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ORIGINAL ARTICLE

Expression of SOD and production of reactive oxygen species in *Acinetobacter calcoaceticus* caused by hydrocarbon oxidation

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Abstract Expression of antioxidant enzymes [superoxide dismutase (SOD) and catalase] and generation of reactive oxygen species (ROS) in the presence of various hydrocarbons were investigated in two strains of hydrocarbon-degrading microorganism *Acinetobacter calcoaceticus* isolated from water (VKPM B-10353) and bottom sediment (EMBM-06). Upon biotransformation of hydrocarbons by the studied strains, superoxide generation strengthening by bacterial cells and accumulation of peroxides in the cultivation medium were observed. The strongest superoxide anion radical generation occurred during the incubation of microorganisms with diesel fuel (increasing 12 times and 2 times), cyclohexane (1.8 times and 1.75 times), a mix of benzene and anthracene (4.4 times and 3.7 times), naphthalene (8 times and 1.9 times) and benz(a)pyrene (2 times and 2.3 times) in strains VKPM B-10353 and EMBM-06 respectively, and anthracene (2.8 times) in strain VKPM B-10353. The accumulation of peroxides in the medium took place upon biotransformation of diesel fuel by both strains. Activity of SOD increased considerably and activity of catalase decreased in cells in the medium with hydrocarbons. Decane and crude oil had the maximum impact on SOD expression. Decane increased SOD expression in strains VKPM B-10353 and EMBM-06 24.4 times and 28.5 times, respectively. Crude oil increased SOD expression in strain VKPM B-10353 19 times, and in strain EMBM-06 16 times. The authors assumed that increased ROS generation and oxidizing stress development in bacteria during the biotransformation of various hydrocarbons can have an adaptive character.

Keywords Hydrocarbons · Oxidative stress · ROS · SOD · Catalase · *Acinetobacter calcoaceticus*

Introduction

In recent years, data from a number of studies has been published stating that the first oxidation stages of aromatic hydrocarbons and their derivatives (Lee 1999; Tamburro et al. 2004), alkanes (Kato et al. 2009; Gogoleva et al. 2012), diphenyls, their chlorine derivatives [polychlorinated biphenyls (PCBs); Ponce et al. 2011], nitrated derivatives of aromatic compounds (Pérez-Pantoja et al. 2013) by various microorganisms can be accompanied by formation of the reactive oxygen species (ROS).

Kato et al. (2009) showed that acetyl-CoA-oxidase, catalase and superoxide dismutase (SOD) are induced in the oil biodegrading thermophilic bacterium *Geobacillus thermoleovorans* B23 during incubation with alkanes. Acetyl-CoA-oxidase forms ROS, catalase and superoxide dismutase which protect the cell from toxic actions of ROS at the initial stage of β-oxidation of alkanes. These processes are functionally similar to the processes which occur in eukaryotic peroxisomes.

Gogoleva et al. (2012) reported that accumulation of peroxides occurs during cultivation of oil-biodegrading microorganisms such as *Gordonia terrae*, *Rhodococcus rubropertinctus* and *Rhodococcus erythropolis* in mineral medium with diesel fuel as the carbon source.

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As it was shown by us earlier, upon cultivation of two strains of *Acinetobacter calcoaceticus* on crude oil-containing medium, levels of free radical processes increased in bacterial cells (Sazykin et al. 2011).

Oxygenases that carry out initial stages of aromatic substrate oxidation and are capable of oxidizing similar substrates produce ROS as a result of the high frequency of faulty reactions (Lee 1999; Pérez-Pantoja et al. 2013). Antioxidants and antioxidant complex enzymes can improve biodegradation of similar substrates (Tamburro et al. 2004; Kang et al. 2007; Ponce et al. 2011).

However, another report stated that oxidation of oil hydrocarbons can be worsened in the presence of antioxidants (Sazykin and Sazykina 2013).

Thus, ROS generated by microorganisms can play a double role in hydrocarbon oxidation.

On the one hand, they cause oxidative stress (Kang et al. 2007) and damage various components of a bacterial cell (Lee 1999), which is what causes cell death and deceleration of microorganism reproduction.

On the other hand, the damage of DNA mediated by ROS provokes a mutagen SOS response and accelerates evolution of the new strains capable of oxidizing substrates inaccessible earlier (Pérez-Pantoja et al. 2013).

Obviously, depending on the qualitative and quantitative structure of pollutants, certain effects can prevail, but, in general, the strategy of excess ROS generation can promote expansion of microorganisms and development of new niches (Lee 1999; Kang et al. 2007; Pérez-Pantoja et al. 2013).

Unfortunately, the number of publications devoted to researching the role of ROS in biotransformation of hydrocarbons is limited, though similar researches can also clarify such an interesting question as a cometabolism of compounds which cannot be utilized by bacteria lacking the corresponding enzymatic systems.

The authors of this work suppose that the increased level of free radical processes upon biotransformation of hydrocarbons and xenobiotics by microorganisms can produce surfactants and increase bioavailability of hydrophobic substrates. Moreover, the oxidative stress can have an adaptive character due to the strengthening of hydrocarbon substrate oxidation with ROS by bacteria lacking the enzymatic systems for hydrocarbons transformation (Pérez-Pantoja et al. 2013).

In this work, possible changes in the expression of SOD and catalase have been examined, as well as the generation level of superoxide anion and hydrogen peroxide upon biotransformation of various hydrocarbons by two strains of oil-degrading microorganism *Acinetobacter calcoaceticus*. According to the authors of this article, the adaptation mechanisms similar to oxidative stress can be an important universal way of oxidizing a variety of substrates insusceptible to enzymatic systems of bacteria.

Methods

Strains and cultivation of microorganisms

In this research, two strains of *Acinetobacter calcoaceticus* isolated from water (VKPM B-10353) and bottom sediment (EMBM-06) of the surf zone of the Kerch Strait of the Azov Sea are used.

Experiments were set up using hydrocarbons or crude oil as a carbon source in a basic mineral salt medium. The composition of the medium is given in Table 1. 2 ml/L of trace element stock solution was added to basic mineral salt medium before use (Table 2).

The initial pH was adjusted to 6.8. The medium was dispensed in 15-mL quantities into 50-mL Erlenmeyer flasks. The flasks were cultivated in a gyratory shaker incubator, Innova 40R (New Brunswick, Canada), programmed at 170 rpm and 30 °C.

The amount of microorganism cells in suspension was estimated by means of the DEN-1B nephelometer (Biosan, Latvia).

Hydrocarbons

In this work, individual hydrocarbons [n-pentane, n-decane, n-hexadecane (HD), oil paraffin, cyclohexane, benzene, naphthalene, anthracene, benz(a)pyrene (BAP) analytical grade ("Aquatest", Russia), crude oil and commercial diesel fuel "Euro C"] were used. Resin and asphaltene fraction was isolated from crude oil with addition of 40-fold volume of pentane with the subsequent centrifugation at 14000 *g* and pellet drying. Solid hydrocarbons, pentane, benzene and cyclohexane were used in the form of solutions in n-hexadecane. Paraffin, naphthalene, pentane, benzene and cyclohexane were in the form of 10 % solutions, anthracene was in the form of a 0.25 % solution, and BAP, resin and asphaltene fraction were in the form of 0.1 % solutions.

Table 1 Composition of basic mineral salt medium

Compound	Concentration (g/L)
$NaNO_3$	2.0
NaCl	0.8
KCl	0.8
$CaCl_2 \cdot 2H_2O$	0.1
KH_2PO_4	2.0
$Na_2HPO_4 \cdot 12H_2O$	2.0
$MgSO_4$	0.2
$FeSO_4 \cdot 7H_2O$	0.001

Table 2 Composition of trace element stock solution

Compound	Concentration (g/L)
$FeCl_3 \cdot 6H_2O$	0.08
$ZnSO_4 \cdot H_2O$	0.75
$CoCl_2 \cdot 6H_2O$	0.08
$CuSO_4 \cdot 5H_2O$	0.075
$MnSO_4 \cdot H_2O$	0.75
H_3BO_3	0.15
$Na_2MoO_4 \cdot 2H_2O$	0.05

SOD and catalase expression by microorganisms

Microorganisms were introduced into 50-ml conical flasks with 15 ml of basic mineral salt medium with addition of 1 % (150 μl) of hydrocarbons. Flasks with basic mineral salt medium with addition of 0.5 % of yeast extract and 0.5 % of tryptone were used as the control. The amount of bacterial cells in the medium before incubation was 1×10^8 cells per ml. Microorganisms were cultivated in a shaker incubator (30 °C, 24 h). Bacterial cells were harvested by centrifugation for 15 min (6000 *g*, 4°C). The cells lysate was prepared by suspending the pellet in an equal volume of 0.85 % NaCl solution with 0.1 % Triton X100 and incubation for 15 min at 37 °C with periodical shaking.

Activity of SOD was estimated according to the Sun Yi et al. method (1988). Catalase activity was estimated according to the Goth method (1991). Activity of enzymes was calculated per 1 mg of total bacterial protein at the moment of incubation termination. Protein concentration in bacterial cell lysate was estimated according to the Lowry method (Lowry et al. 1951). The three independent experiments were carried out in five repetitions each.

Superoxide-anion radical generation by microorganisms

For the experiment, microorganisms were grown for 18 h on the basic mineral salt medium with addition of 0.5 % of yeast extract and 0.5 % of tryptone. Then the microorganism suspension was washed 3 times in basic mineral salt medium and diluted with the same medium to a concentration of 1×10^8 cells per ml.

For evaluation of superoxide generation upon microorganism incubation with hydrocarbons, COSTAR 3632 96-well microplates (USA) were used. 100 μl of culture suspension, 80 μl of basic mineral salt medium, 10 μl of 4-mM deionized water lucigenin solution (Sigma-Aldrich, USA) and 10 μl of the corresponding hydrocarbon were introduced.

One hundred μl of the suspension culture, 90 μl of basic mineral salt medium with addition of 0.5 % of yeast extract, 10 μl of 4-mM solution of lucigenin in deionized water were added into the control. The three independent experiments were carried out in eight repetitions each.

The plate was placed into the Luminoskan Ascent plate luminometer (Thermo Scientific, USA) and incubated for 24 h at a temperature of 30 °C with simultaneous chemiluminescence (CL) intensity measurement every 30 min (48 measurements in total).

Hydrogen peroxide generation by microorganisms

Microorganisms were introduced into 50-ml conical flasks with 20 ml of basic mineral salt medium with addition of 2 % (400 μl) of hydrocarbons. Flasks with basic mineral salt medium with addition of 0.5 % of yeast extract and 1 % of tryptone were used as the control. The amount of bacterial cells in the medium before incubation was 1×10^6 cells per ml. Microorganisms were incubated in a shaker incubator at 30 °C, 170 rpm for 30 days.

For assesement of hydrogen peroxide, samples of cultured liquid were taken from a flask and centrifuged for 5 min at 14100 *g*. 60 μl of supernatant of the culture medium, 100 μl of phosphate-buffered saline (PBS) and 20 μl of luminol were introduced into a plate well. CL measurement was carried out in eight repetitions on a Luminoscan Ascent microplate luminometer; the luminescence of each well was measured within 100 s with the interval of 1 s. At the beginning of measurements, 20 μl of horseradish peroxidase solution (0.01 u/μl) were introduced into each plate by means of a built-in dispenser.

For each measurement, the average CL intensity was calculated and, subsequently, the greatest average CL value was used.

Statistics

All data were analysed using an unpaired student's *t* test in Microsoft Excel; *p* values of less than 0.05 were considered statistically significant.

Results

Changes of catalase and SOD expression

The results of SOD and catalase activity estimation in *A. calcoaceticus* of strains EMBM-06 and VKPM B-10353 are presented in Fig. 1a and b. The maximum level of SOD activity was registered when individual hydrocarbons or crude oil were the only source of carbon in the cultivation medium. In these cases, the specific activity of SOD was higher (2.95–28.5 times for strain EMBM-06 and 2.5–24.4 times for strain VKPM B-10353) than its activity upon cultivation of microorganisms in the medium with yeast extract (control; Fig. 1a and b). The maximum level of SOD activity was reached in the presence of 1 % decane in both strains. SOD expression level greatly increased (16 and 19 times, respectively) with the growth of the studied strains in the presence of 1 % of crude oil (Fig. 1a

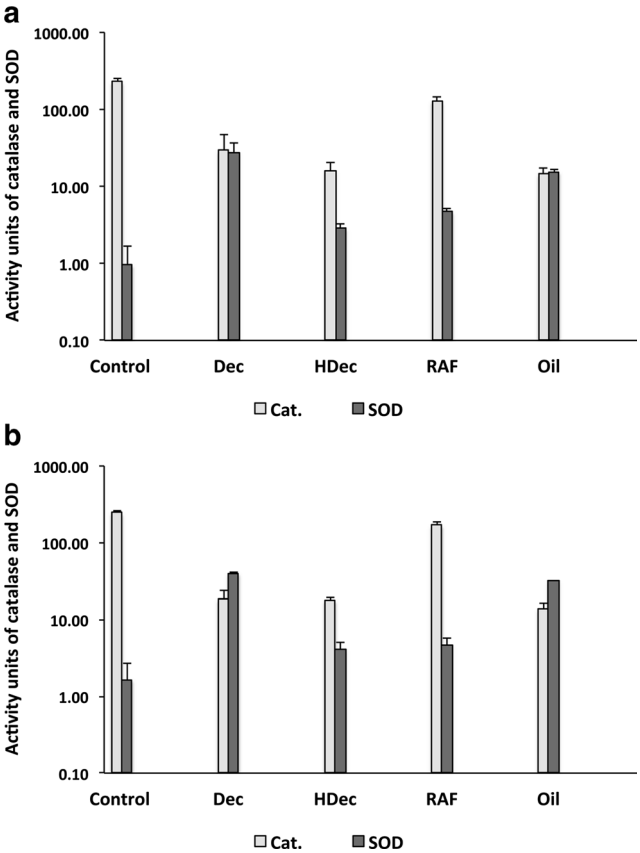

Fig. 1 Activity of catalase and SOD after microorganism incubation with various substrates (logarithmic scale): Cat - catalase specific activity (MME/mg of protein), SOD - superoxide dismutase specific activity (s.u./mg of protein × minutes), Control - basic mineral salt medium with addition of 0.5 % yeast extract and 0.5 % of tryptone, Dec - decane, HDec - hexadecane, RAF - resins and asphaltenes fractions, and Oil - crude oil as the only source of carbon. **a** - *A. calcoaceticus* EMBM-06 **b** - *A. calcoaceticus* VKPM B-10353. Differences from the control for all the data sets are statistically significant ($p < 0.05$)

and b). Unlike SOD, catalase expression level upon cultivation of *A. calcoaceticus* on hydrocarbon substrates was lower. The maximum level of catalase expression was observed upon cultivation of bacteria on basic mineral salt medium with addition of 0.5 % of yeast extract and 0.5 % of tryptone. Catalase activity was higher (7.7–15.7 times for strain EMBM-06 and 13.5–18.1 times for strain VKPM B-10353) in the control (cultivation on the complete medium) than during culturing the strains with hydrocarbons (Fig. 1a and b).

Superoxide generation in the presence of hydrocarbons

The dependence of luminescence intensity of *A. calcoaceticus* strain EMBM-06 on incubation time is shown in Fig. 2a and b. Superoxide generation during the experiment is proportional to luminescence intensity. The obtained data show increasing superoxide generation in the presence of alkanes compared to the control: pentane and diesel fuel (increased 1.4 times and 2 times, respectively), cyclohexane (increased 1.75 times) and aromatic hydrocarbons (hexadecane solutions of benzene and anthracene, naphthalene or benz(a)pyrene; increased 3.7 times, 1.9 times, and 2.3 times, respectively). For other hydrocarbons, the effect was practically absent (Fig. 2a and b). The VKPM B-10353 strain displayed a stronger growth of superoxide generation (Fig. 2c and d). As well as in the case with the previous strain, the most pronounced effect was observed upon incubation with diesel fuel, however, the luminescence intensity values are much higher (4 times higher) in comparison with the strain EMBM-06. The extents of superoxide generation were 12 (diesel fuel), 1.8 (cyclohexane), 8 (naphthalene), 4.4 (mixed benzene and anthracene), 2.8 (anthracene) and 2 (benz(a)pyrene) times higher compared to the control. A similar result is observed in the case with hexadecane solution of naphthalene — the values of CL are more than 3.5 times higher than the similar results of the EMBM-06 strain. CL intensity is much weaker than upon incubation with diesel fuel, but superoxide generation takes more time. Much weaker, but also reliable, effects were registered for hexadecane solutions of benzene and anthracene, of anthracene and of benzapyrene. Other hydrocarbons displayed the luminescence intensity values of almost the same level as the control sample.

Peroxide accumulation in medium when microorganisms were incubated with hydrocarbons

Accumulation of hydrogen peroxide in culture medium was studied during 30 days of incubation with n-decane, n-hexadecane, solutions of n-pentane, oil paraffin, cyclohexane, benzene, naphthalene, anthracene, BAP, resin and asphaltene fractions in n-hexadecane and diesel fuel. Upon incubation with diesel fuel as the only source of carbon, growth of hydrogen peroxide concentration in both studied strains was noted in the second half of the observation period. A two-fold excess over the control level in the EMBM-06 strain was observed from the 18th day of incubation (Fig. 3a), and in the strain VKPM B-10353, from the 19th day of incubation (Fig. 3b). The EMBM-06 strain displayed the stronger accumulation of hydrogen peroxide that exceeded the control values by more than ten times, whereas the values of the VKPM B-10353 strain exceeded the control values by about three times. Upon incubation with other used hydrocarbons, CL values were lower than the control, i.e., accumulation of hydrogen peroxide in both strains of *A. calcoaceticus* was not registered.

Discussion

The processes of microbiological aerobic metabolism of hydrocarbons often include substrate oxidation by oxygenases and hydroxylases as the first stages (Bell et al. 2006; Bell and Wong 2007; Wu et al. 2011; Wang et al. 2011). As a rule, this oxidation is mediated by the radicals formed in the active

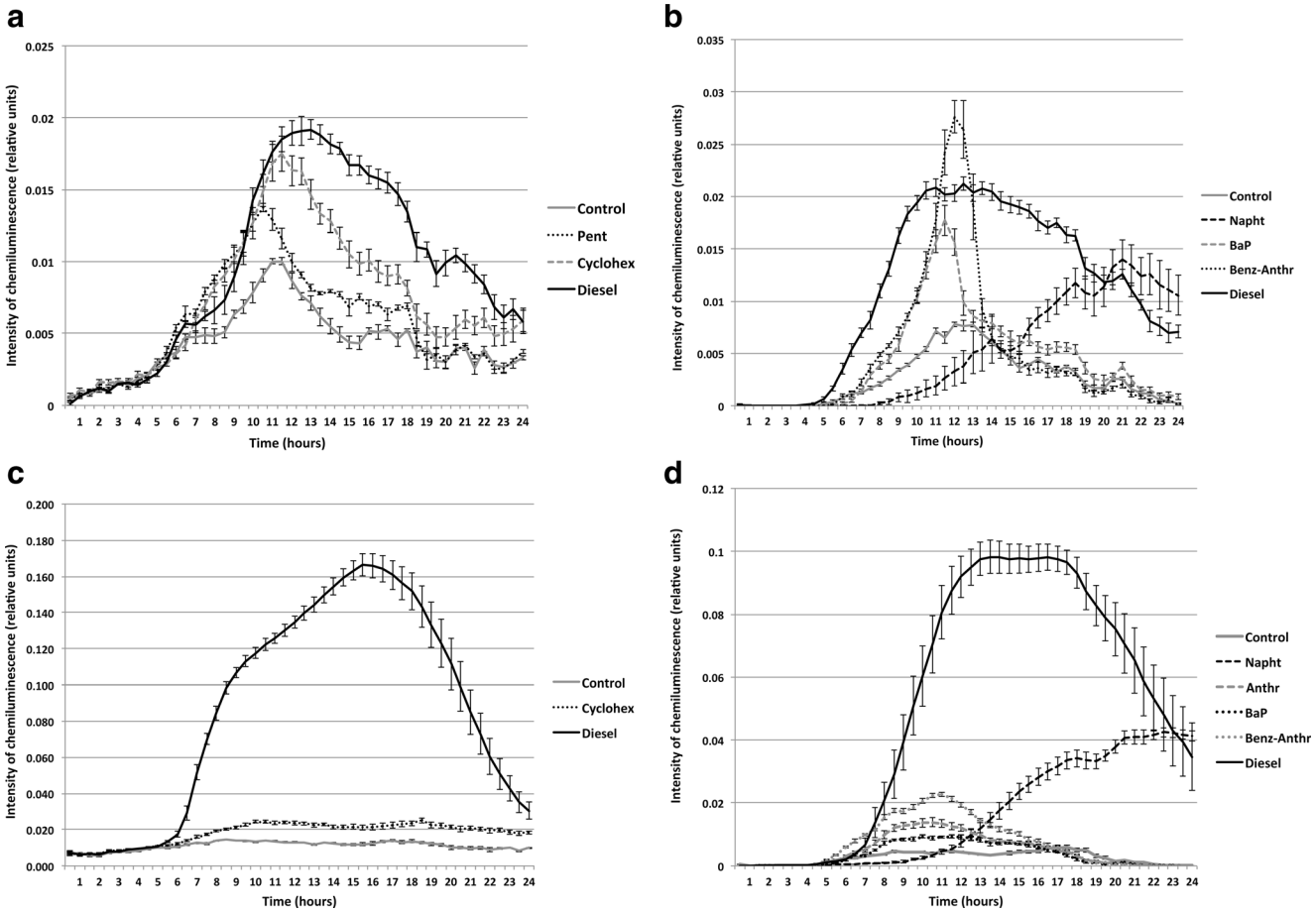

Fig. 2 Intensity of superoxide anion radical generation (measured with lucigenin-activated CL) upon incubation of oil-biodegrading microorganisms within 24 h in basic mineral salt medium with addition of yeast extract or various hydrocarbons as the carbon source: Control - yeast extract and tryptone; Pent - pentane solution in hexadecane; Cyclohex - cyclohexane solution in hexadecane; Benz - benzene solution in hexadecane; Napht - naphthalene solution in hexadecane; Anthr - anthracene solution in hexadecane; Benz-Anthr - benzene and anthracene solution in hexadecane; BaP - benz(a)pyrene solution in hexadecane; Diesel - diesel fuel. **a** - *A. calcoaceticus* EMBM-06 **b** - *A. calcoaceticus* EMBM-06 **c** - *A. calcoaceticus* VKPM B-10353 **d** - *A. calcoaceticus* VKPM B-10353. Differences from the control for all the data sets are statistically significant ($p < 0.05$)

center (Metelitsa 1983; Zenkov and Men'shchikova 1993). Similar enzymatic reactions can be accompanied by enzymatic cycle shunting with formation of ROS (hydrogen peroxide, superoxide anion) or leaking of ROS from the active center.

The probability of enzymatic cycle shunting with ROS formation increases in the absence or the lack of the suitable substrate and in the presence of a "bad" substrate for the enzymes with mild specificity (many hydrocarbon oxygenases).

Thus, ROS are an inevitable byproduct of alkane (Kato et al. 2009), aromatic hydrocarbon (Kang et al. 2007; Lee 1999; Tamburro et al. 2004) and PCB (Ponce et al. 2011) biodegradation by some microorganisms.

As the research results show, oxidation of various hydrocarbon substrates causes considerable strengthening of superoxide anion radical generation in both strains of *A. calcoaceticus*.

We also demonstrated that incubation of the two investigated strains of *A. calcoaceticus* in the medium with hydrocarbons as the only source of carbon lead to the change in expression of native antioxidant enzymes such as SOD and catalase.

In the course of hydrocarbon oxidation, SOD activity significantly increased in both strains. It is obvious that strengthening of superoxide generation increases SOD expression in bacterial cells. This is the way how a microorganism turns superoxide into a more stable ROS form less toxic for a cell.

As has been reported (see "Results" section), catalase activity upon cultivation of *A. calcoaceticus* on hydrocarbon substrates was lower. Decrease of the catalase level upon incubation of oil-biodegrading microorganisms with hydrocarbons was also pointed out in the work of Gogoleva et al. (2012). These authors show the direct dependence between a decrease in catalase activity of hydrocarbon-oxidizing microorganisms and intensity of oil product destruction, and also point out the fact that bacterial destruction of hydrocarbons is accompanied by hydroperoxide formation.

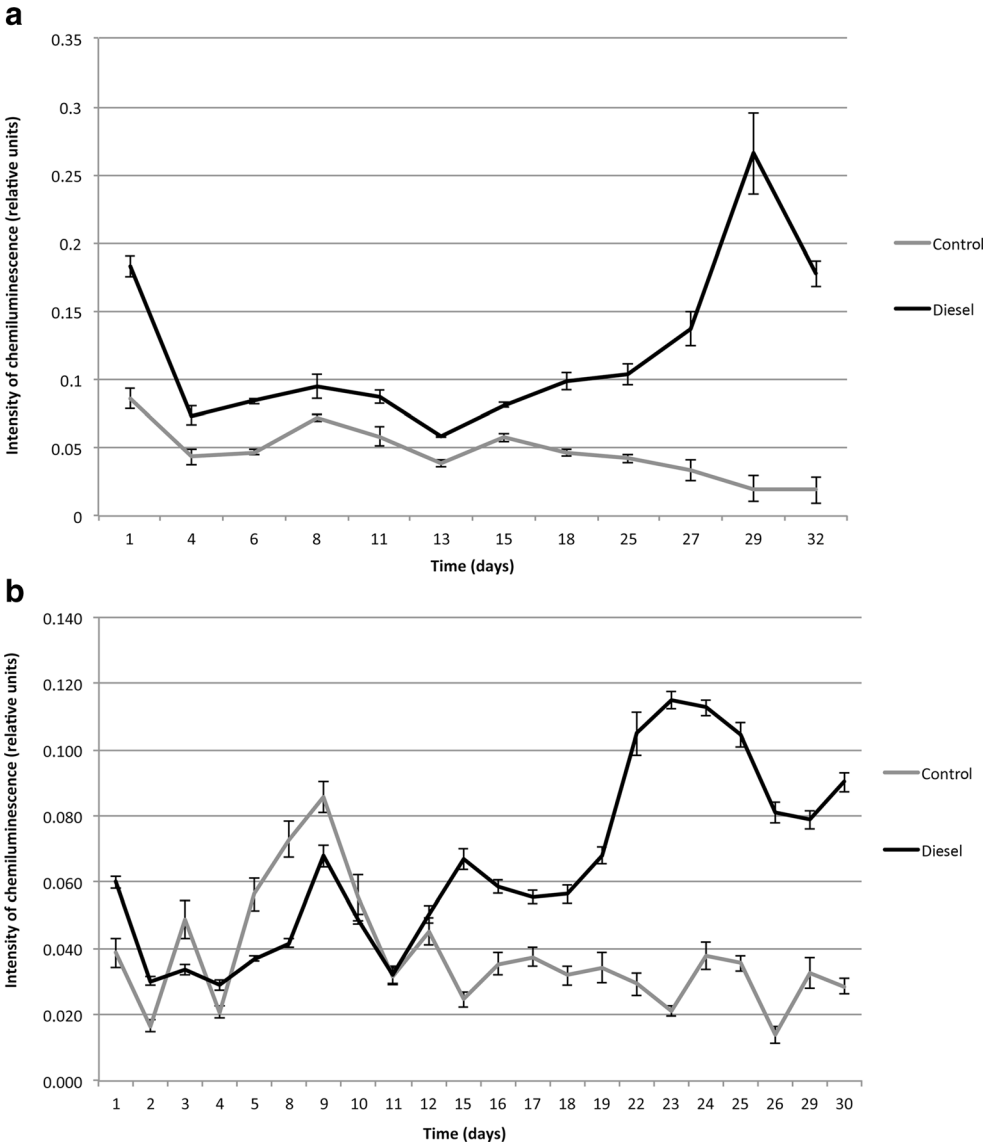

Fig. 3 Intensity of superoxide anion radical generation (measured with lucigenin-activated CL) upon incubation of oil-biodegrading microorganisms within 30 days in basic mineral salt medium with addition of yeast extract or various hydrocarbons as a carbon source: Control - yeast extract and tryptone; Diesel - diesel fuel. CL with other hydrocarbons was lower than the control and is not shown in the figure. **a** - *A. calcoaceticus* EMBM-06 **b** - *A. calcoaceticus* VKPM B-10353. Differences from the control for all the data sets are statistically significant ($p < 0.05$)

Our research, just like the work of Gogoleva et al. (2012), points out the accumulation of peroxides in the medium upon long cultivation of the studied microorganisms on the medium with diesel fuel as the carbon source.

It is likely that the decrease of the catalase activity and accumulation of peroxide compounds in the medium upon biodegradation of hydrocarbons can be explained by some microorganisms having different effective mechanisms of cell protection against excess hydrogen peroxide (Gogoleva et al. 2012).

The authors of this work hypothesized that increased ROS generation and oxidizing stress development in microorganisms in the course of various hydrocarbon substrate and xenobiotic biodegradation is rather a widespread phenomenon that can have an adaptive character. In the case of the oil-degrading strains of *A. calcoaceticus* presented above, we observe a noticeable increase of superoxide anion and SOD levels at various hydrocarbon biotransformations. Hydrogen peroxide accumulates in the cultivation medium as bacterial catalase activity decreases during this process. Formation of the ROS upon oxidation by alkanes, aromatic hydrocarbons, diphenyls and their derivatives is shown for various genera such as *Geobacillus*, *Gordonia*, *Rhodococcus*, *Burkholderia*, *Pseudomonas*, *Ochrobactrum* и *Acinetobacter* (Tamburro et al. 2004; Kang et al. 2007; Kato et al. 2009; Ponce et al. 2011; Sazykin et al. 2011; Gogoleva et al. 2012; Pérez-Pantoja et al. 2013) and it is likely to be a widespread phenomenon among bacteria.

In spite of the fact that oxidative stress leads to cell component damage, delayed reproduction and even death of a part of the bacterial cell population (Lee 1999; Pérez-Pantoja et al. 2013), as a whole, it can give certain advantages. Due to oxidation of extracellular hydrophobic organic compounds, biogenous surfactants (for example, organic peroxides)

increasing the bioavailability of hydrophobic substrates can be formed in the environment (Gogoleva et al. 2012).

ROS can participate in the initial stages of hydrocarbon and xenobiotic oxidation for which microorganisms have no enzymatic systems. ROS damage DNA and, thus, cause a mutagen SOS response during oxidative stress. This, in turn, can lead to accelerated evolution of the enzymatic systems of bacterial cells connected to hydrocarbon and xenobiotic biodegradation (Pérez-Pantoja et al. 2013).

Obtaining access to new substrates, microorganisms have an opportunity to expand their habitat and achieve new ecological niches.

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Compliance with ethical standards

Ethical standards The manuscript does not contain clinical studies or patient data.

Conflict of interest The authors declare that they have no conflicts of interest.

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