Comparative studies on the degradation of guanidino and ureido compounds by *Pseudomonas*

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The utilization of guanidino and ureido compounds was studied in several *Pseudomonas* species. Multiple routes of agmatine catabolism were found. All members of the homology group I of *Pseudomonas* use the initial deamination of agmatine to carbamoylputrescine which is subsequently converted to putrescine. In *Pseudomonas indigofera*, the catabolism of agmatine can also occur via an initial hydrolysis of the amidino group to putrescine catalyzed by an agmatine amidinohydrolase. A third pathway was found in *Pseudomonas cepacia*, namely oxidative deamination producing guanidinobutyraldehyde catalyzed by agmatine dehydrogenase, followed by formation of guanidinobutyrate and removal of urea by guanidinobutyrate amidinohydrolase to produce 4-aminobutyrate. Novel amidinohydrolases were characterized in *P. putida* for the utilization of arcaine and audouine, and in *P. cepacia* for arcaine, homoarginine and guanidinovalerate. Guanidinovalerate amidinohydrolase was also detected in *P. doudoroffii*. Some of these amidinohydrolases accept more than one substrate, e.g. guanidinobutyrate and guanidinovalerate utilization by *P. doudoroffii* and *P. cepacia*, the catabolism of arcaine and audouine by *P. putida*, and the degradation of arcaine and homoarginine by *P. cepacia*.

Introduction

Pseudomonas, a bacterial genus common in soil and water, displays a great versatility in terms of the organic compounds which can serve as sole source of energy, carbon and nitrogen. The spectrum of usable compounds is a characteristic feature of each *Pseudomonas* species. Moreover, in a single species, several pathways may contribute to the catabolism of a specific substrate. For example, the degradation of arginine in *Pseudomonas aeruginosa* and *Pseudomonas putida* proceeds via four different pathways (Stalon & Mercenier, 1984; Stalon *et al.*, 1987; Jann *et al.*, 1988).

Guanidino and ureido compounds are widespread in fungi, plants and animals. A large number of these compounds can serve as growth substrates for various *Pseudomonas* spp. A previous study (Stalon & Mercenier, 1984; see also Cunin *et al.*, 1986) has shown that the genus *Pseudomonas* is heterogeneous with respect to the pathways used for the breakdown of arginine. Here we extend this study to guanidino and ureido compounds in general to evaluate the capacity of *Pseudomonas* to utilize

these naturally occurring compounds and to determine possible variations in the usage of pathways.

Methods

Bacterial strains, media and growth conditions. The bacterial strains from our culture collection used in this work are listed in Table 1. We also included *P. aeruginosa* PAO obtained from Holloway (1955), *P. acidovorans* IP60-18 received from the Institut Pasteur (Paris, France), *P. putida* IRC204 from our strain collection (Stalon *et al.*, 1967) and the *Pseudomonas* species P1855-344 received from Y. Dessaux, Institut des Sciences, Végétales, Gif sur Yvette, France, selected for its ability to use the guanidino compound octopine. They were routinely grown at 30 °C except for *P. aeruginosa* which was cultivated at 37 °C. Nitrogen-free minimal medium 154 was used (Stalon *et al.*, 1967). Substrates used as carbon and/or nitrogen source were added after separate sterilization by filtration. When used as a carbon source, the final concentration of allantoin, allantoinic acid, oxalurate, creatinine, creatine and sarcosine was 40 mM. Other carbon sources were tested at 20 mM. When used as a nitrogen source, substrates were usually included at a final concentration of 10 mM (on a nitrogen content basis) in the presence of 20 mM succinate or citrate. Bacteria were grown in liquid media as previously described (Stalon *et al.*, 1987). The test-tubes were screened after 24 h and examined again after 48 h. When the results were difficult to interpret for slowly growing cultures, the incubation period was prolonged for a further 48 h. For enzyme activity determinations, cultures were grown aerobically as 100 ml batches in 1 litre flasks on a rotatory shaker. Cells from exponentially growing cultures were harvested by centrifugation, washed with NaCl (0.9%) and, unless used

Abbreviations: INT, iodonitrotetrazolium chloride; PMS, phenazine methosulphate; HEV, high voltage paper electrophoresis.

Table 1. Utilization of ureido and guanidino compounds by *Pseudomonas*

–, No growth; NT, not tested. CN, Utilization as carbon and nitrogen source; N, utilization as nitrogen source only. CN italicized (CN), means that 1 mol of urea was excreted per mol of substrate consumed; CN printed in bold type (CN), means that 2 mol of urea were produced per mol of substrate consumed. Substrates tested as a carbon source and used by none of the strains were diguanidinopropane, *N*-methylhydantoine, guanidinocaproate, guanidinosuccinate, carbamoylglutamate, hydantoic acid, methylguanidine and barbituric acid. Substrates used as the only nitrogen source by all strains were urea, carbamoylaspartate and parabanic acid. Substrates used as the sole carbon and nitrogen source by all strains were 4-aminobutyrate, putrescine (except for *P. acidovorans*).

Classification* . . . Growth medium	<i>P. mendocina</i> NCIB 10541 Ia	<i>P. aeruginosa</i> PAO Ib	<i>P. putida</i> IRC204 Ib	<i>P. putida</i> ATCC 12633 Ib	<i>P. fluorescens</i> ATCC 13525 Ib	<i>P. aureofaciens</i> ATCC 13985 Ib	<i>P. doboroffii</i> ATCC 27123 III	<i>P. species</i> P.1855-344 III	<i>P. cepacia</i> NCTC 10743 II	<i>P. cepacia</i> ATCC 17616 II	<i>P. acidovorans</i> IP 60-78 III	<i>P. indigofera</i> ATCC 19706 U
L-Arginine	CN	CN	CN†	CN†	CN	CN	CN	CN	CN	CN	N	CN
2-Oxoarginine	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	N	N
Agmatine	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	–	CN
Succinylagmatine	–	–	–	–	N	–	–	–	CN	–	–	–
Guanidinovalerate	–	–	CN§	N§	–	N§	CN	CN	CN	CN	CN	–
Guanidinobutyrate	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	–
Guanidinopropionate	–	CN	–	–	–	–	–	–	–	–	–	–
Guanidinoacetate	–	–	CN	CN	–	–	CN	–	–	–	–	–
Homoarginine	–	N	N	N	–	–	–	–	CN	–	–	–
Arcaïne	N	CN	CN	CN	N	N	N	–	CN	CN	–	N
Audouine†	–	–	CN	–	–	–	–	–	–	–	–	–
Allantoine	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	N	–
Allantoic acid	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	CN	–
Creatinine	N	–	N	N	–	N	CN	–	NT	NT	–	–
Creatine	N	–	CN†	CN†	–	N	CN	–	–	–	–	–
Citrulline	N	N	CN	CN	N	N	N	N	CN	N	N	N
Carbamoylputrescine	CN	CN	CN	CN	CN	N	CN	CN§	CN§	–	–	–
Carbamoylalanine	–	–	–	–	N	N	–	–	CN	–	N	–

* Taxonomic classification according to Palleroni *et al.* (1974).

† Substrate tested only as carbon source due to the presence of trace contamination by cadaverine.

‡ Abundant urea excretion but not in the ratio 1:1 or 2:1 to substrate consumed.

§ Poor growth even after 7 d culture.

|| Tentatively classified in group I *Pseudomonas* on basis of nutritional spectrum and enzymes content (see results).

immediately, were frozen as a pellet. Cell extracts were prepared at 0–4 °C by sonication of exponential phase cells in 20 mM-potassium phosphate buffer, pH 7.5 (Stalon & Mercenier, 1984). Cell debris was removed by centrifugation at 20 000 *g* for 20 min. Extracts were kept at 4 °C.

Protein concentrations were determined by the Lowry method. Enzymes were assayed at their pH optimum and in the region of proportionality between initial reaction velocity and protein concentration. Michaelis constants were determined using plots of v^{-1} versus s^{-1} and s/v versus s .

Enzyme assays. All enzymes were assayed at 30 °C except for *P. aeruginosa* enzyme activities which were assayed at 37 °C. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 µmol of product h^{-1} .

Amidinohydrolase assay. The method for determining amidinohydrolase is based on the rate of urea formation during hydrolysis of the guanidino compounds. In all cases, the assay mixture contained, in

2.0 ml, 200 µmol glycine/NaOH buffer, pH 9.5, $MnCl_2$, substrate and cell extract. For the determination of *P. cepacia* guanidinobutyrate and guanidinovalerate amidinohydrolases, 0.4 µmol $MnCl_2$ was used whereas 4 µmol $MnCl_2$ were used in all other amidinohydrolase assays. The assay contained 40 µmol guanidinobutyrate, guanidinovalerate, guanidinopropionate or agmatine and 20 µmol arcaïne, audouine or guanidinoacetate.

The reaction was initiated by adding the substrate and stopped after 10–30 min by addition of 2.0 ml 1 M-HCl. Urea was measured by the method of Archibald (1944). In every case it was verified that the reaction was linear with time and enzyme concentration. Creatine amidinohydrolase (EC 3.5.3.3) was assayed in the presence of 100 mM-Tris/malate buffer, pH 8.0, and 25 mM-creatine. Agmatine deiminase (EC 3.5.3.12) was assayed under the conditions given by Mercenier *et al.* (1980), but with 20 mM-agmatine instead of 10 mM.

Agmatine dehydrogenase assay. [Agmatine (acceptor) oxidoreductase deaminating EC 1.4.99.–.] The assay mixture contained, in a final volume of 1 ml, 20 µmol agmatine, 10 µmol KCN, 0.8 µmol iodonitro-

tetrazolium chloride (INT), 0.8 µmol phenazine methosulphate (PMS) and the enzyme. The reaction was initiated by addition of the substrate and the rate of increase in absorbance at 500 nm was followed on a Philips recording spectrophotometer against a blank in which agmatine was omitted. An absorption coefficient of 11.5 mM cm⁻¹ for reduced INT was used for the calculation of reaction rates (Meile & Leisinger, 1982).

Analytical methods. Guanidinoderivatives in culture media were determined by the method of Micklus & Stein (1973); allantoine and allantoic acid were assayed according to Trijbels & Vogels (1966b); creatine was measured by the α -naphthol diacetyl method of Ennor & Atoken (1948); urea was determined using the method of Archibald (1944) and carbamoylderivatives according to Archibald (1944) or Prescott & Jones (1969).

Chemicals. L-Arginine, 4-guanidinobutyrate, 3-guanidinopropionate, guanidinosuccinate, guanidinoacetate, allantoine, allantoic acid, parabanic acid, carbamoylaspartate, carbamoylglutamate, carbamoylalanine, citrulline, creatinine, creatine, sarcosine, 5-aminovalerate, cadaverine, putrescine, 4-aminobutyrate were from Sigma; agmatine, homoarginine and 6-aminocaproate were from Aldrich. Carbamoylputrescine was prepared according to Mercenier *et al.* (1980); 2-oxoarginine was synthesized as described previously (Stalon & Mercenier, 1984).

Arcaine, audouine, guanidinovalerate and guanidinocaproate were synthesized by guanidinylation of agmatine, cadaverine, 5-aminovalerate and 6-aminocaproate respectively, with *O*-methylisourea. The aminoproduct (100 µmol) was dissolved in 20 ml of water adjusted to pH 10 with KOH. The *O*-methylisourea was added in the ratio of 1 mol per mol of free aminogroup. The reaction proceeded for 2–3 h and the pH was maintained at 10 by addition of KOH. The synthesis of the guanidino compound was followed by determination of the guanidino group according to Micklus & Stein (1973). The precipitates formed were collected by filtration and washed twice with 25 ml of ice-cold water. They were then dried and stored at room temperature. Guanidinovalerate, which does not precipitate, was at least 98% pure and was found to contain 2% urea and traces of 5-aminovalerate. The solution was stored at -20 °C after acidification to pH 8.0.

High voltage paper electrophoresis. Synthesized guanidino compounds and products excreted by cells growing at the expense of guanidino and amino compounds were analysed using high voltage paper electrophoresis (HEV). A detailed description of this method was presented in a previous communication (Vander Wauven & Stalon, 1985).

Results

All the strains in this study utilize arginine as the sole energy, carbon and nitrogen source, except for *P. acidovorans*, chosen as a representative of group III *Pseudomonas* as defined by Palleroni *et al.* (1974). We have studied the ability of these strains to use guanidino and ureido compounds and tried to identify the enzymic pathways involved in their utilization.

Whereas 2-oxoarginine, guanidinobutyrate, agmatine, allantoic acid and carbamoylputrescine were used by most of the strains, the utilization of some of the guanidino compounds, as the sole carbon source, was restricted to one species (Table 1); audouine (diguanidinopentane) was used as a carbon source by *P. putida* IRC204 (and not by the neotype strain ATCC 12633),

homoarginine and succinylagmatine by *P. cepacia* NCTC 10743, guanidinopropionate by *P. aeruginosa*, creatinine by *P. doudoroffii* and carbamoylalanine by *P. cepacia* ATCC 17616.

Urea excretion

Since the excretion of urea is generally seen as a characteristic of *Pseudomonas* growing on guanidino and ureido compounds (Table 1) the spent culture liquors of the various strains were analysed to determine whether this metabolite had been excreted. Samples taken at the time of inoculation and at the time of harvesting were assayed for substrate consumption and urea excretion. All strains listed in Table 1 used urea as the sole nitrogen source. However, urea was not consumed when a 10 mM ammonium salt was added to the growth medium, suggesting that urease was repressed by ammonium salts as described for *P. aeruginosa* by Jansen *et al.* (1980). The amount of urea excreted by *Pseudomonas* cells growing on guanidino and ureido compounds as sole carbon source was consequently a rough estimate of the amidohydrolase activities involved in the release of urea. It also provided information concerning the pathway used for their utilization.

For instance, allantoic acid utilization by *P. fluorescens* and *P. aeruginosa* requires two (successive) hydrolases in the catabolic pathway (Trijbels & Vogels, 1966b) and we found 2 mols of urea excreted per mol of substrate consumed (Table 1). On the other hand, in *P. acidovorans* only one amidohydrolase is involved in allantoic acid degradation (Trijbels & Vogels, 1966a) and we observed indeed that 1 mol of urea was produced per mol of substrate consumed. Consequently, except for *P. indigofera* which is unable to use allantoic acid, *Pseudomonas* belonging to groups I and II use this substrate via a reaction sequence identical to that described for *P. aeruginosa* and *P. fluorescens*.

In contrast, the *P. putida* strains excreted substantial amounts of urea from arginine. Analysis of arginine consumed and urea produced showed that 50 to 60% of the guanidino group of arginine was recovered as excreted urea. The explanation is that two pathways participate to the utilization of arginine as a carbon source, the arginine oxidase route in which urea is produced (Miller & Rodwell, 1971; Stalon & Mercenier, 1984) and the arginine succinyltransferase pathway in which it is not (Stalon *et al.*, 1987).

Catabolism of creatine and creatinine

The catabolism of creatine and creatinine (Fig. 1) in *P. doudoroffii* is straightforward since 1 mol of urea is produced for each mol of substrate consumed (Table 1).

Fig. 1. Metabolic pathways of creatinine utilization by *Pseudomonas*. Enzymic steps. Numbers designate enzymes: 1, creatinine amidohydrolase (EC 3.5.2.10); 2, creatine amidinohydrolase (EC 3.5.3.3); 3, sarcosine dehydrogenase (EC 1.5.99.1); 4, creatinine deaminase (EC 3.5.4.21); 5, *N*-methylhydantoinamidinohydrolase (EC 3.5.2.-); 6, carbamoylsarcosine hydrolase (EC 3.5.3.-); 7, creatinine lyase (EC 4.1.3.-); 8, methylguanidine amidinohydrolase (EC 3.5.2.-).

Table 2. *Creatine amidinohydrolase activity in P. doudoroffii and P. putida IRC204 grown in minimal medium 154*

The growth substrates creatine and creatinine were used as the sole carbon source at 40 mM, succinate at 20 mM; nitrogen sources were used at 10 mM. Values are means from duplicate experiments.

Strain	Addition to minimal medium	Doubling time (min)	Creatine amidinohydrolase specific activity [units (mg protein) ⁻¹]
<i>P. doudoroffii</i> ATCC 27123	Succinate + NH ₄ ⁺	80	0.6
	Creatine	97	77.0
	Creatinine	95	52.0
<i>P. putida</i> IRC204	Succinate + NH ₄ ⁺	55	0.1
	Creatine	125	17.0
	Succinate + creatine	50	8.0
	Succinate + creatinine	120	1.0

Table 3. *Enzymes of arcaine and agmatine utilization in P. cepacia NCTC 10743*

Cells were grown in medium 154 supplemented with 20 mM substrate when used as the sole carbon source and 10 mM when used as sole nitrogen source (in the presence of 20 mM-succinate). Enzyme assays were done in duplicate from two independently grown cultures. NT, Not tested.

Growth substrates	Generation time (min)	Enzymes specific activity [units (mg protein) ⁻¹]			
		Arcaine-amidino-hydrolase	Agmatine amidino-hydrolase	Agmatine dehydrogenase	Guanidinobutyrate amidinohydrolase
Succinate + NH ₄ ⁺	102	<0.1	<0.05	<0.1	<0.1
Arcaine	195	17.0	0.9	2.5	14.0
Agmatine	90	0.35	0.2	5.0	18.5
Succinylagmatine	115	NT	0.5	3.0	16.0
Succinate + agmatine	60	<0.1	0.1	2.3	10.3
Guanidinobutyrate	60	<0.1	0.1	<0.1	45.0
Guanidinovalerate	75	NT	0.12	<0.1	7.0
Homoarginine	175	13.5	0.32	<0.1	<0.1

Growth of *P. doudoroffii* at the expense of creatine or creatinine resulted in a fully induced level of creatine amidinohydrolase (EC 3.5.3.-; Table 2), suggesting that creatine is an intermediate in the degradation of creatinine by *P. doudoroffii* as found in other pseudomonads (Appleyard & Woods, 1956; Rikitake *et al.*, 1979).

The utilization of creatine as the sole carbon source by *P. putida* was characterized by the conversion of 50% of the guanidino group into excreted urea (Table 1). Under the conditions used (Table 1), 10 mM-ammonium was present in the growth medium and no urease activity could be detected in cell extracts. Therefore, as in the case of arginine utilization where a fraction only of the amino acid was recovered as urea (Table 1), a second catabolic pathway was contributing to creatine utilization in *P. putida*.

Creatinine (40 mM) supported some growth of *P. putida*; however, the maximum cell yield corresponded to 25% of that obtained with the same concentration of creatine. Creatinine and creatine are in equilibrium in aqueous solution (Rikitake *et al.*, 1979) and, indeed, our creatinine medium was found to contain at least 6 mM-creatine suggesting that the growth of *P. putida* on creatinine resulted from the spontaneous conversion of creatinine to creatine in the medium. Addition of creatinine to succinate medium allowed *P. putida* cells to grow; however, extracts of these cells contained a lower creatine amidinohydrolase activity than cells grown with creatine as sole nitrogen source (Table 2). This low induced level of creatine amidinohydrolase was sufficient to account for growth on creatine formed at a limiting rate from creatinine by enzymic or non-enzymic conversion. Since creatine and creatinine are in equilibrium in aqueous solution, creatine utilization may also involve creatinine as an intermediate. A creatinine degradative pathway was described in a *P. putida* by

Yamada *et al.* (1975). It involves *N*-methylhydantoin-*N*-carbamoylsarcosine and sarcosine as intermediates.

Another possibility for creatine degradation could be the formation of methylguanidine and acetate followed by the release of urea from methylguanidine, as found in *P. stutzeri* (Van Eyck *et al.*, 1968). The two *Pseudomonas* strains used in this study were unable to grow on methylhydantoin or methylguanidine as the sole carbon and nitrogen source. In addition, the methylguanidine pathway appears unlikely for creatine utilization since no methylguanidine could be identified as an excretion product during creatine or creatinine utilization of *P. putida* IRC204; besides, *in vivo* the metabolism of methylguanidine would produce urea. One mol of urea would consequently be formed per mol of creatine used. The lack of growth on a potential intermediate such as methylhydantoin does not, however, conclusively eliminate carbamoylsarcosine as an intermediate of creatine utilization of *P. putida* IRC204. For example, in a previous study it has been shown that succinylarginine, the first intermediate involved in the aerobic degradation of arginine by *Pseudomonas cepacia* (Vander Wauven & Stalon, 1985) and *P. aeruginosa* (Jann *et al.*, 1988) is not taken up by the cells.

Agmatine utilization

Until recently, the accepted physiologically important pathway for agmatine utilization in *Pseudomonas* was that catalysed by agmatine deiminase giving rise to carbamoylputrescine with the concomitant formation of ammonia (Stalon & Mercenier, 1984; see Fig. 2). *P. aureofaciens*, *P. doudoroffii* and *P. species* P1855-344, not previously tested, also have an inducible agmatine deiminase activity (data not shown) and we therefore tentatively include the two latter species in Group I

Fig. 2. Pathways of agmatine catabolism in *Pseudomonas*. Enzymic steps. Numbers designate enzymes: 1, arcainase (arcaine amidohydrolase) (EC 3.5.3.-); 2, succinylagmatine desuccinylase (EC 3.5.1.-); 3, agmatine deiminase (EC 3.5.3.12); 4, carbamoylputrescine hydrolase (EC 3.5.3.-); 5, agmatinase (agmatine amidohydrolase) (EC 3.5.3.11); 6, putrescine transaminase (EC 2.6.1.-); 7, 4-aminobutyraldehyde dehydrogenase (EC 1.2.1.19); 8, agmatine dehydrogenase (EC 1.4.99.-); 9, 4-guanidinobutyraldehyde oxidoreductase (EC 1.2.1.-); 10, guanidinobutyrase (guanidinobutyrate amidohydrolase) (EC 3.5.3.7).

Pseudomonas as defined by Palleroni *et al.* (1974). By contrast, *P. cepacia* belonging to group II as well as *P. indigofera*, produce urea from agmatine (Table 1).

Resting cells of *P. indigofera* incubated with 10 mM-agmatine and 10 mM-aminooxyacetic acid, an inhibitor of pyridoxal phosphate enzymes involved in transamination, accumulated putrescine. Agmatine amidinohydrolase activity was induced 40-fold by growing the cells on agmatine as compared to succinate plus ammonium medium [specific activity 3.5 units (mg protein)⁻¹]. Maximum activity was observed at pH 9.5 in 100 mM-glycine/NaOH buffer in the presence of 2 mM-MnCl₂ and the apparent *K_m* for agmatine was 0.8 mM. Utilization of agmatine by *P. indigofera* appears to proceed by the pathway found in the *Enterobacteriaceae* (Friedrich & Magasanik, 1979).

Resting cells of *P. cepacia* NCTC 10743 grown on agmatine and incubated with 10 mM-agmatine and 10 mM-aminooxyacetic acid produced large amounts of putrescine and 4-aminobutyrate whereas extracts of agmatine-grown cells of *P. cepacia* produced only putrescine. These cell extracts contained a low level of agmatinase activity (Table 3). It is not clear whether the low agmatine amidinohydrolase activity found in cells grown on various guanidino compounds is due to a genuine agmatine amidinohydrolase enzyme or resulted from a non-specific activity of some other amidinohydrolases (see below). Moreover, agmatine-grown cells of *P. cepacia* also contained an inducible guanidinobutyrate amidinohydrolase activity (Table 3). This indicated that as well as agmatinase, another pathway of agmatine utilization may be present, involving guanidinobutyrate as an intermediate. Possible biochemical explanations are oxidation or transamination of agmatine to produce guanidinobutyraldehyde, a precursor of guanidinobutyrate (Fig. 2). Transamination appeared unlikely since *P. cepacia* cells incubated with glutamate, agmatine and aminooxyacetic acid still produced putrescine, urea and 4-aminobutyrate. Agmatine-grown cells were found to contain a high level of agmatine dehydrogenase activity. The artificial electron acceptors PMS and INT were required to obtain enzyme activity *in vitro*. Addition of FAD, FMN, NAD or NADP did not stimulate the reaction. Maximal activity was observed in 100 mM-Tris/HCl buffer, pH 8.25. The apparent Michaelis constant for agmatine was 8 mM. No activity was detected in this extract with arginine, ornithine, putrescine or 4-aminobutyrate used as substrate instead of agmatine. Agmatine dehydrogenase and guanidinobutyrate amidinohydrolase specific activities in cells grown on different media are shown in Table 3. Agmatine and its precursors, arcaine and succinyl-agmatine, induced both activities whereas guanidinobutyrate induced guanidinobutyrate amidinohydrolase

but not agmatine dehydrogenase. It therefore appears that *P. cepacia* has two agmatine degradative pathways. Mutations affecting these pathways are needed to assess the precise function of each pathway in this bacterium. Among the *Pseudomonas* spp. tested, only *P. cepacia* was able to use succinylagmatine. Urea excretion, agmatine dehydrogenase and guanidinobutyrate amidinohydrolase induction by succinylagmatine suggest that the first step in the utilization of this substrate involves agmatine formation. Cell-free extracts of bacteria grown on succinylagmatine catalyse the release of succinate from succinylagmatine. The specific activity of the desuccinylase measured in 50 mM-HEPES/NaOH buffer, pH 7.0, in the presence of 1 mM-CoCl₂ and 10 mM-succinyl-agmatine was 0.9 units (mg protein)⁻¹. Cells grown on succinate contained no detectable succinylagmatine desuccinylase activity indicating that this activity is induced by the substrate present in the growth medium.

Metabolism of arcaine, audouine, homoarginine and guanidinovalerate

Arcaine (diguandinobutane) could serve as the sole carbon and energy source for *P. putida* and *P. cepacia* (Tables 3 and 4). For *P. aeruginosa*, arcaine was a poor carbon source but an excellent nitrogen source (Table 4). Growth of either *P. putida* or *P. aeruginosa* in the presence of arcaine as the sole carbon source was accompanied by the excretion of 1 mol urea for each mol of substrate consumed. This suggests that arcaine utilization occurred through the action of an amidinohydrolase producing agmatine and urea. Cell-free extracts of *P. putida*, *P. aeruginosa* and *P. cepacia* catalysed the release of urea from arcaine. The arcaine amidinohydrolase of *P. putida* showed an absolute requirement for Mn²⁺. Maximum activity was observed at pH 9.5 in 100 mM-glycine/NaOH buffer in the presence of 2 mM-MnSO₄. In crude cell extract the apparent Michaelis constant for arcaine was 0.5 mM. The composition of the growth medium significantly influenced the arcaine amidinohydrolase levels of *P. putida* (Table 4). Arcaine-grown cultures were induced to a high level for arcaine amidinohydrolase and agmatine deiminase activities. Agmatine deiminase was induced to similarly high levels during growth on either agmatine or carbamoyl-putrescine whereas arcaine amidinohydrolase was repressed under these conditions (Table 4). Both enzymes were repressed in the presence of alternative good carbon and nitrogen sources. Arginine, guanidinobutyrate, guanidinoacetate, allantoate and creatine were not inducers of arcaine amidinohydrolase activity (data not shown). Extracts of arcaine- and audouine (diguandinopentane)-grown cells contained high levels of both arcaine amidinohydrolase and audouine amidino-

Table 4. Regulation of arcaine amidinohydrolase and agmatine deiminase synthesis in *P. putida* IRC204 and *P. aeruginosa* grown in medium 154

The growth substrates were used as the sole carbon source at 20 mM, nitrogen sources were used at 10 mM. *P. aeruginosa* grown on arcaine was tested once. Other values are means of at least two determinations. Numbers in parentheses indicate audouine amidinohydrolase specific activity.

Strain	Growth substrates	Generation time (min)	Specific enzyme activity [Units (mg protein) ⁻¹]	
			Arcaine amidino- hydrolase	Agmatine deiminase
<i>P. putida</i> IRC204	Citrate + NH ₄ ⁺	54	<1 (<1)	<1
	Arcaine	126	60 (24)	8.9
	Audouine	600	17.6 (13.9)	<1
	Agmatine	90	<1 (<1)	15.2
	Carbamylputrescine	60	<1	15
	Arcaine + agmatine	54	5.7	11.3
	Arcaine + citrate	90	8.2	6.0
	Arcaine + citrate + NH ₄ ⁺	72	6	4
	Arcaine + citrate + glutamine	54	4.9	2.3
	Arcaine + glutamine	60	8.6	4.2
	Arcaine + NH ₄ ⁺	120	50	9.0
	Arginine	75	<1	<1
<i>P. aeruginosa</i> PAO1	Citrate + NH ₄ ⁺	60	<1	<1
	Arcaine	> 1200	3	49
	Agmatine	72	<1	30
	Citrate + arcaine	280	1.6	18

hydrolase activities. Arcaine amidinohydrolase activity of both cell extracts showed the same apparent Michaelis constant for arcaine. In the same way, audouine amidinohydrolase activity of both cell extracts showed the same apparent K_m value of 0.8 mM for audouine and, in each case, corresponded to 40% of arcaine amidinohydrolase activity. These results suggested that the two activities could be due to the same enzyme. Support for such a bifunctional ureohydrolase came from the observation that the two activities could not be separated by usual protein fractionation methods, namely ammonium sulphate fractionation and molecular sieving, or by affinity chromatography on arginine Sepharose.

The production of 2 mol urea per mol of audouine consumed by *P. putida* cells suggested that, beside audouine amidinohydrolase activity which produces 1-amidinocadaverine, another hydrolase was involved in the release of urea from this compound, or from the subsequent metabolites formed during audouine catabolism. Resting audouine-grown cells incubated with 10 mM each of audouine and aminooxyacetic acid produced cadaverine and traces of an unidentified guanidino compound. Prolonged incubation led to the disappearance of audouine and of the guanidino compound and to an increase of cadaverine formation. This suggested that amidinocadaverine (the presumed guanidino compound) and cadaverine were intermediates of

audouine breakdown (Fig. 3). As amidinocadaverine was not available we were unable to determine whether cadaverine formation resulted from the action of two successive amidinohydrolases or of a single bifunctional enzyme.

During preparation of this manuscript, the properties of arcaine amidinohydrolase from the neotype *P. putida* strain ATCC 12633 were published by Yorifuji's group (Yorifuji *et al.*, 1989). This strain can utilize diguanidino compounds with 3–10 methylene groups as the only nitrogen source. Partially purified preparations of arcaine amidinohydrolase from *P. putida* IRC204 do not show any activity with diguanidinopropane. Arcaine amidinohydrolase from *P. putida* ATCC 12633 apparently has a broader specificity than that of strain IRC204.

Bifunctional amidinohydrolases are probably also present in *P. cepacia* where arcaine amidinohydrolase and homoarginine amidinohydrolase activities were coinduced (Table 3). These activities could not be separated by the usual protein fractionation procedures (data not shown). The homoarginine amidinohydrolase activity was 10–12% of the arcaine amidinohydrolase activity. Unlike the arcaine amidinohydrolase of *P. putida*, that of *P. cepacia* seemed to be devoid of audouine amidinohydrolase activity.

Amidinohydrolases with dual specificities have also been observed in the utilization of guanidinovalerate and

Fig. 3. Catabolism of audouine, homoarginine and guanidinovalerate. Enzymic steps. Numbers designate enzymes: 1, audouinase (audouine amidinohydrolase) (EC 3.5.3.-); 2, amidinocadaverine amidinohydrolase (EC 3.5.3.-); 3, homoarginase (homoarginine amidinohydrolase) (EC 3.5.3.-); 4, lysine decarboxylase (EC 4.1.1.18); 5, guanidinovalerase (guanidinovalerate amidinohydrolase) (EC 3.5.3.-).

guanidinobutyrate (Figs 2 and 3). Guanidinovalerate- and guanidinobutyrate amidinohydrolases activities from *P. cepacia* and *P. doudoroffii* showed the same apparent K_m values for guanidinobutyrate and guanidinovalerate (4 mM for *P. cepacia* amidinohydrolases and 11 mM for the *P. doudoroffii* enzyme). However, this activity was tenfold higher with guanidinobutyrate than with guanidinovalerate. *P. putida* IRC204 utilizes guanidinovalerate as both a nitrogen and a carbon source, whereas *P. putida* ATCC 12633 uses it only as a nitrogen source. Concomitantly, with the guanidinobutyrate amidinohydrolase of *P. putida* IRC204, a guanidinovalerate amidinohydrolase activity representing no more than 2% of the former was also found.

Discussion

Biochemical diversity in metabolic pathways can provide a means of distinguishing between different groups of micro-organisms. We have observed that *Pseudo-*

monas species, as a rule, employ only one of the possible agmatine catabolic pathways. This is, consequently, a biochemical character that may be of taxonomic value. It is interesting to note that the mode of agmatine utilization by species included in our study follows the ribosomal RNA/DNA homology groups delineated by Palleroni *et al.* (1974). All members of homology group I have the inducible agmatine deiminase route. Outside this group, alternative pathways function. Agmatinase is found in group II as represented by *P. cepacia* as well as in the unclassified species *P. indigofera*. However, more than one pathway can exist in one strain since in addition to agmatine amidinohydrolase activity, *P. cepacia* also possesses agmatine dehydrogenase activity.

Amidinohydrolase is the main activity used by *Pseudomonas* to degrade guanidino compounds since amidinohydrolases were characterized for audouine, arcaine, homoarginine, agmatine and guanidinovalerate beside the well known guanidinobutyrate- and guanidinopropionate amidinohydrolase activities (see Chou & Rodwell, 1972; Yorifuji & Shugai, 1978; Yorifuji *et al.*, 1983).

Guanidinopropionate and guanidinobutyrate amidinohydrolases from *P. aeruginosa* have been purified and characterized (Chou & Rodwell, 1972; Yorifuji & Shugai, 1978). These enzymes have been shown to share several molecular and catalytic properties and it has thus been suggested that they have evolved from a common ancestor (Yorifuji *et al.*, 1983). It is interesting to observe that bifunctional amidinohydrolases apparently occur for the dissimilation of audouine and arcaine in *P. putida*, for arcaine and homoarginine in *P. cepacia*, and for guanidinovalerate and guanidinobutyrate in both *P. cepacia* and *P. doudoroffii*. Arcaine amidinohydrolase from *P. putida* strain ATCC 12633 may have a broader specificity than the corresponding enzyme from *P. putida* IRC204 or *P. cepacia* (Yorifuji *et al.*, 1989). However, the hydrolysis of the amidino group to CO₂ and ammonia is the chemical option used by *Pseudomonas* for the utilization of succinylarginine, whereas deamination is used for either arginine or agmatine.

The same diversity appears in the degradation of ureido compounds: ureohydrolysis is observed for allantoic acid and ureidoglycine; hydrolysis of the ureido group to CO₂ and ammonia occurs for carbamoylputrescine (Mercenier *et al.*, 1980; Stalon & Mercenier, 1984), carbamoylalanine (Carvaca & Grisolia, 1958; Campbell, 1960) and carbamoylsarcosine (Kim *et al.*, 1976; Shimizu *et al.*, 1986); phosphorolysis is observed for citrulline in *Pseudomonas* of group I having the arginine deiminase pathway and in other bacterial systems for carbamoylputrescine (Wargnies *et al.*, 1979) and carbamoyloxamate (Vander Wauven *et al.*, 1986).

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