Identification of n-Decane Oxidation Products in Corynebacterium Cultures by Combined Gas Chromatography-Mass Spectrometry

PAOLO BACCHIN, ANDREA ROBERTIELLO, AND AURELIO VIGLIA

SNAM Progetti, LPM Monterotondo, Rome, Italy

Received for publication 13 May 1974

The gas chromatography-mass spectrometry technique was employed to characterize n-decane oxidation products of Corynebacterium strains 7E1C and 269 (SNAM Progetti collection) after 73 h of incubation at 35 C. Corynebacterium 7E1C accumulated consistent amounts of esters of long chain acids with long chain alcohols, mainly decyldecanoate as well as products with mono- and diterminal carboxylic functions. Corynebacterium 269 yielded 1-decanol and 1-10 decanediol as principal oxidation products.

The investigation of microbial hydrocarbon oxidation has expanded in the last decade, especially concerning either the study of the mechanism of hydrocarbon oxidation (2, 3, 7, 12) or the finding of products of industrial interest (9, 11).

Gas chromatography-mass spectrometry appeared to be a very helpful tool in characterizing oxidation products from a fermentation broth and could be employed as a screening technique in investigating microorganisms able to utilize hydrocarbons as sole carbon source.

In this paper we report the results of application of the gas chromatography-mass spectrometry technique to the study of the oxidation products of n-decane by Corynebacterium.

MATERIALS AND METHODS

Organism. The microorganisms studied were Corynebacterium sp. strain 269 (SNAM Progetti collection), isolated from soil in M9 salt medium (6) with n-decane instead of glucose as sole carbon source and Corynebacterium sp. strain 7E1C, kindly provided by A. S. Kester (Department of Biological Sciences, North Texas State University, Denton, Tex.).

Culturing conditions. Medium L of Leadbetter and Foster (8) was used throughout the experiment, and n-decane (Fluka practicum, 95% by gas chromatography) was the sole substrate. The pH of the medium was adjusted at 7 to 7.4. The fermentation experiments were carried out in cotton-plugged 500-ml Erlenmeyer flasks containing 100 ml of medium L. After sterilization for 20 min at 121 C, 4 ml of sterile n-decane was added to the cool medium. After inoculation with a 2-day-old culture of the microorganisms in the same medium, the flasks were incubated for 73 h at 35 C on a rotary shaker at 220 rpm.

An experiment was done by adding, at the beginning of the fermentation, sterile CH3OH (0.5% with respect to n-decane) in order to investigate the influence of a short chain alcohol on the esterase system. The broth was acidified to pH 1.0 H2SO4 and then shaken in a separatory funnel with diethyl ether, previously washed twice with 2 N NaOH. The ether extraction was repeated twice, and the combined ether phases were washed with 15 ml of distilled water.

Analytical methods. The combined ether extract, containing residual n-decane and oxidation products, was brought to volume. Part of this solution was used to determine the total acid formed by titration with 0.01 N NaOH. The remaining part was used to identify the oxidation products.

The aliphatic acids were transformed into their methyl esters using diazomethane (4). For quantitative determination of oxidation products a Hewlett Packard model 7620 gas chromatograph, equipped with a flame ionization detector and automatic integrator, was used. For identification purposes a Varian Mat 111 gas chromatograph-mass spectrometer was used. The operating conditions for the separation of oxidation products were: a stainless-steel column (1.83 m long, 2.2 mm inner diameter) packed with 10% UC-98 W (methyl vinyl silicone); the column temperature was programmed at 80 C for 2 min; from 80 to 110 C at 6 C/min; held isothermally for 2 min; from 110 to 210 C at 8 C/min; from 210 to 250 C at 30 C/min; and held isothermally for 30 min. Injector temperature was 410 C and helium (25 ml/min) was used as a carrier gas. The mass spectra were taken using 70 eV ionizing energy.

RESULTS AND DISCUSSION

The cell yields, amounts of residual n-decane, total oxidation products, and the pH of the medium at the end of the experiment with Corynebacterium strains 7E1C and 269 are reported in Table 1.
Figures 1 and 2 show gas chromatographic patterns of oxidation products from *Corynebacterium* strains 7E1C and 269, respectively. Identities of chromatographic peaks, analyzed by mass spectrometry, are reported in the figure legends.

Mass spectra of the compounds corresponding to peaks 7, 8, 10, and 11 in Fig. 1 are shown in Fig. 3. They show fragmentation patterns of esters of long chain acids with long chain alcohols (1, 10) and all present molecular ions. The m/e of the characteristic fragment ions for ester of monocarboxylic acids are reported in Table 2. A fragment of the general formula [RC(OH) = OH]⁺ was formed after an alkyl oxygen cleavage and the rearrangement of two hydrogen atoms. The [RCO]⁺ and [CO₂R']⁺ ions were obtained from the cleavage of the

**Table 1. Cell yields, total oxidation products, and final pH (73 h) in *Corynebacterium* cultures on n-decane**

| Strain | Dried cells (mg/ml) | Residual n-decane (%) | Oxidation products (%) | pH |
|--------|---------------------|-----------------------|-----------------------|----|
| 7E1C   | 1.41                | 49.15                 | 3.51                  | 6.5|
| 269    | 2.19                | 62.15                 | 4.94                  | 6.3|

*Weight percentage was based on the amount of n-decane supplied. Data was determined by gas chromatography.*

The mass spectrum of the chromatographic peak 12 (Fig. 1) corresponding to the didecyl decane 1–10 dioate is shown in Fig. 4. The molecular ion is at m/e 482. The base peak at m/e 185 [C₁₈H₃₂OCO(CH₂)₈CO]⁺ was formed through α-cleavage of the acyl group. The next interesting peak at high masses is at m/e 325 [C₁₈H₃₀OCO(CH₂)₈CO]⁺ and was produced by eliminating one alkyloxy group from the molecular ion. The fragment ion at m/e 343 was formed by cleavage of one alkyl-oxygen bond and the rearrangement of two hydrogen atoms. The loss of C₁₆H₃₀OCOCH₃— by 2, 3 cleavage originated the peak at m/e 283. Identification of peaks 7 and 8 (Fig. 2) was made possible by comparing their mass spectra to the mass spectra of 1–9 nonanediol and 1–10 decanediol standards, respectively. They show a very low (molecular ion[M] + 1)⁺ peak. The first significant peak, m/e 126 for 1–10 decanediol and m/e 112 for 1–9 nonanediol, is probably due to M — (H₂O + CH₂O). The base peak in both cases is at m/e 55, corresponding to a C₄H₇⁺ fragment.

Monoterminal and diterminal oxidation of n-decane by *Corynebacterium* 7E1C is in agreement with the findings of Kester and Foster (7), except for the absence of 10 hydroxydecanoic bonds adjacent to the carbonyl group. We indicate with R and R' the alkyl moieties of the acyl groups and the alcohol, respectively.
FIG. 2. Gas chromatographic separation of oxidation products of Corynebacterium 269. The following products were identified: peak 1, n-octane; 2, n-nonane; 3, n-decane; 4, methyl octanoate; 5, 1-decanol; 6, methyl decanoate; 7, 1-9 nonanediol; 8, 1-10 decanediol; 9, methyl stearate; 10, decyl decanoate. Products from the first three peaks were initially present in n-decane.

FIG. 3. Mass spectra of compounds 7, 8, 10, and 11 from Corynebacterium 7E1C obtained with a Varian Mat 111 gas chromatograph-mass spectrometer as each of the component emerged from the column. Ionizing energy was 70 eV, and scanning speed was 100 m/s.
acids are formed by biological esterification between n-C₁₀ mono-acid and n-C₁₀ diacid, respectively, and n-decanol, which originated from the biological oxidation of n-decane.

Using the above chromatographic conditions it was not possible to separate the octyldedecanoate-decyltadecanoate and the nonyldedecanoate-decynonanoate mixtures.

Octyldedecanoate can be explained as a condensation product of n-octanol, derived from the oxidation of n-octane present in the n-decane and n-C₁₀ acid produced from n-decane. In decyltadecanoate the acid moiety may arise either from n-octane or from deconoic acid by β-oxidation and the alcohol moiety from oxidation of n-decane. As regards the nonyldecanoate decynonanoate mixture, the n-C₁₅ acid and n-C₁₅ alcohol moieties originated from oxidation of n-decane; the n-C₉ alcohol and n-C₉ acid moieties arose from n-nonane present in the n-decane, the α-oxidation of deconoic acid to nonanoic acid being very unlikely (5). The presence of decylhexadecanoate and decyltadecanoate in strain 7E1C, and the presence of stearic acid in strain 269 cultures, made us think that n-C₁₅ and n-C₁₅ acid moieties were derived from cellular components. Oxidative systems from n-decane to alcohols and from alcohols to acids do not appear to be well balanced in the case of Corynebacterium 269, which accumulated alcohols in high amounts. Among these alcohols it is interesting to note the formation of products with di-terminal alcoholic functions. 1-9 Nonane diol seems to be derived from n-nonane, which is present as an impurity in the n-decane.

LITERATURE CITED
1. Assen, A. J., H. H. Hofstetter, B. T. R. Iyengar, and R. T. Holman. 1971. Identification and analysis of wax esters by mass spectrometry. Lipids 6:502-507.
2. Cardini, G., and P. Jurtshuk. 1968. Cytochrome P-450 involvement in the oxidation of n-octane by cell-free extracts of Corynebacterium sp. strain 7E1C. J. Biol.
VOL. 28, 1974

N-DECANE OXIDATION PRODUCTS IN CORYNEBACTERIUM

3. Gholson, R. K., J. N. Baptist, and M. J. Coon. 1963. Hydrocarbon oxidation by a bacterial enzyme system. Biochem. J. 243:6070-6072.

4. James, A. T., and J. Webb. 1957. The determination of the structure of unsaturated fatty acids on a microscale with the gas-liquid chromatogram. Biochem. J. 66:515-520.

5. Kallio, R. E. 1969. Microbial transformation of alkanes, p. 636. In D. Perlman (ed.), Fermentation advances. Academic Press Inc. New York.

6. Kelner, A., and L. J. Lewis. 1959. Ultraviolet-induced abnormal growth in Escherichia coli: the influence of yeast extract and of photoreactivation. J. Bacteriol. 77:281-295.

7. Kester, A. S., and J. W. Foster. 1963. Diterminal oxidation of long-chain alkanes by bacteria. J. Bacteriol. 85:859-869.

8. Leadbetter, E. R., and J. W. Foster. 1958. Studies on some methane-utilizing bacteria. Arch. Mikrobiol. 30:91-118.

9. Ratledge, C. 1970. Microbial conversion of n-alkanes to fatty acids: a new attempt to obtain economical microbial fats and fatty acids. Chem. Ind. (London) No. 25: 843-854.

10. Ryage, R., and E. Stenhagen. 1959. Mass spectrometric studies. 11. Saturated normal long-chain esters of ethanol and higher alcohols. Ark. Kemi 14:483-495.

11. Shiio, I., and R. Uchio. 1971. Microbial production of long-chain dicarboxylic acids from n-alkanes. Agr. Biol. Chem. 35:2033-2042.

12. Stewart, J. E., R. E. Kallio, D. P. Stevenson, A. C. Jones, and D. O. Schisler. 1959. Bacterial hydrocarbon oxidation. I. Oxidation of n-hexadecane by a gram-negative coccus. J. Bacteriol. 78:441-448.