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Original article

An essential role for arginine catabolism in the acid tolerance of *Lactococcus lactis* MG1363

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Abstract – During anaerobic growth, the neutrophilic bacterium *Lactococcus lactis* produces lactic acid which leads to medium acidification until a pH of about 4.5. *L. lactis* MG1363 can develop an adaptive response to acidity commonly referred to as acid tolerance response (ATR). ATR dramatically improves the acid tolerance of the strain and can be induced by incubation at pH 5. Using two-dimensional protein electrophoresis, we found that ornithine carbamoyl transferase (ArcB) is induced during the development of ATR in chemically-defined medium. To determine its importance in acid tolerance of *L. lactis*, the corresponding gene was mutagenized. We showed that the *arc*B mutant is more sensitive to acidity than the wild-type strain. We also demonstrated that *L. lactis* displays an arginine-dependent ATR in defined medium. Altogether, our results suggest a crucial role for the arginine deiminase pathway in the acid tolerance of *L. lactis* MG1363.

***Lactococcus lactis* / acid tolerance response / two-dimensional electrophoresis / arginine deiminase**

Résumé – Rôle essentiel du catabolisme de l'arginine dans la tolérance à l'acidité de *Lactococcus lactis* MG1363. Pendant la croissance anaérobie, la bactérie neutrophile *Lactococcus lactis* produit de l'acide lactique, conduisant à l'acidification du milieu à un pH d'environ 4,5. *L. lactis* MG1363 peut développer une réponse adaptative à l'acidité couramment appelée « acid tolerance response » (ATR). Le développement de l'ATR augmente fortement la tolérance à l'acidité de la souche et peut être induit suite à une incubation des cellules à pH 5. Une analyse protéomique de l'ATR nous a permis de démontrer l'induction de l'ornithine carbamyl transférase (ArcB) en milieu chimiquement défini. Pour déterminer son importance dans la tolérance de *L. lactis* MG1363 au stress acide, le gène correspondant a été inactivé par recombinaison homologue. Le mutant obtenu est plus sensible à l'acidité que la souche sauvage. Nous avons également démontré que la présence d'arginine accroît la tolérance de *L. lactis* à l'acidité en milieu défini. Nos résultats suggèrent un rôle crucial de la voie de l'arginine déiminase dans la tolérance de *L. lactis* MG1363 au stress acide.

***Lactococcus lactis* / tolérance à l'acidité / électrophorèse bidimensionnelle / arginine déiminase**

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1. INTRODUCTION

In natural ecosystems, as well as during industrial processes, *L. lactis* has to cope with numerous environmental fluctuations. Such stress conditions may affect the growth and/or the survival of the bacterium. In the dairy industry, the lactic acid produced by *L. lactis* acidifies food products to pHs that prevent spoilage (≤4.5). Paradoxically, the acidification generated by *L. lactis* metabolism also leads to its growth arrest. Indeed, like most lactic acid bacteria (LAB), *L. lactis* is a neutrophilic bacterium, displaying an optimal pH of growth between 6.3 and 6.9 [10]. In order to survive these potentially lethal environmental modifications, *L. lactis* has evolved several adaptive strategies that allow it to survive and/or grow in such conditions.

Rallu et al. [19], Hartke et al. [9] and O'Sullivan and Condon [16] showed that several *L. lactis* strains, including MG1363, display an inducible acid tolerance response (ATR) when they encounter a moderate acid pH during logarithmic growth. ATR improves the cell survival in normally lethal acid conditions. A previous proteomic study reported that thirty-three proteins were induced at pH 5.5 in *L. lactis* [9]. Although these proteins are likely to be involved in ATR, their identities and functions still remain unknown.

Only a few of the mechanisms used by *L. lactis* to fight against intracellular acidification caused by the production of lactic acid are known. The expulsion of protons by the F_0F_1–H^+-ATPase and the K^+-ATPase contributes to pH homeostasis and tolerance to medium acidification [15, 17] and like several LAB, *L. lactis* can use specific amino acid catabolism pathways to alkalinize internal pH [23].

As a part of a proteomic analysis of the ATR of *L. lactis* MG1363, the ArcB protein was identified as acid-inducible. This observation strongly suggests that arginine catabolism contributes to the ATR of MG1363. We further demonstrated that ArcB is also involved in the intrinsic acid tolerance of MG1363 (survival to an acid challenge with no acid adaptation step). Altogether, these data establish that arginine catabolism has a major role in the acid tolerance of *L. lactis* strains which possess this pathway.

2. MATERIALS AND METHODS

2.1. Bacterial strains, media and growth conditions

L. lactis ssp. *cremoris* strain MG1363 [8] was grown at 30 °C in M17 [21] or M17/7 supplemented with 0.5% glucose or in SA medium [11], SA/7 or SA/5. M17/7 corresponded to M17 in which β-glycerophosphate was replaced by MOPS (0.2 mol·L^{-1}) and with a pH adjusted to 7.2 with NaOH. Consequently, the medium pH remains above 6.8 throughout the bacterial growth. SA/7 corresponds to the SA medium supplemented with 0.2 mol·L^{-1} of MOPS in order to ensure a pH above 6.8 during bacterial growth. SA/5 and SA/3.8 are SA/7 media which were acidified to pHs of 5.0 and 3.8, respectively, using HCl. M17/3.45 corresponded to M17/7 acidified to pH 3.45 with HCl. *Escherichia coli* strain EC101 was grown at 37 °C in 2TY medium.

For solidified media, agar was added at 15 g·L^{-1}. For *L. lactis*, Erythromycin (Em) and Chloramphenicol (Cm) were added at 2.5 μg·mL^{-1} and 10 μg·mL^{-1}, respectively. For *E. coli*, Em was added at 150 μg·mL^{-1}.

2.2. Proteomic methods

10-mL cultures of *L. lactis* strain MG1363 were maintained overnight in the logarithmic phase in SA/7 as described elsewhere (see Sect. 2.4). When cultures reached an OD_{600} of about 0.1, they were centrifuged, resuspended in SA/5 and incubated for about 120 min with 18.5 MBq [^{35}S]-protein labeling mix (43.5 TBq·$mmol^{-1}$, Nen, Les Ulis, France). Protein extraction and two-dimensional electrophoresis were carried out as described previously [2].

For protein identification, cells from a 250 mL culture in SA medium were washed with Tris 50 mmol·L^{-1}, pH 8 at 4 °C and lysed using a Cell-Disrupter (Constant-Systems, Kenilworth, UK) at 2.7 kbar. Proteins were extracted and separated by 2-DE. After Coomassie blue staining, spots were cut and washed with acetonitrile/ammonium hydrogenocarbonate 50 mmol·L^{-1} (1/1: v/v). Samples were shaken for 15 min and vacuum-dried for 30 min. Digestion of proteins was carried out using 0.5 µg trypsin (Promega, Madison, USA) in 25 µL ammonium hydrogenocarbonate 50 mmol·L^{-1}. Samples were incubated overnight at 37 °C. 2 µL TFA 5% was added to stop the trypsinolysis reaction. Peptides were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Voyager DE super STR, Applied Biosystems, Foster City, USA). The matrix used was the alpha cyano 4 hydroxy cinnamic acid (5 mg·mL^{-1}). The molecular mass range was 500 to 5000 g·mol^{-1}. Peptides obtained were compared with two databases: a specific database of *L. lactis* IL1403 (software Protein Prospector) and Genpeptides.

2.3. Oligonucleotide sequences

The oligonucleotides used to perform the amplification of the *arc*B internal fragment were arcB_F 5'-gtaactgcagtattccaaggtcgcag-3' and arcB_R1 5'-gtttggcatgctgttacgaccatcac-3'. The annealing temperature was 58 °C and the elongation time was 30 s. The oligonucleotides used to verify the chromosomal structure of the *arc*B mutant were UP 5'-gtaaaacgacggccagt-3' and arcB_R2 5'-gaatgctggtaagcagtgc-3'. The annealing was performed at 52 °C and the elongation time was 1 min 30 s.

2.4. Acid challenge

Stress assays were performed with cultures maintained in logarithmic growth for more than 14 h, which were obtained as follows. Dilutions (10^{-2} to 10^{-8}) of a resuspended fresh colony were used to inoculate cultures in M17/7 or SA/7 media. After overnight growth at 30 °C, the culture with an OD_{600} below 0.3 was diluted to a final OD_{600} of 0.01 in fresh medium and incubated until its OD_{600} reached 0.1. Then, cells were harvested by centrifugation and resuspended in M17/3.45 or SA/3.8. Viable cell counts were estimated by plating dilutions on M17 at To (just after resuspension, i.e. without stress) and after 45 min or 120 min. The number of colony-forming units (CFU) were determined after 24 h of incubation. The strain survival is the ratio of the CFU at 45 min or 120 min versus To.

To determine the effect of arginine on the acid tolerance of *L. lactis*, cultures were maintained in the exponential growth phase as described above in SA/7 deprived of arginine, split and incubated in SA/7 without or with 5 mmol·L^{-1} arginine, and then subjected to the acid challenge in SA/3.8.

3. RESULTS

3.1. Proteomic analysis revealed the acid induction of ArcB

In order to identify the cellular modifications allowing *L. lactis* to adapt to acidity, a proteomic analysis was carried out. Bacterial proteins were labeled with [^{35}S]-methionine in cultures grown at neutral pH (SA/7) or adapted at pH 5 (SA/5). About 450 proteins were detected on the 6 electrophoregrams corresponding to the 3 independent samples and gels used for each of the conditions (pH 7 and pH 5). Comparison of the electrophoregrams using the Image Master 2D software revealed that the synthesis rate of 54 proteins increased during the adaptation step compared with the non-adapted control (data not shown). One of these proteins, displaying an apparent molecular mass of 38.0 kg·mol^{-1} and an apparent pI of 5.3, was induced 4.4 (±1.9) times during adaptation (Fig. 1). It was clearly identified by mass spectrometry (with a molecular weight

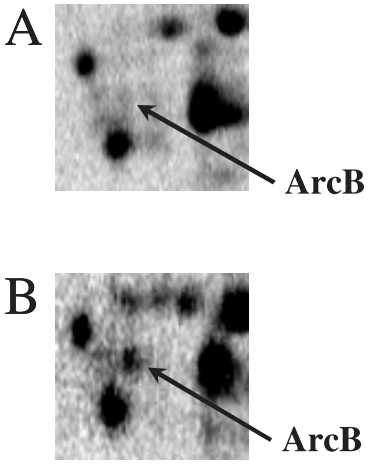

Figure 1. 2-D PAGE analysis of ^{35}S-labeled proteins of *L. lactis* MG1363 at neutral pH (A) or during acid adaptation at pH 5 (B) in SA medium. Three independent experiments were performed for each condition and comparison analysis was performed on all of them using Image Master 2D. Therefore, the two pieces of gels displayed in this figure are only indicative. The ArcB protein, which is induced in acid-adapted cells, is indicated by an arrow. Symbols: ORI indicates the plasmidic replication origin, EmR represents the erythromycin resistance gene.

search (Mowse) score of 2.10^{10}) as the ornithine carbamoyl transferase encoded by the *arc*B gene (data not shown).

To further characterize the role of ArcB in the tolerance of *L. lactis* to acidity, we inactivated the corresponding gene.

3.2. *arc*B gene inactivation

We created an *arc*B insertional mutant by using the conditional replication system pORI19-pVE6007 described by Law et al. [12]. We amplified a 460-pb DNA fragment by PCR using the chromosomal DNA of *L. lactis* MG1363 as template and the oligonucleotides arcB_F and arcB_R1 designed from the genomic sequence of *L. lactis* IL1403 [1] (Fig. 2A). This PCR fragment was cloned into the plasmid pORI19-1 in *E. coli*. The plasmid was then introduced by transformation into *L. lactis* MG1363 in which the thermosensitive plasmid pG$^+$host3 (pVE6007) had previously been introduced [13]. One of the clones, obtained at 30 °C, was grown in M17 to an OD_{600} of 0.3 and then transferred for 3 h at 42 °C for the integration of the pORI19-1_arcB into the chromosomal *arc*B gene (Fig. 2A). The culture was plated on M17 agar medium containing Em. After 48 h incubation at 37 °C, chromosomal DNA of integrants (Em-resistant clones) were analyzed by PCR using the oligonucleotides UP and arcB_R2. The size of the amplified fragment using these primers was about 850 bp, in accordance with the expected size (Fig. 2B).

3.3. Acid-sensitivity of the *arc*B mutant of *L. lactis* MG1363

Growth studies of the *arc*B mutant did not reveal any significant difference, as compared with the wild-type strain, when cultured at 30 °C in M17/7 medium. The generation time was 43 min (data not shown). This result suggested that *arc*B is dispensable for the optimal growth of *L. lactis* in rich medium.

The acid tolerance of the *arc*B mutant was assayed. Both the *arc*B mutant and the wild-type strain were cultivated in M17/7 medium and transferred at OD_{600} = 0.1 in M17 acidified to pH 3.45. The viable cell counts were estimated by plating on M17 agar at To and after 45 min of acid challenge. Under these conditions, the *arc*B mutant appeared 60-fold more sensitive to the acid challenge than the wild-type strain (Fig. 3). This result demonstrated the implication of *arc*B in the acid tolerance of a non-adapted *L. lactis* strain.

3.4. Arginine-dependent acid tolerance in *L. lactis* MG1363

The implication of arginine in the acid tolerance of *L. lactis* MG1363 was further tested. Cells cultivated in SA/7 deprived of

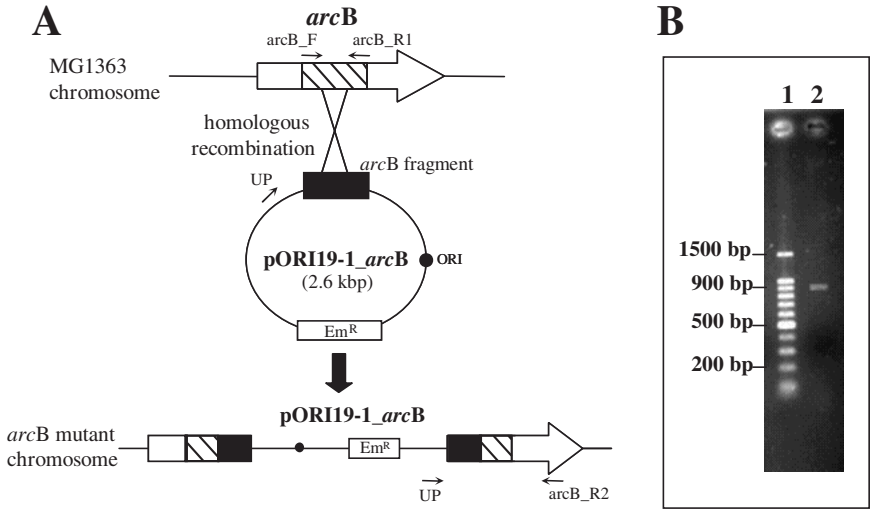

Figure 2. Single crossing-over inactivation of *arc*B. (**A**) Schematic representation of the single crossing-over integration of the vector pORI19-1_arcB (containing a 460 bp *arcB* fragment) into the chromosome of MG1363. Regions homologous to the oligonucleotides are indicated by arrows. (**B**) Verification of the chromosomal structure of the SCO mutant. Agarose gel (1.5%) electrophoresis of the PCR product resulting from the amplification of the *arc*B gene with primers UP and arcB_R2. Lane 1: Standard molecular mass marker; and lane 2: PCR product obtained with the chromosomal DNA of a SCO integrant using the UP and arcB_R2 primers. Note that amplification was not obtained using the MG1363 (wild-type) chromosomal DNA as template (not shown).

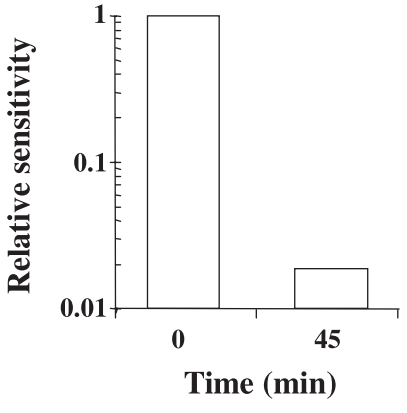

Figure 3. Sensitivity of the *arc*B mutant towards an acid challenge. The acid challenge was performed in M17 at pH 3.45. Viable cell counts were evaluated by plating on M17 agar at time 0 and 45 min after the acid challenge. The relative sensitivity corresponds to the ratio of the survival of the *arc*B mutant to the survival of the wild-type strain.

arginine were split and incubated without or with arginine (5 mmol·L^{-1}) for 1 h. Both samples were then subjected to an acid challenge (pH 3.8) in the presence or not of arginine for 120 min (Fig. 4).

Without arginine, the cell relative survival decreased from 1 to 0.09 after 120 min at pH 3.8. Interestingly, cells which were exposed to arginine displayed a survival rate almost 10-fold higher (0.85). Our results demonstrate that arginine catabolism directly contributes to the tolerance of *L. lactis* MG1363 to acidity.

4. DISCUSSION

In this study, we demonstrated that ArcB is induced during the acid adaptation of *L. lactis*. The analysis of the genomic sequence of *L. lactis* IL1403 revealed that the *arc*B gene is located downstream of

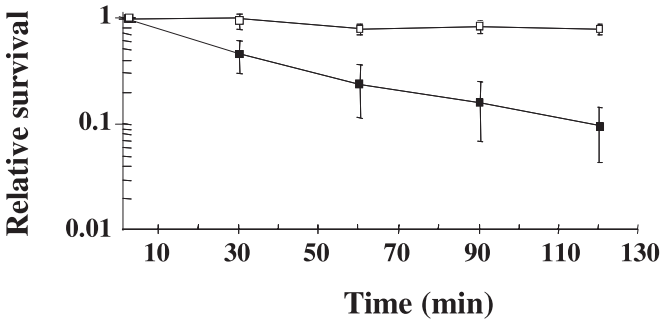

Figure 4. Relative survival of MG1363 of an acid challenge with or without arginine. The acid challenge was performed in SA without (closed symbols) or with arginine (open symbols) and acidified to pH 3.8. The viable cell counts were evaluated by plating on M17 agar at time 0 and after 30, 60, 90 and 120 min of acid challenge. The relative survival corresponds to the ratio of the number of CFU at times 30, 60, 90 or 120 min to the number of CFU measured at time 0.

*arc*A, and upstream of *arc*D1, C1, C2, T and D2 [1]. All these genes are transcribed in the same orientation, and encode putative enzymes involved in the arginine deiminase (ADI) pathway. According to homologies with characterized enzymes, arginine deiminase (ArcA) catalyzes the deimination of arginine to ammonia and citrulline, which is then converted to ornithine and carbamoyl phosphate (a step catalyzed by ornithine carbamoyl transferase (ArcB)). Ornithine is exported via an arginine/ornithine antiporter (ArcD), and the carbamoyl phosphate is dissociated by the carbamate kinase (ArcC) in ammonia and CO_2, generating 1 mol of ATP per mol of arginine consumed [18, 24]. The structure of the ADI gene cluster of *L. lactis* appears quite unusual as compared with other bacteria. Firstly, this cluster contains 2 alleles of the *arc*C gene (65.3% identity) and of the *arc*D gene (65.2% identity). Secondly, it contains an additional gene, *arc*T, which encodes a putative transaminase, previously described in the ADI gene cluster of *Lactobacillus sakei*, but not in that of other bacteria [24]. The role of ArcT in arginine catabolism still remains unknown.

The existence of this ADI pathway has previously been reported in a number of bacterial species, including streptococci and lactobacilli [3–7, 14, 20]. In most cases, this system appeared inducible by arginine but was not systematically induced by acidic pH [4, 6].

Activities of the enzymes corresponding to the ADI pathway were also detected in *L. lactis* ssp. *lactis* but not in all *L. lactis* ssp. *cremoris* [22] although it was established that it exists in the parental strain of MG1363. We demonstrate in this paper that ADI is acid-inducible. It is noteworthy that (i) Turner et al. [22] did not assay acid-stressed cells; and (ii) we detected a negligible amount of the protein ArcB in unstressed cells. As a consequence, we could infer that *L. lactis* ssp. *cremoris* uses the ADI pathway for acid resistance rather than for nutritional purpose, at least in the logarithmic growth phase, a conclusion reinforced by the absence of growth defects of the *arc*B mutant in M17/7 medium.

In spite of the absence of acid induction in some of the species studied so far [4, 6], the involvement of the ADI pathway in acid adaptation seems clear in most cases. For example, addition of arginine to acidified suspensions of *Streptococcus sanguis* prevented lethality [3]. However, to our knowledge, none of these studies included mutational analysis. The acid sensitivity of the *arc*B mutant unambiguously demonstrates

an essential role for the ADI pathway in the acid tolerance of non-adapted *L. lactis* MG1363 in M17 medium. Further experiments will determine its exact involvement in the development of ATR.

It is noteworthy that ornithine can be considered as a precursor of compounds that could participate in the development of flavors of food products, i.e., 2-acetyl-1-pyrroline [6] and polyamines. As a consequence, it would be of particular interest to determine whether the *arc*B mutation influences the technological properties of *L. lactis*. Such a dual role of the ADI pathway would make it an important element that would have to be considered in the selection schemes of technological strains.

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