Sequencing and Transcriptional Analysis of the Biosynthesis Gene Cluster of Putrescine-Producing *Lactococcus lactis*[▽][†]

Victor Ladero,[1] Fergal P. Rattray,[2] Baltasar Mayo,[1] María Cruz Martín,[1]
María Fernández,[1] and Miguel A. Alvarez[1]*

*Instituto de Productos Lácteos de Asturias, IPLA-CSIC, Crta. Infiesto s/n, 33300 Villaviciosa, Asturias,
Spain,[1] and Chr. Hansen A/S, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark[2]*

Lactococcus lactis **is a prokaryotic microorganism with great importance as a culture starter 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* **that 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 (***aguB***,** *aguD***,** *aguA***, and** *aguC***) encoding the catabolic enzymes. These genes are transcribed as an operon that is induced in the presence of agmatine. In some strains, an insertion (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 nonproducing** *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 was lost during the adaptation to the milk environment by a process of reductive genome evolution.**

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 in starter cultures. LAB are mainly used as primary fermentation 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, 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 classified as GRAS (Generally Regarded as Safe) in the United States. A similar concept is used in the EU by the European Food Safety Authority (EFSA), whereby the majority of LAB species are assigned a Qualified Presumption of Safety (QPS) status. Nevertheless, some properties or enzymatic activities can generate undesirable flavors (32) or even toxic compounds as a result of, e.g., 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, hypoten-sion, or respiratory disorders (for a review, see reference 16). In nonfermented 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). The BA-producing LAB can be present in the raw material or introduced during the production process (4, 18, 28). The BA most frequently found 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 was previously reported, with concentrations of up to 0.9 g per kg (8, 16) noted in some cases. Although nondirect 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 production 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 deiminated to ornithine and then decarboxylated to putrescine. In wine and cider studies, putrescine-producing LAB that use the ODC pathway have been previously isolated (17, 25) and the ODC enzyme has been extensively characterized (25, 27). However, no putrescine producers using the ODC pathway have been isolated from dairy products. In contrast, there are a large number of dairy LAB and enterococci belonging to

* Corresponding author. Mailing address: Instituto de Productos Lácteos de Asturias, CSIC, Apdo. de Correos 85, 33300 Villaviciosa, Asturias, Spain. Phone: 34 985 89 21 31. Fax: 34 985 89 22 33. E-mail: maag@ipla.csic.es.
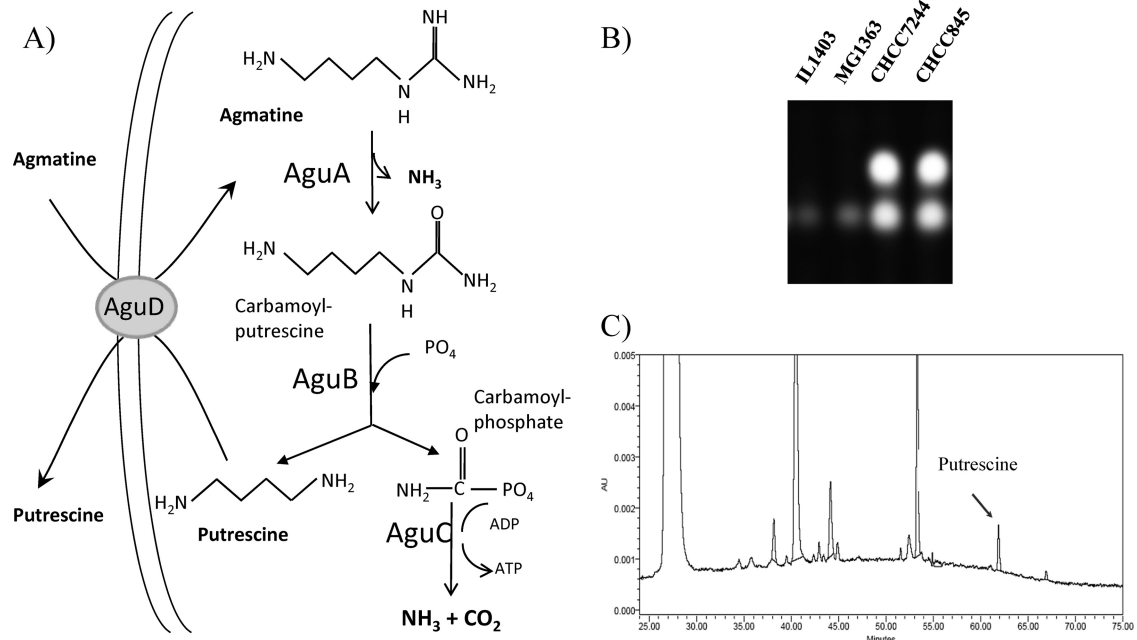
FIG. 1. (A) Scheme for the conversion of agmatine to putrescine via the AGDI metabolic pathway; the reactions catalyzing each enzyme of the route are indicated. (B) TLC analysis of the production of putrescine from agmatine in the IL1403, MG1363, CHCC7244, and CHCC845 strains. (C) HPLC analysis of the production of putrescine from agmatine overnight supernatants of CHCC7244 in M17+A medium. AU, absorbance units.

different species such as *Lactobacillus brevis*, *Lactobacillus curvatus*, and *Enterococcus faecalis* (15, 17, 22) that have been previously identified as putrescine producers operating via the AGDI pathway. Therefore, the main pathway for putrescine biosynthesis in dairy products would appear to be the AGDI pathway (Fig. 1A). To date, no putrescine-producing *L. lactis* strain has been identified. *L. lactis* is the LAB fermentation starter with the greatest importance, as it is used as a primary starter, in either 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 bacterial species (12). *L. lactis* is used for cheese and fermented milk production due to its high 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 it is through this route that *L. lactis* 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 latter by its ability to grow at 40°C and in the presence of 4% NaCl and by 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 comparisons, since they present 0.7% 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 *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains able to produce putrescine from agmatine are 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.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 72 strains of *L. lactis* (45 classified as *L. lactis* subsp. *lactis* and 27 as *L. lactis* subsp. *cremoris*), 30 obtained from the Christian Hansen Culture Collection (CHCC, Hørsholm, Denmark) and 42 from the collection of the Instituto de Productos Lácteos de Asturias (IPLA, Asturias, Spain), were included in this work (see Table S1 in the supplemental material). The strains were identified and genotypically classified to the subspecies level (subsp. *lactis* or subsp. *cremoris*) by comparing the 16S rRNA gene sequences with those in the public databases by the use of the universal primers 27F and 1492R (21). 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 liter^{-1} lactose and glucose. Where indicated, 20 mM agmatine (Sigma-Aldrich, St. Louis, MO) was added to the medium (M17+A). In both cases, the initial pH of the medium was 6.8 ± 0.2. *E. faecalis* BA62 (a putrescine producer) was grown at 30°C in M17 (Oxoid, UK) supplemented with 5 g liter^{-1} lactose and glucose, while *L. brevis* CECT 3810, *L. curvatus* VI14, and *L. collinoides* IPLA11000 (putrescine producers) were grown at 37°C in MRS broth (Oxoid, UK) (pH 6.2 ± 0.2).

Screening for putrescine-producing *L. lactis* strains. Inocula for subsequent experiments were prepared from stock cultures of the above-mentioned collections as follows. Vials were thawed, 20 μl of the contents was 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, and 1 ml of the resulting supernatants was filtered through a 0.2-μm-pore-size Supor membrane (Pall, UK) and analyzed in an initial screening for the presence of putrescine by thin-layer chromatography (TLC) according to the method of García-Moruno et al. (9). Subsequently, the presence of putrescine in the TLC-positive samples was confirmed by reversed-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 C$_{18}$ column in a Waters liquid

TABLE 1. Strains used for validation of the AGDI-PCR test[a]

Species	Strain(s)	Origin	Putrescine production (TLC/HPLC)	Detection of AGDI cluster by primer pair AgmSq1 and AgmSq2	Source(s)
Lactococcus lactis subsp. *lactis*	IL1403, CHCC1915, T2-26	Dairy	All −	All +/IS	3a, CHCC, IPLA
	CHCC845, CHCC7244, T1-48	Dairy	All +	All +	CHCC, IPLA
Lactococcus lactis subsp. *cremoris*	MG1363, CHCC3370, LC144	Dairy	All −	All −	9q, CHCC, IPLA
	2A22, GE214, 3AA9	Dairy	All +	All +	IPLA
Enterococcus faecalis	CNRZ1535, IPLA148T, BA62	Dairy	All +	All +	CNRZ, IPLA
	V583, IPLA67, IPLA55	Human	All +	All +	29a, IPLA
Enterococcus hirae	268	Dairy	All +	All +	IPLA
Lactobacillus brevis	CECT 3810, CECT 3811	Dairy	All +	All +	CECT
	CLC23	Human	All +	All +	J. M. Rodríguez
Lactobacillus curvatus	IPLAVI14, IPLAVI16	Dairy	All +	All +	IPLA
Lactobacillus collinoides	IPLA11000	Cider	+	+	IPLA
Enterococcus faecium	IPLA29, IPLA56	Human	All −	All −	IPLA
Enterococcus durans	IPLA655, IPLA833, IPLA984	Dairy	All −	All −	IPLA
Streptococcus thermophilus	CHCC1524, LMG18311	Dairy	All −	All −	CHCC, LMG
Lactobacillus ruminis	IPLA44	Human	−	−	IPLA
Lactobacillus buchneri	B301	Dairy	−	−	NIZO
Lactobacillus plantarum	LL441	Dairy	−	−	IPLA
Lactobacillus casei	ATCC393	Human	−	−	ATCC

[a] Origin, origin of the sequences and strains used for the design and testing of the AGDI-PCR assay. The presence of the AGDI cluster, the capacity of the strains to produce putrescine in broth, and the results of the specific AGDI-PCR assays are indicated. +/IS indicates a positive PCR result but a nonfunctional AGDI cluster due to the presence of the IS element (see text for details).

chromatograph system controlled by Millenium 32 software (Waters). The gradient and detection conditions were 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, MO) according to the manufacturer's instructions.

One microliter of the DNA was added to the PCR mix containing 5 μl of supplied PCR buffer (10× Dreamtaq buffer; Fermentas, Lithuania), 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.2 mM each oligonucleotide primer, 2 U of DreamTaq DNA polymerase (Fermentas, Lithuania), and water in a final volume of 50 μl. PCR amplification was performed using a DNA thermal cycler (iCycler; Bio-Rad, Hercules, CA) for 35 cycles with the general program (94°C for 30 s, 25 s 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 conditions. PCR products were purified from an agarose gel by the use of a QIAquick gel extraction kit (Qiagen, Germany).

The primer pair PTC2 and AgDdr (unpublished results) (see Table S2 in the supplemental material) was used to amplify by PCR (35 cycles of 94°C for 30 s, 50°C for 25 s, and 72°C for 3 min) a 2.9-kb fragment within the AGDI cluster of the *L. lactis* strains studied in this work (see Table S1 in the supplemental material). 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 software, 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 to those present in the databases. Analysis of transmembrane elements was performed with the TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM/; Danish Technicalt University, Denmark).

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

Northern blot analysis. *L. lactis* subsp. *lactis* T1-48 and CHCC1915 cells were grown in M17 or M17+A to the stationary phase and harvested by centrifugation in a refrigerated benchtop microcentrifuge (Eppendorf, Germany) at maximum speed. Total RNA was purified using an RNA kit (Qiagen, Germany). Cells were mechanically disrupted using glass beads (Sigma, St. Louis, MO) (≤106 μm) 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 in the supplemental material, were radiolabeled by nick translation, incorporating an [α-32P]dATP nucleotide (Perkin-Elmer, Covina, CA), and the signal was obtained from a Typhoon Trio system (GE Healthcare, Piscataway, NJ) after exposure to a storage phosphor screen.

RT-PCR. *L. lactis* cells were grown in M17 and M17+A and harvested under the conditions described previously. Total RNA was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO) as previously described (23). RNA samples (2 μg of total RNA) were treated with 2 U of DNase I (Fermentas, Lithuania) to eliminate any DNA contamination. Then, cDNA was synthesized from total RNA by the use of a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA) with specific primers for each of the analyzed genes (see Table S2 in the supplemental material). PCRs were performed using 2 μl of the cDNA preparation and a 0.4 μM concentration of each gene-specific primer (see Table S2 in the supplemental material). Amplifications were performed for 35 cycles (94°C for 30 s, 50°C for 25 s, and 72°C 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 conditions described above, using the corresponding RNA as the template.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper are available at GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers FR8586582 to FR8586602 (these are detailed in Table S1 in the supplemental material).

RESULTS

Screening for putrescine-producing *L. lactis* strains. From a total of 20 *L. lactis* strains (10 *L. lactis* subsp. *lactis* and 10 *L. lactis* subsp. *cremoris*) from the CHCC collection that were subjected to screening, two *L. lactis* subsp. *lactis* strains, CHCC7244 and CHCC845, gave a positive spot in the TLC analysis of the overnight M17+A culture supernatants (Fig. 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
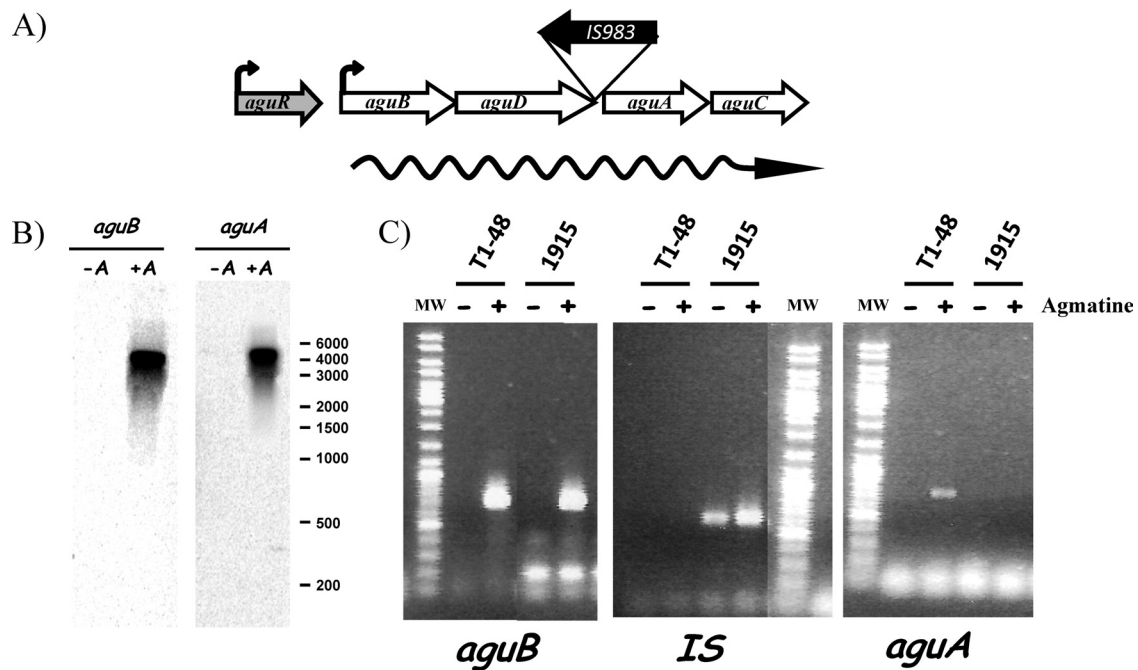
A)

B) C)

FIG. 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 (wavy 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 positions of RiboRuler high-range RNA ladder (Fermentas, Germany) fragments are indicated. (C) RT-PCR amplification with primers designed to amplify the internal regions of the *aguB*, *aguA*, and IS element genes in the absence (−) and presence (+) of agmatine in the T1-48 and CHCC1915 *L. lactis* subsp. *lactis* strains. Lanes MW, molecular weight markers (GeneRuler DNA ladder mix; Fermentas, Lithuania).

HPLC analysis (Fig. 1C); the CHCC845 and CHCC7244 strains produced 0.351 mM and 2.07 mM putrescine, respectively. This is the first report of strains of *L. lactis* being able to produce the biogenic amine putrescine.

In view of this surprising result, we decided to check for the presence of the agmatine deiminase gene (*aguA*) in the genomes of the sequenced strains of *L. lactis* of dairy origin: *L. lactis* subsp. *lactis* IL1403 (GenBank accession no. AE005176.1), *L. lactis* subsp. *cremoris* MG1363 (GenBank accession no. AM406671.1), and *L. lactis* subsp. *cremoris* SK11 (GenBank accession no. CP000425.1). None of these genomes had annotations of the agmatine deiminase genes, although its presence in *L. lactis* subsp. *lactis* IL1403 had been previously suggested (12, 24). A BLAST search using known *aguA* sequences revealed the presence of the gene in the *L. lactis* subsp. *lactis* IL1403 strain but not in the two *L. lactis* subsp. *cremoris* strains, MG1363 and SK11. In addition, the *aguA* gene is also present in the genome of *L. lactis* subsp. *lactis* KF147 (GenBank accession no. CP001834.1), a strain of vegetable origin. A sequence analysis of that genome region in *L. lactis* subsp. *lactis* IL1403 showed the presence of all the genes necessary for the production of putrescine via AGDI pathway, although the results also showed an insertion (IS) element in the cluster in the opposite orientation (Fig. 2A).

To confirm the presence of the cluster in the putrescine-positive strains, PCR amplifications using PTC2 and agDdr primers (see Table S2 in the supplemental material) previously designed in our laboratory based on the *E. faecalis* AGDI cluster genes (unpublished) were performed. The two pro-

ducer strains (*L. lactis* subsp. *lactis* CHCC7244 and CHCC845) yielded a PCR product of the expected size (2.9 kb). *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 nonproducing *L. lactis* subsp. *lactis* CHCC strains, the same 3.9-kb band was obtained. The nonproducing *L. lactis* subsp. *cremoris* CHCC strains and *L. lactis* subsp. *cremoris* MG1363 gave no amplification. As expected, the 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 lactococcus strains analyzed by combining phenotypical (TLC) and molecular (PCR with primer pair PTC2 and agDdr) screening strategies. In this new survey, we added 10 strains from the CHCC collection (for a total of 30 strains) and included 42 strains from the IPLA collection (isolated from different artisanal cheeses) (see Table S1 in the supplemental material). Nineteen strains, all identified as *L. lactis* subsp. *cremoris*, did not produce any PCR product, and 24 strains, all identified as *L. lactis* subsp. *lactis*, produced a 3.9-kb PCR band similar to that of the IL1403 strain; none of the 43 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 for all but one of these PCR-positive strains (the exception was strain 3AA11; see Table S1 in the supplemental material). Among these AGDI-positive strains, 21 (3 from the CHCC collection and 18 from the IPLA collection) were classified as *L. lactis* subsp. *lactis* and 7 (all from
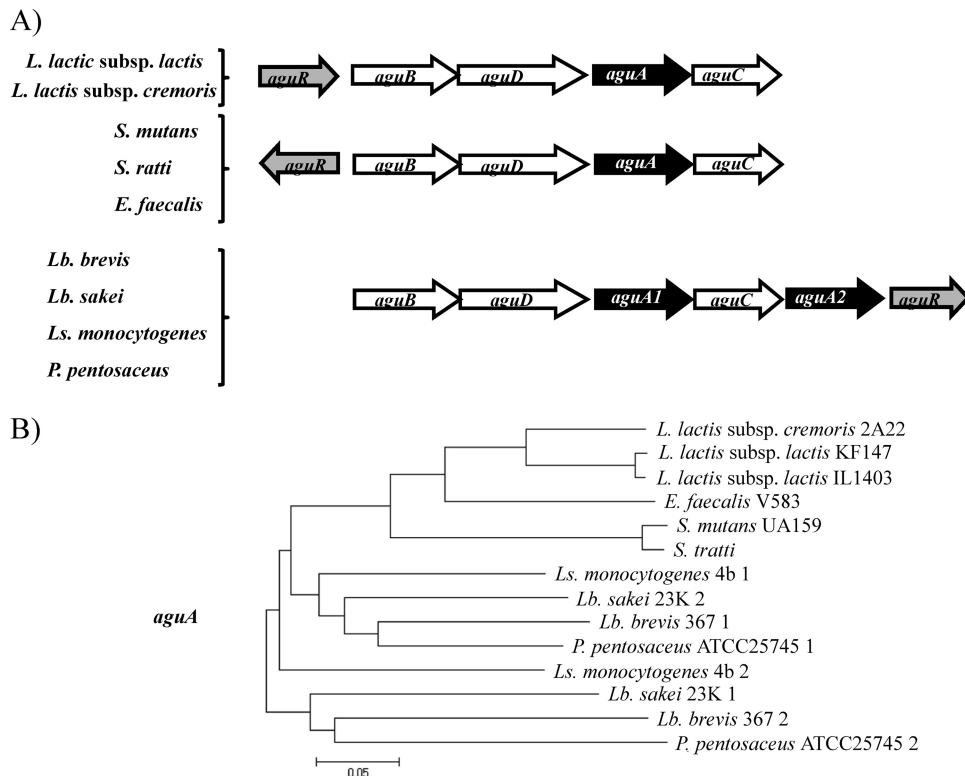
A)

B)

FIG. 3. (A) Comparison of the genetic organizations of the AGDI clusters from different bacteria. Grey arrow, regulator gene; white arrows, structural genes; black arrows, structural genes duplicated in the same genomes. *Lb.*, *Lactobacillus*; *Ls.*, *Listeria*. (B) Phylogenetic tree of the *aguA* genes from different species. Nucleotide sequences were aligned using ClustalW, and the phylogenetic tree was constructed with MEGA version 4 software, using the p-distance as the nucleotide substitution model. Those species with duplicated *aguA* genes are identified with a number (1 or 2) at the end of the strain name.

the IPLA collection) as *L. lactis* subsp. *cremoris*. 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 *L. 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 clusters of 6 of the putrescine-producing isolates were sequenced (see Table S1 in the supplemental material). All the clusters had the same genetic organization, composed of five genes in the same orientation (Fig. 2A).

***aguR* is the first gene in the cluster.** The translated protein showed similarities to 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. The *aguR* gene is preceded by a putative promoter and shows a putative transcriptional terminator.

***aguB*.** This gene encodes a protein that showed similarity to putrescine carbamoyl transferases present in several bacterial species such as *E. faecalis* and *Lb. brevis*. The closest sequence was that of *E. faecalis* V583 (NP_814483), which showed 80% identity. It is preceded by a putative promoter composed of very similar consensus sequences.

***aguD*.** This gene showed similarity to several genes encoding amino acid/biogenic amine antiporters. Analyses of the deduced amino acid sequence performed using the TMHMM tool predicted the presence of 12 transmembrane elements (data not shown).

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

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

The organization of the *L. lactis* AGDI cluster is unique (Fig. 3A), although it did show similarities to those of other Gram-positive cocci such as *E. faecalis*, *Enterococcus gallinarum*, and *Streptococcus mutans*. These species have the same number and order of the genes, but *aguR* is in the opposite orientation (Fig. 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*) (Fig. 3A).

These observed differences in the cluster organization are a reflection of the genetic groups; the differences are also evident in comparisons of the nucleotide sequences of the *aguA* genes of different species (*aguA1* and *aguA2*) (Fig. 3B). We can observe that *L. lactis* forms a separate branch, and although they are very closely related, there are differences between the *aguA* sequences from *L. lactis* subsp. *lactis* and those from *L. lactis* subsp. *cremoris* (Fig. 3B).

IS*983* interrupts the transcription of the AGDI cluster. The presence of the IS element in the 24 *L. lactis* subsp. *lactis* strains that produced 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, IS*983* is located in the middle of the AGDI catabolic genes of *L. lactis* subsp. *lactis* IL1403. In addition, there are 14 other copies of IS*983* spread in different regions of the chromosome. A BLAST database search revealed that IS*983* is present only in *L. lactis* subsp. *lactis* sequences of dairy origin. We have sequenced the complete AGDI clusters of three of these nonputrescine-producing *L. lactis* subsp. *lactis* strains (see Table S1 in the supplemental material). The analysis of the obtained sequences revealed that, in all cases, the IS*983* gene is located between the *aguD* and *aguA* genes and is in the opposite orientation.

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

Subsequently, to test that whether the presence of the IS element is responsible for the inactivation of the AGDI cluster, we used RT-PCR to analyze transcription of two genes upstream (*aguB*) and downstream (*aguA*) of the IS element in two strains: T1-48, a putrescine-producing strain, and CHCC1915, a nonproducing strain harboring the IS element. To avoid 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 (see Table S2 in the supplemental material).

Figure 2C clearly shows that transcription of *aguB* is induced in the presence of agmatine in both strains and that transcription of *aguA* was detected in the 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 the CHCC1915 strain in both the presence and the absence of agmatine (Fig. 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* species, the capacity to produce tyramine and putrescine appears to be species dependent (unpublished). In order to establish whether this is the case in *L. lactis*, a deeper look into the genomic information and the surrounding sequences was undertaken for the genomes of the IL1403, KF147, MG1363, and SK11 strains.

In *L. lactis* subsp. *lactis* IL1403 (dairy strain) and KF147 (vegetable origin), an open reading frame (ORF) that presents similarities to the transcriptional regulators of the LytR family was located upstream of the *aguR* gene (Fig. 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 of *lytR* is *malG*, which encodes an ABC maltose transporter (Fig. 4). The two genes are separated by two and three *orf* genes in *L. lactis* subsp. *cremoris* strains SK11 and MG1363, respectively, and by 17 and 18 *orf* genes—including the AGDI cluster—in *L. lactis* subsp. *lactis* strains KF147 and IL1403, respectively. In the two latter strains, an NADH-dependent oxidoreductase gene (*yrfB*) was found to be located downstream of the AGDI cluster. In the KF147 strain, however, there are 6 additional small ORFs between this gene and *aguC* (Fig. 4).

PCR amplification of the adjacent regions was performed with the 53 strains analyzed in this study that have the AGDI cluster (with or without IS*983*). Two primers were designed to investigate the presence of the *lytR* gene upstream of the AGDI cluster (see Table S2 in the supplemental material). 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* strains showing productive results 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 of the *lytR* gene, indicating that, in *L. lactis* subsp. *cremoris* putrescine-producing strains, the AGDI cluster is located after the amidase gene (Fig. 4). Two *L. lactis* subsp. *lactis* strains (CHCC206 and CHCC3052), which have the AGDI cluster interrupted by IS*983*, also produced a band larger than expected. The sequence of this band revealed the presence of a gene encoding a putative transposase (IS*982*) that can also be found in other locations in the *L. lactis* subsp. *lactis* and subsp. *cremoris* sequenced genomes.

A second pair of primers (see Table S2 in the supplemental material) was designed to test for the presence of *yrfB* downstream of the AGDI cluster; one was designed within *aguC* (*ck3*), and the second (*orf3c*) was designed from an internal sequence of *yrfB* from the *L. lactis* subsp. *lactis* IL1403 genome. When PCR with these primers was performed using the 53 AGDI-positive strains, 42 strains—including *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* strains from both the CHCC and the IPLA collections—yielded a band of the expected size (see Table S1 in the supplemental material). However, 11
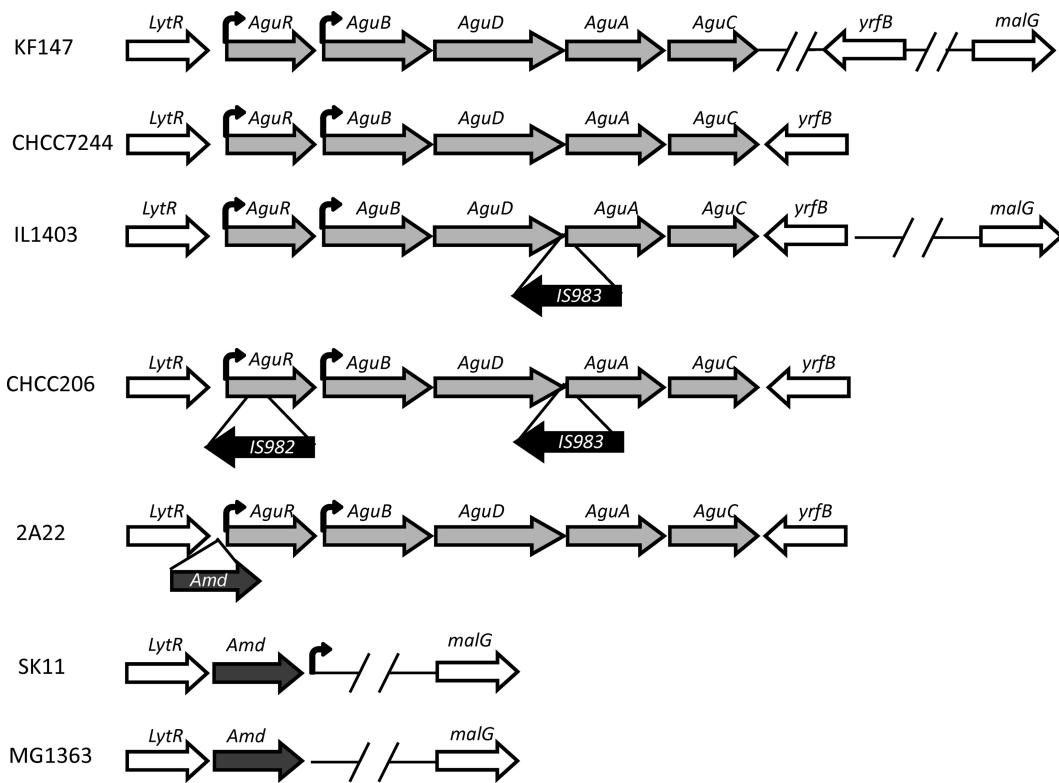
FIG. 4. Scheme for the different genetic organizations of the AGDI clusters found in *L. lactis*. The insertion elements (IS) in the *L. lactis* subsp. *lactis* strains that possess them and the amidase gene in the *L. lactis* subsp. *cremoris* strain that possesses the AGDI cluster are indicated as insertions to facilitate comparisons 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 bent arrows.

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 (locus tag *LLKF_1858*) of *L. lactis* subsp. *lactis* KF147 and used in combination with the *ck3* primer (see Table S2 in the supplemental material). All but 2 of these strains yielded a band (see Table S1 in the supplemental material), 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 G+C content different from that of the surrounding regions of the host species. The G+C content of the AGDI cluster and that of its surrounding regions ranged from 35 to 38%, a percentage range that is similar to that of the *L. lactis* species (around 35%, as calculated from the available sequenced genomes). There were only two exceptions: (i) *aguR*, which showed lower G+C content (28% in *L. lactis* subsp. *lactis* and 30% in *L. lactis* subsp. *cremoris*) and (ii) IS*983*, which showed higher content (44%), suggesting a possible different origin.

PCR assay for detection of putrescine producers in dairy products. Different PCR methods have been described and optimized for 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 results representing false positives due to those *L. lactis* subsp. *lactis* strains that have the AGDI cluster interrupted by IS*983*. To avoid this problem, we designed a new set of primers located in *aguD* and *aguA*, the genes surrounding the IS element, in order to distinguish the strains carrying the functional AGDI cluster from those with the nonfunctional AGDI cluster. In order to extend the method to other strains described as putrescine producers and 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 regions of the AGDI clusters from *Lb. brevis* IOEB 9809 (GenBank accession no. AF446085) and ATCC 367 (NCBI accession no. NC_008497), *L. lactis* subsp. *lactis* IL1403 (NCBI accession no. NC_002662), KF147 (GenBank accession no. NC_013656), and T3-17 (GenBank accession no. FR856588), *L. lactis* subsp. *cremoris* IPLA2A22 (GenBank accession no. FR856590) and GE2-14 (GenBank accession no. FR856601), and *Enterococcus faecalis* V583 (GenBank accession no. AE016830), JH2-2 (GenBank accession no. AF354231), and CNRZ1535 (GenBank accession no. FN392111) allowed the design of degenerate primers (AgmSq1 [5'-CAAGATTTDTTCTGGGCHTTYTTCTC-3'] and AgmSq2 [5'-TTGGHCCACARTCACGAACCCT-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 with those strains known to produce putrescine. As expected, those *L. lactis* subsp. *lactis* strains classified as non-putrescine producers but presenting a nonfunctional AGDI cluster rendered a larger PCR band (1,700 bp) corresponding to the addition of the IS element (see Table S1 in the supplemental material). No amplification was obtained from the nonproducers without the AGDI cluster (Table 1). Since this method could be used to screen large culture collections, the assay was also performed using overnight liquid cultures and isolated colonies picked from plates without the need for a DNA purification step. In these cases, 1 μl from 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 when purified DNA was used as the template (data not shown). The results 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 *L. lactis* subsp. *lactis* and subsp. *cremoris*. We describe the genetic determinants and their organization and surrounding regions. Based on this knowledge, a PCR test for the detection of LAB producing putrescine via the AGDI pathway has been developed.

Putrescine is one of the BA most frequently 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. Two different genetic organizations of the AGDI clusters in those species have been described; it is noteworthy that the organization found in *L. lactis* strains, although resembling that of other cocci such as *Streptococcus mutans*, *Streptococcus ratti*, or *E. faecalis*, is unique (Fig. 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* species, most of the data obtained from the analysis of AGDI cluster organization and sequences indicate that the capacity to produce putrescine from agmatine is not a recent trait acquired from horizontal gene transfer, as it would have been present in the genomes even before the differentiation between *L. lactis* subsp. *lactis* and subsp. *cremoris* 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 (Fig. 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 cocci (Fig. 3B). Sequence comparisons showed 85% identity between the AGDI clusters from *L. lactis* subsp. *lactis* and those from *L. lactis* subsp. *cremoris*. This value is the same as that found in comparisons of the two subspecies at the genomic level (33), and it is in accordance with the hypothesis that *L. lactis* subsp. *lactis* and subsp. *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 having been 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 fermentation starters unable to produce putrescine, a compound that confers undesirable flavor. Moreover, the cluster is located in a conserved region present in all *L. lactis* strains (of the available genomes analyzed), including dairy and nondairy strains, although in different positions of the genomic map, mainly due to the chromosome inversion observed in the 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 (*lytR* and *malG*) were located in all the available *L. lactis* genomes that comprise the AGDI cluster. The *lytR* gene was located upstream in all the analyzed strains, including the *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 that, in *L. lactis* subsp. *cremoris* SK11, a remnant of the putative promoter of the *aguR* gene is still present in the chromosome after the amidase gene (Fig. 4), thus indicating the loss of the AGDI cluster. The downstream region of the AGDI cluster is also well conserved, although greater variability of that region has been observed. In fact, the distance between the cluster and the *malG* gene differs 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, than to other strains of dairy 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 enzymatic properties, usually through the acquisition of plasmids, and, more importantly in the present case, by a process of reductive genome evolution, as indicated by the smaller chromosome size of the dairy strains (11).

The analysis of the sequence revealed (i) a small distance (16 nt on average) between the adjacent catabolic genes of the cluster (*aguB*, *aguD*, *aguA*, and *aguC*), (ii) the absence of clear promoters, and (iii) the presence of consensus ribosome binding sites (AGGAGG) in the intergenic regions. This suggested that all these genes are cotranscribed in the form of a single mRNA molecule, as was shown by the results of Northern blot and RT-PCR analysis in this work (Fig. 2B). The transcription of the catabolic genes of the AGDI cluster as a single mRNA molecule in a study of *S. mutans* UA159 has also been previously reported (10).

The genetic difference found between the putrescine-producing and nonproducing *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 (Fig. 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 that some *L. lactis* subsp. *cremoris* strains have even lost the cluster completely indicates that this cluster is not necessary in dairy environments. The differences between strains such as the presence or absence of a functional AGDI cluster, of an inactivated IS element, or of two inactivated IS elements, evidence of a deleted AGDI cluster together with the presence of a residual promoter, and evidence of a 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; therefore, methods that can rapidly detect BA producers in raw material or foodstuffs are required. Microbiological methods have traditionally been used for 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 detection of the decarboxylation reactions (1). Culture-independent methods based on microbial DNA detection for identification of AGDI-positive strains have been previously described (6, 24). However, these methods are not appropriate for dairy products, since they target the *aguA* gene and therefore give positive results for the *L. lactis* subsp. *lactis* strains with the AGDI cluster inactivated by IS*983*. The PCR method described in this work targets the intergenic region between the *aguD* and *aguA* genes, allowing 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 was shown to possess 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 the use of the strains as fermentation starters resulted in IS inactivation or even the loss of the AGDI cluster in dairy strains. The AGDI-PCR test described here would help to discriminate putrescine-producing strains, not only for dairy products but also for any LAB species, independently of the presence of the fermented food.

REFERENCES

1. **Bover-Cid, S., and W. H. Holzapfel.** 1999. Improved screening procedure for biogenic amine production by lactic acid bacteria. Int. J. Food Microbiol. **53:**33–41.
2. **Burdychova, R., and T. Komprda.** 2007. Biogenic amine-forming microbial communities in cheese. FEMS Microbiol. Lett. **276:**149–155.
3. **Calles-Enríquez, M., et al.** 2010. Sequencing and trancriptional analysis of *Streptococcus thermophilus* histamine biosynthesis gene cluster: factors that affect differential *hdcA* expression. Appl. Environ. Microbiol. **76:**6231–6238.
3a. **Chopin, A., M.-C. Chopin, A. Moillot-Batt, and P. Langella.** 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid **11:**260–263.
4. **Costantini, A., E. Vaudano, V. D. Prete, M. Danei, and E. García-Moruno.** 2009. Biogenic amine production by contaminating bacteria found in starter preparations used in winemaking. J. Agric. Food Chem. **57:**10664–10669.
5. **Coton, E., and M. Coton.** 2009. Evidence of horizontal transfer as origin of strain to strain variation of the tyramine production trait in *Lactobacillus brevis*. Food Microbiol. **26:**52–57.
6. **Coton, M., et al.** 2010. Occurrence of biogenic amine-forming lactic acid bacteria in wine and cider. Food Microbiol. **27:**1078–1085.
7. **Courtney, P. D.** 2000. *Lactococcus lactis* subspecies *lactis* and *cremoris*, p. 1166–1171. *In* R. K. Robinson (ed.), Encyclopedia of food microbiology, 1st ed. Elsevier, Amsterdam, the Netherlands.
8. **Fernández, M., D. M. Linares, B. del Río, V. Ladero, and M. A. Alvarez.** 2007. HPLC quantification of biogenic amines in cheeses: correlation with PCR-detection of tyramine-producing microorganisms. J. Dairy Res. **74:**276–282.
9. **García-Moruno, E., A. V. Carrascosa, and R. Muñoz.** 2005. A rapid and inexpensive method for the determination of biogenic amines from bacterial cultures by thin-layer chromatography. J. Food Prot. **68:**625–629.
9a. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
10. **Griswold, A. R., Y. Y. Chen, and R. A. Burne.** 2004. Analysis of an agmatine deiminase gene cluster in *Streptococcus mutans* UA159. J. Bacteriol. **186:**1902–1904.
11. **Kelly, W. J., L. J. Ward, and S. C. Leahy.** 2010. Chromosomal diversity in *Lactococcus lactis* and the origin of dairy starter cultures. Genome Biol. Evol. **2:**729–744.
12. **Kok, J., G. Buist, A. L. Zomer, S. A. F. T. van Hijum, and O. P. Kuipers.** 2005. Comparative and functional genomics of lactococci. FEMS Microbiol. Rev. **29:**411–433.
13. **Krause, I., A. Bockhardt, H. Neckermann, T. Henleand, and H. Klostermeyer.** 1995. Simultaneous determination of amino acids and biogenic amines by reversed phase high-performance liquid chromatography of the dabsyl derivatives. J. Chromatogr. A **715:**67–79.
14. **Ladero, V., D. M. Linares, M. Fernández, and M. A. Alvarez.** 2008. Real time quantitative PCR detection of histamine-producing lactic acid bacteria in cheese: relation with histamine content. Food Res. Int. **41:**1015–1019.
15. **Ladero, V., E. Sánchez-Llana, M. Fernández, and M. A. Alvarez.** 2011. Survival of biogenic amine-producing dairy LAB strains at pasteurisation conditions. Int. J. Food Sci. Technol. **46:**516–521.
16. **Ladero, V., M. Calles-Enríquez, M. Fernández, and M. A. Alvarez.** 2010. Toxicological effects of dietary biogenic amines. Curr. Nutr. Food Sci. **6:**145–156.
17. **Ladero, V., et al.** 2011. Biogenic amines content in Spanish and French natural ciders: application of qPCR for quantitative detection of biogenic amine-producers. Food Microbiol. **28:**554–561.
18. **Ladero, V., M. Fernández, and M. A. Alvarez.** 2009. Effect of post-ripening processing on the histamine and histamine-producing bacteria contents of different cheeses. Int. Dairy J. **19:**759–762.
19. **Ladero, V., M. Fernández, I. Cuesta, and M. A. Alvarez.** 2010. Quantitative detection and identification of tyramine-producing enterococci and lactobacilli in cheese by multiplex qPCR. Food Microbiol. **27:**933–939.
20. **Landete, J. M., S. Ferrer, and I. Pardo.** 2005. Which lactic acid bacteria are responsible for histamine production in wine? J. Appl. Microbiol. **99:**580–586.
21. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–147. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, NY.
22. **Linares, D. M., M. Martín, V. Ladero, M. A. Alvarez, and M. Fernández.** 2011. Biogenic amines in dairy products. Crit. Rev. Food. Sci. Nutr. **51:**691–703.
23. **Linares, D. M., M. Fernández, M. C. Martín, and M. A. Alvarez.** 2009. Tyramine biosynthesis in *Enterococcus durans* is transcriptionally regulated by the extracellular pH and tyrosine concentration. Microb. Biotechnol. **2:**625–633.
24. **Lucas, P. M., et al.** 2007. Agmatine deiminase pathway genes in *Lactobacillus brevis* are linked to the tyrosine decarboxylation operon in a putative acid resistance locus. Microbiology **153:**2221–2230.
25. **Marcobal, A., B. de las Rivas, M. V. Moreno-Arribas, and R. Muñoz.** 2004. Identification of the ornithine decarboxylase gene in the putrescine-producer *Oenococcus oeni* BIFI-83. FEMS Microbiol. Lett. **239:**213–220.
26. **Marcobal, A., B. de las Rivas, M. V. Moreno-Arribas, and R. Muñoz.** 2006. Evidence for horizontal gene transfer as origin of putrescine production in *Oenococcus oeni* BIFI-83. Appl. Environ. Microbiol. **72:**7954–7958.
27. **Momany, C., S. Ernst, R. Ghosh, N. L. Chang, and M. L. Hackert.** 1995.

Crystallographic structure of a PLP-dependent ornithine decarboxylase from *Lactobacillus* 30a to 3.0 Å resolution. J. Mol. Biol. **252:**643–655.

28. **Novella-Rodríguez, S., M. T. Veciana-Nogués, A. Roig-Sagués, A. Trujillo-Mesa, and M. C. Vidal-Carou.** 2002. Influence of starter and non starter bacteria on the formation of biogenic amines in goat cheese during ripening. J. Dairy Sci. **85:**2471–2478.

29. **Passerini, D., et al.** 2010. Genes but not genomes reveal bacterial domestication of *Lactococcus lactis*. PLoS One **5:**e15306.

29a.**Sahm, D. F., et al.** 1989. In vitro susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrob. Agents Chemother. **33:**1588–1591.

30. **Salama, M. S., W. E. Sandine, and S. J. Giovannoni.** 1991. Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **57:**1313–1318.

31. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

32. **Smit, G., B. A. Smit, and W. J. Engels.** 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. **29:**591–610.

33. **Wegmann, U., et al.** 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. J. Bacteriol. **189:**3256–3270.