2 μg of recombinant protein per lane

and upon fragmentation, an intense fragment ion with *m*/*z* 754.2 was observed. The peptide called CysK(S165-R189) was selected to quantify CysK in cell-free extracts, and the isotopically labeled reference peptide CysK(S165-R189)* was used as an internal standard (Table 3). The data showed that *L. casei* FAM18110 compared to ATCC 334 produced approximately tenfold more CysK.

The SRM method was further used to quantify CysK in cell-free extracts of *L. casei* FAM18110 grown in CDM with and without cysteine. In both cases, equal amounts of CysK were observed, indicating that expression is not regulated by cysteine (data not shown).

Discussion

Hydrogen sulfide is found regularly in the headspace profiles of cheddar cheese, and amounts increase during the first 6 months of ripening (Burbank and Qian 2005; Kristoffersen and Nelson 1955; Manning and Moore 1979; Walker 1959). Hydrogen sulfide has a low odor threshold, and it has been reported that hydrogen sulfide reacted with methionine and caseinate, producing methanethiol (Manning 1979), which is a key flavor in various types of cheese. Thus, the production of hydrogen sulfide may contribute substantially to cheese flavor, but its impact remains to be clarified.

The formation of hydrogen sulfide in cheese is poorly understood. An important factor may be bacterial enzymes that degrade sulfur-containing amino acids. Thus, it has been shown that cystathionine lyases of lactic acid bacteria degraded cysteine, releasing hydrogen sulfide (Aubel et al. 2002; Bruinenberg et al. 1997; de Angelis et al. 2002; Dobric et al. 1998; Irmler et al. 2009; Irmler et al. 2008; Knoll et al. 2010; Martinez-Cuesta et al. 2006; Smacchi and Gobbetti 1998). Other enzymes known to degrade cysteine to hydrogen sulfide are OASSs, examples of which are CysK and CysM from *E. coli* (Awano et al. 2005; Flint et al. 1996). Genomic data from *L. casei* ATCC 334 show that the gene *LSEI_0480* putatively encodes an OASS. Southern hybridization showed that a single-copy gene was present in several *L. casei* strains. Interestingly, the molecular size of the hybridizing band notably varied among the studied strains, which can be explained by restriction site polymorphisms or insertions/deletions present in the gene region of *cysK*. Since the size variation was especially high in the *Eco*RI and *Bam*HI digests, the pattern of the Southern hybridization could be used as a diagnostic tool for the identification of *L. casei* genotypes.

Table 3 Relative quantitation of *cysK* mRNA and CysK protein in various *L. casei* strains

Strain	*cysK* average C_T	*cysK*[a] rel. to ATCC 334	CysK[b] average	CysK rel. to ATCC 334
FAM18099	17.7±0.2	3.2	$(6.2\pm3.7)*10^{-6}$	1.6
FAM18110	15.9±0.4	11.5	$(3.9\pm2.1)*10^{-5}$	9.8
FAM18121	18.3±0.2	2.1	$(6.6\pm3.1)*10^{-6}$	1.7
FAM18149	17.4±0.2	4.1	$(1.3\pm0.6)*10^{-5}$	3.2
FAM18168	18.6±0.3	1.8	$(1.4\pm0.7)*10^{-5}$	3.6
FAM18172	17.9±0.7	2.8	$(2.8\pm0.6)*10^{-6}$	0.7
ATCC 334	19.4±0.2	1.0	$(4.0\pm2.0)*10^{-6}$	1.0

Transcript amounts were determined by real-time RT-PCR analysis and compared with the C_T method. Values represent the mean±S.D. Protein amounts were determined by mass spectrometry. Values represent the mean±S.D.

[a] The value was determined by evaluating the expression $2^{-(C_T - C_{T,ATCC\ 334})}$. Reaction efficiency was 0.995

[b] The value was determined by calculating the ratio of the peak area for the native and internal standard divided by the peak area of an EFTu peptide

Genomic annotation is to a great extent a predictive process. Especially, the assignment of a function to a gene product encoding a pyridoxal-phosphate-dependent enzyme on the basis of sequence homology is a challenge, since these enzymes catalyze a vast array of reactions in amino acid metabolism. This implies that the biochemical characterization of the gene product is still required to understand the function of a gene.

To characterize the enzymatic properties of the putative OASS from *L. casei*, the gene was cloned from *L. casei* FAM18110, expressed in *E. coli*, and the recombinant protein was purified to apparent homogeneity. The purified enzyme exhibited OASS activity at neutral and acidic pH when OAS and sulfide were used as substrates. No activity was detected when OAS was replaced with *O*-phosphoserine, a compound that is present in the caseins at the beginning of cheese ripening. Thiosulfate could not replace sulfide, indicating that the gene is more related to CysK than to CysM. When DTT was used instead of sulfide, a product was detected by thin-layer chromatography analysis using ninhydrin for the detection of amino acids (data not shown). Probably, *S*-(2,3-hydroxy-4-thiobutyl)-cysteine is formed, a product that was formed by CysK from *E. coli* and from *A. pernix* (Flint et al. 1996; Mino and Ishikawa 2003).

In silico sequence analyses of genomes from lactic acid bacteria showed that OASS and CBS subfamilies are closely related (Liu et al. 2008). That CysK enzymes exhibit CBS activity has actually been shown for YrhA from *B. subtilis* and CysK from *A. pernix* (Hullo et al. 2007; Mino and Ishikawa 2003). We observed that *L. casei* strains carrying *ctl1* or *ctl2* grew in a chemically defined medium containing methionine as the sole sulfur source (Irmler et al. 2008; Irmler et al. 2009). This implied that cysteine biosynthesis probably occurred via the reverse

transsulfuration pathway. Whereas CGL activity was found in these *L. casei* strains, the presence of a CBS is disputable. To evaluate whether CysK has cystathionine synthase activity, the enzyme was incubated with homocysteine and serine or OAS. However, no cystathionine synthesis was detected.

With regard to cysteine desulfurization, activity was detected when the protein was separated by native gel electrophoresis. Also, the three cystathionine lyases (MalY, MetB, and Ctl1) from *L. casei* that have recently been shown to break down cysteine showed desulfurization activity when analyzed with this method. The cysteine desulfurization activity of each enzyme could be differentiated by the molecular size of the band and the pH-dependent activity. Thus, MalY degraded cysteine at pH 5.5 and 7.5. MetB and CysK were only active at pH 7.5, and CysK had a lower molecular size than MetB. Ctl1 only showed activity at pH 5.5. In the cell-free extracts of the studied *L. casei* strains except for FAM18099, two bands/activities were observed at pH 7.5. The upper band also appeared at pH 5.5 and was not present in FAM18099. Since the pH dependency was similar to the one from MalY, we concluded that the upper band in the cell-free extracts originated from MalY activity. To obtain further evidence for this hypothesis, *malY* from FAM18099, the strain that did not exhibit the upper band, was sequenced. Indeed, it was found that the gene carried a deletion of 16 bp, leading to a truncated non-functional gene product (data not shown).

The lower band in the cell-free extracts that did not appear a pH 5.5 could result from activity of CysK or MetB. Since recombinant MetB clearly migrated with a higher molecular size than recombinant CysK, we think that the lower band actually originated from CysK activity. Remarkably, the intensity and the molecular size of the band varied between the strains. To understand the nature of these differences, further investigations have to be performed using LC and MS methods.

To address the physiological relevance of the desulfurization reaction, the kinetic parameters of the OAS sulfhydrylation and cysteine desulfurization were determined. The apparent K_M value for OAS (0.6 mM) and cysteine (0.7 mM) indicated that the enzyme shows similar affinity for both substrates. The enzyme probably forms an α-aminoacrylate intermediate, as has been reported for other OASS (Tai and Cook 2000). The α-aminoacrylate that resides quite stably in the enzyme is then released by nucleophiles such as sulfide or DTT. In the case that cysteine is the substrate, production of sulfide only occurs when either sulfide is removed from the reaction (e.g., by lead ions) or a better-competing nucleophile (e.g., DTT) replaces the sulfide. It is probable that hydroxide or water molecules also compete with the sulfide for the α-aminoacrylate, but under physiological conditions, sulfide is the better nucleophile and, therefore, the synthesis of cysteine is favored. That *cysK* is actually involved in cysteine biosynthesis was confirmed by the complementation assay, which demonstrated that an *E. coli* auxotroph lacking OASS genes was complemented with *cysK* from *L. casei*.

In comparison with OASS, cystathionine lyases do not require a nucleophile for the cleavage of cysteine. Therefore, OASS activity certainly plays a minor role in hydrogen sulfide production. However, we were able to detect peptides of CysK in tryptic digests of cell-free extracts of all seven *L. casei* strains by mass spectrometry. Furthermore, when proteins of the cell-free extract were separated by 2D gel electrophoresis and stained by Coomassie Blue, CysK appeared as a prominent protein spot that was reliably detected (data not shown). These observations indicate that CysK is an abundant protein in *L. casei*, and cysteine biosynthesis plays an important role. If *L. casei* also produces high amounts of this protein in a cheese matrix, the desulfurization activity, which is the back reaction, may still contribute to the total amount of hydrogen sulfide production. By quantifying the transcripts of *cysK* and the CysK protein in various strains, we identified that FAM18110 is a naturally occurring mutant overexpressing the gene. Future studies involving this strain will help us to evaluate the impact of *cysK* on the amount of sulfur-containing amino acids and hydrogen sulfide.

The sources of sulfide and OAS required for CysK still remain enigmatic in *L. casei*, especially as the genomic data of *L. casei* ATCC 334 indicate that the genes for sulfate assimilation and reduction are not present (Makarova et al. 2006). Moreover, a gene encoding a serine acetyltransferase, which catalyzes the synthesis of OAS, is apparently not present. In cheese, *L. casei* may acquire cysteine by the uptake of cysteine and peptides containing cysteine derived by the proteolysis of the caseins. However, cysteine is present in lower amounts than methionine in the caseins, and it is probable that cysteine biosynthesis gives a fitness benefit.

With regard to sulfide, we think that *L. casei* cleaves homocysteine, a degradation product of methionine, to yield sulfide. This is achieved by the action of cystathionine lyases, which cleave C–S bonds via α,γ-elimination reactions (e.g., Ctl1 or Ctl2). With regard to OAS synthesis, an open reading frame is located upstream of *cysK*, which is annotated as homoserine *O*-succinyltransferase in ATCC 334. It is possible that this gene is wrongly annotated and represents the missing *cysE* homolog. This hypothesis is supported by the observation that both genes are transcribed together. To obtain further evidence, we have planned to clone the gene and study the enzymatic properties of this gene product.

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