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

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*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-aminobut-2-enoate and two following spontaneous reactions turn the latter compound into 2-oxobutanoate.

Threonine dehydratase (ldvA) converts L-threonine into 2-amino-3-ketobutyrate and two following spontaneous reactions turn the latter compound into 2-oxobutanoate.

In addition to glycine production, threonine can be converted to 2-oxobutanoate by threonine dehydratase (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).

Fig. 1. Potential pathways in LAB for L-threonine and L-serine degradation and glycine biosynthesis. Solid lines represent pathways that are known in Lc. lactis IL1403 based on genome sequence or experimental data. Dashed lines represent pathways that are known in other LAB, but not in Lc. lactis IL1403. Dotted lines represent pathways that are known in other micro-organisms (e.g. E. coli), but not in LAB. The pathways leading to methylglyoxal, pyruvate and BCAA biosynthesis have not been given in detail. Threonine dehydratase converts L-threonine into 2-aminobut-2-enoate and two following spontaneous reactions turn the latter compound into 2-oxobutanoate.

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amino acids (BCAAs) isoleucine, leucine and valine (Fernández & Zúñiga, 2006). However, the strain used in the current study, *L. lactis* IL1403, is known to be auxotrophic for BCAAs (Cocaign-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 *L. 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 *L. 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 *L. 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 glycerol, was as follows (mg l⁻¹): α-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.H₂O, 16; MgSO₄.7H₂O, 200; NaCl, 2900; (NH₄)₂MoO₄.2H₂O, 3; ZnSO₄.7H₂O, 5; thiamine chloride (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 (B₉), 1.21; adenosine, 25; hypoxanthine, 25; lipoic acid, 0.176. These numbers indicate the concentrations that were used in preliminary test 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 bioreactors 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.

**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). After stabilization of the culture, the medium was switched to labelled BS7 medium, which contained 2.5 mM ¹³C,¹⁵N-labelled l-threonine (CortecNet). Sampling is described in the Supplementary Methods.

**Analytical methods.** Glucose and main fermentation end products (lactate, formate, acetate, ethanol) were measured with LC (Alliance 2795 system), a refractive index detector (model 2414) and Empower software, all provided by Waters. The column used was Bio-Rad HPX-87H at 35 °C with isocratic elution of 5 mM H₂SO₄ at a flow rate of 0.6 ml min⁻¹. Acetaldehyde was measured with a specific assay kit provided by Megazyme.

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-hydroxysuccinimidy 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 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 seconds 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 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 *Lc. lactis* IL1403 some of the glycine in biomass proteins is derived via biosynthesis (Lahtvee 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 \((0.144 \pm 0.003 \text{ g g}\text{glucose}^{-1})\) 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 \(0.129 \pm 0.008 \text{ g g}\text{glucose}^{-1}\) by the end of experiment.

The production of lactate (Fig. 2a) was constant in the first half of the experiment \([64.88 \pm 1.73 \text{ mmol g cell dry weight}^{-1}]\) and not dependent on the concentration of L-threonine in the medium. Yet, lactate production increased slightly at higher L-threonine concentrations and was \(74.15 \pm 5.77 \text{ mmol gDW}^{-1}\) 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 gDW\(^{-1}\).

Amino acid utilization and biosynthesis

The production of extracellular glycine was linked to the concentration of L-threonine in the medium and increased
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 (IvA) 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 IlaE (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 13C,15N-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 13C,15N-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.
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 Lc. 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, \textit{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 \textit{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 \textit{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 \textit{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.

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