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YtjE from *Lactococcus lactis* IL1403 Is a C-S Lyase with α,γ-Elimination Activity toward Methionine

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Cheese microbiota and the enzymatic conversion of methionine to volatile sulfur compounds (VSCs) are important factors in flavor formation during cheese ripening and the foci in biotechnological approaches to flavor improvement. The product of *ytjE* of *Lactococcus lactis* IL1403, suggested to be a methionine-specific aminotransferase based on genome sequence analysis, was therefore investigated for its role in methionine catabolism. The *ytjE* gene from *Lactococcus lactis* IL1403 was cloned in *Escherichia coli* and overexpressed and purified as a recombinant protein. When tested, the YtjE protein did not exhibit a specific methionine aminotransferase activity. Instead, YtjE exhibited C-S lyase activity and shared homology with the MalY/PatC family of enzymes involved in the degradation of L-cysteine, L-cystine, and L-cystathionine. YtjE was also shown to exhibit α,γ-elimination activity toward L-methionine. In addition, gas chromatographic-mass spectrometry analysis showed that YtjE activity resulted in the formation of H_2S from L-cysteine and methanethiol (and its oxidized derivatives dimethyl disulfide and dimethyl trisulfide) from L-methionine. Given their significance in cheese flavor development, VSC production by YtjE could offer an additional approach for the development of cultures with optimized aromatic properties.

The enzymatic conversion of amino acids to aroma compounds plays a major role in cheese flavor development. Methionine (Met) catabolism has received particular attention, as it is believed to be the precursor of numerous, diverse, and quantitatively minor volatile sulfur compounds (VSCs) (29) which make important contributions to the overall flavor that is typical in different cheeses (7). Most of these compounds are derived from the degradation of Met to methanethiol (MTL), subsequently leading to a variety of compounds such as dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and *S*-methylthioesters (31).

The role of cheese microbiota, the enzymes involved in the conversion of amino acids, and the regulation of enzymatic conversions are important factors in efforts to control flavor formation during cheese ripening. As the enzymatic conversion of Met is of prime importance in flavor development, the understanding of Met degradation pathways in the cheese ecosystem is of great significance, especially those associated with lactococci which are widely used as starter cultures. Acquiring this knowledge would be constructive in determining flavor contribution pathways and could lead to the development of cultures with optimized aromatic properties.

The Met biosynthetic and catabolic pathways leading to MTL do vary among bacteria (26), as do the enzymes involved and the amount of MTL produced during cheese ripening (13). Two enzymatic pathways in lactococci have been postulated. In the first one, a single-step route, a lyase (cystathionine β- or γ-lyase), catalyzes the simultaneous deamination and demethylthiolation of Met to MTL (2, 10, 14, 17). In *Brevibacterium linens*, disruption of the *mgl* gene encoding L-methionine γ-lyase, which catalyzes the α,γ-elimination of Met to MTL, almost eliminated this strain's considerable capacity to produce VSCs (3). In lactococci, however, cystathionine β- and γ-lyases are thought to have relatively low activities toward Met. The other route involves a two-step mechanism initiated by an aminotransferase that leads to the formation of 4-methylthio-2-oxobutyric acid (KMBA), which is subsequently converted to MTL by either chemical decomposition or enzymatic conversion due to a demethiolating activity. Using nuclear magnetic resonance and gas chromatography-based approaches, this second pathway has been observed in lactococci (19) as well as in several other cheese-ripening bacteria (8).

It has been established that in lactococci, transamination is the first step in Met catabolism. Two major aminotransferases involved in the degradation of Met, the aromatic (AraT) and branched chain (BcaT), have been purified from *Lactococcus lactis* subsp. *cremoris* NCDO 763 and their corresponding genes sequenced (22, 33). However, inactivation of both these genes did not abolish VSC formation from Met, suggesting the involvement of another aminotransferase or pathway (23). Nevertheless, no aminotransferase specific for Met has been cloned and characterized. From the genome of *L. lactis* IL1403, 12 aminotransferases could be predicted, and some of these could initiate degradation of aromatic, branched-chain, and sulfur-containing amino acids important for cheese flavor (6). From these, the product of *ytjE* has been suggested to be specific for Met transamination, as the gene is cotranscribed with the relevant biosynthesis genes (6). More recently, Sperandio et al. (27) proposed that YtjE is involved in cystathionine

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conversion to homocysteine when they studied sulfur metabolism and its regulation in *L. lactis*.

The aim of this study was to characterize YtjE, a putative methionine-specific aminotransferase, and to elucidate its participation in methionine catabolism. For this purpose, the *ytjE* gene isolated from *L. lactis* IL1403 was cloned and expressed in *Escherichia coli* and the YtjE recombinant protein was purified and characterized. The activity of the YtjE protein and its role in VSC production were also investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains DH5α and BL21λ(DE3) (Novagen, Nottingham, United Kingdom), used for cloning and expression of recombinant protein, respectively, were grown aerobically at 37°C in Luria-Bertani (LB) medium. Plasmids were electroporated into *E. coli* (15), and transformants were selected on LB agar (1.5%) plates supplemented with kanamycin (30 μg ml^{-1}) (Sigma, Dorset, United Kingdom) to select for recombinant pET28b plasmid (Invitrogen, Paisley, United Kingdom). *L. lactis* IL1403 (11) and *L. lactis* NCDO 763 (33) were grown at 30°C without agitation in M17 medium supplemented with 0.5% (wt/vol) glucose (G-M17). Growth of bacterial cultures was routinely monitored by measuring the optical density at 600 nm.

Purification of recombinant YtjE protein. Molecular cloning techniques were performed essentially as described by Sambrook et al. (24). To overexpress His-tagged YtjE, a 1.1-kb DNA fragment carrying the *L. lactis* IL1403 *ytjE* gene (GenBank accession number AE006422) was amplified from genomic DNA by PCR using primers YTJEF (5′-CCCATGGGAACAAAATATGATTTTACAA CCATTCC-3′) and YTJER (5′-TTGCGGCCGCTTAAATTTTGAATGTTTT ACGAGTCG-3′), digested with the NcoI and NotI restriction enzymes, and cloned into pET28b (Novagen) to generate pET28b-*ytjE*. Transformants containing pET28b-*ytjE* were identified by colony PCR and restriction enzyme analysis of prepared plasmid DNA. Sequencing confirmed that the YtjE coding sequence was in frame with a carboxyl-terminal His_6 tag encoded by the vector. The pET28b-*ytjE* plasmid was transformed into *E. coli* BL21λ(DE3), and expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG). Protein was extracted, and expression of recombinant YtjE was confirmed using the InVision His tag in-gel stain system (Invitrogen). To optimize YtjE production, cultures were grown at 30°C to an optical density at 600 nm of ~0.5 before we added IPTG (1 mM), and then the cultures were incubated for 3 h at 30°C. Protein was purified using Ni^{2+} affinity chromatography columns (QIAGEN Ltd., Crawley, United Kingdom). Eluted YtjE was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and selected fractions were pooled, dialyzed against a phosphate-buffered saline (pH 7.0) solution containing 10% glycerol, and quantified using the Bradford assay (9).

Preparation of cell samples for enzymatic assays. YtjE activity was assayed using purified His-tagged YtjE protein and cell extracts (CFEs) prepared from IPTG-induced cultures of the *E. coli* pET28b-*ytjE* strain. CFEs prepared from cultures of *E. coli* BL21λ(DE3) carrying empty vector (IPTG induced) and *L. lactis* IL1403 (grown to logarithmic phase) were used as controls. To prepare CFEs, *E. coli* and *L. lactis* cultures (50 ml) were harvested by centrifugation (3,220 × *g*, 15 min) at 4°C. The cell pellets were washed twice in equal volumes of ice-cold potassium phosphate buffer (50 mM; pH 7.0) and resuspended in 2 ml of ice-cold cell suspension buffer (50 mM potassium phosphate, 10 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 100 μM pyridoxal-5′-phosphate [PLP], pH 7.5). A 1-ml aliquot was then transferred to a microcentrifuge tube containing 1 g of sterile glass beads (1-mm diameter) (Sigma) and vortexed (four times for 2 min, with 1-min intervals on ice). The insoluble fraction and beads were removed by centrifugation (10,000 × *g*) for 10 min at 4°C, and the supernatant fraction containing soluble protein was aliquoted, frozen in liquid nitrogen, and stored at −80°C until used in enzyme assays.

Aminotransferase activity. Aminotransferase assays were carried out with the R-Biopharm colorimetric L-glutamic acid assay kit (Glasgow, United Kingdom), using different amino acids as substrates (L-methionine, L-isoleucine, L-valine, L-aspartic acid, and L-cysteine). In this assay, the reduced cofactor (NADH) produced by oxidative deamination of L-glutamic acid reacts with iodonitrotetrazolium in the presence of diaphorase to produce a product that absorbs at a wavelength of 492 nm (A_{492}). One-milliliter reaction mixtures (70 mM Tris HCl, pH 7.8, 10 mM amino acid, 10 mM α-ketoglutarate, 100 μM PLP, and purified YtjE protein or CFEs) were incubated at 37°C for 1 h and 24 h, and then the reactions were stopped by adding sulfosalicylic acid (3% [wt/vol] final concentration). The colorimetric L-glutamic acid assay was carried out using 30 μl of the reaction mixture, and the increase in A_{492} was monitored during incubation at 37°C for 30 min and 3 h using a Bioscreen (Bioscreen C; ThermoLifeSciences, Basingstoke, United Kingdom). A standard curve was obtained using different concentrations of L-glutamic acid. CFEs prepared from *L. lactis* NCDO 763, a strain previously shown to exhibit aminotransferase activity toward Met, were used as a positive control (33). Met aminotransferase activity of the purified YtjE protein was also assayed by measuring the formation of KMBA from L-methionine by reverse-phase high-pressure liquid chromatography (HPLC), using an Agilent HP1100 HPLC system with a PDA UV-vis detector. Reaction products were separated using a Gemini C_{18} column (150 mm by 2.0 mm by 5 μm) (Phenomenex, Macclesfield Cheshire, United Kingdom) thermostated at 30°C. The mobile phase was a linear gradient of acetonitrile plus 0.1% trifluoroacetic acid (solvent B) in Milli-Q water (Millipore Co., Bedford, MA) plus 0.1% trifluoroacetic acid (solvent A) at a flow rate of 0.3 ml min^{-1}. The sample injection volume was 20 μl. The elution profile was monitored at 220 nm, and peaks were identified by comparing retention times with standards.

TABLE 1. Effects of inhibitors on C-S lyase activity of YtjE

Inhibitor	Inhibitor concn	Relative activity (%)[a]
Hydroxylamine	1 mM	1.42 (±0.09)
	10 mM	0.37 (±0.03)
3-Methyl-2-benzothiazolinone hydrazone	1 mM	23.64 (±0.59)
	10 mM	21.71 (±0.49)
Iodoacetic acid	1 mM	24.14 (±1.25)
	10 mM	2.49 (±0.28)
EDTA	1 mM	115.00 (±2.33)
	10 mM	124.00 (±0.38)
NaCl	0.4% (wt/vol)	78.00 (±1.06)
	4% (wt/vol)	22.00 (±0.05)

[a] 100% activity is the enzyme activity toward L-cystathionine (2 mM) with no inhibitor added, using the thiol determination assay. Values are the means of three determinations, and the standard deviations are shown in parentheses.

C-S lyase activity. C-S lyase activity of YtjE was determined by measuring the formation of (i) free thiol groups, (ii) keto acid, and (iii) ammonia, using L-cysteine, L-cystathionine, L-cystine, or L-methionine as the substrate.

The formation of free thiol groups was determined according to a method previously described by Uren (30). Reaction mixtures containing 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 100 μM PLP, substrate (at different concentrations), and either YtjE or CFEs suspended in a 100 mM Tris HCl (pH 8.0) solution were incubated at 37°C for 30 min and 2 h, and the increase in A_{412} was measured at 2-min intervals. The molar absorption coefficient value used for aryl mercaptide was 13,200 liter mol^{-1} cm^{-1}, with 1 enzyme unit representing the formation of 1 μmol aryl mercaptan min^{-1}. The kinetic parameters were computed from the Lineweaver-Burk transformation (V^{-1} versus S^{-1}) of the Michaelis-Menten equation, where V is the formation rate of free thiol groups (μmol min^{-1} mg^{-1} protein) and S is the concentration (mM) of each substrate. To further characterize enzyme activity, the purified YtjE protein was also incubated with different inhibitors (at the indicated concentrations) (Table 1) before the formation of free thiol groups was measured, using L-cystathionine (2 mM) as the substrate.

Keto acid formation was detected as described previously (16). Briefly, the assay was carried out in 1 ml of 100 mM Tris HCl (pH 8.0) containing 100 μM PLP, 2 mM substrate, and various amounts of protein. The reaction was started by adding protein, incubated for 60 min at 37°C, and stopped by adding 0.5 ml of 4.5% trichloroacetic acid. The suspension was centrifuged, and 0.5 ml of the recovered supernatant was added to 0.5 ml of 0.05% 3-methyl-2-benzothiazolinone hydrazone (in 1 M sodium acetate, pH 5.2) and incubated at 50°C for 30 min. The amounts of keto acid produced were spectrophotometrically measured (A_{335}). The formation of pyruvate was also assayed in a lactate dehydrogenase-coupled reaction mixture containing 100 mM Tris HCl (pH 8.0), 2 mM substrate, 100 μM PLP, 200 μM NADH, and 10 μg of lactate dehydrogenase. Reaction mixtures were prewarmed at 37°C prior to the addition of YtjE or CFEs, and the reduction in A_{340} due to oxidation of NADH to NAD was then monitored as a measure of the amount of pyruvate produced.

The formation of ammonia was assayed by measuring the amount of NADH

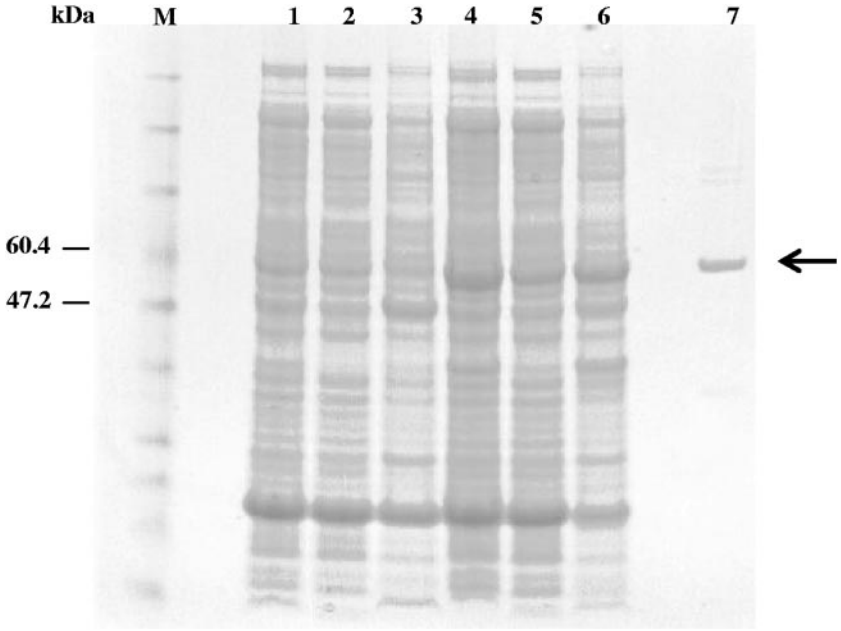

FIG. 1. SDS-PAGE analysis of CFEs prepared from an IPTG-induced culture of *E. coli* pET28-*ytjE* (lanes: 4, total fraction; 5, soluble fraction; 6, insoluble fraction). CFEs prepared from an IPTG-induced culture of *E. coli* carrying empty vector (lanes: 1, total fraction; 2, soluble fraction; 3, insoluble fraction) were included as a control. Purified His-tagged YtjE protein is also shown (arrow; lane 7). The molecular mass markers are also indicated (kDa).

oxidized in the presence of α-ketoglutarate and glutamate dehydrogenase. One-milliliter reaction mixtures containing 100 mM Tris HCl (pH 8.0), 2 mM substrate, 100 μM PLP, and either YtjE or CFEs were incubated at 37°C for 1 h and 24 h. The ammonia assay was carried out using 30 μl of the reaction mixture. The oxidation of NADH was monitored by measuring the decrease in absorbance at 340 nm at intervals of 3 min for 1 h.

Detection of C-S lyase activity by in situ staining. L-Cysteine desulfhydrase activity was confirmed by visualizing enzyme activities (32). YtjE or CFEs from *E. coli* and *L. lactis* were added to 150 μl of a visualizing solution containing 100 mM triethanolamine HCl (pH 7.6), 100 μM PLP, 0.5 mM bismuth trichloride, 10 mM EDTA, 1% Triton X-100, and 5 mM L-cysteine. These mixtures were then incubated at 37°C to detect the development of black precipitate formed by the reaction of sulfide with bismuth. C-S lyase activities (toward L-cysteine) of YtjE and CFEs were also monitored using Tris-glycine gels (Invitrogen) run under nondenaturing conditions. Following electrophoresis (20 mA at 4°C for 2 h), gels were incubated at 37°C in 10 ml of the visualizing solution to detect black precipitate at the position of enzyme activity.

Identification of reaction products of L-cystathionine due to YtjE activity. Amino acid products of YtjE activity toward L-cystathionine were identified by HPLC analysis (at 214 nm) of reaction mixtures containing DTNB derivatives of free thiol group-containing amino acids, as described by Bruinenberg et al. (10).

Determination of cysteine. The specificity of YtjE activity toward L-cystathionine (β/γ) was also assayed by the ninhydrin procedure (18). The formation of cysteine due to YtjE cystathionine γ-lyase activity was determined based on the specific reaction of cysteine with acid nynhydrin reagent (250 mg of ninhydrin in a mixture of 6 ml of acetic acid and 4 ml of HCl) by measuring the increase in absorbance (A_{560}).

VSCs produced by YtjE. YtjE activity toward L-methionine and L-cysteine was also determined by analyzing the production of VSCs. Reactions were carried out in 100 mM Tris HCl (pH 8.0) containing 100 μM PLP and either L-methionine or L-cysteine (5 mM) mixed with YtjE protein or CFEs and incubated at 25°C for 48 h, with samples taken at different time intervals. The products formed were determined by solid-phase microextraction and gas chromatography-mass spectrometry (GC-MS). Samples were allowed to warm to room temperature before the headspace was sampled for 15 min with an 85-μm Carboxen-polydimethylsiloxane fiber (Supelco, Dorset, United Kingdom). The fibers were cleaned by heating at 250°C before use and after injection. Volatile compounds were analyzed by a gas chromatograph (model 6890; Hewlett Packard) connected to an HP 5973 mass selective detector. Samples were injected manually onto a DB-FFAP column (30 m by 0.32 mm; 1-μm film thickness) (Agilent J&W GC; Agilent, Cheshire, United Kingdom) at a helium flow rate of 2 ml min^{-1} for analysis. The oven temperature was held at 40°C for 5 min, then programmed to increase to 100°C at a rate of 5°C min^{-1}, and held at this temperature for 1 min before increasing again to 240°C at a rate of 30°C min^{-1}; the temperature of the column was then held at 240°C for 1 min before returning to 40°C. The injector port was maintained at a temperature of 250°C and operated in the splitless mode. The MS source temperature was 200°C, and the transfer line was 250°C. The mass selective detector was scanned from 30 to 300 *m/z* at a speed of 0.72 scans s^{-1}. Areas of peaks were calculated using ChemStation software (Agilent Technologies UK Ltd., United Kingdom), selecting the beginning and end of molecular peaks manually. Sulfur-containing compounds were identified by comparing retention times and mass spectra with those obtained using standards prepared individually or as mixtures by using pure stocks of DMDS and DMTS (Sigma).

RESULTS

Expression and purification of *L. lactis* IL1403 YtjE. To obtain purified protein for enzymatic analysis, the *ytjE* gene from *L. lactis* IL1403 was cloned and overexpressed in *E. coli* as a C-terminal His-tagged fusion protein. The cloned *ytjE* gene was identical to the native gene, except for the introduction of a triplet of bases (GGA) resulting in the addition of an extra amino acid (Gly) at position 2 at the N terminus of the recombinant YtjE protein. SDS-PAGE analysis and His-tag in-gel staining of IPTG-induced CFE of the recombinant *E. coli* strain confirmed expression of YtjE, which was detected in both the soluble and insoluble fractions, the latter probably due to the formation of insoluble aggregates as a result of the high level of expression (Fig. 1). His-tagged YtjE protein was then purified under native conditions by affinity chromatography on nickel-nitrilotriacetic acid resin, and its purity was confirmed by SDS-PAGE (Fig. 1). SDS-PAGE analysis of the

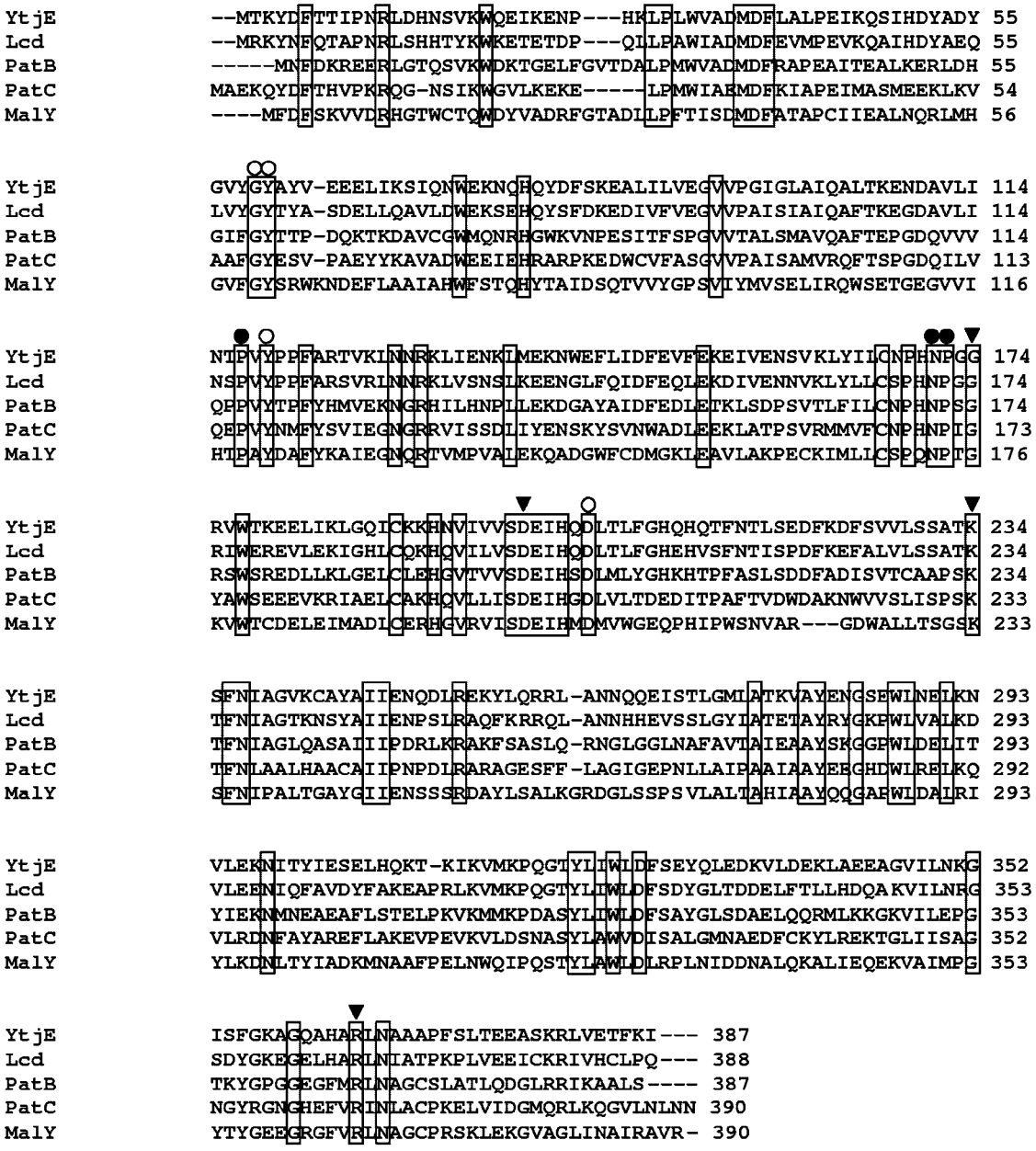

FIG. 2. Alignment of YtjE amino acid sequence from *L. lactis* IL1403 with Lcd from *S. anginosus*, PatB from *B. subtilis*, PatC from *L. delbrueckii*, and MalY from *E. coli*. Boxes indicate identical amino acid residues in all the sequences analyzed. The four residues that are invariant in all aminotransferases (▼) (1) are shown. The Lys_{233} residue is the potential binding site of PLP. Residues typical for aminotransferases (●) and those typical for *trans*-sulfuration enzymes (○) are also indicated.

recombinant protein showed a band of approximately 55 kDa, a molecular mass higher than that predicted using its amino acid sequence (46 kDa).

Methionine aminotransferase activity of YtjE. In the genome of *L. lactis* IL1403, the product of *ytjE* has been suggested to be an aminotransferase specific for Met (6). The putative Met aminotransferase activity of YtjE was therefore assayed by measuring the formation of L-glutamic acid, using Met as the substrate and α-ketoglutarate as an acceptor of the amino group. Similar levels of L-glutamic acid (mean ± standard deviation) were detected in reaction mixtures containing CFEs from the *E. coli* strains expressing (58 ± 9 nmol min^{-1} mg^{-1} protein) or not expressing (51 ± 11 nmol min^{-1} mg^{-1} protein) recombinant YtjE, and no L-glutamic acid could be detected with the purified protein. As expected, high levels of glutamate were obtained when we used CFE from *L. lactis* NCDO 763 (112 ± 10 nmol min^{-1} mg^{-1} protein). When tested, purified YtjE was also shown not to exhibit aminotransferase activity toward L-valine, L-isoleucine, L-phenylalanine, L-cysteine, and L-aspartic acid. To confirm these results, the putative Met aminotransferase activity was also assayed by reverse-phase HPLC analyses as described in Materials and

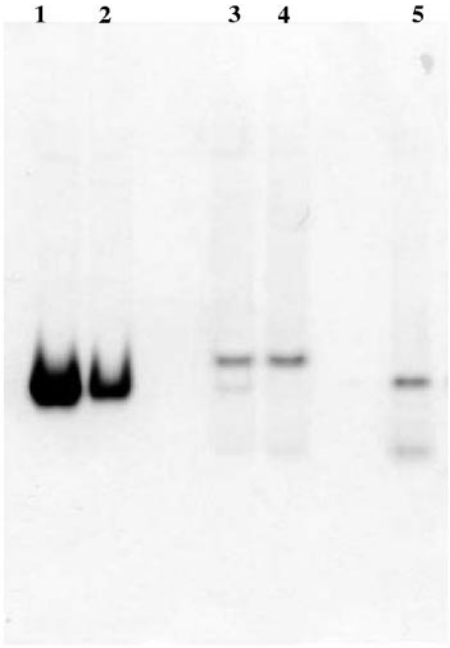

FIG. 3. Cysteine desulfhydrase activity by in situ staining. YtjE activity was monitored in Tris-glycine gels under nondenaturing conditions, using L-cysteine as the substrate. Lanes: 1, 5 μg purified recombinant protein; 2, 2.5 μg purified recombinant protein; 3, CFE from *E. coli* recombinant strain expressing YtjE; 4, CFE from *E. coli* carrying empty vector; 5, CFE from *L. lactis* IL1403.

Methods. KMBA, a product of Met transamination, could not be detected. These results suggest that YtjE is not a Met aminotransferase.

C-S lyase activity. An interrogation of the database, using the deduced YtjE amino acid sequence, revealed relatively high levels of homology to the Lcd protein (59% identity) of *Streptococcus anginosus*, the PatB protein (38%) of *Bacillus subtilis*, the PatC protein (33%) of *Lactobacillus delbrueckii*, and the MalY protein (28%) of *E. coli* (Fig. 2). These proteins have previously been described as C-S lyases with α,β-elimination activity (4, 5, 32, 34). On the basis of this information, the activity of YtjE (in CFE and as purified protein) was investigated by assessing its ability to generate free thiol groups, α-keto acid, and ammonia, using the sulfur-containing compounds L-cysteine, L-cystathionine, L-cystine, and L-methionine as substrates. When their CFEs were compared, the degradation of these substrates measured by the formation of free thiol groups (reacting with DTNB) was higher in the *E. coli* pET28b-*ytjE* strain than in the vector control, thereby suggesting a role for YtjE. This was confirmed using purified YtjE protein, which, under these study conditions, exhibited higher activity toward L-cystine (2,230 ± 120 mU mg^{-1} protein) than toward L-cystathionine (650 ± 10 mU mg^{-1}) and L-methionine (40 ± 1 mU mg^{-1}). The apparent K_m values determined from Lineweaver-Burk plots for L-cystine, L-cystathionine, and L-methionine were 0.46 ± 0.10 mM, 0.27 ± 0.05 mM, and 0.87 ± 0.18 mM, respectively. The relative activities of YtjE toward these substrates were replicated when enzyme activity was determined by measuring the formation of keto acids and ammonia. Furthermore, enzymatic activity toward these substrates always resulted in the formation of a free thiol group, a keto acid component, and ammonia as reaction products, thus confirming the C-S lyase activity of YtjE.

C-S lyase activity toward L-cysteine, also known as cysteine desulfhydrase activity, was assayed by in situ staining. Using this approach, cysteine desulfhydrase activity was conveniently monitored in nondenaturing gels by using L-cysteine as the substrate. Hydrogen sulfide (H_2S) reacts with bismuth to produce an insoluble product which forms brown-to-black bands on the gels (32). Electrophoresis under nondenaturing conditions of purified YtjE revealed a single dark-brown band (Fig. 3), confirming the ability of the YtjE protein to catalyze the α,β-elimination reaction of L-cysteine to H_2S. The same band was also detected in CFEs from a recombinant *E. coli* strain expressing YtjE and *L. lactis* IL1403.

It has been reported that L-cystathionine degradation can lead to the production of different free thiol-containing compounds, homocysteine and cysteine, via two reactions involving α,β-elimination and α,γ-elimination activities, respectively. When tested, homocysteine was identified to be the sole free thiol-containing compound of L-cystathionine degradation due

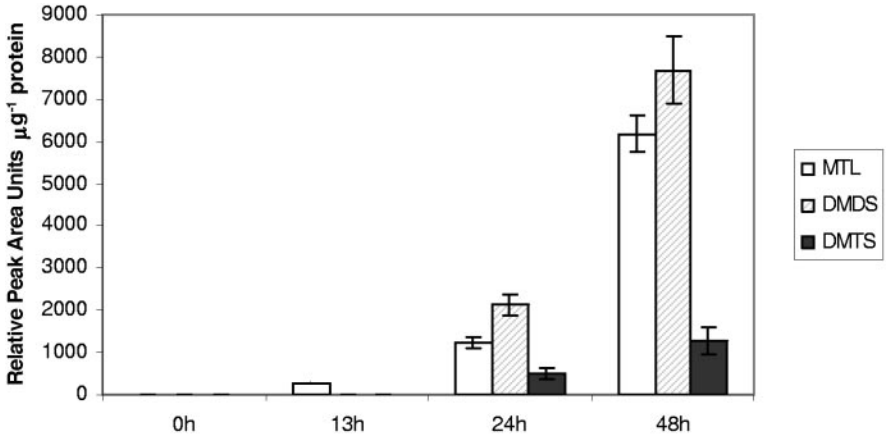

FIG. 4. Production of MTL, DMDS, and DMTS by coincubating L-methionine with the purified YtjE protein. Values are the means of three independent measurements. The standard errors of the means are also indicated.

to the purified YtjE protein by HPLC analysis of DTNB-derivatized reaction products (data not shown). Moreover, the formation of cysteine could not be detected using the ninhydrin method. These results strongly suggest therefore that the YtjE enzyme exhibits only α,β-elimination activity toward L-cystathionine.

The effects of selected inhibitors on YtjE activity, measured by the formation of free thiol groups using L-cystathionine as the substrate, are summarized in Table 1. As with most cystathionine β/γ-lyases (2, 10, 25), YtjE activity was strongly inhibited by carbonyl reagents such as hydroxylamine and 3-methyl-2-benzothiazolinone hydrazone, known inhibitors of PLP-dependent enzymes. The chelating reagent EDTA had no inhibitory effect on activity, while the addition of 4% NaCl resulted in a threefold reduction in enzyme activity. Contrary to the aforementioned cystathionine β/γ-lyases, however, YtjE was also sensitive to the thiol-reactive agent iodoacetic acid.

Formation of volatile sulfur compounds. YtjE activity toward L-cysteine and L-methionine was also assayed by GC-MS in order to identify the VSCs formed. The VSCs formed were H_2S (relative peak area of 110,012 ± 10,810 units μg^{-1} after 24 h of incubation) from L-cysteine and MTL, DMDS, and DMTS from L-methionine (Fig. 4). These compounds could not be detected in control samples incubated in the absence of YtjE. These results indicate that in addition to demonstrating α,β-elimination activity toward L-cysteine, YtjE can also exhibit α,γ-elimination activity with L-methionine as the substrate. As shown in Fig. 4, the formation of MTL increased with incubation time along with the formation of DMDS and DMTS. This is not surprising, as MTL is a highly unstable sulfur compound that quickly reacts to form the oxidized and more stable compound DMDS and, to a lesser extent, DMTS.

DISCUSSION

This work set out to characterize the YtjE protein of *L. lactis* IL1403 and to elucidate its role in methionine catabolism and VSC formation. Therefore, the *ytjE* gene from *L. lactis* IL1403 was cloned in *E. coli* and expressed and purified as a recombinant protein. When tested, the YtjE protein was shown not to possess a specific methionine aminotransferase activity, which disagrees with the published genome analysis of *L. lactis* IL1403. Instead, it was shown in this study that the *ytjE* gene encodes a protein which exhibits C-S lyase activity toward a variety of substrates.

C-S lyases (β/γ) are PLP-dependent enzymes. The β-lyase catalytic activity is associated mainly with the cleavage of L-cystathionine to homocysteine (an α,β-elimination reaction), while the γ-lyase converts L-cystathionine to cysteine (an α,γ-elimination reaction); both cystathionine lyases can degrade Met into MTL, α-ketobutyrate, and ammonia via the α,γ-elimination reaction (10, 13, 14). C-S lyases have also been reported to catalyze α,β elimination of L-cysteine and L-cystine, leading to the production of hydrogen sulfide and thiocysteine, respectively (20, 32). The protein encoded by *ytjE* does not share homology with the MetC group of C-S lyases that have previously been characterized for *L. lactis* (17). The MetC-type proteins are members of the γ family of PLP-dependent enzymes involved in sulfur transfer from cysteine to methionine (21). The deduced YtjE amino acid sequence did, however, reveal high levels of homology (28 to 59%) with the MalY/PatC group of C-S lyases described for *S. anginosus*, *B. subtilis*, *L. delbrueckii*, and *E. coli* (4, 5, 32, 34) that demonstrate weak similarity to aminotransferases belonging to the α family of PLP-dependent enzymes (1, 21). Interestingly, MalY from *E. coli* also shows homology to *trans*-sulfuration enzymes that are also members of the γ family of PLP enzymes, thereby representing an evolutionary link between the two families of PLP-containing enzymes (12). Sequence alignment analysis revealed four amino acid residues in YtjE shown to be invariant in aminotransferases that are members of the α family of PLP-dependent enzymes (1). At the same time, residues typically found in *trans*-sulfuration enzymes of the γ family were also conserved in YtjE (Fig. 2).

As shown previously with MalY from *E. coli*, Lcd from *S. anginosus*, PatB from *B. subtilis*, and PatC from *L. delbrueckii* (4, 5, 32, 34), biochemical characterization of the YtjE protein confirmed it to be a C-S lyase with α,β-elimination activity toward L-cysteine, L-cystathionine, and L-cystine. As with these enzymes, the relative activity of YtjE also favors L-cystine over L-cystathionine. Contrary to that described for Lcd from *S. anginosus* (32), however, YtjE from *L. lactis* IL1403 is also capable of α,γ-elimination activity toward L-methionine. Other PLP-dependent enzymes, such as the L-methionine γ-lyase from *Pseudomonas putida* (28) and the cystathionine β-lyase from *L. lactis* subsp. *cremoris* B78 (2), have also been shown to carry out either α,β- or α,γ-elimination reactions depending on the substrates. A cystathionine β/γ-lyase from *L. lactis* subsp. *cremoris* MG1363 can catalyze both reactions with L-cystathionine (14). While disruption of the encoding gene *metC* did result in a dramatic decrease in this strain's cystathionine lyase activity, no differences were observed when Met was used as the substrate (17), thus suggesting that other lyases such as YtjE might be involved in this reaction. The effects of inhibitors on YtjE activity were comparable to those reported previously for cystathionine lyases in *L. lactis* (2, 10). These enzymes are strongly inhibited by carbonyl reagents but are not inhibited by EDTA, indicating that metal ions are not required for their activity. In contrast to these enzymes, however, YtjE activity was sensitive to sulfhydryl reagents. In this respect, YtjE is similar to the cystathionine lyases of *L. delbrueckii* (PatC) (4) and *Bordetella avium* (20), which also show marked inhibition by the thiol-reactive agent iodoacetic acid.

The formation of VSCs due to YtjE C-S lyase activity toward L-methionine and L-cysteine was also confirmed; GC-MS identified MTL and its autooxidation products DMDS and DMTS, resulting from the degradation of L-methionine and H_2S from L-cysteine. As the impact of these VSCs on the development of flavor in cheese is widely accepted (29), these results indicate that YtjE or similar enzymes in *L. lactis* IL1403 could play an additional role in the formation of flavor components during cheese maturation. YtjE activity toward L-methionine is relatively low compared to the activities shown toward L-cystathionine and L-cystine. Similarly, the efficiency of Met conversion by cystathionine β- and γ-lyases in *L. lactis* has been shown to be about 100-fold less than that of cystathionine (2, 10). Nevertheless, strains that overproduce cystathionine lyases have also been found to degrade Met efficiently (17), and it is well accepted that the organoleptic properties of derived com-

pounds are pronounced at very low concentrations due to their low odor thresholds (3, 13).

In summary, although genome sequence analysis is an invaluable tool for unraveling the function of a gene product, biochemical characterization of the encoded protein is essential for the correct identification of its function. The results shown here demonstrate that YtjE from *L. lactis* IL1403 is a C-S lyase with α,γ-elimination activity that degrades L-methionine. These activities allied to the formation of different VSCs indicate that *L. lactis* YtjE could offer an approach for diversifying and increasing the production of VSCs that are important in cheese flavor development.

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