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EXPERIMENTAL ARTICLES

Use of Glucose and Carbon Isotope Fractionation by Microbial Cells Immobilized on Solid-Phase Surface[1]

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Abstract—By the example of glucose uptake by the soil bacteria *Pseudomonas aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 immobilized on a solid-phase surface (quartz sand), their growth parameters were determined: growth rate (doubling time), total CO_2 production, CO_2 production per cell, lag period with respect to substrate uptake, respiratory quotient. The growth of *P. aureofaciens* and *Rhodococcus* sp. on glucose revealed (1) differences of the lag period with respect to substrate (lag time of ~4 h for *P. aureofaciens* and ~26 h for *Rhodococcus* sp.); (2) differences between the maximal rates of CO_2 production (~50 μg $C–CO_2$ g^{-1} sand h^{-1} for *P. aureofaciens* and ~8.5 μg $C–CO_2$ g^{-1} sand h^{-1} for *Rhodococcus* sp.); (3) differences in CO_2 production per cell (~1.94×10^{-9} μM CO_2/CFU for *P. aureofaciens* and more than ~3.4×10^{-9} μM CO_2 /CFU for *Rhodococcus* sp.). The kinetics of the metabolic CO_2 isotopic composition was shown to be determined by the difference in the carbon isotopic characteristics of products in the cell. Upon introduction of glucose into the medium (the preparatory stage of the metabolism), the uptake of intracellular ^{13}C-depleted products (lipids) is noted; at the stage of the maximal cell growth rate, introduced glucose is mainly metabolized; and at the final stage, upon exhaustion of substrate, the "stored" products—the lipid fraction—get involved in the metabolism. At the maximal rate of glucose uptake, the CO_2 carbon isotopic fractionation coefficient relative to organic products of microbial biosynthesis was determined to be $\alpha = 1.009 \pm 0.002$.

Keywords: pseudomonads, rhodococci, glucose, bacterial growth rate, respiratory quotient, carbon isotope fractionation

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In microbial ecology practice, the basal CO_2 respiration level and microbial activity potential determined as substrate-induced respiration (SIR) are widely used as criteria for the metabolic state of soil microbiota [1–6]. The concept of SIR is based on the assumption that glucose as an easily metabolized substrate can be used by many soil microorganisms, and metabolic CO_2 will, correspondingly, serve as an indicator of their amount and metabolic activity [1]. Under standardized conditions the microbial metabolism of glucose introduced into soil in excess can be limited by only the number of soil microorganisms. The assumption is that the fungal and bacterial components of microbial biomass in soil "react" similarly to the introduction of glucose into soil [7, 8]. It is postulated that within the first 4–8 h after glucose is introduced into soil there is no noticeable increase of microbial population and production of CO_2 (the respiratory response) is proportional to the amount of microbial biomass in soil [1]. It should be noted that this analytically important postulate relative to the uptake of glucose by soil bacteria (pseudomonads and rhodococci) has not yet found a convincing experimental proof. The quantitative assessment of active microbial species in soils is an important indicator of their metabolic potential, which is of special significance for predicting harvests of crops as well as in the development of technologies for biodegradation of organic pollutants in soils.

A metabolic feature of glucose as a test substrate in soils is its uptake by microbial cells immobilized on particles of soil, a solid-phase carrier. Unlike liquid media, where substrate is constantly available, in soils immobilized microbial cells can be limited in provision of substrate. Substrate uptake efficiency and CO_2 production depend in such cases on microbial growth rate and colonization of the solid-phase carrier where substrate is held.

One of the parameters of microbial CO_2 production in soil is that determining the origin and amount of CO_2 formed due to the mineralization of respective organic substrates. The estimations of metabolic CO_2

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produced during the uptake of a test substrate (e.g., glucose) and CO_2 formed due to the degradation of soil organic matter (SOM) are drawn between using ^{13}C- or ^{14}C-labelled glucose and ^{12}C-SOM [9–11]. The assumption is that products of microbial metabolism inherit the carbon isotope composition of substrate to an accuracy of up to the isotopic effects that accompany the uptake of substrate and its metabolic transformations [12].

However, up to the present day all studies on carbon isotope fractionation in microbial uptake of glucose as a catabolic/anabolic metabolism substrate have been conducted in experiments with microorganisms grown in liquid media [12, 13]. No data on the assessment of carbon isotope fractionation in the uptake and metabolism of glucose by microbial cells immobilized on a solid-phase carrier are yet available.

The aim of the work was (1) to determine the factors that affect CO_2 emission and oxygen uptake depending on the amount of microbial cells immobilized on an inert solid-phase carrier and using glucose as a substrate; (2) to assess the ratios of the catabolic and anabolic potentials in the use of glucose by soil bacteria (with pseudomonads and rhodococci as an example); (3) to elucidate the possibilities of using the $^{13}C/^{12}C$ ratios, the values and trend of carbon isotope fractionation in the analysis of the amount and origin of production during the microbial growth on glucose.

The basis for determining the metabolic potential of bacteria that use glucose as an easily metabolized substrate is: (a) the assessment of CO_2 production per one cell; (b) the kinetics of CO_2 emission as the result of the metabolic activity of microbial population. Special attention was given to revealing carbon isotope fractionation of glucose by representatives of the soil microbiota, pseudomonads and rhodococci capable of utilizing glucose as a sole source of carbon and energy. The advisability of using these microorganisms in the experiments was the fact that they are widespread in soils and in the rhizosphere and are of special significance in the development and implementation of biotechnological processes for degradation of toxic organic pollutants in the environment.

EXPERIMENTAL

Subject of study. Bacterial cultures possessing broad substrate specificity were used. The strain *Rhodococcus* sp. 3-30 (isolated in 2005 from fuel oil-polluted soils of the Kola Peninsula, Apatity, Murmansk Region, Russia) is capable of growth both on glucose and petroleum hydrocarbons. The strain *Pseudomonas aureofaciens* BS1393(pBS216), owing to the occurrence of the naphthalene degradation plasmid pBS216 in its cells, can use, along with glucose, polycyclic aromatic hydrocarbons as a carbon and energy source [14].

Experimental design. Experiments in three repeats were conducted under sterile conditions in hermetically sealed glass vessels. The vessels were filled with quartz sand, 100 g each, ignited at a temperature of 560°C (to completely rule out the foreign organic component). The spherical particles of sand were about 0.6–1.0 mm in diameter. The volume of air in experiments was about 600 mL. The used microbial cultures were pregrown for 24 h in 20-mL liquid medium M9 with 0.2% glucose with isotope composition characterized by a value of $\delta^{13}C = -11.2‰$.

Medium M9 was of the following composition, g/L: Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; aqueous solutions, mL/L: 1 M $MgSO_4$, 2; 1 M $CaCl_2$, 1; pH 7.5. Two latter solutions were sterilized separately and were added into the prepared medium [15].

Cells were sedimented by centrifugation, and then the biomass obtained was transferred into 100 mL of fresh medium M9. The amount of bacteria in the medium was determined by the number of colony-forming units (CFUs) using the method of standard serial dilutions and their inoculation onto agarized LB medium [15]. The titres of *P. aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 cells for inoculation were about 6.0×10^8 and 1.8×10^9 CFU/mL, respectively.

A 20-mL inoculate was used to moisten 100-g quartz sand preliminarily mixed with 200 mg of glucose whose carbon isotope composition was characterized by a value of $\delta^{13}C = -11.2‰$. Sand with added to glucose and bacterial suspension was thoroughly mixed. The initial titres of *P. aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 bacteria were about 1.2×10^8 and 3.7×10^8 CFU/g sand, respectively.

Microbial growth. The quantitative characteristics of microbial growth in the experiments were the titres of cells contained in periodically taken samples of sand, the rates of CO_2 production and oxygen uptakes at particular stages of observation.

The metabolic CO_2 production rate was determined by the amount of carbonate formed within the exposure times (Δt_i). For this, 10-mL vials with 2–6 mL aqueous solution of 1 M NaOH to absorb metabolic carbon dioxide were placed inside the vessels. The amount of CO_2 formed was determined by the volume of 1 M HCl consumed for the titration of the remaining part of alkaline absorbent up to the discoloration of phenolphthalein in solution as an indicator test. Carbon dioxide fixed as sodium carbonate was precipitated as $BaCO_3$ after addition of an aqueous solution of $BaCl_2$. Obtained barium carbonate was washed with distilled water, dried and used for the mass spectrometric carbon isotope assay. The extent of CO_2 fixation with alkaline solution was controlled by the amount of the remaining part of CO_2 in the gas phase by molecular mass spectrometry. The sensitivity

of the method by the content of CO_2 in the gas phase was about 0.01% (v/v).

Using the known kinetic methods for the assessment of microbial activity [4, 5, 16–18], we determined the specific microbial growth rates (μ) in experiments with glucose by the production of CO_2 ($CO_2(t)$) with account for the parameters of the approximate kinetic equation (1):

$$CO_2(t) = K + r \cdot \exp(\mu \cdot t), \quad (1)$$

where K reflects the initial rate of CO_2 respiration of quiescent microorganisms upon their application to sand with glucose; r is the initial rate of growing microbial cells actively metabolizing glucose; t is observation time. Duration of the lag period (t_{lag}) was assessed by the time between applying the inoculate to sand and the time when the increase of the CO_2 respiration rate exceeded the initial respiration rate, i.e., $r \cdot \exp(\mu \cdot t) > K$. With account for the kinetic theory of microbial growth [16, 18], the lag period (t_{lag}) was calculated using the parameters of the approximate curve (equation 1), which reflects the CO_2 respiration rate, according to equation (2):

$$t_{lag} = \ln(K/r)/\mu. \quad (2)$$

Following Zavarzin and Kolotilova [19], as an additional microbial growth rate parameter we used the number of cell doublings (n) at the maximal growth-rate time, which was calculated according to expression (3):

$$n = (\log N_t - \log N_0) \times 3.32, \quad (3)$$

where N_0 and N_t are the amounts of microbial cells (colony-forming units, CFU) at the onset of the exposure, $t = 0$, and after a time $t = \Delta t$, respectively.

Uptake of oxygen. The amount of consumed oxygen at particular stages of microbial growth was determined mass spectrometrically from the change of molecular ion peak intensities of oxygen (m/z 32), nitrogen (m/z 28), argon (m/z 40) and carbon dioxide (m/z 44) in the experiments with respect to their amounts in atmospheric air. The base intensity in the calculations was the value of the molecular ion peak for nitrogen (m/z 28) due to its constant concentration in atmospheric air. Uptake of oxygen ($\eta(O_2)$, mMol) in the gas phase in vessels used in the experiments was calculated according to expression (4):

$$q(O_2) = V_{gas} \cdot \eta_{atm}(1 - R_{exp}/R_{atm})/22, \quad (4)$$

where V (mL) is the volume of the gas phase in the experiments; η_{atm}, the fraction of oxygen in atmospheric air; R_{atm} and R_{exp}, the ratios of the molecular ion peak intensities of oxygen (m/z 32) and nitrogen (m/z 28) in the mass spectra of atmospheric air and the gas phase in the experiments, respectively. The composition of the gas phase was assayed by a Varian CH-7 mass spectrometer (Germany).

Periodically after each stage of exposing microorganisms on quartz sand with glucose (about 24 h), the gas phase in the vessels, which was about 600 mL, was replaced with fresh atmospheric air cleared of carbon dioxide.

Determination of the respiratory quotient. The value of the respiratory quotient (RQ) reflecting the molecular concentration ratios of metabolic CO_2 and consumed oxygen at i stages of microbial growth on a medium with glucose was determined according to expression (5):

$$(RQ)_i = q_i/\Delta(O_2)_i, \quad (5)$$

where q_i (mM CO_2) is the amount of produced CO_2 and $\Delta(O_2)_i$ (mM O_2), the amount of consumed oxygen, respectively.

Carbon isotope characteristic. Gaseous CO_2 served as a working gas in mass spectrometric measurements of the $^{13}C/^{12}C$ isotopic abundance ratios. Barium carbonates produced from metabolic carbon dioxide were again decomposed to CO_2 by concentrated orthophosphoric acid, and organic products (glucose, biomass) were burned to CO_2 in the presence of copper oxide at a temperature of 560°C. The ratios of ^{13}C and ^{12}C isotope abundances were measured by a Breath MAT Thermo Finnigan mass spectrometer (Germany). The carbon isotopic ratios in analyzed samples were calculated in relative units ($\delta^{13}C$) according to the generally accepted equation (6):

$$\delta^{13}C = (R_{sa}/R_{st} - 1) \times 1000‰, \quad (6)$$

where $R_{sa} = [^{13}C]/[^{12}C]$ and $R_{st} = [^{13}C]/[^{12}C]$ are the ratios of ^{12}C and ^{13}C carbon isotope abundances in the sample and in the international standard (PDB) [20]. The error of measuring the isotope characteristics was about ±0.1‰.

RESULTS

Bacterial growth. Amounts of cells registered as CFUs after their washout from the surface of quartz sand served as the main growth indices of representatives of soil bacteria (pseudomonads and rhodococci) used in the experiments. Considering the possible differences in their adhesion to the surface of sand carrier, we compared the efficiency of washing out bacterial cells and their subsequent detection. Thus, according to the conditions of an experiment, *P. aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 bacteria were applied in amounts of 1.2×10^{10} and 3.6×10^{10} CFU, respectively, to 100 g quartz sand mixed with glucose. Efficiency of washout from the surface of sand was controlled by comparing the amounts of initially applied bacteria and those determined by the above technique. As it follows from Tables 1 and 2, the amounts of cells found in the case of *P. aureofaciens* and *Rhodococcus* sp. were, respectively, 9.7×10^9 CFU and 2.4×10^9 CFU. Comparison

Table 1. Increase in the amount of *P. aureofaciens* BS1393(pBS216) cells immobilized on quartz sand and consuming glucose as the sole substrate (CFU, 10^7 g^{-1} sand), number of cell doublings (n, h^{-1}) and cell doubling times (t, h)

Time, h	*CFU × $10^7 g^{-1}$ sand	**CFU × $10^7 g^{-1}$ sand	n, h^{-1}	t, h
0	9.7 (±2)***	12 (±2)	–	–
24	158 (±10)	190 (±12)	4.0 (±0.5)	5.9 (±0.6)
72	1.5 (±0.5)	1.8 (±0.7)		

Notes: * The number of CFU was determined by the results of bacterial cell count after inoculation onto a sand-surface washout selective medium (experiment).
** The corrected number of CFU with account for incomplete bacterial washout (experiment).
*** In brackets, standard deviations of three parallel determinations.

Table 2. Growth of *Rhodococcus* sp. str. 3-30 bacterial cells immobilized on quartz sand and consuming glucose as the sole substrate (CFU, 10^7 g^{-1} sand), number of cell doublings (n, h^{-1}) and cell doubling times (t, h)

Time, h	CFU × $10^7 g^{-1}$ sand*	CFU × $10^7 g^{-1}$ sand**	n, h^{-1}	t, h
0	2.4 (±0.5)***	36 (±7)	–	–
48	3.4 (±0.8)	51 (±12)	0.5 (±0.05)	96 (±9)
96	1.9 (±0.5)	29 (±7)		

Notes: * The number of CFU was determined by the results of bacterial cell count after inoculation onto a sand-surface washout selective medium (experiment).
** The corrected number of CFU with account for incomplete bacterial washout (experiment).
*** In brackets, standard deviations of three parallel determinations.

of the amounts of applied cells and those found in the washout indicates that in the case of *Rhodococcus* sp. the registered amount is 15 times as low, whereas in the case of *P. aureofaciens* the decrease was no more than 20%. The defined correction coefficients were used in detection of the real amounts of bacterial cells in the experiments (Tables 1 and 2).

Tables 1 and 2 give the amounts of colony-forming units of bacteria in 1 g sand, which reflect the growth of *P. aureofaciens* BS1393(pBS216) for 72 h and of *Rhodococcus* sp. 3-30 for 96 h on the surface of quartz sand particles with glucose. In addition to these parameters, using expression (3) we calculated the number of cell doublings (n, h^{-1}) and doubling times (t, h) by the moment the maximal growth rate was achieved. Thus, at a maximal growth rate of *P. aureofaciens* BS1393(pBS216) a fourfold doubling of cell amount was observed 24 h after inoculation, and the average doubling time was about 6 h (Table 1). However, after 3 days of exposure we observed a sixfold decrease of the amount of bacteria as compared with their initial amount. In the case of *Rhodococcus* sp. 3-30, by the moment of the maximal growth rate (about 48 h) the number of cell doublings was less than unity, and doubling time was up to 4 days. Herewith, the amount of bacterial cells applied to sand with glucose decreased in 96 h of exposure by only 20% as compared with the initial amount.

In experiments with both bacterial cultures (*P. aureofaciens* and *Rhodococcus* sp.), in accordance with the increase in the amount of their cells we observed an increase in the metabolic CO_2 production rate (Figs. 1a, Fig. 2a). As the amount of cells in sand decreased, a corresponding decrease was observed in production of metabolic CO_2.

The kinetic parameters reflecting the initial specific rates of *P. aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 growth on glucose were calculated from the production of CO_2 according to equations (1) and (2). Table 3 presents the values of parameter K reflecting the rates of CO_2 production at the initial preparatory stage of bacterial growth, and of parameter μ representing the specific rates of CO_2 formation by growing microbial cells, as well as their lag period (t_{lag}) after application of bacterial cells to the substrate-containing medium.

Respiratory quotient. Amounts of metabolic CO_2 formed at particular stages of growth on glucose by *P. aureofaciens* and *Rhodococcus* sp. (Δt_i), and of molecular oxygen consumed therewith, are given in Tables 4 and 5, respectively. Comparing the ratios of molar amounts of CO_2 formed and oxygen consumed therewith, using expression (4) we calculated the respiratory quotient (RQ, mM CO_2/M O_2) (Tables 4 and 5) as an indicator of the metabolic state of investigated cultures at corresponding growth stages. The value of RQ changed within the range of values from 0.6 up to

Table 3. Kinetic parameters characterizing CO_2 production at the initial stage of growth of *P. aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. str. 3-30 cells immobilized on quartz sand and consuming glucose as the sole substrate

Microorganism	K, μg C-CO_2 h^{-1}	r	μ, μg C-CO_2 h^{-1}	t_{lag}, h
P. aureofaciens	10.3 ± 2*	2.4 ± 1	0.34 ± 0.05	4.3
Rhodococcus sp.	152 ± 24	20.2 ± 8	0.078 ± 0.01	26.1

Note: * Variations of the values of the parameters in equations (1) and (2) were obtained in three parallel experiments I, II and III.

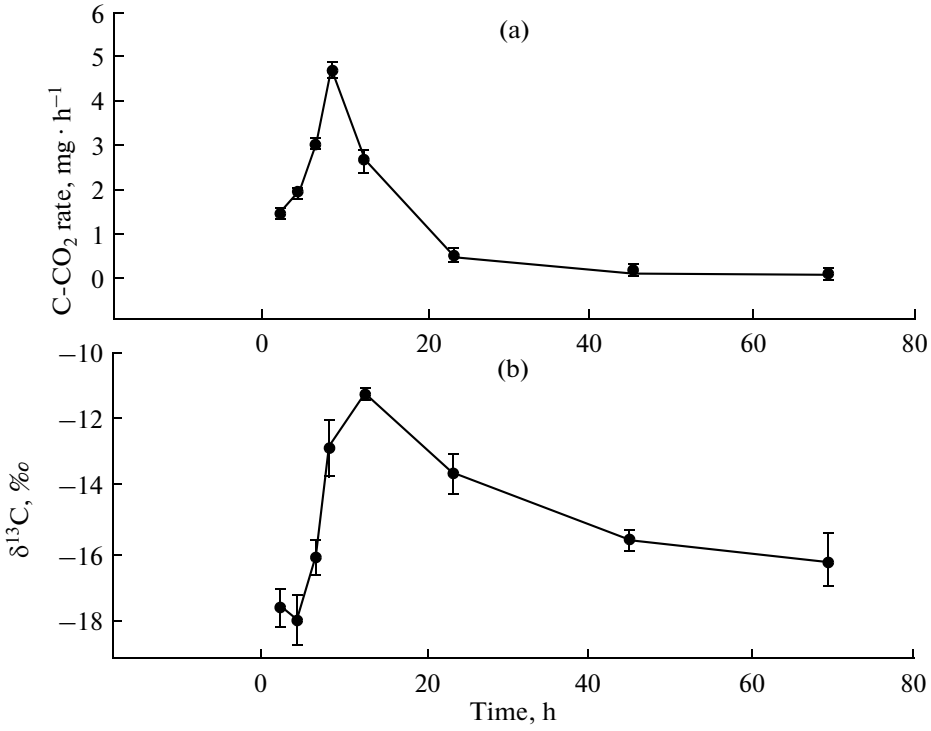

Fig. 1. Dynamics of the change of CO_2 production rate (a) and characteristics of CO_2 carbon isotope composition ($\delta^{13}C$, ‰) (b) during the growth of *P. aureofaciens* BS1393(pBS216) cells immobilized on quartz sand mixed with glucose (data are the result of three parallel experiments).

1.0 mM CO_2/mM O_2 during the growth of both cultures on glucose.

The $\delta^{13}C$ values of microbial products. The initial source of carbon and energy during the growth of the tested bacteria was glucose with $\delta^{13}C = -11.2$‰. The main metabolite formed during the growth of the bacteria was gaseous CO_2, which was continuously fixed initially as sodium carbonate and then was transformed to the water-insoluble salt of barium carbonate. Variations of the $\delta^{13}C$ values for metabolic CO_2 produced by microbial cells immobilized on the surface of quartz sand mixed with glucose are given on Figs. 1b and 2b and in Tables 4 and 5 for particular observation times (Δt, h). To assess the fractionation of CO_2 carbon isotopes as a consequence of its possibly incomplete fixation with alkaline solution, special

Table 4. Amounts of produced CO_2 (mM) and consumed O_2 (mM), the respiratory quotients (RQ) and the CO_2 carbon isotope characteristics ($\delta^{13}C$, ‰) registered within the observation times (Δt, h) during the growth of *P. aureofaciens* BS1393(pBS216) bacteria applied onto the surface of quartz sand

Time, h	[a]Δt, h	[b]CO_2, mM	O_2, mM	RQ	$\delta^{13}C$, ‰
4	4	0.653 (0.023)	1.088 (0.114)	0.60 (0.02)	−17.2 (0.7)
8	4	1.560 (0.054)	1.773 (0.031)	0.88 (0.03)	−12.9 (0.4)
12	4	0.893 (0.113)	1.110 (0.112)	0.81 (0.10)	−11.3 (0.1)
23	11	0.585 (0.044)	0.742 (0.160)	0.79 (0.06)	−13.6 (0.3)
45	21	0.315 (0.100)	0.378 (0.030)	0.83 (0.26)	−15.5 (0.3)
69	23	0.298 (0.210)	0.301 (0.170)	0.99 (0.68)	−16.1 (1.2)
		[c] $\Sigma_{CO_2} = 4.3$ (0.5)	[d] $\Sigma_{O_2} = 5.4$ (0.6)	[e] $RQ_{av} = 0.78$ (0.1)	[f] $\delta^{13}C_{av} = -13.7$ (0.4)

Note: [a] Δ, interval of time within which CO_2 was collected. [b] Amount of metabolic CO_2 carbon, where $q(CO_2) = \Delta t_i \cdot v_i$, and v_i are given in Fig. 1. [c] Σ_{CO_2}, total amount of C-CO_2 collected over 69 h of observation. [d] Σ_{O_2}, total amount of O_2, consumed over 69 h of observation. [e] RQ_{av}, average value of the respiratory quotient. [f] $\delta^{13}C_{av}$, weighted average, where $\delta^{13}C_{av} = (\Sigma q(CO_2)_i \cdot \delta^{13}C(CO_2)_i)/\Sigma_{CO_2}$. Standard deviations of three parallel measurements are indicated in parentheses.

Table 5. Amounts of produced CO_2 (mM) and consumed O_2 (mM), the respiratory quotient (RQ) and the CO_2 carbon isotope characteristic ($\delta^{13}C$, ‰) registered over 115 h of observation during the growth of *Rhodococcus* sp. 3-30 bacteria applied onto the surface of quartz sand

Time, h	[a]Δt_i, h	[b]CO_2, mM	O_2, mM	RQ	$\delta^{13}C$, ‰
4	4	0.072 (0.03)	–	–	–26.1 (0.4)
8	4	0.072 (0.01)	0.30 (0.03)	0.70	–26.3 (0.2)
21	13	0.210 (0.03)	0.38 (0.04)	0.78	–21.0 (1.0)
29	8	0.298 (0.08)	1.30 (0.12)	0.83	–20.1 (0.7)
45	16	1.078 (0.08)	1.12 (0.08)	0.83	–15.1 (0.4)
68	23	0.927 (0.13)	0.65 (0.05)	0.80	–15.6 (0.4)
92	24	0.517 (0.06)	0.50 (0.06)	0.84	–15.5 (0.4)
115	23	0.422 (0.03)			–14.8 (0.2)
		[c]$\Sigma_{CO_2} = 3.6$ (0.5)	[d]$\Sigma_{O_2} = 4.25$ (0.06)	[e]$RQ_{av} = 0.80$	[f]$\delta^{13}C_{av} = -16.5$ (0.4)

Note: [a] Δ, interval of time within which CO_2 was collected. [b] Amount of metabolic CO_2 carbon, where $q(CO_2) = \Delta t_i \cdot v_i$, and v_i are given in Fig. 1. [c] Σ_{CO_2}, total amount of C-CO_2, collected over 115 h of observation. [d] Σ_{O_2}, total amount of O_2 consumed over 69 h of observation. [e] RQ_{av}, average value of the respiratory quotient. [f] $\delta^{13}C_{av}$, weighted average, where $\delta^{13}C_{av} = (\Sigma q(CO_2)_i \cdot \delta^{13}C(CO_2)_i)/\Sigma_{CO_2}$. In brackets, the confidence interval of three parallel measurements.

Table 6. Carbon isotope characteristics of microbial cell biomass ($\delta^{13}C$ (biomass)) grown on glucose and of endogenous metabolic CO_2 ($\delta^{13}C(CO_2)$)

Microorganism	$\delta^{13}C$ (biomass), ‰	$\delta^{13}C$ (CO_2), ‰
P. aureofaciens BS1393(pBS216)	–12.2 (0.5)*	–20.8 (0.4)
Rhodococcus sp. 3-30	–13.5 (0.6)	–21.2 (0.5)

Note: * In brackets, the confidence interval of three parallel measurements.

studies were conducted. From the mass spectrometric assay of the gas phase composition in the experiments we found that the amounts of residual CO_2 not fixed with alkaline solution did not exceed 0.05% of its total amount precipitated as barium carbonate. Therefore, with account of carbon isotope fractionation during the dissolution of CO_2 in water, where $\alpha \approx 1.013$ [21], a possible fractionation of carbon isotopes as the result of incomplete CO_2 fixation was assessed to be about 0.1‰, the value which is on the level of isotope assay error.

In addition to the results obtained in experiments on microbial mineralization of glucose to CO_2 (Tables 4 and 5), the control (Table 6) determined the carbon isotope characteristics of cell biomass grown on glucose ($\delta^{13}C_{biomass,}$ ‰) and "endogenous" CO_2 ($\delta^{13}C_{CO2}$, ‰) produced by cells in the metabolic process using only intracellular products, i.e., in the "starvation" state. The bacterial cells of *P. aureofaciens* and *Rhodococcus* sp. used in the control experiments were sampled at the stage of their exponential growth in liquid mineral medium M9 containing 0.2% glucose with $\delta^{13}C = -11.2$‰. Upon application of cells to clean sand (without glucose), metabolic CO_2 was collected for 24 h and 45 h in the case of the experiments with *P. aureofaciens* and *Rhodococcus* sp. cultures, respectively (Table 6).

DISCUSSION

Microbial cell growth and specific CO_2 production. After the application of *P. aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 inoculates to sand mixed with glucose, the growth rates of the amounts of their cells differed significantly (Tables 1 and 2). Thus, at the maximal growth rates (Table 1, $t =$ 24 h) the amount of *P. aureofaciens* BS1393(pBS216) cells exceeded almost fourfold that of *Rhodococcus* sp. 3-30 with account for the correction coefficient of 15 (Table 2, $t = 48$ h). In accordance with the maximal bacterial growth rates, the metabolic CO_2 production rates were noted to be maximal at the exposure of both cultures. The maximal rate of CO_2 production by the first culture (Fig. 1a) was 6 times as high as the maximal rate of CO_2 production by the second culture (Fig. 2a). It is evident that the total production of CO_2 depended both on the amount of bacterial cells and on

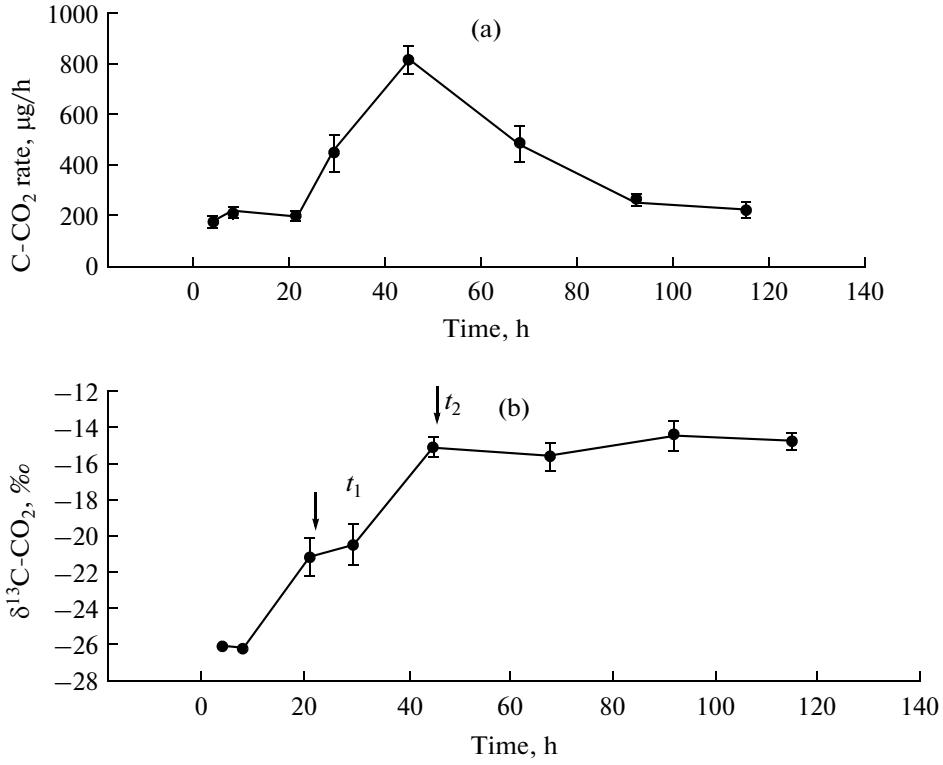

Fig. 2. Dynamics of the change of CO_2 production rate (a) and characteristics of CO_2 carbon isotope composition ($\delta^{13}C$, ‰) (b) during the growth of *Rhodococcus* sp. 3-30 cells immobilized on quartz sand mixed with glucose (data are the result of three parallel experiments). The arrows show the cell growth lag time (t_1) and the time when the CO_2 maximal rate was achieved (t_2).

their substrate-mineralizing activity. For this reason, to characterize the metabolic potential of investigated microorganisms we chose the parameter based on the determination of the amount of CO_2 produced by one bacterial cell, i.e., the specific amounts (per cell or per CFU) of CO_2. Comparison of the maximal amounts of CFUs and CO_2 productions revealed that in the case of the growth of *P. aureofaciens* BS1393(pBS216) on glucose there was 1.94×10^{-9} μM CO_2 per CFU, and in the case of *Rhodococcus* sp. 3-30 about 51×10^{-9} μM CO_2 per CFU and no less than 3.4×10^{-9} μM CO_2 per CFU with account for the correction coefficient. Thus, the mineralization of glucose to CO_2 by *Rhodococcus* sp. 3-30 bacteria per one cell can be from 2 up to 26 times higher than in the case of *P. aureofaciens* BS1393(pBS216). This is evidence of a significant difference in the ratio of the catabolic and anabolic metabolisms of the investigated bacteria during their growth on glucose. Therefore, the currently postulated proportionality between the amount of CO_2 produced and the number of "living" cells using glucose as substrate [1] is not always fulfilled, in particular, for such representatives of soil microorganisms as pseudomonads and rhodococci.

Substrate-induced respiration (SIR). When using glucose as substrate readily metabolized by soil microbiota, the kinetic indices of CO_2 production are some of the important characteristics of microbial activity. The values of the initial CO_2 formation rates (Figs. 1a and 2a) demonstrated significant differences between the representatives of pseudomonads and rhodococci used in the experiments. Thus, in the case of *P. aureofaciens* the maximum of CO_2 production was achieved in 7–8 h, and in the case of *Rhodococcus* sp. only 45 h after the exposure began. Table 3 gives the values of parameters K determined by catabolic processes, and the values of μ reflecting the initial specific rates of CO_2 production by growing microbial cells, as well as their lag periods. As we noted above, a feature of the investigated microbial cultures is a significant difference in their catabolic processes in the uptake of glucose. The value of parameter K reflecting the growth preparation stage of *Rhodococcus* sp. 3-30 is 15 times higher than the value of this parameter for the *P. aureofaciens* BS1393(pBS216) culture (Table 3). At the same time, the specific CO_2 production rate reflecting the exponential growth of *P. aureofaciens* BS1393(pBS216) on glucose (as an anabolic process) was 0.34 ± 0.05 μg C-CO_2 h^{-1} and exceeded almost fivefold the specific growth rate in the case of *Rhodococcus* sp. 3-30, which was about 0.078 ± 0.01 μg C-CO_2 h^{-1}. The noted differences in the values of the kinetic parameters are also consistent with the increased level of the catabolic process revealed above

for *Rhodococcus* sp. as compared with *P. aureofaciens* BS1393(pBS216). A parameter characterizing the growth of bacteria on glucose is the lag time relative to substrate uptake, which in the case of the *P. aureofaciens* BS1393(pBS216) culture was about 4.3 h, and in the case of *Rhodococcus* sp. 3-30 its duration exceeded 26 h (Table 3). Thus, the presented data on the rates of glucose mineralization by the representatives of pseudomonads and rhodococci showed that the use of glucose in SIR at the traditional detection of microbial production of CO_2 for up to 8 h made it possible to reveal only part of the living and active soil microbiota.

Bacterial uptake of glucose. As we noted above, differences in the growth rates of microbial cells were manifested to a significant degree also in the rates of metabolic CO_2 emission (Figs. 1 and 2). Thus, in the case of *P. aureofaciens* BS1393(pBS216) growth the total amount of metabolic CO_2 formed over 72 h of exposure was 4.3 ± 0.5 mM C-glucose or $64 \pm 5\%$ of glucose introduced. The remaining $36 \pm 5\%$ glucose carbon is cell biomass, exometabolites and, possibly, part of unused glucose. It follows from Fig. 1a that the main part of glucose was consumed by microorganisms within 24 h after the experiment began, and the maximal rate of CO_2 production was achieved 8 h after cells were applied to sand to make 5.0 mg C-CO_2 per hour per 100 g sand.

In the case of the growth of the *Rhodococcus* sp. 3-30 culture on glucose, formation of metabolic CO_2 was observed for 115 h. A maximum rate of CO_2 production was registered only 45 h after the application of cells to sand mixed with glucose. The maximal rate was up to 0.85 mg C-CO_2 per hour per 100 g sand (Fig. 2a). From the results of analysis of three parallel experiments with the *Rhodococcus* sp. 3-30 culture the yield of CO_2 over 115 h was 3.6 ± 0.5 mM C-glucose or $54 \pm 5\%$ of introduced glucose. The remaining $46 \pm 5\%$ glucose is cell biomass, exometabolites and part of glucose.

Thus, it follows from the obtained data (Figs. 1a and 2a) that after 24 h in the case of *P. aureofaciens* bacteria and 90 h in the case of *Rhodococcus* sp., production of CO_2 significantly decreases despite the occurrence of about 36% and 46% carbon of introduced glucose, respectively, in the medium. The assumption of the nature of the residual products in the medium (i.e., bacterial biomass and exometabolites) was made based on the isotope composition characteristics, which were determined by means of the isotope mass balance for the registered products, i.e., of CO_2 and substrate (glucose).

Carbon isotope characteristics of microbial products. In experiments with both bacterial cultures growing on glucose (Figs. 1b and 2b, Tables 4 and 5), depending on the metabolic CO_2 production rate, we noted three levels of $\delta^{13}C$ values: (1) changes of $\delta^{13}C$ from the minimal value to a value close to that of glucose (substrate), registered during the lag period and onset of the exponential growth; (2) achievement of $\delta^{13}C$ values close to those of substrate during the time of maximal growth rate and maximal CO_2 production; (3) a decrease of the value of $\delta^{13}C$ in accordance with a decrease of CO_2 production rate. Taking into account the fact that the sole substrate of carbon and energy in the experiments was glucose ($\delta^{13}C = -11.2‰$), the observed changes of the CO_2 carbon isotope characteristics are determined by the fractionation of carbon isotopes during the biosynthesis of intracellular components and possible exometabolites followed by their inclusion into the energy exchange and CO_2 production.

As it follows from Table 4, at the initial stage of *P. aureofaciens* BS1393(pBS216) culture growth, 4 h after cells were applied to sand with glucose, the $\delta^{13}C$ values of metabolic CO_2 were indicative of a decreased content of ^{13}C isotope relative to glucose (i.e., $\delta^{13}C_{CO2} - \delta^{13}C_{glu} = -6‰$). As the production of CO_2 increased and its maximal amount was achieved, this difference decreased almost to 0, i.e., CO_2 inherited the isotope composition of glucose (substrate). At the further decrease of CO_2 production the value of $\delta^{13}C$ demonstrated a tendency to a decrease of the amount of ^{13}C in CO_2, and in 69 h the value of $\delta^{13}C_{CO_2}$ decreased by $-5‰$ as compared with glucose. A similar dependence in the change of CO_2 carbon isotope characteristics was noted earlier for the yeast *Candida lipolytica* grown on glucose. At a decrease of the rate of cell growth and biosynthesis of citric acid as an exometabolite, the amount of ^{13}C isotope in metabolic CO_2 was observed to systematically decrease and the content of this isotope in the exometabolite (citric acid) showed a corresponding increase [12]. For the heterotrophic growth of *E. coli* and *Chlorella pyrenoidosa* bacteria on glucose [13] the lipid fraction of cell biomass was shown to contain 7‰ less ^{13}C isotope as compared with glucose and cell biomass. Therefore, based on the literature data [12, 13] and experimental results (Table 4) the changes of the CO_2 isotope characteristics at various stages of glucose metabolism by *P. aureofaciens* BS1393(pBS216) bacteria can be assumed to be due to the involvement of the lipid fraction of biomass in production of CO_2, as well as the possible production of an exometabolite. It is assumed that bacteria immobilized on sand with glucose consume at the initial growth stage the earlier stored lipid fraction as an energy source required to produce respective enzyme systems, which provide for the transformation of exogenous glucose, e.g., to glucoso-5-phosphate. The occurrence of substrate (glucose) in the medium was an activator of mobilization of intracellular products for the biosynthesis of enzyme systems transforming the substrate into the product used in further metabolism. This process can be a demonstration of the earlier discussed trigger mechanism [22, 23],

owing to which there occurs an additional activation of the mineralization of soil organic matter (SOM) as the result of the introduction of glucose into the soil (the priming effect of glucose). At the subsequent stages of growth, modified glucose and some SOM are used in the biosynthesis of cell components (proteins, lipids, etc.), in production of CO_2 and exometabolites.

The change of the respiratory quotient (RQ) at the initial stage of bacterial growth (Table 4) should be considered as an additional confirmation of the involvement of the lipid fraction in production of CO_2. From the comparison of metabolic CO_2 production and the value of RQ (Table 4) it is seen that 4 h after the beginning of the growth of *P. aureofaciens* BS1393(pBS216) the value of RQ was about 0.6 mM CO_2/mM O_2, and in 8 h at a maximal production of CO_2 the value of RQ reached 0.9 ± 0.1 and was preserved at this level throughout the entire experiment. According to a theoretical estimate, the coefficient reflecting the ratio between the amount of CO_2 formed in the oxidation of lipids to CO_2 and the amount of oxygen (O_2) consumed for this is $RQ_{lipid\text{-}CO_2} = 0.6$ mM CO_2/mM O_2, and in the oxidation of glucose to CO_2 and production of biomass this coefficient is estimated to be $RQ_{glu\text{-}CO_2\text{-}biom} = 1.0$ mM CO_2/mM O_2, respectively. Therefore, the changes of RQ at the initial stage of *P. aureofaciens* BS1393(pBS216) growth on glucose are determined by the involvement of the lipid fraction in the catabolic exchange and the corresponding production of CO_2.

In the case of the second culture, *Rhodococcus* sp. 3-30, at the initial stages of exposure (for 29 h after application of bacteria to sand) the carbon isotope characteristics demonstrated a decreased content of ^{13}C isotope in CO_2 and differed from glucose by −15‰ (Table 5). This result is also considered as evidence of the involvement of the biomass lipid fraction in production of CO_2.

The data of the control experiments with *P. aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 bacteria obtained in the absence of exogenous substrate (glucose), i.e., under conditions of their "starvation", are considered to be a confirmation of the involvement of the lipid fraction in the formation of the isotope characteristics of metabolic CO_2 at the initial stages of bacterial growth on glucose. Thus, metabolic CO_2 formed due to the oxidation of only intracellular metabolites contains 7–8‰ less isotope with respect to carbon of the biomass of cells grown on glucose (Table 6).

It should be noted that upon achievement of the maximal rate of *Rhodococcus* sp. 3-30 growth on glucose (Table 5) the CO_2 carbon isotope characteristics stabilized at the level of $\delta^{13}C = -15$‰, which was indicative of the content of ^{13}C isotope decreased by 4‰ with respect to consumed glucose. With account of the data by Zyakun [12], a possible cause of this feature for the formation of the CO_2 isotope characteristic is the fractionation of carbon isotopes as a consequence of the production of an exometabolite.

Taking into account that 64% of glucose carbon was consumed for production of CO_2 during the growth of *P. aureofaciens,* and the weighted average value of $\delta^{13}C(CO_2) = -13.7$‰ (Table 4), the value of $\delta^{13}C$ characterizing the isotope composition of carbon in the remaining 36% glucose is, according to the isotope mass balance, about −6.8‰.

Performing a similar calculation for the fractionation of glucose carbon isotopes during the growth of *Rhodococcus* sp. bacteria, we find that in the case of the consumption of 54% glucose for production of CO_2 with the weighted average value of $\delta^{13}C(CO_2) = -16.5$‰ (Table 5), the value of $\delta^{13}C$ characterizing the isotope composition of the remaining part of glucose carbon is −5‰. As the carbon isotope characteristics for the remaining products (cell biomass and exometabolites) observed during the growth of both *Pseudomonas aureofaciens* BS1393(pBS216) and *Rhodococcus* sp. 3-30 bacteria on glucose differ significantly from those for glucose ($\delta^{13}C = -11.2$‰), this is considered to be a consequence of carbon isotope fractionation in biosynthetic transformation of glucose carbon into cell biomass and exometabolites.

The value of carbon isotope fractionation factor (ε, ‰) in microbial uptake of glucose in the considered cases is determined by the difference of the carbon isotope characteristics of metabolic CO_2 ($^{13}C(CO_2)$) and organic products of microbial biosynthesis (^{13}C(org.)), i.e., $\varepsilon \approx \delta^{13}C(org.) - \delta^{13}C(CO_2) = 9 \pm 2$‰. Taking into account that $\varepsilon = ((\alpha - 1)/\alpha) \times 1000$‰, the CO_2 carbon fractionation coefficient (α) relative to organic products of microbial biosynthesis was calculated to be $\alpha = 1.009 \pm 0.002$.

Thus, using the carbon isotope characteristic of metabolic CO_2 as an index of glucose and SOM mineralization in the soil microbiota SIR technology, we should take into consideration the extent of glucose uptake and the value of glucose carbon isotope fractionation.

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