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**EXPERIMENTAL ARTICLES**

# Degradation of Machine Oil by Nocardioform Bacteria

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**Abstract**—Gas liquid chromatography (GLC) was used for the first time to screen for machine oil–degrading microorganisms. Oil degradation was evaluated from the microorganism respiratory activity during the utilization of oil as the sole carbon and energy source. The results are consistent with those obtained by the conventional weighing method. Substrate specificity of the active strains with respect to different machine oils was studied. Bacterial communities exhibited the highest activity, whereas a *Rhodococcus erythropolis* strain was the most active among pure cultures. Various stages of bacterial interaction with oil drops were followed by means of fluorescent microscopy.

*Key words*: rhodococci, machine oil utilization, respiration, GLC, fluorescent microscopy, biodiversity, environmental oil pollution.

The environmental risks associated with petroleum product contamination is of a global scale. Different methods (mechanical, physicochemical, chemical, and biological) have been developed for the cleaning of various environments from oil pollution [1]. Microbiological methods are based on the capacity of degradative microorganisms to utilize oil hydrocarbons as the sole carbon and energy source or as a cosubstrate. Hydrocarbon-oxidizing microorganisms were isolated to develop preparations applicable for cleaning soil and water from oil contamination [2, 3]. However, the available microbial preparations oxidize, primarily, oil paraffins [4], while other fractions (cycloparaffins, aromatic hydrocarbons, resins, etc.) are less accessible for microbial enzymes [1, 5].

Various machine oils, which are multicomponent additive-containing liquid mixtures of high boiling temperature hydrocarbons, belong to these difficult-to-oxidize fractions. Because machine oils are often used as lubricants, they are the main soil-polluting agents near oil-processing plants.

Machine oil degradation by microbes has been described in a few reports. To our knowledge, only Pleshakova and Turkovskaya developed biocatalysts for cleaning waste water from mineral oil [6, 7]. These authors developed degradative microbial communities, which, however, comprised members of the genera *Acinetobacter* and *Pseudomonas*, whereas nocardioforms prevail, as a rule, in oil-polluted habitats [4, 8].

In this study, active strains of microorganisms utilizing machine oil were isolated to analyze their biological diversity.

## MATERIALS AND METHODS

### *Isolation and Identification of Microorganisms.*

The microorganisms capable of utilizing machine oil as the sole carbon and energy source were isolated from the oil-polluted soil samples by the enrichment-culture method. The following machine-oil types served as substrates: turbine oil (TuO), motor oil (MO), transformer oil (TO), and compressor oil (CO). Bacteria were cultivated aerobically in flasks on a shaker at 26–28°C in medium no. 1 (Raymond medium [9]) or in medium no. 2 of the following composition (g/l): $K_2HPO_4$, 7; $KH_2PO_4$, 3; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1; $FeSO_4 \cdot 7H_2O$ and $MnCl_2 \cdot 4H_2O$, trace amounts; pH 7.0.

Enrichment cultures were grown for 10 to 14 days; the growing microbial communities were monitored microscopically. Pure cultures were isolated by plating on GPY medium (g/l): glucose, 5; peptone, 10, yeast extract, 5; NaCl, 5; pH 7.2. The isolated strains were maintained on the same medium.

To determine the species affiliation of isolates, their morphological, cultural, physiological, and biochemical properties were studied by currently adopted methods. Cell morphology was examined under a phase-contrast microscope. The cell-wall type was determined by the analysis of mycolic acid isomers and sugar composition [10]. The LCN-A complex (complex of mycolic acids) was determined by the conventional method [10]. Microorganisms were identified according to *Bergey's Manual* [11] and Nesterenko *et al.* [12].

The capacity of the isolates to degrade machine oil was compared with that of the collection strains previously identified in the Laboratory of Oil Microbiology, Institute of Microbiology, Russian Academy of Sci-

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**Table 1.** Substrate specificity of nocardioform bacteria with respect to different machine oils ($CO_2$ concentration, %)

| Microorganism | Substrate | Days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1 | 2 | 2 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| *R. erythropolis* 100 | PAR | 1.3 | 6.1 | – | 6.6 | – | – | 7.9 | – | – |
| | MinO | 0.5 | 4.5 | 6.3 | – | 6.5 | – | 6.9 | – | 6.9 |
| | TuO | 0.5 | 4.1 | 4.9 | 6.1 | 6.8 | – | – | 6.8 | 6.8 |
| | MO | 0.3 | 4.1 | 5.8 | – | 7.0 | 7.1 | – | – | – |
| | TO | 0.3 | 0.5 | 0.8 | – | 1.4 | – | 1.4 | – | 1.4 |
| | CO | 0.2 | 0.6 | 1.4 | – | 1.6 | – | 1.9 | – | 1.9 |
| *D. maris* 101 | TuO | – | 0.8 | – | 4.3 | – | 6.0 | 6.0 | – | 6.0 |
| | TO | – | – | 0.3 | 1.5 | – | – | 3.2 | – | 3.4 |
| | CO | – | – | 0.3 | – | 0.3 | – | – | – | 0.4 |
| *Mycobacterium* 102 | TuO | – | – | 0.3 | – | – | 0.5 | – | – | 0.5 |
| | MO | – | – | 0.3 | – | 0.3 | – | – | – | 0.3 |
| Microbial community | PAR | 0.5 | – | 5.3 | – | – | 6.8 | – | – | – |
| | MinO | 0.5 | – | 5.3 | – | – | 7.3 | – | – | – |
| | TuO | 0.3 | – | 6.2 | – | – | 8.0 | – | – | – |
| | MO | 0.4 | – | 6.4 | – | – | 8.3 | – | – | – |
| | TuO | 0.2 | – | 1.4 | – | – | 2.9 | 3.0 | – | – |
| | CO | 0.2 | – | 3.0 | – | – | 4.5 | 4.7 | – | – |
| Medium + *R. erythropolis* 100 | no | 0.1 | 0.1 | – | 0.1 | – | – | 0.1 | – | 0.1 |
| Medium + microbial community | no | 0.1 | – | 0.2 | – | – | 0.3 | – | 0.3 | – |
| Medium | MinO | 0.1 | 0.1 | – | 0.1 | – | – | 0.1 | 0.1 | – |

Note: Here and in Tables 2 and 3, the sign "–" means "not determined," "no" means "no substrate"; PAR designates a mixture of paraffins; MinO, purified mineral oil; TuO, turbine oil; MO, motor oil; TO, transformer oil; CO, compressor oil.

ences [13]. The collection strains belonged to the species *Rhodococcus erythropolis* (strains 283, 367-6, 12049, sch), *Dietzia maris* (formerly *Rhodococcus maris*) (strains 4-3, 4-2b, 367-5, n-2(4), 2834, 2842r, 7816r, 7824a, 7824b), *Staphylococcus* sp. (strains 2839, 2843, 3011 wh), *Brevibacterium lineus* (strain 21830), *Pseudomonas stutzeri* (strain A), and *Alcaligenes faecalis* (strain 367-1).

### *Measurement of Oil Utilization*

**1. Gas-liquid chromatography (GLC).** Substrate utilization by microorganisms grown on machine oil as the sole carbon and energy source was estimated from their respiratory activity. In addition to the above-mentioned machine oils, purified mineral oil (MinO) (light white oil, ICN Pharmaceutical Inc.) and a mixture of paraffins (PAR) with a chain length of $C_{14}$–$C_{18}$ were also used as substrates.

$CO_2$ release and $O_2$ consumption were measured in hermetically closed 30- or 50-ml flasks containing 10 ml of media no. 1 or 2. Inoculate comprised 2%. The oil content was 0.3%. Cultivation at 26–28°C under aeration on a shaker continued from 3 to 10 days. During cultivation, pH changes were monitored using an EV-74 universal ionometer.

Gases were analyzed on a model 3700 chromatograph equipped with a thermal conductivity detector. Argon was the carrier gas. The temperature was 50°C. To evaluate $CO_2$ evolution, we used a 3.5-m metal column packed with Chromasom 101, 80 mesh. Residual $O_2$ was determined on a 2.0-m metal column (i.d., 2 mm) packed with molecular sieve 5A.

In controls, the respiration activity of microorganisms in the absence of oil (endogenous respiration) and changes in the gas phase in flasks with uninoculated substrates were determined.

All measurements were replicated three to six times.

**2. Weighting method.** Cultures were grown in 3-l flasks containing 300 ml of nutrient media no. 1 or 2 and in hermetically closed 30-ml flasks containing 10 ml of the same media at 26–28°C on a shaker for 7 to 10 days. Oil was extracted with a hexane–acetone (10 : 1) mixture. The extract was evaporated on a rotor

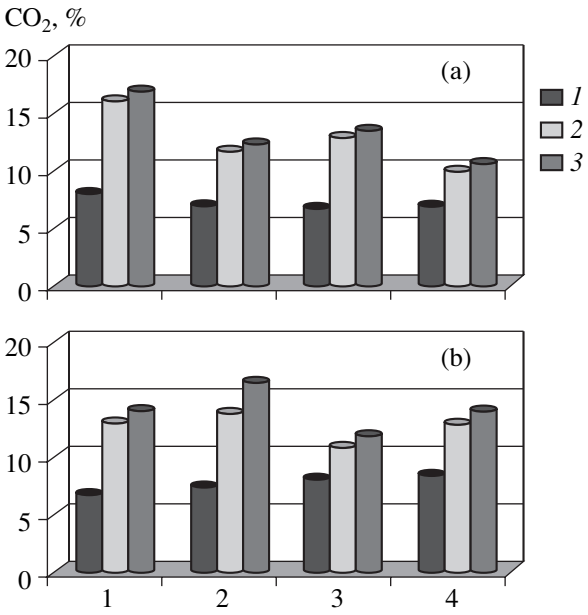



**Fig. 1.** Inhibition of bacterial degradation of machine oils by carbon dioxide: *1*, $CO_2$ concentration in flasks on days 6–7; *2*, $CO_2$ accumulation in flasks upon removal of excess $CO_2$; *3*, continued $CO_2$ emission after repeated $CO_2$ removal. (a) *R. erythropolis*: 1, 2, 3, and 4, microorganisms + PAR, MinO, TuO, and MO, respectively; (b) microbial community: 1, 2, 3, and 4, microbial community + PAR, MinO, TuO, and MO, respectively.

evaporator, and the residual material was weighed to determine the percent of oil utilization [14].

**3. Fluorescent method.** Cell suspension sampled from the culture containing TTO as the substrate was placed on a slide to be stained with an 0.01% acridine orange solution by a conventional technique [10]. The stained specimens were examined under a LUMAM-I2 luminescent microscope. Luminescence was excited using an FS-1-4 filter in combination with a heat-protecting light filter.

## RESULTS AND DISCUSSION

### *Identification of the Isolates*

Six bacterial strains were isolated from mazut-polluted soil (sample no. 1) and from a tank for petroleum product separation of the Kashira heat and electric power plant (sample no. 2).

Based on a combination of the phenotypic properties of strains 100, 101, and 102 isolated from sample no. 1, and in comparison with previously studied strains [13], both generic and species affiliations of the new isolates were determined. To determine the bacterial group to which strains 100, 101, and 102 belong, their cell wall composition was studied and shown to contain meso-diaminopimelic acid, arabinose, and galactose, thus conforming to the type-IV cell wall. The complex of free mycolic acids was determined by the thin-layer chromatography on plates with silica gel. Based on the cell-wall type and mycolic acid composition, the isolates were assigned to group 22, (nocardioform actinomycetes), subgroup 1 (bacteria synthesizing mycolic acids) [11]. Comparison of the mycolic acid $R_f$ values characteristic of collection strains and new isolates showed that strains 100 and 101 were rhodococci, whereas strain 102 contained different mycolic acids typical of mycobacteria.

According to the morphological traits (cell size and shape, developmental cycle), the shape and consistency of colonies, pigmentation, physiological properties (utilization of hydrocarbons, alcohols, organic acids, and nitrogen sources), strain 100 was related to the species *Rhodococcus erythropolis*, and strain 101 to *Dietzia maris*. Strain 102 was identified as *Mycobacterium* sp.

### *Analysis of the Oil-Utilizion Capacity in Microorganisms*

**1. Measurement of oil degradation from bacterial respiration activity by means of GLC.** Strains *R. erythropolis* 100, *D. maris* 101, and *Mycobacterium* sp. 102, as well as strains 3, 4, and 8, isolated from sample no. 2, were capable of growing on paraffins as the sole carbon and energy source. Hence, these strains were assumed to have a capacity for machine oil degradation.

The utilization of different machine oils (TuO, MO, TO, and CO), as well as of PAR and MinO, serving as controls, was measured from the microbial respiration activity. The results obtained are summarized in Table 1. When cultivated in 30-ml flasks, microorganisms utilized oil over seven days, but then the process ceased. The strains showed different substrate specificity with regard to different oils. *R. erythropolis* 100, *D. maris* 101, and a bacterial community utilized TuO and MO more actively than TO and CO. Thus, after a 6-day cultivation of *R. erythropolis* 100 with TTO and MO, the content of $CO_2$ in flasks was about 7%, i.e., the microbial respiration activity was similar to that determined with the more easily oxidized substrates, PAR and MinO. All of the oil substrates studied were more actively degraded in cultures containing bacterial communities obtained from the sample no. 2 than in pure cultures. Strains 3, 4, and 8 isolated from this bacterial community were similar to *R. erythropolis* 100 in their capacity to utilize TuO. Strain *Mycobacterium* sp. 102 showed the lowest activity.

We supposed that the microorganism respiration and oil utilization ceased after six to seven days of incubation (in 30-ml flasks) because of either $O_2$ shortage or of the toxic effect of $CO_2$. To verify these suggestions, *R. erythropolis* was incubated with TuO in 30- and 50-ml flasks, in which the content of residual $O_2$ comprised 3.0–3.4 and 7.0–7.5%, respectively, on the seventh day of incubation. After this incubation period, $CO_2$ emission ceased in both 30- and 50-ml flasks, i.e.,

**Table 2.** Degradative activity of rhodococci with respect to machine oils ($CO_2$ concentration, %)

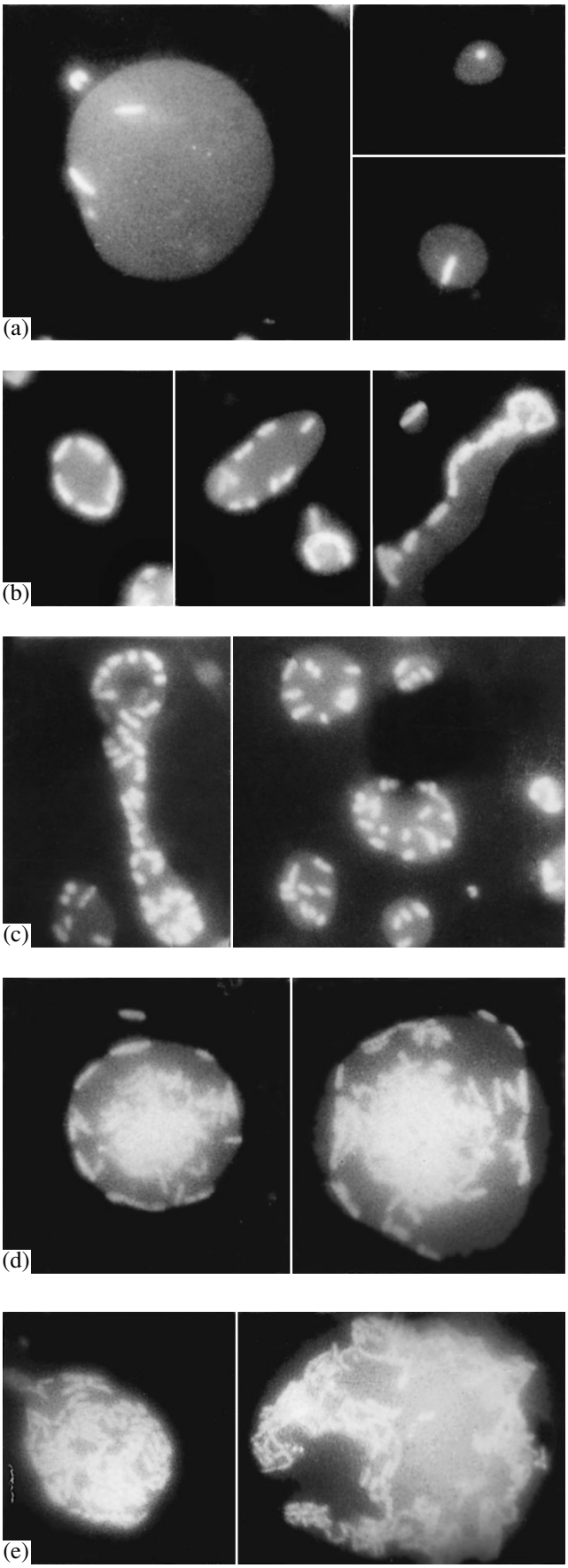
| Species | Strain | Substrate | Days | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 2 | 3 | 4 | 5 | 7 | 9 |
| *R. erythropolis* | 100 | TuO | 4.1 | 4.9 | 6.1 | 6.8 | 6.8 | – |
| | | TO | 0.5 | 0.8 | – | 1.4 | 1.5 | 1.5 |
| | 367-6 | TuO | 2.8 | – | 6.4 | – | 7.0 | – |
| | | TO | 0.8 | – | 0.9 | – | 0.9 | – |
| | 283 | TuO | 1.8 | – | 7.2 | – | 7.3 | 7.3 |
| | | TO | 1.1 | – | 1.4 | – | 1.4 | – |
| | 12049 | TuO | 1.3 | – | 4.4 | – | 7.5 | 7.6 |
| | | TO | 1.4 | – | 2.3 | – | 2.5 | – |
| *D. maris* | 101 | TuO | – | 0.8 | 4.3 | – | 6.0 | 6.0 |
| | | TO | – | 0.3 | 0.8 | – | 3.0 | 3.4 |
| | 4-3 | TuO | – | 0.7 | – | 2.4 | 3.6 | 4.7 |
| | | TO | – | 0.8 | – | 1.3 | 1.5 | 1.7 |
| | 2842r | TuO | – | 1.2 | 2.2 | 2.8 | 3.7 | 4.4 |
| | | TO | – | 0.5 | 0.9 | 1.5 | 1.6 | 1.7 |
| | 7816r | TuO | – | 0.8 | – | 2.7 | 3.5 | 3.9 |
| | | TO | – | 0.8 | – | 1.5 | 1.8 | 2.1 |

**Table 3.** Utilization of machine oils by nocardioform bacteria (the substrate used and $CO_2$ accumulation)

| Microorganism | Substrate | Medium volume of 300 ml, utilization, % | Medium volume of 10 ml | |
|---|---|---|---|---|
| | | | utilization, % | $CO_2$, % |
| *R. erythropolis* 100 | PAR | – | 73 | 15.9 |
| | MinO | 30 | 31 | 9.7 |
| | TuO | 28 | 27 | 8.0 |
| | MO | – | 32 | 8.1 |
| | TO | 16 | 16 | 1.5 |
| | CO | – | 4 | 1.6 |
| | MinO + Na acetate | 28 | – | – |
| | MinO + PAR | 22 | – | – |
| Microbial community | PAR | – | 98 | 14.2 |
| | MinO | 39 | 38 | 16.6 |
| | TuO | 38 | 25 | 11.8 |
| | MO | 37 | 28 | 14.0 |
| | TO | 30 | 30 | 3.0 |
| | CO | 22 | 15 | 4.7 |
| | MinO + Na acetate | 38 | – | – |
| | MinO + PAR | 38 | – | – |

at a different concentration of residual oxygen. Hence, $O_2$ concentration did not limit bacterial oil utilization. The content of $CO_2$ was similar in both kinds of flasks after seven days of incubation (6.0–7.0%) and exceeded 200-fold the $CO_2$ concentration in the air. Hence, it is probably the content of $CO_2$ that is the limiting factor in the system.

The suggestion that $CO_2$ has an effect on microbiological oil degradation was confirmed in a series of experiments with those oil-degrading microorganisms whose activity was suppressed at a $CO_2$ concentration of 6.0–7.0%. After six to seven days of incubation, when $CO_2$ emission stopped, the flasks previously closed hermetically were opened to remove the carbon




dioxide that accumulated. The cultures were aerated over 1.5 to 2 h under sterile conditions, and, after the $CO_2$ concentration in flasks became close to that in the ambient air (0.03%), they were again closed hermetically. This procedure was repeated twice, and, afterwards, oil utilization continued for an additional three to four days, so that total $CO_2$ accumulation in flasks increased significantly to reach 32 to 53% in various experimental versions (Fig. 1).

Endogenous bacterial respiration, as well as carbon dioxide release due to chemical reactions (about 0.3%), had no significant effect on the results. The low level of endogenous respiration in rhodococci agrees well with the data published [1]. The culture medium was insignificantly acidified during the experiments (the pH changed from 7.0 to 6.5).

As determined experimentally, similar acidification of sterile medium with 0.2N $H_2SO_4$ led to an increase in the $CO_2$ concentration in flasks by only 0.3%. In the microorganism-free oil-containing medium, the $CO_2$ content did not exceeded 0.1% after a week of incubation (Table 1).

The oil-utilizing capacity of new isolates, both *R. erythropolis* 100 and *D. maris* 101, was compared with that of the collection species exhibiting hydrocarbon-oxidizing activity [4, 15]. All the strains studied utilized TuO more actively than TO, although different species and strains differed to a certain extent in this property (Table 2). The *R. erythropolis* 100 cultures, as well as the collection strains of the same species, showed the highest activity of TuO degradation. The *D. maris* strains, especially isolate *D. maris* 101, were superior to *R. erythropolis* (except for *R. erythropolis* 12049) in TO utilization.

Thus, the isolates obtained did not differ significantly from the collection strains in the capacity for machine oil degradation, which suggests that the enzymes involved in the substrate oxidation were constitutive.

Some other hydrocarbon-oxidizing collection strains (*R. erythropolis* sch and *D. maris* 4-2b, n-2(4), 2834, 367-5, 7824a, and 7824b) also proved to be capable of machine oil degradation, as determined visually and by microscopic examination. Certain activity was also detected in *Staphylococcus* sp. 2839, 2843, and 3011 wh; *Cytophaga* sp. 3011r; *Pseudomonas* sp. A; and, as mentioned above, in *Mycobacterium* sp. 102. No

**Fig. 2.** Stages of *R. erythropolis* 100 interaction with TuO drops. Fluorescent microscopy, 1300×. (a) On day 1 (initial stage), individual cells attach to the oil drop surface; (b) on days 2 to 3, the number of attached cells increases; (c) on day 4 to 5, bacterial cells penetrate the oil drops and reproduce; (d) on day 6 to 7, the cell number inside the drops increases, the luminescence of the drops enhances; (e) on day 7 to 8 (the final stage in the culture development), microcolonies are formed.

capacity for machine oil utilization was detected in *Brevibacterium lineus* 21830 and *Alcaligenes faecalis* 367-1.

The results obtained suggest that the highest capacity for machine oil degradation is characteristic of *R. erythropolis* strains.

**2. Measurement of bacterial oil degradation by the weighing method.** On days 7 and 8 of the incubation, both microbial communities and *R. erythropolis* 100 cultivated with various machine oils as the sole carbon and energy source utilized from 30 to 39% and from 16 to 30% of the substrate, respectively, when TuO (or MO) and TO (or CO) were added to the cultures. The addition of either sodium acetate or a mixture of paraffins to MinO did not increase the bacterial oil-degrading capacity (Table 3). The medium volume (cf. 3-l and 30-ml flasks) and aeration (cultivation under stationary conditions or on a shaker) were of no importance for oil degradation. The percent of oil utilization by the bacterial community was higher than by the *R. erythropolis* 100 culture, which correlates with the above data obtained by GLC. A correlation was also observed with respect to the substrate specificity to different oils. This can probably be explained by the mechanism of cooxidation underlying the degradation of cyclic alkanes by strains *Pseudomonas aeruginosa* K1 and *Rhodococcus equi* P1 [16]. This was also indirectly supported by different substrate specificities showed by *R. erythropolis* 100 and *D. maris* 101 incubated with TuO and TO (Table 2). Note that the respiration activity level of both the bacterial community and *R. erythropolis* 100 was low (3 and 1.5%, respectively) when the bacteria were cultivated with TO, although significant utilization of this substrate was observed, as determined by the weighing method. TO seemed to be oxidized to some intermediate products but not to $CO_2$, as it was previously found with crude oil [15, 17].

With MinO used as the substrate, significant activity was revealed in *D. maris* 2842 and 367-5 (36 and 33%, respectively); *Cytophaga* sp. 3011r showed a lower activity (28%), and the lowest activity was detected in *Pseudomonas* sp. A (11%).

**3. The study of interaction between oil drops and oil-degrading microorganisms by fluorescent microscopy.** The process of oil utilization by microorganisms was visually monitored under a fluorescent microscope in specimens of *R. erythropolis* 100 culture grown on TuO-containing medium no. 1 for 7 to 8 days.

On the first day of cultivation, individual bacterial cells attached to the oil drop surfaces (Fig. 2a). Over the next 2–3 days, the cell density on each drop increased (Fig. 2b). At this initial stage (1 to 4 days), the cells were oval in shape, $1 \times 3$ μm in size. The next stage (4 to 5 days) was characterized by extensive cell reproduction and penetration into the drops (Fig. 2c). The latter process, which promotes greater surface contact between cells and the substrate; results from an increase in the hydrophobicity of the rhodococcus cell wall due to the synthesis of mycolic acid esters [1]. Upon entering the drops, the cells become finely emulsified, which not only enlarges the bacterium–substrate surface contact, but also improves aeration. Cell penetration into the oil drops was accompanied by an increase in the luminescence of the latter, which increased significantly on the sixth–seventh days of cultivation (Fig. 2d). This can probably be explained by a release of cell metabolic products, including dye-binding emulsifying agents. As seen in Fig. 2d, at this stage of cultivation, the highest cell density within the drops was observed; the cells decreased twice in their size ($1.0 \times 1.5$ μm) and acquired a coccoid shape. At the final stage, the accessible substrate was mostly utilized and the cell-filled drops became peculiar microcolonies, often losing their round shape (Fig. 2e).

Although we did not use any special techniques to discern viable and dead cells, no evident cell lysis was observed in such microcolonies. Most cells seemed to be viable and at rest. Cell growth and division were suppressed; the cell thickness decreased twice and averaged about 0.5 μm, although the original cell length of 3 μm was reached. In old cultures, microcolonies persisted for a long time; only upon culture transfer to a fresh medium did they disintegrate into individual cells.

The duration of the cycle (cell attachment to oil drops, penetration of the latter, and cell reproduction within the drops) depended on the initial drop size and volume and differed in individual oil drops. Because of this, different stages of the cycle can be observed in suspensions of the same age.

The results of this study indicate that nocardioform bacteria synthesizing mycolic acids can efficiently degrade machine oil. *R. erythropolis* showed the highest activity. This is consistent with the published evidence of the domination of *R. erythropolis* representatives in hydrocarbon-oxidizing microbial communities [15, 18]. Some researchers consider *R. erythropolis* to be an indicator species for ecosystem contamination with petroleum products [19]. In our experiments, *R. erythropolis* 100 populations were found to dominate bacterial communities when cocultivated with *D. maris* 101 and *Mycobacterium* sp. 102 and then plated on Petri dishes.

The results obtained with the GLC method used to evaluate bacterial respiration activity during oil degradation correlated with those yielded by the conventional weighing method (with respect to both species composition of active strains and substrate specificity). Monitoring of bacterial growth and development by the GLC method has advantages over the extremely laborious weighing method, as well as over the nephelometric method and protein determination; the latter two methods are difficult to apply in biphasic systems, including the water–oil system.

As determined by both weighing method and GLC, rhodococci utilize only certain oil fractions constituting this multicomponent system.

Using the fluorescent method, we were able to follow different stages of interaction between oil-degrading bacteria and oil drops during substrate utilization. It should be noted that the maximal content of $CO_2$ in the experimental flasks was recorded when the oil drops were filled with bacteria.

Our results on the ability of rhodococci to utilize machine oils as the sole carbon and energy source confirm once more the ecological role of rhodococci as a group of microorganisms highly important in ecosystems contaminated with petroleum products.

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