MICROBIAL PETROLEUM DEGRADATION: THE ROLE OF CLADOSPORIUM RESINAE

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ABSTRACT

Cladosporium resinae *is probably the most prevalent hydrocarbon-utilizing fungus. It has been isolated in fresh water and marine environments. It utilizes aliphatic and aromatic hydrocarbons, as well as alcohols and acids. Growth on aliphatic hydrocarbons is slow, and yields are lower than for cells cultured on acids. However, degradation of hydrocarbons is not slow, since most of the hydrocarbon is mineralized (converted to* CO_2*) and not assimilated to cellular carbon. Growth of the fungus was not supported by organo-phosphorus-, chlorinated hydrocarbon-, or natural pesticides as sole carbon source. However, the fungus was resistant to or stimulated by high concentrations (20,000 ppm) of these pesticides when cultured on hydrocarbons. This suggests that high concentrations of pesticides occurring in oil slicks would not inhibit oil degradation by* Cladosporium resinae. *Hydrocarbon-oxidation is constitutive in* Cladosporium resinae. *Cells oxidize aldehydes, although they did not grow on them. Release of* $^{14}CO_2$ *from D-glucose-1-*^{14}C *labeled cells was not greater in the presence of hydrocarbons than under endogenous conditions, indicating that intermediate and long-chain hydrocarbons are oxidized and do not stimulate endogenous respiration. Comparison of hydrocarbon oxidation by whole cells and cell-free preparations revealed the presence of an efficient cell-free oxidizing system. The pathway of hydrocarbon oxidation has been reported for hydrocarbon-utilizing bacteria and yeasts but not for fungi. Temperature, pH and co-enzyme requirements were determined for the oxidation of hydrocarbons by* C. resinae. *Isolation of intermediates and results of experiments utilizing electron transport inhibitors support the conclusion that in* C. resinae *alkanes are oxidized to their homologous alcohol, aldehyde and acid. The range of hydrocarbon substrates degraded by constitutive enzymes of* Cladosporium resinae *coupled with the ability to degrade hydrocarbons in the presence of high concentrations of pesticides suggests that* C. resinae *may be one of the most important microorganisms capable of degrading oil in the natural environment.*

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INTRODUCTION

The observation by Miyoshi[11] that *Botrytis cinerea* would attack paraffin provided the beginning of hydrocarbon microbiology. Since then many reports of hydrocarbon-utilizing bacteria, yeasts and fungi have become available. However, studies with bacteria and yeasts far exceed those with filamentous fungi. *Cladosporium resinae* has been isolated from sewage sludge[3], asphalt watersheds,[16] air and soil.[12] It corrodes aluminum alloys in fuel systems[8] and it can clog and degrade polymers used in fuel tanks.[4] In addition it is found in the oil contaminated marine and estuarine environment.[1] It is probably the most prevalent hydrocarbon-utilizing fungus, but relatively little information is available on its mode of hydrocarbon metabolism. Parbery[13] recently indicated that "a detailed study of the metabolism of *C. resinae* would seem worthwhile." Moreover, two strains of *C. resinae* have been under investigation in this laboratory for several years and a body of information was available. Therefore, we examined some aspects of hydrocarbon metabolism of *C. resinae* to evaluate its role in petroleum degradation in the natural environment.

Materials and Methods

Organisms

Two strains of *Cladosporium resinae* were examined. They are coded as UD-42 and UD-43 and have been deposited with the American Type Culture Collection where they bear the accession numbers 22711 and 22712, respectively.

Media and Culture Systems

The basal medium consisted of the salts solution of Bushnell and Haas[2] supplemented with 0.1 percent (w/v)

yeast extract, adjusted to pH 5.8 and dispensed in 100 ml volumes to 250 ml flasks. As carbon source solid hydrocarbon (one percent, w/v) was added prior to autoclaving. For liquid hydrocarbons, the salts solution was sterilized by autoclaving and it was then overlaid with ten percent (w/v) filter-sterilized hydrocarbon.

In some experiments individual pesticides were added at a concentration of 20,000 ppm to flasks containing Bushnell-Haas salts solution or to the salts solution plus hexadecane. The culture systems were described elsewhere (Cofone, Walker and Cooney, submitted).

Cell-Free Systems

Washed cells were suspended in citrate-phosphate buffer and disrupted by two passages through a French pressure cell (Aminco, Silver Spring, Md.) at 40,000 p.s.i. After disruption, the preparation was centrifuged at 10,000 X g for ten min. and the supernatant fluid was used to measure hydrocarbon-oxidizing activity. Protein was estimated by the method of Lowry et al.[9] using bovine serum albumin as standard. Isocitrate lyase was assayed in extracts from actively growing cells as described by Dixon and Kornberg.[6]

Hydrocarbon Oxidation

Oxygen consumption by washed intact cells or cell-free preparations was followed using a Model 53 Biological Oxygen Monitor (Yellow Springs, Ohio). Hydrocarbons in citrate-phosphate buffer were added as emulsion prepared by subjecting the mixture to 30 sec. of ultrasound at a setting of eight on a Branson Model S-75 Sonifier (Heat Systems Ultrasonics, Inc., Melville, N.Y.). Cell-free preparation containing 0.25 mg protein per ml and 12 μl of hexadecane per ml were used for respirometric studies to examine several parameters known to influence hydrocarbon-oxidizing systems. Hexadecane was used as substrate because it was readily oxidized by whole cells and cell-free extracts. Coenzyme requirements were examined utilizing NAD, NADP, NADH and NADPH separately at concentrations of five μ moles per ml of extract.

In order to determine the effect of substrates on endogenous metabolism, cells were labeled by culturing them on D-glucose-1-^{14}C (specific activity 14.5 μ Ci/g glucose) and the rate of $^{14}CO_2$ released from washed cells was measured in the absence and in the presence of substrate. Labeled cells were washed with Bushnell-Haas solution three times by centrifugation to remove 99 percent of exogenous label. Washed cells were suspended in 30 ml of Bushnell-Haas salts solution (3 mg dry weight cells/ml) in a 50 ml flask. The suspension was stirred and aerated with sterile air (60 cc/min.) which was forced over the suspension to void any $^{14}CO_2$ released. The $^{14}CO_2$ was bubbled through two traps in series containing ten percent KOH. Negligible radioactivity was detected in the second trap. Samples were withdrawn periodically from the first trap by removing 0.1 ml of the KOH solution and adding it to scintillation fluid in a scintillation vial. Quenching was corrected by using an internal standard (^{14}C-sucrose, 1100 dpm/100 ul). After a steady rate of endogenous respiration was recorded, one percent (v/v) hexadecane was added as a 3 ml emulsion to the cell suspension and the rate of endogenous respiration was determined as described above. In addition to hexadecane, hexane, glucose, hexadecanol, hexadecanal and hexadecanoic acid were examined for their effect on endogenous metabolism.

Spectrophotometric Assays

In order to demonstrate involvement of pyridine nucleotides in hexadecane oxidation reactions, reaction mixtures were maintained at 30 C, at the optimum pH for the reaction being studied. Each mixture contained 12 μl hydrocarbon, 0.2 μ mole NAD or NADH per ml of cell-free preparation (0.25 mg protein) in pH 7.0 citrate-phosphate buffer. NADH was measured at 340 nm and the amount of NADH formed or used was calculated from the extinction coefficient of 6.22×10^6 cm^2 per mole.[7] In some experiments the effects of the electron transport inhibitors, piperonyl butoxide (0.5 M), sodium azide (10 mM) and potassium cyanide (one mM) were examined.

Radioactive Product Determination

In order to determine the pathway of hydrocarbon oxidation in *C. resinae*, cell-free preparations were allowed to oxidize ^{14}C-hexadecane and radioactivity was trapped in a suspected product of hexadecane oxidation. Each of three 125-ml flasks received 37 ml of cell-free extract (0.25 mg protein/ml) plus 250 μ moles NADH. One flask received 500 mg of hexadecanol emulsified in ten ml of buffer while a second and third flask received equal quantities of emulsified hexadecanal and hexadecanoic acid, respectively. Each flask also received 600 μl hexadecane as a 3 ml emulsion. The labeled substrate, 1 μ Ci of *n*-hexadecane-1-^{14}C (specific activity 1 μ Ci/600 μl hexadecane) was added last and duplicate samples were immediately withdrawn from each flask and added to scintillation vials containing 15 ml of scintillation fluid.

The reaction mixture was stirred one hour at 25 C, acidified to pH 2 and extracted twice with diethyl ether. Preliminary experiments demonstrated that one extraction removed 96-99 percent of the hydrocarbons. The aqueous phase was concentrated by evaporation and added to vials containing scintillation fluid. The ether phases from the reaction mixtures containing hexadecanol and hexadecanal were evaporated to dryness. Hexadecanol was converted to its 3,5-dinitrobenzoyl chloride derivative and hexadecanal to its 2,4-dinitrophenylhydrazone derivative as described by Shriner, Fuson and Curtin.[15] When hexadecanoic acid was the trapping agent, it was crystallized directly from ether. Solutions containing the alcohol derivative, the aldehyde derivative or the acid were applied to thin-layer chromatograms of Kieselguhr G. Plates with the alcohol derivative were developed with ethanol, those with the aldehyde derivative were developed with benzene: ethanol: chloroform (2:1:1, v/v) and those with the acid derivative were developed with benzene: methanol: acetic acid (45:8:4, v/v). Plates were examined under ultraviolet light or, for those containing the acid, by spraying with bromocresol green (0.2 percent bromocresol green in *n*-butanol). Products were identified by co-chromatography with authentic compounds. After chromatography, samples were eluted from the adsorbent with methanol and added to vials containing scintillation fluid. Samples were counted in a liquid scintillation detector.

RESULTS AND DISCUSSION

Parbery[13] suggested that erroneous conclusions about the growth of *C. resinae* have occurred because of data based solely on fungal spore counts and work with single isolates. Furthermore, he emphasized weight increase as the preferred method for measuring growth. In the present work two strains of *C. resinae* were used and growth was measured by determining dry weight yields. Data are pre-

sented for UD-42, but strain UD-43 gave similar results except as noted. Values for growth are averages from at least two experiments and growth yields from replicate flasks did not vary more than ten percent.

A variety of hydrocarbons supported growth of *C. resinae* (Table 1). Growth was best on alkanes of intermediate chain length. In most cases growth was accompanied by a decrease in pH, which was proportional to the amount of growth. Growth was slow on hydrocarbons: the data in Table 1 are from 30 day cultures, whereas cells grown on glucose reached stationary phase (140 mg dry cells per 100 ml) in six days.

Filamentous fungi in general do not grow rapidly on *n*-alkanes.[14] For *C. resinae* this is probably because cells oxidize alkanes without assimilating them into cellular material. This is supported by the observation that the ratio of hexadecane oxidized to CO_2 vs. hexadecane assimilated to cellular material in an hour is 97:3 (Walker and Cooney, submitted). Thus, a large amount of alkane can be mineralized without accumulation of a large microbial biomass.

C. resinae degrades 2-methylundecane which has one isopropyl group (Table 1), but not compounds with isopropyl groups at both ends of the molecule. Similar results have been observed for bacteria.[10] When hexadecanoic acid was the substrate for shaken cultures, large yields were produced: 1540 mg per 100 ml (UD-42) and 2200 mg per 100 ml (UD-43).

C. resinae did not grow with aldrin, DDT, dieldrin, diazinon, malathion or rotenone as carbon source in Bushnell-Haas salts medium. None of the pesticides inhibited growth on glucose or hexadecane. At concentrations as high as 20,000 ppm, aldrin and malathion enhanced growth on hexadecane; whereas, aldrin, dieldrin, DDT and malathion enhanced growth on glucose. None of the pesticides affected oxygen uptake on glucose or hexadecane. In natural waters DDT and other pesticides rarely exceed 2 ppm. Much higher concentrations were used here because pesticides can be concentrated in surface oil slicks. Because *C. resinae* is resistant to pesticides, it may be useful in promoting the breakdown of oil in marine and other environments where bacterial degradation is rather slow. In fact, *Cladosporium* is the most prevalent fungus isolated from oil-contaminated marine environments.[1] In addition, hexadecane oxidation was stimulated by compounds which support slight growth (toluene) or which do not support growth (*p*-xylene). Therefore, compounds in petroleum that are not readily degraded may stimulate degradation of paraffins. Maximum hydrocarbon oxidation by *C. resinae*

Table 1: Growth of *C. resinae* (UD-42) after 30 Days in Incubation on Hydrocarbons, Alcohols and Acids in Flasks without Shaking.

Carbon source	Growth (mg dry cells per 100 ml)	pH (stationary phase)
Alkanes		
hexane	3	5.7
heptane	17	5.3
octane	19	5.6
nonane	68	5.2
decane	97	2.7
undecane	132	2.5
dodecane	105	3.0
tridecane	57	3.1
tetradecane	45	3.2
pentadecane	66	3.0
hexadecane	57	3.4
nonadecane	44	3.4
cyclohexane	3	5.4
2-methylundecane	13	5.6
Alkenes		
1-octene	2	4.3
1-decene	8	5.7
1-dodecene	8	5.5
7-tetradecene	15	5.2
cyclohexene	2	5.5
Alcohols		
1-octanol	3	5.5
1-decanol	1	5.4
1-dodecanol	8	5.0
1-tetradecanol	7	5.5
Acids		
octanoic	5	4.3
dodecanoic	51	4.6
tetradecanoic	7	5.3
Aromatic		
benezene	4	5.4
toluene	1	5.4
o-xylene	5	5.3
m-xylene	5	5.3
isopropylbenzene	2	5.7

Table 2: Hydrocarbon Oxidation by *C. resinae* (UD-42) During Various Stages of Growth.

Substrate	Q_{O_2}[a] during Active Growth	Early stationary phase	Late stationary phase
Dodecane	11.0	6.0	1.1
Hexadecane	98.5	11.2	2.2

[a]Expressed as μl O_2 per mg dried cells per hour and corrected for endogenous respiration.

was achieved by actively growing cells (Table 2) although even late stationary phase cells continued to oxidize alkanes. Alkanes, alcohols, aldehydes and acids were oxidized (Figure 1), although the aldehydes did not support growth.

The ability to oxidize hydrocarbons is constitutive in *C. resinae* because: i) cells cultured on glucose consumed oxygen without a lag when transferred to hycrocarbons or their oxygenated derivatives (Figure 1); ii) cycloheximide (at concentrations which inhibited growth) did not inhibit oxygen uptake when cells were transferred from glucose to hydrocarbons; iii) hydrocarbon-grown cells did not have higher Q_{O_2} values on hydrocarbons than did glucose-grown cells which were transferred to hydrocarbons (Figure 2), and iv) isocitrate lyase levels were not higher in hydrocarbon-grown cells than in glucose- or glutamic acid-grown cells.[5] The constitutive ability to oxidize hydrocarbons may be advantageous in a dilute medium such as an aquatic ecosystem which is poor in nitrogen. A constitutive organism can oxidize hydrocarbons without the prior period of protein synthesis required by an inducible organism, and without requiring nitrogen from the environment. Thus, organisms constitutive in their ability to oxidize hydrocarbons may be competitive in a wider variety of habitats than those which do not synthesize the enzymatic machinery until they are faced with the hydrocarbon.

The pathways of hydrocarbon oxidation have been reported for bacteria and yeast, but not for filamentous fungi. *C. resinae* utilizes the following pathway as the major pathway of hydrocarbon degradation:

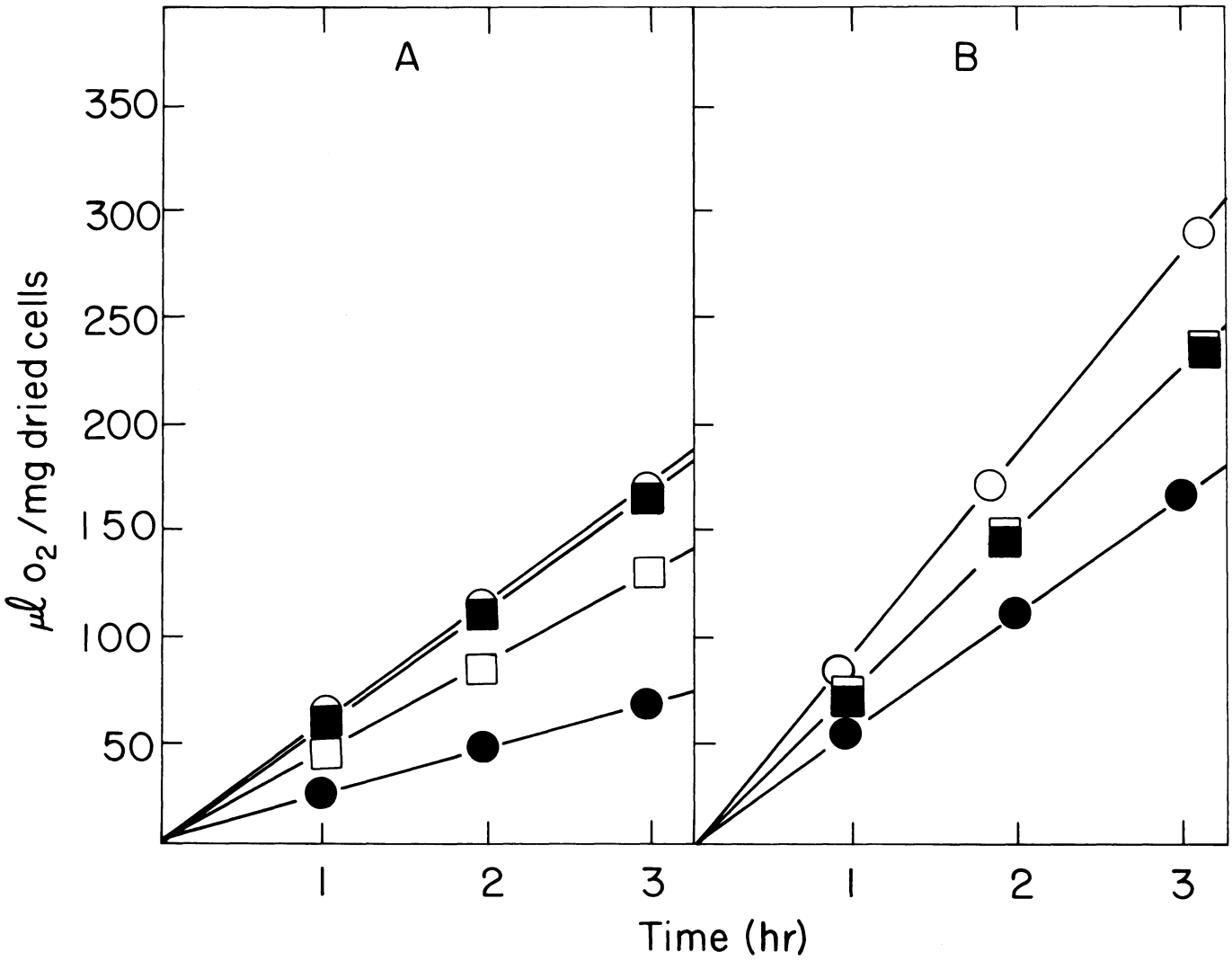

Figure 1: Oxidation of the Alkane (○—○), Alcohol (●—●), Aldehyde (□—□) and Acid (■—■) of Dodecane (A) and Hexadecane (B) by Washed Cells of *C. resinae* (UD-42) Which Were Grown on Glucose. Values Have Been Corrected for Endogenous Oxygen Consumption.

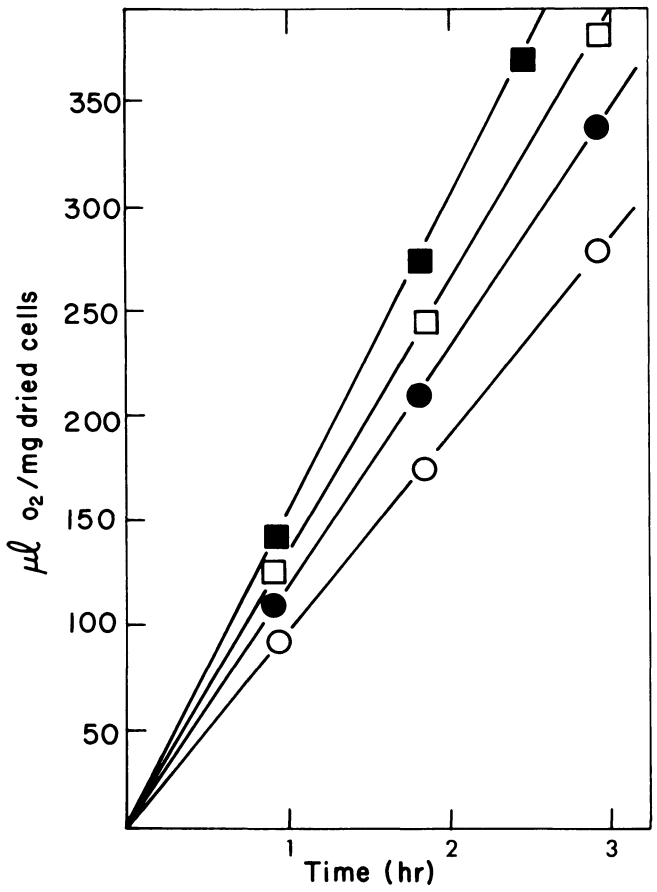

Figure 2: Dodecane Oxidation by Dodecane-Grown (○—○) and Glucose-Grown (●—●) *C. resinae*, and Hexadecane Oxidation by Hexadecane-Grown (□—□) and Glucose-Grown (■—■) *C. resinae* (UD-42). Values Have Been Corrected for Endogenous Oxygen Consumption.

$$RCH_2CH_3 \xrightarrow[O_2]{NADH} RCH_2CH_2OH \xrightarrow{NAD} RCH_2CHO$$

$$\xrightarrow[O_2]{NAD} RCH_2COOH$$

This conclusion is based on: i) oxidation of compounds by whole cells, ii) stimulation of oxygen uptake by the appropriate coenzymes in cell-free preparations, iii) accumulation of the appropriate reduced coenzymes in the presence of cyanide or azide in cell-free preparations and iv) recovery of 65-85 percent of the radioactivity supplied as C^{14}-hexadecane in the presence of trapping amounts of any one of the proposed intermediates.

The alkanes dodecane through hexadecane accumulate in cells (Walker and Cooney, submitted), suggesting that *C. resinae* transports alkanes into the cell and then oxidizes them, rather than oxidizing them at the cell surface.

The wide range of hydrocarbons which supports growth, stimulation of hydrocarbon utilization by pesticides and by hydrocarbons which are not metabolized, and the constitutive nature of alkane-oxidizing ability suggest a prominent role for *Cladosporium resinae* in petroleum degradation in the natural environment.

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