Aliphatic Hydrocarbons of \textit{Cladosporium resinae} Cultured on Glucose, Glutamic Acid, and Hydrocarbons

J. D. WALKER\textsuperscript{1} AND J. J. COONEY

Department of Biology, University of Dayton, Dayton, Ohio 45469

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The carbon source markedly influenced the qualitative and quantitative composition of cellular hydrocarbons in \textit{Cladosporium resinae}. Total lipid and hydrocarbon content was greater in cells grown on \textit{n}-alkanes than in cells grown on glucose or glutamic acid. Glucose-grown cells contained a spectrum of aliphatic hydrocarbons from \textit{C} \textsubscript{7} to \textit{C} \textsubscript{36}; pristane and \textit{n}-hexadecane comprised 98\% of the total. Cells grown on glutamic acid contained \textit{C} \textsubscript{7} to \textit{C} \textsubscript{34} hydrocarbons; \textit{n}-tridecane, \textit{n}-tetradecane, \textit{n}-hexadecane, and pristane made up 74\% of the total. \textit{n}-Decane-grown cells yielded \textit{C} \textsubscript{8} to \textit{C} \textsubscript{32} compounds, and \textit{n}-hexadecane (96\%) was the major hydrocarbon. Cells grown on individual \textit{n}-alkanes from \textit{C} \textsubscript{11} to \textit{C} \textsubscript{15} all contained \textit{C} \textsubscript{11} to \textit{C} \textsubscript{32} hydrocarbons, and cells grown on \textit{n}-hexadecane contained \textit{C} \textsubscript{11} to \textit{C} \textsubscript{32} hydrocarbons. In \textit{n}-undecane-grown cells, \textit{n}-hexadecane and pristane made up 92\% of the total, but in cells grown on \textit{C} \textsubscript{13} to \textit{C} \textsubscript{16} the major cellular hydrocarbon was the one on which the cells were grown. This suggests that cells cultured on \textit{n}-alkanes of \textit{C} \textsubscript{13} or longer accumulate \textit{n}-alkanes prior to oxidizing them.

Cellular hydrocarbons of fungi generally comprise less than 1\% of total cell lipid (5). Cells cultured on carbohydrates contain a spectrum of hydrocarbons with those of chain length \textit{C} \textsubscript{18-22} and \textit{C} \textsubscript{27-31} predominating (9). Despite reports of hydrocarbon oxidation in over 70 genera of microorganisms, there are no reports of cellular hydrocarbons for hydrocarbon-utilizing microorganisms. \textit{Cladosporium resinae} is part of the normal soil microflora and it is prominent in soil and water ecological niches which have been enriched in hydrocarbons. It is a common contaminant of kerosene aviation fuel systems. The biology of the organism and some applied aspects of its growth in hydrocarbon fuel systems were reviewed by Parbery (6). Therefore, we examined the cellular hydrocarbons of \textit{C. resinae} cultured on alkanes, glucose, and glutamic acid.

\textbf{MATERIALS AND METHODS}

\textbf{Growth conditions.} \textit{Cladosporium resinae} ATCC 22711 was maintained and starter cultures for fermentors were prepared as described previously (4). All media were based on the salts solution of Bushnell and Haas (2) supplemented with 0.1\% (wt/vol) yeast extract and adjusted to pH 6.0. At this concentration yeast extract alone supported negligible growth. As carbon source, glucose or glutamic acid (1\% wt/vol) was added to 10 liters of salts solution in a 14-liter fermentor jar prior to autoclaving. For hydrocarbon media, 10 liters of salts solution was prepared and autoclaved. After the solution cooled, 100 ml of an \textit{n}-alkane (\textit{C} \textsubscript{16} through \textit{C} \textsubscript{18}), sterilized by membrane filtration, was added. The fermentor was maintained at 25 \textdegree{}C; agitation and aeration were at 200 rpm and 4,000 cm\textasciicircum{3}/min, respectively. Hydrocarbon cultures were supplemented periodically with additional substrate to maintain a layer of hydrocarbon over the aqueous phase. Growth occurred only in the aqueous phase, and all samples were taken from the aqueous phase. Growth was followed by determining pH of the culture and by cellular dry weight as reported earlier (4). When the dry weight, the pH, or both remained constant for two successive readings, cultures were judged to be in stationary phase. Cells were harvested by centrifugation and washed three times with Bushnell-Haas salts solution. In some experiments, wet cells were analyzed and in other experiments cells were lyophilized and stored under nitrogen at 0 \textdegree{}C prior to analysis.

\textbf{Extraction of hydrocarbon.} Cells were washed once with hexane to remove extracellular hydrocarbons. The hexane washings were examined by gas-liquid chromatography (GLC) and yielded only hex-
ane for glucose- or glutamic acid-grown cells, and hexane plus the hydrocarbon growth substrate for hydrocarbon-grown cells. Additional washings did not remove any hydrocarbons. Thus, one washing with hexane removed extracellular hydrocarbons without removing cellular hydrocarbons.

Washed cells were suspended in CHCl₃-CH₄OH (2:1 vol/vol) which had been adjusted to pH 3 with HCl. The suspension was stirred for 8 h, the cells were removed by filtration, and the extraction was repeated twice. Preliminary experiments indicated that acidified solvents removed more hydrocarbon than neutral solvents. Similar results were reported for bacterial hydrocarbons by Albro and Dittmer (1), who suggested that neutral solvents fail to remove hydrocarbons associated with phospholipids. The combined extracts were washed with 1% aqueous NaCl and evaporated to dryness at 35°C with a rotary evaporator. The residue was dissolved in 1 ml of CHCl₃ and a sample was removed, added to a tared planchet, and dried to constant weight at 100°C.

Another sample was applied to a silica gel thin-layer chromatography plate. In addition, a known fatty acid (lauric acid), fatty acid methyl ester (methyl laurate), triglyceride (tristearin), phospholipid (phosphatidylcholine or phosphatidylethanolamine), and hydrocarbon (hexadecane) were applied to the plate and it was developed with hexane. Compounds were detected by exposing the plates to iodine vapors. Components other than hydrocarbons were not detected, indicating that the hexane eluate was "clean."

Gas chromatography. Most chromatograms were obtained on a Hewlett-Packard model 5750 gas chromatograph equipped with a dual flame ionization detector. Two types of glass columns were used: a column (2 mm by 1.8 m) packed with 10% SE-30 on 60/80 mesh Chromosorb W and a column (2 mm by 2.4 m) packed with 10% EGSS-X on 100/120 mesh. Gas Chrom P. Nitrogen was the carrier gas at a flow rate of 50 cm³/min. Temperature was programmed from 100 to 280°C at 6°C/min, and additional runs were made isothermally at several temperatures between 100 and 280°C for separation on SE-30. The same procedure was followed for EGSS-X with a temperature minimum and maximum of 90 and 200°C, respectively. Temperatures of the injection port and the detector were 360°C. Temperatures of the injection port and the detector were 360°C;

Hydrocarbons were identified by comparison of their retention times with retention times of hydrocarbons in standard mixtures run on each column. Individual hydrocarbons were quantitated by calculating the area under individual peaks as percentage of total peak area. Column efficiency and detector responses were determined with quantitative mixture no. 19251 from Applied Science Laboratories (State College, Pa.) containing C₁₄ to C₃₂ saturated and unsaturated hydrocarbons, yielding a relative error of less than 3% for all components.

Chemicals. Hexane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, and hexadecane were of 99% mol purity and were obtained from

| TABLE 1. Total lipid and hydrocarbon content of C. resineae grown on glucose, glutamic acid, or hydrocarbon |
|---------------------------------------------------------------|
| Carbon source      | Total lipid (mg/g of dried cells) | Total hydrocarbon (mg/g of dried cells) | Hydrocarbon as % of total lipid |
| Glucose           | 29.6                              | 1.0                                      | 3.4                             |
| Glutamic acid     | 30.2                              | 1.2                                      | 4.0                             |
| n-Decane          | 69.5                              | 2.6                                      | 3.7                             |
| n-Undecane        | 70.4                              | 2.6                                      | 3.7                             |
| n-Dodecane        | 75.1                              | 2.8                                      | 3.7                             |
| n-Tridecane       | 70.3                              | 2.7                                      | 3.8                             |
| n-Tetradecane     | 59.9                              | 3.0                                      | 5.0                             |
| n-Pentadecane     | 49.7                              | 2.5                                      | 5.0                             |
| n-Hexadecane      | 59.8                              | 3.0                                      | 5.0                             |

FIG. 1. Gas chromatographic tracing of hydrocarbons from n-decane-grown C. resineae separated on an SE-30 column, programmed from 100 to 280°C at 6°C/min. Numbers refer to number of carbon atoms of normal and branched (br) hydrocarbons.
Phillips Petroleum Co., Bartlesville, Okla. Reagent grade chloroform and methanol were purchased from Matheson, Coleman and Bell, East Rutherford, N.J. Individual hydrocarbon standards, all 99+% pure, as well as quantitative and qualitative mixtures of saturates and unsaturates were purchased from Applied Science Laboratories, Inc., State College, Pa.

RESULTS AND DISCUSSION

*n*-Alkane-grown cells contained about twice as much lipid and two to three times as much total hydrocarbon as cells grown on glucose or glutamic acid (Table 1). Hydrocarbon comprised about the same proportion of cellular

| Hydrocarbon | Glucose | Glutamate | Decane | Undecane | Dodecane | Tridecane | Tetradecane | Pentadecane | Hexadecane |
|-------------|---------|-----------|--------|----------|----------|-----------|-------------|-------------|------------|
| n-7a        | tr      | tr        | tr     | tr       | tr       | tr        | tr          | tr          | tr         |
| n-8         | tr      | tr        | tr     | tr       | tr       | tr        | tr          | tr          | tr         |
| n-9         | tr      | 0.8       | tr     | tr       | tr       | tr        | tr          | tr          | tr         |
| br-9        | tr      | 4.5       | 0.1    | tr       | tr       | tr        | tr          | tr          | tr         |
| n-10        | tr      | 0.1       | 0.4    | tr       | tr       | tr        | tr          | tr          | tr         |
| br-10       | tr      | 0.4       | 0.3    | tr       | tr       | tr        | tr          | tr          | tr         |
| n-11        | tr      | 0.4       | 0.2    | tr       | tr       | tr        | tr          | 5.7         | 0.6 tr     |
| br-11       | tr      | 6.5       | 0.4    | tr       | tr       | tr        | tr          | 0.8 tr      | tr         |
| n-12        | tr      | 0.1       | 0.1    | 77.4     | tr       | tr        | tr          | 0.9         | tr         |
| br-12       | tr      | 0.1       | 0.3    | tr       | tr       | tr        | tr          | tr          | tr         |
| n-13        | tr      | 11.4      | 0.3    | 0.8 tr   | tr       | 78.4      | 4.6 tr      | 0.7         | 0.5        |
| br-13       | tr      | 4.0       | 0.4    | tr       | tr       | tr        | tr          | 3.2         | tr         |
| n-14        | tr      | 28.3      | 0.4    | 2.5      | 0.1      | 1.7       | 65.4        | 2.8         | 1.0        |
| br-14       | tr      | 0.4       | 0.8    | tr       | tr       | tr        | 0.9         | 1.0         | 91.8       |
| n-15        | tr      | 1.3       | 0.5    | tr       | tr       | tr        | tr          | tr          | tr         |
| br-15       | tr      | 39.4      | 22.5   | 96.1     | 45.8     | 5.8       | 7.8         | 4.6         | 61.0       |
| n-16        | tr      | 19.1      | 14.1   | 1.5      | 45.8     | 16.5      | 1.2         | tr          | tr         |
| n-17        | tr      | 1.5       | 10.3   | tr       | tr       | tr        | tr          | 28.5        | tr         |
| Pristane    | 59.1    | 14.1      | 1.5    | 45.8     | 16.5     | 1.2       | tr          | tr          | 28.5       |
| n-18        | tr      | 0.6       | 1.4    | tr       | tr       | tr        | tr          | tr          | 0.8        |
| br-18       | tr      | 1.5       | 0.1    | tr       | tr       | 0.6       | tr          | tr          | tr         |
| n-19        | tr      | tr        | 0.5    | tr       | 1.5      | tr        | tr          | tr          | 0.2        |
| n-20        | tr      | 0.7       | tr     | 0.1      | tr       | 2.3       | tr          | tr          | tr         |
| n-21        | tr      | 0.2       | tr     | 0.2      | tr       | 2.6       | tr          | tr          | 0.5        |
| n-22        | tr      | 0.5       | 0.1    | tr       | tr       | 2.0       | tr          | tr          | tr         |
| n-23        | tr      | tr        | tr     | tr       | tr       | tr        | tr          | tr          | tr         |
| n-24        | tr      | tr        | tr     | tr       | tr       | tr        | tr          | tr          | tr         |
| n-25        | tr      | tr        | 0.2    | tr       | 4.6      | tr         | tr          | tr          | 0.6        |
| n-26        | tr      | tr        | tr     | 0.6      | tr       | tr         | tr          | tr          | 0.5        |
| n-27        | tr      | tr        | tr     | 0.4      | tr       | tr         | tr          | 1.2         | tr         |
| n-28        | tr      | 0.7       | tr     | 0.2      | tr       | 1.7       | tr          | tr          | 1.3        |
| n-29        | tr      | tr        | tr     | tr       | tr       | tr         | tr          | 1.3         | tr         |
| n-30        | tr      | tr        | tr     | tr       | tr       | tr         | tr          | 0.6         | tr         |
| n-31        | tr      | tr        | tr     | tr       | tr       | tr         | tr          | 0.2         | tr         |
| n-32        | tr      | tr        | tr     | tr       | tr       | tr         | tr          | 0.5         | tr         |
| n-33        | tr      | tr        | tr     | tr       | tr       | tr         | tr          | 0.5         | tr         |
| n-34        | tr      | tr        | tr     | tr       | tr       | tr         | tr          | 0.5         | tr         |
| n-35        | tr      | tr        | tr     | tr       | tr       | tr         | tr          | 1.2         | tr         |
| n-36        | tr      | 0.4       | tr     | tr       | tr       | tr         | tr          | 1.7         | tr         |
| Total br    | 59.1    | 31.3      | 2.3    | 47.1     | 16.5     | 1.8       | 1.8         | 3.6         | 28.5       |
| Total odd-C | 59.1    | 45.6      | 2.4    | 48.8     | 16.5     | 83.1      | 27.0        | 93.5        | 33.2       |
| C.P.I.⁴     | 1.0     | 1.12      | 1.0    | 1.0      | 1.0      | 1.12      | 1.0         | 1.0         | 1.25       |

* Calculated as percentage of total peak area obtained from an SE-30 tracing with programming from 100 to 280°C at 6°C/min.
* Normal (n) and branched (br) hydrocarbons—number of carbons.
* Less than 0.1% of total peak area was considered a trace.
* Carbon preference index.
lipids in all cases, indicating that other lipid classes are also affected by growth substrate. Jones (5) reported that hydrocarbons of fungi cultured on complex media comprise 0.04 to 0.7% of the total lipid. For C. resinae the percentage of hydrocarbons was considerably higher than that.

A typical GLC tracing is shown in Fig. 1. Qualitative and quantitative data are summarized in Table 2. Most fungi cultured on carbohydrates contain two predominant groups of hydrocarbons, 

$$C_{18-22}$$ and $$C_{27-31}$$ (9). C. resinae cultured on glucose contained two predominant hydrocarbons that comprised about 98% of the total. In glutamic acid-grown cells, four hydrocarbons comprised 74% of the total, and hydrocarbons longer than $$C_{28}$$ were not detected.

Cells cultured on hydrocarbons all contained $$n$$-alkanes with chain lengths from $$C_{14}$$ through $$C_{28}$$, except for $$n$$-hexadecane-grown cells which lacked $$n$$-20 and $$n$$-22. Short-chain hydrocarbons were absent from cells cultured on undecane through hexadecane.

Glucose-grown cells had a higher percentage of branched hydrocarbons than glutamate-grown cells. Moreover, glucose-grown cells had a higher percentage of odd-chain hydrocarbons than glutamate-grown cells. Cells cultured on odd-chain alkanes had between 48 and 93% odd-chain hydrocarbons, suggesting that cellular hydrocarbons may be synthesized from the odd-chain growth substrate. Stransky, Streibl, and Herout (7) suggested that the carbon preference index (ratio of the number of odd-C to even-C hydrocarbons) approaches unity for lower plants. Our results from cells cultured on a number of substrates (Table 2) support this conclusion. Cells grown on $$n$$-undecane showed little correlation between growth substrate and cellular hydrocarbon. In $$n$$-dodecane through $$n$$-hexadecane-grown cells the major cellular hydrocarbon was the one on which the cells were cultured. This suggests that cells cultured on $$n$$-dodecane through $$n$$-hexadecane may incorporate growth hydrocarbons directly, whereas those cultured on $$n$$-decane and $$n$$-undecane do not.

When a microorganism utilizes a hydrocarbon, the hydrocarbon or an early oxidative intermediate is transported into the cell. Despite the number of reports on hydrocarbon penetration and localization, the mechanism of hydrocarbon transport is unknown and the nature of cellular hydrocarbons of cells cultured on hydrocarbons has not been investigated. To our knowledge, this is the first report of the cellular hydrocarbons of cells cultured on hydrocarbons. Our results support the view that $$C_{12-18} n$$-alkanes are transported directly into the cell rather than being oxidized outside the cell or at the cell surface. R. S. Kennedy and W. R. Finnerty (Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 4, 1972) demonstrated accumulation of $$n$$-alkane growth substrate in the bacterium Micrococcus cerificans.

C. resinae uses constitutive enzyme systems to oxidize $$n$$-alkanes to the corresponding primary alcohol, aldehyde, and mononic acid (8; J. D. Walker and J. J. Cooney, Can. J. Microbiol., in press). There is little correlation between $$n$$-alkane growth substrate and cellular fatty acids, indicating that the fatty acids are metabolized via $$\beta$$-oxidation (4). The present work is consistent with the conclusion that the initial oxidation occurs inside the cell and that acids which accumulate in the medium during growth on $$n$$-alkanes (3) are not derived directly from the alkane. C. Siporin in our laboratory has recently established that the chain length of extracellular fatty acids does not correspond to the chain length of the $$n$$-alkane growth substrate. However, it is possible that some or all hydrocarbons are incorporated directly into cellular structures without being oxidized.

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