Evidence for two functionally distinct ornithine decarboxylation systems in lactic acid bacteria

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Abstract

Biogenic amines are low molecular weight organic bases whose presence in food can result in health problems. The biosynthesis of biogenic amines in fermented foods mostly proceeds through amino acid decarboxylation carried out by lactic acid bacteria (LAB), but not all systems leading to biogenic amine production by LAB have been thoroughly characterized. Here, putative ornithine decarboxylation pathways consisting of a putative ornithine decarboxylase and an amino acid transporter were identified in LAB by strain collection screening and database searches. The decarboxylases were produced in heterologous hosts, purified and characterized \textit{in vitro}, whereas transporters were heterologously expressed in \textit{Lactococcus lactis} and functionally characterized \textit{in vivo}. Amino acid decarboxylation by whole cells of the original hosts was determined as well. It is concluded that two distinct types of ornithine decarboxylation systems exist in LAB. One is composed of an ornithine decarboxylase coupled to an ornithine/putrescine transmembrane exchanger. Their combined activity results in the extracellular release of putrescine. This typical amino acid decarboxylation system is present in only a few LAB strains and may contribute to metabolic energy production and/or pH homeostasis. The second system is widespread among LAB. It is composed of a decarboxylase active on ornithine and L-2,4-diaminobutyric acid (DABA) and a transporter that mediates unidirectional transport of ornithine into the cytoplasm. Diamines that result from this second system are retained within the cytosol.
Amino acid decarboxylation systems contribute to the adaptation of lactic acid bacteria (LAB) to various environments such as fruits, vegetables and animals where LAB occur in the oral and genital cavities and in the digestive tract. LAB are also present in thousands of traditional fermented foods where they contribute to the transformation of raw vegetables, milk or meat into elaborated foods. The presence of LAB carrying amino acid decarboxylation systems may lead to the accumulation of the decarboxylation products, commonly known as biogenic amines. These compounds are occasionally regarded as beneficial, as is the case for γ-amino-butyric acid (42), but more often their presence in food is undesired and results in severe health problems following ingestion (13,23,43).

Along with histamine and tyramine, putrescine (1,4-diaminobutane) is one of the most abundant biogenic amines in several fermented foods, including wine (16,26,29), cheese (30), cider (15,24), sausage (44), fish and meat products (20). Putrescine itself does not seem to possess a directly harmful biologic activity; instead, it enhances the toxic effect of histamine and tyramine (9,18).

The biosynthesis of putrescine occurs through the agmatine deiminase (AgDI) or the ornithine decarboxylase (ODC) pathways. Quite interestingly, the prevalence of either pathway in foodborne LAB strains depends upon the environment. Among cider and cheese LAB strains AgDI has a major role (24,25) while in wine, putrescine production mostly proceeds through ODC (36).

The AgDI system of Lactobacillus brevis IOEB 9809 was already thoroughly characterized in a previous publication (31) while little information is available about the ODC systems of LAB. ODC systems were characterized in detail among Gram-negative Enterobacteriaceae (34,35). Similarly to
other bacterial amino acid decarboxylation systems, they consist of a decarboxylase and a precursor/product transmembrane exchanger (2,39). Their combined action results in amino acid intake, decarboxylation and release of the corresponding amine. The pathway results in alkalisation of the cytosol and generation of a proton motive force, which can be exploited for acid stress resistance and/or the production of metabolic energy in the form of ATP. Decarboxylase and transporter genes are generally organised in clusters located on the bacterial chromosome or on plasmids. Among LAB, only two ornithine decarboxylases were purified and characterized to date. ODC from L. sp. 30a was thoroughly characterized (17). A putative ornithine decarboxylation system was reported in strain Oenococcus oeni RM83 (32), where the corresponding genes were likely acquired by horizontal transfer (33). The same authors also report the existence of a second group of genes in some Lactobacillus species that form a phylogenetically distinct group among ODCs. Biochemical proof about the functionality of these enzymes was lately provided only for the ODC from O. oeni (5).

The aim of this work is to gain insight in putrescine biosynthesis in fermented foods, a so far poorly understood process, especially with respect to the ornithine decarboxylase (ODC) pathway. PCR screening and database searches for ODC homologues revealed several putative decarboxylase and transporter genes. Representative decarboxylases and transporters from diverse LAB were characterized and decarboxylation studies using whole cells were performed. The overall results revealed an unexpected diversity within the ornithine decarboxylation systems of LAB.

MATERIALS AND METHODS
**Bacterial strains and cultures.** The LAB strains employed in this study were either purchased from culture collections or originated from the collection of the Institut d’Œnologie de Bordeaux (IOEB, Bordeaux, France). A complete list of the strains employed is presented in table S1 (supplemental material). Strains were cultured in half-strength Man, Rogosa and Sharpe medium (Beckton Dickinson, Sparks, MD) set at pH 5.0. Growth temperatures ranged from 25°C to 37°C.

**Determination of gene sequences.** PCR products (size ~900 bp) corresponding to an internal segment of putative ODC genes were obtained for some strains. Reaction conditions are described in a previous work (11). In strains Oenococcus oeni IOEB 89006 and Lactobacillus brevis IOEB 9906 the sequences of neighbouring genomic regions were obtained by means of conventional molecular biology techniques. Briefly, the genomic DNA of the two strains was digested and submitted to enzymatic ligation. The ligation mixtures were then purified and employed as matrices for reverse PCR reactions. PCR products were finally sequenced. The sequence obtained with O. oeni consisted of a 3,750 bp product that contained putative ODC and ornithine/putrescine antiporter genes. These displayed 100% identity with ODC and PotE from O. oeni RM83 (CAG 34069 and CAM 07323 respectively). As for L. brevis, a 4,732 bp sequence was obtained. This contained a putative ODC and an ornithine/putrescine antiporter genes and was deposited in GenBank under the accession number JN120479.

**Expression and purification of recombinant decarboxylases.** Decarboxylases were obtained as recombinant His-tagged fusion proteins within *Escherichia coli*. PCR products were submitted to ligase independent directional cloning within pET100/D-TOPO vectors (Invitrogen, Carlsbad, CA). Chemically competent *E. coli* (BL21 Star One Shot, Invitrogen) was employed as expression host. Cloning,
transformation and expression were performed following the protocols provided by the manufacturer. It was verified by sequencing that all products were correctly inserted within the expression construct and that no mutations had intervened throughout the cloning procedure. Cell-free enzymatic extracts were submitted to purification by means of affinity chromatography. Briefly, cell-free enzymatic extracts were submitted to purification by means of affinity chromatography on a BioLogic DuoFlow chromatographic system (Biorad, Marnes-la-Coquette, France) equipped with a HiTrap Chelating HP column (Amersham Biosciences, Uppsala, Sweden). Elution was performed by means of a pH 7.5 potassium phosphate buffer supplemented with increasing amounts of imidazole. SDS-PAGE analysis allowed to isolate fractions containing the pure recombinant enzymes.

Decarboxylation assays on purified enzymes. Enzymatic reactions were performed on 200 μl volumes. Four micrograms of enzyme and 0.4 mM pyridoxal-phosphate (PLP) were employed. Reaction mixtures were incubated for up to one hour at 37°C. Reaction velocities were determined by sampling at regular intervals (typically 10, 30 and 60 minutes). Different substrate concentrations (DABA, lysine or ornithine, at 0.5-100 mM) were employed for the calculation of kinetic parameters. pH activity profiles were established at 10 mM substrate concentrations. Enzymatic activity was stopped by addition of 10 μl trichloroacetic acid (20% v/v) and samples were stored at -20°C until analysis. Kinetic parameters were calculated with the aid of Graphpad Prism 5.04 (Graphpad Software, La Jolla, CA). Assays were performed in 0.05 M buffers. Depending upon the pH of the reaction, different buffers were prepared: citrate (3.0-6.0), phosphate (6.0-8.0), borax (8.0-10.0). Changes in buffer composition had no influence on the enzymatic activity. For modelling pH dependence of enzymatic activity a classical diprotic model was employed (37) and pH activity profiles were established with the aid of GraFit 7.0 (Erithacus Software, Horley, UK).
Decarboxylation assays on whole cells. Strains were cultured until mid-exponential phase (0.5-1.5 OD units ml$^{-1}$, depending upon the strain). Cells were harvested, washed twice with potassium phosphate buffer (0.1 M, pH 5.0) and finally resuspended at 1.0 OD units ml$^{-1}$ in the same buffer containing either ornithine, lysine or diaminobutyric acid at the concentration of 10 mM. Cell suspensions were incubated at 25°C ($O. oeni$), 30°C ($L. brevis$) or 37°C ($L. gasseri$ and $L. casei$). Suspensions were finally centrifuged (10 minutes at 10,000×$g$) and supernatants were directly analysed. Pellets were washed two times with buffer and resuspended in saturated NaHCO$_3$ solution at the final concentration of 10 OD units ml$^{-1}$. Preliminary trials indicated that supplementary extraction treatments of the biomass (e.g. freeze/thaw cycles or incubation with lysozyme) did not significantly improve extraction. Derivatisation and thin layer chromatography (TLC) analysis were then performed as described below.

Thin-layer chromatography (TLC). An analytical method was expressly developed with the aim of detecting and quantifying all decarboxylation products. This was based upon sample derivatisation with fluorescent dansyl chloride, separation by TLC and quantification by densitometric analysis. Fifty microlitre aliquots of each sample were supplemented with 50 μl of a saturated NaHCO$_3$ solution and 100 μl dansyl chloride solution (5 mg ml$^{-1}$ in acetone). Reactions mixtures were incubated at 55°C for 1 hour to allow for the formation of fluorescent adducts. Ten microlitre aliquots of the reaction mixtures were deposed on TLC silica60 plates (Merck, Darmstadt, Germany). Plates were developed for 7 cm in different eluent mixtures, depending upon the products analyzed. Chloroform/triethylamine (4/1) and chloroform/triethylamine/methanol (15/4/1) were used for diamine and agmatine determination respectively. TLC plates were illuminated at a wavelength of 312 nm and images were captured by means of an Infinity-1000 imaging system (Vilber-Lourmat, Marne-
la-Vallée, France). The images were analysed with the aid of BIO-1D software (Vilber-Lourmat). All reagents and standards for TLC analysis were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The method allowed for the quantification of amine amounts as low as 5 μM.

**Cloning and expression of transporter genes.** The gene encoding the PotE homolog of *O. oeni* IOEB 89006 was amplified from chromosomal DNA by PCR with primers potE-fw (5′-gcgaacacatggctcaagagaaaaatgggcgt) and potE-rv (5′-gcgaatctagatctatcaatgctaggtaacagc), introducing NcoI and XbaI restriction sites. Phusion High Fidelity DNA polymerase (Thermo Scientific) was used for amplification, following a procedure provided by the manufacturer. The PCR product was, after digestion with NcoI and XbaI (Fermentas), ligated (T4 DNA ligase, Fermentas) into the NICE system expression vector pNZ8048 (12), which was digested with the same enzymes. The ligation mixture was transformed into *Lactococcus lactis* NZ9000. Chloramphenicol resistant clones were tested for harbouring the correct construct, which was designated pNZpotE. The putative amino acid transporter gene of *L. gasseri* ATCC 33323 was cloned in two steps. The first 1416 bp were amplified using primers lg1880-fw (5′-gcgaacatggtaaaccagatcttataattgactaacccg) and lg1800-rvm (5′-gcgaactgcagaaaacgaatcctaatccaaac) and cloned into pNZ8048, using NcoI and PstI restriction sites. In a second step, the complete gene was amplified using primers lg1880-fw and lg1800-rv (5′-gcgaacctgtgattttattgctacaaaagattgg) followed by restriction with AatII (endogenous restriction site) and PstI. The fragment that included the 200 bp 3′-end of the gene was cloned into the first construct by replacement of the AatII-PstI fragment, resulting in pNZ-lg1880. For gene expression, *L. lactis* cells harbouring the constructs were grown to mid-exponential phase (OD600 ~ 0.6) in M17 supplemented with 0.5% (w/v) glucose, then induced with 5 mg/L of nisin and left to grow for one more hour.
Transport assays. *L. lactis* cells expressing the transporter genes and control cells harbouring the empty vector pNZ8048 were washed and resuspended in 100 mM potassium phosphate buffer, pH 6, to an OD\textsubscript{600} of 2.0. Glucose was added to 0.2% (w/v) and cells were kept on ice until the transport assays. Samples of 100 μl were incubated for 5 min at 30°C with constant stirring. At time zero, 14C-Ornithine (PerkinElmer, Waltham, MA) or 14C-L-putrescine (Amersham, Diegem, Belgium) were added to final concentrations of 17.5 μM and 4.7 μM, respectively. In the exchange experiments, unlabeled putrescine, Ornithine or L-lysine were added to final concentrations as indicated. In competition experiments to determine $K_m$ and $K_i$ values, cells were incubated with 17.5 μM 14C-labelled ornithine and unlabeled substrate at concentrations ranging from 0 to 1 mM (lysine and ornithine) or 0 to 10 mM (DABA). Uptake was stopped by addition of 2 ml of ice-cold 0.1 M LiCl solution, followed by filtration through a 0.45-μM-pore-size nitrocellulose filter (BA 85, Whatman GmbH, Dassel, Germany). The filter was washed once with 2 ml of 0.1 M LiCl and submerged in Emulsifier Scintillator Plus scintillation fluid (Packard Bioscience, Meriden, CT) and radioactivity was measured in a Tri-Carb 2000CA liquid scintillation analyzer (Packard Instruments).

RESULTS

Collection screening and database searches disclose several uncharacterized putative ODC systems among LAB. In a previous work (11) a collection of 275 cider and wine LAB strains was screened for the presence of genes coding for ODCs using primers based upon the gene encoding the well-characterized ODC of *Lactobacillus* sp. 30a (17). The same approach was adopted for the screening of 40 more LAB strains originating from diverse food-related environments (supplemental material,
Table S1). Overall, four strains scored positive: the *L.* sp. 30a control strain, two *O. oeni* strains (namely IOEB 89006 and IOEB 9915) and strain *Lactobacillus brevis* IOEB 9906.

The ODC from *Lactobacillus* sp. 30a was already characterized (17). The other ODC positive strains, namely *O. oeni* IOEB 89006, *O. oeni* IOEB 9915 and *Lactobacillus brevis* IOEB 9906, were investigated in further detail. The complete gene sequences of the putative ODCs and the adjacent amino acid transporters that supposedly are associated with these ODCs (ornithine/putrescine exchangers PotE) were determined. The sequences of the putative ODCs and transporters from the two *O. oeni* strains were identical, so the study of strain IOEB 9915 was abandoned. The *O. oeni* and *L.* brevis ODCs shared 68% and 66% amino acid sequence identity with the *L.* sp. 30a enzyme, respectively. The two transporter proteins shared 84% sequence identity (Fig. 1).

Database searches using BlastP (1) and the *O. oeni*, *L. brevis* and *L.* sp. 30a ODCs as queries revealed no closely related homologues in LAB, but disclosed a large family of more distantly related proteins annotated as putative arginine/lysine/ornithine decarboxylases exclusively found in *Lactobacillus* species. These enzymes shared up to 43% amino acid sequence identity with the *L.* sp. 30a ODC (Fig. 1). To better understand their phylogenetic relationships, all protein sequences were compared in a neighbour-joining tree with the aid of MEGA4 software (47). The tree contained representative amino acid decarboxylases of the aspartate amino-transferase family, including all known substrate specificities (Fig. 2A). Decarboxylases identified in LAB or closely related species included ornithine decarboxylases, glutamate decarboxylases, tyrosine decarboxylases as well as putative arginine/lysine/ornithine decarboxylases. Decarboxylases not described in LAB to date include lysine decarboxylases, arginine decarboxylases, and histidine decarboxylases that are frequently observed in Gram-negative bacteria. Histidine decarboxylation is a trait of LAB as well, but the decarboxylase is from a different family, the pyruvoyl-dependent decarboxylases (14).
Phylogenetic groups accurately reflected the different substrate specificities as already reported (7).

All basic amino acid decarboxylases (i.e. arginine, lysine and ornithine decarboxylases) form separate subgroups. ODCs of the LAB L. sp. 30a, O. oeni, and L. brevis are in the same cluster as ODCs found in Gram-negative bacteria. The putative arginine/lysine/ornithine decarboxylases form a separate cluster that is closely related but distinct from the ODC cluster. Interestingly, in most species the putative decarboxylase genes were adjacent to a putative amino acid transporter gene, even though gene disposition differs from what was observed for O. oeni and L. brevis (Fig. 1). The neighbour-joining tree constructed on the basis of the alignment of the transporter amino acid sequences showed the same clusters which is in line with the functional relation between the decarboxylase and corresponding transporter in one species (Fig. 2B). The most striking difference between the two trees was the phylogenetic relation of the putative arginine/lysine/ornithine decarboxylases and corresponding transporters. While the decarboxylases were close to the ODCs of Gram-positive and Gram-negative species, the transporters seemed to form a separate clade, distant from the ornithine/putrescine exchangers PotE. According to the transporter classification system (41) both transporter types are members of the APC superfamily of transporters, but belong to distinct families (19). The putative amino acid transporters from Lactobacillus spp. associated with the putative L-arginine/L-lysine/L-ornithine decarboxylases are closest to the GGA (Glutamate:GABA Antiporter; TC 2.A.3.7) family, whereas PotE homologues are members of the APA (Basic Amino Acid/Polyamine Antiporter; TC 2.A.3.2) family. The difference between the decarboxylase/transporter pairs is emphasized by the organization on the chromosome. ODCs and PotE are organized in an operon in which the potE gene is downstream of the odc gene. In contrast, the amino acid transporter gene of L. gasseri is located upstream of the decarboxylase and the genes are transcribed divergently (Fig. 1).
Enzymes from *O. oeni* and *L. brevis* are typical ODCs, in contrast to *L. gasseri* and *L. casei* decarboxylases. To better understand the role of the putative decarboxylation systems detected in LAB, the putative ODCs from *L. brevis* IOEB 9906 and *O. oeni* IOEB 89006, and the putative arginine/lysine/ornithine decarboxylases from *Lactobacillus gasseri* ATCC 33323 and *Lactobacillus casei* ATCC 334 were selected for further study as representatives of the two groups of decarboxylases. The four genes were cloned in *Escherichia coli*, in frame with an upstream sequence encoding a His-tag. This allowed for the production of soluble recombinant proteins that were purified by affinity chromatography. For each protein, a pure product was obtained with the expected molecular mass, ranging from 79 to 87 kDa (verified by SDS-PAGE, results not shown). Decarboxylation activity of the four recombinant proteins with the four basic amino acids ornithine, arginine, lysine and L-2,4-diaminobutyric acid (DABA) as substrates was measured by determining the respective decarboxylation products (*i.e.* putrescine, agmatine, cadaverine and diaminopropane) using a method, based upon dansylation and thin-layer chromatography (see experimental section).

The pH of the reaction medium was 5.5 in case of the enzymes from *O. oeni* and *L. brevis* and 7.5 for *L. gasseri* and *L. casei*. These pH values were chosen on the basis of preliminary trials that allowed to establish an approximate range of optimal activity for each recombinant decarboxylase (results not shown). The substrates were added at variable concentrations (0.5 to 100 mM) and initial decarboxylation rates were estimated.

Steady-state kinetic parameters were estimated whenever reliable initial rates could be determined (Table 1). The ODCs from *O. oeni* and *L. brevis* showed a clear preference for ornithine as the substrate. The affinities for ornithine were 0.7 and 1 mM, respectively, while the affinities for DABA and lysine were 5-10 times lower. However, the most prominent difference was observed in the maximal rates resulting in a catalytic efficiency expressed as $k_{cat}/K_m$ of the enzyme from *O. oeni* that
was 500-fold and 1,100-fold higher with ornithine than observed with lysine and DABA, respectively. The decarboxylase from \textit{L. brevis} was somewhat less strict with catalytic efficiencies for lysine and DABA decarboxylation that were 116 and 60 lower than for ornithine, respectively. The putative arginine/lysine/ornithine decarboxylases from \textit{L. gasseri} and \textit{L. casei} displayed substrate preference for DABA. The affinities were in the range between 4 and 12 mM, similar to those observed for the two ODCs for this substrate. A higher maximal rate was observed for the \textit{L. gasseri} enzyme with DABA as substrate resulting in a catalytic efficiency of $1.8 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$ that, nevertheless, was two orders of magnitude lower than observed for the ODCs with ornithine as the substrate. The \textit{L. casei} enzyme appears to have a somewhat stronger preference for DABA over ornithine than the \textit{L. gasseri} enzyme. The rates of lysine decarboxylation by the \textit{L. gasseri} enzyme and lysine and ornithine decarboxylation by the \textit{L. casei} enzyme were too low to allow for an accurate estimation of kinetic parameters (less than 0.1% conversion with 20-80 mM substrate after one hour). In such cases, the catalytic efficiency would be at least 10-fold lower than that obtained for the preferred substrate. In summary, ODCs are ornithine decarboxylases that show a low activity with DABA and lysine as substrates. The enzymes annotated as arginine/lysine/ornithine decarboxylases have a lower specificity, decarboxylating ornithine and DABA, but most of all, a much lower efficiency when compared to the ODCs. Arginine decarboxylation was never observed.

The purified enzymes from the two groups of decarboxylases showed different pH profiles. Fig. 3 shows the profiles for the preferential substrates (\textit{i.e.} ornithine for \textit{O. oeni} and \textit{L. brevis} and DABA for \textit{L. gasseri} and \textit{L. casei}). The pH activity optima of decarboxylases from \textit{O. oeni} and \textit{L. brevis} were significantly lower than those of \textit{L. gasseri} and \textit{L. casei}. The enzyme from \textit{L. brevis} showed a broad profile with an optimum at pH 5.6 which is similar to that of the ODC of \textit{L. sp. 30a} reported before (17). The \textit{O. oeni} decarboxylase reached its peak of activity at pH 5.1 and was significantly less active.
at pH values above 6.0. The pH profiles of the decarboxylases from *L. casei* and *L. gasseri* were relatively narrow and displayed activity optima at pH 6.7 and 7.3 respectively.

**PotE of *O. oeni* catalyzes ornithine/putrescine exchange.** The gene encoding the PotE homologue of *O. oeni*, located downstream the *odc* gene (Fig. 1), was expressed in *L. lactis* NZ9000 using the NICE system (12). At a concentration of 17.5 μM, the initial rate of 14C-labelled L-ornithine uptake by *L. lactis* cells harbouring the empty vector pNZ8048 was 3.0 nmol min⁻¹ mg⁻¹ (Fig. 4A). The background ornithine uptake in control cells is likely mediated by the arginine/ornithine exchangers ArcD1 and/or ArcD2 (6,38) or other endogenous transporters of *L. lactis*. The uptake rate of ornithine increased up to 9.6 nmol min⁻¹ mg⁻¹ in cells expressing *potE* demonstrating functional expression of the transporter of *O. oeni* in *L. lactis*. Similar transport assays with 14C-putrescine as substrate showed an even more distinct effect. When present at 4.7 μM, putrescine was taken up at an initial rate of approximately 12 nmol min⁻¹ mg⁻¹ in cells expressing *potE*, whereas control cells did not show any significant uptake. Convincing evidence for the function of PotE as an ornithine/putrescine exchanger was obtained when an excess of unlabeled ornithine was added to cells that were allowed to take up putrescine for 1 minute. A rapid release of putrescine from the cells was observed (Fig. 4B). Similarly, addition of an excess of unlabeled putrescine led to the release of labelled putrescine, showing putrescine/putrescine exchange.

The affinity of PotE of *O. oeni* for ornithine, putrescine, DABA and lysine was determined from the inhibition of 14C-labelled ornithine uptake by different concentrations of the unlabelled substrates. A *Kₘ* value for ornithine was estimated at 50-75 μM; *Kₘ* values for lysine and DABA were estimated at less than 100 μM and between 1 and 10 mM, respectively (data not shown).
together, the results show that PotE of O. oeni is an ornithine/putrescine exchanger, with affinity for lysine and cadaverine, but not for DABA.

The L. gasseri transporter catalyzes unidirectional ornithine transport. The putative amino acid transporter gene located adjacent to, but divergently transcribed from the putative arginine/lysine ornithine decarboxylase of L. gasseri, was cloned and expressed in L. lactis NZ9000 as described above. A clear increase in initial ornithine uptake rate from 2.4 nmol min⁻¹ mg⁻¹ in control cells to 8.4 nmol min⁻¹ mg⁻¹ in cells expressing the transporter was observed (Fig. 4C), demonstrating transport activity of the cloned gene product with ornithine as the substrate. In contrast, no ¹⁴C-labeled putrescine uptake could be detected (Fig. 4D) and addition of an excess of unlabelled putrescine to cells that were allowed to take up ¹⁴C-ornithine to a steady state level did not result in efflux of accumulated ornithine (Fig. 4C). This shows that the encoded transporter is not an ornithine/putrescine exchanger, but most likely a unidirectional ornithine transporter.

The affinity of the transporter for ornithine was determined by measuring the inhibition of ¹⁴C-labelled ornithine uptake at varying concentrations of unlabelled ornithine. The $K_m$ value was estimated to be 74 μM (not shown).

Following the observation of DABA decarboxylation activity of the putative arginine/lysine/ornithine of L. gasseri, affinity of the transporter for DABA was tested by competition experiments as described above using ¹⁴C-labelled ornithine uptake at a concentration of 17.5 μM and a range of concentrations of unlabelled DABA or lysine. A $K_i$ value was estimated at 12.4 mM, indicating very low affinity of the transporter for DABA. A higher affinity for lysine was observed: ¹⁴C-ornithine uptake was inhibited by unlabelled lysine with an estimated $K_i$ of 1.0 mM.
Decarboxylation products are released by *O. oeni* and *L. brevis*, but not by *L. gasseri* and *L. casei*. Amino acid decarboxylation was determined on resting cells of the four strains. Cell suspensions were incubated in the presence of 10mM ornithine, lysine or DABA. Samples were taken at regular intervals and the decarboxylation products in culture supernatants and in the cytoplasm were determined separately. Analysis of supernatants revealed that *O. oeni* and *L. brevis* were able to release decarboxylation products into the medium (Fig. 5A and 5B). In agreement with the kinetic characteristics of the decarboxylases and transporters, the two bacteria released at least 20-fold more putrescine than cadaverine while diaminopropane was not detected. Analysis of the cell content revealed that in *O. oeni* and *L. brevis* relatively small amounts of putrescine (approximately 1,000-fold less than outside the cell) could be detected in the cytoplasm when ornithine was added.

In contrast to *O. oeni* and *L. brevis*, *L. gasseri* and *L. casei* did not release decarboxylation products into the culture medium. Relatively small amounts (0.3 to 1.2 μmols per gram of wet cells) of putrescine and diaminopropane could be detected within the cytoplasm of both strains, even when no amino acid precursor was added to the medium. When 10 mM DABA was added to *L. gasseri* cell suspensions intracellular concentrations of diaminopropane increased by 0.4 μmol x g⁻¹ and, most importantly, ornithine addition resulted into an almost four-fold increase (from 0.5 to 1.9 μmol x g⁻¹, Fig. 5C) in intracellular putrescine. In *L. casei* both putrescine and diaminopropane could be detected but these did not vary significantly throughout the whole incubation time, regardless of the presence of candidate precursors (Fig. 5D). Cadaverine was not detected inside the cells, in the absence nor presence of lysine.

**DISCUSSION**
The first evidence for LAB ornithine decarboxylases was obtained from strain *L. sp.* 30a (40). A putative ODC system was later detected in *O. oeni* RM83 (32,33). Finally, during the drafting of this manuscript, a partial functional characterization of a recombinant ODC from *O. oeni* was published (5). Overall, the knowledge of this subject remains sparsely distributed.

This work represents the first systematic survey on ornithine decarboxylation by LAB. Our experimentation disclosed two candidate ODC systems in strains *O. oeni* IOEB 89006 and *L. brevis* IOEB 9906, both consisting of a putative decarboxylase gene and a putative transporter gene. The genes found in *O. oeni* IOEB 89006 showed 100% identity to homologues described in *O. oeni* RM83 (32,33). In the present work, thorough functional characterization of the decarboxylase and the ornithine/putrescine transporter were compared with results obtained on whole cells of the same strain. Like its orthologue from *L. sp.* 30a, the enzyme accepted ornithine as the preferred substrate with relatively high specificity and reached its optimum activity at fairly acidic pH (5.1 compared to 5.8 for *L. sp.* 30a). These results are substantially in agreement with those presented in the literature (5), but in addition to preference for ornithine with respect to other substrates, they were accurately expressed in terms of ratios between the respective catalytic efficiencies. The transporter mainly catalysed ornithine/putrescine exchange but also had affinity for lysine and its decarboxylation product cadaverine. The functionality of the ODC system was finally corroborated by the results on whole cells, which confirmed ornithine decarboxylation and putrescine release and again, to a lesser extent, lysine decarboxylation and cadaverine production. The ODC from *L. brevis* IOEB 9906 was also characterized. The overall results suggest that relatively strict specificity for ornithine and fairly low pH activity optimum are common features of ODC systems from LAB. The product encoded by PotE from *L. brevis* was not characterized but the high identity to its homologue from *O. oeni* (84%) and the
results obtained on whole cells (again showing putrescine and cadaverine release) leave little doubt
as to whether this gene actually encodes an ornithine/putrescine antiporter.

This work also demonstrates the existence of a second, functionally distinct group of ornithine
decarboxylase systems in LAB. The enzymes from \textit{L. gasseri} and \textit{L. casei} were proven to be active on
DABA and ornithine. When compared to the ODCs from \textit{O. oeni} and \textit{L. brevis} they showed relatively
low catalytic efficiency and relatively high pH activity optima. The decarboxylase from \textit{L. gasseri}
displayed a unique dual specificity for DABA and ornithine, with a slight preference for the former, a
novel functionality which has not been observed for ornithine decarboxylases before. In contrast, dual
specificity of amino acid decarboxylases for lysine and ornithine have been reported (27,46). Further
experimentation demonstrated that the putative amino acid transporter from \textit{L. gasseri} functions as a
unidirectional basic amino acid transporter, with ornithine as the preferred substrate. Surprisingly, its
affinity for DABA was low. Whether this is physiologically relevant with respect to precursor supply for
the decarboxylase, or whether other mechanisms for DABA supply are present remains unclear.

Nevertheless, the ornithine transport activity of the transporter, the ornithine decarboxylation
activity of the decarboxylase and the adjacent location of the respective genes strongly suggest that
they are part of the same functional pathway. Experiments on whole cells showed that \textit{L. gasseri} was
able to accumulate putrescine within the cytoplasm when ornithine was supplemented to the
medium. This supports the functionality of the decarboxylase/transporter system from \textit{L. gasseri}. As
far as \textit{L. casei} is concerned, intracellular accumulation of diamines was not observed when either
ornithine or DABA were supplied. This is not surprising, given the fact that in this strain the gene
encoding for the decarboxylase doesn’t have a neighbouring putative transporter gene. It is therefore
possible that in \textit{L. casei} amino acid decarboxylation is not supported by direct precursor supply from
outside the cell; the reaction could instead be part of a more complex, multi-reaction pathway.
The existence of a second group of ornithine decarboxylases in LAB was first postulated by Marcobal et al. (2004). To date, putative decarboxylases of this second group are considered to be typical ODCs and the corresponding gene sequences are employed in the design of primers for the detection of putrescine producers (10). On the other hand, the reports concerning the functionality of these enzymes are rare and contradictory. Strain *L. acidophilus* NCFM has putative amino acid decarboxylase and transporter genes (accession YP 193877 and YP 193876 on Fig. 2A and 2B respectively) which are very close to their orthologues from *L. gasseri* ATCC 33323 in terms of sequence and disposition. Inactivation of the genes compromised the natural acid resistance of the strain (4). In the industrial cheese starter *L. helveticus* CNRZ a putative system of the same type is detected (accession YP 001577442 and YP 001577955 on Fig. 2A and 2B respectively), but the strain is not a putrescine producer (8). The data provided here suggest that these decarboxylases, due to their low catalytic efficiency and the absence of a system for putrescine release, cannot account for the production of milligram amounts of putrescine.

Possible physiological functions of the newly discovered DABA/ornithine decarboxylase system may be inferred by analogy with similar systems described in Gram-negative bacteria. In *E. coli* a typical ODC system coexists with a second ornithine decarboxylase, named biosynthetic (34). While the former is related to acid stress resistance, the role of the latter enzyme is to synthesize putrescine, which is retained within the cytoplasm (35). Similarly to the two types of LAB decarboxylases described in this work, the two types of enzyme display different pH optima (3). Intracellular putrescine is partly bound to nucleic acids and its function is possibly related to the modulation of gene expression (21). Putrescine can also be addressed to the synthesis of polyamines, such as spermidine and spermine (45). In other Gram-negative bacterial species (e.g. *Vibrio* and...
Acinetobacter spp.) DABA decarboxylases are found that also function in polyamine biosynthesis (22,28).

Thanks to the present work the search for putrescine producers, which so far mostly relied upon gene annotation, is now more robustly supported by biochemical and microbiological proof. The newly characterized decarboxylase systems provide a basis for the design of more sensitive and less error-prone biomolecular tools for the detection of putrescine producing bacteria in food and other human-related environments.

ACKNOWLEDGEMENTS

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REFERENCES


FIG. 1. Schematic representation of different decarboxylation systems. Percentage identities with amino acid sequences of decarboxylase from L. sp. 30a and transporter from O. oeni are reported.

ODC: Ornithine decarboxylase, PotE: Ornithine/putrescine antiporter, AAT: putative amino acid transporter.

FIG. 2. Neighbour-joining trees based upon the alignment of PLP-dependent amino acid decarboxylases (A) and amino acid transporters (B). Sequences were aligned with the aid of MEGA4 software (44) by employing ClustalW as algorithm. Percentage values from 1000 bootstrap replicates are indicated, values less than 90% are not shown. Representative sequences are presented for each substrate specificity. Non-LAB sequences are shaded. The proteins that were characterized in the present study are marked with a black square.

FIG. 3. pH activity profiles of decarboxylases from O. oeni / L. brevis (top panel) and L. gasseri / L. casei (bottom panel). In each graph the profiles of the enzymes from the opposite group are shown in shaded format. Each enzyme was tested against 10 mM of the preferential substrate. Experimental points represent mean results of triple replicates.

FIG. 4. Ornithine (A and C) and putrescine (B and D) uptake by resting cells of L. lactis NZ9000 expressing potE of O. oeni (▼, A; ○, B) or lgas_1880 of L. gasseri (▼, C; ○, D) or harbouring the empty vector pNZ8048 (●). 14C-labelled Ornithine and putrescine were added to 17.5 μM and 4.7 μM final concentration, respectively. Unlabelled Ornithine and putrescine at a concentration of 1 mM were added after 60 s to cells expressing potE (B; △ and ▼, respectively). Unlabelled putrescine at a
concentration of 1 mM was added after 30 s to cells expressing lgas_1880 (Δ) and control cells harbouring pNZ8048 (○) (C). Experimental points represent mean results of triple replicates.

FIG. 5. Extracellular release of putrescine (□) and cadaverine (■) by *O. oeni* (panel A) and *L. brevis* (panel B). Intracellular contents of putrescine (□) and diaminopropane (▲) in *L. gasseri* (panel C) and *L. casei* (panel D). Values are expressed as mmols or μmols per gram of wet cells. Experimental points represent mean results of triple replicates. Error bars represent standard errors.
TABLE 1. Steady-state kinetic parameters and substrate specificity of the four decarboxylases

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>O. oeni</th>
<th>L. brevis</th>
<th>L. gasseri</th>
<th>L. casei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction pH</td>
<td>5.5</td>
<td>5.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Ornithine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ min$^{-1}$</td>
<td>416 ± 18$^a$</td>
<td>290 ± 13</td>
<td>8.3 ± 0.5</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>$K_m$ mM</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>7.2 ± 1.4</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ M min$^{-1}$</td>
<td>5.5 x 10$^5$</td>
<td>2.8 x 10$^5$</td>
<td>1.2 x 10$^3$</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>DABA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ min$^{-1}$</td>
<td>2.7 ± 0.2</td>
<td>27 ± 4</td>
<td>21 ± 2</td>
<td>3 ± 0.1</td>
</tr>
<tr>
<td>$K_m$ mM</td>
<td>4.9 ± 1.6</td>
<td>11.2 ± 3.6</td>
<td>12.1 ± 2.2</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ M min$^{-1}$</td>
<td>0.5 x 10$^3$</td>
<td>2.4 x 10$^3$</td>
<td>1.8 x 10$^3$</td>
<td>0.7 x 10$^3$</td>
</tr>
<tr>
<td>L-lysine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ min$^{-1}$</td>
<td>5.6 ± 0.6</td>
<td>34 ± 4</td>
<td>ND</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>$K_m$ mM</td>
<td>5.0 ± 1.6</td>
<td>7.2 ± 2.9</td>
<td>ND</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ M min$^{-1}$</td>
<td>1.1 x 10$^3$</td>
<td>4.6 x 10$^3$</td>
<td>ND</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>L-arginine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k_{cat}$ min$^{-1}$</td>
<td>NA$^c$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>$K_m$ mM</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$ Values are means and standard deviations of triplicates

$^b$ ND, low activity did not allow to estimate kinetic parameters

$^c$ NA, no detectable activity
AUTHOR’S CORRECTION

Evidence of Two Functionally Distinct Ornithine Decarboxylation Systems in Lactic Acid Bacteria

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