Metabolism of Both Stereoisomers of Phenylglycine by Different Routes in *Flavobacterium* F24

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Flavobacterium F24 metabolized both stereoisomers of phenylglycine and enzyme studies revealed that L-phenylglycine was transaminated by a constitutive enzyme while the D-stereoisomer was oxidized by a phenazine-methosulphate-dependent D-amino-acid dehydrogenase. This latter enzyme was not induced during growth on L-phenylglycine. Phenylglyoxylate formed in the reactions was decarboxylated by an inducible enzyme to benzaldehyde, which was oxidized mainly by an inducible phenazine-methosulphate-dependent benzaldehyde dehydrogenase not described earlier. Benzoate was further metabolized via 3-hydroxybenzoate to gentisate, which in turn was further degraded through a glutathione-dependent pathway.

INTRODUCTION

The D-enantiomer of 4-hydroxyphenylglycine is an industrially important intermediate in the manufacture of semisynthetic penicillins and cephalosporins. The compound may be synthesized by both chemical and biological procedures. Chemical procedures include the classical resolution of racemic DL-4-hydroxyphenylglycine with *d*-3-bromocamphor-8-sulphonic acid (Yamada *et al.*, 1979) whereas biological methods involve the enantioselective hydrolysis of a derivative such as the amide (Boesten & Meyer-Hoffman, 1975), the *N*-acetyl acid ester (Schutt, 1981; Schutt *et al.*, 1985), the hydantoin (Cecere *et al.*, 1975, 1978; Takahashi *et al.*, 1979; Nakamori *et al.*, 1980; Olivieri *et al.*, 1979, 1981) or the aminonitrile (Arnaud *et al.*, 1980). Nevertheless, it would be very interesting to have an alternative biological method at hand that would allow a direct regio- and stereospecific hydroxylation of phenylglycine yielding D(-)-4-hydroxyphenylglycine. In order to detect such a possible hydroxylation reaction in bacteria we have isolated various organisms on phenylglycine and recently we have described the metabolism of DL-phenylglycine in *Pseudomonas putida* (van den Tweel *et al.*, 1986*a*). The organism however, transaminated both stereoisomers of phenylglycine to phenylglyoxylate and did not hydroxylate phenylglycine.

In this paper we describe the metabolism of both stereoisomers of phenylglycine by a *Flavobacterium* sp. In this strain also no hydroxylation of phenylglycine was observed, but the metabolism of D-phenylglycine was distinct from that in *P. putida* (van den Tweel *et al.*, 1986*a*).

METHODS

Chemicals. Both stereoisomers of phenylglycine, aminooxyacetate (AOA), *N*-ethylmaleimide and phenazine methosulphate (PMS) were products of Aldrich. DL-2-, DL-3- and DL-4-hydroxyphenylglycine were a gift of Océ-Andeno BV, Venlo, The Netherlands. All biochemicals were from Boehringer. All other chemicals were of commercially available analytical grade and were used without further purification.

Isolation and cultivation of strain F24. A mixture of soil and sewage samples was diluted and streaked directly onto agar plates containing a mineral salts medium (van den Tweel *et al.*, 1986*a*) to which D-phenylglycine (1 g l⁻¹) was added. Colonies that had appeared after two weeks of incubation were transferred to agar plates containing

Abbreviations: AOA, aminooxyacetate; PLP, pyridoxal phosphate; PMS, phenazine methosulphate.

the same medium. Strain F24 was isolated by selection of a single colony from these plates. Maintenance and cultivation of strain F24 was as described previously for *P. putida* LW-4 (van den Tweel *et al.*, 1986a).

Simultaneous adaptation experiments. Experiments with washed cells were performed as described previously (van den Tweel *et al.*, 1986a).

Phenylglycine consumption by washed cell suspensions of Flavobacterium F24. The complete reaction mixture (total vol. 5 ml) contained cells, 250 μ mol potassium phosphate buffer pH 7.0 and 5 or 10 μ mol phenylglycine. The reaction tubes were incubated in a shaking water bath (30 °C, 1 Hz). Experiments were performed with or without AOA (15 mM). Samples (0.5 ml), taken at intervals, were analysed at room temperature by reversed phase HPLC using a C-18 column (200 $\times$ 3 mm; Chrompack, Middelburg, The Netherlands) and detected at 216 nm by means of a Perkin-Elmer variable wavelength detector. As an eluent a mixture of 50 mM-potassium phosphate buffer pH 7.0 and methanol (90:10) was used; the flow rate was 0.4 ml min⁻¹. Metabolites were identified by comparison of retention times with authentic samples and by *in situ* scanning of the UV spectra after the flow had been stopped.

Protein determination. Protein contents of crude cell extracts and whole cells were determined by the Lowry method using crystalline bovine serum albumin as a standard.

Separation of the optical isomers of phenylglycine. Samples were separated by HPLC using a chiral stationary phase column (ET 250/8/4 Nucleosil Chiral-1; Macherey-Nagel, Düren, FRG) and detected at 216 nm by means of a Perkin-Elmer variable wavelength detector. The mobile phase was aqueous 1.0 mM-copper acetate pH 5.6, the flow rate was 0.8 ml min⁻¹, and the column temperature was 60 °C. Under these conditions D- and L-phenylglycine had retention times of 9.25 and 8.55 min respectively.

Enzyme assays. All assays were done at 30 °C. Cell extracts were prepared as previously described (van den Tweel *et al.*, 1986a).

(i) *Phenylglycine transaminase.* This enzyme was assayed by measuring the rate of disappearance of phenylglycine by HPLC. The reaction mixture (total vol. 5 ml) contained cell extract, 25 μ mol 2-oxoglutarate, 1 μ mol PLP and 250 μ mol potassium phosphate buffer pH 7.0. The reaction was started by the addition of 5 μ mol phenylglycine. Samples (0.5 ml), taken at intervals, were analysed by HPLC using the same conditions as described for the analysis of the mixture during the incubation of washed cells with phenylglycine.

(ii) *Phenylglycine dehydrogenase (PMS-dependent).* This enzyme was assayed by recording the oxygen uptake with a polarographic oxygen monitor. The reaction mixture (total vol. 3 ml) contained 150 μ mol Tris/HCl buffer pH 9.0, 0.33 μ mol PMS and cell extract. The reaction was initiated by the addition of 3 μ mol substrate. Alternatively, the enzyme activity was recorded by measuring phenylglycine concentrations at various intervals. The complete reaction mixture (total vol. 5 ml) contained cell extract, 250 μ mol Tris/HCl buffer pH 9.0 and 5 μ mol PMS. The reaction was started by adding 10 μ mol phenylglycine. Samples were analysed for phenylglycine by HPLC (conditions as before).

(iii) *Phenylglycine hydroxylase.* The possible presence of phenylglycine hydroxylase activity was investigated using the procedure described by Guroff & Ito (1964) for phenylalanine hydroxylase, except that phenylalanine was replaced by phenylglycine.

(iv) *Phenylglycine dehydrogenase [NAD(P)⁺-dependent].* The presence of an NAD(P)⁺-dependent enzyme was assayed for in a way similar to phenylalanine dehydrogenase (Hummel *et al.*, 1984), except that phenylalanine was replaced by phenylglycine.

(v) *Phenylglycine oxidase.* The presence of a possible phenylglycine oxidase was investigated by means of a polarographic oxygen probe. The reaction mixture (total vol. 3 ml) contained 150 μ mol buffer (either potassium phosphate pH 7.0 or Tris/HCl pH 9.0) and cell extract. The reaction was started by the addition of 3 μ mol phenylglycine.

(vi) *Phenylglyoxylate decarboxylase (Benzoylformate carboxy-lyase, EC 4.1.1.7).* Phenylglyoxylate consumption by cell extracts was measured as described previously (van den Tweel *et al.*, 1986a). Samples were analysed by HPLC using the same conditions as described above.

(vii) *Benzaldehyde dehydrogenase (PMS-dependent).* This enzyme was also assayed by means of a polarographic oxygen probe. The reaction mixture (total vol. 3 ml) contained 150 μ mol Tris/HCl buffer pH 9.0, 0.33 μ mol PMS and cell extract. The reaction was initiated by the addition of 3 μ mol benzaldehyde.

(viii) *Benzaldehyde dehydrogenase [NAD(P)⁺-dependent].* Activity of this enzyme was measured as described previously (van den Tweel *et al.*, 1986a).

(ix) *Gentisate 1,2-dioxygenase (EC 1.13.11.4).* Activity of this enzyme was measured spectrophotometrically as described by Crawford *et al.* (1975).

RESULTS

Taxonomic studies and growth characteristics of strain F24

Strain F24, isolated from soil, was a bright yellow, strictly aerobic, Gram-negative, motile rod, which was not able to grow at 37 °C. It did not produce acid from glucose, was unable to

Table 1. Rates of oxygen uptake by washed cell suspensions of *Flavobacterium* F24 cultivated on various carbon sources

Substrate	Rate of oxygen uptake* [nmol O ₂ min ⁻¹ (mg protein) ⁻¹] by cells grown on		
	D-Phenylglycine	L-Phenylglycine	Succinate
D-Phenylglycine	70	5	0
L-Phenylglycine	35	40	0
DL-2-Hydroxyphenylglycine	5	5	0
DL-3-Hydroxyphenylglycine	5	5	0
DL-4-Hydroxyphenylglycine	5	5	0
Phenylglyoxylate	65	60	0
Benzaldehyde	50	55	0
Benzoate	50	40	0
2-Hydroxybenzoate	0	ND	0
3-Hydroxybenzoate	20	20	0
4-Hydroxybenzoate	0	0	0
Catechol	0	0	0
Gentisate	20	15	0
Protocatechuate	0	0	0
2,3-Dihydroxybenzoate	0	ND	0
Succinate	20	30	50

ND, Not determined.

* Rates are the means of results from at least two separate cell suspensions and are corrected for endogenous oxygen uptake.

form spores, or to denitrify and was both oxidase- and aminopeptidase-positive. Gelatine and aesculin were not hydrolysed and β -galactosidase, urease and arginine dihydrolase were not produced. Strain F24 was able to use the following compounds as sole carbon and energy source: D- and L-phenylglycine, phenylglyoxylate, DL-mandelate, D- and L-phenylalanine, D- and L-tyrosine, 3- and 4-hydroxybenzoate, phenylacetate, acetate, pyruvate, succinate, 3-hydroxybutyrate and glucose. On D- and L-phenylglycine doubling times of 10 and 12 h respectively were observed. Using succinate as carbon source strain F24 doubled in 2.5 h. No growth was observed when using DL-2-, DL-3- or DL-4-hydroxyphenylglycine, benzylalcohol, 2-phenylethanol or malonate as potential growth substrates at 1.0 g l⁻¹. Surprisingly, strain F24 did not grow on benzoate (0.25, 0.5 or 1.0 g l⁻¹) and the addition of benzoate (0.05 g l⁻¹) to a mineral salts medium containing D-phenylglycine (1.0 g l⁻¹) inhibited growth.

Strain F24, on the basis of the above taxonomic results and its growth characteristics, was tentatively identified as a member of the genus *Flavobacterium*.

Incubation experiments with whole cells

The ability of intact cells to oxidize possible intermediates in the metabolism of phenylglycine was tested indirectly by measuring rates of oxygen uptake by intact cells grown on either D- or L-phenylglycine or on succinate (Table 1). Both D- and L-phenylglycine-grown cells showed good initial rates of oxidation of L-phenylglycine, phenylglyoxylate, benzaldehyde, benzoate, 3-hydroxybenzoate and gentisate (2,5-dihydroxybenzoate). D-Phenylglycine-grown cells also readily metabolized D-phenylglycine while L-phenylglycine-grown cells did not. Hydroxylated phenylglycine derivatives, which would be intermediates in the case of an initial hydroxylation, were not oxidized by D- or L-phenylglycine-grown cells. *Flavobacterium* F24 cells grown on succinate did not oxidize the aromatic compounds tested.

Phenylglycine metabolism by washed cells was also tested directly by measuring the disappearance of substrates from incubation mixtures by HPLC. Cells grown on D-phenylglycine readily metabolized both phenylglycine isomers (Fig. 1). The L-stereoisomer, however, was metabolized at about half the rate of D-phenylglycine metabolism (Fig. 1c).

Fig. 1. Phenylglycine consumption (●, ○) and benzoate accumulation (▲, △) by cell suspensions of *Flavobacterium* F24 cultivated on D-phenylglycine. The incubation mixture (5 ml) contained 15.5 mg protein and 10 μ mol phenylglycine: (a) DL-phenylglycine, (b) D-phenylglycine, (c) L-phenylglycine. The incubation was in the presence (○, △) or absence (●, ▲) of AOA.

During the incubations with DL- and D-phenylglycine a product transiently accumulated (Fig. 1a, b) which was identified as benzoate according to its retention time and UV absorption characteristics. No benzoate accumulated during the incubation with L-phenylglycine (Fig. 1c). Similar experiments were also performed in the presence of 15 mM-aminooxyacetate (AOA), a pyridoxal phosphate (PLP) inhibitor (Hotta, 1968), to investigate whether D- and/or L-phenylglycine were initially transaminated as described for *Pseudomonas putida* LW-4 (van den Tweel *et al.*, 1986a). L-Phenylglycine metabolism was completely prevented in the presence of AOA, indicating that a PLP-dependent enzyme is involved in the metabolism of this stereoisomer (Fig. 1c). However, when this experiment was performed with D-phenylglycine as substrate, no inhibition was observed (Fig. 1b). Incubation with DL-phenylglycine in the presence of AOA resulted in phenylglycine metabolism until 50% of the mixture was utilized (Fig. 1a). Different results were obtained with *Flavobacterium* F24 cells cultivated on L-phenylglycine. Such cells consumed about 50% of racemic DL-phenylglycine, did not metabolize D-phenylglycine, and readily metabolized L-phenylglycine. In the presence of the PLP inhibitor AOA, no consumption of DL-phenylglycine or of L-phenylglycine could be measured.

Washed suspensions of D-phenylglycine-grown cells were also incubated with either DL-2-, DL-3- or DL-4-hydroxyphenylglycine but this did not result in a decrease in concentration of these substrates, indicating that no hydroxylated phenylglycine derivative is involved in D-phenylglycine metabolism in this species.

The initial step in the metabolism of D- and L-phenylglycine

From the above results it was suspected that in strain F24 D- and L-phenylglycine are degraded by two different enzymes; this hypothesis was tested in *in vitro* experiments. Cell extracts of D-

Fig. 2. Transamination of phenylglycine (●) and phenylglyoxylate accumulation (□) by cell extracts of *Flavobacterium* F24 cultivated on D-phenylglycine. The reaction mixture contained 15 mg protein. (a) Incubation with DL-phenylglycine; (b) with D-phenylglycine; (c) with L-phenylglycine. Similar results were obtained using extracts of cells grown on L-phenylglycine (data not shown).

Table 2. Specific enzyme activities of some enzymes involved in phenylglycine degradation in *Flavobacterium* F24 cultivated on various carbon sources

Enzyme	Specific enzyme activity* [nmol min ⁻¹ (mg protein) ⁻¹] of extracts of cells grown on		
	D-Phenylglycine	L-Phenylglycine	Succinate
PMS-dependent D-phenylglycine dehydrogenase	45	5	0
L-Phenylglycine transaminase	10	10	10
Phenylglyoxylate decarboxylase	10	5	0
Benzaldehyde dehydrogenase			
NAD ⁺ -dependent	5	5	5
PMS-dependent	225	200	5
Gentisate 1,2-dioxygenase	15	15	0

* The activities are the means of results from two separate extracts.

and L-phenylglycine-grown cells consumed approximately 50% of racemic DL-phenylglycine in the simultaneous presence of PLP and 2-oxoglutarate (Fig. 2a); under these conditions they did not metabolize D-phenylglycine (Fig. 2b) but fully transformed L-phenylglycine (Fig. 2c). Chiral HPLC analysis of the residue after completion of the reaction with DL-phenylglycine (Fig. 2a) showed that only D-phenylglycine was present, indicating that only the L-stereoisomer was transaminated. Replacing 2-oxoglutarate by oxaloacetate, pyruvate or phenylpyruvate also did not result in a transamination of D-phenylglycine. During these incubations (Fig. 2) an almost stoichiometric accumulation of phenylglyoxylate from L-phenylglycine was obtained. Cell extracts of succinate-grown cells were also able to transaminate L-phenylglycine (Table 2). These results show that L-phenylglycine is initially transaminated by a constitutive enzyme, but do not

Fig. 3. PMS-dependent phenylglycine oxidation (●) and phenylglyoxylate accumulation (□) by cell extracts of *Flavobacterium* F24 grown on D-phenylglycine. The reaction mixture contained 4.6 mg protein. (a) Incubation with DL-phenylglycine, (b) with D-phenylglycine, (c) with L-phenylglycine.

reveal the mechanism of D-phenylglycine metabolism. Further experiments with cell extracts showed that no D-phenylglycine hydroxylase, no NAP(P)⁺-dependent dehydrogenase and no oxidase were present in D-phenylglycine-grown cells. However, D-phenylglycine was readily oxidized when PMS was added to cell extracts as measured by oxygen consumption. This rate of oxidation was maximal [$165 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] at pH 9.5 using a 50 mM- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer. Since PMS is readily auto-oxidized at pH 9.5 and above, further experiments were done at pH 9.0 using a Tris/HCl buffer. Fig. 3 shows the results obtained with cell extracts of D-phenylglycine-grown *Flavobacterium* F24 cells during an incubation at pH 9.0 in the presence of PMS. Under these conditions only 50% of the DL-phenylglycine was oxidized and at a relatively low rate (Fig. 3a) when compared with the D-phenylglycine oxidation rate (Fig. 3b), whereas L-phenylglycine was not oxidized (Fig. 3c). Chiral HPLC analysis of the residue after completion of the reaction with DL-phenylglycine (Fig. 3a) showed that only L-phenylglycine was left behind. During all these incubations (Fig. 3) D-phenylglycine was converted stoichiometrically to phenylglyoxylate.

Oxygen uptake experiments in the presence of PMS showed that apart from D-phenylglycine several other D-amino acids were oxidized by cell extracts of D-phenylglycine-grown cells whereas L-amino acids were not (Table 3). Surprisingly, none of the D-hydroxyphenylglycines could be oxidized while other aromatic D-amino acids were readily oxidized. The apparent Michaelis constant for D-phenylglycine of the enzyme system in cell extracts was 0.65 mM.

Phenylglyoxylate decarboxylase

Both the transamination of L-phenylglycine and the PMS-dependent oxidation of D-phenylglycine resulted in the formation of phenylglyoxylate. This latter compound was very slowly decarboxylated by cell extracts as shown by recording phenylglyoxylate disappearance from incubation mixtures by means of HPLC during incubations with thiamin pyrophosphate (Table 2). When *Flavobacterium* F24 was grown on succinate no phenylglyoxylate decarboxylase was present (Table 2).

Table 3. Oxidation of various amino acids by cell extracts of *Flavobacterium* F24 grown on D-phenylglycine

Substrate	Specific activity [nmol O ₂ min ⁻¹ (mg protein) ⁻¹]	Substrate	Specific activity [nmol O ₂ min ⁻¹ (mg protein) ⁻¹]
DL-Phenylglycine	25	DL-Isoleucine	50
D-Phenylglycine	45	DL-Valine	85
L-Phenylglycine	0	DL-Leucine	40
D-Phenylalanine	55	DL-Norleucine	25
D-Tyrosine	50	DL-Norvaline	90
D-Tryptophan	235	DL-Ornithine	15
DL-Histidine	65	DL- α -Aminobutyrate	40
DL-5-Hydroxytryptophan	55		

No activity was observed with the following substrates: DL-2-, DL-3- or DL-4-hydroxyphenylglycine, L-phenylalanine, L-histidine, L-5-hydroxytryptophan, glycine, DL-alanine, DL-glutamine, DL-asparagine, L-isoleucine, L-valine, L-leucine, DL-aspartate, DL-glutamate, DL-threonine, DL-serine, L-ornithine, L-proline, L-arginine, L-lysine, L-methionine, L-hydroxyproline, L-tyrosine and L-tryptophan.

Oxidation of benzaldehyde

Flavobacterium F24 possessed a NAD⁺-dependent benzaldehyde dehydrogenase which was present both in succinate- and D- or L-phenylglycine-grown cells (Table 2). NADP⁺ could not replace NAD⁺. Along with this constitutive NAD⁺-dependent aldehyde dehydrogenase an inducible PMS-dependent benzaldehyde dehydrogenase was present in phenylglycine-grown cells. The latter enzyme showed a pH optimum at pH 9. The activity of this enzyme was not stimulated by the addition of NH₄Cl (15 mM). No activity was lost upon dialysis for 24 h.

Further metabolism of benzoate

Simultaneous adaptation experiments (Table 1) indicated that benzoate was an intermediate in phenylglycine metabolism and that it was metabolized via 3-hydroxybenzoate to gentisate. In spite of this, no enzymic activity for benzoate or 3-hydroxybenzoate oxidation was detected in extracts using either NADH or NADPH as a cofactor. However, experiments with cell extracts confirmed that a gentisate dioxygenase was induced during growth on D- or L-phenylglycine whereas no catechol and protocatechuate dioxygenases were present. The spectral changes observed during cleavage of the benzene nucleus of gentisate at 334 nm were characteristic of a breakdown via maleylpyruvate (Lack, 1959). Further degradation of maleylpyruvate was dependent upon the addition of reduced glutathione, indicating that a GSH-dependent isomerase is present in this species. Moreover, *N*-ethylmaleimide (NEM), a glutathione-trapping agent (Crawford & Frick, 1977), completely inhibited the GSH-dependent degradation of maleylpyruvate; this inhibition could be overcome by adding GSH in slight excess over NEM.

DISCUSSION

The *Flavobacterium* F24 described here grew on both D- and L-phenylglycine albeit slowly (*t*_d 10 and 12 h, respectively) when compared with *P. putida* LW-4 (van den Tweel *et al.*, 1986a), which doubled in 1.5–2.0 h when growing on D-phenylglycine. The low growth rate of *Flavobacterium* F24 on phenylglycine may explain the rather low specific enzyme activities of some enzymes (Table 2) involved in phenylglycine degradation in this organism. Strain F24 degrades D-phenylglycine by a PMS-dependent D-amino-acid dehydrogenase and this enzyme is induced in the presence of D-phenylglycine. No D-phenylglycine transaminase as present in *P. putida* LW-4 (van den Tweel *et al.*, 1986a) was detected in F24 and unfortunately neither was a phenylglycine hydroxylase detected in this organism. Such mono-oxygenase activity might have been expected to be present in view of the rather broad occurrence of a similar mono-oxygenase employed for the hydroxylation of the analogous compound phenylalanine (Guroff & Ito, 1964;

Fig. 4. Proposed pathway for the metabolism of D- and L-phenylglycine in *Flavobacterium* F24. (1) D-Phenylglycine, (2) L-phenylglycine, (3) phenylglyoxylate, (4) benzaldehyde, (5) benzoate, (6) 3-hydroxybenzoate, (7) gentisate. TPP, thiamin pyrophosphate.

Chandra & Vining, 1968; Friedrich & Schlegel, 1972; Nakata *et al.*, 1979). Since a regio- and stereo-selective phenylglycine mono-oxygenase would be of great interest for a biotechnological formation of D(-)-4-hydroxyphenylglycine we are currently screening several other phenylglycine-utilizing bacteria for such an enzyme. Dye-linked D-amino-acid dehydrogenases are involved in the metabolism of various D-amino acids: D-valine (Marshall & Sokatch, 1968), allohydroxy-D-proline (Bater *et al.*, 1977), D-tryptophan (Tsukada, 1966) and D-alanine (Pioli *et al.*, 1976) and in general show a fairly broad substrate specificity. The D-amino acid dehydrogenase in D-phenylglycine-grown *Flavobacterium* F24 cells also oxidized various D-amino acids but it should be emphasized that it is not yet known whether this activity is to be ascribed to only one enzyme. L-Phenylglycine on the other hand was metabolized by a constitutive transaminase which showed no activity with the D-stereoisomer of phenylglycine.

Phenylglyoxylate was identified as the product of both the PMS-dependent D-phenylglycine oxidation and the L-phenylglycine transamination. In extracts, it was slowly decarboxylated in the presence of thiamin pyrophosphate. The activity of the phenylglyoxylate decarboxylase was very low when compared with this enzyme activity in *P. putida* LW-4 grown on either D- or L-phenylglycine (van den Tweel *et al.*, 1986a). No NAD(P)⁺-dependent benzaldehyde dehydrogenase was induced in *Flavobacterium* F24 cells during growth on D- or L-phenylglycine. Instead a PMS-dependent enzyme showing maximal activity around pH 9.0 was detected. Until now, no PMS-dependent aldehyde dehydrogenase has been implicated in the catabolism of aromatic compounds, but several dye-linked aldehyde dehydrogenases are involved in aliphatic aldehyde oxidation (Stirling & Dalton, 1978). One group of these enzymes, the quinoprotein alcohol dehydrogenases (Duine & Frank, 1981), may show a requirement for NH_4^+ (Yamanaka, 1981) but the PMS-dependent benzaldehyde dehydrogenase of *Flavobacterium* F24 did not require NH_4^+ for activity.

Although benzoate presumably is an intermediate in phenylglycine metabolism in *Flavobacterium* F24, it could not support growth. In fact, benzoate inhibited growth above a

concentration of 0.05 g l^{-1} . Inhibition of substrate transport into the cells by benzoate (Freese *et al.*, 1973) may possibly explain this phenomenon.

From simultaneous adaptation experiments a further oxidation of benzoate via 3-hydroxybenzoate to gentisate seems most likely. The fact that neither a benzoate nor a 3-hydroxybenzoate hydroxylase could be measured in cell extracts may result from a low stability of these enzymes *in vitro*. The benzene nucleus of gentisate was cleaved by an inducible dioxygenase yielding maleylpyruvate, which was further degraded by a GSH-dependent reaction sequence. This is in agreement with the fact that until now all Gram-negative organisms have been shown to possess a GSH-dependent gentisate or homogentisate pathway (Hagedorn & Chapman, 1985; Hagedorn *et al.*, 1985; van den Tweel *et al.*, 1986b).

The results presented in this paper show that *Flavobacterium* F24 metabolizes both stereoisomers of phenylglycine by the converging pathway shown in Fig. 4.

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REFERENCES

- ARNAUD, A., GALZY, P. & JALLAGEAS, J.-C. (1980). Production d'acides α -aminés stéréospécifiques par hydrolyse biologique d' α -aminonitriles racémiques. *Société chimique de France* 1-2, 87-90.
- BATER, A. J., VENABLES, W. A. & THOMAS, S. (1977). Allohydroxy-D-proline dehydrogenase. An inducible membrane-bound enzyme in *Pseudomonas aeruginosa* PAO1. *Archives of Microbiology* **112**, 287-289.
- BOESTEN, W. H. & MEYER-HOFFMAN, L. R. M. (1975). Enzymepreparaat met aminopeptidase activiteit. Dutch Patent Application no. 7513551.
- CECERE, F., GALLI, G. & MORISI, F. (1975). Substrate and steric specificity of hydropyrimidine hydrase. *FEBS Letters* **57**, 192-194.
- CECERE, F., GALLI, G., DELLA PENNA, G. & RAPPUOLI, B. (1978). Process for producing D-carbamoyl amino acids and the corresponding D-amino acids. British Patent no. 1506067.
- CHANDRA, P. & VINING, L. C. (1968). Conversion of phenylalanine to tyrosine by microorganisms. *Canadian Journal of Microbiology* **14**, 573-578.
- CRAWFORD, R. L. & FRICK, T. D. (1977). Rapid spectrophotometric differentiation between glutathione-dependent and glutathione-independent gentisate and homogentisate pathways. *Applied and Environmental Microbiology* **34**, 170-174.
- CRAWFORD, R. L., HUTTON, S. W. & CHAPMAN, P. J. (1975). Purification and properties of gentisate 1,2-dioxygenase from *Moraxella osloensis*. *Journal of Bacteriology* **121**, 794-799.
- DUINE, J. A. & FRANK, J. (1981). Methanol dehydrogenase: a quinoprotein. In *Proceedings of the Third International Symposium on Microbial Growth on C₁-compounds*, pp. 31-41. Edited by H. Dalton. London: Heyden.
- FREESE, E., SHEU, C. W. & GALLIERS, E. (1973). Function of lipophilic acids as antimicrobial food additives. *Nature, London* **241**, 321-325.
- FRIEDRICH, B. & SCHLEGEL, H. G. (1972). Die Hydroxylierung von Phenylalanin durch *Hydrogenomonas eutropha* H16. *Archives of Microbiology* **83**, 17-31.
- GUROFF, G. & ITO, T. (1964). Phenylalanine hydroxylation by *Pseudomonas* species (ATCC 11299a). *Journal of Biological Chemistry* **240**, 1175-1184.
- HAGEDORN, S. R. & CHAPMAN, P. J. (1985). Glutathione-independent maleylacetoacetate isomerase in Gram-positive bacteria. *Journal of Bacteriology* **163**, 803-805.
- HAGEDORN, S. R., BRADLEY, G. & CHAPMAN, P. J. (1985). Glutathione-independent isomerization of maleylpyruvate by *Bacillus megaterium* and other Gram-positive bacteria. *Journal of Bacteriology* **163**, 640-647.
- HOTTA, S. S. (1968). Oxidative metabolism of isolated brain mitochondria: changes caused by aminooxyacetate. *Archives of Biochemistry and Biophysics* **127**, 132-139.
- HUMMEL, W., WEISS, N. & KULA, M.-R. (1984). Isolation and characterization of a bacterium possessing L-phenylalanine dehydrogenase activity. *Archives of Microbiology* **137**, 47-52.
- LACK, L. (1959). The enzymic oxidation of gentisic acid. *Biochimica et biophysica acta* **34**, 117-123.
- MARSHALL, V. P. & SOKATCH, J. R. (1968). Oxidation of D-amino acids by a particulate enzyme from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **95**, 1419-1424.
- NAKAMORI, S., YOKOZEKI, K., MITSUGI, K., EGUCHI, E. & IWAGAMI, H. (1980). Method for producing D- α -amino acid. United States Patent no. 4211840.
- NAKATA, H., YAMAUCHI, T. & FUGISAWA, H. (1979). Phenylalanine hydroxylase from *Chromobacterium violaceum*. Purification and characterization. *Journal of Biological Chemistry* **254**, 1829-1833.
- OLIVIERI, R., FASCETTI, E., ANGELINI, L. & DEGEN, L. (1979). Enzymatic conversion of N-carbamoyl-D-amino acids to D-amino acids. *Enzyme and Microbial Technology* **1**, 201-204.
- OLIVIERI, R., FASCETTI, E., ANGELINI, L. & DEGEN, L. (1981). Microbial transformation of racemic hydantoins to D-amino acids. *Biotechnology and Bioengineering* **23**, 2173-2183.

- PIOLI, D., VENABLES, W. A. & FRANKLIN, F. C. H. (1976). D-Alanine dehydrogenase. *Archives of Microbiology* **110**, 287–293.
- SCHUTT, H. (1981). Stereoselective resolution of phenylglycine derivatives and 4-hydroxyphenylglycine derivatives with enzyme resins. United States Patent no. 4260684.
- SCHUTT, H., SCHMIDT-KASTNER, G., ARENS, A. & PREISS, M. (1985). Preparation of optically active D-aryl glycines for use as side chains for semisynthetic penicillins and cephalosporins using immobilized subtilisins in two-phase systems. *Biotechnology and Bioengineering* **27**, 420–433.
- STIRLING, D. I. & DALTON, H. (1978). Purification and properties of an NAD(P)⁺-linked formaldehyde dehydrogenase from *Methylococcus capsulatus* (Bath). *Journal of General Microbiology* **107**, 19–29.
- TAKAHASHI, S., OHASHI, T., KII, Y., KUMAGAI, H. & YAMADA, H. (1979). Microbial transformations of hydantoins to N-carbamyl-D-amino acids. *Journal of Fermentation Technology* **57**, 328–332.
- TSUKADA, K. (1966). D-Amino acid dehydrogenase of *Pseudomonas fluorescens*. *Journal of Biological Chemistry* **241**, 4522–4528.
- VAN DEN TWEEL, W. J. J., SMITS, J. P. & DE BONT, J. A. M. (1986a). Microbial metabolism of D- and L-phenylglycine by *Pseudomonas putida* LW-4. *Archives of Microbiology* **144**, 169–174.
- VAN DEN TWEEL, W. J. J., JANSSENS, R. J. J. & DE BONT, J. A. M. (1986b). Degradation of 4-hydroxyphenylacetate by *Xanthobacter* 124X; physiological resemblance with other Gram-negative bacteria. *Antonie van Leeuwenhoek* **52**, 309–318.
- YAMADA, S., HONGO, C., YOSHIOKA, R. & CHIBATA, I. (1979). Preparation of D-p-hydroxyphenylglycine. Optical resolution of DL-p-hydroxyphenylglycine with d-3-bromocamphor-8-sulfonic acid. *Agricultural and Biological Chemistry* **43**, 395–396.
- YAMANAKA, K. (1981). Comparative aspects of methanol dehydrogenases. In *Proceedings of the Third International Symposium on Microbial Growth on C₁-compounds*, pp. 21–30. Edited by H. Dalton. London: Heyden.