Short Communication

Exposure to an extremely low-frequency electromagnetic field only slightly modifies the proteome of *Chromobacterium violaceum* ATCC 12472

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Abstract

Several studies of the physiological responses of different organisms exposed to extremely low-frequency electromagnetic fields (ELF-EMF) have been described. In this work, we report the minimal effects of *in situ* exposure to ELF-EMF on the global protein expression of *Chromobacterium violaceum* using a gel-based proteomic approach. The protein expression profile was only slightly altered, with five differentially expressed proteins detected in the exposed cultures; two of these proteins (DNA-binding stress protein, Dps, and alcohol dehydrogenase) were identified by MS/MS. The enhanced expression of Dps possibly helped to prevent physical damage to DNA. Although small, the changes in protein expression observed here were probably beneficial in helping the bacteria to adapt to the stress generated by the electromagnetic field.

Keywords: *C. violaceum*, electromagnetic field, proteomic analysis.

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Various biological effects of extremely low-frequency electromagnetic fields (ELF-EMF) (< 0.05 mT) in living organisms have been reported (Berg, 1999; Fojt *et al.*, 2004). However, the biological peculiarities of each organism analyzed and the different experimental conditions used have made it difficult to establish the effects of ELF-EMF in biological systems (Fojt *et al.*, 2004, 2009; Di Campli *et al.*, 2010). Moreover, the increasing use of electric and electronic appliances heightens the need to clarify the effects of such interference. In addition, there is a growing investment in projects related to the generation of electricity through natural sources such as hydroelectric power plants (HPP) that require an extensive network of urban and rural transmission lines that consequently generate ELF-EMF.

Only a few investigations into the effects of ELF-EMF on bacterial cells have been undertaken (Cellini *et al.*, 2008; Huwiler *et al.*, 2012), despite the fact that their short cell cycle and easy handling make bacteria a good model organism for this type of study. *Chromobacterium violaceum* is a free-living bacterium that has been isolated from diverse tropical and subtropical environments around the world (Lima-Bittencourt *et al.*, 2011; Ponnusamy *et al.*, 2011). The genome of strain ATCC 12472 has been sequenced and analyzed by the Brazilian National Genome Consortium (Brazilian National Genome Project Consortium, 2003) and knowledge of its genetic repertoire has led to more detailed studies of the biology of this microorganism (Stauff and Bassler, 2011; Silva-Rocha *et al.*, 2013; Castro-Gomes *et al.*, 2014). *Chromobacterium violaceum* ATCC 12472 has also been tested and analyzed under various stress conditions using high-throughput screening technologies (HTST) such as two dimensional difference gel electrophoresis (2D-DIGE) coupled with mass spectrometry (2D-DIGE-MS/MS) (Baraúna *et al.*, 2011; Ciprandi *et al.*, 2012). The large amount of molecular data generated by this approach provides a basis for the use of *C. violaceum* as a model organism to evaluate biological responses to the magnetic field in prokaryotes. Proteomics provides a useful means of detecting changes in global protein expression and generates crucial information for the identification of new biological targets after exposure to ELF-EMF

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(Wittmann-Liebold *et al.*, 2006; Leszczynski *et al.*, 2012). Technological advances and the importance of omics techniques in ELF-EMF research are indispensable and were emphasized by the World Health Organization and the Radiation and Nuclear Safety and Authority at meetings held in Helsinki, Finland in 2005 (Leszczynski and Meltz, 2006). The aim of this study was to use 2D-DIGE-MS/MS to examine the ability of an electromagnetic field generated by transmission lines to modify the *in situ* expression profile of *C. violaceum* ATCC 12472 at a selected point of the bacterial growth curve.

For bacterial exposure, pre-inocula grown overnight in LB medium were standardized to an optical density at 720 nm (OD_{720}) of 0.04 and exposed to ELF-EMF. The *C. violaceum* cells were exposed *in situ* in a stable environment at the Regional Transmission Station of Pará belonging to Northern Brazil Power Plants S/A (Regional de Transmissão do Pará das Centrais Elétricas do Norte do Brasil S/A - Eletronorte). Flasks containing 50 mL cultures were kept either near the station exit fence (control group) or below the breakers formed by the 500 kV transmission lines of the Tucuruí Hydroelectric Plant (treated group) and were exposed for 7 h without agitation. The electromagnetic field was monitored using an electromagnetic field radiation detector (EMF tester, Lutron). The OD of the samples, used as an indicator of bacterial density, was determined with a Novaspec II spectrophotometer at 720 nm (Pharmacia Biotech). The experiment was carried out in biological triplicate for each condition (control and treated groups) being run on each occasion.

After exposure, cultures were centrifuged (5,000 x *g*, 10 min, 4 °C) and the cell pellets were washed with 50 mM Tris-HCl, pH 7.5 and resuspended in a lysis solution (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris-HCl, pH 7.5) containing a protease inhibitor cocktail (Roche). The bacteria were sonicated and the resulting lysate was centrifuged (21,000 *g*, 1 h, 4 °C). The supernatant containing solubilized proteins was stored at -70 °C until used. Samples were quantified using a 2D Quant kit (GE Healthcare) according to the manufacturer's protocol.

Following protein precipitation by the methanol/chloroform method (Wessel and Flügge, 1984), 54 µg of protein from each sample was labeled with either 400 pmol of Cy3 (control) or Cy5 (treated) dyes for 30 min and the reactions were stopped by adding 10 mM L-lysine. All labeling procedures were done on ice and protected from light. A mixture of all replicates corresponding to 54 µg of protein was used as an internal control and labeled with Cy2. Subsequently, samples for each condition and the internal control were mixed, diluted with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.002% bromophenol blue, 0.5% IPG buffer, 50 mM DTT) and applied to immobilized pH gradient (IPG) strips (24 cm) of pH 4-7 (GE Healthcare), according to the manufacturer's protocol. Three replicates from independent cultures were

analyzed. The strips were rehydrated with each sample for 16 h at room temperature. Isoelectric focusing (IEF) was done using an Ettan IPGphor II apparatus (GE Healthcare) at a total of 115,599 Vh for 18 h. After IEF, the strips were equilibrated and transferred to the top of a 12.5% polyacrylamide gel and the second dimension was run in an Ettan DALTsix system (GE Healthcare) at 5 W per gel for 30 min and then at 17 W per gel until the bromophenol blue reached the bottom of the gel.

Images of DIGE gels were obtained using an Ettan DIGE Imager scanner and analyzed with Image Master 2D Platinum software v.7.0 (all from GE Healthcare). Spot detection was done automatically. Spots with an average relative volume of ± 1.3-fold were considered to be differentially expressed. ANOVA was used to assess the significance of the changes in expression, with $p < 0.05$ indicating significance. After expression analysis, preparative gels containing 450 µg of protein were stained with colloidal Coomassie blue and subsequently used for digestion and identification of differentially expressed protein spots. The images of the preparative gels were aligned with those obtained for the 2D-DIGE analytical gels in the Image Master 2D Platinum program to ensure correct recovery of the differentially expressed spots.

The spots of interest were manually excised from the preparative gels, dehydrated with acetonitrile and incubated with trypsin solution (50 mM ammonium bicarbonate, 10 mM acetic acid and trypsin 20 ng/µL) (Promega) for 1 h on ice. Excess trypsin solution was removed with a pipette and the peptides were digested at 58 °C for 30 min. Subsequently, the digested peptides were extracted from the gels with an ultrasonicator following the addition of 30 µL of 30% formic acid and 50% acetonitrile. The sample was concentrated to approximately 10 µL in a SpeedVac and desalted with a ZipTip (Millipore). For identification, the samples were mixed at a 1:1 ratio with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma) and transferred to the Anchorchip 600 plate of a MALDI-TOF/TOF AutoflexIII (Bruker Daltonics). All spectra were measured in the positive reflector mode. Spectra obtained by this procedure were analyzed using the Mascot server (http://www.matrixscience.com) and compared with the genomic information of the Proteobacteria group deposited in the NCBI nr database (http://www.ncbi.nlm.nih.gov). MS and MS/MS spectra for the identified proteins are shown in Figure S1.

The strength of the electromagnetic field in the control and treated cultures was 0.02 µT and 0.66 µT, respectively, such that the exposure was > 30 times greater in the bacterial cultures near the transmission line; the latter cultures also had a higher bacterial density (greater OD_{720}) at the time of extraction ($p < 0.05$) (Figure S2). In contrast to these findings, studies using different strains of *E. coli* have found no difference in growth when cells were exposed to

Table 1 - *Chromobacterium violaceum* proteins that were differentially expressed during exposure to an electromagnetic field and identified by MS/MS.

Match ID	Locus tag	Protein accession no.	Mascot score	Protein name	Theoretical pI/MW (Da)	Matched peptides
394	CV_2728	34498183	51	Alcohol dehydrogenase	6.12 / 39190	K.AVELIAGFLRR.A
						R.LAADVGIPAGLR.E
458	CV_4253	34499708	112	DNA-binding stress protein	5.59 / 17456	R.ALGHYAPGSYADYAK.L

an electromagnetic field (Mittenzwey *et al.*, 1996; Cellini *et al.*, 2008; Huwiler *et al.*, 2012).

The proteomic analysis detected five spots that were differentially expressed in the cultures near the breaker. Of these, one spot was down-regulated and four were up-regulated (Figure 1 and Table 1); two of these five spots were identified by MS/MS as DNA-binding stress protein (Dps) and alcohol dehydrogenase (Table 1 and Figure S1). These results showed that the exposure of *C. violaceum* to ELF-EMF under the conditions described here caused minimal changes in the bacterial protein expression profile. The two proteins identified here were related to DNA protection and cellular metabolism.

The importance of the versatile Dps protein family in various types of stress, including acidic and oxidative stress, as well as in the physical protection of DNA molecules, has been described (Martinez and Kolter, 1997; Haikarainen and Papageorgiou, 2010; Calhoun and Kwon, 2011). Bacteria have a well-developed mechanism for protecting DNA from physical damage during stress and during exponential growth Dps expression is up-regulated by the hydrogen peroxide-inducible gene activator OxyR, a regulator also found in the genome of *C. violaceum*. The other overexpressed protein was alcohol dehydrogenase. The enhanced expression of this enzyme was most likely

related to an increase in energy production by the bacteria to regenerate NAD$^+$ and was probably related to the greater bacterial growth in the presence of a 0.66 µT magnetic field (Figure S2).

Similar findings to those described here were reported for peripheral human blood lymphocytes and *Saccharomyces cerevisiae* strain DBY747 exposed to electromagnetic fields ranging from 1 to 100 µT (Luceri *et al.*, 2005). There were no DNA strand breaks in either of these cell types, nor was there any variation in the gene expression profile as assessed by microarray experiments (Luceri *et al.*, 2005). Despite the minor influence that ELF-EMF had on global gene expression in *C. violaceum*, certain proteins showed significant changes in their expression levels. Other organisms have also shown only minor physiological and molecular changes after exposure to ELF-EMF. For example, there was a decrease in the viability of *Helicobacter pylori* after exposure to ELF-EMF (Di Campli *et al.*, 2010), whereas *Salmonella enterica* subsp. *enterica* serovar Hadar showed overexpression of the genes *rpoA*, *katN*, and *dnaK* (El May *et al.*, 2009). To date, few studies have used omics techniques to evaluate the gene expression profile of organisms exposed to electromagnetic fields. As shown here using a proteomic approach, the global gene expression of *C. violaceum* was only slightly altered when the bacteria were exposed to ELF-EMF.

Acknowledgments
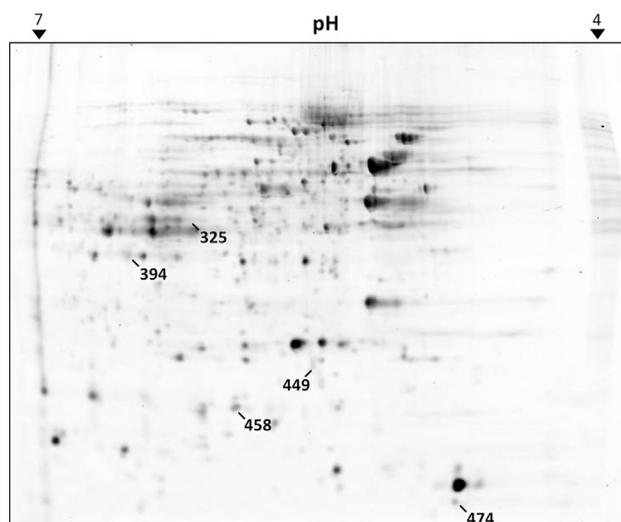
Figure 1 - 2D-DIGE gel of *C. violaceum* bacteria exposed to a low frequency electromagnetic field. The differential spots are indicated by their Match ID. Spot 325 was down-regulated while the others were up-regulated. The scale above the gel indicates the pH range used in the first dimension of 2D-DIGE.

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Supplementary Material

The following online material is available for this article:

Figure S1 - MS and MS/MS spectra generated for spots 394 (a) and 458 (b).

Figure S2 - Cell density and protein concentration of control and exposed cultures.

This material is available as part of the online article from: http://www.scielo.br/gmb

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