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EXPERIMENTAL ARTICLES

The Use of the [^{13}C]/[^{12}C] Ratio for the Assay of the Microbial Oxidation of Hydrocarbons

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Abstract—The study deals with a comparative analysis of the relative abundances of the carbon isotopes ^{12}C and ^{13}C in the metabolites and biomass of the *Burkholderia* sp. BS3702 and *Pseudomonas putida* BS202-p strains capable of utilizing aliphatic (*n*-hexadecane) and aromatic (naphthalene) hydrocarbons as sources of carbon and energy. The isotope compositions of the carbon dioxide, biomass, and exometabolites produced during the growth of *Burkholderia* sp. BS3702 on *n*-hexadecane ($\delta^{13}C = -44.6 \pm 0.2$‰) were characterized by the values of $\delta^{13}C_{CO_2} = -50.2 \pm 0.4$‰, $\delta^{13}C_{biom} = -46.6 \pm 0.4$‰, and $\delta^{13}C_{exo} = -41.5 \pm 0.4$‰, respectively. The isotope compositions of the carbon dioxide, biomass, and exometabolites produced during the growth of the same bacterial strain on naphthalene ($\delta^{13}C = -21 \pm 0.4$‰) were characterized by the isotope effects $\delta^{13}C_{CO_2} = -24.1 \pm 0.4$‰, $\delta^{13}C_{biom} = -19.2 \pm 0.4$‰, and $\delta^{13}C_{exo} = -19.1 \pm 0.4$‰, respectively. The possibility of using the isotope composition of metabolic carbon dioxide for the rapid monitoring of the microbial degradation of petroleum hydrocarbons in the environment is discussed.

Key words: naphthalene, *n*-hexadecane, *Burkholderia* sp., *Pseudomonas putida*, fractionation of carbon isotopes.

The extensive development of transport and power-generating industries is associated with an intense extraction and use of fossil hydrocarbon fuels, such as natural gas and oil, and leads to the ever-increasing pollution of air, water, and land with hydrocarbons [1, 2]. This poses the problem of the rapid monitoring and efficient control of hydrocarbon pollution in the environment.

Microbial populations possess a high potential adaptability to unfavorable environmental conditions. They have evolved unique metabolic systems that allow them to utilize not only natural but also anthropogenic (artificially produced) organic substances as sources of carbon and energy [3]. The development of biotechnologies for the remediation of soils and water bodies faces many problems, such as the search for hydrocarbon-degrading microorganisms and the determination of conditions promoting the degradation of recalcitrant pollutants [4].

The solution of these problems requires a simple and rapid method for determining the efficiency of the microbial utilization of hydrocarbon pollutants. This can be done by monitoring the consumption of pollutants used as the growth substrates and the formation of the respective metabolites. One of the most ubiquitous and universal microbial metabolites is carbon dioxide. However, to be employed for the monitoring of the microbial degradation of petroleum hydrocarbons in soil and water, the metabolic carbon dioxide of microorganisms must reliably be distinguished from the carbon dioxide produced in natural degradation processes, such as the degradation of plant residues. There is evidence that one of the parameters that can be used for the evaluation of the microbial degradation of anthropogenically produced hydrocarbons in the environment is the isotope composition of their carbon atoms [5–7].

As was shown earlier [8, 9], the metabolic carbon dioxide of *Candida* yeasts grown on (C_{19}–C_{23}) alkanes contains 4 to 7‰ less of the isotope ^{13}C than the alkanes. As discussed previously [10], crude oil and its products are characterized by values of $\delta^{13}C$ ranging from –32 to –26‰. Consequently, the metabolic carbon dioxide produced during the microbial oxidation of petroleum products may have $\delta^{13}C$ values ranging from –36 to –30‰ and, hence, may considerably differ in this parameter from the carbon dioxide present in the air and soils.

Modern biotechnologies for the remediation of polluted soils and bodies of water widely employ pseudomonads and corynebacteria, which are able to oxidize many hydrocarbons at temperatures from 4 to 25°C [11]. The fractionation of carbon isotopes by these bacteria grown on particular hydrocarbons has

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not yet been described, which hinders the use of the isotope composition of metabolic carbon dioxide for monitoring bacterial growth on petroleum products.

The aim of this work was to fill this gap.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The bacterial strains *Burkholderia* sp. BS3702 and *Pseudomonas putida* BS202-p used in this work are able to grow on both aliphatic and aromatic hydrocarbons [12]. In our experiments, we used the aliphatic hydrocarbon *n*-hexadecane ($C_{16}H_{34}$) and the aromatic hydrocarbon naphthalene ($C_{10}H_8$) and their mixture (1 : 1) as the growth substrates. The bacteria were grown in sealed flasks containing 100 ml of a mineral medium with 50–100 mg of a hydrocarbon substrate and 0.65 l of air in the headspace.

Analysis of carbon and its isotope composition. To assess carbon balance, the content of carbon was determined in the initial substrate and in the metabolic products of bacteria (biomass, exometabolites, and metabolic carbon dioxide). The amounts of oxygen consumed and carbon dioxide produced were estimated from the composition of the gas phase in the cultivation flasks before and after cultivation. The composition of the gas phase (specifically, the content of nitrogen, oxygen, argon, and carbon dioxide) was determined using an MM8-80 (VG-Analytic Gas) mass spectrometer.

The carbon isotope composition of initial substrate and metabolic products was analyzed using a CH-7 Varian two-channel mass spectrometer and expressed in terms of $\delta^{13}C$ values, defined as

$$\delta^{13}C = (R_{samp}/R_{std} - 1) \times 1000, ‰ \quad (1)$$

where R_{samp} and R_{std} are the ratios of the abundances of $^{13}CO_2$ and $^{12}CO_2$ in a sample and the PDB international standard, respectively.

The isotope composition of carbon in hydrocarbons, biomass, and exometabolites was determined after the complete oxidation of these substances to water and CO_2 at 560°C in the presence of copper oxide as the catalyst. The CO_2 produced was analyzed for isotope composition as described above. Metabolic carbon dioxide was isolated from the gas phase above microbial cultures using low-temperature absorbents. The scheme of experiments is presented in Fig. 1.

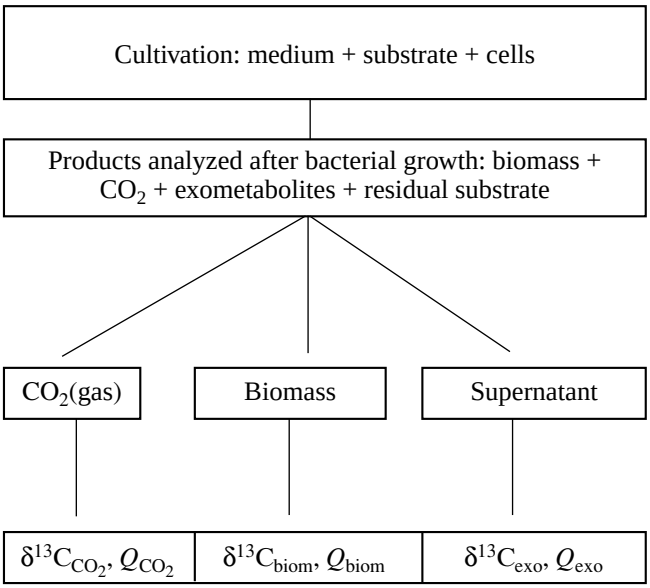

Fig. 1. Scheme of the experiment: $\delta^{13}C_{CO_2}$, $\delta^{13}C_{biom}$, and $\delta^{13}C_{exo}$ are the carbon isotope values and Q_{CO_2}, Q_{biom}, and Q_{exo} are the carbon contents of metabolic carbon dioxide, biomass, and exometabolites, respectively.

RESULTS AND DISCUSSION

Analysis of the metabolic products of *Burkholderia* sp. BS3702 grown on naphthalene. Upon the complete oxidation of naphthalene to CO_2 and water according to the reaction

$$C_{10}H_8 + 12O_2 = 10CO_2 + 4H_2O, \quad (2)$$

parameter Q, defined as the ratio of the molar concentrations of carbon dioxide produced and oxygen consumed, is equal to $Q_{theor} = 0.83$.

The experimental value of parameter Q calculated for the naphthalene-grown *Burkholderia* sp. BS3702 from the data presented in Table 1 turned out to be $Q_{exp} = 0.484$ (during its cultivation, this strain consumed 4.92 mmol O_2 and produced 2.38 mmol CO_2). The difference between Q_{theor} and Q_{exp} can be explained by the fact that some oxygen consumed by the strain was spent for the formation of biomass and exometabolites.

An analysis of the oxidation degree of exometabolites (i.e., the proportion between carbon and oxygen atoms in their molecules) can provide some information on the metabolic pathways in which they were produced. The carbon balance of the *Burkholderia* sp. BS3702 culture grown on naphthalene was as follows: The medium was supplemented with 100 mg naphthalene, i.e., 93.7 mg carbon (C). The CO_2 produced (metabolic CO_2) and the biomass contained 28.8 and 6.8 mg C, respectively. Some amount of C (namely, 33.3 mg) remained in the cultivation medium in the form of nonvolatile compounds. It can easily be calculated that the strain consumed 68.9 mg C, i.e., 73.5% of the carbon present in the added naphthalene.

The oxygen balance was as follows: The strain consumed 4.92 mmol O_2 and incorporated 2.38 mmol O_2 into the metabolic carbon dioxide, 0.28 mmol O_2 into the biomass, and no less than 2.66 mmol O_2 into the exometabolites. Thus, the strain spent 2.7 oxygen

Table 1. The composition of the gas phase during the growth of *Burkholderia* sp. BS3702 on naphthalene and *n*-hexadecane

Gas	Growth on naphthalene (%)		Growth on *n*-hexadecane (%)	
	initial content	final content	initial content	final content
N_2	78.15	85.65	78.15	84.20
O_2	20.65	4.20	20.65	11.90
Ar	1.14	1.27	1.15	1.25
CO_2	0.075	8.90	0.075	2.67

Note: Gas content is expressed as a percent.

atoms per every 2.8 carbon atoms of exometabolites. Consequently, their general formula must be CH_2O, which implies that *Burkholderia* sp. BS3702 grown on naphthalene produces exometabolites predominantly in the form of carbohydrates and/or organic acids, but not alcohols.

Analysis of the metabolic products of *Burkholderia* sp. BS3702 grown on *n*-hexadecane. Upon the complete oxidation of *n*-hexadecane to CO_2 and water according to the reaction

$$2C_{16}H_{34} + 49O_2 = 32CO_2 + 34H_2O. \quad (3)$$

the theoretical value of parameter Q is $Q_{theor} = 0.653$.

The experimental value of parameter Q calculated for the *n*-hexadecane-grown *Burkholderia* sp. BS3702 from the data presented in Table 1 turned out to be $Q_{exp} = 0.26$ (during the cultivation on *n*-hexadecane, this strain consumed 2.81 mmol O_2 and produced 0.726 mmol CO_2). The difference between Q_{theor} and Q_{exp} implies that some oxygen consumed by the strain was spent on the formation of biomass and exometabolites.

The carbon balance of *Burkholderia* sp. BS3702 grown on *n*-hexadecane was as follows: The medium was supplemented with 56.64 mg C of *n*-hexadecane. The metabolic CO_2 and the biomass contained 8.71 and 10.25 mg C, respectively. The amount of C remaining in the cultivation medium in the form of nonvolatile compounds was 5 mg. It can easily be calculated that the strain consumed 23.96 mg of carbon present in the added *n*-hexadecane. The remaining 32.68 mg of the *n*-hexadecane carbon may represent the carbon of the unoxidized *n*-hexadecane and volatile exometabolites.

The oxygen balance was as follows: The strain consumed 2.81 mmol O_2 and incorporated 0.76 mmol O_2 into the metabolic carbon dioxide, 0.42 mmol O_2 into the biomass, and no less than 1.66 mmol O_2 into the exometabolites. Thus, the strain spent no more than one oxygen atom per every carbon atom of exometabolites. Consequently, as in the case of *Burkholderia* sp. BS3702 grown on naphthalene, this bacterium grown on *n*-hexadecane produces exometabolites in the form of carbohydrates and/or organic acids, but not alcohols.

It is known that bacteria grown on *n*-hexadecane produce organic acids mainly in the tricarboxylic acid (TCA) cycle, with the evolution of metabolic carbon dioxide [13]. Based on the earlier observations [9], it can be anticipated that the metabolic CO_2 will be depleted in the ^{13}C isotope, whereas the biomass and organic acids will be enriched in this isotope.

The isotope composition of the metabolic products produced from hydrocarbons. The results of the isotopic analysis of metabolic products produced by bacteria grown on naphthalene and *n*-hexadecane are summarized in Tables 2 and 3. It can be seen that the metabolic carbon dioxide produced by *Burkholderia* sp. BS3702 grown on naphthalene contained 2.9‰ less ^{13}C than naphthalene and 5‰ less ^{13}C than the biomass and the exometabolites (Table 2). The augmented content of the ^{13}C isotope in the exometabolites confirms the above suggestion that they are mainly keto acids, since their formation is accompanied by the abstraction of a CO_2 group, which predominantly contains the ^{12}C isotope.

The same tendencies in the isotope composition were observed for *Burkholderia* sp. BS3702 grown on *n*-hexadecane (Table 3), except for minor differences such as the greater depletion of the biomass in the ^{13}C

Table 2. The fractionation of carbon isotopes during the growth of *Burkholderia* sp. BS3702 on naphthalene

Parameter	Substrate (initial)	CO_2	Biomass	Exometabolites	Substrate consumed, %
Carbon content, mg	93.7	28.8	6.8	33.3	68.9
Value of $\delta^{13}C$, ‰	–21.2	–24.1	–19.2	–19.1	

Table 3. The fractionation of carbon isotopes during the growth of *Burkholderia* sp. BS3702 on *n*-hexadecane

Parameter	Substrate (initial)	CO_2	Biomass	Exometabolites	Substrate consumed, %
Carbon content, mg	56.64	8.71	10.25	25.0	43.96
Isotope effect $\delta^{13}C$, ‰	–44.6	–50.2	–46.6	–41.5	–

isotope as compared with the exometabolites and *n*-hexadecane. In general, the changes observed in the carbon isotope composition of the *n*-hexadecane-grown *Burkholderia* sp. BS3702 metabolites were similar to those described for the yeast *Candida lipolytica* grown on *n*-alkanes [9]. In particular, the metabolic carbon dioxide of *Burkholderia* sp. BS3702 grown on *n*-hexadecane and *C. lipolytica* grown on *n*-alkanes contained, respectively, 8.6 and 9.0‰ less ^{13}C isotope than the exometabolites of these microorganisms. Furthermore, the biomasses of the bacterium and the yeast contained, respectively, 2.0 and 2.2‰ less ^{13}C isotope than the substrates *n*-hexadecane and *n*-alkanes. These data suggest that the fractionation of carbon isotopes in eukaryotic (*C. lipolytica*) and prokaryotic (*Burkholderia*) microorganisms is virtually the same. The enrichment of microbial exometabolites in the ^{13}C isotope is an indication that they are mainly keto acids, whose formation in the TCA cycle is associated with decarboxylation reactions.

The growth of the other bacterium under study, *P. putida* BS202-p, on naphthalene and *n*-hexadecane was characterized by a similar distribution of carbon isotopes, except that the degree of isotope fractionation by the bacterium *P. putida* BS202-p was notably less than in the case of *Burkholderia* sp. BS3702 (Table 4).

The carbon isotope composition of *n*-hexadecane and naphthalene differed by 23.4‰, whereas the difference between the carbon isotope compositions of carbon dioxide produced by *Burkholderia* sp. BS3702 grown on these substrates reached 26‰, i.e., was 2.6‰ greater. In the case of *P. putida* BS202-p, the difference between the carbon isotope compositions of carbon dioxide produced from *n*-hexadecane and naphthalene was 21.6‰, i.e., was 1.4‰ smaller than the difference between the isotope compositions of these substrates. These data allow the important inference to be made that within the accuracy of the isotope effect accompanying the formation of metabolic carbon dioxide (1.4–2.6‰), the latter inherits the isotope composition of the growth substrate. This implies that the microbial consumption of individual substrates from their mixtures can be monitored by analyzing the isotope composition of metabolic carbon dioxide, provided that the substrates differ in their isotope composition.

The feasibility of the proposed approach was confirmed in the following experiment with naphthalene ($\delta^{13}C$ = 21.2‰) and *n*-hexadecane ($\delta^{13}C$ = –44.6‰). The bacteria *Burkholderia* sp. BS3702 and *P. putida* BS202-p were adapted to growth on the individual substrate (either *n*-hexadecane or naphthalene) and then transferred to the growth medium containing their mixture (1 : 1). As follows from the analysis of the carbon isotope composition of metabolic carbon dioxide (Fig. 2), the growth patterns of the *n*-hexadecane-adapted and naphthalene-adapted *Burkholderia* sp. BS3702 cells on the mixture of these hydrocarbons was virtually the same: in both cases, the bacterium first consumed naphthalene and then *n*-hexadecane. At the same time, the growth pattern of *P. putida* BS202-p cells on the hydrocarbon mixture depended on the substrate to which these cells were preadapted. Namely, when the inoculum was

Table 4. Variations in the carbon isotope composition of the metabolic carbon dioxide produced by the bacteria grown on naphthalene and *n*-hexadecane

Bacterial strain	$\delta^{13}C$, ‰	$\delta^{13}C$, ‰ (substrate)
Burkholderia sp. BS3702	$\frac{-(24.4-)23.4}{-23.9}$	–21.2 (naphthalene)
Burkholderia sp. BS3702	$\frac{-(50.4\ldots-48.6)}{-49.5}$	–44.6 (*n*-hexadecane)
P. putida BS202-p	$\frac{-(23.5-23.15)}{-23.3}$	–21.2 (naphthalene)
P. putida BS202-p	$\frac{-(47.9-43.9)}{-45.9}$	–44.6 (*n*-hexadecane)

Note: Data are the means of four independent measurements.

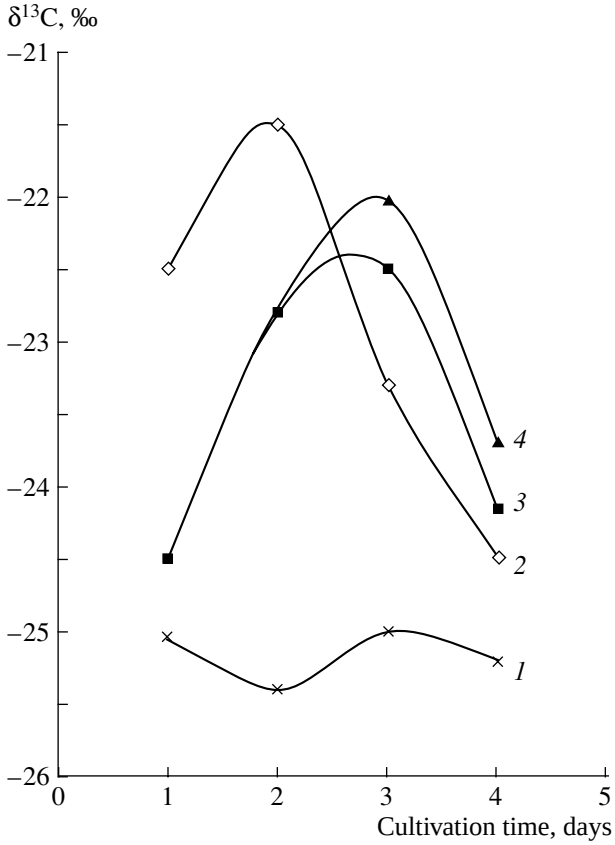

Fig. 2. Changes in the isotope composition of the metabolic carbon dioxide produced during the growth of bacteria on a mixture (1 : 1) of naphthalene and *n*-hexadecane. The cultivation medium was inoculated either with *P. putida* BS202-p cells grown on (*1*) *n*-hexadecane and (*2*) naphthalene or with *Burkholderia* sp. BS3702 cells grown on (*3*) naphthalene and (*4*) *n*-hexadecane.

grown on naphthalene, the *P. putida* BS202-p culture incubated in the medium with the hydrocarbon mixture first consumed naphthalene and then *n*-hexadecane. But when the inoculum was grown on *n*-hexadecane, the *P. putida* BS202-p culture incubated in the medium with the hydrocarbon mixture consumed both naphthalene and *n*-hexadecane at the same time. As is evident from the analysis of the isotope composition of metabolic carbon dioxide, the switching of bacterial metabolism from one substrate to the other was not abrupt in all cases.

To conclude, the analysis of the carbon isotope composition of metabolic carbon dioxide (expressed as the $\delta^{13}C$ value, ‰) allows the observation of the consumption dynamics of individual hydrocarbons from hydrocarbon mixtures, provided that the hydrocarbons differ in isotope composition. This approach can obviously be used not only in the laboratory but also in environmental studies.

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