Characterization of an Arginine:Pyruvate Transaminase in Arginine Catabolism of *Pseudomonas aeruginosa* PAO1

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The arginine transaminase (ATA) pathway represents one of the multiple pathways for l-arginine catabolism in *Pseudomonas aeruginosa*. The AruH protein was proposed to catalyze the first step in the ATA pathway, converting the substrates l-arginine and pyruvate into 2-ketoarginine and l-alanine. Here we report the initial biochemical characterization of this enzyme. The *aroH* gene was overexpressed in *Escherichia coli*, and its product was purified to homogeneity. High-performance liquid chromatography and mass spectrometry (MS) analyses were employed to detect the presence of the transamination products 2-ketoarginine and l-alanine, thus demonstrating the proposed biochemical reaction catalyzed by AruH. The enzymatic properties and kinetic parameters of dimeric recombinant AruH were determined by a coupled reaction with NAD⁺ and l-alanine dehydrogenase. The optimal activity of AruH was found at pH 9.0, and it has a novel substrate specificity with an order of preference of Arg > Lys > Met > Leu > Orn > Gln. With l-arginine and pyruvate as the substrates, Lineweaver-Burk plots of the data revealed a series of parallel lines characteristic of a ping-pong kinetic mechanism with calculated $K_m$ and $k_{cat}$ values of $54.6 \pm 2.5 \mu$mol/min/mg and $38.6 \pm 1.8$ s⁻¹. The apparent $K_m$ and catalytic efficiency ($k_{cat}/K_m$) were 1.6 ± 0.1 mM and 24.1 mM⁻¹ s⁻¹ for pyruvate and 13.9 ± 0.8 mM and 2.8 mM⁻¹ s⁻¹ for l-arginine. When l-lysine was used as the substrate, MS analysis suggested a L-piperidine-2-carboxylate as its transamination product. These results implied that AruH may have a broader physiological function in amino acid catabolism.

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Utilization of arginine as the sole source of carbon and nitrogen by pseudomonads can be mediated by multiple catabolic pathways. The arginine succinyltransferase (AST) pathway is the major route of arginine catabolism under aerobic conditions (4, 13). In the absence of a functional AST pathway, growth on L-arginine remained but was retarded (5, 15). It has been reported that the arginine oxidase pathway is the second route of l-arginine utilization in *Pseudomonas putida* (9, 15). While arginine oxidase activity has never been demonstrated in *Pseudomonas aeruginosa*, the presence of d-arginine dehydrogenase and arginine racemase activities provided an alternative route for l-arginine utilization by this organism (6). Oxidative deamination of arginine by arginine oxidase or dehydrogenase produces 2-ketoarginine, which is subsequently catabolized into succinate by a series of reactions common to both organisms.

Synthesis of 2-ketoarginine from l-arginine can also be accomplished by the transamination reaction (14). In the accompanying paper (17), we reported an arginine-inducible arginine transaminase (ATA) activity in *P. aeruginosa* PAO1, and this activity was completely abolished when the *aroH* gene was deleted. The *aroH* gene was proposed to encode a putative ATA residing in a multigene operon that is highly conserved in *P. aeruginosa*, *P. putida*, and *Pseudomonas fluorescens* (17).

In the present study, we purified the His-tagged AruH protein from *Escherichia coli* and characterized its enzymatic properties. Evidence is provided herein that AruH indeed catalyzes a transamination reaction with pyruvate as the amino acceptor, following a ping-pong bi-bi kinetics model. When l-arginine was used as the amino donor, the products of this transamination reaction, 2-ketoarginine and l-alanine, were confirmed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) and the kinetic properties were analyzed by a coupled reaction with NAD⁺ and l-alanine dehydrogenase.

**MATERIALS AND METHODS**

Expression of *aroH* in *E. coli*. The pBAD protein expression system by arabinose induction (Invitrogen) was employed for overproduction of AruH. The *aroH* structural gene was amplified by PCR from the genomic DNA of *P. aeruginosa* PAO1 using the two following primers to introduce a six-His tag at the N terminus: 5′-CCGCTATGAGACATCATCATCATCATATGCGCTAT

TCCGACTTCA-3′ and 5′-AATCTGCATGCAAGCGCCGGCAACACT-3′. The resulting PCR product was digested with BspHI (NcoI compatible) and PstI, which are unique restriction sites flanking the PCR product as introduced by the primers, and cloned into the NcoI and PstI sites of the expression vector pBAD-HisA. The resulting plasmid, pyZNH3, was introduced into *E. coli* Rosetta (DE3) (EMD Bioscience). For overexpression of *aroH*, the recombinant strain of *E. coli* was grown in LB medium containing ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml) at 22°C until the optical density at 600 nm reached 0.5, at which point 0.2% (wt/vol; final concentration) arabinose was added to the culture for induction. Culture growth was continued for another 18 h under the same conditions, and cells were harvested by centrifugation.

Purification of His-tagged AruH. Recombinant AruH was purified using a HiTrap HP kit (GE Healthcare) according to the manufacturer’s instructions. Briefly, the cell pellet (approximately 10 g) was suspended in 20 ml phosphate buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). EDTA-free protease inhibitor cocktail (two tablets; Roche) was added, and the cells were ruptured by an Amino French pressure cell at 8,000 lb/in². Cell debris was removed by centrifugation at 25,000 × g for 30 min, and the resulting cell-free crude extract was applied to a HiTrap HP column (GE Healthcare) equilibrated with the sodium phosphate buffer described above. After washing away the unbound proteins with equilibration buffer, His-tagged AruH was eluted with a stepwise gradient of 150 mM imidazole in 20 mM phosphate (pH 7.4) and 500 mM NaCl. For further purification, AruH proteins were subjected...

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to anion-exchange chromatography using a Mono Q HR 5/5 column (Pharmacia) equilibrated with 20 mM Tris/HCl (pH 7.4; buffer A). A protein sample was applied to the column and eluted with buffer A, followed by a linear gradient of 0 to 1 M KCl in buffer A over 20 column volumes. Active fractions that were homogeneous, as determined by visual inspection of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels, were pooled and then desalted and concentrated using a Amino Ultra-15 centrifugal filter unit (molecular mass cutoff, 30 kDa; Millipore). UV-visible light absorption spectra of the purified protein (8 mg/ml) in buffer A were recorded at 25°C with a Cary 3E spectrophotometer (Varian). Aliquots of AruH, supplemented with 50 μM pyridoxal 5'-phosphate (PLP) and EDTA-free protease inhibitor cocktail, were stored at 4°C before use for enzyme assays (up to 1 week of storage).

**Gel filtration analysis.** Gel filtration was performed with low- and high-molecular-weight calibration kits (GE Healthcare) by using a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with 50 mM sodium phosphate (pH 7.4) containing 300 mM NaCl. Recombinant AruH (50 μl; 5 mg/ml) was injected into the column and eluted at a flow rate of 0.5 ml/min. The molecular mass standards used were thyroglobulin (669 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa).

**Identification of L-lysine as a product of the AruH reaction by HPLC.** A reaction mixture (1.0 ml) containing 10 μg of recombinant AruH, 20 mM L-arginine, 20 mM pyruvate, and 0.5 mM PLP in 70 mM Tris/HCl buffer (pH 9.0) was incubated for 1 h at 37°C. After incubation, the sample was boiled for 10 min and then filtered using an Ultrafree-0.5 PBCC centrifugal filter unit (molecular mass cutoff, 5 kDa; Millipore). In a negative control experiment, heat-denatured recombinant AruH was used to prepare the reaction mixture. Reaction samples were separated on a Superose 12 HR 10/30 column (Pharmacia) equipped with a Spectra/Por Aqueous C30 column (4.6 by 250 mm; Phenomenex) at a flow rate of 0.5 ml/min. The mobile phase was 0.1 M potassium phosphate (pH 2.0), and elution was monitored by UV detection at 205 nm. Authentic L-lysine, pyruvate, PLP, and L-lysine were used as standards. A protocol described by Jann et al. (6) was followed to prepare 2-ketoarginine.

For the reversible reaction, 2-ketoarginine and L-alanine at 20 mM were used as substrates to replace 20 mM L-arginine and pyruvate under the assay conditions described above, followed by HPLC analysis.

**MS of AruH reaction products.** Samples prepared as described above for HPLC analysis were submitted for electrospray ionization (ESI)-MS analysis at the MS facility of Georgia State University. In the L-lysine experiments, 20 mM L-lysine was used to replace 20 mM L-arginine as the amino donor. The samples were diluted with methanol (1:1, vol/vol) and introduced into a Q-Tof micro MS apparatus (Waters) for analysis using the negative mode by infusion injection at a flow rate of 5 μl/min, with a capillary voltage of 3.5 kV, a sample cone voltage of 25 V, and an extract cone voltage of 1.0 V.

**Enzyme assays.** The arginine:pyruvate transaminase activity of recombinant AruH was measured in two steps. The first step was a transamination reaction catalyzed by recombinant AruH, in which the amino group of L-arginine was transferred to pyruvate, making 2-ketoarginine and L-alanine. In the second step, the amounts of L-alanine formed in the transamination reaction were determined by an enzyme-coupled reaction as previously described (1), in which L-alanine was oxidized to pyruvate and ammonia in the presence of NAD⁺ and L-alanine dehydrogenase.

Briefly, assay mixture I (1.0 ml; final volume) contained 20 mM L-arginine, 20 mM pyruvate, 0.5 mM PLP, and 70 mM Tris/HCl (pH 9.0), unless otherwise noted. Assay mixture I was preincubated for 10 min at 37°C prior to the addition of 10 μg of recombinant AruH, by which the reaction was started. A 4-min period of incubation at 37°C consumed <10% of the substrates. The reaction was stopped by addition of 50 μl anhydrous hydrazine (Sigma), which trapped the remaining substrate pyruvate (also the reaction product 2-ketoarginine) as the hydrazones. The protein precipitates were removed by centrifugation after assay mixture I was boiled for 10 min. Blanks did not contain L-arginine, pyruvate, or recombinant AruH. Assay I was linear with incubation of the assay mixture for up to 5 min and the enzyme concentration up to 20 μg/ml. For the second step, assay mixture II (2.1 ml) contained 1 ml hydrazine/Tris buffer (hydrazine at 1.0 M, Tris base at 40 mM, EDTA at 1.4 mM, adjusted to pH 9.0 with HCl), 1.2 mM NAD⁺, and supernatant from reaction I (0.1 to 0.8 ml containing 10 to 100 μM L-alanine). Assay reaction II was started by the addition of 2 U of L-alanine dehydrogenase (Sigma), and the increase in absorbance at 339 nm was monitored at 25°C until no significant increase could be detected. The molar extinction coefficient (6,300 M⁻¹ cm⁻¹) of NADH was used for the calculation. One unit of enzyme activity was defined as the amount of enzyme that yielded 1 μmol of L-alanine per min under the standard assay conditions described above. Assay II with L-alanine dehydrogenase is specific for L-alanine determination and linear over a range of 5 to 300 μM L-alanine (16). The protein concentration was determined by the method of Bradford (2) with bovine serum albumin as the standard.

**Kinetic studies.** Kinetic assays were performed by the two-step method as described above, with various concentrations of L-arginine (the amino donor, 2.0 to 10.0 mM) and pyruvate (the amino acceptor, 0.3 to 2.0 mM). Using the kinetics module of SigmaPlot (SigmaPlot 2004 for Windows Version 9.01; SPSS Science), apparent kinetic parameters were determined by fitting data to the equations for sequential and ping-pong steady-state mechanisms, respectively.

**Substrate specificity.** Substrate specificity was investigated by the assay method described above but by replacing 20 mM L-arginine with various L-amino acids as the amino donors or replacing 20 mM pyruvate with 20 mM 2-ketoglutarate as the amino acceptor. When 2-ketoglutarate was used as the amino acceptor in the reaction, synthesis of L-glutamate was measured by monitoring the formation of NADH from NAD⁺ in a coupled reaction with L-glutamate dehydrogenase (1). Anhydrous hydrazine (Sigma) was used to trap the remaining substrate 2-ketoglutarate, similar to that used for the arginine:pyruvate transaminase assay.

**pH and temperature studies.** For pH studies, 70 mM potassium phosphate (pH 6.0 to 8.0), Tris/HCl (pH 7.4 to 9.5), and borate/NaOH (9.0 to 10.0) buffers were used in the assay. Similarly, the temperature dependence of AruH activity was evaluated by incubating assay mixture I for 4 min at 25 to 50°C.

**RESULTS**

**Overproduction and purification of recombinant AruH.** The pBAD system was employed for overexpression of recombinant AruH in *E. coli*. However, initial attempts encountered two difficulties, a low expression level and formation of inclusion bodies. We have successfully overcome these problems by integrating several features into the final recombinant strain. As described in Materials and Methods, the *aruH* gene was cloned into the expression vector pBAD-HisA by a strategy that allowed the N terminus of AruH to fuse directly with a His₆ tag rather than through a linker region to minimize interference to the folding or bioactivity of recombinant proteins. The resulting plasmid, pYZNH3, was expressed at 22°C to reduce the formation of inclusion bodies in *E. coli* Rosetta (DE3), which has been proven to be useful in overcoming the codon bias problem. With these modifications, His-tagged AruH was expressed well and became soluble. After two column chromatography steps (affinity and anion exchange), the recombinant AruH protein was purified to homogeneity, as evidenced by SDS-polyacrylamide gel electrophoresis (Fig. 1).
Molecular mass and absorption spectrum. As shown in Fig. 1, a value of 43 kDa for the molecular mass of AruH was determined from a plot of electrophoretic mobility against the logarithm of the molecular masses of known polypeptides. The results of gel filtration column chromatography revealed an apparent molecular mass of 79.3 kDa for native enzymes (data not shown), indicating that His-tagged AruH is a homodimer.

The purified AruH proteins had a distinct yellow color, suggesting the presence of a bound chromophore. As demonstrated below, AruH possessed a PLP-dependent transamination activity. UV-visible light spectroscopic analysis of AruH revealed an absorption peak centered at 425 nm (data not shown), consistent with the presence of PLP.

Assay of a PLP-dependent arginine:pyruvate transaminase activity of AruH. As described in the accompanying paper (17), several lines of genetic and biochemical evidence led us to propose that AruH possesses arginine:pyruvate transaminase activity. In this reaction, L-arginine and pyruvate served as the amino donor and acceptor, respectively, to make 2-ketoarginine and L-alanine. The amount of L-alanine thus synthesized was measured by the generation of NADH in a coupled reaction by a NAD-dependent L-alanine dehydrogenase as described in Materials and Methods. With this assay, it was found that only negligible L-alanine production could be detected in the absence of PLP, indicating that AruH is a PLP-dependent enzyme.

HPLC and MS analyses were employed to demonstrate the generation of 2-ketoarginine and L-alanine in the proposed reaction by AruH. Two transamination reaction mixtures were prepared, one with active AruH and another with heat-inactivated AruH as a negative control. As shown in Fig. 2, the presence of 2-ketoarginine in the reaction mixture was tentatively identified by HPLC; a peak with the same retention time (5.9 min) as the 2-ketoarginine standard appeared only in the reaction mixture containing active AruH and not in the negative control. However, the presence of L-alanine was difficult to demonstrate by HPLC because of its relatively low molar extinction coefficient (79 versus 1,350 M\(^{-1}\) cm\(^{-1}\) for L-arginine) at 205 nm and the closeness of its retention time (3.36 min) to that of L-arginine (3.42 min) under this elution condition.

The same reaction mixtures were also analyzed by MS to detect the presence of L-alanine and 2-ketoarginine. In the mass spectrum of the reaction mixture with active AruH (Fig. 3B), two molecular ion peaks were observed with \(m/z\) values of 88.1 and 172.1, which are identical to those of authentic L-alanine and 2-ketoarginine, respectively. As expected, no L-alanine or 2-ketoarginine was detected in the negative control sample which contained heat-inactivated AruH (Fig. 3A). Taken together, these results indicated that AruH is a PLP-

![FIG. 2. HPLC analysis of AruH reaction products. Chromatograms of the components of the reaction mixture with either heat-inactivated AruH (dashed line) or active AruH (solid line) were recorded at 205 nm as described in Materials and Methods. Signal peaks were individually identified by comparison to the retention times of authentic reference compounds. In this system, the retention times of L-arginine (L-arg), pyruvate (Pyr), 2-ketoarginine (2-KA), L-alanine, and PLP were 3.42, 4.2, 5.9, 3.36, and 10.8 min, respectively.](http://jb.asm.org/)

![FIG. 3. ESI-MS analysis of the AruH reaction products using L-arginine as the substrate. Panels A and B represent analyses of reaction mixtures with heat-inactivated and active AruH, respectively. The signals with \(m/z\) values of 87.0, 88.1, 172.1, and 173.2 were in accordance with the negative ion of pyruvate (Pyr), L-alanine (L-ala), 2-ketoarginine (2-KA), and L-arginine (L-arg).](http://jb.asm.org/)
dependent arginine:pyruvate transaminase that yields 2-ketoarginine and L-alanine.

We also investigated the reverse reaction catalyzed by AruH by using 2-ketoarginine and L-alanine as substrates. No significant production of L-arginine or pyruvate, however, was observed by HPLC analyses as described in Materials and Methods. This could be due to the fact that our assay conditions were not optimal for the reverse reaction.

Optimal pH and temperature. As shown in Fig. 4, the optimal pH for AruH enzyme activity was 9.0. Borate/NaOH buffer, in this case, showed a strong inhibitory effect on the transamination activity of AruH. The optimal temperature for AruH enzyme activity was 42°C; the enzyme was prone to sharp thermal inactivation above this temperature (data not shown).

Steady-state kinetics with L-arginine and pyruvate as substrates. Taking L-arginine and pyruvate as the substrates, Lineweaver-Burk plots of the data revealed a series of parallel lines characteristic of a ping-pong kinetic mechanism (Fig. 5A and B). The calculated $V_{\text{max}}$ and $k_{\text{cat}}$ were 54.6 ± 2.5 μmol/min/mg and 38.6 ± 1.8 s⁻¹, respectively. The apparent $K_m$ and catalytic efficiency ($k_{\text{cat}}/K_m$) for pyruvate were 1.6 ± 0.1 mM and 24.1 mM⁻¹ s⁻¹, respectively. The apparent $K_m$ and catalytic efficiency ($k_{\text{cat}}/K_m$) for L-arginine were 13.9 ± 0.8 mM and 2.8 mM⁻¹ s⁻¹, respectively.

Substrate specificity. AruH was tested with ornithine, D-arginine, and 19 natural amino acids (except L-alanine) as amino donors in the presence of pyruvate as the amino acceptor. While L-arginine was found to be the best substrate among these amino acids, AruH also exhibited the catalytic activity toward L-lysine, L-methionine, L-leucine, ornithine, and L-glutamine with less efficiency (Table 1). In the mass spectrum of the reaction mixture with L-lysine as the substrate (Fig. 6), a molecular ion peak was observed with an $m/z (-)$ value of 126.1, which suggests the production of Δ¹-piperideine-2-carboxylate. As expected, no such signal peak was detected in the negative control sample which contained inactivated AruH (Fig. 6). These results indicated that AruH is a transaminase with broad substrate specificity.

We also tested whether α-ketoglutarate could substitute for pyruvate as an alternative amino acceptor in the AruH-catalyzed deamination of L-arginine and L-lysine. However, no synthesis of glutamate could be detected in a coupled reaction with NAD⁺-dependent glutamate dehydrogenase (data not shown).
as substrates, and the initial reaction rate was measured by analyzing L-alanine production in the coupled reaction as described in Materials and Methods. The activity of AruH with L-arginine as the substrate was defined as 100%. All experiments were performed in duplicate.

Other amino acids tested as the donors included all other proteinogenic L-amino acids (except L-alanine) and D-arginine.

**DISCUSSION**

To our knowledge, this is the first report of an ATA being characterized at the molecular level. We provide solid evidence herein that AruH is a PLP-dependent arginine:pyruvate transaminase. In conjunction with genetic studies in the accompanying paper (17), we established the physiological function of AruH as the first catalytic enzyme of the ATA pathway for L-arginine utilization in *P. aeruginosa*. The high $K_m$ value of AruH for L-arginine (13.9 ± 0.8 mM) might provide an explanation for why the ATA pathway in *P. aeruginosa* is not the preferred pathway and is only active when the major pathway, the AST pathway, is blocked.

In the *Pseudomonas* genome annotation (www.pseudomonas.com), AruH was initially described as an aspartate transaminase (AspTA) because of its significant sequence similarities to other bacterial AspTAs, which belong to subgroup I of the transaminase family (7). The AspTAs in subgroup I may be further subdivided into two subgroups, Ia and Ib, according to their mutual homologies (11). AruH showed significant sequence identities (27 to 39%) and similarities (47 to 57%) to AspTAs of subgroup Ib but not with those of subgroup Ia. For AspTAs, an R-292 residue of subgroup Ia (3) or a K-109 residue of subgroup Ib (10) is highly conserved to interact with the distal carboxyl group of aspartate. None of these two residues is conserved in the corresponding sites of AruH, consistent with our conclusion that AruH is not an aspartate transaminase. Since L-arginine is the best substrate for AruH, one would expect the presence of specific interactions between the guanidino group of L-arginine and the side chains of amino acid residues in the catalytic pocket of this enzyme. Future determination of the molecular structure of AruH is expected to answer these questions about the substrate recognition mechanism. It was noteworthy, however, that those amino acid invariants well conserved in AspTAs of subgroup I (8), including Y70, P138, N194, P195, G197, D222, Y225, K258, R266, G268, and A386 (the residues are numbered according to pig cytosolic AspTA), could still be found in the sequence of AruH. Analogous to the roles played by these residues in the members of transaminase family subgroup I, we propose that K-237 of AruH is the active lysyl residue that binds to the coenzyme PLP, D-204 of AruH may form a hydrogen bond to N(1) of the coenzyme, and R365 of AruH may function in binding the $\alpha$-carboxyl group of substrates.

The conversion of L-arginine into 2-ketoarginine via transamination has been previously reported in *Arthrobacter simplex* (14). In contrast to the arginine:2-ketoglutarate transaminase of *A. simplex*, AruH prefers to use pyruvate as the amino acceptor, not 2-ketoglutarate. Those two enzymes are, however, both PLP dependent and showed an arginineducible expression profile. Compared to the enzyme of *A. simplex*, which could utilize L-arginine (100%) but also L-citrulline (16%) and L-alanine (10%), AruH displayed broader substrate specificity and could utilize L-lysine (51%), L-methionine (44%), L-leucine (24%), and L-ornithine (17%) besides L-arginine (100%) as the amino donor. It would be of interest to explore whether AruH plays a role in the utilization of these other amino acids.

**TABLE 1. Substrate specificity of AruH**

<table>
<thead>
<tr>
<th>Donor amino acid</th>
<th>Relative activity (%)</th>
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<tbody>
<tr>
<td>L-Arginine</td>
<td>100</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>51</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>44</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>24</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>16.5</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>2.3</td>
</tr>
<tr>
<td>Other</td>
<td>NR*</td>
</tr>
</tbody>
</table>

* The pyruvate (20 mM) and amino acid (20 mM) donors indicated were used as substrates, and the initial reaction rate was measured by analyzing L-alanine production in the coupled reaction as described in Materials and Methods. The activity of AruH with L-arginine as the substrate was defined as 100%. All experiments were performed in duplicate.

* Other amino acids tested as the donors included all other proteinogenic L-amino acids (except L-alanine) and D-arginine.

* NR, not reactive or the activities were below the detection limit of the method employed.
amino acids and, reciprocally, whether these amino acids exert any effect on the induction of _auxH_ and other genes in the same locus. For instance, production of Δ¹-piperideine-2-carboxylate via transamination between L-lysine and pyruvate, as demonstrated in this report, might provide an alternative pathway for lysine catabolism (12).

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**REFERENCES**


