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The degradation of 1-phenylalkanes by an oil-degrading strain of *Acinetobacter lwoffi*

O. O. AMUND* and I. J. HIGGINS

Biotechnology Centre, Cranfield Institute of Technology, Cranfield, Bedford, MK43 0AL, U.K.

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An oil-degrading bacterium identified as *Acinetobacter lwoffi* was isolated by elective culture on North Sea Forties crude oil from an activated sludge sample. It grew on a wide range of *n*-alkanes (C_{12}–C_{28}) and 1-phenylalkanes, including 1-phenyldodecane, 1-phenyltridecane and 1-phenyltetradecane. The organism degraded 1-phenyldodecane to phenylacetic acid which was further metabolized via homogentisic acid, whilst 1-phenyltridecane was transformed to *trans*-cinnamic and 3-phenylpropionic acid which were not further metabolized. Evidence is presented for a relationship between aromatic amino acid catabolism and 1-phenyldodecane degradation in this organism.

INTRODUCTION

The metabolic pathways for the degradation of *n*-alkanes and alicyclic compounds by *Acinetobacter* species have been reported (Donoghue and Trudgill, 1975; Finnerty, 1977). Whilst the metabolism of alkylbenzenes by *Nocardia* species has been described (Webley et al., 1956; Davis and Raymond, 1961; Sariaslani et al., 1974), their degradation by *Acinetobacter* species which show similar nutritional versatility has not been previously reported.

Alkylbenzenes are major constituents of oily wastes and pesticides as well as many synthetic detergents, and the biodegradation of such molecules by sewage organisms is therefore of environmental significance. This communication describes the metabolism of 1-phenyldodecane and 1-phenyltridecane by a sewage organism identified as *Acinetobacter lwoffi*.

* To whom correspondence should be addressed. Present address: Department of Biological Sciences, University of Lagos, Lagos, Nigeria.

Materials and Methods

Source of microorganism and substrate range

The organism was isolated by conventional enrichment from activated sludge (Canterbury Sewage Works, Kent, U.K.) using the mineral salts medium of Mulkins-Phillips and Stewart (1974), containing a trace element mixture (Bauchop and Elsden, 1960) and North Sea Forties crude oil (0.1%, v/v) as sole carbon source. The substrate specificity of the isolate was tested by inoculating samples of this medium containing various *n*-alkanes, 1-phenylalkanes, alicyclic and polycyclic hydrocarbons at 0.1% (v/v or w/v if solid) final concentration.

Measurement of growth

Bacterial growth was monitored by absorbance measurement at 600 nm using a Beckman model 34 spectrophotometer. When hydrocarbon substrates were used, organisms were first removed from culture by centrifugation in a Beckman J2-21 refrigerated centrifuge, washed three times in sodium phosphate buffer (0.01 M, pH 7.0) and resuspended in the same buffer before making the determination.

Measurement of oxygen uptake

Oxygen consumption by whole cell suspensions was determined using a thermostatically controlled (30 °C), magnetically stirred oxygen electrode assembly (Rank Bros, Bottisham, Cambridge, U.K.) linked to a recorder (Gallenkamp Euroscribe). Reaction mixtures contained sodium phosphate buffer (0.01 M, pH 7.0), cell suspension (about 10 mg dry wt) and substrate (1.0 mM) in a total volume of 3.0 ml.

Preparation of cell-free extracts

Washed cell suspensions were disrupted in a sonicator (Soniprep, MSE) at 20-sec pulses and 40-sec cooling intervals in an ice bucket for 10 min. The resulting suspensions were centrifuged (30000 × *g*, 1h, 4 °C) in a Beckman L8-55 ultracentrifuge. The pellet fractions were discarded while the soluble fractions were used for enzyme assays.

Protein assay

Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin (Sigma Chemical Co. Ltd, U.K.) as standard.

Enzyme assays

Isocitrate lyase (EC 4.1.3.1) was assayed as described by Dixon and Kornberg (1959). Phenylalkane hydroxylase was measured using the method of Peterson et al. (1967) and homogentisate 1,2-dioxygenase (EC 1.13.11.5) was assayed spectrophotometrically (Chapman and Dagley, 1962). Butyryl-CoA dehydrogenase (EC 1.3.99.2) and thiolase (acetyl-CoA-acetyltransferase, EC 2.3.1.9) were

assayed by the methods of Mahler (1955) and Lynen and Ochoa (1953), respectively.

Gas-liquid chromatography

Aromatic acids were converted into the methylesters by treatment with diazomethane (Sariaslani, 1974) which were separated using a prepacked coiled-glass column (1.83 m × 2.0 mm internal diameter) containing 3.0% (w/v) methylsilicone gum (SE30) on Chromosorb Q (100–120 mesh) or a similar column containing 10% (w/v) polyethylene glycol (PEG 20M) on Diatomite C (100–120 mesh). A Pye model 204 gas chromatograph fitted with flame ionization detector was used. The conditions were as follows: carrier gas (N_2) flow rate, 40 ml·min^{-1}; oven temperature, 185 °C; detector temperature 250 °C; injection temperature 200 °C. Quantitative measurements were carried out with standard methylesters using a Gallenkamp Euroscribe recorder fitted with a computing integrator (Supergrator, Columbia Scientific Instruments Co., U.K.).

Thin-layer chromatography

Culture supernatants were acidified with dilute HCl to pH 3.0 and extracted three times with equal volumes of diethylether. The ethereal extracts were combined and dried over anhydrous $MgSO_4$ before removal of the ether under reduced pressure. The residue was taken up in diethyl ether (5.0 ml) and aliquots (1.0–10.0 µl) were applied to silica gel TLC plates (Eastman-Kodak). Chromatograms were developed for non-hydroxylated aromatic acids in the solvent system: light petroleum (b.p. 60–80 °C) – acetic acid (49:1, v/v), and for hydroxylated acids in the system: light petroleum (b.p. 60–80 °C) – diethylether – formic acid (45:5:1, v/v).

Non-hydroxylated aromatic acids were detected by spraying the plates with a 0.4% (w/v) solution of bromocresol green (pH indicator 3.6–5.2) and they appeared as yellow spots against a blue background. Hydroxylated aromatic acids were detected by spraying the plates with a 1.0% (w/v) ethanolic solution of Gibbs reagent (2,6-dichloro-*p*-benzoquinone-4-chlorimine) followed by a saturated solution of aqueous $NaHCO_3$. The hydroxylated acids were detected as blue spots standing against a white background.

Detection of homogentisic acid

Homogentisic acid was detected in culture supernatants of phenylalanine- and tyrosine-grown cells with Brigg's reagent (Neuberger, 1947).

Batch culture studies

Growth experiments involving metabolite production from pure phenylalkanes were carried out in a bench fermenter (2l, Quickfit Stone, U.K.) in 1-litre volumes, magnetically stirred and with forced aeration (200 ml·min^{-1}). Other batch culture studies were carried out routinely in 250-ml Erlenmeyer flasks

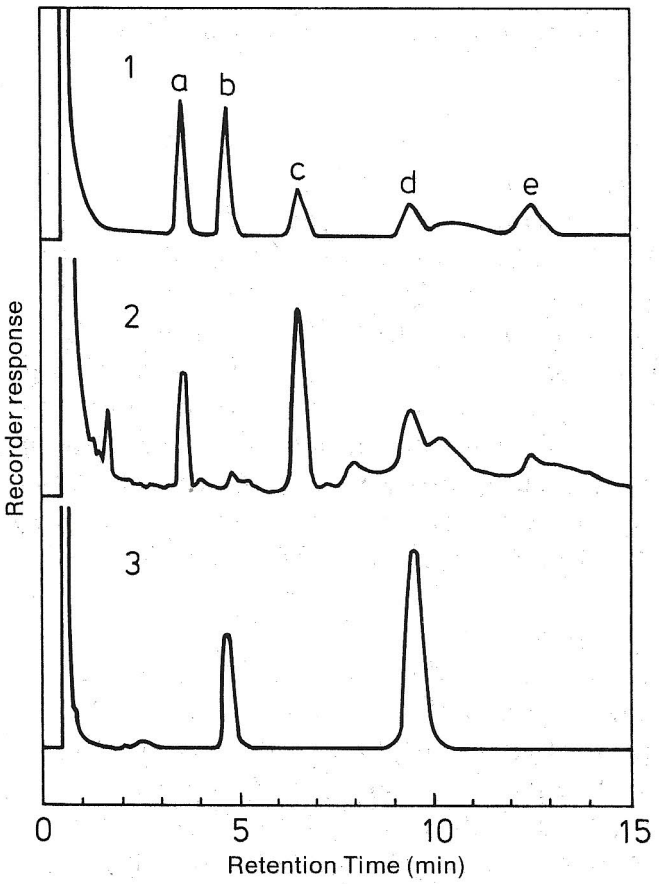

Fig. 1. Gas-chromatographic profiles of the methylesters of aromatic acids produced from 1-phenyldodecane and 1-phenyltridecane by *Acinetobacter lwoffi* AS1 on a PEG 20M column. 1. Aromatic acid standards: (a) phenylacetic acid; (b) 3-phenylpropionic acid; (c) 4-phenylbutyric acid; (d) *trans*-cinnamic acid; (e) 4-phenylbut-3-enoic acid; 2. products from 1-phenyldodecane; 3. products from 1-phenyltridecane.

containing 100-ml volumes of media and agitated on an orbital shaker (Gallenkamp, 200 rpm) at 25°C or 30°C.

Materials

Pure hydrocarbons were obtained from Koch-Light Labs, Colnbrook, Bucks, U.K., while aromatic acid standards were purchased from BDH Chemicals Ltd, Poole, Dorset, U.K. and Sigma Chemical Co. Ltd, U.K. 4-Phenylbut-3-enoic acid was synthesized by Dr F. S. Sariaslani at the University of Kent, Canterbury, U.K.

Results and Discussion

Characteristics of the 1-phenylalkane-utilizing strain

The organism isolated from an activated sludge sample was a gram-negative, non-motile, non-sporeforming, oxidase-negative, catalase-positive, coccoid rod

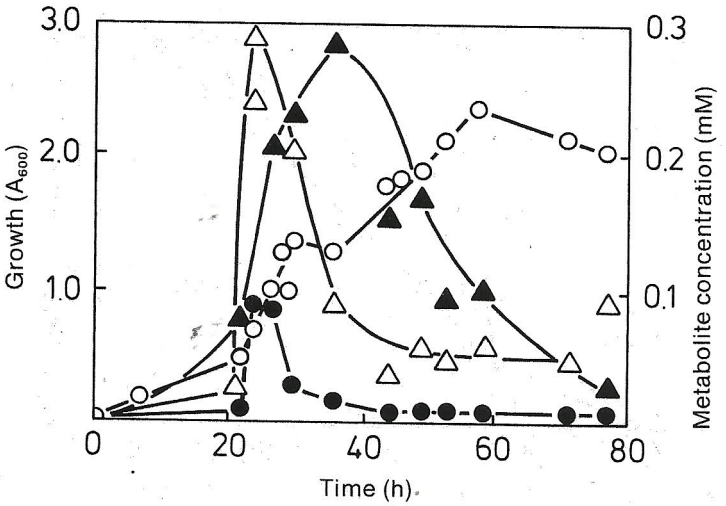

Fig. 2. Accumulation of aromatic acids during the growth of *Acinetobacter lwoffi* AS1 on 1-phenyldodecane. ○, Growth; ●, phenylacetic acid; △, 4-phenylbutyric acid; ▼, *trans*-cinnamic acid.

Table 1. Gas-liquid chromatographic properties of some aromatic acids formed during the growth of *Acinetobacter lwoffi* on 1-phenylalkanes

Product	Properties of methylesters	
	Retention time on methyl silicone gum, SE 30 (min)	Retention time on polyethylene glycol PEG 20M (min)
4-Phenylbutyric acid	4.5	5.7
4-Phenylbut-3-enoic acid	5.7	10.8
3-Phenylpropionic acid	3.2	4.2
trans-Cinnamic acid	4.6	8.2
Phenylacetic acid	2.3	3.2

which could not produce acid from glucose and other carbohydrates and could neither oxidize nor ferment sugars (Hugh and Leifson, 1953). The organism tentatively identified as *Acinetobacter lwoffi* AS1 could grow in mineral salts media containing acetate, succinate, laurate and a wide range of *n*-alkanes (C_{12}–C_{28}) and 1-phenylalkanes including 1-phenyloctane, 1-phenyldodecane, 1-phenyltridecane and 1-phenyltetradecane as sole sources of carbon and energy.

Metabolic products from 1-phenylalkanes

The catabolism of two growth-supporting phenylalkanes was subjected to detailed examination. 1-Phenyldodecane and 1-phenyltridecane were presented as carbon sources at 0.2% (v/v) and after incubation for 48 h, the culture supernatants were analysed by a combination of gas-liquid chromatography (Fig. 1) and thin-layer chromatography. Table 1 summarizes the gas chromatographic

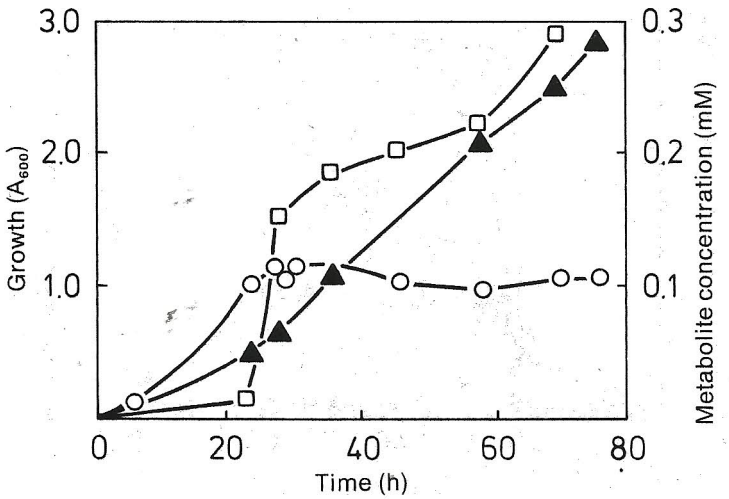

Fig. 3. Accumulation of aromatic acids during the growth of *Acinetobacter lwoffi* on 1-phenyltridecane. ○ , Growth; ▲, *trans*-cinnamic acid; □, 3-phenylpropionic acid.

characteristics of the oxidation products of 1-phenyldodecane and 1-phenyltridecane. Four aromatic acids were identified by means of standards (phenylacetic, 4-phenylbutyric, *trans*-cinnamic and 4-phenyl-but-3-enoic acid) in culture supernatants of phenyldodecane-grown cells. Only two aromatic acids (3-phenylpropionic and *trans*-cinnamic acid) accumulated as oxidation products of 1-phenyltridecane. Two hydroxylated aromatic acids tentatively identified as *o*-hydroxyphenylacetic acid ($R_f = 0.11$) and *o*-hydroxyphenylpropionic acid ($R_f = 0.18$) were detected after chromatography of ethereal extracts of the culture supernatants from 1-phenyldodecane-grown cells.

The formation of aromatic acids from 1-phenylalkanes by *Acinetobacter lwoffi* AS1 suggested a terminal methyl group oxidation to the corresponding phenylalkanoic acids, followed by β-oxidation of the alkyl side-chain. The formation of *trans*-cinnamic acid with an odd-numbered carbon atom side-chain may also suggest the involvement of α-oxidation in 1-phenyldodecane metabolism as appears to be the case for *Nocardia salmonicolor* (Sariaslani et al., 1974). The detection of hydroxylated acids also suggested that the aromatic acid products from 1-phenyldodecane could be further oxidized.

Growth of Acinetobacter lwoffi AS1 on 1-phenylalkanes and the transient accumulation of metabolic intermediates

The growth of *Acinetobacter lwoffi* AS1 on 1-phenyldodecane was diauxic (Fig. 2). During the first growth phase, exponential growth proceeded with a mean generation time of 7.6 h and phenylacetic, 4-phenylbutyric and *trans*-cinnamic acid accumulated, reaching maximum concentrations of 0.10, 0.29 and 0.28 mM respectively. *o*-Hydroxyphenylacetic acid was detected in trace amounts by thin-layer chromatography. During the second growth phase, however, the concentrations of the acids declined rapidly and large amounts of two

Table 2. Oxidation of possible intermediates of 1-phenyldodecane and 1-phenyltridecane metabolism by resting cell suspensions of *Acinetobacter lwoffi* AS1[1]

Test compound	Specific oxygen uptake (nmol·min^{-1}·mg dry wt^{-1}) after growth on various substrates		
	1-Phenyldodecane	1-Phenyltridecane	Succinic acid
Acetic acid	30.8	23.5	41.4
1-Phenyltridecane	n.d.[2]	14.2	n.d.
1-Phenyldodecane	48.9	7.1	7.4
Phenylacetic acid	41.8	7.1	12.4
4-Phenylbutyric acid	10.0	3.5	2.1
3-Phenylpropionic acid	2.7	2.8	0
trans-Cinnamic acid	7.3	7.1	0
Homogentisic acid	10.9	5.7	2.1
3,4-Dihydroxyphenylacetic acid	0	0	0
o-Hydroxyphenylacetic acid	1.8	1.4	4.1
m-Hydroxyphenylacetic acid	0	0	0
p-Hydroxyphenylacetic acid	0	0	0
Catechol	0.9	7.1	8.3
Protocatechuic acid	2.7	3.5	4.1
Benzoic acid	0	0	0
Endogenous rate	5.4	12.5	12.4

[1] See Materials and Methods for experimental details. Oxygen uptake values were corrected for endogenous respiration rates.
[2] n.d., not determined.

hydroxylated aromatic acids tentatively identified as *o*-hydroxyphenylacetic and *o*-hydroxyphenylpropionic acid were detected. There was no diauxie during growth on 1-phenyltridecane, and 3-phenylpropionic and *trans*-cinnamic acid accumulated (Fig. 3), but no hydroxylated acid was produced suggesting that these aromatic acid products are probably not further oxidized.

The diauxic effect observed during growth on 1-phenyldodecane could probably represent an adaptation period during which the enzymes that are required for complete metabolism of the free acids that have accumulated during the first growth phase are synthesized. A similar observation was made by Sariaslani et al. (1974).

Further metabolism of the aromatic acid products

The ability of *Acinetobacter lwoffi* AS1 to utilize the intermediate products of 1-phenylalkane metabolism was tested by inoculating minimal salts medium containing 4-phenylbutyric, 4-phenylbut-3-enoic, 3-phenylpropionic, *trans*-cinnamic and phenylacetic acid with nutrient broth-grown cells. It was observed that only phenylacetic acid was utilized as sole source of carbon and energy. Exponential growth on this substrate proceeded with a mean generation time

Table 3. Activities of key enzymes involved in the catabolism of 1-phenyldodecane in cell-free extracts of *Acinetobacter lwoffi* AS1

Enzyme	Reaction parameter measured	Specific activity (nmol·min^{-1}·mg protein^{-1}) after growth on various substrates		
		1-Phenyl-dodecane	Phenyl-acetic acid	Succinic acid
Phenylalkane-ω-hydroxylase	NADH oxidation at 340 nm	54.6	0.7	0.8
Acyl-CoA dehydrogenase	butyryl-CoA-dependent DCPIP reduction	18.9	16.8	12.8
β-Ketoacyl thiolase	acetoacetyl-CoA breakdown	133.4	46.3	35.4
Homogentisate-1,2-dioxygenase	maleylacetoacetate formation	12.5	7.1	1.7
Isocitrate lyase	glyoxylate-phenylhydrazone formation	70.9	1.0	1.3

of 3.0 h and the examination of culture supernatants by thin-layer chromatography showed that *o*-hydroxyphenylacetic acid was formed.

It was, however, intriguing to note that although *trans*-cinnamic and 3-phenylpropionic acid could not be utilized by the organism, *o*-hydroxyphenylpropionic acid was produced during growth on 1-phenyldodecane. This hydroxylated aromatic acid has been shown to be the first stable intermediate in the bacterial metabolism of *trans*-cinnamic acid (Coulson and Evans, 1959; Blakley and Simpson, 1964) and 3-phenylpropionic acid (Dagley et al., 1963, 1965). A plausible explanation is that a co-oxidation phenomenon could have been at play whereby *trans*-cinnamic acid was converted to *o*-hydroxyphenylpropionic acid in the presence of the utilizable phenylacetic acid. That may also account for the decline in the concentration of *trans*-cinnamic acid during the second phase of growth on 1-phenyldodecane as shown in Fig. 2.

Respirometric and enzymic studies

The results of oxygen uptake measurements comparing the ability of intact cells to oxidize possible intermediates after growth on 1-phenyldodecane, 1-phenyltridecane and succinic acid are shown in Table 2. The initial rates of oxidation of phenylacetic, 4-phenylbutyric and homogentisic acid are much higher for 1-phenyldodecane-grown than for succinic acid-grown cells. Benzoic acid, catechol, as well as 3,4-dihydroxyphenylacetic and *m*- and *p*-hydroxyphenylacetic acid were not readily oxidized.

The activities of some enzymes involved in the degradation of 1-phenyldodecane were measured in cell-free extracts and the results are shown in Table 3.

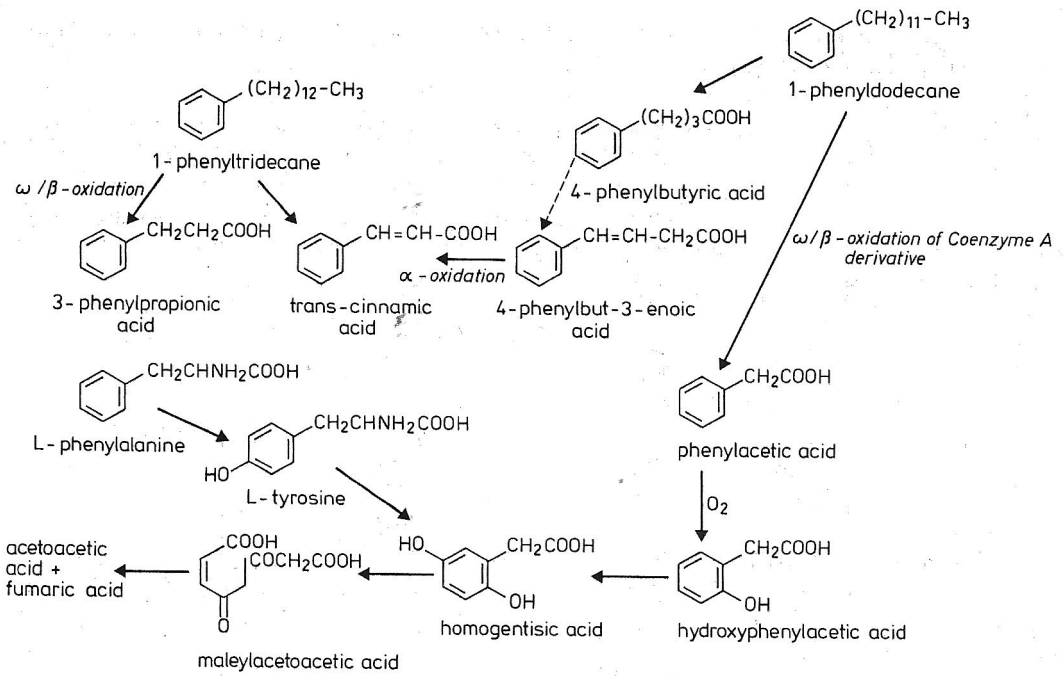

Fig. 4. Proposed scheme for the metabolism of 1-phenyldodecane and 1-phenyltridecane by *Acinetobacter lwoffi* AS1.

Attempts to demonstrate cell-free oxygenation of phenylacetic and *o*-hydroxyphenylacetic acid in cell-free extracts by polarographic oxygen uptake measurements in the presence of NADH or NADPH were unsuccessful. Similar difficulties in demonstrating the cell-free oxidation of these compounds had been previously reported by Blakley et al. (1967). However, organisms cultivated on 1-phenyldodecane produced higher activities of β-oxidation enzymes and isocitrate lyase compared to phenylacetic and succinic acid-grown cells. This observation may be attributed to side-chain degradation eliciting the synthesis of β-oxidation enzymes giving rise to acetate (C-2) units which are subsequently fed into the TCA cycle and glyoxylate by-pass. It is well known that the glyoxylate cycle serves an anaplerotic role when microbes grow on acetate as carbon and energy source (Kornberg, 1966). The involvement of the cycle in microbial *n*-alkane catabolism as evidenced by increased isocitrate lyase activity during growth on these compounds has been previously reported (Trust and Millis, 1970; Kleber and Aurich, 1973; Sariaslani et al., 1975).

Homogentisate 1,2-dioxygenase activity was about tenfold higher in 1-phenyldodecane-grown than in succinic acid-grown cells.

Proposed metabolic routes for the degradation of 1-phenylalkanes in Acinetobacter lwoffi AS1 and relationship with aromatic amino acid catabolism

The probable catabolic routes for 1-phenyldodecane and 1-phenyltridecane

Table 4. Oxygen consumption during the catabolism of aromatic acids and possible intermediates by resting cell suspensions of *Acinetobacter lwoffi*[1]

Test compound	Specific oxygen uptake (nmol·min^{-1}·mg dry wt^{-1}) after growth on various substrates		
	L-Phenylalanine	L-Tyrosine	Succinic acid
L-Phenylalanine	47.8	19.9	0
L-Tyrosine	22.3	46.5	1.6
Phenylpyruvic acid	25.5	66.4	1.6
Phenyllactic acid	12.8	16.6	2.4
Phenylacetic acid	31.9	16.6	3.3
o-Hydroxyphenylacetic acid	14.3	19.9	4.1
m-Hydroxyphenylacetic acid	9.6	26.6	0
p-Hydroxyphenylacetic acid	8.0	33.2	0
Homogentisic acid	28.7	66.4	0
p-Hydroxyphenylpyruvic acid	44.6	73.1	4.1
p-Hydroxyphenyllactic acid	3.2	39.9	1.6
3,4-Dihydroxyphenylacetic acid	9.6	0	3.7
3,4-Dihydroxybenzoic acid	19.1	13.3	0
Catechol	12.8	16.6	3.3
Endogenous rate	22.3	66.4	9.0

[1] Oxygen uptake values were corrected for endogenous respiration rates.

as suggested by product identification, oxygen uptake measurements and enzymic analysis are summarized in Fig. 4. 1-Phenyltridecane is metabolized to 3-phenylpropionic and *trans*-cinnamic acid which probably accumulate in the medium as they could not be further broken down (Figs 1 and 3). The degradation of long-chain *n*-alkylbenzenes with odd-numbered carbon atoms has been reported to result in the formation of *trans*-cinnamic acid which may or may not be further oxidized (Davis and Raymond, 1961; Jigami et al., 1974; Sariaslani et al., 1974). Our results also suggest that 1-phenyldodecane is degraded via phenylacetic, *o*-hydroxyphenylacetic and homogentisic acid and the organism grew readily on these substrates. The probable reason for the accumulation of 4-phenylbutyric acid during microbial growth on 1-phenyldodecane was provided by Sariaslani et al. (1974). These authors presented evidence that the β-oxidation enzyme system had decreased affinity for 4-phenylbutyryl-CoA, probably resulting from steric hindrance owing to the proximity of the aromatic ring. The same argument could account for the accumulation of *trans*-cinnamic acid during the growth of *Acinetobacter lwoffi* AS1 on both 1-phenyldodecane and 1-phenyltridecane.

Respirometric and enzymic studies implicated homogentisic acid as an intermediate in 1-phenyldodecane degradation in *Acinetobacter lwoffi* AS1. Interestingly, this dihydroxylated aromatic acid has been shown to be an intermediate in the bacterial metabolism of tyrosine (Jones et al., 1952) and phenylalanine

(Dagley et al., 1953). Our isolate also grew on L-tyrosine and L-phenylalanine as sole sources of carbon and energy and homogentisic acid was detected in culture supernatants by its characteristic green coloured reaction product with Brigg's reagent. Successive adaptation experiments using the oxygen electrode, however, showed that phenylalanine-grown cells could oxidize tyrosine (Table 4). It is therefore probable that phenylalanine is first hydroxylated to tyrosine before it is further metabolized via homogentisic acid. Since homogentisic acid plays a central role in the bacterial metabolism of phenylalanine, tyrosine and phenylacetic acid, the degradative pathway for 1-phenyldodecane could be envisaged as a composite of the *n*-alkane, and the aromatic amino acid degradative pathways.

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