BACTERIAL FORMATION OF HYDROXYLATED AROMATIC COMPOUNDS

Promotoren: **dr.ir. J.A.M. de Bont,**
hoogleraar in de industriële microbiologie

dr.ir. J. Tramper,
hoogleraar in de bioprocestechnologie

W.J.J. van den Tweel

BACTERIAL FORMATION OF HYDROXYLATED AROMATIC COMPOUNDS

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 8 april 1988
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

1. Zowel de resultaten als de conclusies van Tokarski et al. betreffende de vorming van (+)-2-aminobutyraat uit 2-ketobutyraat met behulp van een transaminase zijn ernstig aan bedenkingen onderhevig.
   - Tokarski, Z., Klei, H.E. & Berg, C.M. (1988). Biotechnol. Lett. 10,7-10

2. De door Tabak et al. veronderstelde aerobe groei op tetrachloormethaan is principieel onjuist.
   - Tabak, H.H., Quave, S.A., Mashni, C.I. & Barth, E.F. (1981). J. Water Poll. Control Fed. 53,1503-1518

3. De veronderstelling van Nilsson et al., dat na activatie met tosylchloride alléén de primaire hydroxygroepen van agarose zijn gesulfoneerd, is gezien hun eigen onderzoeksresultaten onjuist.
   - Nilsson, K., Norrlöw, O. & Mosbach, K. (1981). Acta Chem. Scand. 35,19-27

4. Te vaak wordt vergeten dat ook toegepast onderzoek fundamenteel van karakter kan zijn.

5. Het feit dat Stevenson en Mandelstam geen benzaldehyde dehydrogenase activiteit vonden in celvrije extracten van 4-hydroxybenzoaat-gekweekte *Pseudomonas putida* A.3.12 cellen wordt niet veroorzaakt door instabiliteit van het benzaldehyde dehydrogenase.
   - Stevenson, I.L. & Mandelstam, J. (1965). Biochem. J. 96,354-362
   - dit proefschrift, hoofdstuk 5

6. De persoonlijke mededeling van Lingens aan Reineke dat in *Pseudomonas* sp. CBS3 voor de chloride afsplitsing van 4-chloorbenzoaat moleculaire zuurstof noodzakelijk is, wordt niet onderstreept door eigen bevindingen.
   - Reineke, W. (1984). In: Microbial Degradation of Organic Compounds (Gibson, D.T., Ed.), p. 338. Marcel Dekker Inc., New York
   - Müller, R., Thiele, J., Klages, U. & Lingens, F. (1984). Biochem. Biophys. Res. Commun. 124,178-182

7. De aanschaf van geavanceerde apparatuur resulteert niet automatisch in een vereenvoudiging van het onderzoekswerk.

8. Ten onrechte stellen Wang et al. dat het door hen geïsoleerde flavoproteïne als enig enzym in staat is een aromatische verbinding te hydroxyleren op de paraplaats ten opzichte van een aanwezige hydroxygroep.
   - Wang, L.H., Hamzah, R.Y., Yu, Y. & Tu, S.C. (1987). Biochemistry 26,1099-1104
   - Hareland, W.A., Crawford, R.L., Chapman, P.J. & Dagley, S. (1975). J. Bacteriol. 121,272-285

9. De foto die in vrijwel alle dagbladen is afgedrukt, betreffende het besproeien van aardbeiplanten in Californië met de "ijs minus" mutant van *Pseudomonas syringae* door een vrouw gehuld in een soort "maanpak" (april 1987), draagt niet bij tot een objectief maatschappelijk inzicht in experimenten met genetisch gemanipuleerde microorganismen.

10. De opmerkingen van Molinari en Drioli betreffende de scheiding van substraten en producten van een lipase-gekatalyseerde hydrolyse van olijfolie in een membraanreactor zijn uiterst dubieus.
   - Molinari, R. & Drioli, E. (1986). Proceedings of the Fifth Yugoslavian-Austrian-Italian Chemical Engineering Conference, Portoroz, Yugoslavia, September 16-18

11. Om frustraties achteraf te voorkomen, dienen weddenschappen vooraf duidelijk omschreven te worden vastgelegd.

W.J.J. van den Tweel
Bacterial formation of hydroxylated aromatic compounds
Wageningen, 8 april 1988

Aan Lisette
Aan mijn ouders

DANKWOORD

Gaarne wil ik een ieder bedanken die heeft bijgedragen aan de voltooiing van dit proefschrift.

- Jan Smits voor de wezenlijke bijdrage aan dit boekwerkje.
- Jan de Bont en Hans Tramper voor de vrijheid in onderzoek, begeleiding en vorming.
- Alle Bruggink, Bert Hulshof en Roger Sheldon voor de prettige begeleiding van het project vanuit de industrie.
- Tom Kieboom, Herman van Bekkum en Karel Luyben voor het initiëren van het cis-benzeenglycol-project.
- Jos Bessems, Angelie Beuvink, Rob Broekmeulen, Nico ter Burg, Harrie Hens, Rob Janssens, Jan-Bart Kok, Jan Koppejan, Wim de Laat, Eric Marsman, Philippe Schijns, Dick Smit, Marc Vorage en Myra Widjojoatmodjo voor het aandragen van de vele gegevens.
- Ron Ogg en Jan-Hendrik Roskam voor de goede samenwerking.
- Klaas van 't Riet en Prof. Dr. C.J.E.A. Bulder voor suggesties en voor het corrigeren van de eerste manuscripten.
- Willem van Berkel voor de prettige samenwerking op biochemisch gebied.
- Nees Slotboom en de mensen van de tekenkamer in het Biotechnion voor het tekenwerk.
- Frits Lap en de mensen van de Centrale Dienst van het Biotechnion voor hun vakkundige hulp.
- Bart Geurts en Dick de Bie voor deskundige hulp en advies op organisch-chemisch gebied.
- Piet Stouten voor de hulp bij enkele essentiële HPLC analyses.
- Maarten Posthumus en Kees Teunis voor de massaspectrometrische analyses.
- Mevrouw Tineke de Bruin-Tol voor deskundige hulp bij het determineren en bewaren van diverse geïsoleerde micro-organismen.
- Alex van Neerven voor het maken van diverse electronenmicroscopische foto's en voor de prettige samenwerking.
- Willem van Barneveld en de heer W.C. Nieuwboer voor het vervaardigen van diverse glazen voorwerpen.
- Alle medewerkers en studenten van de sectie Industriële Microbiologie, de vakgroep Microbiologie en de sectie Proceskunde voor de gezelligheid tijdens en na het werk.
- De Stichting voor de Technische Wetenschappen en Andeno B.V. voor de financiële steun.

CONTENTS

Chapter 1

INTRODUCTION

1 GENERAL

The term aromatic was first used in the 19th century to designate a group of compounds derived from plant oils and gums which had pleasant odors. In organic chemistry, however, the term aromatic has a different meaning and relates to the structure of molecules. Benzene is the most known and simplest aromatic compound which was first obtained in the beginning of the 19th century by destructive distillation of coal. The C_6H_6-unit of benzene presented a major challenge to organic chemists for many decades. In 1865 the German chemist Kekulé proposed that benzene consists of a ring containing six carbon atoms connected by alternating single and double bonds. However, benzene does not undergo the addition reactions characteristic of alkenes; instead, it exhibits a stability akin to that of alkanes. A more satisfactory representation for the benzene structure became available with the concept of resonance. In resonance terminology, benzene is a hybrid of the two Kekulé structures (Figure 1). The π-electrons are delocalized in a "π cloud" distributed over the six carbon atoms of the ring, resulting in a remarkable thermodynamic stability. Compounds containing a planar cyclic system with $(4n + 2)$ π-electrons, where $n = 0, 1, 2, 3, ...$, are referred to as aromatic compounds.

Several aromatic compounds and hydroxylated aromatic compounds (phenols) are produced on a large scale and they are used for various purposes (Table 1). Benzene, toluene and other aromatic compounds were originally obtained by the distillation of coal tar. Today these compounds are mainly produced from petroleum.

In addition to the phenolic bulk compounds shown in Table 1 also many specialty hydroxylated aromatic compounds are of great importance for agrochemical, pharmaceutical and petrochemical industries

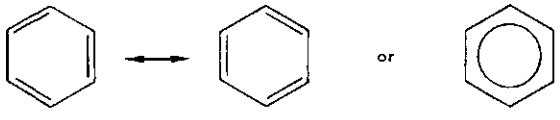

Figure 1. Representation of the benzene ring.

Table 1. Industrial production and main application of various aromatic compounds.

Compound	Production (tons)	Uses
Benzene[a]	$5.5 \cdot 10^6$	solvent, detergents, resins, polymers
Toluene[b]	$>11.8 \cdot 10^6$	explosives, resins, polymers
Ethylbenzene[b]	$>13.0 \cdot 10^6$	precursor for styrene
Styrene[b]	$>11.5 \cdot 10^6$	plastics, resins, insulators
o-Xylene[b]	$> 2.0 \cdot 10^6$	plasticizers, pigments
m-Xylene[b]	$1.6 \cdot 10^6$	polyesters, precursor for fungicides
p-Xylene[b]	$> 5.4 \cdot 10^6$	polyesters, fibers, films, resins
Phenol[b]	$> 3.7 \cdot 10^6$	resins, caprolactam
Catechol[a]	$> 2.0 \cdot 10^4$	photographic developers, UV absorbers and optical brighteners, tire adhesives, dye stuffs
Resorcinol[a]	$3.5 \cdot 10^4$	
Hydroquinone[a]	$> 4.0 \cdot 10^4$	

[a] Estimated for 1979
[b] Estimated for 1981
Source: Encyclopedia of Chemical Technology (1982)

(Table 2). These compounds, however, can not be isolated in sufficient amounts from natural resources. Consequently, they have to be synthesized. Chemically this can be achieved in two ways, either by a direct hydroxylation reaction or by an indirect synthesis. Due to the aromatic ring-structure, direct hydroxylation of aromatics is a difficult task in preparative organic chemistry (Olah et al., 1981; Gunstone, 1960; March, 1985). The problems associated with direct chemical hydroxylation reactions are particularly severe when the compounds to be hydroxylated (or their products) are optically active and/or unstable, since in these instances the reaction should be conducted

Table 2. The use of certain specialty hydroxylated aromatic compounds.

Compound	Uses
D-4-Hydroxyphenylglycine	precursor for antibiotics
4-Hydroxyphenylacetic acid	precursor for antibiotics and pharmaceuticals
L-3,4-Dihydroxyphenylalanine	drug
Indigo	dye
Substituted catechols	precursors for synthetic flavors
4,4-Dihydroxybiphenyl	momomer for polymers
Dihydroxyphenylacetic acids	building blocks for pharmaceuticals and dyes
5-Hydroxytryptophan	drug
4-Hydroxystyrene	fragrance
L-Tyrosine	infusion
α-Tetralol	fragrance
Hydroxybenzaldehydes	building block for pharmaceuticals

rapidly and under mild conditions in order to prevent racemization and decomposition. In case of certain products the direct hydroxylation can be circumvented by performing an indirect multi-step chemical synthesis (e.g. the chemical synthesis of D-4-hydroxyphenylglycine by means of the Strecker-reaction [Dahlmans et al., 1980] or the catalytic asymmetric production of L-3,4-dihydroxyphenylalanine [Knowles et al., 1977]). However, such multi-step processes are mostly not economically attractive. As a result of the shortcomings of both the direct and the indirect chemical formation of hydroxylated aromatics, alternative production processes are of great interest.

Biotechnological production methods for hydroxylated aromatic compounds are a promissing alternative to the cumbersome organic chemical endeavours. In nature, aromatic compounds and hydroxylated forms thereof are abundantly present and are derived via various biological ways often starting from simple compounds. They are formed and degraded by many biological systems and cells or enzymes have been considered for the bioproduction of hydroxylated aromatic compounds (Table 3). This thesis is concerned with aspects of the

Table 3. Formation of hydroxylated aromatics by biological systems.

Biocatalyst	Product	Reference
Plant cells	L-3,4-dihydroxyphenylalanine	Huizing & Wichers (1984)
Yeast	4-hydroxybiphenyl	Schwartz (1981)
Fungi	4,4-dihydroxybiphenyl	Dodge et al. (1979) Schwartz et al. (1980)
	5-hydroxybenzimidazole	Seigle-Murandi et al. (1986)
	L-3,4-dihydroxyphenylalanine	Haneda et al. (1973)
	5-(p-hydroxyanilino)-1,2,3,4-thiatriazole	Theriault & Longfield (1971, 1973)
Bacteria	L-3,4-dihydroxyphenylalanine	Para et al. (1984)
	2- and 5-hydroxyacetanilide	Theriault & Longfield (1967)
	catechol	Shirai (1987)
	5-hydroxytryptophan	Mitoma et al. (1956)
	6-hydroxynicotine acid	Lehky & Kulla (1985)
	4-hydroxybenzoate	van den Tweel et al. (1986c)
	indigo	Ensley et al. (1983)
	D-4-hydroxyphenylglycine	Olivieri et al. (1981)
	para-cresol	Hagedorn (1983)
Enzymes	L-3,4-dihydroxyphenylalanine	Vilanova et al. (1984)
	D-3,4-dihydroxyphenylglycine	Klibanov et al. (1981)
	D-4-hydroxyphenylglycine	van den Tweel et al. (1987c)

biotechnological formation of hydroxylated aromatics by bacteria. Bacteria in principle can achieve the bioformation of hydroxylated aromatic compounds in four different ways: biosynthetic routes, direct hydroxylation methods mediated either by monooxygenases or dioxygenases, replacement of substituents by hydroxyl groups, and the addition and/or modification of side-chains. These four different approaches are discussed in sections 2, 3, 4 and 5 of this introduction.

Once a bacterium is available which catalyzes the desired biotransformation, various process engineering aspects have to be dealt with in order to obtain a good process. The activity and stability of the biocatalyst should be high to obtain a maximal volumetric productivity of the bioreactor. From the point of view of down-stream processing, a high product concentration is generally desirable. Moreover the specificity of the biocatalyst should also be high to facilitate product recovery and to prevent product contamination. Immobilization of the biocatalyst may be useful to enhance stability, and to make a continuous process economically more attractive, while the use of organic solvents can result in a shift of the equilibrium in the desired direction, in a reduction of substrate and/or product inhibition, and may facilitate product recovery. These bioengineering aspects will be briefly discussed in section 6 of the introduction. A general outline of this thesis is given in section 7.

2 BACTERIAL BIOSYNTHESIS OF HYDROXYLATED AROMATIC COMPOUNDS

In nature several metabolic pathways lead to the biosynthesis of aromatic compounds (Weiss & Edwards, 1980). The branched pathway for the biosynthesis of L-phenylalanine, L-tyrosine and L-tryptophan is unquestionable the most significant and interesting one since it leads to the synthesis of alkaloids, various cinnamic acid derivatives, certain hormones, lignin etc. Chorismic acid is the common non-aromatic precursor from which two branches arise. One branch leads to tryptophan via anthranilic acid and the other via prephenic acid to phenylalanine and tyrosine, respectively (Figure 2). Some bacteria are devoid of prephenic acid dehydrogenase activity and synthesize tyrosine via another intermediate called arogenic acid (Stenmark et al., 1974). Subsequent research has shown that various *Pseudomonas* spp. synthesize phenylalanine and tyrosine either via arogenic acid or via, respectively, phenylpyruvic acid and 4-hydroxyphenylpyruvic acid (Keller et al., 1982, 1983; [Figure 2]). L-Tyrosine may also be formed directly through a regio- and stereoselective hydroxylation of phenylalanine, and it has been shown that various bacteria are able to

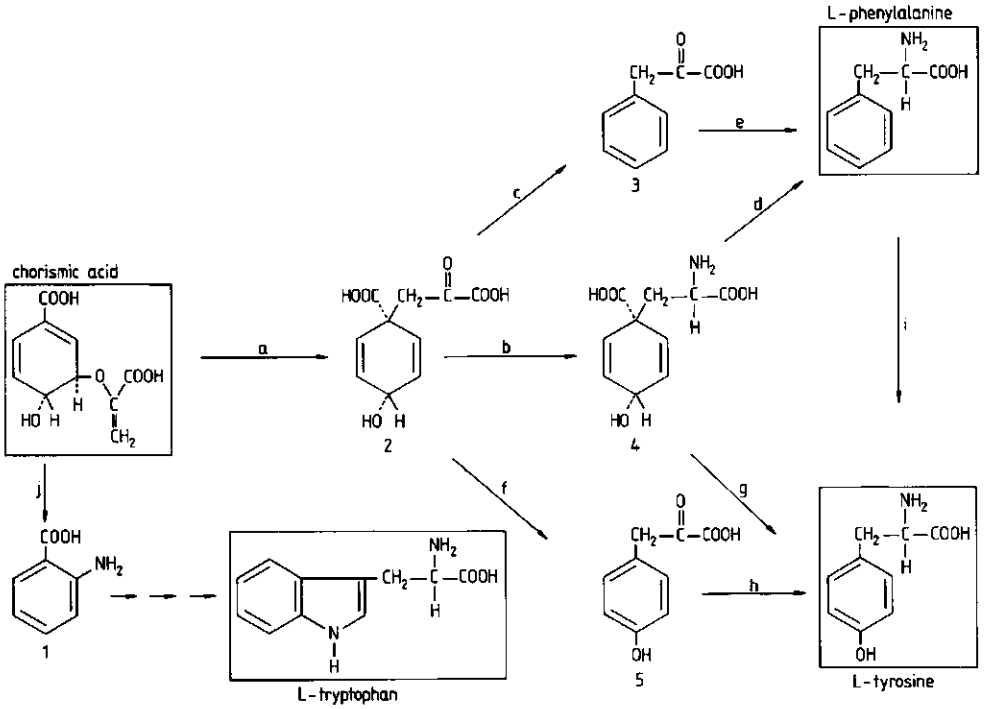

Figure 2. Biochemical routes of L-tryptophan, L-phenylalanine, and L-tyrosine biosynthesis. Anthranilic acid (1), prephenic acid (2), phenylpyruvic acid (3), arogenic acid (4), 4-hydroxyphenylpyruvic acid (5). Enzymes: chorismate mutase (a), prephenate aminotransferase (b), prephenate dehydratase (c), arogenate dehydratase (d), phenylalanine aminotransferase (e), prephenate dehydrogenase (f), arogenate dehydrogenase (g), tyrosine aminotransferase (h), phenylalanine hydroxylase (i), anthranilate synthase (j).

perform this hydroxylation (Chandra & Vining, 1968; Friedrich & Schlegel, 1972).

In bacteria many other hydroxylated aromatic compounds can be derived from the forementioned aromatic amino acids. For instance, 4-hydroxyphenylglycine which is used as a building block for the natural antibiotics enduracidin and nocardicin A is probably synthesized from L-tyrosine (Hatano et al., 1984), while L-5-hydroxytryptophan, the precursor of the bacterial pigment violacein is synthesized by a hydroxylation of L-tryptophan (Mitoma et al., 1956).

3 HYDROXYLATION OF AROMATIC COMPOUNDS

Compounds containing a benzene ring are abundantly present in nature, and to a large extent the continuous operation of the carbon

cycle depends upon the catabolism of these compounds by microorganisms. The ubiquitous distribution of aerobic soil bacteria capable of metabolizing aromatic compounds was already demonstrated in 1928 (Gray & Thornton, 1928). These authors examined 245 soil samples and showed that 146 of the samples contained bacteria capable of oxidizing naphthalene, phenol, or cresol. Subsequent work during the last thirty years has revealed that oxygenases play a very important role in the aerobic degradation and transformation of aromatic compounds. Oxygenases catalyze the incorporation of either one or two atoms of molecular oxygen into their substrates. Therefore, they are classified into two major groups, monooxygenases and dioxygenases.

3.1 HYDROXYLATION OF AROMATIC COMPOUNDS BY MONOOXYGENASES

Monooxygenase catalyze the incorporation of one oxygen atom of molecular oxygen into an organic substrate; the other oxygen atom is reduced to water by a reductant. Hydrogen donors are mainly reduced NAD and NADP. Aerobic bacteria possess various monooxygenases which can hydroxylate aromatic compounds. Some of these enzymes are rather selective for one substrate while others exhibit a relaxed substrate specificity.

3.1.1 SUBSTRATE SELECTIVE MONOOXYGENASES

Bacterial substrate-selective monooxygenases include the pteridine- and flavin-requiring monooxygenases which are important both for anabolic and catabolic reactions. Benzoic acid, L-phenylalanine, and L-mandelic acid can all be hydroxylated on the para position by monooxygenases which require Fe^{2+} and tetrahydropteridine (Figure 3). The benzoate 4-hydroxylase of a *Pseudomonas* sp. also oxidized 4-amino-, 4-nitro-, and 4-chlorobenzoate, however, the hydroxylated products were not characterized (Reddy & Vaidyanathan, 1976). Vaidyanathan and coworkers have also isolated a *Pseudomonas convexa* that degraded L-mandelic acid via L-4-hydroxymandelic acid (Bhat & Vaidyanathan, 1976a). The initial step in this degradative pathway was catalyzed by a pteridine-requiring monooxygenase, which was highly specific for the L-isomer of L-mandelic acid (Bhat & Vaidyanathan, 1976b). The para hydroxylation of L-phenylalanine in various bacteria is also catalyzed by a pteridine-requiring monooxygenase (Guroff & Rhoads, 1967; Nakata et al., 1979). Although phenylalanine hydroxylase also slowly catalyzes the hydroxylation of L-tryptophan to 5-hydroxytryptophan (Nakata et al., 1979), no hydroxylation of the analogous compound DL-phenylglycine by the phenylalanine monooxygenase of *Pseudomonas acidovorans* ATCC 11299a has

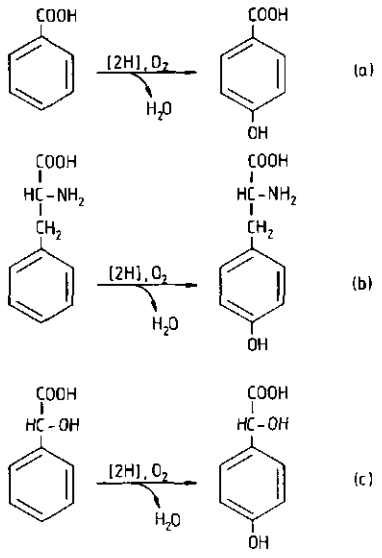

Figure 3. Para-hydroxylation of benzoic acid (a), L-phenylalanine (b), and L-mandelic acid (c) by bacterial, pteridin requiring monooxygenases.

been observed (van den Tweel & Smit, unpublished results). Currently there is a great industrial interest in both hydroxylated products; 5-hydroxy-L-tryptophan is a precursor of the central-nervous-system transmitter 5-hydroxytryptamine (serotonin), while D-4-hydroxyphenylglycine is an important building block of semisynthetic antibiotics. Already in 1956 Mitoma et al. reported that *Chromobacterium violaceum* converted L-tryptophan to L-5-hydroxytryptophan, however, the yields were low. Later on the yield was increased significantly by using *Bacillus subtilus* ATCC 21733 and by adding Fe^{2+} to the fermentation broth (Daum & Kieslich, 1974a; 1974b). In striking contrast to the bacterial hydroxylation of L-phenylalanine and L-tryptophan, so far no bacteria have been found that are able to hydroxylate D-phenylglycine to D-4-hydroxyphenylglycine (van den Tweel et al., 1986a; van den Tweel & de Bont, 1987).

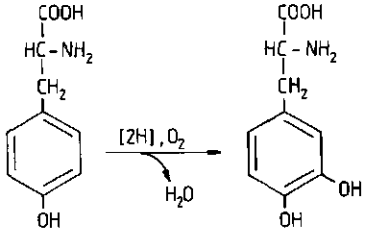

Figure 4. Hydroxylation of L-tyrosine to L-DOPA.

L-3,4-Dihydroxyphenylalanine (L-DOPA) is another important compound that can be synthesized by a hydroxylation of an amino acid (Figure 4). This dihydroxy compound is an efficaceous pharmacological agent in the treatment of Parkinson's disease. Extensive screening procedures have shown that various bacteria are able to hydroxylate L-tyrosine to L-DOPA (Singh et al., 1973; Yoshida et al., 1973, 1974; Pshirkov et al., 1982). For this screening a very simple selection procedure, based on a violet-black color formation on agar plates by the reaction of the accumulating L-DOPA with ferrous iron ions, was developed (Tanaka et al., 1974).

Flavin-dependent monooxygenases play a very important role in the bacterial degradation of hydroxylated benzoic and phenylacetic acids. All isolated bacterial flavoproteins are very substrate specific and incorporate one atom of molecular oxygen into the substrate, either on the position ortho of the existing hydroxyl group (Adachi et al., 1964; Yamamoto et al., 1965; Hosokawa & Stanier, 1966; Michalover & Ribbons, 1973) or on the para-position (Hareland et al., 1975; Wang et al., 1987). In the bacterial cell the resulting dihydroxy aromatic acids are subsequently ringcleaved by intra- or extradiol dioxygenases. In the presence of the metal chelator 2,2-dipyridyl, various bacteria are able to accumulate 2,5- or 3,4-dihydroxyphenylacetic acid from 4-hydroxyphenylacetic acid in almost stoichiometric amounts (van den Tweel et al., 1986b, 1987b, 1988a; [Figure 5]). The bacterial formation of 2,5- and 3,4-dihydroxyphenylacetic acid was investigated because these compounds can be used as building blocks in the synthesis of various pharmaceuticals (Suh & Skorcz, 1969; Kanamaru et al., 1979), dye developers (Lestina & Bush, 1976), and hair dyes (Bachmann & Portmann, 1981).

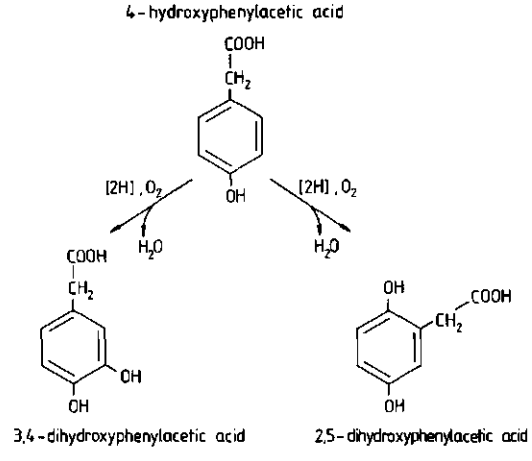

Figure 5. Bioconversion of 4-hydroxyphenylacetic acid to either 3,4-dihydroxyphenylacetic acid (Klebsiella oxytoca) or 2,5-dihydroxyphenylacetic acid (Xanthobacter 124X, Flavobacterium JS-7).

3.1.2 MONOOXYGENASES WITH RELAXED SUBSTRATE SPECIFICITY

Oxygenases which show a relaxed substrate specificity are mostly multi-component enzyme complexes. Recently a new pathway has been described for bacterial toluene degradation. Finette et al. (1984) have isolated a *Pseudomonas mendocina* strain that metabolizes toluene by an initial oxidation of the benzene ring to para-cresol (Figure 6A). In this strain a multi-component, plasmid encoded monooxygenase is responsible for the initial hydroxylation step (Yen et al., 1986). These authors did not report on the substrate specificity of the enzyme but in view of the relaxed substrate specificity of multi-component oxygenases in general, this para-hydroxylating enzyme might be very valuable for the bioformation of hydroxylated aromatic compounds. Bacteria can also degrade toluene via other routes (Figure 6). Well-known is the degradation of toluene, meta- and para-xylene by bacteria carrying the TOL plasmid (Williams & Worsey, 1976). The *XylA* gene of this plasmid specifies xylene oxygenase, a relaxed specificity

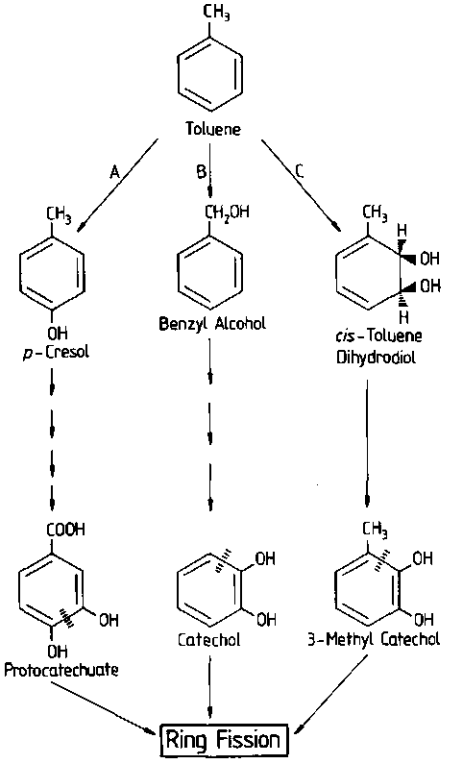

Figure 6. Various pathways used by bacteria for the oxidation of toluene to ring fission substrates.

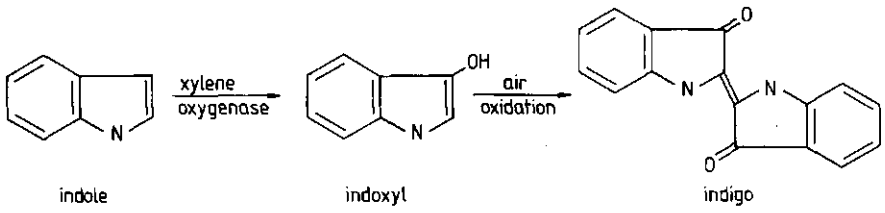

Figure 7. Indigo formation catalyzed by the TOL plasmid-encoded xylene oxygenase.

enzyme that hydroxylates or monooxygenates toluene and meta- and para-xylene and their corresponding alcohols (Harayama et al., 1986; [Figure 6B]). Timmis and coworkers have shown that this monooxygenase also might be applicable for the production of indigo, a brilliant blue pigment which is used extensively in the dyeing of cotton and wool fabrics. Indole is hydroxylated by the xylene oxygenase to indoxyl which oxidizes spontaneously to form indigo (Mermod et al., 1986; [Figure 7]). Evidently, from the viewpoint of manufacturing hydroxylated aromatics, bacteria which are able to degrade toluene may be very useful.

In addition to the already described oxygenases which are employed specifically for the metabolism of aromatic compounds, also other enzymes may gratuitously hydroxylate aromatics. Well known is the fortuitous oxidation of aromatics by methane-utilizing bacteria (Stirling et al., 1979; Higgins et al., 1980; Patel & Hou, 1983). During the last decade a number of patents has been filed concerning the application of these bacteria for the production of commercially useful chemicals including hydroxylated aromatics (Higgins, 1979, 1981, 1982; Hall, 1982). For example, benzene can be converted to phenol, phenol to hydroquinone, toluene to para-cresol and to benzoic acid, and naphthalene to 1-naphthol (Figure 8). Fundamental studies have shown that *Methylosinus trichosporium* OB3b contains both a soluble and a particulate methane monooxygenase (Scott et al., 1981a,b), and that only the soluble enzyme which is formed under conditions of copper deficiency, oxidizes aromatic compounds (Burrows et al., 1984). Another multi-component monooxygenase, the alkane monooxygenase of *Pseudomonas oleovorans*, also oxidizes various substrates, however, aromatic compounds as for instance benzene and toluene were not oxidized (McKenna & Coon, 1970).

The membrane bound ammonia monooxygenase of *Nitrosomonas europaea* is also able to hydroxylate aromatics. Hyman et al. (1985) have shown that this ammonia-oxidizing organism hydroxylates benzene to phenol, which in turn can be oxidized to hydroquinone. Catechol and resorcinol were not detected as products of phenol oxidation,

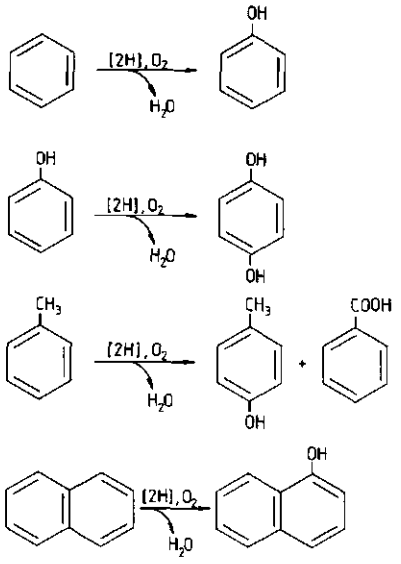

Figure 8. Hydroxylation of various aromatic compounds by the methane monooxygenase of Methylosinus trichosporium OB3b.

implying that the hydroxylation was para-directed. The oxidation rates for benzene were surprisingly high: 100 nmol min^{-1} mg $protein^{-1}$ (initial benzene concentration 4 mM, and hydrazine as reductant). Whether or not other aromatic compounds are hydroxylated by these cells is yet unknown.

3.2 HYDROXYLATION OF AROMATIC COMPOUNDS BY DIOXYGENASES

Dioxygenases are defined as a group of enzymes that catalyze the incorporation of two atoms of molecular oxygen into the substrate. This group can be subdivided in dioxygenases which catalyze so-called double hydroxylation reactions yielding cis-dihydrodiols and in dioxygenases which accomplish the cleavage of the aromatic ring by the insertion of two atoms of molecular oxygen.

3.2.1 DIOXYGENASES INVOLVED IN THE FORMATION OF CIS-DIHYDRODIOLS AND CATECHOLS

Various bacteria initially oxidize the most simple aromatic compound, benzene, by means of a dioxygenase to cis-1,2-dihydroxy-cyclohexa-3,5-diene (cis-benzeneglycol)(Gibson et al., 1970b; Högn &

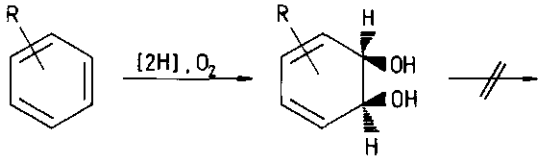

Figure 9. Bioformation of cis-dihydrodiols.

Jaenicke, 1972; [Figure 9]). Subsequently, cis-benzeneglycol is oxidized to catechol which is further degraded by the well-established reactions of either the β-ketoadipate pathway or the "meta" fission-pathway (Dagley, 1971). Similar steps are involved in the bacterial catabolism of toluene (Gibson et al., 1970a; [Figure 6C]), halogenated benzenes (Reineke & Knackmuss, 1984; de Bont et al., 1986; Schraa et al., 1986; Spain & Nishino, 1987), ethyl benzene (Gibson et al., 1973a), ortho-xylene (Schraa et al., 1987), 2-phenylbutane and 3-phenylpentane (Baggi et al., 1972), isobutylbenzene and isopropylbenzene (Jigami et al., 1975; Eaton & Timmis, 1986), benzoic acids (Reiner, 1971: Reiner & Hegeman, 1971; Whited et al., 1986), phthalic acid (Martin et al., 1987), 3-phenylpropionic acid (Burlingame & Chapman, 1983), naphthalene (Catterall et al., 1971; Jeffrey et al., 1975), biphenyl (Gibson et al., 1973b), phenanthrene (Koreeda et al., 1978), and anthracene (Evans et al., 1965; Jerina et al., 1976); by means of an oxygenase one molecule of oxygen is incorporated yielding a cis-dihydrodiol which is subsequently dehydrogenated to the corresponding catechol. Currently there is a huge industrial interest in these aromatic dioxygenase enzymes for the production of the cis-dihydrodiol intermediates, since such diols may be useful starting compounds for the production of synthetic polymers (Ballard et al., 1983) and certain pharmaceuticals (Ley et al., 1987). Moreover, the relaxed substrate specificity of these multi-component enzymes (Axcell & Geary, 1975; Yeh et al., 1977), also enables the formation of a wide range of substituted cis-dihydrodiols, thus making a commercial application more attractive (Gibson et al., 1968; 1974; Ziffer et al., 1977; Taylor, 1983; Ensley et al., 1983; Reiner & Hegeman, 1971; Zeyer et al., 1985a). It should be noted however, that in order to prevent further metabolism of the produced cis-dihydrodiols, mutants lacking a functional cis-dihydrodiol dehydrogenase, have to be used (Figure 9).

In addition to the cis-dihydrodiol intermediates also the catechol intermediates are of some interest for various industries. Consequently, research is done to achieve bacterial formation of catechols from benzene (Shirai, 1986,1987) and from various benzoates (Zeyer et al., 1985a). The internal regeneration of cofactors (reduction equivalents

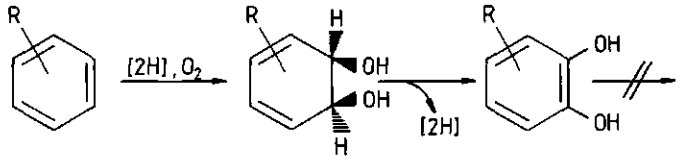

Figure 10. Bioformation of catechols.

needed for the initial hydroxylation step can be regenerated by dehydrogenation of the cis-dihydrodiol), might be a welcome advantage of such processes (Figure 10). However, a major problem encountered with these bioconversions is the toxic effect of the labile catechols (Shirai, 1987).

3.2.2 AROMATIC RING CLEAVAGE BY DIOXYGENASES

From the foregoing discussion, it is apparent that the degradation of aromatic compounds leads to the formation of dihydroxy aromatics as terminal benzenoid compounds. Dioxygenases initiate the degradation of such compounds by the insertion of two atoms of molecular oxygen, either between the two hydroxylated carbon atoms or adjacent to a hydroxylated carbon atom (Figure 11). Most of the ring cleaving dioxygenases contain non-heme iron as the sole cofactor (Nozaki,

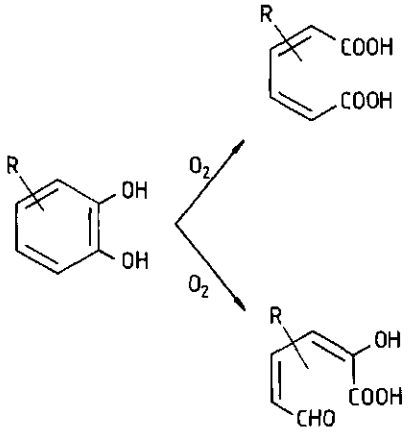

Figure 11. Ring fission of catechols by an intra- or extradiol cleavage yielding either muconic acids or hydroxymuconic semialdehydes.

1979). Consequently, these enzymes are inhibited in the presence of metal chelators, thus creating a possibility to produce dihydroxy aromatic compounds (section 3.1.1; Figure 5).

The product of the intradiol cleavage, muconic acid, is very useful for the synthesis of surfactants, anti-oxidants and flame retardants. Maxwell (1982) has manipulated a *Pseudomonas putida* in that it is able to produce muconic acid from toluene. All naturally occurring bacteria, on the other hand, degrade toluene by means of the extra diol cleavage (Figure 6). Hagedorn (1984) used mutants of *Pseudomonas putida* which lacked active 2-hydroxymuconic semialdehyde-metabolizing enzymes, to produce 2-hydroxymuconic semialdehyde or substituted analogues thereof from compounds such as toluene and para-xylene. Subsequently, the products were chemically converted to picolinic acids and pyridines. These specialty heterocyclic aromatic chemicals are mainly used in the production of adhesives, pesticides and vitamins.

4 REPLACEMENT OF SUBSTITUENTS BY HYDROXYL GROUPS

A quite different method to obtain hydroxylated aromatic compounds is the replacement of substituents of the aromatic ring by hydroxyl groups. Recent publications have shown that many bacteria can replace various substituents by hydroxyl groups: some reactions are catalyzed by hydrolytic enzymes, others by oxygenases.

4.1 HYDROLYTIC DEHALOGENATION

In view of the high barrier of activation energy of the aromatic ring and the requirement for strong nucleophilic catalysts, it was considered unlikely some years ago that bacteria had evolved enzymes for the direct hydrolysis of the aromatic carbon-halogen bond (Knackmuss, 1981). However, during the last decade many bacteria have been described which are able to replace the halogen by a hydroxyl group (Figure 12). Such reaction has been observed in a *Pseudomonas* species growing on 3-chlorobenzoate (Johnston et al., 1972), in various organisms utilizing 4-chlorobenzoate (Ruisinger et al., 1976; Klages et al., 1979; Keil et al., 1981; Zaitsev and Karasevich, 1981a, 1981b; Marks et al., 1984a; van den Tweel et al., 1987e), and in a pentachlorophenol degrading bacterium (Apajalahti & Salkinoja-Salonen, 1987a,b). The precise nature of the reaction is yet unknown, but two groups of researchers have shown independently and for different bacteria that the oxygen atom in 4-hydroxybenzoate is derived from water rather than from molecular oxygen (Marks et al., 1984b; Müller et al., 1984). Similar results were very recently obtained by Apajalahti & Salkinoja-

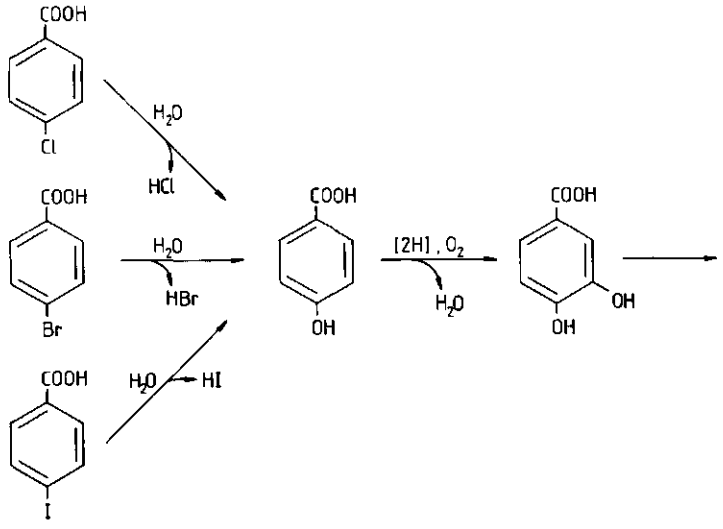

Figure 12. Hydrolytic dehalogenation of various halobenzoates.

Salonen (1987a) for the conversion of tri- and tetrachlorophenols to trichlorohydroquinone. The use of such hydrolytic dehalogenation reactions yielding hydroxyaromatics may be an interesting alternative to existing biotransformations yielding hydroxylated aromatics. A major advantage of such a bioconversion is that further oxidation of the hydroxylated product, either chemically or biologically, can easily be prevented since such a hydrolytic dehalogenation theoretically can be achieved in the absence of oxygen. Preliminary experiments with both free and immobilized bacterial cells containing the 4-halobenzoate dehalogenase, have indeed shown that a formation of 4-hydroxybenzoate from 4-halobenzoates is feasible (van den Tweel et al. 1986c, 1987d). Surprisingly however, no bioconversion took place when oxygen was absent. The presence of an energy-dependent uptake system may explain this rather unexpected anomalous result (Groenewegen et al., 1987). So far, only 4-halobenzoate and polychlorophenol dehalogenases have been found, however, for industrial purposes it would be attractive to obtain hydrolytic dehalogenases which act on other haloaromatic compounds as well.

4.2 OXIDATIVE REPLACEMENT

In contrast to the forementioned hydrolytic dehalogenation of haloaromatic compounds, also oxidative dehalogenation reactions

yielding hydroxy aromatics have been described. Almost all of these reactions are attributed to the non-specific action of either a monooxygenase or a dioxygenase. For instance, the enzyme phenylalanine hydroxylase is able to hydroxylate 4-fluorphenylalanine yielding tyrosine in a NADH-dependent reaction (Kaufman, 1961). Another non-specific enzymatic reaction resulting in halide release from an aromatic compound is when 3-fluorobenzoate is oxidized by the benzoate dioxygenase. In the adapted strain *Pseudomonas* B13-2 the regioselectivity of dioxygenation was changed in such way that only 2-fluoro-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid resulted from dioxygenation of 2-fluorobenzoate, whereas the 6-flouro analog was no longer formed (Engesser et al., 1980). This fluorodiol spontaneously rearranged to catechol under loss of fluoride and CO_2. A situation that may, or may not, be comparable to the benzoate dioxygenase situation was described for a *Pseudomonas* species able to grow on 4-chlorophenylacetate (Klages et al., 1981). It was observed that 3,4-dihydroxyphenylacetate was formed from 4-chlorophenylacetate in a reaction probably catalyzed by a dioxygenase (Markus et al., 1984).

Other substituents attached to aromatic rings that can be converted to hydroxyl groups are amino and nitro groups. Zeyer and coworkers have isolated various bacteria which grow on either haloanilines or substituted nitroaromatic compounds as sole carbon and nitrogen source. A *Moraxella* sp. which was able to grow on 4-haloanilines, converted these compounds by an aniline oxygenase to 4-chlorocatechol (Zeyer et al., 1985b), while a *Pseudomonas putida* which was isolated on 2-nitrophenol, oxidized this compound by a nitrophenol oxygenase to nitrite and catechol (Zeyer et al., 1986). Another *Pseudomonas* sp. which utilized 4-chloro-2-nitrophenol only as sole nitrogen source, converted this compound quantitatively to 4-chlorocatechol (Nörtemann et al., 1986).

Sulfonated aromatics are anthropogenic compounds which are widely used as emulsifiers and wetting agents. In spite of the fact that these chemicals are only recently released in the environment, many bacteria can use these compounds as sole sulfur source and/or carbon source for growth. Using sulfonated naphthalenes as sole sulfur source, several bacteria were isolated (Zürrer et al., 1987). One of them, a *Pseudomonas* sp., converted 1- and 2-naphthalenesulfonic and benzenesulfonic acid quantitatively to 1- and 2-naphthol and phenol, respectively. Experiments with labeled oxygen have shown that also in this case the hydroxyl group was derived from molecular oxygen. Bacteria, however, which utilize either 1- or 2-naphtalenesulfonic acid as sole carbon source, degrade these compounds via the known catabolic sequences of naphthalene (Brilon et al., 1981). By means of an enzymic 1,2-dioxygenation and a spontaneous rearomatization, the carbon-sulfur bond is cleaved yielding 1,2-dihydroxynaphthalene and

sulfite.

Demethylation of aromatic ethers, metabolites of biological lignin degradation, to hydroxy aromatics is another example of oxygenolytic replacement (Cartwright et al., 1971). In case of 4-methoxybenzoate, a rather aspecific monooxygenase converted 4-methoxybenzoate to 4-hydroxybenzoate and formaldehyde (Bernhardt et al., 1973).

5 ADDITION AND/OR MODIFICATION OF THE SIDE CHAIN

The formation of hydroxylated aromatic compounds can also be achieved by bacterial enzymes that are different from the enzymes described above. Yamada and coworkers have shown that the pyridoxal phosphate requiring β-tyrosinase (tyrosine phenol-lyase) and tryptophanase which initiate the degradation of respectively, L-tyrosine and L-tryptophan in some bacteria, may be useful for this purpose. Both enzymes catalyse a variety of α,β-elimination (eq. 1), β-replacement (eq. 2) and the reverse of the α,β-elimination (eq. 3) reactions as follows (Yamada & Kumagai, 1978; Yamada & Shimizu, 1985):

$$\text{L-RCH}_2\text{CH(NH}_2\text{)COOH} + \text{H}_2\text{O} \Rightarrow \text{RH} + \text{CH}_3\text{COCOOH} + \text{NH}_3 \quad (1)$$

$$\text{L-RCH}_2\text{CH(NH}_2\text{)COOH} + \text{R}^1\text{H} \Rightarrow \text{L-R}^1\text{CH}_2\text{CH(NH}_2\text{)COOH} + \text{RH} \quad (2)$$

$$\text{R}^1\text{H} + \text{CH}_3\text{COCOOH} + \text{NH}_3 \Rightarrow \text{L-R}^1\text{CH}_2\text{CH(NH}_2\text{)COOH} + \text{H}_2\text{O} \quad (3)$$

where for β-tyrosinase: R= phenolyl, -OH, -SH, -Cl; R^1= phenolyl; and for tryptophanase: R = indolyl, -OH, -SH, -Cl; R^1= indolyl.

Screening has shown that both enzyme activities are found in various bacteria, most of which belonged to the *Enterobacteriaceae*; especially to the genera *Escherichia, Proteus and Erwinia* (Enei et al., 1972; Nakazawa et al., 1972b). Whole cells of *Erwinia herbicola* (either free or immobilized), containing high β-tyrosinase activity transformed pyruvate, ammonia and phenol or catechol to L-tyrosine or L-DOPA respectively, via the reverse of the α,β-elimination reaction ([eq. 3]; Enei et al., 1973b; Yamada et al., 1978). As a result of the low solubility of L-tyrosine and L-DOPA the apparent equilibrium, in the presence of large amounts of substrates, declined towards the synthetic rather than towards the degradative direction. During L-DOPA synthesis a large amount of byproducts was formed as a result of a non-enzymic reaction of L-DOPA with pyruvate (Enei et al., 1973b). An alternative way to synthesize L-DOPA by means of the β-tyrosinase is by using DL-serine instead of pyruvate and ammonia (the

β-replacement reaction; Enei et al., 1973a; Para et al., 1984). Like the β-tyrosinase reaction, L-tryptophan or L-5-hydroxytryptophan were efficiently synthesized from pyruvate, ammonia, indole or 5-hydroxyindole when tryptophanase containing cells were used as the catalyst (Nakazawa et al., 1972a; Yamada & Kumagai, 1978).

D-4-Hydroxyphenylglycine (D-4-HPG) is a commercially important building block for the production of the semisynthetic penicillin amoxicillin. The chemical synthesis of D-4-HPG (production of racemic 4-HPG, followed by diastereomeric salt separation) is complex and therefore several biological production methods have been developed. Some of these processes have been or will be commercialized (Figure 13). A very nice chemoenzymatic process for the production of D-4-HPG, based on the stereoselective resolution of the racemic hydantoin, is operated on an industrial scale by the Japanese firm Kanegafuchi ([Figure 13A]; Kanegafuchi, 1977). The racemic 5-(4-hydroxyphenyl)-hydantoin is converted by (immobilized) cells of *Bacillus brevis* containing a D-specific hydantoinase to a mixture of N-carbamoyl-D-4-HPG and L-5-(4-hydroxyphenyl)hydantoin. A major advantage of this process is the rapid spontaneous racemization of the unreacted L-hydantoin *in situ*, resulting theoretically in a 100% yield of the N-carbamoyl-D-acid on basis of the DL-hydantoin. The resulting N-carbamoyl-D-acid can be converted to the corresponding D-amino acid by reaction with nitrous acid without changing the configuration (Takahashi et al., 1979). More recently, it has been shown that the hydrolysis of the N-carbamoyl-D-amino acid can also be accomplished by bacteria (Nakamori et al., 1980; Yokozeki et al., 1987). An *Agrobacterium radiobacter* that contained both a D-hydantoinase and a N-carbamoyl-D-amino acid amidohydrolase converted DL-5-(4-hydroxyphenyl)hydantoin to free D-4-HPG in a single process step (Figure 13B; Olivieri et al., 1979, 1981). Industrial application seems therefore most promising, especially when the specific activities of the microbial cells are enhanced by strain improvement procedures.

DSM has developed a biotechnological process for the production of a wide variety of optically active amino acids based on a L-specific aminopeptidase (Boesten & Meyer-Hoffman, 1975; Meyer et al., 1985). In order to produce D-4-HPG by this method (Figure 13C), the DL-4-hydroxyphenylglycine amide is hydrolyzed by *Pseudomonas putida* cells containing a high L-aminopeptidase activity, yielding L-4-HPG and the D-4-hydroxyphenylglycine amide. The recovery of the nearly insoluble D-4-hydroxyphenylglycine amide is accomplished by simple filtration, and subsequently D-4-HPG is obtained by either chemical or enzymic hydrolysis. The L-isomer of 4-HPG on the other hand, has to be racemized and recycled.

Another process that might be useful for the biosynthesis of D-4-HPG is the hydrolysis of the aminonitril compound yielding either the

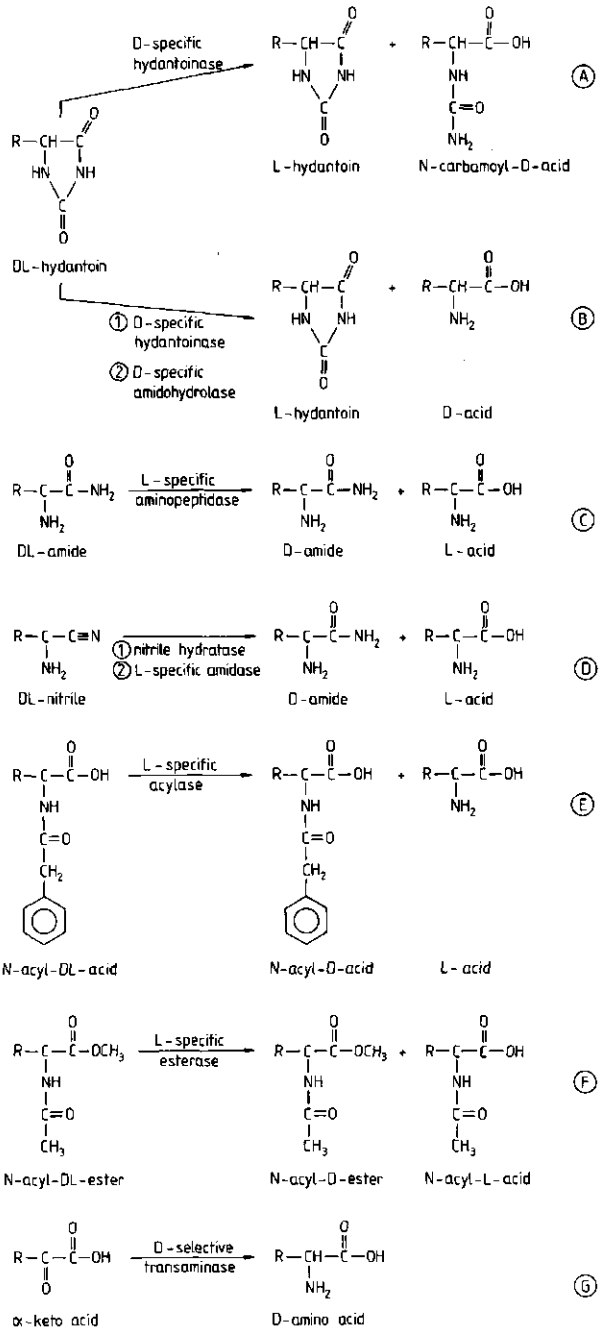

Figure 13. Various biotransformations which can be applied to manufacture the D-enantiomer of 4-hydroxyphenylglycine. R = HO-C_6H_4-.

amino amide or the amino acid ([Figure 13D]; Arnaud et al., 1980). However, the use of aminonitril as substrate in this bioprocess is hampered by the partly reversal of the Strecker-reaction.

Stereoselective hydrolysis either of N-acyl-DL-4-hydroxyphenylglycine by an *Escherichia coli* containing high acylase activity ([Figure 13E]; Cole & Utting, 1974) or of N-acyl-DL-4-hydroxyphenylglycine methyl ester by various esterases ([Figure 13F]; Schutt, 1981) has also been described. A major drawback of the acylase method is that the preparation of the N-acyl-DL-amino acid has to occur via the relatively expensive DL-amino acid in most cases (Cole & Utting, 1974), while the esterase procedure is hampered by the slow chemical hydrolysis of the substrate under the described reaction conditions (Schutt, 1981). Recently, Schutt et al. (1985) have shown that the enantioselective ester hydrolysis of DL-2-acetamido-4-hydroxyphenylacetic acid methyl ester can be very efficiently performed by immobilized subtilisin in the presence of organic solvents. Besides the fact that the substrate solubility was enhanced also the rate of non-enzymatic hydrolysis was reduced.

Another promising method for the bioformation of D-4-HPG is the enantioselective transamination of 4-hydroxyphenylglyoxylate ([Figure 13G]; van den Tweel et al., 1987c, 1988c). By means of a reversible D-selective transaminase of a *Pseudomonas putida*, 4-hydroxyphenylglyoxylate and L-glutamate are transformed to α-ketoglutarate and D-4-HPG. Since the starting compounds are relatively inexpensive (L-glutamate is a very cheap bulk product while 4-hydroxphenylglyoxylate can easily be prepared chemically from inexpensive DL-4-hydroxymandelate), this process might be a welcome alternative to other production processes for D-4-HPG.

6 SOME TECHNOLOGICAL ASPECTS OF THE BIOPRODUCTION OF HYDROXYLATED AROMATIC COMPOUNDS

In order to develop a commercial biotechnological process for the manufacture of hydroxylated aromatics, not only biological aspects of the desired biotransformation have to be studied, but also technological aspects should be taken into account. The selectivity, activity and stability of the biocatalyst should be high to obtain a maximal volumetric productivity of the bioreactor. Moreover, a high product concentration is desirable since it generally facilitates down-stream processing. In case of biotransformations by bacterial cells, which are based on oxygenases, the activity and stability of the (immobilized) biocatalyst is strongly dependent upon the availability of reduction equivalents, needed for the hydroxylation reaction. In order to enhance

the activity and stability of the biotransformation, cosubstrates can be added to the bioreactor (Taylor, 1985). Another approach to meet this demand for reducing power in intact bacteria, is by using starting compounds which are more reduced than the compound to be hydroxylated (van den Tweel et al., 1988b). In this way the reduction equivalents which result from the oxidation reaction, may be used to achieve the hydroxylation reaction of the aromatic compound.

Aromatic substrates and/or products often have an inhibitory effect on both bacterial growth and biocatalysis (Jenkins & Dalton, 1985; Jenkins et al., 1987; van den Tweel et al., 1987a, 1988b). Because of the apolar nature of most aromatics the application of water-immiscible organic solvents might be useful to decrease the toxic effect of the aromatic compounds. The influence of many water-immiscible solvents on retention of activity of immobilized *Mycobacterium* cells was determined by Brink and Tramper (1985), and it was found that retention of activity is usually favored by low solvent polarity in combination with a high molecular weight. LogP, which is defined as the logarithm of the partition coefficient of a given compound in a standard octanol-water two phase system is a very useful parameter to describe a correlation between biocatalytic activity and solvent properties (Laane et al., 1985). Solvents with a logP smaller than 2 were least suitable for biocatalysis while solvents having a logP above 4 were readily applicable. Preliminary experiments have shown that also for the production of cis-benzeneglycol from benzene, the addition of hexadecane (logP 8.8) is useful to circumvent the inhibitory effect of benzene, however, because of the polarity of cis-benzeneglycol, the addition of water-immiscible solvents will not reduce the inhibitory effect of the product (van den Tweel et al., 1987a). A potential danger for the biocatalyst in the hexadecane/water biphasic system is denaturation at the liquid/liquid interface. However, if required, this problem can be overcome by immobilizing the biocatalyst in the aqueous phase.

Another possible advantage of the application of water-immiscible solvents is the integration of the actual biocatalysis and the downstream processing, as the product can be easily removed from the bioreactor via the organic solvent.

A problem encountered during the bioformation of some hydroxylated aromatics, is a further transformation, either biologically or chemically, of the desired product. By using mutants (Shirai, 1987), or by working under conditions of low and controlled oxygen tensions (van den Tweel et al., 1986c), or by the addition of antioxidants (Para et al., 1984), the oxidation of the accumulating hydroxylated product can be (partially) circumvented. In case of the hydroxylation of L-tyrosine to L-DOPA, the undesired deamination of both the substrate and the product could be prevented by introducing suitable N-blocking

groups (Sih et al., 1969; Sih, 1972).

7 OUTLINE OF THIS THESIS

The present research was initiated together with Andeno B.V. The aim was to investigate and to ultimately exploit the divers biochemical potential of bacteria to develop biotechnological processes for the manufacture of hydroxylated aromatic compounds.

Initially, the emphasis was on bacteria which would hydroxylate DL-phenylglycine regio- and stereospecifically yielding D-4-hydroxyphenylglycine. For this, several bacteria were isolated on D-phenylglycine as sole carbon and energy source, and subsequently it was investigated whether these organisms metabolized phenylglycine via an initial hydroxylation reaction (chapters 2 and 3). Unfortunately, none of the studied bacteria were able to hydroxylate phenylglycine. However, one of the isolates, *Pseudomonas putida* LW-4, also grew on D-4-hydroxyphenylglycine but not on L-4-hydroxyphenylglycine, and it was shown that the D-isomer was initially degraded by means of a D-selective transaminase. By using this transaminase in the reverse direction we were able to produce D-4-hydroxyphenylglycine from 4-hydroxyphenylglyoxylate (chapter 4). Another *Pseudomonas putida*, on the other hand, which degraded both stereoisomers of 4-hydroxyphenylglycine, possessed both a D- and a L-specific transaminase (chapter 5).

Other compounds of interest were hydroxylated phenylacetic acids. In chapter 6 the catabolism of 4-hydroxyphenylacetic acid by a *Xanthobacter* species is described, and it is shown that whole cells in the presence of 2,2-dipyridyl, convert 4-hydroxyphenylacetic acid to 2,5-dihydroxyphenylacetic acid. In chapter 7 the degradation of DL-phenylhydracrylic acid and metabolites thereof, by a *Flavobacterium* is described.

The bioformation of cis-benzeneglycol from benzene was investigated in collaboration with the Chemistry Department of the Technical University Delft. Chapter 8 deals with the continuous production of cis-benzeneglycol from benzene by a mutant, whereas chapter 9 describes the application of organic solvents for this biotransformation. In this latter chapter also some preliminary results on the use of a newly described liquid-impelled loop reactor are presented.

The hydrolytic dehalogenation of haloaromatic compounds is a fully different approach to form hydroxylated aromatics. In chapter 10 and 11 the formation of 4-hydroxybenzoate from various halobenzoates is described, while in chapter 12 some kinetic aspects of this bioconversion by immobilized cells are presented.

Finally in chapter 13 some concluding remarks are presented concerning the application of bacteria in general to synthesize hydroxylated aromatic compounds.

REFERENCES

Adachi, K., Takeda, Y., Senoh, S. & Kita, H. (1964). Metabolism of p-hydroxyphenylacetic acid in Pseudomonas ovalis. Biochimica et Biophysica Acta 93,483-493

Apajalahti, J.H.A. & Salkinoja-Salonen, M.S. (1987a). Dechlorination and para-hydroxylation of polychlorinated phenols by Rhodococcus chlorophenolicus. Journal of Bacteriology 169,675-681

Apajalahti, J.H.A. & Salkinoja-Salonen, M.S. (1987b). Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced Rhodococcus chlorophenolicus. Journal of Bacteriology 169,5125-5130

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

Axcell, B.C. & Geary, P.J. (1975). Purification and some properties of a soluble benzene-oxidising system from a strain of Pseudomonas. Biochemical Journal 146,173-183

Bachmann, H. & Portmann, P. (1981). Agent for oxidative dyeing of hair. German Offenlegungsschrift 2,939,303

Baggi, G., Catelani, D., Galli, E. & Treccani, V. (1972). The microbial degradation of phenylalkanes. Biochemical Journal 126,1091-1097

Ballard, D.G.H., Courtis, A., Shirley, I.M. & Taylor, S.C. (1983). A biotech route to polyphenylene. Journal of the Chemical Society, Chemical Communications pp. 954-955

Bernhardt, F.-H., Erdin, N., Staudinger, H. & Ullrich, V. (1973). Interactions of substrates with a purified 4-methoxybenzoate monooxygenase system (O-demethylating) from Pseudomonas putida. European Journal of Biochemistry 35,126-134

Bhat, S.G. & Vaidyanathan, C.S. (1976a). Involvement of 4-hydroxymandelic acid in the degradation of mandelic acid by Pseudomonas convexa. Journal of Bacteriology 127,1108-1118

Bhat, S.G. & Vaidyanathan, C.S. (1976b). Purification and properties of L-mandelate-4-hydroxylase from Pseudomonas convexa. Archives of Biochemistry and Biophysics 176,314-323

Boesten, W.H.J. & Meyer-Hoffman, L.R.M. (1975). Enzympreparaat met aminopeptidase activiteit. Dutch Patent Application 7,513,551

Brilon, C., Beckmann, W. & Knackmuss, H.-J. (1981). Catabolism of naphthalenesulfonic acids by Pseudomonas sp. A3 and Pseudomonas sp. C22. Applied and Environmental Microbiology 42,44-55

Brink, L.E.S. & Tramper, J. (1985). Optimization of organic solvent in multiphase biocatalysis. Biotechnology and Bioengineering 27,1258-1269

Burlingame, R. & Chapman, P.J. (1983). Catabolism of phenylpropionic acid and its 3-hydroxy derivative by Escherichia coli. Journal of Bacteriology 155,113-121

Burrows, K.J., Cornish, A., Scott, D. & Higgins, I.J. (1984). Substrate specificities of the soluble and particulate methane mono-oxygenase of Methylosinus trichosporium OB3b. Journal of General Microbiology 130,3327-3333

Cartwright, N.J., Holdom, K.S. & Broadbent, D.A. (1970). Bacterial attack on phenolic ethers. Dealkylation of higher ethers and further observations on O-demethylases. Microbios 3,113-130

Catterall, F.A., Murray, K. & Williams, P.A. (1971). The configuration of the 1,2-dihydroxy-1,2-dihydronaphthalene formed in the bacterial metabolism of naphthalene. Biochimica et Biophysica Acta 237,361-364

Chandra, P. & Vining, L.C. (1968). Conversion of phenylalanine to tyrosine by microorganisms. Canadian Journal of Microbiology 14,573-578

Cole, M. & Utting, K. (1974). Enzymic resolution of racemic N-acyl-DL-amino acids. Britisch Patent 1,369,462

Dahlmans, J.J., Boesten, W.H.J. & Bakker, G. (1980). Enzymatische scheiding van D- en L-aminozuren op technologische schaal. Chemisch Magazine m322-323

Dagley, S. (1971). Catabolism of aromatic compounds by microorganisms. Advances in Microbial

Physiology 6,1-46
Daum, J. & Kieslich, K. (1974a). Process for the preparation of 5-hydroxy-L-tryptophan. United States Patent 3,830,696
Daum, J. & Kieslich, K. (1974b). Darstellung von 5-Hydroxytryptophan durch mikrobiologische Hydroxylierung von L-Tryptophan. Naturwissenschaften 61,167-168
de Bont, J.A.M., Vorage, M.J.A.W., Hartmans, S. & van den Tweel, W.J.J. (1986). Microbial degradation of 1,3-dichlorobenzene. Applied and Environmental Microbiology 52,677-680
Dodge, R.H., Cerniglia, C.E. & Gibson, D.T. (1979). Fungal metabolism of biphenyl. Biochemical Journal, 178,223-230
Eaton, R.W. & Timmis, K.N. (1986). Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in Pseudomonas putida RE204. Journal of Bacteriology 168,123-131
Enei, H., Matsui, H., Yamashita, K., Okumura, S. & Yamada, H. (1972). Distribution of tyrosine phenol lyase in microorganisms. Agricultural and Biological Chemistry 36,1861-1868
Enei, H., Matsui, H., Nakazawa, H., Okumura, S. & Yamada, H. (1973a). Synthesis of L-tyrosine or 3,4-dihydroxyphenyl-L-alanine from DL-serine and phenol or pyrocatechol. Agricultural and Biological Chemistry 37,493-499
Enei, H., Nakazawa, H., Okumura, S. & Yamada, H. (1973b). Synthesis of L-tyrosine or 3,4-dihydroxyphenyl-L-alanine from pyruvic acid, ammonia and phenol or pyrocatechol. Agricultural and Biological Chemistry 37,725-735
Engesser, K.-H., Schmidt, E. & Knackmuss, H.-J. (1980). Adaptation of Alcaligenes eutrophus B9 and Pseudomonas sp. B13 to 2-fluorobenzoate as growth substrate. Applied and Environmental Microbiology 39,68-73
Ensley, B.D., Ratzkin, B.J., Osslund, T.D., Simon, M.J., Wackett, L.P. & Gibson, D.T. (1983). Expression of naphthalene oxidation genes in Escherichia coli results in the biosynthesis of indigo. Science 222,167-169
Evans, W.C., Fernley, H.N. & Griffiths, E. (1965). Oxidative metabolism of phenanthrene and anthracene by soil Pseudomonads. Biochemical Journal 95,819-831
Finette, B.A., Subramanian, V. & Gibson, D.T. (1984). Isolation and characterization of Pseudomonas putida PpF1 mutants defective in the toluene dioxygenase enzyme system. Journal of Bacteriology 160,1003-1009
Friedrich, B. & Schlegel, H.G. (1972). Die hydroxylierung von phenylalanin durch Hydrogenomonas eutropha H16. Archives of Microbiology 83,17-31
Gibson, D.T., Koch, J.R., Schuld, C.L. & Kallio, R.E. (1968). Oxidative degradation of aromatic hydrocarbons by microorganisms. Metabolism of halogenated aromatic hydrocarbons. Biochemistry 7,3795-3802
Gibson, D.T., Hensley, M., Yoshioka, H. & Mabry, T.J. (1970a). Formation of (+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by Pseudomonas putida. Biochemistry 9,1626-1630
Gibson, D.T., Cardini, G.E., Maseles, F.C. & Kallio, R.E. (1970b). Incorporation of oxygen-18 into benzene by Pseudomonas putida. Biochemistry 9,1631-1635
Gibson, D.T., Gschwendt, B., Yeh, W.K. & Kobal, V.M. (1973a). Initial reactions in the oxidation of ethylbenzene. Biochemistry 12,1520-1528
Gibson, D.T., Roberts, R.L., Wells, M.C. & Kobal, V.M. (1973b). Oxidation of biphenyl by a Beijerinckia species. Biochemical and Biophysical Research Communications 50,211-219
Gibson, D.T., Mahadevan, V. & Davey, J.F. (1974). Bacterial metabolism of para- and meta-xylene: oxidation of the aromatic ring. Journal of bacteriology 119,930-936
Gray, P.H.H. & Thornton, H.G. (1928). Soil bacteria that decompose certain aromatic compounds. Zentralblatt für Bakteriologie 73,74-96
Groenewegen, P.E.J., van den Tweel, W.J.J. & de Bont, J.A.M. (1987). Bioformation of 4-hydroxybenzoate from 4-halobenzoates by Alcaligenes denitrificans NTB-1. Proceedings of the 4th European Congress on Biotechnology 3,464
Gunstone, F.D. (1960). Hydroxylation methods. Advances in Organic Chemistry 1,103-147
Guroff, G. & Rhoads, C.A. (1967). Phenylalanine hydroxylase from Pseudomonas species (ATCC 11299a). Journal of Biological Chemistry 242,3641-3645
Hagedorn, S. (1983). Production of para-cresol. European Patent Application 0,105,630
Hagedorn, S. (1984). Construction of novel mutant micro-organisms. European Patent Application 0,138,391
Hall, M.C. (1982). Microbiological process for the preparation of hydroquinone. European Patent

Application 0,073,134
Haneda, K., Watanabe, S. & Takeda, I. (1973). Production of L-3,4-dihydroxyphenylalanine from L-tyrosine by microorganisms. Journal of Fermentation Technology 51,398-407
Harayama, S., Leppik, R.A., Rekik, M., Mermod, N., Lehrbach, P.R., Reineke, W. & Timmis, K.N. (1986). Gene order of the TOL catabolic plasmid upper pathway operon and oxidation of both toluene and benzylalcohol by the xylA product. Journal of Bacteriology 167,455-461
Hareland, W.A., Crawford, R.L., Chapman, P.J. & Dagley, S. (1975). Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from Pseudomonas acidovorans. Journal of Bacteriology 121,272-285
Hatano, K., Nogami, I., Higashide, E. & Kishi, T. (1984). Biosynthesis of enduracidin: origin of enduracidin and other amino acids. Agricultural and Biological Chemistry 48,1503-1508
Higgins, I.J. (1979). Biotransformations. United Kingdom Patent Application 2,024,205
Higgins, I.J., Best, D.J. & Hammond, R.C. (1980). New findings in methane-utilising bacteria highlight their importance in the biosphere and their commercial potential. Nature 286,561-564
Higgins, I.J. (1981). Microbiological oxidations. United Kingdom Patent Application 2,081,306
Higgins, I.J. (1982). Biotransformations using methane-utilising bacteria. United States Patent 4,323,649
Högn, T. & Jaenicke, L. (1972). Benzene metabolism of Moraxella species. European Journal of Biochemistry 30,369-375
Hosokawa, K. & Stanier, R.Y. (1966). Crystallization and properties of p-hydroxybenzoate hydroxylase from Pseudomonas putida. Journal of Biological Chemistry 241,2453-2460
Huizing, H.J. & Wichers, H.J. (1984). Production of L-DOPA by Mucuna pruriens cell suspension cultures through accumulation or by biotransformation of tyrosine. Progress in Industrial Microbiology 20,217-228
Hyman, M.R., Sansome-Smith, A.W., Shears, J.H. & Wood, P.M. (1985). A kinety study of benzene oxidation to phenol by whole cells of Nitrosomonas europaea and evidence for the further oxidation of phenol to hydroquinone. Archives of Microbiology 143,302-306
Jeffrey, A.M., Yeh, H.J.C., Jerina, D.M., Patel, T.R., Davey, J.F. & Gibson, D.T. (1975). Initial reactions in the oxidation of naphthalene by Pseudomonas putida. Biochemistry 14,575-584
Jenkins, R.O. & Dalton, H. (1985). The use of indole as a spectrophotometric assay substrate for toluene dioxygenase. FEMS Microbiology Letters 30,227-231
Jenkins, R.O., Stephens, G.M. & Dalton, H. (1987). Production of toluene cis-glycol by Pseudomonas putida in glucose fed-batch culture. Biotechnology and Bioengineering 29,873-883
Jerina, D.M., Selander, H., Yagi, H., Wells, M.C., Davey, J.F., Mahadevan, V. & Gibson, D.T. (1976). Dihydrodiols from anthracene and phenanthrene. Journal of the American Chemical Society 98,5988-5996
Jigami, Y., Omori, T. & Minoda, Y. (1975). The degradation of isopropylbenzene and isobutylbenzene by Pseudomonas sp. Agricultural and Biological Chemistry 9,1781-1788
Johnston, H.W., Briggs, G.G. & Alexander, M. (1972). Metabolism of 3-chlorobenzoic acid by a Pseudomonad. Soil Biology and Biochemistry 4,187-190
Kanamaru, T., Ishimaru, T. & Okazaki, H. (1979). ω-(3,4-Dihydroxyphenyl)alkanoic acid antifibriotic agents. United Kingdom Patent Application 2,022,414
Kanegafuchi (1977). Process for preparing D-(-)-N-carbamoyl-2-(phenyl or substituted phenyl) glycines. Britisch Patent 1,564,982
Kaufman, S. (1961). The enzymic conversion of 4-fluorophenylalanine to tyrosine. Biochimica et Biophysica Acta 51,619-621
Keil, H., Klages, U. & Lingens, F. (1981). Degradation of 4-chlorobenzoate by Pseudomonas sp. CBS3: induction of catabolic enzymes. FEMS Microbiology Letters 10,213-215
Keller, B., Keller, E., Salcher, O. & Lingens, F. (1982). Arogenate (pretyrosine) pathway of tyrosine and phenylalanine biosynthesis in Pseudomonas aureofaciens ATCC 15926. Journal of General Microbiology 128,1199-1202
Keller, B., Keller, E., Klages, U. & Lingens, F. (1983). Aromatic amino acid biosynthesis in a 4-chlorobenzoic acid degrading Pseudomonas species; phenylalanine and tyrosine synthesis via arogenate. Systematic and Applied Microbiology 4,27-33
Klages, U. & Lingens, F. (1979). Degradation of 4-chlorobenzoic acid by a Nocardia species. FEMS Microbiology Letters 6,201-203
Klages, U., Markus, A. & Lingens, F. (1981). Degradation of 4-chlorophenylacetic acid by a Pseudomonas sp. Journal of Bacteriology 146,64-68

Klibanov, A.M., Berman, Z. & Alberti, B.N. (1981). Preparative hydroxylation of aromatic compounds catalysed by peroxidase. Journal of the American Chemical Society 103,6263-6264
Knackmuss, H.-J. (1981). Degradation of halogenated and sulfonated hydrocarbons. In: Microbial Degradation of Xenobiotics and Recalcitrant Compounds (Leisinger, T., Cook, A.M., Hütter, R. & Nüesch, J., Eds.), pp. 189-212. Academic Press, London
Knowles, W.S., Sabacky, M.J. & Vineyard, B.D. (1977). L-DOPA process and intermediates. United States Patent 4,005,127
Koreeda, M., Akhtar, M.N., Boyd, D.R., Neill, J.D., Gibson, D.T. & Jerina, D.M. (1978). Absolute stereochemistry of cis-1,2-, trans-1,2-, and cis-3,4-dihydrodiol metabolites of phenanthrene. Journal of Organic Chemistry 43,1023-1027
Laane, C., Boeren, S. & Vos, K. (1985). On optimisation organic solvents in multi-liquid-phase biocatalysis. Trends in Biotechnology 3,251-252
Lehky, P. & Kulla, H. (1985). Verfahren zur Herstellung von 6-Hydroxynikotinsäure. European Patent Application 0,152,948
Lestina, G.J. & Bush, W.M. (1976). Stabilized oxichromic compounds. United States Patent 3,935,262
Ley, S.V., Sternfeld, F. & Taylor, S.C. (1987). Microbial oxidation in synthesis: a six step preparation of (+)-pinitol from benzene. Tetrahedron Letters 28,225-226
March, J. (1985). Advanced Organic Chemistry. Reactions, Mechanisms, and Structure. p. 498. Wiley & Sons, New York
Marks, T.S., Smith, A.R.W. & Quirk, A.V. (1984a). Degradation of 4-chlorobenzoic acid by Arthrobacter sp. Applied and Environmental Microbiology 48,1020-1025
Marks, T.S., Wait, R., Smith, A.R.W. & Quirk, A.V. (1984b). The origin of the oxygen incorporated during the dehalogenation/hydroxylation of 4-chlorobenzoate by an Arthrobacter sp. Biochemical and Biophysical Research Communications 124,669-674
Markus, A., Klages, U., Krauss, S. & Lingens, F. (1984). Oxidation and dehalogenation of 4-chlorophenylacetate by a two-component enzyme system from Pseudomonas sp. strain CBS3. Journal of Bacteriology 160,618-621
Martin, R.E., Baker, P.B. & Ribbons, D.W. (1987). Biotransformations of fluoroaromatic compounds: accumulation of hydroxylated products from 3-fluorophthalic acid using mutant strains of Pseudomonas testosteroni. Biocatalysis 1,37-46
Maxwell, P.C. (1982). Construction of microorganisms. European Patent Application 0,071,446
Mckenna, E.J. & Coon, M.J. (1970). Enzymatic ω-oxidation. Purification and properties of the ω-hydroxylase of Pseudomonas oleovorans. Journal of Biological Chemistry 245,3882-3889
Mermod, N., Harayama, S. & Timmis, K.N. (1986). New route to bacterial production of indigo. Bio/Technology 4,321-324
Meyer, E.M., Boesten, W.H.J., Schoemaker, H.E. & van Balken, J.A.M. (1985). In: Biocatalysis in Organic Syntheses (Tramper, J., van der Plas, H.C. & Linko, P., Eds.), pp. 135-156. Elsevier Science Publishers, Amsterdam
Michalover, J.L. & Ribbons, D.W. (1973). 3-Hydroxybenzoate 4-hydroxylase from Pseudomonas testosteroni. Biochemical and Biophysical Research communications 55,888-896.
Mitoma, C., Weissbach, H. & Udenfriend, S. (1956). 5-Hydroxytryptophan formation and tryptophan metabolism in Chromobacterium violaceum. Archives of Biochemistry and Biophysics 63,122-130
Müller, R., Thiele, J., Klages, U. & Lingens, F. (1984). Incorporation of [^{18}O]water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from Pseudomonas spec. CBS3. Biochemical and Biophysical Research Communications 124,178-182
Nakamori, S., Yokozeki, K., Mitsugi, K., Eguchi, C. & Iwagami, H. (1980). Method for producing D-α-amino acid. United States Patent 4,211,840
Nakata, H., Yamauchi, T. & Fujisawa, H. (1979). Phenylalanine hydroxylase from Chromobacterium violaceum. Journal of Biological Chemistry 254,1829-1833
Nakazawa, H., Enei, H., Okumura, S., Yoshida, H. & Yamada, H. (1972a). Enzymatic preparation of L-tryptophan and 5-hydroxy-L-tryptophan. FEBS Letters 25,43-45
Nakazawa, H., Enei, H., Okumura, S. & Yamada, H. (1972b). Synthesis of L-tryptophan from pyruvate, ammonia and indole. Agricultural and Biological Chemistry 36,2523-2528
Nörtemann, B., Bruhn, C. & Knackmuss, H.-J. (1986). Recruitment of complementary catabolic activities for mineralization of aminonaphthalene sulfonates and chloronitrophenols. EMBO Workshop: Genetic Manipulation of Pseudomonads - Applications in Biotechnology and Medicine

Nozaki, M. (1979). Oxygenases and dioxygenases. Topics in Current Chemistry 78,145-186
Olah, G.A., Fung, A.P. & Keumi, T. (1981). Oxyfunctionalisation of hydrocarbons. Hydroxylation of benzene and Alkylbenzenes with hydrogen peroxide in hydrogen fluoride/boron trifluoride. Journal of Organic Chemistry 46,4305-4306
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
Para, G., Rifai, S. & Baratti, J. (1984). Production of L-DOPA from pyrocatechol and DL-serine by bioconversion using immobilized Erwinia herbicola cells. Biotechnology Letters 6,703-708
Patel, R.N. & Hou, C.T. (1983). Enzymatic transformation of hydrocarbons by methanotrophic organisms. Developments in Industrial Microbiology 24,141-163
Pshirkov, S.Y., Boiko, O.I., Kiprianova, E.A. & Starovoitov, I.I. (1982). Transformation of L-tyrosine into L-dihydroxyphenylalanine by Pseudomonas cultures. Mikrobiologiya 51,272-274
Reddy, C.C. & Vaidyanathan, C.S. (1976). Purification and properties of benzoate-4-hydroxylase from a soil Pseudomonad. Archives of Biochemistry and Biophysics 177,488-498.
Reineke, W. & Knackmuss, H.-J. (1984). Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Applied and Environmental Microbiology 47,395-402
Reiner, A.M. (1971). Metabolism of benzoic acid by bacteria: 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid is an intermediate in the formation of catechol. Journal of Bacteriology 108,89-94
Reiner, A.M. & Hegeman, G.D. (1971). Metabolism of benzoic acid by bacteria. Accumulation of (-)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid by a mutant strain of Alcaligenes eutrophus. Biochemistry 13,2530-2536
Ruisinger, S., Klages, U. & Lingens, F. (1976). Abbau der 4-Chlorbenzoesäure durch eine Arthrobacter-species. Archives of Microbiology 110,253-256
Schraa, G., Boone, M.L., Jetten, M.S.M., van Neerven, A.R.W., Colberg, P.J. & Zehnder, A.J.B. (1986). Degradation of 1,4-dichlorobenzene by Alcaligenes sp. strain A175. Applied and Environmental Microbiology 52,1374-1381
Schraa, G., Bethe, B.M., van Neerven, A.R.W., van den Tweel, W.J.J., van der Wende, E. & Zehnder, A.J.B. (1987). Degradation of 1,2-dimethylbenzene by Corynebacterium strain C125. Antonie van Leeuwenhoek 53,159-170
Schutt, H. (1981). Stereoselective resolution of phenylglycine derivatives and 4-hydroxyphenylglycine derivatives with enzyme resins. United States Patent 4,260,684
Schutt, H., Schmidt-Kastner, G., Arens, A. & Preiss, M. (1985). Preparation of optically active D-arylglycines for use as side chains for semisynthetic penicillins and cephalosporins using immobilized subtilisins in two-phase systems. Biotechnology and Bioengineering 27,420-433
Schwartz, R.D. (1981). Novel reaction: meta hydroxylation of biphenyl by an actinomycete. Enzyme and Microbial Technology 3,158-159
Schwartz, R.D., Williams, A.L. & Hutchinson, D.B. (1980). Microbial production of 4,4'-dihydroxybiphenyl: biphenyl hydroxylation by fungi. Applied and Environmental Microbiology 39,702-708
Scott, D., Brannan, J. & Higgins, I.J. (1981a). The effect of growth conditions on intracytoplasmatic membranes and methane mono-oxygenase activities in Methylosinus trichosporium OB3b. Journal of General Microbiology 125,63-72
Scott, D., Best, D.J. & Higgins, I.J. (1981b). Intracytoplasmic membranes in oxygen-limited chemostat cultures of Methylosinus trichosporium OB3b: biocatalytic implications of physiologically balanced growth. Biotechnology Letters 3,641-644
Seigle-Murandi, F., Steiman, R., Chapelle, F. & Luu Duc, C. (1986). 5-Hydroxylation of benzimidazole by micromycetes. Optimisation of production with Absidia spinosa. Applied Microbiology and Biotechnology 25,8-13
Shirai, K. (1986). Screening of microorganisms for catechol production from benzene. Agricultural and Biological Chemistry 50,2875-2880
Shirai, K. (1987). Catechol production from benzene through reaction with resting and immobilized cells of a mutant strain of Pseudomonas. Agricultural and Biological Chemistry

51,121-128
Sih, C.J., Foss, P., Rosazza, J. & Lemberger, M. (1969). Microbial synthesis of L-3,4-dihydroxyphenylalanine. Journal of the American Chemical Society 91,6204
Sih, C.J. (1972). Method of preparing L-DOPA. United States Patent 3,671,397
Singh, D.V., Mukherjee, P.P., Pal, S.P. & Bhattacharyya, P.K. (1973). Microbial synthesis of L-DOPA (L-3,4-dihydroxy L-phenylalanine) by a Pseudomonad mutant D101. Journal of Fermentation Technology 51,713-718
Spain, J.C. & Nishino, S.F. (1987). Degradation of 1,4-dichlorobenzene by a Pseudomonas sp. Applied and Environmental Microbiology 53,1010-1019
Stenmark, S.L., Pierson, D.L., Glover, D.J., Jensen, R.A. (1974). Blue-green bacteria synthesize L-tyrosine by the pretyrosine pathway. Nature 247,290-292
Stirling, D.I., Colby, J. & Dalton, H. (1979). A comparison of the substrate and electron-donor specificities of the methane mono-oxygenases from three strains of methane-oxidizing bacteria. Biochemical Journal 177,361-364
Suh, J.T. & Skorcz, J.A. (1969). Central nervous system stimulating 1,4-benzodioxan-6-yl amino acids. United States Patent 3,478,059
Takahashi, S., Ohashi, T., Kii, Y., Hidehiko, K. & Yamada, H. (1979). Microbial transformation of hydantoins to N-carbamyl-D-amino acids. Journal of Fermentation Technology 4,328-332
Tanaka, Y., Yoshida, H. & Nakayama, K. (1974). Production of L-DOPA by mutants of Pseudomonas melanogenum. Agricultural and Biological Chemistry 38,633-639
Taylor, S.C. (1983). Biochemical process. European Patent Application 0,076,606
Taylor, S.C. (1985). Enzymic synthesis of 5,6-dihydroxycyclohexa-1,3-diene. In: Enzymes in Organic Synthesis. Ciba Foundation Symposium III, pp. 71-75. Pitman, London
Theriault, R.J. & Longfield, T.H. (1967). Microbial conversion of acetanilide to 2'-hydroxyacetanilide and 4'-hydroxyacetanilide. Applied Microbiology 15,1431-1436
Theriault, R.J. & Longfield, T.H. (1971). 5-(Hydroxyanilino)-1,2,3,4-thiatriazoles and method of making same. United States Patent 3,592,735
Theriault, R.J. & Longfield, T.H. (1973). Microbial hydroxylation of 5-anilino-1,2,3,4-thiatriazole. Applied Microbiology 25,606-611
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 144,169-144
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
van den Tweel, W.J.J., ter Burg, N., Kok, J.B. & de Bont, J.A.M. (1986c). Bioformation of 4-hydroxybenzoate from 4-chlorobenzoate by Alcaligenes denitrificans NTB-1. Applied Microbiology and Biotechnology 25,289-294
van den Tweel, W.J.J. & de Bont, J.A.M. (1987). Metabolism of both stereoisomers of phenylglycine by different routes in Flavobacterium F24. Journal of General Microbiology 133,745-754
van den Tweel, W.J.J., Marsman, E.H., Vorage, M.J.A.W., Tramper, J. & de Bont, J.A.M. (1987a). The application of organic solvents for the bioconversion of benzene to cis-benzeneglycol. In Bioreactors and Biotransformations (Moody, G.W. & Baker, P.B., Eds.) pp.231-241. Elsevier Applied Science Publishers, London
van den Tweel, W.J.J., Smits, J.P., Tramper, J. & de Bont, J.A.M. (1987b). Biosynthesis of hydroxylated aromatic compounds. Proceedings of the 4th European Congress on Biotechnology 2,172-174
van den Tweel, W.J.J., Ogg, R.L.H.P. & de Bont, J.A.M. (1987c). Werkwijze voor de bereiding van een D-α-aminozuur uit het overeenkomstige α-ketozuur. Dutch Patent Application 8,702,449
van den Tweel, W.J.J., de Laat, W.T.A.M., ter Burg, N. & Tramper, J. (1987d). Kinetic aspects of the bioconversion of 4-chlorobenzoate to 4-hydroxybenzoate by Alcaligenes denitrificans NTB-1 immobilized in carrageenan. Biocatalysis 1,161-172
van den Tweel, W.J.J., Kok, J.B. & de Bont, J.A.M. (1987e). Reductive dechlorination of 2,4-dichlorobenzoate to 4-chlorobenzoate and hydrolytic dehalogenation of 4-chloro-, 4-bromo-, and 4-iodobenzoate by Alcaligenes denitrificans NTB-1. Applied and Environmental Microbiology 53,810-815
van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1988a). Catabolism of DL-α-phenylhydracrylic, phenylacetic and 3- and 4-hydroxyphenylacetic acid via homogentisic acid in a

Flavobacterium sp. Archives of Microbiology 149,207-213
van den Tweel, W.J.J., Vorage, M.J.A.W., Marsman, E.H., Koppejan, J., Tramper, J. & de Bont, J.A.M. (1988b). Continuous production of cis-1,2-dihydroxycyclohexa-3,5-diene (cis-benzeneglycol) from benzene by a mutant of a benzene-degrading Pseudomonas sp. Enzyme and Microbial Technology 10,134-142
van den Tweel, W.J.J., Smits, J.P., Ogg, R.L.H.P. & de Bont, J.A.M. (1988c). The involvement of an enantioselective transaminase in the metabolism of D-3- and D-4-hydroxyphenylglycine in Pseudomonas putida LW-4. Submitted for publication
Vilanova, E., Manjon, A. & Iborra, J.L. (1984). Tyrosine hydroxylase activity of immobilized tyrosinase on enzacryl-AA and CPG-AA supports: stabilization and properties. Biotechnology and Bioengineering 26,1306-1312
Wang, L.-H., Hamzah, R.Y., Yu, Y. & Tu, S.-C. (1987). Pseudomonas cepacia 3-hydroxybenzoate 6-hydroxylase: induction,purification, and characterization. Biochemistry 26,1099-1104
Weiss, U. & Edwards, J.M. (1980). The biosynthesis of aromatic compounds. John Wiley & Sons, New York
Whited, G.M., McCombie, W.R., Kwart, L.D. & Gibson, D.T. (1986). Identification of cis-diols as intermediates in the oxidation of aromatic acids by a strain of pseudomonas putida that contains a TOL plasmid. Journal of Bacteriology 166,1028-1039
Williams, P.A. & Worsey, M.J. (1976). Ubiquity of plasmids in coding for toluene and xylene metabolism in soil bacteria: evidence for the existence of new TOL plasmids. Journal of Bacteriology 125,818-828
Yamada, H. & Kumagai, H. (1978). Microbial and enzymatic processes for amino acid production. Pure and Applied Chemistry 50,1117-1127
Yamada, H., Yamada, K., Kumugai, H., Hino, T. & Okumura, S. (1978). Immobilization of β-tyrosinase cells with collagen. Enzyme Engineering 3,57-62
Yamada, H. & Shimizu, S. (1985). Microbial enzymes as catalysts for synthesis of biologically useful compounds. In: Biocatalysts in Organic Syntheses (Tramper, J., van der Plas, H.C. & Linko, P., Eds.), pp. 19-37. Elseviers Science Publishers, Amsterdam
Yamamoto, S., Katagiri, M., Maeno, H. & Hayaishi, O. (1965). Salicylate hydroxylase, a monooxygenase requiring flavin adenine dinucleotide. Purification and properties. Journal of Biological Chemistry 240,3408-3413
Yeh, W.K., Gibson, D.T. & Liu, T.-N. (1977). Toluene dioxygenase: a multicomponent enzyme system. Biochemical and Biophysical Research Communications 78,401-410
Yen, K.-M., Blatt, L.M., Whited, G.M. & Gibson, D.T. (1986). Cloning of the toluene monooxygenase genes from a new TOL plasmid in Pseudomonas mendocina. EMBO Workshop: Genetic Manipulation of Pseudomonads - Application in Biotechnology and Medicine
Yokozeki, K., Nakamori, S., Eguchi, C., Yamada, K. & Mitsugi, K. (1987). Screening of microorganisms producing D-p-hydroxyphenylglycine from DL-5-(p-hydroxyphenyl)hydantoin. Agricultural and Biological Chemistry 51,355-362
Yoshida, H., Tanaka, Y. & Nakayama, K. (1973). Production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and its derivatives by Vibrio tyrosinaticus. Agricultural and Biological Chemistry 37,2121-2126
Yoshida, H., Tanaka, Y. & Nakayama, K. (1974). Production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) by Pseudomonas melanogenum. Agricultural and Biological Chemistry 38,455-462
Zaitsev, G.M. & Karasevich, Y.N. (1981a). Utilization of 4-chlorobenzoic acid by Arthrobacter globiformis. Mikrobiologiya 50,35-40
Zaitsev, G.M. & Karasevich, Y.N. (1981b). Preparative metabolism of 4-chlorobenzoic acid in Arthrobacter globiformis. Mikrobiologiya 50,423-428
Zeyer, J., Lehrbach, P.R. & Timmis, K.N. (1985a). Use of cloned genes of Pseudomonas TOL plasmid to effect biotransformation of benzoates to cis-dihydrodiols and catechols by Escherichia coli cells. Applied and Environmental Microbiology 50,1409-1413
Zeyer, J., Wasserfallen, A. & Timmis, K.N. (1985b). Microbial mineralization of ring-substituted anilines through an ortho-cleavage pathway. Applied and Environmental Microbiology 50,447-453
Zeyer, J., Folsom, B. & Timmis, K.N. (1986). Degradation of substituted ortho-nitrophenols by Pseudomonas putida strain B2. EMBO Workshop: Genetic Manipulation of Pseudomonads-Applications in Biotechnology and Medicine
Ziffer, H., Kabuto, K., Gibson, D.T., Kobal, V.M. & Jerina, D.M. (1977). The absolute stereochemistry of several cis-dihydrodiols microbiologically produced from substituted benzenes.

Tetrahedron 33,2491-2496
Zürrer, D., Cook, A.M. & Leisinger, T. (1987). Microbial desulfonation of substituted naphthalenesulfonic acids and benzenesulfonic acids. Applied and Environmental Microbiology 53,1459-1463

Chapter 2

MICROBIAL METABOLISM OF D- AND L-PHENYLGLYCINE BY *PSEUDOMONAS PUTIDA* LW-4

W.J.J. van den Tweel, J.P. Smits and J.A.M. de Bont

SUMMARY

A strain of *Pseudomonas putida* capable of utilizing both stereoisomers of phenylglycine as the sole carbon and energy source was isolated from soil. No phenylglycine racemase was detected in cells grown on either stereoisomer. In an initial reaction each stereoisomer of phenylglycine was transaminated yielding phenylglyoxylate which was further metabolized via benzaldehyde to benzoate. Subsequently, benzoate was further degraded via an ortho-cleavage of catechol.

Archives of Microbiology (1986) 144,169-174

INTRODUCTION

Aerobic microbial degradation of aromatic compounds can be accomplished along various ways. Generally, the side-chain is manipulated first to yield a substrate for ring cleavage, but a few cases have been described in which hydroxylation of the benzene nucleus occurs prior to side-chain modification. Both phenylalanine (Guroff & Ito, 1964) and mandelate (Bath et al., 1973) are examples of compounds that can be hydroxylated prior to side-chain modification. Our ultimate aim is to find an organism which is able to hydroxylate phenylglycine regio- and stereospecificly yielding D-(-)-4-hydroxyphenylglycine. The latter compound is used as a building block of semisynthetic antibiotics.

Presently, hardly anything is known about the microbial degradation of phenylglycine and its hydroxylated derivatives, but the metabolism of the analogous compounds mandelate (Bath et al., 1973; Stevenson & Mandelstam, 1965) and phenylalanine has been studied in detail. For phenylalanine various initial degradation steps have been reported (Figure 1): transamination (Fujioka et al., 1970), oxidation

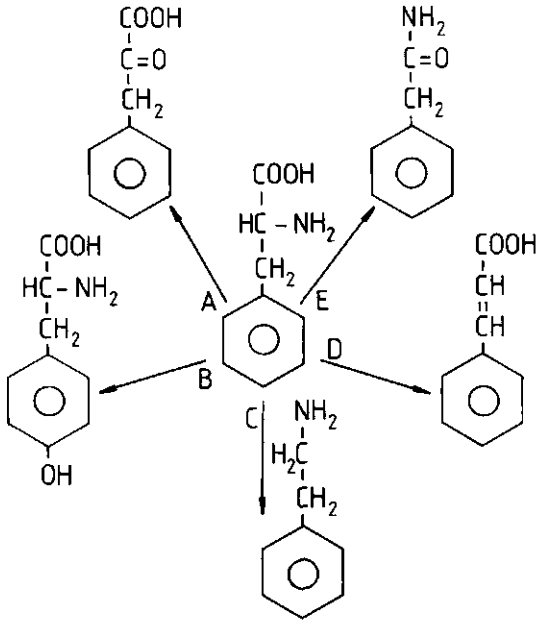

Figure 1. Possible initial degradation steps in the catabolism of phenylalanine in various microorganisms. Transamination, dehydrogenation or oxidation (A), hydroxylation (B), decarboxylation (C), deamination (D) and oxidative decarboxylation (E).

(Koyama, 1982), dehydrogenation (Hummel et al., 1984), decarboxylation (Boeker & Snell, 1972), hydroxylation (Guroff & Ito, 1964), deamination (Ogata et al., 1966), and oxidative decarboxylation (Koyama, 1982).

In order to obtain information about the metabolism of phenylglycine, and especially about the initial degradation step involved, microorganisms were isolated on D-phenylglycine as sole carbon and energy source. This paper describes the results obtained for phenylglycine catabolism in *Pseudomonas putida* LW-4.

MATERIALS AND METHODS

Organism and media. Pseudomonas putida LW-4 was isolated from soil. Details of the isolation are given under Results. The organism was maintained on agar slopes of 5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract medium to which Oxoid no. 3 agar (15 g l^{-1}) had been added. The organism was routinely grown in a mineral salts medium containing in 1 l of deionized water: K_2HPO_4, 1.55 g; NaH_2PO_4, 0.85 g; NH_4Cl, 2.0 g; $(NH_4)_2SO_4$, 0.1 g; $MgCl_2.6H_2O$, 0.075 g; yeast extract, 0.1 g and 0.2 ml of a trace elements solution as described by Vishniac and Santer (1957). Carbon sources were added to this mineral medium at 1 g l^{-1}.

Suspensions of washed cells and cell-free extracts. Cells were harvested in the mid-exponential growth phase by centrifugation (16,000 **g** for 10 min at 4°C), washed with potassium phosphate buffer pH 7.0 (50 mM) and resuspended in the same buffer. For the preparation of cell-free extracts, the cells were disrupted by ultrasonic disintegration (12x15 s). The resulting homogenate was centrifuged at 27,000 **g** for 20 min at 4°C and the supernatant, containing 10-15 (mg protein) ml^{-1}, was the crude cell-free extract. A cofactor-free extract was obtained by pumping a crude cell-free extract over a Sephadex G-25 column (20 cm x 1.5 cm). Protein fractions collected directly after the void volume were pooled and used as the cofactor-free extract.

Oxygen uptake by whole cells. Endogenous oxygen uptake by washed cells (3 ml) was measured for at least 3 min at 30°C using a polarographic oxygen probe. Subsequently, 0.1 ml of a substrate solution (30 mM) was added and oxygen uptake was followed for at least 5 min.

Protein determination. Protein contents of cell-free extracts and whole cells were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Enzyme assays. All assays were performed at 30°C.

Phenylglycine racemase. This assay was based on analysing for both stereoisomers of phenylglycine by means of gaschromatography.

The reaction mixture (total volume 5 ml) contained cell-free extract, 50 nmol pyridoxal phosphate and 250 μmol potassium phosphate buffer pH 7.0. The reaction was initiated by adding 25 μmol of a stereoisomer of phenylglycine. At various times samples were withdrawn from the reaction mixture and the reaction was stopped by boiling. After a brief centrifugation to remove the precipitated protein the supernatant was concentrated by vacuum evaporation at 35°C. To the dry sample 1 ml 1N HCl in isopropanol was added. Esterification was allowed to proceed for 1 h at 110°C after which the sample was concentrated again by vacuum evaporation. Subsequently, 250 μl ethylacetate and 50 μl perfluoropropionic anhydride were added. Derivatization of the amino group was performed for 10 min at 110°C. Solvent and reagent were removed by vacuum evaporation. The residue was dissolved in 1 ml dichloromethane. These samples (1 μl) were analysed on a Chirasil-L-val capillary column (25 m x 0.22 mm fused silica WCOT; Chrompack, Middelburg, The Netherlands). The column temperature was 140°C and the carrier gas was N_2. Under these conditions D- and L-phenylglycine had retention times of 5.52 and 6.00 min, respectively.

Phenylglycine transaminase. The enzyme was assayed by measuring by means of HPLC the rate of disappearance of phenylglycine. The reaction mixture (total volume 10 ml) contained cell-free extract; 20 μmol α-ketoglutarate; 0.2 μmol pyridoxal phosphate and 500 μmol potassium phosphate buffer pH 7.0. The reaction was started by the addition of 10 μmol phenylglycine. Samples, taken at intervals, were separated by reverse-phase ion-pairing chromatography and detected at 207 nm by means of a Perkin-Elmer variable wavelength detector. As an eluent a mixture of 10 mM tetra-n-butyl ammonium hydroxide in 10 mM sodium carbonate buffer pH 10.0 and methanol was used (80:20 [v/v]). 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.

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

Phenylglycine dehydrogenase. This possible enzyme activity was assayed for in a way similar to phenylalanine dehydrogenase (Hummel et al., 1984), except that phenylalanine was replaced by phenylglycine.

Phenylglyoxylate decarboxylase (EC 4.1.1.7.). Phenylglyoxylate consumption by cell-free extracts was measured by the spectrophotometric assay as described by Hegeman (1970). The reaction mixture for the HPLC method (total volume 10 ml) contained: cell-free extract; 500 μmol potassium phosphate buffer pH 7.0 and 0.5 μmol thiamine pyrophosphate. The reaction was initiated by adding 10 μmol phenyl-

glyoxylate. Samples, taken at various times, were fractionated by reverse-phase chromatography using a mixture of 50 mM potassium phosphate buffer pH 7.0 and methanol (90:10 [v/v]) as an eluent and detected at 207 nm.

Benzaldehyde dehydrogenase. Activity of this enzyme was assayed for spectrophotometrically by following at 340 nm the rate of reduction of NAD^+ and $NADP^+$ in cell-free extracts in the presence of benzaldehyde. The reaction mixture (total volume 1 ml) contained cell-free extract, 0.2 μmol $NAD(P)^+$ and 50 μmol potassium phosphate buffer pH 8.0. The reaction was started by adding 0.1 μmol benzaldehyde.

Catechol 1,2-dioxygenase (EC 1.13.11.1). Whether or not a catechol 1,2-dioxygenase was involved, was investigated as described by Hayaishi et al. (1957). The activity of this enzyme was measured by means of a polarographic oxygen probe.

Catechol 2,3-dioxygenase (EC 1.13.11.2). Activity of this enzyme was measured spectrophotometrically as described by Kojima et al. (1961).

Chemicals. Both stereoisomers of phenylglycine and aminooxyacetic acid were a product of Aldrich. D-3-, D-4-, L-4- and DL-2-hydroxyphenylglycine were a gift of Océ-Andeno B.V., Venlo, The Netherlands. Tetra-n-butyl ammonium hydroxide was purchased at Fisons, Loughborough, England. Perfluoropropionic anhydride was a product of Fluka, Buchs, Switzerland. All biochemicals were from Boehringer, Mannheim, FRG. All other chemicals were of commercially available analytical grade and were used without further purification.

RESULTS

Isolation and characterization of strain LW-4

Enrichment cultures could be obtained readily from various soil and sewage samples using D-phenylglycine as the sole carbon and energy source. Material taken from enrichment cultures was streaked onto agar plates with D-phenylglycine, and various microorganisms were isolated by selection of single colonies. One isolate designated strain LW-4 doubled in 1.5-2.0 h at 30°C when grown on either D- or L-phenylglycine. This strain was a motile, polarly flagellated, arginine dihydrolase and oxidase positive, Gram-negative straight rod. It did not grow at 41°C, could not denitrify, was strictly aerobic and formed a fluorescent pigment. Strain LW-4 was able to use the following compounds as sole carbon and energy source: D- and L-phenylglycine, D-3- and D-4-hydroxyphenylglycine, phenylglyoxylate, benzylalcohol,

benzaldehyde, 3- and 4-hydroxybenzaldehyde, benzoate, 3- and 4-hydroxybenzoate, catechol, DL-mandelate, DL-phenylalanine, DL-tyrosine, phenylacetate, benzylamine, glucose, acetate, pyruvate and succinate. No growth was observed using trehalose, DL-2-hydroxy-phenylglycine, L-4-hydroxyphenylglycine or 2-hydroxybenzoate as potential growth substrate.

This strain was identified as belonging to *Pseudomonas putida* according to Bergey's Manual of Determinative Bacteriology (eighth edition).

Oxygen uptake by washed cell suspensions

The effect of possible intermediates of phenylglycine on oxygen uptake by washed cell suspensions of *Pseudomonas putida* LW-4 cultured on both stereoisomers of phenylglycine or on succinate was recorded. Whole cells grown on either the D- or L-phenylglycine readily oxidized the following compounds: D- and L-phenylglycine, phenylglyoxylate, benzaldehyde, benzoate and catechol (Table 1).

Table 1. Rates[a] of oxygen uptake by washed cell suspensions of Pseudomonas putida cells grown on various carbon sources.

Substrate	Carbon source for growth		
	D-Phenyl-glycine	L-Phenyl-glycine	Succinate
D-Phenylglycine	200	210	0
L-Phenylglycine	90	120	0
D-4-Hydroxyphenylglycine	20	10	0
L-4-Hydroxyphenylglycine	0	0	0
D-3-Hydroxyphenylglycine	50	30	0
DL-2-Hydroxyphenylglycine	0	10	0
Phenylglyoxylate	700	360	0
Benzaldehyde	750	460	60
Benzoate	500	320	0
2-Hydroxybenzoate	40	40	0
3-Hydroxybenzoate	50	40	0
4-Hydroxybenzoate	30	40	0
Catechol	230	190	10
Protocatechuate	10	0	0
Gentisate	10	0	0
Succinate	30	20	1,600

[a] Rates of oxygen uptake are expressed in nmol O_2 consumed min^{-1} (mg protein)$^{-1}$ after subtraction of the endogenous oxygen uptake rate and are the average of results from at least two separate cell suspensions

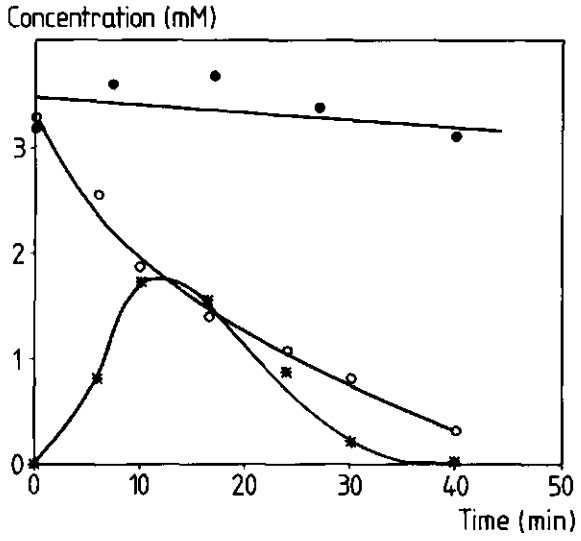

Figure 2. Benzoate (*) formation from DL-phenylglycine (○) by cell suspensions of Pseudomonas putida LW-4 cultivated on D-phenylglycine. The complete reaction mixture (total volume 5 ml) contained cells (total protein 3.5 mg), 250 μmol potassium phosphate buffer pH 7.0 and 17.5 μmol DL-phenylglycine. The reaction tube was incubated in a shaking water bath (30 °C, 1 Hz); samples were withdrawn periodically for analyses by HPLC. In the presence of 10 mM aminooxyacetic acid DL-phenylglycine (●) consumption could hardly be detected. Similar results were obtained with cells grown on L-phenylglycine.

Excretion of intermediary products of D- and L-phenylglycine catabolism

A racemic mixture of phenylglycine was readily metabolized to completion by washed cells grown on either D- or L-phenylglycine (Figure 2). During this incubation a transient accumulation of benzoate was observed. When this incubation was performed in the presence of 10 mM aminooxyacetic acid, an inhibitor of pyridoxal phosphate dependent enzymes (Hotta, 1968), hardly any phenylglycine consumption could be detected (Figure 2).

Activities of enzymes in cell-free extracts

Inhibition of phenylglycine metabolism by aminooxyacetic acid (Figure 2) indicates that a pyridoxal phosphate dependent enzyme is involved in the metabolism of phenylglycine. Therefore, it was investigated whether a pyridoxal phosphate-dependent phenylglycine transaminase was present. The simultaneous addition of α-ketoglutarate and pyridoxal phosphate to cell-free extracts of cells grown on either stereoisomer of phenylglycine indeed resulted in a rapid decrease of

the phenylglycine concentration. Pyruvate and oxaloacetate could not replace α-ketoglutarate. D-phenylglycine was transaminated faster than L-phenylglycine (Table 2).

Table 2. Specific enzyme activities of the enzymes involved in phenylglycine degradation of Pseudomonas putida cells grown on various substrates.

Enzymes	Growth substrate		
	D-Phenylglycine	L-Phenylglycine	Succinate
D-Phenylglycine transaminase[a]	50	40	< 5
L-Phenylglycine transaminase[a]	15	20	< 5
Phenylglyoxylate decarboxylase[a]	1,400	1,000	< 5
Benzaldehyde dehydrogenase[b]			
NAD^+-dependent	185	175	< 5
$NADP^+$-dependent	280	300	< 5
Catechol 1,2-dioxygenase[c]	370	490	10

[a] Activities are expressed in nmol substrate consumed min^{-1} (mg protein)$^{-1}$
[b] Rates in nmol $NAD(P)^+$ reduced min^{-1} (mg protein)$^{-1}$
[c] Rates in nmol O_2 consumed min^{-1} (mg protein)$^{-1}$

It was also investigated whether cells grown on D- or L-phenylglycine possessed a phenylglycine racemase. Cell-free extracts of cells grown on either stereoisomer were incubated with either D- or L-phenylglycine; no α-ketoglutarate was added to these incubation mixtures. Samples were taken at various times and analysed for D- and L-phenylglycine by gaschromatography. The results showed that these cell-free extracts were not able to racemize either stereoisomer of phenylglycine. Neither did the simultaneous addition of pyridoxal phosphate result in a racemization of D- and L-phenylglycine.

Cell-free extracts of cells grown on phenylglycine were also checked for the presence of a hydroxylase and dehydrogenase that would catalyse reactions analogous to reactions involved in phenylalanine metabolism (Figure 1). However, neither a hydroxylase nor a dehydrogenase could be detected in cells grown on phenylglycine.

When transamination was studied in cell-free extracts of cells grown on either D- or L-phenylglycine, both stereoisomers of phenylglycine were converted almost stoichiometrically to benzaldehyde

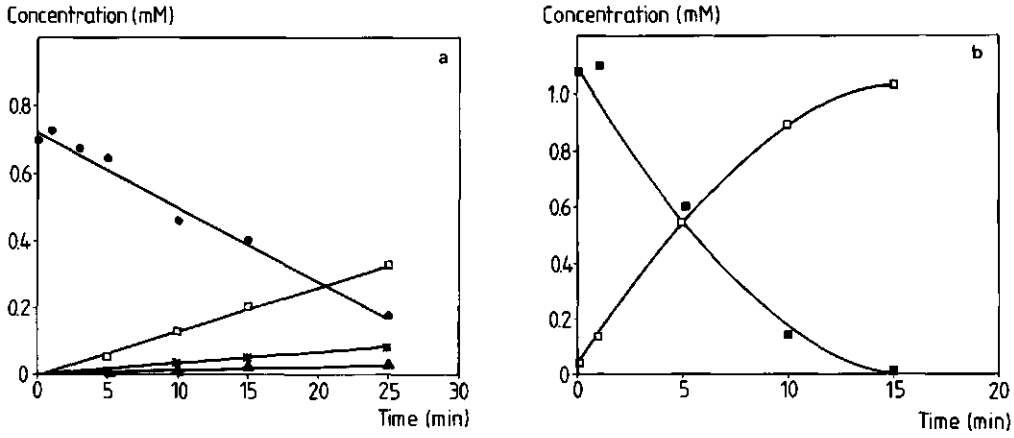

Figure 3. Consumption of D-phenylglycine (**a**) and phenylglyoxylate (**b**) by cofactor-free extracts of Pseudomonas putida cells grown on D-phenylglycine. **a** For transamination the reaction mixture (total volume 10 ml) contained cell-free extract (6.5 mg protein), 50 μmol α-ketoglutarate, 100 nmol pyridoxal phosphate, 500 μmol potassium phosphate buffer pH 7.0 and 7.5 μmol D-phenylglycine. **b** For decarboxylation the reaction mixture (total volume 10 ml) contained cofactor free extract (1.2 mg protein), 1 μmol thiamine pyrophosphate, 500 μmol potassium phosphate buffer pH 7.0 and 11 μmol phenylglyoxylate. (●), D-phenylglycine; (□), benzaldehyde; (▲), benzylalcohol; (*), benzoate; (■), phenylglyoxylate.

(Figure 3A). Apart from benzaldehyde, also small amounts of benzoate and benzylalcohol accumulated.

Although no phenylglyoxylate was detected during incubation of cell-free extracts with phenylglycine it was expected from simultaneous adaptation experiments that this compound probably would be the product of the transamination. Phenylglyoxylate was indeed readily decarboxylated by cofactor-free extracts of cells grown on phenylglycine yielding almost stoichiometrical amounts of benzaldehyde (Figure 3B), and here also very small amounts of benzylalcohol and benzoate were detected in the samples. The decarboxylation rate increased threefold when thiamine pyrophosphate was added to the reaction mixture. Optimal activity of this decarboxylase was measured using a potassium phosphate buffer pH 6.4. The product of this reaction was benzaldehyde and this compound was oxidized by a dehydrogenase in the presence of either NAD^+ or $NADP^+$ as cofactor (Table 2). The rate of benzaldehyde oxidation was higher when $NADP^+$ rather than NAD^+ was used as a cofactor (Table 2). An assay with the two nucleotides together did not result in a doubling of the individual values. Variation of pH had almost no effect on the activity ratio $NADP^+/NAD^+$ which was about 1.5-2.0.

A catechol 1,2-dioxygenase was induced during growth of *Pseudo-*

monas putida LW-4 on either stereoisomer of phenylglycine (Table 2). The enzyme showed an optimal reaction rate at pH 7.5. No catechol 2,3-dioxygenase activity could be measured in cells grown on both stereoisomers.

DISCUSSION

Phenylglycine, although not a natural occurring amino acid, was readily degraded by various microorganisms which could be isolated easily. The here described *Pseudomonas putida* LW-4 initially transaminates both stereoisomers of phenylglycine. No racemase, as being employed in D-mandelate degradation (Hegeman et al., 1970) could be detected in cells grown on either stereoisomer; neither could a pyridoxal phosphate dependent racemase as described by Soda and Osumi (1969) be found.

During growth on phenylglycine no hydroxylase and dehydrogenase as found in phenylalanine metabolism (Figure 1) could be detected. The presence of a phenylglycine oxidase has to be excluded as well, since in the absence of externally added cofactors no phenylglycine consumption by cell-free extracts occurred.

The transamination rate was higher with the D-stereoisomer of phenylglycine than with the L-stereoisomer and this was not dependent upon the isomer used as a carbon source for cultivation. This result was not expected, since usually transaminases acting on natural L-amino acids show no or almost no activity with D-amino acids. For instance, the transaminase of phenylalanine in *Achromobacter eurydice* showed no activity with the D-stereoisomer (Fujioka et al., 1970); similar results were obtained with the aromatic amino acid transaminase from rat brain (Tangen et al., 1965). On the other hand, some amino acids used for cell wall synthesis are formed by highly specific D-amino acid transaminases (Thorne et al., 1955). The transamination of D- and L-phenylglycine resulted in the formation of phenylglyoxylate which in turn was decarboxylated yielding benzaldehyde. The pH-profile for this decarboxylation in cell-free extracts showed an optimum at a pH value of 6.4. This pH optimum is similar to that reported for the phenylglyoxylate decarboxylase in *Pseudomonas putida* A.3.12 (ATCC 12633) (Hegeman, 1970). In this latter organism benzaldehyde is oxidized by two different enzymes, one being NAD^+ and the other $NADP^+$ dependent (Stevenson & Mandelstam, 1965). In our *Pseudomonas putida* strain no proof was obtained for two separate enzymes, although in cell-free extracts both NAD^+ and $NADP^+$ could serve as a cofactor.

A further catabolism of benzoate could not be measured in cell-free extracts. However, washed cell suspensions showed no

increased oxygen consumption in the presence of 2-, 3- and 4-hydroxybenzoate (Table 1), but readily oxidized catechol. Therefore, an oxidation of benzoate via 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid to catechol is most likely (Reiner & Hegeman, 1971).

Catechol was cleaved by an 1,2-dioxygenase showing an optimal reaction rate at pH 7.5.

From the results obtained a fully inducible pathway for the degradation of both stereoisomers of phenylglycine in *Pseudomonas putida* LW-4 as shown in Figure 4 is proposed.

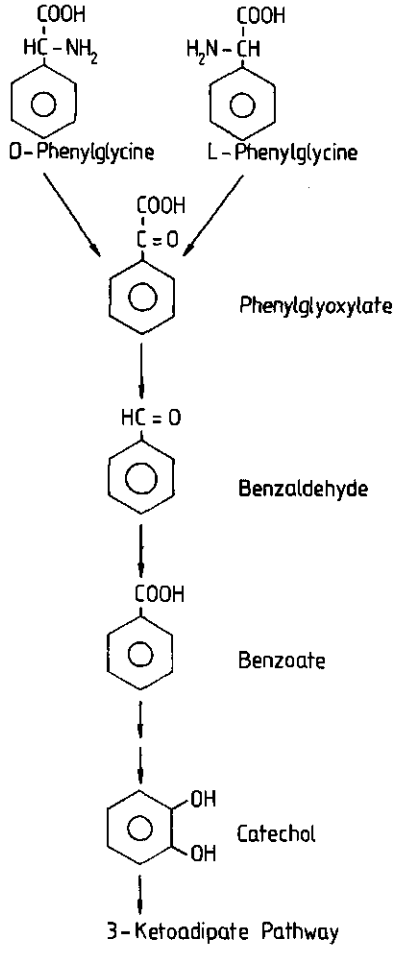

Figure 4. Proposed pathway for the metabolism of both stereoisomers of phenylglycine in Pseudomonas putida LW-4.

ACKNOWLEDGEMENTS

The authors are grateful to Professor C.J.E.A. Bulder, Professor K. van 't Riet, Dr. J. Tramper and Dr. A. Bruggink (Océ-Andeno B.V.) for their advice and helpful discussions in preparing the manuscript. These investigations were supported in part by the Netherlands Technology Foundation (STW).

REFERENCES

Bhat, S.G., Ramanarayanan, M. & Vaidyanathan, C.S. (1973). Mandelic acid-4-hydroxylase, a new inducible enzyme from Pseudomonas convexa. Biochemical and Biophysical Research Communications 52,834-842

Boeker, E.A. & Snell, E.E. (1972). Amino acid decarboxylases. In: The Enzymes (Boyer, P.D., Ed.), Vol 6, pp. 217-253. Academic Press, London, New York

Fujioka, M., Morino, Y. & Wada, H. (1970). Metabolism of phenylalanine (Achromobacter eurydice). In: Methods in Enzymology (Tabor, H. & White-Tabor, C., Eds.), Vol 17, pp. 585-589. Academic Press, London, New York

Guroff, G. & Ito, T. (1964). Phenylalanine hydroxylation by Pseudomonas species (ATCC 11299a). Journal of Biological Chemistry 240,1175-1184

Hayaishi, O., Katagiri, M. & Rothberg, S. (1957). Studies on Oxygenases: Pyrocatechase. Journal of Biological Chemistry 229,905-920

Hegeman, G.D. (1970). Mandelate decarboxylase (Pseudomonas putida). In: Methods in Enzymology (Tabor, H. & White-Tabor, C., Eds.), Vol 17, pp. 674-678. Academic Press, London, New York

Hegeman, G.D., Rosenberg, E.Y. & Kenyon, G.L. (1970). Mandelic acid racemase from Pseudomonas putida. Purification and properties of the enzyme. Biochemistry 9,4029-4036

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

Kojima, Y., Itada, N. & Hayaishi, O. (1961). Metapyrocatechuase: a new catechol-cleaving enzyme. Journal of Biological Chemistry 236,2223-2228

Koyama, H. (1982). Purification and characterization of a novel L-phenylalanine oxidase (deaminating and decarboxylating) from Pseudomonas sp. P-501. Journal of Biochemistry 92,1235-1240

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Ogata, K., Uchiyama, K. & Yamada, H. (1966). Microbial formation of cinnamic acid from phenylalanine. Agricultural and Biological Chemistry 30,311-312

Reiner, A.M. & Hegeman, G.D. (1971). Metabolism of benzoic acid by bacteria. Accumulation of (-)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid by a mutant strain of Alcaligenes eutrophus. Biochemistry 10,2530-2536

Soda, K. & Osumi, T. (1969). Crystalline amino acid racemase with low substrate specificity. Biochemical and Biophysical Research Communications 35,363-368

Stevenson, I.L. & Mandelstam, J. (1965) Induction and multi-sensitive end-product repression in two converging pathways degrading aromatic substances in Pseudomonas fluorescens. Biochemical Journal 96,354-362

Tangen, O., Fonnum, F. & Haavaldsen, R. (1965). Separation and purification of aromatic amino acid transaminases from rat brain. Biochimica et Biophysica Acta 96,82-90

Thorne, C.B., Gomez, C.G. & Housewright, R.D. (1955). Transamination of D-amino acids by Bacillus subtilis. Journal of Bacteriology 69,357-362

Vishniac, W. & Santer, M. (1957). The Thiobacilli. Bacteriological Reviews 21,195-213

Chapter 3

METABOLISM OF BOTH STEREOISOMERS OF PHENYLGLYCINE BY DIFFERENT ROUTES IN *FLAVOBACTERIUM* F24

W.J.J. van den Tweel and J.A.M. de Bont

SUMMARY

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.

Journal of General Microbiology (1987) 133,745-754

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 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., 1986a). 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 it will be demonstrated that the metabolism of D-phenylglycine in this species is distinct from the metabolism in *Pseudomomas putida* (van den Tweel et al.,1986a).

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., 1986a) 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 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 *Pseudomonas 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). Protein contents of whole cells and crude cell extracts were determined by the Lowry method using crystalline bovine serum albumin as a standard.

Phenylglycine consumption by washed cell suspensions of Flavobacterium F24. The complete reaction mixture (total volume 5 ml) contained cells, 250 μmol potassium phosphate buffer pH 7.0 and 5 or 10 μmol phenylglycine. The reaction tubes were incubated in a shaking water bath (30°C, 1 Hz). Experiments were performed with or without aminooxyacetate (15 mM). Samples (0.5 ml), taken at intervals, were analysed at room temperature by reverse-phase HPLC using a C-18 column (200 by 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 [v/v]) 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.

Separation of the optical isomers of phenylglycine. Samples were analysed by HPLC using a chiral stationary phase (ET 250/8/4 Nucleosil Chiral-1 column; 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, 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 means of HPLC. The reaction mixture (total volume 5 ml) contained cell extract, 25 μmol 2-oxoglutarate, 1 μmol PLP and 250 μmol potassium phosphate buffer pH 7.0. The reaction was started by the addition of 5 μmol 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 volume 3 ml) contained 150 μmol Tris/HCl buffer pH 9.0, 0.33 μmol PMS and cell extract. The reaction was initiated by the addition of 3 μmol substrate. Alternatively, the enzyme activity was recorded by measuring phenylglycine concentrations at various intervals. The complete reaction mixture

(total volume 5 ml) contained cell extract, 250 μmol Tris/HCl buffer pH 9.0 and 5 μmol PMS. The reaction was started by adding 10 μmol phenylglycine. Samples were analysed for phenylglycine by means of HPLC (conditions as before).

(iii) Phenylglycine hydroxylase. The presence of possible 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 a $NAD(P)^+$-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 volume 3 ml) contained 150 μmol 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 μmol phenylglycine.

(vi) Phenylglyoxylate decarboxylase (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 means of HPLC using the same conditions as described before.

(vii) Benzaldehyde dehydrogenase (PMS-dependent). This enzyme was also assayed by means of a polarographic oxygen probe. The reaction mixture (total volume 3 ml) contained 150 μmol Tris/HCl buffer pH 9.0, 0.33 μmol PMS and cell extract. The reaction was initiated by the addition of 3 μmol 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 form spores, or to denitrify and was both oxidase- and aminopeptidase-positive. Gelatine and aesculin were not hydrolysed and β-galactosidase, urease or 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 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

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.

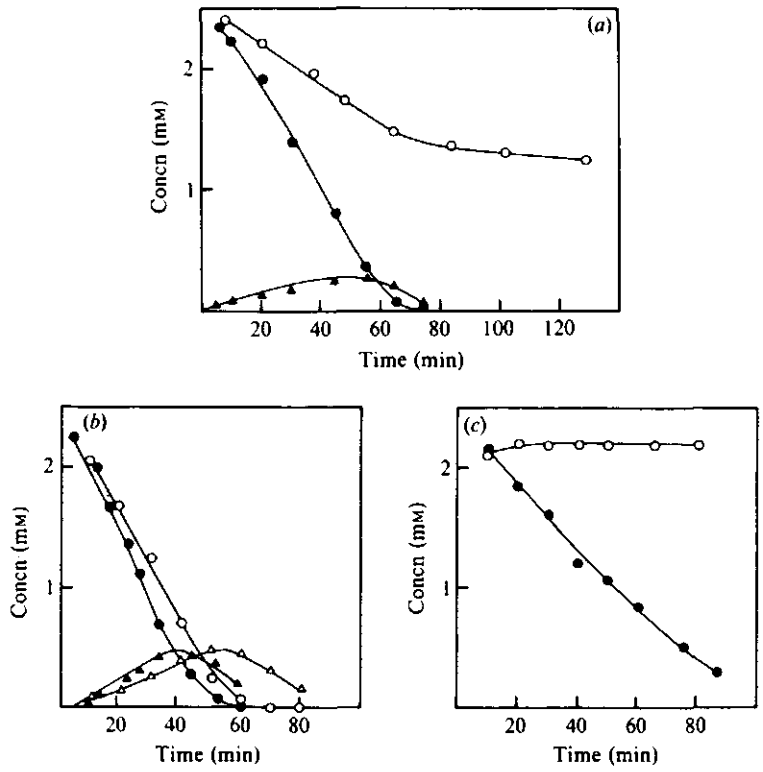

Figure 1. Phenylglycine consumption (●,○) and benzoate accumulation (▲,△) by cell suspensions of Flavobacterium F24 cultivated on D-phenylglycine. The incubation mixture (5 ml) contained 15.5 mg protein and 10 μmol phenylglycine: (a) DL-phenylglycine; (b) D-phenylglycine; (c) L-phenylglycine. The incubation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

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 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 (Figure 1). The L-stereoisomer, however, was metabolized at about half the rate of D-phenylglycine metabolism (Figure 1c). During the incubations with DL- and D-phenylglycine a product transiently accumulated (Figure 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 (Figure 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 (Figure 1c). However, when this experiment was performed with D-phenylglycine as substrate, no inhibition was observed (Figure 1b). Incubation with DL-phenylglycine in the presence of AOA resulted in phenylglycine metabolism until 50% of the mixture was utilized (Figure 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- and L-phenylglycine-grown cells consumed approximately 50% of racemic phenylglycine in the simultaneous presence of PLP and 2-oxoglutarate (Figure 2a), under these conditions they did not metabolize D-phenylglycine (Figure 2b) but fully transformed L-phenylglycine (Figure 2c). Chiral HPLC analysis of the residue after completion of the reaction with DL-phenylglycine (Figure 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 (Figure 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 reveal the mechanism of D-phenylglycine metabolism. Further experiments with cell extracts

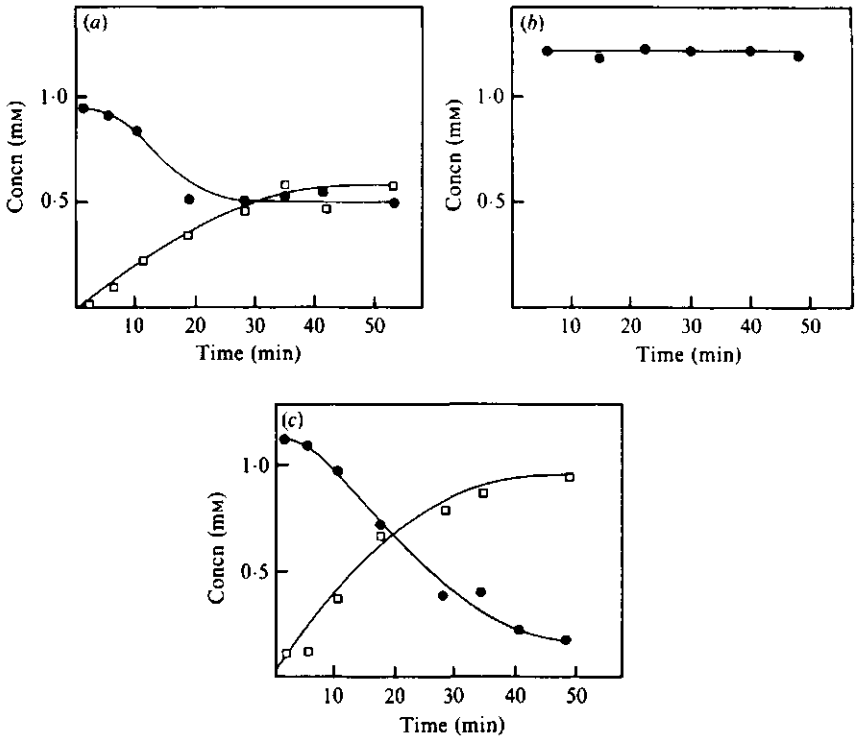

Figure 2. Transamination of phenylglycine (•) and phenylglyoxylate accumulation (□) by cell extracts of Flavobacterium F24 cultivated on D-phenylglycine. The reaction mixture contained 15 mg protein. (a) Incubation with DL-phenylglycine; (b) with D-phenylglycine; (c) with L-phenylglycine. Similar results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

showed that no D-phenylglycine hydroxylase, no $NAD(P)^+$-dependent dehydrogenase and no oxidase were present in D-phenylglycine grown cells. However, D-phenylglycine was readily oxidized when phenazine methosulphate (PMS) was added to cell extracts as measured by oxygen consumption. This rate of oxidation was maximal [165 nmol min^{-1} (mg protein)$^{-1}$] at pH 9.5 using a 50 mM $Na_2CO_3/NaHCO_3$ 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. Figure 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 (Figure 3a) when compared with the D-phenylglycine oxidation rate (Figure 3b), whereas L-phenylglycine was not oxidized (Figure 3c). Chiral HPLC analysis of the residue after completion of the reaction with DL-phenylglycine (Figure

3a) showed that only L-phenylglycine was left behind. During all these incubations (Figure 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-Menten 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 thiamine pyrophosphate (Table 2). When *Flavobacterium* F24 was grown on succinate no phenylglyoxylate decarboxylase was present (Table 2).

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^+.

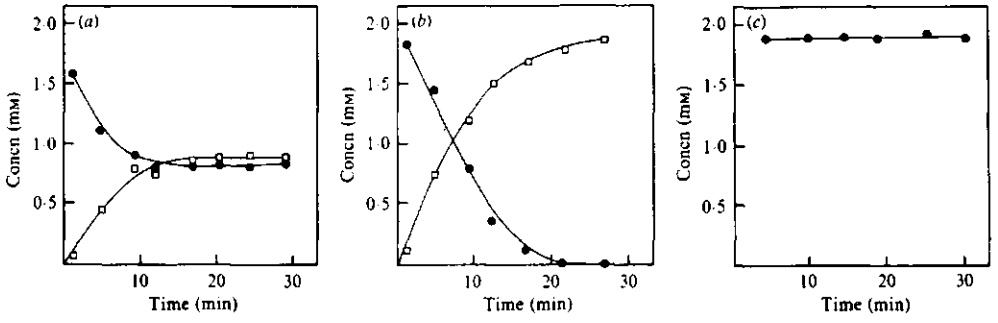

Figure 3. PMS-dependent phenylglycine oxidation (•) and phenylglyoxylate accumulation (□) by cell extracts of Flavobacterium F24 grown on D-phenylglycine. The reaction mixture contained 4.6 mg protein. (a) Incubation with DL-phenylglycine; (b) with D-phenylglycine; (c) with L-phenylglycine.

Table 2. Specific enzyme activities of some enzymes involved in phenylglycine degradation in Flavobacterium F24 grown 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.

Along with this constitutive NAD^+-dependent aldehyde dehydrogenase an inducible PMS-dependent benzaldehyde dehydrogenase was present in phenylglycine-grown cells. This latter enzyme showed a pH optimum at pH 9. The activity of this enzyme was not stimulated by the addition of NH_4Cl (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 dioxygenase 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.

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.

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 *Pseudomonas 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 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 activity as present in *Pseudomonas 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; Chandra & Vining, 1968; Friedrich & Schlegel, 1972; Nakata et al., 1979). Since a regio- and stereoselective 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 relaxed substrate specificity. The

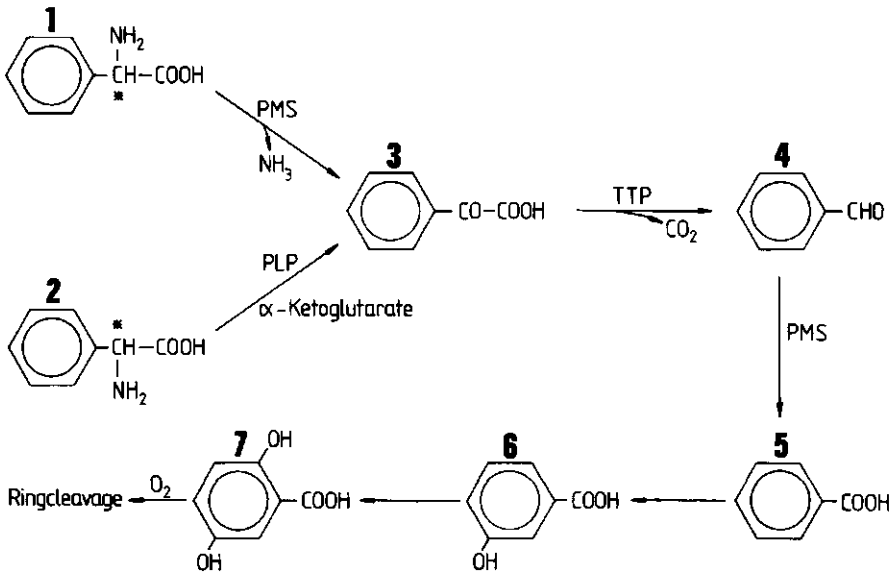

Figure 4. Proposed pathway for the metabolism of D- and L-phenylglycine in Flavobacterium F24. (1) D-Phenylglycine, (2) L-phenylglycine, (3) phenylglyoxylate, (4) benzaldehyde, (5) benzoate, (6) 3-hydroxybenzoate, (7) gentisate. TPP, thiamine pyrophosphate.

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 thiamine pyrophosphate. The activity of the phenylglyoxylate decarboxylase was very low when compared with this enzyme activity in *Pseudomonas 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 Figure 4.

ACKNOWLEDGEMENTS

We are grateful to Professor C.J.E.A. Bulder, Professor K. van 't Riet, Dr. J. Tramper and Dr. A. Bruggink (Océ-Andeno BV) for advice in preparing the manuscript. These investigations were supported in part by the Netherlands Technology Foundation (STW).

REFERENCES

Arnaud, A., Galzy, P. & Jallageas, J.-C. (1980). Production d'acides α-aminés stéréospécifiques par hydrolyse biologique d'α-amino-nitriles 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 PA01. Archives of Microbiology 112,287-289

Boesten, W.H.J. & Meyer-Hoffman, L.R.M. (1975). Enzympreparaat met aminopeptidase activiteit. Dutch Patent Application 7,513,551

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 1,506,067

Chandra, P. & Vining, L.C. (1968). Conversion of phenylalanine to tyrosine by microorganisms. Canadian Journal of Microbiology 14,573-578

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

Crawford, R.L. & Frick, T.D. (1977). Rapid spectrophotometric differentiation between gluta thione-dependent and glutathione-independent gentisate and homogentisate pathways. Applied and Environmental Microbiology 34,170-174

Duine, J.A. & Frank, J. (1981). In: Proceedings of the Third International Symposium on Microbial Growth on C_1-compounds (Dalton, H., Ed.), pp. 31-41. Heyden & Son, London

Freese, E., Sheu, C.W. & Galliers, E. (1973). Function of lipophilic acids as antimicrobial food additives. Nature 241,321-325

Friedrich, B. & Schlegel, H.G. (1972). Die Hydroxylierung von Phenylalanin durch Hydrogemonas 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., 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

Hagedorn, S.R. & Chapman, P.J. (1985). Glutathione-independent maleylacetoacetate isomerase in Gram-positive bacteria. Journal of Bacteriology 163,803-805

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 4,211,840

Nakata, H., Yamauchi, T. & Fujisawa, 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 4,260,684

Schutt, H., Schmidt-Kastner, G., Arens, A. & Preiss, M. (1985). Preparation of optically active D-arylglycines 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-phydroxyphenylglycine with d-3-bromocamphor-8-sulfonic acid. Agricultural and Biological Chemistry 43,395-396

Yamanaka, K. (1981). In: Proceedings of the Third International Symposium on Microbial Growth on C_1-compounds (Dalton, H., Ed.), pp. 21-30. Heyden & Son, London

Chapter 4

THE INVOLVEMENT OF AN ENANTIOSELECTIVE TRANSAMINASE IN THE METABOLISM OF D-3- AND D-4-HYDROXYPHENYLGLYCINE IN *PSEUDOMONAS PUTIDA* LW-4

W.J.J. van den Tweel, J.P. Smits, R.L.H.P. Ogg
and J.A.M. de Bont

SUMMARY

Pseudomonas putida LW-4, isolated on D-phenylglycine as sole carbon and energy source, was also able to grow on D-3- and D-4-hydroxyphenylglycine. Both D-3- and D-4-hydroxyphenylglycine were initially converted to the corresponding hydroxyphenylglyoxylates by means of an enantioselective transaminase. Subsequently, the hydroxyphenylglyoxylates were decarboxylated and then oxidized to 3- and 4-hydroxybenzoate, respectively. These latter compounds in turn were oxidized by NADPH-dependent hydroxylases to protocatechuate, which was further oxidized via an intradiol cleavage. Preliminary experiments with cell extracts in which the 4-hydroxyphenylglyoxylate decarboxylase was partially removed by an ammonium sulfate fractionation showed that D-4-hydroxyphenylglycine could be formed from 4-hydroxyphenylglyoxylate by the enantioselective transaminase.

Submitted for publication

INTRODUCTION

D-(-)-4-hydroxyphenylglycine (D-4-HPG) is used for the production of certain semisynthetic penicillins and cephalosporins, especially for amoxicillin, a broad spectrum antibiotic. The compound may be obtained from a racemic mixture both by the classical resolution with *d*-3-bromocamphor-8-sulfonic acid (Yamada et al., 1979), and by biological resolutions. These 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), the N-acyl acid (Cole & Utting, 1974), or the aminonitrile (Arnaud et al., 1980). In spite of this diversity in concepts for the synthesis of D-4-HPG, there still is a great need for other production processes. We previously have considered a regio- and stereoselective microbial hydroxylation of phenylglycine (van den Tweel et al., 1986; van den Tweel & de Bont, 1987). In the present paper we describe the catabolism of hydroxylated phenylglycine derivatives in *Pseudomonas putida* LW-4, and demonstrate the involvement of an enantioselective transaminase which can be used to synthesize D-4-HPG from 4-hydroxyphenylglyoxylate.

MATERIALS AND METHODS

Organism and media. Pseudomonas putida LW-4, biotype A (NCIB 12565) was routinely grown batchwise in a mineral salts medium (van den Tweel et al., 1986) to which carbon sources were added at 1.0 g l^{-1}. The organism was maintained on agar slopes containing glucose (5.0 g l^{-1}), yeast extract (3.5 g l^{-1}) and Oxoid no. 3 agar (15 g l^{-1}).

Suspensions of washed cells and cell extracts. Organisms were harvested in the mid-exponential growth phase by centrifugation (10 min, 16,000 g), washed with potassium phosphate buffer pH 7.0 (50 mM), and resuspended in the same buffer. For the preparation of cell extracts, the cells were disrupted by ultrasonic disintegration (12 x 15-s bursts at 0°C) followed by centrifugation at 27,000 g for 15 min at 4°C. The clear supernatant, containing 10-20 mg protein ml^{-1} was used as the crude cell extract.

Ammonium sulfate fractionation. The crude cell extract was fractionated by stepwise addition of a saturated ammonium sulfate solution (pH 7.0) to 10, 20, 25, 30, 35, 40, 45, 50, 60 and 70% saturation. After each step the precipitate was collected by centrifugation for 15 min at 27,000 g, dissolved in potassium phosphate buffer (pH 7.0, 50 mM), and assayed for activity.

Simultaneous adaptation experiments. Endogenous oxygen uptake

by suspensions of freshly washed cells (total volume 3 ml) was measured for at least 3 min at 30°C using a YSI model 53 monitor equipped with a YSI model 5331 polarographic oxygen probe (Yellow Springs Instruments Co., Yellow Springs, Ohio, USA). Subsequently, 0.1 ml of a substrate solution (30 mM) was added and the oxygen uptake was followed for at least another 5 min.

Hydroxyphenylglycine consumption by whole cells. The complete reaction mixture (total volume 5 ml) contained cells (9.5 mg protein), 500 μmol potassium phosphate buffer pH 7.0 and 20 μmol substrate. The reaction tubes were incubated in a shaking water bath (30°C, 1 Hz). Samples (0.5 ml) taken at intervals, were analyzed at room temperature by reverse-phase HPLC as described hereafter. 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 cell extracts and whole cells were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Separation of the enantiomers of 4-HPG. Two different methods were used to analyse the enantioselectivity of the transamination reaction. For the first method samples were analyzed directly by HPLC using a chiral stationary phase (Chiral Pro-Cu column, 250x4.6 mm; Serva Feinbiochimica, Heidelberg, FRG). Detection was at 220 nm by means of a variable wavelength detector. The mobile phase was 1 mM $CuSO_4$, flowrate was 1 ml min^{-1}, and the column temperature was 60°C. Under these conditions D- and L-4-HPG had retention times of 8.5 and 7.7 min, respectively. The second method which also proved to be very satisfying is recently described by Buck and Krummen (1984). The samples were derivatized with o-phthaldialdehyde and Boc-L-cysteine prior to chromatography as described by Buck and Krummen. The resulting diastereoisomers were then separated on a C-18 column (100x3 mm; Chrompack, Middelburg, The Netherlands). A mixture of potassium phosphate buffer (pH 7.0, 50 mM) and methanol (1:1 [v/v]) was used as eluent at a flow of 0.5 ml min^{-1}. Detection was by fluorescence using a Hewlett Packard 1046A programmable fluorescence detector (excitation 344 nm; emission 443 nm). Under these conditions the derivatized L-enantiomer had a retention time of 5.8 min, whereas the diastereoisomer of D-4-HPG eluted after 8.6 min.

Enzyme assays. All assays were done at 30°C. Spectrophotometric assays were performed with a Perkin-Elmer 550A spectrophotometer. Transamination of HPG and decarboxylation of 4-hydroxyphenylglyoxylate was measured by means of HPLC as described previously (van den Tweel et al., 1986). Samples, taken at intervals, were centrifuged and the resulting supernatants were subsequently analysed on a C-18 column (200x3 mm; Chrompack, Middelburg, The Netherlands). The

mobile phase was 50 mM potassium phosphate buffer pH 7.0, flowrate was 0.5 ml min^{-1}, and detection was by UV absorbance at 216 nm (model LC-85B variable wavelength detector, Perkin-Elmer, Gouda, The Netherlands). During these assays hydroxybenzaldehydes accumulated which were detected as described hereafter. Hydroxybenzaldehyde dehydrogenase was assayed by following the decrease of hydroxybenzaldehyde by means of HPLC. The reaction mixture (total volume 10 ml) contained cell extract, 10 μmol $NAD(P)^+$ and 500 μmol potassium phosphate buffer pH 7.0. The reaction was started by adding 10 μmol hydroxybenzaldehyde. At various times, samples were withdrawn from the reaction mixture and analysed directly by reverse-phase HPLC with the above C-18 column. As an eluent 50 mM potassium phosphate buffer pH 7.0 and methanol (80:20 [v/v]) was used, flowrate was 0.4 ml min^{-1}, and detection was at 225 nm. PMS-dependent hydroxybenzaldehyde dehydrogenase was assayed for in a way similar to the PMS-dependent phenylacetaldehyde dehydrogenase (van den Tweel et al., 1988), except that phenylacetaldehyde was replaced by either 3- or 4-hydroxybenzaldehyde and that the reaction was performed at pH 7.0 (potassium phosphate buffer, 50 mM). 3-Hydroxybenzoate hydroxylase (EC 1.14.13.23) and 4-hydroxybenzoate hydroxylase (EC 1.14.13.2) were assayed spectrophotometrically by observing the initial rate of hydroxylase-catalyzed oxidation of NAD(P)H at 340 nm. The reaction mixture (total volume 1.0 ml) contained cell extract, 0.1 μmol NAD(P)H and 50 μmol potassium phosphate buffer pH 7.0. The reaction was started by the addition of 1 μmol substrate. Hydroxylase activities were corrected for oxidation of NAD(P)H occurring in the absence of substrate. Whether protocatechuate was oxidized by an intradiol or extradiol cleavage was investigated spectrophotometrically, protocatechuate 3,4-dioxygenase (EC 1.13.11.3) was measured at 290 nm (MacDonald et al., 1954), while protocatechuate 4,5-dioxygenase (EC 1.13.11.8) was assayed at 410 nm (Dagley et al., 1960). However, specific activities of protocatechuate 3,4-dioxygenase were calculated by measuring the oxygen uptake with a polarographic oxygen probe. The reaction mixture (total volume 3 ml) contained cell extract and 150 μmol potassium phosphate buffer pH 7.0. The reaction was started by adding 3 μmol protocatechuate.

Chemicals. DL-2-, DL-3-, D-3-, DL-4- and L-4-HPG were a gift of Andeno B.V., Venlo, The Netherlands. D-4-HPG was a product of Sigma Chemical Co., St. Louis, MO, USA. The sodium salt of 4-hydroxyphenylglyoxylic acid was obtained from Aldrich Chemie, Brussels, Belgium. All biochemicals were from Boehringer, Mannheim, FRG. All other chemicals were of commercially available analytical grade and were used without further purification.

Table 1. Rates[1] of oxygen uptake by washed cell suspensions of Pseudomonas putida LW-4 grown on various carbon sources.

Substrate	Carbon source for growth		
	D-3-HPG	D-4-HPG	Succinate
DL-2-HPG	<5	<5	<5
D-3-HPG	325	<5	<5
D-4-HPG	<5	260	<5
L-4-HPG	<5	<5	<5
4-Hydroxyphenylglyoxylate	<5	300	<5
3-Hydroxybenzaldehyde	550	35	ND[2]
4-Hydroxybenzaldehyde	85	295	30
2-Hydroxybenzoate	<5	<5	<5
3-Hydroxybenzoate	420	5	<5
4-Hydroxybenzoate	10	400	<5
Catechol	10	<5	10
Protocatechuate	415	315	<5
Gentisate	<5	<5	<5
Succinate	50	20	900

1 Rates of oxygen uptake are expressed in nmol O_2 min^{-1} $(mg\ protein)^{-1}$ after subtraction of the endogenous oxygen uptake rate and are the means of results from three separate cell suspensions

2 Not determined

RESULTS

Incubation experiments with whole cells.

Pseudomonas putida LW-4 grew on D-3-HPG and D-4-HPG, but not on DL-2-HPG and L-4-HPG. Simultaneous adaptation experiments showed that cells grown on D-4-HPG readily oxidized the following compounds: D-4-HPG, 4-hydroxyphenylglyoxylate, 4-hydroxybenzaldehyde, 4-hydroxybenzoate and protocatechuate (Table 1). Cells grown on D-3-HPG oxidized the 3-hydroxy-substituted aromatics but no significant oxygen uptake was recorded with the 4-hydroxy-substituted compounds (Table 1). The oxidation of L-3-HPG and 3-hydroxyphenylglyoxylate could not be tested since these compounds were not commercially available. L-4-HPG was not oxidized by cells grown on D-4-HPG (Table 1). HPG metabolism by washed cells was also tested directly by measuring the disappearance of the substrate by means of HPLC. Cells grown on D-4-HPG completely metabolized D-4-HPG while L-4-HPG was not metabolized (Figure 1). During the incubation with D-4-HPG a product accumulated which was identified as 4-hydroxybenzoate according to its retention time and UV absorption characteristics. During an incubation with DL-4-HPG only 50% of the racemic 4-HPG was metabolized (Figure 2). Again a transient accumulation of

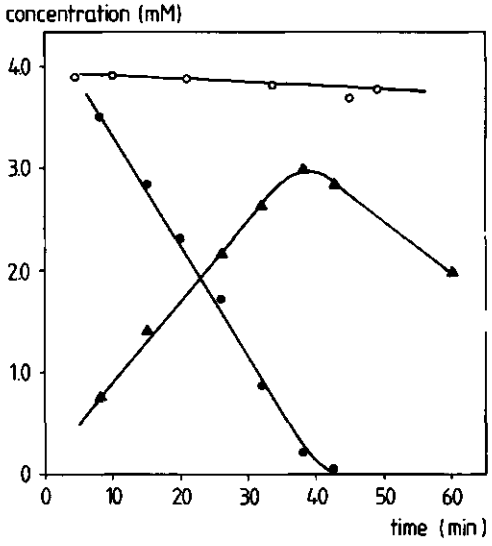

Figure 1. Consumption of D-4-HPG (•) and L-4-HPG (○) by cell suspensions of Pseudomonas putida LW-4 cultivated on D-4-HPG. ▲, 4-Hydroxybenzoate accumulation.

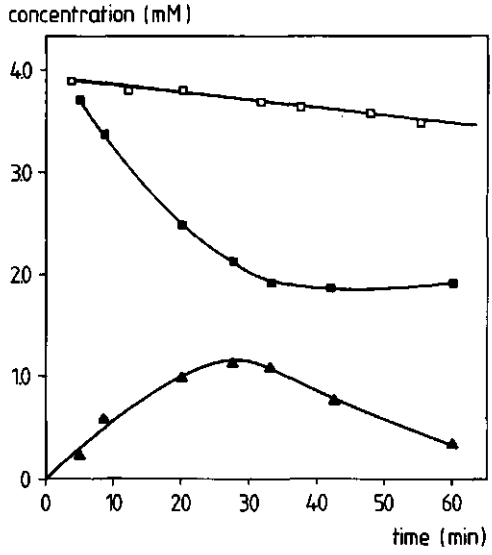

Figure 2. Metabolism of DL-4-HPG by D-4-HPG-grown cells in the presence (□) and absence (■) of 10 mM aminooxyacetate. ▲, 4-hydroxybenzoate accumulation.

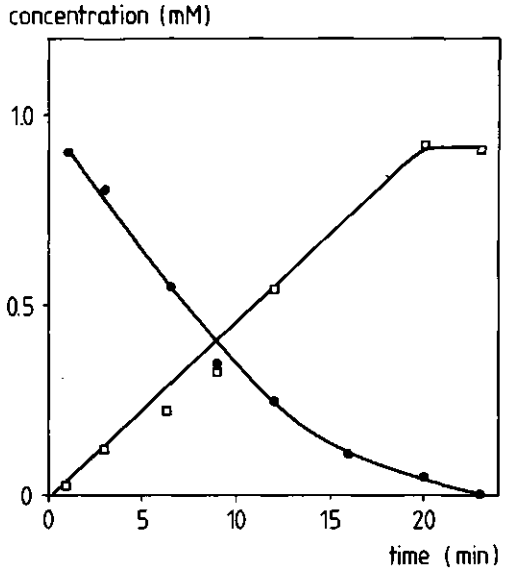

Figure 3. Transamination of D-4-HPG (•) by crude cell extracts of D-4-HPG-grown LW-4 cells. The incubation mixture (total volume 5 ml) contained cell extract (2.25 mg protein), 25 μmol α-ketoglutarate, 1 μmol pyridoxal phosphate, 500 μmol potassium phosphate buffer pH 7.0 and 5 μmol D-4-HPG. □, 4-hydroxybenzaldehyde formation.

4-hydroxybenzoate was observed. Chiral HPLC analyses of the residue after completion of the reaction with DL-4-HPG (Figure 2) showed that only L-4-HPG was present, indicating that only the D-stereoisomer was metabolized. In the presence of 10 mM aminooxyacetate, an inhibitor of pyridoxal phosphate dependent enzymes (Hotta 1968), almost no DL-4-HPG consumption was detected (Figure 2). Similar experiments were done with cells grown on D-3-HPG. D-3-HPG was completely metabolized (38 nmol min^{-1} (mg protein)$^{-1}$), while racemic 3-HPG was metabolized for 50% (37 nmol min^{-1} (mg protein)$^{-1}$). During these incubations 3-hydroxybenzoate accumulated from D-3-HPG. Again no substrate was consumed in the presence of aminooxyacetate.

Enzyme activities in cell extracts.

From the inhibition of D-3- and D-4-HPG metabolism by aminooxyacetate it was suspected that pyridoxal phosphate dependent enzymes are involved in the metabolism of these compounds. Simultaneous addition of α-ketoglutarate and pyridoxal phosphate to extracts of cells grown on D-4-HPG indeed resulted in a decrease of D-4-HPG concentration (Figure 3). During this incubation a product accumulated which was identified as 4-hydroxybenzaldehyde (Figure 3). No accumulation of the suspected intermediate 4-hydroxyphenylglyoxylate

was observed due to the high specific activity of 4-hydroxyphenylglyoxylate decarboxylase in the assay mixture (Table 2). Optimal activity of the D-4-HPG transamination was around pH 8.0. In the absence of either α-ketoglutarate or pyridoxal phosphate no D-4-HPG was consumed. D-3-HPG was also transaminated by crude extracts of D-4-HPG-grown cells. During this incubation 3-hydroxybenzaldehyde was formed in almost stoichiometric amounts. DL-2-HPG and L-4-HPG, on the other hand, were not metabolized (Table 2). Similar results were observed with cell extracts of D-3-HPG-grown cells (Table 2).

Although no 4-hydroxyphenylglyoxylate was detected during the incubation of cell extracts with D-4-HPG it was expected from simultaneous adaptation experiments that this compound was the product of

Table 2. Specific enzyme activities[1] of the enzymes involved in D-3-HPG and D-4-HPG degradation in Pseudomonas putida LW-4.

Enzyme	Growth substrate		
	D-3-HPG	D-4-HPG	Succinate
Hydroxyphenylglycine transaminase			
- DL-2-HPG	<5	<5	<5
- D-3-HPG	20	40	<5
- D-4-HPG	60	170	<5
- L-4-HPG	<5	<5	<5
4-Hydroxyphenylglyoxylate decarboxylase	135	300	<5
Hydroxybenzaldehyde dehydrogenase			
NAD^+-dependent			
- 3-Hydroxybenzaldehyde	30	ND	<5
- 4-Hydroxybenzaldehyde	ND	35	<5
$NADP^+$-dependent			
- 3-Hydroxybenzaldehyde	45	ND	<5
- 4-Hydroxybenzaldehyde	ND	70	<5
PMS-dependent			
- 3-Hydroxybenzaldehyde	35	ND	40
- 4-Hydroxybenzaldehyde	ND	55	45
Hydroxybenzoate hydroxylase (NADPH-dependent)			
- 3-Hydroxybenzoate	120	<5	<5
- 4-Hydroxybenzoate	<5	105	<5
Protocatechuate 3,4-dioxygenase	170	215	<5

1 Rates in nmol min^{-1} $(mg\ protein)^{-1}$ are the means of results of two separate extracts
2 Not determined

the transamination. Cell extracts of D-4-HPG-grown cells indeed readily decarboxylated 4-hydroxyphenylglyoxylate, this in contrast to cell extracts of succinate-grown cells.

Owing to the high absorption of the hydroxybenzaldehydes at 340 nm an assay by means of HPLC was used to investigate the $NAD(P)^+$-dependent oxidation of the formed hydroxybenzaldehydes. As was the case for phenylglycine degradation in this *Pseudomonas* sp. (van den Tweel et al., 1986) both NAD^+ and $NADP^+$ could serve as cofactors for the oxidation of 3- and 4-hydroxybenzaldehyde (Table 2). In addition to the $NAD(P)^+$-dependent hydroxybenzaldehyde oxidation also a PMS-dependent oxidation of the hydroxybenzaldehydes was observed. However, extracts of succinate-grown cells also contained this enzyme activity (Table 2). Further metabolism of both 3- and 4-hydroxybenzoate proceeded by its conversion to protocatechuate (Table 2). The enzyme catalyzing the hydroxylation of 4-hydroxybenzoate showed no activity when 4-hydroxybenzoate was replaced by benzoate, 2- or 3-hydroxybenzoate. Similar results were obtained with the enzyme converting 3-hydroxybenzoate to protocatechuate. Both enzymes showed an absolute requirement for NADPH as a reductant; NADH could not replace NADPH. The converging product, protocatechuate, was oxidized by an intradiol cleavage (Table 2); no extradiol cleaving enzyme was

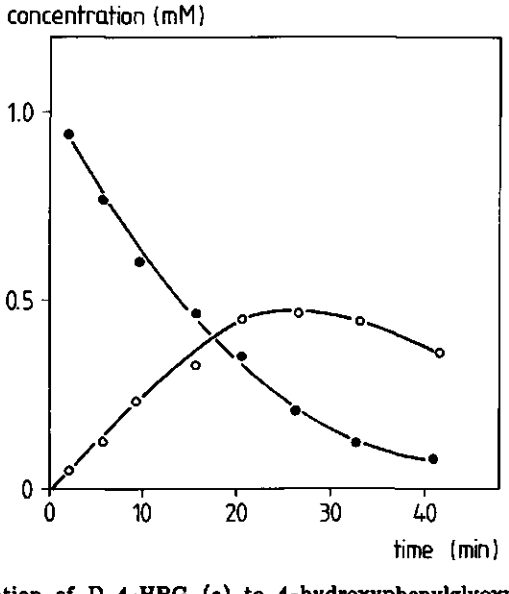

Figure 4. Transamination of D-4-HPG (•) to 4-hydroxyphenylglyoxylate (○) by a partially purified extract obtained during 30-35% ammonium sulfate saturation. The incubation mixture (total volume 5 ml) contained: 0.6 mg protein, 5 μmol D-4-HPG, 25 μmol α-ketoglutarate, 1 μmol pyridoxal phosphate and 500 μmol potassium phosphate buffer pH 7.0.

detected in cells grown on either D-3-HPG or D-4-HPG. The activity of the inducible protocatechuate 3,4-dioxygenase steadily increased up to pH 9.0.

Formation of D-4-HPG from 4-hydroxyphenylglyoxylate.

The reverse reaction of the enantioselective transamination of 4-HPG was studied in partially purified extracts since 4-hydroxyphenylglyoxylate was very rapidly decarboxylated to 4-hydroxybenzaldehyde by crude cell extracts, also when no thiamine pyrophosphate was added (Figure 3). This removal of decarboxylase activity was achieved by ammonium sulfate fractionation. Analysis of the protein fractions, obtained after a stepwise addition of ammonium sulfate, showed that the precipitate obtained during 30-35% saturation had the highest ratio D-4-HPG transaminase/4-hydroxyphenylglyoxylate decarboxylase activity (285 and 95 nmol min^{-1} (mg protein)$^{-1}$, respectively). During the transamination of D-4-HPG by this partially purified extract a product accumulated which was identified according to its retention time and UV-characteristics as 4-hydroxyphenylglyoxylate (Figure 4). When this partially purified extract was incubated with 4-hydroxyphenylglyoxylate in the presence of pyridoxal phosphate and excess L-glutamate, the desired product, 4-HPG, accumulated at an initial rate

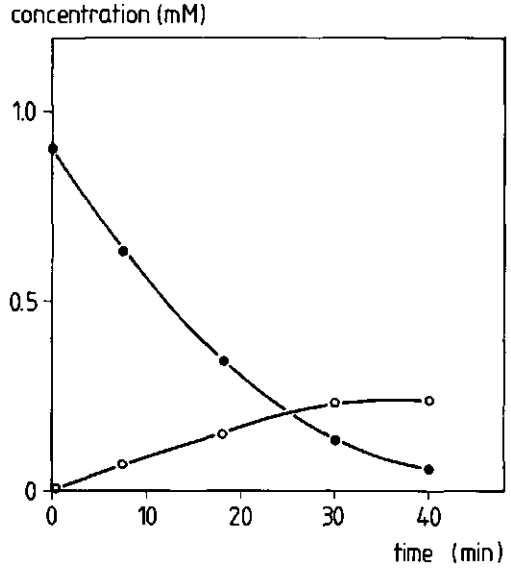

Figure 5. Formation of D-4-HPG (o) from 4-hydroxyphenylglyoxylate (•) by the protein fraction which precipitated during 30-35% ammonium sulfate saturation. The incubation mixture (total volume 5 ml) contained: 2.0 mg protein, 4.5 μmol 4-hydroxyphenylglyoxylate, 300 μmol L-glutamate, 1 μmol pyridoxal phosphate and 500 μmol potassium phosphate buffer pH 7.0.

of 21 nmol min^{-1} (mg protein)$^{-1}$ (Figure 5). By means of the described HPLC analyses it was shown that the D-stereoisomer was formed, while no L-4-HPG could be detected.

DISCUSSION

In a previous paper we have shown that *Pseudomonas putida* LW-4 is able to transaminate both stereoisomers of phenylglycine to phenylglyoxylate which in turn was decarboxylated to benzaldehyde (van den Tweel et al., 1986). This latter compound, after being oxidized to benzoate, was further degraded via the ortho-cleavage of catechol.

The results presented in this paper clearly demonstrate that *Pseudomonas putida* LW-4 degrades D-3-HPG and D-4-HPG in a similar way. Both compounds were initially transaminated in the presence of α-ketoglutarate and pyridoxal phosphate to the corresponding hydroxyphenylglyoxylates (Figures 1-3). Surprisingly only the D-stereoisomers were transaminated while L-3-HPG and L-4-HPG were not metabolized (Figure 1). The involvement of an enantioselective transaminase in the metabolism of 4-HPG in *Pseudomonas putida* LW-4 may also explain the fact that LW-4 was not able to grow on L-4-HPG (van den Tweel et al., 1986). At present we do not know whether the transamination of D-3-HPG and D-4-HPG is catalyzed by the same enzyme. To reveal this we currently are isolating the D-4-HPG transaminase. The products of the initial transamination reaction, 3- and 4-hydroxyphenylglyoxylate, were in turn decarboxylated to 3- and 4-hydroxybenzaldehyde, respectively. Further oxidation of these hydroxybenzaldehydes resembled the oxidation of benzaldehyde in this species in that both NAD^+ and $NADP^+$ could serve as a cofactor (van den Tweel et al., 1986). A more thorough investigation of benzaldehyde oxidation in *Pseudomonas putida* LW-4 grown on D-phenylglycine has recently revealed that along with the $NAD(P)^+$-dependent benzaldehyde oxidation, also a PMS-dependent benzaldehyde dehydrogenase was present (van den Tweel, unpublished results). Maximal activity was measured at pH 9.0, and cell extracts of D-phenylglycine- and succinate-grown cells had activities of 110 and 35 nmol min^{-1} (mg protein)$^{-1}$, respectively. A similar enzyme was also present in cells grown on D-3-HPG and D-4-HPG, however, the PMS-dependent oxidation of the hydroxybenzaldehydes in these cells was as high as in succinate-grown cells (Table 2). The resulting products 3- and 4-hydroxybenzoate were hydroxylated to protocatechuate. The 4-hydroxybenzoate hydroxylase of LW-4 resembled the 4-hydroxybenzoate hydroxylase of other *Pseudomonas* spp. in that only NADPH could act as a reductant and that the enzyme was highly specific for 4-hydroxybenzoate (Entsch et al., 1976;

Hosokawa & Stanier, 1966; Hesp & Calvin, 1969). The hydroxylation of 3-hydroxybenzoate yielding protocatechuate has also been investigated in a *Pseudomonas testosteroni* (Michalover & Ribbons, 1973). Similar to our results only NADPH could serve as a cofactor to hydroxylate 3-hydroxybenzoate at the 4-position.

The converging product protocatechuate was further oxidized by an inducible ortho-fission enzyme, protocatechuate 3,4-dioxygenase, to β-carboxymuconate. The pH profile of this enzyme strongly resembled that of the protocatechuate 3,4-dioxygenase of *Pseudomonas putida* A.3.12 (Stanier & Ingraham, 1954). The induction of this ortho-fission enzyme is in agreement with the results of Stanier et al. (1966) that all fluorescent Pseudomonads during growth on 4-hydroxybenzoate induce a protocatechuate 3,4-dioxygenase whereas 4-hydroxybenzoate-grown non-fluorescent Pseudomonads induce the meta-fission enzyme, protocatechuate 4,5-dioxygenase.

On the basis of the foregoing results an inducible pathway for the degradation of D-3-HPG and D-4-HPG in *Pseudomonas putida* LW-4 as shown in Figure 6 is proposed. The most interesting step involved in this pathway is the D-selective transamination of 4-HPG since such bioconversion might be used in the reverse direction to synthesize specifically D-4-HPG from 4-hydroxyphenylglyoxylate. Preliminary experiments with a fractionated cell extract of *Pseudomonas putida* LW-4 indeed confirmed our hypothesis that D-4-HPG could be produced from 4-hydroxyphenylglyoxylate (Figure 5). The observation that not all 4-hydroxyphenylglyoxylate was converted to D-4-HPG is due to the presence of 4-hydroxyphenylglyoxylate decarboxylase activity in the extract. A similar reaction may also take place in *Streptomyces fungicidus* B-5477 which synthesizes an unique cyclic antibiotic, enduracidin, containing two molecules of both D-4-HPG and L-4-HPG (Hatano et al., 1984). Tyrosine is probably metabolized via 4-hydroxyphenylpyruvate, 4-hydroxyphenylacetate and 4-hydroxymandelate to 4-hydroxyphenylglyoxylate which in turn is converted to 4-HPG by a transaminase (Hatano et al., 1984). Nocardicin A, a monocyclic β-lactam antibiotic, also contains L-4-HPG (Hosoda et al., 1977). It was suggested that 4-HPG is synthesized from tyrosine as described above (Hosoda et al., 1977).

The formation of D-amino acids from α-keto-acids by a D-amino acid transaminase from *Bacillus licheniformis* ATCC 9945 has been described in a recent patent application (Aretz & Sauber, 1986), however, the D-transaminase of this strain did not catalyze the transamination of D-4-HPG (van den Tweel et al., 1987). The same concept has also been applied for the biosynthesis of L-phenylalanine. Several research groups studied either free or immobilized cells to produce L-phenylalanine by a transamination from phenylpyruvate (Asai et al., 1959; Bulot & Cooney, 1985; Calton et al., 1986, Ziehr et al.,

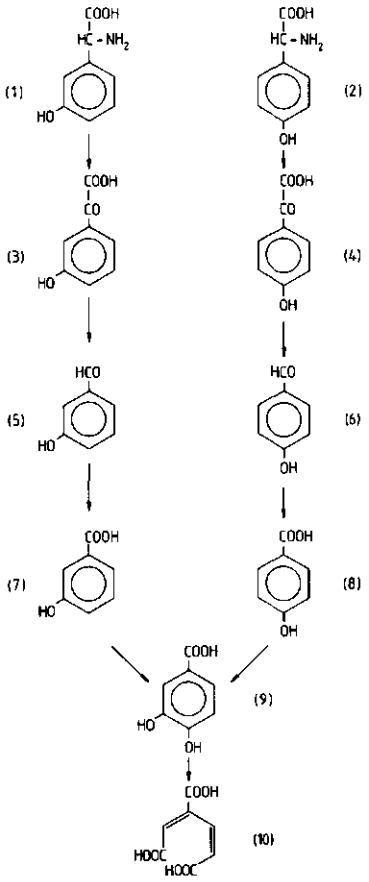

Figure 6. Proposed pathway for the converging metabolism of D-3-HPG and D-4-HPG in Pseudomonas putida LW-4. 1, D-3-HPG; 2, D-4-HPG; 3, 3-hydroxyphenylglyoxylate; 4, 4-hydroxyphenylglyoxylate; 5, 3-hydroxybenzaldehyde; 6, 4-hydroxybenzaldehyde; 7, 3-hydroxybenzoate; 8, 4-hydroxybenzoate; 9, protocatechuate; 10, β-carboxymuconate.

1987; Evans et al., 1987). An even more complex bioconversion by whole cells has been described by Wada (1974) who isolated a *Pseudomonas denitrificans* which converted DL-2-hydroxy-3-phenylpropionate to L-phenylalanine. This bioconversion was catalysed by two enzyme systems, a DL-2-hydroxy-3-phenylpropionate dehydrogenase and a transaminase (Wada, 1974). Kitai et al. (1962) on the other hand, performed this bioconversion with three enzymes: a transaminase for the conversion of phenylpyruvate in L-phenylalanine, a glutamate dehydrogenase to regenerate glutamate and an ethanol dehydrogenase for NADPH regeneration.

The bioformation of D-4-HPG from 4-hydroxyphenylglyoxylate in a fashion analogous to the forementioned methods, may be a welcome

alternative for the production of D-4-HPG and we are currently investigating this bioconversion in more detail.

ACKNOWLEDGEMENTS

The authors thank P. Stouten (State Institute for Quality Control of Agricultural Products, Wageningen) for performing some of the chiral HPLC analyses, Dr. A. Bruggink and Dr. L.A. Hulshof (Andeno B.V., Venlo), and Prof. Dr. J. Tramper for advice in preparing the manuscript. This investigation was partially supported by Andeno B.V.

REFERENCES

Aretz, W. & Sauber, K. (1986). Neue D-Aminosäure-Transaminase und ihre Verwendung. German Patent Application 3,447,023

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

Asai, T., Aida, K. & Oishi, K. (1959). On the enzymatic preparation of L-phenylalanine. Journal of General and Applied Microbiology 5,150-152

Boesten, W.H.J. & Meyer-Hoffman, L.R.M. (1975). Enzympreparaat met aminopeptidase activiteit. Dutch Patent Application 7,513,551

Buck, R.H. & Krummen, K. (1984). Resolution of amino acid enantiomers by high-performance liquid chromatography using automated pre-column derivatisation with a chiral reagent. Journal of Chromatography 315,279-285

Bulot, E. & Cooney, C.L. (1985). Selective production of phenylalanine from phenylpyruvate using growing cells of Corynebacterium glutamicum. Biotechnology Letters 7,93-98

Calton, G.J., Wood, L.L., Updike, M.H., Lantz, L. & Hamman, J.P. (1986). The production of L-phenylalanine by polyazetidine immobilized microbes. Bio/Technology 4,317-320

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. Britisch Patent 1,506,067

Dagley, S., Evans, W.C. & Ribbons, D.W. (1960). New pathways in the oxidative metabolism of aromatic compounds by micro-organisms. Nature 188,560-566

Entsch, B., Ballou, D.P. & Massey, V. (1976). Flavin-oxygen derivatives involved in hydroxylation by p-hydroxybenzoate hydroxylase. Journal of Biological Chemistry 251,2550-2563

Evans, C.T., Peterson, W., Choma, C. & Misawa, M. (1987). Biotransformation of phenylpyruvic acid to L-phenylalanine using a strain of Pseudomonas fluorescens ATCC 11250 with high transaminase activity. Applied Microbiology and Biotechnology 26,305-312

Hesp, B. & Calvin, M. (1969). Studies on p-hydroxybenzoate hydroxylase from Pseudomonas putida. Journal of Biological Chemistry 244,5644-5655

Hatano, K., Nogami, I., Higashide, E. & Kishi, T. (1984). Biosynthesis of enduracidin: origin of enduracidin and other amino acids. Agricultural and Biological Chemistry 48,1503-1508

Hosoda, J., Tani, N., Konomi, T., Ohsawa, S., Aoki, H. & Imanaka, H. (1977). Incorporation of ^{14}C-amino acids into nocardicin A by growing cells. Agricultural and Biological Chemistry 41,2007-2012

Hosokawa, K. & Stanier, R.Y. (1966). Crystallization and properties of p-hydroxybenzoate hydroxylase from Pseudomonas putida. Journal of Biological Chemistry 241,2453-2460

Hotta, S.S. (1968). Oxidative metabolism of isolated brain mitochondria: changes caused by aminooxyacetate. Archives of Biochemistry and Biophysics 127,132-139

Kitai, A., Kitamura, J. & Miyachi, N. (1962). The formation of L-amino acid through the conjugated reaction system. I. The formation of L-alanine and L-phenylalanine by the alcohol dehydrogenase conjugated system. Hakko To Taisha 5,61-65

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

MacDonald, D.L., Stanier, R.Y. & Ingraham, J.L. (1954). The enzymatic formation of β-carboxymuconic acid. Journal of Biological Chemistry 210,809-820

Michalover, J.L. & Ribbons, D.W. (1973). 3-Hydroxybenzoate 4-hydroxylase from Pseudomonas testosteroni. Biochemical and Biophysical Research Communications 55,888-896

Nakamori, S., Yokozeki, K., Mitsugi, K., Eguchi, E. & Iwagami, H. (1980). Method for producing D-α-amino acid. United States Patent 4,211,840

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 hydantoines to D-amino acids. Biotechnology and Bioengineering 23,2173-2183

Schutt, H. (1981). Stereoselective resolution of phenylglycine derivatives and 4-hydroxyphenylglycine derivatives with enzyme resins. United States Patent 4,260,684

Schutt, H., Schmidt-Kastner, G., Arens, A. & Preiss, M. (1985). Preparation of optically active D-arylglycines for use as side chains for semisynthetic penicillins and cephalosporins using immobilized subtilisins in two-phase systems. Biotechnology and Bioengineering 27,420-433

Stanier, R.Y. & Ingraham, J.L. (1954). Protocatechuic acid oxidase. Journal of Biological Chemistry 210,799-808

Stanier, R.Y., Palleroni, N.J. & Doudoroff, M. (1966). The aerobic Pseudomonads: a taxanomic study. Journal of General Microbiology 43,159-271

Takahashi, S., Ohashi, T., Kii, Y., Kumagai, H. & Yamada, H. (1979). Microbial transformations of hydantoines to N-carbamoyl-D-amino acids. Journal of Fermentation Technology 57,328-332

van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1986). Microbial metabolism of D- and L-phenylglycine by Pseudomonas putida LW-4. Archives of Microbiology 144,169-174

van den Tweel, W.J.J. & de Bont, J.A.M. (1987). Metabolism of both stereoisomers of phenylglycine by different routes in Flavobacterium F24. Journal of General Microbiology 133,745-754

van den Tweel, W.J.J., Ogg, R.L.H.P. & de Bont, J.A.M. (1987). Werkwijze voor de bereiding van een D-α-aminozuur uit het overeenkomstige α-ketozuur. Dutch Patent Application 8,702,449

van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1988). Catabolism of DL-α-phenylhydracrylic, phenylacetic and 3- and 4-hydroxyphenylacetic acid via homogentisic acid in a Flavobacterium sp.. Archives of Microbiology 149,207-213

Wada, H. (1974). Studies of cultural conditions and mechanisms for the production of L-phenylalanine from its hydroxy analogue. Nihon Nogei Kagakkai-shi 48,351-357

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

Ziehr, H., Kula, M.-R., Schmidt, E., Wandrey, C. & Klein, J. (1987). Continuous production of L-phenylalanine by transamination. Biotechnology and Bioengineering 29,482-487

Chapter 5

DL-4-HYDROXYPHENYLGLYCINE CATABOLISM IN *PSEUDOMONAS PUTIDA* MW27

W.J.J. van den Tweel, M.N. Widjojoatmodjo and J.A.M. de Bont

SUMMARY

Thirteen bacteria were isolated on D-4-hydroxyphenylglycine as sole carbon and energy source. Seven strains transaminated only the D-enantiomer while the other six isolates transaminated both enantiomers of 4-hydroxyphenylglycine. One of the six strains utilizing both enantiomers was characterized as a *Pseudomonas putida*. This strain, MW27, employed two enantioselective transaminases to catalyze the initial step in the metabolism of DL-4-hydroxyphenylglycine. The product of the transamination, 4-hydroxyphenylglyoxylate, was further metabolized via 4-hydroxybenzaldehyde and 4-hydroxybenzoate to protocatechuate. Preliminary results indicate that both transaminases are co-ordinately synthesized together with the 4-hydroxyphenylglyoxylate decarboxylase and the $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenase.

Submitted for publication

INTRODUCTION

D-(-)-4-hydroxyphenylglycine (D-4-HPG) is used in large quantities as the side chain of semisynthetic penicillins and cephalosporins and its bioproduction has been studied extensively (Cole & Utting, 1974; Boesten & Meyer-Hoffman, 1975; Cecere et al., 1975; Arnaud et al., 1980; Schutt, 1981; van den Tweel & de Bont, 1987). Recently we discovered an enantioselective D-4-HPG transaminase in *Pseudomonas putida* LW-4 that is able to convert 4-hydroxyphenylglyoxylate in D-4-HPG (van den Tweel et al., 1988b). Currently, we are studying 4-HPG metabolism in several other D-4-HPG-degrading isolates to reveal whether microorganisms in general transaminate only the D-enantiomer, and also to investigate whether different enzymes are used by microorganisms to degrade D-4-HPG.

The thirteen isolates tested could be divided in two distinct groups i.e. a group resembling the previously described *Pseudomonas putida* LW-4 and a group that transaminated both enantiomers of 4-HPG. This paper describes the metabolism of DL-4-HPG in *Pseudomonas putida* MW27 which is a representative strain of the second group.

MATERIALS AND METHODS

Isolation, maintenance and cultivation. Various soil and sewage samples were diluted and streaked directly onto agar plates containing a mineral salts medium (van den Tweel et al., 1986) to which D-4-HPG (2.0 g l^{-1}) was added. Colonies that appeared were transferred to agar plates containing the same medium. Subsequently, thirteen bacteria were isolated by selection of single colonies from these plates. Maintenance and cultivation of the isolates was as described previously for *Pseudomonas putida* LW-4 (van den Tweel et al., 1986). All media were routinely sterilized by heat treatment (20 min, 120°C), however, media containing 4-HPG as growth substrate were filter sterilized (0.2 μm), since after thermal sterilization 4-HPG was partially deaminated to 4-hydroxymandelate.

Determination of the maximal growth rate. Doubling times of strain MW27 on D-4-HPG and L-4-HPG were measured as described previously (van den Tweel et al., 1988a).

Preparation of washed cell suspensions and cell extracts. Washed cells and cell extracts were prepared as described previously (van den Tweel et al., 1986).

Ammonium sulfate fractionation. A crude cell extract of D-4-HPG-grown MW27 cells was fractionated by stepwise addition of a saturated ammonium sulfate solution (pH 7.0) to 15, 25, 35, 40, 45, 50,

55, 60, 65, 70 and 100% saturation. After each step the precipitate was collected by centrifugation for 15 min at 27,000 g, dissolved in potassium phosphate buffer (pH 7.0, 50 mM), and assayed for activity.

Measurement of substrate uptake by washed cells suspensions. Oxygen uptake rates by washed cells were measured polarographically with an oxygen probe as described previously (van den Tweel et al., 1986). Consumption of 4-HPG by whole cells was tested by adding washed cells (5.0 mg protein) to 7.5 ml of a 2 mM 4-HPG solution in potassium phosphate buffer pH 7.0. The reaction tubes were incubated in a shaking water bath (30°C, 1 Hz), and samples taken at intervals were analyzed as described before (van den Tweel et al., 1988b).

Enzyme assays. All assays were performed at 30°C. Transamination of 4-HPG was measured by a spectrophotometric assay which is based on the fact that the product of the transamination, 4-hydroxyphenylglyoxylate, possesses a molar absorption coefficient of 4500 M^{-1} cm^{-1} at 340 nm (pH 7.0), while 4-HPG under these conditions absorbs no light. This assay is also valid in case 4-hydroxyphenylglyoxylate is decarboxylated to 4-hydroxybenzaldehyde since the latter compound possesses a molar absorption coefficient equal to that of 4-hydroxyphenylglyoxylate under the stated conditions. For the transaminase assay the reaction mixture (total volume 1 ml) contained: cell extract, 0.1 μmol pyridoxal phosphate, 15 μmol α-ketoglutarate (or other α-keto acids), and 100 μmol potassium phosphate buffer pH 7.0. The reaction was initiated by adding 5 μmol 4-HPG, and the increase of absorbance at 340 nm was recorded. Whether or not the initial step in the degradation of 4-HPG was catalyzed by an oxidase, a PMS- or a $NAD(P)^+$-dependent dehydrogenase was investigated as described for phenylglycine degradation in a *Flavobacterium* sp., except that phenylglycine was replaced by 4-HPG (van den Tweel & de Bont, 1987). 4-Hydroxyphenylglyoxylate decarboxylase, 4-hydroxybenzaldehyde dehydrogenase (either $NAD(P)^+$- or PMS-dependent), 4-hydroxybenzoate-3-hydroxylase (EC 1.14.13.2), protocatechuate 3,4-dioxygenase (EC 1.13.11.3), and protocatechuate 4,5-dioxygenase (EC 1.13.11.8) were measured as described previously (van den Tweel et al., 1988b). The oxidation of DL-4-hydroxymandelate was routinely assayed with a YSI 53 monitor equipped with a YSI 5331 polarographic oxygen probe (Yellow Springs Instruments Co., Yellow Springs, Ohio, USA). The reaction mixture (total volume 3 ml) contained cell extract, 150 μmol potassium phosphate buffer (pH 7.0), 0.33 μmol phenazine methosulphate (PMS), and 30 μmol DL-4-hydroxymandelate.

Analytical methods. The presence of flagella was demonstrated by using the flagella-staining procedure described by Mayfield et al. (1977). Protein contents of cell extracts and whole cells were determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a standard. API 20 NE test strips (Analytical Profile

Index system, Montalieu-Vercieu, France) were used for identification of strain MW27.

Materials. DL-2-HPG, D-3-HPG, D-4-HPG, L-4-HPG and the sodium salt of DL-4-hydroxymandelate were gifts of Andeno B.V., Venlo, The Netherlands. 4-Hydroxyphenylglyoxylate, 4-hydroxybenzaldehyde and PMS were products of Aldrich Chemical Co., Milwaukee, Wisconsin, USA. All other chemicals were commercially available analytical grade products and were used without further purification. The biochemicals used were obtained from Boehringer, Mannheim, FRG.

RESULTS

The initial step of DL-4-HPG catabolism in the isolates

Thirteen D-4-HPG-utilizing bacteria isolated from various habitats were tested for growth on D-4-HPG as well as on L-4-HPG. Seven strains grew on the D-stereoisomer only while the six remaining strains utilized both the D- and the L-enantiomer of 4-HPG. The seven strains using only the D-stereoisomer all contained a D-specific 4-HPG transaminase, thus resembling *Pseudomonas putida* LW-4. The six strains utilizing both isomers were studied in more detail and extracts of these cells were assayed for 4-HPG dehydrogenase activity, using either $NAD(P)^+$ or PMS as electron acceptor, 4-HPG oxidase and 4-HPG transaminase activity. In all cases only transaminase activity was observed. Strain MW27 was chosen for a more detailed study of the metabolism of DL-4-HPG.

Characterization of strain MW27

Strain MW27 was a catalase and oxidase positive, Gram-negative straight rod, which formed a fluorescent pigment. It was motile and possessed several polar flagella. An API 20 NE test showed that MW27 did not denitrify, produced no acid from glucose and no indole from tryptophan, contained arginine dihydrolase and urease, produced no β-galactosidase, and did not hydrolyse aesculin and gelatin. Furthermore, the following substrates were assimilated: glucose, mannose, gluconate, caprate, malate, citrate, and phenylacetate; no growth was observed on arabinose, mannitol, N-acetyl-glucosamine, maltose, and adipate. Based on the forementioned characteristics and on the results from the API test, and the inability to grow at 41°C, the organism was tentatively identified as a *Pseudomonas putida.*

Growth of Pseudomonas putida MW27 on various aromatic substrates

Pseudomonas putida MW27 was tested for growth on a number of aromatic compounds to obtain more information about its ability to metabolize DL-4-HPG and related compounds. 4-Hydroxyphenylglyoxylate, 4-hydroxybenzaldehyde and 4-hydroxybenzoate, intermediates of D-4-HPG degradation in *Pseudomonas putida* LW-4 (van den Tweel et al., 1988b), supported growth. During growth on 4-hydroxybenzaldehyde the concentration of the substrate was kept below 1.0 g l^{-1} to prevent inhibition of growth. D- and L-phenylglycine, D- and L-mandelate, D-3-HPG, and DL-4-hydroxymandelate could also serve as sole carbon and energy source for strain MW27. No 4-hydroxymandelate was detected in the supernatant of the culture after growth on DL-4-hydroxymandelate, apparently both stereoisomers were metabolized. No growth was observed on DL-2-HPG, phenoxyacetate and 2-phenylpropionic acid. On D-4-HPG as well as on L-4-HPG doubling times of about 60 min were observed.

Table 1. Oxygen uptake rates of washed cell suspensions of Pseudomonas putida MW27 cells grown on various carbon sources.

Substrate	Growth substrate						
	D-4-HPG	L-4-HPG	DL-4-hydroxy-mandelate	4-Hydroxy-phenyl-glyoxylate	4-Hydroxy-benzal-dehyde	4-Hydroxy-benzoate	Succinate
D-4-HPG	390	600	230	165	<5	<5	<5
L-4-HPG	140	415	225	290	<5	<5	<5
DL-4-Hydroxy-mandelate	30	5	320	30	<5	<5	<5
4-Hydroxy-phenyl-glyoxylate	260	530	180	420	25	20	<5
4-Hydroxy-benzaldehyde	275	750	510	700	100	560	35
4-Hydroxy-benzoate	380	550	320	590	60	570	<5
Proto-catechuate	410	420	320	410	380	465	<5

Rates of oxygen uptake (nmol O_2 min^{-1} (mg protein)$^{-1}$) are the means of results of two separate cell suspensions and are corrected for endogenous oxygen uptake

Substrate dependent oxygen uptake

The oxygen uptake by washed cell suspensions of strain MW27, grown on certain carbon sources, was tested with a number of possible intermediates of DL-4-HPG metabolism (Table 1). Cells grown on either D-4-HPG or L-4-HPG oxidized both enantiomers of 4-HPG; for both cell suspensions D-4-HPG was a better substrate than L-4-HPG. 4-Hydroxyphenylglyoxylate, 4-hydroxybenzaldehyde, 4-hydroxybenzoate and protocatechuate were also readily oxidized by these cells, while DL-4-hydroxymandelate was not. DL-4-hydroxymandelate was only oxidized by DL-4-hydroxymandelate-grown cells. Cells cultivated on either DL-4-hydroxymandelate or 4-hydroxyphenylglyoxylate also metabolized D- and L-4-HPG, however, these cells did not prefer D-4-HPG above L-4-HPG. 4-Hydroxybenzaldehyde-, 4-hydroxybenzoate- and succinate-grown cells oxidized no D- or L-4-HPG, DL-4-hydroxymandelate or 4-hydroxyphenylglyoxylate. 4-Hydroxybenzoate-grown cells, surprisingly, oxidized 4-hydroxybenzaldehyde at a rate equal to 4-hydroxybenzoate oxidation (Table 1).

4-HPG consumption by washed cell suspensions

The foregoing experiments have shown that cells grown on either D-4-HPG or L-4-HPG oxidized both stereoisomers of 4-HPG. In order

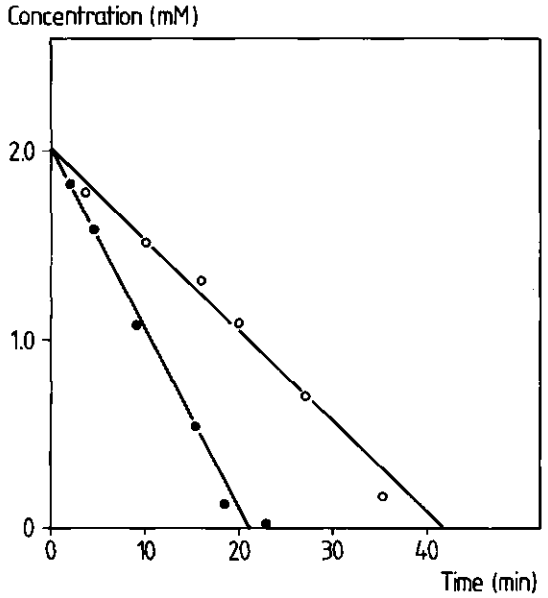

Figure 1. Consumption of D-4-HPG (•) and L-4-HPG (○) by D-4-HPG-grown Pseudomonas putida MW27 cells.

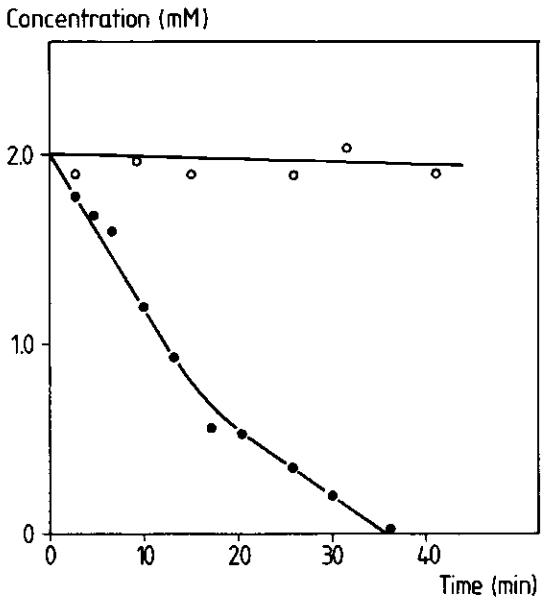

Figure 2. Consumption of DL-4-HPG in the absence (•) or presence (o) of 15 mM aminooxyacetate by Pseudomonas putida MW27 cells grown on D-4-HPG.

to measure the consumption of each enantiomer more directly, the consumption of these substrates was measured by means of HPLC. As before, cells grown on D-4-HPG metabolized both 4-HPG-isomers (Figure 1). The rate of L-4-HPG consumption was about half the rate of D-4-HPG uptake. Almost identical results were obtained for L-4-HPG-grown cells. Racemic 4-HPG was readily metabolized until approximately 50% of the substrate was consumed; at that point the metabolism of 4-HPG slowed down (Figure 2). When this latter incubation was performed in the presence of aminooxyacetate, an inhibitor of pyridoxal phosphate-dependent enzymes (Hotta, 1968), no 4-HPG was consumed (Figure 2).

Enzymes involved in DL-4-HPG degradation

Experiments with crude extracts of 4-HPG-grown MW27 cells showed that the initial step in D- and L-4-HPG metabolism was a pyridoxal phosphate-dependent transamination with α-ketoglutarate as keto-donor (Table 2). In striking contrast to experiments with whole cells, the transaminase activity was highest for L-4-HPG. No transaminase activity was observed when α-ketoglutarate was replaced by pyruvate, phenylpyruvate, oxaloacetate or 2-oxo-butyrate, independent whether D-4-HPG or L-4-HPG was used as a growth substrate. Cell extracts of D-4-HPG- and L-4-HPG-grown cells contained an inducible

Table 2. Enzyme levels[1] of various enzymes involved in the catabolism of both stereoisomers of 4-HPG by Pseudomonas putida MW27.

Enzyme	Growth substrate						
	D-4-HPG	L-4-HPG	DL-4-hydroxy-mandelate	4-Hydroxy-phenyl-glyoxylate	4-Hydroxy-benzal-dehyde	4-Hydroxy-benzoate	Succinate
DL-4-hydroxy-mandelate dehydrogenase	<5	<5	50	<5	<5	<5	<5
D-4-HPG transaminase	250	135	640	265	<5	<5	<5
L-4-HPG transaminase	350	345	980	585	<5	<5	<5
4-Hydroxy-phenylglyoxylate decarboxylase	490	580	1200	600	<5	20	ND[2]
Benzaldehyde dehydrogenase							
PMS-dep.	20	30	35	40	15	35	20
$NADP^+$-dep.	250	230	540	400	<5	25	ND
4-Hydroxyben-zoate-3-hydroxylase	95	120	45	240	60	175	<5
Protocate-chuate 3,4-dioxygenase	550	465	575	825	400	635	5

1 The activities are the means of results from two separate extracts and are expressed in nmol min^{-1} $(mg\ protein)^{-1}$

2 Not determined

4-hydroxyphenylglyoxylate decarboxylase (Table 2). The product of this decarboxylation, 4-hydroxybenzaldehyde, in turn was oxidized by a $NADP^+$-dependent, as well as by a PMS-dependent 4-hydroxybenzaldehyde dehydrogenase (Table 2). No NAD^+-dependent 4-hydroxybenzaldehyde dehydrogenase was present. 4-Hydroxybenzoate was subsequently hydroxylated by an inducible NADPH-dependent 4-hydroxybenzoate-3-hydroxylase to protocatechuate (3,4-dihydroxybenzoate). No activity was observed when NADH was used instead of NADPH. In analogy with other fluorescent *Pseudomonas* spp. the benzene ring of protocatechuate was cleaved by an intradiol dioxygenase (Table 2).

Transamination of D-4-HPG and L-4-HPG

From the above results it is evident that both enantiomers of 4-HPG are transaminated in an initial reaction, however, it is not yet clear whether only one or whether more transaminases are responsible for this phenomenon. To elucidate this, a crude cell extract of D-4-HPG-grown cells was fractionated by the addition of ammonium sulfate (Figure 3). By doing this, the D-4-HPG transaminase activity which mainly precipitated at 45-50% saturation, was separated from the L-4-HPG transaminase activity which precipitated at higher ammonium sulfate concentrations. In case of the transamination of L-4-HPG, 94% of the total activity before precipitation was recovered, while in case of D-4-HPG transamination only 65% was recovered.

Enzyme regulation

To study the regulation of the enzymes involved in DL-4-HPG metabolism, MW27 was grown on various carbon sources and certain specific enzyme activities were measured (Table 2). In addition to the established intermediates of 4-HPG metabolism, MW27 was also grown

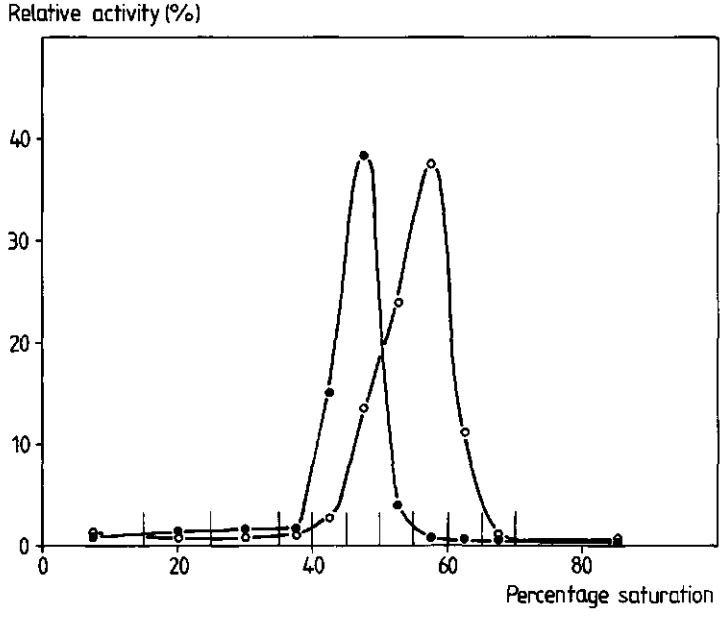

Figure 3. Separation of the transaminase activity for D-4-HPG (•) and L-4-HPG (○) by ammonium sulfate fractionation. The activity in the separate fractions is presented as a percentage of the total activity before ammonium sulfate addition. The bars in this figure indicate the ammonium sulfate saturation steps.

on DL-4-hydroxymandelate, for it is known that some bacteria degrade this compound by co-ordinately regulated enzymes via 4-hydroxyphenylglyoxylate (Stevenson & Mandelstam, 1965; Kennedy & Fewson, 1968). During growth on DL-4-HMA, in addition to the PMS-dependent hydroxymandelate dehydrogenase which used for the conversion of 4-hydroxymandelate to 4-hydroxyphenylglyoxylate, also the transaminases for D-4-HPG and L-4-HPG were induced. In contrast to other *Pseudomonas* spp. again no NAD^+-dependent 4-hydroxybenzaldehyde dehydrogenase was present in DL-4-hydroxymandelate-grown cells (Table 2). No $NAD(P)^+$-dependent DL-4-hydroxymandelate dehydrogenase or oxidase was present in DL-4-hydroxymandelate-grown MW27 cells. A high transaminase activity for both D- and L-4-HPG was also measured in cells grown on 4-hydroxyphenylglyoxylate, however no DL-4-hydroxymandelate dehydrogenase activity was present in such cells (Table 2). The DL-4-hydroxymandelate dehydrogenase, both 4-HPG transaminases, the 4-hydroxyphenylglyoxylate decarboxylase, and the $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenase were not synthesized during growth on 4-hydroxybenzaldehyde, 4-hydroxybenzoate and succinate (Table 2). The activity of the PMS-dependent 4-hydroxybenzaldehyde dehydrogenase, on the other hand, was not markedly depended upon the growth substrate.

DISCUSSION

Recently we have reported on a D-4-HPG-degrading *Pseudomonas putida* sp. (van den Tweel et al., 1988b). This organism, strain LW-4, converted D-4-HPG by means of an enantioselective transaminase to 4-hydroxyphenylglyoxylate which in turn was further oxidized via 4-hydroxybenzaldehyde and 4-hydroxybenzoate to protocatechuate. L-4-HPG was not metabolized by LW-4. The here presented results demonstrate that seven of the thirteen strains isolated on D-4-HPG strongly resemble *Pseudomonas putida* LW-4 in that only the D-stereoisomer of 4-HPG supported growth and that an enantioselective transaminase was responsible for the initial step in D-4-HPG metabolism. The other six isolates, on the other hand, metabolized both enantiomers of 4-HPG and in case of both isomers the initial step was a transamination.

Pseudomonas putida MW27 was one of the isolates which degraded both stereoisomers of 4-HPG. Both enantiomers of 4-HPG were degraded via a route identical to the one described for D-4-HPG in *Pseudomonas putida* LW-4 (Tables 1 and 2). D-4-HPG as well as L-4-HPG were initially metabolized by enantioselective transaminases as was shown by separation of the activities by ammonium sulfate fractionation (Figure 3).

The degradation of DL-4-hydroxymandelate by microorganisms is

well-documented and in some bacteria certain enzymes responsible for DL-4-hydroxymandelate degradation are co-ordinately controlled (Stevenson & Mandelstam, 1965; Rosenberg, 1971; Livingstone & Fewson, 1972). In *Pseudomonas putida* MW27 both transaminases were also synthesized during growth on DL-4-hydroxymandelate and 4-hydroxyphenylglyoxylate (Table 2), indicating that these transaminases might be co-ordinately regulated together with specific enzymes responsible for DL-4-hydroxymandelate and 4-hydroxyphenylglyoxylate degradation. Both enantiomers of 4-HPG and 4-hydroxyphenylglyoxylate, however, did not elecit DL-4-hydroxymandelate dehydrogenase synthesis (Table 2). Similar results were obtained for L-mandelate degradation in a *Pseudomonas aeruginosa* strain: after growth on phenylglyoxylate no L-mandelate dehydrogenase was present (Rosenberg, 1971). In *Pseudomonas putida* A.3.12, on the other hand, the 4-hydroxymandelate dehydrogenase was also induced during growth on 4-hydroxyphenylglyoxylate (Hegeman, 1966a; Stevenson & Mandelstam, 1965). Simultaneously with both transaminases and the 4-hydroxyphenylglyoxylate decarboxylase also a $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenase was present in cell extracts of MW27 (Table 2). Moreover, the differential rates of the activities of these four enzymes were proportional to one another during growth on various substrates. During growth on 4-hydroxybenzaldehyde, 4-hydroxybenzoate and succinate the four enzymes were not synthesized (Table 2). These preliminary results indicate that the D-4-HPG and L-4-HPG transaminase, the 4-hydroxyphenylglyoxylate decarboxylase and the $NADP^+$-dependent 4-hydroxybenzaldehyde dehydrogenase seem to be co-ordinately regulated. However, in order to obtain more conclusive data, experiments with gratuitous inductors (Hegeman, 1966a; Livingstone & Fewson, 1972), repressors (Mandelstam & Jacoby, 1965) and/or mutants (Hegeman, 1966b,c; Livingstone & Fewson, 1972) need to be done.

In contrast to other *Pseudomonas putida* spp. no NAD^+-dependent 4-hydroxybenzaldehyde dehydrogenase was detected (Stevenson & Mandelstam, 1965; van den Tweel et al., 1988b). Also in this respect, MW27 resembled the forementioned *Pseudomonas aeruginosa* strain (Rosenberg, 1971). In addition to the $NADP^+$-dependent dehydrogenase MW27 possessed a constitutive PMS-dependent 4-hydroxybenzaldehyde dehydrogenase (Table 2). Obviously, during growth on 4-hydroxybenzaldehyde the PMS-dependent enzyme takes care of the initial oxidation of 4-hydroxybenzaldehyde, since the $NADP^+$-dependent aldehyde dehydrogenase was not synthesized (Table 2). The presence of this constitutive enzyme also explains why 4-hydroxybenzoate-grown cells readily oxidized 4-hydroxybenzaldehyde (Table 1). Succinate-grown cells, on the other hand, also possess this enzyme but the cells can not further oxidize the formed 4-hydroxybenzoate; consequently less

oxygen is consumed (Table 1). A similar phenomenon has been observed in the well-studied *Pseudomonas putida* A.3.12 (Stevenson & Mandelstam, 1965). In the presence of 4-hydroxybenzaldehyde the $NAD(P)^+$-dependent benzaldehyde dehydrogenase was repressed, while whole cells still readily oxidized 4-hydroxybenzaldehyde. Surprisingly however, no additional aldehyde dehydrogenase was detected in cell extracts. On basis of their results Stevenson and Mandelstam (1965) concluded that the additional benzaldehyde dehydrogenase was destroyed during preparation of the cell extract. Recent studies with *Pseudomonas putida* A.3.12, however, have revealed that also in this strain a PMS-dependent enzyme is present (van den Tweel & Hens, unpublished results). *Pseudomonas putida* LW-4 contained a similar PMS-dependent aldehyde dehydrogenase (van den Tweel et al., 1988b).

ACKNOWLEDGEMENT

This investigation was financially supported by Andeno B.V. and by the Netherlands Technology Foundation (STW).

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

Boesten, W.H.J. & Meyer-Hoffman, L.R.M. (1975). Enzympreparaat met aminopeptidase activiteit. Dutch Patent Application 7,513,551

Cecere, F., Galli, G. & Morisi, F. (1975). Substrate and steric specificity of hydropyrimidine hydrase. FEBS Letters 57,192-194

Cole, M. & Utting, K. (1974). Enzymic resolution of racemic N-acyl-DL-amino acids. Britisch Patent 1,369,462

Hegeman, G.D. (1966a). Synthesis of the enzymes of the mandelate pathway by Pseudomonas putida. I. Synthesis of enzymes by the wild type. Journal of Bacteriology 91,1140-1154

Hegeman, G.D. (1966b). Synthesis of the enzymes of the mandelate pathway by Pseudomonas putida. II. Isolation and properties of blocked mutants. Journal of Bacteriology 91,1155-1160

Hegeman, G.D. (1966c). Synthesis of the enzymes of the mandelate pathway by Pseudomonas putida. III. Isolation and properties of constitutive mutants. Journal of Bacteriology 91,1161-1167

Hotta, S.S. (1968). Oxidative metabolism of isolated brain mitochondria: changes caused by aminooxyacetate. Archives of Biochemistry and Biophysics 127,132-139

Kennedy, S.I.T. & Fewson, C.A. (1968). Metabolism of mandelate and related compounds by bacterium NCIB 8250. Journal of General Microbiology 53,259-273

Livingstone, A. & Fewson, C.A. (1972). Regulation of the enzymes converting L-mandelate into benzoate in Bacterium N.C.I.B. 8250. Biochemical Journal 130,937-946

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,1235-1240

Mandelstam, J. & Jacoby, G.A. (1965). Induction and multi-sensitive end-product repression in the enzymic pathway degrading mandelate in Pseudomonas fluorescens. Biochemical Journal 94,569-577

Mayfield, C.I. & Inniss, W.E. (1977). A rapid, simple method for staining bacterial flagella. Canadian Journal of Microbiology 23,1311-1313

Rosenberg, S.L. (1971). Regulation of the mandelate pathway in Pseudomonas aeruginosa. Journal of Bacteriology 108,1257-1269

Schutt, H. (1981). Stereoselective resolution of phenylglycine derivatives and 4-hydroxyphenylglycine derivatives with enzyme resins. United States Patent 4,260,684

Stevenson, I.L. & Mandelstam, J. (1965). Induction and multi-sensitive end-product repression in two converging pathways degrading aromatic substances in Pseudomonas fluorescens. Biochemical Journal 96,354-362

van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1986). Microbial metabolism of D- and L-phenylglycine by Pseudomonas putida LW-4. Archives of Microbiology 144,169-144

van den Tweel, W.J.J. & de Bont, J.A.M. (1987). Metabolism of both stereoisomers of phenylglycine by different routes in Flavobacterium F24. Journal of General Microbiology 133,745-754

van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1988a). Catabolism of DL-α-phenylhydracrylic, phenylacetic and 3- and 4-hydroxyphenylacetic acid via homogentisic acid in a Flavobacterium sp. Archives of Microbiology 149,207-213

van den Tweel, W.J.J., Smits, J.P., Ogg, R.L.H.P. & de Bont, J.A.M. (1988b). The involvement of an enantioselective transaminase in the metabolism of D-3- and D-4-hydroxyphenylglycine in Pseudomonas putida LW-4. Submitted for publication

Chapter 6

DEGRADATION OF 4-HYDROXYPHENYLACETATE BY *XANTHOBACTER* 124X
Physiological resemblance with other Gram-negative bacteria

W.J.J. van den Tweel, R.J.J. Janssens and J.A.M. de Bont

SUMMARY

Xanthobacter 124X when grown on 4-hydroxyphenylacetate was able to hydroxylate this compound yielding homogentisate. Ring fission of this latter compound gave maleylacetoacetate which was isomerized to fumarylacetoacetate. The isomerase involved resembled maleylacetoacetate isomerases in Gram-negative bacteria in that glutathione was required for activity. Fumarate and acetoacetate were both detected as products of the hydrolysis of fumarylacetoacetate.

Antonie van Leeuwenhoek (1986) 52,309-318

INTRODUCTION

Members of the genus *Xanthobacter* grow on a wide variety of compounds (Wiegel et al., 1978) including unusual substrates as for instance gaseous alkenes (van Ginkel & de Bont, 1986), halogenated aliphatic compounds (Janssen et al., 1985) and cyclohexane (Trower et al., 1985). However, the ability of this genus to degrade aromatic compounds has not been investigated. Recently, we have isolated a *Xanthobacter* sp. on styrene and this organism grew on several other aromatic compounds including 4-hydroxphenylacetate (4-HPA). We decided to study the metabolism of this compound by our isolate since nothing is known about the metabolic pathways for the degradation of aromatics in these organisms. Another important reason for this study is our ultimate goal to apply microbial hydroxylation reactions to synthesize valuable hydroxylated aromatic compounds (van den Tweel et al., 1986).

Microorganisms may degrade 4-HPA along two different metabolic routes. One route involves the introduction of a hydroxyl group ortho to the first hydroxyl group yielding 3,4-dihydroxyphenylacetate (homoprotocatechuate). Subsequently, the benzene nucleus of homoprotocatechuate may be cleaved by a dioxygenase either between C2 and C3 (Adachi et al., 1964; Blakley et al., 1967; Sparnins et al., 1974) or between C4 and C5 (Dagley & Wood, 1965). Alternately, Chapman and Dagley (1962) have presented evidence for a hydroxylation of 4-HPA at 1-position to give 2,5-dihydroxyphenylacetate (homogentisate). Ring fission of this latter compound resulted in a formation of maleylacetoacetate (Chapman & Dagley, 1962). This ring-fission product is either hydrolysed directly yielding maleate and acetoacetate (Crawford, 1976) or is isomerized into fumarylacetoacetate which in turn yields fumarate and acetoacetate (Chapman & Dagley, 1962). This isomerization may or may not be glutathione-(GSH)-dependent as demonstrated recently by Hagedorn and Chapman (1985). The Gram-positive bacteria tested by these authors possessed a GSH-independent maleylacetoacetate isomerase while the tested Gram-negative bacteria had a glutathione-dependent enzyme.

In the present paper we describe the degradation of 4-HPA by our Gram-negative isolate designated *Xanthobacter* 124X.

MATERIALS AND METHODS

Isolation procedure and growth conditions. For enrichment, a mineral salts medium (van den Tweel et al., 1986) was used to which styrene (1 g l^{-1}) was added. A sewage sample was used as inoculum. After several weeks material taken from the enrichment culture was

streaked onto agar plates containing the same mineral salts medium. These plates were placed in a desiccator (10 l) to which styrene (150 µl) was added. A yellow organism, strain 124X, was isolated. It was able to use styrene as sole carbon and energy source. This organism was maintained on agar slopes of 5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract medium to which Oxoid no. 3 agar (15 g l^{-1}) had been added. Strain 124X was routinely grown at 30°C and at pH 7.0 in a chemostat at a dilution rate of 0.04 h^{-1} under carbon-limited conditions. The above mentioned mineral salts medium was used and carbon sources were added at a concentration of 1 g l^{-1}.

Suspensions of washed cells and cell-free extracts. Cells were harvested by centrifugation (16,000 **g** for 10 min at 4°C), washed with potassium phosphate buffer pH 7.0 (50 mM) and resuspended in the same buffer. For the preparation of cell-free extracts, cells were disrupted by ultrasonic disintegration (12x15 s). The resulting homogenate was centrifuged at 27,000 **g** for 15 min at 4°C and the supernatant, containing 10-20 mg protein ml^{-1}, was the crude cell-free extract.

Oxidation of substrates by washed cells. Oxidation of various possible intermediates was assayed by measuring the rate of disappearance of these compounds when given as a substrate by means of high-performance liquid chromatography (HPLC). The reaction mixture (total volume 10 ml) contained: washed cells, 30 µmol substrate, and 500 µmol potassium phosphate buffer pH 7.0. The reaction tubes were incubated in a shaking water bath (30°C, 1 Hz). Samples, taken at intervals, were separated on a CP-Spher Si-column (Chrompack, Middelburg, The Netherlands) and detected at 220 nm by means of a Perkin-Elmer variable-wavelength detector. As an eluent 50 mM potassium phosphate buffer pH 7.0 was used. 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.

Analyses. The yellow pigment was extracted with acetone and analysed by the procedure of Hertzberg et al. (1976). The presence of mycolic acids was investigated as described by Minnikin et al. (1975). *Xanthobacter autotrophicus* JW33 (DSM 1618), *Mycobacterium fortuitum* and *Nocardia vaccinii* were used as reference strains. Acetoacetate was determined by coupling with diazotized p-nitroaniline to give a coloured substituted formazan (Walker, 1954). The amount of glutathione present in cell extracts was determined by the procedure of Fahey et al. (1978) with glutathione reductase. Protein contents of crude cell-free extracts and whole cells were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Fumarate and maleate were analysed by HPLC at 206 nm using an Organic Acid Column (Chrompack, Middelburg, The Nether-

lands). The column temperature was 65°C and as an eluent 0.01 N H_2SO_4 was used. Under these conditions maleate and fumarate had retention times of 5.28 and 7.62 min, respectively.

Enzyme assays. All assays were performed at 30°C.

4-HPA hydroxylase. Activity of this enzyme was assayed spectrophotometrically by following at 340 nm the rate of oxidation of NADPH. Reactions were performed in 1.0 ml 50 mM Tris/HCl buffer pH 8.0 containing crude cell-free extract and 0.1 μmol NADPH. The reaction was initiated by adding 1 μmol 4-HPA.

Homogentisate dioxygenase. This assay also was done spectrophotometrically by measuring the formation of maleylacetoacetate at 330 nm ($\epsilon_{330}=1.4\cdot10^4$ l mol^{-1} cm^{-1}; Knox & Edwards, 1955). The reaction mixture (total volume 1.0 ml) contained 50 μmol potassium phosphate buffer pH 7.0 and crude cell-free extract. The reaction was started by the addition of 0.1 μmol homogentisate.

Maleylacetoacetate isomerase. After completion of the homogentisate dioxygenase assay this enzyme was assayed for by the addition of 0.1 μmol glutathione resulting in a decrease of maleylacetoacetate.

Chemicals. Glutathione reductase, glutathione, flavine adenine dinucleotide, NAD(P)H and dithiothreitol were obtained from Boehringer (Mannheim, FRG). Homogentisate, 2,2-dipyridyl, tiron and N-ethylmaleimide were products of Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). All other chemicals were of the highest grade commercially available.

RESULTS

Characterization of strain 124X

Strain 124X formed slimy, yellow rounded colonies when grown on nutrient agar or on solid medium with 4-HPA as the carbon source. The yellow color was due to the presence of zeaxanthin dirhamnoside as was shown by extracting this water-insoluble pigment with acetone and comparing its spectrum (λ_m: [429], 454 and 481 nm) with the spectrum of the carotenoid of *Xanthobacter autotrophicus*, formerly *Corynebacterium autotrophicum* (Hertzberg et al., 1976). The cell morphology of strain 124X was similar to *Xanthobacter* cells (Malik & Claus, 1979; Wiegel et al., 1978) in that cells showed irregularly shaped rods, some branched with typical swollen ends. Strain 124X was non-motile, oxidase positive and the Gram stain was positive. No mycolic acids were detected in strain 124X or in the type strain *Xanthobacter autotrophicus* JW33; the reference strains *Nocardia vaccinii* and *Mycobacterium fortuitum* possessed several mycolic acids. Atmospheric nitrogen was fixed in nitrogen deficient media at a

Table 1. The ability of Xanthobacter 124X, Xanthobacter autotrophicus JW33 and Xanthobacter PY2 to grow on various compounds as sole carbon and energy source.

Compound	Strain		
	124X	JW33	PY2
Methanol	+	+	+
Ethanol	+	+	+
1-Propanol	+	+	+
Propene	–	–	+
H_2/CO_2	+	+	+
Styrene	+	–	–
Benzene	–	–	–
Ethylbenzene	+	–	–
Toluene	+	–	–
2-Phenylethanol	+	+	+
Phenylacetate	+	+	+
2-HPA	–	–	–
3-HPA	–	–	–
4-HPA	+	+	+
DL-Phenylalanine	–	–	–
DL-Histidine	–	–	–
D-Phenylglycine	–	–	–
Benzoate	–	–	–
2-Phenylpropionate	–	–	–
3-Phenylpropionate	–	–	–
DL-Mandelate	–	–	–
Maleate	–	–	–

+, growth. –, no growth.

reduced oxygen level. On the basis of these properties strain 124X was assigned to the genus *Xanthobacter*.

Table 1 shows the ability of *Xanthobacter* 124X, *Xanthobacter autotrophicus* JW33 (DSM 1618) and *Xanthobacter* PY2 (van Ginkel & de Bont, 1986) to grow on various carbon sources. Only *Xanthobacter* 124X was able to use styrene, toluene and ethylbenzene as sole carbon and energy source. Surprisingly, all three strains grew on 2-phenylethanol, phenylacetate and 4-HPA. Both strain 124X and JW33 were unable to grow on propene as sole carbon and energy source.

Oxidation and accumulation of metabolites by whole cells

Washed cell suspensions of *Xanthobacter* 124X, after growth on 4-HPA, metabolized 4-HPA and homogentisate with activities of 80 and 15 nmol min^{-1} (mg protein)$^{-1}$, respectively. Homoprotocatechuate, 2- and 3-HPA were not metabolized by these cells. In the presence of 10

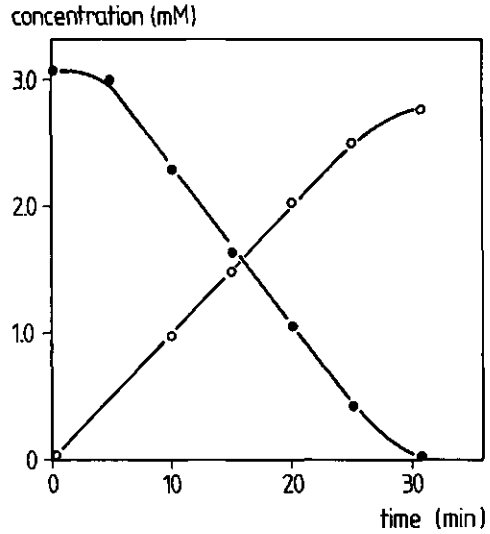

Figure 1. Conversion of 4-HPA (•) into homogentisate (○) by washed cells of 4-HPA-grown Xanthobacter 124X. The assay mixture (total volume 10 ml) contained: washed cells (7.0 mg protein), 30 μmol 4-HPA, 100 μmol 2,2-dipyridyl and 500 μmol potassium phosphate buffer pH 7.0.

mM 2,2-dipyridyl, a Fe^{2+}-complexing agent, 4-HPA was almost stoichiometrically hydroxylated to homogentisate (Figure 1). These results suggest that 4-HPA is oxidized through the homogentisate pathway of metabolism.

4-HPA hydroxylase

The incubation of crude cell-free extracts with NADPH and 4-HPA demonstrated a 4-HPA-dependent NADPH oxidation (Table 2). Maximal activity was found using a 50 mM Tris/HCl buffer, pH 8.0. The addition of Mg^{2+} (2 mM), flavine adenine dinucleotide (5 μM) or dithiothreitol (1 mM) did not improve the recoverable activity. No activity was observed when NADH was used instead of NADPH as the electron donor.

Homogentisate dioxygenase

Dialysed cell-free extracts of *Xanthobacter* 124X grown on 4-HPA readily oxidized homogentisate giving a ring-fission product with spectral characteristics identical with those established for maleylacetoacetate (Knox & Edwards, 1955) with a maximum at 330 nm at pH 13, and disappearing on acidification. Using published extinction coefficients (Knox & Edwards, 1955), an almost stoichiometric conversion of homogentisate to maleylacetoacetate was observed (Figure 2).

Table 2. Enzyme activities[a] in crude cell-free extracts of Xanthobacter 124X cells grown on 4-HPA or on succinate.

Enzyme activity assayed	Growth substrate	
	4-HPA	Succinate
4-HPA-1-hydroxylase[b]	15	0
Homogentisate dioxygenase[c]	100	0
Maleylacetoacetate isomerase[d]	120	

[a] Activities are expressed in nmol min^{-1} (mg protein)$^{-1}$ and are the average of at least two separate extracts.

[b] Rate of 4-HPA-dependent oxidation of NADPH.

[c] Rate of homogentisate dependent uptake of oxygen.

[d] Decrease of maleylacetoacetate at 330 nm after addition of GSH.

Fe^{2+} was required for dioxygenase activity and maximal homogentisate dioxygenase activity was measured at pH 7.5 in the presence of 0.5 mM $FeSO_4$. 2,2-Dipyridyl (1.0 mM) completely inhibited enzyme activity while tiron (4,5-dihydroxy-m-benzenedisulfonate; 10 mM), an Fe^{3+}-chelating agent, had almost no effect on dioxygenase activity.

Glutathione-dependent maleylacetoacetate degradation

Further degradation of maleylacetoacetate was dependent upon the addition of reduced glutathione indicating that a GSH-dependent isomerase is present in this *Xanthobacter* species (Figure 2). In addition, N-ethylmaleimide (NEM), a glutathione trapping agent (Crawford & Frick, 1977), completely inhibited the GSH-dependent degradation of maleylacetoacetate; this inhibition could be overcome by adding GSH in slight excess over NEM (Figure 2B).

Fumarate was identified by HPLC as a product of homogentisate oxidation by crude cell-free extracts of *Xanthobacter* 124X in the presence of glutathione. When a crude cell-free extract (2.8 mg protein) was incubated with 0.4 mM homogentisate, an accumulation of 0.04 mM fumarate was observed. Maleate was not detected. During this incubation, acetoacetate accumulated as was shown qualitatively using the diazo-coupling method as described by Walker (1954).

Fumarate was metabolized at a rate of 80 nmol min^{-1} (mg protein)$^{-1}$ by a crude cell-free extract of 4-HPA-grown cells. Maleic acid, when included in cell-free extracts of cells grown on either 4-HPA or succinate, was slowly metabolized at a rate of 5 nmol min^{-1} (mg protein)$^{-1}$ and no fumarate formation was observed during such incubations.

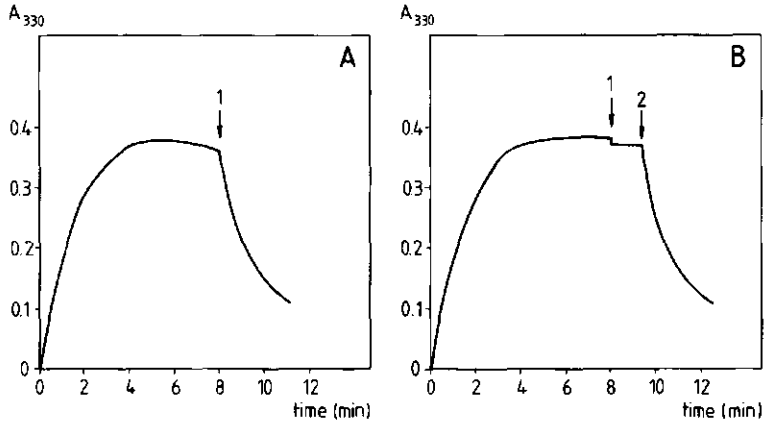

Figure 2. **Spectral changes during the oxidation of homogentisate by a crude cell-free extract of 4-HPA-grown Xanthobacter 124X in the absence (A) and in the presence (B) of NEM (1.0 μmol). The reactions were carried out in 1.0 ml 50 mM potassium phosphate buffer pH 7.0 containing 0.2 mg protein. Reactions were initiated by the addition of 0.03 μmol homogentisate. At the times indicated by the arrows, the following additions were made: 1, 0.1 μmol GSH; 2, 1.0 μmol GSH. Reference cuvettes received all additions except homogentisate. A, absorbance.**

Glutathione content of 4-HPA-grown Xanthobacter cells

Examining the glutathione content of *Xanthobacter* 124X cells was of interest since Hagedorn and Chapman (1985) have correlated the presence of a glutathione-dependent maleylacetoacetate isomerase in Gram-negative organisms with the presence of glutathione in extracts of these organisms. Using the procedure of Fahey et al. (1978), it was shown that *Xanthobacter* 124X, grown on 4-HPA, possessed a readily detectable level of glutathione (3.9 μmol of GSH + $^{1}/_{2}$GSSG [g dry mass]$^{-1}$).

Induction of the enzymes catalysing 4-HPA catabolism

A comparison of the activities of some enzymes responsible for 4-HPA metabolism in crude cell-free extracts of *Xanthobacter* 124X grown upon either 4-HPA or succinate is given in Table 2. These results demonstrate that both the 4-HPA-hydroxylase and homogentisate dioxygenase are inducible.

DISCUSSION

Strain 124X was identified as a *Xanthobacter* species according to cell morphology, mycolic acid content, pigmentation, the ability to fix

molecular nitrogen and the pattern of substrates utilized. This genus is considered Gram-type-negative on the basis of the ultrastructure of the cell wall, although Gram stains are often positive or variable (Wiegel, 1981). Our species also showed a positive Gram stain.

So far, the ability of *Xanthobacter* spp. to grow on aromatic compounds has not been investigated. Out of 35 strains tested by Wiegel et al. (1978) only one strain grew on benzoate, one on mandelate and one on phenylalanine. The type strain JW33 as well as PY2 and 124X were all able to grow on 2-phenylethanol, phenylacetate and 4-HPA. However, only strain 124X was able to use styrene, toluene and ethylbenzene as sole carbon and energy source. None of these strains was able to grow on benzene. The *Xanthobacter* described by Trower et al. (1985) did not grow on either toluene or benzene. *Xanthobacter autotrophicus* GJ10 (Janssen et al., 1985), however, also

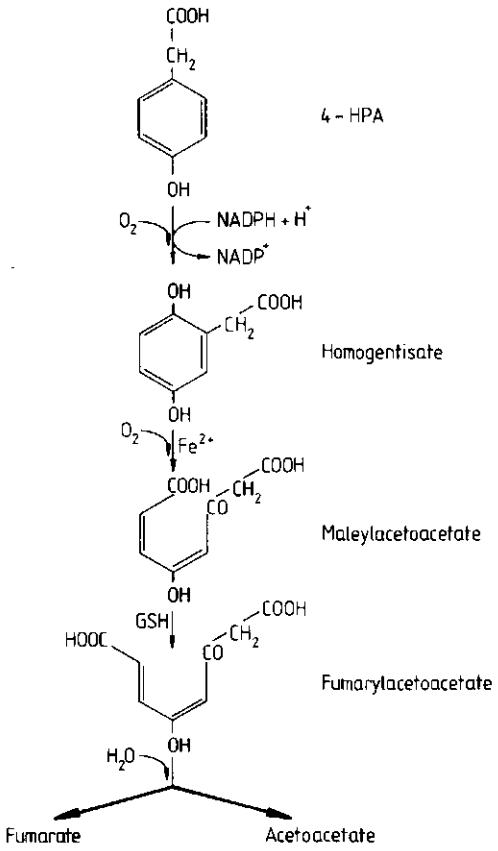

Figure 3. Pathway of 4-HPA degradation in Xanthobacter 124X.

was able to use toluene as sole carbon and energy source while benzene did not support growth. In *Xanthobacter* 124X, the initial reaction in the 4-HPA metabolism was a NADPH-dependent hydroxylation yielding homogentisate. In the presence of 2,2-dipyridyl an almost stoichiometrical bioconversion of 4-HPA into homogentisate was obtained. The benzene nucleus of homogentisate was cleaved by a Fe^{2+}-dependent dioxygenase. According to its spectral characteristics, the fission product was identified as maleylacetoacetate. Further metabolism of maleylacetoacetate was dependent upon the addition of GSH, this in contrast to the GSH-independent maleylacetoacetate hydrolase in *Bacillus* B11c (Crawford, 1976) and GSH-independent maleylacetoacetate isomerases in some Gram-positive organisms (Hagedorn & Chapman, 1985).

Both fumarate and acetoacetate were detected as products of the metabolism of homogentisate by crude cell-free extracts of *Xanthobacter* 124X grown on 4-HPA while no maleate formation was detected. These results indicate that maleylacetoacetate is isomerized GSH-dependently to fumarylacetoacetate which is hydrolysed yielding fumarate and acetoacetate. That maleate is no intermediate being rapidly isomerized to fumarate, was confirmed, since maleate was metabolized much slower than fumarate. Moreover, in extracts no fumarate accumulated from maleate. Since extracts of cells grown on succinate also metabolized maleate, it is likely that this compound is not specifically involved in 4-HPA catabolism.

The involvement of a GSH-dependent isomerase resembled the degradation of homogentisate in other Gram-negative bacteria (Hagedorn & Chapman, 1985). Cells of *Xanthobacter* 124X also resembled Gram-negative organisms in that they contained a readily detectable amount of GSH. The level of GSH in Gram-positive organisms has been shown to be much lower (Fahey et al., 1978; Hagedorn et al., 1985). From the results presented, a degradation route as given in Figure 3, is proposed for 4-HPA catabolism in *Xanthobacter* 124X.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. A. Bruggink (Océ-Andeno B.V.), Prof. C.J.E.A. Bulder, Prof. K. van 't Riet and Dr. J. Tramper for advice in preparing the manuscript. *Mycobacterium fortuitum* and *Nocardia vaccinii* were a generous gift of Prof. D. Jones. These investigations were supported in part by the Netherlands Technology Foundation (STW).

REFERENCES

Adachi, K., Takeda, Y., Senoh, S. & Kita, H. (1964). Metabolism of p-hydroxyphenylacetic acid in Pseudomonas ovalis. Biochimica et Biophysica Acta 93,483-493

Blakley, E.R., Halvorson, H. & Kurz, W. (1967). The microbial production and some characteristics of δ-carboxymethyl-α-hydroxymuconic semialdehyde. Canadian Journal of Microbiology 13,159-165

Chapman, P.J. & Dagley, S. (1962). Oxidation of homogentisic acid by cell-free extracts of a vibrio. Journal of General Microbiology 28,251-256

Crawford, R.L. (1976). Degradation of homogentisate by strains of Bacillus and Moraxella. Canadian Journal of Microbiology 22,276-280

Crawford, R.L. & Frick, T.D. (1977). Rapid spectrophotometric differentiation between glutathione-dependent and glutathione-independent gentisate and homogentisate pathways. Applied and Environmental Microbiology 34,70-174

Dagley, S. & Wood, J.M. (1965). Oxidation of phenylacetic acid by a Pseudomonas. Biochimica et Biophysica Acta 99,383-385

Fahey, R.C., Brown, W.C., Adams, W.B. & Worsham, M.B. (1978). Occurrrence of glutathione in bacteria. Journal of Bacteriology 133,1126-1129

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

Hertzberg, S.H., Borch, G. & Liaaen-Jensen, S. (1976). Bacterial carotenoids. Absolute configuration of zeaxanthin dirhamnoside. Archives of Microbiology 110,95-99

Janssen, D.B., Scheper, A., Dijkhuizen, L. & Witholt, B. (1985). Degradation of halogenated aliphatic compounds by Xanthobacter autotrophicus GJ10. Applied and Environmental Microbiology 49,673-677

Knox, W.E. & Edwards, S.W. (1955). The properties of maleylacetoacetate, the initial product of homogentisate oxidation in liver. Journal of Biological Chemistry 216,489-498

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Malik, K.A. & Claus, D. (1979). Xanthobacter flavus, a new species of nitrogen-fixing hydrogen bacteria. International Journal of Systymatic Bacteriology 29,283-287

Minnikin, D.E., Alshamaony, L. & Goodfellow, M. (1975). Differentation of Mycobacterium, Nocardia and related taxa by thin-layer chromatography analysis of whole-organism methanolysates. Journal of General Microbiology 88,200-204

Sparnins, V.L., Chapman, P.J. & Dagley, S. (1974). Bacterial degradation of 4-hydroxyphenylacetic acid and homoprotocatechuic acid. Journal of Bacteriology 120,159-167

Trower, M.K., Buckland, R.M., Higgins, R. & Griffin, M. (1985). Isolation and characterization of a cyclohexane-metabolizing Xanthobacter sp. Applied and Environmental Microbiology 49,1282-1289

van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1986). Microbial metabolism of D- and L-phenylglycine by Pseudomonas putida LW-4. Archives of Microbiology 144,169-174

van Ginkel, C.G. & de Bont, J.A.M. (1986). Isolation and characterization of alkene utilizing Xanthobacter spp. Archives of Microbiology 145,403-407

Walker, P.G. (1954). A colorimetric method for the estimation of acetoacetate. Biochemical Journal 58,699-704

Wiegel, J. (1981). Distinction between the Gram reaction and the Gram type of bacteria. International Journal of Systematic Bacteriology 31,88

Wiegel, J., Wilke, D., Baumgarten, J., Opitz, R. & Schlegel, H.G. (1978). Transfer of the nitrogen-fixing hydrogen bacterium Corynebacterium autotrophicum Baumgarten et al. to Xanthobacter gen. nov. International Journal of Systematic Bacteriology 28,573-581

Chapter 7

CATABOLISM OF DL-α-PHENYLHYDRACRYLIC, PHENYLACETIC AND 3- AND 4-HYDROXYPHENYLACETIC ACID VIA HOMOGENTISIC ACID IN A *FLAVOBACTERIUM* SP.

W.J.J. van den Tweel, J.P. Smits and J.A.M de Bont

SUMMARY

A degradation pathway for DL-α-phenylhydracrylic, phenylacetic, 3- and 4-hydroxyphenylacetic acid by a *Flavobacterium* is presented. Experiments with washed cells and enzyme studies revealed that DL-α-phenylhydracrylic acid in an initial reaction was oxidatively decarboxylated to phenylacetaldehyde. Whole cells oxidized both stereoisomers of phenylhydracrylic acid at different rates. The product phenylacetaldehyde in turn was oxidized to phenylacetic acid. No hydroxylation of phenylacetic acid was detected in cell extracts, but on the basis of experiments with washed cells it is assumed that phenylacetic acid is mainly metabolized via 3-hydroxyphenylacetic acid. This latter product was subsequently hydroxylated yielding the ring-cleavage substrate homogentisate. 4-Hydroxyphenylacetic acid was also degraded via homogentisate. Ringcleavage of homogentisate gave maleylacetoacetate which was further degraded through a glutathione-dependent pathway. Homoprotocatechuate was not an intermediate in the metabolism of DL-α-phenylhydracrylic, phenylacetic, 3- and 4-hydroxyphenylacetic acid metabolism, but it could be hydroxylated aspecifically to 2,4,5-trihydroxyphenylacetic acid by the action of the 3-hydroxyphenylacetic acid-6-hydroxylase.

Archives of Microbiology (1988) 149,207-213

INTRODUCTION

Certain hydroxylated derivatives of phenylacetic acid (PA) and of phenylmalonic acid (PMA) can be used as building blocks in the synthesis of semisynthetic antibiotics, analgesics and inflammation inhibitors (Roy & Bruggink, 1981; Bouchara, 1971) and we currently are investigating bacterial hydroxylation reactions that would produce such hydroxylated compounds from PA or PMA (van den Tweel et al., 1987). Such hydroxylation reactions are mediated by monooxygenases and these enzymes require reducing equivalents. To meet this demand for reducing power in an intact microorganism excreting hydroxylated products, it is necessary to supply the cell with a co-substrate. Alternatively, it would be possible to meet this demand by having a degradative pathway starting with a compound that is more reduced than PA or PMA and that is degraded via these acids to a desired hydroxylated product. Phenylhydracrylic acid (PHA) is such a compound and we therefore have isolated an organism on this compound and studied its metabolism that was found to proceed via PA but not via PMA.

PA has been recognized as an intermediate in the aerobic microbial metabolism of many other compounds as for instance phenylalanine (Kunita, 1956; Kishore et al., 1976; Lee & Desmazeaud, 1986; Yuasa et al., 1975; Pometto & Crawford, 1985), some ω-phenyl substituted fatty acids (Webley et al., 1955) and 1-phenylalkanes (Amund & Higgins, 1985; Sariaslani et al., 1974). Metabolism of PA has been studied in *Aspergilli* and pathways have mainly been based on the isolation of mono- and dihydroxyphenylacetic acids which accumulated in small amounts during incubations with PA. As early as 1951, Kluyver and Van Zijp observed the production of 2,5-dihydroxyphenylacetic acid (homogentisate) from PA by *Aspergillus niger*. Later on, it was proposed that some other *Aspergillus* spp. initiated PA degradation by hydroxylation to give 2-hydroxyphenylacetic acid (2-HPA) which was subsequently hydroxylated to homogentisate (Yuasa et al., 1975; Ueno et al., 1973; Bocks, 1967; Sugumaran & Vaidyanathan, 1978). Sugumaran et al. (1973), however, proposed a quite different pathway for PA metabolism in non-sporulating *Aspergillus niger* cells involving a hydroxylation to 3-hydroxyphenylacetic acid (3-HPA) which in turn was decarboxylated and oxidized yielding 3-hydroxybenzoic acid. Other *Aspergillus niger* cells metabolized PA via 4-hydroxyphenylacetic acid (4-HPA) to 4-hydroxymandelate (Kishore et al., 1976). 2,6-Dihydroxyphenylacetic acid has also been reported to accumulate from PA, however, its significance in the degradative pathway of PA metabolism is not known yet (Yoshizako et al., 1977). In contrast to fungal PA degradation, only one paper deals with the catabolism of PA in yeast. Anderson and Dagley (1980) have shown that *Trichosporon cutaneum*

metabolizes PA via 3-HPA to homogentisate. Information about bacterial PA metabolism is also scant. In 1955 Kunita has reported on the degradation of PA in two distinct *Pseudomonas fluorescens* strains. Based on simultaneous adaptation experiments and on the isolation of 4-HPA and 3,4-dihydroxyphenylacetic acid (homoprotocatechuate) he proposed that one strain oxidized PA to homoprotocatechuate through 4-HPA (Kunita, 1955a). From the adaptive pattern of the other strain, however, he concluded that PA in this organism was metabolized via 2-HPA to homogentisate (Kunita, 1955b). A similar pathway for PA degradation was proposed for a *Pseudomonas* sp. that was able to grow on styrene (Baggi et al., 1983), and also for a *Nocardia salmonicolor* (Sariaslani et al., 1974). However, in the latter strain PA catabolism might also occur via homoprotocatechuate. Another *Pseudomonas* sp. described by Blakley et al. (1967) metabolized PA again via 4-HPA to homoprotocatechuate.

Presently, only very little is known about the bacterial degradation of PHA. In the culture supernatant of a *Corynebacterium* growing on PHA both phenylacetaldehyde and PA have been detected (Niemer et al., 1959). Similar results were obtained with a *Pseudomonas putida*, which was also able to grow on PHA, in that some mutants of this strain converted PHA to PA (Stevens & Rörsch, 1971). In the present paper we report on the metabolism of DL-PHA in a *Flavobacterium* sp. as well as on the metabolism of PA by this microorganism and we furthermore discuss the formation and degradation of hydroxylated derivatives of PA by this bacterium.

MATERIALS AND METHODS

Media and culture conditions of strain JS-7. For isolation of strain JS-7 a mineral salts medium as described previously was used (van den Tweel et al., 1986a). The organism was maintained on agar slopes of 5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract medium to which Oxoid no. 3 agar (15 g l^{-1}) had been added. Strain JS-7 was routinely grown at 30°C and at pH 7.0 in a chemostat at a dilution rate of 0.04 h^{-1} under carbon-limited conditions. The above mentioned mineral salts medium was used and carbon sources were added at a concentration of 1 g l^{-1}.

Determination of the maximal growth rate. The doubling time of strain JS-7 on various substrates was determined by measuring the increase in turbidity with an EEL nephelometer (Evans Electroselenium, Ltd., Halstead, England). For this purpose Erlenmeyer flasks (250 ml) were equipped with a side arm to allow turbidity measurements at various time intervals.

Suspensions of cells and cell extracts. Suspensions of cells, cell

extracts and cofactor-free extracts were prepared as described previously (van den Tweel et al., 1986a).

Simultaneous adaptation experiments. Oxygen uptake by washed cell suspensions was done as described previously (van den Tweel et al., 1986a).

Oxidation of substrates by washed cells. Oxidation of various possible intermediates was assayed by measuring in time concentrations of these compounds by means of high-performance liquid chromatography (HPLC). Unless stated otherwise, the reaction mixture (total volume 5 ml) contained washed cells, 5 μmol substrate, 500 μg chloramphenicol and 250 μmol potassium phosphate buffer pH 7.0. The reaction tubes were incubated in a shaking water bath (30°C, 1 Hz). Samples, taken at intervals, were separated on a C-18 column (200x3 mm, Chrompack, Middelburg, The Netherlands). The mobile phase was methanol-potassium phosphate buffer 50 mM pH 7.0 (20:80 [v/v]), flow rate was 0.4 ml min^{-1}, and detection was by UV absorbance at 210 nm. Accumulating intermediates were identified by comparison of the retention times with authentic samples and by *in situ* scanning of the UV spectra after the flow had been stopped. Under these conditions the following retention times (min) were observed: DL-PHA, 3.45; PA, 4.38; 3-HPA, 3.03; 4-HPA, 2.78; homoprotocatechuate, 2.45 and homogentisate, 2.65.

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

Isolation of the product which accumulated during the incubation with homoprotocatechuate and mass spectrum analysis of this product. Cells grown on PA were incubated with 10 mM homoprotocatechuate in a 100 mM potassium phosphate buffer, pH 7.0. Cells were removed by centrifugation after 8 mM homoprotocatechuate had been consumed and the supernatant was acidified with concentrated HCl to pH 4.0 and extracted twice with an equal volume of ethylacetate. The combined ethylacetate extracts were dried over anhydrous Na_2SO_4, and the solvent was evaporated at reduced pressure. The mass spectrum of the residue was recorded on an AEI MS-9 mass spectrometer.

Enzyme assays. All assays were done at 30°C. Spectrophotometric assays were performed with a Perkin-Elmer 550A spectrophotometer. Oxygen uptake experiments were done with a YSI model 53 monitor equipped with a YSI 5331 polarographic oxygen probe (Yellow Springs Instruments Co., Yellow Springs, Ohio, USA). Activities are expressed as nmol substrate consumed min^{-1} (mg protein)$^{-1}$.

(i) DL-PHA dehydrogenase (NAD^+-dependent). The reaction mixture (total volume 1 ml) contained cell extract, 1 μmol NAD^+ and 100 μmol Tris/HCl, pH 9.0. The reaction was started by the addition of 1 μmol DL-PHA and the rate of NADH formation was recorded.

(ii) DL-PHA dehydrogenase (PMS-dependent). This enzyme was assayed by recording oxygen uptake. The reaction mixture (total volume 3 ml) contained cell extract, 0.33 μmol PMS and 150 μmol potassium phosphate buffer, pH 8.0. The reaction was initiated by the addition of 3 μmol DL-PHA.

(iii) DL-PHA oxidase and DL-PHA decarboxylase. To investigate whether a DL-PHA oxidase and/or decarboxylase was involved in DL-PHA metabolism, cell extracts were incubated with DL-PHA without cofactors added. The reaction mixture (total volume 5 ml) contained cell extract, 250 μmol potassium phosphate buffer pH 7.0 and 5 μmol DL-PHA. The decrease in DL-PHA concentration and formation of intermediates was measured by periodically withdrawing samples for HPLC analysis.

(iv) Phenylacetaldehyde dehydrogenase (NAD^+-dependent; EC 1.2.1.39). This enzyme was assayed by measuring phenylacetaldehyde-dependent NADH formation as described by Fujioka et al. (1970). As a buffer 50 mM Tris/HCl, pH 9.0 was used.

(v) Phenylacetaldehyde dehydrogenase (PMS-dependent). This enzyme activity was assayed for in a way similar to the PMS-dependent DL-PHA dehydrogenase assay. The reaction was initiated by the addition of 1.5 μmol phenylacetaldehyde. Rates were corrected for the autooxidation of phenylacetaldehyde in the presence of PMS.

(vi) 2-Phenylethanol dehydrogenase. The presence of a possible PMS-dependent 2-phenylethanol dehydrogenase was investigated using the assay as described for the PMS-dependent phenylacetaldehyde dehydrogenase, except that phenylacetaldehyde was replaced by 2-phenylethanol.

Whether or not a $NAD(P)^+$-dependent 2-phenylethanol dehydrogenase was involved was investigated by measuring a 2-phenylethanol-dependent NAD(P)H formation at 340 nm as described for the NAD^+-dependent phenylacetaldehyde dehydrogenase.

(vii) PA hydroxylase. Attempts to assay for PA hydroxylase activity were by measuring PA concentrations in various incubation mixtures. The standard mixture (total volume 5 ml) contained cell extract, 10 μmol NAD(P)H and 150 μmol potassium phosphate buffer, pH 7.0; various cofactors like flavine adenine dinucleotide (FAD; 0.05 μmol) and tetrahydropteridine (4 μmol), either in the presence or absence of Fe^{2+} (2.5 μmol), were added to this mixture.

(viii) 3-HPA-6-hydroxylase. Two assays for this enzyme were employed. In the spectrophotometric assay the initial rate of oxidation of NADH was determined from the decrease in absorbance at 340 nm. The reaction mixture (total volume 1 ml) contained cell extract, 0.01 μmol FAD, 50 μmol potassium phosphate buffer pH 7.0 and 0.5 μmol 3-HPA. Alternatively, consumption of O_2 as dependent on substrate and NAD(P)H was determined in a reaction mixture (3 ml)

that was the same as that used in the spectrophotometric assay.

(ix) 4-HPA-1-hydroxylase (EC 1.14.13.18). This enzyme was assayed using the same spectrophotometric procedure as described for the 3-HPA-6-hydroxylase, except that 4-HPA was used instead of 3-HPA.

(x) Homoprotocatechuate dioxygenase (EC 1.3.11.7 or EC 1.3.11.15). This dioxygenase activity was determined by measuring oxygen uptake. The reaction mixture (total volume 3 ml) contained cell extract and 150 μmol potassium phosphate buffer, pH 7.0. The reaction was started by adding 3 μmol homoprotocatechuate.

(xi) Homogentisate dioxygenase (EC 1.13.11.5). This enzyme was assayed as described previously (van den Tweel et al., 1986b).

Chemicals. DL-PHA (tropic acid, 99% pure), PA, 3- and 4-HPA, phenylmalonic acid, homogentisate, homoprotocatechuate, N-ethylmaleimide, 2,2-dipyridyl, ortho-phenanthroline and phenazine methosulphate were all products of Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Glutathione, FAD, chloramphenicol, $NAD(P)^+$ and NAD(P)H were obtained from Boehringer (Mannheim, FRG). Tetrahydropteridine (2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine hydrochloride) was a product of Janssen Chimica (Beerse, Belgium). All other chemicals were of commercially available analytical grade and were used without further purification.

RESULTS

Isolation and characterization of strain JS-7

For enrichment a mixture of various soil samples was incubated in the mineral salts medium with DL-PHA at a concentration of 1.0 g l^{-1}. After two weeks the enrichment culture, kept without shaking at 30°C in the dark, showed good growth. Material taken from this enrichment culture was streaked onto agar plates with the same medium composition, and three different organisms were isolated by selection of single colonies. One isolate, strain JS-7, was a bright yellow, Gram-negative rod measuring 0.5-0.8 x 1.1-1.8 μm. Transmission electron microscopic photographs showed that this motile strain possessed up to 5 polair flagella. It was unable to denitrify, was oxidase positive, and did not grow at 37°C. Gelatine and aesculin were not hydrolysed and no acid was formed from glucose. According to these results strain JS-7 was tentatively identified as a *Flavobacterium* species (Holmes et al., 1984).

Using DL-PHA as sole carbon and energy source, *Flavobacterium* JS-7 doubled in 5.5 h. On succinate, PA, 3- and 4-HPA doubling times of 2.5, 7.0, 6.5 and 6.5 h, respectively, were observed. Pyruvate, ace-

tate and glucose were also able to support growth. No growth was observed using 2-HPA, PMA, 2-phenylethanol, D-phenylglycine or D-4-hydroxyphenylglycine as potential growth substrates.

Simultaneous adaptation experiments

After growth on DL-PHA, washed cells of *Flavobacterium* JS-7 readily oxidized the growth substrate and also phenylacetaldehyde, PA and 3-HPA; however, other possible intermediates like 4-HPA, homogentisate, homoprotocatechuate, DL-mandelate and 2-phenylethanol were oxidized at a much lower rate (Table 1). PMA, another possible intermediate in DL-PHA catabolism, was not oxidized by these cells. When grown on PA, cells readily oxidized PA, 3- and 4-HPA, homogentisate and homoprotocatechuate. However, no oxidation of DL-PHA was observed; evidently growth on PA fails to induce enzymes responsible for initial attack upon DL-PHA. 3-HPA-grown cells oxidized 3- and 4-HPA, homogentisate and homoprotocatechuate, this in contrast to 4-HPA-grown cells which showed very low oxidation rates with 3-HPA and homoprotocatechuate (Table 1).

Table 1. Rates[a] of oxygen uptake by washed cell suspensions of Flavobacterium JS-7 grown on various carbon sources.

Substrate[b]	Carbon source for growth				
	DL-PHA	PA	3-HPA	4-HPA	Succinate
DL-PHA	22	< 1	< 1	< 1	< 1
Phenylacetaldehyde	41	10	15	10	10
PA	25	41	< 1	< 1	< 1
2-HPA	< 1	5	5	< 1	< 1
3-HPA	25	51	50	3	< 1
4-HPA	7	16	38	49	< 1
Homogentisate	9	27	15	8	< 1
Homoprotocatechuate	2	25	15	< 1	< 1
DL-Mandelate	5	< 1	5	< 1	ND[c]
2-Phenylethanol	5	3	< 1	< 1	< 1
Succinate	15	19	42	32	55

[a] Rates of oxygen uptake are corrected for endogenous oxygen uptake and are expressed in nmol O_2 consumed min^{-1} (mg protein)$^{-1}$

[b] No activity [< 1 nmol min^{-1} (mg protein)$^{-1}$] was also observed with the following substrates: PMA, phenylglyoxylate, catechol, 2,3-dihydroxybenzoate, gentisate and protocatechuate

[c] ND: not determined

Oxidation and accumulation of metabolites by washed cell suspensions

In addition to the foregoing oxygen uptake experiments, the metabolism of DL-PHA, PA, 3- and 4-HPA, and metabolites thereof, was also tested directly by measuring the disappearance of the respective substrates from incubation mixtures by means of HPLC (Table 2). Initial experiments showed that during prolonged incubations (>0.5 h) *de novo* enzyme synthesis readily occurred resulting in unreliable data. To overcome this disadvantage all incubation experiments were performed in the presence of chloramphenicol. Cells grown on DL-PHA metabolized DL-PHA, PA, 3-HPA and homogentisate while 4-HPA and homoprotocatechuate were also metabolized albeit at much lower rates (Table 2). PMA and 2-phenylethanol were not oxidized, indicating that neither an initial decarboxylation of PHA yielding 2-phenylethanol, nor an oxidation route via PMA is involved in DL-PHA catabolism in *Flavobacterium* JS-7.

Racemic PHA was readily metabolized until approximately 50% of the substrate was metabolized; at that point the metabolism of PHA significantly slowed down (Figure 1). During this incubation a transient accumulation of PA was observed, reaching a maximum at the moment the rate of PHA catabolism decreased (Figure 1). Identical results were obtained when the initial PHA concentration was doubled, indicating that this phenomenon might be caused by differences in oxidation rates of the enantiomers of PHA rather than by a K_m effect.

Table 2. Consumption rates[a] of possible intermediates of DL-PHA, PA, 3- and 4-HPA by Flavobacterium JS-7 cells grown on various substrates.

Substrate[b]	Growth substrate				
	DL-PHA[c]	PA	3-HPA	4-HPA	Succinate
DL-PHA	15	<0.5	<0.5	<0.5	<0.5
PA	9	11	<0.5	<0.5	<0.5
3-HPA	9.5	18	12	<0.5	<0.5
4-HPA	1	2.5	12	12	<0.5
Homogentisate	3	7	4	4	<0.5
Homoprotocatechuate	1	5	2	<0.5	<0.5

[a] Rates are the means of results of two separate cell suspensions and are expressed in nmol min^{-1} (mg protein)$^{-1}$

[b] 2-HPA, PMA and 2-phenylethanol were not metabolized [<0.5 nmol min^{-1} (mg protein)$^{-1}$] by these cells

[c] Initial consumption rates

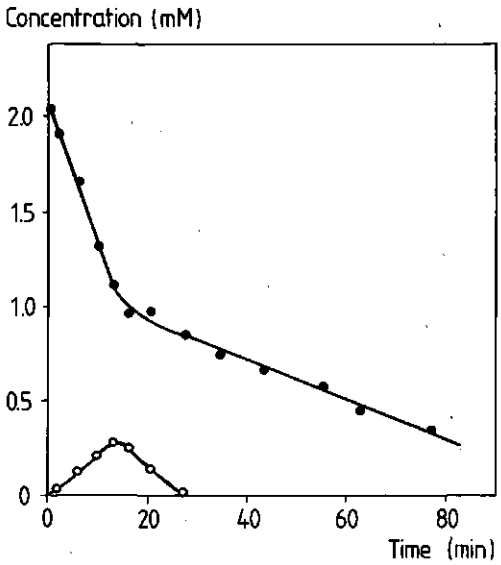

Figure 1. Transient accumulation of PA (○) from DL-PHA (●) by washed cell suspensions of Flavobacterium JS-7 cultivated on DL-PHA. The complete reaction mixture (total volume 5 ml) contained 4.8 mg protein ml^{-1}.

Unfortunately, this hypothesis could not be verified using each enantiomer of PHA seperately since these compounds are not commercially available.

During incubations of DL-PHA-grown cells with PA no accumulating intermediates were detected; this in contrast to incubations of these cells with 3-HPA during which homogentisate transiently accumulated (Figure 2A). Similar results for homogentisate accumulation from 3-HPA were obtained for incubations with PA-grown or 3-HPA-grown cells. When incubations were done in the presence of 2,2-dipyridyl, a known inhibitor of homogentisate dioxygenase (van den Tweel et al., 1986b), 3-HPA was converted quantitatively to homogentisate (Figure 2B). DL-PHA-grown and PA-grown cells also consumed 4-HPA but 4-HPA was a very poor substrate for these cells when compared with 3-HPA (Table 2); evidently PA is mainly metabolized via 3-HPA to homogentisate. Surprisingly, cells grown on 3-HPA metabolized 4-HPA and 3-HPA at equal rates, while *Flavobacterium* cells grown on 4-HPA metabolized no 3-HPA (Table 2).

Homogentisate was also quantitatively recovered from 4-HPA using the inhibitor 2,2-dipyridyl in incubations with both 3-HPA- or 4-HPA-grown cells (plots not shown). During these incubations no accumulation of homoprotocatechuate was detected. Nevertheless, *Flavobacterium* cells grown on either DL-PHA, PA or 3-HPA were able to metabolize homoprotocatechuate. During these incubations with homoprotocatechuate a product (r_t 2.12 min) accumulated (Figure 3)

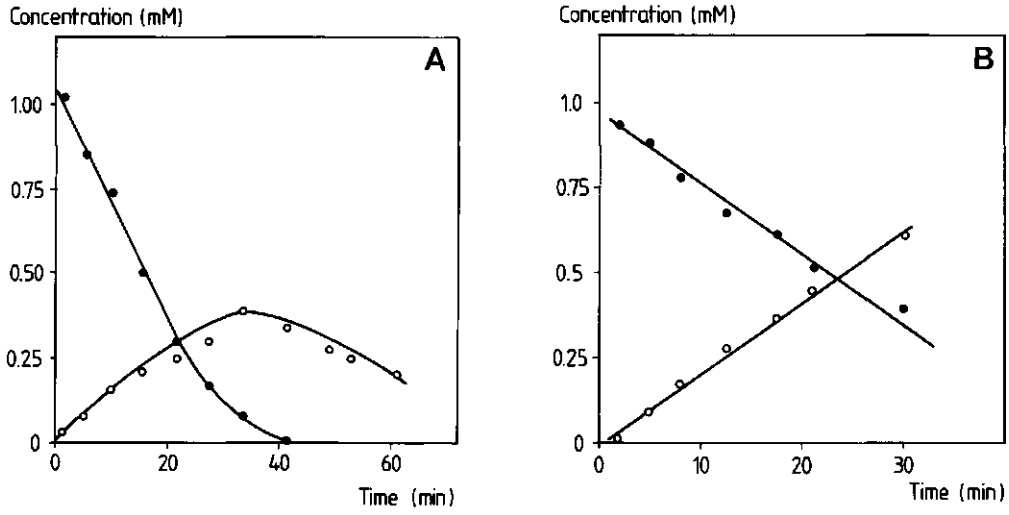

Figure 2. Metabolism of 3-HPA (•) by DL-PHA-grown cells (3.2 mg protein ml^{-1}) either in the absence (A) or presence (B) of 2,2-dipyridyl (10 mM). During these experiments homogentisate (○) accumulated.

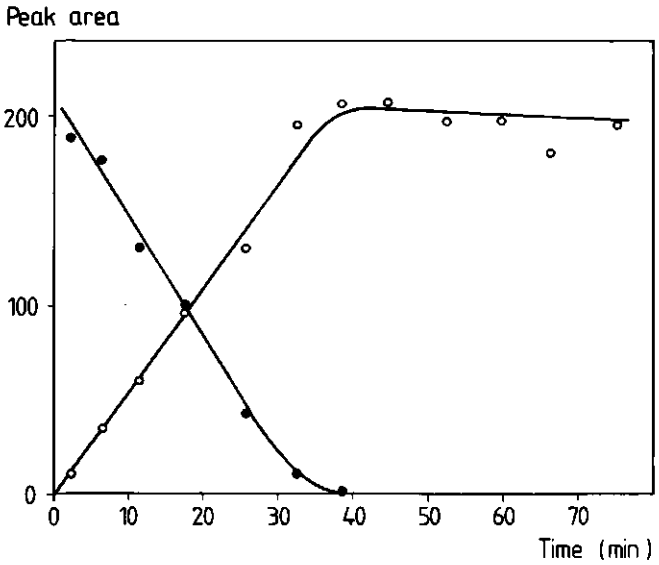

Figure 3. Conversion of homoprotocatechuate (•) to a more polar product (○) by PA-grown cells (4.9 mg protein ml^{-1}). The initial concentration of homoprotocatechuate was 0.6 mM.

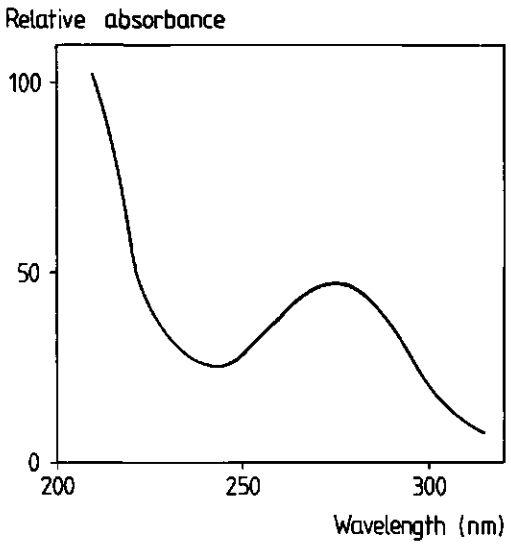

Figure 4. The UV-absorbance spectrum of the unknown compound which accumulated during the incubation of whole cells with homoprotocatechuate (Figure 3).

and the UV absorption spectrum of this unknown compound exhibited a maximum at 275 nm (Figure 4). Simultaneously with the formation of this product the incubation mixture began to turn red. This aqueous red solution upon acidification turned yellow and a subsequent addition of zinc dust resulted in total decolorization. Almost identical spectral properties have been described for the 2,5-quinone of 2,4,5-trihydroxyphenylacetic acid (Wada & Fellman, 1973; Isono, 1958), indicating that homoprotocatechuate was hydroxylated to 2,4,5-trihydroxyphenylacetic acid which compound is very unstable and rapidly oxidized to the hydroxyquinone. Further proof for the identity of the unknown compound was obtained by extracting it with ethylacetate from the aqueous phase and subsequent evaporation of the solvent resulted in a red residue. The mass spectrum of the residue had a parent ion with the expected value of 182 m/e.

Experiments with cell extracts

From the foregoing experiments with whole cells it is assumed that DL-PHA is degraded in an initial oxidative reaction via phenylacetaldehyde. Cell extracts of DL-PHA-grown cells indeed contained a NAD^+-dependent PHA dehydrogenase (Table 3). $NADP^+$ could not replace NAD^+. Maximal activity was observed using a 50 mM Tris/HCl buffer at pH 9.0. The activity of the enzyme was also measured by recording the disappearance of DL-PHA in the presence of NAD^+ from the incubation mixture by means of HPLC. DL-PHA was rapidly con-

Table 3. Specific enzyme activities[a] of some enzymes involved in DL-PHA and PA catabolism in Flavobacterium JS-7 grown on various substrates.

Enzyme	Growth substrate				
	DL-PHA	PA	3-HPA	4-HPA	Succinate
PHA dehydrogenase					
NAD^+-dependent	155	5	<1	<1	<1
Phenylacetaldehyde dehydrogenase					
NAD^+-dependent	260	25	5	10	10
PMS-dependent	20	40	30	15	20
3-HPA hydroxylase[b]	50	240	50	<1	<1
4-HPA hydroxylase[b]	10	40	50	25	<1
Homoprotocatechuate hydroxylase[b]	20	120	20	<1	<1
Homogentisate dioxygenase	15	70	80	55	<1

[a] Activities in nmol min^{-1} (mg protein)$^{-1}$
[b] Activities were measured in the presence of FAD with NADPH as a reductant

verted to stoichiometric amounts of PA until about half of the racemic PHA was oxidized while the remaining PHA was oxidized only very slowly (Figure 5). No accumulation of possible aldehyde intermediates was observed. Spectrophotometric analysis of the NADH content showed that during the initial rapid oxidation phase 2 mol NADH were produced per mol PHA oxidized. Cofactor-free extracts, when supplied with NAD^+, also converted DL-PHA quantitatively to PA.

Cell extracts of DL-PHA-grown cells were also checked for other enzyme activities acting on PHA; no non-oxidative decarboxylase, oxidase or PMS-dependent dehydrogenase were detected. Neither was a $NAD(P)^+$- or PMS-dependent 2-phenylethanol dehydrogenase detected in DL-PHA-grown cells.

Cell extracts of DL-PHA-grown cells also contained an inducible NAD^+-dependent phenylacetaldehyde dehydrogenase with maximal activity at pH 9.5 and a constitutive PMS-dependent phenylacetaldehyde dehydrogenase with maximal activity at pH 9.0 (Table 3). $NADP^+$ was not a substrate for the NAD^+-dependent enzyme. The PMS-dependent enzyme was routinely assayed at pH 8.0 since the addition of PMS to a mixture of Tris/HCl buffer pH 9.0 and phenylacetaldehyde resulted in its rapid autooxidation. It was confirmed by means of HPLC that PA was indeed the product formed from phenylacetaldehyde in the NAD^+- and PMS-dependent enzyme reactions in the extracts.

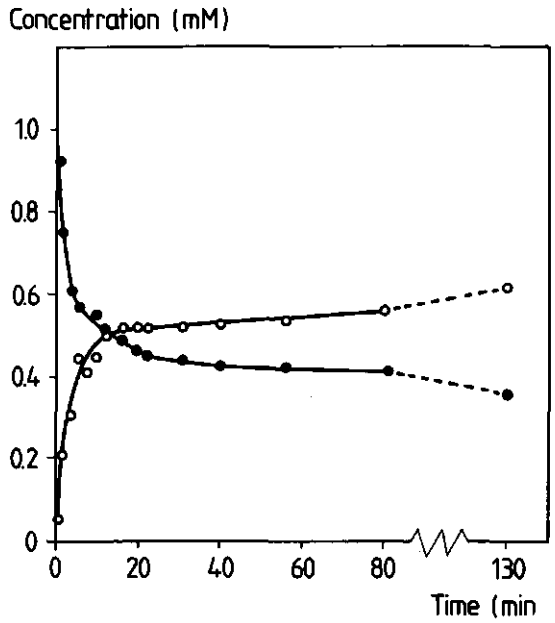

Figure 5. Oxidation of DL-PHA (•) by a cell extract of DL-PHA-grown Flavobacterium JS-7 cells. The complete reaction mixture (total volume 7 ml) contained: cell extract (13.3 mg protein), 21 μmol NAD^+, 7 μmol DL-PHA and 350 μmol Tris/HCl buffer pH 9.0. During this incubation PA (○) accumulated.

On the basis of the results presented it is concluded that PHA is indeed oxidized via phenylacetaldehyde to PA. Since whole cells grown on either DL-PHA or PA readily consumed 3-HPA it was investigated whether a PA hydroxylase was present in these cell extracts. Unfortunately, however, no PA hydroxylase was detected although various cofactors (NAD(P)H, FAD and tetrahydropteridine) either in the presence or absence of Fe^{2+} were tested. Cells grown on either DL-PHA, PA or 3-HPA, however, contained an inducible 3-HPA hydroxylase which showed maximal activity in the simultaneous presence of FAD and NADPH (Table 3). Under identical assay conditions also a hydroxylation of 4-HPA and homoprotocatechuate was observed; again the addition of FAD enhanced the hydroxylation rate. The carbon source used for growing the cells had no effect on the ratio of the rates of 3-HPA and homoprotocatechuate which was always 2.0-2.5 (Table 3). Replacement of NADPH by NADH caused an approximately five fold decrease in hydroxylation activity both when 3-HPA, 4-HPA or homoprotocatechuate were used as substrates. Consistently with the oxidation of 3- and 4-HPA by whole cells (Tables 2 and 3), extracts of 3-HPA-grown cells hydroxylated 3- and 4-HPA at equal rates, while extracts of 4-HPA-grown cells only hydroxylated 4-HPA (Table 3).

The stoichiometry of the hydroxylation of 3-HPA to homogen-

tisate was investigated by inhibiting further oxidation of the product by ortho-phenanthroline (Hareland et al., 1975). Following oxygen uptake with an oxygen probe and measuring decrease in absorbance at 340 nm showed that the hydroxylation of 250 nmol 3-HPA required 235 nmol O_2 and 255 nmol NADPH.

Activity for ring cleavage enzymes in extracts was detected for a homogentisate dioxygenase which was induced during growth on either DL-PHA, PA, 3- or 4-HPA but not for a homoprotocatechuate dioxygenase. Ringcleavage of homogentisate required an equimolar amount of O_2 and the increase of absorbance at 330 nm was indicative for a breakdown of homogentisate via maleylacetoacetate (Knox & Edwards, 1955). Subsequent addition of reduced glutathione (GSH) to the incubation mixture resulted in a rapid decrease in absorbance. Moreover, N-ethylmaleimide (NEM), a GSH trapping agent (Crawford & Frick, 1977) completely inhibited the GSH-dependent degradation of maleylacetoacetate. This inhibition by NEM could be overcome by adding GSH in slight excess over NEM, demonstrating a GSH-dependent isomerase is present in this *Flavobacterium* sp.

DISCUSSION

Flavobacterium JS-7 oxidized DL-PHA to PA. Experiments with whole cells showed (Figure 1) that both enantiomers were oxidized albeit at different rates, whereas in cell extracts (Figure 5) only one stereoisomer was oxidized at an appreciable rate. It thus seems that more than one enzyme is involved in the initial breakdown of both enantiomers of PHA, but only a NAD^+-dependent dehydrogenase was detected. Since no indications were obtained for 2-phenylmalonate semialdehyde to be an intermediate in PHA metabolism, an oxidative decarboxylation of DL-PHA yielding phenylacetaldehyde is proposed.

Phenylacetaldehyde in turn was oxidized by an inducible NAD^+-dependent phenylacetaldehyde dehydrogenase. Maximal activity of this enzyme was measured at pH 9.5 and in this respect the enzyme resembled the phenylacetaldehyde dehydrogenase described by Fujioka et al. (1970). Next to this inducible NAD^+-dependent dehydrogenase also a constitutive PMS-dependent phenylacetaldehyde dehydrogenase was found in cell extracts. The pH optimum of this enzyme resembled the pH optimum of the PMS-dependent benzaldehyde dehydrogenase which was involved in the metabolism of both enantiomers of phenylglycine in another *Flavobacterium* sp. (van den Tweel & de Bont, 1987).

No PA hydroxylase was detected in cell extracts of *Flavobacterium* JS-7 cells grown on either DL-PHA or PA. Difficulties in demonstrating the oxidation of PA by cell extracts of bacteria have

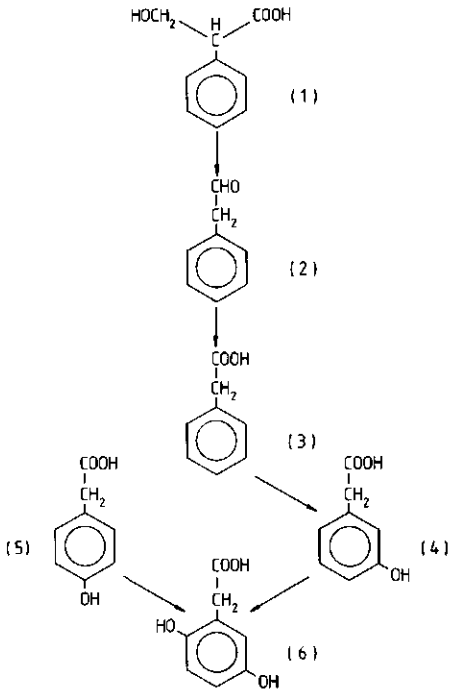

Figure 6. Proposed pathway for the metabolism of DL-PHA, PA and 3- and 4-HPA in Flavobacterium JS-7. (1) DL-PHA, (2) phenylacetaldehyde, (3) PA, (4) 3-HPA, (5) 4-HPA, (6) homogentisate.

already been reported by several research groups (Sariaslani et al., 1974; Blakley et al., 1967; Baggi et al., 1983; Lee & Desmazeaud, 1986), this in contrast to the hydroxylation of PA by *Aspergillus niger* (Sugumaran & Vaidyanathan, 1978) and by *Rhizoctonia solani* (Kohmoto & Nishimura, 1975). From experiments with whole cells (Tables 1 and 2) and from the induced enzyme activities (Table 3) it is concluded that PA is hydroxylated mainly via 3-HPA to homogentisate. 3-HPA was not metabolized via homoprotocatechuate as described for an *Escherichia coli* (Cooper & Skinner, 1980). The hydroxylation of 3-HPA to homogentisate was catalysed by a NAD(P)H-dependent monooxygenase which was stimulated by the addition of FAD. Surprisingly, cells possessing this latter enzyme were also able to hydroxylate homoprotocatechuate. Moreover, the activity ratio 3-HPA/homoprotocatechuate was not dependent on the carbon source used for growth of the cells and was also constant when changing the composition of the assay mixture indicating 3-HPA-6-hydroxylase also attacks homoprotocatechuate. From the absorbance spectrum and from the mass spectrum of the accumulating product it is concluded that homoprotocatechuate

is hydroxylated yielding the unstable 2,4,5-trihydroxyphenylacetic acid (Wada & Fellman, 1973; Isono, 1958). A similar conversion has also been observed in *Trichosporon cutaneum* (Sparnins et al., 1978). However, in this yeast the hydroxylation of homoprotocatechuate to 2,4,5-trihydroxyphenylacetic acid was involved in 4-HPA metabolism. In *Flavobacterium* JS-7, this reaction has no metabolic significance.

Flavobacterium JS-7 degraded 4-HPA also via homogentisate. Surprisingly, 4-HPA-1-hydroxylase catalyzing the initial step in the oxidation of 4-HPA was also induced in 3-HPA-grown cells (Table 3). Apparently, in addition to 4-HPA, can also 3-HPA serve as an inducer for this latter enzyme. This in contrast to the forementioned 3-HPA-6-hydroxylase which is only induced by 3-HPA (Table 3). Figure 6 shows the degradation pathway for DL-PHA, PA, 3-HPA and 4-HPA to homogentisate in *Flavobacterium* JS-7.

Homogentisate, obtained from both 3-HPA and 4-HPA, was cleaved by an inducible dioxygenase to maleylacetoacetate. Further metabolism of maleylacetoacetate was by a GSH-dependent enzyme, this in contrast to the GSH-independent maleylacetoacetate hydrolase in *Bacillus* B11c (Crawford, 1976) and GSH-independent maleylacetoacetate isomerase in some Gram-positive organisms (Hagedorn & Chapman, 1985). Previously we have shown that another *Flavobacterium* sp. (van den Tweel & de Bont, 1987) possessed a GSH-dependent gentisate pathway. These findings are in keeping with the fact that Gram-negative bacteria possess a GSH-dependent (homo)gentisate pathway (Hagedorn & Chapman, 1985; Hagedorn et al., 1985; van den Tweel et al., 1986b). Since our main interest is in the bioformation of hydroxylated aromatic compounds, we currently are isolating the aspecific 3-HPA-6-hydroxylase to study its substrate specificity in more detail.

ACKNOWLEDGEMENTS

We are grateful to C.J. Teunis for performing the mass spectrum analyses and to A.R.W. van Neerven for making electron microscopic photographs. These investigations were supported in part by the Netherlands Technology Foundation (STW).

REFERENCES

Amund, O.O. & Higgins, I.J. (1985). The degradation of 1-phenylalkanes by an oil-degrading strain of Acinetobacter Iwoffi. Antonie van Leeuwenhoek 51,45-56

Anderson, J.F. & Dagley, S. (1980). Catabolism of aromatic acids in Trichosporon cutaneum. Journal of Bacteriology 141,534-543

Baggi, G., Boga, M.M., Catelani, D., Galli, E. & Treccani, V. (1983). Styrene catabolism by a strain of Pseudomonas fluorescens. Systematic and Applied Microbiology 4,141-147

Blakley, E.R., Kurz, W., Halvorson, H. & Simpson, F.J. (1967). The metabolism of phenylacetic acid by a Pseudomonas. Canadian Journal of Microbiology 13,147-157

Bocks, S.M. (1967). Fungal metabolism. III. The hydroxylation of anisole, phenoxyacetic acid, phenylacetic acid and benzoic acid by Aspergillus niger. Phytochemistry 6,785-789

Bouchara, E. (1971). Analgesic and antiinflammatory substituted phenylacetic acids. French Patent 2,054,532

Cooper, R.A. & Skinner, M.A. (1980). Catabolism of 3- and 4-hydroxyphenylacetate by the 3,4-dihydroxyphenylacetate pathway in Escherichia coli. Journal of Bacteriology 143,302-306

Crawford, R.L. (1976). Degradation of homogentisate by strains of Bacillus and Moraxella. Canadian Journal of Microbiology 22,276-280

Crawford, R.L. & Frick, T.D. (1977). Rapid spectrophotometric differentiation between glutathione-dependent and glutathione-independent gentisate and homogentisate pathways. Applied and Environmental Microbiology 34,70-174

Fujioka, M., Morino, Y. & Wada, H. (1970). Metabolism of phenylalanine (Achromobacter eurydice). In: Methods in Enzymology (Tabor, H. & White-Tabor, C., Eds.), Vol. 17, pp. 585-596. Academic Press, New York

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

Hagedorn, S.R. & Chapman, P.J. (1985). Glutathione-independent maleylacetoacetate isomerase in Gram-positive bacteria. Journal of Bacteriology 163,803-805

Hareland, W.A., Crawford, R.L., Chapman, P.J. & Dagley, S. (1975). Metabolic function and properties of 4-hydroxyphenylacetic acid 1-hydroxylase from Pseudomonas acidovorans. Journal of Bacteriology 121,272-285

Holmes, B. Owen, R.J. & McMeekin, T.A. (1984). Flavobacterium. In: Bergey's Manual of Systematic Bacteriology (Krieg, N.R. & Holt, J.G., Eds.), Vol. I, pp 353-361. Williams & Wilkins, Baltimore

Isono, M. (1958). Oxidative metabolism of phenylacetic acid by Penicillium chrysogenum Q176. Part XIII. Isolation and identification of an intermediate metabolite of p-hydroxyphenylacetic acid. Nippon Nogei Kagaku Kaishi 32,256-259

Kishore, G., Sugumaran, M. & Vaidyanathan, C.S. (1976). Metabolism of DL-(±)-phenylalanine by Aspergillus niger. Journal of Bacteriology 128,182-191

Kluyver, A.J. & van Zijp, J.C.M. (1951). The production of homogentisic acid out of phenylacetic acid by Aspergillus niger. Antonie van Leeuwenhoek 17,315-324

Knox, W.E. & Edwards, S.W. (1955). The properties of maleylacetoacetate, the initial product of homogentisate oxidation in liver. Journal of Biological Chemistry 216,489-498

Kohmoto, K. & Nishimura, S. (1975). Phenylacetic acid meta-hydroxylase from Rhizoctonia solani. Phytochemistry 14,2131-2133

Kunita, N. (1955a). Bacterial oxidation of phenylacetic acid. I. The pathway through homoprotocatechuic acid. Medical Journal of Osaka University 6,697-702

Kunita, N. (1955b). Bacterial oxidation of phenylacetic acid. II. The pathway through homogentisic acid. Medical Journal of Osaka University 6,703-708

Kunita, N. (1956). Evidence for alternative pathways for the oxidation of phenylalanine by Pseudomonas fluorescens. Medical Journal of Osaka University 7,203-215

Lee, C.-W. & Desmazeaud, M.J. (1986). Evaluation of the contribution of the tyrosine pathway to the catabolism of phenylalanine in Brevibacterium linens 47. FEMS Microbiology Letters 33,95-98

Niemer, H., Bucherer, H. & Kohler, A. (1959). Über den Abbau von Atropin durch Corynebacterium belladonnae. Zentrallblatt für Physiologische Chemie 317,328-242

Pometto, A.L. & Crawford, D.L. (1985). L-Phenylalanine and L-tyrosine catabolism by selected Streptomyces species. Applied and Environmental Microbiology 49,727-729

Roy, P.D. & Bruggink, A. (1981). p-Hydroxyphenylmalonic acid derivatives. European Patent Application 61,219

Sariaslani, F.S., Harper, D.B. & Higgins, I.J. (1974). Microbial degradation of hydrocarbons. Catabolism of 1-phenylalkanes by Nocardia salmonicolor. Biochemical Journal 140,31-45

Sparnins, V.L., Anderson, J.J., Omans, J. & Dagley, S. (1978). Degradation of 4-hydroxyphenylacetic acid by Trichosporon cutaneum. Journal of Bacteriology 136,449-451

Stevens, W.F. & Rörsch, A. (1971). The breakdown of tropic acid in Pseudomonas putida strain L. I. Utilization of various substrates; the conversion of tropic acid into phenylacetic acid. Biochimica et Biophysica Acta 230,204-211

Sugumaran, M., Ramanarayanan, M., & Vaidyanathan, C.S. (1973). Involvement of protocatechuic acid in the metabolism of phenylacetic acid by Aspergillus niger. FEBS Letters 29,69-72

Sugumaran, M. & Vaidyanathan, C.S. (1978). Metabolism of phenylacetic acid in Aspergillus niger. Journal of the Indian Institute of Science 60,125-141

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

van den Tweel, W.J.J. & de Bont, J.A.M. (1987). Metabolism of both stereoisomers of phenylglycine by different routes in Flavobacterium F24. Journal of General Microbiology 133,745-754

van den Tweel, W.J.J., Smits, J.P., Tramper, J. & de Bont, J.A.M. (1987). Biosynthesis of hydroxylated aromatic compounds. Proceedings of the 4th European Congress on Biotechnology 2,172-174

Ueno, T., Yoshizako, F. & Nishimura, A. (1973). The formation of homogentisic acid from phenylacetic acid by an Aspergillus sp. Canadian Journal of Microbiology 19,393-395

Wada, G.H. & Fellman J.H. (1973). 2,4,5-Trihydroxyphenylacetic acid. A metabolite of L-3,4-dihydroxyphenylalanine. Biochemistry 12,5212-5217

Webley, D.M., Duff, R.B. & Farmer, V.C. (1955). Beta-oxidation of fatty acids by Nocardia opaca. Journal of General Microbiology 13,361-369

Yoshizako, F., Chubachi, M., Nishimura, S. & Ueno, T. (1977). The metabolism of phenylacetic acid by Aspergillus fumigatus ATCC 28282: identification of 2,6-dihydroxyphenylacetic acid. Canadian Journal of Microbiology 23,1140-1144

Yuasa, K., Ishizuka, K., Kaburaki, S. & Sakasai, T. (1975). Metabolism of phenylalanine in Aspergillus sojae. Agricultural and Biological Chemistry 39,2199-2206

Chapter 8

CONTINUOUS PRODUCTION OF CIS-1,2-DIHYDROXYCYCLOHEXA-3,5-DIENE (CIS-BENZENEGLYCOL) FROM BENZENE BY A MUTANT OF A BENZENE-DEGRADING *PSEUDOMONAS* SP.

W.J.J. van den Tweel, M.J.A.W. Vorage, E.H. Marsman,
J. Koppejan, J. Tramper and J.A.M. de Bont

SUMMARY

A *Pseudomonas* sp. able to grow on benzene as sole carbon and energy source was isolated from a mixture of soil and water samples. Experiments with whole cells and enzyme studies showed that benzene was metabolized via cis-1,2-dihydroxycyclohexa-3,5-diene (cis-benzeneglycol) to catechol. A mutant of this strain, which lacked a functional cis-benzeneglycol dehydrogenase, converted benzene almost quantitatively to cis-benzeneglycol. Addition of benzene to a carbon-limited chemostat in which this mutant was growing on succinic acid, resulted in an accumulation of cis-benzeneglycol in the medium. Under the conditions used cis-benzeneglycol was produced at a rate of about 84 nmol min^{-1} (mg protein)$^{-1}$. In order to predict the cis-benzeneglycol concentration at various times, a mathematical model is described that fitted rather well for both benzene-transport-limited and kinetically-limited production conditions. However, no prolonged continuous production process was achieved under carbon-limited conditions, because the mutant cells were outcompeted by revertants after about three days. To circumvent this problem, the chemostat was operated under nitrogen-limited conditions, which resulted in a very stable continuous cis-benzeneglycol production process.

Enzyme and Microbial Technology (1988) 10,134-142

INTRODUCTION

Currently there is a great industrial interest in aromatic dioxygenase enzymes for the production of cis-dihydrodiols, since such diols may be useful starting compounds for the production of synthetic polymers (Ballard et al., 1983) and various pharmaceuticals (Ley et al., 1987). By the action of the NADH-requiring dioxygenases, two oxygen atoms are incorporated into the aromatic nucleus and a cis-dihydrodiol is formed (Gibson et al., 1970b; Högn & Jaenicke, 1972; Jeffrey et al., 1975). In 1970 Gibson and coworkers isolated a mutant of *Pseudomonas putida* that was able to convert benzene to cis-1,2-dihydroxycyclohexa-3,5-diene [cis-benzeneglycol (CBG)]. Further investigations have shown that the broad substrate specificity of these dioxygenases also enabled the formation of a range of substituted cis-dihydrodiols (Gibson et al., 1970a,1973,1974; Ziffer et al., 1977; Taylor, 1983; Jenkins & Dalton, 1985). Well known is the oxidation of indole by the naphtalene or toluene dioxygenase to cis-indole-2,3-dihydrodiol which, after a spontaneous elimination of water and oxidation by air, gives rise to indigo formation (Ensley et al., 1983).

Cis-dihydrodiols can also be formed by dioxygenases acting on benzoates and derivatives thereof. A mutant of *Alcaligenes eutrophus* accumulated dihydrodihydroxybenzoic acid (DHB) from benzoate (Reiner & Hegeman, 1971). As a result of the relaxed specificity of the benzoate dioxygenase of this mutant various halo- and methyl-substituted DHB's were also formed (Reineke et al., 1978). Recently, two research groups have shown that also toluate-1,2-dioxygenase encoded by the *XylD* gene of the Tol plasmid can be used for the formation of substituted DHB's (Whited et al., 1986; Zeyer et al., 1985).

In spite of the huge interest in these dihydrodiols, very little information is available on bioproduction processes for such compounds. Currently ICI is investigating the bioformation of CBG from benzene by means of a mutant of *Pseudomonas putida* (Taylor, 1983, 1985). As far as we know, their production process is based on non-growing cells, which under restricted supply of nitrogen and an excess amount of co-substrate for NADH regeneration, convert benzene in CBG (Taylor, 1985). More recently Jenkins et al. (1987), working with a *Pseudomonas* strain of ICI, have described a fed-batch process for the production of cis-tolueneglycol [(+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene] from toluene. To investigate in more detail some intriguing aspects of the forementioned bioconversions (enzyme induction, cofactor regeneration, toxicity of substrate and/or product, and stability of the mutant), we have also isolated a mutant of a benzene degrading microorganism and have studied the continuous conversion of benzene in CBG by cells growing on succinate in a chemostat.

MATERIALS AND METHODS

Isolation, maintenance and cultivation of strain 50. Strain 50 was isolated from an inoculum obtained from a mixture of various soil and water samples using benzene as sole carbon and energy source. Enrichment of the organism was conducted in a 250 ml Erlenmeyer flask that contained 50 ml of a mineral salts medium (van den Tweel et al., 1986). Benzene (50 μl) was added to the gaseous phase of this enrichment culture as described previously (de Bont et al., 1986). The flask was incubated at 30°C. Upon growth, subcultures were made and serial dilutions were plated on mineral medium plates to which benzene was supplied in the vapor phase. After incubation one organism was isolated and designated as strain 50. Strain 50 was maintained on agar slopes of 5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract medium to which Oxoid no. 3 agar (15 g l^{-1}) had been added. Larger quantities of cells were grown in 5 l Erlenmeyer flasks equipped with a tube mounted in a rubber stopper (de Bont et al., 1986); these flasks contained 500 ml of mineral salts medium to which 0.5 g benzene was added via the vapor phase.

Isolation of mutants defective in the CBG dehydrogenase enzyme system. Pseudomonas 50 was grown to stationary phase in 25 ml mineral salts medium supplemented with 1 g l^{-1} arginine. The cells were washed twice with 100 mM sodium citrate buffer (pH 5.5) and resuspended in 25 ml of the same buffer containing 2.5 mg of N-methyl-N'-nitro-N-nitrosoguanidine (NTG). After standing at room temperature for 15 min, the cells were washed twice with 50 mM potassium phosphate buffer (pH 7.0), suspended again in the forementioned arginine medium, and allowed to grow for 8 h at 30°C. Subsequently, cells were washed twice with the mineral salts medium and allowed to grow in the presence of benzene for 4 h before addition of filter sterilized penicilline G (5000 units ml^{-1}). After 5 h the cells were washed with potassium phosphate buffer and plated onto mineral salts indicator plates which contained 20 g l^{-1} agar, 0.2 g l^{-1} arginine and 25 mg l^{-1} 2,3,5-triphenyltetrazolium chloride (TTC). The plates were incubated in a desiccator to which 40 μl benzene l^{-1} was added, and the cells were allowed to grow for 72 h at 30°C. Small colonies that were white were isolated, and tested for growth on glucose, benzene and catechol. Strains unable to grow on benzene but still able to grow on catechol were further screened for the accumulation of CBG by measuring the absorption of the culture fluid at 262 nm. For this, the presumptive CBG dehydrogenase-negative strains were grown in 100 ml Erlenmeyer flasks that contained 20 ml arginine medium. Benzene (20 μl) was added to the tube which was mounted in the rubber stopper sealing the flask. The tube had a small opening inside the flask (de Bont et al., 1986). After one day of incubation at

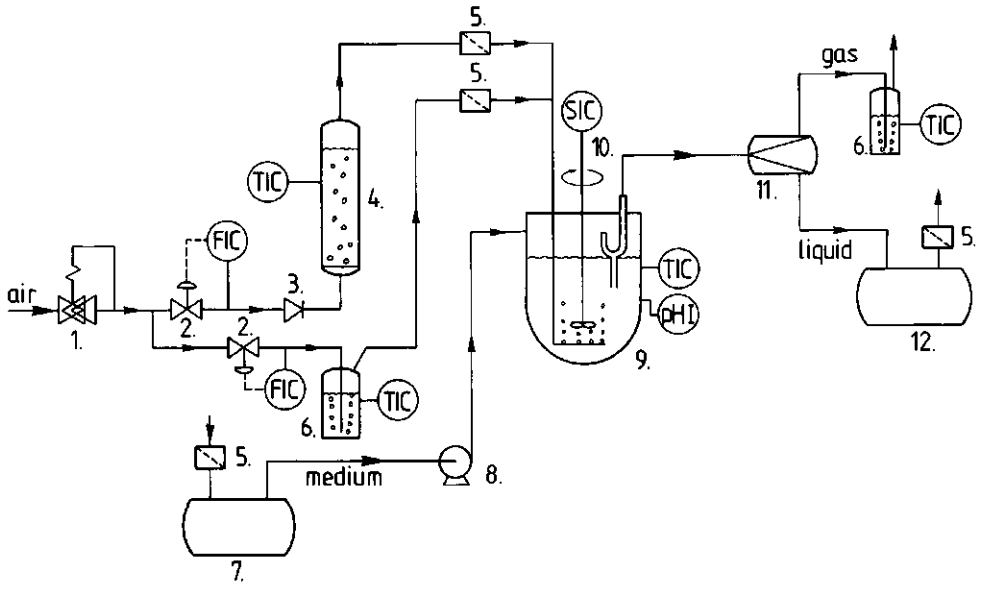

Figure 1. Schematic representation of the chemostat used for continuous CBG production. Pressure reducer, 1; mass flow controller, 2; one-way valve, 3; benzene column, 4; air filter, 5; gas washer, 6; medium tank, 7; pump, 8; fermentor vessel, 9; stirrer, 10; separator, 11; effluent tank, 12. TIC, FIC, SIC and pHIC; temperature, flow rate, stirring speed and pH indication and control.

30°C, the increase of absorbance at 262 nm was measured.

Oxygen uptake experiments. Simultaneous adaptation experiments with washed cell suspensions were done as described previously (de Bont et al., 1986).

Continuous bioproduction of CBG. Continuous formation of CBG was achieved in a chemostat (Applikon, Schiedam, The Netherlands) with peripherals (Figure 1). Mutant strain Mt 92 was routinely grown on succinate in this fermentor (working volume 0.93 l) at pH 7.0 and at a dilution rate of 0.085 h^{-1} under either carbon- or nitrogen-limited conditions. The temperature of the vessel was kept constant at 30°C by a thermocontrolled recycling waterbath, and the impeller speed was kept constant at 850 rpm. Air at a rate of 60 ml per minute was supplied to the fermentor through an inlet beneath the stirrer. The dissolved oxygen concentration (DOC) of the culture was continuously monitored with a steam sterilizable oxygen electrode (Applikon, Schiedam, The Netherlands). At steady state (after 3 to 5 volume changes, when the DOC and the turbidity were constant) a part of the air was passed through a column containing benzene. The flow rates of the air stream and of the air/benzene flow were maintained constant (total flow 60 ml per min) with mass flow meters/controllers (Brooks

type 5850 TR). The benzene column, kept at 20°C, was equipped with graduation marks from which the amount of benzene entering the fermentor was calculated. To prevent loss of benzene by adsorption to the tubings, viton tubings were used (Iso-vernic, Rubber B.V., Hilversum, The Netherlands). The benzene concentration in the medium was determined by high performance liquid chromatography (HPLC). To determine the benzene concentration in the outcoming gas, the gas stream was passed through a water column kept at 30°C. At various times the benzene concentration in the water, which was in equilibrium with the outcoming gas, was analysed by HPLC and from the determined partition coefficient of benzene between water and air at 30°C the benzene concentration in the outcoming gas flow was calculated.

For carbon-limited growth of Mt 92 a mineral salts medium was used containing in 1 l of deionized water: K_2HPO_4, 1.55 g; NaH_2PO_4, 0.85 g; NH_4Cl, 2.0 g; $(NH_4)_2SO_4$, 0.1 g; $MgCl_2.6H_2O$, 0.075 g; yeast extract, 0.2 g and 0.2 ml of a trace elements solution as described by Vishniac and Santer (1957). Succinic acid was added to this mineral medium at 1 g l^{-1}. Nitrogen-limited growth was achieved using the same medium except that 0.125 g l^{-1} Na_2SO_4 was used instead of $(NH_4)_2SO_4$, only 0.15 g l^{-1} NH_4Cl was added, and that the succinic acid concentration was either 2.5 g l^{-1} or 7.5 g l^{-1}.

Analytical methods. Protein concentrations were measured by the method of Lowry et al. (1951). Benzene concentrations were determined by reverse-phase HPLC on a C-18 column (particle size 3 μm, 30x3 mm; Perkin-Elmer, Gouda, The Netherlands) and detection was at 210 nm by means of a variable-wavelength detector. As an eluent (0.6 ml min^{-1}) methanol:50 mM potassium phosphate buffer pH 7.0 (1:2 [v/v]) was used. Under these conditions benzene showed a retention time of 6.6 min. CBG and phenol were analysed on a ChromSpher-Si column (200x3 mm; Chrompack, Middelburg, The Netherlands). The mobile phase was 50 mM potassium phosphate buffer pH 7.0, the flow rate was 0.4 ml min^{-1}, and detection was at 210 nm. Under the stated conditions CBG and phenol had retention times of 3.35 and 3.10 min, respectively. CBG and phenol were further identified by *in situ* scanning of the UV absorption spectra after the flow had been stopped. Whether or not succinate was present in the culture supernatant was investigated at 45°C by HPLC using an Aromatic Acids column (100x6.5 mm; Chrompack, Middelburg, The Netherlands). As an eluent (0.6 ml min^{-1}) 0.01 N H_2SO_4 was used, and detection was again at 210 nm. Under these conditions succinate had a retention time of 3.65 min. Total organic carbon (TOC) analysis of the culture supernatant, to verify carbon-limited growth-conditions, was done with a Xertex DC-50 TOC analyzer (Dohrman, Santa Clara, CA, USA). The growth rate of strain 50 on benzene was determined by measuring

the increase of turbidity by means of an EEL nephelometer (Evans Electroselenium Ltd., Halstead, UK) as described previously (de Bont et al., 1986). In a similar way the change of biomass was registrated during the CBG production experiments. The total amount of nitrogen in the culture-medium, to check nitrogen-limited growth conditions, was analysed by a method based on the reaction of Berthelot as described by Novozamsky et al. (1974).

Estimation of the air-water benzene transfer coefficient and determination of the partition coefficient of benzene between water and air. The benzene transfer coefficient at the air water interface, $(k_La)_2$, was calculated from Equation 1 as described by Ichikawa et al. (1981):

$$(k_La)_2 = (D_2/D_1)^{0.5} (k_La)_1 \qquad (1)$$

where $(k_La)_1$ is the gas-liquid oxygen transfer coefficient, D_1 and D_2 are the diffusion coefficients for oxygen and benzene, respectively. The volumetric gas-liquid oxygen transfer coefficient, $(k_La)_1$, for our system was found to equal 0.0402 s^{-1} using a dynamic oxygen electrode method. The diffusivity of benzene in water ($0.99 \cdot 10^{-9}$ m^2 s^{-1}) was estimated from the molecular weight as described by Perry and Chilton (1969). For the diffusivity of oxygen in water a value of $2.4 \cdot 10^{-9}$ m^2 s^{-1} was used (Brink & Tramper, 1986). Substitution of these data in Equation 1 yielded $(k_La)_2 \approx 0.026$ s^{-1}. The partition coefficient of benzene between water and air at 30°C ($K = c_l/c_g \approx 3$) was determined by means of HPLC. For this, benzene flows with varying concentrations of benzene (c_g) were supplied to the fermentor which contained sterile medium. After reaching an equilibrium between the gas and liquid phase the benzene concentration in the water phase (c_l) was analysed. Up to a c_g of 2.5 mM a linear relationship was found between c_g and c_l.

Enzyme assays. All assays were performed with a Perkin-Elmer 550A UV spectrophotometer at 30°C. Cell extracts were prepared as described previously (de Bont et al., 1986). CBG dehydrogenase (EC 1.3.1.19) was assayed by measuring the NADH formation at 340 nm as described previously (de Bont et al., 1986). Catechol-1,2- (EC 1.13.11.1) and catechol-2,3-dioxygenase (EC 1.13.11.2) were assayed as described by Hayaishi et al. (1957) and Kojima et al. (1961), respectively.

Materials. The following materials were obtained from the sources indicated: Benzene (Merck-Schuchard, Hohenbrunn, FRG), penicillin G (Gist-Brocades, Delft, The Netherlands), TTC (Sigma Chemical Co., St Louis, MO, USA) and NTG (Aldrich Chemie, Brussels, Belgium). CBG was synthesized according to the method described by Nakajima et al. (1959). All other chemicals were commercially available analytical grade products and were used without further purification.

RESULTS AND DISCUSSION

Characterization of strain 50

The benzene-utilizing isolate strain 50 was an oxidase-positive, Gram-negative rod. Transmission electron microscopic photographs showed that this motile strain possessed up to 8 polair flagella (Figure 2). It was unable to denitrify and did not grow at 41°C. Gelatine was not hydrolysed and no β-galactosidase and arginine dihydrolase were produced. The strain was not able to grow autotrophically with H_2 as the energy source and no fluorescence on yeast extract-glucose plates was observed. On the basis of the above characteristics, the organism was tentatively classified as a *Pseudomonas* sp. At an initial concentration of 1.5 mM benzene in the water phase (c_l) *Pseudomonas* 50 showed a doubling time of 125 min. Increasing the initial c_l, the growth rate of the organism decreased while no growth was observed above 2.5 mM. Similar results have been described for a monochlorobenzene-degrading organism (Reineke & Knackmuss, 1984). Pyruvate, succinate, gluconate, malate, citrate, phenylacetate and glucose were also able to support growth. No growth was obtained using toluene or styrene as potential growth substrates.

The ability to grow on benzene was not lost after successive transfers of *Pseudomonas* 50 for 2 years on a yeast extract/glucose medium. It thus is likely that the ability to grow on benzene is

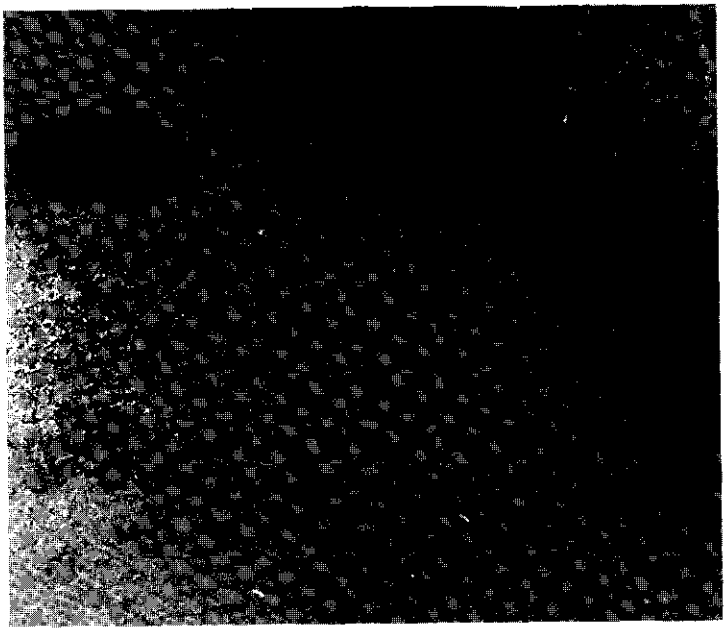

Figure 2. Transmission electron micrograph of Pseudomonas 50. The bar represents 2 μm.

chromosomally encoded. Recently Yarmoff et al. (1986) proposed that in their *Pseudomonas mendocina* the benzene genes are indeed chromosomally encoded. Similar results have also led Finette et al. (1984) to the conclusion that the genes responsible for enzymes of the dihydrodiol pathway for toluene degradation in their *Pseudomonas putida* are located on the chromosome. This is in contrast to the location in many *Pseudomonas* spp. of genes coding for the enzymes involved in the degradation of aromatic hydrocarbons and related compounds on transmissible plasmids that may be lost from a population (Haas, 1983).

Benzene metabolism by Pseudomonas 50

Simultaneous adaptation experiments showed that washed cell suspensions of benzene-grown cells readily oxidized benzene, CBG and catechol (rates: 40, 70 and 105 nmol O_2 min^{-1} (mg protein)$^{-1}$, respectively). Phenol was not metabolized by these cells. Succinate-grown cells did not oxidize the forementioned substrates. During growth on benzene a NAD^+-dependent CBG dehydrogenase, a catechol-1,2- and a catechol 2,3-dioxygenase were induced (specific activities: 240, 160 and 80 nmol min^{-1} (mg protein)$^{-1}$, respectively). These results clearly demonstrate that *Pseudomonas* 50 degraded benzene by means of an inducible pathway via CBG and catechol (Figure 3).

Isolation of CBG dehydrogenase deficient mutants

Mutants of *Pseudomonas* 50 were made with NTG as described in Materials and Methods. Indicator plates, containing TTC, were used to discriminate between organisms which can or can not grow on benzene (Bochner & Savageau, 1977). Performing the described procedure, colonies of organisms able to grow on benzene were large and red, while non-benzene utilizing organisms formed only small white colonies. Similar results with such indicator plates have recently been

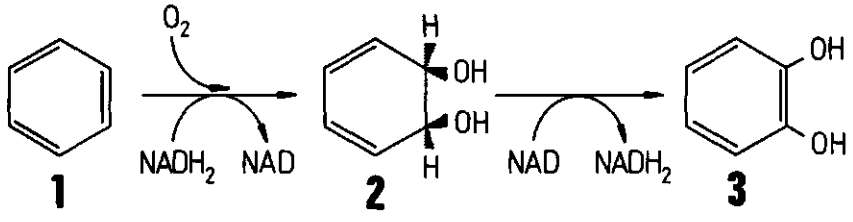

Figure 3. Initial steps of benzene degradation by Pseudomonas 50. 1, benzene; 2, CBG; 3, catechol.

described for the isolation of mutants of a *Pseudomonas putida* which were defective in the toluene dioxygenase enzyme system (Finette et al., 1984). Small white colonies were subsequently tested for growth on glucose, benzene and catechol. Out of 400 colonies tested, 103 mutants did not grow on benzene while they showed good growth on glucose and catechol. These 103 mutants were grown in the arginine medium in the presence of benzene. After one day the extinction of the supernatant was measured at 262 nm and 10 mutants had developed a significant increase of absorbance at this wavelength. Analysis by HPLC showed that 2 mutants (Mt 19 and Mt 92) had indeed accumulated CBG from benzene. For further studies Mt 92 was used because this mutant had accumulated more CBG than Mt 19.

Identification of the formed product

First, the accumulating product was analysed by means of HPLC using a variable wavelength detector. The retention time for the product was identical to the retention time of authentic CBG, and a mixture of the two showed only one peak. Moreover, stopflow scanning showed that the formed product possessed the same UV absorbance spectrum as published and measured for CBG (Nakajima et al., 1959). Acid catalysed dehydration of both the accumulating product and of authentic CBG resulted in the formation of phenol as was also shown by HPLC.

Bioproduction of CBG under carbon-limited conditions

To study the continuous production of CBG, Mt 92 was initially grown in a chemostat (Figure 1) under carbon-limited conditions. At steady state, varying amounts of benzene were supplied to the culture. After a short period of enzyme induction, CBG started to accumulate in the medium, reaching a maximum after about 40 h (Figure 4). The addition of 0.69 mmol benzene h^{-1} to the culture resulted in a CBG concentration of 7.2 mM after 40 h (Figure 4A). During the production phase no benzene was detected in the culture fluid ($c_l \approx 0$ mM); evidently, all benzene transferred to the water phase was converted to CBG. Although some benzene was present in the outcoming gas, the concentration of benzene (c_g) was too low to allow accurate measurements. From the total amount of benzene provided and the amount of CBG produced, a yield of 83% was calculated. Under the specified conditions probably no dehydration of CBG occurred since no phenol was detected in the culture supernatant. Similar results were obtained when 1.03 mmol benzene h^{-1} was supplied to the culture. Again c_l equalled zero, c_g was too low to obtain an accurate value, the CBG concentration was now 10 mM and the yield (78%) was similar (Figure

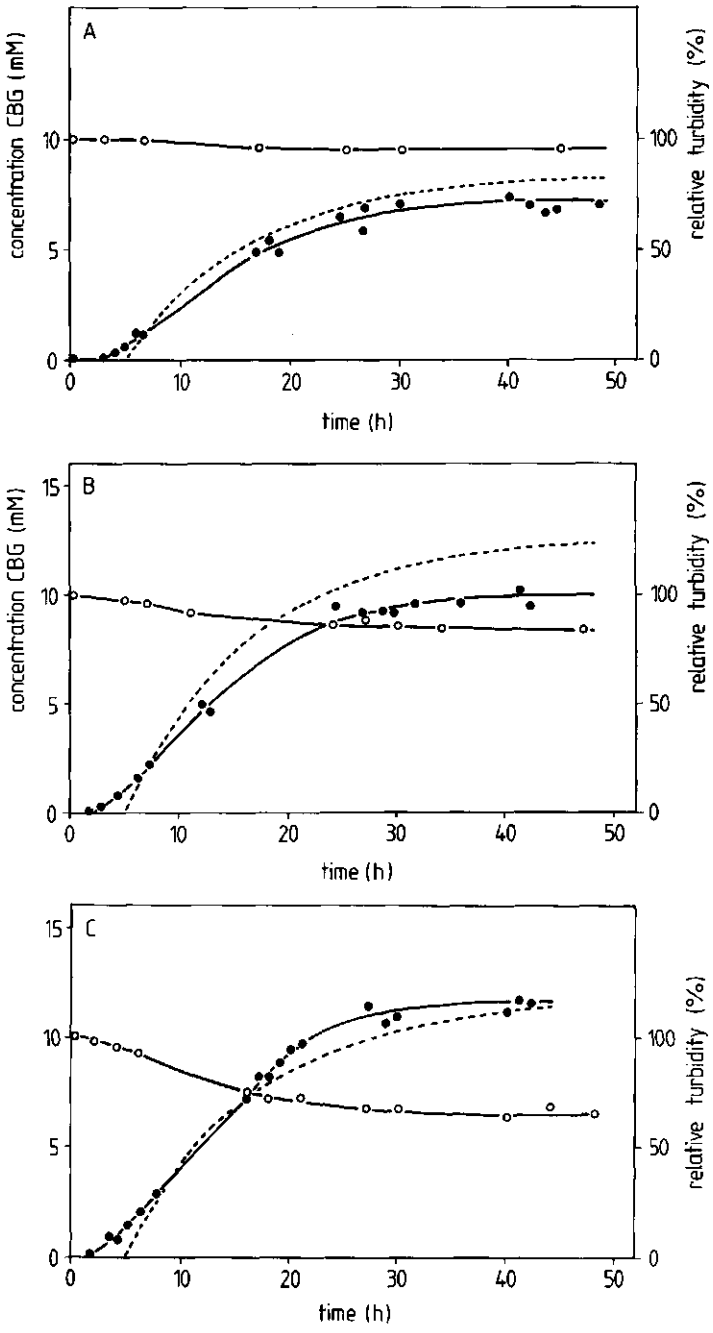

Figure 4. CBG (•) production by Mt 92 growing under carbon-limited conditions in the chemostat. At time zero (protein content 285 mg l^{-1}) varying amounts of benzene were supplied via the air stream to the fermentor (A, 0.69 mmol benzene h^{-1}; B, 1.03 mmol h^{-1}; C, 2.24 mmol h^{-1}). o, turbidity of the culture; ----- , production model predictions.

4B). From these results it is expected that under these two specified conditions transport of benzene to the liquid was the rate-limiting step. To verify this, the benzene supply in a separate experiment was increased to 2.24 mmol h^{-1}. After 40 h a CBG concentration of 11.7 mM was reached (Figure 4C). In contrast to the foregoing experiments, detectable concentrations of benzene were present both in the culture supernatant and in the gas phase ($c_l \approx 0.85$ mM and $c_g \approx 0.29$ mM). From the amount of benzene added to the culture, 92% was recovered either as benzene itself or as the product CBG. The yield of CBG, on basis of the total amount of benzene added to the culture during this experiment, sharply decreased to about 42%. These results indicate that in the latter experiment the rate of CBG production was restricted by a biological factor rather than by mass transport of benzene. If in this case the activity of the pertinent dioxygenase would be maximal, an even higher benzene input should not result in an increase of the CBG concentration. To verify this 4.4 mmol benzene h^{-1} was supplied to the culture. After the introduction of benzene a rapid decrease of turbidity was observed, surprisingly, however, almost no CBG was produced. Probably as result of a toxic effect of the high benzene concentration ($c_l \approx 2.9$ mM) no enzyme induction took place. After decreasing the benzene input to again 2.24 mmol h^{-1} the culture slowly regained its capacity to bioconvert benzene to CBG and eventually a CBG concentration of 11.6 mM was reached.

During the described experiments a significant decrease in biomass, measured as turbidity, was observed (Figure 4). This effect, which was enhanced at increasing benzene flows, very likely arises from the fact that under the stated carbon-limited conditions an essential part of the provided substrate is used to generate reduction equivalents for the oxidation of benzene to CBG. Assuming that 1 mol NADH is needed for the oxidation of 1 mol of benzene to CBG, a hypothetical amount of NADH resulting from 1 mol of succinic acid can be calculated. After continuously adding 1.03 mmol benzene h^{-1} 0.8 mmol CBG h^{-1} was formed (Figure 4B). During this process the biomass concentration decreased about 17% (Figure 4B). Assuming that under such conditions also 17% of the supplied succinic acid (0.68 mmol h^{-1}) is used for CBG formation, a NADH/succinic acid ratio of about 6.6 was calculated. This value corresponds rather well with the theoretical expected value. The decrease of biomass after adding 0.69 mmol benzene h^{-1} (Figure 4A), however, was too low to calculate a reliable NADH/succinic acid ratio, while from the data of Figure 4C (succinic acid supply, 0.68 mmol h^{-1}; benzene addition, 2.24 mmol h^{-1}; CBG formation, 0.93 mmol h^{-1}; biomass decrease, about 33%) a value of about 4.5 was calculated. Probably as a result of the presence of benzene during the latter experiment (Figure 4C; $c_l \approx 0.85$ mM) a bigger decrease of biomass and thus a lower NADH/succinic acid ratio was

obtained than for the experiment of Figure 4B during which almost no benzene was present in the culture medium.

Production of CBG under carbon-limited conditions was only possible for a limited period. After about 65 h the CBG concentration started to decrease, which was accompanied by an increase in biomass concentration and a decrease in the dissolved oxygen concentration of the cultures. Experiments with washed cells and with cell extracts showed that cells had reverted to the wild type and had outcompeted the mutant cells as a result of further metabolism of accumulated CBG and complete utilization of benzene.

Continuous formation of CBG under nitrogen-limited conditions

To prevent further oxidation of CBG by revertants the chemostat was operated under nitrogen-limited conditions. It was expected that under such conditions the revertant would have no advantage over Mt 92, resulting in a prolonged CBG production. The medium composition was changed as described in Materials and Methods with 7.5 g l^{-1} succinic acid to obtain nitrogen limitation. However, after addition of 2.8 mmol benzene h^{-1} to the culture, almost no CBG accumulation was observed. HPLC analysis of the culture-medium showed that next to other organic acids still 2.4 g l^{-1} succinic acid was present in the medium. Separate growth experiments of the wild type, *Pseudomonas* 50, on succinate and benzene simultaneously, showed that under such conditions diauxie occurred. First, succinic acid was utilized and then,

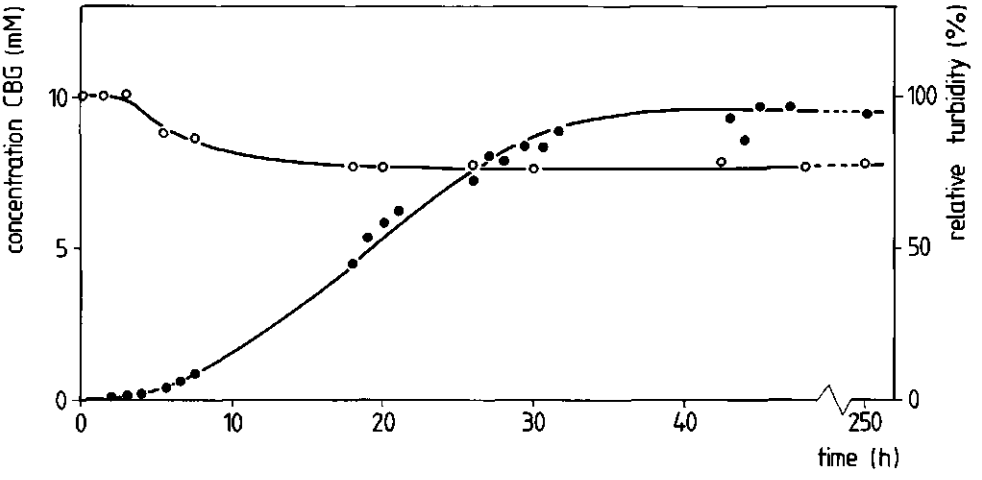

Figure 5. Continuous CBG (•) production by Mt 92 growing under nitrogen-limited conditions in the chemostat. At time zero (protein content 210 mg l^{-1}) 2.8 mmol benzene h^{-1} was supplied via the air stream to the fermentor. ○, turbidity of the culture.

after a subsequent induction phase, benzene. Evidently, in the presence of succinic acid, repression of induction of the benzene-degrading enzymes occurred. To prevent this repression, the succinic acid concentration in the supplied medium was reduced to 2.5 g l^{-1}. At steady state no succinic acid was present in the culture-medium, but other organic acids were still detected. No ammonia was detected in the culture fluid. Supplying 2.8 mmol benzene h^{-1} to this culture resulted in CBG production (Figure 5). Under these nitrogen-limited conditions no repression of enzyme induction occurred; a longer enzyme-induction phase than that found for carbon-limited conditions was observed (Figure 4C), possibly because the nitrogen needed for enzyme formation is not available in sufficient amounts. After about 40 h a CBG concentration of 9.6 mM was obtained and this level was maintained for more than 10 days (Figure 5). A very stable population was therefore obtained under nitrogen-limited conditions with no revertants interfering in the process. During this CBG-production process the turbidity again decreased after benzene introduction. This effect may result from the fact that an appreciable amount of benzene was present in the water phase ($c_l \approx 1.25$ mM). It should be noted, however, that under nitrogen-limited conditions the cells may accumulate large amounts of storage material which might invalidate the turbidity as a measure of biomass.

Theoretical considerations concerning the continuous bioproduction of CBG

A model was developed to give a valuable prediction about the accumulation of CBG in time. To create this model, a distinction had to be made between two production processes. During the experiments described in Figures 4A and 4B the rate of CBG formation was limited by transport of benzene to the liquid, whereas Figure 4C describes the results for a biologically-limited production process. A model will be given first for the benzene-transport-limited CBG production process and second for the biologically-limited process. Establishing a mass balance for benzene over the bioreactor, the following Equation is obtained:

$$F_g c_{g,i} = k_L a(c_l^* - c_l)V + F_g c_g \qquad (2)$$

where F_g is the gas flow rate, $c_{g,i}$ is the benzene concentration in the ingoing gas, $k_L a$ is the volumetric gas-liquid benzene transfer coefficient, c_l is the benzene concentration in the culture fluid, c_l^* is the benzene concentration at the gas liquid interface, V is the working volume of the fermentor, and c_g is the benzene concentration in the gas phase of the fermentor. In Equation 2 it is assumed that

the flows of in- and out-flowing gas are equal and this assumption is valid because the flows did not differ by more than 5 percent from each other as was shown experimentally. According to the film theory:

$$c_l^* = c_g K \tag{3}$$

where K is the partition coefficient of benzene between the water phase and the gas phase. Substitution of Equation 3 into Equation 2 gives:

$$F_g c_{g,i} = k_L a(c_g K - c_l)V + F_g c_g \tag{4}$$

During the experiments described in Figures 4A and 4B, c_l equalled zero. Substitution of $c_l \approx 0$ and rearrangement of Equation 4 gives:

$$c_g = c_{g,i}\left(\frac{F_g}{k_L aKV + F_g}\right) \tag{5}$$

Using this Equation the amount of benzene transferred to the culture medium, which is equal to the amount of CBG produced, can be described as follows:

$$F_g(c_{g,i} - c_g) = \frac{c_{g,i}}{\frac{1}{F_g} + \frac{1}{k_L aKV}} \tag{6}$$

Using Equation 6 the increase of the CBG concentration in time (dx/dt) after introduction of benzene in the system can be calculated using the following material balance:

$$\frac{dx}{dt} \cdot V = \left(\frac{c_{g,i}}{\frac{1}{F_g} + \frac{1}{k_L aKV}}\right) - F_l x \tag{7}$$

in which F_l is the liquid flow rate. Solution of this differential mass balance with $x(0)=0$ yields:

$$x_l = \frac{c_{g,i}}{F_l} \cdot \left(\frac{1}{\frac{1}{F_g} + \frac{1}{k_L aKV}}\right) \cdot [1 - \exp\{-(F_l/V) \cdot t\}] \tag{8}$$

With Equation 8 the CBG concentration profile can be described for benzene-transport-limited production processes (Figures 4A and 4B). However, directly after benzene introduction no CBG will accumulate since the appropriate enzymes have to be synthesized first. Taking into account an induction phase of 5 h, and substitution of the appropriate values for the various parameters in Equation 8, the model predictions as shown in Figures 4A and 4B are obtained. The fact that the theoretical values are higher than the experimental data probably results from the assumption that $c_l \approx 0$. However, because of the nature of benzene it is very difficult to accurately measure its concentration in the water phase.

In case the CBG production is limited by the maximal activity of the biocatalyst, the material balance for CBG over the reactor is given by Equation 9:

$$\frac{dx}{dt} V = V_m P - F_l x \tag{9}$$

where V_m is the maximal CBG formation activity and P is the total protein content in the culture vessel. Solution of this differential mass balance with $x(0)=0$ gives Equation 10:

$$x(t) = \frac{V_m P}{F_l} \cdot [1 - \exp\{-(F_l/V) \cdot t\}] \tag{10}$$

Assuming that the protein content for the biologically-limited production process described by Figure 4C is reduced according to the turbidity (≈33%) a V_m of about 84 nmol min^{-1} (mg protein)$^{-1}$ [1.4 10^{-6} mmol s^{-1} (mg protein)$^{-1}$] was calculated from the obtained CBG concentration after 40 h. Introducing again an enzyme induction phase of 5 h the CBG-formation curve for this biologically-limited production was simulated by means of Equation 10 (Figure 4C).

CONCLUSIONS

Mt 92, which is derived from a *Pseudomonas* sp. able to grow on benzene, can be employed to achieve a continuous production process based on growing cells for the conversion of benzene to CBG. The process described was based on the addition of benzene to a chemostat that was operated under either carbon- or nitrogen-limited conditions. Under carbon-limited conditions the production of CBG resulted in a decrease of biomass, evidently a significant amount of the reduction equivalents obtained from succinic acid oxidation was used for the

dioxygenation of benzene. To predict the concentration of CBG a mathematical model was developed that, taking into account an enzyme induction phase of 5 h, agreed rather well with the experimental data. Operation under nitrogen-limited conditions is favored because under such conditions revertants to the wild type did not have an advantage over Mt 92. For the experiments described herein, succinic acid was used as the growth substrate, however, because of repression of enzyme induction at detectable succinic acid concentrations the use of other growth substrates might be favorable. Growing Mt 92 on succinate we were able to produce about 1 mol CBG per mol succinic acid. Since benzene concentrations above 2.5 mM were inhibitory to Mt 92, the supply of benzene had to be controlled very strictly. The introduction of an organic solvent with a high logP value as described by Rezessy-Szabó et al. (1987) is probably very suitable to circumvent benzene toxicity. In this respect, we currently are investigating this bioconversion in a liquid-impelled loop reactor (Tramper et al., 1987). To study a possible inhibition of the bioconversion by CBG (Jenkins & Dalton, 1985) and to facilitate CBG recovery, we currently are also investigating this bioconversion at higher CBG concentrations.

ACKNOWLEDGEMENTS

This project is done in cooperation with Prof. A.P.G. Kieboom and Prof. H. van Bekkum, Department of Organic Chemistry, Technical University Delft, and with Prof. K.Ch.A.M. Luyben, Department of Chemical Engineering, Technical University Delft. We are grateful to H. Hey (Department of Soil Science and Plant Nutrition, Agricultural University Wageningen) for analysing the nitrogen content of the culture-medium, and to A. van Neerven for making electron microscopic photographs. These investigations were supported in part by the Netherlands Technology Foundation (STW).

NOMENCLATURE

- a gas-liquid specific surface area (m^{-1})
- $c_{g,i}$ benzene concentration of the incoming gas (mM)
- c_g benzene concentration in the gas phase of the reactor (mM)
- c_l benzene concentration in the water phase (mM)
- c_l^* benzene concentration at the gas liquid interface (mM)
- D diffusion coefficient of either oxygen or benzene in water (m^2 s^{-1})
- F_g ingoing gas flow rate ($l\ s^{-1}$)
- F_l liquid flow rate ($l\ s^{-1}$)

K partition coefficient of benzene between the water phase and the gas phase at 30°C (-)
k_L mass transfer coefficient in the liquid film adjacent to the gas liquid interphase (m s^{-1})
P total amount of protein in the culture vessel (mg protein)
t time (s)
V working volume of the reactor (l)
V_m maximal CBG production rate (mmol s^{-1} (mg protein)$^{-1}$)
x_t CBG concentration (mM)

REFERENCES

Ballard, D.G.H., Courtis, A., Shirley, I.M. & Taylor, S.C. (1983). A biotech route to polyphenylene. Journal of the Chemical Society, Chemical Communications, pp. 954-955

Bochner, B.R. & Savageau, M.A. (1977). Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. Applied and Environmental Microbiology 33,434-444

Brink, L.E.S. & Tramper, J. (1986). Modelling the effects of mass transfer on kinetics of propene epoxidation of immobilized Mycobacterium cells: pseudo-one-substrate conditions and negligible product inhibition. Enzyme and Microbial Technology 8,281-288

de Bont, J.A.M., Vorage, M.J.A.W., Hartmans, S. & van den Tweel, W.J.J. (1986). Microbial degradation of 1,3-dichlorobenzene. Applied and Environmental Microbiology 52,677-680

Ensley, B.D., Ratzkin, B.J., Osslund, T.D., Simon, M.J., Wackett, L.P. & Gibson, D.T. (1983). Expression of the naphtalene oxidation genes in Escherichia coli results in the biosynthesis of indigo. Science 222,167-169

Finette B.A., Subramanian, V. & Gibson, D.T. (1984). Isolation and characterization of Pseudomonas putida PpF1 mutants defective in the toluene dioxygenase enzyme system. Journal of Bacteriology 160,1003-1009

Gibson, D.T., Hensley, M., Yoshioka, H. & Mabry, T.J. (1970a). Formation of (+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by Pseudomonas putida. Biochemistry 7,1626-1630

Gibson, D.T., Cardini, G.E., Maseles, F.C. & Kallio, R.E. (1970b). Incorporation of Oxygen-18 into benzene by Pseudomonas putida. Biochemistry 9,1631-1635

Gibson, D.T., Gschwendt, B., Yeh, W.K. & Kobal, V.M. (1973). Initial reactions in the oxidation of ethylbenzene by Pseudomonas putida. Biochemistry 12,1520-1528

Gibson, D.T., Mahadevan, V. & Davey, J.F. (1974). Bacterial metabolism of para- and meta-xylene: oxidation of the aromatic ring. Journal of Bacteriology 119,930-936

Haas, D. (1983). Genetic aspects of biodegradation by Pseudomonads. Experientia 39,1199-1213

Hayaishi, O., Katagiri, M. & Rothberg, S. (1957). Studies on oxygenases: pyrocatechuase. Journal of Biological Chemistry 229,905-920

Högn, T. & Jaenicke, L. (1972). Benzene metabolism of Moraxella species. European Journal of Biochemistry 30,369-375

Ichikawa, Y., Sato, S. & Takahashi, J. (1981). Effect of pressure on biomass production from n-butane. Journal of Fermentation Technology 59,269-273

Jeffrey, A.M., Yeh, H.J.C., Jerina, D.M., Patel, T.R., Davey, J.F. & Gibson, D.T. (1975). Initial reactions in the oxidation of naphthalene by Pseudomonas putida. Biochemistry 14,575-584

Jenkins, R.O. & Dalton, H. (1985). The use of indole as a spectrophotometric assay substrate for toluene dioxygenase. FEMS Microbiology Letters 30,227-231

Jenkins, R.O., Stephens, G.M. & Dalton, H. (1987). Production of toluene cis-glycol by Pseudomonas putida in glucose fed-batch culture. Biotechnology and Bioengineering 29,873-883

Kojima, Y., Hada, N. & Hayaishi, O. (1961). Metapyrocatechuase: a new catechol-cleaving enzyme. Journal of Biological Chemistry 236,2223-2228

Ley, S.V., Sternfeld, F. & Taylor, S.C. (1987). Microbial oxidation in synthesis: a six step preparation of (+)-pinitol from benzene. Tetrahedron Letters 28,225-226

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Nakajima, M., Tomida, I. & Takei, S. (1959). Darstellung von cis-Benzolglykol, Stereoisomeren Mucondialdehyden und Kondurit-F. Chemische Berichte 92,163-172

Novozamski, I, van Eck, R., van Schouwenburg, J.C. & Walinga, I. (1974). Total nitrogen determination in plant material by means of the indophenolblue method. Netherlands Journal of Agricultural Sciences 22,3-5

Perry, R.H. & Chilton, C.H. (1969). Chemical Engineerings Handbook, fifth edition. McGraw-Hill Inc., New York

Reineke, W., Otting, W. & Knackmuss, H.-J. (1978). cis-Dihydrodiols microbially produced from halo- and methylbenzoic acids. Tetrahedron 34,1707-1714

Reineke, W. & Knackmuss, J.-H. (1984). Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Applied and Environmental Microbiology 47,395-402

Reiner, A.M. & Hegeman, G.D. (1971). Metabolism of benzoic acid by bacteria. Accumulation of (-)-3,5-cyclohexadiene-1,2-diol-1-carboxylic acid by a mutant strain of Alcaligenes eutrophus. Biochemistry 10,2530-2536

Rezessy-Szabó, J.M., Huijberts, G.N.M. & de Bont, J.A.M. (1987). Potential of organic solvents in cultivating microorganisms on toxic water-insoluble compounds. In: Biocatalysis in Organic Media. Studies in Organic Chemistry 29, pp. 295-302. Elsevier, Amsterdam

Taylor, S.C. (1983). Biochemical process. European Patent Application 0,076,606

Taylor, S.C. (1985). Enzymic synthesis of 5,6-dihydroxycyclohexa-1,3-diene. In: Enzymes in Organic Synthesis, Ciba Foundation Symposium, III, pp. 71-75. Pitman, London

Tramper, J., Wolters, I. & Verlaan, P. (1987). The liquid-impelled loop reactor: a new type of density-difference mixed bioreactor. In: Biocatalysis in Organic Media. Studies in Organic Chemistry 29, pp. 311-316. Elsevier, Amsterdam

van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1986). Microbial metabolism of D- and L-phenylglycine by Pseudomonas putida LW-4. Archives of Microbiology 144,169-174

Vishniac, W. & Santer, M. (1957). The Thiobacilli. Bacteriological Reviews 21,195-213

Whited, G.M., McCombie, W.R., Kwart, L.D. & Gibson, D.T. (1986). Identification of cis-diols as intermediates in the oxidation of aromatic acids by a strain of Pseudomonas putida that contains a TOL plasmid. Journal of Bacteriology 166,1028-1039

Yarmoff, J.J., Kawakami, Y., Yago, T. & Nishimura, H. (1986). Benzene degradation and cis-benzene glycol production. Cloning of the benzene degradation genes. EMBO Workshop: Genetic Manipulation of Pseudomonads-Application in Biotechnology and Medicine.

Zeyer, J., Lehrbach, P.R. & Timmis, K.N. (1985). Use of cloned genes of Pseudomonas TOL plasmid to effect biotransformation of benzoates to cis-dihydrodiols and catechols by Escherichia coli cells. Applied and Environmental Microbiology 50,1409-1413

Ziffer, H., Kabuto, K., Gibson, D.T., Kobal, V.M. & Jerina, D.M. (1977). The absolute stereochemistry of several cis-dihydrodiols microbially produced from substituted benzenes. Tetrahedron 33,2491-2496

Chapter 9

THE APPLICATION OF ORGANIC SOLVENTS FOR THE BIOCONVERSION OF BENZENE TO CIS-BENZENEGLYCOL

W.J.J. van den Tweel, E.H. Marsman, M.J.A.W. Vorage, J. Tramper and J.A.M. de Bont

SUMMARY

Benzene was oxidized to cis-benzeneglycol (CBG) by a mutant of a *Pseudomonas* sp. (strain Mt 92). A prolonged continuous production of CBG from benzene was achieved by supplying benzene to Mt 92 cells growing on succinate under nitrogen-limited conditions in a chemostat. During such continuous production experiments the benzene concentration should be kept low to minimize the toxic effect of benzene. Incubation experiments with Mt 92 showed that n-hexadecane is a suitable solvent to circumvent benzene toxicity during the bioformation of CBG from benzene. Moreover, the addition of n-hexadecane did not significantly effect the rate of CBG formation. Since the bioconversion could easily be performed in the presence of n-hexadecane the feasibility of a recently described liquid-impelled loop reactor was investigated. Preliminary experiments showed that such bioreactor can indeed be used for the conversion of benzene to CBG.

In: Bioreactors and Biotransformations (Moody, G.W. & Baker, P.B., Eds.), pp. 231-241. Elsevier Applied Science publishers, London

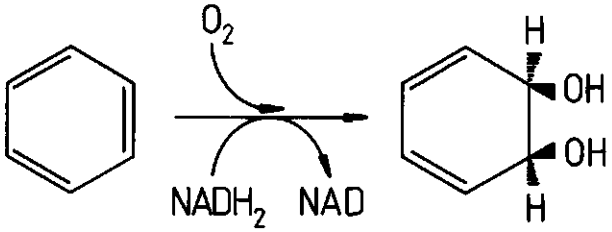

Figure 1. Bioconversion of benzene to CBG.

INTRODUCTION

CBG and other cis-dihydrodiols may be useful building blocks for the production of synthetic polymers (Ballard et al., 1983) and various pharmaceuticals (Ley et al., 1987). Already in 1970 Gibson and coworkers isolated a mutant of *Pseudomonas putida* which was able to convert benzene to CBG (Figure 1). Using this mutant ICI was able to produce CBG in kilogram quantities for the production of polymerization monomers (Taylor, 1985). Moreover, the broad substrate specificity of the pertinent dioxygenase also enabled the production of a range of substituted cis-dihydrodiols (Taylor, 1983). In spite of the huge interest in CBG only minor information is available on bioproduction processes for CBG (Jenkins et al., 1987; van den Tweel et al., 1988). Both biological and process engineering aspects that limit the exploitation of the CBG production process apparently need more detailed investigations. In the present work we have concentrated on the aspect of benzene toxicity towards the biocatalyst and it was attempted to overcome this toxicity by using n-hexadecane as a second liquid phase. The influence of many water-immiscible solvents on retention of activity of immobilized *Mycobacterium* cells was determined by Brink and Tramper (1985) and it was found that retention of activity is usually favoured by low solvent polarity in combination with a high molecular weight. LogP, which is defined as the logarithm of the partition coefficient of a given compound in a standard octanol-water two phase system is a very useful parameter to describe a correlation between biocatalytic activity and solvent properties (Laane et al., 1985). Solvents with a logP smaller than 2 were least suitable for biocatalysis while solvents having a logP above 4 were readily applicable.

This paper shows that n-hexadecane, which has a logP value of 8.8, is a suitable solvent to circumvent the inhibitory effect of benzene on the biocatalyst. Moreover, preliminary experiments are presented on the use of a liquid-impelled loop reactor, a new type of density-difference-mixed bioreactor (Tramper et al., 1987), for the bioformation of CBG from benzene.

MATERIALS AND METHODS

Organism. The isolation and characterization of Mt 92, which is able to convert benzene stoichiometrically to CBG, will be described elsewhere (van den Tweel et al., 1988).

Continuous bioproduction of CBG. Continuous formation of CBG was achieved by Mt 92 cells growing on succinic acid under nitrogen-limited conditions in a chemostat (working volume 0.93 l, 30°C, pH 7.0, dilution rate 0.085 h^{-1}) (Figure 2; van den Tweel et al., 1988). Air at a rate of 60 ml per minute was supplied to the fermentor through an inlet beneath the stirrer. Strain Mt 92 was routinely grown in a mineral salts medium containing in 1.0 l of deionized water: K_2HPO_4, 1.55 g; NaH_2PO_4, 0.85 g; NH_4Cl, 0.15 g; Na_2SO_4, 0.125 g; $MgCl_2.6H_2O$, 0.075 g; yeast extract, 0.2 g and 0.2 ml of a trace element solution (Visniac & Santer, 1957). Succinic acid was added to this mineral salts medium at 2.5 g l^{-1}. At steady state a part of the air was passed through a column containing benzene (van den Tweel et al., 1988). As a result of the appearance of benzene in the culture medium the benzene dioxygenase was induced and CBG started to accumulate.

Incubation experiments with induced Mt 92 cells. The effluent culture medium of the continuous bioproduction experiment was collected in a tank which was kept at 0°C. After one day these induced cells were harvested by centrifugation (16,000 g for 5 min at 4°C), washed with potassium phosphate buffer pH 7.0 (50 mM), resuspended in the same buffer and used for the incubation experiments. Incubations were performed at 30°C in 315 ml serum bottles containing 19 ml of the following nitrogen-free mineral salts medium: K_2HPO_4, 1.55 g l^{-1}; NaH_2PO_4, 0.85 g l^{-1}; Na_2SO_4, 0.1 g l^{-1}; $MgCl_2.6H_2O$, 0.075 g l^{-1} and 0.2 ml l^{-1} of the forementioned trace element solution. Unless stated otherwise 0.5 g l^{-1} succinic acid was added to the medium for cofactor regeneration. After addition of the specified amount of benzene the incubation was initiated by the addition of 1 ml induced Mt 92 cells (total protein 14 mg). In some experiments 20 ml n-hexadecane was added to the serum bottles prior to benzene addition. Samples, taken at various times from the water phase, were analysed for the concentration of CBG by HPLC.

Production of CBG in the liquid-impelled loop reactor. Figure 6 gives a schematic representation of the employed liquid-impelled loop reactor. The height of the reactor was 60 cm, the internal diameter of the downcomer and riser was 25 and 40 mm, respectively. The total volume of the reactor was 1.25 l (1.0 l water and 0.25 l n-hexadecane). The design and principles of mixing of the liquid-impelled loop reactor are the same as applied in the airlift loop reactor. In the liquid-impelled loop reactor the density-difference mixing was created by

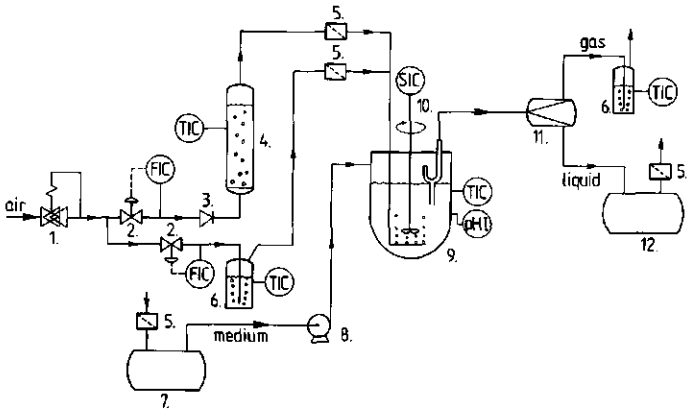

Figure 2. Schematic representation of the chemostat used for continuous CBG production. Pressure reducer, 1; mass flow controller, 2; one-way valve, 3; benzene column, 4; air filter, 5; gas washer, 6; medium tank, 7; pump, 8; fermentor vessel, 9; stirrer, 10; separator, 11; effluent tank, 12. TIC, FIC, SIC and pHIC; temperature, flow rate, stirring speed and pH indication and control.

injection of n-hexadecane (19 l h^{-1}) which has a density (0.773 g ml^{-1} at 20°C) smaller than that of water. To supply oxygen to Mt 92 cells the recirculating n-hexadecane was aerated in an aeration flask containing 350 ml n-hexadecane. The temperature of the bioreactor was kept constant at 30°C by a thermocontrolled recycling waterbath. For the CBG production experiment the forementioned nitrogen-free mineral salts medium was used to which 0.5 g l^{-1} succinic acid was added. After adding 5 ml benzene to the hexadecane phase, the experiment was started by adding induced Mt 92 cells (total protein 2.5 g). Samples were taken from the water phase and analysed for the amount of CBG.

Analyses. Protein concentrations were measured by the method of Lowry et al. (1951). Benzene and CBG concentrations were determined by reverse-phase HPLC (van den Tweel et al., 1988).

Materials. Benzene was purchased from Merck-Schuchard, Hohenbrunn, FRG. n-Hexadecane was a product from J.T. Baker Chemicals, Deventer, The Netherlands. All other chemicals were commercially available analytical grade products and were used without further purification.

RESULTS AND DISCUSSION

Continuous bioproduction of CBG

In order to investigate in more detail some intriguing aspects of the bioconversion of benzene to CBG (Figure 1), e.g. enzyme induction,

cofactor regeneration, stability of the mutant, toxicity of substrate and/or product, we have studied the continuous production of CBG by Mt 92 cells growing on succinic acid in a chemostat (van den Tweel et al., 1988). Since production of CBG under carbon-limited conditions was only possible for a limited period as a result of revertation of the mutant to the wild type, the bioreactor (Figure 2) was operated under nitrogen-limited conditions as described in Materials and Methods. At steady state, 2.8 mmol benzene h^{-1} was supplied to the culture (Figure 3). After a period of enzyme induction CBG started to accumulate in the medium reaching a maximum concentration of 9.6 mM. Under these conditions a very stable bioproduction process was obtained, even after 10 days the CBG concentration was maintained maximal (Figure 3). During such continuous CBG production processes the benzene concentration was a very crucial parameter. Concentrations above about 2.5 mM had an inhibitory effect on both growth and induction of the benzene dioxygenase (van den Tweel et al., 1988). Consequently, the benzene concentrations should be carefully controlled to minimize the toxic effect of benzene.

During the continuous processes only about one mol of CBG was produced per mol of succinic acid utilized. Effluent cells which still have the ability to convert benzene to CBG, however, may be reused, either batchwise or continuously, to give a more economic process. Since benzene will also inhibit such bioconversion, its effect on induced cells was studied in more detail.

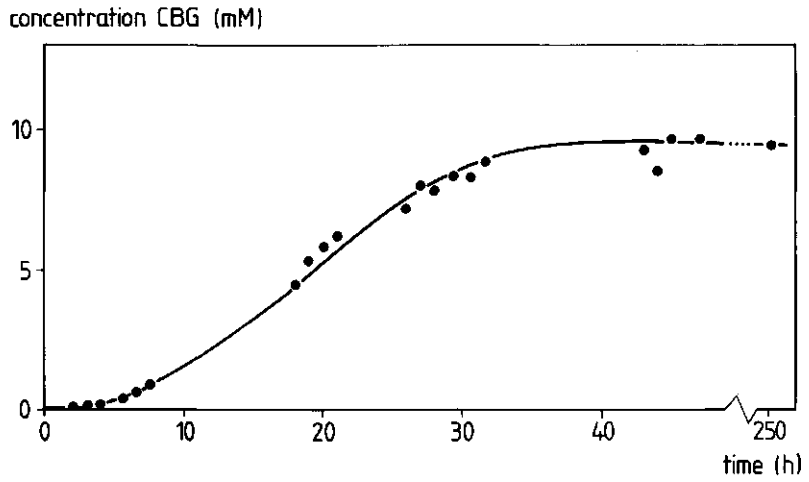

Figure 3. Continuous CBG (•) production by Mt 92 growing under nitrogen-limited conditions on succinic acid in the chemostat. At time zero (protein content 210 mg l^{-1}) 2.8 mmol benzene h^{-1} was supplied via the air stream to the fermentor.

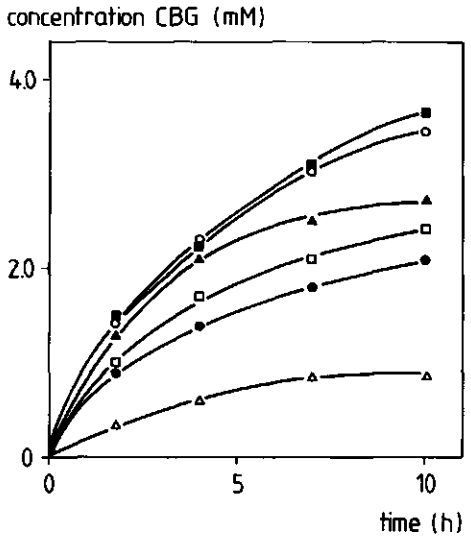

Figure 4. Effect of the amount of benzene on the formation of CBG by Mt 92. Incubations were performed as described under Materials and Methods without any hexadecane added. ■, 10; ○, 25; ▲, 50; □, 75; ●, 100; and △, 150 μl benzene.

The effect of benzene on the bioformation of CBG by induced Mt 92 cells

To study the effect of benzene on the activity of induced Mt 92 cells, incubation experiments were performed in the presence of increasing amounts of benzene (Figure 4). In order to prevent growth during these experiments the incubations were done in a nitrogen-free mineral salts medium. Under the specified conditions the addition of 10 μl benzene resulted in a benzene concentration in the water phase of 0.95 mM. The best results for the bioformation were obtained using 10 and 25 μl benzene (initial benzene concentrations 0.95 and 2.38 mM, respectively). After an initial CBG formation activity of about 19 nmol min^{-1} (mg protein)$^{-1}$ the rate significantly decreased, probably as a result of the depletion of reduction equivalents. In the presence of 100 μl benzene (initial concentration 9.5 mM) still a fairly good CBG production was observed. This is in contrast to growth of the wild type which was inhibited above benzene concentrations of 2.5 mM (van den Tweel et al., 1988). Evidently, CBG formation by induced cells is less susceptible to high benzene concentrations than is growth. To obtain an optimal bioconversion process with nongrowing cells the benzene concentration in the water phase on the one hand should be minimal, whereas on the other hand a permanent supply of benzene to the cells is also required.

The use of n-hexadecane for the bioconversion of benzene to CBG by induced Mt 92 cells

Recently, studies by Rezessy-Szabó et al. (1987) have demonstrated the potential of organic solvents with high logP values (e.g. n-hexadecane and dibutylphtalate) to circumvent benzene toxicity during growth of the wild type of Mt 92 (*Pseudomonas* 50). In order to investigate the potential of organic solvents to reduce the inhibitory effect of benzene on the bioformation of CBG from benzene, incubation experiments were done with induced Mt 92 cells in the presence of n-hexadecane (50% [v/v]). In contrast to foregoing experiments high amounts of benzene in the presence of n-hexadecane did not influence the bioformation of CBG (Figure 5). Moreover, the CBG formation rate for all additions tested was about the same and almost equalled the rate which was obtained in the absence of n-hexadecane after adding 10 µl benzene (Figure 4). HPLC analyses showed that after addition of n-hexadecane no detectable concentrations of benzene were present in the water phase. Evidently, by reducing the benzene concentration in the water phase the inhibitory effect of benzene was circumvented. The results show furthermore that the activity of the biocatalyst was limiting the CBG formation rate rather than benzene mass transfer from the organic solvent to the water phase. Similar results have also been obtained by Furuhashi et al. (1986), in that the addition of n-hexadecane reduced both the toxicity of the substrate (styrene) and of the product (styreneoxide)

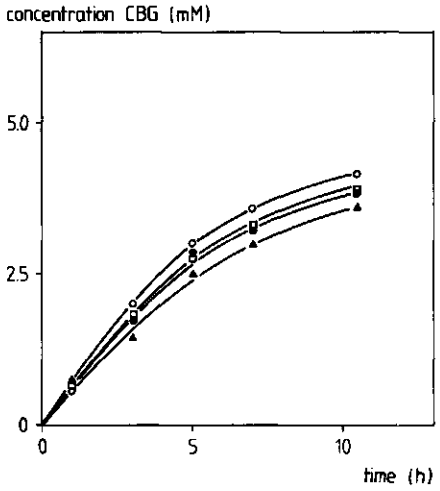

Figure 5. The use of hexadecane to decrease the toxic effect of increasing amounts of benzene on the bioconversion of benzene to CBG by Mt 92. Incubations were performed as described under Materials and Methods in the presence of hexadecane. ●, 50; □, 100; ▲, 200; and ○, 400 µl benzene.

which resulted in an enhanced production of styreneoxide from styrene by *Nocardia corallina* cells.

Bioformation of CBG in the liquid-impelled loop reactor

Recently Tramper et al. (1987) have introduced a new type of bioreactor: the liquid-impelled loop reactor. In its simplest configuration this reactor contains two phases, a water phase and a water-immiscible organic solvent phase. Density-difference mixing can be obtained by the injection of an organic solvent with a density smaller than water at the bottom of the riser. Since the use of such bioreactor might be advantageous for the bioproduction of CBG (reduction of the inhibitory effect of benzene, good mass tranfer characteristics, and the possibility to enhance the supply of oxygen since oxygen is better soluble in many organic solvents than in water) some preliminary experiments were performed in a n-hexadecane-impelled loop reactor. Initial experiments were performed in a bioreactor with an external loop since phase separation was easier with such a configuration (Tramper et al., 1987). Oxygen was indirectly supplied to this bioreactor by blowing air through the recirculating n-hexadecane solution as schematically shown in Figure 6. Moreover, the aeration flask can also be used to add other components to the bioreactor. Under the specified conditions the density difference between water and n-hexadecane and the recirculating flow rate of n-hexadecane were high enough to achieve a good circulation of the water phase and to allow a good phase separation. After the addition of benzene to the hexadecane phase, the experiment was started by adding induced Mt 92 cells (total protein 2.5 g) to the water phase.

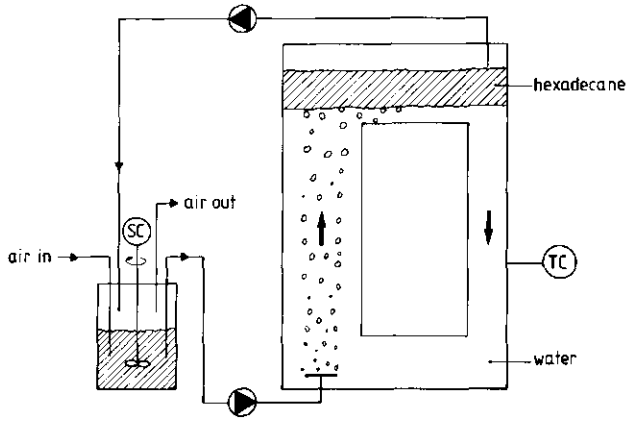

Figure 6. Schematic representation of the liquid-impelled loop reactor.

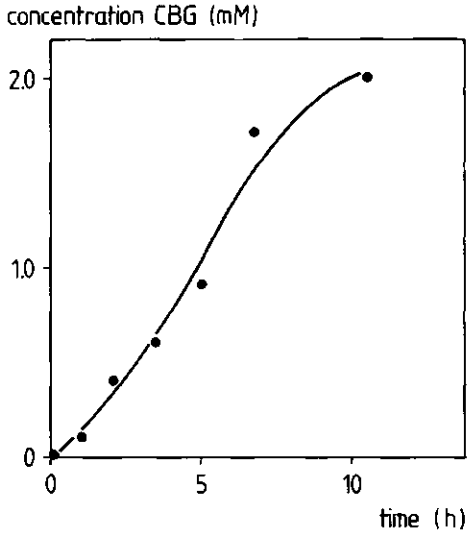

Figure 7. Formation of CBG (•) by induced MT 92 cells in the described liquid-impelled loop reactor.

Immediately CBG started to accumulate in the water phase reaching a concentration of 2 mM after about 10 h (Figure 7). Currently we are investigating the use of the liquid-impelled loop reactor more fundamentally and simultaneously we are optimizing the bioformation of CBG in this new type of bioreactor.

CONCLUSIONS

Continuous bioformation of CBG from benzene by Mt 92 cells growing on succinate under nitrogen-limited conditions in a chemostat can easily be achieved. However, a low benzene concentration is a prerequisite for good performance of the bioconversion process. In order to make the continuous process economically more attractive, it is also necessary to reuse the produced cells. Again a low benzene concentration is a requirement to be met for an optimal bioformation of CBG under such non-growth conditions. The addition of an organic solvent with a high logP value such as n-hexadecane can be very useful to circumvent the toxic effect of benzene on the production of CBG by either growing or non-growing cells. CBG itself also might have an inhibitory effect on the bioformation (Jenkins et al., 1987; Jenkins & Dalton, 1985), however, because of its good solubility in water the addition of a water-immiscible organic solvent probably will not reduce this product inhibition.

Preliminary experiments with a liquid-impelled loop reactor have shown that such type of bioreactor might be useful for the bioformation of CBG. Currently we are studying this possibility in more detail.

REFERENCES

Ballard, D.G.H., Courtis, A., Shirley, I.M. & Taylor, S.C. (1983). A biotech route to polyphenylene. Journal of the Chemical Society, Chemical Communications pp. 954-955

Brink, L.E.S. & Tramper, J. (1985). Optimization of organic solvent in multiphase biocatalysis. Biotechnology and Bioengineering 27,1258-1269

Furuhashi, K., Shintani, M. & Takagi, M. (1986). Effects of solvents on the production of epoxides by Nocardia corallina B-276. Applied Microbiology and Biotechnology 23,218-223

Gibson, D.T., Cardini, G.E., Maseles, F.C. & Kallio, R.E. (1970). Incorporation of oxygen-18 into benzene by Pseudomonas putida. Biochemistry 9,1631-1635

Jenkins, R.O. & Dalton, H. (1985). The use of indole as a spectrophotometric assay substrate for toluene dioxygenase. FEMS Microbiology Letters 30,227-231

Jenkins, R.O., Stephens, G.M. & Dalton, H. (1987). Production of toluene cis-glycol by Pseudomonas putida in glucose fed-batch culture. Biotechnology and Bioengeneering 29,873-883

Laane, C., Boeren, S. & Vos, K. (1985). On optimizing organic solvents in multi-liquid-phase biocatalysis. Trends in Biotechnology 3,251-252

Ley, S.V., Sternfield, F. & Taylor, S.C. (1987). Microbial oxidation in synthesis: a six step preparation of (+)-pinitol from benzene. Tetrahedron Letters 28,225-226

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Rezessy-Szabó, J.M., Huijberts, G.N.M. & de Bont, J.A.M. (1987). Potential of organic solvents in cultivating microorganisms on toxic water-insoluble compounds. In: Biocatalysis in Organic Media. Studies in Organic Chemistry 29, pp. 295-302. Elsevier, Amsterdam

Taylor, S.C. (1985). Enzymic synthesis of 5,6-dihydroxycyclohexa-1,3-diene. In: Enzymes in Organic Synthesis. Ciba Foundation Symposium III, pp. 71-75. Pitman, London

Taylor, S.C. (1983). Biochemical process. European Patent Application 0,076,606

Tramper, J., Wolters, I. & Verlaan, P. (1987). The liquid-impelled loop reactor: a new type of density-difference mixed bioreactor. In: Biocatalysis in Organic Media. Studies in Organic Chemistry 29, pp. 311-316. Elsevier, Amsterdam

van den Tweel, W.J.J., Vorage, M.J.A.W., Marsman, E.H., Koppejan, J., Tramper, J. & de Bont, J.A.M. (1988). Continuous production of cis-1,2-dihydroxycyclohexa-3,5-diene (cis-benzeneglycol) from benzene by a mutant of a benzene-degrading Pseudomonas sp. Enzyme and Microbial Technology 10,134-142

Vishniac, W. & Santer, M.(1957). The Thiobacilli. Bacteriological Reviews 21,195-213

Chapter 10

BIOFORMATION OF 4-HYDROXYBENZOATE FROM 4-CHLORO-BENZOATE BY *ALCALIGENES DENITRIFICANS* NTB-1

W.J.J. van den Tweel, N. ter Burg, J.B. Kok and J.A.M. de Bont

SUMMARY

Strain NTB-1, identified as an *Alcaligenes denitrificans*, was isolated from a mixture of soil and sewage samples using 4-chlorobenzoate as sole carbon and energy source. Simultaneous adaptation experiments and enzyme studies revealed that 4-chlorobenzoate was converted to 4-hydroxybenzoate which was further oxidized yielding 3,4-dihydroxybenzoate. Bioformation of 4-hydroxybenzoate from 4-chlorobenzoate by *Alcaligenes denitrificans* NTB-1 was obtained when 4-chlorobenzoate-grown cells were incubated with 4-chlorobenzoate under conditions of low and controlled oxygen concentrations.

Applied Microbiology and Biotechnology (1986) 25,289-294

INTRODUCTION

The risks and hazards associated with halogenated aromatic compounds introduced by man into the environment have in recent years incited many microbiological studies to assess the extent of biodegradation of these compounds. The microbiological degradation of these pollutants has been studied in many ecosystems, and organisms that are able to modify the halogenated aromatic compounds by cometabolism or that may grow at the expense of such compounds have been isolated or constructed (Ghoshal et al., 1985; Knackmuss, 1981). From physiological studies with pure cultures of aerobic organisms it has been possible to define as a general principle that in aerobic catabolism of halogenated aromatics the benzene nucleus is first transformed into an ortho-dihydroxy aromatic compound which is then ringopened by a dioxygenase type of reaction. The resulting non-aromatic halogenated compound may then eventually be hydrolysed to eliminate the halide (Ghoshal et al., 1985; Knackmuss, 1981).

However, exceptions to this general rule, that halide release occurs after ring fission, have been noted for some isolated cases, and these reactions may be attributed to the non-specific action of either a monooxygenase or a dioxygenase (Kaufman, 1961; Guroff et al., 1966; Engesser et al., 1980; Markus et al., 1984; Faulkner & Woodcock, 1961; Shailubhai et al. 1983).

A second and extremely interesting type of reaction also resulting in halide release from a halogenated aromatic compound while forming a hydroxylated aromatic compound is quite different from the above mentioned reactions in that it is O_2-independent (Marks et al., 1984b; Müller et al., 1984). Such reactions have been observed in a *Pseudomonas* species growing on 3-chlorobenzoate (Johnston et al., 1972) and in various organisms utilizing 4-chlorobenzoate (Keil et al., 1981; Klages et al., 1979; Marks et al., 1984a; Ruisinger et al., 1976; Zaitsev & Karasevich, 1981a, 1981b). This novel type of hydrolytic dehalogenation of halogenated aromatic compounds is of great interest because such enzymes yield a hydroxylated aromatic compound from a halo-aromatic. Many hydroxylated aromatic compounds are very important in the pharmaceutical and petrochemical industries but there are no readily available preparative chemical reactions for direct specific aromatic hydroxylation on an industrial scale (Olah et al., 1981). Consequently, the hydroxyl group has to be introduced by an expensive series of reactions. Therefore, many attempts are made to produce such compounds in biological systems by using either enzymes (Klibanov et al., 1981; Vilanova et al., 1984) or whole cells (Baklashova et al., 1984; Daum & Kieslich, 1974; Hagedorn, 1983,1984; Kulla & Lehky, 1985; Taylor, 1982; Theriault & Longfield, 1967, 1973; Yoshida et al., 1973, 1974; Pshirkov et al., 1982) that hydroxylate aromatics.

The alternative direct dehalogenation reaction now available, that would produce hydroxylated aromatics from haloaromatics, has prompted us to start a search for this type of enzyme in various haloaromatic degrading bacteria. To this end, we are currently isolating various haloaromatic degrading organisms on various compounds and investigating whether an initial catabolic reaction involves the novel type of dehalogenase reaction (de Bont et al., 1986). Moreover, it would be very relevant to demonstrate that organisms containing the appropriate enzyme would indeed be applicable as a biocatalyst for the production of hydroxylated aromatic compounds.

In this paper we describe the isolation of an *Alcaligenes denitrificans* sp. that is able to metabolize 4-chlorobenzoate (4-CBA) via 4-hydroxybenzoate (4-HBA) and it is demonstrated that the excretion and accumulation of 4-HBA by this organism takes place under conditions of controlled oxygen limitation.

MATERIALS AND METHODS

Isolation, maintenance and culture of strain NTB-1. For enrichment the following mineral salts medium was used: K_2HPO_4 (1.55 g l^{-1}), $NaH_2PO_4.H_2O$ (0.85 g l^{-1}), $(NH_4)_2SO_4$ (3 g l^{-1}), $MgSO_4.7H_2O$ (0.1 g l^{-1}), yeast extract (0.1 g l^{-1}) and 0.2 ml of a trace element solution (Vishniac & Santer, 1957). 4-CBA (0.4 g l^{-1}) and a mixture of various soil and sewage samples were added to this mineral salts medium. After several months the enrichment culture was plated on a mineral salts agar containing 4-CBA (0.2 g l^{-1}). Plates containing a mineral salts agar without 4-CBA were used as reference. Individual colonies growing at the expense of 4-CBA were picked and checked for purity. One isolate designated NTB-1 was used throughout this investigation. This organism was maintained on agar slopes of 5 g l^{-1} glucose and 3.5 g l^{-1} yeast extract medium to which Oxoid no. 3 agar (15 g l^{-1}) had been added. Cultivation of strain NTB-1 was routinely conducted in a chemostat (dilution rate 0.03 h^{-1}) under carbon limited conditions. The continuous culture (30°C, pH 7) was supplied with the above mineral salts medium to which 4-CBA was added at 1 g l^{-1}. Alternatively, cells were grown batchwise in a 30 l fermentor (4-CBA concentration 1 g l^{-1}). The 4-CBA degrading activity was lower in batch-grown cells than in chemostat-grown cells (5 and 40 nmol 4-CBA consumed min^{-1} (mg protein)$^{-1}$, respectively).

Experiments with whole cells. Simultaneous adaptation experiments were performed as described previously (van den Tweel et al., 1986). Incubation experiments at controlled oxygen concentrations in the gas phase were performed in serum bottles (total volume 130 ml) with washed cells (8.8 mg protein in 7.5 ml 100 mM potassium phosphate

buffer pH 7.0) that had been grown on 4-CBA in chemostat culture. After flushing the bottles for 10 min with N_2 a specified amount of O_2 was added. The reaction was initiated by the addition of 11.25 mmol 4-CBA (unless stated otherwise). The bottles were incubated in a shaking water bath (30°C, 1 Hz). Samples were periodically withdrawn from the incubation mixture and were analysed by means of high-performance liquid chromatography. The oxygen concentration in the gas phase did not significantly decrease during these experiments, as was shown by gas chromatography. Incubation experiments under controlled dissolved oxygen concentrations were performed in a 1.0 l fermentor (30°C, 1000 rpm) equipped with a YSI model 5331 polarographic oxygen probe (Yellow Springs Instruments Co., Yellow Springs, Ohio, USA). The vessel contained washed cells (180 mg protein in 600 ml 100 mM potassium phosphate buffer pH 7.0) that had been grown batch-wise on 4-CBA. The dissolved oxygen concentration was regulated by continuously passing a mixture of N_2 and air (total flow 45 l h^{-1}) through the suspension.

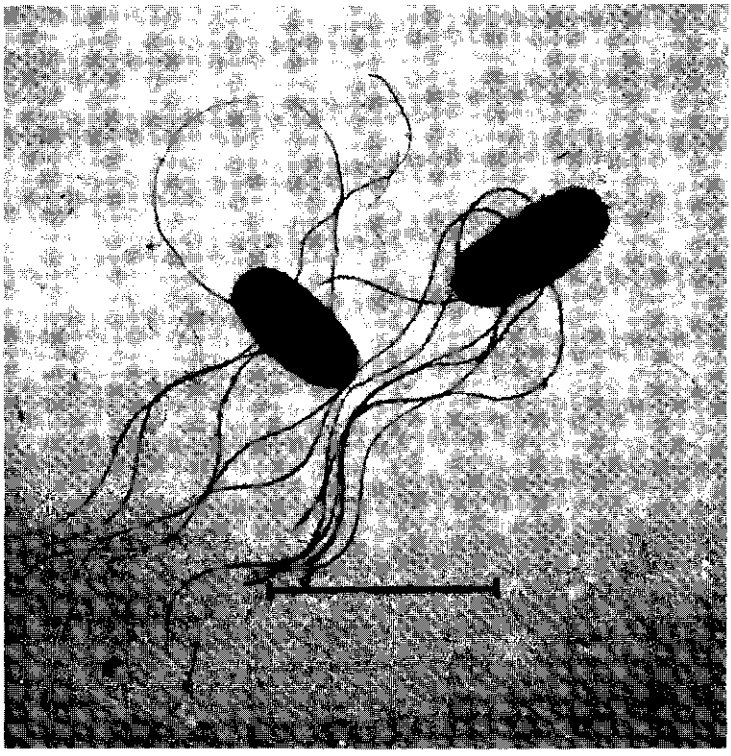

Figure 1. Transmission electron microscopic photograph of strain NTB-1. The bar represents 2 μm.

Enzyme assays. Cell extracts were prepared by ultrasonic treatment as described previously (van den Tweel et al., 1986). All assays were performed at 30°C. 4-HBA hydroxylase was determined by means of 4-HBA-dependent NADPH oxidation (Marks et al., 1984a). Protocatechuate dioxygenase was measured by means of a polarographic oxygen probe (Marks et al., 1984a).

Analytical methods. Chloride ion concentrations were determined with a Marius Micro-chlor-o-counter (Marius, Utrecht, The Netherlands). Protein contents of whole cells and cell extracts were determined by the Lowry method (Lowry et al., 1951). The concentration of 4-HBA and 4-CBA was quantitatively analysed by means of reverse-phase high-performance liquid chromatography as described by Marks et al. (1984a). Samples were injected directly after cells had been removed by centrifugation. The product which accumulated from 4-CBA was identified as 4-HBA by comparison of the retention time with authentic 4-HBA and by *in situ* scanning of the UV spectrum after the flow had been stopped. Oxygen concentrations in the gas phase were determined with a Packard 427 gas chromatograph (Packard Instrument Co., Downers Grove, Illinois, USA) fitted with a molecular sieve; column temperature was 100°C and the carrier-gas was helium.

Chemicals. 4-CBA and 4-HBA were obtained from Janssen Chimica, Beerse, Belgium and were used without further purification. All other chemicals were of the highest purity commercially available and were also used without further purification.

RESULTS AND DISCUSSION

Characterization of strain NTB-1

Strain NTB-1 was a Gram-negative motile rod possessing peritrichious flagella (Figure 1). It did not produce acid from glucose or xylose and was both oxidase and aminopeptidase positive. Gelatine and aesculin were not hydrolysed; no β-galactosidase, urease and arginine dihydrolase were produced by this organism, which was able to grow at 37°C. NTB-1 was able to denitrify, and atmospheric nitrogen was formed from nitrate. No coloured pigment was formed. On the basis of these taxonomical characteristics NTB-1 was identified as belonging to *Alcaligenes denitrificans* (Kersters & de Ley, 1984).

When NTB-1 was grown at various 4-CBA concentrations it was observed that the doubling time was dependent upon the concentration of 4-CBA. Using a 4-CBA concentration of 2 mM a doubling time of 10 h was observed while on 4 mM 4-CBA NTB-1 doubled in 20 h. Moreover, a much longer lag-phase was observed at higher 4-CBA concentrations. Similar results have been described for a monochloro

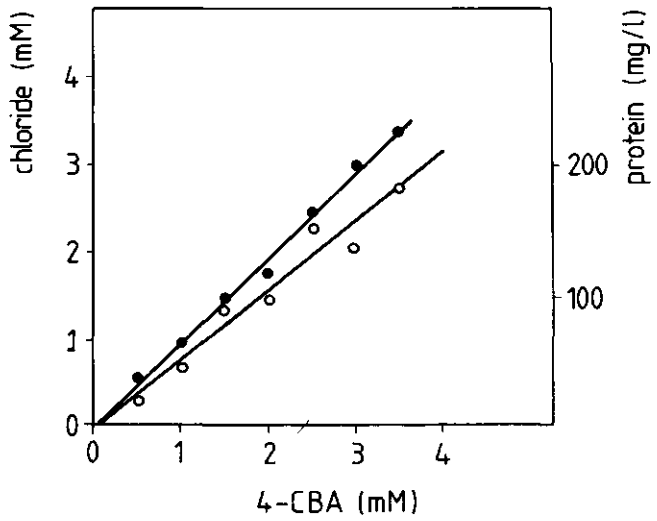

Figure 2. Protein concentration (o) and chloride release (•) after growth of Alcaligenes denitrificans NTB-1 at various concentrations of 4-CBA.

benzene degrading organism, strain WR 1306 (Reineke & Knackmuss, 1984) for which both the growth rate and the lag-phase were dependent upon the concentration of monochlorobenzene supplied. A linear relationship between 4-CBA added and the amount of protein formed was obtained (Figure 2) in spite of the noted effect of 4-CBA concentration on growth rate. At all concentrations, 4-CBA was totally degraded because growth at the expense of 4-CBA was accompanied by the elimination of stoichiometric amounts of chloride (Figure 2).

Degradation of 4-CBA by Alcaligenes denitrificans NTB-1

Simultaneous adaptation experiments showed that washed cell suspensions of NTB-1 grown on 4-CBA readily oxidized 4-CBA, 4-HBA and 3,4-dihydroxybenzoate (rates: 100, 90 and 20 nmol O_2 min^{-1} (mg protein)$^{-1}$, respectively), while succinate-grown cells were not able to oxidize these compounds. Moreover, cell extracts of 4-CBA-grown cells contained a 4-HBA-3-monooxygenase activity which was active in the presence of NADPH (20 nmol min^{-1} (mg protein)$^{-1}$), while also a protocatechuate dioxygenase activity was present (175 nmol min^{-1} (mg protein)$^{-1}$). However, no dehalogenase activity was detected in extracts. These results are indicative for an initial conversion of 4-CBA into 4-HBA which in turn may be further oxidized to 3,4-dihydroxybenzoate. The fact that no dehalogenase activity was recorded in cell extracts may be caused by the low stability of the enzyme (Marks et al., 1984a).

Bioformation of 4-HBA from 4-CBA

Although the precise mechanism of the initial reaction involved in 4-CBA degradation in strain NTB-1 is not yet known, it is expected that a similar reaction takes place as described in two other bacteria able to grow on 4-CBA (Marks et al., 1984b; Müller et al., 1984). Recently, it has been shown that in these organisms the biological displacement of the halogen of 4-CBA by a hydroxyl group involves a hydrolytic process showing no requirement for molecular oxygen (Marks et al., 1984b; Müller et al., 1984). When the first step in the metabolism of 4-CBA in NTB-1 indeed would be hydrolytic, it should be possible to form 4-HBA out of 4-CBA at reduced oxygen levels, since no further hydroxylation can take place in the absence of molecular oxygen. The results presented in Figure 3 indeed show this to be the case. At high concentrations of oxygen in the gas phase no 4-HBA formation was observed, whereas at reduced oxygen levels significant 4-HBA formation was achieved. Conversion was optimal at 1.2% oxygen in the gas phase resulting in a conversion of 4-CBA to 80% 4-HBA with 100% chloride release (Figure 4). However, at an oxygen concentration in the gas phase of almost zero a significant decrease in 4-CBA degradation and consequently 4-HBA formation was observed. This decrease of activity was unexpected because of the presumed hydrolytic nature of the initial step. However, it still is possible that energy and thus oxygen is needed for transport of 4-CBA.

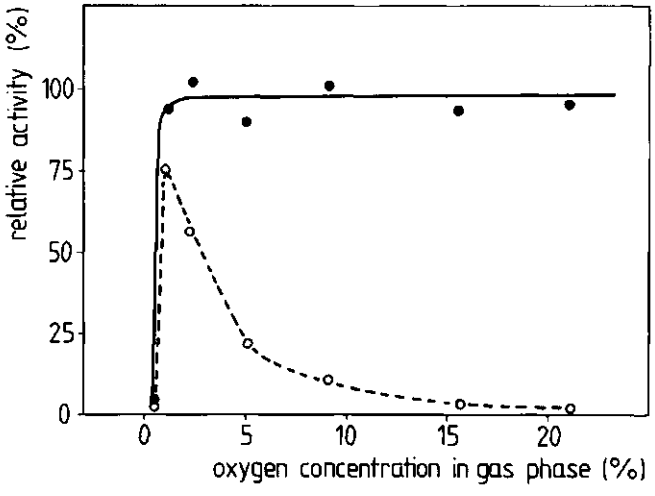

Figure 3. Effect of the oxygen concentration in the gas phase on the rate of 4-CBA consumption (•) and 4-HBA formation (○) by Alcaligenes denitrificans NTB-1 cells. A rate of 100% represents an activity of 40 nmol min^{-1} (mg protein)$^{-1}$.

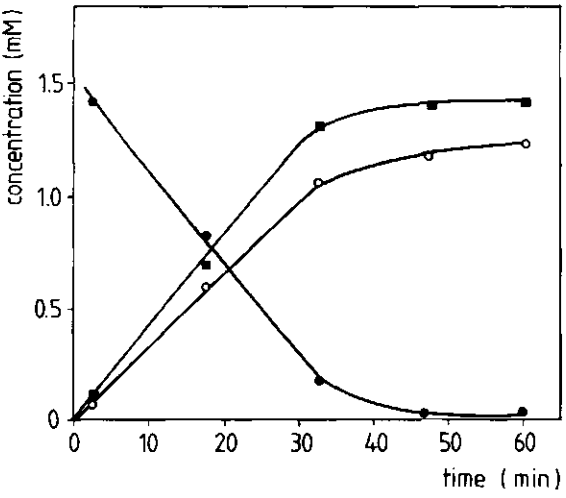

Figure 4. Conversion of 4-CBA (•) into 4-HBA (○) and chloride (▪) by Alcaligenes denitrificans NTB-1 cells at 1.2% oxygen in the gas phase.

In order to biologically produce large amounts of 4-HBA from 4-CBA it may be advantageous to use a high starting concentration of 4-CBA. Experiments showed that increasing 4-CBA concentrations up to 20 mM did not effect the rate of 4-CBA degradation and 4-HBA formation (Figure 5). Although *Alcaligenes denitrificans* NTB-1 was not able to grow at these relatively high concentrations it was still able

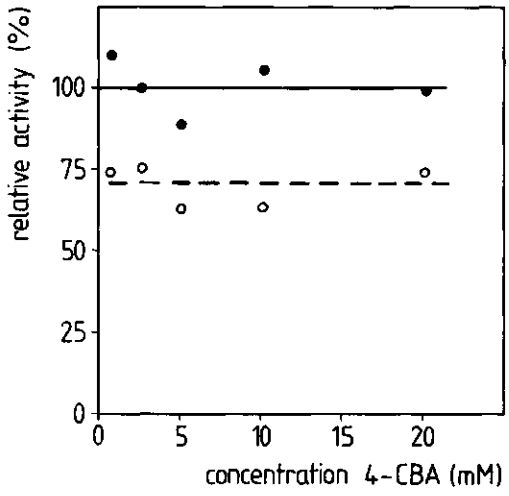

Figure 5. Effect of increasing 4-CBA concentrations on the consumption rate of 4-CBA (•) and the formation rate of 4-HBA (○) by Alcaligenes denitrificans NTB-1 cells at 1.2% oxygen in the gas phase. A rate of 100% represents an activity of 40 nmol min^{-1} (mg protein)$^{-1}$.

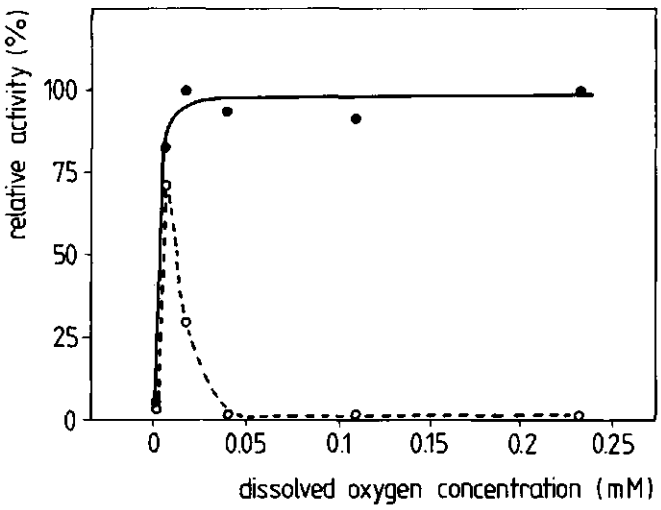

Figure 6. Effect of the dissolved oxygen concentration on the rate of 4-CBA consumption (•) and the rate of 4-HBA formation (○) by Alcaligenes denitrificans NTB-1 cells. A rate of 100% represents an activity of 5 nmol min^{-1} $(mg\ protein)^{-1}$.

to convert 4-CBA into 4-HBA. The above experiments were done at controlled oxygen concentrations in the gas phase. However, since the bioconversion takes place in the water phase, similar experiments were performed during which the dissolved oxygen concentration was monitored by means of an oxygen probe. The results of these experiments are shown in Figure 6. Only at a very low dissolved oxygen concentration (3.3 μM) a good conversion of 4-CBA in 4-HBA was observed, whereas at zero oxygen levels and at a concentration of 40 μM O_2 almost no 4-HBA was formed from 4-CBA. These results are in agreement with the results presented in Figure 3. The discrepancy between no bioformation at 40 μM dissolved oxygen (Figure 6) and a fairly good bioformation rate at the corresponding concentration of oxygen in the gas phase of 3.6% (Figure 3) is probably caused by the fact that the oxygen concentration in the aqueous phase is lower than the expected equilibrium concentration since the microorganisms continuously consume oxygen.

From the results presented it may be concluded that it is feasible to synthesize 4-HBA from 4-CBA under strictly controlled oxygen conditions. Further research will concentrate on screening for other micro-organisms and enzymes able to produce hydroxylated aromatic compounds from various haloaromatics.

ACKNOWLEDGEMENT

We wish to thank Alex van Neerven for making electron microscopic photographs.

REFERENCES

Baklashova, T.G., Koshcheenko, K.A. & Skryabin, G.K. (1984). Hydroxylation of indolyl-3-acetic acid by immobilized mycelium of Aspergillus niger. Applied Microbiology and Biotechnology 19,217-223

Daum, J. & Kieslich, K. (1974). Process for the preparation of 5-hydroxy-L-tryptophan. United States Patent 3,830,696

de Bont, J.A.M., Vorage, M.J.A.W., Hartmans, S. & van den Tweel, W.J.J. (1986). Microbial degradation of 1,3-dichlorobenzene. Applied and Environmental Microbiology 52,677-680

Engesser, K.-H., Schmidt, E. & Knackmuss, H.-J. (1980). Adaptation of Alcaligenes eutrophus B9 and Pseudomonas sp. B13 to 2-fluorobenzoate as growth substrate. Applied and Environmental Microbiology 39,68-73

Faulkner, J.K. & Woodcock, D. (1961). Fungal detoxification. Part V. Metabolism of o- and p-chlorophenoxyacetic acids by Aspergillus niger. Journal of the Chemical Society 5397-5400

Ghoshal, D., You, I.-S., Chatterjee, D.K. & Chakrabarty, A.M. (1985). Microbial degradation of halogenated compounds. Science 228,135-142

Guroff, G., Kondo, K. & Daly, J. (1966). The production of meta-chlorotyrosine from parachlorophenylalanine by phenylalanine hydroxylase. Biochemical and Biophysical Research Communications 25,622-628

Hagedorn, S. (1983). Production of para-cresol. European Patent Application 0,105,630

Hagedorn, S. (1984). Construction of novel mutant micro-organisms. European Patent Application 0,138,391

Johnston, H.W., Briggs, G.G. & Alexander, M. (1972). Metabolism of 3-chlorobenzoic acid by a Pseudomonad. Soil Biology and Biochemistry 4,187-190

Kaufman, S. (1961). The enzymic conversion of 4-fluorophenylalanine to tyrosine. Biochimica et Biophysica Acta 51,619-621

Keil, H., Klages, U. & Lingens, F. (1981). Degradation of 4-chlorobenzoate by Pseudomonas sp. CBS3: induction of catabolic enzymes. FEMS Microbiology Letters 10,213-215

Kersters, K. & de Ley, J. (1984). Genus Alcaligenes. In: Manual of Systematic Bacteriology (Krieg, N.R., Ed.), Vol I, pp. 361-373. Williams & Wilkins, Baltimore

Klages, U. & Lingens, F. (1979). Degradation of 4-chlorobenzoic acid by a Nocardia species. FEMS Microbiology Letters 6,201-203

Klibanov, A.M., Berman, Z. & Alberti, B.N. (1981). Preparative hydroxylation of aromatic compounds catalysed by peroxidase. Journal of the American Chemical Society 103,6263-6264

Knackmuss, H.-J. (1981). Degradation of halogenated and sulfonated hydrocarbons. In: Microbial Degradation of Xenobiotics and Recalcitrant Compounds (Leisinger, T., Cook, A.M., Hütter, R. & Nüesch, J., Eds.), pp. 189-212. Academic Press Inc., London

Kulla, H. & Lehky, P. (1985). Verfahren zur Herstellung von 6-Hydroxynikotinsäure. European Patent Application 0,152,948

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Markus, A., Klages, U., Krauss, S. & Lingens, F. (1984). Oxidation and dehalogenation of 4-chlorophenylacetate by a two-component enzyme system from Pseudomonas sp. CBS3. Journal of Bacteriology 160,618-621

Marks, T.S., Smith, A.R.W. & Quirk, A.V. (1984a). Degradation of 4-chlorobenzoic acid by Arthrobacter sp. Applied and Environmental Microbiology 48,1020-1025

Marks, T.S., Wait, R., Smith, A.R.W. & Quirk, A.V. (1984b). The origin of the oxygen incorporated during the dehalogenation/hydroxylation of 4-chlorobenzoate by an Arthrobacter sp. Biochemical and Biophysical Research Communications 124,669-674

Müller, R., Thiele, J., Klages, U. & Lingens, F. (1984). Incorporation of [^{18}O]water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from Pseudomonas spec. CBS3. Biochemical and Biophysical Research Communications 124,178-182

Olah, G.A., Fung, A.P. & Keuni, T. (1981). Oxyfunctinalisation of hydrocarbons. 11. Hydroxylation of benzene and alkylbenzenes with hydrogen peroxide in hydrogen fluoride/Boron trifluoride. Journal of Organic Chemistry 46,4305-4306

Pshirkov, S.Y., Boiko, O.I., Kiprianova, E.A. & Starovoitov, I.I. (1982). Transformation of L-tyrosine into L-dihydroxyphenylalanine by Pseudomonas cultures. Mikrobiologiya 51,272-274

Reineke, W. & Knackmuss H.-J. (1984). Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Applied and Environmental Microbiology 47,395-402

Ruisinger, S., Klages, U. & Lingens, F. (1976). Abbau der 4-Chlorbenzoesäure durch eine Arthrobacter-species. Archives of Microbiology 110,253-256

Shailubhai, K., Sahasrabudhe, S.R., Vora, K.A. & Modi, V.V. (1983). Degradation of chlorinated derivatives of phenoxyacetic acid and benzoic acid by Aspergillus niger. FEMS Microbiology Letters 18,279-282

Taylor, S.C. (1982). Biochemical process. European Patent Application 0,076,606

Theriault, R.J. & Longfield, T.H. (1967). Microbial conversion of acetanilide to 2'-hydroxyacetanilide and 4'-hydroxyacetanilide. Applied Microbiology 15,1431-1436

Theriault, R.J. & Longfield, T.H. (1973). Microbial hydroxylation of 5-anilino-1,2,3-thiatriazole. Applied Microbiology 25,606-611

van den Tweel, W.J.J., Smits, J.P. & de Bont, J.A.M. (1986). Microbial metabolism of D- and L-phenylglycine by Pseudomonas putida LW-4. Archives of Microbiology 144,169-174

Vilanova, E., Manjon, A. & Iborra, J.L. (1984). Tyrosine hydroxylase activity of immobilised tyrosinase on enzacryl-AA and CPG-AA supports: stabilization and properties. Biotechnology and Bioengineering 26,1306-1312

Vishniac, W. & Santer, M. (1957). The Thiobacilli. Bacteriological Reviews 21,195-213

Yoshida, H., Tanaka, Y. & Nakayama, K. (1973). Production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and its derivatives by Vibrio tyrosinaticus. Agricultural and Biological Chemistry 37,2121-2126

Yoshida, H., Tanaka, Y. & Nakayama, K. (1974). Production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) by Pseudomonas melanogenum. Agricultural and Biological Chemistry 38,455-462

Zaitsev, G.M. & Karasevich, Y.N. (1981a). Utilization of 4-chlorobenzoic acid by Arthrobacter globiformis. Mikrobiologiya 50,35-40

Zaitsev, G.M. & Karasevich, Y.N. (1981b). Preparative metabolism of 4-chlorobenzoic acid in Arthrobacter globiformis. Mikrobiologiya 50,423-428

Chapter 11

REDUCTIVE DECHLORINATION OF 2,4-DICHLOROBENZOATE TO 4-CHLOROBENZOATE AND HYDROLYTIC DEHALOGENATION OF 4-CHLORO-, 4-BROMO-, AND 4-IODOBENZOATE BY *ALCALIGENES DENITRIFICANS* NTB-1

W.J.J. van den Tweel, J.B. Kok and J.A.M. de Bont

SUMMARY

Alcaligenes denitrificans NTB-1, previously isolated on 4-chlorobenzoate, also utilized 4-bromo-, 4-iodo-, and 2,4-dichlorobenzoate but not 4-fluorobenzoate as sole carbon and energy source. During growth, stoichiometric amounts of halide were released. Experiments with whole cells and cell extracts revealed that 4-bromo- and 4-iodobenzoate were metabolized like 4-chlorobenzoate, involving an initial hydrolytic dehalogenation yielding 4-hydroxybenzoate, which in turn was hydroxylated to 3,4-dihydroxybenzoate. The initial step in the metabolism of 2,4-dichlorobenzoate was catalysed by a novel type of reaction for aerobic organisms, involving inducible reductive dechlorination to 4-chlorobenzoate. Under conditions of low and controlled oxygen concentrations, *Alcaligenes denitrificans* NTB-1 converted all 4-halobenzoates and 2,4-dichlorobenzoate almost quantitatively to 4-hydroxybenzoate.

Applied and Environmental Microbiology (1987) 53,810-815

INTRODUCTION

Halogenated compounds have been produced synthetically on a large scale during the last few decades, and they have entered the biosphere either by accidental spillage or by deliberate release. In many cases these chemicals may be transformed or fully eliminated from the environment by microbial degradation.

Aerobically, the majority of haloaromatic compounds are degraded by microorganisms that leave the carbon-halogen bond intact until chlorocatechols are formed and the ring is cleaved by a dioxygenase (de Bont et al., 1986; Hartmann et al., 1979; Zaitsev & Baskunov, 1985; Dorn et al., 1974; Harper & Blakley, 1971; Reineke & Knackmuss, 1984; You & Bartha, 1982; Steiert & Crawford, 1985). However, aerobic catabolism of haloaromatic compounds may also proceed along an initial oxidative or hydrolytic dehalogenation reaction. For instance, 4-fluorophenylalanine was oxidized by a monooxygenase to tyrosine (Kaufman, 1961). In a similar way 2-fluorobenzoate (Engesser et al., 1980) and 4-chlorophenylacetate (Markus et al., 1984) were oxidized by a dioxygenase yielding catechol and 3,4-dihydroxyphenylacetate, respectively. An analogous reaction is probably involved in the metabolism of 2-chlorobenzoate (2-CBA) in *Pseudomonas cepacia* INMI-KZ-2 (Zaitsev & Karasevich, 1984). Hydrolytic dehalogenation reactions have been reported for the metabolism of 3- and 4-chlorobenzoates (3-CBA and 4-CBA)(Zaitsev & Karasevich, 1981; Ruisinger et al., 1976; Marks et al., 1984a; Keil et al., 1981; Klages et al., 1979; Johnston et al., 1972). Aerobic metabolism of halogenated aliphatic compounds also may involve either oxidative or hydrolytic dehalogenation reactions. Methylchloride was oxidized by a methanotroph via the methane monooxygenase activity present, although methylchloride did not serve as a carbon source for growth (Stirling & Dalton, 1979). Dichloromethane dehalogenation in a *Hyphomicrobium* strain was catalysed by a reduced glutathione-dependent dehalogenase, with formaldehyde as a product (Kohler-Staub & Leisinger, 1985). Glutathione-independent hydrolytic dehalogenation of halogenated aliphatic compounds has also been observed (Motsosugi et al., 1982; Janssen et al., 1985). To our knowlegde, however, there are no reports on the aerobic metabolism of halogenated aliphatic or aromatic compounds involving reductive dehalogenation.

Under anaerobic conditions, haloaromatic compounds are degraded by reductive dechlorination before ringcleavage, as shown for chlorinated phenols (Boyd & Shelton, 1984) and chlorinated benzoates (Shelton & Tiedje, 1984; Horowitz et al., 1983). Reductive dehalogenation was also responsible for the degradation of halogenated aliphatic hydrocarbons under methanogenic conditions (Vogel & McCarthy, 1985; Bouwer & McCarthy, 1983a) and under denitrifying

conditions (Bouwer & McCarthy, 1983b).

Recently we reported on the bioformation of 4-hydroxybenzoate (4-HBA) from 4-chlorobenzoate (4-CBA) by *Alcaligenes denitrificans* NTB-1 (van den Tweel et al., 1986b). The results described in this paper demonstrate that this species also grows on 2,4-dichlorobenzoate (2,4-DCBA), 4-bromobenzoate (4-BBA), and 4-iodobenzoate (4-IBA). 4-CBA, 4-BBA and 4-IBA were all hydrolytically dehalogenated to 4-HBA, and evidence will now be presented for a reductive dehalogenation reaction under aerobic conditions. This novel type of reaction is involved in the catabolism of 2,4-DCBA via 4-CBA to 4-HBA.

MATERIALS AND METHODS

Media and culture conditions. Alcaligenes denitrificans NTB-1 was routinely grown in a chemostat (volume, 1.0 l; dilution rate 0.03 h^{-1}) under carbon-limited conditions. The continuous culture (30°C, pH 7) was supplied with a mineral salts medium (van den Tweel et al., 1986b) to which 2,4-DCBA, 4-CBA or 4-BBA was added at 1.0 g l^{-1}, and 4-IBA at 0.5 g l^{-1}.

Experiments with whole cells. Simultaneous adaptation experiments and incubation experiments at controlled oxygen concentrations were performed as described previously (van den Tweel et al., 1986b).

Enzyme assays. Cell extracts were prepared by ultrasonic treatment as described previously (van den Tweel et al., 1986a). All assays were performed at 30°C. Attempts to assay for 2,4-DCBA dehalogenase activity were done by measuring 2,4-DCBA concentrations in various incubation mixtures in the presence of cell extract and NAD(P)H. 4-CBA dehalogenase was assayed by measuring the conversion of 4-CBA in 4-HBA. 4-HBA hydroxylase was determined by means of 4-HBA-dependent NADPH oxidation (Marks et al., 1984a). Protocatechuate dioxygenase was measured by means of a polarographic oxygen probe (Marks et al., 1984a).

Analytical methods. Chloride, bromide, and iodide concentrations were determined with a Micro-chlor-o-counter (Marius, Utrecht, The Netherlands). Protein contents of whole cells and cell extracts were determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as the standard. Concentrations of 4-fluorobenzoate (4-FBA), 4-CBA, 4-BBA, 2,4-DCBA and 4-HBA were quantitatively analysed by means of reverse-phase high-performance liquid chromatography (HPLC) with a C-18 column (200 by 3 mm; Chrompack, Middelburg, The Netherlands). The mobile phase was methanol-water-acetic acid (60:40:1 [v/v]), the flow rate was 0.4 ml min^{-1}, and detection was by UV absorbance at 254 nm. Under these conditions the following

retention times (in minutes) were observed: 4-FBA, 4.1; 4-CBA, 8.5; 4-BBA, 11.4; 4-IBA, 10.6; 2,4-DCBA, 9.8; and 4-HBA, 2.9. Accumulating metabolites were initially identified by comparison of retention times with those of authentic samples and by *in situ* scanning of the UV spectra after the flow had been stopped. Ether extraction of the accumulating products was done as described by Marks et al. (1984b). The resulting ether extracts were methylated with diazomethane (Vogel, 1985) and analysed by gas liquid chromatography/mass spectrometry (GC-MS). Samples were analysed on a capillary column (SIL19B; 26 m by 0.22 mm inner diameter; Chrompack, Middelburg, The Netherlands). The temperature was programmed to rise from 120°C (2 min) to 260°C at a rate of 4°C min^{-1}. The methyl esters of 2,4-DCBA, 4-CBA, and 4-HBA under these conditions had retention times of 6.95, 4.43 and 12.52 min, respectively. Mass spectra of these compounds were obtained on a VG MM7070F mass spectrometer at 70 eV electron impact. The oxygen concentration in the gas phase was measured as described previously (van den Tweel et al., 1986b).

Chemicals. All biochemicals were from Boehringer, Mannheim, Federal Republic of Germany. 2-Chloro-4-hydroxybenzoate and 2,3-dichlorobenzoate (2,3-DCBA) were from ICN Biomedicals Inc., Plainview, New York, USA. 4-CBA, 4-FBA and 2,4-DCBA were purchased from Janssen Chimica, Beerse, Belgium. 4-BBA was a product from Fluka, Buchs, Switzerland. All other chemicals were of commercially available analytical grade and were used without further purification.

Table 1. Rates of oxygen uptake by washed-cell suspensions of Acaligenes denitrificans NTB-1 grown on various carbon sources.

Substrate[a]	Oxygen uptake[b] (nmol of O_2 consumed/min per mg of protein) with indicated carbon source					
	4-CBA	4-BBA	4-IBA	2,4-DCBA	4-HBA	Succinate
4-FBA	<5	10	<5	5	5	<5
4-CBA	100	170	45	45	20	<5
4-BBA	75	140	60	50	25	<5
4-IBA	100	100	50	35	15	<5
2,4-DCBA	50	80	30	30	5	<5
4-HBA	90	220	55	40	20	<5
Protocatechuate	20	60	5	10	5	<5
Succinate	<5	<5	<5	<5	<5	55

[a] No activity was observed with the following substrates: 2-chloro-4-hydroxybenzoate, 3,5-dichlorocatechol, gentisate, benzoate, 2- or 3-HBA, 2- or 3-CBA, 2-FBA, 3-BBA, 2,6-DCBA, 2,5-DCBA, 3,4-DCBA, 3,5-DCBA, catechol, or 2,3-dihydroxybenzoate.

RESULTS

Growth of Alcaligenes denitrificans NTB-1 on various substrates

Alcaligenes denitrificans NTB-1 was able to use the following compounds as sole carbon and energy sources: 4-CBA, 2,4-DCBA, 4-BBA, 4-IBA, 4-HBA, 3-hydroxybenzoate (3-HBA), benzoate, phenylacetate, glucose, succinate, pyruvate, acetate and ethanol. No growth was observed with 2-CBA, 3-CBA, 2-HBA, 3,5-DCBA, 3,4-DCBA, 2,5-DCBA, 2,6-DCBA, 4-FBA, 4-chloro- or 4-fluorophenylacetate or 2-chloro-4-hydroxybenzoate as substrates (substrate concentrations 2 mM). Previous results have shown that both the lag phase and the growth rate of *Alcaligenes denitrificans* NTB-1 during growth on 4-CBA were dependent upon the concentration of 4-CBA (van den Tweel et al., 1986b). Similar results were obtained during growth on 4-BBA and 2,4-DCBA. At a 2 mM concentration of these two compounds, doubling times of 12 and 13 h, respectively, were observed while with 4-mM 4-BBA or 2,4-DCBA, strain NTB-1 doubled in 68 and 26 h, respectively. No growth of NTB-1 on 4-IBA (2 mM) was observed in batch culture. However, by growing NTB-1 in a chemostat under carbon-limited conditions (dilution rate 0.03 h^{-1}) good growth was obtained. During all growth experiments the halide was quantitatively released, as determined by measuring halide accumulation.

Simultaneous adaptation experiments

To investigate the metabolism of 4-CBA, 4-BBA, 4-IBA, and 2,4-DCBA, cells were grown on several substrates and incubated with possible intermediates, and rates of oxygen uptake were recorded. Cells grown on halobenzoates oxidized 4-CBA, 4-BBA, 4-IBA, 2,4-DCBA, 4-HBA, and 3,4-dihydroxybenzoate but not 4-FBA (Table 1). Succinate-grown cells did not oxidize 4-CBA, 4-BBA, 4-IBA, or 2,4-DCBA, but surprisingly, 4-HBA-grown cells oxidized these halogenated compounds. 2-Chloro-4-hydroxybenzoate, an intermediate of 2,4-DCBA metabolism in case of an initial hydrolytic dehalogenation at the 4-position, was not oxidized by 2,4-DCBA-grown cells, nor was 3,5-dichlorocatechol, an intermediate in case of an initial dioxygenation, oxidized by washed cells grown on 2,4-DCBA (Table 1).

Catabolism of halobenzoates and excretion of intermediary products from these halobenzoates by washed cell suspensions

To study the metabolism of the halobenzoates more directly, the disappearance of various halobenzoates from incubation mixtures was studied by HPLC. 4-CBA-grown cells readily catabolized 4-CBA, 4-BBA,

Table 2. Dehalogenation rates of various substrates by Alcaligenes denitrificans NTB-1 cells grown on various carbon sources.

Substrate	Dehalogenation rate (nmol of substrate consumed/min per mg of protein) with indicated carbon source					
	4-CBA	4-BBA	4-IBA	2,4-DCBA	4-HBA	Succinate
4-FBA	<1	<1	<1	<1	<1	<1
4-CBA	18	11	12	28	8	<1
4-BBA	15	11	12	30	8	ND[a]
4-IBA	18	9	10	28	7	ND
2,4-DCBA	11	7	6	20	5	<1

[a] ND, Not determined.

4-IBA and 2,4-DCBA but not 4-FBA (Table 2). Similar results were obtained for cells grown on 4-BBA, 4-IBA, 2,4-DCBA, and 4-HBA (Table 2). Although the activities varied depending on the carbon source used for growth, no significant variation was observed in the relative rates of consumption of 4-CBA, 4-BBA, and 4-IBA (Table 2).

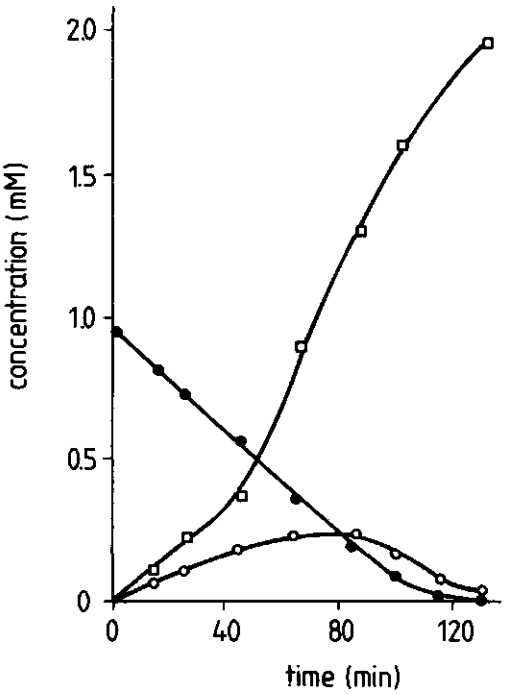

Figure 1. Formation of 4-CBA (○) and chloride (□) from 2,4-DCBA (●) by 4-HBA-grown Alcaligenes denitrificans NTB-1 cells. Total volume of the reaction mixture was 10 ml, containing 17.5 mg of protein.

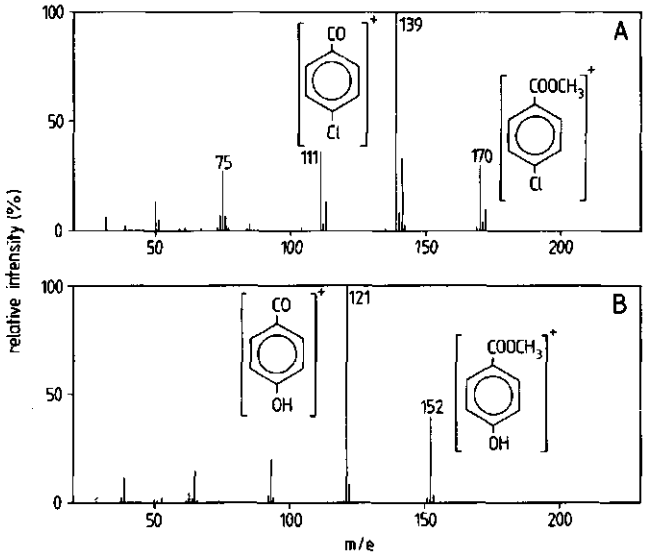

Figure 2. Mass spectrum of methylated 4-CBA (A), produced by 4-HBA-grown cells from 2,4-DCBA, and of methylated 4-HBA (B), formed from 2,4-DCBA at 1.2% oxygen in the gas phase.

Furthermore, the ratio of 4-halobenzoate to 2,4-DCBA consumption rate for cells grown on various haloaromatics was in all cases about 0.65. An interesting observation was made with 4-HBA-grown cells. These cells, when incubated with 2,4-DCBA, transiently accumulated a product (Figure 1) which, when analysed by HPLC, had the same retention time and UV characteristics as authentic 4-CBA. After methylation of the reaction mixture sampled at 60 min (Figure 1), two compounds were detected by GC/MS analysis. One compound showed the mass spectrum of methylated 2,4-DCBA and the other compound showed a mass spectrum (Figure 2A) identical to the mass spectrum of authentic 4-chlorobenzoic acid methylester. This accumulation of 4-CBA from 2,4-DCBA was not expected since the rate of 4-CBA consumption by whole cells was greater than the rate of 2,4-DCBA consumption (Table 2). However, when 4-CBA and 2,4-DCBA were added simultaneously, it was observed that the 4-CBA concentration remained constant until 2,4-DCBA was metabolized to leave a concentration of approximately 0.25 mM (Figure 3).

Previous results concerning the metabolism of 4-CBA by *Alcaligenes denitrificans* NTB-1 had shown that 4-CBA was almost stoichiometrically converted to 4-HBA under conditions of low oxygen concentration (van den Tweel et al., 1986b). Similar experiments have now been done with cells grown on 4-BBA, 4-IBA and 2,4-DCBA with the same substrates (Figure 4). In addition, the product obtained from the 2,4-DCBA incubation mixture was methylated and GC/MS analysis

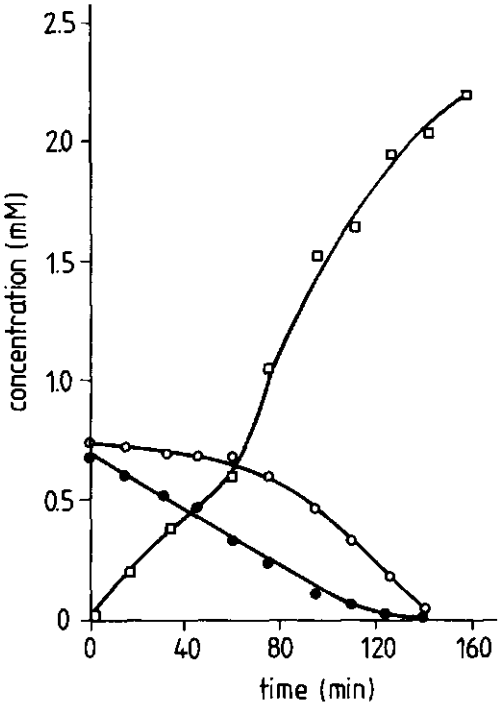

Figure 3. Simultaneous consumption of 4-CBA (○) and 2,4-DCBA (●) by 4-HBA-grown NTB-1 cells. □, chloride release. Total volume of the reaction mixture was 10 ml, containing 17.5 mg of protein.

showed that this methylated product was indeed 4-hydroxybenzoic acid methylester (Figure 2B). The effect of the oxygen concentration in the gas phase on the rate of 2,4-DCBA consumption and the rate of 4-HBA formation was identical to the effect of oxygen on 4-CBA consumption rate and 4-HBA formation, as shown previously (van den Tweel et al., 1986b), in that the conversion was maximal at about 1.2% oxygen in the gas phase while under anaerobic conditions almost no 2,4-DCBA consumption and hence no 4-HBA formation was obtained.

No decrease in concentration as measured by HPLC and no halide release was observed when 2,4-DCBA-grown NTB-1 cells were incubated under normal atmospheric conditions with the following compounds: 2-chloro-4-hydroxybenzoate, 4-chlorophenylacetate, 2-CBA, 3-CBA, 4-FBA, 2,6-DCBA, 2,5-DCBA, 3,4-DCBA, or 3,5-DCBA.

Experiments with cell extracts

The feasibility of a degradation route of 2,4-DCBA involving 4-CBA and subsequent metabolism of 4-CBA was further investigated, as was the metabolism of 4-BBA and 4-IBA, by measuring specific

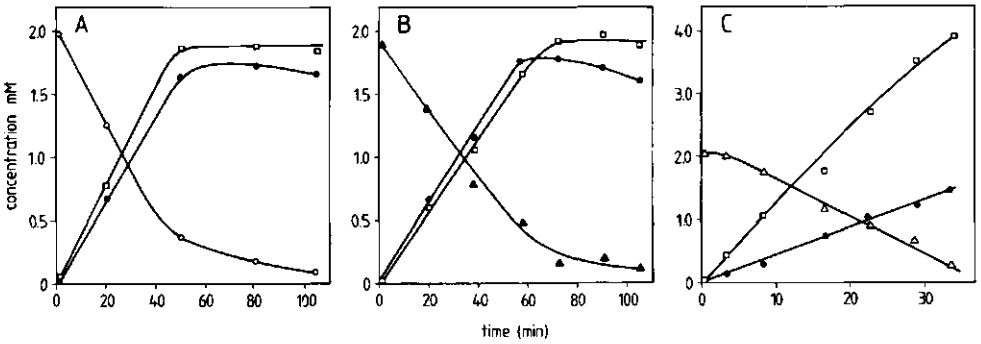

Figure 4. Bioconversion of 4-BBA (A), 4-IBA (B) and 2,4-DCBA (C) to 4-HBA by 4-BBA-, 4-IBA-, and 2,4-DCBA-grown cells, respectively, at 1.2% oxygen in the gas phase. Symbols: ○, 4-BBA; ▲, 4-IBA; △, 2,4-DCBA; ●, 4-HBA; □, halide. All incubation mixtures (total volume 10 ml) contained 29.5 mg of protein.

enzyme activities in cell extracts. NTB-1 cells grown on either 4-HBA, 4-CBA, 4-BBA, 4-IBA, or 2,4-DCBA had a NADPH-dependent 4-hydroxybenzoate hydroxylase and also a 3,4-dihydroxybenzoate dioxygenase, whereas succinate-grown cells did not contain these enzymes (Table 3). Several attempts were made to show the presence of a reductive 2,4-DCBA dehalogenase and a hydrolytic 4-CBA dehalogenase in extracts of 2,4-DCBA-grown cells, but no activities were detected.

Table 3. Specific activities of the 4-HBA hydroxylase (NADPH-dependent) and the 3,4-dihydroxybenzoate dioxygenase of Alcaligenes denitrificans NTB-1 after growth on various substrates.

Growth substrate	Sp act (nmol/min per mg of protein)	
	4-HBA hydroxylase	3,4-Dihydroxybenzoate dioxygenase
4-CBA	20	175
4-BBA	30	180
4-IBA	15	175
2,4-DCBA	15	210
4-HBA	15	190
Succinate	<1	<1

DISCUSSION

Aerobic microorganisms isolated on 4-CBA hydrolytically dehalogenate this substrate to 4-HBA (Zaitsev & Karasevich, 1981; Ruisinger et al., 1976; Marks et al., 1984a; Keil et al., 1981; Klages et al., 1979, van den Tweel et al., 1986b), in contrast to the dioxygenase reactions used for the initial step in the catabolism of various other halobenzoates in several organisms (Harper & Blakley, 1971; Zaitsev & Baskunov, 1985; Dorn et al., 1974). Microorganisms which degrade 4-CBA via 4-chlorocatechol so far have not been isolated by classical enrichment techniques, probably because the benzoate 1,2-dioxygenase of ordinary benzoate degraders is not active on 4-CBA (Knackmuss, 1975; Reineke & Knackmuss, 1978). Some years ago Hartmann et al. (1979) isolated a *Pseudomonas* sp. by continuous enrichment which degraded 3-CBA, 4-CBA, and 3,5-DCBA via the corresponding chlorocatechols. They assumed that this strain was naturally evolved from two defined strains, *Pseudomonas* B13 and *Pseudomonas* mt-2, which were used as coinocula. Later on, Reineke and Knackmuss (1980) confirmed this hypothesis by constructing *in vitro* a 4-CBA degrader by combining the genes of the 3-CBA pathway of *Pseudomonas* B13 and some genes of the TOL plasmid of *Pseudomonas* mt-2.

Alcaligenes denitrificans NTB-1 is no exception to the general rule for 4-CBA metabolism (van den Tweel et al., 1986b), and apart from 4-CBA it also metabolized 4-BBA and 4-IBA to 4-HBA. 4-FBA, however, did not serve as a growth substrate, nor was it defluorinated by cells grown on 4-CBA. In contrast, *Arthrobacter* TM-1, grown on 4-CBA was able to defluorinate 4-FBA (Marks et al., 1984a).

Strain NTB-1 was also able to grow on 2,4-DCBA. Bacteria degrading 2,4-DCBA have also been isolated by Vandenbergh et al. (1981), but in these strains the catabolic route of 2,4-DCBA has not been studied. Zaitsev and Karasevich (1985) isolated a *Corynebacterium* sp. which was able to convert 2,4-DCBA to 4-HBA. They considered three possible pathways for 2,4-DCBA metabolism: (i) reductive dechlorination to 4-CBA, which in turn would be dechlorinated to 4-HBA; (ii) hydrolytic dechlorination, yielding 2-chloro-4-hydroxybenzoate, followed by reductive dechlorination; (iii) a simultaneous dechlorination of both chlorine atoms, yielding 4-HBA. Zaitsev and Karasevich (1985) suggested that the third pathway was the most probable, although on the basis of their results the two other pathways can not be excluded.

Experiments with whole NTB-1 cells showed that 2-chloro-4-hydroxybenzoate was not an intermediate in the metabolism of 2,4-DCBA in our strain, nor was 2,4-DCBA degraded via 3,5-dichlorocatechol, a theoretical route resembling the aerobic degradation pathway of many haloaromatic compounds (de Bont et al., 1986;

Hartmann et al., 1979; Zaitsev & Baskunov, 1985; Dorn et al., 1974; Harper & Blakley, 1971; Reineke & Knackmuss, 1984; You & Bartha, 1982; Steiert & Crawford, 1985). Instead, from incubation experiments with 4-HBA-grown cells it is concluded that 2,4-DCBA is initially reduced to 4-CBA. To our knowlegde this is the first report on aerobic metabolism of halogenated compounds involving reductive dehalogenation. Washed cells grown on 4-HBA had a higher consumption rate for 4-CBA than for 2,4-DCBA but nevertheless accumulated 4-CBA from 2,4-DCBA. Inhibition of the 4-CBA dehalogenase by 2,4-DCBA (Figure 3) may explain this anomaly. Surprisingly, however, no 4-CBA accumulated from 2,4-DCBA with NTB-1 cells grown on 4-CBA, 4-BBA, 4-IBA, or 2,4-DCBA, although the ratio of 2,4-DCBA to 4-CBA consumption in these cells was comparable to this ratio in 4-HBA-grown cells (Table 2). We have not yet been able to measure a 2,4-DCBA reductive dehalogenase in cell extracts, although cell extracts have been prepared and tested in the presence and absence of oxygen with both NADH and NADPH as reductants.

4-HBA was produced from 2,4-DCBA when NTB-1 cells were incubated under conditions of low oxygen concentrations, which is in keeping with 4-CBA dehalogenation as a hydrolytic process (Marks et al., 1984b; Müller et al., 1984). Surprisingly, however, under anaerobic conditions almost no dehalogenation was observed, and presently it is investigated whether this behaviour is due to an energy-dependent active transport of 2,4-DCBA.

On the basis of the results presented, a degradation route as shown in Figure 5 is proposed for halobenzoate metabolism in *Alcaligenes denitrificans* NTB-1.

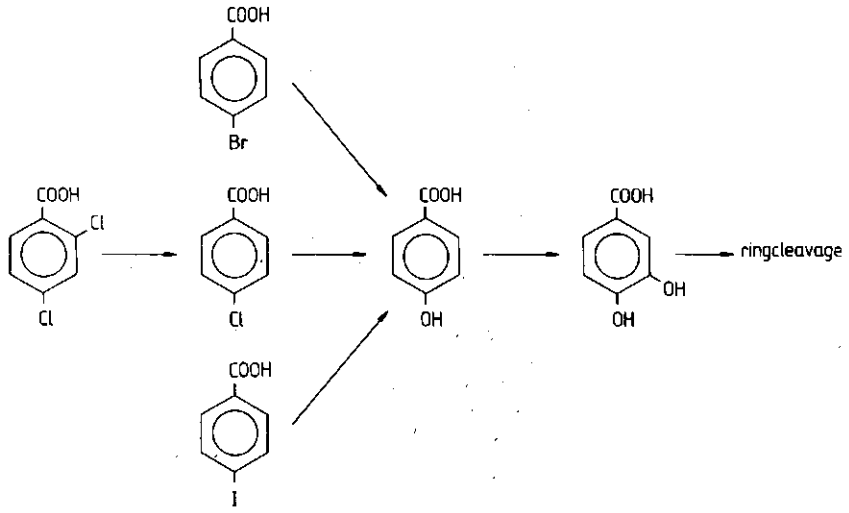

Figure 5. Proposed catabolic pathway of 2,4-DCBA, 4-CBA, 4-BBA, and 4-IBA in Alcaligenes denitrificans NTB-1.

ACKNOWLEDGEMENTS

We are grateful to Dr. M.A. Posthumus for performing the MS analyses and to G. Geurtsen and Dr. D.A. de Bie for advice and technical assistance during the methylation procedure.

REFERENCES

Bouwer, E.J. & McCarty, P.L. (1983a). Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Applied and Environmental Microbiology 45,1286-1294

Bouwer, E.J. & McCarty, P.L. (1983b). Transformations of halogenated organic compounds under denitrification conditions. Applied and Environmental Microbiology 45,1295-1299

Boyd, S.A. & Shelton, D.R. (1984). Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Applied and Environmental Microbiology 47,272-277

de Bont, J.A.M., Vorage, M.J.A.W., Hartmans, S. & van den Tweel, W.J.J. (1986). Microbial degradation of 1,3-dichlorobenzene. Applied and Environmental Microbiology 52,677-680

Dorn, E., Hellwig, M., Reineke, W. & Knackmuss, H.-J. (1974). Isolation and characterization of a 3-chlorobenzoate degrading Pseudomonad. Archives of Microbiology 99,61-70

Engesser, K.-H., Schmidt, E. & Knackmuss, H.-J. (1980). Adaptation of Alcaligenes eutrophus B9 and Pseudomonas sp. B13 to 2-fluorobenzoate as growth substrate. Applied and Environmental Microbiology 39,68-73

Harper, D.B. & Blakley, E.R. (1971). The metabolism of p-fluorobenzoic acid by a Pseudomonas sp. Canadian Journal of Microbiology 17,1015-1023

Hartmann, J., Reineke, W. & Knackmuss, H.-J. (1979). Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a Pseudomonad. Applied and Environmental Microbiology 37,421-428

Horowitz, A., Suflita, J.M. & Tiedje, J.M. (1983). Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Applied and Environmental Microbiology 45,1459-1465

Janssen, D.B., Scheper, A., Dijkhuizen, L. & Witholt, B. (1985). Degradation of halogenated aliphatic compounds by Xanthobacter autotrophicus GJ10. Applied and Environmental Microbiology 49,673-677

Johnston, H.W., Briggs, G.G. & Alexander, M. (1972). Metabolism of 3-chlorobenzoic acid by a Pseudomonad. Soil Biology and Biochemistry 4,187-190

Kaufman, S. (1961). The enzymic conversion of 4-fluorophenylalanine to tyrosine. Biochimica et Biophysica Acta 51,619-621

Keil, H., Klages, U. & Lingens, F. (1981). Degradation of 4-chlorobenzoates by Pseudomonas sp. CBS3: induction of catabolic enzymes. FEMS Microbiology Letters 10,213-215

Klages, U. & Lingens, F. (1979). Degradation of 4-chlorobenzoic acid by a Nocardia sp. FEMS Microbiology Letters 6,201-203

Knackmuss, H.-J. (1975). Über den Mechanismus der biologischen Persistenz von halogenierten aromatischen Kohlenwasserstoffen. Chemiker Zeitung 9,213-219

Kohler-Staub, D. & Leisinger, T. (1985). Dichloromethane dehalogenase of Hyphomicrobium sp. strain DM2. Journal of Bacteriology 162,676-681

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurements with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Marks, T.S., Smith, A.R.W. & Quirk, A.V. (1984a). Degradation of 4-chlorobenzoic acid by Arthrobacter sp. Applied and Environmental Microbiology 48,1020-1025

Marks, T.S., Wait, R., Smith, A.R.W. & Quirk, A.V. (1984b). The origin of the oxygen incorporated during the dehalogenation/hydroxylation of 4-chlorobenzoate by an Arthrobacter sp. Biochemical and Biophysical Research Communications 124,669-674

Hartmann et al., 1979; Zaitsev & Baskunov, 1985; Dorn et al., 1974; Harper & Blakley, 1971; Reineke & Knackmuss, 1984; You & Bartha, 1982; Steiert & Crawford, 1985). Instead, from incubation experiments with 4-HBA-grown cells it is concluded that 2,4-DCBA is initially reduced to 4-CBA. To our knowlegde this is the first report on aerobic metabolism of halogenated compounds involving reductive dehalogenation. Washed cells grown on 4-HBA had a higher consumption rate for 4-CBA than for 2,4-DCBA but nevertheless accumulated 4-CBA from 2,4-DCBA. Inhibition of the 4-CBA dehalogenase by 2,4-DCBA (Figure 3) may explain this anomaly. Surprisingly, however, no 4-CBA accumulated from 2,4-DCBA with NTB-1 cells grown on 4-CBA, 4-BBA, 4-IBA, or 2,4-DCBA, although the ratio of 2,4-DCBA to 4-CBA consumption in these cells was comparable to this ratio in 4-HBA-grown cells (Table 2). We have not yet been able to measure a 2,4-DCBA reductive dehalogenase in cell extracts, although cell extracts have been prepared and tested in the presence and absence of oxygen with both NADH and NADPH as reductants.

4-HBA was produced from 2,4-DCBA when NTB-1 cells were incubated under conditions of low oxygen concentrations, which is in keeping with 4-CBA dehalogenation as a hydrolytic process (Marks et al., 1984b; Müller et al., 1984). Surprisingly, however, under anaerobic conditions almost no dehalogenation was observed, and presently it is investigated whether this behaviour is due to an energy-dependent active transport of 2,4-DCBA.

On the basis of the results presented, a degradation route as shown in Figure 5 is proposed for halobenzoate metabolism in *Alcaligenes denitrificans* NTB-1.

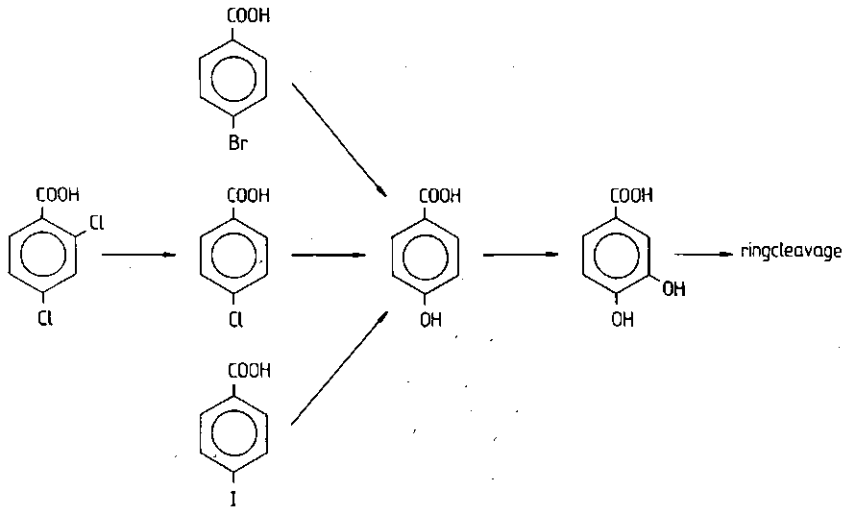

Figure 5. Proposed catabolic pathway of 2,4-DCBA, 4-CBA, 4-BBA, and 4-IBA in Alcaligenes denitrificans NTB-1.

ACKNOWLEDGEMENTS

We are grateful to Dr. M.A. Posthumus for performing the MS analyses and to G. Geurtsen and Dr. D.A. de Bie for advice and technical assistance during the methylation procedure.

REFERENCES

Bouwer, E.J. & McCarty, P.L. (1983a). Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Applied and Environmental Microbiology 45,1286-1294

Bouwer, E.J. & McCarty, P.L. (1983b). Transformations of halogenated organic compounds under denitrification conditions. Applied and Environmental Microbiology 45,1295-1299

Boyd, S.A. & Shelton, D.R. (1984). Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Applied and Environmental Microbiology 47,272-277

de Bont, J.A.M., Vorage, M.J.A.W., Hartmans, S. & van den Tweel, W.J.J. (1986). Microbial degradation of 1,3-dichlorobenzene. Applied and Environmental Microbiology 52,677-680

Dorn, E., Hellwig, M., Reineke, W. & Knackmuss, H.-J. (1974). Isolation and characterization of a 3-chlorobenzoate degrading Pseudomonad. Archives of Microbiology 99,61-70

Engesser, K.-H., Schmidt, E. & Knackmuss, H.-J. (1980). Adaptation of Alcaligenes eutrophus B9 and Pseudomonas sp. B13 to 2-fluorobenzoate as growth substrate. Applied and Environmental Microbiology 39,68-73

Harper, D.B. & Blakley, E.R. (1971). The metabolism of p-fluorobenzoic acid by a Pseudomonas sp. Canadian Journal of Microbiology 17,1015-1023

Hartmann, J., Reineke, W. & Knackmuss, H.-J. (1979). Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a Pseudomonad. Applied and Environmental Microbiology 37,421-428

Horowitz, A., Suflita, J.M. & Tiedje, J.M. (1983). Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Applied and Environmental Microbiology 45,1459-1465

Janssen, D.B., Scheper, A., Dijkhuizen, L. & Witholt, B. (1985). Degradation of halogenated aliphatic compounds by Xanthobacter autotrophicus GJ10. Applied and Environmental Microbiology 49,673-677

Johnston, H.W., Briggs, G.G. & Alexander, M. (1972). Metabolism of 3-chlorobenzoic acid by a Pseudomonad. Soil Biology and Biochemistry 4,187-190

Kaufman, S. (1961). The enzymic conversion of 4-fluorophenylalanine to tyrosine. Biochimica et Biophysica Acta 51,619-621

Keil, H., Klages, U. & Lingens, F. (1981). Degradation of 4-chlorobenzoates by Pseudomonas sp. CBS3: induction of catabolic enzymes. FEMS Microbiology Letters 10,213-215

Klages, U. & Lingens, F. (1979). Degradation of 4-chlorobenzoic acid by a Nocardia sp. FEMS Microbiology Letters 6,201-203

Knackmuss, H.-J. (1975). Über den Mechanismus der biologischen Persistenz von halogenierten aromatischen Kohlenwasserstoffen. Chemiker Zeitung 9,213-219

Kohler-Staub, D. & Leisinger, T. (1985). Dichloromethane dehalogenase of Hyphomicrobium sp. strain DM2. Journal of Bacteriology 162,676-681

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurements with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Marks, T.S., Smith, A.R.W. & Quirk, A.V. (1984a). Degradation of 4-chlorobenzoic acid by Arthrobacter sp. Applied and Environmental Microbiology 48,1020-1025

Marks, T.S., Wait, R., Smith, A.R.W. & Quirk, A.V. (1984b). The origin of the oxygen incorporated during the dehalogenation/hydroxylation of 4-chlorobenzoate by an Arthrobacter sp. Biochemical and Biophysical Research Communications 124,669-674

Markus, A., Klages, U., Krauss, S. & Lingens, F. (1984). Oxidation and dehalogenation of 4-chlorophenylacetate by a two-component enzyme system from Pseudomonas sp. strain CBS3. Journal of Bacteriology 160,618-621

Motosugi, K., Esaki, N. & Soda, K. (1982). Purification and properties of a new enzyme, DL-2-haloacid dehalogenase, from Pseudomonas sp. Journal of Bacteriology 150,522-527

Müller, R., Thiele, J., Klages, U. & Lingens, F. (1984). Incorporation of [^{18}O]water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from Pseudomonas spec. CBS 3. Biochemical and Biophysical Research Communications 124,178-182

Reineke, W. & Knackmuss, H.-J. (1978). Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of benzoic acid. Biochimica et Biophysica Acta 542,421-423

Reineke, W. & Knackmuss, H.-J. (1980). Hybrid pathway for chlorobenzoate metabolism in Pseudomonas sp. B13 derivatives. Journal of Bacteriology 142,467-473

Reineke, W. & Knackmuss, H.-J. (1984). Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Applied and Environmental Microbiology 47,395-402

Ruisinger, S., Klages, U. & Lingens, F. (1976). Abbau der 4-Chlorbenzoesäure durch eine Arthrobacter-species. Archives of Microbiology 110,253-256

Shelton, D.R. & Tiedje, J.M. (1984). Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Applied and Environmental Microbiology 48,840-848

Steiert, J.G. & Crawford, R.L. (1985). Microbial degradation of chlorinated phenols. Trends in Biotechnology 3,300-305

Stirling, D.I. & Dalton, H. (1979). The fortuitous oxidation and cometabolism of various carbon compounds by whole-cell suspensions of Methylococcus capsulatus (Bath). FEMS Microbiology Letters 5,315-318

Vandenbergh, P.A., Olsen, R.H. & Colaruotolo, J.F. (1981). Isolation and genetic characterization of bacteria that degrade chloroaromatic compounds. Applied and Environmental Microbiology 42,737-739

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., ter Burg, N., Kok, J.B. & de Bont, J.A.M. (1986b). Bioformation of 4-hydroxybenzoate from 4-chlorobenzoate by Alcaligenes denitrificans NTB-1. Applied Microbiology and Biotechnology 25,289-294

Vogel, A.I. (1956). Practical Organic Chemistry, p. 973. Longman, London

Vogel, T.M. & McCarty, P.L. (1985). Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Applied and Environmental Microbiology 49,1080-1083

You, I.-S. & Bartha, R. (1982). Metabolism of 3,4-dichloroaniline by Pseudomonas putida. Journal of Agricultural Food Chemistry 30,274-277

Zaitsev, G.M. & Karasevich, Y.N. (1981). Preparative metabolism of 4-chlorobenzoic acid. Mikrobiologiya 50,423-428

Zaitsev, G.M. & Karasevich, Y.N. (1984). Utilization of 2-chlorobenzoic acid by Pseudomonas cepacia. Mikrobiologiya 53,75-80

Zaitsev, G.M. & Baskunov, B.P. (1985). Utilization of 3-chlorobenzoic acid by Acinetobacter. Mikrobiologiya 54,203-208.

Zaitsev, G.M. & Karasevich, Y.N. (1985). Preparatory metabolism of 4-chlorobenzoic and 2,4-dichlorobenzoic acids in Corynebacterium sepedonicum. Mikrobiologiya 54,356-359

Chapter 12

KINETIC ASPECTS OF THE BIOCONVERSION OF 4-CHLOROBENZOATE TO 4-HYDROXYBENZOATE BY *ALCALIGENES DENITRIFICANS* NTB-1 IMMOBILIZED IN CARRAGEENAN

W.J.J. van den Tweel, W.T.A.M. de Laat, N. ter Burg and J. Tramper

SUMMARY

Alcaligenes denitrificans NTB-1 converts 4-chlorobenzoate to 4-hydroxybenzoate by means of a hydrolytic dehalogenation. In the presence of oxygen the product is further metabolized by the cells. Although it has been shown that the dehalogenation does not require oxygen, no bioproduction of 4-hydroxybenzoate was achieved by whole cells when oxygen was absent. An energy-dependent active transport may explain this anomaly. Little activity was lost after immobilization of whole cells in carrageenan. Diffusion of oxygen within the carrageenan beads rapidly limited the rate of dechlorination. This effect was magnified with increasing bead diameters and cell loadings. External transport of oxygen, on the other hand, did not decrease the reaction rate, except at extremely low oxygen concentrations. Maximal dehalogenation activity was observed at 35°C and at pH 8.0 and this was independent of whether free or immobilized cells were used. 4-Hydroxybenzoate significantly inhibited the rate of dechlorination, while the chloride formed and the substrate 4-chlorobenzoate did not show inhibitory effects on dechlorination. The yield of this bioconversion was mainly dependent on the oxygen concentration. In case of free cells, 4-hydroxybenzoate was produced only at very low oxygen concentrations, while immobilized cells still produced 4-hydroxybenzoate at air saturation as a result of oxygen diffusion limitation.

Biocatalysis (1987) 1,161-172

INTRODUCTION

Many hydroxylated aromatic compounds are widely used in various industries. However, no direct preparative chemical reactions exist for regiospecific aromatic hydroxylation on an industrial scale. Consequently, the hydroxyl group has to be introduced indirectly by an often expensive and laborious series of reactions. Therefore, many attempts are made to produce such compounds biologically by using either whole cells or enzymes that hydroxylate aromatic compounds (Theriault & Longfield, 1967, 1973; Yoshida et al., 1973, 1974; Daum & Kieslich, 1974; Klibanov et al., 1981; Pshirkov et al., 1982; Taylor, 1982; Hagedorn, 1983, 1984; Baklashova et al., 1984; Vilanova et al., 1984; Kulla & Lehky, 1985; Shirai, 1986; van den Tweel et al., 1987b).

Recently, it was demonstrated that organisms growing on 4-chlorobenzoic acid (4-CBA) form 4-hydroxybenzoic acid (4-HBA) in an initial hydrolytic reaction. This latter product is then further metabolized (Marks et al., 1984; Müller et al., 1984). The use of such hydrolytic dehalogenation reactions yielding hydroxyaromatics from the corresponding haloaromatics might be an interesting alternative to existing biological hydroxylation reactions. A major advantage of such a bioconversion is that further oxidation of the hydroxylated product either chemically or biologically, can easily be prevented since such a hydrolytic dehalogenation theoretically can be achieved in the absence of oxygen. Recently we have shown that *Alcaligenes denitrificans* NTB-1 is able to convert 4-CBA almost quantitatively to 4-HBA under conditions of low and controlled oxygen concentrations (van den Tweel et al., 1986). However, no reaction took place when oxygen was completely absent. The presence of an energy-dependent uptake system for 4-CBA may explain this rather unexpected anomalous result (Groenewegen et al., 1987).

In order to study the feasibility of continuous bioproduction of hydroxyaromatics from haloaromatics, the bioconversion of 4-CBA to 4-HBA by *Alcaligenes denitrificans* NTB-1 cells immobilized in carrageenan was used as a model system.

MATERIALS AND METHODS

Materials. 4-CBA (99% pure) and 4-HBA (99% pure) were obtained from Janssen Chimica, Beerse, Belgium, and were used without further purification. All other chemicals were of commercially available analytical grade. Solutions were made up in demineralized water.

Cultivation of Alcaligenes denitrificans NTB-1. Alcaligenes denitrificans NTB-1, an organism previously used (van den Tweel et al., 1986, 1987a), was routinely grown fed-batchwise in a fermentor (10

l) using a mineral salts medium (van den Tweel et al., 1986) to which 4-CBA was added at 5 g l^{-1}. Continuously, 3 l medium per day was pumped in the fermentor and each day 3 l was withdrawn. Under these conditions the 4-CBA concentration was always kept below 0.5 mM. Cells withdrawn from the fermentor were harvested by centrifugation (16,000 g for 10 min), washed once with potassium phosphate buffer (50 mM, pH 7.0), resuspended in the same buffer, and stored at -20°C until needed.

Immobilization procedure. Carrageenan (2.75 g; Genugel X0828, A/S Kobenhavns Pektinefabrik, Skensved, Denmark) was dissolved in demineralized water (100 ml) at 60°C. After cooling to 27°C the solution was mixed with cell suspension (20 ml; about 50 g protein l^{-1}). The resulting homogeneous mixture was extruded dropwise through a thin needle into a potassium phosphate buffer (500 mM, pH 8.0). The size of the drops formed at the needle tip was controlled by a water-saturated longitudinal airflow. After hardening for 2.5 h at room temperature, the beads were placed in a potassium phosphate buffer (50 mM, pH 8.0) and stored in this buffer at 4°C until use.

Analyses. The concentration of 4-HBA and 4-CBA was quantitatively analysed by means of HPLC as described previously (van den Tweel et al., 1987a). Chloride ion concentrations were determined with a Marius Micro-chlor-o-counter (Marius, Utrecht, The Netherlands). Protein contents of cell suspensions were determined by the Lowry method (Lowry et al., 1951). Oxygen concentrations in the gas phase were determined as described previously (van den Tweel et al., 1986).

Activity assays. Dehalogenation of 4-CBA was assayed either by measuring initial chloride release by means of a Micro-chlor-o-counter or by measuring initial 4-CBA consumption by means of HPLC. The reaction volume (total volume 10 ml) contained either free or immobilized cells, 4-CBA (20 μmol) and potassium phosphate buffer (500 μmol, pH 8.0), unless stated otherwise. The reaction tubes were incubated in a shaking waterbath (30°C, unless stated otherwise; 120 r.p.m.). Samples (0.5 ml) taken at intervals were analysed for either chloride or 4-CBA. Incubation experiments at controlled oxygen concentrations were performed as described previously (van den Tweel et al., 1986, 1987a).

Kinetic parameters for oxygen were determined at 30°C with a YSI 53 monitor equipped with a YSI 5331 polarographic oxygen probe (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA). An appropriate amount of either free or immobilized cells was suspended in potassium phosphate buffer (10 ml, 50 mM, pH 8.0) and the stirred suspension saturated with pure oxygen. Subsequently, 4-CBA (20 μmol) was added and the decrease in oxygen concentration monitored by a recorder.

Data reduction. Initial slopes were used for determination of the

activity. The activity was plotted as a function of pH and temperature. In the experiments with the beads of varying diameter or varying cell load, the substrate concentration was followed until depletion. From these experimentally obtained concentration versus time curves the kinetic parameters and diffusion effectiveness factors were calculated by means of a computer program (Tramper et al., 1983). The apparent V'_{max} and K'_m were obtained by fitting the experimental concentration versus time curve with the integrated Michaelis-Menten equation, minimizing the sum of squares (see van Ginkel et al. (1983) for examples of fitting). Subsequently, the external effectiveness (η_e) was calculated. This η_e is defined as the ratio of the reaction rate at the particle surface concentration ($r(S_S)$) and the rate at bulk concentration ($r(S_b)$):

$$\eta_e = r(S_s)/r(S_b) \tag{1}$$

Substitution of the Michaelis-Menten Equation ($r=V_{max}S/(K_m+S)$) gives Equation 2:

$$\eta_e = S_s(K_m + S_b)/\{S_b(K_m + S_s)\} \tag{2}$$

The substrate concentration at the surface (S_S) was calculated by means of the film theory from the bulk concentration (S_b) and the observed substrate consumption rate (r'_S) according to Equation 3:

$$r'_s = k_L \cdot A(S_b - S_s) \tag{3}$$

The mass-transfer coefficient in the liquid film adjacent to the liquid-solid interface, k_L, was calculated as described by Brink and Tramper (1986), whereas A, the liquid-solid specific surface area, was derived from Equation 4.

$$A = \frac{\pi \cdot d_p^2}{\frac{1}{6}\pi \cdot d_p^3}(1-\varepsilon) = \frac{6(1-\varepsilon)}{d_p} \tag{4}$$

with d_p the diameter of the spheres and ϵ the liquid holdup.

The internal effectiveness factor (η_i) was calculated by means of an iteration procedure from the Thiele-modulus (ϕ) as described by Froment and Bischoff (1979). Substitution of the substrate consumption rate ($r'_S=\eta_i(V_{max}S_S)/(K_m+S_S)$) in the integrated equation for ϕ (Froment & Bischoff, 1979) gives:

$$\phi = \frac{d_p}{6} \cdot \frac{[r'_s/\eta_i]^{1/2}}{\left[2D_{eff}(K_m + S_s)\left(1 - \frac{K_m}{S_s}\ln\left(1 + \frac{S_s}{K_m}\right)\right)\right]^{1/2}} \tag{5}$$

with D_{eff} the effective diffusion coefficient in carrageenan.

Starting the forementioned iteration with η_i=1 and calculating $\eta_{i,0}$ and $\eta_{i,1}$ as the internal effectiveness factors for, respectively zero and first order reaction kinetics according to Kobayashi and Laidler (1973) and Froment and Bischoff (1979), η_i was calculated from Equation 6:

$$\eta_i = \theta\eta_{i,0} + (1-\theta)\eta_{i,1} \qquad (6)$$

in which θ is a weight factor defined as $S_S/(K_m+S_S)$. Subsequently, the calculation was restarted until η_i converged.

The calculation of the overall effectiveness factor (η) and the intrinsic kinetic parameters was performed as follows:

1. Calculation of the effectiveness factor $\eta=\eta_i\eta_e$ as a function of the substrate concentration using the apparent parameters;
2. Correction of the experimental rate (r'_S) with the effectiveness factor: $r_S=r'_S/\eta$ (pseudo-experiment);
3. Calculation of the intrinsic V_{max} and K_m with data of the pseudo-experiment. With these calculated intrinsic values the calculations were restarted until the intrinsic values converged.

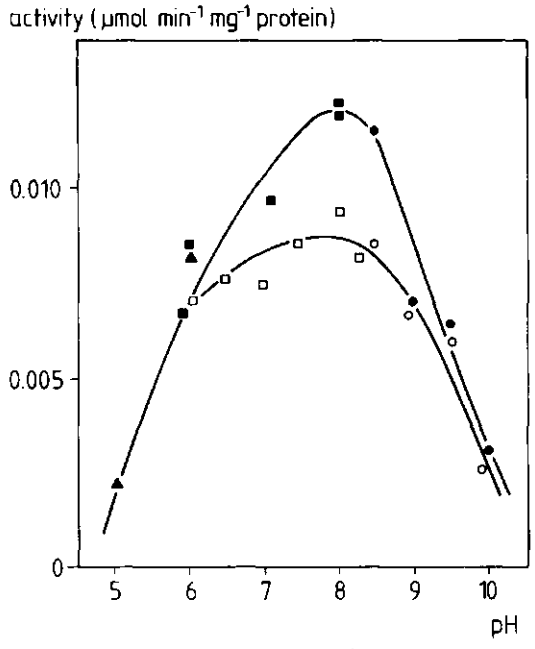

Figure 1. Dehalogenation activity of free (▲, ■, ●) and immobilized (□, ○; 2.4 mm bead diameter, 3.2 g protein l^{-1} gel) Alcaligenes denitrificans NTB-1 cells as a function of pH. Activity was assayed by measuring 4-CBA consumption by HPLC. Buffers used (50 mM): ▲, citric acid-Na_2HPO_4; □, ■, potassium phosphate; ○, ●, glycine-NaOH.

RESULTS AND DISCUSSION

Initial dehalogenation rates as a function of pH and temperature

In order to define maximal dehalogenation conditions, the most appropriate pH and temperature for the pertinent dehalogenation reaction were determined. The activity of free and immobilized cells as a function of pH is shown in Figure 1. Free cells showed maximal dehalogenation activity at pH 8.0. For immobilized cells, activity was optimal between 7.5 and 8.5. The profile of the immobilized cells was much broader than that of free cells, and the activity at the optimum was significantly lower. The apparent retention of activity after immobilization increased, however, when going from pH 8.0 to either lower or higher pH values, and was about 100% at pH 6.0 and pH 9.5. Similar results were obtained for the temperature-activity profile for free and immobilized cells (Figure 2). Again a much flatter profile and an activity equal to that of free cells at high and low temperatures were found for the immobilized cells. Diffusion limitation of one of the substrates to the biocatalyst may cause the flattening of the curves for the immobilized cells. During the experiments with

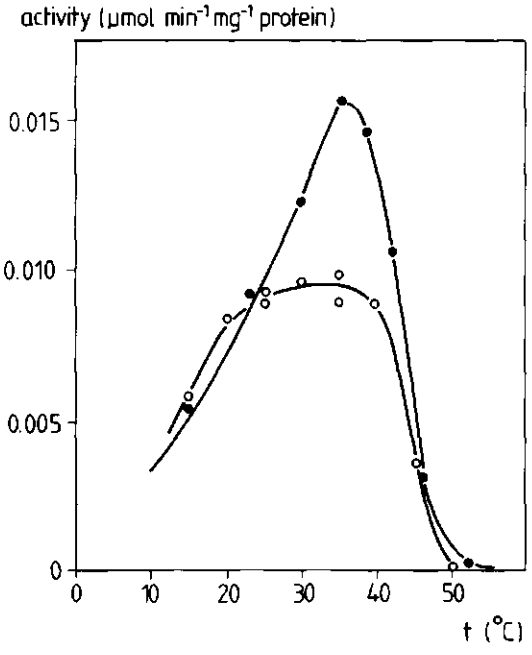

Figure 2. Activity of free (•) and immobilized (○; 2.4 mm bead diameter; 3.5 g protein l^{-1} gel) Alcaligenes denitrificans NTB-1 cells as a function of temperature. Activity was assayed by measuring 4-CBA consumption by HPLC.

immobilized cells over a range of pH (pH 7.0-8.0; Figure 1) and temperatures (25°C-35°C; Figure 2), significant amounts of 4-HBA accumulated from 4-CBA. As production of 4-HBA from 4-CBA by free cells was achieved only at low oxygen concentrations (van den Tweel et al., 1986, 1987a), diffusion limitation of oxygen in case of the immobilized cells was probably responsible for the lower dehalogenation rates. Experiments were performed in which free and immobilized cells (2.9 mm bead diameter; 8.4 g protein l^{-1} gel) were incubated at pH 8.0 and at 35°C with oxygen instead of air. Under these conditions immobilization resulted in only a 10% decrease in dehalogenation. Since best results were obtained with a potassium phosphate buffer (50 mM, pH 8.0), this buffer was used in subsequent experiments. However, although the dehalogenation rate was maximal at 35°C, further experiments were performed at 30°C for practical reasons.

Apparent kinetic constants for 4-CBA and oxygen

The following experiments were performed to quantify the effect of diffusion limitation of the substrates on the kinetic parameters. First, the apparent K'_m and V'_{max} for 4-CBA were determined. For this, beads varying in diameter and in protein loading were incubated at air saturation, and the 4-CBA concentration was assayed by means of HPLC until depletion. The kinetic constants for 4-CBA were determined from these concentration versus time curves as described in Materials and Methods. The apparent K'_m values for 4-CBA were about the same for all particle sizes and protein loadings and were almost equal to the K_m of free cells (about 19 μM). Evidently, neither internal nor external 4-CBA diffusion limitation existed. The apparent V'_{max} showed a small decrease with increasing particle sizes and increasing cell loads, probably as a result of oxygen diffusion limitation, which may occur under the conditions stated, as will be shown later on.

These results strongly suggested that diffusion of oxygen was the most essential factor in the bioconversion of 4-CBA in 4-HBA by immobilized cells. Therefore, the kinetics for oxygen were studied in more detail. First, the diameter of the biocatalyst beads was varied while the cell loading was kept constant. The consumption of oxygen was recorded from pure oxygen saturation to depletion, and the kinetic parameters for oxygen were calculated as before. The results obtained are shown in Figure 3. The strong increase of the apparent K'_m values with diameter indicates that control of the reaction rate by diffusion limitation of oxygen must certainly be taken into account in this system, especially when large biocatalyst beads are used at low oxygen concentrations. Similarly, the apparent K'_m increased (Figure 4) when the gel loading (protein l^{-1} gel) is increased, indicating that the

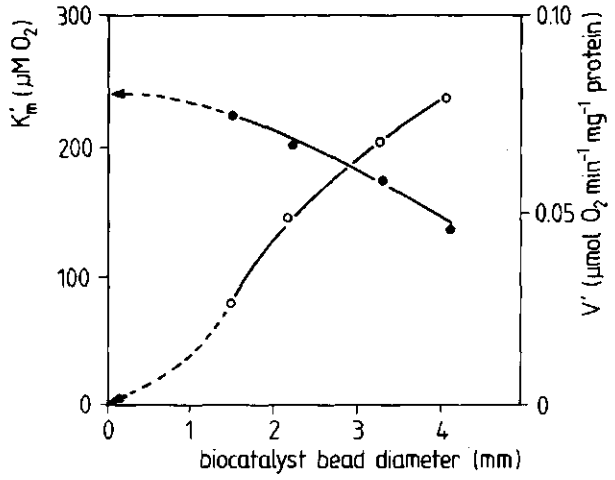

Figure 3. Influence of bead diameter on apparent kinetic constants for oxygen (V'_{max}, •; K'_m, ○) of immobilised Alcaligenes denitrificans NTB-1 cells (8.2 g protein l^{-1} gel). The arrows indicate the intrinsic K_m (5 μM) and V_{max} (0.08 μmol min^{-1} (mg protein)$^{-1}$) for free cells.

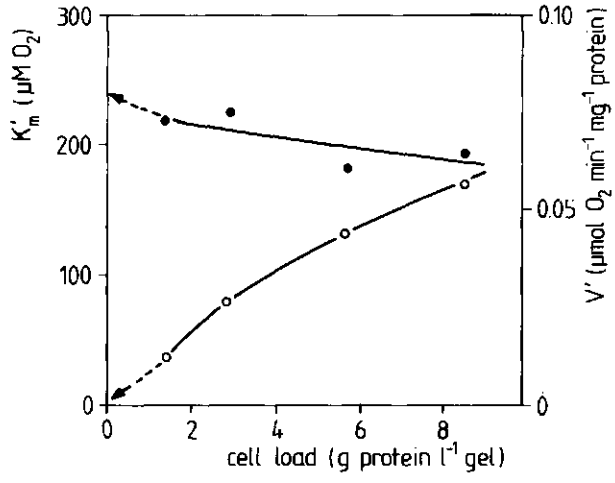

Figure 4. Effect of the cell load on apparent kinetic constants for oxygen (V'_{max}, •; K'_m, ○) of immobilized Alcaligenes denitrificans NTB-1 cells (3.2 mm bead diameter). The arrows indicate the intrinsic K_m (5μM) and V_{max} (0.08 μmol min^{-1} (mg protein)$^{-1}$) for free cells.

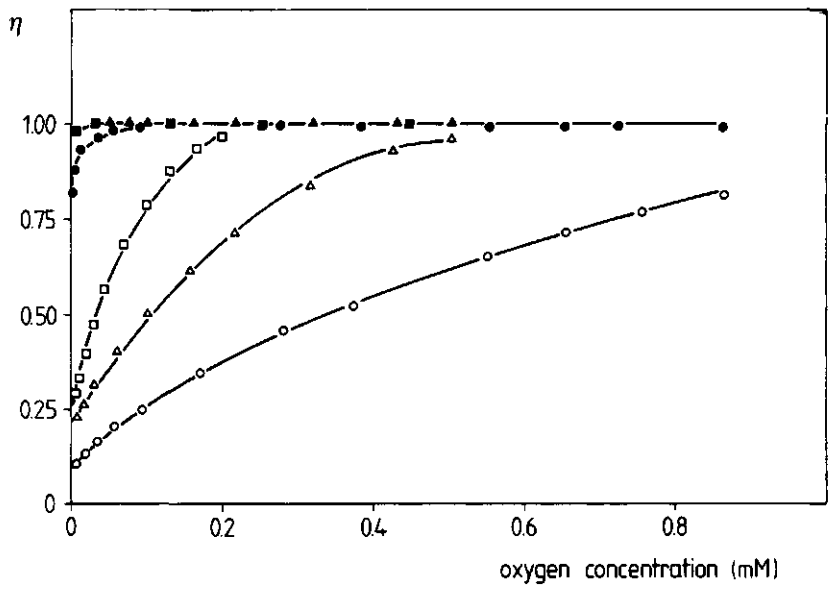

Figure 5. Variation of the internal (η_i; □, △, ○) and external (η_e; ■, ▲, ●) efficiency factors for immobilized cells (3.2 mm bead diameter) at varying cell loads as a function of the oxygen concentration. □, ■; 1.4; △, ▲, 2.9 and ○, ●, 8.6 g protein l^{-1} gel.

efficiency of the immobilized biocatalyst is controlled by transport of oxygen. Using the apparent kinetic parameters of Figure 4, the internal and external effectiveness factors were calculated as a function of the oxygen concentration (Figure 5), as described in the Materials and Methods section. Figure 5 clearly demonstrates the increasing importance of diffusion limitation of oxygen as the concentration of this substrate drops during the course of the experiment. The overall efficiency factor is determined mainly by internal transport limitation. An effect of external transport limitation is observed only when rather large beads (3.2 mm) with a high protein content (8.6 g protein l^{-1} gel) are used at very low oxygen concentrations (≤0.1 mM; Figure 5). Increasing either the diameter of the spheres or the cell load had no significant effect on the calculated intrinsic K_m and V_{max} of the immobilized cells; in all experiments (Figures 3 and 4) these parameters were almost equal to the K_m and V_{max} of free cells (5 μM and 0.08 μmol O_2 min^{-1} (mg protein)$^{-1}$, respectively).

Effect of the oxygen concentration in the gas phase on the conversion of 4-CBA to 4-HBA

The foregoing experiments showed that diffusion of oxygen has a great impact on the dechlorination of 4-CBA by immobilized cells. To exemplify this, the effect of the oxygen concentration in the gas phase on the bioconversion of 4-CBA to 4-HBA was investigated by means of HPLC. As shown before (van den Tweel et al., 1986) no dehalogenation occurred below approximately 1% of oxygen in the gas phase, using free or immobilized cells (Figures 6A and 6B). Above this level dechlorination was practically always maximal for free cells (Figure 6A), whereas for immobilized cells the rate of dehalogenation steadily increased with increasing oxygen concentrations until the maximum was reached (Figure 6B). Apparently, at low oxygen concen-

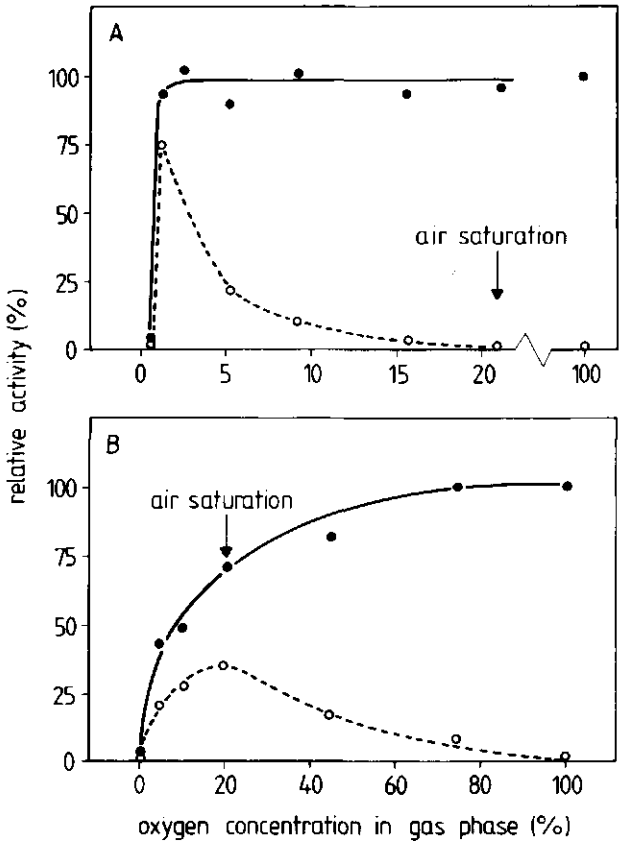

Figure 6. Effect of the oxygen concentration in the gas phase on the rate of 4-CBA consumption (•) and 4-HBA formation (○) by free (A) and immobilized (B; 3.1 mm bead diameter, 6.8 g protein l^{-1} gel) Alcaligenes denitrificans NTB-1 cells. A rate of 100% represents an activity of 0.036 and 0.033 μmol min^{-1} (mg protein)$^{-1}$ for free and immobilized cells, respectively.

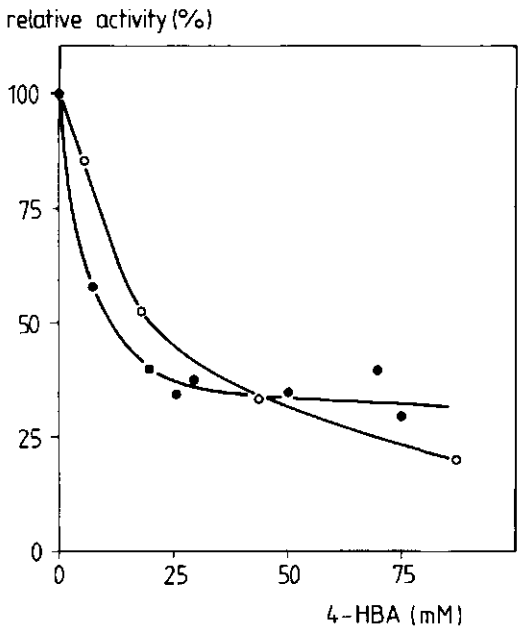

Figure 7. Inhibition of dehalogenation of free (•) and immobilized (○; 2.5 mm bead diameter; 12 g protein l^{-1} gel) Alcaligenes denitrificans NTB-1 cells by 4-HBA. Activity was assayed by measuring chloride release. A rate of 100% represents an activity of 0.039 and 0.033 μmol min^{-1} $(mg\ protein)^{-1}$ for free and immobilized cells, respectively.

trations only a small proportion of the immobilized cells is fully active due to depletion of oxygen in the core of the beads. Because of this oxygen limitation within the support, 4-HBA was still produced at oxygen concentrations up to 75% in the gas phase (Figure 6B). Free cells, on the other hand, did not form any 4-HBA above air-saturation. Evidently, a shortage of oxygen is needed to achieve the bioproduction of 4-HBA from 4-CBA; whenever oxygen is in excess, no 4-HBA will accumulate as a result of complete mineralization.

Inhibition of dehalogenation

A thorough knowledge about the inhibition of dehalogenation is essential for the bioconversion process. Van den Tweel et al. (1986) demonstrated that 4-CBA concentrations up to 20 mM did not effect the dehalogenation of 4-CBA by free cells. Almost identical results were obtained using immobilized *Alcaligenes denitrificans* NTB-1 cells. Chloride concentrations up to 500 mM also had no effect on the dehalogenation rate by either free or immobilized cells. The product 4-HBA, however, had a great impact on the dehalogenation rate (Figure 7). At a product concentration of 25 mM the dehalogenation

rate was decreased about threefold, in both free and immobilized cells. Relative slow diffusion of 4-HBA out of the support, resulting in a concentration gradient in the support, is thus another factor that could cause an apparent low activity after immobilization. However, since Figures 1 and 2 represent initial rates, it is unlikely that build-up of 4-HBA concentration occurred in the relevant assays. Moreover, in view of the very low initial substrate concentration, in addition to the very good fit of the integrated Michaelis-Menten equation on the experimental data, inhibition of dehalogenation by 4-HBA can be neglected under the conditions specified.

Storage stability

The storage stability of free and immobilized *Alcaligenes denitrificans* NTB-1 cells in potassium phosphate buffer (50 mM, pH 8.0) at 4°C was also studied. Immobilization did not improve the stability of dechlorination activity. After about 13 days both free and immobilized cells had lost half of their dechlorination activity. From the data obtained a first order rate constant of inactivation of 0.053 day^{-1} was calculated. Storage of free cells at 20°C for 5 days resulted in a complete loss of activity, while immobilized cells under such conditions still retained 70% of their activity.

CONCLUSIONS

Alcaligenes denitrificans NTB-1 cells can easily be immobilized in carrageenan with high retention of dechlorination activity. Maximal activity for both free and immobilized cells was obtained at 35°C with a potassium phosphate buffer (50 mM, pH 8.0). Under practical conditions the rate of dechlorination was rapidly limited by internal oxygen transport. However, high oxygen concentrations resulted in maximal 4-CBA dehalogenation, while 4-HBA formation under these conditions was negligible. Consequently, the oxygen concentration has to be strictly controlled to obtain a good production of 4-HBA at an acceptable rate. In order to optimize the bioconversion of 4-CBA to 4-HBA we are currently modelling this process, taking also in account the inhibition by 4-HBA.

ACKNOWLEDGEMENT

The authors thank the Netherlands Technology Foundation (STW) for financial support.

REFERENCES

Baklashova, T.G., Koshcheenko, K.A. & Skryabin, G.K. (1984). Hydroxylation of indolyl-3-acetic acid by immobilized mycelium of Aspergillus niger. Applied Microbiology Biotechnology 19,217-223

Brink, L.E.S. & Tramper, J. (1986). Modelling the effects of mass transfer on kinetics of propene epoxidation of immobilised Mycobacterium cells: pseudo-one-substrate conditions and negligible product inhibition. Enzyme and Microbial Technology 8,281-288

Daum, J. & Kieslich, K. (1974). Process for the preparation of 5-hydroxy-L-tryptophan. United States Patent 3,830,696

Froment, G.F. & Bischoff, K.B. (1979). Chemical Reactor: Analysis and Design. Wiley & Sons, New York

Groenewegen, P.E.J., van den Tweel, W.J.J. & de Bont, J.A.M. (1987). Bioformation of 4-hydroxybenzoate from 4-halobenzoates by Alcaligenes denitrificans NTB-1. Proceedings of the 4th European Congress on Biotechnology 3,464

Hagedorn, S. (1983). Production of para-cresol. European Patent Application 0,105,630

Hagedorn, S. (1984). Construction of novel mutant micro-organisms. European Patent Application 0,138,391

Klibanov, A.M., Berman, Z. & Alberti, B.N. (1981). Preparative hydroxylation of aromatic compounds catalysed by peroxidase. Journal of the American Chemical Society 103,6263-6264

Kobayashi, T. & Laidler, K.J. (1973). Kinetic analysis for solid-supported enzymes. Biochimica et Biophysica Acta 302,1-12

Kulla, H. & Lehky, P. (1985). Verfahren zur Herstellung von 6-Hydroxynikotinsäure. European Patent Application 0,152,948

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193,265-275

Marks, T.S., Wait, R., Smith, A.R.W. & Quirk, A.V. (1984). The origin of the oxygen incorporated during the dehalogenation/hydroxylation of 4-chlorobenzoate by an Arthrobacter sp. Biochemical and Biophysical Research Communications 124,669-674

Müller, R., Thiele, J., Klages, U. & Lingens, F. (1984). Incorporation of [^{18}O]water into 4-hydroxybenzoic acid in the reaction of 4-chlorobenzoate dehalogenase from Pseudomonas spec. CBS3. Biochemical and Biophysical Research Communications 124,178-182

Pshirkov, S.Y., Boiko, O.I., Kiprianova, E.A. & Starovoitov, I.I. (1982). Transformation of L-tyrosine into L-dihydroxyphenyl-alanine by Pseudomonas cultures. Mikrobiologiya 51,272-274

Shirai, K. (1986). Screening of microorganisms for catechol production from benzene. Agricultural and Biological Chemistry 50,2875-2880

Taylor, S.C. (1982). Biochemical process. European Patent Application 0,076,606

Theriault, R.J. & Longfield, T.H. (1967). Microbial conversion of acetanilide to 2'-hydroxyacetanilide and 4'-hydroxyacetanilide. Applied Microbiology 15,1431-1436

Theriault, R.J. & Longfield, T.H. (1973). Microbial hydroxylation of 5-anilino-1,2,3-thiatriazole. Applied Microbiology 25,606-611

Tramper, J., Luyben, K.Ch.A.M. & van den Tweel, W.J.J. (1983). Kinetic aspects of glucose oxidation by Gluconobacter oxydans cells immobilized in calcium alginate. European Journal of Applied Microbiology and Biotechnology 17,13-18

van den Tweel, W.J.J., ter Burg, N., Kok, J.B. & de Bont, J.A.M. (1986). Bioformation of 4-hydroxybenzoate from 4-chlorobenzoate by Alcaligenes denitrificans NTB-1. Applied Microbiology and Biotechnology 25,289-294

van den Tweel, W.J.J., Kok, J.B. & de Bont, J.A.M. (1987a). Reductive dechlorination of 2,4-dichlorobenzoate to 4-chlorobenzoate and hydrolytic dehalogenation of 4-chloro-, 4-bromo-, and 4-iodobenzoate by Alcaligenes denitrificans NTB-1. Applied and Environmental Microbiology 53,810-815

van den Tweel, W.J.J., Smits, J.P., Tramper, J. & de Bont, J.A.M. (1987b). Biosynthesis of hydroxylated aromatic compounds. Proceedings of the 4th European Congress on Biotechnology 2,172-174

van Ginkel, C.G., Tramper, J., Luyben, K.Ch.A.M. & Klapwijk, A. (1983). Characterization of Nitrosomonas europaea immobilized in calcium alginate. Enzyme and Microbial Technology 5,297-303

Vilanova, E., Manjon, A. & Iborra, J.L. (1984). Tyrosine hydroxylase activity of immobilized tyrosinase on enzacryl-AA and CPG-AA supports: stabilization and properties. Biotechnology and Bioengineering 26,1306-1312

Yoshida, H., Tanaka, Y. & Nakayama, K. (1973). Production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and its derivatives by Vibrio tyrosinaticus. Agricultural and Biological Chemistry 37,2121-2126

Yoshida, H., Tanaka, Y. & Nakayama, K. (1974). Production of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) by Pseudomonas melanogenum. Agricultural and Biological Chemistry 38,455-462

Chapter 13

CONCLUDING REMARKS

In view of the problems associated with the chemical synthesis of some hydroxylated aromatic compounds, biological production processes may be a welcome alternative. As stated in chapter 1 the bacterial formation of hydroxylated aromatics in principle can be accomplished in four different ways: along biosynthetic routes, by means of direct hydroxylation methods, by replacement of substituents by hydroxyl groups, and by addition and/or modification reactions of side-chains. In spite of this diversity in concepts, at present commercial processes for the bioformation of hydroxylated arqmatics are rather scarce.

Nowadays, biosynthetic routes are merely useful for the synthesis of natural bulk products as for instance amino acids, however, as a result of progressing molecular biology techniques also specialty hydroxylated aromatic compounds may be synthesized from simple starting compounds. By combining pathways for the degradation of naphthalene from a *Pseudomonas* and tryptophan from an *E. coli*, Ensley (1986) has constructed organisms which form indigo and other pigments during growth on simple organic compounds. In spite of the ingenuity of such concepts the biosynthesis of specialty hydroxylated aromatics from simple starting compounds has still a long way to go.

Hydroxylation reactions of aromatic compounds mediated either by monooxygenases or by dioxygenases have a huge potential in industrial organic synthesis. So far, however, there exists only one commercial process. This process is based on the hydroxylation of benzene or substitutes thereof to cis-dihydrodiols by intact *Pseudomonas putida* cells which possess a dioxygenase with relaxed substrate specificity. Currently Imperial Chemical Industries (ICI) operates a fed-batch process to produce cis-dihydrodiols in multi-kilogram quantities. One of the problems in this type of bioconversion is cofactor regeneration. To enhance the stability of the process co-substrates are added for NADH regeneration (Taylor, 1985). Another problem associated with this process is the inhibitory effect of both the substrate and the product. It has been shown that the cis-dihydrodiol of toluene irreversibly inhibits the dioxygenase activity, whereas toluene and its cis-dihydrodiol in a cooperative manner reduce the viability of the cells (Jenkins et al., 1987). Consequently the production phase is rather limited. The application of water-immiscible solvents may be useful to maintain a low substrate concentration in the biocatalytic water phase (chapter

9), however, as a result of the polar nature of the cis-dihydrodiols other solutions have to be found to overcome the toxic effect of the product. Another enzyme which might be useful to hydroxylate aromatic compounds is the methane monooxygenase. Currently some research groups are investigating the application of this aspecific enzyme in a similar way. The fact that at present only one process has been commercialized is mainly caused by a lack of suitable biocatalysts and/or the instability of the hydroxylation process. To obtain a wide range of potential biocatalysts, extensive goal-orientated screening programs have to be developed (Cheetham, 1987). As a result of the lability of oxygenases in general and their dependence upon cofactors, applications of oxygenases probably have to be based on the use of whole cells. In this way the oxygenase is most stable and cofactor regeneration can easily be accomplished by adding cosubstrates.

The replacement of substituents by hydroxyl groups also might be a very attractive technique to synthesize hydroxylated aromatics. Of special interest is the hydrolytic dehalogenation reaction, since such reaction theoretically can be achieved in the absence of oxygen; thus preventing a further oxidation of the product. In chapters 10, 11 and 12 of this thesis it is stated that an *Alcaligenes denitrificans* catalyzes the bioformation of 4-hydroxybenzoate from various 4-halobenzoates. Recent work at our department, however, has revealed that the strain used was no pure culture (Groenewegen et al., to be published). Apart from the *Alcaligenes* strain the culture also contained a slow-growing Gram-positive rod. Growth experiments with both strains have revealed that only the Gram-positive organism was able to grow on 4-hydroxy-, 2,4-dichloro-, 4-chloro-, 4-bromo-, and 4-iodobenzoate. The fact that the experiments described in chapter 10, 11 and 12 were done with a bi-culture had no impact on the results as was confirmed by recent experiments with the pure culture of the Gram-positive bacterium. Incubation experiments at varying oxygen tensions surprisingly revealed that under anaerobiosis no conversion of 4-halobenzoates in 4-hydroxybenzoate occurred (chapters 10, 11 and 12). Similar results were recently obtained by Apajalahti and Salkinoja-Salonen (1987a) for the para-hydroxylation reactions of polychlorophenols. The degradation of tetrachlorohydroquinone by whole cells, on the other hand, was not dependent on the presence of molecular oxygen (Apajalahti & Salkinoja-Salonen, 1987a, 1987b). In order to explain these anomalous results, these biological dehalogenation processes have to be studied in more detail. Currently only hydrolytic dehalogenases have been described which act on 4-halobenzoates and polychlorophenols, however, for industrial purposes other hydrolytic dehalogenases are needed. Screening programs therefore are also necessary in this field of biocatalysis.

Biological addition and/or modification reactions of side chains are readily applicable processes for the industrial production of specialty hydroxylated aromatics. A great advantage of some of these processes is their cofactor independency. Nowadays, the Japanese firm Kanegafuchi produces D-4-hydroxyphenylglycine by an enantioselective hydrolysis of the corresponding racemic hydantoin. Another promissing method for the production of D-4-hydroxyphenylglycine is the enantioselective transamination of 4-hydroxyphenylglyoxylate (chapter 4). Currently this biotransformation is being patented in collaboration with Andeno B.V. in Venlo, The Netherlands. A major problem associated with this bioconversion is its unfavorable equilibrium. To obtain a complete bioconversion, one of the reaction products has to be removed. The use of aspartate as an amino donor may be very useful in this respect since the deaminated product, oxaloacetate, can be rapidly decarboxylated, enzymatically or chemically, to pyruvate. A similar process for the production of L-phenylalanine has recently been commercialized (Calton et al., 1986). In addition to these cofactor-independent reactions also cofactor-dependent modifications of aromatic side-chains in future may be very valuable to manufacture specialty hydroxylated aromatic compounds. Nowadays such processes catalyzed by dehydrogenases are already being developed for the production of various L-amino acid and hydroxy acids from the corresponding α-keto acids (Wandrey, 1987).

REFERENCES

Apajalahti, J.H.A. & Salkinoja-Salonen, M.S. (1987a). Dechlorination and para-hydroxylation of polychlorinated phenols by Rhodococcus chlorophenolicus. Journal of Bacteriology 169,675-681

Apajalahti, J.H.A. & Salkinoja-Salonen, M.S. (1987b). Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced Rhodococcus chlorophenolicus. Journal of Bacteriology 169,5125-5130

Calton, G.J., Wood, L.L., Updike, M.H., Lantz II, L. & Hamman, J.P. (1986). The production of L-phenylalanine by polyazetidine immobilized microbes. Bio/Technology 4,317-320

Cheetham, P.S.J. (1987). Screening for novel biocatalysts. Enzyme and Microbial Technology 9,194-213

Ensley, B.D., Ratzkin, B.J., Osslund, T.D., Simon, M.J., Wackett, L.P. & Gibson, D.T (1983). Expression of naphthalene oxidation genes in Escherichia coli results in the biosynthesis of indigo. Science 222,167-169

Jenkins, R.O., Stephens, G.M. & Dalton, H. (1987). Production of toluene cis-glycol by Pseudomonas putida in glucose fed-batch culture. Biotechnology and Bioengineering 29,873-883

Taylor, S.C. (1985). Enzymic synthesis of 5,6-dihydroxycyclohexa-1,3-diene. In: Enzymes in Organic Synthesis. Ciba Foundation Symposium III, pp. 71-75. Pitman, London

Wandrey, C. (1987). Fine Chemicals. Proceedings of the 4th European Congress on Biotechnology 4,171-188

Chapter 14

SUMMARY

As stated in the introduction of this thesis, hydroxylated aromatic compounds in general are of great importance for various industries as for instance pharmaceutical, agrochemical and petrochemical industries. Since these compounds can not be isolated in sufficient amounts from natural resources, they have to be synthesized. Chemical synthesis of hydroxylated aromatics is often a difficult task. Direct hydroxylation methods can only be achieved under extreme conditions, while indirect methods often are laborious multi-step processes. Biotechnological formation methods for hydroxylated aromatic compounds are a promissing alternative to the cumbersome organic chemical endeavours. The bioformation of hydroxylated aromatics in principle can be accomplished in four different ways: along biosynthetic routes, by means of direct hydroxylation methods, by replacement of substituents by hydroxyl groups, and by addition and/or modification reactions of side-chains. This research was done to investigate the potential of bacteria or enzymes thereof to form hydroxylated aromatics.

To obtain a microorganism which hydroxylates D-phenylglycine regio- and stereospecifically yielding D-4-hydroxyphenylglycine, various bacteria were isolated on D-phenylglycine as sole carbon and energy source. Unfortunately, however, none of the isolates was able to hydroxylate phenylglycine (chapters 1 and 2). Experiments with whole cells and cell extracts showed that the side chain was modified before hydroxylation of the aromatic ring occurred. One of the isolates, *Pseudomonas putida* LW-4, was also able to grow on D-4-hydroxyphenylglycine and it was shown that this compound was initially degraded by means of an enantioselective transaminase. Preliminary experiments with partially purified extracts have demonstrated that this reversible enzyme can be used to form D-4-hydroxyphenylglycine from 4-hydroxyphenylglyoxylate (chapter 4). To investigate D-4-hydroxyphenylglycine degradation in general, also other bacteria were isolated on D-4-hydroxyphenylglycine as sole carbon and energy source. One of these isolates, *Pseudomonas putida* MW27, possessed a D-selective as well as a L-selective 4-hydroxyphenylglycine transaminase (chapter 5). Evidently some microorganisms transaminate both enantiomers of 4-hydroxyphenylglycine and thus are less suitable for the formation of D-4-hydroxyphenylglycine by means of a trans-

amination.

To apply bacteria or enzymes thereof for the hydroxylation of phenylacetate and/or certain hydroxyphenylacetates a thorough knowledge concerning the bacterial metabolism of these compounds is needed. In chapter 6 the degradation of 4-hydroxyphenylacetate by a *Xanthobacter* species is described and it is shown that this strain can convert 4-hydroxyphenylacetate to 2,5-dihydroxyphenylacetate (homogentisate). To accomplish a formation of homogentisate by whole cells, further degradation of homogentisate had to be blocked by metal-chelators. In chapter 7 the degradation of DL-phenylhydracrylic acid and metabolites thereof, by a *Flavobacterium* species is described. In the presence of dipyridyl these cells converted both 3- and 4-hydroxyphenylacetate to homogentisate. As stated in chapter 7, the internal regeneration of reduction equivalents by using starting compounds which are more reduced than the compound to be hydroxylated, might be an interesting alternative to the simple addition of cosubstrates.

The bioformation of cis-1,2-dihydroxycyclohexa-3,5-diene (cis-benzeneglycol) from benzene illustrates the potential of biotransformations. The chemical synthesis of cis-benzeneglycol consists of several steps with a very low yield, whereas the biological formation is a one step process with a high yield. Continuous bioformation of cis-benzeneglycol from benzene by mutant cells growing on succinate under nitrogen-limited conditions in a chemostat, was easily achieved (chapter 8). In order to predict the cis-benzeneglycol concentration at various times, a mathematical model was developed that fitted rather well for both benzene-transport-limited and kinetically limited production conditions. This continuous process, however, resulted in two products: cis-benzeneglycol and cells. In order to make the continuous process economically more attractive, it is necessary to reuse the produced cells. Another problem encountered during the bioproduction of cis-benzeneglycol was the toxicity of benzene; a low benzene concentration was a prerequisite for good performance of the bioconversion process. Incubation experiments with the cis-benzeneglycol-producing mutant showed that hexadecane is a suitable solvent to circumvent benzene toxicity (chapter 9). Moreover, the addition of hexadecane did not significantly effect the rate of cis-benzeneglycol formation.

Chapters 10, 11 and 12 deal with the bioformation of 4-hydroxybenzoate from various 4-halobenzoates. Bioformation of 4-hydroxybenzoate was only achieved when whole cells were incubated with the specified 4-halobenzoates under conditions of low and controlled oxygen concentrations. Surprisingly no formation of 4-hydroxybenzoate occurred under anaerobic conditions, this in spite of the fact that such dehalogenases have been demonstrated to be hydrolytic. 4-Hydroxybenzoate was also formed from 2,4-dichlorobenzoate. This

latter compound was initially reductively dechlorinated to 4-chlorobenzoate which in turn was converted to 4-hydroxybenzoate (chapter 11). In order to study the feasibility of continuous bioproduction of hydroxyaromatics from haloaromatics, the bioconversion of 4-chlorobenzoate to 4-hydroxybenzoate by cells immobilized in carrageenan was used as a model system. At air saturation the rate of dechlorination was rapidly limited by internal oxygen transport. However, high oxygen concentration resulted in maximal 4-chlorobenzoate dehalogenation, while 4-hydroxybenzoate formation under these conditions was negligible. Consequently, the oxygen concentration has to be strictly controlled to obtain a good production of 4-hydroxybenzoate at an acceptable rate.

SAMENVATTING

In de inleiding van dit proefschrift wordt aangegeven dat een groot aantal gehydroxyleerde aromaten van groot belang is voor de farmaceutische, agrochemische en petrochemische industrie. Daar deze verbindingen niet uit natuurlijke bronnen kunnen worden geïsoleerd, dienen zij te worden gesynthetiseerd. Organisch-chemisch is de synthese van gehydroxyleerde aromaten meestal niet eenvoudig. Een directe hydroxylering van aromaten kan alleen onder extreme condities plaatsvinden, terwijl een indirecte synthese vaak uit meerdere reactiestappen bestaat en dus economisch gezien niet zo aantrekkelijk is. Een veelbelovend alternatief voor de organisch-chemische synthese is de biologische vorming van gehydroxyleerde aromaten. De biologische vorming van gehydroxyleerde aromaten kan in principe op vier verschillende manieren geschieden: middels een biosynthetische route, door een directe hydroxylering, door het vervangen van een substituent door een hydroxygroep, of door een additie en/of modificatie van een zijketen. Het doel van het hier beschreven onderzoek is een beter inzicht te verkrijgen in het gebruik van bacteriën bij de vorming van gehydroxyleerde aromaten.

Met als doel een micro-organisme te verkrijgen dat in staat is fenylglycine te hydroxyleren tot D-4-hydroxyfenylglycine, werden verscheidene bacteriën geïsoleerd op D-fenylglycine en werd onderzocht op welke manier fenylglycine wordt gemetaboliseerd (hoofdstukken 2 en 3). Helaas was geen van de isolaten in staat fenylglycine te hydroxyleren. Middels experimenten met hele cellen en celvrije extracten werd aangetoond dat eerst de zijketen wordt gemodificeerd alvorens de benzeenring wordt gehydroxyleerd. Verder onderzoek wees uit dat een van de isolaten ook op D-4-hydroxyfenylglycine kan groeien en dat deze verbinding initieel met behulp van een enantioselectief transaminase wordt gemetaboliseerd. Inleidende experimenten met gedeeltelijk gezuiverde extracten hebben laten zien dat dit reversibel enzym in principe kan worden gebruikt voor het maken van D-4-hydroxyfenylglycine uit 4-hydroxyfenylglyoxylaat (hoofdstuk 4). Vervolgens werd onderzocht op welke manier andere bacteriën 4-hydroxyfenylglycine afbreken. Een van de bacteriën die op D-4-hydroxyfenylglycine geïsoleerd zijn, *Pseudomonas putida* MW27, metaboliseerde zowel D- als L-4-hydroxyfenylglycine. Dit in tegenstelling tot *Pseudomonas putida* LW-4 die wél op D- maar niet op L-4-hydroxyfenylglycine groeit. Experimenten met celvrije extracten hebben aangetoond dat *Pseudomonas putida* MW27 zowel een D-selectief als een L-selectief 4-hydroxyfenylglycine transaminase bezit. Blijkbaar kunnen sommige micro-organismen beide enantiomeren van 4-hydroxy-

fenylglycine transamineren en zijn dus minder geschikt voor het maken van D-4-hydroxyfenylglycine met behulp van een transaminering.

Een goede kennis betreffende de bacteriële afbraak van fenylazijnzuur en haar derivaten is onontbeerlijk indien men bacteriën of enzymen daaruit wil gebruiken voor het produceren van gehydroxyleerde fenylazijnzuren. In hoofdstuk 6 is de afbraak van 4-hydroxyfenylazijnzuur door een *Xanthobacter* species beschreven, terwijl in hoofdstuk 7 het catabolisme van DL-fenylhydracrylzuur, fenylazijnzuur, 3- en 4-hydroxyfenylazijnzuur door een *Flavobacterium* species wordt behandeld. In de aanwezigheid van dipyridyl, een chelator van ijzerionen, kunnen beide organismen 2,5-dihydroxyfenylazijnzuur ophopen uit de genoemde hydroxyfenylazijnzuren. Zoals voorgesteld in hoofdstuk 7, kan interne cofactor regeneratie, door gebruikmaking van een substraat dat meer gereduceerd is dan het te hydroxyleren substraat, een mogelijk alternatief zijn voor het toevoegen van een cosubstraat.

De bacteriële vorming van cis-1,2-dihydroxycyclohexa-3,5-diëen (cis-benzeenglycol) uit benzeen is een illustratief voorbeeld van de potentie van biotransformaties. De chemische synthese van cis-benzeenglycol bestaat uit verschillende reactiestappen met een laag rendement, terwijl de biologische vorming een éénstapsproces is met een hoge opbrengst. Onder stikstof gelimiteerde condities tijdens groei op succinaat kon met behulp van een mutant op relatief eenvoudige wijze cis-benzeenglycol continu worden geproduceerd (hoofdstuk 8). De concentratie van cis-benzeenglycol op diverse tijdstippen kon middels een hiervoor ontwikkeld wiskundig model vrij nauwkeurig worden berekend voor condities met benzeentransport limitatie evenals voor kinetisch gelimiteerde productiecondities. Het continuproces heeft als nadeel dat twee producten werden gevormd: cis-benzeenglycol en biomassa. Economisch gezien is hergebruik van de biomassa zeer wenselijk. Een ander probleem vormde de toxiteit van benzeen; een lage benzeenconcentratie was een vereiste voor een goede bioconversie. Incubatieexperimenten met een mutant die cis-benzeenglycol produceerde, hebben aangetoond dat hexadecaan een geschikt oplosmiddel is voor het omzeilen van de toxiteit van benzeen, temeer daar de toevoeging van hexadecaan de vormingssnelheid van cis-benzeenglycol niet noemenswaardig beïnvloedde (hoofdstuk 9).

Hoofdstukken 10, 11 en 12 behandelen de biologische vorming van 4-hydroxybenzoaat uit 4-chloor-, 4-broom- en 4-joodbenzoaat. Alleen onder lage en gecontroleerde zuurstofconcentraties vormden hele cellen 4-hydroxybenzoaat uit de genoemde 4-halobenzoaten. Verrassenderwijs trad geen 4-hydroxybenzoaatvorming op tijdens anaerobe incubaties, dit ondanks het hydrolytische karakter van zulke dehalogenases. 4-Hydroxybenzoaat kon ook worden gevormd uit 2,4-dichloorbenzoaat. 2,4-Dichloorbenzoaat werd namelijk initieel reductief gedechloreerd tot 4-chloorbenzoaat en vervolgens hydrolytisch

gedechloreerd tot 4-hydroxybenzoaat (hoofdstuk 11). Met het oog op een mogelijke continue productie van hydroxyaromaten uit haloaromaten, is de vorming van 4-hydroxybenzoaat uit 4-chloorbenzoaat door in carrageen geïmmobiliseerde bacteriën bestudeerd (hoofdstuk 12). Dechlorering van 4-chloorbenzoaat door geïmmobiliseerde bacteriën werd snel gelimiteerd door interne zuurstofdiffusie. Hoge externe zuurstofconcentraties waren dus nodig voor een maximale dechlorering, echter onder zulke condities was de 4-hydroxybenzoaatvorming verwaarloosbaar. Voor het verkrijgen van een redelijk rendement met een acceptabele vormingssnelheid is dus ook met geïmmobiliseerde cellen een stringente controle van de zuurstofconcentratie een vereiste.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 25 november 1957 te Limbricht (Limburg). Na het behalen van het gymnasium-β diploma aan het Bisschoppelijk College te Sittard in 1976 werd in datzelfde jaar een aanvang gemaakt met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool in Wageningen. Het vakkenpakket in de doctoraalfase bestond uit technische microbiologie (verzwaard hoofdvak) en proceskunde (hoofdvak). De praktijktijd werd verricht in de Verenigde Staten bij Prof. Dr. W.H. Scouten, Chemistry Department, Bucknell University, Lewisburg, Pennsylvania. Na afronding van de studie (maart 1983) startte de auteur met een vierjarig, door STW gefinancierd promotieonderzoek bij de Vakgroep Microbiologie en de sectie Proceskunde. Prof. Dr. J.A.M. de Bont en Prof. Dr. J. Tramper begeleidden dit onderzoek. Sedert april 1987 is de auteur werkzaam bij de secties Industriële Microbiologie en Proceskunde van de Landbouwuniversiteit aan een door Andeno B.V. gesubsidieerd project.