L-Arginine Utilization by *Pseudomonas* Species

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The utilization of arginine was studied in several different *Pseudomonas* species. The arginine decarboxylase and agmatine deiminase pathways were found to be characteristic of *Pseudomonas* species of group I as defined by Palleroni *et al.* (1974). *Pseudomonas putida* strains had three distinct arginine catabolic pathways initiated by arginine decarboxylase, arginine deiminase and arginine oxidase, respectively. The two former routes were also present in *P. fluorescens* and *P. mendocina* and in *P. aeruginosa* which also used arginine by a further unknown pathway. None of these pathways occurred in *P. cepacia* strains; agmatine catabolism seemed to follow an unusual route involving guanidinobutyrate as intermediate.

INTRODUCTION

Several different pathways for arginine utilization by micro-organisms have been described (Abdelal, 1979) and are outlined in Fig. 1. These will be referred to as (i) the arginine oxidase pathway, (ii) the arginine decarboxylase pathway, (iii) the arginine deiminase pathway and (iv) the arginase pathway. In some bacteria, more than one pathway for arginine utilization has been observed. For example, *Bacillus licheniformis* and the cyanobacterium *Aphanocapsa* use the arginase and the arginine deiminase routes (Broman *et al.*, 1978; Weathers *et al.*, 1978). Arginine can be deiminated and decarboxylated by *Pseudomonas aeruginosa* (Mercenier *et al.*, 1980*a, b*) and by *Aeromonas formicans* (Stalon *et al.*, 1982). In *Klebsiella aerogenes*, the arginine decarboxylase/agmatinase route and an unknown arginine amidinotransferase pathway have been reported (Friedrich and Magasanik, 1978; 1979).

In our study of arginine utilization by prokaryotes, we investigated the occurrence of these catabolic pathways in various species of *Pseudomonas*. Growth of the strains on potential intermediates of arginine metabolism was assessed either by the analysis of the products which accumulated in the culture medium or by measurement of enzyme activities.

METHODS

Chemicals. Arginine, ornithine, citrulline, putrescine and guanidinobutyrate, and L-amino acid oxidase from *Crotalus adamenteus*, were obtained from Sigma. DL[1-¹⁴C]Arginine.HCl was purchased from CEA-IRE, Fleurus (Belgium). Agmatine was obtained from Aldrich and found to be free from putrescine. N-Carbamoylputrescine and N-[carbamoyl-¹⁴C]carbamoylputrescine were prepared as previously described (Mercenier *et al.*, 1980*a*). 2-Ketoarginine was synthesized according to Meister (1952, 1953). Arginine was oxidized enzymically by L-amino acid oxidase for 12 h, after which the solution was filtered using a Diaflo apparatus, the enzyme recovered, and the filtrate was concentrated under reduced pressure, 2-Ketoarginine was crystallized at 4 °C and the precipitate redissolved in hot water. Residual arginine was separated from 2-ketoarginine by chromatography on Dowex 50 WX8 (H⁺ form). The column (2.5 × 30 cm) was washed with 1 l water, which eluted the 2-ketoarginine, the arginine remaining adsorbed. The eluate was concentrated and 2-ketoarginine was recrystallized at 4 °C. A 5% (w/v) solution was found essentially free of arginine as determined by amino acid analysis (Mercenier *et al.*,

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Fig. 1. Pathways of arginine catabolism. The enzymes involved are denoted by the numbers beside the arrows: 1, arginine deiminase (EC 3.5.3.6); 2, catabolic ornithine carbamoyltransferase (EC 2.1.3.3); 3, carbamate kinase (EC 2.7.2.2); 4, arginine decarboxylase (EC 4.1.1.19); 5, agmatine deiminase (EC 3.5.3.12); 6, *N*-carbamoylputrescine hydrolase (EC 3.5.3.-); 7, ornithine decarboxylase (EC 4.1.1.17); 8, agmatinase (EC 3.5.3.11); 9, arginase (EC 3.5.3.1); 10, arginine oxidase (EC 1.4.3.-); 11, 2-ketoarginine decarboxylase (EC 4.1.1.-); 12, 4-guanidinobutyraldehyde oxidoreductase (EC 1.2.1.-); 13, guanidinobutyrase (EC 3.5.3.7); 14, agmatine oxidase (EC 1.4.3.-); 15, putrescine transaminase (EC 2.6.1.-); 16, putrescine oxidase (EC 1.4.3.10); 17, aminobutyraldehyde dehydrogenase (EC 1.2.1.19).

1980a). 2-Ketoarginine visualized as a guanidino or 2-keto derivative migrated as a single spot on descending paper chromatography according to Miller & Rodwell (1971).

Bacterial strains. The strains and their origins are listed in Table 1. These strains were routinely grown at 30 °C, on nitrogen-free minimal 154 (Stalon *et al.*, 1967). Substrates used as sources of carbon or nitrogen were added after separate sterilization. Unless otherwise stated, the final concentration of metabolites was 10 mM. Rich medium was mineral medium 154 supplemented with yeast extract 0.4% (w/v).

Growth of the bacteria. In order to screen bacteria able to assimilate potential intermediates of arginine catabolism, each strain was cultivated at 30 °C with aeration for 1 to 3 d in 5 ml liquid medium supplemented with the substrate. Bacterial growth was followed by measuring the optical density at 660 nm with a Beckman photometer. For enzyme activity determinations the cells were grown in 100 ml medium in 1 l flasks with vigorous shaking. The cells were collected after 12 h of growth. The method used for cell growth under limited oxygen tension was carried out as previously described (Mercenier *et al.*, 1980b).

Enzyme assays. All enzymes were assayed at 37 °C. Catabolic ornithine carbamoyltransferase of *Pseudomonas* was determined as previously described (Stalon *et al.*, 1972), except that the buffer was adjusted to pH 7.5. Catabolic ornithine carbamoyltransferase of *Aeromonas* was determined according to Stalon *et al.* (1982). The *Pseudomonas* arginine decarboxylase assay of Mercenier *et al.* (1980a) was modified by using 0.5 mM-arginine. The *Aeromonas* arginine decarboxylase determination was according to Stalon *et al.* (1982). Arginine deiminase was determined according to Stalon *et al.* (1972), agmatine deiminase following Mercenier *et al.* (1980a), guanidinobutyrase according to Chou & Rodwell (1972), and agmatinase by the method of Friedrich & Magasanik (1979). Qualitative *in vitro* assays for *N*-carbamoylputrescine hydrolase were carried out by adapting the method described by Tabor *et al.* (1976) for the detection of ¹⁴CO₂. Single clones were grown at 30 °C in separate wells of microtitre plates; each well contained 100 µl rich medium with 50 µM-¹⁴C-carbamoylputrescine (10⁶ c.p.m. µmol⁻¹). A Ba(OH)₂ impregnated Whatman paper no. 3 was placed over each plate. After 24 h, the paper was removed, dried and autoradiographed to assess the production of ¹⁴CO₂ by each culture.

The protein of the culture extracts was determined by the Lowry method. Specific activity is defined as the amount of enzyme catalysing the formation of 1 μ mol product h^{-1} (mg protein^{-1}).

Analytical methods. The presence of intermediary products of arginine catabolism was assayed in the culture supernatants by means of an amino acid analyser (Mercenier *et al.*, 1980a). Citrulline, *N*-carbamoylputrescine and urea were also detected by the colorimetric method of Archibald (1944).

RESULTS AND DISCUSSION

The aerobic pseudomonads have been divided, on the basis of RNA homology, into five groups (Palleroni *et al.*, 1974), four of which contain the species tested in our study (Table 1). Group I is subdivided into three DNA homology groups, one subgroup corresponds to *P. fluorescens* and *P. putida*, another to *P. aeruginosa*, *P. stutzeri*, *P. mendocina*, *P. pseudoalcaligenes*, and the third to *P. syringae*. *Pseudomonas cepacia* is in group II. *Pseudomonas acidovorans* and *P. testosteroni* have been assigned to group III and group V contains *P. maltophilia*.

The nutritional characteristics which we observed for the *Pseudomonas* strains (Table 1) were consistent with the data of Stanier *et al.* (1966) with only minor discrepancies. For example, *P. pseudoalcaligenes* was found to use ornithine only as a nitrogen source although it had been reported to grow on this metabolite as a carbon source. With respect to substrates utilization, variations appeared between species of the same subgroup (Table 1) and also between strains of the same species. The two *P. cepacia* strains displayed different phenotypes: one used citrulline and *N*-carbamoylputrescine as carbon sources, whereas the other used them only as nitrogen sources. Utilization of citrulline as a carbon source appeared to be restricted to relatively few strains. It has been suggested that the rate of citrulline uptake in *P. aeruginosa* may be the rate-limiting step for its utilization (Rahman *et al.*, 1980). The utilization of arginine as a carbon source was restricted to some of the species assigned to group I and to *P. cepacia* (Table 1). Among the other group I species, *P. stutzeri*, *P. pseudoalcaligenes* and *P. syringae* used arginine only as a nitrogen source. The group III species, *P. testosteroni* and *P. acidovorans*, did not grow on arginine and utilized only a few of the potential arginine catabolites and thus appear less versatile than the other *Pseudomonas* species. It should be noted that the *P. acidovorans* strains are now classified as *Xanthomonas* (J. De Ley, personal communication).

The arginine oxidase pathway and 2-ketoarginine utilization

Pseudomonas putida P₂ ATCC 25571 catabolizes arginine by oxidative deamination leading to 2-ketoarginine; this step is followed by a decarboxylation giving guanidinobutyraldehyde, which is then converted to guanidinobutyrate (Fig. 1, enzymes 10, 11, 12) (Miller & Rodwell, 1971; Chou & Rodwell, 1972; Vanderbilt *et al.*, 1975). *Pseudomonas* strains that utilized 2-ketoarginine and guanidinobutyrate were characterized by an abundant release of urea into the culture medium. They exhibited a guanidinobutyrase activity (enzyme 13) induced during growth of the cells on these two substrates (Table 2). All the *P. putida* strains excreted urea and also had a high level of guanidinobutyrase when grown on arginine. The induction of arginase activity in *Bacillus licheniformis* during growth on arginine leads to the production of urea (Broman *et al.*, 1978). *Escherichia coli* also produces urea when grown on arginine as nitrogen source and this results from the formation of agmatine which is cleaved into putrescine and urea by an agmatinase (enzyme 8) (Morris & Pardee, 1971). Thus, only *P. putida* appears to use arginine oxidase as the first step for the catabolism of arginine. Although *P. stutzeri* ATCC 17588, *P. testosteroni* and *A. formicans* could use 2-ketoarginine as nitrogen source, no guanidinobutyrase was detected either in crude extracts or in toluenized cells.

The arginine decarboxylase activity

Arginine decarboxylase (enzyme 4) activity has been observed in cell-free extracts of *E. coli* (Morris & Pardee, 1971), *P. aeruginosa* (Mercenier *et al.*, 1980a) and *Aeromonas formicans* (Stalon *et al.*, 1982), grown in the presence of arginine. All the *Pseudomonas* species of group I exhibited arginine decarboxylase activity (Table 3). The enzyme was stimulated by both 0.5 mM-

Table 1. *Ability of Pseudomonas to use the potential arginine catabolites as sole source of carbon and/or nitrogen*

Medium 154 was supplemented with 10 mm of each substrate. Succinate was used as carbon source for *Pseudomonas* strains using arginine catabolites as nitrogen source. Methionine (1 mm) was used as supplement to the growing cultures of *P. maltophilia*. Glucose was the carbon source for *E. coli*, *Klebsiella*, and *Bacillus* strains. CN, utilization as carbon and nitrogen source; N, utilization as nitrogen source only; —, no growth.

Strain	Taxonomic classification*	L-Arginine	2-Keto arginine	4-Guanidino- butyrate	4-Amino- butyrate	Agmatine	N-Carbamoyl-putrescine	Putrescine	Citrulline	Ornithine
<i>P. putida</i> P ₂ ATCC 25571	I	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. putida</i> ATCC 12663	I	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. putida</i> IRC 204	I	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. fluorescens</i> ATCC 13525	I	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. aeruginosa</i> PAO	I	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. mendocina</i> NCIB 10541	I	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. stutzeri</i> ATCC 11607	I	N	—	—	CN	CN	CN	CN	CN	CN
<i>P. stutzeri</i> ATCC 17588	I	N	N	N	CN	CN	CN	CN	CN	CN
<i>P. pseudocaligenes</i> ATCC 17440	I	N	N	N	CN	CN	CN	CN	CN	CN
<i>P. syringae</i> ATCC 19310	I	N	—	—	CN	CN	CN	CN	CN	CN
<i>P. cepacia</i> NCTC 10743	II	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. cepacia</i> ATCC 17616	II	CN	CN	CN	CN	CN	CN	CN	CN	CN
<i>P. testosteroni</i> ATCC 11996	III	N	N	—	—	—	—	—	—	—
<i>P. acidovorans</i> IP 60-78	III	—	CN	CN	CN	—	—	—	—	—
<i>P. acidovorans</i> ATCC 15688	III	—	N	N	N	—	—	—	—	—
<i>P. maltophilia</i> ATCC 13637	V	N	N	N	N	N	N	N	N	N
<i>Escherichia coli</i> P4X (this laboratory)		N	—	—	—	—	—	—	—	—
<i>Klebsiella aerogenes</i> (Dr Magasanik)		CN	—	—	CN	CN	—	CN	CN	CN
<i>Bacillus licheniformis</i> ATCC 14580		CN	—	—	CN	—	—	CN	CN	CN
<i>Aeromonas formicans</i> NCIB 9232		CN	N	—	—	CN	CN	CN	CN	CN

* According to Palleroni *et al.* (1974).

Table 2. *Guanidinobutyrase activity in Pseudomonas species grown on different media*

Bacteria were grown on medium 154 with 10 mM of the different substrates as sole source of carbon and nitrogen. Enzyme was assayed as described in Methods. Specific activities are recorded as μmol product formed $(\text{mg protein})^{-1} \text{ h}^{-1}$.

Strain	Specific activity of guanidinobutyrase after growth on:			
	Minimal medium*	Arginine	2-Ketoarginine	Guanidinobutyrate
<i>P. aeruginosa</i> PAO	0.4	0.9	110	110
<i>P. putida</i> ATCC 12633	<0.2	40	63	63
<i>P. putida</i> ATCC 25571	<0.2	32	45	74
<i>P. putida</i> IRC 204	<0.2	34	70	50
<i>P. mendocina</i> NCIB 10541	<0.2	2.6	60	50
<i>P. fluorescens</i> ATCC 13525	<0.2	1	129	150
<i>P. cepacia</i> NCTC 10743	<0.2	0.5	14	60
<i>P. cepacia</i> ATCC 17616	<0.2	0.2	60	50
<i>P. acidovorans</i> IP 60-78	<0.2	NT	58†	56†
<i>P. syringae</i> ATCC 19310	<0.2	0.2†	NT	3†

NT, Not tested.

* Minimal medium was mineral salt 154 supplemented with 10 mM-succinate and 5 mM- $(\text{NH}_4)_2\text{SO}_4$.

† Culture was grown in the presence of 10 mM-succinate.

Table 3. *Formation of arginine decarboxylase and agmatine deiminase in Pseudomonas*

Enzymes were assayed as described in Methods. Specific activities are recorded as μmol product formed $(\text{mg protein})^{-1} \text{ h}^{-1}$.

Strain	Growth medium	Specific activity of arginine decarboxylase		Specific activity of agmatine deiminase		
		Minimal*	Arginine†	Minimal*	Arginine†	Agmatine‡
<i>P. putida</i> ATCC 25571		0.2	0.8	0.2	0.6	2.2
<i>P. putida</i> ATCC 12633		0.02	0.6	0.1	0.7	5.2
<i>P. putida</i> IRC 204		0.02	0.9	0.2	0.6	4.0
<i>P. fluorescens</i> ATCC 13525		0.05	0.5	0.4	0.7	2.5
<i>P. aeruginosa</i> PAO		0.02	1.9	0.8	1.0	30.0
<i>P. mendocina</i> NCIB 10541		0.2	0.6	0.4	4.4	25.0
<i>P. pseudoalcaligenes</i> ATCC 17440		0.3	0.8‡	0.3	1.6	4.0‡
<i>P. stutzeri</i> ATCC 11607		0.5	0.5‡	0.3	2.0	9.2‡
<i>P. syringae</i> ATCC 19310		0.06	0.05‡	0.4	0.8	4.6‡
<i>Aeromonas formicans</i> NCIB 9232		0.05	0.3	0.5	0.4	6.0
<i>Escherichia coli</i> P4X		NT	0.6§	NT	NT	NT

* Minimal medium was mineral salt medium 154 supplemented with 10 mM-succinate and 5 mM- $(\text{NH}_4)_2\text{SO}_4$ for *Pseudomonas* strains; glucose was used instead of succinate for *E. coli*.

† Arginine and agmatine media were medium 154 supplemented 10 mM-arginine or 10 mM-agmatine, respectively.

‡ Cultures were grown in the presence of 10 mM-succinate.

§ Cultures were grown in the presence of 10 mM-glucose.

pyridoxal phosphate and magnesium ions, but thiamin pyrophosphate, the co-factor of the 2-ketoarginine decarboxylase (enzyme 11), was without effect.

Pseudomonas stutzeri ATCC 11607 had constitutive arginine decarboxylase activity but with the other species the synthesis of arginine decarboxylase was induced by growth in the presence of arginine (Table 3). *Pseudomonas testosteroni* and *P. cepacia* had no arginine decarboxylase, although *P. cepacia* could grow on the enzyme product, agmatine.

The agmatine deiminase pathway

Since agmatine was utilized for growth by most of the *Pseudomonas* strains of group I, we investigated whether they excreted urea or *N*-carbamoylputrescine. Agmatine utilization by *E.*

Table 4. Regulation of catabolic ornithine carbamoyltransferase activity in *Pseudomonas* species

Strain	Oxygen tension ...	Ornithine carbamoyltransferase specific activity after growth on:			
		Citrate/NH ₄ ⁺		Arginine	
		High	Low	High	Low
<i>P. aeruginosa</i> PAO1		15	800	12	860
<i>P. putida</i> IRC 204		8	1990	25	1400
<i>P. putida</i> P ₂ ATCC 25571		7	1310	18	730
<i>P. putida</i> ATCC 12633		9	1440	20	740
<i>P. fluorescens</i> ATCC 13525		7	390	6	125
<i>P. mendocina</i> NCIB 10541		22	270	15	250
<i>Aeromonas formicans</i> NCIB 9232		40	12	110	4300
<i>Bacillus licheniformis</i> IRC7		25	15	1	1800

Medium 154 was supplemented with 25 mM-citrate and 10 mM-(NH₄)₂SO₄, or 25 mM-arginine as sole carbon and nitrogen source. High oxygen tension means air saturation in the fermenter, low oxygen tension means <1% saturation (see Mercenier *et al.*, 1980*b*). The enzyme specific activity is expressed as $\mu\text{mol citrulline formed h}^{-1} (\text{mg protein})^{-1}$.

coli and *K. aerogenes* is characterized by urea excretion resulting from agmatinase activity (enzyme 8), whereas *P. aeruginosa* excretes carbamoylputrescine (Mercenier *et al.*, 1980*a*). With the exception of *P. cepacia*, all *Pseudomonas* strains able to use agmatine as growth substrate excreted some *N*-carbamoylputrescine produced by the action of an induced agmatine deiminase (enzyme 5) (Table 3). Arginine induced agmatine deiminase at low levels in most of the *Pseudomonas* strains, although the level was slightly higher in *P. pseudoalcaligenes* and was 10-fold higher in *P. mendocina* and *P. stutzeri*. The *in vivo* tests revealed that all the strains exhibiting agmatine deiminase activity also possessed a *N*-carbamoylputrescine hydrolase (enzyme 6). *Pseudomonas cepacia*, *E. coli*, *K. aerogenes* and *B. licheniformis* gave no positive spots in this test (see Methods). *Pseudomonas cepacia* excreted urea when grown on agmatine as did *E. coli* and *K. aerogenes*. In contrast to the last two strains, no agmatinase activity was detected in crude extracts of *P. cepacia* after growth on agmatine; the only enzyme consistently detected was guanidinobutyrase (enzyme 13). The high level of this enzyme in cultures grown in the presence of agmatine indicated that agmatine acts as an inducer of guanidinobutyrase. The results suggest that these strains metabolize agmatine through a transaminase or an oxidase as reported for various fungi (Isobe *et al.*, 1982). In addition, it should be noted that *P. cepacia* NCTC 10743 was able to use *N*-carbamoylputrescine as a carbon source and that *B. licheniformis* could utilize *N*-carbamoylputrescine as a nitrogen source. No putrescine carbamoyltransferase was detected in the strains listed in Table 1. The pathway of *N*-carbamoylputrescine utilization in *P. cepacia* and *B. licheniformis* has yet to be investigated.

The arginine deiminase pathway

Previous work in our laboratory showed that the arginine deiminase pathway in *P. aeruginosa* PAO or *Aeromonas formicans* was induced by particular growth conditions such as a transition from high to low oxygen tension or depletion of carbon and phosphate sources (Mercenier *et al.*, 1980*b*; Stalon *et al.*, 1982). We detected the arginine deiminase pathway in all the fluorescent species of group I and in *P. mendocina*. In the presence of oxygen, the enzymes of the arginine deiminase pathway (enzymes 1, 2, 3) remained at a low level of activity but were induced when the oxygen tension was lowered even in the absence of arginine in the growth medium (Table 4). It should be noted that the maximum specific activity of the catabolic ornithine carbamoyltransferase and the two other enzymes of the pathway, were generally lower for *Pseudomonas* cultures growing on arginine as sole carbon and nitrogen source than on citrate plus ammonia. Growth on arginine stopped prematurely due to an excessive increase in pH of the medium (between 8.2 and 8.5), in spite of the buffering capacity of the medium, caused by the excretion of ammonia and ornithine. Under low oxygen tension, all the strains possessing the arginine

deiminase pathway converted 5–15% of the available arginine into citrulline and 85–90% into ornithine. When grown in the presence of arginine under high oxygen tension, they did not produce measurable levels of the two metabolites. In *A. formicans* and *B. licheniformis* however, both exogenous arginine and stress conditions were needed to induce the arginine deiminase pathway. Arginine deiminase and the inducible ornithine carbamoyltransferase were not demonstrated in the other *Pseudomonas* species listed in Table 1, even under conditions of energy depletion.

CONCLUSIONS

The present study has established that the genus *Pseudomonas* is heterogeneous with respect to the mechanism of arginine dissimilation and further, that heterogeneity exists within the individual subgroups.

The arginine decarboxylase/agmatine deiminase pathway is a characteristic of the *Pseudomonas* strains of group I as defined by Palleroni *et al.* (1974). This enzymic sequence fulfils the polyamine requirement when arginine is abundant. However, this pathway may also be a major route of arginine dissimilation in *P. mendocina*, *P. stutzeri* and *P. pseudoalcaligenes* since growth on arginine elicited the synthesis of agmatine deiminase in these strains.

The arginine oxidase pathway is probably the main arginine catabolic pathway during aerobic growth of *P. putida* strains, since exogenous arginine induced the synthesis of guanidinobutyrase and 90% of arginine consumed could be recovered as urea in the culture medium.

The arginine deiminase route was characteristic of the fluorescent group of *Pseudomonas* as well as *P. mendocina*. The arginine deiminase pathway in *B. licheniformis* (Broman *et al.*, 1978), *P. aeruginosa* (Mercenier *et al.*, 1980*b*), *Streptococcus faecalis* (Simon *et al.*, 1982) and *Aeromonas formicans* (Stalon *et al.*, 1982) serves to generate ATP under energy depletion conditions. *Pseudomonas putida* possesses at least three pathways for arginine utilization. It seems likely that there may also exist three arginine catabolic pathways in *P. aeruginosa*, since mutants blocked in both the arginine decarboxylase and arginine deiminase pathways and also defective in guanidinobutyrase were able to use arginine as sole carbon and nitrogen source (D. Haas, personal communication). The nature of this unknown arginine catabolic pathway remains to be discovered. With the exception of 2-ketoarginine and agmatine utilization, none of the arginine catabolic pathways described in this study were detected in *P. cepacia* strains, and the route for the catabolism of arginine in *P. cepacia* remains to be determined.

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