Distribution of the Arginine Oxygenase Pathway among Coryneform Bacteria

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Of 14 coryneform and 2 Micrococcus strains tested, Arthrobacter globiformis IFO 12137, A. simplex IFO 12069, and Brevibacterium helvolum IFO 12073 utilized L-arginine as a sole carbon and nitrogen source, and synthesized the enzymes specific for the arginine oxygenase pathway when grown on L-arginine. The first step reaction was stimulated by FAD and aeration, and the enzyme responsible was shown to be arginine 2-monooxygenase (EC 1.13.12.1). High activities of five enzymes, including guanidinobutyramidase and guanidinobutyrase (EC 3.5.3.7), were detected in the extract of L-arginine-grown A. simplex cells. The enzymes in the last two steps, 4-aminobutyrate aminotransferase (EC 2.6.1.19) and succinate-semialdehyde dehydrogenase (EC 1.2.1.16), of B. helvolum were also induced by putrescine. These results indicate that some bacteria belonging to the coryneform group employ the arginine oxygenase pathway as a major route for L-arginine metabolism, L-arginine being degraded to succinate via 4-guanidinobutyramide and 4-guanidinobutyrate. The last part of the pathway may be common to the pathway for putrescine degradation.

Recent studies have revealed the remarkable diversity in bacterial L-arginine degradation. The reaction sequences of the arginine deiminase, arginase, arginine oxygenase, arginine decarboxylase, arginine succinyltransferase, and arginine oxidase pathways have been elucidated, and a few other pathways have also been reported. The former three pathways are distributed among Gram-positive bacteria and actinomycetes. The arginine oxygenase pathway was found by Thoai and co-workers in Streptomyces griseus. The first step is catalyzed by arginine 2-monoxygenase (EC 1.13.12.1), L-arginine being oxidized to 4-guanidinobutyramide and carbon dioxide. Guanidinobutyramidase, guanidinobutyrase (EC 3.5.3.7), and 4-aminobutyrate aminotransferase (EC 2.6.1.19) convert 4-guanidinobutyramide to succinate-semialdehyde via 4-guanidinobutyrate and 4-aminobutyrate. The arginine oxidase pathway is one of the three or four L-arginine degradation pathways in fluorescent species of Pseudomonas, including P. putida (recently, Jann et al. reported that a racemase which converts L-arginine to D-arginine catalyzes the first step of this pathway in P. aeruginosa). Both oxygenase and oxidase pathways involve 4-guanidinobutyrate as an intermediate, but the reactions leading to this compound are quite different (Fig. 1). Both pathways use the same reactions for the conversion of 4-guanidinobutyrate to succinate-semialdehyde and the latter may be converted to succinate.

Many strains in the non-pathogenic coryneform group have been assigned to genera Arthrobacter and Brevibacterium. Recent extensive phylogenetic studies of bacteria have confirmed the close relationship between coryneform bacteria and actinomycetes. Although the nutritional versatility of non-pathogenic coryneform bacteria is well known, little is known about the degradation of L-arginine. L-Arginine aminotransferase has been found in...
Fig. 1. Arginine Oxygenase and Arginine Oxidase Pathways.

Solid line arrows, arginine oxygenase pathway of Streptomyces and coryneform bacteria; broken line arrows, the arginine oxidase pathway of fluorescent Pseudomonas. The enzymes are denoted by numbers beside the arrows: 1, arginine 2-monooxygenase (EC 1.13.12.1); 2, guanidinobutyramidase; 3, guanidino- butyrase (EC 3.5.3.7); 4, 4-aminobutyrate aminotransferase (EC 2.6.1.19); 5, succinate-semialdehyde dehydrogenase (EC 1.2.1.16); 6, arginine oxidase (for the enzymes in Pseudomonas aeruginosa, see ref. 9); 7, a-keto-arginine decarboxylase; 8, guanidinobutyraldehyde dehydrogenase and/or aminobutyraldehyde dehydrogenase (EC 1.2.1.19).

Arthrobacter simplex, but the metabolic fate of α-ketoarginine, the product of the enzyme reaction, remains unknown. Studies on the distribution of the arginine degradation pathway in coryneform bacteria in connection with the phylogenetic structure of these organisms, and comparative enzymological studies on the arginine oxygenase and arginine oxidase pathways may provide some insight into the evolution of the metabolic versatility of bacteria.

We recently found that some non-pathogenic coryneform bacteria could grow on l-arginine or 4-guanidinobutyrate as a sole nitrogen or a sole carbon and nitrogen source. This paper describes the ability of these bacteria to utilize L-arginine and related compounds, and the induction of the five enzymes of the arginine oxygenase pathway. Our results indicate that the enzyme in the first step of L-arginine degradation is arginine 2-monooxy-

Materials and Methods

Chemicals. 4-Guanidinobutyramide was prepared from 4-guanidinobutyric acid methyl ester by the method of Thoai and Olomucki. The ester was synthesized by introducing dry hydrogen chloride into a suspension of 4-guanidinobutyric acid in methanol. Succinate-semialdehyde was prepared from monosodium L-glutamate by Strecker degradation with an equimolar amount of chloramine T and then purified by Dowex 1 × 8 (Cl⁻) chromatography. 4-Guanidinobutyric acid was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and L-arginine hydrochloride and 4-aminobutyric acid from Nacalai Tescue (Kyoto, Japan).

Organisms and culture methods. The coryneform and Micrococcus strains were obtained from the Institute for Fermentation (Juso-honmachi, Osaka, Japan). Pseudomonas sp. ATCC 14676 was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.)

The basal medium for the growth tests with l-arginine and related metabolites contained 0.2% K₂HPO₄, 0.1% NaCl, 0.02% MgSO₄·7H₂O, 0.01% yeast extract, 0.0001% biotin, and 0.1% (v/v) trace element solution in distilled water (pH, about 7). l-Arginine hydrochloride, 4-guanidinobutyrate, or 4-aminobutyrate was added to the basal medium at a concentration of 0.1%, when tested as a carbon and nitrogen source. For testing each compound as a nitrogen source, the basal medium was supplemented with monosodium succinate, disodium succinate, and the compound in question at the concentrations of 0.01, 0.09, and 0.1%, respectively. The bacterial cells were inoculated into an L-shaped culture tube containing 8.0 ml of medium and then grown at 30°C in a shaking incubator. The turbidity was monitored at 570 nm with a Hitachi EPO-B colorimeter.

The arginine medium used for examination of the distribution of the arginine oxygenase pathway contained 0.5% L-arginine hydrochloride, 0.1% K₂HPO₄, 0.2% NaCl, 0.02% MgSO₄·7H₂O, and 0.05% yeast extract (pH, about 7). Bacterial cells were grown in a 2-l shaking flask containing 900 ml of the medium at 30°C for 12 hr on a reciprocal shaker.

Cells of A. simplex used for the induction experiment on the arginine oxygenase pathway enzymes were grown in 15 l of asparagine-succinate medium containing 0.1% l-asparagine, 0.05% monosodium succinate, 0.45% disodium succinate, 0.1% K₂HPO₄, 0.02% MgSO₄·7H₂O, and 0.05% yeast extract (pH, about 7). The cells were grown in a 20-l polyethylene bottle at 30°C under aeration. After 9 hr cultivation, the cells in 5 l of culture broth were collected by centrifugation and retained for...
measurement of the constitutive levels of enzymes. To the remaining 101 of the culture was added L-arginine hydrochloride to a concentration of 0.1% and the cultivation was continued for 8 hr, and then the cells were harvested by centrifugation.

The peptone-glycerol medium used for the induction experiment on 4-aminobutyrate aminotransferase and succinate-semialdehyde dehydrogenase with B. helvolum contained 0.05% peptone, 0.5% glycerol, 0.2% K₂HPO₄, 0.1% KH₂PO₄, 0.02% NaCl, 0.01% MgSO₄·7H₂O, and 0.05% yeast extract. The cells were grown in 15l of the medium in a 20-l polyethylene bottle at 30°C for 12 hr with aeration, and then harvested by continuous centrifugation. The B. helvolum cells used for the purification of arginine 2-monooxygenase or the preparation of the analytically used guanidinobutyramidase and succinate-semialdehyde dehydrogenase were similarly grown in the medium. After cultivation for 6 hr, L-arginine hydrochloride (for the induction of the oxygenase and the amidase) or 4-aminobutyrate (for the induction of the dehydrogenase) was added at a concentration of 0.1%. The cultivation was continued for a further 6 hr and then the cells were harvested by centrifugal treatment.

Preparation of enzymes. All operations were carried out at 0 ~ 5°C, unless stated otherwise.

Arginine 2-monooxygenase was partially purified as follows. Cells (85 g wet weight) of B. helvolum were disrupted with a Kaijo Denki sonic disintegrator in 170 ml of 25 mM KPO₄ buffer (pH 8.0) at 5 ~ 10°C. The sonicate was centrifuged and then the supernatant, which contained 3,000 mg of protein and 380 units of the oxygenase, was diluted with 160 ml of 20 mM KPO₄ buffer (pH 8.0) to lower the protein concentration to about 10 mg/ml. The enzyme (280 ml) was brought to 30% saturation with ammonium sulfate (40.0 g). The precipitated material was removed by centrifugation and then the supernatant was brought to 50% saturation with ammonium sulfate (40.0 g). The precipitated fraction, which contained 300 units of the oxygenase was thoroughly dialyzed against 20 mM KPO₄ buffer (pH 8.0) and then applied to a column (3 x 29 cm) of DEAE-Toyopearl 650M column (1.6 x 39 cm) equilibrated with the dialysis buffer. After washing the column with 500 ml of the buffer containing 0.05 M KCl, the amidase was eluted with the buffer containing 0.1 M KCl; 10 ml fractions were collected. The active fractions (fr. 6 ~ 9), which contained 12.1 mg of protein and 180 units of the amidase, were pooled and stored under nitrogen in a freezer at ~20°C. The guanidinobutyrase used for the oxygenase assay was prepared from 4-guanidinobutyrate-grown cells of Pseudomonas sp. ATCC 14676, as described previously. The purified enzyme had a specific activity of above 150 units/mg and exhibited no arginase activity. The succinate-semialdehyde dehydrogenase of B. helvolum used for the 4-aminobutyrate aminotransferase assay was purified to homogeneity. The details of the purification procedure will be published elsewhere. The purified dehydrogenase exhibited a specific activity of above 80 units/mg.

Assay of enzymes. The reaction mixture for the assay of arginine 2-monooxygenase (1.0 ml) contained 50 mM NaBicine buffer (pH 8.0), 100 μM FAD, 100 μM L-arginine, and the enzyme. The mixture was incubated at 30°C for 60 min with aeration on a vortex mixer at intervals of 10 min for 10 sec each time. The reaction was terminated by heating the mixture for 5 min in a boiling water bath. The 4-guanidinobutyramide formed was subjected to enzymic hydrolysis to 4-aminobutyrate, ammonia, and urea; the mixture (1.0 ml) contained a 0.2-mI aliquot of the oxygenase reaction mixture, 50 mM Na-Bicine buffer (pH 8.0), 0.1 mM MnCl₂, 1.0 unit of guanidinobutyramidase, and 1.0 unit of guanidinobutyrase. The reaction was allowed to proceed at 30°C for 60 min and then terminated by the addition of 0.25 ml of 12.5% trichloroacetic acid. The urea formed was determined colorimetrically.

Guanidinobutyramidase assay mixture (1.5 ml) contained 33.0 mM Na-Bicine buffer (pH 9.0), 167 mM hydroxylamine, 10 mM 4-guanidinobutyramide, and the enzyme. The reaction was allowed to proceed at 30°C for 30 min and then the 4-guanidinobutyrohydroxamate formed was measured colorimetrically.

Guanidinobutyrase was assayed as described previously; the reaction was allowed to proceed in 100 mM Tris-HCl buffer, pH 9.0, without added divalent metal ions.

4-Aminobutyrate aminotransferase and succinate-semialdehyde dehydrogenase were assayed by the methods of Jakoby. The aminotransferase reaction mixture (1.0 ml) contained 100 mM Na-Bicine buffer (pH 8.5), 50 mM 4-aminobutyrate, 5.0 mM α-ketoglutarate, 0.1 mM pyridoxal 5'-phosphate, 0.5 mM NADP⁺, 0.1 unit of pu-
rifled B. helvolum succinate-semialdehyde dehydrogenase, and the enzyme. The reaction was allowed to proceed at 25°C in a Hitachi 100-40 spectrophotometer and the absorbance at 340 nm was recorded every 15 sec using an NEC PC-8801 personal computer. The dehydrogenase assay mixture (1.0 ml) contained 100 mM Na-Bicine buffer (pH 9.5), 0.5 mM NADP^+, 0.5 mM succinate-semialdehyde, and the enzyme. The reaction was allowed to proceed at 25°C and the absorbance at 340 nm was measured as described above.

The urease assay mixture (1.0 ml) contained 50 mM Na-Bicine buffer (pH 9.0), 25 mM urea, and the enzyme. The reaction was allowed to proceed at 30°C for 120 min. It was terminated by the addition of 0.25 ml of 12.5% trichloroacetic acid and then the ammonia formed was measured.

One enzyme unit for the enzymes described above was defined as the amount of each enzyme which produced one jumol of the analyzed product per min under the conditions described. Specific activity is expressed as units per mg of protein.

Analytical methods. Protein in crude extracts or preparations after batch-wise purification steps was measured by the method of Lowry et al. with bovine serum albumin as the standard, and that in fractions obtained on column chromatography was estimated from the absorbance at 280 nm using an E(1%)-value of 10.0. Urea was measured colorimetrically according to Prescott and Jones using diacetylmonooxime and antipyrine. Guanidino compounds were measured colorimetrically according to Rosenberg et al. using diacetyl (2,3-butanedione) and a-naphthol. 4-Guanidinobutyrohydroxamate was measured by the method of Lipmann and Tuttle. Ammonia was measured by the method of Moore and Stein.

Results

Growth of coryneform bacteria on arginine, guanidinobutyrate, and aminobutyrate

The growth of 14 strains of Arthrobacter and Brevibacterium, and 2 strains of Micrococcus was examined on the medium containing L-arginine, 4-guanidinobutyrate, or 4-aminobutyrate (Table I). A. globiformis IFO 12136, A. globiformis IFO 12137, A. simplex IFO 12069, and B. helvolum IFO 12073 could utilize L-arginine as a carbon and nitrogen source. B. lipolyticum grew slowly on the amino acid as the carbon and nitrogen source, and rather well when succinate was added to the medium as the carbon source. These bacteria also grew well on 4-guanidinobutyrate and 4-aminobutyrate. The two Micrococcus strains only grew well on L-arginine when succinate was present. B. ammoniagenes, B. lines, and B. stationis could not utilize L-arginine, but could utilize 4-guanidinobutyrate and 4-aminobutyrate. A. citreus and five other strains could not utilize any of the compounds tested.

Distribution of enzymes of the arginine oxygenase pathway

The strains capable of utilizing L-arginine...
were grown in the arginine medium. Cell extracts were prepared and then guanidinobutyramidase activity was assayed. The extracts of \textit{A. globiformis} IFO 12137, \textit{A. simplex}, \textit{B. helvolum}, and \textit{B. lipolyticum} were active; trace or no activity was detected in the extract of \textit{A. globiformis} IFO 12136 and the two \textit{Micrococcus} strains. Each of the amidase-active extracts was fractionated on a column of DEAE-Toyopearl and then the fractions were assayed for arginine 2-monoxygenase, guanidinobutyramidase, and guanidinobutyrase. Figure 2 shows an example; guanidinobutyramidase, which catalyzes the second step of the pathway, was separated almost completely from the other two enzymes, which allowed us to estimate the precise activities of these enzymes in the crude extract. The specific activity was calculated from the total units of each enzyme eluted and the amount of protein applied to the column (Table II). The specific activities of the three enzymes in the extracts of \textit{A. globiformis} IFO 12137, \textit{A. simplex}, and \textit{B. helvolum} were found to be relatively high.

**Induction of all enzymes of the arginine oxygenase pathway in \textit{A. simplex}**

Cells of \textit{A. simplex} were grown in 15 l of the asparagine-succinate medium. After 9 hr cultivation, cells in a part (5 l) of the culture broth, which were in the middle logarithmic phase, were harvested and retained for examination of the constitutive levels of the enzymes. L-Arginine was added to the remaining broth at a concentration of 0.1% and then the cultivation was continued. During cultivation for

### Table II. Induction of Enzymes of the Initial Three Steps of the Arginine Oxygenase Pathway

<table>
<thead>
<tr>
<th>Strain</th>
<th>Arginine 2-monoxygenase (units/mg)</th>
<th>Guanidinobutyramidase (units/mg)</th>
<th>Guanidinobutyrase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{A. globiformis} IFO 12136</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{A. globiformis} IFO 12137</td>
<td>0.02</td>
<td>0.03</td>
<td>4.4</td>
</tr>
<tr>
<td>\textit{A. simplex} IFO 12069</td>
<td>0.05</td>
<td>0.03</td>
<td>2.2</td>
</tr>
<tr>
<td>\textit{B. helvolum} IFO 12073</td>
<td>0.07</td>
<td>0.82</td>
<td>5.5</td>
</tr>
<tr>
<td>\textit{B. lipolyticum} IFO 12678</td>
<td>0.0004</td>
<td>0.007</td>
<td>1.6</td>
</tr>
</tbody>
</table>

### Table III. Induction of Enzymes of the Arginine Oxygenase Pathway in \textit{A. simplex}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>\textit{t}-Asparagine (Specific activity, units/mg)</th>
<th>\textit{t}-Arginine (Specific activity, units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine 2-monoxygenase</td>
<td>0.005</td>
<td>0.045</td>
</tr>
<tr>
<td>Guanidinobutyramidase</td>
<td>0.004</td>
<td>0.13</td>
</tr>
<tr>
<td>Guanidinobutyrase</td>
<td>0.07</td>
<td>0.69</td>
</tr>
<tr>
<td>4-Aminobutyrate aminotransferase</td>
<td>0.013</td>
<td>0.35</td>
</tr>
<tr>
<td>Succinate-semialdehyde dehydrogenase</td>
<td>0.004</td>
<td>0.21</td>
</tr>
<tr>
<td>Urease</td>
<td>0.66</td>
<td>0.58</td>
</tr>
</tbody>
</table>
8 hr, the turbidity at 570 nm increased from 0.7 to 2.0 and about 80% of the L-arginine was consumed. Cell extracts were prepared from asparagine-grown and arginine-grown cells, and then the arginine oxygenase pathway enzymes in the two extracts were assayed. It was found that L-arginine elicited the biosynthesis of all enzymes of the arginine oxygenase pathway (Table III). The specific activities of arginine 2-monooxygenase and succinate-semialdehyde dehydrogenase in the extract of L-arginine-grown cells were 9 and 50 times, respectively, as much as those in the case of the asparagine-grown cells. A high level (about 0.6 units/mg) of urease was detected in both extracts, indicating that this organism produced the enzyme constitutively. These results indicated that all of the arginine oxygenase pathway enzymes were induced by L-arginine and strongly suggested that this pathway operated as a major degradation pathway for L-arginine in this organism.

*Induction of 4-aminobutyrate aminotransferase and succinate-semialdehyde dehydrogenase in B. helvolum*

4-Aminobutyrate is a common intermediate of the arginine oxygenase, arginine oxidase, and arginine decarboxylase pathways (see Fig. 1). Putrescine is an intermediate of biosynthesis and degradation of polyamines in many microorganisms. In *Pseudomonas aeruginosa*, 4-aminobutyrate aminotransferase is induced by putrescine, as well as by L-arginine, 4-guanidinobutyrate, and 4-aminobutyrate, and the enzyme also participates in the conversion of putrescine to 4-aminobutyraldehyde, which may be converted to 4-aminobutyrate. We examined the induction of 4-aminobutyrate aminotransferase and succinate-semialdehyde dehydrogenase by putrescine and other compounds to determine whether or not these enzymes are involved in putrescine metabolism in coryneform bacteria. Cells of *B. helvolum* grown in the peptone-glycerol medium were incubated at 30°C for 5 hr in a medium containing 0.2% K₂HPO₄, 0.2% KH₂PO₄, and any one of the inducers at a concentration of 20 mM. Extracts were prepared from cells incubated with each inducer and from ones incubated without any inducers, and then the enzyme activities were measured. Putrescine was a good inducer of both enzymes; the activities of both enzymes induced by putrescine were about the same as those induced by L-arginine, 4-guanidinobutyrate, or 4-aminobutyrate (Table IV). These results indicated that the aminotransferase and the dehydrogenase participate in both L-arginine and putrescine degradation.

**Partial purification of arginine 2-monooxygenase, and the effects of FAD and aeration on the enzyme reaction**

Arginine 2-monooxygenase was partially purified from L-arginine-grown *B. helvolum* cells as described under Materials and Methods (Table V). We could not obtain a homogeneous enzyme because the partially purified

<table>
<thead>
<tr>
<th>Table IV. Induction of Aminobutyrate Aminotransferase and Succinate-Semialdehyde Dehydrogenase in B. helvolum</th>
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</thead>
<tbody>
<tr>
<td><strong>Inducer</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>L-Arginine</td>
</tr>
<tr>
<td>4-Guanidinobutyrate</td>
</tr>
<tr>
<td>4-Aminobutyrate</td>
</tr>
<tr>
<td>Putrescine</td>
</tr>
<tr>
<td>5-Aminovalerate</td>
</tr>
<tr>
<td>None</td>
</tr>
</tbody>
</table>

* Not tested.

<table>
<thead>
<tr>
<th>Table V. Purification of Arginine 2-Monooxygenase of B. helvolum</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step</strong></td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
</tr>
<tr>
<td>Toyopearl</td>
</tr>
</tbody>
</table>
The arginine 2-monooxygenase reaction mixture containing 0.018 unit of the partially purified enzyme (specific activity, 1.64) was placed in a 15-ml test tube with a screw cap. The gas phase of the test tube was air (O) or nitrogen (●). The mixture was incubated in a water bath at 30°C and agitated at regular intervals, for 5 sec each time, on a vortex mixer during incubation for 60 min. The values on the abscissa indicate the total time of agitation of the reaction mixture in 60 min. After 60 min incubation, the reaction was terminated and the 4-guanidinobutyramide formed was measured.

enzyme was rather unstable. The purified enzyme was usually used within 10 days after preparation. The reaction with the purified enzyme was stimulated by FAD, but not by FMN. The enzyme was nearly saturated with 100 µM FAD. Compared with the activity observed with the saturating concentration of FAD, 80 and 10% were observed at the coenzyme concentrations of 10 and 1 µM, respectively. The enzyme reaction was remarkably stimulated when the reaction mixture was frequently aerated on a vortex mixer and almost completely depressed when the mixture was agitated under nitrogen during the reaction (Fig. 3). From these results it was confirmed that the first step of L-arginine degradation was catalyzed by arginine 2-monooxygenase. This enzyme seemed to require a relatively high concentration of FAD for its full activity.

Discussion

A. simplex IFO 12069, A. globiformis IFO 12137, and B. helvolum IFO 12073 can utilize L-arginine and produce the enzymes specific for the arginine oxygenase pathway. Cells of A. simplex grown on L-arginine possess all the enzymes for the degradation of L-arginine to succinate. 4-Aminobutyrate aminotransferase and succinate-semialdehyde dehydrogenase of B. helvolum are also induced by putrescine, indicating that the last two enzymes of the pathway also participate in putrescine degradation in these coryneform bacteria. The specific activities of these enzymes seem to be enough to support growth on L-arginine. These results suggest that this pathway is a major route for L-arginine degradation in these bacteria. L-Arginine is not always a good inducer of enzymes of its degradation pathway; it is a somewhat poor inducer of 4-aminobutyrate aminotransferase12) and agmatine deiminase,7) which are involved in the arginine decarboxylase pathway of fluorescent Pseudomonas. The induction profile of the coryneform arginine oxygenase pathway is similar to that of the arginine oxidase pathway of P. putida and related organisms,7,8) in that L-arginine induces significant amounts of all of the pathway enzymes.

Our arginine 2-monooxygenase assay procedure measures the urea formed from the reaction product, 4-guanidinobutyramide, through the action of guanidinobutyramidase and guanidinobutyrase. Guanidinobutyramidase obtained from A. simplex exhibits rather high specificity for 4-guanidinobutyramide; aliphatic amides are inert as substrates (data not shown). Guanidinobutyrase of Pseudomonas is highly specific for 4-guanidinobutyrate11); 5-guanidinovalerate and 6-guanidinocaproate are very poor substrates of the enzyme and the other guanido compounds tested are all inert. No urea is formed when L-arginine is incubated with the arginine 2-monooxygenase preparation alone. The enzyme reaction is oxygen-dependent and is stimulated by FAD, this being in agreement with the case of arginine 2-monooxygenase of Streptomyces3) and lysine 2-monooxygenase of Pseudomonas.29) These results indicate that 4-guanidinobutyramide is formed from L-arginine and that the enzyme in the first step
is arginine 2-monooxygenase. Guanidinobutyrase of *A. simplex* and *A. globiformis* slowly forms urea from L-arginine, *i.e.*, this enzyme exhibits arginase activity, although it is very weak (data not shown), and therefore it is disadvantageous to use the enzyme of coryneform bacteria for the arginine 2-monooxygenase assay. The oxygenase of *B. helvolum* is considerably unstable. We are now trying to purify the more stable enzyme of *A. simplex*.

The last part of the arginine oxygenase pathway, from 4-aminobutyrate to succinate, is common to the putrescine degradation pathway in *B. helvolum*. This type of putrescine degradation pathway is known in many bacteria and thus the distribution of the pathway may be very wide. Guanidinobutyrase also introduces 4-guanidinobutyrate to the common pathway at the point of 4-aminobutyrate. This enzyme is distributed somewhat widely; it participates in the arginine oxidase pathway, which is found in fluorescent *Pseudomonas* and is present in 4-guanidinobutyrate-grown cells of *Serratia marcescens* (T. Yorifuji and C. Tokuda, unpublished observation). Thus only the earlier parts of the oxygenase and oxidase pathways are specific for each pathway. Comparison of the properties and the structures of guanidinobutyrases between coryneform bacteria and Gram-negative bacteria should be the subject of future research to shed light on the evolution of the enzymes.

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References