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Review

Lactobacilli evolve by cumulative DNA degeneration

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Abstract – Lactic acid bacteria require rich media since, due to mutations in their biosynthetic genes, they are unable to synthesise numerous amino acids and nucleobases. The extent of genetic lesions was investigated in two biosynthetic pathways for 150 *Lactobacillus plantarum* isolates from various origins. Arginine biosynthesis and pyrimidine biosynthesis share a common intermediate, carbamoyl phosphate (CP). No pyrimidine auxotrophs were detected and only 7 *L. plantarum* strains required arginine for growth. Arginine auxotrophs were more frequently found in *L. plantarum* isolated from milk products than from fermented plant products or humans: association with dairy products might favour arginine auxotrophy. The *argCJBDF* genes were functional in most strains and when inactive, only one gene was mutated in more than half of the arginine auxotrophs. Random mutations may have generated these auxotrophs since different *arg* genes were inactivated. Analysis of the sequenced *L. plantarum* genome revealed the presence of 6 sets of duplicated genes in the arginine and pyrimidine biosynthetic pathways. Among the three copies of the CP synthetase large sub-unit encoding gene, *pyrAb2* harboured frame-shift mutations and may be a pseudogene. These data support the hypothesis that lactic acid bacteria have adapted to specific habitats by progressively losing unnecessary genes and their genome has evolved through cumulative DNA degeneration.

Lactobacillus **/ evolution / adaptation / mutation / auxotrophy**

Résumé – Évolution de certains lactobacilles par dégénérescence cumulée de l'ADN. Les bactéries lactiques nécessitent des milieux riches pour leur croissance car des mutations dans des gènes impliqués dans la biosynthèse de nombreux acides aminés, de vitamines ou de nucléobases ont invalidé leurs capacités à synthétiser ces composés. La nature et la fréquence des lésions génétiques responsables de ces auxotrophies naturelles ont été recherchées dans les gènes codant pour la synthèse de deux voies de biosynthèse, celle de l'arginine et celle des pyrimidines. Ces deux voies possèdent un intermédiaire de biosynthèse commun, le carbamyl phosphate (CP). Une collection de 150 souches de *Lactobacillus plantarum* isolées de produits fermentés et de niches écologiques diverses a été testée. Aucune souche n'était auxotrophe vis-à-vis des pyrimidines et 7 souches seulement présentaient une auxotrophie vis-à-vis de la citrulline, un précurseur de l'arginine. Les auxotrophes Arg^- étaient plus fréquemment rencontrés parmi les isolats laitiers que parmi des isolats végétaux ou d'origine humaine. Nous faisons l'hypothèse que l'association des lactobacilles aux produits laitiers favoriserait la sélection d'auxotrophes vis-à-vis de l'arginine. Les gènes impliqués dans la biosynthèse de la citrulline sont regroupés dans l'opéron *argCJBDF* et sont fonctionnels dans la plupart

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des souches étudiées. En cas d'inactivation, seul un gène est altéré dans plus de la moitié des auxotrophes Arg⁻ examinés. Dans trois cas, l'inactivation de gène résulte d'évènements de mutation ponctuelle de type transversion. Dans les quatre autres auxotrophes, des altérations non réversibles de type délétion ou insertion ont été mis en évidence. L'absence de cible privilégiée pour les mutations suggère qu'un processus de mutagenèse aléatoire est à l'origine de l'apparition de ces auxotrophes naturels. Une analyse in silico du génome séquencé de *L. plantarum* a permis la mise en évidence de 6 groupes de copies de gènes impliqués dans la biosynthèse de l'arginine et des pyrimidines. Les gènes codant l'enzyme impliquée dans la biosynthèse du CP, le précurseur des deux voies métaboliques, sont présents en trois exemplaires. Parmi les copies du gène codant la grande sous-unité de cette enzyme, le gène *pyrAb2* revêt les caractéristiques d'un pseudogène, porteur de plusieurs mutations qui changent le cadre de lecture et écourtent la protéine. Ainsi, ces résultats appuient l'hypothèse selon laquelle les bactéries lactiques et plus particulièrement *L. plantarum*, évoluent en s'adaptant à leurs habitats. Le génome de *L. plantarum* semble dégénérer par accumulation de lésions génétiques. La diversité métabolique rencontrée au sein des bactéries lactiques peut s'expliquer par ces modifications intra-génomiques. Cependant, l'apport d'information par des mécanismes de transferts horizontaux joue également un rôle crucial dans l'évolution des génomes de ces bactéries.

Bactérie lactique / évolution / adaptation / mutation / auxotrophie

1. INTRODUCTION

Genetic biodiversity is the result of exogenous gene acquisition or of intragenomic modifications. The genetic diversity found in microorganisms is essential for their survival and adaptation to continuous environmental changes and stresses. Lactic acid bacteria (LAB) are Gram-positive bacteria that have adapted to rich environments. As a result they have lost the ability to synthesise many amino acids (aa) and vitamins. Among the LAB growing in rich specialised habitats, *Lactobacillus johnsonii* is found in mammalian digestive tracts. Genome sequencing of *L. johnsonii* revealed the lack of all the aa biosynthesis pathways [8]. On the other hand, *Lactobacillus plantarum* is one of the few ubiquitous LAB species. So, *L. plantarum* may be exposed to prototrophic conditions, and subsequently might not have accumulated as many genetic lesions [11]. As a matter of fact, all genes encoding aa biosynthetic pathways were present except those of branched-chain aa biosynthesis [8]. However, 12 aa [10] were needed for *L. plantarum* to grow on defined minimal media, which suggests that some genes in *L. plantarum* genome harboured mutations. The LAB's complex nutritional needs may be the result of two opposing evolutionary processes. A primitive LAB may have had restricted metabolism and gradually acquired new enzymatic activities. Or, a chemo-organotrophic ancestor with many biochemical abilities may have evolved by progressively losing unnecessary genes upon closer association with plants, animals or humans. By analysing the genetic lesions found in naturally-occurring auxotrophs of LAB related to *L. plantarum*, we intend to characterise the first steps in the process of genome evolution towards cumulative DNA degeneration.

2. ANALYSIS OF THE NUTRITIONAL NEEDS WITH RESPECT TO STRAIN ORIGIN

The extent of genetic lesions was evaluated in a collection of *L. plantarum* strains with clear taxonomical status and from various origins [3]. The genetic lesions were examined in two biosynthetic pathways where most genes have been characterised in *L. plantarum* (Fig. 1). Arginine biosynthesis and pyrimidine biosynthesis have a

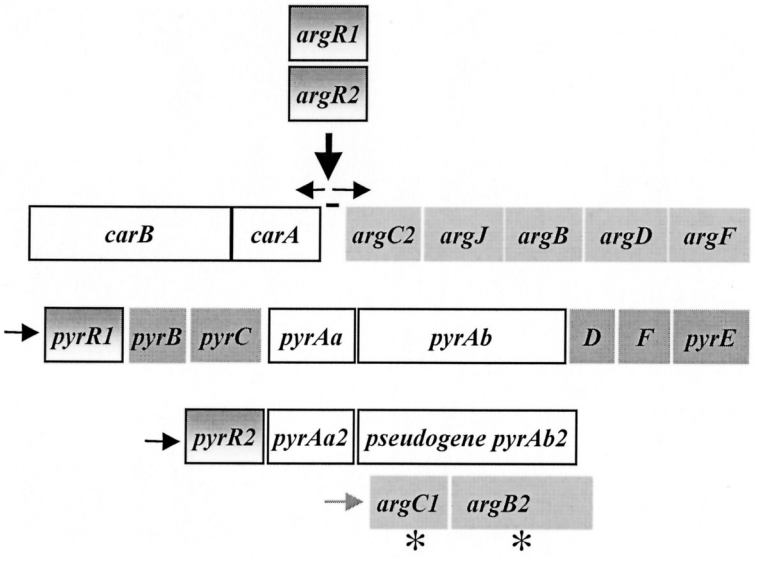

Figure 1. Genes involved in the pyrimidine and arginine biosynthetic pathways in *L. plantarum*: presence of duplicated genes, gene inactivation and strain-specific genes. The pyrimidine and the arginine biosynthetic pathways have a common intermediate: carbamoyl phosphate. Genes involved in the biosynthesis of citrulline, an arginine precursor, are clustered in two divergently transcribed operons [2], whose transcription is repressed by two repressors (ArgR1/ArgR2) in the presence of arginine [12]; and personal communication). The *pyr* operon contains the pyrimidine de novo pathway encoding genes and is regulated by transcriptional attenuation via the PyrR1 regulatory protein [5] in the presence of UMP. The sequence of the *L. plantarum* WCFS1 genome revealed two gene cluster duplications [9] with loss of synteny: [*argC1-argB2*] and [*pyrR2-pyrAa1-pyrAb2*]. The aster designs genes found in strain WCFS1 but not detected in other *L. plantarum* strains (CCM1904 and FB400; PCR data not shown).

common precursor, carbamoyl phosphate (CP). Nutritional requirement for arginine and the pyrimidines was analysed on defined media DLA agar-plates incubated at 30 °C in 4% CO_2-enriched air. Among the 150 *L. plantarum* tested, no pyrimidine auxotrophs and only seven arginine auxotrophs were detected [1]. The analysis of the nutritional needs with respect to strain origin was established on the basis of three sources of strains: fermented plant products; dairy products; and human isolates. Of the arginine auxotrophs, five originated from cheese and two from pickled vegetables (Fig. 2). The percentages of arginine auxotrophs isolated from dairy products (5 out of 25), from plant products (2 out of 89 strains) and from humans (0 out of 16 strains) were 20, 2 and 0% of the corresponding populations, respectively. Our data suggest that *L. plantarum* associated with dairy products are more likely to lose the ability to synthesise arginine than *L. plantarum* strains isolated from plant products or humans. In other LAB, the occurrence of auxotrophies was correlated with specific growth factors present in a given habitat, including orotic acid present in milk for *Lactobacillus delbrueckii* subsp. *bulgaricus* [15] and D-mevalonic acid for rice wine spoilage lactobacilli [16]. In milk, the amounts of branched-chain amino acids are growth-limiting. However, 94% of the *Lc. lactis* strains isolated from dairy products have auxotrophies for branched-chain amino acids while most strains isolated from fermented plant products are prototrophs [7]. Thus, it is not always possible

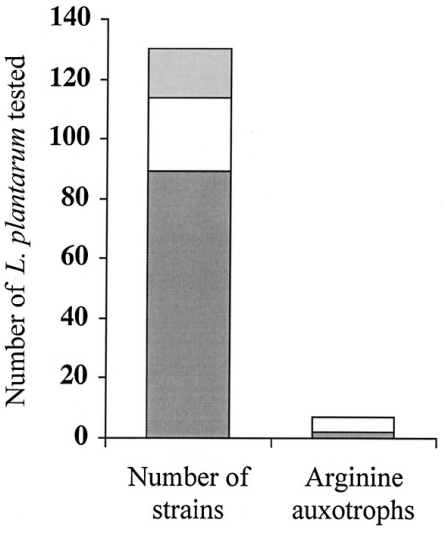

Figure 2. Analysis of the nutritional needs for arginine with respect to strain origin. Three source criteria were used: in dark grey, fermented plant products (89 strains); in white, dairy products (25 strains); and in light grey, human isolates (16 strains). The 20 strains with unknown origin were not represented on this figure. Among the 150 *L. plantarum* tested, seven arginine auxotrophs were found. All *L. casei* strains were arginine auxotrophs (data not shown).

to correlate specific LAB auxotrophy with growth factor availability. This may be the result of the numerous metabolite exchanges between organisms simultaneously present in fermented foods or ecological niches. Moreover, the metabolic routing of substrates depends on the genetic context of the cell present in a given habitat.

3. CHARACTERISATION OF NON-FUNCTIONAL *L. PLANTARUM* BIOSYNTHETIC GENES SUGGEST RANDOM MUTATIONS

To identify the mutations responsible for arginine auxotrophy, several strategies were used. The analysis of spontaneous reversion to prototrophy identified three revertible and 4 non-revertible lesions. All the arginine auxotrophs were rescued by citrulline, an arginine precursor. Genetic complementation with functional *arg* genes confirmed that the lesions were present on the *argCJBDF* cluster and not within the *argGH* genes encoding for the catalysis of citrulline to arginine. From the known sequence of an arginine prototroph (accession number X99978; *argCJBDF* sequence of strain CCM 1904 equivalent to ATCC 8014), primers were designed. The *arg* operon was PCR amplified in the seven auxotrophs and in their arginine prototrophic derivatives when available. The PCR amplified products were sequenced. These sequences were compared with a functional *arg* cluster of the prototroph CCM 1904. The data are summarised in Table I. The three arginine auxotrophs, which could revert to protrophy, harboured single transversion point mutations. These point mutations were demonstrated to be solely responsible for arginine auxotrophy by comparing the sequence found in the arginine auxotrophs and their spontaneous prototrophic derivatives. In two arginine auxotrophs, two different genes were mutated by a single point mutation, which introduced a stop codon. The resulting ArgB protein in strain FB400 and ArgF in strain CCM 3626 were truncated. Another gene was mutated by a single point mutation in strain NCFB 772, where a transversion mutation introduced a missense mutation (G333E) resulting in a non-functional ArgC. The four other arginine auxotrophs harboured non-revertible lesions. A deletion of 82 nucleotides (nt) in *argC* was identified in three strains (NCFB 963; NCFB 965; and NCFB 2171) which were considered to be isogenic based on identity of sequences, physiological parameters, and source of isolation. A 3nt insertion was found in strain KOG5 combined with missense mutations in the *argJB* genes [1]. Thus, mutations occurred in different genes, suggesting a random mutation process in the shutoff of arginine biosynthesis in *Lactobacillus*.

Table I. The molecular basis of arginine auxotrophy in seven *L. plantarum.*

Allele	# of strains	Type of mutation	Impact on protein[a]
argC2	3	82nt deletion	Protein truncation: M14-P341del
	1	Transversion G>A	Missense mutation: G333E
argB	1	Transversion C>T	Amber mutation: Q51-V248del
argD	1	3nt insertion	Amino acid addition: 47insY
argF	1	Transversion C>T	Amber mutation: Q290-N340del

[a] The numbers refer to the amino acid position in proteins of the arginine biosynthetic gene cluster in the prototroph strain CCM1904 (accession number X99978).

4. DUPLICATED GENES AND PSEUDOGENES IN *L. PLANTARUM*

L. plantarum with 3.3 Mbp has the largest of the known LAB genome. Recently, the genome sequence was completed and led to the identification of 3052 predicted proteins. Amongst these proteins, 38 were putative pseudogenes and 1443 proteins belonged to 440 multigene families [9]. From genetic and physiological studies of the arginine and pyrimidine biosynthetic pathways in the prototroph CCM 1904, we identified 14 different genes with three sets of paralogs (*pyrAa*/*carA*; *pyrAb*/*carB*; and *argR1*/*argR2*) [5, 12, 13]. Other lactic acid bacteria such as *Enterococcus faecalis* harboured up to four *argR* copies [4]. Unlike strain CCM 1904, the sequenced strain is not prototrophic for arginine and the pyrimidines in standard growth conditions. The sequenced strain WCFS1 is a clone of strain NCIMB 8826, whose growth depends on inorganic carbon supply (CO_2 or bicarbonate) in the absence of arginine or pyrimidines [1]. All the 14 genes identified in CCM 1904 were found in *L. plantarum* WCFS1 with less than 1% nucleic acid divergence. No insertion or deletion were detected in these genes which would have explained the conditional auxotrophy phenotype of strain NCIMB 8826 (data not shown).

The complete genome was explored for the presence of additional gene duplications or the presence of pseudogenes in the arginine and pyrimidine biosynthetic pathways. This approach led to the identification of two additional gene clusters; [*pyrR2-pyrAa2-pyrAb2*] and [*argC1-argB2*]. The aa identity ranged from 60% between PyrR1 and PyrR2 to as low as 24% between ArgB and ArgB2 (Tab. II). The *pyrAb2* gene may be a pseudogene. No ATG initiation codon was present. If an alternative translation starting codon (GTG) was used, a shorter protein (853 aa) than the known functional CPS large chain putative protein (about 1000 aa) would be expressed (Tab. II). Only part of the 853 aa protein has significant similarity to known CPS large chains, which suggests several frame-shift mutations. We searched for *pyrAb2* in the prototroph CCM 1904 using PCR amplifications with specific primers deduced from the corresponding locus in WCFS1. The [*pyrR2-pyrAa2-pyrAb2*] cluster was detected in both strains (data not shown). Sequencing of the amplified products is necessary to determine the *pyrAb2* divergence between the two strains.

The case of the [*argC*1-*argB2*] cluster is different. When the two proteins were compared with the proteins found in the databases, the best hits were obtained with proteins of *Archaebacteria*. These genes are part of a larger cluster (*rimK* to lp_0493), which is thought to have been acquired by horizontal gene transfer after analysis of the base frequency deviation index [9]. By using specific primers,

Table II. Comparative analysis of duplicated genes involved in the arginine and pyrimidine biosynthetic pathways in *L. plantarum*. [a] Protein names as defined in the WCFS1 *L. plantarum* genome (http://www.cmbi.kun.nl/lactobacillus). [b] Carbamoyl phosphate is synthesised by CP synthetases (CPS) (EC 6.3.5.5). CPS contains two chains: the small (or glutamine) chain (GLN) promotes the hydrolysis of glutamine to ammonia, which is used by the large (or ammonia) chain (SYN) to synthesise CP. CPS-A is the arginine-regulated CPS and CPS-P is the pyrimidine regulated CPS [13]. A third gene cluster, *pyrAa2-pyrAb2*, has been revealed by the genome sequence. [c] *pyrAb2* is a pseudogene. If the codon initiation GTG is used, a putative protein of 853 aa shared 33% identity with CarB on its first 666 aa. [d] Unlike in strain WSFS1, in strain CCM 1904, the *argC1* locus was not detected by PCR amplifications. [e] Unlike in strain WCFS1, in strain CCM 1904, the gene corresponding to lp_0488 was not detected by PCR amplifications. In strain WCFS1, lp_0488 is a bifunctional protein (EC 2.7.2.8/ EC 3.5.1.16) so that only the first 260 aa were compared with ArgB.

Gene name	Protein name[a]		Function	Protein size in aa	Amino acid identity with	%	Reference
carA	lp_0527	CPS[b] sub-units	CPS-A (GLN)	355	CarA	100	[13]
pyrAa	lp_2701		CPS-P (GLN)	364		44	[5]
pyrAa2	lp_1783		unknown	361		36	[9]
carB	lp_0526		CPS-A (SYN)	1020	CarB	100	[13]
pyrAb	lp_2700		CPS-P (SYN)	1058		62	[5]
pyrAb2[c]	lp_1784		unknown	853		33[b]	[9]
argR2	lp_1604	regulators	arginine repressor	153	ArgR2	100	Accession number AF451891
argR1	lp_1411		arginine repressor	152		35	[9]
pyrR1	lp_2704		pyrimidine regulator	180	PyrR1	100	[5]
pyrR2	lp_1782		unknown	174		60	[9]
argC2	lp_0528	other enzymes	EC 1.2.1.38	341	ArgC2	100	[1]
argC1[d]	lp_0487		unknown	339		30	[9]
argB	lp_0530		EC 2.7.2.8	248	ArgB	100	[1]
argB2[e]	lp_0488		unknown	632		24	[9]

we PCR-amplified the [*argC1-argB2*] locus in WCFS1 but not in other *L. plantarum* strains (CCM 1904 and FB400) (data not shown), which suggested that this gene transfer in WCFS1 may have occurred recently.

The regulators of both the pyrimidine and the arginine biosynthesis pathways were duplicated. The regulatory functions represent a large class (8.5%) of the total predicted proteins in *L. plantarum*, which is only similar to that found in other bacteria (*Pseudomonas aeruginosa*; *Listeria monocytogenes*) characterised by their ability to adapt [9] to many different environments.

5. CONCLUSIONS

L. plantarum is ubiquitous in a variety of rich natural niches. Few isolates of *L. plantarum* (7 out of 150 tested) have lost the ability to synthesise citrulline, a precursor of arginine. The impaired genes were identified. Since no preferred gene was inactivated, DNA degeneration by random mutation may have inactivated unnecessary genes during their adaptation to specific habitats. This may also be the case of the *pyrAb2* locus, one of the three copies of the CPS large sub-unit gene found in *L. plantarum*. Unlike the other two copies (*pyrAb* and *carB*) [13], the third copy, *pyrAb2*, may be a pseudogene, which would support the hypothesis of cumulative DNA degeneration of unnecessary genes. Adaptative mutagenesis has been documented in *L. plantarum* [11, 17]. In starved or stressed culture conditions, a small subpopulation may become hypermutable and involved in adaptive (stationary-phase) mutagenesis [6, 14]. Thus, stationary-phase stress may trigger *L. plantarum* adaptive mutation and reactivate cryptic pathways or inactivate unnecessary metabolic pathways. *L. plantarum* are found in ecological niches with abundant nutrients, complex microbial population interactions, long-term incubations and many kinds of stress. These factors favour adaptive mutagenesis and contribute to LAB biodiversity. Of course, LAB, and in particular *L. plantarum*, also evolve by acquiring "alien" genes via horizontal gene transfer between bacteria. This transfer has been highlighted in recent *L. plantarum* genome analysis [9] and our analysis of the [*argC1-argB2*] cluster in different *L plantarum* strains.

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