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Chemiluminescence Analysis of Oil Oxidizing Bacteria *Actinetobacter calcoaceticus* Extracts: Effects of the Extracts on pSoxS-lux Biosensor

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Abstract—A comparative H_2O_2-luminol- and Fe(II)-induced chemiluminescence analysis of extracts of two strains of marine oil oxidizing bacteria *Actinetobacter calcoaceticus* cultivated either in the presence or absence of oil was carried out. Effects of these extracts on *E. coli* MG1655 biosensor (pSoxS-lux) were studied. Activation of H_2O_2-induced chemiluminescence in the presence of oil was observed. This suggests activation of free radical lipid peroxidation. Aqueous extracts of microorganisms cultivated in the presence of oil were shown to activate reactive oxygen species production (ROS) in Fe(II)-induced chemiluminescence reaction mixture. Acetone–ethanol extracts induced antioxidative systems of both strains. Chemiluminescence analysis in a biological system carried utilizing *E. coli* MG1655 (pSoxS-lux) revealed that aqueous extracts of the strains cultivated in the absence of oil contained potential antioxidants.

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INTRODUCTION

Recent studies have suggested combined implementation of both biological and chemical approaches for remediation of hydrocarbon polluted soils [1–3]. A chemical approach implicates pollutant oxidation with either hydrogen peroxide or during the processes similar to Fenton's reaction that occur under natural for soil pH values. These processes are known to be the source of hydroxyl (•OH), superoxide anion-radical $\left(O_2^{\bullet-}\right)$, and hydroperoxide radical $\left(HO_2^{\bullet}\right)$. These studies showed that single-type ways of hydrocarbon degradation, i.e., either chemical or microbiological, are far less effective compared to complex treatment that includes both free radical oxidation and the following biodegradation. Moreover, it was observed that such complex treatment sharply increases biological accessibility and degradation level of heavy hydrocarbon fractions. In these studies, complex biochemical treatment was carried out under natural soil pH values using indigenous microorganisms as biodestructors.

Hydrocarbon reducing microorganisms that release hydrogen peroxide into the environment are well known. This mechanism is doubtlessly important for interspecific competition. It is shown to be involved in biofilm formation [4]. However, the role of this mechanism in nonenzymatic hydrocarbon oxidation has not been proven thus far. Transfer of two electrons on the oxygen molecule that results in formation of hydrogen peroxide is provided by several flavin-containing enzymes, such as glucose oxidase, xanthine oxidase, and amino acid oxidases. These enzymes reduce O_2 to form peroxide ion O_2^{2-}, which, when reacting with protons, forms hydrogen peroxide (H_2O_2). Apparently, a variety of organisms can produce considerable amounts of hydrogen peroxide and may be potentially involved in hydrocarbon degradation, because many of them contain flavin-dependent enzymes.

The phenomenon of production of ROS by the oil reducing microorganisms is indirectly confirmed by the presence of hydrocarbon-inducible peroxidases that are involved in hydrocarbon metabolism [5–7].

Kato et al. (2009) showed that incubation of thermophilic bacterium *Goebacillus thermoleovorans* B23 in the presence of alkanes led to induction of acetyl-CoA oxidase, catalase, and superoxide dismutase (SOD). ROS were shown to be formed at initial stages of alkane β-oxidation catalyzed by acetyl-CoA oxidase, while catalase and SOD were shown to protect cells against toxic effects of ROS. These processes are functionally similar to those occurring in eukaryotic peroxisomes.

Therefore, it is considered to be especially interesting to study prooxidants and antioxidants that are produced by oil oxidizing microorganisms.

Here, we present the results of the study that was aimed at the analysis of the contribution of free radical oxidation processes in microbiological degradation of oil. The main goal of this work was to assess total

prooxidant and antioxidant potential of *Actinetobacter calcoaceticus*, strains 6 and 13.

EXPERIMENTAL

Two strains collected in the area the wreckage of a tanker in Kerch Strait in November 2007 were identified as *Actinetobacter calcoaceticus*, strains 6 and 13. Strains were isolated by incubation of water samples and aqueous extracts of bed silts in Voroshilova–Dianova's mineral medium [9] (1 g/l NH_4NO_3, 1 g/l K_2HPO_4, 1 g/l KH_2PO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.02 g/l $CaCl_2 \cdot 6H_2O$) supplemented with 3% crude oil obtained from "Oktyabyrsky" oil deposit in the Rostov Region as the only carbon source for 10 days. After that, solid medium obtained by addition of 2% agar, 0.1% Tween-80, and 2% crude oil was inoculated with the material in order to obtain pure cultures. Both strains were catalase positive.

Microorganisms were identified at the All-Russia Collection of Industrial Microorganisms (Scientific Center, Genetica Russian Federation Research Institute for Genetics and Selection of Industrial Microorganisms) by sequencing of 16S ribosomal RNA variable motifs.

Cell-free extracts were prepared by treatment of microorganisms with physiological solution and organic solvents. To perform this procedure, microbial cultures were cultivated in a liquid medium on a thermostatic shaker (Biosan, Latvia) at 30°C and 200 rpm for 72 h. Rich LB medium was used for cultivation (10 g/l tripton, 5 g/l yeastrel, 10 g/l sodium chloride) [10]. To investigate hydrocarbon effects, LB medium was supplemented with 2% oil. Bacterial biomass was collected by 15 min centrifugation at 6500 g at 4°C. The collected cells were washed thrice with 0.85% NaCl, so as to be further used in experiments. Washed cells of both strains were used for preparation of aqueous (extraction mixture: 0.85% NaCl and 0.1% Triton X100 in deionized water) or organic (extraction mixture: 1 : 1 acetone–ethanol) extracts. The biomass was homogenized in a porcelain mortar with grinded glass in the presence of extractants at +4°C for 15 min.

Intensity of H_2O_2-luminol-induced chemiluminescence in bacterial extracts was performed using a special device for chemiluminescence registration constructed on the basis of mass-spectrometer scintillometer RFT 22028 (RFT, Germany) according to Shestakov's method [11].

Fe(II)-induced chemiluminescence was registered as follows: the suspension of yolk lipoproteins was obtained by dilution of yolk with distilled water to a 1 : 10 ratio. Chemiluminescence was initiated by supplementation of the reaction mixture with rhodamine 6G fluorescent dye [12].

In the measuring vessel, 3 ml of 25 mM PBS, pH 7.7; 50 μl of 1 mM rhodamine 6G; and 100 μl of bacterial extract normalized with respect to protein concentration were mixed together. To perform the control experiment, the same reaction mixture containing 100 μl of distilled water instead of bacterial extract was used. Samples were thermostated at 37°C for 100 s with continuous mixing to register the background luminescence. After that, the reaction mixture was supplemented with 500 μl of 0.02 M aqueous solution of $Fe_2SO_4 \cdot 7H_2O$ and chemiluminescence was registered until the maximum of the slow flash was reached. $Fe_2SO_4 \cdot 7H_2O$ solution was prepared right before use and acidized with 0.1 N HCl (200 μl HCl per 10 ml of $Fe_2SO_4 \cdot 7H_2O$ aqueous solution) in order to avoid self oxidation.

Aqueous extracts were tested with *Escherichia coli* MG 1655 biosensors (pSoxS-lux) [13] in the presence of 1×10^{-2} M methyl viologen, which effectively induced oxidative stress in this type biosensor cells. The bacterial biosensor was kindly provided by I.V. Man ukhov (Scientific Center of Russian Federation, Genetika Research Institute for Genetics and Selection of Industrial Microorganisms, Moscow, Russia). Bacterial luminescence was measured in a LM-01T microplate luminometer (Immunotech, Czech Republic)

All data presented are the results of thrice repeated measurements. Statistical analysis was performed by Kokunin's method [14].

RESULTS AND DISCUSSION

H_2O_2-Luminol-Induced Chemiluminescence Analysis

We hypothesized that free radicals produced by a bacterial culture are implicated in microbial degradation of oil derivatives. Therefore, it should have been expected that stability of lipid components of bacterial extracts would have increased alongside with activation of free radical oxidation processes. To verify this suggestion, aqueous and acetone-ethanol extracts of *A. calcoaceticus*, strains 6 and 13, cultivated on a standard type medium in the presence (experimental group) or absence (control group) of 2% oil were investigated.

Analysis of aqueous extracts of bacterial strains cultivated on oil containing medium revealed activation of H_2O_2-luminol-induced chemiluminescence, whereby both fast flash height (by 208–700%) and total light yield in 100 s and 500 s increased in comparison with microorganisms cultivated without oil (Table 1).

Analysis of acetone-ethanol extracts of bacteria cultivated on oil containing medium also revealed activation of H_2O_2-luminol-induced chemiluminescence, whereby total light yield increased by 53% for strain 6 and by 250% for strain 13 in 500 s (Table 2).

Therefore, cultivation of bacteria on oil containing medium led to the increase in prooxidant properties of bacterial extracts and, apparently, to hyperproduction of ROS in bacterial cells.

Table 1. H_2O_2-luminol-induced chemiluminescence of aqueous extracts of *A. calcoaceticus*, strains 6 and 13

Sample		fast flash, arbitrary units	Total light yield, s	
			100	500
Strain no. 6	Control	240 ± 35.3	51 ± 6.8	166 ± 18.8
	Experimental	740 ± 86.4*	131 ± 15.7*	318 ± 37.4*
Strain no. 13	Control	18 ± 2.9	2 ±0.01	10 ± 1.3
	Experimental	151 ± 20.7*	13 ± 2.3*	23 ± 2.65*

Note: * signifies statistically significant differences with respect to control values (*t*-test, $p < 0.05$).

Table 2. H_2O_2-luminol-induced chemiluminescence of acetone-ethanol extracts of *A. calcoaceticus*, strains 6 and 13

Sample		fast flash, arbitrary units	Total light yield, s	
			100	500
Strain no. 6	Control	103 ± 11.3	291 ± 33.4	1006 ± 88.8
	Experimental	108 ± 12.4	253 ± 26.2	1543 ± 137.7*
Strain no. 13	Control	95 ± 8.7	112 ± 10.8	532 ± 75.6
	Experimental	101 ± 9.9	166 ± 17.7	1332 ± 150.0*

Note: * signifies statistically significant differences with respect to control values (*t*-test, $p < 0.05$).

Fe(II)-Induced Chemiluminescence Analysis

There should be quite a powerful antioxidative protection system in order to sustain dynamic equilibrium between free radical oxidation and antioxidative mechanisms that would allow oil hydrocarbon degrading without negative consequences for a bacterial cell. To verify this suggestion, aqueous extracts of *A. calcoaceticus*, strains 6 and 13, cultivated on a standard type medium in the presence (experimental group) or absence (control group) of 2% oil were investigated. Comparative analysis of chemiluminescence dynamics revealed that the total light yield for strains 6 and 13 increased by 63 and 41%, respectively, which might suggest an increase in concentration of lipid peroxides (Table 3).

On the other hand, the induction time of chemiluminescence for the strains 6 and 13 were shortened by 74 and 87% respectively, which suggested an increase of Fe^{2+} oxidation rate.

The time required to reach the maximum of luminescence and total light yield until the maximum of slow flash is reached was increased by 15 and 26% for strain 6 and by 140 and 400% for strain 13, respectively. The maximum of the slow flash was increased by 15 and 300% for strains 6 and 13, respectively.

Therefore, aqueous extracts of bacterial cultures grown on oil containing medium activate chemiluminescence response of the slow flash. These observa-

Table 3. Fe(II)-induced chemiluminescence of aqueous extracts of *A. calcoaceticus*, strains 6 and 13

Sample		Induction period	Total light yield, s	Time required to reach the maximal luminescence of the slow flash, s	Total light yield of the slow flash until the maximum	Chemiluminescence yield for 500 s of the slow flash, arbitrary units	slow flash height, arbitrary units	Total light yield in 100 s since Fe^{2+} supplementation
Strain no. 6	Control	1560 ± 200.4	59 ± 6.8	4000 ± 387.4	832 ± 94.7	6 ± 1.0	65 ± 9.3	19 ± 2.8
	Experimental	400 ± 68.8*	50 ± 7.2	4600 ± 510.3	1049 ± 121.1	6 ± 1.0	75 ± 9.9	31 ± 2.9*
Strain no. 13	Control	2300 ± 388.5	56 ± 7.1	1200 ± 158.5	150 ± 23.4	8 ± 1.2	35 ± 7.8	22 ± 2.3
	Experimental	300 ± 51.3*	46 ± 5.4	2900 ± 360.3*	750 ± 83.2*	10 ± 0.8	140 ± 23.1*	31 ± 3.6

Note: * signifies statistically significant differences with respect to control values (*t*-test, $p < 0.05$).

Table 4. Fe(II)-induced chemiluminescence of acetone-ethanole extracts of *A. calcoaceticus*, strains 6 and 13

Sample		Induction period, s	Total light yield, s	Time required to reach the maximal luminescence of the slow flash, s	Total light yield of the slow flash until the maximum	Chemiluminescence yield for 500 s of the slow flash, arbitrary units	slow flash height, arbitrary units	Total light yield in 100 s since Fe^{2+} supplementation
Strain no. 6	Control	4200 ± 408.1	36 ± 5.5	1600 ± 180.0	90 ± 12.4	17 ± 12.5	28 ± 3.5	0
	Experimental	5400 ± 520.0	3 ± 0,6*	1500 ± 167.0	7 ± 0.9*	1 ± 1.1*	2 ± 0.28*	0
Strain no. 13	Control	4140 ± 400.0	30 ± 3.9	800 ± 100.0	125 ± 16.4	30 ± 4.2	35 ± 4.8	3 ± 0.45
	Experimental	5500 ± 510.5	15 ± 2.1*	600 ± 69.9	20 ± 2.9*	10 ± 1.6*	15 ± 1.9*	0*

Note: * signifies statistically significant differences with respect to control values (*t*-test, $p < 0.05$)

tions suggest that efficacy of formation of free radicals that facilitate oil hydrocarbon degradation increased.

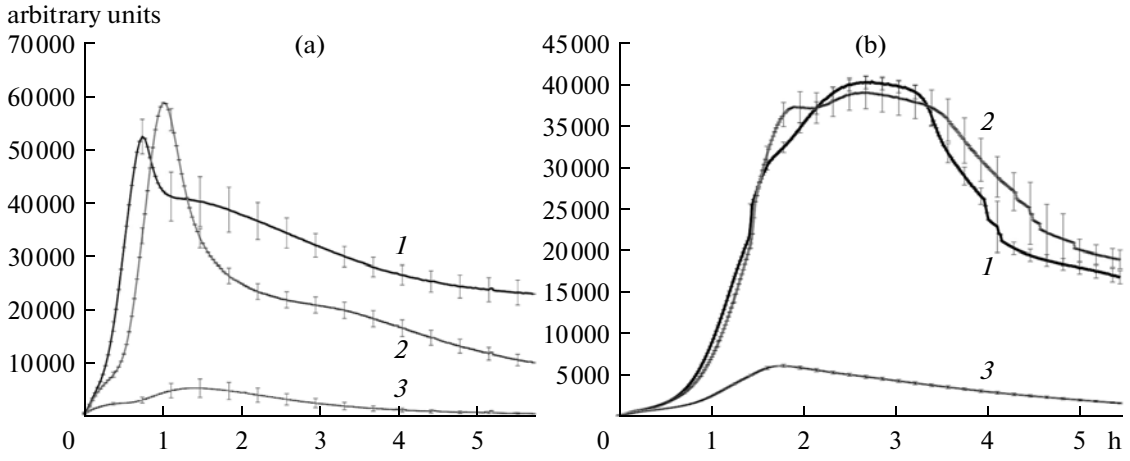
In contrast to microorganisms cultivated in the absence of oil, the study of chemiluminescence intensity in acetone-ethanol extracts of *A. calcoaceticus* strains cultivated on medium containing oil revealed an increase in antioxidative activity for both strains 6 and 13 that appeared as an increase in the induction period by 29–33% with simultaneous decrease in the other chemiluminescence criteria (Table 4).

Aqueous extracts of the strains studied were also tested in biological registration system with *E. coli* MG1655 biosensor (pSoxS-lux), which reacts to the increase in intracellular concentration of superoxide, i.e., substances inducing oxidative stress.

It is important to note that aqueous extracts of bacteria that were cultivated in the absence of oil considerably inhibit generation of ROS induced by methyl viologen in Sox-lux biosensor cells. Extract of strain 6 decreased the response of *E. coli* MG1655 biosensor (pSoxS-lux) to methyl viologen 10-fold (figure a), whereas the extract of strain 13 decreased it 8-fold (figure b). Extracts of these strains cultivated in the presence of oil did not inhibit oxidative stress compared to the control experiment and even increased biosensor response in certain periods of time.

Although the obtained results seem to contradict the data of chemiluminescence analysis of cell-depleted extracts, it may be suggested that constitutive synthesis of antioxidants protects cells against ROS-induced injury. It was shown that cultivation of microorganisms in the presence of oil might lead to decrease in the level of antioxidants due to neutralization of ROS. On the other hand, the effects of antioxidants might be compensated by increased synthesis of prooxidants.

Therefore, it may be suggested that both strains of *A. calcoaceticus* being cultivated on the crude oil con-



Methyl viologen activated chemiluminescence of *E. coli* MG1655 biosensor (pSoxS-lux) in the absence (control, *1*) or presence of extracts of *A. calcoaceticus*, strains (a) 6 and (b) 13 cultivated in the (*2*) presence or (*3*) absence of oil.

taining medium synthesize prooxidants that produce ROS and induce oxidative stress in Sox-lux biosensor cells. On the other hand, these strains synthesize antioxidants that protect biosensor cells against oxidative stress and downregulate Fe(II)-induced chemiluminescence response. Apparently, the function of these antioxidants is to protect oil degrading microorganisms against their own ROS.

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