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Biohydrogen production by purple non-sulfur bacteria *Rhodobacter sphaeroides*: Effect of low-intensity electromagnetic irradiation

Lilit Gabrielyan [a,b], Harutyun Sargsyan [b], Armen Trchounian [a,b,*]

[a] Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia
[b] Research Institute of Biology, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia

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ABSTRACT

The present work was focused on the effects of low-intensity (the flux capacity was of 0.06 mW cm^{-2}) electromagnetic irradiation (EMI) of extremely high frequencies or millimeter waves on the growth and hydrogen (H_2) photoproduction by purple non-sulfur bacteria *Rhodobacter sphaeroides* MDC6521 (from Armenian mineral springs). After exposure of *R. sphaeroides*, grown under anaerobic conditions upon illumination, to EMI (51.8 GHz and 53.0 GHz) for 15 min an increase of specific growth rate by ~1.2-fold, in comparison with control (non-irradiated cells), was obtained. However, the effect of EMI depends on the duration of irradiation: the exposure elongation up to 60 min caused the delay of the growth lag phase and the decrease specific growth rate by ~1.3-fold, indicating the bactericidal effect of EMI. H_2 yield of the culture, irradiated by EMI for 15 min, determined during 72 h growth, was ~1.2-fold higher than H_2 yield of control cells, whereas H_2 production by cultures, irradiated by EMI for 60 min was not observed during 72 h growth. This difference in the effects of extremely high frequency EMI indicates a direct effect of radiation on the membrane transfer and the enzymes of these bacteria. Moreover, EMI increased DCCD-inhibited H^+ fluxes across the bacterial membrane and DCCD-sensitive ATPase activity of membrane vesicles, indicating that the proton F_oF_1-ATPase is presumably a basic target for extremely high frequency EMI related to H_2 production by cultures.

1. Introduction

Millimeter waves (MMW) are the new and widespread factor in the environment, the level of which is increased every year with development of technology [1,2]. MMW are short waves (with wavelengths from 0.1 mm to 10 mm) of electromagnetic energy varying in a frequency between 30 and 300 GHz.

Nowadays, the most common source of MMW is various satellite telecommunications, cellular and cordless phones, microwave ovens, dish antennas, and traffic and military radars [1–4] MMW does not affect the chemical bonds, but able to break down hydrogen bonds [5].

Effects of electromagnetic irradiation (EMI) of extremely high frequencies at low intensity on microorganisms have attracted the attention of scientists from various countries, because microorganisms living in various ecological conditions are exposed to different doses of MMW. During irradiation MMW have shown two types of effects: thermal and non-thermal [3,6]. Thermal effects are a result of absorption of MMW energy by cell molecules, producing the basic heating of the cells [2,4,7]. The non-thermal effects of MMW have been obtained from various experiments, which reported an increase of the bacterial growth induced by MMW [4,8].

EMI of extremely high frequencies has non-thermal effects on various microorganisms' growth properties and survival, metabolic activity, and bacterial sensitivity to antibiotics. Previously in our laboratory the inhibitory effects of extremely high frequency EMI have been reported for *Enterococcus hirae*, *Escherichia coli* and *Lactobacillus acidophilus* [2,9–11]. These effects depend on various conditions such as intensity of radiation, duration of exposure, bacterial culture growth conditions, pH of growth medium and others [2,9–11]. Among cellular targets are molecules of water, cytoplasmatic membrane and genome [1,2]. Change of bacterial membrane properties such as ions transfer and enzyme activity can serve as a basis for effects of EMI on various bacteria [2,11]. The H^+-translocating F_oF_1-ATPase has been suggested as one of the probable target for EMI [2,11]. This radiation has also been studied as a pretreatment process [7,12,13]. It has been shown, that microalgae pretreatment by microwave irradiation enhanced biogas production rate and final biogas yield [7]. However, effects of EMI of extremely high frequencies on phototrophic purple bacteria have not been reported yet.

In the present work the effects of EMI of extremely high frequency (51.8 and 53 GHz) or MMW (5.79 and 5.66 mm wavelengths) on the growth properties and hydrogen (H_2) photoproduction by purple non-sulfur bacterium *Rhodobacter sphaeroides* have been reported. The

* Corresponding author at: Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, 1 A. Manoukian Str., 0025 Yerevan, Armenia.
E-mail address: Trchounian@ysu.am (A. Trchounian).

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effects of these EMI on the H^+ flux though bacterial membrane and the F_OF1-ATPase activity were determined.

2. Materials and Methods

2.1. Bacterial Strain and Cultivation Conditions

R. sphaeroides strain MDC6521 (Microbial Depository Center, National Academy of Sciences of Armenia, Yerevan, Armenia, WDCM803), which was isolated from the Arzni mineral spring in the Armenian mountain (above sea level 1250 m), was used in the study [14,15]. The bacterium was cultivated in anaerobic conditions in batch culture (150 mL thick wall glass bottles) upon illumination with a light intensity of ~36 W m^{-2} in Ormerod medium containing carbon source – succinate (3.54 g L^{-1}) and nitrogen source – yeast extract (0.2%), as described previously [14,15]. Halogen lamp (60 W) was used for illumination. Light intensity was measured by a lux-meter LM37 (Carl Roth, Germany). Bacterial growth was recorded by changes in optical density (OD_{660}) using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA), and by determining dry weight (DW) of bacterial biomass (g L^{-1}), which was correlated with OD_{660} according the equation: $DW = 0.50 \times OD_{660}$. Lag phase of bacterial growth was measured graphically (intersection of tangent to growth curves) as time interval, during which cell number remains relatively constant (time before doubling of OD). Specific growth rate was determined as the quotient of ln2 division on doubling time of OD over the interval, when the logarithm of OD of the culture at 660 nm increased with time linearly (logarithmic growth phase), and it was expressed as h^{-1} [14,15].

Cells grown were harvested by centrifugation (6000 rot per min) during 20 min, washed and diluted in distilled water. Then, the bacterial suspension was divided into three parts: the first part served as a control (non-irradiated) and the other two were transferred into plastic plate (Petri dish) for irradiation with 51.8 GHz and 53 GHz.

2.2. Electromagnetic Irradiation of R. sphaeroides

The irradiation of *R. sphaeroides* suspension was performed by EMI generator (model G4–141) with conical antenna (State Scientific-Production Enterprize "Istok", Fryazino, Moscow Region, Russia), as described [9,11]. Bacterial suspension (10 mL) with thickness of ~1 mm (cell density was ~10^7 cell per mL) was exposed to EMI with the frequency of 51.8 GHz and 53 GHz in the option of amplitude modulation with frequency of 1 Hz (frequency stability was 0.05%); the flux capacity was of 0.06 mW cm^{-2} [9,11]. After direct irradiation of bacterial suspension for 15 min and 60 min, cells were immediately transferred into the fresh growth medium (concentrated cells volume was 1.5% of the growth medium) or the assay medium.

2.3. The Medium pH, Redox Potential Determinations and H_2 Assay

The initial pH of the medium was maintained at 7.5 ± 0.1 by 0.1 M NaOH or 0.1 M HCl. pH was determined during bacterial growth at certain time intervals (from 0 h to 96 h) by a pH-meter (HANNA Instruments, Portugal) with appropriate selective pH electrode (HJ1131B), as described before [15].

The value of medium redox potential (E_h) was determined during *R. sphaeroides* anaerobic growth using a pair of redox (platinum (Pt) and titanium-silicate (Ti–Si)) electrodes, as described [14,15]. Ti–Si electrode measured the overall E_h, whereas Pt electrode (sensitive to O_2 and H_2) under anaerobic conditions detected only H_2. E_h of both electrodes were tested in the control solution as described [15]: E_h at 25 °C was of 245 ± 10 mV. E_h kinetics determined using pair of redox electrodes during culture growth gives information about main redox processes and also H_2 generation [15]. The H_2 yield was evaluated by the drop of E_h to low negative values during bacterial growth, as described [14], and expressed in mmol L^{-1}. This determination of H_2 was close to the method with Clark-type electrode employed by other authors [16].

In addition, H_2 evaluation in bacterial suspension was confirmed by the chemical method based on the bleaching of solution of potassium permanganate in H_2SO_4 in the presence of H_2 [17].

2.4. Determination of H^+ Flux Through Bacterial Membrane

The flux of H^+ through the bacterial membrane in whole cells of *R. sphaeroides* was determined using appropriate selective electrode (HJ1131B, HANNA Instruments, Portugal), as described [9,11]. Bacterial cells (irradiated and non-irradiated) were transferred into the assay medium – 50 mM Tris-phosphate buffer (pH 7.5), containing 0.4 mM $MgSO_4$, 1 mM KCl and 1 mM NaCl, and then energy source – succinate was added. The H^+ flux was expressed as the change in the external activity of the ion in mmol min^{-1} per g DW. The bacterial culture was incubated for 10 min with 0.2 mM *N,N′*-dicyclohexylcarbodiimide (DCCD) in order to study the effects of this inhibitor on H^+ flux.

2.5. ATPase Activity Assay

Bacterial membrane vesicles were prepared by the Kaback method, as described before [14,15]. ATPase activity was measured by amount of inorganic phosphate (P_i), liberated after adding 3 mM ATP to membrane vesicles of *R. sphaeroides* [14,15]. P_i was determined by the method of Tausski and Shorr (1953) using a Spectro UV–Vis Auto spectrophotometer (Labomed, USA), as described previously [11,15]; corrections were made for blanks without ATP or membrane vesicles. ATPase activity was expressed in nmol P_i per μg protein per min. The assay mixture of 50 mM Tris-HCl buffer (pH 8.0), containing 0.4 mM $MgSO_4$, was used. When used, membrane vesicles were pre-incubated with DCCD for 10 min. Note, DCCD is known as inhibitor for the H^+-translocating F_OF_1-ATPase in bacteria, including *R. sphaeroides* [14].

2.6. Reagents, Data Processing and Others

Yeast extract, Tris (amino-methane) from Carl Roth GmbH (Germany); DCCD, ATP (Tris salt), sodium succinate from Sigma Aldrich (USA), and other reagents of analytical grade were used. Each experiment was repeated three times; error bars were presented on figures. Standard errors such as standard deviation were calculated using appropriate function of Microsoft Excel 2013, as described previously [14]. The changes were validated by calculation of Student's validity criteria (P) [9]; the differences between experiments (irradiated and non-irradiated cells) were valid, if $P < 0.05$.

3. Results and Discussion

3.1. Effects of Extremely High Frequency EMI on R. sphaeroides Growth Properties

R. sphaeroides was exposed to EMI with extremely high frequencies of 51.8 GHz and 53 GHz during 15 min and 60 min. The EMI of 51.8 GHz frequency is resonant for water (effects might be mediated by water), and it has been shown, that 53 GHz frequency can have resonant-like bactericidal effects on *E. coli*, which probably occurs by their direct interaction with bacteria [1–3]. Moreover, these were distinguishing effects [1,2,9–11].

After irradiation of *R. sphaeroides* by extremely high frequency EMI (51.8 GHz and 53 GHz) for 15 min an increase of specific growth rate by ~1.2-fold ($P < 0.02$) for EMI at the both frequencies, in comparison with non-irradiated cells (control), has been observed (Fig. 1a). However, exposure to EMI depended on the duration of irradiation: the elongation of exposure up to 60 min led the decrease in bacterial growth rate by ~1.3-fold ($P < 0.05$), indicating the bactericidal effect of the extremely high frequency EMI (Fig. 1a). The maximal inhibitory effect has been

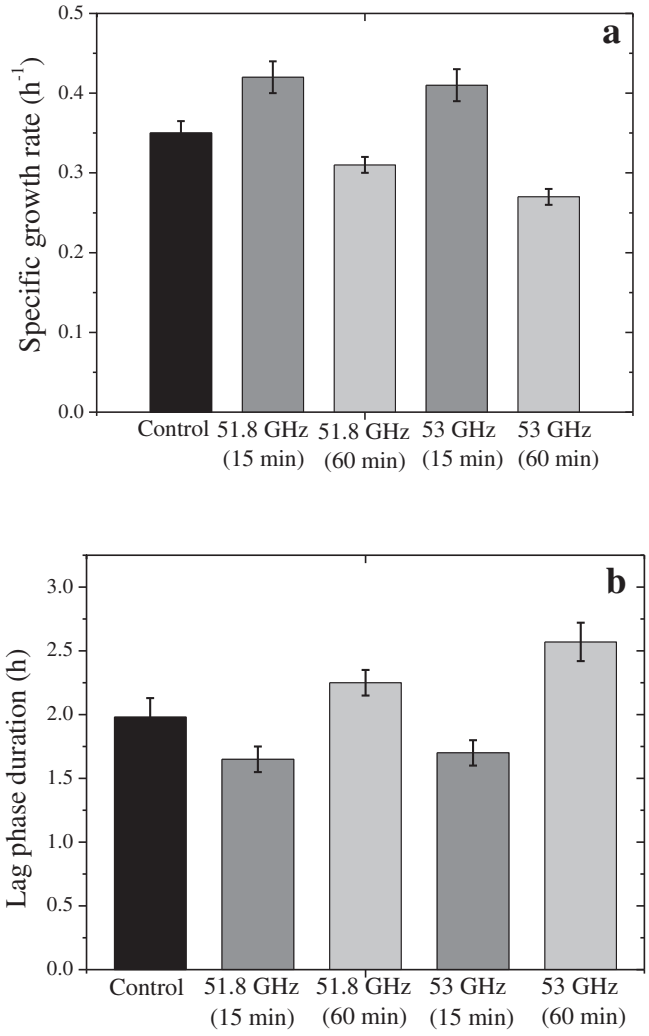

Fig. 1. The effects of 51.8 and 53 GHz frequencies EMI on *R. sphaeroides* MDC6521 growth properties: specific growth rate (a) and lag phase duration (b). Control was without irradiation.

obtained at the frequency of 53 GHz. The duration of latent (lag) growth phase was prolonged ~1.14-fold ($P < 0.01$) and ~1.3-fold ($P < 0.05$) for EMI at 51.8 GHz and 53 GHz, respectively (Fig. 1b). The results indicated antibacterial effect of EMI on *R. sphaeroides*.

3.2. Effects of Extremely High-Frequency EMI on Redox Potential Kinetics and H_2 Production by R. sphaeroides

It is known, that redox potential (E_h) is very important factor of the environment, which can be defined as the biological system ability to reduce or oxidize various compounds. The anaerobic growth of *R. sphaeroides* is coupled with a drop of E_h from the positive to the low negative values, which determine the bacterial anaerobic growth [15,18,19].

To reveal the action mechanisms of EMI on *R. sphaeroides*, E_h kinetics during bacterial growth was studied. *R. sphaeroides* MDC6521 control cells grew well under anaerobic conditions upon illumination, resulting a drop of E_h from the positive ($+130 \pm 5$ mV) values at the beginning of lag growth phase to the low negative (-517 ± 25 mV) values upon transition to a stationary phase (Fig. 2). E_h of bacterial medium has gradually decreased during the 72 h anaerobic growth up to -700 ± 5 mV and -677 ± 20 mV for EMI at 51.8 GHz and 53 GHz for 15 min, respectively; whereas E_h of control non-irradiated cells has decreased up to -637 ± 20 mV (Fig. 2). This drop indicates the enhancement of

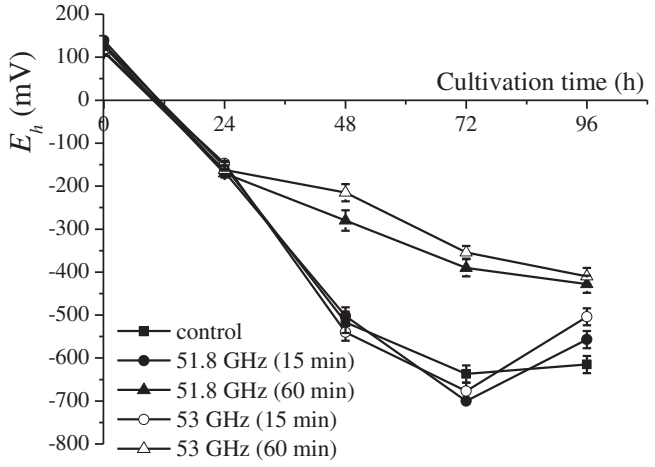

Fig. 2. The effects of 51.8 and 53 GHz frequencies EMI on E_h kinetics during *R. sphaeroides* MDC6521 anaerobic growth in batch culture upon illumination.

reduction processes, which characterize bacterial metabolism under anaerobic conditions, and generation of H_2 upon the photo-fermentation [14,15]. The prolongation of exposure up to 60 min resulted in a delayed decrease of E_h: for EMI at 51.8 GHz E_h decreased up to -390 ± 20 mV during the 72 h bacterial growth, whereas for EMI at 53.0 GHz E_h drop was -354 ± 15 mV (Fig. 2). The inhibition of bacterial growth after prolongation of exposure of extremely high frequency EMI up to 60 min can be coupled with E_h or with direct effect of EMI on bacterial membrane.

A relationship between decreasing of E_h to low negative values and H_2 generation was shown for various bacteria: the electron flow can be shifted toward the reduction of protons to H_2 under strong reducing conditions [15,18].

R. sphaeroides has been shown to produce H_2 by nitrogenase, which is dependent on supplying electrons from reduced ferredoxin and requires large amount of ATP [14,20]. The nitrogenase-encoding gene (responsible for H_2 generation in these bacteria) is expressed in anaerobic conditions under light; and nitrogenase catalyzes the conversion of H^+ to H_2 [20,21].

In control non-irradiated cells H_2 production has been obtained after 48 h anaerobic growth and increased ~4 fold ($P < 0.05$) during the growth up to 96 h (Fig. 3). The increase of H_2 generation might be coupled with activity of H_2-producing enzymes, which are involved in H_2 metabolism in purple bacteria. H_2 yield of the culture, irradiated by

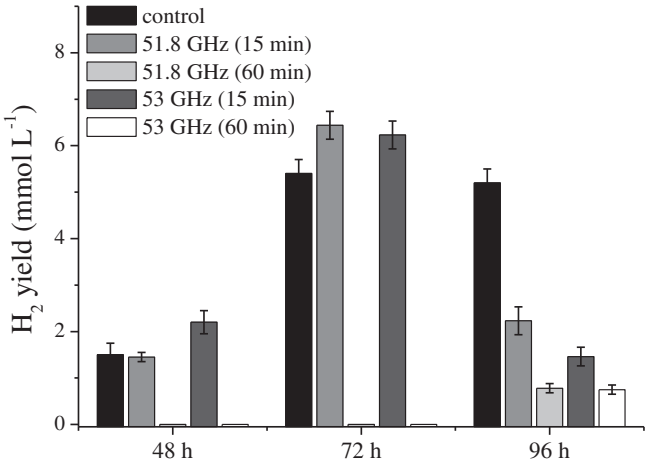

Fig. 3. The effects of 51.8 and 53 GHz frequencies EMI on H_2 photoproduction during *R. sphaeroides* MDC6521 anaerobic growth in batch culture.

extremely high frequency EMI at both frequencies for 15 min, determined during 72 h anaerobic growth, was ~1.2-fold ($P < 0.01$) higher than H_2 yield of non-irradiated cells; whereas H_2 production by *R. sphaeroides* determined during 96 h growth was ~2.3-fold ($P < 0.02$) and 3.5-fold ($P < 0.05$) lower in cells, irradiated by EMI at 51.8 GHz and 53 GHz, respectively, in comparison with control (Fig. 3). H_2 production by cultures, irradiated by EMI at both frequencies for 60 min was not observed during 72 h growth and decreased ~7-fold ($P < 0.05$) after 96 h growth, in comparison with non-irradiated control (Fig. 3). The difference in the effects of extremely high frequency EMI indicated the direct effect of radiation on the membrane transport and the enzymes of these bacteria. This is in favor with results obtained for the other bacteria [1–3].

3.3. Effects of Extremely High-Frequency EMI on H^+ Fluxes Through the Bacterial Membrane and on Membrane-Associated F_oF_1-ATPase Activity

Proton-coupled membrane transport and ATPase activity have been determined in the culture of *R. sphaeroides*, irradiated by extremely high frequency EMI. Exposure to EMI (51.8 GHz and 53.0 GHz) of *R. sphaeroides* for 15 min did not affect energy-dependent H^+ fluxes through bacterial membrane of whole cells (Fig. 4a). But it has been shown, that extremely high frequency EMI at 51.8 and 53 GHz increased H^+ fluxes in the presence of DCCD, the specific inhibitor of H^+-translocating systems, in ~2- and 1.5-folds ($P < 0.02$), respectively (Fig. 4b). The results obtained indicate that the F_oF_1-ATPase might be a target in *R. sphaeroides* for extremely high frequency EMI.

It is known, that H_2 generation in purple bacteria is mediated by nitrogenase, requiring the energy of ATP produced by the F_oF_1-ATPase [14,20]. Some conformational change in the F_oF_1-ATPase, leading to modulation of activity by EMI, is possible. EMI at 51.8 and 53 GHz decreased of overall membrane-associated ATPase activity in 1.5-fold ($P < 0.05$) (Fig. 5a). Indeed, DCCD-sensitive ATPase activity of membrane vesicles has been increased ~1.4- and 1.2-fold ($P < 0.05$) in 51.8 and 53 GHz irradiated cells, in comparison with non-irradiated control (Fig. 5b). The results indicate the effect of extremely high frequency EMI on F_oF_1-ATPase and suggest that this ATPase is a sensitive target for EMI. The similar data were obtained for *E. coli* and *E. hirae* [10,11].

4. Conclusion and Significance

Extremely high frequency EMI can change metabolic activity of bacteria, especially connected with their membranes. EMI can interact with the free and bounded water molecules of bacterial membranes and change their structure; as well as changes in the bacterial membrane properties, such as H^+ fluxes across the membrane and the membrane-associated ATPase activity were observed [1,2,9–11].

The effects of extremely high frequency EMI at 51.8 and 53 GHz on growth properties and H_2 yield in purple non-sulfur bacterium *R. sphaeroides* have been studied for the first time. Novel and significant

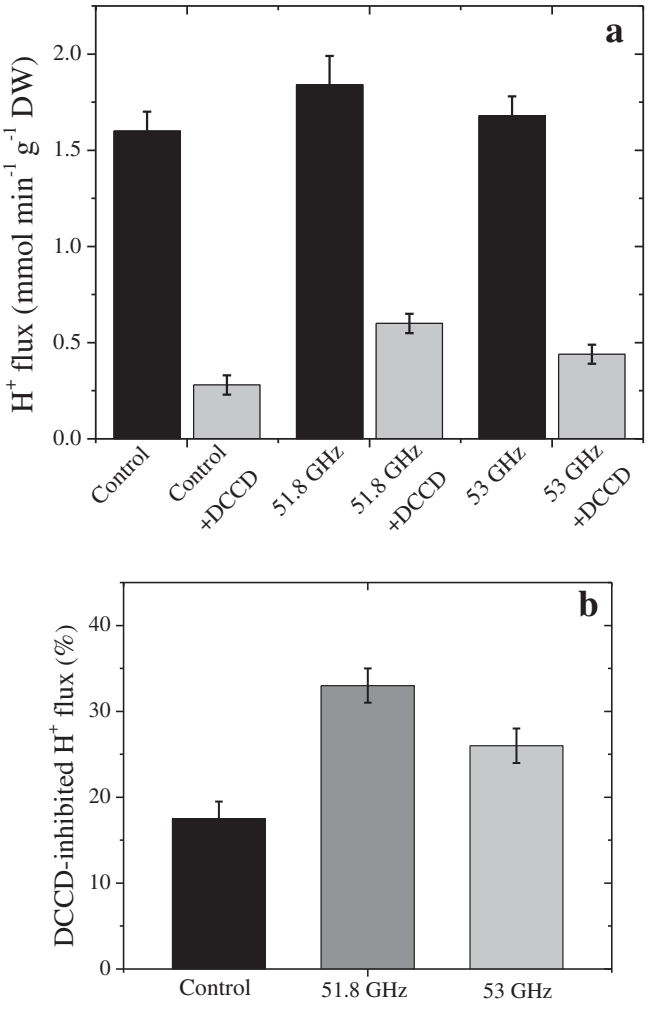

Fig. 4. The effects of 51.8 and 53 GHz frequencies EMI on energy-dependent total (a) and DCCD-sensitive (b) H^+ fluxes through *R. sphaeroides* MDC6521 membrane. Control was without irradiation, 0.2 mM DCCD was present in the assay medium, when indicated.

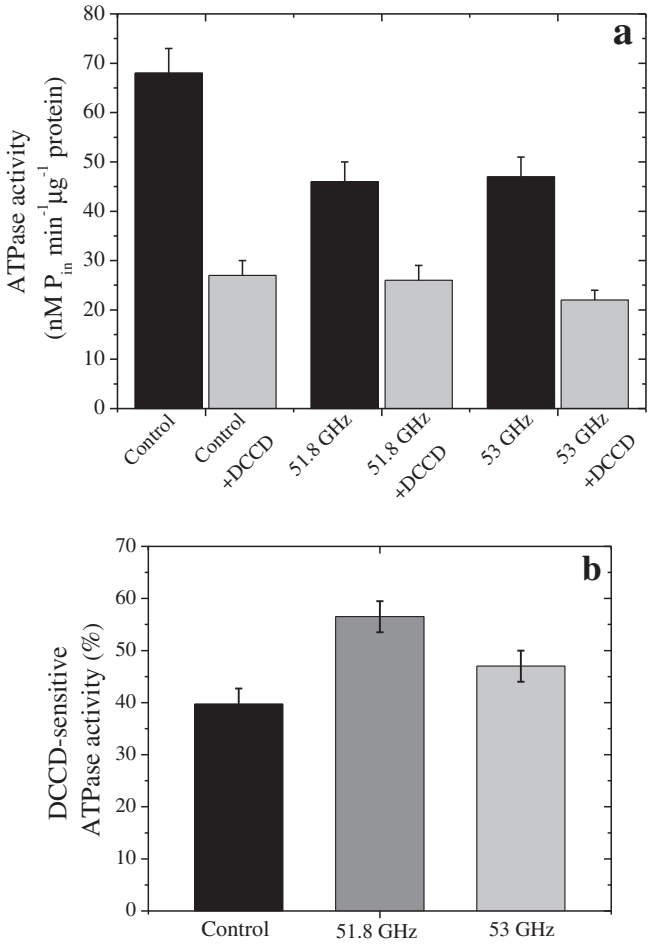

Fig. 5. (a) The effects of 51.8 and 53 GHz frequencies EMI and DCCD on ATPase activity of *R. sphaeroides* MDC6521 membrane vesicles. Control was without irradiation, 0.2 mM DCCD was present in the assay medium, when indicated. (b) The effects of 51.8 and 53 GHz frequencies EMI on DCCD-inhibited ATPase activity of *R. sphaeroides* membrane vesicles.

data about the EMI effects on H^+ flux though bacterial membrane and the F_oF_1-ATPase activity in *R. sphaeroides* have been obtained.

The results obtained indicate that the effect of extremely high frequency EMI depends on the duration of irradiation. Irradiation of EMI for 15 min increased the specific growth rate at both frequencies, whereas the extension of exposure up to 60 min showed the bactericidal effect of the EMI on *R. sphaeroides*. It is expressed in the delayed duration of growth lag phase and inhibition of bacterial growth rate. The findings are similar to effects of EMI on other bacteria such as *E. coli*, *E. hirae* and lactic acid bacteria [2,9–11]. The stimulatory effects of EMI at 30–60 GHz were also reported for nitrogen-fixing bacteria such as *Azotobacter* sp. [8]. By small exposure time (5–10 min) the increase of bacterial cell's number was obtained [8]. The effect of short time exposure of EMI might be coupled with direct or indirect activation of compensatory mechanisms or bacterial repair systems.

Changes of membrane properties as EMI possible targets are discussed. It has been shown, that extremely high frequency EMI affects E_h of bacterial medium: this parameter has gradually decreased, indicating the enhancement of reduction processes and H_2 production by *R. sphaeroides*. H_2 production by culture, irradiated by EMI at both frequencies for 15 min was ~1.2-fold higher than H_2 yield of non-irradiated cells (72 h growth). Prolongation of irradiation duration up to 60 min led to H_2 production inhibition.

The difference in the effects of EMI indicate a direct effect of radiation on the H^+ fluxes across the bacterial membrane and the membrane-associated ATPase activity. It has been shown, that extremely high frequency EMI increases DCCD-inhibited H^+ fluxes across the membrane and DCCD-sensitive F_oF_1-ATPase activity of membrane vesicles. These results point out the role of the F_oF_1-ATPase as a target for extremely high frequency EMI. Moreover, the changes of ATPase activity can be a result of E_h variation [19]. The similar data on direct or indirect effects on enzymes and other membrane-associated activity such as ATPase activity were obtained for various bacteria [2,9–11].

This work might reveal new perspectives in regulation of growth and hydrogen metabolism in phototrophic purple bacteria those are important in application of these bacteria in biotechnology. Understanding the mechanisms of action of EMI on purple bacteria is very important for the possible future use of EMI technology.

Acknowledgments

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