Production of cetyl palmitate from \textit{n}-hexadecane by \textit{mycobacterium}, strain 3'.

By N.A. Krasil'nikov and T.V. Koronelli

Original title: Образование \textit{tsetilpal'mitata} iz \textit{n}-\textit{geksadekana} kul'tuvoi \textit{Myobacterium} shtamm 3'.


Translated by the Translation Bureau (JD)
Foreign Languages Division
Department of the Secretary of State of Canada

Fisheries Research Board of Canada
Halifax Laboratory
Halifax, N.S.

1970

11 pages typescript
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Obrazovanie tsetil'pal'mitata iz n-geksadekana kul'turoi Mycobacterium shtamm 31.

Mikrobiologiya

Microbiology

Publisher - Éditeur

DATE OF PUBLICATION

DATE DE PUBLICATION

PLACE OF PUBLICATION

LIEU DE PUBLICATION

USSR

USSR

1969

XXVIII

5

Microbiology

757-760

11

DATE OF REQUEST

DATE DE LA DEMANDE

16.4.70

TRANSLATOR (INITIALS)

TRADUCTEUR (INITIALES)

J.D.

DATE COMPLETED

ACHEVÉ LE

JUL 1.0 1976

CAS 1485
Mikrobiologiya /Microbiology/, Vol. XXXVIII (1969)  
No.: 5, pages 757-760.  
UDK 576.852.2.098:547.915.5

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PRODUCTION OF CETYL PALMITATE FROM n-HEXADECANE BY THE CULTURE MYCOBACTERIUM STRAIN 3'.

The lipids of the culture Mycobacterium strain 3' accumulated during growth on the medium with n-hexadecane, were studied. It was established that the lipids were derivatives of palmitic acid and consisted of 45% wax, which was pure cetyl palmitate. Part of the wax was excreted into the culture medium. The methods of GLC and mass spectrometry were employed for identification of cetyl palmitate.
In a series of publications (Nette et al. 1965, Norenkova 1966, Lukins and Foster 1963, Traxler 1966), it was shown that many representatives of the genus *Mycobacterium* are capable of oxidizing various hydrocarbons and using them for construction of a cellular mass. The study of the lipids of such cultures is of great interest, as the process of assimilation of hydrocarbons by various microorganisms follows different pathways and can change the composition of cellular fats. However, the lipids of the paraffin oxidizing mycobacteria have not yet been satisfactorily investigated. In the work reported here, lipids of the culture *Mycobacterium strain* 3', isolated from soil enriched with petroleum products, were studied.

**Experimental Method**

The cultures of *Mycobacterium strain* 3' were grown for 5 days in the liquid medium prepared according to Chapek, with \((NH_4)_2SO_4\) containing 1% by volume of n-hexadecane as the source of carbon. Conditions of cultivation and treatment of the liquid culture medium were described in a previous communication (Koronelli, 1968). The total lipids were extracted from the crushed
cellular mass with a chloroform-methanol mixture (3:1). The extract was washed twice with water, the solvents distilled off in vacuum and the residue weighed. Gas-liquid Chromatography (GLC) using a Chrom-2 chromatograph was used for the identification of the fatty acids. The stationary phase was polyethylene glycol adipate, the column length 1.7 m, the carrier gas - N₂ at 50 ml./min, and the temperature 180°.

Results and Discussion.

The authors described, earlier, a series of cultures of paraffin-oxidizing Mycobacteria which accumulated significant quantities of the biomass and lipids during growth on the medium with n-hexadecane. One of these cultures, Mycobacterium strain 3', was worthy of special attention as it did not grow on the liquid medium prepared with glucose according to Tschapek. During the time since the first communication, the quantity of biomass accumulated by this culture with the use of n-hexadecane increased from 1.1 to 3.1 g/l., while the lipid content remained nearly unchanged and accounted for 14% of the weight of the dry cells.
After subjecting all the lipids to methanolysis (Vaver et al. 1965) and subsequent GLC analysis, we detected on the chromatogram only one peak, which corresponded to the methyl ester of palmitic acid (Figure). Hence it follows that the palmitic acid having the same number of carbon atoms as the original hydrocarbon, is the fundamental fatty acid constituting the lipids of *Mycobacterium strain 3'*.

In this respect the studied culture is similar to Nocardia, the lipids of which also contained primarily palmitic acid formed as a result of the oxidation of the end methyl group of n-hexadecane (Davis, 1964). *Mycobacterium strain 3'* differs from the yeast *Candida sp.*, grown in the same carbon substrate, in that it does not form unsaturated acids (Dyatlovitskaya et al., 1965).

Using preparative thin-layer chromatography on silica gel in the system hexane-ether-glacial acetic acid (89:10:1), we isolated and determined the quantities of the individual fractions in the total mass of the lipids. The lipids were first dissolved in a chloroform-methanol mixture (3:1) and then extracted with diluted ammonia in order to obtain the free fatty acids. The ammoniacal extract was acidified with 6 n-hydrochloric acid and washed twice.
with ether in a separating funnel. The ether extract was dried over calcined sodium sulfate, the ether distilled off in vacuum and the residue weighed. The solution of lipids remaining after removal of the fatty acids, was fractionated using thin-layer chromatography.

Gas-liquid chromatogram of the methyl esters of fatty acids of a culture of *Mycobacterium strain 3".*

1 - solvent
2 - methyl ester of palmitic acid
All of the isolated fractions were solid substances at ambient temperature. From the table it can be seen that the wax is the main component of the cellular lipids of the studied culture. White shining crystals, melting point 49°C, were obtained after recrystallizing the isolated wax from glacial acetic acid. Using thin layer figure chromatography (Bergelson et al., 1963) on silica gel in carbon tetrachloride and in a mixture of carbon tetrachloride and chloroform (9:1) we proved conclusively that the isolated crystalline product is a single substance. Because palmitic acid is the fundamental fatty acid of the lipids of Mycobacterium strain 3', it was apparent that the studied wax was an ester of palmitic acid with a melting point close to that of cetyl palmitate, which melts, according to the literature data, at 51-52°C (Whitby, 1926).

In fact, as a result of methanolysis of the wax, the methyl ester (identified with a known sample by the GLC method) of palmitic acid was isolated with a recovery of 86% of the theoretical. Mass spectrometry was used for further identification of the wax. 

1 The mass spectrum was recorded in the laboratory of mass spectrometry, Institute of the Chemistry of Natural Compounds, Academy of Sciences of the USSR.
The strongest peaks on the mass spectrum were those of the molecular ion with an m/e\(^*\) of 480, corresponding to the calculated molecular weight of cetyl palmitate, and peaks with m/e\(^*\) of 257 and 224 corresponding to the fragments C\(_{16}\)H\(_{33}\)O\(_2^+\) and C\(_{16}\)H\(_{32}^+\). The spectrum was typical of high molecular weight esters (Ryhage and Stenhagen, 1963) and fully coincided with that of cetyl palmitate as described in the literature (Stewart et al., 1959).

Thus, a significant portion of the cellular lipids of the culture of Mycobacterium strain 3' growing on n-hexadecane, is represented by cetyl palmitate – a compound in which both the alcoholic part (cetyl alcohol) and the acid (palmitic acid) each contain 16 carbon atoms and reflect the structure of the original hydrocarbon.

### Composition of the lipids of Mycobacterium strain 3'.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Quantity (as % of the sum of the lipids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polar lipids</td>
<td>27.7</td>
</tr>
<tr>
<td>(R(_f) ≤ 0.1)</td>
<td></td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>2.6</td>
</tr>
<tr>
<td>Glycerides (R(_f) ≈ 0.5)</td>
<td>24.2</td>
</tr>
<tr>
<td>Wax (R(_f) ≈ 0.8)</td>
<td>45.5</td>
</tr>
</tbody>
</table>

*Revisor's note.* The abbreviation "m/e" is given as it appeared in the original text.
The extracellular lipids of this culture were studied as well.

The following method was used in this study:
2 litres of centrifugated culture liquid were evaporated under vacuum to 0.5 lit. and extracted twice with ether. The ether extract was dried over calcined sodium sulfate, filtered, and the ether distilled in a vacuum. The residue was dissolved in a small amount of chloroform and chromatographed preparatively on a silica gel coated plate in a 9:1 hexane-ether system, the borders of the plate being shown with an alcoholic solution of phosphomolybdic acid.

It was found that practically the only component of the extracellular lipids was the wax (Rf = 0.8) which after being recrystallized from glacial acetic acid melted at 49°. There was no observed decrease in the melting point, when the mixture of crystals obtained was melted together with cetyl palmitate separated from the cells, which testifies as to the identity of both substances. Two litres of centrifugate yielded 90 mg of the cetyl ester of palmitic acid.
Intensive production of wax was earlier observed by Raymond and Davis (1960) during the oxidation of n-hexadecane by the culture Nocardia. Glycerides accounted for 60%, and wax - pure cetyl palmitate - for 40% of the lipids isolated from the cells by these authors.

However, there is no data dealing with intracellular accumulation of cetyl palmitate by other microorganisms grown on n-hexadecane. Thus, triglycerides are the main components of the lipids of the yeast of the genus Candida, and wax of unknown composition is present only in small quantities (Greshnykh et al., 1968). In the cells of Pseudomonas aeruginosa, wax was not present at all (Romero and Brenner, 1966). It is necessary to note that a culture of gram-negative micrococci has been described that also produces significant amounts of cetyl palmitate during growth on n-hexadecane, but in the opinion of the authors excretes it fully into the surrounding medium (Stewart et al., 1959). However, the authors did not isolate the cellular lipids, and their conclusion as to their absence was based only on microscopic observation of the cells.
Raymond and Davis (1960) assumed that the accumulating wax serves as a reserve cellular substance. We believe that its role is not limited to this. The molecules of the n-hexadecane substrate being oxidized, which is insoluble in an aqueous culture medium, are more easily dissolved and maintained on the surface of cells the membranes of which are saturated with wax. In this connection, it should be noted that the cells of Nocardia, according to the observations of Raymond and Davis (1960) did not produce wax during growth on the glucose-containing medium. The culture that we investigated - Mycobacterium strain 3' - essentially does not grow in a liquid medium containing glucose.

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Received for publication on 4 June 1968.

Literature


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PRODUCTION OF CETYL PALMITATE FROM N-HEXADECANE
BY THE MYCOBACTERIUM, STRAIN 3'

N. A. Krassilnikov and T. V. Koronelli

Lipids accumulated by the Mycobacterium, strain 3', during its growth on the medium with n-hexadecane, were studied. These lipids were found to be derivatives of palmitic acid and to consist by 45% of wax (pure cetyl palmitate). Some wax was released into the growth medium. Cetyl palmitate was identified by the methods of gas-liquid chromatography and mass spectrometry.