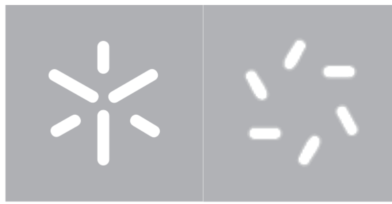

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Characterization of cell integrity of Planctomycetes species: signaling and DNA protection

Tese de Mestrado
Genética Molecular

Trabalho efectuado sob a orientação do
Doutor Rui Oliveira
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Resumo

Os Planctomycetes são um grupo notável de bactérias que devido às suas características invulgares: divisão por gemulação, paredes celulares sem peptidoglicano e estrutura celular compartimentalizada apresentam relevância crescente em vários domínios científicos como a evolução, biologia celular, ecologia e genómica. Estudos de ecologia molecular microbiana evidenciaram que os Plancomycetes são organismos abundantes em habitats terrestres e aquáticos, desempenhando um papel preponderante nos equilíbrios dos mesmos. Embora seja conhecida a sua grande dispersão, os processos fisiológicos e metabólicos subjacentes a esta ubiquidade permanecem por esclarecer. As vias de sinalização celular permitem a adaptação a ambientes em constante mudança e são determinantes para sobrevivência dos organismos. Para os Planctomycetes, um aspecto importante a ter em conta no que se refere à ecologia prende-se com a resistência a radiação Ultravioleta (UV), principalmente para as espécies marinhas.

O presente trabalho pretende caracterizar aspectos relacionados com a integridade celular de espécies marinhas de Planctomycetes após a exposição a radiação UV. Foi desenvolvido um novo método para detecção de danos de DNA em Planctomycetes, utilizado na caracterização da resistência à radiação UV de várias estirpes. Este trabalho foi complementado com testes de sobrevivência. O potencial efeito protector de extractos etanólicos de pigmentos de Planctomycetes foi também avaliado. Como as vias de sinalização mediadas por *Mitogen-activated protein kinase* (MAPK) regulam a resposta a diversos tipos de stress, espécies mutantes para componentes da via da Protein kinase C foram rastreados para resistência a radiação UVC para avaliar um possível envolvimento desta via na resposta à radiação UV. Potenciais homólogos de Planctomycetes foram também analisados. Este estudo multidisciplinar permitiu a caracterização de várias espécies marinhas de Planctomycetes quanto a danos de DNA e sobrevivência. Foi ainda demonstrado um possível envolvimento da via de sinalização mediada pela PKC na resposta de *Saccharomyces cerevisiae* a radiação UV. Os valores elevados de homologia entre os genes da levedura e de Planctomycetes podem indicar que este grupo depende de vias de sinalização complexas na manutenção da integridade celular.

Abstract

The phylum Planctomycetes is a remarkable group of bacteria with increasing relevance in numerous fields of research such as evolution, cell biology, ecology and genomics due to its unusual characteristics: division by budding, peptidoglycan-less cell walls and compartmentalized cell structure. Molecular microbial ecology studies repeatedly provided evidence that Planctomycetes are abundant in terrestrial and marine habitats, underlying that these organisms play an important role in the ecology of the habitats. Although this wide known dispersion, still little is known about the physiology and metabolism underlying the geographic ubiquity of Planctomycetes. Signalling pathways are essential for adaptation to a constantly changing environment that imposes exposure to several kinds of stress and are essential for organism's survival. One important aspect of Planctomycetes ecology is ultra-violet radiation (UV) resistance, at least in marine species.

In this work, we aimed to characterize aspects of cell integrity of marine Planctomycetes species upon exposure to UV radiation related to DNA damage and signalling. A new DNA damage detection method was developed for Planctomycetes and used to evaluate DNA damage resistance of several strains together with survival tests. In addition, the potential protective role of ethanolic extracts of Planctomycetes pigments was also evaluated. As signalling pathways mediated by Mitogen-activated protein kinase (MAPK) regulate response to several types of stress, several mutant strains for the Protein kinase C cascade components were screened for UV resistance to evaluate the possible involvement on this pathway in UV response. Furthermore, several predicted homologous kinases of Planctomycetes were analysed. This multi disciplinary approach allowed us to characterize several marine Planctomycetes strains comparatively to DNA damage and survival. Furthermore, we were able to demonstrate that PKC mediated pathway is probably involved in UV response in Saccharomyces cerevisiae. The high homology values between Planctomycetes and Saccharomyces cerevisiae may indicate that Planctomycetes rely on evolved signalling pathways to maintain cell integrity.

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Abbreviations List

CPD - Cyclobutane-pyrimidine dimers

DNA - Deoxyribonucleic acid

ELK - Eukaryotic like kinase

ERK - Extracellular signal-regulated kinase

GTP – Guanosine triphosphate

HZO - hydrazine oxidoreductase

ICM - intracytoplasmatic membrane

LB – Luria Bertani

LMA – Low melting agarose

MAPK - Mitogen-activated protein kinase

MAPKK - Mitogen-activated protein kinase kinase

MAPKKK - Mitogen-activated protein kinase kinase kinase

MEK - mitogen-activated ERK-activating kinase

MEKK - MEK kinase

NER – Nucleotide excision repair

NMA – Normal melting agarose

OD – Optical density

PBS - Phosphate saline buffer

PCR – Polymerase chain reaction

PKC – Protein kinase C

RNA - Ribonucleic acid

SOC - Super optimal composite

TAE - Tris acetate EDTA

TLC – Thin layer chromatography

UV – Ultra-violet

UVA – Ultra-violet **A**

UVB – Ultra-violet B

UVC – Ultra-violet C

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1. Introduction

1.1 Bacteria that make the difference: the Planctomycetes

1.1.1 Historical context, taxonomy and phylogeny

The Planctomycetes, phylum *Planctomycetes*, order *Planctomycetales*, family *Planctomycetaceae* (Schlesner and Stackebrandt 1986; Garrity *et al.*, 2004), constitute an unusual and intriguing distinctive group of bacteria that forms a divergent phylum of the Domain Bacteria. In 1924, Gimesi observed *Planctomyces bekefii* for the first time in a pond (Lake Lágymányos) at Budapest, Hungary and described it as a planktonic fungus due to its morphological characteristics (hence the genus name *Planctomyces*) (Gimesi, 1924) (Fig. 1).

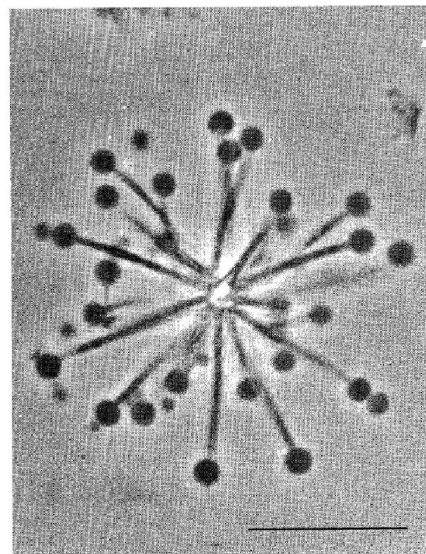


Fig. 1: Phase contrast micrograph of a rosette of *Pl. bekefii.* Adapted from Fuerst, 1995. Bar 10µm.

Posteriorly, Henrici and Johnson (1935) described the same organism but named it as *Blastocaulis sphaerica*, later classified as a synomym of *Pl. bekefii* on the basis of literature (Hirsch, 1972). Schmidt and Starr (Schmidt and Starr, 1980) confirmed those results after a comparative structural study of both samples. Since these early studies, numerous authors have reported the occurrence of organisms with similar characteristics from all over the world. Due to their unique position within the domain Bacteria, in 1986 a new order

Planctomycetales and family *Planctomycetaceae* were proposed based on 16S rDNA sequences. Since then several new genera and species have been isolated and described. To date the phylum *Planctomycetes* comprises 9 genera with only 11 validly described species: *Pl. maris*, *Gemmata obscuriglobus*, *Pl. limnophilus*, *Pl. brasiliensis*, *Blastopirellula marina* (previously designated *Pirellula marina*), *Pirellula staley, Isosphaera pallida*, *Rhodopirellula baltica* and the more recently *Singulisphaera acidiphila*, *Schlesneria paludicula* and *Zavarzinella formosa* (Bauld and Staley, 1976; Franzmann and Skerman, 1984; Hirsch and Müller, 1985; Schlesner, 1986; Giovannoni *et al.*, 1987c; Schlesner, 1989; Schlesner *et al.*, 2004; Kulichevskaya *et al.*, 2007; Kulichevskaya *et al.*, 2008; Kulichevskaya *et al.*, 2009). There are also 5 genera at *Candidatus* status (terminology used when referring to well characterized but yet uncultured organisms): Scalindua, Kuenenia, Brocadia, Anammoxoglobus and Jettenia (Strous *et al.*, 1999; Schmid *et al.*, 2000; Kuypers *et al.*, 2003; Kartal *et al.*, 2007; Quan *et al.*, 2008) that exist only in bioreactor mixed cultures and that are responsible for the anammox process (<u>an</u>aerobic <u>amm</u>onium <u>ox</u>idation). More recently a new family was proposed based on the description of the novel species *Phycisphaera mikurensis* (Fukunaga *et al.*, 2009). Several other Planctomycetes have been isolated in pure culture but never named taxonomically (Schmidt, 1978; Stackebrandt *et al.*, 1986a; Fuerst *et al.*, 1991; Sittig and Schlesner, 1993; Schlesner, 1994; Wang *et al.*, 2002; Pimentel-Elardo *et al.*, 2003; Gade *et al.*, 2004). Nevertheless, environmental 16S rRNA clones revealed that many more strains remain to be isolated and that this group is probably much more diverse than the diversity observed in isolated cultured strains suggests. Due to all this, the phylogeny of Planctomycetes is still a challenging and controversial question.

1.1.2 General Characteristics

The phylum Planctomycetes comprises bacteria with unique features, some of them unexpected for prokaryotic organisms. Bacteria are known to possess peptidoglycan as the main component of their cell wall with the only exceptions to this postulation being members of *Chlamydia*, *Mycoplasma* and *Planctomycetes* (Vollmer *et al.*, 2008). The cell wall of Planctomycetes is a proteinaceous structure and the amino-acid composition varies among species (Konig *et al.*, 1984; Liesack *et al.*, 1986). The cell wall can be rich in glutamate, proline and cystine displaying a significant degree of disulphide bonds that stabilize its structure. The lack of peptidoglycan is consistent with the reported considerable resistance of these bacteria to antibiotics acting on peptidoglycan synthesis (Konig *et al.*, 1984; Liesack *et al.*, 1986; Stackebrandt *et al.*, 1986b; Giovannoni *et al.*, 1987a) and also with the osmotic instability showed by some strains (Schmidt, 1978). Curiously, despite the absence of peptidoglycan in the cell wall, genes involved in the synthesis of this polymer are present in the genome of *Rhodopirellula baltica* (*Pirellula* sp. strain1) (Glockner *et al.*, 2003).

When it comes to morphology, Planctomycetes are a quite diverse and heterogeneous group. Cells can present a polar organization and can be ovoid, spherical or pear-shaped. Moreover, cells can be arranged in rosettes (the most common conformation) (Fig. 2a, b and c), form filaments or appear as unicellular (Ward *et al.*, 2006). Crateriform structures are another unusual characteristic of Planctomycetes. In electron microscopy, these structures appear at the cell surface as electron-dense circular regions when the cells are submitted to negative staining. They can appear either uniformly distributed through the cell surface or confined to the area of the reproductive pole (Fig. 2d). The function of these structures remains unknown, but they are probably associated with the presence of fimbria (Liesack *et al.,* 1986). In addition, although not common among all members, some strains display some non-prosthecate appendages known as polar holdfast or stalk structures (Fig. 2b and c). The presence of these structures facilitates the attachment to surfaces and the formation of rosettes (Fuerst, 1995). The holdfast is of glycoproteic nature (Olga Lage personal communication). Planctomycetes can be sessile or present motility by means of a flagellum. The colonies of *Isosphaera pallida* can present gliding motility, which is a phototactic behavior previously unreported for a budding bacterium (Giovanonni *et al.*, 1987a)

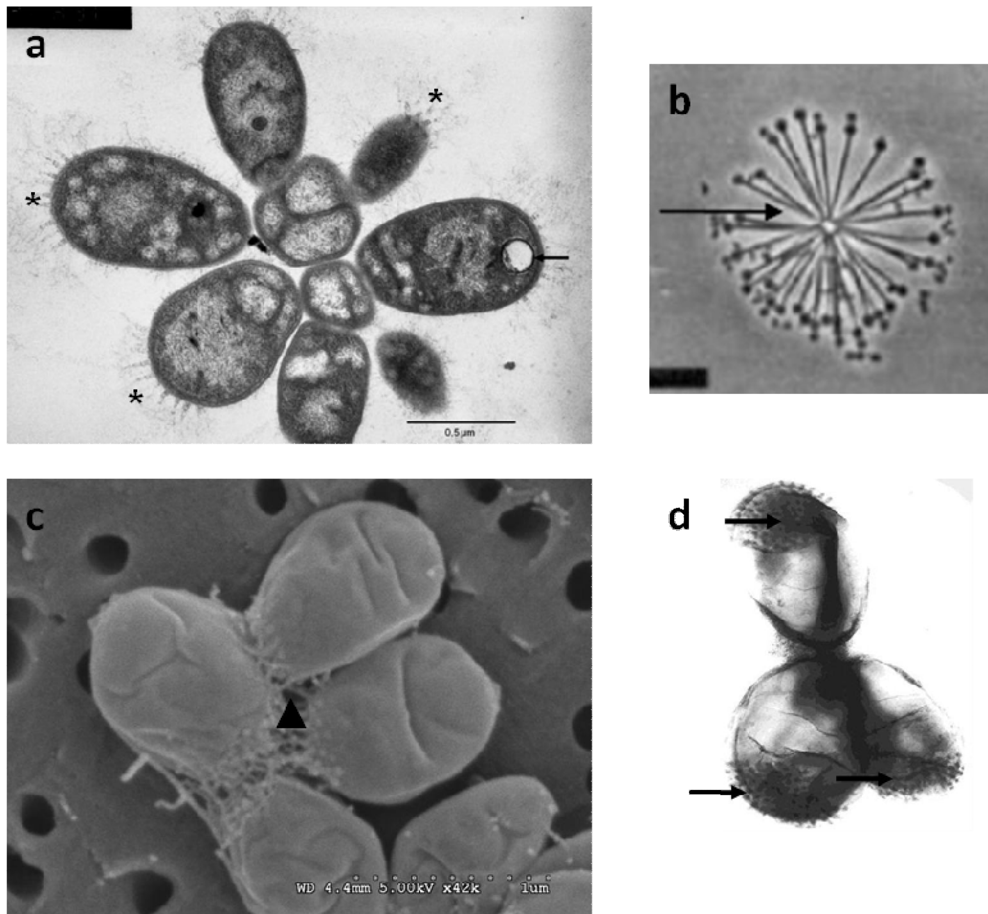


Fig. 2: Several morphological aspects of Planctomycetes. a) TEM micrograph *Pirellula* spp. rosette-like arrangements. Fimbria can be observed (*); b) *Pl. bekefii* rosette where the spherical cells are joined together by stalks. (arrow) (adapted from Ward *et al.*, 2006). c) SEM micrograph of *Pirellula* spp.evidencing the holdfast (▲); d) TEM negative staining of *Pirellula* spp. evidencing the crateriform pits (arrows). a, c and d: courtesy of Olga Lage.

Members of this group reproduce by a yeast-like budding process. Despite this remarkable similarity little is known about the reproduction process in Planctomycetes. With the exception of *Gemmata*, *Singulisphaera* and *Isosphaera*, Planctomycetes display a dimorphic life cycle resembling that of *Caulobacter crescentus* (phylum Proteobacteria, family Caulobacteraceae) that begins when the sessile mother cell produces the bud in the polar or sub-polar region. The bud increases in size and produces a flagellum. Then separates from the mother cell and becomes sessile. The flagellum loss is usually accompanied by pilus synthesis (Tekniepe *et al.*, 1981; Glöckner *et al.*, 2003; Gade *et al.*, 2005a; Ward *et al.*, 2006; Kulichevskaya *et al.*, 2008). Multiple daughter cells can arise from one region of the mother

cell. Recently, the cell cycle of Gemmata obscuriglobus was described (Lee *et al.*, 2009). In this genus, daughter cells do not produce flagella.

Some of the larger genome sizes known in bacteria, for instance 5.2 Mb (million base pairs) for *Pl. limnophilu*s, 7.15 Mb for *R. baltica* and 9Mb for *G. obscuriglobus*, are other characteristic of Planctomycetes. These values are usually correlated with microorganisms exposed to changing environmental conditions, or with the ability to undergo complex differentiation, most probably due to the dimorphic life style and stalk, holdfast, fimbrae and flagella synthesis of Planctomycetes (Glöckner *et al.*, 2003; Ward *et al.*, 2006).

Despite this unique set of morphological features, the most striking and distinctive trait of Planctomycetes is their exceptional cell plan with several membranes within the cytoplasm (Lindsay *et al.*, 2001; Fuerst, 2005). A basic cell plan occurs in all species but variation is found among genera. Typical basic cell plan includes peripheral paryphoplasm and an inner ribosome-containing riboplasm that includes the nucleoid. As revealed by thin sections obtained via cryotechniques, all Planctomycetes present cells divided in compartments by a major intracytoplasmatic membrane (ICM). Variation is related to the nature of the compartment defined by the ICM and the occurrence or not of other membranes inside the ICM. (Lindsay *et al.*, 1997; Lindsay *et al.*, 2001; Fuerst, 2005; Fuerst, 2006b). In *Pirellula*-like species, all cells possess a major compartment bounded by the ICM that contains the highly condensed DNA and ribosome-like particles. All other genera possess an equivalent compartment denominated riboplasm. In *Gemmata* species this compartment is surrounded by two membranes in close proximity and separated by an electron-transparent space, resembling a eukaryotic nucleus with a surrounding double membrane system (Fuerst and Webb, 1991). Another characteristic compartment in Planctomycetes is the paryphoplasm which surrounds the ICM and is surrounded by the cytoplasmatic membrane. The anammox-performing Planctomycetes present a specific compartment, the anammoxosome, which harbors the enzymes responsible for the annamox process and is where part of the process takes place (Lindsay *et al.*, 2001; Fuerst, 2006a). The major enzyme responsible for this conversion is the hydrazine oxidoreductase (HZO), which is exclusively found within the anammoxosome (Jetten *et al.*, 2005; Op den Camp *et al.*, 2006). Another unusual characteristic of Planctomycetes is the unique linearly concatenated cyclobutane chain lipids denominated "ladderane lipids" present on the membrane of this compartment that probably confer impermeability to the compartment membrane protecting the rest of

the cell from toxic intermediates of the process, such as hydrazine (DeLong, 2002; Damsté, 2002; Boumann *et al.*, 2007).

These kinds of intracellular structures pose an interesting challenge to the traditional dichotomy between prokaryotes and eukaryotes based on cell organization. Eukaryotic cells essentially differ from the prokaryotic by their possession of membrane-bound compartments, as these are highly unusual in prokaryotes. Regardless the true phylogeny of the membrane-bounded genome of Planctomycetes, this feature gives this group a great phylogenetic importance that, in association with other characteristics, places it at the center of evolutionary biology and defies the actual definition of prokaryote and eukaryote (Fuerst, 1995; Lindsay *et al.*, 2001; Fuerst, 2004; Fuerst, 2005).

1.1.3 Ecological relevance

Planctomycetes are ubiquitous microorganisms that have been described to be widely distributed in all kinds of habitats such as marine, freshwater, soil, hypersaline, hypothermal, brackish and even acidic environment. (Staley, 1973; Starr and Schmidt 1989b; Schlesner, 1994; Fuerst, 1995; Neef *et al.*, 1998; Wang *et al.*, 2002). Several evidences point out associations with invertebrates (Fuerst *et al.*, 1991, Fuerst *et al.*, 1997) Mediterranean sponges (Friedrich *et al.*, 1999, Friedrich *et al.*, 2001; Webster *et al.*, 2001), cyanobacteria and microalgae(Ward *et al.*, 2006) and plants (Fuerst, 1995; Derakshani *et al.*, 2001; Kulichevskaia *et al.*, 2006) . Close association with macroalgae has been shown by Lage and Bondoso (2007). Additionally, they have been found in marine snow aggregates (marine sediments rich in organic substances) (DeLong *et al.*, 1993) and marine sediments (Llobet-Brossa *et al.*, 1998). This broad distribution suggests the existence of several physiological mechanisms that allow Planctomycetes to adapt and colonize such diverse ecosystems. Due to this and their exceptional nutritional versatility Planctomycetes have been proposed to be responsible for carbon remineralization in these aggregates, thus playing an important role in the global carbon cycle (Gade *et al.*, 2005a). The numerous sulphatase-encoding genes reported especially in aquatic species (Jenkins *et al.*, 2001; Bauer *et al.*, 2004; Chistoserdova *et al.*, 2004) suggest that they can play an important role in the degradation of sulphated

glycopolymers which are abundant in marine environments in the form of phytodetrital macroaggregates ("marine snow").

All the species known to date are chemoheterotrophs with the exception of the anammox bacteria that are chemoautotrophs. A random sequencing approach to the genomes of *Gemmata obscuriglobus* and *Pirellula marina* showed a high number of genes involved regulation, transport, translation and DNA replication (Jenkins *et al.*, 2001). Several genes involved in C1 metabolism were identified in *Rhodopirellula baltica, Gemmata obscuriglobus, Blastopirellula marina* and *Planctomyces maris* (Glockner *et al.*, 2003; Bauer *et al.*, 2004; Christoserdova *et al.*, 2004; Woebken *et al.*, 2007). All these data suggest an important role of Planctomycetes in global biogeochemical cycles. Furthermore, analysis of *Rhodopirellula baltica* proteome revealed several proteins with signal peptides and kinase domains (Studholme *et al.*, 2004; Gade *et al.*, 2005; Hieu *et al.*, 2008). Modern genomics developments demonstrated that complexity in signal transduction systems seems to reflect the organism's life style. The concept was denominated "Bacterial IQ" and reflects how organisms adapt to diverse habitats and stimuli. Apparently, this quotient (fraction of the genes dedicated to signal transduction) is higher for organisms that colonize diverse and complex habitat (Koonin and Wolf, 2008).

Despite all aspects of Planctomycetes that reinforce their great value for cell biology, evolution and ecology, molecular studies in this group have been relatively few. Nonetheless, the availability of the complete genome sequence of some Planctomycetes species and genome drafts of other members allows new molecular approaches for a better understanding of this (still) relatively unknown phylum.

A comprehensive knowledge of the ecological role of this group in the environment is still incomplete, mostly because of the relatively few species present in pure culture. The physiology and metabolism of Planctomycetes underlying the geographic ubiquity is also not well understood. Even though, several genomes of Planctomycetes, including that of *Rhodopirellula baltica* (Glöckner *et al.*, 2003), have been published or are under current sequencing, there is a need for functional analysis on the transcriptional and proteomic level in order to define which of the predicted genes can be expressed in principle and the physiological conditions inducing their expression and foresee the function of undefined genes.

1.2 Marine environments: from "desert" to "rainforest"

The marine ecosystem is the largest one in the world: looking back from the space we don't see planet "earth" but rather planet "ocean". The oceans cover up to 70% of the earth's surface and their biodiversity is the least well understood. Furthermore, in terms of productivity it has been calculated that marine organisms produce as much as or, even more, as the terrestrial ecosystems (Fontaubert *et al.*, 1996). Marine heterotrophs, of which bacteria are a major component, are the key players in the recycling of this huge productivity and consequently control several crucial biogeochemical cycles. According to Peter Burkill from the Plymouth Marine Laboratory "The role that the ocean plays in structuring the Earth's climate is largely driven by microorganisms". Despite all these facts bacteria (taxonomy, biodiversity or function) are still an unknown world in all marine provinces. Since the introduction of marine molecular microbiology techniques, several progresses have been made in describing the wide diversity of marine microbes and in understanding their fundamental role in the biogeochemical cycles and equilibrium of the whole system inclusively in stress situations. However molecular studies based on PCR amplification approaches have shown that the microorganisms abundant in the sea are not related to any microbe currently in culture (Rappé and Giovannoni, 2003). Without cultures it is difficult to investigate bacterial biodiversity, its functional role and interaction with other players in the ecosystem. One of these groups that shows potential in the global C, N and S cycles and as a scientific model are the Planctomycetes for which all studies in the last decades are starting to unveil their importance. Members of this group are widespread in systems under constant pressures and stress, highly polluted and in extreme environmental conditions. The so-called extreme microorganisms, extremophiles, are considered valuable models for basic research since understanding the molecular and physiological basis underlying their adaptation to extreme conditions could be useful in the development of new biotechnological applications. As biodiversity is a key aspect to take into account in the search for new biotechnological applications, oceans are prominent as they are brimming with biodiversity. When it comes to bacteria representatives, oceans display around 3×10^{28} – that is about 100 million more than the number of stars visible in the universe.

A better understanding of the physiology and metabolism underlying the ubiquity of these microorganisms may provide striking clues on several aspects that have high impact on the global climate. Besides that, a better comprehension of all the adaptation mechanisms to several and constant changing conditions may allow us to explore these metabolic features in biotechnological applications.

1.3 Sensing the changing environment: function of the Mitogen-activated kinase (MAPK) signaling pathways

Adaptation to a constantly changing environment figures among one of the critical attributes contributing to survival of all living organisms. Complex signal transduction pathways are responsible for sensing and react to rapid and extreme changes, ensuring the activation of cellular mechanisms to promptly respond and adapt. MAPK phosphorylation cascades have a prominent function when it comes to respond to environmental stimuli and stresses. Highly conserved among eukaryotes, pathways dependent on the activity of these proteins are not exclusive of these organisms (Heinisch et al., 1999). In fact, prokaryotes also exhibit a family of protein kinases with high sequence similarities to their eukaryotic relatives, denominated ELK (eukaryotic like kinase). The first ELK to be reported was in *Myxococcus xanthus* and posteriorly, several others were identified. ELKs were mentioned to play important roles in several pathways involving development, virulence, metabolism and stress adaptation (Munoz-Dorado *et al.*, 1991; Zhang *et al.*, 1998; Av-Gay and Everett, 2000; Inouye *et al.*, 2008; Wehenkel *et al.*, 2008). Several signal peptides are also associated with regulatory pathways mediated by protein kinases and the analysis of ELKs and signal peptides may provide new insights into signal transduction systems in prokaryotes.

MAPK cascades are a key element in transducing very precisely the detected external stimuli into cellular responses. The core signaling module is composed of three protein kinases: MAPK (also known as extracellular signal-regulated kinase [ERK]) that will activate a MAPK kinase (MAPKK, also known as mitogen-activated, ERK-activating kinase [MEK]) which activates a MAPK kinase kinase (MAPKKK, also known as MEK kinase [MEKK]) (Banuett, 1998). Many extracellular and intracellular signals modulate transcription of specific genes through activation or inhibition of MAPK cascades. The tandem arrangement of the three

kinases leads to the signal amplification and high responsiveness and allows integration of several distinct upstream signals. Initiation of the signal transduction cascade is achieved by several events that can lead to the activation of the MAPKKK, as occupancy of receptors coupled to heterotrimeric G proteins by their cognate agonists and the binding of the appropriate ligands to other classes of receptors that stimulate production of activated monomeric G-proteins, or both. The phosphorylation sequence is then initiated and leads to the activation of the MAPK that phosphorylate a wide range of well characterized substrates such as transcription factors, translational regulators, MAPK-activated protein kinases (MAPKAP kinases), phosphatases, and other classes of proteins. These pathways allow cells to regulate metabolism, cellular morphology, cell cycle progression and gene expression in response to a wide variety of stresses (Chen and Torner, 2007).

When it comes to eukaryotic cells, it is easy to assume that the complex cellular structure implies a tightly coordinated and controlled association of events. Signal transduction pathways based mainly on kinases assure the control of these events in means of time and space. Therefore, taking into account the Planctomycetes cell structure, it becomes quite evident that these microorganisms are supposed to rely on several signal transduction pathways due to their complex organization and its close association with functional cell biology. For instance, the analysis of *Pirellula sp*. strain 1 genome provided evidence for the existence of high number of signal peptides. In addition, more than 15 ELKS were identified (Pérez *et al.*, 2008). Even though Two-Component systems are the main controllers of signal transduction in bacteria, ELKS are probably associated with other more sophisticated mechanisms of signal transduction.

1.4 UV radiation and DNA damage

All living organisms inhabiting a constantly changing environment are exposed to several types of stress that alter the stability of cellular components and affect cells integrity. Although all cell molecules are affected by innumerous hazardous environmental agents, due to its physicochemical constitution, DNA becomes the main target for physical and chemical genotoxic agents able to alter its structure and eventually giving rise to mutations

that can potentially lead to cell death. As DNA carries all the hereditary genetic information in the cells, its structure maintenance is decisive, ensuring the correct functioning of cells and transmission of genetic information. Among all the endogenous and exogenous genotoxic agents that can alter the proper structure and integrity of DNA, ultra-violet (UV) radiation figures as one of the most common environmental mutagen agents. All living organisms are daily exposed to constant UV radiation as it represents 45% of the total sunlight spectrum and defense mechanisms allow them to minimize the outcome harmful effects (Zion *et al.*, 2006; Batista *et al.*, 2009). Decrease in growth and survival, pigment bleaching, protein deprivation and photoinhibition of photosynthesis figure among several biological effects of UV radiation in living organisms. UV radiation can be divided in three segments according to the wavelength: UVA, ranging from 320 to 400 nm, UVB, ranging from 295 to 320 nm and finally UVC, ranging from 100 to 295 nm. UV wavelength is known to play a significant role in the rate and type of damage. When it comes to cellular DNA damage, UVB and UVC are the most effective since UVA radiation is poorly absorbed by nucleobases. Nevertheless, UVA rays can still induce secondary photoreactions on DNA existing photoproducts or cause indirect damage by indirect photosensitizing reactions causing oxidative damage to DNA. Although UVA radiation only causes indirect damage to DNA, UVB and UVC can cause direct and indirect damage due to the high absorption by nucleobases. UV radiation is known to cause two of the most mutagenic and cytotoxic lesions on DNA: cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) and their Dewar valence isomers, with both lesions causing the distortion of the DNA helix (Ravanat *et al.*, 2001; Zion *et al.*, 2006; Goosen *et al.*, 2008; Batista *et al.*, 2009) (Fig. 3).

As a battery of lesions can arise from all these types of radiations, including single and double strand DNA breaks, UV-photoproducts and base analogues, cells have evolved several mechanisms to assess DNA damage that allow efficient repair of the diverse lesions enabling organisms to survive. In addition to all these mechanisms, other physiological, morphological and behavioral aspects are thought to have influence in the resistance to UV radiation such as presence of pigments, DNA condensation, G+C content, phototaxis and cell morphology and physical protection of the genomic material (Jeffrey *et al.*, 2000; Minsky *et al.*, 2002, Levin-Zaidman *et al.*, 2003; Zimmerman and Battista, 2005).

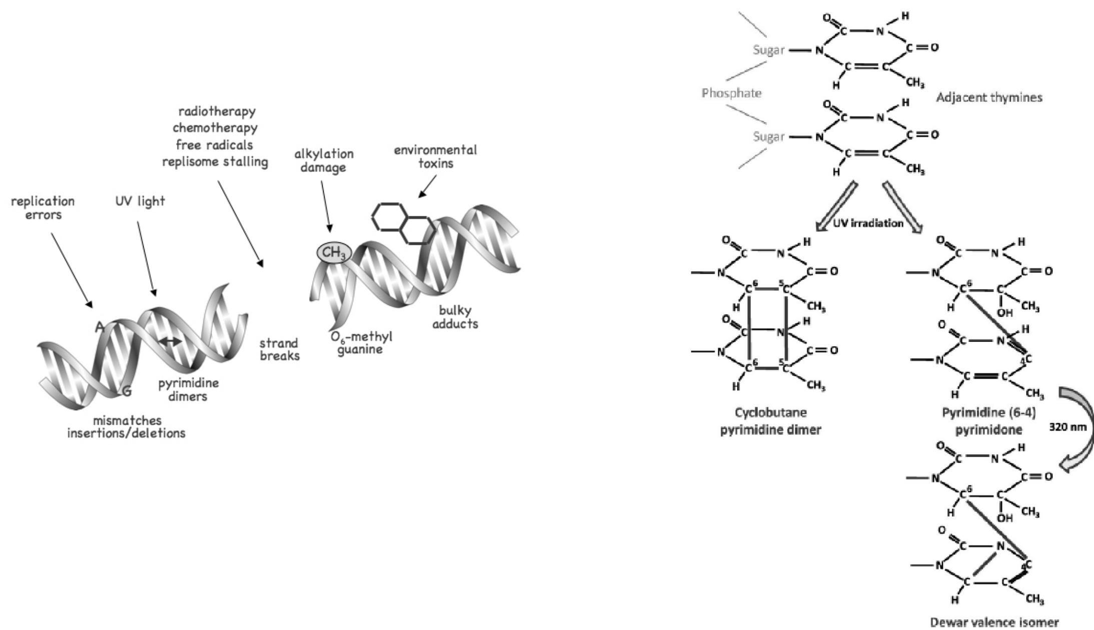


Fig. 3: A) Several types of DNA damage that can occur upon exposure to UV radiation (adapted from http://www.ppu.mrc.ac.uk/research/profiles/7/img/slide1.jpg); B) Chemical structure of the most mutagenic and citotoxic DNA lesions that can occur due to UV radiation exposure: cyclobutane pyrimidine dimmers and 6-4 photoproducts and correspondent Dewar valence isomers. (adapated from Batista *et al.*, 2009).

Several studies claim that organisms are only subjected to UVA and UVB radiation as stated by Asif Khan: "Earth's ozone layer completely blocks solar light of very low ultraviolet wavelengths. Biological organisms on Earth have therefore never developed a tolerance for this 'UVC' radiation, and artificially generated UVC light has become a useful tool in the treatment and destruction of bacteria, yeast, viruses and fungi." (Khan, 2006) Although it is true that ozone layer blocks UVC rays preventing them from reaching the earth surfaces, several organisms have inhabited earth before the existence of an oxygen atmosphere that could afford the protection and had been exposed for long time to short wavelength radiation. Mechanisms of protection and recovery have been developed by living organisms belonging to the 3 biological kingdoms through evolutionary history. With the emergence of the ozone layer, the evolutionary pressure continued as damage caused by UVB radiation resembles the one caused by UVC: both cause similar photoproducts differing only on the relative proportion. Besides that, since the ozone layer is currently experiencing continuous depletion, more deleterious UV rays are able to reach the planet surface. Despite all that, even though UVC radiation is not of high environmental significance, the fact that the

maximum absorption peak of the DNA molecule is 260 nm placed UVC radiation has a powerful tool in laboratory studies. Irradiation with different UV spectra leads to different biological consequences but many of the same lesions in DNA are produced at longer wavelengths of UV radiation, including UVA and UVB. However, due to the high energy of UVC radiation, these damages are more efficiently produced by this type of UV spectra (Bender *et al.*, 1997; Cleaver, 2006).

Due to all these aspects and regarding its evolutionary and environmental importance, UV radiation is one of the most studied DNA damaging agents. Notwithstanding all the knowledge about the mechanisms involved in the response to UV radiation, these remain unknown for Planctomycetes. Moreover, the signaling and mechanisms associated with the response to this type of radiation may include several other molecular and cellular pathways not discovered till now, composing a more complex response that what we might expect.

1.5 Saccharomyces *cerevisiae*: the "top" model organism?

Saccharomyces cerevisiae was the first eukaryotic organism to have its genome fully sequenced (Goffeau *et al.*, 1996). The sequence of 12,068 kilobases presents approximately 6000 potential protein-encoding genes, 140 genes specifying ribosomal RNA, 40 genes for small nuclear RNA molecules, and 275 transfer RNA genes. The introduction of the genome sequence changed the yeast investigation environment and lead to the increasing significance of yeast as model organism. By the application of new molecular techniques, innumerous genes and several cellular networks were identified (Botstein *et al.*, 1997; Game, 2002; Dolinski and Botstein, 2005).

Nowadays, *S. cerevisiae* genome is available online in a database that allows search for information concerning mutant phenotypes and other relevant information related to genes. Moreover, the *Saccharomyces* Genome Deletion Project Consortium has created a complete mutant collection, each lacking the function for one gene or ORF in a haploid or diploid genetic background (Winzeler *et al.*, 1999). A marker of resistance to the antibiotic geneticin allows the easy selection of the mutant strains by the simple use of a selective growth medium (Game, 2002). The availability of these mutants provides a powerful

experimentation tool to analyze and identify several genetic mechanisms controlling many cellular pathways. Despite some exceptions, these mutants allow the establishment of a correlation between the observed phenotype and the deleted gene. However, it is worth noting that for many genes little is known about their biological function (Dolinski and Botstein, 2005).

In the past years, a rapid progression has taken the investigation around *S. cerevisiae* towards understanding of interaction networks. Drubin's statement "The powerful genetics of yeast provides the means for bridging the gap between the test tube and the cell."(Drubin, 1989) illustrates the capacity of combination of genetic, biochemical and physiological studies using yeasts making them a suitable model organism to address several biological questions. Yeast presents several characteristics very appealing for experimental use: easy and cheap maintenance, easy manipulation, physiological and genetic simplicity and short generation times. In addition, due to extensive protein conservation, the results obtained can be easily extrapolated to other organisms. For instance, higher eukaryotes and yeast present striking similarities when it comes to fundamental cellular processes, such as transcription, translation, replication, and DNA repair. All these characteristics place yeast in a prominent place as a valuable tool to study the effect of stress agents on several aspects of cellular biology and to evaluate the potential protective effect of several chemical compounds. The easy manipulation of yeast genes through molecular techniques also allows a further understanding of all these processes at a molecular level.

1.5.1 Yeast stress response: the PKC1-mediated pathway role

Yeast cells are frequently exposed to extreme and rapid changes of environmental conditions depending on response pathways to adapt. As discussed before, MAPK pathways figure as key signaling pathways in coordinating cell responses to the dynamic environment that surrounds them. In budding yeast, mating, filamentation/invasion, high osmolarity, and cell integrity pathways are under the control of four MAPKs (Fus3, Kss1, Hog1, and Slt2/Mpk1 respectively) that are activated in response to mating pheromones, starvation, osmolarity, and cell wall damage (Qi and Elion, 2005). An additional pathway is known to

participate only in sporulation of yeast, being under the control of the MAPK Smk1 and controlling the spore cell wall assembly (Gustin *et al.*, 1998).

Cell wall has a leading role in yeast cell integrity as it is necessary not only for the maintenance of the cell shape but also to protect cells from extreme conditions, like osmotic stress. The macromolecular organization of this structure confers strength and a dynamic frame that allows adaption according to the surrounding conditions (Levin, 2005; Lesage et *al.*, 2006). Cell wall is involved in the transduction of the external information to the cell and thus controls several intracellular processes through a feedback loop (Lesage and Bussey, 2006). Despite that, cell wall construction is tightly controlled by molecular pathways and closely coordinated with the cell cycle progression (England and Gober, 2001). The major signaling pathway coordinating cell wall assembly is Protein kinase C (PKC) cell integrity pathway (Lesage *et al.*, 2006). Notwithstanding, since its components are connected to many cellular functions, this pathway is also involved in the response to a wide variety of stresses as heat-shock, hypoosmotic shock, nutritional stress, and those associated with any other injuries that alter the integrity of the outer layer of cells (Gustin *et al.*, 1998). More recently, this signaling pathway has been described as required to overcome oxidative stress in *S. cerevisiae* cells (Villela *et al.*, 2005). In yeast, this pathway is also involved through Pkc1 and other signaling branches in diverse cellular functions as biosynthesis of several cell wall components, biosynthesis of phospholipids, secretion, mitotic recombination, cell cycle G2/M progression and assembly of the mitotic spindle (Levin, 2005; Lesage and Bussey, 2006). The PKC integrity pathway participates in these mitosis-related events as it is involved in actin cytoskeleton reorganization during cell cycle being this process interrelated with polarized secretion and cell wall remodeling (Levin, 2005).

The Pkc1-mediated signaling pathway depends on a family of cell wall surface sensors coupled to the G-Protein Rho1. When bounded to GTP it associates and activates Pkc1 triggering the MAP kinase cascade that comprises the protein kinase C Pkc1, an MAPKKK (Bck1), a pair of redundant MAPKK (Mkk1/2) and a MAP kinase (Mpk1/Slt2). Activated Slt2 phosphorylates downstream transcription factors, the MADS box family member Rlm1, and the SBF subunits Swi4 and Swi6 (Fig. 4).

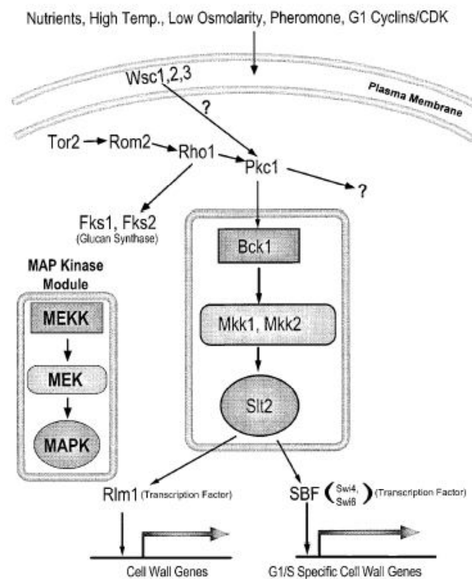


Fig. 4: PKC mediated pathway/ Cell integrity pathway in *Saccharomyces cerevisiae*: the signaling cascade triggering is mediated by Pkc1 activation (adapted from Gustin *et al.*, 1998).

In yeast, Pkc1-mediated signaling is a well known pathway when it comes to its components and cascade activation. Furthermore, the signals to which it responds leading to the activation of the cascade and several connections to other signaling pathways are well characterized, highlighting the importance of this signaling pathway in the complex web of the cell integrity pathway. However, as stress response is essential for survival and cells are daily exposed to several types of harmful agents, the interconnection between this signaling branches may be more complex than what we expect in order to enable a much more quick and effective response apart the type of stress. As MAPK signaling cascades are essential in this type of multivariate response to stress it is expected that they might be involved in other still unknown response mechanisms. For instance, in mammalian cells, UV radiation leads to transcription of a large set of genes that trigger a specific cellular reaction to UV designated by UV response. In these cells, this reaction is coordinated by the MAPK c-Jun N-terminal kinase. As it happens for other signaling pathways activated by MAP kinases, this pathway also regulates cell proliferation, cell death, inflammation and cell fate determination (Ip and Davis, 1998). When it comes to yeast, UV response also exists but a possible role for MAP kinases in this mechanism remains questionable. In 2004, Bryan and co-workers (Bryan *et al.*, 2004) reported that the MAPK Slt2 is involved in yeast response to stress as cells lacking Slt2 have reduced ability to respond to UV radiation. As mentioned before, Slt2 is the MAPK activated by the Pkc1-mediated signaling pathway. Nevertheless, the probable involvement of the signaling pathway and its components remains to evaluate.

2. Project aims

The aim of this project is to assess several aspects related to cell integrity of marine Planctomycetes species with focus in signaling and DNA protection. Planctomycetes are a quite ubiquitous group but metabolical and physiological aspects underlying this wide dispersion remain unclear. As key players on diverse habitats, it is expected that this group has developed several survival strategies that allow them do adapt to several dynamic environments and to diverse stress agents. As UV radiation plays an important role on the ecology and survival of marine organisms, we aimed to evaluate the impact of this mutagen on several marine Planctomycetes strains by developing a new DNA damage assay in order to evaluate the genotoxic effects of UVC radiation on the selected species. The assay will also be validated to use with other genotoxic agents. In addition, survival will also be evaluated as it depends not only on physico-chemical characteristics (DNA condensation, G+C content, cellular structures, and pigments) but also on repair pathways. Besides that, the development of a new DNA damage detection assay for Planctomycetes will facilitate further genotoxic studies on this group.

As some of the strains selected present pigmentation and pigments are thought to play a role on protection against UV radiation, ethanolic pigment extracts of Planctomycetes will be assayed for their possible protective effect on DNA damage mediated by UVC radiation. For this purpose two distinct methods will be used: the comet assay applied to the model organism *S. cerevisiae* and a cell-free assay based on the transformation ability of plasmid pET25. Moreover viability assays will also be performed with *S. cerevisiae* cells pre and co-incubated with the ethanolic extracts and posteriorly exposed to UVC radiation. These methods will allow us to analyze the possible protection against UV radiation provided by the ethanolic extracts in different aspects: DNA damage and viability.

As little is known about Planctomycetes UV resistance at cellular and molecular level, we will also try to elucidate some aspects related to signaling response to this type of stress. For eukaryotes, MAP kinase mediated pathways are essential in the response to different types of stress. As mentioned before, for *S. cerevisiae*, Pkc1 mediated pathway plays an important role on the maintenance of cell integrity regulating several aspects of yeast cell biology through interfaces with other signaling pathways. Besides that, there are reports suggesting the possible participation of this pathway on yeast cellular response to UV radiation. The availability of several Planctomycetes genomes including that of *Rhodopirellula baltica* allows us to do functional analysis at transcriptional and proteomic level: a bioinformatic

approach will be used to analyze potential homologues of Planctomycetes for yeast PKC pathway components. PCR amplification will be performed allowing the posterior use of these sequences for heterologous expression in corresponding mutant strains of *S. cerevisiae* (phenotype complementation). As PKC mediated pathway is essential in the maintenance of cell integrity for yeast, we intend to unveil more about the signaling pathways involved in this type of response in Planctomycetes. The majority of the described phenotypes for mutants in the PKC signaling pathway are related to sensibility to cell wall antagonists. However, as this signaling pathway was reported to have a possible connection to UV response, screening of the mutant's resistance to UVC radiation, before and after the transformation, will also be performed.

This multidisciplinary and integrated approach will allow us to unveil about the Planctomycetes response to UV radiation at cellular, physiological and molecular level as several biological aspects as DNA damage, signaling pathways, pigment influence will be evaluated:. As UV radiation figures as a key genotoxic agent on marine habitats this study will permit to evaluate the impact of this harmful stressor on this microbial group. Furthermore, a new method for DNA damage detection in Planctomycetes will be validated to facilitate further toxicological studies in this group.

3. Materials and methods

3.1 Planctomycetes strains, media and growth conditions

The Planctomycetes strains used during this work were strains Cor3, Gr7, Fc9.2 and FF15. All these strains are part of the OJF collection (Lage & Bondoso, 2007) and were previously isolated from the surface of marine macroalgae (respectively from *Corallina* sp. - Cor, *Gracillaria bursa-pastoris* - Gr and *Fucus spiralis* – F) collected from different locations in Portugal. Strain *Rhodopirellula baltica / Pirellula* sp. strain 1 was also used. Cultures were maintained on solid M607 medium (agar 1.5% w/v, Table 1) at 26ºC and then kept at 4ºC for storage for longer periods. Liquid cultures were incubated in an orbital shaker at 26ºC, 200rpm, with a ratio flask volume/medium of 10/1. Growth was monitored by optical density measured at 600nm (OD_{600}). Throughout the different experiments axenicity of the cultures was assessed microscopically. The same growth conditions were used for *Arthrobacter* sp.

Table 1: M607 medium composition. Modified after Schlesner, 1986.

	M607
Peptone	0,025%
Yeast Extract	0,025%
Glucose[1,4]	0,025%
Tris-HCl	50mL/L
Hutner's basal salts[2,4]	20mL/L
Vitamin solution[3,4]	10mL/L
Natural sea water[5]	0,9%
Deionized water	20mL/L

[1]Solution at 2,5%

[2] Modified from Cohen-Bazire *et al.*, 1957. The modified Hutner's Basal Salts Solution is composed by $MgSO_4.7H_2O$, 29.7 g; nitrilotriacetic acid (NTA), 10.0 g; $CaCl_2.2H_2O$, 3.3 g; $FeSO_4.7H_2O$, 99 mg; $NaMoO_4.2H_2O$, 12.7 mg; "metals 44", 50 mL, per liter of solution. The "metals 44" solution contains, per liter, $ZnSO_4 \cdot 7H_2O$, 10.95 g; $FeSO_4 \cdot 7H_2O$, 5.0 g; Na_2-EDTA, 2.5 g; $MnSO_4 \cdot 7H_2O$, 1.54 g; $CuSO_4 \cdot 5H_2O$, 392 mg; $CoCl_2$ 6 $6H_2O$, 203 mg; H_3BO_3, 114.7 mg.

[3]Modified after Staley, 1968. The modified vitamin solution no. 6 contains, per liter, nicotinamide, 10.0 mg; calcium pantothenate, 5.0 mg; riboflavin, 5.0 mg; thiamide-hydrochloric acid, 5.0 mg; biotin, 2.0 mg; folic acid, 2.0 mg; B12, 0.1 mg.

[4]Added aseptically to autoclaved media

[5]Previously filtered at 0,22µL

3.2 Yeast strain, media and growth conditions

All yeast strains used on this work were in the haploid *Saccharomyces cerevisiae* strain BY4741 background (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* (Brachmann *et al.*, 1998). Strain genotypes are documented in Table 2.

Table 2: Yeast strains used in this work and corresponding genotypes. The accession number is the reference of each strain on the Euroscarf archive.

Accession number	Strain	Genotype
Y01328	*bck1Δ*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YJL095w::kanMX4
Y02487	*mkk1Δ*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YOR231w::kanMX4
Y02112	*mkk2Δ*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YPL140c::kanMX4
Y00993	*slt2Δ*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YHR030c::kanMX4
Y06158	*rad4Δ*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YER162c::kanMX4
Y00278	*rad23Δ*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YEL037c::kanMX4
Y01643	*rev1Δ*	BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YOR346w::kanMX4

Stock cultures of these strains were maintained on solid YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar) at 4°C. Cells were grown in liquid

YPD medium, using an orbital shaker at 30°C, 200 rpm, with a ratio flask volume/medium of 10/1. Growth was monitored by optical density measured at 600nm (OD_{600}).

For the assays using mutant strains, another additional strategy was used. Stock cultures of all strains were maintained on liquid YNBD (Yeast Nitrogen Base without aminoacids, Difco), with 2% agar and 2% glucose, in which the required aminoacids, leucine and uracil, were added to a final concentration of 20μg/mL. Cells were grown overnight on liquid YNBD using an orbital shaker at 30°C, 200rpm with a ratio flask volume/medium of 10/1. Growth was monitored by optical density measured at 600nm. These cultures were kept at 4°C and used as pre-inoculums.

3.3 Optimization of a DNA damage detection assay in Planctomycetes

The assay protocol for DNA damage detection in Planctomycetes species was designed based on the comet assay protocol used for *S. cerevisiae* (Azevedo *et al.*, 2010) and the protocol described by Fernandez *et al.*, 2008. Several optimization steps were followed (Table 7, see Results) and the final protocol was established as stated below. Planctomycetes cells were inoculated in 10mL of M607 liquid medium and allowed to grow overnight at 26°C, 200rpm until OD_{600} of 0.1-0.2.Cells from one milliliter of each culture were harvested by centrifugation at 15000rpm, 4°C for 5 minutes, ressuspended in papain buffer (10mg/mL papain (Sigma), 1x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4), 38mM EDTA, 10mM LD-cysteine) and incubated 1 hour at 37°C, 200rpm to ensure cell wall digestion. Afterwards cells were harvested by centrifugation at 15000rpm, 4°C for 5 min and ressuspended in equal volume of S buffer (1M sorbitol, 25 mM KH2PO4, pH=6.5).

The washing process was then repeated and cells ressuspended in 100μL of S buffer and then distributed in 25μL aliquots. Sixty microliters of 1.5% LMA (low-melting agarose, w/v in S buffer) (37°C) was then added to each sample and 20μL of the mixture were placed on 0.5% NMA (normal-melting agarose, w/v in deionized water) pre-coated glass slides, covered with cover slips and allowed to solidify at 4°C for 5 min. The cover slips were then removed and the slides were subjected to the genotoxic treatment. Immediately after exposure the slides were immersed in fresh lysis solution (30mM NaOH, 1M NaCl, 0.05% (w/v) laurylsarcosine, 50mM EDTA, 10mM Tris-HCl, pH=10) previously warmed at 37°C. The

slides were incubated in the dark at 37°C for 20 min and then washed 3 times on a tray with abundant cold deionized water. To prevent DNA stretching this procedure was executed with the slides in the horizontal position. Fixation was performed by 3 sequential washes in cold ethanol (-20°C) at 76% v/v, 96% v/V and 100% v/v, 3 min each. Posteriorly slides were dried overnight at 80°C and then stored at 4°C until observation. For fluorescence microsopy analysis slides were previously stained with 10µL of GelRed (1:3000 w/v in deionized water).

3.4 Comet Assay optimization for UVC mediated DNA damage detection in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae strain BY4741 cells from a stock culture were inoculated in 5mL of liquid YPD medium and allowed to grow overnight in an orbital shaker at 30°C, 200rpm. An appropriate volume of this culture was diluted in fresh YPD medium in order to obtain an OD_{600} of 0.1 and incubated under the same conditions until OD_{600} 0.4-0.6. Cells were harvested by centrifugation at 5000 rpm, 4°C for 2 min and washed twice with the same volume of deionized H_2O at 4°C. The pellet was then ressuspended in the same volume of S buffer. The needed amount of cells was then obtained by centrifugation at 15300 rpm, 4°C for 2 min and then resuspended in lyticase buffer (2mg/mL lyticase, 500µL S buffer 2x, 300µL deionized H_2O and 50mM β-mercaptoethanol; final volume 1mL) and incubated at 30°C, 200 rpm for 30 min in order to ensure cell wall digestion. Posteriorly cells were harvested by centrifugation at 15300 rpm, 4°C for 2 min and ressuspended in the same volume of S buffer. Several steps were then optimized in order to assure correct evaluation of DNA damage (Table 9, see Results). After these optimization steps the final protocol was established as stated below.

Cells from 80µL aliquots were harvested by centrifugation at 15300 rpm, 4°C for 2 min and ressuspended in 80µL of 1.5% LMA (w/v in S buffer). Twenty microliters of this mixture were then placed in 0.5% NMA (w/v in deionized water) pre-coated glass slides, covered with cover slips and allowed to solidify at 4°C for 5 min. Afterwards the cover slips were removed and the slides were subjected to the genotoxic treatment. Immediately after exposure the slides were immersed in fresh lysis solution and kept in the dark at 4°C for 20 min. The slides were then washed in electrophoresis buffer (30mM NaOH, 10mM EDTA,

10mM Tris-HCl, pH=10) for 20 min and then submitted to electrophoresis in this buffer at 0.7 V/cm (distance measured between electrodes) for 10 min. The mini gels were then neutralized by immersion in neutralization buffer (10mM Tris-HCl, pH=7.4) and fixed in ethanol 76% v/v and 96%v/v, for 10 min each. Drying was carried at room temperature on a laminar flow chamber and the slides were stored at 4°C until visualization. For fluorescence microscopy analysis slides were previously stained with 10µL of GelRed (1:3000 w/v in deionized water).

3.5 Genotoxic Treatments

The slides were subjected to UVC (254nm) irradiation using Stratalinker® UV Crosslinker (Stratagene) at the desired dose (J/m^2). For Planctomycetes strains the doses applied were: 100, 300, 500, 700 and 1000J/m^2. For the assays with yeast doses applied were: 50, 100 and 150 J/m^2.

For the hydrogen peroxide treatment the solution (10mM H_2O_2) was applied directly on top of the cells in each gel and incubated 20 min at 4°C.

3.6 Viability assays

3.6.1 Viability assay of Planctomycetes strains after exposure to UVC radiation

Planctomycetes cells were inoculated in 10mL of M607 liquid medium and allowed to grow overnight at 26°C, 200rpm until an OD_{600} of 0.1-0.2 was achieved. With a sterile toothpick the cultures were inoculated by striking on M607 solid medium, allowed to dry and then plates were irradiated using Stratalinker® UV Crosslinker (Stratagene) with the following UVC dosages: 100, 300, 500, 700 and 1000J/m^2. Plates were then incubated for 10 days at 26°C in the dark.

3.6.2 Viability assay of *Saccharomyces cerevisiae* cells treated with ethanolic extracts of Planctomycetes and submitted to UVC radiation: evaluation of a potential protective effect of ethanolic extracts of Planctomycetes

3.6.2.1 Pigment-ethanolic extracts attainment

Pigment extraction was performed by a colleague (Nogueira, 2010, unpublished) following the subsequent resumed protocol. Pigment extracts were prepared in order to evaluate potential protective effect against DNA damage mediated by UVC radiation on *S. cerevisiae* cells. The selected strain Cor3 was cultivated in one liter of liquid M607 medium for 15 days at 26°C, 200rpm. The culture was then harvested by centrifugation and frozen overnight. Ethanol 99% was added and the samples were sonicated several times. Ethanol was allowed to evaporate until the cellular mass ran out of color. The supernatant was separated from the cellular debris and the remaining ethanol was allowed to evaporate. The remaining sediment was then ressuspended in a dichloromethane/ methanol solution (9:1) and separated by thin layer column chromatography (TLC) in order to analyze the several fractions. The fractions with the bands of interest were then evaporated and the two separate fractions were ressuspended in 500µL of cold 100% ethanol. In order to allow the complete homogenization of the samples all were kept for two hours in the dark at 4°C with agitation (150rpm). All the samples were then held in reserve in the dark at -20°C.

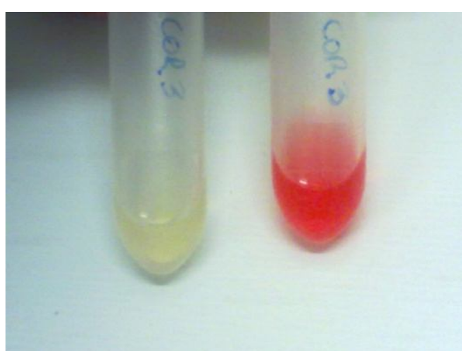


Fig. 5: Pigment Ethanolic extracts of Planctomycetes: COR I (left) and COR II (right).

3.6.2.2 Viability assay

S. cerevisiae cells from a stock culture were inoculated in 5mL of liquid YPD medium and allowed to grow overnight in an orbital shaker at 30°C, 200rpm. An appropriate volume

of this culture was diluted in fresh YPD medium in order to obtain an OD_{600} of 0.1 and incubated under the same conditions until an OD_{600} of 0.4-0.6 was obtained and diluted to OD_{600} 0.1. For UVC sensibility screening cells were subjected to serial dilutions (described below). For pre incubation studies each extract (40x diluted) was added to cell suspension and cells were incubated at 30°C for 20 min. Cells were then harvested by centrifugation at 15300 rpm, 4°C for 2 min and ressuspended in YPD medium maintaining the initial cell concentration. For co-incubation studies extracts were added to the cell suspension in the same concentration as for pre-incubation assays. For positive control, absolute ethanol was added in the same concentration of the extracts as it was the solvent in which extracts were dissolved. Serial dilutions were then performed on YPD medium (0, 10^{-1}, 10^{-2}, 10^{-3}) and sets of 5μL drops were place in solid YPD medium. Plates were allowed to dry and then exposed without lid to the desired doses of UVC radiation (10, 30, 50, 70, 90, 100, 150 J/m^2) using Stratalinker® UV Crosslinker (Stratagene). Posteriorly, plates were incubated at 30°C, in the dark for one day. Images were then obtained using Molecular Imager ChemiDoc XRS System (Bio-Rad) and the Quantity One 1-D image analysis software.

3.7 Transformation efficiency of plasmid pET25 after exposure to UVC radiation: evaluation of a potential protective effect of ethanolic extracts of Planctomycetes

Plasmid pET25 was dissolved in ultra pure water at final concentration of 10ng/μL. Extracts were added to the samples at final concentration of 2% (v/v). A control with ethanol in the same concentration was used as ethanol is the solvent in the extracts. Plasmid pET25 suspensions were then exposed to UVC radiation in open eppendorfs tubes using Stratalinker® UV Crosslinker (Stratagene) with the following dosages: 75, 150 and 300J/m^2. A final amount of 10ng or 1ng irradiated DNA was used to transform 200μL competent cells of *Escherichia coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^q ZΔM15* Tn*10* (Tet^r)] (Transformation Protocol on section 3.8.4). The transformed *E.coli* cells were then plated on solid LB (Luria-Bertani) medium (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl, 2% w/v agar) containing 100μg/mL ampicilin and incubated overnight at 37°C. Counting of colonies was performed using Molecular Imager ChemiDoc XRS System (Bio-Rad) and the Quantity One 1-D image analysis software.

3.8 Evaluation of a possible connection of PKC mediated pathway and the response to UVC radiation in *Saccharomyces cerevisiae*

Yeast cultures were grown as described above (section 3.2). Both for the tests on YPD as for the tests with YNBD, a set of 4 dilutions (0, 10^{-1}, 10^{-2}, 10^{-3}) was prepared and 5µL drops of each strain were spotted on plates with solid YPD or YNBD medium and allowed to dry. Plates were then exposed to the desired doses of UVC radiation (10, 30, 50, 70, 90, 100 and 150 J/m^2) using Stratalinker® UV Crosslinker (Stratagene) as posteriorly incubated in the dark at 30ºC for one day. Images were then obtained using Molecular Imager ChemiDoc XRS System (Bio-Rad) and the Quantity One 1-D image analysis software.

3.9 Bioinformatic query for potential Planctomycetes homologues of yeast PKC pathway

3.9.1 Search of homologies and selection of the genes of interest

The search for homologies was based on the PKC pathway components found in yeast. The aminoacidic sequences were obtained from SGD (*Saccharomyces* genome database - www.yeastgenome.org). Based on the sequences retrieved, the predicted homologous sequences for *Rhodopirellula baltica* were found using the KEGG online database. The similarity between the sequences was analyzed using the BLAST algorithm available on two different online databases: SGD and NCBI (National Center for Biotechnology Information). From the several predicted homologous sequences, the ones with lower *e* values were selected for PCR amplification. Using the MEGA software, the codon usage from the different sequences was calculated to avoid expression problems that could outcome from future expression of bacterial genes in eukaryotic systems.

Table 3: Potential Planctomycetes homologues of yeast PKC pathway. KEGG refers to the online Database KEGG. The *e* value was obtained using BLAST tool from the NCBI online database.

Rhodopirellula baltica **reference name (KEGG)**	**Gene (*Rhodopirellula baltica*)**	***Saccharomyces cerevisiae*** **PKC pathway homologue**	***e*** **value**
RB1821	*pknB*	*bck1*	$7{,}4\ e^{-18}$
RB2162	*pknH*	*bck1*	$7{,}9\ e^{-18}$
RB2855	*pknB*	*mkk1*	$8{,}9\ e^{-14}$
RB7928	*pknB*	*mkk1/2*	$3{,}9\ e^{-17}$
RB10133	*pknH*	*bck1*	$1{,}1\ e^{-26}$

3.9.2 Primers Design

Primers for PCR amplification of the selected sequences were designed based on the primer design tool available online on NCBI, PrimerBLAST. From the several primer pairs retrieved by the program one was chosen for each of the sequences following several parameters such as primer length, GC/AT content, annealing temperature, self dimer formation and primer extremities stability in order to minimize mispriming.

Table 4: Primers obtained using primer design tool PrimerBLAST for the selected homologous sequences.

Sequence to amplify	**Primer name**	**Sequence (5'-3')**
RB1821	RB1821F	ACG GGT GAA CAT GCC GCC AC
	RB1821R	GCA GCG GTT TCA GGT GGG CT
RB2162	RB2162F	GAA GGA CGC TGG GCG GTC AC
	RB2162R	GAT GCG TGG GTC ACG GTG GG
RB2855	RB2855F	CGC ACA TCC GAT CTC GCC CC
	RB2855R	ACG CGG ACA AGG CCA TCG TG
RB7928	RB7928F	TGG CGG CCA CAG TTG AAG ACG
	RB7928R	CCG GGC AGG TGG TTT GGT GG
RB10133	RB10133F	CCG TCG CCG TAA GCA CAG CA
	RB10133R	GGC CAG CGA CTC GCA CTC AA

3.9.3 Polymerase Chain Reaction amplification

PCR amplification was only performed for the RB2162 sequence. The reaction was carried out using different master mix solutions with different concentrations of the typical PCR reaction reagents according the template DNA (*Rhodopirellula baltica* or Cor3). The primer set used was the same for the two reactions as the sequence of Cor3, a *Rhodopirellula* sp. is unknown. The Master Mix solutions and PCR program are described below in Tables 5 and 6.

Table 5: Master Mix composition for RB2162 and COR2162 amplification. Taq buffer supplied by Fermentas.

	Final Concentration
$MgCl_2$	3mM
Taq Buffer	1x
Taq Polymerase	1U
dNTPs	200mM
Primer F	0,2µM
Primer R	0,2µM
DNA	100ng
dH_2O	Until 25µl

Table 6: PCR program used for RB2162 and COR2162 amplification.

Nº of cycles	Step	Duration	Temperature
1x	Initial denaturation	4’	96ºC
30x	Denaturation	60’’	96ºC
	Annealing	60’’	60ºC
	Extension	120’’	53ºC
1x	Final extension	10’	74ºC

After PCR amplification, PCR products were analyzed by agarose (0,7% w/v in 1x Tris-acetate-EDTA (TAE)(10 mM Tris, pH=7.5; 1mM EDTA pH=8.0) buffer) gel electrophoresis and then purified using E.Z.N.A.® Gel Extraction Kit. DNA concentration and purity was evaluated using NanoDrop® ND-1000. PCR products were kept at -20ºC.

3.9.4 Ligation of the PCR amplicons to pGEM and *E.coli* transformation

The ligation reaction was performed following the instructions given by the manual provided with pGEM®-T Easy Vector Systems (Promega) using a PCR product/vector 3:1 concentration ratio. The reaction was incubated overnight at 4ºC and the ligation products were then used to transform *E. coli* XL1-Blue competent cells (see Annex I: protocol for competent cells preparation). Two hundred microliters aliquots of competent cells were allowed to defrost on ice, ligation product was then added in the maximum final ratio of 1:10 (Ligation product/ cell volume) and the mixture was incubated for 30 min on ice. The samples were then submitted to a 90 seconds heat shock at 42ºC followed by incubation on ice for 10 min. Eight hundred microliters of SOC (2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) medium was added to each mixture and cells were incubated 1 h at 37ºC, 200rpm. Cells were then harvested by centrifugation, 1minute at maximum velocity, part of the supernatant was discarded (about 850µL), the pellet ressuspended on the remaining supernatant and then plated on solid LB medium supplemented with 100µg/mL ampicilin in order to allow selection of transformants. Plates were incubated overnight at 37ºC.

3.9.5 MidiPreps and Sequencing

For MidiPrep reactions, one colony of the transformation reaction was inoculated overnight in 50mL LB liquid medium supplemented with 100µg/mL ampicilin, at 37ºC 200rpm. Cultures were harvested by centrifugation and the MidiPrep reactions were performed using the PureYield™ Plasmid Midiprep System (Promega) following the centrifugation protocol advised on the kit. The ligation product containing COR2162 was

then sequenced. Sequencing was performed using the M13 pair of primers. The retrieved sequences were then analysed using the software DNADynamo from BlueTractorSoftware.

4. Results and discussion

4.1 UVC radiation effect on marine Planctomycetes strains

4.1.1 Optimization of a DNA damage detection assay in Planctomycetes

Planctomycetes are key players on several ecosystems where their actions translate into vital events to the equilibrium of those ecosystems. Their ubiquitous distribution depends on physiological and metabolical pathways that allow them to inhabit and adapt to a constantly changing surrounding world. Although in recent years several progresses have been made, a lot remains to clarify when it comes to the physiological properties underlying this wide dispersion. Furthermore, little is known about resistance of these microorganisms to genotoxic agents that may affect and alter their DNA. Analysis of DNA damage caused by several stress agents can be made by several laboratorial techniques. Among the numerous strategies to detect DNA damage in cells, comet assay figures as one of the most popular method due to its relative celerity and easiness. In addition, the capacity of being applied in almost all types of cells along with the single-cell result obtained has increased its application in diverse fields of biological study (Dhawan *et al.*, 2009). Despite this, to our knowledge, application of the comet assay to bacterial cells has only been reported in two scientific papers (Singh *et al.*, 1999; Férnandez *et al.*, 2008).

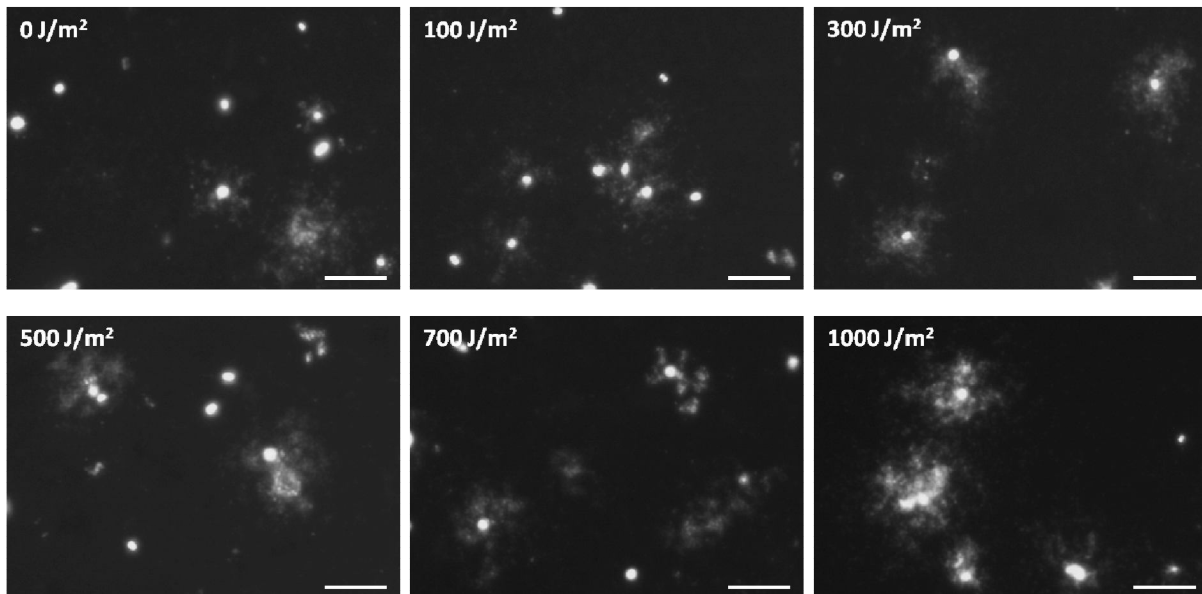
We developed an improved assay for DNA damage detection in Planctomycetes. As UV radiation is a key stressor in marine environments, we intended to develop an assay that would allow us to evaluate its impact on Planctomycetes when it comes to DNA damage caused by this type of agent. We based ourselves on the Comet assay optimized for *S. cerevisiae* in our lab (Azevedo *et al.*, 2010) and on the assay described by Fernandez *et al.*, 2008. Several optimization steps were performed in order to obtain an assay optimized for Planctomycetes (Table 7).

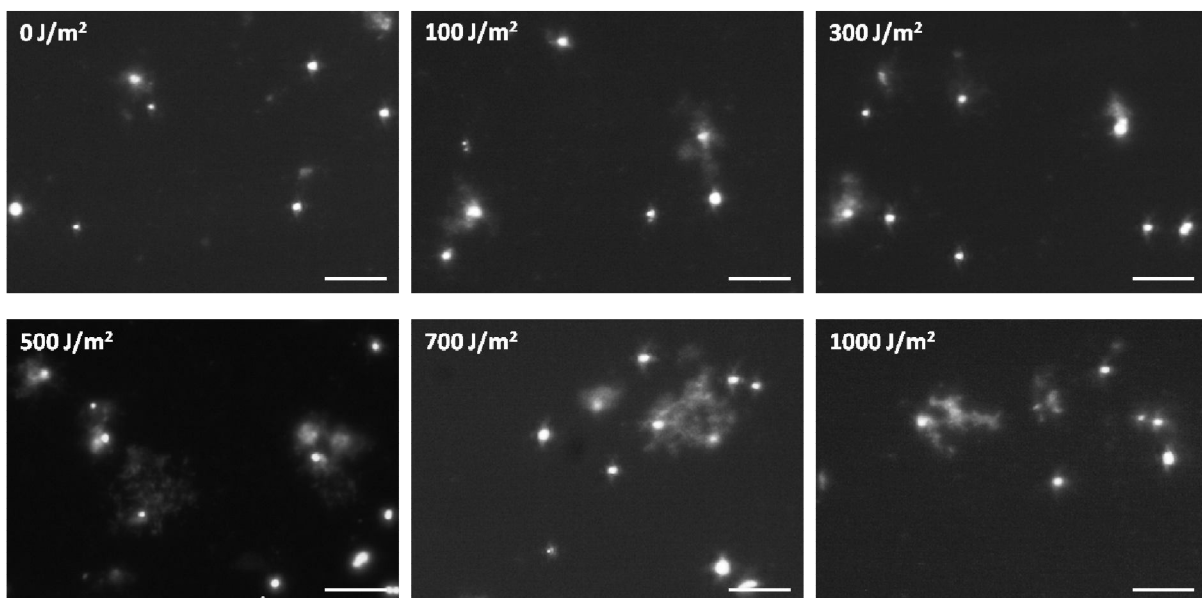
Table 7: Optimization steps performed for the DNA damage detection assay. For each step the tested conditions and the optimum final one are shown.

Step	Tested conditions	Final
Cell wall digestion : enzyme	Papain and Proteinase K	Papain
Cell wall digestion: Papain concentration	5 and 10mg/ml	10mg/ml
Cell wall digestion: duration	1 and 2 hours	1 hour
Lysis	4ºC and 37ºC (pre-heated solution)	37ºC (pre-heated solution)
Electrophoresis: duration and voltage	5 and 10 minutes, 0,7 and 1,4V/cm	10minutes, 1,4V/cm
Slides dehydration	Ethanol 76% and 96%, 10 minutes each ; Ethanol 70%, 90% and 100%, 3 minutes each	Ethanol 70%, 90% and 100%, 3 minutes each
Drying (slides)	Overnight: room temperature and 80ºC	Overnight, 80ºC

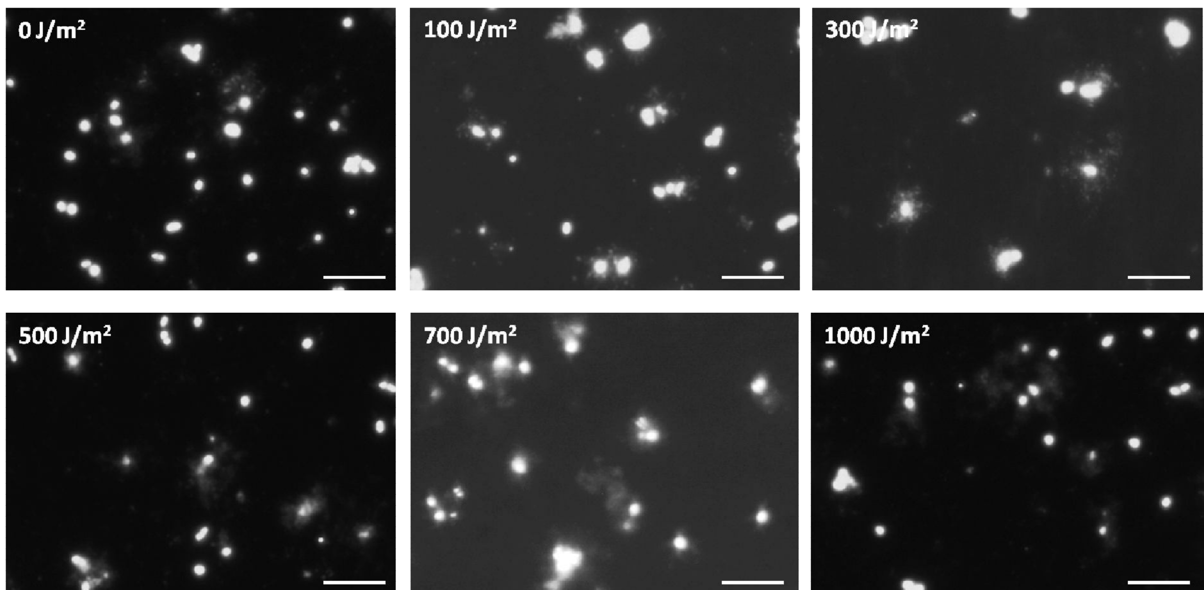
Due to the cell wall composition, the digestion of cell wall step was of crucial importance. As Planctomycetes possess a proteinaceous cell wall, proteinase K and papain were used in order to evaluate the most adequate enzyme to perform cell wall digestion. The two enzymes were tested in different concentrations and with different digestion times. Both temperature and other parameters as pH were taken into account in order to ensure the best performance of each. Our results showed that papain retrieved best results after one h of cell wall digestion, as cells appeared with no cell wall but intact cellular membrane. Digestions with proteinase K led to incomplete digestion of some cell walls and disruption of other cells, fact that would compromise the results of our assay. This evaluation was performed by observation under optical microscopy. For lysis step, two different temperatures were tested, 4°C and 37°C. Lysis step leads to cell wall removal and the eventual removal of other proteins. The higher temperature seemed to indulge lysis and was used in the trials that followed. Electrophoresis was optimized too, with different times and voltages tested in contrast with elimination of this procedure. As the avoidance of this step did not affected DNA damage detection, this step was eliminated. Furthermore, the optimization of the assay led to the elimination of the neutralization step and a dehydration step corresponding to set of three washes in cold (-20°C) 70%, 90% and 100% (v/v) ethanol

for 3 min each. Elimination and alteration of these steps perform decreased the time length of the protocol and increased the simplicity of the overall procedure. Besides that, the introduction of the step of dehydration of the slides overnight at 80ºC greatly improved the quality of our results when it comes to image attainment and posterior analysis (Fig. 6).

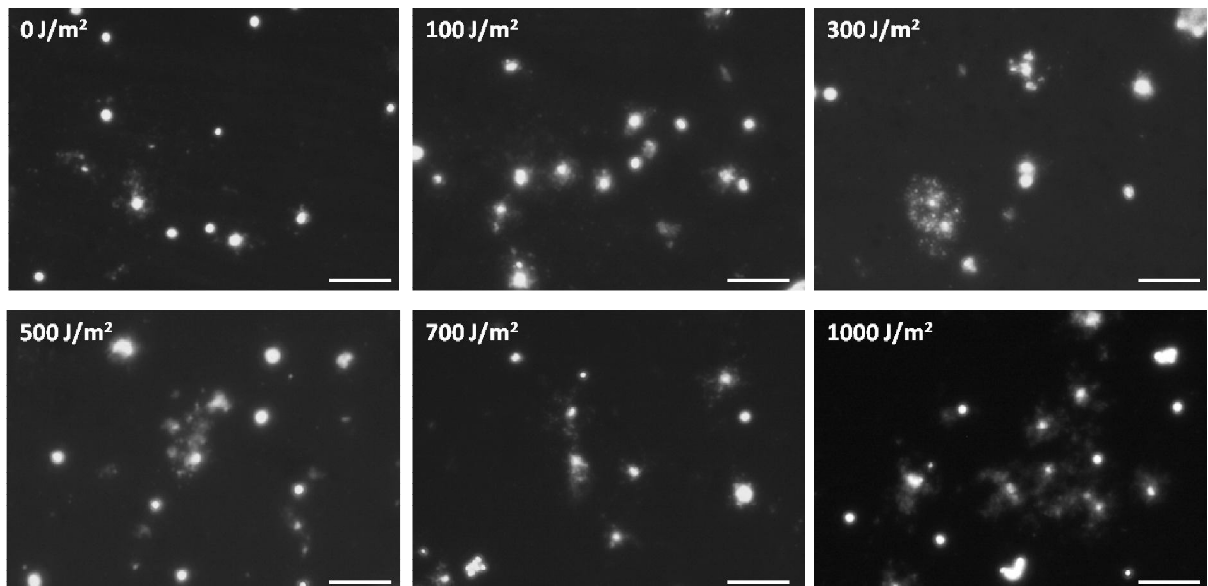




OJF7



Fc9.2



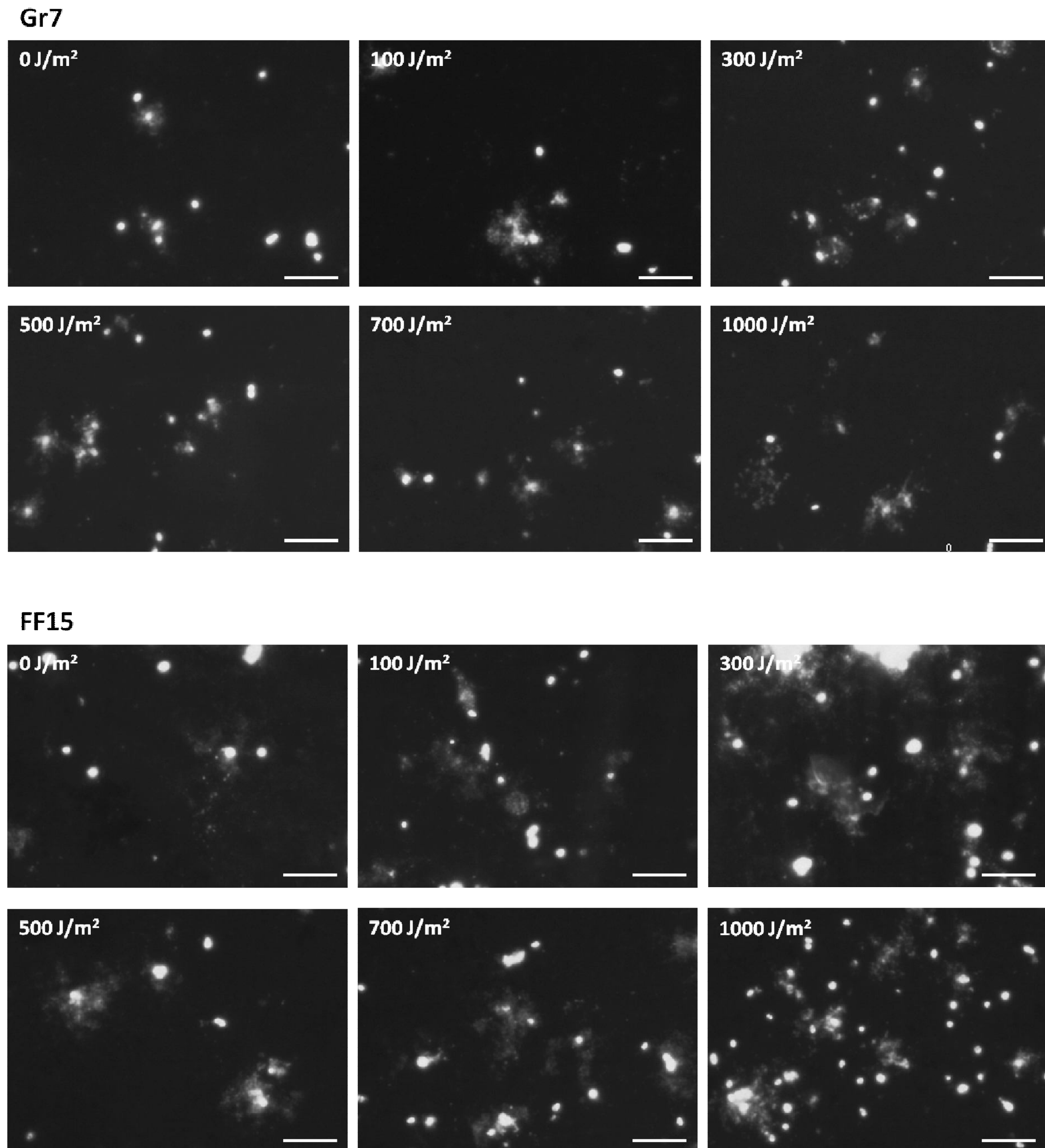


Fig. 6: Microphotographs of the DNA damage assay optimized for Planctomycetes strains Cor3, *R.baltica*, OJF7, Fc9.2, Gr7 and FF15. All strains were exposed to increasing doses of UVC radiation. The diverse morphology of the halos of fragmented DNA can be observed. DNA was stained using 1:3000 GelRed (Biotum). All images were obtained with 400x magnification. Bar 10µm.

In order to validate the assay for the detection of DNA damage caused not only by UVC radiation, the assay was performed using hydrogen peroxide (10mM) as genotoxic agent (Fig. 7).

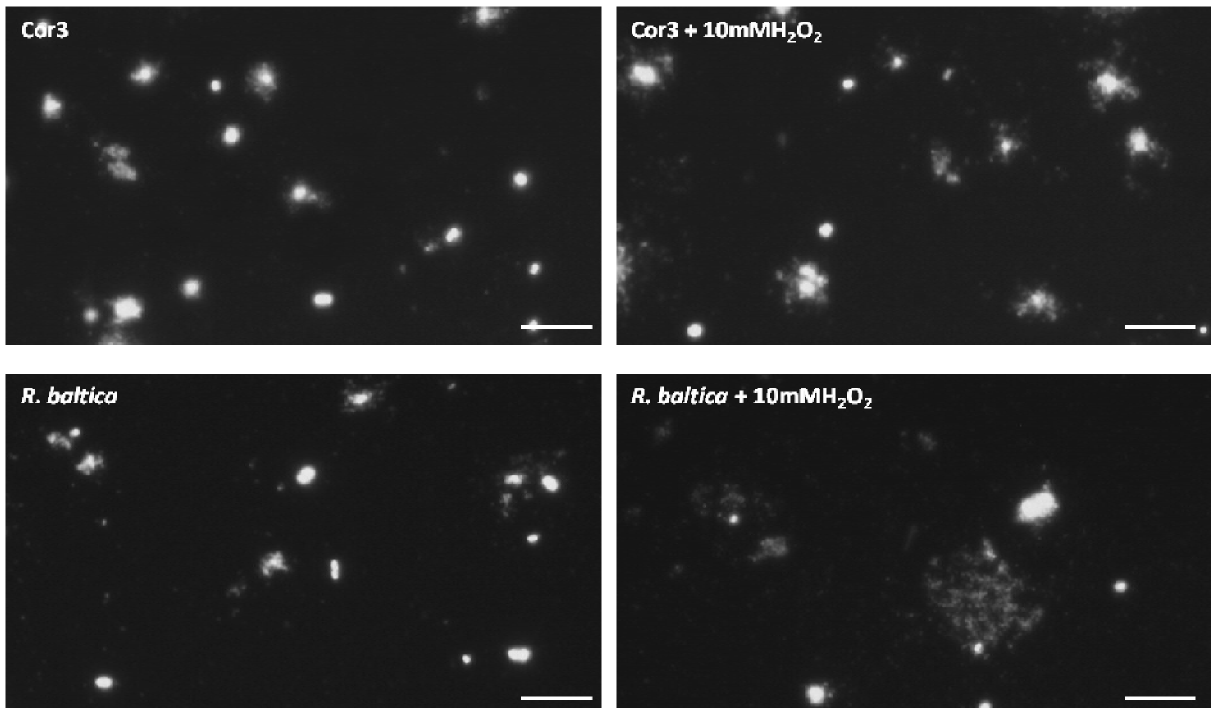


Fig. 7: Microphotographs of the DNA damage assay optimized for Planctomycetes strains. Strains Cor3 and *R. baltica* were exposed to 10mM hydrogen peroxide. The diverse morphology and increase of the halos of fragmented DNA can be observed when compared to control. DNA was stained using 1:3000 GelRed (Biotum). All images were obtained with 400x magnification. Bar 10μm.

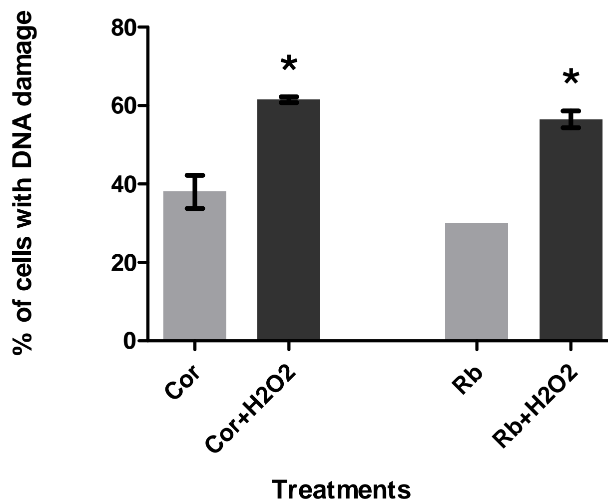


Fig. 8: Evaluation of the DNA damage detection assay of Planctomycetes exposed to 10mM H2O2. Cells were considered to present damage when fragmented DNA was observed dispersed around a central core forming a halo. The experiments were done in triplicate and results are presented as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison. Asterisks indicate differences considered statistically significant: * means p <0.05 when compared to the respective control.

When cells of the Planctomycetes strains were exposed to the genotoxic agents, the proportion of cells considered with DNA damage increased (Figs.6, 7, 8 and 9 (see section 4.2.1). DNA damage was identified through observation of halos of DNA damage around a central core. The morphology of these halos is quite variable among the strains and the diverse conditions tested (Figs. 6 and 7). Our results shown that by using this assay we were able to detect DNA damage caused by both UVC radiation as for hydrogen peroxide. Therefore this assay is suitable to detect DNA damage caused by two different kinds of genotoxic agents, fact which validates our assay as a consistent and trustworthy method for DNA damage detection in these bacteria. UVC radiation and H_2O_2 cause DNA damage through indirect and direct action (respectively) that finally led to strand breaks detectable by our assay. Even though the increase verified on the DNA damage upon exposure to the genotoxic agents, a clear relationship dose/effect was not established and should be optimized in order to obtain the maximum information with the assay. Due to Planctomycetes ubiquity, these organisms are potential "sentinels" for monitoring the health of different ecosystems. Several aspects of biomonitors are of major interest in this kind of studies, being resistance to stress factors one of the key considerations to take into account. As mentioned before, DNA is one of the main targets of several environmental stress agents and the maintenance of its integrity is fundamental for living organisms. Our assay provides an easy and rapid method for DNA damage detection that can be of great importance when using these organisms as biological monitors.

4.1.2 UVC radiation effects on Planctomycetes: DNA and viability are differently affected and vary among strains

Aquatic species inhabiting the water column face different types of stress daily as they are exposed to constant oscillations of temperature, salinity, turbidity and radiation. As bacteria account for 90% of the cellular DNA on aquatic environments and DNA figures as a key target of UV radiation, the study of the impact of UV radiation on marine bacteria is of primary importance (Zennoff *et al.*, 2006). Survival may depend not only on repair mechanisms but also in physic characteristics that allow integrity maintenance of several

cellular components as cellular structures or pigmentation. Radiation has potential deleterious effects on bacteria as they display simple haploid genomes and their small size prevents cellular shading and the potential benefits of pigmentation (Garcia-Pichel, 1994). Besides that, bacterioplancton seems to lack UV screening compounds (Garcia-Pichel, 1994; Karentz, 1994). UV stress has proved to have significant impact on marine bacterial communities and consequently on the equilibrium of their environments (Herndl *et al.*, 1993; Helbling *et al.*, 1995; Joux *et al.*, 1999; Hernd *et al.*, 2000; Arrieta *et al.*, 2000; Vincent and Neale, 2000; Buma *et al.*, 2001; Winter *et al.*, 2001; Biggs and Moody, 2003; Agogue *et al.*, 2005).

To evaluate the potential genotoxic effects of UVC radiation on marine Planctomycetes strains we used the assay described above. DNA damage levels vary according to the dosage and according to the strains. Although a dose increase has led to a consequent increase of DNA damage in all strains, the levels of damage attained were different among the tested strains (Fig. 9).

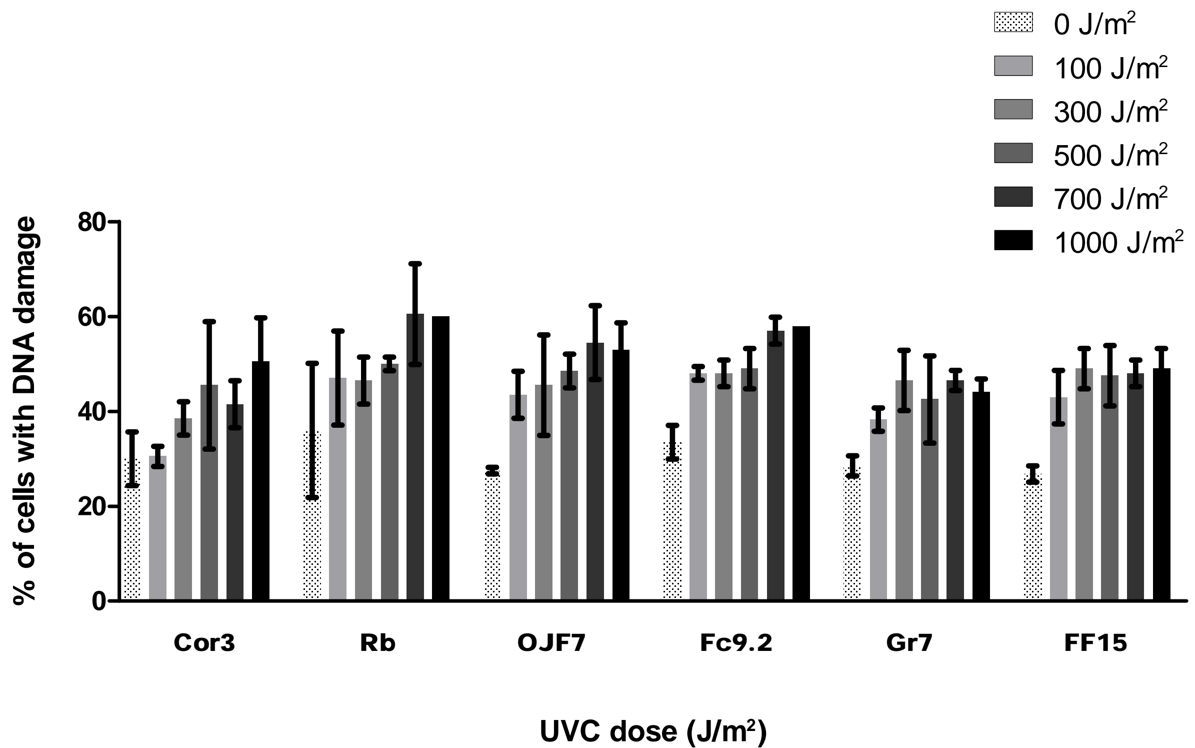


Fig. 9: Evaluation of the DNA damage detection in marine Planctomycetes strains exposed to several doses of UVC radiation. Cells were considered to present damage when fragmented DNA was observed dispersed around a central core forming a halo. Experiments were done in triplicate and results are presented as a mean ± standard deviation (SD).

Even though, an increase on the UVC radiation dose has led to a higher percentage of cells with DNA damage for all strains, strains Cor3, *R. baltica*, Fc9.2 and OJF7 appear to be slightly more sensitive to DNA damage caused by this type of radiation as they achieve higher damage levels and a higher increase of DNA damage after exposure to UVC radiation. For strains Gr7 and FF15 the levels of DNA damage did not altered significantly above low dosages. However, among all the strains tested, the resistance to UVC-mediated DNA damage did not differ significantly as all the strains show quiet similar values of damage. In addition, our results show that DNA damage and viability (Table 8) are differently affected. High doses of UVC radiation (>500 J/m2) had a strong impact on the viability of the majority of the strains as only Fc9.2 and Cor3 were able to resist.

Table 8: Marine Planctomycetes strains resistance to UVC radiation evaluated by a viability test. *Arthrobacter* sp. was used as qualitative control as it is considered a UVC resistant bacterium.

UVC dose/Strain	0J/m²	100J/m²	300J/m²	500J/m²	700J/m²	1000J/m²
FF15						
Fc9.2						
Cor3						
R.baltica						
OJF7						
Gr7						
Arthrobacter sp.						

For viability assays, *Arthrobacter* sp. was used as control since it was described as a UVC resistant bacterium (Kuhlman *et al.*, 2005). When comparing with this strain, Planctomycetes seem to be very resistant to UVC radiation. The number of viable cells is strongly affected by radiations dosages above 100J/m^2. For strains OJF7, Fc9.2, Gr7 and FF15

the outcome of the viability assay tally the results from the DNA damage assay. The same does not happen for Cor3 and *R. baltica*. Strain FF15 attained the maximum level of DNA damage at low UVC doses, which is in accordance with the results observed in survival assays, where viability is strongly affected at the same dose. The same is observed for strain Gr7 but for dosages of 300J/m^2. These two strains are the most sensitive ones and lower dosages of UVC radiation significantly affect their survival. Cor3, *R. baltica*, OJF7 and Fc9.2 seem to be less affected by the increase on the radiation dosage, being Cor3 and Fc9.2 the most resistant ones. For these strains, the increase on the percentage of DNA damage is coincident with the increase of the radiation dosage. So, there seems to be a different behavior between the strains: the first two seem to be irreversibly affected by lower dosages of UVC but these results are not reproduced on the DNA damage increase. The second group seems to be more resistant to UVC as higher dosages are needed to significantly affect viability. The increased resistance of these strains seems to allow the detection of an increase in the percentage of cells with DNA damage in accordance with the increase on irradiation dosage as the higher values of DNA damage were attained by the most resistant strains. These results indicate that although the assay is efficient in detecting DNA damage, does not allow the easy establishment of dose/effect relation. Further optimization and tests could allow the more accurate detection of DNA damage and improve the outcomes. Furthermore, as UV radiation affects not only DNA but also other cellular components, and cell survival depends on multiple factors such as repairing pathways and physical properties, these results may indicate that the different aspects have different impact on the response of each strain to UVC exposure. In the DNA damage assay, the procedure after exposure to UVC radiation prevents repair mechanisms as cells are immediately immersed on lysis solution preventing possible repair enzymes of acting on DNA damage. In addition, this procedure is performed in obscurity giving additional "protection" as photolyase, an enzyme responsible for DNA repair upon exposure to UV light (photorepair) requires visible light for activation (Sancar *et al.*, 1994; Suter *et al.*, 1997; Joux *et al.*, 1999). However, in viability assays, although the plates were kept in obscurity, other enzymes might be repairing DNA in the viable cells. Our data suggest that some of the tested strains may present other characteristics that confer them more survival capacity when exposed to this UVC radiation, even when presenting higher DNA damage values. Furthermore, some studies assert that for bacteria, survival after UV exposure will depend mostly on maintenance of the proteins

integrity than that of DNA (Daly *et al.*, 2007) fact that may explain the discrepancy observed in our results for DNA damage and viability. In this study, the authors refers that several microorganisms die when exposed to radiation causing little DNA damage. In addition, a study using *Gemmata obscuriglobus* suggested that highly packed chromatin organization might enhance radiation tolerance (Lieber *et al.*, 2009). As Planctomycetes present highly ordered condensed chromatin, this fact might confer them more resistance to radiation.

Some studies suggest that more pigmented strains could be more resistant to UV exposure although a relationship between pigmentation and resistance was never demonstrated (Hermansson *et al.*, 1987; Arrage *et al.*, 1993; Joux et al., 1999; Kolber *et al.*, 2000; Beja *et al.*, 2000; Beja *et al.*, 2002; de la Torre *et al.*, 2003). Nevertheless our data indicates that, although the non pigmented strain FF15 is the more sensitive strain, this aspect does not seem to influence our strains resistance as there is no evidence of a linear pigment/resistance relation. Furthermore, Fc9.2 is one of the less pigmented strains and still the more resistant one. In addition, Cor3 presents a highly similar pigmentation to the one of *R.baltica*, but higher resistance.

Another aspect thought to be involved in UV resistance relates to the habitat of each organism: organisms that are more exposed to radiation are generally more resistant (Jeffrey *et al.*, 2000) Our strains were isolated from the surface of different macroalgae collected in different locations along the Portuguese shore: Gr7 was isolated from a lagoon with continuous interchanges with the sea in Aveiro, Fc9.2 from a rocky beach in Carreço, Viana do Castelo and all the others at a rocky beach on Foz, Porto (see Annexe II). Although isolated from different locations all our strains deal with daily oscillations of UV exposure on their natural environments as all are subjected to the constant water levels oscillations that alter the potential exposure of these microorganisms along with time. In addition, some studies suggest that organisms that inhabit polluted environments are usually more resistant to several environmental stressors. Our data differ from this hypothesis as the most resistant strain Fc9.2 was isolated from the less polluted sampled habitats. The phylogenetic relationship between all the strains (Fig. 10) is another aspect to take into account.

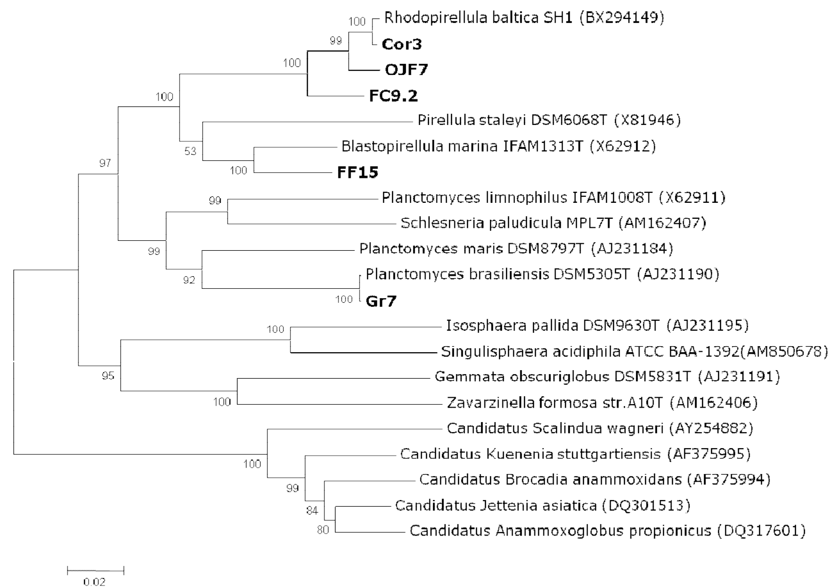


Fig. 10: 16S rDNA phylogenetic tree generated by the Neighbor-Joining method indicates the relationship of the isolates (bold) to members of the order Planctomycetales. Bootstrap values were calculated using 1000 replicates.

Among the strains tested, the ones related to *R. baltica* cluster appear to present more resistance to UVC damage. Still, different resistances are observed among them.

Our results do not seem to indicate a possible biogeographical correlation with resistance to UV radiation when it comes to the tested strains. Furthermore, for the strains tested, pigmentation does not appear to have significant influence on UVC resistance although it may be relevant as the non-pigmented strain FF15 is the less viable. The difference observed between the DNA damage levels and strain survival may indicate that the survival capacity upon UV exposure depends not only on avoiding DNA damage but most likely on the repair mechanisms that are responsible for maintenance of DNA integrity. In addition our results suggest that there is a wide variety of response to UVC damage among the tested bacteria allowing them to respond differently to this genotoxic agent.

4.2 Evaluation of a potential protective effect of ethanolic extracts of Planctomycetes to UVC-mediated damage: studies on DNA damage and viability

4.2.1 Yeast Comet assay does not allow detection of DNA damage mediated by UVC radiation on yeast cells

In order to evaluate a potential protective effect of the pigment ethanolic extracts of Planctomycetes on DNA damage mediated by UVC radiation, we tried to optimize the Comet Assay previously developed in our lab to allow the detection of this type of damage on *S. cerevisiae.*

Table 9: Optimization steps performed for the yeast comet assay. For each step the tested conditions and the optimum final one are shown.

Step	Tested conditions	Final
LMA concentration	1,5% and 3%	1,5%
Electrophoresis: duration and voltage	0,7 and 1,4V/cm, 5 and 10 minutes	0,7V/cm, 10minutes
Aditional step: recovery	10, 20, 30 minutes recovery time after UVC exposition	-

Although several aspects of the assay were subjected to optimization (Table 9), the assay did not allow the detection of DNA damage caused by this type of radiation above a certain dosage as an increase of the dosage did not led to an increase on tail length (Figs. 11, 12 and 13). Furthermore, even for doses where a linear response dose/damage was observed (50J/m^2) the tail length did not increase significantly.

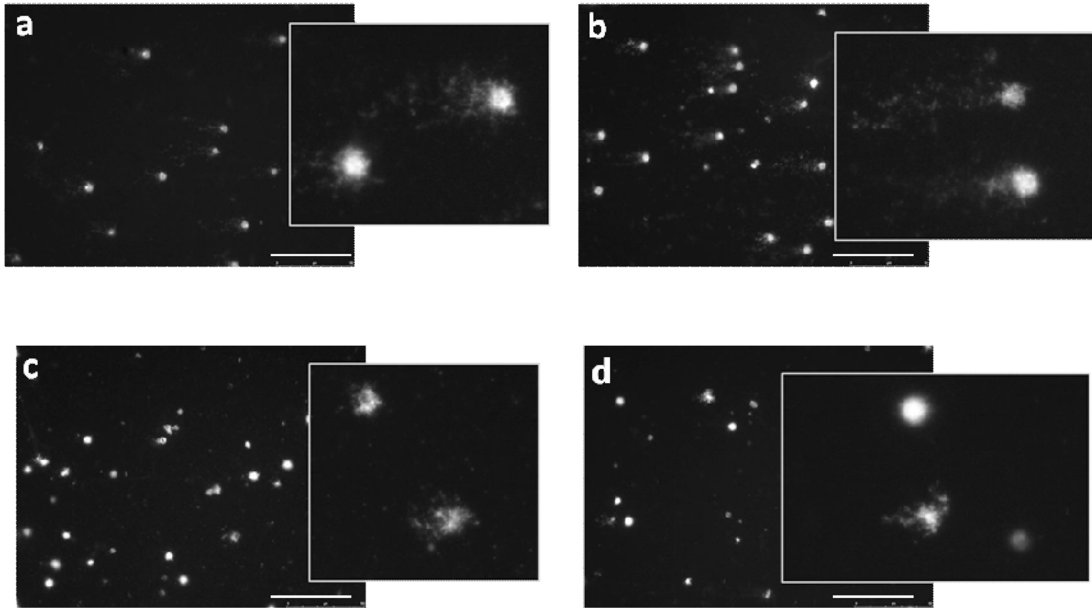


Fig. 11: Microphotographs of the Yeast Comet assay. DNA was stained using 1:3000 GelRed (Biotum). A) *S. cerevisiae* strain BY4741; B) *S. cerevisiae* strain BY4741 + 50 J/m^2 UVC; C) *S. cerevisiae* strain BY4741 + 100 J/m^2 UVC; D) *S. cerevisiae* strain BY4741 + 150 J/m^2 UVC. Although, the assay was effective for detecting DNA damage caused by low UVC doses, higher doses led to a decrease on tail length. All images were obtained with 400x magnification. Bar 50μm.

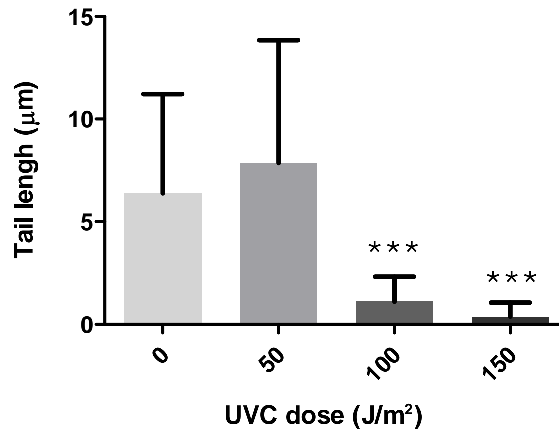


Fig. 12: Yeast comet assay results from *S. cerevisiae* cells irradiated with different doses of UVC radiation. Tail length was calculated using the CometScore software. Tail length was the chosen parameter to evaluate the level of DNA damage. The experiments were done in triplicate and results are presented as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison. Asterisks indicate differences considered statistically significant: *** means $p < 0.01$ when compared to the respective control.

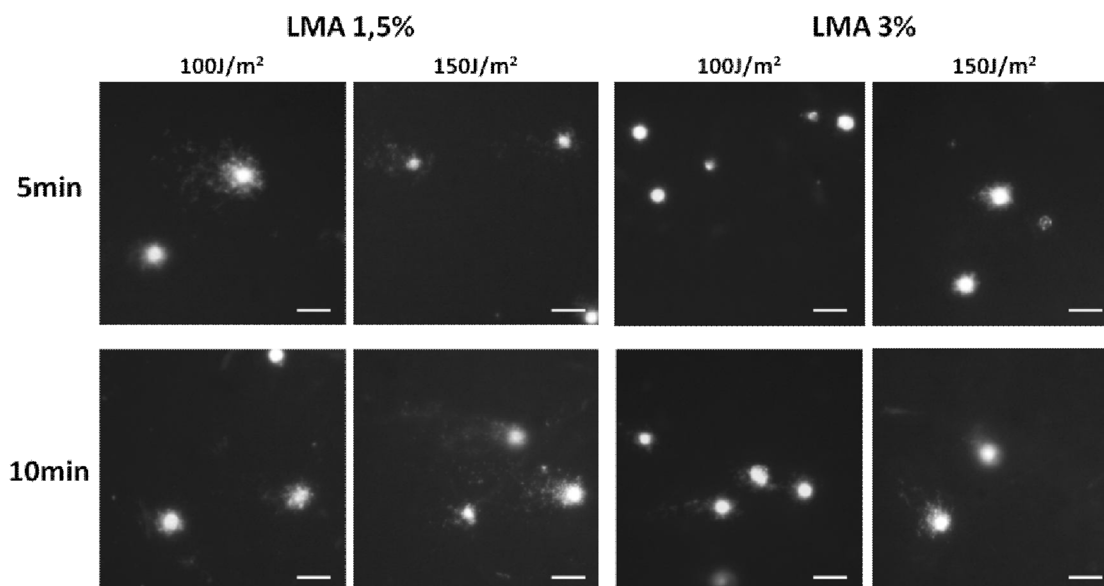


Fig. 13: Microphotographs of the Yeast Comet assay in *S. cerevisiae*. Different LMA concentrations (1,5% and 3%) and electrophoresis times (5 and 10 min) were tested for cells exposed to different UVC doses. DNA was stained using 1:3000 GelRed (Biotum). All images were obtained with 400x magnification. Bar 10µm.

In order to discard the possibility that the UV dosage was not sufficient for affecting yeast survival, viability was also evaluated (Fig. 14).

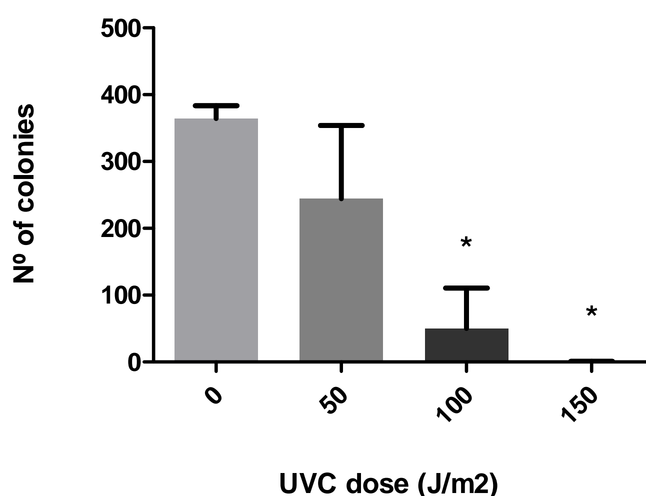


Fig. 14: Impact of UVC radiation on *S.cerevisiae* viability. Doses of 100 J/m^2 UVC radiation or higher greatly reduce survival. The experiments were done in triplicate and results are presented as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison. Asterisks indicate differences considered statistically significant: * means $p < 0.05$ when compared to the respective control.

The data obtained suggests that *S. cerevisiae* is strongly affected by increasing UVC dosages as survival is highly affected by UVC doses >100J/m^2. Our results suggested that the Comet Assay is not an effective method for UVC mediated DNA damage detection in *S. cerevisiae*. Several steps of the assay were modified taking into account that UVC damage is different from the damage caused by hydrogen peroxide, which was the DNA damage causing agent that was used for the development of yeast comet assay in our laboratory. As UVC radiation could cause a higher number of breaks leading to small fragments of DNA, shorter electrophoresis runs and higher concentration of LMA were used in order to assure the "trapping" of these fragments so that they could be visualized. The results obtained showed no difference from the ones obtained with the previous conditions (Fig...). Tail length decreased upon exposure to higher doses of UVC radiation and comet heads displayed a diffuse and irregular aspect and "inter-comet" spread material appeared stained, which may indicate the possibility that many small fragments are formed and dispersed in the mini gel. Furthermore, reports suggest that detection of DNA damage mediated by UVC using the comet assay requires incubation after exposure to radiation so that the activation of the repair mechanisms may occur (Wojewódzka *et al.*, 2002). In yeast, UVC repair mechanisms essentially rely on nucleotide excision repair (NER) (Goosen, 2010). The first step is mediated by AP lyase that performs the incision on the DNA strand leading to unwinding of DNA that would form the "tail".

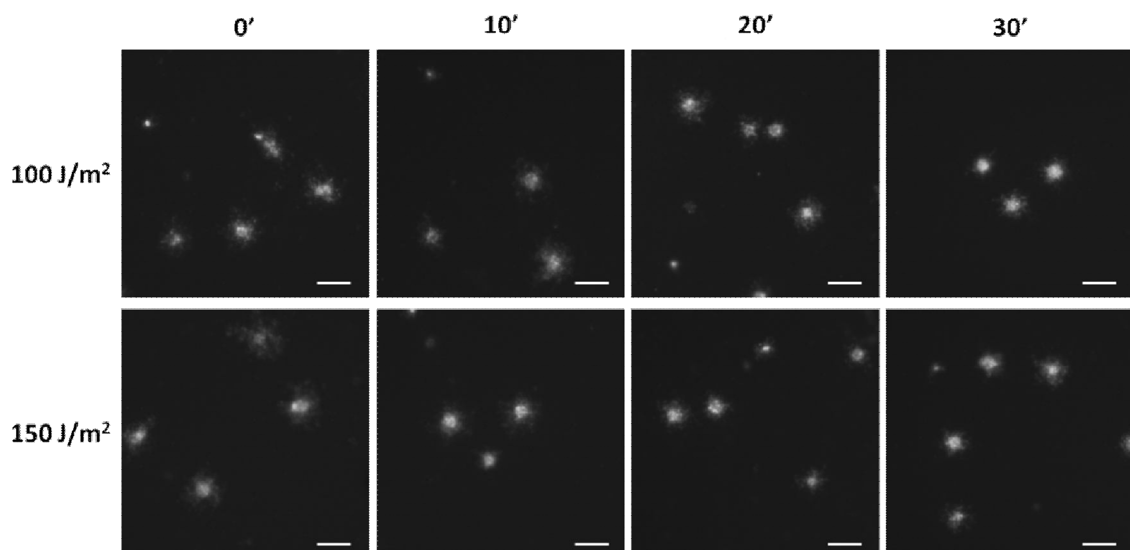


Fig. 15: Yeast comet assay microphotographs. After UVC exposure, cells were allowed to recover for different periods of time (10, 20 and 30 min). DNA was stained using 1:3000 GelRed (Biotum). All images were obtained with 400x magnification. Bar 10μm.

Our results showed that despite we have allowed the onset of NER by incubation for different periods of time, the results obtained with the assay did not lead to an increase on DNA damage with the increase on UVC dosage (Fig. 15). In addition, several parameters of damage evaluation described for Comet assay were also tested as comet length could not be the more accurate for measurement of this kind of damage. Nonetheless, none of the parameters was able to establish a linear relationship between dose/damage. As we were not able to optimize Yeast comet assay for detection of UVC mediated DNA damage, other assays were used to evaluate the potential protective effect ethanolic extracts of Planctomycetes pigments against UVC radiation.

4.2.2 Pre and co-incubation of *Saccharomyces cerevisiae* cells with ethanolic extracts of Planctomycetes prior to UVC radiation does not improve survival

S. cerevisiae was used as model organism to evaluate the potential protective effect of ethanolic extracts of Planctomycetes pigments against UVC mediated damage. Pre and coincubation of yeast cells with the extracts as performed (Fig. 16).

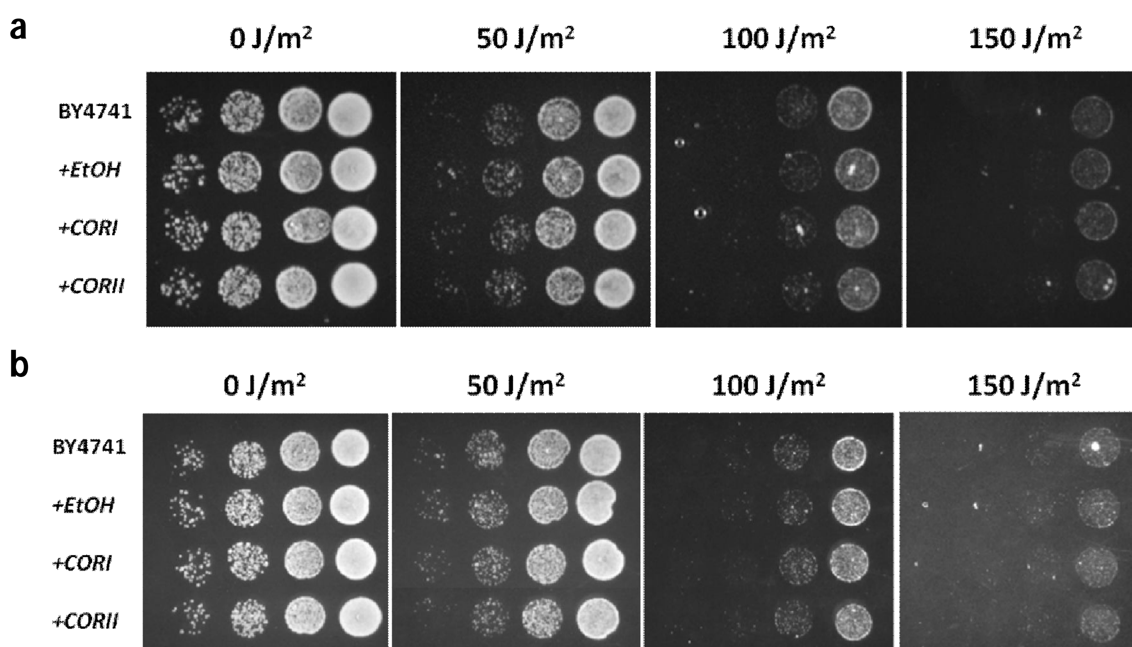


Fig. 16: Drop growth test results of yeast cells incubated with each one of the ethanolic extracts of Planctomycetes pigments. a)Pre-incubation, b)Co-incubation.

None of the extracts seems to have protective effect against UVC radiation both in co as in pre incubation. Some studies claim that pigments influence cell resistance to UV radiation by capturing damaging radiation and enabling it from reaching cellular structures that could be affected and altered by it (Cockell and Knowland, 1999). These results have to be analyzed cautiously because, it is important to take into account that the true composition and concentration of the pigments is undefined and the protective effect depends upon the nature of the pigments. Therefore, the influence of pigments on UVC resistance could be important since some pigments of these strains could not be efficiently incorporated in the final extract. As Cor3 was one of the most resistant strains, we opted to extract and test its extracts in order to evaluate its possible influence on the resistance shown by the strain. Nonetheless the results obtained, it is important to take into account that the true composition of the pigments is not know as well as the concentration of the potential protective compounds, facts which may have influence in the results.

4.2.3 Co-incubation of plasmid pET25 with ethanolic extracts of Planctomycetes prior to the exposure to UVC radiation does not improve its transformation ability

Cells have several repair pathways to counteract DNA damage provoked by UV radiation enabling them to detect and darn damage. In addition, several intracellular substances also play a role in the DNA protection against this type of radiation. In order to avoid these aspects and to evaluate the potential protective effect of the ethanolic extracts of Planctomycetes pigments against DNA damage caused by UV radiation, a cell-free assay was used, which is based on the transformation ability of plasmid DNA. Plasmid pET25 pre and co incubated with Planctomycetes pigments ethanolic extracts was UVC irradiated and posteriorly used to transform *E.coli* competent cells. Transformation ability was calculated by colony counting.

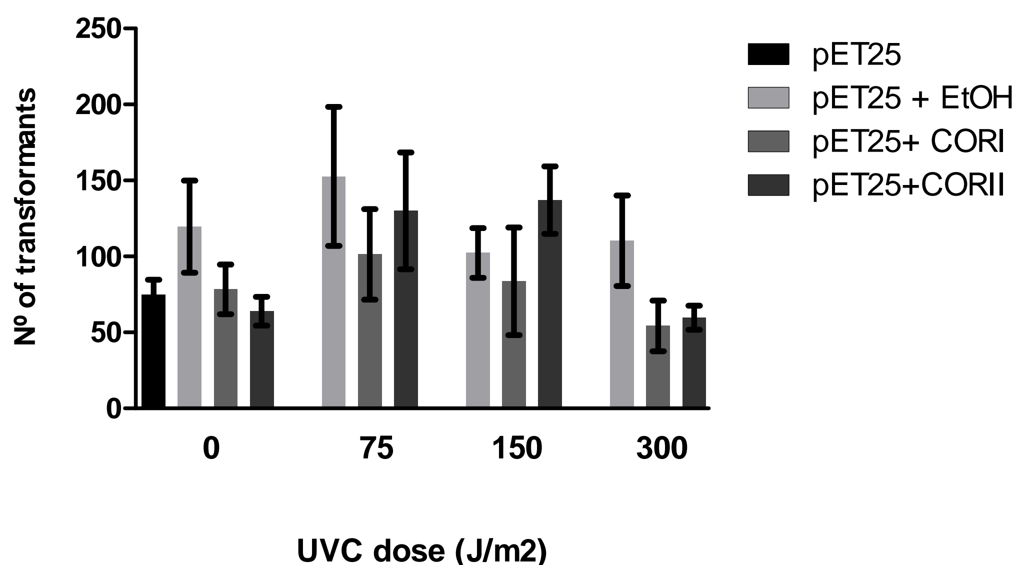


Fig. 17: Plasmid pET25 transformation ability after UVC irradiation with and without presence of ethanolic pigment extracts of Planctomycetes. The experiments were done in triplicate and results are presented as a mean ± standard deviation (SD).

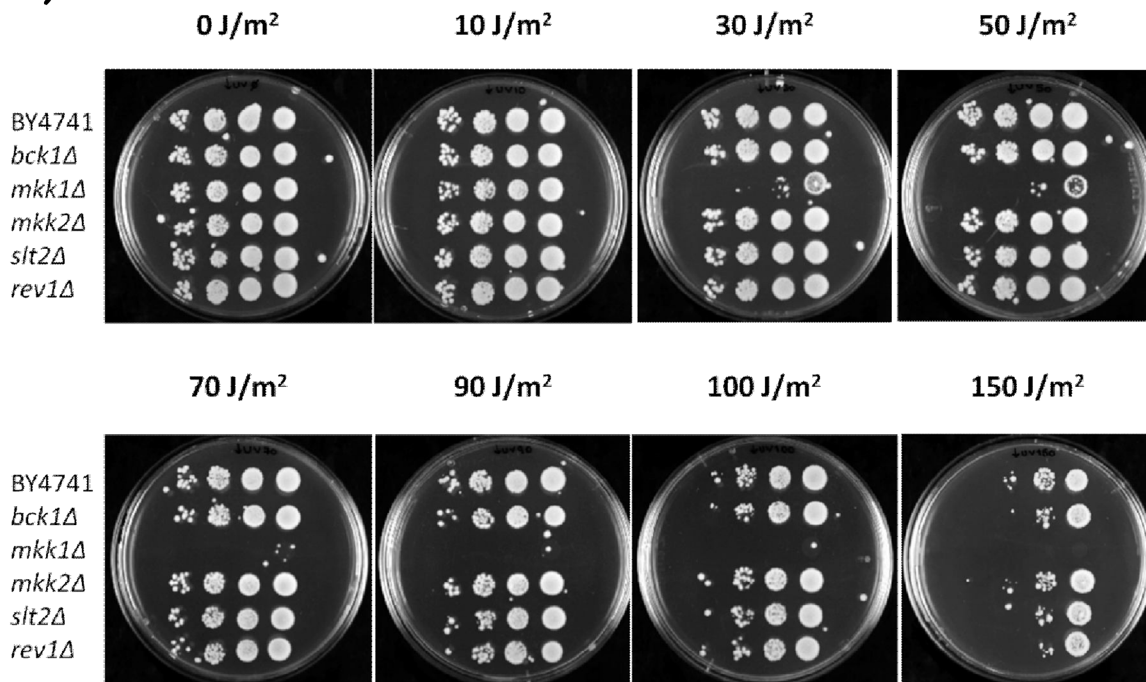
None of the extracts seemed to have a protective effect on DNA damage mediated by UVC radiation. (Fig. 17). Extracts do not seemed to have toxic effects on plasmid DNA as the transformation efficiency is not significantly altered on non-irradiated extract-treated plasmid assays. An unusual result refers to the irradiated control samples (without extracts) as it was expected that irradiated plasmid would have decreased transformation efficiency due to the DNA mutations and strand breaks caused by UV irradiation. However some studies claim that damaged plasmid DNA can be repaired in the host cells (Strike and Roberts, 1982). In addition, studies of human cells transfection with irradiated plasmid reported that a higher yield in the number of transformants was observed with irradiation dose increasing until 1kJ/m^2, even though the increase varies among experiments (Spivak *et al.*, 1984), which is in accordance with our results. As ethanol was used as solvent to the pigments extracts, the presence of this component during transformation could have some influence on the results observed as for control (0J/m^2) the number of transformants was higher than for the control with no ethanol. Ethanol effect on transformation efficiency is controversial as in some studies it is thought to reduce transformation efficiency (Sarkar *et al.*, 2002) and in others to improve it (Sharma *et al.*, 2005). Although the assay suggests that the Planctomycetes extracts do not protect DNA from UVC radiation, further optimization of

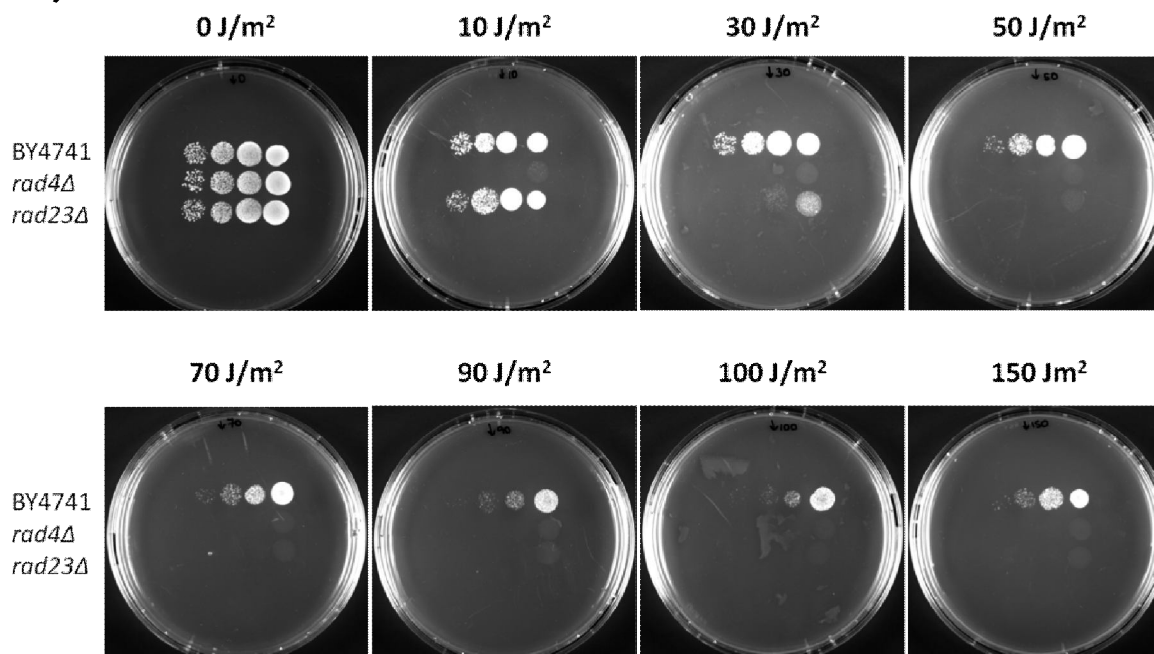
the assay should be done in order to correct the control results allowing the accurate evaluation of the results. As DNA is the main target of UV radiation and DNA integrity is essential for cellular stability and survival, it is important that DNA damage is correctly estimated. Besides that, when testing a eventual protective effect of any compound it is important that the results are the more accurate possible. This assay provides a straightforward functional test for evaluation of protective properties of new compounds against DNA damage.

4.3 The PKC-mediated pathway is involved on yeast response to UV damage probably through Mkk1

All living organisms display several mechanisms that respond to all types of stress to which they are exposed. In eukaryotes like *S. cerevisiae* several signalling pathways coordinate cell response to stress in an integrated and cooperative way. Among these pathways, the PKC pathway figures as a key signalling pathway in maintenance of yeast cell integrity. Although implicated in response to several kinds of stress and in the regulation of several cellular events, only one study reports the possible involvement of the PKC pathway on yeast response to UV radiation (Bryan *et al.*, 2004). For yeast, UV radiation response depends fundamentally on NER mechanisms (Guzder *et al.*, 1995). It is thought that DNA damage recognition is mediated by the protein complex Rad4.Rad23 in interaction with other recognition proteins as Rad7 and Rad14 (Min and Pavletich, 2007; Guzder *et al.*, 1995; Jansen *et al.*, 1998).

Trying to unveil more about the possible involvement of this signalling pathway in the response to UV radiation, a set of *S.cerevisiae* mutant strains for the several components of the PKC signalling cascade were screened for sensitivity to UV radiation (Fig. 18).


a)
0 J/m²
10 J/m²
30 J/m²
50 J/m²
70 J/m²
90 J/m²
100 J/m²
150 J/m²
BY4741
bck1Δ
mkk1Δ
mkk2Δ
slt2Δ
rev1Δ


b)
0 J/m²
10 J/m²
30 J/m²
50 J/m²
70 J/m²
90 J/m²
100 J/m²
150 Jm²
BY4741
rad4Δ
rad23Δ

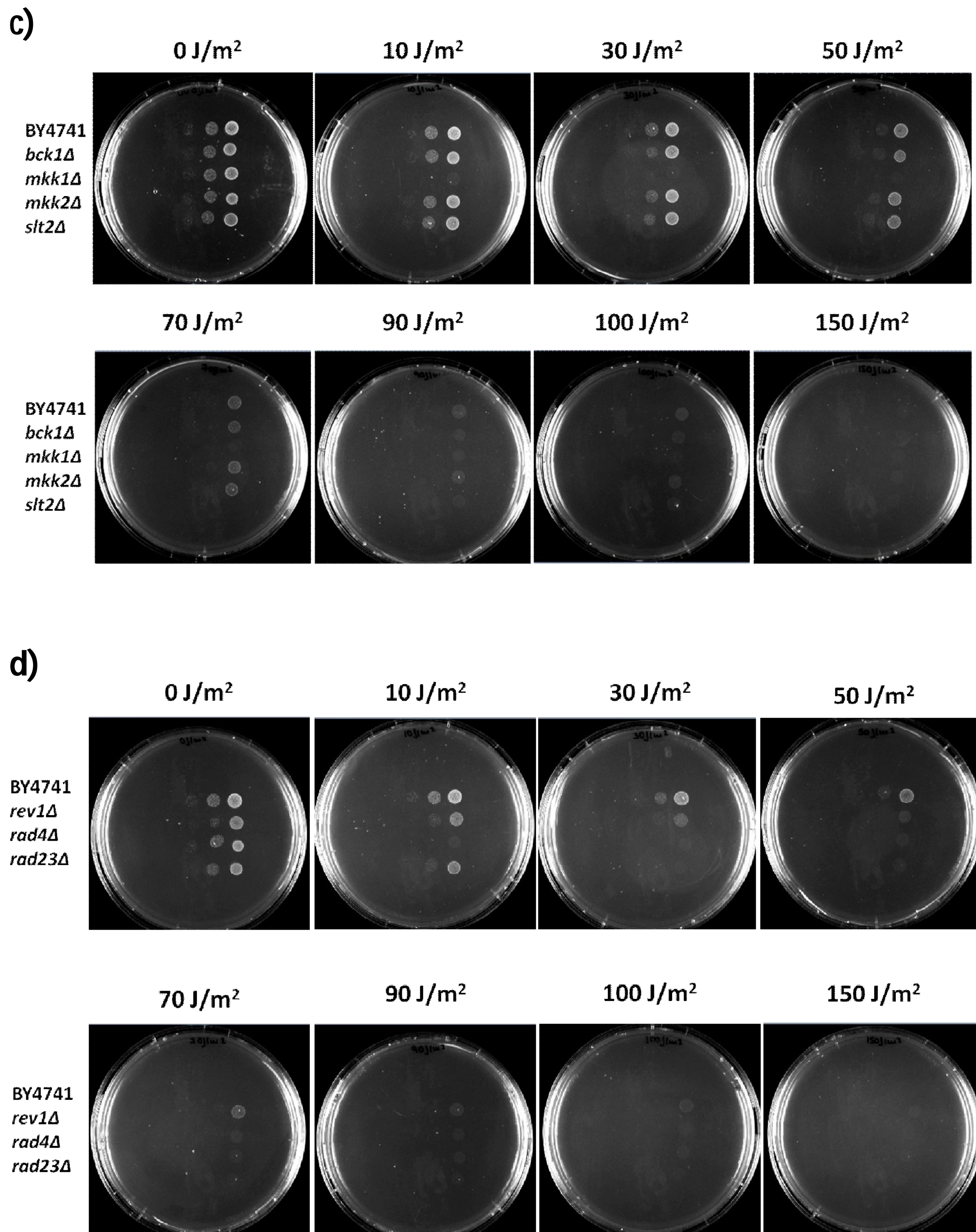


Fig. 18: Screening of *S. cerevisiae* strains UV sensibility. The several mutant strains for the PKC mediated pathway components and mutant strains for *rad4*, *rad23* and *rev1* (genes involved in UV response) were exposed to several UVC doses in complete YPD (a and b) and minimum YNBD media (c and d). Plates were photographed after one day of growth after exposure.

The same results were obtained in complete (YPD) and in minimum media (YNBD supplemented with the necessary aminoacids): all mutant strains shown similar sensibilities to UV irradiation with exception to *mkk1*Δ. Mutant *rad* strains were used as control as well as *rev1* mutant which is involved on stress response pathways including UV, displaying a moderate sensibility to UVC. (Guzder *et al.*, 1995; Jansen *et al.*, 1998; Heidenreich *et al.*, 2006; Min and Pavletich, 2007; Sabbioneda *et al.*, 2007) The data obtained is not in accordance with the results obtained by Bryan and co-workers as they described *slt2*Δ as sensitive to a UV dose of 100J/m^2. Our results place this strain as non-sensitive to UVC radiation as it shows the same level of resistance demonstrated by the non-mutant strain BY4741. In our results, only *mkk1*Δ showed higher sensitivity to low doses of UVC radiation resembling the values demonstrated by the *rad* mutants used as control. As Rad4 and Rad23 proteins are involved in the response to UV damage on yeast, it was expected that UVC exposure seriously affect viability of strains affected in the corresponding genes. Furthermore, *mkk1*Δ presented a similar phenotype to the one of *rad4*Δ which displays high sensitivity to UVC radiation (Birrell *et al.*, 2001). *rad23*Δ was reported as moderately sensitive as we showed in our results (Birrell *et al.*, 2001). One unusual aspect of the sensitivity of *mkk1* due to the described functional redundancy of *MKK1* and *MKK2* as *mkk2*Δ did not presented the expected similar sensitivity. Redundancy refers to homologous genes that by means of partial overlap in functions compensate for each other's loss. It is though that redundancy is advantageous for biological systems as it may be interpreted as mechanisms by which functional overlap contributes to/or facilitates signal transduction (Kafri *et al.*, 2009). As redundant proteins, the cascade triggering leads to the indiscriminate activation of one or another. However, this characteristic is not confirmed by our results as the sensitivity to UVC radiation displayed by the two proteins strongly differs. Trying to find a possible explanation for this fact we consider the transcription factors that may depend on activation by the two proteins. Using the YEASTRACT online database we analysed the documented and potential transcription factors associated with each gene. Although there are some common transcription factors, several unusual differences are found (see ANNEX III: list of the different transcription factors). Among the several predicted transcription factors for *MKK1* figure some related to checkpoints of the cell cycle and to the associated cyclins as is the case of Ace2 and Swi5. Both transcription factors are involved in the regulation of genes expressed at M/G1 and early G1 phases and are dependent on Cdc28, a

kinase that regulates several steps in the yeast cell cycle. Accordingly, this kinase connection to the PKC mediated pathway was already described by Marini and co-workers (Marini et al., 1996). Cdc28 is essential for cell to entry S phase being also required for S phase progression and G2/M entry. All these processes are coordinated by interaction with cyclins and constitute important checkpoints on the cell cycle (Marini et al., 1996; Heinisch *et al.,* 1999). Nonetheless, the process by which the kinase activates the pathway remains unknown. As transcription factors interacting with Cdc28 are potentially associated with Mkk1 it is possible that this consists in another interaction point of the kinase with this signalling pathway. Furthermore, these data suggest that Mkk1 interacts with other signalling pathways related to cell cycle regulation. Checkpoints of the cell cycle are crucial when it comes to cell damage mediated by stress agents, deciding cell fate. This may explain the phenotype presented by *mkk1Δ* strain as a possible involvement with these checkpoints could explain the increased sensibility. UVC radiation is a genotoxic stress agent causing several damages to cells that seriously affect viability. Cells stop cell cycle when errors are detected at checkpoints so that they can be repaired. If Mkk1 has a key role on the connection of PKC signalling pathway to cell cycle regulation, cells lacking this gene may not display the essential interactions necessary for activation of pathways for UV response upon damage occurrence. In addition, our data indicate that, nonetheless the way that this interaction is established, Mkk1 is probably involved on cross-talk between PKC mediated pathway and other signalling pathways involved on UV response. Connections with other pathways related to nutrient sensing and cytoskeleton dynamics are known (Heinisch *et al.*, 1999) but to our knowledge, the possible connection of PKC pathway with UV response has been reported once. However, the way this connection is established, which pathway is activated and what components are involved in the cross-talk are unknown.

4.4 Planctomycetes have potential yeast PKC homologues that may indicate the presence of complex signalling pathways

Sensing the environment is of fundamental importance for all living organisms. As mentioned before, MAPK pathways are central when it comes to stress response and signalling on eukaryotes. In addition, the underlying principles are similar and highly conserved between eukaryotes. On the other hand, the potential presence of this kind of signalling pathways is controversial for the prokaryotes due to the “simple” cell plan displayed by these organisms. Until now, several kinases are known for prokaryotes but their function on signalling remains unclear. In order to evaluate the potential presence of complex signalling pathways on Planctomycetes a bioinformatic analysis was made. Based on the aminoacidic sequences of several *R. baltica* kinases, a BLAST analysis was performed against *S. cerevisiae* genome. The search returned several kinases that display low *e* values indicating high homology values to the yeast PKC mediated pathway components (see Table 3 on section 3.8.1). The obtained sequence COR2162 presents relevant similarity to the one of *R. baltica*. (Fig...)

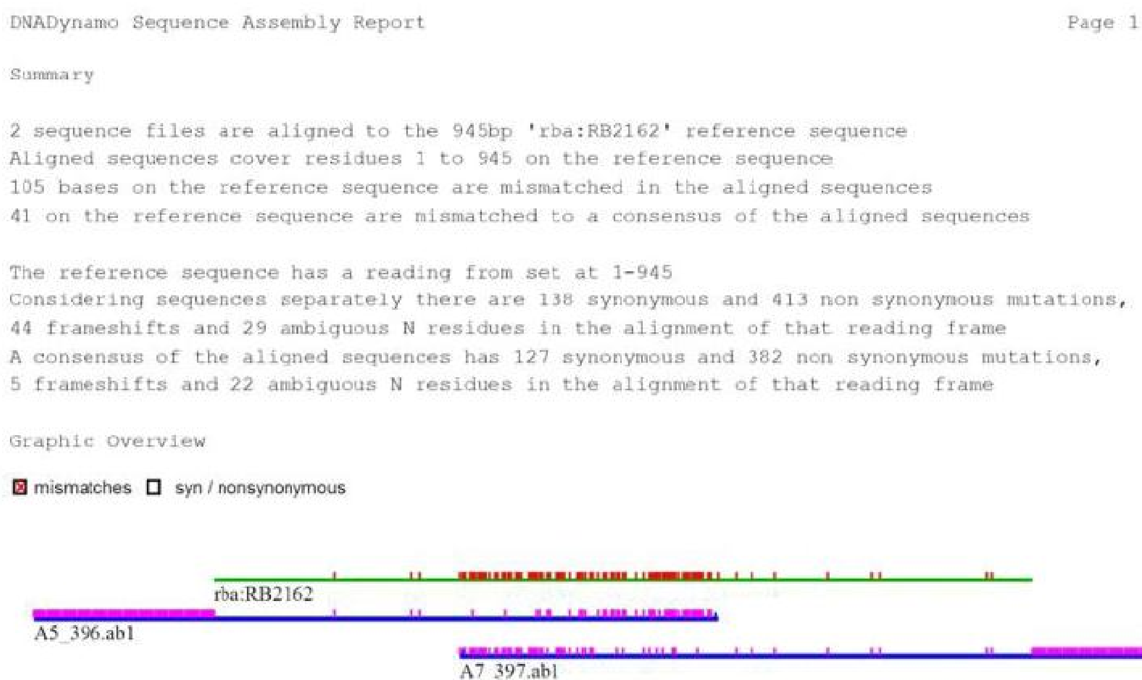


Fig. 19: Report from the alignment of the sequences of COR2162 obtained by sequencing (A5_396 and A7_397) and the sequence of RB2162. The alignment was effectuated using the Software DNADynamo.

This result may indicate that *R. baltica* relies on complex signalling pathways potentially similar to MAPK coordinated pathways. Taking into account the cell plan of Planctomycetes, it is probable that more complex signalling pathways are displayed by these organisms as the existence of membrane systems implies signalling in a different magnitude and coordination that those involved in conventional prokaryotes.

5. Conclusions and future perspectives

This work intended to unveil some aspects related to cell integrity of Planctmycetes when it comes to DNA damage and survival upon UVC exposure. Being UV radiation a key stressor to marine environments, it is important to evaluate the impact of this radiation on the marine organisms and ecosystems. A new DNA damage detection assay was developed and optimized for Planctomycetes. This assay allows the detection of DNA damage provoked not only by UV radiation as also by hydrogen peroxide. Nonetheless, a dose/effect response is not clearly observable which may indicate that the assay needs furthermore optimizations. As Planctomycetes are key players in several ecosystems and potential "biomonitors" this assay can be used as a tool for quick and easy evaluation of DNA damage evaluation, a fundamental aspect when it comes to this kind of studies. Some aspects of the UVC response of marine species of Planctomycetes where characterized suggesting that Planctomycetes seem to be highly resistant to UVC radiation when comparing to *Arthrobacter* sp., a strain described as UVC resistant. The cluster related to *R. baltica* was the most resistant one, being Fc9.2 the more resistant strain. In contrast, FF15 was the most sensitive strain. The outcomes from the viability tests and the DNA damage assay are not in concordance for all the tested strains, which suggest that several characteristics are determinant for survival upon DNA damage. Besides that, our study indicates that among the tested strains there are wide variations in the UV response that determine their resistance to this kind of radiation.

Pigments of Planctomycetes were evaluated for their potential protective action against UVC radiation using viability and cell-free assays. None of the pigments demonstrated protective action. However, as composition and concentration of the pigments is undefined and the protective effect depends upon the nature of the pigments further analysis of these pigments should be performed to allow a more clear evaluation of this effect.

Furthermore, we intended to characterize signaling pathways involved in cellular integrity of Planctomycetes as little about these mechanisms for this group is known. Homology between *R. baltica* kinases and the components of yeast PKC pathway were evaluated and low *e* values suggest that signaling in Planctomycetes might relay in evolved signaling pathways. The cell plan of Planctomycetes and the existence of membrane systems probably imply signalling in a different magnitude and coordination that those involved in conventional prokaryotes. Future work should involve cloning Planctomycetes homologues in the corresponding yeast mutants. Phenotype complementation should allow a more

accurate characterization of Planctomycetes kinases and integrity signalling pathways. Furthermore, as yeast PKC pathway is fundamental for yeast cellular integrity, we evaluated its possible connection to UV response. Mutant strains for the PKC pathway components were tested for their UVC resistance and our results suggest that not only this pathway is probably involved in UV response to cross-talking with other signalling mechanisms, as also, *MKK1* might play a key role in this connection. Mkk1 deletion seriously affected yeast survival upon exposure to low doses of UVC radiation and this results poses a challenging question as *mkk1* is reported as redundant to *mkk2*, which did not demonstrated to be as sensitive. In future works, cloning of yeast *mkk1* into the mutant strain should be performed in order to evaluate the probable phenotype complementation.

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ANNEXES

ANNEX I

Protocol for $CaCl_2$ XL1-Blue *E. coli* competent cells preparation

XL1-Blue *E. coli* cells were inoculated in 10mL of liquid LB medium and grown overnight at 37°C, 200rpm. One millilitre of this pre-inoculum was added to 100mL of liquid LB medium and incubated at 37°C, 200rpm until an 0,4-0,6 OD_{600} was obtained. Cells were cooled down on ice for 10 min and posteriorly 80mL of the culture was harvested on falcons by centrifugation at 4000rpm, 4°C for 10 min. Supernatant was discarded and cells were gently ressuspended in 20mL/falcon of sterile cold 0,1M $MgCl_2$. Cells were harvested by centrifugation at 4000rpm, 4°C for 10 min, ressuspended in 2mL/falcon falcon of sterile cold 0,1M $MgCl_2$ and kept on ice for 2 h. Aliquots of 200µL were distributed in eppendorf tubes, flash-frozen on liquid nitrogen and kept at -80°C.

ANNEX II

Planctomycetes strains sampling locations and associated macroalgae

The Planctomycetes strains used in this work were all isolated in LEMUP lab and are part of the OJF culture collection. The several strains were isolated from the surface of different macroalgae collected at different locations along the Portuguese shore: Carreço, Porto and Aveiro.

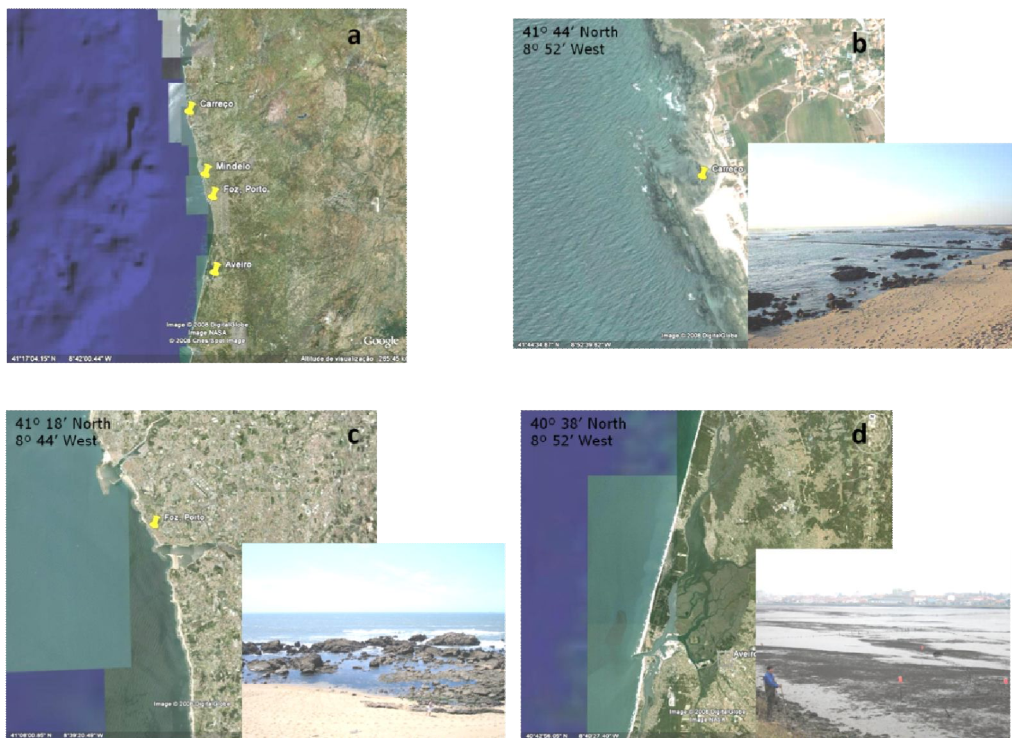

Fig. 20: a)Sampling locations along the Portuguese shore from where the macroalgae were collected: b) Carreço; c) Porto d) Aveiro. The GPS coordinates are mentioned for each place.

Fig. 21: Macroalgae from where the Planctomycetes strains were isolated.

ANNEX III

Mkk1 potential transcription factors

Ace2p: Transcription factor that activates expression of early G1-specific genes, localizes to daughter cell nuclei after cytokinesis and delays G1 progression in daughters, localization is regulated by phosphorylation; potential Cdc28p substrate.

Gcn4p: Transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation; expression is tightly regulated at both the transcriptional and translational levels.

Gsm1p: Putative zinc cluster protein of unknown function; proposed to be involved in the regulation of energy metabolism, based on patterns of expression and sequence analysis.

Hac1p: bZIP transcription factor (ATF/CREB1 homolog) that regulates the unfolded protein response, via UPRE binding, and membrane biogenesis; ER stress-induced splicing pathway utilizing Ire1p, Trl1p and Ada5p facilitates efficient Hac1p synthesis.

Rlm1p: MADS-box transcription factor, component of the protein kinase C-mediated MAP kinase pathway involved in the maintenance of cell integrity; phosphorylated and activated by the MAP-kinase Slt2p.

Sfl1p: Transcriptional repressor and activator; involved in repression of flocculation-related genes, and activation of stress responsive genes; negatively regulated by cAMP-dependent protein kinase A subunit Tpk2p.

Stp1p: Transcription factor, activated by proteolytic processing in response to signals from the SPS sensor system for external amino acids; activates transcription of amino acid permease genes and may have a role in tRNA processing.

Stp2p: Transcription factor, activated by proteolytic processing in response to signals from the SPS sensor system for external amino acids; activates transcription of amino acid permease genes.

Swi5p: Transcription factor that activates transcription of genes expressed at the M/G1 phase boundary and in G1 phase; localization to the nucleus occurs during G1 and appears to be regulated by phosphorylation by Cdc28p kinase.

Yap1p: Basic leucine zipper (bZIP) transcription factor required for oxidative stress tolerance; activated by H2O2 through the multistep formation of disulfide bonds and transit from the cytoplasm to the nucleus; mediates resistance to cadmium.

Mkk2 potential transcription factors

Cup2p: Copper-binding transcription factor; activates transcription of the metallothionein genes CUP1-1 and CUP1-2 in response to elevated copper concentrations.

Fkh1p: Forkhead family transcription factor with a minor role in the expression of G2/M phase genes; negatively regulates transcriptional elongation; positive role in chromatin silencing at HML and HMR; regulates donor preference during switching.

Gat1p: Transcriptional activator of genes involved in nitrogen catabolite repression; contains a GATA-1-type zinc finger DNA-binding motif; activity and localization regulated by nitrogen limitation and Ure2p.

Gln3p: Transcriptional activator of genes regulated by nitrogen catabolite repression (NCR), localization and activity regulated by quality of nitrogen source.

Gzf3p: GATA zinc finger protein and Dal80p homolog that negatively regulates nitrogen catabolic gene expression by competing with Gat1p for GATA site binding; function requires a repressive carbon source; dimerizes with Dal80p and binds to Tor1p.

Hap2p: Subunit of the heme-activated, glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex, a transcriptional activator and global regulator of respiratory gene expression; contains sequences sufficient for both complex assembly and DNA binding.

Mbp1p: Transcription factor involved in regulation of cell cycle progression from G1 to S phase, forms a complex with Swi6p that binds to MluI cell cycle box regulatory element in promoters of DNA synthesis genes.

Nrg1p: Transcriptional repressor that recruits the Cyc8p-Tup1p complex to promoters; mediates glucose repression and negatively regulates a variety of processes including filamentous growth and alkaline pH response.