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Cysteine biosynthesis in Lactobacillus casei: identification and characterization of a serine acetyltransferase

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One sentence summary: A gene that is annotated as homoserine succinyltransferase actually encodes a serine acetyltransferase in Lactobacillus casei.

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ABSTRACT

In bacteria, cysteine can be synthesized from serine by two steps involving an L-serine O-acetyltransferase (SAT) and a cysteine synthase (CysK). While CysK is found in the publicly available annotated genome from Lactobacillus casei ATCC 334, a gene encoding SAT (cysE) is missing. In this study, we found that various strains of L. casei grew in a chemically defined medium containing sulfide as the sole sulfur source, indicating the presence of a serine O-acetyltransferase. The gene lying upstream of cysK is predicted to encode a homoserine trans-succinylase (metA). To study the function of this gene, it was cloned from L. casei FAM18110. The purified, recombinant protein did not acylate L-homoserine in vitro. Instead, it catalyzed the formation of O-acetyl serine from L-serine and acetyl-CoA. Furthermore, the plasmid expressing the L. casei gene complemented an Escherichia coli cysE mutant strain but not an E. coli metA mutant. This clearly demonstrated that the gene annotated as metA in fact encodes the SAT function and should be annotated as cysE.

Keywords: Lactobacillus casei; serine acetyltransferase; cysE; cysteine biosynthesis

INTRODUCTION

Lactobacillus casei is often present in the non-starter microbiota of cheese at the end of ripening (Beresford and Williams 2004). This implies that this species plays a significant role in proteolysis and flavor development during cheese ripening and that it could be used as adjunct culture with starter bacteria to improve ripening control and enhance flavor intensity. Knowledge of metabolic activities and pathways is advantageous in the selection of proper strains. The increasing number of newly sequenced genomes allows for the prediction of phylogenetic relatedness and metabolic pathways (Siezen et al. 2004; Liu et al. 2008). However, annotation of these genomes relies mostly on sequence and structural comparisons, which may lead to incorrect functional gene assignments and consequently to incorrect metabolic pathway predictions.

The biosynthesis of cysteine in cheese-inhabiting bacteria is of particular interest since cysteine is, unlike methionine, present in lower amounts in caseins (Farrell et al. 2004) and could therefore be a growth-limiting source. Furthermore, cysteine and methionine are precursors for the formation of odor-active volatile sulfur compounds during cheese ripening (Landaud, Helinck and Bonnarme 2008).
Cysteine can be synthesized from serine or homocysteine (Fig. 1). The biosynthesis from serine is well characterized in Enterobacteriaceae (Kredich 1996). First, serine is acetylated to O-acetyl-L-serine (OAS) by a serine acetyltransferase (SAT, EC 2.3.1.30), which is encoded by cysE. Second, cysteine synthase (EC 2.5.1.47) encoded by cysK replaces the acetyl group by sulfide to form cysteine. We have recently demonstrated that the orthologous cysK gene from L. casei FAM18110 actually synthesizes cysteine from OAS and sulfide (Bogicevic et al. 2012). Surprisingly, the annotated genome of L. casei ATCC 334 apparently does not carry a gene encoding SAT, which is necessary for the formation of OAS (Makarova et al. 2006; Liu et al. 2008). In connection with the characterization of cysK from L. casei, we observed that the gene was co-transcribed with the coding sequence that lies upstream of cysK (Bogicevic et al. 2012). This gene is annotated as homoserine O-succinyltransferase (Meta, EC 2.3.1.46) in various L. casei genomes that are deposited in the GenBank database (Table S1, Supporting Information). Meta is an enzyme that catalyzes the acylation of homocysteine (Fig. 1) and should therefore be involved in methionine biosynthesis (Born and Blanchard 1999).

In this paper, we provide functional data showing that L. casei synthesizes cysteine from serine. First, the growth of various L. casei strains with sulfide as the sulfur source was determined. Furthermore, the gene encoding the putative meta was cloned and the recombinant protein was used for enzymatic assays. Finally, complementation tests of Escherichia coli mutants were performed to confirm enzymatic results.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, DNA manipulation and culture media**

The strains and plasmids used in this study are listed in Table 1. Genomic and plasmid DNA were prepared as described previously (Bogicevic et al. 2012). Escherichia coli strains were grown at 37°C in LB broth (Sambrook, Fritsch and Maniatis 1989). When necessary, ampicillin (amp) was added at a concentration of 100 μg mL⁻¹. Lactobacillus casei strains were grown at 30°C in MRS broth (de Man, Rogosa and Sharpe 1960).

The chemically defined medium (CDM) described by Christensen and Steele (2003) was used to study the dependency on various sulfur sources. CDM containing either sodium sulfide, L-cysteine or L-cysteine together with L-methionine at a final concentration of 0.7 mM was inoculated with L. casei (1% v/v). After incubation for 3 days at 30°C, the optical density at 600 nm (OD₆₀₀) of the cultures was determined using a spectrophotometer (LKB Biochrom 4050 Ultrospec II).

**Cloning, expression and purification of His-tagged CysE**

The orthologous LSEI_0479 gene was amplified from genomic DNA of L. casei FAM18110 with the primer pair LSEI_0479_F1/LSEI_0479_R (Table 1) and cloned into the pEXP5-CT/TOPO plasmid vector (Life Technologies, Zug, Switzerland) according to the manufacturer’s instructions. The plasmid containing the gene in the proper orientation was named pEXP5-CT/cysE and maintained in E. coli BL21(DE3). Expression of recombinant protein was performed as described previously (Bogicevic et al. 2012).

The recombinant protein was purified with Protino Ni-TED 1000 packed columns kit (Machery-Nagel, Düren, Germany) according to the manufacturer’s instructions. Therefore, bacterial cells were suspended in 1 mL of LEW buffer provided in the kit and disrupted with glass beads (212–300 μm in diameter, 0.6 g) and an Omni Bead Ruptor 24 (Omni International, Kennesaw, US). After elution, the buffer of the purified protein was exchanged to 20 mM sodium phosphate using PD-10 columns (GE Healthcare, Little Chalfont, UK). Finally, the protein was concentrated with Ultracel 30K centrifugal filters (Millipore, Cork, Ireland). The concentration and purity of the purified protein was determined with the Qubit Protein Assay Kit (Life Technologies) and with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by colloidal Coomassie staining, respectively.
Enzyme assays

The acylation of L-serine, D-serine and L-homoserine was studied using a method based on mass spectrometry: for this, an enzymatic assay was performed in a syringe at RT containing 200 μL of 50 mM ammonium acetate (pH 7.4), 1 mM amino acid, 0.06 mM acyl-CoA (acetyl-CoA, propionyl-CoA, butyryl-CoA or succinyl-CoA) and recombinant enzyme. To test inhibition, 1 μM L-methionine was added.

The reaction mixture was analyzed via continuous flow injection at 5 μL min⁻¹ into a high-resolution Bruker maXis 4G+ QTOF-mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. The mass spectrometer was operated in positive ion mode with a capillary voltage of 4 kV, an endplate offset of –500 mV and a temperature of 1250 °C.

Data acquisition, processing and reporting were achieved using DataAnalysis 4.2 software (Bruker Daltonics GmbH, Bremen, Germany). The observed m/z window was in the range from 75 to 1250.

A photometric assay was used to determine the kinetic parameters. The release of coenzyme A from the acetylated form was measured with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction mixture contained 0.2 mL of 50 mM ammonium acetate buffer (pH 7.4), 1 mM DTNB, 1 mM EDTA, various concentrations of L-serine (0.1–10 mM) and various concentrations of acetyl-CoA (0.01–0.16 mM). The Km for L-serine was determined with a fixed concentration of acetyl-CoA (0.06 mM), while the Km for acetyl-CoA was determined with a fixed concentration of L-serine (1 mM). The reaction was initiated by the addition of purified recombinant protein and carried out at 25 °C. The change of absorbation at 412 nm was recorded at 5 min intervals for 60 min. Km was determined by using the Hanes–Woolf transformation (S versus S v⁻¹), where v is the formation rate of CoA and S is the concentration of each substrate.

Complementation of E. coli auxotrophs

The plasmids pEXP5-CT/cysE and pEXP5-NT/CALML3 (Table 1) were used to transform the cysteine auxotroph E. coli JM39 and the methionine auxotroph E. coli DL41. Transformants were selected on LB/amp agar plates, checked for the presence of the plasmids and finally grown in LB/amp to yield stocks of the transformed bacteria. Transformed E. coli were grown in LB/amp medium for 6 h. After the determination of OD600, the cells were harvested by centrifugation (3000 g, 10 min), washed once with 1X M9 salts and then resuspended in 1X M9 salts to a final OD600 of 2. Transformed E. coli JM39 (50 μL) were plated on M9 minimal medium (Sambrook, Fritsch and Maniatis 1989) containing glucose (0.4%), IPTG (1 mM) and ampicillin (100 μg mL⁻¹) in the absence and presence of L-cysteine (1 mM). Transformed E. coli DL41 were plated as described above but in the absence and presence of L-methionine (1 mM). All plates were incubated at 37 °C for 3 days.

RESULTS

Growth with various sulfur compounds

We studied the requirement for various sulfur sources by inoculating a CDM containing either sulfide, cysteine or cysteine and methionine with various L. casei strains (Table 1). Medium that did not contain any of these sulfur compounds served as negative control. All strains tested grew in medium containing cysteine and methionine (Fig. 2), whereas no growth was observed in medium lacking the sulfur compounds. All strains also showed proper growth, when either sulfide or cysteine was
Cloning of the putative cysE gene

The genome of six _L. casei_ strains deposited in GenBank contains an 816-bp coding sequence, annotated as homoserine succinyltransferase, which lies upstream of cysK (Table S1, Supporting Information). A comparison of these genes showed a high nucleotide sequence identity (above 98%). Based on this information, we designed the primer pair LSEI_0479_F1/R (Table 1) to amplify the gene from _L. casei_ FAM18110. A PCR product of approximately 800 bp was obtained and cloned into the expression vector pEXP5-CT/TOPO, thereby adding a 6× His tag to the C-terminus of the predicted protein.

The deduced amino acid sequence of the amplified gene shared a high sequence identity (above 98%) with the putative homoserine succinyltransferases from other _L. casei_ strains (Fig. 3). Since we assumed that this gene in fact encodes a serine O-acetyltransferase, we call the gene cysE and the protein SAT in the remainder of this report. The nucleotide sequence of the cysE gene was deposited at GenBank under the accession number KU216159.

Heterologous expression of pEXP5-CT/cysE in _E. coli_ BL21(DE3) yielded a soluble recombinant protein, which was purified by added as the sole sulfur source, except ATCC 334 and FAM18172 which failed to grow under both conditions.
Substrate specificity and kinetic parameters of the putative CysE

To test whether the purified enzyme exhibited acylation activity, the enzyme was incubated together with various amino acids (L-serine, D-serine and L-homoserine) and acyl-CoAs (acetyl-CoA, propionyl-CoA, butyryl-CoA and succinyl-CoA). The enzymatic assay was continuously injected into a mass spectrometer. When the enzyme was incubated with L-serine and acetyl-CoA, a peak with an m/z of 148 appeared (Fig. 4A). This peak represents the protonated OAS (M+H)+. When succinyl-CoA or propionyl-CoA was used together with serine, the enzyme formed O-succinyl serine (m/z 206; Fig. 4B) and O-propionyl serine (m/z 162, Fig. 4C), respectively. The intensities of both peaks were lower than that for OAS, demonstrating that propionyl-CoA and succinyl-CoA were not the preferred acyl donor substrates. The formation of O-butyryl serine (m/z 176) was not observed when butyryl-CoA was used as the acyl donor (Fig. 4D). Further, no product formation was observed when D-serine or L-homoserine was used as acyl acceptors (data not shown).

When L-cysteine was added to the enzymatic reaction as a putative enzyme inhibitor, the peak corresponding to OAS (m/z 148.0) was still clearly detected by MS (Fig. S3, Supporting Information).

The Km for the acetylation of serine was determined by a photometric assay that contained Ellman’s reagent. This compound reacts with the sulfhydryl group of the released CoA, yielding a yellow color. Thus, we determined a Km of 1.13 (±0.13) mM and 0.021 (±0.004) mM for serine and acetyl-CoA, respectively. Again, when L-serine was replaced by D-serine or L-homoserine, no color formation was observed (data not shown).

Functional analysis by genetic complementation

Each of the expression plasmids listed in Table 1 was introduced into the cysteine auxotroph E. coli JM39 (cysE mutant) and into the methionine auxotroph mutant E. coli DL41 (metA mutant). Transformation of E. coli JM39 with pEXP5-CT/cysE resulted in the growth of the strain in the absence of L-cysteine (Fig. 5). Transformed E. coli DL41 failed to grow in the absence of L-methionine in the culture medium (Fig. 5).

DISCUSSION

The genome of L. casei ATCC 334 was the first strain of this species to be sequenced (Makarova et al. 2006). With regard
to the two-step synthesis of cysteine from serine, the gene encoding cysteine synthase is found in the annotated genome, but a gene encoding the enzyme that provides the substrate OAS for the cysteine synthase is missing. This implies that L. casei cannot synthesize cysteine from serine. However, when we inoculated a CDM containing only sulfide as the sole sulfur source with various L. casei strains, we observed growth in seven out of nine strains. This indicated that L. casei, in general, possesses the genes necessary for the synthesis of cysteine from serine and sulfide. The two strains that did not grow with sulfide—ATCC 334 and FAM18172—also did not grow when we used cysteine as the sole sulfur source, but they did grow very well when cysteine and methionine were present (Fig. 2). This indicates that the pathway for the conversion of cysteine to methionine is inactive. Previously, we showed that two genes from L. casei FAM18168, named metB and malY, encode cystathionine synthase and cystathionine lyase, respectively (Irmler et al. 2008). We assume that both genes constitute the transsulfuration pathway which converts cysteine to homocysteine, the precursor of methionine. We sequenced both genes from L. casei FAM18172 and FAM18110 and compared the deduced amino acid sequences with the one of FAM18168 and ATCC 334 (Figs S1 and S2, Supporting Information). We found that ATCC 334 possesses a strain-specific glutamine to proline amino acid substitution (Q169P) in MalY. A slight truncation of the MetB protein as a result from a nonsense mutation in the corresponding gene is specific for FAM18172. Whether these genetic changes render the strains auxotrophic for methionine needs further investigations.

With regard to the synthesis of cysteine from serine, we assumed that the metA gene that forms an operon with cysK in fact encodes a SAT protein. To study our hypothesis, we cloned the putative metA from L. casei FAM18110 and expressed it heterologously in E. coli. We found that the purified recombinant protein clearly acetylated L-serine. Slight acylation activity was also observed when propionyl-CoA and succinyl-CoA were used as substrates. The enzyme did not show acylation activity when D-serine or L-homoserine was used as acyl donors. Taken together with the complementation of an E. coli cysE mutant strain, this study clearly showed that the L. casei gene encodes SAT and therefore should be named cysE and not metA.

Liu et al. (2012) made a comprehensive in silico comparison of genes involved in sulfur metabolism. The authors describe that the genomes of other LAB species such as L. acidophilus, L. delbrueckii subsp. bulgaricus, L. rhamnosus, L. helveticus, L. fermentum, L. kefiranaeii, L. reuteri and L. salivarius possess a cysK gene but not a cysE gene. Moreover, cysK is co-localized with a

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**Figure 5.** Growth properties of transformed E. coli JM39 and E. coli DL41 bearing either plasmid pEXP5-CT/cysE (cysE) or pEXP5-NT/CALML3 (CALML3) on minimal medium (MM) agar plates. When indicated (right-hand side), the medium was supplemented with sulfur-containing amino acid.
metA gene in the genomes of this species. Consequently, these species should not be able to synthesize cysteine from serine. The situation resembles the one of L. casei and considering the genetic organization we assume that metA in these LAB species could also encode a SAT. Our assumption could be clarified by studying growth of these bacteria in the presence and absence of various sulfur compounds.

It has been reported that SAT activity in various organisms is inhibited by cysteine (Johnson, Roderick and Cook 2005). We did not observe this kind of feedback inhibition with the L. casei SAT in vitro. The SATs from other bacteria, such as E. coli, Salmonella enterica, Thermus thermophilus, Paracoccus denitrificans and Haemophilus influenzae, have a Km for serine of 0.11, 0.77, 0.013, 0.4 and 4.7 mM and for acetyl-CoA of 0.56, 0.1, 0.011, 0.1 and 0.7 mM, respectively (Kredich and Tomkins 1966; Burnell and Whatley 1977; Baecker and Wedding 1980; Kobayashi et al. 2004; Guan et al. 2008). The L. casei SAT showed affinity for both substrates within the same order of magnitude (Km of 1.1 mM for serine and 0.02 mM for acetyl-CoA).

According to Johnson, Roderick and Cook (2005), acyltransferases can be divided into hexapeptide and non-hexapeptide proteins. This classification is based on the presence or absence of imperfect tandem repeats of a hexapeptide sequence. While Meta from E. coli and a homoserine acetyltransferase from H. influenzae belong to the non-hexapeptide acetyltransferases, SATs generally possess this structural motif that can be found using InterProScan (IPR001451). We searched for these hexapeptide sequences in the L. casei SAT protein but did not detect this structural motif (data not shown). Therefore, the L. casei SAT is, to our knowledge, the first SAT to be described as a non-hexapeptide acetyltransferase.

With the advent of next-generation sequencing, the amount of assembled and annotated genome data of microbes is rapidly accumulating. Currently, the annotation of genomes is mainly based on sequence and structural comparisons. This study shows that functional confirmations are very valuable, especially when metabolic pathways that are predicted from genome data seem to be incomplete.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSLE online.

**Conflict of interest.** None declared.

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