Metabolic Engineering of Acetaldehyde Production by
Streptococcus thermophilus

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The process of acetaldehyde formation by the yogurt bacterium Streptococcus thermophilus is described in this paper. Attention was focused on one specific reaction for acetaldehyde formation catalyzed by serine hydroxymethyltransferase (SHMT), encoded by the glyA gene. In S. thermophilus, SHMT also possesses threonine aldolase (TA) activity, the interconversion of threonine into glycine and acetaldehyde. In this work, several wild-type S. thermophilus strains were screened for acetaldehyde production in the presence and absence of l-threonine. Supplementation of the growth medium with l-threonine led to an increase in acetaldehyde production. Furthermore, acetaldehyde formation during fermentation could be correlated to the TA activity of SHMT. To study the physiological role of SHMT, a glyA mutant was constructed by gene disruption. Inactivation of glyA resulted in a severe reduction in TA activity and complete loss of acetaldehyde formation during fermentation. Subsequently, an S. thermophilus strain was constructed in which the glyA gene was cloned under the control of a strong promoter (P_LacA). When this strain was used for fermentation, an increase in TA activity and in acetaldehyde and folic acid production was observed. These results show that, in S. thermophilus, SHMT, displaying TA activity, constitutes the main pathway for acetaldehyde formation under our experimental conditions. These findings can be used to control and improve acetaldehyde production in fermented (dairy) products with S. thermophilus as starter culture.

Yogurt is a product obtained through milk fermentation with a specific yogurt starter culture consisting of a mixture of two species of lactic acid bacteria (LAB), Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus (23). The main roles of this mixed starter in the production of yogurt are (i) acidification through the conversion of lactose into lactic acid, (ii) creation of the viscous texture by the production of exopolysaccharides, and (iii) development of the typical yogurt flavor (29).

The typical yogurt flavor is caused by lactic acid, which imparts an acidic and refreshing taste, and a mixture of various carbonyl compounds like acetate, diacetyl, and acetaldehyde, the latter of which is considered the major flavor component (10, 11, 21, 29). The relatively high concentration of acetaldehyde (in the range of 5 to 21 mg/liter) found in yogurt must be due to a low utilization rate of this metabolite since the yogurt bacteria lack the main enzyme for acetaldehyde conversion into ethanol, alcohol dehydrogenase (12).

The production of acetaldehyde by LAB seems to be strain dependent. L. delbrueckii subsp. bulgaricus has been reported by some authors to be a greater acetaldehyde producer than S. thermophilus, whereas other authors have reported the contrary (21, 27). Although it is presently unclear what the major pathway for acetaldehyde production by LAB is, several metabolic pathways have been shown to lead to its formation (5, 24) and it is possible that more than one metabolic pathway operate simultaneously (Fig. 1). During yogurt fermentation, acetaldehyde can be produced directly from lactose metabolism as a result of pyruvate decarboxylation. It can be produced (i) directly via pyruvate decarboxylase or pyruvate oxidase or (ii) indirectly through the formation of the intermediate acetyl coenzyme A by pyruvate dehydrogenase or pyruvate formate lyase. Furthermore, acetaldehyde can be formed by the activity of deoxyriboaldolase, which degrades thymidine into acetaldehyde and glyceraldehyde-3-phosphate. Finally, while several amino acids can be converted into acetaldehyde via pyruvate as a metabolic intermediate, threonine can be directly converted into acetaldehyde and glycine by the activity of threonine aldolase (TA) (29).

In the yogurt bacterium S. thermophilus, the only enzyme with TA activity (interconversion of threonine into acetaldehyde and glycine) seems to be the serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). This is an important enzyme involved not only in the formation of glycine and serine but also in the turnover of folate in all organisms (3, 28). However, the role of TA in acetaldehyde formation in mixed yogurt cultures is still not fully understood (32).

The aim of this work was to investigate the role and importance of SHMT in the production of acetaldehyde by S. thermophilus. It was observed that the main pathway for acetaldehyde formation in this microorganism is through the activity of SHMT since the inactivation of the encoding glyA gene led to the almost complete abolition of TA activity. This result indicates the absence of an alternative pathway for acetaldehyde production and thus establishes the importance of SHMT in
the production of this key aroma compound. Overexpression of the glyA gene showed an increase in TA activity and in acetaldehyde and folic acid formation. These results indicate that, in *S. thermophilus*, SHMT with its TA activity constitutes the main pathway for acetaldehyde formation under our experimental conditions. These findings could be used to control and improve acetaldehyde production in fermented (dairy) products by using *S. thermophilus* as starter culture. They also allow the selection of natural *S. thermophilus* variants with improved flavor-forming characteristics and the improvement of acetaldehyde formation through the metabolic engineering of SHMT.

**MATERIALS AND METHODS**

**Strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. The *S. thermophilus* strains were routinely grown in M17 medium (Brunschwig Chemie, Amsterdam, The Netherlands) supplemented with 1% (wt/vol) lactose (LM17 medium) at 42°C unless stated otherwise. When indicated, the medium was supplemented with 10 or 25 mMM threonine. *Escherichia coli* was routinely grown in Luria broth-based medium at 37°C with aeration. When appropriate, the medium contained chloramphenicol (5 μg ml⁻¹ for *E. coli*). For the growth curve experiments, *S. thermophilus* strains were grown overnight (knockout strains with 2.5 μg of chloramphenicol ml⁻¹, overexpression strains with 5 μg of chloramphenicol ml⁻¹) and then diluted 100-fold in fresh LM17 medium.

**DNA isolation, manipulation, and PCR.** Isolation of *E. coli* plasmid DNA and standard recombinant techniques were performed by following established protocols (26). Large-scale isolation of *E. coli* plasmid DNA for nucleotide sequence analysis was performed with the Jetstar plasmid maxprep kit (Genomed GmbH, Bad Oeynhausen, Germany) system following the instructions of the manufacturer. Plasmid and chromosomal DNA from *S. thermophilus* was isolated as described previously for *Lactococcus lactis* (30). Electroporation of *S. thermophilus* was done according to the method of Mollet et al. (18), with the modification that cells were incubated in electroporation buffer at 4°C for 4 h prior to electroporation. Restriction enzymes, *Taq* polymerase, and T4 DNA ligase were used as recommended by the suppliers (R-Biopharm GmbH [formerly Boehringer Mannheim], Darmstadt, Germany; Gibco Invitrogen Corporation, Breda, The Netherlands).

**Construction of plasmids for disruption of the glyA gene.** An internal fragment of the glyA gene (505 bp) from *S. thermophilus* NIZOB130 was obtained directly from the genomic DNA by PCR amplification. The degenerated oligonucleotides used (5'-ACNAAYAARTAYGCNGARGG-3' and 5'-GGNCCNCKNA RNSWYTTYRTG-3', where N is A, C, G, or T; Y is C or T; R is A or G; K is G or T; W is A or T; and S is G or C) were designed based on the sequences of the known GlyA protein from different microorganisms (Fig. 2). The PCR product obtained was cloned in pGEM-T, generating the plasmid pNZ2300. The nucleotide sequence of this cloned fragment was analyzed with an ALFred DNA sequencer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Sequencing reactions were performed with an AutoRead sequencing kit and initiated by using Cy5-labeled universal and reverse primers following the instructions of the manufacturer (Amersham Pharmacia Biotech). Sequence data were assembled and analyzed using the CLONE program, version 1. Homology searches were performed by using the sequence similarity search program BLAST (22). The CLUSTAL method (8) was used for multiple alignments of...
sequences. The internal fragment of the glyA gene was isolated from plasmid pNZ2300 as an Apal-SpeI fragment and then cloned into the vector pG* host9 previously digested with the same enzymes, yielding the plasmid pNZ3310. The vector pG* host9 contains a thermostable replicon (15).

**Gene disruption.** *S. thermophilus* strains NIZOB130 and AO54 were transformed with pNZ2330, and the transformants were selected at 28°C on M17 sucrose (SM17) agar supplemented with erythromycin. In order to facilitate the integration of pNZ2330, overnight cultures grown on SM17 medium at 28°C with erythromycin were diluted 100-fold into fresh medium and then grown at 28°C until early exponential growth phase, when the growth temperature was shifted to 42°C. At 42°C, growth was continued until stationary phase and cells were plated at appropriate dilutions on SM17 agar with erythromycin. These plates were incubated at 42°C; the primary integrates appeared as erythromycin-resistant colonies after 24 to 48 h. In order to confirm the correct integration at the glyA locus, Southern hybridization was performed with the 505-bp glyA fragment as a probe. The glyA mutants of the strains NIZOB130 and AO54 were designated respectively as *S. thermophilus* NZZ310 (NIZOB130ΔglyA) and NZZ311 (AO54ΔglyA), and they were exclusively grown at 42°C to ensure stable integration of the vector.

**Acetaldehyde determination.** Acetaldehyde was measured spectrophotometrically by using an acetaldehyde determination kit based on the enzymatic (acetaldehyde dehydrogenase) reduction of NAD to NADH (R-Biopharm GmbH). The acetaldehyde level was determined after 20 h of fermentation at 42°C; the inoculum was 1.0% of a fresh overnight culture. All assays were performed at least in triplicate.

**Enzymatic assay.** TA activity of SHMT was monitored with L-threonine as a substrate, and the enzyme activity towards *t*-threonine was measured as described by Wilkins et al. (33) with some modifications. This method basically measures the threonine-dependent formation of acetaldehyde over time by headspace gas chromatography. Cells were harvested in the exponential growth phase by centrifugation, resuspended in phosphate buffer, and subsequently lysed with a French pressure cell at constant pressure (900 lb/in²). The cell extracts should be kept on ice from this point until the end of the enzymatic assay. Cell debris was removed by centrifugation (4°C, 10,000 × g for 10 min), and the cell extract was used for the enzymatic assay. The reaction was performed at 42°C and terminated by the addition of 600 µl of hydrochloric acid (1 M). The acetaldehyde formed was measured spectrophotometrically (see above), and the specific TA activity was expressed in milliunits (1 mU is 1 nmol of acetaldehyde formed per minute per milligram of protein). Protein concentration was determined using a protein assay kit based on the method of Bradford (2) with bovine serum albumin as the standard (Bio-Rad Laboratories BV, Veenendaal, The Netherlands).

**Folic acid determination.** The folic acid present in the cells was measured by a microbiological assay with a folate auxotrophic *Lactobacillus casei* strain (7). The method was adjusted for use with microtiter plates as described previously (9, 19).

**Construction of a plasmid with the glyA gene under the control of the lacA promoter.** Isolation of the complete coding sequence of the glyA gene from *S. thermophilus* NIZOB130 was obtained directly from the genomic DNAs by PCR amplification. The reaction was carried out in a mixture containing 50 µl of 10 mM Tris-Cl (pH 8.55), 25 mM KCl, 5 mM (NH₄)₂SO₄, 2 mM MgSO₄, a 0.1 mM concentration of each deoxynucleoside triphosphate, 20 ng of each primer, 1 µg of the genomic DNA, and 0.5 U of Pwo polymerase (Roche Diagnostics Nederland BV, Almere, The Netherlands) at 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min for a total of 30 cycles. The 3’ and 5’ ends of the primer had, based on published glyA gene sequences, the sequences 5’-GGCGAACAACCTGCAAGGTTCTTCCGCAATCCACATTGGACAACAGG-3’ and 5’-TATCTCGCTCTCAGTTAATAGATGGGAAAGC-3’, with restriction enzyme sites (XhoI and PstI) introduced to allow insertion into the vector pNZZ276. The amplified PCR product was digested with XhoI and PstI, separated by agarose gel electrophoresis, and purified with the Jetquick gel extraction kit (Genomed GMBH). The amplified DNA fragment (1.2 kb) containing the glyA gene sequence starting at the ribosome binding site was inserted downstream of the lacA promoter of pNZ276, yielding the plasmid pNZZ305. This construction was stabilized in *E. coli*, and the nucleotide sequence of the amplified glyA gene was determined (as described previously) to confirm that no mismatching had occurred during PCR amplification. The plasmid was isolated in large scale by using the Jetstarp plasmid maxprep kit (Genomed GMBH), and the pure plasmid was electroporated into the two different strains of *S. thermophilus*, NIZOB130 and AO54.

**Overexpression of the glyA gene in *S. thermophilus***. A fermentation experiment was conducted with four strains of *S. thermophilus*, the wild-type strains NIZOB130 and AO54 and both of these strains harboring plasmid pNZZ305. They were all fermented in 1 liter of LM17 medium with 5 µg of chloramphenicol/ml when necessary during a 24-h period at 42°C. The decrease of the pH during incubation was continuously monitored with a pH meter microprocessor (WTW, Weinheim, Germany). In order to follow the growth of *S. thermophilus*, several samples were taken during the incubation period and serial dilutions were made in sterile physiological salt solutions and deep-plated on...
LM17 agar. Plates were incubated for 48 h at 42°C, and microbial count data were expressed as the log of CFU per milliliter. Samples were taken (i) for the TA assay at the end of the exponential growth phase and (ii) to measure folic acid and acetaldehyde production after 24 h of incubation. All experiments and analyses were performed in triplicate.

RESULTS

Acetaldehyde production and TA activity. Acetaldehyde-producing capacity was determined in several wild-type S. ther-
mophilus strains. In a first screening experiment, acetaldehyde production was compared for all S. thermophilus strains from the NIZO Food Research collection grown in LM17 medium either with (10 or 25 mM) or without L-threonine added (Fig. 3). A clear difference in the amount of acetaldehyde formed could be detected among the different strains. While some strains produced no detectable amounts of acetaldehyde, other strains produced a considerable amount of this product. The level of acetaldehyde formation could be increased in all

FIG. 2. Multiple alignments of the amino acid sequences encoded by glyA genes from S. thermophilus NIZOB130 (STH; a partial sequence with 178 amino acids); A. actinomycetemcomitans (ACTAC; 420 amino acids), B. subtilis (BACSU; 415 amino acids), B. japonicum (BRAJA; 432 amino acids), and E. coli (ECOLI; 417 amino acids). EMBL database accession numbers are shown in parentheses. Asterisks denote residues conserved among all sequences, and dots represent gaps introduced into a sequence for alignment. The amino acid sequences used for the design of degenerated primers to obtain a part of the glyA gene from S. thermophilus are boxed (highly conserved regions).
strains by adding L-threonine to the culture medium, suggesting the involvement of a TA. The amount of increase appeared to be strain specific and variable. A total of 10 strains, varying in acetaldehyde production between no detectable amounts to relatively high levels (200 μM), were selected for further studies. TA activity and acetaldehyde levels were measured in the selected strains of *S. thermophilus*. The results shown in Fig. 4 reveal that strains producing high levels of acetaldehyde during fermentation also contained high TA activity. This suggests that the TA pathway is responsible for acetaldehyde production in *S. thermophilus* (Fig. 1).

**Disruption of the glyA gene in S. thermophilus.** An internal fragment of the *glyA* gene from *S. thermophilus* strains NIZOB130 and AO54 was amplified by PCR with degenerated primers. The resulting PCR product was cloned in pGEM-T, generating the plasmid pNZ2300. Nucleotide sequencing revealed the presence of a single open reading frame of 505 bp that showed high homology with GlyA proteins from other microorganisms. A multiple-amino-acid sequence alignment that includes this internal fragment of GlyA from *S. thermophilus* with known GlyA proteins from various microorganisms is shown in Fig. 2. The sequence of the *S. thermophilus* GlyA fragment showed clear homologies with GlyA from *Bacillus subtilis* (69% identity), *Bradyrhizobium japonicum* (69% identity), *Actinobacillus actinomycetemcomitans* (67% identity), and *E. coli* (66% identity). The highest homology (80% identity) with the *S. thermophilus* GlyA protein was found with a putative GlyA-encoding sequence from another LAB, *L. lactis*, of which the genome sequence was recently published (1; http://www.spock.jouy.inra.fr).

To study the physiological role of the *glyA* gene, a knockout mutant was constructed. The chromosomal *glyA* gene was inactivated by gene disruption with site-specific, temperature-sensitive integration vector pNZ2310 in these two strains of *S. thermophilus*, NIZOB130 and AO54. The *glyA* mutation was confirmed in cells that were able to grow at 42°C under antibiotic selection. One colony of each strain was collected and checked by Southern blot analysis (the hybridization patterns...
obtained confirmed the integration of pNZ2310 at the glyA locus [data not shown], and the collected mutants were named NZ2330 and NZ2331. TA activities and acetaldehyde production were determined for both glyA mutants in comparison with those of the respective parental strains (Table 2). Inactivation of the glyA gene resulted in the almost complete inactivation of TA activity in both strains, indicating that TA activity in S. thermophilus resides in SHMT encoded by the glyA gene. Furthermore, no detectable amount of acetaldehyde was produced by these strains during fermentation. The disruption of the glyA gene resulted in a sixfold reduction of the growth rate relative to those of the parental strains (data not shown). Moreover, the glyA mutation resulted in reduced acidification, reflected by a final pH of the culture of 5.2 relative to that of the wild type, which reached a final pH of around 4.5. The large amount of glycine present in LM17 medium (930 mg/liter) seems to preclude glycine limitation as the cause for this reduced growth rate and acidification. This conclusion was supported by the failure to restore the wild-type growth characteristics of the mutants upon the addition of extra glycine to the growth medium. The observed growth defect in the glyA mutants could be related to a malfunctioning in folate metabolism, since SHMT is a key enzyme in the regeneration of methyltetrahydrofolate from tetrahydrofolic acid (3, 27). In fact, SHMT is an important enzyme involved in the folate-dependent interconversion of serine and glycine. Interestingly, the S. thermophilus strains that showed no detectable acetaldehyde formation during fermentation are the same strains that show low production of folic acid (34). SHMT is the most widespread glycine biosynthetic enzyme among plants, animals, and microorganisms. The conversion of serine to glycine serves as an important source of acetaldehyde production by S. thermophilus. In a previous paper, we have shown that TA is the major enzyme involved in acetaldehyde production in the yogurt bacterium S. thermophilus. The screening experiment showed that supplementation of the growth medium with L-threonine led to an increase in acetaldehyde production and that the differences in acetaldehyde formation were correlated to the differences in TA activity. The disruption of the glyA gene, coding for the SHMT enzyme with TA activity, resulted in the abolishment of acetaldehyde production. In addition, by higher production of SHMT in S. thermophilus, it was possible to achieve an increased production of acetaldehyde. This provides the ultimate evidence that the previously described process for acetaldehyde formation by S. thermophilus and L. delbrueckii subsp. bulgaricus through the breakdown of threonine to glycine (12, 13, 16, 28, 32) is a really important source of acetaldehyde production by S. thermophilus. SHMT (EC 2.1.2.1) is a pyridoxal phosphate- and tetrahydrofolate acid-dependent enzyme. TA (EC 4.1.2.5) is also a pyridoxal phosphate-dependent enzyme and catalyzes the interconversion of threonine and glycine plus acetaldehyde (31). The physiological role of TA is generally believed to be in the production of glycine (4). The observation that L. lactis strain Z8, which lacks TA activity, requires glycine for growth support's this hypothesis (25). SHMT is an important enzyme involved in the folate-dependent interconversion of serine and glycine. Interestingly, the S. thermophilus strains that showed no detectable acetaldehyde formation during fermentation are the same strains that show low production of folic acid (34). SHMT is the most widespread glycine biosynthetic enzyme among plants, animals, and microorganisms. The conversion of serine to glycine serves as the major source of one-carbon units that are essential for the biosynthesis of purine, thymidylate, and methionine. Although SHMT from rabbit liver and lamb was shown to have TA activity, SHMT in mung beans, in E. coli, and in rats had no detectable TA activity. Therefore, the activity of cleaving threonine to glycine and acetaldehyde is not a general property of SHMT (20).

Several TAs (EC 4.1.2.5) encoded by the itAE gene have been isolated from different microorganisms. Amino acid sequence comparison of the encoded enzymes with SHMT enzymes encoded by glyA show that these different enzymes are not related (20). In order to detect and clone an itAE-like gene from S. thermophilus strains NIZOB130 and AO54, PCR was carried out with chromosomal DNA by using degenerate primers that were derived from alignments of different itAE genes. Although, different PCR conditions were tried, we were not able to amplify the itAE gene in the S. thermophilus strains. In addition, a BLAST search was done (http://www.biol.ucl.ac.be/gene/genome) in the almost complete genome sequence of S. thermophilus and this did not result in the identification of an itAE-like gene (Pascal Hols, personal communication). Once we were unable to detect itAE-encoded TA, it seemed logical to postulate that SHMT would be the only source of TA activity

**DISCUSSION**

In this paper, we have shown that TA is the major enzyme activity involved in acetaldehyde production in the yogurt bacterium S. thermophilus. The screening experiment showed that supplementation of the growth medium with L-threonine led to an increase in acetaldehyde production and that the differences in acetaldehyde formation were correlated to the differences in TA activity. The disruption of the glyA gene, coding for the SHMT enzyme with TA activity, resulted in the abolishment of acetaldehyde production. In addition, by higher production of SHMT in S. thermophilus, it was possible to achieve an increased production of acetaldehyde. This provides the ultimate evidence that the previously described process for acetaldehyde formation by S. thermophilus and L. delbrueckii subsp. bulgaricus through the breakdown of threonine to glycine (12, 13, 16, 28, 32) is a really important source of acetaldehyde production by S. thermophilus. SHMT (EC 2.1.2.1) is a pyridoxal phosphate- and tetrahydrofolate acid-dependent enzyme. TA (EC 4.1.2.5) is also a pyridoxal phosphate-dependent enzyme and catalyzes the interconversion of threonine and glycine plus acetaldehyde (31). The physiological role of TA is generally believed to be in the production of glycine (4). The observation that L. lactis strain Z8, which lacks TA activity, requires glycine for growth supports this hypothesis (25).

SHMT is an important enzyme involved in the folate-dependent interconversion of serine and glycine. Interestingly, the S. thermophilus strains that showed no detectable acetaldehyde formation during fermentation are the same strains that show low production of folic acid (34). SHMT is the most widespread glycine biosynthetic enzyme among plants, animals, and microorganisms. The conversion of serine to glycine serves as the major source of one-carbon units that are essential for the biosynthesis of purine, thymidylate, and methionine. Although SHMT from rabbit liver and lamb was shown to have TA activity, SHMT in mung beans, in E. coli, and in rats had no detectable TA activity. Therefore, the activity of cleaving threonine to glycine and acetaldehyde is not a general property of SHMT (20).

**Overexpression of the glyA gene in S. thermophilus.** In a number of fermentation experiments, the behavior of the four S. thermophilus strains NIZOB130, AO54, NIZOB130 harboring pNZ2305, and AO54 harboring pNZ2305—the last two of which overexpress glyA—was compared. The acidification and the growth rate of all four strains were nearly identical. Furthermore, the following observations were made (Table 2): (i) an increase in acetaldehyde production of the glyA-overexpressing strains exhibited in strain NIZOB130 harboring pNZ2305 (about 82%) and strain AO54 harboring pNZ2305 (about 91%), (ii) an increase in folic acid production exhibited in strain NIZOB130 harboring pNZ2305 (20%) and strain AO54 harboring pNZ2305 (100%), and (iii) an increase in TA activity exhibited in strain NIZOB130 harboring pNZ2305 (61%) and strain AO54 harboring pNZ2305 (70%).

**Acetaldehyde production in the wild-type S. thermophilus strains NIZOB130 and AO54, the glyA-knockout strains NZ2310 and NZ2311, and the glyA-overexpressing mutants harboring pNZ2305**

<table>
<thead>
<tr>
<th>S. thermophilus strain</th>
<th>TA-sp act (mU/mg)</th>
<th>Acetaldehyde production (µM. ppm)</th>
<th>Folate production (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIZOB130</td>
<td>35 ± 4</td>
<td>4.8 ± 0.5, 109</td>
<td>158 ± 10</td>
</tr>
<tr>
<td>NZ2310</td>
<td>2 ± 2</td>
<td>0 ± 0.5, 0</td>
<td>238 ± 20</td>
</tr>
<tr>
<td>NIZOB130 + pNZ2305</td>
<td>58 ± 4</td>
<td>8.5 ± 0.5, 193</td>
<td>186 ± 10</td>
</tr>
<tr>
<td>AO54</td>
<td>35 ± 6</td>
<td>3.9 ± 0.5, 89</td>
<td>78 ± 5</td>
</tr>
<tr>
<td>NZ2311</td>
<td>2 ± 2</td>
<td>0 ± 0.5, 0</td>
<td>135 ± 10</td>
</tr>
<tr>
<td>AO54 + pNZ2305</td>
<td>62 ± 6</td>
<td>7.4 ± 0.5, 168</td>
<td>183 ± 15</td>
</tr>
</tbody>
</table>
in *S. thermophilus*. This hypothesis was tested by disruption of the *glyA* gene. This disruption in *S. thermophilus* led to (i) a complete loss of detectable TA activity, suggesting that *S. thermophilus* contains no other enzymes with TA activity, and (ii) a significant reduction in the growth rate, which could not be restored by supplementing the medium with glycine. Because SHMT plays a central role in folate and one-carbon-unit metabolism, this growth defect observed is most probably due to a disturbance in the supplying of one-carbon units for the biosynthesis of purines, thymidylate, and methionine (29).

Although some researchers suggest that the physiological role of SHMT may be related to the production of glycine for growth, in this work, the inactivation of the *glyA* gene did not lead to a specific requirement for glycine. Supplementation of LM17 medium with glycine could not restore growth. In *S. thermophilus*, the role of the *glyA* gene must be different from its role in *E. coli*. In *E. coli*, disruption of the *itaE* gene alone did not affect the growth rate while disruption of the *itaE* gene in combination with the *glyA* gene caused a significant decrease in the growth rate. This suggests that TA is not the major source of cellular glycine in the wild type but that it catalyzes an alternative pathway for glycine when SHMT is knocked out (14). In *S. thermophilus*, however, SHMT seems not to be involved in glycine supply, as our growth experiments clearly show.

In conclusion, we have shown that acetaldehyde formation in *S. thermophilus* is catalyzed by a secondary activity (TA) of SHMT, the key enzyme in folate turnover and one-carbon-unit metabolism. This finding enabled us to enhance TA activity and also acetaldehyde and folic acid production by the overexpression of *glyA*, the gene encoding SHMT. Future work is directed to further explore this enzyme activity and to make use of these findings for the improvement of fermented dairy products.

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REFERENCES