Epiphytic Planctomycetes from macroalgae: insights of their morphology, physiology and ecology

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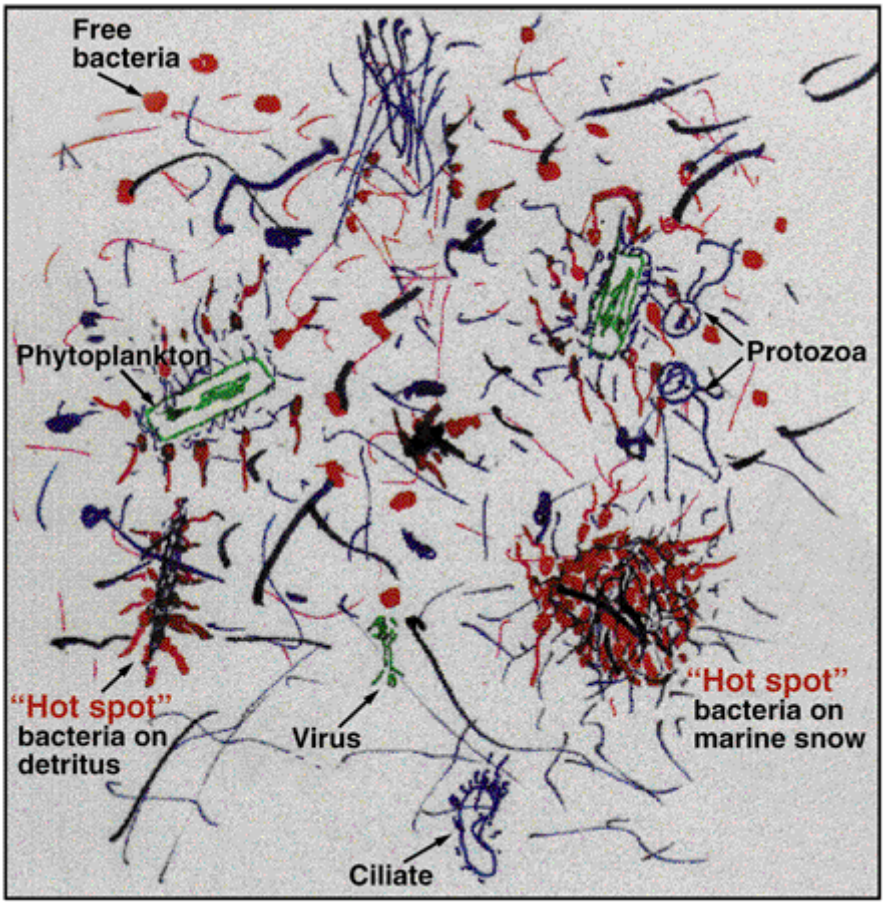
Funding

To my mom
and to Carlos
for their endless love and
support



The *microbial loop*: *impressionist version*. From Farooq Azam

"The Earth is a microbial planet, on which macroorganisms are recent additions - highly interesting and extremely complex in ways that most microbes aren't, but in the final analysis relatively unimportant in a global context."
Mark Wheelis as quoted by Carl Woese

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Summary

Marine macroalgae are widely colonized by a variety of macro and micro-organisms like invertebrates, diatoms, fungi and bacteria. Of these, the association bacteria-macroalgae is the one most studied and it has been described for over a century. Research in this area has mainly been focused on reproductive and nutritional aspects and on the antimicrobial properties of the macroalgae polymers that protect them from biofouling microorganisms. However, studies to investigate the microbial community associated with macroalgae and its dynamics are still reduced. In the last 5 years, we have assisted to a high increase in molecular studies addressing the diversity of bacteria associated with macroalgae. These showed that *Planctomycetes*, a very particular phylum of *Bacteria*, has a widespread distribution among macroalgae. Furthermore, these bacteria were reported to be the dominant group in the kelp *Laminaria hyperborea* which suggests an important potential ecological role in these habitats. The present study, focused on the *Planctomycetes*-macroalgae association, aims to enlarge our knowledge on this ecosystem by characterizing new planctomycetes previously isolated from the macroalgae biofilm community and also their geographic and temporal distribution on several macroalgae.

A previous study based on culture dependent methods showed a high diversity of unknown planctomycetes associated with different macroalgae from the north coast of Portugal. Based on 16S rRNA gene analysis, 39 strains, representing five novel taxa of planctomycetes, were selected for further characterization. The strains were phylogenetically related to the genus *Rhodopirellula* that only comprised one species validly described, *R. baltica* SH1^T. The taxa were taxonomically characterized through a polyphasic approach, *i.e.*, by combining a wide range of morphological, metabolic, chemotaxonomic and genetic characteristics that allowed separating them from the type species *R. baltica* SH1^T. *Rhodopirellula lusitana*, a novel species with 97.6 % similarity in the 16S rRNA gene to *R. baltica* SH1^T, was one of the most abundant taxa recovered from all the macroalgae sampled and appeared to be a specific group found only in macroalgae. *Rhodopirellula rubra* shares ~98 % similarity in the 16S rRNA gene to *R. baltica* SH1^T and was also found in association with other macroalgae in Sweden and with marine sponges. *Rhodopirellula formosa* was isolated from the surface of *Fucus spiralis* and *Ulva* sp. and shares 96.6 % 16S rRNA sequence gene similarity with *R. baltica* SH1^T. The two novel genera, *Roseimaritima and Rubripirellula*, shared less than 94 % similarity in the 16S rRNA sequence gene to *R. baltica* SH1^T and also appeared to be specific to macroalgae. The polyphasic characterization allowed to

distinguish all the taxa between them and from *R. baltica* SH1^T supporting the creation of novel species and genera. The physiological and biochemical characterization of these organisms showed their high capacity to metabolize a wide range of carbohydrates, many of them found in the sulphated polymers produced by macroalgae. This result suggests a specialization of planctomycetes as macroalgae colonizers.

In order to determine in more detail the relationships between the isolates belonging to the novel taxa under study, the application of a different molecular marker was applied to the *Planctomycetes* group as a complement of the 16S rRNA gene and as a possible substitute of DNA-DNA hybridization (DDH). DDH is required for species description when the 16S rRNA gene similarity between two strains is higher than 97 % as it is the case of *R. rubra* and *R. lusitana*. The gene encoding for the beta subunit of the RNA polymerase (*rpoB*), a conserved and widespread gene among *Bacteria*, was analysed and applied to the order *Planctomycetales*. Based on a partial sequencing of this gene, it was determined that novel planctomycetes species are delimitated by a 96.3 % threshold value in the *rpoB* gene sequence similarity and that intraspecific relations among strains are defined by a value higher than 98.2 %. These threshold values were applied to the isolates under taxonomic identification and allowed to determine their phylogenetic position and their taxonomic affiliation to *R. baltica* SH1^T supporting the creation of the novel taxa without the need of DDH evaluation.

The analysis of the planctomycetes communities associated with six different macroalgae (*Chondrus crispus*, *Fucus spiralis*, *Mastocarpus stellatus*, *Porphyra dioica*, *Sargassum muticum* and *Ulva* sp.) through Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA gene clone libraries showed that these communities are host-specific and do not present significant geographical and temporal variations. Furthermore, results evidenced that specific species of planctomycetes are associated with each macroalgae.

The 16S rRNA gene clone libraries constructed for the planctomycetes communities of *C. crispus*, *F. spiralis* and *Ulva* sp., revealed the presence of 51 unique Operational Taxonomic Units (OTU) at a 97 % cut-off value of which 31 % were found to be exclusively associated with macroalgae. The diversity obtained through molecular methods was much higher than the one obtained from culturing methods. The obtained clones were related to the *Pirellula-Rhodopirellula-Blastopirellula* genera (74.1 %) and to the uncultured class OM190 (16.6 %). The genus *Planctomyces* represented only 5.7 % of the total OTUs. The highest richness of planctomycetes was found in the macroalgae *C. crispus*, which also had the highest number of unique OTUs (14). *F.*

spiralis had the lowest richness. The community of planctomycetes harboured by *C. crispus* is more closely related to the one existing in *Ulva* sp. as shown by DGGE fingerprinting and taxonomic composition.

The results presented in this thesis showed that the application of molecular techniques is fundamental for an in deep characterization of microbial communities. DGGE fingerprinting and 16S rRNA gene clone libraries provided new insights into the diversity and ecology of planctomycetes associated with macroalgae and gave further support to a host-specific association of bacteria with macroalgae. Macroalgae revealed to be a promising source of novel planctomycetes that remains to be isolated in pure culture.

Resumo

As macroalgas são amplamente colonizadas por uma grande variedade de macro e microorganismos, tais como invertebrados, diatomáceas, fungos e bactérias. Destas, a associação mais estudada é a existente entre bactérias e macroalgas, a qual foi descrita há mais de um século. Os estudos efectuados até à data têm-se focado essencialmente em aspectos reprodutivos e nutricionais e nas propriedades antimicrobianas de polímeros produzidos pelas macroalgas para sua proteção contra *biofouling* por microrganismos. Contudo, estudos realizados para determinar a comunidade microbiana na superfície das macroalgas e sua dinâmica são ainda escassos. Nos últimos 5 anos, tem-se assistido a um aumento considerável de estudos moleculares que abordam a diversidade de bactérias associadas às macroalgas. Estes estudos mostraram que os planctomycetes, um filo muito peculiar do reino *Bacteria*, estão amplamente distribuídos em macroalgas. Para além disso, estas bactérias são o grupo dominante na alga *Laminaria hyperborea*, o que sugere um importante papel ecológico nestes habitats. O presente estudo, focado na associação planctomycetes-macroalgas, tem como objectivo alargar o nosso conhecimento neste ecossistema particular através da caracterização de novos planctomycetes previamente isolados da comunidade presente nos biofilmes de macroalgae e, também, da sua distribuição geográfica e temporal em várias macroalgas.

Um estudo anterior, baseado em métodos dependentes de cultivo, revelou uma grande diversidade de novos planctomycetes associados com diferente macroalgas da costa norte de Portugal. Com base no gene 16S rRNA, 39 estirpes, que representam cinco novos grupos de planctomycetes, foram seleccionadas para caracterização. As estirpes são filogeneticamente relacionadas com o género *Rhodopirellula*, que contém apenas uma espécie descrita, *R. baltica* SH1^T. Os novos grupos foram caracterizados taxonomicamente através de uma abordagem polifásica, *i.e.*, combinando diversas características morfológicas, metabólicas, quimiotaxonómicas e genéticas que permitiram a sua separação da espécie *R. baltica* SH1^T. *Rhodopirellula lusitana*, uma nova espécie de *Rhodopirellula* com 97.6 % de semelhança no 16S rRNA com *R. baltica* SH1^T, foi um dos grupos mais abundantes isolados a partir de todas as macroalgas amostradas e aparenta ser exclusivo deste habitat. *Rhodopirellula rubra* apresenta uma semelhança de cerca de 98 % no gene do 16S rRNA com *R. baltica* SH1^T, e foi observada associada a macroalgas amostradas na Suécia e também a esponjas marinhas. *Rhodopirellula formosa* foi isolada da superfície de *Fucus spiralis* e

Ulva sp., e tem uma semelhança de 96.6 % no gene do 16S rRNA com *R. baltica* SH1^T. Os dois géneros novos, *Roseimaritima* e *Rubripirellula*, possuem uma semelhança inferior a 94 % com o gene do 16S rRNA *de R. baltica* SH1^T e também parecem ser específicos de macroalgas. A caracterização polifásica permitiu a distinção dos grupos entre si e de *R. baltica* SH1^T, apoiando a criação dos novos géneros e espécies. A caracterização fisiológica destes novos planctomycetes mostrou que estes são capazes de utilizar uma grande variedade de carbohidratos, muitos dos quais podem ser encontrados nos polissacarídeos sulfatados produzidos pelas macroalgas. Estes resultados sugerem uma especialização dos planctomycetes como colonizadores de macroalgas.

De modo a determinar com mais detalhe as relações entre os isolados pertencentes aos novos taxa em estudo, aplicou-se um marcador molecular diferente como complemento do gene do 16S rRNA e como possível substituto da hibridização DNA-DNA. A realização de hibridização DNA-DNA é necessária para a descrição de espécies quando a semelhança do gene 16S rRNA entre duas estirpes é maior que 97 %, como é o caso de *R. rubra* e *R. lusitana*. O gene que codifica a subunidade beta da RNA polimerase (*rpoB*), um gene conservado e presente nas *Bacteria*, foi analisado e aplicado à ordem *Planctomycetales*. Com na sequenciação parcial deste gene, determinou-se que uma espécie nova de *Planctomycetes* é delimitada pelo valor de 96.3 % de semelhança na sequência do gene do *rpoB* e as relações intra-específicas são definidas por valores superiores a 98.2 %. Estes valores limites foram aplicados às estirpes a serem identificadas taxonomicamente, permitindo determinar a sua posição filogenética e afiliação taxonómica em relação a *R. baltica* SH1^T e validando a criação dos novos taxa sem a necessidade de realização de hibridização DNA-DNA.

A análise das comunidades de *Planctomycetes* associadas com seis macroalgas diferentes (*Chondrus crispus*, *Fucus spiralis*, *Mastocarpus stellatus*, *Porphyra dioica*, *Sargassum muticum* e *Ulva* sp.) através de DGGE (Electroforese em Gel de Gradiente Desnaturante) e bibliotecas genómicas do gene do 16S rRNA mostrou que estas comunidades apresentam especificidade no hospedeiro e não variam significativamente com o tempo ou o espaço geográfico. Para além disso, os resultados evidenciaram que existem determinadas espécies de planctomycetes associadas com cada macroalga.

As bibliotecas genómicas construídas a partir das comunidades de planctomycetes associadas com *C. crispus*, *F. spiralis* e *Ulva* sp. mostraram a existência de 51 unidades taxonómicas operacionais (OTU) definidas com base no valor de 97 %. Destas, 31 % são encontrados exclusivamente associados a

macroalgas. A diversidade obtida através deste método molecular foi bastante superior à obtida através de métodos de cultura. Os clones obtidos estão relacionados com os géneros *Pirellula, Rhodopirellula* e *Blastopirellula* (74.1 %) e com o grupo OM190 (16.6 %) que não possui nenhum organismo cultivado. O género *Planctomyces* representa apenas 5.7 % dos clones obtidos. *C. crispus* foi a macroalga com maior riqueza de planctomycetes e com o maior número de OTUs únicos (14). *F. spiralis* apresentou a menor riqueza em OTUs. A comunidade de planctomycetes presente em *C. crispus* é mais relacionada com a comunidade existente em *Ulva* sp., como demostrado pelos perfis de DGGE e composição taxonómica.

Os resultados apresentados nesta tese demonstraram que a aplicação de técnicas moleculares é fundamental para uma caracterização mais aprofundada de comunidades microbianas. Os perfis de DGGE e as bibliotecas genómicas do gene do 16S rRNA revelaram novos conhecimentos acerca da diversidade e ecologia de planctomycetes associados com macroalgas e suportam a existência de uma associação específica de bactérias com macroalgas. As macroalgas mostraram ser uma fonte promissora para isolamento de novos planctomycetes que, até à data, não existem em culturas puras.

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List of publications

This thesis is based on the following original articles

Chapter 3 - Joana Bondoso, Luciana Albuquerque, M. Fernanda Nobre, Alexandre Lobo-da-Cunha, Milton S. da Costa and Olga Maria Lage. *Roseimaritima ulvae* gen. nov., sp. nov. and *Rubripirellula obstinata* gen. nov., sp. nov. two novel planctomycetes isolated from the epiphytic community of macroalgae. Accepted for publication in Systematic and Applied Microbiology

Chapter 4 - Joana Bondoso, Luciana Albuquerque, Alexandre Lobo-da-Cunha, Milton S. da Costa, Jens Harder and Olga Maria Lage. *Rhodopirellula lusitana* sp. nov. and *Rhodopirellula rubra* sp. nov., isolated from the surface of macroalgae. Accepted for publication in Systematic and Applied Microbiology

Chapter 5 – Joana Bondoso, Luciana Albuquerque, Alexandre Lobo-da-Cunha, Milton S. da Costa, Jens Harder and Olga Maria Lage. *Rhodopirellula formosa* sp. nov.. a novel *Rhodopirellula* associated with macroalgae. Manuscript in preparation

Chapter 6 - Joana Bondoso, Jens Harder and Olga Maria Lage. (2013). *rpoB* gene as a novel molecular marker to infer phylogeny in *Planctomycetales*. Antonie van Leeuwenhoek 104: 477-488.

Chapter 7 - Joana Bondoso, Vanessa Balagué, Josep M. Gasol and Olga Maria Lage. Community composition of the *Planctomycetes* associated with different macroalgae. Submitted for publication to FEMS Microbial Ecology

Chapter 8 - Joana Bondoso, Filipa Godoy-Vitorino, Vanessa Balagué, Josep M. Gasol, Jens Harder and Olga Maria Lage. Epiphytic *Planctomycetes* associated with three main lineages of macroalgae. Manuscript in preparation.

Chapter 1.

Literature Review

1.1 The Phylum *Planctomycetes*

The *Planctomycetes* is an unusual and intriguing phylum of the Domain *Bacteria* that possesses a unique combination of physiological, morphological and genetic features that sets them apart from the common bacteria. They are a widespread group usually found in aquatic and terrestrial habitats, as well in association with a number of diverse eukaryotic organisms. In the last decade, special importance has been given to this group in the field of evolutionary biology because of the unusual presence of characteristics usually found in eukaryotic cells only (Devos and Reynaud, 2010; Fuerst and Sagulenko, 2012). This subject has been widely debated and the acceptance of planctomycetes as a transition from the prokaryote to the eukaryote form has not been achieved so far (Forterre and Gribaldo, 2010; Forterre, 2011; McInerney et al., 2011; Vesteg and Krajcovic, 2011).

Phylogenetically, the phylum *Planctomycetes* is placed in the large monophyletic PVC super phylum, together with the *Chlamydiae*, *Verrucomicrobia, Lentisphaerae,* and the Candidate *Poribacteria*, Candidate phylum OP3 and Candidate division WWE (Wagner and Horn, 2006)

In the last decade there has been a great increase in *Planctomycetes* studies (Fig. 1.1) mainly focused on their phylogenetic position, evolution, nutritional role in the ecosystem and unusual cellular organization which indicate the increasing relevance of this group in actual science. Albeit their recognized importance in several fields of research, only a reduced number of species are isolated in pure culture, which limits the knowledge on this interesting group. Furthermore, molecular ecology studies have reported the existence of an unknown diversity of *Planctomycetes* that can reveal new physiological, morphological and genetic aspects of these organisms.

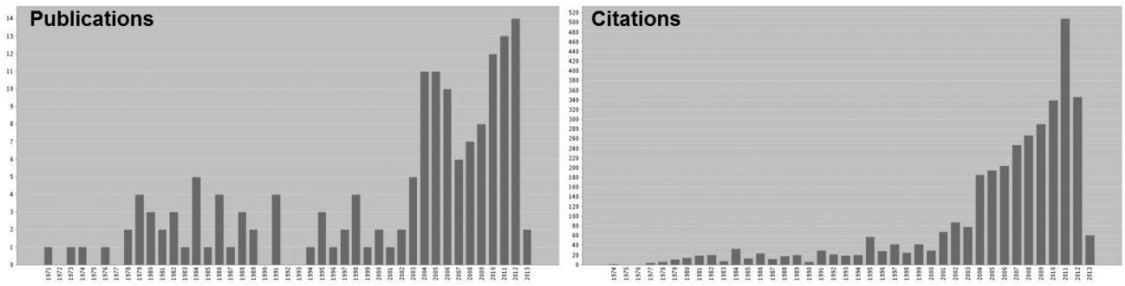

Fig. 1. 1 Publications and citations records on planctomycetes. Results were extracted from the ISI Web of Knowledge by searching keywords related to planctomycetes.

1.1.1 Taxonomy and Phylogeny

The history of *Planctomycetes* started back in 1924, when they were observed, for the first time, by Gimesi in the Lake Lágymányos, Budapest, Hungary (Gimesi, 1924). Originally, Gimesi described the observed organism as a fungus because of its morphological resemblances to this group (Fig. 1.2a) and named it as a novel *Fungi* genus, *Planctomyces* (Planc.to.my'ces. Gr. adj. planktos wandering, floating; Gr. masc. n. mukês fungus; N.L. masc. n. *Planctomyces* floating fungus) and *Planctomyces bekefii* was designated the type species. Albeit the wide distribution of this organism in several aquatic habitats (Hirsch, 1974) it was never isolated in pure culture, which hampered its correct identification. Thus, it was considered a fungus for almost fifty years. Only in 1972 Hirsch assigned *Pl. bekefii* to the *Bacteria* (Hirsch, 1972) after realized its similarities to *Blastocaulis sphaerica*, a freshwater budding bacterium described in 1935 by Henrici and Johnson (1935). Several other budding bacteria resembling *Pl. bekefii* were observed and assigned as novel species to the genus *Planctomyces* but they were also never isolated. Examples are *Pl. condensatus, Pl. stranskae, Pl. subulatus, Pl. ferrimorula, Pl. crassus* (Fig. 1.2b) and *Pl. guttaeformis* (Wawrik, 1952; Skuja, 1964; Hortobágyi, 1965). It was only in 1972 that the first planctomycete was isolated from a creek and a lake (Staley, 1973). However, it was incorrectly identified as *Pasteuria ramosa*, which is a parasite of *Daphnia* and it was only assigned to the genus *Planctomyces* ten years after (Starr et al., 1983). In 1987 its name was changed to *Pirellula staley* (Schlesner and Hirsch, 1987), its current taxonomic name (Fig. 1.2c). The first validly described planctomycete was a marine organism, *Planctomyces maris* (Fig. 1.2d), isolated in 1976 by Bauld and Staley (1976). With the introduction of the 16S rRNA sequencing technique, Schlesner and Stackebrandt (Schlesner and Stackebrandt, 1986) described the family *Planctomycetaceae* and the order *Planctomycetales,* but planctomycetes were only recognized as an independent phylum within the *Bacteria* in 2001 (Garrity and Holt, 2001).

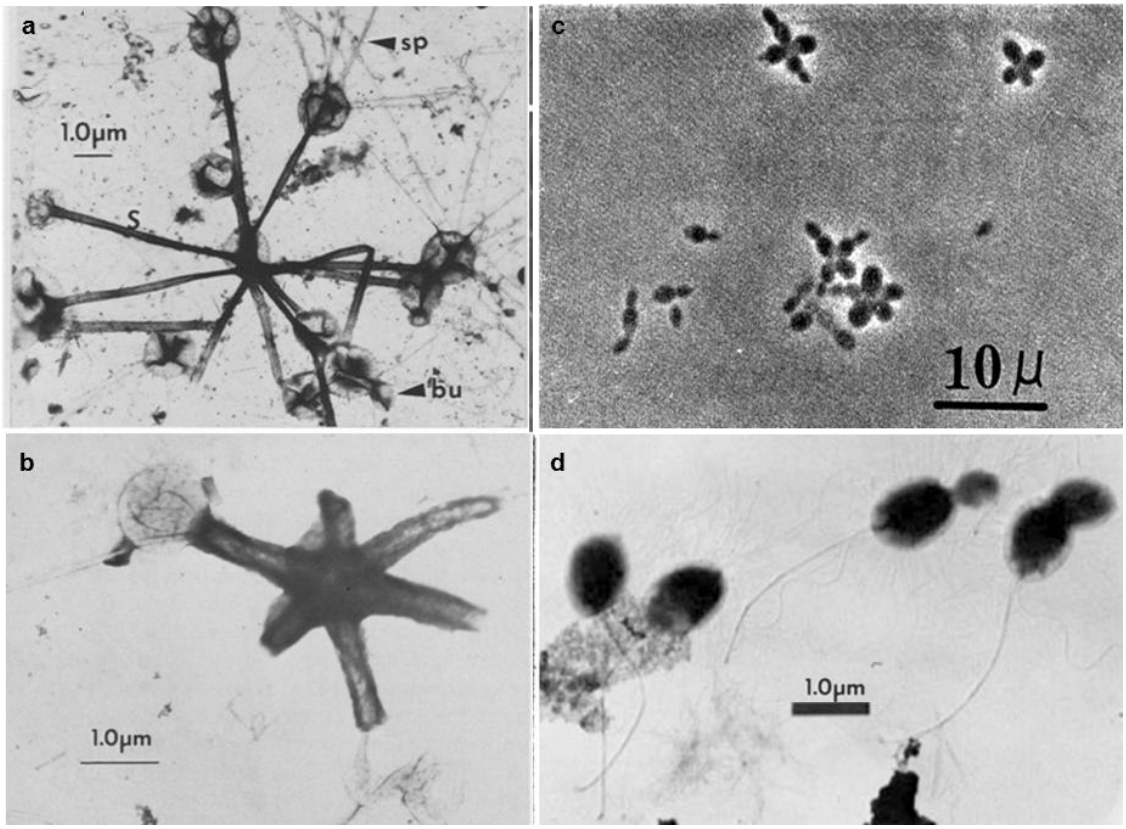

Fig. 1. 2 Microphotographs of several morphotypes of *Planctomycetes* observed in environmental samples. (a) Rosettes of *Pl. bekefii* observed in a pond in Kiwanis Park, Arizona (Schmidt et al., 1981); (b) *Pl. crassus* from the railroad-bridge pond, Budapest, Hungary (Schmidt et al., 1981); (c) Optical microscopy images of rosettes of *P. staleyi*, the first isolated planctomycete (Staley 1973); (d) Shadowed electron micrograph of cells of *Pl. maris*, the first described planctomycete (Ward et al., 2006).

Presently, the phylum *Planctomycetes* is formed by two classes: *Planctomycetacia*, with the order *Planctomycetales* that contains the majority of the taxa isolated and the order "*Candidatus* Brocadiales" which includes the anaerobic ammonium-oxidizing (anammox) bacteria; and the class *Phycisphaerae* with the order *Phycisphaerales* (Fig. 1.3). Of the twelve described genera, the majority includes only one species, in a total of fifteen validly described species. The majority of the species are aquatic and freshwater organisms. The order "*Candidatus* Brocadiales" has no cultured representatives because, so far, the efforts to isolate strains in pure culture have been unsuccessful and so these bacteria only exist in mixed cultures in bio-reactors.

In spite of the relative low number of described species in comparison with other groups of *Bacteria*, the isolation and description of novel planctomycetes has recently increased. In the last 4 years, one new class and order, five new genera, and six species have been described (Fig. 1.3, indicated by *). The isolation of novel planctomycetes has also increased in the last years through the use of selective isolation media with relatively low levels of nutrients and supplemented with antibiotics

(Winkelmann and Harder, 2009; Bondoso et al., 2011; Lage and Bondoso, 2011; Lage et al., 2012). Although this increase, the number of isolated strains does not yet reflect the diversity evidenced by culture independent studies. In August 2013, an analysis performed in the Ribosomal Database Project (RDP; Cole et al., 2009) showed the presence of 11045 clone sequences belonging to *Planctomycetes* of which only 291 (2 %) have been isolated in pure culture. The isolation of novel strains would contribute for a better understanding of the cellular biology of this group and to reveal its ecological role in the wide variety of habitats where they are found.

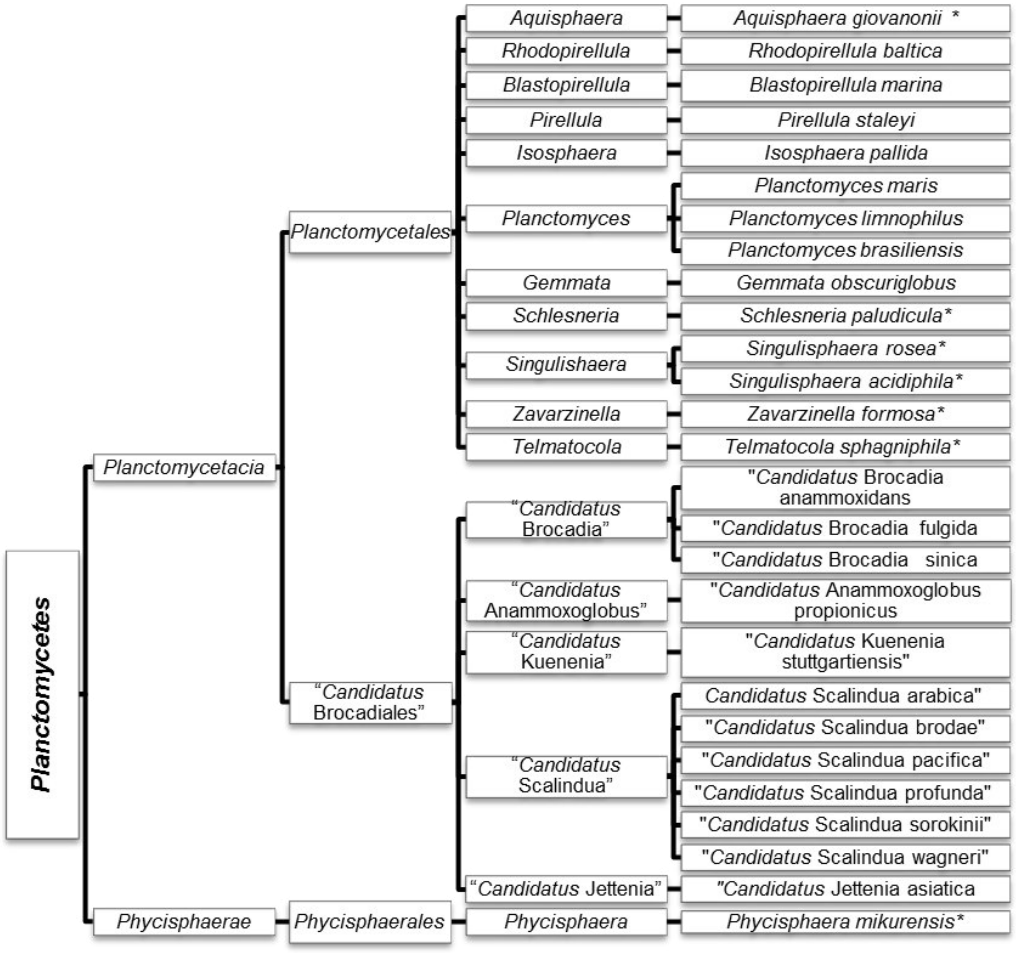

Fig. 1. 3 Diagram of the taxonomic outline of the phylum *Planctomycetes* showing the genera and species current and validly described.

The isolated and monophyletic position of the *Planctomycetes* is clear and it is confirmed by the 16S and 23S rRNA gene sequence analyses (Ward et al., 2000), the sequencing of the β-subunit of the ATPase (Rönner et al., 1985), the heat shock protein (HSP70) (Ward-Rainey et al., 1997), the elongation factor Tu (Jenkins and Fuerst, 2001), concatenated protein-coding gene sets (Strous et al., 2006) and whole-genome analyses (Jun et al., 2010). These studies suggest an early evolutionary origin

of *Planctomycetes* albeit their still controversial and uncertain phylogenetic history. Based on the 16S rRNA gene, they were recently grouped in the PVC superphylum together with phyla *Verrucomicrobia*, *Chlamydiae*, *Lentisphaerae* and the Candidate phyla *Poribacteria*, *OP3* and Candidate division WWE (Wagner and Horn, 2006). Although bearing huge differences in terms of their lifestyles, which range from free-living organisms to obligate pathogens, their relationships are supported by phylogenetic analyses with the 23S rRNA gene (Pilhofer et al., 2008; Glockner et al., 2010), concatenated sets of protein-coding genes (Kamneva et al., 2010; Gupta et al., 2012) and by whole genome analysis (Kamneva et al., 2012). However some studies based on 16S and 23S rRNA genes (Ward et al., 2000) and concatenated dataset of proteins (Ciccarelli et al., 2006; Griffiths and Gupta, 2007) do not confirm the PVC phylogenetic relations, probably because of a restricted number of sequences analysed. In Figure 1.4, it is possible to visualize the grouping of the phyla in the PVC super-phylum and its separation from the remnant bacteria.

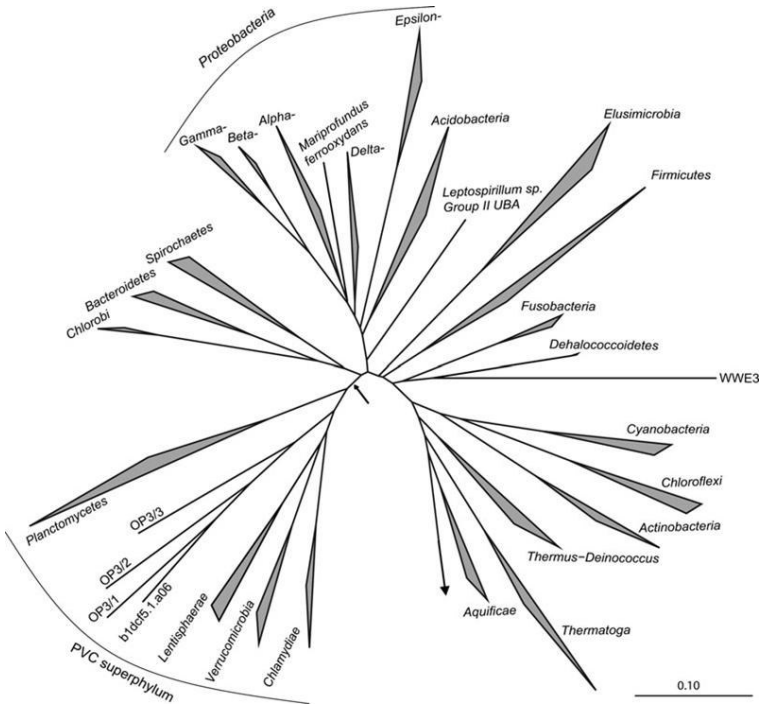

Fig. 1.4 23S rRNA gene phylogenetic tree showing the phylogenetic position of members of the Planctomycetes–Verrucomicrobia–Chlamydiae (PVC) super phylum. Adapted from Glockner et al. (2010).

Despite their differences, members of PVC super-phylum share some eukaryotic-like features such as compartmentalization of the cell, absence of peptidoglycan and an FtsZ-less cell division (Devos and Reynaud, 2010; Reynaud and Devos, 2011) which places them as candidates in the prokaryote/eukaryote transition. Since the discovery of a complex cell organization, resembling that of eukaryotes, that the

Planctomycetes have been involved in the search of the last universal common ancestor (LUCA) in order to understand the origin and evolution of *Eukarya*, one of the main gaps in Life history. However, the *Planctomycetes* position in the tree of Life and its evolutionary relationship has always been debated and remains a controversial subject. While some authors consider that planctomycetes and other PVC bacteria are intermediates in the transition from prokaryote to eukaryote (Devos and Reynaud, 2010) others claim that the eukaryotic-like features found in planctomycetes and in other PVC members are analogous to the ones found in eukaryotes, rejecting any homology and evolutionary relationships between them (McInerney et al., 2011). Phylogenetic reconstructions have not been helpful solving this issue. On Brochier and Philippe's reconstruction of the *Bacteria* phylogeny based on conserved positions in ribosomal RNA identified by a "slow-fast" method, *Planctomycetes* emerged at the base of *Bacteria* (Brochier and Philippe, 2002). Latter, di Gulio (Di Giulio, 2003), with the same sequence data set used by Brochier and Philippe but considering different conserved positions, showed that the phylum *Planctomycetes* was not the first line of divergence. Another phylogenetic analysis, with a set of 20 concatenated protein confirmed this result (Barion et al., 2007). A whole genome based comparison with four planctomycetes genomes was done to evaluate their phylogenetic position (Fuchsman and Rocap, 2006) and showed that *Planctomycetes* do not share an unusually large number of genes with the *Archaea* or *Eukarya* as it was thought, comparatively to other bacteria. More recently, two studies based on proteome analysis using two different approaches, have reached two different opposed conclusions. One, conducted by Nasir and collaborators (Nasir et al., 2011) based on annotation of protein domains concluded that the PVC superphylum appeared not different from other bacteria. The other compared feature frequency profiles of whole proteomes and showed that *Planctomycetes* are placed at the basal position of the *Bacteria* domain (Jun et al., 2010).

Whether *Planctomycetes* are the ancestors of bacteria or not, and it is evident that results varied according to the method used, one must agree that this phylum should be studied more deeply because of its particularities, in order to resolve its position in the Tree of Life.

1.1.2 Morphology and cellular biology

Planctomycetes combine a number of morpho-physiological features that are unusual in the bacterial domain.

One of the most striking features of *Planctomycetes* is the presence of an internal membrane system comparable to the membrane configuration in the eukaryotes that contributes to their compartmentalized cell plan (Fuerst, 2005) and is also present in members of the phylum *Verrucomicrobia* (Lee et al., 2009). This feature contributed to the alternative theory that planctomycetes are key organisms in the eukaryogenesis (Forterre and Gribaldo, 2010; Fuerst and Sagulenko, 2011, 2012). Although some bacteria display internal membrane bound structures where specific metabolic processes take place (Kerfeld et al., 2010), bacteria are usually seen as non-compartmentalised organisms, without organization in organelles and with the DNA dispersed in the cytoplasm. This organization separates them from the eukaryotes whose cells contains a nucleus (consisting of an envelope and structured chromosomes), endoplasmic reticulum, peroxisomes, mitochondria and plastids in phototrophic organisms. According to Lindsay et al., (2001), planctomycetes possess an internal organization due to an intracytoplasmic membrane (ICM) that divides the cell in two compartments, the paryphoplasm, and the riboplasm or pirellulosome. The cell plan organization varies among different taxonomic groups (Fig. 1.5). The paryphoplasm, a ribosome-free space, can vary in size and invagination. It is surrounded on one side by the cytoplasmic membrane that is closely apposed to the cell wall. It surrounds the pirellulosome, an organelle that contains the highly condensed DNA and is rich in ribosome like particles. This is the most basic organization. Furthermore, the anammox bacteria and the genus *Gemmata* have a more complex organization that includes, respectively, the presence of an anammoxosome (where the anammox process takes place) or a nucleoid surrounded by a double membrane containing the condensed DNA, which resembles the nucleus of a eukaryotic cell.

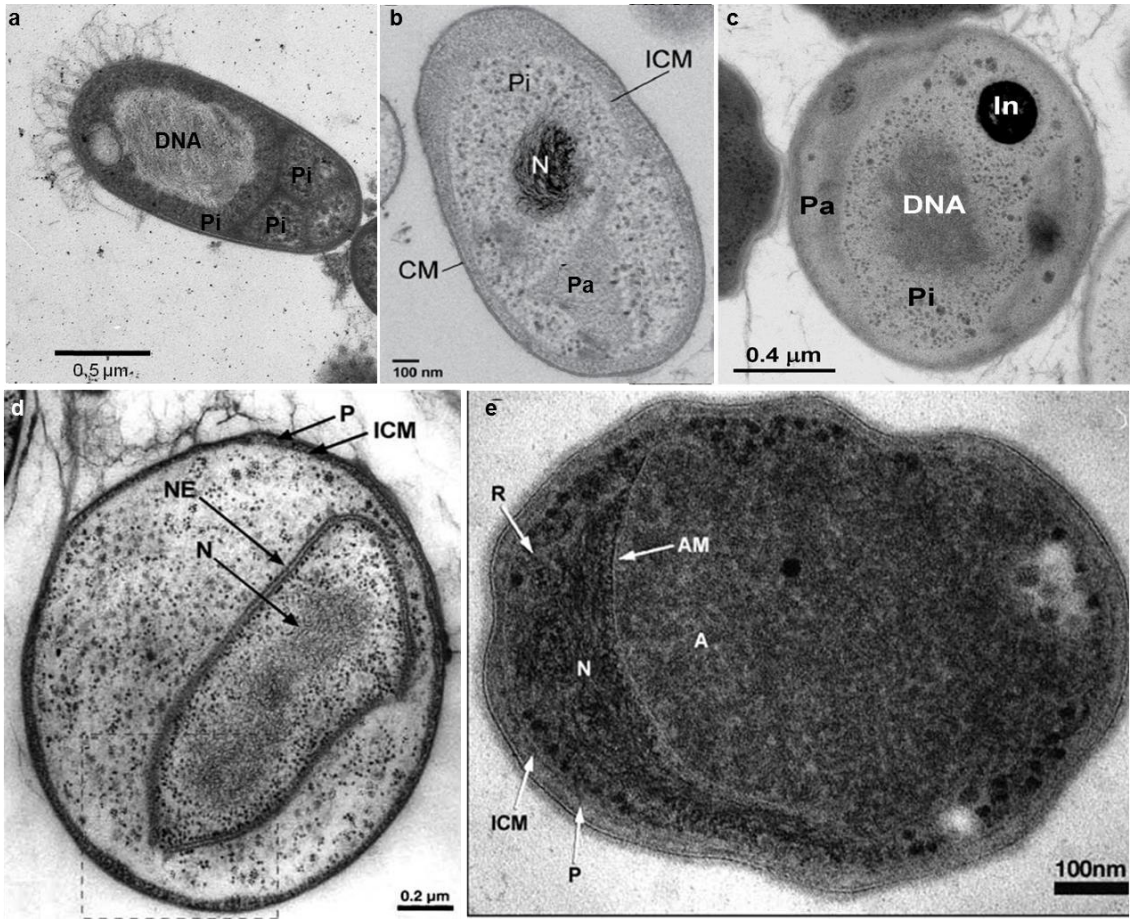

Fig. 1. 5 Cell plan organization of planctomycetes. Thin sections obtained by transmission electron microscopy from a) *Rhodopirellula* sp. Cor3, courtesy of Olga Lage; b) *Planctomyces limnophilus* (Jogler et al., 2011); c) *Aquisphaera giovanonni*, (Bondoso et al., 2011); d) *Gemmata obscuriglobus*, (Fuerst, 2005); e) "*Candidatus* Brocadia anammoxidans" (Fuerst, 2006),. Pi/R, Pirellulosome or riboplasm, Pa, paryphoplasm, CM, cytoplasmic membrane, ICM, intra cytoplasmic membrane, N, nucleoid, NE, nuclear envelope.

The presence of a complex cellular organization in planctomycetes has been widely accepted for more than ten years, but recent studies in the "nucleated" *Gemmata obscuriglobus* suggest that the cell plan in *Planctomycetes* might be similar to the one of a Gram negative bacterium. Using electron tomography, Santarella-Mellwig et al. (2013) showed that *Gemmata obscuriglobus* cells are not compartmentalized and do not possess a nucleoid. Instead, they present a complex endomembrane system with invaginations of the cell membrane that are interconnected and do not form any close compartment. Thus, planctomycetes probably possess an outer membrane and an inner membrane (cytoplasmic membrane), the so-called intracytoplasmic membrane, which can be more or less invaginated. The space between those two membranes is the bacterial periplasm and not the paryphoplasm. The presence of several marker genes associated with the outer membrane in several planctomycetes genomes corroborates this hypothesis (Speth et al. 2012).

Recently, it was demonstrated via green fluorescent protein (GFP) that endocytosis is present in planctomycetes, particularly in *Gemmata obscuriglobus* (Lonhienne et al., 2010). Endocytosis is a universal eukaryotic process not identified in *Bacteria* or *Archaea* by which cells incorporate molecules such as proteins through a membrane-trafficking system and recycle them back to the surface or sort them to lysosomes for degradation. This feature is consistent with the presence of membrane coat (MC) – like proteins in planctomycetes and other compartmentalized members of the PVC phylum. These proteins are homologous to the MC proteins of eukaryotes that are known to induce coated vesicle formation associated with endocytosis (Santarella-Mellwig et al., 2010). Both endocytosis and the presence of MC-like proteins have evolutionary implications and suggest that *Planctomycetes* and other PVC members are indeed involved in eukaryogenesis.

Planctomycetes do not possess the universal peptidoglycan layer in the cell wall, a feature also shared by *Chlamydiae*, archaea and eukaryotes. *Planctomycetes* possess a proteinaceous cell wall composed by amino-acids that vary among species. The cell wall can be rich in glutamate (König et al., 1984) or in proline and cystine/cysteine (Liesack et al., 1986). These particular characteristics of the cell wall allow strains to be resistant to ampicillin and other β-lactamic antibiotics that target peptidoglycan synthesis like penicillin-G, D-cycloserine, cephalotin and vancomycin (Schmidt, 1978; König et al., 1984), which have been successfully used in specific isolation experiments of planctomycetes (Schlesner, 1994; Winkelmann and Harder, 2009; Bondoso et al., 2011; Lage and Bondoso, 2011).

The majority of planctomycetes divide by budding, a way of reproduction rare in bacteria. These normally divide by binary fission with the intervention of GTPase FtsZ protein. *Planctomycetes*, as well as the sister phylum *Chlamydiae*, lack the tubulin like protein FtsZ (Pilhofer et al., 2008) which is also absent in eukaryotes and in the archaeal group *Crenachaeota* (Vaughan et al., 2004). The FtsZ protein is also absent in planctomycetes that do not divide by budding as, for example, *Phycisphaera mikurensis* that reproduces by binary fission (Fukunaga et al., 2009) and anammox bacteria, which display 'constrictive' binary fission (van Niftrik et al., 2009). The mechanism underneath the reproduction in planctomycetes and chlamydia has not been so far discovered.

Another unusual trait characteristic of all planctomycetes is the presence of crateriform structures on the cell surface, whose function is still unknown (Fig. 1.6 a and b). These structures appear as electron-dense circular depressions after negative staining when viewed in transmission electron microscopy (Fig. 1.6a). They can be

uniformly distributed on the entire cell surface or only located on the reproductive pole. Most probably associated with the crateriform pits is the presence of fimbriae that emerge to the outside of the cell.

Presence of non-prosthecate stalks or appendages can be present in planctomycetes although this is not a universal feature among all the members. In some strains, the stalks lead to the formation of spherical rosettes (Fig. 1.6 c and e). In the *Pirellula-Blastopirellula-Rhodopirellula* (PRB) group, the cells secrete a fibrillar material, the holdfast, that groups the cells in the rosette (Fig. 1.6 c and d) and, most possibly, also facilitates the attachment to surfaces in the natural habitat. The holdfast is of glycoproteic nature (Lage, 2013).

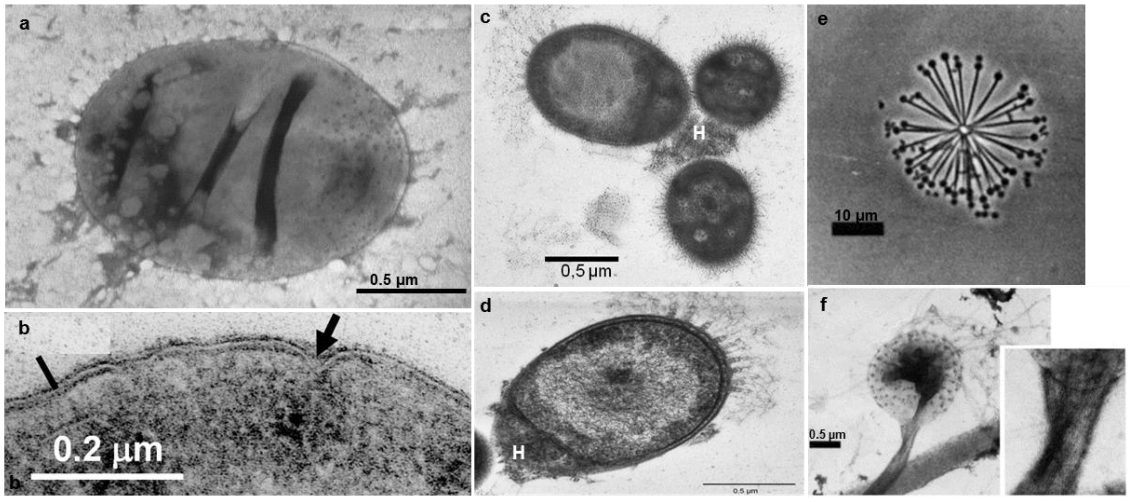

Fig. 1. 6 Non-prosthecate appendages present in planctomycetes. a) – scanning electron photograph of a *Pirellula* spp. cell evidencing the crateriform pits (courtesy of Olga Lage); b) Ultrathin section of the cell wall of *Aquisphaera giovannonii* evidencing the crateriform pits (arrow) (Bondoso et al., 2011); c and d) rosette and individual cell of *Pirellula* spp. cell evidencing the holdfast (H) (courtesy of Olga Lage). e) Rosette of *Planctomyces bekefii*, consisting of many spherical cells joined together at the distal tips of their stalks (Ward et al., 2006). f) Fibrillar stalk of *Pl. bekefii* (Ward et al., 2006)

Cellular shape and arrangement are very diverse in planctomycetes. Cells can be pear-shaped, ovoid or spherical. Cells are normally arranged in rosettes (Fig. 1.6 c and e), that is a characteristic feature of many planctomycetes and is common among members of the PRB group. Some species are unicellular but filaments also exist (Ward et al., 2006). Planctomycetes may or may not display motility. They can be motile by means of a flagellum or by gliding motility, as *Isosphaera pallida* (Giovannoni et al., 1987a).

1.1.3 Physiology and ecology of *Planctomycetes*

Planctomycetes present some metabolic diversity. With the exception of the order *Candidatus* Brocadiales that contains bacteria responsible for the ANaerobic AMMonium OXidation (anammox), all planctomycetes described so far are chemoheterotrophs, with carbohydrates serving as prime sources of carbon. The anammox are chemoautotrophic planctomycetes that form a deep branching group within the *Planctomycetes* and were only identified in 1999 (Strous et al., 1999). They play an important role in the nitrogen cycle, converting ammonium and nitrite to dinitrogen gas with nitric oxide and hydrazine as intermediates (Strous et al., 2002). The majority of planctomycetes existing in pure culture are mesophilic and neutrophilic but some extremophile planctomycetes were also isolated. *Isosphaera pallida* is the only thermophilic planctomycete, isolated from a hot spring (Giovannoni et al., 1987b). Several acidophilic strains isolated from acidic peatlands were described in the last six years (Kulichevskaya et al., 2007; Kulichevskaya et al., 2008; Kulichevskaya et al., 2009; Kulichevskaya et al., 2012b; Kulichevskaya et al., 2012a; Kulichevskaya et al., 2012c). A halotolerant planctomycete, *Pl. brasiliensis*, was isolated from a hypersaline water in a salt pit in Brazil (Schlesner, 1989). Most of the members of this group are aerobes or facultative anaerobes, while the anammox are anaerobic.

However, the ecophysiological diversity of cultured planctomycetes is not at all representative of the one revealed by molecular studies. This group has a widespread distribution as they can be found in all kinds of habitats including marine (Vergin et al., 1998; Kirkpatrick et al., 2006; Martín-Cuadrado et al., 2007; Shu and Jiao, 2008; Pizzetti et al., 2011b; Shu et al., 2011), brackish (Schlesner, 1994; Kan et al., 2006; Zeng et al., 2013a) and freshwaters (Pizzetti et al., 2011a; Pollet et al., 2011; Steven et al., 2011; Wu et al., 2012) and terrestrial ecosystems (Wang et al., 2002; Buckley et al., 2006; Tsai et al., 2009; Zhou et al., 2009; Faoro et al., 2010; Michel and Williams, 2011; Chen et al., 2012; Miyashita et al., 2013) which suggests that they can adapt and colonize a diverse range of ecological niches. Planctomycetes can be found in upwelling systems (Woebken et al., 2007; Allen et al., 2012), which are rich in nutrients and have a high abundance of phytoplankton. Other studies reported higher densities of planctomycetes after diatom (Morris et al., 2006; Tadonleke, 2007; Pizzetti et al., 2011b) or cyanobacterial blooms (Eiler and Bertilsson, 2004) suggesting a possible association of planctomycetes with phytoplankton. Planctomycetes are reported to prefer an attached lifestyle rather than occurring as free-living organisms. In the marine environment, DeLong and co-workers (DeLong et al., 1993) found that planctomycetes

were more abundant in clone libraries from marine aggregate (marine snow) attached bacteria than the ones from bacterioplankton. Allgaier and Grossart (2006) reported that planctomycetes were absent on the free-living bacteria clone libraries but were present in the clone libraries of particle-associated bacteria. Similar results were obtained in deep sea enviroment (Eloe et al., 2011) and in the Black Sea (Fuchsman et al., 2011). Thus, it is not surprising that they are usually found in sediments (Kim et al., 2004; Mu et al., 2005; Inagaki et al., 2006; Liang et al., 2007; Dang et al., 2009; Li et al., 2009a; Polymenakou et al., 2009; Ghosh et al., 2010; Divya et al., 2011; Du et al., 2011; Durbin and Teske, 2011; Liao et al., 2011; Diaz et al., 2013; Li and Wang, 2013; Qiu et al., 2013) and are part of several biofilm communities (Baumgartner et al., 2009b; Boomer et al., 2009; Pašić et al., 2010; Kriwy and Uthicke, 2011; Bartrons et al., 2012; Borsodi et al., 2012; Liu et al., 2012; Tang et al., 2012; Huang et al., 2013; Kostanjšek et al., 2013).

They can be found in several extreme environments such as desert soils (Abed et al., 2010; Andrew et al., 2012), hypersaline environments (Burns et al., 2004; Lefebvre et al., 2006; Baumgartner et al., 2009a; Schneider et al., 2013), hot springs (Kanokratana et al., 2004; Portillo et al., 2009; Tekere et al., 2011; Bohorquez et al., 2012), acidophilic habitats (Hao et al., 2007; Xie et al., 2011; Ivanova and Dedysh, 2012; Lucheta et al., 2013), glacial waters (Liu et al., 2006; Zeng et al., 2013b) and Antarctic soils and waters (Christner et al., 2003; Newsham et al., 2010; Piquet et al., 2010; Huang et al., 2013). They were found to be one of the dominant groups on hydrocarbon polluted environments (Abed et al., 2011) accounting for more than 20 % of the total bacterial community. Several other studies reports of the presence of planctomycetes on other polluted habitats (Miskin et al., 1999; Reed et al., 2002; Chouari et al., 2003; Caracciolo et al., 2005; Abed et al., 2007; Akob et al., 2007; Hao et al., 2009; Halter et al., 2011; Yu et al., 2011; Han et al., 2012), suggesting a possible role on the degradation of hydrocarbons and other pollutants.

Several molecular studies showed that planctomycetes are associated with eukaryotic hosts, like ants (Eilmus and Heil, 2009), invertebrates (Fuerst et al., 1997), sponges (Webster et al., 2001; Pimentel-Elardo et al., 2003; Mohamed et al., 2008; Zhu et al., 2008; Ouyang et al., 2010; Sun et al., 2010; Webster et al., 2011; Costa et al., 2013), ascidians (Oliveira et al., 2013) and corals (Yakimov et al., 2006; Webster and Bourne, 2007; Duque-Alarcón et al., 2012). They can be also in association with macrophytes (Hempel et al., 2008; He et al., 2012), lichens (Bjelland et al., 2011; Grube et al., 2012), sphagnum peat bogs (Kulichevskaia et al., 2006) and the rizosphere of several plants (Jackson et al., 2006; Jenkins et al., 2006; Jensen et al.,

2007; Zhao et al., 2010; Zhang et al., 2011; Zhang et al., 2013). They are also frequent in the microbiota of several organisms like the human gut (Cayrou et al., 2013), intestinal tract of the Black tiger shrimp (Fuerst et al., 1997; Chaiyapechara et al., 2012), fecal samples of wild gorilla (Frey et al., 2006), stomach of oysters (King et al., 2012), intestinal track of termites (Shinzato et al., 2005; Mackenzie et al., 2007; Köhler et al., 2008; Makonde et al., 2013), intestinal tract of the polychaete *Neanthes glandicincta* (Li et al., 2009b), foregut of the dromedary camel (Samsudin et al., 2011), gastrointestinal tract of carp (van Kessel et al., 2011). Recently, molecular studies revealed that planctomycetes are widespread in the biofilm community of several species of macroalgae and present a high diversity. They are the dominant group in the biofilm community of the kelp *Laminaria hyperborea* with values that can reach 51 % of the total bacterial community (Bengtsson and Ovreas, 2010). Planctomycetes are also frequent colonizers of *Ulvacean* algae including *Ulva compressa* (Hengst et al., 2010), *Ulva intestinalis* (Hengst et al., 2010; Lachnit et al., 2011), *Ulva australis* (Longford et al., 2007; Burke et al., 2011b) and *Ulva profilera* (Liu et al., 2010). Several isolates have also been obtained from *Ulva* sp. and *Ulva intestinalis* (Lage and Bondoso, 2011). This group was also reported to be present in the green macroalgae *Chara aspera* (Hempel et al., 2008) and *Caulerpa taxifolia* (Meusnier et al., 2001). Epiphytic planctomycetes were also found in the red algae *Porphyra umbilicalis* (Miranda et al., 2013), *Laurencia dendroidea* (de Oliveira et al., 2012), *Delisea pulchra* (Longford et al., 2007) and *Gracilaria vermiculophylla* (Lachnit et al., 2011). Isolates were retrived from *C. crispus*, *Mastocarpus stellatus*, *Gracilaria bursa-pastoris*, *Gelidium pulchellum*, *Grateloupia turuturu* and *Porphyra dioica* (Lage and Bondoso, 2011). A novel order of planctomycetes containing one species isolated from *Porphyra* sp. has been described (Fukunaga et al., 2009) 16S rRNA clone libraries from the brown algae *Fucus vesiculosus* releaved a great diversity of planctomycetes (Lachnit et al., 2011). Isolated planctomycetes were obtained from other brown algae like *Fucus spiralis*, *Sargassum muticum*, *Laminaria* sp. (Lage and Bondoso, 2011) and *Laminaria hyperborea* (Bengtsson and Ovreas, 2010).

1.2 Microbial systematics

Systematics is "the scientific study of the kinds and diversity of organisms and of any and all relationships among them" (Emerson, 1961) and the ultimate goal of this scientific field is the definition of a species. Taxonomy is generally taken as a synonym of systematics and it consists in the classification, naming (nomenclature) and identification of organisms (Cowan, 1968), which are interdependent areas. Classification is the organization of organisms into groups or taxa; nomenclature is the assignment of names to taxa and identification consists in determining the identity of an isolate and its consequent allocation to a taxon. The first classification system was introduced by Linnaeus, in 1735 and divided the natural world into the animal kingdom, the plant kingdom, and the mineral kingdom (Linnaeus, 1735). Bacterial classification started in the end of the 18th century with the work of Otto Müller which created two genera based on morphological differences, *Monas* and *Vibrio.* However, it was only in 1866 that bacteria were separated from plants and included in the Kingdom *Protista*, phylum *Moneres* (Haeckel, 1867). In 1872, Ferdinand Cohn recognized that bacteria were highly diverse and arranged them in six form genera creating the first system for classifying bacteria according to their morphology (Cohn, 1872). For several years, the classification systems were based solely on morphological characteristics, the so-called artificial classification. In the 70s, Carl Woese introduced the ribosomal RNA molecule analysis which revolutionized the microbial systematics field and in 1990 he proposed a three-domain classification system based on phylogeny and evolution, in which organisms were divided in the domains *Archaea*, *Bacteria*, and *Eucarya* (Woese, 1990). Prokaryotes are then classified into the domains *Archaea* or *Bacteria* and assigned to the lower hierarchical ranks 'phylum', 'class', 'order', 'family', 'genus' and 'species'. The most widely accepted classification system for prokaryotes is the *Bergey's Manual of Systematic Bacteriology*, which provides the basis for nomenclature, classification, and descriptions of bacteria. Nomenclature has been called the 'handmaid of taxonomy' (Sneath, 1989) and in prokaryote systematic it is regulated by a system called *International Code of Nomenclature of Bacteria*, which is also known as the *Bacteriological Revised Code* published in 1975 (Lapage et al., 1976). This system started in 1980 with the publication of the Approved Lists of Bacterial Names (Skerman et al., 1980) which contained all bacterial names to date and it marked a new starting point in bacterial nomenclature. The list contained approximately 2000 bacterial names and, since then, more than 13000 species were validly published (http://www.bacterio.net/number.html#AL). Bacterial species are

named according to the binomial nomenclature established by Carl Linnaeus (Linnaeus, 1735) and names are Latinised. Species names consist of two parts, the first name is the genus and the second is the specific epithet. For the correct identification of an organism this must be compared to the most closely related strains already classified using phenotypic and genotypic properties in order to place it into particular taxa. When it is not possible to achieve an accurate identification, the unknown organism should be characterized in order to describe the new taxon based on the existing nomenclature. The characterization and nomenclature of a novel bacterium should follow several criteria in order to be validly published (discussed below), which are regulated by the *ad hoc* committee of the *International Committee for the Systematics of Prokaryotes*, (Tindall et al., 2010).

1.2.1 Description and characterization of novel species

When a strain or a group of strains are shown not to belong to any other taxa already described in the literature, it must be extensively characterized in order to the new name to be validly published. The first problem that arises when describing a new organism is the definition of what is a species. This concept has been under debate for several years but, so far, there is no consensus on this definition. Several concepts have been suggested for microbial species but none is widely accepted. The most pragmatic and widely accepted definition states that a species can be defined as 'a category that circumscribes a (preferably) genomically coherent group of individual isolate strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions' (Rossello-Mora and Amann, 2001). For taxonomists, however, it is important to define how to delineate a species. In that way, the *ad hoc* committee of the *International Committee for the Systematics of Prokaryote* proposed that a species is a group of strains sharing 70 % or greater DNA-DNA reassociation values and 5 °C or less ΔTm (the difference in melting temperature between the heterologous and homologous DNA hybrids) and more than 97 % identity in 16S rRNA gene (Stackebrandt, 2002). Phenotypic and chemotaxonomic features should agree with this definition. For several years, prokaryotes were described and identified based only on their morphological characteristics, such as cellular shape and colony colour and morphology. Latter, due to the work of Robert Koch that allowed a larger number of bacteria to be isolated in pure culture, the physiological features were also included in the characterization of microorganisms and several tests were developed to rapidly identify them (Tindall et al., 2007). In the late 1950s, numerical taxonomy was introduced as an objective identification and classification system of

prokaryotes that was possible through the use of computers (Sneath and Sokal, 1973). The system established phenotypic relationships between the organisms based on similarity matrixes constructed based on a set of characters. At the same time, chemotaxonomy and analysis of lipids, cell wall amino acids and whole protein content were also introduced in bacterial systematics. In the 1960s, genome-based methods were developed to determine intraspecies relationships like DNA base composition (mol % G+C) and DNA-DNA hybridization (DDH). This technique allows determining the whole genome similarity of two strains based on the re-association of the single strands of DNA from each strain and has been used in the last 50 years as a criterion for species delineation based on a 70 % DNA-DNA binding value (Wayne et al., 1987). In the late 1970s, the work of Carl Woese and colleagues on the small subunit rRNA sequences (Woese and Fox, 1977) has revolutionized the microbial taxonomy. It was shown that the 16S rRNA gene could be used to infer phylogenies between microorganisms and could be used as a molecular chronometer due to its ubiquity and conservation among organisms (Woese, 1987). With the advances in DNA sequencing, that has made this technique a rapid and cost-effective method, the analysis of the 16S rRNA gene became a gold standard for species delineation in substitution of DDH, which is a much more laborious and expensive technique. It was shown that when two isolates have less than 97 % similarity in the 16S rRNA gene usually they share less than 70 % DDH and should be different species (Stackebrandt and Goebel, 1994). Nowadays, sequencing of the almost complete 16S rRNA gene is required for the classification and identification of a new species (Stackebrandt, 2002). Nevertheless, the 16S rRNA gene has been shown to lack enough discriminatory power to delineate bacterial species (Fox, 1992). DDH remains the best method for the correct species delineation but it is only necessary when the 16S rRNA gene sequence between two isolates is higher than 97 % (Tindall et al., 2010). A recent study where Stackebrandt and Ebers (2006) tried to correlate published DDH values with 16S rRNA gene showed that when two organisms share less than 98.5 % similarity in this gene, it is unlikely that they share more than 60 to 70 % DNA similarity. However this value has not been applied so far. Nevertheless, and because DDH is a very laborious and time-consuming technique, the current recommendations for the taxonomic characterization of prokaryotes allows DDH substitution by the sequencing and analysis of other genes with high resolution power (Tindall et al., 2010). Currently, a novel species of bacteria is described through a polyphasic approach that combines morphological, biochemical, chemotaxonomic and genetic characteristics (Fig. 1.7) in order to retrieve as much information as possible (Vandamme et al., 1996).

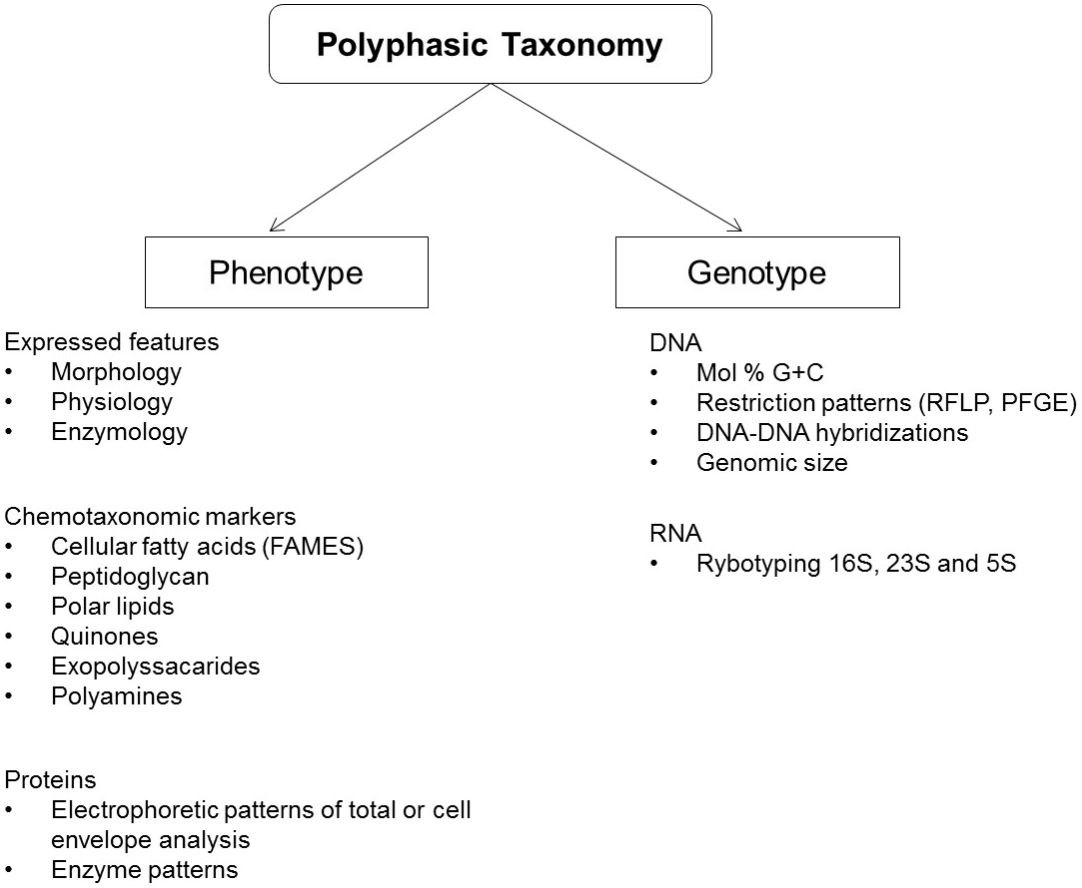

Fig. 1. 7 Schematic representation of the techniques used in polyphasic taxonomy. Adapted from Vandamme et al. 1996.

The term "polyphasic taxonomy" was introduced in 1970 by Colwell (1970) and it consists in the integration of different types of data that includes phenotypic and genotypic data as well as phylogenetic information that allow the classification of the biological entities. Genotypic data derives from nucleic acids (DNA and RNA) whereas the phenotypic data comes from proteins and their functions, different chemotaxonomic markers, and a wide range of other expressed features. In practice, a polyphasic taxonomic classification of a new isolate or group of isolates consists in: i) phylogenetic placement of the isolates and identification of the closest neighbours (classification) through 16S rRNA gene; ii) determination of the genomic relationship between the isolates and their closest neighbours through DDH or other fine typing method when the 16S rRNA gene similarities are higher than 97 %; iii) morphological, biochemical, chemotaxonomic and genetic characterization of the isolates.

The description of higher taxonomic ranks such as genus and families should also follow the same rules as defined above. However, when compared to species, higher

taxa are more difficult to delineate and there is not a clear taxonomic definition and rules to define them. In those cases, the phylogenetic position of the isolates is of main importance to determine their separation from the closest representatives. The stability of the phylogenetic position of the group in question is tested by comparing genotypic, chemotaxonomic, and phenotypic profile with that of its closest relatives. When the results obtained from this comparison support the phylogenetic group, a new genus should then be created (Gillis et al., 2005).

The process of species or genus description involves a long and time-consuming process but it is essential for Microbiology. For example, in medical microbiology it is crucial to identify novel pathogens; in food microbiology is important to identify microbial contaminants, while in microbial ecology it is fundamental to identify key microorganisms responsible for environmental processes. A polyphasic taxonomical approach allows obtaining an almost complete description of an organism and is currently the widely accepted among bacterial taxonomists for bacterial characterization.

1.3 Bacterial diversity and community structure

Bacteria represent by far the most abundant and diverse group of organisms on Earth, reaching up to 10^9 total different species (Dykhuizen, 1998) and playing key roles in biochemical and geochemical cycles in the ecosystems. Bacterial populations are influenced by many physical, chemical and biological factors and they respond to environmental alterations by changing their richness and evenness (Sigler et al., 2002). Consequently, the knowledge about the structure of the microbial communities, *i.e.*, the different kinds of organisms that are present and their abundances is important to understand the ecosystems functioning. The study of a bacterial community structure can be used to i) determine microbial distribution patterns; ii) predict interactions between co-occurring microorganisms; iii) examine seasonal and geographical variations of the bacterial community; and iv) link community structure to particular functions based on the presence of certain groups of bacteria (e.g., photosynthetic or nitrogen-fixing bacteria) (Fuhrman, 2009). The assessment of the diversity of microorganisms in the environment was, initially and exclusively based on 'culture dependent' methods. The application of the 16S rRNA gene to microbial ecology and the advances in DNA sequencing technology in the last decades, such as 454 pyrosequencing, allowed researchers to characterize the general biodiversity, genetic potential and abundant members of whole bacterial communities (Giovannoni et al.,

1990; Fierer and Jackson, 2006; Rusch et al., 2007; Costello et al., 2009; Caporaso et al., 2011) through the so called 'culture independent' methods.

1.3.1 Methods to assess bacterial community structure

The diversity of bacterial communities can be analysed through different methods that are sub-divided in two major categories: 'culture dependent' and 'culture independent' techniques (Dahllof, 2002).

Culture dependent methods were used exclusively for decades. However, it is known that less than 1 % of the total bacteria can be isolated through cultivation (Staley and Konopka, 1985). For instance, only 0.001 % to 0.1 % of seawater bacteria can be isolated in agar plates (Amann et al., 1995). Nevertheless, bacterial pure cultures are essential to understand the metabolic and physiological role of bacteria in the ecosystems. An excellent example was the isolation of *Pelagibacter ubique (Rappé et al., 2002)*, the first cultivated member of the ubiquitous SAR11 clade, and its subsequent genome sequencing that enabled to answer to ecological questions, such as the need of exogenous reduced sulphur compounds for a strain without the genes for assimilatory sulphate reduction (Joint, 2008).

Because a great diversity of bacteria remains to be isolated, culture independent methods have been used to characterize whole bacterial communities' structures. Such methods allow the identification of single bacterial species in sample material without the cultivation of the organisms (Muyzer et al., 1993; Amann et al., 2001). Most of the culture independent methods used are based in PCR amplification of target genes of which the 16S rRNA gene is the most commonly used. During the past decades, different methods based on the 16S rRNA gene were developed for studying microbial assemblages (Head et al., 1998). The analyses of this gene started to be applied in the 1980s by Pace et al. (1986) and since then hundreds of environments have been characterized by 16S rRNA gene analyses. For example, it was demonstrated that bacterioplankton diversity follows a latitudinal gradient and the richness is primarily determined by temperature (Fuhrman et al., 2008). Other study using 16S rRNA pyrosequencing showed that bacterial taxa in a marine coastal site follow a seasonal pattern (Gilbert et al., 2012). Furthermore, a great unknown diversity of bacteria in the most varied environments has been revealed (Giovannoni et al., 1990; Pace, 1997; Eckburg et al., 2005; Sogin et al., 2006).

The most common strategy to analyse bacterial communities is the construction of 16S rRNA gene clone libraries (Rappé and Giovannoni, 2003). This method consists in the extraction of the total DNA from an environmental sample and subsequent PCR

amplification of microbial sequences with 16S rDNA primers that are usually 'universal' to cover all bacterial lineages. When searching for a particular group of interest, specific primers are used. The resulting PCR amplicons are cloned in a host, normally *E. coli*, and sequenced. The cloned sequences are compared to previously published sequences (Cole et al., 2009) and their phylogenetic position is determined allowing to assess the diversity and richness of the sample. The limitations of this approach includes i) the more abundant sequences are preferentially amplified, and low-abundance sequences are usually underrepresented; ii) underestimation of the true diversity of the natural environment as the number of clones sequenced are usually low (under 1000) (Pedrós-Alió, 2006). Furthermore is a very laborious, time consuming and it can be an expensive technique especially when a large number of clones and samples have to be examined. In the last years, the construction of clone libraries has been replaced by high-throughput DNA sequencing such as '454' pyrosequencing technology that generates larger amounts of data at a reasonable cost and time consumption than the traditional Sanger sequencing (Margulies et al., 2005; Sogin et al., 2006). However, fragments generated with this methods varies from 100 to 400 bp which do not allow a full taxonomic assignment while 16S rRNA clone libraries generates almost full length sequences (~1500 bp) and therefore it still remains the 'gold standard' for the identification of novel bacterial lineages within a community and its full characterization (Tringe and Hugenholtz, 2008).

Several molecular studies of bacterial communities aim to determine population dynamics such as temporal and spatial variations or to assess community structure changes in response to various perturbations which involve the analyses of multiple samples. In this case, clone libraries construction is not a feasible approach due to the large number of samples resulting in a very time and labour consuming process. Fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE, (Muyzer et al., 1993)), temperature gradient gel electrophoresis (TGGE, (Muyzer and Smalla, 1998)), terminal restriction fragment length polymorphism (T-RFLP, (Avaniss-Aghajani et al., 1994)) and amplified ribosomal DNA restriction analysis (ARDRA, (Massol-Deya et al., 1995) can easily provide profiles of the bacterial community structure and are especially suitable for studies comparing a large number of samples. DGGE is one of the most used fingerprinting techniques and it consists in the separation of a mixture of PCR-amplified DNA fragments of equal length based on different nucleotide composition. The resulting PCR products are loaded in a polyacrylamide gel containing a denaturing gradient (urea and formamide) that will cause partial denaturation of the PCR duplexes. Fragments with different sequences

denature at different positions in the gel, based on their melting properties and the concentration of denaturants. A GC-clamp (a GC-rich sequence usually 30-50 nucleotides) is attached to the 5'-end of one of the primers to stop the complete dissociation of the double-stranded DNA (Muyzer and Smalla, 1998). The resulting profiles are analysed on the basis of its banding pattern (presence/absence of bands) allowing a rapid comparison of the different bacterial communities (Zoetendal et al., 2001). In addition, each band can be excised and sequenced allowing to determine the phylogenetic composition of the population (Ferris et al., 1996). DGGE has been successfully applied to study the spatial and temporal variations of bacterial communities (Murray et al., 1998; Riemann et al., 1999; van der Wielen et al., 2002; Kan et al., 2006), to detect differences between distinct communities (Murray et al., 1996; Casamayor et al., 2000; McCaig et al., 2001), to investigate communities diversity (Garbeva et al., 2001; Li et al., 2006; Li et al., 2012) and to determine communities changes in response to environmental disturbances (Ferris et al., 1997; Westergaard et al., 2001; Müller et al., 2002). However it also possesses some technical limitations such as restricted phylogenetic information that do not allow a full taxonomic assignment due to the small size of the PCR fragments which, due to technique characteristics, have ~500bp (Muyzer et al., 1998) and underestimation of the diversity of the population because, often, bands with different nucleotide composition can migrate to the same position (Ercolini, 2004). Furthermore, DGGE profiles only represent a small fraction of the whole bacterial community which can be overcome by applying specific primers to assess the diversity of particular groups of bacteria (Muhling et al., 2008). Other variations of DGGE fingerprinting based on other functional genes, like *rpoB* (Dahllof et al., 2000), have been developed to avoid 16S rRNA gene intraspecies heterogeneity that originate different bands belonging to the same organism. This allows to assess specific bacterial groups such as denitrifying bacteria (Throback et al., 2004; Kjellin et al., 2007), methanotrophs (Iwamoto et al., 2000) and ammonia-oxidizing bacteria (Nicolaisen and Ramsing, 2002). Even though, 16S rRNA gene-DGGE is still utilized because of the large sequence database of this gene.

A widely used method for visualization and quantification of different bacterial groups from environmental samples is fluorescence *in situ* hybridization (FISH). This method consists in the construction of 16S rRNA-targeted nucleic acid probes specific to the organisms of interest that are fluorescently labelled and will hybridize with the complementary regions of the ribosomal RNA within the cell (Amann et al., 1995). The main advantage of this method is that it allows *in situ* enumeration of the cells in an

environmental sample, without the need of PCR and therefore eliminating its associated biases. It has been successfully applied to various habitats including lakes and oceans (Glockner et al., 1999; Kloep et al., 2006; Morris et al., 2006; Vila-Costa et al., 2008), soil (Caracciolo et al., 2005), sediments (Ishii et al., 2004) and wastewaters treatment tanks (Egli et al., 2001; Egli et al., 2003). However FISH also presents its limitations. It was noticed that the numbers of cells that hybridize with the probes vary with the fluorochrome type and the hybridization stringency (Bouvier and Del Giorgio, 2003). Furthermore, less active cells, either because they are slow growing or starving, have low rRNA levels and thus present low FISH intensity signals. This problem has been surpassed by the use of catalysed reporter deposition (CARD) FISH in which the hybridization signal is enhanced through the use of tyramide-labelled fluorochromes and probes labelled with horseradish peroxidase (Pernthaler et al., 2002).

There is a wide range of methods (microbiological or molecular) to assess the bacterial structure and diversity. However, all the methods have their own limitation and they should be applied according to the samples under study and the questions to be answered. If possible, different methods should be combined to provide a deep perspective of the bacterial population under study.

1.3.2 Bacterial communities on macroalgae

Macroalgae are ecologically relevant and play an important role in the marine environment in primary production and determination of the physical structure of the habitat (Schiel and Foster, 2006). Macroalgae are be classified in three phyla: *Chlorophyta* (green algae), *Heterokontophyta*, *Phaeophyceae* (brown algae) and *Rhodophyta* (red algae). These organisms can be used as a food source , for industrial applications such as biofuel productions and as fertilizers (El Gamal, 2010). Furthermore, macroalgae are known producers of biactive secondary metabolites with anti-inflammatory, cytotoxic and immunosuppressive activity. Several antibacterial, antiviral, antiplasmodial and anti fungal compounds have been isolated from macroalgae (Bhakuni and Rawat, 2005). Macroalgae provide rich habitats for different epiphytic, endophytic and epizoobenthic organisms that can benefit from the ready availability of oxygen and a range of organic carbon sources produced by the algae (Armstrong et al., 2000; Goecke et al., 2010). Therefore, macroalgae can be widely colonized by a wide range of organisms including bacteria, microalgae, fungi,and protists (Wahl et al., 2012). Bacteria are the primary colonizers of macroalgae surfaces and they can reach densities of $10^2 – 10^7$ cells cm^{-2} depending on the macroalgae species (Largo et al., 1997; Dobretsov and Qian, 2002; Tujula et al., 2006; Bengtsson

et al., 2010; Wahl et al., 2010). The epiphytic bacteria can have positive effects on the macroalgal host, assuming protective and nutritional roles (Wahl, 2008; Goecke et al., 2010) . However, they can also have negative effects like induction of diseases and decomposition (Vairappan et al., 2001; Ivanova et al., 2002). Bacteria can also prevent the biofouling and invasion of pathogens and protect the macroalgae against toxic substances (Holmstrom et al., 1996; Wiese et al., 2009). They produce growth factors and are involved in nitrogen fixation which is important for the algae (Dimitrieva et al., 2006). Furthermore, bacteria are essential in inducing normal morphology of the macroalgae (Nakanishi et al., 1999; Matsuo et al., 2003; Marshall et al., 2006) They are a source of novel bioactive products with antibacterial and anticancer activity (Egan et al., 2008; Goecke et al., 2010; Villarreal-Gómez et al., 2010) as well as toxins and signalling compounds (Armstrong et al., 2001; Penesyan et al., 2009). Therefore, it is of major importance to better understand the extent of the association bacteria-macroalgae because it represents a promising and rich source of novel bioactive molecules.

The study of the bacterial communities associated with macroalgae has been done mainly by culture dependent methods and these studies revealed that macroalgae are also a source for the discovery of novel taxa (Goecke et al., 2013; Hollants et al., 2013). A survey of 16S rRNA gene sequences present in GenBank (Goecke et al., 2013) showed the existence of 82 new bacterial species isolated from macroalgae which belong to *Bacteroidetes* and *Proteobacteria* (the most abundant groups) and to *Firmicutes*, *Actinobacteria*, *Verrucomicrobia* and *Planctomycetes*. The isolation of bacteria from macroalgae remains important because their physiological characterization (e.g. polysaccharide degradation and antibacterial activity) will allow tounderstand their ecological roles on the macroalgae surfaces and the factor that determine their distribution on the different macroalgae.

Molecular analyses of the bacterial communities on macroalgae are still scarce when compared to other well studied bacterial associations like, for instance, sponges (Webster and Taylor, 2012) and corals (Rohwer et al., 2002). In fact, the epiphytic communities from macroalgae only started to be effectively studied by culture independent methods in the last 5 years (Egan et al., 2012). The main groups of bacteria found in molecular studies are *Proteobacteria* (55 %), *Bacteroidetes* (20 %), *Firmicutes* (10 %), *Actinobacteria* (9 %), *Planctomycetes* (4 %), *Cyanobacteria* (1 %), *Verrucomicrobia* (1 %), *Chloroflexi*, *Deinococcus-Thermus*, *Fusobacteria*, *Tenericutes* and the candidate division OP11,each with less than 1 % (Hollants et al., 2013). Although these groups were found associated with all the three lineages of macroalgae

it was noticed that green macroalgae possess more representatives of the *Bacteroidetes* and *Alphaproteobacteria* than red or brown algae while these two harbour more *Firmicutes*, *Actinobacteria* and *Planctomycetes* species (Hollants et al., 2013). These phylogenetic studies also provided insights in the distribution of bacteria among the macroalgae species. It was shown by DGGE and 16S rRNA clone libraries that the macroalgae *Laminaria saccharina*, *Laminaria hyperborean* and *Ulva australis* harbour specific bacterial communities that are clearly different from the surrounding seawater (Staufenberger et al., 2008; Lachnit et al., 2009; Bengtsson et al., 2010; Burke et al., 2011a) or other co-occurring organisms (Longford et al., 2007a). Furthermore, several studies suggest the existence of a host specific bacterial community associated with each macroalgae species. Different macroalgae from the same habitat exhibit distinct bacterial communities composition (Longford et al., 2007a; Lachnit et al., 2009; Hengst et al., 2010; Nylund et al., 2010; Barott et al., 2011; Trias et al., 2012; Vega Thurber et al., 2012) suggesting that each algal host provides a unique niche for microbial colonization. Furthermore, the same macroalgae species from different locations showed high similar bacterial communities (Staufenberger et al., 2008; Lachnit et al., 2009; Sneed and Pohnert, 2011; de Oliveira et al., 2012). Nevertheless, there is no clear evidence of core community of bacterial species unique to each macroalgal host most probably due to the restricted number of studies done so far. Recently, a metagenomic study from *Ulva australis* (Burke et al., 2011b) identified a set of core functions that was consistently present in different individuals even though there was no evident core community bacterial species among those different individuals (Burke et al., 2011a).This result probably indicates that the differences in the bacterial communities between different macroalgae species depend on the microbial functioning rather than on their phylogeny (Burke et al., 2011b). However, a transcriptomic study of the microbiome of *Laurencia dendroidea* (de Oliveira et al., 2012) reinforced the existence of a specific bacterial community as individuals from different sampling sites exhibited a high taxonomic similarity. The data, thus, suggests that host-specificity is only present in some macroalgae while others present variable communities.

Bacterial communities on macroalgae were also shown to be temporally variable, following the common patterns of bacterioplankton (Fuhrman et al., 2006). Lachnit et al. (2011) reported seasonal changes between summer and winter in the bacterial communities associated with *Fucus vesiculosus*, *Gracilaria vermicuphylla* and *Ulva intestinalis* and these changes were reproducible over consecutive years. However, none of the OTUs reported showed to be consistently found in summer and winter.

DGGE profiles of the green macroalga *Ulva australis* showed that the bacterial community is variable over seasons, but that there is a stable core community (Tujula et al., 2010).

In conclusion, the association of bacteria with macroalgae is complex and further studies are essential to understand the nature and the factors involved in these interactions. These studies should not only include taxonomic and phylogenetic identification of the bacterial populations but should also include functional studies such as metagenomics, transcriptomics, proteomics and metabolomics.

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Chapter 2.

Aims and Thesis Outline

The remarkable and unique characteristics of *Planctomycetes* motivated us, in the last years, to in depth our knowledge about this group. In an initial approach, the isolation of a great collection (more than 150 strains) of *Planctomycetes* was obtained especially from the epiphytic community of macroalgae of the North coast of Portugal (Lage and Bondoso, 2011). This environment proved, thus, to be an important habitat for *Planctomycetes* that needed further attention and was worth to be studied. Furthermore, in the last years several studies reported planctomycetes as part of the bacterial communities of macroalgae suggesting that they play an important ecological role on these surfaces.

The two major aims in this thesis were: a) to characterize and taxonomically describe new taxa of *Planctomycetes* that have been previously isolated from the surface of macroalgae and b) to study the planctomycetes communities associated with several macroalgae belonging to the three main lineages. The results presented in this thesis are organized in chapters 3 to 7. These are written in the form of manuscripts, of which some are already published, some are submitted for publication and others are still under preparation.

Thirty nine strains of planctomycetes were selected for characterization based on the 16S rRNA gene similarities to the closest cultured representative, *Rhodopirellula baltica*. A polyphasic approach, consisting in a wide range of techniques, was applied to describe the isolated taxa. Furthermore, the phylogeny and the taxonomic placement of these novel isolates were confirmed by sequencing and analysis of an alternative molecular marker, the β-subunit RNA polymerase encoding gene (*rpoB*). **Chapter 3** comprises the description of two novel genera of *Planctomycetes*, *Roseimaritima* and *Rubripirellula* and **Chapters 4 and 5** include the description of three new species belonging to the genus *Rhodopirellula* which were named *R. rubra*, *R.lusitana* and *R. formosa*. The application of the *rpoB* gene sequencing and analysis as a suitable complement to the 16S rRNA gene and substitute of DNA-DNA hybridization in the *Planctomycetales* is reported in **Chapter 6.**

In order to achieve a more comprehensive overview of the phylogenetic composition of the planctomycetes community in the macroalgae biofilms, culture independent methods, namely 16S rRNA gene clone libraries and DGGE fingerprinting, were applied to six macroalgae representing the main lineages (*Ulva* sp., *Fucus spiralis*, *Chondrus crispus*, *Sargassum muticum*, *Porphyra dyoica*, and *Mastocarpus stellatus*). To date, there is only one molecular study that specifically assessed the planctomycetes diversity on macroalgae and it was performed in the kelp *Laminaria hyperborea*. Therefore, this work intends to extend the knowledge on the association

planctomycetes-macroalgae by characterizing other epiphytic planctomycetes communities. In **Chapter 7** the existence of possible host-specificity of the planctomycetes communities was assessed by DGGE fingerprinting. Specific primers for *Planctomycetes* were applied and the DGGE profiles of the planctomycetes communities from *Ulva* sp., *F.spiralis*, *C. crispus*, *S. muticum*, *P. dyoica*, and *M. stellatus* were compared and analysed statistically. Spatial variation of the same species of macroalgae was also determined by sampling in two rocky beaches, one located in Porto and the other about 60 km north, in Carreço, Viana do Castelo. The diversity and phylogenetic affiliation of the planctomycetes associated with the above macroalgae species was assessed through bands excision and sequencing. As DGGE do not allow a full taxonomic assignment and therefore limits the knowledge of the true diversity of the bacterial communities, 16S rRNA gene clone libraries were constructed to fully characterize the planctomycetes communities on macroalgae and results are shown in **Chapter 8.** In this chapter, three macroalgae, *Ulva* sp., *F. spiralis* and *C. crispus* were sampled in Porto and Carreço during one year in four different occasions (autumn, winter, spring and summer). DGGE profiles were performed to determine the existence of spatial and temporal variation of the planctomycetes communities as well as a probable host-specific association with the macroalgal host. The 16S rRNA gene clone libraries were constructed only for the winter and summer seasons as they represent more extreme environmental conditions. Therefore it would be possible to determine if the taxonomic composition of the planctomycetes changed with different environmental conditions.

The integration of all the results obtained will be discussed in **Chapter 9**, and **Chapter 10** addresses research perspectives for future implementation.

Chapter 3.

Roseimaritima ulvae gen. nov., sp. nov. and *Rubripirellula obstinata* gen. nov., sp. nov. two novel planctomycetes isolated from the epiphytic community of macroalgae[1]

Abstract

Four isolates, belonging to the deep-branching phylum *Planctomycetes*, were recovered from the biofilm of two marine macroalgae, *Ulva* sp. and *Laminaria* sp., from the Northern coast of Portugal. These strains were light pink- or red-pigmented; the cells were variable in shape and usually organized in rosettes. They had a dimorphic cell cycle with budding reproduction. The organisms were chemoheterotrophic, strictly aerobic and mesophilic. The 16S rRNA gene sequence analysis showed that the strains belong to the family *Planctomycetaceae* with *Rhodopirellula* as the closest genus. The isolates form two separate branches (strain LF1^T forms one branch and the strains UC8^T, UF3 and UF42 form a second branch) clearly separated from *Rhodopirellula baltica* with 94.2 % and 93.8 % 16S rRNA gene sequence similarity, respectively.

Based on differential characteristics that distinguish the novel genera from *R. baltica* SH1^T, such as cell size and shape, ultrastructure, enzymatic activities, substrate utilization pattern, fatty acid composition, phospholipid profiles and phylogeny we propose that the isolates represent two novel genera of the order *Planctomycetales*, *Roseimaritima ulvae* gen. nov., sp. nov. (type strain is UC8^T) and *Rubripirellula obstinata* gen. nov., sp. nov. (type strain is LF1^T).

[1] The results present in this chapter were accepted for publication in Systematic and Applied Microbiology
Joana Bondoso, Luciana Albuquerque, M. Fernanda Nobre, Alexandre Lobo-da-Cunha, Milton S. da Costa and Olga Maria Lage. Roseimaritima ulvae gen. nov., sp. nov. and Rubripirellula obstinata gen. nov., sp. nov. two novel planctomycetes isolated from the epiphytic community of macroalgae.

Introduction

The *Planctomycetes* represent a deep-branching group of *Bacteria* phylogenetically related to the phyla *Verrucomicrobia*, *Chlamydiae* and *Lentisphaerae* (Wagner and Horn, 2006). Several characteristics of the planctomycetes are unique in prokaryotic organisms; these include absence of peptidoglycan, a proteinaceous cell wall and compartmentalized cell structure (Fuerst and Sagulenko, 2011). In general, they are metabolically diverse and widespread. The *Planctomycetes* comprise the order *Planctomycetales,* with eleven genera that comprise fifteen species described (Ward, 2010; Bondoso et al., 2011; Zaicnikova et al., 2011; Kulichevskaya et al., 2012a; Kulichevskaya et al., 2012b), the order *Phycisphaerales* comprising one genus with only one species (Fukunaga et al., 2009) and the '*Candidatus* Brocadiales' with five candidate genera (Jetten et al., 2010).

In recent years, several studies reported the association of planctomycetes with marine macroalgae (Lage and Bondoso, 2011; Friedrich, 2012). The kelp *Laminaria hyperborean* possess a biofilm community dominated by these bacteria (Bengtsson and Ovreas, 2010). A new order of *Planctomycetes,* the *Phycisphaerales*, was proposed to include a novel isolate obtained from the surface of a *Porphyra* sp. (Fukunaga et al., 2009). Isolation of epiphytic planctomycetes of macroalgae reveals a great phylogenetic diversity (Lage and Bondoso, 2011). Here, we describe two novel species based on four isolates designated UC8^T, UF3, UF42 and LF1^T from the epiphytic bacterial community of *Ulva* sp. and *Laminaria* sp.

Methods

The strains described in this study were isolated by Lage and Bondoso (2011) in Carreço (UC8^T from *Ulva* sp.) and Porto (UF3 and UF42 from an *Ulva* sp. and LF1^T from a *Laminaria* sp.). Strain UC8^T was isolated from the aqueous extract of *Ulva* sp. on modified M629 agar (5 g l^{-1} peptone, 0.5 g l^{-1} yeast extract, 20 ml l^{-1} Hutner's basal salts, 10 ml l^{-1} vitamin solution in 90 % natural seawater – NSW). Strains UF3 and UF42 were isolated on modified M629 broth inoculated with pieces of *Ulva* sp. and strain LF1^T was isolated in modified M13 broth (0.25 g l^{-1} peptone, 0.25 g l^{-1} yeast extract, 0.25 g l^{-1} glucose, 20 ml l^{-1} Hutner's basal salts, 10 ml l^{-1} vitamin solution in 90 % natural seawater – NSW buffered with 5 mM Tris-HCl, pH 7.5) inoculated with portions of *Laminaria* sp. Strains were routinely maintained in modified M13 at 26 ºC (LF1^T) or 30 ºC (UC8^T, UF3 and UF42), in the dark. For long term storage, isolates were stored at -80 ºC in sterile natural seawater with 20 % (w/v) glycerol. Unless stated otherwise, morphological, biochemical and physiological tests were performed at 26 ºC

for LF1^T and 30 ºC for UC8^T, UF3 and UF42 in M13 modified media with 1.6 % agar or in liquid cultures (200 rpm). The type strain of *Rhodopirellula baltica* SH1^T(DSM 10527^T), obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, was used for comparative purposes.

Cell morphology and motility were observed by optical microscopy during the exponential phase of growth. For scanning electron microscopy (SEM), exponentially growing cells were fixed in 2.5 % glutaraldehyde in Marine buffer, pH 7.0 (Watson et al., 1986) for 2.5 h, dehydrated through a graded ethanol series, critical point dried and observed with a HITACHI S-570. For transmission electron microscopy (TEM), cells were harvested from 5 day old plates and cryopreserved as described before (Bondoso et al., 2011).

Growth temperature range was examined from 5 to 45 ºC in 5 ºC intervals and NaCl tolerance was determined in artificial sea water based media ASW; (Lyman and Fleming, 1940) supplemented with NaCl up to concentrations ranging from 3.5 to 10 %. Minimal and maximum salinity requirement for growth was examined in ASW-based media with increasing proportions of ASW ranging from 0-50 % and 90-300 % (100 % ASW corresponds to 34.5 ‰ salinity). The requirement for seawater salts was performed by replacing the ASW by 3.3 % of NaCl in distilled water. The pH range for growth was determined in liquid media using 10 mM of each of the following buffers: MES for pH 4.5, 5.0, 5.5, 6.0 and 6.5, Tris-HCl for pH 7.5 and 8.5, CHES for pH 9.0, 10.0, 10.5 and CAPS for pH 11.0. Vitamin requirement was determined in M20c medium (Schlesner et al., 2004) prepared with 90 % natural seawater to which different vitamin solutions (1 % [v/v] omitting one vitamin at a time) were added. Results were considered positive after two transfers in the same medium.

Hydrolysis of starch, casein, elastin, alginate and carboxymethyl-cellulose, oxidase and catalase activities were determined using standard methods (Skerman, 1969; Tindall et al., 2007). Cellulase activity was detected by the liquefaction of carboxymethyl-cellulose based medium around the colonies. Other enzymatic activities were evaluated with the API ZYM and API 20NE systems (bioMérieux) according to manufacturer's instructions with the exception that cell suspensions were prepared in 20 ‰ marine salts (MacDonell et al., 1982) and results were recorded after 10 days for API 20 NE and 48 h for API ZYM. Carbon metabolism was studied by oxidation (BIOLOG GN2 MicroPlate), acidification (API 50 CH) and assimilation of carbon sources by conventional methods (basal medium supplemented with each carbon source). Single-carbon source assimilation tests were determined for the type strains UC8^T and LF1^T in 20 ml screw capped tubes containing 7 ml of liquid medium

composed of 90 % NSW buffered with 5 mM Tris-HCl, pH 7.5, to which filter-sterilized Hutner's basal salts (20 ml l^{-1}), $NH_4(SO4)_2$ (0.5 g l^{-1}), Na_2HPO_4 (0.05 g l^{-1}), vitamin solution (10 ml l^{-1}) and the filter-sterilized carbon source (1.0 g l^{-1}) were added. Growth was evaluated by measuring the turbidity of the cultures at 600 nm. To inoculate GN2 MicroPlate test strips, liquid cultures were grown for 48h, centrifuged and resuspended in sterile 0.75 % ASW without $CaCl_2$ (Makemson et al., 1998) and results were examined after 10 days. API 50 CH system was inoculated with 48h-liquid cultures resuspended in glucose-free medium M13 prepared with 50 % ASW and supplemented with 0.01 g l^{-1} phenol red. Results were recorded after 60 h of incubation. Nitrogen sources utilization was determined in medium containing 90 % NSW, glucose (0.5 g l^{-1}), Na_2HPO_4 (0.05 g l^{-1}), Hutner's basal salts (20 ml l^{-1}) and vitamin solution (10 ml l^{-1}), buffered with 5 mM Tris-HCl pH 7.5 and supplemented with 1.0 g l^{-1} of each of the twenty natural amino acids, as well as peptone, yeast extract, casamino acids, N-acetyl-glucosamine (NAG), urea, ammonium, nitrate or nitrite. Turbidity of the cultures was measured at 600 nm. Anaerobic growth was tested in anaerobic chambers (GENbox anaer; bioMérieux) and results were recorded after three weeks.

Cultures for chemotaxonomic analysis were grown in liquid modified M13 medium until late exponential phase at 26 ºC for all the strains. Cells were harvested by centrifugation and washed in Tris-HCl 0.1 M, pH 7.5 before subsequent analyses. Polar lipids were extracted from freeze-dried cells as described previously (da Costa et al., 2011a). Individual polar lipids were separated by two-dimensional thin-layer chromatography (TLC) and visualized as described previously. Respiratory quinones were extracted from freeze-dried cells, purified by TLC and separated by HPLC as described by da Costa et al. (da Costa et al., 2011b). Fatty acid methyl esters (FAMEs) were obtained from fresh wet biomass, separated, identified and quantified with the standard MIS Library Generation Software (Microbial ID Inc.) as described previously (da Costa et al., 2011c).

For G+C content, DNA was isolated as described before (Nielsen et al., 1995) and the content was determined by HPLC (Mesbah et al., 1989). The almost complete 16S rRNA gene of $UC8^T$, UF3, UF42 and $LF1^T$ was amplified and analysed as described by Bondoso et al. (Lage and Bondoso, 2011). Sequences were assembled with Vector NTI (Invitrogen), manually examined and aligned with closely related sequences from NCBI database with ClustalW (Thompson et al., 1994). Phylogenetic trees were generated in MEGA version 5.03 (Tamura et al., 2011) using different calculation methods including neighbour joining, maximum parsimony and maximum likelihood to test for the stability of the tree.

BOX-PCR and ERIC-PCR of strains UC8^T, UF3 and UF42 were performed as described, respectively, by Winkelman et al. (Winkelmann et al., 2010) and by Lage et al. (Lage et al., 2012) using 100 ng of genomic DNA per 25 µl PCR reaction. Fingerprinting profiles were visualised after separation by electrophoresis in a 2 % agarose gel at 60V for 90 min in Tris–acetate–EDTA buffer. The gel was post-stained with ethidium bromide for 45 min and gel images were acquired in a GE Typhoon gel scanner.

Results and discussion

Colonies of strains UC8^T, UF3, UF42 and LF1^T were circular, small, convex, and translucent and light pink or almost red in LF1^T on M13 agar. Strains UC8^T, UF3 and UF42 attached to surfaces when grown in liquid media as is seen with other planctomycetes (Bondoso et al., 2011). Cells of strains UC8^T, UF3 and UF42 were circular to ovoid and never pear-shaped like *R. baltica* SH1^T, and organized in rosettes of large numbers of cells (Fig. 3.1a). Cell size was also smaller than that of *R. baltica* SH1^T. LF1^T cells were ovoid to pear-shaped, usually organized in rosettes of 3 up to 10 cells (Fig. 3.1c). All isolates reproduced by budding (Fig. 3.1).

SEM observation of UC8^T and LF1^T revealed the presence of fimbriae (Fig. 3.1b, d) and a flagellum on the reproductive pole of the cells. TEM of the strains UC8^T and LF1^T confirmed the distinct morphological differences between the new strains and the closest species, *R. baltica* SH1^T, although the characteristic *Planctomycetes* cell structure was present (Fig. 3.2). Cells had fimbriae in the apical and reproductive pole and the holdfast was present on the opposite pole. Crateriform pits on the cell surface, typical of *Planctomycetes,* were distributed in the reproductive pole. Compartmentalisation was seen in the paryphoplasm and the pirellulosome, the latter with ribosomes, storage inclusions and condensed DNA forming a visible nucleoid. UC8^T cells had an extensive paryphoplasm with granular appearance and a small pirellulosome while the paryphoplasm is fibrillar and the pirellulosome is more prominent in LF1^T. The pirellulosome was normally close to the apical pole. Strain LF1^T possessed a robust holdfast and the cells were surrounded by a thick cell wall and a glycocalyx, characteristics that differentiate it from *R. baltica* SH1^T. Some LF1^T cells had hump-like protrusions similar to those seen in *Pirellula staleyi* ATCC 35122 (Butler et al., 2002).

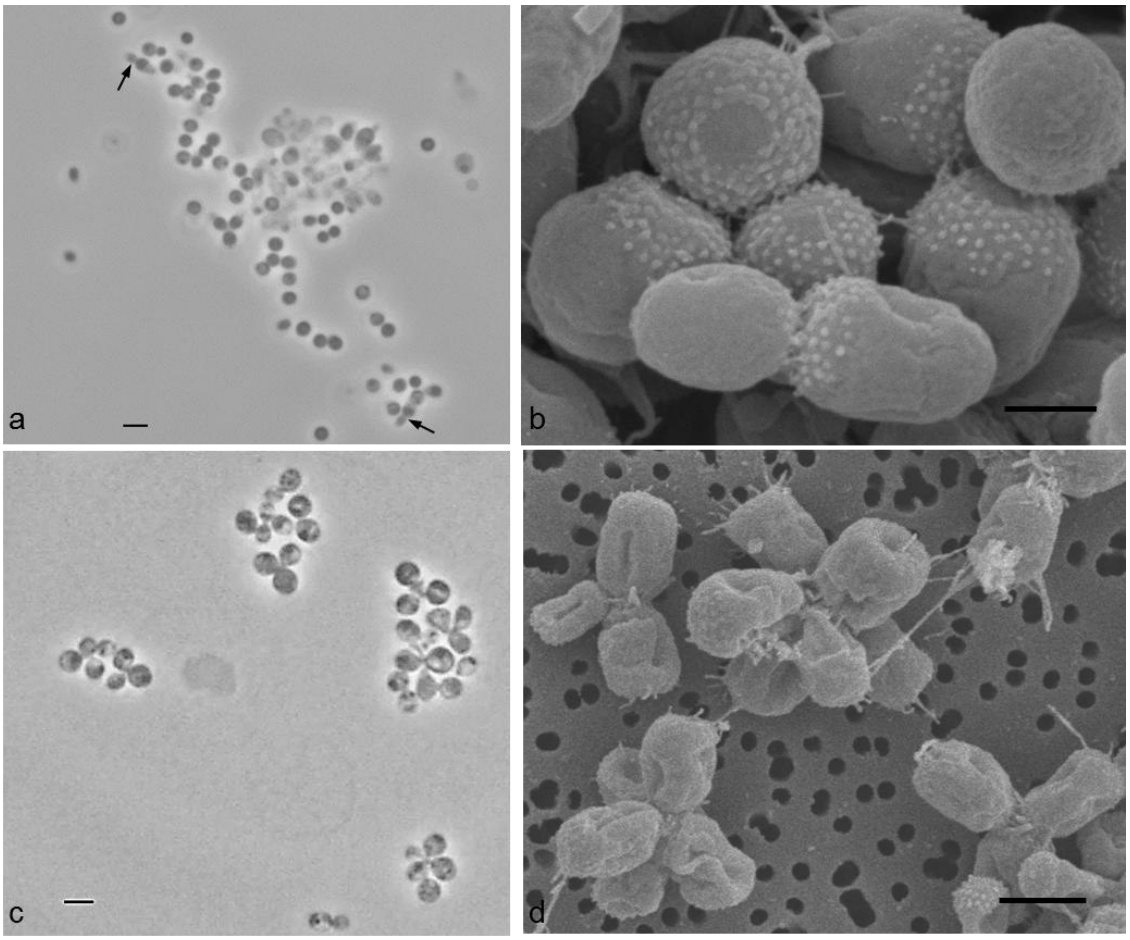

Fig. 3. 1 Morphological characteristics of strains UC8^T and LF1^T; (a and c) show phase-contrast and (b and d) show electron microscopy. Cells of strain UC8^T are spherical to ovoid, with budding (arrows) and the insertion of fimbriae in the reproductive pole (b) is observed. Cells of strain LF1^T are ovoid to pear shaped, usually organized in rosettes. Cells are attached by the holdfast (d). Bars - a) and c) 2 µm; b) 0.5 µm; d) 1 µm.

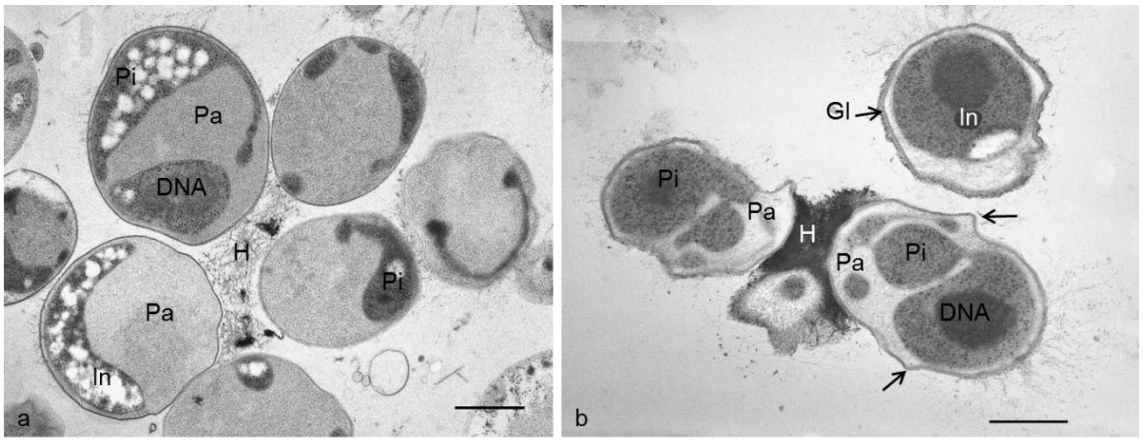

Fig. 3. 2 Transmission electron microscopy of strains UC8^T (a) and LF1^T (b). Typical planctomycete cell plan divided into the paryphoplasm (Pa) and the pirellulosome (Pi) is observed. Cells are bound by the holdfast (H) and LF1^T is surrounded by a glycocalyx (Gl). Inclusions (In) and condensed DNA can be seen. LF1^T presents hump-like protusions (arrows). Bars – 0.5 µm.

Optimum growth temperature was about 30 ºC for the isolates represented by strain UC8^T and 25 ºC for strain LF1^T. All strains required seawater for growth. The temperature, salinity tolerance and pH ranges are shown in Table 3.1. Differential characteristics between strains, including the type strain *R. baltica* SH1^T with API 50CH, Biolog GN2 and API ZYM are shown in Table 3.2. Members of the UC8^T group oxidized twenty (of 28) carbohydrates in the BIOLOG GN2 and produced acid from thirty-four of 49 carbohydrates in the API 50 CH. Strain LF1^T oxidized only 10 substrates in the Biolog GN2 and produced acid from only 20 carbohydrates. *R. baltica* SH1^T was the strain that oxidized more carbohydrates (22 in the BIOLOG GN and 40 in the API 50CH). All strains examined used carbohydrates as single carbon and energy sources but did not assimilate the amino acids or organic acids.

Strains UC8^T, UF3 and UF42 were cytochrome oxidase and catalase positive and LF1^T was cytochrome oxidase positive and catalase negative. Strains UC8^T, UF3 and UF42 were able to hydrolyse esculin, starch but not urea, gelatine, alginate, casein and elastin. Strain LF1^T only hydrolysed starch. Nitrate was reduced to nitrite by all isolates.

The strains utilised several amino acids and proteinaceous supplements as sources of nitrogen. Nitrate and ammonium were also utilized but urea was not. Nitrite was only utilised by strain UC8^T. In contrast to the majority of the other planctomycetes described, LF1^T was unable to utilize NAG at 1 % (w/v) concentration (Table 3.1). Of the vitamins tested, all strains required vitamin B12. Anaerobic growth was not observed.

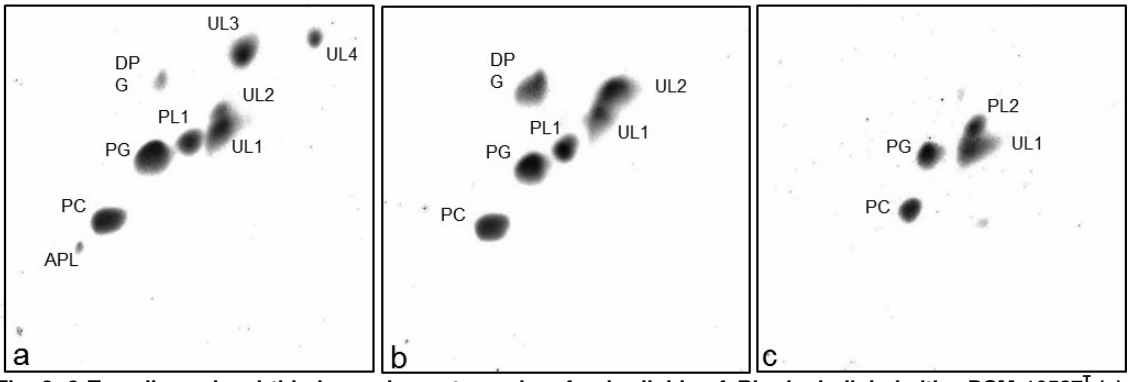

Fig. 3. 3 Two-dimensional thin-layer chromatography of polar lipids of *Rhodopirellula baltica* DSM 10527^T (a), strain UC8^T (b) and strain LF1^T (c) at 26ºC. The lipids were stained by spraying with 5 % molybdophosphoric acid in ethanol followed by heating at 160 ºC. PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; APL, aminophospholipid; PL1,2, phospholipid 1,2; UL1,2,3,4; unknown lipid 1,2,3,4.

The polar lipid composition of strains UC8^T, LF1^T and *R. baltica* SH1^T by TLC indicated that diphosphatidylglycerol (DPG) was one of the major polar lipids of UC8^T but was absent from LF1^T (Fig. 3.3). An aminophospholipid and unknown lipids 3 and 4 were only present in *R. baltica* SH1^T and phospholipid 2 was only present in LF1^T.

Table 3. 1 Differential characteristics of the two novel genera *Roseimaritima*, *Rubripirellula* and the closest genus *Rhodopirellula*.

	Roseimaritima	***Rubripirellula***	***Rhodopirellula***
Cell size (µm)	1.1-1.8 x 0.9-1.5	1.5-2 x 1.3-1.7	1.0–2.5x1-2–2-3
Cell shape	Spherical to ovoid	Pear-shaped to ovoid	Pear-shaped to ovoid
Cell arrangement	Rosettes 20-40 cells	Rosettes 2-10 cells	Rosettes of variable number of cells
Pigmentation	Light Pink	Red	Pink to Red
Salinity tolerance range (%):			
ASW range (v/v)	20-175	50-125	10-175
Maximum NaCl (w/v)	5	4	5
Temperature for growth (ºC):			
Range	15-35	10-30	5-30
Optimum	30	25	28
pH range	6.5-10	7.5-10.5	5.5-10.5
Carbon sources			
NAG	+	-	+
Ribose	-	-	+
Raffinose	+	-	-
Arabinose	+	-	-
Sucrose	+	-	+
Maltose	+	-	+
Lactose	+	-	+
Trehalose	+	-	+
Mannitol	+	-	-
Lactulose	+	-	-
Dextran	+	-	+
Nitrogen sources			
Alanine	-	-	+
Asparagine	-	-	+
Phenylalanine	-	-	+
Threonine	+	-	-
Proline	+	-	+
NAG	+	-	+
Nitrite	+	-	-
Hydrolysis of:			
Starch	+	+	+
Esculin	+	-	+
Catalase	+	-	+
FAMEs			
$C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c.	2.4	8.4	17.9
$C_{16:0}$	35.1	17.3	26.8
$C_{17:1}\omega$8c	2.0	12.0	5.1
Polar lipids			
Diphosphatidylglycerol	++	-	+

+, positive; -, negative; ++, strongly positive

All the strains assimilated galactose, rhamnose, glucose and xylose. None of the strains utilize casamino acids, fucose, sorbose, raffinose, ribitol, sorbitol, m-inositol, erithritol, arabitol, glycerol, succinate, ketoglutarate, malate, pyruvate, citrate, acetate, benzoate, fumarate, formate and inulin. All the strains utilized aspartate, arginine, glutamine, casamino acids, yeast extract, peptone and ammonium as nitrogen sources. The following were not utilized: gluconate, glutamate, cysteine, cystine, guanine, histidine, lysine, methionine, ornithine, serine, tyrosine, tryptophan, valine and urea.

Table 3. 2 Differential characteristics of strains UC8^T, UF3, UF42, LF1^T and *R. baltica* SH1^T in API 50CH, API ZYM and Biolog GN2.

	UC8^T	UF3	UF42	LF1^T	*R. baltica* SH1^T
API 50CH					
D-arabinose	+	+	+	-	+
L-arabinose	+	+	+	-	+
D-ribose	+	+	+	-	+
L-rhamnose	+	+	-	-	+
Methyl-ßD-xylopyranoside	-	-	-	-	w
D-mannitol	+	+	+	-	+
Methyl-ßD-Mannopyranoside	w	+	w	-	+
Amygdalin	w	+	+	-	+
D-melibiose	+	+	+	-	+
D-saccharose (sucrose)	+	+	+	-	+
D-trehalose	+	+	+	-	+
D-melezitose	-	+	-	-	+
D-raffinose	w	+	w	-	+
xylitol	-	+	-	-	+
D-turanose	+	+	+	-	+
D-tagatose	+	+	+	-	+
D-fucose	+	+	+	-	+
L-fucose	+	+	+	-	+
D-arabitol	-	w	-	-	-
L-arabitol	-	w	-	-	+
Potassium 2-ketogluconate	w	-	-	-	+
API ZYM					
α-galactosidase	+	+	+	-	+
ß -galactosidase	-	-	+	-	+
α -glucosidase	+	+	+	-	+
ß-glucosidase	-	-	-	-	+
N-acetyl-ß-glucosaminidase	-	-	-	-	+
Trypsin	-	-	+	-	+
Biolog GN					
Dextrin	-	-	-	-	+
Glycogen	-	+	-	-	-
N-Acetyl-DGalactosamine	+	+	+	-	+
N-Acetyl-DGlucosamine	+	+	+	-	+
L-Arabinose	-	-	+	-	+
D-Arabitol	-	-	+	-	-
L-Fucose	+	+	+	-	+
D-Mannitol	+	+	+	-	+
D-Melibiose	+	+	+	-	+
β-Methyl-D-Glucoside	+	+	+	-	+
D-Psicose	+	+	-	-	-
L-Rhamnose	-	+	-	+	+
D-Sorbitol	-	+	-	-	-
Sucrose	+	+	+	-	+
D-Trehalose	+	+	+	-	+
Turanose	+	+	+	-	+
Pyruvic Acid Methyl Ester	-	-	-	-	+
Succinic Acid Mono-Methyl-Ester	-	-	-	-	+
Acetic Acid	+	-	-	+	+
D-Galacturonic acid	-	+	-	-	-
D-Gluconic acid	+	+	+	-	+
D-Glucosaminic acid	+	-	-	-	-
Succinic Acid	+	-	+	-	-

Propionic Acid	-	-	+	-	-
Glucuronamide	+	+	+	-	+
L-Glutamic Acid	-	+	+	-	-
Inosine	-	-	-	+	-
Glycerol	+	+	+	-	+
D,L-α-Glycerol Phosphate	-	+	+	-	-
α-D-Glucose-Phosphate	+	+	+	-	-

+, positive; -, negative; w, weakly positive.
All the strains produced acid from D-xylose, L-xylose, D-galactose, D-glucose, D-frutose, D-mannose, D-sorbose, D-rhamnose, methyl-αD-glucopyranoside, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, gentiobiose, D-lyxose and potassium 5-ketogluconate. Acid was not produced from glycerol, erythritol, D-adonitol, dulcitol, inositol, inulin, starch, glycogen and potassium gluconate.
All the strains were positive for alkaline phosphatase, esterase C4, esterase lipase C8, leucine, valine and cystine arylamidase, acid phosphatase. All the strains were negative for lípase C14, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, ß-glucuronidase, α-mannosidase, α-fucosidase.
All the strains oxidized D-cellobiose, D-frutose, D-galactose, gentiobiose, α-D-Glucose, α-D-lactose, lactulose, maltose, D-mannose, D-glucuronic acid, D,L-lactic acid. None of the strains oxidized tween 40 and 80, adonitol, erythritol, m-inositol, xylitol, Cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxy-phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, malonic acid, quinic acid, D-saccharic acid, sebacic Acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, uridine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D-glucose-6-phosphate.

The major respiratory lipoquinone of all planctomyces examined was menaquinone 6 (MK-6) as is usual in this phylum. The fatty acid composition of strains UC8^T, UF3 and UF42 showed a predominance of $C_{18:1}\omega 9c$ and $C_{16:0}$ which accounted for about 43-48 % and 33-35 % of the total fatty acids, respectively (Table 3.3). The major fatty acid of strain LF1^T was C_{18}:$_1\omega 9c$ (42 %) with smaller amounts of $C_{16:0}$ (17 %) and $C_{17:1}\omega 8c$ (12 %). These profiles were distinct from that of *R. baltica* SH1^T, which had predominantly $C_{18:1}\omega 9c$ (43 %), $C_{16:0}$ (27 %) and summed feature 3 ($C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$) (18 %). All new strains had $C_{18:0}$ 3-OH and $C_{20:1}\omega 9c$ fatty acids which were absent in *R. baltica* SH1^T.

Phylogenetic analysis of the nearly complete 16S rRNA gene showed the affiliation of UC8^T, UF3, UF42 and LF1^T to the *Planctomycetes*, with *R. baltica* SH1^T as the most closely related organism (Fig. 3.4). The phylogenetic distance of the 16S rRNA gene between members of the UC8^T group and LF1^T was around 4 %. The 16S rRNA gene sequence similarity was 93.6 % between the UC8^T group and *R. baltica* SH1^T and 94.2 % between strain LF1^T and *R. baltica* SH1^T. Strains belonging to the UC8^T group shared 100 % pairwise sequence similarity among isolates and were most closely related (99 %) to an uncultured bacterial clone SBS-FW-053 isolated from the biofilm of seawater reverse osmosis (SWRO) membranes. The closest cultured planctomycete to this group was *Pirellula* sp. 158, isolated from the Kiel Fjord, with 97.0 % sequence similarity. Strain LF1^T had no cultured relatives; the closest being an uncultured clone from marine sediments and from the sponge *Astrosclera willeyana* with 95.0 % 16S rRNA gene sequence similarity. Phylogenetic analysis using the neighbour-joining,

maximum-likelihood and parsimony methods showed that both groups form two branches in a separate cluster from *R. baltica* SH1^T(Fig. 3.4).

Table 3. 3 Fatty acid composition of strains UC8 ^{T}, UF3, UF42, LF1 ^{T} and type strain of *Rhodopirellula baltica* SH1^T grown in liquid M13 medium at 26 ºC.

Fatty acid	ECL[a]	*R. baltica* SH1^T	UC 8 ^{T}	UF 3	UF 42	LF 1^T
Unknown ECL 11.980	11.980	0.6	0.4	0.6	0.4	-
$C_{14:0}$	14.000	0.5	0.7	0.8	0.7	0.4
$C_{15:0}$	15.000	0.5	0.3	0.3	0.3	1.0
Summed feature 3	15.816	17.9	2.4	2.3	2.3	8.4
$C_{16:0}$	16.000	26.8	35.1	33.4	34.2	17.3
$C_{17:1}$ ω8c	16.791	5.1	2.0	1.8	1.7	12.0
$C_{17:0}$	17.000	1.0	2.1	1.7	2.1	4.5
$C_{16:0}$ 2-OH	17.234	-	-	-	-	1.1
$C_{18:1}$ ω9c	17.769	42.6	47.0	42.5	48.1	41.5
Summed feature 8	17.822	2.8	1.1	0.9	1.2	0.9
$C_{18:0}$	18.000	1.3	3.1	2.7	3.1	5.6
Unknown ECL 18.558	18.558	-	-	7.5	-	-
Unknown ECL 18.796	18.796	0.6	0.6	0.5	0.5	-
Summed feature 7	18.866	-	-	-	-	1.1
$C_{19:0}$	19.000	-	-	-	-	2.1
$C_{18:0}$ 3-OH	19.548	-	1.2	1.2	1.1	1.3
C $_{20:1}$ ω 9c	19.769	-	3.0	3.7	3.4	0.6
$C_{20:0}$	20.000	-	-	-	-	0.7

[a]ECL, equivalent chain length. Summed features represent groups of two or three fatty acid that could not be separated by GLC with MIDI System: summed feature 3 comprises $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c; summed feature 7 comprises cyclo-$C_{19:0}$ ω10c/19 ω6; summed feature 8 comprises $C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c.

Fingerprinting profiles with ERIC and BOX primers revealed that isolates UC8^T and UF42 were genetically identical (Supplementary Fig. S1), even though they were isolated from different sites but from the same species of macroalga. Although similar, strain UF3 had a different profile from UC8^T and UF42.

The mole G+C content of the DNA of group UC8^T and LF1^T was 57-59 % and 56.1 %, respectively, which were higher than the mole G+C content of *R. baltica* SH1^T (54.1 %).

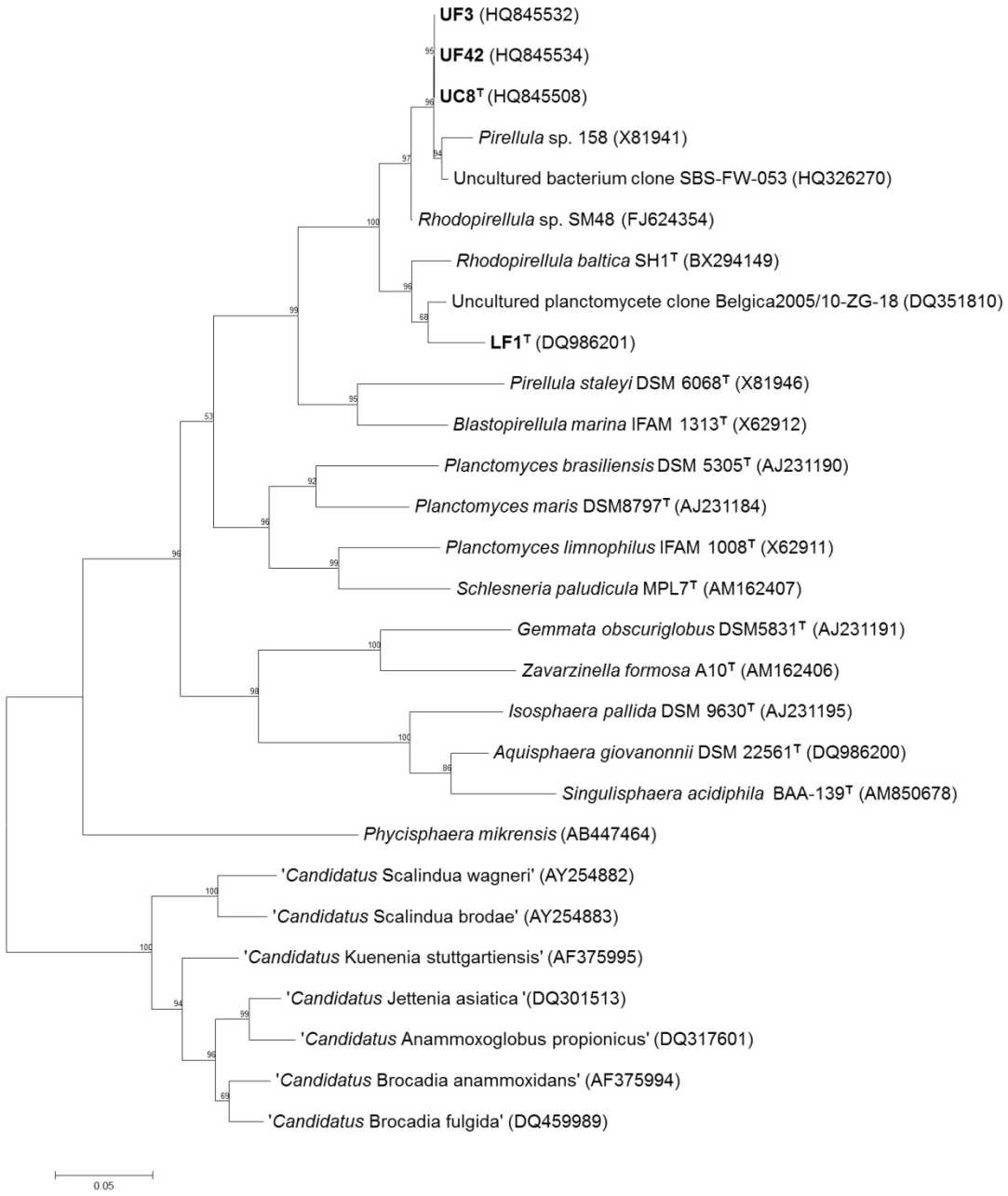

Fig. 3. 4 Optimal maximum likelihood 16S rRNA gene phylogenetic tree showing the relationships between strains UC8^T, UF3, UF42 and LF1^T and other representatives of the phylum *Planctomycetes* (accession numbers are shown in parenthesis). The tree was based on the Jukes-Cantor model. Numbers on the tree refer to bootstrap values based on 1000 replicates. Only values above 50 % are shown. *"Candidatus"* genera Anammox were used as outgroup. Bar - 0.05 substitutions per nucleotide position.

Conclusions

Group UC8^T forms a cluster phylogenetically apart from *R. baltica* and strain LF1^T with more than 6 % dissimilarity in the 16S rRNA gene sequence, which supports the separation of a novel genus within the *Planctomycetes*. Furthermore, it possesses morphological and chemotaxonomic characteristics that clearly distinguish it from the most closely related genera. UC8^T forms small spherical-shape cells with a different ultrastructure compared to the pear-shaped cells characteristics of the PRB group. The phospholipid profile of UC8^T is similar to that of *R. baltica* SH1^T, but diphosphatidylglycerol (DPG) is one of the major phospholipids of UC8^T which is a minor component in *R. baltica* SH1^T. Furthermore, there are two additional major unknown lipids in type species *R. baltica* SH1^T that are not present in UC8^T. UC8^T possesses higher amounts of C16:0 fatty acid (35 %) and lower amounts of C16:1 ω7c and/or C16:1 ω6c (2.4 %) in comparison to *R. baltica* SH1^T (26.8 % and 17.9 %). Moreover, UC8^T possesses C18:0 3-OH and C 20:1 ω9c fatty acids that are absent in *R. baltica* SH1^T.

Isolate LF1^T shares only 94.2 % similarity with the 16S rRNA gene of *R. baltica* SH1^T, which once again is indicative of a novel genus. Other characteristic that distinguishes strain LF1^T from *Rhodopirellula* includes a distinct phospholipid profile from that of *R. baltica* SH1^T with undetectable diphosphatidylglycerol as well as other lipids present in the new organism. The fatty acid profile of both strains is also distinct; there are substantial differences in the levels of C16:0 (17 %) in LF1^T and (28 %) in *R. baltica* SH1^T; cyclo-C19:0 ω10c/19 ω6, C20:1 ω9c and C20:0 are absent in *Rhodopirellula* and UC8^T but are present in strain LF1^T. Other striking difference in LF1^T is very restricted carbon sources utilization when compared to *Rhodopirellula* and UC8^T. Also, LF1^T is not able to utilize NAG as carbon and nitrogen sources, a common feature among planctomycetes.

Based on the distinctive morphological, metabolic and chemotaxonomic characteristics that discriminate the two novel isolates from *Rhodopirellula*, we are of the opinion that the organism represent two new species of two novel genera for which we propose the name *Roseimaritima ulvae* gen. nov., sp. nov. (UC8^T) and *Rubripirellula obstinata* gen. nov., sp. nov. (LF1^T).

Description of *Roseimaritima* gen. nov.

Roseimaritima (*ro.se.i.ma.ri'ti.ma*. L. adj. roseus, rose-coloured; L. adj. *maritimus -a -um*, of the sea, marine; N.L. fem. n. *Roseimaritima*, the marine rose-coloured bacterium.)

Cells have peptidoglycan-less cell walls, form primarily spherical but some cells are ovoid, form rosettes of variable number of cells. Colonies are light pink. Crateriform pits at the reproductive pole and holdfast in the opposite pole. Stalks are absent. Reproduce by budding. Chemoheterotrophic, strictly aerobic, catalase- and cytochrome oxidase- positive. The major respiratory quinone is MK-6. The major fatty acids are $C_{18:1}\omega9c$ and $C_{16:0}$. The predominant polar lipids are phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol. This genus is a member of the family *Planctomycetaceae*. The type species is *Roseimaritima ulvae.*

Description of *Roseimaritima ulvae* sp. nov.

Roseimaritima ulvae (ul'va.e. N.L. gen. n. *ulvae*, of *Ulva*, the generic name of the host alga, *Ulva* sp. - the source of isolation). Cells are spherical to ovoid with 1.1-1.8 x 0.9-1.5 µm in diameter, possess a dimorphic life cycle with a motile phase. Colonies in M13 medium are translucent and smooth. Optimum growth temperature is about 30 ºC (temperature range is between 15 ºC and 35 ºC), pH optimum is about 7.5 (pH range is from 6.5 to 10). Requires sea salts for growth with a minimum of 20 %-25 % of ASW salinity. The maximum salinity for growth is 175 % ASW. Vitamin B12 is required for growth. Major fatty acids are $C_{18:1}\omega9c$ (43-48 %) and $C_{16:0}$ (33-35 %). Starch, esculin and carboxymethyl cellulose are degraded; urea, casein, elastin and alginate are not. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamilase, valine arylamidase, cysteine arylamidase, acid phosphatase, α-glucosidase and α-galactosidase are positive in the API ZYM; other activities are negative. Indole is not produced. Arginine dihydrolase is absent. Nitrate is reduced to nitrite. N-acetylglucosamine, D-galactose, L-rhamnose, D-arabinose, D-glucose, xylose, sucrose, maltose, lactose, D-cellobiose, D-trehalose, D-mannitol, lactulose, dextran are assimilated. Amino acids, casamino acids, D-fructose, L-fucose, D-ribose, L-sorbose, D-raffinose, ribitol, sorbitol, *myo*-inositol, erythritol, D-arabitol, glycerol, succinate, α-ketoglutarate, malate, citrate, benzoate, fumarate, formate, acetate, pyruvate and inulin are not assimilated. Acid is produced from D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, methyl-α-D-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-

lactose, D-melibiose, D-sucrose, D-trehalose, D-raffinose, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose and potassium 5-ketogluconate in the API 50 CH. Oxidizes N-acetylglucosamine, N-acetylgalactosamine, cellobiose, D-fructose, L-fucose, D-galactose, gentibiose, D-glucose, α-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melobiose, β-methylglucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, acetic acid, gluconic acid, D-glucuronic acid, D,L-lactic acid, succinic acid, glycerol, glucoronamide, glucose-1-phosphate in the BIOLOG GN2 System. Aspartate, arginine, glutamine, threonine, peptone, yeast extract, casamino acids, NAG, ammonia, nitrate and nitrite serve as sources of nitrogen. Urea is not utilized.

The G+C mole content of the DNA of the type strain is 57.0 ± 0.6 % (HPLC method).

The type strain is UC8^T isolated from the epiphytic community of *Ulva* sp.

Description of *Rubripirellula* gen. nov.

Rubripirellula (Ru.bri.pi.rel'lul.a. L. adj. *ruber -bra -brum*, red; N.L. fem. n. *Pirellula* name of a bacterial genus; N.L. fem. n. *Rubripirellula*, red-colored *Pirellula*).

Colonies are red colored and cells are pear-shaped to ovoid forming rosettes; cells do not possess peptidoglycan in their cell wall. Crateriform pits in the reproductive pole and holdfast in the opposite pole. Reproduce by budding. Chemoheterotrophic with restricted carbohydrate utilization, strictly aerobic, catalase positive and cytochrome oxidase negative. The major respiratory quinone is MK-6. The major fatty acid is $C_{18:1}\omega 9c$. The predominant polar lipids are phosphatidylcholine and phosphatidylglycerol. This genus is a member of family *Planctomycetaceae*. The type species is *Rubripirellula obstinata.*

Description of *Rubripirellula obstinata* sp. nov.

Rubripirellula obstinata (ob.sti.na'ta; L. fem. adj. *obstinata*, stubborn, obstinate due to an inconstant growth)

Cells are pear-shaped to ovoid with 1.5-2.0 x 1.3-1.7 µm, possess a dimorphic life cycle with a motile phase. Cells are surrounded by a glycocalyx. Colonies in M13 are translucent and smooth. Optimum growth temperature is about 25 ºC (temperature range for growth is between 10 ºC and 30 ºC); the optimum pH for growth is about 7.5 (pH range for growth is from 6.5 to 10). Requires sea salts for growth and a minimum salinity (ASW) of 50 %. Maximum salinity for growth is 125 % ASW. Vitamin B12 is required. Major fatty acids are $C_{18:1}\omega 9c$ (42 %), $C_{16:0}$ (17 %) and $C_{17:1}$ $\omega 8c$ (12 %). Starch is degraded; esculin, cellulose, urea, casein, elastin and alginate are not degraded. Indole is not produced. Arginine dihydrolase is absent.

Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamilase, valine arylamidase, cystine arylamidase, acid phosphatase are positive in API ZYM; other activities are negative. Nitrate is reduced to nitrite. D-fructose, D-galactose, L-rhamnose, D-glucose and xylose are assimilated. Amino acids, NAG, casamino acids, L-fucose, D-ribose, D-arabinose, L-sorbose, sucrose, maltose, lactose, D-cellobiose, D-trehalose, lactulose, D-raffinose, D-mannitol, dextran, ribitol, sorbitol, *myo*-inositol, erythritol, D-arabitol, glycerol, succinate, α-ketoglutarate, malate, citrate, benzoate, fumarate, formate, acetate, pyruvate and inulin are not assimilated. Acid is produced from D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, methyl-αD-glucopyranoside, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, gentiobiose, D-lyxose and potassium 5-ketogluconate in the API 50 CH.

The organism oxidizes D-cellobiose, D- fructose, D-galactose, gentibiose, D-glucose, α-lactose, lactulose, maltose, D-mannose, L-rhamnose, acetic acid, D-glucuronic acid, D,L-lactic acid and inosine in BIOLOG GN2 System. Aspartate, arginine, peptone, yeast extract, casamino acids, ammonia, nitrate serve as sources of nitrogen. Urea, NAG and nitrite are not utilized.

The G+C mole content of the DNA is 56.1 ± 0.2 % (HPLC method).

The type strain is LF1^T isolated from the epiphytic community of *Laminaria* sp.

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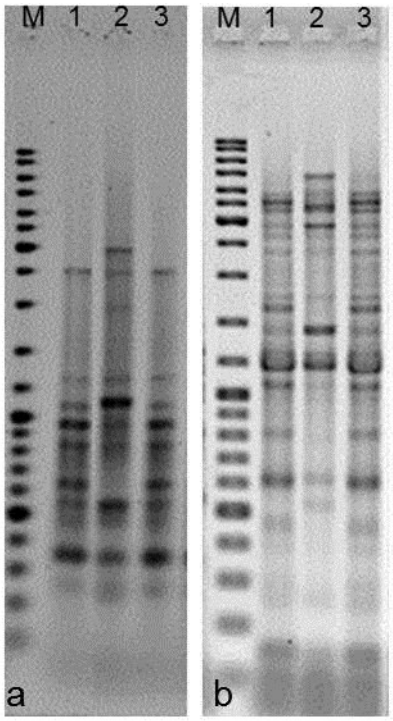
Supplementary material



Supplementary Fig. S 1 ERIC-PCR (a) and BOX-PCR (b) profiles of strains UC8^{T,} UF3 and UF42. Lanes: M, DNA marker Gene Ruler Ladder Mix (Fermentas™); 1, UC8^{T}; 2, UF3; 3, UF42.

Chapter 4.

Rhodopirellula lusitana sp. nov. and *Rhodopirellula rubra* sp. nov., isolated from the surface of macroalgae[2]

Abstract

Twenty two strains of *Rhodopirellula* were previously isolated from the epiphytic community of several marine macroalgae and separated into two groups, designated as group B and group C. In this study, we characterized these groups as two novel species belonging to the genus *Rhodopirellula*.

These strains were represented by pleomorphic cells that were organized in rosettes and formed pink- or red pigmented colonies. The organisms were chemoorganotrophic and required vitamin B_{12} for growth. Their optimal temperature for growth was around 25 ºC. Major fatty acids were $C_{18:1}$ ω9*c*, $C_{16:0}$ and $C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c*. Phosphatidylcholine and phosphatidylglycerol were the major polar lipids. Unidentified phospholipids were also present. The 16S rDNA sequence analysis confirmed the affiliation of these organisms to the order *Planctomycetales,* genus *Rhodopirellula*, with *R. baltica* SH1^T, the only described species of the genus, as the closest phylogenetic relative. The analysis of a partial sequence of the gene encoding the β-subunit of RNA polymerase (*rpoB*) confirmed the phylogenetic separation of the isolates into two different species of the genus *Rhodopirellula*. Available 16S rRNA sequences of strains of group B demonstrate an occurrence across the world, whereas group C was not observed before.

On the basis of physiological, biochemical, chemotaxonomic and genetic characteristics we propose that our isolates represent two new species of *Rhodopirellula, Rhodopirellula rubra* sp. nov. (type strain is LF2^T) and *Rhodopirellula lusitana* sp. nov. (type strain is UC17^T).

[2] The results present in this chapter were accepted for publication in Systematic and Applied Microbiology.
Joana Bondoso, Luciana Albuquerque, Alexandre Lobo-da-Cunha, Milton S. da Costa, Jens Harder and Olga Maria Lage. Rhodopirellula lusitana sp. nov. and Rhodopirellula rubra sp. nov., isolated from the surface of macroalgae. Systematic and Applied Microbiology

Introduction

Rhodopirellula is a widespread marine genus of *Planctomycetes* as revealed by molecular techniques as well as isolation and cultivation studies. This genus was only proposed in 2004 emerging from the genus *Pirellula* (Schlesner et al., 2004). Successful isolation of strains of *Rhodopirellula* has been achieved from samples from brackish and marine waters, sediments, macroalgae biofilms and invertebrates (Schlesner, 1994; Fuerst et al., 1997; Pimentel-Elardo et al., 2003; Gade et al., 2004; Winkelmann and Harder, 2009; Lage and Bondoso, 2011; Lage et al., 2012) but so far only one species was described (Schlesner et al., 2004). *R. baltica* remains one of the best studied planctomycetes and was the first planctomycete with a complete genome sequenced (Glockner et al., 2003). Further characterization of new species within this genus will elucidate the ecological role of these organisms in the marine environment. A large genetic diversity of strains, not yet studied, has been encountered in this genus, as revealed previously by Winkelmann et al. (Winkelmann et al., 2010) and Lage and Bondoso (Lage and Bondoso, 2011). In this study we characterize two novel species of the genus *Rhodopirellula.* The strains were isolated from the epiphytic community of macroalgae from the north coast of Portugal and represent groups B (type species is LF2^T) and C (type species is UC17^T) as designated in Lage and Bondoso (Lage and Bondoso, 2011).

Material and methods

The organisms used in this study were strains LF2 ^{T}, FC3, FC19, Gr18, MsF5, and UC9 (representing group B), UC17^T, CcC6, CcC8, FC25, FC26, Sm2, Sm4, UC13, UC16, UC20, UC22, UC28, UC31, UC33, UC36 and UF6 (representing group C). Sampling locations, isolation media and macroalgal hosts are shown in Supplementary Table S1. Cultures were routinely maintained on modified M13 agar medium (Lage and Bondoso, 2011) at 25 ºC in the dark. The type species *Rhodopirellula baltica* SH1^T (DSM 10527^T) was used for comparison.

Morphology was observed by optical microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) during exponential phase of growth in M13 medium. For SEM, cells were fixed in 2.5 % glutaraldehyde in Marine buffer (Watson et al., 1986), pH 7.0 for 2.5 h, dehydrated through a graded ethanol series, critical point dried and observed in a HITACHI S-570. For TEM, cells were processed as described by Bondoso et al.(Bondoso et al., 2011).

Unless otherwise stated, all physiological tests were performed in triplicate, in modified M13 medium at 25 ºC. Liquid cultures were agitated at 200 rpm. Results were

recorded visually after seven days or by measuring the turbidity of liquid cultures at 600 nm. The growth temperature was tested in 5 ºC increments ranging from 5 to 45 ºC. The pH range for growth was determined in 20 mL tubes using 10 mM MES for pH 4.5, 5.0, 5.5, 6.0 and 6.5, Tris-HCl for pH 7.5 and 8.5, CHES for pH 9.0, 10.0, 10.5 and CAPS for pH 11.0. The ability to grow with NaCl instead of seawater salts was performed by replacing Artificial Sea Water (ASW; (Lyman and Fleming, 1940)) by 3.3 % NaCl. Growth at different salinities was assessed in ASW based M13 media with increasing proportions of ASW ranging from 0 to 300 % (100 % ASW corresponds to 34.5 ‰ salinity) and with ASW supplemented with NaCl ranging from 3.5 to 10 %. Medium M20c (Schlesner et al., 2004) was used to test requirement for vitamin and results were observed after two transfers.

Enzymatic profiles of the strains were determined using API ZYM and API 20NE systems and standard plate methods (Skerman, 1969; Tindall et al., 2007) for starch, carboxymethyl cellulose, elastin, alginate, casein and xylan. Inoculation of the test strips was done as described by the manufacturer except that the cells were resuspended in 20 ‰ marine salts (Sigma) (MacDonell et al., 1982) and the results were observed after 10 days for API 20NE and 48 h for API ZYM. Catalase and oxidase activities were determined using traditional methods (Tindall et al., 2007). Assimilation of single-carbon sources was tested in strains LF2^T and UC17^T in liquid medium composed of 90 % Natural Sea Water (NSW) buffered with 5 mM Tris-HCl, pH 7.5, 0.05 % $NH_4(SO4)_2$, and 0.05 % Na_2HPO_4 supplemented with filter-sterilized Hutner's basal salts (20 ml l^{-1}), vitamin solution (10 ml l^{-1}) and 0.1 % (w/v) of the carbon source. The ability to oxidize carbohydrates was evaluated with the BIOLOG GN2 MicroPlate System using 150 µL of exponentially growing cultures (OD_{590} =0.3), previously resuspended in sterile 0.75 % ASW without $CaCl_2$ (Makemson et al., 1998) and results were observed after 10 days. Production of acid from carbohydrates was determined with the API 50 CH system (bioMérieux) using cells resuspended in glucose-free M13 medium (OD_{600} =0.3) prepared with 50 % ASW and supplemented with 0.01 g l^{-1} phenol red. The production of acid was observed after 60 h. Growth under anoxic conditions was assessed on modified M13 media agar plates (Lage and Bondoso, 2011) in anaerobic chambers (GENboxanaer; bioMérieux) for three weeks.

Nitrogen source utilization by the isolates LF2^T and UC17^T was determined in 20 ml screw capped tubes with medium containing 90 % NSW buffered with 5 mM Tris-HCl, pH 7.5, 0.05 % glucose, 0.005 % Na_2HPO_4, supplemented with Hutner's basal salts (20 ml l^{-1}), vitamin solution (10 ml l^{-1}), and 0.1 % of each of the twenty natural amino acids,

as well as peptone, yeast extract, casamino acids, N-acetyl-glucosamine (NAG), urea, ammonium, nitrate or nitrite.

Cultures for chemotaxonomic analysis were grown in modified M13 medium at 26 ºC until late exponential phase. Cells for polar lipid analysis were harvested by centrifugation, washed in Tris-HCl 0.1 M, pH 7.5 and resuspended in the same buffer. Lipid extraction and two dimensional thin-layer chromatography (TLC) were performed as described previously (da Costa et al., 2011a). Lipoquinones were extracted from lyophilised cells, purified by thin-layer chromatography and separated by high-performance liquid chromatography (HPLC) (da Costa et al., 2011b). Fatty acid methyl ester (FAMEs) compositions were obtained from fresh wet biomass, separated, identified and quantified using the standard MIS Library Generation Software (Microbial ID) as described previously (da Costa et al., 2011c) .

Determination of the G+C content was performed by HPLC (Mesbah et al., 1989) after DNA extraction (Nielsen et al., 1995).

The phylogeny and taxonomic position of the novel isolates was assessed by means of 16S rRNA gene sequencing as described by Lage and Bondoso (Lage and Bondoso, 2011). In order to confirm the genetic distinctiveness of the novel isolates from the closest species *R. baltica* SH1^T, a 1200-bp fragment of the gene encoding the RNA polymerase beta subunit (*rpoB*) of strains LF2^T and UC17^T was amplified with the pair of primers *rpoB* 2001F (5´ ATGGGITCIAGCARCG 3') and *rpoB* 3302R (5´ ATCTGICCCACGTTCATMCG 3') as described by Bondoso et al. (Bondoso et al., 2013). Genomic fingerprinting profiles of the strains were obtained by amplifying genomic DNA with the pair of primers ERIC1 and ERIC2 as described by Lage et al. (Lage et al., 2012).

For DNA-DNA hybridisation (DDH), cells were disrupted by using a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (Cashion et al., 1977). DDH was carried out as described by De Ley et al. (De Ley et al., 1970) with the modifications described by Huss et al. (Huss et al., 1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with in-situ temperature probe (Varian).

Results and discussion

Based on the 16S rDNA sequence analysis, the strains were phylogenetically distinct from *R. baltica* SH1^T (Fig. 4.1). Isolates of groups B and C shared, respectively, 97.8-98 % and 97.6-97.7 % in the 16S rRNA gene sequence similarity with *R. baltica* SH1^T and formed two separated branches in the phylogenetic tree with high bootstraps values. Isolates within each of groups B and C shared, respectively, 99.4-100 % and 99.6-100 % pairwise sequence similarity in the 16S rRNA gene, thus representing several strains inside the same group. Genetic discrimination was achieved with the ERIC-PCR, which confirmed the uniqueness of each strain under study (Supplementary Fig. S2).

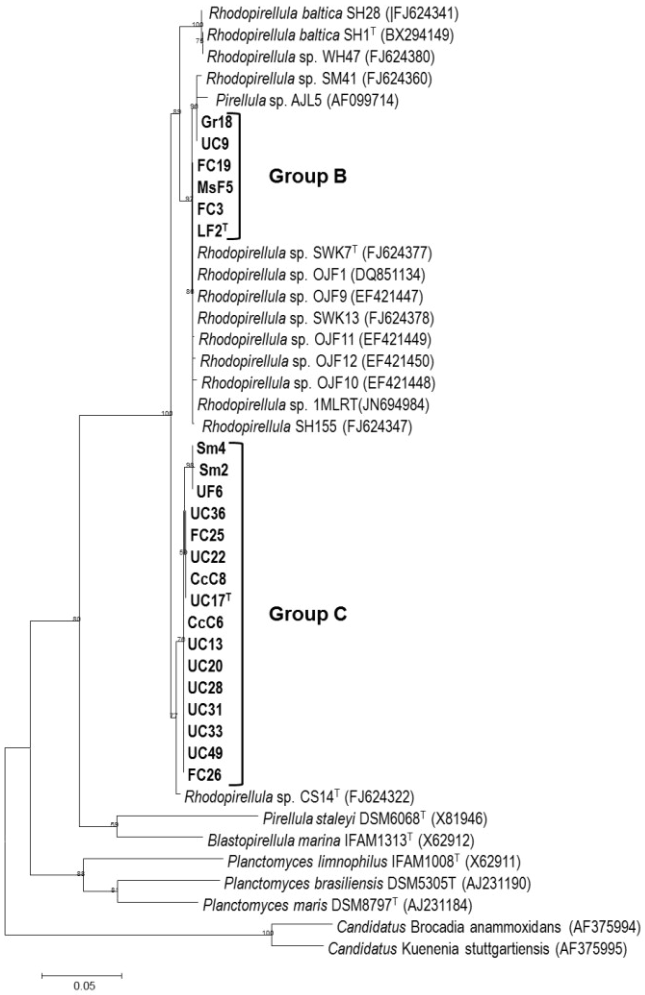


Fig. 4. 1 Maximum likelihood 16S rRNA gene phylogenetic tree showing the relationships of strains belonging to groups B and C with other representatives of the phylum *Planctomycetes* (accession numbers are shown in parenthesis). Numbers on the tree refer to bootstrap values based on 1000 replicates, only values above 50% are shown. Planctomycetes of the 'Anammox' group were used as outgroup. Bar, 0.05 substitutions per nucleotide position.

A partial fragment of the *rpoB* gene was amplified in order to confirm the separation of strains LF2^T and UC17^T in two distinct species of *Rhodopirellula*, since the 16S rRNA gene pairwise value of both strains to *R. baltica* SH1^T was higher than the 97 % cut-off previously suggested for species separation (Tindall et al., 2010). Based on several studies that used the *rpoB* gene as an identification tool for bacteria, Adekambi (2009) (Adékambi et al., 2006) suggested that the species delineation cut-off value should be around 96-97 % when at least an 825-bp fragment is analysed. The 1200-bp fragment of the *rpoB* gene of strains LF2^T and UC17^T shared only 77 % and 80 % to the partial *rpoB* of *R. baltica* SH1^T and 79 % between them and therefore confirmed the separation of two novel species.

While group C strains were never isolated before or found in other molecular studies, group B appears to be a widespread group of planctomycetes. It also comprises strains OJF1 and OJF9-12, which were isolated from the sediment of a water recycling treatment tank of a fish farm (Lage et al., 2012) and shared a 16S rRNA gene sequence similarity higher than 99.8 % with strains of group B. Strains SWK7 and SWK13, isolated by Winkelmann et al. (Winkelmann and Harder, 2009) from algae samples from Tjärnö, Sweden, were also included in group B sharing a 16S rRNA gene similarity of 99.2 % with our strains. The close relationship of these strains was confirmed by DNA-DNA hybridization (DDH) for the pair of strains LF2^T and SWK13 leading to a similarity of 67.7 %, which is close to the 70 % limit separation of two species (Goris et al., 2007). Analysis of the partial *rpoB* gene supported the inclusion of strains LF2^T, SWK7 and SWK13 in the same species, with a pairwise sequence similarity of 99 %. Furthermore, the genome of SWK7 shared a 70.42 % average nucleotide identity (ANI) to the genome of *R. baltica* SH1^T which confirmed the separation of group B in a different species (Richter and Rossello-Mora, 2009).

The morphology of colonies as well as their pigmentation varied among the strains. When growing in modified M13 agar medium, the majority of the isolates presented small, granular and convex colonies while the isolates CcC8, FC25, Sm2 and MsF5 formed irregular and mucilaginous colonies. The isolates belonging to group B formed reddish colonies, while in group C the pigmentation of the colonies was pink. All the isolates formed *Pirellula*-like cells that reproduced by budding (Fig. 4.2). Differences in the cellular morphology were visible in the shape, size and clustering. In group B, cells were pear- and club-shaped forming aggregates with a large number of cells (>50 cells) (Fig. 4.2a), while group C cells varied from ovoid to pear-shaped and rosettes usually varied from 4 to 25 cells (Fig. 4.2b), resembling the general shape of *R. baltica* SH1^T.

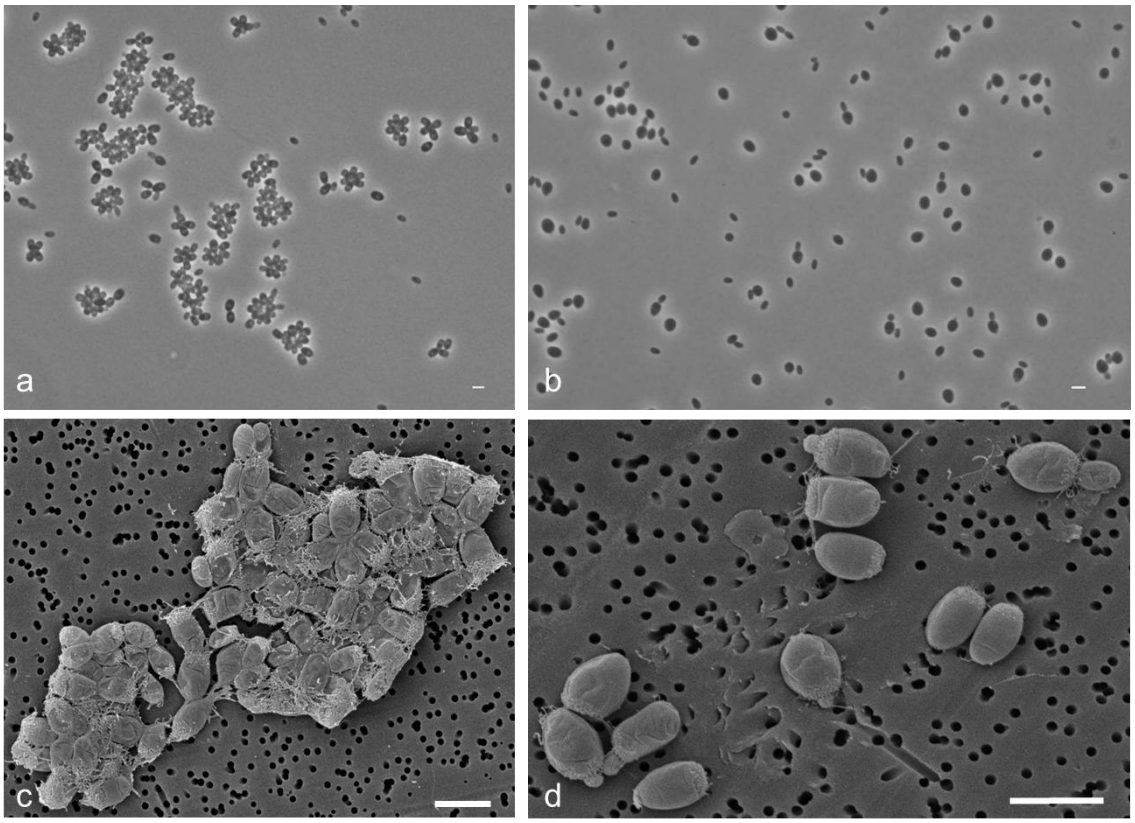


Fig. 4. 2 Morphological characteristics of strains LF2^T (a and c) and UC17^T (b and d) observed by optical microscopy (a and b) and scanning electron microscopy (c and d). Cells of the strains are ovoid to pear-shaped and several budding cells are seen. Characteristically, strain LF2^T presents rosettes with a higher number of cells (a and c) and prominent fimbriae (c).

In group B, rosettes of 4-8 cells were visible in the beginning of the exponential phase (Fig. 4.2a), while in group C the cells were predominantly single (Fig. 4.2b); rosettes were only seen in late exponential phase. As observed by SEM, both groups possessed fimbriae on the reproductive pole of the cells and a holdfast on the opposite pole where the cells joined to form rosettes (Fig. 4.2a and 4.2c). Fimbriae in LF2^T, located at the reproductive pole were prominent, large and possessed a ring-like structure at their base and insertion in the cell (Fig. 4.3a). Thinner pili were also present. In UC17^T, fimbriae were less evident and mainly composed by smaller pili (Fig. 4.3b). LF2^T had very electron transparent and extensive paryphoplasm (Pa). The pirellulosome, often divided in several sections, had condensed DNA, ribosomes and several inclusions of unknown nature. Strain UC17^T had a smaller electron dense paryphoplasm and a well-developed pirellulosome (Fig. 4.3b). Crateriform pits, typical of *Planctomycetes* were clearly seen on the reproductive pole of group B (Fig. 4.3a) but not detected by TEM in group C.

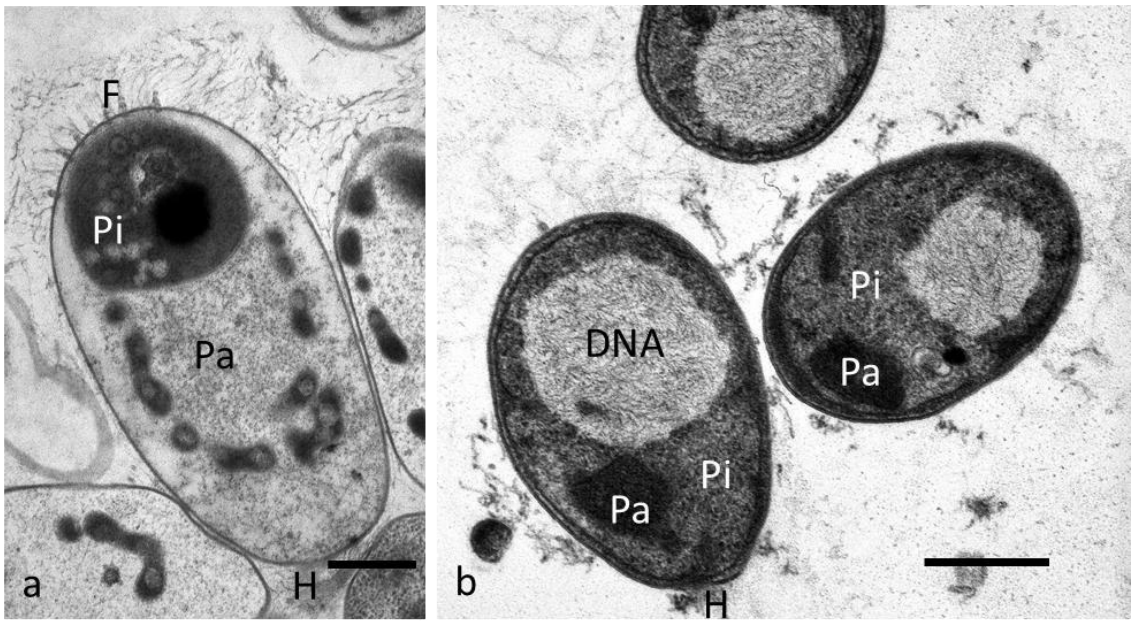


Fig. 4. 3 Transmission electron microscopy of strains LF2^T (a) and UC17^T (b). Typical planctomycete cell plan divided in the paryphoplasm (Pa) and the pirellulosome (Pi) is evident. The polar fimbriae are well evident in strain LF2^T. Holdfast (H) and condensed DNA can also be seen.

Both groups of strains had doubling times between 8.5 and 14 hours. Minimal temperature for growth was 5 ºC and strains grew up to 30 or 35 ºC. Seawater was essential for growth and a minimum of salinity from 10 to 25 % ASW was required. The maximum ASW for growth was 150-175 % and 4 % NaCl in group C and 4 or 5 % in group B. Group B had a lower requirement for salinity as well as a higher NaCl tolerance. Strains from group B and C grew from pH 5 to 10.5.

The isolates were cytochrome oxidase and catalase positive and hydrolysed esculin but not urea, casein, elastin, alginate or xylan. Group B isolates had the ability to degrade gelatine, with the exception of strain FC3. The majority of the strains from group C as well as strain MsF5 did not hydrolyse starch, while LF2^T, UC9 and FC19 did. Carboxymethyl cellulose was only degraded by strains of group C and MsF5. Production of indole from tryptophan and arginine dihydrolase were negative in all the strains. Nitrate was reduced to nitrite with the exception of strains Sm2, Sm4, UF6 and FC19.

API ZYM enzymatic profiles of strains of group B were very similar in all the strains with the exception of α-galactosidase, β-glucosidase and N-acetyl-β-glucosamidase activities; in strains of group C, differences in enzymatic activities were more evident (Supplementary Table S2). Comparatively to the type strain *R. baltica* SH1^T some differences were observed (Table 4.1) regarding trypsin, β-glucosidase, N-acetyl-β-glucosaminidase and α-fucosidase. With the exception of strains FC25 and CcC8 as

well as the type strain *R. baltica* SH1^T, the ability to grow under anaerobic conditions on the GENbox anaer system (bioMérieux) was observed. Glucose was not fermented by any strain.

Table 4. 1 Differential characteristics between the two novel species R*hodopirellula lusitana* and *Rhodopirellula rubra* and *Rhodopirellula baltica*.

	Rhodopirellula baltica	***Rhodopirellula rubra***	***Rhodopirellula lusitana***
Cell size (µm)	1.0–2.5x1-2–2-3[a]	1.3-2.5 x 0.9-1.7	1.2-2.1 x 0.8-1.7
Cell shape	Pear-shape to ovoid [a]	Pear-shape	Pear-shape to ovoid
Pigmentation	Pink to Red [a]	Red	Pink to Red
Seawater requirement	Yes	Yes	Yes
Salinity tolerance range:			
SW range (% v/v)	10-175	5-150	15-150
NaCl tolerance (% w/v)	5	5	4
Temperature for growth (ºC):			
Range	5-30	5-35	5-30
Optimum	28[a]	25	25
pH range	5.5-10.5	5-9.5	5-10
Vitamin requirement	B12	B12	B12
Carbon sources			
Ribose	+	-	-
Raffinose	-	+	-
Mio-inositol	-	-	+
Dextran	+	+	-
Mannitol	-	+	+
Nitrogen sources			
Glutamic acid	-	-	+
Methionine	-	-	+
Arginine	+	+	-
Ornithine	-	+	-
Proline	+	-	+
Serine	-	+	-
Nitrate	-	+	+
Nitrite	-	-	+
API 50CH			
Methyl-ßD-Xylopyranoside [b]	w	+	-
L-Arabinose	-	+	+
Glycogen [b]	+	-	-
Xylitol [b]	+	-	-
Sorbitol	-	+	+
potassium5-KetoGluconate [b]	w	+	-
API ZYM			
ß-glucosidase [b]	+	-	-
Trypsin [b]	+	-	-
N-acetyl-ß-glucosaminidase	+	+	-
α-fucosidase [b]	-	-	+
Hydrolysis of			
Starch	+	+	-

Carboxymethyl cellulose	-	-	+
Gelatine	-	+	-
Biolog GN			
Dextrin [b]	+	-	-
L-arabinose [b]	+	+	-
Mannitol	+	-	+
D-psicose	-	+	-
D-galactonic acid lactone	-	+	+
Succinic Acid [b]	-	+	+
α-keto glutaric acid [b]	-	+	+
Succinamic Acid [b]	-	+	-
L-alaninamide [b]	-	+	-
L-alanine [b]	-	+	-
L-alanylglycine	+	+	-
Glycil-L-glutamic acid	-	+	-
Uridine [b]	-	+	+
Glycerol	+	+	-
FAMEs			
$C_{12:0\ 3OH}$	-	0.1	0.4
$C_{15:0}$	0.5	0.6	0.3
$C_{16:0}$	26.8	24.1	22.9
summed feature 3 [b]	17.9	16.7	18.8
summed feature 3 [d]	-	3.8	-
$C_{17:1\omega8c}$	5.1	3.2	2.7
$C_{17:0}$	1.0	0.5	0.5
$C_{18:1\omega9c}$	41.6	42.7	46.4
$C_{18:0}$	1.3	2.6	3.2
Phospholipids			
PC	+	+	+
PG	++	++	++
DPG	++	+	++
PL1	+	+	++
UL1	+	+	++
UL2	+	+	-
UL3	+	+	+
UL4	+	-	+
APL	+	-	-
DNA G+C content	54.1 %	56.1 %	54.6 %
Nitrate reduction to nitrite	-	+	+
Anaerobic growth	No	Yes	Yes

+, positive; -, negative; ++, strongly positive
All the strains assimilate NAG, fructose, galactose, rhamnose, glucose, xylose, sucrose, maltose, celobiose, trehalose and lactulose. None of the strains utilize casamino acids, fucose, arabinose, sorbose, ribitol, sorbitol, erithritol, arabitol, glycerol, succinate, ketoglutarate, malate, pyruvate, citrate, acetate, benzoate, fumarate, formate and inulin. All the strains utilize as nitrogen sources aspartate, alanine, asparagine, phenylalanine, glutamine, casamino acids, yeast extract, peptone and ammonium. The following were not utilized: gluconate, cysteine, cystine, guanine, histidine, lysine, tyrosine, tryptophan, valine, urea and nitrite.
[a]Data from Schlesner et al., (2004)
[b] indicates characteristics that can vary among strains
[c,d]summed feature 3 comprises $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c.

Strains were able to oxidize several carbon substrates using the BIOLOG GN2, but not polymers, brominated chemicals and amines. Differences observed among strains are shown in Supplementary Table S2. The general carbohydrate oxidation profile of the two groups is similar to the one of the type strain of *R. baltica* with a few differences shown in Table 4.1. All strains of groups B and C produced acid from 31 and 32

carbohydrates using the API 50 CH, respectively (Supplementary Table S2). Many carbohydrates served as single carbon and energy sources for strains LF2^T and UC17^T but organic acids or amino acids did not. However, the organisms utilised several amino acids and proteinaceous compounds as a source of nitrogen. Ammonium and nitrate were utilized but urea was not. Nitrite was only utilized by strain UC17^T.

Phospholipid profiles of *R. baltica* SH1^T, LF2^T and UC17^T were similar; phosphatidylcholine (PC), phosphatidylglycerol (PG) and phospholipid 1 were major polar lipids (Fig. 4.4). UC17^T also possessed unknown lipids 1, 2, 3, 4 and diphosphatidylglycerol (DPG) and LF2^T possessed unknown lipid 1, 2, 3 and DPG that was also a major component.

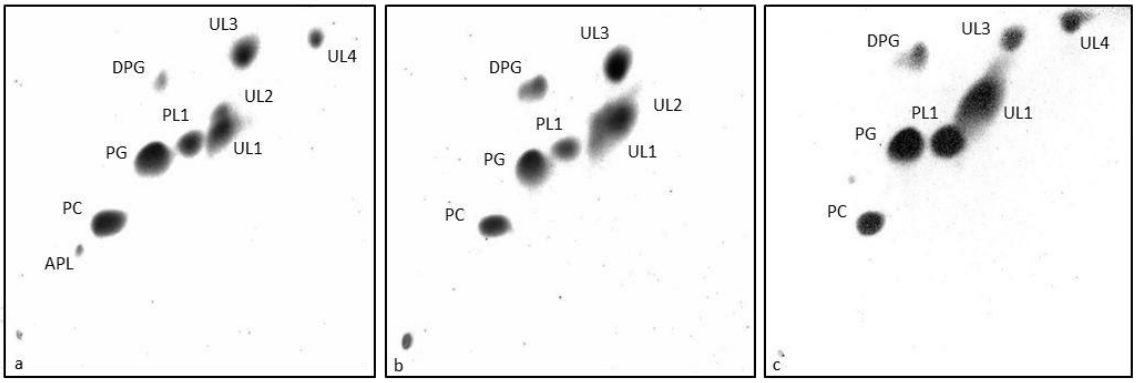


Fig. 4. 4 Two-dimensional thin-layer chromatography of polar lipids of *Rhodopirellula baltica* DSM 10527T (a), strain LF2^T (b) and strain UC17^T (c) at 26 ºC in modified M13. The lipids were stained by spraying with 5% molybdophosphoric acid in ethanol followed by heating at 160 ºC. PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; APL, aminophospholipid; PL1, phospholipid 1; UL1,2,3,4, unknown lipid 1,2,3,4.

The only respiratory lipoquinone detected in all the strains was menaquinone 6 (MK-6). The fatty acid composition of strains of both groups showed a predominance of $C_{18:1}$ ω9*c,* $C_{16:0}$ and $C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c* (Supplementary Table S3). These fatty acids were also the three major fatty acids of *R. baltica* SH1^T, although their relative abundances were different in each group (Table 4.1).

The mole G+C content of the DNA of groups LF2^T and UC17^T varied between 55.7 and 56.1 % and 54.6 and 55.1 %, respectively. *R. baltica* SH1^T had a mole G+C content of 54.1 % which is close to the 55 % value described in the literature (Schlesner et al., 2004).

Conclusions

Based on phylogenetic distinctiveness evaluated by the 16S rRNA and *rpoB* genes sequences similarities that confirmed the genetic separation of the two groups from *R. baltica* SH1^T, ultrastructural differences, chemotaxonomic differences of the phospholipid profiles and fatty acid composition, and metabolic properties namely nitrate reduction to nitrite and microaerobic growth (summarized in Table 4.1), we are of the opinion that groups B and C represent novel species within the genus *Rhodopirellula*, for which we propose the names *Rhodopirellula rubra* sp. nov. with LF2^T as the type strain and *Rhodopirellula lusitana* sp. nov. with UC17^T as the type strain, respectively.

Emended description of the genus *Rhodopirellula* (Schlesner et.al. 2004)

Rhodopirellula [*Rho.do.pi.rel'lu.la*. Gr. neut. n. *rhodon* a rose; N.L. fem. n. *Pirellula* name of a bacterial genus; N.L. fem. n. *Rhodopirellula* a red *Pirellula*.]

Cells are round, ovoid, ellipsoidal or pear-shaped, occurring singly or in rosettes by attachment at the smaller cell pole. Buds are formed at the broader cell pole. Buds may have a single flagellum inserted subpolarly at the proximal pole. Adult cells are immobile. Crateriform structures and fimbriae are found in the upper cell region. Colonies are pink to red in colour. Non-sporulating. Strictly aerobic or facultative anaerobic. Catalase- and cytochrome oxidase-positive. The proteinaceous cell wall lacks peptidoglycan. The major polyamines are putrescine, cadaverine and sym-homospermidine. The major menaquinone is MK-6. The major fatty acids are 16 : 1Δ9, 16 : 0,17 : 1Δ9, 17 : 0, 18 : 1Δ9, 18 : 1Δ11 and 18 : 0. The major phospholipids are phosphatidylcholine and phosphatidylglycerol. Additional, unidentified polar lipids are also present. This genus is a member of the phylum *Planctomycetes*, order *Planctomycetales*, family *Planctomycetaceae*, as currently defined primarily on the basis of 16S rRNA gene sequence analysis. The type species is *Rhodopirellula baltica*.

Description of *Rhodopirellula rubra* sp. nov.

Rhodopirellula rubra [ru' bra L. fem. adj. *rubra*, red, the color of cell colonies of the type strain].

The species possesses the following characteristics in addition to those described for the genus. Cells are pear- or club-shaped with 1.3-2.5 x 0.9-1.7 µm. Form rosettes with up to 50 cells. Colonies in M13 are red and variable in morphology and consistency. Optimum growth temperature is about 25 ºC (temperature range between 5 ºC and 35 ºC). pH range is between 5 and 9.5; optimum pH is around 7. Require sea salts to grow (minimum 5 % ASW) and maximum salinity tolerated is 150 %. Alkaline

phosphatase, esterase (C4), esterase lipase (C8), leucinearylamilase, valinearylamidase, cysteine arylamidase, acid phosphatase, β-galactosidase, α-glucosidase are positive in API ZYM. Some strains were also positive for α-galactosidase, β-glucosidase, N-acethyl-β-glucosaminidase and α-fucosidase. Hydrolyses esculin but not urea, casein, elastin, xylan and alginate. Starch, carboxymethyl cellulose and gelatine are degraded in some strains. Indole is not produced. Arginine dihydrolase is absent. The ability to reduce nitrate to nitrite is present in some strains. N-acetylglucosamine, D-fructose, D-galactose, L-rhamnose, D-glucose, xylose, raffinose, sucrose, maltose, lactose, D-cellobiose, D-trehalose, D-mannitol, lactulose and dextran are assimilated. L-fucose, D-ribose, D-arabinose, L-sorbose, ribitol, sorbitol, myo-inositol, erythritol, D-arabitol, glycerol, succinate, α-ketoglutarate, lactate, malate, citrate, benzoate, fumarate, formate, acetate, pyruvate and inulin are not assimilated. Amino acids and casamino acids are not assimilated. Acid is produced from D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, methyl-βD-xylanopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, D-melizitose, D-raffinose, gentiobiose, D-turanose, D-lyxose, D,L-fucose and potassium 5-ketogluconate in the API 50 CH. Some strains also produce acid from D-manitol, methyl-α-D-mannopyranoside, starch, xylitol, D-tagatose and L-arabitol. The organism oxidizes N-acetylglucosamine, cellobiose, D-fructose, L-fucose, D-galactose, gentibiose, D-glucose, α-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melobiose, β-methylglucoside, D-psicose, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, succinic acid mono-methyl-ester, D-gluconic acid, D-glucoronic acid, D,L-lactic acid, succinic acid, succinamic acid, glucoronamide, alaninamide, L-alanine, L-alanylglycine, glycyl-L-glutamic acid uridine and glycerol on the BIOLOG GN2 System. Some strains also oxidize N-acetyl-D-galactosamine, L-arabinose, D-sorbitol, acetic acid, D-galactonic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, L-proline, L-pyroglutamic acid, L-serine, thymidine, 2,3-butanediol, D,L-α-glycerol phosphate and α-D-glucose-phosphate. Aspartate, asparagine, arginine, alanine, phenylalanine, glutamine, ornithine, serine, NAG, peptone, yeast extract, casaminic acids, nitrate and ammonium serve as sources of nitrogen. Urea and nitrite are not utilized. Vitamin B12 is required for growth. Some strains are able to grow under microaerophilic conditions. Major fatty acids are $C_{18:1}\omega9c$, $C_{16:0}$ and $C_{16:1}\ \omega7c$/$C_{16:1}\ \omega6c$.

Major polar lipids are phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol. Unknown lipids can also be present. The G+C mole content of the DNA of the type strain is 56.1 % ± 0.8. The type strain is LF2^T isolated from the epiphytic community of the macroalgae *Laminaria* sp..

Description of *Rhodopirellula lusitana sp. nov*.

Rhodopirellula lusitana [lu.si.ta'na. L. fem. adj. *lusitana*, pertaining to Lusitania, the Roman province in western Iberia].

The species possesses the following characteristics in addition to those described for the genus. Cells are ovoid or pear-shape with 1.2-2.1 x 0.8-1.7 µm and possess a dimorphic life cycle. Form rosettes of 4 to 25 cells. Colonies in M13 are pink and variable in morphology and consistency. Optimum growth temperature is about 25 ºC (temperature range between 5 ºC and 30 ºC). pH range in between 5 and 10; optimum pH about 7. Require sea salts to grow (minimum of 15 % ASW) and maximum salinity tolerated is 150 %. Esculin and carboxymethyl cellulose are degraded; casein, elastin, gelatine, starch, urea, xylan and alginate are not degraded. Alkaline phosphatase, leucine arylamilase, valine arylamidase, acid phosphatase are positive in API ZYM. Some strains were also positive for esterase lipase, lipase, cysteine arylamidase, trypsin, α-galactosidase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-fucosidase. Indole is not produced. Arginine dihydrolase is absent. Some strains reduce nitrate to nitrite. N-acetylglucosamine, D-fructose, D-galactose, L-rhamnose, D-glucose, xylose, sucrose, maltose, lactose, D-cellobiose, D-trehalose, D-mannitol, *myo*-inositol, lactulose are assimilated. L-fucose, D-ribose, D-arabinose, L-sorbose, D-raffinose, ribitol, sorbitol, erythritol, D-arabitol, glycerol, succinate,α-ketoglutarate, lactate, malate, citrate, benzoate, fumarate, formate, acetate, pyruvate, dextran, amino acids, casaminic acids and inulin are not assimilated. Acid is produced from D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-turanose, D-lyxose and L-fucose in the API 50 CH. Some strains also produce acid from D-sorbitol, methyl-βD-xylanopyranoside, D-manitol, methyl-αD-mannopyranoside, D-trehalose methyl-αD-glucopyranoside, D-melizitose, D-raffinose, starch, glycogen, gentiobiose, D-tagatose, D-fucose, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate. Oxidizes N-acetylglucosamine, N-acetylgalactosamine, cellobiose D- fructose, L-fucose, D-galactose, gentibiose, D-glucose, α-lactose,

lactulose, maltose, D-mannitol, D-mannose, D-melobiose, β-methylglucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, D-glucuronic acid and D,L-lactic acid on the BIOLOG GN2 System. Some strains also oxidize dextrin, sorbitol, turanose, psicose, xylitol, pyruvic acid methyl ester, acetic acid, D-galactonic acid lactone, γ-hydroxybutyric acid, D-gluconic acid, α-ketoglutaric acid, D,L-lactic acid, succinic acid, succinamic acid, glucoronamide, L-alaninamide, L-alanine, L-aspartic acid, L-glutamic acid, L-proline, L-serine, uridine, thymidine, 2,3-butanediol, α-D-glucose-phosphate and D-glucose-6-phosphate. Aspartate, glutamine, asparagine, alanine, phenylalanine, glutamine, methionine, proline, NAG, peptone, yeast extract, casaminic acids, ammonia, nitrate, nitrite and ammonium serve as sources of nitrogen. Urea is not utilized. Vitamin B12 is required for growth. Some strains can grow under microaerophilic conditions. Major fatty acids are $C_{18:1}\omega9c$, $C_{16:0}$ and $C_{16:1}\ \omega7c$/$C_{16:1}\ \omega6c$. Major polar lipids are phosphatidylcholine, phosphatidylglycerol and diphosphatidylglycerol. Unknown lipids are also present. The G+C mole content of the DNA of the type strain is 54.6 % ± 0.9.The type strain is UC17^{T}isolated from the epiphytic community of the green macroalgae *Ulva* sp..

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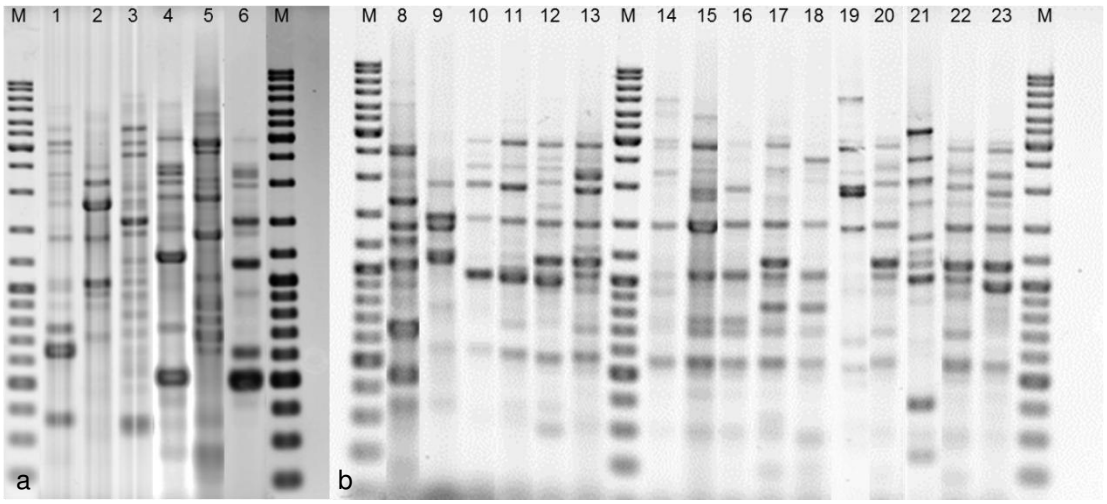
Supplementary material



Supplementary Fig. S 2 ERIC-PCR profiles of strains from group B (a) and group c (B). Lanes: M, DNA marker Gene Ruler Ladder Mix (Fermentas™); 1, LF2^{T}; 2, FC3; 3, FC19; 4, UC9; 5, MsF5; 6, Gr18; 8, Sm2; 9, FC25; 10, FC26; 11, UC13; 12, UC16; 13, UC17 ^{T}; 14, UC20; 15, UC22; 16, UC28; 17, UC31; 18, UC33; 19, UC36; 20, UF6; 21, CcC6; 22, CcC8.

Supplementary Table S 1 Strains under study designation and details of the isolation

Isolate	Macroalga host	Collection site	Isolation media[a]
LF2^T	*Laminaria* sp.	Foz, Porto	M13
FC3	*Fucus spiralis*	Carreço, Viana do Castelo	M514
FC19	*Fucus spiralis*	Carreço, Viana do Castelo	M13
Gr18	*Gracilaria bursa-pastoris*	Ria de Aveiro, Aveiro	M13
MsF5	*Mastocarpus stellatus*	Foz, Porto	M13
UC9	*Ulva* sp.	Carreço, Viana do Castelo	M13
Sm2	*Sargassum muticum*	Foz, Porto	M13
Sm4	*Sargassum muticum*	Foz, Porto	M590
FC25	*Fucus spiralis*	Carreço, Viana do Castelo	M629
FC26	*Fucus spiralis*	Carreço, Viana do Castelo	M629
UC13	*Ulva* sp.	Carreço, Viana do Castelo	M514
UC16	*Ulva sp.*	Carreço, Viana do Castelo	M629
UC17^T	*Ulva sp.*	Carreço, Viana do Castelo	M629
UC20	*Ulva sp.*	Carreço, Viana do Castelo	M629
UC22	*Ulva sp.*	Carreço, Viana do Castelo	M629
UC28	*Ulva sp.*	Carreço, Viana do Castelo	M13
UC31	*Ulva sp.*	Carreço, Viana do Castelo	M13
UC33	*Ulva sp.*	Carreço, Viana do Castelo	M13
UC36	*Ulva sp.*	Carreço, Viana do Castelo	M13
CcC6	*Chondrus crispus*	Carreço, Viana do Castelo	M629
CcC8	*Chondrus crispus*	Carreço, Viana do Castelo	M13
UF6	*Ulva sp.*	Foz, Porto	M629

[a] According to Lage and Bondoso (2011)

Supplementary Table S 2 Differential features of group B and C strains and *R. baltica* SH1^T (RB) in API 50CH, API ZYM and Biolog GN2.

	LF2 [1]	FC3	FC19	UC9	MsF5	Gr18	UC17[1]	SM2	SM4	FC25	FC26	UC13	UC16	UC20	UC22	UC28	UC31	UC33	UC36	CcC6	CcC8	UF6	RB
API 50 CH																							
Methyl-ßD-xylopyranoside	+	+	+	+	+	+	-	w	w	+	+	w	-	w	w	-	+	w	+	+	w	+	w
D-mannitol	-	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	w
D-sorbitol	-	-	-	-	-	-	-	w	-	-	-	w	w	-	-	-	w	-	-	-	-	-	-
Methyl-αD-mannopyranoside	-	-	+	+	+	+	+	w	-	+	+	w	+	+	w	+	+	w	-	w	w	-	+
Methyl-αD-glucopyranoside	+	+	+	+	+	+	+	w	-	+	w	w	+	+	w	+	+	w	-	w	w	-	+
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-melizitose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
Starch	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+
Xylitol	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Gentiobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	w	+
D-turanose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	w	+
D-tagatose	-	-	+	+	-	-	+	w	w	-	-	w	+	-	-	-	w	-	-	-	-	-	w
D-fucose	+	+	+	+	+	+	+	w	w	+	+	+	+	+	+	+	+	+	+	+	+	-	+
D-arabitoL	-	-	-	-	-	-	-	-	-	-	-	--	-	-	-	-	+	-	-	-	-	-	-
L-arabitoL	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	w
potassium 2-ketogluconate	-	w	-	-	-	w	-	w	w	-	w	w	w	-	w	-	+	w	w	w	w	w	w
potassium 5-Ketogluconate	+	+	+	+	+	w		w	w	w	-	w	-	+	w	+	+	w	-	w	w	-	w
Biolog GN2																							
Dextrin	-	-	-	-	-	-	-	+	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	+
N-Acetyl-D-galactosamine	-	-	+	-	-	-	+	+	+	+	nd	+	+	+	+	+	+	-	+	+	+	+	+
L-arabinose	+	+	+	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	+
D-psicose	+	+	+	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	+	+	+	+	+	-
D-sorbitol	-	+	+	+	-	+	+	+	-	-	nd	+	+	-	+	+	+	-	+	+	-	-	-
Turanose	+	+	+	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	-	+	+	+	+	+
Xylitol	-	-	-	-	-	-	-	-	-	-	nd	+	-	-	-	-	+	-	-	-	-	-	-
Pyruvic acid methyl ester	+	+	+	+	+	+	+	+	+	+	nd	+	-	+	+	-	+	-	-	-	+	+	+
Acetic acid	+	+	-	+	+	+	+	+	+	+	nd	+	+	+	-	-	+	+	+	+	+	+	+
D-galactonic acid lactone	+	+	+	+	-	+	+	-	-	+	nd	+	-	-	-	+	+	-	-	-	+	-	-
D-gluconic acid	+	+	+	+	+	+	+	-	-	+	nd	+	+	-	-	+	+	-	-	+	+	+	+
βhydroxybutyric acid	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
γHydroxybutyric acid	-	-	+	-	-	-	-	-	+	-	nd	-	-	-	-	-	-	-	-	-	-	-	-
α-Keto glutaric acid	+	+	+	+	+	+	+	-	+	-	nd	+	-	+	+	+	+	-	+	-	-	+	-
D,L-lactic acid	+	+	+	+	+	+	+	-	+	+	nd	+	+	+	+	+	+	-	+	+	+	+	+

Succinic acid	+	+	+	+	+	+	+	+	+	+	nd	-	+	+	-	+	+	+	+	-	+	+	-
Succinamic acid	+	+	+	+	+	+	-	+	+	-	nd	+	-	-	-	-	-	-	+	-	-	+	-
Glucuronamide	+	+	+	+	+	+	+	+	+	+	nd	+	-	+	+	+	+	-	+	-	-	+	+
L-alaninamide	+	+	+	+	+	+	-	-	-	-	nd	+	-	-	-	-	-	-	-	-	-	+	-
L-alanine	+	+	+	+	+	+	-	-	-	-	nd	-	-	-	+	-	-	-	-	-	-	-	-
L-alanylglycine	+	+	+	+	+	+	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	+
L-aspartic Acid	+	+	-	-	+	-	-	-	+	-	nd	+	-	-	-	-	+	-	-	-	-	+	-
L-glutamic acid	+	+	+	-	-	+	-	-	-	+	nd	-	-	-	-	-	-	-	-	-	-	+	-
Glycyl-L-aspartic acid	+	+	+	-	+	-	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-
Glycyl-L-glutamic acid	+	+	+	+	+	+	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-
L-proline	+	+	-	+	-	+	-	-	-	-	nd	-	-	+	+	-	-	-	-	-	-	-	-
L-pyroglutamic acid	+	-	-	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-
L-serine	+	-	+	-	-	-	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	+	-
Uridine	+	+	+	+	+	+	+	+	+	+	nd	+	+	+	+	+	+	-	+	w	+	+	-
Thymidine	+	+	-	+	+	-	+	+	+	+	nd	+	+	+	+	+	+	-	+	w	+	+	-
2,3-Butanediol	+	-	+	+	-	-	-	+	+	-	nd	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	+
D,L-α-glycerol phosphate	+	+	-	+	+	+	-	-	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-
α-D-glucose-phosphate	+	+	-	+	+	-	+	+	+	-	nd	+	+	+	-	-	+	-	+	-	-	+	-
D-glucose-6-phosphate	-	-	-	-	-	-	+	+	+	-	nd	+	+	+	-	-	+	+	+	-	-	+	-
API ZYM																							
Esterase (C4)	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase Lipase (C8)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
Lipase (C14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Cystine arylamidase	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Trypsin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
α-galactosidase	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+
ß-galactosidase	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+
α-glucosidase	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ß-glucosidase	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
N-acetyl-ß-glucosaminidase	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+
α-fucosidase	-	-	-	+	-	+	+	-	-	-	+	-	+	+	+	+	-	+	+	+	+	-	-

ND- not determined

W- weakly positive

All the strains produced acid from D-arabinose, L-arabinose D-ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-frutose, D-mannose, D-sorbose, D-rhamnose, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, L-fucose, L-lyxose, D-melibiose D-saccharose. Acid was not produced from glycerol, erythritol, D-adonitol, dulcitol, inositol, inulin and glycogen.

All the strains oxidized N-acetyl-D-glucosamine, D-cellobiose, D-frutose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, D-glucuronic acid,

None of the strains oxidized α-cyclodextrin, glycogen, tween 40 and 80, adonitol, D-arabitol, erythritol, m-inositol, Cis-aconitic acid, citric acid, formic acid, D-glucosaminic acid α-hydroxybutyric acid, p-hydroxy-phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, L-alaninamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, uridine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D-glucose-6-phosphate.

All the strains were positive for alkaline phosphatase, leucine and valine arylamidase, acid phosphatase. All the strains were negative for α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, ß-glucuronidase and α-mannosidase.

Supplementary Table S 3 Fatty acid composition of strains grown in liquid M13 medium at 26 ºC.

Data are percentages of total fatty acids. Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system.

Fatty acid	ECL[a]	RB	SM2	SM4	FC25	FC26	UC13	UC16	UC17[T]	UC20	UC22	UC28	UC31	UC33	UC36	CcC6	CcC8	UF6	LF2[T]	FC3	FC19	UC9	Gr18
UN 11.980[b]	11.980	0.6	0.4	0.3	2.0	1.0	0.5	0.7	0.5	0.8	0.5	0.7	0.5	0.5	0.7	0.8	0.8	0.4	0.4	1.1	0.4	1.2	1.5
12:0 3OH	13.455	-	0.2	0.2	-	0.4	0.3	0.3	0.4	0.4	0.2	0.4	0.3	0.3	0.4	0.3	0.3	0.3	0.1	0.3	0.2	-	-
14:00	14.000	0.5	0.8	0.9	1.1	0.6	0.6	0.8	0.6	0.7	0.7	0.8	0.6	0.7	0.7	0.7	0.7	0.8	0.8	0.6	0.5	1.3	1.4
15:00 isso	14.623	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15:00	14.999	0.5	0.2	0.2	1.4	-	0.7	0.3	0.3	0.3	0.4	0.3	0.4	0.3	0.5	0.4	0.6	0.2	0.6	0.6	1.0	0.9	1.1
16:0 N alcohol	15.549	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-
Sum in feature 3[c]	15.817	17.9	18.9	23.2	22.4	19.3	18.7	19.4	18.8	19.7	19.6	19.1	19.9	19.1	18.4	17.6	19.0	19.8	16.7	14.3	12.9	19.8	21.1
Sum in feature 3[c]	15.853	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.8	4.6	6.5	5.6	7.3
16:00	16.001	26.8	26.9	23.8	22.2	23.0	22.6	23.4	22.9	22.6	24.2	23.6	22.7	23.8	23.2	23.6	23.1	26.1	24.1	23.0	22.2	27.3	24.3
17:1anteiso ω9c	16.518	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-
17:1ω8c	16.792	5.1	1.3	1.5	8.9	2.6	5.0	2.1	2.7	2.3	2.7	2.1	3.3	2.3	3.5	3.0	4.1	1.4	3.2	3.9	6.2	3.0	3.5
17:00	17.000	1.0	0.5	0.3	1.4	0.6	0.8	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.6	0.9	0.4	0.5	0.8	1.1	0.7	1.2
16:0 2OH	17.233	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-
UN 17.365	17.365	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.5	-	-	-
18:1ω9c	17.770	42.6	44.2	41.8	36.2	46.9	43.4	45.8	46.4	46.5	45.0	45.6	46.3	47.3	46.1	45.6	43.9	43.4	42.7	42.1	42.4	37.4	35.6
Sum in feature 8[d]	17.824	2.8	1.5	1.5	1.6	1.9	2.0	2.9	1.9	2.6	2.3	2.3	1.6	1.8	1.7	2.2	2.7	1.8	1.5	3.2	1.5	1.4	1.3
17:0 ISI 2OH	17.875	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18:00	17.999	1.3	2.5	2.1	1.9	3.2	3.7	3.5	3.2	2.8	2.9	4.1	3.1	2.6	3.1	3.7	3.0	2.2	2.6	4.0	3.5	1.4	1.7
UN 18.796[e]	18.797	0.6	0.2	0.2	1.1	0.6	1.2	0.4	0.5	0.5	0.5	0.4	0.7	0.4	0.7	0.6	0.9	0.2	0.2	-	0.4	-	-
Sum in feature 7[f]	18.868	-	1.6	1.4	-	-	0.3	-	0.3	0.5	0.3	0.4	-	0.4	0.4	-	-	1.7	0.8	1.0	1.0	-	-
18:1 2OH	19.089	-	0.1	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.3	-	0.2	-	-
18:0 2OH	19.298	-	0.3	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.6	-	-	-	-
17:0 ISI 2OH		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19:00		-	-	-	-	-	-	-	-	-	--	-	-	-	-	-	-	-	-	-	0.2	-	-
20:1 ω9c	19.770	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-
20:00	20.000	-	0.5	1.5	-	-	0.2	-	-	-	0.3	0.3	-	-	-	-	-	0.6	0.1	-	-	-	-

[a] ECL, equivalent chain length.
[b] Unknown fatty acid with an equivalent chain length of 11.980.
[c] Sum Feature 3, 16:1 w7c/16:1 w6c
[d] Sum Feature 8, 18:1 w7c/18:1 w6c
[e] Unknown fatty acid with an equivalent chain length of 18.797.
Sum Feature 7, 19:0 cyclo w10c/19w6

Chapter 5.

Rhodopirellula formosa sp. nov., a novel species of *Rhodopirellula* isolated from macroalgae surfaces[3]

Abstract

Marine macroalgae are a known source of new bacteria. In this work we describe three isolates that have been recovered from the biofilm of two marine macroalgae from the north coast of Portugal. The isolates belong to the phylum *Planctomycetes*, as determined by the 16S rDNA sequence analysis, and are more closely related to the *Rhodopirellula* genus with 96.6 % pairwise similarity.

Colonies of the new strains are light pink in colour and the cells are round in shape and organized in rosettes with a large number of cells. Reproduction occurs by budding. They are facultative anaerobes, chemoheterotrophs and mesophiles with an optimum temperature of 30 ºC. They have a broad tolerance to salts and do not required sea salts to grow. Major polar lipids are phosphatidylcholine (PC), phosphatidylglycerol (PG). Major fatty acids are $C_{18:1}$ ω9*c* and $C_{16:0.}$ They also have $C_{16:1}$ ω11*c* and small amounts of 2- and 3- hydroxy fatty acids.

On the basis of physiological, biochemical chemotaxonomic and genetic characteristics we are of the opinion that the isolates represents new species of the genus *Rhodopirellula* for which we propose the name *Rhodopirellula formosa* sp. nov (type species is $FF4^T$).

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[3] The results present in this chapter are under preparation for submission

Introduction

Planctomycetes are part of the PVC superphylum and together with the *Verrucomicrobia*, *Chlamydiae* and *Lentisphaerae* (Wagner and Horn, 2006) have a deep-branching position within the *Bacteria*. The importance of this group lays down on several characteristics that are unexpected in prokaryotic organisms, like proteinaceous cell walls without peptidoglycan, budding reproduction, compartmentalized cell structure, endocytosis-like protein uptake and membrane coat-like proteins (Lonhienne et al., 2010; Fuerst and Sagulenko, 2012) (Santarella-Mellwig et al., 2010). Planctomycetes are cosmopolitan and have been described in association with eukaryotic hosts, like micro and macroalgae, invertebrates and sponges (Fuerst et al., 1997; Pimentel-Elardo et al., 2003; Tadonleke, 2007; Hempel et al., 2008; Kohler et al., 2008; Bengtsson and Ovreas, 2010; Bengtsson et al., 2010; Burke et al., 2011; Lachnit et al., 2011). Reports from culture dependent and independent methods studies showed that planctomycetes are extensively associated with several marine macroalgae (Egan et al., 2012), and, in some cases, they can be the dominant bacteria present, like in *Laminaria* sp. (Bengtsson and Ovreas, 2010). Recently a novel planctomycete with distinctive characteristics was isolated from the surface of *Porphyra* sp., and was placed in a new order, *Phycisphaerales* (Fukunaga et al., 2009). Although a considerable number of strains have been recently isolated, the characterization of new species or genera is still scarce. Thus, *Planctomycetes* as a group possess a relatively small number of taxa. Here, we describe three novel strains of *Planctomycetes* isolated from the epiphytic bacterial community of *Fucus spiralis* and *Ulva* sp. from the north coast of Portugal, belonging to the genus *Rhodopirellula*.

Material and Methods

The strains under study have been previously isolated (Lage and Bondoso, 2011) from the surface of *Fucus spiralis* (Strains FF4^T and FC92) and from the surface of *Ulva* sp. (UF2) . Strains FF4^T and FC92 were isolated in liquid M629 medium (5 g L^{-1} peptone, 0.5 g L^{-1} yeast extract, 20 ml L^{-1} Hutner's basal salts, 10 ml L^{-1} vitamin solution in 90 % natural seawater – NSW), and UF2 was isolated from liquid M514 medium (5 g L^{-1} peptone, 1 g L^{-1} yeast extract in 90 % natural seawater). The cultures were routinely maintained in modified M13 agar medium (Lage and Bondoso 2011) at 30 ºC, in the dark. The isolates were stored at -80ºC in sterile natural seawater - NSW with 20 % (w/v) glycerol. For comparison purposes *Rhodopirellula baltica* (DSM 10527^T- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was used.

Unless otherwise stated, all morphological, biochemical and physiological tests were performed in triplicate at 30ºC in M13 modified media with 1.6 % agar. Liquid cultures were agitated at 200 rpm. Results were usually recorded after 7-10 days. Cell morphology and motility was observed by optical microscopy during the exponential phase of growth and examined in a Zeiss Axioplan microscope. For scanning electron microscopy (SEM), exponential growing cells in liquid M13 medium were fixed in 2.5 % glutaraldehyde in Marine buffer (Watson et al., 1986), pH 7.0 for 2.5 h, dehydrated through a graded ethanol series, critical point dried and observed in a HITACHI S-570. For transmission electron microscopy (TEM), cells were harvested from 5 days old agar cultures, cryopreserved and further treated as described before (Bondoso et al., 2011). The growth temperature range was tested from 5 to 45 ºC in 5 ºC intervals in solid media. The NaCl tolerance was determined in solid medium prepared with Artificial SeaWater (ASW; (Lyman and Fleming, 1940)) and supplemented with NaCl concentrations ranging from 3.5 to 10 %. Salinity range was examined in solid medium with increasing proportions of ASW from 0-50 % and 90-300 % (100 % ASW corresponds to 34.5 ‰ salinity). The requirement for seawater was assessed by replacing the ASW solid medium by 3.3 % NaCl. The pH range for growth was determined in liquid medium using 10 mM of MES for pH 4.5, 5.0, 5.5, 6.0 and 6.5, Tris-HCl for pH 7.5 and 8.5, CHES for pH 9.0, 10.0, 10.5 and CAPS for pH 11.0 and was assessed by measuring the absorbance at 600nm. Vitamin requirement was determined in M20c medium (Schlesner et al., 2004) consisting of 0.1 % (w/v) glucose, 0.1 % (w/v) casamino acids and 2 % (v/v) Hutner's basal salts with 90 % (v/v) natural seawater. Different vitamins solutions (1 % (v/v), omitting one of each co-factors at a time) were added to media. Results were recorded after two transfers in the same medium to avoid false positives due to potential cell storage of the vitamins. Oxidase and catalase, hydrolysis of starch, casein, Tween 20, Tween 80, esculin, elastin, arbutin and xylan were determined using standard methods (Skerman, 1969; Tindall et al., 2007). Additional enzyme activities were assessed with the API ZYM and API 20NE systems (bioMérieux). Cell suspensions for API 20 NE and API ZYM were done in 20 ‰ marine salts (MacDonell et al. 1982) with a density of, respectively, 0.5 and 6 McFarland. Results were observed after 10 days and 48 h, respectively. Single-carbon source assimilation tests were performed in a liquid medium composed of 90 % NSW buffered with 5 mM Tris-HCl, pH 7.5, to which filter-sterilized 20 ml L^{-1}Hutner's basal salts, 0.5 g $L^{-1}NH_4(SO4)_2$, 0.05 g L^{-1} Na_2HPO_4, 10 ml L^{-1} vitamin solution and 1.0 g l^{-1} of the filter-sterilized carbon source. Growth of the strains on single carbon sources was

examined by measuring the turbidity of the cultures (600 nm) in 20 ml screw capped tubes containing 7 ml of medium for up to two weeks. Carbon source oxidation was assessed with the BIOLOG GN2 MicroPlate System. To inoculate the GN2 MicroPlate, liquid cultures were grown for 48h, centrifuged and suspended in sterile 0.75 % ASW without $CaCl_2$ (Makemson et al. 1998). The results were assessed after 10 days. Acid production from carbohydrates was determined by using API 50 CH system (bioMérieux) according to the manufacturer's instructions, using M13 medium without the addition of glucose, prepared in 50 % ASW and supplemented with 0.01 g L^{-1} phenol red. Results were recorded after 60 h. Anaerobic growth was assessed in cultures in solid medium incubated in anaerobic chambers (GENbox anaer; bioMérieux) for 3 weeks. Nitrogen source utilization was determined in medium containing 90 % NSW, 0.5 g L^{-1}glucose, 0.05 g L^{-1} Na_2HPO_4, 20 ml L^{-1} Hutner's basal salts and 10 ml L^{-1}vitamin solution, buffered with 5 mM Tris-HCl pH 7.5, and supplemented with 1.0 g L^{-1} of each of the twenty natural amino acids, as well as peptone, yeast extract, casamino acids, N-acetyl-glucosamine (NAG), urea, ammonium, nitrate or nitrite. The tests were performed in 20 ml screw capped tubes containing 7 ml of medium for one week. Growth was assessed by measuring the absorbance at 600 nm. Cultures for chemotaxonomic analysis were grown in liquid medium until late exponential phase at 26 ºC. Cells for polar lipid analysis were harvested by centrifugation, washed in 0.1 M Tris-HCl, pH 7.5 and suspended in the same buffer. Lipid extraction and two dimensional thin-layer chromatography (TLC) were performed according to da Costa et al. (2011a). Lipoquinones were extracted from lyophilised cells, purified by thin-layer chromatography and separated by high-performance liquid chromatography (HPLC) as described by da Costa et al. (2011b). Fatty acid methyl ester (FAMEs) composition was determined and quantified using the standard MIS Library Generation Software (Microbial ID) as described previously (da Costa et al., 2011c). For G+C content, DNA was isolated as described (Nielsen et al., 1995) and the content was determined by HPLC (Mesbah et al., 1989). The phylogeny and taxonomic position of the novel isolates was performed through 16S rRNA sequencing as described by Lage and Bondoso (2011).

Results and Discussion

Strains FF4^T, FC92 and UF2 form small, circular, convex and translucent colonies of light pink pigmentation in M13 medium (Fig. 5.1a). Cells were smaller than the ones of *R. baltica* SH1^T and mainly round in shape with 0.9-1.75 µm in diameter, although some pear-shaped cells were observed. The cells were organized in rosettes of a large number of cells (Fig. 5.1b). Reproduction occurs by budding (Fig. 5.1b and Fig. 5.2a).

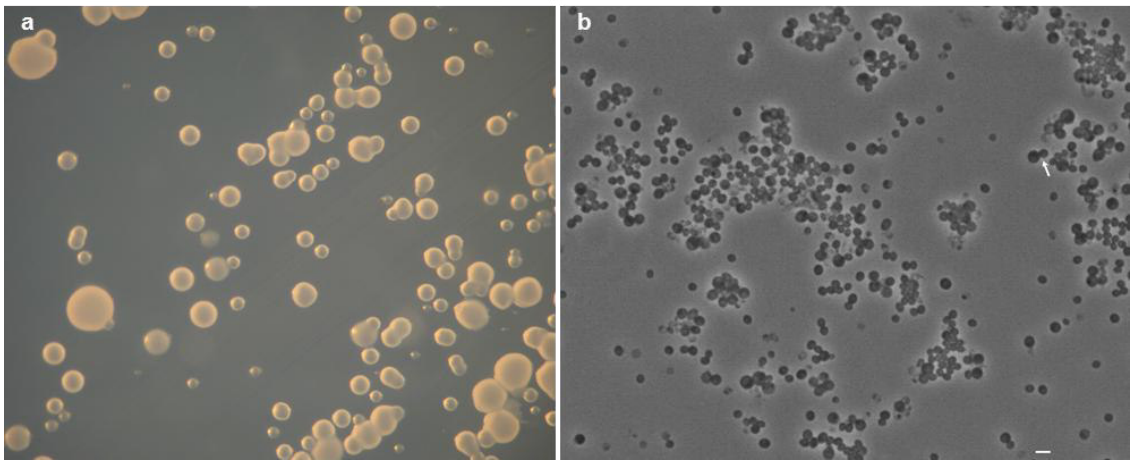

Fig. 5. 1 Morphological characteristics of strain FF4^T. (a) Colonies pink colored grown in M13 medium; (b) cell morphology under phase-contrast microscopy showing the round shape of the cells and the aggregates of variable number of cells. Arrow indicates budding. Bar, 2 mm.

SEM micrographs of strain FF4^T evidenced the round shaped of the cells (Fig. 5.2a) and the clustering of cells in the typical rosettes. Ultrathin sections obtained by TEM revealed that cells had the usual planctomycetes cell plant, divided in an electron transparent paryphoplasm and the pirellulosome surrounded by the cytoplasmic membrane. The DNA is condensed inside the pirellulosome that also contains ribosome-like particles and inclusions.

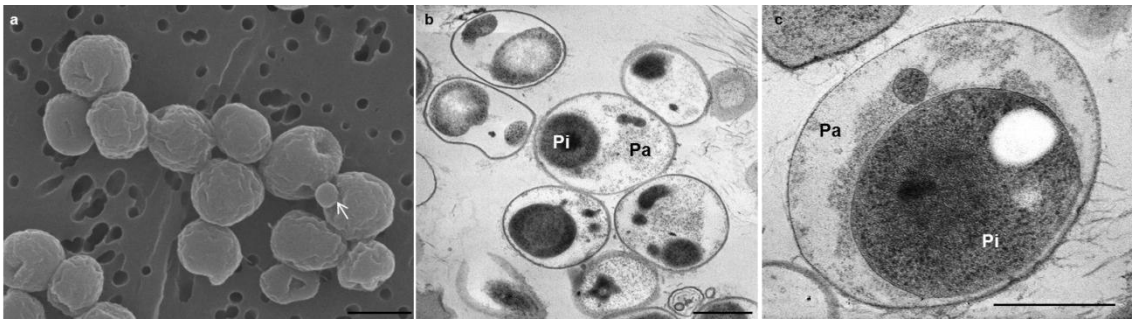

Fig. 5. 2 Scanning (a) and transmission (b and c) electron micrographs of cells of strain FF4^T. (a) Rosette of round shaped cells with a bud being formed (arrow). Ultrathin sections showing the paryphoplasm (Pa), pirellulosome (Pi), DNA, inclusions (In) and cell wall (CW). Cells possess fimbriae with a ring-like structure. In (c) it is visible the separation of the paryphoplasm from the pirellulosome by the cytoplasmastic membrane (CM).

The optimum temperature for growth was around 30 ºC, and strains were able to grow from 5 to 35 ºC. The pH range for growth was from 6.5 to 10, with an optimal at pH 7.5. Maximum salinity tolerated was 225 % of ASW and 7 of NaCl. Seawater salts were not required for the growth of the strains. Differential physiological characteristics between strains, including the type strain *R. baltica* SH1^T, obtained with API 50CH, Biolog GN2 and API ZYM are shown in Table 5.1. BIOLOG GN2 profiles showed that the preferred carbon sources are carbohydrates (21 positives out of 28). Alcohols, amino-acids, aromatic and brominated chemicals and amines were not oxidized. Strains acidified 33 carbohydrates as observed in the API 50 CH. N-acetylglucosamine, D-galactose, L-rhamnose, D-frutose, D-arabinose, D-glucose, xylose, raffinose, sucrose, maltose, lactose, D-cellobiose, D-trehalose, D-mannitol, lactulose and dextran are used as single carbon sources by the strain FF4^T but not amino acids and organic acids. Strains FF4^T, FC92 and UF2 were cytochrome oxidase and catalase positive and hydrolysed esculin, starch and carboxy-methylcellulose. FF4^T and FC92 were also able to hydrolyse gelatine. None of the strains were able to hydrolase urea, elastin, casein or alginate. API ZYM enzymatic profiles were similar for all the strains with exception of trypsin, α-chymotrypsin, ß-glucosidase and naphthol-AS-BI-phosphohydrolase. Nitrate was reduced to nitrite. Anaerobic growth was observed.

The strains utilised several amino acids and proteinaceous molecules as a source of nitrogen. Nitrate, nitrite and urea were not utilized. Of the vitamins tested, only vitamin B12 was required.

Table 5. 1 Differential features of strains FF4^T, FC92, UF2 and *R. baltica* SH1^T in API 50CH, API ZYM and Biolog GN2.

	FF4^T	FC92	UF2	*R. baltica* SH1^T
API 50CH				
D-adonitol	+	+	-	-
Methyl-ßD-xylopyranoside	-	-	-	+
D-galactose	-	-	+	+
Xylitol	-	-	-	+
Potassium 2-ketogluconate	-	-	-	+
API ZYM				
Trypsin	+	-	-	+
α-chymotrypsin	+	-	-	-
Naphthol-AS-BI-phosphohydrolase	+	+	-	-
ß-glucosidase	+	-	-	+
N-acetyl-ß-glucosaminidase	-	-	-	+
α-fucosidase	+	+	+	-
Biolog GN				
Dextrin	+	-	-	+
L-Arabinose	-	-	-	+
D-Psicose	+	-	+	-
Xylitol	-	+	+	-
Pyruvic Acid Methyl Ester	-	+	+	+
Succinic Acid Mono-Methyl-Ester	-	-	-	+
Acetic Acid	-	-	-	+
D-Gluconic acid	-	+	-	+
D-Glucosaminic acid	+	-	-	-
Succinic Acid	-	-	+	-
L-Alaninamide	+	+	+	-
Glycerol	-	+	+	+
α-D-Glucose-Phosphate	+	+	+	-
D-Glucose-6-Phosphate	-	+	+	-

All the strains produced acid from D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, Methyl-αD-mannopyranoside, Methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, gentiobiose D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol and potassium 5-ketogluconate

Acid was not produced glycerol, erythritol, dulcitol, inositol, D-sorbitol, inulin, starch, glycogen and potassium gluconate

All the strains were positive for alkaline phosphatase, esterase C4, esterase lipase C8, leucine, valine and cystine arylamidase, acid phosphatase, α-glucosidase, ß-galactosidase and α-galactosidase. All the strains were negative for Lipase (C 14), ß-glucuronidase and α-mannosidase.

All the strains oxidized N-Acetyl-D-galactosamine, N-Acetyl-D-glucosamine, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, D-glucuronic acid, D,L-lactic acid and glucuronamide. None of the strains oxidized α-cyclodextrin, tween 40 and 80, glycogen, adonitol, D-arabitol, erythritol, m-inositol, D-sorbitol, Cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-glucosaminic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxy-phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic Acid, succinamic acid, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, D,L-carnitine, γ-Amino Butyric Acid, urocanic acid, inosine, uridine, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol.

Polar lipids bidimensional profile (Fig. 5.3) revealed that phosphatidylcholine (PC) and, phosphatidylglycerol (PG) were the major polar lipids of FF4^T as well as in *R. baltica* SH1^T. Diphosphatidylglycerol (DPG) was also present in small proportions in both FF4^T and *R. baltica* SH1^T. In contrast to *R. baltica* SH1^T, FF4^T lacks PL1 and the unidentified lipids UL3 and UL4 and has additional lipids PL2 and an unidentified lipid UL5.

The major respiratory quinone of all the strains was menaquinone 6 (MK-6), as observed in all *Planctomycetes*.

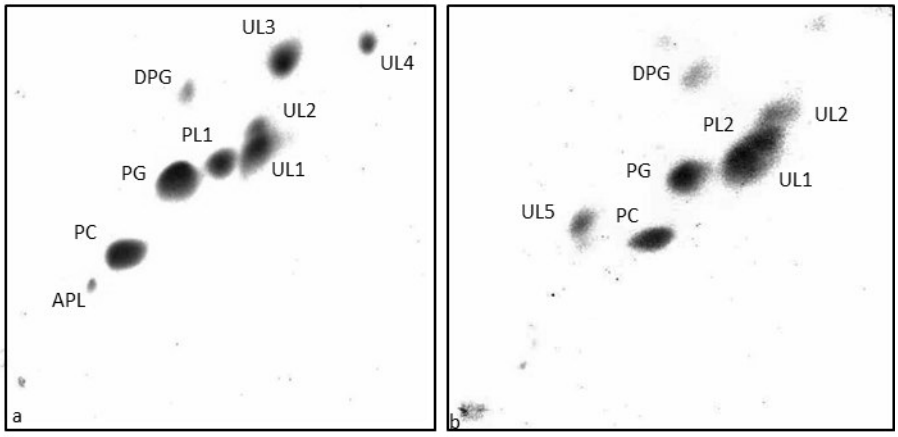

Fig. 5. 3 Two-dimensional thin-layer chromatography of polar lipids of *Rhodopirellula baltica* DSM 10527^T (a) and strain FF4^T (b) at the optimum growth temperature. The lipids were stained by spraying with 5% molybdophosphoric acid in ethanol followed by heating at 160 ºC. PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; APL, aminophospholipid; PL1,2, phospholipid 1,2; UL1,2,3,4,5; unknown lipid 1,2,3,45.

The fatty acid composition of strains FF4^T, FC92 and UF2 showed a predominance of $C_{18:1}$ ω9c and $C_{16:0}$ which accounted for 53-54 % and 28-30 %, respectively (Table 5.2). In contrast to *R. baltica* SH1^T, strains FF4^T, FC92 and UF2 only possessed small amounts of $C_{16:1}$ w7c/$C_{16:1}$ w6c (3.4-2.4 %) which in *R. baltica* SH1^T accounts for 15.4 %. The novel isolates also showed $C_{16:1}$ ω11c fatty acids (4-5 %) and small amounts of 2-hydroxy acids ($C_{16:0}$ -2OH and $C_{18:0}$ -2OH) that were absent in *R. baltica* SH1^T.

Table 5. 2 Fatty acid composition of strains FF4^T, FC92, UF2 and type strain *R. baltica* SH1^T grown in liquid M13 medium at 26 ºC.

Fatty acid	ECL[a]	*R. baltica* SH1^T	FF4^T	FC92	UF2
$C_{10:0}$	10.000	0.1	0.4	0.5	0.2
$C_{10:0\ 3OH}$	11.422	-	0.1	0.2	0.2
Unknown ECL 11.980[b]	11.980	0.6	0.3	0.3	0.3
$C_{12:0\ 3OH}$	13.455	0.1	-	0.1	-
$C_{14:0}$	14.000	0.5	0.3	0.3	0.3
$C_{15:0}$	15.000	0.5	0.1	0.1	-
$C_{16:0\ N\ alcohol}$	15.549	-	-	0.2	-
$C_{16:1\ \omega11c}$	15.757	-	4.3	4.7	4.0
Summed feature 3[c]	15.186	17.9	2.7	3.4	2.4
$C_{16:0}$	16.000	26.8	29.7	27.7	29.3
$C_{17:1\omega8c}$	16.792	5.1	1.2	1.1	1.3
$C_{17:0}$	17.000	1.0	0.7	0.6	0.9
$C_{16:0\ 2OH}$	17.232	-	0.3	0.2	0.3
$C_{18:1\omega9c}$	17.772	42.6	52.9	54.0	53.5
Summed feature 8[e]	17.823	2.8	1.2	0.7	1.2
$C_{18:0}$	17.999	1.3	3.2	3.9	3.7
$C_{19:0\ iso}$	18.633	-	0.2	-	0.2
Summed feature 7[f]	18.866	-	0.4	0.9	0.4
$C_{19:0}$	19.002	-	-	0.1	0.1
$C_{18:0\ 2OH}$	19.267	-	0.4	0.2	0.4
$C_{20:1\ \omega9c}$	19.771	-	0.6	0.3	0.4
$C_{20:0}$	19.999	-	0.8	0.6	0.8

[a] ECL, equivalent chain length.
[b] Unknown fatty acid with an equivalent chain length of 11.980.
[c] Sum Feature 3, 16:1 w7c/16:1 w6c.
[d] Sum Feature 8, 18:1 w7c
[e] Sum Feature 7, 19:0 cyclo w10c/19w6

Phylogenetic analysis of the almost complete 16S rRNA gene revealed that the strains FF4^T, FC92 and UF2 belong to the genus *Rhodopirellula* (Fig. 5. 4). The isolates formed a separated branch, supported by a high bootstrap value, from the *R. baltica* SH1^T, confirming the phylogenetic distinctiveness of these strains. The 16S rRNA gene of the isolates shared a similarity of 96.6 % to *R. baltica* SH1^T which, in accordance to the defined 97 % cut-off, is indicative of a novel species. The isolates shared 99.8 % pairwise sequence. The group of strain FF4^T also contains strains *Rhodopirellula* sp. SM1 and SM24 isolated from the sediments from Mallorca and Philippines, respectively (Frank et al., unpublished). The 16S rRNA sequence similarity between them is 99.4 %, indicating that they belong to the same species. Winkelman and Harder isolated several strains belonging to this cluster, from several locations on Europe, indicating that this is a widespread species.

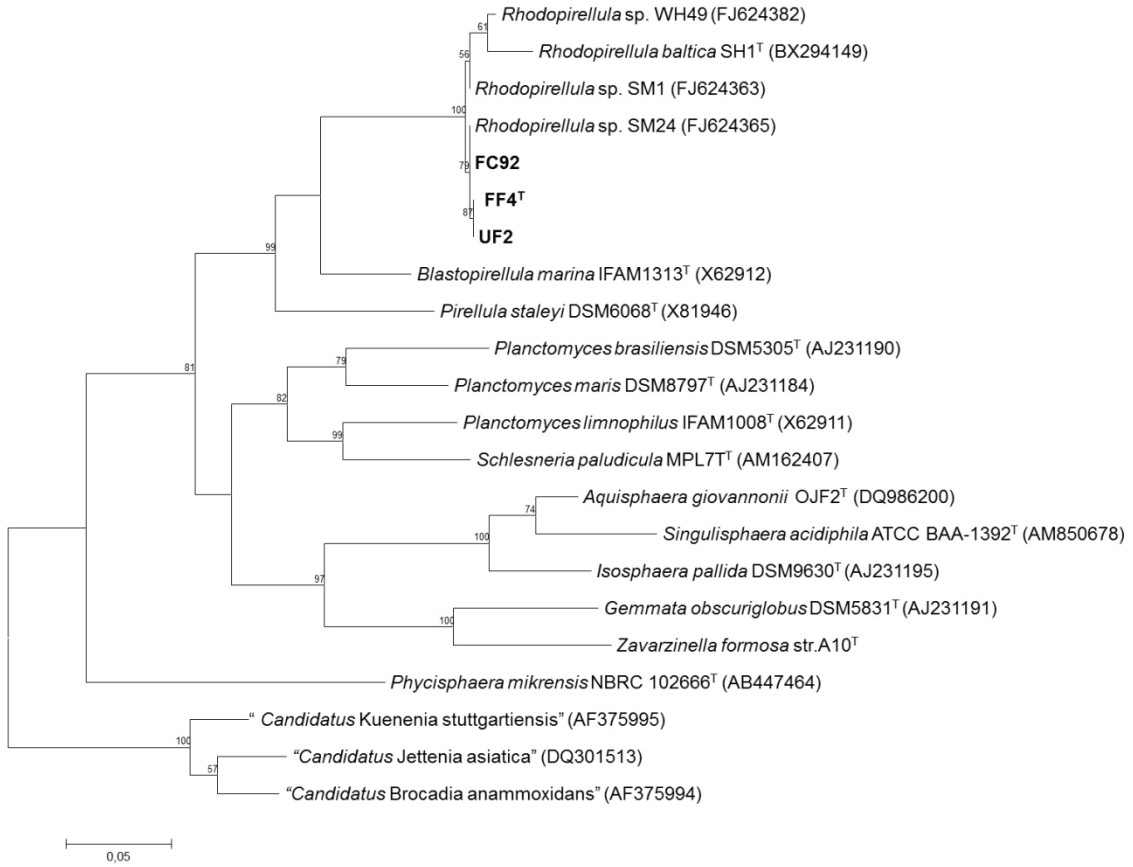

Fig. 5. 4 Maximum likelihood 16S rRNA gene phylogenetic tree showing the relationships between strains FF4^T, FC92 and UF2 and other representatives of the phylum *Planctomycetes* (accession numbers are shown in parenthesis). The tree was based on the Jukes-Cantor model. Numbers on the tree refer to bootstrap values based on 1000 replicates. Only values above 50 % are shown. *"Candidatus"* genera Anammox were used as outgroup. Bar - 0.05 substitutions per nucleotide position.

The mole G+C content of the DNA of strains FF4^T, FC92 and UF2 vary from 53.4 to 54.5 %, very similar to the one determined for *R. baltica* SH1^T (54.1 %).

Conclusions

Based on phylogenetic distinctiveness, chemotaxonomic parameters that show differences in the fatty acid composition such as the presence of $C_{16:1}$ ω11*c* and 2-hydroxy acids that are absent in *R. baltica* SH1^T and the low amount of $C_{16:1}$ ω7*c*/$C_{16:1}$ ω6*c* that is one of the major fatty acids in *R. baltica* SH1^T, phospholipid profiles and morphological and biochemical characteristics (Table 5.3) we are of the opinion that strains FF4^T, FC92 and UF2 represent a novel species within the order *Planctomycetales*, for which we propose the name *Rhodopirellula formosa* sp. *nov*..

Table 5. 3 Differential characteristics that distinguish the novel species *Rhodopirellula formosa* from *Rhodopirellula baltica*.

	Rhodopirellula formosa	***Rhodopirellula baltica***
Cell size (µm)	1.1-1.75 x 0.9-1.5	1.0–2.5x1-2–2-3
Cell shape	Circular to pear-shaped	Pear-shape to ovoid
Pigmentation	Light Pink	Pink to Red
Seawater requirement	No	Yes
Salinity tolerance range:		
SW range (% v/v)	5-225	10-175
NaCl tolerance (% w/v)	7	5
Temperature for growth (ºC):	30	28-30
Range	5-35	5-30
Optimum	30	28-30
pH range	6.5-10	5.5-10.5
Carbon sources		
Ribose	-	+
Arabinose	+	-
Rafinose	+	-
Trehalose	+	+
Mannitol	+	-
Lactate	+	-
Nitrogen sources		
Aspartic acid	+	-
Asparagine	-	+
Ornithine	+	-
Proline	-	+
Serine	+	-
Nitrate	-	+
Nitrite	-	+
Hydrolysis of:		
Carboxymethyl cellulose	+	-
Gelatine	+	-
FAMEs		
14:0	-	0.5
16:1 ω11c	4.3	-
16:0	29.7	26.8
16:1ω7c/16:1ω6c	2.7	17.9
17:1ω8c	1.2	5.1
18:1ω9c	52.9	42.6
18:0 3OH	1.3	-
20:1 ω9c	0.6	-
20:0	0.8	-
Phospholipids		
PL2	-	+
UL3	-	+
UL4	+	-
UL5	+	-
DNA G+C content	53.4 %	54.1 %
Anaerobic Growth	Yes	No
Nitrate reduction to nitrite	+	-

Description of *Rhodopirellula formosa* sp. nov.

Rhodopirellula formosa [for.mo'sa L. fem. adj. *formosa*, beautiful, beautifully formed, finely formed.]

The species possesses the following characteristics in addition to those described for the genus. Cells are spherical to pear-shaped with 1.1-1.75 x 0.9-1.5 µm and form rosettes of a variable number of cells. Colonies in M13 are light pink, translucent and smooth. Optimum growth temperature is 30 ºC (temperature range between 5 ºC to 35 ºC). pH range in between 6.5 to 10.5. Sea salts are not required to grow Maximum salinity tolerated is 225 % of ASW and 7 % of NaCl. Major fatty acids are $C_{18:1}\omega 9c$ (53-54 %), $C_{16:0}$ (27-29 %), $C_{16:1}\omega 11c$ (4-5 %) and $C_{18:0}$ (3-4 %). Hydroxy fatty acids are present as well. Starch, cellulose, esculin and carboxy-methylcellulose are degraded; some strains also degrade gelatine. Urea, casein, elastin and alginate are not degraded.

Alkaline phosphatase, esterase C4, esterase lipase C8, leucine, valine and cystine arylamidase, acid phosphatase, α-chymotrypsin, α-glucosidase, ß-galactosidase, α-galactosidase and α-fucosidase are positive in API ZYM Lipase (C 14), ß-glucuronidase, α-mannosidase and N-acetyl-ß-glucosaminidase are negative. Indole is not produced. Arginine dihydrolase is absent. Nitrate is reduced to nitrite. N-acetylglucosamine, D-galactose, L-rhamnose, D-frutose, D-arabinose, D-glucose, xylose, raffinose, sucrose, maltose, lactose, D-cellobiose, D-trehalose, D-mannitol, lactulose and dextran are assimilated. Amino acids are not assimilate as well as Casaminic acids, L-fucose, D-ribose, L-sorbose, ribitol, sorbitol, *myo*-inositol, erythritol, D-arabitol, glycerol, succinate, α-ketoglutarate, malate, citrate, benzoate, fumarate, formate, acetate, pyruvate and inulin. Acid is produced from D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, D-mannitol, Methyl-αD-mannopyranoside, Methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, gentiobiose D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol and potassium 5-ketogluconate. Acid was not produced from Methyl-ßD-xylopyranoside, glycerol, erythritol, dulcitol, inositol, D-sorbitol, inulin, starch, glycogen or potassium gluconate. The organism N-Acetyl-D-galactosamine, N-Acetyl-D-glucosamine, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, D-glucuronic acid, D,L-lactic

acid, L-Alaninamide, glucuronamide and α-D-Glucose-Phosphate. on the BIOLOG GN2 System. Some strains also oxidized dextrin, D-psicose, xylitol, pyruvic acid methyl ester, D-gluconic acid, D-glucosaminic acid, succinic acid, glycerol and D-glucose-6-phosphate. Other carbon compounds are not oxidized on the BIOLOG GN2 system. Glutamate, alanine, phenylalanine, glutamine, ornithine, serine as well as yeast extract, casaminic acids, NAG and ammonia serve as sources of nitrogen. Urea, nitrate, nitrite are not utilized. Vitamin B12 is required for growth.

The G+C mole content of the DNA of the type strain is 53.4 % ± 0.5

The type strain is $FF4^T$ isolated from the epiphytic community of *Fucus spiralis.*

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Chapter 6.

rpoB gene as a novel molecular marker to infer phylogeny in *Planctomycetales*[4]

Abstract

The 16S rRNA gene has been used in the last decades as a gold standard for determining the phylogenetic position of bacteria and their taxonomy. It is a well conserved gene, with some variations, present in all bacteria and allows the reconstruction of genealogies of microorganisms. Nevertheless, this gene has its limitations when inferring phylogenetic relationships between closely related isolates. To overcome this problem, DNA-DNA hybridization appeared as a solution to clarify interspecies relationships when the sequence similarity of the 16S rRNA gene is above 97 %. However, this technique is time consuming, expensive and laborious and so, researchers developed other molecular markers such as housekeeping or functional genes for accurate determination of bacterial phylogeny. One of these genes that have been used successfully, particularly in clinical microbiology, codes for the beta subunit of the RNA polymerase (*rpoB*). The *rpoB* gene is sufficiently conserved to be used as a molecular clock, it is present in all bacteria and it is a mono-copy gene.

In this study, *rpoB* gene sequencing was applied to the phylum *Planctomycetes*. Based on the genomes of 19 planctomycetes it was possible to determine the correlation between the *rpoB* gene sequence and the phylogenetic position of the organisms at a 95-96% sequence similarity threshold for a novel species. A 1200-bp fragment of the *rpoB* gene was amplified from several new planctomycetal isolates and their intra and inter-species relationships to other members of this group were determined based on a 96 % species border and 98.7 % for intraspecies resolution.

[4] The results present in this chapter were published in the following manuscript:
Joana Bondoso, Jens Harder and Olga Maria Lage. (2013) rpoB gene as a novel molecular marker to infer phylogeny in Planctomycetales. Antonie van Leeuwenhoek 104: 477-488.

Introduction

Bacterial taxonomy has been revolutionized with the introduction of the ribosomal RNA molecule for species cataloguing (Woese and Fox, 1977; Woese, 1987). Because of its ubiquity and degree of conservation among microorganisms, the 16S rRNA gene has been used as a gold standard for determining the phylogenetic position of bacteria. As a rule, two strains that share less than 97 % similarity in the 16S rRNA gene should be considered as two different species (Stackebrandt and Goebel, 1994). This 3 % dissimilarity cut-off has been used in the last decades as a criterion in the delineation of microbial species (Tindall et al., 2010). For 16S rRNA gene values of similarity higher than 97 %, DNA-DNA hybridization (DDH) is necessary to clarify the intraspecific relationships, whereas values ≥ 70 % indicate that two strains belong to the same species (Wayne et al., 1987). Although the 16S rRNA gene is very useful in assigning novel isolates to orders and genera, it has some limitations when delineating novel species. For example, two organisms can have 16S rRNA gene sequences almost identical and still can be two different species based on DDH (Fox, 1992). In 2006, Stackebrandt and Ebers (2006) compared data from taxonomic publications and based on 16S rRNA gene similarities and DDH values, they recommended a higher cut-off value between 98.7 and 99 % in 16S rRNA gene sequence similarity for which DDH would be necessary. These values were supported by other studies (Konstantinidis and Tiedje, 2005a, b; Konstantinidis et al., 2006; Meier-Kolthoff et al., 2013), but, so far, they were not adopted by all taxonomists. Alternatively, the sequence identity between two strains, which is referred as average nucleotide identity (ANI) of common genes, can be calculated. The availability of a large number of microbial genomes allowed the calculation of the relationship between the values of ANI and the ones of DDH. The 70 % cut-off value of DDH corresponds approximately to 94-95 % ANI (Konstantinidis and Tiedje, 2005a; Goris et al., 2007). Albeit the advantages of both DDH and ANI are known, they present some disadvantages. DDH is a very laborious and time-consuming technique and presents several drawbacks (Konstantinidis et al., 2006); ANI determination implies the availability of whole-genome sequences, or at least 20 % of a draft genome (Richter and Rossello-Mora, 2009; Klenk and Goker, 2010), of the strains under comparison, which is still limited.

To overcome these technical problems, scientists established other molecular marker genes to resolve bacteria phylogenetic relationships based on gene sequencing. The analysis of the *rpoB* gene, encoding for the β-subunit of the RNA polymerase, has been used in microorganisms, especially in clinical microbiology

(Adékambi et al., 2006a). Correlations between the sequence similarity of this gene and DDH or ANI values were established (Adékambi et al., 2008), which showed that the analysis of the *rpoB* gene sequence is a good and easy alternative to DDH determinations and whole genome sequencing. The *rpoB* gene is a universal gene among the bacteria and is sufficiently conserved to be used in evolutionary relationships. Contrary to the multiple copies of the 16S rRNA gene, the *rpoB* is a one copy gene, which eliminates sequence variations in a single organism. Furthermore, it allows the discrimination at the intraspecific level. For over twenty years, the *rpoB* gene has been applied more and more often for clarification of the taxonomy and phylogeny of several bacteria, e.g. for *Aeromonas* (Kupfer et al., 2006), *Corynebacterium* (Khamis et al., 2004), *Staphylococcus* (Drancourt and Raoult, 2002), *Mycobacterium* (Adékambi et al., 2003; Adékambi et al., 2006b), *Leptospira* and *Acinetobacter* (La Scola et al., 2006b; La Scola et al., 2006a), enteric bacteria (Mollet et al., 1997), spirochetes and *Bartonellas* (Renesto et al., 2000; Renesto et al., 2001) and *Rickettsia* (Drancourt and Raoult, 1999).

Planctomycetes together with the sister phyla *Verrucomicrobia*, *Chlamydiae* and *Lentisphaerae* belong to the PVC super-phylum (Wagner and Horn, 2006). Their phylogeny was not always consensual and several studies were needed to determine their exact position and relationship to other groups of bacteria (Ciccarelli et al., 2006; Barion et al., 2007; Pilhofer et al., 2008; Nasir et al., 2011). Besides the 16S rRNA gene, several other molecular markers have been applied in planctomycetal taxonomy. Bomar and collaborators (1988) analysed the phylogenetic position of members of *Planctomycetales* based on the 5S rRNA gene. Butler and Fuerst (2004) used the ribonuclase P RNA to differentiate *Planctomycetes* from other *Bacteria* and to determine their relative phylogenetic position. Recently, a multilocus sequence analysis (MLSA) was applied to the *Rhodopirellula* genus for discrimination of related strains and to correlate diversity with the biogeography (Winkelmann et al., 2010; Cayrou et al., 2013).

The *rpoB* gene was never studied in *Planctomycetes*, as well as its applicability for the identification of new isolates in this group. In this study, the *rpoB* gene was applied to a group of previously isolated planctomycetes (Lage and Bondoso, 2011; Lage et al., 2012) aiming to determine their intraspecific relationship and to establish their taxonomy within this group. Thus, the hypervariable regions of this gene flanked by conserved regions were identified and a 1200-bp fragment was amplified in 38 planctomycetes. The availability of several *Planctomycetes* with sequenced genomes

allowed the correlation of the *rpoB* gene with the 16S rRNA gene and with ANI. Based on these analyses, it was possible to define genus and species threshold values for the *rpoB* gene and to resolve intraspecies relationships that were not possible by the analysis of the 16S rRNA gene. An *rpoB* gene sequence database was constructed for *Planctomycetales*, which will allow the use of this gene as an alternative to the 16S rRNA sequencing or as a complement for a correct taxonomic evaluation of novel isolates.

Material and Methods

Bacterial strains

A total of 38 strains of planctomycetes, previously isolated from the surface community of macroalgae, corresponding to groups A, B, C and D as described by Lage and Bondoso (2011) and the sediments of a water treatment recycling tank of a marine fish farm, belonging to group B (Lage et al., 2012) were used in this study (Table 6.1). Strains were grown on modified agar M13 marine medium (Lage and Bondoso, 2011) at 26 ºC in the dark. Furthermore, 19 planctomycetes with whole genome sequenced were included in this study (Table 6.1).

Amplification and sequencing of partial rpoB gene

Degenerated primers for the partial amplification of the *rpoB* gene in planctomycetes were designed using the Sequence VARiability Analysis Program (SVARAP) (Colson et al., 2006). *rpoB* gene sequences from the genomes of the type strains *Rhodopirellula baltica*, *Blastopirellula marina*, *Gemmata obscuriglobus*, *Planctomyces limnophylus* and *Planctomyces maris* (the ones available at the beginning of the study) were downloaded from the NCBI database. The primers designed were pla2001F (5´ ATGGGITCIAGCARCG 3') and pla3302R (5´ ATCTGICCCACGTTCATMCG 3') and for sequencing purposes the primer pla2304F (5' CAACTWYGARCGATCA 3'). Primers were obtained from the consensus *rpoB* gene sequence of the available planctomycetes genomes and were designed in the less variable regions flanking the most hypervariable regions (2000-3300 bp, Fig. 6.1), allowing the amplification of a ~1200 bp amplicon. The partial *rpoB* gene sequences were submitted to GenBank (accession number KC810968, KC810969 and KF286572 to KF286607).

Genomic DNA was extracted with FastDNA SPIN Kit for Soil (MPBio) according to manufacturer instructions. For the amplification of the *rpoB* gene, the primers described above were used. The PCR mixture consisted of 1 x PCR master mix (Promega), 25 pmol of each primer and 100 ng of genomic DNA to a final volume of 25 µl. Gradient PCRs determined the optimal annealing temperature for the pair of primers used. PCR

reactions were performed in an Eppendorf ThermoCycler with the following thermal PCR profile: initial denaturing step of 5 min at 95 ºC, 30 cycles of 1 min at 94 ºC, 1 min at 62 ºC and 90 sec at 72 ºC, and a final extension of 10 min at 72 ºC. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

PCR products were purified by Sephadex G-50 and both strands were sequenced by a dye-labelled dideoxy termination method (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems). Analyses were performed in duplicate in an automated DNA capillary sequencer Applied Biosystems 3130XL Genetic Analyzer after Sephadex purification of the sequencing reaction.

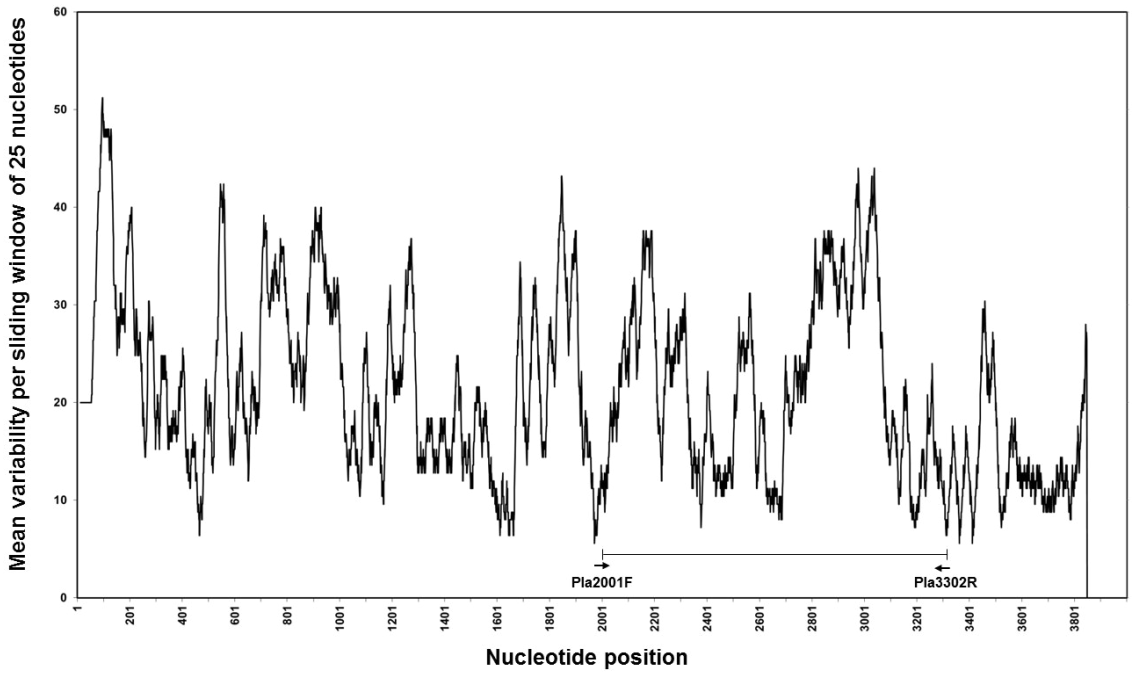

Fig. 6. 1 Mean variability for successive windows of 25 nucleotide position along the complete *rpoB* gene of *Rhodopirellula baltica*, *Blastopirellula marina*, *Planctomyces maris*, *Planctomyces limnophilus* and *Gemmata obscuriglobus* showing the ~1200 bp amplicon amplified and the primers designed. Analyses were performed in the SVARAP program.

rpoB sequence analysis and phylogeny reconstruction

The obtained nucleotide sequences of the partial *rpoB* gene were processed with the Sequencing Analysis 5.2 (Applied Biosystems) and assembled with Vector NTI Advance™ 10.3. Multiple alignments of the partial *rpoB* gene with known sequences from the GenBank database (NCBI) were carried out in ClustalW (Thompson et al., 1994). The *rpoB* sequences from genomic data were trimmed after multiple alignments to correspond to the ~1200 fragment amplified.

The 16S rRNA and *rpoB* gene datasets were subjected to phylogenetic and molecular evolutionary analysis in MEGA version 5.03 (Tamura et al., 2011). Pairwise sequence similarities based on Jukes-Cantor model were calculated in MEGA. Alignment gaps and missing data were not included in the calculations.

In silico analysis of the complete rpoB and 16S rRNA genes

Complete 16S rRNA and *rpoB* genes from nineteen genomes of planctomycetes (Table 6.1) were recovered from PATRIC web resource (http://www.patricbrc.org/portal/portal/patric/Home) and aligned with ClustalW within the package MEGA 5.03. Pairwise sequence analysis and phylogenetic reconstructions were performed as described above.

ERIC-PCR fingerprinting

DNA of the isolates was extracted using E.Z.N.A Bacterial DNA Isolation Kit (Promega) according to the manufacturer's instructions from 7-days cultures on agar plates. ERIC-PCR with the pair of primers ERIC1R and ERIC2 was performed as described previously (Lage et al., 2012). The fingerprinting profiles were visualized by post-staining with ethidium bromide for 45 min after separation by electrophoresis in a 2 % agarose gel at 60 V for 90 min in Tris–acetate–EDTA buffer.

Average nucleotide identity (ANI) calculation

The ANI between each pair of genomes was determined by using the *in silico* DNA–DNA hybridization method of the JSpecies software (Richter and Rossello-Mora, 2009).

Table 6. 1 *Planctomycetes* used in the study of 16S rRNA and *rpoB* genes.

Strain	16S accession number	*rpoB* accession number
Rhodopirellula baltica SH1[T]	NC_005027	NC_005027
Rhodopirellula baltica SH28	FJ624341	AMCW00000000
Rhodopirellula baltica SWK14	FJ624373	AMWG00000000
Rhodopirellula baltica WH47	AFAR01000001	AFAR01000142
Rhodopirellula 6C	FJ624304	ANMO00000000
Rhodopirellula SH398	FJ624343	ANOF00000000
Rhodopirellula SM41	FJ624360	ANOH00000000
Rhodopirellula SWK7	FJ624377	ANOQ00000000
Rhodopirellula SM1	FJ624363	ANOG00000000
Blastopirellula marina DSM 3645	NZ_CH672377	NZ_CH672377
Pirellula staleyi DSM6068	NC_013720	NC_013720
Planctomyces maris DSM 8797	NZ_ABCE01000003	NZ_ABCE01000024
Planctomyces limnophilus DSM 3776	NC_014148	NC_014148
Planctomyces brasiliensis DSM 5305	NC_015174	NC_015174
Schlesneria paludicula DSM 18645	AHZR01000092	AHZR01000071
Gemmata osbcuriglobus UQM 2246	NZ_ABGO0100019248	NZ_ABGO01000071
Zavarzinella formosa DSM 19928	AIAB01000000	AIAB01000143
Isosphaera pallida ATCC 43644	NC_014962	NC_014962
Singulisphaera acidiphila DSM 18658	AGRX01000088*	AGRX01000097
FC2	HQ845435	This study
FC29	HQ845457	This study
MsF2	HQ845493	This study
UF7	This study	This study
UF8	This study	This study
LF2	HQ845500	This study
FC3	HQ845436	This study
FC17	HQ845449	This study
FC19	HQ845451	This study
OJF1	DQ851134	This study
OJF9	EF421447	This study
OJF10	EF421448	This study
OJF11	EF421449	This study
OJF12	EF421450	This study
UC9	HQ845509	This study
Gr18	HQ845486	This study
MsF5	HQ845495	This study
CcC31	HQ845422	This study
CcC5	EF589352	This study
CcC6	HQ845424	This study
CcC8	HQ845424	This study
FC24	HQ845454	This study
FC25	EF589353	This study
FC26	HQ845455	This study
UC13	HQ845513	This study
UC14	HQ845514	This study
UC17	EF589351	This study
UC20	HQ845516	This study

UC22	HQ845518	This study
UC28	HQ845522	This study
UC31	HQ845523	This study
UC33	HQ845524	This study
UC36	HQ845525	This study
UC38	HQ845526	This study
UC49	HQ845530	This study
Sm2	HQ845501	This study
FF4	JN236500	This study
FC92	This study	This study
UF2	HQ845531	This study

Results

Correlation between *rpoB* and 16S rRNA gene sequences

Complete *rpoB* and 16S rRNA genes sequences of the available nineteen whole genomes of *Planctomycetes* (Table 6.1) were recovered from PATRIC database. A maximum likelihood phylogenetic tree was constructed for each gene (Fig. 6.2) and the topology of the trees was compared. The phylogenetic relationships shown in the 16S rRNA gene tree were retained in the *rpoB* gene tree with high bootstrap values. Exceptions were *B. marina/P. staleyi* and *Pl. brasiliensis/Pl. maris* that did not cluster together when the *rpoB* gene was analysed.

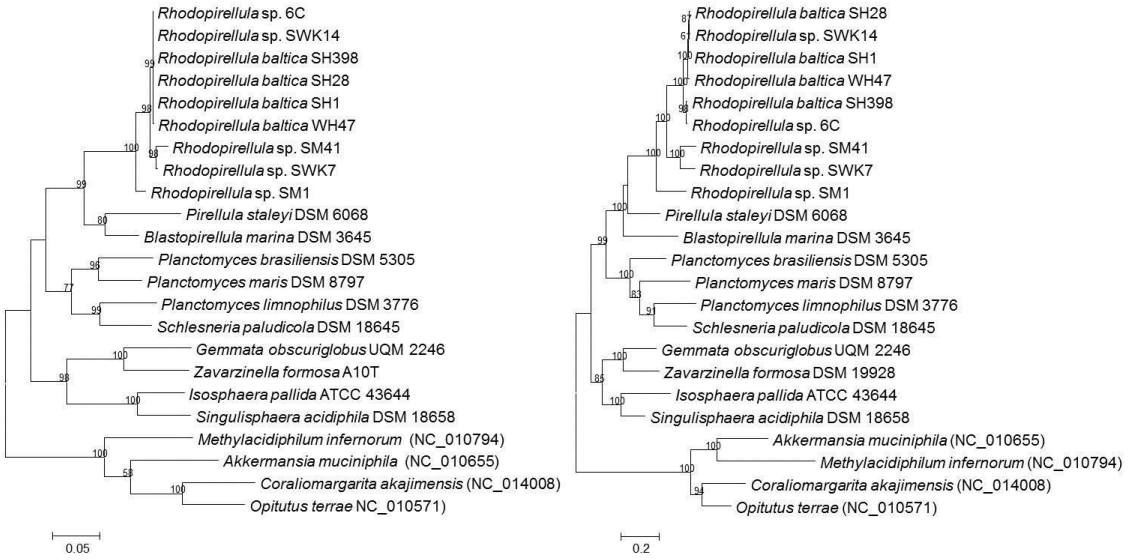

Fig. 6. 2 Comparison between the 16S rRNA gene (on the left) and *rpoB* gene (on the right) phylogenetic trees for the order *Planctomycetales*. Both trees were obtained with the maximum likelihood method based on the general time-reversible (GTR) model. The numbers beside nodes are the percentages for bootstrap analyses; only values above 50% are shown. Scale bar= 0.05 substitutions per 100 nucleotides. *Verrucomicrobia* sequences were used as outgroup.

The *rpoB* gene showed a higher discriminatory power when analysing closely related species, as in the case of the genus *Rhodopirellula.* Whilst in the 16S rRNA gene analyses the pairwise similarity of each strain to the type species *R. baltica* SH1^T

varied between 100 and 97.2 %, in the *rpoB* gene the similarities varied from 98.4 to 75.3 % (Table 6.2). Furthermore, *Rhodopirellula* sp. 6C and SH398, which were shown not to belong to species *R. baltica* on the basis of DDH (55 % to *R. baltica* SH1^T) and ANI (89.4 %), appear separated from the remaining strains of the group on the *rpoB* gene tree, confirming the DDH and ANI previously obtained (Winkelmann et al. 2010, Frank et al. unpublished). In this case the *rpoB* gene diverged in ~5 % while similarity values for the 16S rRNA gene were higher than 99.9 %, which, according to the values described in the literature, corresponds to the same species as *R. baltica* SH1^T. Within a species, the *rpoB* gene showed a divergence higher than 1.3 %.

Table 6. 2 Pairwise similarities in the 16S rRNA and *rpoB* genes of strains of genus *Rhodopirellula* to *R. baltica* SH1^T. DDH and ANI values are also shown.

Strain	Species	16S rRNA (%)	*rpoB* (%)	DDH[a]	ANI (%)
SWK14	*Rhodopirellula baltica*	100	98.70	nd	97.25
SH28	*Rhodopirellula baltica*	99.93	98.94	>70	97.05
WH47	*Rhodopirellula baltica*	99.93	98.70	nd	97.35
6C	*Rhodopirellula europaea*	99.93	95.38	55-70	88.38
SH398	*Rhodopirellula europaea*	99.93	95.23	49.3-58.9	88.48
SWK7	*Rhodopirellula rubra*	98.80	78.91	nd	70.42
SM41	*Rhodopirellula sallentina*	97.74	78.69	nd	70.47
SM1	*Rhodopirellula maiorica*	97.20	75.27	38	68.73

[a]Data from Winkelmann et al., (2010) and Frank et al. (unpublished)

In order to facilitate the use of the *rpoB* gene sequencing for phylogenetic determinations, a partial fragment of this gene (~1200 bp), corresponding to the PCR amplicon amplified with the designed pair of primers, was studied. The pairwise similarity values of the complete *rpoB* gene were plotted against the values of the corresponding 1200-bp gene fragment (Fig. 6.3) and an almost linear relationship between the two parameters was obtained ($r^2 = 0.9794$).

The pairwise similarities of the 16S rRNA gene and the corresponding partial 1200 bp fragment of *rpoB* gene obtained from the 19 planctomycetal species referred in Fig. 6.2 were correlated and plotted (Fig. 6.4). Species and genera were categorized based on the taxa already described and validated and on the DDH values from the literature (Winkelmann and Harder, 2009; Ward, 2010; Winkelmann et al., 2010). The relationship between both genes is described by the linear equation 16S rDNA

similarity = 1.4528 (*rpoB* similarity) – 60.697. Based on this data, two strains should be placed in separate species when the similarity in the partial *rpoB* gene sequence is lower than 95.5 %. Values higher than 98.2 % indicate that two strains should be grouped in the same species. The three species of the genus *Planctomyces*, *Pl. maris*, *Pl. limnophilus* and *Pl. brasiliensis* were clearly in the region of different genera, which was defined by a value of pairwise similarity in the *rpoB* gene below 72 %. The 16S rRNA gene pairwise sequence similarity between these strains ranged from 84.31 to 89.15 % and the partial *rpoB* gene similarity was between 62.53 and 64.73 %.

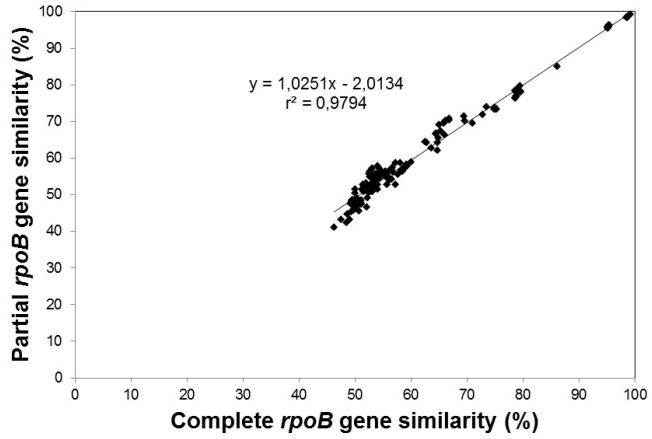

Fig. 6. 3 Correlation between the pairwise similarity values of the complete *rpoB* gene sequence and the 1200-bp gene fragment.

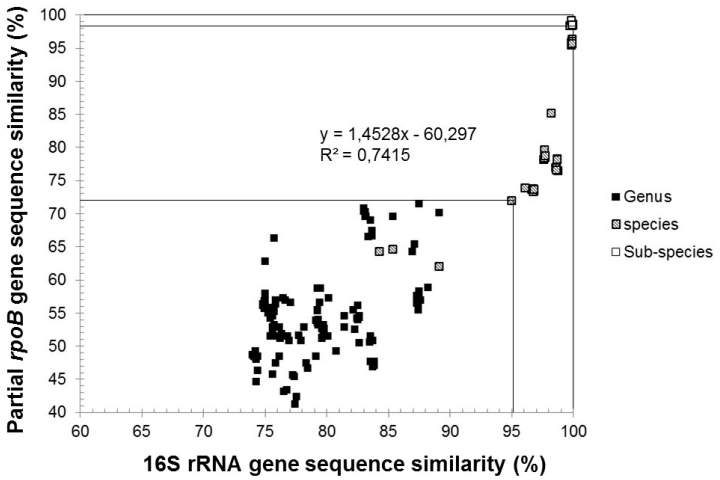

Fig. 6. 4 Scatter plot representing the correlation between the partial rpoB gene sequence (~1200bp) similarity and the 16S rRNA gene sequence similarity. The dataset was composed by 171 data points. Each data point represents a pairwise sequence comparison of the rpoB gene (y-axe) plotted against the 16S rRNA gene sequence similarity (x-axe) between two strains.

Correlation between *rpoB* and ANI

The correlation between the *rpoB* sequences similarities and the ANI values (Fig. 6.5) is also high (r^2= 0.76) with a correlation described by the linear equation ANI value = 1.5892 (*rpoB* similarity) – 46.643. The analysis of Figure 6.5 indicates that species could be defined by a value between ~74 % and 95.4 % which corresponds to an ANI value between 69 and 90 %. For two strains to be considered the same species, the *rpoB* sequence similarity should be higher than 98.6 %, which corresponds to an ANI value of 95.8 %. These values are similar to the ones obtained in the correlation of the partial *rpoB* and 16S rRNA genes (Fig. 6.4). Once again, the species *Pl. maris*, *Pl. limnophilus* and *Pl. brasiliensis* appear in the genera region. When analysing only data from *Rhodopirellula* genus, a higher correlation (r^2 = 0.958) was obtained, suggesting that *rpoB* gene is adequate for intrageneric delineation.

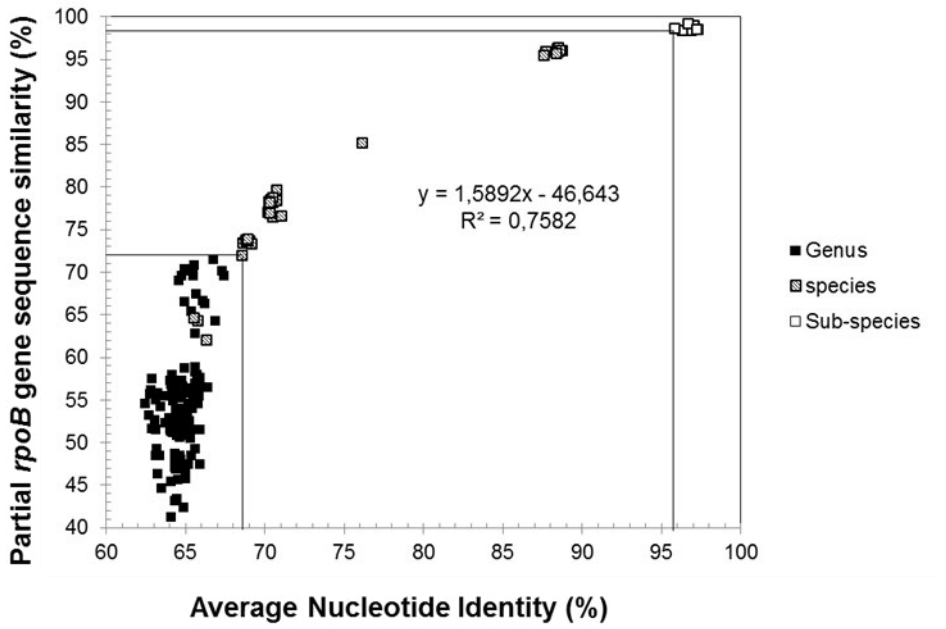

Fig. 6. 5 Scatter plots representing the correlation between the partial *rpoB* gene sequence (~1200bp) similarity and the ANI. Dataset was composed of 171 data points. Each data point represents a pairwise sequence comparison of the *rpoB* gene (y-axe) plotted against the ANI (x- axes) between two strains.

Amplification of *rpoB* gene in *Planctomycetales*

In order to complement the taxonomy and phylogeny of novel *Planctomycetales* strains previously isolated, the amplification of a ~1200 bp fragment of the *rpoB* gene was done. Using the pair of primers pla2001F/pla3302R, a new database based on the *rpoB* gene sequences (38 planctomycetes) was created for the order *Planctomycetales*.

The strains under study were previously identified on the basis of the 16S rRNA sequence and formed four different clusters (A, B, C, and D) in the phylogenetic tree (Fig. 6.6a), closely related to the genus *Rhodopirellula*. Group A contains strains that share more than 99 % similarity in the 16S rRNA gene to *R. baltica* SH1^T, thus being considered the same species. Group B and C share between 97.4 % and 97.9 % to the 16S rRNA gene of *R. baltica* SH1^T, and group D shows a similarity of 96.5 % with the 16S rRNA gene of *R. baltica* SH1^T. Although it was clear, by the analysis of the 16S rRNA gene, that group D should be considered a novel species of *Rhodopirellula*, the same was not true for groups B and C. Additional information was needed to determine their exact taxonomic relationship to *R. baltica* SH1^T.

Based on the partial sequences of the *rpoB* gene obtained for the isolates under study, a maximum likelihood tree was constructed (Fig. 6.6b). The topology of the *rpoB* gene phylogenetic tree was similar to the 16S rRNA gene tree, evidencing the same clusters A, B, C and D with high bootstrap values. In both trees, groups B and C were closely related to each other and phylogeneticaly related to *R. baltica* (group A). It was also possible to observe that the *rpoB* gene was more discriminative than the 16S rRNA gene. While in the 16S rRNA tree there was not a clear distinction of the strains within each group, in the *rpoB* gene tree it was possible to divide the groups in several sub-groups (Fig. 6.6). Furthermore, the 16S rRNA gene pairwise similarity values varied ~0.5 % within each group and ~4.5 % between the groups while the *rpoB* gene pairwise similarity values diverged between 0 to 9 % within groups and more than 15 % between the groups (Supplementary Table S4).

Identification of novel isolates

Based on the cut-off values defined by the *in silico* analysis of the partial *rpoB* gene sequence of the already described planctomycetes and its correlation with the ANI and 16S rRNA gene similarities, it was possible to assign taxonomically the novel isolates. Similarity values higher than 98 % represent the same species and values lower than 95.5 % indicate novel species. Group B shares a similarity in the *rpoB* gene to *R. baltica* SH1^T between 78-76%, which is indicative of a different species. Group C and

R. baltica SH1^T presents *rpoB* gene similarities in the range of 80-81 %, being thus a novel species of *Rhodopirellula*. Group D was confirmed to be a novel species as the *rpoB* gene similarity values obtained were ~74 % to *R. baltica* SH1^T. Through the analysis of pairwise *rpoB gene* sequence similarities it was also possible to define potential novel species within each group. Within group A, there were three different groups for candidate species, FC29, FC2/MsF2 and UF7/UF8, besides 6C/SH398 which were confirmed to be a different species of *Rhodopirellula* through ANI and DDH (Frank et al. unpublished). In group B, three possible species were identified, and in groups C and D, there were two possible novel species in each.

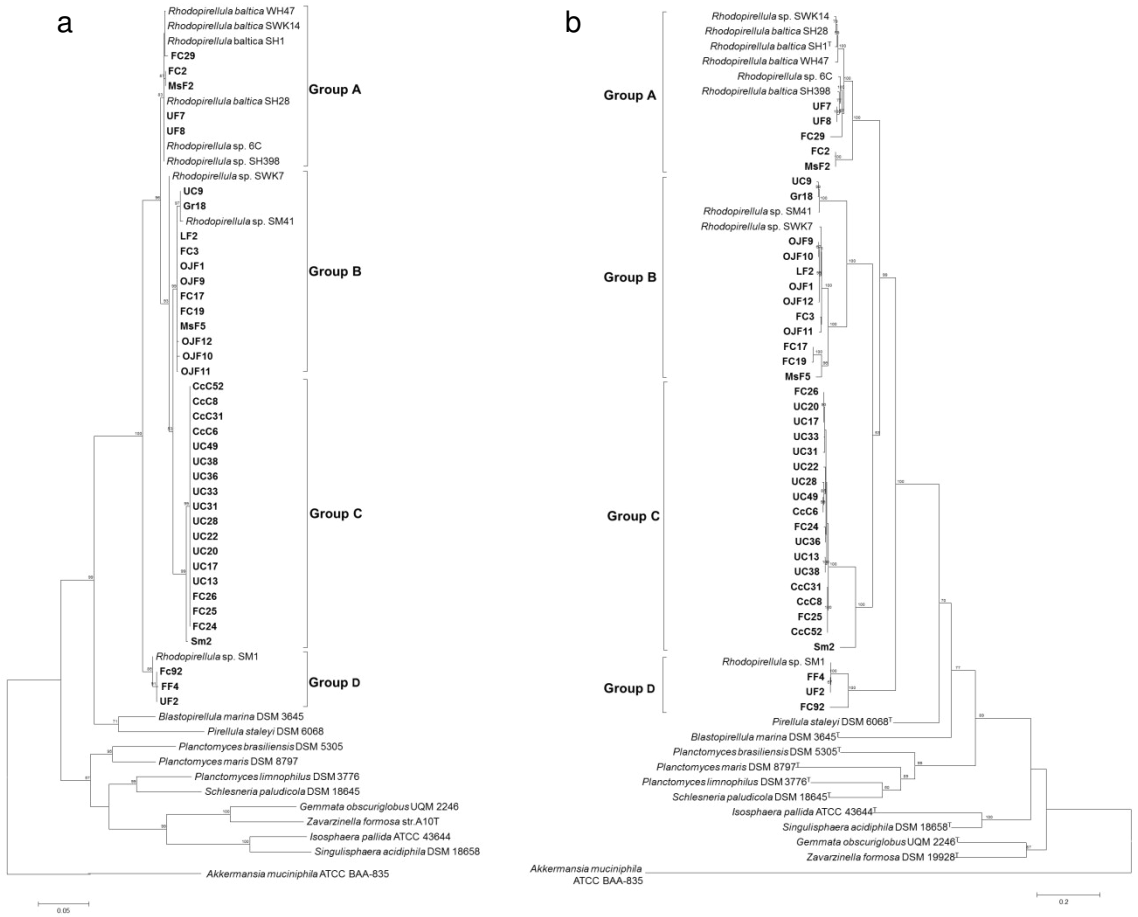

Fig. 6. 6 Maximum-Likelihood tree of members of the order *Planctomycetales* based on the 16S rRNA gene (a) and *rpoB* gene (b) sequences. Evolutionary analysis was based on the GTR+GI model. Strains in bold represent the isolates from Lage and Bondoso (2011) and Lage et al. (2012). The final dataset consisted of a total of 1224 (a) and 1079 (b) nucleotide positions. The numbers beside nodes are the percentages for bootstrap analyses; only values above 50% are shown. Scale bar= 0.05 substitutions per 100 nucleotides. The different ribotypes (groups A–D) are evidenced. *Verrucomicrobia* 16S rRNA gene sequences were used as an outgroup.

Comparison between *rpoB* gene sequences and genetic profile by ERIC-PCR data

In order to validate the use of *rpoB* gene as a genetic tool for the determination of intraspecific relations, similar isolates were analysed with ERIC-PCR fingerprinting (Fig. 6.7). ERIC-profiles revealed that the pairs FC2/MsF2 (Group A, Fig. 6.7a), FC17/FC19 (Group B, Fig. 6.7b) and CcC31/CcC52/CcC8 (Group C, Fig. 6.7c) were genetically very similar. For these strains, *rpoB* gene sequence similarity varied between 99.78 and 100 %.

Strain FC29 that belonged to the same ribotype of FC2/MsF2 (99.6 %) but to a different *rpoB* group (90.32 %) had a different ERIC-profile confirming the genetic discrimination power of *rpoB* gene.

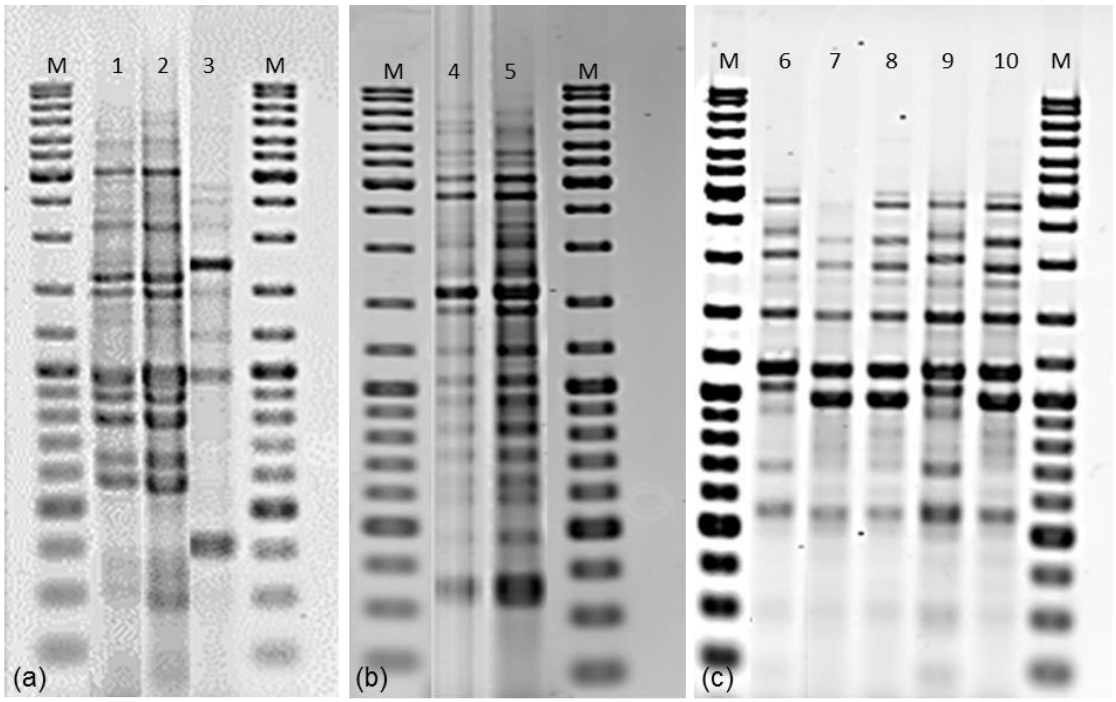

Fig. 6. 7 ERIC-PCR fingerprinting profiles of strains of groups A (a), B (b) and C (c). Lanes: M, DNA marker Gene Ruler Ladder Mix (Fermentas™); 1, FC2; 2, MsF2; 3, FC29; 4, FC17; 5, FC19; 6, UC49; 7, CcC32; 8, CcC51; 9, CcC6; 10, CcC8.

Discussion

Our results demonstrated that the *rpoB* gene sequencing and analysis is a powerful molecular tool to discriminate planctomycetal strains at the species level. The analysis of the complete *rpoB* gene showed that this gene has more discriminatory power than the widely used 16S rRNA gene when analysing closely related strains. Intraspecific relationships in the *Planctomycetes* were delineated by a threshold value in the *rpoB* gene in the range of 98.2 and 98.6 %, which is in agreement with the 98.2 % value reported in the literature (Adékambi et al., 2008). This threshold value is consistent with DDH data obtained previously and reported in Table 6.2 (Frank et al. unpublished). Isolates 6C and SH398 were shown to have a value of DNA reassociation with *R. baltica* SH1^T lower than 70% - indicative of a novel species (Wayne et al. 1987) – and 95.4 % similarity in the *rpoB* gene to *R. baltica* SH1^T. Isolate SH28 and *R. baltica* SH1^T exhibited a DDH higher than 70 % indicating that both belong to the same species, which is in agreement with an *rpoB* gene similarity of 98.9 %. In a survey of data available in the literature, Adékambi et al. (2008) also found a correlation between DDH and the *rpoB* gene and defined a value of 97.7 % for the delineation of novel species. In the present study, the cut-off value for *Planctomycetes* was found to be 95.5 %, value lower than the one reported by Adékambi et al. (2008). However, this threshold value was defined based on only one genus (*Rhodopirellula*) and six different species. So far, the majority of *Planctomycetes* genera consist of only one species which limited the analysis. For values between 95.5 and 98.2 % special care should be taken when defining a novel species. Further *rpoB* gene sequence data of novel species is essential to improve this threshold value.

For the analysis of the *rpoB* gene we propose the amplification of a hypervariable region between 2300-3300 bp, to avoid the sequencing of the entire gene (~3500bp). The sequencing of this region proved to allow accurate identification and discrimination of isolates in several groups of bacteria, like in *Enterobacteriaceae* (Mollet et al., 1997), *Mycobacterium* (Adékambi et al., 2003), *Corynebacterium* species (Khamis et al., 2004) and *Salmonella enterica* (Kwon et al., 2001). *Planctomycetes in silico* analyses revealed that the partial *rpoB* gene was correlated with the 16S rRNA gene and ANI data (Fig. 6.3 and 6.4) and allowed to define cut-off values for intra and interspecific relationships. Based on this study, a novel species of *Planctomycetes* can be defined by an *rpoB* gene sequence similarity lower than 95.5 % in the gene region 2300-3300bp. Similarities lower than 72 % indicated a novel genus, which matched with the 95 % 16S rRNA gene similarity, used for genus delineation (Goris et al., 2007; Tindall

et al., 2010). Intraspecific relations between the isolates were delineated by an *rpoB* gene sequence value higher than 98.6 % which corresponds to an ANI value of 95.8 % (Fig. 6.5). The ANI can be used to separate bacterial species as an alternative to DDH, and 70% DDH corresponds approximately to 95% ANI (Konstantinidis and Tiedje, 2005a; Goris et al., 2007). The existent congruence between *rpoB* gene data and the 16S rRNA gene or the ANI indicates that the partial sequencing and analysis of the *rpoB* gene is a good and viable alternative to conventional methods.

The novel primers designed, specific to *Planctomycetes,* allowed the construction of a database of 38 partial *rpoB* gene sequences and the threshold values defined above were applied to the taxonomy of the isolates under study. According to the 16S rRNA gene analysis, those isolates belong to the genus *Rhodopirellula*. Applying a 97 % similarity threshold (Stackebrandt and Goebel, 1994), the 16S rRNA gene could not discriminate between groups A, B, C and *R. baltica,* but separated group D in a new species of *Rhodopirellula*. However, applying the threshold values defined above for the *rpoB* gene, groups B and C clearly represent novel species of *Rhodopirellula* as the values of similarity in the partial *rpoB* gene varied between 78 and 80 %. Strains belonging to group A should also be placed in a separate species since they shared less than 95.5 % with the *rpoB* gene of *R. baltica* SH1^T. Furthermore, inside each group – defined as a group of strains where the 16S rRNA gene has insufficient resolving potential for the taxonomic question - the values of divergence in the *rpoB* gene varied from 0 to 15 %, indicating the existence of different species. It is worth noting that a species is a group of strains sharing 70% or greater DNA-DNA reassociation values and 5°C or less ΔTm and the phenotypic and chemotaxonomic features should agree with this definition (Stackebrandt, 2002). Although the strains might be genetically different because of different environment adaptations, they could present similar phenotypic and biochemical characteristics which are not sufficient to separate them into different species. The *rpoB* gene could be used as a tool to define and propose novel species but this approach has to be complemented with additional phenotypic information that confirms the phylogenetic result.

In this study, we suggest the use of the *rpoB* gene analysis as an alternative method to the low discriminatory power 16S rRNA gene for taxonomic affiliation of *Planctomycetes* strains. This technique allowed the discrimination of isolates at the intraspecific level. Furthermore, it can substitute the laborious DDH technique for the discrimination of two species.

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Supplementary Table S 4 Pairwise similarities of *rpoB* gene sequences (lower left) and 16S rRNA gene (bottom right) between the strains under study and *Rhodopirellula* spp.

		Group A											Group B												
		SH1	WH47	SWK1	SH28	FC29	UF7	UF8	6C	SH398	FC2	MsF2	LF2	FC3	OJF1	OJF9	OJF10	OJF11	OJF12	SWK7	FC17	FC19	MsF5	UC9	Gr18	SM41
Group A	SH1	-	99,92	100	99,92	99,68	99,92	99,92	99,92	99,92	99,76	99,76	97,88	97,88	97,88	97,88	97,63	97,80	97,71	98,70	97,88	97,88	97,88	97,55	97,55	97,88
	WH47	98,31	-	99,92	99,84	99,60	99,84	99,84	99,84	99,84	99,68	99,68	97,80	97,80	97,80	97,80	97,55	97,71	97,63	98,62	97,80	97,80	97,80	97,47	97,47	97,80
	SWK14	98,50	98,50	-	99,92	99,68	99,92	99,92	99,92	99,92	99,76	99,76	97,88	97,88	97,88	97,88	97,63	97,80	97,71	98,70	97,88	97,88	97,88	97,55	97,55	97,88
	SH28	98,88	98,31	99,25	-	99,60	100	100	100	100	99,84	99,84	97,96	97,96	97,96	97,96	97,71	97,88	97,80	98,78	97,96	97,96	97,96	97,63	97,63	97,80
	FC29	94,02	93,52	93,92	94,32	-	99,60	99,60	99,60	99,60	99,60	99,60	97,71	97,71	97,71	97,71	97,47	97,63	97,55	98,37	97,71	97,71	97,71	97,38	97,38	97,71
	UF7	95,81	95,41	95,71	95,91	95,41	-	100	100	100	99,84	99,84	97,96	97,96	97,96	97,96	97,71	97,88	97,80	98,78	97,96	97,96	97,96	97,63	97,63	97,80
	UF8	95,81	95,41	95,71	95,91	95,41	100,0	-	100	100	99,84	99,84	97,96	97,96	97,96	97,96	97,71	97,88	97,80	98,78	97,96	97,96	97,96	97,63	97,63	97,80
	6C	95,91	95,51	95,81	96,00	95,71	98,41	98,41	-	100	99,84	99,84	97,96	97,96	97,96	97,96	97,71	97,88	97,80	98,78	97,96	97,96	97,96	97,63	97,63	97,80
	SH398	96,20	95,81	96,10	96,30	95,61	98,79	98,79	98,60	-	99,84	99,84	97,96	97,96	97,96	97,96	97,71	97,88	97,80	98,78	97,96	97,96	97,96	97,63	97,63	97,80
	FC2	91,47	91,05	91,47	91,57	90,32	91,37	91,37	91,47	91,37	-	100	97,80	97,80	97,80	97,80	97,55	97,71	97,63	98,62	97,80	97,80	97,80	97,47	97,47	97,63
	MsF2	91,47	91,05	91,47	91,57	90,32	91,37	91,37	91,47	91,37	100	-	97,80	97,80	97,80	97,80	97,55	97,71	97,63	98,62	97,80	97,80	97,80	97,47	97,47	97,63
Group B	LF2	76,97	76,84	76,33	76,33	76,84	77,72	77,72	77,34	77,84	77,34	77,34	-	100	100	100	99,76	99,92	99,84	99,19	100	100	100	99,68	99,68	99,35
	FC3	77,09	76,97	76,46	76,46	76,84	77,72	77,72	77,59	77,84	76,97	76,97	98,79	-	100	100	99,76	99,92	99,84	99,19	100	100	100	99,68	99,68	99,35
	OJF1	76,97	76,84	76,33	76,33	76,84	77,72	77,72	77,34	77,84	77,34	77,34	100,00	98,79	-	100,0	99,76	99,92	99,84	99,19	100	100	100	99,68	99,68	99,35
	OJF9	76,84	76,71	76,21	76,21	76,71	77,59	77,59	77,47	77,72	77,22	77,22	99,91	98,69	99,91	-	99,76	99,92	99,84	99,19	100	100	100	99,68	99,68	99,35
	OJF10	76,84	76,71	76,21	76,21	76,71	77,59	77,59	77,47	77,72	77,22	77,22	99,91	98,69	99,91	100	-	99,68	99,60	98,95	99,76	99,76	99,76	99,44	99,44	99,11
	OJF11	76,97	76,84	76,33	76,33	77,09	77,72	77,72	77,59	77,84	77,22	77,22	98,50	98,97	98,50	98,41	98,41	-	99,76	99,11	99,92	99,92	99,92	99,60	99,60	99,27
	OJF12	76,84	76,71	76,21	76,21	76,71	77,59	77,59	77,22	77,72	77,22	77,22	99,91	98,69	99,91	99,81	99,81	98,41	-	99,03	99,84	99,84	99,84	99,52	99,52	99,19
	SWK7	76,97	76,84	76,33	76,33	77,09	77,97	77,97	77,84	78,09	77,22	77,22	98,97	98,88	98,97	99,07	99,07	98,60	98,88	-	99,19	99,19	99,19	98,87	98,87	98,54
	FC17	77,84	77,72	77,47	77,47	78,22	77,97	77,97	77,97	77,97	78,46	78,46	93,01	93,72	93,01	92,91	92,91	93,32	92,91	93,21	-	100	100	99,68	99,68	99,35
	FC19	78,22	78,09	77,84	77,84	78,34	78,09	78,09	78,34	78,09	78,59	78,59	92,71	93,42	92,71	92,60	92,60	93,01	92,60	92,91	99,72	-	100	99,68	99,68	99,35
	MsF5	76,71	76,59	76,46	76,46	77,34	77,47	77,47	77,47	77,47	78,59	78,59	94,02	94,52	94,02	93,92	93,92	94,32	93,92	94,02	95,71	95,41	-	99,68	99,68	99,35
	UC9	77,72	77,47	77,59	77,09	77,09	77,97	77,97	77,34	78,59	77,72	77,72	85,19	85,19	85,19	85,08	85,08	85,08	85,08	84,97	85,42	85,30	84,40	-	100,0	99,68
	Gr18	78,22	77,97	78,09	77,59	77,34	78,46	78,46	77,84	79,08	78,22	78,22	85,42	85,42	85,42	85,30	85,30	85,30	85,30	85,19	85,64	85,53	84,62	99,44	-	99,68
	SM41	78,46	78,46	78,59	78,09	77,84	78,95	78,95	78,34	79,57	78,34	78,34	85,53	85,53	85,53	85,42	85,42	85,42	85,42	85,30	85,75	85,64	84,51	99,07	99,44	-
Group C	FC24	80,65	80,65	80,41	80,29	80,29	80,05	80,05	80,65	80,65	80,41	80,41	78,95	79,20	78,95	79,08	79,08	79,44	78,83	79,32	78,59	78,22	79,20	78,34	78,83	78,83
	FC26	80,53	80,53	80,53	80,17	80,29	79,93	79,93	80,65	80,53	80,05	80,05	78,95	79,20	78,95	79,08	79,08	79,44	78,83	79,32	78,22	77,97	79,08	78,34	78,83	78,83
	UC13	80,77	80,77	80,53	80,41	80,41	80,05	80,05	80,41	80,65	80,29	80,29	79,20	79,44	79,20	79,08	79,08	79,57	79,08	79,32	78,34	78,09	79,32	78,71	79,20	79,20
	UC17	80,65	80,65	80,65	80,29	80,41	80,05	80,05	80,77	80,65	80,17	80,17	79,08	79,32	79,08	79,20	79,20	79,57	78,95	79,44	78,34	78,09	79,20	78,46	78,95	78,95
	UC20	80,65	80,65	80,65	80,29	80,41	80,05	80,05	80,77	80,65	80,17	80,17	79,08	79,32	79,08	79,20	79,20	79,57	78,95	79,44	78,34	78,09	79,20	78,46	78,95	78,95
	UC22	80,65	80,41	80,17	80,29	79,93	79,69	79,69	80,17	80,17	80,29	80,29	79,93	79,93	79,93	80,05	80,05	80,05	79,81	80,05	78,83	78,59	79,69	78,09	78,59	78,59
	UC28	80,77	80,77	80,53	80,65	80,05	80,05	80,05	80,77	80,65	79,93	79,93	80,17	80,41	80,17	80,29	80,29	80,53	80,05	80,53	79,69	79,44	80,17	78,59	79,08	79,08
	UC31	80,89	80,89	80,89	80,53	80,53	80,29	80,29	80,89	80,77	80,29	80,29	79,44	79,69	79,44	79,57	79,57	79,93	79,32	79,81	78,71	78,46	79,32	78,71	79,20	79,20
	UC33	80,53	80,65	80,65	80,29	80,53	80,05	80,05	80,77	80,65	80,05	80,05	79,08	79,32	79,08	79,20	79,20	79,44	78,95	79,44	78,46	78,22	79,32	78,59	79,08	79,08
	UC36	80,89	80,89	80,65	80,53	80,53	80,17	80,17	80,77	80,77	80,41	80,41	79,08	79,32	79,08	79,20	79,20	79,57	78,95	79,44	78,83	78,59	79,44	78,46	78,95	78,95
	UC38	80,77	80,77	80,53	80,41	80,41	80,05	80,05	80,41	80,65	80,41	80,41	79,32	79,57	79,32	79,20	79,20	79,69	79,20	79,44	78,46	78,22	79,44	78,83	79,32	79,32
	UC49	80,77	80,77	80,53	80,65	80,29	80,05	80,05	80,77	80,65	80,05	80,05	79,81	80,05	79,81	79,93	79,93	80,29	79,69	80,17	79,20	78,95	79,81	78,34	78,83	78,83
	CcC6	80,77	80,77	80,53	80,65	80,29	80,05	80,05	80,77	80,65	80,05	80,05	79,81	80,05	79,81	79,93	79,93	80,29	79,69	80,17	79,20	78,95	79,81	78,34	78,83	78,83
	CcC31	81,01	81,01	80,77	80,65	80,41	80,17	80,17	80,77	80,77	80,53	80,53	78,83	79,08	78,83	78,71	78,71	79,32	78,71	78,95	78,46	78,46	78,83	77,84	78,34	78,34
	CcC52	81,01	81,01	80,77	80,65	80,41	80,17	80,17	80,77	80,77	80,53	80,53	78,83	79,08	78,83	78,71	78,71	79,32	78,71	78,95	78,46	78,46	78,83	77,84	78,34	78,34
	CcC8	81,01	81,01	80,77	80,65	80,41	80,17	80,17	80,77	80,77	80,53	80,53	78,83	79,08	78,83	78,71	78,71	79,32	78,71	78,95	78,46	78,46	78,83	77,84	78,34	78,34
	FC25	80,89	80,89	80,65	80,53	80,29	80,05	80,05	80,65	80,65	80,41	80,41	78,71	78,95	78,71	78,59	78,59	79,20	78,59	78,83	78,34	78,34	78,71	77,72	78,22	78,22
	Sm2	81,01	81,01	81,01	80,77	80,77	82,08	82,08	82,31	82,20	81,96	81,96	80,41	80,05	80,41	80,53	80,53	79,93	80,29	80,53	79,81	80,05	79,81	79,32	79,32	79,81
Group D	Fc92	74,79	74,53	75,05	75,05	74,14	74,92	74,92	74,92	74,66	73,62	73,62	73,09	73,09	73,09	73,09	73,09	72,69	72,95	73,48	72,95	73,22	73,09	70,39	70,66	71,34
	FF4	73,35	73,22	73,62	73,62	72,82	73,22	73,22	73,62	73,35	73,88	73,88	74,01	73,75	74,01	74,01	74,01	73,75	73,88	73,88	75,18	74,92	74,92	71,34	71,61	72,02
	UF2	73,35	73,22	73,62	73,62	72,82	73,22	73,22	73,62	73,35	73,88	73,88	74,01	73,75	74,01	74,01	74,01	73,75	73,88	73,88	75,18	74,92	74,92	71,34	71,61	72,02

		Group C																		Group D		
		FC24	FC26	UC13	UC17	UC20	UC22	UC28	UC31	UC33	UC36	UC38	UC49	CcC6	CcC31	CcC52	CcC8	FC25	Sm2	Fc92	FF4	UF2
Group A	SH1	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,71	96,63	96,46	96,63
	WH47	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,30	97,63	96,55	96,38	96,55
	SWK14	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,71	96,63	96,46	96,63
	SH28	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,80	96,55	96,38	96,55
	FC29	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,55	97,88	96,46	96,29	96,46
	UF7	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,80	96,55	96,38	96,55
	UF8	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,80	96,55	96,38	96,55
	6C	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,80	96,55	96,38	96,55
	SH398	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,47	97,80	96,55	96,38	96,55
	FC2	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,63	96,38	96,21	96,38
	MsF2	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,63	96,38	96,21	96,38
Group B	LF2	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,13	95,36	95,19	95,36
	FC3	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,13	95,36	95,19	95,36
	OJF1	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,13	95,36	95,19	95,36
	OJF9	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,13	95,36	95,19	95,36
	OJF10	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,80	97,88	95,10	94,93	95,10
	OJF11	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	98,05	95,27	95,10	95,27
	OJF12	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,88	97,96	95,36	95,19	95,36
	SWK7	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	97,96	98,13	96,04	95,87	96,04
	FC17	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,13	95,36	95,19	95,36
	FC19	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,13	95,36	95,19	95,36
	MsF5	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,05	98,13	95,36	95,19	95,36
	UC9	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,96	95,19	95,36	95,19
	Gr18	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,71	97,96	95,19	95,36	95,19
	SM41	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,38	97,63	95,36	95,53	95,36
Group C	FC24	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	FC26	99,25	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC13	98,79	98,79	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC17	99,35	99,91	98,88	-	100	100	100	100	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC20	99,35	99,91	98,88	100	-	100	100	100	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC22	98,79	98,79	98,50	98,88	98,88	-	100	100	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC28	98,22	98,41	98,12	98,50	98,50	98,69	-	100	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC31	98,79	99,35	98,60	99,44	99,44	98,69	98,60	-	100	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC33	98,97	99,54	98,88	99,63	99,63	98,88	98,69	99,35	-	100	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC36	99,44	99,25	99,16	99,35	99,35	99,16	98,79	99,07	99,35	-	100	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC38	98,88	98,88	99,91	98,97	98,97	98,60	98,22	98,69	98,97	99,25	-	100	100	100	100	100	100	99,52	94,76	94,58	94,76
	UC49	98,50	98,88	98,60	98,97	98,97	98,79	99,35	99,07	98,97	99,07	98,69	-	100	100	100	100	100	99,52	94,76	94,58	94,76
	CcC6	98,50	98,88	98,60	98,97	98,97	98,79	99,35	99,07	98,97	99,07	98,69	100,0	-	100	100	100	100	99,52	94,76	94,58	94,76
	CcC31	98,60	98,60	98,60	98,69	98,69	98,31	98,12	98,69	98,50	98,79	98,69	98,41	98,41	-	100	100	100	99,52	94,76	94,58	94,76
	CcC52	98,60	98,60	98,60	98,69	98,69	98,31	98,12	98,69	98,50	98,79	98,69	98,41	98,41	100	-	100	100	99,52	94,76	94,58	94,76
	CcC8	98,60	98,60	98,60	98,69	98,69	98,31	98,12	98,69	98,50	98,79	98,69	98,41	98,41	100	100	-	100	99,52	94,76	94,58	94,76
	FC25	98,50	98,69	98,50	98,60	98,60	98,22	98,03	98,60	98,41	98,69	98,60	98,31	98,31	99,91	99,91	99,91	-	99,52	94,76	94,58	94,76
	Sm2	88,40	88,07	87,96	88,18	88,18	87,96	87,96	88,61	88,18	88,29	88,07	88,18	88,18	88,18	88,18	88,18	88,07	-	95,27	95,10	95,27
Group D	Fc92	72,95	72,82	73,35	72,95	72,95	72,82	73,48	73,22	72,95	73,22	73,22	73,48	73,48	73,35	73,35	73,35	73,22	75,44	-	99,84	100,00
	FF4	73,22	72,82	72,95	72,95	72,95	72,82	73,48	73,09	73,09	73,35	72,82	73,48	73,48	72,82	72,82	72,82	72,69	76,59	89,26	-	99,84
	UF2	73,22	72,82	72,95	72,95	72,95	72,82	73,48	73,09	73,09	73,35	72,82	73,48	73,48	72,82	72,82	72,82	72,69	76,59	89,26	100	-

Chapter 7.

Community composition of the *Planctomycetes* associated with different macroalgae[5]

Abstract

Insights into the diversity of marine natural microbial biofilms, as for example those developing at the surface of marine macroalgae, can be obtained by using molecular techniques based on rRNA genes. We applied DGGE with 16S rRNA genes specific primers for *Planctomycetes* to compare the communities of these organisms developing on six different macroalgae (*Chondrus crispus*, *Fucus spiralis*, *Mastocarpus stellatus*, *Porphyra dioica*, *Sargassum muticum* and *Ulva* sp.) sampled in spring 2012 in two rocky beaches in the north of Portugal. *Planctomycetes* can be one of the dominant organisms found in the epibacterial community of macroalgae and we wanted to determine the degree of specificity and the spatial variation of these group. Shannon diversity indexes obtained from the comparison of DGGE profiles was similar in all the macroalgae and in both sites. *F. spiralis* was the algae presenting lower planctomycetes diversity while *M. stellatus* and *P. dioica* from Porto showed the highest diversity. The analysis of DGGE profiles including ANOSIM statistics indicate the existence of a specific planctomycetes community associated with the algal host, likely independent of geographical variation. Sequencing of DGGE bands indicated that planctomycetes communities were highly diverse and some OTUs seemed to be specifically associated to each macroalgae.

[5] The results present in this chapter were submitted for publication in FEMS Microbial Ecology in the following manuscript
Joana Bondoso, Vanessa Balagué, Josep M. Gasol and Olga Maria Lage. Community composition of the Planctomycetes associated with different macroalgae

Introduction

Planctomycetes is a widespread phylum of *Bacteria* present in many diverse habitats although, in general, it appears in relatively low abundances in environmental samples (Rusch et al., 2007). They are usually found in association with eukaryotic hosts, like ants (Eilmus and Heil, 2009), invertebrates (Fuerst et al., 1991; Chaiyapechara et al., 2012), sponges (Pimentel-Elardo et al., 2003; Mohamed et al., 2008; Zhu et al., 2008; Ouyang et al., 2010; Sun et al., 2010; Sipkema et al., 2011; Webster et al., 2011; Costa et al., 2012), ascidians (Oliveira et al., 2013), corals (Webster and Bourne, 2007), macrophytes (Hempel et al., 2008; He et al., 2012), lichens (Grube et al., 2012), *Sphagnum* peat bogs (Kulichevskaya et al., 2006), and with the rizosphere of several plants (Nunes Da Rocha et al., 2009). They are also known to be frequent in the epibacterial community of several macroalgae (Longford et al., 2007; Bengtsson et al., 2010; Burke et al., 2011; Lachnit et al., 2011; Lage and Bondoso, 2011). An advantage for this colonization is the presence in planctomycetes of a high number of sulfatase genes (Wegner et al., 2013), which are involved in the degradation of the sulphated polymers produced by the algae.

Denaturing Gradient Gel electrophoresis (DGGE), is a molecular technique that separates similar sized DNA fragments. It was first described by Muyzer and collaborators (1993) as a community fingerprinting technique using the 16S rRNA gene to estimate bacterial diversity in environmental samples and identify the dominant uncultivable taxa. It is a relatively easy, reproducible, reliable, and fast technique. Although the DGGE does not allow a full taxonomic assignment, it has successfully been applied in the comparison of bacterial communities as they vary through time and space. DGGE has been used with marine bacterial assemblages (Murray et al., 1996; Moeseneder et al., 1999; Riemann et al., 1999; Schauer et al., 2000), marine picoeukaryotic assemblages (Díez et al., 2001), with the bacteria of the surface mucus layer of coral species (Morrow et al., 2012), the cyanobacterial epiphytes on macroalgae (Ohkubo et al., 2006) or the microbial community inhabiting sponges (Li et al., 2007; Thiel et al., 2007a; Thiel et al., 2007b) and corals (Webster and Bourne, 2007).

Mühling et al. (2008) developed primers to apply the technique to particular bacterial groups. One of the primer sets was developed for *Planctomycetes* and was further used by Pollet et al. (2011). Here, we optimized the *Planctomycetes*-specific PCR-DGGE developed by Mühling et al. (2008) and Pollet et al. (2011) to investigate the host-specific association of planctomycetes with six different macroalgae belonging

to the phyla *Heterokontophyta*, *Chlorophyta* and *Rhodophyta* and their spatial variation in two nearby locations of the north coast of Portugal.

Material and methods

Macroalgae sampling and site locations

Macroalgae were collected in May 2012 in tidal pools of the beaches in Porto (41º09'N, 8º40'W) and Carreço (41º44'N, 8º52'W). Fresh vegetative thalli of *Chondrus crispus*, *Fucus spiralis*, *Mastocarpus stellatus, Porphyra dioica, Sargassum muticum* and *Ulva sp.* were collected in triplicate in sterile plastic bags with seawater and transported to the lab within 1-2 hours. The algae used in this study were phylogenetically affiliated to *Heterokontophyta* (*F. spiralis* and *S. muticum*), *Chlorophyta* (*Ulva* sp.) and *Rhodophyta* (*C.crispus*, *M. stellatus* and *P. dioica*). The algae *Mastocarpus stellatus* was sampled only in Porto, as it was absent in Carreço. However, it was used for comparison with the other macroalgae from Porto. Temperature, salinity and pH were measured at the sampling sites. Once in the laboratory, the algae were rinsed in sterile natural seawater to remove loosely attached bacteria and frozen at -20ºC until DNA extraction was performed.

DNA extraction

Genomic DNA of the epiphyte bacterial communities was extracted with UltraClean® Soil DNA Isolation Kit (MoBio laboratories, Inc.). Ten circles from each specimen were cut with a circular 0.5 cm diameter cork borer and used for extraction. DNA extraction was performed according to manufacturer's instructions with the exception that the tubes containing the beads solution and the macroalgae pieces were initially vortexed for 15 min in a Disruptor Cell Genie.

PCR-DGGE fingerprinting

Geographical and macroalgae host variations of the planctomycetes communities were assessed by DGGE with the *Planctomyces*-specific pairs of primers described in Table 7.1. To determine the best method to visualize the DGGE profiles, three approaches were tested: 1) an initial PCR with the specific planctomycetes pair of primers 352F/920R, followed by a nested PCR with the pair of primers 518f-GC/907R, as described by Mühling et al. (2008); 2) direct PCR with the pair of primers 352F-GC/920R, as described by Pollet et al. (2011) and 3) an initial PCR with the pair of universal primers 9bfm/1512R, followed by a nested PCR with the primers 352F with a GC clamp and 920R using the previous PCR product diluted 200x as template. The

DGGE profiles were then compared and the method that yielded more defined and clear bands was selected.

Table 7. 1 Oligonucleotides used for PCR-DGGE

Primer	Sequence (5'-3')	Target organism	Reference
9bfm	GAGTTTGATYHTGGCTCAG	*Bacteria*	Mühling et al. (2008)
1512R	ACGGHTACCTTGTTACGACTT	Universal (bacteria and archaea)	Mühling et al. (2008)
PLA352F	GGC TGC AGT CGA GRA TCT	*Planctomycetales*	Mühling et al. (2008)
PLA920R	TGT GTG AGC CCC CGT CAA	*Planctomycetales*	Mühling et al. (2008)
518f-GC	CCAGCAGCCGCGGTAATCGCCC GCCGCGCGCGGCGGGCGGGGC GGGGGCACGGGGGGG	*Bacteria*	Muyzer et al. (1993)
907r	CCGTCAATTCMTTTGAGTTT	*Bacteria*	Muyzer et al. (1998)
GC-tail*	CGC CCG CCG CGCCCC GCG CCC GTC CCG CCG CCC CCC GGG CG		Pollet et al. (2011)

* Used in combination with the primer PLA352F

The variability of the planctomycetes microbial community on macroalgae was analysed by performing DGGE on 16S rDNA fragments of three individuals of the same algae collected in both locations. PCR reactions were performed in 50 µL mixtures containing 1x Green GoTaq® Flexi Buffer, 2 mM $MgCl_2$, 200 µM of each deoxynucleotide, 1 mM of each primer, 1 mg mL^{-1} of bovine serum albumin, 2 units of GoTaq® DNA Polymerase and 20 ng of DNA template. PCR conditions for each pair of primers described above were performed according to Mühling et al. (2008) and Pollet et al. (2011). The PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen). About 800 ng of the PCR products from these mixtures were run in a DGGE gel at 60 °C with a CBS Scientific system as previously described by Pollet et al. (2011) using a 50 to 70 % gradient (6 % acrylamide) at 120 V (18 h). A ladder made from a mixture of individual isolates of *Planctomycetes* previously isolated from macroalgae was also loaded in the extremities of the gel. The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1× Tris-acetate-EDTA buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized under UV light in a ChemiDoc system (Bio-Rad). The DGGE images were analysed using the QuantityOne software (Bio-Rad).

Sequence and analysis of DGGE bands

Representative DGGE bands were excised from the gel and re-amplified with the pair of primers 352F/920R with the PCR conditions described above. The resulting PCR product was purified and sequenced at Macrogen Europe using the primer 352F.

The sequences obtained from the DGGE gels were manually cleaned in Sequence Analysis 5.2 and blasted against the 16S rRNA gene database in RDP. The closest relatives were downloaded and aligned with the band sequences in ClustalW. The resulting alignment was used to construct an optimum maximum likelihood tree to determine the phylogenetic position of the obtained sequences.

Data analysis and statistical treatment of DGGE profiles

Digitized DGGE images were analysed using the QuantityOne software (BIORAD, USA). Similarity of resulting banding patterns was assessed by constructing a matrix taking into account the presence or absence of individual bands in each sample and their relative intensity. Based on this matrix, a Bray-Curtis similarity matrix was produced and then two types of analyses were run in order to plot the results. A MDS analysis modelled the variability of the patterns by representing them as points in a lower-dimensional space. On the other hand, the Cluster analysis grouped the samples on a dendrogram. These statistical analyses were run using the software tool PRIMER6 (Plymouth Routines in Multivariate Ecological Research). Comparisons between the sampling sites and macroalgae hosts were made using analyses of similarity (ANOSIM) in which an R value of 1 indicates maximum variation between groups and an R value of 0 indicates no differences between groups.

Results

DGGE profiles of *Planctomycetes* associated with the macroalgae

The first approach to study the planctomycetes communities in the surface of macroalgae following the PCR method described by Mühling et al. (2008) (method 1) appeared not to be appropriate for our samples. The resulting PCR amplicons yielded multiple faint and undefined bands when visualized in the agarose gel (details not shown). With the other two approaches, the resulting PCR product showed only one clear band with the expected size. The band obtained with the nested PCR with the pair of primers 352F-GC/920R (method 2) was more intense than the one from the direct PCR (method 3; details not shown). Furthermore, the number of bands obtained in the DGGE profiles with the nested PCR protocol was higher than the one obtained with the direct PCR approach, although these were apparently more defined and

without noise background (Fig. 7.1a). Some of the representative bands from both, the nested and direct PCR-DGGE, were sequenced and all matched to the *Planctomycetes* (details not shown). Further analyses with PCR-DGGE of the planctomycetes community on macroalgae were thus based on the nested PCR approach.

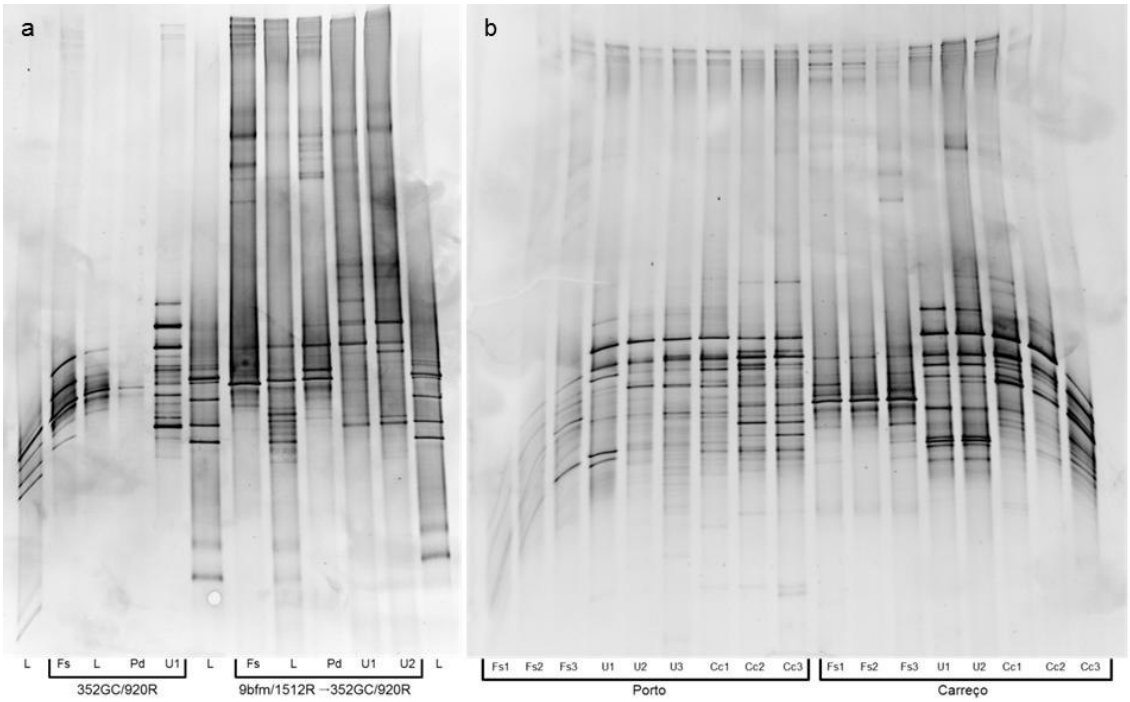

Fig. 7. 1(a) DGGE profiles of 16S rDNA amplified from different algae with the direct PCR protocol and specific pair of primers for *Planctomycetes* (left), or the nested approach (right). F- *Fucus spiralis*, L – *Laminaria* sp., P – *Porphyra dioica*, U1 and U2 – *Ulva* sp.. L – Ladder (b) DGGE fingerprinting profiles of the planctomycetes community associated with three individuals (1, 2 and 3) of *Fucus spiralis* (F), *Ulva* sp. (U) and *Chondrus crispus* (C)

The intra-species variability of the planctomycetes community between different individuals of the same macroalgae is shown in Figure 7.1b which shows DNA extracted and amplified from three individuals of the algae *C. crispus*, *F. spiralis* and *Ulva* sp. sampled at two different sites. Overall, the DGGE profiles of the triplicates of each alga were similar, with the exception of individuals U1 and CcC1 from Porto, and the relative band abundances were also similar, suggesting that the planctomycetes communities are consistent within different individuals of the same species of algae and do not present large intra-individual variations.

The DGGE profiles of the planctomycetes communities associated with the six different macroalgae from Carreço and Porto are shown in Figure 7.2. A total of 53 bands were identified in the gel. The bands identified in each DGGE profile were assumed to be different Operational Taxonomic Units (OTUs) and the intensity of each band was considered to provide the relative abundance of each OTU.

Based on these assumptions, the number of dominant OTUs (S) and the Shannon diversity index was determined for each macroalga (Table 7.2). The highest planctomycetes diversity was found in the red macroalgae *Porphyra dioica* (H' = 3.12) and *Mastocarpus stellatus* (H' = 3.02), both sampled in Porto. The other macroalgae showed lower planctomycetes diversity with Shannon indexes ranging from 2.4 to 2.9. Overall, there were no evident differences in the diversity of the planctomycetes communities in Carreço (mean H' = 2.71) and Foz (mean H' = 2.75) among different macroalgal species.

The analysis of the DGGE gel showed the existence of planctomycetes strains that are present in the majority of the macroalgae such as bands 10/28, 7, 25 and 4/12 while others were only found associated with a specific algal species in both locations, like band 23 (*P. dioica*) and bands 16/30 (*F. spiralis*).

Table 7. 2 Number of bands (S) observed in each macroalga and respective Shannon diversity index (H').

Macroalgae	Site	OTUs (S)	Shannon index (H')
Sargassum muticum	Carreço	18	2.85
	Porto	19	2.82
Porphyra dioica	Carreço	14	2.48
	Porto	25	3.12
Chondrus crispus	Carreço	19	2.85
	Porto	15	2.62
Ulva sp.	Carreço	19	2.84
	Porto	15	2.56
Fucus spiralis	Carreço	14	2.54
	Porto	12	2.38
Mastocarpus stellatus	Porto	23	3.02

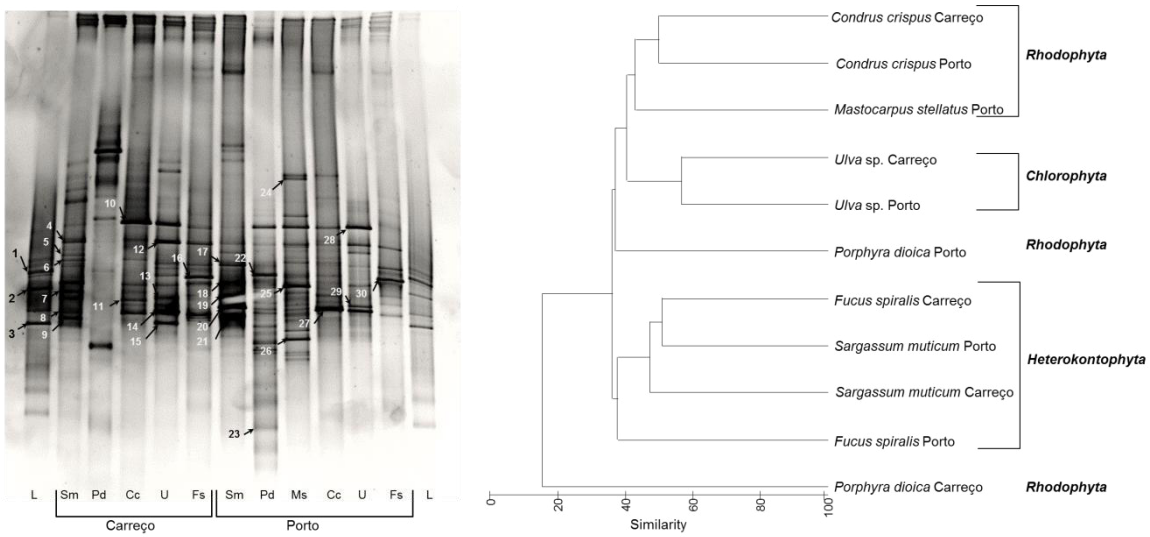

Fig. 7. 2 (a) DGGE fingerprinting profiles of the planctomycetes community associated with *Sargassum muticum* (Sm), *Porphyra dioica* (Pd), *Chondrus crispus* (Cc), *Ulva* sp. (U) *and Fucus spiralis* (Fs) from Carreço and Porto. L – Ladder. The arrows refer to the bands excised and sequenced. (b) Dendrogram of DGGE profiles of the planctomycetes communities, based on Bray-Curtis similarity.

Host-specific *Planctomycetes* community

The band profile of the DGGE gel was used to construct a resemblance matrix that originated a dendrogram showing the clustering of the samples (Fig. 7.2b). With the exception of *Porphyra dioica,* the community profiles of planctomycetes associated with the different macroalgae were clustered according to the algal host phylum and not according to the sampling site. *Ulva* sp. showed a high similarity between individuals from both locations (>60 %) while *S. muticum* and *C. crispus* exhibit a similarity of ~50 % between samples. In Figure 7.2b it is possible to visualize two major branches, one containing the DGGE profiles of *Rhodophyta* (*C. crispus* and *Mastocarpus stellatus*) and *Chlorophyta* algae and another one consisting of algae from the phylum *Heterokontophyta*. The profiles from the red algae *C. crispus* and the green algae *Ulva* sp. were grouped according to the host species and did not change with the geographical location. *P. dioica* and *F.spiralis* harboured a specific community that differed according to the habitat sampled. These findings were confirmed by statistical analysis with ANOSIM (a test to verify significant differences between two or more groups of samples) that confirmed the results discussed above. Samples were grouped by “Site” and “macroalgae species” as factors. There were no statistical differences in the DGGE profiles between both sites ($R = -0.056$, $p = 0.5$) while between the different algae the profiles were significantly distinct ($R=0.536$, $p=0.006$).

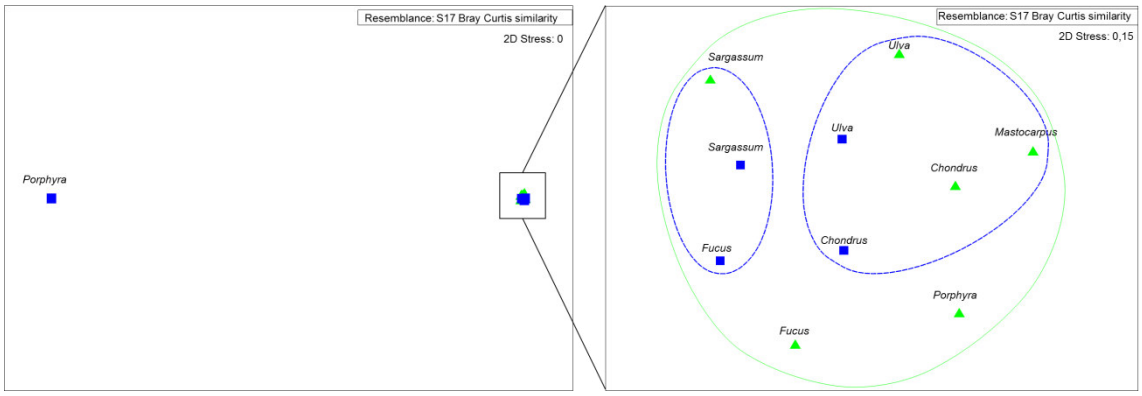

Fig. 7. 3 nMDS plots evidencing the clustering of the macroalgae-inhabiting *Planctomycetes* samples based on the DGGE profiles. (a) General MDS plot with all the samples. (b) MDS subgroup plot from samples inside the square in (a). ■ - Carreço, ▲ - Porto. — 20 % similarity; --- 40 % similarity;

A better visualization of the similarity between the planctomycetes communities in each macroalgae was obtained with a non-metric multidimensional scaling (nMDS, Fig. 7.3). The nMDS plot showed two distinctly separated groups. The planctomycetes community on *P. dioica* from Carreço was clearly distinct from the other macroalgae communities (Fig. 7.3). This group was expanded in a second nMDS plot that shows two clusters and isolated samples from the macroalgae *P. dioica* (Porto) and *F.spiralis* (Porto) with no similarity to the other groups. *Ulva* sp. (Porto and Carreço), *C. crispus* (Porto and Carreço) and *M.stellatus* (Porto) were grouped together with a similarity of 40 % and *S. muticum* (Porto and Carreço) shared a similarity of 40 % with *F. spiralis* (Carreço).

Taxonomic affiliation of the bands

In order to identify the major groups of *Planctomycetes* associated with the macroalgae, the most defined and representative bands were extracted from the gel and sequenced. Sequences that showed double peaks were eliminated from the analysis. A total of 30 band sequences were included in the final dataset. The sequences were all phylogenetically affiliated to the phylum *Planctomycetes* confirming the specificity of the primers used (Supplementary Table S5). Furthermore, some bands in different lanes but in the same position were cut and sequenced to confirm that they represented the same OTU. This was the case of the pair of bands 4/12, 10/28 and 16/30 that appeared in the phylogenetic tree with a value of similarity higher than 99.6 %. The majority of the bands sequenced represented distinct OTUs, with the exception of the bands 12, 14 and 27 that shared a similarity higher than 99.4 % and 20/21 that shared 100 % similarity in the 16S rRNA gene.

The closest relatives of the bands were mainly uncultured planctomycetes obtained from the surface of *Laminaria hyperborea*, *F.vesiculosus* and *Ulva australis*

(Supplementary Table S5). The closest cultured relatives were isolated strains from macroalgae surface (Lage and Bondoso, 2011) including strain Pd1 (from *Porphyra dioica*), strains LF1 and LF2 (from *Laminaria* sp.), strains FC18 and FF4 (from *F.spiralis*), and strain UC8 (from *Ulva* sp.).

The phylogenetic tree obtained (Fig. 7.4) showed that the OTUs were distributed in four major clusters (groups A-D) and were affiliated with genera *Rhodopirellula*, *Planctomyces* and with two unclassified planctomycetes genera. For an easier interpretation of the results, the 16S rRNA gene sequences were grouped at 98 % similarity, value indicative of "species". Group A can be divided in two different 'species' (A1 and A2) and contained the majority of the bands sequenced. OTUs belonging to this group were found in all the algae sampled suggesting a widespread distribution. It includes strains mainly isolated from macroalgae and it is phylogenetically related to planctomycete sp. FC18 that was originally isolated from the surface of *F. spiralis* from Carreço (Lage and Bondoso, 2011). 'Species' A1 can be found in a wide variety of habitats, including macroalgae, microbial mats, methane seep sediments, seafloor lavas, sponges and oil-polluted sediments indicating that these strains are widely distributed and also that they can adapted to extreme and polluted environments. However, 'species' A2 was only found in *Laminaria hyperborea*, *F. spiralis* and ocean water around *Enteromorpha prolifera*, suggesting that these strains are specifically associated with macroalgae. Group B sequences were phylogenetically related to the genus *Rhodopirellula* and contain three different OTUs closely related to other clones obtained from several habitats including macroalgae and sponges. Group C contains 'species' related to *Planctomyces maris* and other clones mainly described from macroalgae. OTUs represented by bands 6 and 7 were only found in the macroalga *Laminaria hyperborea* and were present in all the DGGE profiles indicating a possible specific association of these OTUs with macroalgae. Group D consisted of two different 'species' that were phylogenetically close to the Anammox genera (responsible for ANaerobic AMMonium Oxidation (Strous et al., 1999)) and presented less than 80 % similarity in the 16S rRNA gene to the described genera of *Planctomycetes*. This low value could be indicative of a distinct order of *Planctomycetes* that remain yet to be isolated.

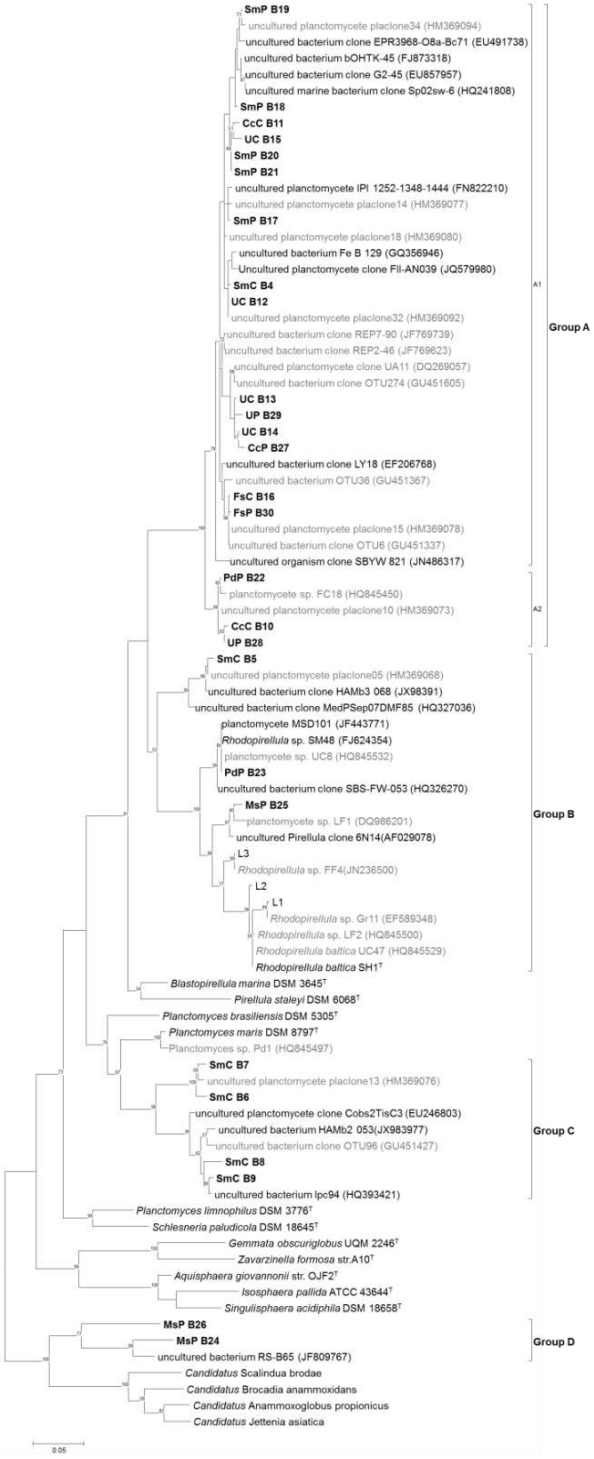

Fig. 7. 4 Maximum-Likelihood tree of 16S rRNA gene sequences extracted from DGGE bands (in bold) and their phylogenetic relation to other members of Planctomycetes and closest uncultured representatives. Strains in grey represent the clones identified from the surface of macroalgae. The numbers beside nodes are the percentages for bootstrap analyses; only values above 50 % are shown. Scale bar= 0.05 substitutions per 100 nucleotides. The different groups are presented on the right. Anammox 16S rRNA gene sequences were used as outgroup.

Discussion

This is the first culture-independent study exclusively focused on the distribution of planctomycetes in the epiphytic microbial community of several co-occurring macroalgae. In the last years, members of the *Planctomycetes* have been reported to be associated with macroalgae (Longford et al., 2007; Fukunaga et al., 2009; Bengtsson and Ovreas, 2010; Bengtsson et al., 2010; Burke et al., 2011; Lachnit et al., 2011; Lage and Bondoso, 2011; Miranda et al., 2013). Several novel taxa of planctomycetes have been isolated from the surface of macroalgae (Fukunaga et al., 2009; Lage and Bondoso, 2011), but it is well known that isolated strains do not always represent the whole community (Rappe and Giovannoni, 2003). With this study we aim to characterize the whole planctomycetes community associated with macroalgae. *Planctomycetes* are known to contain a high number of sulfatases genes that could play a major role in the degradation of the sulphated polysaccharides abundant in the algae walls (Wegner et al., 2013), which prompted us to investigate the planctomycetes community in these hosts. PCR-DGGE fingerprinting has been used in the microbial ecology study of bacterial communities associated with eukaryotic hosts, like sponges (Webster et al., 2011), algae (Lachnit et al., 2009; Tujula et al., 2010) and corals (Webster and Bourne, 2007) allowing to address questions such as the spatial and temporal variations and the determination of the host-specific bacterial community. In the present study we used PCR-DGGE with specific primers for planctomycetes to explore the epiphytic community of this group on different co-occurring macroalgae from different phyla and from two different locations. We showed that there were significant differences in the DGGE profiles of the six macroalgae, indicating the existence of a *Planctomycetes* specific community associated with each macroalgae. The results also indicated that individuals from the same algae but different locations were more similar to each other than to other algae in the same location. Furthermore it was shown that the intra-species variability of the planctomycetes communities within the same species was not significant, although *Ulva* sp. and *C. crispus* showed some variation. Similar banding patterns of DGGE of the whole bacterial community on individuals of the same algae species in the same habitat have been reported previously. Longford et al. (2007) showed a minimum of 60 % similarity between the bacterial communities in the epiphyte communities of the algae *Delisea pulchra* and 70 % similarity in *Ulva* sp. Tujula et al. (2010) reported that the differences in the individuals DGGE profiles of *Ulva australis* from different tidal pools where not greater than the differences between individuals collected in the same tidal pool. The results

presented in this study extend the absence of intraspecific variation within a given macroalgae to a specific group of bacteria.

By using PCR-DGGE of the 16S rRNA gene we determined the composition of each planctomycete community profile of six different co-occurring macroalgae. Each band was assigned to a different OTU, although we found that in some cases different bands were very similar (higher than 99.4 %). This does not exactly mean that they represent clones of the same species. In the case of planctomycetes it was already shown that they present a high genetic diversity at the ecotype level, determined by Multilocus Sequence Analysis (MLSA) and Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR), even when the 16S rRNA gene similarity between two isolates is higher than 99.5% (Winkelmann et al., 2010; Lage et al., 2012; Cayrou et al., 2013). However, we treated each band as a distinct OTU. In terms of richness there was not a significant difference among the algae, indicating that planctomycetes can easily colonize all the host species and there is a high diversity of species. This is in agreement with results obtained from culture- dependent methods in which different taxa of planctomycetes were isolated from different macroalgae, independently of the host species (Lage and Bondoso, 2011). Culture independent methods also have shown also that planctomycetes can be found in association with *F.vesiculosus*, *Gracilaria vermicuphylla*, *Ulva intestinalis* (Lachnit et al., 2011), *Ulva* spp. (Hengst et al., 2010), *Macrocystis pyrifera* (Michelou et al., 2013), *Ulva australis* (Longford et al., 2007; Burke et al., 2011), *Delisea pulchra* (Longford et al., 2007), *Laminaria hyperborea* (Bengtsson and Ovreas, 2010) and *Porphyra umbilicalis* (Miranda et al., 2013). Interestingly, they were absent in other algae, e.g. *Saccharina japonica* (Balakirev et al., 2012), *Laminaria saccharina* (Staufenberger et al., 2008). This can be due to the primers used, as some are known to contain mismatches to the phylum *Planctomycetes* or PCR conditions. As an example, an in-depth study where a large number (~16000 sequences) of clones from the whole bacterial community of *Ulva australis* (Burke et al., 2011) was sequenced showed the presence of 3.4 % planctomycetes clones, while a PCR-DGGE study made in the same algae indicated the absence of this phylum (Tujula et al., 2010).

Cluster analysis of the DGGE profiles showed the existence of a host-specific community of planctomycetes. The banding patterns of the algae *C. crispus*, *Ulva* sp. and *S. muticum* were more similar to each other than to the ones from other algae in the same location. Furthermore, they were grouped according to the phyla of host macroalgae, with the exception of *P. dioica*, suggesting the existence of epiphytic

planctomycetes shared among taxonomically closely related hosts. The influence of the host in the bacterial community of macroalgae has been reported previously, although these studies address the whole bacterial community and not only the planctomycetes. For example, *Fucus serratus, Fucus vesiculosus*, *Laminaria saccharina*, *Ulva compressa*, *Delesseria sanguinea* and *Phycodrys rubens* from North and Baltic Seas have epibacterial communities that differed less between both locations than between algae from the same place (Lachnit et al., 2009). These authors also found that DGGE profiles from the algae under study were grouped according to the host phylum in both locations. In our study, the DGGE fingerprinting profiles exhibited by *Rhodophyta* (with the exception of *Porphyra dioica* from Carreço) were more similar to the ones of *Chlorophyta*. This finding was confirmed also by 16S rRNA clone libraries of *C. crispus* and *Ulva* sp. that showed several OTUs shared in common by both algae (Bondoso et al., unpublished). In the same Lachnit et al. (2009) study, the DGGE profiles of the bacterial community associated with *Rhodophyta* were more similar to the ones of *Chlorophyta*. Other studies on bacterial epiphytes from macroalgae also found host-specificity of the bacterial communities. The red alga *Bonnemaisonia asparagoides* exhibited a different profile of bacterial species from other two coexisting red algae, *Lomentaria clavellosa* and *Polysiphonia stricta*, and there was not significant intraspecific differences between localities (Nylund et al., 2010). The bacterial composition on *Laminaria saccharina* and *Dyctyosphaeria ocellata* were very similar between different habitats (Staufenberger et al., 2008; Sneed and Pohnert, 2011). Hengst and collaborators (2010) reported a strong effect of the algal hosts *Ulva* spp., *Scytosiphon lomentaria* and *Lessonia nigrescens* in the bacterial community associated with these species. Host-specific associations have also been reported for sponges (Hentschel et al., 2002; Taylor et al., 2007) and diatoms (Grossart et al., 2005). Although a recent metagenome study on *Ulva australis*-associated bacteria suggested that the differences among macroalgae are dependent on bacterial species functionality and not on its taxonomy, our results reinforce the importance of the host phylogeny in other macroalgae-associated bacterial communities and suggest that macroalgae modulate the bacterial community associated with their surface through different intrinsic biological, physical and chemical characteristics. Furthermore, the planctomycetes communities from the surface of macroalgae would follow the same pattern as the whole bacteria community, presenting a host-specific association rather than a spatial specific distribution.

The phylogenetic composition of the planctomycetes community in the macroalgae was found to be very diverse. Eleven different ‘species’ were retrieved from the DGGE

extracted bands, based on a 98 % threshold. However, not all the bands could be extracted and sequenced, which could influence the results obtained indicating that not all the planctomycetes community was covered. The most abundant group was group A, composed of two distinct 'species' and 11 OTUs. 16S rRNA clone libraries obtained from *Laminaria hyperborea* surfaces also showed prevalence of strains belonging to this group, which accounted for 72 % to 97.8 % in the libraries. Similarly, in *Fucus vesiculosus*, 16S rRNA clone libraries revealed that the majority of the planctomycetes clones belonged to this group. So far, there is only one cultured representative in this group, isolated from the surface of *F.spiralis* from Carreço, *Planctomycete* sp. FC18 (Lage and Bondoso 2011). FC18 exhibited a 16S rRNA gene similarity between 94 and 98 % to the uncultured strains of this group. Some of the OTUs present in group A, in particular 'species' A2, appeared to be present only in macroalgae samples thus suggesting a specific association to macroalgae of these planctomycetes. Group B, closely related to *Rhodopirellula* spp., contains three different 'species' and were phylogenetically closer to other cultured planctomycetes isolated from the surface of macroalgae which are currently being described as novel genera. Although *Rhodopirellula* sp. and in particular *R. baltica* have been shown to be widely associated with macroalgae, surprisingly none of the DGGE bands matched this group. One possibility for this fact could be that not all the DGGE bands could be sequenced. 'Species' belonging to group C, affiliated to *Planctomyces maris*, were also retrieved from *Laminaria hyperborea*, *F.vesiculosus* and *P. dioica*. Two of the OTUs were exclusively found in *Laminaria hyperborea.* Group D was composed of two species loosely related with the order *Planctomycetales,* probably indicating the presence of a novel order of *Planctomycetes* that would be found mainly associated with macroalgae. The closest relative was only one uncultured sequence, with 93-94 % similarity in the 16S rRNA gene to group D OTUs.

The results presented in this study showed a host-specific community of planctomycetes associated with macroalgae. Furthermore, the planctomycetes communities were highly diverse and some of the OTUs were found to be specifically associated with macroalgae. Because DGGE does not allow a full taxonomic study and assignment of the communities, further studies are needed to investigate whether these specific OTUs are associated with a specific host, or are widely distributed among the macroalgae.

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Supplementary Table S 5 Taxonomic affiliation of the DGGE bands sequences with the closest uncultured and isolated organisms

BAND	Group	Closest organism (Uncultured)	Source	% similarity	Closest organism (isolate)	% similarity	RDP classification
L1					*Rhodopirellula* sp. Gr11; EF589348	0.990	*Rhodopirellula*
L2					*Pirellula* sp.; Schlesner 1; X81938	0.990	*Rhodopirellula*
L3					*Planctomyces* sp. UF2; HQ845531	0.99	*Rhodopirellula*
SmC B4	A1	uncultured planctomycete; placlone32; HM369092	Surface of *Laminaria hyperborea*	0.992	*Planctomyces* sp. FC18; HQ845454	0.953	unclassified_Planctomycetaceae
SmC B5	B	uncultured planctomycete; placlone05; HM369068	Surface of *Laminaria hyperborea*	0.990	*Rhodopirellula* sp. LF2; HQ845500	0.903	*Rhodopirellula*
SmC B6	C	uncultured planctomycete; placlone13; HM369076	Surface of *Laminaria hyperborea*	0.984	*Planctomyces* sp. Pd1; HQ845497	0.883	*Planctomyces*
SmC B7	C	uncultured planctomycete; placlone13; HM369076	Surface of *Laminaria hyperborea*	0.977	*Planctomyces* sp. Pd1; HQ845497	0.875	*Planctomyces*
SmC B8	C	uncultured bacterium; HAMb2_053; JX983977	biofilm developed from HAM lagoon water, Magdalen Islands	0.961	*Planctomyces maris* ^{T}; DSM 8797T; AJ231	0.898	*Planctomyces*
SmC B9	C	uncultured bacterium; lpc94; HQ393421	intestinal microflora of *Haliotis diversicolor*	0.992	*Planctomyces maris* ^{T}; DSM 8797T; AJ231	0.894	*Planctomyces*
CcC B10	A2	uncultured planctomycete; placlone10; HM369073	Surface of *Laminaria hyperborea*	0.982	planctomycete sp. FC18; HQ845450	0.973	unclassified_Planctomycetaceae
CcC B11	A1	uncultured bacterium; OTU36; GU451367	Surface of *Fucus vesiculosus*	0.981	planctomycete sp. FC18; HQ845450	0.953	unclassified_Planctomycetaceae
UC B12	A1	uncultured planctomycete; placlone32; HM369092	Surface of *Laminaria hyperborea*	0.996	planctomycete sp. FC18; HQ845450	0.953	unclassified_Planctomycetaceae
UC B13	A1	uncultured bacterium; OTU34; GU451365	Surface of *Fucus vesiculosus*	0.982	planctomycete sp. FC18; HQ845450	0.947	unclassified_Planctomycetaceae
UC B14	A1	uncultured planctomycete; UA11; DQ269057	Surface of *Ulva australis*	0.982	planctomycete sp. FC18; HQ845450	0.949	unclassified_Planctomycetaceae
UC B15	A1	uncultured bacterium; OTU36; GU451367	Surface of *Fucus vesiculosus*	0.971	planctomycete sp. FC18; HQ845450	0.944	unclassified_Planctomycetaceae
FC B16	A1	uncultured planctomycete; placlone15; HM369078	Surface of *Laminaria hyperborea*	0.988	planctomycete sp. FC18; HQ845450	0.947	unclassified_Planctomycetaceae
SmP B17	A1	uncultured planctomycete; placlone14; HM369077	Surface of *Laminaria hyperborea*	0.986	planctomycete sp. FC18; HQ845450	0.955	unclassified_Planctomycetaceae
SmP B18	A1	uncultured bacterium; bOHTK-45; FJ873318	sediments from the Okhotsk Sea	0.973	planctomycete sp. FC18; HQ845450	0.947	unclassified_Planctomycetaceae
SmP B19	A1	uncultured planctomycete; placlone34; HM369094	Surface of *Laminaria hyperborea*	0.988	planctomycete sp. FC18; HQ845450	0.955	unclassified_Planctomycetaceae
SmP B20	A1	uncultured bacterium; OTU36; GU451367	Surface of *Fucus vesiculosus*	0.952	planctomycete sp. FC18; HQ845450	0.953	unclassified_Planctomycetaceae
SmP B21	A1	uncultured bacterium; OTU36; GU451367	Surface of *Fucus vesiculosus*		planctomycete sp. FC18; HQ845450	0.955	unclassified_Planctomycetaceae
PdP B22	A2	uncultured planctomycete; placlone10; HM369073	Surface of *Laminaria hyperborea*	0.990	planctomycete sp. FC18; HQ845450	0.988	unclassified_Planctomycetaceae
PdP B23	C				planctomycete sp. UC8; HQ845508	0.988	*Rhodopirellula*
MsP B24	D	uncultured bacterium; RS-B65; JF809767	Medea hypersaline basin, Mediterranean Sea"	0.932	*Pirellula* sp.; Schlesner 158; X81941	0.821	*Rhodopirellula*
MsP B25	B	uncultured Pirellula clone 6N14; AF029078		0.979	planctomycete LF1; DQ986201	0.973	*Rhodopirellula*
MsP B26	D	uncultured bacterium; HglFeb6H7m; JX017243	marine bulk water	0.973	planctomycete FF4; JN236500	0.793	*Rhodopirellula*
CcP B27	A1	uncultured bacterium; Fe_B_129; GQ356946	methane seep sediment	0.981	planctomycete sp. FC18; HQ845450	0.946	unclassified_Planctomycetaceae
UP B28	A2	uncultured planctomycete; placlone10; HM369073	Surface of *Laminaria hyperborea*	0.981	planctomycete sp. FC18; HQ845450	0.971	unclassified_Planctomycetaceae
UP B29	A1	uncultured bacterium; OTU34; GU451365	Surface of *Fucus vesiculosus*	0.981	planctomycete sp. FC18; HQ845450	0.942	unclassified_Planctomycetaceae
FsP B30	A1	uncultured planctomycete; placlone15; HM369078	Surface of *Laminaria hyperborea*	0.990	planctomycete sp. FC18; HQ845450	0.949	unclassified_Planctomycetaceae

Chapter 8.

Seasonal and geographical variation of epiphytic *Planctomycetes* associated with three main lineages of macroalgae[6]

Abstract

Macroalgae provide rich habitats for different epibiotic microbial communities that can benefit from a range of organic carbon sources produced by the algae. In the last decades several phylogenetic studies have been performed in order to better understand the microbial composition of the macroalgae biofilm. Previous studies have demonstrated that planctomycetes are usually associated with several distinct macroalgae. In this study we assessed the dynamics of the planctomycetes epiphytic communities on three distinct macroalgae, *Fucus spiralis* (*Heterokontophyta*), *Ulva* sp. (*Chlorophyta*) and *Chondrus crispus* (*Rhodophyta*) sampled in two locations, through the combination of denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries. The temporal, geographical and host-specific variations of the planctomycetes epiphytic communities were assessed by DGGE through a nested PCR-approach with specific primers and their composition and diversity were determined through 16S rRNA gene clone sequencing in winter and summer samples. The planctomycetes communities on the macroalgae studied showed no significant variation during one year sampling but they were specifically associated with the host macroalga, independently of the sampling location. A total of 51 Operational Taxonomic Units (OTU), based on a cut-off of 97 %, were recovered from eleven 16S rRNA gene clone libraries. The majority of the clones were related to the *Pirellulales* which includes the genus *Pirelulla*, *Blastopirellula* and *Rhodopirellula* (74.1 %) and to the uncultured class OM190 (16.6 %). The genus *Planctomyces* represents only 5.7 % of the total OTUs. *Ulva* sp. and *C. crispus* showed a higher diversity than *F. spiralis.* None of the OTUs was shared by the three macroalgae, confirming the macroalgal host-specificity of the planctomycetes communities. The molecular studies presented in this work allowed a more in-depth characterization of the planctomycetes communities associated with macroalgae by identifying a high diversity that was not possible by culture dependent methods. This study provided a more comprehensive picture of the association planctomycetes-macroalgae enabling a better understanding of the nature of this interaction.

[6] The results present in this chapter are under preparation for submission

Introduction

Bacteria are frequent colonizers of living surfaces in the marine environment, by a process referred as epibiosis (Wahl, 2008; Wahl et al., 2012). These surfaces provide rich habitats for settlement and development of different epibiotic bacterial communities. Many eukaryotic hosts, such as sponges, corals, ascidians, bryozoans, micro- and macroalgae, have established a wide number of interactions with the microbial world that have been widely investigated because of the potential production of new bioactive products (Egan et al., 2008). Research on the biofilm microbial community associated with macroalgae has increased in the last years but the understanding of its ecology and microbial interactions is still scarce when compared, for example, to the sponge-bacteria associations. Epiphytic bacteria can have positive effects on the macroalgal host, assuming protective and nutritional roles (Wahl, 2008; Goecke et al., 2010) like preventing the biofouling and invasion of pathogens and protecting the macroalgae against toxic substances (Holmstrom et al., 1996; Maximilien et al., 1998; Egan et al., 2008; Wiese et al., 2009). Bacteria isolated from macroalgae are known to produce growth factors and are involved in nitrogen fixation which is important for the algae (Dimitrieva et al., 2006; Goecke et al., 2010). However, bacteria can also have negative effects on macroalgae by inducing diseases and decomposition (Vairappan et al., 2001; Ivanova et al., 2002; Michel et al., 2006). Molecular studies revealed that the main groups of bacteria usually found associated with macroalgae are α- and γ –*Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria* (Goecke et al., 2010; Friedrich, 2012; Hollants et al., 2013). Other phyla that have also been reported include members of *Cyanobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Chloroflexi*, *Deinococcus-Thermus*, *Fusobacteria*, *Tenericutes*, and the candidate division OP11 (Hollants et al., 2013).

Planctomycetes is an interesting group of bacteria due to their peculiar characteristics that usually are unforeseen in other bacteria. The wide association of planctomycetes with macroalgae was proved by culture dependent methods (Lage and Bondoso, 2011) and a novel order, *Phycisphaerales*, which contains an isolate obtained from the surface of the macroalgae *Porphyra* sp., was described (Fukunaga et al., 2009). Further culture independent studies reported the presence of planctomycetes in the microbial community of macroalgae (Meusnier et al., 2001; Longford et al., 2007; Bengtsson et al., 2010; Hengst et al., 2010; Liu et al., 2010; Burke et al., 2011; Lachnit et al., 2011; Miranda et al., 2013). The importance of this group of bacteria in these communities was observed in the kelp *Laminaria*

hyperborean where planctomycetes account for 51–53 % of the total bacteria (Bengtsson and Ovreas, 2010). Due to their capability to attach to surfaces, planctomycetes are, in fact, good candidates to live in the biofilm community of macroalgae. Furthermore, genome sequencing of planctomycetes revealed the existence of a high number of genes expressing sulfatases, which are involved in the degradation of polysaccharides produced by macroalgae (Wegner et al., 2013). This may constitute an advantage for planctomycetes in the biofilm community.

In the present study we assessed the seasonality, geographical and host variation of the planctomycetes epiphytic community on three distinct macroalgae, *Fucus spiralis* (*Heterokontophyta*), *Ulva* sp. (*Chlorophyta*) and *Chondrus crispus* (*Rhodophyta*) sampled in two 50 km distant geographical locations of the North coast of Portugal, through the combination of denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries.

Material and methods

Macroalgae sampling

Fresh vegetative thalli of *F. spiralis*, *Ulva* sp. and *C. crispus* were collected in two rocky beaches in the North coast of Portugal; Porto (41º09'N, 8º40'W) and Carreço (41º44'N, 8º52'W), between October 2010 and August 2011 in four different occasions with 3 month intervals, corresponding to the annual seasonal cycle. Once in the laboratory, three individuals of each alga were rinsed with sterile natural seawater to remove loosely attached bacteria and frozen at -20ºC until DNA extraction was performed.

DNA extraction

Genomic DNA of the macroalgae bacterial community was extracted with UltraClean® Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA). Ten circles from each specimen were cut with a circular 0.5 cm diameter cork borer. DNA extraction was performed according to manufacturer's instructions with the exception that the tubes containing the beads solution and the macroalgae pieces were initially vortexed for 15 min in a Disruptor Cell Genie (Scientific Industries Inc., Springfield, MA).

PCR-DGGE fingerprinting

Geographical and temporal variation of the *Planctomycetes* community was evaluated by covering the four sampling events through nested PCR-DGGE with the pairs of primers described in Table 8.1. The PCR with the pair of primers 9bfm/1512R was performed according to Muhling et al. (2008). The nested PCR with the planctomycetes specific primer 352F-GC/920R was done as described by Pollet et al. (2011) using as template the PCR product obtained with the pair of primers 9bfm/1512R diluted 200x. All PCR reactions were performed in 50 µL mixtures containing 1x Green GoTaq® Flexi Buffer, 2mM $MgCl_2$, 200µM of each deoxynucleotide, 1mM of each primer, 1mg/mL of bovine serum albumin, 2 units of GoTaq® DNA Polymerase (Promega, Madison, WI) and 20 ng of template DNA. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen, Carlsbad, CA). PCR products obtained from these mixtures (800 ng) were run in a DGGE gel at 60°C with a CBS Scientific system (CBS Scientific Co., Del Mar, CA) as previously described by Pollet et al. (2011) using a 50 to 70 % gradient (6 % acrylamide) at 120 V (18 h). The gel was stained with SybrGold (Molecular Probes-Invitrogen, Carlsbad, CA) for 45 min, rinsed with 1× Tris-acetate-EDTA buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Chemi Doc system (Bio-Rad, Hercules, CA). DGGE images were analysed using the Quantity One software (Bio-Rad, Hercules, CA) in order to detect the different bands present in the gels. DGGE bands were excised and reamplified with the pair of primer 352F/ 920R, without the GC clamp. The resulting PCR product was purified and sequenced at Macrogen (www.macrogen.com) using the primer 352F without the clamp.

16S rRNA clone libraries

In order to investigate the composition and diversity of the community of planctomycetes in the studied algae, 16S rRNA clone libraries from *C. crispus*, *F. spiralis* and *Ulva* sp. collected from Porto and Carreço in February 2011 (winter) and August 2011 (summer) were constructed with the *Planctomycetes* specific pair of primers PLA46F/P1390R (Table 8.1). PCR amplifications of the 16S rRNA gene were performed in 50 µL reactions with four microliters of pooled DNA from the three individuals. PCR reactions were carried out in a MyCycler thermocycler (BioRad Laboratories, Hercules, CA) and each consisted of 1x PCR Master Mix (Promega, Madison, WI), 100 µmol of each primer and 40 µg of bovine serum albumin. Thermal cycling was as described by Pollet et al. (2011). In order to minimize PCR bias in

cloning, PCR reactions were done in triplicate and pooled for library construction. Cloning, plasmid transformation and clone picking were performed by Macrogen (www.macrogen.com) with pTOP TA V2 vector. Ninety-six clones were selected from each library and both strands of each clone were sequenced with the universal primers M13F and M13R.

Table 8. 1 Oligonucleotides used for PCR-DGGE and 16S rRNA clone libraries

Primer	Sequence (5'-3')	Target organism	Reference
9bfm	GAGTTTGATYHTGGCTCAG	*Bacteria*	(Muhling et al., 2008)
1512R	ACGGHTACCTTGTTACGACTT	Universal (bacteria and archaea)	(Muhling et al., 2008)
PLA352F	GGC TGC AGT CGA GRA TCT	*Planctomycetales*	(Muhling et al., 2008)
PLA920R	TGT GTG AGC CCC CGT CAA	*Planctomycetales*	(Muhling et al., 2008)
PLA46F	GGA TTA GGC ATG CAA GTC	*Planctomycetales*	(Chouari et al., 2003)
P1390R	GAC GGG CGG TGT GTA CAA	Bacteria	(Chouari et al., 2003)
GC-tail	CGC CCG CCG CGCCCC GCG CCC GTC CCG CCG CCC CCC GGG CG		(Pollet et al., 2011c)

Data analyses and statistical treatment

Digitized DGGE images were analysed using the Quantity One software (Bio-Rad, Hercules, CA). To avoid variations among the gels, only data obtained in each gel was analysed together. Similarity of resulting banding patterns was assessed by constructing a matrix taking into account the presence or absence of individual bands in each sample and their relative intensity. Based on this matrix, a Bray-Curtis similarity matrix was produced and then two types of analysis were run in order to plot the results. An MDS analysis modelled the spatial variability of the patterns by representing them as points in a lower-dimensional space. On the other hand, the Cluster analysis grouped samples on a dendrogram, pointing significant differences ($p<0.05$) among them. These statistical analyses were run using the software PRIMER6 (Plymouth Routines in Multivariate Ecological Research). Comparisons between the sampling sites and macroalgae hosts were made using analyses of similarity (ANOSIM) to verify significant differences between two or more groups of samples.

Sequences derived from the 16S rDNA clone libraries were edited with Sequencing Analysis 5.2 (Applied Biosystems), assembled with Vector NTI Advance

11.5 and checked manually for errors. Sequence analyses were done using the QIIME pipeline, Quantitative Insight Into Microbial Ecology, (Caporaso et al., 2010). The sequences were clustered into operational taxonomic units (OTUs) at 97 % similarity using cd-hit (Li and Godzik, 2006), corresponding to the species level, and assigned taxonomically using the Greengenes database (DeSantis et al., 2006). The reference sequence of each OTU was used to construct a maximum likelihood tree with the closest neighbours determined by SeqMatch in the RDP database (Cole et al., 2007).

To evaluate the amount of diversity contained within samples (alpha diversity), we constructed rarefaction plots based on the phylogenetic diversity measurements within QIIME. To determine the amount of diversity shared between two samples (beta diversity) we employed the UniFrac metric and plotted a dendrogram of samples (Lozupone et al., 2006). The OTU network was visualized using Cytoscape (Saito R et al., 2012).

Results

DGGE fingerprinting profiles of the planctomycetes communities

In total, 24 DGGE profiles, visualized in five different gels (Supplementary Fig. S3 and S4), were obtained from the three macroalgae sampled in two locations and in four different occasions. Replicates where not used since it was demonstrated that intra-variability among individuals of the same algae was low (data not shown).

The possible existence of a specific association of planctomycetes with the macroalgae host and the temporal variation was visualized in two different DGGE gels, each one corresponding to a geographical site (Supplementary Fig. S3). The geographical variation of each alga was visualized in three different DGGE gels, each corresponding to the algae under study (Supplementary Fig. S4). The band pattern of the DGGE profiles was used to construct dendrograms evidencing the clustering of the different samples and non-metric multidimensional scaling figures were done for a better visualization (Fig. 8.1 and 8.2). The data obtained was statistically analysed through ANOSIM to confirm the separation of groups. Due to the low number of profiles analysed and absence of replicates, some values of p from the ANOSIM test were very high and therefore they should be analysed carefully. Figure 8.1 shows that, in general, the DGGE profiles of the planctomycetes communities were grouped according to the algae sampled, independently of the sampling season. These findings are supported by ANOSIM statistical test (Table 8.2) in which R values higher than 0.75 indicate clearly separated groups (Clarke and Warwick, 2001) proving that

the communities varied significantly with the macroalgal host independently of sampling season. The communities of *F. spiralis* were the most distinct in both locations with similarities values to the communities of the other two macroalgae lower than 30 % (Fig. 8.1 a and c) and forming a clear separated group in the nMDS plots (Fig. 8.1 b and d). The communities of *Ulva* sp. and *C. crispus* were grouped together and shared a similarity between 40 and 60 % (Fig. 8.1 a and c). The ANOSIM test (Table 8.2) showed that these communities formed barely indistinguishable groups ($R < 0.385$) whereas *F. spiralis* communities are clearly different from the ones of *Ulva* sp. and *C. crispus* ($R \cong 1$). In Carreço, the communities of *Ulva* sp. and the ones of *F. spiralis* were grouped and formed two separated groups in the nMDS plots (Fig 8.1b), what was not observed in *C. crispus* communities. In Porto, the communities of each macroalga were clustered together, with the exception of *C. crispus* sampled in summer and *Ulva* sp. sampled in winter (Fig. 8.1d).

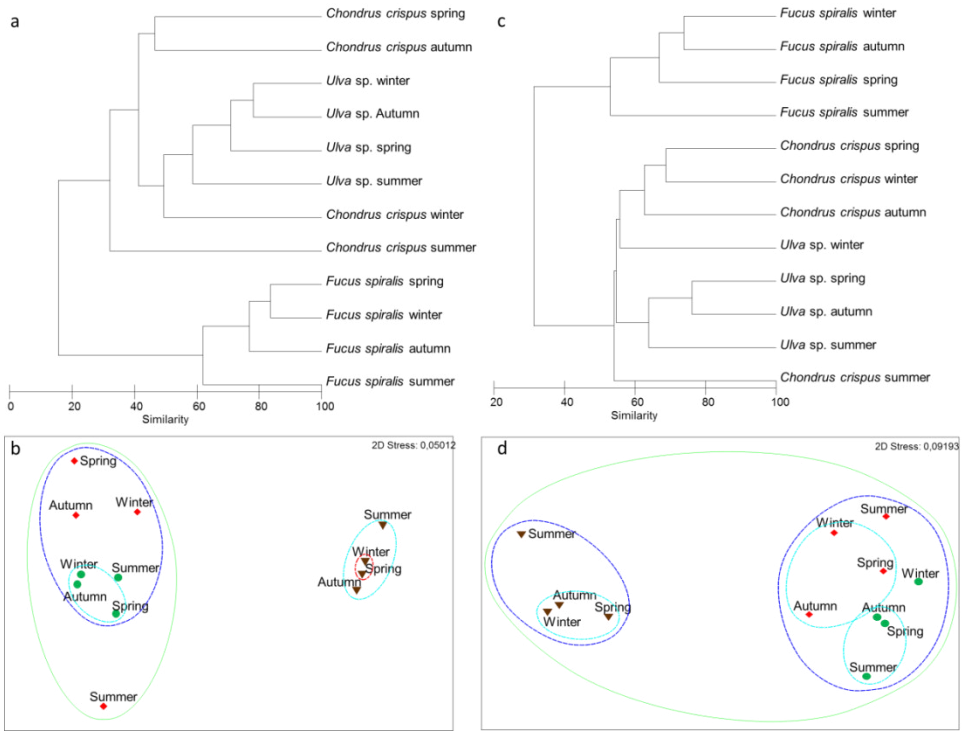

Fig. 8. 1 Dendrograms of DGGE profiles (a and c) based on Bray-Curtis similarity (a and c) and respective nMDS plots (b and c) evidencing the clustering of the samples of the planctomycetes communities associated with the macroalgae from Carreço (a and b) and Porto (c and d). ▼- *Fucus spiralis*; ● – *Ulva* sp.; ♦ - *Chondrus crispus* — 20 % similarity; --- 40 % similarity; --- 60 % similarity; --- 80 % similarity

The DGGE profiles with the variation of the planctomycetes communities (Supplementary Fig. S4) through the four one-year sampling, showed that the ones associated with *Ulva* sp. were very similar as well as the ones from *C. crispus* as confirmed by ANOSIM (Table 8.2) and evidenced in the dendrograms and nMDS plots (Fig. 8.2 c-e). Regarding *F. spiralis*, the planctomycetes communities were grouped according to the geographical site with the exception of the ones sampled in summer (Fig. 8.2 a and b). However, the ANOSIM test does not show any geographical influence. These results suggest that there is a stable community of planctomycetes associated with each macroalgae that is kept regardless of the geographical location and the time of the year.

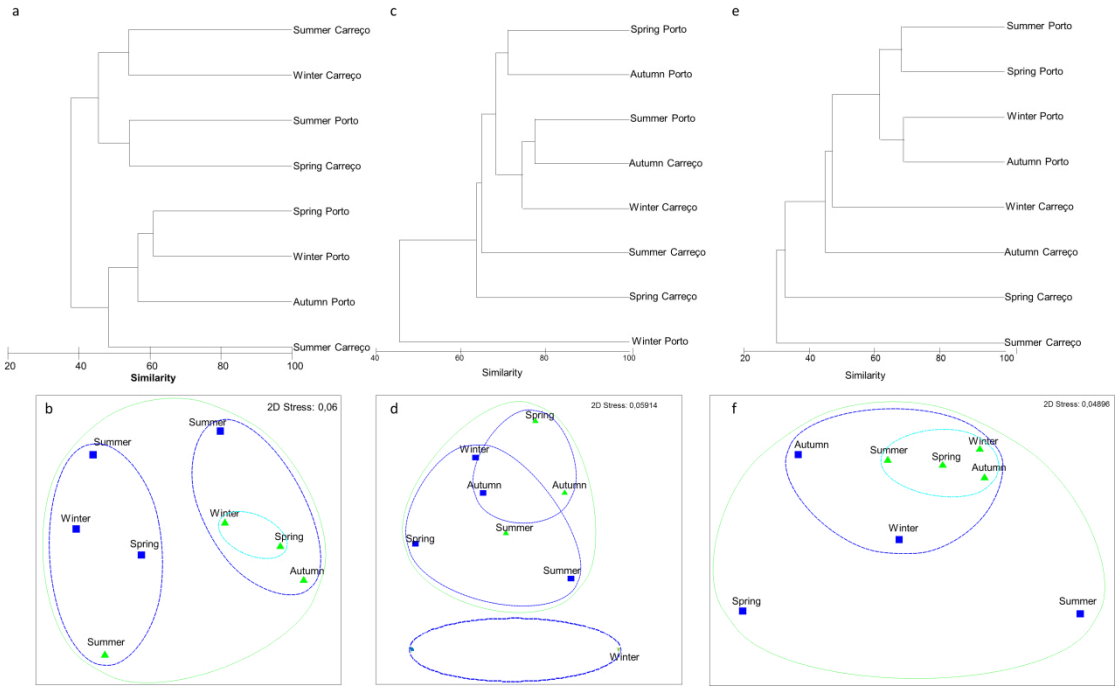

Fig. 8. 2 Dendrograms of DGGE profiles (a, c and e) based on Bray-Curtis similarity and respective nMDS plots (b, d and f) evidencing the clustering of the samples of the planctomycetes communities associated with *Fucus spiralis* (a and b), *Ulva* sp. (c and d) and *Chondrus crispus* (e and f). ■ - Carreço, ▲ - Porto

Table 8. 2 Comparative analysis of similarity (ANOSIM) of planctomycetes communities on the surfaces of *F. spiralis*, *Ulva* sp. and *C. crispus*. DGGE profiles were grouped by "Site", "Season" and "macroalgae species" as factors.

Factor	Algae/Site	R	p
Site	*C. crispus*	0.302	0.05*
	F. spiralis	0.292	0.05*
	Ulva sp.	-0.042	0.6
Season	*C. crispus*	-0.396	1
	F. spiralis	-0.125	0.6
	Ulva sp.	0.021	0.5
	Carreço	-0.275	0.9
	Porto	-0.235	0.9
Macroalgae	Carreço	0.813	0.001*
	Fucus, Ulva	1	0.02
	Fucus, Chondrus	0.958	0.02
	Ulva, Chondrus	0.292	0.02
	Porto	0.789	0.001*
	Fucus, Ulva	1	0.02
	Fucus, Chondrus	0.99	0.02
	Ulva, Chondrus	0.385	0.08

* Significant results

Overview of the 16S rRNA gene clone libraries

For an in-depth study of the composition and diversity of the epiphytic planctomycetes communities, eleven clone libraries were constructed for the macroalgae sampled in both sites in summer and winter. These seasons were chosen due to more extreme different environmental conditions. For technical reasons it was not possible to perform a 16S rRNA gene clone library of *C. crispus*, Carreço, winter.

A total of 1003 sequences were obtained and from these, 729 (~73 %) were affiliated to the *Planctomycetes* (Supplementary Table S5). The non planctomycetes clones were mainly *Proteobacteria* (15 %), *Verrucomicrobia* (2 %) and *Bacteroidetes* (2 %). Other phyla included *Deinococcus-Thermus*, *Actinobacteria* and the candidate division OD1. Macroalgae chloroplasts represented 2 % of the clones obtained. The sequences represented 51 OTUs at a 97 % identity cut-off value on the 16S rRNA gene (representing different species). The values of the alpha diversity metrics and predicted OTUs (Chao1) for each clone library are shown in Supplementary table S5. The diversity as well as richness was higher for communities associated with the macroalgae *C. crispus* (Table 8.3 and Supplementary Fig. S5). There were no differences in diversity between winter and summer although richness was higher on winter. Porto showed a higher diversity and richness of species than Carreço. With the exception of *F. spiralis* clone libraries, the observed rarefaction curves (Supplementary Fig. S5) were lower than the estimated ones.

Table 8. 3 Clone libraries diversity and richness estimates grouped by algae, site and season

Group	# sequences	# OTUs	Shannon	Simpson	Evenness	Chao1
F. spiralis	271	14	1.17	0.49	0.44	5.2
Ulva sp.	223	26	2.42	0.87	0.74	13.9
C. crispus	227	33	2.67	0.89	0.76	25.1
Carreço	321	27	2.02	0.78	0.61	8.8
Porto	400	39	2.80	0.91	0.76	15.3
Winter	348	36	2.55	0.87	0.69	15.7
Summer	373	36	2.50	0.86	0.71	10.8

Taxonomic composition of the planctomycetes community

According to the Greengenes taxonomic outline (McDonald et al., 2012), the cloned sequences were distributed across two classes (*Planctomycetacia* and OM190) and four different orders of *Planctomycetes* (Fig. 8.3). The order *Pirellulales*, which includes the genera *Pirellula*, *Rhodopirellula* and *Blastopirellula* according to the Bergey's Taxonomy (Ward, 2010), was the most abundant with 76.4 % of the total clones. Their representatives were present in all the samples in numbers ranging from 47.1 % to 100 %. The macroalga *F. spiralis* was the one showing a higher presence of *Pirellulales*, and in Carreço it was the only group found. The class OM190 with the orders CL500-15 and agg227 and formed by uncultured organisms, represented 17.5 % of the sequences obtained and was present in all the macroalgae with the exception of *F. spiralis* sampled in Carreço. The less abundant taxon was represented by the genus *Planctomyces* (5.7 %), which appeared only in some clone libraries of *Ulva* sp. and *C. crispus.*

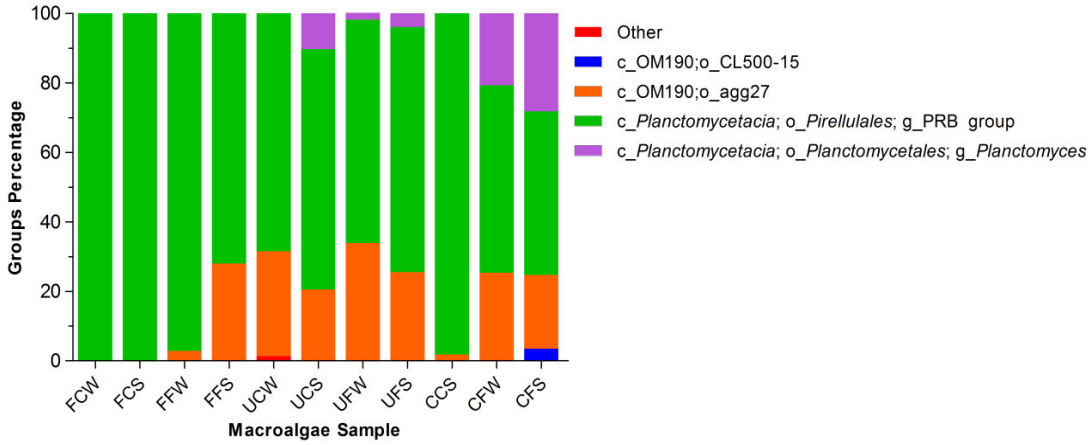

Fig. 8. 3 Taxonomic distribution of the OTUs in the 16S rRNA gene clone libraries. *c* stands for class, *o* stands for order and *g* stands for genera. FC – *F.spiralis*, Carreço; FF - *F.spiralis*, Porto, UC – *Ulva* sp., Carreço, UF – *Ulva* sp., Porto, CC – *C. crispus*, Carreço, CF – *C. crispus*., Porto; W – winter, S - summer

The detailed taxonomic classification can be found in Supplementary Table S6 and the relative abundance of each OUT is represented in the heatmap of Supplementary Figure S6. About 59 % of the clones obtained were related to clones associated with macroalgae, including *Fucus vesiculosus, Gracilaria vermicuphylla*, *Laminaria hyperborea* and *Ulva australis*, and the majority shared similarities higher than 97 % in the 16S rRNA gene (Supplementary Table S6). Moreover, several of the OTUs sequenced were related to cultured strains previously isolated from the surface of several macroalgae, including *Planctomyces* sp. Pd1, planctomycete sp. UC8, UF2, FC18 and LF1, *Rhodopirellula* sp. LF2, FC3 and UC16 and *R. baltica* sp. CcC1 (Lage and Bondoso, 2011). The majority of the 16S rRNA sequences similarities of the OTUs relatively to cultured representatives of *Planctomycetes* was under 96 %, indicating the existence of an unknown diversity of this phylum inhabiting macroalgae. The most abundant OTU was OTU30 representing 30 % of the total clones, followed by OTUs 34 (11 %) and 45 (9 %). These OTUs were taxonomically related to the planctomycete FC18, isolated from the surface of *F. spiralis* (Lage and Bondoso, 2011), with similarities in the 16S rRNA gene ranging from 93.3 to 98.1 %.

The phylogenetic tree obtained from representative sequences of the 51 different OTUs (Supplementary Fig. S7) showed the existence of 6 major phylogenetic clusters (Groups A, B, C, D, E and F) which can be divided in minor sub-groups. Some of these sub-groups are found exclusively in association with macroalgae (Supplementary Fig. S5, highlighted in pink) and account for 31 % of the total OTUs sequenced suggesting the existence of specific taxa of planctomycetes that are exclusively found in these biofilm communities. Group A contains 11 OTUs mainly

found in *C. crispus* and *Ulva* sp. They are phylogenetically related to the genus *Rhodopirellula*, sharing between 93.7 and 98.8 % similarity in the 16S rRNA gene. Many of these OTUs are closely related to strains isolated from the surface of macroalgae (Lage and Bondoso, 2011) forming several sub-groups within group A and sharing similarities between 96.3 and 99.7 % in the 16S rRNA gene. Group B is the most abundant one and contains 20 OTUs, many of which were found only associated with *F. spiralis*. It contains mainly uncultured strains from macroalgae, suggesting that it is a widespread group preferentially associated with macroalgae. The closest cultured relative is planctomycete FC18, isolated from *F. spiralis* in Carreço, with a similarity in the 16S rRNA gene ranging from 83 % to 99.1 %. The phylogenetic position of this group is uncertain as the bootstrap values on the phylogenetic tree are low (under 50 %). They appear more related to *Pirellula staleyi* in the phylogenetic tree although they share between 92 and 75 % similarities values in the 16S rRNA gene to *R. baltica*, *B. marina* and *P. staleyi*. Group C contains only one OTU found in only one sample from *C. crispus* and is closely related to an uncultured clone from ocean water around the macroalgae *Enteromorpha prolifera*. It is phylogenetically affiliated to *Blastopirellula marina* with 91 % similarity in the 16S rRNA gene and the closest cultured representative is a planctomycete isolated from the sponge *Niphates* sp. In Group D there are 9 OTUs, three of which were only found in macroalgae. They are associated with *C. crispus* and *Ulva* sp. and are phylogenetically related to *Planctomyces maris* with similarities in the 16S rRNA gene of around 90 %. Groups E and F are clearly separated from the members of the order *Planctomycetales* sharing 16S rRNA gene similarity values lower than 81 %.

The distribution of the 51 different OTUs among the different macroalgae clone libraries was investigated and an OTU network was constructed evidencing the shared and unique OTUs (Fig. 8.4). The macroalgae *C. crispus* had more exclusive OTUs (not shared with any other algae type), whilst *F. spiralis* had the lowest number of unique OTUs. *C. crispus* and *Ulva* sp. shared more OTUs between them, in agreement with results from DGGE fingerprinting analyses, than with *F. spiralis*. None of the OTUs was found to be present in all the clones, indicating the absence of a common core community of planctomycetes among macroalgae. Nevertheless, some OTUs were present in a large number of the clone libraries (OTUs 30, 34, 36, 37 and 45) and were closely related to uncultured planctomycetes from the surface of the macroalgae *Fucus vesiculosus* and *Laminaria hyperborea*, with shared similarities ranging from 98 to 99.7 % in the 16S rRNA gene.

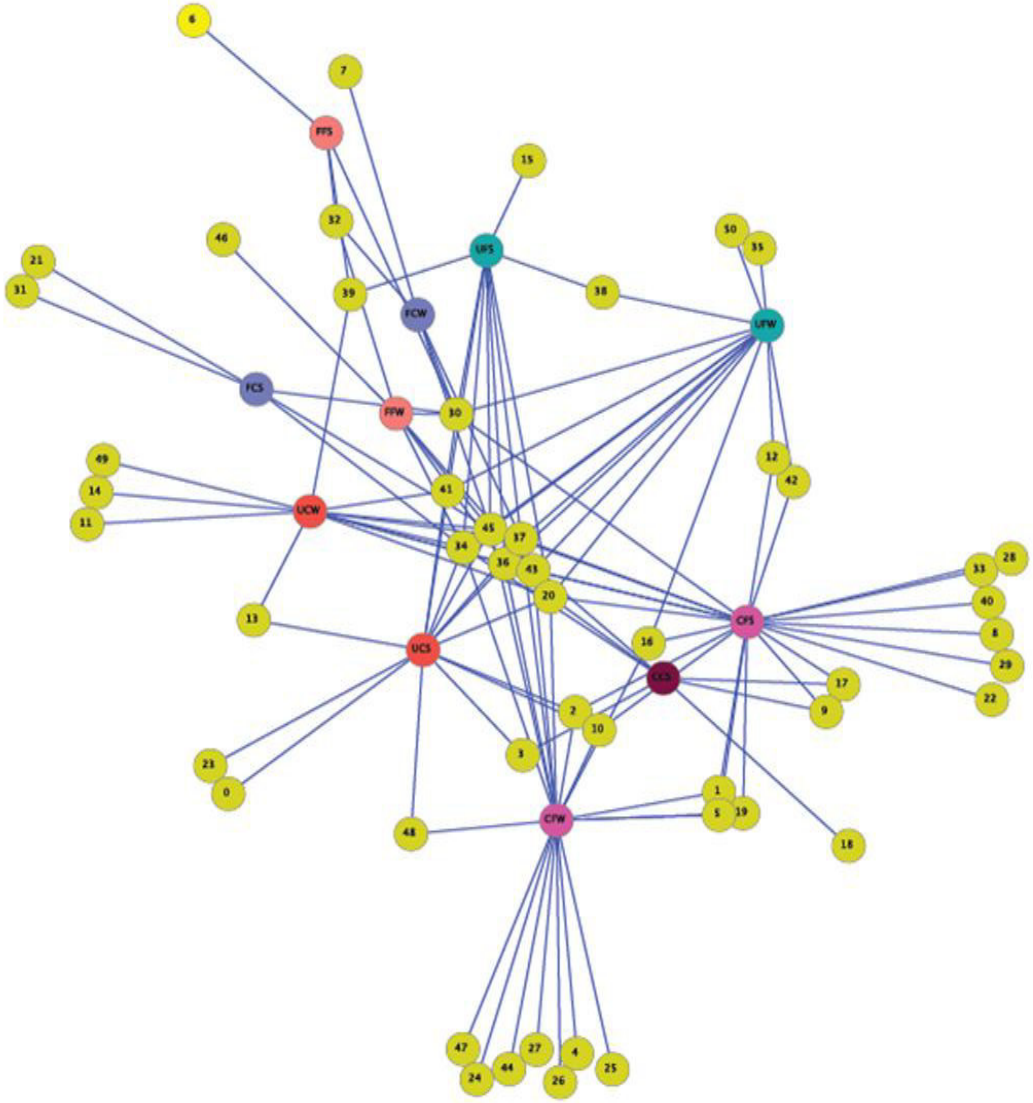

Fig. 8. 4 OTU network map showing the shared OTUs among the clone libraries. FC – F. *spiralis*, Carreço; FF - *F.spiralis*, Porto, UC – *Ulva* sp., Carreço, UF – *Ulva* sp., Porto, CC – *C. crispus*, Carreço, CF – *C. crispus*., Porto; W – winter, S - summer

The phylogenetic relationship among the planctomycetes communities based on their taxonomic composition was obtained by analysing betadiversity through clustering (Fig. 8.5a) and Principal Coordinates Analysis (PCoA, Fig. 8.5b). The planctomycetes populations seem to be significantly associated with the macroalga type regardless of some seasonal changes, namely the ones from summer sampled in Carreço. *F. spiralis* samples harbour communities that are very different from those of *Ulva* sp. and *C. crispus* showing a distant placement in the betadiversity plots (Fig. 8.4b) as shown also by DGGE profiles analyses. The communities of *Ulva* sp. and *C. crispus* were more related between them than to ones of *F. spiralis* (Fig. 8.4a). This result supports the DGGE data. The differences obtained among the clone libraries

were due to 13 OTUs that were found to be differentially abundant between the host, season and sampling site (Table 8.5). Statistical analysis using ANOVA test were performed to determine whether OTUs relative abundance was significantly different between the categories used (host, season or sampling site). None OTU differed significantly with season or sampling site, while some showed a strong association with the macroalga species. OTUs 20 and 36 which belong to the deep branching group F were found mainly associated with *Ulva* sp. and *C. crispus*. OTU 30 from group B and phylogenetically related to planctomycete FC18 was present in higher abundances in *F. spiralis* than in the other two macroalgae and OTU 32, also belonging to group B, was found exclusively associated with *F. spiralis*. OTU 37, from group A and phylogenetically related to *Rhodopirellula* sp., was only present in *Ulva* sp. and OTU 41, also from group A, was present in more abundance in this macroalgae.

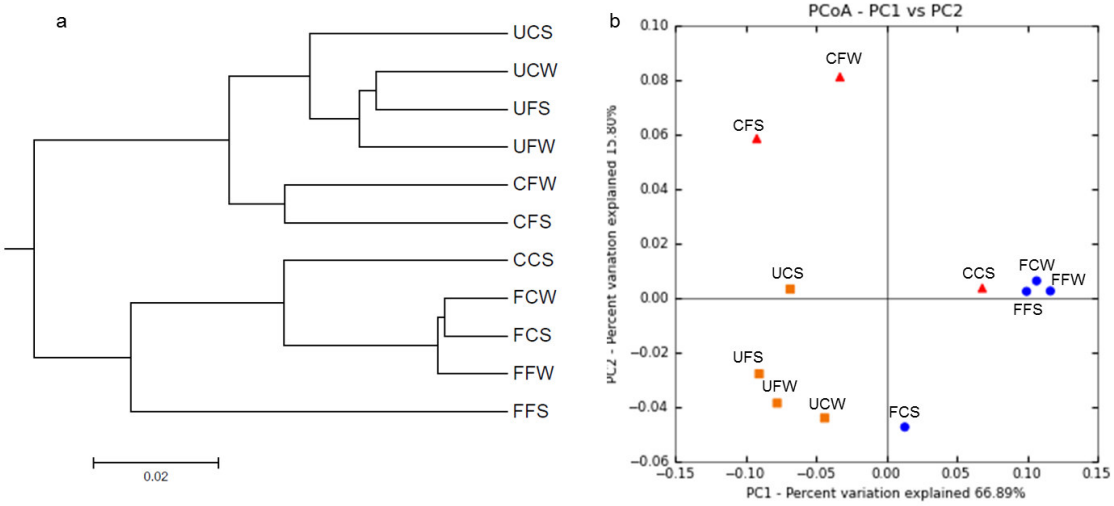

Fig. 8. 5 UPGMA dendrogram with weighted UniFrac (a) considering the relative abundance of each OTU in each clone libraries and respective Principal coordinate analysis (PCoA) (b). FC – *F.spiralis*, Carreço; FF - *F.spiralis*, Porto, UC – *Ulva* sp., Carreço, UF – *Ulva* sp., Porto, CC – *C. crispus*, Carreço, CF – *C. crispus*., Porto; W – winter, S - summer

Table 8. 4 Taxonomic classification of the 13 OTUs that contributed to the differences among clone libraries and their relative abundance

OTU	Total abundance (%)	Relative abundance (%)											p values		
		FCW	FCS	FFW	FFS	UCW	UCS	UFW	UFS	CCS	CFW	CFS	Host	Season	Site
2	1.6	np	np	np	np	np	4.7	np	np	np	2.3	13.8	0.25	0.54	0.32
10	0.5	np	np	np	np	np	4.7	np	np	np	1.1	2.3	0.5	0.67	0.32
16	0.3	np	np	np	np	np	np	1.53	np	np	1.1	1.1	0.32	0.08	0.35
20	4	np	np	np	np	11.5	9.3	6.2	1.9	3.4	11.5	12.6	0.03*	0.59	0.71
30	20	45.6	84.9	65.7	60.0	np	2.6	1.8	3.9	np	np	1.2	0.00006*	0.67	0.92
32	1.1	1.7	np	8.5	7.1	np	np	np	np	np	np	np	0.05*	0.24	0.73
34	11	N	2.2	5.7	np	31.6	5.1	17.5	21.6	85.5	11.5	np	0.30	0.33	0.73
36	5.2	np	np	2.9	np	19.7	10.3	21.1	23.5	np	5.7	2.4	0.0004*	0.58	0.52
37	3.3	1.1	np	np	np	6.6	12.8	10.5	7.8	0.0	5.7	8.2	0.005*	0.69	0.96
39	3.9	np	3.6	np	np	3.8	np	np	3.8	np	np	np	0.5	0.84	0.84
41	1.4	np	np	np	np	3.8	6.9	4.6	9.6	np	np	np	0.0005*	0.93	0.65
43	2.6	np	np	1.4	np	6.9	np	1.5	np	np	19.5	4.6	0.18	0.30	0.21
45	9.3	15.6	10.8	14.3	np	11.8	12.8	14.0	21.6	1.8	10.3	18.8	0.73	0.84	0.39

FC – *F.spiralis*, Carreço; FF - *F.spiralis*, Porto, UC – *Ulva* sp., Carreço, UF – *Ulva* sp., Porto, CC – *C. crispus*, Carreço, CF – *C. crispus*., Porto; W – winter, S - summer

Discussion

Previous studies of bacterial populations inhabiting the surface of macroalgae have shown that planctomycetes are part of this community in numbers ranging from values as low as 1 % in *Ulva prolifer*a to 53 % in the kelp *Laminaria hyperborea*. Planctomycetes present several characteristics that allow them to colonize these surfaces. These include (1) the presence of a glycoproteic holdfast (Lage, 2013) that helps in the attachment, (2) the presence of a high number of sulfatases genes responsible for the degradation of sulphated polysaccharides which are abundant in the macroalgae cell walls (Wegner et al., 2013) and (3) the resistance to some antibiotics (Schlesner, 1994; Cayrou et al., 2010; Lage and Bondoso, 2011) that can be produced by the algae or by other bacteria in the biofilm communities.

In this study we analysed the temporal and geographical variation of the planctomycetes communities on three different macroalgae and the existence of a possible association of this group to specific macroalgae. DGGE fingerprinting showed that the planctomycetes communities of macroalgae did not vary significantly during one-year sampling. This finding is supported by the similar diversities and richness of OTUs obtained in winter and summer clone libraries (Table 8.3). Furthermore, none OTU presented significant seasonal changes (Table 8.5). Furthermore, none OTU presented significant seasonal changes (Table 8.5). Contrary to our observations, previous studies indicated that planctomycetes communities associated with macroalgae presented temporal variations (Bengtsson and Ovreas,

2010; Lachnit et al., 2011). However, these findings were based only on OTU occurrence analyses without statistical support. Furthermore, the reported temporal fluctuation of the planctomycetes associated with the macroalgae *Fucus vesiculosus*, *Ulva intestinalis* and *Gracilaria vermiculophylla* was only based on a restricted number of planctomycetes sequences (33) (Lachnit et al., 2011) influencing the results obtained. Differences in abundance, OTU composition and diversity of the *Planctomycetes* communities' composition of the kelp *Laminaria hyperborea* across three sampling times were observed, although these differences were possibly related to the senescence of the kelp tissue as it aged (Bengtsson and Ovreas, 2010). Other studies based on DGGE analyses of the whole bacterial community on *Ulva australis* suggested the existence of a core community stable over time (Longford et al., 2007; Tujula et al., 2010). Our results also suggest a stable planctomycetes community associated with *Ulva* sp. through time and space. The phylogenetic composition in *Ulva* sp. clone libraries was also very similar, with the exception of the one sampled in Carreço in summer. Indeed, the clone libraries from all the macroalgae sampled in Carreço in summer were phylogenetically more distant from the other clone libraries. This result could be related with abnormal conditions that induced stress in the communities and changed their composition. Further temporal analyses performed in consecutive years would be necessary to determine if the planctomycetes communities in summer are truly distinct from the other seasons.

The results from the present study also indicated that the geographical location do not influence the distribution of the planctomyces communities associated with macroalgae. DGGE profiles of the planctomycetes communities showed no significant differences between the ones from Porto and the ones from Carreço. Only *F. spiralis* appear to have communities with some geographical dependence. These results are in accordance with a previous DGGE fingerprinting study of seven different algae sampled in Spring 2012 in the same locations (Bondoso et al., unpublished). Lachnit et al. (2009) also showed that the whole epibacterial communities from macroalgae sampled in the Baltic and North Seas were more similar between representatives of same species than between macroalgae in the same location. Similar findings were also reported for *Bonnemaisonia asparagoides* (Nylund et al., 2010*), Laminaria saccharina* (Staufenberger et al., 2008), *Dyctyosphaeria ocellata* (Sneed and Pohnert, 2011), *Ulva* spp., *Scytosiphon lomentaria* and *Lessonia nigrescens* (Hengst et al. 2010). The clone sequencing evidenced that the communities' composition of each macroalga was more similar between the sampling sites than with other co-

occurring macroalgae species. Furthermore, none of the sequenced OTUs showed a geographical dependence. However, the planctomycetes populations from macroalgae sampled in Porto presented a higher diversity, richness and evenness, which could be related with the number of clones sequences obtained from Porto (400) that was superior to the ones of Carreço (321). The sampling place situated in Porto, which is the second largest city in Portugal, is much more affected by anthropogenic factors than the one in Carreço. Pollution usually leads to a decrease in bacterial diversity (Torsvik et al., 1998) but Cho and Kim (2000) showed that aquifers with livestock wastewater input had higher diversity. *Planctomycetes* are able to utilize a wide range of organic substrates and strains previously isolated from macroalgae showed physiological tolerances, for example, to heavy metals (Lage et al., 2012) and UVs (Viana et al., 2013) which are characteristics of bacteria usually found in disturbed microbial assemblages (Atlas et al., 1991).

The existence of specific communities of planctomycetes associated with each macroalgae was demonstrated both by DGGE fingerprinting and clone sequencing. Host-specificity of microbial communities has been shown in other macroalgae (Longford et al., 2007; Lachnit et al., 2009; Hengst et al., 2010; Barott et al., 2011; Trias et al., 2012; Vega Thurber et al., 2012). This highly specific association of bacteria with macroalgae is mostly due to a combination of physiological and biochemical properties of the macroalgae (Goecke et al., 2010) that probably also influences the community of planctomycetes. In the present study, we also showed that there is a clear separation of the planctomycetes communities of *F. spiralis* relatively the other two macroalgae that presented similar DGGE profiles and taxonomic composition. In the other DGGE study from 2012 (Bondoso et al., unpublished), it was found that *Rhodophyta* macroalgae DGGE profiles were more similar to the ones of *Ulva* sp. (*Chlorophyta*). Similar findings were also reported by Lachnit et al. (2009) which may indicate that these macroalgae groups present similar chemical surface characteristics and intrinsic mechanisms that modulate the epibacterial community on their surfaces. The absence of a core community of planctomycetes among the three macroalgae, shown by the clone libraries analyses, supports the hypothesis of host-specificity. Furthermore, statistical analyses showed the existence of OTUs specifically associated with each macroalga species. Hollants et al. (2013) reported that, in many cases, the differences among different macroalgae species and similarities within the same species are evident at the phylum or class level but not at the genera or species level. With this work we proved

the existence of particular species of planctomycetes associated with each species of macroalgae. Goecke et al. (2010) suggested that the specific associations existent between bacteria and macroalgae are mainly due to substrate preferences of the bacterial strains and to antifouling and antimicrobial metabolites produced by the macroalgae (Goecke et al., 2010). Macroalgae are known to produce a wide range of secondary metabolites in order to chemically control the epibiosis on their surface and, therefore, modulate their bacterial communities (Hellio et al., 2001; Nylund et al., 2005; Wahl et al., 2010; Sneed and Pohnert, 2011). A study made to investigate the antibacterial and antifungal activity of 82 macroalgae from the Iberian Peninsula showed that *F. spiralis* was effective in inhibiting the growth of several Gram + and Gram- bacteria while *Ulva rigida* was not (Salvador et al., 2007). Although in the same study several *Rhodophyceae* algae showed the highest activity against bacteria, *C. crispus* has been reported to have low antimicrobial activity (Hellio et al., 2001; Nylund et al., 2005; Cox et al., 2010). These data could explain why, of all the three macroalgae studied, *F. spiralis* showed the lowest diversity and richness of planctomycetes. The ability to utilize the macroalgae exudates as well as the structural polysaccharides of their cells walls by the associated bacteria is also an important factor that modulates the communities on macroalgae (Goecke et al., 2010; Lachnit et al., 2010). The composition of the macroalgae cells walls and the polysaccharides produced vary among the main lineages of these organisms. Brown algae are producers of fucoidan and laminarin, red algae produce alginate and carrageenan and green algae contain ulvan. The majority of these polymers are sulphated polysaccharides and *Planctomycetes* are known to contain a high abundance of genes expressing sulfatases (Wegner et al., 2013), which allow them to utilize these substrates. Furthermore, different species and genera of *Planctomycetes* possess different abundance of genes expressing sulfatases: 104-196 in *Rhodopirellula* species, 40 in *B. marina*, 83 in *P. maris* and 101 in *P. brasiliensis* (Wegner et al., 2013). However, *Rhodopirellula* species only have in common about 60 sulfatase genes, which mean that each species harbours a specific set of sulfatases that is probably related with their ecological niche. Therefore, the different chemical composition of the polysaccharides secreted by each macroalgae should determine the different distribution of planctomycetes species on the macroalgae surface.

The taxonomic composition of the planctomycetes communities on the different macroalgae revealed the existence of 51 different OTUs which formed 6 major clusters in the phylogenetic tree (Supplementary Fig. S7). Only three of these groups

(Groups A, B and C) contained isolated planctomycetes, thus suggesting that macroalgae harbour a great unknown diversity of planctomycetes species and genera that remain to be isolated. Furthermore, several of the OTUs sequenced formed phylogenetic clusters exclusively found in macroalgae suggesting the existence of unique planctomycetes species associated only with macroalgae. The OTUs were taxonomically related to the genus *Blastopirellula*, *Rhodopirellula, Planctomyces* and to an uncultured deep-branching lineage OM190. Clones related to these genera have been usually found in association with other macroalgae such as *Delisea pulchra* and *Ulva australis* (Longford et al., 2007)*, Laminaria hyperborea* (Bengtsson and Ovreas, 2010c), *Ulva intestinalis*, *Gracilaria vermiculophylla* and *Fucus vesiculosus* (Lachnit et al., 2011) suggesting that they are frequent colonizers of the macroalgae surface. The most abundant group (Group B) consists of OTUs more related to the planctomycete FC18, which was isolated from the surface of *F. spiralis* from Carreço (Lage and Bondoso, 2011). However, the lineage of this group is not clear as shown by the low bootstrap values in the phylogenetic tree. Furthermore, it appears more closely related to *Pirellula staleyi* in the phylogenetic tree, although the pairwise similarity values of 16S rRNA gene to *B. marina* or *R. baltica* are smaller than to *P. staleyi*. This cluster is composed by OTUs that are widespread among all the algae under study and contains the majority of clones found in *F. spiralis*, including several OTUs exclusively associated with it. Group B was also reported to be the most abundant one in the kelp *Laminaria hyperborea* and in the brown algae *Fucus vesiculosus* (Bengtsson and Ovreas, 2010; Lachnit et al., 2011). These findings suggest that members of this group are most adapted to brown algae probably specialized in degrading fucoidan, the main polysaccharide found in the cell wall matrix of brown algae, which is mainly composed by f α-1,3-L-fucose (Holtkamp et al., 2009). In agreement with this, the OTUs found specifically associated with *F. spiralis* belonged to group B. Group A, the second most abundant, contains OTUs closely related to several isolated strains obtained in the culture dependent study conducted previously (Lage and Bondoso, 2011) which were mainly found in *Ulva* sp., indicating the preference of these strains for colonization of this macroalgae. In the same isolation experiment (Lage and Bondoso, 2011), *Ulva* sp. was the algae that provided a higher number of *Rhodopirellula* species which supports the data from the present study. The deep-branching uncultured groups E and F (class OM190) are widespread among all the macroalgae and have been reported in several other habitats. The *Planctomyces*-related group (Group D), the least abundant, was absent

in *F. spiralis*, which could be related to the fact that *Planctomyces* spp. are not able to grow on fucose (Ward 2010).

Conclusions

In summary, the results presented in this study support the existence of host-specific communities of macroalgae, which occurs at lower taxonomic levels such as species and genera. There was no core community of planctomycetes associated with the three macroalgae studied, which are representatives of the main lineages of macroalgae. Based on these results and data from the literature, we suggest that the specific association to the macroalgal host is determined by the excreted polymers from the algae and by the set of sulfatases expressing genes that each planctomycetes possess. Our data showed that the planctomycetes communities associated with macroalgae do not present significant temporal or geographical variations. Similar results were obtained with PCR-DGGE and 16S rRNA gene clone libraries indicating that the analysis of planctomycetes communities associated with macroalgae can be performed by fast and inexpensive techniques like DGGE. Moreover, the results shown here are consistent with another DGGE study of seven macroalgae sampled in 2012, confirming that the relationships between planctomycetes and macroalgae are maintained in consecutive years.

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Supplementary material

Supplementary Table S 6 Clone libraries description and estimates of diversity and richness

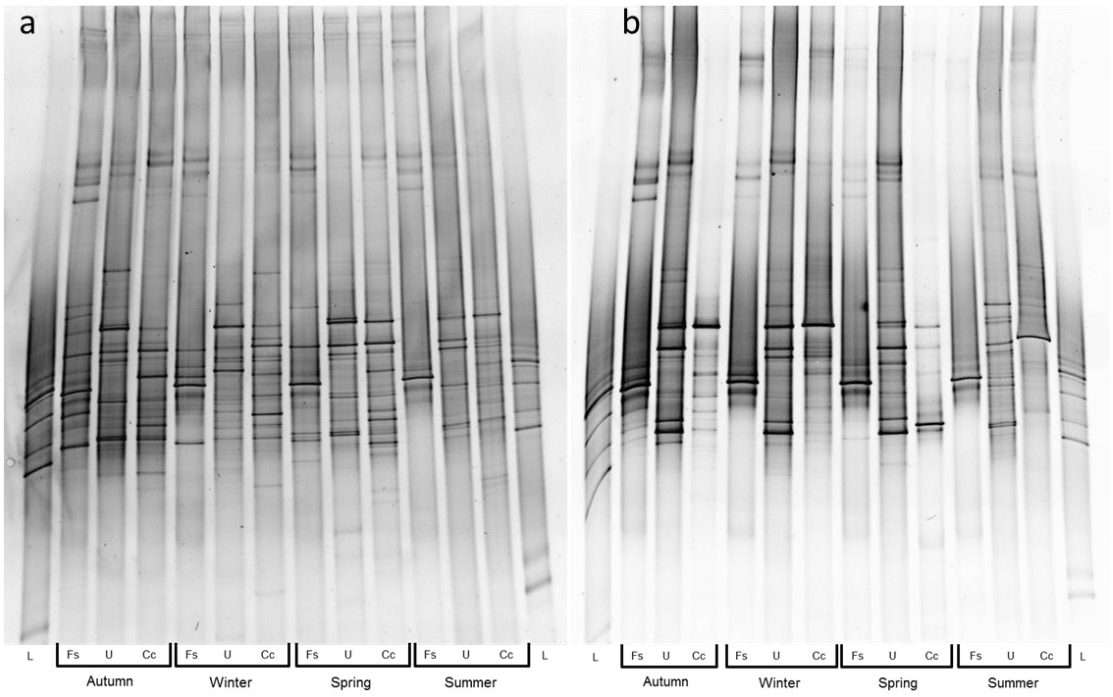
Host	Season	Site	Acronym	# Clones	*Planctomycetes* clones	# OTUs	Unique OTUs	Observed species	Chao 1
Fucus spiralis	Winter	Carreço	FCW	90	58 (64.4%)	6	0	5.0	8.0
Fucus spiralis	Summer	Carreço	FCS	94	93 (98.9%)	5	2	3.8	5.0
Fucus spiralis	Winter	Porto	FFW	95	70 (73.7%)	7	1	6.3	6.8
Fucus spiralis	Summer	Porto	FFS	92	50 (54.3%)	4	1	4.0	4.0
Ulva sp.	Winter	Carreço	UCW	90	76 (84.4%)	11	3	11.1	12.5
Ulva sp.	Summer	Carreço	UCS	81	39 (48.2%)	11	3	14.0	14.9
Ulva sp.	Winter	Porto	UFW	88	57 (50.2%)	14	2	13.8	21.1
Ulva sp.	Summer	Porto	UFS	90	51 (56.7 %)	10	1	10.0	10.4
Chondrus crispus	Summer	Carreço	CCS	91	55 (60.4%)	7	1	6.5	14.5
Chondrus crispus	Winter	Porto	CFW	96	87 (90.3%)	19	7	16.4	31.4
Chondrus crispus	Summer	Porto	CFS	96	85 (88.5%)	19	6	18.6	25.5

Supplementary Table S 7 Taxonomic affiliation of the representative OTUs sequences with the closest uncultured and isolated organisms

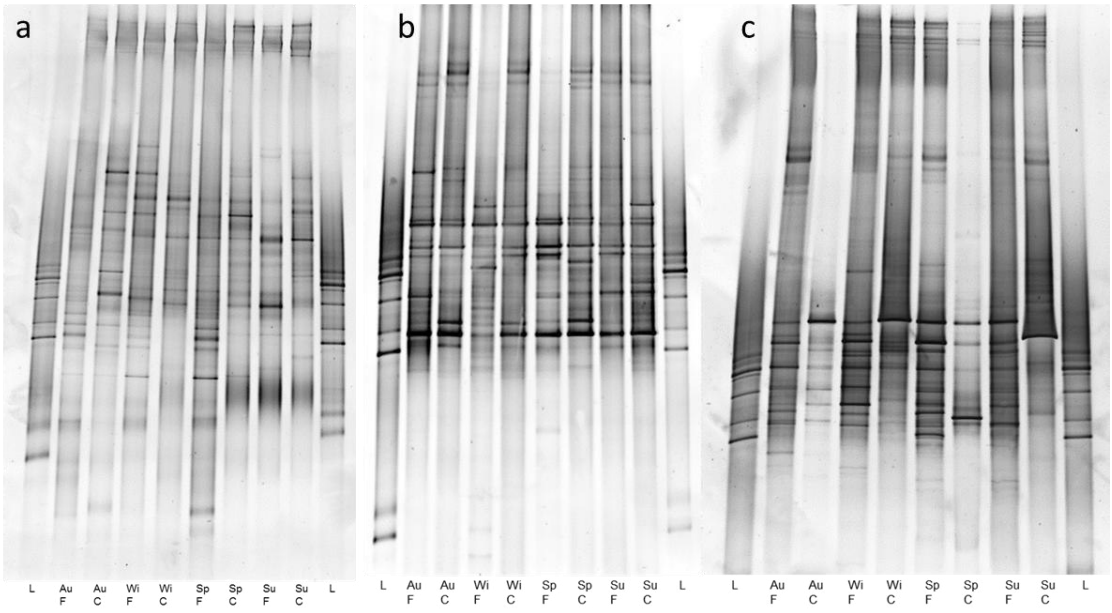
OTU#	Closest organism (Uncultured)	Source	% similarity	Closest organism (isolate)	% similarity	RDP classification	Greengenes classification
0	uncultured bacterium; V1SC07b186	white filamentous microbial mat in shallow hydrothermal vent	96.1	planctomycete MSC318; JF443744	92.3	*Blastopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Blastopirellula; Unclassified; otu_2433
1	uncultured planctomycete; placlone03	Surface of *Laminaria hyperborea*	98.8	*Planctomyces* sp. Pd1; HQ845497	88,0	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
2	uncultured planctomycete; UA12	Surface of *Ulva australis*	98.6	planctomycete DDSW1318; JF443789	89.5	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
3				planctomycete sp. UC8; HQ845508	99.2	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
4	uncultured bacterium; OTU184;	Surface of *Gracilaria vermiculophylla*	97.9	bacterium WH5-6; JQ269310	91.8	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
5	uncultured bacterium; OTU126;	Surface of *Gracilaria vermiculophylla*	99.1	*Planctomyces* sp. Pd1; HQ845497	87.6	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
6	uncultured planctomycete; placlone07	Surface of *Laminaria hyperborea*	95.2	planctomycete sp. UF2; HQ845531	79.2	unclassified Planctomycetaceae	NA
7	uncultured bacterium; OTU6;	Surface of *Fucus vesiculosus*	94.3	planctomycete sp. FC18; HQ845450	88.2	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
8	uncultured bacterium; Ax26_F6	Sponge *Axinella corrugata*	97.1	anaerobic ammonium-oxidizing planctomycete KOLL2a; AJ250882	81.7	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__agg27; o__CL500-15; otu_2405
9				planctomycete LF1; DQ986201	98.3	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
10	uncultured planctomycete; placlone23	Surface of *Laminaria hyperborea*	99.1	planctomycete sp. FC18; HQ845450	94.3	*Blastopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Blastopirellula; Unclassified; otu_2433
11	uncultured Planctomycetales bacterium; Alchichica_AL67_2_1B_16	microbialites from Alchichica alkaline lake	99.3	*Marichromatium* sp. 09261-374; EU700084	79.3	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__agg27; o__OM190; otu_2406
12	uncultured Planctomycetales bacterium; SWB-Pla-37	Sponge *M. laxissima*	96.3	planctomycete sp. UF2; HQ845531	80.5	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__agg27; o__OM190; otu_2406
13	uncultured bacterium; OTU34	Surface of *Fucus vesiculosus*	97.2	planctomycete sp. FC18; HQ845450	93.9	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
14	uncultured bacterium; V1B07b6	white filamentous microbial mat in a shallow hydrothermal vent	95.2	planctomycete sp. UF2; HQ845531	94,0	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; s__Rhodopirellula baltica; otu_2436
15	uncultured planctomycete; UA12;	Surface of *Ulva australis*	97.1	planctomycete DDSW1318; JF443789	89.6	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
16	uncultured planctomycete; placlone05	Surface of *Laminaria hyperborea*	99,0	bacterium WH5-6; JQ269310	90.3	*Blastopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Blastopirellula; s__Blastopirellula marina; otu_2434
17	uncultured planctomycete; placlone05	Surface of *Laminaria hyperborea*	97.2	*Pirellula* sp. AGA/M12 ; X86391	90.3	*Blastopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Blastopirellula; Unclassified; otu_2433
18	uncultured bacterium; OTU31	Surface of *Fucus vesiculosus*	96.3	planctomycete sp. FC18; HQ845450	90.7	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
19	uncultured planctomycete; JdFBGBact_42	basalt glass from Cobb Seamount Juan de Fuca	98.2	anaerobic ammonium-oxidizing planctomycete KOLL2a; AJ250882	81.7	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__agg27; o__OM190; otu_2406

20	uncultured planctomycete; placlone07	Surface of *Laminaria hyperborea*	98,0	planctomycete A-2; AM056027	80.8	unclassified Planctomycetaceae	NA
21	uncultured bacterium; NEP1-27	ocean water at the surface around normal *Enteromorpha prolifera*	94,0	planctomycete sp. FC18; HQ845450	89.7	unclassified Planctomycetaceae	
22	uncultured planctomycete; placlone13	Surface of *Laminaria hyperborea*	99,0	planctomycete MS3054; JF443788	88.5	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
23	uncultured bacterium; Hg5a2A6	*Haliclona* cf. *gellius* sp. (marine sponge)	97.3	*Planctomyces* sp. Pd1; HQ845497	89,0	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
24	uncultured bacterium; lHE5_03	ventral setae of the crab *Shinkaia crosnieri*	96.9	anaerobic ammonium-oxidizing planctomycete KOLL2a; AJ250882	91.4	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__agg27; o__OM190; otu_2406
25	uncultured Pirellula clone 5H12	marine water at a depth of 200 m	95.1	planctomycete sp. FC18; HQ845450	93.5	*Blastopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Blastopirellula; Unclassified; otu_2433
26	uncultured planctomycete; PRPR64	Hawaiian marine sponge *Suberites zeteki*	97,0	planctomycete sp. UF2; HQ845531	80.9	unclassified Planctomycetaceae	k__Bacteria; p__Planctomycetes; c__agg27; o__OM190; otu_2406
27	uncultured planctomycete; Cobs2TisC3	hermit crab residing on the coral *Pacillopora meandrina*	97,0	planctomycete MS3054; JF443788	89.7	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
28	planctomycete MS3047	sponge *Niphates* sp.	98.2	planctomycete MS3047; JF443773	98.2	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
29	uncultured planctomycete; pltb-vmat-53	microbial mat at a shallow submarine hot spring within a Coral Reef	98,0	planctomycete str. 529; AJ231169	96.2	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; s__Rhodopirellula baltica; otu_2436
30	uncultured bacterium; OTU6	Surface of *Fucus vesiculosus*	99.7	planctomycete sp. FC18; HQ845450	93.3	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; s__Rhodopirellula baltica; otu_2436
31	uncultured bacterium; REP2-46	ocean water around normal *Enteromorpha prolifera*"	95.4	planctomycete sp. FC18; HQ845450	91.1	unclassified Planctomycetaceae	
32	uncultured bacterium; OTU34	Surface of *Fucus vesiculosus*	99.4	planctomycete sp. FC18; HQ845451	94.2	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
33	uncultured Planctomycetales bacterium; TDNP_USbc97_109_1_7	upper sediment from the semiarid 'Tablas de Daimiel National Park' wetland	92.6	planctomycete A-2; AM056027	81.5	unclassified_Planctomycetaceae	NA
34	uncultured bacterium; OTU31	Surface of *Fucus vesiculosus*	99.6	planctomycete sp. FC18; HQ845451	94.1	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
35	uncultured bacterium; HAMb1_088	biofilm om HAM lagoon water	96.3	planctomycete MS3054; JF443788	87,0	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
36	uncultured planctomycete; placlone07	Surface of *Laminaria hyperborea*	98.7	*Rhodopirellula baltica* CcC11; HQ845417	79.1	unclassified Planctomycetaceae	NA
37	uncultured planctomycete; placlone06	Surface of *Laminaria hyperborea*	98,0	planctomycete LF1; DQ986201	97.7	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
38				*Rhodopirellula* sp. UC16; EF589350	98.2	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
39	planctomycete str. 529	Methane seeps	97.1	planctomycete str. 529; AJ231169	97.1	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435

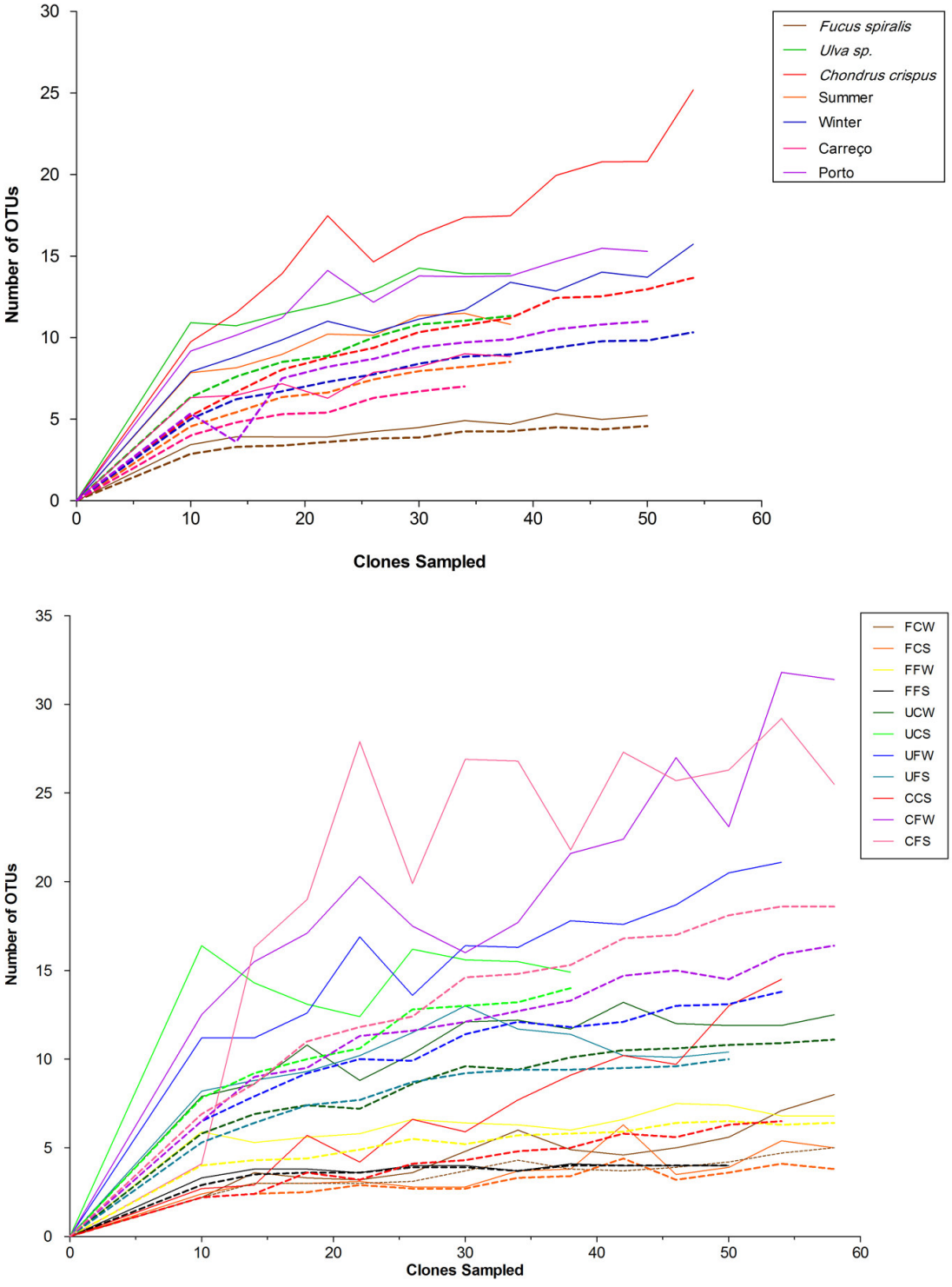
40	uncultured bacterium; NEP4-219	ocean water around normal E*nteromorpha prolifera*"	96.6	planctomycete MS1399; JF443756	95,0	*Blastopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Blastopirellula; Unclassified; otu_2433
41				*Rhodopirellula* sp. LF2; HQ845500	95.1	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
42				*Rhodopirellula* sp. LF2; HQ845500	96.4	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; s__Rhodopirellula baltica; otu_2436
43	uncultured planctomycete; placlone34	Surface of *Laminaria hyperborea*	99.1	planctomycete sp. FC18; HQ845450	93.3	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
44	uncultured planctomycete; Cobs2TisC3	abdominal flora of hermit crab residing on the coral *Pacillopora meandrina*	97.8	planctomycete MS3054; JF443788	88.3	*Planctomyces*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Planctomycetales; f__Planctomycetaceae; g__Planctomyces; s__Planctomyces maris; otu_2444
45	uncultured planctomycete; placlone24	Surface of *Laminaria hyperborea*	98.8	planctomycete sp. FC18; HQ845450	98.1	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Blastopirellula; Unclassified; otu_2433
46	uncultured planctomycete; placlone10	Surface of *Laminaria hyperborea*	97.4	planctomycete sp. FC18; HQ845450	97.6	unclassified Planctomycetaceae	NA
47	uncultured planctomycete; placlone05	Surface of *Laminaria hyperborea*	96.3	*Rhodopirellula* sp. FC3; HQ845436	88.8	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
48	uncultured planctomycete; placlone01	Surface of *Laminaria hyperborea*	96.8	planctomycete sp. FC18; HQ845450	94.4	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; s__Rhodopirellula baltica; otu_2436
49				planctomycete LF1; DQ986201	89.7	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435
50	uncultured bacterium; Hg1a1G5	*Haliclona* cf. *gellius* sp.	96.2	planctomycete str. 529; AJ231169	95.8	*Rhodopirellula*	k__Bacteria; p__Planctomycetes; c__Planctomycea; o__Pirellulales; f__; g__Rhodopirellula; Unclassified; otu_2435



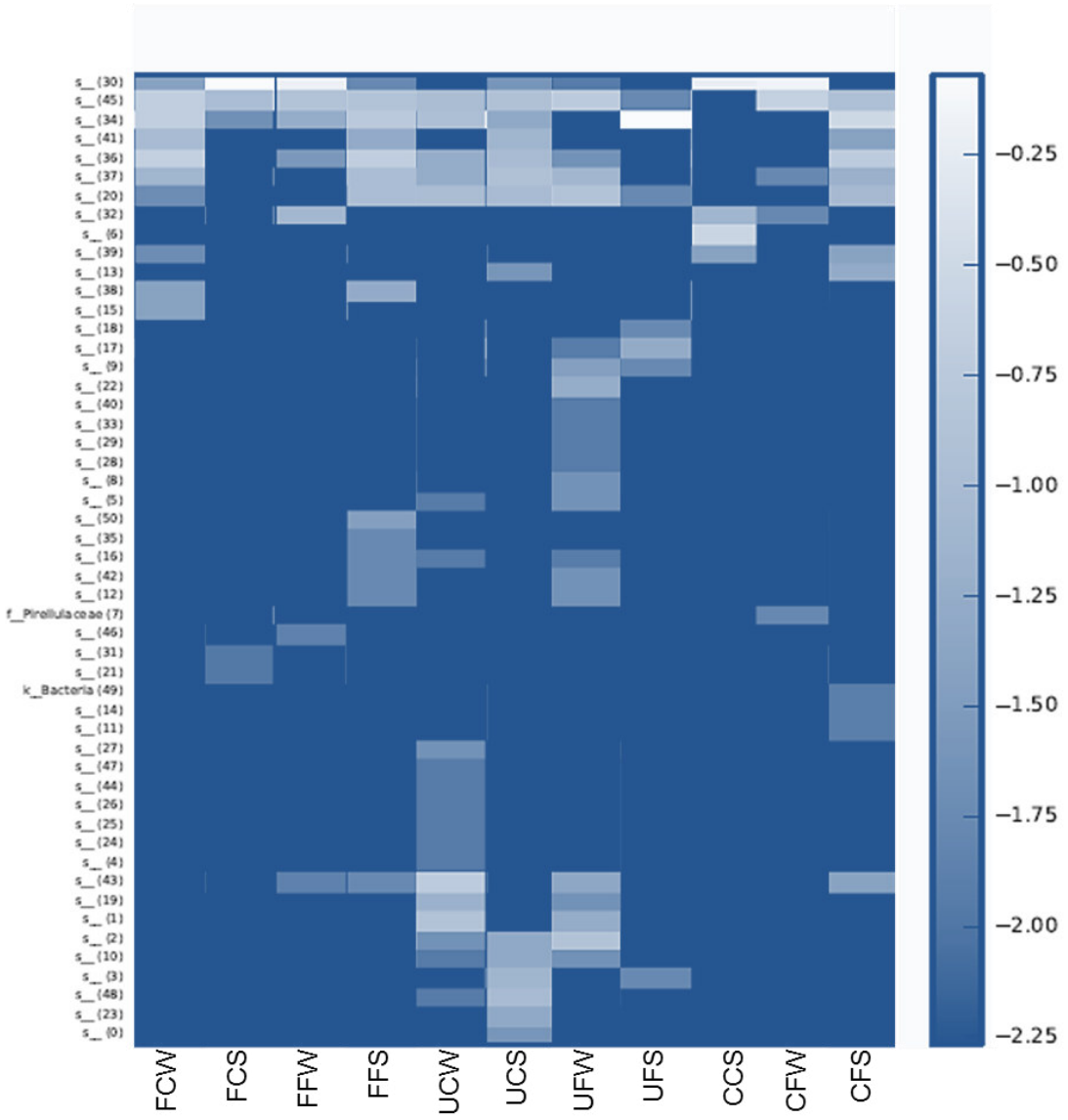
Supplementary Fig. S 3 DGGE fingerprinting profiles of planctomycetes community associated from *Fucus spiralis* (Fs), *Ulva* sp. (U) and *Chondrus crispus* (Cc) from Porto (a) and Carreço (b) L – Ladder included a mixture of individual species of *Planctomycetes* previous isolated.



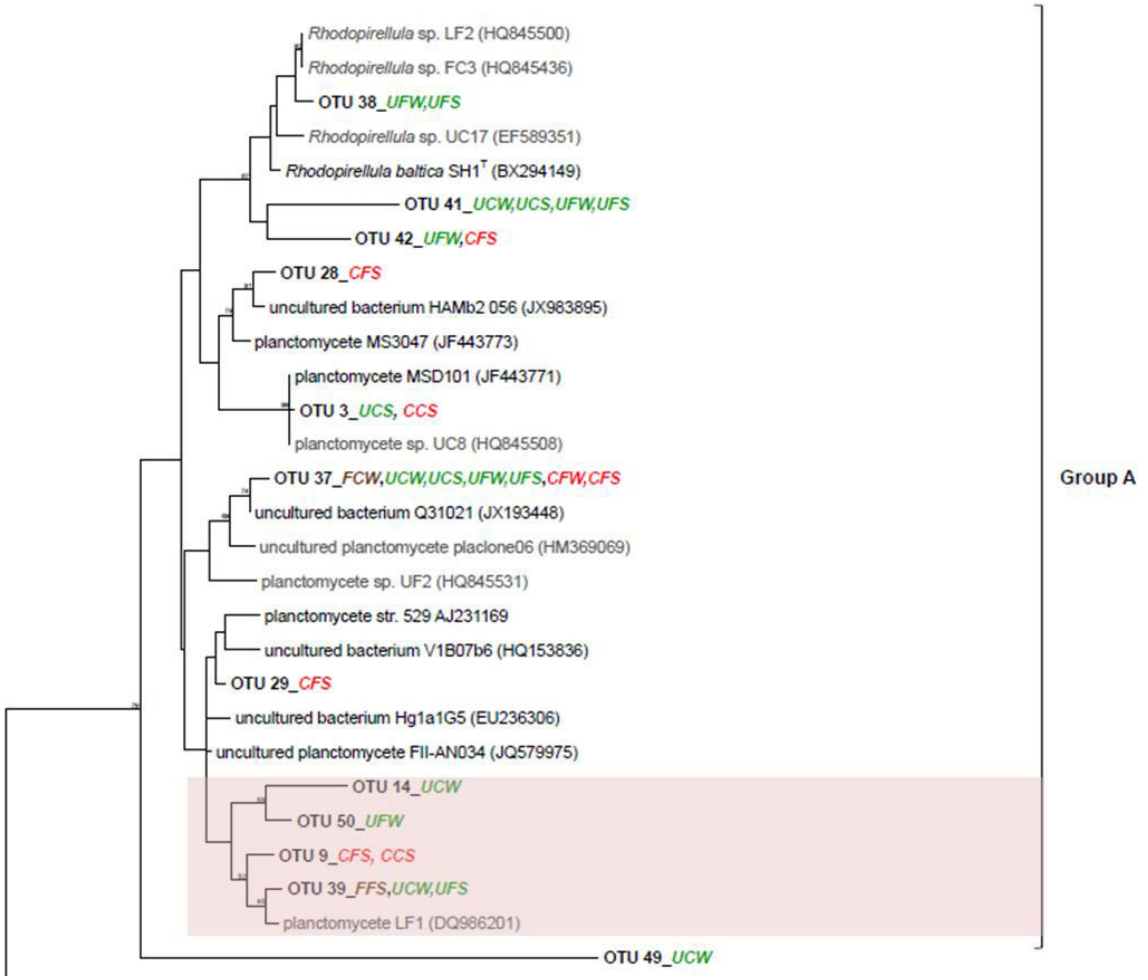
Supplementary Fig. S 4 DGGE fingerprinting profiles of planctomycetes community associated from *Fucus spiralis* (a), *Ulva* sp. (b) and *Chondrus crispus* (c) in Autumn (Au), Winter (Wi), Spring (Sp) and Summer (Su) from Porto (F) and Carreço (C) L – Ladder included a mixture of individual species of *Planctomycetes* previous isolated.

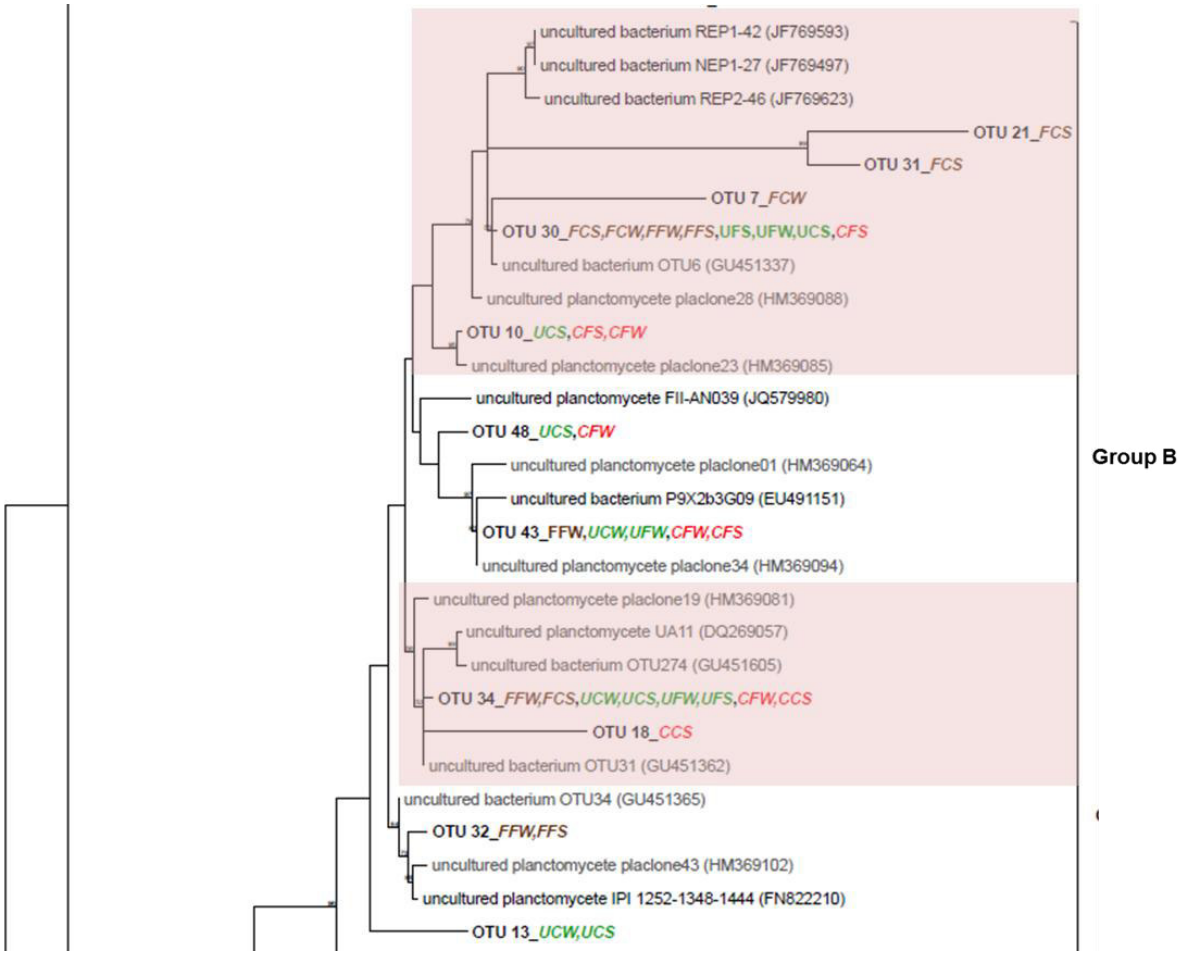


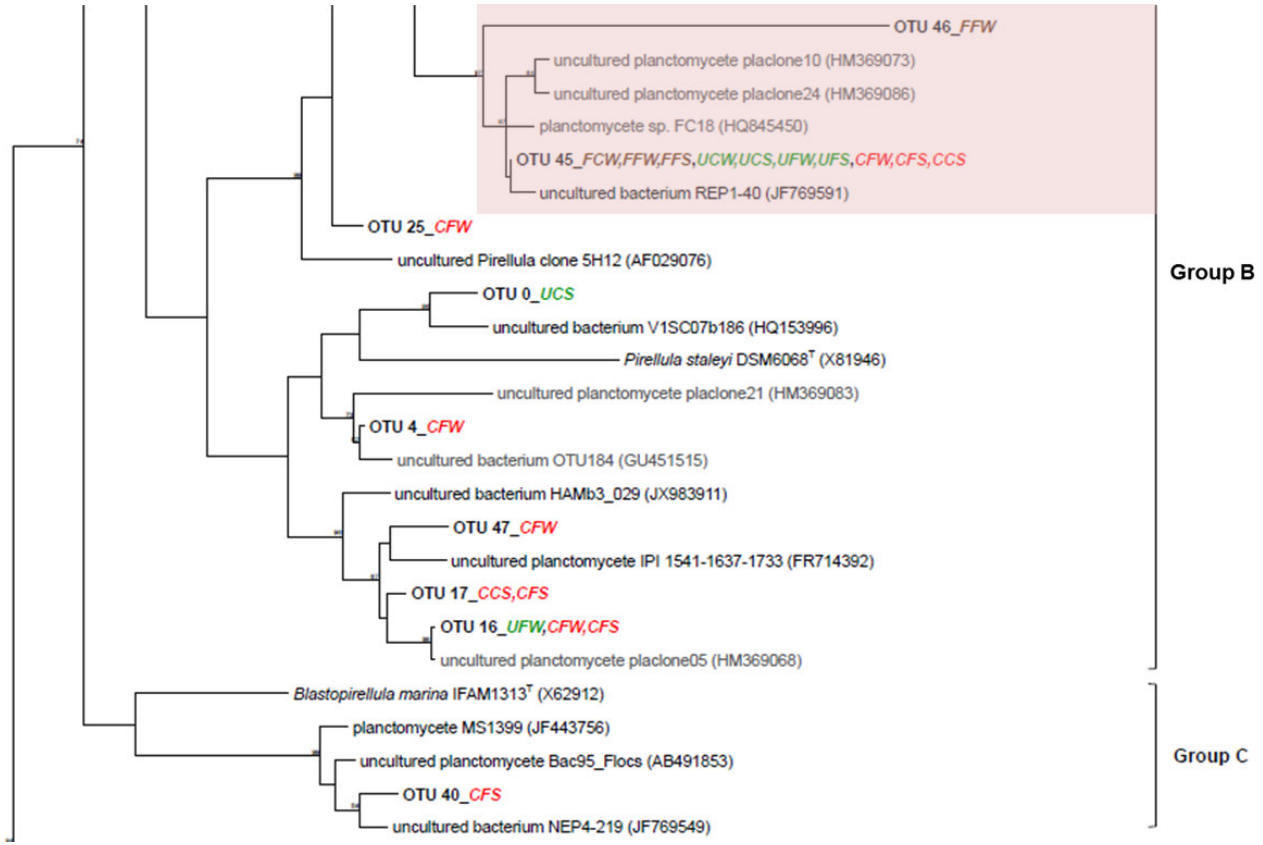
Supplementary Fig. S 5 Observed (solid lines) and estimated (dotted lines) clone library-based rarefaction curves at 97 % sequence homology. (a) Clone libraries grouped per algae type sampling site and sampling season. (b) Individual clone libraries. Curves were based on 1003 cloned sequences in total and 58 per each clone library. Detailed values of observed and estimated richness for each clone library are in Supplementary Table S1.

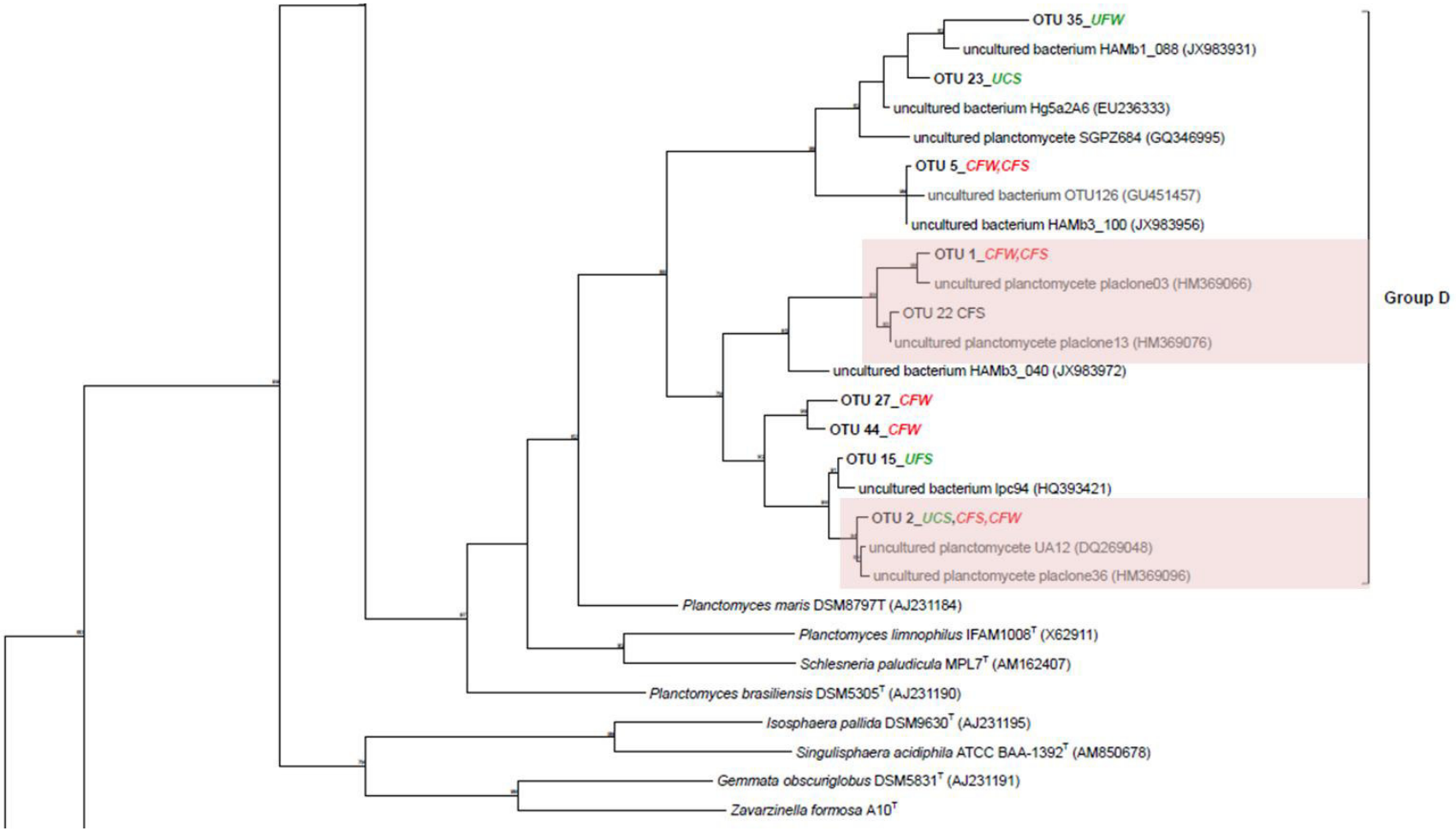


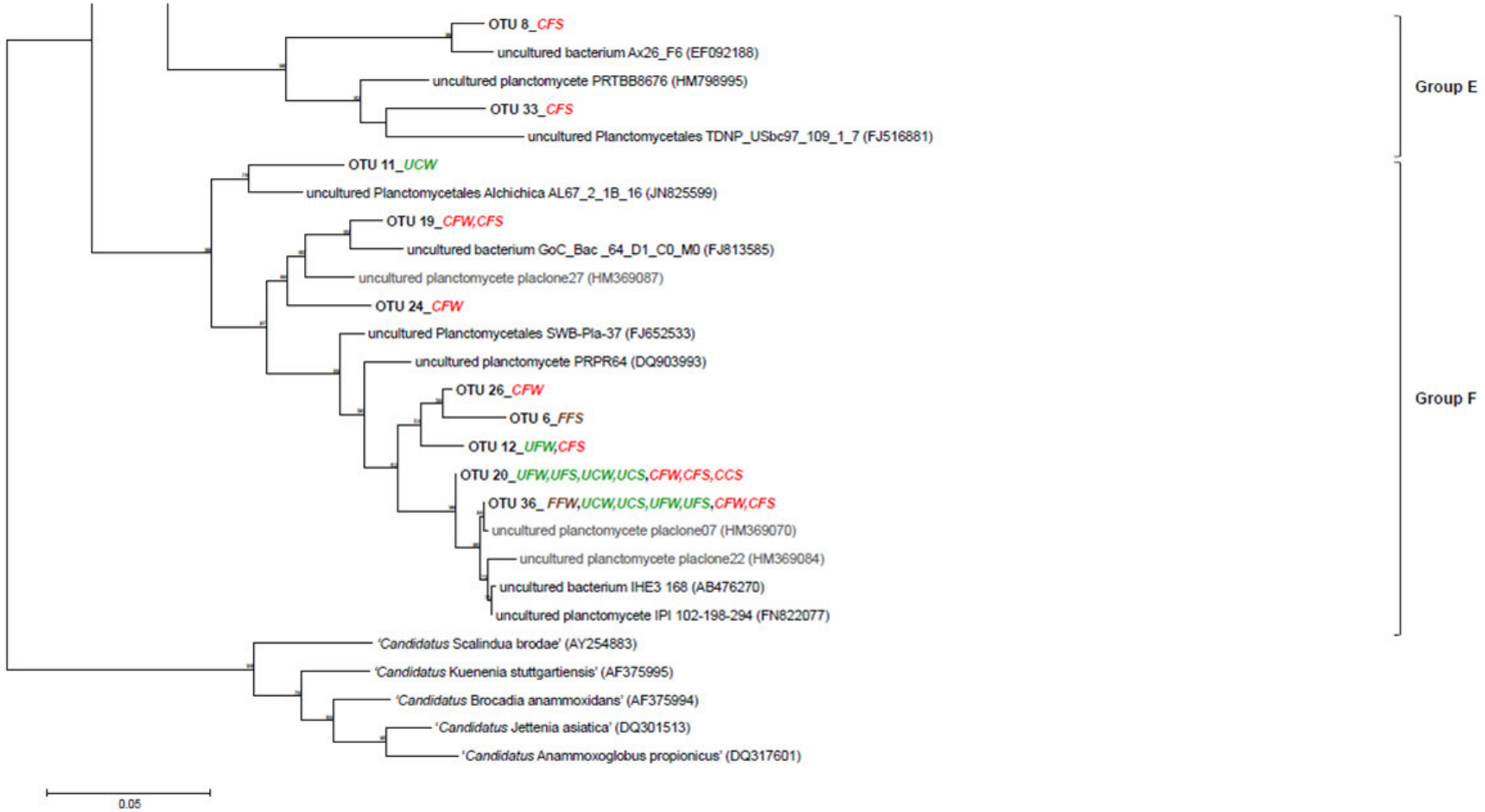
Supplementary Fig. S 6 OTU heatmap displaying raw OTU counts per sample. FC – *F.spiralis*, Carreço; FF - *F.spiralis*, Porto, UC – *Ulva* sp., Carreço, UF – *Ulva* sp., Porto, CC – *C. crispus*, Carreço, CF – *C. crispus*., Porto; W – winter, S - summer


Rhodopirellula sp. LF2 (HQ845500)
Rhodopirellula sp. FC3 (HQ845436)
OTU 38_UFW,UFS
Rhodopirellula sp. UC17 (EF589351)
Rhodopirellula baltica SH1T (BX294149)
OTU 41_UCW,UCS,UFW,UFS
OTU 42_UFW,CFS
OTU 28_CFS
uncultured bacterium HAMb2 056 (JX983895)
planctomycete MS3047 (JF443773)
planctomycete MSD101 (JF443771)
OTU 3_UCS, CCS
planctomycete sp. UC8 (HQ845508)
OTU 37_FCW,UCW,UCS,UFW,UFS,CFW,CFS
uncultured bacterium Q31021 (JX193448)
uncultured planctomycete placlone06 (HM369069)
planctomycete sp. UF2 (HQ845531)
planctomycete str. 529 AJ231169
uncultured bacterium V1B07b6 (HQ153836)
OTU 29_CFS
uncultured bacterium Hg1a1G5 (EU236306)
uncultured planctomycete FII-AN034 (JQ579975)
OTU 14_UCW
OTU 50_UFW
OTU 9_CFS, CCS
OTU 39_FFS,UCW,UFS
planctomycete LF1 (DQ986201)
OTU 49_UCW
Group A


uncultured bacterium REP1-42 (JF769593)
uncultured bacterium NEP1-27 (JF769497)
uncultured bacterium REP2-46 (JF769623)
OTU 21_FCS
OTU 31_FCS
OTU 7_FCW
OTU 30_FCS,FCW,FFW,FFS,UFS,UFW,UCS,CFS
uncultured bacterium OTU6 (GU451337)
uncultured planctomycete placlone28 (HM369088)
OTU 10_UCS,CFS,CFW
uncultured planctomycete placlone23 (HM369085)
uncultured planctomycete FII-AN039 (JQ579980)
OTU 48_UCS,CFW
uncultured planctomycete placlone01 (HM369064)
uncultured bacterium P9X2b3G09 (EU491151)
OTU 43_FFW,UCW,UFW,CFW,CFS
uncultured planctomycete placlone34 (HM369094)
uncultured planctomycete placlone19 (HM369081)
uncultured planctomycete UA11 (DQ269057)
uncultured bacterium OTU274 (GU451605)
OTU 34_FFW,FCS,UCW,UCS,UFW,UFS,CFW,CCS
OTU 18_CCS
uncultured bacterium OTU31 (GU451362)
uncultured bacterium OTU34 (GU451365)
OTU 32_FFW,FFS
uncultured planctomycete placlone43 (HM369102)
uncultured planctomycete IPI 1252-1348-1444 (FN822210)
OTU 13_UCW,UCS
Group B


OTU 46_FFW
uncultured planctomycete placlone10 (HM369073)
uncultured planctomycete placlone24 (HM369086)
planctomycete sp. FC18 (HQ845450)
OTU 45_FCW,FFW,FFS,UCW,UCS,UFW,UFS,CFW,CFS,CCS
uncultured bacterium REP1-40 (JF769591)
OTU 25_CFW
uncultured Pirellula clone 5H12 (AF029076)
OTU 0_UCS
uncultured bacterium V1SC07b186 (HQ153996)
Pirellula staleyi DSM6068T (X81946)
uncultured planctomycete placlone21 (HM369083)
OTU 4_CFW
uncultured bacterium OTU184 (GU451515)
uncultured bacterium HAMb3_029 (JX983911)
OTU 47_CFW
uncultured planctomycete IPI 1541-1637-1733 (FR714392)
OTU 17_CCS,CFS
OTU 16_UFW,CFW,CFS
uncultured planctomycete placlone05 (HM369068)
Blastopirellula marina IFAM1313T (X62912)
planctomycete MS1399 (JF443756)
uncultured planctomycete Bac95_Flocs (AB491853)
OTU 40_CFS
uncultured bacterium NEP4-219 (JF769549)
Group B
Group C


OTU 35_UFW
uncultured bacterium HAMb1_088 (JX983931)
OTU 23_UCS
uncultured bacterium Hg5a2A6 (EU236333)
uncultured planctomycete SGPZ684 (GQ346995)
OTU 5_CFW,CFS
uncultured bacterium OTU126 (GU451457)
uncultured bacterium HAMb3_100 (JX983956)
OTU 1_CFW,CFS
uncultured planctomycete placlone03 (HM369066)
OTU 22 CFS
uncultured planctomycete placlone13 (HM369076)
uncultured bacterium HAMb3_040 (JX983972)
OTU 27_CFW
OTU 44_CFW
OTU 15_UFS
uncultured bacterium lpc94 (HQ393421)
OTU 2_UCS,CFS,CFW
uncultured planctomycete UA12 (DQ269048)
uncultured planctomycete placlone36 (HM369096)
Group D
Planctomyces maris DSM8797T (AJ231184)
Planctomyces limnophilus IFAM1008T (X62911)
Schlesneria paludicula MPL7T (AM162407)
Planctomyces brasiliensis DSM5305T (AJ231190)
Isosphaera pallida DSM9630T (AJ231195)
Singulisphaera acidiphila ATCC BAA-1392T (AM850678)
Gemmata obscuriglobus DSM5831T (AJ231191)
Zavarzinella formosa A10T



Supplementary Fig. S 7 Maximum-Likelihood tree of 16S rRNA gene OTUs sequences (in bold) found associated with Fucus spiralis, Ulva sp. and Chondrus crispus. Sequences were grouped by ≥ 97 % sequence identity. Sequences from F. spiralis are highlighted in brown, Ulva sp. in green and C. crispus in red. Sequences in grey represent the clones identified from the surface of macroalgae. FC – F.spiralis, Carreço; FF - F.spiralis, Porto, UC – Ulva sp., Carreço, UF – Ulva sp., Porto, CC – C. crispus, Carreço, CF – C. crispus., Porto; W – winter, S - summer . The numbers beside nodes are the percentages for bootstrap analyses; only values above 50 % are shown. Scale bar= 0.05 substitutions per 100 nucleotides. The different groups are presented on the right. Anammox 16S rRNA gene sequences were used as outgroup.

Chapter 9.

General Discussion

The aim of this study was to characterize the planctomycetes communities living in the surface of macroalgae. Previous isolation experiments showed that the surface of macroalgae harbours an unknown biodiversity of planctomycetes (Lage and Bondoso, 2011). Molecular studies have also shown that these bacteria are widespread among several macroalgae species representing 4 % of the total clones associated with macroalgae (Hollants et al., 2013) and are the dominant group in the kelp *Laminaria hyperborea* (Bengtsson and Ovreas, 2010). Planctomycetes present several characteristics that allow them to colonize these surfaces, which are the presence of glycoproteic (Lage, 2013) holdfasts that helps in the attachment, the presence of a high number of sulfatases genes (Glockner et al., 2003; Wegner et al., 2013) involved in the degradation of sulphated polysaccharides, which are abundant in the macroalgae walls and resistance to ultraviolet radiation (Viana et al., 2013), to which the marine ecosystem is highly exposed, and to antibiotics (Schlesner, 1994; Lage and Bondoso, 2011), that can be produced by the macroalgae or by other bacteria in the biofilm communities.

Two approaches were done to achieve the aim of this work. One that consisted in the characterization of novel isolates previously obtained through culture-dependent methods and another one where the planctomycetes communities associated with distinct macroalgae was studied through culture independent molecular methods. Thus, five novel taxa of *Planctomycetes* were characterized through a polyphasic approach in order to propose novel genera and species of this scarcely studied phylum. Furthermore, the spatiotemporal changes and the degree of specificity of the planctomycetes communities associated with different macroalgae from the North coast of Portugal were studied through PCR-DGGE fingerprinting based on the 16S rRNA gene. The diversity and phylogenetic structure of these epiphytic communities was assessed by the sequencing analyses of 16S rRNA gene clone libraries.

Novel taxa isolated from the surface of macroalgae

The isolation of novel taxa of bacteria is very important because it allows understanding the role of bacteria in their natural environment through their physiological and biochemical characterization. The phylum *Planctomycetes* comprises a restricted number of isolated species (15) that do not reflect the diversity obtained in molecular studies. Nevertheless, in the last years several novel taxa of planctomycetes have been isolated, which revealed new metabolisms, such as acidophily (Kulichevskaya et al., 2007; Kulichevskaya et al., 2008; Kulichevskaya et al., 2009; Kulichevskaya et al., 2012a; Kulichevskaya et al., 2012b) and cellular

features like fission division (Fukunaga et al., 2009) that contrasts to the budding division typical of the majority of planctomycetes (Fuerst, 2004). Thus, it is of great importance the isolation and the morpho-physiological and metabolic characterization of novel planctomycetes strains.

In the present study, five different taxa of planctomycetes, previously isolated from macroalgae (Lage and Bondoso, 2011), were characterized through a polyphasic approach. These strains were phylogenetically close to the genus *Rhodopirellula*, a widespread group of marine planctomycetes that was only proposed as a separate genus in 2004 (Schlesner et al., 2004). So far, there is only one species described, *R. baltica* SH1^T, the type species of the genus, that was the first planctomycete with a genome sequenced (Glockner et al., 2003). In 2009, Winkelmann and Harder (2009) reported the existence of a large number of *Rhodopirellula* spp. isolates in several aquatic habitats and showed a huge diversity among them (Winkelmann et al., 2010). In the framework of this PhD thesis, three novel species of *Rhodopirellula*, *R. lusitana, R. rubra* and *R. formosa* (Chapters 4 and 5) were described. They were first identified on the basis of the 16S rRNA gene and share a similarity in this gene between 96.6 and 98 % to *R. baltica* SH1^T. Furthermore two novel genera, *Roseimaritima ulvae* and *Rubripirellula obstinata* were described (Chapter 3) which share less than 94 % similarity in the 16S rRNA gene sequence to *R. baltica* SH1^T. Based on the 97 % cut- off in the 16S rRNA gene proposed for the delineation of novel species (Stackebrandt and Goebel, 1994), only *R. formosa*, that shares a 96.6 % similarity in this gene to *R. baltica* SH1^T, should be considered a novel species. The other two taxa, *R. lusitana* and *R. rubra* share more than 97.6 % similarity in the 16S rRNA gene to *R. baltica* SH1^T and thus they should be considered strains of this species. However, it is recognized that the 16S rRNA gene alone is not enough to describe taxonomically a novel species and additional methods should be used to ensure a correct taxonomic assignment, especially when the sequence similarity of the 16S rRNA gene is higher than 97 %. In these cases, DNA-DNA hybridization (DDH) should be performed and a 70 % value in the DNA reassociation between two isolates is used for species separation. Also other genes sequences, usually housekeeping genes, can be analysed with more resolution power than the 16S rRNA gene for species discrimination (Tindall et al., 2010). To confirm the exact affiliation of the isolates of the proposed species, *R. lusitana* and *R. rubra*, a phylogenetic marker based on the beta subunit of the RNA polymerase was developed and studied in the phylum *Planctomycetes* (Chapter 6). The analysis of a partial fragment of this gene (~1200bp) showed that two strains should be considered to belong to the same

species when the similarity of the partial *rpoB* gene is higher than 98.7 %. This value was supported by DDH and ANI data previously obtained (Schlesner et al., 2004; Winkelmann et al., 2010) and it is similar to the one described in the literature for other bacterial groups (Adékambi et al., 2008). The similarity values in the *rpoB* gene to the type species *R. baltica* SH1^T obtained for *R. lusitana* and *R. rubra* varied from 78 to 80 %, confirming their separation into different species. *R. formosa* was also confirmed to be a novel species of *Rhodopirellula* with an *rpoB* gene sequence similarity with *R. baltica* SH1^T of 74 %. However, for the validly description of a novel species, the morphological, biochemical and chemotaxonomic data should be in accordance with the phylogenetic data from the 16S rRNA or other genes and DDH and must provide a clear distinction from the closest described species which, in this case, is *R. baltica* SH1^T. For a better visualization and comprehension, the results obtained on the polyphasic study of all the species under characterization in this thesis were summarized in Supplementary Table S8. These form granular, pink-colored and translucent colonies and the cells are organized in rosettes, similarly to *R. baltica* SH1^T and other *Rhodopirellula* related strains (Fuerst et al., 1997; Pimentel-Elardo et al., 2003; Gade et al., 2004; Schlesner et al., 2004; Winkelmann and Harder, 2009; Bengtsson and Ovreas, 2010). The colonies of *R. rubra* and *Rubripirellula obstinata* were almost red in color whereas *R. formosa* and *Roseimaritima ulvae* present light pink colonies. One of the characteristics of *Rhodopirellula*-like strains is the shape of the cells that is ovoid or pear-shaped. This feature was observed in the majority of the isolates with the exception of the genus *Roseimaritima* which presented round cells of relatively small size. As observed in members of the order *Planctomycetales*, all the strains reproduced by budding. Ultrastructurally, it was possible to distinguish the different species on the basis of relative size of the paryphoplasm (periplasm) and pirellulosome (cytoplasm) and their electron-density, extracellular matrix, fimbriae and holdfast and separate them from the type species *R. baltica* SH1^T. Closely related species within a group of bacteria can be differentiated ultrastructurally such as methanogenic bacteria which showed to be an ultrastructurally diverse group with differences in granular inclusions, reserve materials, and intracytoplasmic membranes that were unique to certain species (Zeikus and Bowen, 1975). *Thiobacillus* species present obvious differences in the cell envelope and inclusions that are specific of certain species (Shively et al., 1970). In *Bacteroides melaninogenicus*, subspecies can be distinguished on the basis of morphological features like extramural structures and intracellular matrix (Listgarten

and Lai, 1979). The characteristic complex cell plan of planctomycetes divided in pirellulosome (with ribosomes, storage inclusions and condensed DNA) and paryphoplasm (Fuerst and Sagulenko, 2013) was present in all the isolates. Crateriform pits were also present in the reproductive pole as observed in all members of the *Pirellula-Rhodopirellula-Blastopirellula* (PRB) group. The holdfast was present in all the isolates. Species belonging to the genus *Rhodopirellula* presented fimbriae with a ring-like structure which seems to be a common feature of this group. It has also been observed in *R. baltica* SH1^T, *Rhodopirellula* sp. 6C (Winkelmann et al., 2010), *Rhodopirellula* sp. strain SH796 (Gade et al., 2004) but absent in *Pirellula* and *Blastopirellula* (Lindsay et al., 1997; Butler, 2002). The ultrastructure of the novel species *R. rubra* was the one most resembling the one of *R. baltica* SH1^T (Schlesner et al., 2004), although some differences were observed. *R. rubra* showed a wide and very electron transparent paryphoplasm and a pirellulosome often divided in small compartment-like structures. Fimbriae and thinner pilli were present and were very prominent and longer than the ones present in *R. baltica* SH1^T and the other species under description. *R. lusitana* has a smaller electron dense paryphoplasm when compared to the other isolates and a well-developed pirellulosome. Fimbriae were less evident and mainly composed of smaller pilli. *R. formosa* showed an electron transparent fibrillar paryphoplasm that can vary in size. The novel genus *Rubripirellula obstinata* presents clear differences from *Rhodopirellula* species. It possesses an electron transparent fibrillar paryphoplasm, a convoluted pirellulosome, a thick cell wall and an evident glycocalyx surrounding the cell which was not observed in any other species. The robust holdfast was clearly different from the ones of *Rhodopirellula* spp. and *Roseimaritima ulvae*. Some cells also showed hump-like protrusions similarly to *Pirellula staleyi* ATCC 35122 (Butler, 2002). *Roseimaritima ulvae* presented an extensive electron transparent paryphoplasm with granular appearance and a comparatively smaller pirellulosome with a large number of storage substances.

The isolates under study are heterotrophic and mesophilic with optimal growth temperatures between 25 and 30 ºC, similarly to the species of the PRB group. *Roseimaritima ulvae* and *Rubripirellula obstinata* showed a more restricted growth temperature range that varied from 15 ºC to 35 ºC and 10 to 30 ºC, respectively, contrasting with the 5 to 32 ºC of *R. baltica* SH1^T. Salinity is required by all the strains as well as *R. baltica* SH1^T, however *R. formosa* does not need sea salts for growth. Carbon utilization profiles revealed that the isolates are specialized in sugar metabolism (hexoses, pentoses and some polysaccharides) and of the carbon

sources tested sugar alcohols (except mannitol), amino-acids and organic acids were not utilized. In the Biolog GN system, no oxidation of polymers, brominated chemicals and amines were obtained. These results were in agreement with data previously obtained for *R. baltica* SH1^T and related strains (Schlesner et al., 2004). The annotation of *R. baltica* SH1^T genome revealed that it can utilize a broad spectrum of monosaccharides and some di- and polysaccharides, which was confirmed by proteomic studies (Gade et al., 2005). These showed that almost all the enzymes of the glycolysis and TCA cycle and the ones of the oxidative branch of the pentose phosphate were present. *Rubripirellula obstinata* showed a very restricted utilization of carbohydrates in contrast to the described marine planctomycetes to date (Schlesner et al., 2004; Lee et al., 2012) and to the other species under this study. The carbon profile utilization showed that they were able to utilize the majority of the monomers that constitute the main polysaccharides secreted by macroalgae. Brown algae possess fucoidan, composed by fucose, uronic acids, galactose and xylose; laminarin, which is composed by β-glucan, and alginate that is formed by mannuronic and guluronic acid (Rioux et al., 2007). *Ulva* sp. and other green algae possesses ulvan, which is mainly composed by rhamnose, xylose and uronic acid (Ray and Lahaye, 1995) and red algae possess mainly carrageenan and agar composed by sulphated galactans (Usov, 1998). The great majority of the strains utilized fucose, galactose, xylose and rhamnose which indicate that they can obtain their nutritional requirements from the macroalgae through the hydrolysis of the complex sulphated polymers (due to sulfatases genes) and utilizing the resulting carbohydrates as energy and carbon source. They were also able to utilize mannitol, with the exception of *Rubripirellula obstinata*, that is one of the main food reserves present in brown algae (Davison and Reed, 1985). Analysis of the hydrolytic metabolism of the isolates showed their ability to degrade complex polysaccharides and revealed differences among them. Esculin was the only polymer degraded by all the isolates. *R. rubra* was not able to degrade cellulose but was the only able to hydrolyse gelatine. *R. lusitana* was the only species in which starch was not degraded. In the API ZYM system differences were evident between and within the species. Catalase activity was positive in all except in *Rubripirellula obstinata.* The isolates were able to reduce nitrate to nitrite and some could grew under anaerobic conditions, features never observed in *R. baltica* SH1^T.

Chemotaxonomic differentiation of the species here described was also possible. The species described in this work possess menaquinone 6 (MK-6). So far, this has

been the only quinone present in *Planctomycetes*, which makes unfeasible the use of this molecule as chemotaxonomic marker. Even species from different classes have MK-6, as it is the case of the *Phycisphaera mikurensis* that belongs to the class *Phycisphaerae* (Fukunaga et al., 2009). Fatty acid profiles allowed a good definition of the novel genera *Rubripirellula* and *Roseimaritima* and the species *R. formosa* whereas species *R. rubra* and *R. lusitana* only showed a few differences in comparison to *R. baltica* SH1^T, in agreement with the 16S rRNA gene sequence similarities. The major fatty acids found in all strains were C 18:1ω9c (oleic acid) and C16:0 (palmitic acids), which are also the most abundant in the majority of other planctomycetes (Kerger et al., 1988; Ward et al., 2006). These fatty acids are more commonly found in eukaryotic organisms but have also been reported in other bacteria (O'Leary, 1962). The relative abundance of C16:0 allows discrimination at the genus level when comparing *Rhodopirellula* (22-30 %), *Rubripirellula* (17.3 %) and *Roseimaritima* (33-35 %). The most evident difference between *R. baltica* SH1^T and the other taxa was the absence of 2-OH or 3-OH fatty acids on *R. baltica* SH1^T. The presence of the short chain hydroxy acid C12:0 3-OH allowed the separation of *R. baltica* SH1^T from *R. rubra* and *R. lusitana*. *R. formosa* was the only species with C16:1ω11c and *Roseimaritima* presented C 20:1 ω9c which was absent or in very low amounts in the other taxa. *Rubripirellula* has the highest amount of C17:1ω8c (12 %) whereas in the other species it varied from 1 to 5 %. In general, the fatty acid composition is very useful for the discrimination of closely related species. This was not the case of, for example, species of the genus *Meiothermus* that have similar fatty acids profiles with few small differences, namely the absence of 2-OH fatty acids (Albuquerque et al., 2009; Albuquerque et al., 2010). In *Planctomycetes*, this chemotaxonomic marker seems to be important in the delineation of different genera but it does not present significant variation when analysing closely related species, as it is the case of *R. baltica* SH1^T, *R. rubra* and *R. lusitana*. *R. formosa*, that showed a similarity lower than 96 % in the 16S rRNA gene to *R. baltica* SH1^T, showed more differences in the fatty acids profiles. Overall, the polar lipid content of the taxa under description was very similar with the exception of the one of *Rubripirellula* that was more distinct from the other strains, suggesting that this marker do not varies much. *Rubripirellula* did not contain diphosphatidylglycerol (DPG) which is also absent in *B. marina*, *Pl. limnophilus* and *Pl. brasiliensis*. Phosphatidylglycerol (PG) is a common polar lipid among planctomycetes (Ward et al., 2006) and was present in all the strains under characterization. Phosphatidylcholine (PC) is also present in all the proposed species. However this is not a common lipid among members of

Planctomycetes as is absent in the closely related genera *Blastopirellula* and *Pirellula*.

G+C content was not helpful in the discrimination of the species as it showed only 3 % variation among the taxa under study and therefore cannot be used as intragenic or intraspecific marker. This was shown before by Schlesner (2004) that also found little variation among genera (from 53.6 to 57.4 %).

On the basis of morphological, physiological and chemotaxonomic as well as phylogenetic data from 16S rRNA gene and *rpoB* gene it was possible to delineate the novel taxa and clearly separate them from the closest cultured type species, *R. baltica* SH1.

Population dynamics of planctomyces associated with macroalgae

Even though isolation and characterization of novel taxa of bacteria are very important to understand the role of bacteria in their natural environment, culture-dependent methods are selective because, normally, they only allow retrieving the most abundant organisms. Furthermore, they do not provide information on the structure and functioning of the bacterial populations in a given ecological niche. The total bacterial diversity on Earth is estimate to be 10^9 different bacterial species (Dykhuizen, 1998) whereas the oceans can harbour 10^6 (Curtis et al., 2002). However, only 10^3 species of bacteria were described so far (http://www.bacterio.net/number.html#total). The introduction of molecular techniques based on the 16S rRNA gene has revolutionized the microbial ecology. The dynamics and structure of microbial communities have been revealed through the application of PCR based-DGGE (Denaturing Gradient Gel Electrophoresis) (Muyzer et al., 1993) and several novel taxa of bacteria have been discovered by the construction of clone libraries (Giovannoni et al., 1990). The present work applied PCR-DGGE and 16S rRNA clone libraries and comprises the first extensive culture-independent study exclusively focused on the composition and distribution of planctomycetes in the epiphytic microbial community of several co-occurring macroalgae.

The construction of the 16S rRNA clone libraries to assess the composition and dynamics of the planctomycetes communities associated with three distinct macroalgae was performed through the application of the specific primers 46F/1390R. This allowed the obtainment of a higher diversity of planctomycetes which was never retrieved in other molecular studies with macroalgae. Planctomycetes are usually underrepresented in 16S rRNA gene clone libraries as they contain mismatches to the commonly used universal primers 27F and 1492R

(Vergin et al., 1998; Blackwood et al., 2005). Furthermore, they are usually less abundant in environmental samples which difficult their detection in clone libraries. As an example, planctomycetes were dominant in the kelp *Laminaria hyperborea* (Bengtsson and Ovreas, 2010) but they were absence in a related species of kelp *Laminaria saccharina* (Staufenberger et al., 2008). In the present study, 51 different taxa, based on a 97 % 16S rRNA gene cut-off (representing species), were retrieved from 11 clone libraries which varied from 4 OTUs in *F. spiralis* to 19 in *C. crispus*. With general bacterial primers, Lachnit et al. (2011) only obtained 17 planctomycetes OTUs based on a 99 % threshold, from 12 different clone libraries of three different macroalgae and Longford et al. (2007) only obtained 2 and 7 OTUs from *Ulva australis* and *Delisea pulchra* clone libraries, respectively. In contrary, three clone libraries of *Laminaria hyperborea* (Bengtsson and Ovreas, 2010) constructed with specific primers to *Planctomycetes*, revealed 23 OTUs at 98 % sequence. For studying the dynamics and variations of this group in association with the macroalgae, DGGE was performed with specific planctomycetes primers. DGGE has successfully been applied for the comparison of bacterial communities as they vary through time and space. However, this technique has restricted detection of the total bacterial diversity in a sample and, usually, does not detect less abundant groups (Muyzer et al., 1993). As an example, an in-depth study where a large number (~16000 sequences) of clones from the whole bacterial community of *Ulva australis* (Burke et al., 2011) was sequenced showed the presence of 3.4 % planctomycetes clones, while a PCR-DGGE study made with the same macroalgae indicated the absence of this phylum (Tujula et al., 2010). To overcome this problem, DGGE should be performed using pair of primers designed for specific groups. Muhling et al. (2008) successfully used a three-step nested-PCR-DGGE with the pair of primers 352F/920R for the specific amplification of *Planctomycetes* sequences on the DGGE profiles. Pollet et al. (2011a) further applied this pair of primers in a one-step direct PCR-DGGE for the study of the spatiotemporal changes of the planctomycetes communities on two freshwater lakes by DGGE profiling. In the present study we developed a two-step based PCR-DGGE on the basis of the work performed by Muhling et al. (2008) and Pollet et al. (2011a). The three-step nested-PCR performed on the DNA extracted from the surface of the macroalgae as described by Muhling et al. (2008) lead to several non-specific PCR amplicons, while with the direct PCR done according to Pollet et al. (2011a) some of the DNA samples did not amplified or the resulting PCR products showed faint bands on agarose gel. Using a two-step nested PCR, where the DNA was first amplified with the pair of primers 9bfm/1512uR

(Muhling et al., 2008) and then with the specific pair of primers 352F-GC/920R (Pollet et al., 2011a), the resulting PCR bands were clear and defined. When comparing DGGE profiles of the two-step nested and the direct PCRs, less background was obtained with the direct PCR but the nested PCR-DGGE showed more and defined bands and was thus selected. Another main disadvantages of DGGE is the impossibility of a full taxonomic assignment of microorganisms (Muyzer et al., 1998). Therefore, 16S rRNA gene clone libraries were constructed in order to obtain an in-depth taxonomic characterization of the planctomycetes communities associated with three macroalgae that represented the three main lineages: *Chondrus crispus* (red algae), *Fucus spiralis* (brown algae) and *Ulva* sp. (green algae). Both PCR-DGGE and 16S rRNA gene clone libraries provided similar results, namely in the OTUs composition retrieved and in the spatial-temporal-host variation of the planctomycetes communities. A comparison between the diversity obtained with the pair of primers 352F/920R and the one obtained with the pair of primers 46F/1390R showed that number of OTUs and the composition of the planctomycetes communities were similar, albeit the amplicon generated by the pair 352F/920R was lower in size (Pollet et al., 2011b). This suggests that DGGE can be a rapid and less expensive technique to study the dynamics of planctomycetes populations on the macroalgae surface in alternative to full 16S rRNA gene sequencing.

In the present studies (Chapters 7 and 8), DGGE fingerprinting analyses showed that the planctomycetes populations are specifically associated with the macroalgal host, independently of its geographical location, and were temporally stable. Further support of these results were given by the analyses of 16S rRNA gene clone libraries (Chapter 8) that evidenced a close phylogenetic relationship between clone libraries of the same macroalgae from two different locations sampled in winter and summer and showed the inexistence of a core community of planctomycetes associated with all the macroalgae. Furthermore, statistical analyses based on ANOVA showed that none of the OTUs sequenced were geographical or temporal dependent while some were significantly associated with the host. The effect of the host on the whole bacterial community associated with macroalgae has been reported in other studies for a wide range of macroalgae (Longford et al., 2007; Lachnit et al., 2009; Hengst et al., 2010; Barott et al., 2011; Trias et al., 2012; Vega Thurber et al., 2012). It has been was also demonstrated that the same species of macroalgae from different localities exhibited similar bacterial communities composition (Staufenberger et al., 2008; Lachnit et al., 2009; Sneed and Pohnert, 2011; de Oliveira et al., 2012). This

data suggests that the planctomycetes communities of macroalgae follow the same pattern as the whole bacterial community. Therefore, the factors that determine bacterial host-specificity probably also influence the planctomycetes populations. It has been suggested that host-specificity is related with the chemical composition of the macroalgae cell walls and their secreted polysaccharides and secondary metabolites (Goecke et al., 2010). Macroalgae produce mainly sulphated polysaccharides that are different in the three main lineages: fucoidan and laminarin are produced by brown algae; ulvan is present only in green algae and red algae produce carrageenan. Planctomycetes possess a high number of sulfatases genes involved in the degradation of these sulphated polysaccharides (Glockner et al., 2003; Wegner et al., 2013). It was shown that the abundance of these genes is species dependent and each species harbours a specific set of gene encoding sulfatases (Wegner et al., 2013) which can be the reason of a differential distribution of planctomycetes species among macroalgae. This hypothesis is supported by the results presented in Chapter 7, in which it was shown that the planctomycetes communities were also host phylum-dependent. The DGGE profiles from *C. crispus* and *M. stellatus* (*Rhodophyta)* were more similar between them than to the ones of other macroalgae. Similar results were obtained for *F. spiralis* and *Sargassum muticum* (*Heterokontophyta*). The macroalgae *Porphyra dioica* was the only exception as it presented distinct communities according to the sampling location. This hypothesis was supported by the existence of specific OTUs associated preferentially or exclusively with *Fucus spiralis* and with *Ulva* sp.

Contrary to results from other studies that suggested that the communities of planctomycetes associated with macroalgae are temporally dynamic (Bengtsson and Ovreas, 2010; Lachnit et al., 2011), the present results showed no significant differences between the DGGE profiles obtained in the four different sampling times (Chapter 8). The taxonomic composition of the populations as well as diversity and richness of OTUs obtained from clone sequencing were also stable between winter and summer. Nevertheless, the absence of temporal shifts in whole bacterial communities has been reported (Longford et al., 2007; Tujula et al., 2010). These studies reported the existence of a core community of bacteria that is stable through time. The results from the present study suggest that planctomycetes could probably be part of this stable core community.

The unknown epiphytic planctomycetes community

The clone sequencing of the eleven 16S rRNA gene clone libraries (Chapter 8) revealed 51 distinct OTUs, grouped at a similarity higher than 97 %, indicating that macroalgae harbour a huge diversity of planctomycetes. Furthermore, they also possess a high number of unique planctomycetes OTUs (31 %) found exclusively associated with macroalgae suggesting that these taxa are particularly adapted to these micro-niches. The present study showed that some OTUs were species-specific as they were only found in association with one of the macroalgae studied. So far, none of the molecular studies analysing epiphytic bacterial communities of macroalgae have proved the existence of specific bacterial species associated with particular species of macroalgae although specific phyla and classes have been identified (Hollants et al., 2013). In this study we have demonstrated that planctomycetes species are particularly associated with a specific macroalgae.

The phylogenetic distribution of the clones showed the existence of 6 major clusters (groups A-F, Supplementary Fig. S7) related to genera *Rhodopirellula*, *Blastopirellula*, *Planctomyces* and to an uncultured deep-branching lineage OM190. These groups have been usually found in association with other macroalgae like *Delisea pulchra* and *Ulva australis* (Longford et al., 2007), *Laminaria hyperborea* (Bengtsson and Ovreas, 2010) and *Ulva intestinalis*, *Gracilaria vermiculophylla* and *Fucus vesiculosus* (Lachnit et al., 2011) suggesting that they are frequent colonizers of macroalgae surface. In a previous culture dependent study based on 12 different macroalgae (Lage and Bondoso, 2011), the isolated strains were also related to genera *Rhodopirellula Blastopirellula* and *Planctomyces* even though a lower diversity was obtained (10 OTUs) in comparison with 16S rRNA sequencing. These isolated strains, some of which were taxonomically described in Chapters 3-5, are mainly within group A, which contains clones related to the genus *Rhodopirellula*. Group A was the second most abundant group, which is in agreement with the data from Lage and Bondoso (2011) that reported a high number of *Rhodopirellula* related strains. This group also contains two OTUs, 37 and 41, that were specifically associated with *Ulva* sp. Surprisingly, OTUs related to the widespread species *Rhodopirellula rubra* were only found in *Ulva* sp. from Porto and *Rhodopirellula lusitana* was not at all detected. A possible explanation could the set of primers used that could not amplify correctly these taxa. OTUs related to *Rhodopirellula formosa* were found in all macroalgae, suggesting a widespread distribution. The novel genus *Roseimaritima*, which was suggested to be specifically associated with *Ulva* sp. (Lage and Bondoso,

2011), was also found in *C. crispus*. Several OTUs related to the novel genus *Rubripirellula* were found in association with all the macroalgae sampled and they were not found in any other study thus indicating that this group is probably specific to macroalgae and geographical localized. The most abundant group (B) obtained was related to planctomycete FC18, that was isolated from *Fucus spiralis* from Porto (Lage and Bondoso, 2011). The planctomycetes communities present in the kelp *Laminaria hyperborea* (Bengtsson and Ovreas 2010) and in the brown algae *Fucus vesiculosus* (Lachnit et al., 2011) also possess a high abundance of this group. In the present study, this group was the most abundant one in *F. spiralis* and two of the OTUs from this group, 30 and 32, were found to be significantly associated with this macroalgae, suggesting that members of this group are most adapted to brown algae probably specialized in degrading fucoidan and laminarin. Group D, which contains OTUs related to genus *Planctomyces* was absent in *F. spiralis*. Species of the genus *Planctomyces*, are not able to degrade fucose (Ward 2010), the main component of fucoidan (Holtkamp et al., 2009), which can explain their absence in this macroalgae. The uncultured deep-branching group OM190 (Groups E and F) was found in all the macroalgae here studied and has been reported in several other habitats suggesting a wide ecological adaptation of member of this group.

The cultured independent strategies applied for the study of the epiphytic planctomycetes allowed an in-depth characterization of these communities that was not possible through cultivation. Nevertheless, isolation experiments are needed to get insights into these very diverse planctomycetes communities in order to analyse their physiology and metabolism which will allow a better understanding of the interactions planctomycetes-macroalgae.

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Supplementary Table S 8 Differential characteristics between the novel taxa described in this work and *Rhodopirellula baltica.*

	Rhodopirellula baltica	***Rhodopirellula rubra***	***Rhodopirellula lusitana***	***Rhodopirellula formosa***	***Roseimaritima***	***Rubripirellula***
Cell size (µm)	1.0–2.5x1-2–2-3[a]	1.3-2.5 x 0.9-1.7	1.2-2.1 x 0.8-1.7	1.1-1.75 x 0.9-1.5	1.1-1.8 x 0.9-1.5	1.5-2 x 1.3-1.7
Cell shape	Pear-shape to ovoid Rosettes	Pear-shape	Pear-shape to ovoid	Circular to ovoid	Spherical to ovoid	Pear-shaped to ovoid
Cell arrangement	with variable number of cells [a]				Rosettes 20-40 cells	Rosettes 2-10 cells
Pigmentation	Pink to Red [a]	Red	Pink to Red	Light Pink	Light Pink	Red
Seawater requirement	Yes	Yes	Yes	No	Yes	Yes
Salinity tolerance range:						
SW range (% v/v)	10-175	5-150	15-150	5-225	20-175	50-125
NaCl tolerance (% w/v)	5	5	4	6	5	4
Temperature for growth (ºC):						
Range	5-30	5-35	5-30	5-35	15-35	10-30
Optimum	28[a]	25	25	30	30	25
pH range	5.5-10.5	4.5-9.5	4.5-10	6.5-10	6.5-10	7.5-10.5
Carbon sources						
Arabinose	-	-	-	+	+	-
Dextran	+	+	-	+	+	-
Lactate	-	-	-	+	-	-
Lactose	+	+	+	+	+	-
Lactulose	-	+	+	+	+	-
Maltose	+	+	+	+	+	-
Mannitol	-	+	+	+	+	-
Mio-inositol	-	-	+	+	-	-
NAG	+	+	+	+	+	-
Raffinose	-	+	-	-	+	-
Ribose	+	-	-	-	-	-
Sucrose	+	+	+	+	+	-
Trehalose	+	+	+	+	+	-

Nitrogen sources				+	+	-
Alanine	+	+	+	+	-	-
Arginine	+	+	-	+	+	-
Asparagine	+	+	+	+	-	-
Aspartic acid	+	+	+	+	+	+
Glutamic acid	-	-	+	+	+	-
Methionine	-	-	+	-	+	-
NAG	+	+	+	+	+	-
Nitrate	-	+	+	-	+	+
Nitrite	-	-	-	-	+	-
Ornithine	-	+	-	-	+	-
Phenylalanine	+	+	+	+	-	-
Proline	+	-	+	-	+	-
Serine	-	+	-	+	+	-
Threonine	-	-	-	-	+	-
API 50CH				-	-	-
D-adonitol	-	-	-	Variable (+)	-	-
Amygdalin	+	+	+	+	+	-
D-arabinose	+	+	+	+	+	-
D-fucose	+	+	Variable (+)	+	+	-
D-mannitol	-	Variable	Variable (+)	+	+	-
D-melezitose	+	+	Variable (+)	+	Variable (-)	+
D-melibiose	+	+	+	+	+	-
D-raffinose	+	+	Variable (+)	+	+	-
D-ribose	+	+	+	+	+	-
D-saccharose (sucrose)	+	+	+	+	+	-
D-tagatose	W	Variable (-)	Variable (-)	+	+	-
D-trehalose	+	+	Variable (+)	+	+	-
D-turanose	+	+	Variable (+)	+	+	-
Galactose	+	+	+	-	+	+
Glycogen	+	-	Variable (-)	-	+	-
L-Arabinose	+	+	+	+	+	-
L-arabitol	+	Variable (-)	Variable (-)	+	-	-
L-fucose	+	+	+	+	+	-
L-rhamnose	+	+	+	+	Variable (+)	-

Methyl-ßD-Mannopyranoside	+	Variable (-)	Variable (+)	+	Variable (-)	-
Methyl-ßD-Xylopyranoside	w	+	Variable (-)	-	--	-
Potassium 2-ketogluconate	+	-/w	Variable (-)	-	-	-
potassium 5-KetoGluconate	w	+	Variable (-)	+	+	-
Sorbitol	-	-	-/W	-	+	-
Xylitol	+	Variable (-)	-	-	+	-
API ZYM						
ß-glucosidase	+	variable	-	Variable (-)	-	-
Trypsin	+	-	Variable (-)	Variable (-)	Variable (-)	-
N-acetyl-ß-glucosaminidase	+	Variable (+)	Variable (-)	-	-	-
α-fucosidase	-	Variable (-)	Variable (+)	+	-	-
α-galactosidase	+	Variable (+)	Variable (-)	+	+	-
ß -galactosidase	+	+	Variable (-)	+	Variable (+)	-
α -glucosidase	+	+	Variable (+)	+	+	-
α-chymotrypsin	-	-	-	+	-	-
Naphthol-AS-BI-phosphohydrolase	-	-	-	Variable (+)	-	-
Catalase	+	+	+	+	+	-
Hydrolysis of					++	-
Starch	+	+	-	+	+	+
Cellulose	-	-	+	+	+	+
Gelatine	-	+	-	Variable (+)	-	-
FAMEs				+		
$C_{10:0\ 3OH}$	-	-	-	0.1-0.2		
$C_{12:0\ 3OH}$	-	0.1-0.4	0.2-0.4	-		
$C_{14:00}$	0.5	0.5-1.4	0.6-0.9	0.3	0.7-0.8	0.4
$C_{15:00}$	0.5	0.6-1.1	0.2-1.4	0-0.1	0.3	1.0
$C_{16:00}$	26.8	22.2-27.3	22.2-26.9	27.7-29.7	33.4-35.1	17.3
$C_{16:1\omega11c}$	-	-	-	4.0-4.7		
summed feature 3 [b]	17.9	12.9-21.1	17.6-23.2	2.4-3.4	2.3-2.4	8.4
summed feature 3 [c]	-	3.8-7.3	-			
$C_{17:1\omega8c}$	5.1	3.0-6.2	1.3-8.9	1.1-1.3	1.7-2.0	12.0
$C_{17:00}$	1.0	0.5-1.2	0.3-1.4	0.6-0.9	1.7-2.1	4.5

$C_{16:00\ 2OH}$	-	0-0.2	-	0.2-0.3		1.1
$C_{18:1\omega9c}$	41.6	35.6-42.7	36.2-46.9	52.9-54.0	42.5-48.1	41.5
Summed feature 8 [d]	2.8	1.3-3.2	1.5-2.7	0.7-1.2	0.9-1.2	0.9
$C_{18:0}$	1.3	1.4-4.0	2.1-3.7	3.2-3.9	2.7-3.1	5.6
Unknown ECL 18.558					0-3.5	-
Unknown ECL 18.796	0.6	0-0.5	0.2-1.1		0.5-0.6	-
Summed feature 7 [e]	-	0-1.0	0-1.7	0.4-0.9	-	1.1
$C_{19:0}$	-	0-0.2	-	0-0.1	-	2.1
$C_{18:00\ 2OH}$				0.2-0.4		
$C_{18:00\ 3OH}$	-	-	-	0.3-0.6	1.1-1.2	1.3
$C_{20:1\ \omega9c}$	-	0-0.2	0-0.2		3.0-3.7	0.6
$C_{20:0}$	-	0-0.1	0-1.5	0.6-0.8		0.7
Phospholipids						
PC	+	+	+	+	+	+
PG	++	++	++	++	+	+
DPG	++	+	++	+	++	-
PL1	+	+	++	+	+	-
PL2	-	-	-	+	-	+
UL1	+	+	++	++	+	++
UL2	+	+	-	-	++	-
UL3	+	+	+	-	-	-
UL4	+	-	+	-	-	-
UL5	-	-	-	+	-	-
APL	+	-	-	-	-	-
DNA G+C content	54.1 %	56.1 %	54.6 %	53.4%	57.0%	56.1%
Nitrate reduction to nitrite	-	+	+	+	+	+
Anaerobic growth	No	Yes	Yes	Yes	No	No

+, positive; -, negative; ++, strongly positive; Variable (+), majority positive; Variable (-), majority negative

[a]Data from Schlesner et al., (2004(

[b,c]summed feature 3 comprises $C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c.

[d] summed feature 8 comprises $C_{18:1}$ ω7c

[e] summed feature 7 comprises $C_{19:0}$ cyclo ω10c/C_{19} ω6

Chapter 10.
Concluding remaks and future perspectives

The work presented in this thesis is a relevant contribution for the *Planctomycetes* taxonomy. In particular, it allowed to increase our knowledge in the widespread *Rhodopirellula* genus and to further understand its ecology and physiology. The use of a polyphasic taxonomy approach for the description of the novel strains of planctomycetes presented in this study allowed their separation from the closest cultured representative, *Rhodopirellula baltica* SH1^T and their classification into novel taxa of the phylum *Planctomycetes*. The phylogenetic positioning of some strains was not resolved by the 16S rRNA gene analysis, as they shared similarities higher than 97 % to *R. baltica* SH1^T, and, therefore, a molecular marker based on the β-subunit of the RNA polymerase (*rpoB*) was developed. The partial sequencing of this gene proved to have enough resolution power to discriminate closely related species of the order *Planctomycetales* allowing the replacement of DDH. This study could be, hopefully, helpful for future taxonomic assignments and novel taxa descriptions.

With culture independent molecular studies, an in-depth characterization of the planctomycetes communities associated with macroalgae was achieved. Host-specific planctomycetes communities were observed in all the macroalgae tested. Moreover, these communities were more similar on macroalgae species belonging to the same phyla. These results support the hypothesis that the distinct distribution of bacterial species on macroalgae surfaces is determined by the chemical composition of macroalgae cell walls and their exudates. In general, the communities from the same macroalgae but from different localities were more similar between them than to other co-occurring macroalgae species indicating the absence of a geographical pattern. Furthermore, no temporal variation pattern was also obtained. 16S rRNA gene clone libraries of macroalgae proved the existence of specific species associated with a particular macroalga, one OTU with *F. spiralis* and one with *Ulva* sp. However, none was specific to *C. crispus*, but two OTUs were found to be preferentially associated with both *C. crispus* and *Ulva* sp. that shared communities more closely related. A great diversity of planctomycetes related to the genera *Rhodopirellula*, *Blastopirellula* and *Planctomyces* and to the uncultured group OM190 was found in these communities. Macroalgae harbour a high diversity of planctomycetes not yet retrieved in pure culture. Furthermore, many of the OTUs obtained are unique and only found in association with macroalgae suggesting particular adaptations of these organisms to colonize macroalgae.

Based on the present results and data from the literature, it can be hypothesized that the association planctomycetes-macroalgae is possibly driven by the chemical

nature of the polysaccharides produced by the macroalgae and the core set of sulfatase genes specific of each planctomycetes species.

This multidisciplinary study revealed important aspects of the interaction between planctomycetes and macroalgae and some hypothesis have been raised. However, some questions arose from this work that deserve to be addressed:

1) Is the similarity between planctomycetes communities of specific macroalgae from different locations maintained, even in relatively distant geographical places with different environmental conditions? Does this geographical pattern apply to all macroalgae?

DGGE profiles can be used to rapidly assess the planctomycetes communities in the same species of macroalgae from several different locations.

2) Is host-specificity common to all the planctomycetes communities from different macroalgae? Do each macroalgae harbour specific species?

A more in-depth study comprising all the macroalgae occurring in the same habitat should be performed. Again, DGGE profiles can provide a first insight in the host specificity. Taxonomic diversity of the communities will allow determining the existence of specific species associated with each macroalgae. With the new generation sequencing techniques, such as 454 pyrosequencing, the whole community can be fully characterized and the less abundant taxa can be retrieved.

3) What would be the characteristics of the unique planctomycetes species found in association with macroalgae?

For this we should performed selective isolation experiments, based on macroalgae extracts, in order to isolate these novel taxa. Their biochemical and physiological characterization would hopefully allow better understanding the association planctomycetes-macroalgae and their interactions. Partial or complete genome sequencing will also allow determining genetic properties, such as the presence of sulfatase genes, involved in this association.

4) Now that we know “who is there” remains to know “what are they doing there”.

Physiological experiments, with the strains already isolated from macroalgae, directed to the utilization profile of the several polysaccharides extracted from macroalgae and their exudates would help determining nutritional factors involved in this association. Proteomics studies would allow determining the proteins expression with different substrates.