Pseudomonas aeruginosa Mutants Defective in Heptane Oxidation

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SUMMARY

Fourteen mutants of *Pseudomonas aeruginosa* ATCC17423, isolated for their inability to grow rapidly on heptane while still growing normally on heptanoic acid, were all also able to grow on heptanol or heptanal. A study of heptane oxidation in extracts showed differences between the mutants, which were resolved into four groups (A to D). Group A or B alone could not oxidize heptane, but a mixture of the extracts was effective, and either A or B stimulated the slight activity of extracts from group D mutants. Extracts from group C mutants were inactive in virtually all circumstances. The mutants therefore lack, in different ways, one or more of the three components demonstrated to be necessary for heptane oxidation, but no mutant totally lacking rubredoxin was demonstrated.

INTRODUCTION

Alkane oxidation in *Pseudomonas aeruginosa* (van Eyk & Bartels, 1970) appears generally similar in mechanism to that in *P. oleovorans* (Baptist, Gholson & Coon, 1963; Gholson, Baptist & Coon, 1963) and *P. denitrificans* (Kusunose *et al.* 1967). Enzymological investigations have shown a three-component alkane hydroxylase, yielding the corresponding primary alcohol; this is oxidized to the aldehyde and then the acid by dehydrogenases. Mutants of *P. denitrificans* lacking the ability to grow on hexadecane, but still able to grow in hexadecanoic acid, were isolated by Illarionov (1972). Some of these mutants grew on hexadecanol or hexadecanal, some on hexadecanal but not hexadecanol, and a large number (50% of all alkane-defective mutants) on hexadecanol but not hexadecanal. Jenkins, Raboin & Moran (1972) isolated mutants of *Mycobacterium rhodochrous* B29 which normally grows on any of several alkanes. Some mutants no longer grew on any alkane tested; some no longer grew on certain alkanes, but could grow on others. One mutant, unable to grow on octane, still possessed octane hydroxylase activity in extracts of organisms grown on other alkanes. This paper describes the *n*-heptane oxidizing system of *P. aeruginosa* ATCC17423 and the defective systems of mutants derived from this strain.

METHODS

Organism. *Pseudomonas aeruginosa* ATCC17423 was kindly provided by Professor R. Y. Stanier, University of California, Berkeley, U.S.A. Stock cultures were maintained on nutrient agar slopes at room temperature.

Growth of bacteria. Bacteria were grown at 30 °C in 2 l flasks containing 500 ml liquid mineral medium on a rotary shaker. The medium contained: 35 mM- Na_2HPO_4 ; 15 mM- $(\text{NH}_4)_2\text{SO}_4$; 18 μM - FeSO_4 ; 1.9 μM - ZnSO_4 ; 360 nM- MnSO_4 ; 200 nM- Na_2MoO_4 ; 160 nM- CuSO_4 ; 160 nM- CoCl_2 ; 1.6 μM - MgCl_2 ; and 260 nM- CaCl_2 . The last two components were

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autoclaved separately and added immediately before inoculation. Heptane (2 ml) was added to some cultures as carbon and energy source and the growing culture was harvested by centrifuging after 24 to 36 h incubation. Alternatively, cultures were supplied with 10 mM-sodium malonate in the mineral medium. After 16 h further 10 mM-sodium malonate (7.5 ml of 10 %, w/v, solution) was added to the 500 ml culture, with 2 mM-diethoxymethane, a gratuitous inducer of the alkane oxidizing system (van Eyk & Bartels, 1968). After a further 6 h incubation, the bacteria were harvested by centrifuging. This latter method was preferred because extracts were free from the traces of heptanoic acid sometimes present in extracts of heptane-grown organisms.

Mutant isolation. Bacteria taken in the exponential phase of growth from a nutrient broth culture were resuspended at about 5×10^7 organisms/ml in 0.1 M-citrate buffer pH 6.0 and then 10 μ g *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine/ml were added. After 15 min at room temperature, the bacteria were centrifuged, resuspended in nutrient broth and left, unshaken, at 30 °C for 5 h. The viable count was determined in a small sample and the remainder was stored at 3 °C for 24 h. It was then diluted in mineral medium to give 1000 organisms/ml and 0.1 ml samples were spread on agar plates containing mineral medium solidified with 1.5 % Difco Special Agar-Noble. The plates were incubated at 30 °C in sealed tins in an atmosphere containing heptane, supplied by pipetting 50 μ l on a Whatman No. 1 filter paper in each Petri dish lid. After 2 days' incubation, when wild-type organisms had formed large colonies, all small colonies were picked off and those that grew on nutrient agar at the same rate as the wild-type organism were tested for growth on heptane, heptanol, heptanal and heptanoic acid by incubating in an atmosphere of the substrate as described above. To confirm that the isolates were derived from *P. aeruginosa* ATCC 17423, the characteristic pigment formation was noted and their growth responses to phenylalanine and butan-2,3-diol were tested. The wild-type organism, unlike most of the *P. aeruginosa* strains tested by Stanier, Palleroni & Doudoroff (1966), grew on the former but not on the latter.

Preparation of cell-free extracts. A suspension of 1.5 g (wet) bacteria in 0.1 M-tris-chloride buffer pH 7.6, total volume 6 ml, was disrupted ultrasonically, using an MSE 100 W ultrasonic disintegrator, for 3 min. The extract was centrifuged at 105000 g for 20 min at 4 °C and the supernatant fluid was used for studies of enzymic heptane oxidation. In studies on the possible involvement of particulate components, this centrifugation was preceded by one at 17000 g for 25 min and followed by one at 200000 g for 120 min, both at 4 °C.

Assay of heptane oxidizing activity. Crude extract, containing up to 10 mg protein, was added to an incubation mixture containing 70 μ mol tris-chloride buffer pH 7.6, 3 μ mol NADH and 10 μ mol MgCl_2 in a 25 ml conical flask, so that the total volume was 1.0 ml, and prewarmed at 30 °C for about 3 min. Heptane (340 μ mol) was then added to the flask, which was immediately stoppered and incubated with shaking at 30 °C for 15 min. The reaction was stopped by cooling the flask rapidly in melting ice. The products of heptane oxidation were assayed by injecting 1 μ l samples of the incubation mixture into a Perkin-Elmer F11 Mk II gas chromatograph using a glass column, 915 $\times$ 2 mm, packed with Porapak Q (80 to 100 mesh; Waters and Associates, Stockport, Cheshire) and operating at 230 °C. A glass capillary tube to trap non-volatile materials was used in the injection port, which was kept at 260 °C. The carrier gas, N_2 , flowed at 22 cm³/min. Two products were detected in such assays, and had retention times identical with heptan-1-ol and heptanoic acid. The latter type predominated, typically comprising 90 % of the total products. Proportionality between product concentration and recorded peak height was established for each. Heptane oxidizing activity was expressed in units (u) of μ mol total products formed/min.

Fig. 1. Alcohol dehydrogenase activities in extracts of heptane-grown and nutrient broth-grown organisms. Extracts were prepared by ultrasonic disruption and assayed for dehydrogenase activity in the presence of each alcohol from methanol to hexanol (10 mM) or heptanol (1 mM). The specific activity with each alcohol is shown for (■) heptane-grown and (□) nutrient broth-grown bacteria.

Spectrophotometric assays. Ethanol, heptanol and heptanal dehydrogenase activities were assayed at room temperature by measuring ΔE_{340} caused by the enzymic reduction of NAD^+ to NADH. For ethanol and heptanol dehydrogenases, the standard reaction mixtures, in total volumes of 1.0 ml, were 70 μmol sodium pyrophosphate buffer pH 9.0, 1.5 μmol NAD^+ , bacterial extract, and 1 mmol ethanol or 1 μmol heptanol; for heptanal dehydrogenase they were 70 μmol tris-chloride pH 8.8, 1.5 μmol NAD^+ , bacterial extract, and 1 μmol heptanal. Heptanol and heptanal were added as solutions in the presence of 0.05 % Triton X-100, which had no effect either alone or on the ethanol dehydrogenase assay. The reactions were started by the addition of the alcohol or aldehyde substrate.

Protein estimations. Protein concentrations were estimated by a modification of the method of Lowry, Rosebrough, Farr & Randall (1951) described by Kennedy & Fewson (1968).

Chemicals. DEAE-cellulose was from BioRad Laboratories, Richmond, California, U.S.A.; lysozyme, nicotinamide coenzymes and triethanolamine from Boehringer, Mannheim, Germany; diethoxymethane from Fluka AG, Buchs, Switzerland; *n*-alkanes, heptan-1-ol, reduced glutathione, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire.

RESULTS AND DISCUSSION

The mutagenic treatment killed about 90 % of the bacteria. Of approximately 25000 survivors screened, about 1 % gave small colonies. Sixteen mutants were isolated which grew very slowly or not at all on heptane but which could still grow on heptanoic acid; all could grow on heptanol or heptanal. This corresponds to the finding that all 11 dodecane-mutants of *P. aeruginosa* ATCC 15692 were able to grow on dodecanol or dodecanal (Macham and Heydemann, unpublished observation). Nearly all investigations have implicated the corresponding alcohol, aldehyde and fatty acid as intermediates in alkane catabolism, and

Table 1. *Stimulation of heptane oxidation by cations*

Metal chlorides were added, at the indicated final concentrations, to 1 ml crude extract + 3 μ mol NADH + 340 μ mol heptane. Heptane-oxidizing activity is given relative to a value of 26.5 mu/mg protein without added cations.

Cation	Concentration (mM)	Relative rate (%)
None	—	(100)
Sr ²⁺	10	164
	1	119
Mg ²⁺	10	193
	1	118
Ca ²⁺	10	198
	1	123
Ba ²⁺	10	169
	1	151

extracts of heptane-grown *P. aeruginosa* ATCC 17423 contained heptanol and heptanal dehydrogenase activities of 49 and 310 mu/mg protein, respectively. As the method of mutant selection might be expected to yield mutants defective in any of the steps in the pathway, the existence of more than one route of heptanol catabolism was considered.

Three alcohol dehydrogenases of different specificities were found in *P. aeruginosa* by van der Linden & Huybregste (1969). To test whether *P. aeruginosa* ATCC 17423 had more than one alcohol dehydrogenase with activity for heptanol, the dehydrogenase activity for various alcohols was measured in extracts from organisms grown on different substrates (Fig. 1). Although the extract from organisms grown in nutrient broth had similar specific activities for ethanol and pentanol, that from heptane-grown organisms was more than three times as active with pentanol as with ethanol. As a single enzyme would always have the same ratio of specific activities for a given pair of alcohols, this indicates that there is in *P. aeruginosa* ATCC 17423 more than one alcohol dehydrogenase. As no single mutation could cause the loss of all alcohol dehydrogenase activity, this is why no mutant with this deficiency was found. The heptanol dehydrogenase may itself be specified by two genes, one chromosomal and one plasmid-borne, as is octanol dehydrogenase in *P. oleovorans* (Chakrabarty, Chou & Gunsalus, 1973), but this was not investigated. A recent report of more than one aldehyde dehydrogenase in *P. aeruginosa* (Bertrand, Gallo & Azoulay, 1973) suggests a similar explanation for the failure to find mutants unable to grow on heptanal.

All mutants were thus deficient in the conversion of heptane to heptanol. Suitable assay conditions for this heptane-oxidizing system were established, using extracts of the wild type. The total amount of products formed was proportional to time over the first 15 min and proportional to extract concentration up to 9 mg protein/assay (3.5 μ mol products/15 min). Aeration was essential for heptane oxidation, and the addition of NAD⁺, NADPH or, especially, NADH, increased the rate. Activity was independent of the amount of heptane added between 68 and 1360 μ mol. Of buffers tested, tris-HCl and triethanolamine-HCl gave the greatest activity. Maximum activity of from 20 to 30 mu/mg protein was observed at pH 7.6; activity was at least 90 % of the maximum within the range pH 7.0 to 7.8. Of the metal chlorides tested, only Sr²⁺, Mg²⁺, Ca²⁺ or Ba²⁺ stimulated activity (Table 1). Extracts dialysed at 3 °C for 4 h against 500 vol. of 0.1 M-tris-HCl, pH 7.6, lost all heptane-oxidizing activity but it was restored by adding 10 mM-MgCl₂ to the assay mixture; this addition was therefore routinely made to ensure that full activity was expressed in the assays. Stimulation

Fig. 2. Release of heptane-oxidizing activity during ultrasonic disruption. Samples were removed from a suspension of *P. aeruginosa* ATCC 17423 after different times of ultrasonic treatment, and centrifuged. The supernatant fluids were assayed for heptane-oxidizing activity (□), alcohol dehydrogenase activity (○) and protein (●). For methods, see text.

by, but not absolute dependence on, Mg^{2+} was reported by van Eyk & Bartels (1970); they did not investigate the effects of other group IIA cations.

Heptane-oxidizing activity was unaffected by 25 % carbon monoxide in the gas phase of the assay system, thereby substantially discounting the involvement of cytochrome P-450 in the system, in common with other pseudomonad alkane-oxidizing systems (Peterson, Basu & Coon, 1966). The heptane-oxidizing activity of extracts diminished rapidly when they were stored; typically about 85 % was lost in 20 h at 3 °C. Of the thiol reagents tested, only thioglycollate, at 2 mM, preserved activity during storage; dithiothreitol, glutathione, L-cysteine and 2-mercaptoethanol were ineffective. Van Eyk & Bartels (1970) were unable to protect the alkane hydroxylase activity in extracts of *P. aeruginosa* ATCC 473 using thiol reagents, but thioglycollate was not tested by them.

During a study of the optimal conditions for preparation of the cell-free extract, two procedures yielded results indicating that the heptane oxidation system, or part of it, could be particulate or membrane-bound. During ultrasonic disruption, the release of the heptane-oxidizing system into the supernatant fluid was subject to a lag (Fig. 2), although total protein and ethanol dehydrogenase activity were released without a lag. This suggested that a component requiring more extensive disruption was necessary for heptane-oxidizing activity. That the system comprised more than one component was shown when a partial separation of activity was achieved after centrifuging at 200000 *g* for 120 min, when the supernatant fluid lost about 65 % of activity but only about 35 % of the protein (Table 2). The pellet, though itself inactive, restored most of the lost activity to the supernatant fraction when recombined with it. As full activity was not restored it may be that the prolonged centrifugation partially inactivated a component of the system. The residual activity in the supernatant fraction suggested that the sedimented component was either not essential or only incompletely removed.

Table 2. *Effect of centrifuging on the heptane-oxidizing system*

A crude extract prepared by ultrasonic disruption of *P. aeruginosa* ATCC 17423 was centrifuged at 17000 *g* for 25 min. The supernatant fluid was treated and the heptane-oxidizing system assayed in the fractions as summarized in the Table.

Centrifugation procedure summary	Heptane-oxidizing activity (mu/ml)	Heptane-oxidizing system specific activity (mu/mg)	Activity after remixing (mu/ml)
Crude (17000 <i>g</i> supernatant)	196	27.2	
↓			
105000 <i>g</i> 20 min			
→ Pellet	0	0	190
→ Supernatant	192	30.0	
↓			
200000 <i>g</i> 2 h			
→ Pellet	0	0	140
→ Supernatant	68	16.2	

Table 3. *Heptane oxidation by mixed extracts*

Extracts were prepared and assayed for activity as described in Methods, but using mixtures of equal amounts of two extracts. When the activity of the mixture exceeded the sum of the activities of the two extracts assayed separately by 80 % or more, the result was scored + ; otherwise 0. A, B, C and D are mutant groups, and the numbers are mutant numbers.

	A				B	C			D			Wild type
	15	26	66	80	91	61	115	129	8	11	23	
A {	15	0	0	0	+	0	0	0	0	0	+	0
	26		0	0	+	0	0	0	+	+	+	+
	66			0	+	0	0	0	+	+	+	+
	80				+	0	0	0	+	+	+	+
B {	91					0	0	0	+	+	+	0
C {	61						0	0	0	0	+	0
	115							0	0	0	+	0
	129								0	0	0	0
D {	8									+	+	0
	11										+	0
	23											0

The assay system, standardized as for wild-type extracts, was used to investigate extracts of the mutants. Extracts were prepared from bacteria grown on malonate with diethoxymethane. Of the sixteen mutants, two had high reversion rates, two, which may be permeability mutants, grew slowly on heptane but had heptane-oxidizing activity approaching that of the wild type, and one did not grow on malonate; these were not further investigated. Three mutants, 8, 11 and 23, grew very slowly on heptane and had variable, but little, heptane-oxidizing activity, never exceeding 25 % that of the wild type. No other mutant had detectable heptane-oxidizing activity.

Samples of extracts from each mutant were assayed together with extract from each other mutant in turn (Table 3) or with extract from the wild type. The extracts complemented each other in some mixtures: the activity of the mixture exceeded the sum of the activities of the two extracts separately. Although the activity in many of the mixings varied from one batch of organisms to another, the presence or absence of complementation was consistent.

From the complementation pattern, the mutants may be resolved into four groups. Group A comprised mutants 26, 66 and 80 which were the only ones which significantly stimulated the wild-type extract, and, when mixed with extract from mutant 91, generated heptane-oxidizing activity about equal to that of wild-type extract. Mutant 15 also complemented 91, although the activity produced was very low, and was also included in group A. Group B mutant 91 was unique in possessing no heptane-oxidizing activity and complementing extracts of group A.

Mutants 61, 115 and 129 were classified together as group C: they were unable to stimulate wild-type extract activity, and did not complement extracts from either group A or group B. Only two positive interactions resulted from all mixing experiments involving group C: 61 plus 23, and 115 plus 23. Some of the mutants in group C may lack activity because the inducer failed to enter the organisms, or, having entered, failed to induce the enzymes.

Mutants 8, 11 and 23, which possessed residual activity when assayed individually, yielded complex complementation results. In general, they interacted with both groups A and B, but not with C. They were therefore classed as group D. For all mutant extracts the ability to complement other extracts in mixing assays was destroyed by prior heat treatment at 80 °C for 20 min.

The factors F_A and F_B , addition of which produced activity in group A and group B extracts respectively, were partially purified. Assay of the factors was made possible by finding that the activity of mixtures of A and B extracts was proportional to the concentration of either extract when tested with an excess of the other. F_A was fractionated from type B extracts by ammonium sulphate treatment. Although a third of F_A activity was lost, about 90 % of the remainder precipitated between 25 and 35 % saturation. Investigations of wild-type extracts had suggested that a component of the heptane-oxidizing system might be located in the membrane fraction but attempts to separate the system's components by lysis of wild-type organisms, using EDTA and lysozyme, resulted in almost total loss of heptane-oxidizing activity. When the type-B mutant was lysed by the same method and the particulate fraction collected, it contained considerable F_A activity. In contrast, studies on F_B were restricted by its lability. Nearly all activity was lost during overnight storage and also during ammonium sulphate fractionation. When type-A mutant extract was mixed with DEAE cellulose in the presence of 20 mM-tris-chloride buffer pH 7.4, all F_B was removed from solution. About 40 % of the F_B was then eluted from the DEAE cellulose with 300 mM-buffer. The recovery of additional F_B by elution at higher buffer concentrations was difficult to assess because such concentrations were found to be inhibitory in the assay system. Conventional column chromatography took so long that most of the F_B activity was lost.

The heptane-oxidizing system of *P. aeruginosa* ATCC 17423 thus possessed at least two components, membrane-bound F_A and labile F_B . The pseudomonad alkane-oxidizing systems studied by Peterson, Kusunose, Kusunose & Coon (1967) and by van Eyk & Bartels (1970) have each been shown to comprise three components: a membrane-bound hydroxylase, a very labile NADH-rubredoxin reductase and rubredoxin. In view of the similarities between the known properties of the *P. aeruginosa* ATCC 17423 heptane-oxidizing system and these other systems, it was thought possible that a third, probably rubredoxin-type, component could be involved, and that a lack of it might be the defect in group D mutants. Were this so, then if both group A and group B extracts contained this rubredoxin, the observed stimulation of group D mutants by them would be accounted for. This hypothesis was discounted, however, when it was found that ammonium sulphate treatment of group A extracts greatly diminished their ability to stimulate group D extracts. Residual activity

was in the F_B fraction. In view of the known stability of rubredoxin-type proteins to ammonium sulphate fractionation, the factor in group A extracts which stimulated group D extracts was probably the same factor that complemented group B extract, and not a rubredoxin. Group D mutants were therefore probably partially defective with respect to both F_A and F_B , perhaps because of a defect in the induction transcription system for the relevant operon. Attempts to show the involvement of a third factor by purification of F_A and F_B were unsuccessful: further purification of F_B beyond DEAE adsorption treatment was restricted by its lability, and when F_B partially purified to this stage was combined with ammonium sulphate-fractionated F_A , heptane-oxidizing activity was still present.

Although no direct evidence for a third component was found, its involvement could not be excluded. The results of the complementation experiments cannot readily be explained in terms of a two-component system. The failure to isolate mutants lacking rubredoxin may have been due to the small total number of mutants selected. Alternatively, rubredoxin may fulfil another, unknown, metabolic function so vital that a rubredoxin-deficient mutant could not live.

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