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Comparative Study of the Ability of Three *Xanthobacter* Species To Metabolize Cycloalkanes

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The ability of three species of *Xanthobacter* to metabolize cyclohexane and its derivatives has been compared. *Xanthobacter flavus* was unable to utilize any of the cycloalkanes under investigation. *X. autotrophicus* was unable to utilize cyclohexane but was able to grow with a limited range of substituted cycloalkanes, including cyclohexanol and cyclohexanone. Comparison of a previously isolated cyclohexane growing *Xanthobacter* sp. with *X. flavus* and *X. autotrophicus* indicated it to be closely related to *X. autotrophicus*. Studies with cell-free extracts have indicated that the route of metabolism for cyclohexanol by *X. autotrophicus* is the same as that shown for the cyclohexane growing *Xanthobacter* sp., proceeding via cyclohexanol→cyclohexanone→ ε-caprolactone→ → adipic acid. A comparison of the cyclohexanol dehydrogenase found in *X. autotrophicus* with that found in the cyclohexane-growing *Xanthobacter* sp. indicated these enzymes to be distinctly different from one another on the basis of substrate specificity, molecular weight, and pH optima. The cyclohexanone monooxygenase enzymes found in the two bacteria were also found to be different when the pH optima and cofactor specificity of the two enzymes were compared. Preliminary genetic studies on the cyclohexane-growing *Xanthobacter* sp. have indicated that there are no plasmids present in this bacterium. The presence of RP4 in the *Xanthobacter* sp. can be detected following its conjugation with an RP4-carrying *Escherichia coli* strain.

Members of the genus *Xanthobacter* are gram-negative, nitrogen-fixing, hydrogen-oxidizing, nonmotile bacteria. To date, only two species have been described in *Bergey's Manual of Systematic Bacteriology* (22); these are *Xanthobacter autotrophicus*, previously known as *Corynebacterium autotrophicum* (23), and *Xanthobacter flavus*, known as *Mycobacterium flavum* prior to reclassification by Malik and Claus (15). However, DNA-DNA hybridization studies of the genus *Xanthobacter* performed by Jenni et al. (12) have indicated that there may be several different species clustered within this genus.

A *Xanthobacter* species capable of growth on cyclohexane as the sole carbon and energy source was isolated and characterized in this laboratory by Trower et al. (20). The isolation of an organism with an efficient metabolic pathway for the degradation of cyclohexane is both novel and unusual since there have been few reports of microorganisms capable of growth upon this hydrocarbon (1, 18). Enzymatic studies with cell extracts of this *Xanthobacter* sp. showed that cyclohexane is degraded by a route that involves the initial hydroxylation of cyclohexane to cyclohexanol. Further oxidation of cyclohexanol occurs via cyclohexanone to 1-oxa-2-oxocycloheptane (ε-caprolactone) to 6-hydroxyhexanoate (6-hydroxycaproate) and finally to adipic acid (20). This pathway is analogous to that described for the two other cyclohexane-growing organisms, a *Nocardia* species (18) and a pseudomonad (1).

In the following study we compared the ability of three different species of *Xanthobacter* to metabolize cyclohexane and its related compounds since such an unusual genetic trait may be a feature common to this newly characterized genus. Furthermore, any differences seen in the ability of these bacteria to metabolize cycloalkanes may be a useful feature in distinguishing the different subgroups which may exist in this genus. In addition, we have carried out preliminary investigations into the genetic organization of cyclohexane degradation to establish whether the genes responsible for this catabolic pathway are carried on a plasmid or are chromosomally encoded.

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MATERIALS AND METHODS

Isolation, maintenance, and culture of microorganisms. A species of *Xanthobacter* capable of growth on cyclohexane was isolated from the soil of local Nottinghamshire forests by classical enrichment techniques, using cyclohexane as a sole carbon source (20). *X. autotrophicus* (strain DSM 431) was obtained from the Deutsche Sammlung von Mikroorganismen (DSM), Gottingen, Federal Republic of Germany. *X. flavus* was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland. The bacteria were grown on either liquid or solid mineral salts medium (MSM) containing the required carbon source as described by Trower et al. (20). For maintenance of bacteria on solid MSM *Xanthobacter* sp. was grown on cyclohexane, *X. autotrophicus* was grown on cyclohexanol, and *X. flavus* was grown on succinate. For growth of *X. flavus* the MSM also contained the following vitamins: thiamine, riboflavin, nicotinic acid, pyridoxin-HCl, and calcium pantothenate, each at a concentration of 1 μg/ml; and vitamin B_{12}, biotin, and folic acid, each at 0.1 μg/ml.

The requirement of complex growth factors by the cyclohexane-growing *Xanthobacter* sp. was tested by the method of Holding and Collee (10). Determination of the possession of lecithinase, urease, and phosphatase activity by the cyclohexane-growing *Xanthobacter* sp. and of the ability of this bacterium to hydrolyze geletin and casein was carried out by the NCIMB.

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 ultrasonication (MSE Soniprep 150) for a total of 2 min (20-s bursts; amplitude, 6 μm 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 Gornall et al. (5), with bovine serum albumin as a standard.

Measurement of oxygen uptake. Oxygen consumption by whole cells was measured polarographically, using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C. Incubation mixtures contained (in 3 ml) 60 μmol of phosphate buffer (pH 7.0), 20 μmol of substrate, and 50 μl of cell suspension. Cyclohexane and other water-immiscible substrates (2 μl) were added directly to the incubation mixtures.

Enzyme assays. Cyclohexane hydroxylase activity was assayed polarographically by measuring the NADPH-dependent oxidation of cyclohexane in the oxygen electrode. Reaction mixtures contained (in 3 ml) 240 μmol of phosphate buffer (pH 6.5 or 7.5), 0.3 μmol of NADPH, 1.0 to 3.0 mg of protein, and 2 μl of cyclohexane.

Cyclohexanol dehydrogenase activity was measured spectrophotometrically (Beckman Du-7) as previously described (2). The reaction mixture contained (in 1 ml) 40 μmol of glycine-NaOH buffer (pH 10.2) for the *Xanthobacter* sp. or 40 μmol of glycine-NaOH buffer (pH 9.2) for *X. autotrophicus*, 0.3 μmol of NAD^+, 0.1 to 0.5 mg of protein, and 1 μmol of cyclohexanol.

Cyclohexanone monooxygenase activitiy was assayed polarographically in the oxygen electrode by measuring the cyclohexanone-dependent oxidation of NADH or NADPH. Reaction mixtures contained (in 3 ml) 480 μmol of glycine-NaOH buffer (pH 8.8) and 0.3 μmol of NADPH for the *Xanthobacter* sp. or 150 μmol of phosphate buffer (pH 7.5) and 0.3 μmol of NADH for *X. autotrophicus*, 0.5 mg of protein, and 2 μmol of cyclohexanone.

1-Oxa-2-oxocycloheptane hydrolase activity was measured by the method of Norris and Trudgill (16). 1-Oxa-2-oxocycloheptane (50 μmol) in distilled water was adjusted to pH 8.0 and then made up to a total volume of 10 ml. The reaction was started by the addition of 0.1 to 0.5 mg of protein, and the reaction mixture was maintained at pH 8.0 by constant titration of 10 mM NaOH against the acidic reaction product.

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

Polyacrylamide gel electrophoresis. Cell extracts (100,000 × *g* supernatant) were fractionated by polyacrylamide rod gel electrophoresis (8.5% [wt/vol] polyacrylamide) (8). For measurement of in situ cyclohexanol dehydrogenase activity a technique similar to that of Grell et al. (6) was used but with a modified staining solution. The staining solution (in 22 ml) contained 17 mM glycine-NaOH buffer (pH 10.2) for *Xanthobacter* sp. cell extracts or 17 mM glycine-NaOH buffer (pH 9.2) for *X. autotrophicus* cell extracts, 0.1 M cyclohexanol, 2 mM NAD^+, 0.4 mM Nitro Blue Tetrazolium, and 0.1 mM phenazine methosulfate. The gels were stained for 3 to 4 h. For detection of protein gels were stained for 1 h in 0.2% (wt/vol) Coomassie brilliant blue G in aqueous 7.5% (vol/vol) glacial acetic acid–18% (vol/vol) methanol.

Molecular weight determination. Molecular weight determinations were performed by gel filtration, using Ultrogel AcA 44 (LKB Instruments Ltd., Selsdon, Croydon, Surrey, England). Cell extracts (100,000 × *g* supernatant) were eluted from the column (65 by 1.6 cm) with 20 mM phosphate buffer (pH 7.0). The column was calibrated with a Pharmacia gel filtration molecular weight calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden) containing RNase A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), and bovine serum albumin (67,000).

Isolation of plasmid DNA. DNA was isolated from cyclohexane-grown *Xanthobacter* sp. by the method of Wheatcroft and Williams (21). Plasmid DNA preparations were purified by using freeze-thaw-generated sucrose gradients as described by Wheatcroft and Williams (21). Other large-scale plasmid isolations used included the methods of Hansen and Olsen (9) and Guerry et al. (7).

Small-scale plasmid DNA isolations were performed on single colonies of *Xanthobacter* sp. from MSM plates with cyclohexane as the growth substrate or 1-ml MSM cultures grown with cyclohexane, using the methods of Kado and Liu (13) and an adaption of the Wheatcroft and Williams method (21).

Agarose gel electrophoresis. DNA fragments were separated by agarose gel electrophoresis, using Tris-borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0), on a horizontal apparatus at 75 V for 3 h or 20 V overnight, using 0.7% (wt/vol) agarose (agarose "10"; BDH Chemicals Ltd.) and 0.5 μg of ethidium bromide per ml. The loading buffer (added at 0.1 volume of sample) consisted of 30% (wt/vol) Ficoll, 0.2 M EDTA, and 50 μg of bromophenol blue per ml. Gels were visualized on a transilluminator (Ultraviolet Products Ltd.). Photographs were taken with type 667 Polaroid film.

Conjugation. Matings were performed with overnight nutrient broth donor cultures and late-log-phase recipient cultures of *Xanthobacter* sp. in cyclohexane MSM. Filter matings were carried out by mixing 25 μl of both donor and recipient cells on a filter (0.45-μm pore size) and placing the filter on nutrient agar. The procedure was the same for donor and recipient controls. Plates were incubated overnight at 25°C, after which cells were washed from the filter with saline and dilutions were plated to selective media. Viable counts were performed on donor cultures.

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, and *cis,trans*-1,4-diol were purchased from Aldrich Chemical Co., Gillingham, Dorset, England. 2-Hydroxycyclohexanone was purchased from Pfaltz and Bauer, Inc., Stamford, Conn. 1-Oxa-2-oxycycloheptane was obtained from Koch-Light, Coinbrook, England. NADPH, NADH, $NADP^+$, and NAD^+ were supplied by Boehringer Mannheim, Lewes, East Sussex, England. Nitro Blue Tetrazolium and phenazine methosulfate were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, England.

RESULTS

Distinguishing microbiological characteristics of the different species of *Xanthobacter*. Comparison of the microbiological characteristics of the cyclohexane-growing *Xanthobacter* sp. with those previously obtained for *X. flavus* and *X. autotrophicus* indicated this new species to be more closely related to *X. autotrophicus* than to *X. flavus* (Table 1). As a strain of *X. autotrophicus* it is, however, relatively atypical since it demonstrates the ability to hydro-

lyze gelatin and casein, possesses a lecithinase and a urease, lacks phosphatase activity, and has the ability to use malonate and maltose as carbon sources.

Growth of the different *Xanthobacter* species on cyclohexane and related compounds. A comparison of the ability of the three different species of *Xanthobacter* to grow on cyclohexane and related compounds indicated that only the *Xanthobacter* sp. was capable of growth with the recalcitrant hydrocarbon cyclohexane. *X. autotrophicus*, when tested for growth on a range of substituted cycloalkanes, was able to utilize cyclohexanol, cyclohexanone, and cyclohexan-1,2-diol as the sole carbon source. Unlike the cyclohexane-growing *Xanthobacter* sp. (20), *X. autotrophicus* was unable to utilize methylcyclohexane, cyclohexan-1,3-diol, cyclohexan-1,4-diol, or cyclohexan-1,2-dione as a carbon source. *X. flavus*, unlike *X. autotrophicus*, was unable to utilize any of the substituted cycloalkanes tested.

The ability of the three species of *Xanthobacter* to grow on the aromatic hydrocarbons benzene and toluene and the straight-chain alkanes (C_6 and C_{16}) was also tested, but none of the *Xanthobacter* species were able to utilize these compounds as growth substrates.

Comparison of the route of metabolism for cyclohexanol by *X. autotrophicus* and the cyclohexane-growing *Xanthobacter* sp. Since both the cyclohexane-growing *Xanthobacter* sp. and *X. autotrophicus* appeared to be closely related with respect to not only their ability to metabolize specific organic acids and sugars but also their ability to metabolize substituted cycloalkanes, comparative studies on these two bacteria were further extended.

(i) Metabolite oxidation. The ability of washed cells of the cyclohexanol-grown *X. autotrophicus* to oxidize potential cyclohexanol metabolites and other substituted cycloalkanes is shown in Table 2. Comparison of these results with those obtained from the cyclohexane-grown *Xanthobacter* sp. indicated considerable similarity in their ability to oxidize cyclohexanol, cyclohexanone, 1-oxa-2-oxocycloheptane (ε-caprolactone), and cyclohexanol-1,2-diol. Neither *X. autotrophicus* nor the cyclohexane-grown *Xanthobacter* sp. was found to be capable of oxidizing adipic acid.

TABLE 1. Differential characteristics of the species of the genus *Xanthobacter*

Characteristic	*Xanthobacter* sp.[a]	*X. autotrophicus*[b]	*X. flavus*[c]
Growth factor requirement	−	−	+
Utilization of			
Fructose	+	+	(+)
Propionate	+	+	−
Malonate	+	−	+
Maltose	+	−	+
Ribose	−	−	+
Phenylalanine	−	−	+
Histidine	−	−	+
Acetate	+	+	(+)
Glucose	−	−	+
Hydrolytic activity on gelatin and casein	+	−	−
Lecithinase activity	+	−	−
Urease activity	+	+[d]	−
Phosphatase activity	−	+	+
Autotrophic growth	+	+	−[e]

[a] Tests on *Xanthobacter* sp. were carried out according to the procedures described in Materials and Methods.
[b] Data from *Bergey's Manual of Systematic Bacteriology* (22).
[c] Data from Malik and Claus (15). (+) Poor growth.
[d] 11 to 89% of strains are positive.
[e] Autotrophic growth occurs only below 5% O_2 atmosphere.

(ii) Enzyme activities in cell extracts of cyclohexanol-grown *X. autotrophicus*. To confirm that the major routes of metabolism of cyclohexanol by *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. were comparable and to assess any similarities in the key enzymes involved in this pathway, studies were undertaken with *X. autotrophicus*, using crude cell extracts.

Incubation of cell extracts (10,000 × *g* and 100,000 × *g* supernatants) from the cyclohexanol-grown *X. autotrophicus* with either NADH or NADPH in the presence of cyclohexane failed to cause any cyclohexane stimulation in O_2 consumption when measured in the oxygen monitor. This confirmed the studies with whole cells in that cyclohexane hydroxylase is unlikely to be present in *X. autotrophicus*.

Enzyme assays carried out to determine the presence of a cyclohexanol dehydrogenase, a cyclohexanone oxygenase, and a ε-caprolactone hydrolase indicated each of these enzymes to be present in the cell extracts (10,000 × *g* supernatant) of the cyclohexanol-grown *X. autotrophicus* (Table 3). The finding that much lower levels (20- to 100-fold) of enzyme activity were detected in cells grown with succinate as the carbon source (data not shown) indicated the inducible nature of these enzymes.

Preliminary studies with the cyclohexanol dehydrogenase and the cyclohexanone oxygenase concerning their cofactor specificity and pH optimum indicated that these enzymes were unlikely to be closely related to the same enzymes found in the cyclohexane-grown *Xanthobacter* sp. (Table 3). Comparison of these enzymes from the two different *Xanthobacter* spp. indicated that cyclohexanol dehydrogenase demonstrated greater specificity for NAD^+ than $NADP^+$ in each case. However, the cyclohexanol dehydrogenase from the cyclohexane-grown *Xanthobacter* sp. displays a pH optimum of 10.1 to 10.5, whereas the optimum pH found for the *X. autotrophicus* enzyme was 9.0 to 9.4. Comparison of the cyclohexanone oxygenase isolated from the two species of *Xanthobacter* also indicated that this enzyme was different in the two microorganisms. The enzyme isolated from the cyclohexanol-grown *X. autotrophicus* is specific for NADH and has a pH optimum of 7.2 to 7.7. In contrast, the enzyme isolated from the cyclohexane-grown *Xanthobacter* sp. is specific for NADPH and displays a pH optimum of 8.5 to 9.0.

Characterization of cyclohexanol dehydrogenase from *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. A comparison of the substrate specificities of the cyclohexanol dehydrogenase present in cell extracts (100,000 × *g* supernatant) of the cyclohexanol-grown *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. is shown in Table 4. The substrate which gave rise to the greatest activity for both enzymes was cyclopentanol. The cyclohexanol dehydrogenase from the *Xanthobacter* sp. was found to have a broad specificity for secondary alcohols, and the primary alcohol butan-1-ol was also oxidized by this enzyme. In contrast, the cyclohexanol dehydrogenase from *X. autotrophicus* was found to have a relatively narrow substrate range and demonstrated no activity towards the cyclohexandiols or butan-1-ol.

Further differences in the two cyclohexanol dehydrogenase enzymes was shown in their respective molecular weights and in their relative mobilities following separation by polyacrylamide gel electrophoresis.

The separation of cell extracts (100,000 × *g* supernatant)

TABLE 2. Comparitive oxidation of potential cyclohexanol metabolites and other hydrocarbons by washed cell suspensions of *Xanthobacter* sp. and *X. autotrophicus*[a]

Substrate	Rate of oxygen uptake (μmol of O_2 h^{-1} mg [dry wt] of organisms^{-1})	
	Xanthobacter sp. (cyclohexane grown)	*X. autotrophicus* (cyclohexanol grown)
Cyclohexane	7.5	0.3
Cyclohexanol	5.4	4.6
Cyclohexanone	5.0	3.4
1-Oxa-2-oxocycloheptane	3.5	2.0
1-Methylcyclohexanol	3.5	3.6
2-Methylcyclohexanol	3.3	1.1
3-Methylcyclohexanol	5.3	2.6
4-Methylcyclohexanol	6.1	1.7
Cyclohexan-1,2-diol	8.6	2.7
Cyclohexan-1,3-diol	5.0	1.3
Cyclohexan-1,4-diol	6.0	1.1
Toluene	0.7	0.4
Heptane	<0.05	<0.05
Decane	<0.05	<0.05
Dodecane	<0.05	<0.05
Succinate	<0.05	<0.05
Adipate	<0.05	<0.05

[a] Values shown were calculated by subtracting the endogenous rate from the rate recorded with the substrate present. Incubation mixtures contained (in 3 ml) 60 μ mol of phosphate buffer (pH 7.0), 100 μl of cell suspension (2.5 mg, dry weight), and 20 μ mol of substrate. Water-immiscible substrates (2 μl) were added directly to the incubation chamber.

from the cyclohexanol-grown *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. by polyacrylamide gel electrophoresis followed by in situ staining for cyclohexanol dehydrogenase is shown in Fig. 1. Only one major staining band was found in the cell extracts obtained from either organism, but the different relative mobilities of these two bands with reference to the bromophenol blue markers indicated the two enzymes to be dissimilar. A further very faint staining band was also found in each of the cell extracts; interestingly, the faint band displayed by the *Xanthobacter* sp. proved to have a relative mobility similar to that of the major staining band found in *X. autotrophicus*.

Estimation of the molecular weights of the two cyclohexanol dehydrogenase enzymes was obtained by comparing the elution volumes of the different enzymes with those obtained for protein standards of known molecular weight following separation of cell extracts (100,000 × *g* supernatant) by Ultrogel AcA44 chromatography. By using this technique the molecular weight of the cyclohexanol dehydrogenase from the cyclohexanol-grown *X. autotrophicus* was found to be 20,000 ± 3,000 (n = 3), while that from the *Xanthobacter* sp. was shown to be 43,000 ± 3,000 (n = 3). Only one band of cyclohexanol dehydrogenase activity could be detected in the column eluents obtained from cell extracts of either *Xanthobacter* species.

Preliminary genetic studies with the cyclohexane-grown *Xanthobacter* sp. The scarcity of reports concerning the ability of microorganisms to grow on cyclohexane compared with those found to grow on cyclohexanols suggests that this unusual metabolic ability may be related to the possession of a degradative plasmid by these microorganisms. Such a finding is not without precedent since plasmids of this kind have been shown to be responsible for a number of degradative pathways (4). In this report, two closely related species of *Xanthobacter* have been demonstrated: one grows on cyclohexanol; the other has the capacity to grow on cyclohexane. It is therefore possible that either the gene(s) responsible for the cyclohexane hydroxylase or other genes that may be required for cyclohexane degradation are present on a degradative plasmid in the cyclohexane-growing *Xanthobacter* sp.

To test this hypothesis a variety of techniques suitable for the isolation of large degradative plasmids, including the Hansen and Olsen procedure (9), the cleared lysate procedure (7), and the procedure of Wheatcroft and Williams (21), were used to detect the presence of plasmid DNA in extracts of the cyclohexane-grown *Xanthobacter* sp. By these techniques no plasmid DNA was detected in this organism. Figure 2 shows agarose gel electrophoresis of DNA extracted from *Xanthobacter* sp. by the Wheatcroft and Williams method (21). The results obtained from this technique clearly show the presence of only chromosomal DNA following separation of the extracted DNA by sucrose gradient centrifugation. Furthermore, no plasmids were detected in *Xanthobacter* sp. by using the rapid plasmid screening procedures of Kado and Liu (13), Eckhardt (3), and Holmes and Quigley (11).

To confirm the methodology used for plasmid isolation and, perhaps more importantly, its application to the *Xanthobacter* species of interest, the broad-host-range plasmid RP4 was conjugated into the cyclohexane-growing *Xanthobacter* sp. from an *Escherichia coli* strain, J53. Transconjugants were selected by growth on cyclohexanone in the presence of kanamycin (25 μg/ml) and tetracycline (10 μg/ml). The presence of RP4 in the *Xanthobacter* recipients

TABLE 3. Comparison of enzymes involved in cyclohexane/cyclohexanol degradation of *Xanthobacter* sp. and *X. autotrophicus* after growth on cyclohexane and cyclohexanol, respectively[a]

Enzyme	*Xanthobacter* sp.[b]			*X. autotrophicus*		
	pH optimum	Cofactor specificity	Activity (U/mg of protein)[c]	pH optimum	Cofactor specificity	Activity (U/mg of protein)[c]
Cyclohexane hydroxylase	6.2–6.7	NADPH	7.5×10^{-3}			
Cyclohexanol dehydrogenase	10.1–10.5	NAD^+	0.87	9.0–9.4	NAD^+	0.73
		$NADP^+$	0.05		$NADP^+$	$<0.1 \times 10^{-3}$
Cyclohexanone monooxygenase	8.5–9.0	NADPH	0.15	7.2–7.7	NADH	0.42
					NADPH	$<0.9 \times 10^{-3}$
1-Oxa-2-oxo cycloheptane hydrolase	7.8–8.1		21.3	8.0		21.6

[a] Enzyme activity and pH optima were measured according to the procedures described in Materials and Methods.
[b] Data from Trower et al. (20).
[c] One unit of enzyme activity equals 1 μmol of substrate utilized per min.

could be clearly detected (data not shown) by the rapid screening procedure of Wheatcroft and Williams (21).

DISCUSSION

Comparison of a variety of microbiological characteristics obtained for the cyclohexane-growing *Xanthobacter* sp. with those reported for *X. autotrophicus* (23) and *X. flavus* (15) indicate this new *Xanthobacter* sp. to be closely related to *X. autotrophicus*.

This relationship to *X. autotrophicus* is further reflected in the ability of these two species of *Xanthobacter* to use substituted cycloalkanes as growth substrates, a property which is not shown by *X. flavus*. The metabolism of cycloalkanes or their substituted analogs may therefore be a characteristic trait of differing strains of *X. autotrophicus*. Indeed *X. autotrophicus* NCIMB 10811 is also capable of growth on cyclohexanol (NCIMB, personal communication).

None of the species of *Xanthobacter* were able to grow with either the straight-chain alkanes (C_6 and C_{16}) or the aromatic hydrocarbons toluene and benzene as growth substrates. Growth with short-chain alkanes (14) and, more recently, short-chain alkenes (C. G. van Ginkel, K. A. Dekker, and J. A. M. de Bont, Proc. 100th Ordinary Meet. Soc. Gen. Microbiol., 1984) has, however, been reported for *Xanthobacter*-like bacteria and may be a separate characteristic representative of other as yet uncharacterised species of this genus.

Similarities between *X. autotrophicus* and the cyclohexane-grown *Xanthobacter* sp. could also be demonstrated with respect to their mode of metabolism of cyclohexanol. Like the cyclohexane-grown *Xanthobacter* sp., the cyclohexanol-grown *X. autotrophicus* possesses an inducible cyclohexanol dehydrogenase, a cyclohexanone oxygenase, and a ε-caprolactone hydrolase. The presence of these enzymes indicate that the route of metabolism for cyclohexanol is likely to proceed via oxidation of cyclohexanol ⟶ cyclohexanone → ε-caprolactone ⟶ adipic acid. This route of metabolism is the major pathway used by the *Xanthobacter* sp. during growth on cyclohexane (20) and has been demonstrated for other cycloalkanol-utilizing microorganisms (16).

TABLE 4. Relative activity of the cyclohexanol dehydrogenase from *Xanthobacter* sp. and *X. autotrophicus* with different substrates[a]

Substrate	% Activity	
	Xanthobacter sp.	*X. autotrophicus*
Cyclohexanol	100	100
Cyclopentanol	174	168
Butan-1-ol	22	0
Butan-2-ol	52	130
1-Methylcyclohexanol	52	0
2-Methylcyclohexanol	65	8
3-Methylcyclohexanol	99	41
4-Methylcyclohexanol	91	10
Cyclohexan-1,2-diol	24	0
Cyclohexan-1,3-diol	27	0
Cyclohexan-1,4-diol	30	0
2-Hydroxycyclohexanone	0	0

[a] Cyclohexanol dehydrogenase activity was measured according to the procedures described in Materials and Methods. Reactions were performed at the pH optimum for each enzyme. Substrates were added at a concentration of 1 mM. Values represent a percentage of the rate measured for each enzyme with cyclohexanol as the substrate.

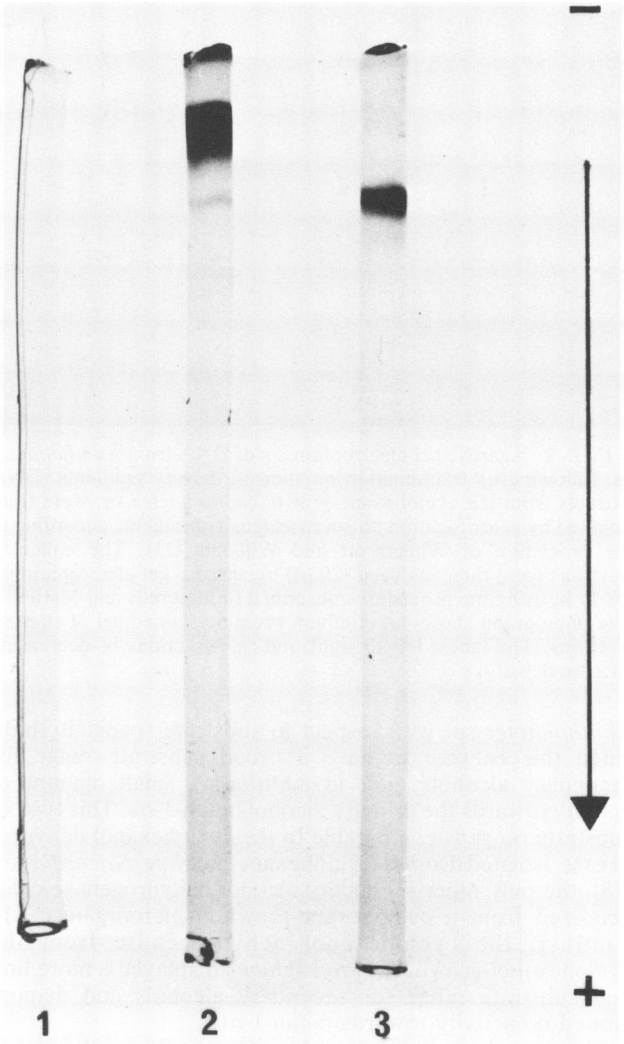

FIG. 1. In situ activity staining for cyclohexanol dehydrogenase following separation of crude cell extracts by polyacrylamide electrophoresis. Cell extracts (100,000 × *g* supernatant containing 50 to 100 μg of protein) were fractionated by polyacrylamide gel electrophoresis by the procedure described in Materials and Methods, using a current of 3 mA per gel tube. The photograph shows gels stained for cyclohexanol dehydrogenase activity according to the procedures described in the text. Gel 1, Control gel of fractionated extracts from the cyclohexanol-grown *Xanthobacter* sp. stained for cyclohexanol dehydrogenase activity but without addition of cyclohexanol; gel 2, cell extracts from the cyclohexane-grown *Xanthobacter* sp.; gel 3, cell extracts from the cyclohexanol-grown *X. autotrophicus*.

Characterization of the cyclohexanol dehydrogenase from each of the species of *Xanthobacter* with respect to pH optima and molecular weight indicated the two enzymes to be distinctly different from one another. A similar feature shared by the two enzymes was their preferred requirement of NAD^+ for enzyme activity, a property displayed by other secondary alcohol dehydrogenases which have so far been characterized (16, 17). Analysis of cell extracts from the cyclohexane-grown *Xanthobacter* sp. and the cyclohexanol-grown *X. autotrophicus* by polyacrylamide gel electrophoresis and gel filtration chromatography indicated only one major cyclohexanol dehydrogenase enzyme to be present. Characterization of the enzyme from the cyclohexane-grown

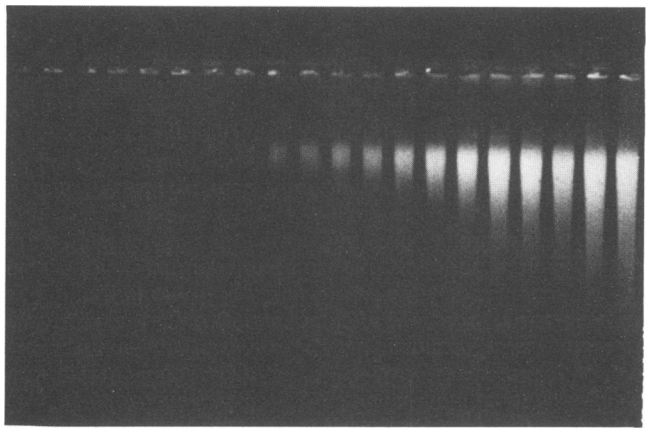

FIG. 2. Agarose gel electrophoresis of DNA from *Xanthobacter* sp. following its fractionation on sucrose density gradients. DNA extracts from the cyclohexane-grown *Xanthobacter* sp. were fractionated by centrifugation on sucrose density gradients according to the procedure of Wheatcroft and Williams (21). The collected fractions were then analyzed (20 μl) by agarose gel electrophoresis for 15 h, using the procedures described in Materials and Methods. The photograph shows an ethidium bromide-stained gel of sucrose fractions. The lanes, left to right, indicate fractions of decreasing sucrose density.

Xanthobacter sp. with respect to substrate specificity indicated this enzyme to have a broad substrate range for secondary alcohols and, in addition, a small amount of activity towards the primary alcohol butan-1-ol. This type of substrate range is comparable to the cyclohexanol dehydrogenase isolated from a cyclohexane-growing *Nocardia* sp. (18), the only other secondary alcohol dehydrogenase characterized from a cyclohexane-growing microorganism. In contrast, the cyclohexanol dehydrogenase from the cyclohexanol-grown *X. autotrophicus* displayed a more limited substrate range for secondary alcohols and demonstrated no activity towards butan-1-ol.

The other key enzyme involved in cyclohexanol metabolism, the lactone-forming cyclohexanone oxygenase, was also found to be different in the two *Xanthobacter* species. The most striking difference was the finding that the *X. autotrophicus* enzyme was specific for NADH. This is a particularly interesting feature since of the lactone- and ester-forming oxygenases so far characterized only one other oxygenase, the 2,5-diketocamphane monooxygenase from *Pseudomonas putida*, is specific for NADH. The latter enzyme is unusual with respect to other lactone-forming monooxygenases in a number of features since it carrys flavin mononucleotide rather than flavin adenine dinucleotide as the prosthetic group and is a multicomponent enzyme possessing separable reductase and oxygenase components (19). Interestingly, the cyclohexanone monooxygenase isolated from the cyclohexane-growing *Xanthobacter* sp. also possesses flavin mononucleotide as its prosthetic group (20) but, unlike the 2,5-diketocamphane monooxygenase, is specific for NADPH and not NADH and is not a multicomponent enzyme. Hence the cyclohexanone oxygenase enzymes isolated from the two *Xanthobacter* species, although different from one another, each appear to show unusual characteristics for this group of enzymes and merit further investigations with regard to their properties.

Our inability to detect the presence of plasmid DNA in the cyclohexane-growing *Xanthobacter* sp. would indicate that the genetic information responsible for the added capacity of this particular *Xanthobacter* sp. to grow on cyclohexane is not carried on a degradative plasmid. However, the presence of a large undetected plasmid cannot be excluded. It is also possible that such a plasmid may be destroyed during the physical procedures used to isolate it; however, our ability to detect the presence of plasmid RP4 in the *Xanthobacter* sp. would seem to dispel this idea.

The finding that shuttle vectors, such as RP4, may be transferred into this *Xanthobacter* sp. by conjugation with RP4-carrying *E. coli* J53 is, however, an important discovery in itself, since this vector or its derivatives have the potential to be used as broad-host-range cloning vehicles in the further characterization of the genes responsible for cyclohexane degradation.

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