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Effects of americium-241 and humic substances on *Photobacterium phosphoreum*: Bioluminescence and diffuse reflectance FTIR spectroscopic studies

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ABSTRACT

The integral bioluminescence (BL) intensity of live *Photobacterium phosphoreum* cells (strain 1883 IBSO), sampled at the stationary growth stage (20 h), was monitored for further 300 h in the absence (control) and presence of ^{241}Am (an α-emitting radionuclide of a high specific activity) in the growth medium. The activity concentration of ^{241}Am was 2 kBq l^{-1}; [^{241}Am] = 6.5×10^{-11} M. Parallel experiments were also performed with water-soluble humic substances (HS, 2.5 mg l^{-1}; containing over 70% potassium humate) added to the culture medium as a possible detoxifying agent. The BL spectra of all the bacterial samples were very similar ($\lambda_{max} = 481 \pm 3$ nm; FWHM = 83 ± 3 nm) showing that ^{241}Am (also with HS) influenced the bacterial BL system at stages prior to the formation of electronically excited states. The HS added per se virtually did not influence the integral BL intensity. In the presence of ^{241}Am, BL was initially activated but inhibited after 180 h, while the system ^{241}Am + HS showed an effective activation of BL up to 300 h which slowly decreased with time. Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, applied to dry cell biomass sampled at the stationary growth phase, was used to control possible metabolic responses of the bacteria to the α-radioactivity stress (observed earlier for other bacteria under other stresses). The DRIFT spectra were all very similar showing a low content of intracellular poly-3-hydroxybutyrate (at the level of a few percent of dry biomass) and no or negligible spectroscopic changes in the presence of ^{241}Am and/or HS. This assumes the α-radioactivity effect to be transmitted by live cells mainly to the bacterial BL enzyme system, with negligible structural or compositional changes in cellular macrocomponents at the stationary growth phase.

1. Introduction

Bacterial bioluminescence (BL) [1,2] assays are widely used to monitor environmental toxicity of various contaminants [2–6]. In such assays, the bioluminescent intensity of bacterial cells or bacterial enzyme (luciferase) based preparations (see, e.g. [7] and references reported therein) is the tested parameter which can easily be measured instrumentally.

The currently increasing environmental contamination with radionuclides, including low-level radiation of different kinds, is of growing environmental concern (see, e.g. [8–13] and references reported therein). Therefore, relevant studies involving luminescent bacterial cells are of significant interest both for applied fields such as optimisation of BL-based assays [7,8] and for basic research on elucidating bacterial metabolic responses to such hazardous environmental factors as radionuclide traces [9–13].

Previously, several bioluminescent assay systems in vivo and in vitro were shown [7] to be sensitive to solutions of the α-emitting radionuclide americium-241 (^{241}Am) in the activity range 0.16–6.67 kBq l^{-1}. Adding ^{241}Am salt to bacterial assay systems was found to result in an initial activation of the BL intensity, which was followed by its inhibition. In addition, humic substances (HS) were reported to be capable of detoxifying various contaminants as demonstrated by bioluminescent monitoring (see, e.g. [14–16] and references reported therein), including their detoxification effects on low-level α-radiation [16].

In the present work, the integral BL intensity of live *Photobacterium phosphoreum* cells, sampled at the stationary growth stage (20 h), was monitored for further 300 h in the absence (control) and presence of ^{241}Am. Parallel experiments were also performed with water-soluble humic substances added to the culture medium as a possible detoxifying agent (reported to be effective against

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α-radioactivity [16]). BL spectra of the samples were monitored to control possible impacts of ^{241}Am and/or HS on the formation of electronically excited states [17,18]. Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy was used to check the possibilities of any macroscopic changes in bacterial cell composition and/or fine structural rearrangements of cellular macrocomponents as metabolic responses to the external factors [19,20].

2. Materials and methods

Ph. phosphoreum (strain 1883 IBSO) was taken from the Collection of the Institute of Biophysics SB RAS, Krasnoyarsk, Russia (CCIBSO 863). The bacterium was cultured at 22 °C on a rotary shaker (130 rpm) as described earlier [21]. The aqueous nutrient medium used for bacterial growth included (g l^{-1}): NaCl, 30; KH_2PO_4, 1; $(NH_4)_2HPO_4$, 0.5; $Na_2HPO_4{\cdot}12H_2O$, 6; $MgSO_4{\cdot}7H_2O$, 0.2; peptone, 5; with 3 ml l^{-1} glycerol. Bacterial suspensions both for BL intensity and for DRIFT spectroscopic measurements were sampled at the stationary stage of growth (20 h).

Along with the control samples, those with ^{241}Am and/or water-soluble humic substances (HS, 2.5 mg l^{-1}; containing over 70% potassium humate) were separately studied. As a source of HS, the Gumat-80 preparation ("Gumat", Russia) produced by non-extracting treatment of coal with alkali [14,15] was used.

The radioactivity of ^{241}Am-containing samples was measured with a Wallac Wizard 1480 gamma-counter (PerkinElmer, Finland). The activity concentration of the culture medium, 2 kBq l^{-1} ([^{241}Am] = 6.5 × 10^{-11} M), was created by adding $^{241}Am^{III}$ nitrate.

The integral BL intensity was measured at room temperature (20 °C) by a standard procedure [3,6] using a TriStar Multimode Microplate Reader LB 941 (Berthold Technologies, Germany). Optical density of bacterial suspensions was registered using a KFK-2MP colorimeter (Russia). The number of cells was counted by a Zeiss Axioskop 40 fluorescence microscope with a Filter Set 02 (C. Zeiss, Germany).

After specified periods of time, samples (0.2 ml each) of the bacterial suspension (kept at 4 °C between BL measurements) were placed into microplates, incubated for 5 min at room temperature (20 °C), and then the stabilised values of their integral BL intensity were measured at 20 °C. Relative bioluminescence intensity (I_{rel}) was calculated as follows:

$$I_{rel} = \frac{I_{rad}/N_{rad}}{I_{contr}/N_{contr}}.$$

Here, I_{rad} and I_{contr} are the bioluminescent intensities of radioactive and control samples, respectively; N_{rad} and N_{contr} are the numbers of cells in the radioactive and control samples, respectively. Parallel measurements of BL intensity were performed in quadruplicate; mean values of I_{rel} with standard errors were plotted as a function of time.

Bioluminescence spectra were measured using a Fluorolog 3-22 spectrofluorimeter (Horiba Jobin Yvon, France) with the option of single photon counting.

For DRIFT spectroscopic measurements, the bacterial suspensions for each of the four samples (control, with HS, with ^{241}Am, and with HS + ^{241}Am) were repeatedly centrifuged (6000 × *g*, 4 °C, 15 min) and washed three times from the culture medium (and from ^{241}Am when applicable) with 2% NaCl aqueous solution. The final centrifuged cell pellets were dried under vacuum (6.67 Pa; 0.05 mmHg) at room temperature for 2 h and, prior to measurements, powdered in an agate mortar. DRIFT spectra were recorded and processed as reported elsewhere [19,20] using a Nicolet 6700 Fourier transform infrared (FTIR) spectrometer (Thermo Electron Corporation, USA) with a DRIFT accessory (DTGS detector; KBr beamsplitter; with 100 accumulated scans and a resolution ±4 cm^{-1}) using a Micro sampling cup (Spectra-Tech Inc., USA).

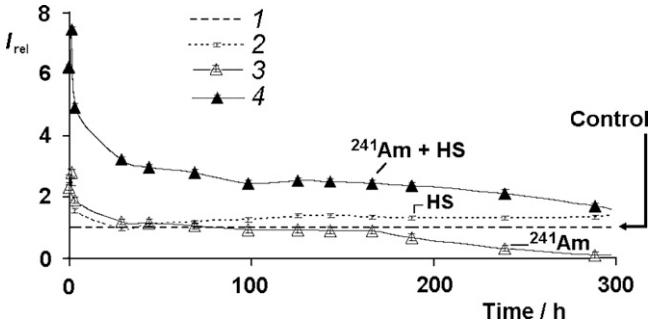

Fig. 1. Relative bioluminescence intensity (I_{rel}) as a function of time for *Photobacterium phosphoreum* (strain 1883 IBSO) sampled at the stationary growth stage (20 h of growth): (*1*) control cells (I_{rel} normalized to unity) and in the presence of (*2*) humic substances (HS), (*3*) americium-241 (^{241}Am), (*4*) ^{241}Am + HS.

3. Results and discussion

3.1. Bioluminescence studies

The values of relative bacterial BL intensity (I_{rel}) as a function of time for control cells, as well as in the presence of HS only, ^{241}Am only or HS + ^{241}Am, are presented in Fig. 1. In all the cases with the additives, as can be seen from curves 2 to 4, the period of the first few hours is characterized by a sharp leap in BL intensity as compared to the control, with an abrupt decrease. This is typical for initial periods of short exposures of luminous bacterial cells to low-level radioactivities and to HS and can be ascribed to the first steps of gradual uptake of ^{241}Am by cells or other applied moderate stresses [7,9,16]. This phenomenon (hormesis), attributed to triggering cell defense responses under the influence of low concentrations of toxic compounds, low-dose radiation and other stressors, is known for various organisms (see, e.g. [9] and references therein). For the sample with HS alone (curve 2), this leap of I_{rel} was the weakest, and further the addition of HS at its low concentration applied (2.5 mg l^{-1}) resulted in a weak activation of the BL intensity by up to maximum 40%.

In the presence of americium only (2 kBq l^{-1}; curve 3), after a short activation period, the BL intensity was close to that of the control (cf. line 1) and, after 180 h, was gradually inhibited virtually down to zero. However, interestingly, in the presence of both ^{241}Am (2 kBq l^{-1}) and HS (2.5 mg l^{-1}; see curve 4), an effective BL activation was observed (up to 750% within the initial period) throughout the whole period of measurements (300 h), gradually and slowly decreasing with time. Thus, even this very low concentration of HS can efficiently detoxify the radiological effect of ^{241}Am.

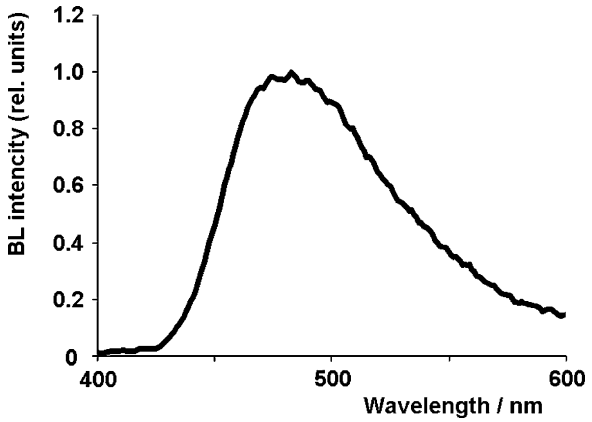

Fig. 2. Bioluminescent spectrum for live cell suspension of *Photobacterium phosphoreum* (strain 1883 IBSO).

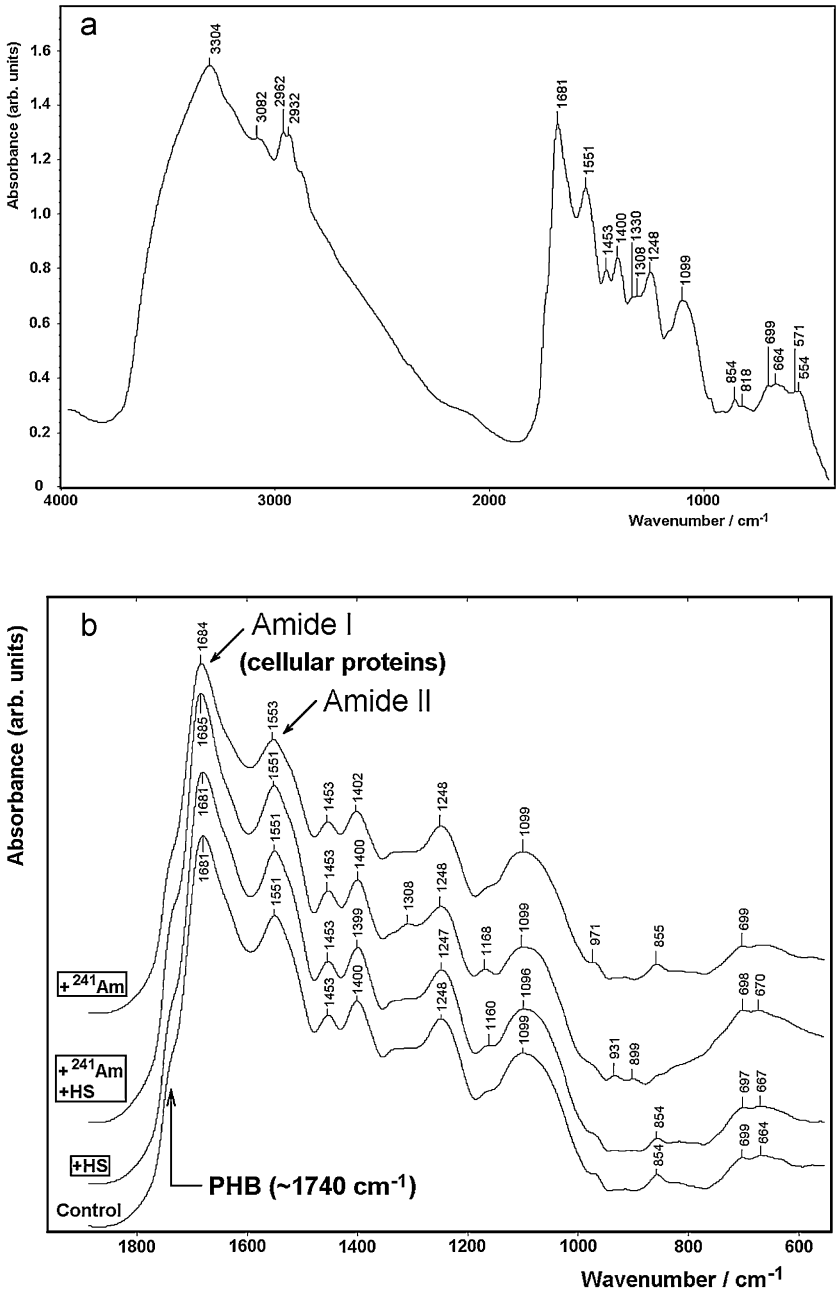

Fig. 3. Diffuse reflectance infrared Fourier transform (DRIFT) spectra of dry cell biomass of *Photobacterium phosphoreum* (strain 1883 IBSO) (*a*) in the mid-IR region (for control cells) and (*b*) in the fingerprint region (1800–600 cm^{-1}) for control cells (control) as well as in the presence of humic substances (+HS), americium-241 ($+^{241}Am$) and both ($+^{241}Am$+HS). In plot (*b*), the spectra are vertically offset for clarity; the positions of the amide I and amide II bands of cellular proteins and of the shoulder at ca. 1740 cm^{-1} (ester C═O stretching mode typical for poly-3-hydroxybutyrate, PHB) are indicated with arrows.

It should be emphasized that the applied concentration of americium(III) in the suspensions (6.5×10^{-11} M) was evidently too low to exert any chemical toxic effect. Note for comparison that the "chemical" toxicity of another actinide, uranium, has recently been for the first time reported [22] to be related to direct inhibition of pyrroloquinoline quinone (PQQ)-dependent bacterial growth and metabolism by uranyl cations ($[UO_2]^{2+}$, excluding coordinated water). However, this molecular mechanism is evidently attributed to the specific "chemical nature" of the uranium(VI) dioxo-cation [8,13,22] and cannot be a priori extended to other radionuclides.

Thus, in full agreement with the literature data [7–9,16], it can be reasoned that most of the initially activating and further inhibiting action of the ^{241}Am radionuclide on the bacterial BL was of radioactive nature. The detoxifying action of the low HS concentration may therefore be attributed to HS-induced deactivation of peroxide compounds appearing in aqueous medium as secondary products induced by ionising radiation (in the presence of α-emitting ^{241}Am) [9], resulting in a weaker damage to cells [16]. This is facilitated by the well-known ability of HS to form relatively strong complexes with actinides [23,24], thus shielding the radionuclides and secondary products of their radioactive decay from live cells, as well as by the reducing activity of HS [14,15].

The BL spectra of the bacterial samples, monitored to control possible impacts of ^{241}Am and/or HS on the formation of electronically excited states, were very similar and did not change with time (not shown). Each spectrum contained a structureless broad band with a flat maximum at 481 ± 3 nm and a full width at half maximum ca. 83 ± 3 nm. A typical BL spectrum (for the control sample)

is shown in Fig. 2. Full similarity of the BL spectra both for the control sample and in the presence of HS and/or ^{241}Am shows that the additives influenced the bacterial BL system at stages prior to the formation of electronically excited states [17,18].

3.2. Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopic studies

To control possible metabolic responses of the live bacteria to the α-radioactivity stress induced by ^{241}Am and its alleviation in the presence of HS, DRIFT spectroscopy was used. The DRIFT methodology, although measuring the IR irradiation reflected from the surface of powdered materials, features the IR absorption in the material [19,20,25,27]. Recently, DRIFT spectroscopy of dry bacterial cell biomass samples has been shown to be sensitive to fine structural rearrangements of cellular proteins, e.g. changes in the amide I band profile of cellular proteins (within the region 1620–1690 cm^{-1}), implying a redistribution of their secondary structure components (compared to the spectroscopic data in the control cells), as a response to stress and other external factors [19,25]. In addition, DRIFT spectroscopy is also sensitive to possible changes in the carbonyl stretching region (around 1740 cm^{-1}) related to the accumulation of intracellular biopolyesters, reserve materials found in many bacteria under unfavourable conditions (see [19,20,25–27] and references reported therein).

It has to be mentioned that the DRIFT methodology allows spectra to be obtained without the use of KBr (which is a common IR-transparent matrix used for mixing with the sample powder and pressing in a pellet for obtaining FTIR spectra in the conventional transmission-absorption mode). This is especially important for analysing polar functional groups, particularly those with H-bonds, such as proteins, since the polar matrix of KBr can affect the exact positions of their vibrational modes [19,20] and thus hamper their correct structural analysis.

A typical DRIFT spectrum in the mid-IR region (4000–400 cm^{-1}) which, to the best of our knowledge, is reported for the first time for photobacteria and, in particular, for *Ph. phosphoreum* (Fig. 3a), consists of a number of bands characteristic of major cellular constituents of Gram-negative bacteria (for details, see, e.g. [19,20,27]). The DRIFT spectra were very well reproducible and appeared to be very similar for all the samples studied (Fig. 3b). In particular, the amide I band (featuring mainly the stretching C=O vibration mode in peptide moieties of cellular proteins, sensitive to their secondary structure [27]) had a very similar shape with a maximum only slightly shifting from 1681 cm^{-1} (for control cells or with HS only) to 1684 and 1685 cm^{-1} (for the samples with ^{241}Am). This shift of +3 to +4 cm^{-1} in the amide I band maximum is yet too low to be reliably interpreted as a specific metabolic response. Note that our preliminary study of *Ph. phosphoreum* cells (sampled at the exponential growth stage (10 h) at which cells might have been more sensitive to environmental factors), in the presence of tritium, ^{3}H, showed a higher amide I band shift of +6 cm^{-1} as compared to the control and in the presence of HS [28]. Such a shift already implied an increase in the proportion of β-structured proteins, similar to that which had earlier been observed in other bacteria under stressed conditions [19,25]. It has to be emphasized that the overall noticeably nonsymmetrical shape of the amide I bands (see Fig. 3b), being similar in all the samples, with maxima within 1681–1685 cm^{-1}, implies a superposition of various secondary structure components (virtually unaffected by HS and/or ^{241}Am), with a considerable contribution from β-structures [19,27] (while a dominating α-helix commonly shows a maximum within 1655–1660 cm^{-1}).

All the samples (see Fig. 3b) showed a similarly weak but noticeable shoulder at ca. 1740 cm^{-1} (stretching C=O mode) typical of polyester moieties [19,20,25–27]. Note that ubiquitous cellular polyesters such as lipopolysaccharides and phospholipids also show a weak absorption in this region. Nevertheless, their total cellular content (a few percent of cell dry mass) and low molar percentage of the ester C=O moieties in these macromolecules usually give a much weaker shoulder. Thus, any noticeable increase in the absorption at ca. 1740 cm^{-1} is known to be caused by intracellular polyhydroxyalkanoates (PHAs). Boyandin et al. [21] showed that *Ph. phosphoreum* strain 1883 under nitrogen deficiency in the growth medium (a common factor inducing PHA accumulation in bacteria) can accumulate a homopolymer, poly-3-hydroxybutyrate (PHB), similar to many other bacteria [19,20,25–27], in low quantities only, up to a few percent of dry cell mass. Under our experimental conditions, the intensity of the shoulders at ca. 1740 cm^{-1} in Fig. 3b indeed corresponds to a few percent of cell dry mass of PHB (as estimated from DRIFT spectra using the procedure described in [20]). The approximately identical intensities of the shoulders at ca. 1740 cm^{-1} relative to the main bands of cellular proteins, amide I and amide II (around 1680 and 1550 cm^{-1}), imply that HS and/or ^{241}Am do not appreciably influence the PHB accumulation rate.

Thus, the similarity of DRIFT spectra in Fig. 3b implies a steadily low content of poly-3-hydroxybutyrate and no or negligible other compositional changes in the presence of ^{241}Am and/or HS. This suggests that by the stationary growth stage (20 h of growth), the α-radioactivity influences live *Ph. phosphoreum* cells at the level of the cellular BL enzyme system only, without other apparent metabolic responses involving noticeable structural and/or quantitative changes in cellular constituents.

4. Conclusions

Live cells of *Ph. phosphoreum* (strain 1883 IBSO), sampled at the stationary growth stage (20 h), were found to be highly sensitive to low-level α-radiation (^{241}Am; 2 kBq l^{-1}) showing an initial activation of BL intensity followed by its gradual decrease and, finally, inhibition. Humic substances added at a low concentration (2.5 mg l^{-1}) were found to efficiently alleviate the ^{241}Am radiotoxicity and resulted in a noticeable and prolonged bacterial BL activation. Besides the ecologically relevant radioprotection effect of HS, this could be beneficial for a further development of bacterial BL assays. Invariability of the bacterial BL spectra in the presence of HS and/or ^{241}Am shows that the additives influenced the bacterial BL system at stages prior to the formation of electronically excited states.

The DRIFT spectra of dry cell biomass of *Ph. phosphoreum*, reported for the first time for photobacteria, were found to be very similar both for the control cells and in the presence of HS and/or ^{241}Am, in particular, in the structurally and/or compositionally sensitive regions featuring cellular proteins and reserve polyesters. This suggests that by the stationary growth stage (20 h of growth), the α-radioactivity influences the bacterial cells mainly at the level of the cellular BL enzyme system, without any appreciable structural and/or quantitative changes in cellular macrocomponents detectable by DRIFT spectroscopy.

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