The Catabolism of Phenanthrene and Naphthalene by Bacteria

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Thirteen strains of bacteria able to grow on phenanthrene were isolated from soil; they included fluorescent and non-fluorescent pseudomonads, vibrios and unidentified bacteria. Two of the pseudomonads, like *Aeromonas* sp. s45P1, also grew on naphthalene. In all strains, growth on phenanthrene induced the enzyme responsible for the conversion of 1-hydroxy-2-naphthoate to 2-carboxybenzaldehyde, NAD-dependent 2-carboxybenzaldehyde dehydrogenase and protocatechuate oxygenase, but not salicylate hydroxylase, catechol oxygenase or NAD(P)H-dependent 1-hydroxy-2-naphthoate hydroxylase. Growth on naphthalene induced salicylate hydroxylase and catechol oxygenase. It is suggested that the catabolism of phenanthrene occurs via protocatechuate in all these bacteria, and that the pathways for degradation of phenanthrene and naphthalene are separate.

INTRODUCTION

With the development of the petroleum industry, there has been an increase in the amount of polyaromatic hydrocarbons released into the environment. As part of a study of the mechanism of degradation of hydrocarbons by micro-organisms, we have examined the catabolism of phenanthrene by bacteria. The enzymic steps involved in the conversion of phenanthrene to 1-hydroxy-2-naphthoate by a soil pseudomonad were elucidated by Evans, Fernley & Griffiths (1965). The naphthoate was then presumed to be oxidatively decarboxylated to 1,2-dihydroxynaphthalene which would be further degraded through the naphthalene-catabolizing pathway described by Davies & Evans (1964). However, *Aeromonas* sp. s45P1, which was isolated from soil by Kiyohara, Nagao & Nomi (1976), did not convert 1-hydroxy-2-naphthoate to 1,2-dihydroxynaphthalene but instead metabolized it to 2-carboxybenzaldehyde which was then oxidized to *o*-phthalate, by an NAD-dependent dehydrogenase, and finally to protocatechuate (Fig. 1). The cleavage of the naphthoate (Kiyohara & Nagao, 1977) involves the fission of the bond between the carbon atoms bearing the hydroxyl and carboxyl groups by an intradiol type of dioxygenase which acts in a similar way to gentisate oxidase (EC 1.13.1.4) (Sugiyama *et al.*, 1958).

The work described in the present paper shows that the protocatechuate pathway is the only pathway for degradation of phenanthrene in 13 independent isolates of soil bacteria. The relationship between the pathways for degradation of phenanthrene and naphthalene is also described.

METHODS

Bacterial strains and media. Bacteria able to grow on phenanthrene were isolated from soil by enrichment culture in phenanthrene/salts medium, followed by plating on *Pseudomonas* F agar (Difco). The salts medium contained (% w/v, in tap water): $(\text{NH}_4)_2\text{HPO}_4$, 0.5; KH_2PO_4 , 0.15; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.15; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; NaCl , 0.05; the pH was 7.3 without adjustment. Phenanthrene (0.3%, w/v) was added to the medium before autoclaving at 120 °C for 15 min. The phenanthrene was melted by the heat but was finely dispersed by vigorously shaking the flasks after cooling. Naphthalene, which was crystallized from

Fig. 1. Pathways proposed for the degradation of phenanthrene and naphthalene: ---, Evans *et al.* (1965); - - - - -, Davies & Evans (1964); —, Kiyohara *et al.* (1976).

Enzymes: A, 1-hydroxy-2-naphthoate-degrading enzyme; B, 2-carboxybenzaldehyde dehydrogenase; C, protocatechuate 3,4-oxygenase; D, protocatechuate 4,5-oxygenase; E, 1-hydroxy-2-naphthoate hydroxylase; F, salicylaldehyde dehydrogenase; G, salicylate hydroxylase; H, catechol 1,2-oxygenase; I, catechol 2,3-oxygenase.

acetone-water and aseptically collected by filtration, was added to salts medium after autoclaving. Heat-labile substrates were sterilized by membrane filtration (Sartorius, 0.2 μ m pore size); other substrates were autoclaved in the salts medium.

Diagnostic tests on the isolated strains were carried out according to Pelczar (1957), Hasegawa (1975), Holding & Collee (1971), Gibbs & Skinner (1966) and Stanier, Palleroni & Doudoroff (1966). Identifications were according to Buchanan & Gibbons (1974).

Preparation of extracts. Bacteria (2 ml suspension), which had been subcultured in phenanthrene/salts medium (10 ml) in a large test tube for 2 d at 30 °C, were inoculated into salts medium (100 ml) containing 0.3% (w/v) carbon source in 500 ml conical flasks and shaken (155 reciprocations min^{-1}) for 18 to 20 h at 30 °C. The bacteria were centrifuged at 20000g for 20 min at 5 °C, washed twice with sterile 10 mM- $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 7.1, and suspended in the same buffer (approx. 8 mg dry wt ml^{-1}). This cell suspension was used for measurements of O_2 uptake. To prepare extracts, the suspension was homogenized by sonication at 20 kHz (type T-A-4201 sonic oscillator; Marine Instruments Co., Tokyo, Japan) for three periods of 5 min at 5 °C and centrifuged at 106 000g for 30 min. The supernatant liquid was used as the extract for enzyme assays.

Enzyme assays. Published procedures were used for assay of the following enzymes: catechol 1,2-oxygenase [catechol:oxygen 1,2-oxidoreductase (deacyclizing); EC 1.13.11.1] (Hegeman, 1966); catechol 2,3-oxygenase [catechol:oxygen 2,3-oxidoreductase (deacyclizing); EC 1.13.11.2] (Feist & Hegeman, 1969); protocatechuate 3,4-oxygenase [protocatechuate:oxygen 3,4-oxidoreductase (deacyclizing); EC 1.13.11.3] (Fujisawa & Hayaishi, 1968); protocatechuate 4,5-oxygenase [protocatechuate:oxygen 4,5-oxidoreductase (deacyclizing); EC 1.13.11.8] (Ono, Nozaki & Hayaishi, 1970); protocatechuate 2,3-oxygenase (Crawford, 1975); salicylaldehyde dehydrogenase (Shamsuzzaman & Barnsley, 1974); salicylate hydroxylase [salicylate,

NADH: oxygen oxidoreductase (1-hydroxylating, decarboxylating); EC 1.14.13.1] (Yamamoto *et al.*, 1965); and 1-hydroxy-2-naphthoate-degrading enzyme (Kiyohara & Nagao, 1977). Catechol 1,2-oxygenase and salicylate hydroxylase could not be assayed directly in extracts with a high activity of catechol 2,3-oxygenase and in these cases the 2,3-oxygenase was inactivated by treating the extracts with 30 mM-H₂O₂ for 1 min at 30 °C. Protocatechuate 3,4-oxygenase was assayed after inactivation of the 4,5-oxygenase in the same manner.

1-Hydroxy-2-naphthoate hydroxylase was assayed by following the formation of NADH or NADPH under the same conditions as were used for salicylate hydroxylase except that 1-hydroxy-2-naphthoate was used as substrate.

2-Carboxybenzaldehyde dehydrogenase was measured spectrophotometrically by following the formation of NADH at 30 °C in a 1 cm light-path cuvette. The reaction mixture (3.0 ml) contained: 0.5 ml extract; 0.5 ml 1.2 mM-NAD; 1.5 ml 48.3 mM-Na₂HPO₄ (pH 8.0); 0.5 ml 2.4 mM-2-carboxybenzaldehyde was added to initiate the reaction.

Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine albumin as a standard.

Oxygen uptake by intact bacteria. The capacity of intact bacteria to oxidize substrates was measured by conventional manometric techniques. Each Warburg vessel contained 10 mmol substrate and chloramphenicol (final concn 100 µg ml⁻¹) in 2.0 ml NaH₂PO₄/K₂HPO₄ buffer, pH 7.1, in the main compartment; 1 ml cell suspension (approx. 8 mg dry wt) in the side-arm; and 0.2 ml 20% (w/v) KOH in the centre well. Rates of O₂ uptake are expressed as µl O₂ consumed h⁻¹ (mg dry wt)⁻¹.

Chemicals. All chemicals used were obtained commercially: phenanthrene (99% purity), 1-hydroxy-2-naphthoic acid (extra pure), protocatechuic acid (extra pure), catechol (guaranteed grade), disodium *o*-phthalate (guaranteed grade), benzoic acid (guaranteed grade) and disodium succinate (extra pure) from Tokyo Chemical Industry Co., Tokyo, Japan; naphthalene (guaranteed grade) and sodium salicylate (guaranteed grade) from Ishizu Pharmaceutical Co., Osaka, Japan; crystalline bovine albumin from Seikagaku Kogyo Co., Tokyo, Japan; NAD (98% purity), NADH (98% purity), NADPH (98 to 99% purity) and FAD (94 to 99% purity) from Sigma.

RESULTS AND DISCUSSION

We isolated 13 strains of bacteria able to grow on phenanthrene. All were Gram-negative, motile, polar-flagellate rods. Six of these strains (s15P1, s47P1, s179P3, s179P21, s182P1 and s185P1) produced diffusible, fluorescent pigment on *Pseudomonas* F agar; tests for catalase, oxidase, reduction of nitrate and denitrification were positive; Voges-Proskauer and H₂S formation tests were negative; no growth was observed at 41 °C. These strains were therefore termed 'fluorescent pseudomonads'. Three strains (s175P1, s184P1 and s187P1) differed only in their failure to produce pigment; they were termed 'non-fluorescent pseudomonads'. Two strains (s176P1 and s183P1) were termed 'vibrios' because they formed luminescent colonies and attacked glucose oxidatively and fermentatively without the evolution of gas. s183P1 produced indole and H₂S and gave a positive Voges-Proskauer test. The other two isolates could not be assigned to any group. In addition, strain s7K5 was isolated from soil by using kerosene as sole source of carbon and energy and was a non-fluorescent pseudomonad.

All strains able to grow on phenanthrene also grew on *o*-phthalate and *p*-hydroxybenzoate (Table 1). Only *Aeromonas* sp. s45P1 and the fluorescent pseudomonads s15P1 and s47P1 grew on naphthalene as well as on phenanthrene. All strains gave a yellowish orange colour in phenanthrene/salts media after about 15 h growth. The phenotype of the ability to assimilate naphthalene was represented as Nah⁺ by Dunn & Gunsalus (1973) and we have expressed the ability to assimilate phenanthrene as Phn⁺. According to this designation, s45P1, s47P1 and s15P1 were Phn⁺Nah⁺, s7K5 was Phn⁻Nah⁺, and the other strains were Phn⁺Nah⁻. These characteristics were unstable. s47P1 lost the Nah⁺ character to become Phn⁺Nah⁻ and both s45P1 and s15P1 gave rise to Phn⁻Nah⁺ and Phn⁺Nah⁻ phenotypes in the course of this work. Both characters were liable to disappear during prolonged storage on nutrient agar slants or during transfer to different growth substrates. In particular, Nah⁺ was completely lost by growing on benzoate but Phn⁺ and the ability to grow on salicylate were not lost under these conditions. It is possible that Nah⁻ isolates might have lost their Nah⁺ character during enrichment with phenanthrene.

Table 1. *Growth of strains on aromatic compounds as sole sources of carbon*

Organisms were inoculated into basal medium (10 ml) containing different carbon compounds (0.3 %, w/v) and shaken at 30 °C for 7 d.

Group	Strain	Growth on:					
		Phenan- threne	<i>o</i> - Phthalate	<i>p</i> -Hydroxy- benzoate	Naphtha- lene	Salicylate	Benzoate
<i>Aeromonas</i> sp.	s45P1	+	+	+	+	+	+
Fluorescent	s15P1, s47P1	+	+	+	+	+	+
<i>Pseudomonas</i> sp.	s179P3, s182P1	+	+	+	—	+	+
	s179P21, s185P1	+	+	+	—	—	—
Non-fluorescent	s7K5	—	—	—	+	+	—
<i>Pseudomonas</i> sp.	s175P1	+	+	+	—	+	+
	s184P1, s187P1	+	+	+	—	—	—
<i>Vibrio</i> sp.	s176P1	+	+	+	—	—	—
	s183P1	+	+	+	—	+	+
Unidentified	s172P12, s190P2	+	+	+	—	—	—

Table 2. *Oxidation of possible metabolic intermediates of phenanthrene and naphthalene by bacteria which had been grown on phenanthrene*

Bacteria (approx. 8 mg dry wt) which had been grown on phenanthrene (0.3 %, w/v) for 18 to 20 h were tested manometrically for their ability to oxidize substrates (10 mmol) in the presence of chloramphenicol (100 µg ml⁻¹). Phenanthrene and naphthalene were used as fine powders. Measurements were corrected for endogenous respiration [approx. 0.9 µl O₂ h⁻¹ (mg dry wt)⁻¹].

Rates of oxidation [µl O₂ uptake h⁻¹ (mg dry wt)⁻¹] of:

Strain	Phenan- threne	1- Hydroxy- 2-naph- thoate	2- Carboxy- benzalde- hyde	<i>o</i> - Phthalate	Protocate- chuate	Naphtha- lene	Salicyl- aldehyde	Salicylate	Catechol
s45P1	27.0	20.7	14.4	20.0	26.6	0.8	1.8	1.0	2.2
s15P1	19.3	41.3	NT	17.7	12.2	4.3	NT	2.0	5.3
s47P1	16.6	24.9	NT	12.8	7.6	6.8	NT	3.0	1.4
s179P3	33.6	35.1	35.7	7.7	26.1	11.9	5.2	6.5	4.5
s179P21	21.6	42.9	32.9	16.9	36.6	23.9	1.0	6.1	6.4
s182P1	32.6	37.4	60.7	20.6	29.0	34.2	4.6	4.0	5.7
s185P1	37.8	28.1	30.9	15.6	31.0	4.5	4.3	—	2.4
s175P1	34.4	36.4	38.0	18.9	54.7	5.0	10.5	1.8	8.2
s184P1	27.8	48.6	25.8	17.4	20.0	4.0	9.9	0.9	5.9
s187P1	40.1	37.7	32.2	12.3	13.4	32.7	10.2	7.8	5.5
s176P1	34.4	26.3	57.0	25.1	45.8	8.2	9.4	0.7	5.4
s183P1	25.7	45.0	57.1	22.0	52.9	40.1	8.6	4.5	7.0
s172P12	32.4	36.4	34.4	5.4	33.4	1.8	—	1.5	—
s190P2	33.5	37.3	8.1	10.1	11.5	19.7	0.5	0.8	—

—, No significant net O₂ uptake; NT, not tested.

All Phn⁺ strains which had been grown on phenanthrene oxidized 1-hydroxy-2-naphthoate, 2-carboxybenzaldehyde, *o*-phthalate and protocatechuete but showed little O₂ uptake with salicylaldehyde, salicylate and catechol (Table 2). These results suggest that in all these strains, phenanthrene is catabolized through 1-hydroxy-2-naphthoate, 2-carboxybenzaldehyde, *o*-phthalate and protocatechuete, as has previously been shown for *Aeromonas* sp. s45P1 (Kiyohara *et al.*, 1976). Five Nah⁻ strains (s179P21, s182P1, s187P1, s183P1 and s190P2) oxidized naphthalene as well as phenanthrene. The supernatants after oxidation of naphthalene by these strains gave a yellow colour in alkali ($\lambda_{\max}$ = 420 nm), which is similar to that of *cis-o*-hydroxybenzalpyruvate (Davies & Evans, 1964). This suggests that

Table 3. *Oxidation of possible metabolic intermediates of phenanthrene and naphthalene by Nah⁺ strains which had been grown on naphthalene*

Bacteria (approx. 8 mg dry wt) which had been grown on naphthalene (0.3%, w/v) for 18 to 20 h were tested manometrically for their ability to oxidize substrates (10 mmol) in the presence of chloramphenicol (100 $\mu\text{g ml}^{-1}$). Phenanthrene and naphthalene were used as fine powders. Measurements were corrected for endogenous respiration [approx. 2.0 $\mu\text{l O}_2 \text{ h}^{-1} (\text{mg dry wt})^{-1}$].

Strain	Rates of oxidation [$\mu\text{l O}_2$ uptake $\text{h}^{-1} (\text{mg dry wt})^{-1}$] of:						
	Phenanthrene	1-Hydroxy-2-naphthoate	<i>o</i> -Phthalate	Protocatechuete	Naphthalene	Salicylate	Catechol
s45P1	1.2	1.8	NT	1.2	32.1	24.5	17.9
s15P1	1.4	0.9	0.7	3.5	35.5	14.0	4.2
s7K5	6.1	—	—	—	67.5	56.8	57.2

—, No significant net O_2 uptake; NT, not tested.

the enzymes oxidizing phenanthrene in these strains also oxidize naphthalene. On the other hand, Nah⁺ strains s45P1, s15P1 and s7K5 after growth on naphthalene oxidized salicylate and catechol but not phenanthrene, 1-hydroxy-2-naphthoate, *o*-phthalate or protocatechuete (Table 3), suggesting that these strains catabolized naphthalene through catechol as proposed by Davies & Evans (1964).

The measurements of O_2 uptake by intact bacteria were confirmed by the results of enzyme assays (Table 4). All Phn⁺ strains grown on phenanthrene contained 1-hydroxy-2-naphthoate-degrading enzyme, 2-carboxybenzaldehyde dehydrogenase and protocatechuete oxygenase, but no significant activities of salicylate hydroxylase, catechol oxygenase or 1-hydroxy-2-naphthoate hydroxylase. The salicylaldehyde dehydrogenase activity may have been due to cross-specificity of 2-carboxybenzaldehyde dehydrogenase. 1-Hydroxy-2-naphthoate-degrading enzyme and 2-carboxybenzaldehyde dehydrogenase were not present in bacteria grown on succinate.

Nah⁺ strains which had been grown on naphthalene had salicylate hydroxylase and catechol oxygenase activities (Table 5). There was no detectable activity of 1-hydroxy-2-naphthoate-degrading enzyme, 2-carboxybenzaldehyde dehydrogenase or protocatechuete oxygenase. Strain s7K5, the only strain isolated as Phn⁻, had especially high activities of salicylate hydroxylase and catechol 2,3-oxygenase, and even showed small but detectable activities of these enzymes after growth on succinate. These results confirm the suggestion that these strains metabolize naphthalene through the pathway proposed by Davies & Evans (1964).

Protocatechuete and catechol are cleaved in three ways: by *meta*-cleavage, *ortho*-cleavage or both. *Aeromonas* sp. s45P1 metabolized protocatechuete and catechol by both the *meta*- and *ortho*-cleavage pathways but *Pseudomonas* sp. s15P1 used only the *ortho*-cleavage pathway. Phn⁺ strains (s179P21, s182P1, s185P1 etc.) oxidized protocatechuete by the *meta*-cleaving enzyme (Table 4). No Phn⁺ strain had a protocatechuete 2,3-oxygenase similar to that found in *Bacillus* species by Crawford (1975).

None of the strains we isolated oxidized phenanthrene through salicylate and catechol or produced an NAD(P)H-dependent 1-hydroxy-2-naphthoate hydroxylase, which would catalyse the decarboxylation of the naphthoate. In Phn⁺Nah⁺ strains, the pathways for degradation of phenanthrene and naphthalene seem to be independent of each other, and not connected in the metabolic process of aromatic ring-fission. We conclude that the mechanism for catabolism of phenanthrene, which we proposed for *Aeromonas* sp. s45P1, occurs widely in bacteria.

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Table 4. *Activities of enzymes in bacteria which had been grown on phenanthrene*

Activities were assayed in extracts prepared from bacteria which had been grown for 18 to 20 h in basal medium containing phenanthrene (0.3 % w/v).

Strain	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]							
	1-Hydroxy- 2-naphthoate- degrading enzyme	2-Carboxy- benzaldehyde dehydrogenase	Proto- catechuate 3,4-oxygenase	Proto- catechuate 4,5-oxygenase	1-Hydroxy- 2-naphthoate hydroxylase	Salicylaldehyde dehydrogenase	Salicylate hydroxylase	Catechol 1,2-oxygenase Catechol 2,3-oxygenase
s45p1	179	174	17	83	—	—	—	—
s15p1	304	183	7	—	—	—	—	—
s47p1	74	34	5	24	—	—	—	—
s179p21	61	106	—	28	—	19	—	1
s182p1	14	183	—	32	—	—	1	1
s185p1	61	106	—	83	—	72	—	1
s175p1	37	183	—	119	—	137	—	1
s184p1	362	200	21	—	—	72	—	—
s187p1	392	149	6	—	—	48	—	—
s176p1	74	174	—	129	—	130	—	1
s183p1	64	84	—	107	—	58	—	1
s172p12	34	106	67	137	—	—	—	1
s190p2	642	87	3	—	—	39	—	—

The value of 1 is not significant; —, no activity detected.

Table 5. Activities of enzymes of the pathway for naphthalene degradation in extracts of *Nah*⁺ strains which had been grown on naphthalene or on succinate

Activities were assayed in extracts prepared from bacteria which had been grown for 18 to 20 h in basal medium containing naphthalene (0.3 %, w/v) or succinate (10 mM).

Strain	Growth substrate	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]		
		Salicylate hydroxylase	Catechol 1,2-oxygenase	Catechol 2,3-oxygenase
s45P1	Naphthalene	48	19	550
	Succinate	NT	—	—
s15P1	Naphthalene	61	4	1
	Succinate	NT	—	—
s7K5	Naphthalene	771	—	242
	Succinate	NT	—	12

The value of 1 is not significant; —, no activity detected; NT, not tested.

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