Putrescine producing *Lactococcus lactis*: Sequencing and transcriptional analysis of the biosynthesis gene cluster

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Abstract

*Lactococcus lactis* is a prokaryotic microorganism with great importance as a starter culture and has become the model species among the lactic acid bacteria. The long and safe history of use of *L. lactis* in dairy fermentations has resulted in the classification of this species as GRAS (General Regarded As Safe) or QPS (Qualified Presumption of Safety). However, our group has identified several strains of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* which are able to produce putrescine from agmatine via the agmatine deiminase (AGDI) pathway. Putrescine is a biogenic amine that confers undesirable flavor characteristics and may even have toxic effects. The AGDI cluster of *L. lactis* is composed of a putative regulatory gene, *aguR*, followed by the genes encoding the catabolic enzymes- *aguB*, *aguD*, *aguA* and *aguC*. These genes are transcribed as an operon, which is induced in the presence of agmatine. In some strains, an IS element interrupts the transcription of the cluster, which results in a non-putrescine producing phenotype. Based on this knowledge, a PCR-based test was developed in order to differentiate non-producing *L. lactis* strains from those with a functional AGDI cluster. The analysis of the AGDI cluster and their flanking regions revealed that the capacity to produce putrescine via the AGDI pathway could be a specific characteristic that has been lost during the adaptation to the milk environment by a process of reductive genome evolution.
Introduction

Lactic acid bacteria (LAB) play an essential role in the production of fermented dairy products, with *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus helveticus* being the species most frequently used as starter cultures. LAB are mainly used as primary starters for the production of a large diversity of dairy products all over the world. Their principal role in dairy fermentations is the rapid production of lactic acid from lactose, resulting in a lowering of the pH, and thus inhibiting the growth of spoilage and pathogenic microorganisms. In addition, they possess other metabolic activities that confer the flavor and texture characteristics of the final product.

The long and safe history of use of LAB in dairy fermentations has resulted in the majority of species being considered as GRAS (Generally Regarded as Safe) in the USA. A similar concept is used in the EU by the European Food Safety Authority (EFSA), whereby the majority of LAB species have a Qualified Presumption of Safety (QPS) status. Nevertheless, some properties or enzymatic activities can generate undesirable flavors (32) or even toxic compounds such as the production of biogenic amines (BA) (22).

BA are nitrogenous compounds that are formed mainly in foodstuffs through the decarboxylation of certain amino acids by particular bacteria. The ingestion of BA-rich foods may lead to several toxicological problems such as tachycardia, hypotension or respiratory disorders (for a review see 16). In non-fermented foods, such as fish, BA are formed by the action of contaminating gram-negative bacteria, while in fermented products (wine, meat, cheese and cider) the production is principally due to LAB (2, 14, 17, 19, 20). These BA-producing LAB can be present in the raw material or introduced during the production process (4, 18, 28). The most frequent BA in fermented dairy products are histamine and tyramine, but putrescine is also commonly detected (8). A great variability in the levels of putrescine in cheeses has been reported, with concentrations up to 0.9 g per Kg (8, 16) noted in some cases. Although non-direct toxic effects have been described for putrescine itself, it is also able to increase the toxic
effect of other BA. In addition, putrescine can participate directly in the promotion of malignancy, due to its role in the regulation of cell growth and the transformation of cells, or indirectly since it can give rise to secondary amines that can combine with nitrites to generate nitrosamines (16). Putrescine is produced from arginine through two successive catabolic reactions: decarboxylation and deamination. Depending on the order of these reactions, two different routes have been described: the agmatine deiminase (AGDI) pathway, in which arginine is first decarboxylated to agmatine and then deaminated to putrescine; and the ornithine decarboxylase (ODC) pathway, in which arginine is first deaminated to ornithine and then decarboxylated to putrescine. In wine and cider, putrescine-producing LAB using the ODC pathway have been isolated (17, 25) and the ODC enzyme has been characterized extensively (25, 27). However, no putrescine producers using the ODC pathway have been isolated from dairy products. In contrast, there is a large number of dairy LAB and enterococci belonging to different species such as Lactobacillus brevis, Lactobacillus curvatus, Enterococcus faecalis, and Enterococcus faecium (15, 17, 22) identified as putrescine producers via the AGDI pathway. Therefore, the main pathway for putrescine biosynthesis in dairy products would appear to be through the AGDI pathway (Figure 1A). To date, no putrescine-producing L. lactis has been identified. L. lactis is the LAB starter with the greatest importance as it is used as a primary starter, either in pure or mixed cultures, for the production of a large diversity of cheeses and fermented milks. Moreover, L. lactis has become a model organism among the LAB and it is the second-best studied gram-positive bacteria (12). L. lactis is used for cheese and fermented milk production due to its fast growth rate and rapid production of lactic acid in the dairy environment. In nature, this species is found on plant surfaces, from which it can reach the milk after being swallowed by ruminants. It is believed that through this route it has adapted to the dairy environment. L. lactis is divided in two subspecies, L. lactis subsp. lactis and L. lactis subsp. cremoris, with the former subspecies distinguished from the later by its ability to grow at 40°C, growth in the presence of 4% NaCl and the production of ammonia from arginine (7). Nevertheless, several strains are atypical and cannot be distinguished from each other based on these simple phenotypic assays. However, they can be classified at the genetic level by 16S rRNA gene sequence comparison since they present a 0.7% of dissimilarity (30).
In this work, *L. lactis* strains from artisanal cheeses and industrial collections were screened for putrescine production. The identification and characterization of several *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains able to produce putrescine from agmatine is described. The genes responsible for putrescine biosynthesis were sequenced and their transcription analyzed. A PCR-based test was developed for the identification of strains with a functional AGDI cluster that could be used for the selection of non-putrescine producing *L. lactis* strains.

**MATERIAL AND METHODS**

**Bacterial strains and culture conditions.**

A total of 72 strains of *L. lactis* (47 classified as *L. lactis* subsp. *lactis* and 29 as *L. lactis* subsp. *cremoris*), 30 obtained from the Christian Hansen Culture Collection (CHCC, Hørsholm, Denmark), and 42 from the Instituto de Productos Lácteos de Asturias (IPLA, Asturias, Spain) collection were included in this work (Table S1). The strains were identified and genotypically classified to subspecies level (subsp. *lactis* or subsp. *cremoris*) by comparing the 16S rRNA gene sequences using the universal primers 27F and 1492R (21) with those in the public databases. In addition, the four *L. lactis* genomes available in databases were also included in this study.

All *L. lactis* strains were grown at 30°C without aeration in M17 medium (Oxoid, UK) supplemented with 5 g l\(^{-1}\) lactose and glucose. Where indicated, 20 mM agmatine (Sigma Aldrich, St. Louis, USA) was added to the medium (M17+A). In both cases the initial pH of the medium was 6.8±0.2. *Enterococcus faecalis* BA62 (putrescine producer) was grown at 30°C in M17 (Oxoid, UK) supplemented with 5 g l\(^{-1}\) lactose and glucose, while *Lactobacillus brevis* CECT 3810, *Lactobacillus curvatus* VI14 and *Lactobacillus collinoides* IPLA1100 (putrescine producers) were grown at 37°C in MRS pH 6.2±0.2 (Oxoid, UK).
Screening for putrescine producing *L. lactis* strains

Inoculua for subsequent experiments were prepared from stock cultures of the above mentioned collections as follows. Vials were thawed, and 20 μl were used to inoculate 2 ml of M17+A, and cultures were incubated at 30°C for 24 h. Cultures were then centrifuged at 2,250 g for 10 min, 1 ml of the resulting supernatants filtered through a 0.2 μm Supor membrane (Pall, UK) and analyzed in an initial screening for the presence of putrescine by TLC according to the method of García Moruno et al. (9). Subsequently, the presence of putrescine in the TLC positive samples was confirmed by reverse-phase high performance liquid chromatography (RP-HPLC) as previously described (18). Briefly, supernatants were derivatized using dabsyl chloride, and injected onto a Waters Nova-pack C18 column in a Waters liquid chromatograph system controlled by Millenium 32 software (Waters, USA). The gradient and detection conditions were those as described previously (13).

DNA manipulation and PCR amplification

Total DNA from all the strains used in this work was isolated using a genomic DNA Purification kit (Sigma Aldrich, St. Louis, USA) according to the manufacturer’s instructions.

One microliter of this DNA was added to the PCR mix containing 5 μl of supplied PCR buffer (10x Dreamtaq Buffer, Fermentas, Lithuania), 0.2 mM dNTPs, 0.2 mM of each oligonucleotide primer, 2 units of DreamTaq DNA polymerase (Fermentas, Lithuania) and H2O in a final volume of 50 μl. PCR amplification was performed in a DNA thermal cycler (iCycler™, Bio-Rad, Hercules, USA) for 35 cycles with the general program (94 °C for 30 seconds, 25 seconds at the suitable annealing temperature of the primers used, and 72 °C for 1 min per Kb of DNA to be amplified). DNA was separated on a 0.8 % agarose gel in TAE buffer (40 mM Tris/acetate, 1 mM EDTA; pH 8.0) and...
visualized after ethidium bromide staining under UV light. PCR products were purified from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Germany).

The primer pair PTC2 and AgDdr (Unpublished results; Table S2) was used to amplify by PCR (35 cycles of 94 °C for 30 seconds, 50 °C for 25 seconds, and 72 °C for 3 min) a 2.9Kb fragment within the AGDI cluster of the L. lactis strains studied in this work (Table S1). Subsequently, the amplified fragments were sequenced using the PCR primers. The obtained sequences were used together with the sequences of AGDI clusters available in the public genome databases as a basis for further primer design for a progressive “genome walking” sequencing approach. The various PCR fragments generated were sequenced by Macrogen Inc (Korea), and assembled using Vector NTI Advance program, version 9.1 (Invitrogen, CA). Sequence analysis was performed using the University of Wisconsin Genetics Computer Group software package. BLAST and BLASTP programs were used to determine the similarities of the deduced amino acid sequences with those present in the databases. Analysis of transmembrane elements was performed with the TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM/; Danish Technical University, Denmark).

AgmSq1 and AgmSq2 primers pair designed in the present work were used to screen for the presence of the AGDI cluster in a battery of LAB strains (Table 1) using the PCR mix composition mentioned above and the following PCR program: 35 cycles of 94 °C for 30 seconds, 52 °C for 25 seconds, and 72 °C for 2 min.

**Northern blot**

*L. lactis* subsp. *lactis* T1-48 and CHCC 1915 cells were grown in M17 or M17+A to stationary phase and harvested by centrifugation in a refrigerated benchtop microcentrifuge (Eppendorf, Germany) at maximum speed. Total RNA was purified using a RNA Kit (Qiagen, Germany). Cells were mechanically disrupted using glass beads (≤ 106 µm; Sigma, St. Louis, USA) and the recommended Qiagen buffer (Qiagen, Germany). The tubes were then vigorously shaken three times for 1 min at high speed in a bead beater (FastPrep-24 system, MP Biomedicals, Illkirch, France). RNA was
subjected to electrophoresis as previously described (3). Transfers and hybridizations were performed using standard protocols (31). DNA probes, obtained by PCR using the primers described in Table S2, were radio-labeled by nick translation, incorporating [$\alpha$-$^{32}$P] dATP nucleotide (Perkin-Elmer, Covina, USA) and the signal was obtained from a Typhoon TRIO (GE Healthcare, Piscataway, USA) after exposure to a storage phosphor screen.

Reverse transcription PCR (RT-PCR)

*L. lactis* cells were grown in M17 and M17+A and harvested in the same conditions described previously. Total RNA, was extracted using TRI Reagent (Sigma Aldrich, St. Louis, USA) as previously described (23). RNA samples (2 µg of total RNA) were treated with 2 U of DNaseI (Fermentas, Lithuania) to eliminate any DNA contamination. Then, cDNA was synthesized from total RNA using a High capacity cDNA reverse transcription kit (Applied Biosystem, CA, USA) with specific primers for each of the analyzed genes (Table S2). PCR reactions were performed using 2 µl of the cDNA preparation and 0.4 µM of each gene specific primer (Table S2). Amplifications were performed for 35 cycles (94 ºC for 30 seconds, 50 ºC for 25 seconds, and 72 ºC for 1 min), and samples were analyzed on a 0.8% agarose gel in TAE buffer. The absence of contaminating DNA was checked by non reverse-transcribed PCR, which was performed under the same conditions described above, using the corresponding RNA as template.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this paper are available at GenBank (http://www.ncbi.nlm.nih.gov) with the various accession numbers detailed in Table S1.
RESULTS

Screening for putrescine producing \textit{L. lactis} strains

From a total of 20 \textit{L. lactis} strains screened from the CHCC collection (10 \textit{L. lactis} subsp. \textit{lactis} and 10 \textit{L. lactis} subsp. \textit{cremoris}), two subsp. \textit{lactis} strains, CHCC7244 and CHCC845, gave a positive spot in the TLC analysis of the overnight M17+A culture supernatants (Figure 1B), indicating that they were able to produce putrescine from agmatine through deamination. The capability of these two strains to produce putrescine was confirmed and quantified by HPLC analysis (Figure 1C), CHCC845 and CHCC7244 strains produced 0.351 mM and 2.07 mM putrescine, respectively. This is the first report of strains of \textit{L. lactis} being able to produce the biogenic amine putrescine.

In view of this surprising result, we decided to check the presence of the agmatine deiminase gene (\textit{aguA}) in the genomes of the sequenced strains of \textit{L. lactis} of dairy origin; \textit{L. lactis} subsp. \textit{lactis} IL1403 (GenBank AE005176.1), \textit{L. lactis} subsp. \textit{cremoris} MG1363 (GenBank AM406671.1) and \textit{L. lactis} subsp. \textit{cremoris} SK11 (GenBank CP000425.1). None of these genomes had annotated the agmatine deiminase genes, although its presence had been suggested for \textit{L. lactis} subsp. \textit{lactis} IL1403 (12, 24). A BLAST search using known \textit{aguA} sequences revealed the presence of the gene in the \textit{L. lactis} subsp. \textit{lactis} IL1403 strain, but not in the two \textit{L. lactis} subsp. \textit{cremoris} strains MG1363 and SK11. In addition the \textit{aguA} gene is also present in the genome of \textit{L. lactis} subsp. \textit{lactis} KF147 (GenBank CP001834.1), a strain of vegetable origin. A sequence analysis of that genome region in \textit{L. lactis} subsp. \textit{lactis} IL1403 showed the presence of all the necessary genes for the production of putrescine via AGDI pathway, although with a IS element inserted in the cluster in the opposite orientation (Figure 2A).

To confirm the presence of the cluster in the putrescine positive strains, PCR amplifications using PTC2 and agDdr primers (Table S2) previously designed in our laboratory based on the \textit{Enterococcus faecalis} AGDI cluster genes (Unpublished results) were performed. The two producer strains (\textit{L. lactis} subsp. \textit{lactis} CHCC 7244 and CHCC845) yielded a PCR product with the expected size (2.9 Kb). \textit{L. lactis} subsp.
lactis IL1403 yielded a 1 kb larger band (3.9 Kb), which is consistent with the presence of the IS element. In the eight non-producing L. lactis subsp. lactis CHCC strains the same 3.9 Kb band was obtained. The non-producing L. lactis subsp. cremoris CHCC strains and L. lactis subsp. cremoris MG1363 gave no amplification. As expected, IL1403 and MG1363 strains did not produce putrescine from agmatine (data not shown).

In view of these results we decided to increase the number of lactococci strains analyzed by combining phenotypical (TLC) and molecular screening strategies (PCR with primer pair PTC2/agdDr). In this new survey we added 10 strains from the CHCC collection (total of 30 strains), and included 42 strains from the IPLA collection (isolated from different artisanal cheeses) (Table S1). Nineteen strains, all identified as L. lactis subsp. cremoris did not produce any PCR product and twenty four strains, all identified as L. lactis subsp. lactis, produced a 3.9 Kb PCR band similar to that of IL1403 strain; none of these forty three strains produced putrescine. However, 29 out of the 72 analyzed strains, produced the 2.9 Kb PCR band corresponding to the AGDI cluster. Putrescine production from agmatine was confirmed by TLC and HPLC in all but one of these PCR+ strains (3AA11 strain, Table S1). Among these AGDI+ strains, 21 were classified as L. lactis subsp. lactis (3 from CHCC collection and 18 from IPLA collection), and 7 strains as L. lactis subsp. cremoris (all from IPLA collection). Interestingly, the agmatine deiminase genes were not present in the previously sequenced genomes of L. lactis subsp. cremoris MG1363 and SK11.

Sequence analysis of the AGDI cluster of Lactococcus lactis

To obtain the sequence of the AGDI cluster, some primers were designed based on the sequence of L. lactis subsp. lactis IL1403, to render several overlapping PCR products that were sequenced and assembled. The whole AGDI cluster was sequenced in 6 of the putrescine-producing isolates (Table S1). All the clusters had the same genetic organization, composed of five genes in the same orientation (Figure 2A).

aguR: Is the first gene in the cluster. The translated protein showed similarities with the transcriptional regulators of the LuxR family and had the highest amino acid identity (41%) with the product of the orthologous gene present in the AGDI clusters of several
E. faecalis strains. *aguR* gene is preceded by a putative promoter and shows a putative transcriptional terminator.

*aguB*: Encodes a protein that showed similarity with putrescine carbamoyl transferases present in several bacterial species such as *E. faecalis* and *L. brevis*. The closest sequence was that of *E. faecalis* V583 (NP_814483) and showed an 80% identity. It is preceded by a putative promoter composed of very close consensus sequences.

*aguD*: This gene showed similarity with several genes encoding amino acid/biogenic amine antiporters. Analyses of the deduced amino acid sequence using the TMHMM tool predicted the presence of twelve transmembrane elements (data not shown).

*aguA*: This gene encodes the putative agmatine deiminase and its amino acid sequence presented the highest identity, 83%, with that of *E. faecalis* V583 (NP_814483).

*aguC*: The deduced protein of the last gene of the cluster has similarities with carbamate kinases belonging to AGDI clusters (56% amino acid identity with *L. brevis* ATCC27305), but also with those associated with the arginine deamination pathway (ADI). In fact, the highest amino acid identity (71%) was found with the carbamate kinase associated to the ADI cluster which is present in each of the *L. lactis* subsp. *lactis* available genomes.

The organization of the *L. lactis* AGDI cluster is unique (Figure 3A), although it did show similarities with those of other gram positive cocci such as *E. faecalis*, *Enterococcus gallinarum* and *Streptococcus mutants*. These species have the same number and order of the genes, but *aguR* is in the opposite orientation (Figure 3A). In the case of *Lactobacillus*, *Pediococcus* and *Listeria* the order is different, with the putative regulator located at the end of the cluster in the same orientation. In addition, this group has a second putative agmatine deiminase gene (*aguA2*) (Figure 3A).

These observed differences in the cluster organization are a reflection of the genetic groups, which is also evident when comparing the nucleotide sequence of the *aguA* genes of different species (*aguA1 and aguA2*) (Figure 3B). We can observe that *L. lactis* form a separate branch, and although very close, there are differences between the *aguA* sequences from *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (Figure 3B).
**IS983 interrupts the transcription of the AGDI cluster**

The presence of the IS element in the 24 *L. lactis* subsp. *lactis* strains, that rendered a 3.9 Kb PCR band, seems to be the explanation for the inability of these strains to produce putrescine from agmatine. As has been mentioned before, IS983 is located in the middle of the AGDI catabolic genes of *L. lactis* subsp. *lactis* IL1403. In addition, there are 14 other copies of IS983 spread in different regions of the chromosome. A BLAST search in databases revealed that IS983 is present only in *L. lactis* subsp. *lactis* sequences of dairy origin. We have sequenced the complete AGDI cluster in three of these non-putrescine producing *L. lactis* subsp. *lactis* strains (Table S1). The analysis of the obtained sequences revealed that in all cases the IS983 gene is located between the *aguD* and *aguA* genes and in the opposite orientation.

The fact that the IS element is located between the *aguD* and *aguA* genes but in opposite orientation suggests that the transcription of the downstream genes may be interrupted if the catabolic cluster is transcribed as an operon (a mRNA covering *aguB*, *aguD*, *aguA* and *aguC*; see Figures 1A and 2A), as seems to be the situation based on the sequence analysis. In order to verify if the catabolic genes are expressed in the same mRNA, Northern blot analysis was performed. After the hybridization of the total RNA from CHCC7244 (growth in the presence of agmatine) with two probes matching the first catabolic gene (*aguB*) and the first gene after the IS (*aguA*), a unique band of the size corresponding to the four genes was obtained (Figure 2B) confirming that they constitute a single operon. In contrast, when the total RNA was obtained from the same strain grown in absence of agmatine, no hybridization signal was obtained, indicating that the transcription of the polycistronic mRNA is induced by the presence of the substrate of the AGDI pathway.

Subsequently, to test that the presence of the IS element is responsible for the inactivation of the AGDI cluster, we analyzed by RT-PCR the transcription of a gene upstream (*aguB*), and downstream (*aguA*) of the IS element in two strains: T1-48, a putrescine producing strain, and CHCC1915 a non-producing strain harbouring the IS element. To avoid the synthesis of cDNA from other copies of the IS element from different regions of the genome or interference from transcription from the IS promoter,
the cDNA was synthesized with specific primers located in front of each gene (Table S2).

Figure 2C clearly shows that transcription of aguB is induced in the presence of agmatine in both strains, while the transcription of aguA is detected in presence of agmatine in the producer strain, whereas no transcription was detected in the IS-harboring strain. aguA expression is interrupted by the presence of the IS element, which is in fact transcribed in CHCC 1915 strain, both in the presence and absence of agmatine (Figure 2C).

Chromosomal surrounding regions of the AGDI cluster.

The presence of BA producing genes in LAB has been postulated to be a strain specific trait acquired through horizontal transfer (5, 26). However, in Enterococcus the capacity to produce tyramine and putrescine appears to be species dependent (unpublished results). In order to establish if this is the case in L. lactis, a deeper look into the genomic information and the surrounding sequences was undertaken for the genomes of IL1403, KF147, MG1363 and SK11 strains.

In L. lactis subsp. lactis IL1403 (dairy strain) and KF147 (vegetable origin) an ORF that presents similarities with the transcriptional regulators of the LytR family was located upstream the aguR gene (Figure 4). In the case of L. lactis subsp. cremoris MG1363 and SK11 (both dairy strains) without the AGDI cluster, we also found the lytR gene. A search in the four genomes showed that the first common gene downstream lytR is malG, which encodes for an ABC maltose transporter (Figure 4). These two genes are separated by two and three orfs in L. lactis subsp. cremoris strains SK11 and MG1363 respectively and by seventeen and eighteen orfs -including the AGDI cluster in L. lactis subsp. lactis strains KF147 and IL1403, respectively. In these two latter strains a NADH-dependent oxidoreductase gene (yrfB) was located downstream of the AGDI cluster. Although in the KF147 strain there are 6 additional small ORFs between this gene and aguC (Figure 4).

A PCR amplification of the adjacent regions was performed in the 53 strains analyzed in this study that have the AGDI cluster, with and without IS983. Two primers were
designed to investigate the presence of the lytR gene upstream the AGDI cluster (Table S2). The first primer was designed based on the internal sequence of the lytR gene (orf20) and a second primer (aguR1c) was designed within the aguR gene. All the strains gave lytR positive amplification upstream of the AGDI cluster. However, all the L. lactis subsp. cremoris producing strains yielded a band larger than expected. The sequence of this band revealed the presence of an amidase gene between lytR and aguR. This gene is present in the genomes of SK11 and MG1363, just downstream the lytR gene, indicating that in L. lactis subsp. cremoris putrescine-producing strains the AGDI cluster is located after the amidase gene (Figure 4). Two L. lactis subsp. lactis strains (CHCC206 and CHCC3052), which have the AGDI cluster interrupted by IS983, also produced a band larger than expected. The sequence of this band revealed the presence of a gene encoding for a putative transposase (IS982) which can also be found in other locations of the L. lactis subsp. lactis and subsp. cremoris sequenced genomes.

A second pair of primers (Table S2) was designed to test the presence of yrfB downstream the AGDI cluster, one was designed within aguC (ck3) and the second (orf3c) was designed from an internal sequence of yrfB from L. lactis subsp. lactis IL1403 genome. When PCR with these primers was performed on the 53 AGDI+ strains, 42 strains -including L. lactis subsp. lactis and L. lactis subsp. cremoris from both CHCC and IPLA collections- yielded a band of the expected size (Table S1). However, 11 strains, (9 L. lactis subsp. lactis and 2 L. lactis subsp. cremoris, all from the IPLA collection) did not produce a PCR band. In order to analyze these strains, a new primer (Lcorf4C) was designed based on the sequence of the downstream gene (LLKF_1858) of L. lactis subsp. lactis KF147 and used in combination with ck3 primer (Table S2). All but 2 of these strains yielded a band (Table S1), indicating that these strains isolated from artisanal cheeses showed higher similarity to the strains of vegetable origin. All these results together indicate that the genomic regions surrounding the AGDI cluster are highly conserved.

One of the characteristics of heterologous acquired sequences is that they have a different G+C content to that of the surrounding regions of the host species. The GC content of the AGDI cluster and that of its surrounding regions ranged from 35 to 38 %.

A percentage that is similar to that of the L. lactis species (around 35 % as calculated from the sequenced genomes available). There were only two exceptions: (i) aguR, that
presented a lower G+C content (28% in *L. lactis* subsp. *lactis* and 30% in *L. lactis* subsp. *cremoris*) and (ii) *IS983* that showed a higher content (44%), suggesting a possible different origin.

**PCR assay for putrescine producers detection in dairy products**

Different PCR methods have been described and optimized for the detection of putrescine producing LAB via the AGDI pathway (6, 24). All of them use the *aguA* gene as the target for detection. However, in dairy products this target would lead to the detection of false positives due to those *L. lactis* subsp. *lactis* strains which have the AGDI cluster interrupted by *IS983*. To avoid this problem we designed a new set of primers located in the genes surrounding the IS element, *aguD* and *aguA*, in order to distinguish the strains carrying the functional AGDI cluster from those with the non-functional AGDI cluster. In order to extend the method to other strains described as putrescine producers, belonging to different species present in fermented foods, sequences from *aguD* and *aguA* genes present in databases and those obtained in this work were used (Table 1). The alignment of the corresponding region of the AGDI clusters from *L. brevis* IOEB 9809 (AF446085), ATCC 367 (NC_008497), *Lactococcus lactis* subs *lactis* IL1403 (NC_002662), KF147 (NC_013656), T3-17 (FR856588), *Lactococcus lactis* subs *cremoris* IPLA 2A22 (FR856590), GE2-14 (FR856601), *Enterococcus faecalis* V583 (AE016830), JH2-2 (AF354231) and CNRZ1535 (FN392111) allowed the design of degenerate primers (AgmSq1 [5´-CAAGATTDTDCTGGCHTTYTCTC-3´] / AgmSq2 [5´-TTGGHCCACARTCACGAACCC-3´]). These primers were tested against a battery of LAB belonging to different genera with or without the ability to produce putrescine from agmatine (Table 1). A PCR fragment of around 700 bp was obtained in those strains known to produce putrescine. As expected, those *L. lactis* subsp. *lactis* classified as non-putrescine producers but presenting the non-functional AGDI cluster rendered a larger PCR band (1700 bp) corresponding to the addition of the IS element (Table S1). No amplification was obtained from the non-producers without the AGDI cluster (Table 1). Since this method could be used to screen large culture collections, the assay was also performed in overnight liquid cultures and from isolated colonies picked from
plates without the need for a DNA purification step. In these cases, 1 μl of an overnight culture, or from a colony previously resuspended in 50 μl of sterile water, was directly used in the PCR assay. The same amplification profile was obtained as with purified DNA as template (data not shown). The results obtained demonstrate that this test could be used for the screening of microbial collections and other isolates for putrescine producers.

Discussion

Society is increasingly concerned with the issue of food safety, with ever increasing demands from the consumer for higher quality and safer foods. Thus, a great deal of effort has been made in the development of techniques to detect contaminants, such as biogenic amines, in foodstuffs. The synthesis and accumulation of BA in food requires the presence of microorganisms with the capacity to produce these toxic compounds. In this work we report the identification of several putrescine producing *L. lactis* strains belonging to the subspecies *lactis* and *cremoris*. We describe the genetic determinants, their organization and surrounding regions. Based on this knowledge, a PCR test for the detection of putrescine-producing LAB via the AGDI pathway has been developed.

Putrescine is one of the most frequent BA detected in dairy products (8) and can be formed by the decarboxylation of arginine to produce agmatine, which in turn yields putrescine by deamination. Several species of LAB have this pathway and thus are able to produce putrescine from agmatine. Among them, two different genetic organizations of the AGDI cluster were described and it is noteworthy that the organization found in *L. lactis* strains, although resembling that of other cocci such as *Streptococcus mutants*, *Streptococcus ratti* or *E. faecalis*, is unique (Figure 3A). Several authors have suggested that the capacity to produce BA by LAB has been acquired recently through horizontal gene transfer (5, 26). However, in the case of *Lactococcus* most of the data obtained from the analysis of AGDI cluster organization and sequence indicate that the capacity to produce putrescine from agmatine is not a recent trait acquired from horizontal gene transfer, as it would be present in the genomes even before the differentiation between *lactis* and *cremoris* subspecies occurred. The data from the AGDI cluster organization in *L. lactis* reveal that it is unique among LAB, with the regulatory gene located
upstream of the cluster and orientated in the same direction as the catabolic genes (Figure 3A). A phylogenetic tree, based on *aguA* sequences, clustered the genes from *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* together and next to those of other coccii (Figure 3B). The sequence comparison shows 85% identity between the AGDI clusters from *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. This value is the same as that found between both subspecies at the genomic level (33) and it is in accordance with the hypothesis that the subspecies *lactis* and *cremoris* constitute two different genetic lineages that diverged a long time ago (11, 29). In addition, the overall G+C content of the cluster is similar to that of the genome, around 35%. All these data support our hypothesis that the AGDI cluster has been present in the genome of *L. lactis* for a long time, rather than being recently acquired. In some strains, it would have been lost during the adaptation to the milk environment or even due to an empirical selection of starters unable to produce putrescine, a compound that confers undesirable flavor.

Moreover, the cluster is located in a conserved region present in all the *L. lactis* (of available genomes analyzed), including dairy and non-dairy strains, although in different positions of the genomic map, mainly due to the chromosome inversion observed in MG1363 strain and the different genome sizes (11). The surrounding regions of the AGDI are very well conserved in all the analyzed strains. Two common genes were located in all the *L. lactis* available genomes (*lytR* and *malG*) that comprise the AGDI cluster. The *lytR* gene was located upstream in all the analyzed strains, including *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains; although in the latter case an amidase gene was found between *lytR* and *aguR*. It is remarkable in *L. lactis* subsp. *cremoris* SK11 that a remnant of the putative promoter of the *aguR* gene is still present in its chromosome after the amidase gene (Figure 4), thus indicating the loss of the AGDI cluster. The downstream region of the AGDI cluster is also well conserved although a greater variability has been observed. In fact, the distance between the cluster and the *malG* gene varies between the genomes, and is lower in the case of the *L. lactis* subsp. *cremoris* strains. Interestingly, there was a group of strains isolated from artisanal cheeses in which the downstream sequence was more similar to that of *L. lactis* subsp. *lactis* KF147, a strain of vegetable origin. This sequence was not detected in any of the strains of industrial origin; again indicating that these strains are better adapted to the milk environment. This adaptation has been related to the acquisition of properties, usually through the acquisition of plasmids, and, more important in the
The genetic difference found between the putrescine-producing and non-producing *L. lactis* subsp. *lactis* strains was due to the presence of an insertion element between *aguD* and *aguA* genes. In addition, this IS element was orientated in the opposite direction, suggesting the existence of transcription interference. RT-PCR experiments showed that the presence of the IS element, impeded the transcription of the *aguA* gene (Figure 2C) and resulted in an inability to produce putrescine. The fact that in some *L. lactis* subsp. *lactis* strains the AGDI cluster is silenced by the occurrence of one or two IS elements, and some *L. lactis* subsp. *cremonis* have even lost the cluster completely, indicates that this cluster is not necessary in dairy environments. The differences between strains such as functional AGDI cluster, IS inactivated, two IS inactivated, deleted AGDI cluster but with a residual promoter, and completely deleted AGDI cluster reflect different steps in the evolution of *L. lactis* dairy strains.

The presence of BA producing microorganisms is a negative trait that can affect the safety and quality of dairy products and therefore, methods that can rapidly detect BA producers in raw material or foodstuffs are required. Traditionally microbiological methods have been used for the detection of this capability (1). However, in the case of the detection of putrescine producers from agmatine (deamination reaction) no microbiological methods have been described so far, since all of them are based on the detection of the decarboxylation reactions (1). Culture-independent methods based on microbial DNA detection have been described for the detection of AGDI+ strains (6, 24). However, these methods are not appropriate for dairy products, since they target the *aguA* gene and therefore recognize as positive the *L. lactis* subsp. *lactis* strains with the
AGDI cluster inactivated by IS983. The PCR method described in this work targets the intergenic region between the *aguD* and *aguA* genes, allowing the discrimination of those strains that possess the AGDI cluster but do not produce putrescine.

In summary, this work describes the existence of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains with the capability to produce putrescine from agmatine. The gene cluster of this pathway has been characterized and had a unique organization suggesting that, together with other characteristics of the sequence, it has not been recently acquired by *L. lactis*. It seems that the adaptation to the milk environment and/or the selective pressure exerted because of their use as starters resulted in IS inactivation or even the loss of the AGDI cluster on dairy strains. The AGDI-PCR test described here will help to discriminate putrescine producing strains, not only for dairy products but for any LAB species, independently of the fermented food.

Acknowledgments

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References


Figure and Table Legends

Figure 1: A) Scheme for the conversion of agmatine to putrescine via de AGDI metabolic pathway, the reaction catalyze for each enzyme of the route is indicated. B) TLC analysis of the production of putrescine from agmatine in IL1403, MG1363, CHCC7244 and CHCC845 strains. C) HPLC analysis of the production of putrescine from agmatine from an overnight supernatants of CHCC7244 in M17+A.

Figure 2: Genetic organization and transcriptional analysis of the AGDI cluster in L. lactis. A) Scheme for the genetic organization of the AGDI cluster in L. lactis, the regulatory gene (aguR), the catabolic genes (aguB, aguD, aguA and aguC), the insertion element (IS) found in some strains and the putative promoters (bend arrow) are indicated. B) Northern blot analysis of the catabolic genes in the AGDI cluster, in the absence (-A) and presence (+A) of agmatine. Internal fragments of the aguB and aguA genes were used as probes. The position of the RiboRuler™ High Range RNA Ladder (Fermentas, Germany) fragments is indicated. C) RT-PCR amplification with primers designed to amplify the internal regions of the aguB, aguA and IS genes in the absence (-) and presence (+) of agmatine in T1-48 and CHCC1915 L. lactis subsp. lactis strains. MW: Molecular weight markers (GeneRuler DNA ladder Mix; Fermentas, Lithuania).

Figure 3: A) Comparison of the genetic organizations of the AGDI cluster from different bacteria. Stripped arrow, aguR; grey arrow aguA. B) Phylogenetic tree of the aguA genes from different species. Nucleotide sequences were aligned using ClustalW and the phylogenetic tree was constructed with MEGA V.4 software package using the p-distance as nucleotide substitution model. In those species with duplicated aguA genes they are identified with a number (1 or 2) at the end of the strain name.

Figure 4: Scheme for the different genetic organization of the AGDI cluster found in L. lactis. The insertion elements (IS) in the L. lactis subsp. lactis strains that possess them
and the amidase gene in the *L. lactis* subsp. *cremoris* strain that present the AGDI cluster are indicated as insertions to facilitate the comparison between clusters. Common and flanking genes within all strains are shown. Truncated horizontal lines indicate that other orfs have been located in that region. Putative promoters are indicated as a bend arrow.

**Table 1:** Origin of the sequences and strains used for the design and test of the AGDI-PCR assay. The origin of the strains, the presence of the AGDI cluster, their capacity to produce putrescine in broth and the result of the specific AGDI-PCR assays are indicated. +/-IS indicates a positive result of the PCR but unfunctional AGDI cluster due to the presence of the IS element (see text for details).
Table 1: Strains used for the validation of the AGDI-PCR test.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Origin</th>
<th>Putrescine production (TLC/HPLC)</th>
<th>AgmSQ1/2</th>
<th>Source</th>
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<tr>
<td>Lactococcus lactis</td>
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<td>CHCC &amp; IPLA</td>
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<td>J. M. Rodriguez</td>
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IS: presence of the IS element.