Microbial Metabolism of Alicyclic Hydrocarbons: Isolation and Properties of a Cyclohexane-degrading Bacterium

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SUMMARY

A micro-organism which grows on cyclohexane as sole carbon and energy source has been isolated from estuarine mud flats. It has been tentatively identified as a *Nocardia*. The organism, which is auxotrophic for biotin, grows on cyclohexane (supplied as a vapour) with a mean generation time of about 10 h and a Y_{SUB} of 59.9 g dry wt (mol cyclohexane)⁻¹. Growth on cyclohexane leads to the production of intracytoplasmic membrane structures which are not present after growth on succinate. Growth, respiration and enzyme studies are consistent with the degradation of cyclohexane via cyclohexanol, cyclohexanone, ϵ -caprolactone and 6-hydroxycaproate. Extracts of organisms grown on cyclohexane contained cyclohexanol dehydrogenase, cyclohexanone monooxygenase and ϵ -caprolactone hydrolase; these enzymes are absent from, or at very low activity in, extracts of organisms grown on succinate.

INTRODUCTION

Little is known about the microbial degradation of the alicyclic hydrocarbons cyclohexane and methylcyclohexane. They appear to be resistant to microbial attack and isolation of pure cultures able to utilize these substances is extremely difficult. Imelik (1948) described the isolation of a strain of *Pseudomonas aeruginosa* that grew on cyclohexane, but gave few experimental details, and growth of this bacterium on cyclohexane has not been further studied. More recently, a mixed bacterial culture from soil has been reported to grow on cyclohexane (Jones & Edington, 1968) whilst Tonge & Higgins (1974) described an unidentified soil isolate and *Nocardia petroleophila* which grew on methylcyclohexane. *Nocardia petroleophila* probably catabolizes the hydrocarbon via 3-methylcyclohexanol and 3-methylcyclohexanone.

Cyclohexane co-oxidation was described by Beam & Perry (1973) and a more detailed study by de Klerk & van der Linden (1974) showed that it can be co-oxidized to cyclohexanol by a pseudomonad growing on heptane. Mixed cultures of this bacterium growing on heptane together with another pseudomonad isolate degraded cyclohexane completely. Methylcyclohexane can be co-oxidized to 4-methylcyclohexanone by a soil isolate growing on 2-methylbutane (Ooyama & Foster, 1965).

In this paper we report the isolation and properties of a pure culture of a bacterium that grows on cyclohexane or methylcyclohexane.

METHODS

Isolation, maintenance and culture of the micro-organism. The organism was isolated from estuarine mud flats near Sittingbourne, Kent, by classical enrichment techniques using methylcyclohexane vapour as sole carbon source. It was maintained on Lab-Lemco (Oxoid) agar slopes (2.3 %, w/v) and grown routinely at 30 °C in a medium containing (g l⁻¹): Na₂HPO₄, 4.2; KH₂PO₄, 2.8; MgSO₄·7H₂O, 0.2; NH₄NO₃, 2.0; plus 1 ml of trace element solution containing (g l⁻¹): FeCl₃, 17; CaCl₂, 0.6; ZnSO₄, 0.2; CuSO₄·7H₂O, 0.2; MnSO₄, 0.2; CoCl₂, 0.8; H₃BO₃, 0.1; Na₂MoO₄·2H₂O, 0.3. All the constituents were mixed and autoclaved at 121 °C for 15 min. In addition, autoclaved culture supernatant solutions (4 %, v/v) from exponential phase cultures of a succinate-grown pseudomonad isolate were added. For growth studies, hydrocarbons were supplied in the vapour phase from centre-well reservoirs (containing 5 to 10 ml liquid substrate) in 250 ml and 2 l conical flasks containing 50 and 500 ml medium respectively. Other growth substrates were dissolved in the minimal medium (1 %, w/v) and flasks were incubated on a gyratory shaker at 300 rev. min⁻¹.

Organisms used for the preparation of washed suspensions and extracts were grown in batch cultures (10 l) in an impeller-agitated fermenter (Biotech, South Croydon; 10 l capacity, air flow rate 0.4 l min⁻¹, mean generation time 9 to 11 h). Cyclohexane was supplied as vapour by means of a gassing bottle, containing cyclohexane, in the air line.

Measurement of growth. Growth was determined by relating the E_{610} of cultures to the dry weight of organisms by a standard curve prepared using cyclohexane-grown washed suspensions. Growth yields were determined in sealed 250 ml Monod flasks containing 50 ml minimal medium. Carbon-limited growth was followed until there was no further increase in turbidity over a period of 12 h, when the flasks were opened and the dry weights were measured by direct weighing of washed cells.

Preparation of washed suspensions of bacteria. Organisms were harvested by centrifuging (30 000 g, 20 min, 25 °C), washed twice in 67 mM-KH₂PO₄/KOH buffer, pH 7.0, and re-suspended in the same buffer.

Preparation of extracts. Washed suspensions were disrupted by one passage through a French pressure cell (Aminco, Maryland, U.S.A.; 69 MPa, 0 °C) before centrifuging (30 000 g, 30 min, 5 °C) to remove unbroken organisms and wall debris.

Measurement of oxygen uptake. Oxygen consumption was measured at 30 °C by conventional Warburg manometry or using an oxygen electrode (Rank Bros, Bottisham, Cambridgeshire). Incubation mixtures contained (in a volume of 3 ml): 165 µmol KH₂PO₄/KOH buffer, pH 7.0; 20 µmol substrate (with the Warburg apparatus) or 2 µmol substrate (with the oxygen electrode); and 4 to 8 mg dry wt bacteria. Hydrocarbons were added as sonicated emulsions in water.

Enzyme assays. Cyclohexanol dehydrogenase was measured as cyclohexanone reduction by following NADPH oxidation at 340 nm. Reaction mixtures contained (in 3 ml): 67 µmol KH₂PO₄/KOH buffer, pH 7.0; 0.5 µmol NADPH; 2 µmol cyclohexanone; and extract (0.5 to 1.0 mg protein). Cyclohexanol oxidation was followed by 2,6-dichlorophenolindophenol reduction measured at 600 nm. Reaction mixtures contained (in 3 ml): 200 µmol glycine/NaOH buffer, pH 10.0; 0.3 µmol 2,6-dichlorophenolindophenol; 2.8 µmol KCN; 0.5 µmol NADP⁺; 2 µmol cyclohexanol; and extract (0.5 to 1.0 mg protein).

Cyclohexanone monooxygenase was measured as described by Norris & Trudgill (1971). Reaction mixtures contained (in 3 ml): 67 µmol KH₂PO₄/KOH buffer, pH 7.0; 0.5 µmol NADPH; 2 µmol cyclohexanone; and extract (0.5 to 1.0 mg protein).

ϵ -Caprolactone hydrolase was measured by the method of Norris & Trudgill (1971). Reaction mixtures contained (in 1 ml): 34 μ mol KH_2PO_4 /KOH buffer, pH 7.0; 10 μ mol ϵ -caprolactone; and extract (0.1 to 0.2 mg protein).

All enzyme assays were done at 30 °C.

Protein estimation. The protein content of extracts was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Gas-liquid chromatography. A coiled glass column (1.83 m $\times$ 4 mm internal diam.) packed with 10% Carbowax on Chromasorb W was used in a Varian Aerograph Series 1400 gas chromatograph fitted with a flame ionization detector. Operating conditions were: carrier gas (N_2) flow rate, 40 ml min⁻¹; oven temperature, 100 °C; detector temperature, 200 °C.

Electron microscopy. Freshly harvested organisms were prefixed in 0.1% (w/v) OsO_4 in Kellenberger buffer (Ryter & Kellenberger, 1958) before fixing overnight in 1% (w/v) OsO_4 in the same buffer. Fixed organisms were washed with Kellenberger buffer and embedded in agar. The agar blocks were stained for 2 h at room temperature with 0.1% (w/v) uranyl acetate in Kellenberger buffer. After dehydration in a graded series of ethanol/water mixtures and treatment with propylene oxide, the agar blocks were embedded in Araldite. Ultra-thin sections were cut with an LKB ultramicrotome and poststained with saturated uranyl acetate/lead citrate (Reynolds, 1963). Sections were examined in a Zeiss E.M.9 electron microscope.

Chemicals. AnalaR cyclohexane (99.8% pure) and methylcyclohexane (98% pure) were from BDH; NADP^+ and NADPH were from Boehringer. All other chemicals were of the highest commercial quality and were obtained from BDH or Koch-Light. The commercial cyclohexanediols used were all *cis-trans* mixtures.

RESULTS

Culture, isolation and growth studies

Several soil and mud samples were examined for methylcyclohexane-utilizing micro-organisms but only two (both from estuarine mud flats) proved positive. In both cases, cyclohexane also served as a growth substrate, but isolation of a pure culture was hindered by the production of large amounts of capsular slime. When finally obtained, pure cultures failed to grow for more than a few generations when transferred to fresh minimal media whatever the carbon source. In contrast, mixed cultures of the organism able to degrade cyclohexane together with one other bacterial isolate from the original mixed cultures grew readily on minimal media.

One strain of the organism able to utilize cyclohexane was chosen for detailed study. It was Gram-variable, non-motile, pleiomorphic and composed of branching rods. On solid media, colonies were bright-yellow, gelatinous, circular and convex. It was tentatively identified as a *Nocardia*. Electron micrographs of the cyclohexane-grown organism, whilst confirming the isolation of pure cultures, also revealed internal membrane structures and spherical electron-dense hydrocarbon inclusions (Fig. 1*a*) similar to those described recently in a hydrocarbon-grown *Acinetobacter* (Kennedy & Finnerty, 1975); these membranes were not present after growth on succinate (Fig. 1*b*). The other organism in the mixed culture was not able to utilize cyclohexane directly and was a Gram-negative rod, tentatively identified as a pseudomonad.

When culture supernatant solution, obtained after growth of the pseudomonad on succinate minimal medium, was autoclaved and added to the *Nocardia* culture medium,

Fig. 1. Sections of the *Nocardia* isolate after growth on (a) cyclohexane or (b) succinate. Bar markers represent 0.1 μm .

vigorous growth of the pure culture occurred on a variety of substrates. The growth factor accumulated during growth of the pseudomonad and reached a maximum concentration just before the onset of the stationary phase. Addition of autoclaved, late-exponential phase pseudomonad culture supernatant solution to the *Nocardia* medium at 0.2 % (v/v) was insufficient to support growth, whilst 4 % (v/v) supported vigorous growth; there was no further stimulation on adding larger proportions. Sterilized supernatant solutions from cultures of other bacteria (e.g. *Escherichia coli*, *Klebsiella aerogenes*, *Bacillus subtilis*) grown on minimal medium were equally effective growth promoters. Growth studies indicated that the isolate was able actively to concentrate the growth factor. The *Nocardia* was eventually found to be auxotrophic for biotin and autoclaved pseudomonad medium could be replaced by 10 ng biotin per ml growth medium.

When tested for growth on cyclohexane derivatives and other potential carbon sources, the *Nocardia* grew readily on cyclohexanol, cyclohexanone, 1,2-, 1,3- and 1,4-cyclohexanediols, ϵ -caprolactone, ethylcyclohexane and isopropylcyclohexane, but not on cyclohexene oxide, 2-hydroxycyclohexanone, hexane, hex-1-ene, octane, oct-1-ene, heptane, decane, dodecane, hexadecane, cyclopentane, cycloheptane, adipate, glucose or glycerol.

On transfer from succinate- to cyclohexane-minimal medium, there was a variable adaptation period of 20 to 40 h before exponential growth commenced (mean generation time, approx. 10 h). Growth substrate yield coefficients (Y_{SUB}) for cyclohexane and succinate were

Table 1. *The effect of growth substrate on the capacity of the Nocardia isolate to oxidize cyclohexane, its analogues and possible catabolic intermediates*

Oxygen consumption rates were measured by conventional Warburg manometry or with an oxygen electrode* as described in Methods. Rates of oxygen uptake were corrected for the endogenous rate (shown in the last line).

Substrate	Growth substrate ...	Rates of oxygen uptake [$\mu\text{l O}_2 \text{ min}^{-1} (\text{mg dry wt organisms})^{-1}$]	
		Cyclohexane	Succinate
Cyclohexane		44	0
Cyclohexene		0*	NT
Cyclohexanol		40	0
Cyclohexanone		80	10
1,2-Cyclohexanediol		71	6
1,3-Cyclohexanediol		51	0
1,4-Cyclohexanediol		91	0
1,2-Cyclohexanedione		8*	NT
Cyclohexene oxide		32	0
Methylcyclohexane		36*	NT
Ethylcyclohexane		29*	NT
Isopropylcyclohexane		11*	NT
ϵ -Caprolactone		83	0
Adipate		31	3
Succinate		47	80
None		0.6	2

NT, Not tested.

59.9 and 51.8 g dry wt (mol substrate) $^{-1}$ respectively. The yield coefficients were constant over a range of substrate concentrations (0.2 to 1.1 mg ml $^{-1}$ for succinate and 0.1 to 0.4 mg ml $^{-1}$ for cyclohexane).

Respiratory studies with washed suspensions

The ability of washed suspensions of cyclohexane-grown *Nocardia* to oxidize cyclohexane and some of its analogues and possible catabolic intermediates was compared with that of succinate-grown organisms (Table 1). Organisms grown on cyclohexane were adapted to metabolize a variety of these compounds but organisms grown on succinate oxidized only cyclohexanone and 1,2-cyclohexanediol.

Enzyme activities in extracts

Several methods for preparing extracts and of assaying activity were tried, but cyclohexane oxidation could not be detected in cyclohexane-grown bacteria. However, these extracts did contain cyclohexanol dehydrogenase, cyclohexanone monooxygenase and ϵ -caprolactone hydrolase activities (Table 2). Cyclohexanol dehydrogenase and cyclohexanone monooxygenase activities were not detectable in succinate-grown organisms and the ϵ -caprolactone hydrolase activity was only 3 % of that in cyclohexane-grown organisms.

Cyclohexanol dehydrogenase was first detected in the *Nocardia* extracts while searching for cyclohexanone monooxygenase activity. Under anaerobic conditions (a control for the oxygenase activity), cyclohexanone-dependent oxidation of NADPH occurred with concomitant disappearance of cyclohexanone and formation of cyclohexanol, which were followed by gas chromatography as described in Methods. NADPH could not be replaced

Table 2. *Activities of enzymes likely to be involved in cyclohexane catabolism in extracts of the Nocardia isolate grown on cyclohexane or succinate*

Extracts were prepared and enzyme activities assayed as described in Methods and in the text.

Enzyme	Growth substrate ...	Enzyme activities [$\mu\text{mol min}^{-1}$ (mg protein) $^{-1}$]	
		Cyclohexane	Succinate
Cyclohexanol dehydrogenase (cyclohexanol oxidation)		0.02	ND
Cyclohexanol dehydrogenase (reduction of cyclohexanone)		0.35	ND
Cyclohexanone monooxygenase		0.07	ND
ϵ -Caprolactone hydrolase		12.0	0.36

ND, Not detected.

by NADH. Activity could only be measured in the direction of cyclohexanol to cyclohexanone in the presence of 2,6-dichlorophenolindophenol and NADP⁺; the reaction rate was only 5.7% of that in the reverse direction (Table 2). The formation of cyclohexanone was confirmed by gas chromatography.

Because of the competition for NADPH between cyclohexanol dehydrogenase and cyclohexanone monooxygenase, the monooxygenase was measured with an oxygen electrode (see Methods). There was no stimulation of endogenous NADH oxidase activity by cyclohexanone, which indicates that this enzyme also is NADPH-specific.

DISCUSSION

Despite difficulties experienced by ourselves and other workers, the results described in this paper show that pure cultures of bacteria able to utilize cyclohexane as sole carbon and energy source can be isolated. As the elective culture techniques used were standard, it is difficult to identify the reason for our success. Initial election for growth on methylcyclohexane may be an important factor but it seems unlikely that cyclohexane utilization is usually associated with auxotrophy.

The internal membrane structures are similar to those in a hydrocarbon-grown *Acinetobacter* (Kennedy & Finnerty, 1975) and *Mycobacterium rhodochrous* (Cattell, 1972). Membrane structures are well authenticated in some autotrophs and in methane-utilizing bacteria. The significance of intracytoplasmic membranes in the *Nocardia* and other hydrocarbon utilizers remains to be determined although we have noted that some hydrocarbon utilizing strains do not appear to contain such membranes (unpublished observations).

The respiration and enzyme studies are consistent with a catabolic route for cyclohexane involving initial oxidation to cyclohexanol followed by degradation of cyclohexanol by the route identified in *Nocardia globerula* and *Acinetobacter* (Norris & Trudgill, 1971; Donoghue & Trudgill, 1975); i.e. oxidation of cyclohexanol by a dehydrogenase to cyclohexanone followed by a monooxygenase-catalysed ring insertion of oxygen to form ϵ -caprolactone and further breakdown of the lactone to acetate and succinate via 6-hydroxycaproate and adipate. The growth yield on cyclohexane is consistent with the involvement of two energetically-wasteful monooxygenase reactions.

The ability of washed organisms to oxidize the cyclohexanediols, 1,2-cyclohexanedione and cyclohexene oxide, does not necessarily indicate that these compounds are catabolic intermediates but may indicate a lack of specificity of the enzymes involved. Inability to observe cyclohexane oxygenase activity was not unexpected since hydrocarbon oxygenases are notoriously difficult to detect and further attempts are in hand using radioactive cyclohexane. The properties of the cyclohexanol dehydrogenase, especially the need to add an artificial electron acceptor to measure cyclohexanol oxidation, may reflect a disruption of an enzymic complex during extraction.

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