Flávia Viana[1,3], Olga Maria Lage[2,3] and Rui Oliveira[1]

High ultraviolet C resistance of marine Planctomycetes

[1] Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

[2] Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/nº 4169-007 Porto, Portugal

[3] CIMAR/CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental – Universidade do Porto, Rua dos Bragas, 289, 4050-123 Porto, Portugal

Corresponding author

Olga Maria Lage

Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre s/nº 4169-007 Porto, Portugal

Phone: +351 220402724

Fax: +351 253678980

E-mail: olga.lage@fc.up.pt

Abstract

Planctomycetes are bacteria with particular characteristics such as internal membrane systems encompassing intracellular compartments, proteinaceous cell wall, cell division by yeast-like budding and large genomes. These bacteria inhabit a wide range of habitats, including marine ecosystems, in which ultra-violet radiation has a potential harmful impact in living organisms. To evaluate the effect of ultra-violet C on the genome of several marine strains of planctomycetes, we developed an easy and fast DNA diffusion assay in which the cell wall was degraded with papain, the wall-free cells were embedded in an agarose microgel and lysed. The presence of double strand breaks and unwinding by single strand breaks allows DNA diffusion, which is visible as a halo

upon DNA staining. The number of cells presenting DNA diffusion correlated with the dose of ultra-violet C or hydrogen peroxide. From DNA damage and viability experiments, we found evidence indicating that some strains of planctomycetes are significantly resistant to ultra-violet C radiation, showing lower sensitivity than the known resistant *Arthrobacter* sp. The more resistant strains were the phylogenetically closer to *Rhodopirellula baltica*, suggesting that these species are adapted to habitats under the influence of ultra-violet radiation. Our results provide evidence indicating that the mechanism of resistance involves DNA damage repair and/or other DNA ultra-violet C-protective mechanism.

Key words: Planctomycetes, UVC resistance, Comet assay, DNA diffusion assay, Genotoxicity

41

42 **1. Introduction**

43 The planctomycetes constitute an unusual and distinctive group of bacteria that forms a divergent
44 phylum of the Domain Bacteria. They possess unique features such as a proteinaceous cell wall
45 lacking peptidoglycan, in general reproduction by a yeast-like budding process, large genome sizes
46 and an exceptional cell plan with a complex internal membrane structuring (Fuerst 2005; Ward et
47 al. 2006). Planctomycetes are ubiquitous microorganisms that have been described to be widely
48 distributed in very different habitats (Ward et al. 2006) such as marine, brackish, freshwater, soil
49 and sediments; hypersaline, hypothermal and acidic environments; and in association with other
50 organisms such as invertebrates (Fuerst et al. 1991;1997), namely sponges (Friedrich et al. 1999;
51 2001; Webster et al. 2001), cyanobacteria and microalgae (Ward et al. 2006), macroalgae (Lage
52 and Bondoso 2011) and plants (Fuerst 1995; Derakshani et al. 2001; Kulichevskaia et al. 2006).
53 This broad distribution suggests the existence of a wide physiological diversity that allows
54 planctomycetes to adapt and colonize such diverse ecosystems. Planctomycetes are believed to
55 play an important role in the global biogeochemical cycles, namely the carbon and nitrogen cycles
56 through *e.g.*, the mineralization of marine snow particles (DeLong et al. 1993). *Rhodopirellula*
57 *baltica* is well characterized as a polysaccharide degrader (Rabus et al. 2002; Glöckner et al. 2003;
58 Gade et al. 2005). In addition, the presence of a high number of genes encoding for proteins with
59 significant homology to sulfatases in several planctomycetes, namely *Rhodopirellula baltica*
60 (Glöckner et al. 2003), suggests the involvement of these organisms in the transformation of
61 inorganic sulphur (Hieu et al. 2008; Wegner et al. 2013). Planctomycetes are the unique living
62 organisms known to carry out the anaerobic ammonium oxidation, also known as the anammox
63 process, in which nitrite and ammonium are converted to dinitrogen gas (Stous et al. 1999).These
64 observations are indicative that planctomycetes are key players on several ecosystems where their
65 activity translates into vital events to the overall equilibrium.
66 Marine microorganisms are exposed to variable ultra-violet radiation (UV) depending on their
67 position in the water column. Besides direct damage on DNA, decrease in growth and survival,
68 pigment bleaching and photoinhibition of photosynthesis have been identified among several

69 biological effects of UV (Sinha and Häder 2002; Batista et al. 2009). This radiation is divided,
70 according to the wavelength, in three segments, UVA (400-315 nm), UVB (315-280 nm) and UVC
71 (280-100 nm). UVB and UVC are the most energetic and harmful ones, causing direct and indirect
72 damage due to the high absorption by nucleobases (Mitchell and Karentz 1993). However, UVC
73 does not reach the Earth's surface due to the absortion effect of the ozone layer. Microorganisms in
74 the water column are under the influence of the detrimental effect of UV, depending on the
75 atmospheric conditions and the clarity of water. In addition, in shallow areas and in the intertidal
76 zone, UV can be very high. In all cases, UV irradiation can oscillate significantly during the day,
77 which imposes dynamic stress responses to organisms. Ultra-violet radiation constrains the
78 distribution of microorganisms such as planctomycetes, which resistance and response
79 mechanisms have only recently began to be investigated (Lieber et al. 2009).
80 Marine organisms have inhabited earth before the existence of oxygen in the atmosphere that
81 could afford the protection and had been fully exposed to short wavelength radiation in shallow
82 areas and in intertidal habitats. Therefore, mechanisms of protection and recovery from UVC have
83 been developed by living organisms through evolutionary history (Garcia-Pichel 1998). In
84 addition, other factors may also contribute to UV irradiation survival under planetary anoxic
85 conditions: the presence of high numbers of cells and the formation of clusters may provide
86 shielding of cells against UV and resistance against extreme stress may also provide cross
87 protection against UV (Cockell et al. 2011). With the emergence of the ozone layer, the
88 evolutionary pressure continued as damage caused by UVB radiation resembles the one caused by
89 UVC, with both causing similar photoproducts. (Schuch and Menck 2010).
90 In this work we evaluated the impact of UVC on several marine planctomycetes strains isolated
91 from the surface of macroalgae, which are normally exposed to high radiation levels. Analysis of
92 DNA damage caused by several stress agents can be made by several laboratory techniques.
93 Among the numerous methods to detect DNA damage in cells, the comet assay figures as one of
94 the most popular method due to its relative celerity and easiness. In addition, the capacity of being
95 applied to almost all types of cells, along with the single-cell result obtained, has increased its
96 application in diverse fields of biological study (Dhawan et al. 2009). The comet assay (Östling

and Johanson 1984) and the DNA diffusion assay (Singh 2000) have been extensively used as reliable tools for the assessment of DNA damage in many biological systems. Despite this, to our knowledge, application of the comet assay to bacterial cells has only been reported in a limited number of scientific papers (Singh et al. 1999; Fernández et al. 2008). By developing an improved DNA damage assay based on the single cell gel electrophoresis or comet assay, here designated by DNA diffusion assay, the genotoxic effects of UVC on selected species and the potential protective role of planctomycetes extracts against the noxious effects of this radiation were evaluated. The applicability of this assay in prokaryotes has been restricted possibly due to the usual small size of their genomes. Here we demonstrate the applicability of an adaptation of the comet assay, the DNA diffusion assay, to planctomycetes, which are among the prokaryotic organisms with larger genomes as suggested by the 7,145 megabases genome of the recently sequenced genome of *Rhodopirellula baltica* (Glöckner et al. 2003).

2. Materials and Methodology

2.1 Strains, media and growth conditions

The planctomycetes used in this work were the pigmented *Rhodopirellula baltica* (Schlesner at al. 2004) and strains Cor5, LF2, Gr7, FC9.2 and FF15 (Fig. 1) that were previously isolated from the surface of marine macroalgae, namely, *Corallina* sp. (Cor5), *Laminaria* (LF2), *Gracillaria bursa-pastoris* (Gr7) and *Fucus spiralis* (FC9.2 and FF15), collected from different locations in the northern coast of Portugal (Lage and Bondoso 2011). *Rhodopirellula baltica* was chosen as a model in this study due to its phylogenetic proximity to several of the other strains under study and to the known adaptation to environmental stressors (Wecker et al. 2009). In UV viability experiments we have also used a pigmented strain of *Arthrobacter* sp. isolated in our laboratory from the surface of macroalgae. Cultures were maintained on modified solid M13 medium (Lage and Bondoso 2011) at 26 ºC and stored at 4 ºC. Liquid cultures were incubated in an orbital shaker at 26 ºC, 200 revolutions per min (rpm), with a ratio flask volume/medium of 10/1. Growth was monitored by optical density measured at 600 nm (OD_{600}). Stock cultures of *Saccharomyces cerevisiae* strain BY4741 (*MAT*a, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*) (Brachmann et al. 1998)

were grown on solid YPD medium (1 % w/v yeast extract, 2 % w/v peptone, 2% w/v glucose and 2 % w/v agar) at 30 ºC for 2 days and then stored at 4 ºC for 2 weeks. For experiments, yeast cells were grown in liquid YPD medium, using an orbital shaker at 30 ºC, 200 rpm, with a ratio flask volume/medium of 10/1. Growth was monitored by optical density at 600 nm.

2.2 Optimization of a DNA damage detection assay in planctomycetes

The improved assay for DNA damage detection in planctomycetes species was designed based on the comet assay protocol used for *Saccharomyces cerevisiae* (Azevedo et al. 2011) and the protocol of DNA diffusion assay for bacteria described by Fernández et al. (2008). For the degradation of the proteinaceous cell wall we tested different concentrations and digestion times of proteinase K and papain. Observation of samples after alkaline lysis treatment in optical microscopy evidenced that in papain-treated samples, a larger proportion of cells were lysed than in the case of proteinase K treatment (data not shown). Discrimination of lysed cells without genotoxic treatment was possible by blurry edges and by a considerably higher length (at least 2.5 μm) than normal cells (around 1 to 1.5 μm; our unpublished data). In addition, to try to improve the efficiency of the lysis step, we have also increased the temperature of incubation from 4 ºC to 37 ºC so that DNA unwinding could become more pronounced. We have applied the electrophoresis step of the comet assay in order to obtain comet tails with length directly propotional to DNA damage. However we obtained only halos of diffusin of DNA (not shown). Therefore, we have excluded this step in the optimization of the assay. The presence of a fluorescent halo dispersed from the cell was considered as DNA-damaged cell in opposition to cells without surrounding fluorescence bearing non-damaged DNA (Fig. 2a). Furthermore, we have excluded the neutralization step after alkaline lysis and decreased the DNA fixation time with ethanol, the final steps of the comet assay procedure, without affecting results. Finally, we have improved sharpness of images from samples by including an overnight, 80 ºC incubation after DNA fixation. Based on the above-mentioned observations the final protocol was established as follows. Cultures of planctomycetes were grown overnight in modified M13 liquid medium at 26 ºC, 200 rpm, until OD_{600} 0.1-0.2. Cells from one milliliter of each culture were harvested by

153 centrifugation at 15000 rpm, 4 ºC for 5 min, ressuspended in papain buffer (10 mg mL^{-1} papain,
154 Sigma; 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4; 38 mM
155 ethylenediaminetetraacetic acid -EDTA-; 10 mM DL-cysteine) and incubated 1 h at 37 ºC, 200
156 rpm to ensure cell wall digestion. Cells were harvested by centrifugation at 15000 rpm, 4 ºC for 5
157 min, washed twice with the same volume of ice-cold S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH
158 6.5), resuspended in 100 µL of S buffer and then distributed by 25 µL aliquots. Sixty microliters of
159 1.5 % low-melting agarose (w/v in S buffer) at 37 ºC was then added to each sample and 20 µL of
160 the mixture were placed on glass slides (pre-coated with 0.5 % w/v normal-melting agarose),
161 covered with cover slips and allowed to solidify at 4 ºC for 5 min. The cover slips were then
162 removed and the samples were subjected to the genotoxic treatment (see 2.3). Immediately after
163 exposure, the slides were immersed in fresh lysis solution (30 mM NaOH, 1 M NaCl, 0.05% w/v
164 laurylsarcosine, 50 mM EDTA, 10 mM Tris-HCl, pH 10) previously warmed at 37 ºC. The slides
165 were incubated in the dark at 37 ºC for 20 min and then washed three times on a tray with
166 abundant ice-cold deionized water. Fixation was performed by three sequential washes in cold
167 ethanol (-20 ºC) at 70 % (v/v), 90 % (v/v) and 100 %, 3 min each. Subsequently, slides were dried
168 overnight at 80 ºC and then stored at 4 ºC until observation. For fluorescence microscopy analysis,
169 slides were previously stained with 10 µL of GelRed® (1:3000 v/v).
170
171 **2.3 Genotoxic treatments**
172 Agarose-embedded cells on the slides and cells in inoculated solid media were subjected to UVC
173 (254 nm) irradiation using the Stratalinker® UV Crosslinker (Stratagene) at 100, 300, 500, 700
174 and 1000 J m^{-2} (for planctomycetes) or 10, 30, 50, 70, 90, 100 and 150 J m^{-2} (for yeast). In all
175 cases, irradiated cells were immediately placed in the dark in order to avoid photoreactivation of
176 the enzyme photolyase responsible for the conversion of pyrimidine dimers into pyrimidines. For
177 chemical genotoxic treatments, 10 mM H_2O_2 was applied directly on top of the minigel containing
178 the cells and incubated 20 min at 4 ºC.
179
180 **2.4 Viability assay of planctomycetes strains after exposure to UVC**

Planctomycetes viability was determined in modified M13 solid media, inoculated by streaking with a sterile toothpick from a log phase liquid culture (OD_{600} of 0.1-0.2) in modified M13 at 26 ºC, 200 rpm. After incubation at room temperature to dry the surface of the medium, plates were irradiated without lid with the different UVC doses. Plates were then incubated for 10 days at 26 ºC in the dark. Images were obtained using a zoom stereomicroscope (Wild Heerbrugg M8).

2.5 Analysis of the UVC protective effect of ethanolic extracts of planctomycetes

2.5.1 Ethanolic extraction of pigments

For this experiment strain Cor5 was chosen due to its high resistance to UVC observed in this study. A culture of strain Cor5 was prepared in 1 L liquid modified M13 medium, incubated 8 days at 26 ºC, 200 rpm and cells were harvested by centrifugation at 15000 rpm, 4 ºC for 5 min and frozen at -20 ºC overnight. Ethanol 99 % (v/v) was added and the samples were sonicated several times until the cellular mass ran out of color. The supernatant was separated by decantation from the cellular debris and the ethanol was allowed to evaporate on a rotary evaporator at 38 ºC. The remaining sediment was then dissolved in a dichloromethane/methanol solution (9:1) and pigments were separated by column chromatography with silica gel as the solid adsorbent in stationary phase. The presence of pigments in the various fractions was assessed by thin layer chromatography. Two bands were obtained (CORI and CORII) and each fraction was dissolved in 500 µL of ice-cold absolute ethanol. In order to allow the complete homogenization, samples were kept for two hours in the dark at 4 ºC with agitation (150 rpm) and, subsequently, stored in the dark at -20 ºC.

2.5.2 Viability assay of *Saccharomyces cerevisiae*

A culture of *Saccharomyces cerevisiae* in YPD was incubated overnight in an orbital shaker at 30 ºC, 200 rpm, diluted in fresh YPD medium to OD_{600} 0.1, incubated under the same conditions for two generations (until OD_{600} 0.4) and diluted back to OD_{600} 0.1 with fresh YPD. The suspension was serially diluted with YPD (1, 10^{-1}, 10^{-2} and 10^{-3} fold concentration) and sets of 5 µL drops were placed on solid YPD medium. Plates were allowed to dry and then exposed without lid to the

desired doses of UVC. Subsequently, plates were incubated at 30 ºC, in the dark for 48 h and images were then obtained using the Molecular Imager ChemiDoc XRS System (Bio-Rad) and analyzed with the Quantity One 1-D image analysis software.

For assessment of the potential UVC protective effect of pigments from planctomycetes, ethanolic extracts of planctomycetes (see 2.5.1) were added to cell suspensions (40x extract final dilution) just before the serial dilution step (see above) and incubated at 30 ºC for 20 min. Cells were harvested by centrifugation at 15300 rpm, 4 ºC for 2 min and resuspended in YPD medium in order to maintain the initial cell concentration. Cell suspensions were serially diluted and viability was assessed as stated above. A similar assay was done without the washing step with fresh YPD to keep the extracts in contact with cells during UVC exposure (co-incubation). For control, the same volume of absolute ethanol was added to cell suspensions, as it was the solvent in which extracts were dissolved.

2.6 Statistical analysis

All data was obtained from at least two independent experiments. Results presented in photographs are from representative experiments. One-way analysis of variance (ANOVA) was used for comparison using the GraphPad Prism version 5 software.

3. Results

3.1 Effects of genotoxic agents in planctomycetes

UV radiation is a major stressor in marine environments. Marine planctomycetes living in the intertidal zone experience daily oscillations of UV light exposure. To estimate the impact of this radiation on planctomycetes we have selected a species isolated from the water column (*Rhodopirellula baltica*) and several from the macroalgae surface (strains Cor5, LF2, Gr7, FC9.2 and FF15). The phylogenetic relationships of the planctomycetes, based on the 16S rDNA, are presented in Fig. 1. Cor 5 is a strain of *R. baltica* and LF2 and FC9.2 are two different species of *Rhodopirellula* sharing, respectively, 97.9 and 96.6 % in the 16S rRNA gene sequence similarity

236 with *R. baltica* SH1^T. FF15 is phylogenteically related to *Blastopirellula marina* and Gr7 to
237 *Planctomyces brasiliensis*. The selected strains cover different members of Planctomycetales.
238 In this study, we developed an assay for detection of DNA damage in these microorganisms. In
239 this assay, cells treated with a genotoxic agent are embedded in an agarose microgel, lysed and the
240 damaged genomic DNA diffuses as a result of double strand breaks and unwinding induced by
241 single strand breaks. For most of the strains, the proportion of cells considered to present DNA
242 damage correlated with increasing UV dosages (Figs. 2b and 3). Only in strains Gr7 and FF15, the
243 levels of DNA damage did not alter significantly above low dosages, suggesting that the dosage of
244 100 J m^{-2} was sufficient to cause a maximum of DNA damage. In order to verify the applicability
245 of the DNA diffusion assay to chemical genotoxic agents and further validate its applicability, we
246 used hydrogen peroxide (10 mM) as genotoxicant. The proportion of cells with DNA damage has
247 also increased with this genotoxic agent (Fig. 4), suggesting that this assay can detect DNA
248 damage caused by different kinds of genotoxic agents (UVC and oxidative damage) in these
249 bacteria.
250
251 **3.2. UVC effects on viability of planctomycetes**
252 The impact of UV on viability was investigated by exposing the several planctomycetes under
253 study to UVC. The number of viable cells was clearly affected by radiation dosages equal and
254 above 100 J m^{-2}, the lowest level assayed (Fig. 5). Strains FF15 and Gr7 were the most susceptible
255 ones while Cor5 and FC 9.2 were the most resistant, being the only strains able to resist UVC
256 doses above 500 J m^{-2}. *Arthrobacter* sp. was used as reference for UVC sensitivity since it was
257 described as a UVC resistant bacterium (Kuhlman et al. 2005; Osman et al. 2008). Our results
258 suggest that, in general, the marine planctomycetes used in this study are quite resistant to UVC,
259 especially strains FC9.2, Cor5, *Rhodopirellula baltica* and LF2 as they display at least similar
260 sensitivity to UVC as *Arthrobacter* sp.
261
262 **3.3. Evaluation of a potential protective effect of ethanolic extracts of planctomycetes to**
263 **UVC-mediated DNA damage**

264 Some studies suggest that more pigmented strains could be more resistant to UV exposure
265 although a relationship between pigmentation and resistance has never been demonstrated
266 (Hermansson et al. 1987; Arrage et al. 1993; Joux et al. 1999; Kolber et al. 2000; Béjà et al. 2000;
267 de la Torre et al. 2003). In our work, a clear correlation between pigmentation and UVC resistance
268 could not be established even though Cor5, *Rhodopirellula baltica* and LF2 are highly resistant
269 strains that display pigmented colonies (Fig. 5). In order to evaluate a potential protective effect of
270 pigments from planctomycetes against UVC, *Saccharomyces cerevisiae* was used as experimental
271 model due to the simplicity of laboratorial manipulation and well-established techniques for
272 evaluation of UV resistance (Hanway et al. 2002). We selected Cor5, one of the most UVC
273 resistant pigmented strains, extracted its pigments and assayed its influence on viability of yeast
274 cells exposed to UVC. The potential protective effect of the ethanolic extracts of planctomycetes
275 pigments against UVC was assessed with pre- and co-incubation of yeast cells with the extracts.
276 None of the extracts seemed to have protective effect against UVC in both incubation conditions
277 (data not shown).
278
279 **4. Discussion**
280 With the DNA diffusion assay here developed for planctomycetes we were able to detect cells with
281 damaged DNA in a given population. However, higher sensitivity would be required for the
282 quantitative assessment of the extent of DNA damage in each cell. A possible reason for the low
283 sensitivity may be the limited diffusion of DNA of small genomes such as the ones of bacteria.
284 Due to the ubiquity of planctomycetes, these organisms could be potential proxies for the good
285 state of different ecosystems. Resistance to stress factors is a key aspect for biomonitoring studies.
286 Our assay provides an easy and rapid method for DNA damage detection that can be of great
287 importance when using these organisms as biological monitors.
288 Our results of resistance to UVC (Fig. 5) are in accordance with the ones obtained with *Gemmata*
289 *obscuriglobus* (D10 of 675.8 J m^{-2}) (Lieber et al. 2009), indicating that planctomycetes are a group
290 of bacteria with high resistance to UVC. Interestingly, among the strains tested, FC 9.2, sharing
291 about 96 % sequence similarity in the 16S rDNA to *R. baltica*, was the most resistant. The strains

292 in the *R. baltica* cluster (Fig. 1) also presented high levels of resistance against UVC damage while
293 strains phylogenetically more distant from *R. baltica*, FF15 and Gr7, were the most susceptible
294 ones. As depicted in Figure 1, Cor5 is a strain of the species *Rhodopirellula baltica*. Both
295 presented comparable resistances against UVC (Figs. 3, and 5) even though they were isolated,
296 respectively, from the macroalgae surface and from the water column in places geographically
297 distant. These results seem to indicate a uniform behavior towards environmental stresses, such as
298 UVC and oxidative stress, within this species. In a transcriptional study of *R. baltica* SHI^T to
299 changing environmental conditions (salinity and temperature) this planctomycetes revealed to
300 possess a high responsivity and a high number of genes were affected by the changes induced
301 (Wecker at al. 2009).
302 Although all strains presented similar levels of DNA damage, in terms of viability the strains
303 displayed a diverse range of sensitivity (Figs. 3 and 5). Therefore, our results suggest that several
304 types of cellular damage could mediate the effect of UVC on planctomycetes viability. Survival
305 after UV exposure could depend more on avoiding proteins carbonylation than on DNA
306 degradation, since microorganisms lose viability when exposed to radiation causing low DNA
307 damage (Daly et al. 2007; Krisko and Radman 2010). However, other molecules, such as lipids,
308 could also be targetted by UV or by the cellular byproducts caused by this radiation. In addition,
309 the highly packed chromatin organization of planctomycetes such as *Gemmata obscuriglobus* has
310 been suggested to enhance radiation tolerance (Lieber et al. 2009) by limiting diffusion of DNA
311 fragments when double stand breaks are generated, which would facilitate the repair (Coxx and
312 Battista 2005).
313 Besides DNA protection, DNA repair mechanisms can also contribute to high UVC resistance. In
314 the DNA diffusion assay, the procedure after exposure to UVC prevents the activity of DNA repair
315 mechanisms as cells are immediately immersed in lysis solution, in order to denature enzymes. In
316 addition, this procedure is performed in the dark, which does not allow photoreactivation of
317 photolyase, an enzyme responsible for repair of UV-induced DNA damage upon exposure to
318 visible light (Joux et al. 1999; Sancar 1994; Suter et al. 1997). However, in viability assays,
319 although the cultures were kept in the dark, enzymes of the nucleotide excision repair might be

repairing DNA in the viable cells. Our data suggest that some of the tested strains, such as *R. baltica* and strains LF2 and FC9.2, may present characteristics that confer them best survival capacities when exposed to UVC, even when presenting a high proportion of the population with DNA damage. Such characteristics might include high proportion of Mn (II)/Fe (II). Since Mn (II) has less reactivity with O_2 or H_2O_2 than Fe (II), production of reactive oxygen species would be considerably less than in cells with lower Mn (II)/Fe (II) (Daly et al. 2007). Another mechanism of protection against UV was suggested by Krisko and Radman (2010) in bacteria, based on the protection by low molecular weight cytosolic compounds that would avoid protein carbonylation. On the other hand these strains could possess efficient mechanisms of protein and lipid turnover, so that degraded proteins and lipids could be replaced rapidly, or efficient protection mechanisms of these molecules.

Some studies claim that pigments play a role in cell resistance to UV by capturing damaging radiation and avoiding the harmful effects on cellular structures (Cockell and Knowland 1999). Our results suggest that planctomycetes pigments do not protect cells from UVC-induced DNA damage, although more detailed extraction and analysis of pigments is required to confirm this.

4. Conclusion

This work intended to unveil aspects related to cell integrity of planctomycetes regarding DNA damage and survival upon UVC exposure. Since UV is a key stressor in marine environments, it is important to evaluate the impact of this radiation in marine organisms and ecosystems. A new DNA damage detection assay, a DNA diffusion assay, was developed and optimized for planctomycetes, bacteria with large genome sizes. This assay allows the detection of DNA damage induced not only by UV but also by hydrogen peroxide. Some of the planctomycetes studied seem to be highly resistant to UVC possessing equal or higher resistance levels than the UVC resistant *Arthrobacter* sp.The more resistant strains were members of the *R. baltica* cluster. In contrast, the colorless FF15 was the most sensitive strain. Resistance to UVC measured as viability, did not closely correlate with DNA damage, suggesting that mechanisms such as DNA repair might be important for planctomycetes defenses against radiation.

348

Acknowledgments

This research was supported by the European Regional Development Fund (ERDF) through the COMPETE - Operational Competitiveness Programme and national funds through FCT – Foundation for Science and Technology, under the projects Pest-C/BIA/UI4050/2011 and PEst-C/MAR/LA0015/2013. We are grateful to Cátia Moreira for helping with the extraction of the pigments.

357

358 **5. References**

359 Arrage AA, Phelps TJ, Benoit RE, White DC (1993) Survival of subsurface microorganisms
360 exposed to UV and hydrogen peroxide. Appl Environ Microbiol 59:3545-3550.

361 Azevedo F, Marques F, Fokt H, Oliveira R, Johansson B. (2011) Measuring oxidative DNA
362 damage and DNA repair using the yeast comet assay. Yeast 28:55-61.

363 Batista LF, Kaina B, Meneghini R, Menck CF (2009) How DNA lesions are turned into
364 powerful killing structures: insights from UV-induced apoptosis. Mutat Res 681:197-
365 208.

366 Béjà O, Aravind L, Koonin EV, et al. (2000) Bacterial rhodopsin: evidence for a new type of
367 phototrophy in the sea. Science 289:1902-1906.

368 Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998) Designer
369 deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains
370 and plasmids for PCR-mediated gene disruption and other applications. Yeast 14:115-
371 132.

372 Cockell CS, Knowland J (1999) Ultraviolet radiation screening compounds. Biol Rev Camb
373 Philos Soc 74:311-345.

374 Cockell CS, Rettberg P, Rabbow E, Olsson-Francis K (2011) Exposure of phototrophs to 548
375 days in low Earth orbit: microbial selection pressures in outer space and on early earth.
376 ISME J 5:1671-1682.

377 Coxx MM, Battista JR (2005) *Deinococcus radiodurans* - the consummate survivor. Nat Rev
378 Microbiol 3:882-892.

379 Daly MJ, Gaidamakova EK, Matrosova VY, et al. (2007) Protein oxidation implicated as the
380 primary determinant of bacterial radioresistance. PLoS Biol 5:e92.

381 de la Torre JR, Christianson L, Béjà O, Suzuki MT, Karl D, Heidelberg JF (2003)
382 Proteorhodopsin genes are distributed among divergent bacterial taxa. Proc Natl Acad
383 Sci USA 100:12830–12835.

384 DeLong EF, Franks DG, Alldredge L (1993) Phylogenetic diversity of aggregate-attached vs.
385 free-living marine bacterial assemblages. Limnol Oceanogr 38:924-934.
386 Derakshani M, Lukow T, Liesack W (2001) Novel bacterial lineages at the (sub)division level
387 as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil
388 and rice roots of flooded rice microcosms. Appl Environ Microbiol; 67: 623-631.
389 Dhawan A, Bajpayee M, Parmar D (2009) Comet assay: a reliable tool for the assessment of
390 DNA damage in different models. Cell Biol Toxicol 25:5-32.
391 Fernández JL, Cartelle M, Muriel L, Santiso R, Tamayo M, Goyanes V, Gosálvez J, Bou G
392 (2008) DNA fragmentation in microorganisms assessed *in situ*. Appl Environ Microbiol
393 74:5925-5933.
394 Friedrich AB, Fischer I, Proksch P, Hacker J, Hentschel U (2001) Temporal variation of the
395 microbial community associated with the Mediterranean sponge *Aplysina aerophoba*.
396 FEMS Microbiol Ecol 38:105-113.
397 Friedrich AB, Merkert H, Fendert T, Hacker J, Proksch P, Hentschel U (1999) Microbial
398 diversity in the marine sponge *Aplysina cavernicola* (formerly *Verongia cavernicola*)
399 analyzed by fluorescence in situ hybridization (FISH). Mar Biol 134:461-470.
400 Fuerst JA (1995) The planctomycetes: emerging models for microbial ecology, evolution and
401 cell biology. Microbiology 141:1493-1506.
402 Fuerst JA (2005) Intracellular compartmentation in planctomycetes. Annu Rev Microbiol 59:
403 99-328.
404 Fuerst JA, Gwilliam HG, Lindsay M, Lichanska A, Belcher C, Vickers JE, Hugenholtz P
405 (1997) Isolation and molecular identification of planctomycete bacteria from postlarvae
406 of the giant tiger prawn, *Penaeus monodon*. Appl Environ Microbiol 63:254-262.
407 Fuerst JA, Sambhi SK, Paynter JL, Hawkins JA, Atherton JG (1991) Isolation of a bacterium
408 resembling *Pirellula* species from primary tissue culture of the giant tiger prawn
409 (*Penaeus monodon*). Appl Environ Microbiol 57:3127-3134.
410 Gade D, Gobom J, Rabus R: (2005) Proteomic analysis of carbohydrate catabolism and
411 regulation in the marine bacterium *Rhodopirellula baltica*. Proteomics 5:3672-3683.

412 Garcia-Pichel F. (1998) Solar ultraviolet and the evolutionary history of cyanobacteria. Orig
413 Life Evol Biosph 28:321-347.
414 Glöckner FO, Kube M, Bauer M, Bauer M, Teeling H, Lombardot T, Ludwig W, Gade D,
415 Beck A, Borzym K, Heitmann K, Rabus R, Schlesner H, Amann R, Reinhardt R (2003)
416 Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. Proc
417 Natl Acad Sci USA 100:8298-8303.
418 Hanway D, Chin JK, Xia G, Oshiro G, Winzeler EA, Romesberg FE (2002) Previously
419 uncharacterized genes in the UV- and MMS-induced DNA damage response in yeast.
420 Proc Natl Acad Sci USA 99:10605-10610.
421 Hermansson M, Jones GW, Kjelleberg S (1987) Frequency of antibiotic and heavy metal
422 resistance, pigmentation, and plasmids in bacteria of the marine air-water interface.
423 Appl Environ Microbiol 53:2338-2342.
424 Hieu CX, Voigt B, Albrecht D, Becher D, Lombardot T, Glöckner FO, Amann R, Hecker M,
425 Schweder T: (2008) Detailed proteome analysis of growing cells of the planctomycete
426 *Rhodopirellula baltica* SH1. Proteomics 8:1608-1623.
427 Joux F, Jeffrey WH, Lebaron P, Mitchell DL (1999) Marine bacterial isolates display diverse
428 responses to UV-B radiation. Appl Environ Microbiol 65:3820-3827.
429 Kolber ZS, Van Dover CL, Niederman RA, Falkowski PG (2000) Bacterial photosynthesis in
430 surface waters of the open ocean. Nature 407:177-179.
431 Krisko A, Radman M. (2010) Protein damage and death by radiation in *Escherichia coli* and
432 *Deinococcus radiodurans*. PNAS 107:14373-14377
433 Kuhlman KR, Allenbach LB, Ball CL, Fusco WG, La Duc MT, Kuhlman GM, Anderson RC,
434 Stuecker T, Erickson IK, Benardini J, Crawford RL (2005) Enumeration, isolation, and
435 characterization of ultraviolet (UV-C) resistant bacteria from rock varnish in the
436 Whipple Mountains, California. ICARUS 174:585-595.
437 Kulichevskaia IS, Pankratov TA, Dedysh SN (2006) Detection of representatives of the
438 Planctomycetes in *Sphagnum* peat bogs by molecular and cultivation methods.
439 Microbiology 75:329-335.

Lage OM, Bondoso J (2011) Planctomycetes diversity associated with macroalgae. FEMS Microbiol Ecol 78:366-375.

Lieber A, Leis A, Kushmaro A, Minsky A, Medalia O (2009) Chromatin organization and radio resistance in the bacterium *Gemmata obscuriglobus*. J Bacteriol 191:1439-1445.

Mitchell DL, Karentz D (1993) The induction and repair of DNA photodamage in the environment. In: Young AR, Bjorn LO, Moan J, Nultsch W (eds) Environmental UV Photobiology. Plenum Press New York, pp 345-377.

Osman S, Peeters Z, La Duc MT, Mancinelli R, Ehrenfreund P, Venkateswaran K. (2008). Effect of shadowing on survival of bacteria under conditions simulating the Martian atmosphere and UV radiation. Appl. Environ. Microbiol. 74:959–970.

Östling O, Johanson KJ (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123:291-298.

Rabus R, Gade D, Helbig R, Bauer M, Glöckner FO, Kube M, Schlesner H, Reinhardt R, Amann R. (2002) Analysis of N-acetylglucosamine metabolism in the marine bacterium *Pirellula* sp. strain 1 by a proteomic approach. Proteomics 2:649-655.

Sancar A (1994) Structure and function of DNA photolyase. Biochemistry 33:2-9.

Schlesner H, Rensmann C, Tindall BJ, Gade D, Rabus R, Pfeiffer S, Hirsch P (2004) Taxonomic heterogeneity within the Planctomycetales as derived by DNA-DNA hybridization, description of *Rhodopirellula baltica* gen. nov., sp. nov., transfer of *Pirellula marina* to the genus *Blastopirellula* gen. nov. as *Blastopirellula marina* comb. nov. and emended description of the genus *Pirellula*. Int J Syst Evol Microbiol 54:1567-1580.

Schuch AP, Menck CFM (2010) The genotoxic effects of DNA lesions induced by artificial UV-radiation and sunlight. J Photochem Photobiol B 99:111-116.

Singh NP (2000) A simple method for accurate estimation of apoptotic cells. Exp Cell Res 256:328-337.

Singh NP, Stephens RE, Singh H, Lai H (1999) Visual quantification of DNA double-strand breaks in bacteria. Mutat Res 429:159-168.

Sinha RP, Häder DP. (2002) UV-induced DNA damage and repair: a review. Photochem Photobiol Sci 1: 225-236.

Strous M, Fuerst JA, Kramer EH, Logemann S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MS (1999) Missing lithotroph identified as new planctomycete. Nature 400:446-449.

Suter B, Livingstone-Zatchej M, Thoma F (1997) Chromatin structure modulates DNA repair by photolyase in vivo. EMBO J 16:2150-2160.

Ward N, Staley JT, Fuerst JA, Giovannoni S, Schlesner H, Stackebrandt E (2006) The order Planctomycetales, including the genera *Planctomyces*, *Pirellula*, *Gemmata* and *Isosphaera* and the Candidatus genera *Brocadia*, *Kuenenia* and *Scalindua*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) The Prokaryotes. Springer. New York, pp 757-793.

Webster NS, Wilson KJ, Blackall LL, Hill RT (2001) Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. Appl Environ Microbiol 67:434-444.

Wecker P, Klockow C, Ellrott A, Quast C, Langhammer P, Harder J, Frank Oliver Glöckner. FO (2009) Transcriptional response of the model planctomycete *Rhodopirellula baltica* SH1T to changing environmental conditions. BMC Genomics 10:410.

Wegner CE, Richter-Heitmann T, Klindworth A, Klockow C, Richter M, Achstetter T et al (2013). Expression of sulfatases in *Rhodopirellula baltica* and the diversity of sulfatases in the genus *Rhodopirellula*. Marine Genomics 9:51-61.

Figure captions

Fig. 1. Phylogenetic 16S rDNA tree generated by neighbor-joining analysis indicating the relationship of the isolates (in bold) to the members of the order Planctomycetales. Phylogenetic trees were generated using different calculation methods including neighbor joining, maximum parsimony and maximum likelihood to test for the stability of the tree. The anammox "Candidatus" genus was used as an outgroup. The numbers beside nodes are the percentages for bootstrap analyses, only values above 50% are shown. Bar: 0.02 substitutions per 100 nucleotides.

Fig. 2. DNA-damaged cells assessed by the DNA diffusion assay. a DNA-damaged (left) and native DNA (right) cells of *Rhodopirellula baltica* treated with 100 µM hydrogen peroxide. b DNA damage induced by UVC radiation in strain Cor5. Cells were exposed to increasing doses of UVC as mentioned in each panel. For both experiments DNA damage was analyzed with the DNA diffusion assay, samples were stained with GelRed and observed in fluorescence microscopy with 400x magnification. Bar: 10 µm.

Fig. 3. Evaluation of the DNA damage detection in strains of marine planctomycetes exposed to several doses of UVC radiation with the DNA diffusion assay. In each sample, at least 50 cells were counted and cells presenting a fluorescence halo were counted as DNA-damaged. Percentages were calculated in relation to the total number of cells counted. Experiments were done in triplicate and results are presented as mean values ± standard deviation. One-way analysis of variance (ANOVA) was used for comparison. * $p < 0.05$ and ** $p < 0.01$.

Fig. 4. Evaluation of the DNA damage detection in planctomycetes exposed to 10 mM hydrogen peroxide with the DNA diffusion assay (see 2.2 and 2.3). In each sample, at least 50 cells were counted and cells presenting a fluorescence halo were counted as DNA damage. Percentages were calculated in relation to the total number of cells counted. Experiments were done in triplicate and results are presented as mean values ± standard deviation. One-way analysis of variance (ANOVA) was used for comparison. ** $p < 0.01$.

Fig. 5. Sensitivity of marine planctomycetes to increasing UVC radiation dose using a viability test. *Arthrobacter* sp. was used as a qualitative control as it is considered a UVC resistant bacterium. These results are from a representative experiment from three independent replicates.

Figure 1
Click here to download high resolution image

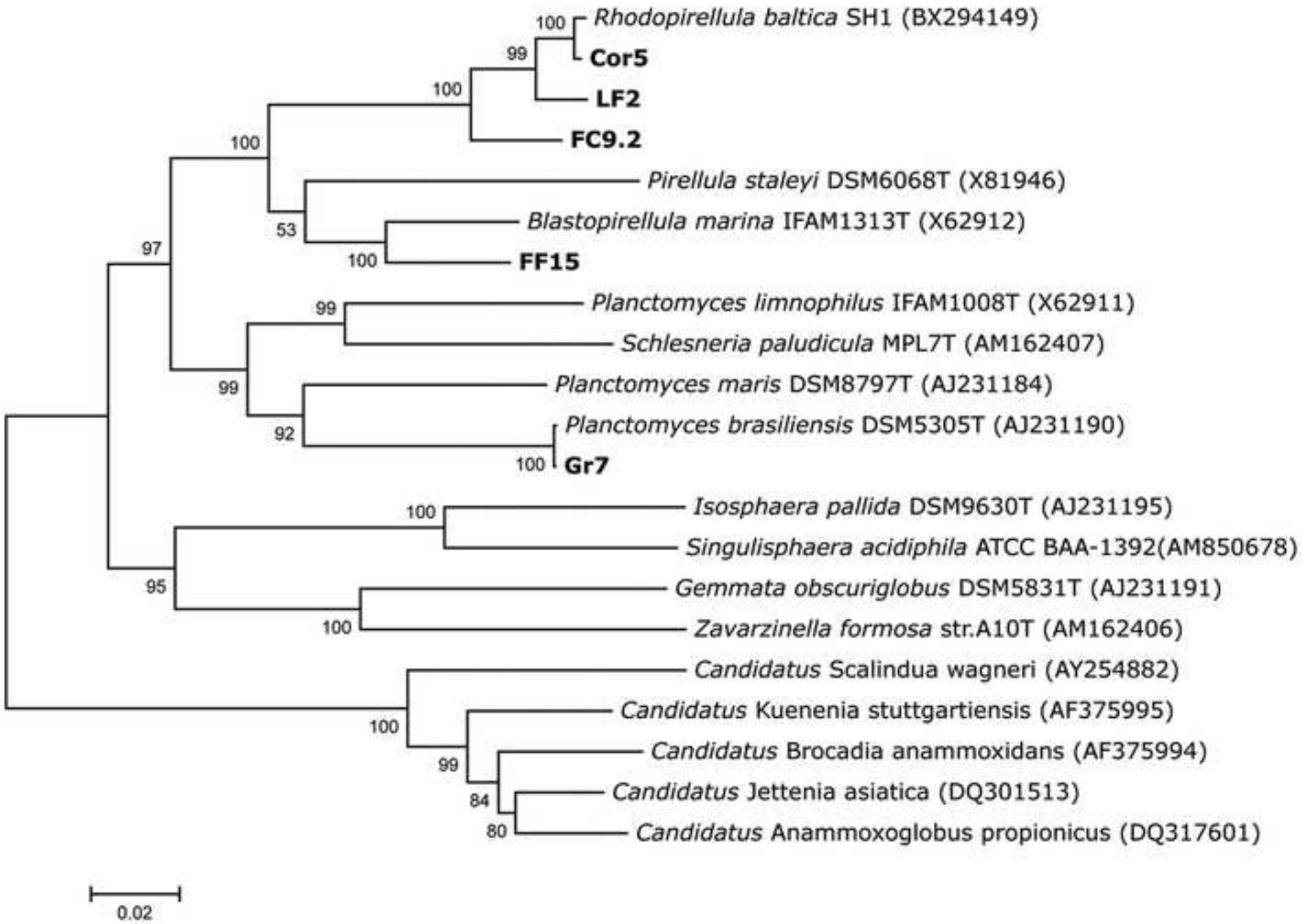

Figure 2
Click here to download high resolution image

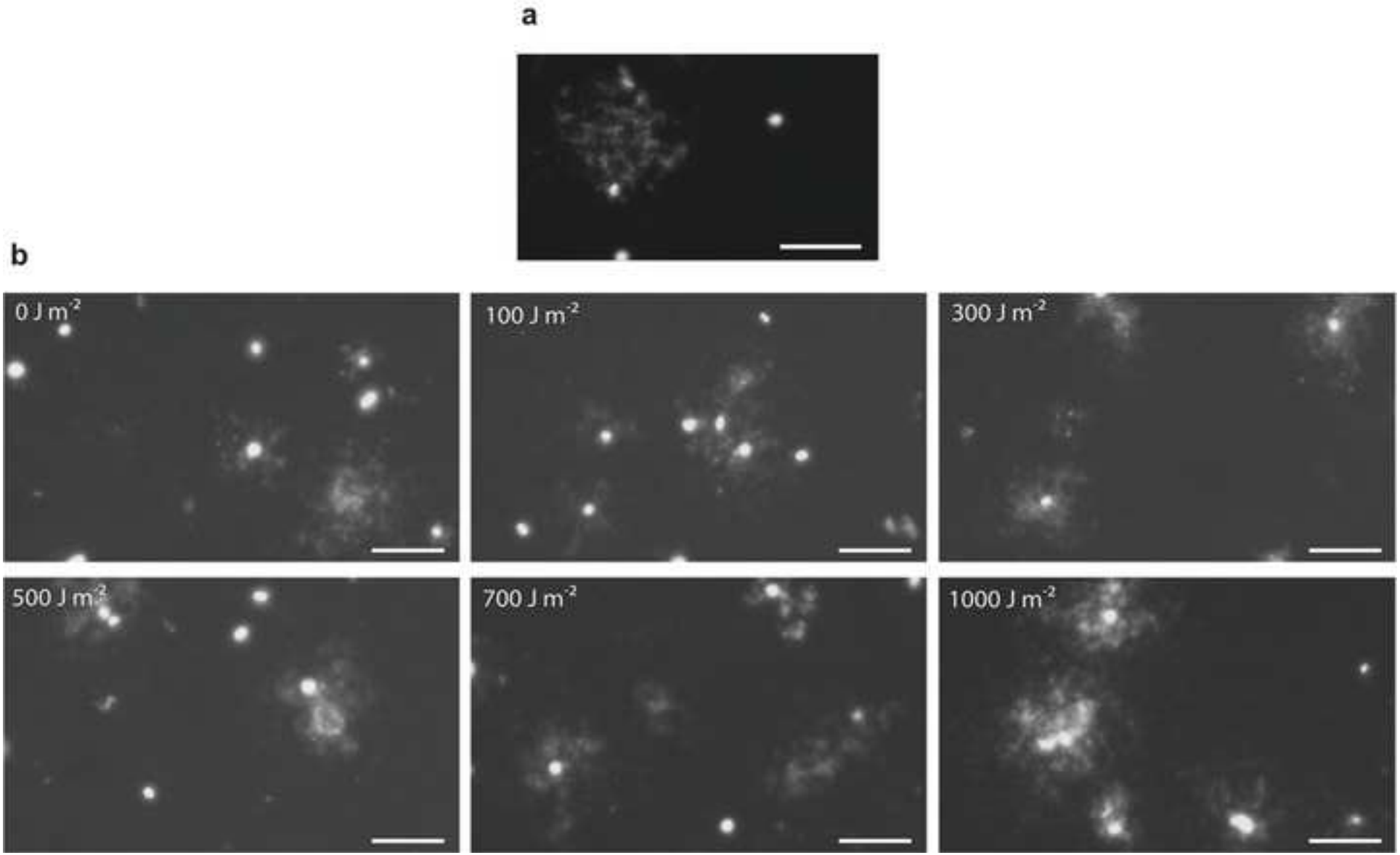

Figure 3
Click here to download high resolution image

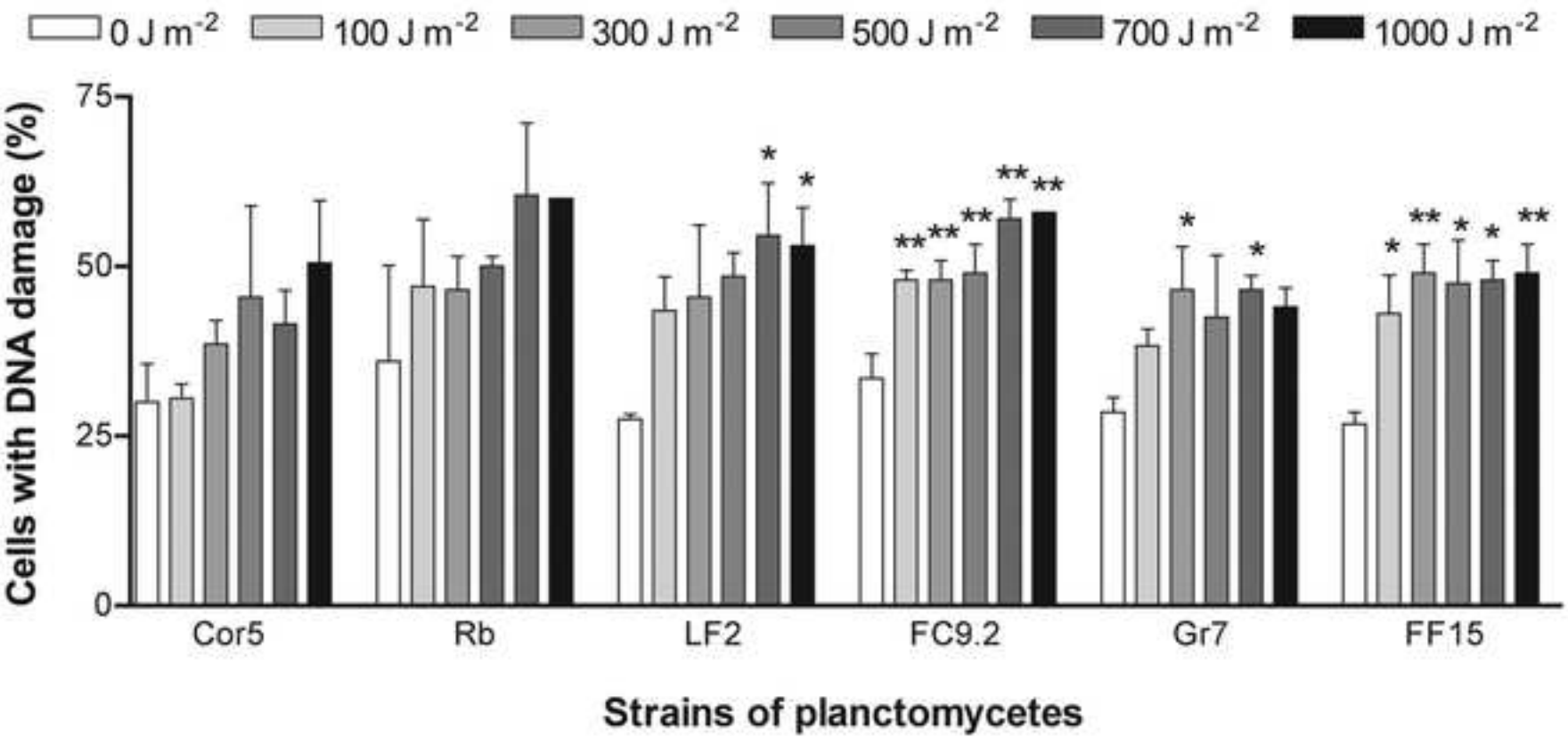

Figure 4
Click here to download high resolution image

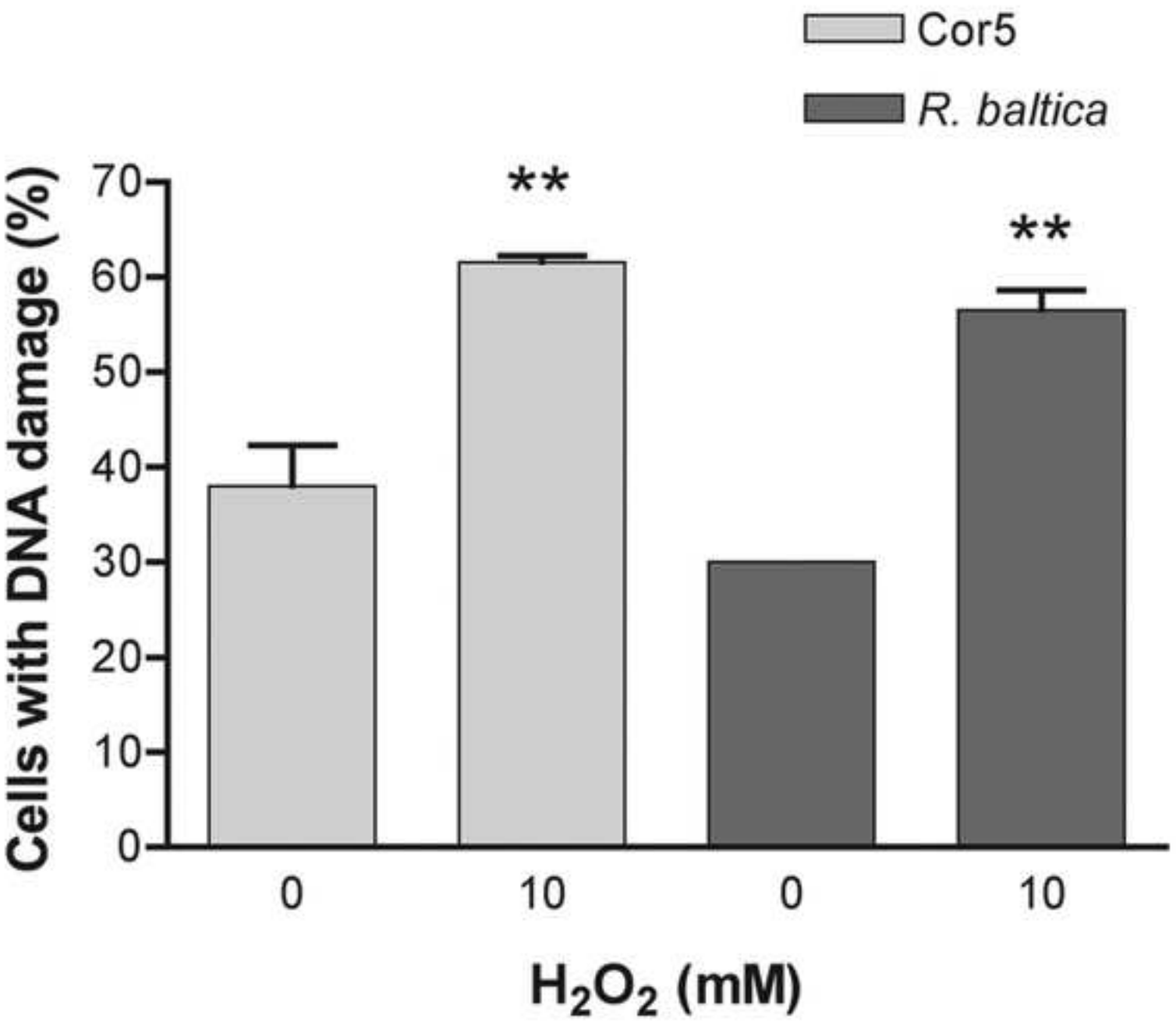

Figure 5
Click here to download high resolution image