Excess of threonine compared with serine promotes threonine aldolase activity in
Lactococcus lactis IL1403

Kadri Aller,1,2 Kaarel Adamberg,1,2,3 Indrek Reile,4 Veronica Timarova,1,2
Kari Peebo1,2 and Raivo Vilu1,2

1Competence Center of Food and Fermentation Technologies, Akadeemia tee 15A, Tallinn 12618,
Estonia
2Tallinn University of Technology, Department of Chemistry, Akadeemia tee 15, Tallinn 12618,
Estonia
3Tallinn University of Technology, Department of Food Processing, Ehitajate tee 5, Tallinn 19086,
Estonia
4National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia.

Lactococcus lactis is an important lactic acid starter for food production as well as a cell factory
for production of food grade additives, among which natural flavour production is one of the main
interests of food producers. Flavour production is associated with the degradation of amino acids
and comprehensive studies are required to elucidate mechanisms behind these pathways. In this
study using chemically defined medium, labelled substrate and steady-state cultivation, new data
for the catabolism of threonine in Lc. lactis have been obtained. The biosynthesis of glycine in this
organism is associated with the catabolic pathways of glucose and serine. Nevertheless, if
threonine concentration in the growth environment exceeds that of serine, threonine becomes the
main source for glycine biosynthesis and the utilization of serine decreases. Also, the conversion
of threonine to glycine was initiated by a threonine aldolase and this was the principal pathway
used for threonine degradation. As in Streptococcus thermophilus, serine
hydroxymethyltransferase in Lc. lactis may possess a secondary activity as threonine aldolase.
Other catabolic pathways of threonine (e.g. threonine dehydrogenase and threonine dehydratase)
were not detected.

INTRODUCTION
Lactic acid bacteria (LAB) are essential in the food
industry, particularly in the production of dairy products,
as they contribute to the aroma and texture of products
and prevent spoilage of food (Price et al., 2012). Moreover,
the technological advances in the last decade have created
the opportunity to exploit LAB for the bioproduction
of value-added chemicals, e.g. vitamins, polysaccharides,
low-calorie sweeteners (polyols), flavour compounds and
ethanol (Gaspar et al., 2013).

The catabolism of threonine in LAB (Fig. 1) has attracted
interest owing to the fact that this amino acid can be
degraded to acetaldehyde and glycine by threonine aldolase
(TA) (Fernández & Zúñiga, 2006). Acetaldehyde, as well as
acetone and diacetyl, are important flavour components in
yogurt. In fact, acetaldehyde can be produced by several
other biochemical pathways in LAB (e.g. by pyruvate
decarboxylation), which can operate simultaneously (Ott
et al., 2000; Chaves et al., 2002). However, the yield of
acetaldehyde during fermentation of Lactococcus lactis is
low (Bongers et al., 2005), as opposed to species used in
yogurt starter culture, Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus (Chaves et al., 2002).
Acetaldehyde can be further metabolized into ethanol by
alcohol dehydrogenase, and the gene encoding this enzyme
(adhE) is present in Lc. lactis (Christensen et al., 1999; Bolotin
et al., 2001).

TA, which catalyses the conversion of threonine to
acetaldehyde and glycine, has been characterized as the
basic catabolic pathway of threonine in LAB (Christensen
et al., 1999; Fernández & Zúñiga, 2006). TA activity in
bacteria can be associated with two enzymes: serine
hydroxymethyltransferase (SHMT), encoded by glyA, and
a low-specificity TA (Liu et al., 1998; Chaves et al., 2002; Simic et al., 2002; Fernández & Zúñiga, 2006). The principal role of SHMT is catalysing the reversible formation of glycine and 5,10-methylenetetrahydrofolate from serine (Fernández & Zúñiga, 2006). Novak & Loubiere (2000) proved with labelled substrates that, if Lc. lactis is cultivated in a simple synthetic medium, 68 % of the glycine in cellular proteins is derived from glucose and 32 % from serine. Interestingly, some SHMTs have a secondary activity as TA, e.g. in S. thermophilus (Chaves et al., 2002), although this is not a ubiquitous property amongst LAB (Ogawa et al., 2000). A low-specificity TA (encoded by ltaE), which catalyses the cleavage of L-threonine or L- allo-threonine to glycine and acetaldehyde, has been found in Escherichia coli (Liu et al., 1998). Yet, no homologues of ltaE have been found in LAB (Fernández & Zúñiga, 2006).

A well-described route for threonine catabolism in both prokaryotes and eukaryotes is the conversion of threonine to 2-amino-3-ketobutyrate by threonine dehydrogenase (TDH), followed by the formation of acetyl-CoA and glycine (initiated by 2-amino-3-ketobutyrate-CoA ligase) or aminoacetone and CO₂ (spontaneous decarboxylation) (Marcus & Dekker, 1993). This pathway has been investigated the most in E. coli, where the genes associated with this route are tdh and kbl (Epperly & Dekker, 1991; Marcus & Dekker, 1993; Schmidt et al., 2001). Based on annotated genomes, the first reaction in this pathway (conversion of threonine to 2-amino-3-ketobutyrate) may exist in such LAB as Oenococcus oeni (Borneman et al., 2012), Streptococcus suis (Hu et al., 2011) and S. thermophilus (Sun et al., 2011).

In addition to glycine production, threonine can be converted to 2-oxobutanoate by threonine dehydratase (and two spontaneous reactions) and the gene encoding this enzyme (ilvA) exists in Lc. lactis (Bolotin et al., 2001; Fernández & Zúñiga, 2006). 2-Oxobutanoate is the precursor in the biosynthetic pathway of branched-chain amino acids.
amino acids (BCAAs) isoleucine, leucine and valine (Fernández & Zúñiga, 2006). However, the strain used in the current study, Lc. lactis IL1403, is known to be auxotrophic for BCAAs (Cocaïn-Bousquet et al., 1995; Zhang et al., 2009; Aller et al., 2014), and so this pathway might not be functional in IL1403, although the genes associated with the reactions have been characterized (Bolotin et al., 2001). Moreover, neither of the previously mentioned catabolic reactions of threonine (catalysed by TA and TDH) has been characterized in Lc. lactis IL1403.

A chemically defined medium (CDM) is indispensable in studying the metabolic pathways and physiology of microorganisms, because unlike in rich media, all components in a CDM are defined (Zhang et al., 2009). Thus, experiments with CDM produce more comprehensible data, as the substrates and bioproducts can be easily quantified by analytical methods. Therefore, this article focuses on determining the degradation routes of threonine in Lc. lactis IL1403 using a CDM (BS7) developed in-house.

**METHODS**

Methods are given in full detail in the Supplementary Methods, available in the online Supplementary Material.

**Micro-organism.** The strain used in all experiments is Lc. lactis subsp. lactis IL1403, which was provided by INRA. Inoculum was prepared from lyophilized stock culture stored at −80 °C, which was pre-grown twice in test tubes at 34 °C on CDM BS7.

**Media.** CDM BS7, which has been designed specifically for the better detection of amino acids and quantification of full amino acid composition, was used for cultivation. The composition of BS7, which did not contain glycine, was as follows (mg l−1): t-glucose, 4500; K₂HPO₄, 900; KH₂PO₄, 750; MOPS, 7500; l-alanine, 78; l-arginine, 185; l-asparagine, 74; l-aspartic acid, 72; l-cysteine, 64; l-glutamic acid, 70; l-glutamine, 132; l-histidine, 60; l-isoleucine, 102; l-leucine, 207; l-lysine, 158; l-methionine, 41; l-phenylalanine, 86; l-proline, 92; l-serine, 163; l-threonine, 76; l-tryptophan, 16; l-tyrosine, 29; l-valine, 107; CaCl₂, 50; CoSO₄.7H₂O, 3; CuSO₄.5H₂O, 3; FeSO₄.7H₂O, 1.4; MnSO₄.7H₂O, 16; MgSO₄.7H₂O, 200; NaCl, 2900; (NH₄)₂MoO₄.2H₂O, 12; ZnSO₄.7H₂O, 5; thiamine hydrochloride (B₁), 0.51; riboflavin (B₂), 0.326; nicotinamide (B₃), 0.325; d-pantothenate (B₅), 0.65; pyridoxine (B₆), 0.642; biotin (B₇), 0.305; folic acid (B₈), 1.21; adenine, 25; hypoxanthine, 25; lipoic acid, 0.176. These numbers indicate the concentrations that were used in preliminary test tube experiments and for growing inoculum for continuous cultivations. In the case of continuous cultivation, a different buffer composition was used (mg l⁻¹): K₂HPO₄, 3000; KH₂PO₄, 2500; no MOPS.

Chemicals were obtained from Serva Electrophoresis and Sigma-Aldrich and were mixed together in the following order (to avoid precipitation): sugar, buffers, amino acids, minerals, vitamins, additional components. All media were sterilized by filtration, using stainless steel filter holders and nitrocellulose membrane filters (0.22 μm), provided by Merck Millipore.

**Continuous cultivation.** Continuous cultivation was carried out using the D-stat method and 1.25 l Biobundle bioreactors with EZ-Control biocontrollers and BioXpert Pharma 3.80 M XP control program, all provided by Applikon Biotechnology. The system was equipped with pH, O₂ and temperature sensors. Two BS7 media with different threonine concentrations (0.3 and 5 mM) were simultaneously pumped into the fermenter, whereas after stabilization was complete, the ratio of these media was changed over time (using computer algorithms), leading to the constant increase of threonine concentration in the feed. Three parallel experiments with a dilution rate of 0.2 h⁻¹ were carried out, in which the concentration of threonine in the fermenter inflow was changed from 1.24 to 2.58 mM (one experiment) or 4.13 mM (two experiments). The cultivation temperature in all experiments was 34 °C, agitation speed 300 r.p.m. and cultivation volume 300 ml. The pH was kept at 6.4 by addition of 2 M NaOH. The gas environment was kept anaerobic by flushing the bioreactors with N₂. Sampling is described in the Supplementary Methods.

**Labelling experiment.** In order to correctly determine l-threonine degradation products, we carried out a D-stat experiment at 0.2 h⁻¹ with BS7 medium, where the concentration of l-threonine was 2.5 mM. The equipment and environmental conditions were the same as in previous experiments (see Continuous cultivation, above).

Free amino acids were detected with an amino acid analyser Acquity UPLC. The samples were derivatized beforehand for 10 min at 55 °C, using AccQ-Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) and then loaded on an AccQ-Tag Ultra column at 55 °C. Amino acids were separated using a gradient of AccQ-Tag Ultra eluents A and B. They were detected with a photodiode array detector, and data were processed with Empower software. In order to measure labelled l-threonine or glycine in the labelling experiment, an LCT Premier™ XE ESI TOF MS was used, where amino acids were separated as described above and then sprayed directly into an MS operated in positive ionization mode (solvent vapour temperature 300 °C, source temperature 120 °C, capillary voltage 2.5 kV). MassLynx V4.1 software was used for data processing. All the equipment, reagents and eluents were provided by Waters.

For intracellular amino acid measurements, biomass was hydrolysed with 6 M HCl for 24 h at 105 °C. Amino acids were measured as described above. l-Tryptophan and l-cysteine could not be detected with this method, because they are degraded during hydrolysis. Also, l-asparagine is converted to l-aspartate and l-glutamine to l-glutamate during hydrolysis.

NMR spectra were measured on a Bruker Avance III 800 MHz spectrometer equipped with a He cryoprobe. Samples were prepared by adding 60 μl D₂O to 540 μl supernatant (10% D₂O concentration). ¹³C spectra were measured at 15 °C sample temperature with 30 ° flip angle pulses and proton decoupling. Ten thousand scans with 2 s relaxation delay were collected for each sample. All spectra were frequency-referenced according to the lactate signal and intensities were scaled to constant lactate signal intensity. Spectral assignment is based on chemical shifts, integrals and ¹³C-¹³C couplings and was confirmed with a separate gated decoupling ¹³C experiment.

**Proteome analysis.** Samples from two D-stat experiments were analysed in single runs. Peptides were separated on an Ultimate 3000 RSLCnano system (Dionex; Fisher Scientific) using a cartridge trap-column in backflush configuration and an analytical 50 cm Easy-Spray column (Thermo Fisher Scientific). Peptides were eluted at
200 nl min\(^{-1}\) with an 8–40 % buffer B gradient (buffer B, 80 % acetonitrile + 0.1 % formic acid; buffer A, 0.1 % FA) to a Q Exactive MS/MS operating with a top-10 strategy and a maximum cycle time of 1 s. The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno \textit{et al.}, 2013) with the dataset identifier PXD001602. Raw MS files were analysed by the MaxQuant software package version 1.3.0.5 (Cox \& Mann, 2008). Proteome analysis is described in full detail in the Supplementary Methods.

**RESULTS**

**Biomass and end-product yields at various L-threonine concentrations**

We have observed previously that in \textit{Lc. lactis} IL1403 some of the glycine in biomass proteins is derived via biosynthesis (Lahtvee \textit{et al.}, 2011) and that biosynthesis of glycine is preferred over utilization mainly at lower specific growth rates, i.e. at 0.1–0.2 h\(^{-1}\). Preliminary test tube experiments (Fig. S1a and S1b) with varied concentrations of L-serine and L-threonine indicated that the sum of glycine in cellular proteins and in extracellular secretion was up to 31 % higher in media with additional L-threonine (Fig. S1c). The utilization of L-serine was decreased when the concentration of L-threonine was higher than that of L-serine, and vice versa (Fig. S1d). In order to study this effect in detail, continuous cultivations were carried out in bioreactors.

Three parallel D-stats with dilution rate 0.2 h\(^{-1}\) were carried out where the concentration of L-threonine in the feed was gradually raised from 1.24 to 4.13 mM. Biomass yield, \(Y_{XS}\) (Fig. 2a), was constant in the first half of the experiment (0.144 \(\pm\) 0.003 g \(g_{\text{glucose}}\)\(^{-1}\)) and not dependent on the concentration of L-threonine in the medium. However, \(Y_{XS}\) started to drop slightly when L-threonine concentration in the medium was higher than 3 mM, and reached 0.129 \(\pm\) 0.008 g \(g_{\text{glucose}}\)\(^{-1}\) by the end of experiment.

The production of lactate (Fig. 2a) was constant in the first half of the experiment [64.88 \(\pm\) 1.73 mmol (g cell dry weight, g \(_{\text{DW}}\)\(^{-1}\)] and not dependent on the concentration of L-threonine in the medium. Yet, lactate production increased slightly at higher L-threonine concentrations and was 74.15 \(\pm\) 5.77 mmol g \(_{\text{DW}}\)\(^{-1}\) by the end of the experiment. Production of acetate, formate and ethanol (Fig. 2b) was constant throughout the experiments. The production of acetaldehyde was never higher than 0.05 mmol g \(_{\text{DW}}\)\(^{-1}\).

**Amino acid utilization and biosynthesis**

The production of extracellular glycine was linked to the concentration of L-threonine in the medium and increased

---

**Fig. 2.** Growth characteristics in D-stat experiments (mean results of three fermentations). (a) \(\bullet\), Biomass yield \(Y_{XS}\) [g (g glucose consumed)\(^{-1}\)]; \(\bigcirc\), production of lactate. (b) Production of formate (\(\square\)), acetate (\(\blacksquare\)) and ethanol (\(\blacksquare\)). (c) Production of glycine (\(\bigtriangleup\)) and utilization of L-serine (\(\blacktriangleleft\)) and L-threonine (\(\blacktriangleleft\)). (d) Utilization of L-isoleucine (\(\bigcirc\)), L-leucine (\(\bigcirc\)) and L-valine (\(\bigcirc\)).

\(C_{\text{Thr}}\) concentration of threonine.
from $0.34 \pm 0.03$ to $1.34 \pm 0.08$ mmol g$^{-1}$DW (Fig. 2c; mean of three experiments), whereas the intracellular concentrations of glycine and all other amino acids in biomass proteins remained constant throughout the experiments (Table 1). The consumption of L-serine (Fig. 2c) in these fermentations decreased from $0.84 \pm 0.19$ to $0.53 \pm 0.07$ mmol g$^{-1}$DW, which constitutes a decrease of $34 \pm 6$ to $19 \pm 2$% from the amount of L-serine in the medium. This suggests that glycine was produced mostly from L-threonine, rather than from L-serine, since L-threonine utilization (Fig. 2c) increased from $1.09 \pm 0.04$ to $2.08 \pm 0.15$ mmol g$^{-1}$DW, which constitutes a reduction of $57 \pm 2$ to $30 \pm 1$% from the amount of L-threonine in the medium. However, the consumption of L-threonine was higher than the biosynthesis of glycine. LAB can degrade threonine into 2-oxobutanoate, which is a precursor in the biosynthesis of BCAAs. However, there was no downtrend in the utilization of external L-isoleucine, L-leucine and L-valine pools at different L-threonine concentrations (Fig. 2d).

The utilization of several amino acids, e.g. L-arginine (1.11-fold), L-asparagine (1.34-fold), L-cysteine (1.46-fold) and L-glutamine (1.31-fold), and the production of L-glutamate (1.19-fold) and L-ornithine (1.17-fold) were increased at higher L-threonine concentrations in feed (Fig. S2).

Table 1. Amino acid concentrations in cellular proteins during D-stat experiments

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration mmol g$^{-1}$DW</th>
<th>SE (%)</th>
<th>% Total cellular protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>0.495 ± 0.024</td>
<td>4.8</td>
<td>8.3</td>
</tr>
<tr>
<td>Arg</td>
<td>0.142 ± 0.007</td>
<td>4.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Asn + Asp</td>
<td>0.430 ± 0.021</td>
<td>5.0</td>
<td>11.5</td>
</tr>
<tr>
<td>Gln + Glu</td>
<td>0.511 ± 0.021</td>
<td>4.2</td>
<td>15.4</td>
</tr>
<tr>
<td>Gly</td>
<td>0.335 ± 0.017</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>His</td>
<td>0.066 ± 0.006</td>
<td>9.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Ile</td>
<td>0.209 ± 0.009</td>
<td>4.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Leu</td>
<td>0.313 ± 0.013</td>
<td>4.3</td>
<td>8.3</td>
</tr>
<tr>
<td>Lys</td>
<td>0.337 ± 0.014</td>
<td>4.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Met</td>
<td>0.067 ± 0.011</td>
<td>16.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Phe</td>
<td>0.142 ± 0.006</td>
<td>4.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Pro</td>
<td>0.135 ± 0.006</td>
<td>4.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Ser</td>
<td>0.204 ± 0.011</td>
<td>5.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Thr</td>
<td>0.227 ± 0.009</td>
<td>4.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.093 ± 0.003</td>
<td>3.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Changes in protein expression

Proteome analysis was done for two D-stat experiments; expression was measured at three L-threonine concentrations in the feed (1.24, 2.86 and 3.99 mM) and compared with the first point. For instance, the expression of SHMT (GlyA) was increased at higher L-threonine concentrations, whereas the expressions of alcohol dehydrogenase (AdhE) and threonine dehydratase (IvwA) were decreased (Fig. 3a). L-Threonine as a substrate was definitely abundant in the medium, as the expression of threonine synthase (ThrC) was virtually unchanged during the experiment (Fig. 3a).

As for other notable changes at the proteome level, the expression of a betaine ABC transporter permease (BusAB), which is involved in osmotic stress, was increased (Fig. 3a). The expression of CysM, which is involved in cysteine biosynthesis from serine, was at first increased but then decreased by the end of the experiment (Fig. S2). Additionally, the expression of an enzyme involved in riboflavin biosynthesis (RibAB) was immensely increased. However, the fold change varied greatly between parallel experiments: it was 5.43 or 6.18 for one fermentation and 1.22 or 2.01 for the other.

Expression of some hypothetical proteins with the highest fold changes can be seen in Fig. 3b. Nevertheless, according to BLAST search, the nucleotide sequences of the genes encoding these proteins show no similarity to ltaE (a low-specificity TA) or tdh and kbl (involved in the conversion of L-threonine to 2-amino-3-ketobutyrate and subsequent production of glycine) in E. coli K-12 MG1655.

Experiment with $^{13}$C,$^{15}$N-labelled L-threonine

In order to exactly determine L-threonine degradation products, a D-stat experiment was carried out at 0.2 h$^{-1}$ with a feed containing 2.5 mM L-threonine. After stabilization was complete, the medium was switched to a feed that contained 2.5 mM $^{13}$C,$^{15}$N-labelled L-threonine. Supplementary Movie S1 shows that after switching to a labelled medium Lc. lactis gradually incorporates labelled L-threonine into cells, converts it into glycine and secretes the latter amino acid to the environment. Note that L-threonine is the main source of extracellular glycine (Supplementary Movie S1).

Moreover, NMR analysis showed that other products biosynthesized from labelled L-threonine were acetaldehyde, acetaldehyde hydrate and ethanol (Fig. S3). The ratio of these three products (in sum) was virtually 1:1 with glycine (Fig. 4). Additionally, small labelled peaks were seen in NMR spectra, but the concentration of this unknown product was too low to adequately identify it. The peak was compared with an aminoacetone standard, but it did not match (Fig. S4). When samples were lyophilized, the unknown peak was no longer present, hinting that the compound might have been volatile or unstable.

http://mic.sgmjournals.org
DISCUSSION

Based on the results presented herein it can be concluded that if L-threonine is in excess in the growth environment it is primarily degraded to glycine and acetaldehyde, followed by the partial conversion of acetaldehyde to ethanol (Fig. 4). Lactate does not directly or indirectly originate from L-threonine; this was proved with the labelling experiment (Fig. S3) indicating that over-consumed serine was degraded via serine hydratase to pyruvate followed by lactate or acetate synthesis. The comparison of utilized L-serine (Fig. 2c) and L-serine in total cellular protein (Table 1) indicates that 60–74% of consumed L-serine was directed to the biosynthesis of various substances. Decrease of the utilization of L-serine (Fig. 2c) might be related to a common transporter for serine and threonine. Noens & Lolkema (2014) showed that SerP1 transports L-serine, L-threonine and L-cysteine with high affinity. The decrease in L-serine consumption probably evoked the increase in L-asparagine utilization (Fig. S2) and degradation to L-aspartate followed by degradation to pyruvate. L-Asparagine degradation to L-aspartate occurs via L-asparaginase (AsnB), which was slightly upregulated (Fig. S2). In addition, the lower consumption of L-serine is apparently responsible for the elevated utilization of L-cysteine (Fig. S2), as the biosynthesis of the latter amino acid from L-serine is hampered (see expression of CysM in Fig. S2) and cells rely more on external L-cysteine pools.

SHMT might have TA activity, as shown by Chaves et al. (2002), although it is not a common feature in LAB (Ogawa et al., 2000). Similarly, this activity of SHMT may be present in L. lactis, as the expression of SHMT (GlyA) was increased at higher L-threonine availability (Fig. 3a). Also, as several hypothetical proteins were upregulated at higher L-threonine concentrations (Fig. 3b), these may also be involved in the degradation pathways of L-threonine. In addition, higher L-threonine availability might increase intracellular pools of acetaldehyde, which in turn would induce the build-up of osmotic pressure [similarly to lactate in Loubiere et al. (1997) and Pieterse et al. (2005)].
This would explain the reduction in biomass yield (Fig. 2a) and the increase in BusAB expression (Fig. 3a). Also, in order to alleviate stress conditions, Lc. lactis increases the consumption of additional carbon and nitrogen sources (amino acids), as seen in Fig. S2. L-Arginine, for example, can be utilized to produce additional ATP for growth (Fernández & Zúñiga, 2006).

The production of glycine in unlabelled D-stat experiments increased at higher L-threonine availability, whereas the biosynthesis of ethanol was constant and acetaldehyde was present in minute amounts. This suggested that there might be an alternative glycine biosynthesis pathway present in Lc. lactis (the production of glycine from L-threonine rather than from 2-amino-3-oxobutanoate). However, a D-stat experiment with labelled L-threonine proved that the ratio of glycine and ethanol/acetaldehyde/acetaldehyde hydrate was almost 1:1, excluding this hypothesis. The conversion of acetaldehyde to ethanol by an alcohol dehydrogenase (AdhE) might be limited, which would explain why the production of ethanol was constant in unlabelled experiments. This is also supported by the fact that the expression of AdhE was reduced at higher L-threonine availability (Fig. 3a). When higher amounts of acetaldehyde are produced, the conversion rate of this compound to ethanol might stay constant and the residual acetaldehyde probably evaporates.

The utilization of L-threonine was on average twofold higher than the biosynthesis of glycine (Fig. 2c). Even if intracellular L-threonine and glycine concentrations (Table 1) are taken into account, 14% of consumed L-threonine, on average, is degraded via unknown pathways. Thus, low amounts of other compounds were certainly produced from L-threonine as well. Regrettably, these metabolites could not be accurately identified, their concentrations being too low (Fig. S4). If L-threonine was converted to BCAAs, the bioavailability of these compounds should be increased, and thus the utilization of L-isoleucine, L-leucine and L-valine from the medium should be decreased. On the contrary, results show that the utilization of BCAAs is slightly increased at higher L-threonine availability (Fig. 2d) and the expression of the enzyme involved in the first reaction of BCAA biosynthesis (IlvA) is reduced (Fig. 3a). Hence, it is certain that BCAAs are not the compounds of interest.

Lastly, low amounts of produced glycine might have been directed to the biosynthesis of purines, which are precursors in the production of riboflavin (Burgess et al., 2004). This claim is based on proteome results, which show that enzymes involved in riboflavin biosynthesis (RibAB) were upregulated at higher L-threonine availability. The positive effect of threonine supplementation and TA overexpression on riboflavin production has also been noticed in experiments with the filamentous hemiascomycete Ashbya gossypii (Monschau et al., 1998). The facilitated production of riboflavin is also indicated by an increase in L-glutamine utilization (Fig. S2), since the latter amino acid is involved in the biosynthesis of nucleotides (Kilstrup et al., 2005).

In conclusion, it was experimentally proven herein that a TA activity exists in Lc. lactis IL1403 and might be associated with GlyA. Incidentally, this is the principal pathway for L-threonine degradation in this particular strain. Moreover, L-threonine is preferred over L-serine as a substrate for glycine biosynthesis if the concentration of L-threonine is higher than that of L-serine, suggesting that they share a common transporter. Also, the conversion of acetaldehyde to ethanol is limited at higher L-threonine availability.

ACKNOWLEDGEMENTS

The financial support for this research was provided by the Enterprise Estonia project EU29994, and the Ministry of Education, Estonia, through the institutional research funding grants IUT 1927 and IUT 23-7. The proteome data deposition to the ProteomeXchange Consortium was supported by the PRIDE team. The authors also thank Andres Maser, Mikk Oun and Marc Krenkel for helping with sampling during fermentations, Sten Erm and Ranno Nahku for their guidance in setting up computer algorithms for D-stat experiments, and Mary-Liis Kutt for her help in LC-MS data analysis. Kadri Aller designed and carried out the experiments, conducted analytical measurements and wrote the manuscript. Kaarel Adamberg and Raivo Vilu supervised the experiments and revised the manuscript. Indrek Reile was responsible for NMR analysis and Karl Pleebo for proteomics. Veronica Timarova helped to carry out continuous cultivations.

REFERENCES


