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Identification of Ecologically Significant *Rhodococcus* Species Based on Free Fatty Acid Composition

The fatty acid composition depends to a greater extent on the age of a bacterial culture and cultivation conditions, i.e. medium composition, pH, and temperature. However, if to comply strictly with the general requirements to culture growth, highly standardized conditions for microorganism cultivation, and standardized test material (stationary growth phase cells should be used to analyze fatty acids), the qualitative composition of fatty acids is rather constant. Implementation of these rules depends entirely on the researcher and should be under constant control. The fatty acid composition in individual bacterial species could be so characteristic that when identified, it casts no doubt on species affiliation of cultures. However, members of different species of a singular genus or even different genera have quite often similar fatty acid profiles. In case of polyphasic taxonomy, a fatty acid analysis as a rapid and relatively cheap method is often useful to classify a large number of strains and to obtain significant information on characterization and identification of bacterial cultures.

According to the data obtained in the laboratory of alkanotrophic microorganisms, Institute of Ecology and Genetic of Microorganisms, Ural Branch of the Russian Academy of Sciences (*Ivshina et al.*, *Microbiology* (*in Russ*). *1994*. *64:118−128*), *Rhodococcus* actinobacteria, irrespective of their species affiliations, are characterized by elevated contents of palmitic ($C_{16:0}$), palmitoleic ($C_{16:1}$) and oleic ($C_{18:1}$) acids, as well as by permanently present tuberculostearic ($10MeC_{18:0}$) acid. The predominant components of rhodococcal fatty acids are saturated straight-chain (43.7 to 68.0% of the total fatty acids detected) acids with even (73.9 to 98.1%) carbon atoms.

At that, the fatty acid diversity in rhodococcal strains of various species represents quite a complex mixture, whereas their qualitative composition is almost identical. Significant differences are first of all in the ratio of individual acids. Thus, based on the ratio of myristic ($C_{14:0}$) and pentadecanoic ($C_{15:0}$) acids, ecologically significant rhodococcal species are classified into two groups. Group **I** rhodococci (*R. erythropolis*, *R. rhodochrous*, *R. ruber*) are characterized by $C_{14:0}$ and $C_{15:0}$ ratio of >1, and the ratio of <1 is characteristic of group **II** (*R. opacus*).

The ‘marker’ cyclopropane fatty acid $C_{17\nabla}$ (2.5 to 3.9%) is typically present in *R. erythropolis* representatives. By that feature, they differ from strains of other ecologically significant rhodococcal species in which this fatty acid is not detected. Another distinctive feature of *R. erythropolis* cultures is the elevated contents of myristic ($C_{14:0}$) and tuberculostearic acids.

Distinctive features of difficult-to-identify taxa *R. rhodochrous* and *R. ruber* are revealed by comparison of their fatty acid profiles. Thus, *R. ruber* representatives are successfully distinguished by the minimum (3.4%) percentage of tuberculostearic acid

and its homologues ($10MeC_{16:0}$, $10MeC_{17:0}$) compared to that of *R. rhodochrous* and *R. erytrhopolis* (9.7 and 10.8%, respectively). Originality of the fatty acid composition in *R. opacus* representatives is reflected in large (10.1%) total amounts of tuberculostearic acid and its homologues. Isolates of *R. fascians* are not unambiguously differentiated by the fatty acid profiles. It was observed, however, that they are characterized by the greatest (up to 35.6%) levels of palmitic acid.

Specific fatty acid features determined in rhodococcal cells were used to develop a particular taxonomic system allowing *Rhodococcus* differentiation at the species level.

Identification of free fatty acids in ecologically significant *Rhodococcus* species

1. Studied material. A reaction mixture of total cell lipids of natural isolates and collection cultures of various *Rhodococcus* species after alkaline hydrolysis.

2. Reagents. An absolute methanol – water (9:1) mixture. 1% Phenolphthalein solution in 90% ethanol. 6N of HCl. Petroleum ether (40−70°C). 2.5% Hydrogen chloride solution in methanol. A standard mixture of fatty acid methyl esters ("SUPELCO", USA).

3. Materials. A round bottom flask with a sidearm. A mechanical 1-channel variable volume dispenser (working volume of 1−5 ml) with a set of tips. A separating funnel. A glass-stoppered flask. A vacuum desiccator. Rubber gloves. Safety goggles.

4. Method.

4.1. *Preparation of cell extracts***.** 50 mg of dry biomass + 3.75 ml of chloroform – methanol (2:1) mixture are kept for 2 h at vigorous shaking.

4.2. *Preparation of total crude lipids.* Chloroform − methanol extracts are centrifuged (3,000 rpm, 15 min) to precipitate the bacterial cells. The supernatant is filtered through a paper filter into another centrifuge tube. For re-extraction, the bacterial cells are re-suspended in 4.75 ml of the chloroform − methanol − water mixture (1:2:0.8); the obtained mixture is shaken and centrifuged. 2.5 ml of chloroform and 2.5 ml of water are added to the combined supernatant. The chloroform and water − methanol layers are separated by centrifugation (3,000 rpm, 15 min). The lower chloroform layer is decanted into a pre-weighed evaporating ground joint flask, diluted with an equal volume of benzene and evaporated to dryness under nitrogen steam on a rotary evaporator at 30−35°C. After evaporation, the flask is placed into a vacuum desiccator with KOH until a constant weight. The flask containing the residue of total crude lipids is weighed using an analytical balance. After weighing, the lipids are imbedded in the chloroform − methanol mixture (2:1).

4.3. *Determination of free fatty acids.* An aliquot of the solution containing 15−30 mg of total crude lipids is transferred into a side-armed flask, and 5.0 ml of 0.3N NaOH solution in methanol is added. The mixture is boiled, and then cooled for 2 h. Three drops of the phenolphthalein solution and 90% methanol solution are added to the reaction mixture of total lipids for the reaction product solution to fill the entire volume of the flask's side arm. By adding phenolphthalein, the mixture turns crimson indicating the successful alkaline hydrolysis. The resultant solution is transferred to a separating funnel, and the petroleum ether is added by portions (3 to 4 times, 5 ml per portion) to extract the unsaponifiable compounds (glycerol alkene-1-esters, sterols, hydrocarbons, carotenoids, higher alcohols, etc.). The upper layer (unsaponifiable compounds) is constantly decanted into a pre-weighed glass-stoppered flask. The water alcohol phase (the lower layer) is acidified with 6N HCl (0.3 ml) until the solution is decolorized, and the free fatty acids are extracted by adding the portions of petroleum ether (3 to 4

times, 5 ml per portion). The upper layer (free fatty acids) is constantly decanted into a pre-weighed glass-stoppered flask.

The combined extracts of unsaponifiables and free fatty acids are evaporated to dryness under the nitrogen steam, and dried in a vacuum desiccator over NaOH. The residue is weighed, and the contents of the resultant compounds are calculated by the formulas:

Cell fatty acids, % = (fatty acid weight, mg / sample weight, mg) x 100.

Unsaponifiable compounds, % = (Unsaponifiable content, mg/sample weight, mg) x100.

The obtained preparation of free fatty acids is embedded in the fresh chloroform − methanol mixture (2:1) and stored at -4°C with no time limits.

For identification, free fatty acids are converted into methyl esters via acid hydrolysis (2.5% hydrogen chloride solution in methanol, 60°C, for 1 h) and analyzed by GC-MS using an Agilent 6890N gas chromatograph and an Agilent MSD 5973N quadruple mass-spectrometer ("Agilent Technologies", USA). A RTX-5MS capillary column (30 m/0.25 mm/0.25 μm with a 5 m pre-column) is used for the analysis.

5. General labor safety measures. All manipulations should be performed in a fume hood and using rubber gloves. Be careful when working with hydrochloric acid and a mixture of alcohol and chloroform (eyes should be protected with safety goggles). The working table should be disinfected not only before, but also after work. Use 0.5% aqueous chloramine solution to treat the working table.

6. Data processing. Based on the obtained results and using the Key to *Rhodococcus* species identification, group the strains according to the fatty acid content and conclude about the species affiliation of the cultures studied.

7. A key to the identification of ecologically significant *Rhodococcus* species

1. The concentration ratio of myristic acid $C_{14:0}$ to pentadecanoic acid $C_{15:0}$ is above 1 – ***R. erythropolis***, ***R. rhodochrous***, ***R. ruber***.

A. Cyclopropane fatty acid $C_{17\nabla}$ is present – ***R. erythropolis***.

B. The percentage of tuberculostearic acid $10MeC_{18:0}$ and its homologues $10MeC_{16:0}$, $10MeC_{17:0}$ is minimum, less than 3 – ***R. ruber***.

C. The percentage of tuberculostearic acid $10MeC_{18:0}$ and its homologues $10MeC_{16:0}$, $10MeC_{17:0}$ is high, greater than 9 – ***R. rhodochrous***.

2. The concentration ratio of myristic acid $C_{14:0}$ to pentadecanoic acid $C_{15:0}$ is less than 1 – ***Rhodococcus* sp., *R. opacus***.

A. The percentage of tuberculostearic acid $10MeC_{18:0}$ and its homologues $10MeC_{16:0}$, $10MeC_{17:0}$ is less than 4 – ***Rhodococcus* sp.**

B. The percentage of tuberculostearic acid $10MeC_{18:0}$ and its homologues $10MeC_{16:0}$, $10MeC_{17:0}$ is greater than 10 – ***R. opacus***.

8. References

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