Carbamoylphosphate synthetase activity is essential for the optimal growth of *Streptococcus thermophilus* in milk

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Running title: CO_2 metabolism in *S. thermophilus*

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ABSTRACT

Aim: To study the role of the carbon dioxide metabolism in *Streptococcus thermophilus* through investigation of the phenotype of a carbamoylphosphate synthetase negative mutant.

Methods and results: The effect of carbon dioxide on the nutritional requirements of *S. thermophilus* DSM20617^T and its derivative; carbamoylphosphate synthetase-negative mutant A17(*ΔcarB*), was investigated by cultivating the strain in a chemically defined medium under diverse gas compositions and in milk. The results obtained revealed that CO_2 depletion or *carB* gene inactivation determined the auxotrophy of *S. thermophilus* for L-arginine and uracil. In addition, the parent strain grew faster than the mutant, even when milk was supplemented with uracile or arginine.

Conclusions: Milk growth experiments underlined that carbamoylphosphate synthetase activity was essential for the optimal growth of *S. thermophilus* in milk.

Significance and impact of the study: The study of the carbon dioxide metabolism in *S. thermophilus* revealed new insights in regards to the metabolism of this species which could be useful for the optimization of dairy fermentation processes.

Introduction

In all organisms, pyrimidine metabolism is required to supply the cell with building blocks for the synthesis of DNA, RNA, and certain coenzymes needed in the central metabolic pathway (Martinussen *et al*. 1998).

The first step in the pyrimidine biosynthetic pathway is the synthesis of carbamoylphosphate (CP) formed by fusing bicarbonate with an ammonium group from glutamine, and a phosphate group obtained from ATP. A second ATP molecule is hydrolyzed to donate sufficient energy to drive the reaction (Kilstrup *et al.* 2005). CP is also precursor for the biosynthesis of the amino acid arginine. The formation of CP is catalyzed by carbamoylphosphate synthetase (CpS) (Martinussen *et al*. 2001). Prokaryotic CpS are allosteric heterodimeric enzymes composed of a small glutaminase subunit encoded by *carA* and a large synthetase subunit encoded by *carB* (Nicoloff *et al*. 2001). The two genes are commonly organized as an operon with the gene order *carAB* (Nicoloff *et al*. 2000).

Streptococcus thermophilus is a major component of dairy starters used for the manufacture of yogurt and cheeses. One of the main roles of *S. thermophilus* is to provide rapid acidification as a consequence of the production of lactic acid. Despite the relevance of amino acids and nucleic acid biosynthetic pathways in the adaptation of *S. thermophilus*, to dairy environments, only the functionality of genes involved in branched-chain amino acids, proline and glutamine biosynthesis have been experimentally investigated in this species (Limauro *et al.* 1996; Garault *et al.* 2000; Monnet *et al.* 2005). More recently, the aspartate biosynthesis of *S. thermophilus* was studied with a primary focus on the first step of the pathway, in particular, the fixation of CO_2 by a phosphoenolpyruvate carboxylase. This study demonstrated that this enzymatic activity is fundamental for the growth of *S. thermophilus* in milk and that aspartate availability modulates the level of urease activity (Arioli *et al.* 2007) (Fig. 1). The genes coding for the enzymes involved in the anaplerotic reactions described in Figure 1 are core genes, *i.e.* genes which are present in all dairy *S. thermophilus* strains and are presumed to be essential for survival under conditions normally encountered by *S.*

thermophilus in dairy environments (Rasmussen *et al.* 2008). In this study, we investigated the carbon dioxide metabolism of *S. thermophilus*, by analyzing the phenotype of a CpS-negative mutant obtained by replacement of a functional *carB* gene with a deleted and inactive version, *ΔcarB*. We showed that CpS-negative mutant is auxotrophe for both L-Arg and uracil, the final products of the metabolic pathway governed by CpS. Moreover, the inactivation of *carB* resulted in a significant reduction of growth rate of the mutant in milk.

Materials and Methods

Bacterial strains, growth conditions, and reagents

Wild-type *S. thermophilus* DSM 20617^T and its CpS-negative derivative, A17(Δ*carB*), were maintained in M17 broth (Difco Laboratories, Detroit, Mich.) at 37 °C. Plasmid-containing *S. thermophilus* strains were maintained in M17 broth (10 g l^{-1}, lactose) supplemented with 5 μg of erythromycin per ml at 28 °C, while strains containing the pG$^+$host9 derived vector integrated into the chromosome were maintained in the same medium supplemented with 2 μg of erythromycin per ml at 42 °C. *Escherichia coli* strains were routinely maintained in aerobic conditions in Luria broth at 37 °C supplemented with 10 μg of kanamycin per ml and when necessary with 200 μg of erythromycin per ml. The auxotrophy for L-Arg and/or uracil and the growth behavior of *S. thermophilus* wild-type and A17(Δ*carB*) in the absence and in the presence of uracil and/or L-Arg was evaluated in a chemically-defined medium (CDM) containing lactose (10 g l^{-1}), derived from the medium described by Reiter and Oram (1962) deprived of both uracil and arginine. A mixture containing lactose, salts and vitamins was prepared at a concentration double that in the CDM. Whenever requested, this medium was supplemented with L-Arg and/or uracil, using a filter sterilized concentrated solution. Milk cultures were performed with reconstituted (10% w/v) skimmed milk (SM) (Difco Laboratories, Detroit, Mich.) that had been heat treated at 80 °C for 10 min.

The effect of carbon dioxide availability on the growth yield of *S. thermophilus* was investigated on CDM agar on Petri dishes. Briefly, cells grown in M17 broth at 37 °C for 18 h, were harvested by centrifugation, washed twice in sterile NaCl solution (9 g l^{-1}), resuspended in the same solution and inoculated at a concentration equivalent to 0.07 absorbance unit (575 nm) in CDM agar plates containing 5 ml of solid media with or without the addition of 0.1 mmol l^{-1} L-Arg plus 0.2 mmol l^{-1} uracil. Inoculated Petri plates were incubated at 37 °C for 24 h in 2.5 l anaerobic jars under N_2 atmosphere or in CO_2 enriched atmosphere (18 % v/v) obtained using one Anaerocult A (Merk KGaA, Milan, Italy) for each jar. After an incubation period of 24 h at 37 °C, the culture grown on the plates was collected using sterile NaCl 9 g l^{-1} and concentrated by centrifugation to a 1 ml final volume, using the same solution. The cell density was evaluated spectrophotometrically at 575 nm.

PCR protocols and DNA sequencing

Total bacterial DNA was extracted starting from 100 μl of M17 broth culture as previously described (Arioli *et al.* 2007). A PCR approach for the amplification of the *carB* gene was developed on the basis of the genome sequence of *S. thermophilus* LMG13811, CNRZ1066 and LMD9 (entry CP000023, CP000024 and CP000419 respectively) (Bolotin *et al.* 2004; Makarova *et al.* 2006). The amplification of a DNA region of about 3200 bp encompassing the entire *carB* gene was performed as recommended by the supplier (Takara Bio Inc., Shida, Japan) using 0.5 μmol l^{-1} of primers CarBF 5'- GACAACCAGAGAGTTGTC -3' and CarBR 5'- ACAAGAAGCTCTGGAGAGA -3' and 2 U of ExTaq DNA polymerase. The PCR conditions were: 35 cycles at 94°C for 1 min, 60 °C for 35 s and 72 °C for 2 min and a single final extension at 72 °C for 7 min. All amplification reactions were performed in a Mastercycler (Eppendorf Italia s.r.l., Milan, Italy). The PCR product was purified (NucleoSpin Extract, Machery-Nagel GmbH and Co, Düren, Germany) and sequenced using the CarBF and CarBR primers followed by primer walking. The sequencing reactions were analyzed in a 310 automatic DNA sequencer (Applera, Monza, Italy) with fluorescent dideoxy chain

terminators (Big Dye Terminertor Cycle Sequencing kit V 2.0, Applera, Monza, Italy). The sequence obtained was analyzed with ORF Finder and BLAST services at the National Center for Biotechnology Information and subsequently manually aligned with the homologous *carB* gene of *S. thermophilus* LMG13811, CNRZ1066 and LMD9.

Replacement of *carB* gene with a deleted version *ΔcarB* by allelic exchange

DNA manipulation of pG$^+$host9 vector and derivatives was carried out in *Escherichia coli* VE7108. Plasmid isolation was performed using the Nucleospin plasmid Kit according to the manufacture's instructions (Machery-Nagel GmbH and Co, Düren, Germany).

Streptococcus thermophilus strain A19 contains a deletion of 1687 bp in the *carB* gene referred to as *ΔcarB*. The *ΔcarB* gene, was obtained by PCR as previously described (Mora *et al*. 2004). Briefly, DNA fragments located upstream and downstream of the 1687 bp deletion were independently amplified using the CarB1-CarB2 and CarB3-CarB4 primer pairs (CarB1 CTATTATCATTGGTCAGGCT; CarB2 GTTACATACCACAAGAGCAT; CarB3 GCGTGATGCCGAAGACAATGCTCTTGTGGTATGTAAC TCCAAGAGACTATCGCTGA; CarB4 TATCAAGGGCTGTGAAGAGT). Primer CarB3 has a 37 bp 5'-region complementary to the 5' region of an amplified product obtained using CarB1-CarB2 primer set. To generate a new template DNA containing a deleted version of the *carB* gene, these 2 PCR fragments were diluted to a final amount of 100 fmol and mixed with 5 μl of 10X PCR reaction buffer, 200 μmol l^{-1} of each dNTP and 1.5 U of Taq DNA polymerase in 50 μl (Amersham-Pharmacia Biotech, Milan, Italy) and subjected to a single cycle of the following thermal protocol: denaturation at 94 °C for 2 min, reassociation at 40 °C for 5 min and extension at 72 °C for 10 min. Following this step, primers CarB5 and CarB6 (CarB5 TTATTACTGCAGCTGCAGGCAAGCTTGCTTGGCTTTGA; CarB6 TTATTACTGCAGCTGCAGAGCTGCTCCATCTTCGTCAT) carrying a *Pst*I site at their 5' ends were added to the reaction mixture at the final concentration of 0.5 μmol l^{-1} and subjected to the following amplification protocol: 40 cycles consisting of 94°C for 45 s, 58 °C for 35 s and 72 °C for 50 s and followed by a final extension at 72 °C for 10 min (*data not*

shown, Figure II only for reviewers). The resulting PCR fragment, *ΔcarB* was ligated into the dephosphorylated-*Pst*I site of pG$^+$host9, generating pMI76. pMI76 was introduced into *S. thermophilus* wild-type, using a previously described protocol (Arioli *et al*. 2007). The procedure of gene replacement described by Biswas and coworkers (1993) was then applied to the *carB* gene. The resulting carbamoylphosphate synthetase negative mutant was named A17(*ΔcarB*) and analyzed by PCR using the primer set CarBF-CarBR to verify the presence of the recombinant *ΔcarB* gene *(data not shown, Figure III only for reviewers)*.

Growth curves and measurement of acidifying activity

Growth curves of DSM 20167^T and the A17 derivative mutant were evaluated in triplicate in CDM and in pasteurized skimmed milk (SM) at 37°C, as described by Arioli *et al.* (2007). Briefly, cells from of an overnight M17 culture were harvested by centrifugation, washed twice and resuspended in sterile NaCl 9 g l^{-1} and inoculated at a concentration equivalent to 0.7 absorbance unit (575 nm) in CDM or in reconstituted SM. Sterile 8-ml tubes were filled with 7.8 ml of inoculated CDM or milk and hermetically sealed. The growth in CDM was measured spectrophotometrically. The growth in SM was measured with a microplate-reader M680 (Bio-Rad Laboratories, Hercules, CA, USA). A clarification procedure was necessary for the evaluation of growth in milk (Chen and Steele 1998). Specifically, 0.5 ml of skim milk culture was mixed with 0.5 ml of 2 mol l^{-1} borate-200 mmol l^{-1} EDTA (pH 8.0) and incubated at 55°C for 10 min. The cells were then harvested by centrifugation and washed once with 1.0 ml of 2 mol l^{-1} borate-200 mmol l^{-1} EDTA (pH 8.0). The cell pellet was washed twice with 100 mmol l^{-1} BisTris buffer (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane) (pH 6.5), and the absorbance at 575 nm was determined. Some experiments were performed in the presence of L-Arg and/or uracil. The growth rates of *S. thermophilus* strains were determined from the linear portion of the line of ln(OD) vs. time, which corresponds to a cell density lower than 0.4 OD. For evaluating the acidifying activity of *S. thermophilus* in milk, the pH was measured continuously during 24 h using a CINAC apparatus (Ysebaert, Frépillon, France).

Results

Effect of carbon dioxide availability on arginine and uracil auxotrophy

In accordance with the annotated genomes of *S. thermophilus* (Bolotin *et al.* 2004; Hols *et al.* 2005, Makarova *et al*. 2006), the main anaplerotic reactions involved in carbon dioxide fixation are shown in Fig. 1. Besides the biosynthetic pathway involving phosphoenolpyruvate carboxylase (PpC), whose physiological role in the metabolism of *S. thermophilus* was previously investigated (Arioli *et al.* 2007), the putative genes involved in arginine and uracil biosynthetic pathways and carbamoylphosphate synthetase were also present in the core genome of this species. To investigate the role of carbamoylphosphate synthetase in *S. thermophilus* metabolism, the microorganism was grown in chemically defined medium (CDM) medium agar plates with and without supplementation of arginine and uracil in enriched carbon dioxide or under nitrogen atmosphere. The results obtained (Fig. 2) clearly showed that carbon dioxide depletion determined arginine and uracil auxotrophy. Under nitrogen atmosphere *S. thermophilus* was not able to grow in CDM without supplementation of arginine and uracil, while under a carbon dioxide enriched atmosphere arginine and uracil significantly enhanced the growth yield (Fig. 2).

Sequence analysis of the *carB* gene

Primers designed on the basis of the sequences of the genomes of strains LMG13811, CNRZ1066 and LMD9 allowed the amplification of a 3200 bp fragment coding for a 1059-amino-acid CpS protein with a predicted molecular mass of 116.44 kDa. *Streptococcus thermophilus* DSM 20617^T *carB* gene sequence (GenBank accession No. AM983544) revealed a high similarity (99%) with the *carB* gene sequences from strains LMG13811, CNRZ1066 and LMD9. The average percent G+C of the *carB* gene was higher, 43.4%, than expected for the *S. thermophilus* species, 39% (Bolotin et al, 2004; Hols et al, 2005, Makarova *et al.* 2006).

Carbamoylphosphate synthetase-negative mutant auxotrophy for arginine and uracil

To evaluate the phenotypic effect of the *carB* deletion in *S. thermophilus* and to confirm the auxotrophy for arginine and uracil underlined culturing the wild-type strain in carbon dioxide depleted atmosphere, the mutant A17(*ΔcarB*) characterized by a truncated *carB* gene was obtained by gene replacement and compared with the wild type strain DSM 20617^T in CDM. As shown in Fig. 3A, the growth rate of the wild-type (0.62 h^{-1}) was significantly enhanced by the addition of 0.1 to 2.4 mmol l^{-1} arginine to the medium (1.02 h^{-1} and 0.99 respectively). The addition of 0.2-2 mmol l^{-1} uracil resulted in a lower stimulation of the growth rate of the wild-type strain (0,75 and 0.68 respectively). As expected, the mutant A17(*ΔcarB*) was unable to grow in the absence of arginine and uracil (Fig. 3B) and no growth was observed when the medium was supplemented with arginine alone or uracile alone (data not shown). The addition of both 0.1 mmol l^{-1} arginine and 0.2 mmol l^{-1} uracil to the medium allowed the mutant strain to reach a growth rate higher than that measured for the wild-type strain (1.23 h^{-1}). Increasing the concentration of arginine from 0.1 to 2.4 mmol l^{-1} did not prove to have a significant effect on the growth rate of the mutant, while the increase of uracil concentration from 0.2 mmol l^{-1} to 2 mmol l^{-1} determined a significant reduction of the growth rate of the mutant both in presence of 0.1 and 2.4 mmol l^{-1} (0.54 and 0.57 h^{-1} respectively).

Carbamoylphosphate synthetase activity is essential for the optimal growth and acidification of *S. thermophilus* in milk

The effect of the inactivation of CpS on the growth of *S. thermophilus* in milk was evaluated. Comparative experiments carried out with the wild-type and the A17(*ΔcarB*) mutant in reconstituted skimmed milk (RSM) revealed a significant reduction of growth and acidification rate in absence of a functional CpS activity (Fig. 4). Interestingly, the addition of arginine and uracil to the milk did not have any positive effect on the growth of the A17(*ΔcarB*) mutant but determined an unexpected reduction of growth and acidification rate both in the wild-type and in the mutant (Fig. 4 and *data not shown Figure I only for reviewers*). When uracil and

245 arginine were added to the milk individually it was observed that uracil determined the
246 highest reduction of the acidification rate both in the wild-type and in the A17 mutant.

Discussion

The carbon dioxide metabolism of *S. thermophilus* was investigated with respect to the carbamoylphosphate synthetase activity which is involved in the metabolic pathway for the biosynthesis of arginine and uracil (Fig. 1). The metabolic relevance of this pathway was highlighted by culturing *S. thermophilus* in a CDM under nitrogen or carbon dioxide enriched atmosphere. Indeed, the microorganism became auxotroph for arginine and uracil in absence of carbon dioxide. A carbon dioxide concentration-dependent auxotrophy for arginine and uracil has also been reported for some strains of *Lactobacillus plantarum* (Nicoloff *et al.*, 2001). In this species, carbamoylphosphate, a precursor for both pyrimidine and arginine synthesis (Fig. 1), is synthesized by two CpSs, CpS-P and CpS-A encoded by *pyrAaAb* and *carAB*, respectively, whose transcription is regulated by pyrimidines and arginine availability (Nicoloff *et al.* 2000, 2001, 2005). *L. plantarum* strains showing a high-CO_2-requiring prototrophy are able to synthesize sufficient arginine and pyrimidines only in CO_2-enriched air. Unlike *L. plantarum*, *S. thermophilus* has only one CpS encoded by a *carB* gene and that represents a key node in arginine and pyrimidine biosynthesis. The mutant A17, in which the wild-type gene *carB* was inactivated with a deleted and inactive version *ΔcarB*, was auxotroph for arginine and uracil. Interestingly, while arginine and uracil showed a positive effect on the growth rate of the wild-type strain in a CDM, the addition of the two molecules in milk resulted in a significant reduction of growth and acidification. The comparison between the growth of the wild-type strain and the mutant A17 showed that carbamoylphosphate synthetase activity is essential for the optimal growth of *S. thermophilus* in milk. The mutant A17 had a reduced growth in comparison to the wild type strain, even when the milk was supplemented with arginine and uracil. This result was unexpected and may be due to a limitation transport of these nutrients across the membrane. For example, arginine and uracil may compete with other related molecules on common membrane transporters, resulting in a nutrient starvation. Similar observations have been previously reported by Braquart *et al.* 1998 and Arioli *et al.* 2007 concerning the growth-inhibiting effect

of high aspartate concentrations in the culture media. During the growth of *S. thermophilus* in milk, glutamate is required in large amounts for the optimal growth of *S. thermophilus*, which is auxotrophic for this amino acid. The presence of an excess of aspartic acid saturates the entry sites, which reduces glutamate uptake and therefore the bacterial growth (Bracquart *et al.* 1998).

In conclusion, this study revealed that arginine and uracil biosynthesis is essential for the optimal growth and acidification of *S. thermophilus* in milk.

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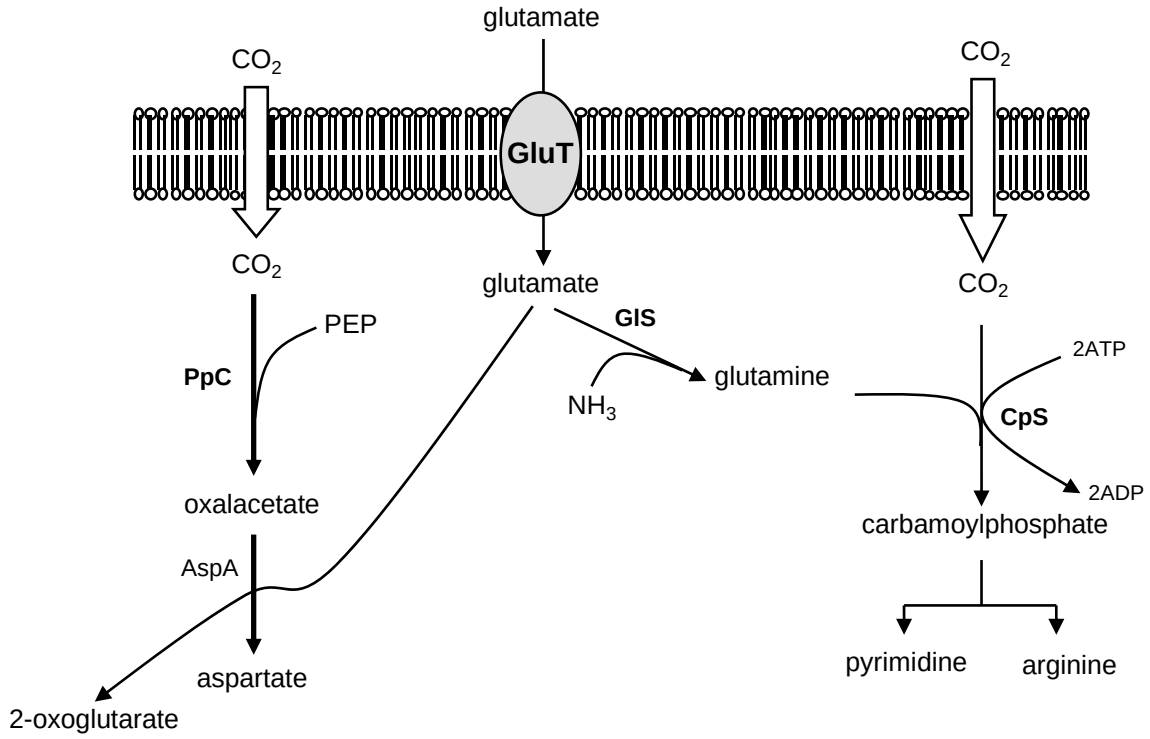
Figure 1 Schematic representation of the anaplerotic reactions involved in carbon dioxide fixation and in aspartate, arginine and uracil biosynthesis as deduced from the annotated genomes of *Streptococcus thermophilus* (Bolotin *et al.*, 2004). PpC, phosphoenolpyruvate carboxylase, CpS, carbamoylphosphate synthetase, AspA, aspartate aminotransferase, GlS, glutamine synthetase, GluT, hypothetical glutamate membrane transporter. The shaded box indicates the enzymatic activity inactivated in the mutant A17(*ΔcarB*).



373 **Figure 2** Growth of the *S. thermophilus* wild-type strain in CDM under nitrogen or
374 carbon dioxide enriched atmosphere in CDM (white bars), or CDM supplemented with 0.1
375 mM L-Arg and 0.2 mM uracil (CDM-au) (black and grey bars). All cultures were repeated
376 three times and the standard deviation values were always < 0.1 O.D. $_{600\ nm}$ units.
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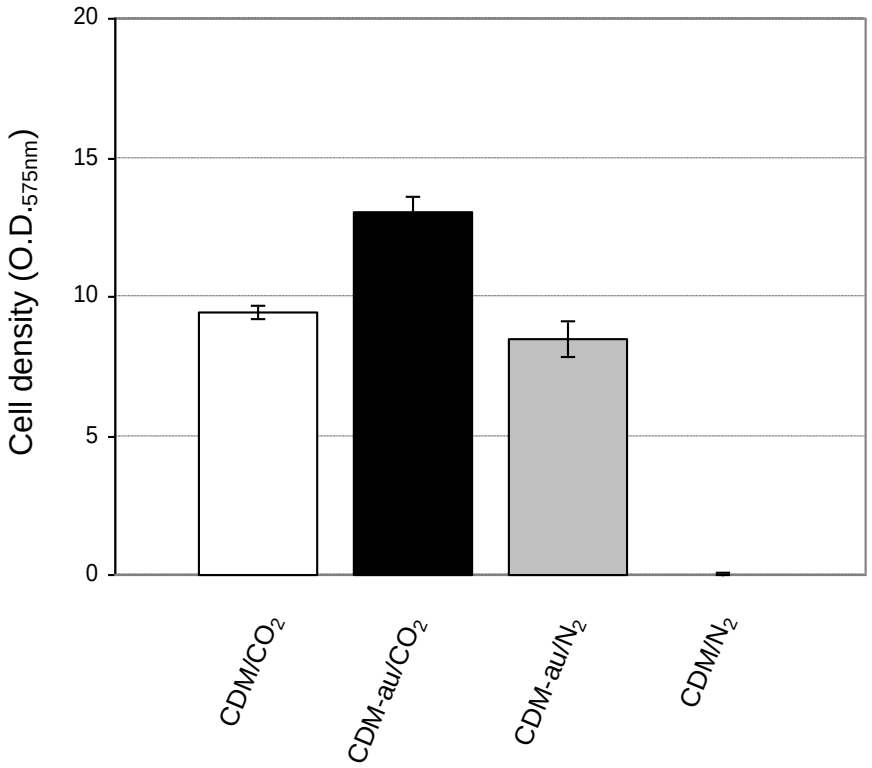

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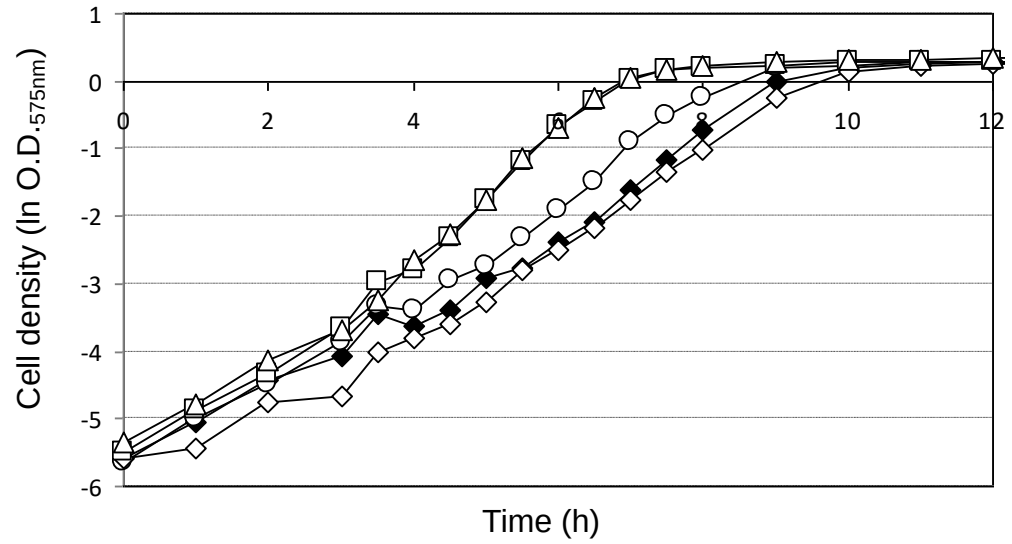
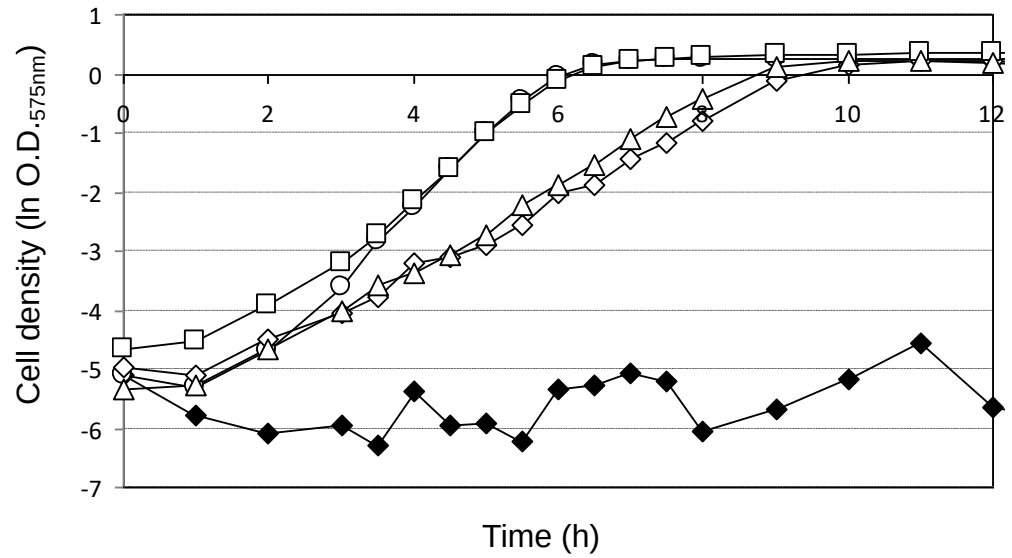
Figure 3 (a) Growth of *S. thermophilus* wild-type in CDM (◆), CDM supplemented with 0.2 mM uracil (○), and 2 mM uracil (◇), 0.1 mM L-Arg (□) and 2.4 mM L-Arg (△). (b) Growth of the *S. thermophilus* A17(*ΔcarB*) mutant in CDM (◆), CDM supplemented with 0.2 mM uracil plus 0.1 mM L-Arg (○), 2 mM uracil plus 0.1 mM L-Arg (◇), 0.2 mM uracil plus 2.4 mM L-Arg (□),and 2 mM uracil plus 2.4 mM L-Arg (△). All CDM cultures were repeated three times and the standard error values of the growth rates and the final biomass concentrations were lower than 3%.

(a)


Cell density (ln O.D.575nm)
Time (h)
1
0
-1
-2
-3
-4
-5
-6
0
2
4
6
8
10
12

(b)


Cell density (ln O.D.575nm)
Time (h)
1
0
-1
-2
-3
-4
-5
-6
-7
0
2
4
6
8
10
12

389

390

Figure 4 (a) Growth of the *S. thermophilus* wild-type strain (◆) and the A17(*ΔcarB*) mutant (■) in reconstituted skimmed milk without (black symbols) and with (white symbols) the addition of 0.2 mM uracil plus 2,4 mM L-Arg. (b) Milk acidification curves in the same experimental condition as above described. All milk cultures were repeated three times and the standard deviation values were always < 0.08 O.D. $_{600\ nm}$ units and < 0.08 pH units.