The relevance of carbon dioxide metabolism in
Streptococcus thermophilus

Stefania Arioli,1 Paola Roncada,2 Anna Maria Salzano,3 Francesca Deriu,4 Silvia Corona,5 Simone Guglielmetti,1 Luigi Bonizzi,4 Andrea Scaloni3 and Diego Mora1

Correspondence
Diego Mora
diego.mora@unimi.it

1Department of Food Science and Microbiology, Milan, Italy
2Istituto Sperimentale Italiano Lazzaro Spallanzani, sezione di Proteomica, Facoltà di Medicina Veterinaria, Milan, Italy
3Proteomics and Mass Spectrometry Laboratory, ISPAAM, National Research Council, Naples, Italy
4Department of Veterinary Pathology, Hygiene and Public Health, University of Milan, Milan, Italy
5IRCCS Policlinico San Matteo, Pavia, Italy

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, 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.

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; Arse`ne-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...
bicarbonate (Louailche 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 DSM20617T 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 20617T, its PpC- derivative A18(ApPC) (Arioli et al., 2007), and the PpC- CpS- double mutant A19(ApPCΔcarB), 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 pG13 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(ApPC) and A19(ApPCΔcarB) 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; **ureABCDEF**, operon encoding urease; **GluT**, hypothetical glutamate membrane transporter. The genes **carB** and **ppc**, in bold boxes, were inactivated in the mutant A19(ApPCΔcarB).
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 CP0000419, 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'-GGAAATGCGTTAGAAGATG-3') and CarBR (5'-CTGCTACGTTTTCTAATCGT-3') at 0.5 μmol l⁻¹, and 2 U Ex Taq 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 Biosystems) with fluorescent dideoxy chain terminators (Big Dye Terminator Cycle Sequencing kit version 2.0; Applied Biosystems). 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 ΔcarB in the mutant A18(Δppc).** DNA manipulation of pGFP 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 ΔcarB. The ΔcarB 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'-CAAGGGCTGTGAAGATG-3'; CarB2, 5'-CTGCTACGTTTTCTAATCGT-3'; CarB3, 5'-CGCGTTAGCAGGCAAGCATGCTTGTGGTATGTAACCTCAAGAGATACCTGCTGA-3'; CarB4, 5'-TATCAAGGGCTGTGAAAGT-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'-TTATATCATTGGCTAGGCTT-3') and CarB6 (5'-TATACACGACTGAGCGACTGC-3'), carrying a PstI site at the 5' end, were added to the reaction mixture to produce 1 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 ΔcarB was ligated into the dephosphorylated PstI site of pGFP 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 carboxamyl-phosphate-synthase-negative mutant was named A19(ΔppcΔcarB).

**Growth curves and measurements.** Growth curves of DSM 20167ᵀ and the A19 derivative mutant were evaluated in triplicate at 37 °C. Briefly, cells from of 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 15000 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 p-nitrophenol at 420 nm (molar extinction coefficient (ε) 18.30 × 10⁵ 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 × 10⁵ 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 (Manchini et al., 1985), without RNase treatment. Total extracted nucleic acid was recovered and in water, and used for the evaluation of ¹³C/¹²C stable isotope ratio by isotope ratio MS. The ¹³C/¹²C (%v) 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 18000 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/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%, w/v, DTT; 10 mM Tris; 2%, w/v, ampholine; pH 4.0–6.5). A 100 μg protein quantity 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 equilibration, 1% DTT was added; to the second equilibration, 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.

RESULTS

The effect of CO2 availability on the metabolism of S. thermophilus was evaluated by growing strain DSM 20617T 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.
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(ΔppcΔcarB) of the wild-type DSM 20617T, 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(ΔppcΔcarB) 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 enzymatic 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(ΔppcΔcarB) 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.

**Fig. 2.** Transmission electron micrographs showing S. thermophilus wild-type and A19(ΔppcΔcarB) 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 (aau). Bars, 0.2 μm.

**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 (± 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 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 $-23.14 \pm 0.7$, mean ± SD) compared with those fed with standard urea (13C/12C % value equal to $-27.10 \pm 0.7$). 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 $-23.49 \pm 0.7$).

**Proteomic analysis of wild-type S. thermophilus and A19($\Delta$ppc$\Delta$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 \textit{S. thermophilus} was evaluated by growing the wild-type strain and the A19(Δ\textit{ppcΔcarB}) mutant in an enriched CO\textsubscript{2} atmosphere or a N\textsubscript{2} 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 CO\textsubscript{2} atmosphere (data not shown). 2D Electrophoresis maps from the wild-type strain and the A19(Δ\textit{ppcΔcarB}) mutant grown in an enriched CO\textsubscript{2} atmosphere or a N\textsubscript{2} 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 μLC-ESI-IT-MS-MS approaches (Table 1).

Despite the relevant effects of CO\textsubscript{2} 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 CO\textsubscript{2} atmosphere compared with cultivation under N\textsubscript{2} (wild-type/CO\textsubscript{2} versus wild-type/N\textsubscript{2}) (Fig. 4a). Interestingly, most of the proteins upregulated in the CO\textsubscript{2}-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 CO\textsubscript{2}. The activation of bioenergy metabolism in the presence of a high availability of CO\textsubscript{2} was in agreement with the large increment of cell dry weight under CO\textsubscript{2} (0.1581 g) compared with the cell biomass obtained under a N\textsubscript{2} 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 CO\textsubscript{2}-enriched atmosphere, while the more basic isoform (spot 39) was more abundant in cells cultured under a N\textsubscript{2} 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(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 \textit{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 N\textsubscript{2}-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 \textit{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 inattivated in the mutant A19(Δ\textit{ppcΔcarB}) have a potentially high relevance to the CO\textsubscript{2} metabolism of \textit{S. thermophilus}. To corroborate this hypothesis, the proteome of the wild-type grown under CO\textsubscript{2} 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 N\textsubscript{2} (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 under 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 CO\textsubscript{2} depletion as a result of the presence of N\textsubscript{2} (wild-type/N\textsubscript{2}) or the inactivation of the anaplerotic reactions catalysed by PpC and Cps [A19(Δ\textit{ppcΔcarB})/CO\textsubscript{2}] (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

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>pI*</th>
<th>M*</th>
<th>Swiss-Prot code</th>
<th>Strain</th>
<th>Method†</th>
<th>Peptides</th>
<th>Sequence coverage (%)</th>
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<tr>
<td>1</td>
<td>ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones</td>
<td>5.08</td>
<td>77 153</td>
<td>Q03J94</td>
<td>LMD-9</td>
<td>MS/MS</td>
<td>15</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>ATP-binding subunit of Clp protease and DnaK/DnaJ chaperones</td>
<td>5.08</td>
<td>77 153</td>
<td>Q03J94</td>
<td>LMD-9</td>
<td>PMF</td>
<td>7/9</td>
<td>14</td>
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<td>5.08</td>
<td>77 153</td>
<td>Q03J94</td>
<td>LMD-9</td>
<td>PMF</td>
<td>22/28</td>
<td>35</td>
</tr>
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<td>Q03MR6</td>
<td>LMD-9</td>
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<td>64 756</td>
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<td>LMD-9</td>
<td>PMF</td>
<td>11/12</td>
<td>25</td>
</tr>
<tr>
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<td>36 022</td>
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<td>Putative uncharacterized protein (str1986)</td>
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<td>20 176</td>
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<td>PMF</td>
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<td>PMF</td>
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<td>PMF</td>
<td>8/20</td>
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<tr>
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<td>Q03LT1</td>
<td>LMD-9</td>
<td>PMF</td>
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<td>8919</td>
<td>Q03K41</td>
<td>LMD-9</td>
<td>PMF</td>
<td>5/9</td>
<td>41</td>
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<td>42</td>
<td>Phosphotransferase system, HPr-related protein</td>
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<td>8919</td>
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<td>PMF</td>
<td>7/12</td>
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<td>10 517</td>
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<td>LMD-9</td>
<td>PMF</td>
<td>14/26</td>
<td>64</td>
</tr>
</tbody>
</table>

*Theoretical values.
†PMF, Peptide mass fingerprinting.
Milk acidification, \( \beta \)-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 \( CO_2 \) atmosphere compared with a \( N_2 \) atmosphere (Fig. 4a). On the other hand, the observed prevalence of \( HP(r)(Ser-P) \) in cells grown in \( CO_2 \) suggested that, under this condition, the culture was in a status of high energy (Fig. 5). Otherwise, the high prevalence of \( HP(r)(His-P) \) in the proteome of the culture grown under a \( N_2 \) atmosphere reflected a low level of energy status of the culture. According to observations by others (van den Boggaard et al., 2000), the relative amount of \( HP(r)(Ser-P) \) 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 \( N_2 \) atmosphere showed a significantly higher acidification rate compared with cells grown in enriched \( CO_2 \) atmosphere (Fig. 6a). In fact, cells grown under \( N_2 \) were able to acidify reconstituted pasteurized milk to a pH value below 5 (4.90 ± 0.03, mean ± st) after 10 h of incubation at 37 °C, while cells grown in \( CO_2 \) reached pH 4.96 ± 0.06 after 24 h of incubation.

To substantiate the effect of \( CO_2 \) availability on the energy metabolism of \( S. \) thermophilus in milk, and the role of \( HP(r)(Ser-P) \) on the regulation of \( lacSZ \) operon and \( ldh \), biomass samples used to inoculate milk samples were also subjected to \( \beta \)-galactosidase and lactate dehydrogenase activity measurements. Wild-type cells grown under \( N_2 \) showed a \( \beta \)-galactosidase activity (252 ± 12 U mg\(^{-1}\), mean ± sd) that was more than twofold higher than cells growth under enriched \( CO_2 \) (113 ± 9 U mg\(^{-1}\)). Conversely, the lactate dehydrogenase activity showed the higher values in wild-type cells grown under \( CO_2 \) (3593 ± 359 U mg\(^{-1}\)), while in cells grown under \( N_2 \) this enzymic activity was 60% lower (2144 ± 214 U mg\(^{-1}\)). Unlike the wild-type, the basic isofrom of \( HP(r) \) (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 \( \beta \)-galactosidase and lactate dehydrogenase activity values measured for mutant A19(\( Appc\Delta carB) \) were the same irrespective of the gas composition used for micro-organism growth (\( \beta \)-galactosidase: 298 ± 12 and 297 ± 15 U mg\(^{-1}\); lactate dehydrogenase: 3577 ± 360 and 3715 ± 420 U mg\(^{-1}\); in \( N_2 \) and \( CO_2 \), 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(\( Appc\Delta carB) \) is not known at present, and needs further investigation.

**DISCUSSION**

Despite the industrial importance of \( S. \) thermophilus in dairy fermentation processes, the effect of \( CO_2 \) on the overall metabolism of this micro-organism has been poorly investigated (Louailche et al., 1993, 1996; Arioli et al., 2007). In this study, the relevance of \( CO_2 \) metabolism in \( S. \) thermophilus needs further investigation.
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 normal S. thermophilus cell morphology characterizing the culture grown under a N2 atmosphere was similar to the cell morphology of the mutant A19(ΔppcΔcarB) 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
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 *pyrAAb* 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 A19Δ(ppc;ΔcarB) 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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