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Isolation and Characterization of a Cyclohexane-Metabolizing *Xanthobacter* sp.

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An unusual *Xanthobacter* sp., capable of independent growth on cyclohexane as the sole source of carbon and energy, has been isolated from soil by using classical enrichment techniques. The mean generation time for growth on cyclohexane was 6 h. The microorganism showed a limited ability to utilize hydrocarbons, with only alicyclic hydrocarbons closely related to cyclohexane supporting growth. Ultrastructural studies indicated the presence of electron-transparent vesicles in the cyclohexane-grown *Xanthobacter* sp., but the presence of complex intracytoplasmic membranes could not be identified. A soluble inducible enzyme capable of oxidizing cyclohexane was identified in cell extracts. This enzyme had a pH optimum of 6.5, an absolute specificity for NADPH, and a stoichiometric requirement for molecular O_2 which was consistent with the formation of cyclohexanol. The enzyme showed no activity towards straight chain alkanes and only a limited activity towards unsaturated ring compounds. Enzymatic studies with cell extracts have indicated the main route of metabolism of cyclohexane by this *Xanthobacter* sp. to proceed via cyclohexane → cyclohexanol → cyclohexanone → 1-oxa-2-oxocycloheptane (ε-caprolactone) → 6-hydroxyhexanoate (6-hydroxycaproate) → → adipic acid. Alternative routes involving initial double hydroxylation of the cyclohexane ring may operate fortuitously but are unlikely to represent major pathways for the dissimilation of cyclohexane by this microorganism.

The scarcity of reports concerning the microbial oxidation of cyclohexane by pure strains of the microorganisms gives some indication to the recalcitrant nature of this naturally abundant cycloalkane. Many workers have searched unsuccessfully for microorganisms capable of automonous growth on cyclohexane (2, 3, 10, 11, 18, 22, 23), a finding which has led some to investigate the degradation of this compound through commensalism and cooxidation (2, 3, 10, 15).

The growth of a microorganism upon cyclohexane was first reported by Imelik (14), but only recently have there been two further accounts of growth upon this alicyclic hydrocarbon: a *Nocardia* sp. isolated by Stirling et al. (27) and a *Pseudomonas* sp. isolated in our own laboratory (1). In each case, a route of oxidation for cyclohexane was identified that involved the oxidation of cyclohexane to cyclohexanol and the further metabolism of cyclohexanol to adipic acid by a route previously documented by Donoghue et al. (11) for the oxidation of cycloalkanols. However, neither Stirling et al. (27) nor Anderson et al. (1) investigated the possibility of a degradative pathway involving a double hydroxylation of the cycloalkane ring, even though respiration rates comparable to those of the growth substrate cyclohexane were obtained for the cyclohexandiols with washed cells.

To some extent, these studies were limited by the inability of Stirling et al. (27) to find cyclohexane hydroxylase activity in cell extracts and the lability of the cyclohexane hydroxylase activity found in the cell extracts of the cyclohexane-grown *Pseudomonas* sp. (1).

Routes of metabolism for the degradation of cyclohexandiols such as *trans*-cyclohexan-1,2-diol have been reported by Yugari (29) and later by Davey and Trudgill (9). The route proposed by Yugari (29) and later suggested by Murray et al. (20) is fundamentally different from that found by Davey and Trudgill (9) and those previously reported for the degradation of cycloalkanols by Donoghue et al. (11) in that ring cleavage is achieved by hydrolysis of the carbon-carbon bond of cyclohexan-1,2-dione. For the degradation of cyclohexane, this route provides a feasible alternative to the formation of a lactone in the mechanism of ring cleavage.

In this report, we describe the isolation of an unusual *Xanthobacter* sp., which is capable of growth upon cyclohexane, as the sole source of carbon. Alternative pathways for the degradation of cyclohexane by this bacterium have been thoroughly investigated. Furthermore, we provide preliminary data on the properties of the initial cyclohexane hydroxylase enzyme.

MATERIALS AND METHODS

Isolation, maintenance, and culture of cyclohexane-degrading microorganisms. Bacteria capable of growth upon cyclohexane were first isolated as mixed cultures from the soil of local Nottinghamshire forests by classical enrichment techniques with cyclohexane vapor as the carbon source. A bacterium, a *Xanthobacter* sp. (identification and classification: NCIB [National Collection of Industrial Bacteria], Torry Research Station, Aberdeen AB9 8DG, Scotland), was isolated from one of these mixed cultures and found to grow in pure culture with cyclohexane as the sole source of carbon and energy. The bacterium was maintained on either liquid or solid mineral salts medium with cyclohexane as the sole carbon source. The composition of liquid medium was (in grams per liter) as follows: $Na_2HPO_4 \cdot 12H_2O$, 4.5; KH_2PO_4 (anhydrous), 1.0; $(NH_4)_2SO_4$, 1.8; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2 \cdot 2H_2O$, 0.1. For solid medium, the composition was (in grams per liter) as follows: NaH_2PO_4, 1.56; K_2HPO_4, 1.9; $(NH_4)_2SO_4$, 1.8; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; and plain agar (Difco Laboratories, Detroit, Mich.), 12.5. One milliliter of a trace element solution per liter was added to both the liquid and solid media. The composition was (in grams per liter) as follows: $FeSO_4 \cdot 7H_2O$, 1.0; $CuSO_4 \cdot 5H_2O$, 0.02; H_3BO_3, 0.014; $MnSO_4 \cdot 4H_2O$, 0.10;

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$ZnSO_4 \cdot 7H_2O$, 0.10; $Na_2MoO_4 \cdot 2H_2O$, 0.02; and $CaCl_2 \cdot 6H_2O$, 0.02. Stock cultures of the bacterium were kept on slopes of solid mineral salts medium maintained in cyclohexane vapor at 4°C and were subcultured when necessary. Cyclohexane was sterilized by passage through a polycarbonate 0.2-μm (pore size) bacterial filter (Nuclepore Corp., Pleasanton, Calif.). For growth in liquid medium, slopes were inoculated into 250-ml conical flasks containing 100 ml of sterile growth medium. Cyclohexane (0.1 ml) was added to the medium, and flasks were incubated and maintained as previously described (1). For larger quantities of cells, bacteria were grown in batch culture in 2-, 5-, or 20-liter impeller-agitated fermenters (L. H. Engineering, Stoke Poges, Buckinghamshire, England). Cyclohexane was provided as a vapor through the main air supply (0.5 or 1.0 liters $\cdot$ min^{-1}). For growth on organic acids, sugars, and amino acids, the liquid medium contained 2 g $\cdot$ $liter^{-1}$ of the respective carbon source.

For growth studies upon other hydrocarbons, nonvolatile carbon sources were added directly to mineral salts medium (final concentration, 1.0 g $\cdot$ $liter^{-1}$). Volatile carbon sources were added directly to liquid medium (0.1 ml/100 ml) or mineral salts medium or were provided in the vapor phase by addition to the center wells of Erlenmeyer flasks. For growth on volatile substrates on solid medium, hydrocarbons were absorbed onto sterile filter papers placed inside the lids of petri dishes which were then sealed.

Preparation of washed cell suspensions. The cell cultures were harvested in the late log phase of growth by centrifugation (10,000 × *g* for 10 min at 4°C), washed twice in 20 mM KH_2PO_4-Na_2HPO_4 buffer (pH 7.0), and then suspended in the same buffer (1.5 times the cell volume). Suspended cells were either used directly or stored at −20°C until required.

Preparation of cell extracts. Resuspended cell suspensions were disrupted by ultra-sonication (MSE Soniprep 150) for a total of 2 min (20-s bursts; 6 μm peak-to-peak at 4°C). Cell debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C (10,000 × *g* supernatant). Soluble (10,000 × *g* supernatant) and membrane fractions were prepared by centrifugation of the 10,000 × *g* supernatant at 100,000 × *g* for 60 min at 4°C. Protein concentrations were determined by the biuret assay of Gornal et al. (12) with bovine serum albumin as a standard.

Measurement of O_2 uptake. Oxygen consumption by whole cells or cell extracts was measured polarographically with a Clark type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C. For the oxidation of substrates by whole cells, incubation mixtures contained (in 3 ml) 60 μl of phosphate buffer (pH 7.0) and 20 μmol of substrate. Water-immiscible substrates such as cyclohexane (2 μl) were added directly to the incubation mixture.

Enzyme assays. Cyclohexane hydroxylase was assayed by measuring the NADPH-dependent oxidation of cyclohexane in the O_2 monitor. Reaction mixtures contained (in 3 ml) 150 μmol of phosphate buffer (pH 6.5), 5 mg of protein, 0.5 μmol of NADPH, and 2 μl of cyclohexane. In experiments for the measurement of reaction, stoichiometry cyclohexane was dissolved in dimethylformamide before addition to the reaction mixture.

Cyclohexanol dehydrogenase was assayed spectrophotometrically (Beckman Du-7) as previously described (11); the reaction mixture contained (in 1 ml) 20 μmol of glycine-NaOH buffer (pH 10.3), 0.1 to 0.5 mg of protein, 0.5 μmol of cyclohexanol, and 0.2 μmol of NAD^+. For the measurement of cyclohexan-1,2-diol, cyclohexan-1,3-diol, and cyclohexan-1,4-diol dehydrogenase activity, the assay was the same, except that 5 μmol of these substrates were added to the reaction mixture. 2-Hydroxycyclohexanone dehydrogenase activity was assayed in either the direction of 2-hydroxycyclohexanone → cyclohexan-1,2-dione as detailed for cyclohexanol dehydrogenase activity (but utilizing 5 μmol of 2-hydroxycyclohexanone) or in the reverse direction (cyclohexan-1,2-dione → 2-hydroxycyclohexanone) as described by Davey and Trudgill (9). Experiments involving the measurement of reaction stoichiometry were performed under anaerobic conditions with Thunberg cuvettes and involved the addition of various amounts of cycloalkanol (0.05 to 0.25 μmol) to reaction mixtures containing a fixed amount of NAD^+ (0.5 μmol).

Cyclohexanone monooxygenase activity was measured either spectrophotometrically by following the cyclohexanone-dependent oxidation of NADPH at 340 nm (21) or polarographically in the oxygen monitor. Reaction mixtures contained (in a volume of 1 ml) 200 μmol of glycine-NaOH buffer (pH 8.8), 0.05 to 1.0 mg of protein, 0.3 μmol of cyclohexanone. For the assay of cyclohexanone monooxygenase in the oxygen electrode, reaction mixtures contained (in 3 ml) 600 μmol of glycine-NaOH buffer (pH 8.8), 0.5 to 1.0 mg of protein, and 1.0 μmol of cyclohexanone. For the assay of cyclohexan-1,2-dione, cyclohexan-1,4-dione, and 2-hydroxycyclohexanone monooxygenase activity, the procedures were the same, except 2 μmol of cyclohexan-1,2-dione was added to the reaction mixtures. Experiments involving the measurement of reaction stoichiometry were carried out in the oxygen electrode so that oxygen consumption could be monitored for the various additions (0.15 to 0.3 μmol) of the cycloalkanones with a fixed amount of NADPH (0.5 μmol). For the measurement of reaction stoichiometry between NADPH and cyclohexanone, the amount of NADPH (0.3 μmol) oxidized by the addition of various amounts of cyclohexanone (0.025 to 0.15 μmol) was measured spectrophotometrically.

1-Oxa-2-oxocycloheptane hydrolase activity was measured by the methods of Norris and Trudgill (21). For the assay involving the measurement of residual lactone by alkaline hydroxamate formation, the reaction mixture contained (in 2 ml) 1 mmol of Tris-hydrochloride buffer (pH 8.0), 0.2 mg of protein, and 10 μmol of 1-oxa-2-oxocycloheptane. For the assay with a pH stat, an unbuffered system (initial volume, 5 ml) containing 0.2 to 0.5 mg of protein and 10 μmol of 1-oxa-2-oxocycloheptane was maintained at pH 8.0 by constant titration of 1 mM NaOH against the acidic reaction product.

6-Hydroxyhexanoate dehydrogenase activity was determined as described by Donoghue et al. (11) by measuring the reduction of NAD^+ at 340 nm under anaerobic conditions. Reaction mixtures contained (in 1 ml) 20 μmol of glycine-NaOH buffer (pH 9.9), 0.5 to 1.0 mg of protein, 0.3 μmol of NAD^+, and 2 μmol of 6-hydroxyhexanoate.

Estimation of enzyme pH optima. Enzyme pH optima were determined as described previously (1).

Identification and determination of reaction products. Cyclohexanol, cyclohexan-1,2-diol, cyclohexan-1,3-diol, cyclohexan-1,4-diol, cyclohexanone, cyclohexan-1,2-dione, cyclohexan-1,3-dione, cyclohexan-1,4-dione, 2-hydroxycyclohexanone, 1-oxa-2-oxocycloheptane, 6-hydroxyhexanoate, and adipic acid were detected by gas-liquid chromatography (GLC) with a Perkin-Elmer F-33 gas chromatograph. For the detection of all alicyclic compounds except 6-hydroxyhexanoate and adipic acid, 2-m columns packed with 6% (wt/wt) Silar 5HC on Chromosorb P (injector temperature, 175°C; oven temperature, 110°C), 15%

(wt/wt) Carbowax 20 M on Chromosorb W (injector temperature, 170°C; oven temperature, 120°C), or 10% (wt/wt) Reoplex 2M on Chromosorb W (injector temperature, 175°C; oven temperature, 165°C) were used. Hexan-1-ol was utilized as the internal standard. Samples to be analyzed were acidified to pH 2.0 with 2M HCl, and precipitated protein was removed by centrifugation. After the addition of an internal standard, the reaction mixture was extracted three times with diethyl ether, dried over anhydrous Na_2SO_4, and then concentrated by the passage of N_2 gas before column injection. 6-Hydroxyhexanoate and adipic acid were detected on a 2-m glass column packed with 8% (wt/wt) FFAP on acid-washed Chromosorb W (injector temperature, 250°C; oven temperature, 225°C). Samples for analysis were again acidified to pH 2.0; the protein precipitate was removed by centrifugation and, when necessary, reduced in volume by freeze-drying before direct injection onto the GLC column.

In all GLC analyses, peaks were integrated with a Spectra-Physics SP4270 integrator, and retention times were compared with authentic standards.

1-Oxa-2-oxocycloheptane was also measured by alkaline hydroxamate formation, acidification conversion to the ferric hydroxamate, and measurement of absorbance at 510 nm as described by Cain (6).

6-Hydroxyhexanoate and adipic acid were also identified by comparison with authentic standards by using thin-layer chromatography (TLC). Systems used were those described by Donoghue et al. (11) and Blundstone (5).

Electron microscopy. Fermentor-grown *Xanthobacter* cells were prepared for electron microscopy as described by Stirling et al. (27). Ultra-thin sections were cut with a Reichert 802 microtome and after post-staining examined in an AEI 802 transmission electron microscope.

Preparation of compounds. 6-Hydroxyhexanoate was prepared by alkaline hydrolysis of 1-oxa-2-oxocycloheptane as described by Norris and Trudgill (21). The preparation of fresh solutions of cyclohexan-1,2-dione (monoenol form) or equilibrium mixtures containing the monohydrate and the monoenol form of cyclohexan-1,2-dione were carried out as described by Davey and Trudgill (9).

Materials. Aristar and Analar cyclohexane were obtained from BDH (British Drug House), Poole, Dorset, England. Cyclohexanol, cyclohexanone, *cis,trans*-cyclohexan-1,2-diol, *cis,trans*-cyclohexan-1,3-diol, *cis,trans*-cyclohexan-1,4-diol, cyclohexan-1,2-dione, cyclohexan-1,3-dione, and cyclohexan-1,4-dione were purchased from Aldrich Chemical Co., Gillingham, Dorset, England. 2-Hydroxycyclohexanone was purchased from Pfaltz and Bauer, Inc., Stamford, Conn. 1-Oxa-oxocycloheptane was obtained from Koch-Light, Coinbrook, England. NADPH, NADH, $NADP^+$, and NAD^+ were supplied by Sigma Chemical Co. Ltd., Poole, Dorset, England.

RESULTS

Isolation and characterization of the cyclohexane-utilizing bacterium. Soil samples from various wooded areas around Nottingham have previously been examined for cyclohexane-utilizing organisms, and several mixed cultures capable of sustained growth upon cyclohexane have been isolated.

Further investigation of these mixed cultures revealed a gram-negative, nonmotile bacterium which was capable of independent growth on solid or liquid medium with cyclohexane as the sole carbon and energy source. This organism, when growing on nutrient agar medium or on solid medium with cyclohexane as the carbon source, assumes smooth, yellow, rounded convex colonies. An examination of these colonies under the light microscope after growth on nutrient agar indicated a cell morphology that is peculiar to the newly formed genus *Xanthobacter* (19, 28) in that cells showed irregularly shaped rods, some branched with typical swollen ends.

The growth of this organism required no complex growth factor as determined by the methods of Holding and Collee (13). In common with *Xanthobacter* sp., this bacterium showed a limited ability to utilize both sugars and amino acids as sole carbon sources with only glutamate, fructose, maltose, and sucrose supporting growth. However, the bacterium was able to grow on the alcohols, methanol, ethanol, and propanol and a wide range of organic acids which included pyruvate, succinate, propionate, acetate, citrate, malonate, fumarate, lactate, and gluconate. Identification of this bacterium as a member of the genus *Xanthobacter* was confirmed by the NCIB.

Growth studies with this *Xanthobacter* sp. on a variety of hydrocarbons indicated that cyclopentane, cycloheptane, methylcyclohexane, ethylcyclohexane, cyclohexanone, and cyclohexan-1,2-diol also supported growth. Poor growth was recorded upon cyclohexene oxide, cyclohexanol, cyclohexan-1,3,diol, cyclohexan-1,4-diol, cyclohexan-1,2-dione, and cyclohexan-1,4-dione. No growth was found to occur when cyclooctane, cyclodecane, cyclohexene, 1-oxa-2-oxocycloheptane, 6-hydroxyhexanoate, adipic acid, 2-hydroxycyclohexanone, cyclohexan-1,3-dione, the aromatic hydrocarbons benzene and toluene, and *n*-alkanes (C_6 to C_{16}) were used as substrates.

With cyclohexane as the growth substrate, the adaption period (as measured by an increase in viable colony count after a transfer from succinate media) was 12 to 16 h. The mean generation time was determined as 6 h for cyclohexane and 7 h for succinate as the growth substrate.

Metabolite accumulation. An initial screen for potential pathway intermediates involved in cyclohexane metabolism was carried out by GLC analysis of extracts of growth media from cyclohexane-grown cells harvested in the late log phase of growth. Media analyzed in this way revealed the presence of both cyclohexanol and cyclohexanone in the concentration range from 0.05 to 0.1 μM. No cyclohexan-1-2-diol, cyclohexan-1,3-diol, cyclohexan-1,4-diol, or 2-hydroxycyclohexanone was detected.

Metabolite oxidation. In contrast to the limited range of cycloalkane derivatives supporting growth of the *Xanthobacter* sp., washed cells of the microorganism previously grown upon cyclohexane were capable of oxidizing a wide range of these compounds (Table 1). Cyclohexanol, cyclohexanone, 1-oxa-2-oxocycloheptane, and the various cyclohexandiols were all oxidized at rates comparable to or greater than the growth substrate cyclohexane. No activity was detected for the *n*-alkanes C_7, C_{10}, and C_{12}. Washed cells previously grown upon succinate as the carbon source demonstrated a limited ability to oxidize cyclohexanone, 1-oxa-2-oxocycloheptane, and cyclohexan-1,2-diol; these oxidation rates were relatively small when compared with those obtained for the cyclohexane-grown cells.

Enzyme activities in cell extracts of the cyclohexane-grown *Xanthobacter* sp. The incubation of crude cell extracts (10,000 × *g* supernatant) with NADPH and cyclohexane demonstrated a cyclohexane-dependent stimulation in O_2 consumption with a pH optimum of 6.5 (Table 2). The addition of glycerol (10% [vol/vol]), dithiothreitol (1.0 mM), glutathione (1.0 mM), or Fe^{3+} (0.1 mM) to the breakage medium did not improve recoverable activity. No activity was observed when either NADH or ascorbate was replaced as the elec-

TABLE 1. Oxidation of various substrates by whole-cell suspensions of *Xanthobacter* sp. grown on cyclohexane and succinate and measured polarographically with a Clark type oxygen electrode

Substrate	Rate of oxygen uptake[a]	
	Cyclohexane	Succinate
Cyclohexane	5.6	<0.05
Cyclohexene	2.4	<0.05
Cyclohexene oxide	2.3	<0.05
Cyclohexanol	9.5	<0.05
Cyclohexanone	8.5	0.8
1-Oxa-2-oxocycloheptane	5.5	0.8
Cyclohexan-1,2-diol	8.6	0.3
Cyclohexan-1,3-diol	5.0	0.1
Cyclohexan-1,4-diol	6.5	0.1
2-hydroxycyclohexanone	2.1	<0.05
Cyclohexan-1,2-dione	4.9	<0.05
Cyclohexan-1,3-dione	0.6	<0.05
Cyclohexan-1,4-dione	1.8	<0.05
Cyclopentane	1.1	<0.05
Cycloheptane	1.4	<0.05
Cyclooctane	<0.05	<0.05
Methylcyclohexane	3.1	<0.05
Ethylcyclohexane	1.0	<0.05
Benzene	0.7	<0.05
Toluene	0.7	<0.05
Heptane	<0.05	<0.05
Decane	<0.05	<0.05
Dodecane	<0.05	<0.05
Adipate	<0.05	<0.05
Succinate	0.9	5.2
Endogenous	0.4	0.7

[a] Values shown (micromoles of O_2 per hour per milligram [dry weight] of organisms) was calculated by subtracting the endogenous rate from the rate recorded with the substrate present. Incubation mixtures contained (in 3 ml) 60 μmol of phosphate buffer (pH 7.0), 20 μl of cell suspension (5 mg [dry weight]), and 2.0 μmol of substrate. Water-immiscible substrates (2 μl) were added directly to the incubation chamber.

tron donor. Further centrifugation of the crude cell extracts at 100,000 × *g* for 60 min and an assay of the resuspended sediment- and particle-free supernatant indicated the enzyme activity to be present in the particle-free supernatant. After storage for 1 month at −20°C, the enzyme kept more than 90% of its activity, but when stored at −4°C, the enzyme lost more than 50% of its activity in a 72-h period. To stabilize the enzyme, a variety of compounds were added to cell extracts, but of those tested, only NADPH (0.1 mM) or cyclohexane (10 μl of cell extract per ml) proved successful, with full enzyme activity remaining in the presence of these substrates when stored over a 72-h period at 4°C.

GLC analysis of extracts obtained from the incubation of 100,000 × *g* supernatant (10 to 15 mg of protein) with cyclohexane (5 μl) and NADPH (5 μmol) have so far proved unsuccessful in demonstrating the formation of either cyclohexanol or mixtures of cyclohexandiols that might be likely products of cyclohexane hydroxylase. Suspicions that these product(s) may be further oxidized to other metabolites in the cyclohexane degradation pathway were confirmed when incubation extracts were analyzed by both TLC and GLC. By our using these techniques, the product of the reaction was found to cochromatograph with 6-hydroxyhexanoate. No products corresponding to cyclohexandiones, 2-hydroxycyclohexanone, 1-oxa-2-oxo-cycloheptane, adipate, or compounds which might be representative of other acidic products could be identified. The incubation of either cyclohexanol (5 μmol) or cyclohexanone (5 μmol) with 100,000 × *g* supernatant under the same conditions (with NADPH as the oxogenous cofactor) also resulted in the formation of 6-hydroxyhexanoate.

Further confirmation of a major route of oxidation of cyclohexane via cyclohexanol → cyclohexanone → 6-hydroxyhexanoate rather than via cyclohexandiol formation was obtained by stoichiometry experiments (Table 3), which showed that 2 mol of oxygen were consumed for every mole of cyclohexane added and that 1 mol of oxygen was consumed per mole of cyclohexanol.

Our initial attempts to purify cyclohexane hydroxylase either by gel filtration (Sephacryl S200) or ion-exchange (DEAE-cellulose) chromatography have so far resulted in the loss of enzyme activity, perhaps indicating the multimeric nature of this enzyme. Further characterization of the enzyme with respect to substrate specificity was therefore carried out with 100,000 × *g* supernatant. Reactions were carried out at pH 6.5, the pH optimum of the enzyme. Under these conditions, the initial hydroxylation is the rate-limiting step for any further enzyme-coupled reactions and is therefore representative of cyclohexane hydroxylase activity. This was tested by demonstrating a stimulation in reaction rate by the addition of either cyclohexanol or cyclohexanone to the reaction mixture. When assayed under these conditions, cyclohexane hydroxylase demonstrated activity towards related cycloalkanes, alkyl-substituted cycloalkanes, and, to a lesser extent, the aromatic hydrocarbons benzene and toluene. No activity could be obtained towards a series of straight chain *n*-alkanes of chain length C_7 to C_{16} (Table 4).

Cyclohexanol dehydrogenase. Cell extracts (100,000 × *g* supernatant) catalyzed the reduction of NAD^+ in the presence of cyclohexanol, *cis,trans*-cyclohexan-1,2-diol, *cis,trans*-cyclohexan-1,3-diol, or *cis,trans*-cyclohexan-1,4-diol (Table 2). No activity was observed with either 2-hydroxycyclohexanone or cyclohexan-1,2-dione (equilibrium mixture) when the reaction was monitored in the reverse direction with NADH as a cofactor. Cyclohexanol dehydrogenase activity displayed a pH optimum of 10.1 to 10.5. When assayed at pH 10.3 under anaerobic conditions, the increase in absorbance at 340 nm was found to be linear. The substitution of $NADP^+$ for NAD^+ gave rise to a small but detectable activity (Table 2). The ability of cyclohexanol dehydrogenase to utilize $NADP^+$ as a cofactor therefore confirms our previous finding that the product from the incubation of 100,000 × *g* supernatant with cyclohexane and NADPH could be further metabolized.

The measurement of reaction stoichiometry for the oxidation of various cycloalkanols indicated the reduction of ca. 1 mol of NAD^+ per mol of substrate when cyclohexanol or the *cis-trans* mixture of cyclohexan-1,2-diol was used as a substrate. For the *cis-trans* mixtures of cyclohexan-1,3-diol and cyclohexan-1,4-diol, a reaction stoichiometry of ca. 2 mol of NAD^+ reduced per mole of substrate oxidized was calculated, indicating the likely products of these reactions to be cyclohexan-1,3-dione and cyclohexan-1,4-dione, respectively.

The ability of the cyclohexanol dehydrogenase to oxidize not only cyclohexanol but also the cyclohexandiols led us to determine the relative affinities of the enzyme for these substrates by calculation of their respective apparent K_m values (Table 5). Comparison of the data indicated an apparent K_m for cyclohexanol 2 orders of magnitude lower than for each of the cyclohexandiols. These results therefore suggest that the preferred substrate for the dehydrogenase is cyclohexanol.

TABLE 2. pH optima, cofactor specificity, and activities of enzymes involved in cyclohexane degradation by cell extracts of *Xanthobacter* sp. after growth upon cyclohexane and succinate

Enzyme[a]	pH optimum	Cofactor specificity	Substrate	Activity (μmol · min^{-1} [mg of protein]$^{-1}$)	
				Cyclohexane	Succinate
Cyclohexane hydroxylase	6.2-6.7	NADPH	Cyclohexane	7.5×10^{-3}	$<0.9 \times 10^{-3}$
Cyclohexanol dehydrogenase	10.1-10.5	NAD^+	Cyclohexanol	0.87	$<0.1 \times 10^{-3}$
			Cyclohexan-1,2-diol	0.73	NT[b]
			Cyclohexan-1,3-diol	0.92	NT
			Cyclohexan-1,4-diol	2.10	NT
			2-Hydroxycyclohexanone	$<0.1 \times 10^{-3}$	NT
		$NADP^+$	Cyclohexanol	0.05	NT
Cyclohexanone monooxygenase	8.5-9.0	NADPH	Cyclohexanone	0.15	0.01
			Cyclohexan-1,2-dione	0.10	NT
			Cyclohexan-1,3-dione	$<0.9 \times 10^{-3}$	NT
			Cyclohexan-1,4-dione	0.13	NT
			2-Hydroxycyclohexanone	0.11	NT
1-Oxa-2-oxocycloheptane hydrolase (ε-caprolactone hydrolase)	7.8-8.1		1-Oxa-2-oxocycloheptane	21.30	<0.5
6-Hydroxyhexanoate dehydrogenase	9.5-10.0	NAD^+	6-Hydroxyhexanoate	0.025	$<0.1 \times 10^{-3}$

[a] Enzyme activity was measured as described in the text.
[b] NT, Not tested.

Incubation of the 100,000 × *g* supernatant (1 mg of protein) with NAD^+ (5 μmol) and cyclohexanol (5 μmol) under aerobic conditions, followed by extraction and identification of the reaction product by GLC, indicated the product of this reaction to be cyclohexanone.

Ring cyclohexan-1,2-dione hydratase. When 10,000 × *g* supernatant from cyclohexane-grown cells was assayed for its ability to catalyze a decrease in the absorbance of cyclohexan-1,2-dione (monoenol) at 262 nm (9), no change in absorbance above that of endogenous rates occurred which would be indicative of cyclohexan-1,2-hydratase. Furthermore, incubation of 10,000 × *g* supernatant with either cyclohexan-1,2-dione or 2-hydroxycyclohexanone in a pH stat at pH 7.0 or 8.0 failed to demonstrate the formation of any acidic products. These results, therefore, provide no evidence for the presence of a cyclohexan-1,2-dione hydratase in crude cell extracts of the *Xanthobacter* sp.

Cyclohexanone monooxygenase. When the 100,000 × *g* supernatant was incubated with NADPH, a cyclohexanone-stimulated consumption of O_2 occurred. No activity could be detected when NADPH was replaced by NADH. The pH optimum for this reaction was found to be 8.5 to 9.0 (Table 2). When assayed at this pH, the back reaction to cyclohexanol catalyzed by cyclohexanol dehydrogenase was found to be negligible. A further study of the substrate specificity of this enzyme indicated that it was also capable of oxidizing cyclohexan-1,2-dione (equilibrium mixture), cyclohexan-1,4-dione, and 2-hydroxycyclohexanone. No activity was recorded for cyclohexan-1,3-dione. When the reaction stoichiometry for these various substrates was measured in the oxygen electrode, ca. 1 mol of O_2 was consumed for each mole of cyclohexanone, cyclohexan-1,4-dione, or 2-hydroxycyclohexanone oxidized. The oxidation of ca. 1 mol of cyclohexanone was also accompanied by the consumption of 1 mol of NADPH, which indicated the enzyme to be

TABLE 3. Reaction stoichiometry for the consumption of oxygen in the oxidation of cyclohexane and cyclohexanol by cell extracts of *Xanthobacter* sp.[a]

Substrate	Cyclohexane added (μmol)	Oxygen consumed (μmol)	Ratio (cyclohexane added/oxygen consumed)
Cyclohexane	0.025	0.051	2.05
	0.050	0.103	2.06
	0.100	0.200	2.00
	0.200	0.390	1.95
Cyclohexanol	0.05	0.052	1.04
	0.10	0.100	1.00
	0.20	0.210	1.05
	0.40	0.380	0.95

[a] Reaction mixtures contained (in 3 ml) 150 μmol of phosphate buffer (pH 6.5), 100,000 × *g* supernatant (5 mg of protein), and 0.5 μmol of NADPH. Reactions (at 30°C) were started by the addition of either cyclohexane or cyclohexanol and followed until the substrate had been completely oxidized. So that accurate additions of cyclohexane could be obtained, cyclohexane was first dissolved in dimethylformamide to give a final concentration of 0.025 M.

TABLE 4. Substrate specificity of cyclohexane hydroxylase[a]

Substrate	Sp act (nmol · min^{-1} [mg of protein]$^{-1}$)
Cyclopentane	11.0
Cyclohexane	7.6
Cycloheptane	4.6
Cyclooctane	2.0
Methylcyclohexane	5.9
Ethylcyclohexane	2.5
Cyclohexene	0.9
Benzene	1.5
Toluene	1.6
Heptane	<0.9
Octane	<0.9
Undecane	<0.9
Dodecane	<0.9
Hexadecane	<0.9
NADPH oxidase	2.3

[a] Reaction mixtures contained (in 3 ml) 150 μmol of phosphate buffer (pH 6.5), 100,000 × *g* supernatant (5 mg of protein), and 0.5 μmol of NADPH. Reactions (at 30°C) were started by the addition of the substrate (2 μl), and the initial velocity was measured by following polarographically O_2 consumption in the O_2 monitor.

TABLE 5. Kinetic evaluation of substrates for cyclohexanol dehydrogenase and cyclohexanone monooxygenase from cell extracts of *Xanthobacter* sp. grown on cyclohexane[a]

Enzyme	Substrate	Apparent K_m (μM)[b]
Cyclohexanol dehydrogenase	Cyclohexanol	1.3
	Cyclohexan-1,2-diol	410.0
	Cyclohexan-1,3-diol	500.0
	Cyclohexan-1,4-diol	590.0
Cyclohexanone monooxygenase	Cyclohexanone	<0.5
	Cyclohexan-1,2-dione	143.0
	Cyclohexan-1,4-dione	19.0
	2-Hydroxycyclohexanone	9.3

[a] For cyclohexanol dehydrogenase activity, reaction mixtures contained (in 1 ml) 20 μmol of glycine-NaOH buffer (pH 10.3), 100,000 × *g* supernatant (0.05 to 1.0 mg of protein), and 0.2 μmol of NAD^+. Reactions (at 30°C) were started by the addition of the cycloalkanol, and the initial reaction velocity was determined by following the increase in absorbance at 340 nm. For cyclohexanone monooxygenase activity determination, reaction mixtures contained (in 1 ml) 200 μmol glycine-NaOH buffer (pH 8.8), 100,000 × *g* supernatant (0.06 to 1.0 mg of protein), and 0.3 μmol of NADPH. Reactions (at 30°C) were started by the addition of the cycloalkanone, and the initial velocity was measured by following the decrease in absorbance at 340 nm.

[b] The Michaelis constant for the various substituted cycloalkanes was calculated as described in the procedure of Lineweaver-Burk.

a typical monooxygenase. Measurement of the apparent K_m values of this enzyme for the cycloalkanones strongly suggested that cyclohexanone was the preferred substrate (Table 5).

When 100,000 × *g* supernatant (1 mg of protein) was incubated with 5 μmol of NADPH and 4 μmol of cyclohexanone, extraction and analysis of the reaction product by TLC and GLC indicated it to be 6-hydroxyhexanoate.

1-Oxa-2-oxocycloheptane hydrolase (ε-caprolactane hydrolase). The presence of an active 1-oxa-2-oxocycloheptane hydrolase in 100,000 × *g* supernatant, which would preclude any possibility of accumulating 1-oxa-2-oxocycloheptane (the reaction product of cyclohexanone monooxygenase) was demonstrated by incubation of 100,000 × *g* supernatant with 1-oxa-2-oxocycloheptane. The measurement of enzyme activity indicated a specific activity 10-fold greater than that measured for cyclohexanone monooxygenase (Table 2). An investigation of the stoichiometry by the titration method revealed equimolar consumption of NaOH for a range of concentrations (25 to 100 μmol) of 1-oxa-2-oxocycloheptane added to the reaction mixture. The expected product of the reaction, 6-hydroxyhexanoate, was identified by TLC and GLC after an extraction of incubation mixture containing 100 μmol of 1-oxa-2-oxocycloheptane and 100,000 × *g* supernatant (1 mg of protein), which was followed to completion by titration with 10 mM NaOH.

6-Hydroxyhexanoate dehydrogenase. The 100,000 × *g* supernatant from cyclohexane-grown cells catalyzed the reduction of NAD^+ in the presence of 6-hydroxyhexanoate. The reaction, which was measured anaerobically, was found to be nonstoichiometric and had a pH optimum of 9.5 to 10.0. NAD^+ could not be substituted by $NADP^+$, nor did further addition of $NADP^+$ to an incubation mixture previously incubated with NAD^+ cause any increase in absorbance at 340 nm. When an incubation mixture containing 10 μmol of 6-hydroxyhexanoate, 10 μmol of NAD^+, and 100,000 × *g* supernatant (5 mg of protein) was incubated under anaerobic conditions, the product of the reaction cochromatographed with adipic acid when analyzed by TLC.

Induction of the enzymes catalyzing cyclohexane oxidation. A comparison of the activities of the enzymes responsible for cyclohexane metabolism in the crude cell extracts of the *Xanthobacter* sp. grown upon either cyclohexane or succinate is given in Table 2. These results clearly demonstrate that the enzymes of cyclohexane degradation are inducible.

Ultrastructural studies on the *Xanthobacter* sp. Ultrastructural studies of the *Xanthobacter* cells were made after their growth on cyclohexane and succinate. Transmission electron micrographs (data not shown) of sections of cyclohexane-grown cells showed no evidence of any complex intracytoplasmic membrane system, although the cells showed large electron-transparent inclusions, usually one or two per cell. These electron-transparent inclusions had well-defined limits and may be typical of those found in other hydrocarbon-growing bacteria in which they have been reported to contain unmodified hydrocarbon growth substrate (25). However, although fewer in number, electron-transparent inclusions were also found in electron micrographs of sections from succinate-grown cells in which they are likely to be representative of storage material such as poly-β-hydroxybutyrate (28).

DISCUSSION

Xanthobacter is a genus of nitrogen-fixing hydrogen bacteria of which only two species have so far been well defined (19, 28). The isolation of a *Xanthobacter* sp. which is capable of growth on cyclohexane is therefore both novel and unusual, considering the relatively few microorganisms which have so far been reported to have the metabolic capacity to grow upon this hydrocarbon.

Our studies with cell extracts of this organism are consistent with a main route for cyclohexane degradation via cyclohexane → cyclohexanol → cyclohexanone → 1-oxa-2-oxocycloheptane → 6-hydroxyhexanoate → → adipic acid (Fig. 1). However, the ability of the *Xanthobacter* sp. to grow on cyclohexan-1,2-diol, cyclohexan-1,3-diol, and cyclohexan-1,4-diol, together with the rapid oxidation observed for these cyclohexandiols by washed cyclohexane-grown cells, leaves open the possibility that, in some instances, oxidation of cyclohexane may occur via minor routes involving double hydroxylation of the ring. This rapid oxidation of cyclohexandiols was also observed in washed cells of a cyclohexane-grown *Nocardia* sp. (27) and in a cyclohexane-grown *Pseudomonas* sp. (9). However, our inability to find a 2-hydroxy-cyclohexanone dehydrogenase, or a cyclohexan-1,2-dione hydratase in the crude cell extracts of cyclohexane-grown cells seems to rule out a pathway of cyclohexane metabolism involving ring cleavage through the hydrolysis of the carbon-carbon bond as suggested by Yugari (29) and later by Murray et al. (20). Our studies with cyclohexane-grown *Xanthobacter* sp. suggests that if cyclohexan-1,2-diol is an intermediate in cyclohexane metabolism, then ring cleavage would proceed via the formation of an unstable lactone (9; Fig. 1).

Substrate specificity studies upon both cyclohexanol dehydrogenase and the cyclohexanone monooxygenase indicate that cyclohexanol and cyclohexanone, respectively, are the preferred substrates for these two enzymes. However, the finding that cyclohexanol dehydrogenase can readily oxidize the cyclohexandiols and that cyclohexanone monooxygenase can oxygenate the products of these reactions, apart from cyclohexan-1,3-dione, stresses the importance of the need to thoroughly investigate the initial oxidation products of the cyclohexane hydroxylase enzyme before any major pathway for cyclohexane metabolism can be assumed. For example, our findings of the absence of

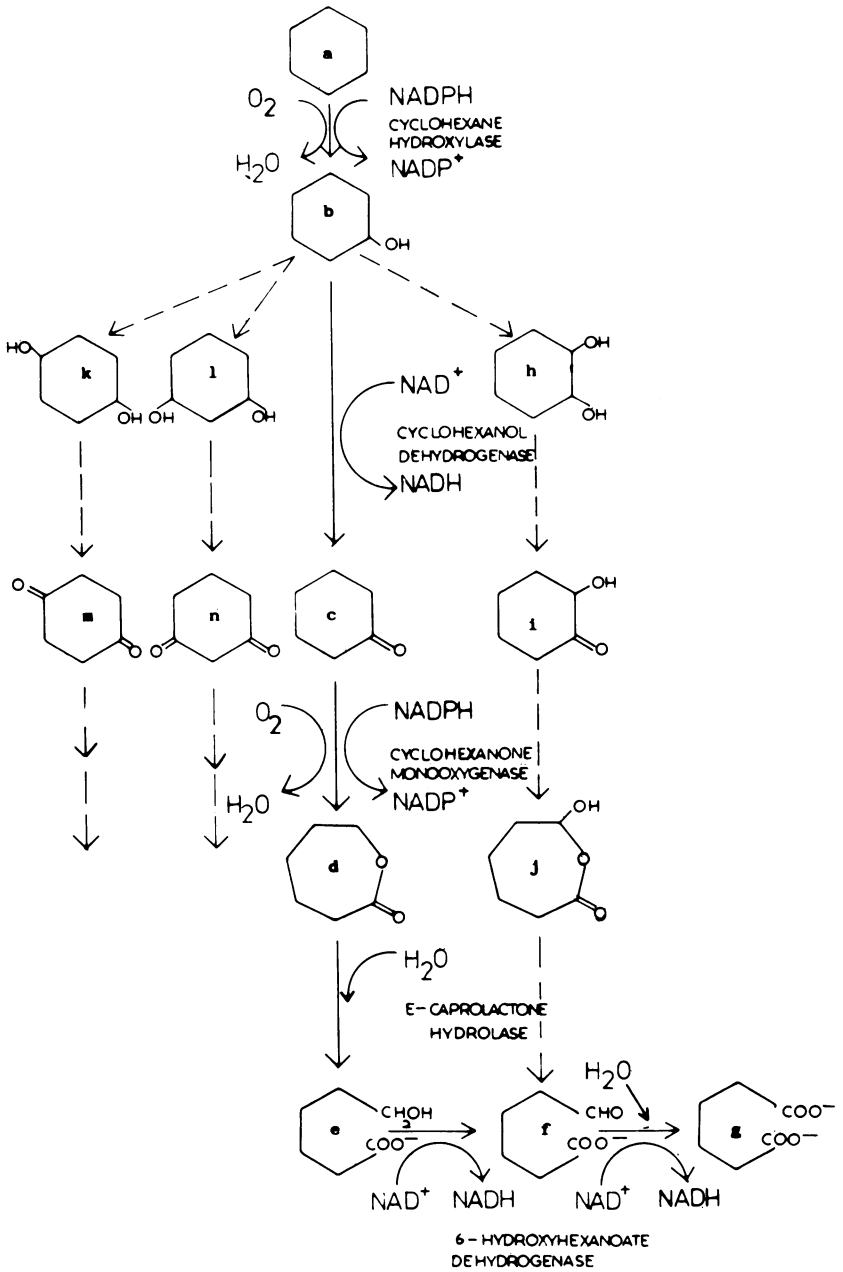

FIG. 1. Proposed route for the oxidation of cyclohexane by *Xanthobacter* sp. Major route (⟶); minor routes (– – →). Compounds: (a) cyclohexane, (b) cyclohexanol, (c) cyclohexanone, (d) 1-oxa-2-oxocycloheptane (ε-caprolactone), (e) 6-oxohexanoate, (f) 6-hydroxyhexanoate, (g) adipate, (h) cyclohexan-1,2-diol, (i) 2-hydroxycyclohexan-1-one, (j) 1-oxa-2-oxo-7-hydroxycycloheptane, (k) cyclohexan-1,4-diol, (l) cyclohexan-1,3-diol, (m) cyclohexan-1,4-dione, and (n) cyclohexan-1,3-dione.

cyclohexandiols in the growth medium of the cyclohexane-grown *Xanthobacter* sp., our inability to detect other products apart from 6-hydroxyhexanoate in the extracts from incubations containing cyclohexane, NADPH, and cell extracts, and the stoichiometric relationships between cyclohexane and oxygen in the formation of 6-hydroxyhexanoate indicated that, for this *Xanthobacter* sp., oxidation of cyclohexane through cyclohexandiol intermediates is unlikely to represent a major route of degradation.

Our findings with this new species of *Xanthobacter* are therefore consistent with previous reports (1, 27) and confirm that the major route of cyclohexane dissimilation proceeds via a pathway involving two monooxygenase reactions, one catalyzing the formation of cyclohexanol and the other carrying out a biological Baeyer-Villiger reaction to form 1-oxa-2-oxocycloheptane.

An interesting aspect of this work is the finding of a soluble, active cyclohexane hydroxylase. This NADPH-dependent enzyme is much more stable than the enzyme isolated from a cyclohexane-grown *Pseudomonas* sp. (1) which lost all of its activity over a 24-h period when stored at 4°C.

Compared with the rate of oxidation of cyclohexane by whole cells, the specific activity of cyclohexane hydroxylase

in 100,000 × *g* supernatants is relatively low; however, this is not unusual for this type of enzyme. The loss of enzymatic activity of cyclohexane hydroxylase after its passage through either an ion-exchange or gel filtration column suggests that it may share similar characteristics with the soluble methane and 5-exo-camphor hydroxylase enzymes (7, 16) in being a dissociable multicomponent system. Investigations to confirm this are presently under way.

Unlike the methane hydroxylases (8), cyclohexane hydroxylase appears to have a restricted substrate range and shows no activity towards straight chain *n*-alkanes and only a limited activity towards unsaturated ring compounds.

Interestingly, the complex intracytoplasmic membranes present in the cyclohexane-growing *Nocardia* sp. (27) and other hydrocarbon-utilizing microorganisms (4, 17, 24) were not found in the cyclohexane-grown *Xanthobacter* sp. Since these intracellular membrane structures may be associated with membrane-bound hydroxylase activity, our results are therefore in keeping with the soluble nature of the cyclohexane hydroxylase found in this *Xanthobacter* sp. Whether cyclohexane hydroxylase activity becomes membrane associated, resulting in the concomitant appearance of intracytoplasmic membranes in the *Xanthobacter* sp. when grown under limiting oxygen conditions as reported for the methane monooxygenase from *Methylosinus trichosporium* (26), poses an interesting question.

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