The relevance of carbon dioxide metabolism in *Streptococcus thermophilus*

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Streptococcus thermophilus is a major component of dairy starter cultures used for the manufacture of yoghurt and cheese. In this study, the CO₂ metabolism of *S. thermophilus* DSM 20617^T, grown in either a N₂ atmosphere or an enriched CO₂ atmosphere, was analysed using both genetic and proteomic approaches. Growth experiments performed in a chemically defined medium revealed that CO₂ depletion resulted in bacterial arginine, aspartate and uracil auxotrophy. Moreover, CO₂ depletion governed a significant change in cell morphology, and a high reduction in biomass production. A comparative proteomic analysis revealed that cells of *S. thermophilus* showed a different degree of energy status depending on the CO₂ availability. In agreement with proteomic data, cells grown under N₂ showed a significantly higher milk acidification rate compared with those grown in an enriched CO₂ atmosphere. Experiments carried out on *S. thermophilus* wild-type and its derivative mutant, which was inactivated in the phosphoenolpyruvate carboxylase and carbamoyl-phosphate synthase activities responsible for fixing CO₂ to organic molecules, suggested that the anaplerotic reactions governed by these enzymes have a central role in bacterial metabolism. Our results reveal the capnophilic nature of this micro-organism, underlining the essential role of CO₂ in *S. thermophilus* physiology, and suggesting potential applications in dairy fermentation processes.

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INTRODUCTION

Streptococcus thermophilus is a food micro-organism that is widely used in several milk fermentation processes. *S. thermophilus* is a Gram-positive lactic acid bacterium characterized by an anaerobic metabolism based on glycolysis and homolactic fermentation. The main role of *S. thermophilus* in dairy fermentations is the rapid conversion of lactose into lactate, and also the production of other compounds that contribute to food flavour and texture. Its capacity to ferment milk is strictly related to the ability of *S. thermophilus* to take up lactose from milk, and

to catabolize its glucose moiety. Despite the anaerobic nature of this species, the role of CO₂ on the overall metabolism of *S. thermophilus* has been investigated rarely. While a CO₂-enriched atmosphere is used effectively in foodstuff preservation against bacterial spoilage, a growth-stimulating effect of CO₂ has been reported for a limited number of lactic acid bacterial species. Detailed studies are available for *Lactobacillus plantarum* and *Lactococcus lactis* only (Wang *et al.*, 1998, 2000; Nicoloff *et al.*, 2000, 2005; Arsène-Ploetze *et al.*, 2006). The high atmospheric concentration of CO₂ could have several effects on cellular physiology because it alters membrane properties, probably modifies the intracellular pH, and interferes with carboxylation reactions. In lactic acid bacteria, and in almost all organisms, CO₂ is used as a substrate for several carboxylation reactions, such as anaplerotic reactions, and amino acid and pyrimidine biosynthesis pathways. Regarding *S. thermophilus*, data available on CO₂ metabolism are related to the ability of this bacterium to fix

Abbreviations: CpS, carbamoyl-phosphate synthetase; HPr(His-P), histidine phosphorylated HPr; HPr(Ser-P), serine phosphorylated HPr; μ LC-ESI-IT-MS-MS, micro liquid chromatography coupled with electrospray ionization ion trap tandem mass spectrometry; pI, isoelectric point; PpC, phosphoenolpyruvate carboxylase.

The GenBank/EMBL/DDBJ accession number for the sequence determined in this work is AM983544.

bicarbonate (Louaileche *et al.*, 1993, 1996). These studies have demonstrated that CO₂ and sodium bicarbonate stimulate growth and exert a marked influence on the metabolic activities of *S. thermophilus*. In analyses of the bacterial genome of two strains (Bolotin *et al.*, 2004; Hols *et al.*, 2005), there has been observation of two main anaplerotic pathways that are governed by enzymic activities able to fix CO₂ to organic molecules. These pathways are involved in amino acid and pyrimidine synthesis, and are governed by phosphoenolpyruvate carboxylase (PpC) and carbamoyl-phosphate synthase (CpS), which catalyse the fixation of CO₂ to phosphoenolpyruvate and glutamine, respectively (Fig. 1). Recently, the CO₂ metabolism of *S. thermophilus* has been investigated by evaluating the phenotype of a PpC⁻ mutant (Arioli *et al.*, 2007). That work demonstrated that the fixation of CO₂ by PpC activity is fundamental for the biosynthesis of aspartic acid, and for the growth of *S. thermophilus* in milk (Arioli *et al.*, 2007). That study also reported that the availability of aspartic acid modulates the level of urea amidohydrolase activity, underlining the existence of a link between CO₂ and N₂ metabolism in this species.

In the current work, the CO₂ metabolism of *S. thermophilus* DSM20617^T was investigated by taking advantage of our previous experience in proteomic analysis of this bacterium (Arena *et al.*, 2006; Salzano *et al.*, 2007). To emphasize the effect of this gas on bacterial metabolism, *S. thermophilus* cells were grown on solid media under an enriched CO₂ atmosphere or a N₂ atmosphere, and comparatively evaluated. A differential analysis was also performed between the wild-type and a mutant that was obtained by inactivating the two enzymes involved in CO₂ fixation to phosphoenolpyruvate and glutamine. Our results underline the central role of CO₂ in the overall metabolism of *S. thermophilus*, and provide additional information on the physiology of this industrially important lactic acid bacterium.

METHODS

Bacterial strains, growth conditions and reagents. Wild-type PpC⁺CpS⁺ *S. thermophilus* DSM 20617^T, its PpC⁻ derivative A18(Δ ppc) (Arioli *et al.*, 2007), and the PpC⁻CpS⁻ double mutant A19(Δ ppc Δ carB), were maintained in M17 broth (Difco Laboratories) at 37 °C. Plasmid-containing *S. thermophilus* strains were maintained in M17 broth (10 g lactose l⁻¹) supplemented with 5 µg erythromycin ml⁻¹, at 28 °C, while strains containing the pG⁺host9-derived vector integrated into the chromosome were maintained in the M17 broth supplemented with 2 µg erythromycin ml⁻¹, at 42 °C. *Escherichia coli* strains were routinely maintained in Luria-Bertani broth at 37 °C with aeration; the medium was supplemented with 10 µg kanamycin ml⁻¹ and, when necessary, with 200 µg erythromycin ml⁻¹. The auxotrophy for aspartic acid, arginine and uracil of *S. thermophilus* wild-type, A18(Δ ppc) and A19(Δ ppc Δ carB) was evaluated in a chemically defined medium (CDM) containing lactose (10 g l⁻¹); this medium was derived from that described by Reiter & Oram (1962), and modified as described by Arioli *et al.* (2007). A mixture containing lactose, salts and vitamins was prepared at a concentration that was 2× the concentration in CDM. After adjusting the pH to 7 using NaOH, the mixture was autoclaved for 15 min at 110 °C. The amino acids were prepared as a 5× concentrated solution that was filter sterilized (0.22 µm) after adjustment of its pH to 7. When necessary, this medium was supplemented with 2.25 mM L-aspartate, 0.1 mM L-arginine, 0.2 mM uracil, 1–5 mM urea and 1–50 mM sodium bicarbonate.

Cell biomass for proteomic studies was obtained by growing *S. thermophilus* on M17 agar plates. Briefly, cultures grown on solid medium were collected using sterile NaCl solution (9 g l⁻¹), harvested by centrifugation, washed twice, resuspended in sterile NaCl solution, and inoculated at a concentration equivalent to an OD₅₉₅ of 0.7 on M17 agar containing 2% lactose, pH 6.8. Inoculated agar was incubated at 37 °C for 24 h in a 2.5 l anaerobic jar in a N₂ atmosphere, or in a CO₂-enriched atmosphere (18%, v/v) obtained using one Anaerocult A sachet (Merck KGaA) in each jar. To investigate the experimental conditions under which a spontaneous release of CO₂ occurred during the bacterial growth, inoculated plates were incubated on M17 medium containing 1 mM urea, under a N₂ atmosphere (Mora *et al.*, 2004, 2005).

PCR protocols and DNA sequencing. Total bacterial DNA was extracted from 100 µl M17 broth culture, as previously described (Mora *et al.*, 2004). A PCR approach for the amplification of the *carB*

Fig. 1. Schematic representation of the anaplerotic reactions and related biosynthetic pathways involved in aspartate, arginine and pyrimidine biosynthesis, and urea metabolism, in *S. thermophilus*. *ppc*, Gene encoding phosphoenolpyruvate carboxylase; *carB*, gene encoding carbamoyl-phosphate synthase; *aspA*, gene encoding aspartate aminotransferase; *glnA*, gene encoding glutamine synthase; *urelABCDEFG*, operon encoding urease; GluT, hypothetical glutamate membrane transporter. The genes *carB* and *ppc*, in bold boxes, were inactivated in the mutant A19(Δ ppc Δ carB).

gene was developed on the basis of the genome sequences of *S. thermophilus* strains LMG 18311, CNRZ1066 and LMD-9 (GenBank accession numbers CP000023, CP000024 and CP000419, respectively). The amplification of a DNA region of about 3200 bp, encompassing the whole *carB* gene, was performed as recommended by the supplier, using primers CarBF (5'-GGAAATCGGTGAGAAAGCT-3') and CarBR (5'-CTGCTCAGCTTTTCAATCGT-3') at 0.5 µmol l⁻¹, and 2 U *ExTaq* DNA polymerase (Takara Bio). 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). The PCR product was purified (NucleoSpin Extract; Machery-Nagel), and sequenced using the CarBF and CarBR primers, followed by primer walking. The sequence reactions were analysed in a 310 automatic DNA sequencer (Applied) with fluorescent dideoxy chain terminators (Big Dye Terminator Cycle Sequencing kit version 2.0; Applied). The sequence obtained (GenBank accession no. AM983544) was analysed with ORF Finder and BLAST services at the National Center for Biotechnology Information (NCBI), and then manually aligned with the homologous *carB* genes of *S. thermophilus* LMG 18311, CNRZ1066 and LMD-9.

Replacement of *carB* gene with a deleted version $\Delta carB$ in the mutant A18(Δppc). DNA manipulation of pG⁺host9 vector and derivatives was carried out in *E. coli* VE7108 (Biswas *et al.*, 1993; Mora *et al.*, 2004). Plasmid isolation was performed using a Nucleospin plasmid kit (Machery-Nagel), according to the manufacturer's instructions.

Strain A19 contains a deletion of 1687 bp in the *carB* gene, referred to as $\Delta carB$. The $\Delta carB$ gene was obtained by PCR, as previously described (Arioli *et al.*, 2007; Mora *et al.*, 2004). Briefly, DNA fragments located upstream and downstream of the 1687 bp deletion were independently amplified using CarB1–CarB2 and CarB3–CarB4 primer pairs (CarB1, 5'-CTATTATCATTTGGTCAGGCT-3'; CarB2, 5'-GTTACATACCACAAGAGCAT-3'; CarB3, 5'-GCGTGATGCCGAAGACAATGCTCTTG-TGGTATGTAAGTCCAAGAGACTATCGCTGA-3'; CarB4, 5'-TATCAAGGGCTGTGAAGAGT-3'). Primer CarB3 has a 37 bp 5' region complementary to the 5' region of the amplified product obtained using the CarB1–CarB2 primer set. To generate a new template DNA containing a deleted version of the *ppc* gene, these two PCR fragments were diluted to a final concentration of 100 fmol, and mixed with 5 µl 10× PCR buffer, 200 µM each dNTP, and 1.5 U *Taq* DNA polymerase in 50 µl (Amersham-Pharmacia Biotech), and subjected to the following thermal protocol: denaturation at 94 °C for 2 min, re-association at 40 °C for 5 min, and extension at 72 °C for 10 min. Following this step, primers CarB5 (5'-TTATTACTGCAGCTGCAGGCAAGCTTGCTTG-GCTTTGA-3') and CarB6 (5'-TTATTACTGCAGCTGCAGAGCTGCTCCATCTTCGTCAT-3'), carrying a *Pst*I site at the 5' end, were added to the reaction mixture to a final concentration of 0.5 µM, and subjected to the following amplification protocol: 40 cycles of 94 °C for 45 s, 58 °C for 35 s and 72 °C for 50 s, followed by a final extension at 72 °C for 10 min. The resulting PCR fragment $\Delta carB$ was ligated into the dephosphorylated *Pst*I site of pG⁺host9, to generate pMI76. pMI76 was introduced into *S. thermophilus* A18(Δppc) (Arioli *et al.*, 2007) by using a previously described protocol (Arioli *et al.*, 2007; Mora *et al.*, 2004). The procedure of gene replacement described by Biswas and coworkers (Biswas *et al.*, 1993) was then applied to the *carB* gene. The resulting carbamoyl-phosphate-synthase-negative mutant was named A19($\Delta ppc\Delta carB$).

Growth curves and measurements. Growth curves of DSM 20167^T and the A19 derivative mutant were evaluated in triplicate at 37 °C. Briefly, cells from an overnight M17 culture were harvested by centrifugation, washed twice, suspended in sterile NaCl solution (9 g l⁻¹), and inoculated at a concentration equivalent to an OD₆₀₀ of 0.7 in CDM. Sterile 8 ml tubes were filled with 7.8 ml inoculated CDM, and hermetically sealed. The growth was measured

spectrophotometrically at 600 nm. The effect of gas composition on the growth level of *S. thermophilus* was evaluated by inoculating 50 µl cell suspension, prepared as described above, on the surface of plates containing 5 ml CDM. After incubation of 24 h at 37 °C, the culture grown on the plates was collected into sterile NaCl (9 g l⁻¹), and concentrated by centrifugation to a 1 ml final volume in NaCl. The cell density was evaluated spectrophotometrically at 600 nm.

Milk acidification rate, and enzymic activity measurements. For milk acidification experiments, cells were collected as described for proteomic analysis, washed in NaCl solution to reach a final density of 1.2 (OD₆₀₀), and inoculated (5%, v/v) in reconstituted skimmed milk (Difco Laboratories) that had been pasteurized at 80 °C for 10 min. Acidification measurements were followed by using a standard pH meter for 24 h at 37 °C. The cell suspension used to inoculate the milk was also subjected to evaluation for β -galactosidase and lactate dehydrogenase activities. Cell suspensions, washed in 50 mM Tris/HCl buffer, pH 7, and concentrated, were subjected to mechanical disruption using a Precellys bead beater (Advanced Biotech). After centrifugation at 15 000 g at 4 °C, the protein content within total cell extract was measured using the Bradford method (Bradford, 1976), with BSA as the standard. β -Galactosidase activity was evaluated in 50 mM Tris/HCl buffer, pH 7, using 10 µg protein and 0.7 mg 2-nitrophenyl β -D-galactopyranoside ml⁻¹ (Sigma-Aldrich). Samples were incubated at 37 °C for 20 min, and the β -galactosidase activity was expressed as absorbance units min⁻¹ (mg of protein)⁻¹ by measuring the absorbance of o-nitrophenol at 420 nm [molar extinction coefficient (ϵ) 18.30 × 10³ l mol⁻¹ cm⁻¹]. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol product min⁻¹. The results are expressed as the means (±SD) of four independent determinations. Lactate dehydrogenase activity was determined by measuring the rate of NADH oxidation at 340 nm (ϵ 6.22 × 10³ l mol⁻¹ cm⁻¹), essentially as described by Gaspar *et al.* (2007) and Garrigues *et al.* (1997). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmol product min⁻¹. Lactate dehydrogenase activity is expressed as the mean (±SD) of four independent determinations.

¹³C/¹²C stable isotope ratio experiments. *S. thermophilus* was grown in M17 broth for 16 h in the presence of urea or [¹³C]urea (4 mM). Cells were collected by centrifugation, washed 10 times in distilled water and either: (i) lyophilized, and used for the determination of the ¹³C/¹²C stable isotope ratio; or (ii) subjected to a modified alkaline cell lysis protocol (Manachini *et al.*, 1985), without RNase treatment. Total extracted nucleic acid was recovered in water, and used for the evaluation of ¹³C/¹²C stable isotope ratio by isotope ratio MS. The ¹³C/¹²C (‰) values obtained are expressed as the means of three independent determinations (±SD).

Electron microscopy. Bacterial cells were collected by centrifugation (150 g), and the resulting pellet was processed for transmission electron microscopy. The extract treated bacterial cells were fixed in 2.5% glutaraldehyde, and later post-fixed with 1% osmium tetroxide (in 0.1 M cacodylate buffer, pH 7.2) for 2 h, at room temperature. After eliminating the remaining osmium tetroxide, the samples were dehydrated in a graduated cold ethanol series (35–100%); each step was performed for about 10–15 min, at room temperature. The fixed cells were embedded in Epon 812. Blocks were cut with an ultramicrotome (Ultracut; Reichert), and collected on nickel grids. Sections were post-stained with 5% uranyl acetate for 5 min at room temperature, and treated with lead citrate for 1 min. Sections were observed and photographed with a Philips CM 12 electron microscope and a Zeiss 900.

2D Electrophoresis. Three independent biological replicates of each bacterial growth experiment were harvested at 18 000 g, at 4 °C for 10 min, and quickly washed five times with cold PBS. Cellular pellets

were suspended in a solution containing 8 M urea, 4 % CHAPS, 1 % DTT, 10 mM Tris/HCl, and a cocktail of protease inhibitors (GE Healthcare), and disrupted five times by sonication for 4 min at maximum power. Cell debris was removed by centrifugation at 18 000 g at 20 °C for 60 min. The supernatant was precipitated, and delipidated with cold tri-*n*-butyl phosphate:acetone:methanol (1:12:1, v/v) (Mastro & Hall, 1999). Cellular pellets were resuspended in 8 M urea, 4 % CHAPS, 1 % DTT, 10 mM Tris/HCl and 2 % ampholine, pH 4.0–6.5, containing nuclease mix (GE Healthcare) to remove nucleic acids. The protein concentration within samples was determined using a 2-D Quant kit (GE Healthcare).

Home-made immobilized pH gradient (IPG) strips (13 cm), with a linear pH range of 4.0–5.5, were rehydrated overnight in a dedicated buffer (8 M urea; 4 %, w/v, CHAPS; 1 %, v/v, DTT; 10 mM Tris; 2 %, w/v, ampholine; pH 4.0–6.5). A 100 µg quantity protein sample was loaded on to each IPG strip via cathodic cup loading. IEF was applied using an Ettan IPGphor IEF system (GE Healthcare) at 20 °C, with a current of 160 µA per strip. The following protocol was applied: 30 V (4 h), 50 V (3 h), 100 V (3 h), 500 V (3 h), 1000 V (3 h), 3000 V (3 h), 4000 V (3 h), 6000 V (3 h) and 8000 V (8 h). After the first dimension, IPG strips were equilibrated twice with a solution containing 6 M urea, 2 % SDS, 50 mM Tris/HCl, pH 8.8, and 30 % (v/v) glycerol, for 15 min. To the first equilibrium, 1 % DTT was added; to the second equilibrium, 2.5 % iodoacetamide and a trace of Bromophenol Blue were added. The second dimension was performed by the use of home-made 8–15 % acrylamide gradient vertical SDS-PAGE slab gels (13 × 13 × 0.1 cm), using a Protean II xi cell (Bio-Rad); the gels were run at 15 mA per gel for 20 min, and then at 50 mA per gel. Then, gels were stained with colloidal Coomassie Blue, and scanned with a Pharos FX Plus Laser Imaging System (Bio-Rad). 2D Electrophoresis was performed in triplicate for each sample. Image analysis was performed using ImageMaster 2D Platinum version 6.0.1 software (GE Healthcare). Images were analysed for detection and quantification of the spot intensities, gel matching and statistical analysis. To correct for overall intensity differences between the gels, each gel was normalized to the standard according to the total quantity of valid spots. Differential analysis was limited to spots that were either unique to one set of gels or changed in intensity by a factor of at least two. Within the experimental replicates of the same sample, no percentage volume variations higher than 15 % were detected for the spots analysed.

MS analysis, and protein identification. Spots of interest were excised manually from 2D electrophoresis gels, destained, in-gel reduced, S-alkylated, and digested with trypsin, as previously reported (Talamo *et al.*, 2003). Digests were extracted with 25 mM NH₄HCO₃:acetonitrile (1:1, v/v) by sonication, and concentrated. Samples were desalted using µZipTipC18 pipette tips (Millipore) before MALDI-TOF MS analysis, and/or direct analysis by micro liquid chromatography coupled with electrospray ionization ion trap tandem mass spectrometry (µLC-ESI-IT-MS-MS).

Peptide digests were loaded on the MALDI target, together with α -cyano-4-hydroxycinnamic acid as the matrix. Samples were analysed with a Voyager-DE PRO spectrometer (Applera), as previously reported (Rocco *et al.*, 2006). Mascot software (Matrix Science) (Perkins *et al.*, 1999) was used to identify spots from a NCBI non-redundant database by using peptide mass fingerprinting experiments in reflectron mode. Candidates with program scores >79 were further evaluated by the comparison with M_r and isoelectric point (pI) experimental values from 2D electrophoresis.

Peptide digests were also analysed by µLC-ESI-IT-MS-MS using a LCQ Deca Xp Plus MS equipped with an electrospray source connected to a Phoenix 40 pump (ThermoFinnigan), as previously reported (Talamo *et al.*, 2003; D'Ambrosio *et al.*, 2006; Rocco *et al.*, 2006). Proteins were identified by the SEQUEST algorithm (MacCoss *et al.*, 2002) by

comparison of tryptic peptide product ion mass spectra against those generated from a database containing the annotated *S. thermophilus* LMG 13811, CNRZ1066 and LMD-9 genome sequences (Bolotin *et al.*, 2004; Makarova *et al.*, 2006), together with trypsin and keratins. The SEQUEST parameters included selection of trypsin with up to two missed cleavage sites, and static and dynamic mass modification associated with cysteine carbamidomethylation and methionine oxidation, respectively. Identified proteins were ranked in ascending order according to consensus scores, and false-positive identifications were minimized by filtration against four of the following criteria: Xcorr >2, ΔC_n >0.2, Sp >400, rsp <5, ions >30 % (Link *et al.*, 1999). Where appropriate, protein identifications were checked manually to provide for a false positive rate of <1 %, using Xcorr and ΔC_n values described and validated elsewhere (Peng *et al.*, 2003). Proteins with more than two identified peptides were considered as reliably identified proteins.

RESULTS

The effect of CO₂ availability on the metabolism of *S. thermophilus* was evaluated by growing strain DSM 20617^T on solid medium in an anaerobic jar, under an enriched CO₂ concentration (18 % v/v) or under a N₂ atmosphere. After incubation for 24 h at 37 °C, the biomass was collected, and analysed by electron microscopy and proteomic analysis. Prolonging the incubation to 36 or 48 h did not result in an increase in biomass yield; therefore, the cultures were considered to be in the stationary phase of growth after incubation for 24 h. The choice of solid medium to perform the experiments reported in this study was dictated by the difficulty in obtaining complete removal of CO₂ from liquid medium. Moreover, all the attempts to provide liquid medium that was CO₂ free for the time necessary to perform the experiments were ineffective.

Effect of CO₂ availability on cell morphology

Electron microscopy of *S. thermophilus* cells grown in the presence of different CO₂ availabilities revealed significant differences in cell morphology. Under a N₂ atmosphere, cells that were abnormally shaped were detected with high frequency. The cells were abnormally elongated, and were most probably the result of a defect in cell division (Fig. 2). When *S. thermophilus* was cultured under a N₂ atmosphere on solid medium supplemented with 1 mM urea, the normal ovoid shape was partially restored (Fig. 2d). This phenomenon is associated with the slow release of CO₂ during the growth of the micro-organism in the presence of urea (Mora *et al.*, 2004); in fact, it has been reported that bacterial urea amidohydrolase (EC 3.5.1.5) hydrolyses urea to NH₃ and carbamate, which spontaneously decomposes to yield a second molecule of NH₃ and CO₂. The changes in cell morphology observed in the CO₂-starved culture prompted us to investigate the metabolic activities involved in CO₂ utilization.

Role of the anaplerotic reactions catalysed by phosphoenolpyruvate carboxylase and carbamoyl-phosphate synthase in CO₂ metabolism

We have identified two main metabolic pathways involved in CO₂ utilization from the annotated genomes of *S.*

Fig. 2. Transmission electron micrographs showing *S. thermophilus* wild-type and A19($\Delta ppc\Delta carB$) mutant after growth in M17 medium under an enriched CO₂ atmosphere and a N₂ atmosphere. (a) Wild-type grown under enriched CO₂. (b) and (c) Wild-type grown under N₂. (d) Wild-type grown under N₂ on medium supplemented with 1 mM urea. (e) Mutant grown under enriched CO₂. (f) Mutant grown under enriched CO₂ on medium supplemented with aspartate, arginine and uracil (aa). Bars, 0.2 μ m.

thermophilus genome (Bolotin *et al.*, 2004; Hols *et al.*, 2005); these pathways are governed by PpC and CpS (Fig. 1), and are involved in amino acid and pyrimidine biosynthesis. To understand the relevance of these pathways to bacterial metabolism, the double mutant A19($\Delta ppc\Delta carB$) of the wild-type DSM 20617^T, presenting inactivation of both PpC and CpS, was obtained from the strain A18(Δppc) (Arioli *et al.*, 2007). On the basis of the reaction scheme shown in Fig. 1, and the data reported for strain A18(Δppc), evaluation of the growth of mutant A19 on CDM revealed its expected auxotrophy for aspartic acid, arginine and uracil, which are the final products of the pathways governed by PpC and CpS (Fig. 3). Interestingly, the cell morphology of the mutant A19($\Delta ppc\Delta carB$) grown in the CO₂-enriched atmosphere was very similar to that of the wild-type grown in the absence of CO₂ (under a N₂ atmosphere) (Fig. 2e). This observation underlined the fact that the two anaplerotic pathways inactivated in the double mutant have a central role in the CO₂ metabolism of *S. thermophilus*. In fact, despite the high availability of CO₂ in the growth environment, the mutant A19 was unable to use CO₂ through the anaplerotic reactions based on PpC and CpS; on the other hand, the wild-type strain

was unable to conduct its metabolism through the same enzymic reactions when cultured in the absence of CO₂. In the light of these considerations, the stress conditions determining the abnormal cell morphology in the wild-type strain cultured under a N₂ atmosphere could be due to the limited availability of aspartic acid, arginine and pyrimidine. This hypothesis was corroborated by the observation that the wild-type cell morphology was restored when the A19($\Delta ppc\Delta carB$) mutant was grown on M17 medium supplemented with aspartic acid, arginine and uracil (Fig. 2f). When the wild-type was grown under a N₂ atmosphere on M17 medium supplemented with aspartic acid, arginine and uracil, no changes in cell morphology were observed. Therefore, we conclude that CO₂ depletion in *S. thermophilus* determines arginine, aspartic acid and uracil auxotrophy.

CO₂ depletion, arginine, aspartic acid and uracil auxotrophy, and urea metabolism, in *S. thermophilus*

To confirm our hypothesis on CO₂-depletion-dependent auxotrophy for arginine, aspartate and uracil, *S. thermophilus*

wild-type was grown on CDM, CDM supplemented with arginine, aspartate and uracil (CDM-aau), CDM supplemented with sodium bicarbonate (CDM-HCO₃), and CDM supplemented with urea (CDM-urea), either in an enriched CO₂ atmosphere or in a N₂ atmosphere. Growth was measured as the mean ($\pm$ SD) of three cell density (OD₆₀₀) measurements. After incubation for 24 h at 37 °C, bacterial growth was detected on all the media incubated in a

Fig. 3. Growth of *S. thermophilus* wild-type (●) and mutant A19(Δ ppc Δ carB) (▲) in CDM, and growth of the mutant in CDM supplemented with 2.25 mM aspartate, 0.1 mM arginine and 0.2 mM uracil (□). The values are the means of three replicates, and for each value the SD was <0.05 OD₆₀₀ units. (b) Growth of *S. thermophilus* wild-type in CDM, CDM supplemented with 2.25 mM L-Asp, 0.1 mM L-Arg and 0.2 mM uracil (CDM-aau), CDM supplemented with 5 mM NaHCO₃ (CDM-HCO₃), and CDM supplemented with 5 mM urea (CDM-urea). The experiments were performed under a carbon dioxide or nitrogen atmosphere as indicated. All CDM cultures were repeated three times. SD values are indicated.

CO₂-enriched atmosphere, but only on CDM-aau and CDM-HCO₃ under a N₂ atmosphere (Fig. 3). When *S. thermophilus* was grown on CDM under N₂, no bacterial growth was detected. These results clearly suggest that CO₂ is essential to feed the anaplerotic reactions governed by PpC and CpS, and therefore for the biosynthesis of arginine, pyrimidine and aspartate. Interestingly, aspartate, arginine and uracil auxotrophy generated by CO₂ depletion (in a N₂ atmosphere) did not occur when CDM was supplemented with bicarbonate (Fig. 3). The addition of urea did not allow the growth of the strain on CDM under N₂. The lack of growth on CDM-urea under N₂ was explained by the presence of a level of urease activity in the inoculated cells that was unable to hydrolyse the urea supplement in the medium and, therefore, to produce enough CO₂ to feed the anaplerotic reactions. It is known that urease biogenesis is strongly induced in the early stage of growth when the environmental pH reaches a subacid value, and it decreases in the late-exponential phase of growth (Mora *et al.*, 2005). In this context, it is reasonable that the cells inoculated on CDM-urea under N₂, unable to grow as result of their auxotrophy for aspartate, arginine and uracil, did not acidify the medium to a level sufficient for the induction of urease biogenesis.

The addition of bicarbonate or urea to the medium resulted in a significant increment of growth yield when *S. thermophilus* was cultivated under an enriched CO₂ atmosphere (Fig. 3). The growth enhancement observed on CDM-urea under a CO₂ atmosphere suggested that CO₂ generated by urease activity may be directly metabolized by the micro-organism. When *S. thermophilus* was grown on M17 medium supplemented with [¹³C]urea, the evaluation of the ¹³C/¹²C stable isotope ratio in the biomass, and in the nucleic acid extracts, revealed that labelled CO₂ generated by urea hydrolysis was actively metabolized. In fact, a significant increase in the ¹³C/¹²C ratio was detected in the biomass of the cells fed with [¹³C]urea (¹³C/¹²C ‰ value equal to -23.14 ± 0.7 , mean $\pm$ SD) compared with those fed with standard urea (¹³C/¹²C ‰ value equal to -27.10 ± 0.7). Likewise, the evaluation of the ¹³C/¹²C ratio carried out on a total nucleic acid extraction revealed a significant increase of ¹³C in the nucleic acids derived from cells fed with [¹³C]urea (¹³C/¹²C ‰ value equal to $+17.04 \pm 0.5$) compared with those derived from cells fed with standard urea (¹³C/¹²C ‰ value equal to -23.49 ± 0.7).

Proteomic analysis of wild-type *S. thermophilus* and A19(Δ ppc Δ carB) mutant grown under different gas atmospheres

Proteomic approaches have been widely used to monitor global changes of gene expression patterns during bacterial growth/starvation, or following environmental stresses, and they have provided a comprehensive view in understanding microbial physiology or adaptation to external stimuli (Arena *et al.*, 2006; Bernhardt *et al.*, 2003; Eymann *et al.*, 2004; Salzano *et al.*, 2007). Thus, the effect of different CO₂

availability on the proteome of *S. thermophilus* was evaluated by growing the wild-type strain and the A19($\Delta ppc\Delta carB$) mutant in an enriched CO₂ atmosphere or a N₂ atmosphere. The cells were collected from solid M17 medium after incubation for 24 h, and total protein extracts were separated by 2D electrophoresis in the pH range 4.5–5.5. Almost 540 protein spots were detected in the proteomic map of the wild-type strain grown under the CO₂ atmosphere (data not shown). 2D Electrophoresis maps from the wild-type strain and the A19($\Delta ppc\Delta carB$) mutant grown in an enriched CO₂ atmosphere or a N₂ atmosphere were subjected to densitometric analysis to determine quantitative differences within bacterial proteomic repertoires. Protein spots that varied among the experimental conditions, and some that were constant (used as a reference), were excised from the gels, and identified by combining peptide mass fingerprinting MALDI-TOF and μ LC-ESI-IT-MS-MS approaches (Table 1).

Despite the relevant effects of CO₂ availability on bacterial morphology, only a limited number of proteins appeared to be up- or downregulated in the wild-type strain grown in the enriched CO₂ atmosphere compared with cultivation under N₂ (wild-type/CO₂ versus wild-type/N₂) (Fig. 4a). Interestingly, most of the proteins upregulated in the CO₂-enriched atmosphere are involved in bioenergy metabolism (Fig. 4a); they were identified as glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (spot 14), 6-phosphofructokinase (spot 19), triosephosphate isomerase (spot 25) and L-lactate dehydrogenase (spot 18), confirming that glycolysis and homolactic fermentation were actively stimulated in presence of CO₂. The activation of bioenergy metabolism in the presence of a high availability of CO₂ was in agreement with the large increment of biomass dry weight obtained when *S. thermophilus* was grown under the same experimental conditions, i.e. 72 % of cell dry weight under CO₂ (0.1581 g) compared with the cell biomass obtained under a N₂ atmosphere (0.0437 g).

It is also worth mentioning the gas-dependent behaviour of the two HPr isoforms present within the bacterial proteomic maps of the wild-type strain. The more acidic isoform (spot 38) was dominant in the CO₂-enriched atmosphere, while the more basic isoform (spot 39) was more abundant in cells cultured under a N₂ atmosphere (Figs 4a and 5a, b). HPr is a phosphate-transfer protein of the phosphoenolpyruvate phosphotransferase system involved in the uptake of carbohydrates, and it regulates lactose permease (LacS) expression and activity, which contribute to the fast cellular response to alterations in the external lactose concentration. In several bacteria, Hpr, together with the catabolite control protein CcpA, also play a central role in a transcriptional regulation system that ensures an efficient rate of carbohydrate uptake. The serine-phosphorylated form of HPr [HPr(Ser-P)], dominant in the exponential phase of growth of various bacteria, acts as a co-repressor of CcpA, and controls transcriptional repression of *lacSZ* (Gunnewijk & Poolman, 2000a; van den Bogaard *et al.*, 2000); the histidine-phosphorylated

form of HPr [HPr(His-P)], dominant in the stationary phase, phosphorylates the hydrophilic IIA domain of LacS, thereby affecting the transport (Gunnewijk & Poolman, 2000b). After the mid-exponential phase of growth, there is an increase in the level of HPr(His-P) and a decrease in the level of HPr(Ser-P), resulting in increased transport activity, and an increase in the biosynthesis of LacS, thereby compensating for the decrease in the level of substrate (lactose), and the increase in the level of inhibitory end product (galactose) in the medium (Gunnewijk & Poolman, 2000a). Similar to problems encountered during analysis of other lactic acid bacteria, our attempts to identify structural differences between spots 38 and 39 failed, probably as result of signal suppression phenomena during MS analysis of phosphopeptides. Based on their pI differences, relative stability under 2D electrophoresis, and previous observations on HPr counterparts from *Lactococcus lactis* (Palmfeldt *et al.*, 2004), spot 38 was identified as HPr(Ser-P), while spot 39 was associated to HPr(His-P) and HPr (Fig. 5a, b).

All the proteins upregulated in the wild-type strain under a N₂-enriched atmosphere displayed a chaperone activity, or behaved like folding templates, towards denatured polypeptides; in particular, they were identified as GroEL, DnaK, GrpE and translation elongation factor Tu (spots 7, 5, 28 and 10, respectively) (Fig. 4a, Table 1). Their increased abundance during the response of *S. thermophilus* and other lactic acid bacteria to various environmental stresses has been reported, and it has been associated with an attempt by the bacterium to protect itself from the accumulation of toxic protein waste (Arena *et al.*, 2006).

Diagrams and data displayed in Figs 1–3 reveal that the anaplerotic reactions inactivated in the mutant A19($\Delta ppc\Delta carB$) have a potentially high relevance to the CO₂ metabolism of *S. thermophilus*. To corroborate this hypothesis, the proteome of the wild-type grown under CO₂ was compared with the proteome obtained by growing the A19 mutant under the same experimental conditions. This comparison revealed that a number of proteins were up- or downregulated in the mutant, and these proteins were very similar to those whose expression level changed in the wild-type strain growing in N₂ (Fig. 4, Table 1). In fact, a similar quantitative trend was observed for glyceraldehyde-3-phosphate dehydrogenase (spot 14), 6-phosphofructokinase (spot 19), L-lactate dehydrogenase (spot 18), DnaK (spot 5), GrpE (spot 28), translation elongation factor Tu (spot 10) and HPr (spots 38 and 39). The basic isoform of HPr (spot 39) was dominant in the mutant in the two atmospheric conditions used (Fig. 5). Considering that the wild-type and A19 mutant were cultured under the same experimental conditions, the observed relative abundance of the two HPr isoforms seems to reflect the similar effect on energy metabolism induced by CO₂ depletion as a result of the presence of N₂ (wild-type/N₂) or the inactivation of the anaplerotic reactions catalysed by PpC and CpS [A19($\Delta ppc\Delta carB$)/CO₂] (Fig. 5). On the other hand, the abundance of specific proteins was changed in the mutant

Table 1. Proteins identified in the 2D electrophoresis maps of *S. thermophilus* wild-type strain and A19 mutant grown under different gas atmospheres

Spot	Protein	pI*	M _r *	Swiss-Prot code	Strain	Method†	Peptides	Sequence coverage (%)
1	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	5.08	77 153	Q03J94	LMD-9	MS/MS	15	49
2	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	5.08	77 153	Q03J94	LMD-9	PMF	7/9	14
3	ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones	5.08	77 153	Q03J94	LMD-9	PMF	22/28	35
4	Molecular chaperone, DnaK	4.62	64 756	Q03MR6	LMD-9	PMF	26/35	55
5	Molecular chaperone, DnaK	4.62	64 756	Q03MR6	LMD-9	PMF	11/12	25
6	60 kDa chaperonin, GroEL	4.70	56 923	Q03MK3	LMD-9	PMF	21/30	53
7	60 kDa chaperonin, GroEL	4.70	56 923	Q03MK3	LMD-9	PMF	37/49	61
8	FKBP-type peptidyl-prolyl <i>cis-trans</i> isomerase	4.41	46 682	Q03MQ9	LMD-9	PMF	16/17	35
9	Uncharacterized conserved protein	4.84	8502	Q03KC4	LMD-9	PMF	4/4	52
10	Elongation factor Tu	4.89	43 867	Q03LX0	LMD-9	PMF	12/16	38
11	Phosphoglycerate kinase	4.85	42 217	Q5LY23	CNRZ1066	PMF	11/14	44
12	Ribosomal protein S6	4.97	11 111	Q03IV3	LMD-9	PMF	9/21	84
13	Enolase	4.72	46 977	Q03LI0	LMD-9	PMF	27/35	69
14	Glyceraldehyde-3-phosphate dehydrogenase	5.26	36 022	Q03IS2	LMD-9	PMF	14/20	32
15	Glyceraldehyde-3-phosphate dehydrogenase	5.26	36 022	Q03IS2	LMD-9	PMF	12/17	32
16	Glyceraldehyde-3-phosphate dehydrogenase	5.26	36 022	Q03IS2	LMD-9	PMF	17/23	44
17	Glyceraldehyde-3-phosphate dehydrogenase	5.26	36 022	Q03IS2	LMD-9	PMF	8/9	29
18	L-Lactate dehydrogenase	4.92	35 429	Q03K30	LMD-9	PMF	6/9	28
19	6-Phosphofructokinase	5.09	35 982	Q03KB7	LMD-9	PMF	24/29	62
20	Fructose-1-phosphate kinase	4.77	32 541	Q5M169	CNRZ1066	PMF	6/8	20
21	ABC-type transport system involved in Fe-S cluster assembly, ATPase component	4.95	33 451	Q03MN3	LMD-9	PMF	11/12	37
22	Fructose-bisphosphate aldolase	4.81	31 525	Q03IH9	LMD-9	MS/MS	3	23
23	Transcriptional regulator/sugar kinase	4.51	28 256	Q03LA1	LMD-9	PMF	7/10	25
24	Biotin carboxyl carrier protein	4.45	16 945	Q03M49	LMD-9	PMF	4/4	25
25	Triosephosphate isomerase	4.70	26 629	Q03LW9	LMD-9	PMF	8/12	37
26	Putative uncharacterized protein (str1986)	4.98	20 176	Q5LXL5	CNRZ1066	PMF	6/15	27
27	Putative uncharacterized protein (str1986)	4.98	20 176	Q5LXL5	CNRZ1066	PMF	10/20	61
28	Molecular chaperone GrpE	4.42	23 411	Q03MR7	LMD-9	MS/MS	2	19
29	ATP-dependent Clp protease proteolytic subunit	4.72	21 578	Q03M78	LMD-9	MS/MS	3	22
30	Single-stranded DNA-binding protein	5.01	18 680	Q03IV4	LMD-9	PMF	9/9	48
31	50S Ribosomal protein L5	9.34	19 830	Q03IG3	LMD-9	PMF	7/7	48
32	Transcription elongation factor greA	4.65	17 443	Q03MH4	LMD-9	PMF	6/9	51
33	DNA-binding ferritin-like protein	4.59	19 221	Q03LA4	LMD-9	PMF	6/6	34
34	10 kDa Chaperonin	4.56	9912	Q03MK4	LMD-9	PMF	8/20	66
35	50S Ribosomal protein L10	5.36	17 463	Q03LT2	LMD-9	PMF	8/10	41
36	30S Ribosomal protein S17	9.92	10 027	Q03IG0	LMD-9	MS/MS	2	24
36	Ribosomal protein L18	10.40	12 923	Q03IG7	LMD-9	MS/MS	3	32
36	50S Ribosomal protein L19	10.78	13 146	Q03KD6	LMD-9	MS/MS	4	25
36	50S Ribosomal protein L21	9.86	11 184	Q03M33	LMD-9	MS/MS	2	29
37	Ribosomal protein L7/L12	4.42	12 360	Q03LT1	LMD-9	PMF	9/13	74
38	Phosphotransferase system, HPr-related protein	4.88	8919	Q03K41	LMD-9	PMF	5/9	41
39	Phosphotransferase system, HPr-related protein	4.88	8919	Q03K41	LMD-9	PMF	7/12	44
40	Ribosomal protein S15P/S13E	10.18	10 517	Q03MP3	LMD-9	PMF	14/26	64

*Theoretical values.

†PMF, Peptide mass fingerprinting.

Fig. 4. Graphical representation of proteins differentially regulated in wild-type and *S. thermophilus* cells grown in different atmospheres. (a) The wild-type grown in an enriched CO₂ atmosphere (white bars) and a N₂ atmosphere (black bars). (b) The wild-type (white bars) and mutant A19($\Delta ppc\Delta carB$) (black bars) cultures grown in enriched CO₂ atmosphere. No percentage volume variations higher than 15% were detected for the spots analysed within three experimental replicates of the same sample.

(Fig. 4); these proteins were putative uncharacterized protein str1986 (spot 26), single-stranded DNA-binding protein (spot 30), ribosomal protein L7/L12 (spot 37), and an uncharacterized conserved protein (spot 9). Putative uncharacterized protein str1986 belongs to a family of small proteins associated with alkaline shock response, and it was downregulated in the mutant A19. Single-stranded DNA-binding protein, ribosomal protein L7/L12 and the uncharacterized conserved protein were upregulated; the reason for their abundance in a mutant auxotroph for arginine, aspartic acid and uracil is not known at present.

Milk acidification, β -galactosidase and lactate dehydrogenase activity of *S. thermophilus* grown under different gas atmospheres

Gel densitometric analysis of some glycolytic enzymes and L-lactate dehydrogenase in the wild-type strain suggested that

glycolysis and homolactic fermentation were actively stimulated in the presence of an enriched CO₂ atmosphere compared with a N₂ atmosphere (Fig. 4a). On the other hand, the observed prevalence of HPr(Ser-P) in cells grown in CO₂ suggested that, under this condition, the culture was in a status of high energy (Fig. 5). Otherwise, the high prevalence of HPr(His-P) in the proteome of the culture grown under a N₂ atmosphere reflected a low level of energy status of the culture. According to observations by others (van den Bogaard *et al.*, 2000), the relative amount of HPr(Ser-P) and its interaction with CcpA should result in an increase of lactose transport efficiency, and in *lacSZ* operon derepression, and in *ldh* repression. On the basis of these considerations, the milk acidification rate of cells grown under the two gas atmospheres was evaluated. The results obtained revealed that cells grown under a N₂ atmosphere showed a significantly higher acidification rate compared with cells grown in enriched CO₂ atmosphere (Fig. 6a). In fact, cells grown under N₂ were able to acidify reconstituted pasteurized milk to a pH value below 5 (4.90 ± 0.03 , mean $\pm$ SD) after 10 h of incubation at 37 °C, while cells grown in CO₂ reached pH 4.96 ± 0.06 after 24 h of incubation.

To substantiate the effect of CO₂ availability on the energy metabolism of *S. thermophilus* in milk, and the role of HPr(Ser-P) on the regulation of *lacSZ* operon and *ldh*, biomass samples used to inoculate milk samples were also subjected to β -galactosidase and lactate dehydrogenase activity measurements. Wild-type cells grown under N₂ showed a β -galactosidase activity (252 ± 12 U mg⁻¹, mean $\pm$ SD) that was more than twofold higher than cells grown under enriched CO₂ (113 ± 9 U mg⁻¹). Conversely, the lactate dehydrogenase activity showed the higher values in wild-type cells grown under CO₂ (3593 ± 359 U mg⁻¹), while in cells grown under N₂ this enzymic activity was 60% lower (2144 ± 214 U mg⁻¹). Unlike the wild-type, the basic isoform of HPr (Fig. 5) was dominant in the A19 mutant independent of the atmospheric composition used for bacterial growth (Fig. 5). For these reasons, it was not surprising to find that β -galactosidase and lactate dehydrogenase activity values measured for mutant A19($\Delta ppc\Delta carB$) were the same irrespective of the gas composition used for micro-organism growth (β -galactosidase: 298 ± 12 and 297 ± 15 U mg⁻¹; lactate dehydrogenase: 3577 ± 360 and 3715 ± 420 U mg⁻¹; in N₂ and CO₂, respectively). Interestingly, activities for both enzymes measured in A19 cells were higher than those measured for wild-type cells. The reason for the upregulation of these enzymes in mutant A19($\Delta ppc\Delta carB$) is not known at present, and needs further investigation.

DISCUSSION

Despite the industrial importance of *S. thermophilus* in dairy fermentation processes, the effect of CO₂ on the overall metabolism of this micro-organism has been poorly investigated (Louaileche *et al.*, 1993, 1996; Arioli *et al.*, 2007). In this study, the relevance of CO₂ metabolism in *S.*

Fig. 5. Cropped images from 2D electrophoresis highlighting the distribution of the HPr isoforms in the proteomic map of *S. thermophilus* cells grown in different atmospheres. Wild-type *S. thermophilus* cells grown under (a) enriched CO₂, and (b) N₂. A19(Δ*ppc*Δ*carB*) mutant grown under (c) enriched CO₂, and (d) N₂. Spots 38 and 39 were tentatively identified as HPr(Ser-P) and HPr(His-P)/HPr, respectively.

thermophilus was studied by evaluating the cell morphology and the proteomic repertoire of cultures grown under atmospheres with a different gas composition. Unlike most lactic acid bacteria, *S. thermophilus* maintains several biosynthetic pathways involved in amino acid and pyrimidine synthesis that are in some cases governed by anaplerotic reactions using CO₂ as a substrate. Previous studies have reported that the metabolic pathway involved in aspartate biosynthesis (Fig. 1) has an essential role for the optimal growth of *S. thermophilus* in milk (Arioli *et al.*, 2007), thus revealing the importance of CO₂ in the general metabolism of this bacterium. For these reasons, a double mutant, in

which the genes encoding PpC and CpS were inactivated, was constructed, and compared with the wild-type strain.

A limited CO₂ availability for *S. thermophilus* resulted in a marked alteration in cell morphology, and a notable reduction in biomass production. The stress condition induced by CO₂ starvation was evident from the dramatic changes in cell morphology of *S. thermophilus* cells grown under N₂ (Fig. 2), when compared with those grown under the enriched CO₂ atmosphere. Abnormally elongated *S. thermophilus* cells have been observed in a *recA* mutant subjected to heat shock stress, and in a *recA* mutant of *Bacillus subtilis* in response to nutrient starvation (Sciocchetti *et al.*, 2001; Giliberti *et al.*, 2002). Interestingly, the abnormal *S. thermophilus* cell morphology characterizing the culture grown under a N₂ atmosphere was similar to the cell morphology of the mutant A19(Δ*ppc*Δ*carB*) grown under a CO₂ atmosphere, but was unable to use the inorganic carbon as a result of the genetic inactivation of PpC and CpS activity. Therefore, it was argued that the anaplerotic reactions catalysed by PpC and CpS have a key role in the CO₂ metabolism of *S. thermophilus*. Moreover, the normal ovoid-shaped cell morphology of *S. thermophilus* was restored in the culture grown in N₂, but only when the medium was supplemented with pyrimidine and the amino acid end products of the biosynthetic pathways governed by CpS and PpC (Fig. 1), or when the medium was supplemented with sodium bicarbonate. Interestingly, the normal cell morphology was also partially restored under a N₂ atmosphere when the medium was supplemented with urea, which is a CO₂-generating substrate. When *S. thermophilus* was cultured under N₂, a significant reduction of biomass production (72 % of dry weight) was observed; this clearly demonstrated that CO₂ starvation has a dramatic effect on the energy metabolism of the micro-organism. The use of a

Fig. 6. Acidification curves of milk samples inoculated with *S. thermophilus* wild-type cells grown for 24 h at 37 °C in an enriched CO₂ atmosphere (●) and under N₂ (○). The values are the means of three replicates, and for each value the SD was <0.08 pH units.

chemically defined medium under a N₂ or an enriched CO₂ atmosphere revealed that CO₂ depletion determined aspartic acid, arginine and uracil auxotrophy, thus confirming the key role of PpC and CpS in *S. thermophilus* metabolism. It was also concluded that the decarboxylating reactions potentially active in *S. thermophilus* metabolism were not able to efficiently feed the CO₂-consuming reactions.

S. thermophilus wild-type was able to grow in CDM broth deprived of arginine, aspartate and uracil under a N₂ atmosphere, while it showed a clear auxotrophy for these nutrients when it was cultured on CDM agar under N₂. Previous observations (Arioli *et al.*, 2007) have revealed that addition of urea and bicarbonate to CDM broth does not have any effect on the growth of either the parental or the phosphoenolpyruvate-carboxylase-negative mutant strain, indicating that CO₂ dissolved in the liquid medium is not present in limiting concentration.

A CO₂-concentration-dependent auxotrophy for arginine and uracil has also been reported for some strains of *L. plantarum* (Nicoloff *et al.*, 2005). In *L. plantarum*, carbamoyl phosphate, the precursor of both pyrimidine and arginine synthesis (Fig. 1), is synthesized by two CpSs, namely CpS-P and CpS-A, encoded by *pyrAaAb* and *carAB*, respectively, whose transcription is regulated by pyrimidine and arginine availability (Nicoloff *et al.*, 2000, 2005). *L. plantarum* strains showing a high-CO₂-requiring auxotroph are able to synthesize sufficient arginine and pyrimidines in CO₂-enriched air only. Unlike *L. plantarum*, *S. thermophilus* has only one CpS, which is encoded by a *carB* gene (Hols *et al.*, 2005), and this represents a key node in arginine and pyrimidine biosynthesis (Fig. 1). Moreover, in *L. plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus*, inorganic carbon has been proposed to be the growth-limiting factor in normal air by limiting the synthesis of carbamoyl phosphate (Nicoloff *et al.*, 2005); however, arginine and pyrimidine prototrophs requiring high concentrations of CO₂ have not been found in *S. thermophilus*.

In the available genome of lactic acid bacterial species, phosphoenolpyruvate carboxylase and carbamoylphosphate synthase genes have been identified in *Lactobacillus* spp. *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. gasseri*, *L. helveticus* and *L. johnsonii*, while *Lactococcus lactis*, *L. plantarum*, *L. brevis* and *L. salivarius* are characterized by the presence of pyruvate carboxylase (E.C. 6.4.1.1) and/or phosphoenolpyruvate carboxykinase (E.C. 4.1.1.49) and carbamoyl-phosphate synthase. *Lactobacillus fermentum* shows only a putative phosphoenolpyruvate carboxylase, and *Lactobacillus casei* and *Lactobacillus sakei* have a putative carbamoyl-phosphate synthase. Other lactic acid bacterial species do not appear to have genes encoding enzymic activities involved in the anaplerotic assimilation of CO₂ (Bolotin *et al.*, 2001; Kleerebezem *et al.*, 2003; Pridmore *et al.*, 2004; Chaillou *et al.*, 2005; Van de Guchte *et al.*, 2006; Altermann *et al.*,

2005; Makarova *et al.*, 2006; Callanan *et al.*, 2008; Claesson *et al.*, 2006; Morita *et al.*, 2008).

The comparative proteomic analysis carried out between cultures of *S. thermophilus* grown under enriched CO₂ and those grown in a N₂ atmosphere confirmed that CO₂ stimulates energy metabolism, as revealed by the upregulation of enzymes involved in glycolysis and homolactic fermentation (Fig. 4). This suggests that cells become metabolically active. Further evidence to support the stimulating effect of CO₂ on energy metabolism in *S. thermophilus* was obtained by the demonstration of the distribution of the two HPr isoforms (Fig. 5). The observed prevalence of HPr(Ser-P) in cells grown in an enriched CO₂ atmosphere again suggested that, under this condition, the culture was in a high level of energy status. In contrast, the high prevalence of HPr(His-P) in the proteome of culture grown under a N₂ atmosphere seems to reflect that the culture was in a low level of energy status. Considering that, in the two experimental conditions analysed, *S. thermophilus* was cultured on the same medium, the transition from HPr(Ser-P) to HPr(His-P) seems to reflect the metabolic stress induced by CO₂ depletion, i.e. as argued above, depending on arginine, aspartic acid and uracil starvation. The indirect influence of the biosynthetic pathways governed by PpC and CpS on the phosphorylation status of HPr was highlighted by analysing the proteome of mutant A19. In this case, HPr(His-P) was the dominant phosphorylated form of HPr for all the experimental conditions applied (Fig. 5). These data are in agreement with the observation that the genetic inactivation of PpC and CpS in the mutant A19 caused an auxotrophy for aspartate, arginine and uracil, irrespective of the availability of CO₂. Another study (van den Bogaard *et al.*, 2000) has reported the role of the catabolite control protein A (CcpA) on the transcriptional regulation of the *lacSZ* operon and *ldh* gene, thereby determining direct control of lactose transport, β -galactosidase activity and glycolysis. Published data on *S. thermophilus* show that the regulatory role of CcpA is presumably enhanced by the interaction with HPr(Ser-P) (van den Bogaard *et al.*, 2000). Our results show a direct correlation between the distribution of the HPr-phosphorylated isoforms and the measured levels of β -galactosidase and lactate dehydrogenase activities (Figs 5 and 6), and their relevance in the milk acidification process.

The key role of PpC and CpS activity in the CO₂ metabolism of *S. thermophilus* was further corroborated when the proteomes of the wild-type and the mutant A19(Δ p_{ppC} Δ carB) grown under CO₂ were compared (Fig. 4). These comparative experiments confirmed previous observations of comparisons between the proteome of the wild-type grown in CO₂ versus that of the wild-type grown in N₂, i.e. that CO₂ depletion produces a modulation of energy metabolism. In conclusion, this study highlights the relevance of CO₂ availability for the metabolism of *S. thermophilus*, revealing new insights in the physiology of this micro-organism, and providing new opportunities for industrial applications.

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