Fatty Acid Composition of *Cladosporium resinae* Grown on Glucose and on Hydrocarbons

J. J. COONEY AND C. M. PROBY

Department of Biology, University of Dayton, Dayton, Ohio 45409

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*Cladosporium resinae* was grown in submerged cultures on glucose; on Jet-A commercial aviation fuel; and on a series of n-alkanes, n-decane through n-tetradecane. Cell yield was greatest on glucose and least on Jet-A; n-alkanes were intermediate. Among n-alkanes cell yield decreased as chain length increased, except for n-dodecane, which supported less growth than n-tridecane or n-tetradecane. The total fatty acids of stationary-phase cells were analyzed by gas-liquid chromatography. In all cases the predominant fatty acids were 16:0, 18:1, and 18:2. The fatty acid composition of glucose-grown cells was similar to that of hydrocarbon-grown cells. Cells grown on n-tridecane or n-tetradecane yielded small amounts of acids homologous to the carbon source, but a similar correlation was not noted for n-decane, n-undecane, or n-dodecane. Cells grown on n-undecane or n-tridecane contained more odd-carbon fatty acids than cells grown on the other substrates, and the effect was more pronounced in n-tridecane-grown cells. Thus, the fatty acids of this organism are derived chiefly from de novo synthesis rather than from direct incorporation of oxidized hydrocarbons. The extent of direct incorporation increases as the chain length of the hydrocarbon growth substrate is increased.

When grown on hydrocarbons as sole carbon source, some microorganisms oxidize specific n-alkanes or alkenes to the homologous alkanolic acids and incorporate them directly into cellular lipids (8-10, 17, 19). In some cases direct incorporation is limited to hydrocarbons of specific chain lengths (10). Other microorganisms show little or no correlation between hydrocarbon substrate and cellular fatty acids (3, 12, 14, 28); presumably they metabolize the products of initial oxidation via β-oxidation to acetyl CoA and synthesize cellular fatty acids de novo.

Direct incorporation is indicated by an increase in intracellular fatty acid homologous to the hydrocarbon growth substrate. As cells contain few odd-carbon fatty acids, an increase in odd-carbon acids after growth on an odd-carbon hydrocarbon is also taken as evidence for direct incorporation and may indicate 2-carbon additions to or subtractions from the acid incorporated.

In contrast to the lipids of other microorganisms, relatively little is known of the lipids of filamentous fungi, and hydrocarbon-using fungi have received little attention. The organism used in this study was isolated from a contaminated hydrocarbon fuel system (11). It was identified for us as *Cladosporium resinae* by D. G. Parbery, University of Melbourne, Parkville, Victoria, Australia. *C. resinae*, the "kerosene-fungus," is widely distributed as a component of the soil microflora (26). Parbery (25) observed and described the perfect state of the organism and classified it as *Amorphotheca resinae* (gen. nov., sp. nov.).

**MATERIALS AND METHODS**

**Organism.** The strain of *C. resinae* is coded UD-42 in our collection. It was maintained in Sabouraud dextrose agar. Starter cultures were prepared in 250-ml flasks with 100 ml of the appropriate medium per fermentor. Flask cultures were incubated at 25 C on a reciprocal shaker.

**Media.** All media were based on the salts solution of Bushnell and Haas (6) which contains ammonium nitrate as nitrogen source. When glucose was the carbon source, 10 liters of salts solution in a 14-liter fermentor jar was supplemented with 2% glucose and 0.01% (w/v) yeast extract. The pH was adjusted to 5.7, the jar was sealed with provisions made for venting, and it was autoclaved for 90 min at 15 psi. For hydrocarbon media, a 10-liter salts solution was prepared and sterilized in the same fashion. After the solution cooled, 100 ml of a hydrocarbon sterilized by membrane filtration was added: n-decane, n-undecane, n-dodecane, n-tridecane, n-tetradecane, or Jet-A commercial jet fuel. Such kerosene-type fuels contain over 5,000 different hydrocarbons of which paraffins and cycloparaffins comprise 75-90%. Additions of hydrocarbons did not alter the pH of the aqueous phase.
Culture conditions. The microferm fermentor (New Brunswick Scientific Co., New Brunswick, N. J.) was maintained at 25 C; agitation and aeration were at 200 rev/min and 4,000 ml/min, respectively. Hydrocarbon cultures were supplemented with additional substrate periodically to insure that growth was not limited by exhaustion of the carbon source.

Immediately after inoculation and at intervals thereafter, 50- to 100-ml samples were removed from the culture. Each sample was tested for contamination by examining an air-dried smear stained with safranine. The pH of the sample was determined. A known volume of sample was centrifuged at 27,000 x g and the cells were washed three times with water. Washed cells were transferred to a tared planchet, dried to constant weight at 110 C, and weighed to determine cell dry weight. Difficulties were encountered in sedimenting cells grown on hydrocarbons. A surface scum was always present in the supernatant fluid after centrifugation, and a firm pellet was not obtained. Therefore, an alternate technique was employed using preweighed Whatman no. 50 filter paper. A known volume of culture was filtered, the cells were washed on the filter, and the paper was dried to constant weight at 110 C and subsequently weighed.

When the dry weight, the pH, or both remained unchanged for two successive readings, cultures were judged to be in the stationary phase. Cells were harvested at 27,000 x g at 5 C. Cells that adhered to the fermentor parts below the level of the medium were collected by scraping and added to cells from centrifugation. Cells were then washed three times and dried in a model FDC Thermovac lyophlizzer.

Preparation of fatty methyl esters. Duplicate 400-mg samples of dried cells were weighed, and 1 mg of lauric acid was added to one sample as an internal standard. Cellular fatty acids were extracted and converted to their corresponding methyl esters by direct transesterification with BF₃ in methanol (22) as modified by Dunlap and Perry (9). Methyl esters were then taken up in 1.0 to 2.0 ml of hexane for gas-liquid chromatography (GLC). Prior to analysis, samples were stored at -20 C under nitrogen. With lauric acid as standard, yields of 94 to 119% were obtained as methyl laurate.

Gas-liquid chromatography. All chromatograms were obtained on an F & M model 700 laboratory chromatograph equipped with a dual flame ionization detector, and an F & M model 240 temperature programmer (F & M Scientific Corp., Avondale, Pa.). Two types of columns were used: a glass column (8 ft by 0.25 in.) packed with 10% EGSS-X on 100 to 120 mesh on Gas Chrom P (Supelco, Inc., Bellefonte, Pa.) and a prepacked stainless steel column (6 ft by 0.165 in.) containing 10% SE-30 on 80 to 100 mesh Gas Chrom S (Applied Science Laboratory, State College, Pa.). The carrier gas was helium at a flow rate of 50 ml/min for EGSS-X and 27 ml/min for SE-30. Separation on EGSS-X was isothermal at each of several temperatures from 150 to 200 C. Temperature was programmed from 150 to 250 C at 5 C/min for separation on SE-30. Temperatures of the injection port and the detector were 250 C.

Methyl esters were identified by comparison of retention times with retention times of methyl esters in standard mixtures run on each of the two types of column. Esters in each sample were quantitated by calculating the area under individual peaks as percentage of total peak area. Column efficiency and detector response of both types of column were determined with National Institutes of Health reference mixture D (Applied Science Laboratories, State College, Pa.), which gave a relative error of less than 7% for major components and less than 6% for minor components.

Unsaturated methyl esters were confirmed by catalytic hydrogenation with 5% platinum on charcoal as catalyst (5). Disappearance of suspected unsaturated methyl esters from chromatograms and enhancement of peaks for their saturated homologues was taken as further evidence of unsaturation.

Chemicals. All chemicals were reagent or pure grade and were obtained from the following sources: 14% (w/v) boron trifluoride-methanol (Applied Science Laboratories, State College, Pa.); n-hexane, petroleum ether, 5% platinum on charcoal, and methanol (Matheson, Coleman and Bell, Cincinnati, O.); n-decane, n-undecane, n-dodecane, n-tridecane, and n-tetradecane (Phillips Petroleum Co., Bartlesville, Okla.); chloroform (Malinckrodt Chemical Works, St. Louis, Mo.); and Jet-A commercial jet fuel, courtesy of Trans World Airlines, Dayton, Ohio.

RESULTS

Among the substrates used, glucose supported the most rapid growth and yielded the largest mass of cells (Table 1). The pH fell in hydrocarbon cultures, indicating that extracellular organic acids were produced. Because cells tended to adhere to the fermentor, pH proved to be a more reliable measure of growth than cell dry weight. C. resinae grew more rapidly and more extensively on the shorter chain n-alkanes than on the longer chain n-alkanes. n-Dodecane was an exception since less total cell mass was obtained than on n-tridecane or n-tetradecane, and stationary phase was reached in the shortest time of all hydrocarbon cultures. On Jet-A, the organism had an intermediate growth rate but the poorest cell yield. Examination of dried smears indicated that conidial and mycelial forms were present in all cultures; no attempt was made to separate them.

A typical GLC tracing is presented in Fig. 1. Fatty acids longer than 20 carbons or shorter than 11 carbons were not detected. A number (5 to 10) of spurious peaks was detected which eluted after the polyunsaturated acid 18:3. Such peaks were broad and most were asymmetrical even with temperature programming. None of them corresponded to any fatty acid methyl ester standard with a chain length as long as 24 carbons, saturated or unsaturated. Purification of solutions of methyl esters prior to GLC by thin-layer chromatography eliminated these peaks. Fulk and Shorb (15) reported production of a
TABLE 1. Growth of Cladosporium resinae on glucose and on hydrocarbons

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Days to reach stationary phase</th>
<th>pH at harvest</th>
<th>Total yield of dry cells (g/liter)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5</td>
<td>5.40</td>
<td></td>
</tr>
<tr>
<td>n-Decane</td>
<td>10</td>
<td>3.5</td>
<td>0.92</td>
</tr>
<tr>
<td>n-Undecane</td>
<td>10</td>
<td>3.7</td>
<td>0.47</td>
</tr>
<tr>
<td>n-Dodecane</td>
<td>6</td>
<td>5.1</td>
<td>0.14</td>
</tr>
<tr>
<td>n-Tridecane</td>
<td>39</td>
<td>3.6</td>
<td>0.26</td>
</tr>
<tr>
<td>n-Tetradecane</td>
<td>35</td>
<td>3.7</td>
<td>0.29</td>
</tr>
<tr>
<td>Jet-A</td>
<td>12</td>
<td>4.2</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Corrected for water evaporation during incubation.

saturated artifact from oleic acid (18:1) by boron trifluoride-methanol. The artifact eluted from an EGS-S-X column with a retention time similar to that of peak "A" (Fig. 1).

Table 2 summarizes qualitative and quantitative data obtained from GLC analyses of the total cellular fatty acids of C. resinae. In all cases the predominant fatty acids were 16:0, 18:1, and 18:2, which represented 84 to 92% of the total. In glucose-grown cells odd-carbon fatty acids comprised 2.2% of the total, and 77.8% of the fatty acids present were unsaturated.

In n-decane-grown cells odd-carbon acids represented 1.8%; unsaturated acids comprised 75.9%. No 10:0 was detected, but a trace of 20:0 was present. In cells cultured on n-undecane odd-carbon fatty acids comprised 3.2% of the total, and unsaturates comprised 62.9%. A trace of 11:0 was detected. Fatty acids from n-dodecane-grown cells included 1.9% odd-carbon acids. Unsaturated acids accounted for 69.5% of the total, and a trace of 12:0 was detected. n-Tridecane-grown cells yielded the highest content of odd-carbon fatty acids (8.4%) and a small amount (1.2%) of 13:0 was detected. Unsaturated acids represented 68.1%. Only trace amounts of odd-chain fatty acids were detected in cells grown on n-tetradecane, and, as with n-decane and n-dodecane-grown cells, odd-chain acids represented less than 2% of the total. Unsaturated acids comprised 74.5%, and 14:0 represented 6.45% of the total. A peak corresponding to n-tetradecane was observed on chromatograms. In Jet-A-grown C. resinae the odd-carbon fatty acids represented 2.7% of the total, unsaturated acids accounted for 76.3%, and no 18:3 was detected.

DISCUSSION

Cell yields of 10 g (dry weight) per liter are not uncommon for fungi grown on carbohydrates. Thus, the yield of stationary-phase cells obtained on glucose is low, and growth was significantly less with hydrocarbons as sole sources of organic carbon. Bekhtereva et al. (2) noted decreased cell yield when n-hexadecane or kerosene was added to glucose cultures of Blakesleea trispora. However, in the present work low yields appear to be related to use of deep culture techniques because the organism develops a luxurious mycelial mat in quiescent culture on any of three kerosene-type jet fuels (7), including Jet-A, which supported the least growth in the present work. Ratledge (29) obtained better growth of a Candida species on hydrocarbons in shake flasks than in a fermentor unless an apparent excess of hydrocarbon was present in the fermentor and unless inoculum, agitation rate, and ionic strength were optimum.

Filamentous fungi in general do not grow rapidly on single n-alkanes (28). Dependence of rate and amount of growth on the chain length of n-alkane substrate is not unusual. Iizuka et al. (16) examined two fuel isolates identified as a Cladosporium species and Hormodendrum hordei, respectively, for ability to use n-alkanes from C-8 through C-18. Each isolate grew well on the series C-9 through C-18; stationary cultures yielded 3 to 6 g of dried cells per liter. Rate and
amount of growth were decreased at the ends of the series. *H. hordei* achieved more growth on the 10-, 11-, 14-, and 15-carbon *n*-alkanes than on the intermediate length, 12- or 13-carbon compounds. In the present work rate and amount of growth decreased as the *n*-alkane chain length increased. *n*-Dodecane was an exception. It supported less growth than shorter chain compounds, and cultures reached a stationary phase sooner. A similar finding was reported by Markovetz et al. (21), who observed that a *Cladosporium* species grew on *n*-decane and *n*-tetradecane but not on *n*-dodecane. Some strains of *Fusarium*, *Aspergillus*, and *Penicillium* which will not grow on one *n*-alkane grow profusely on the *n*-alkane which has one more carbon (23). It may be significant that lauric acid, the expected product of monomterial oxidation of *n*-dodecane, inhibited growth of *Myco- torula japonica* on *n*-hexadecane. The growth of hydrocarbon-using bacteria is also related to substrate chain length. For example, *Micrococcus cerificans* will not grow on alkenes of less than 10 carbons, and growth increases with chain length through 18 carbons (19).

The fatty acid composition of *C. resinae* is similar to that of other fungi, which contain 16:0, 18:1, and 18:2 as their principal fatty acids (4, 13, 18, 24, 27, 30). Branched, cyclopropane, or hydroxy fatty acids, which are often found in bacteria, were not detected. We are not aware of any previous attempt to correlate fatty acid composition of hydrocarbon-using filamentous fungi with the hydrocarbon substrate.

The extent of direct incorporation into cellular lipids varies with the organism and with the hydrocarbon being utilized. The fatty acid composition of cells grown on *n*-decane, *n*-undecane, or *n*-dodecane gave little indication that the fatty acids were synthesized other than via de novo synthesis. *n*-Tridecane- and *n*-tetradecane-grown cells contained fatty acids homologous to the growth substrate. In addition to the presence of tridecanoic acid in *n*-tridecane-grown cells, there was an increase in fatty acids with odd-numbered carbon skeletons, from less than 2% in cells grown on other hydrocarbons to 8.4% in cells grown on *n*-tridecane. Thus, the fatty acids of *C. resinae* are derived chiefly from de novo synthesis. There is only limited direct incorporation into lipids, but the extent of incorporation increases with the length of the *n*-alkane substrate.

<table>
<thead>
<tr>
<th>Fatty acid methyl ester</th>
<th>Carbon source</th>
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<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>10:0*</td>
<td>tr</td>
</tr>
<tr>
<td>11:0</td>
<td>tr</td>
</tr>
<tr>
<td>12:0</td>
<td>tr</td>
</tr>
<tr>
<td>13:0</td>
<td>tr</td>
</tr>
<tr>
<td>14:0</td>
<td>tr</td>
</tr>
<tr>
<td>14:1</td>
<td></td>
</tr>
<tr>
<td>15:0</td>
<td>0.66</td>
</tr>
<tr>
<td>15:1</td>
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</tr>
<tr>
<td>16:0</td>
<td>18.46</td>
</tr>
<tr>
<td>16:1</td>
<td>0.93</td>
</tr>
<tr>
<td>17:0</td>
<td>0.62</td>
</tr>
<tr>
<td>17:1</td>
<td>0.94</td>
</tr>
<tr>
<td>18:0</td>
<td>1.67</td>
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<tr>
<td>18:1</td>
<td>37.20</td>
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<tr>
<td>18:2</td>
<td>33.15</td>
</tr>
<tr>
<td>18:3</td>
<td>5.60</td>
</tr>
<tr>
<td>20:0</td>
<td>tr</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated as percentage of total peak area obtained from an EGGSS-X tracing.

<sup>b</sup> Carbon number : number of double bonds.

<sup>c</sup> Less than 0.5% of total peak area was considered trace (tr).

<sup>d</sup> Determined from a single culture.

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**LITERATURE CITED**


FATTY ACIDS OF C. RESINAE