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Low-intensity electromagnetic irradiation of 70.6 and 73 GHz frequencies enhances the effects of disulfide bonds reducer on *Escherichia coli* growth and affects the bacterial surface oxidation–reduction state

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ABSTRACT

Low-intensity electromagnetic irradiation (EMI) of 70.6 and 73 GHz frequencies (flux capacity – 0.06 mW cm^{-2}) had bactericidal effects on *Escherichia coli*. This EMI (1 h) exposure suppressed the growth of *E. coli* K-12(λ). The pH value (6.0–8.0) did not significantly affect the growth. The lag-phase duration was prolonged, and the growth specific rate was inhibited, and these effects were more noticeable after 73 GHz irradiation. These effects were enhanced by the addition of DL-dithiothreitol (DTT), a strong reducer of disulfide bonds in surface membrane proteins, which in its turn also has bactericidal effect. Further, the number of accessible SH-groups in membrane vesicles was markedly decreased by EMI that was augmented by *N,N′*-dicyclohexycarbodiimide and DTT. These results indicate a change in the oxidation–reduction state of bacterial cell membrane proteins that could be the primary membranous mechanism in the bactericidal effects of low-intensity EMI of the 70.6 and 73 GHz frequencies.

1. Introduction

A complex network of sensing and responding to physical and chemical factors is used by living cells, especially by bacteria, to communicate with each other and to survive under different environmental conditions [1]. It was suggested that electromagnetic irradiation (EMI) of extremely high frequency (30–300 GHz) with low energy (low intensity) at specific resonant frequencies can affect bacteria in the manner of energy transformation into informative signals [2]. This is interesting due to the initial absence of EMI from the environment, the use of EMI in mobile and low-orbital cosmic communication systems [3] and its wide ranges of applications – in agriculture, medicine, food science, ecology and more [4,5].

Rapidly accumulating data describe the potential of low-intensity coherent EMI of narrow ranges (resonant frequencies) to cause stimulating or depressing effects on *Escherichia coli* which is considered the best-characterized bacteria and a model organism [6–9]. These effects mainly depend on irradiation intensity and exposure, the composition of growth and irradiation media, the genetic features of strains, the peculiarities of bacterial metabolism and other factors [10–12]. Additionally, these effects can regulate the reciprocal reaction of organisms against impact of physical and chemical factors (antibiotics and other chemicals) [12,13]. Alterations in the growth cycle of bacteria is possible due to metabolic processes or mechanical resonances [2,18]. However, the primary mechanisms of these effects are not clear yet.

From the literature and our earlier works it is known that *E. coli* growth can be depressed at specific frequencies of low-intensity EMI from the ranges of 45–53 GHz and of 70–75 GHz [8–13]. Simultaneously, that effect occurs with the lytic cycle of lambda bacteriophage at the frequencies of 41.3, 51.8 and 70.5 GHz [7,14]. Therefore, genome targeting is one of the possible interaction mechanisms with such EMI. However, the energy produced by these frequencies is not sufficient to break a chemical bond in DNA. It is possible that EMI at these levels can generate oxygen radicals, or disturb DNA-repair processes [14].

The elastic forces in cell membranes or walls help to dampen oscillatory forces by taking part in coherent self-sustained oscillations, resulting in possible macromolecular conformational transitions that are fed with metabolic energy [15–17]. They are biologically driven and require ATP. Thus, the proton F_0F_1-ATPase, the main enzymatic complex of the bacterial membrane, can play a key role in membranous mechanisms of EMI action. The latter has been proven with the changes of irradiated bacterial cell sensitivity to N,N′-dicyclohexycarbodiimide (DCCD) – an inhibitor of the F_0F_1-ATPase [12,13,18,19]. The change in the oxidation–reduction potential (E_h) of the bacterial surface, which plays an exclusive role in where bacteria can survive and especially in the regulation of the F_0F_1-ATPase is another findings [18]. Moreover, EMI effects on bacteria can be mediated by water molecules at their own

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resonant frequencies (41.5, 51.8 and 53 GHz) [20], and a dramatic decrease in E. coli growth was shown for these frequencies [10,12,13,18]. The oscillation of water molecules can alter protein conformation and the degree of hydration and other properties of proteins [6,7,10,11]. The effects on E. coli growth and on properties of water molecules have been recently reported for 70.6 and 73 GHz EMI [10]. However, more intensive studies are needed to derive clear explanations and applications.

In this paper, the effects of low-intensity 70.6 and 73 GHz EMI on *E. coli* growth under different oxidation–reduction conditions were monitored. Thus, non-molecule-based triggering of changes in bacterial cell growth at pH of 6.0–8.0, the growth correlation with the effect of a reducer of disulfide bonds – DL-dithiothreitol (DTT), [21], and changes in the number of surface-accessible SH-groups by EMI of those frequencies were significant.

2. Materials and methods

2.1. Bacteria, growth and preparation to assays

The wild-type strain of *E. coli*, K-12(λ), was grown in peptone medium (0.2% peptone, 0.5% NaCl and 0.2% K_2HPO_4, pH 7.3) in anaerobic conditions by fermenting glucose (0.2%). Then bacteria were harvested and concentrated by centrifugation. Next the bacteria were resuspended in bi-distilled water, and the suspension was divided into two parts. The first part was used as a control (non-irradiated). The second part was transferred onto a plate for subsequent irradiation [10–13,18,19]. Control and irradiated bacteria were transferred into the growth medium at pH 6.0–8.0 or a DTT concentration of 1 or 3 mM (pH 7.3). The number of SH-groups was also determined. The pH was adjusted by small amounts of 0.01 M NaOH and 0.01 N HCl.

Bacterial growth was monitored for ~12–14 h (in the same conditions, see above) with a Spectro UV–Vis Auto spectrophotometer (Labomed, USA) by obtaining the absorbance changes at a wavelength of 600 nm. The lag phase duration and specific growth rate were determined as described elsewhere [10–13].

Membrane vesicles were isolated from spheroplasts treated with lysozyme by the method of osmotic lysis. The protein content was determined by the Lowry method using bovine serum albumin as a standard [12,18,22,23].

2.2. Electromagnetic irradiation procedure

The bacterial suspensions on plates (thickness ~1 mm) were irradiated with a coherent electromagnetic field of 70.6 or 73 GHz frequencies (4.23 or 4.11 mm wavelengths, respectively) using a generator G4–142 type (Russian made). The amplitude-modulation frequency was 1 Hz (frequency stability was 0.05%) and the distance from the conical antenna to the object of irradiation was ~20 cm (the far zone). For this distance, the power flux density measured using a power meter (Russian made) was 0.06 mW cm^{-2} (power was equally-distributed in the exposed sample and power reflected to the waveguide system was insignificant). The overall procedure was the same as that described elsewhere [11–13].

2.3. The determination of the number of SH-groups

The number of accessible SH-groups of membrane vesicles were determined by reactivity with Ellmann reagent (5,5′-dithiobis-2-nitrobenzoic acid) using the Spectro UV–Vis Auto spectrophotometer (Labomed, USA) as described [22] using glutathione as a standard. The assay mixture was 50 mM Tris–Cl buffer with 0.4 mM $MgSO_4$ and 100 mM KCl (pH 8.0). Appropriate corrections were made for blanks without membrane vesicles. Assays were conducted in different conditions – non-treated and treated with DTT (3 mM), and/or with DCCD (0.2 mM) treated membrane vesicles [22]. For treatment, vesicles were incubated with reagents for 10 min prior to the assays. In the case of DTT and DCCD, the two incubations were done sequentially. The amount of SH groups were expressed in nmol (mg protein)$^{-1}$. The effects of DCCD and DTT were the measured as the differences between the amounts of SH-groups found in their presence and in their absence.

2.4. Data processing

Three replicates of each data point were taken at 37 °C. The SigmaPlot software was used to determine the standard errors (<3%) and the Student's t test calculation with the validity criteria (p) (validity for the difference between the changed values for each average data with irradiated bacteria and the non-irradiated control; if not mentioned, $p \leqslant 0.001$) [11].

3. Results

3.1. 70.6 and 73 GHz EMI effects on E. coli growth at different pH values

E. coli growth depression by low-intensity 70.6 and 73 GHz EMI has been determined in our previous work (at pH 7.5) [11]. Inhibition of *E. coli* growth by EMI at other pH values was demonstrated in this work. At pH 7.3 (similar to pH 7.5), the growth specific rate was depressed ~1.3-fold and ~1.4-fold by 70.6 and 73 GHz EMI, respectively, but at pH 6.0 and 8.0 the inhibitions were lower – ~1.1-fold and ~1.2-fold ($p = 0.002$) compared with the control (Fig. 1A). Moreover, bacteria exposed to 70.6 GHz EMI had a ~1.5-fold prolonged lag-phase compared with non-exposed cells at these pH values ($p = 0.003$). However, the bactericidal effect of 73 GHz EMI was clearly observed at pH 7.3, when the lag-phase was prolonged by ~2-fold ($p = 0.05$). In the cases of pH 6.0 and 8.0, the prolongation of lag-phases was ~1.9-fold and ~1.7-fold, respectively (Fig. 1B). Most likely, lag-phase prolongation indicated about unfavorable conditions that still permit growth (nutrients are available; pH is not extreme; etc.).

These data demonstrated pH-dependent effects of EMI on bacterial growth, resulting stronger bactericidal action at pH 7.3. These results confirmed earlier findings [11] of bactericidal effects of EMI at both frequencies, especially at 73 GHz. Such effects at 70.6 and 73 GHz EMI might indicate their resonant interaction with bacteria. Changes in the properties of water molecules at these frequencies have been shown before [11], but bactericidal effects are complex and not associated with those changes alone. Alterations in the conformations and functions of membrane proteins might be involved as well.

3.2. DTT effect on irradiated E. coli growth

DTT action on irradiated *E. coli* growth was established only at pH 7.3 because the effect of DTT is independent of pH [21]. DTT in concentrations of 1 (not shown) and 3 mM (Fig. 2) had a depressive effect on *E. coli* growth compared with the control. The more effective concentration for DTT was 3 mM. DTT led to a~1.2-fold decrease of the growth specific rate ($p = 0.003$) (Fig. 2A) and to a ~1.6-fold prolongation of the lag-phase ($p = 0.003$) (Fig. 2B) in non-irradiated bacteria. These values were even higher for irradiated bacteria. The inhibitory effect of DTT on bacterial growth is not new: this study confirmed the results reported before [22,24]. However the enhancement of DTT's effect on bacteria by EMI was novel. Certainly, EMI changed bacterial sensitivity toward

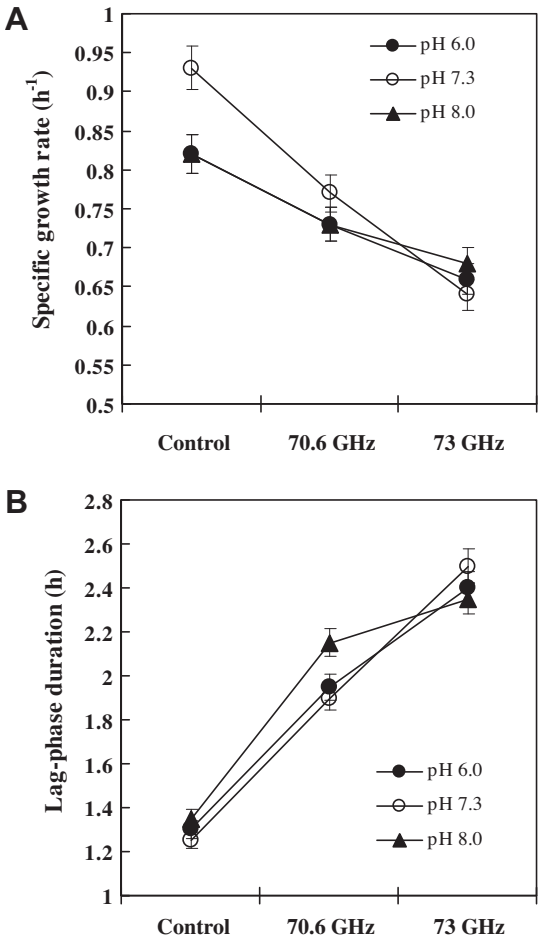

Fig. 1. *E. coli* K12(λ) growth specific rate (A) and lag-phase duration (B), in the control and after EMI of 70.6 and 73 GHz frequencies in peptone medium at different pH. Irradiation time was 1 h, also see Section 2.

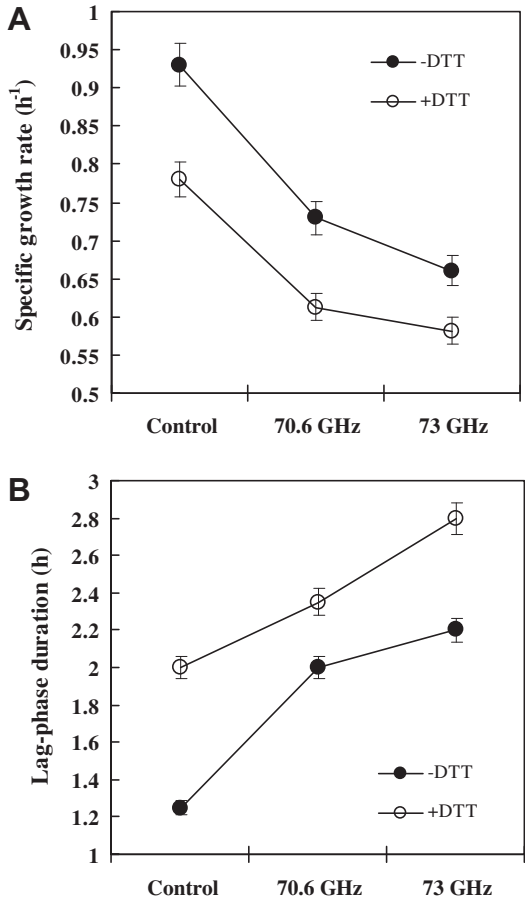

Fig. 2. The effects of 70.6 and 73 GHz EMI in combination with DTT on *E. coli* K12(λ) specific growth rate (A) and lag-phase duration (B). DTT (3 mM) was added into the growth medium (pH 7.3) immediately before inoculation. Control was without EMI and DTT. Also, see legends to Fig. 1.

DTT, which was more effective in the case of 73 GHz, as the growth specific rate was decreased by ~1.6-fold ($p = 0.002$) and the lag-phase was prolonged by ~2.2-fold compared with the control. With 70.6 GHz, the changes in these growth characteristics were lower: the decreases were by ~1.5-fold ($p = 0.002$) and by ~1.9-fold, respectively.

DTT, as a strong reducer, resulted in the loss of activity or specificity of membrane surface proteins to alter cell surface oxidation–reduction state or to induce oxidative stress with subsequent dramatic alterations within the cell [22,24]. Perhaps the inhibition of the growth rate and the prolongation of the lag-phase duration in controls and in EMI exposed bacteria were results of DTT stimuli, which elicited complex but functionally-coordinated alterations in the functions of membrane proteins.

3.3. Changes in the number of accessible SH-groups of membrane vesicles by EMI

To understand the role of membranes in the primary targeting of 70.6 and 73 GHz EMI signals, the number of accessible SH-groups of membrane vesicles was determined for non-irradiated (control) and irradiated bacteria. The suggestion that the effects of low-intensity EMI might be related to modifications in the structure, stability, interactions with each other, enzymatic activity and other functions of membrane proteins [12,17–19] could be supported by the decreased number of SH-groups in irradiated bacteria. Both 70.6 and 73 GHz EMI lowered this number by ~1.4 and ~1.6 folds, respectively, compared with the control ($p = 0.003$) (Fig. 3A). Furthermore, it was shown that ATP increased the number of SH-groups in the control and in bacteria exposed to 70.6 GHz by ~1.2-fold, and in bacteria exposed to 73 GHz by ~1.1-fold. The treatment of membrane vesicles with DCCD also had an enhancing effect on the number of SH-groups, but in the control, it was less (an increase of ~1.1-fold) than in the 70.6 and 73 GHz irradiated samples, which increased by – ~1.2-fold. After DCCD treatment and subsequent ATP addition, this number was decreased by ~1.3-fold compared with the initial level in the control (in membranes without ATP and DCCD). In vesicles of bacteria exposed to 73 GHz this number was increased by ~1.2-fold. The number in bacteria exposed to 70.6 GHz was unchanged. These data might be a result of changed intra- and inter-disulfide-dithiol transitions and conformational changes in membrane proteins, especially those caused by EMI.

Similarly, the number of SH-groups in membrane vesicles was determined after DTT treatment at the same conditions as those described above. The effective concentration of DTT required to decrease the level of SH-groups was 3 mM (Fig. 3B) (effects with lower concentration of DTT were not shown). In this case, the number of SH-groups was ~5.8-fold lower than in the control ($p = 0.003$), and in bacteria irradiated with 70.6 and 73 GHz, the numbers were – ~10 and 4.7-fold less, respectively (see Fig. 3). However, DTT cannot reduce buried (solvent-inaccessible) disulfide bonds, so this could mean that 70.6 GHz, but not 73 GHz EMI decreased the accessibility of intra- and inter-molecular SH-groups.

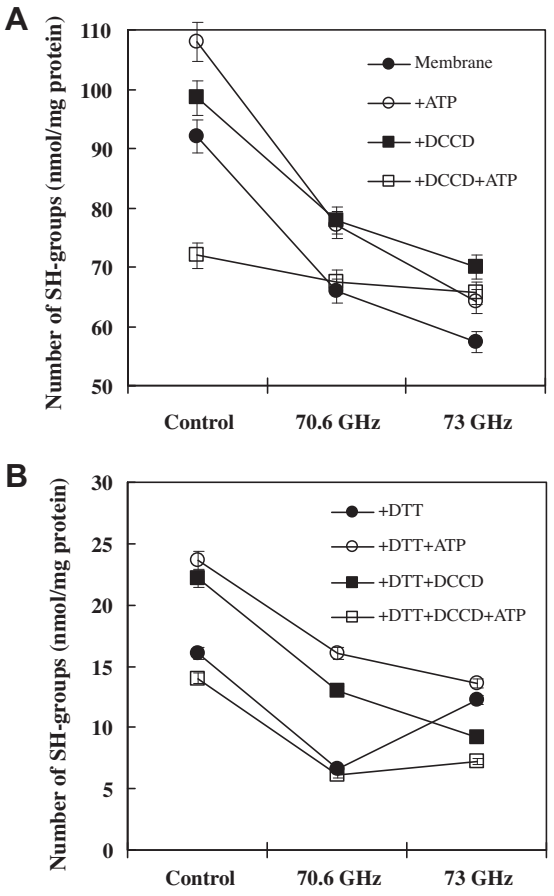

Fig. 3. The changes in the number of accessible SH-groups in *E. coli* K12(λ) membrane vesicles after 70.6 and 73 GHz EMI determined without (A) and with DTT (B). Membrane vesicles were treated with DCCD (0.2 mM) and DTT (3 mM) for 10 min prior to the assays, and ATP (3 mM) was added when mentioned. In case DTT + DCCD incubation was done consequently. The value was a difference between the values in presence and absence of membranes in assay medium. For others, see Section 2.

Moreover, the number of SH-groups was increased after ATP addition by ~2.4-fold in the 70.6 GHz irradiated bacteria, whereas in the control and in 73 GHz irradiated cells, the increases were only ~1.5 and ~1.1-fold, respectively (see Fig. 3). Thus, the ATP-dependent increase of the level of SH-groups was a result of thiol restoration in membrane proteins, especially in the F_0F_1-ATPase, by DTT, as previously suggested [22–25]. Similar effects were determined with DCCD-treated controls and 70.6 GHz irradiated cells, but not 73 GHz irradiated vesicles. DCCD and ATP together returned the level of accessible SH groups approximately to their initial values – when treated only with DTT in the cases of the control and 70.6 GHz irradiation. However, in the case with 73 GHz irradiation the number was ~1.7-fold lower.

Therefore, EMI of these frequencies most likely not only affected the F_0F_1-ATPase, but also changed the interaction between membrane proteins, especially through disulfide cross links, by modifying their structure and stability.

4. Discussion

The effects of 70.6 and 73 GHz EMI on *E. coli* growth under different conditions have been established previously [11] and were further investigated in this work to better understand the exact action mechanisms. These frequencies were determined to be resonant frequencies for *E. coli* because of their bactericidal action. More depressive effect on bacterial growth had 73 GHz.

Due to the insufficiency of the available technique, the exact mechanisms of EMI action on bacteria have not been clarified yet [11]. We have tried to find a link between EMI and alterations in the oxidation–reduction state of the bacterial cell surface. It is known that bacterial growth regulation depends on E_h, which plays an essential role in the activity of membrane proteins and cellular metabolism [21,24,25]. Because the membrane potential linked to outside conditions, the oxidation–reduction state of the bacterial surface could be changed by alterations of the external pH and by the addition of DTT [21,24].

The pH that most clearly demonstrated the bactericidal effect of EMI was 7.3 (see Fig. 1). A link between anaerobic growth of bacteria, the external pH and an oxidation–reduction state is suggested; at alkaline pHs, the growth is coupled with a shift of E_h from the positive to the low negative values [21,24,25]. Therefore, with a lowering of the pH, a larger E_h decrease can be observed. This is an unusual finding according to the theory of oxidation–reduction processes, but it has been experimentally proven with various bacterial species [21,24,25]. Thus, for irradiated *E. coli*, the surface oxidation–reduction state most likely had been changed by our treatment, and the bacteria could not regulate it or restore it to a sufficient level.

Moreover, in anaerobic conditions, the addition of DTT depressed *E. coli* growth. DTT as a strong reducer dropped E_h and amplified reduction processes in the cell by directly changing the state of thiol groups in membrane proteins [21,22]. This might affect the affinity and activity of membrane proteins and their interactions with each other, especially that the interaction between the F_0F_1-ATPase and formate hydrogen lyase [22,23]. EMI increased sensitivity toward DTT. This could be a result of deeper changes in proteins, in their interactions with each other and in their functions. The enhanced growth inhibition by EMI under anaerobic conditions upon sugar fermentation and at alkaline pHs (see Fig. 2) could be a consequence of bacterial inability to regulate oxidation–reduction processes.

The decrease in the number of accessible SH-groups in membrane vesicles irradiated by 70.6 and 73 GHz EMI suggests the membrane proteins are the primary target of the radiation (see Fig. 3). This change was a consequence of rearrangement in intra- and inter-molecular disulfide bonds in membrane proteins [21].

This study may lead to a clear understanding of the strict primary action mechanisms of extremely high frequency EMI on the cellular level and may be useful in determining more effective bactericidal conditions and in developing new antibacterial therapies and for food protection.

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