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Arginine Catabolism by Sourdough Lactic Acid Bacteria: Purification and Characterization of the Arginine Deiminase Pathway Enzymes from *Lactobacillus sanfranciscensis* CB1

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The cytoplasmic extracts of 70 strains of the most frequently isolated sourdough lactic acid bacteria were screened initially for arginine deiminase (ADI), ornithine transcarbamoylase (OTC), and carbamate kinase (CK) activities, which comprise the ADI (or arginine dihydrolase) pathway. Only obligately heterofermentative strains such as *Lactobacillus sanfranciscensis* CB1; *Lactobacillus brevis* AM1, AM8, and 10A; *Lactobacillus hilgardii* 51B; and *Lactobacillus fructivorans* DD3 and DA106 showed all three enzyme activities. *Lactobacillus plantarum* B14 did not show CK activity. *L. sanfranciscensis* CB1 showed the highest activities, and the three enzymes were purified from this microorganism to homogeneity by several chromatographic steps. ADI, OTC, and CK had apparent molecular masses of ca. 46, 39, and 37 kDa, respectively, and the pIs were in the range of 5.07 to 5.2. The OTCs, CKs, and especially ADIs were well adapted to pH (acidic, pH 3.5 to 4.5) and temperature (30 to 37°C) conditions which are usually found during sourdough fermentation. Internal peptide sequences of the three enzymes had the highest level of homology with ADI, OTC, and CK of *Lactobacillus sakei*. *L. sanfranciscensis* CB1 expressed the ADI pathway either on MAM broth containing 17 mM arginine or during sourdough fermentation with 1 to 43 mM added arginine. Two-dimensional electrophoresis showed that ADI, OTC, and CK were induced by factors of ca. 10, 4, and 2 in the whole-cell extract of cells grown in MAM broth containing 17 mM arginine compared to cells cultivated without arginine. Arginine catabolism in *L. sanfranciscensis* CB1 depended on the presence of a carbon source and arginine; glucose at up to ca. 54 mM did not exert an inhibitory effect, and the pH was not relevant for induction. The pH of sourdoughs fermented by *L. sanfranciscensis* CB1 was dependent on the amount of arginine added to the dough. A low supply of arginine (6 mM) during sourdough fermentation by *L. sanfranciscensis* CB1 enhanced cell growth, cell survival during storage at 7°C, and tolerance to acid environmental stress and favored the production of ornithine, which is an important precursor of crust aroma compounds.

Two routes for bacterial degradation of arginine have been described: the arginine-urease pathway, which involves the enzyme arginase with the formation of ornithine and urea, and, more commonly, the arginine deiminase (ADI) (or dihydrolase) pathway (13, 32, 47). The ADI pathway is comprised of three enzymes: ADI (EC 3.5.3.6), which degrades arginine into citrulline and ammonia; ornithine transcarbamoylase (OTC) (EC 2.1.3.3), which cleaves citrulline into carbamoyl phosphate and ornithine; and carbamate kinase (CK) (EC 2.7.2.2), which produces ATP, ammonia, and carbon dioxide through dephosphorylation of carbamoyl phosphate. A fourth membrane transport protein, which catalyzes an electroneutral exchange between arginine and ornithine, is also fundamental for this pathway. The genetic organization of the ADI pathway has been studied largely either with gram-negative bacteria (18, 19, 41) or with gram-positive bacteria such as *Clostridium perfringens* (39), *Bacillus licheniformis* (35), *Streptococcus sanguis* (8), *Lactobacillus sakei* (48), and *Oenococcus oeni* (47). Overall, sequence analysis showed high similarities among species and revealed that genes are clustered to form the operon *arcABCTD* and encode ADI (*arcA*), OTC (*arcB*), CK (*arcC*), and the membrane transport protein (*arcD*). Additionally, a putative transaminase-encoding gene (*arcT*) is located in this region. A new protein, ArcR, of the Crp-Fnr family, encoded by a gene located 109 bp downstream from ArcC, was found in *B. licheniformis* and may serve as an activator of the ADI pathway (34). Several regulation mechanisms were shown for arginine degradation. Oxygen, carbohydrate metabolism, arginine concentration, pH, repression by glucose, and intracellular ATP, NADH, or other intermediate metabolites seemed to be signals for ADI expression, with an influence which depended on the bacterial species (10).

In spite of these genetic and physiological characterizations, the practical significance of the ADI pathway is still debated and uncertain. The ADI pathway may fulfill various roles: to provide ATP for microbial growth under a variety of environmental conditions, especially when carbohydrate is not available or at low concentration; to supply carbamoyl phosphate for biosynthesis of citrulline or pyrimidines; and to protect

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bacteria against damage caused by, e.g., acid and/or starvation environmental stresses (9). Therefore, the expression of the ADI pathway in industrial microorganisms, such as lactic acid bacteria (LAB), could be of great practical significance. Only certain LAB degrade arginine: *O. oeni* and some related wine species (32), *Lactococcus lactis* subsp. *lactis* (12), *L. sakei* (10), *Enterococcus faecalis* (45), *S. sanguis* and some related oral bacteria (9), and *Lactobacillus plantarum* (3). The increase in acid resistance of LAB may be due to the restoration of the optimum intracellular pH through arginine utilization and NH_3 production (37, 42). *O. oeni*, and especially heterofermentative lactobacilli, which are generally less acid tolerant, may derive energy and ammonia from arginine catabolism, thus becoming more competitive in the stressful (acid and alcohol) environment of wine (32). The extra energy (ATP) produced via the ADI pathway enables extrusion of cytoplasmic protons by the F_0F_1 ATPase (42) and may help cells to survive longer after depletion of the primary energy source (3, 32, 40, 46, 48).

Except for a short communication by Ograbek et al. (D. Ograbek, M. J. Brandt, and W. P. Hammes, XVII Int. Conf. Commun., p. 173, 1999), no studies have considered the ADI pathway in sourdough lactobacilli. Panettone, Colomba, Pandoro, and various types of rye and wheat breads are made by using sourdough. The predominant sourdough LAB belong to the genus *Lactobacillus* and the key role of *Lactobacillus sanfranciscensis* is well recognized. Most of the positive effects of sourdough on the organoleptic, nutritional, and shelf-life properties of baked goods are attributed to the metabolic activities of LAB (20, 26). Nevertheless, during routine use, sourdough LAB are subjected mainly to cold and acid stresses, which are inherent to refrigerated storage at 4 to 7°C, and to high acidity at the end of sourdough fermentation (pH of about 3.4 to 4.0). The survival of *L. sanfranciscensis* decreased dramatically when cells grown at constant pH (6.4) were transferred suddenly to pH 3.4. The tolerance to low pH was increased after protein synthesis during initial exposure to sublethal acid conditions (pH 5 for 60 min), and constitutively acid-tolerant mutants were isolated (17). The expression of the ADI pathway in sourdough lactobacilli may be considered to be another important tool for increasing their adaptive response to environmental stress conditions with practical repercussions on the constant microbial composition and performance of sourdough.

This paper describes the activity of the ADI pathway in sourdough LAB; the purification and characterization of ADI, OTC, and CK enzymes from *L. sanfranciscensis* CB1; and the use of *L. sanfranciscensis* CB1 as starter for sourdough to evaluate the activity of ADI pathway enzymes during fermentation.

MATERIALS AND METHODS

Microorganisms, culture conditions, and subcellular fractionation. Seventy strains of LAB, previously isolated from Italian sourdoughs from central and southern Italy, were used in this study (14, 23). The species used were those commonly identified in sourdoughs: *L. sanfranciscensis* (strains CB1, E15, E17, 174, A79, A15, H4, BB12, 2A, 5D, E20, H10, 7A, H7, 9F, 57, 77St, H3, 14C, A22, A1, A4, 7N, and E3), *Lactobacillus alimentarius* (1A, 1B, 2B, 8D, 5α, 15A, 15F, 15 M, 15R, DA70, 2S, and 5A), *Lactobacillus brevis* (AM1, AM8, H12, 1F, 1D, 6 M, 10A, 14G, 25K, and 21S), *Lactobacillus fermentum* (6E, I2, and CD5), *Lactobacillus hilgardii* (51B and 52B), *L. plantarum* (DC400, AD4, B14, 17N, 18E, 19A, 20B, AD4, and 21B), *Lactobacillus farciminis* (5C1, 10xF6, 5xF14, and I2), *Lactobacillus fructivorans* (DD3, DA106, and DD10) and *Weissella confusa* (14A, 14R, and 8V).

Strains were propagated routinely for 24 h at 30 or 37°C in modified MRS broth (Oxoid, Basingstoke, Hampshire, England) with the addition of fresh yeast extract (5%, vol/vol) and 28 mM maltose, with a final pH of 5.6. Before used to assay arginine catabolism, cells were always subcultured (30°C for 24 h) three times in MAM broth (tryptone, 10 g; yeast extract, 5 g; arginine, 3 g; KH_2PO_4, 0.5 g; $MgSO_4$, 0.2 g; $MnSO_4$, 0.05 g; Tween 80, 1 ml; glucose, 5 g; and H_2O_2, 1,000 ml [pH 6.0]) (48) with (adapted cells) or without (control) 17 mM arginine added. This was to induce arginine catabolism. After cultivation, cells were harvested by centrifugation, washed with sterile distilled water, and resuspended in sterile distilled water to an optical density at 620 nm (OD_{620}) of ca. 2.5. This bacterial suspension was used to inoculate (4%, vol/vol) MAM broth with or without 17 mM arginine added, which was incubated statically at 30 or 37°C for different times. When the growth was assayed under aerobiosis, incubation in MAM broth was carried out by shaking the flasks at 150 rpm.

To determine enzyme activities, 12-h-old cultures were subjected to subcellular fractionation by lysozyme treatment in 50 mM Tris-HCl buffer (pH 7.5) containing 24% (wt/vol) sucrose, as described by Crow et al. (15). The only modification was that spheroplasts resuspended in isotonic buffer were sonicated for two cycles for 40 s (Sony Prep model 150; Sanio, Tokyo, Japan) (22). Two cellular fractions, cell wall and cytoplasm, were recovered and used for enzyme assays. Both fractions were dialyzed for 24 h at 4°C against 20 mM phosphate buffer (pH 7.0) and concentrated ca. 20-fold by freeze-drying (MOD E1PTB; Edwards, Milan, Italy). Protein concentrations in the enzyme preparations and during purification steps were determined by the Bradford method (6), using bovine serum albumin as a standard.

Cell growth was estimated by measuring the OD_{620} and directly by plating on Sour-Dough Bacteria agar medium (28).

Enzyme activities. The assay for ADI activity was based on the method of Zúñiga et al. (48) with some modifications. Under standard conditions, the reaction mixture consisted of 150 μl of 50 mM arginine, 2.3 ml of 50 mM acetate buffer (pH 5.5), 50 μl of cell wall or cytoplasm preparation, and 3.6 μl of sodium azide (final concentration, 0.05% [wt/vol]). Controls without substrate and without enzyme were included. After incubation at 37°C for 1 h, the reaction was stopped by adding 0.5 ml of a solution of 2 N HCl, and precipitated protein was removed by centrifugation. The citrulline content of the supernatant was determined by the method of Archibald (2). One milliliter of supernatant was added to 1.5 ml of an acid mixture of H_3PO_4-H_2SO_4 (3/1, vol/vol) and 250 μl of diacetyl monoxime (1.5% 2,3 butanadiona monoxime) (Sigma Chemical Co., St Louis, Mo.) in 10% (vol/vol) methanol, mixed, and boiled in the dark for 30 min. After cooling for 10 min, the absorbance at 460 nm was measured.

OTC activity was determined by the method described by Ruepp et al. (41). The assay mixture contained 80 μl of 50 mM potassium phosphate (KP_i) buffer (pH 7.0), 20 μl of 25 mM L-ornithine (Sigma Chemical Co), 75 μl of 133 mM carbamoyl phosphate (Sigma Chemical Co), 120 μl of cell wall or cytoplasm preparation, and 300 μl of distilled water. After incubation at 37°C for 2 h, citrulline was determined as described above (2).

CK activity was determined by the method of Liu et al. (33) with some modifications. A mixture containing 650 μl of 50 mM acetate buffer (pH 5.5), 100 μl of 50 mM ADP (Sigma Chemical Co), and 100 μl of 73 mM $MgCl_2$ was equilibrated at room temperature for 10 min, and then 100 μl of 133 mM carbamoyl phosphate was added with further incubation at 37°C for 10 min. The enzymatic reaction was initiated by the addition of 50 μl of cell wall or cytoplasm preparation. After incubation at 37°C for 15 min, the ammonia liberated was measured by an enzymatic method (kit ammonia, cat. no. E1112732; DHFFCHAMB, Milan, Italy). During growth in MAM broth, ornithine was determined either by the colorimetric method with ninhydrin, as described by Chinard (11), or by high-performance liquid chromatography analysis (see below).

Enzyme purification. Five liters of a 12-h-old culture of adapted cells of *L. sanfranciscensis* CB1 cultivated in MAM medium containing 17 mM arginine was harvested, and the cytoplasmic extract was prepared as described above (15, 22). After freeze-drying, the cytoplasmic extract was resuspended in 50 mM KP_i buffer (pH 7.0) and applied to a DEAE-cellulose anion-exchange column (55 by 1.6 cm [inside diameter]) (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a linear NaCl gradient (0 to 0.5 M) in 50 mM KP_i buffer (pH 7.0) at a flow rate of 90 ml/h. Fractions with the highest ADI, OTC, or CK activities were pooled, dialyzed, and concentrated 10-fold by freeze-drying. The fractions were separately redissolved in 50 mM KP_i buffer (pH 6.5) containing 0.15 M NaCl and further purified by gel filtration on a fast protein liquid chromatography (FPLC) Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) that had been equilibrated with 50 mM KP_i buffer (pH 6.5)

containing 0.15 M NaCl. The same buffer at a flow rate of 0.3 ml/min was used to elute the proteins. The most active fractions from gel filtration were pooled. After dialysis against distilled water at 4°C for 24 h, the 10-fold-concentrated fractions with ADI or CK activity were purified further on an FPLC phenyl-Superose 5/5 hydrophobic interaction column (Amersham Pharmacia Biotech) previously equilibrated with 50 mM KP_i buffer (pH 7.0) containing 1.7 M $(NH_4)_2SO_4$. Enzyme fractions were resuspended in the same buffer and separately applied to the column. Proteins were eluted with a reverse linear gradient of 1.7 to 0 M $(NH_4)_2SO_4$, at a flow rate of 0.4 ml/min. Pooled ADI-active fractions were desalted by dialysis against distilled water. For CK activity, fractions from the phenyl-Superose column were desalted before enzyme assay. After freeze-drying and resuspension in 50 mM KP_i buffer (pH 6.5), the samples containing ADI or CK activity were loaded into a Mono Q HR 5/5 column (Amersham Pharmacia Biotech). Elution was with a linear gradient of 0 to 0.5 M NaCl in 50 mM KP_i (pH 6.5) at a flow rate of 0.4 ml/min. Active fractions were pooled, dialyzed, and lyophilized. Purification of the CK to homogeneity required a further elution from the MonoQ column, which was performed as described previously. After gel filtration, the fractions with OTC activity were subjected to protein precipitation in the presence of 1.7 mM of $(NH_4)_2SO_4$. The supernatant was used for further chromatographic steps as described for ADI.

Units of ADI and OTC enzyme activity were calculated as micromoles of citrulline liberated in 10 min. Units of CK enzyme activity were calculated as micromoles of NH_3 liberated in 10 min. The specific activities of the enzymes were calculated as units per milligram of protein. Data for enzyme activity, as well as cell numbers and metabolite concentrations, were evaluated for their significance ($P < 0.05$) by one-way analysis of variance (43).

Characterization of the ADI pathway enzymes. The apparent molecular masses of the purified enzymes were estimated both by FPLC gel filtration on a Superose 12 HR 10/30 column (50 mM KP_i buffer [pH 8.0] containing 0.15 M NaCl) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (30). The gel contained 12% acrylamide (separation distance, 10 cm; gel thickness, 1 mm), and electrophoresis was performed with the Mini Protean II apparatus (Bio-Rad, Milan, Italy). The proteins were stained with Coomassie brilliant blue. Molecular mass marker proteins (Sigma Chemical Co.) were used as references.

The isoelectric points (pI) of ADI, OTC, and CK were determined by two-dimensional (2D)-electrophoresis with amyloglucosidase (pI 3.8), ovalbumin (pI 5.1), carbonic anhydrase (pI 7), and myoglobin (pI 7.6) as markers (Sigma Chemical Co.). Each purified enzyme was resuspended directly in the denaturing buffer containing 8 M urea, 4% CHAPS (3,3-cholamidopropyl dimethylammonium-1-propane sulfonate), 40 mM Tris base, and 65 mM dithioerythritol (DTE). 2D electrophoresis was performed by using the Immobiline-polyacrylamide system, essentially as described by Görg et al. (25) and Hochstrasser et al. (27). Isoelectric focusing was carried out on Immobiline strips (IPG strip), providing a nonlinear pH gradient from pH 3 to 10 (Amersham Pharmacia Biotech) by isoelectric focusing using IPG-phore, at 15°C. The voltage was increased from 300 to 5,000 V during the first 5 h and then stabilized at 8,000 V for 5 h. After electrophoresis, IPG strips were equilibrated for 12 min against 6 M urea–30% (vol/vol) glycerol–2% (wt/vol) SDS–0.05 M Tris-HCl (pH 6.8)–2% (wt/vol) DTE and for 5 min against 6 M urea–30% (vol/vol) glycerol–2% (wt/vol) SDS–0.05 M Tris-HCl (pH 6.8)–2.5% (vol/vol) iodioacetamide–0.5% (wt/vol) bromophenol blue. Electrophoresis in the second dimension was carried out by using the buffer system of Laemmli (30) on 9 to 16% polyacrylamide gels (18 cm by 20 cm by 1.5 mm) with a 40-mA/gel constant current and at 10°C for approximately 5 h until the dye front reached the bottom of the gel. The gels were silver stained as described by Hochstrasser et al. (27) and Oakley et al. (38). The protein maps were scanned with a laser densitometer (Molecular Dynamics 300s) and analyzed with the Image Master 2D elite computer software (Pharmacia).

The optimum pHs for ADI, OTC, and CK were determined at 37°C in the pH range of 3.5 to 9 by use of a universal buffer composed of 57 mM boric acid, 33 mM citric acid, 33 mM NaH_2PO_4, 1 M NaOH, and various amount of 0.1 M HCl. The temperature dependence was determined at pH 5.5 for ADI and CK and at pH 7.0 for OTC in the range of 7 to 55°C.

To assay the effects of inhibitors and divalent cations, a mixture containing solutions of the purified enzyme and 2.0 mM (final concentration) chemical reagents or divalent cations in 50 mM KP_i buffer (pH 6.5) was incubated for 30 min at 30°C. The reaction was initiated by adding the appropriate substrates, and enzyme activity was assayed under standard conditions. Controls to eliminate the interference of inhibitors or divalent cations were included.

Peptide amino acid sequences. Purified proteins in SDS-polyacrylamide gels were cut out and subjected, as tryptic digests, to amino acid sequencing by using an automatic protein-peptide sequencer (model 477A; Applied Biosystems Inc., Foster City, Calif.) connected on-line with a phenylthiohydantoin-amino acid analyzer model 120A and a control/data module model 900A (Applied Biosystems Inc.). Sequence comparison was performed by using the SWISS-PROT/EMBL/GenBank/DDBJ databases.

2D analysis of arginine-induced modifications in ADI pathway enzyme expression. After incubation at 30°C for 24 h in MAM, with or without arginine (17 mM), cells were harvested by centrifugation, washed in 50 mM Tris-HCl (pH 7.5) containing 0.1 mg of chloramphenicol per ml, centrifuged (15,000 × *g* for 10 min), and frozen or resuspended directly in denaturing buffer containing 8 M urea, 4% CHAPS, 40 mM Tris base, and 65 mM DTE. Cells were disrupted by using a Sony Prep model 150 (Sanio, Tokyo, Japan) in four cycles of sonication (20 s each). After unbroken cells were pelleted (15,000 × *g* for 10 min at 4°C), the protein content was measured by the method of Bradford (6). The same amount of 60 μg of total protein was used for each electrophoretic run. 2D gel electrophoresis was performed as described previously. Three gels were analyzed and spot intensities were normalized as reported by Bini et al. (4). In particular, the spot quantification for each gel was calculated as relative volume; the relative volume was the volume of each spot divided by the total volume over the whole image. In this way, differences in the color intensities among the gels were eliminated (1). The induction factor for individual proteins was expressed as the ratio between the spot intensities of the same protein in the cells cultivated in MAM broth with or without arginine (17 mM). All of the induction factors were calculated based on the average of the spot intensities of each of the three gels, and the standard deviation was calculated. Only induction factors with a statistical significance at a *P* value of <0.05 were considered.

Sourdough fermentation. The characteristics of the wheat flour used were as follows: moisture, 12.8%; protein (N × 5.70), 10.7% of dry matter (d.m.); fat, 1.8% of d.m.; and ash, 0.6% of d.m. Wheat flour (100 g), 45 ml of sterile water (with or without arginine added), and 15 ml of a cellular suspension (10^8 CFU/ml) of 24-h-old cells were used to produce 160 g of dough (dough yield of 160) with a continuous high-speed mixer (dough mixing time, 5 min). This dough contained 1.5% total soluble carbohydrates. When arginine was added to the dough (1 to 43 mM), adapted cells of *L. sanfranciscensis* CB1 were used. The pH of the arginine solution added to the dough was set to 5.8. Doughs were incubated at 30°C for 6 h. After fermentation, aliquots of sourdoughs (36 g) were stored at 7°C for different times and subsequently added as starter to 80 g of wheat flour and 44 ml of sterile water (with or without added arginine) to produce new sourdoughs.

The CFU per gram was estimated by planting on Sour-Dough Bacteria agar medium. The concentrations of lactic acid, acetic acid, and ethanol were determined by enzymatic methods as described by Gobbetti et al. (21).

Determination of free amino acids. The concentration of free amino acids in the water extracts of sourdoughs was determined. Ten grams of dough was diluted with 50 ml of distilled water, homogenized with a Classic Blender (PBI International, Milan, Italy), and incubated with shaking (100 rpm) at 30°C for 30 min. After centrifugation at 12,000 × *g* for 15 min, the supernatant was freeze-dried. Extract (20 mg) was resuspended in 6 ml of distilled water and filtered through a filter membrane with a 500-Da cutoff. The permeate was previously derivatized in a 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate precolumn and then used for high-performance liquid chromatography analysis (AccQ-Tag method [Waters Associates]). Chromatographic separation was carried out on a Waters AccQ-Tag column at 37°C, and elution was performed at a flow rate of 1 ml/min with a ternary gradient composed of 50 mM acetate buffer (pH 5.0) containing phosphoric acid (i), acetonitrile (ii), and water (iii). A fluorescence detector was used at 250-nm excitation and 395-nm emission wavelengths. Identification and quantification of amino acids were carried out by comparison with a standard mixture of amino acids (Sigma Chemical Co.).

Statistical analysis. Enzymatic measurements from three independent replicates were subjected to one-way analysis of variance at the Computer Centre of the University of Perugia by using SAS (43); for multiple comparison, the Tukey test was used, and the alpha value for all experiments was set at 0.05.

RESULTS

Arginine catabolism in sourdough LAB. Seventy strains of sourdough LAB were preliminarily screened for the three enzyme activities which comprise the ADI pathway. Cells were subjected to subcellular fractionation, and cell wall and cytoplasmic fractions were used for enzyme assays. Activity was detected in cytoplasm extracts only. Eight of 70 sourdough LAB strains possessed ADI pathway enzymes under our experimental conditions (Table 1). Great variability was found

TABLE 1. Specific enzyme activities in cytoplasm extracts of sourdough LAB

Strain	Sp act (U/mg)[a]		
	ADI	OTC	CK
L. sanfranciscensis CB1	86.3A	145.6A	90.0A
L. plantarum B14	28.8D	63.6E	ND[b]
L. brevis AM1	48.4C	72.8D	20.1D
L. brevis AM8	64.7B	85.4C	68.3B
L. brevis 10A	30.9D	62.5E	56.0C
L. hilgardii 51B	47.7C	97.8B	20.5D
L. fructivorans DD3	23.0E	42.5F	70.4B
L. fructivorans DA106	23.3E	24.7G	52.1C

[a] For specific activity determination, see Materials and Methods. Values in the same column without a common uppercase letter were significantly different ($P < 0.05$).

[b] ND, not detected under our experimental conditions.

within the species. Of the 24 strains of *L. sanfranciscensis*, only CB1 had ADI pathway enzyme activities. No activity was detected in *L. alimentarius*, *L. fermentum*, and *W. confusa* strains, while the only strain (B14) of *L. plantarum* which showed activity seemed to lack CK. Three strains, AM1, AM8, and 10A, of *L. brevis* were found to be active. The highest activity of all three ADI pathway enzymes was found in *L. sanfranciscensis* CB1. For this strain, the ratios between the enzyme activities in cytoplasmic extracts of cells subcultured three times in MAM broth with (adapted cells) and without (control) 17 mM added arginine were 129 ± 4.4, 47 ± 1.5, and 3 ± 0.08 for ADI, OTC, and CK, respectively. Lower ratios, especially for ADI and OTC, were found for the other seven strains selected (data not shown).

The eight sourdough LAB strains which possessed detectable enzyme activities were grown in MAM broth containing 27 mM glucose, with or without 17 mM added arginine. The kinetics of OD_{620}, pH, and citrulline, ammonia, and ornithine metabolites for *L. sanfranciscensis* CB1 are shown in Fig. 1. As expressed by OD_{620}, differences in cell growth started after 6 h of incubation, reaching the highest difference of ca. 3 (MAM with 17 mM added arginine) versus ca. 2.5 (MAM without arginine) at 20 h. These OD_{620} values corresponded to ca. log 9.0 versus ca. log 8.1 CFU/ml, respectively. Similar kinetics were found for pH, which at 20 h showed very large differences of pH 6.73 versus 4.19. Degradation of 17 mM arginine in MAM broth started at the beginning of the culture incubation, before differences in cell growth or pH became evident. In particular, the content of citrulline increased up to 8 h (ca. 15 mM), after which it decreased to ca. 3.5 mM at the end of incubation. Concomitantly, at 8 h a sudden increase in the ornithine concentration was found, which continued for a further 24 h (ca. 21.5 mM). The concentration of ammonia increased constantly during incubation up to ca. 41 mM. After 24 h of incubation, arginine was depleted. Since ca. 8.5 mM NH_3 was also produced in the MAM broth without added arginine, the net amount of NH_3 synthesized in the presence of 17 mM arginine added was ca. 32.5 mM, which, as expected from the stoichiometries, corresponded to a ratio of about 1 mM arginine consumed to 2 mM NH_3 produced. The same results were found when 13.5 mM maltose was substituted for glucose in MAM broth (data not shown). Although differences for OD_{620} and pH were smaller, which agreed with the results

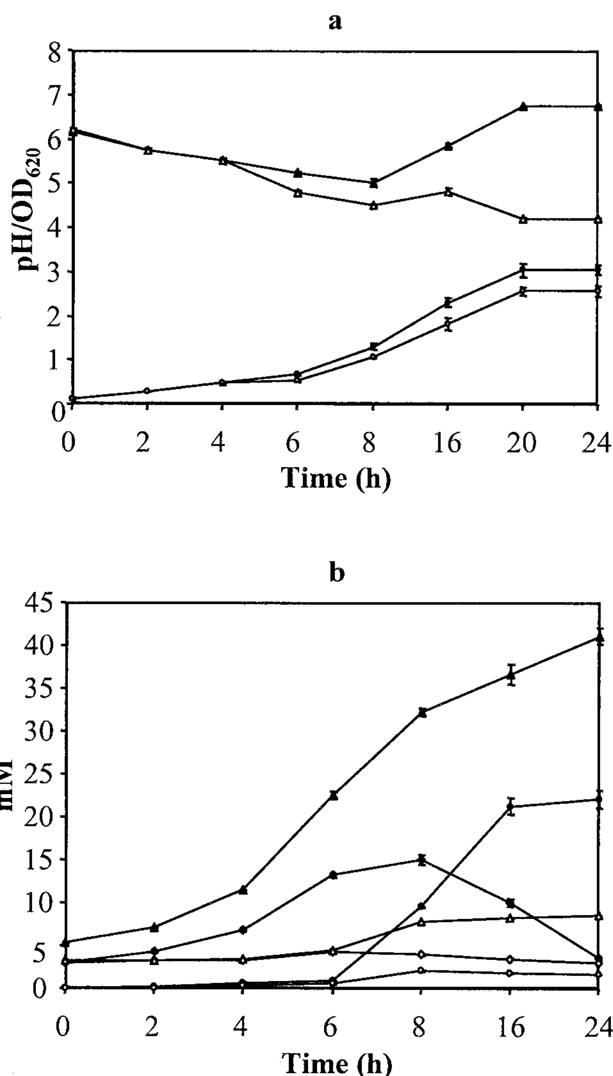

FIG. 1. Kinetics of OD_{620} (circles) and pH (triangles) (a) and of citrulline (diamond), ammonia (triangles), and ornithine (circles) metabolites (b) for *L. sanfranciscensis* CB1 cells cultivated in MAM broth with (filled symbols) and without (empty symbols) 17 mM arginine added.

found for enzyme activities (Table 1), the same kinetics were found for *L. brevis* strains AM1, AM8, and 10A and *L. fructivorans* strains DD3 and DA106. No differences between cells cultivated in MAM broth with or without 17 mM added arginine were found for *L. plantarum* B14 and *L. hilgardii* 51B. As previously shown, *L. plantarum* B14 seemed to lack CK activity, while *L. hilgardii* 51B showed a lower growth in MAM broth than the other strains. The determination of urea gave negative results for all eight strains assayed, thus excluding the presence of the arginine-urease pathway (data not shown).

Based on these results and since *L. sanfranciscensis* is a fundamental microorganism for sourdough properties (20), we decided to investigate further the ADI pathway enzymes of strain CB1.

Purification and characterization of ADI enzymes from *L. sanfranciscensis* CB1. The purification of ADI, OTC, and CK from *L. sanfranciscensis* CB1 required several chromato-

TABLE 2. Purification of ADI, OTC, and CK from *L. sanfranciscensis* CB1

Enzyme and purification step	Amt of protein (mg)	Total activity (U)	Sp act (U/mg)[a]	Purification (fold)	Activity yield (%)
ADI					
Cytoplasmic extract	1,370	22,198	16.20	1	100
DEAE-cellulose	141	12,320	87.37	5.39	55.50
Superose 12	17.1	4,031	235.73	14.55	18.15
Phenyl-Superose	10.2	2,838	278.23	17.17	12.78
Mono-Q	1.8	1,064	591.11	36.48	4.79
OTC					
Cytoplasmic extract	1,370	41,530	30.31	1	100
DEAE-cellulose	24.2	6,221	257.06	8.48	14.98
Superose 12	0.47	385	818.72	27.01	0.92
Precipitation by $(NH_4)_2SO_4$	0.12	201	1,675	55.26	0.48
Mono-Q	0.07	137	1,960	64.66	0.33
CK					
Cytoplasmic extract	1,370	1,461	1.06	1	100
DEAE-cellulose	37.4	231	6.17	5.82	15.79
Superose 12	1.83	28	15.08	14.22	1.89
Phenyl-Superose	0.51	15	30	28.30	1.04
Mono-Q	0.12	7	60	56.60	0.49
Mono-Q	0.03	2	80	75.47	0.16

[a] For determination of specific activity, see Materials and Methods.

graphic steps, which in part differed depending on the type of enzyme (Table 2). ADI, OTC, and CK were purified 36-, 65-, and 75-fold, with recoveries of 4.8, 0.3, and 0.2%, respectively. After the final anion-exchange chromatography, single bands at apparent molecular masses of ca. 46, 39, and 37 kDa were found by SDS-PAGE for ADI, OTC, and CK proteins, respectively (Fig. 2a). The same apparent molecular masses were determined by gel filtration on a Superose 12 HR 10/30 column (data not shown). As shown by 2D analysis (Fig. 2b), the whole-cell extract from cells grown with 17 mM arginine added showed induction factors of 10 ± 0.87, 4 ± 0.2, and 2 ± 0.1 for ADI, OTC, and CK, respectively, compared to whole-cell ex-

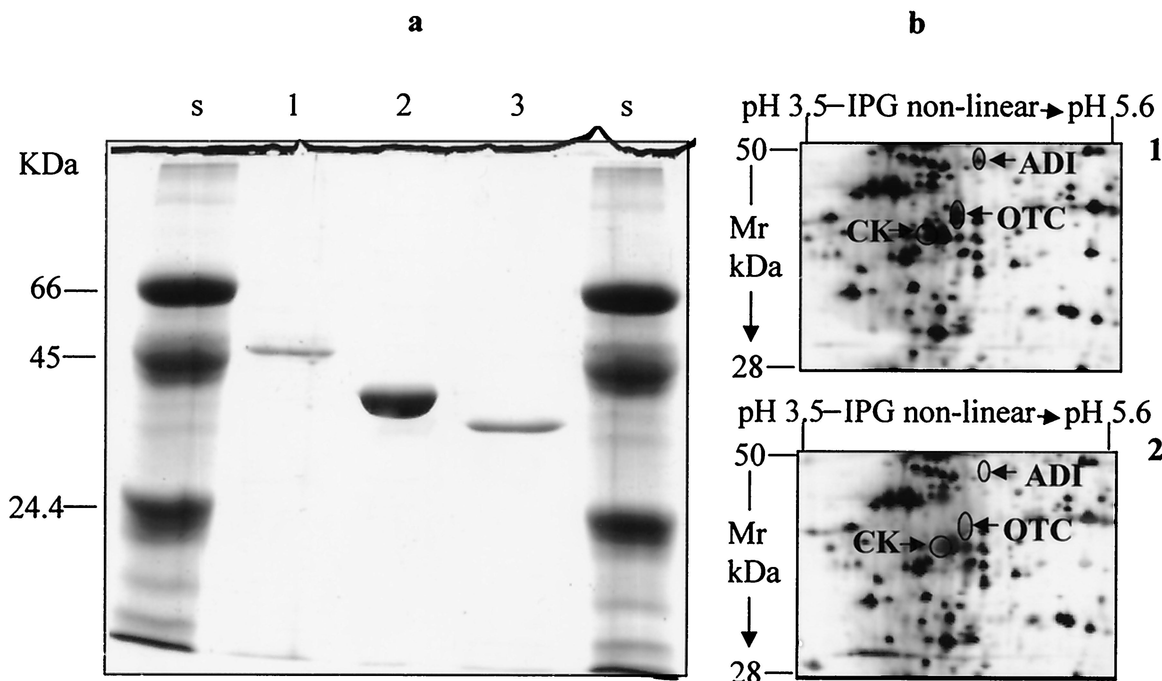

FIG. 2. (a) SDS-PAGE of the purified enzymes of *L. sanfranciscensis* CB1. Lanes: s, reference proteins (see Materials and Methods); 1, ADI; 2, OTC; 3, CK. (b) 2D electrophoresis analysis of protein expression in *L. sanfranciscensis* CB1. Panel 1, cells subcultured three times in MAM broth with 17 mM arginine added (adapted cells) and then cultivated in MAM broth with 17 mM arginine added for 24 h at 30°C. Panel 2, cells subcultured three times in MAM broth (control) and then cultivated in MAM broth for 24 h at 30°C. The positions of enzymes ADI (46 kDa, pI 5.18), OTC (39 kDa, pI 5.2), and CK (37 kDa, pI 5.07) are indicated.

TABLE 3. Effect of chemical reagents and divalent cations on the activities of ADI, OTC, and CK from *L. sanfranciscensis* CB1

Inhibitor or cation (2 mM)	Relative activity[a] (%)		
	ADI	OTC	CK
NEM	90D	140A	10L
Iodacetamide	84E	100D	15I
Cysteine	127A	110C	108AB
PMSF	0H	0M	91C
EDTA	103C	40I	20H
DL-Penicillamine	88E	58G	100B
DL-Propargylglycine	121A	93E	0N
DTT	87D	46H	110A
Mg^{2+}	98C	100D	100B
Cu^{2+}	0H	100D	85D
Co^{2+}	100C	100D	30G
Hg^{2+}	12G	71F	20H
Ni^{2+}	91D	33L	4M
Mn^{2+}	116B	122B	90C
Ca^{2+}	109B	100D	105B
Fe^{2+}	83E	0M	35F
Zn^{2+}	23F	100D	40E

[a] The activity of the control in the absence of chemical reagents and divalent cations was taken as 100%. Values in the same column without a common uppercase letter are significantly different ($P < 0.05$).

tract from cells grown in MAM broth without arginine. The pure enzyme preparations were used for further characterization.

The activity of ADI was optimal at pH 5.0, with 80% of the maximum activity at pH 3.0. ADI activity decreased rapidly when the pH was raised to neutrality and was less than 25% at pH 7.5. The pH optima for OTC and CK were found to be 6.5 and 6.0, respectively. A decrease of the activity similar to that found for ADI was observed when the pH was increased from the optimum, while OTC and CK seemed to have a lower relative activity at acidic pH values: 30 and 50% at pH 4.0, respectively. The optimum temperature for ADI and CK was found to be 30°C, while 40°C was optimal for OTC. All three enzymes had very high activity in the range 30 to 38°C, which coincided with the temperature used in sourdough fermentation (20). The sensitivity of the three ADI pathway enzymes to 2 mM concentrations of inhibitors and divalent cations varied (Table 3). ADI and OTC were totally inhibited by the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF). The metal chelator EDTA and the reducing agent dithiothreitol (DTT) also caused considerable inhibition of OTC. The sensitivity of CK was completely different, since it was markedly inhibited by sulfhydryl-modifying agents such as *N*-ethylmaleimide (NEM) and iodoacetate, by EDTA, and by DL-propargylglycine. ADI was strongly inhibited by Cu^{2+}, Hg^{2+}, and Zn^{2+}, OTC was strongly inhibited by Ni^{2+} and Fe^{2+}, and CK was strongly inhibited by all of the divalent cations used with the exceptions of Mg^{2+} and Ca^{2+}. As determined by 2D electrophoresis, the isoelectric points of ADI, OTC, and CK from *L. sanfranciscensis* CB1 were found to be in the range of 5.07 to 5.2.

Peptide sequences of ADI enzymes from *L. sanfranciscensis* CB1. The tryptic digests of the three ADI enzymes from *L. sanfranciscensis* CB1 were analyzed by using an automatic protein-peptide sequencer (model 477A; Applied Biosystems Inc.). Two fragments for each protein were identified, and all showed the highest homology with sequences of the same enzymes of *L. sakei* (EMBL accession number AJ001330), as determined by sequence comparison performed by using the SWISS-PROT/EMBL/GenBank/DDBJ databases (Table 4). Lower levels of homology were detected with the ADI enzymes of *L. lactis* subsp. *lactis* (5) (data not shown).

TABLE 4. Peptide sequences of ADI, OTC, and CK from *L. sanfranciscensis* CB1

Enzyme	Sequences	Protein identified and related homology (%)[a]
ADI	RDNGN, LLKRPGK	ADI, *L. sakei* (80 and 100)
OTC	GLAEK, SAFTTA	OTC, *L. sakei* (80 and 83.3)
CK	SATAQT, HGNGPQV	CK, *L. sakei* (66.6 and 100)

[a] Sequence comparison was performed by using the SWISS-PROT/EMBL/GenBank/DDBJ databases.

Sourdough fermentation. *L. sanfranciscensis* CB1 cells subcultured three times in MAM broth containing 17 mM arginine (adapted cells) were used to produce sourdoughs in the presence of various amounts of arginine (Table 5). Compared with the sourdough started in the absence of arginine (control), all of the concentrations of arginine used (1 to 43 mM) favored an increase in pH, starting between 2 and 4 h and becoming more evident after 6 h of fermentation, which coincided with the more usual time of dough leavening (20). Compared to the control dough, the increase of pH was in part proportional to the amount of arginine added and NH_3 produced, ranging from 4.13 to 6.20 after 6 h. As shown by the pH values determined at 24 h, the buffering capacity due to the degradation of arginine was maintained during the prolonged incubation. When nonadapted cells of *L. sanfranciscensis* CB1 were used to ferment a dough with 43 mM added arginine, they did not have the same capacity to degrade arginine as adapted cells. Nevertheless, after nonadapted cells were propagated three times in sourdoughs with 6 to 24 mM added arginine, they acquired the capacity to degrade arginine like adapted cells produced in MAM broth (data not shown). Two subculturings in MAM broth before sourdough fermentation decreased the capacity to degrade arginine by ca. 30%.

After 6 h of fermentation, the control (ca. pH 3.91) and the sourdough with 6 mM added arginine (ca. pH 4.57) were compared for several characteristics (Table 6). In spite of the differences in pH, the concentrations of lactic and acetic acids

TABLE 5. Effect of arginine addition on the pH of sourdough during 24 h of fermentation by *L. sanfranciscensis* CB1

Arginine (mM)	pH[a] at h:				
	0	2	4	6	24
0	5.86A	5.30C	4.38D	3.91E	3.69C
3	5.88A	5.64B	4.77C	4.13D	3.69C
6	5.86A	6.27A	5.38B	4.57C	3.70C
14	5.84A	6.27A	6.0A	5.83B	4.50B
28	5.78A	6.29A	6.20A	6.12AB	4.71A
43	5.85A	6.35A	6.27A	6.18A	4.77A
43[b]	5.80A	5.25C	4.39D	3.92E	3.70C

[a] Values in the same column without a common uppercase letter are significantly different ($P < 0.05$).

[b] Sourdough fermented by *L. sanfranciscensis* CB1 cells not subcultured three times in MAM containing 17 mM arginine.

TABLE 6. Cell numbers, consumption of arginine, and concentration ADI pathway metabolites in sourdoughs after 6 h of fermentation by *L. sanfranciscensis* CB1

Parameter (unit)	Value[a] in:		Ratio (R+/R−)
	Sourdough without arginine (R−)	Sourdough with 6 mM arginine (R+)	
Lactic acid (mM)	50A	49A	0.98
Acetic acid (mM)	10A	10A	0.9
Ethanol (mM)	46A	45A	0.93
Log CFU/g	9.5A	9.5A	1
Arginine consumed (mM)[b]	0.2B	5.8A	29
Citrulline (mM)	0.08B	0.15A	1.87
Ornithine (mM)	0.6B	2.6A	4.3
NH_3 (mM)	12B	38.9A	4.1

[a] Values in the same row without a common uppercase letter were significantly different ($P < 0.05$).
[b] A 0.5 mM concentration of arginine were naturally contained in the dough.

TABLE 7. Main characteristics of doughs started with sourdoughs previously fermented by *L. sanfranciscensis* CB1 with or without addition of 1 to 6 mM arginine and stored at 7°C for 12 h[a]

Arginine (mM)	Log CFU/g	pH	Lactic acid (mM)	Acetic acid (mM)	Ethanol (mM)
0	9.0B	3.79B	53B	9.1A	44C
1	9.11BA	3.83BA	54BA	8.8A	44.6C
3	9.27AB	3.9AB	56A	9A	47.2B
6	9.5A	4.11A	57A	9.4A	50.4A

[a] The doughs were fermented for 6 h in the presence of the concentration of arginine indicated. Values in the same column without a common uppercase letter were significantly different ($P < 0.05$).

and ethanol did not vary between the two sourdoughs, being constant at ca. 50, 10, and 46 mM, respectively. Also, the numbers of *L. sanfranciscensis* CB1 cells in the two sourdoughs were the same, at ca. log 9.5 CFU/g. The 6 mM arginine added to the sourdough was almost completely consumed by *L. sanfranciscensis* CB1, while the limited amount of arginine naturally present in the dough was used only in part by nonadapted cells. In these cases, the ratio for arginine consumption was ca. 29, meaning that adaptation was indispensable to induce arginine degradation. This consideration reflected on the several metabolites of arginine catabolism. In agreement with the results found in MAM broth (Fig. 1b), citrulline, one of the end products of ADI activity, did not accumulate in the sourdough (ca. 0.15 mM) but was degraded to ornithine by OTC enzyme. The concentration of ornithine was markedly higher in the sourdough with 6 mM added arginine compared to the control (ca. 2.6 versus 0.6 mM). The same was found for NH_3, which is an end product for ADI and CK. The NH_3 accumulation exceeded that theoretically produced by 6 mM arginine, and NH_3 production from asparagine and glutamine deiminase activity also cannot be excluded. These sourdoughs were stored at ca. 7°C for 12 h, as is usual in the daily production of leavened baked goods. After storage, the survival of *L. sanfranciscensis* CB1 cells increased from ca. log 8.6 to log 9.4 CFU/g from the control to the sourdough with 6 mM arginine added.

After storage for 12 h at 7°C, control sourdough and sourdoughs fermented with 1 to 6 mM arginine by *L. sanfranciscensis* CB1 were reused as starters for dough fermentation at 30°C for 6 h (Table 7). The number of *L. sanfranciscensis* CB1 cells increased proportionally to the amount of arginine in the dough, from ca. log 9.0 CFU/g in the control to ca. log 9.5 CFU/g in the presence of 6 mM added arginine. In spite of the increase in pH (3.79 ± 0.15 versus 4.11 ± 0.12), an increase in the concentration of lactic acid (53 ± 0.41 versus 57 ± 0.38 mM) was found in the same two sourdoughs. No difference was found for acetic acid concentration.

DISCUSSION

Arginine catabolism by the ADI pathway has been studied in several bacterial species, mainly regarding genetic and physiological aspects. *O. oeni* (32), *L. lactis* subsp. *lactis* (12), *L. sakei* (10), *E. faecalis* (45), *S. sanguis* (9), and *L. plantarum* (3) were the main LAB considered. More knowledge about the practical significance of the ADI pathway is required, especially for LAB, which populate different food environments. In this study, arginine catabolism by sourdough lactobacilli was studied. Only obligately heterofermentative sourdough strains such as *L. sanfranciscensis* CB1; *L. brevis* AM1, AM8, and 10A; *L. hilgardii* 51B; and *L. fructivorans* DD3 and DA106 showed all of the ADI, OTC, and CK activities. *L. plantarum* B14 did not show CK activity. Among LAB, the ADI pathway may be absent, partially present, or totally present (7). With few exceptions, wine-homofermentative lactobacilli (e.g., *L. plantarum*) did not degrade arginine, while heterofermentative lactobacilli (e.g., *L. brevis* and *L. hilgardii*) degraded arginine. In contrast, *L. plantarum* strains isolated from orange degraded arginine to citrulline, ornithine, and ammonia (3). In agreement with our findings, wide variation among strains of the same species was generally found (32). Except for a short communication by Ograbeck et al. (XVII Int. Conf. Commun.), this is the first study which characterized the enzymology of the ADI pathway in sourdough LAB and investigated its repercussions during sourdough fermentation

The molecular masses of ADI and OTC of *L. sanfranciscensis* CB1 agreed well with those reported for *L. lactis* subsp. *lactis* (45.98 and 39.45 kDa, respectively) (5), *L. sakei* (45.91 and 37.77 kDa) (48), *Staphylococcus aureus* (46.94 and 37.77 kDa) (29), and *B. licheniformis* (47.42 and 37.65 kDa) (35). The two peptide fragments of ADI and OTC revealed homologies of 80 and 100% and of 80 and 83.3% with sequences in the domain structures of the ADI and OTC, respectively, of *L. sakei* (48). The molecular mass of CK in *L. sanfranciscensis* CB1 was slightly higher than those found for these other strains, in which it ranged from 33.65 to 35.53 kDa. The two peptide fragments sequenced from CK had homologies of 66.6 and 100% with the same enzyme of *L. sakei* (48). The pIs of ADI pathway enzymes have always been reported to be in the range of 5.09 to 5.28. Except for the ADI system of oral streptococci (9), a biochemical characterization of these enzymes has not been reported in the literature. In agreement with the results found for *S. sanguis* (9), OTC, CK, and especially ADI of *L. sanfranciscensis* CB1 were well adapted to acidic (pH 3.5 to 4.5) and temperature (30 to 37°C) conditions which are usually found during fermentation of baked goods (20). The serine proteinase inhibitor PMSF, the metal chelator EDTA, and the reducing agent DTT caused a marked inhibition of OTC, indicating that this is a metalloenzyme with a

functional sulfhydryl group near or at its active site. Specific inhibition of CK also by NEM and iodoacetamide showed that disulfide bridges are important in maintaining an active conformation of this enzyme. Only PMSF seemed to be inhibitory to ADI.

Concerning the mechanisms of regulation of the ADI pathway, it was found that *L. sanfranciscensis* CB1 (i) was stimulate by arginine (24), (ii) did not grow in MAM broth with arginine as the sole carbon source, (iii) started the ADI pathway activity from the first 2 h of incubation when the pH was still ca. 5.7, (iv) needed the presence of a carbon source (27 mM glucose or 13.5 mM maltose) and was not inhibited by glucose at up to ca. 54 mM (data not shown), and (v) expressed arginine catabolism during sourdough fermentation where the dough contained 1.5% of soluble carbohydrates.

Other LAB, such as *Streptococcus* (16) and *L. sakei* (10), showed repression of the ADI pathway by glucose or galactose rather than by glucose or lactose as for *Lactobacillus leichmanii* (36). In contrast, strains of *Carnobacterium* (31) and *O. oeni* (47) were reported to degrade arginine even at high concentrations of glucose. Like for *L. sakei* (10), we also found that arginine is necessary to over induce the ADI pathway. By using an L-lactate dehydrogenase mutant of *L. sakei*, which was unable to lower the pH, Champomier Vergès et al. (10) showed that low pH values were not responsible for the induction of the ADI pathway. For *L. sanfranciscensis* CB1, we found that arginine catabolism started at very high pH values. The same authors suggested that together with arginine, another unidentified environmental factor, different from low pH and probably linked to the metabolic state of the cells, was needed to promote this degradation. On MAM broth containing 17 mM arginine and 27 mM glucose under aerobiosis, the growth of *L. sanfranciscensis* CB1 was higher than that under anaerobiosis (OD_{620} of ca. 4.5 versus 3.0). The final pH reached was 6.73 in anaerobiosis versus 6.0 in aerobiosis. As shown for *L. sakei* (10), this indicated that the arginine catabolism in *L. sanfranciscensis* CB1 is highly expressed under anaerobiosis conditions.

As shown for wine strains (32), LAB able to derive ATP from arginine catabolism may be more competitive in the stressful environment of sourdough than those strains that are unable to do so. In addition, the ADI pathway may be a mechanism for pH homeostasis, since the NH_3 produced favors the neutralization of the environment and the concomitant ATP generation enables expulsion of cytoplasmic protons by the F_0F_1 ATPase (42). In this study, it was possible to vary the pH of the sourdough fermented by adapted cells of *L. sanfranciscensis* CB1 as a function of the amount of arginine added (3 to 43 mM). While the more elevated concentrations of arginine (14 to 43 mM) gave pH values too high to be compatible with a sourdough, 6 mM arginine was considered a small amount to be added to the dough which still favored optimal acidic conditions. After 6 h of fermentation, no differences in the concentration of heterolactic fermentation end products for the control sourdough (pH 3.91) and the sourdough with 6 mM added arginine (pH 4.57) were found. As expected, metabolites of the ADI pathway, especially ornithine and NH_3, accumulated in the dough with added arginine. Ornithine is an important precursor of 2-acetyl-1-pyrroline, which is formed during baking and gives a highly appreciated roasty, popcorn-like smell to the wheat bread crust (44).

The survival of *L. sanfranciscensis* CB1 cells in the sourdough fermented with arginine was about 2 log units higher than that in the control during storage for 144 h at 7°C (data not shown). As reported for *L. sakei* (10), *L. lactis* subsp. *lactis* (42), and *S. sanguis* (9), the degradation of arginine by *L. sanfranciscensis* CB1 is clearly associated with a higher survival after the stationary phase of growth is reached. This might be attributed to the increase of pH due to NH_3 production by the ADI pathway, since the survival of the same CB1 strain considerably decreased as a consequence of the acid stress during sourdough fermentation (17).

The presence of a carbon source such as glucose and/or maltose, a low arginine supply (6 mM), a low oxygen concentration, and cell adaptation are all conditions leading to the expression of the ADI pathway in *L. sanfranciscensis* CB1 which are compatible with its natural sourdough environment. Under these conditions, the expression of the ADI pathway by *L. sanfranciscensis* CB1 during sourdough fermentation promotes (i) enhanced cell growth and survival which positively interfere with the constant microbial composition, (ii) enhanced tolerance to acid environmental stress, and (iii) greater production of ornithine, which improve the organoleptic characteristics of the sourdough.

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