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Role of inorganic carbon in lactic acid bacteria metabolism

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Abstract – Capnophiles are bacteria stimulated by bicarbonate and CO_2, the two major forms of inorganic carbon (IC) in physiological neutral liquids. Capnophiles are often pathogenic heterotrophs found in IC-rich ecological niches such as human cavities. Like capnophiles, the growth of lactic acid bacteria (LAB) such as *Lactobacillus plantarum* and *Enterococcus faecalis* is stimulated by IC. CO_2 or HCO_3^- are substrates in carbamoyl phosphate (CP) synthesis and other carboxylation reactions in amino acid and nucleotide biosynthesis. When media were supplemented with nucleotides and all the amino acids, potassium bicarbonate still stimulated *L. plantarum* growth. This suggests that IC may be involved in other aspects of *L. plantarum* physiology besides its implication as a substrate in carboxylation reactions. Carbonic anhydrase (CA) catalyses the hydration of CO_2 into bicarbonate. Since inorganic carbon stimulated *L. plantarum* growth, we searched for CA encoding genes in LAB genomes. CA can be classified into three classes according to their protein relatedness: α, β and γ. A class α CA was found in the *L. plantarum, Leuconostoc mesenteroides, Streptococcus thermophilus, Oenococcus oeni, Enterococcus faecalis* and *Enterococcus faecium*. These enterococci harboured a second CA encoding gene belonging to the γ class. No CA encoding gene was found in the *Lactococcus lactis* genome. These observations are discussed with regard to LAB evolution and ecological niches, which are often rich in IC.

Lactic acid bacteria / *Lactobacillus plantarum* / carbon dioxide / bicarbonate / carbonic anhydrase

Résumé – Effet du carbone inorganique sur le métabolisme des bactéries lactiques. Le carbone inorganique est trouvé principalement sous deux formes dans la plupart des milieux biologiques, le CO_2 (sous forme de gaz ou dissous) et le bicarbonate (HCO_3^-). Le carbone inorganique stimule ou inhibe la croissance d'un certain nombre d'organismes. Le terme de capnophiles désigne les bactéries dont la croissance est facilitée ou nécessite des concentrations de CO_2 plus élevées que celle de l'air. Ces bactéries à Gram négatif ou positif, chimio-organotrophes hétérotrophes, sont souvent trouvées dans la flore commensale ou pathogène de l'homme, et présentent un métabolisme aérobie strict ou anaérobie facultatif. Comme les capnophiles, les bactéries lactiques sont retrouvées dans divers environnements, souvent enrichis en carbone inorganique (tractus intestinal et vaginal, végétaux en décomposition ou fermentés). Le CO_2 stimule la croissance de certaines bactéries lactiques (*Lactobacillus*, *Enterococcus faecalis*) et de bactéries relativement proches phylogénétiquement (*Streptococcus pneumoniae*). Mais le rôle du carbone inorganique dans le métabolisme de ces bactéries a été très peu étudié. On considérait que l'effet du CO_2 chez ces hétérotrophes s'expliquait par son rôle de substrat dans les réactions de carboxylation. En analysant en détail l'effet du CO_2 et du bicarbonate (HCO_3^-) sur sa croissance, nous avons pu proposer que les réactions de carboxylation seules n'expliquaient pas l'effet du carbone inorganique sur la croissance de *L. plantarum*, bactérie lactique que nous avons définie comme capnophile.

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Une stimulation de la croissance a été observée à la fois par enrichissement en CO_2 dans la phase gazeuse ou par ajout de HCO_3^- dans le milieu. Nous avons recherché la présence de l'anhydrase carbonique (CA), une enzyme ubiquitaire chez de nombreux organismes, qui catalyse l'hydratation du CO_2 en HCO_3^-. Trois classes, α, β et γ sont définies à partir de leur homologie de séquence. Nous avons trouvé en recherchant des séquences présentant des homologies significatives avec des CA connues, des gènes codant pour une anhydrase carbonique de classe α chez *L. plantarum, L. mesenteroides, Oenococcus oeni, Streptococcus thermophilus, E. faecalis* et *E. faecium*, ces deux derniers organismes possédant également une anhydrase carbonique de classe β. Aucune ORF ne présentant une homologie significative avec une anhydrase carbonique n'a été trouvée chez *Lactococcus lactis*. Cette observation nous conduit à proposer que certaines bactéries lactiques ont évolué vers d'autres stratégies d'hydratation du carbone inorganique que celles impliquant la CA.

Bactérie lactique / *Lactobacillus plantarum* / gaz carbonique / bicarbonate / anhydrase carbonique

1. INTRODUCTION

Inorganic carbon (IC) corresponds to CO_2 as a gas (CO_2(g)) or dissolved in the aqueous phase (CO_2(aq)), and its hydrated or dissociated forms (HCO_3^-, CO_3^{2-} and H_2CO_3). The concentration of inorganic carbon and the ratio of the IC species vary in different environments. In the atmosphere, CO_2 concentration (expressed in % or ppm: part per million) is much lower today (360 ppm) than it was 3 billion years ago when the first bacteria, low G+C % Gram-positive bacteria, appeared [14]. The CO_2(g) concentration in the soil is higher (>0.1%, 1000 ppm) than in the atmosphere [10, 36]. In aqueous phases, CO_2(g) can dissolve in CO_2(aq) according to the following equilibrium:

$$CO_2(g) + H_2O \leftrightarrow CO_2(aq).$$

The CO_2(aq) concentration ([CO_2]aq expressed in mol·L^{-1}) varies with CO_2 solubility as expressed by Henry's law:

$$[CO_2]aq = K\,\rho CO_2$$

where K depends on the medium and the temperature, and ρCO_2 is the partial pressure of CO_2 in the gas phase (expressed in atm, 1 atm = 10^6 ppm). When the partial pressure of CO_2 is kept constant, CO_2 hydration and dissociation in water is a function of the pH and other factors such as salinity [35].

$$\underset{}{CO_2(aq) + H_2O} \underset{(1)}{\leftrightarrow} \underset{}{H_2CO_3} \underset{(2)}{\leftrightarrow} HCO_3^- + H^+ \underset{(3)}{\leftrightarrow} CO_3^{2-} + 2H^+.$$

It is analytically difficult to distinguish between the species CO_2(aq) and H_2CO_3. For this reason, the term CO_2(aq) is often used to express the sum of CO_2(aq) sensu stricto and H_2CO_3. However, the equilibrium for reaction (1) favours CO_2(aq) formation (ratio of [CO_2]aq/[H_2CO_3]: 1/1000). In most biological conditions, pH values are less than 8, so that the reaction (3) is inefficient, and the concentration of carbonate ions (CO_3^{2-}) may be neglected. The sum of CO_2 (aq) and HCO_3^- concentrations is considered to correspond to the total IC concentration in most biological systems.

IC is used as a carbon source by autotrophs, as an electron acceptor for methanogens, and as a substrate by almost all organisms for several carboxylation reactions such as anaplerotic reactions, amino acids or pyrimidine biosynthesis pathways. Lactic acid bacteria (LAB) are heterotrophic Gram-positive bacteria with a low G+C% content. Variable concentrations of inorganic carbon are found in LAB ecological niches. Many fermented products contain high levels of inorganic carbon due to CO_2 production by LAB or other microorganisms. When associated with CO_2-producing

eukaryotes, as in the vicinity of intestinal epithelium, LAB are also exposed to rich IC amounts. For example, in the human epithelial lumen, the IC concentration is estimated to be 20.6 $\mu mol \cdot L^{-1}$ [7, 18]. LAB associated with plants also encounter daily variable exposure to IC concentrations.

Carbonic anhydrase (CA), which catalyses the reversible hydration of CO_2 to bicarbonate (reactions (1) and (2)), is a ubiquitous, essential enzyme. Based on sequence similarities, three classes of CA have been found in a lot of bacteria and Archaebacteria. Some bacteria possess more than one CA encoding gene [39, 40], which may or may not belong to the same class. This enzyme seems to have appeared at the beginning of life on earth [40]. Thus, the conversion of CO_2 to bicarbonate is important for prokaryote and eukaryote survival and may be implied in their central metabolism.

High or low levels of IC concentrations induce different responses in heterotrophic bacteria. High atmospheric CO_2 concentration inhibits microbial growth, although the extent of the inhibition depends on the microorganism and on growth conditions such as the culture medium (for a review see [6]). CO_2 alters membrane properties, probably modifies the intracellular pH, and interferes with carboxylation reactions. As a consequence, CO_2 is effectively used in foodstuff preservation against bacterial spoilage. On the other hand, a low concentration of IC is necessary for the growth of several organisms [28, 32–34, 45–47]. CO_2 alone stimulated LAB growth and in particular *L. plantarum* growth [13]. However, to our knowledge, the effect of non-lethal inorganic carbon concentration on LAB metabolism has never been analysed in detail. Recently, Amanatidou et al. [2] studied the effect of CO_2 and O_2 enrichment or depletion in the gas phase on the growth of several microorganisms including three LAB: *L. lactis*, *Leuconostoc mesenteroides* and *L. plantarum*. CO_2 was found to stimulate growth of *L. plantarum* but not that of *L. lactis* or *L. mesenteroides*. In this study, the effect of CO_2 was not studied alone but in combination with O_2.

For autotrophs, the role of CO_2 in metabolism has been extensively studied (for a review [37]). For heterotrophs, in 1941 Krebs described that CO_2 was required in *Escherichia coli* for carboxylation reactions such as amino acid biosynthesis pathways and anaplerotic reactions [22]. In *E. faecalis* and *L. mesenteroides*, some auxotrophies were suppressed when strains were incubated in a CO_2-enriched atmosphere [24, 25]. In *L. plantarum,* auxotrophies for phenylalanine, arginine, tyrosine and pyrimidines were observed in several strains incubated under normal air but not when incubated in a CO_2-enriched air [4, 25, 29]. CO_2 may be required in these organisms for carboxylation reactions like in *E. coli*. However, in *E. coli,* the positive effect of CO_2 could not be restricted to the carboxylation reactions [21]. In this enterobacteria, cyanase catalyses the transformation of cyanate with bicarbonate to give NH_4 and CO_2, which diffuse out of the cell [8]. A CA deleted mutant would not grow in the presence of cyanate because of a depletion of cellular bicarbonate. The authors attempted to relieve this growth inhibition by adding metabolites whose synthesis are known to depend on carboxylation reactions, but the growth inhibition could not be completely overcome. In *L. plantarum*, it was proposed that IC enrichment was required for efficient carbamoyl-phosphate synthetase (CPS) activity [29]. However, as demonstrated in *E. coli*, CO_2 could be important for LAB metabolism besides the sensu stricto carboxylation reactions.

This study focused on how CO_2 stimulates growth in LAB. We analysed the effect of both increasing ρCO_2 in the gas phase and addition of bicarbonate to the medium, on the growth of *L. plantarum* in several media. Our data suggested that in both cases an effect on *L. plantarum* metabolism was observed. Genome databases were

analysed for the presence of carbonic anhydrase encoding genes in *L. plantarum* and other LAB whose genomes have been sequenced. Such genes were found in all LAB except *Lc. lactis*. The consequences of these observations will be discussed.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

The CCM 1904 and NCIMB 8826 *L. plantarum* strains were grown on rich defined medium, DLA [5], or minimal medium [26], complemented or not with arginine and uracil at a final concentration of 50 $\mu g \cdot mL^{-1}$. Precultures were incubated overnight at 30 °C without shaking and used to inoculate 20 mL of the same medium in a Klett flask (total volume 130 mL) to obtain an initial $OD_{600\ nm}$ of 0.05. The flasks were closed with gas-tight corks to prevent CO_2 loss during shaking. A ρCO_2 of 4% (40 µatm) was obtained by injecting through the cork 4.8 mL of pure CO_2 gas with a sterile syringe. Final potassium bicarbonate concentrations between 1 and 15 $g \cdot L^{-1}$ were obtained by adding a filter-sterilised solution of 100 $g \cdot L^{-1}$ of potassium bicarbonate after autoclaving and before inoculation. To adjust the pH value to 6.5, HCL 6N was used. Cell cultures were incubated at 28 °C with shaking and growth was measured using a Klett-Summerson apparatus.

2.2. Sequence analysis

Amino acid sequences deduced from putative ORFs were compared with those of CA in the PUBmed nucleotide databank using either BLASTp or tBLASTn [1]. A significant identity was estimated if the putative protein harboured putative conserved domains found in one of the three classes of known CA. Sequence data for *Streptococcus thermophilus* were obtained from the UCL Life Sciences Institute (ISV) (Belgium) website at http://www.biol.ucl.ac.be/gene/genome/. The putative ribosome binding site and promoter were searched for using Patscan (http://ir2lcb.cnrs-mrs.fr/cgi-bin/patscan.cgi).

3. RESULTS

The effect of IC on *L. plantarum* growth was tested. The aim of our study was to test if IC-mediated stimulation was restricted to its role as a substrate in biosynthetic carboxylation reactions of amino acids and of pyrimidines. We analysed the effect of increasing ρCO_2 and adding $KHCO_3$ to the medium on *L. plantarum* growth in rich or minimal media, with different amino acid or nucleotide compositions. First, the effect of inorganic carbon as carbon dioxide gas was tested: 0.035% (as found in normal air), 4% and 10%. Secondly, bicarbonate ions were added directly to the liquid media, by adding different potassium bicarbonate concentrations. In each case, the pH was fixed at 6.5 before inoculation. *L. plantarum* growth was tested in liquid media in hermetically-closed flasks to prevent loss of inorganic carbon. The effect of inorganic carbon concentrations on two *L. plantarum* strains was tested. Strain NCIMB 8826, the *L. plantarum* whose genome has been sequenced, requires CO_2-enriched air to grow on defined media plates when arginine and pyrimidines are absent [4]. The other strain, CCM 1904 (equivalent to ATCC 8014), is prototrophic for arginine and pyrimidines and grew in normal air.

3.1. Inorganic carbon stimulated *L. plantarum* growth in the absence of arginine and pyrimidines

We measured the effect of a CO_2-enriched atmosphere on the growth of NCIMB 8826 and CCM 1904 in DLA liquid medium. This medium contains all the amino acids except arginine and pyrimidines. As expected from its phenotype on agar-plates, in the absence of arginine and pyrimidines (uracil), NCIMB 8826 grew only when the

atmosphere was enriched with CO_2. A growth rate of 0.41 h^{-1} was obtained when air was supplemented with 4% CO_2. In the DLA media, growth of the prototroph CCM 1904 occurred at all the tested gas phase CO_2 concentrations. With a ρCO_2 of 0.035% as found in normal air, a specific growth rate of 0.33 h^{-1} was obtained. With 4% or 10% ρCO_2, the growth rate was 0.41 h^{-1}. Increasing the CO_2 tension significantly stimulated the growth of both *L. plantarum* strains. The effect of variable $KHCO_3$ concentration (from 0 to 15 $g \cdot L^{-1}$) on strain CCM 1904 growth, in the same medium (in the absence of arginine and uracil) was measured to determine the growth rate, the lag phase and the maximal yield (Tab. I). In defined rich media, DLA (Fig. 1 and Tab. I), and in minimal media, MM (data not shown), without arginine and pyrimidines, CCM 1904 growth depended on bicarbonate concentration. The best yield at the stationary phase and the optimal growth rate were observed in DLA with bicarbonate concentration of 2 $g \cdot L^{-1}$ (Fig. 1). In minimal medium, optimal growth was at 2 $g \cdot L^{-1}$ $KHCO_3$ (data not shown). To test if the bicarbonate-mediated growth stimulation in *L. plantarum* was pH-dependent, we analysed the effect of bicarbonate on *L. plantarum* growth rates at different pH between 5 and 7 in DLA. The tested pH had no effect on bicarbonate growth stimulation (data not shown).

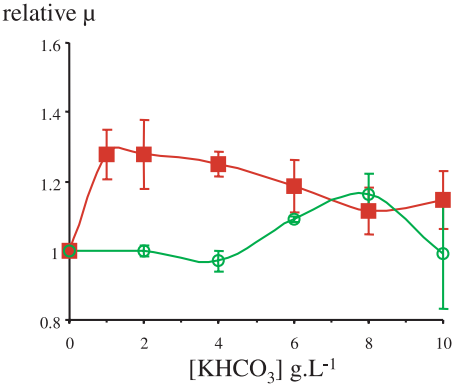

Figure 1. Effect of the $KHCO_3$ concentration on the growth rate (µ) in *L. plantarum* CCM 1904. The growth rate (µ) obtained in the absence of $KHCO_3$ was used as a reference to express the relative µ (µ obtained in the presence of $KHCO_3$ divided by µ obtained in the absence of $KHCO_3$). Experiments were performed in defined rich medium DLA. Open circles: in the presence of arginine and pyrimidines; closed squares: in the absence of arginine and pyrimidines. The data represent the mean value of at least 4 independent experiments.

3.2. Addition of potassium bicarbonate stimulated *L. plantarum* growth in media supplemented with arginine and uracil

IC stimulation of *L. plantarum* growth may be explained by the requirement of CO_2 as a substrate for the carboxylation reaction of the CPS (EC 6.3.5.5) involved in both arginine and pyrimidine biosynthesis pathways [29]. To test this hypothesis, we analysed the prototroph CCM 1904's growth with respect to IC supply, in the presence of arginine (A) and uracil (U) in different media: minimal medium, MM, or rich defined medium, DLA. Bicarbonate concentrations had no effect on the lag phase (data not shown). Growth stimulation depended on bicarbonate concentration: an optimal effect was obtained at 1 to 2 $g \cdot L^{-1}$ of bicarbonate in minimal medium, complemented with A (data not shown), and at 8 $g \cdot L^{-1}$ in DLA complemented with A and U (Fig. 1 and Tab. I). When the cultures were performed in the open air and not in hermetically-closed flasks, growth was not stimulated because bicarbonate was transformed into CO_2 which subsequently diffused into the air. Thus, even in DLA supplemented with A and U, and in the presence of all the 20 common amino acids and pyrimidines, bicarbonate stimulated *L. plantarum* growth. The Ymax obtained in the stationary phase was reduced at 8 $g \cdot L^{-1}$ of $KHCO_3$ as compared with culture with less than 8 $g \cdot L^{-1}$ of $KHCO_3$. This was observed in DLA, independent of

Table I. Effect of bicarbonate on *L. plantarum* growth in DLA medium complemented or not with arginine and uracil.

Medium	$[KHCO_3]$ ($g \cdot L^{-1}$)	μ (h^{-1})	Ymax *
DLA	0	0.34 ± 0.05	100
	2	0.44 ± 0.06	107 ± 1
	8	0.40 ± 0.05	93 ± 4
DLA+AU	0	0.46 ± 0.04	100
	2	0.49 ± 0.03	100 ± 1
	8	0.56 ± 0.04	91 ± 5

Specific growth rate (μ) is the mean value of at least four independent experiments. *: maximum population density (Ymax) is the Klett unit percentage obtained at stationnary phase as compared with the 0 $g \cdot L^{-1}$ of $[KHCO_3]$ condition.

A and U's presence. Additional experiments such as viability testing would be necessary to confirm this observation.

3.3. The carbonic anhydrase enzyme in LAB

Carbonic anhydrase catalyses the reversible hydration of CO_2 to bicarbonate [39]. Since growth was stimulated in response to IC, the presence of such an enzyme is likely in *L. plantarum*. Indeed, a protein called Lp2736, homologous to carbonic anhydrase and encoded by the *cah* gene was found in the *L. plantarum* WCFS1 genome [15]. In front of the *cah* gene, a consensus for a good putative ribosome binding site and a promoter were found (data not shown). This gene encoded a 211 amino acid-long protein belonging to the α class of known carbonic anhydrase and shared more than 47% similarity with the CA of *O. oeni*, *Vibrio parahaemolyticus, Xanthomonas campestris*, and *Bacillus halodurans* (for the most homologous proteins). α class CA are not often found in bacteria. Therefore, to test if α class CA were commonly found in LAB, we searched for putative CA proteins with significant identity with known CA in LAB whose genomes have been sequenced: *E. faecalis, E. faecium, L. lactis* subsp. *lactis, Lactobacillus gasseri, L. mesenteroides* subsp. *mesenteroides* ATCC 8293, *Oenococcus oeni* MCW and *S. thermophilus* and in Bifidobacteria (*Bifidobacterium longum* DJO10A and *Bifidobacterium longum* biovar *longum*). ORFs with deduced amino acid sequences showing identity with the *L. plantarum cah*-encoded protein were found in *L. mesenteroides* subsp. *mesenteroides* (Lmes0773), *O. oeni* (Ooen0510), *E. faecalis* (EF1711), *E. faecium* (Efae2400) and *S. thermophilus* (C79-115). These ORFs shared 29, 32, 34, 25 and 31% identity, respectively with the *L. plantarum* CA. The genetic contexts of the putative CA encoding gene were compared in the different LAB (Fig. 2). We found that CA genes were linked to genes with different functions, such as regulators, transporters, catabolic or anabolic enzymes and proteases. In *L. plantarum*, the *cah* gene was found in the vicinity of a cell surface hydrolase encoding gene (Lp2737) (Fig. 2). Only in the two *Enterococcus* strains, which are closely related phylogenetically, did we detect synteny. In these cases, the *cah* gene was linked to a gene encoding a LysR family regulator (Fig. 2). In all the other LAB tested, no gene clusters in the vicinity of the CA-related genes were conserved even when they belonged to the same class of CA.

In some bacteria, more than one putative encoding CA gene was detected, and two out of three known classes of CA were found. Therefore, we analysed if CA encoding genes belonging to other classes were found in LAB. Using the *E. coli paaY* gene, two genes of the γ class CA were found in *E. faecalis* (EF2918) and *E. faecium* (Efae1549). Using the *E. coli cynT* gene or the *Oceanobacillus iheyensi* OB1097 locus, two genes of the β class CA were found in *Bifidobacterium longum* NCC2705 (*icfA*) and DJO10A. Finally, in the *Lc. lactis* subsp. *lactis* complete genome and in the *L. gasseri* partially-sequenced genome, we found no genes presenting significant homology with the

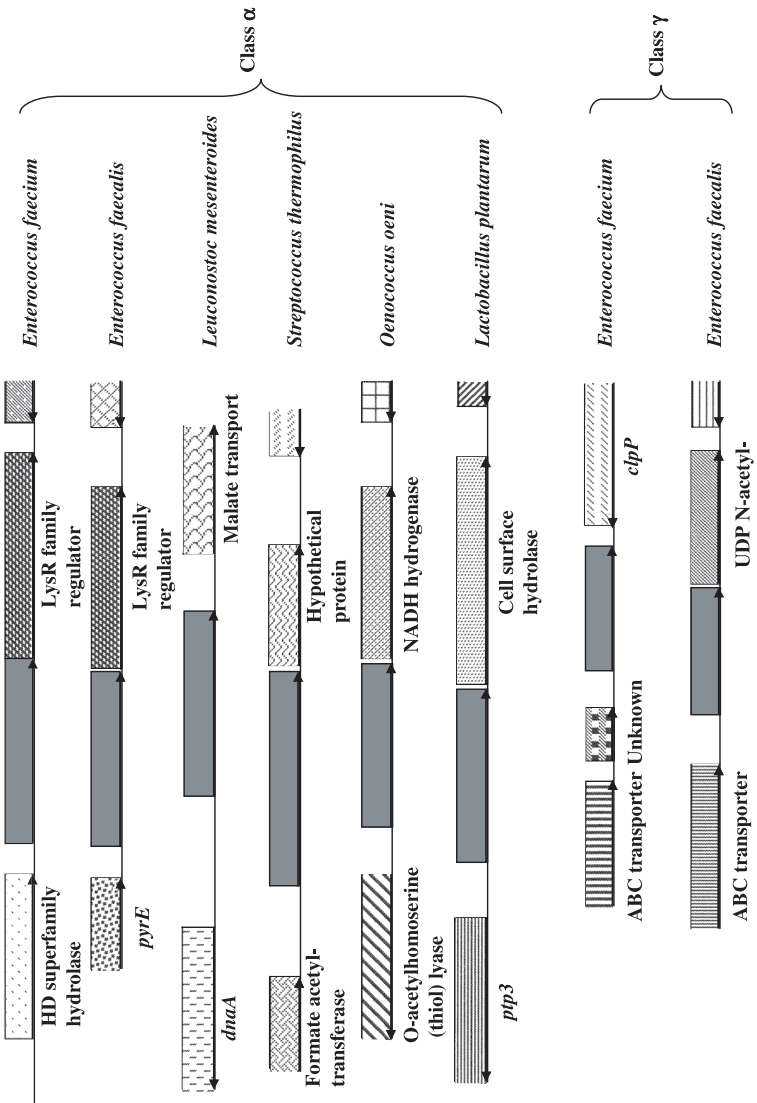

Figure 2. Genomic organisation of carbonic anhydrase encoding genes in 6 lactic acid bacteria. The carbonic anhydrase (CA) is represented by a grey rectangle. The arrows indicate the transcription orientation of the different genes schematised by rectangles. The CA class were determined by sequence homology (see Materials and methods).

three classes of CA encoding genes from a variety of origins (*L. plantarum* class α *cah* gene; *E. faecium* class γ CA encoding gene; *E. coli cynT, cah*, or *caiE*; the *O. iheyensis* OB1097 locus; or the *B. longum* β class CA encoding genes). Indeed, the absence of CA-related genes in the *L. gasseri* genome should be confirmed when its genome is completely sequenced.

4. DISCUSSION

The aim of our work was to study the effect of non-lethal IC concentration on *L. plantarum* metabolism in order to understand better how IC stimulates growth in LAB. Our results showed that either gas phase CO_2 enrichment or addition of bicarbonate increased the growth rate of *L. plantarum* in rich defined medium or in minimal medium, whether or not arginine or pyrimidines were present: 20% in the presence of arginine and pyrimidines and 30% in the absence of arginine and pyrimidines. Heterotroph bacteria were designated as capnophilic bacteria on the basis of their CO_2 growth stimulation on solid medium. Such bacteria (for example, *Capnocytophaga* and *Actinobacillus*) are pathogens found in CO_2-rich human cavities [3, 12, 38, 41]. On the other hand, heterotrophs showing a reduced lag phase in the presence of IC but no change in the growth rate, were not defined as capnophiles (case of *E. coli*) [32–34]. On the basis of the observed IC growth stimulation, we propose to define *L. plantarum* as a capnophilic bacterium.

4.1. How does inorganic carbon stimulate *L. plantarum* growth?

CO_2 is required for carboxylation reactions since mutants of CPS encoding genes in *L. plantarum* required CO_2-enriched atmosphere to grow [30]. High-CO_2-requiring natural auxotrophs are prevalent in natural isolates of *L. plantarum* without arginine and pyrimidine supplements [4]. This suggests that inorganic carbon is required as a substrate of CPS. However, in our experiments, growth stimulation was still observed in a defined rich medium containing all the amino acids and nucleotides. This suggests that IC plays a broader role than simply an intermediate in amino acid or nucleotide biosynthesis pathways. In LAB, decarboxylation reactions are important for pH homeostasis and proton motive force energy storage [19, 20]. Decarboxylation of histidine to histamine in *Lactobacillus buchneri* [27] or glutamate to γ-aminobutyrate in *Lactobacillus* sp. strain E1 [11] led to proton extrusion. It is still not clear if both species HCO_3^- and CO_2(aq) (comprising H_2CO_3) are equally required for growth stimulation. However, growth stimulation was observed at pH 5, when CO_2(aq) is the most abundant species, and at pH 7, when HCO_3^- is the most abundant species. Based on these observations, carbonic anhydrase may be involved in controlling the amounts of both species at various pH, since this enzyme converts CO_2 to HCO_3^-.

4.2. Carbonic anhydrase is not ubiquitous in LAB and may play different physiological roles

Analysis of LAB complete genomes revealed the presence of putative carbonic anhydrase (CA) encoding genes in five LAB genera: *Leuconostoc, Oenococcus, Enterococcus, Streptococcus*, and *Lactobacillus*, but not in *Lactococcus*. CA is widespread in organisms, which suggests that this enzyme plays a fundamental role in prokaryotes and in eucaryotes. Very low amino acid identity between different CA suggests that this enzyme is ancient [39, 40]. Low amino acid identity was also observed between CA from LAB (25 to 35% of identity). A single copy of a CA encoding gene of the α class was found in *L. plantarum, L. mesenteroides*, *S. thermophilus* and *O. oeni* and of the β class in Bifidobacteria. Two copies of CA encoding genes, one homologous to the α class and the other to the γ class, were found in *E. faecalis* and in *E. faecium*. The α class

CA enzymes are widespread in mammals and unicellular green algae [39, 40]. Thus, even though only a few prokaryotes harbour the α class of CA, this class of CA was always found in LAB harbouring at least one CA. α class CA of *Neisseria* or *Anaebaena* contain a peptide signal, suggesting that this protein is secreted or associated with the inner membrane [39], perhaps for the transport of inorganic carbon. A peptide signal was found in CA from *S. thermophilus,* but not from other LAB. The different classes of CA in LAB may play different roles, as suggested in other microorganisms by Smith et al. [39], although the physiological role of the CA enzyme is not clear in microorganisms. CA could be involved in CO_2 or HCO_3^- transport and modulate enzymatic reactions by variation of its substrate or product concentrations (CO_2 or HCO_3^-). In *E. coli*, cyanate degradation would require CA activity to prevent HCO_3^- depletion during this process [8, 9]. In some pathogens such as *Salmonella typhymurium*, this enzyme would be important for bacterial survival in the host [39]. The β and γ classes of CA are involved in the carbon concentrating mechanism (ccm) in photosynthetic bacteria. Genes homologous to the carboxysome components were found in two LAB studied: *E. faecalis* and *E. faecium* (data not shown). In *S. thyphimurium* [17], the carboxysome-component encoding genes are probably involved in ethanolamine catabolism, which suggests a similar role in enterococci. The genetic context of these genes may help to determine the role of these CA in LAB. However, we did not observe any conservation in gene organisation around CA encoding genes in LAB. This may reflect the fact that CA is an ancient enzyme [40].

Unlike the other LAB tested, in *Lc. lactis* no CA encoding gene was found. In other bacteria such as *Borrelia burgdoerferi*, *Chlamydia trachomatis* or *Mycoplasma pneumoniae*, no ORF with identity with known classes of CA was found [39]. This would suggest that CA is not an ubiquitous enzyme. Smith et al. [39, 40] suggested that CA could play a role in removing CO_2 produced by decarboxylation, by conversion to HCO_3^- to drive the decarboxylation reaction forward. Since these decarboxylation reactions are important in *Lc. lactis* [19, 20], we were surprised not to find any CA encoding gene in this bacterium. It is possible that in *Lc. lactis* the sequence of the CA gene has diverged from the other known CA so that we did not detect the corresponding gene. CA may also not be needed for *Lc. lactis* survival. In fact, while *Lc. lactis* is mostly found in milk products, the other LAB studied are found in more diverse environments where CA expression may be essential. In *Lc. lactis* a different strategy for CO_2/bicarbonate interconversion may have evolved. Enzymatic dosage would be necessary to confirm the absence of CA in *Lc. lactis*.

4.3. Is there CO_2-regulated gene expression in LAB?

The growth stimulation by IC in complete media suggested that CO_2-regulation would exist in *L. plantarum*. CO_2-mediated gene regulation was observed in heterotrophs [42] such as *Pseudoalteromonas* ([43], unknown function); the *cad* operon in *E. coli* [44]; and virulence genes in pathogenic bacteria such as *Bacillus anthracis*, *Actinobacillus actinomycetemcomitans*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and enteropathogenic *E. coli* [16, 42]. In Cyanobacteria, LysR-type regulators are known to regulate gene expression in response to CO_2, including genes encoding the carboxysome components [23, 31]. We found that in *E. faecalis*, two LysR regulators are clustered with a γ class CA encoding gene, perhaps in an operon. This CA may be CO_2-regulated, as observed in cyanobacteria. A homologue of this gene (Lp1857) was found in *L. plantarum.* A combination of proteomic and genetic experiments would be required to identify CO_2-regulated genes in LAB.

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