

## Expression of the *pyr* Operon of *Lactobacillus plantarum* Is Regulated by Inorganic Carbon Availability through a Second Regulator, PyrR<sub>2</sub>, Homologous to the Pyrimidine-Dependent Regulator PyrR<sub>1</sub><sup>∇</sup>

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**Inorganic carbon (IC), such as bicarbonate or carbon dioxide, stimulates the growth of *Lactobacillus plantarum*. At low IC levels, one-third of natural isolated *L. plantarum* strains are nutritionally dependent on exogenous arginine and pyrimidine, a phenotype previously defined as high-CO<sub>2</sub>-requiring (HCR) prototrophy. IC enrichment significantly decreased the amounts of the enzymes in the pyrimidine biosynthetic pathway encoded by the *pyrR<sub>1</sub>BCAa<sub>1</sub>Ab<sub>1</sub>DFE* operon, as demonstrated by proteomic analysis. Northern blot and reverse transcription-PCR experiments demonstrated that IC levels regulated *pyr* genes mainly at the level of transcription or RNA stability. Two putative PyrR regulators with 62% amino acid identity are present in the *L. plantarum* genome. PyrR<sub>1</sub> is an RNA-binding protein that regulates the *pyr* genes in response to pyrimidine availability by a mechanism of transcriptional attenuation. In this work, the role of PyrR<sub>2</sub> was investigated by allelic gene replacement. Unlike the *pyrR<sub>1</sub>* mutant, the  $\Delta$ *pyrR<sub>2</sub>* strain acquired a demand for both pyrimidines and arginine unless bicarbonate or CO<sub>2</sub> was present at high concentrations, which is known as an HCR phenotype. Analysis of the IC- and pyrimidine-mediated regulation in *pyrR<sub>1</sub>* and *pyrR<sub>2</sub>* mutants suggested that only PyrR<sub>2</sub> positively regulates the expression levels of the *pyr* genes in response to IC levels but had no effect on pyrimidine-mediated repression. A model is proposed for the respective roles of PyrR<sub>1</sub> and PyrR<sub>2</sub> in the *pyr* regulon expression.**

*Lactobacillus plantarum* is an aerotolerant, homofermentative lactic acid bacterium (11). Its growth is stimulated by increasing supplies of inorganic carbon (IC), such as carbon dioxide or its predominant soluble form in cells, bicarbonate. An optimal growth rate was obtained when 4% CO<sub>2</sub> was present in the gas phase or when 2 g/liter KHCO<sub>3</sub> was directly added to the culture media (1). *L. plantarum* strains can be naturally present along with CO<sub>2</sub>-producing cells, such as heterofermentative lactic acid bacteria, yeast in sourdough or beer, or eukaryotic cells in the gastrointestinal tract. *L. plantarum* is a versatile bacterium with metabolic abilities that favor its adaptation to a large range of biotopes (17). The nutritional richness of its biotopes has allowed *L. plantarum*, like other lactic acid bacteria, to evolve toward auxotrophy for several amino acids, vitamins, and nucleobases. When the arginine and pyrimidine requirements of 150 *L. plantarum* strains isolated from various origins were analyzed, nutritional needs were found to be strain specific and modulated by the addition of bicarbonate or CO<sub>2</sub>. While most strains were prototrophs, one-third of the strains were defined as high-CO<sub>2</sub>-requiring (HCR) prototrophs for both arginine and pyrimidine, i.e., these strains were prototrophs only when CO<sub>2</sub> partial pressure was increased or bicarbonate was added to the medium (5).

These observations suggested that IC would modulate *L. plantarum* arginine and pyrimidine metabolism either by changing the activities of enzymes with CO<sub>2</sub>-bicarbonate-related substrates or products or by regulating the expression levels of the enzyme-encoding genes.

Bicarbonate is the substrate for carbamoyl phosphate synthesis, an enzymatic step common in the biosynthesis pathways of arginine and pyrimidine. This step is catalyzed by the heterodimeric carbamoyl phosphate synthases (CPS) (Fig. 1A). Two CPS are functional in *L. plantarum*: CPS-P, encoded by *pyrAa<sub>1</sub>Ab<sub>1</sub>*, and CPS-A, encoded by *carAB* (20). The expression of CPS-P is regulated by pyrimidine availability, whereas CPS-A expression is subject to regulation by arginine, both at the transcriptional level. In the arginine pathway, carbamoyl phosphate is a substrate for citrulline synthesis catalyzed by the ornithine transcarbamoylase ArgF. In UMP synthesis, carbamoyl phosphate is fused to aspartate, yielding carbamoyl aspartate catalyzed by the aspartate transcarbamoylase PyrB. The genes involved in arginine and pyrimidine biosynthesis have been genetically characterized in the prototrophic strain CCM 1904 and mutant derivatives (4, 18–20). Bicarbonate concentrations affected the activities of CPS-A and CPS-P differently, as demonstrated with  $\Delta$ *pyrAa<sub>1</sub>Ab<sub>1</sub>* and  $\Delta$ *carAB* mutants. At low CO<sub>2</sub> concentrations, such as those in ordinary air, CPS-A activity was too low to provide carbamoyl phosphate for arginine and pyrimidine biosynthesis, as the  $\Delta$ *pyrAa<sub>1</sub>Ab<sub>1</sub>* mutant FB331 did not grow. On the other hand, CPS-P could satisfy the carbamoyl phosphate requirement for both arginine and pyrimidine biosynthesis pathways since the  $\Delta$ *carAB* mutant

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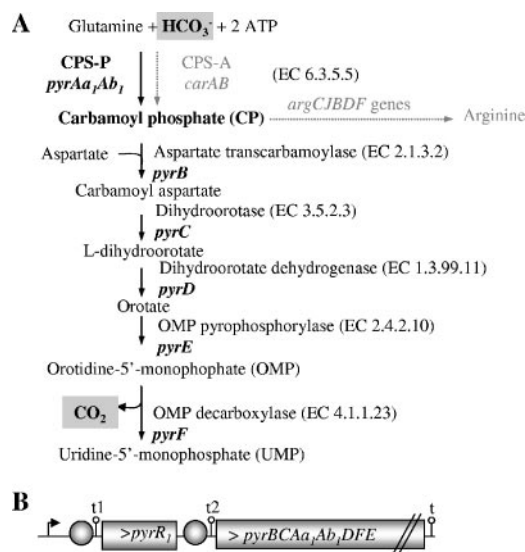


FIG. 1. UMP and arginine biosynthetic pathways in *L. plantarum*. (A) The *pyrBCAa<sub>1</sub>Ab<sub>1</sub>DFE* operon codes for the enzymes involved in UMP synthesis. For each step, the corresponding enzyme and gene are indicated. CPS-P stands for carbamoyl phosphate synthase pyrimidine-regulated protein. The *carAB* operon codes for CPS-A, the carbamoyl phosphate synthase arginine-regulated protein (20). (B) Genetic organization of *pyr* genes. Flag, transcriptional start; t, transcriptional terminator; circle, transcriptional attenuators as defined previously (19).

FB335 was a prototroph. When CPS-P expression was repressed by uracil, the wild-type prototroph strain CCM 1904 was unable to grow in the absence of arginine. The resulting phenotype, referred to as the uracil-sensitive phenotype, is due to cellular arginine depletion (20). In addition, the growth rate of the *L. plantarum* prototroph strain was reduced in ordinary air compared to that in bicarbonate-enriched air (1). All these observations revealed that the limiting reactant in carbamoyl phosphate synthesis in ordinary air may result from limiting amounts of either the substrate bicarbonate or the enzyme CPS-P.

The CPS-P-encoding *pyrAa<sub>1</sub>Ab<sub>1</sub>* is cotranscribed with the *pyrR<sub>1</sub>BCAa<sub>1</sub>Ab<sub>1</sub>DFE* operon, encoding the enzymes catalyzing UMP synthesis (Fig. 1B) (19). The first gene in the operon encodes the transcriptional repressor PyrR<sub>1</sub>. This protein was demonstrated to regulate the transcription of the *pyr* operon in response to pyrimidine availability in *L. plantarum*, most probably by transcriptional attenuation (19), a mechanism first elucidated in *Bacillus subtilis* (23). A second copy of the *pyrR* gene

was found in the *L. plantarum* genome (12). PyrR<sub>2</sub> shares 62% identity at the amino acid level with PyrR<sub>1</sub> (19), suggesting that PyrR<sub>2</sub> might also be a *pyr* operon regulator. In *B. subtilis*, a single copy of PyrR is present, and when activated by UMP, PyrR is able to stabilize the formation of an RNA loop (the anti-antiterminator loop) formed upstream of the coding sequence. This binding favors the formation of a terminator loop leading to transcriptional termination. When UMP pools are low, PyrR binding is less efficient and the formation of the antiterminator is favored, preventing termination. In *L. plantarum*, two such attenuators have been identified upstream of *pyrR<sub>1</sub>* and between *pyrR<sub>1</sub>* and *pyrB*. A similar attenuator was also found upstream of *pyrP*, a monocistronic, pyrimidine-repressed gene located elsewhere in the genome that codes for a uracil permease (2). Low pyrimidine-independent expression of the *pyr* operon was obtained by antiterminator site-directed mutagenesis. The resulting strain, AE1023, had reduced UTP and CTP pools and could grow in the absence of pyrimidines and arginine only if the bicarbonate concentration was high, which corresponds to the HCR phenotype (19). Therefore, repression of the *pyr* operon prevents the ability of *L. plantarum* to grow at low carbon dioxide levels (19). Since the HCR phenotype was linked to lower *pyr* expression, it became evident that it was necessary to investigate whether CO<sub>2</sub>-bicarbonate concentration affects *pyr* gene expression in *L. plantarum*.

In this work, *pyr* gene expression in response to CO<sub>2</sub>-bicarbonate concentrations was tested using a proteomic approach as well as by quantification of *pyr* transcripts in the wild type and mutants of each of the two PyrR regulator-like orthologs. PyrR<sub>2</sub> was found to regulate *pyr* genes in response to IC independently of pyrimidine availability.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *L. plantarum* subsp. *plantarum* CCM 1904 and NC8 are wild-type arginine and uracil prototrophs; they and their derivatives (Table 1) were grown on MRS agar plates (Difco Laboratories) at 30°C. Liquid cultivations were performed in agitated DLA chemically defined medium (4). When required, uracil and arginine were added at 50 µg/liter. IC-enriched medium was obtained either by adding KHCO<sub>3</sub> at a final concentration of 2 g/liter, with pH adjusted to 6.5 by using 2 M HCl, or by increasing the CO<sub>2</sub> tension in the gas phase to 4%. To prevent CO<sub>2</sub> loss during incubation when CO<sub>2</sub> or KHCO<sub>3</sub> was added, the Erlenmeyer flasks were closed with gas-tight corks. To obtain 4% CO<sub>2</sub> in the gas phase, 10.8 ml of pure CO<sub>2</sub> gas was injected with a sterile syringe through the gas-tight cork of an Erlenmeyer flask (internal volume, 320 ml; volume of gas phase, 270 ml; liquid volume, 50 ml). For RNA and protein studies, cells were first cultivated in DLA without uracil in normal-air-agitated precultures. These precultures were used as an inoculum for 50-ml cultures in DLA: under "growing" conditions, the inoculum was added to obtain

TABLE 1. List of studied strains

Strain	Genotype	Comment(s) <sup>a</sup>	Source or reference
AE1026	<i>pyrR<sub>1</sub></i>	PyrR <sub>1</sub> D104Y mutation in CCM 1904	19
CCM 1904	Wild type	Prototroph	5
FB335	$\Delta$ <i>carAB</i>	Site mutagenesis in CCM 1904	20
U17	$\Delta$ <i>carAB</i> $\Delta$ <i>pyrR<sub>1</sub></i>	Derivative of FB335	19
U32	$\Delta$ <i>carAB pyrR<sub>1</sub></i>	PyrR <sub>1</sub> truncated from Q17-E180 in FB335	19
NC8	Wild type	Prototroph	5
FB421	<i>pyrR<sub>1</sub></i>	PyrR <sub>1</sub> D104Y mutated in NC8	This work
FB422	$\Delta$ <i>pyrR<sub>2</sub></i>	Entire <i>pyrR<sub>2</sub></i> coding sequence deletion in NC8	This work

<sup>a</sup> Sequence data refer to EMBL accession number Z54240.

TABLE 2. Primer list

Genetic locus <sup>a</sup>	PCR product parameter		Primer names and sequences (5'→3') <sup>b</sup>
	Use	Size (bp)	
<i>pyrP</i> (AJ012720)	P probe and RT-PCR	298	<i>pyrP</i> -g4, AATCATTACAAAAAGGGGTGCG (F); <i>pyrP</i> -d2, TCATCAGAGACTGCA TCACAACG (R)
<i>pyrR<sub>2</sub></i> (AJ617795)	<i>pyrR2</i> mutant	596	lp1781f1, ACTTACTGCACTACGTCGCACTCGTTCACGGAG (F); Δ <i>pyrR2r</i> , TGA CT TTGTCGTAAATTTGCCCTCCCAATTTGTGG (R)
	<i>pyrR2</i> mutant	579	Δ <i>pyrR2f</i> , TGGGAGGCAATTTACGACAAAGTCAAACAGATG (F); lp1783r1, GCCA AAGAATTCGATGACCACGACATTGAGC (R)
<i>pyrR<sub>1</sub></i> (Z54240)	R1 probe	240	2072, CTGACAACTAATGGCAGC (F); 2068, ACCGCATGATGATCATCCCCG (R)
	RT-PCR	379	<i>pyrR631</i> , CAATTAACAAGCACCTTTAAC (F); 2068, ACCGCATGATGATCAT CCCC (R)
<i>pyrAb<sub>1</sub></i> (Z54240)	RT-PCR or Ab <sub>1</sub> #1 probe	364	N58, CAGCTTCTGGTATTGCGC (F); 2005, ATCTTCAAAGCAGCATCCCCG (R)
	RT-PCR or Ab <sub>1</sub> #2 probe	291	N49', TCAAAGTTGAATCGTGTGG (F); 2019, AATCATGCTCGCCACTATCG (R)
<i>pyrB</i> (Z54240)	B probe and RT-PCR	260	N1, TGAGAATAGCACCCGCACAC (F); 2042, TGTACGGATGTGCCCCAC (R)
<i>pyrD</i> (Z54240)	D probe	313	N35, TGATGAATACGTGGCAGTGC (F); N41, GATAAACCCCGCTACCATG (R)
<i>rm</i> (AL935263)	RT-PCR or <i>rm</i> probe	400	530f, GTGCCAGCAGCCGCGG (F); 907r, CCGTCAATTCCTTTGAGTTT (R)

<sup>a</sup> The EMBL accession numbers are shown in parentheses.

<sup>b</sup> F, Forward primer; R, reverse primer. Underlined sequences show the PstI and EcoRI restriction sites.

an optical density at 600 nm (OD<sub>600</sub>) of 0.05 and the culture was stopped when the OD<sub>600</sub> reached 0.5 (approximately 4 h and two generations in the exponential growth phase); under "nongrowing" conditions (with the presence of uracil and no inorganic carbon enrichment), cells were inoculated to obtain an OD<sub>600</sub> of 0.4 and incubated for 4 h.

**Mutagenesis by allelic exchange in NC8.** Mutagenesis by allelic replacement of *pyr* genes has been successfully used for *L. plantarum* with pGID023-derived plasmids such as plasmid pAE1020, which harbors the *pyrR<sub>1</sub>* mutation (19). Another derivative of pGID023, plasmid pΔ*pyrR2*, was designed to delete *pyrR2* from its start codon until its stop codon. This plasmid was constructed as follows. Two amplifications, using *L. plantarum* CCM 1904 genomic DNA template and primer set Lp1781f1/Δ*pyrR2r* or primer set Δ*pyrR2f*/Lp1783r1, were performed. Since primers Δ*pyrR2r* and Δ*pyrR2f* have an overlapping 25-nucleotide-long sequence (Table 2), the two PCR products were merged by a second PCR with primers Lp1781f1/Lp1783r1. The resulting 1.15-kb PCR product was PstI-EcoRI restricted and cloned into the linearized PstI-EcoRI pGID023 vector. For allelic gene replacement, the Δ*pyrR2* allele present in plasmid pΔ*pyrR2* was electroporated into the two wild-type prototroph *L. plantarum* strains, NC8 and CCM1904. However, no transformants were obtained with CCM 1904, which has reduced electro-competence. The selection of the recombinants harboring the mutated allele was performed as previously described (20). The constructs were confirmed by sequencing the corresponding PCR-amplified chromosome locus. The NC8-derivative strains FB421 and FB422 contained the *pyrR<sub>1</sub>* and *pyrR<sub>2</sub>* mutations, respectively.

**Protein extractions and separations.** Cells were grown in 50 ml DLA medium (see above) and harvested by centrifugation at an OD<sub>600</sub> of 0.5. The cell pellet was washed once in 10 mM Tris, 1 mM EDTA, pH 7.6, and resuspended in 600 μl of the same buffer. Cells were disrupted by grinding them with glass beads (0.3 mm in diameter; 1 g per 0.6-ml extract) at the maximal speed in an MM2 mixer mill (Retsh Haan, Germany).

For first-dimension (1D) gel electrophoresis, the cells were disrupted by grinding them for 6 min, followed by centrifugation. Proteins (40 μg) of the supernatant were diluted in Laemmli buffer (50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 1% sodium dodecyl sulfate [SDS], 1% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) (1:1), separated by electrophoresis in 10% acrylamide gel, and stained using Coomassie blue (Amersham Pharmacia Biotech, General Electric, Germany).

For two-dimensional (2D) gel electrophoresis studies, prior to being ground (six cycles, each with 1 min shaking and a 1-min pause at 4°C), cells were incubated for 30 min at 4°C in the presence of 1 mM phenylmethylsulfonyl fluoride and benzamide (348 units; Roche) to prevent protease degradation and nucleic acid contamination. The lysate was decanted overnight at 4°C, and the clear lysate was centrifuged at 14,000 × g for 1 h at 4°C. Protein concentration in the supernatant was quantified using the method of Bradford (3), standardized with known concentrations of bovine serum albumin, and subsequently adjusted to 3 mg/ml using Tris-EDTA (TE) buffer. Two hundred fifty microliters of rehydration buffer (7 M urea, 2 M thiourea) purified by mixing for 1 hour with 10 g/liter Amberlite IRN-150L (Amersham Pharmacia Biotech, Germany),

2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.5% immobilized pH gradient (IPG) buffer (pH range, 4 to 7), 3.4 mg/ml dithiothreitol (DTT), and a few bromophenol blue grains was added to the protein extracts (100 μl). Proteins were first separated according to pI. Proteins (300 μg) were loaded on 18-cm IPG strips (linear gradient pH, 4 to 7) with the IPGphor isoelectric focusing system as recommended by the manufacturer (Amersham Pharmacia Biotech, Germany). Rehydration (6 h at 0 V and 6 h at 30 V) was followed by four 2-hour increments in which the voltage was increased stepwise to 150 V, 500 V, 1,000 V, and 3,000 V. Finally, separation was obtained using 8,000 V until a minimum of 45,000 V-h was reached. Strips were frozen at -80°C for at least 1 day, thawed at room temperature, and placed in the equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and traces of bromophenol blue) two times for 15 min, in the presence of 10 mg/ml DTT and then in the presence of 25 mg/ml iodoacetamide. The second-dimension gel experiment was performed with an 11.5% SDS-polyacrylamide gel electrophoresis (PAGE) gel. Proteins were stained with brilliant blue G-collodial (SIGMA). Computer analysis was performed using the ImageMaster 2D platinum program (Amersham Pharmacia Biotech); 16 gels were grouped in two classes according to the two tested conditions (ordinary air or a 4% CO<sub>2</sub>-enriched atmosphere), and each class contained 8 independent gels. After spot detection (around 300 spots per gel), gels were matched with one reference gel (extracts from cells cultivated in ordinary air). For each spot, the relative volume corresponded to the normalized volume of the spot compared to the normalized volume of the entire gel coloration. The statistical analysis was performed by calculating for each spot the Student *t* value and the "ratio" (the average for 8 relative volumes obtained at low CO<sub>2</sub> concentrations divided by the average for 8 relative volumes obtained at high CO<sub>2</sub> concentrations). Only spots sharing Student *t* values higher than 1.4 (corresponding to a *P* value of <0.1) and ratios higher than 2 were analyzed by mass spectrometry (MS). The selected spots were cut from 1D or 2D gel and stored at -20°C.

**Protein digestion and mass spectrometry.** For in-gel digestion, picked spots were washed with 100 μl of 25 mM NH<sub>4</sub>HCO<sub>3</sub> buffer and dehydrated with 100 μl of acetonitrile (ACN), and the two operations were repeated twice. The samples were vacuum dried for 10 min, reduced (100 μl of 10 mM 1,4-DTT-25 mM NH<sub>4</sub>HCO<sub>3</sub> buffer at 56°C for 1 h), and alkylated (100 μl of 25 mM iodoacetamide-25 mM NH<sub>4</sub>HCO<sub>3</sub> buffer at room temperature in the dark for 1 h). After three washes for 5 min each in 25 mM NH<sub>4</sub>HCO<sub>3</sub> and ACN alternately, samples were vacuum dried and rehydrated overnight at room temperature in the presence of trypsin (3 volumes of 12.5 ng trypsin [V5111; Promega]/μl in 25 mM NH<sub>4</sub>HCO<sub>3</sub> buffer freshly diluted). Tryptic peptides were extracted from the gels by sonication for 30 min in 5 μl of 35% H<sub>2</sub>O-60% ACN-5% HCOOH. Mass measurements were performed with a BIFLEX III matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS (Bruker Daltonics, Bremen, Germany) equipped with SCOUT high-resolution optics with an X-Y multi-sample probe and griddle reflectors. This instrument was used in positive-ion-reflector mode with a maximum accelerating potential of 19 kV. A saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, Saint Louis, MO) in acetone was used as a matrix. Spreading and fast evaporation of 0.5 μl of matrix solution

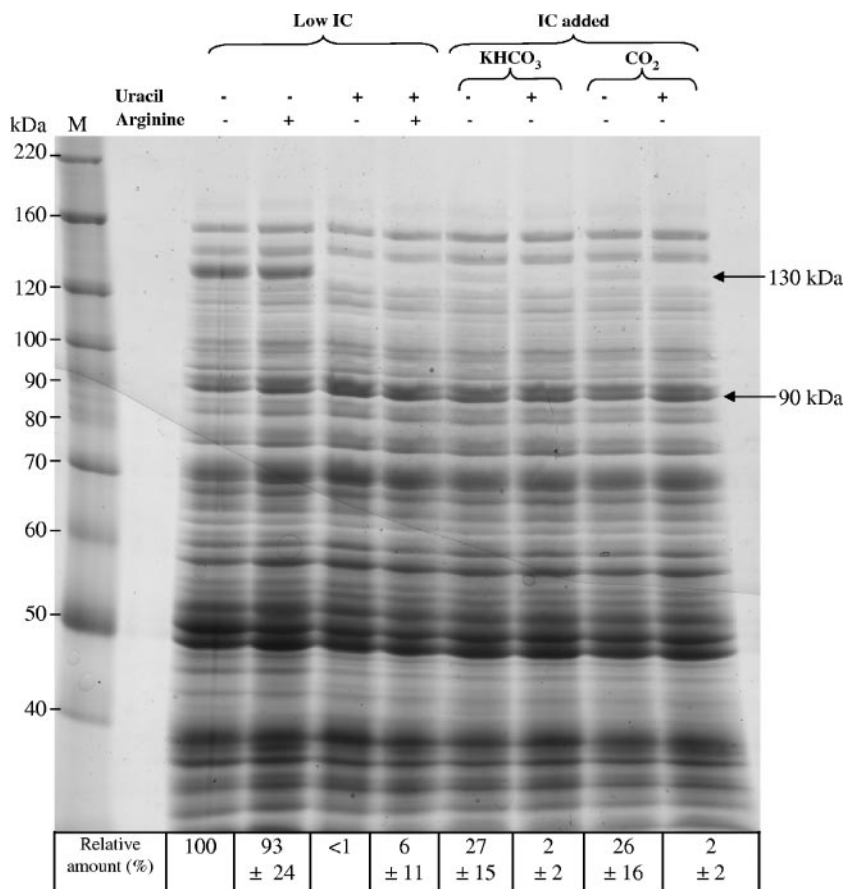


FIG. 2. SDS-PAGE protein patterns in response to inorganic carbon, arginine, and uracil. Proteins were extracted from *L. plantarum* wild-type strain CCM 1904 under different growth conditions: in ordinary air (Low IC), with  $\text{KHCO}_3$  added at 2 g/liter, in a 4%  $\text{CO}_2$ -enriched atmosphere, or with arginine and uracil (+). M, protein size marker (BenchMark protein ladder; Invitrogen). The 90-kDa constitutive band of an unknown protein served as the internal standard for quantification of the signal corresponding to PyrAb<sub>1</sub> (band shown at 130 kDa). Numbers indicate the relative amounts of the PyrAb<sub>1</sub> polypeptide expressed as percentages of the value obtained for cells cultivated at low IC levels, in the absence of arginine and uracil. The values shown are averages for at least four independent experiments.

yielded a fine layer of crystals, on which a droplet of 0.5  $\mu\text{l}$  of 5% aqueous HCOOH solution was mixed first with 0.5  $\mu\text{l}$  of peptide-containing digest medium and then with 0.3  $\mu\text{l}$  saturated matrix solution (in 50%  $\text{H}_2\text{O}$ -50% ACN). The preparation was vacuum dried and washed once with 0.7  $\mu\text{l}$  of 5% aqueous HCOOH. Mass spectra were internally calibrated with trypsin autolysis peaks ( $m/z = 842.510$  and  $m/z = 2,211.105$ ). Monoisotopic peptide masses were assigned, and the peak list was transferred to the MS BioTools program (Bruker Daltonics, Bremen, Germany) and then compared to data in the NCBI protein database by using MASCOT software (Matrix Science, London, United Kingdom). Tryptic mass searches retained only data with up to one missed tryptic cleavage and optional methionine oxidation, with mass accuracy limited to 50 ppm.

**Transcription analysis.** RNA extraction and semiquantitative reverse transcription (RT)-PCR protocols have been previously described (18). RT-PCR was performed with primers listed in Table 2, using optimized amounts of total RNA to target different transcripts (20 ng for *pyrAb*<sub>1</sub> and *rm*, 30 ng for *pyrP*, and 150 ng for *pyrR*<sub>1</sub> and *pyrB*). For Northern hybridizations, DNA probes were PCR amplified (95°C for 1 min, followed by 35 three-step cycles at 94°C for 40s, 50°C for 40s, and 72°C for 2 min; the program was completed with a postelongation step at 72°C for 10 min), using primers listed in Table 2. The PCR products were digoxigenin labeled using a digoxigenin-labeling kit (Roche). For each probe, the optimal amount of total RNA to be used was tested (0.1  $\mu\text{g}$  for *rm*, 5  $\mu\text{g}$  for *pyrD*, 2  $\mu\text{g}$  for *pyrAb*<sub>1</sub> probe Ab<sub>1</sub>#1 [see Fig. 4A] or Ab<sub>1</sub>#2, and 10  $\mu\text{g}$  for *pyrR*<sub>1</sub> and *pyrB*). Slot blot hybridizations were performed and quantified as described previously (2), with Quantity One software (Bio-Rad). At least three independent hybridizations were performed for each tested condition. The background level was subtracted, and the relative signal was calculated as follows: the measured

signal for each probe was divided by the signal obtained with the *rm* probe to obtain the relative amount for each *pyr* gene. Under the tested conditions (uracil and IC supplementation or not), the constitutive expression levels of the *rm* genes were checked using the *upp* gene, whose transcription is not regulated by IC and pyrimidine availability (2; data not shown).

**Nucleotide sequence accession number.** The *pyrR*<sub>1</sub>*BCAa*<sub>1</sub>*Ab*<sub>1</sub>*DFE* operon and *pyrR*<sub>2</sub> genes were sequenced in strain NC8. These sequences have been submitted to the EMBL database and assigned the accession numbers AM228716 and AM228717, respectively.

## RESULTS

**The expression of the *pyr* operon is repressed at high bicarbonate concentrations.** The effect of IC supplementation on gene expression was first analyzed by 1D gel electrophoresis (Fig. 2). Proteins were extracted from *L. plantarum* CCM 1904 cultivated in DLA medium with either  $\text{CO}_2$  or  $\text{KHCO}_3$  in combination with uracil and arginine. The amount of one protein with an apparent mass of 130 kDa decreased fourfold upon addition of  $\text{CO}_2$  or  $\text{KHCO}_3$ . Arginine addition had no effect, whereas exogenous uracil addition led to a 10- to 100-fold decrease in the concentration of the 130-kDa protein. The 130-kDa protein band obtained under three different growth conditions was cut from the gel for mass spectrometry protein

TABLE 3. Mass spectrometry analysis of IC-regulated proteins observed either by 1D or 2D gel electrophoresis

Protein <sup>a</sup>	Gene <sup>a</sup>	Spot <sup>b</sup>	Gel type	Protein characteristic <sup>c</sup>		Mass spectrometry value <sup>d</sup>			Air/IC ratio (P) <sup>f</sup>
				pI	MM (Da)	Error (ppm)	Coverage (%)	Score	
Carbamoyl phosphate synthase (large subunit)	<i>pyrAb<sub>1</sub></i>	1	1D	4.93	115,757	20	46	484	–
		2	1D	4.93	115,757	22	56	429	–
		5	1D	4.93	115,757	25	47	305	–
		1	2D	4.93	115,757	31	42	334	4.7 (1.5)
		2	2D	4.93	115,757	42	54	371	4.7 (1.5)
Dihydroorotase	<i>pyrC</i>	3	2D	4.93	115,757	42	56	458	4.7 (1.5)
		4	2D	5.76	45,677	40	52	123	4.3 (1.9)
		5	2D	5.76	45,677	44	64	130	4.3 (1.9)
Carbamoyl phosphate synthase (small subunit)	<i>pyrAa<sub>1</sub></i>	6	2D	5.67	40,143	49	48	212	2.5 (1.8)
Aspartate carbamoyltransferase	<i>pyrB</i>	7	2D	6.06	34,689	50	63	189	5.4 (2.2)
		8	2D	6.06	34,689	38	62	205	5.4 (2.2)
Dihydroorotate dehydrogenase	<i>pyrD</i>	9	2D	6.14	31,318	51	53	139	5.5 (2.6)
Orotate phosphoribosyltransferase	<i>pyrE</i>	10	2D	5.64	22,691	50	72	147	3.3 (3.4)
Transcriptional repressor	<i>pyrR<sub>1</sub></i>	11	2D	5.64	19,812	ND <sup>e</sup>	71	127	2.0 (1.6)

<sup>a</sup> EMBL accession no. Z54240.

<sup>b</sup> For 1D gels, numbers refer to growth conditions (1, no IC added; 2, with arginine; 5, addition of 2g KHCO<sub>3</sub>/liter), and for 2D gels, numbers refer to spots shown in Fig. 3.

<sup>c</sup> pI, Isoelectric point; MM, molecular mass.

<sup>d</sup> Score, probability-based Mowse score calculated using MASCOT software (Matrix Science, London, United Kingdom); error, mass accuracy; coverage, percentage of the protein sequence covered by the matched peptide.

<sup>e</sup> Internal calibration not determined.

<sup>f</sup> P values were determined by Student's *t* test. –, results are shown in Fig. 2.

identification (Table 3). In all three cases, the best scores matched those of the *L. plantarum* large-chain carbamoyl phosphate synthase PyrAb<sub>1</sub> (EMBL database accession no. P77886). In a second set of experiments, proteins were separated using 2D gel electrophoresis (Fig. 3). Two growth conditions for the wild-type strain CCM 1904 were compared: a 4% CO<sub>2</sub>-enriched atmosphere and a low-IC condition found in ordinary air. Spots with the most-significant differential expression levels were excised from the gel. The corresponding proteins were identified using MALDI-TOF mass spectrometry. Among those, the PyrAb<sub>1</sub> protein previously identified in the 1D gel experiment was found (Table 3, spots 1, 2, and 3). Moreover, the expression levels of all the other proteins encoded by the *pyrR<sub>1</sub>BCAa<sub>1</sub>Ab<sub>1</sub>DFE* operon, except PyrF, were

decreased in response to IC (Table 3, spots 4 to 11). With a pI of 7.7, PyrF was excluded from the gels that targeted proteins with pI values between 4 and 7.

These results clearly suggest that the expression levels of the genes in the *pyr* operon are subject to regulation by bicarbonate-CO<sub>2</sub> availability in either a direct or an indirect manner.

**IC-dependent transcription of the *pyr* genes.** The *pyrR<sub>1</sub>BCAa<sub>1</sub>Ab<sub>1</sub>DFE* and *pyrP* genes are part of the *pyr* regulon, whose transcription is regulated in response to exogenous pyrimidine (2, 19). Transcription regulation of the *pyr* regulon in response to IC concentration was assessed using slot blot hybridization and RT-PCR amplifications. For this study, five *pyr* genes were chosen because of their genetic positions: four genes within the *pyr* operon, *pyrR<sub>1</sub>* located upstream of the first attenuator, *pyrB*

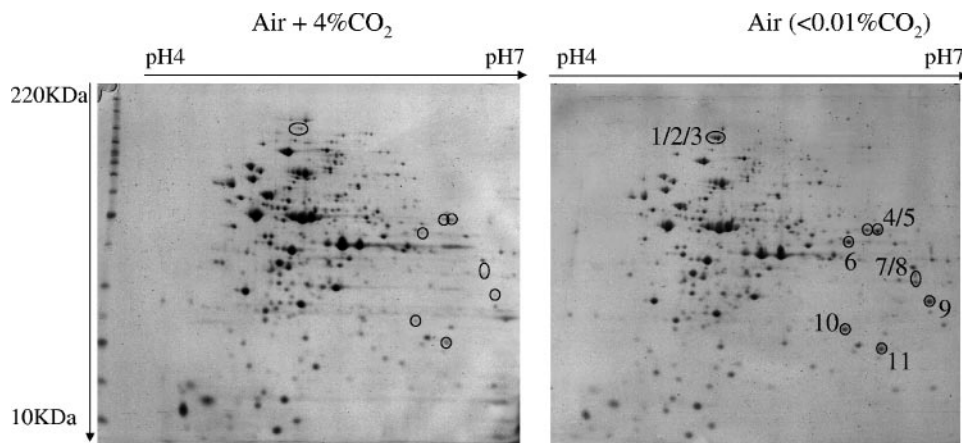


FIG. 3. Comparison of proteomic profiles at low and high inorganic carbon levels. *L. plantarum* wild-type strain CCM 1904 was cultivated in defined DLA medium (in the absence of arginine or uracil), with agitation in CO<sub>2</sub>-enriched or ordinary air. Spots corresponding to the proteins encoded by the *pyr* genes are circled and identified by numbers referenced in Table 3. Sizes of protein markers (BenchMark protein ladder; Invitrogen) are shown in 10-kDa increments from 10 to 220 kDa.

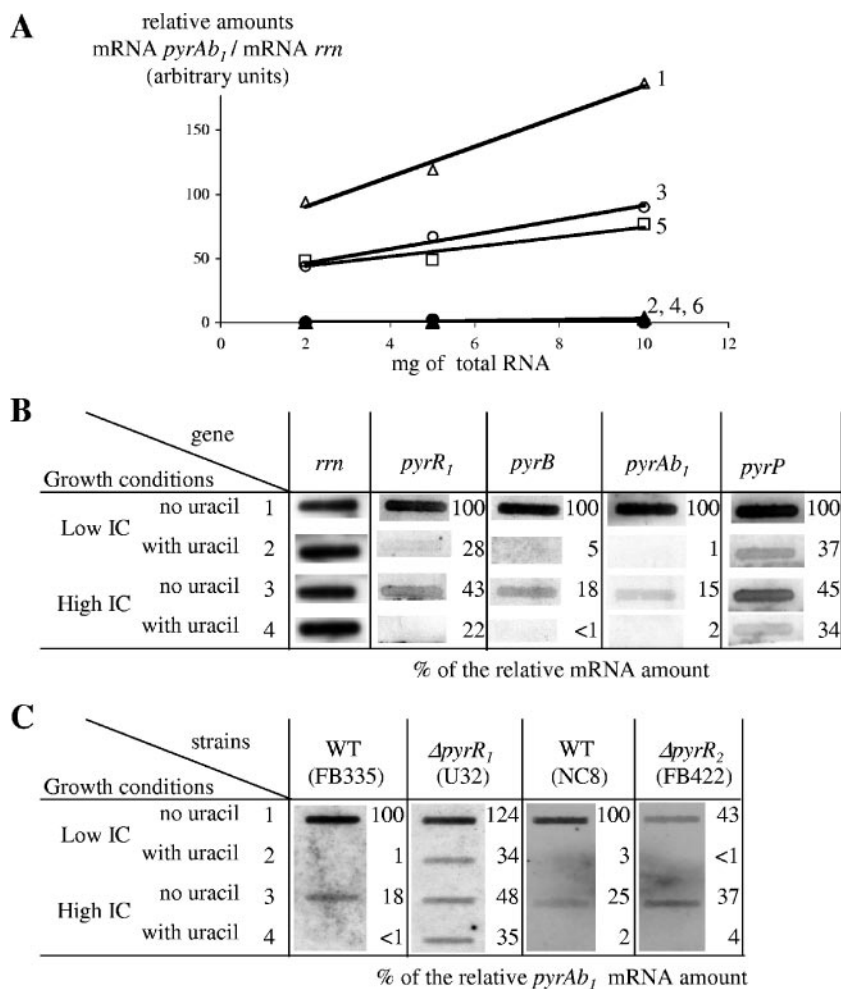


FIG. 4. Transcription of *pyr* genes in response to IC and uracil. Slot blot hybridizations detecting *pyr* mRNA were performed with probes specific to *pyr* genes (described in Table 2) and standardized with probe *rrm*, specific to 16S RNA encoding genes. The relative amounts (arbitrary units) were calculated by dividing the signal for each *pyr* probe by the signal obtained with probe *rrm*. RNAs were extracted from cells cultivated in DLA under different conditions, in normal air (1 and 2), in a 4% CO<sub>2</sub>-enriched atmosphere (3 and 4), with KHCO<sub>3</sub> added at 2 g/liter (5 and 6), without uracil (1, 3, and 5), or with uracil (2, 3, and 6). (A) Quantification test with probe Ab<sub>1</sub>#1, with different amounts of total RNA prepared from strain CCM1904. (B) Transcription of different *pyr* genes in the wild-type strain CCM 1904. (C) Gene *pyrAb<sub>1</sub>* transcription efficiencies in strains harboring wild-type or mutated *pyrR<sub>1</sub>* or *pyrR<sub>2</sub>* genes. Relative amounts calculated for different growth conditions were compared to relative amounts obtained for the corresponding wild-type isogenic strain grown at low IC levels without uracil added (condition marked 1). Each “% of the relative mRNA amount” is therefore expressed as a percentage of the amount obtained for condition 1, defined as 100%. Names of strains are indicated in parentheses.

located just after the second attenuator, and *pyrAb<sub>1</sub>* and *pyrD* located in the middle and at the end of the operon, respectively. The last gene was *pyrP*, a gene not found in the *pyr* operon (Fig. 1B). For each probe, the intensity of the signal was proportional to the amount of spotted RNA (Fig. 4A for *pyrAb<sub>1</sub>*, data not shown for other *pyr* genes). For each *pyr* gene, the detected mRNA amount decreased significantly when RNA originated from cells incubated under IC enrichment conditions, either by increasing the gas phase CO<sub>2</sub> partial pressure or by adding KHCO<sub>3</sub> to the medium (Fig. 4A for *pyrAb<sub>1</sub>*; also Fig. 4B, compare condition 1 and 3 or 5). After quantification, the “IC regulation factor” (the relative amount obtained at low IC levels divided by the relative amount obtained at high IC levels) was in the order of five- to sixfold for the *pyrBCA<sub>1</sub>Ab<sub>1</sub>DFE* operon and of twofold for both the *pyrR<sub>1</sub>*

and the *pyrP* genes (Fig. 4B). Arginine addition had no detectable effect on the transcription levels of the tested *pyr* genes (data not shown). As expected from characterized uracil-repressed genes, in the presence of exogenous uracil, transcription of *pyrAb<sub>1</sub>*, *pyrB* (Fig. 4, compare conditions 1 and 2) and *pyrD* (data not shown) was repressed in the order of 20- to 100-fold, whereas *pyrR<sub>1</sub>* and *pyrP* transcription was reduced 3-fold. Similar IC-mediated repression levels were obtained using semiquantitative RT-PCR experiments (data not shown), with specific primer sets detecting *pyrAb<sub>1</sub>*, *pyrB*, *pyrP*, and *pyrR<sub>1</sub>* transcripts. These results are in agreement with the observations made by proteomics that the expression of the *pyr* regulon responds to changes in bicarbonate and CO<sub>2</sub> concentration. This finding adds to the previously known regulation that occurs in response to pyrimidine availability.

**IC-responding regulation of the *pyr* genes did not require a functional PyrR<sub>1</sub>.** Both pyrimidine and IC concentrations regulate the transcription of the *pyr* genes. Since PyrR<sub>1</sub> is known as the repressor of the *pyr* operon in response to exogenous pyrimidines (19), the role of PyrR<sub>1</sub> in IC-responding regulation was first analyzed by comparing the phenotypes of the wild-type and *pyrR*<sub>1</sub> strains in the genetic context of two prototroph strains, CCM 1904 and NC8 (Table 1). PyrR<sub>1</sub> displays 99.4% amino acid identity in these two strains. Like wild-type strains, *pyrR*<sub>1</sub> mutants were prototrophs and had similar nutritional IC requirements (19). The expression levels of the *pyr* genes in response to IC levels were estimated by quantification of the amounts of PyrAb<sub>1</sub> after SDS-PAGE. Several *pyrR*<sub>1</sub> mutants were tested in the genetic context of CCM 1904 and its  $\Delta$ *carAB*-derivative strain FB335; the CCM 1904-derivative strain AE1026 harbors the D104Y mutation in PyrR<sub>1</sub>, and strains U32 and U17 are  $\Delta$ *pyrR*<sub>1</sub>  $\Delta$ *carAB* mutants (19). The same patterns of regulation were obtained for strains CCM 1904 and FB335, suggesting that deletion of the *carAB* genes had no detectable effect on the tested IC- and uracil-responding regulation. In all three *pyrR*<sub>1</sub> mutants, the regulations in response to uracil or IC levels were similar (data not shown). In addition, similar results were also obtained when wild-type strains NC8 or CCM1904 or their *pyrR*<sub>1</sub> derivatives (AE1026 and FB421, respectively) were compared (data not shown). Thus, PyrR<sub>1</sub> plays the same role in the *L. plantarum* wild-type prototroph CCM 1904 and NC8 strains.

We used strain U32 ( $\Delta$ *pyrR*<sub>1</sub>) to quantify the amounts of *pyrBCAa*<sub>1</sub>*Ab*<sub>1</sub>*DFE* transcript by using slot blot hybridization with probes specific to *pyrAb*<sub>1</sub> and *pyrB* (Fig. 4C and data not shown). The IC regulation factors were similar in the reference strain FB335 (factors 5 and 6) and in the *pyrR*<sub>1</sub> U32 mutant (factors 3 to 5) both at the protein and at the mRNA levels. Nevertheless, in the absence of uracil and in normal air, we observed that *pyr* expression in the *pyrR*<sub>1</sub> mutant was increased compared to that in the wild type, indicating that the *pyr* operon is not fully induced in the absence of pyrimidines. As expected from previous work, uracil-dependent repression was found to be PyrR<sub>1</sub> dependent: a 100-fold repression was observed with the wild-type strain (Fig. 4C, compare lanes 1 and 2), but only a 3- to 4-fold repression was observed with mutated *pyrR*<sub>1</sub>. Finally, the amounts of detected *pyrAb*<sub>1</sub> mRNA in *pyrR*<sub>1</sub> mutants in the presence of uracil were similar whether the cells grew at low or high IC concentrations (Fig. 4C, compare lanes 2 and 4), as observed in wild-type strains. These observations suggested that PyrR<sub>1</sub> has no effect on *pyr* operon repression in response to IC concentration and, moreover, is not the sole sensor of pyrimidine availability.

***pyrR*<sub>2</sub> deletion confers higher CO<sub>2</sub> needs for growth in the absence of arginine and uracil.** A *pyrR*<sub>2</sub> mutant was constructed to test the effect of PyrR<sub>2</sub> on the ability of *L. plantarum* to grow at low IC concentrations and its role in IC-dependent regulation. The function of *pyrR*<sub>2</sub> was analyzed in strain FB422, a  $\Delta$  *pyrR*<sub>2</sub> derivative of NC8. The effects of the addition of CO<sub>2</sub>, arginine, and uracil on the growth of CCM1904 and NC8 were analyzed. The  $\Delta$ *pyrR*<sub>2</sub> mutant was an HCR prototroph (5), since it was unable to grow in the absence of uracil and arginine in ordinary air but grew in 4% CO<sub>2</sub>-enriched air. Thus, *pyrR*<sub>2</sub> deletion confers the HCR phenotype.

**IC-mediated regulation of *pyr* genes depends on a functional PyrR<sub>2</sub>.** The impact of *pyrR*<sub>2</sub> deletion on *pyrBCAa*<sub>1</sub>*Ab*<sub>1</sub>*DFE* operon expression was assessed both at the protein level by comparing the PyrAb<sub>1</sub> polypeptide expression pattern (data not shown) and at the transcriptional level by quantifying transcripts with probes specific for *pyrAb*<sub>1</sub> (Fig. 4C) or *pyrB* (data not shown). In the mutant strain in ordinary air, the expression levels of these genes, and by assumption that of the entire *pyr* operon, were reduced compared to that of the wild type (Fig. 4C, condition 1). Moreover, whereas the expression of the *pyr* genes was two- to sixfold repressed by IC in the wild-type strain, their level of expression in the *pyrR*<sub>2</sub> mutant was unaffected by changes in IC availability (Fig. 4C, strain FB422 in conditions 1 and 3). Deletion of the *pyrR*<sub>2</sub> gene resulted in reduced *pyr* operon transcription under low-IC conditions, with loss of the observed IC-dependent regulation. This suggested that PyrR<sub>2</sub> is required for maximal *pyr* gene expression when IC concentration is low.

**A functional PyrR<sub>2</sub> is not involved in the pyrimidine-dependent repression of the *pyr* operon.** The role of PyrR<sub>2</sub> in pyrimidine dependent repression of the *pyr* operon was analyzed by quantification of the protein PyrAb<sub>1</sub> (SDS-PAGE data not shown) and of the transcripts of genes *pyrAb*<sub>1</sub> (Fig. 4C) and *pyrB* (data not shown). In the presence of uracil, the same level of repression was obtained for the wild type and for the  $\Delta$ *pyrR*<sub>2</sub> strains (Fig. 4C). These results showed that *pyrR*<sub>2</sub> is not involved in pyrimidine-dependent repression of the *pyr* operon.

## DISCUSSION

The transcription-dependent regulation of the *pyr* operon in response to exogenous pyrimidines has been extensively studied (23). Using proteomic and transcriptional quantitative analysis, this work gives evidence of transcription regulation of the *pyr* operon in response to inorganic carbon but not to arginine.

**Inorganic carbon levels modulate the transcription of *pyr* genes.** Similar levels of regulation were obtained either by adding CO<sub>2</sub> to the gas phase or by adding KHCO<sub>3</sub> to the medium. The values for IC-mediated repression calculated at the levels of protein and RNA were similar (four- to fivefold for the *pyrBCAa*<sub>1</sub>*Ab*<sub>1</sub>*DFE* operon, twofold for *pyrR*<sub>1</sub> and *pyrP*). Thus, inorganic carbon concentrations are sensed by the cell to regulate *pyr* gene expression mainly at the level of transcription or RNA stability for at least two noncontiguous loci (the *pyr* operon and the *pyrP* gene). Regulation of gene expression in response to IC has been observed in phototrophs and chemolithotrophs (*Rhodobacter sphaeroides*, *Ralstonia eutropha*, and cyanobacteria) in genes involved in inorganic carbon assimilation (14) but also in chemoorganotrophs (*Bacillus anthracis*, *Escherichia coli*, group A streptococci, *Pseudomonas* spp., and *Staphylococcus aureus*) (10, 13, 22), in general stress or in purine metabolism in cyanobacteria (21, 26), and in amino acid decarboxylation in *E. coli* (24). To our knowledge, this report constitutes the first description that proteins involved in pyrimidine biosynthesis are regulated in response to IC. Differential proteomic experiments with increased detection thresholds (two-dimensional differential gel electrophoresis) are currently in progress to further characterize IC-responding genes in *L. plantarum*.

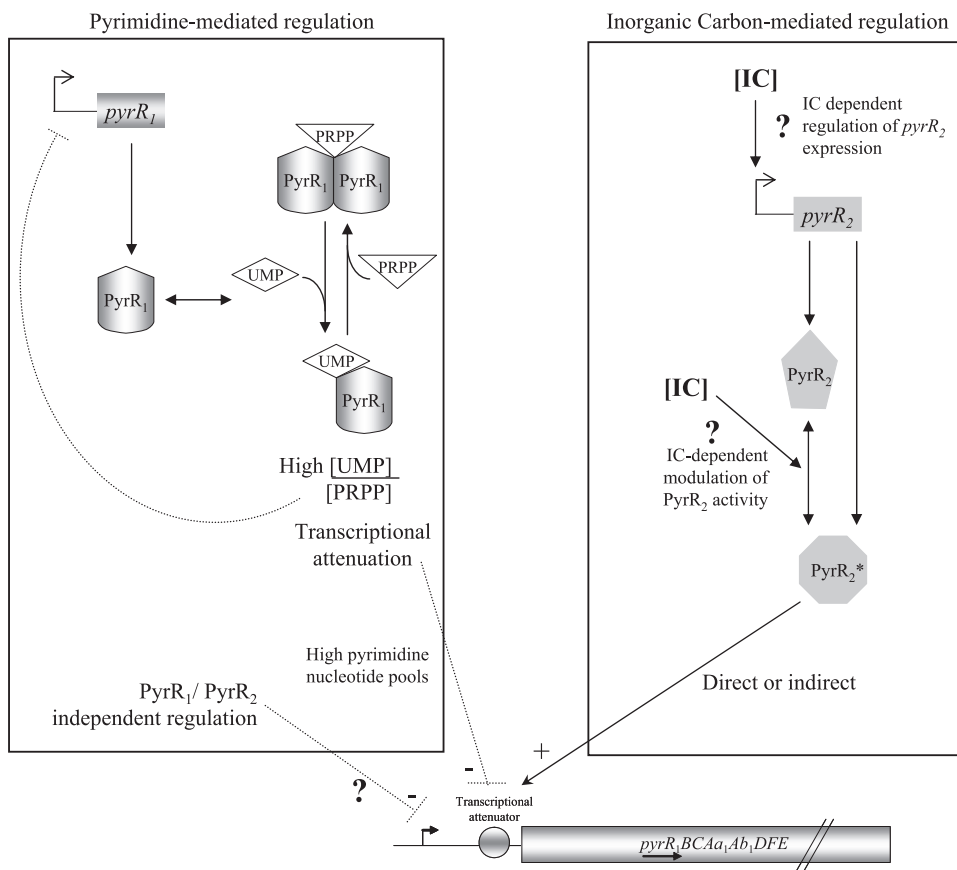


FIG. 5. Model for PyrR<sub>1</sub> and PyrR<sub>2</sub> regulation of *L. plantarum* *pyr* regulon in response to pyrimidine availability and inorganic carbon. The regulated *pyr* genes studied include the *pyrR<sub>1</sub>BCAa<sub>1</sub>Ab<sub>1</sub>DFE* operon and *pyrP*. The gray circle schematizes *pyr* gene *cis* transcription regulatory elements that are involved in response to pyrimidine availability and IC at the DNA or the mRNA level. The activity of the RNA-binding PyrR<sub>1</sub> protein is regulated by binding to antagonist effectors such as 5-phospho-D-ribose-1-pyrophosphate (PRPP) and UMP. The UMP-PyrR<sub>1</sub> complex binds to an attenuation site of the *pyr* mRNA, leading to terminated transcription (19). Another unknown mechanism independent of PyrR<sub>1</sub> and PyrR<sub>2</sub> activity operates under conditions of elevated intracellular pyrimidine nucleotide levels. IC regulation may occur at the level of *pyrR<sub>2</sub>* expression or the gene product activity. PyrR<sub>2</sub>\* represents the functional PyrR<sub>2</sub> regulator.

**Only PyrR<sub>2</sub> is involved in the IC-mediated regulation of *pyr* gene expression.** Analysis of knockout mutants in each gene of the two *L. plantarum* PyrR homologs shows that PyrR<sub>1</sub> is involved in the uracil-mediated repression but not in the IC response (model proposed in Fig. 5). As a *pyrR<sub>1</sub>* mutant does not lead to total loss of pyrimidine-dependent regulation (19) (Fig. 4C), another unknown mechanism controls *pyr* mRNA levels in response to intracellular pyrimidine nucleotide pools. Neither of the two pyrimidine-responding regulations involved PyrR<sub>2</sub>. It is not uncommon to find two different regulatory mechanisms that control CPS expression in response to pyrimidine availability. For instance, in *Escherichia coli*, two independent, pyrimidine-specific regulatory systems controlling *carAB* have been identified: UTP-sensitive reiterative transcription, which accounts for 30% of the repression (9), and a multiprotein-dependent regulation at the level of transcription initiation (7).

PyrR<sub>2</sub> positively regulates *pyr* gene expression in response to a decrease in IC concentrations. The involvement of a regulator belonging to the PyrR regulator family in IC-responding regulation has never been described. It is not clear how *L. plantarum* PyrR<sub>2</sub> senses the IC level or how the IC response is

mediated. A first hypothesis would be that PyrR<sub>2</sub> modulates PyrR<sub>1</sub> activity by forming heteromers, as proposed for the other regulators found at two copies in *L. plantarum*, ArgR<sub>1</sub> and ArgR<sub>2</sub>. Both ArgR<sub>1</sub> and ArgR<sub>2</sub> are required for the arginine-responding repression of the *arg* regulon, probably by forming a heterohexamer (18). PyrR homologs are known to repress gene expression by binding to RNA loops, leading to transcriptional termination. PyrR from *Bacillus caldolyticus*, *B. subtilis*, *Thermus thermophilus*, or *Mycobacterium tuberculosis* forms dimers with identically concave, basic surfaces, presumably the RNA-binding sites (6). Thus, it could be speculated that *L. plantarum* PyrR<sub>1</sub>/PyrR<sub>2</sub> form a heterodimer that is unable to promote transcriptional termination, leading to increased expression of the *pyr* operon. This hypothesis predicts that a *pyrR<sub>2</sub>* mutant would have reduced *pyr* gene transcription, as more PyrR<sub>1</sub> homodimer would be available to favor termination. Another prediction is that at low IC levels, this mutant would have altered pyrimidine-mediated regulation, but such phenotype was not observed. Reciprocally, in a *pyrR<sub>1</sub>* mutant, a loss of the IC response would be expected but was not observed. Since an important clue came from the observation that the IC dependent regulation is independent of PyrR<sub>1</sub>, if a



PyrR<sub>1</sub>/PyrR<sub>2</sub> heterodimer were formed, it would have no major role in IC-mediated or pyrimidine-mediated regulation. PyrR<sub>2</sub> is highly similar to PyrR<sub>1</sub>, suggesting that they both recognize the mRNA. Since PyrR<sub>2</sub> acts as an activator, one could speculate that PyrR<sub>2</sub>, instead of stabilizing the anti-antiterminator loop as PyrR<sub>1</sub>, prevents the formation of the anti-antiterminator, thus favoring antitermination.

Our model proposes that PyrR<sub>1</sub> and PyrR<sub>2</sub> regulate the expression levels of *pyr* genes by two independent mechanisms (Fig. 5). Two possible mechanisms are proposed to explain the contribution of PyrR<sub>2</sub> to the increased expression of the *pyr* regulon at low IC levels. In addition to the above-proposed model in which PyrR<sub>2</sub> directly prevents the formation of the anti-antiterminator, PyrR<sub>2</sub> could indirectly modulate the transcription of the *pyr* genes, by modulating the expression of a regulator specifically dedicated to IC response, which in turn would regulate *pyr* gene transcription. To date, gene transcription in response to IC in bacteria involves transcription activators: regulators of the LysR-family, such as CbbR (14) and AtxA (8, 16), or two-component signal transducing systems, such as MgA (15). Since PyrR homologs have been described as repressors (25), PyrR<sub>2</sub> may be a repressor that controls the expression of another repressor when IC is present at low concentrations.

Based on this model, a double *pyrR<sub>1</sub> pyrR<sub>2</sub>* mutant would have the HCR phenotype with reduced *pyr* gene expression independently of the IC concentration in the media. Moreover, *pyr* genes would be expressed even in the presence of uracil. As previously stated, residual pyrimidine regulation is observed in the *pyrR<sub>1</sub>* mutant. Therefore, the levels of *pyr* gene expression in the *pyrR<sub>1</sub> pyrR<sub>2</sub>* double mutant would be similar in the presence and in the absence of IC, whereas the residual pyrimidine regulation would still be present. Further experiments are required to identify the molecular mechanism of PyrR<sub>2</sub>-mediated regulation in response to IC concentration.

**Is IC-mediated regulation of the *pyr* operon impaired in HCR auxotrophs?** IC availability is a limiting parameter for pyrimidine and arginine metabolism in lactobacilli since HCR prototrophs were commonly isolated in natural environments (5). These isolates require high CO<sub>2</sub> levels in the absence of arginine and pyrimidine for their growth. Under low-IC conditions (ordinary air), it was assumed that the CPS-A activity was too low to produce enough carbamoyl phosphate for arginine or pyrimidine biosynthesis pathways, and the CPS-P produced the majority of carbamoyl phosphate for both pathways (20). Recently, mutations were introduced in the regulatory region of the *pyr* operon, encoding the CPS-P. These mutations led to constitutive repression of *pyr* gene expression (19). The resulting strain (strain AE1023) had an HCR phenotype. Therefore, it was proposed that constitutively low *pyr* gene expression prevented growth at low IC concentrations. In this work, we have shown that *pyr* gene expression is higher when IC concentration is low. One could suppose that under this condition, the level of the CPS-P enzymatic reaction is lower because the concentration of one of its substrates is reduced. Therefore, the amount of the CPS-P enzyme required to produce enough carbamoyl phosphate would be larger under conditions of low IC than under those of enriched IC. Reduced *pyr* gene expression under low-IC conditions (as, for example, in mutant AE1023) reduces CPS-P amounts, result-

ing in a shortage of the carbamoyl phosphate required for arginine and pyrimidine biosynthesis, which confers the HCR phenotype. In this work, we have shown that PyrR<sub>2</sub> is required to reach maximal transcriptional efficiency when the IC level is low. Moreover, the  $\Delta$ *pyrR<sub>2</sub>* mutant has the HCR phenotype with a reduced amount of CPS-P compared to that in the wild type strain when the IC level is low. All these observations are in agreement with the hypothesis that reduced carbamoyl phosphate pools due to low *pyr* gene expression would lead to the HCR phenotype. Therefore, the impaired integrity of the *pyrR<sub>2</sub>* gene as well as the activity of the PyrR<sub>2</sub> protein in the HCR auxotrophs may be the molecular basis that confers the HCR phenotype in some auxotrophs isolated from natural environments (5).

In this work, we demonstrated that the two copies of PyrR homologs in *L. plantarum* were functional and responded to different stimuli. To unravel microbial adaptation to IC environment, future work should investigate the role of PyrR-like proteins in microorganisms with several PyrR homologs, as detected not only in the genome of *L. plantarum* but also in a few other gram-positive species (data not shown), all found so far to belong to the *Lactobacillus* genus, such as *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, and *Lactobacillus johnsonii*.

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