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EXPERIMENTAL ARTICLES

Morphological, Physiological, and Biochemical Characteristics of a Benzoate-Degrading Strain *Rhodococcus opacus* 1CP under Stress Conditions

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Abstract—Ability of actinobacteria *Rhodococcus opacus* 1CP to survive under unfavorable conditions and retain its biodegradation activity was assessed. The morphological and ultrastructural features of *R. opacus* 1CP cells degrading benzoate in the presence of oxidants and stress-protecting agents were investigated. The cells of *R. opacus* 1CP were resistant to oxidative stress caused by up to 100 mM H_2O_2 or up to 25 μM juglone (5-oxy-1,4-naphthoquinone). After 2 h of stress impact, changes in the fatty acid composition, increased activity of antioxidant enzymes, and changes in cell morphology and ultrastructure were observed. The strain retained its ability to degrade benzoate. Quercetin had a protective effect on benzoate-degrading cells of *R. opacus* 1CP. The strategy for cells survival under unfavorable conditions was formulated, which included decreased cell size/volume and formation of densely-packed cell conglomerates, in which the cells are embedded into a common matrix. Formation of conglomerates may probably be considered as a means for protecting the cells against aggressive environmental factors. The multicellular conglomerate structure and the matrix material impede the penetration of toxic substances into the conglomerates, promoting survival of the cells located inside.

Keywords: Rhodococcus opacus 1CP, benzoate, degradation, oxidative stress, starvation, dehydration, immobilization, ultrastructural organization, cell morphology

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Developing microorganisms are exposed to various stress factors, which affect living organisms of different level of organization. Cyclic or unscheduled changes in environmental conditions (temperature, humidity, radiation, the presence of toxicants) affect the survivability of a microbial population as a whole. Population growth and development depend on availability of carbon and energy sources, cell density, and the presence of ecological substitutes and antagonists. Due to continuously proceeding biochemical processes, live cells are exposed to reactive oxygen species (ROS). These are generated in the course of normal oxygen metabolism and due to the action of diverse biotic and abiotic stressors. High ROS concentrations cause oxidative stress. They may damage biomolecules (membrane lipids, proteins, and DNA), although at low concentrations they may function as secondary messengers (Storz and Imlay, 1999; Uzilday et al., 2014). The most common ROS are hydrogen peroxide (H_2O_2), hypochlorite (OCl^-), peroxynitrate (ONO_2^-), and hydroxyl radical ($HO^\bullet$). Low-molecular antioxidants and antioxidant enzymes are responsible for decreasing their intracellular level. In bacteria, autooxidation of H_2O_2 by nonrespiratory flavoproteins results in the formation of $O_2^{\bullet-}$ (Korshunov and Imlay, 2010). The level of $O_2^{\bullet-}$ toxic for the cells decreases under the action of superoxide dismutase resulting in formation of intracellular peroxide and oxygen. Catalase converting H_2O_2 to O_2 and H_2O, as well as peroxidase, are responsible for peroxide degradation.

Bacterial response to oxidative stress was studied in detail in *Escherichia coli* (Farr and Kogoma, 1991), *Acinetobacter oleivorans* DR1 (Kim et al., 2015), in pseudomonades (Kim and Park, 2014), in a number of other bacteria, as well as in yeasts (Buryukova et al., 2006; Liu et al., 2012). Among the factors associated with oxidative stress, the authors note biodegrading activity, biofilm formation, interaction with plants, and the impact of heavy metals and antibiotics.

As to the strains degrading various pollutants, there is growing understanding of the fact that successful

application of biological preparations is hampered by transition of microorganisms from laboratory conditions to real environmental conditions, which are not always favorable for survival. One of the causes complicating the use of laboratory strains under natural conditions is the amount of moisture available to the organisms (Moreno-Forero et al., 2016). In nature, when conditions unfavorable for growth occur, microorganisms form dormant forms (spores, cysts), which are well studied in fungi and spore-forming bacteria (Liu et al., 2015). The forms in which non-spore-forming bacteria survive unfavorable conditions and their ability to maintain the species abundance remain insuficiently studied. Under unfavorable conditions, non-spore-forming bacteria are known to form cyst-like cells, which subsequently germinate, results in the development of a vegetative culture (Loiko et al., 2011; Mulyukin et al., 2014). The degree to which the cells are able to retain their biodegradation potential after cessation of unfavorable exposures is little understood.

The goal of the present work was to investigate the stress impact on the population of an actinobacterium *Rhodococcus opacus* 1CP degrading aromatic compounds: its survival, biodegrading and metabolic activity.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The gram-positive non-spore-forming bacterial strain *Rhodococcus opacus* 1CP isolated from an enrichment culture on 2,4-dichlorophenol and able to grow on a number of aromatic compounds, including benzoate, was the study subject (Gorlatov et al., 1989). The spore-forming *Bacillus* sp. strain from the laboratory collection was used as the control when cell survival during storage was assessed.

The liquid mineral medium of the following composition (g/L) was used for cultivation on benzoate: Na_2HPO_4, 0.73; KH_2PO_4, 0.35; $MgSO_4 \cdot 7H_2O$, 0.1; $NaHCO_3$, 0.25; $MnSO_4$, 0.002; NH_4NO_3, 0.75; $FeSO_4 \cdot 7H_2O$, 0.02. The amount of sodium benzoate introduced into the medium was 100–250 mg/L. Cultivation was also carried out in diluted (10%, vol/vol) Luria-Bertani (LB) rich beef-peptone medium. The biomass grown on agar medium was washed off with liquid medium and used as inoculum. Cultivation was performed in 750-mL Erlenmeyer flasks containing 200 mL of the medium at 29°C on a shaker at 220 rpm. Methods of feed-batch cultivation and batch cultivation with different initial substrate concentrations were used for growing the bacteria.

Cell immobilization. The cell suspension of *R. opacus* 1CP (100 mg of wet biomass/mL) was immobilized on Whatman glass fiber paper using the physical sorption method: 10 μL of cell suspension was applied to a paper fragment (4 × 4 mm^2) as a drop 3–4 mm in diameter. The element obtained was air-dried for 30–40 min and fixed on the surface of a Clark oxygen electrode for making measurements or stored in a refrigerator in a dry state at 4°C.

Stress impacts. The effect of stressors was assessed by simulating various unfavorable environmental conditions (starvation, oxidative stress, and desiccation) and by the parameters of cell survival and their ability to degrade benzoate. The activity of the enzyme of initial benzoate attack, benzoate-1,2-dioxygenase (BDO) (EC 1.14.12.10), possessing a narrow substrate specificity was used as a marker of biodegradation activity. The metabolic activity of the cells was assessed by recording respiratory activity (O_2 consumption) of the cells resuspended in 50 mM Tris-HCl buffer.

Cell cultures were desiccated in three variants. (1) The cells were grown in flasks containing mineral medium (100 mL) with benzoate (200 mg/L) or 4-chlorophenol (50 mg/L) as the sole growth substrate to attain OD_{545} = 1.5–1.8 U on a shaker at 220 rpm. The flasks remained on the shaker until the medium dried completely and were subsequently stored at 4°C. (2) The cells were grown on LB slants at 28°C and incubated at this temperature until the agar medium was completely dry. (3) The cells were grown in a liquid mineral medium with benzoate (200 mg/L) to attain OD_{545} = 1.5 at 28°C. A series of sequential dilutions in sterile distilled water to 10^{-9}–10^{-11} was then made, and petri dishes with LB agar were inoculated with 100 μL of cell suspensions. Single grown colonies were air-dried at room temperature for 2–4 months.

For the cells to germinate, each flask was supplemented with 100 mL of sterile mineral medium containing benzoate (200 mg/L) or 4-chlorophenol (50 mg/L) and incubated at 28°C at 220 rpm. Mineral medium (15 mL) and benzoate (200 mg/L) or 4-chlorophenol/2,4,6-trichlorophenol (50 mg/L) were added to the test tubes with desiccated slant agar. Petri dishes were poured over with 15 mL of mineral medium, and after 2 h the agar plate was transferred into the flask containing 100 mL of mineral medium and benzoate (100 mg/L) or 4-chlorophenol/2,4,6-trichlorophenol (50 mg/L). Growth was assessed by the OD_{545} increase and substrate consumption.

Impact of the toxic action of solvents (benzene, toluene, styrene, hexane, dimethylformamide (DMFA)) and hexadecane was assessed by detection of nucleic acids in the culture liquid (CL) (Gaur and Khare, 2009). *R. opacus* 1CP cells were grown on benzoate (200 mg/L) to attain optical density (OD_{545}) of 0.6 U; the solvents were added (the final concentration 1%); and after 1 and 24 h the nucleic acid content in CL was determined at 260 nm.

The impact of oxidative stress was investigated by growing the cells in the mineral medium with benzoate as growth substrate. Benzoate was introduced fractionally (100 or 200 mg/L) as it was consumed. Hydrogen peroxide (H_2O_2) was added to the cultures to the

final concentration of 25, 50, 100, 150, or 200 mM. Juglone (5-oxy-1,4-naphthoquinone) was added for the final concentration of 5, 10, or 25 mM. Menadione (2-methyl-1,4-naphthoquinone) was added to the cultures at 0.1, 1, or 10 mM; quercetin (3,3',4',5,7-pentahydroxyflavon), at 0.1, 1, or 10 mM. The oxidizers were added at different stages of cultivation: (1) immediately after inoculation (the initial OD_{545} = 0.1 U) and (2) into the culture with OD_{545} = 0.6 U (corresponded to the first third–half of the exponential growth phase). The cells were preadapted to H_2O_2 by introducing it into a benzoate-grown (100 mg/L) culture with OD_{545} = 1.0 U at the final concentration of 1 or 5 mM for 1 h. The biomass from these flasks was used to inoculate the flasks with the mineral medium containing benzoate (100 mg/L) and H_2O_2 (25, 50, 100, 150, or 200 mM). Oxidizer-free cultures (without H_2O_2) served as the control.

Polarographic determination of the respiratory activity and BDO activity. A Clark oxygen electrode was used with the Ingold 5313/10 O_2 amplifier (Instrumentation laboratory (MI), Switzerland–United States). The signal was recorded with an XY Recorder-4103 (Laboratorium přistroje Praha, Czechoslovakia). In order to determine the respiratory activity and BDO activity, the cells freshly grown on benzoate (200–250 mg/L) were harvested by centrifugation (16000 *g*, 15 min, 4°C), washed with 50 mM Tris-HCl buffer (pH 7.6), resuspended in the same buffer, and used immediately for analysis. The measurements were made in 50 mM Tris-HCl buffer (pH 7.6) air-saturated with oxygen at room temperature in an open 5-mL cuvette equipped with a stirrer. To determine the respiratory activity after recording basic cell respiration, stirring was stopped and the change in the oxygen concentration was recorded using the Clark oxygen electrode. The recoded signal reflected the respiratory rate (respiratory activity) of the culture. The respiratory rate was expressed in μg O_2/(L s). To determine the BDO activity, a change in oxygen consumption by cells in the presence of a benzoate or substituted benzoate solution was measured with the Clark oxygen electrode after recording base cell respiration. The recorded signal reflected the enzyme reaction rate—the reaction between BDO and benzoate or a benzoate derivative. The reaction rate was expressed in pA/s (1 pA/s ~ 0.153 μg O_2/(L s)).

In order to determine the fatty acid composition of the cells, they were grown on a rich medium (LB) to attain OD_{545} = 1.5–2.0 U, with the subsequent addition of H_2O_2 to 50 or 100 mM. After 2 h of incubation, the cells were harvested by centrifugation; the lipids were isolated using the Bligh–Dyer method (Bligh and Dyer, 1959); and fatty acids were identified as their methyl esters. Fatty acid methyl esters were obtained in the reaction with acetyl chloride (6 vol %) in methanol (Christie, 1993). The cells of the culture grown on benzoate served as the control. The fatty acid composition of the cells was determined with chromatography-mass spectrometry on a 6890N gas chromatograph (Agilent Technologies, United States) with a 5973 N mass-detector (Agilent Technologies, United States), an HP-1 column (with the length of 50 m, diameter of 0.32 mm, and the immobile phase layer of 0.52 nm thick) at an injector temperature of 250°C. Thermostat temperature programing was as follows: 80°C, the isotherm 1 min; linear growth at 10°C/min to 290°C. The chromatograph was equipped with the NIST98 mass-spectrometry database.

Determination of the enzyme activity. The activity of the enzymes involved in the degradation of benzoate, catechol-1,2-dioxygenase (Cat-1,2-DO), and protocatechuate-3,4-dioxygenase (PCA-3,4-DO) of *R. opacus* strain 1CP was determined as described earlier (Solyanikova et al., 2015). The catalase (EC 1.11.1.6) activity was determined according to Aebi method (Aebi, 1984); the superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured using the indirect spectrophotometric assay proposed by Kostyuk et al. (Kostyuk et al., 1990); the ascorbate peroxidase (EC 1.11.1.11) and peroxidase (EC 1.11.1.7) activities were determined as described earlier (Habib et al., 2014); the glutathione reductase (EC 1.6.4.2) activity was measured using the method described by Li et al. (Li et al., 2003).

Light microscopy. Light microscopy under phase contrast was conducted using a Nikon Eclipse Ci microscope with a Jenoptic ProgRes®SpeedXT^{core}5 camera.

Electron microscopy. In order to prepare ultrathin sections, the cells were concentrated by centrifugation (10000 *g*, 15 min) and fixed in a 0.05 M cacodylate buffer (pH 7.2) containing 2% glutaraldehyde. The material was then washed thrice in 0.05 M cacodylate buffer (pH 7.2) and additionally fixed with 2% OsO_4 in the same buffer for 4 h at 18–20°C. The dehydrated material was then embedded in Epon 812 epoxy resin. The sections were mounted on support grids, contrasted for 30 min in 70% alcohol containing 3% uranyl acetate, and additionally contrasted with lead citrate according to Reynolds (Reynolds, 1963). Ultrathin sections were examined under a JEM-1200EX transmission electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

RESULTS AND DISCUSSION

Viability of *R. opacus* 1CP cells in suspension. Storage of *R. opacus* 1CP cells as suspension in 50 mM Tris-HCl buffer, pH 7.6, at 4°C resulted in cell respiration decreasing uniformly during starvation, irrespective of the growth substrate (benzoate) concentration: from 250 μg/L to 6 g/L. As may be seen from the data shown on Fig. 1a, respiratory decline did not exceed 25% over seven days. Senescence of the cell suspension was accompanied by a gradual decrease in the BDO activity, which makes perfect sense (Fig. 1b).

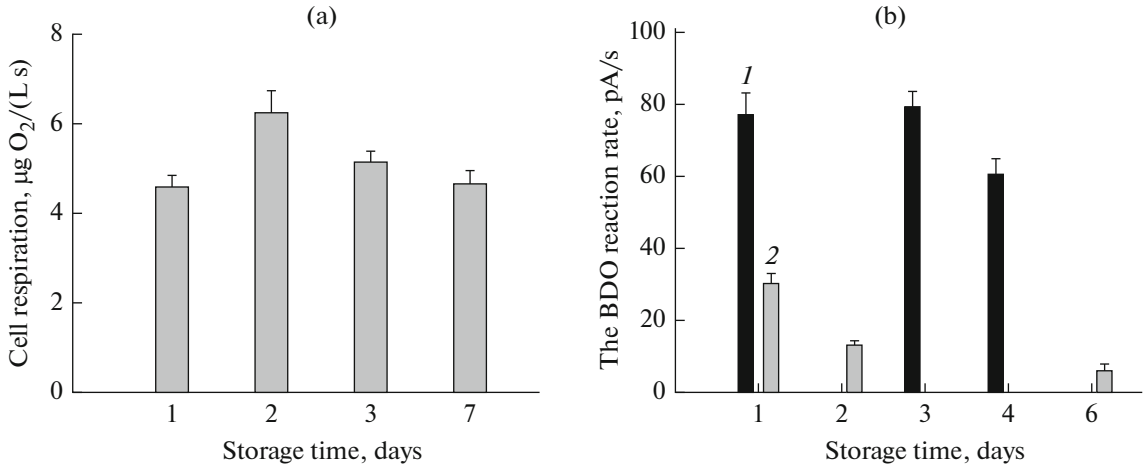

Fig. 1. Change in respiration (a) and the reaction rate of benzoate 1,2-dioxygenate (reaction when 3.47 mM benzoate was added to the reaction cuvette) (b) of *R. opacus* 1CP cells during storage of actinobacterial suspension (4°C): the cells were grown in medium with 2 g/L benzoate (*1*) and with 4 g/L benzoate (*2*).

After 14 months of storage of cell suspension (the resting stage) grown in the culture with 200 mg/L benzoate, the BDO reaction rate was 21 pA/s; respiration was retained at a low but stably reproducible level.

Cell storage at a higher temperature (25°C) led to a complete decline in BDO activity. However, the effect of this temperature was reversible. Addition of benzoate (300 mg/L) to the cells stored at 25°C for four months in which no BDO activity was observed resulted in their increased respiratory activity. The process was accompanied by BDO induction: the activity increased 40-fold over the first 24 h after addition of the growth substrate (Fig. 2). Induction by benzoate also activated cell response to other possible BDO substrates (table).

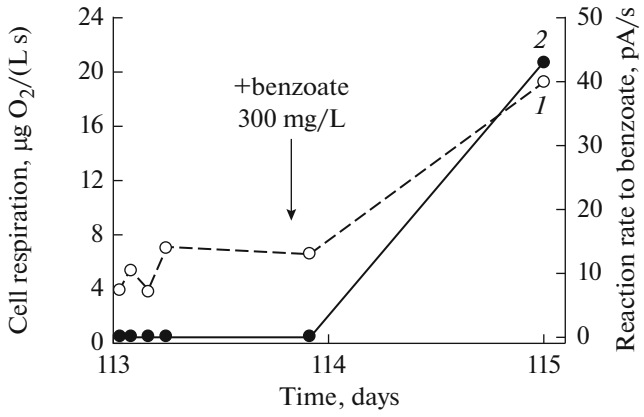

Fig. 2. Change in respiration (*1*) and reaction of benzoate-1,2-dioxygenase (BDO) with benzoate (*2*) of the cells of *R. opacus* 1CP in the course of BDO induction by benzoate. The moment of injection of benzoate into the suspension is indicated with an arrow.

Effect of desiccation on the viability of *R. opacus* 1CP cells. Desiccation of *R. opacus* 1CP culture cells on agarized LB medium at elevated temperature (30°C) without immobilization led to a considerable loss of the number of viable cells. Only in two of fourteen cases, the cells of *R. opacus* 1CP desiccated and stored for 0.5–2 years resumed growth on addition of a liquid LB medium or a mineral medium with 2,4,6-trichlorophenol after 1 and 3 months of incubation, respectively. In the control variant (the spore-forming *Bacillus* sp. strain), growth began after 48 h of cultivation in liquid LB medium.

Fig. 3 presents the data on the respiration rate and the BDO activity of *R. opacus* 1CP cells immobilized on GF glass fiber paper, dried to air-dry state at 20°C, and stored at 4°C for 10 days in comparison to the cells of the suspension used for immobilization. After immobilization of the cells of the suspension stored for 14 months at 4°C, the response of immobilized cells to benzoate substantially exceeded the response obtained for the suspension.

In the opinion of Urbano et al., rhodococci are highly resistant to desiccation (Urbano et al., 2014). Desiccation is accompanied by formation of reactive oxygen species causing oxidative stress, which results in damage to cell structures. The authors demonstrated a relationship between lipid metabolism and response to oxidative stress in extremophilic rhodococci. LeBlanc et al. showed a possible involvement in the regulation of response to cell desiccation of a number of additional genes, including the gene *dps1* encoding the defense protein against oxidative stress, the function of which was previously not associated with desiccation, and two genes encoding the sigma factors SigF1 and SigF3 (LeBlanc et al., 2008).

Effect of solvents on the viability of *R. opacus* 1CP cells. The effect of organic solvents—aromatic and aliphatic compounds (benzene, toluene, styrene, hex-

Rate of response to substrates/substrate analogs for BDO of *R. opacus* 1CP cells (113 days of storage at 25°C)

Substrate	Rate of response (%) of cell suspensions to the substrate after	
	22 h of aeration	20 h of induction by benzoate (300 mg/L)
Benzoate	0	100
2-Chlorobenzoate	ND	20
3-Chlorobenzoate	ND	21
4-Chlorobenzoate	0	18
3-Methylbenzoate	ND	47
4-Methylbenzoate	0	38

ND stands for "not determined." 100% = 43.08 ± 0.12 pA/s.

ane, hexadecane) and DMFA on the cells of *R. opacus* 1CP was studied. *R. opacus* 1CP did not appear to be capable of growth on benzene, toluene, and hexane (1%). The culture grew and degraded styrene and hexadecane (1%). While DMFA at a concentration of up to 1% did not exert a negative influence on cell growth with phenol and 4-chlorophenol, it did not stimulate growth and was not used as a growth substrate.

The toxic effect of aromatic and aliphatic compounds on the cells of *R. opacus* 1CP growing on benzoate was estimated using the changes in the cell membrane permeability to nucleic acids. It is assumed that the solvents with different values of $\log P_{ow}$ are known to have a different effect on cell survival (Gaur and Khare, 2009). It is assumed that the lower this value (e.g., 2.64 for toluene), the more toxic the compound is. In the case of *R. opacus* 1CP, OD_{260} changed insignificantly in the control (from 0.210 to 0.276 after 24 h). In all experimental variants absorption was reliably higher than in the control. Thus, addition of hexane ($\log P_{ow}$ = 3.86) resulted in absorption increasing to 0.282 after an hour and to 0.356 after 24 h. Toluene caused more pronounced changes: OD_{260} after 1 and 24 h was 0.611 and 0.852, respectively. Styrene ($\log P_{ow}$ = 2.86), which was a growth substrate for *R. opacus* strain 1CP, also caused release on nucleic acids into the medium (OD_{260} = 0. 561 after an hour). This value, however, did not increase after 24 h of growth. This may be explained by induction of the enzymes involved in styrene degradation by this strain (Tischler et al., 2010). For hexadecane, no significant OD increase was found after 24 h of exposure to this compound. It is possible that, as in the case of styrene, this is explained by the ability of the strain *R. opacus* 1CP to degrade hexadecane.

The impact of oxidative stress on the degrading activity of *R. opacus* 1CP. Behavior of the culture of *R. opacus* 1CP was investigated in the presence of different oxidizing agents (H_2O_2, juglone, and menadione) during growth on benzoate, including the presence of quercetin.

The bactericidal and bacteriostatic effects depended on the oxidizer concentrations. The culture of *R. opacus* 1CP is able to degrade benzoate at a concentration of up to 10 g/L (Solyanikova et al., 2016). The lag period for the cells inoculated into a liquid medium with 100 mg/L benzoate was 8 h (OD_{545} upon inoculation ~0.1). Under such conditions, simultaneous introduction of benzoate and H_2O_2 in the 50–200 mM concentration range inhibited cell growth completely.

Introduction of potential inhibitors and protectors 24 h after inoculation of the benzoate medium, when the culture was at the beginning of the logarithmic growth phase, made it possible to differentiate the action of the compounds added. For example, the cells grown on mineral medium with benzoate (200 mg/L) did not differ significantly in the maximal specific growth rate and the doubling time values upon introduction of benzoate (200 mg/L) or LB (10% vol/vol) 24 h after the beginning of cultivation (Fig. 4, curves *1, 2*; an interval of 24–30 h from the beginning of the experiment). The maximal optic density of the culture depended on the amount of the growth substrate introduced (Fig. 4, curves *1, 2*; an interval of 30–50 h

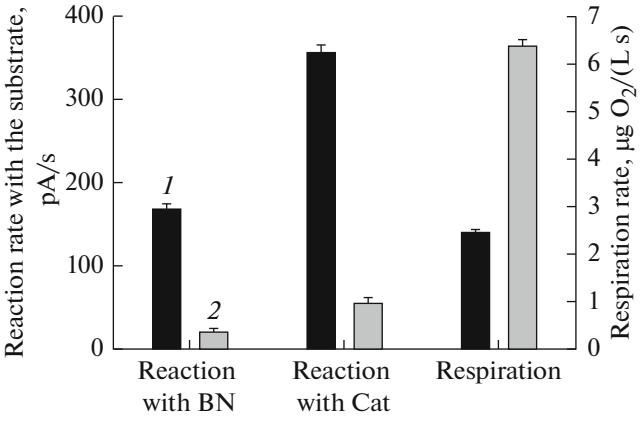

Fig. 3. Comparison between respiration, BDO activity with benzoate (BN), and reaction with catechol (Cat) for *R. opacus* 1CP cell suspension grown on benzoate (*2*) and for the same cells immobilized on carier and stored during 10 days (*1*).

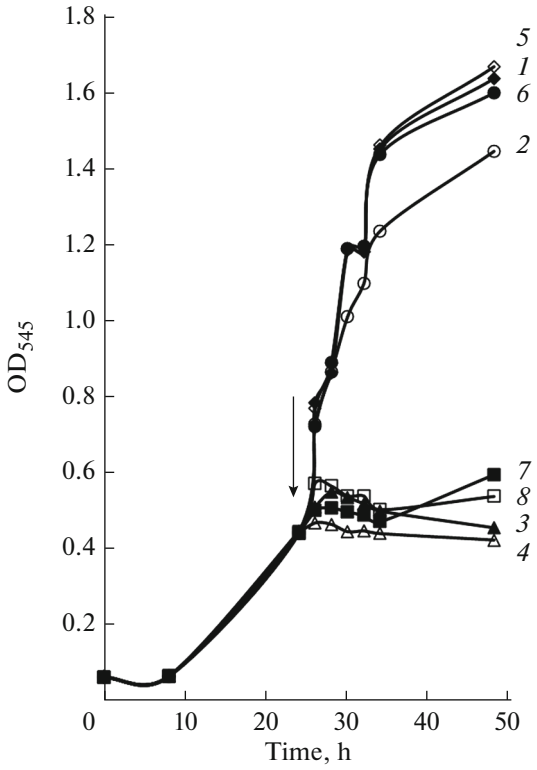

Fig. 4. Growth curves of *R. opacus* 1CP cells in mineral medium with benzoate (200 mg/L) with the subsequent addition after 24 h of: benzoate (200 mg/L) (*1*), 10% (vol/vol) LB (*2*), benzoate (200 mg/L) + 100 mM H_2O_2 (*3*), 1 mM quercetin (*4*), benzoate (200 mg/L) + 0.1 mM quercetin (*5*), benzoate (200 mg/L) + 1 mM quercetin (*6*), benzoate (200 mg/L) + 0.1 mM quercetin + 100 mM H_2O_2 (*7*), and preadaptation at 10 mM H_2O_2 with the subsequent addition of benzoate (200 mg/L) + 100 mM H_2O_2 (*8*).

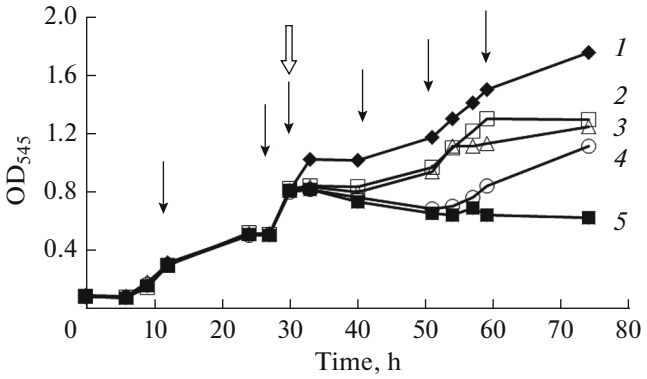

Fig. 5. Relationship between growth (OD_{545} increase) of the *R. opacus* 1CP culture growing on benzoate (fractional addition of 200 mg/L) and the H_2O_2 concentration, mM: 0 (*1*), 25 (*2*), 50 (*3*), 100 (*4*), and 200 (*5*). Benzoate was added at 0 h and hereinafter as indicated with thin arrows. The moment of hydrogen peroxide introduction is designated with an empty arrow.

from the beginning of the experiment). No resumption of culture growth was observed in the variant of oxidative stress development (200 mg/L benzoate + 100 mM H_2O_2) (Fig. 4, curve *3*).

The cells of the culture grown on benzoate to OD_{545} = 0.6–0.8 (the total substrate consumption was 300 mg/L) exhibited increased tolerance to the stress effect of H_2O_2, namely, complete inhibition of growth was noted on addition of 200 mM H_2O_2 (Fig 5). A decrease in the H_2O_2 concentration to 150 mg/L also induced irreversible cessation of growth of nonadapted cells (data not shown).

Thus, 150 mM H_2O_2 appeared to be the critical concentration for the culture not adapted to oxidative stress: its introduction suppressed cell growth and it did not resume. Other H_2O_2 concentrations (25, 50, and 100 mM) inhibited growth of the cells in the delayed linear growth phase, up to 20 h; however, later OD_{545} of the culture increased, although it remained lower than in the control (without H_2O_2) (Fig. 5).

Preliminary cultivation of the cells of *R. opacus* 1CP in the presence of low H_2O_2 concentrations resulted in cell preadaptation to the concentrations causing the death of nonadapted cells. For example, irreversible cessation of growth of nonadapted cells was observed at 150 mg/L H_2O_2. Preliminary cultivation on benzoate (200 mg/L) in the presence of 1 or 5 mM H_2O_2 for 1 h allowed the cells to retain their capacity for growth in the presence of H_2O_2 at concentrations of up to 150 mM inclusive (data not shown). Increasing the inducing H_2O_2 concentration to 10 mM also produced a positive effect on the ability of the cells to degrade benzoate. The data presented on Fig. 4 show that the culture to which 100 mM H_2O_2 was added after 24 h of growth on benzoate did not resume growth over the subsequent 24 h (Fig. 4, curve *3*). On the contrary, in the case when after 24 h of growth 10 mM H_2O_2 and after 2 h 100 mM H_2O_2 was introduced into culture, OD_{545} increased over the subsequent 22 h (48 h from the onset of the experiment) (Fig. 4, curve *8*). Further cultivation in the presence of benzoate (200 mg/L) (72 h from the beginning of cultivation, the data are not shown) was accompanied by an increase in OD_{545}; however, this value was lower than in the benzoate (200 mg/L) or 5% LB variants (Fig. 4, curves *1*, *2*). A positive influence of preliminary cultivation with sublethal hydrogen peroxide concentrations on survival of the cells was shown for yeasts (Biryukova et al., 2006; Liu et al., 2012). For example, incubation of *Candida oleophila* with 5 mM H_2O_2 increased tolerance of this yeast not only to oxidative stress but to high temperature (40°C) and low pH values (Liu et al., 2012).

Cytological investigations showed that in the control variant, when *R. opacus* 1CP was cultivated in a rich organic medium, the culture was characterized by the presence of branching cell forms (Fig. 6a). Bacterial growth in the mineral medium with benzoate in the absence of H_2O_2 was accompanied by formation of

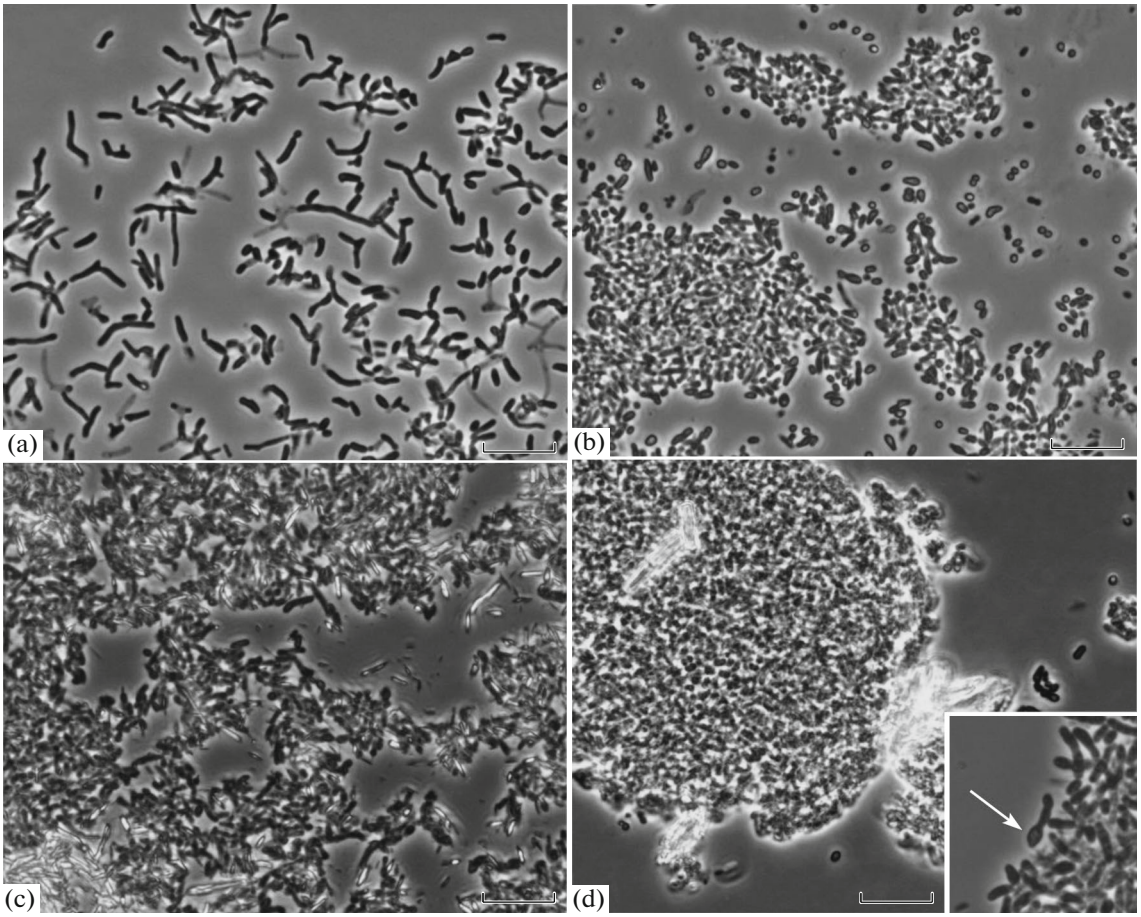

Fig. 6. *R. opacus* 1CP grown in LB medium (a), on benzoate at a concentration of 100 mg/L (b), on benzoate in the presence of 10 mM quercetin (c), and on benzoate in the presence of 10 mM menadione (2-methyl-1,4-naphthoquinone) (d). Inset: a fragment with club-shaped cells at 0.1 mM menadione. Light microscopy. Phase contrast. The bar scale length is 10 μm.

cell conglomerates consisting of refractory cells in the form of short rods or ovoids (Fig. 6b). The population as a whole was characterized by slightly marked cell polymorphism.

Investigation of the ultrstructure of the cells grown on benzoate showed that a feature of these cells was the appearance of single electron-dense polyphosphate (PP) inclusions and the presence in the cytoplasm of numerous electron-transparent polyhydroxyalkanoate (PHA) inclusions, which were probably responsible for the cell refractoriness (Fig. 7).

Under the conditions of growth on benzoate in the presence of 50 and 100 mM H_2O_2, the populations of *R. opacus* 1CP were characterized by smaller sizes of both individual and aggregated cells, compared to the populations growing in the absence of hydrogen peroxide. The cells in the form of short rods or ovoids, often with a high degree of refractoriness, were predominant in it. When the culture grew on benzoate in the presence of H_2O_2 at a concentration of 100 mM, lysed cells in the form of empty envelopes (sheaths) appeared in the culture. Investigation of stress-affected cells of *Candida albicans* showed that adaptation to oxidative stress resulted in increased general stress tolerance (Jakab et al., 2014). However, the data on fungal cell morphology obtained by the authors did not reveal such a variety of the forms obtained when we studied the bacterium *R. opacus* 1CP.

The electron microscopic study of ultrathin sections of the cells grown in the presence of 50 mM H_2O_2 showed that the cytoplasm of the cells grown under these conditions, apart from electron-transparent PHA inclusions, was almost completely filled with small electron-dense polyphosphate (PP) inclusions, which abounded in both the nucleoid zone and on the periphery of the cytoplasm (Fig. 8). In the presence of 100 mM H_2O_2, small electron-dense PP granules aggregated to form large conglomerates at the cell poles (Fig. 8).

The influence of juglone, a stronger oxidizer than H_2O_2, on the cells of *R. opacus* 1CP (the initial $OD_{545} = 0.25$) was studied. Earlier, it was established that while H_2O_2 at a concentration higher than 120 mM completely inhibited yeast growth, juglone did it at lower concentrations of 30 μM and higher

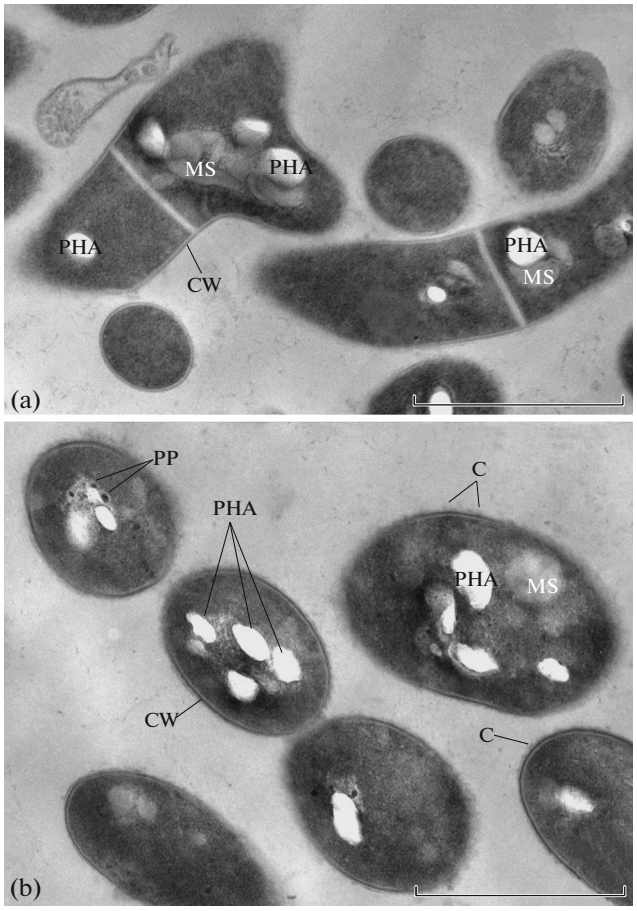

Fig. 7. Ultrathin sections of the cells of *R. opacus* strain 1CP upon growth in LB medium (a) and medium with benzoate (b). Designations: CW, cell wall; C, capsule; PHA, polyhydroxyalkanoate inclusions; PP, polyphosphate granules; MS, myeline-like structures. The bar scale length is 1 μm.

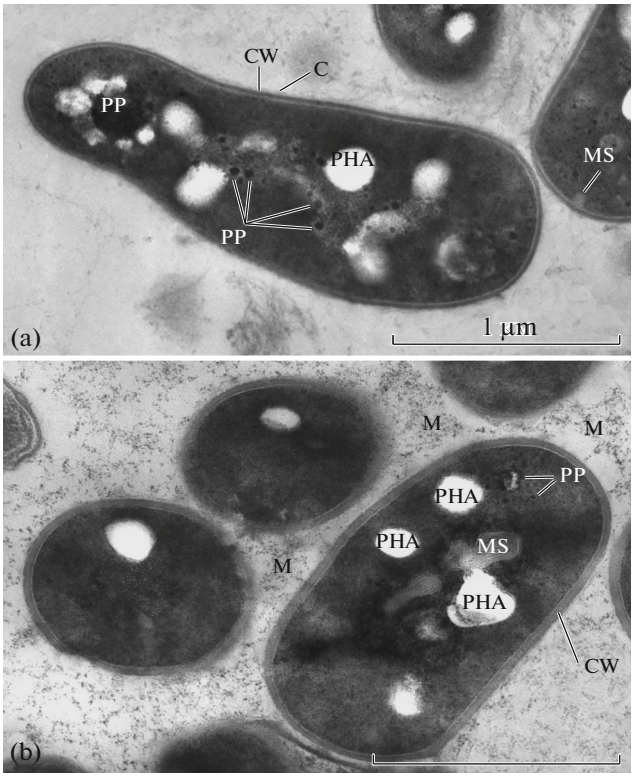

Fig. 8. Ultrathin sections of the cells of strain 1CP upon growth in the medium with benzoate in the presence of 50 mM H_2O_2 (a) and in the medium with benzoate in the presence of 1 mM menadione (b). Designations: CW, cell wall; C, capsule; PHA, polyhydroxyalkanoate inclusions; PP, polyphosphate granules; MS, myeline-like structures; M, intercellular matrix. The bar scale length is 1 μm.

(Biryukova et al., 2006). By analogy, in order to reveal the toxic action of juglone on the cells of the strain studied, oxidizer was used in the 5–25-μM concentration range. No toxic effects of juglone on the cells of *R. opacus* 1 CP were revealed in this concentration range.

After that, the impact of quercetin on the growth and ultrastructural organization of *R. opacus* 1CP cells was investigated. Quercetin is a plant flavonoid whose role is widely discussed, although no unambiguous conclusion as to the vector of its effect on organisms was reached (Harwood et al., 2007). Many publications give evidence of the antioxidant effect of quercetin on eukaryotes (Periasamy et al., 2016). However, several authors question the presence of a positive influence of quercetin on the organisms of eukaryotes and prokaryotes. For example, the antimicrobial action of quercetin (Cushnie and Lamb, 2005) based on inhibition of the activity of bacterial gyrase, which is responsible for DNA supercoiling (Plaper et al., 2003), on induction of DNA cleavage and, additionally, inhibition of the functions of the plasma membranewas also were shown (Fetzner, 2012). The preliminary results obtained by us show that quercetin behaves as a stress protector in our case. This was manifested by the lack of inhibition of the rate of benzoate degradation by the cells and by the changes occurring in the cells at the ultrastructural level. First, it was shown that the cells were not able to grow on quercetin as a growth substrate (Fig. 4, curve *4*). Note that cell cultivation on benzoate in the presence of quercetin did not result in significant deviations from the growth curve for benzoate alone (Fig. 4, curves *5, 6*). It was also established that the cytoplasm of the cells grown on benzoate in the presence of quercetin (Fig. 6c) contained single small electron-transparent PHA inclusions and small electron-dense PP inclusions (Fig. 9). Thus, in the presence of quercetin, cell morphology was similar to their state in the absence of oxidizing agents. Moreover, addition of quercetin to the cells cultivated with benzoate in the presence of 100 mM H_2O_2 led to faster resumption of growth (Fig. 4, curve *7*) compared to the variant where quercetin was absent (Fig. 4, curve *3*). This pattern was revealed for both young cells (Fig. 4) and those grown on benzoate to the last third of the exponential phase (48 h),

after which H_2O_2 or H_2O_2 + quercetin additives were introduced (data not shown).

Bacterial growth in the mineral medium with benzoate (200 mg/L) in the presence of 0.1 mM menadione was associated with emergence of large club-shaped cell forms inside the conglomerate and on its surface (Fig. 6d). Analysis of the cell phase contrast images showed that, under these conditions, characteristic polar swellings were formed as a result of formation and local accumulation of multiple small spherical refractory inclusions at one of the poles. Increasing the menadione concentration to 1 mM resulted in formation of cell conglomerates predominantly consisting of large, long, curved refractory cell forms both on the conglomerate surface and inside it; the conglomerates were characterized by densely packed cells.

The data obtained allow us to propose using *R. opacus* 1CP as a producer for the development of the technology for the manufacture of industrially significant compounds, in particular PHA using a cheap growth substrate (benzoate) in the presence of H_2O_2.

Impact of oxidative stress on the cell fatty acid composition. Saturated and unsaturated fatty acids were detected in the cell composition. Exposure to hydrogen peroxide caused a decrease in fatty acids: $C_{14:0}$ (from 3.1 to 2.8%), 15-methyl-$C_{16:0}$ (from 11.1 to 9.7%), and $C_{19:0}$ (from 15.8 to 13.7%). The content of unsaturated fatty acids increased: 11-methyl-$C_{14:1}$ (from 5.6 to 6.5%), 9-$C_{16:1}$ (from 15.3 to 18.3%), 8-$C_{18:1}$ (from 6.5 to 7.3%). In the variants of peroxide-treated cultures, there was a 2% increase in the content of saturated acids, such as 14-methyl-$C_{16:0}$ (from 1.9 to 3.9%) and $C_{18:0}$ (from 0 in the control 2%). Thus, a general tendency for the relative content of saturated fatty acids to decrease and the content of unsaturated fatty acids to increase was observed. Comparison between the fatty acid compositions of the cells exposed to 50 and 100 mM H_2O_2 did not, on the whole, reveal any correlation between changes in the fatty acids content and the H_2O_2 concentration, except for the unsaturated 9-$C_{16:1}$ acid, when its content increased with a rise in the H_2O_2 concentration: the control, 15.5%; 50 mM H_2O_2, 16.4%; 100 mM H_2O_2, 18.3%.

Thus, apart from a change in cell morphology, a change in the fatty acid composition of peroxide-treated cells was revealed. Interesting results were obtained when the effect of salt stress on the cells of *Rhodococcus erythropolis* strain was studied (de Carvalho et al., 2014). The authors noted synthesis of polyunsaturated fatty acids (in our case, an increase in the synthesis of methylated monounsaturated acids) in response to the stress impact of sodium chloride after a short-term exposure (up to 35 min) to this stress agent. However, the authors, having identified the fatty acids and investigating the processes of cell adaptations, did not provide the the data on cell morphology within the population.

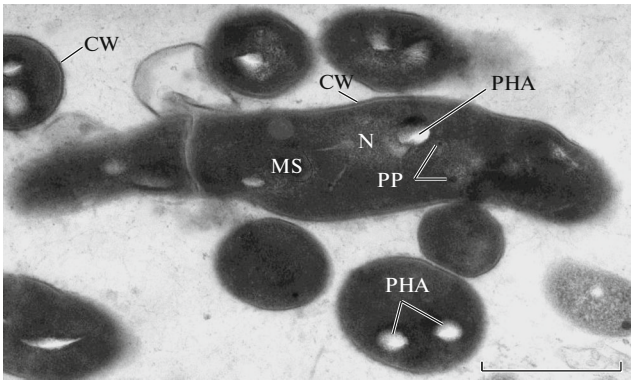

Fig. 9. Ultrathin sections of the cells of *R. opacus* strain 1CP upon growth in the medium with benzoate in the presence of quercetin. Designations: CW, cell wall; PHA, polyhydroxyalkanoate inclusions; PP, polyphosphate granules; MS, myelin-like structures; N, nucleoid. The bar scale length is 1 μm.

Enzyme activity. Activity of the enzymes involved in protection against peroxide radicals, namely, catalase, superoxide dismutase, ascorbate peroxidase, and peroxidase, was determined in the cells. It was found that in the cells grown in rich medium (LB), the activity of these enzymes was not detected, except for catalase which was reliably detected, although specific activity varied 1.5–3-fold in parallel samples. In benzoate-grown cells, the ascorbate oxidase activity was absent, and the catalase activity was 2–3 times higher than in the cells grown in LB. Incubation of benzoate-grown *R. opacus* 1CP cells with H_2O_2 resulted in an increase of the catalase activity by two orders of magnitude over 4 h (to 0.142 μmol/min mg protein at 25 mM H_2O_2). No correlation was found between the specific catalase activity value and increasing H_2O_2 concentrations.

Investigating the response of *Delftia acidovorans* MC1 to the chemical stress induced by 2,4-dichlorophenoxypropionic acid, Benndorf and Babel revealed an interesting fact (Benndorf and Babel, 2002). Contrary to the long-held belief that lipophilic or reactive compounds induce the synthesis of the heat shock or oxidative stress proteins, they did not observe induction of the GroEL, DnaK, and AhpC proteins, which are considered to be the markers of induction of heat shock and oxidative stress response. Note that the activity of two chlorocatechol-1,2-dioxygenases catalyzing conversion of 3,5-dichlorocatechol to 2,4-dichloro-*cis*,*cis*-muconate was revealed. This led the authors to the conclusion that induction of the biodegradation enzymes was the main mechanism of resistance to this compound for bacterium *D. acidovorans* MC1. It was shown earlier that activity of the enzymes induced in *R. opacus* 1CP upon growth on benzoate, catechol-1,2-dioxygenase (Cat-1,2-DO),

and protocatechuate-3,4-dioxygenase (PCA-3,4-DO) varied, depending on the growth substrate concentration within the range of 0.22–0.25 and 0.09–0.44 U/mg protein, respectively (Solyanikova et al., 2016). Determination of the activity of these enzymes in benzoate-grown cells exposed to 25 mM H_2O_2 showed that the activity of PCA-3,4-DO remained in the same range (0.118 ± 0.008 U/mg protein). The activity of Cat-1,2-DO was 1.5 times higher (0.362 ± 0.040 U/mg protein) than in benzoate-grown cells not exposed to peroxide. Thus, an increase in the biodegradation enzyme activity is one of the consequences of the oxidative stress impact.

In this work, we studied the ability of the cells of a nonmycelial actinobacterium *R. opacus* 1CP to retain its viability under conditions of starvation, oxidative stress, low temperature (4°C), and dehydration. Even under unfavorable conditions, the rhodococcal cells were shown to retain their metabolic activity for a long time. As to cell constitution, benzoate as a sole substrate exerted an influence on the morphology of *R. opacus* 1CP cells, which was, in particular, shown up in their mild polymorphism and the absence of branching cell forms. The additional stress factor in the form of H_2O_2 resulted in emergence of smaller size cells compared to the cells growing on benzoate. The general strategy for survival of the cells under unfavorable stress conditions included their decreased size and formation of densely packed cells conglomerate. Apparently, conglomerate formation may be considered as one of the means of bacterial protection against the aggressive environmental factors. The multicellular conglomerate structure and the matrix material impede the penetration of toxic substances into the conglomerates, thereby promoting survival of the cells located inside. It should be noted that all the above-mentioned structural and morphological changes (decreased sizes, cell rounding, and formation of multicellular conglomerates) are well-known signs of transition of the cells to a dormant state. It is possible that, in response to oxidative stress, the cells may switch on the mechanism of structural arrangements of transition to rest without switching off their metabolic activity. Such a strategy is likely to protect actively metabolizing cells against the injurious aggressive environmental factors.

Impacts of stress of any character on microorganisms result in a sharp change in their physiological, morphological, and biochemical features, on the whole mobilizing the metabolic processes. Actinobacteria with their high ability to survive unfavorable conditions are well suited for application in biotechnological processes, which require long-term maintenance of cell metabolic activity, for example, in the technologies for decontamination of soils and polluted ecotopes under unfavorable environmental conditions, which was once again shown by our investigations.

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