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Three-Component Lysine/Ornithine Decarboxylation System in Lactobacillus saerimneri 30a

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Lactic acid bacteria play a pivotal role in many food fermentations and sometimes represent a health threat due to the ability of some strains to produce biogenic amines that accumulate in foods and cause trouble following ingestion. These strains carry specific enzymatic systems catalyzing the uptake of amino acid precursors (e.g., ornithine and lysine), the decarboxylation inside the cell, and the release of the resulting biogenic amines (e.g., putrescine and cadaverine). This study aimed to identify the system involved in production of cadaverine from lysine, which has not been described to date for lactic acid bacteria. Strain Lactobacillus saerimneri 30a (formerly called Lactobacillus sp. 30a) produces both putrescine and cadaverine. The sequencing of its genome showed that the previously described ornithine decarboxylase gene was not associated with the gene encoding an ornithine/putrescine exchanger as in other bacteria. A new hypothetical decarboxylation system was detected in the proximity of the ornithine decarboxylase gene. It consisted of two genes encoding a putative decarboxylase sharing sequence similarities with ornithine decarboxylases and a putative amino acid transporter resembling the ornithine/putrescine exchangers. The two decarboxylases were produced in Escherichia coli, purified, and characterized in vitro, whereas the transporter was heterologously expressed in Lactococcus lactis and functionally characterized in vivo. The overall data led to the conclusion that the two decarboxylases and the transporter form a three-component decarboxylation system, with the new decarboxylase being a specific lysine decarboxylase and the transporter catalyzing both lysine/cadaverine and ornithine/putrescine exchange. To our knowledge, this is an unprecedented observation of a bacterial three-component decarboxylation system.

Actinic acid bacteria (LAB) are considered to be the most important human-related bacterial group, due to their ability to colonize mucosal tissues and their involvement in a wide variety of fermented foods. Amino acid decarboxylation systems represent one of the keys to LAB adaptability. Decarboxylation systems consist of a decarboxylase and a precursor/product transmembrane exchanger (1, 2). Their combined action results in amino acid intake, decarboxylation, and release of the corresponding biogenic amine. The pathway results in alkalinization 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 organized in clusters located on the bacterial chromosome or on plasmids. A decarboxylase with a given amino acid specificity is adjacent to an exchanger with an equally strict amino acid/biogenic amine specificity, as was observed for histidine/histamine (3), tyrosine/tyramine (4), aspartate/alanine (5), ornithine/putrescine (6), and glutamate/y-aminobutyrate (7) decarboxylation systems.

Among biogenic amines, histamine, tyramine, putrescine, cadaverine, and β-phenyl-ethylamine are most widely encountered in foods and beverages (8). These compounds are detrimental to food, as their ingestion results in health problems (9). In food, cadaverine occurs in amounts ranging from a few hundred micrograms to more than one gram per kilogram (or liter). This compound was detected in wine (10, 11), cheese (12), cider (13), sausage (14), and fishery products (15). In spite of the wide occurrence in different food matrices, the detection of cadaverine-producing strains is limited almost exclusively to ham and sausage. These producers are usually Enterobacteriaceae, Bacillus, Staphylococcus, and Lactobacillus spp. (14, 16). Some cadaverine-producing Oenococcus oeni strains were detected in wine as well (17), but further observations suggest that this trait is relatively infrequent among wine bacteria (18).

To date, the biosynthesis of cadaverine by LAB is poorly understood. Even though cadaverine (1,5-diamino-pentane) and putrescine (1,4-diamino-butane) are structurally related, as are their respective precursors lysine and ornithine, the well-characterized ornithine decarboxylase (ODC) of Lactobacillus saerimneri 30a (formerly known as Lactobacillus sp. 30a) is specific for ornithine and does not produce cadaverine from lysine (19). Recently, the characterization of the ODC system of Oenococcus oeni confirmed that both the decarboxylase and ornithine/putrescine exchanger show measurable affinity for lysine but that the main activity was clearly with ornithine (6). In Gram-negative Enterobacteriaceae, separate lysine and ornithine decarboxylation systems are common, and both are known to contribute to extreme acid resistance (20, 21, 22, 23). This suggests that also in cadaverine-producing Gram-positive LAB it is likely that a specific lysine decarboxylase (LDC) exists, although no LDC system, or the genes encoding it, was ever reported among LAB.

Strain L. saerimneri 30a was isolated from horse stomach. A complex amino acid decarboxylation metabolism appeared to be one of its most distinctive traits (24). In fact, L. saerimneri 30a is
known to be capable of decarboxylating histidine, ornithine, and lysine into the corresponding amines (25). The histidine and ornithine decarboxylases of this strain were already characterized (26, 27). While the genetic environment of the latter is unknown, the presence of an ornithine/putrescine exchanger gene in downstream position is to be expected by analogy to other known ODC systems (6). To date, no information is available about the genes and proteins responsible for lysine decarboxylation by L. saerimneri 30a. The ability of L. saerimneri 30a to synthesize up to several grams of cadaverine per liter (24, 25) makes this strain a good model candidate for the characterization of LDC systems of LAB.

This work was initiated with the aim to identify the LDC system of L. saerimneri 30a. In a preliminary phase, attempts were made by means of conventional molecular biology techniques to detect the genes of the LDC system, which, unfortunately, did not give conclusive results (results not shown). A primer pair proposed for LDC gene amplification in Gram-positive bacteria (28) did not yield a PCR product (18). A significant leap forward was made by the analysis of the whole genome sequence of the strain, which is presented elsewhere (29). First, it was determined that no transporter gene was present downstream of odc. Second, a putative decarboxylation system consisting of a decarboxylase and an amino acid transporter was identified approximately 20 kb apart from odc. The three proteins, i.e., the ODC, the putative decarboxylase, and the amino acid transporter, were functionally characterized. The results revealed a unique three-component decarboxylation system made of two decarboxylases and a single dual-specificity transporter for ornithine and lysine decarboxylation.

MATERIALS AND METHODS

Bacterial strains and cultures. Lactobacillus saerimneri strain 30a (ATCC 33222) was obtained from the ATCC collection (LGC Standards, Mol- skeim, France). The microorganism was cultured at 37°C in half-strength Man, Rogosa, and Sharpe medium (Becton Dickinson, Sparks, MD) set at pH 5.0.

Decarboxylation assays on whole cells. Lactobacillus saerimneri strain 30a was cultured until mid-exponential phase (1.5 to 2.0 optical density [OD] units ml⁻¹), and cells were harvested, washed twice with potassium phosphate buffer (0.1 M, pH 5.0), and finally resuspended at 1.0 OD unit ml⁻¹ in the same buffer containing either ornithine or lysine at the concentration of 10 mM. Cell suspensions were incubated at 37°C, with incubation times ranging from 0.5 to 22 h. Upon harvesting, cell suspensions were centrifuged (10 min at 10,000 g), and supernatants were directly analyzed for biogenic amines by means of thin-layer chromatography/densitometry, as described previously (31). The results were analyzed for biogenic amines by means of thin-layer chromatography/densitometry, as previously described (6).

Expression and purification of recombinant decarboxylases. Decarboxylases were obtained as recombinant His-tagged proteins in Esche- richia coli. PCR products comprising the ODC and LDC open reading frames were introduced in pET100/D-TOPO vectors (Invitrogen, Carlsbad, CA) by ligase-independent directional cloning. Chemically competent E. coli (BL21 Star One Shot; Invitrogen) was employed as an 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. Proteins were purified by affinity chromatography using a BioLogic DuoFlow chromatographic system (Bio-Rad, Marnes-la-Coque, France) equipped with a HitTrap chelating HP column (Amer- sham Biosciences, Uppsala, Sweden) pretreated with 0.1 M NiCl₂. Elution was performed by means of a pH 7.5 potassium phosphate buffer supplemented with increasing amounts of imidazole. The recombinant enzymes were identified in the fractions by SDS-PAGE.

Decarboxylation assays on purified enzymes. Reactions were performed in 200-μl volumes in 0.05 M citrate buffer (pH 5.5) containing 4 μg of enzyme and 0.4 μm pyridoxal phosphate (PLP) at 37°C. Enzymes were incubated with lysine, ornithine, 2,4-diaminobutyric acid, or arginine at concentrations in the range of 0.5 to 100 mM. Initial reaction rates were determined by sampling at regular intervals (typically 0, 10, 30, and 60 min) over the first hour of incubation, during which reaction rates were constant. pH and temperature activity profiles were established by calculating initial reaction rates at 10 mM substrate concentrations. Depending upon the pH, citrate (pH 3.0 to 6.0), phosphate (pH 6.0 to 8.0), and borax (pH 8.0 to 10.0) buffers were used at 0.05 M concentrations. Changes in buffer composition had no influence on the enzymatic activity. The reactions were stopped by addition of 10 μl trichloroacetic acid (20%, vol/vol), and samples were stored at −20°C until analysis. Product concentrations were determined by thin-layer chromatography/densitometry, as previously described (6). Kinetic parameters were estimated using GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA). For modeling the pH dependence of the enzymatic activity, a classical biproic model was employed (30), and pH activity profiles were established with the aid of GraFit 7.0 (Erithacus Software, Horley, United Kingdom).

Cloning and expression of the transporter gene. The gene encoding the putative amino acid transporter was amplified using primers L30Aaat-fw (GGGAAAACCATGGCTGAATCTTCAAGCC) and L30Aaat-rv (GGGAA ATCTAGATATGAAATAGTATGGACCG), containing NcoI and XbaI restriction sites for cloning in the NICE system expression vector pNZ8048 (31). The resulting plasmid, pNZ-L30Aaat, was transformed to Lactococcus lactis NZ9000. To express the gene from the nisin-inducible promoter Fneo, cells were grown in rich M17 medium containing 10 mM glucose to mid-exponential phase (OD at 600 nm [OD₆₀₀] of ~0.6) and induced with 5 ng/ml nisin. After 1 h of further incubation, cells were harvested and washed for transport assays.

Transport assays in whole cells. L. lactis NZ9000 cells containing pNZ-L30Aaat or the empty vector pNZ8048 were washed and resuspended in ice-cold 50 mM KP, (pH 6.0) to an OD₆₀₀ of 2.0. Glucose was added to a concentration of 10 mM. After 5 min of incubation at 30°C with stirring, 12.5 μM l-[U-¹⁴C]ornithine was added to 100 μl of cell suspension. For chase experiments, 1 mM unlabeled putrescine, cadaverine, or lysine was added after 1 min. Uptake was stopped by addition of 2 ml of ice-cold 0.1 M LiCl, followed by filtration through a 0.45-mm-pore-size nitrocellulose filter (BA85; Schleicher & Schuell GmbH). The filter was washed once with 2 ml of ice-cold 0.1 M LiCl and submerged in Emulsifier Scintillator Plus scintillation fluid (Packard Bioscience). Retained radioactivity was counted in a Tri-Carb 2000CA liquid scintillation analyzer (Packard Instrumentation).

RESULTS

Ornithine and lysine decarboxylase activities of resting cells of Lactobacillus saerimneri 30a. In a previous screening, a total of 275 cider and wine LAB strains were tested for different amino acid decarboxylase activities (18). Only strain Lactobacillus saer- imneri 30a scored positive for lysine decarboxylase activity. This strain showed also histidine and ornithine decarboxylase activities. Cells of L. saerimneri 30a were harvested at mid-exponential phase and resuspended (pH 5.0, 37°C) in the presence of either ornithine or lysine at a concentration of 10 mM. The initial rates of conversion of ornithine and lysine were 0.33 and 0.05 mmol g⁻¹ h⁻¹, respectively. After 22 h of incubation, 1.57 mmol putrescine per gram of wet cells and 0.59 mmol cadaverine g⁻¹ were obtained (Fig. 1). L. saerimneri 30a was therefore able to decarboxylate both ornithine and lysine, with a somewhat higher catalytic efficiency for the former. Similar experiments reported for other ODC-positive LAB strains showed at least 20 times more putrescine than cadaverine under the same conditions (6).
A putative decarboxylase system is encountered in the proximity of odc. The draft genome sequence of L. saerimneri 30a (29) revealed the well-characterized odc in close vicinity to a new putative amino acid decarboxylase gene at a distance of 24 kb (Fig. 2). Surprisingly, no transporter gene encoding an ornithine/putrescine exchanger was found adjacent to odc, while an amino acid transporter homologue gene was found downstream of the putative decarboxylase gene, suggesting that they form a new amino acid decarboxylation system. The two genes of this putative system are oriented in the same direction and possibly cotranscribed (Fig. 2). In contrast, the distant gene encoding the ODC is transcribed separately. The genomic region between the odc and the newly identified decarboxylase genes comprised 23 predicted open reading frames (ORFs) coding for proteins involved in diverse cellular processes, i.e., chromosome partitioning proteins, acetyl coenzyme A (acetyl-CoA) carboxylase subunits, ABC transporters, etc., but none showed any apparent relation to a decarboxylase function. The region did not contain any gene of transposase, phage, or plasmid origin, and its GC percentage was not significantly different from the average GC content of the L. saerimneri 30a genome (Fig. 2).

The amino acid sequence encoded by the new putative decarboxylase gene displayed relatively high (52 to 60%) identity to known ODCs from Firmicutes and Enterobacteriaceae (Fig. 3), while it exhibited lower (38 to 45%) identity to a group of recently described basic amino acid decarboxylases from Lactobacillus spp. (6). Sequence identity with LDC sequences from Gram-negative bacteria was quite poor (e.g., 28% with LDC from E. coli). Similarly, the amino acid sequence encoded by the putative transporter gene had highest identity with the putrescine/ornithine exchanger PotE from Firmicutes and Enterobacteriaceae (56 to 59% identity) and significantly lower identity with the lysine/cadaverine exchanger CadB found in Gram-negative bacteria. All these transporters contain 13 putative transmembrane segments. No significant sequence identity was observed with the ornithine symporter that is associated with the recently described basic amino acid decarboxylases from Lactobacillus spp. mentioned above.

The putative decarboxylase gene encodes a decarboxylase strictly specific for lysine. The odc and putative decarboxylase gene were cloned in Escherichia coli in frame with an upstream sequence encoding a His tag that enabled purification of the gene products by Ni-nitrilotriacetic acid (NTA) affinity chromatography. For each protein, a pure product was obtained with the ex-
TABLE 1 Steady-state kinetic parameters and substrate specificities of the two decarboxylases from *L. saerimneri* 30a

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Parameter (unit)</th>
<th>Value for KDC</th>
<th>Value for ODC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine</td>
<td>( k_{cat} ) (min(^{-1}))</td>
<td>NA</td>
<td>586 ± 29</td>
</tr>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>1.6 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>( k_{cat}/K_m ) (M(^{-1}) min(^{-1}))</td>
<td>NA</td>
<td>3.6 × 10(^5)</td>
</tr>
<tr>
<td>Lysine</td>
<td>( k_{cat} ) (min(^{-1}))</td>
<td>101 ± 17</td>
<td>15 ± 3</td>
</tr>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>12.7 ± 4.2</td>
<td>10.3 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>( k_{cat}/K_m ) (M(^{-1}) min(^{-1}))</td>
<td>8 × 10(^5)</td>
<td>1.5 × 10(^5)</td>
</tr>
<tr>
<td>2,4-Diaminobutyric acid</td>
<td>( k_{cat} ) (min(^{-1}))</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>( k_{cat}/K_m ) (M(^{-1}) min(^{-1}))</td>
<td>NA</td>
<td>ND</td>
</tr>
<tr>
<td>Arginine</td>
<td>( k_{cat} ) (min(^{-1}))</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>( K_m ) (mM)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Values are means and standard errors of triplicates. KDC, lysine decarboxylase; ODC, ornithine decarboxylase; NA, not applicable (no product formation was observed); ND, not determined (due to low activity, it was not possible to estimate kinetic parameters).

The putative amino acid transporter displays dual preference for ornithine/putrescine and lysine/cadaverine. The putative amino acid transporter gene downstream of the lysine decarboxylase gene was cloned and expressed in *L. lactis* using the NICE expression system (31). Uptake of \(^{14}\)C-labeled ornithine was tested in resting cells expressing the transporter gene or harboring the empty vector pNZ8048 (Fig. 5). At a concentration of 12.5 mM, the initial rate of uptake of ornithine in control cells, mediated by one or more endogenous transport systems in *L. lactis*, was approximately 2.4 nmol min\(^{-1}\) mg\(^{-1}\) protein. Cells expressing the transporter showed a strongly increased initial ornithine transport rate of at least 16 nmol min\(^{-1}\) mg\(^{-1}\) protein. When an excess of preferred substrates (i.e., ornithine for ODC and lysine for KDC) were measured in the pH range between 3 and 10 at a temperature of 37°C and in the temperature range between 15 and 50°C at a pH of 5.5 (Fig. 4). In agreement with the results for the enzyme isolated from *L. saerimneri* 30a (19), the recombinant ODC from *L. saerimneri* 30a displayed an optimum at pH 5.9. The pH optimum of the KDC was slightly lower, at a value of 5.2. Furthermore, while the ODC showed at least 40% of the maximal activity at pH values ranging from 4 to 9, the KDC displayed a much narrower profile (Fig. 4A). The temperature profile (Fig. 4B) was again very broad for ODC, whose initial reaction rate remained essentially unchanged between 37 and 50°C. The profile of the KDC exhibited a peak at 37°C, with a steep decrease in the higher range of temperatures.

**FIG 4** pH (A) and temperature (B) activity profiles of KDC (○) and ODC (●). Each enzyme was tested against a 10 mM concentration of the preferential substrate. Relative activity is expressed in each panel with respect to the highest average activity measured for the given enzyme and conventionally fixed at 100%. Background activity measured in enzyme-free buffers was always below 2% and was subtracted. Experimental points represent mean results of triplicate replicates. Error bars represent standard errors.
unlabeled putrescine was added to cells that were allowed to accumulate $^{14}$C-ornithine for 1 min, cells expressing the transporter rapidly released the ornithine, whereas control cells did not release any ornithine at all (Fig. 5A, closed triangles and squares, respectively). This demonstrates that the transporter is able to exchange ornithine with putrescine, similar to what was observed for PotE homologues which are part of ornithine decarboxylation systems such as in Staphylococcus epidermidis (33) and O. oeni (6). When instead of putrescine an excess of cadaverine or lysine was added, a similar rapid release of ornithine was observed in cells expressing the transporter (Fig. 5A and B, respectively). Control cells showed no release of ornithine upon cadaverine addition and only a relatively slow release when lysine was added. The results demonstrate that the transporter efficiently catalyzes an exchange mode of transport and that it has affinity for ornithine and putrescine as well as lysine and cadaverine. Efficient exchange of ornithine and cadaverine and of ornithine and lysine warrants efficient exchange of cadaverine and lysine, and therefore, the transporter can take part in an ornithine as well as a lysine decarboxylation pathway.

**DISCUSSION**

Degradative basic amino acid decarboxylase systems are quite common among Gram-negative Enterobacteriaceae, where they play a role in acid stress resistance (34, 35, 36). Different systems consisting of a decarboxylase and a precursor/product exchanger have been found for lysine/cadaverine (LDC) (cadAB), arginine/putrescine (adiAC), and ornithine/putrescine (ODC) (speP/potE). Among LAB and phylogenetically related bacteria, arginine is degraded via the arginine deiminase (ADI) pathway (37) and cadAB have not been identified, whereas ODC pathways have been reported in O. oeni (6, 38), Lactobacillus brevis (6), Staphylococcus epidermidis (33), and Staphylococcus lugdunensis (39). The patchwork distribution of ODC among strains and species, along with the high level of sequence identity with homologues from Gram-negative bacteria and the occurrence of elements that are distinctive of genetic mobility, strongly suggests that these systems originate from horizontal gene transfer from Enterobacteriaceae.

The biochemical or genetic basis of cadaverine production from lysine observed for some LAB, among which is L. saerimneri 30a, was never demonstrated. Here, we describe a decarboxylase encoded in the genome of L. saerimneri 30a with a strict substrate preference for lysine. The designation KDC is proposed for this enzyme. To the best of our knowledge, this is the first report of a specific lysine decarboxylase from lactic acid bacteria. The encoding gene is adjacent to a transporter gene that encodes a lysine/cadaverine exchanger. When the amino acid sequences of the decarboxylase and transporter were compared to sequence data in public databases, a clear similarity to ODCs and ornithine/putrescine exchangers emerged, whereas sequence identity to LDCs and lysine/cadaverine exchangers of Gram-negative bacteria was definitely lower (Fig. 3A and B). This suggests that the KDC system of L. saerimneri 30a, unlike the ODC systems from LAB, is not the mere result of horizontal transfer from Gram-negative bacteria. Instead, the system has likely evolved from an amino acid decarboxylation system of different specificity (i.e., an ODC system). The gene symbol kde is proposed for the sequence of this decarboxylase to emphasize the different phylogenetic origin (29). This sequence provides means for the improvement of molecular tools (i.e., PCR and quantitative PCR [qPCR] primers) aimed at the detection of food-borne cadaverine producers.

The newly characterized KDC and the adjacent transporter are part of a larger degradative decarboxylation system for both lysine and ornithine. The system consists of a decarboxylase specific for lysine, another decarboxylase specific for ornithine, and an exchanger that has affinity for both the lysine/cadaverine and ornithine/putrescine pairs. The system is unique in that two different decarboxylases make use of one and the same transporter for the uptake and excretion of substrate and products, respectively. On the genome of L. saerimneri 30a, the ornithine decarboxylase is found approximately 20 kb away from kde. The ODC sequence is highly identical to those of ODCs found in other LAB (Fig. 3A), but in contrast to these, no neighboring transporter gene is present. Different mechanisms can be proposed for the evolutionary origin of this unique decarboxylation system. The relative proximity between the genes encoding the ODC, the KDC, and the dual-specificity transporter suggests that the key event could have been the duplication of the decarboxylase gene within a formerly strictly substrate-specific ODC system. On the other hand the 22-kb region between the genes for the ODC and the exchanger does not show apparent signs of gene mobility and/or plasticity in either the gene annotation or the general pattern of the GC content (Fig. 2). Alternatively, the three-component system could be the remainder of two distinct ODC systems that underwent a process of gene elimination and further evolution that eventually led to the development of novel substrate specificities.

The study of the kinetic properties of the KDC from L. saerimneri 30a showed that this enzyme displays a narrow pH profile and rather low affinity and maximal turnover rate. Possibly, this relates to the threat of lysine depletion that the activity of a lysine decarboxylase system poses to cellular metabolism. In E. coli, lysine decarboxylase is controlled by multiple mechanisms of regulation that prevent lysine depletion under conditions of poor nutrient availability (32) or at neutral or mildly acidic pH (40, 41). The distinctive kinetic properties of the decarboxylase from L. saerimneri 30a probably represent an effective, however rudimentary, mechanism that prevents lysine depletion. It cannot be excluded that more refined regulation mechanisms (i.e., acting at a transcriptional or conformational level) also exist in L. saerimneri 30a.
ACKNOWLEDGMENT

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