Isolation and Characterization of Novel Planctomycetes from Svarthammarhola Ice Cave with Potential for Production of Bioactive Molecules

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Master's Thesis in Microbiology

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June 1st, 2018

Acknowledgements

This master's thesis was carried out in the period from January 2017 to June 2018 under the supervision of Professor Lise Øvreås, in the research group Marine Microbiology, a part of the Department of Biological Sciences at the University of Bergen.

First of all, I would like to thank you Lise for an amazing and exciting thesis, and the opportunity to work with these peculiar organisms. I very much appreciate all those times you have neglected the mountain on your desk, called your "to do list", just to help me with my struggles. I would also like to thank you for giving me the opportunity to present my work at the PVC conference in Porto, Portugal, and to accompany you at the research group meeting in Romania.

A big thank you to all the people at the lab that have helped me with all my strange inquiries and experiments. Especially Hilde Rief Armo and Julia Endresen Storesund, who have always been available for questions, tips and assistant during my lab work and the writing of this thesis.

Also, I would like to thank Professor Lars Herfindal and the rest of his research group for hosting me at your lab at the Center for Pharmacy, Department of Clinical Sciences at University of Bergen. You welcomed me into your group and assisted with my work. Especially in the beginning, when I did not know what an adherent cell was.

Finally, I would like to thank my friends, family and girlfriend for always supporting me and showing interest in my experiments and endeavours while writing this master's thesis. I could not have done this without you.

Abstract

Planctomycetes represent a phylum of bacteria that possess peculiar characteristics. These include reproduction by budding, crateriform structures, rosette formation and an intricate cellular plan. They are considered important contributors in the global carbon and nitrogen cycle and have in recent years been proposed to have potential for various biotechnological applications. The Planctomycetes are ubiquitous bacteria, and has been reported in diverse and extreme environments, both terrestrial and aquatic. These extreme environments include cave ecosystems, as well as ice and glacier ecosystems. However, these reports have been based solely on molecular studies, and no Planctomycetes have been described in axenic culture from either cave or frozen environments. In this study the presence of Planctomycetes in perennial ice from the Svarthammarhola ice cave is elucidated. Drilled ice cores from the distinct layered ice mass were collected. Thawed ice was used as inoculum in enrichments for isolation of Planctomycetes strains. Isolated strains were then subjects for characterization, utilizing molecular and cultivation-based techniques. Four Planctomycetes strains were obtained in axenic culture, and all strains showed both morphological and physiological differences. Phylogenetic analyses of the strains' 16S rRNA gene revealed that 3 of the strains shared phylogenetic similarities of 99 and 98%, and their closest described species was 90% similar. The fourth strain was 88 and 87% similar towards the other strains, and displayed 90% similarity towards its closest described relative, thus the isolated strains represent two novel genera, within the phylum Planctomycetes.

To elucidate the strains' ability to produce bioactive molecules, two of the strains were screened against the two cancerous cell lines Molm13 and PC3, as well as the normal healthy rat kidney cell line, NRK. Aqueous and organic compounds were extracted from cell cultures and used to treat the cell lines for 72 hours. The cell lines were then investigated for apoptosis induced by the extracts. The screening revealed that the strains were able to produce compounds with intermediate cytotoxicity towards the human AML cell line Molm13. Thus, displaying that the strains might be able to produce bioactive molecules that provide advantages towards other organisms competing for resources in their indigenous environment.

Table of Contents

1. Introduction

1.1 Cave Systems

Caves can be defined as “any natural space below the surface that extends beyond the twilight zone, and that is accessible to humans” (Northup and Lavoie, 2001). Several different types of caves exist, and they are classified according to bedrock type and formation methods (Palmer, 1991). Caves formed in limestone and other calcareous rocks, and lava tubes in basaltic rocks are the most common type of caves (Northup and Lavoie, 2001). Other cave types such as gypsum, granite and ice also exist, but their distribution is more limited. There are many caves spread across the face of our planet, however only a few of these have been studied. In Europe and North America alone, it is estimated that only 50% of all caves have been explored. Considering the entire globe, only about 10% of all caves have been discovered (Lee et al. 2012), where only 350 have been described in detail (Onac and Forti, 2011). Caves represent a type of natural, rocky subsurface habitat that host some of the most exotic landscapes, minerals and mineral formations (White, 1988; Ford and Williams, 2007; Palmer, 2007), as well as microorganisms (Culver and Pipan, 2009).

1.1.1 Cave Formation

There are several types of caves, and their formation is usually caused by the dissolution of sedimentary rocks (Palmer, 2007; White and Culver, 2000). One type of cave is Karst caves, which are formed by the presence of soluble rocks, where systems of cracks and fissures are developed by aggressive water flow that dissolves the soluble rocks (Ford and Williams, 1989; Kruber, 1915; Sokolov, 1962; Thornbury, 1954). Another type is tectonic caves, these caves form as the bedrock shifts along cracks and faults in the Earth’s crust, creating openings and fractures as the bedrock moves. There are also thermokarst caves, which forms in rocks containing ice (Kotlyakov, 1984). The cavity formation is caused by small amounts of ice that are frozen inside rocks and creates cracks and fissures. The shape of this ice is determining the shape of the cavity. There are also glacial caves, which can be formed inside the ice, firn, and the snow of glaciers or snowfields (Ezhov, 1990; Mavlyudov, 1992, 2006, 2018). Causes for the cave

formation are the same as for karst caves, except for the chemical dissolution of rocks, which in glacial cavities are caused by ice thermoerosion (Mavlyudov, 2018)

1.1.2 Ice Caves

Ice caves are caves hosting perennial ice accumulations and are considered the most enigmatic and least well-known part of the global cryosphere (Persoiu and Lauritzen, 2018). They occur in places were the combination of cave morphology and climate conditions make favourable habitats for the formation of ice and allows it to persist. Ice caves have proven to be sensitive to today's increasing global temperatures and climatic changes. In fact, many of present-day ice caves are threatened by these factors, which are jeopardizing their existence in the future (Kern and Persoiu, 2013). This is very unfortunate considering that they hold valuable information regarding historical climate variations, as well as possibly hosting valuable biodiversity (Persoiu and Lauritzen, 2018; Purcarea, 2018).

1.1.3 Cave Glaciation

For development of perennial ice inside a cave certain conditions from the outside climate and the cave climate must be fulfilled. It is necessary that the temperature outside the cave is below 0°C at least some part of the year. Inside the cave, the air and rocks must also have temperatures below 0°C. Ice can also form if there is a difference in temperature between the rock and the outside air, as well as when bedrock temperatures are above 0°C. Flow of external air can still cool the cave walls to freezing temperatures during winter months and force the formation of ice (Mavlyudov, 1989, 2008). The nature of the cavity determines the air circulation in the cave, as well as which zones of the cave remains cooled (Mavlyudov, 1994). It is in such cooling zones that the mean annual air temperature (MAAT) and the rock temperature are below 0°C, and lower than other areas of the cave. These zones are favourable areas for ice development. For the formation of ice to happen it is also necessary for the frozen cavity to be penetrated by water. Ice formation occurs when the heat brought in by the water is less than the cold reserve in the cave (Mavlyudov, 2018). The morphology, distribution, ice reserve and ice mass balance are defined by the amount of water entering the cave.

Most perennial ice hosting caves have a MAAT higher than 0°C, therefore certain requirements regarding climate must be met to either maintain ice, or for the formation of new or more ice in the cave. One requirement is that there is undercooling during winter months, the other, a mechanism for the preservation of negative temperatures during summer months. Undercooling can be achieved by conductive heat transfer, driven by temperature differences between the cave and the outside environment. The cooling of a cave can also be achieved by pressure fluctuations, gravitational settling, and by diphasic flow due to water circulation (Persiou, 2018).

1.1.4 Svarthammarhola Ice Cave

Svarthammarhola (N67.13'E15.31' at 295 m a.s.l.) is an ice cave located near Fauske, north of the Arctic circle, in Norway. It is the largest ice cave in Fennoscandia and contains the largest cave chamber (300x90x40 m/lwh) in this region, possibly also in Northern Europe (Lauritzen et al., 2018). The cave has two major entrances (Figure 1.1), one situated at 245 m a.s.l and the other at 295 m a.s.l. It is described as a simple dynamic cave, with congelation ice accumulation and ablation near the lower entrance, driven by Balch-ventilation, with the availability of intruding water (Luetscher and Jeannin, 2004; Lauritzen et al., 2018). Near the lower entrance is a horizontal tunnel where the ice mass is situated (Figure 1.1). The ice mass has a horizontal surface which is 160 m long and 25 m wide, with an exposed flat surface of 3350 m^2. Near the downstream end there is an 18 m high ablation wall into a lower gallery where the ice is exposed (Figure 1.1). An ablation tunnel (3 m in diameter) has formed under the ice, penetrating the ablation wall (Lauritzen et al., 2018). From the lowest lobe in the western ablation wall to the top surface of the present ice the total maximum stratigraphic thickness of the ice mass is 27 m.

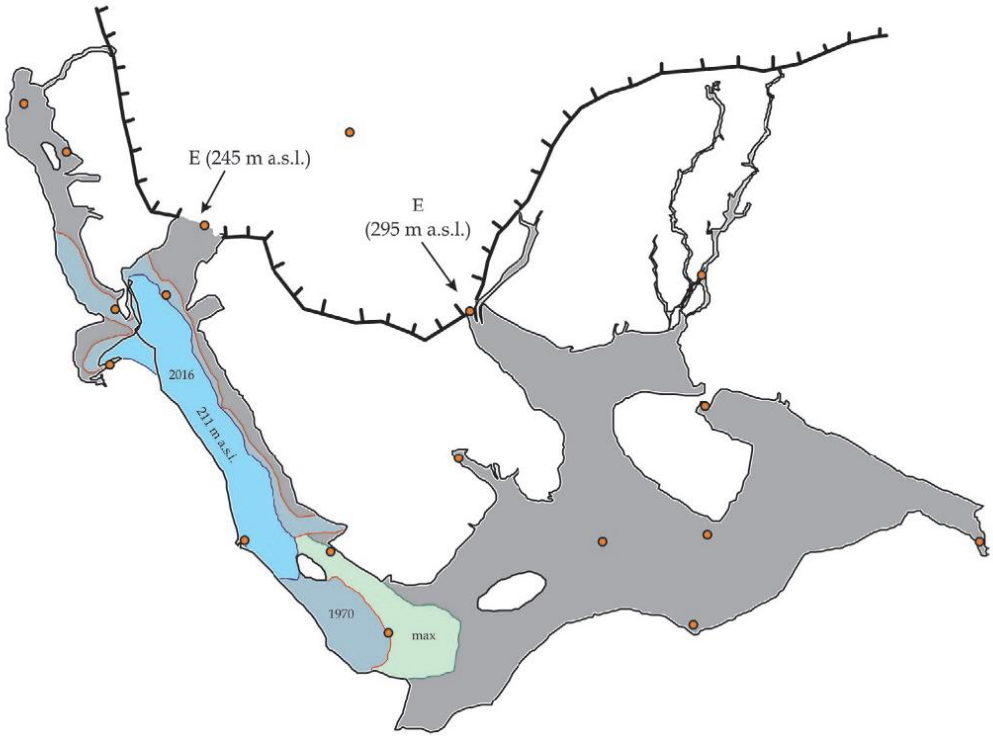


Figure 1.1: Svarthammarhola, plan. E: Major entrances. Red *dots*, data logger stations; ice block extents: *blue*, 2016 (Sampling site), 1970; *green*: oldest extent (of unknown age) as traved by cryogenic precipitates. Source: Lauritzen et al., 2018.

Over the past decades since the cave first was discovered in 1969, there has been some significant changes to the cave. The first cave survey lead by Heap (1970), reported on a much more extensive ice surface than present today (5450 m^2) (Lauritzen et al., 2018). The large ice surface at the upstream end is the part that have been impacted the most. Especially during the 1980s when it disintegrated and transformed into an ice dammed lake, which disappeared completely (Lauritzen et al., 2018). In 1976, photographs were taken of the ice, these were used to compare the ice in 2016. It was revealed that the downstream ice was reduced with about 5 m and the upstream end close to 2 m (Lauritzen et al., 2018). Measurements from 2005 to 2011, combined with historical data, reveals that the ice surface has declined with an average rate of 0,11-0,15 m/year since 1975 (Lauritzen et al., 2018). In the oblation wall, organic matter like plant remains and sediments occurs at various levels in the ice mass. By using ^{14}C dating of plant fragments it has been revealed that the base of the ice mass can be dated back to AD 1365 ± 75, making the base of the ice mass <650 years old (Lauritzen et al., 2018). As previously mentioned Svarthammarhola is

significantly affected by Balch-ventilation, with winds of up to 8 m/s (Baastad, 2006; Lauritzen et al., 2018). The wind direction depends on the difference in temperature between the cave and the surface. When the cave is warmer than the surface the wind moves in the cave's upward direction (chimney effect), when the cave is colder than the surface, the wind moves downwards. Between these two conditions, a significant oscillation is displayed by the wind (Baastad, 2006; Lauritzen et al., 2018). Based on ventilation the energy flux of the cave was calculated to be -421 GJ between 2005 and 2006 (Baastad, 2006), meaning that the cave consumed energy and that the ice mass was ablating (Lauritzen et al., 2018). Lauritzen and collaborates (2018) states that: "From the historical data, the ice mass appears to have ablated monotonically, and possibly at an increasing rate since the cave was discovered in 1969".

1.2 Cave Microbiology

1.2.1 History of Cave Microbiology

There has been an increased interest in microbial communities from dark and frozen environments over the past decade. However, prior to 1997, only a few publications described the microbiology of caves, were most of these studies were based on microscopic descriptions of cultured bacteria (Faust, 1949; Caumartin, 1963; Brigmon et al., 1994; Rusterholtz and Mallory, 1994; Mikell et al., 1996). Between the 1900s to the 1940s there was an emphasis to whether saltpetre and carbonate speleothems was a microbial component to cave formation or of microbial origin as secondary nitrate deposits (Faust, 1949; Hess, 1900; Dudich, 1932; Høeg, 1949). From 1960s to early 1990s the focus of cave microbiology turned into the cultivation and linking microbial processes to mineralogical phenomena (Caumartin, 1963; Thrailkill, 1964; Smyk and Drzal, 1964; Went, 1969; Caumartin, 1968; Hubbard et al., 1986). Besides these main focuses microbial cave research has made efforts in describing metabolism related to food web structure and development of cave ecosystems. These earlier studies lead to the conclusion that microorganisms could not have an important role in most caves, and that they would typically be inactive or in low biomass due to nutrient limitations. It has also been emphasized that microorganisms in caves have identical or similar functions, to those in soil communities (Caumartin, 1963; Northup and Lavoie, 2001; Barton and Northup, 2007).

1.2.2 Conditions for Microbial Life in Caves

Living in the subsurface differs in many ways from life on the surface. There are both advantages and disadvantages connected to subsurface microbial life. The organisms are living in the dark and cannot benefit from the sunlight energy. On the other hand, they are protected from the damaging effects caused by ultraviolet radiation, extreme weather conditions, desiccation and temperature fluctuations. The cave environment is relatively stable and represents a unique subset of the deep subsurface environment on Earth (Boston et al., 2001). All caves are different when it comes to biological, chemical and physical characteristics (Onac and Forti, 2011). Rock type and its physical and chemical properties, including the geological and hydrologic characteristics, are influencing the diversity of organisms found in cave systems (Engel, 2015). The trophic

structure is often less complex in caves than in other surface environments due to absence of light, stable temperatures and humidity, as well as low variations in nutrient sources (Ghosh et al. 2017). Although nutrients are limited in caves, diverse microorganisms have been reported (Cheeptham, 2013; Culver and Pipan, 2009).

Carbon and other nutrients are obtained by cave microorganisms primarily by two mechanisms. One being the conversion of inorganic carbon to organic carbon by photosynthesis or chemosynthesis performed by autotrophs. The other being heterotrophic assimilation of already existing organic carbon (Engel, 2015). Near the entrance there will be a possibility for photosynthesis, however, deeper and darker into the cave, the presence of photosynthetic autotrophs will diminish (Ghosh et al., 2017; Barton, 2015; Cheeptham, 2013). Microorganisms can be brought deep inside the cave by wind and water, as reported by Ogórek and collaborators, were fungal spores from the same species were found both deep inside and outside an ice cave in Slovakia (Ogórek et al., 2017). When brought deep into the cave by wind or water the microorganisms may not survive the conditions but can still be preserved and retain their genetic information (Engel, 2015). Some shallow cave systems can also provide allochthonous energy and nutrients by the penetration of the subsurface by plant roots. In this way, dissolved or particulate organic matter can be provided to the subsurface (Engel, 2015). Alterations in the method and the amount of surface materials supplied to the cave ecosystems can result in energy and nutrient limitations and can further lead to oligotrophic conditions in some caves (Poulson and Lavoie, 2000). Microorganisms are known to be at the energetic and nutritional base of the cave ecosystems. However, microbial diversity and the microorganisms' role in cave ecosystems have not been fully understood. Many types of caves have been discovered and studied worldwide, yet our knowledge about colonization, diversity and metabolic function is very limited (Lee et al., 2012).

1.2.3 Microorganisms from Cold Environments

The Earth can be regarded a cold planet. Ocean covers 70% of the Earth and deep water with temperatures below 5°C makes up the majority of the ocean. In addition to this, large parts of the biosphere are periodically permanently frozen and as much as 24% of the exposed land surface of the Northern Hemisphere are permanently frozen

(Zhang et al., 2003). The microorganisms that live in these permanently cold environments are referred to as psychrophiles and their habitats vary from the deep ocean, the polar regions, glaciers, caves and to the upper atmosphere (Feller and Gerday, 2003; Feller, 2013)

Microorganisms found in these areas are cold adapted and considered psychrophilic, which means that they grow optimally in temperatures less than 15°C. Psychrotolerant organisms are organisms that survive at temperatures below 0°C but grow optimally at 20-25°C (Morita, 1975). Over the course of time these microorganisms have evolved several adaptive strategies to maintain their vital metabolic functions under such extreme conditions (D'amico et al., 2006). Life in cold and icy environments depends on several adapted traits to counter stress factors like low temperatures, pH fluctuation, high osmotic pressure and limited nutrient availability (D'amico et al., 2006; Margesin and Miteva, 2011; De Maayer et al., 2014). These factors will place physiochemical constraint on cellular functions by negatively influencing cell integrity, as well as water viscosity, solute diffusion rates, membrane fluidity, enzyme kinetics and macromolecular interactions (Rodrigues and Tiedje, 2008; Piette et al., 2011). This have led to the evolution of several adaptations on the cellular level, including production of cryoprotectants and antifreeze proteins such as glycine and betaine which lowers the cytoplasmic freezing point, protecting against ice crystal formation in the cytoplasm (Casanueva et al., 2010; Cowan, 2009). Some psychrophiles also produce antifreeze or ice binding proteins that bind to ice crystals accumulated in the cell and lowers their freezing point (Celik et al., 2013).

In the recent years, the main emphasis of microorganisms in cold environments has been on their mechanisms of adaptation, as well as their structural and functional diversity in these environments (Price, 2007; Priscu et al., 2007; Margesin and Miteva, 2011; Gunde-Cimerman et al., 2012). These frozen environments include polar ice sheets and glaciers (Miteva et al., 2004; Lanoil et al., 2009; Rehakova et al., 2010; Anesio and Laybourn-Parry, 2012), permafrost (Rivkina et al., 2004), mountain glacier forefields (Lapanje et al., 2012; Zumsteg et al., 2012), frozen lakes (Felip et al., 1995), sea ice (Deming, 2002), Arctic (Varin et al., 2010; Adams et al., 2014) and Antarctic permanent lake ice (Priscu et al., 1998; Dieser et al., 2010; Murray et al., 2012).

Antarctic volcanic ice caves on Mount Erebus has recently been studied, focusing on the microbial communities. The study revealed low bacterial diversity as well as low fungal diversity (Tebo et al., 2015).

1.2.4 Ice Cave Microbiology

Ice caves are represented by unique and secluded icy habitats with light-deprived ecosystems of low nutrient content and constant low temperatures, that contrast to other parts of the Earth's cryosphere (Purcarea, 2018). The first report of microorganisms in an ice cave can be dated back to 1949, when Pop (1949) detected the presence of nitrifying bacterial communities in sediments in the Scărișoara ice cave in Romania. In 2003, Margesin and collaborates isolated the first bacterial strain from an ice cave in the Austrian alps (Margesin et al., 2003, 2004). Microbial research involving ice caves have since then been very limited, however, recently, the Scărișoara ice cave has been host of several recent studies on microbial communities. In 2013 Hillebrand-Voiculescu and co-workers did cultivation experiments on one-year old ice stalagmites in the cave, and reported cultured strains affiliating to the *Pseudomonas*, *Bacillus* and *Paenibacillus* genera. One year later the presence of bacterial and eukaryotic SSU rRNA were reported in samples collected from 1400 and 900-year-old ice block layers (Hillebrand-Voiculescu et al., 2014). Since then cultured characterization of cultured bacteria from sequential ice has been done using PCR-DGGE, finding bacterial amplicons belonging to Gammaproteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, with varying distribution along the chronological ice layers (Iţcuş et al., 2016).

1.2.5 The Importance of Studying Ice Caves

The inaccessibility and the limited impact on human daily life and the environment, have led to a constrained interest in microbial cave research (Purcarea, 2018). However, this reduced interest has paradoxically lead to conserved microbial diversity in these caves. There is now a growing interest to revisit these habitats, much due to recent studies on potential drug discovery and antibiotic resistance of cave microorganisms (Pawlowski et al., 2016; Ghosh et al., 2017). Microorganisms found in caves have also displayed various enzymatic and antimicrobial activities, that contrast

to those observed in other extreme environments (Barton, 2006; Cheeptham, 2013; Lavoie, 2015; Man et al., 2015).

At the same time, frozen and cold environments have been proposed as habitats hosting microbial communities with potential for numerous biotechnological resources. Especially enzymes derived from cold-adapted prokaryotes is proving to be ideal tools for several biotechnological applications. This includes applications in many diverse industries like food and beverages, pharmaceuticals, detergents and biosurfactants, biofilm removal, personal care and cosmetics, molecular biology, diagnostics, therapeutics and is still spreading to new areas of the industry (Huston, 2008; Karan et al., 2012; Perfumo et al., 2018; Antranikian et al., 2004; Muller-Greven, et al., 2012; Awazu et al., 2011; Celik and Yetis, 2012; Fornbacke and Clarsund 2013; Barroca et al., 2017).

We are considered to live in a 'post antibiotic era' (Alanis, 2005) with an increasing number of reports of multi-resistant pathogenic bacteria, where few or no antibiotics are available (Wright, 2016). However, antibiotic resistance mechanisms predate our use of antibiotics, and is an ancient and naturally occurring phenomenon, widespread in the environment (D'Costa et al., 2011). Antibiotic resistance can occur through a variety of molecular mechanisms, including decreased drug permeability, active efflux, alteration or bypass of the drug target, antibiotic-modifying enzymes, and physiological states such as biofilm. These mechanisms are often classified as either mechanisms that have evolved specifically to detoxify specific antibiotics, or mechanisms that are intrinsic to specific bacteria that have the effect of resistance, but not targeted to an individual antibiotic (Wright, 2016). To minimize the development of antibiotic resistant pathogens, there is a need for both understanding the mechanisms behind the resistance and the discovery of new antibiotic compounds (Andersson, 2003). Today most antimicrobial compounds are derived from tropical and warm environments, therefore, compounds deriving from cold-adapted microorganisms are likely to be different from many of the classes of antimicrobials currently in use (Borchert et al., 2017). Hence, researchers turn to other environments, cold and dark environments. In the past decade several novel antimicrobials have been discovered in cold- adapted microorganisms, including synoxazolidinone A and B

(Tadesse et al., 2010), serraticin A (Sánchez et al., 2010), subtilomycin (Phelan et al., 2013) and lobophorin H and I (Pan et al., 2013).

Ice caves could host very interesting microbiomes from a biotechnological point of view, considering that ice caves represent dark, cold and preserved communities of microorganisms. Recently, ice caves were also recognized as a proxy for paleoclimate reconstruction (Persoiu and Onac, 2012), this means that studying the ice cave microbiome can lead to discovery of microbial biomarkers for climate variations (Purcarea, 2018). Very few microbiological studies have been performed on ice caves. Based on the biotechnological potential of microorganisms from cave ecosystems and cold environments, ice caves could be a good habitat for microorganisms possessing traits of biotechnological value.

1.3 Planctomycetes

Planctomycetes are a division of peculiar bacteria within the PVC superphylum that also include Verrucomicrobia, Chlamydiae and Lentisphaerae, as well as the candidate phyla Poribacter (Wagner and Horn, 2006). Planctomycetes were at first described as eukaryotes (Gimesi, 1924), but was later acknowledged as bacteria (Hirsch, 1972), and were first isolated in pure culture by Staley (1973). Despite this, proposals have been made that Planctomycetes share some sort of evolutionary link with eukaryotes (Fuerst and Sagulenko, 2011; Forterre and Gribaldo, 2010; Devos and Reynaud, 2010). Much due to their intricate cellular plan and that their genomes encode proteins with high structural similarity to membrane coat proteins in eukaryotes, which no other bacteria or archaea do (Santarella-Mellwig et al., 2010).

1.3.1 Phylogeny

Planctomycetes are known for having large genomes (Jeske et al., 2013; Kim et al., 2016), like *Roseimaritima ulvae* (8,130 MB) (Faria et al., 2017), *Singulisphaera acidiphila* (9,76 MB) (Kulichevskaya et al., 2008), and *Zavarzinella formosa* with a genome size of 9,86 MB (Kulichevskaya et al., 2009). The branching of the bacterial tree of life has been subject to much debate regarding Planctomycetes. Together with thermophiles, Planctomycetes has been proposed as some of the deepest branching bacteria (Stackebrandt et al., 1984; Brochier and Philippe, 2002). In recent years, Planctomycetes have been proposed to belong at the deepest branch among the phyla of the domain bacteria in the tree of life (Jun et al., 2010; Fuerst and Sagulenko, 2011), as shown in Figure 1.2.

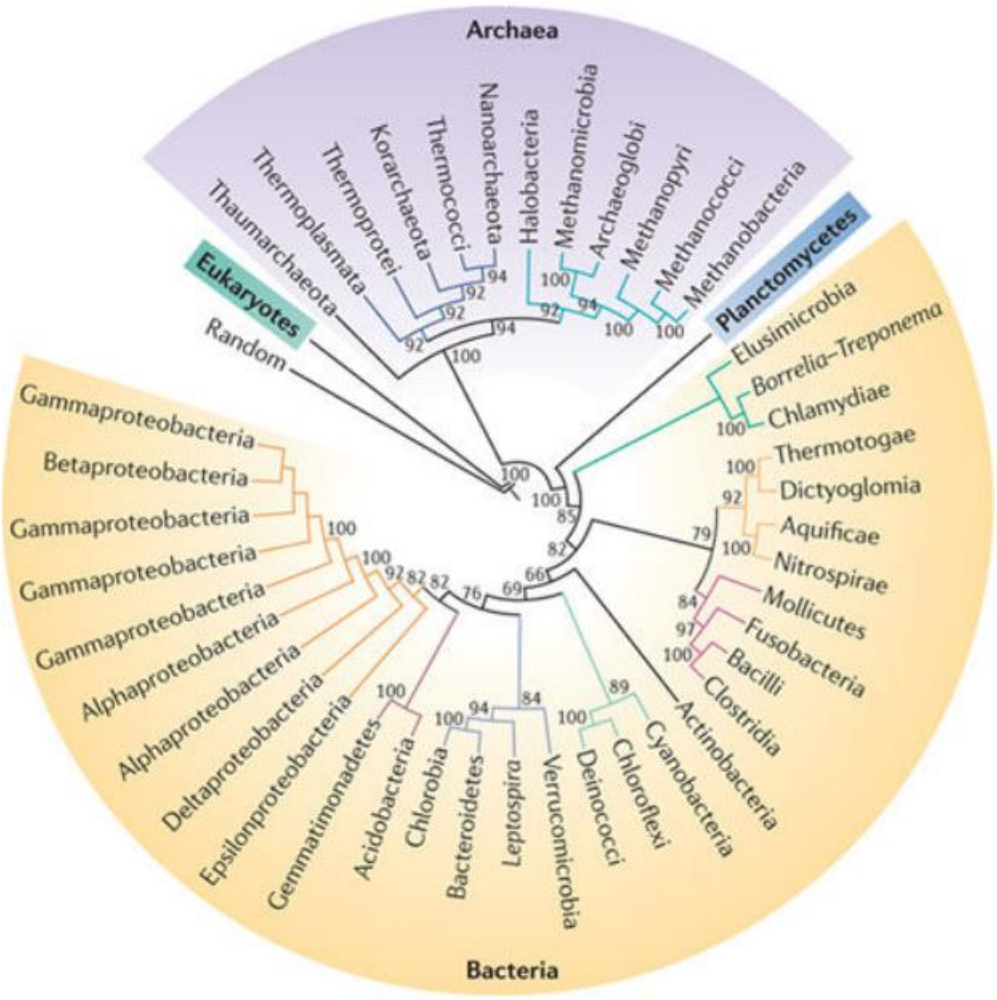


Figure 1.2: A tree of representatives of the domains Bacteria, Archaea and Eykarya, constructed by comparing feature frequency profiles of whole proteomes, and showing a deep-branching position for Planctomycetes relative to other bacterial phyla. The colouring of branches indicates 'supraclass' groups, which are defined by statistical support values of >82, except for in the Archaea, for which there are three clear clades according to this analysis. The numbers indicate the jack-knife monophyly index (%). Source: Fuerst and Sagulenko, 2011.

Planctomycetes together with Verrucomicrobia and Chlamydiae (PVC) has consistently been recovered as a monophyletic group in trees, based on different phylogenetic estimation methods (Wagner and Horn, 2006; Pilhofer et al., 2008; Kamneva et al., 2010). The phylum of Planctomycetes branches into two different classes; Phycisphaerae and Planctomycetia. These further branches into 4 different orders, 6 families, 35 genera and 58 described species, where 18 of these have the status as *Candidatus* (NCBI Taxonomy Browser, retrieved 6.5.18).

1.3.2 Distribution in the Environment

Since their first discovery, Planctomycetes have been observed in a wide range of terrestrial and aquatic habitats. These habitats include marine, hypersaline, hyperthermal, brackish and fresh water, as well as in many terrestrial environments including soils and acidic environments (Fuerst 1995; Neef et al., 1998; Schlesner, 1994; Lage and Bondoso, 2012). They have been found in biofilms on kelp and seaweed surface (Bengtsson and Øvreås, 2010; Fukanaga et al., 2009; Lage and Bondoso, 2011; Burke et al., 2011; Kim et al., 2016), and in association with several eukaryotic organisms like prawns, sponges and crabs (Fuerst et al., 1991, 1997; Pimentel-Elardo et al., 2003; Kohn et al., 2016). Planctomycetes have also been found in cave environments (Pašić et al., 2009; Borsodi et al., 2012; De Mandal et al., 2014), as well as in cold environments like glaciers, sea ice and on Mt. Erebus, Antarctica (Rysgaard and Glud, 2004; Zeng et al., 2013; Boetius et al., 2015; Tebo et al., 2015; Yang et al., 2016).

1.3.3 Physiology

Most Planctomycetes are chemoheterotrophic aerobes, and are considered oligotrophs that grow slowly (Fuerst, 2017). There have also been reports of Planctomycetes isolated and cultured anaerobically with sulphur, that probably ferment carbohydrates (Elshahed et al., 2007). The majority of Planctomycetes described in axenic culture are mesophilic, however, thermophile species have also been reported (Giovannoni et al., 1987; Slobodkina et al., 2016). One of these species are *I. pallida*, isolated from a hot spring, and has a maximum growth temperature of 55°C (Giovannoni et al., 1987). Several acidophilic strains have been reported from acidic wetlands in Russia (Kulichevskaya et al., 2007, 2008; Dedysh and Kulichevskaya, 2013). Among these are *S. acidiphila* and *S. paludicola* which are capable of growth at pH as low as 4,2. There are several reports of Planctomycetes inhabiting marine microalgae, which have been used as both a source of inoculum for isolation, and as growth media (Bengtsson and Øvreås 2010; Lage and Bondoso, 2014). They are believed to have a central role in algal heteropolysaccharide degradation including exopolysaccharides produced by other bacteria (Fuerst, 2017).

Some Planctomycetes are anammox bacteria and possess the ability to oxidize ammonium anaerobically and autotrophically (Kartal et al., 2012; van Niftrik and Jetten, 2012). These Planctomycetes possess anammoxosomes, compartments where the ammonium oxidation takes place and has been called a 'bacterial mitochondrion' (Jogler, 2014; Neumann et al., 2014).

A much wider diversity of Planctomycetes have been recorded by molecular methods than recorded based on axenic cultures (Yilmaz et al., 2015). This suggests that a much wider physiological diversity may be explored and applied in the future (Fuerst, 2017).

1.3.4 Cell Morphology

Members of the Planctomycetes division possess a number of morphological traits distinguishing them from other bacteria, such as non-prosthecate stalks, crateriform-like structures, budding reproduction and rosette formation (Fuerst, 1995). At the same time some species and strains display fimbria, fascicles and holdfast-structures (Fuerst, 1995).

Crateriform structures have been reported to be homogeneously distributed over the cell surface of Planctomycetes (Fuerst, 1995), and has an average opening of 35 nm, except around the neck where the budding happens (Santarella-Mellwig et al., 2013). Recently, nuclear pore-like structures have been found in *Gemmata obscuriglobus*. These pore-like structures were found to occur in internal membranes and are similar to the nuclear pores found in eukaryotes (Sagulenko et al., 2017).

Most Planctomycetes display a distinctive cell structure, where spherical, ovoid, ellipsoidal, tear-drop and pear-like shapes are the most common (Fuerst, 1995). Non-prosthecate stalks are observed in some species and are used as holdfast structures, either for the cells to adhere to a surface, or for cells to attach to one another and form rosette formations (Figure1.3 a and b) (Fuerst, 1995). Some Planctomycetes also display flagella which are often sheathed and originating from a cellular pole (Fuerst, 1995).

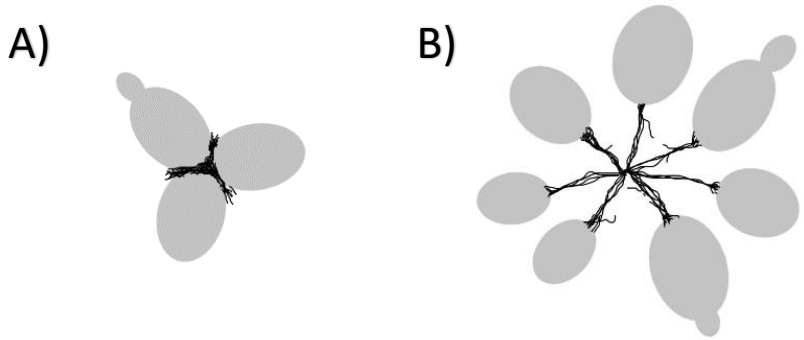


Figure 1.3: Planctomycetes cell arrangement**.** A) Cells attach to one another *via* non-prosthecate stalks and form rosette formations. B) Rosette formation with cells attaching to the surface *via* their stalks. Figures have been made based on data from Fuerst, 1995.

1.3.5 Cell Division and Life Cycle

One of the hallmark traits of the Planctomycetes are that they reproduce by a budding process (Fuerst, 1995). Most Planctomycetes have budding reproduction from a reproductive pole, although there have been reports of budding occurring from the side of the cell (Christian Jogler, personal communication, May 10, 2017). Planctomycetes are thought to lack the FtsZ protein (Glöckner er al., 2003), which are considered an important protein for binary fission (Margolin, 2000). As opposed to division by binary fission, budding reproduction is a process where a small bud forms at one end of the mother cell. As the bud grows, the size of the mother cell remains the same, while the bud enlarges. When the bud reaches about the same size as the mother cell, the bud detaches (Tekniepe et al., 1981). A significant difference to reproduction by binary fission is that the mother cell often has different properties than the bud. This is represented in Figure 1.4, where the life cycle of a typical Planctomycetes cell is illustrated. A Pirellula-like, flagellated cell attaches to a surface before budding reproduction follows, producing a flagellated swarmer cell (Franzmann and Skerman, 1984; Tekniepe et al., 1981).

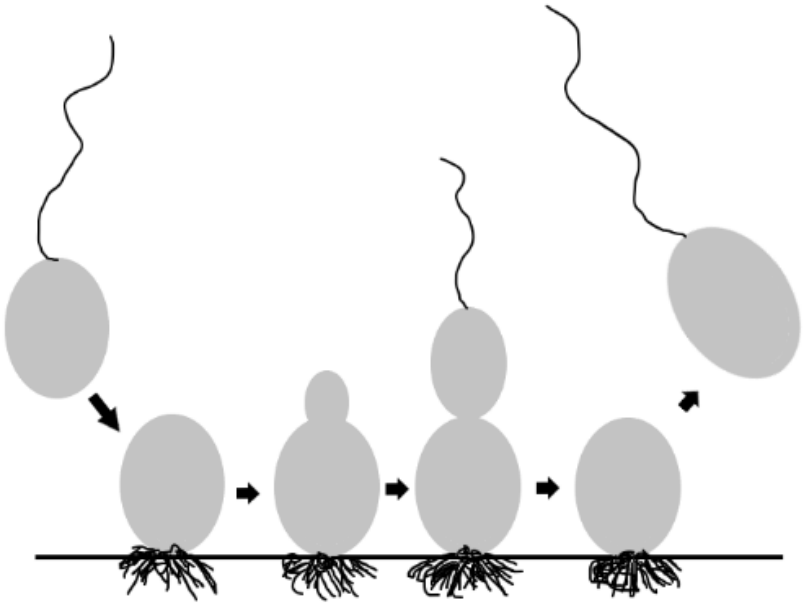


Figure 1.4: Generalized life-cycle for Planctomycetes. Mature swarmer cell fastens to surface by its holdfast-structure, followed by budding from a reproductive pole. The daughter cell matures and develops flagella, before budding is completed, and the daughter cell becomes swarmer cell. (Based on Fuerst, 1995; Franzmann and Skerman, 1984; Tekniepe et al. 1981).

In 2009, Lee and collaborates showed that the life cycle of Gemmata obscuriglobus is a complex process. The formation and development of the bud starts from a reproductive pole. As the bud grows, chromosomal nucleoid DNA is transported to the daughter cell. The transferred DNA is initially naked and not surrounded by a membrane. Eventually a complete nucleoid envelope is formed, consisting of two closely apposed membranes, deriving from the intracytoplasmic membranes of both the mother and daughter cell. Budding reproduction can be performed repeatedly by a single mother cell (Lee et al., 2009).

1.3.6 Cell Plan

Planctomycetes were thought to possess a compartmentalized cytosol, separated by an intracytoplasmic membrane that makes up the paryphoplasm and pirellulosome (Lindsay et al., 1997). However, this has been challenged by recent studies (Speth et al., 2012; Santarella-Mellwig et al., 2013; Boedeker et al., 2017). The planctomycetal cell plan is now thought to be of a more Gram-negative nature (Boedeker et al., 2017), than previously assumed (Fuerst and Sagulenko, 2011). Recent studies have shown that Planctomycetes possess a typically Gram-negative cell envelope with an outer

membrane, a peptidoglycan layer and a cytoplasmic membrane (Jeske et al., 2015). Their cytoplasm is an enclosed compartment in its entirety that follows the outer shape of the cell with invaginations of enlarged periplasm (Figure 1.5), rather than being fully compartmentalized (Boedeker et al., 2017). The only exceptions are anammox Planctomycetes, which possess anammoxosomes (Jogler, 2014). The invaginations of the periplasm have shown to be interconnected, however, the occurrence of the invaginations vary from cell to cell, and species to species (Boedeker et al., 2017). In 2013, Santarella-Mellwig and collaborates showed that the Planctomycete *Gemmata obscuriglobus* are not compartmentalized as invaginations in the membrane are not closed, but rather interconnected on a three-dimensional basis (Santarella-Mellwig et al., 2013).

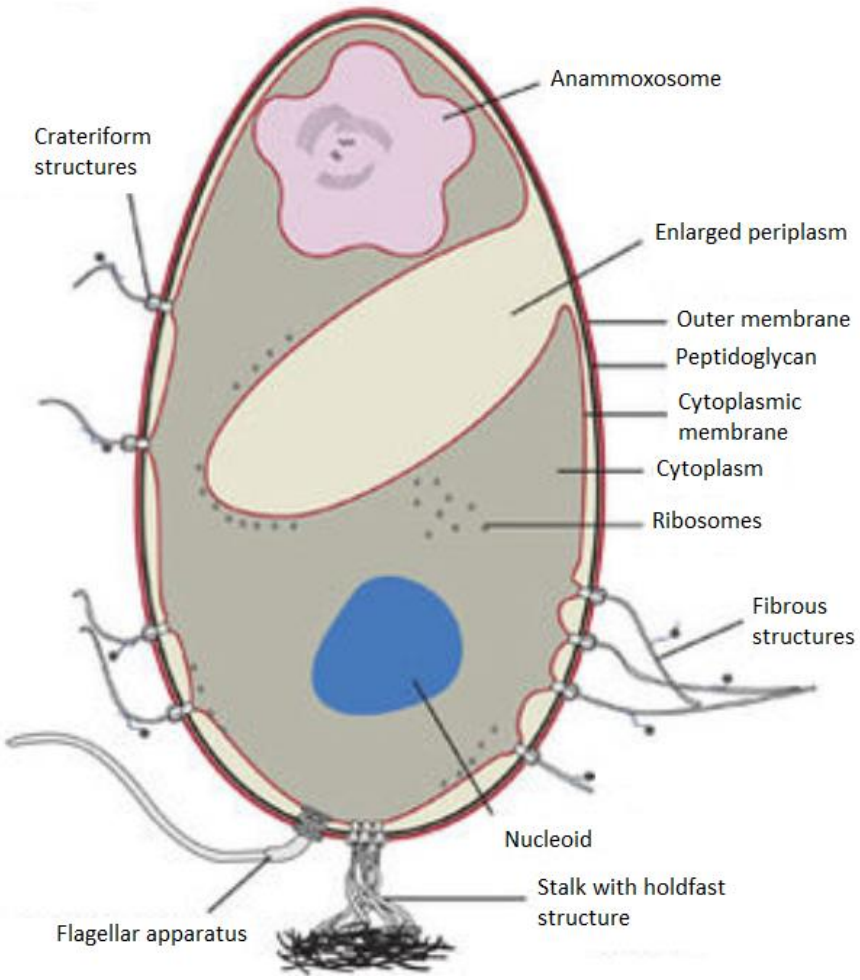


Figure 1.5: Overview of the planctomycetal cell plan**.** Showing tendency of massive invaginations. Crateriform structures, anammoxosome (only applicable for anammox Planctomycetes), enlarged peroplasm, outer membrane, peptidoglycan layer, cytoplasmic membrane, cytoplasm, ribosomes and fibrous structures. Source: Boedeker et al., 2017.

1.3.7 Cell Wall

The planctomycetal cell wall have been of much debate over the past decades. Initially Planctomycetes was thought to have a proteinaceous cell wall (König et al., 1984; Liesack et al., 1986), as well as lacking peptidoglycan and the ability to synthesize the FtsZ protein essential for cell division (Fuerst and Sagulenko, 2011; Jogler et al., 2012; Pilhofer et al., 2008). Planctomycetes were also suggested to support endocytosis-like uptake of macromolecules into the paryphoplasma of the species *Gemmata obscuriglobus* (Santarella-Mellwig et al., 2010), which would be the first occurrence of a vesicle-based uptake system outside the eukaryotic domain (Lonhienne et al., 2010; Jermy, 2010). This supplemented to the link between Planctomycetes and eukaryotes. In 2006, Wagner and Horn described Planctomycetes as Gram-negative bacteria, although it has been pointed out that their cell plan differs from the classical Gram-negative cell plan (Fuerst and Sagulenko, 2011; Devos, 2014). In recent years, peptidoglycan has been found in several Planctomycetes even though the bacterial cell division protein FtsZ is absent (van Teeseling et al., 2015; Jeske et al., 2015), questioning the previous consensus. Other studies have shown that it is possible for bacterial cell division in the absence of the FtsZ protein (Leaver et al., 2009), and peptidoglycan has also been found in Chlamydiae, which also lacks the FtsZ protein (Pilhofer et al., 2013). Recent bioinformatic studies and chemical analysis has proposed a more Gram-negative cell plan (Speth et al., 2012; Mahat et al., 2015). In 2017 an extensive study by Boedeker and collaborates, utilizing both bioinformatic and microscopic techniques, supported a more typical Gram-negative cell plan for Planctomycetes than previously assumed (Fuerst and Sagulenko, 2011). Where they emphasize that a Gram-negative cell plan challenges the existence of an endocytosis-like process (Boedeker et al., 2017). They point out that there still is a notable difference between the planctomycetal cell plan and a traditional Gram-negative one. Whereas most Gram-negative bacteria produce outer membrane vesicles (Schwechheimer and Kuehn, 2015), invaginations in their outer membrane and formation of periplasmic vesicles is stopped by its asymmetrical architecture and the peptidoglycan cell wall (van Teeseling et al., 2015; Jeske et al., 2015; Boedeker et al., 2017).

1.4 The importance of Planctomycetal Studies

Planctomycetes have been proposed to possess several traits of biotechnological value (Fuerst, 2017). Much due to their large genome sizes, as well and their ecological role. From an evolutionary point of view, Planctomycetes are especially interesting because of their deep phylogenetic branching in the bacterial domain (Fuerst and Sagulenko, 2011).

1.4.1 Potential Producers of New Antibiotics

We are entering an era were the need to find new 'natural' antibiotics to fight multidrug resistant pathogens is of the utmost importance. The discovery of new antibiotic compounds correlates to the phylogenetic distance between the microorganism under study and the known producers (Müller and Wink, 2014). This means that bacteria with a phylogenetically distinct lineage could be a good source for secondary metabolites and antibiotic compounds. The most potent antibiotic producers are also characterized by large genomes, often more than 8 MB, and complex life styles (Müller and Wink, 2014). Taking this into consideration, Planctomycetes could potentially be excellent producers of novel antibiotic compounds. Recent discoveries have proven that Planctomycetes are able to produce antibiotics and antifungal molecules and their potential in biotechnological applications have been shown through genome mining (Donadio et al., 2007; Jeske et al., 2013; Graça et al., 2016; Boedeker et al., 2017).

1.4.2 Role in Ecosystems Exploited for Biotechnological Purposes

As previously mentioned, Planctomycetes are found in a wide range of environments, and are of clear environmental importance as they are known to play key roles in global carbon and nitrogen cycles (Fuerst and Sagulenko, 2011; Kartal et al., 2013). This knowledge has led to exploitation of metabolic processes for biotechnological application. For example, the use of anammox Planctomycetes in wastewater treatment, where they anaerobically oxidize ammonium (Kartal et al., 2013). This has been utilized in large industrial scale bioreactors (van Niftrik and Jetten, 2012). Furthermore, the biotechnological application of planctomycetal enzymes such as sulfatases as biocatalysts has been demonstrated (Wallner et al., 2005). Also, Planctomycetes has been proposed as new models for cell complexity and activities,

and is considered a significant source for new lipids, enzymes, and for new types of waste remediation technology (Fuerst, 2017).

1.4.3 Potential for Anticancer Activity

Cancer is expected to become the leading cause of death in near future (Heron and Anderson, 2016). With both an increasing and aging population, new challenges on society emerges, in respect to the need for novel therapeutics with less harmful side-effects. The majority of new developed therapeutics are derived from natural compounds (Imhoff et al., 2011). Bacterial groups such as *Actinobacteria, Myxobacteria,* and *Cyanobacteria* are already well-known producers of bioactive secondary metabolites (Jeske et al., 2016). It is known that studying novel bacterial phyla can lead to the discovery of new bioactive compounds (Bredholt et al., 2008). Therefore, it seems reasonable that Planctomycetes could be a potential source of such compounds. In 2016, Jeske et al., showed that planctomycetal strains might be able to produce several secondary metabolites and postulated that Planctomycetes are an untapped source of novel bioactive molecules (Jeske et al., 2016). Planctomycetes have recently been proven to possess secondary metabolite genes and clusters that are related to pathways for production of various bioactive compounds, including antitumor compunds like epothilone (Graça et al., 2016).

1.4.4 An Understudied Division of Bacteria

Despite their importance for environmental microbiology, biotechnology and cell biology, few planctomycetal strains has been obtained as axenic cultures. Thus, from a phylogenetic point of view the phylum is very undersampled, and only a few representatives are taxonomically characterized in detail (Ward, 2010; Fuerst and Sagulenko, 2011). As previously mentioned, Planctomycetes has been reported both in cave environments and in frozen environments (Pašić et al., 2009; Borsodi et al., 2012; De Mandal et al., 2014; Rysgaard and Glud, 2004; Zeng et al., 2013; Boetius et al., 2015; Tebo et al., 2015; Yang et al., 2016). However, these studies have been based on the structure of the microbial communities and not being designated Planctomycetes studies. No Planctomycetes has previously been obtained in culture from these environments and no planctomycetes have been reported from an ice cave. Cultured organisms can provide a substantial amount of information about their role in the

environment, as well as making it possible for *in vitro* studies addressing their potential as producers of bioactive molecules.

2. Aims

The aim for this thesis was elucidate the presence and success of Planctomycetes in glacier cave ecosystems at the Svarthammarhola Ice Cave in Norway. For this purpose, three hypotheses were evaluated in more detail:

1) Planctomycetes are indigenous members of the glacier cave microbiome
2) Planctomycetes are active and alive members of the glacier cave microbiome
3) The production of secondary metabolites permits Planctomycetes to actively defend their habitats and resources towards other faster growing heterotrophic bacteria

3. Sample Material

The sample material used for this study was obtained from a glacier inside the cave Svarthammarhola (N67.13'E15.31' at 295 m a.s.l.) near Fauske, Norway (Figure 3.1). Samples were collected in October 2016 as a part of an expedition lead by Øvreås, Lauritzen and Purcarea affiliated to the CaveIce project (http://www.ibiol.ro/proiecte/Cavice/trips.htm).

The cave has two entrances as indicated by arrows in Figure 3.1. Near the lower entrance (245 m a.s.l) in a horizontal tunnel, a large ice mass is found, represented by the blue area in Figure 3.1. The supposed flat surface of the ice is found to be 3350 m^2, with a horizontal surface 160 m long and up to 25 m wide (Lauritzen et al., 2018). Samples for this study was gathered from this ice mass.

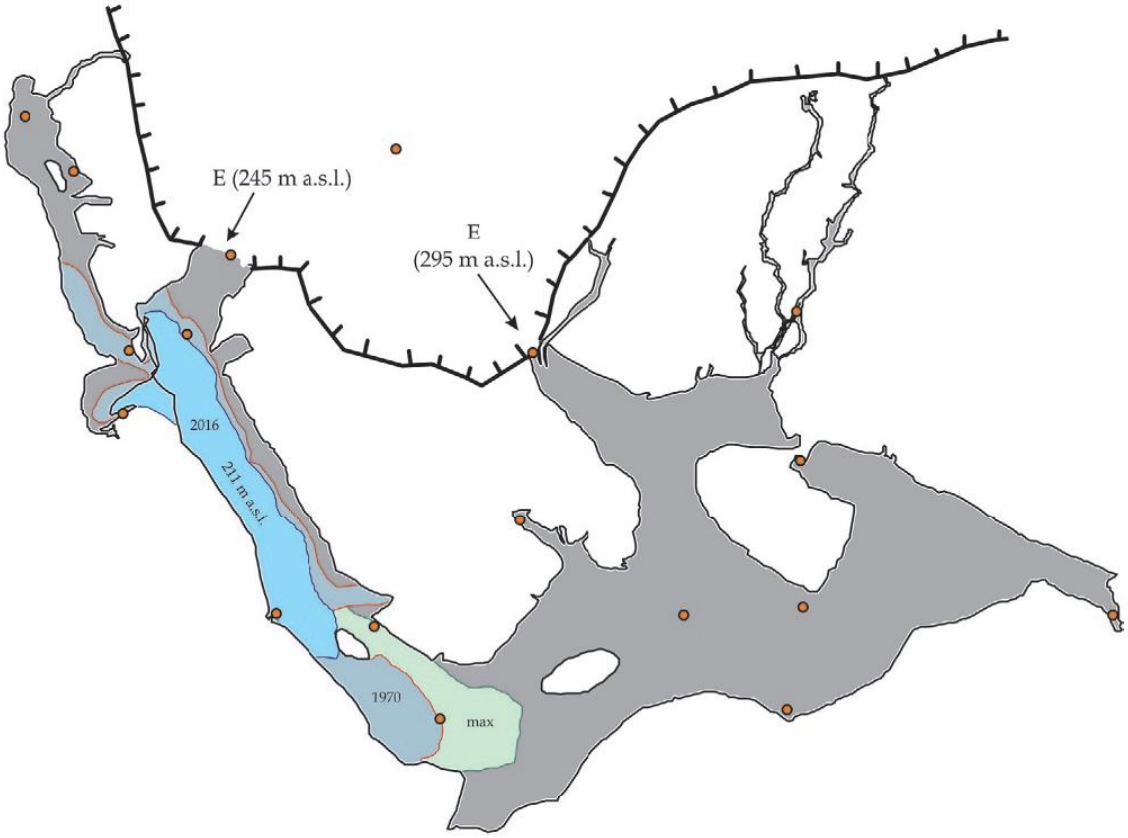


Figure 3.1: Svarthammarhola, plan. E: Major entrances. Red *dots*, data logger stations; ice block extents: *blue*, 2016 (Sampling site), 1970; *green*: oldest extent (of unknown age) as traved by cryogenic precipitates. Source: Lauritzen et al., 2018.

The ice mass was divided into 7 different layers and sample sites, based on prior knowledge about the stratification and dating of the ice (Lauritzen et al., 2018). In that

way the samples would represent a chrono sequence, ranging from oldest ice samples near the cave floor, and the youngest at the top of the ice mass (Figure 3.2).

Figure 3.2: Sampling site. Showing the foot of the ice mass and how the different layers in the ice was divided into sampling sites. Photo: Stein Erik Lauritzen.

Samples were named P1-7, whereas P1 was taken from the layer closest to the cave floor (Figure 3.2), thereby the oldest part of the ice. Sample P7 was taken from the layer closest to the top of the ice mass (youngest). An overview of the different elevations the samples were collected from, and the samples pH values are represented in Table 3.1

Table 3.1: Sampling details.

SAMPLE	ICE BLOCK ELEVATION (m)	ICE VOLUME (L)	pH
P1	0.45-0.63	5	8,92
P2	2.60 – 2.80	4	8,18
P3	3.77 – 4.06	3	6,8
P4	6.40 – 6.70	3	6,78
P5	7.64 - 7.90	3	8,75
P6	9.03 – 9.30	3	6,57
P7	12	3	6,44

3.1.1 Sample Collection

At the ice mass, samples were collected as ice cores. This was done by sterilizing the surface of the ice using a torch burner. Then, 10 cm of the surface ice was scraped off using an ice axe. The new surface was then sterilized by again using a torch burner. Ice cores were then drilled from the ice mass, using a modified power drill. The Power drill was modified with a hollow cylindrical drill bit, with an internal diameter of 17 mm and a length of 10 cm. In that way it was possible to retrieve the ice cores at the drill site. The equipment was sterilized using 100% ethanol and a flame between each sample being gathered. A flame sterilized spatula was used to remove the ice cores from the drill and directly into sterile plastic bottles. For each sample site 2 sterile plastic bottles were used (2 L and 1 L). The plastic bottles containing ice cores were kept in a cooling bag, were they would remain at temperatures below 0°C in the dark while they were transported to the lab at the University of Bergen. An average of 90 ice cores were collected from each sampling site.

At the lab, ice cores were thawed at 4°C in the dark. Upon thawing it was discovered that samples P2, P3 and P6 contained a lot of sediments and organic matter (Figure 3.3). Therefore, these samples were centrifuged, and the remaining pellets of sediments and soils was transferred to separate falcon tubes. Thus, dividing the samples into P2 and P2S, P3 and P3S, P6 and P6S whereas sample P2S, P3S and P6S contained the sediments. Also, sample P1 contained more sample material than the others, and was therefore split up into P1a and P1b.

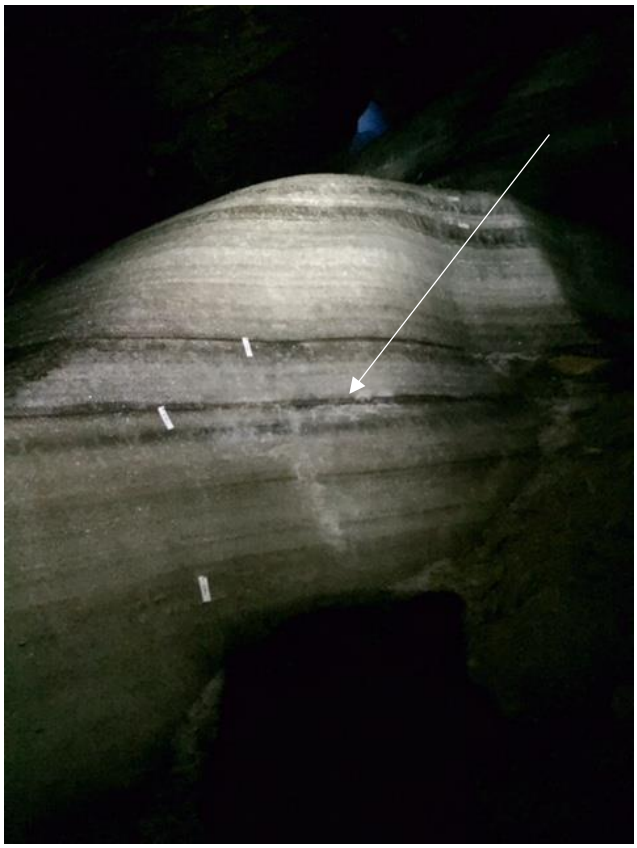


Figure 3.3: Sample site P2. This sample site was at the right side of the glacier. The location of this sample site was decided as this covers the first dominant organic band located at position 2,71 m (indicated by arrow). Photo: Stein Erik Lauritzen.

Five mL of sample was set aside for enrichment and isolation experiments, whilst the rest of the samples were used for filtering with Millipore® Sterivex™ filters (Sigma-Aldrich, Z359920), and then prepared for sequencing using Illumina 'MiSeq sequencing system'.

3.1.2 Sample Processing

Thawed samples were filtered through Millipore® Sterivex™ filters with a pore size of 0,2 µm. This was done by attaching the filters to tubes connected to a MasterFlex pump (Cole-Parmer, Chicago, IL, USA), and pump the samples through the filters. When the filtration was complete, the filters were stored at -80°C until further DNA extraction.

DNA and RNA were extracted from the filters using the Allprep RNA/DNA extraction kit from Qiagen (catalogue no. 80204), following the manufacturer's instructions. For samples P2S, P3S and P6S, which contained soils and sediments, the Mo Bio Power Soil

DNA isolation kit (catalogue no. 12888-100) was used, following the manufacturer's instructions.

Amplification of 16S rRNA gene for analysis of the microbial communities from the ice was done using the MiSeq v2 kit (Illumina Inc. CA, USA), with a two-PCR step approach. Finally, the samples were sent for sequencing analyses using the Illumina 'MiSeq sequencing system' (Illumina Inc. CA, USA) at the Norwegian High-throughput sequencing centre, Oslo (Norway).

3.1.3 Planctomycetes Presence in the Ice Mass

Sequencing of 16S rRNA obtained from thawed ice cores revealed that Planctomycetes was present in the ice (Figure 3.4). The highest abundance of Planctomycetes was found in samples P1a, P1b and P7, with approximately 10,5, 13 and 10,5% of the reads being of planctomycetal origin, respectively. The lowest abundances were found in samples P3 and P6, where approximately 2,1% of the reads was Planctomycetes. All samples were dominated by sequences belonging to the Planctomycetacia class, while Phycisphaerae was the second most represented class. The average of Planctomycetes reads in all samples were 6,4%. At the same time, the average for all the filtered samples (samples P1a-P7) was 6,6%, while the average for the samples containing soils (samples P2S, P3S and P6S) was 5,8%. This indicated a higher presence of Planctomycetes in the ice than in the organic layers.

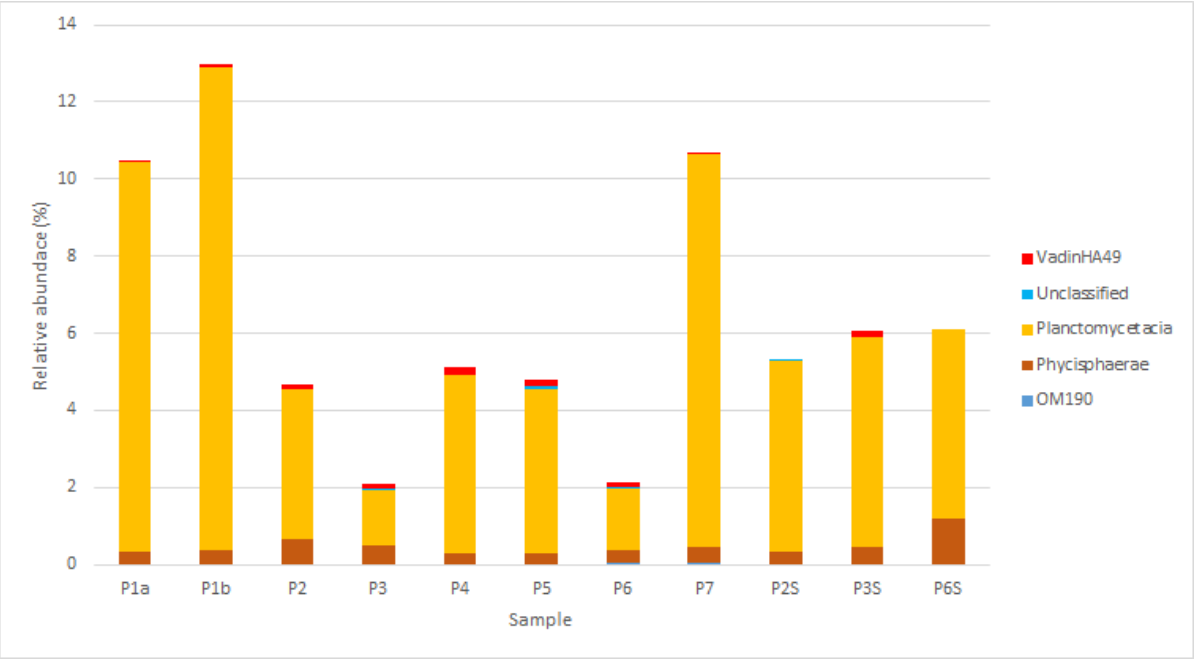


Figure 3.4: Showing relative abundance (%) for Planctomycetes classes in the different samples from Svarthammarhola ice cave. The highest abundance was found in sample P1b, where ~13% of the total reads were Planctomycetes. The lowest abundance of Planctomycetes was found in samples P3 and P6 (~2,1%). The most abundant class of Planctomycetes in all samples were Planctomycetacia.

4. Methods

From the initial sample material from Svarthammarhola ice cave, enrichments were prepared in culture media. If Planctomycetes-like cells were to be observed in the enrichment cultures, efforts were made to obtain them in axenic cultures. For proper diphasic characterization of novel Planctomycetes strains a variety of molecular and experimental techniques were used. Also, isolated strains were subjects to be screened for bioactive molecules as a part of a project involving Planctomycetes' ability to produce bioactive molecules and induce apoptosis in cancerous cell lines.

4.1 Enrichment Media

To stimulate growth of a broad range of Planctomycetes including terrestrial, marine and freshwater bacteria, M30 media containing 18% SW was prepared. M30 18% SW media is a modified version of M30 (Schlesner 1994), which is a common growth and isolation media used for Planctomycetes, where all the essential salts are provided. M30 18% SW media was prepared in two solutions, Solution 1 containing the liquid components, and Solution 2 containing the solid components. Solution 1 was prepared by adding the following (per litre) to a glass flask; 700 mL RO-water, 180 mL aged sea water, 20 mL Hutner's basal salts (Appendix I) (Cohen-Bazire et al. 1957), and 50 mL Tris buffer 0.1 M pH 7.5. The solution was then autoclaved. Solution 2 was prepared by dissolving 2 g/L N-acetyl-glucosamine, 200 mg/L ampicillin sodium salt, 10 mg/L Na_2HPO_4 x $2H_2O$, and 1 mL/L Vitamine solution no. 6 10x sol. (Appendix II) (Staley 1968), in 50 mL RO-water. Solution 2 was filtered through a 0,2 µm filter and then added to the autoclaved Solution 1. M30 18% SW media was stored at 5°C in the dark

When preparing gelrite plates for cultivation the same procedure was followed as for the liquid media, only by adding 5 g/L Gelrite (Gellan Gum) to Solution 1 before autoclaving. Solution 2 was then added to the autoclaved solution 1 and mixed, before 20 mL was poured into petri dishes. M30 18% SW plates were made under sterile conditions. Plates were stored upside down in sealed plastic bags at 5°C in the dark.

4.2 Enrichment and Isolation

Liquid from the thawed ice cores were added to glass reagent tubes with caps, containing 5 mL of liquid M30 18% SW media. Samples were added in dilutions of 1:10 and 1:100, hence, 500 and 50 µL of sample. Two parallels were prepared for each dilution, one kept at 10°C and one at room temperature in the dark. After inoculation the glass tubes were capped and wrapped with parafilm to prevent evaporation. The inoculations were carefully monitored by light microscopy (Leitz Wetzlar HM-LUX), looking for cells with Planctomycetes characteristics. When Planctomycetes-like cells were observed, 20, 30 and 50 µL of the inoculum was plated onto M30 18% SW plates (gelrite) to obtain single colonies. The same incubation temperatures were used as previously. The plates were examined daily and colonies that appeared were studied by light microscopy. If colonies that showed Planctomycetes-like characteristics appeared, these were re-streaked on fresh plates to get pure single colonies of Planctomycetes. Isolated strains were obtained by repeated colony picking and streaking on new solid media.

4.3 Characterization of Isolated Strains

To provide characterization of the strains' morphological and physiological attributes several experimental techniques were used.

4.3.1 Morphological Analysis

The external morphology of the isolated strains was examined using a Leica DM750 (Leica microsystems) phase contrast light microscope and photographed with a Leica EC3 camera (Leica microsystems) mounted on the light microscope. Phase contrast 3 was used under a magnification of 100 x. Cell size was determined by measuring cells using the same light microscope with a Leica eyepiece HC PLAN 10x/20 BR.M (Leica microsystems).

Internal structures were investigated by examining ultrathin cross sections viewed in a Jeol JEM-1230 TEM microscope and photographed using a Gatan Multiscan camera. Sample preparation and transmission electron microscopy was carried out at the Molecular Imaging Center (Fuge, Norwegian Research Council), University of Bergen. All isolated strains were cultivated on M30 18% SW gelrite plates, incubated at room temperature for 12 days. Samples were prepared for ultrathin cross sectioning by chemical fixation using, 1,5 – 2% glutaraldehyde in 0,1 M Na-cacodylate buffer, then 1% osmium-tetroxide in 0,1 M Na-cacodulate buffer was added, before being embedded in Agar 100 Resin. Ultrathin cross sections were cut and collected on grids, before being stained with 1% uranyl-acetate and Reynolds lead-citrate. Samples were then loaded into the TEM (Jeol JEM-1230) and photographed (Gatan multiscan camera).

4.3.2 Temperature Range and Optimum

Temperature optimum and growth range were measured to get a better understanding of the different strains most preferred growth conditions. This was obtained by cultivating the isolated strains in M30 18% SW at 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C. Regular M30 18% SW was used as a blank. One hundred mL of cell culture were centrifuged in an Avanti® J-26 XP (Beckman Coulter, IN, USA) using JA-12 rotors, for 15min at 9500 x g. The supernatant was discarded, and the pellet resuspended in 5 mL sterile dH_2O. M30 18% SW was then added to washed and autoclaved glass reagent tubes in volumes of 4,4 mL. Three parallels were prepared for

each isolate at each temperature. Cell suspension were added to the reagent tubes so that the optical density (OD) of the inoculated tubes resembled that of 0,05 at 600nm, as proposed by Hall et al (2014), using a Spectronic 21 spectrophotometer (Milton Roy). The tubes were gently vortexed after the cell suspension was added. Uninoculated tubes with M30 18% SW were used as a blank and to calibrate the spectrophotometer. The tubes were kept in separate incubators at their respective temperatures with shaking at 120 rpm. The cultures' OD were measured once every day using the same spectrophotometer. The tubes were gently vortexed before measuring OD to ensure proper homogeneity. The length of incubation varied between the different temperatures, depending on the time before the OD started to decline and the cultures entering the death phase. Growth curves were plotted in Microsoft Excel. Growth rates were calculated using the formula described in Friedrich Widdel's Theory and Measurement of bacterial growth [Equation 1 (Widdel, 2007)].

$$\mu = \frac{\ln OD_2 - lnOD_1}{t_2 - t_1} \quad (1)$$

Growth rate is represented in μ, the last and the first point in the exponential phase is represented in OD_1 and OD_2 with their corresponding time points T_1 and T_2. The data were then plotted in Microsoft Excell.

4.3.3 Salinity Range and Optimum

Salinity range and optimum were tested to gain insights to which NaCl concentrations the strains found most favourable for growth. Isolated strains were cultivated in M30 media where aged seawater was substituted with NaCl concentrations of 0%, 0,1%, 0,5%, 1%, 1,5%, 2%, 2,5%, 3% and 3,5% (w/v), and incubated at 25°C, with shaking at 120 rpm. The same procedure was the used for preparation and measurements as in the procedure used when measuring temperature range. Measurements were done daily for 23 days. The data was plotted in Microsoft Excel and growth rates were calculated using Equation 1.

4.3.4 pH Range and Optimum

pH range and optimum were tested to gain insights to which pH levels the strains found most favourable for growth. Isolated strains were cultivated in M30 18% SW modified using 1 M HCl and NaOH to adjust the pH. The pH of the media used ranged

from 5 to 9, with intervals of 0,5 measured by using a PHM210 Standard pH meter (Radiometer Analytical, CO, USA). The same procedure was then used for preparation and measurements as in the procedure used for measuring temperature range. Measurements were done daily for 23 days. The data was plotted in Microsoft Excel and growth rates were calculated using Equation 1.

4.3.5 Utilization of Carbon Sources

The different strains were examined for which carbon sources they could utilize, this was done to elucidate the strains ecological role, as well as to characterize them in more detail. All strains were cultivated in M30 18% SW, were N-acetyl-glucosamine was substituted with different substrates. In the absence of N-acetyl-glucosamine, 0,1 g/L $(NH_4)SO_4$ was added in order to supply nitrogen to the medium. The following substrates were added then (0,025% w/v): mannose, L-sorbose, D-raffinose, carboxymethyl cellulose, cellulose, D-arabinose, D-cellobiose, D-glucose, D-maltose, sucrose, D-fructose, D-galactose, L-rhamnose, chitin, D-mannitol and D-xylose. Media containing different carbon sources was pipetted into washed and autoclaved glass reagent tubes in volumes of 4,4 mL. One hundred mL of cell culture were centrifuged in an Avanti® J-26 XP (Beckman Coulter, IN, USA) using JA-12 rotors, for 15min at 9500 x g. The supernatant was discarded, and the pellet resuspended in sterile 5 mL dH_2O. Cell suspension were added to the reagent tubes so that the OD of the inoculated tubes resembled that of 0,05 at 600nm as proposed by Hall et al., (2014) when performing growth experiments. The OD of the cultures were measured by using a Spectronic 21 spectrophotometer (Milton Roy). The tubes were then gently vortexed and incubated at 25°C with shaking at 120 r.p.m. Three parallels were prepared for each strain for all carbon sources. Uninoculated tubes with M30 18% SW with no carbon source were used as negative controls and to calibrate the spectrophotometer. When evaluating growth in the tubes a Spectronic 21 spectrophotometer (Milton Roy) was used to measure OD. Growth measurements were performed every second day until day 8, and then on day 11, 14, 16 and 18. Measurements were plotted in Microsoft Excel.

4.3.6 Enzymatic Activity

The enzymatic activities of the isolated strains were analysed using API ZYM (bioMérieux, France) to gain insights to which enzymes the strains possess. API ZYM is a method developed for the study of enzymatic activities and are based on strips containing cupules with different substrates added. The different substrates allow for different enzymatic reactions to take place. Enzymatic activities are revealed in the strips by coloured reactions when reagents are added.

One hundred mL of cell culture grown in M30 18% SW was centrifuged at 9500 x g for 15min in an Avanti® J-26 XP (Beckman Coulter, IN, USA) using JA-12 rotors. The supernatant was discarded, and the pellet resuspended in 5 mL dH_2O to a turbidity of 5-6 McFarland (McFarland J, 1907) as described in the manufacturer's instructions. The wells in the incubation box were filled with MilliQ water to supply a humid atmosphere before the API ZYM strip were placed in the box. Further, 65 µL of cell suspension were pipetted into each cupule in 3 parallel strips and incubated at 25°C for 6 hours. After incubation, each cupule was added 1 drop of reagent ZYM A and ZYM B (bioMérieux, France). After 5 minutes the strips were placed under light and the strips were scored according to the manufacturer's instructions.

4.3.7 Antibiotic Resistance and Sensitivity

All isolated strains were tested for antibiotic resistance and sensitivity. Colonies were picked from M30 18% SW plates and prepared in a suspension with dH_2O to an inoculum density of 0,5 McFarland (McFarland J, 1907) as proposed in the manufacturer's instructions (Oxoid). Then, 50 µL of the inoculum was plated onto 25 mL M30 18% SW gelrite plates using a sterile glass rod to disperse the inoculum. One antibiotics disc was placed in the middle of each plate. The different antibiotics discs used was: ampicillin (10 µg), chloramphenicol (10 µg), kanamycin (30 µg), streptomycin (10 µg), penicillin G (10 µg), trimethoprim (2,5 µg), ofloxacin (5 µg), erythromycin (10 µg), vancomycin (5 µg), nalidixic acid (30 µg) and tetracycline (10 µg) (Oxoid Microbiology Products, Thermo Scientific). Plates were incubated at room temperature in the dark and inhibition zones were measured after 12 days of incubation.

4.4 Molecular Based Methods

The small subunit (SSU) ribosomal 16S rRNA gene is the most commonly used phylogenetic marker for microbial diversity studies (Rosello-Mora and Amann, 2001). The gene is highly conserved across the three domains and is the most commonly used evolutionary chronometer in microbial studies. The use of 16S rRNA gene allows for identification of the organism, which 16S rRNA gene is being studied, as well as evolutionary and phylogenetic comparison based on differences and changes in the 16S rRNA gene (Rosello-Mora and Amann, 2001).

4.4.1 Polymerase Chain Reaction

In order to gain insight to the phylogenetic relationships of the isolated strains, they were prepared for 16S rRNA gene sequencing. Colonies that showed Planctomycetes-like characteristics was transferred to PCR-tubes containing 10 µL PCR-water by using autoclaved toothpicks. The tubes were then kept on an 80°C heat block for 2 minutes and then on ice for 2 minutes. This was repeated two times in order to achieve proper cell lysis. The lysed cells were further used as template in the preparation of PCR product using Planctomycetes specific forward primers. One µL template were added to a mastermix containing the following reagents; 10 µL HotStarTaq DNA polymerase, 0,5µl BSA 100%, 6,5µl PCR water, 1 µL Pla46f 10µM primer (sequence 5' – 3', GGATTAGGCATGCAAGTC) (Neef et al., 1998) and 1 µL H1542r 10µM primer (sequence 5' – 3', AAGGAGGTGATCCAGCCGCA) (Pantos et al., 2003) per sample. PCR program for the samples were then run on a Veriti™ 96 well thermal cycler (Applied Biosystems, CA, USA), and is described in Table 4.1.

Table 4.1: PCR program for HotStarTaq DNA polymerase with Planctomycetes specific primers.

Step	**°C**	**Minutes**
1.	95	15:00
2.*	95	00:45
3.*	60	00:45
4.*	72	01:00
5.	72	10:00
6.	4	∞

**Steps 2 to 4 were repeated 30x.*

The expected PCR product should be around 1500 bp long. PCR products were visualized on a 1.5% agarose gel in Tris-acetate-EDTA. For DNA staining, 2 µL GelRed™ (10000x in H_2O. Biotium) was added to the gel. One µL GeneRuler 1kb DNA ladder (Thermo Scientific) was used as ladder, and 3 µL PCR product mixed with 6x loading dye was loaded into the wells. The gel was run at 150 V for 40 minutes in gel electrophorese, allowing the bands to separate properly. DNA fragments in the gel were visualized by using Bio-Rad ChemiDoc™ XRS+ (Bio-Rad, CA, USA) gel dock and compared to a GeneRuler 1kb ladder. If the samples showed clear amplicon they were selected for further purification of PCR product.

4.4.2 Purification of PCR Product and Preparation for Sequencing

PCR products were purified to ensure proper and clean DNA using Illustra™ ExoProStar™. Five µL of PCR product was aliquoted into PCR tubes and added 2 µL ExoProStar. PCR program for purification of PCR products with Illustra ExoProStar were run on a Veriti™ 96 well thermal cycler (Applied Biosystems, CA, USA) for 15 minutes at 37°C, followed by 15 minutes at 80°C.

Purified PCR products were then prepared for sequencing with BigDye 3.1. For each sample, 1 µL BigDye 3.1, 1 µL sequencing buffer, 3,2 µL primer (1 µM), 5 µL dH_2O and 2 µL purified PCR product was added. The PCR program used for sequencing preparation is described in Table 4.2.

Table 4.2: PCR program for sequence preparation for BigDye 3.1 sequencing

Step	**°C**	**Minutes**
1.	96	05:00
2.*	96	00:10
3.*	50	00:05
4.*	60	04:00
5.	4	∞

**Steps 2 to 4 were repeated 25x.*

The samples were then brought to the Sequencing Facility at the University of Bergen (http://www.uib.no/en/seqlab) for SANGER sequencing using a JANUS Automated Workstation, PerkinElmer & 3730xl DNA Analyzer (Applied Biosystems).

4.4.3 Phylogenetic Analysis

In order to investigate and compare the different strains similarities in the 16S rRNA gene, the sequences from the different strains were aligned and compared. Corresponding sequences (primers Pla46f and H1542r) were aligned and combined into a single contig using BioEdit v7.0.5.3 (Hall 1999) and MEGA v7.0.26 (Kumar et al., 2015). Alignment of the sequences were done using the ClustalW function in the BioEdit software (Hall, 1999; Hall 2007).

Merged sequences of 16S rDNA of isolated strains were analysed using the Nucleotide BLAST function available in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), to find the closest matching 16S rDNA sequences. The Nucleotide BLAST function was also used to compare different strains' 16S rRNA gene sequences for identities.

4.4.4 Phylogenetic Tree

A phylogenetic tree was made to compare and visualize the phylogenetic relationships of the strains and other sequences of planctomycetal descendance. The tree was built using MEGA v7.0.26 (Kumar et al., 2015). Sequences for isolated strains were aligned with their 3 most identical hits from BlastN as well as selected Planctomycetes species using the BioEdit software (Hall, 1999; Hall, 2007). This was done by using the ClustalW function (Thompson et al., 1994). The alignment was then proceeded to MEGA v7.0.26 and a maximum-likelihood tree was built with Tamura-Nei model (Tamura and Nei, 1993). The robustness of the tree was weighed using 100 bootstrap replications.

4.5 Screening for Bioactive Molecules

Two of the isolated strains from Svarthammarhola ice cave was used in a screening for bioactive molecules. This was done as a part of a conjoined project between the University of Bergen and the University of Porto, where the experiments took place at the Centre for Pharmacy, Department of Clinical Science, University of Bergen. In this project extracts from different strains of Planctomycetes from various habitats and environments, were examined for their ability to induce apoptosis in cancerous cell lines.

4.5.1 Preparation of Planctomycetes Cultures

The two strains were cultured for 7 days in 250 mL M30 18% SW at 25°C in a shaking incubator at 120 rpm. The cultures were then centrifuged at 10 000 x g for 15 minutes at 10°C using an Avanti® J-26 XP (Beckman Coulter, IN, USA) fitted with a JA12 rotor. The supernatant was discarded before the pellets were resuspended in sterile dH_2O, then centrifuged again. This was repeated twice. The pellets were freeze-dried in an Edwards Modulyo (IMA Life, Italy) for 18 h with a pressure of approximately 4×10^{-2} atm and a temperature below -40°C. The freeze-dried pellets were weighed and stored at -20° until further processing.

4.5.2 Preparation of Extracts for Cell Line Experiments

While being kept on ice, 6 mL of methanol: MiliQ water: chloroform (1:1:1) were added to each freeze-dried pellet in falcon tubes. First chloroform, then MiliQ and methanol mixed together. One falcon tube without any biomass was also added the mixture, to be used as an extraction control. The contents were then sonicated at 20 000 rpm in two cycles of 30 seconds to ensure homogenization, using a Tempest Virtishear I.Q sonicator (Virtis, Gardiner NY) fitted with a 10 mm rotor. The samples were cooled on ice in between each cycle. After proper homogenization, the samples were left to extract for 1 h at 4°C, including agitation after 30 minutes. The tubes were then centrifuged at 2000 rpm for 30 minutes, in that way allowing the samples to separate into the organic and the aqueous phases. The bottom phase (Organic) and the top phase (Aqueous) was carefully removed by pipetting and transferred to different Eppendorf tubes. The remaining contents were then centrifuged again at 10 000 rpm for 15 minutes, for further separation of the phases, before being

transferred to the rest of the extracts. All the extracts were evaporated to dryness in a vacuumed centrifuge (Eppendorf concentrator plus, Eppendorf AG, Hamburg, Germany). The evaporated extracts were then added 25 µL DMO for each 10 mg of initial freeze-dried biomass. For the aqueous extracts 75 µL of MilliQ water was also added per 10 mg of initial biomass.

4.5.3 Preparation of Cell Lines for Screening

The extracts were tested for their ability to induce apoptosis in 3 different cell lines. One being the normal epithelial rat kidney cell line NRK (ATCC no: CRL-6509), which would work as a control cell line for healthy normal cells as opposed to the two other cell lines. The two other cell lines were the human AML cell line Molm13 (Matsuo et al. 1997, Quentmeier et al. 2003) and the human prostate cancer cell line PC3 (ATCC no: CRL-1435). Molm13 cells were cultured in RPMI medium (Sigma R5886), added 10% (w/v) fetal calf serum (Sigma F7524) and 0,2 mM L-glutamine added 50 IU/mL penicillin and 0,1 mg/mL streptomycin. Culturing density was about 8 – 80 x 10^4 cells/mL and adjusted by adding fresh medium. NRK and PC3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM – Sigma D-6429), added 10% (v/v) fetal calf serum (Sigma F7524) and 0,2 mM L-glutamine added 50 IU/mL penicillin and 0,1 mg/mL streptomycin. NRK and PC3 cells are adherent cells, and at 90% confluence, the cells were detached by mild trypsin treatment. This was done by discarding of the existing medium, leaving only attached cells, before washing the cells two times with 5 mL PBS. The cells were then added 2 mL PBS and 6 mL trypsin. The cells were then left in the incubator at 37°C for 5 minutes. The detached cells were then resuspended carefully in 6 mL of fresh medium, before being transferred to a 15 mL Corning tube. The tube was then centrifuged at 150 x g and the supernatant discarded, before being reseeded in fresh medium with supplements at 40 – 50% confluence. All the cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2. All media, serum, supplements and reagents were from Sigma-Aldrich, St. Louis, MO, USA.

4.5.4 Cytotoxic Assays Using Planctomycetes Extracts

In 96-well plates, 100 µL cell suspension containing 6000 cells of NRK or PC3 cell line were added to each well, 24 h prior to the addition of the extracts. In that way, the adherent cells could attach to the substratum. After 24 h the medium was discarded carefully by pipetting, before adding 99 µL of fresh medium to the wells for organic extracts and 96 µL fresh media to the wells for aqueous extracts. At the same time suspensions containing 8 x 10^4 cells/mL of Molm13 cell line was added to 96-well plates. This was done in volumes of 99 µL in wells for organic extract treatment, and 96 µL in wells for aqueous extracts. For all the cell lines 1 µL of the organic extracts and 4 µL of the aqueous extracts were added respectively, to 99 µL and 96 µL of cell culture. DMSO was used as a solvent control in the same volumes as in the extracts. A negative control of 100 µL cell culture was also prepared for each cell line. The spaces between the wells were then filled with sterile dH_2O to avoid evaporation in the wells. There were 3 parallels for each extract treatment prepared for the NRK and PC3 cell lines and 4 parallels for the Molm13 cell line. All parallels were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 72 h.

The viability of the cells was first monitored using the WST-1 assay metabolic assay (Roche diagnostics, Catalogue number 11644807001, Germany) following the manufacturer's instructions. Each well containing cells and extracts was added 10 µL of the WST-1 reagent, that was previously heated to 37°C. All plates were then placed back into the incubator for 2 h. After incubation, the plates were examined using a multiwell plate reader with an absorbance of 450 nm and a reference set to 620 nm.

After WST-1 assays were performed, the wells were added 100 µL fixator, containing 2% buffered formaldehyde (pH 7,4) added 0,01 mg/L of the DNA-specific fluorescent dye, Hoechst 33342. The plates were then left at room temperature for 1 h in the dark. Each well in the 96-well plates were then examined using a Nikon Diaphot 300 fluorescent microscope fitted with a 40x Flu-Phase contrast lens and a DS-Fi3 camera, as described by Prestegard et al (2009). The morphology and the nuclei of the cells were of special interest, were a condensed nucleus with strong fluorescence would represent an apoptotic cell. Three hundred cells from each well were counted and used to determine cell death. To calculate an apoptosis percentage in each well, the

negative control was taken into consideration. Equation 2 was used to calculate the apoptosis percentage with respect to those cells that could have died by natural causes.

$$x - \left(y \left(\frac{100 - x}{100 - y} \right) \right) \quad (2)$$

Were x being the counted number of apoptotic cells out of 100 cells and y is the counted number of apoptotic cells per 100 cells in the negative control.

5. Results

As described in Sample Material (section 3), the ice mass in Svarthammarhola was revealed to host Planctomycetes. Samples P1a, P1b and P7 displayed the highest abundance of Planctomycetes, while samples P3 and P6 had the lowest abundance (Figure 3.4).

Isolated strains from initial sample enrichments were subject to diphasic characterization utilizing both molecular and experimental methods. As well as being screened for bioactive molecules by treatment of cancerous cell lines with extracts from the strains.

5.1 Isolation of Strains

All enrichments were thoroughly examined by phase contrast light microscopy, searching for cells with Planctomycetes characteristics, like budding reproduction and rosette formation. Several potential isolates were targeted but failed to be isolated and characterized. At the end 4 different isolates were obtained from 3 different enrichment cultures inoculated with liquid from the thawed ice cores.

5.1.1 Enrichment Culture P2S

Strain P2S was isolated from sediments and soils from sample P2 inoculated in M30 18% SW. After 20 days of enrichment, planctomycetes-like cells were observed in enrichment culture P2S, 1:10 dilution, incubated at room temperature. In this inoculation yeast like cells were also observed. After the initial enrichment, culture was plated on M30 18% SW plates, and pale pink colonies appeared. By inspecting these colonies by phase contrast light microscopy, they seemed to contain both Planctomycetes-like cells and cells resembling eukaryotic yeast cells, as shown in Figure 5.1. The Planctomycetes-like cells showed signs of budding and rosette formation (Figure 5.1). Sequencing using Planctomycetes specific forward primer (Pla46) for the 16S rRNA gene of the colonies confirmed that it was indeed cells of planctomycetal origin. The colonies were re-streaked onto new fresh media until the colonies were pure and separated from the yeast-like cells. When the strain appeared in pure culture and no yeast-like cells were to be observed, the colour of the colonies

seemed more white and pale than previously, as well as having a creamier consistency. The strain was named after the sample origin, thus, naming it strain P2S.

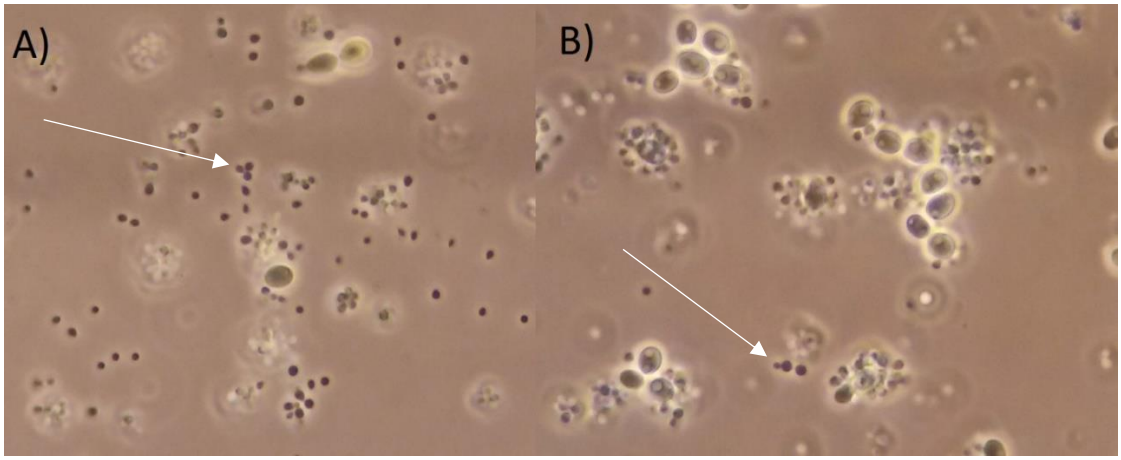


Figure 5.1: Showing phase contrast light microscopy images of Planctomycetes-like cells, as well as eukaryotic yeast-like cells from enrichment culture P2S, plated on M30 18% SW plates. A) Arrow indicating rosette formation in Planctomycetes-like cells. B) Arrow indicating Planctomycetes-like cells expressing budding reproduction.

5.1.2 Enrichment Culture P6

After 23 days of enrichment planctomycetal-like cells were observed in 2 of the parallels of enrichment P6, in both parallels kept at 10°C, 1:10 dilution, and at room temperature, 1:10 dilution. There were only few cells in each parallel, but those observed showed clear signs of rosette formation and the cells displayed the characteristic Planctomycetes ovoid cell shape. Enrichments were plated onto fresh M30 18% SW plates and pinkish red colonies appeared on the plates from both parallels after 10 days. Sequencing of the 16S rRNA gene revealed that both isolates were affiliated with Planctomycetes. Colonies were re-streaked until pure strains were obtained. The strains isolated from sample P6 kept on room temperature was named strain P6.1, and the strain isolated from sample P6 kept on 10°C was named P6.2. Strain P6.2 formed very dense colonies appearing to resemble that of a biofilm when observed with phase contrast microscopy, whilst strain P6.1 formed smaller aggregates of cells which were not as dense as recorded for strain P6.2. On M30 18% SW plates strain P6.2 formed large rubber-like colonies with a clear red colour, that were hard to pull apart, whereas P6.1 formed smaller colonies with a pink colour.

5.1.3 Enrichment Culture P1a

Planctomycetes-like cells appeared in enrichment culture P1a kept at room temperature, in the 1:100 dilution, after 173 days incubation. The enrichment was clearly dominated by cells with Planctomycetes characteristics showing clear ovoid-shaped cells and signs of budding reproduction, as well as rosette formations. Enrichments were plated on fresh M30 18% SW plates and 16S rRNA gene sequencing of the colonies showed that the colonies were of planctomycetal origin. Colonies were re-streaked on fresh media until pure, and named after the sample of origin, thus strain P1a. The strain formed pale grey-white colonies with creamy consistency on plates.

5.1.4 Other Enrichment Cultures

Other enrichment cultures also seemed to contain cells with Planctomycetes characteristics. These include enrichment culture P3 kept at room temperature, dilutions 1:10 and 1:100, and enrichment culture P2 kept at room temperature, dilution 1:100. However, when these were plated onto fresh plates of M30 18% SW, no colonies appeared in the gelrite plates. Plating onto new fresh plates were tried repeatedly, however no colonies appeared. Therefore, no further attention was given to these enrichment cultures.

5.2 Cell Morphology

Both internal and external cell morphology and structure was examined for all 4 strains. Examination was done both by phase contrast light microscope and by transmission electron microscope.

5.2.1 Strain P1a

When in pure culture, colonies on solid media of strain P1a appeared grey-white in colour with a creamy consistency. In liquid media the strain appeared as a homogenized cell suspension, however when entering the stationary phase of culture growth, the cells tended to aggregate into flakes that appeared grey-white in colour and was suspended in the culture.

When studied under by phase contrast light microscopy cells of strain P1a appeared to group together in large cell aggregates and consortia, in rosette formations or as singular swarmer cells, as shown in Figure 5.2a. Cells of strain P1a are spherical to ovoid in shape and exhibit budding reproduction, however it is unclear whether budding happens from a reproductive pole (Figure 5.4b). The cell size ranged from 0,7 - 1,2 µm x 0,7 - 0,9 µm and the average size of the cells was calculated to be 1,0 x 0,8 µm.

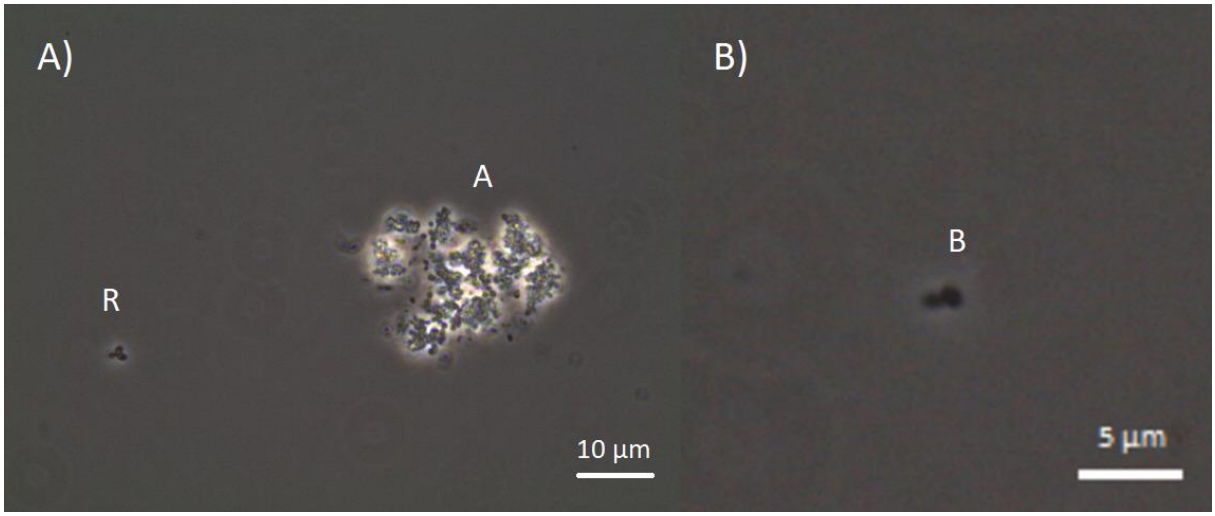


Figure 5.2: Phase contrast light microscope images of strain P1a. A) Showing cell aggregates (A) and cells arranged in rosette formation (R). bar 10 µm. B) Showing budding cell (B), budding does not seem to happen from a reproductive pole. bar 5 µm.

Ultrathin cross sections of the strain studied by transmission electron microscopy showed that strain P1a had cells arranged in rosette formations (Figure 5.3a).

Furthermore, cells showed signs of budding, however, this did not seem to happen from a reproductive pole (Figure 5.3b). TEM analyses showed that budding occurred from the side of the cell, rather than from one of the poles. The daughter cell seemed to already have genetic material transferred. Strain P1a displayed large invaginations of the periplasm (Figure 5.3c), and the invagination seemed to be surrounded by a membrane. Fibrous structures were also observed in strain P1a (Figure 5.3d), as well as large crateriform structures.

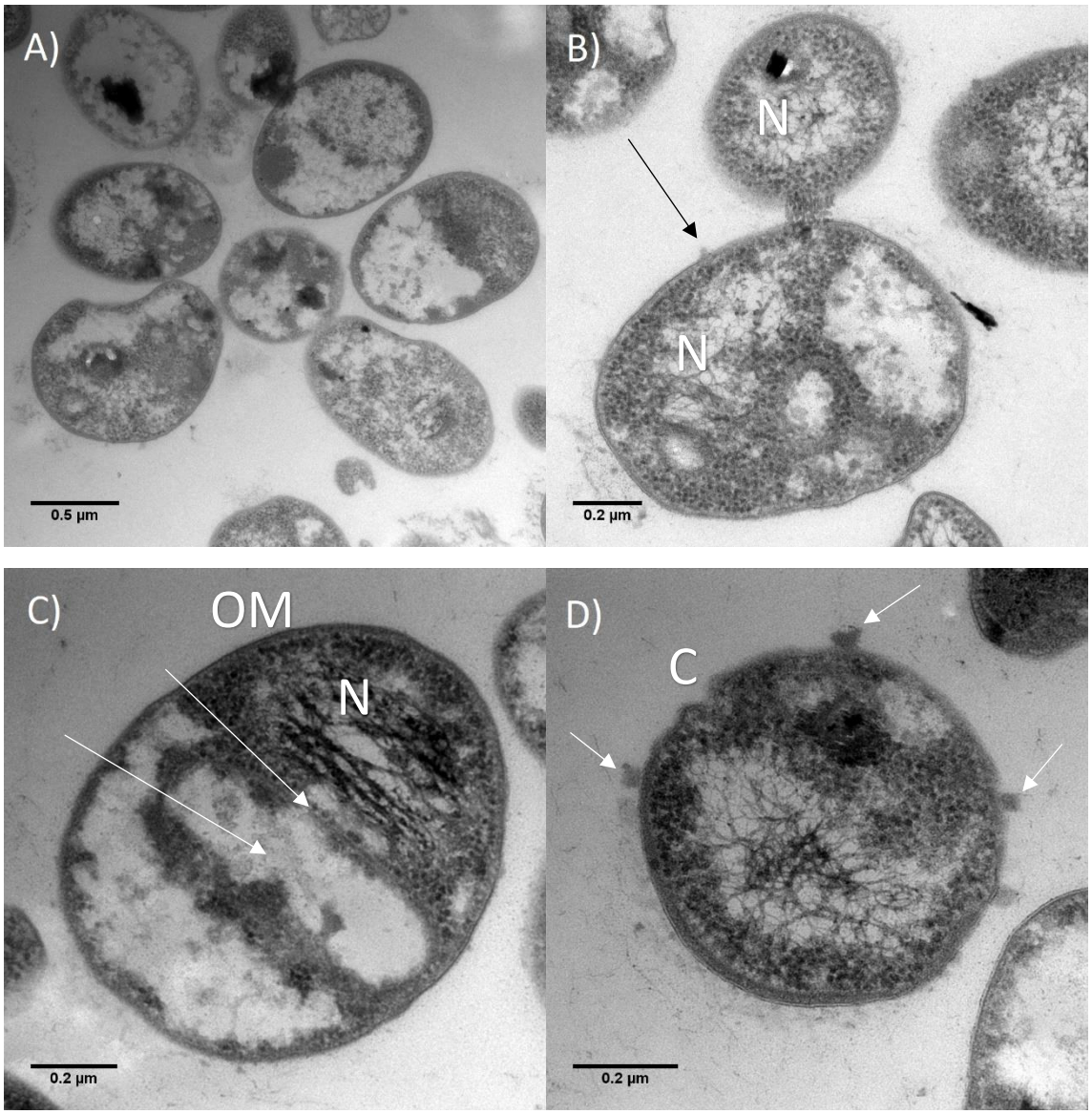


Figure 5.3: Transmission electron micrographs of ultrathin sections of strain P1a. A) Showing cells arranged in rosette formation. bar 0,5 µm. B) Showing budding cell with highly condensed nucleotide (N) transferred to the daughter cell, fibrous structure (arrow). bar 0,2 µm. C) Showing cell of strain P1a with highly condensed nucleotide (N), outer membrane and invaginations of periplasm (arrow) surrounded by a membrane (arrow). bar 0,2 µm. D) Showing cell with fibrous structures (arrows) and large crateriform structure (C). bar 0,2 µm.

5.2.2 Strain P2S

On M30 18% SW plates strain P2S displayed white colonies with creamy consistency that was easy to pick from the plate and to homogenize in liquid culture. When grown in liquid media the cells appeared to be dispersed throughout the culture giving a white transparent colour to the entire culture.

When studied under phase contrast microscope strain P2S appeared as single motile swarmer cells or in rosette-like formations of no more than 3 cells (Figure 5.4 a and b). Cells were spherical and ovoid shaped and showed sign of budding from a reproductive pole (Figure 5.4c). Cells in rosette formation and occurrences of two swarmer cells seemed to be interconnected *via* tubular-looking structures (Figure 5.4 b and d). Strain P2S did not show any signs of rather large aggregations of cells, when most cells appeared as single swarmer cells and in rosette formations of 2 and 3 cells. Cells size were measured to range from 0,6 - 1,4 µm x 0,7 - 1,6 µm and the average size of the cells was calculated to be 1 x 1,1 µm.

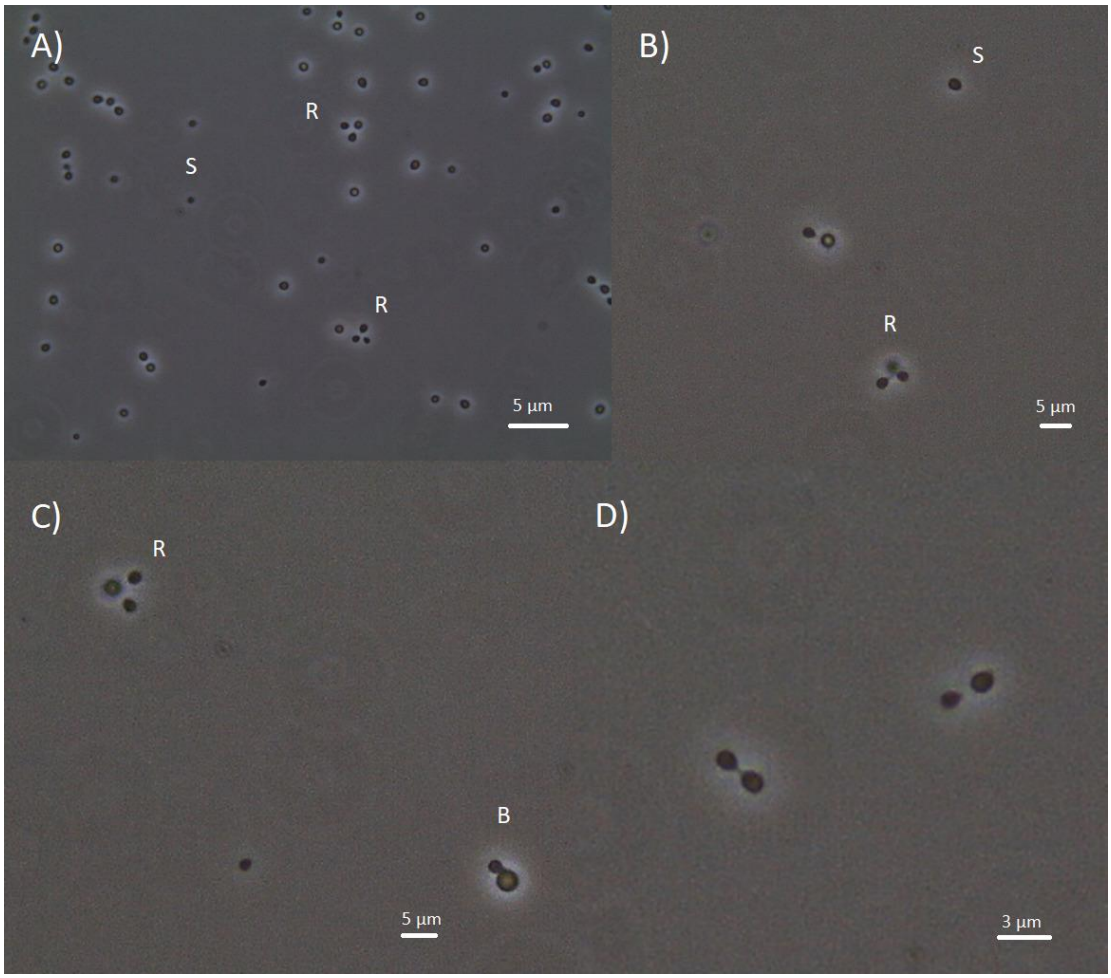


Figure 5.4: Showing phase contrast light microscope images of strain P2S. A) Cells appearing as swarmer cells (S), or in rosette-like formations (R) of 2 or 3 cells. bar 5 µm. B) Showing cells as swarmer cells (S) and in rosette-like formation (R). bar 5 µm C) Showing budding cell (B) and cells in rosette-like formation (R). bar 5 µm. D) Showing cells interconnected by tubular-looking structure (arrow). bar 3 µm.

Ultrathin cross sections of strain P2S revealed that the strain exhibited highly condensed nucleotides surrounded by an electron dense membrane, indicating the presence of ribosomes (Figure 5.5). The strain displayed a clearly defined outer membrane, as well as large invaginations of the periplasm that were surrounded by a membrane (Figure 5.5 a and b). Figure 5.5b shows that the strain exhibited fibrous structures along the outer membrane.

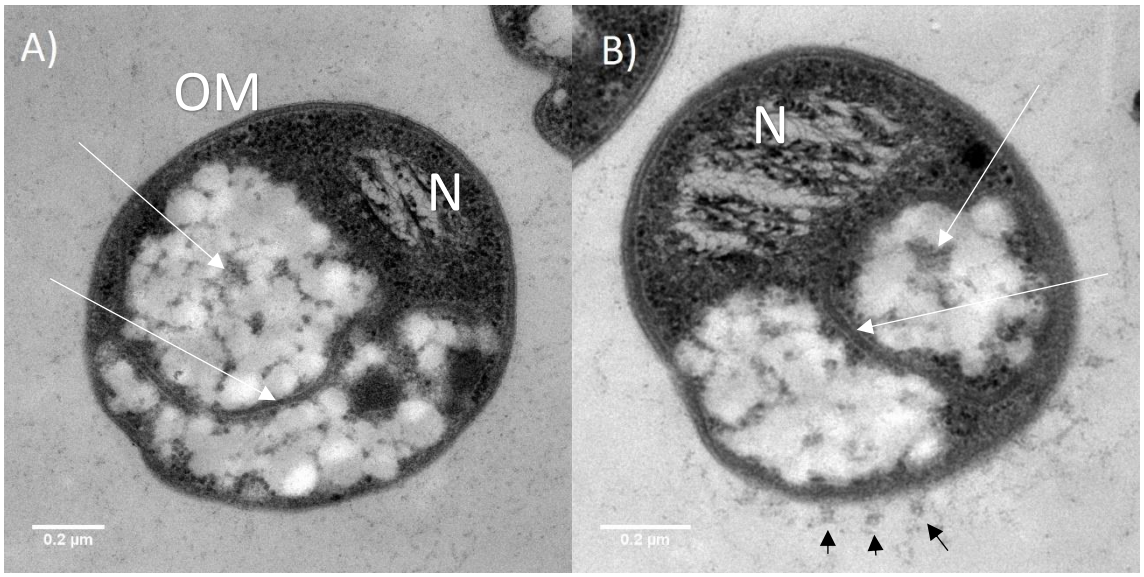


Figure 5.5: Transmission electron micrographs of ultrathin sections of strain P2S. A) Showing cell with highly condensed nucleotides (N) surrounded by electron dense membrane containing ribosomes, as well as a clearly defined outer membrane (OM), Large invaginations of the periplasm (arrow) surrounded by a membrane (arrow). bar 0,2 µm. B) Showing cell with highly condensed nucleotides (N) and invaginations of the periplasm (arrow, white) surrounded by a membrane (arrow, white). Fibrous structures along the outer membrane indicated by black arrows. bar 0,2 µm.

5.2.3 Strain P6.1

On solid culture media strain P6.1 formed small pink colonies with rubber-like consistency. In liquid culture the strain formed pink aggregates of cells that either sunk to the bottom of the container or attached to the container at the surface of the liquid media.

Colonies from plates examined by phase contrast light microscopy showed that cells gathered in large aggregates as well as displaying single swarmer cells (Figure 5.6a). The cells appeared to have spherical to ovoid shapes and swarmer cells were not motile. Budding reproduction was displayed, as shown in Figure 5.6b, and it seems like the budding happens from a reproductive pole. The cell size ranged from 0,6 - 1,2 µm x 0,7 x 1,4 µm and the average size of the cells was calculated to be 0,9 x 1,1 µm.

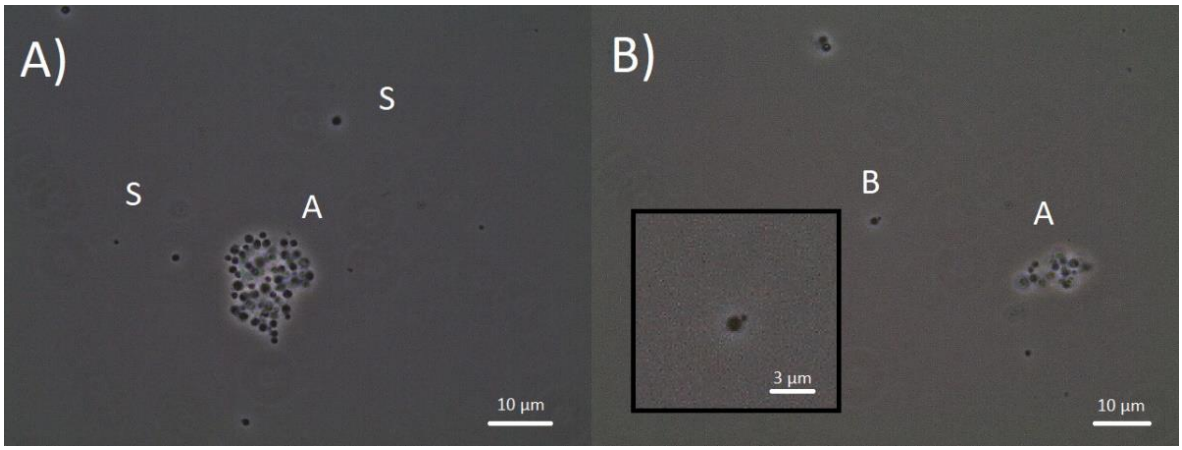


Figure 5.6: Phase contrast light microscope images of strain P6.1. A) Showing aggregate of cells (A) and single swarmer cells (S). bar 10 µm. B) Showing budding reproduction (B) and from what seems to be a reproductive pole, and aggregation of cells (A). bar 10 µm and 3 µm for the zooming in on the budding.

TEM analyses of ultrathin sections revealed some cells were arranged in a rosette-like formations (Figure 5.7 a and c). The cells appeared to be attached to a joined holdfast structure in the centre of the rosette forming cells. Shapes varied from spherical to oval. Most of the cells also showed fibrous structures on the outside of the outer membrane (OM), as well as highly condensed nucleotides within a nucleoid. The majority of the cells displayed an area of low electron density between the OM and the cytoplasm, the periplasm. Crateriform structures was observed in many cells (Figure 5.7b) in connection to the fibrous structures. The cell displayed in Figure 5.7 b and d, shows a budding cell, were the genetic material already has been transferred from the

mother cell. The budding seems to be happening from a reproductive pole and the bud appears to be ready to detach, considering the already established OM, nucleotides and cytoplasm.

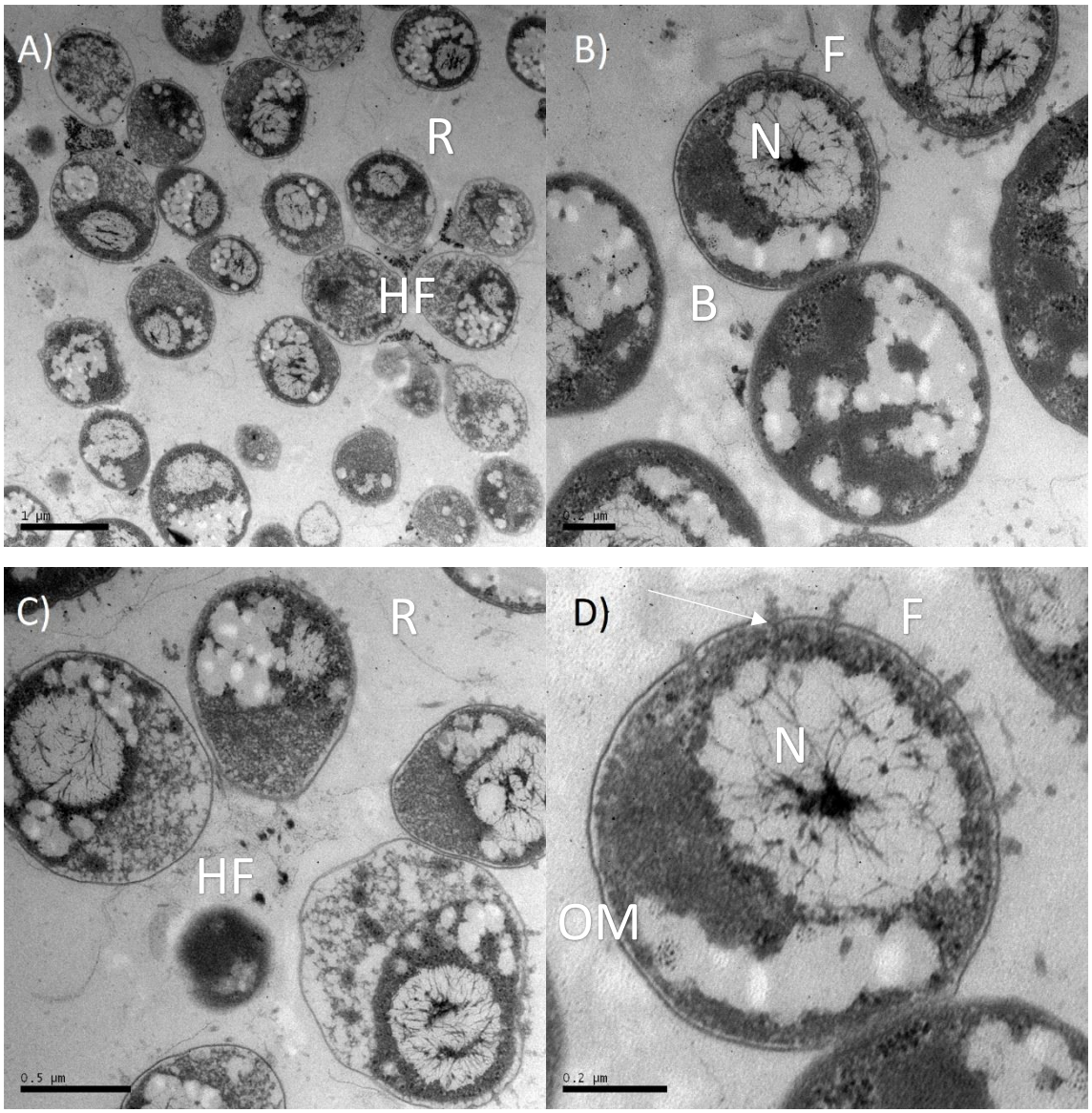


Figure 5.7: Transmission electron micrographs of ultrathin sections of strain P6.1. A) Cells showing rosette formation (R) and holdfast structures (HF). bar 1 µm. B) Budding cell, daughter cell showing highly condensed nucleotide (N), fibrous structures (F) and outer membrane (OM), as well as the budding itself (B). bar 0,2 µm. C) Rosette formation of cells (R) via holdfast structures (HF). bar 0,5 µm. D) Budding cell, crateriform structures (arrow) affiliated with fibrous structures (F), outer membrane (OM) and highly condensed nucleotides (N). bar 0,2 µm.

5.2.4 Strain P6.2

Colonies on M30 18% SW plates appeared as big red rubber-like clumps on M30 18% SW plates. The colonies were hard to pick from the plates and difficult to homogenize when transferred to liquid media. When the strain was grown in liquid culture, the cells aggregated and tend to gather at the bottom of the container. It also occurred that the cells started to grow and attach to the container, along the surface of the liquid media.

Cells of strain P6.2 were spherical to ovoid shaped and expressed large cell aggregates with high cell density as well as swarmer cells in liquid culture (Figure 5.8a). Some cells were arranged in rosette-like formations of a few cells, as shown in Figure 5.8a, and some cells exhibited budding reproduction from a reproductive pole (Figure 5.8b). The cell size ranged from 0,8 - 1,4 µm x 0,8 - 1,6 µm and the average size of the cells was calculated to be 1,1 x 1,3 µm.

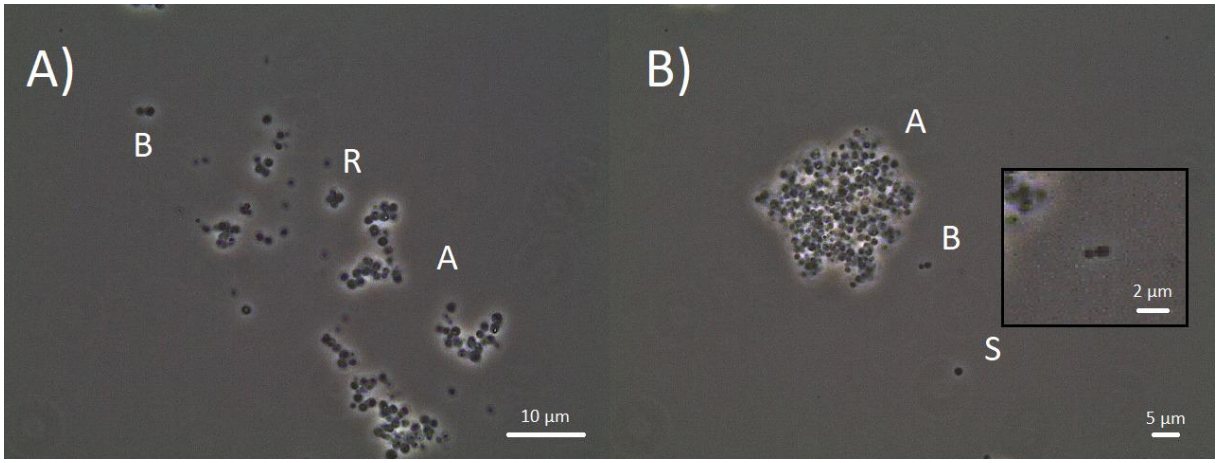


Figure 5.8: Phase contrast light microscope images of strain P6.2, magnification 100x. A) showing budding reproduction (B), rosette formation (R) and cell aggregates (A). bar 10 µm. B) Showing aggregation of cells (A), budding reproduction (B) and single swarmer cells (S). bar 5 µm, and 2 µm for the zooming in on the budding.

As shown in Figure 5.9 a and b, strain P6.2 had cell shapes of a spherical to ovoid nature and showed a clearly defined nucleoid with highly condensed nucleotides, surrounded by an electron dense membrane, resembling ribosomes. The cells displayed a clearly defined outer membrane (OM), with periplasm in the area of low electron density between the OM and the cytoplasm. Crateriform structures was

observed in connection to fibrous structures (Figure 5.9a). Ribosomes seemed to be concentrated around the nucleoid, as well as around invaginations of the periplasm and along the cytoplasmic membrane, indicated by arrowhead in Figure 4.9b.

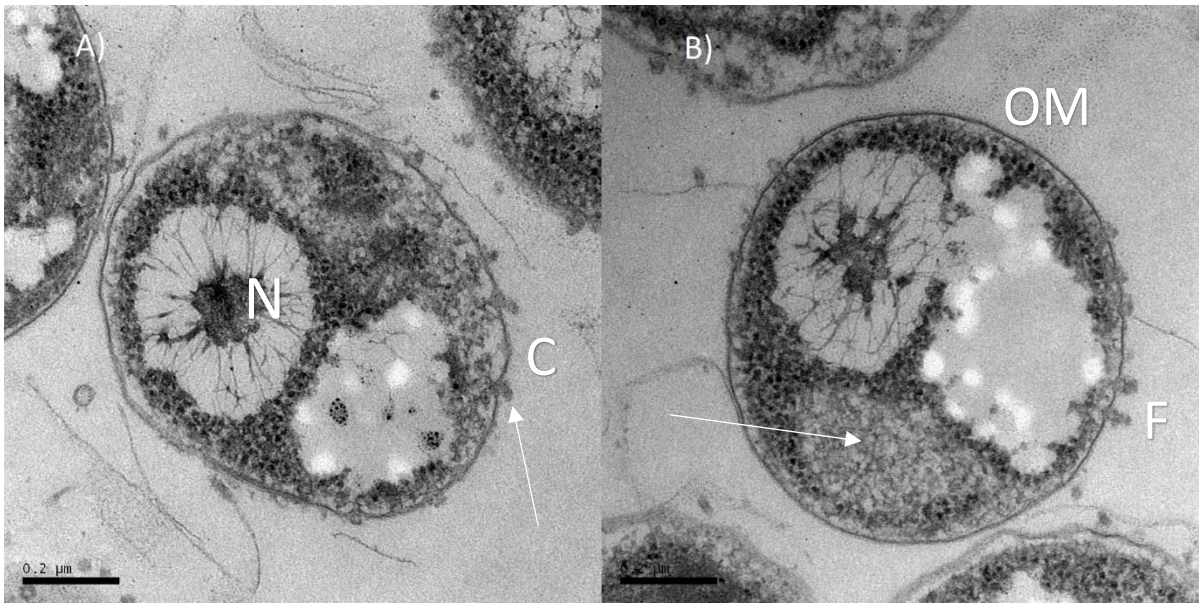


Figure 5.9: Transmission electron micrographs of ultrathin sections of strain P6.2. A) Showing cell of strain P6.2 with highly condensed nucleotides (N), crateriform structures (C) connected to fibrous structures (arrow). bar 0,2 µm. B) Cross section of cell showing a clearly defined outer membrane (OM), fibrous structures (F) along the OM and large invaginations of the periplasm (arrow). bar 0,2 µm.

5.3 Physiological Characterization

Various physiological characteristics were investigated, and growth rates were plotted to determine the most favourable conditions for growth of the three strains P6.1, P6.2 and P2S. Growth during the exponential phase was used to calculate the growth rates using the formula described by Widdel (2007). Parameters under which growth was monitored includes temperature, salinity concentrations (%NaCl w/v) and pH. Conditions for growth and growth rates were not investigated for strain P1a because this strain was obtained in pure culture at a much later stage than the other strains, and thus not enough time for describing as part of this current thesis.

5.3.1 Temperature Range and Optimum

Temperature experiments were performed, ranging from 5 – 40°C. Temperature range for strains P6.1 and P6.2 were shown to be from 5 to 35 °C, when cultured at 40°C both strains exhibited declining optical density (OD) (Figure 5.10). Strain P2S displayed growth at temperatures between 5 and 30 °C, including very limited growth at 35 °C (µ/day = 0,0115). Negative growth was shown by strain P2S when cultivated at 40 °C. Optimum growth for all strains was 25 °C. All 3 strains appear as psychrotolerant due to their ability to grow at temperatures as low as 5°C.

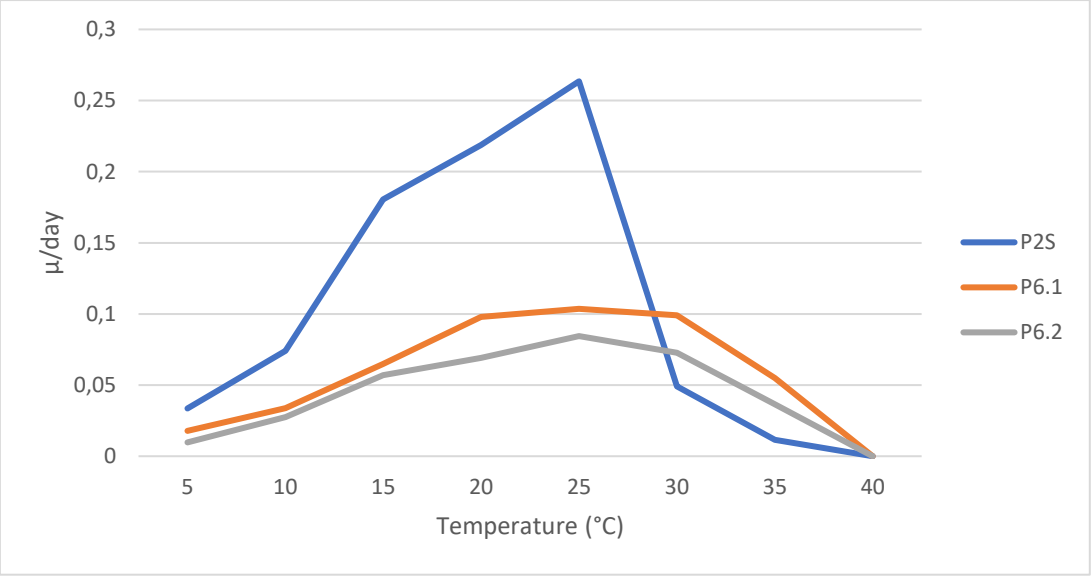


Figure 5.10: Growth rates (µ/day) displayed for strains P6.1, P6.2 and P2S when cultivated in M30 18% SW with temperatures ranging from 5 to 40 °C.

When cultivated at 5°C, all strains exhibited rather slow growth, strains P2S and P6.1 did not reach the double of initial OD before 13 and 14 days, respectively. Strain P6.2 used 29 days to double its initial OD. All strain kept a slow growth rate at this temperature before starting to enter death phase, strain P2S on day 58, while strains P6.1 and P6.2 both enter death phase on day 75. When cultivated at 10 and 15°C, strain P2S seemed to be the fastest grower, and reached the highest OD. Strain P6.2 was the slowest and reached the lowest OD of the strains. When cultivated at optimum temperature (25°C), all strains enter exponential phase after day 3. Strain P2S reached its highest cell density (OD) on day 10, before it immediately entered the death phase. Both strains P6.1 and P6.2 entered stationary phase around day 15, before OD slowly declined. At 30°C or higher P6.1 was the fastest growing strain and when cultivated at 35°C P2S barely exhibit any growth. The two other strains showed a quick burst of growth before rapidly entering the death phase. None of the strains displayed any growth when grown on 40°C. When cultivated at temperatures 30 and 35 °C strains P6.1 and P6.2 tended to grow and attach to the glass tube in the surface of the growth media. Therefore, it was necessary to vortex the tubes extra to ensure proper homogenization upon measurements.

5.3.2 Salinity Range and Optimum

Strains P6.1 and P6.2 was able to grow from 0 to 0,5% NaCl (w/v). However, strain P6.1 did show weak growth at 0,5% NaCl (w/v) (μ/day = 0,019), strain P6.2 also showed slight growth when cultivated at 1% NaCl (w/v) (μ/day = 0,017). Growth range for strain P2S was determined to be between 0 and 3,5% NaCl (w/v), however growth at 3 and 3,5% NaCl (w/v) was limited, with growth rates of 0,023 and 0,019, respectively. Salinity growth optimum for strain P6.1 was between 0 and 0,1% NaCl (w/v), 0,1% NaCl (w/v) for strain P6.2 and 0,5% NaCl (w/v) for strain P2S (Figure 5.11).

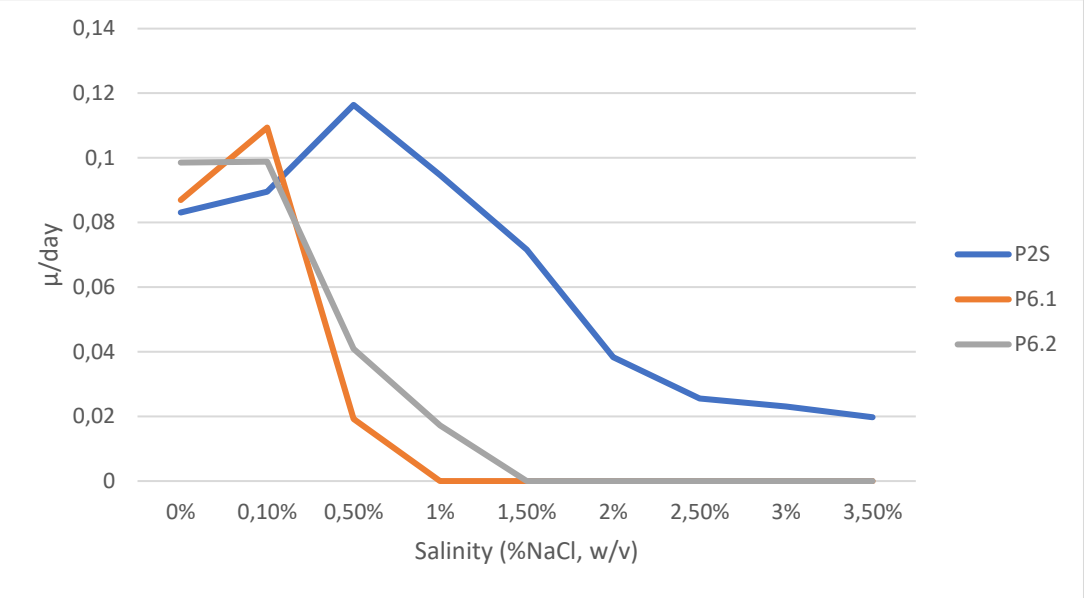


Figure 5.11: Growth rates (μ/day) displayed for strains P6.1, P6.2 and P2S when cultivated in M30 with salinity adjusted to range from 0 to 3,5% NaCl (w/v).

All 3 strains were able to grow when there was no NaCl available, as well as when there was NaCl added to the media. This indicates that the strains are halotolerant, where strains P6.1 and P6.2 only tolerates low NaCl concentrations (<1% NaCl w/v), and strain P2S tolerates NaCl concentrations of up to 3,5% NaCl (w/v).

5.3.3 pH Range and Optimum

Strains P2S, P6.1 and P6.2 were able to grow in pH ranging from 5 to 9 when cultured in M30 18% SW when pH was adjusted. Both strains P6.1 and P6.2 showed the highest growth rate when cultivated at pH = 7,5, whereas strain P2S displayed the highest growth rate when cultivated at pH = 8 (Figure 5.12). The pH optimum of the strains indicate that they are neutrophilic but are still able to grow in media as acidic as pH = 5.

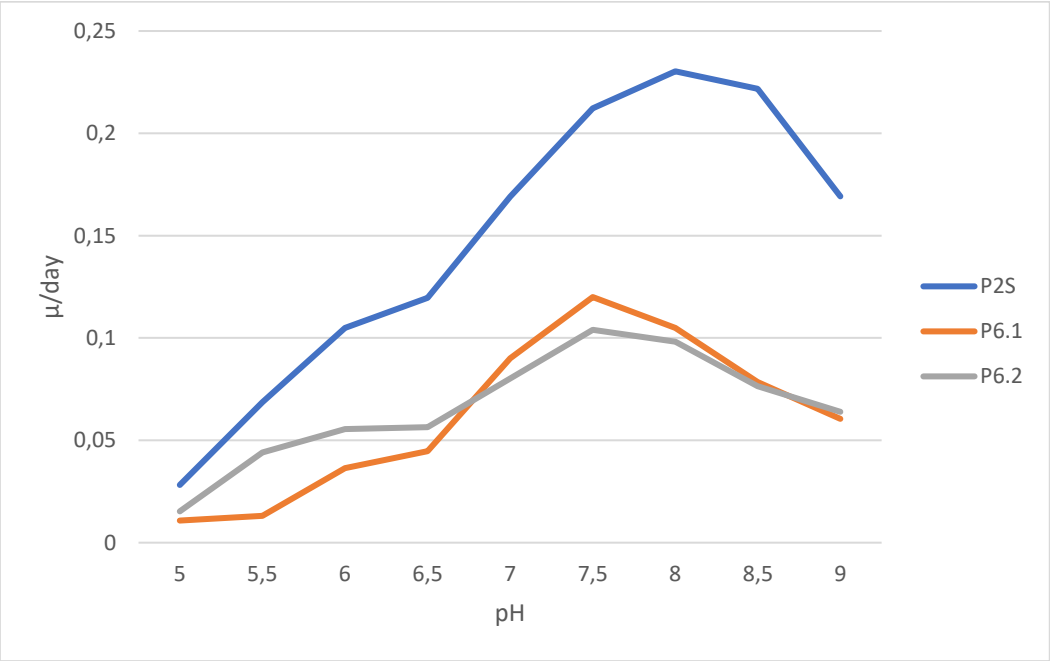


Figure 5.12: Growth rates (µ/day) displayed for strains P6.1, P6.2 and P2S when cultivated in M30 18% SW with pH adjusted to range from 5 to 9.

5.4 Physiological Attributes

The physiology of the strains was examined by utilizing different techniques. This includes analysis for carbon utilization, enzymatic repertoire and antibiotic resistance and sensitivity.

5.4.1 Utilization of Carbon Sources

The different strains were cultured with different carbon sources added to growth media to gain insights to which carbon sources the different strains were able to metabolize. Growth was measured as changes in optical density (OD). Initial OD for strains P1a, P6.1 and P6.2 were 0,05 and 0,06 for strain P2S. The growth experiments were carried out for 11 days.

All strains were capable of utilizing a variety of carbon sources, listed in Table 5.1. All strains exhibited good growth on N-acetyl-D-glucosamine, D-maltose, D-glucose and D-cellobiose. Strains P1a, P6.1 and P2S also showed good growth when grown on D-galactose. P1a and P2S exhibited growth when grown on D-xylose and D-mannose, as well as sucrose, L-rhamnose and D-raffinose for strain P2S. Strain P6.1 grew well on sucrose, carboxymethyl cellulose, D-xylose, D-mannitol and D-mannose, and displayed limited growth on L-sorbose and L-rhamnose. As mentioned, strain P6.2 exhibited good growth on N-acetyl-glucosamine, D-maltose, D-glucose and D-cellobiose, but did also show some growth on sucrose, D-galactose, D-mannitol and D-mannose, as well as slight growth on D-arabinose and D-xylose. Strain P1a also exhibited growth when cultured in media containing D-arabinose.

Table 5.1: Showing optical density (OD) for strains P1a, P2S, P6.1 and P6.2 when grown on different carbon sources. Initial OD for strains P1a, P6.1 and P6.2 was 0,05 and 0,06 for strain P2S. Measurements displayed are from day 11 after inoculation. Green indicates strong growth, yellow indicates intermediate growth, while red indicated negative growth.

Carbon sources	P1a	P2S	P6.1	P6.2
Sucrose	0,06	0,17	0,1	0,1
L-Sorbose	0,03	0,065	0,06	0,04
D-Xylose	0,18	0,21	0,08	0,06
D-Raffinose	0,03	0,11	0,03	0,03
D-Cellobiose	0,18	0,24	0,15	0,1
D-Galactose	0,21	0,21	0,12	0,08
D-Fructose	0,03	0,075	0,04	0,02
D-Mannitol	0,03	0,06	0,09	0,09
D-Arabinose	0,17	0,075	0,05	0,065
D-Mannose	0,16	0,18	0,085	0,085
Carboxymethyl cellulose	0,03	0,05	0,1	0,05
D-Glucose	0,2	0,27	0,12	0,12
Cellulose	0,025	0,065	0,04	0,04
N-acetyl-D-glucosamine	0,2	0,26	0,15	0,14
D-Maltose	0,17	0,26	0,14	0,1
Chitin	0,02	0,05	0,05	0,04
L-Rhamnose	0,06	0,23	0,06	0,045
Control	0,01	0,02	0,015	0,02

The experiment shows that the strains are capable of utilizing a variety of different carbon sources. The control which contained M30 18% SW without any carbon source, showed an OD well below the initial OD for all strains, indicating that the cells were not metabolizing any remaining carbon from previous cultivation. All strains displayed highest OD when grown on reducing sugars, or derivatives from reducing sugars. These include N-acetyl-D-glucosamine, as well as D-cellobiose for strain P6.1, and D-maltose and D-glucose for strain P2S. Strain P1a exhibited the highest OD when grown on D-galactose, D-glucose and N-acetyl-D-glucosamine.

5.4.2 Enzymatic Activity

The API-ZYM test was used to reveal the enzymatic activities of the strains. API-ZYM strips where inoculated with cell suspension and scoring of the strips were done after 6 h of incubation.

The enzymatic repertoire of all strains includes esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. Strain P1a was also positive for lipase (C14), esterase (C4), valine arylamidase, cystine arylamidase and acid phosphatase (Table 5.2). Strain P2S tested positive for alkaline phosphatase, valine arylamidase and α-glucosidase. The enzymatic repertoire of strain P6.1 also included alkaline phosphatase, esterase (C4) and leucine arylamidase. While strain P6.2 was positive for alkaline phosphatase, esterase (C4), leucine arylamidase and valine arylamidase.

Table 5.2: Showing the enzymatic repertoire tested with API ZYM of strains P1a, P2S, P6.1 and P6.2. Green colour indicates positive result, while red indicates negative result.

Enzyme	P1a	P2S	P6.1	P6.2
Control	+	+	+	+
Alkaline phosphatase	-	+	+	+
Esterase (C4)	+	-	+	+
Esterase Lipase (C8)	+	+	+	+
Lipase (C14)	+	-	-	-
Leucine arylamidase	-	-	+	+
Valine arylamidase	+	+	-	+
Cystine arylamidase	+	-	-	-
Trypsin	-	-	-	-
α-chymotrypsin	-	-	-	-
Acid phosphatase	+	-	-	-
Naphtol-AS-BI-phosphohydrolase	+	+	+	+
α-galactosidase	-	-	-	-
β-galactosidase	-	-	-	-
β-glucuronidase	-	-	-	-
α-glucosidase	-	+	-	-
β-glucosidase	-	-	-	-
N-acetyl-β-glucosaminidase	-	-	-	-
α-mannosidase	-	-	-	-
α-fucosidase	-	-	-	-

+, positive; -, negative

The experiment showed that the strains possess different enzymes. Strain P6.1 and P6.2 had identic profiles except that strain P6.1 was negative for valine arylamidase. P2S was the only strain positive for α-glucosidase, whereas strain P1a was the only one positive for lipase (C14), cystine arylamidase and acid phosphatase. Strain P1a tested positive for 7 different enzymes, whereas strain P6.1 tested positive for 5 enzymes.

Both strains P2S and P6.2's enzymatic repertoire includes 6 out of 19 different enzymes tested for in this experiment. All strains showed positive results in the positive control indicating that the experiment was successful.

5.4.3 Antibiotic Resistance and Sensitivity

All strains were tested for their susceptibility towards 11 different types of antibiotics. The strains were plated onto fresh media and one antimicrobial disc were placed in the middle of each plate, before being incubated at room temperature for 12 days.

All the strains exhibited resistance towards ampicillin, streptomycin, penicillin G, vancomycin and nalidixic acid, as well as showing sensitivity towards erythromycin (Table 5.3). Strains P6.1, P6.2 and P2S displayed sensitivity towards chloramphenicol, whilst strain P1a was resistant. Tetracycline seem to limit the growth of strains P6.1 and P2S, whereas the other two strains showed no signs of inhibition zone. Both strains P2S and P1a displayed an inhibition zone of 12 and 11 mm, respectively, in the presence of ofloxacin. Strain P2S was the only strain that was sensitive towards trimethoprim and kanamycin.

Table 5.3: Antibiotic impact on the growth of strains P1a, P2S, P6.1 and P6.2. Inhibition zone measured in mm, resistence (R). Green colour indicates resistance, while red colour indicates sensitivity.

Antibiotics	P6.1	P6.2	P2S	P1a
Ampicillin (10µg)	R	R	R	R
Chloramphenicol (10 µg)	7 mm	6 mm	18 mm	R
Kanamycin (30µg)	R	R	12 mm	R
Streptomycin (10µg)	R	R	R	R
Penicillin G (10 µg)	R	R	R	R
Trimethoprim (2,5 µg)	R	R	11 mm	R
Ofloxacin (5 µg)	R	R	12 mm	11 mm
Erythromycin (10 µg)	12 mm	10 mm	18 mm	4 mm
Vancomycin (5 µg)	R	R	R	R
Nalidixic acid (30 µg)	R	R	R	R
Tetracycline (10 µg)	6 mm	R	9 mm	R

R, resistence; inhibition zone measured in mm.

Three of the strains were resistant towards more antibiotics than they are sensitive towards. Strain P2S was sensitive to 6 out of 11 antibiotics used in this experiment, therefore this strain appears to be the most sensitive strain. Strains P1a and P6.2 were only sensitive towards 2 out of 11 antibiotics, therefore they are the most resistant strains in this experiment. Both strains P6.1 and P6.2 showed the highest sensitivity towards erythromycin (10 µg) with an inhibition zone of 12 mm and 10 mm, respectively. Strain P2S displayed highest sensitivity towards chloramphenicol (10 µg) and erythromycin (10 µg), with inhibition zones of 18 mm.

5.5 Phylogeny

Strains P2S, P6.1, P6.2 and P1a all belong to the kingdom Bacteria, phylum Planctomycetes, class Planctomycetia, order Planctomycetales, family Planctomycetacea. The assembled 16S rRNA sequences for strain P2S was 1,388 bp, for strain P6.1 was 1,392 bp, for strain P6.2 was 1,397 bp and for strain P1a was 1,393 base pairs long (Appendix III).

5.5.1 Alignment of the 4 Strains

Strains P6.1 and P6.2 showed a similarity of 99% in their 16S rRNA sequences. Strain P1a showed an 87% similarity towards P2S and P6.2, whilst being 88% similar to P6.1. Strain P2S show a 98% similarity to both strains P6.1 and P6.2, as shown in Table 5.4. Summarized, strains P6.1, P6.2 and P2S share similarities of 98% or higher between each other, whilst strain P1a are 88% or less similar to the other strains in the 16S rRNA gene.

Table 5.4: Showing similarities in the 16S rRNA gene between strains isolated from Svarthammarhola Ice Cave.

	P1a	**P2S**	**P6.1**	**P6.2**
P1a	**100%**	87%	88%	87%
P2S	87%	**100%**	98%	98%
P6.1	88%	98%	**100%**	99%
P6.2	87%	98%	99%	**100%**

Alignment of the 4 strains' 16S rRNA gene sequences, showed that there are especially some regions where there are differences (Supplementary Figure 1). First of all, strains P2S, P6.1 and P6.2 display gaps during the first and last 10 bp, where strain P1a does not have any gaps. Overall, irregularities in the alignment occur at spread regions throughout the 16S rRNA. However, strain P1a show dissimilarities towards the other strains mainly in regions 340-380, 720-745 (V4), 900-945 (V5), 1330-1350 and 1370-1390 (V8).

5.5.2 Closest Relatives

Searches with BlastN revealed that strain P1a's closest cultured relative was the planctomycete strain 292 retrieved from Lake Fuhlensee near Kiel, Germany (Griepenburg et al., 1999) with a similarity of 92%. The most similar uncultured sequence for strain P1a with a similarity of 99% was the bacterium clone MPB2-197, originated from a freshwater lake in east Antarctica, as shown in Table 5.5.

Table 5.5: Relationship between the 16S rRNA gene sequence of strain P1a and its three closest uncultivated (1-3) and its three closest cultivated relatives (4-6) from the NCBI database.

Blast hit	Identity	Acc. Nr.	Reference	Habitat
1. Uncultered bacterium clone: MPB2-197	99%	AB630873.1	Nakai et al., 2012	Freshwater lake, Easte Antarctica.
2. Uncultured Planctomycetaceae clone: B08-03C	99%	FJ543048.1	Rattray et al., 2010	Earthworm intestine.
3. Uncultured Planctomycetales clone: ROM 42	99%	HE575398.1	Chiellini et al., 2012	Purification plant, Poggibonsi, Italy.
4. Planctomycete strain 292	92%	AJ231182.1	Griepenburg et al., 1999	Lake Fuhlensee, Kiel
5. Pirellula sp. Schlesner 678	91%	X81947.1	Ward et al., 1995	Lake Fuhlensee, Kiel
6. *Bythopirellula goksoyri*	90%	NR_118636.1	Storesund and Øvreås, 2013	AMOR, Norwegian-Greenland Sea

Search with BlastN revealed that the closest cultured relative to strain P2S with a similarity of 91%, was strain Schlesner 302 obtained from a Fjord Schlei in the Baltic Sea (Ward et al., 1995), as shown in Table 5.6. Strain P2S showed 98% similarity to the uncultured bacterium clone R1-9 (Chen et al., 2015), as well as 98% similarity to the uncultured bacterium clones F11 and 96 (Li et al., 2011; Zhao et al., 2011), both affiliated to wastewater in China (Table 5.6).

Table 5.6: Relationship between the 16S rRNA gene sequence of strain P2S and its three closest uncultivated (1-3) and the three closest cultivated relatives (4-6) from the NCBI database.

Blast hit	Identity	Acc. Nr.	Reference	Habitat
1. Uncultured Planctomycetacea clone: R1-9	98%	KP717533.1	Chen et al., 2015	Yanshi WWTP, China.
2. Uncultured bacterium clone: F11	98%	FJ230909.1	Li et al., 2011	WWTP, Hebei, China.
3. Uncultured bacterium clone: 96	98%	JF828764.1	Zhao et al., (unpublished)	Waste water, China.
4. Pirellula sp. Schlesner 302	91%	X81942.1	Ward et al., 1995	Fjord Schlei, Baltic Sea
5. Rhodopirellula sp. SM49	91%	FJ624355.1	Winkelmann and Harder, 2009	Dar Es Salaam, Tanzania
6. Pirellula sp. Schlesner 139	90%	X81945.1	Ward et al., 1995	Kiel fjord

The most similar uncultured sequence for strains P6.1 with a similarity of 99% was bacterium clone BJGMM-3s-145, obtained from Yellow river delta in China (Jia et al., unpublished). The strain also showed a 99% similarity to bacterium clones 101-75 and F11 (Kim and Crowley, 2007; Li et al.,2011) which originated from tar pits in California, USA, and a wastewater treatment in China (Table 5.7). Searches with BlastN in the NCBI database also revealed that the closest related cultured specimen for strain P6.1 was the Pirellula sp. Schlesner 302 strain from Fjord Schlei (Ward et al., 1995), with similarities of 92% (Table 5.7).

Table 5.7: Relationship between the 16S rRNA gene sequence of strain P6.1 and its three closest uncultivated (1-3) and the three closest cultivated relatives (4-6) from the NCBI database.

Blast hit	Identity	Acc. Nr.	Reference	Habitat
1. Uncultured bacterium clone: BJGMM-3s-145	99%	JQ800921.1	Jia, et al., (unpublished)	Yellow river delta, China.
2. Uncultured bacterium clone: 101-75	99%	EF157256.1	Kim and Crowley, 2007	Rancho La Brea tar pits, CA, USA.
3. Uncultured bacterium clone: F11	99%	FJ230909.1	Li et al., 2011	WWTP, Hebei, China.
4. Pirellula sp. Schlesner 302	92%	X81942.1	Ward et al., 1995	Fjord Schlei, Baltic Sea
5. Pirellula sp. Schlesner 516	91%	X81940.1	Griepenburg et al., 1999	Sewage sludge
6. Planctomycete strain 543	91%	AJ231173.1	Griepenburg et al., 1999	Sewage sludge

Strain P6.2 showed 99% similarity towards the uncultured bacterium clones BJGMM-3s-145, 101-75 and F11 (Table 5.8). The closest related cultured specimens were Pirellula sp. Schlesner 302 and Schlesner 516, both with a 91% similarity in the 16S rRNA gene. Strain P6.2 also displayed a 90% similarity towards the Planctomycete strain 543 obtained by Griepenburg and collaborates (1999) from sewage sludge Table 5.8).

Table 5.8: Relationship between the 16S rRNA gene sequence of strain P6.2 and its three closest uncultivated (1-3) and the three closest cultivated relatives (4-6) from the NCBI database.

Blast hit	Identity	Acc. Nr.	Reference	Habitat
1. Uncultured bacterium clone: BJGMM-3s-145	99%	JQ800921.1	Jia, et al., (unpublished)	Yellow river delta, China.
2. Uncultured bacterium clone: 101-75	99%	EF157256.1	Kim and Crowley, 2007	Rancho La Brea tar pits, CA, USA.
3. Uncultured bacterium clone: F11	99%	FJ230909.1	Li et al., 2011	WWTP, Hebei, China.
4. Pirellula sp. Schlesner 302	91%	X81942.1	Ward et al., 1995	Fjord Schlei, Baltic Sea
5. Pirellula sp. Schlesner 516	91%	X81940.1	Griepenburg et al., 1999	Sewage sludge
6. Planctomycete strain 543	90%	AJ231173.1	Griepenburg et al., 1999	Sewage sludge

Both strains P6.1 and P6.2 showed very similar relatives when searched in the Blast database. The only difference was the similarity towards Pirellula sp. Schlesner 302, which was 91% for strain P6.2 and 92% for strain P6.1. As well as the Planctomycete strain 543, where strain P6.1 showed 91% similarity and strain P6.2 showed 90% similarity.

5.5.3 Phylogenetic Tree

A phylogenetic tree was made to analyse the phylogenetic distances between the strains and their closest related sequences, as well as to analyse their relationship to other described species of Planctomycetes.

Construction of a maximum likelihood tree (Figure 5.13) clustered strains P2S, P6.1 and P6.2 into a monophyletic clade, together with their closest uncultured sequences, as well as their closest related cultured sequences from Tables 5.6–5.8. The closest related described Planctomycetes species for these 3 strains were *Rhodopirellula baltica* from the Baltic Sea with a similarity of 90%, and *Rubripirellula obstinata*, originated from kelp surface with a similarity of 89%. Strain P1a formed a monophyletic clade together with its most closely related uncultured and cultured sequences (Table 5.5), including *Bythopirellula goksoyri*, isolated from AMOR in the Norwegian-Greenland Sea with a similarity of 90%. The described Planctomycetes species included in this tree was chosen to provide an overview of the Svarthammarhola strains' phylogenetic position and their relationship to different genera within the Planctomycetes division.

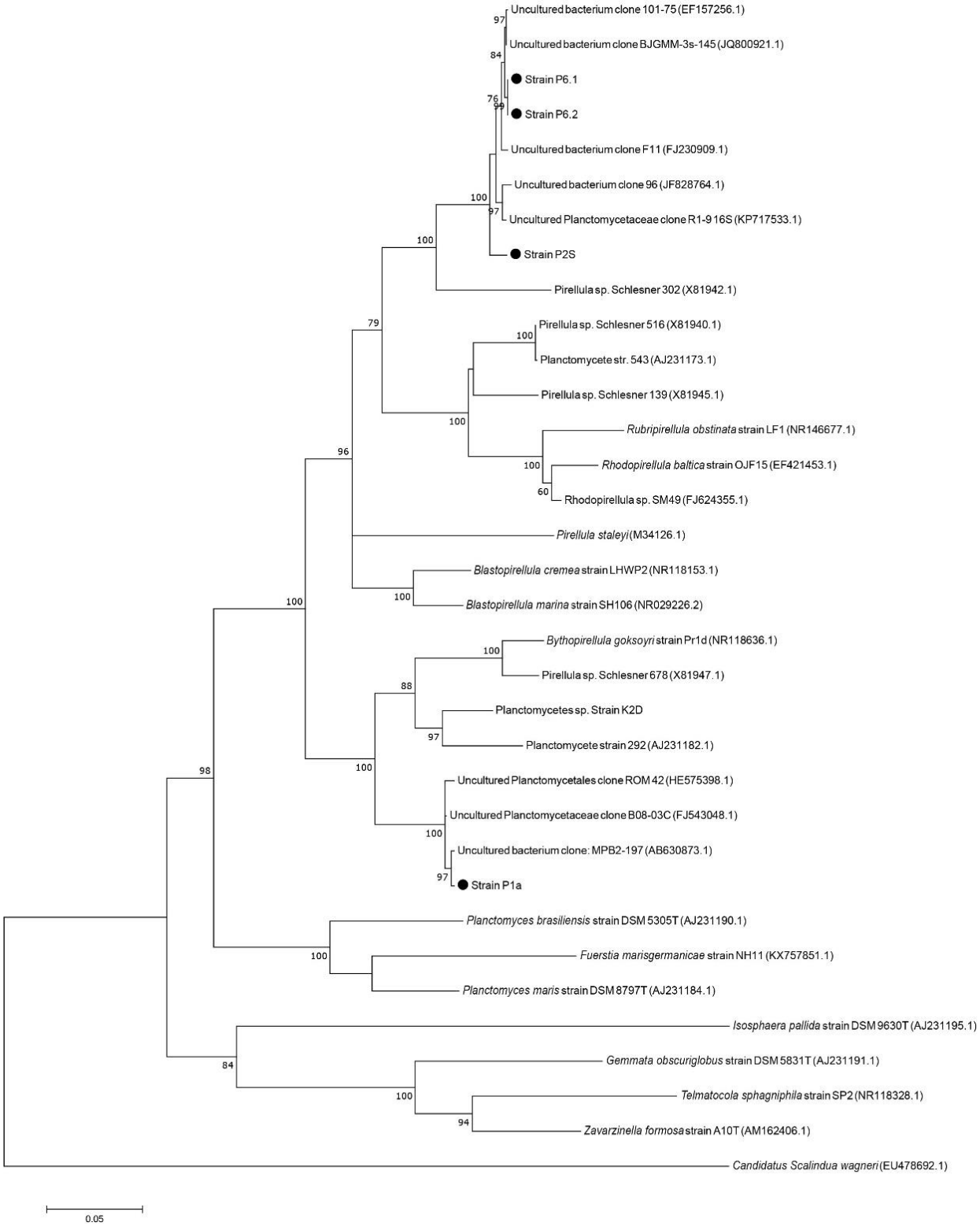


Figure 5.13: Maximum likelihood 16S rRNA gene phylogenetic tree showing the relationships between strains from Svarthammarhola (highlighted with black dots), their closest uncultured and cultured relatives, as well as selected Planctomycetes species (accession numbers shown in parenthesis). The tree was based on the Tamura-Nei model (Tamura and Nei, 1993), with discrete Gamma distribution used for model of evolutionary rate differences among sites. Numbers on the tree refer to bootstrap values, based on 100 replicates. Only values above 50% are shown. The anammox Planctomycete *Candidatus Scalindua wagneri* were used as outgroup. Bar – 0,05 substitutions per nucleotide position.

5.6 Summary of Traits and Comparison to *R. obstinata*

The Planctomycetes species *Rubripirella obstinata* was chosen for comparison of morphological and physiological traits for the 4 strains. There were other species with higher similarities in the 16S rRNA gene, however the comparison data for *R. obstinata* involved more similar experiments and comparable data. Data for comparison of *R. obstinata* was retrieved from the description performed by Bondoso and collaborates (2015).

Morphologically the Svarthammarhola strain differ from *R. obstinata* (Table 5.9). Cell size are in general smaller and vary more. The smallest cells of *R. obstinata* are 1,5 x 1,3 µm, whereas the smallest measured cells from Svarthammarhola are cells from strains P2S and P6.1, with a size of 0,6 x 0,7 µm. Strains from Svarthammarhola all have spherical to ovoid cells, where *R. obstinata* have pear-shaped to ovoid cells. Strains P2S, P6.1, P6.2 all have the same temperature optimum as *R. obstinata*, however growth range differs, as well as pH range.

Table 5.9: Comparison of morphological and physiological traits of strains P1a, P2S, P6.1, P6.2 and *R. obstinata*.

Morphology	**Strain P1a**	**Strain P2S**	**Strain P6.1**	**Strain P6.2**	***R. obstinata***
Cell size (µm)	0,7-1,2 x 0,7-0,9	0,6-1,4 x 0,7 - 1,6	0,6-1,2 x 0,7-1,4	0,8-1,4 x 0,8-1,6	1,5-2 × 1,3-1,7
Cell shape	Spherical to ovoid	Spherical to ovoid	Spherical to ovoid	Spherical to ovoid	Pear shaped to ovoid
Cell arrangement	Aggregates, rosette or single cells	Rosette of 2-3 cells or single cells	Aggregates, rosette or single cells	Aggregates, rosette or single cells	Rosette of 2-10 cells
Pigmentation	Grey-white	White	Pink	Red	Red
Salinity tolerance	*NA*	0-3,5% NaCL (w/v)	0-0,5% NaCl (w/v)	0-1% NaCl (w/v)	50-125% ASW
Temperature range (°C)	*NA*	5-35	5-35	5-35	10-30
Temperature optimum (°C)	*NA*	25	25	25	25
pH range	*NA*	5-9	5-9	5-9	6,5-10
pH optimum	*NA*	8	7,5	7,5	7,5

NA, not available.

Comparison of different carbon sources utilized are displayed in Table 5.10. The Svarthammarhola strains are capable of utilizing a broader spectrum of carbon sources than *R. obstinata*, considering the carbon sources used in this experiment.

Table 5.10: Comparison of carbon sources utilized by strains P1a, P2S, P6.1, P6.2 and *R. obstinata*.

Carbon source	**Strain P1a**	**Strain P2S**	**Strain P6.1**	**Strain P6.2**	***R. obstinata***
Sucrose	*w*	+	+	+	-
L-Sorbose	-	-	*w*	-	-
D-Xylose	+	+	+	*w*	+
D-Raffinose	-	+	-	-	-
D-Cellobiose	+	+	+	+	-
D-Galactose	+	+	+	+	+
D-Fructose	-	*w*	-	-	+
D-Mannitol	-	-	+	+	-
D-Arabinose	+	*w*	-	*w*	-
D-Mannose	+	+	+	+	+
Carboxymethyl cellulose	-	-	+	-	-
D-Glucose	+	+	+	+	+
Cellulose	-	-	-	-	+
N-acetyl-D-glucosamine	+	+	+	+	+
D-Maltose	+	+	+	+	-
Chitin	-	-	-	-	*NA*
L-Rhamnose	*w*	+	-	-	+

+, positive; -, negative; w, weak; NA, not available.

The enzymatic repertoire of *R. obstinata* does not differ much from that of the Svarthammarhola strains (Table 5.11). No enzymes are displayed by *R. obstinata* that the strains do not possess. However, it does test positive for cysteine arylamidase and acid phosphatase which only strain P1a test positive for among the Svarhammarhola strains.

Table 5.11: Comparison of the enzymatic repertoire of strains P1a, P2S, P6.1, P6.2 and *R. obstinate* tested with API ZYM.

Enzyme	**Strain P1a**	**Strain P2S**	**Strain P6.1**	**Strain P6.2**	***R. obstinata***
Alkaline phosphatase	-	+	+	+	+
Esterase (C4)	+	-	+	+	+
Esterase lipase (C8)	+	+	+	+	+
Lipase (C14)	+	-	-	-	-
Leucine arylamidase	-	-	+	+	+
Valine arylamidase	+	+	-	+	+
Cystine arylamidase	+	-	-	-	+
Trypsin	-	-	-	-	-
α-chymotrypsin	-	-	-	-	-
Acid phosphatase	+	-	-	-	+
Naphtol-AS-BI phosphohydrolase	+	+	+	+	-
α-galactosidase	+	-	-	-	-
β-galactosidase	-	-	-	-	-
β-glucuronidase	-	-	-	-	-
α-glucosidase	-	+	-	-	-
β-glucosidase	-	-	-	-	-
N-acetyl-β-glucosaminidase	-	-	-	-	-
α-mannosidase	-	-	-	-	-
α-fucosidase	-	-	-	-	-

+, positive; -, negative.

5.7 Screening for Bioactive Molecules

Strains P6.1 and P6.2 were screened for bioactive molecules. At the time when the screening was performed, strain P2S was not obtained in pure culture, and strain P1a was not yet discovered. The screening was done as part of a collaborative project between the University of Bergen, and the University of Porto. The aim of the Project was to screen different strains of Planctomycetes from different habitats for bioactive molecules. Extracts from the different strains were obtained, and these were used to treat the different cancerous cell lines. The cell lines used was the human AML cell line (Molm13) and human prostatic cancer cell line (PC3), as well as the normal rat kidney epithelial cell line (NRK) as a control. All the different cell lines were analysed for metabolic activity using the WST-1 assay, which measures enzymatic conversion of tetrazolium salt into coloured metabolites. However, Planctomycetes, including strains P6.1 and P6.2, possess pigmentation that interferes with the WST-1 colorimetric assay, therefore results from this assay was not applicable. Consequently, only cytotoxicity was assessed by studying the nuclear morphology of the treated cell lines. The results from the project now being in the process of being published. The manuscript is available in Appendix IV (Calisto et al., 2018).

5.6.1 Determination of Induced Apoptosis

Apoptotic cells appeared under the fluorescence microscope as condensed nucleuses exhibiting strong fluorescence when stained with DNA-specific dye Hoechst 33342 (Figure 5.14b). Healthy and viable cells appeared as whole cells without fluorescent dye staining their nuclei (Figure 5.14a). The cytotoxic potential of the extracts was divided into categories, including high – H (>70% cell death), intermediate – I (40-70% cell death), low – L (10-40% cell death) or no (<10% cell death). The different cell lines were screened under the microscope for apoptotic nuclei and the percentage of apoptotic cells were calculated using Equation 2.

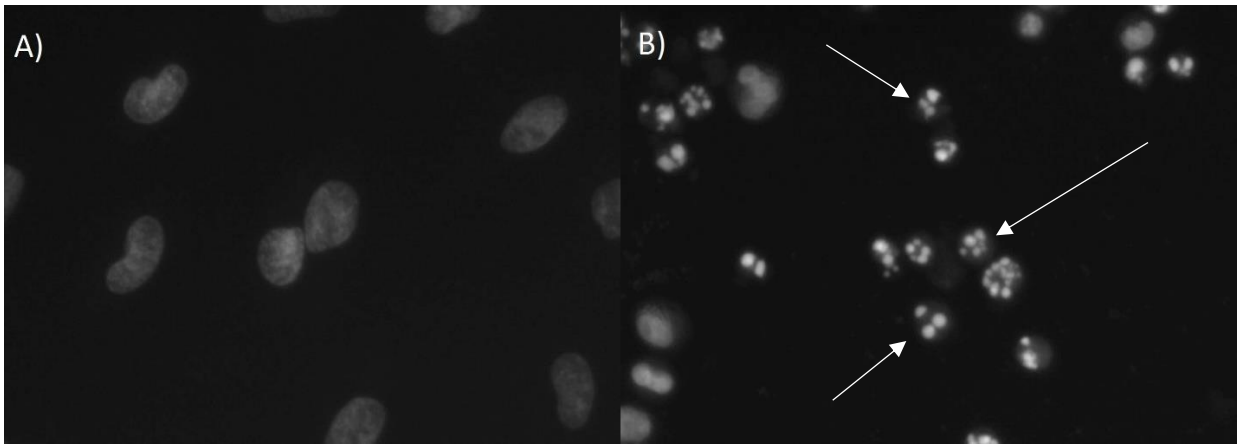


Figure 5.14: Comparison of healthy viable and apoptotic Molm13 cells. A) Showing control Molm-13 cells treated with only DMSO and no planctomycetal extracts. Providing an indication of how healthy and viable cells appeared under fluorescent microscope. B) Showing Molm13 cells treated with aqueous extracts from strain P6.1, incubated for 72 h. Arrows indicating apoptotic cells by distinctive staining of condensed nucleuses.

5.6.2 Cytotoxic Assays

Both the aqueous and organic extracts from strain P6.1 showed intermediate activity towards Molm13 cells, with 64 and 56% cell death, respectively (Figure 5.15). These extracts also showed no cytotoxicity towards the PC3 and NRK cell line, except for the aqueous extract on PC3 cells, which displayed 12,66% apoptotic cells, hence, low cytotoxicity. Strain P6.2 showed intermediate cytotoxicity towards Molm13 in the aqueous extracts, and low toxicity in the organic extracts. There was no cytotoxicity shown against PC3 cells in either of the extracts and low toxicity was displayed in NRK cells for both extracts.

It was shown that strain P6.1 displayed a higher cytotoxicity than strain P6.2 in both extracts. Both strains show higher toxicity in their aqueous extracts towards Molm13 cells than in the organic extracts. None of the strains showed higher than low cytotoxicity towards any of the adherent cell lines, where strain P6.1 showed low toxicity towards PC3 cells in the aqueous extracts, and strain P6.2 showed low cytotoxicity towards NRK cells in both aqueous and organic extracts. Both strains display intermediate cytotoxicity towards Molm13 cells.

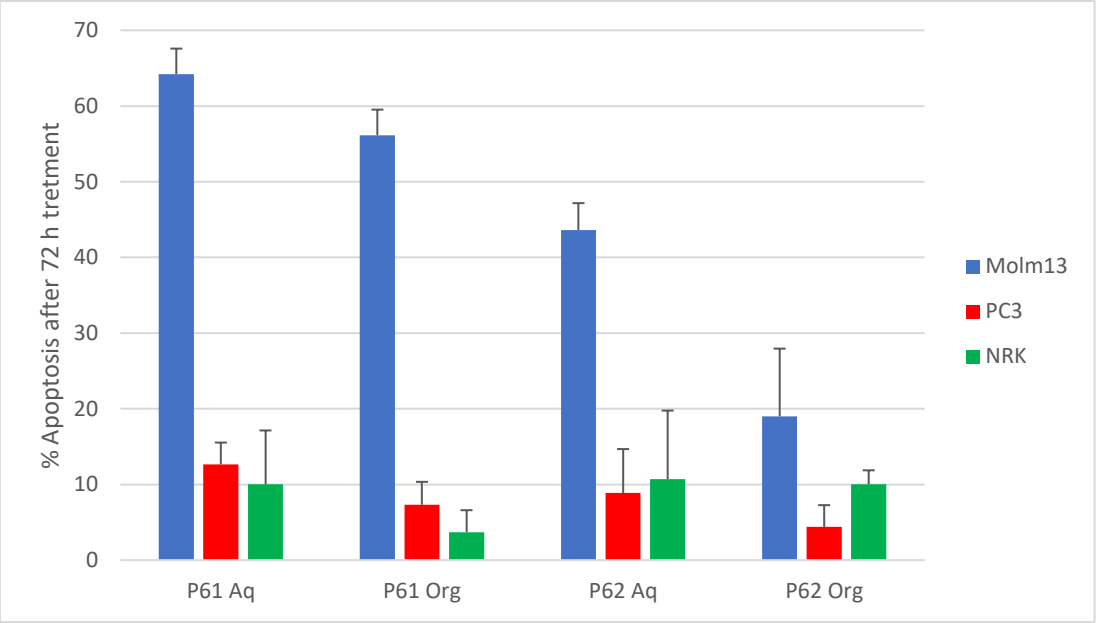


Figure 5.15: Showing calculated induced apoptosis (%) after 72h treatment on 3 different cell lines with both aqueous and organic extracts from strains P6.1 and P6.2. Values represented are calculated based on the results from 3 parallels on the PC3 and NRK cell lines and 4 parallels on the Molm13 cell line.

The screening of these strains' extracts was performed as part of a collaborative project, involving several other strains than strain P6.1 and P6.2. The ability to induce apoptosis in Molm13, PC3 and NRK cell lines for all involved strains are represented in Appendix IV (Calisto et al., 2018). When the effect of extracts from strains P6.1 and P6.2 are compared to the rest of the strains' extracts, it is clear that they are not the most potent producers of bioactive molecules. However, extracts from strains P6.1 and P6.2 still induce higher percentage of apoptosis towards Molm13 cells than most of the extracts from other strains. Only a few of the strains involved in the experiment exhibited higher than low toxicity towards the PC3 cell line. The same is applicable for the strains' toxicity towards the NRK cell line.

6. Discussion

Planctomycetes have previously been reported in caves and frozen environments (Pašić et al., 2009; Borsodi et al., 2012; De Mandal et al., 2014; Rysgaard and Glud, 2004; Zeng et al., 2013; Boetius et al., 2015; Tebo et al., 2015; Yang et al., 2016). However, these were solely on molecular experiments, and not dedicated Planctomycetes studies. Planctomycetes have also never been isolated in axenic cultures from either ice or cave ecosystems.

In the descriptive microbial community analysis (Figure 3.4), it was shown that Planctomycetes were present in the ice mass of Svarthammarhola ice cave. Though varying abundances from 2,1 – 13% were shown to be present. The dominant group in all samples were the Planctomycetacia, but also Phycisphaera were documented.

In this study 11 thawed ice samples from Svarthammarhola ice cave was used as inoculum for enrichment cultures using a modified version of M30 media spanning the entire chrono sequence of the ice mass. The M30 media has previously shown to be successful in the isolation of Planctomycetes (Schlesner, 1994). The enrichments were followed closely by microscopy and 4 novel strains were obtained in pure cultures. Further, these 4 strains were subject to diphasic characterization using both molecular and conventional cultivational based techniques. Two of the strains were further used in a screening assay for bioactive molecules, together with several different Planctomycetes strains from various habitats.

6.1 Isolation

Four novel Planctomycetes strains were isolated from the initial enrichment cultures. The strains were isolated from enrichment cultures containing sample material from samples P1a and P6, as well as soil and organic matter from sample P2, sample P2S. Considering the abundances of Planctomycetes in the different samples (Figure 5.2), it is not surprising that we find Planctomycetes strains in samples P1a and P2S, given their relative abundances of 10,5 and 5,3%, respectively (Figure 3.4). However, in sample P6 one of the lowest abundances of Planctomycetes was recorded. Only about 2,1% of the reads were Planctomycetes. Therefore, it was unexpected that 2 of 4 strains originated from this sample. Cells with Planctomycetes-like characteristics was also observed in enrichment culture P3, where the abundance of Planctomycetes was found to be 2,1% (Figure 3.4). However, no axenic Planctomycetes culture was obtained from this enrichment culture, as no further growth was detected when the cells were transferred to fresh media.

Initially strain P2S seemed to be growing together with yeast like-cells (Figure 5.1), and in some cases the Planctomycetes cells seemed to be attached to the eukaryotic cell, almost feeding off them. However, after repeated streaking onto fresh solid media strain P2S was obtained in axenic culture.

Cells of strain P1a was not discovered in the initial enrichment culture before 173 days after the initial inoculation. When the strain first was observed, the culture was very dense and dominated by Planctomycetes cells. It was still hard to obtain them in axenic culture, as they did not respond well when supplemented with fresh media. Several different variations of M30 was tried including, M30 18% SW without ampicillin and M30 with no aged seawater added. Finally, the strain appeared in colonies on M30 18% SW gelrite plates and were further streaked onto fresh media until obtained in axenic culture.

6.2 Morphology

All strains displayed different internal and external morphological structure. When cultured in liquid media strain P1a appeared to be evenly suspended in the media, but in later growth stages formed small flake-like aggregates of cells that appeared grey-white in colour. In contrast strain P2S was suspended in the liquid media throughout incubation and gave the culture a transparent white colour. Strains P6.1 and P6.2 formed pink and red aggregates that gathered at the bottom of the tubes, as well as growing attached to the tubes near the surface of the liquid media. None of the other strains displayed any growth attached to the tubes when cultured in liquid media.

When grown on solid media, strain P1a formed grey-white colonies with creamy consistency. This was also true for strain P2S, except that the colour of the colonies was white. Strain P6.1 formed small pink colonies that were easy to detach from the solid media. In contrast strain P6.2, formed big red colonies that were hard to detach from the solid media, as well as hard to homogenize when transferred to liquid media.

6.2.1 Strain P1a

When studied by phase contrast light microscopy, strain P1a had cells that appeared to be spherical to ovoid in shape. Strain P1a formed mainly large aggregates of cells, but swarmer cells were also observed, as well as occurrences of rosette formation and budding reproduction (Figure 5.2). Indications of budding reproduction not happening from a reproductive pole were raised during phase contrast light microscopy (Figure 5.2b). This was again observed during transmission electron microscope analysis of the strain (Figure 5.3b). This is not the first time non-polar budding reproduction has been reported in Planctomycetes (Christian Jogler, personal communication, May 10, 2017). Furthermore, TEM analyses revealed that strain P1a had highly condensed nucleotides, as well as crateriform and fibrous structures along the outer membrane (Figure 5.3d). The cytosol of the strain was situated close in proximity to the outer membrane with very thin layer of periplasm (Figure 5.3b, c and d), in contrast to what displayed by strains P6.1 and P6.2. Strain P1a exhibited large invaginations of the periplasm surrounded by a membrane (Figure 5.3c). The invagination appears as a compartmentalized structure, however because of the way the cross sections are cut,

it is more likely to be an invagination of the periplasm like described by Boedeker and collaborates (2017).

6.2.2 Strain P2S

Strains P2S appeared as single motile swarmer cells or in rosette formations of no more than 2-3 cells (Figure 5.4). Cells that were arranged in rosette formations seemed to be interconnected *via* tubular-like structure (Figure 5.4), possibly non-prosthecate stalks used as holdfast structures, as described by Fuerst (1995). The motile swarmer cells moved with “wriggling” motions, indicating the presence of flagella, however no such structures were observed when studied under the transmission electron microscope. The fact that strain P2S appear as single cells or in rosette formations of no more than 3 cells, as well as being motile, might explain how the strain remains suspended in liquid culture and not as aggregates as the other strains. TEM analyses of strain P2S revealed highly condensed nucleotides, surrounded by electron dense material, indicating the presence of ribosomes (Figure 5.5). The strain also displayed the same invaginations of the periplasm as strain P1a, also with a clearly defined membrane surrounding the invagination. Furthermore, strain P2S exhibited an outer membrane situated very close to the cytosol, in the same way as strain P1a.

6.2.3 Strain P6.1

When observed by phase contrast light microscopy strain P6.1 showed clear signs of rosette formation and budding reproduction (Figure 5.6). The strain also exhibited motile swarmer cells, possibly daughter cells, and that the mother cells stay attached and arranged in rosette formations, as described by Fuerst (1995). The budding shown by the strain seemed to happen from a reproductive pole (Figure 5.6b). Furthermore, TEM analyses of the strain revealed that cells arranged in rosette formations were held together by holdfast structures (Figure 5.7 a and c). Budding reproduction was displayed during TEM analyses (Figure 5.7 b and d), showing budding during the later stages due to the already transferred genetic material (Lee et al., 2009). The strain also displayed highly condensed nucleotides, as well as well-defined fibrous structures. The fibrous structures seemed to be connected to the outer membrane through crateriform structures (Figure 5.7d), this match what proposed by Boedeker and

collaborates (2017). Strain P6.1 displayed a large space between the outer membrane and the cytosol, the periplasm, which differs to what found in strains P1a and P2S.

6.2.4 Strain P6.2

Strain P6.2 displayed large, rubber-like colonies on solid media that were hard to detach. When studied under phase contrast light microscope strain P6.2 exhibit large cell aggregates, were the cells are arranged very dense (5.8). The large aggregates and the dense cell arrangement could possibly explain why colonies were hard to detach from solid media and to homogenize in liquid culture. Furthermore, phase contrast light microscopy revealed that strain P6.2 form rosette formation of several cells, as well as budding reproduction (Figure 5.8b). Swarmer cells were also observed, however, they did not appear to be motile. Transmission electron microscopy analyses revealed that strain P6.2 had highly condensed nucleotides as well as crateriform and fibrous structures (Figure 5.9). The strain also displayed large invaginations of the periplasm (Figure 5.9b), however, where strains P1a and P2S had a clearly defined membrane surrounding the invaginations, strain P6.2 have electron dense material, resembling ribosomes, surrounding its invaginations. As for strain P6.1, strain P6.2 also have a clearly defined space between the outer membrane and the cytosol. As shown by Boedeker and collaborates (2017), this area resembles the periplasm, and it appears to be larger in strains P6.1 and P6.2, than in strains P1a and P2S.

6.3 Physiological Characterization

Temperature optimum and range were determined for strains P2S, P6.1 and P6.2. Strain P1a was not included, because it was obtained in axenic culture at a later stage of this study. Temperature optimum for all strains were determined to be 25°C (Figure 5.10), similar to the closely related *R. obstinata* (Bondoso et al., 2015). The strains were able to grow in temperatures ranging from 5-35°C, however, strain P2S exhibited very limited growth at 35°C. The temperature range of the strains indicate that they are phychrotolerant (Morita, 1975). The strains were able to grow at temperatures both lower and higher than *R. obstinata*. Growth at low temperatures have previously been reported in several Planctomycetes including; *R. baltica* (5-30°C) (Bondoso et al., 2015), *T. sphagniphila* (6-30°C) (Kulichevskaya et al., 2012B), and *S. acidiphila* (4-33°C)

(Kulichevskaya et al., 2008). Growth at higher temperatures have also been recorded previously in Planctomycetes, ranging as high as 55°C (Giovannoni et al., 1987).

The salinity optimum of strains was determined to be 0,1% (NaCl w/v) for P6.1, between 0 and 0,1 (NaCl w/v) for P6.2, and 0,5% (NaCl w/v) for strain P2S (Figure 5.11). Strains P6.1 and P6.2 displayed a salinity growth range between 0 and 0,5% (NaCl w/v), while strain P2S were able to grow in salinity concentrations ranging from 0 to 3,5% (NaCl w/v), indicating that the three strains are halotolerant (Ollivier et al., 1994). Halotolerance in terrestrial Planctomycetes has previously been recorded including; *S. paludicola* and *S.* singulisphaera (0-0,5% NaCl w/v) (Kulichevskaya et al., 2007, 2008), *Z. Formosa* (0-0,6 NaCl w/v) (Kulichevskaya et al., 2009), and *S. rosea* (0-1% NaCl w/v) (Kulichevskaya et al., 2012A). Planctomycetes with high salinity tolerance have also been recorded in *R. brasiliensis* isolated from salt pit water, able to grow in salinity concentrations of 10% NaCl (w/v) (Schlesner, 1989). The Svarthammarhola strains' tolerance towards NaCl could be caused by the use of aged seawater in the enrichment media, however it is also possible that salts were present in the cave and ice mass, especially in the soil and organic matter where strain P2S originated from.

The pH optimum for growth of strain P6.1 and P6.2 was determined to be at 7,5, while strain P2S exhibited the strongest growth when cultivated in media with pH adjusted to 8 (Figure 5.12). All strains were able to grow on all the different pH values growth were tested, hence a growth range of pH ranging from 5 to 9. The optimum and growth range of the strains indicate that they all are neutrophilic (Tortora et al., 2015). Considering the pH of the initial ice samples (Table 3.1), the pH optimum of strains P6.1 and P6.2 deviate from the pH of the initial sample. The pH of the initial sample was 6,57, however the strains might have adapted considering the pH of the enrichment media. The pH of sample P2 was 8,18, and the pH optimum for strain P2S (pH = 8) does not deviate much from this, however strain P2S was isolated from soils and organic matter in sample P2, where pH was not measured. Broad pH growth range have previously been recorded in Planctomycetes, the closely related *R. baltica* has been shown able to grow in media with pH ranging from 5,5 to 10,5.

6.4 Physiological Attributes

6.4.1 Utilization of Carbon sources

It was shown that the Svarthammarhola strains were able to utilize a variety of carbon sources. The substrates that seemed to provide the strongest growth for the strains was D-cellobiose, D-glucose, N-acetyl-D-glucosamine and D-maltose, as well as D-galactose for strain P1a (Table 5.1). These are all sugars based on or derived from glucose. D-Cellobiose and D- maltose are both disaccharides consisting of two glucose molecules, whereas N-acetyl-D-glucosamine is a derivative of glucose. The assimilation of these carbons has frequently been reported in Planctomycetes, including; Rhodopirellula *baltica* (Schlesner et al., 2004), *Telmatocola sphagniphila* (Kulichevskaya et al., 2012B), *Roseimaritima ulvae* and *Rubripirellula* obstinata (Bondoso et al., 2015), as well as in *Fuerstia marisgermanicae* (Kohn et al., 2016). In 2015 Bondoso and collaborates (2015) showed that *R. ulvae* was able to assimilate 15 out of the 17 carbon sources tested in this experiment. However, utilization of some of these substrates was determined by using the GN2 Microplates (Biolog, USA), which is not optimized for environmental bacteria.

The Svarthammarhola strains were also shown to assimilate sucrose, D-galactose and D-mannose (Table 5.1). Strain P2S were also able to grow on xylose derived from wood, raffinose a trisaccharide and rhamnose, a deoxy sugar, indicating the ability to assimilate complex sugars. Planctomycetes have previously been proposed to be able to assimilate complex carbon sources (Lage and Bondoso, 2012). Strains P6.1 and P6.2 also displayed growth when grown on the sugar alcohol D-mannitol which is produced by several organisms, including plants (Song and Vieille, 2009). D-Mannitol is also an isomer to sorbose, which all the strains exhibited limited growth when cultured in (Table 5.1).

6.4.2 Enzymatic Activity

By using the API ZYM test strips, it was shown that the Svarthammarhola strains possessed different enzymatic activities. However, two enzymes were shown to be present in all strains, these include, Esterase lipase (C8) and Naphthol-AS-BI-phosphohydrolase (Table 5.2). Otherwise alkaline phosphatase was found in all strains except P1a. Which is an enzyme common in both eukaryotes and prokaryotes and

provides a source of inorganic phosphate when the environment does not provide one (Sharma et al., 2014). Also, esterase (C4) and valine arylamidase are found in 3 of the strains (Table 4.2). Lipase (C14) was only reported in strain P1a, lipase hydrolyses lipids for nutritional purposes and are essential for intracellular processes like transport and processing of lipids (Svendsen, 2000). Strain P1a was also the only strain that were positive for acid phosphatase, which frees attached phosphoryl groups from other substances. Acid phosphatase has a low pH optimum and are used by soil microorganisms to access organically bound phosphate (Bull et al., 2002). Strains P1a's negative results for alkaline phosphatase and positive result for acid phosphatase might indicate that the strain obtains vital phosphate only from organic sources.

All strains except P2S tested negative for α-glucosidase (Table 5.2), which is an enzyme that breaks down starch and disaccharides into glucose (Chiba, 1997). Considering that all strains exhibited good growth when grown in media containing disaccharides (sucrose, cellobiose and maltose) this seem a bit strange. At the same time there are other enzymes that can be used to in the hydrolysis of disaccharides like maltase and sucrase. None of the strains were positive for α-mannosidase, which cleaves the α-form of mannose (Li, 1966), although all strains displayed growth when cultured in media containing mannose.

When compared to other Planctomycetes, the Svarthammarhola strains, seems to have a more limited enzymatic repertoire. Strain P1a had the broadest enzymatic repertoire and tested positive for 7 enzymes. *Fuerstia marisgermanicae* were positive for 11 enzymes from the API ZYM test (Kohn et al., 2016), *R. obstinata* for 7 enzymes (Bondoso et al., 2015), and *R. baltica* were positive for 13 enzymes (Schlesner et al., 2004).

6.4.3 Antibiotic Resistance and Sensitivity

By cultivating the Svarthammarhola strains on M30 18% SW plates with antimicrobial susceptibility disks (Oxoid), the resistance and sensitivity of the Svarthammarhola strains were displayed. Strains P1a, P6.1 and P6.2 were shown to be resistant towards 9, 8 and 9, respectively, out of the 11 antibiotics used in this experiment (Table 5.3). Strain P2S was resistant towards 5 of the antibiotics. This indicates that the strains from Svarthammarhola are Planctomycetes with resistance towards a broad spectrum

of antibiotics. Planctomycetes have previously shown to be good representatives for broad-spectrum antibiotic-resistant organisms as they are naturally resistant to some antibiotic families (Cayrou et al., 2010).

Planctomycetes has been found to be resistant towards β-lactam antibiotics (Cayrou et al., 2010), the same was true for the Svarthammarhola strains, as all of them displayed resistance towards ampicillin and penicillin G (Table 5.3). All strains were sensitive towards erythromycin which binds to the 50S unit of the bacterial rRNA and inhibits protein synthesis, as well as structural and functional processes (Trevor et al., 2015A). Furthermore, all strains except strain P1a showed sensitivity towards chloramphenicol, which is a bacteriostatic and inhibits protein synthesis by binding to the 23S and 50S rRNA, preventing peptide bond formation (Schifano et al., 2013). In 2010, Cayrou and collaborates reported that most Planctomycetes were resistant to chloramphenicol, this contradicts what found in the Svarthammarhola strains. Strains P6.1 and P6.2 were the only strains sensitive to tetracycline, which binds to the 30S rRNA and prevents the introduction of new amino acids to peptide chains, hence, inhibiting protein synthesis (Connell et al., 2003). All strains were also resistant towards streptomycin, a protein inhibitor that binds to the 16S and 30S rRNA subunit (Sharma et al., 2007), nalidixic acid, a bacteriostatic inhibiting growth by acidification or inhibition of DNA gyrase (Trevor et al., 2015B), and vancomycin, which inhibits cell wall synthesis in Gram-positive bacteria (Trevor et al., 2015C).

In this study it has been shown that the strains isolated from Svarthammarhola represents antibiotic resistance of a broad spectrum. Especially strains P1a and P6.2 are of interest, considering that they showed resistance towards 9 out of 11 antibiotics used in this study.

6.5 Phylogeny

The 16S rRNA gene was sequenced for all 4 strains by using the Planctomycetes specific forward primer Pla46f (Neef et al., 1998) and the general prokaryotic reverse primer H1552r (Pantos et al., 2003). This proved successful as almost entire 16S rRNA genes was obtained for all strains. Differences in the 16S rRNA gene between the strains was displayed, as well as differences towards their closest relatives.

As shown in Table 5.4, strains P2S, P6.1 and P6.2 displayed 98 and 99% similarity towards one another. Despite their genetic similarities, the 3 strains showed morphological and physiological differences. The closest cultured relative of the three strains was strain Schlesner 302 (Ward et al., 1995), with similarities of 92 and 91% (Tables 5.6-8), and the strains' closest described species were *R. baltica* and *R. lusitana* (Schlesner et al., 2004; Bondoso et al., 2014), with similarities of 90%. Strain P1a showed similarities towards strains P2S, P6.1 and P6.2 of 87, 88 and 87%, respectively (Table 5.4). The closest cultured relative of the strain was Planctomycete strain 292 (Griepenburg et al., 1999), and the closest described species were *B. goksoyri* (Storesund and Øvreås, 2013), with a similarity of 90% (Table 5.5). Considering the proposed taxonomic boundaries for genus of cultured bacterial 16S rRNA gene sequences being 94,5% (Yarza et al., 2014), strains P2S, P6.1 and P6.2 represent one novel genus, while strain P1a represent another novel genus.

In Figure 5.13, the maximum likelihood 16S rRNA gene phylogenetic tree shows strains P2S, P6.1 and P6.2 forming a monophyletic group together with their closest uncultured relatives, branching away from strain Schlesner 302, with a bootstrap value of 100%. Furthermore, strain P1a forms a monophyletic group together with its closest uncultured relatives, branching away from its closest cultured relatives, including *B. goksoyri*. The bootstrap value of the branch was 100%.

There are always biases included in datasets based solely on one gene. The phylogenetic tree reflects the evolution of the 16S rRNA genes but not necessary the true phylogeny of the organisms from which the genes are obtained. However, the 16S rRNA gene is considered a very slow evolving gene and is conserved across the domains (Rosello-Mora and Amann, 2001).

6.6 Screening for Bioactive Molecules

In this study, the ability of strains P6.1 and P6.2 to induce intermediate apoptosis in the cancerous cell line Molm13 was demonstrated (Figure 5.15). The two strains also showed low to no toxicity towards the human prostatic cancer cell line PC3 and the normal healthy rat kidney cell line NRK. The screening of bioactive molecules for these two strains were part of a project including extracts from several Planctomycetes strains (Appendix IV). When compared to the other Planctomycetes in this project, the extracts from strains P6.1 and P6.2 showed a higher toxicity towards Molm13 cells than most of the other strains' extracts. However, there were also strains that displayed higher cytotoxicity than strains P6.1 and P6.2.

Knowledge regarding bioactive substances in Planctomycetes is very limited. However, recently Planctomycetes have been recognized as important producers of bioactive compounds by *in silico* genome mining, as well as molecular screenings. In these studies, the presence of genes associated with different pathways for production of several bioactive molecules, like the antitumor compound epothilone (Donadio et al., 2007; Graça et al., 2013; Wang et al., 2014; Graça et al., 2016; Ivanova et al., 2017; Vollmers et al., 2017). Therefore, it is very promising that strains P6.1 and P6.2, as well as several of the strains included in Appendix IV, showed significant cytotoxic activity towards the Molm13 cells.

The cytotoxicity the extracts from strains P6.1 and P6.2 showed against Molm13 cells (Figure 5.15) is especially interesting and can provide indication of how the strains interact in the microbial ecosystem of the ice mass in Svarthammarhola ice cave. Considering that the strains were rather slow growing, the ability to produce bioactive compounds that is cytotoxic to other cells may provide an advantage for the strains in their natural habitat. In that way they might be able to compete with other faster growing heterotrophic bacteria for resources and habitat.

6. Conclusion

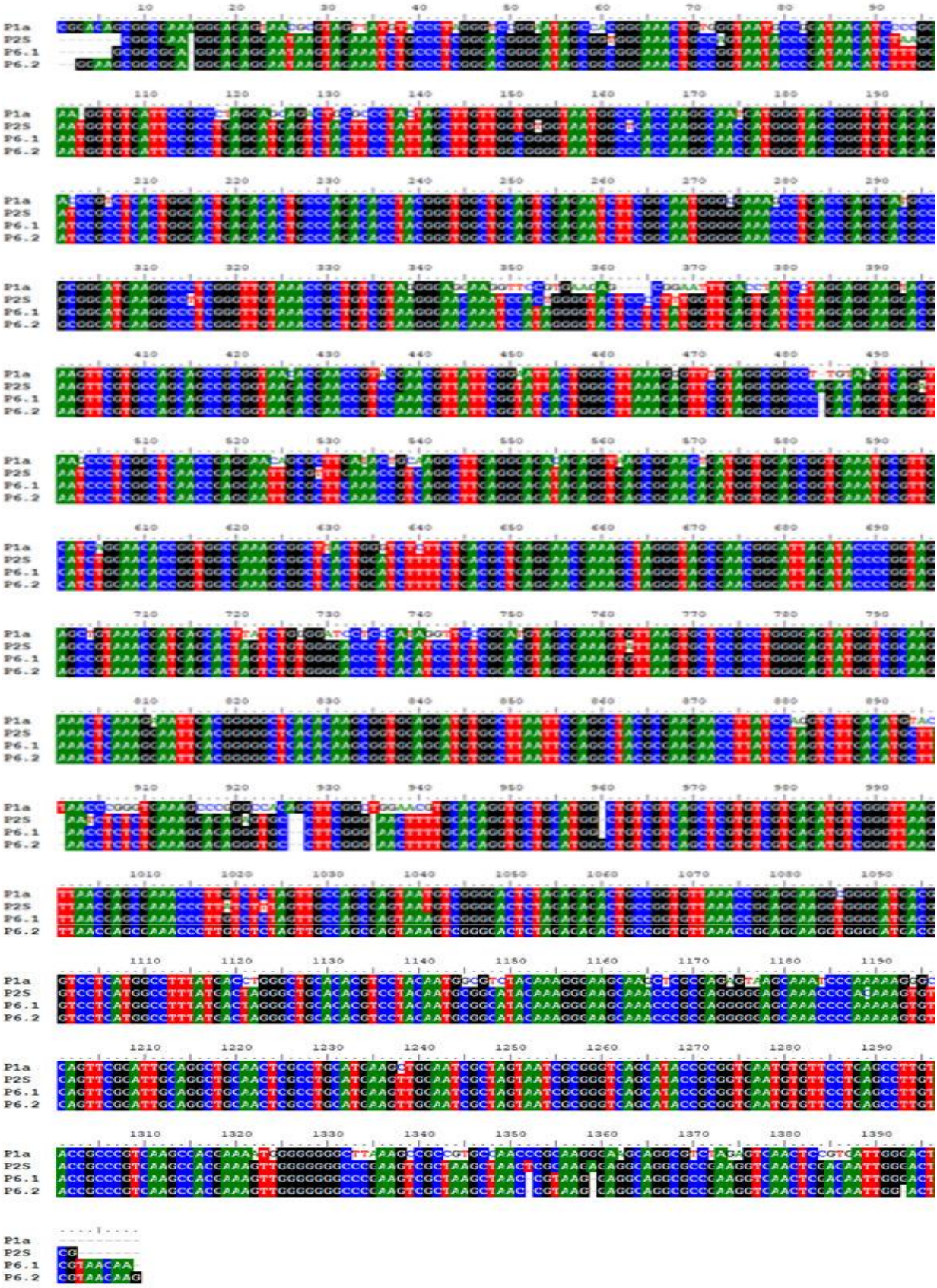
In this study it has been shown that Planctomycetes are present in the ice mass of Svarthammarhola ice cave. Four novel Planctomycetes strains were obtained from the ice and their characteristics and phylogenetic relationship propose that Planctomycetes are indigenous in the cave microbiome. Whether the strains were active in the ice mass is hard to say, but they are definitely alive and conserved. By screening two of the strains' extracts for bioactive molecules it has been shown that they produce secondary metabolites displaying cytotoxicity. These metabolites can possibly be used actively by the strains to defend their habitats and resources towards other faster growing heterotrophic bacteria. As these compounds can be used for medical purposes, this study shows the importance of targeting extreme and remote habitats, like ice caves, as a source for novel bacteria with potential of biotechnological applications. It is important to keep studying such environments, as knowledge regarding the ecology, metabolism and function of inhabiting organisms can lead to discoveries of great societal value.

7. Suggestions for Future Work

In future studies of the Svarthammarhola strains, their genomes should be further investigated. A complete genome sequencing of the strains would provide insights to their metabolic features as well as their functionality. Furthermore, physiological characterization regarding growth conditions was not provided for strain P1a. This should be further tested in order to provide a more thorough characterization. It would also be interesting to cultivate the strains at temperatures lower than 5°C, and even lower than 0°C, to see if the strains can grow in conditions similar to that in frozen ice. The strains should also be examined by scanning electron microscopy, to further investigate on the presence of flagella, as well as gaining further insights to the budding reproduction process. Strain P1a displayed indications of budding occurring from the side of the cell, this needs to be further investigated. It would also be interesting to elucidate on the strains resistance and sensitivity towards antibiotics, by conducting the same experiments using other antibiotics.

Further studies should also target the bioactive compounds found in the Svarthammarhola strains. In that way, gaining insights to which molecules are causing the cytotoxicity displayed towards cancerous cell lines used in this study.

Supplementary Figures



Supplementary Figure 1.1: Alignment of 16S rRNA gene of strains P1a, P2S, P6.1 and P6.2. Alignment was performed in the BioEdit software (Hall, 1999; Hall 2007) using ClustalW.

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Appendix I: Hutner's Basal Salts

Component	Amount
Nitrilotriacetic acid	5 g
$MgSO_4$ x $7H_2O$	13,85 g
CaCl x $2H_2O$	1,67 g
$NaMoO_4$ x $2H_2O$	6,3 mg
$FeSO_4$ x $7H_2O$	49,5 mg
Metal salts solution «44»	50 ml
Double distilled water	450 ml

Metal salts solution "44"

Component	Amount
Ethylene diamino tetra acetate (EDTA)	25 mg
$ZnSO_4$ x $7H_2O$	109,5 mg
$FeSO_4$ x $7H_2O$	50 mg
$MnSO_4$ x H_2O	15,4 mg
$CuSO_4$ x $5H_2O$	3,9 mg
$CoCl_2$ x $6H_2O$	2,0 mg
$Na_2B_4O_7$ x $10H_2O$	1,8 mg
Double distilled water	100 ml

Appendix II: Vitamin Solution No. 6 – 10x Solution

Component	Amount
Biotin	4 mg
Pyridoxine hydrochloride	20 mg
Thiamine hydrochloride	10 mg
Ca pantothenate	10 mg
p-Aminobenzoic acid	10 mg
Folic acid	4 mg
Riboflavin	10 mg
Nicotinic acid	10 mg
Vitamin B12	0,2 mg
Double distilled water	100 ml

Appendix III: 16S rRNA Gene Sequences

>P1a 16S rRNA Gene Sequence

CGGACAGCGGCGAAAGGGAGAGTAACGCGTAGTTATGTACCCTAGGGTCCGGAATAGCCA

CGGGAAACTGTGGGTAATGCCGGATAACATCCCCGGATCAAAGGTGTGATTCCGCCCTAG

GAGCAGACTGCGCCCTACTAGCTTGTTGGTGGGGTAATGGCCCACCAAGGCAATGATGGG

TAGCGGGTGTGAGAGCATGACCCGTCTCACTGGGACTGAGACACTGCCCAGACACCTACG

GGTGGCTGCAGTCGAGAATCTTCGGCAATGGGCGAAAGCCTGACCGAGCGATGCCGCGTG

CGGGATGAAGGCCCTCGGGTTGTAAACCGCTGTCGTAGGGGAGGAAGGTTCCGTGAAGAG

CGGAATTTGACCTATCCTAGGAGGAAGTACGGGCTAAGTTCGTGCCAGCAGCCGCGGTAA

CACGAACCGTACGAACGTTATTCGGAATTACTGGGCTTAAAGGGTTTGTAGGCGGCCTTG

TAAGTCAGGTGTGAAAGCCCTCGGCTCAACCGAGGAACAGCGCTTGATACTGCAAGGCTT

GAGGGAGACAGAGGTAAGCGGAACTGATGGTGGAGCGGTGAAATGCGTTGATATCATCAG

GAACACCGGTGGCGAAAGCGGCTTACTGGGTCTCTTCTGACGCTGAGGAACGAAAGCTAG

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GGGATCCTCCCATAGGTTCCCGGATGTAGCGAAAGTGTTAAGTGCTCCGCCTGGGGAGTA

TGGTCGCAAGGCTGAAACTCAAAGAAATTGACGGGGGCTCACACAAGCGGTGGAGGATGT

GGCTTAATTCGAGGCTACGCGAAGAACCTTATCCAGGTCTTGACATGTACGGATTAACCC

GGGTGAAAGCCCGGGCCACAGCTTCGGCTGGAACGTGCACAGGTGCTGCATGGCTGTCGT

CAGCTCGTGTCGTGAGATGTCGGGTTAAGTCCCTTAACGAGCGAAACCCTTGTCTCTAGT

TGCCAGCGAGTAATGTCGGGGACTCTAGAGAGACTGCCGGTGTTAAACCGGAGGAAGGCG

GGGATGACGTCAAGTCCTCATGGCCTTTATGACCTGGGCTGCACACGTCCTACAATGGCG

TCTACAAAGGGAAGCAAGCTCGCGAGAGTAAGCAAATCCCAAAAAGGGCGCCTCAGTTCG

GATTGCAGGCTGCAACTCGCCTGCATGAAGCTGGAATCGCTAGTAATCGCGGGTCAGCAT

ACCGCGGTGAATGTGTTCCTGAGCCTTGTACACACCGCCCGTCAAGCCACGAAAATGGGG

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ATTGGGACTAAGT

>P2S 16S rRNA Gene Sequence

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TGTGAGAGCACGATCCGCCTCACTGGGACTGAGACACTGCCCAGACACCTACGGGTGGCT

GCAGTCGAGAATCTTCGGCAATGGGGGAAACCCTGACCGAGCGACGCCGCGTGCGGGATG

AAGGCCTTCGGGTTGTAAACCGCTGTCGTAAGGGAACAAATCCACTGGGGTACTCCCCTT

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GAACCGTCCGAACGTTATTCGGTATTACTGGGCTTAAAGAGTTCGTAGGCGGCCCAGACA

GGTCAGATGTGAAATCCCTCGGCTCAACCGAGGAATTGCGTTTGAAACCGTCAGGCTTGA

GGGAGATAGAGGTGAGCGGAACAGATGGTGGAGCGGTGAAATGCGTTGATATCATCTGGA

ACACCGGTGGCGAAAGCGGCTCACTGGATCTTTTCTGACGCTGAGGAACGAAAGCTAGGG

TAGCGAACGGGATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGAGCACTAGTCTGTG

GGGACCCTCACATCCTCTCGGACGTAGCGAAAGTATTAAGTGCTCCGCCTGGGGAGTATG

GTCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCTCACACAAGCGGTGGAGGATGTGG

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GAAAGGAGAGAGTGCCTTCGGGAACTTTTGCACAGGTGCTGCATGGCTGTCGTCAGCTCG

TGTCGTGAGATGTCGGGTTAAGTCCCTTAACGAGCGAAACCCTTATCTTTAGTTGCCAGC

GAGTAATGTCGGGGACTCTAGAGAGACTGCCGGTGTTAAACCGGAGGAAGGTGGGGATGA

CGTCAAGTCCTCATGGCCTTTATGACTAGGGCTGCACACGTCCTACAATGCGGCATACAA

AGGGAAGCAAACCCGCGAGGGGGAGCAAACCCCACAAAGTGTCGCTCAGTTCGGATTGCA

GGCTGCAACTCGCCTGCATGAAGTTGGAATCGCTAGTAATCGCGGGTCAGCATACCGCGG

TGAATGTGTTCCTGAGCCTTGTACACACCGCCCGTCAAGCCACGAAAGTTGGGGGGGCCC

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CTAAGTCG

>P6.1 16S rRNA Gene Sequence

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TCTACTTCCTATTAGCTTGTTGGCGGGGTAATGGCCCACCAAGGCAACGATGGGTAGCGG

GTGTGAGAGCACGATCCGCCTCACTGGGACTGAGACACTGCCCAGACACCTACGGGTGGC

TGCAGTCGAGAATCTTCGGCAATGGGGGAAACCCTGACCGAGCGACGCCGCGTGCGGGAT

GAAGGCCCTCGGGTTGTAAACCGCTGTCGTAAGGGAACAAATCCATAGGGGTACTCCTCT

ATGGTTGAGTGATCTTAGGAGGAAGGACGGGCTAAGTTCGTGCCAGCAGCCGCGGTAAGA

CGAACCGTCCAAACGTTATTCGGTATCACTGGGCTTAAAGAGTTCGTAGGCGGCCCGACA

GGTGAGGTGTGAAATCCCTCGGCTCAACCGAGGAATTGCGCTTCAAACCGTCAGGCTTGA

GGGAGATAGAGGTGAGCGGAACAGATGGTGGAGCGGTGAAATGCGTTGATATCATCTGGA

ACACCGGTGGCGAAAGCGGCTCACTGGATCTTTTCTGACGCTGAGGAACGAAAGCTAGGG

TAGCGAACGGGATTAGATACCCCGGTAGTCCTAGCCGTAAACGATGAGCACTAGTCTGTG

GGGACCCTCACATCCTCTCGGACGTAGCGAAAGTGTTAAGTGCTCCGCCTGGGGAGTATG

GTCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCTCACACAAGCGGTGGAGGATGTGG

CTTAATTCGAGGCTACGCGAAGAACCTTATCCTAGTCTTGACATGCTTAAGAACCTCTCT

GAAAGGAGAGGGTGCCTTCGGGAACTTTTGCACAGGTGCTGCATGGCTGTCGTCAGCTCG

TGTCGTGAGATGTCGGGTTAAGTCCCTTAACGAGCGAAACCCTTGTCTCTAGTTGCCAGC

GAGTAAAGTCGGGGACTCTAGAGAGACTGCCGGTGTTAAACCGGAGGAAGGTGGGGATGA

CGTCAAGTCCTCATGGCCTTTATGACTAGGGCTGCACACGTCCTACAATGCGGCATACAA

AGGGAAGCAAACCCGCGAGGGGGAGCAAACCCCAAAAAGTGTCGCTCAGTTCGGATTGCA

GGCTGCAACTCGCCTGCATGAAGTTGGAATCGCTAGTAATCGCGGGTCAGCATACCGCGG

TGAATGTGTTCCTGAGCCTTGTACACACCGCCCGTCAAGCCACGAAAGTTGGGGGGGCCC

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>P6.2 16S rRNA Gene Sequence

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TGAGTCTACTTCCTATTAGCTTGTTGGCGGGGTAATGGCCCACCAAGGCAACGATGGGTA

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TGGCTGCAGTCGAGAATCTTCGGCAATGGGGGAAACCCTGACCGAGCGACGCCGCGTGCG

GGATGAAGGCCCTCGGGTTGTAAACCGCTGTCGTAAGGGAACAAATCCATAGGGGTACTC

CTCTATGGTTGAGTGATCTTAGGAGGAAGGACGGGCTAAGTTCGTGCCAGCAGCCGCGGT

AAGACGAACCGTCCAAACGTTATTCGGTATCACTGGGCTTAAAGAGTTCGTAGGCGGCCC

GACAGGTGAGGTGTGAAATCCCTCGGCTCAACCGAGGAATTGCGCTTCAAACCGTCAGGC

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TATGGTCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCTCACACAAGCGGTGGAGGAT

GTGGCTTAATTCGAGGCTACGCGAAGAACCTTATCCTAGTCTTGACATGCTTAAGAACCT

CTCTGAAAGGAGAGGGTGCCTTCGGGAACTTTTGCACAGGTGCTGCATGGGCTGTCGTCA

GCTCGTGTCGTGAGATGTCGGGTTAAGTCCCTTAACGAGCGAAACCCTTGTCTCTAGTTG

CCAGCGAGTAAAGTCGGGGACTCTAGAGAGACTGCCGGTGTTAAACCGGAGGAAGGTGGG

GATGACGTCAAGTCCTCATGGCCTTTATGACTAGGGCTGCACACGTCCTACAATGCGGCA

TACAAAGGGAAGCAAACCCGCGAGGGGGAGCAAACCCCAAAAAGTGTCGCTCAGTTCGGA

TTGCAGGCTGCAACTCGCCTGCATGAAGTTGGAATCGCTAGTAATCGCGGGTCAGCATAC

CGCGGTGAATGTGTTCCTGAGCCTTGTACACACCGCCCGTCAAGCCACGAAAGTTGGGGG

GGCCCGAAGTCGCTAAGCTAACCGTAAGGAGGCAGGCGCCGAAGGTCAACTCGACAATTG

GACTAAGTCGTAACAAG

Appendix VII: Anticancer Activity in Planctomycetes

Anticancer activity in Planctomycetes

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Abstract

There is a strong need to develop new drugs against many severe diseases. Therapy resistance is a major problem in for instance infectious diseases and cancer. Drug discovery has again turned to nature to search for molecules that can become drug leads. Although many bacterial phyla are extensively studied, some, like the *Planctomycetes*, remain largely unexplored as potential sources of new leads. Planctomycetes form a diverse group of bacteria with peculiar characteristics such as division by polar budding and absence of the FtsZ gene. Furthermore, they exhibit large genomes up to 12.5 Mb, and possess a high number of secondary metabolites as assessed by *in silico* genomic analysis. These characteristics have also revealed the presence of potential anticancer activity. Based on these promising characteristics, we wanted to investigate planctomycetes as a source for natural products with anticancer properties. Organic and aqueous extracts were obtained from cultivated planctomycetes strains originated from a variety of habitats such as marine systems (free living or attached to marine algae), deep marine iron hydroxide deposits, brackish water and glacier ice system. The extracts were screened for ability to inhibit cell growth, or induce cell death on two cancer cell lines, the human prostatic cancer cell line PC3, and human acute myeloid leukaemia (AML) cell line MOLM-13, as well as normal rat kidney epithelial cell line (NRK). Out of 39 strains, five exhibited cytotoxicity towards NRK cells, whereas 32 of the strains were toxic to the AML cell line, and four were toxic to the PC3 cell line. Four strains showed high toxicity and selectivity towards either one, or both of the cancer cell line

over the NRK-cells, and are potential producers of anti-cancer compounds. We found no correlation between bioactivity and strains habitat and geographic location but regarding phylogeny some *Rhodopirellula* spp. showed higher toxicity toward MOLM-13 cells. These results from the first anticancer screening with planctomycetes showed that these peculiar microorganisms should be further explored for anti-cancer compounds and that more effort must be put in providing culture collections for drug development purposes.

Introduction

Biotechnological search for new natural products is of utmost importance for the well-being and sustainability of humanity in the fight against many deadly diseases such as cancer and bacterial infections due to resistant strains (O'Neill, 2016). In Europe, cancer represents the second most important cause of death and morbidity with more than 3.7 million new cases and 1.9 million deaths each year (WHO, 2012). In the USA, values of 1 688 780 new cancer cases diagnosed and 600 920 cancer deaths were estimated for 2017 (Siegel Rebecca *et al.*, 2017). As the prophylactic treatment to prevent cardiovascular diseases improves, it is expected that cancer will become the leading cause of death within few years (Heron, 2016). This increase in cancer incidents represents new challenges. As the population ages, there will be a need for novel therapeutics with less harmful side-effects like cardiac failure or bone marrow depletion, often associated with therapy-induced mortality in elderly patients.

In the development of new therapeutics, the majority of drug candidates are natural derived compounds (Imhoff *et al.*, 2011). Some phyla of bacteria, *Actinobacteria*, *Myxobacteria* and *Cyanobacteria,* are well known producers of secondary metabolites (Jeske *et al.*, 2016) and have been extensively studied for bioactive compounds. Traditionally, terrestrial organisms have been targeted for the search of novel antibacterial compounds and secondary metabolites. Therefore, special attention is directed towards marine biological sources (Joseph & Sujatha, 2010) and less explored bacterial phyla. The marine environment is a huge ecosystem still highly underexplored. It thus represents a vast source for novel bioactive molecules that can fulfil the ever-increasing need for new therapeutics to meet the increasing demand of pharmaceuticals. The oceans are inhabited by an immense diversity of microorganisms that only recently started to be unveiled. Estimations foresee numbers exceeding 10^{29} bacterial cells in the open ocean, with an average cell concentration of 10^{6} per millilitre of seawater (Whitman *et al.*, 1998,

Amaral-Zettler *et al.*, 2010). It has become notorious that the study of novel bacterial phyla could lead to new bioactive molecules (Bredholt *et al.*, 2008). One of these phylogenetic groups are the *Planctomycetes*, bacteria that have an intricate, still not fully understood, cell biology with complex life cycles and large genomes. Recently, Planctomycetes have proven the capacity to produce antibiotics and antifungal molecules and also genome mining has demonstrated their promising biotechnological potential (Donadio *et al.*, 2007, Jeske *et al.*, 2013, Graça *et al.*, 2016, Jeske *et al.*, 2016, Boedeker *et al.*, 2017) . This is due to the presence of secondary metabolite genes or clusters related to various pathways for the production of several bioactive molecules, including some antitumor compounds like epothilone (Graça *et al.*, 2016).

Besides the *in silico* study of its potential, no study has hitherto addressed the anti-cancer proprieties of planctomycetes. In fact, the study presented here is the first anticancer screening performed with planctomycetes assessing the induction of apoptosis and the decrease of growth in cancer cell lines. Acute myeloid leukaemia (AML) cell lines were chosen because AML is one of the most aggressive forms of leukaemia and is associated with high rate of chemo resistant relapse, and dose-limiting side-effects. Moreover, therapies developed towards leukaemia have high translational value to other cancers. We also tested for activity towards a solid tumour derived cell line, using prostate cancer (PC) cell lines since PC is one of the most common cancer types and with high mortality rates, especially in western countries. Prostate cancer is the leading cause of death from cancer in USA (Siegel Rebecca *et al.*, 2017) and the third most common cause of death in Europe (Bray & Kiemeney, 2017). The results obtained with both cancer cell lines (AML and PC) were compared with the ability to induce cell death in a normal epithelial cell line. Our results show that planctomycetes represent a promising source for novel molecules that can become anticancer drug leads.

Material and Methods

Bacterial collections

The planctomycetes strains used in this study were obtained from several different habitats (Table 1), namely marine iron hydroxide deposits (13 strains), marine water column (1 strain), macroalgal surface (18 strains), a meromictic lake (5 strains) and a

glacier ice inside a cave (2 strains). The strains affiliate to diverse phylogenetic clusters within the *Planctomycetes* phylum (Supplementary Fig. 1).

Cultivation of planctomycetes

Pure cultures of planctomycetes were cultivated in 250 mL M13 or M30 media with 70 – 90 % seawater with the exception of the ice derived isolates that were cultivated in M30 with 18 % seawater (Table 1) at 25 ^{0}C, and 120 r.p.m. After 7 days incubation, the cultures were centrifuged for 15 min at 10 000 x g., and the supernatant discarded. The cell pellets were washed twice with sterile water and freeze-died for approximately 18 h (with temperature below - 40 ^{0}C and about 4 × 10 $^{-2}$ atm pressure). The final biomass was weighted and kept at -20 ^{0}C until further processing.

Preparation of planctomycetes extracts for cell lines experiments:

Six mL of methanol: MilliQ water: chloroform (1:1:1) were added to each freeze-dried pellet. Six mL of the mixture without cells were used as an extraction control. The samples were homogenized (Tempest Virtishear I.Q. from Virtis, Gardiner NY fitted with 10 mm rotor), at 20000 r.p.m., for two periods of 30 s, with cooling on ice for 10 s between the cycles. The samples were allowed to extract for 1 h at 4°C, with agitation after 30 min, and then centrifuged at 450 x g for 30 min at 4°C, to separate the aqueous and organic phases. For each sample: (i) the organic phase (bottom phase) was carefully transferred to a separate tube and evaporated to dryness in a vacuumed centrifuge (Eppendorf concentrator plus, Eppendorf AG, Hamburg, Germany). The dried extract was dissolved in DMSO in a ratio of 10 mg of initial freeze-dried biomass of the bacterial culture to 25 µL of DMSO. (ii) the aqueous phase (top phase) was treated in a similar way as the organic extract with the exception of the final step. The aqueous extracts were added 75 µL of MilliQ water, in addition to 25 µL DMSO for each 10 mg initial freeze-dried biomass.

Cancer cell lines and cytotoxicity assays:

Planctomycetes extracts were tested for their capacity to induce apoptosis in the normal epithelial rat kidney cell line NRK (ATCC no: CRL-6509), the human AML cell line MOLM-13 (Matsuo *et al.*, Quentmeier *et al.*, 2003) and in the human prostate cancer cell line PC3 (ATCC no.: CRL-1435).

MOLM-13 cells were cultured in RPMI medium (Sigma R5886), supplemented with 10% (v/v) foetal calf serum (Sigma F7524), 0.2 mM L-glutamine and added 50 IU/mL penicillin and 0.1 mg/mL streptomycin. The cells were cultured to a density between 8 - 80 x 10^4 cells/mL, and diluted by adding fresh medium with supplements. NRK and PC3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM - Sigma D-6429), with the same supplements as for MOLM-13 cells. The PC3 and NRK cells are adherent, and at 90% confluence, the cells were detached by mild trypsin treatment (0.30 mg/mL trypsin for 5 min at 37 °C) and reseeded in fresh medium with supplements at 40-50% confluence. Cells were incubated at 37 °C, in a humidified atmosphere with 5% CO_2. All media, serum and supplements, and reagents were from Sigma-Aldrich, St. Louis, MO, USA.

The cytotoxicity experiments were performed in 96-well plates. One hundred µL containing 6000 cells of NRK or PC3 cell lines were placed in each well 24 h before the addition of the extracts to allow cells attachment to the substratum. After 24 h the medium was replaced by fresh medium and the extracts were added. MOLM-13 cell suspensions (20 000 cells/well in 100 µL) were added to the plates at the same time as the extracts. For all cell lines, one µL of the organic extracts or 4 µL of the aqueous extracts were added, respectively, to 99 µL or 96 µL of cell culture. The same volume of DMSO was used as solvent control and a negative control of 100 µL cell culture was also made. Cells were incubated with the extracts for 72h. The viability of the cells was first monitored using the WST-1 assay metabolic assay (Roche, Mannheim, Germany) following the manufacturers instruction. Thereafter, the cells were fixed with 2% buffered formaldehyde (pH 7.4) containing 0.01 mg/mL of the DNA-specific fluorescent dye, Hoechst 33342 and morphology of the nuclei was visualised by fluorescence microscopy (Nikon Diaphot 300 fitted with a 40× Flu-Phase contrast lens and a DS-Fi3 camera) as described by Prestegard et al (2009). A minimum of hundred cells from each cell line were used to determine cell death microscopically. All extracts were tested for cytotoxic activity 3-5 times for each concentration.

Statistical analysis

Statistical analyses and visualization was done in R (R Core Team 2017) using the vegan (Oksanen *et al.*, 2015), ggplot2 (Wickham, 2009) and pheatmap packages (Kolde, 2015). To see if the toxic effect of Planctomycetes on the different cancer cell lines was related to strain phylogeny (*Blastopirellula, Gimesia, Mariniblastus, Rhodopirellula sp.*,

Rhodopirellula lusitana, Roseimaritima, Rubinisphaera or Pir 4) or original habitat (ice, iron hydroxides, meromictic lake, macroalgal surface or seawater) we used analysis of similarities (ANOSIM, vegan). The response variable was a dissimilarity matrix of individual strain effects (aqueous and organic, n=6) on the three cancer cell lines, and the predictors were strain phylogenetic affiliation and original habitat. A PCA analysis (999 perm) was used to further examine correlations between strain toxicity and the different cell lines.

RESULTS

A total of 39 planctomycetes were screened for cytotoxic bioactivity towards human AML cell line (MOLM-13) and human prostatic cancer cell line (PC3). The normal rat kidney epithelial cell line (NRK) was used as control. All cells were analysed for metabolic activity using the WST-1 assay which measures enzymatic conversion of a tetrazolium salt into a coloured metabolite. However, as several of the planctomycetes possess coloration that interferes with the WST-1 colorimetric assay, cytotoxicity was therefore only assessed through the nuclear morphology (see Supplementary Figure 2)

All the planctomycetes were cultivated for 7 days except strain L2, which was also grown for 15 days to assess a longer stationary phase on the potential production of bioactive molecules.

The extracts were tested at 1% for the organic extracts and 4% for the aqueous extracts, both corresponding to extracts from 0.4 mg freeze-dried material per 0.1 mL cell culture medium. First, it was tested whether the extracts exhibited cytotoxicity during 24-hours incubation. However, we noticed only modest cytotoxicity for all extracts after this incubation time. Since many cytostatics, like metabolic inhibitors, have a protracted cytotoxic effect, we decide to incubate the cells with the planctomycetes extracts for 72 hours before assessing for cell death. We divided the cytotoxic potential of the extracts into high - H (> 70 % cell death), intermediate - I (40-70% cell death), low - L (10-40 % cell death) or no (<10 % cell death) (Supplementary Table 1). In total, 40 aqueous and 40 organic extracts were analysed. The different cell lines were screened under the microscope for apoptotic nuclei and their percentage calculated (Supplementary Fig 2). Based on these values, a heat-map of the cytotoxic effects was constructed (Fig. 1). The organic extracts showed higher cytotoxic capacity than the aqueous extracts both in number (55% of the organic and 35% of the aqueous) and intensity (mean and median

cell death was, respectively, 26.4% and 12.10% for the organic and 13.6% and 6.0% for the aqueous extracts) (Figure 1 and Supplementary Table 1).

Only five extracts showed intermediate or high activity towards NRK cells; the aqueous extract from strain Plm2, and the organic extracts from strains 7mR, SH1, UC49.1 and Sm4. High or intermediate activity towards PC3 prostate cancer cells was detected only in the organic extracts from strains CcC6, L1, SH1, Sm4, UC16, and UC49.1. Of these, strains SH1, Sm4 and UC49.1 were also cytotoxic towards NRK cells, but strains CcC6 and L1 appeared selective towards PC3 cells over NRK cells.

In general the MOLM-13 cells were much more sensitive towards the planctomycetal extracts than the other two cell lines. When counting strains causing high and intermediate cytotoxicity, 17.5% of the strains were H, and 42.5 % I in the organic extracts, and 12.5% were H, and 25 % I in the aqueous extracts. Only strains P6.1, VLbF2, 1mW and VLsL2h produced cytotoxic activity in both extracts, the remaining strains had cytotoxicity in only one of the extracts.

From strain L2, extracts were obtained after 7 and 15 days of cultivation. After 7 days the organic extract was bioactive against MOLM-13 with low cytotoxicity (24 %) but after the 15 days a 3-fold increase in cytotoxicity (70 %) was seen (Fig. 1, Supplementary Table 1).

Some strains stood out as particularly promising regarding anticancer activity. The organic extracts from the *Rhodopirellula lusitana* strains CcC6 (H - 82.7% PC3 and I - 59% MOLM-13) and UF6 (L – 26% PC3 and H – 97% MOLM-13) and *Rhodopirellula* sp. L1 (H – 87% PC3 and I – 59% MOLM-13) induced cell death in the cancer cell lines selectively, i.e. strong activity towards both cancer cell lines, but not NRK-cells (Figure 1 and Supplementary Table 1). Another striking feature was that several planctomycetes were selective towards MOLM-13 cells only, such as *Rhodopirellula lusitana* strain CcC8 (I – 67%), UC13 (H – 91%), L2 (H – 70%) and *Planctomyces maris* VlrD4 (I – 44%) only in the organic phase. Furthermore, *Rhodopirellula* sp. FF4 (L (aq) – 26% and I (org) – 65%), *Rubinisphaera* sp. 1MW (H (aq) – 78% and I (org) – 46%), *Planctomyces maris* VLbF2 (H (aq) – 76% and I (org) – 64%) and *Blastopirellula marina* (H (aq) – 73% and L (org) –33%) showed activity both in aqueous and organic phases. *R. lusitana* seems to be highly bioactive in the organic extracts but not in the aqueous extracts (Fig. 2). The most cytotoxic strains belong to *Rhodopirellula* spp. (Fig. 2)

In this study, a broad range of phylogenetic different planctomycetes was selected covering diverse habitats and geographical origins (Table 1, Supplementary Fig. 1).

Effects of individual strains on cancer cell lines differed between different phylogenetic groups (ANOSIM, $p < 0.005$), with *Rhodopirellula* sp. and *R. lusitana*, in particular, showing higher toxicity toward MOLM-13 cells. However, no distinct patterns regarding habitat or geographical location (data not shown) were identified. In line with the ANOSIM analysis, PCA analysis also showed that the organic extracts from *Rhodopirellula* sp. and *R. lusitana* as well as *Rubrinisphaera* strains had stronger effects on MOLM-13 cells than aqueous extracts (Fig. 3). However, the opposite was the case for the extracts from *Blastopirellula*, *Gimesia*, *Pir 4* and *Rubinisphaera* strains (Fig. 3).

Discussion

In this study, we have demonstrated the capacity of planctomycetes to induce apoptosis and decrease cell growth of two cancer cell lines; the acute myeloid leukaemia MOLM-13 and the human prostatic PC3. A considerable high number of planctomycetes were able to affect the MOLM-13 cells (32 strains) whereas a lower number affected PC3 cells (six strains).

Little is known about bioactive substances in planctomycetes, as compared to other microorganisms such as actinobacteria and, in particular, streptomycetes, myxobacteria, and cyanobacteria. However, planctomycetes have recently been recognized as important producers of bioactive compounds by both *in silico* genome mining analyses and molecular screenings. These analyses revealed the presence of genes related to various pathways for the production of several bioactive molecules, including some antitumor compounds like epothilone (Donadio *et al.*, 2007, Graça *et al.*, 2013, Wang *et al.*, 2014, Graça *et al.*, 2016, Ivanova *et al.*, 2017, Vollmers *et al.*, 2017). It is therefore very promising that several of our planctomycetes strains isolated from various ecosystems showed significant cytotoxic activity towards the MOLM-13 cells and the human prostatic PC3 cells.

The cultivation of planctomycetes is challenging as they are slow growing bacteria and therefore production of intermediate and high scale of biomass is not always straightforward. In this study we were able to establish growth conditions that allowed considerable biomass production. For strain L2 a prolonged growth period (15 days instead of 7 days) was included and results from this experiment seemed to favour the formation of bioactive molecules (Fig. 1). It should be emphasized that in both situations the cultures were in the same stationary growth phase. The only differences are the duration for the production of bioactive molecules or an increased stress by the aging of

the culture. Planctomycetal growth time is therefore an important parameter to be considered in future work.

Four strains induced high toxicity (> 70%) against NRK control cells. Interestingly, strain UC49 was highly toxic towards all cell lines in the organic extract, but produced no toxic substances in the aqueous extracts towards any of the cell lines. This demonstrates that the two obtained extracts, aqueous and organic, were different regarding the composition on planctomycetes metabolites, similar to what has been demonstrated for cyanobacteria (Liu *et al.*, 2014, Humisto *et al.*, 2015). Still seven strains showed activity in both extracts (>40%), which is likely to be caused by compounds with intermediate hydrophobicity present in both extracts.

The MOLM-13 cells were in general more sensitive towards the extracts compared to the prostate cancer cells (Fig. 1). We have previously observed that the activity towards the MOLM-13 cells in the aqueous extracts can be due to high levels of adenosine (Prestegard *et al.*, 2009, Liu *et al.*, 2014). Adenosine has been shown to induce apoptosis in MOLM-13 cells (Tanaka et al., 1994). However, in our study, we used non-heat inactivated foetal bovine serum, where adenosine deaminase is intact, and able to convert adenosine into the inactive metabolite inosine. Thus, the anti-AML activity seen in the aqueous extracts in our experimental assays could not be due to adenosine. The selectivity towards AML compared to NRK or PC3 could be due to the more rapid proliferation rate of MOLM-13 cells, and could point towards a mode of action related to DNA replication or the mitotic machinery.

The most promising results regarding the anticancer activity were obtained with the organic extracts from the *R. lusitana* strains (CcC6, L1 and UF6, Fig. 1). They showed activities towards both MOLM-13 cells and PC3 cells, but not towards the NRK cells. Interestingly the phylogenetic related *R. baltica* also produced extracts showing high toxicity towards the cancer cell lines but also against the NRK cells. This demonstrates the importance of a broad culture collection for bioprospecting purposes, since even highly related strains can give very different bioactivities.

Planctomycetes attached to algal surfaces seemed to be most active towards the cell lines tested as they produced the most potent anti-cancer compounds in this study. This could be explained by the high level of competition in macroalgal biofilms against eukaryotic cells such as microalgae, which trigger Planctomycetes production of secondary metabolites. These metabolites could selectively target cell signalling component

exclusively found in eukaryotes. If these targets are evolutionary conserved, they might even be present in mammalian cancer cells.

We did not find a strong correlation between biogeography and habitat of the most promising candidates, as L1 was isolated from a kelp surface from Kongsfjorden in the Arctic (Svalbard, Norway), whereas the CcC6 was isolated from the surface of *Chondrus crispus* of the Coast of Portugal. The cytotoxicity-producing planctomycetes were ubiquitously distributed and adapted to tolerate fluctuations in salinity, temperatures, light and nutrient regimes.

This work further confirms the potential of Planctomycetes as producers of important bioactivities by extending them to the production of anti-cancer compounds. Living in complex biodiverse environments, Planctomycetes are obliged to fight for their niche against many eukaryotic cells. To overcome their slow growth rate, they produce chemicals towards their competitors. This can explain why the Planctomycetes produce substantial bioactive substances that also might be targeting important processes in eukaryotic cells. As these molecules can be used for medical purposes, this study enlightens the importance of exploring molecules produced by planctomycetes for drug development. In the light of the evolutionary history, large genome size and diverse metabolic pathways, we foresee that several unique and important compounds may appear in these enigmatic bacteria.

Acknowledgment

This research was partially supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020, the Structured Program of R&D&I INNOVMAR—Innovation and Sustainability in the Management and Exploitation of Marine Resources (reference NORTE-01-0145-FEDER-000035, Research Line NOVELMAR), funded by the Northern Regional Operational Program (NORTE2020) through the European Regional Development Fund (ERDF), the ERA-netLAC project “Cave ice microbiom: metabolic diversity and activity in response to climate dynamics and anthropogenic pollution CAVICE (DCC-0178)” funded by the Norwegian Research Council (project number 256162) and the EU H2020-TWINN-2015, BLUEandGREN - Boosting scientific

excellence and innovation capacity in biorefineries based on marine resources (project number 692419).

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Captions:

Figure 1 - Heat-map showing the different degrees of toxicity that organic and aqueous extracts of planctomycetes induced in NRK, MOLM-13 and PC3 cell lines. Heat-map scale: 0-20% -no toxicity; 20-40%- low toxicity; 40-70% - intermediate toxicity; 70-100% high toxicity. White boxes – Not evaluated. Graph was created in R studio programme, using pheatmap package. Cluster analysis was based on Eucledian Distance

Figure 2 - Boxplots indicate the effects in % cell death on cancer cell lines (PC3 and MOLM-13) and controls (NRK) in aqueous (A) and organic phase (O) of strains affiliating with different subgroups of planctomyctetes. The band inside each box represents the median and the black points indicate values for individual strains.

Figure 3 - Principal Component Analyses (PCA) constructed from a distance matrix based on the effect of the individual strains on the different cell lines. The figures

illustrate the relationship between strains, their phylogenetic affiliation, their original environment and activity against the different cell lines in aqueous and organic phases. Axis 1 and 2 explains 50 % and 23 % respectively of the observed variance. Arrows indicates the correlation between strains and activity against cell lines.

Table 1 – Phylogeny, habitat and location of planctomycetes strains used in this study.

Supplementary Figure 1 - Phylogenetic 16S rRNA gene tree generated by maximum-likelihood analysis based in General Time Reversible model and Gamma distributed with Invariant sites (G+I) indicating the relationship of the planctomycetes strains tested in this work. Bar – 0.05 substitutions per 100 nucleotides. The planctomycete *Candidatus* Scalinda brodae was used as outgroup.

Supplementary Figure 2 - Examples of MOLM-13 cells stained with the DNA binding specific Hoechst dye. A - control cells and B – cells exposed to organic extracts of strain UC49.1. The apoptotic nuclei are bright, condensed or fragmented.

Supplementary Table 1 – Data based on the different degrees of toxicity used for the construction of the heat-map.

Table 1 – Phylogeny, habitat and location of planctomycetes strains used in this study.

Strain	***Phylogeny***	***Habitat***	***Location***	***Country***
MgM4h	*Blastopirellula cremea*	Iron hydroxide deposits	South Pacific	Fidji/Tonga
TBK2h	*Blastopirellula marina*	Iron hydroxide deposits	South Pacific	Fidji/Tonga
VLsL2h	*Blastopirellula* sp.	Iron hydroxide deposits	South Pacific	Fidji/Tonga
Vloj2h	*Blastopirellula* sp.	Iron hydroxide deposits	South Pacific	Fidji/Tonga
VLoJ4h	*Blastopirellula* sp.	Iron hydroxide deposits	South Pacific	Fidji/Tonga
FC18	*Mariniblastus fucicola*	*Fucus spiralis*	Carreço	Portugal
K2D	Pir 4 group	Iron hydroxide deposits	South Pacific	Fidji/Tonga
P6.1	Pir 4 (Ice cluster)	Cave Ice	Svarthamarhola	Norway
P6.2	Pir 4 (Ice cluster)	Cave Ice	Svarthamarhola	Norway

VlrD4	*Planctomyces maris*	Iron hydroxide deposits	South Pacific	Fidji/Tonga
VL6F2	*Planctomyces maris*	Iron hydroxide deposits	South Pacific	Fidji/Tonga
Plm2	*Planctomyces maris*	Iron hydroxide deposits	South Pacific	Fidji/Tonga
9mWe	*Planctomyces* sp.	Meromictic lake	Fana	Norway
UC49.1	*Rhodopirellula baltica*	*Ulva* sp.	Carreço	Portugal
SH1	*Rhodopirellula baltica*	Water column	Baltic Sea	Germany
UC21	*Rhodopirellula baltica*	*Ulva* sp.	Carreço	Portugal
L1	*Rhodopirellula islandica*	*Saccharina nigripes*	Kongsfjorden	Norway
CcC6	*Rhodopirellula lusitana*	*Chondrus crispus*	Carreço	Portugal
CcC8	*Rhodopirellula lusitana*	*Chondrus crispus*	Carreço	Portugal
Sm4	*Rhodopirellula lusitana*	*Sargassum moticum*	Porto	Portugal
UC13	*Rhodopirellula lusitana*	*Ulva* sp.	Carreço	Portugal
UC16	*Rhodopirellula lusitana*	*Ulva* sp.	Carreço	Portugal
UF6	*Rhodopirellula lusitana*	*Ulva* sp.	Porto	Portugal
L2	*Rhodopirellula lusitana*	*Saccharina nigripes*	Kongsfjorden	Norway
UC9	*Rhodopirellula rubra*	*Ulva* sp.	Carreço	Portugal
7mR	*Rhodopirellula* sp.	Meromictic lake	Fana	Norway
FC9.2	*Rhodopirellula* sp.	*Fucus spiralis*	Carreço	Portugal
FF4	*Rhodopirellula* sp.	*Fucus spiralis*	Porto	Portugal
TBK1	*Rhodopirellula* sp.	Iron hydroxide deposits	South Pacific	Norway
VLsL4lr	*Rhodopirellula* sp.	Iron hydroxide deposits	*South Pacific*	Fiji/Tonga
VLsK4lR	*Rhodopirellula* sp.	Iron hydroxide deposits	*South Pacific*	Fidji/Tonga
VLpG4r	*Rhodopirellula* sp.	Iron hydroxide deposits	*South Pacific*	Fidji/Tonga
UC8	*Roseimaritima ulvae*	*Ulva* sp.	Carreço	Portugal

UF3	*Roseimaritima ulvae*	*Ulva* sp.	Porto	Portugal
UF4.1	*Roseimaritima ulvae*	*Ulva* sp.	Porto	Portugal
Gr7	*Rubinisphaera brasiliensis*	*Gracilaria bursa-pastoris*	Aveiro	Portugal
1mW	*Rubinisphaera* sp.	Meromictic lake	Fana	Norway
8mW	*Rubinisphaera* sp.	Meromictic lake	Fana	Norway
15mW	*Rubinisphaera* sp.	Meromictic lake	Fana	Norway

Supplementary Table 1 – Data based on the different degrees of toxicity used for the construction of the heat-map.

Phylogeny	Strain	NRK Organic	PC3 Organic	MOLM-13 Organic	NRK Aqueous	PC3 Aqueous	MOLM-13 Aqueous
Blastopirellula cremea	MgM4h	9.15	5.66	39.76	0.00	4.71	0.00
Blastopirellula marina	TBK2h	14.69	7.49	33.33	16.35	6.80	73.15
Blastopirellula sp.	VLsL2h	2.52	11.11	53.46	37.61	8.01	69.12
Blastopirellula sp.	VLoj2h	3.34	7.23	40.93	6.51	11.88	44.07
Blastopirellula sp.	VLoj4h	5.98	9.21	38.70	3.22	5.08	54.63
Mariniblastus fucicola	FC18	4.51	15.90	17.18	9.87	5.98	73.25
Pir 4	K2D	0.00	14.96	7.02	7.19	0.00	53.36
Pir 4	P6.1	3.70	7.32	56.15	10.02	12.66	64.21
Pir 4	P6.2	10.02	4.39	19.01	10.72	8.87	43.62
Planctomyces maris	VLrD4	7.37	0.00	43.52	5.65	4.21	3.87
Planctomyces maris	Plm2	7.03	2.06	2.01	44.11	5.25	78.97
Planctomyces maris	VLbF2	6.68	5.94	64.20	18.98	8.95	75.83
Planctomyces sp.	9mWe	9.32	8.01	32.88	14.06	15.59	66.89
Rhodopirellula baltica	SH1	79.36	42.35	53.91	5.22	14.10	8.44
Rhodopirellula baltica	UC49.1	75.63	78.09	80.20	0.38	0.31	10.93
Rhodopirellula baltica	UC21	19.66	6.60	28.60	3.79	2.17	4.19
Rhodopirellula islandica	L1	4.56	87.03	59.18	13.84	2.95	1.06
Rhodopirellula lusitana	CcC6	15.50	82.70	71.77	9.67	7.53	2.83
Rhodopirellula lusitana	CcC8	18.15	ND	66.86	0.84	9.71	1.02
Rhodopirellula lusitana	L2	ND	9.49	23.67	2.68	0.00	0.00
Rhodopirellula lusitana	L2(2)	15.32	16.06	70.44	5.02	ND	2.50
Rhodopirellula lusitana	Sm4	88.09	47.31	71.19	17.35	6.09	0.83
Rhodopirellula lusitana	UC13	15.21	1.99	91.22	1.11	0.94	3.80

Rhodopirellula lusitana	UC16	35.07	60.78	96.52	1.92	0.03	0.02
Rhodopirellula lusitana	UF6	5.46	26.02	96.91	7.73	9.63	10.69
Rhodopirellula rubra	UC9	7.97	0.84	24.13	1.43	4.83	3.80
Rhodopirellula sp.	7mR	79.36	21.50	66.11	0.00	2.99	13.51
Rhodopirellula sp.	FC 9.2	1.53	3.68	8.74	1.98	0.00	3.55
Rhodopirellula sp.	FF4	9.60	9.08	65.05	11.31	2.71	26.06
Rhodopirellula sp.	TBK1	13.09	0.94	64.32	5.42	4.57	0.00
Rhodopirellula sp.	VLsL4lr	2.46	3.10	64.65	0.82	3.96	21.92
Rhodopirellula sp.	VLsK4lR	5.45	3.53	48.09	0.00	5.25	12.75
Rhodopirellula sp.	VLpG4r	7.38	4.39	43.17	14.06	6.28	50.33
Roseimaritima ulvae	UC8	8.45	13.48	58.76	0.00	ND	0.00
Roseimaritima ulvae	UF3	9.49	0.16	55.49	0.78	5.95	0.00
Roseimaritima ulvae	UF4.1	5.09	0.00	22.12	0.00	20.52	0.89
Rubinisphaera brasiliensis	Gr7	6.48	4.08	19.77	26.69	2.01	53.00
Rubinisphaera sp.	8mW	11.12	8.55	5.29	0.00	8.66	7.16
Rubinisphaera sp.	15mW	7.85	0.00	1.88	12.76	8.20	17.32
Rubinisphaera sp.	1mW	3.52	2.32	45.86	14.41	22.48	78.08

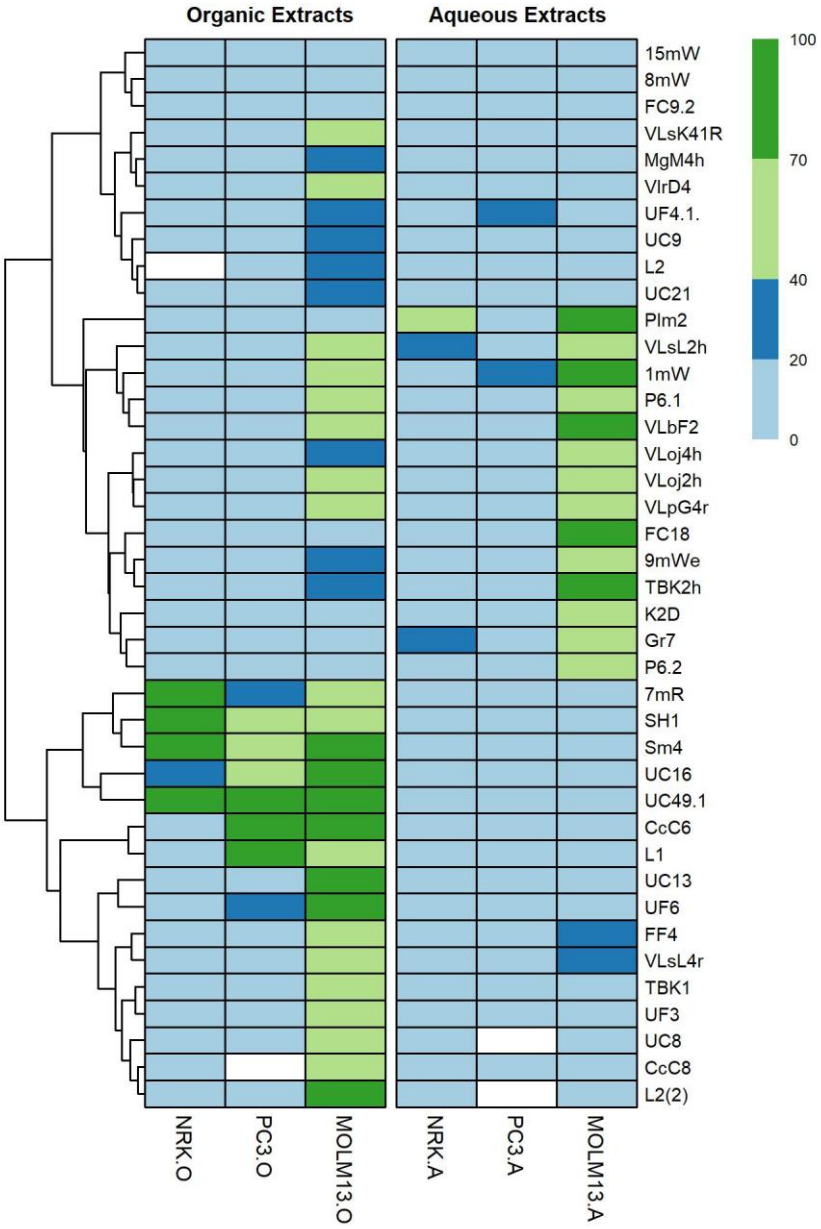
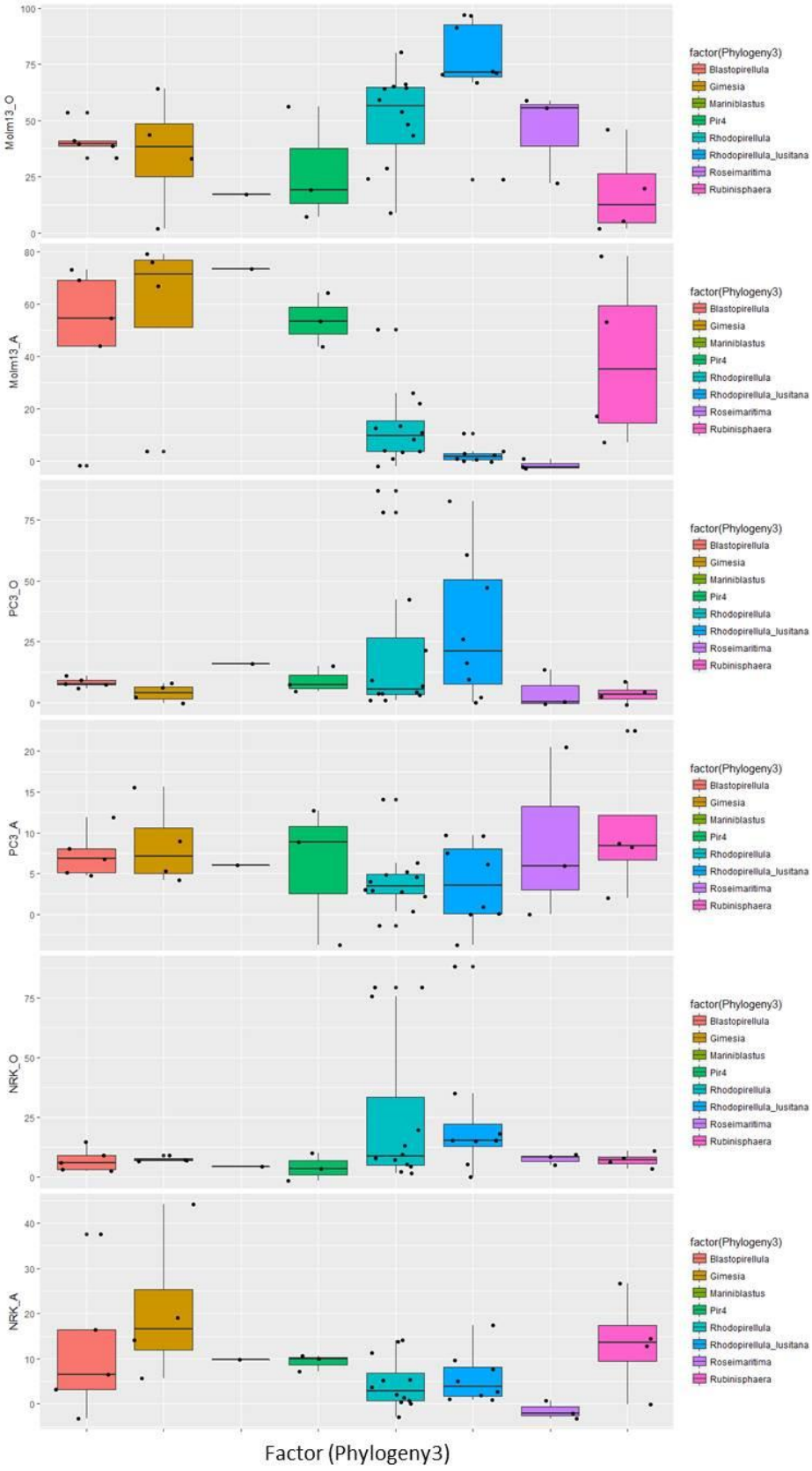


Figure 1 - Heat-map showing the different degrees of toxicity that organic and aqueous extracts of planctomycetes induced in NRK, MOLM-13 and PC3 cell lines. Heat-map scale: 0-20% -no toxicity; 20-40%- low toxicity; 40-70% - intermediate toxicity; 70-100% high toxicity. White boxes – Not evaluated. Graph was created in R studio programme, using pheatmap package. Cluster analysis was based on Eucledian Distance



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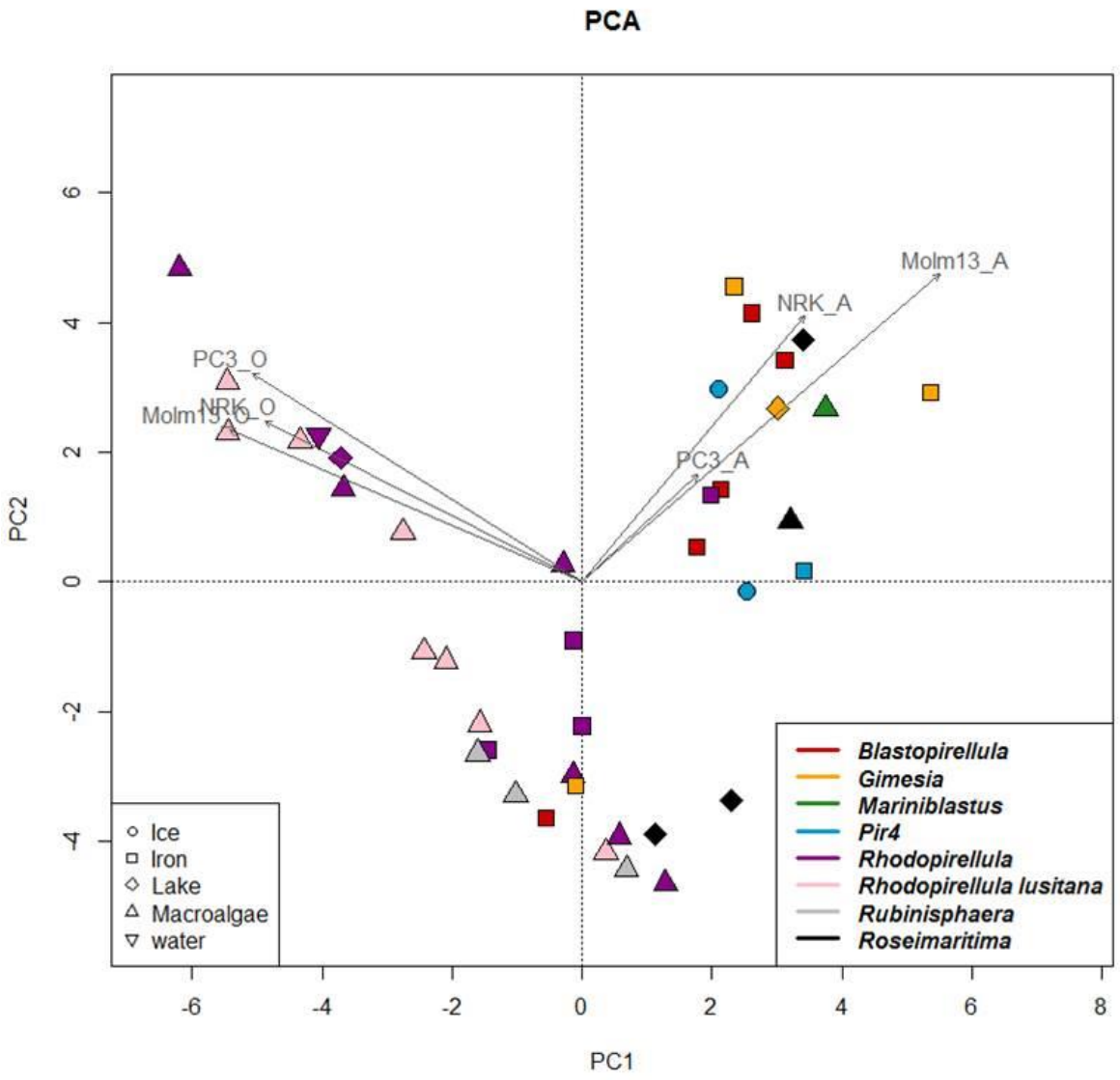

Figure 3 - Principal Component Analyses (PCA) constructed from a distance matrix based on the effect of the individual strains on the different cell lines. The figures illustrate the relationship between strains, their phylogenetic affiliation, their original environment and activity against the different cell lines in aqueous and organic phases. Axis 1 and 2 explains 50 % and 23 % respectively of the observed variance. Arrows indicates the correlation between strains and activity against cell lines.