RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY (2010) 13:169-177
DOI: 10.2436/20.1501.01.123 ISSN: 1139-6709 **www.im.microbios.org**

INTERNATIONAL MICROBIOLOGY

The role of two families of bacterial enzymes in putrescine synthesis from agmatine via agmatine deiminase

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Received 22 September 2010 · Accepted 29 December 2010

Summary. Putrescine, one of the main biogenic amines associated to microbial food spoilage, can be formed by bacteria from arginine via ornithine decarboxylase (ODC), or from agmatine via agmatine deiminase (AgDI). This study aims to correlate putrescine production from agmatine to the pathway involving *N*-carbamoylputrescine formation via AdDI (the *aguA* product) and *N*-carbamoylputrescine amidohydrolase (the *aguB* product), or putrescine carbamoyltransferase (the *ptcA* product) in bacteria. PCR methods were developed to detect the two genes involved in putrescine production from agmatine. Putrescine production from agmatine could be linked to the *aguA* and *ptcA* genes in *Lactobacillus hilgardii* X_1B, *Enterococcus faecalis* ATCC 11700, and *Bacillus cereus* ATCC 14579. By contrast *Lactobacillus sakei* 23K was unable to produce putrescine, and although a fragment of DNA corresponding to the gene *aguA* was amplified, no amplification was observed for the *ptcA* gene. *Pseudomonas aeruginosa* PAO1 produces putrescine and is reported to harbour *aguA* and *aguB* genes, responsible for agmatine deiminase and *N*-carbamoylputrescine amidohydrolase activities. The enzyme from *P. aeruginosa* PAO1 that converts *N*-carbamoylputrescine to putrescine (the *aguB* product) is different from other microorganisms studied (the *ptcA* product). Therefore, the *aguB* gene from *P. aeruginosa* PAO1 could not be amplified with *ptcA*-specific primers. The *aguB* and *ptcA* genes have frequently been erroneously annotated in the past, as in fact these two enzymes are neither homologous nor analogous. Furthermore, the *aguA*, *aguB* and *ptcA* sequences available from GenBank were subjected to phylogenetic analysis, revealing that gram-positive bacteria harboured *ptcA*, whereas gram-negative bacteria harbour *aguB*. This paper also discusses the role of the agmatine deiminase system (AgDS) in acid stress resistance. [**Int Microbiol** 2010; 13(4):169-177]

Keywords: agmatine · putrescine · agmatine deiminase · *N*-carbamoylputrescine amidohydrolase · putrescine carbamoyltransferase

Introduction

Putrescine, 1,4-diaminobutane, is a polyamine present in almost all living cells [11]. High putrescine concentrations in food are strongly correlated to the amino acid decarboxylase activity of undesirable bacteria. However, low putrescine levels are considered physiological, as a precursor of spermidine and spermine, and consequently play a metabolic role [36]. Putrescine presence in food is undesirable because it modifies aroma and flavour [18]. Putrescine content may be considered as a potential freshness marker or quality indicator of deterioration in food [8,28]. Moreover, from the safety standpoint, putrescine increases the adverse effects of histamine, tyramine and phenylethylamine, because it interferes with the enzymes that metabolise these other amines [34,35].

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Putrescine can be synthesized either directly from ornithine by ornithine decarboxylase (ODC, the *speC* product) or indirectly from arginine via arginine decarboxylase (ADC, the *speA* product) (Fig 1). ADC converts arginine in agmatine, then agmatine deiminase (the *aguA* product) and *N*-carbamoylputrescine amidohydrolase (the *aguB* product) or putrescine carbamoyltransferase (the *ptcA* product), biosynthetically converting agmatine to putrescine via the AgDI pathway (Fig. 1). Naumoff et al. [25] identified a set of genes that have been erroneously annotated and thus confirmed that two different enzymes can convert *N*-bamoylputrescine to putrescine. One enzyme converts *N*-carbamoylputrescine to putrescine and carbamoylphosphate (the *ptcA* product), whereas other enzyme converts *N*-carbamoylputrescine to putrescine, CO_2 and NH_3 (the *aguB* product). Therefore, these enzymes are neither homologous nor analogous, they are different enzymes.

Agmatine can be used as a precursor of polyamines and other important metabolites depending on the microorganism in question [20]. Various groups of bacteria can metabolise agmatine; however, only a few microorganisms are reported as able to do so. L-Arginine utilization by *Pseudomonas* species was demonstrated by Stalon and Mercenier in 1984 [32]. The presence of the AgDI pathway has been reported in *P. aeruginosa* PAO1 [23,24] and *E. faecalis* ATCC 11700 [30,31]. Moreover, Ivanova et al. [12] reported a putative agmatine deiminase gene in *B. cereus* and Arena et al. [4] also showed putrescine production from agmatine by *Lb. hilgardii* X_1B. The effect of different metabolites on putrescine synthesis in *Lb. hilgardii* X_1B has also been studied [3]. More recently, an operon encoding enzyme of the AgDS (agmatine deiminase system) was identified in *Streptococcus mutans* UA159 [9].

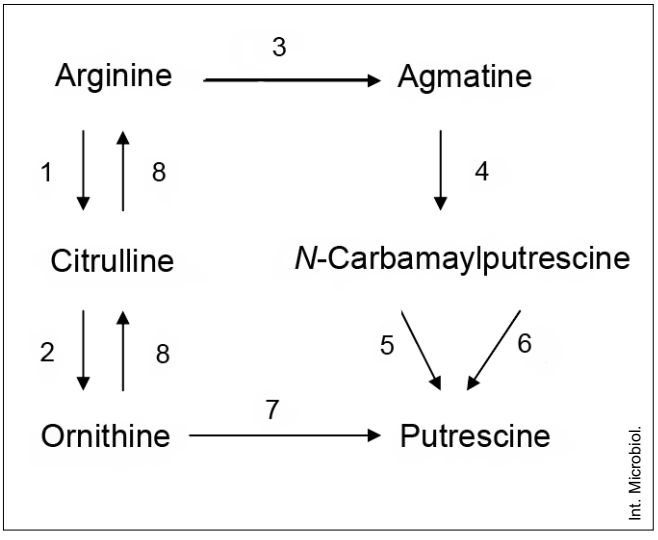

Fig 1. Pathways of agmatine metabolism in bacteria: (1) Arginine deiminase. (2) Ornithine transcarbamylase. (3) Arginine decarboxylase. (4) Agmatine deiminase. (5) *N*-carbamoylputrescine amidohydrolase. (6) Putrescine carbamoyltransferase. (7) Ornithine decarboxylase. (8) Anabolic system.

In the food industry, the detection of biogenic amine-producing bacteria is important to avoid poisoning. Therefore, rapid methods are required for the early detection of these bacteria. Molecular methods for the detection and identification of food-borne bacteria are becoming widely accepted as an alternative to traditional culture methods. Thus, PCR is commonly used for the rapid, sensitive, and specific detection of target genes. PCR techniques targeting bacterial amino acid decarboxylase genes responsible for biogenic amine production have been designed previously [6, 15–17,22]. In this study, we developed a molecular tool to detect putrescine-producing bacteria via AgDI on the basis of agmatine deiminase and putrescine carbamoyltransferase gene sequence comparison. The study also reveals there is a correlation of putrescine production with the presence of the aforementioned genes.

Materials and methods

Microorganisms. *Lactobacillus hilgardii* X_1B was isolated and identified from an Argentinean wine [33]. Other bacteria were obtained from the Spanish Type Culture Collection (CECT): *Psudomonas aeruginosa* PAO1 (CECT 4122), *Bacillus cereus* ATCC 14579 (CECT 148T) and *Enterococcus faecalis* ATCC 11700 (CECT 5143). *Lactobacillus sakei* 23K was kindly provided by Monique Zagorec from the French National Institute for Agronomical Research (INRA) [5]. *Pediococcus parvulus* P339 isolated from wine [27] was used as negative control.

Growth and culture conditions. *Lactobacillus hilgardii* X_1B, *Lb. sakei* 23K and *Pd. parvulus* P339 were grown in MRS broth (Scharlab, Sentmenat, Spain). *Enterococcus faecalis* ATCC 11700 was grown in tryptone soya broth containing in g/l: tryptone 15, soya peptone 5, and NaCl 5. *Bacillus cereus* ATCC 14579 was grown in Nutrient I, containing in g/l: beef extract 5, peptone 10, and NaCl 5. Finally, *P. aeruginosa* PAO1 was grown in Nutrient II, containing in g/l: beef extract 1, yeast extract 2, agmatine 1, peptone 10, and NaCl 5. Culture media were adjusted to pH 6.5 with 1 mM KOH before sterilisation by autoclave at 121ºC for 20 min. Microorganisms were grown statically at 28ºC. Biogenic amine standards and the compounds contained in the different media were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Putrescine production by resting cells. Cultures were harvested after 24 h incubation statically at 28ºC. Cells were washed with sodium phosphate buffer 0.2 M pH 6.5. Centrifuged cells were resuspended in the same buffer to $OD_{600} = 0.7$. This OD_{600} of 0.7 corresponds to $3.4 \pm 0.4 \times 10^8$ cfu/ml for *Lb. hilgardii* X_1B, $3.2 \pm 0.3 \times 10^8$ for *Lb. sakei* 23K, $5.65 \pm 0.6 \times 10^8$ cfu/ml for *E. faecalis* ATCC 11700, $1.7 \pm 0.2 \times 10^8$ cfu/ml for *B. cereus* ATCC 14579 and $1.87 \pm 0.2 \times 10^8$ cfu/ml for *P. aeruginosa* PAO1.

The reaction mixture to determine putrescine formation had the following composition in a final volume of 2 ml: 0.5 ml L-agmatine solution (to a final concentration of 1 g/l); 1 ml sodium phosphate buffer (0.2 M) pH 6.5; and 0.5 ml cell suspension at 0.7 units of optical density. These compounds were obtained from Sigma. The mixture was incubated for 8 h at 28 ºC. The samples were then centrifuged at 13,400 ×*g* for 3 min at 4ºC, and the biogenic amines were immediately detected and quantified in the supernatant.

Biogenic amine determination. HPLC analyses were carried out with a reverse phase column by using an elution gradient composed of methanol and phosphate buffer 0.2 M. For analysis, the samples were diluted with phosphate buffer at 1:10 ratio. Agmatine and putrescine were precolumn derivatized with *o*-phthaldialdehyde (OPA) (Sigma). The reaction solution consisted of 200 mg OPA in 9 ml methanol, 1 ml 0.4 M sodium borate pH 10, and 160 µl 2-mercaptoethanol (MCE) (Sigma). OPA and MCE were prepared 24 h before utilization.

The samples were injected into the HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with an L-Intelligent pump (Merck-Hitachi), AS-2000A Autosampler (Merck-Hitachi), T-6300 column thermostat (Merck-Hitachi) and an L7485 LaChrom fluorescence spectrophotometer (Merck-Hitachi); the excitation wavelength was set at 335 nm, and emission wavelength was at 450 nm. A 100 RP-18 column, (Merck-Hitachi) (25 cm × 5 µm) was used for the stationary phase with a flow of 1.0 ml/min. The column was thermostated at 40ºC.

A gradient of solvent A (2.268 g KH_2PO_4 and 14.968 g $Na_2HPO_4 \cdot 12H_2O$ adjusted to pH 5.8 with H_3PO_4 and filled up with deionized water to 1 litre) and solvent B (100% methanol) was applied to a 100 RP-18 column (Merck -Hitachi) (25 cm × 5 µm) as follows: 0–20 min, 40–55% B linear gradient, 1 ml/min; 20–45 min, 55–85% linear gradient, 1 ml/min.

Statistical analysis. The MINITAB Student test was used to validate the method. Three replicate determinations were carried out for three independent resting cells. Relative standard deviations for amine concentrations were ≤5%.

Detection of *aguA* and *ptcA* genes. Bacterial DNA for partial *aguA* and *ptcA* gene amplification was obtained by using the Microbial DNA isolation Kit (MoBio Laboratories). Sequence alignments to design oligonucleotide primers were determined using Mega 4.0, ClustalW2 on EBI [http://www.ebi.a-c.uk], and BLAST software [1]. To amplify the internal part of the aguA gene (830 bp) we used the primer pair aguA-F (5´-GAC TGG AC(AGT) TT(CT) AAG G(GC/CT) TGG GG-3´) and aguA-R (5´-TG(CT) TG(AG) GT(AG) AT(AG) CA(AG) TG(AG)-3´). To amplify the internal part of *ptcA* (350 bp) we used the primer pair ptcA-F (5´-CC(AGT) GA(AG) (AC)T(AG) TGG A(AC)(AGT) AC(AGC) GG-3´) and ptcA-R (5´-GG(AG) AA(AG) CGA A(GT)(AG) TCA TA(AG) CAA AT-3´). The amplification conditions for the aguA-F/aguA-R pair were 5 min at 95°C for denaturation, and subsequently, 40 cycles at 94°C for 1 min, 1 min at 53°C, and 72°C for 2 min followed by 10 min at 72°C. The amplification with the ptcA-F and ptcA-R pair was performed as follows: 5 min at 95°C for denaturation and 40 cycles at 94°C for 1 min, 0.5 min at 53°C, and 1 min at 72°C followed by 10 min at 72°C. The PCR products were separated by gel electrophoresis in a 1.2% agarose gel using Tris-borate EDTA 0.5 M as buffer system [29]. The gels were analysed after staining with ethidium bromide.

Analysis of the genetic organization of the agmatine deiminase gene clusters. To design the genetic organization of the agmatine deiminase gene clusters we referred to the information on nucleotide sequences available at [http://www.ncbi.nlm.nih.gov] for *Lb. hilgardii* ATCC 8290 (NZ ACGP00000000), *E. faecalis* V583 (NC 004668), S. *mutans* UA159 (NC 004350), *Lb. sakei* 23K (NC 007576), *Lactobacillus brevis* ATCC 367 (NC 008997.1), *Pediococcus pentosaceus* ATCC 25745 (NC 008525.1) and *P. aeruginosa* PAO1 (NC 002526). ClustalW2 on EBI [http://www.ebi.a-c.uk], Microbesonline resources [http://www.microbesonline.org] and BLAST programs [1] were used for sequence alignment and to search similar sequences.

Evolutionary relationships between bacterial genes involved in putrescine production from agmatine. The *aguA*, *aguB*, and *ptcA* sequences, available from GenBank, were subjected to phylogenetic analysis. Several reconstruction methods (neighbour-joining and maximum likelihood) were applied using the BioNumerics V2.5 (Applied Maths) software package to infer the phylogeny of those genes. Pairwise distances were calculated with the Mega 4.0 software.

Results

Detection of *aguA* and *ptcA* genes. Information about of nucleotide sequences available in the literature was used to design the oligonucleotide primer pair to detect the *aguA* and *ptcA* genes. We aligned the nucleotide sequences from *P. aeruginosa* PAO1 (NC_002516), *Lb. sakei* 23K (NC_007576), *Streptomyces avermitilis* (NC_003155) and *Lactococcus lactis* II1403 (NC_002662) to design the primer pair to amplify *aguA*. Also, sequence alignment of *Bacillus halodorurans* C-125 (NC_002570), *Lb. plantarum* WCFS1 (NC_004567), *L. mesenteroides* ATCC 8293 (NZ_AABH00000000) and *B. cereus* ATCC 10987 (NC_003909) was performed to design primer pairs to detect *ptcA*. Two conserved domains of both genes were selected in

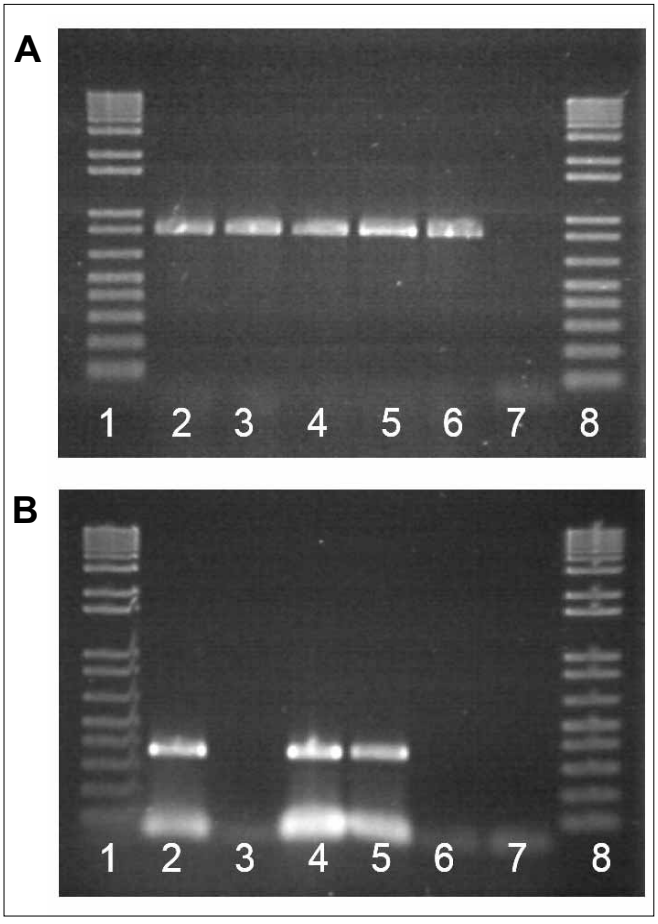

Fig 2. (A) PCR amplification of an *aguA* gene fragment with primers AguAF and AguAR. 1 Kb Plus Ladder (Invitrogen) (1, 8), *Lb. hilgardii* X_1B (2), *Lb. sakei* 23K (3), *E. faecalis* ATCC 11700 (4), *B. cereus* ATCC 14579 (5), *P. aeruginosa* PAO1 (6), *Pd. parvulus* P339 (7) used as a negative control. **(B)** PCR amplification of a *ptcA* gene fragment with primers ptcA-F and ptcA-R. 1 Kb Plus Ladder (Invitrogen) (1, 8), *Lb. hilgardii* X_1B (2), *Lb. sakei 23K* (3), *E. faecalis* ATCC 11700 (4), *B. cereus* ATCC 14579 (5), *P. aeruginosa* PAO1 (6), *Pd. parvulus* P339 (7) used as a negative control.

Table 1. Comparative survey of presence of implicated genes in putrescine production from agmatine and activity in *Lactobacillus hilgardii* X_1B, *Lactobacillus sakei* 23K, *Enterococcus faecalis* ATCC 11700, *Bacillus cereus* 148T and *Pseudomonas aeruginosa* PAO1

Microorganism	PCR *aguA*	PCR *ptcA*	Putrescine production	*N*-Carbamoylputrescine production	References
Lb. hilgardii X_1B	+	+	+	+	[3,4]
Lb. sakei 23K	+	–	–	?	[5]
E. faecalis ATCC 11700	+	+	+	+	[30,31]
B. cereus 148 T	+	+	+	+	[12]
P. aeruginosa PAO1	+	–	+	+	[23,24]

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each case to design the synthetic primers aguA-F and aguA-R, which amplified an 830-bp *aguA* DNA fragment, and the synthetic primers ptcA-F and ptcA-R, which amplified a 350-bp *aguB* DNA fragment. When these primers were used, under the conditions described in Material and methods, only the expected 830-bp and 350-bp fragments were obtained (Fig. 2A,B). However, annealing temperatures below 53ºC yielded unspecific fragments. *Lactobacillus hilgardii* X_1B, *E. faecalis* ATCC 11700, *B. cereus* ATCC 14579, *P. aeruginosa* PAO1 and *Lb. sakei* 23K showed an amplification band of the *aguA* fragment of the expected size, whereas the negative control (*Pd. parvulus* P339) did not show the amplification band (Fig. 2A, Table 1). PCR of *ptcA* revealed an amplification band of the expected size for *Lb. hilgardii* X_1B, *E. faecalis* ATCC 11700 and *B. cereus* ATCC 14579. However *P. aeruginosa* PAO1, *Lb. sakei* 23K and the negative control (*Pd. parvulus* P339) did not display this band (Fig. 2B, Table 1).

Putrescine production analyses. The analyses of putrescine production from agmatine revealed that *Lb. hilgardii* X_1B, *E. faecalis* ATCC 11700, *B. cereus* ATCC 14579 and *P. aeruginosa* PAO1 all produced putrescine. However, *Lb. sakei* 23K and the negative control were unable to do so (Fig. 3, Table 1). Figure 3 shows that *P. aeruginosa* PAO1 produced between 2.5- and 5-fold more putrescine than the other microorganisms. The amounts of putrescine produced by the gram-positive bacteria *E. faecalis* ATCC 11700 and *Lb. hilgardii* X_1B were similar; both producing twice as much putrescine as *B. cereus* CECT 148T. When the microorganisms were precultured with agmatine, putrescine production increased in all the microorganisms tested (data not shown), with the highest increase observed in *P. aeruginosa* PAO1 followed by *Lb. hilgardii* X_1B, *B. cereus* CECT 148T and *E. faecalis* ATCC 11700, in decreasing order.

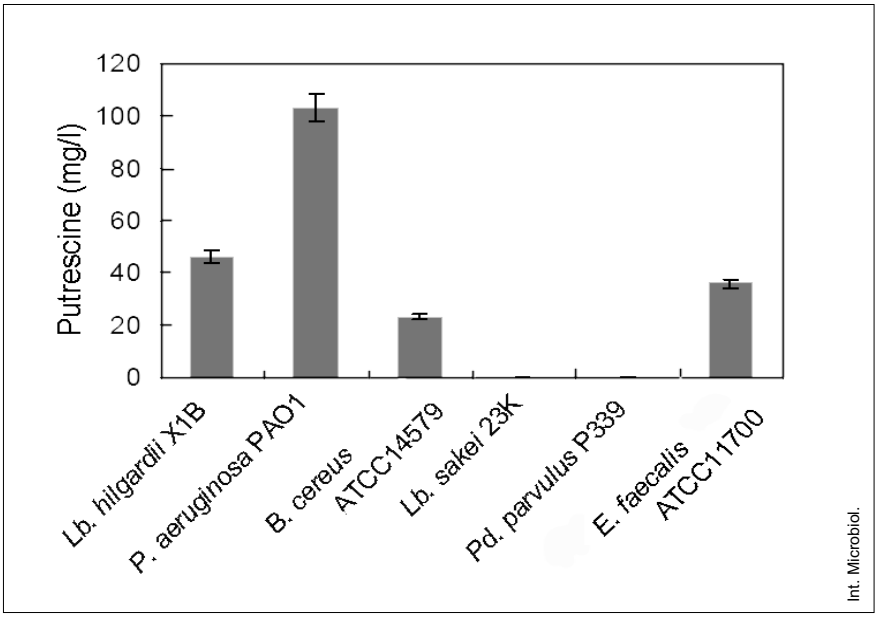

Fig 3. Production of putrescine from agmatine deamination by resting cells of *Lb. hilgardii* X_1B, *Lb. sakei* 23K, *E. faecalis* ATCC 11700, *B. cereus* ATCC 14579, *P. aeruginosa* PAO1 and *Pediococcus parvulus* P339 as negative control.

Genetic organization of the agmatine deiminase gene clusters. Sequence analysis up- and downstream from the agmatine deiminase gene of *Lb. hilgardii* ATCC 8290, *E. faecalis* V583, *S. mutans* UA159, *Lb. sakei* 23K, *Lb. brevis* ATCC 367, *Pd. pentosaceus* ATCC 25745 and *P. aeruginosa* PAO1 revealed the presence of agmatine deiminase and a transcriptional regulator in all the microorganisms studied (Fig. 4). Gram-positive microorganisms shown in Fig. 4 harbour a carbamate kinase and a putrescine carbamoyltransferase, while *N*-carbamoylputrescine amidohydrolase is present in *P. aeruginosa* PAO1. The *Lb. hilgardii* ATCC 8290 AgDI gene cluster contains a second putative agmatine deiminase (*aguA2*), both agmatine deiminases of *Lb. hilgardii* ATCC 8290 show a similarity of 57.5% and 47.4% for amino acid and nucleotide sequences, respectively. The *Lb. hilgardii* ATCC 8290 AgDI gene cluster contains a two-component system (TCS) upstream and transcribed separately the agmatine deiminase gene cluster. The TCS showed high similarity with the TCS involved in citrate/malate metabolism of *Clostridium carboxidovorans* P7.

Streptococcus mutans UA159 and *E. faecalis* V583 contain the gene of a transcriptional regulator of the LysR family located upstream and transcribed separately. While *Lb. sakei* 23K, *Lb. brevis* ATCC 367, *Pd. pentosaceus* ATCC 25745, and *Lb. hilgardii* ATCC 8290 have a gene-encoding transcriptional regulator of the RpiR family downstream of the cluster and in the same transcription direction. *Pseudomonas aeruginosa* PAO1 contains the gene of a transcriptional regulator of the TetR family located downstream of the cluster. Moreover, *Lb. sakei* 23K also contains a putative peptidil arginine deiminase and a putative antiporter amino acid/polyamine.

Evolutionary relationships between bacterial *aguA* and *ptcA/aguB*. Phylogenetic trees were constructed to establish the evolutionary relationships between the genes responsible for agmatine transformation into

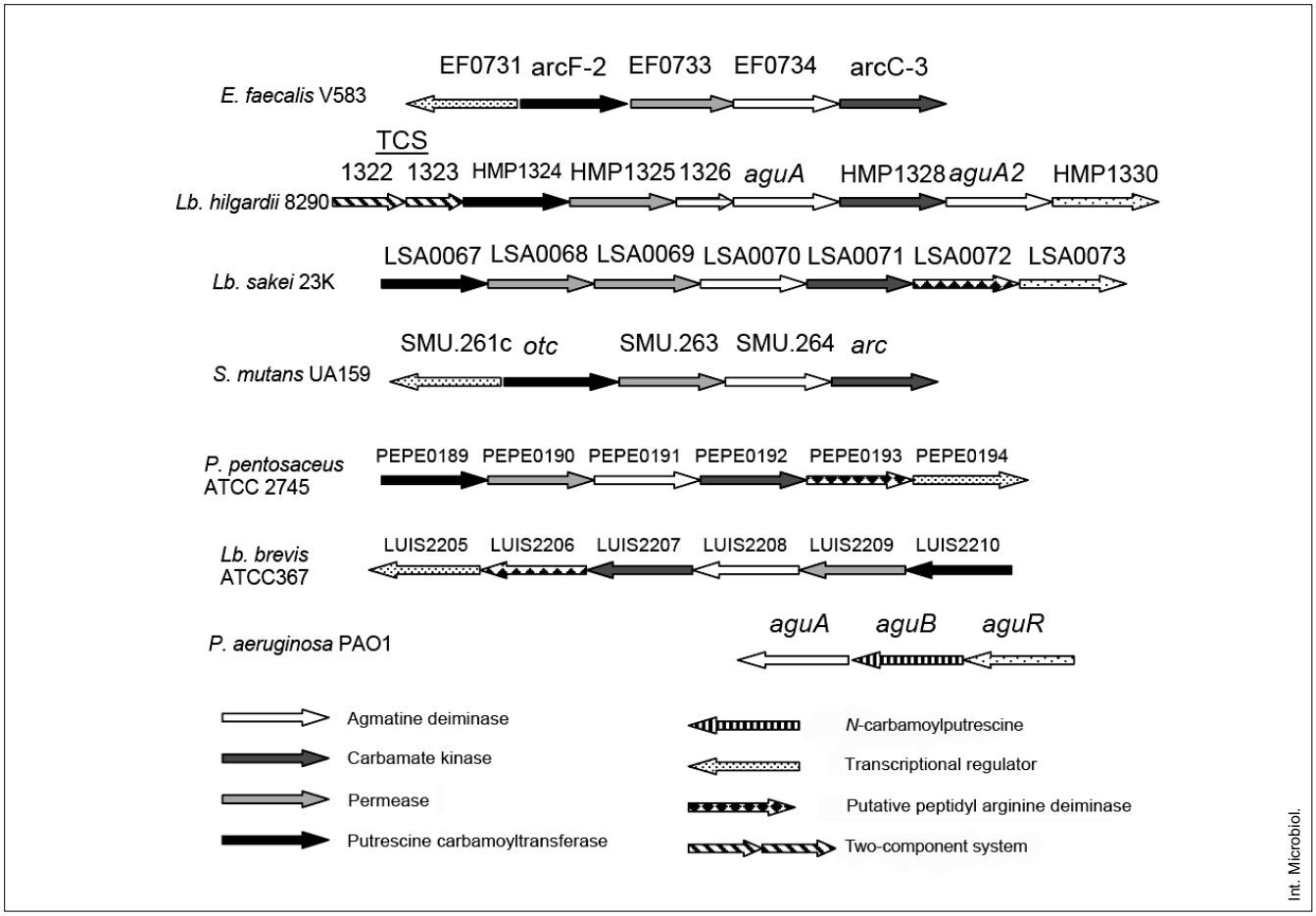

Fig 4. Analyses of sequences up- and downstream from the agmatine deiminase gene of *Lb. hilgardii* ATCC8290 (NZ ACGP00000000), *E. faecalis* V583 (NC 004668), *S. mutans* UA159 (NC 004350), *Lb. sakei* 23K (NC 007576), *Lb. brevis* ATCC 367 (NC 008997.1), *Pd. pentosaceus* ATCC 25745 (NC 008525.1) and *P. aeruginosa* PAO1 (NC 002526).

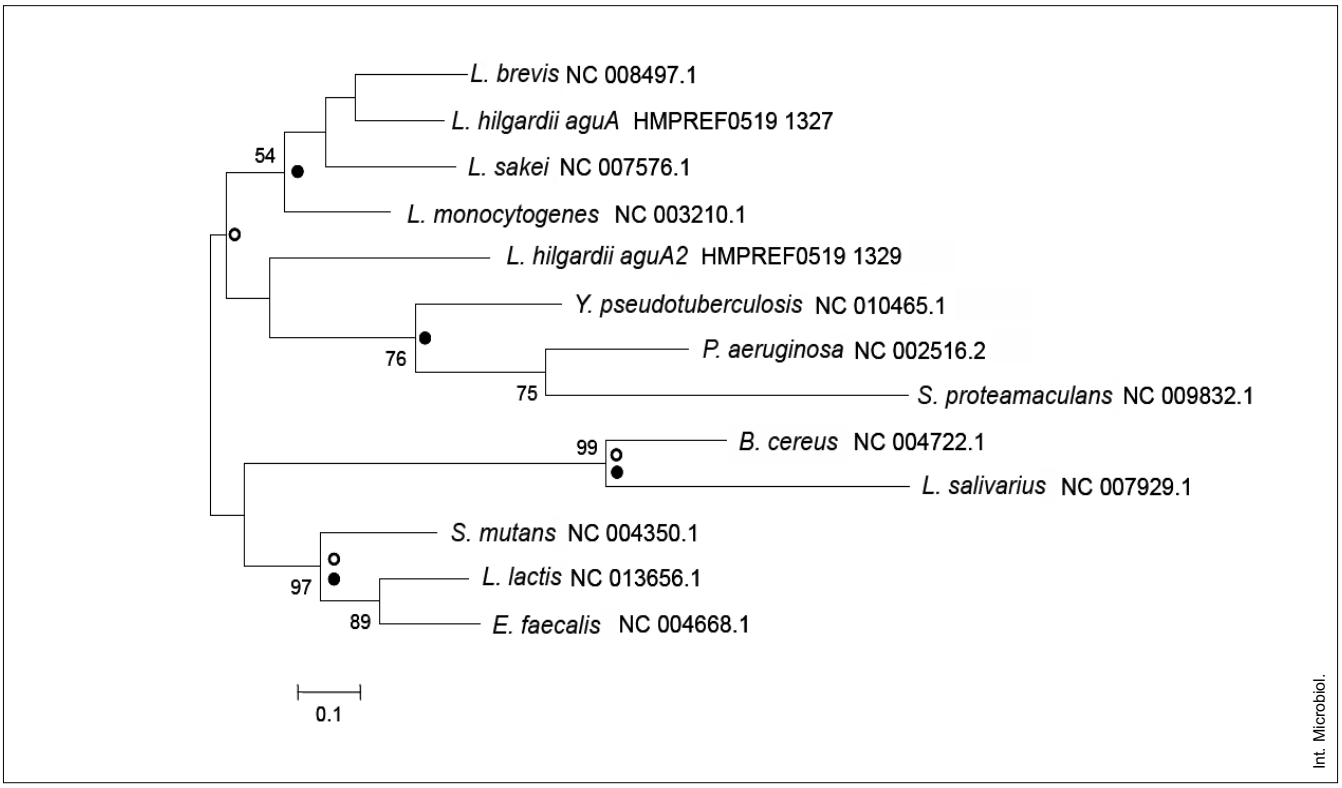

Fig 5. Maximum likelihood trees based on *aguA* gene sequences. Bootstrap values (1000 replications) above 50% are shown. Filled and open circles respectively indicate nodes recovered reproducibly by neighbour-joining and maximum likelihood. Bar, substitutions per nucleotide position. Sequences of *aguA* from: *Listeria monocytogenes* EGD-e (NC_003210.1), *Lactococcus lactis subsp. lactis* KF147 (NC_013656.1), *E. faecalis* V583 (NC_004668.1), *S. mutans* UA159 (NC_004350.1), *Yersinia pseudotuberculosis* YPIII (NC_010465.1), *Bacillus cereus* ATCC 14579 (NC_004722.1), *P. aeruginosa* PAO1 (NC_002516.2), *Lb. salivarius* UCC118 (NC_007929.1), *Lb. sakei* subsp. *sakei* 23K (NC_007576.1), *Lb. brevis* ATCC 367 (NC_008497.1), *Serratia proteamaculans* 568 (NC_009832.1), *Lb. hilgardii* ATCC 8290, (ZP_03953667) were accessible from GenBank.

putrescine. The phylogenetic analyses confirmed the existance of a unique gene that conversts agmantine into *N*-carbamoylputrescine in bacteria. Figure 5 shows the agmantine deiminase gene is the same from gram-positive and gram-negative bacteria. The figure demonstrates that both *aguA* (HMPREF0519, 1327) and *aguA2* (HMPREF0519, 1329) from *L. hilgardii* ATCC 8290 are agmatine deiminase genes.

However, we confirmed the existence of two families of genes that convert *N*-carbamoylputrescine into putrescine, these gene are *N*-carbamoylputrescine amidohydrolase (*aguB*) and putrescine carbamoyltransferase (*ptcA*), proposed on the basis of phylogenetic studies. Figure 6 shows how *aguB* and *ptcA* genes are different. Gram-positive bacteria harbour *ptcA*, while gram-negative bacteria hold *aguB*; this correlation has also been found in Microbes Online [http://www.microbesonline.org]. These results suggest that *aguA* evolved together in gram-positive and gram-negative bacteria, whereas *ptcA* and *aguB* clearly evolved separately in each type of microorganism.

Discussion

In general, it is assumed that biogenic amines present in food are produced by microbial decarboxylation of amino acids [7,13–15]. However, one of the main contamination-associated biogenic amines, putrescine, and the polyamines spermine and spermidine, can be formed also through another pathway involving the deamination of agmatine [20]. The ornithine decarboxylase gene (*odc*) is the responsible for putrescine production from ornithine, and it has been sequenced from different microorganisms such as *Oenococcus oeni*, *Lactobacillus* 30a, *Vibrio cholerae*, *Escherichia coli*, *Yersinia pestis*, *P. aeruginosa*, *P. putida* (see sequences published in GenBank). PCR-based detection of microorganisms capable of producing putrescine from ornithine is also reported in the literature [22].

Putrescine production was studied in a pathway that involves the deamination of agmatine. Both *aguA* and *ptcA* genes could be amplified in *E. faecalis* ATCC 11700, *B. ce-*

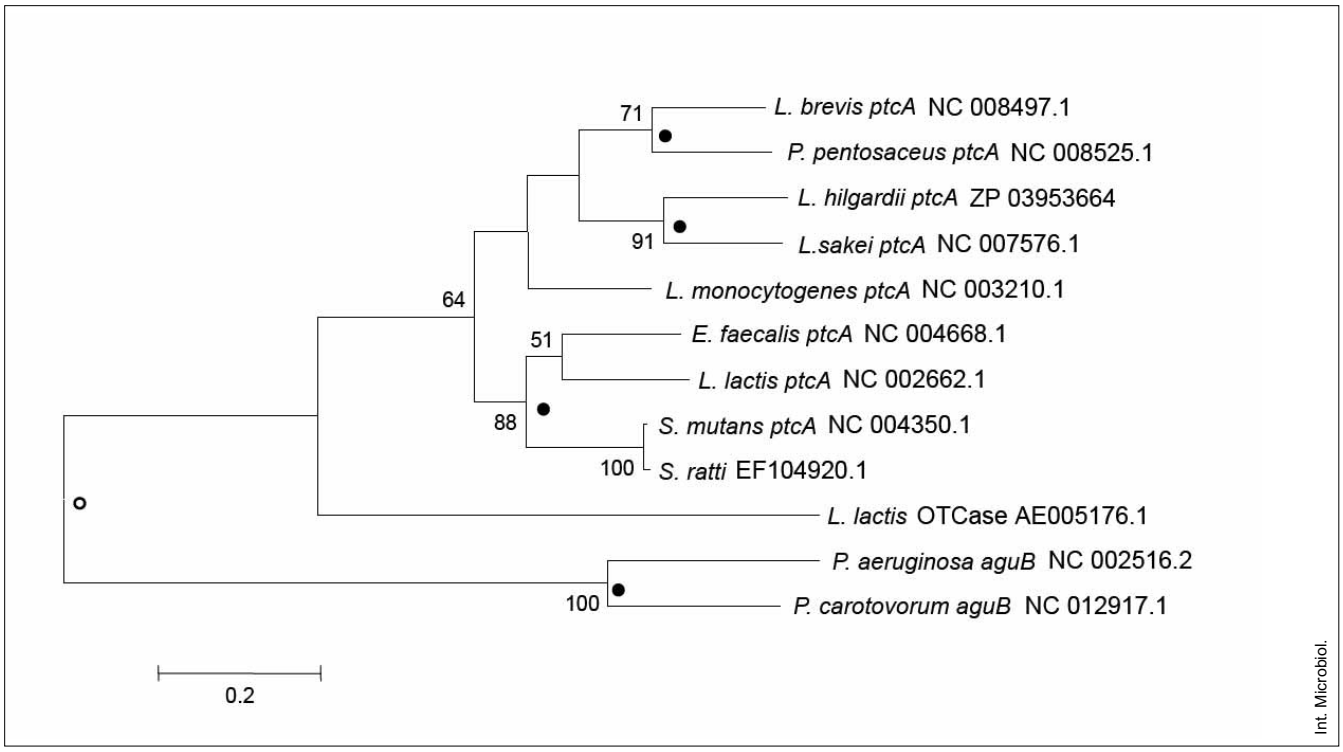

Fig. 6. Maximum likelihood trees based on *aguB* and *ptcA* gene sequences. Bootstrap values (1000 replications) above 50% are shown. Filled and open circles respectively indicate nodes recovered reproducibly by neighbour-joining and maximum likelihood. Bar, substitutions per nucleotide position. Sequences of *aguB* and *ptcA* from: *P. aeruginosa* PAO1 (NC_002516.2), *Pectobacterium carotovorum subsp. carotovorum* PC1 (NC_012917.1), *Listeria monocytogenes* EGD-e (NC_003210.1), *Lb. brevis* ATCC 367 (NC_008497.1), *E. faecalis* V583 (NC_004668.1), *Lc. lactis subsp. lactis* Il1403 (NC_002662.1), *S. mutans* UA159, (NC_004350.1), *P. pentosaceus* ATCC 25745 (NC_008525.1), *Lb. sakei subsp. sakei* 23K (NC_007576.1), *Streptococcus ratti* FA-1 (EF104920.1), *Lb. hilgardii* ATCC 8290 (ZP_03953664) were accessible from GenBank.

reus ATCC 14579 and *Lb. hilgardii* X_1B and produced putrescine from agmatine. Only *aguA* could be amplified in *Lb. sakei* 23K and this organism was unable to produce putrescine. A putative agmatine deiminase protein was found in *Lb. sakei* 23K in the locus Q8RPX2 [5]. In this paper, we have reported that *Lb. sakei* 23K is unable to produce putrescine from agmatine. Moreover, *Lb. sakei* 23K did not show an amplification band of the *ptcA* fragment, although a putative putrescine carbamoyltransferase is present in the agmatine deiminase operon [5]. On the other hand, this microorganism can utilize arginine via the arginine deiminase pathway [37]. We suggest that the putrescine carbamoyltransferase from *Lb. sakei* 23K has lost this function, because *Lb. sakei* 23K was unable to produce putrescine from agmatine.

The *aguA* gene was amplified in *P. aeruginosa* PAO1, which produces putrescine from agmatine; however, *ptcA* could not be amplified. Although the presence of *aguB* in *P. aeruginosa* has been reported [23], *aguB* and *ptcA* are clearly different genes coding for different activities: *N*-carbamoylputrescine amidohydrolase for *aguB* and putrescine carbamoyltransferase for *ptcA*.

Lactobacillus hilgardii X_1B, *E. faecalis* ATCC 11700, *B. cereus* ATCC 14579, *P. aeruginosa* PAO1 and *Lb. sakei* 23K harbour the agmatine deiminase gene (*aguA*) (Fig. 5): all microorganisms have the same PCR-amplifiable gene. However, as *P. aeruginosa* PAO1 has *aguB* but not *ptcA* (Fig. 6), thus we could not amplify *ptcA* with the ptcA-F and ptcA-R primers. In the case of *Lb. hilgardii* X_1B, we have demonstrated that this organism produces putrescine from agmatine via AgDI, via carbamoylputrescine as intermediary [3].

Landete et al. [16] demonstrated different species-dependent behaviours. *Pseudomonas aeruginosa* PAO1 showed a different response to gram-positive bacteria when succinate, spermine, spermidine, histamine, tyramine and sugars on agmatine deamination were tested. Arginine and agmatine influence inhibition and stimulation of pathway agmatine deamination in *P. aeruginosa* PAO1 to a lower extent than in the other species. Nevertheless, this microorganism produced more putrescine than the other bacteria. Although the effects of spermine, spermidine and succinic acid were different for *Lb. hilgardii* X_1B, *E. faecalis* ATCC 11700 and *B. cereus* CECT 148T, a similar proportional

response was obtained for each product in each microorganism. This could mean that a general mechanism regulates putrescine synthesis in these three microorganisms.

Therefore, the control of putrescine production in the AgDI pathways appears to be similar between microorganisms that are more closely related phylogenetically, and differ in microorganisms with a more distant relationship, like *P. aeruginosa*. These data suggest that there should be a different regulation mechanism for *P. aeruginosa* and the other microorganisms studied. The enzyme that converted *N*-carbamoylputrescine to putrescine (the *aguB* or *ptcA* product) was a different enzyme to *P. aeruginosa* PAO1 and the other microorganisms studied here; moreover, the agmatine deiminase gene cluster of *P. aeruginosa* PAO1 differs from other microorganisms (Fig. 4).

Phylogenetic trees were constructed to ascertain the evolutionary relationships between these genes. Two clearly separate clusters can be observed in Fig. 6, suggesting that the *P. aeruginosa* PAO1 *aguB* gene evolved separately from the other *ptcA* genes analysed. Therefore, we confirmed the existence of two families of enzymes that convert *N*-carbamoylputrescine into putrescine. These enzymes are *N*-carbamoylputrescine amidohydrolase (the *aguB* product) and putrescine carbamoyltransferase (*ptcA*), proposed on the basis of phylogenetic studies. This has also been found on MicrobesOnline (data not shown). Thus, it would now seem that a significant number of sequences annotated as ornithine carbamoyltransferase (OTCases) or *N*-carbamoylputrescine amidohydrolase (*aguB*) in the past are in fact putrescine carbamoyltransferase (*ptcA*). The reannotation of this clade of the OTCase sequence as PTCases was confirmed by a comparative neighbourhood study of their encoding genes in fully sequenced and published genomes (*E. faecalis*, *Lb. hilgardii*, *Lb. sakei*, *Lc. lactis*, *S. mutans* and *L. monocytogenes*) [26, and this paper]. These genes successively encode a transcriptional regulator, the reannotated PTCase, an amino acid permease (probably antiporter), and finally the carbamate kinase. When the agmatine deiminase gene cluster has been analysed in other genomes, such as *Pd. pentosaceus* and *Lb. brevis*, the same gene order has been found [21].

Applebaum et al. [2] observed how putrescine plays a role in regulating intracellular pH in *E. coli*. The AgDS yields ammonia, CO_2 and ATP while converting agmatine to putrescine, and it has been proposed to increase acid-resistance properties of *S. mutans* [10]. More recently, Lucas et al. [21] demonstrated that the agmatine deiminase cluster of *Lb. brevis* 9809 belongs to a genomic region providing acid resistance. Liu and Burne [19] showed how three two-component systems influence AgDS gene expression in response to acidic stress. These works have demonstrated that the AgDS plays a role in acid stress resistance. Moreover, the analyses of the genomic region in *Lb. hilgardii* ATCC 8290 demonstrated the presence of a TCS (Fig. 4), which might be involved in acid-stress resistance. The *aguR* (SMU.261.c) gene of *S. mutans*, which encodes a trans-acting factor required for efficient AgDS expression [19], is a putative LuxR-like transcriptional regulator belonging to the FixJ-NarL superfamily; SMU.261.c is required for *S. mutans* AgDS induction by low pH and agmatine [19]. Transcriptional regulators analogous to SMU.261c were found in the agmatine deiminase gene clusters of other microorganisms, as shown in Fig. 4.

In this paper, we have demonstrated that PCR is a useful and rapid method to identify the presence of bacteria able to form putrescine. We have developed the first method—as far as we know—able to detect the *aguA* and *ptcA* genes responsible for putrescine production from agmatine.

Acknowledgements. The authors are grateful to Monique Zagorec (Unité Flore Lactique et Environnement Carné (FLEC), INRA. Domaine de Vilvert, 78 350, Jouy en Josas, France) for providing the *Lb. sakei* 23K strain. The authors gratefully acknowledge the support of the Comisión Interministerial de Ciencia y Tecnología (CICYT), Ministerio de Educación y Ciencia, Spain (Project AGL2000-0827-CO2-01), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT, Argentina) and the Agencia Española de Cooperación Internacional (Spain). English text revised by F. Barraclough.

References

1. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402
2. Applebaum D, Sabo DL, Fisher EH, Morris DR (1975) Biodegradative ornithine decarboxylase of *Escherichia coli*. Purification, properties and pyridoxal 50-phosphate binding site. Biochem 14:3675-3681
3. Arena ME, Landete JM, Manca de Nadra MC, Pardo I, Ferrer S (2008) Factors affecting the production of putrescine by *Lactobacillus hilgardii* X_1B isolated from wine. J Appl Microbiol 105:158-165
4. Arena ME, Manca de Nadra MC (2001) Biogenic amine production by *Lactobacillus*. J Appl Microbiol 90:158-162
5. Chaillou S, Champomier-Verges MC, Cornet M, et al. (2005) The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. Nat Biotechnol 23:1494-1495
6. Coton M, Coton E, Lucas P, Lonvaud A (2004) Identification of the gene encoding a putative tyrosine decarboxylase of *Carnobacterium divergens* 508. Development of molecular tools for the detection of tyramine-producing bacteria. Food Microbiol 21:125-130
7. Farías M, Manca de Nadra MC, Rollan GC, Strasser de Saad AM (1993) Histidine decarboxylase activity in lactic acid bacteria. J Int Sci Vigne Vin 27:191-193
8. Frébort I, Skoupa L, Pec P (2000) Amine oxidase-based flow biosensor for the assessment of fish freshness. Food Control 11:13-18
9. Griswold AR, Chen Y-YM, Burne RA (2004) Analysis of an agmatine deiminase gene cluster in *Streptococcus mutans* UA159. J Bacteriol 186:1902-1904

10. Griswold AR, Jameson-Lee M, Burne RA (2006) Regulation and physiologic significance of the agmatine deiminase system of *Streptococcus mutans* UA159. J Bacteriol 188:834-841
11. Igarashi K, Kashiwagi K (2000) Polyamines: mysterious modulators of cellular functions. Biochem Biophys Res Comum 271:559-564
12. Ivanova N, Sorokin A, Anderson I, et al. (2003). Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature 423:87-91
13. Kalac P, Savel J, Krízek M, Pelikánová T, Prokopová M (2002) Biogenic amines formation in bottled beer. Food Chem 79:431-434
14. Kalac P, Krausová P (2005) A review of dietary polyamines: formation, implications for growth and health and occurrence in foods. Food Chem 90:219-223
15. Landete JM, Ferrer S, Pardo I (2005) Which are the lactic acid bacteria responsible for histamine production in wine? J Appl Microbiol 99:580-586
16. Landete JM, Arena ME, Pardo I, Manca de Nadra MC, Ferrer S (2008) Comparative survey of putrescine production from agmatine deamination in different bacteria. Food Microbiol 25:882-887
17. Le Jeune C, Lonvaud-Funel A, ten Brink, Hofstra H, van der Vossen JMBM (1995) Development of a detection system for histidine decarboxylating lactic acid bacteria on DNA probes, PCR and activity test. J Appl Bacteriol 78:316-326
18. Lehtonen P (1996) Determination of amines and amino acids in wine—a review. Am J Enol Vitic 47:127-133
19. Liu Y, Zeng L, Burne RA (2009) AguR is required for induction of the *Streptococcus mutans* agmatine deiminase system by low pH and agmatine. Appl Environ Microbiol 75:2629-2637
20. Lu CD, Itoh Y, Nakada Y, Jiang Y (2002) Functional analysis and regulation of the divergent *spuABCDEFGH-spuI* operons for polyamines uptake and utilization in *Pseudomonas aeruginosa* PAO 1. J Bacteriol 184:3765-3773
21. Lucas PM, Blancato VS, Claisse O, Magni C, Lolkema JS, Lonvaud-Funel A (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
22. Marcobal A, de las Rivas B. Moreno-Arribas MV, Muñoz R (2004) Identification of the ornithine decarboxylase gene in the putrescine-producer *Oenococcus oeni* BIFI-83. FEMS Microbiol Lett 239:213-220
23. Nakada Y, Itoh Y (2003) Identification of the putrescine biosyntesis genes in *Pseudomonas aeruginosa* and characterization of agmatine deiminase and *N*-carbamoyl putrescine amidohydrolase of the arginine decarboxylase pathway. Microbiology 149:707-714
24. Nakada Y, Jiang Y, Nishijyo T, Itoh Y, Lu C-D (2001) Molecular characterization and regulation of the *aguBA* operon, responsible for agmatine utilization in *Pseudomonas aeruginosa* PAO1. J Bacteriol 183: 6517-6524
25. Naumoff DG, Xu Y, Stalon V, Glansdorff N, Labedan B (2004) The difficulty of annotating genes: the case of putrescine carbamoyltransferase. Microbiology comment. Microbiology 150:3908-3911
26. Naumoff DG, Xu Y, Glansdorff N, Labedan B (2004) Retroviewing sequence of enzyme experimentally characterized but erroneously annotated: the case of the putrescine carbamoyltransferase. BMC Genomics 5:52
27. Rodas AM, Ferrer S, Pardo I (2003) 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. Syst Appl Microbiol 26:412-422
28. Rokka S, Eerola M, Smolander H-L, Alakomi, Ahvenainen R (2004) Monitoring of the quality of modified atmosphere packaged broiler chicken cuts stored in different temperature conditions. B. Biogenic amines as quality indicating metabolites. Food Control 15:601-607
29. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press , Cold Spring Harbor, NY, USA
30. Simon JP, Stalon V (1982) Enzymes of agmatine degradation and the control of their synthesis in *Streptococcus faecalis*. J Bacteriol 152: 676-681
31. Simon JP, Wargnies B, Stalon V (1982) Control of enzyme synthesis in the arginine deiminase pathway of *Streptococcus faecalis*. J Bacteriol 150:1085-1090
32. Stalon V, Mercenier A (1984) L-Arginine utilization by *Pseudomonas* species. J Gen Microbiol 130:69-76
33. Strasser de Saad AM, Manca de Nadra MC (1987) Isolation and identification of the lactic acid bacteria from Cafayate (Argentina) wines. Microbiol Aliments Nutr 5:5-49
34. Straub BW, Kicherer M, Schilcher SM, Hammes WP (1995) The formation of biogenic amines by fermentation organisms. Z Lebensm Unters Forsch 201:79-82
35. ten Brink B, Damink C, Joosten HMLJ, Huis in´t Veld JHJ (1990) Occurrence and formation of biologically active amines in foods. Int J Food Microbiol 11:73–84
36. Yoshida M, Kashiwagi K, Shigemasa A, et al. (2004) A unifying model for the role of polyamines in bacterial cell growth, the polyamine modulon. J Biol Chem 279:46008-46013
37. Zúñiga M, Champomier-Verges M, Zagorec M, Pérez-Martínez G (1998) Structural and functional analysis of the gene cluster encoding the enzymes of the arginine deiminase pathway of *Lactobacillus sakei*. J Bacteriol 180:4154-4159