Phylogenetic and Phenotypic Studies of the Extremophile *Chroococcidiopsis* (Cyanobacterium). A Foundation for Synthetic Biology in Space.

By

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CHAPTER 1: INTRODUCTION

CHROOCOCCIDIOPSIS AND OTHER CYANOBACTERIA IN EXTREME ENVIRONMENTS

Introduction

The broad goal of this thesis was to survey the natural diversity of *Chroococcidiopsis,* an organism known for its radiation and desiccation tolerance and its ability to survive in extreme environments, in order to understand the mechanisms it uses. The research was devised to build a foundation that would allow the design of extremophiles for use in space, such as for the production of food, or for use in life support systems. Finding the mechanisms by which organisms live in extreme environments on Earth should lead to the ability to engineer organisms for survival in space. To this end, the thesis details how 15 species of *Chroococcidiopsis* (Geitler, 1933), were surveyed for survival and growth under a range of conditions. In this introductory chapter, I first outline a grand vision for using genetically engineered organisms in space. I then contrast extreme environments on Earth with those found in space and outline what is known from previous experiments investigating survival in space environments. I then detail the current understanding of the taxonomy, morphology and phylogeny of the cyanobacteria in general. I describe the distribution and diversity of the cyanobacteria in order to place *Chroococcidiopsis* in a wider context. I also contrast the traditional morphological classification with that of the molecular sequence data from genes and whole genomes. I then detail a number of mechanisms by which the cyanobacteria, *Chroococcidiopsis* and other extremophiles are known to deal with environmental extremes. These extreme conditions include hot and cold temperatures, high levels of salinity, oxidative damage, radiation and desiccation. Finally, I outline the role that synthetic biology could play in enhancing natural biology for use in industrial applications in space.

The mass problem

The US space shuttle program was recently retired after 30 years of service. Per-launch costs have been measured at around $1.5 billion (Pielke and Byerly, 2011), by dividing the lifetime cost of the program ($192 billion, adjusted for inflation), by the total number of times it flew (134 times). The shuttle had a capacity of 30,000 Kg, making the cost of sending a kilogram from Earth into space approximately $50,000. Although initial planned costs were much lower than this at $1,401 per Kg, this high cost of sending things into space is still a large part of the budget of any space mission. For example, the average human needs 674 g of dry food per day in order to eat a healthy diet plus 2.77 Kg of water (Allen et al., 2003). Just for the cost of transporting food and water (3.4 Kg) from Earth to the International Space Station via the shuttle, for a single person, would cost between $4,763 per day at the low end of planned costs and $170,000 per day at the high end (these estimates assume no recycling and no water for hygiene). Transport on the Russian Soyuz rockets or from new companies such as SpaceX, offer slightly cheaper alternatives, perhaps by as much as 10-fold cheaper. But it is still clear that the cost of sending anything into space is prohibitive because of the cost of launch from Earth. This launch cost is due to the large amount of energy and infrastructure needed to escape the Earth's gravity. These high costs may be bearable for a government space exploration program, but for a long-term settlement program, this cost is impractical. However, if humanity is to begin living permanently off of the Earth then we need to solve this mass problem. One way to reduce the cost of supplies advocated in this thesis, is to make use of the resources in space (*in situ*), rather than by taking supplies from the Earth.

Biology is the solution to the mass problem

Biology offers a solution to the mass problem described above. In the lab, a single cell, such as from the bacterium *Escherichia coli* that doubles every 20 minutes, can produce over a billion copies of itself in a 24 hour period. This example highlights the self-replicating nature of biology, showing that a small initial mass such as a single cell, could grow and replicate from the resources around it. But now let us think about something more useful than a billion *E. coli* cells. Biology has been used as a natural manufacturing technology on Earth for many years for a number of industrial applications: such as for the production of food, lumber, rubber, natural dyes and many other materials and chemicals. Recent advances in genetic engineering and synthetic biology have shown that humans are rapidly increasing the range of products capable of being produced, including organisms to produce synthetic biofuels, drugs and biomaterials (reviewed in Khalil and Collins, 2010). If we now link these three abilities of biology, that of natural manufacturing, self-replication and some form of engineer-able control, then we have a very powerful potential for a low mass, self-replicating manufacturing technology that could one day revolutionize the conversion of raw elements into useful products in space.

Life in space

The idea of sending life into space has long been dreamed of in science fiction, but also conjures up the ethical fear of sending life into a possibly pristine solar system. There has yet to be a definitive answer to the question of whether life exists now or has existed in the past, elsewhere in our solar system. Because of this, planetary protection programs have been established in order to prevent the contamination of space (Barengoltz, 1983;

Conley, 2008; Horneck, 2008). The main worry is that premature importation of organisms to a planet could potentially contaminate or destroy endogenous life, whether related to Earth life or of an independent origin.

Even if no life were to be found on Mars, there still exists the ethical debate as to if humanity should be settling the solar system or not. Evidence from the geological record is clear however, that a number of large-scale impact events from meteorites have hit the Earth, wiping out ecosystems on a regular basis ever since it formed (e.g., Sleep et al., 1989). History is likely to repeat itself, and at some point, the Earth will once again be hit by a catastrophic event (Chapman and Morrison, 1994). I write this thesis with an optimistic view however. I support the view held by Chris McKay, that we should first search for life, and if it is found to exist elsewhere in the solar system, then we should support it as best we can, so that it can thrive (McKay, 2001). If no life is found elsewhere, then I think it is humanity's destiny to go explore, settle and spread throughout our solar system and eventually the universe.

Sending any biology into space and have it survive is no easy task. Previous experiments have exposed organisms to a number of different space environments with varying degrees of survival. A number of organisms have been flown in low Earth orbit such as the orbit of the International Space Station for example, which is an environment still inside the Earth's magnetic field, so low in gamma and X-ray radiation, but high in UVR as it is outside the Earth's atmosphere. Low Earth orbit is a desiccated environment with

no water available and temperatures can vary widely from -120 °C to more than +120 °C (reviewed in Horneck et al., 2010) making it a challenging environment for life.

Spores of *Penicillium roqueforti* (Hotchin et al., 1968), the eukaryotes *Saccharomyces cerevisiae* and *Aspergillus ochraceous*, and the prokaryotes *Bacillus subtilis* and *Deinococcus radiodurans* (reviewed in Horneck et al., 2010) have all been flown in these conditions. These organisms showed high lethality when fully exposed to the space environment (Horneck et al., 1994; 1995; 2001; Dose et al., 1995). For example, 1% of spores of *B. subtilis* were shown to survive exposure onboard NASA's Long Duration Exposure Facility (LDEF) in low Earth orbit for up to six years (Horneck et al., 1994). Survival increased dramatically to 80% when the spores were shielded from UVR, indicating the large amount of damage that UVR can cause. On the European Space Agency's Biopan satellite missions, a number of organisms have also been exposed. Survivors included two species of lichen, *Rhizocarpon geographicum* and *Xanthoria elegans, which* were found to restore their metabolism after both a 14-day and 10-day flight in space (Sancho et al., 2007; de La Torre et al., 2010; de Vera et al., 2010). The cyanobacterium *Synechococcus sp*. (Nageli) also survived a Biopan mission, but only when protected by gypsum-halite crystals (Mancinelli et al., 1998). In this experiment, the authors also assayed DNA damage via ^{32}P labeling and concluded that the major cause of cell death was via DNA damage.

The EXPOSE facility on the International Space Station is the most recent platform for experiments that expose organisms to space environments. Cockell et al. (2011) exposed

a mixed cyanobacterial rock-dwelling community outside of the International Space Station for 548 days before returning it to Earth. The natural community contained a number of species that included the cyanobacterium *Nostoc commune*. The samples were also augmented with other cyanobacteria that included *Chroococcidiopsis* (029, Negev, Israel). Whilst a number of species died, including *N. commune*, *Chroococcidiopsis* was among the species that survived and could be cultured again after the sample was returned to Earth. The experiment demonstrated that the space environment is capable of acting as a selective pressure on a microbial community and also highlighted the suitability of *Chroococcidiopsis* as a hardy organism for space applications.

The idea of creating artificial ecosystems (ecopoiesis) or Earth like ecosystems (terraforming) on other planets has gained traction both in science (e.g., McKay et al., 1991) and science fiction (e.g. Robinson, 1995). If one wanted to take Earth based life to Mars, for example, what would it look like? Friedmann originally proposed that *Chroococcidiopsis* would be a suitable candidate for the terraforming of Mars due to its ability to survive in the cold, irradiated, desiccated, nutrient deprived desert of the McMurdo Dry Valley in Antarctica (Friedmann, 1995). Based on these Earth analogs of Mars (Horowitz et al., 1972), it is thought that organisms such as *Chroococcidiopsis* could currently survive there if buried underneath the Martian permafrost (Friedmann, 1986; Cockell et al., 2005).

Research goals

The goal of this thesis was to find mechanisms in extremophiles that could be engineered into other organisms to allow them to live in extreme environments, such as those found in space. The strategy adopted was to survey existing extremophiles on Earth, to characterize unknown species and to identify possible mechanisms of tolerances to extremes. The plan was that these mechanisms could then be transferred to other organisms in order to enhance their abilities to survive in extreme environments. Although the goal of creating a synthetic extremophile was not completed, a number of potential target organisms were characterized that showed interesting phenotypes that may be suitable for space bound organisms in the future. The cyanobacterium *Chroococcidiopsis* was chosen for this study because of the previous work that had identified it as a hardy genus found in a range of extreme environments (Friedmann, 1985). The goal was to map the ability to survive in extreme environments onto the phylogeny of the genus. This next section details our current understanding of the phylogeny of the cyanobacteria and *Chroococcidiopsis*.

Introduction to the cyanobacteria and *Chroococcidiopsis.*

The cyanobacteria are one of the most important phyla when it comes to the evolution of life on Earth. Oxygenic photosynthesis, the adaptation that allowed for the use of water as an electron donor, dramatically increased the efficiency of these primary producers enabling them to rapidly diversify and consume the resources around them. One such resource was diatomic nitrogen, which the cyanobacteria are able to fix themselves rather than relying on the less abundant but more readily usable forms of fixed nitrogen. During

photosynthesis, cyanobacteria release O_2 as a waste product, which dramatically increased the amount of O_2 in the Earth's atmosphere, leading to the formation of an oxygen-rich atmosphere and the stratospheric ozone layer. This then enabled, among other evolutionary innovations, the origin of eukaryotes, and in particular animals. History has shown that these primary producers have played a large role in shaping the carbon, nitrogen and oxygen cycles of the Earth and they continue to do so today (Liu et al., 1997; Capone et al., 1997). The symbiosis of a cyanobacterium with a protist to create the ancestor of the present day chloroplast (Margulis, 1992; Delwiche, 1997) was also a major step in the history of life, leading to the algae and plants, and again highlights the vital role that the cyanobacteria have played in the evolution of life on Earth (see Herrero and Flores, 2010).

Chroococcidiopsis is identified within the cyanobacteria, primarily by its pattern of cell division. The genus divides by repeated binary fissions producing many smaller cells called baeocytes contained within a larger mother cell with a fibrous cell wall (Waterbury, 1989; Rippka et al., 2001). Historically, *Chroococcidiopsis* has been grouped as a monophyletic clade based on its morphology. However, molecular sequence data has since shown that it is actually polyphyletic (Rudi et al., 1997; Garcia-Pichel et al., 1998; Norris and Castenholz, 2006; Fewer et al., 2002), meaning that the ability to divide by multiple divisions has evolved independently a number of times.

The name "*Chroococcidiopsis"* is a graphic description: "chroa" is Greek for skin, "kokkidios" is the presumed diminutive of "kokkos" meaning a small seed or grain and

"opsis" means the appearance of, so *Chroococcidiopsis* is defined as a genus with "small cells appearing in skin or coating" (Waterbury, 1989; Rippka et al., 2001). Phylogenetically, the genus forms a sister taxon to the heterocyst-forming cyanobacteria, discussed later, and it is hypothesized that the ability to perform cellular differentiation may be involved in the survival of the organisms in extreme conditions (Fewer et al., 2002), as *Chroococcidiopsis* is known to form resting cells when faced with environmental stress (Billi and Caiola, 1996).

The genus is found all over the world, both in fresh water, salt water and on land (Rippka and Herdman, 1992; Andersen et al., 1997). It is found in tropical climates, polar climates and hot and cold deserts. When found on land, it lives both on, and sometimes inside of rocks (Rippka and Herdman, 1992). *Chroococcidiopsis* became of interest to the astrobiology community after it was found growing in crystalline cavities between porous rocks in the McMurdo Dry Valleys of Antarctica (Friedmann and Ocampo, 1976). Culture of, and experimentation with, *Chroococcidiopsis* in the laboratory has revealed that a small selection of species were both radiation and desiccation tolerant, but not psychrophilic (Ocampo-Friedmann et al., 1988; Caiola et al., 1993; Billi et al., 2000b), as discussed shortly.

Phylogeny, taxonomy and morphological diversity

The phylogeny of the cyanobacteria can be seen on the tree shown in Fig. 1.1. They form a monophyletic clade and are the only prokaryotes capable of oxygenic photosynthesis (Wu et al., 2009). Morphologically based classification has grouped the cyanobacteria

into five subsections, examples of which are illustrated in Fig. 1.2 (Rippka et al., 1979; Waterbury, 1989; Rippka et al., 2001). Subsection I (*Chroococcales*) are unicellular and coccoidal, Subsection II (*Pleurocapsales*) are similar to Subsection I but reproduce by rapid binary fission producing large cells containing many smaller cells called baeocytes. Subsections III, IV and V are the filamentous cyanobacteria. Subsection III (*Oscillatoriales*) are filamentous species that only produce vegetative cells. Subsection IV (*Nostocales*) and V (*Stigonematales*) are filamentous species that can differentiate into heterocysts, distinct cell types capable of fixing nitrogen (Bryant, 1994), or akinetes, specialized resting cells (Herdman, 1987). Subsection V is further distinguished by complex branching patterns. *Chroococcidiopsis* is a member of subsection II (*Pleurocapsales)*, which are organisms that divide by multiple fissions and produce baeocytes. Subsection II is further split into Subgroup I (containing *Cyanocystis, Dermocarpella, Stanieria* and *Xenococcus*) and Subgroup II (containing *Chroococcidiopsis*, *Myxosarcina* and *Pleurocapsa*) the morphology of which are detailed shortly. Whilst morphology has been a useful tool for the classification of cyanobacteria, it often has not agreed with molecular sequence data. Schirrmeister and colleagues (2011) mapped these various morphologies to a phylogenetic tree and showed an early origin of multicellularity in the cyanobacteria with the single cell phenotype having been gained and lost multiple times. These results indicate that characterizing clades based on morphology alone may not be the most suitable trait by which to assign species.

Chroococcidiopsis is a member of Subsection II of the cyanobacteria and identified by its division pattern. Within subsection II there are two main groups of division patterns

(Komarek and Anagnostidis, 1989). Subsection II, Group I has a pattern whereby reproduction takes place by multiple fissions only, or in combination with a limited number of binary fissions. *Chroococcidiopsis* is a member of Subsection II, group II, where reproduction takes place by extensive binary fissions, followed by multiple fissions (Waterbury and Stanier, 1978; Kunkel, 1984; Komarek and Anagnostidis 1989; Waterbury, 1989; Rippka et al., 2001). Baeocytes, the small cells produced by *Chroococcidiopsis* are shown in Fig. 1.5. Vegetative *Chroococcidiopsis* cells vary between 1 and 10 μM in diameter depending on the species and stage of cell cycle, whilst baeocytes are between 1 and 3 μM in diameter (Waterbury, 1989; Rippka et al., 2001). Groups of baeocytes are enclosed in a gelatinous mother cell. When the mother cell bursts and baeocytes are released, the replication process begins over again with binary fission (Waterbury, 1989; Rippka et al., 2001).

Phylogeny inferred from molecular sequence data

Using molecular sequence data is a more accurate way to produce phylogenetic trees and assign species than by phenotype alone. When compared with other genetic markers in the cyanobacteria, the 16S rRNA has been shown to be congruent with some markers (e.g., Seo and Yokota, 2003; Tomitani et al., 2006) and incongruent with others (e.g., Rudi et al., 1998, Leikoski et al., 2005). This makes phylogenetic trees constructed from more than one marker a valuable tool for increasing the resolution of the phylogeny. However, the 16S rRNA remains useful as the primary marker used in prokaryotic phylogenetics, due to the low interspecies transfer, presumed low environmental selection and the fact that it is found in all prokaryotes studied thus far.

The cyanobacterial subsections described earlier from morphology alone have received mixed support from multiple sequence analyses (Giovannoni et al., 1988; Wilmotte et al., 1994; Turner et al., 1999; Wilmotte and Herdman, 2001; Fewer et al., 2002; Tomitani et al., 2006). Subsection I is consistently polyphyletic. Subsection II has been found to be polyphyletic, with baeocyte formation known to have evolved at least twice, or have been passed on by horizontal gene transfer (Fewer et al., 2002). Subsection III has also been shown to be polyphyletic (Fewer et al., 2002). However, subsections IV and V have shown monophyletic support in a number of studies (Turner et al., 1999; Fewer et al., 2002; Sanchez-Baracaldo et al., 2005; Shi and Falkowski, 2008; Swingley et al., 2008; Doolittle and Zhaxybayeva, 2009).

Although sequence data is a more powerful tool for inferring phylogeny than morphology alone, sequence data does not necessarily simplify the task of inferring phylogenies. One complication to cyanobacterial genomics is that some species are known to possess multiple copies of their genomes (e.g., Man and Carr, 1974; Binder and Chisholm, 1990; Castenholz, 1992; Whitton and Potts, 2000). It is currently unclear if this is due to increased polyploidy and all copies are the same or if each copy of the genome is an individual chromosome and is responsible for increased complexity. Another problem when inferring phylogenies is horizontal gene transfer. The sequencing of whole genomes has shown a large amount of horizontal gene transfer in the prokaryotes and in the cyanobacteria (Gogarten and Townsend, 2005; Zhaxybayeva et al., 2006). This has led some to propose a network model of prokaryotic evolution rather than a branching

tree (Hilario and Gogarten, 1993; McInerney et al., 2008; Doolittle and Zhaxybayeva, 2009). For example, Shi and Falkowski (2008) quantified horizontal gene transfer by analyzing 682 orthologous genes that were present in 13 complete cyanobacterial genomes. They showed that 52% of these genes had undergone some horizontal gene transfer and the phylogenetic trees produced from these were incongruent. However, trees constructed with the remaining 48% of genes showed a broad phylogenetic agreement. The authors then characterized this core set of genes and found agreement with previous studies showing that housekeeping genes, such as enzymes involved in photosynthesis, ribosomal genes and essential metabolic pathways are less susceptible to horizontal gene transfer than other genes (Jain et al., 1999). This result shows why it is important to use housekeeping genes when constructing trees with multiple phylogenetic markers. One of the mechanisms known for horizontal gene transfer is via cyanophage (e.g., Millard et al., 2004; Lindell et al., 2004; Coleman et al., 2006). However, despite the known existence of cyanophage and their role in gene transfer, they have not yet been used for the genetic manipulation of cyanobacteria.

History of cyanobacteria in the fossil records

In order to understand the phylogeny of the cyanobacteria, it helps to use evidence from the fossil record to try to calibrate the phylogeny. There are various lines of evidence indicating different date ranges for the evolution of the cyanobacteria (e.g., see Blank and Sanchez-Baracaldo, 2010). Most evidence points to a period of time between the Proterozoic and Archean era (between 2.5-3.5 Gya). The earliest evidence of fossilized *Chroococcidiopsis*, or something similar in morphology, dates back to the Early

Devonian Rhynie Chert from 400 Mya (Taylor et al., 1995). Cyanobacterial microfossils and microbialites are most abundant in the fossil record during the Proterozoic Era 2500–570 Mya (Schopf and Walter, 1982). Carbon isotopes consistent with RuBisCO activity have been measured dating back to the same period as some of the oldest forms of life (Hayes and Waldbauer, 2006), although some other prokaryotes also have this enzyme. The cyanobacterial biomarker 2-methylhopanoids have been found as far back as 2.7 Gya (Brocks, 1999; Brocks et al., 2003). However, it is possible that cyanobacteria are not the only producer of this biomarker. It is thought that the cyanobacteria were responsible for the rapid oxygenation of the Earth's atmosphere as the first organisms to evolve oxygenic photosynthesis. This likely occurred around 2.32 Gya (Bekker et al., 2004) although oxygen would have accumulated in the atmosphere only when the production of O_2 from oxygenic photosynthesis was greater than the amount of O_2 being consumed by physical and biological processes. The evidence of such absorption of molecular oxygen can be seen in banded iron formations, which are thin layers of iron oxides found in Precambrian sedimentary rocks and are seen in the fossil record in the years preceding the oxidation of the atmosphere (e.g., Bekker et al., 2004).

A number of phenotypic characteristics have been mapped onto the known phylogeny of the cyanobacteria, including metabolism and cell morphology. For example, Tomitani et al. (2006) proposed that the clade of heterocyst (differentiated cells for nitrogen fixation) and akinete (survival cells) forming cyanobacteria evolved once between 2.45 and 2.1 Gya. They coupled this to the rise in atmospheric oxygen and the need to separate nitrogen fixation from the oxidative environment. Other work suggests that early

cyanobacterial lineages lived in freshwater ecosystems until at least 2.4 Gya and only later did they diversify into salt waters (Garcia-Pichel et al., 1998; Blank and Sanchez-Baracaldo, 2010).

Traits such as nitrogen fixation (Blank, 2004), salt tolerance, filamentous morphology (Schirrmeister et al., 2011), and tolerance of higher temperatures (Sanchez-Baracaldo et al., 2005), appear to have evolved independently on a number of occasions. This convergent evolution and horizontal gene transfer discussed above, highlights the difficulties in tracing complex patterns of trait evolution in the cyanobacteria and other prokaryotes.

Geographical and environmental diversity of life on Earth and in space

In this section, I outline the various extreme environments on Earth that cyanobacteria have been found in. I then compare and contrast this with a number of space environments such as the moon and Mars. In each section I also detail some of the known mechanisms used by organisms to survive in such extremes including what is known about *Chroococcidiopsis* in particular.

The distribution of cyanobacteria in various geographical locations, both on land and at sea, in hot and cold climates, both extreme and temperate, have shown that they live in virtually all ecosystem habitats studied (Geitler, 1933; Golubic, 1980; Friedmann, 1980; Bryant, 1994; Tang et al., 1997; Papke et al., 2003; Cockell and Stokes, 2004; Ward et al., 2006; Bhaya et al., 2007; Jungblut et al., 2010; Harding et al., 2011).

Chroococcidiopsis is found in a range of environments from the hot Negev desert in Israel to the cold deserts of Antarctica (Friedmann and Ocampo-Friedmann, 1985; Büdel and Wessels, 1991). Cold ecosystems are normally either characterized by perennially cold temperatures (e.g., Margesin and Schinner, 1999) or by freeze thaw cycles (e.g., Zakhia et al., 2008). Water activity, the amount of water available for biological use, is defined as the vapor pressure of a liquid divided by the vapor pressure of pure water at the same temperature. Water activity is a known limiting factor in dry ecosystems (Pointing et al., 2007; Warren-Rhodes et al., 2007). In such harsh environments as the hyper arid deserts, the cyanobacteria are often the principal primary producers and their distribution and colonization is most strongly correlated with the amount of moisture in the environment (Warren-Rhodes et al., 2007). PCR amplification from different areas of the McMurdo Dry Valleys in Antarctica, a cold hyperarid desert have found that specialized communities form in distinct niches (Pointing et al., 2009), indicating that adaptation is occurring on a local level in these deserts.

Hypoliths and endoliths in hot and cold deserts

Organisms that grow inside of rocks are called endoliths or cryptoendoliths if they occupy porous cavities. It is known that the ability of cyanobacteria to live in such a range of environments is partly due to their strategy of seeking protection by sheltering in and under rocks (Friedmann, 1980; Friedmann et al., 1993, Wierzchos et al., 2006). Organisms that live under rocks are called hypoliths (Golubic, 1980), literally "hypo" meaning below, and "lith", meaning rock. An example of photosynthetic hypoliths is shown in Fig. 1.3 from the Gobabeb desert, Namibia. Communities of

Chroococcidiopsis have been found living in these conditions in deserts such as the Atacama Desert in Chile (Schlesinger et al., 2003; Warren-Rhodes et al., 2006). Cyanobacteria such as *Chroococcidiopsis* have also been found inside porous rocks or at the boundary between stone and soil (Friedmann and Ocampo, 1976). In these conditions cells can survive in a dry ametabolic state for much of the year (Friedmann et al., 1993; Warren-Rhodes et al., 2006). Fig. 1.4 (Whitton and Potts, 2000) shows a broken away view of a cryptoendolithic community from the Negev desert. Other organisms are known to actively bore into rock in order to seek shelter (Cockell and Herrera, 2008). Organisms that live on and inside of rocks are of great interest because if there were life on Mars at some point in the past, then organisms might have taken refuge underground as the planet became dry and cold (Rothschild, 1990; Caiola and Billi, 2007).

Mechanisms of phenotypic diversity in the cyanobacteria and other extremophiles

In order to engineer organisms for living in extreme environments, I wanted to gain an understanding of the current limits of life on Earth. The section below deals with the limits of organisms found in salts and brines, cold, hot, dry and high radiation environments. The known limits for each of these environments is discussed, as are the known mechanisms that organisms use for survival (reviewed in Rothschild, 2009).

Halophiles: Life in salts and brines

Halophilic organisms are those that require salt for growth and halotolerant are those that tolerate salt but can grow without it (Golubic, 1980; Rothschild and Mancinelli, 2001). Feldman (1951) classified the cyanobacteria according to their tolerance for salt and

species have been documented from a range of salinities (Thajuddin and Subramanian, 1992; Rothschild, 1995). The distribution of salt tolerance can be seen mapped to a phylogeny in Fig.1.1, and shows that the trait likely has evolved independently multiple times or been transferred by horizontal gene transfer.

Chroococcidiopsis has been found in both fresh and saltwater (~ 0.5 M sea salt) environments (Dor et al., 1991). Golubic (1980) studied different communities along a salt gradient and showed that taxa had colonized various niches at different salinity levels, indicating local adaptation to different extremes. For example, *Spirulina subsalsa* is a halophilic cyanobacterium commonly found in saltpans and grows in water up to 24 g·L (~ 3 M) salt (Nubel et al., 2000; Thajuddin and Subramanian, 1992). Cyanobacteria are difficult to segregate according to true marine and freshwater species as it has been previously reported that many species recorded as freshwater also grow on seawater media (Thajuddin and Subramanian, 1992).

A number of salt tolerant mechanisms are known. In order to regulate internal salt concentrations, cells have evolved ion channels and pumps to remove salt from cells (Gabbay-Azaria and Tel-Or, 1991). They have also evolved ion pumps to move ions inside of the cell in order to maintain osmotic equilibrium and cell turgor (Miller et al., 1976). In addition, cells often accumulate or synthesize osmotic solutes in order to sequester excess salt and stop it from causing internal damage. Some examples of solutes used are sugars, the amino acids proline, mycosporine-like amino acids (MAAs) and

glycine betaine (Pessaarakli, 1995; Chen et al., 2007; Ferjani et al., 2003). These solutes could possibly be overexpressed in other organisms in order to increase tolerance to salt.

Salt evaporites and organisms such as cyanobacteria that live within them (Rothschild et al., 1994; Tosca et al., 2008) are of particular interest to the space community due to their potential as a refuge for life on Mars (Rothschild, 1990). Small amounts of salt have been found in the past on Mars, for example perchlorate was found by the Phoenix mission (Hecht et al., 2009). However, because it is known that liquid water once flowed on Mars (e.g., Masursky et al. 1979, Squyres et al., 2004), as this liquid water dried up it is hypothesized that evaporites of high salt concentrations could have formed (Rothschild, 1990).

Psychrophiles: Life in cold environments

Organisms with a peak growth rate around 15 °C that are unable to grow above 20 °C are called psychrophiles, whilst those that only tolerate low temperatures but grow faster at higher temperatures are called psychrotrophs (Friedmann et al., 1987b). The lowest recorded biological activity reported was that of cells found in an ice core at -25 °C (Junge et al., 2004). The authors assayed oxidative respiration in *Cytophaga-Flavobacteria-Bacteroides* and archaea and showed that the activity level decreased with temperature. Growth and replication at this temperature however, was not reported. Compared to space environments, the surface of Mars has an average temperature of -46 °C (Williams, 2004) which is similar to the moon's average surface temperature of -53 °C (Vasavada et al., 1999). Whilst there has been no recoded biological activity at

this temperature range, organisms are known to survive at temperatures lower than this when frozen and recovered. One such organism is the tardigrade (water bear), a multicellular animal, which when exposed in a desiccated state, can survive being frozen in liquid nitrogen, (freezing at around −210 °C) as first shown by Becquerel (1950). Mammalian and bacterial cell lines are also regularly frozen in liquid nitrogen and are then subsequently revived. Such organisms are normally stored with glycerol or other cryoprotectants however, and cells have a range of survival rates depending on the cell line and the length of time frozen.

Although many species of cyanobacteria are found in polar climates, studies that have looked at growth rates show that they have not adapted to grow faster at colder temperatures. For example, Tang and colleagues (1997) looked at 27 isolates of mat-forming cyanobacteria (*Oscillatoriaceae)* from Arctic and Antarctic water systems and found that the majority of species studied were not adapted to grow faster at lower temperatures. Antarctic desert species of *Chroococcidiopsis* have been isolated from locations where the outside temperature drops to -48 °C in winter (Friedmann et al., 1987b). These species also did not show that their growth rate had adapted to grow faster in colder temperatures (Ocampo-Friedmann et al., 1988). This could be because *Chroococcidiopsis* is shielded from the environment by living underneath rocks. Friedmann (1982) found that certain types of porous rocks in Antarctica had a narrow subsurface zone that trapped moisture within it and provided a favorable microclimate for bacteria to live.

An alternative hypothesis is that cells have evolved mechanisms that allow them to rapidly thaw when the temperature increases and then to freeze again in a controlled fashion, without cellular damage when the temperature drops again (Friedmann et al., 1987a). A number of genetic mechanisms are known to allow psychrophiles to live in cold environments. A number of genetic elements have been identified that could potentially be used to engineer psychrophilic organisms. Examples include cold adapted chaperones (Ferrer et al., 2003), antifreeze proteins (DeVries, 1971), ice-nucleating/ice binding proteins (Raymond et al., 2009), heat shock proteins (Hossain and Nakamoto, 2002) and enzymes that increase membrane fluidity (Finegold et al., 1990; Russell, 1997; Huston et al., 2004; Methé et al., 2005; Montooth et al., 2006).

Billi et al. (2011) investigated freeze-thawing in two species of *Chroococcidiopsis* and observed reduced survival and DNA damage after 50 repeat freeze-thaw cycles between -20 and +20 °C. This study performed freeze-thawing 50 times and then measured cell survival and inferred DNA damage from PCR fingerprinting assays. Cell survival ranged from 69% (species *Chroococcidiopsis sp*. CCMEE 123) to 0% (*Chroococcidiopsis sp*. CCMEE 134) although PCR fingerprinting assays showed damaged genomic template, it was shown to occur similarly in both species. The authors just looked at a single freeze-thaw temperature however, and did not measure survival during the repeated cycles, rather just a single time point at the end of the assay.

Thermophiles: Life in hot environments

Thermophiles are organisms that live at 45 ° C and up (Madigan et al., 2010). Although this study is primarily concerned with life at cold temperatures, thermophilic cyanobacteria such as *Synechococcus sp.* have been studied in hot spring communities along temperature gradients (Reviewed in Castenholz, 1969; Ward, 2000). The highest temperature recorded for a living cyanobacterium was *Synechococcus cf. lividus,* isolated from a 73°C hot spring (Miller and Castenholz, 2000). This is also the theoretical maximum temperature for photosynthetic life because chlorophyll breaks down at this temperature (Brock, 1967; Castenholz, 1969; Rothschild and Mancinelli 2001).

A number of mechanisms are known that allow organisms to live in thermal environments. For example, desaturases are enzymes that remove double bonds from lipid membranes causing them to become more rigid and less flexible. Having a rigid membrane would be an advantage in thermal environments as they would help keep the cell structure together. Having a looser membrane might be an advantage in a colder environment in order to allow the cell to be more flexible during freezing. This is supported by the genome sequence of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1, which was found to lack desaturase genes that were present in the genomes of other mesophilic cyanobacteria (Nakamura et al., 2002). Other studies have shown that the genomes of thermophiles encode fewer bulky amino acid residues in their proteins (Dutta and Chaudhuri, 2010). This indicates that tighter protein folding likely takes place in these organisms to counter the increased thermal energy. Although species of *Chroococcidiopsis* have been found in hot springs (e.g.,

Billi et al., 2000b), no studies to date have investigated the mechanism of thermal tolerance in the genus.

Xerophiles: Life in desiccated environments

Xerophiles are organisms that can live and reproduce in very dry conditions where water activity (water available for biological use), is limited. Desiccation tolerant organisms can survive drying to 10% water content (0.1g $H_2O \cdot g$ dry mass) whilst desiccation sensitive organisms die when dried to 20% water content (Alpert, 2005). Desiccation tolerance is a widespread trait that has been found in a number of organisms, including invertebrate animals such as tardigrades (Keilin, 1959) rotifers (Ricci, 2005) as well as in plants, seeds, lichens, algae, fungi (reviewed in Alpert, 2005) and a number of bacteria and cyanobacteria including *Chroococcidiopsis* (Billi, 2002; Büdel et al., 2002; la Torre et al., 2003). Animals, such as the tardigrade have been known to survive for almost a decade in a desiccated state (Guidetti and Jönsson, 2002).

Water is a solvent for all known metabolism because enzymes and membranes use it in order to function properly. Metabolism likely stops once water falls below 10%, as not enough water is available to surround membranes and proteins (Alpert, 2006). Desiccation resistant organisms tend to tolerate drought by drying in a controlled fashion and preventing cell death (Alpert, 2005). Many desiccation tolerant organisms perform anhydrobiosis, a process whereby an organism can enter a state of dormancy upon desiccation, and resume metabolic activity upon rewetting (Clegg, 1973; Billi, 2002; Alpert, 2006).

Viable *Chroococcidiopsis* cells have been recovered from laboratory-dried cultures isolated from the Negev desert after 5.5 years (Caiola et al., 1993). A number of mechanisms are though to explain why these and other cyanobacteria are so desiccation tolerant. Close examination of desiccated *Chroococcidiopsis* cells revealed that they had thicker cellular envelopes than non-desiccated cells and it was hypothesized that this helped to prevent water loss (Caiola et al., 1996a). These cell membranes have been characterized during desiccation and have been found to contain higher levels of polysaccharides (Caiola et al., 1993; 1996a). Many desiccation tolerant organisms have been found to produce increased amounts of sugars during desiccation (Hershkovitz et al., 1991; Potts, 1994). These sugars are known to be able to substitute for the structural components in place of water (Alpert and Oliver, 2002) and can prevent damage occurring during drying (Crowe et al., 1997). In *Chroococcidiopsis,* it has also been hypothesized that the thick cellular envelope may be part of a resting state that cells enter into during environmental stress (Caiola et al., 1993).

Space environments such as the Moon or Mars are known to be extremely dry, even though water has been found in both locations. On the Moon, water ice has been found inside of dark polar craters (Colaprete et al., 2010), and on Mars it has been found in the subsurface permafrost (Kostama et al., 2006). Although the temperature can rise above freezing in both locations, water could not remain in a liquid state in either because any melted ice would immediately sublime from solid to gas (Leighton and Murray, 1966).

Radiation resistance

Radiation resistant organisms can survive exposure to radiation. There are a number of different types of radiation as discussed below, but this thesis mainly deals with UVR (Ultraviolet Radiation). The ultraviolet spectrum is categorized into UVA, UVB and UVC, according to its wavelength. UVB and UVC are the most damaging forms of UV because nucleotides absorb at 260 nm and amino acids at 280 nm. The stratospheric ozone layer blocks all UVC (280-100 nm) and the majority of UVB (315-280 nm) from reaching the Earth today. UVA (400-315 nm) is not blocked by the ozone layer although it is less harmful than UVC and UVB. Because the cyanobacteria evolved before the ozone layer formed, their UVR protection mechanisms may be left over from before that time.

Although the atmosphere on Mars is thin, it is 95% CO_2 and shields out the majority of UVR wavelengths below ~200 nm (reviewed in Olsson-Francis et al., 2009a). Because there is no atmosphere on the Moon, UVR is not shielded at all. In Chapter 3 of this thesis, a monochromatic UVC source of 254 nm is used for exposure of organisms to UVR. Although not ideal, as it just represents one wavelength of UVR, it has been used in previous studies to successfully determine response to UVR exposure (e.g., Leuko et al., 2011).

Gamma and X-ray radiation come from solar winds and galactic cosmic rays. On Earth, most of this radiation is shielded by the Van Allen belt, the magnetic field around the planet. The Moon and Mars however, lack a magnetic field and receive the full spectrum

of gamma and X-ray radiation (Baglioni et al., 2007). Because most X-ray and gamma radiation is blocked from the Earth, it is hypothesized that resistance to these forms of radiation may be a byproduct of the ability to repair damage caused by UVR (Billi et al., 2000b, Cockell et al., 2005). This study primarily focuses on ultraviolet radiation, rather than gamma or X-ray because UVC was readily available in the laboratory.

Many types of DNA damage have been shown to occur following UVR (reviewed in Rothschild and Cockell, 1999; Sinha and Häder 2002). Depending on the range of UVR and dosage, these can include single strand breaks, double strand breaks, DNA-protein crosslinks and modified bases such as thymine dimers (Ehling-Schulz and Scherer, 1999; Goodsell, 2001). One DNA repair mechanism studied in this thesis is photoreactivation, which repairs DNA by the use of a photolyase enzyme. Photolyase enzymes use light energy to break bonds between cyclobutane pyrimidine dimers, which are caused by UVR. These dimers are recognized and bound by the photolyase enzyme that then converts them back into monomers (Ehling-Schulz and Scherer, 1999; Frohnmeyer, 2003).

Chroococcidiopsis has been previously tested for survival under a number of radiation environments. Billi et al. (2000b) exposed ten species to ionizing X-ray irradiation, and four species were found to survive up to 15 KGy, species CCMEE 015 (Negev), CCMEE 057 (Sinai), CCMEE 101 (Sonoran) and CCMEE 171 (Antarctica). The authors showed DNA damage by running genomic extractions on an ethidium bromide TAE agarose gel, and showed that breakages were repaired after 24 hours (Billi et al., 2000b). DNA damage from UVR and subsequent repair, has also been demonstrated via nicked-end

labeling assays (Billi et al., 2009b) and DNA fingerprinting (Billi et al., 2011), however direct measurement of other types of DNA damage, such as modified bases, has never been performed in *Chroococcidiopsis.* Just how widespread UVR resistance is within the genus and measurement of modified bases after UVR exposure is detailed in Chapter 3 of this thesis.

The link between desiccation and radiation resistance

Desiccation is known to cause breakages in genomic DNA (Potts, 1994; Rebecchi et al., 2009). The critical time for a cell to repair this damage, if it is to survive, is during the rehydration process (Dose, 1992). This type of DNA damage, consisting of breakages in genomic DNA is similar to damage induced by radiation. It is thought that radiation tolerance is therefore a byproduct of desiccation tolerance. This link has been demonstrated in *Deinococcus radiodurans,* as mutants deficient in DNA repair also have impaired desiccation and radiation resistance (Mattimore and Battista, 1996; Cox and Battista, 2005; Jonsson, 2005).

Another link between desiccation and radiation tolerance is that they both produce damage from reactive oxygen species (ROS). ROS are formed during desiccation because proteins involved in key cellular functions, such as electron transport, can no longer function properly without water as a solvent (Kranner, 2005). Likewise, UVR produces ROS from over-excited photosynthetic pigments (He and Häder, 2002) and from damaged electron transport chains (He and Häder, 2002; Pattanaik et al., 2007).

Because ROS can damage DNA and other biomolecules (Cooke, 2003), cells have evolved antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase to remove free radicals and reduce oxidative damage. SODs, for example, are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (McCord and Fridovich, 1988; He and Hader, 2002). *Chroococcidiopsis* may use SOD as a protection against oxidative damage, as cells have been shown to express SOD in their membranes after long-term desiccation (Caiola et al., 1996b; Billi, 2009b).

In the above sections, I have described a range of extreme environments that life on Earth has been found in. I described what is known about life in these environments and also outlined what is known about the mechanisms by which organisms can survive in them. Next I will turn to the question of synthetic biology and what the future may hold in terms of our resource utilization and our ability to engineer organisms for extreme environments.

Synthetic biology solves the mass problem, as a tool for resource utilization in space

Synthetic biology has been defined as the process of making biology easier to engineer via the design and construction of biological systems not found in nature (Endy, 2005). Recent advances in the synthesis of whole genomes (Gibson et al., 2010), novel applications of synthetic biology (Khalil and Collins, 2010) and the changing economics of DNA synthesis (Carlson, 2009) have enabled a move away from chemical manufacturing towards biological manufacturing. Recent examples range from fuels (Schirmer et al., 2010, Steen et al., 2010), to chemicals (Wang et al., 2009) and drugs (Ro

et al., 2006). With this growing ability to harness organisms for the production of resources on Earth, comes the possibility of using synthetic biology in space to do the same. Synthetic biology offers a potential ability to produce food, fuel and materials from *in situ* resources in space environments, such as the Moon and Mars. Such ability would offer a lightweight, self-replicating system that could be programmed, and re-programmed, for the production of consumables as part of a long-term human presence in the solar system. This kind of *in situ* resource utilization solves the mass problem described at the beginning of this chapter, because these organisms could produce things from the resources found around them rather than sending everything from Earth. But does the solar system really have the resources that biology needs to support such an endeavor, or is it a dry barren place?

Resources in space

The view of the resources available in space has changed radically in recent years. The abundance of water ice on Mars has long been known, but the availability of water on the moon and on asteroids has only recently been shown. Until this time, most space biological studies had focused on using biology for recycling resources for closed loop life support, such as the re-processing of CO_2 produced by humans (Gòdia et al., 2002; Poughon et al., 2009). Biological *in situ* resource utilization has been suggested for the moon in the past (e.g., Johansson, 1995; Brown et al., 2008), but a NASA workshop in 2008 concluded that biological productivity in lunar regolith would not be feasible due to the lack of oxygen and CO_2 available (Dalton and Roberto, 2008).

The Moon Mineralogy Mapper (Pieters et al., 2009) and the Lunar Crater Observation and Sensing Satellite (LCROSS) mission (Colaprete et al., 2010; Schultz et al., 2010) however, have since dramatically changed the view of the moon. The LCROSS mission provided direct spectroscopic evidence of water and other volatiles by crashing an empty rocket stage into a permanently shadowed crater on the South Pole of the Moon (Colaprete et al., 2010, Schultz et al., 2010). The source of water ice found is thought to have arrived on the moon from impact events from outer solar system bodies such as from asteroids and comets (Papike, et al., 1982). Table 1 shows the spectra of the various volatiles found in the lunar ice. Everything that biology needs to survive (C, H, N, O, S, K, Ca), is present, including a fixable carbon source (carbon dioxide) and a nitrogen source (ammonia). The only element missing is phosphorus. Phosphorus, however, has already been found in lunar regolith in a fraction referred to as KREEP (Potassium, Rare Earth Elements and Phosphorus) (Warren and Wasson, 1979). Light hydrocarbons including methane, methanol and ethylene were also present, as were the sulfur-bearing species hydrogen sulfide and sulfur dioxide. The water vapor in the instrument's view was ~155 kilograms, estimating the total water content at 5.6 ± 2.9% by mass (Colaprete et al., 2010). This updated view of the resources on the Moon, make the prospect of biological *in situ* resource utilization (BISRU) a more promising reality there.

An extremophile chassis organism

Specialized organisms for the production of particular chemicals and foods are often used in industrial microbiology. For example, the yeast *Saccharomyces cerevisiae* is used in brewing and bread making and *E. coli* is regularly used for the production of drugs such

as recombinant insulin (reviewed in Black, 1989). The concept of a "chassis organism" is one that is designed to be easily tractable to genetic manipulation. In the future, chassis organisms hardened to extreme environments could be developed for the production of consumables in space. One idea to begin the development of a chassis organism for space is to look at organisms in extreme environments on Earth as a toolkit for synthetic biology (Rothschild, 2010). It is questionable if *Chroococcidiopsis* would be suitable as a chassis organism, but initial studies have demonstrated that basic genetic engineering is possible in the organism. A novel DNA extraction method was shown by Billi et al. (1998), and a plasmid transfer system by Billi et al. (2001). More recently, Billi (2009a) demonstrated homologous recombination with a mobilizable plasmid in *Chroococcidiopsis*. In addition, Huang et al. (2010) recently developed a number of tools for synthetic biology in cyanobacteria including BioBrick shuttle vectors, reporter proteins, a range of promoters and a targeted protein degradation system demonstrated in *Synechocystis* and *Nostoc* species. These first few examples of genetic tools for cyanobacteria demonstrate a growing potential to engineer these organisms for future applications, both on Earth and in space.

Spirulina (Arthrospira platensis / *Arthrospira maxima)* is a cyanobacterium grown on Earth as a human health food supplement (Janssen et al., 2010). *Spirulina* uses photosynthesis to fix carbon dioxide to make sugar. It is also a complete protein source and is approximately 50% protein by mass (Pandey and Tiwari, 2010). As a simple example, if *Spirulina* could be engineered to survive the extreme environment of the lunar surface, or shielded from this environment underground or in a bioreactor, then the

lunar carbon dioxide and other resources described above could one day be used as a feedstock to grow food for human consumption. This example assumes a plentiful power source such as from solar or nuclear, in order to keep liquid water available. This demonstrates how natural biology could be used for the production of consumables in space. Synthetic biology could then add to this ability by engineering organisms for the production of other compounds, such as biomaterials, chemicals and fuels.

Can we engineer organisms for extreme environments?

A number of genetic engineering projects have demonstrated the engineering of extremophilic traits into non-extremophiles. For example, preliminary work has taken place on the genetic engineering of desiccation tolerance in *E. coli* using parts from cyanobacteria. Because of the known role that sugar plays in acting as a substitute for water during desiccation, cells were made to express a sucrose synthase taken from the *Synechocystis* genome and showed a 10,000-fold increase in desiccation tolerance (Billi et al., 2000a). The cold tolerant phenotype has also been engineered into *E. coli,* allowing them to grow 141-fold faster at 8 °C than the unmodified organisms (Ferrer et al., 2003). The authors had hypothesized that protein folding was a limiting function at lower temperatures and therefore overexpressed a chaperone protein, taken from the psychrophilic bacterium *Oleispira antarctica* (Ferrer et al., 2003). In another example, from the yeast *S. cerevisiae, c*ells were made to withstand high levels of salt in the media by directed evolution (Dhar et al., 2011). The authors sequenced the newly evolved organisms and found an increased ploidy in their genomes, indicating that genome size correlated with salt tolerant ability. These preliminary examples in single celled

organisms show that it is already possible to engineer or evolve organisms for survival and productivity under a range of extreme conditions.

Thesis goals and unanswered questions

Given what we know about the resources available in the solar system, extreme environments, current and potential capabilities of synthetic biology and the mechanisms that organisms use to survive in extremes, I will now return to the goals of this thesis. To recap, the goals were to survey the cyanobacterial genus *Chroococcidiopsis*, to characterize unknown species, to identify possible mechanisms of tolerance to extreme environments, and finally to validate and build a synthetic extremophile capable of surviving in a space environment. *Chroococcidiopsis* was investigated as a potential chassis organism for biological *in situ* resource utilization in space. In order to evaluate its suitability there are a number of unanswered questions that should first be addressed.

Before I begin making inferences about the evolution of extremophilic traits, it is important to know if the current phylogeny of *Chroococcidiopsis* is accurate, as phylogeny should be based on evolution. Would the current phylogeny of *Chroococcidiopsis* change if more markers were included and how might the existing 16S rRNA trees differ from trees produced with other markers? If species sampling were to be increased to include species from different environments, where would these previously unsequenced species of *Chroococcidiopsis* map onto the current phylogeny of the genus? Would the newly characterized strains be resistant to extreme conditions as well? How would these new species survive at different temperatures and how well

would they tolerate UVR, salt, desiccation and oxidative damage? Would these traits correlate with the polyphyly of the genus? To build a toolkit for extremophile synthetic biology, it would be important to find out the mechanisms that these organisms use in order to survive in extreme environments. For example, do these organisms have mechanisms to protect themselves against damage, or repair it? In the future, could these traits be transferred to other organisms to allow them to live in extreme environments?

This thesis now begins to answer these questions. Chapter 2 is a methods chapter and details the development of a growth assay for *Chroococcidiopsis.* This assay was designed to produce growth rates and thermal reaction norms by measuring growth in different species at different temperatures. In order to study growth rates, a high throughput optical density assay in 96 well plates was designed, however a number of problems were encountered with the amount of cell aggregation and media evaporation taking place in the wells. *Chroococcidiopsis* morphology, where a mother cell can contain a number of smaller cells, and cells can aggregate together and form biofilms, make it difficult to work with. The assay was unsuccessful in measuring growth rates via optical density. This highlights a problem when working with extremophiles, which is that common techniques may not be as amenable for use with non-model organism. Eventually, instead of reading optical density, an alternative assay was developed for measuring survival and cell density on agar plates and this assay is outlined in detail in the next chapter.

In Chapter 3, in order to understand the geographical distribution of *Chroococcidiopsis*, I obtained 15 different species from a number of culture collections from around the world that had been isolated from a wide range of environments. These environments included hot deserts such as the Negev in Israel, cold deserts, such as in Antarctica, and also species from fresh and saltwater habitats.

Historically, and in the majority of culture collections, *Chroococcidiopsis* had been identified by morphology alone. DNA sequencing has since allowed the genus to be identified by sequence homology to other known species. However, previous phylogenies reconstructed from sequence data have shown that although two species may look the same, they may have different evolutionary histories. As the main goal of the thesis was to try to map phenotypic characteristics onto a phylogeny of the genus, it was important to have the best phylogeny possible given the resources available. Phylogenies constructed from 16S rRNA genes are common in the literature, but it is known that multiple genetic markers will produce more accurate phylogenetic trees.

In order to understand the evolutionary history of the *Chroococcidiopsis* genus, I sequenced four different markers from each of the 15 species of *Chroococcidiopsis* being surveyed. The chapter represents the highest resolution of the phylogeny of the *Chroococcidiopsis* genus yet published. I then began to map different traits to this phylogeny. The first trait studied was that of halotolerance, the ability of the organisms to grow in saltwater. The main finding from this chapter was that a previously unidentified clade of saltwater *Chroococcidiopsis* existed, that was distinct from the

freshwater species. This is the first example of the polyphyly of *Chroococcidiopsis* being linked to a particular phenotype. Although the salt tolerant phenotype is not particularly useful in a space environment, this result still helps to elucidate the different characteristics of these organisms and the phylogeny then continued to be used in Chapter 4 of the thesis.

In Chapter 4, I down-selected from the 16 initial species to eight species of *Chroococcidiopsis* based on phylogeny, isolation environment and location. I then characterized these eight species in detail, by studying their ability to tolerate UVC radiation, desiccation and freeze thawing. Two previously uncharacterized species were able to survive greater levels of UVC radiation and freeze thawing than other species. These two species were of interest because they are closely related phylogenetically but phenotypically divergent. In order to begin to understand the mechanisms that these organisms use to tolerate extreme conditions, I then characterized their DNA damage and subsequent repair ability via a thymine dimer assay. I performed experiments both in the light and in the dark to see if light dependent repair was occurring. I also characterized their oxidative damage tolerance via resistance to H_2O_2-induced damage in order to understand if there was a possible role for antioxidants in the protection of the cells. In addition to DNA repair, I studied the role that protective pigments could play, by performing pigment extractions and spectral scans which showed the absorbance wavelengths of different pigments produced by the organisms.

In the final Chapter, I summarize the work done in this thesis and conclude by linking together the results from the previous chapters. Although the grand goal of the thesis, to design a synthetic extremophile, has not yet been achieved, the results are described and their significance is discussed. I finally conclude the thesis by making a number of recommendations for future areas of study that could lead from the work presented here. Overall, this work lays a foundation for the further study of *Chroococcidiopsis* and in particular the traits and mechanisms responsible for survival in extreme environments.

Figures in Chapter 1.

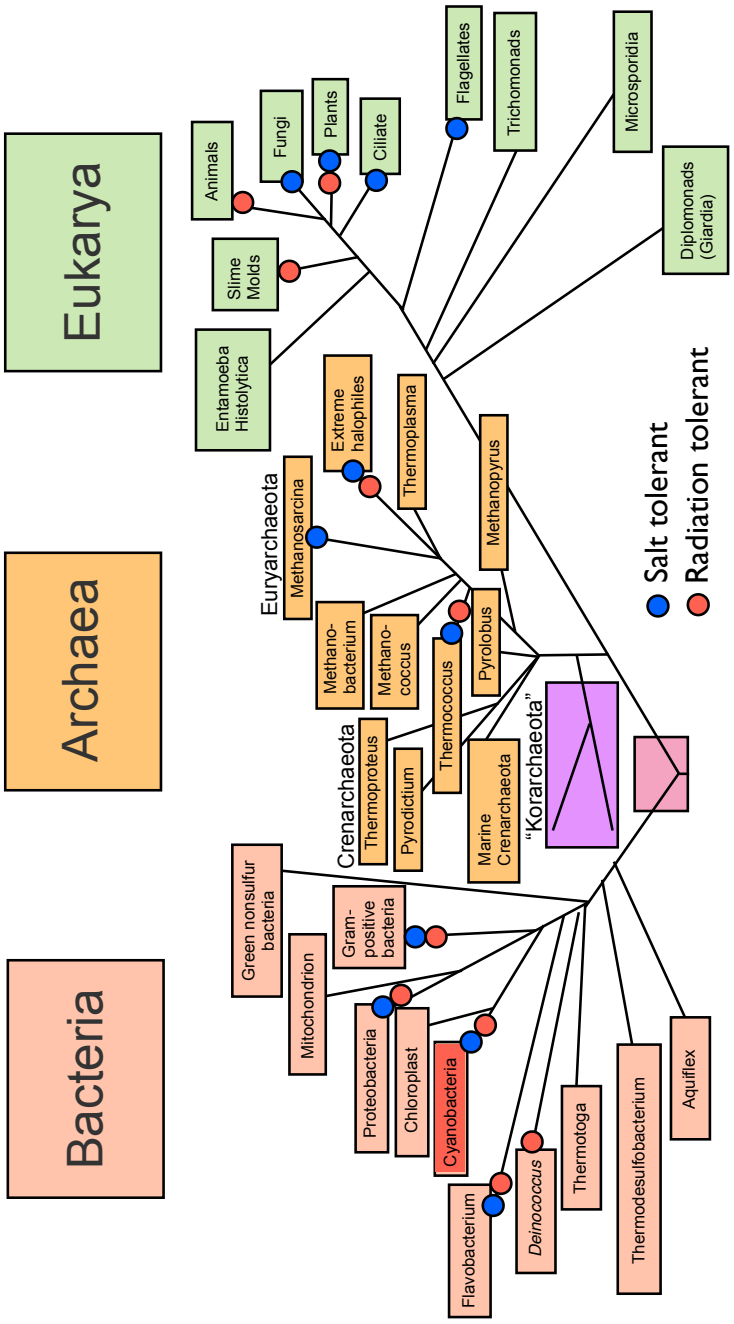


Fig. 1.1: Distribution of salt tolerance and radiation tolerance in the three domains of life. This diagram is based on (Blankenship, 2010; Madigan et al., 2010; Oren, 2008 [which describes organisms capable of surviving in saltwater environments]; Pace, 1997; Cox and Battista, 2005 [which describes organisms capable of surviving high levels of radiation]). The diagram indicates that they may have evolved independently on multiple occasions and that radiation resistance and salt tolerance may be linked by a common mechanism.

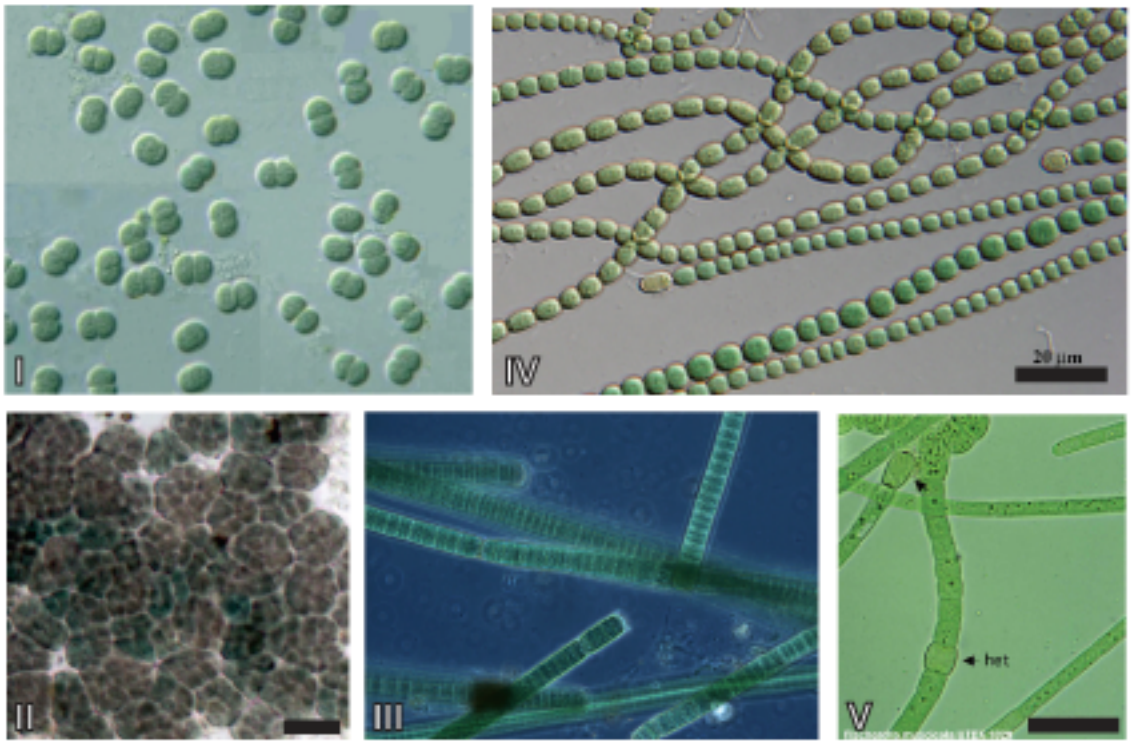


Fig. 1.2: Example morphological diversity of the five cyanobacterial subsections.

I: *Synechocystis.* Credit: Microbial Digital Specimen Archives.
II: *Chroococcidiopsis polansiana* (CCMP 1489), scale bar 20 µM
III: *Oscillatoria.* Collected from Chocolate Pots thermal spring, Yellowstone National Park, Fall 2004. Credit: Lynn Rothschild.
IV: *Anabaena sp*. (Tischer/PCC 7937 /UTEX 1444) Credit: Culture Collection of Autotrophic Organisms (CCALA), scale bar 20 µM.
V: *Fischerella muscicola,* (UTEX 1829): Credit: Instituto de Bioquímica Vegetal y Fotosíntesis, scale bar 20 µM.

Fig. 1.3: Image of hypolithic cyanobacteria found growing under a rock in the Gobabeb desert in Namibia. Photograph courtesy of Matthew Reyes (Exploration Solutions Inc.)

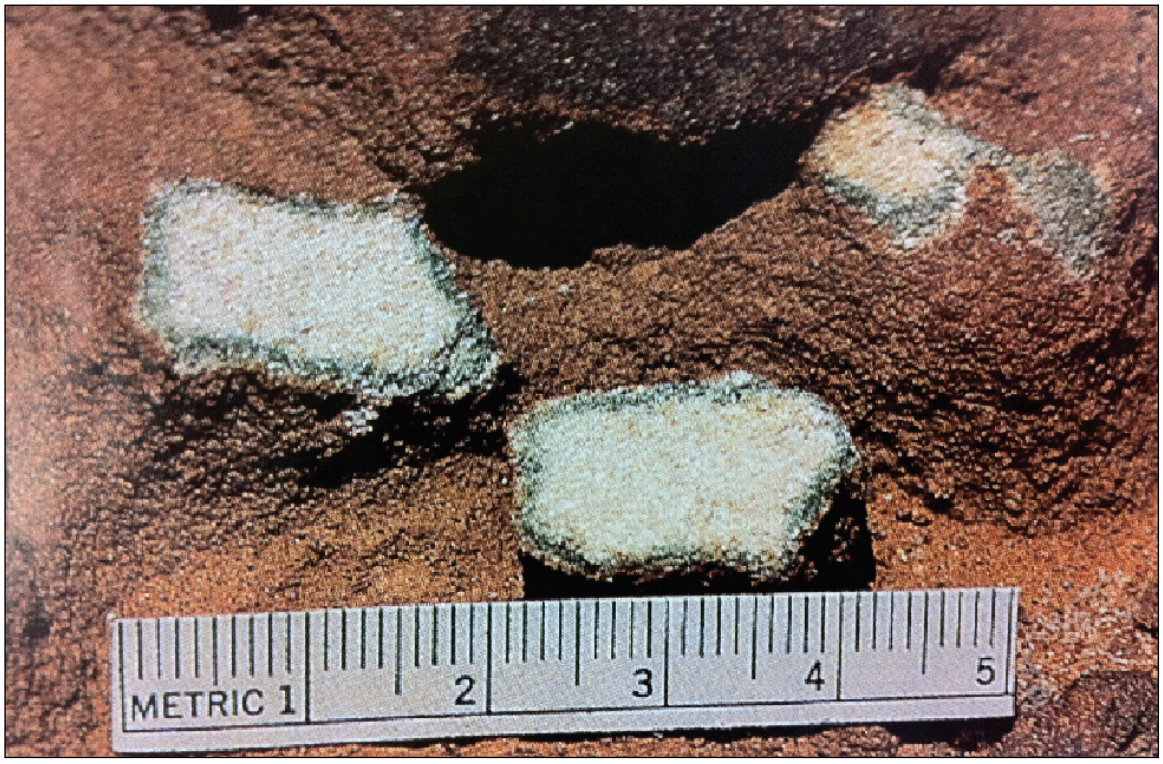


Fig. 1.4: Image of Nubian sandstone from the Negev Desert, Israel that has been chipped away to reveal a community of cryptoendolithic cyanobacteria living underneath the surface. Photograph from Whitton and Potts (2000).

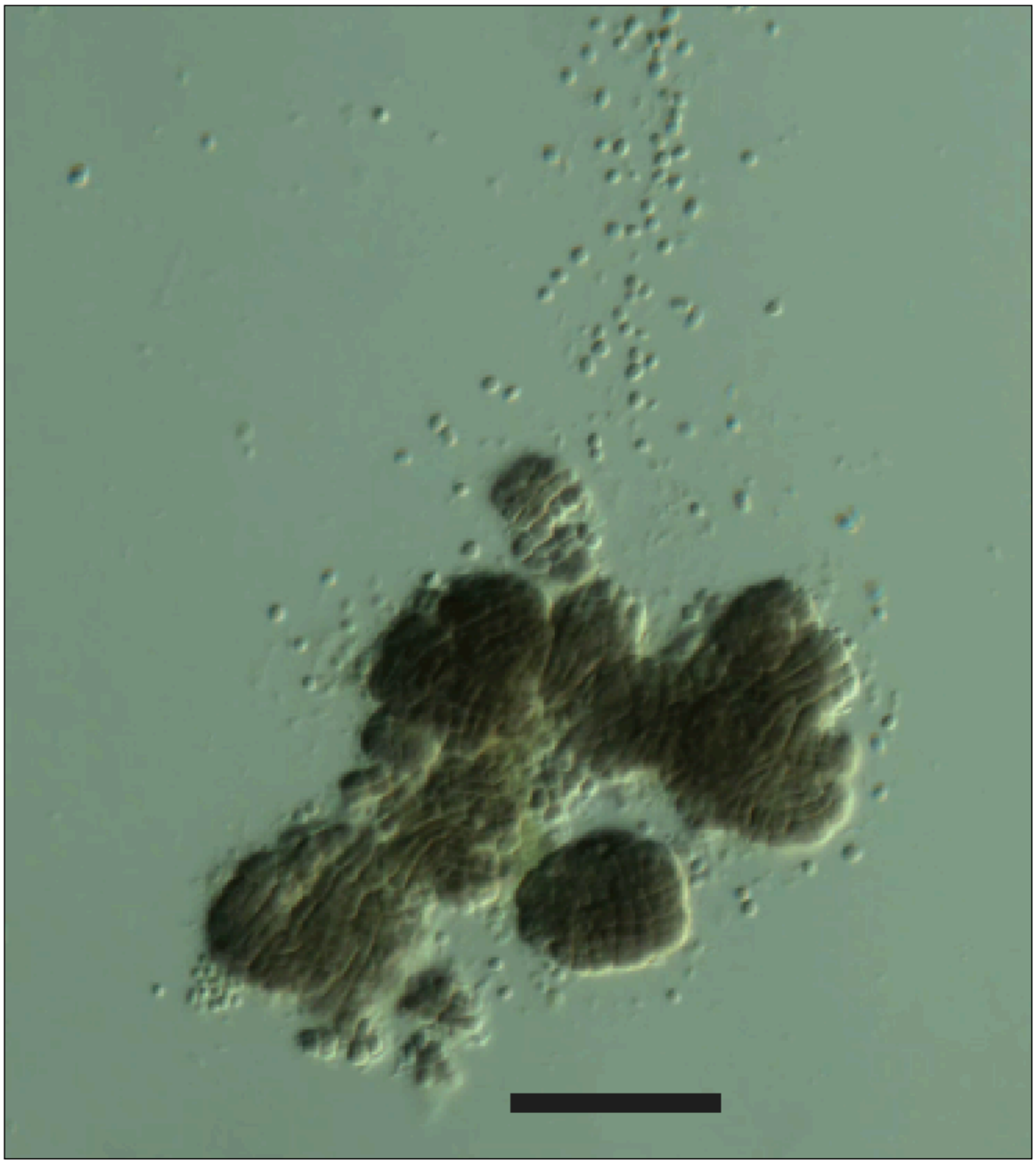


Fig. 1.5: Light micrograph of species CCMP 1991 (Hawaii) showing baeocyte morphology, scale bar = 50 μM.

Tables in Chapter 1.

Compound	Chemical name	% Relative to H_2O	% by total mass
H_2O	Water	100	5.60
H_2S	Hydrogen sulfide	16.75	0.94
NH_3	Ammonia	6.03	0.34
SO_2	Sulfur dioxide	3.19	0.18
C_2H_4	Ethylene	3.12	0.17
CO_2	Carbon dioxide	2.17	0.12
CH_3OH	Methanol	1.55	0.09
CH_4	Methane	0.65	0.04
OH	Hydroxide	0.03	0.002

Table 1: Composition of lunar ice: compounds identified by spectroscopy in the LCROSS ejecta plume (Colaprete et al., 2010).

CHAPTER 2

METHODS FOR MEASURING GROWTH IN *CHROOCOCCIDIOPSIS*

Contributions

In this chapter, I developed the growth assays and performed the experiments and data collection. I had help from Mike Grace with cell culture and optical density readings and from Norman Wang in initially setting up the MySQL database for the data processing.

Introduction

In order to understand the mechanisms of life at cold temperatures, one of the goals of this thesis was to survey a number of species of *Chroococcidiopsis* for their ability to survive and be productive at a range of different temperatures. If life is to be engineered to survive the extreme cold temperatures of the moon or Mars, then an understanding of how organisms survive and live in cold environments on Earth is a good starting point. I hypothesized that particular species from colder climates would have adapted to grow faster at colder temperatures than species from warmer climates. In previous studies of Antarctic cyanobacteria, Tang et al. (1997) had taken isolates from field samples and characterized their ability to grow at seven different temperatures between 5 and 35 °C. The growth rates at different temperatures were then plotted, showing change in growth rate against change in temperature. From this, the growth temperature optimum for each species was identified. Such a plot, where changes in an environmental variable correspond to changes in a phenotype is called a reaction norm, as shown in Fig. 2.1.

A number of previous studies have investigated thermal reaction norms, how growth rates change with temperature and how organisms adapt and display trade-offs between growth rate and survival. There are two competing hypotheses, one is named “hotter is

better" which says that the rate-depressing affects of lower temperature cannot be overcome by physiology or genetic adaptation (Hamilton, 1973; Bennett, 1987; Knies et al., 2009). The second hypothesis called "perfect-compensation" says that genetic adaptation is capable of circumventing the temperature dependence of biochemical kinetics (Clarke, 2003). It was because of the existence of this theoretical framework that I wanted to use reaction norms as a way to understand cold growth phenotypes in *Chroococcidiopsis*. I had hoped that this analysis would be the first step towards understanding the mechanisms of temperature adaptation in these organisms, as has been demonstrated in earlier work. Knies et al. (2006) previously investigated growth rate changes in populations of phage infected *E. coli* grown at different temperatures. The authors identified a number of genetic mutations that had occurred in the phage that corresponded with changes in the thermal reaction norms. Other studies have found generalist vs. specialist trade-offs between temperature range and optimum growth rate at different temperatures (Gilchrist, 1995; Izem and Kingsolver, 2005; Knies et al., 2006). For example, an organism adapted to a narrow temperature range may have optimized enzymatic activity within that range, thereby giving an increased fitness at that temperature. However the same organism may then have a fitness disadvantage growing at a temperature outside of this range (Hochachka and Somero, 2002).

To characterize thermal reaction norms and identify adaptation to different temperatures I needed to perform growth assays at a range of different temperatures. I wanted to develop a quantitative measure of the growth rate of *Chroococcidiopsis* for all 15 species being studied. I therefore tried to develop a high throughput growth assay in 96 well

plates. However, due to the unusual morphology and cell aggregation, optical density proved to be a difficult variable to measure. The process of developing this assay is documented here, and the problems encountered, such as cell aggregation and media evaporation are outlined, as are a number of solutions to these problems. Unfortunately, I was not able to produce suitable growth rates from this assay, and an agar plating assay was developed instead, which is detailed in Chapter 3. However, a number of new ideas are proposed that may provide future avenues for success for those wishing to continue the development of a quantitative growth assay in the future.

Measuring growth in bacteria

Bacterial fission is the process of organismal growth, where a cell first replicates its DNA, then divides in two, either by budding or by symmetrical division. In some bacteria such as *Chroococcidiopsis*, the pattern of division creates a mother cell, with a number of smaller cells inside of it called baeocytes, as described in Chapter 1. Measuring cell number in baeocyte forming organisms can be challenging, due to the variation in cell number because there are multiple cells located inside a mother cell. Optical density is a common measure of cell growth as media turbidity is correlated positively with cell abundance (Wyman and Fay, 1986). However, cell aggregates can increase variance in optical density making it a problematic assay for some genera such as *Chroococcidiopsis*.

Optical density measurements are normally performed at 600 nm for organisms such as *E. coli* or yeast because this wavelength is a suitable measurement to infer growth, as

cells change the turbidity of the medium. However, for photosynthetic organisms such as cyanobacteria, 600 nm is avoided because cyanobacteria tend to have pigments such as phycobilins that absorb near to 600 nm and can interfere with the turbidity readings. Instead, a wavelength of 730 nm is more commonly used for photosynthetic organisms as it is outside the absorbance peak of any of the known photosynthetic pigments and is therefore also a suitable wavelength for measuring cell turbidity (Wyman and Fay, 1986).

Initial growth in Erlenmeyer flasks

To begin investigating growth rates in *Chroococcidiopsis,* I started by repeating optical density growth assays from previous studies (Billi et al., 1996). I cultured cells in 250 mL Erlenmeyer flasks as shown in Fig 2.2 and 2.3. Cultures were grown in 100 mL of BG11 media (for the freshwater species CCMEE 246, Negev, Israel) and L1 (for the saltwater species CCMP 3184, Samoa). I measured optical density at the start of the experiment and then every two days, by taking 200 μL of culture and reading the optical density at 730 nm on a Spectramax 384 spectrophotometer (Molecular Devices, Sunnyvale, CA.). A plot of optical density changing over time is shown in Fig 2.4 (top). I also performed cell counts using a hemocytometer, after first sonicating 200 μL of cell culture (Ultrasonic processor XL, Misonix, tip size 1/16, setting three for three seconds), to separate single cells in order to be able to count them. Cells were then counted in 10 μL samples. Cells were also grown on a shaking platform (135 rpm) and non-shaking platform to see if shaking increased growth rate. The results are shown in Fig. 2.4 and the increased aeration is likely responsible for the increased growth. Initially, single measurements were taken for each culture, but to estimate the variance in the

measurements, duplicate measurements were later taken. The large error bars, which represent the mean and the variance for each data point, are symptomatic of non-homogeneous cell cultures such as *Chroococcidiopsis.*

Originally, I had planned that optical density growth curves would be the main assay for measuring changes in response to different temperatures. But growing large number of cultures in Erlenmeyer flasks takes up a lot of space. A gradient incubator was used that contained five growth chambers, each capable of maintaining growth at a different temperature, as shown in Fig. 2.5. However each compartment of the gradient incubator could only hold a limited number of Erlenmeyer flasks at once. The process of subsampling from multiple cultures at multiple time points and the repeated opening and closing of cultures increased the risk of contaminating cultures during the assay. I therefore attempted to develop a high-throughput assay in order to read optical density and infer growth curves from cultures growing in 96 well plates. This had the advantage of taking up less space in the incubator and also being able to read the optical density of the culture, without having to open the culture and take a sub-sample. I planned to automate this growth assay in order to increase the number of experiments that I could perform inside the gradient incubator. Such an automated assay in 96 well plates would have allowed for more conditions to be tested, more replicates per sample, and smoother growth curves due to reduced variance and increased accuracy per sample.

Software

I collected optical density data from 96 well plates using a plate reader that saved the data as an XML or text file that could later be processed. Because high-throughput assays

such as this produce large amounts of data, I wrote scripts using the statistical package R (r-project.org), to automate the processing of this data file. The goal was to put a plate into the plate reader, read the optical density, add the results to a mySQL database, process the data, take averages and then display growth curves for each series. The plots were then produced using ggplot2 (Wickham, 2009), a package available for the R platform, and the scripts to produce them can be downloaded from: http://openwetware.org/wiki/Cumbers:Software. The SoftMax Pro software that the machine runs on also contains a scripting environment and this was also used for some analysis.

Growth assay in 96 well plates

To infer growth curves from optical density of cells in 96 well plates, cultures were added to the plates in replicates of 8 wells. Each well contained 200 μL of cells of a starting culture of $1x10^6$ cells·mL. Optical density at 730 nm was then measured on the plate reader every few days. A row of blank media was also added to the plate, and the average of this reading was subtracted from each of the sample wells. Fig. 2.6. shows multiple plates growing in a growth incubator and Fig. 2.7. shows a close-up view of a number of species grown at different temperatures. Initial growth curves at a single temperature showed little variability between wells, as shown in Fig. 2.8 (left). However, these results were obtained from SAG 39.79 (Cuba), a species with a homogenous growth characteristic where cell aggregates were not observed. The same assay performed with a culture showing cell aggregates (CCMP 3184, Samoa) showed a very different result as shown in Fig. 2.8 (right). As the goal of the assay was to be able to

compare growth rates in different species, I needed the assay to be able to read growth curves from all species being studied. I hypothesized that the well by well variability observed was likely caused by the cellular aggregates, as detailed below.

Problems encountered with aggregating cells

Chroococcidiopsis is known to form aggregates and biofilms when grown in Erlenmeyer flasks, as can be seen in Fig. 2.9. Cell aggregates can also be seen when cells are grown in 96 well plates, as shown in Fig. 2.10. These images demonstrate the difficulty of growing *Chroococcidiopsis* in liquid media as it is hard to ensure that growth measurements are started from a homogeneous culture and cell aggregates can also form as cells grow. Cell aggregates are problematic as they distort the path of the light more than homogeneous cultures do, creating more variability in optical density. The error bars in Fig. 2.8 represent the average and standard deviation of three repeat reads of the same plate on the plate reader. In between each read, the plate was automatically shaken for three seconds to ensure proper mixing of the cells. The error bars on the species that did not show cell aggregates (SAG 39.79, Cuba) in Fig. 2.8 (left) are much smaller than those of the species that showed larger cell aggregates (CCMP 3184, Samoa) Fig. 2.8 (right) Also note that species (SAG 39.79, Cuba) in Fig.2.8 (left) shows each well starting at a similar optical density whilst the species with larger cell aggregates (CCMP 3184, Samoa) shown in Fig. 2.8 (right) show a much larger variation in the starting optical density. The different starting optical densities for each of the curves also creates asynchronous starting points for each growth curve. Statistical methods exist for dealing with such noisy data, which are discussed later. In order to try and reduce the problems

that cell aggregates create in optical density readings, a number of techniques were applied to try and separate the cells without damaging them. Detergent methods, such as adding 10% SDS and vortexing were not able to separate them. However, gentle sonication of the cells did successfully separate them (Ultrasonic processor XL, microtip size 1/16, repeated rounds of three seconds, Misonix Inc, Farmingdale, NY, USA), producing relatively homogeneous cultures of single cells. However, when grown in liquid cultures, the cells would re-aggregate again after a few days of separation, making this technique impractical.

Problems encountered with evaporation in 96 well plates

In addition to cell aggregates being a cause of variance in optical density readings I also noticed that the evaporation of media was occurring in each well. The 96 well plates used in this assay were clear flat bottom plates with low-evaporation Perspex lids (BD Falcon™ Cat No. 353072, BD, NJ, USA). These plates allowed for the optical density of the plate to be read without removing the lid which reduced evaporation, but it was still present, particularly at temperatures above 26 °C. This evaporation proved to be a large problem when producing growth curves from cultures grown at different temperatures as can be seen in Fig. 2.11. I initially thought that as long as each column of wells and the blank media evaporated at the same rate, then this evaporation would not have a major effect on the growth curves. However, I suspected that evaporation was also affecting the variability of the growth curves. I later observed that the evaporation was not uniform across the plate and the wells closer to the outside of the plate evaporated faster than the wells on the inside. Evaporation of water can cause two problems, the first problem is

that increased evaporation results in an increase in salt concentration in the remaining medium. These changes in salinity can affect growth and survival of cyanobacteria, which is described in more detail in Chapter 3. The second problem is that optical density readings are normalized to the path length through which the light is traveling. When optical density is read in a cuvette, the distance is the same each time, and is set by the width of the cuvette. When optical density is measured through a 96 well plate, there are two options for setting the path length of the light. The first is to manually set the path length to match that of the volume of the liquid. The second is to use the Pathcheck function, which is available with some spectrophotometers such as the SpectraMax Plus 384. Pathcheck works by using an infrared sensor to measure the volume of liquid in a well, once the volume is known, the path length can be adjusted to the volume of the well. However, Pathcheck could not be used in this assay, because it does not produce accurate readings when non-homogeneous cultures such as *Chroococcidiopsis* are measured, because the cell aggregates interfere with the infrared reading (SpectraMax Plus 384 User Manual, Molecular Devices, Sunnyvale, CA, USA). One possibility to solve this problem would be to top up each well to the original volume of 200 µL with sterile water. Another option would be to measure the volume in each well manually and to normalize to this result. However, because evaporation varied in different wells by different amounts, measuring the path length of each well manually or topping up each well to the original volume would have been impractical and defeated the objective of developing a high throughput assay.

I tried a number of methods to reduce evaporation in the 96 well plates. I first sealed the Perspex lids to the top of the plates with parafilm, however evaporation then formed in between the wells of the plates. To try to prevent this, I then covered the plates with an adhesive breathable membrane (Breathe Easy, Cat: 9123-6100, USA Scientific, USA) and adhered this to the plate with a hand roller. This membrane allowed for gas exchange between the cells and the environment. However, evaporation still formed above each well. I could remove this evaporation by spinning the plate in a centrifuge for 1 minute at 500 RPM. Despite all of these different attempts to prevent water loss, evaporation still occurred at different rates, in different wells, and caused differences in optical density readings because of it. The removal and reapplication of the breathable membrane during the assay was also an added cause of potential contamination.

Statistical smoothing of time series data

Smoothing is a statistical technique that has been implemented in the R package "ggpplot2" (Wickham, 2009) and is designed to be applied to noisy time series data. Normally, when growth curves are being plotted, each past observation is treated equally. However, with an exponential smoother applied to a data series, the weight of each data point in the series decreases over time. Fig. 2.11 shows an example smoother applied to a noisy dataset from a species of *Chroococcidiopsis* known to produce cell aggregates (EE8 246, Negev, Israel). The figure shows optical density readings from the plate reader for each time point in the series and I fitted a statistical smoother to the growth curve shown. Even with the smoother applied, the data shows erratic changes in optical

density through the time series, which is likely due to both cell aggregation and media evaporation, as I observed both in this experiment.

Sonication and plating cells onto agar plates

After the numerous attempts to solve the problems of evaporation and cell aggregation described above, I decided to begin investigating alternative assays for measuring growth and survival. After the sonication step described earlier, which effectively separated cell aggregates into homogeneous cultures of single cells, I realized that I could now measure cell density after growth directly on agar plates, which is described in more detail in Chapter 3. To test this assay on the cold growth phenotype of interest, I initially plated out three species at five different temperatures as shown in Fig. 2.12. I then plated out the remaining species until all 15 species had been grown on agar plates at eight different temperatures, as shown by cutting and pasting the images into a table in Fig. 2.13. Although this assay did not produce data suitable for measuring growth curves, it did allow a relatively fast way to assay for growth and survival at a range of temperatures. The results show that there was very limited growth at low temperatures in all of the species being studied, and none of the species assayed showed optimal growth below 26 °C. I performed a more detailed microscopic analysis of one species, PCC 8201, as shown in Fig. 2.14 and confirmed that although cells survived at 5 °C, growth was very limited. The main goal of this study was to find organisms that grew well at low temperatures and to understand the mechanism of this ability. However, these results showed that the ability to grow at cold temperatures was not a trait found in any of these species. After I obtained these results, I decided to switch the focus of the study away

from cold growth phenotypes and towards other traits of interest, such as tolerance to salt and radiation. I found that I could measure these traits by growing cells on agar plates rather than inferring growth curves from optical density readings. It was because of this shift in the focus of the study that I stopped developing the high throughput growth assay using optical density.

Lessons learned: Developing more effective strategies for the future

Despite the failure to develop a suitable optical density growth assay for *Chroococcidiopsis*, I learned a number of lessons from the problems that I encountered. If I were to repeat this study again, or want to develop an assay to measure growth curves from newly discovered organisms in the future, then there are a number of strategies that I would adopt and new approaches that I would try. One problem with the approach in the work described above, was that I was premature in developing a high throughput assay before a low throughput assay was working effectively. In hindsight, rather than spend time trying to automate the plate-reading assay, I should have started by more closely monitoring the variance in optical density and cell counts of cultures growing in larger Erlenmeyer flasks, as this would have identified the problem of biofilms and aggregates earlier. Only if these were successful should I have moved on to the 96 well plate assays. When monitoring the 96 well plates, I should have measured the growth of individual wells and plotted growth curves using excel, rather than spend time writing an automated script, for a protocol that had not yet been demonstrated to work with all of the species being assayed. Also, rather than picking a single organism to optimize the protocol for, or trying to optimize the protocol for all the species at once, I should have chosen a representative sample of three or four species to perform these initial studies on

and then, once optimized, checked that the assay worked with the rest of the organisms being studied.

Growth conditions

To aid in the discussion of developing novel growth assays for newly discovered organisms, I will use as an example, a hypothetical field sample collected from an Antarctic ice core. The goal is to characterize the distribution of cyanobacteria or other organisms in the ice core, to isolate those organisms and then to find their optimum growth temperature. Some organisms found in permanently cold environments are known to die when moved to warmer temperatures (Friedman, 1993), so it would be important to store the frozen ice core at a similar temperature as the location that it was sampled from. Samples could then be taken from the ice core and initial observations made under the microscope to assess the number and diversity of organisms present. These, presumably mixed cultures, could then be grown in Erlenmeyer flasks and sampled frequently and any changes observed and noted. Initially, to maximize the chances of organism survival and successful culturing, the organisms could be grown at two temperatures, a psychrophilic temperature, such as 4 °C and a mesophilic temperature such as 25 °C, and differences noted between the two samples. Samples could be taken regularly for optical density and microscopic analysis and this could be repeated for the various depths of the ice core, to ascertain if there were different organisms growing at different depths.

Growth medium

The growth medium used would need to be chosen carefully, by first studying the literature to see if organisms from similar ice cores had been analyzed before, and starting with that growth medium first. An alternative strategy to inform the search for a suitable growth medium would be to analyze the contents of the ice core via HPLC to find the ratios of the different elements present. A growth medium could then be made to mimic the elements in the ice core and samples cultured in this liquid medium.

Measuring optical density

Different tools could be used for monitoring optical density. Initially, for low throughput growth assays, side arm flasks could be used. These are glass Erlenmeyer flasks that contain a glass appendage through which optical density can be read without removing or opening the flask, as shown in Fig. 2.15, this speeds up the measurement time and reduces potential contamination of the cultures. However, growth readings of side arm flasks require a special spectrophotometer.

Obtaining monocultures

Separating mixed cultures into pure cultures is a prerequisite for understanding the biochemistry and genetics of an organism. A fluorescence activated cell sorter (FACS) could be utilized to both count the organisms present in an environmental sample and to separate them into single wells of a 96 well plate. Each well could then be monitored for growth and larger cultures grown up from these. However, this technique would only work if the organisms are autofluorescent. Non-autofluorescent organisms could be

stained with a nucleic acid stain such as DAPI or SYBR Gold, however, these are destructive assays and would kill the organisms. Other reliable ways to remove contaminants such as with antibiotics or repeated streaking of cells until single colonies can be isolated could also be used, as detailed in Vaara et al. (1979) and Choi et al. (2007). Finally, once I obtained monocultures, growth assays could be performed at a wider range of temperatures to assay the minimum, maximum, range and optimum growth temperatures for the organisms.

Reducing error in sampling

A 96 well plate assay is likely not suitable for slow growing organisms such as those that would be isolated from an ice core. This is because the maximum volume that 96 well plates can hold is only 300 μL. Due to the surface to volume ratio of the well, this volume can rapidly evaporate causing the problems described earlier. One alternative is to use larger plates such as 12 and 24 well, as these would have a lower surface to volume ratio and evaporation would be reduced. Unfortunately, the SpectraMax Plus 384 plate reader that I used in this study could not read plates with larger volume wells. In the future, using plates with larger well volumes would increase the size of each sample and therefore give a better estimate of the variance in the optical density. Repeat reads of a single well followed by the shaking of the plate between reads, would help show how much optical density variation is caused by cell aggregation. The related problem, of having different rates of evaporation within a single plate, could be solved by having a whole plate dedicated to each condition being tested. This would allow an average

reading from all of the wells to be calculated. Error due to outliers or instrument bias could then easily be identified and removed.

Dealing with cell aggregates

In any environmental sample, there may be a number of species that produce biofilms, such as those shown in Fig. 2.9, meaning that not all organisms found in environmental samples will be amenable to optical density readings. If the newly discovered organisms form cell aggregates, then rather than trying to separate them, an alternative strategy would be to characterize the aggregates and the individual cells within them. Assuming that the organisms produce autofluorescent pigments, modern FACS systems are capable of differentiating between single cells, two cells in active fission and clusters of numerous cells. Other flow cytometers, such as the ImageStreamX (Amnis Inc., Seattle, USA) are capable of taking micrographs of cells as they pass through the sorter. From these micrographs, morphology, cell size, aggregate number and fluorescence could all be obtained, which could aid in analysis and identification of organisms in the samples.

Part of the reason that aggregating cultures are so difficult to work with, particularly for comparative studies, is that the level of aggregation varies between species. Using the high throughput techniques described above, histograms showing number of cells, average number of cells per aggregate and cell size could be used to describe and define a standard assay for measuring the level of cell aggregation. For example, a scale of one could be defined as single cells that do not aggregate, whilst 10 could represent densely packed cells. Such a scale could be used as a standard way for researchers working with

such organisms to be able to characterize them and may aid in future comparative analyses. For example, this scale could be used to describe the aggregation level at which organisms are not emendable to optical density assays because the cell aggregates cause too much variance.

One alternative to assaying growth without breaking up cell aggregates is to observe the growth of cells in Cooper dishes. Cooper dishes are similar to petri dishes but have a recessed lid and allow direct observation of cell growth via microscopy (Cooper, 1961). Many of the morphological descriptions of the cyanobacteria described in Bergey's Manual of Systematic Bacteriology (Rippka et al., 2001) were obtained from observations of cells grown in Cooper dishes. Cooper dish culturing could also be a suitable strategy for the monitoring of cell growth and morphology for slow growing organisms, as individual cell size and replication patterns could be tracked over time using software such as ImageJ (Abramoff et al., 2004).

Alternative assays

Another alternative to ascertaining growth is to grow the organisms in a closed bioreactor or chemostat. This has the advantage of being able to feed a constant flow of fresh nutrients into, and remove waste from, the growth chamber, thereby keeping the volume constant and optimizing the growth conditions (Novick and Szilard, 1950). Closed chemostats have the advantage of being able to monitor a number of parameters including optical density, pH, temperature and elemental analysis whilst minimizing the

variance caused by other factors such as evaporation. Such a controlled static chemical environment is not possible in an Erlenmeyer flask.

Another method for inferring growth, other than by optical density is by total cell mass. This can be done, by sampling from a culture periodically, passing the sample through a filter paper, drying and then weighing the change in mass of the filter paper. This technique however, is not suitable for a high-throughput assay.

Culture independent methods and metagenomic analysis

An alternative to culturing organisms from novel environmental samples is to sequence the DNA of the organisms without first culturing them, a method known as metagenomics (Handelsman et al., 1998). DNA extractions could be performed on samples from different levels of the ice core and metagenomic libraries constructed and sequenced. Alignment and assembly of these results would show the degree of genetic and organismal diversity present within the ice core. These results might lead to more informed choices in the decisions made when attempting to culture the organisms, such as finding similar growth media that the organisms are already known to grow on. The metagenomic analysis would be particularly useful if the organisms in the ice cannot be cultured from the methods described previously and may also reveal new organisms previously unidentified. If the FACS technique described earlier was successful in isolating single cells, then growing up monocultures of these organisms or performing single cell genomics via Multiple Displacement Amplification (MDA) (Spits et al., 2006) could be performed to identify individual organisms via whole genomic sequencing.

Conclusions

This chapter has detailed the various approaches taken in developing a growth assay for measuring growth rates in *Chroococcidiopsis* at different temperatures. The goal was to be able to measure growth in order to produce thermal reaction norms to analyze adaptation by different species to different temperatures. The goal of this thesis is to understand how life on Earth survives in extreme environments, such as the extreme cold of Antarctica, in order to be able to engineer life for survival in space.

After introducing the methods used for measuring growth, I detailed the development of a high-throughput optical density assay in 96 well plates. Two main problems were encountered during the development of this assay, cell aggregation and evaporation. Each problem was discussed in detail, along with a number of attempted solutions to solve these problems. Growth on agar plates showed that none of the species of *Chroococcidiopsis* used in this study grew well at cold temperatures, which lead me to investigate other phenotypes such as salt and radiation tolerance. Although I failed to develop a high throughput assay for reading growth rates, I learned a number of lessons, described above, that could be used to develop more effective strategies for measuring growth in *Chroococcidiopsis* and other organisms in the future. The goals of this thesis were to understand the distribution and limits of life so that life could be engineered for extreme environments. Measuring growth via optical density is not a prerequisite for this but this Chapter has highlighted some of the problems of working with extremophiles and non-model organisms.

Figures in Chapter 2.

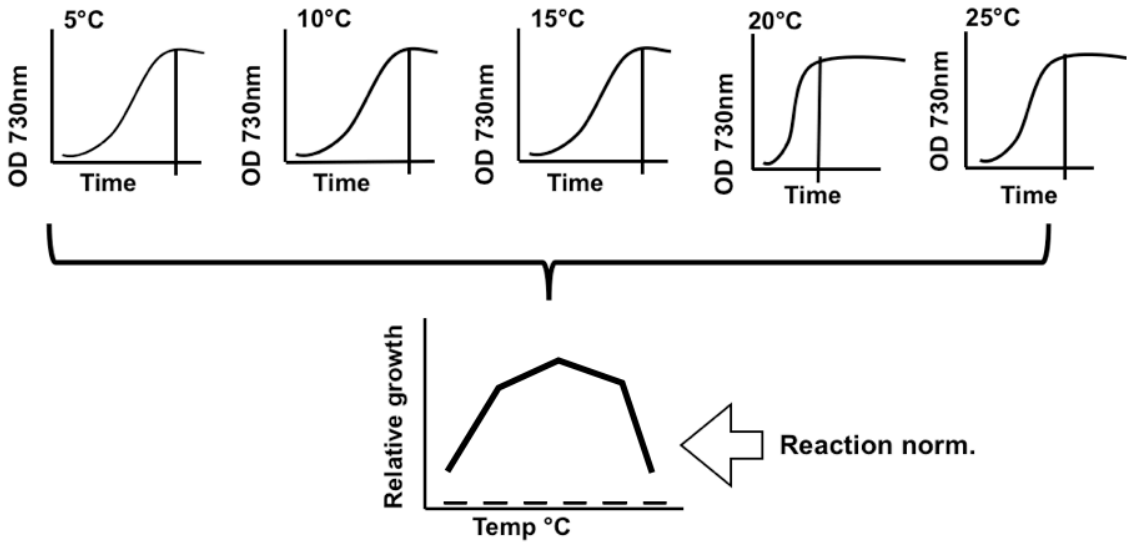
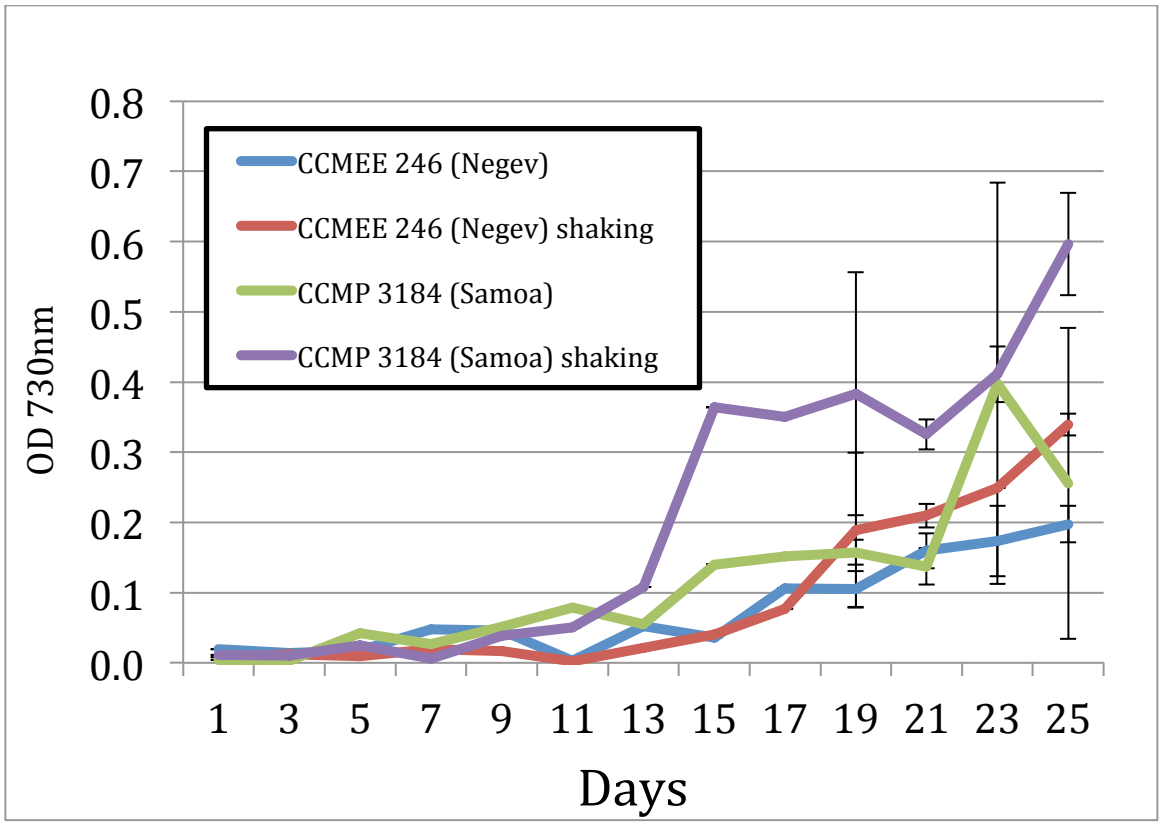


Fig. 2.1. A thermal reaction norm depicted in diagram form. The organism would be grown at a number of different temperatures, examples are shown along the top of the image. From these curves, the growth rate could be calculated and then plotted against temperature, as shown in the bottom image. This image represents a thermal reaction norm from which the shape and peak growth temperature can be calculated.

Fig. 2.2. Three week old culture of CCMEE 246 (Negev, Israel). Shown growing in BG11 media.

Fig. 2.3. Cultures growing in Erlenmeyer flasks. Here, cells can be seen growing in liquid media on a shaking platform at 135 rpm, under light of 20 µmol photons·m^{-2}·s^{-1}.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD 730nm
CCMEE 246 (Negev)
CCMEE 246 (Negev) shaking
CCMP 3184 (Samoa)
CCMP 3184 (Samoa) shaking
1 3 5 7 9 11 13 15 17 19 21 23 25
Days

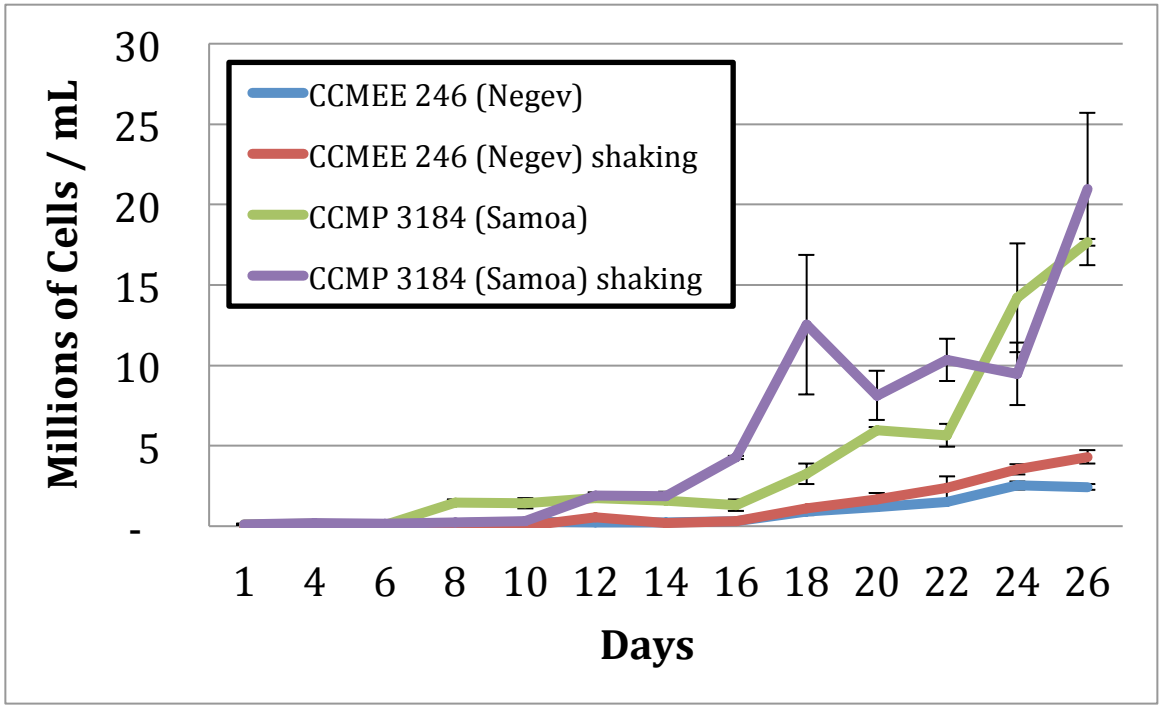

30
25
20
15
10
5
-
Millions of Cells / mL
CCMEE 246 (Negev)
CCMEE 246 (Negev) shaking
CCMP 3184 (Samoa)
CCMP 3184 (Samoa) shaking
1 4 6 8 10 12 14 16 18 20 22 24 26
Days

Fig. 2.4. Optical density assays (bottom) and hemocytometer counts (top). These were performed on two species, CCMEE 246 (Negev, Israel) and CCMP 3184 (Samoa) at 25°C. The chart shows both cells grown on a shaking platform (135 rpm) and non-shaking. Growth flasks are shown in Fig. 2.2. and cultures were grown in 100 mL of BG11 media (CCMEE 246) and L1 (CCMP3184) in 250 ml Erlenmeyer flasks. Cells were counted at the start and every two days by taking 2 x 200 μL of culture and reading the optical density (OD) at 730 nm as measured by spectrophotometer. Single samples were taken until day 19, when duplicate samples were taken to improve accuracy. For the hemocytometer readings, 200 μL was first sonicated (Ultrasonic processor XL, Misonix, tip size 1/16, setting three for three seconds) to separate cells that had aggregated together, 10 μL of cells were then counted on a hemocytometer in duplicate. The error bars in each chart show mean and variance.

Fig. 2.5. The Rexmed RIT 450 (Rexmed Inc. Taiwan) gradient incubator. This was used as it has five compartments capable of each holding a different temperature. Aluminum foil was used to normalize the light in each chamber. The light cycle was a 16h:8h light/dark cycle and a mix of Philips TLD36W/840 (cool white) and Philips TLD36w (aqua) fluorescent light at 54±2 µmol photons·m^{-2}·s^{-1} as measured with a PAR detector (PMA2100/PMA2132, Solar Light Company, PA, USA).

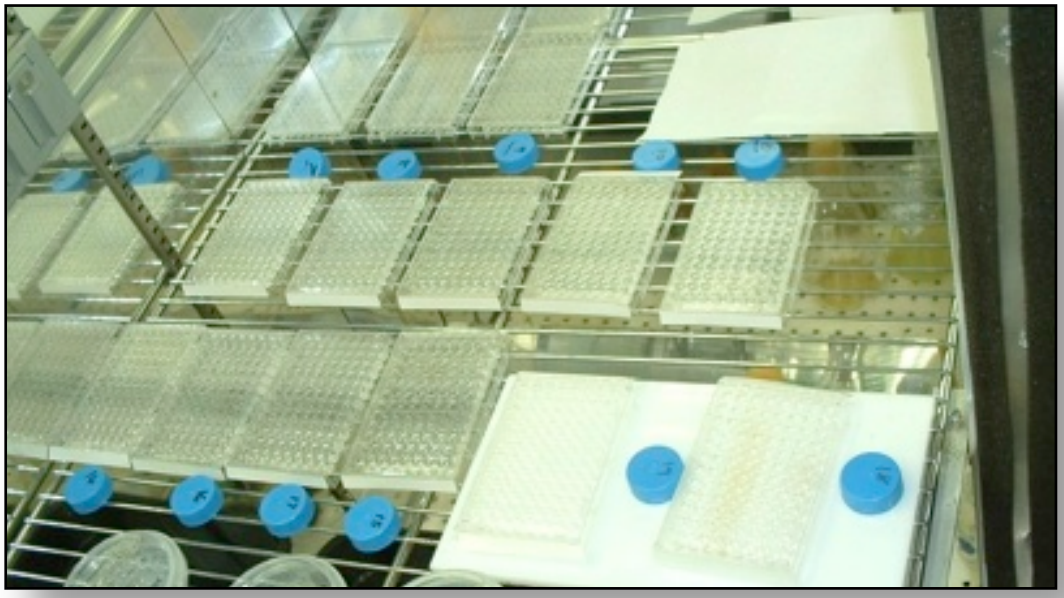


Fig. 2.6. Plates of 96 wells growing in an incubator. Each plate is covered with adhesive breathable membrane (Breathe Easy, Cat: 9123-6100, USA Scientific, USA) to try and reduce evaporation.

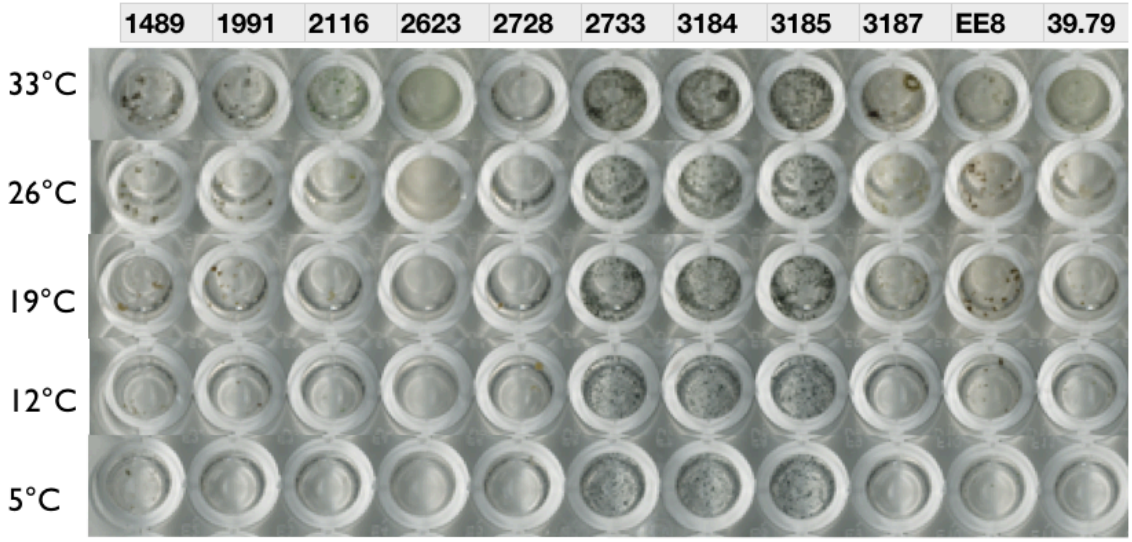


Fig. 2.7. A close-up view of a series of 96 well plate. Cells were grown here at five different temperatures. These images have been pasted together to form a single image and in general show denser growth of cells at higher temperatures. The species numbers are shown along the top of the image and can be referenced to their location with Table 2.1.

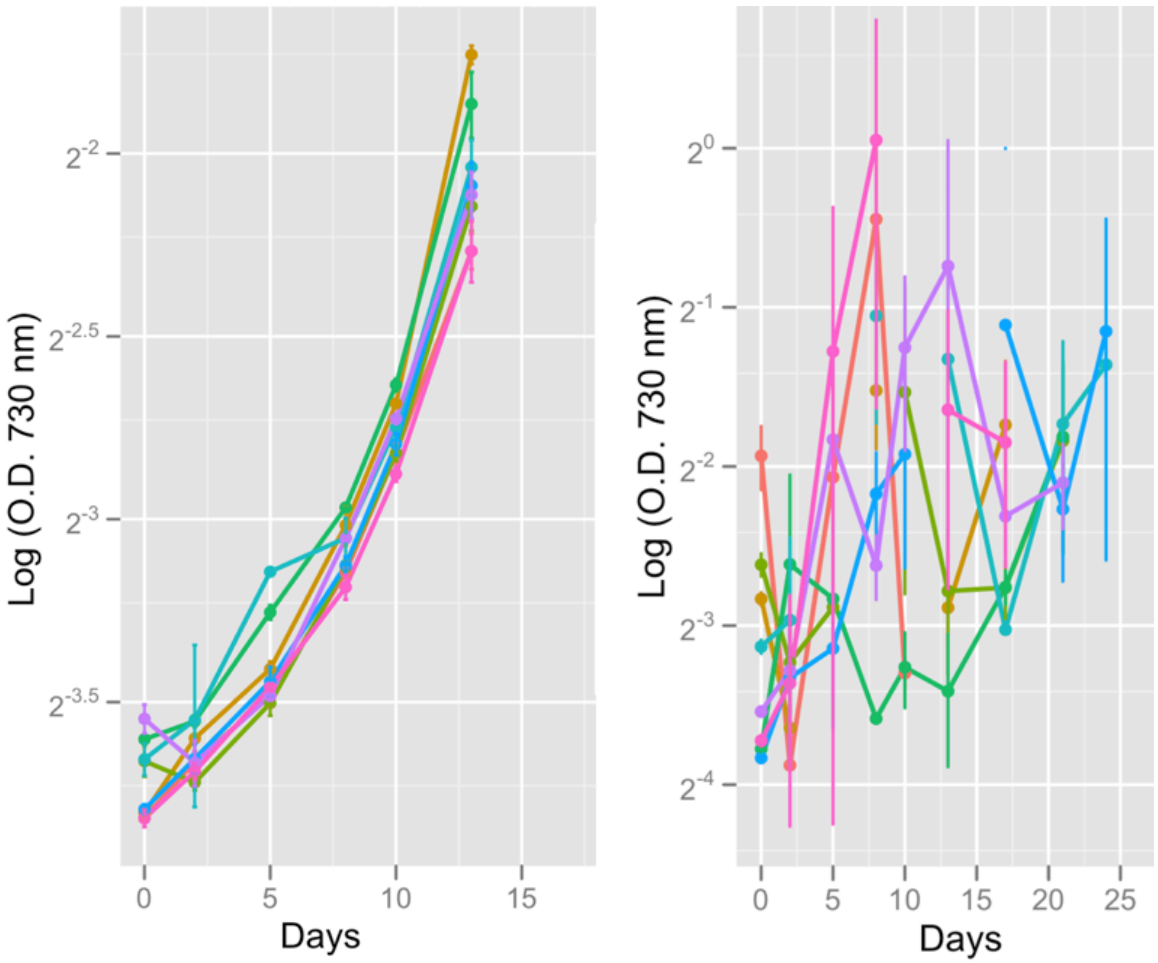


Fig 2.8. *Left*: Initial results from 96 well plate growth assays showing a species that does not form cell aggregate SAG 39.79 (Cuba). Eight replicate wells were monitored and each color represents a different well of the repeat. Each well was read three times on the spectrophotometer and the plate was automatically shaken for three seconds in between each read. All growth was performed at 26 °C. *Right*: The same assay was performed with CCMP 3184 (Samoa), a species that forms cell aggregates. This image highlights how the 96 well plate assay is not suitable for all of the species being assayed. The large variation in the CCMP 3184 (Samoa) plates is likely due to cell aggregates and different levels of evaporation in the plate.

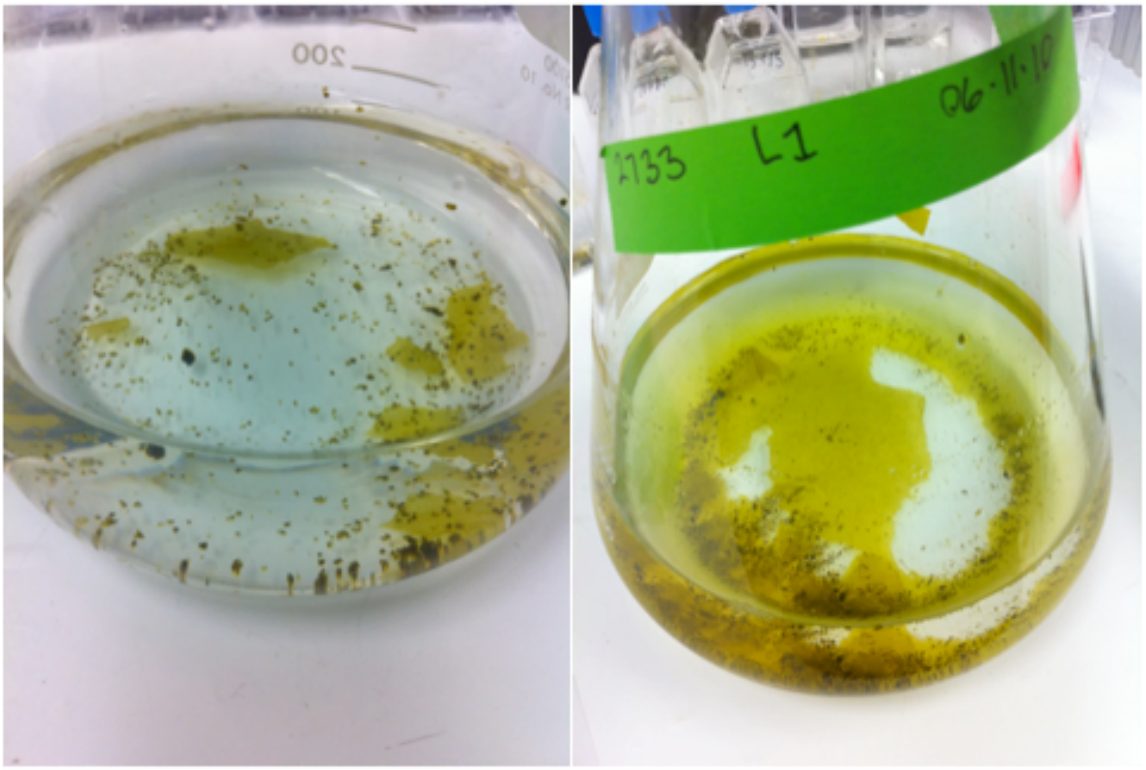


Fig. 2.9. *Chroococcidiopsis* shown forming biofilms and cell aggregates in 500 mL Erlenmeyer flasks. The left shows CCMP 1489 (C. polansiana, Galapagos) and the right shows CCMP 2733 (Micronesia).

Fig. 2.10. Cell aggregates of *Chroococcidiopsis* forming in 96 well plates. Cell aggregates increase the variance in optical density making some species unsuitable for this assay. The left well shows SAG 39.79 (Cuba) which does not show cell aggregates, the middle well contains 3184 (Samoa) which shows medium sized cell aggregates and the right shows CCMEE 246 (Negev Israel) which shows the largest cell aggregates. The diameter of each well is 0.8 cm.

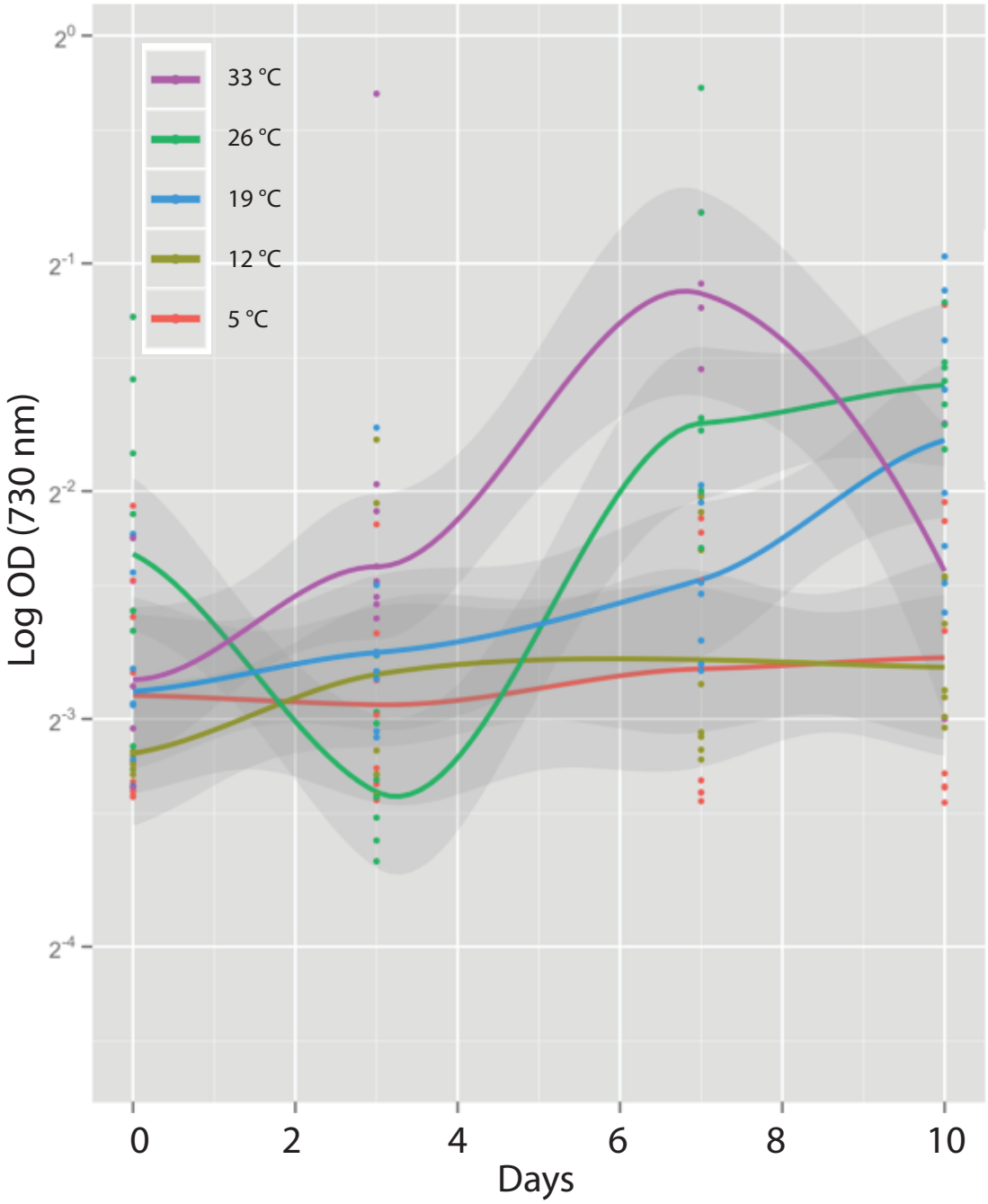


Fig. 2.11. A statistical smoother applied to temperature growth data. Reliable growth curves could not be obtained from a number of cell cultures such as EE8 246 (Negev, Israel) shown above. In this example six wells were read at each time point and the smoother fitted to those data points, indicating the line of best fit and the shaded area representing the variance observed, which is also smoothened. Cells were grown at five different temperatures as shown.

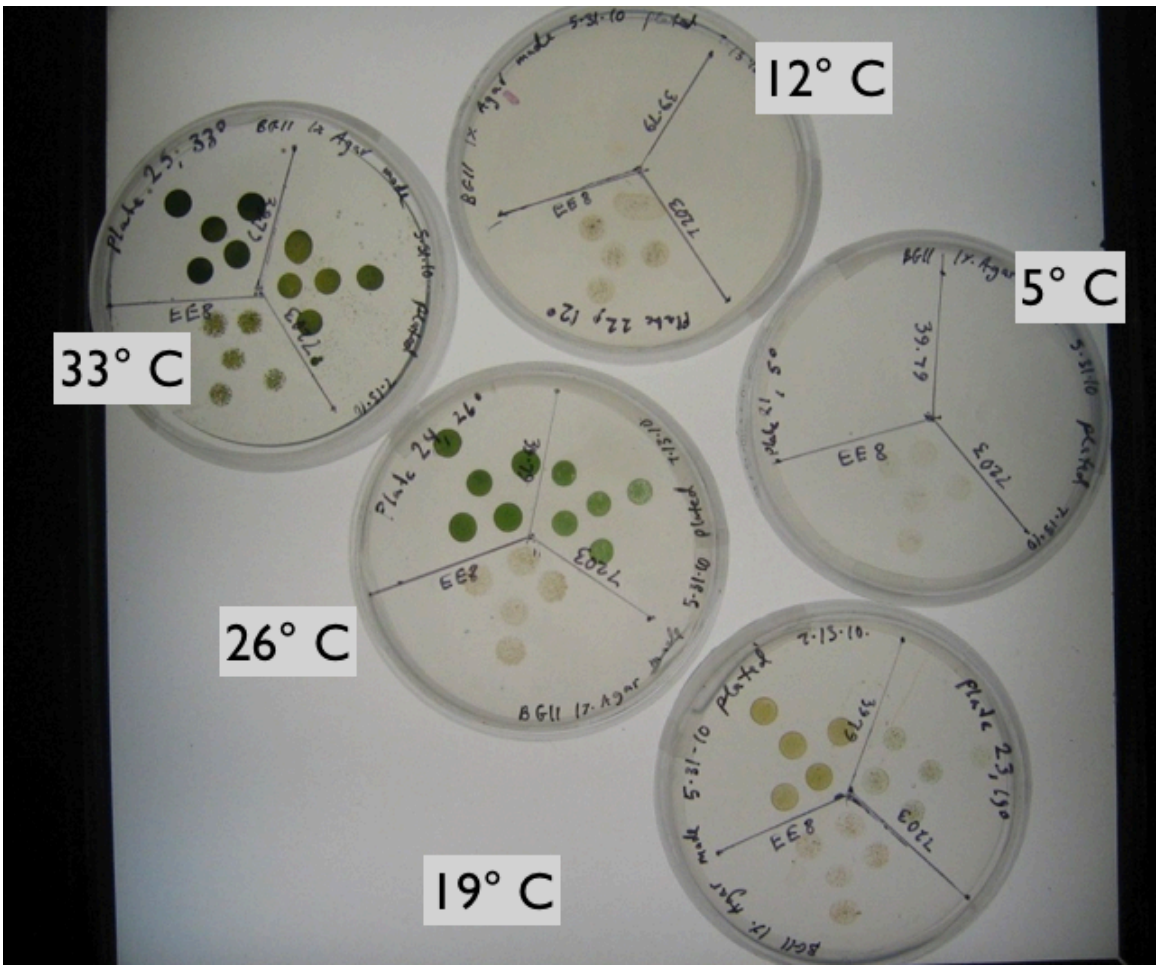


Fig. 2.12. Preliminary growth assays on agar plates. Three different species of *Chroococcidiopsis* are shown growing at five different temperatures. This was one of the first demonstrations of the agar plating assay which is described in detail in Chapter 3. Five replicate 10 µL spots are added to each segment of the agar plate and left to grow fortwoweeks at the given temperature. A representative sample from each group shown here was collated into the chart in Fig. 2.13 along with the results from the remainder of the species.

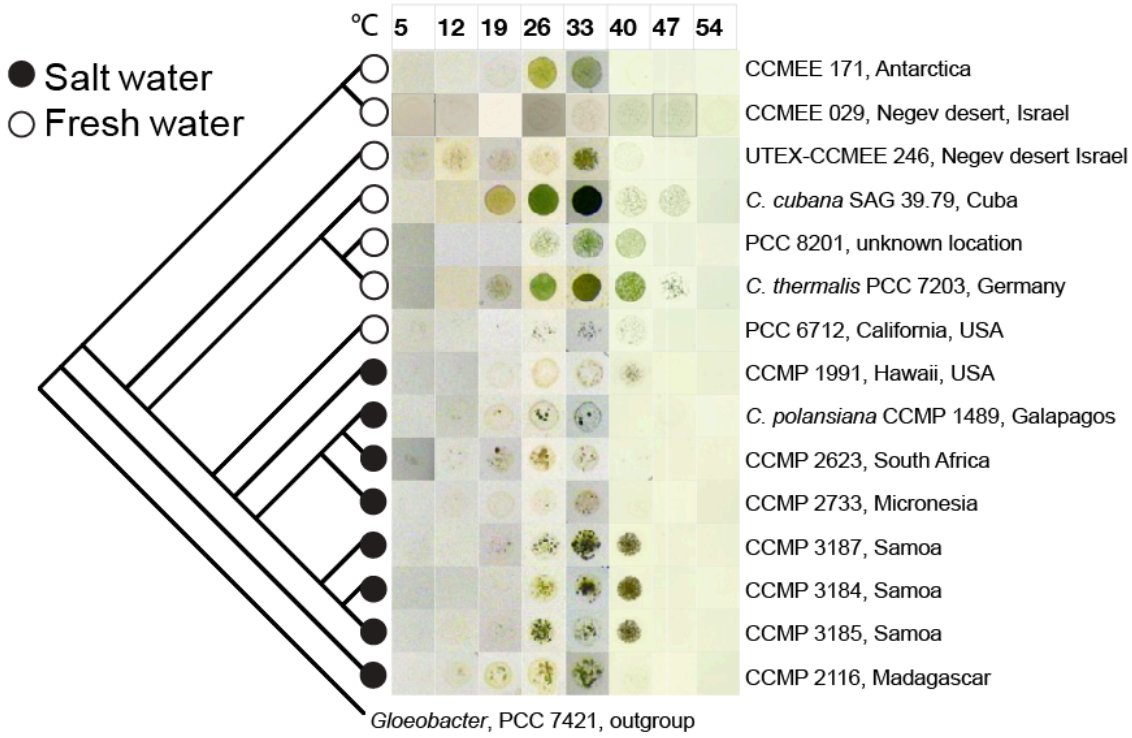


Fig. 2.13. A collated version of the cold growth plate assay. Image shows all 15 species growing at 8 different temperatures.

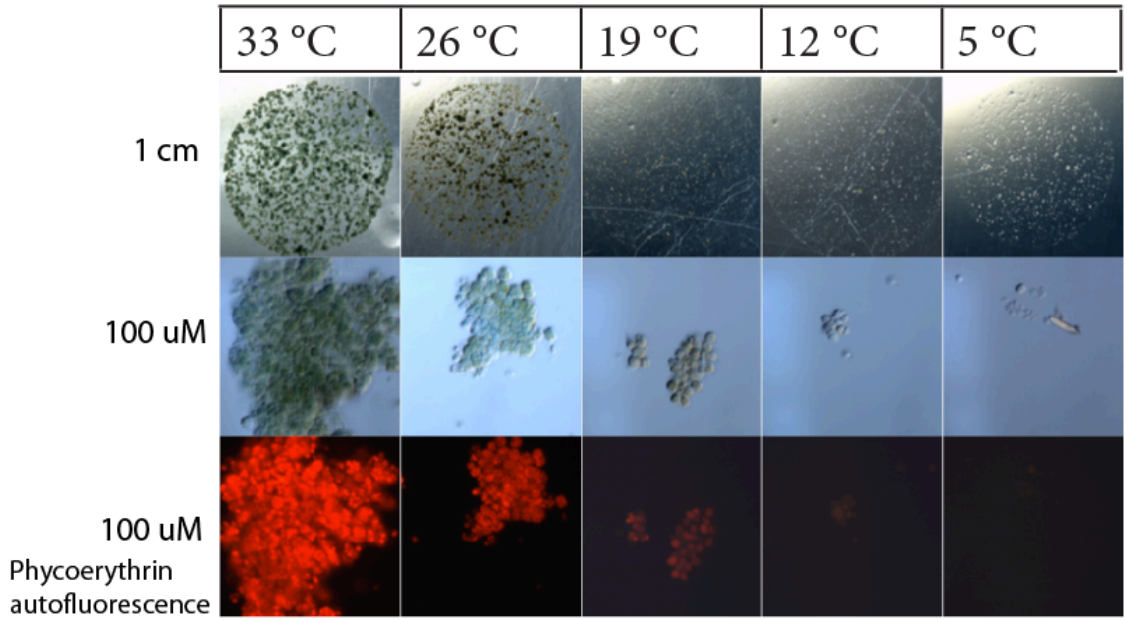


Fig. 2.14. PCC 8201 growing at five different temperatures. Microscopy of cell growth and survival at different temperatures. The top row shows cells growing on an agar plate on the binocular scope. The middle row shows a sample of each under the light microscope. The bottom row shows a filter showing phycoerythrin autofluorescence for easier identification of cells.

Fig. 2.15. A photograph of a side arm flask and optical density reader. (Photo source: http://biotech.matcmadison.edu)

CHAPTER 3

SALT TOLERANCE AND POLYPHYLY IN THE CYANOBACTERIUM *CHROOCOCCIDIOPSIS (PLEUROCAPSALES)*

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Contributions

In this chapter, I devised the research along with Lynn Rothschild and my committee members. I designed primers for the markers not previously published, optimized the PCR conditions for each marker, and performed cloning and sequencing of the DNA. I developed the growth assay on agar plates and grew the cultures under various salt conditions. I analyzed the data and also performed all of the bioinformatics and phylogenetic analysis. I wrote the chapter with input from Lynn Rothschild. Mike Grace helped with making media, maintaining stock cultures, microscopy and optimizing the PCR conditions for the rbcL sequences.

Abstract

Chroococcidiopsis (Geitler, 1933) is a genus of cyanobacteria known to contain desiccation and radiation resistant species. Various species live in diverse habitats ranging from extreme deserts to fresh and saltwater environments. The cell division pattern and morphology have historically been used to define the genus. To better understand the phenotypic diversity of the genus and how it maps to the genotypic diversity, 15 species were selected that had been previously isolated from different locations, including salt and freshwater environments. The 16S rRNA, rbcL, desC1 and gltX genes were sequenced from all species and phylogenetic trees made. All markers showed strong support for the monophyly of a clade of saltwater *Chroococcidiopsis*. Most markers supported the monophyly of a freshwater clade, and placed it as sister to the salt clade. Both clades were well supported from 16S rRNA and rbcL trees made from larger taxon cyanobacterial trees, demonstrating the polyphyletic nature of the genus. All 15 species were grown on 9 different sea salt concentrations to understand the

range of requirements for salt. The results showed that all species isolated from salt waters required salt for growth whilst freshwater species were halo-intolerant. Two species did not fall within the salt or freshwater clades. This study shows an increased resolution of the phylogeny of *Chroococcidiopsis* and clearly demonstrates for the first time, the polyphyly of this genus linked to a specific phenotype.

Introduction

The cyanobacterial genus *Chroococcidiopsis* (Geitler, 1933) has been isolated from a diverse range of habitats. Some species are particularly hardy, found in locations ranging from cold deserts such as the Dry Valleys, Antarctica (Friedmann and Ocampo, 1976) to hot deserts such as the Atacama in Chile (Warren-Rhodes et al., 2006; 2007). Other species are found in the sea or in brackish waters (Dor et al., 1991; Billi et al., 2000). Species have been found in a wide variety of climates and some are known to survive extreme desiccation, X-ray radiation (Billi et al., 2000) and simulated Martian UV flux (Cockell et al., 2005; Billi et al., 2011). Cells are often found growing in or on rocks, offering some thermal, desiccation and radiation protection, (Vogel, 1955; Friedmann et al., 1967; Cockell and Stokes, 2004; Cockell et al., 2005) and as symbionts with lichens (Büdel and Rhiel, 1985).

Because of the ability to survive in extreme environments, *Chroococcidiopsis* is of great interest to the space community in terms of understanding the limits of life on Earth. *Chroococcidiopsis* has also been proposed in the past as an organism suitable for the terraforming of Mars (Friedmann and Ocampo-Friedmann, 1995). Recent advances in synthetic biology, such as whole genome synthesis (Gibson et al., 2010) could mean that

our ability to rationally design and control biological systems will increase dramatically in the coming years. The goal of this thesis is to be able to design and engineer organisms to live in extreme environments. Currently, the distribution of such traits that enable the genus *Chroococcidiopsis* to survive extreme conditions and the genetic mechanisms behind these adaptations remains largely unknown. It has been proposed that the genomes of organisms could be thought of as a toolkit for synthetic biology (Rothschild, 2010). If the mechanisms for tolerances to different environments can be understood then one day, this toolkit could be used to design organisms that live in extreme environments. This study begins to tackle this problem by characterizing the distribution, phylogeny and salt tolerance of *Chroococcidiopsis*. Organisms adapted to different levels of salt are of particular interest due to the presence of salt deposits and evaporites on Mars (Tosca et al., 2008) as these locations have been suggested as a potential refuge for life there (Rothschild, 1990).

Chroococcidiopsis is a member of subsection II (*Pleurocapsales*) of the cyanobacteria (Waterbury, 1989; Rippka et al., 2001), a subsection defined by the presence of single coccoidal cells that reproduce internally by binary fission, forming small cells (baeocytes) contained within the enlarged mother cell. The genus was first defined on the basis of morphological studies well before the advent of molecular phylogeny (Geitler, 1933). Molecular sequence data has since indicated that the genus is polyphyletic (Rudi et al., 1997; Garcia-Pichel et al., 1998; Fewer et al., 2002; Norris and Castenholz, 2006) and therefore that the ability to divide via the production of multiple internal cells must have either arisen on more than one occasion, been lost in the majority of extant taxa, or been transferred by horizontal gene transfer. It was once thought that

Chroococcidiopsis was the most primitive extant cyanobacterium because of its ability to withstand high levels of radiation, presumed to be a harsh condition found on the early Earth (Friedmann et al., 1994). Molecular data and phenotypic data now suggest that *Gloeobacter violaceus* is more primitive because it lacks photosynthetic thylakoids (Rippka et al., 1974).

In order to understand the evolution of the *Chroococcidiopsis* genus, Fewer et al. (2002) sequenced the 16S rRNA of seven species of *Chroococcidiopsis* as well as the 16S rRNA from a number of other species from subsection II (Pleurocapsales). The phylogenetic tree produced showed that five *Chroococcidiopsis* species clustered together. Fewer et al. (2002) also sequenced the 16S rRNA from a number of filamentous heterocyst-forming cyanobacteria and the trees showed strong support for the monophyly of subsections V (*Stigonematales*) and IV (*Nostocales*). This *Chroococcidiopsis* clade lay sister to the heterocyst-forming clade and not with the rest of the *Pleurocapsales*. However, these authors also identified two *Chroococcidiopsis* species that did not fall in the same clade, but clustered with the rest of the subsection II members. These were the saltwater species CCMP 1489 (Galapagos) and the freshwater species PCC 6712 (California). Fewer et al. (2002) previously suggested that the resolution of these and other isolates as different species or genera could be resolved with an increased sample size and additional phenotypic characterization.

Myxosarcina is another member of the *Pleurocapsales* that has a somewhat similar morphology to *Chroococcidiopsis* but is not closely related phylogenetically. It is similar

to *Chroococcidiopsis*, apart from that it has motile baeocytes that move by a gliding mechanism and it has a fibrous wall (Waterbury, 1989; Rippka et al., 2001). It remains unclear if the similar distinctive morphology arose through convergent evolution, or horizontal gene transfer. Other cases of convergent evolution have also been noted; Norris and Castenholz, (2006) previously identified a morphotype of *Chroococcidiopsis,* species CCMEE 6048. These two examples show that species of *Chroococcidiopsis* can be easily wrongly assigned therefore the species in this study were chosen to broaden the diversity of sequenced species in order to see where any new species fell on the molecular phylogeny and to better understand the evolution of the genus.

Billi et al. (2001) investigated the genetic diversity of desert-dwelling *Chroococcidiopsis* by 16S rRNA sequencing and their results suggested that hot and cold isolates were closely related. A recent study by Bahl et al. (2011) looked at the ecology of cyanobacteria found on and inside desert rocks resulting in the most thorough sequencing of desert cyanobacteria to date. Bahl's sequences were mainly from environmental samples so it is unclear if the morphology corresponding to the reported species is the same as the main cluster of *Chroococcidiopsis* species identified by Fewer (2002). Bahl viewed all species sequenced as a monophyletic clade and concluded that hot and cold desert ecotypes had not mixed with each other genetically and that no modern dispersal had taken place. It is currently unclear if the hot and cold clades represent distinct species of different origin.

Most *Chroococcidiopsis* species available in public culture collections have been isolated from land or from freshwater. It has been previously reported (Thajuddin and Subramanian, 1992) that many cyanobacterial species reported as freshwater species also grow on seawater media (Banerjee et al., 2009), making it difficult to segregate cyanobacteria into true marine and freshwater species. The ability of some cyanobacteria to adapt to extreme salinity is well documented. *Spirulina subsalsa*, a known halophile, can grow in up to 3 M salt (an equivalent of ~240 $g \cdot L^{-1}$) and is commonly found in salt pans (Thajuddin and Subramanian, 1992; Nübel et al., 2000). By comparison, seawater is about 0.5 M salt containing approximately 33 $g \cdot L^{-1}$, of which 30 g is NaCl. A number of saltwater isolates of *Chroococcidiopsis* exist although few studies have been performed on these species and few species have been sequenced. Dor et al. (1991) had previously isolated species of *Chroococcidiopsis* that were halotolerant, but molecular sequencing was not performed and these species appear to no longer be available.

The main salt in seawater and the main salt used in this study is sodium chloride. A high concentration of salt can put ionic stress onto organisms, largely as Na^+ (and Cl^-) are known to cause necrosis, chlorosis and inhibit enzymes and metabolic processes such as protein synthesis (Burg et al., 2007). Halophilic or halotolerant organisms are salt loving or salt tolerant organisms, respectively (Rothschild and Mancinelli, 2001). Moderate halophiles are organisms that require salt to survive and grow, but cannot live in high salt concentrations.

To better understand the evolution of the traits that allow *Chroococcidiopsis* to survive in such a wide variety of environments, a phylogenetic and phenotypic survey was performed using 15 species of the genus. Species were chosen to broaden the number of sequenced isolates and to see where new unsequenced species fell on the molecular phylogeny. This survey lays the foundation for the identification of the mechanisms of salt tolerance and moves toward the larger goal of building a toolkit for the engineering of life for extreme environments. The first environmental variable tested, and reported in this study was salt tolerance, because it was hypothesized that species may have varying abilities to tolerate different salinities. It was hypothesized that salt tolerance might correlate to phylogeny which would increase our understanding of the evolution of the trait within the genus.

Materials and Methods

Cyanobacterial cultures and culturing

The 15 species used in this study were unialgal but a number of cultures were non-axenic. Efforts were made to remove other bacteria associated with the cyanobacteria by repeated streaking and by plating on antibiotics but a small number still remained. An isolate from PCC 6712 matched the 16S rRNA sequence for *Roseobacter denitrificans* and species CCMEE 029 (Negev, Israel) contained an isolate with a close match to the 16S rRNA of *Pseudomonas mendocina*. Previous studies have reported symbiotic bacteria living within the cellular envelope of *Chroococcidiopsis* (Billi et al., 1998) so it is currently unclear if these bacteria are symbionts or contaminants.

The 15 species used in this study and the source of each species are shown in Table 3.1 and mapped to their location of isolation according to the culture collections (Fig. 3.1). Species were selected to represent a geographical and environmental diversity. Three species were selected from Samoa (CCMP 3184, 3185 and 3187) in order to assess the degree of local diversity. Freshwater species were grown in BG11 growth medium (Stanier et al., 1971), made from 50x concentrate (Sigma-Aldrich Inc. St. Louis, MO, USA). The medium was supplemented with HEPES buffer to 20 mM in order to buffer against pH change as cultures were also being grown at different temperatures as part of a separate study. The BG11 was adjusted to pH 7.4 before filter sterilization. Saltwater species were grown in the same medium but supplemented with 4% (40 $g \cdot L^{-1}$) sea salt (Sigma-Aldrich Inc. St. Louis, MO, USA cat. S9883) as a substitute for seawater according to the manufacturer's instructions. According to the product specification, the final sea salt medium contained per liter approximately 30 g of sodium chloride, 2.6 g sulfate, 1.32 g magnesium, 0.42 g potassium, 0.4 g calcium plus other trace elements. Initially, saltwater species were grown in the saltwater medium L1 as recommended by the CCMP (Guillard and Hargraves, 1993), but in order to normalize any effect of the medium, all species were grown on BG11 as described above and no noticeable difference in physiology or growth was observed between the BG11 with sea salt and L1 medium.

Some species of *Chroococcidiopsis* grow in cellular aggregates making plating of cells difficult. In order to break up cell aggregates, liquid cultures in mid exponential phase were first gently sonicated (Ultrasonic processor XL, microtip size 1/16, repeated rounds

of three seconds, Misonix Inc, Farmingdale, NY, USA) until optical density (730 nm) had plateaued, indicating that large clumps of cells had separated. Cells were then centrifuged at 1000 x g, supernatant discarded and cells resuspended in fresh media to a concentration of 10^6 cells·mL as confirmed by hemocytometer. Salt tolerance assays were performed in triplicate by plating 10 µL of cells onto agar plates containing varying percentages of added sea salt. Plates were wrapped in parafilm to prevent evaporation before being placed in a growth incubator (Rexmed Inc. Taiwan) at 25°C under a 16h:8h light/dark regime and a mix of Philips TLD36W/840 (cool white) and Philips TLD36w (aqua) fluorescent light at 54±2 µmol photons·m^{-2}·s^{-1} as measured with a PAR detector (PMA2100 / PMA2132, Solar Light Co. PA, USA).

In order to reduce possible trace metal contamination from the media that might change the growth conditions of the cultures, care was taken to wash all glassware and anything in contact with the media in 10% nitric acid overnight followed by rinsing three times with distilled Barnstead water (at 18 mega-ohms) according to the USGS protocol (Harris et al., 1997).

Choice of markers

To produce the phylogenetic trees, four markers were chosen. The 16S rRNA was chosen, as it is the most commonly used marker for genotyping of bacteria and many sequences are already available in sequence databases. The 16S rRNA is not expected to be under phenotypic selection for novel function but is known to contain suitable variation for identification purposes. Likewise rbcL, the large subunit of RuBisCO

(ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39), the enzyme that catalyzes the first major step of carbon fixation, was also chosen as a housekeeping gene that was expected to be relatively conserved and not under selection for any of the phenotypes being studied. gltX (glutamyl-tRNA synthetase, EC 6.1.1.17) is the enzyme that charges the glutamic acid tRNA and was chosen as a third housekeeping gene. The gene desC1 is a Δ9 acyl-lipid desaturase, (EC 1.14.19.-) and was chosen as it was previously known to be involved in high temperature adaptation in cyanobacteria (Chintalapati et al., 2006) and therefore might be under selection in the different temperature environments that the species were isolated from. It was expected that the trees produced from the 16S rRNA, rbcL and gltX would be of a similar structure, whilst the tree produced by desC1 would show a different topology due to some selection pressure depending on the temperature of the isolated environment. The response to temperature change in *Chroococcidiopsis* is not covered in this study.

DNA extraction, PCR amplification and sequencing

DNA was extracted from the cultures using the DNeasy Plant Mini Kit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instructions. The primers used are shown in Table 3.2. The 16S rRNA primers were designed by downloading *Chroococcidiopsis* 16S rRNA sequences annotated as high quality in the SILVA database (arb-silva.de, Pruesse et al., 2007). These sequences were pre-aligned in SILVA, which takes account of the rRNA secondary structure. The alignment was loaded into Geneious (Drummond et al., 2010) and Primer3 (Rozen and Skaletsky, 2000) was used to design primers covering homologous regions at the beginning and end of the 16S rRNA region.

Primers for the large subunit of RuBisCO were the same as previously reported for other cyanobacteria (Tomitani et al., 2006). Primers for the desC1 and gltX genes were designed by downloading existing cyanobacterial annotated sequences from GenBank, aligning them using MUSCLE (Edgar, 2004) through Geneious and creating degenerate primers covering conserved regions of the gene using Primer3.

Each template and primer pair needed optimizing for PCR annealing temperature and Mg^{2+} concentration. Gradients of temperature (50°C to 72°C) and Mg^{2+} (1.5 mM to 6 mM) were performed until a single band of the correct size was produced or could be cut from the gel. GoTaq (Promega Inc. Madison, WI, USA) or Platinum taq (Invitrogen Inc. Carlsbad, CA, USA) master mixes were used to perform the PCR. Amplicons were then cloned into the pGEM-T vector (Promega Inc. Madison, WI, USA) and colonies were picked and sequenced to three times coverage (Elim, Hayward, CA, USA). Vectors were trimmed from the sequences with the UniVec tool in Geneious and each sequence was checked for chimeric PCR products using the Bellerophon tool (Huber et al., 2004). Each protein coding sequence was annotated to the correct reading frame and trimmed to the nearest codon before alignments were performed.

Phylogenetic analysis

Alignments were created for all 15 species for each of the four genetic markers sequenced plus *Gloeobacter violaceus* PCC 7421 which was included as an outgroup as this genus lacks thylakoid membranes (Rippka et al., 1979) and is generally accepted to be the earliest diverging cyanobacterium (Nelissen et al., 1995). Individual trees were

produced for each marker as well as a combined analysis of all four markers and all three markers without rbcL as discussed later. A combined analysis was performed by concatenating all sequences into a partitioned alignment file and performing the analysis in the same was as the single gene analysis, detailed below.

The 16S rRNA sequences were aligned using the NAST on-line aligner through GreenGenes and hyper-variable regions were excluded using lane mask (DeSantis et al., 2006). The rbcL, desC1 and gltX sequences were each translation aligned with the MUSCLE aligner (Edgar, 2004) and nucleotide sequences were analyzed for tree building. The best-fit model of nucleotide substitution for each alignment was selected with ModelTest (Posada and Crandall, 1998). GTR+γ+I model was used for rbcL, desC1 and for all three and all four marker combined analysis, as discussed later. The GTR+γ was chosen as the best model for the gltX and 16s rRNA alignments. A maximum likelihood analysis was performed using RaxML (Stamatakis et al., 2005) with 2000 bootstrap replicates. Using the RaxML tree as a starting tree, a bayesian analysis was performed using MrBayes (Huelsenbeck and Ronquist, 2001) to calculate the posterior probabilities of the branches. Some analyses were performed on the CIPRES portal (phylo.org). Trees were then imported into R (r-project.org) for further analysis and Adobe Illustrator for annotations and marking of salt and fresh species. In order to see the evolutionary relationships of these 15 species in the context of the broader phylogeny of other species of *Chroococcidiopsis* not sequenced in this study and other cyanobacteria, trees were made from further 16S rRNA and rbcL sequences already available in the sequence databases. Unfortunately these databases did not contain

enough cyanobacterial desC1 or gltX sequences in order to make larger trees for those markers as well. The search for additional *Chroococcidiopsis* sequences was limited to organisms that were available in the culture collections and had been identified by morphology. Environmental samples were not included so as to only include species of *Chroococcidiopsis* that had been identified by morphology and not by sequence similarity alone. An additional five *Chroococcidiopsis* sequences were included in the larger 16S rRNA tree and 33 other cyanobacteria were included. All sequences were marked as high quality and taken from the SILVA database of 16S rRNA (Pruesse et al., 2007). For the large taxon rbcL tree, no additional *Chroococcidiopsis* rbcL sequences have been published, but Genbank was used to find 27 additional cyanobacterial rbcL sequences. Not all sequences found in Genbank were chosen for analysis, but a selection were chosen to include sequences from type species of all five subsections of the cyanobacterial taxonomy.

Microscopy

Agar plates were individually photographed on a light table using a digital camera. Individual spots were cut out using Photoshop (Adobe, San Jose, CA, USA) and images assembled using Numbers (Apple, Cupertino, CA, USA). Micrographs were taken using a Zeiss Axio Imager Z1 with a DIC filter and a binocular microscope, Zeiss Stemi 2000-c (Carl Zeiss MicroImaging, Thornwood, NY, USA).

Statistical methods

To assess how well the salt tolerant phenotype mapped to the phylogeny, Blomberg's K was used. This is a statistical method for the measurement of phylogenetic signal, which is the tendency for related species to resemble each other (Blomberg et al., 2003). Blomberg's K was calculated using the R package Picante (Kembel et al., 2010). The higher the K statistic is, the greater the phylogenetic signal for the trait. This method normalizes branch lengths using phylogenetically independent contrasts (Felsenstein, 1985). To test for greater phylogenetic signal than expected, the method produces random trees in order to create a null distribution. This distribution can then be used as a 1-tailed P-value. Traits with $p < 0.05$ have non-random phylogenetic signal.

Results

In total, 57 novel sequences from this study were deposited in Genbank under accession numbers: JF810025-JF810082. The 16S rRNA sequences were obtained from all 15 species. Five of these sequences were already available in Genbank and these sequences were confirmed to be associated with the physical cultures by re-sequencing. *Chroococcidiopsis cubana*, PCC 39.79 (Cuba) was re-submitted to Genbank because the new sequence (JF810080) contained five base pairs that were different from the sequence originally deposited (AJ344558). Sequences obtained from species PCC 6712 (California, AJ344557) and *Chroococcidiopsis thermalis* (PCC 7203, Germany, AB039005) were identical to those in Genbank, and so were not resubmitted. Only 480 bp of the 16S was available in Genbank for species CCMEE 029 (Negev, Israel, AF279107) and CCMEE 171 (Antarctica, AF279110) so these were re-sequenced and

submitted. Novel sequences for the three markers rbcL, desC1 and gltX were obtained from each of the 15 species and submitted.

All phylogenetic trees produced during the analysis showed strong support for the monophyly of a clade of saltwater species of *Chroococcidiopsis, except for CCMP 2116 (Madagascar)*. These trees included the four individual markers (Figs. S2.1 and S2.2), the combined phylogram from all four markers (Fig. 3.2), and the larger taxon analysis from the 16S rRNA (Fig. 3.3) and rbcL tree (Fig. 3.4). For the individual analyses of each genetic marker, most markers also showed support for the monophyly of the freshwater species of *Chroococcidiopsis* and placed it sister to the saltwater clade (Fig. S3.1 and S3.2). The trees made from a larger number of taxa using 16S rRNA (Fig. 3.3) and rbcL (Fig. 3.4) are congruent with the individual marker trees with respect to the monophyly of the saltwater clade. These two trees are incongruent with respect to the freshwater clade however. Whilst the 16S rRNA tree (Fig. 3.3) supports the monophyly of the freshwater species, the rbcL tree (Fig. 3.4) does not.

A number of species do not cluster with the salt and fresh water clades. Both larger trees (Fig. 3.3 and Fig. 3.4) show that CCMP 2116 (Madagascar) does not cluster with the rest of the *Chroococcidiopsis* species. Species PCC 6712 (California) is a freshwater species that is consistently sister to the saltwater clade, rather than with the rest of the freshwater species. Species CCMP 1991 (Hawaii) is consistently part of the saltwater clade, but is always sister to the rest of the species in the clade. Both larger taxon trees (Fig. 3.3 and 3.4) show support for the monophyly of the heterocyst forming cyanobacteria. The

diversity of *Chroococcidiopsis* morphology is shown in Fig. 3.5 mapped to a cladogram of the four marker combined tree as shown in Fig. 3.2.

The results of growing each of the 15 species in media containing 9 different salt concentrations are shown in Fig. 3.6. The salt tolerance is mapped to a cladogram of the four marker combined tree represented as the phylogram shown in Fig. 3.2. Species isolated from saltwaters are moderate halophiles and require salt for growth. Species isolated on land or in freshwater are halointolerant, show reduced colony forming ability as salt concentration is increased and fail to produce any colonies at concentrations above 250 mM of added sea salt. Conversely, species from the saltwater clade all show little or no ability to form colonies when plated on media containing no added sea salt. The only saltwater species to grow on media containing no added sea salt was CCMP 1991. These results from the agar plates were replicated in BG11 liquid media containing either 4% added sea salt or no added sea salt. Survival was measured by microscopy after one month. Again, only the salt water species CCMP 1991 (Hawaii) was able to survive in freshwater media whereas none of the freshwater species were able to survive in BG11 + 4% added sea salt media.

The salt tolerance phenotype when mapped to the four marker combined phylogram (Fig. 3.2) correlates with the phylogeny, and the significance of the salt and fresh clades are supported by Blomberg's K. The results, which are based on the minimum, maximum and optimum sea salt concentration for colony forming ability, as confirmed by microscopy, are shown in Table 3.3. The results indicate that there is a statistically

significant phylogenetic signal amongst all three observed traits, minimum salt ($p = 0.012$), maximum salt ($p = 0.001$) and optimum salt ($p = 0.001$) required for growth. The strongest phylogenetic signal comes from the maximum salt concentration that allows for growth ($K = 1.16$), indicating that this trait maps to the phylogeny the best.

Discussion

The objective of the study was to better understand the phylogeny of the genus *Chroococcidiopsis* and the distribution of the ability to survive in diverse environments. Although salt tolerant *Chroococcidiopsis* were known to exist previously (Dor et al., 1991) there was a lack of molecular data to show where they fitted into the phylogeny. These results reveal a distinct moderate halophilic clade of *Chroococcidiopsis* and demonstrate that CCMP 1489 *C. polansiana* (Galapagos), identified previously as a possible new species or genus (Fewer et al., 2002), is not a distinct phylogenetic strain but is in fact part of a much larger clade of moderately halophilic *Chroococcidiopsis.*

The morphology of the genus (Fig. 3.5) shows that the majority of the freshwater species look similar in morphology to *C. thermalis* PCC 7203 (Germany), one of the species identified by Fewer (2002) in the main *Chroococcidiopsis* clade. The saltwater species are more diverse however. Species CCMP 2116 (Madagascar), which consistently fell as a sister to the other species in the phylogeny shows smaller and more densely packed cells than the other species. Species CCMP 1991 (Hawaii), which was part of, but consistently sister to the rest of the salt clade, also forms tighter clumps of small cells. Species *C. polansiana* CCMP 1489 (Galapagos), CCMP 2623 (South Africa) and CCMP

2733 (Micronesia) all form cells of similar morphology. On closer examination of the morphology of the freshwater species, whilst all of them showed division by multiple fission, only species CCMEE 246, (Negev, Israel) showed smaller baeocytes inside of a mother cell, which is a defining feature of the genus (Rippka et al., 2001, Waterbury, 1989). This could be the true morphology of these species, or it could be a case of phenotypic plasticity where division involving an outer mother cells appears only under certain conditions or in particular environments. Future studies detailing the morphology and growth patterns could reveal this.

Previous work (Blank and Sanchez-Baracaldo, 2010) indicated that saltwater species of cyanobacteria are derived from freshwater species. Only one marker sequenced, rbcL, indicated that the freshwater clade gives rise to the saltwater clade (Fig. S3.1, bottom), it also indicated that the freshwater clade is paraphyletic, however, the larger rbcL analysis does not agree with this (Fig. 3.4). If a tree is made without the rbcL marker included, then the two clades are again shown (Fig. S3.3 and Fig. S3.4). The individual phylograms for the markers 16S rRNA (Fig. S3.1, top), desC1 (Fig. S3.2, top) and gltX (Fig. S3.2, bottom) show support for the salt clade, but do not indicate any evolution of salt from fresh.

A number of anomalies appear in the data, the saltwater species CCMP 2116 (Madagascar) lies in the salt clade for the desC1 and 16S rRNA trees (Fig. S3.1, top and Fig. S3.2, top), but does not lie in the salt clades for the gltX and rbcL trees (Fig. S3.1, bottom and Fig. S3.2, bottom). The freshwater species PCC 6712 (California) is sister to

the salt clade in the large and small 16S rRNA analyses (Fig. 3.3 and Fig. S3.1, top). Neither of these species fall in the main clades in the larger taxa analyses and could be revealed as separate clades or even genera given increased sampling and sequencing.

The study aimed to survey *Chroococcidiopsis* for the distribution of extreme traits and to use this survey to uncover the genetic mechanisms behind adaptations to extreme environments. Although the species in the salt clade appear only moderately halophilic they may provide clues to the mechanism of halotolerance. Higher tolerances to salt is an important factor in biotechnology for the engineering of salt tolerant plants that can grow in a wider range of environments and have larger yields. Halotolerant organisms may also be of use for the production of biofuels in saltwater, in which case they do not compete for food crop land use.

Halophiles are found in all three domains of life indicating that this trait likely has multiple origins or has evolved by horizontal gene transfer (Oren, 2008). Considering that all four markers show distinct saltwater clades for *Chroococcidiopsis*, it seems unlikely that horizontal gene transfer is responsible for the evolution of such a complex trait. Future work can begin to understand the mechanism of adaptations to salt environments. Given that changing salinity levels in the environment can drive adaptations to genes, genomes and pathways (Oren, 2008), there are likely a number of mechanisms that might have evolved to deal with increased salinity.

Halotolerant organisms need to uptake some salt in order to keep an osmotic potential that is lower than the media around them to ensure they can still take up water. However, high concentrations of salt inside the cell can be damaging. Cells have evolved proteins such as ion channels and pumps to remove salt from cells (Gabbay-Azaria and Tel-Or, 1991), they have also evolved mechanisms to sequester excess salt and prevent it from causing internal damage. Plants are known to store salt in vacuoles for example. Cells have also evolved ways to accumulate cytoplasmic osmotic solutes such as the amino acid proline, glycine betaine or sugars (Chen et al., 2007; Hagemann, 2010; Oren, 2002; Pessaarakli, 1995; Whitton and Potts, 2000). The cyanobacterium *Synechocystis sp.* PCC 6803 is known to accumulate glucosylglycerol and sucrose under salt stress and the glucosylglycerol-phosphate synthase gene (ggpS) is known to play a role in salt tolerance (Ferjani et al., 2003). A number of other adaptations to salt have been previously identified such as the evolution of proteins involved in DNA binding in response to higher environmental salt levels (Bergqvist et al., 2003). It is possible that one or more of these mechanisms may be responsible for the moderately halophilic phenotype of the salt clade of *Chroococcidiopsis* and further study could uncover this mechanism.

In the future, a full comparative genomic or transcriptomic investigation could reveal the mechanism of salt adaptation in this genus and in cyanobacteria in general. A thorough analysis measuring the salt growth phenotype of a larger range of cyanobacteria would aid in this search and would provide a more accurate classification of the salt tolerance of cyanobacteria in general. This study provides the first example of the polyphyly of the genus linked to a particular phenotype, moderate halophily. It also demonstrates that

genera classified by traditional methods such as morphology, can sometimes confuse the true evolutionary relatedness of species.

The goal of this thesis was to understand the distribution of extremophilic traits in *Chroococcidiopsis* so that other organisms could one day be engineered to survive in extreme environments found in space. This study of halotolerance is of interest because it helps to understand the polyphyly of the genus. However the salt tolerant phenotype uncovered is not of much utility in space. Organisms have been found to survive on Earth in water up to 3 M NaCl, whilst the *Chroococcidiopsis* species studied here were shown to survive to only 1 M in added sea salt. There is no evidence suggesting that space environments would contain a higher salt concentration than Earth indicating that salt will likely not be a difficult challenge for Earth organisms in space.

Acknowledgments

Thanks to J. M. Grace, D. Rogoff and S. Leuko for help and technical assistance and thanks to R. Castenholz, A. Coleman, C. Dunn, M. Tatar, D. Weinreich and G. Wessel for commenting on the manuscript. Thanks to R. Castenholz for the kind gift of species from the CCMEE, to R. Anderson for the kind gift of species from the CCMP and to C. Kerfeld for sequence data from *Chroococcidiopsis* PCC 7203 (Germany) which aided primer design. Special thanks to S.P. Worden and the NASA Ames Synthetic Biology Initiative. JC was funded as part of a cooperative agreement between Brown University and NASA Ames Research Center, NASA Award Number NNX08AZ52A.

Figures in Chapter 3

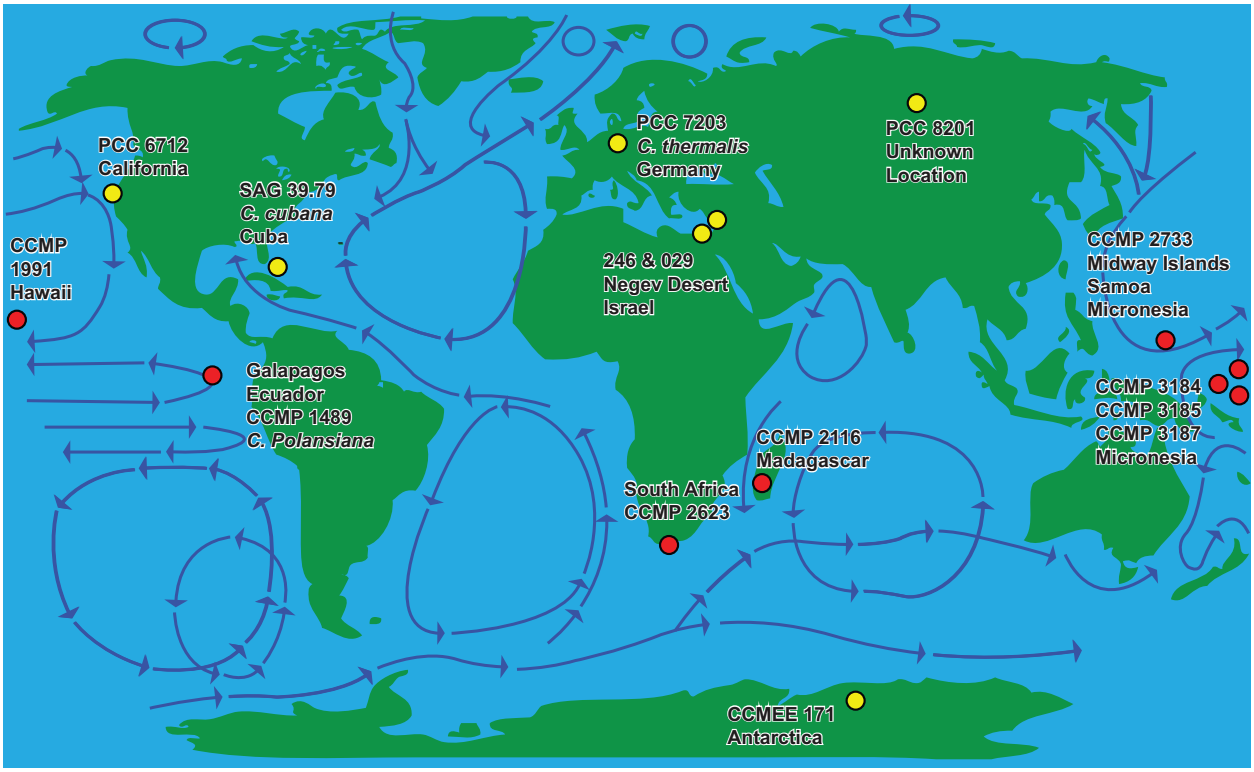

Fig. 3.1. World map showing the 15 species of *Chroococcidiopsis* used in this study. Red dots indicate species isolated from saltwaters and yellow dots indicate species isolated from freshwater or land. Arrowed lines indicate current flows although there is no apparent correlation between current flow and phylogeny.

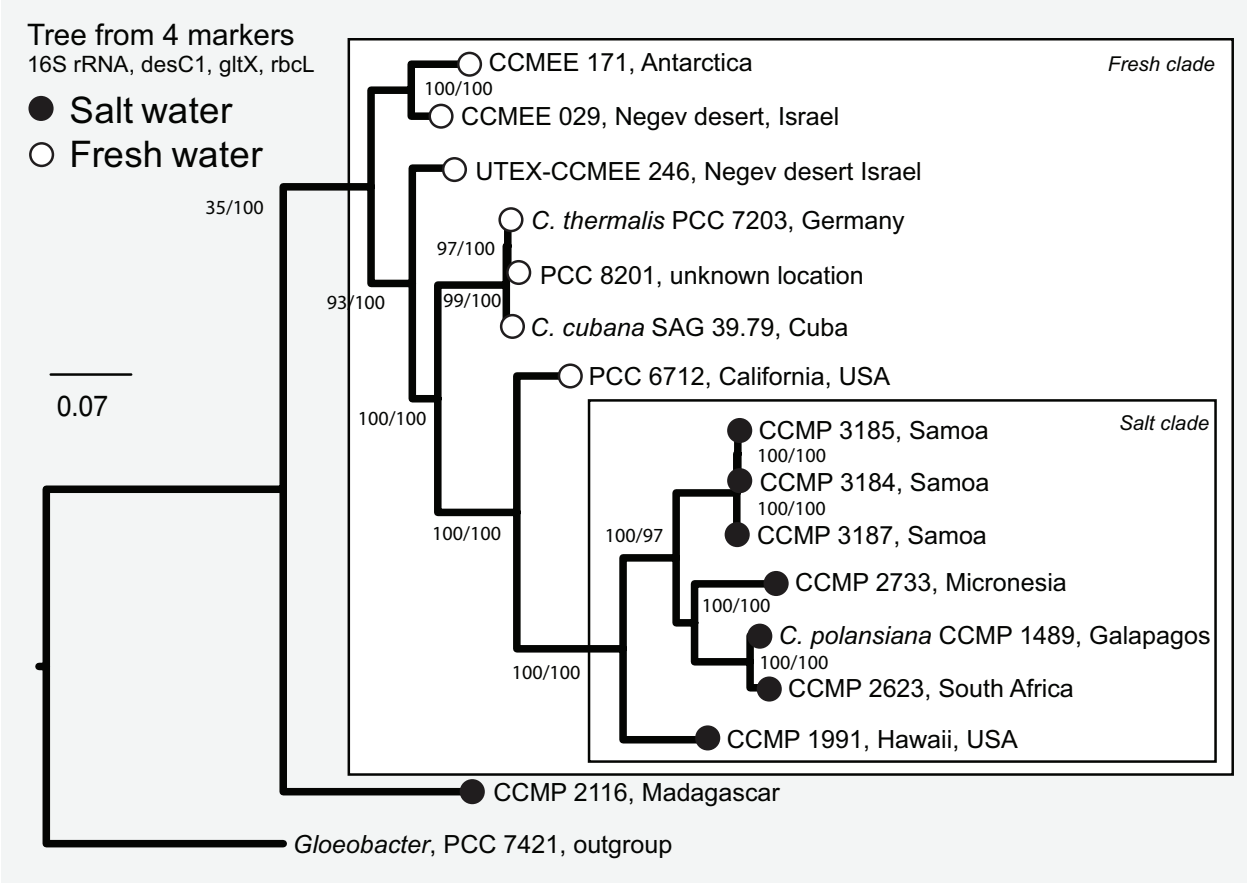

Fig. 3.2. RaxML phylogram showing a joint tree constructed from 16S rRNA, gltX, desC1 and rbcL from the 15 *Chroococcidiopsis* species studied. *Gloeobacter violaceus* was used as an outgroup. The maximum likelihood analyses was conducted under the GTR+Γ+I model. The tree topology and branch lengths are for the sampled tree that has the highest likelihood (2,000 searches, log likelihood -8826.62). Support values on the left are from 2000 bootstrap replicates. Numbers on the right are posterior probabilities from the MrBayes analysis using the raxML tree as the starting tree (10,000,000 generations, sampled every 100 generations, four chains, temperature 0.2, burn-in of 25% of the trees sampled). The salt and fresh species and clades are labeled with Adobe Illustrator. The scale bar represents substitutions per site

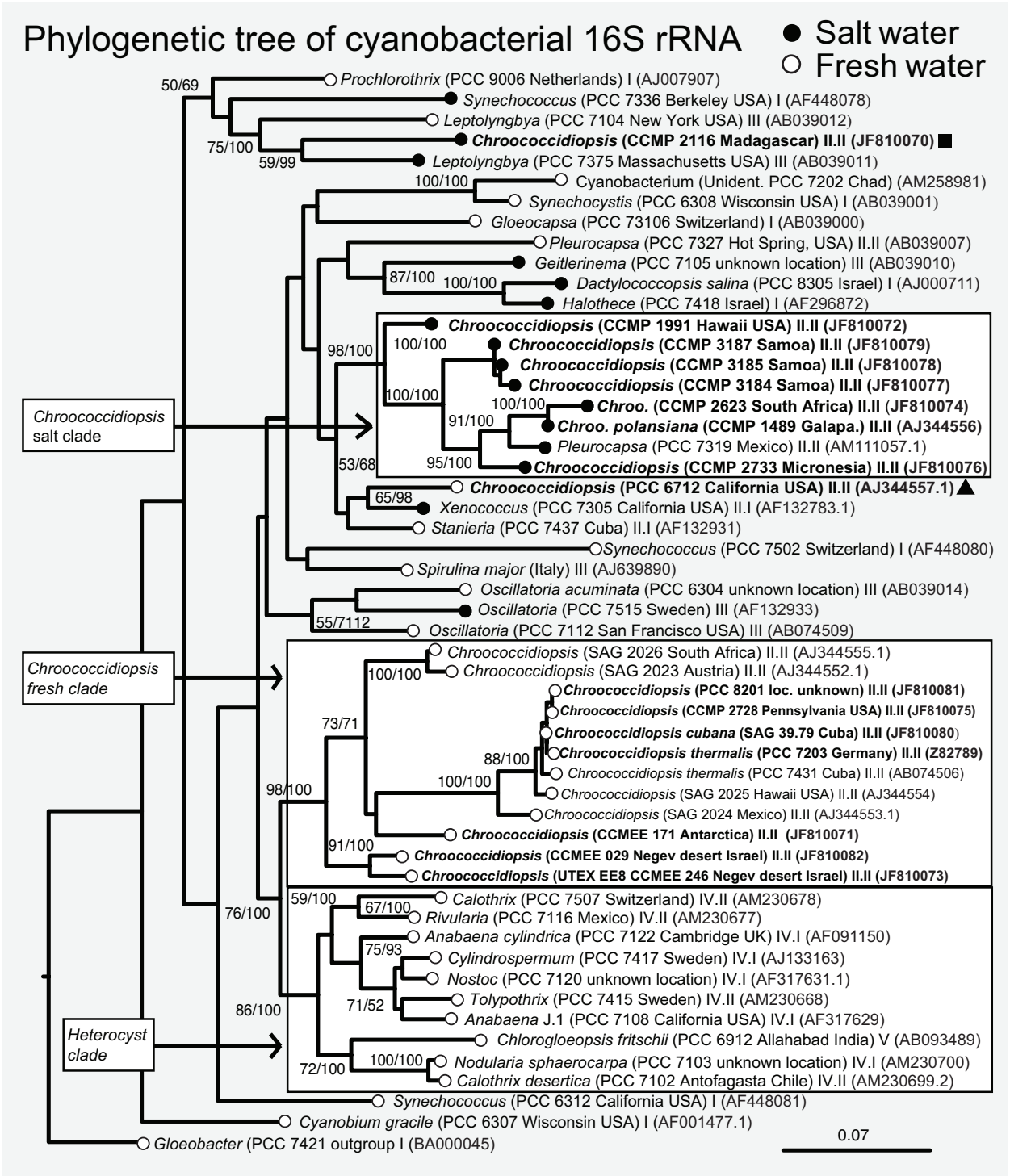

Fig. 3.3. RaxML phylogram showing 16S rRNA sequences for 15 *Chroococcidiopsis* species studied (marked in bold), five other *Chroococcidiopsis* species and a representative sample of 33 other cyanobacteria from Subsections I through V (labeled). *Gloeobacter violaceus* is used as an outgroup. The maximum likelihood analyses was conducted under the GTR+Γ+I model. The tree topology and branch lengths are for the sampled tree with the highest likelihood (2,000 searches, log likelihood -12,991).

Support values on left are from 2,000 bootstrap replicates where support was greater than 50%. Numbers on right are posterior probabilities from a MrBayes analysis using the raxML tree as the starting tree (10,000,000 generations, sampled every 100 generations, four chains, temperature 0.2, burn-in of 25% of the trees sampled). The scale bar represents substitutions per site. The salt and fresh species and clades are labeled with Adobe Illustrator.. For species in bold the salt tolerance was measured empirically, for others the assignment was taken from the suggested media from the culture collection literature. The heterocyst forming clade is also labeled. *Chroococcidiopsis* (CCMP 2116 Madagascar) is marked with a square symbol and *Chroococcidiopsis* (PCC 6712 California USA) is marked with a triangle, see text for more details.

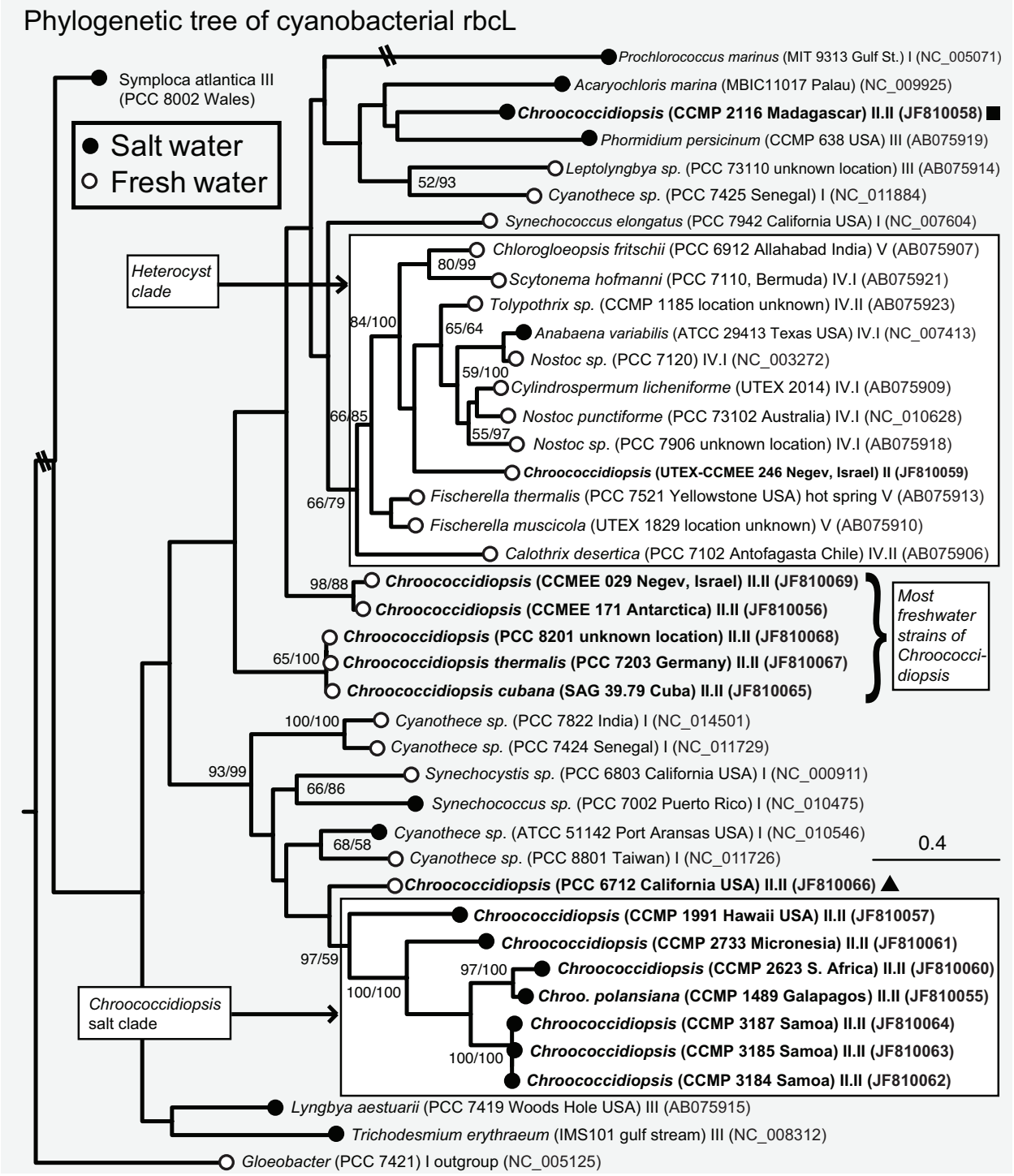

Fig. 3.4. RaxML phylogram showing rbcL sequences for 15 *Chroococcidiopsis* species studied (marked in bold) and a representative sample of 27 cyanobacteria from Subsections I through V (labeled). *Gloeobacter violaceus* is used as an outgroup. The maximum likelihood analyses was conducted under the GTR+Γ+I model. The tree topology and branch lengths are for the sampled tree with the highest likelihood (2,000 searches, log likelihood -8,112). Support values on left are from 2,000 bootstrap

replicates where support was greater than 50%. Numbers on right are posterior probabilities from a MrBayes analysis using the raxML tree as the starting tree (10,000,000 generations, sampled every 100 generations, four chains, temperature 0.2, burn-in of 25% of the trees sampled). The scale bar represents substitutions per site. The saltwater clade is labeled as is the heterocyst forming clade but the freshwater species do not form a separate clade. For the species marked in bold the salt tolerance was measured empirically, for others the assignment was taken from the culture collections suggested growth media. *Chroococcidiopsis* (CCMP 2116, Madagascar) is marked with a square symbol and *Chroococcidiopsis* (PCC 6712, California) is marked with a triangle, see text for more details. Salt and fresh species and clades are labeled with Adobe Illustrator.

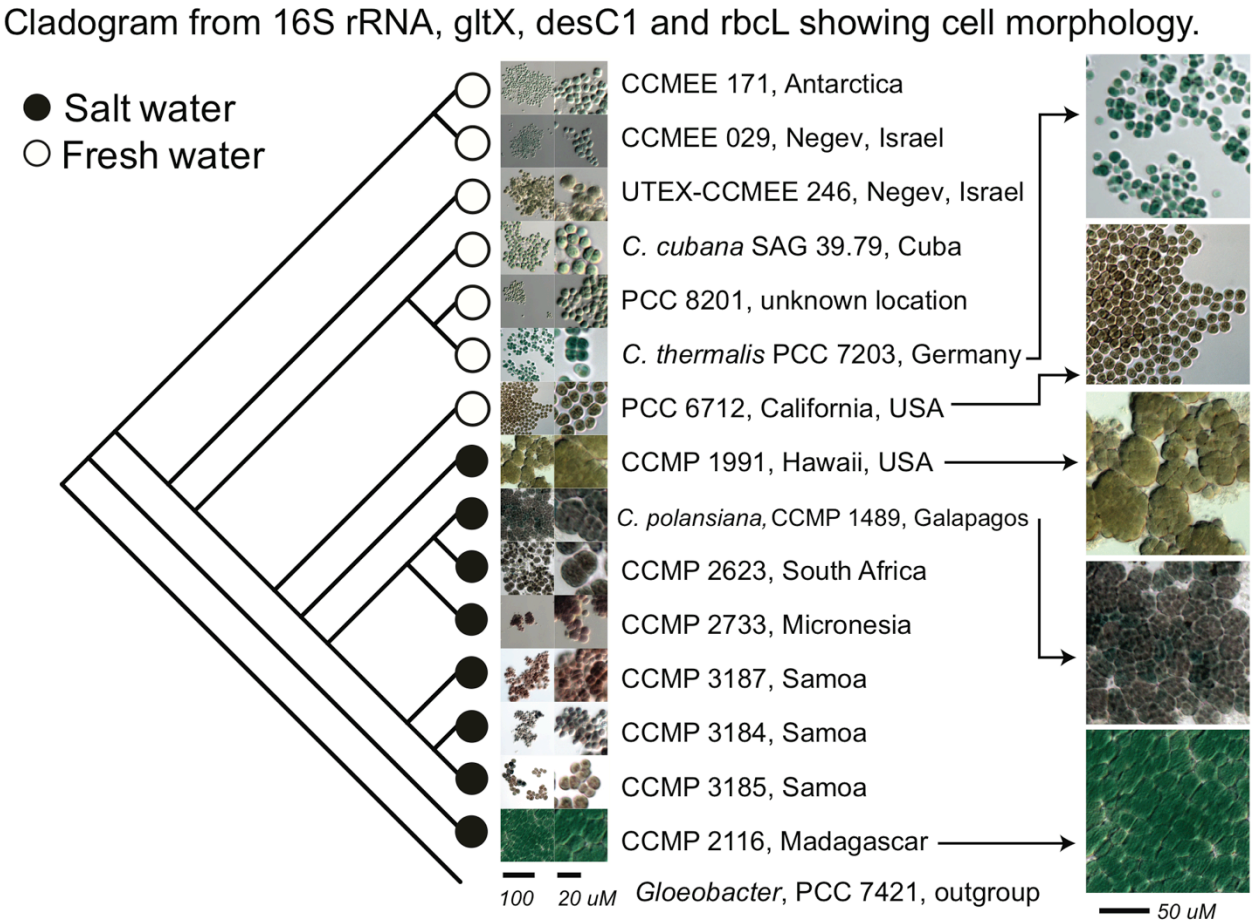

Fig. 3.5. Micrographs of the 15 species of *Chroococcidiopsis* from this study mapped to the four marker combined cladogram (see Fig. 3.2). Enlarged images of select species

are shown on the right hand side of the figure and demonstrate the diversity of morphology within the genus.

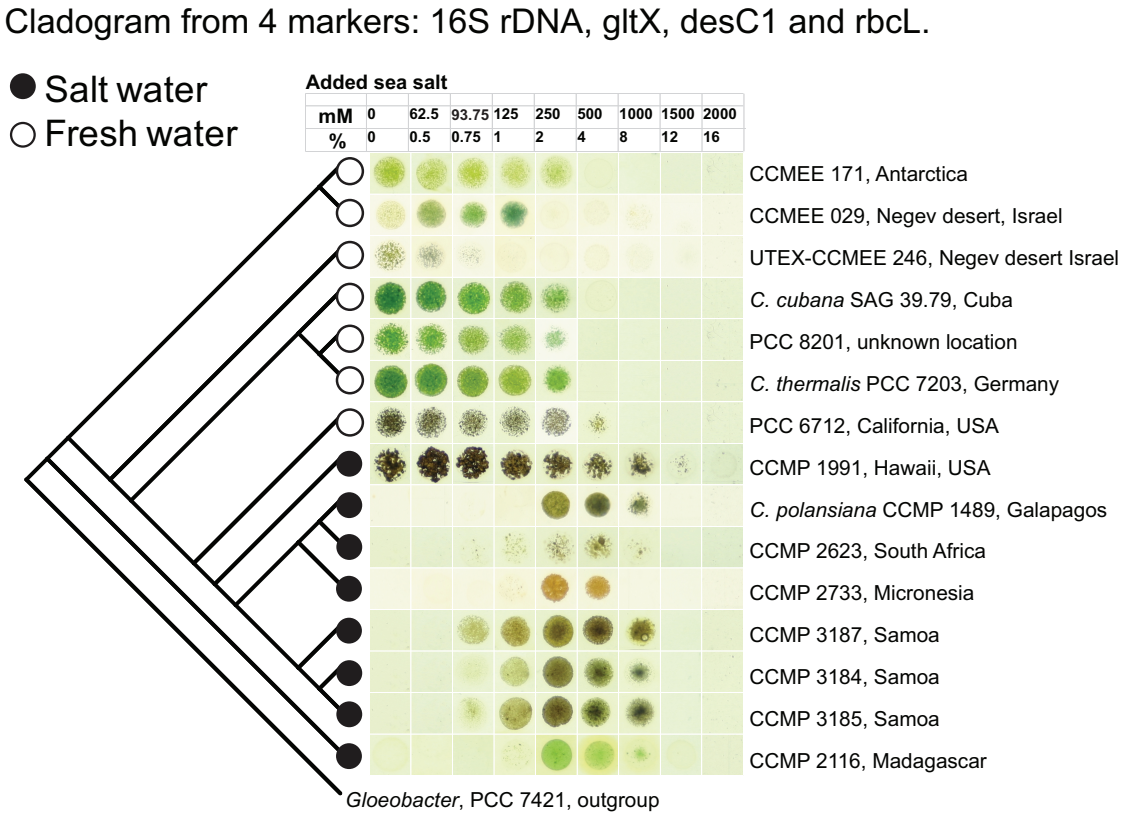

Fig. 3.6. Salt requirements for growth mapped to the four marker combined cladogram (see Fig. 3.2) of the 15 species of *Chroococcidiopsis* used in this study. Cells were plated onto agar plates containing varying amounts of added sea salt as shown.

Tables in Chapter 3

Table 3.1: *Chroococcidiopsis* species used in this study

Name	Original growth medium	location discovered
CCMEE 246 and UTEX N6910b19 EE8	BG11	Negev Desert, Israel
CCMP 1991	L1	Hawaii
CCMP 2116	L1	Madagascar
CCMP 2623	L1	Simon's Town, South Africa
CCMP 2733	L1	Pavillion Beach, Sand Island, Midway Atoll, Midway Islands, Micronesia
CCMEE 171-A789-2	BG11	University Valley, Antarctica
CCMP 1489, *C. polansiana*	L1	Fernandina Island, Galapagos station 8
CCMP 3184	L1	Vailela Beach, Island of Savaii, Western Samoa, micronesia
CCMP 3185	L1	Vailela Beach, Island of Savaii, Western Samoa, Micronesia
CCMP 3187	L1	Vailela Beach, Island of Savaii, Western Samoa
PCC 6712	BG11	Marin County, California
SAG 39.79, *C. cubana*	BG11	Cuba, Pinar del Rio, dry soil

PCC 7203, *C. thermalis*	BG11	East Germany
PCC 8201	BG11	Unknown location (Previously misidentified as "cyanelle from *Glaucocystis nostochinearum*"
CCMEE 029	BG11	Negev Desert, Israel

Abbreviations for culture collections: Provasoli-Guillard National Center for Culture Collection of Marine Phytoplankton (CCMP) (Andersen et al., 1997), the Pasteur Culture Collection of Cyanobacteria (Rippka et al., 1979), The University of Oregon's Culture Collection of Microorganisms from Extreme Environments (CCMEE) (http://cultures.uoregon.edu) and Sammlung von Algenkulturen (SAG) at the University of Göttingen (Göttingen, Germany).

Table 3.2: PCR primers used in this study

Marker	Primer name	Fwd / Rev	Sequence 5' to 3'	Best annealing temp.	Best Mg^{2+} conc.	Expected prod. size	Ref.
16S rRNA	chroR-A7	Rev	AGG CGA GTT GCA GCC TGC A	58°C	1.5 mM	1400 bp	This work
16S rRNA	chroF-D3	Fwd	CTC AGG ATG AAC GCT GGC	58°C	1.5 mM	1400 bp	This work

			GGT AT				
desC1	desC1-Cy-R	Rev	GCW ACC CAC CAR CAR TT	56-59°C	3-6 mM	500 bp	This work
desC1	desC1-Cy-F	Fwd	GTY WCC CAY CGC AGY TTY	56-59°C	3-6 mM	500 bp	This work
rbcL	rbcL-GR-D	Rev	TGC CAI ACG TGG ATA CCA CC	50-65°C	4.5 mM	960 bp	(Tomitani 2006)
rbcL	rbcL-GF-AB	Fwd	GAR TCT TCI ACY GGT ACY TGG AC	50-65°C	4.5 mM	960 bp	(Tomitani 2006)
gltX	gltX-F-8	Fwd	GCT GGG CAT GAA CTG GGA YGA RGG	53-65°C		489 bp	This work
gltX	gltX-R-8	Rev	CGT ACA GCA GGA TTT GTT TGG CNG TRT TNG C	53-65°C		489 bp	This work
gltX	gltX-F-7	Fwd	GCT GGC TGG GCA TGA AYT GGG AYG ARG	53-65°C		530 bp	This work

Table 3.3: Statistical significance of salt tolerance mapped to the four-marker tree shown in Fig. 3.2.

	K	P value
Min salt	0.28	0.012
Max salt	1.16	0.001
Optimum salt	0.81	0.001

Supplementary materials in Chapter 3.

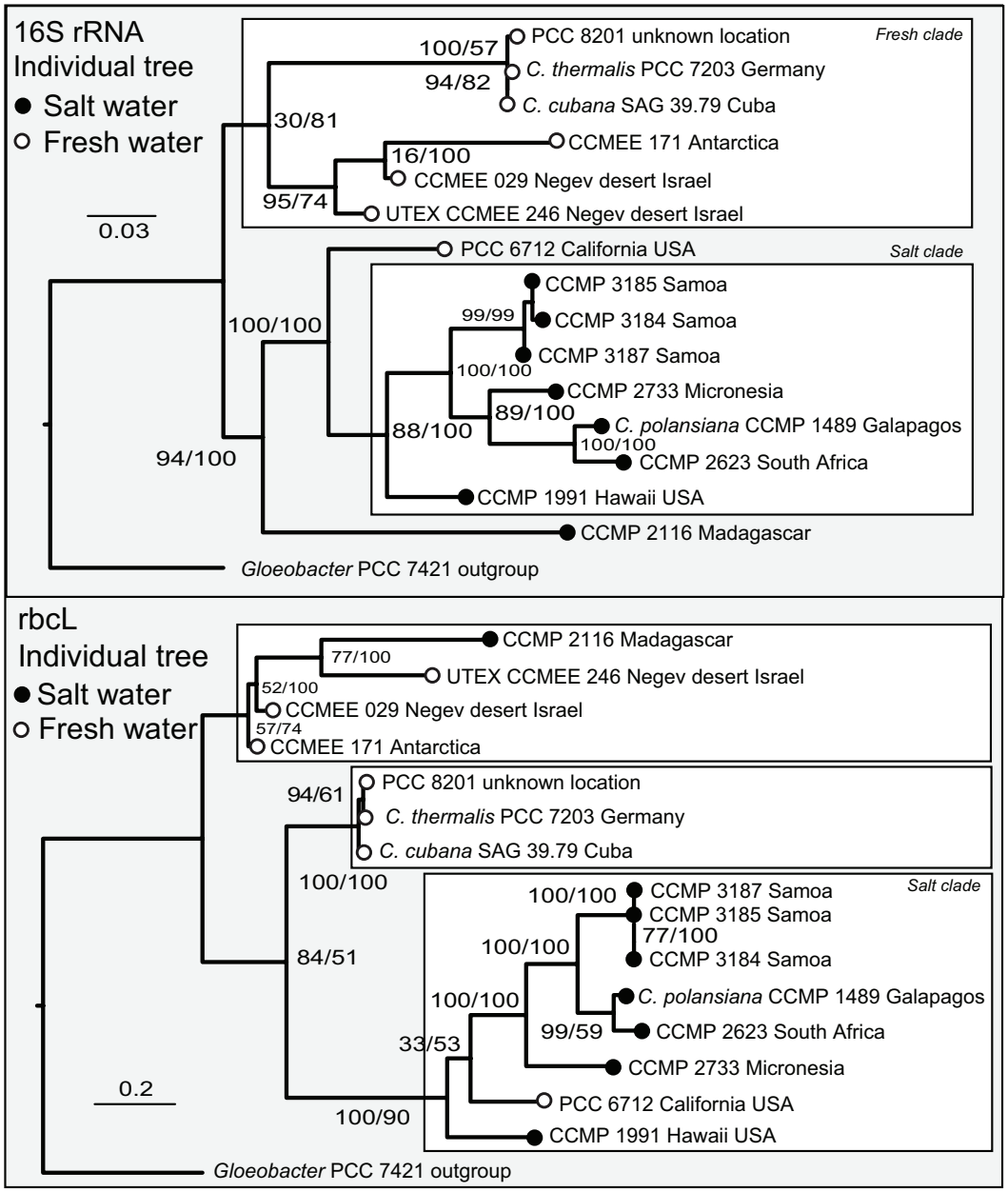

Fig. S3.1: RaxML Phylogram showing 16S rRNA and rbcL individual trees from *Chroococcidiopsis* species studied. *Gloeobacter violaceus* is used as an outgroup. The maximum likelihood analyses was conducted under the GTR + Γ + I model. The tree topology and branch lengths are for the sampled tree that has the highest likelihood (2,000 searches, log likelihood -4798.11 (16S rRNA) and -3273.45 (rbcL). Number on left are support values from 2000 bootstrap replicates and scale bar represents substitutions per site. Numbers on right are posterior probabilities from MrBayes analysis, 10,000,000 generations, sampled every 100 generations, four chains, temperature 0.2, burn-in 25% of the trees sampled.

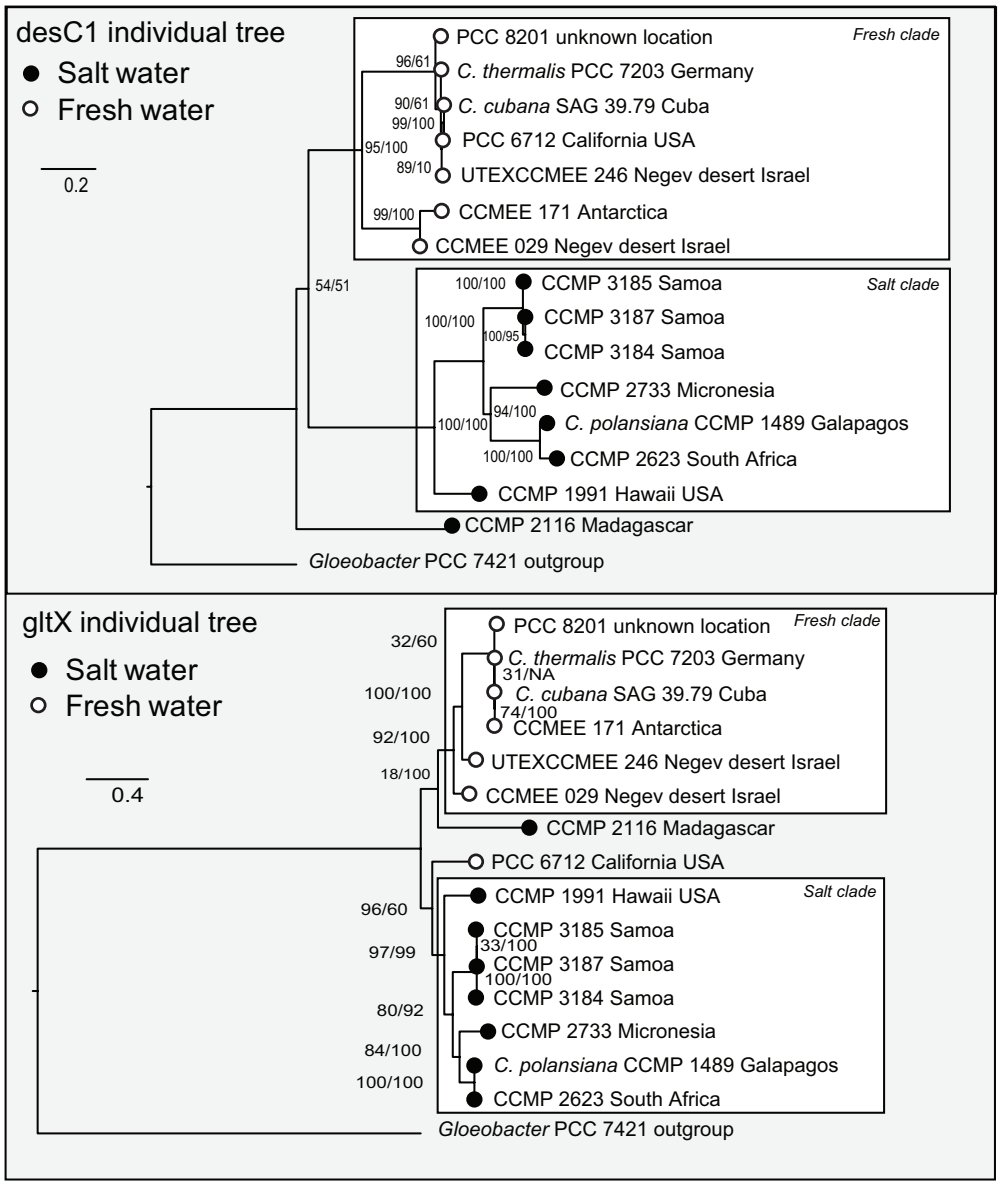

Fig. S3.2: RaxML Phylogram showing desC1 and gltX individual trees from *Chroococcidiopsis* species studied. *Gloeobacter violaceus* was used as an outgroup. The maximum likelihood analyses was conducted under the GTR + Γ + I model. The tree topology and branch lengths are for the sampled tree that has the highest likelihood (2,000 searches, log likelihood -3790.17 (gltX) and -3303.88 (desC1). Support values on the left are from 2000 bootstrap replicates. Numbers on right are posterior probabilities from MrBayes analysis, 10,000,000 generations, sampled every 100 generations, four chains, temperature 0.2, burn-in 25% of the trees sampled. The scale bar represents substitutions per site.

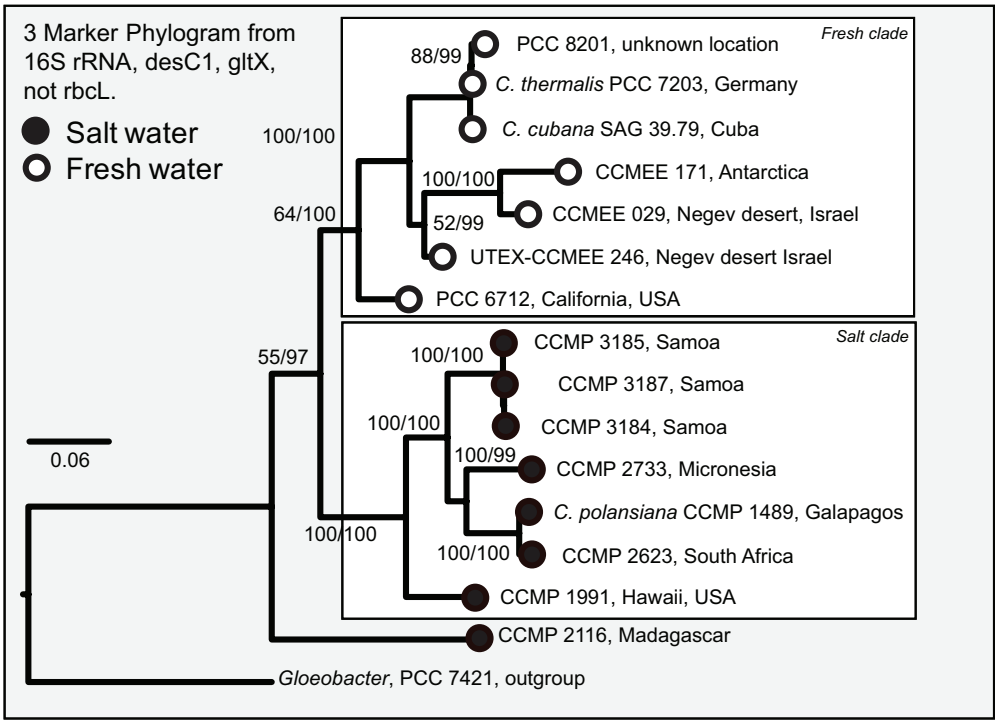

Fig. S3.3: RaxML Phylogram showing a joint tree constructed from 16S rRNA, gltX and desC1 from the 15 *Chroococcidiopsis* species studied. *Gloeobacter violaceus* was used as an outgroup. The maximum likelihood analyses was conducted under the GTR + Γ + I model. The tree topology and branch lengths are for the sampled tree that has the highest likelihood (2,000 searches, log likelihood -12617). Support values are from 2000 bootstrap replicates and scale bar represents substitutions per site. The salt and type clades are labeled. Numbers on right are posterior probabilities from MrBayes analysis, 10,000,000 generations, sampled every 100 generations, four chains, temperature 0.2, burn-in 25% of the trees sampled. The scale bar represents substitutions per site.

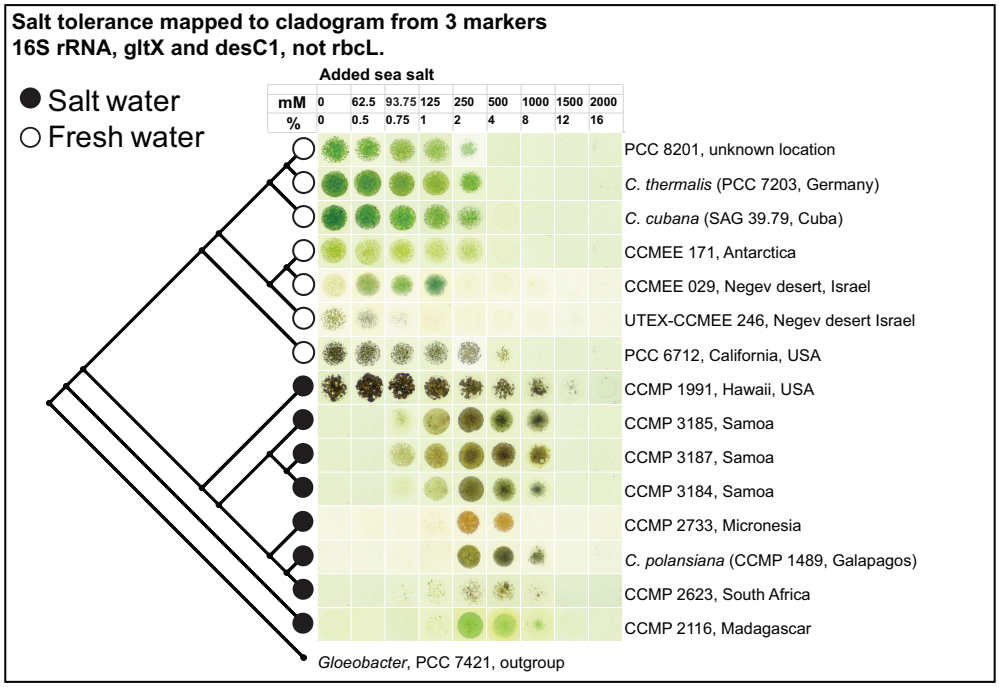

Fig. S3.4: Salt tolerance mapped to cladogram of the 15 species of *Chroococcidiopsis* used in this study. The cladogram is made from three markers, 16S rRNA, gltX, desC1 but not rbcL.

CHAPTER 4

CHARACTERIZATION OF *CHROOCOCCIDIOPSIS* (*PLEUROCAPSALES*) IN EXTREME CONDITIONS.

Contributions

In this chapter, I devised the research along with Lynn Rothschild and my committee members. I performed the experiments and analyzed the data. I wrote the chapter with input from Lynn Rothschild. Mike Grace helped with plating out of cells, repetition of the freeze-thaw assay, making media and maintaining stock cultures.

Abstract

Eight species of *Chroococcidiopsis* (Geitler, 1933), a cyanobacterium often found in extreme environments, were characterized for their ability to survive UVC (254 nm) radiation, desiccation and freeze/thawing. The study identified two previously uncharacterized saltwater species of *Chroococcidiopsis* that were more radiation resistant than the majority of the other species. Species CCMP 1991 from Hawaii survived up to 1750 $J \cdot m^2$ and species CCMP 3184 from Samoa survived up to 1000 $J \cdot m^2$ (254 nm UV) compared with 250 $J \cdot m^2$ for most other species tested. These two UV radiation resistant species were of interest because they are closely related phylogenetically but phenotypically divergent. Each was characterized for their ability to repair DNA damage as assessed by UV-induced thymine dimers and for oxidative damage tolerance via resistance to H_2O_2-induced damage. The Hawaiian species was more tolerant to H_2O_2 exposure than the Samoan species, indicating a possible role for antioxidants in the protection of the cell. Both species were more tolerant than others to freeze/thawing in liquid nitrogen, which is also known to cause DNA damage. Both species repaired thymine dimers faster in the light than in the dark, with the Hawaiian species repairing faster than the Samoan species in the light. Spectral absorbance scans were performed to

identify pigments in each of the species. The Hawaiian species contained a pigment that absorbed at around 315 nm that none of the other species showed. Although this pigment is outside the range of UVC absorbance, it is hypothesized that it may play a role in DNA protection as a UV sunscreen or as an antioxidant.

Introduction

Species of the cyanobacterium *Chroococcidiopsis* have been found in locations ranging from the cold Dry Valleys in Antarctica (Friedmann and Ocampo, 1976) to hot deserts such as the Atacama in Chile (Warren-Rhodes et al., 2006; 2007). The genus has acquired various traits to facilitate living in such inhospitable environments, such as an efficient DNA repair mechanism to survive in high UV environments (Billi et al., 2000b) and desiccation tolerance to survive in low water environments (Caiola et al., 1996a). Because of these abilities, *Chroococcidiopsis* has been proposed as a suitable organism for terraforming the planet Mars (Friedmann, 1995). Whilst terraforming is a controversial topic, the genetic engineering of extremophiles for the production of resources in space is a current area of interest to NASA (Cumbers and Rothschild, 2010). Synthetic biology, the process of making biology easier to engineer (Endy, 2005), extends genetic engineering by creating tools to rationally design genetic circuits and organisms. Whole genome synthesis was recently demonstrated (Gibson et al., 2010), as were a range of novel applications (Reviewed in Khalil and Collins, 2010). If biology can be rationally designed and logically controlled then synthetic biology could offer a light-weight self-replicating technology that could be programmed for life support functions such as food and biomaterial production in space. Such a technology could be

used to support a long-term human presence on the moon or Mars (Cumbers and Rothschild, 2010). The goal of this thesis is to engineer extremophiles for space by first surveying the natural diversity of life in extremes on Earth. The objective is to characterize unknown species in order to find novel mechanisms that allow life to survive in extremes on Earth in order to transfer these mechanisms to other organisms (Rothschild, 2010).

Although numerous species of *Chroococcidiopsis* have been identified and isolated, and despite the fact that the genus is famous for its extremophilic capabilities, only a small number of species have been studied for their abilities to survive in different extreme conditions. Friedmann was a pioneer in the study of *Chroococcidiopsis*, particularly from hot and cold desert environments (Friedman, 1993), where he discovered a number of microorganisms living in subsurface ecosystems (Friedmann, 1980). The radiation resistant ability of the genus was first demonstrated by Billi et al., (2000a), who compared a number of different species for their ability to survive X-ray radiation. *Chroococcidiopsis* has also been studied when subjected to solar ultraviolet radiation (UVR) in the open environment (Cockell et al., 2008) and under a simulated Martian UVR environment in the laboratory (Cockell et al., 2005; Billi et al., 2011). Cells only survived the Martian UV flux when protected by a 1 mm layer of Martian regolith that had been sieved to a grain size of <45 μM. Other studies that exposed cells to a simulated Martian UV flux showed that *Chroococcidiopsis* had a greater resistance than *Anabaena cylindrica* akinetes (resting cells), (Olsson-Francis and Cockell, 2009b) and spores of *Bacillus subtilis* (Schuerger et al., 2003).

UVR is well known to cause a range of different stresses to biological organisms (Franklin and Forster, 1997). UVR damages biomolecules such as DNA and proteins, which absorb radiation in the UV range. One known form of damage is the formation of thymine dimers, where two adjacent thymines are fused together on a strand of DNA (Goodsell, 2001). UVR also damages other biological molecules in a variety of ways, as reviewed in Rothschild and Cockell (1999) and Sinha and Häder (2002). UVR can inhibit growth and enzymatic activity (Kumar et al., 1996; Wu et al., 2005) and is also known to cause damage to cell membranes, pigments and signal transduction pathways (Vincent and Roy, 1993; Häder et al., 2003; Cadoret, 2005; Portwich and Garcia-Pichel, 2007). Reactive oxygen species (ROS) are also a byproduct of UVR (He and Häder, 2002; Pattanaik et al., 2007) as discussed in more detail below.

Cyanobacteria are responsible for the great oxidation event that led to the Earth's oxygenated atmosphere. The ozone layer, which formed around the same time, by absorbing wavelengths below 320 nm, shielded the Earth from the majority of harmful UVR (UVB and UVC) from the sun. Because the cyanobacteria evolved during a time when the ozone layer did not exist, they likely have a number of historical and present day UV-protection mechanisms (Kasting, 1987). In general these mechanisms can be classified as avoidance, shielding, repair and antioxidant production.

When avoiding UVR, cells can actively move away from the source of the radiation (Bebout and Garcia-Pichel, 1995) using methods such as gliding or by changing their buoyancy (Brookes, 2001). One shielding mechanism that cells use is self-shading by

neighboring cells. Cells that form aggregates or multilayers survive preferentially due to the upper layers of cells protecting the lower layers (Horneck et al., 2001). For example, lichens exposed to space environments show preferential survival of the lower layers of cells (Sancho et al., 2007; la Torre et al., 2010). Sunscreen pigments can also shield cells by absorbing UVR (Ehling-Schulz and Scherer, 1999), thus protecting the molecules beneath them. Melanin is a well-known protective pigment in humans that also occurs in some bacteria (Geng et al., 2008). Some cyanobacteria are also known to shield themselves by producing extracellular polysaccharides (EPS), sugars (Ehling-Schulz and Scherer, 1997) and mycosporine-like amino acids (Sinha, 2000).

A large range of DNA repair strategies have evolved to counter the effects of UVR (Ehling-Schulz and Scherer, 1999; Friedberg et al., 2005). For example, photolyase enzymes are light activated DNA repair enzymes, known to work by recognizing cyclobutane-type pyrimidine dimers such as thymine dimers and converting them back into monomers (Ehling-Schulz and Scherer, 1999; Frohnmeyer, 2003).

Reactive oxygen species such as free radicals and peroxidases (Allan, 1983) are a byproduct of metabolism and there is a constant low level of damage occurring in the cell because of them. Because of this, there are also constant repair and protection mechanisms at work (Franca et al., 2007). DNA in particular is a target of damage caused by ROS (Cooke, 2003). A number of biological mechanisms are known to exist to detoxify reactive intermediates or to repair the damage that they cause. Antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase

remove free radicals and reduce oxidative stress (McCord and Fridovich, 1988; He and Hader, 2002). Some photopigments such as bacterioruberin and the carotenoids are also know to act as antioxidants (Karsten and Garcia-Pichel, 1996; Shahmohammadi et al., 1998)

Like the radiation-resistant bacterium *Deinococcus radiodurans* (Mattimore and Battista, 1996), it has been proposed that *Chroococcidiopsis* is able to withstand high levels of X-ray radiation and desiccation due to an efficient DNA repair mechanism (Billi et al., 2000b). It is thought that this mechanism to repair damage caused by X-ray radiation could be the same as that which is responsible for UVR resistance (Cockell et al., 2005; Billi, 2009a) as both forms of radiation are known to cause strand breakage in DNA. Double-strand breaks in the genome of a number of species, including 029 (Negev) and 171 (Antarctica) were observed following X-ray irradiation by fragmented DNA on an agarose gel (Billi et al., 2000b). This damage was repaired within 24 hours, demonstrating that an efficient DNA repair system existed. Irradiation of 029 (Negev) cells by a simulated Martian UV flux (Cockell et al., 2005) showed cellular damage but DNA damage was not measured directly. PCR methods to detect UVR damage based on inhibition of DNA polymerase has been demonstrated in species 029 (Negev) by Billi et al. (2011). The quantification of nicked-ends following radiation has also been shown in this species (Billi, 2009b), but no measure of other types of DNA damage such as modified bases has been demonstrated.

The link between desiccation resistance and radiation resistance in *Chroococcidiopsis* is not yet clear. If the two traits were linked by a common DNA repair system, then one would expect to find DNA damage in desiccated cells. However, Billi (2009b) did not find genomic fragmentation in 029 (Negev) cells that had survived desiccation for four years. Billi (2009b) did show that dried 029 (Negev) cells protected themselves by producing antioxidant enzymes such as superoxide dismutase (Caiola et al., 1996b, Billi, 2009b), which is known to reduce oxidative stress. Combined with the evidence of DNA repair, it seems that *Chroococcidiopsis* may survive in extreme conditions by both DNA protection from antioxidants and by DNA repair (Billi et al., 2011).

Whilst it is known that some species of *Chroococcidiopsis* are resistant to extreme environments, the mechanisms by which they survive in different environments remain largely unknown. The objective of this study was to survey the phenotypic diversity of *Chroococcidiopsis* from across the known phylogeny of the genus and to characterize previously unstudied species for their ability to tolerate UV radiation, desiccation, freeze/thawing and oxidative damage. Understanding the distribution and various abilities of the species to survive in extreme environments could identify useful organisms for future comparative genomic and transcriptomic studies to understand the mechanisms of such traits in more detail. The goal of the study was to find members of the genus that exhibited phenotypes that could one day be useful for the engineering of organisms for resource utilization in space.

Materials and methods

Cyanobacterial cultures and culturing

Eight species were used in this study and are shown in Table 3.1. Each species was obtained as a unialgal culture from the culture collections specified. The species chosen represent a geographical, environmental and phylogenetic diversity of the genus *Chroococcidiopsis* (see Chapter 3). Freshwater species were grown in BG11 growth medium (Stanier et al., 1971), made from 50x concentrate (Sigma-Aldrich Inc. St. Louis, MO, USA). The BG11 was adjusted to pH 7.4 before filter sterilization. Saltwater species were grown in the same medium but supplemented with 4% (40 g·L-1) sea salt (Sigma-Aldrich Inc. St. Louis, MO, USA cat. S9883) as a substitute for seawater according to the manufacturer's instructions.

Some species of *Chroococcidiopsis* grow in cellular aggregates making plating of homogeneous monolayers difficult. In order to break up cell aggregates, liquid cultures in mid exponential phase were first gently sonicated (Ultrasonic processor XL, microtip size 1/16, repeated rounds of three seconds, Misonix Inc, Farmingdale, NY, USA) until optical density (730 nm) had plateaued, indicating that large clumps of cells had separated. Cells were then centrifuged at 1000·g, supernatant discarded and cells resuspended in fresh media to a concentration of $1 \cdot 10^6$ colony forming units per mL as confirmed by hemocytometer analysis. Cells were plated on agar plates of the same media and checked the next day to confirm that the sonication had produced a homogeneous monolayer of viable cells. As with previous studies of *Chroococcidiopsis*, a single cell or cell aggregate was counted as a colony forming unit (Billi et al., 2000b).

As species 1991 (Hawaii) had a morphology showing smaller, denser cell aggregates than 3184 (Samoa), to compare the DNA repair ability of these two species, cells were normalized to $6 \cdot 10^7$ cells·mL rather than normalizing to colony-forming units.

All growth and survival assays were performed by spotting replicates of 10 µL of cells onto agar plates, unless otherwise stated in the figure legends. This technique has been demonstrated previously for salt tolerance assays (see Chapter 3). Plates were sealed with parafilm to reduce evaporation before being placed in a growth incubator (Rexmed Inc. Taiwan) at 25°C under a 16h:8h light/dark regime and a mix of Philips TLD36W/840 (cool white) and Philips TLD36w (aqua) fluorescent light at 54±2 µmol photons·m^{-2}·s^{-1} as measured with a PAR detector (PMA2100/PMA2132, Solar Light Company, PA, USA).

Microscopy and image analysis

Agar plates were individually photographed on a light table using a digital camera. Micrographs were taken using a Zeiss Axio Imager Z1 with a DIC filter and a binocular microscope, Zeiss Stemi 2000-c (Carl Zeiss MicroImaging, Thornwood, NY, USA). ImageJ (Abramoff et al., 2004) was used to perform quantification of cell density. Each agar plate image was cropped to size and converted to a 32 bit grey scale image. Background was subtracted and light density was measured using the Gel Analyzer tool in ImageJ (Abramoff et al., 2004).

UVR tolerance

To determine the UVR tolerance of each species, cells were first plated onto agar plates in 10 μL spots as described above. Cells were then covered with petri dish lids and left for 24 hours before irradiation. After this time, lids were removed and cells were placed in a sterile hood 44 cm beneath a UVC light source of 254 nm for different lengths of time corresponding to different dosages of radiation. The intensity of the beam was 42 $\mu Ws \cdot cm^2$ at 254 nm, measured with a high resolution spectrometer (HR4000, Ocean Optics, Dunedin, FL, USA). Dosages were also measured in real time using a radiometer (PMA2100, Solar Light Company, Glenside, PA, USA). For comparison, *E. coli* (species TOP10, Invitrogen, Carlsbad, CA USA) was also irradiated after plating cells on LB medium in the same manner as the *Chroococcidiopsis*.

Freeze/thaw tolerance

To measure tolerance to repeated freeze thawing, 70 μL of each species was placed into a PCR tube. The PCR tube was then flash frozen by placing the tube into an ice bucket containing liquid nitrogen for 30 seconds. The tube was then removed and left to thaw at room temperature. Cells were then repeatedly re-frozen or plated in 10 μL replicates and growth was assayed as described above. The same protocol was followed for freeze thawing in the dry ice/ethanol slurry.

Hydrogen peroxide tolerance

To assess the tolerance of species to H_2O_2, dilutions were freshly prepared from a 9.7 M, 30% v/v stock of H_2O_2 (Sigma-Aldrich Inc. St. Louis, MO, USA) in fresh media. Cells

were mixed 1:1 in each dilution in PCR tubes, plated and growth assayed as described above.

Desiccation tolerance

Desiccation tolerance was assessed in 9 month-old cultures, kept in the dark at room temperature, according to Billi (2009b) and Caiola et al. (1993). After 9 months, cells were rehydrated by suspension in 50 µL of sterile water rather than fresh medium, in order to keep the solute concentration as it was before desiccation. The cells were re-plated and their colony-forming ability assayed via microscopy.

Thymine dimer detection

Thymine dimers were measured using a dot blot according to Sinha et al. (2001) as modified by Leuko et al. (2011). DNA from irradiated cells was extracted using a DNeasy Plant Mini Kit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Quality and quantity was measured on a Nanodrop (ThermoScientific, Wilmington, DE, USA) and samples were diluted to 2 ng·µL. NaOH was added to $1/10^{th}$ volume and samples were denatured at 80° C for 30 mins. Samples were then blotted onto a Hybond membrane (RPNLFP L/02/10) (GE Healthcare, Piscataway, NJ, USA) using a Minifold I dot blot system followed by a wash with 400 µL TE per well. The membrane was dried for 30 minutes at 80° C in a drying oven before being blocked with 5% ECL advanced blocking reagent (GE Healthcare, Piscataway, NJ, USA) for one hour. The unwashed membrane was incubated in mouse anti-thymine dimer primary antibody (#MC-062, Kamiya Biomedical, Seattle, WA,

USA) at a 1:3,000 dilution in PBS-T buffer for two hours at room temperature. The membrane was then washed twice with PBS-T followed by a one hour incubation in a secondary antibody, sheep anti-mouse IgG with Cy3 labeling (Kamiya Biomedical Seattle, WA, USA) at a 1:1,000 dilution in PBS-T with 5% ECL advanced blocking reagent. The membrane was then washed twice in PBS-T before being visualized on a Typhoon Trio Scanner (GE Healthcare, Piscataway, NJ, USA) and quantified with ImageJ (Abramoff et al., 2004).

Pigment extraction and absorbance measurements

Pigments were extracted in the dark by first spinning down 1.5 mL of $1 \cdot 10^6$ cells·mL at 4000·g for three minutes. Supernatant was removed leaving behind ~50 µL of liquid and ~25 mg of wet weight. Tubes were then placed in ice and cells were ground with an electric pellet pestle motor (Kontes/Sigma-Aldrich Inc. St. Louis, MO, USA) in a 1.5 mL centrifuge tube in repetitions of 30 seconds until cells were broken, as confirmed by microscopy. Methanol was added to 250 µL and mixed. Cells were then spun down at 10,000·g for 10 minutes. The supernatant containing the extracted pigment was removed and absorbance was measured on a Spectramax scanning spectrophotometer (Sunnyvale, CA, USA) from 190 to 1000 nm.

Results

Dose response curves for UVC are plotted in Fig. 4.1. Both figures show that a range of UV tolerances exist amongst the species tested. A number of *Chroococcidiopsis* species died after the first dosage tested of 250 $J \cdot m^2$. *E. coli* (species TOP10) was also irradiated for comparative purposes and also did not survive the dosage of 250 $J \cdot m^2$. Two species

survived similar large doses of radiation, 1991 (Hawaii) which survived up to 1750 $J \cdot m^2$ and 029 (Negev, Israel) which survived up to 1500 $J \cdot m^2$. Species 3184 (Samoa) showed a moderate radiation resistance and survived up to a dosage of 750 $J \cdot m^2$.

Cell survival after 9 months of desiccation is shown in Table 4.2. When desiccated cells were re-suspended, only species PCC 6712 (California) and CCMP 1489 *C. polansiana* (Galapagos) failed to produce colonies, indicating that the majority of species studied are desiccation tolerant.

Repeated freeze-thawing of cells in liquid nitrogen (Fig. 4.2) showed that 1991 (Hawaii) and 3184 (Samoa) both withstood multiple freeze thawing of up to 16 rounds, whilst the majority of other species died after 12 rounds. Species 7203 *C. thermalis* died after a single round of freeze thawing in liquid nitrogen. To investigate freeze thawing in freezing conditions that were not as cold as liquid nitrogen (approximately -196 °C), species 1991 (Hawaii) and 3184 (Samoa) were subjected to freeze thawing in an ethanol/dry ice slurry (approximately -68 °C). Fig. S4.1 shows that both species survived up to 16 rounds of freeze thawing in the ethanol/dry ice slurry but showed reduced colony forming ability with freeze-thaw cycles when frozen in liquid nitrogen.

Previous work has shown that CCMP 1991 (Hawaii) and CCMP 3184 (Samoa) are phylogenetically similar clustering close to each other in a saltwater clade of *Chroococcidiopsis* (see Chapter 2). Because these two species showed similar phylogeny but different radiation phenotypes, both were investigated in more detail. When both

species were exposed to 250 $J \cdot m^2$ of UVC (Table 3.3), species CCMP 1991 (Hawaii) received 73,697 ± 1.7% thymine dimers per Mb of DNA, 8.4% more than CCMP 3184 (Samoa) which received 67,958 ± 2%. The repair of these thymine dimers was investigated in these two species over time as shown in Fig. 4.3. Thymine dimer repair occurred faster in species CCMP 1991 (Hawaii) than in species CCMP 3184 (Samoa). After four hours species CCMP 1991 (Hawaii) had repaired 51% of the thymine dimers, whereas only 20% of the thymine dimers had been repaired in CCMP 3184 (Samoa). Fig. 4.4 shows that in both species, thymine dimer repair takes place faster when cells are left to recover in the light than in the dark**.**

Species 1991 (Hawaii) was more tolerant to H_2O_2 than species 3184 (Samoa) as shown in Fig. 4.5. Whilst species 1991 (Hawaii) survived up to 100 mM species 3184 (Samoa) survived an order of magnitude less, up to 10 mM H_2O_2.

The absorbance spectrum of the 8 species studied is shown in Fig. 4.6 after extraction in methanol. Species 1991 (Hawaii) shows a putative pigment absorbing around 315 nm that none of the other species show.

Discussion

The data presented in this study indicate that there is a diversity of phenotypes within the genus *Chroococcidiopsis*. The results show a range of UV tolerances showing that two of the species in particular are more tolerant to UV than the rest of the species. Species 1991 (Hawaii) and 029 (Negev) both show similar radiation resistance as seen in Fig. 4.1.

Species 029 (Negev) is a well studied species of *Chroococcidiopsis* and has been shown to be also resistant to X-ray irradiation and desiccation (Billi et al., 2000b) via mechanisms that involve both DNA repair (Billi et al., 2000b) and anti-oxidant production (Caiola et al., 1996b). Species 029 (Negev) has also been shown to be UV radiation resistant (Cockell et al., 2005), so it is therefore not surprising that this species shows high radiation resistance in this study too. Two species of *Chroococcidiopsis* that were previously uncharacterized have been identified as UV radiation resistant. Species 1991 (Hawaii) is of a similar resistance to 029 (Negev) and species 3184 (Samoa) is about half as radiation resistant in comparison.

The results indicate that the reason for this ability of the two previously uncharacterized species to survive UV radiation may involve a combination of DNA protection and repair mechanisms. Species 1991 (Hawaii) is more resistant to H_2O_2 indicating that it may contain enzymes such as SOD that play a role in reducing oxidative damage (Caiola et al., 1996b; He and Häder, 2002). Species 1991 (Hawaii) is also faster at repairing thymine dimers in its DNA than species 3184 (Somoa) indicating that it may have a better DNA repair mechanism.

The large error bars on the dose response curves are due to the clumping of the *Chroococcidiopsis* cells. In general, larger cellular aggregates were observed to contain more cells per aggregate which would likely decrease the chance of genome damage occurring per aggregate and increase the chance of survivability of the colony forming unit. This makes *Chroococcidiopsis* cells difficult to work with, particularly for

comparative studies. The sonication method used in this study is an effective way to reduce cellular aggregates and create homogeneous cultures for plating experiments. However, it has been noted in previous studies (Billi et al., 2000b) that the morphology of the genus, where mother cells contain daughter cells (baeocytes) at various stages of active cell division, may be part of the reason that the cells are able to survive such extreme conditions. Centrifugation was unable to effectively separate single cells from multiple cells, likely due to variable cell sizes, growth stage and internal buoyancy systems that certain species such as 1991 (Hawaii) appeared to have. Species 1991 (Hawaii) has tighter packed cells than species 3184 (Samoa) which could contribute to the enhanced DNA repair ability observed. *D. radiodurans* is also known to form cellular aggregates which again indicates that this may be part of the DNA protection and/or repair mechanism (Cox and Battista, 2005).

DNA repair was shown to take place faster in the light than in the dark in both species. This could be due to a light-dependent DNA repair mechanism. Photolyase genes such as the photorepair gene phrA (Hendrischk et al., 2007) could be responsible for such a repair system, although it is likely not the only mechanism given that some DNA repair still occurred in the dark in both species. In particular, in species CCMP 1991 (Hawaii) there appears to be a fast DNA repair mechanism occurring in the firsttwo hours that is not as apparent in the first two hours of DNA repair in the Samoan (CCMP 3184) species. This indicates that there could be two different DNA repair mechanisms at work in the two different species. Although CCMP 1991 (Hawaii) received more thymine dimers per Mb of DNA than the Samoan species CCMP 3184 (Table 4.3), it was able to

repair these thymine dimers faster, indicating that DNA repair may be more important than DNA protection in its ability to survive UV radiation.

A number of anomalies exist in the data. Species 171 from Antarctica has been previously shown to be resistant to X-ray radiation (Billi et al., 2000b), but in this study, it died after a relatively low UV dosage of 250 $J \cdot m^2$ (Fig. 4.1). The species was also not particularly resistant to freeze/thawing in liquid nitrogen (Fig. 4.2) but the opposite might be expected from a species living in an environment such as Antarctica that undergoes regular freeze thawing. It has been noted in previous studies that culturing of *Chroococcidiopsis* in the laboratory can, over time, make it less resistant to UV radiation and desiccation (Olsson-Francis and Cockell, 2009a). This could be the reason for the lack of radiation resistance in 171 (Antarctica), as according to the CCMEE culture collection, it was first isolated in 1971.

The pigment scans in Fig. 4.6 show a putative pigment in species 1991 (Hawaii) that has an absorption peak around 315 nm. This absorption is on the border of UVA and UVB, but not close to 254 nm (UVC), which was the wavelength of the radiation source used in this study. This suggests that this pigment is unlikely to play a role in the protection of the organism from UVC. However, the pigment may still play a role in protection of the organism from UVB. The pigment shows a similar absorbance profile to that of the carotene lycopene (Sicilia et al., 2005) and the carotenoid 4-ketomyxol 2′-fucoside (Takaichi, 2005). Further experiments, such as HPLC could identify what this pigment is and if it plays a role as a sunscreen pigment. Pigments, such as carotenoids are also

known to reduce reactive oxygen species (He and Hader, 2002) so this putative pigment in 1991 (Hawaii) may be an antioxidant.

The linkage between radiation and desiccation tolerance is known to be through DNA repair (Mattimore and Battista, 1996) as discussed earlier. However a link between freeze/thaw tolerance and DNA repair has not been established but could be an important future avenue of research. DNA repair may be a common link between many forms of environmental stresses faced by organisms due to the central role that the molecule plays as an information carrier in the cell. This study focused only on cell survival and DNA damage/repair and this was limited to thymine dimer damage. Future studies could also investigate other forms of DNA damage that are known to take place (Friedberg, 2005) as well as protein oxidation, which has also been implicated in bacterial radiation resistance (Daly et al., 2007).

However, the conditions tested here are much milder than the organisms might find in a real space environment. The polychromatic Martian UV flux tested by (Cockell et al., 2005) or a simulated flux from 1.5 years of exposure on board the International Space Station as tested by (Billi et al., 2011) would be a better test for space conditions in the future. However, the species showing exceptional ability in this study, could now be characterized under these environments too.

This study provides a foundation for performing comparative genomics on species of *Chroococcidiopsis* that are phylogenetically similar, but phenotypically diverse, such as

CCMP 1991 (Hawaii) and CCMP 3184 (Samoa). These two species showed high levels of UV radiation resistance and both prove interesting candidates for future studies. Full genome sequencing or transcriptomics under DNA repairing conditions could reveal which mechanisms are at play and the relative contributions of DNA protection and repair. Both could lead to a fuller understanding of how *Chroococcidiopsis* survives in extreme environments and how other organisms might one day be engineered to do the same.

The goals of this thesis as outlined in the introductory chapter, were to build a foundation for the engineering of extremophiles for space applications. The goal of the study was to find the genetic mechanisms responsible for survival in extreme environments and to use those as a toolkit to engineering other organisms for survival under such conditions. This was attempted by characterizing the response of different species to the environmental stresses of UVR, desiccation and freeze-thawing. The results indicated that resistance to extreme environments such as UV radiation is not as widespread in the genus as may have been previously thought. Two species were examined in detail for their ability to repair thymine dimers in DNA following radiation and for their ability to survive exposure to H_2O_2. Although neither of the mechanisms for these traits have been elucidated, a number of interesting species were identified and preliminary work on dissecting the mechanisms of DNA protection and repair were demonstrated.

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List of figures in Chapter 4.

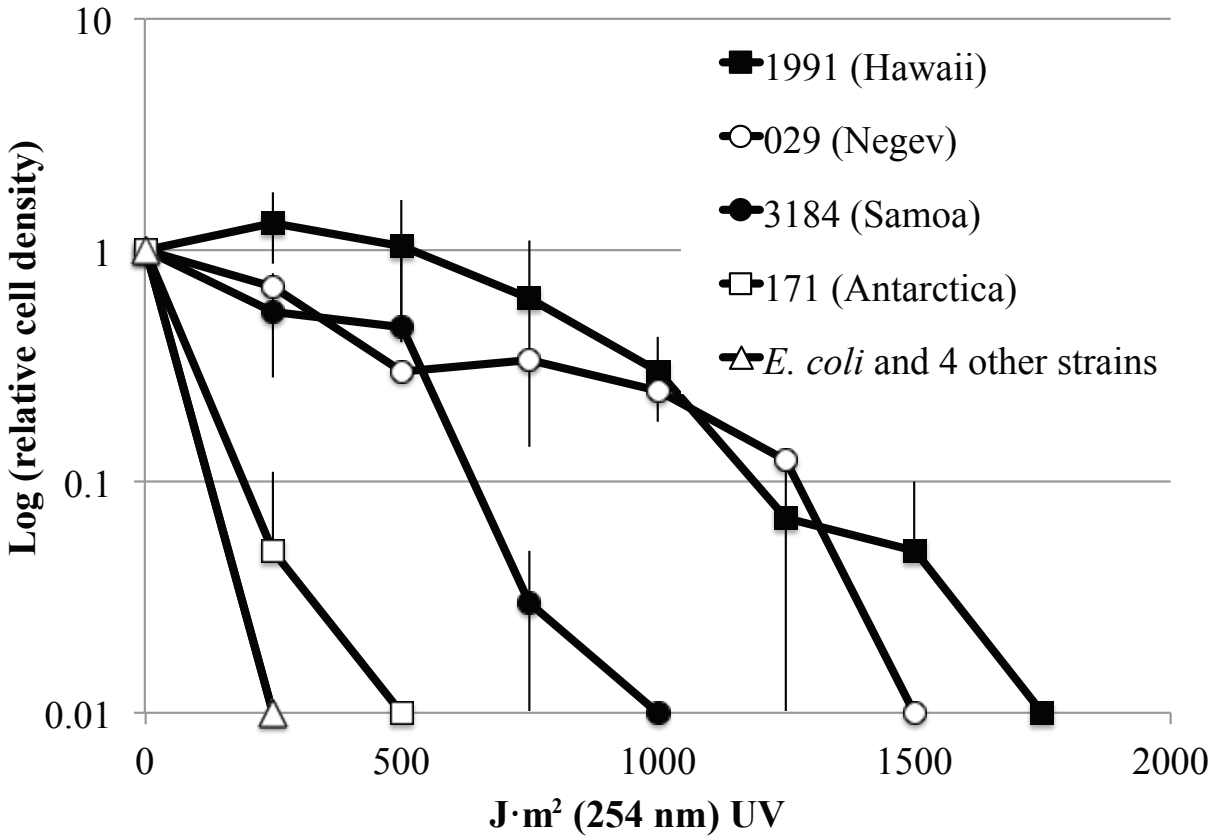


Fig. 4.1: Dose response curves of species exposed to different dosages of UVC (254 nm). Species CCMP 1991 (Hawaii) and CCMEE 029 (Negev) have similar radiation resistance. Species CCMP 3184 (Samoa) has moderate radiation resistance. Cells were irradiated on agar plates and incubated as described above. Cell density was then measured and normalized to the density of un-irradiated cells. Values are means of two independent trials with six replicates per trial.

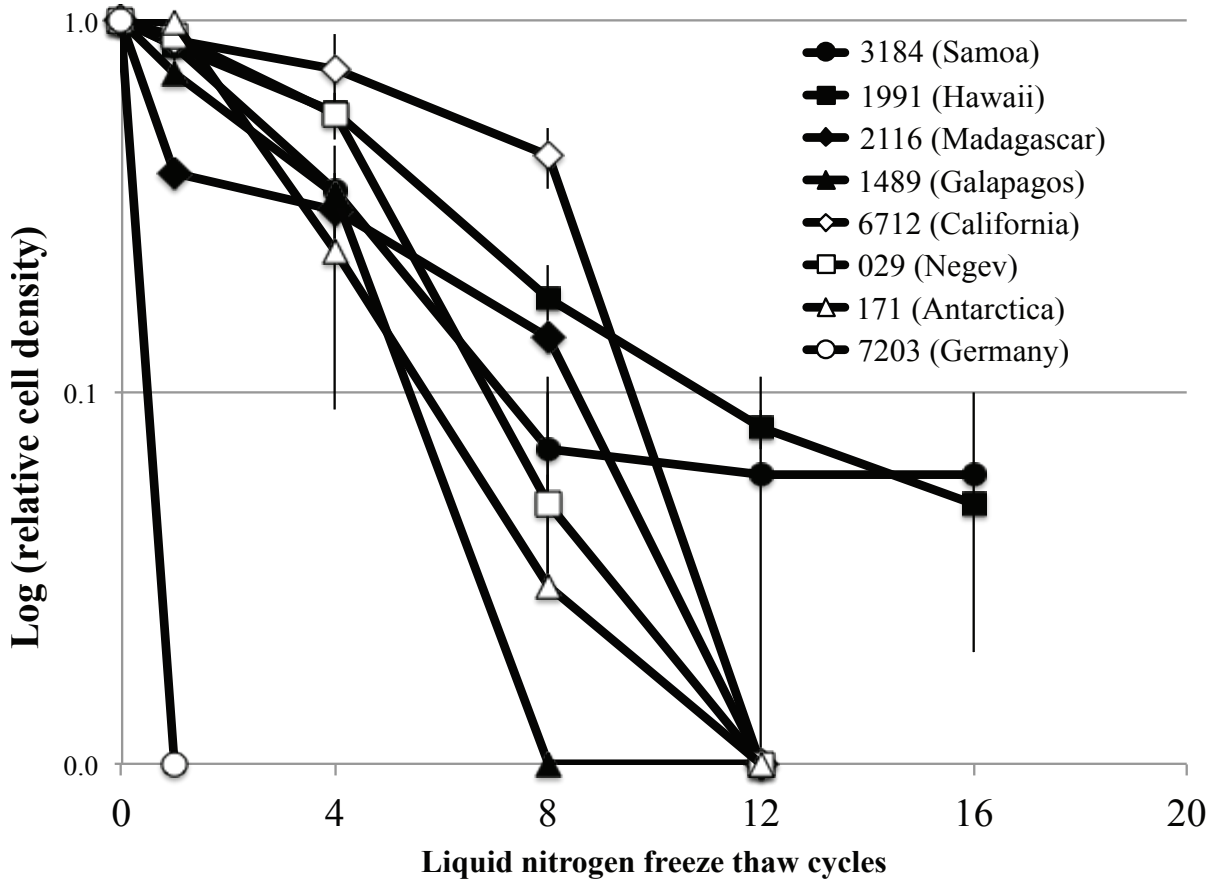


Fig. 4.2: Repeated freeze thawing of cells in liquid nitrogen followed by plating and growth on agar plates. The results show that species 1991 (Hawaii) and 3184 (Samoa) are more resistant to freeze thawing than the other species. Density of cells was assayed and normalized relative to the cell density of control cells. Mean values are shown from two independent trials with six replicates per trial. Species 1489 (Galapagos) and 2116 (Madagascar) are from a single trial with six replicates.

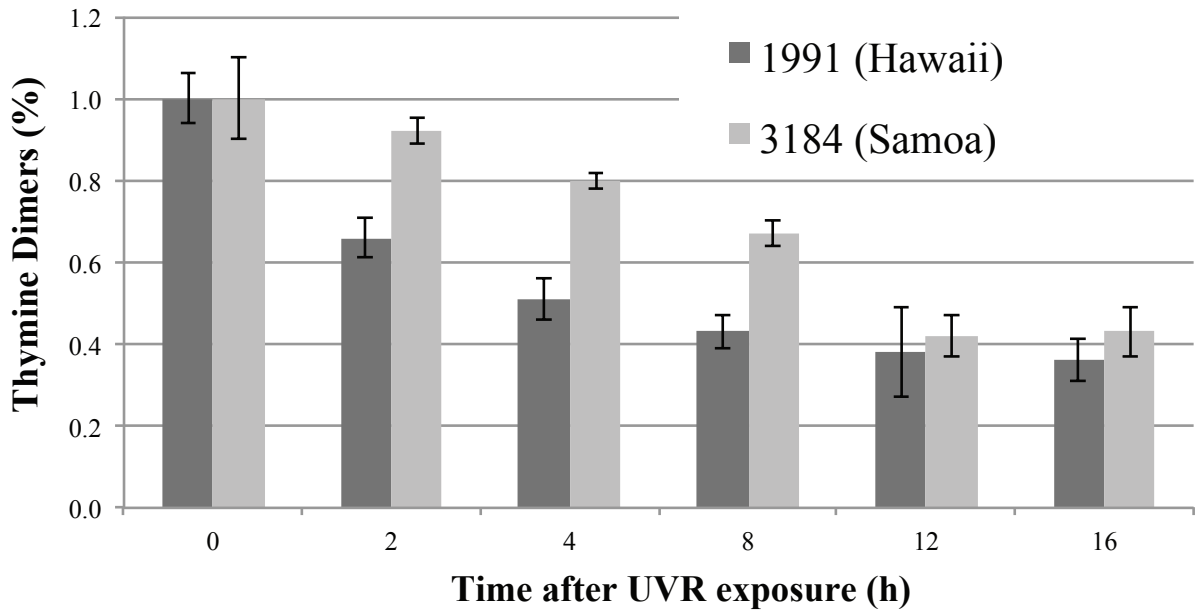
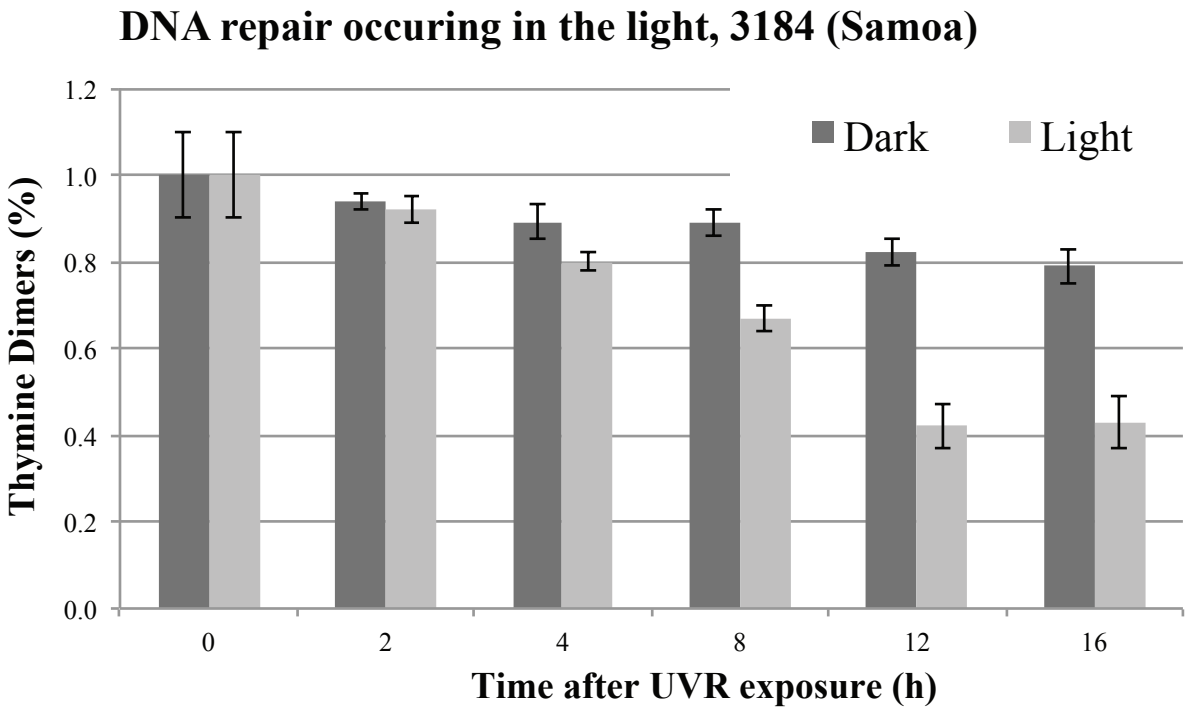


Fig. 4.3: DNA repair of thymine dimers occurs faster in species 1991 (Hawaii) than in species 3184 (Samoa). Cells were irradiated with 250 $J \cdot m^2$ of UV radiation and left to recover under normal light conditions. Samples were taken periodically during recovery and DNA was extracted and run on a dot blot to measure the number of thymine dimers, mean values are from three analyses.


DNA repair occuring in the light, 3184 (Samoa)
Thymine Dimers (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dark
Light
0
2
4
8
12
16
Time after UVR exposure (h)

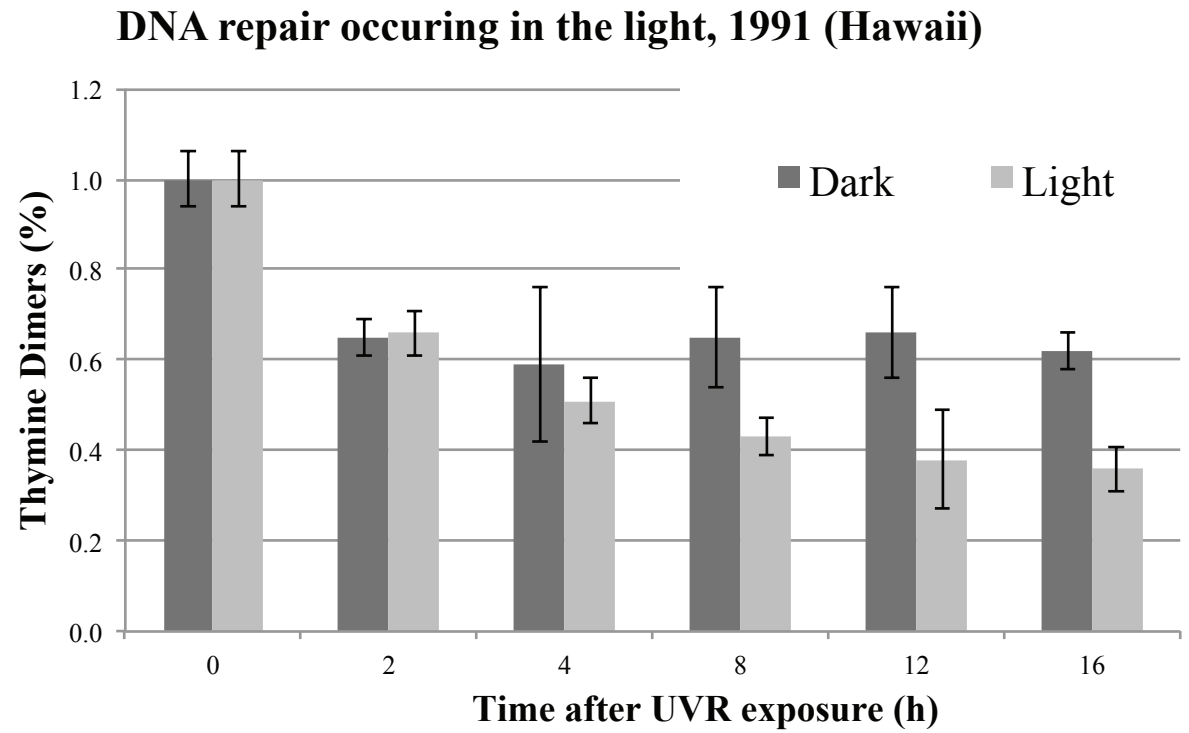

DNA repair occuring in the light, 1991 (Hawaii)
Thymine Dimers (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Dark
Light
0
2
4
8
12
16
Time after UVR exposure (h)

Fig. 4.4: DNA repair takes place faster in the light than in the dark in both 1991 (Hawaii) and 3184 (Samoa). As in Fig. 4.3, but cells were left to recover in the dark as well as the light, mean values are from three analyses.

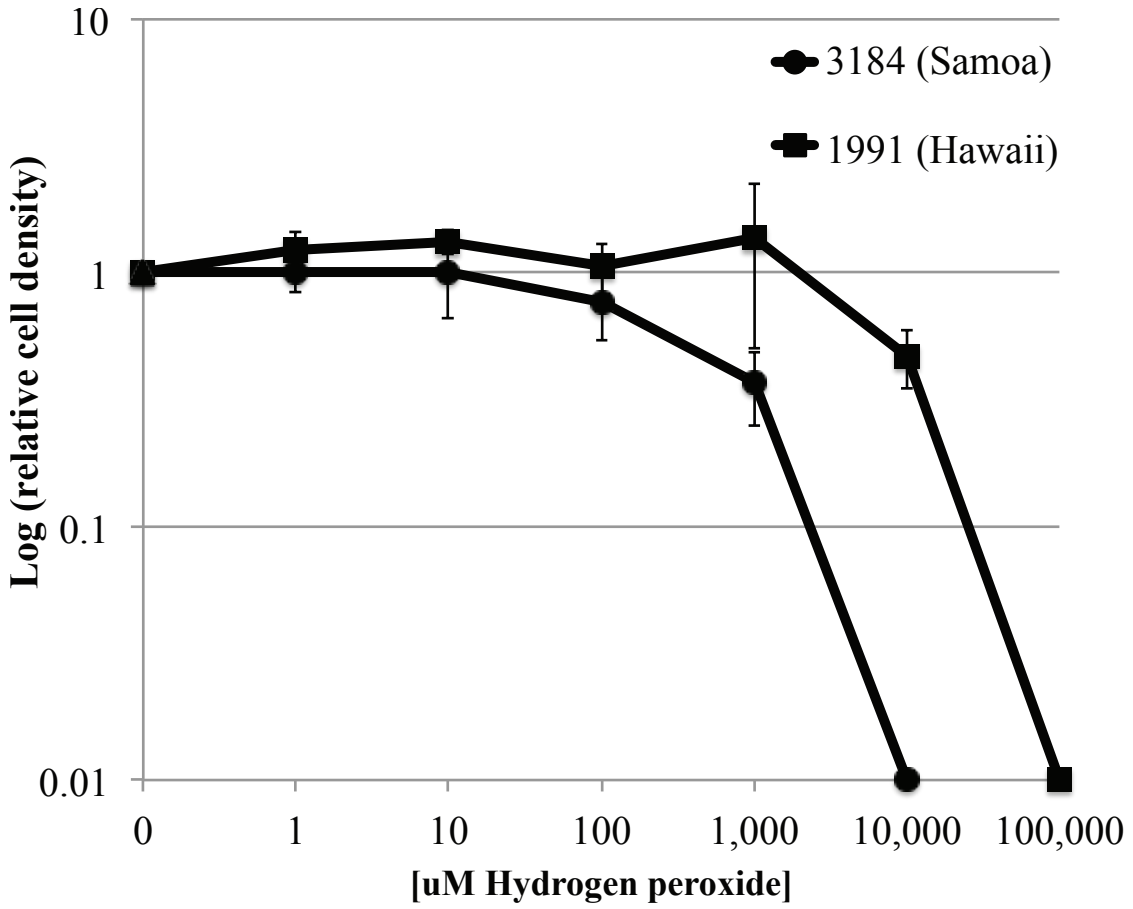


Fig. 4.5: Hydrogen peroxide tolerance in 1991 (Hawaii) and 3184 (Samoa) shows that 1991 (Hawaii) is more tolerant to H_2O_2 than 3184 (Samoa). Cells were diluted 1:1 in H_2O_2 and plated onto agar plates for growth. Cell density was then measured and normalized relative to the density of un-exposed cells. Mean values are shown from three independent trials with six replicates per trial.

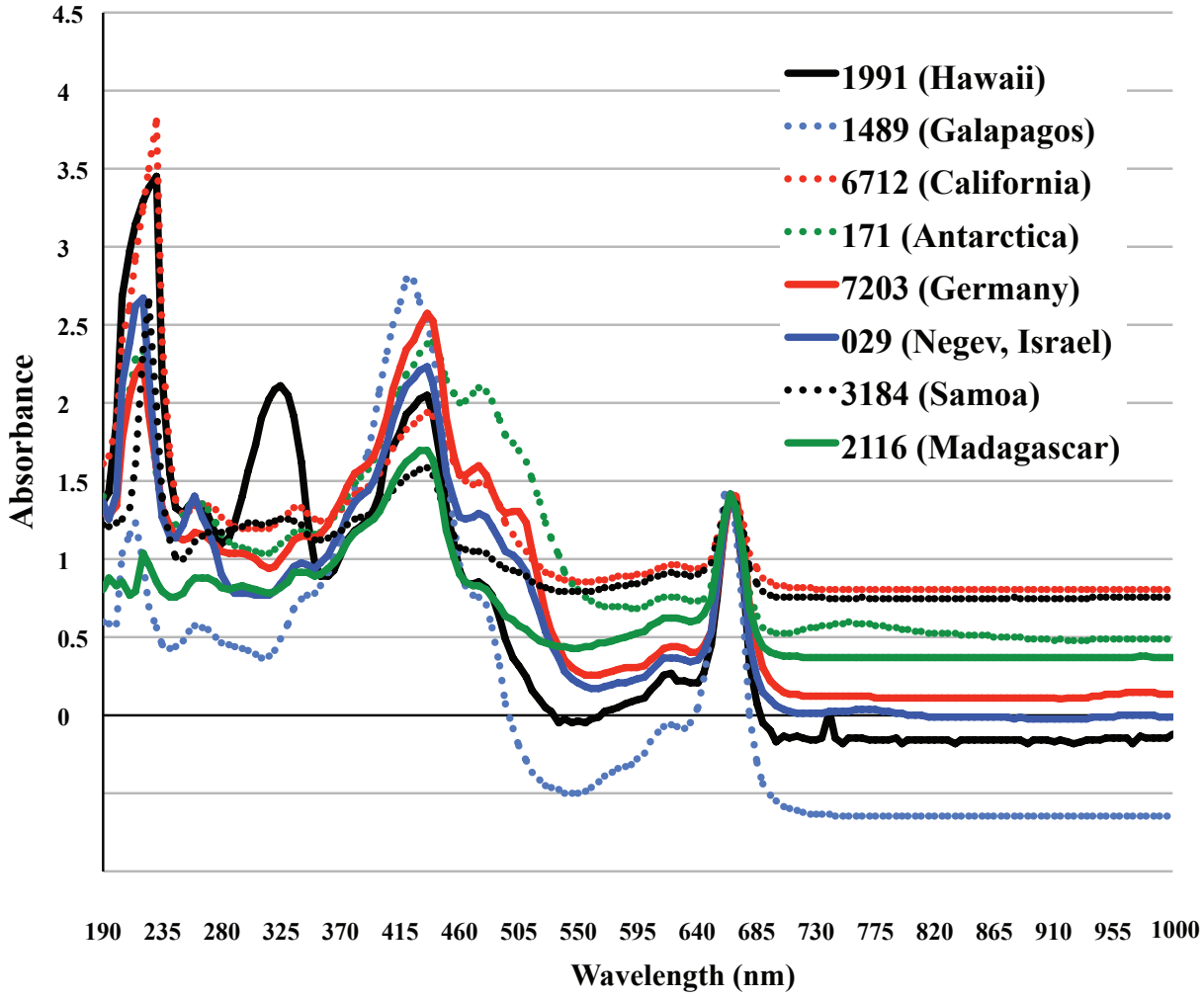


Fig. 4.6: Spectral absorbance scans of pigments extracted in methanol shows that species 1991 (Hawaii) has a pigment absorbing around 315 nm that is absent in the other species. Characteristic peaks of chlorophyll a can be seen around 430 and 662 nm and carotenoids around 500 nm.

List of tables in Chapter 4.

Table 4.1: *Chroococcidiopsis* species used in this study

Name	Growth medium	Location isolated
CCMP 1991	BG11 + 4% sea salt	Hawaii
CCMP 2116	BG11 + 4% sea salt	Madagascar
CCMP 1489, *C. polansiana*	BG11 + 4% sea salt	Fernandina Island, Galapagos station 8
CCMP 3184	BG11 + 4% sea salt	Vailela Beach, Island of Savaii, Western Samoa, Micronesia
PCC 6712	BG11	Marin County, California
PCC 7203, *C. thermalis*	BG11	East Germany
CCMEE 029	BG11	Negev Desert, Israel
CCMEE 171-A789-2	BG11	University Valley, Antarctica

Abbreviations for culture collections: Provasoli-Guillard National Center for Culture Collection of Marine Phytoplankton (CCMP) (Andersen et al., 1997), the Pasteur Culture Collection of Cyanobacteria (Rippka et al., 1979), The University of Oregon's Culture Collection of Microorganisms from Extreme Environments (CCMEE) (http://cultures.uoregon.edu) and Sammlung von Algenkulturen (SAG) at the University of Göttingen (Göttingen, Germany).

Table 4.2: Colony forming ability after 9 months of desiccation.

Species	Desiccation tolerant?
CCMEE 171 (Antarctica)	+
CCMEE 029 (Negev, Israel)	+
PCC 7203 (*C. thermalis*)	+
PCC 6712 (Marin)	-
CCMP 1991 (Hawaii)	+
CCMP 1489, *C. polansiana* (Galapagos)	-
CCMP 3184 (Samoa)	+
CCMP 2116 (Madagascar)	+

Table 4.3: Thymine dimer accumulation after 250 $J \cdot m^2$ of UV radiation and before DNA repair, from triplicate analyses.

	Thymine dimers / Mb DNA	error	% error
CCMP 1991 (Hawaii)	73,697	1,272	± 1.7
CCMP 3184 (Samoa)	67,958	1,358	± 2

Supplementary materials in Chapter 4.

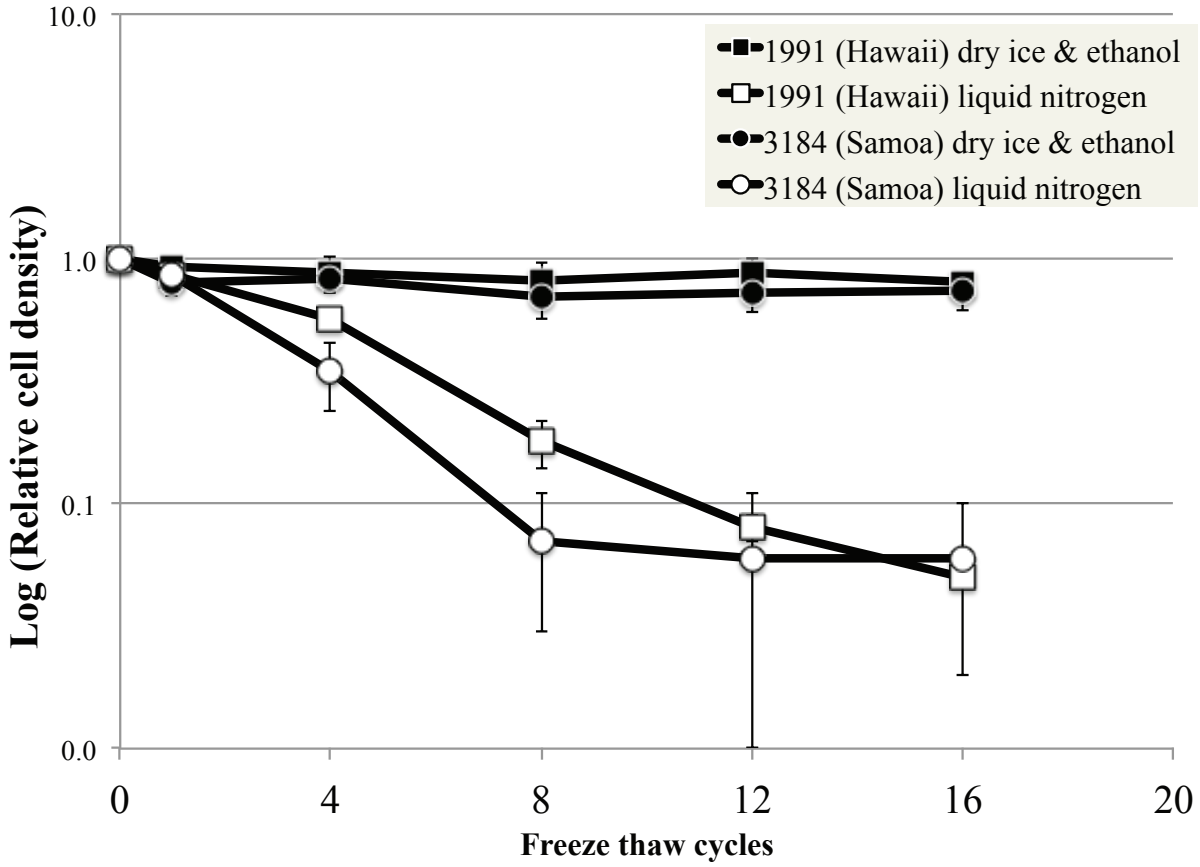


Fig. S4.1: A comparison of repeated freeze thaw cycles of 1991 (Hawaii) and 3184 (Samoa). Solid markers represent freeze thaw cycles in a dry ice/ethanol slurry at -68 °C compared with freeze thawing in liquid nitrogen at ~ -196 ° C. Both species appeared unaffected by the dry ice/ethanol freezing when compared to the liquid nitrogen freezing.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The work described in this thesis has provided a foundation for understanding the distribution and mechanisms of tolerances to extreme environments. Chapter 1 provided a broad review of mechanisms that cyanobacteria and other organisms found in extreme environments use for survival. Chapter 2 detailed the development of a high-throughput optical density growth assay for *Chroococcidiopsis.* Although the assay was unsuccessful and an alternative assay was developed for measuring growth on agar plates, a number of lessons were learned for working with organisms such as *Chroococcidiopsis* in the future. In Chapter 3, fifteen species of *Chroococcidiopsis* were analyzed in a multi-gene phylogenetic study, the largest study of its type in the genus. The results identified a previously unknown moderately halophilic clade of *Chroococcidiopsis* and showed that the genus is polyphyletic based on this trait. In Chapter 4, a number of *Chroococcidiopsis* species were selected for further analysis to assess the distribution of tolerance to desiccation, ultraviolet radiation (UVR), freeze/thawing and oxidative damage. Two species that had not been previously characterized, CCMP 1991 (Hawaii) and CCMP 3184 (Samoa) were found to be more resistant to ultraviolet radiation than the other species. A number of experiments were performed to begin to understand the mechanism of this radiation resistance. The most radiation resistant species CCMP 1991 (Hawaii) was also more resistant to oxidative damage and a possible protective role of antioxidants is hypothesized. CCMP 1991 (Hawaii) may also be protected by a pigment that it expressed, that was not found in any other species studied. A fast DNA repair system, as measured by thymine dimer repair was also identified in species CCMP 1991 (Hawaii) and this DNA repair ability is

hypothesized to be linked to its survival in liquid nitrogen, possibly due to a common form of DNA damage.

One question that emerges from looking at the results of each chapter is if there is a common mechanism of stress tolerance in some species? Fig 1.1 shows salt tolerance and radiation resistance mapped to the tree of life. The data points are taken from two similar diagrams in two different review papers, one on salt tolerance (Oren, 2008) and one on radiation resistance (Cox and Battista, 2005). The figure shows a correlation between salt and radiation tolerant organisms. Two out of three UVC radiation resistant species of *Chroococcidiopsis*, characterized in Chapter 4, were saltwater species. Species CCMP 1991 (Hawaii) and species 3184 (Samoa), the other radiation resistant species was 029 (Negev), a freshwater species. However, the phylogeny of these species, detailed in Chapter 3 (e.g., see Fig. 3.2 and Fig. 3.3), shows that the freshwater species is not closely related to the saltwater species, increasing the likelihood that the mechanism of radiation resistance in the saltwater species could be distinct from that of the freshwater species. Fig. 4.6 shows that CCMP 1991 (Hawaii) has a broad range of salt tolerances ranging from 0 to 1.5 M added sea salt. CCMP 1991 (Hawaii) was the only saltwater species that was able to survive on media without added salt. It is hypothesized that the ability that allows the two saltwater species to tolerate both salt and radiation may be linked, or that it could be part of a general stress tolerance mechanism.

The link between salt tolerance and radiation resistance is already well known. Kiss et al. (1978) investigated five radiation resistant species of *Clostridium botulinum* and showed

that radiation resistance correlated with NaCl tolerance possibly due to a DNA repair ability. In another study, expression of the *D. radiodurans* IrrE gene, a known regulator of radiation resistance, conferred enhanced salt tolerance in *E. coli* (Pan et al., 2009). The IrrE protein is known to activate transcription of the DNA repair protein recA following irradiation (Hua et al., 2003), supporting the hypothesis that DNA repair is the common link.

Taken together, these chapters and the results within them highlight both the range of extreme phenotypes that life is capable of tolerating and the diversity of possible mechanisms that could be responsible for them. A number of questions still remain unanswered however, and a number of future avenues have been opened for further investigation as detailed below.

What will whole genome sequences reveal about the phylogeny of extremophiles?

Although this study presents the most detailed view of the *Chroococcidiopsis* genus to date, it still only represents the evolutionary history of four genes out of a genome of likely 3,000 or more genes (an estimate based on the number of genes in the *Synechocystis* sp. PCC 6803 genome, genome.kazusa.or.jp). As genome sequencing costs fall, it is now becoming commonplace to sequence whole microbial genomes. Projects such as the Genomic Encyclopedia of Bacteria and Archaea (GEBA, jgi.doe.gov/programs/GEBA) are underway at the Joint Genome Institute, the Department of Energy's sequencing facility in Walnut Creek, CA. GEBA is sequencing a wide range of cyanobacteria, including a number of *Chroococcidiopsis* species such as

PCC 7203 (Germany), which was used in this study. When these sequences are published and analyzed, a more holistic view of cyanobacterial evolution will be available. A whole genome analysis of all the species used in this study may not be a good use of resources at this time, but it would provide a fuller picture of the phylogeny of the genus and could strengthen the conclusions presented from the four marker analysis. The availability of whole genome sequences could also answer a number of other questions, such as how many copies of the genome does *Chroococcidiopsis* have? Does the genome have more than one chromosome and how might these factors affect its radiation resistant ability? Whole genome sequencing could also reveal the true amount of horizontal gene transfer that has taken place in the genus, which could also shed light on how the organism has adapted to different environments. However, a full genomic analysis could also make the phylogenetic analysis more challenging. A comparative genomic analysis from sequencing the whole genome of species such as the radiation resistant CCMP 1991 (Hawaii) could help to identify possible DNA repair and pigment synthesis pathways and also identify if homologs of known antioxidant enzymes exist, such as superoxide dismutase. Comparative genomics could also aid in dissecting the mechanisms of salt tolerance and other traits studied here, for example, by looking for distinct sets of genes or regulatory pathways that are present in the salt tolerant species, but absent in the freshwater species. Once elucidated, it may then be possible to use these mechanisms for the genetic engineering of other organisms to live in extreme environments.

What is the relative role of protection vs. repair in radiation resistant extremophiles?

In Chapter 4 of this thesis, I identified two previously uncharacterized species of *Chroococcidiopsis* that were UVR resistant. Both these species showed efficient DNA repair mechanisms following UVR. The experiments in Chapter 4 began to dissect the possible mechanisms responsible for radiation resistance in terms of DNA protection from pigments and DNA repair via light and dark repair mechanisms. It is already known in many extremophiles that both protection and repair are responsible for the ability of organisms to survive in extreme conditions (reviewed in Rothschild and Mancinelli, 2001). The question remains as to the relative roles of protection versus repair. To investigate this further, the different types of damage and repair occurring could be separated and studied independently. For example, DNA damage was investigated in this study, but damage to proteins and lipids could also be investigated in the future. Pigments could also be investigated further both *in vitro* and *in vivo* by measuring the amount of protection that they provide from UVR. Possible pigment synthesis and mRNA up-regulation during UVR could also be assessed. Likewise, antioxidant enzymes could be assayed for their possible regulation and efficiency in reducing reactive oxygen species following UVR.

What are the limits for life in space and can we design synthetic extremophiles?

A significant number of challenges have been identified in this thesis that would need to be addressed before the goal of sending engineered life into space could be achieved. In the introduction, I identified a number of extreme conditions that organisms face, on

Earth and in space. A number of environments and conditions have been tested in this thesis, including salt tolerance, desiccation, temperature, freeze-thawing and radiation. However, these conditions vary in terms of their severity and danger that they pose to life in space.

Salt does not appear to be a problematic factor for life on Earth as many examples of halophiles tell us that high salt is not a problem. In Chapter 3, a number of *Chroococcidiopsis* species were shown to survive up to 1 M sea salt, and I described organisms that have been found living in high salt environments up to 3 M NaCl. There is no evidence indicating that space environments such as the moon or Mars would be more salty than this environment, indicating that salt would likely not represent a physical challenge for organisms in space.

Regarding desiccation, the goal of this thesis was to design organisms to be productive in space, rather than just to survive there. Desiccation is a concern in most space environments such as the moon or Mars. However, the example of food production on the moon from *in situ* carbon and water given in Chapter 1, presumed a source of energy in order to make liquid water available for the organisms to grow. The availability of water as a solvent will be the primary concern for organisms to be productive in space, so whilst desiccation is a concern for survival, it is not a major concern for the goal of this thesis.

The temperature variations of space described in Chapter 1 such as those found on the surface of the moon would either kill organisms due to the high temperatures in full sunlight (116.85 °C, Vasavada et al., 1999), or freeze them due to the extremely low temperatures when in darkness (-200 °C). As reviewed in Chapter 1, cyanobacteria can survive on Earth at 73 °C in hot springs (Miller and Castenholz, 2000) and can also survive when frozen in liquid nitrogen as detailed in Chapter 4. However, although life may be able to survive these extremes, it would not necessarily be productive at the high temperatures and would most certainly not be whilst frozen. For example, in Chapter 2 it was demonstrated that although *Chroococcidiopsis* survived when grown at 5 °C, none of the 15 species grew well at temperatures below 19 °C. Shielding the organisms from space environments, such as inside a bioreactor, would likely be the most appropriate protection from extremes of temperature.

Out of all the environments mentioned, radiation presents the biggest challenge for biology in space. The UVR tested in Chapter 4 was just a single wavelength rather than a polychromatic spectrum of UVR that one would find on Mars. However, both the experiments in Chapter 4, and previous studies looking at simulated Martian UVR (e.g., Cockell et al., 2005) indicate that biology could not survive unshielded on the Martian surface. If Earth-based life were to be sent to Mars now, it is clear from this and previous work that physical protection from radiation would be a key to survival. However, some protection from a thin layer of sand or salt in an evaporite may provide enough protection (Rothschild, 1990).

What are the challenges in dissecting complex phenotypes?

Despite the challenge of radiation discussed above, in this thesis a number of extremophilic traits were identified in earth organisms that could be of use to engineering organisms for extreme environments. But the dissection of complex traits remains a bottleneck in molecular biology. As with all complex phenotypes in biology, evolution has not made the code 'human readable', and the dissection of this complexity will need a concerted effort. As discussed earlier, modern tools in molecular biology such as comparative genomics or transcriptomics are two techniques that could be applied to understand what genes are present and being regulated in extremophiles. Until now, systematic approaches for characterizing microbial genes have progressed slowly, usually occurring by mutating of genes one by one. This is often a tedious, manual process involving the creation of thousands of different species. However, new tools for the dissection of complex phenotypes are being rapidly developed. For example, recent work from George Church and colleagues (Warner et al., 2010) demonstrated a method that the authors named "trackable multiplex recombineering" (TRMR), pronounced 'tremor'. They constructed a library of *E. coli* mutants that contained a rationally modified version of nearly every single gene. This was accomplished within one week and at a cost of around $1 per gene (Tipton and Dueber, 2010). These types of tools for genome engineering are making the dissection of the mechanisms of previously intractable phenotypes much easier. If methods such as TRMR could be applied to extremophiles, then the genetic elements responsible for extreme traits could be identified more rapidly, which could greatly enhance our ability to engineer organisms for space applications.

Should we evolve or engineer extremophiles?

Throughout this thesis I have made reference to synthetic biology and the rational design and engineering of particular functions. The idea of standardized genetic parts that can be rapidly rearranged to design novel genetic circuits (Endy, 2005) has yet to fully materialize (Kwok, 2010), but over recent years, the development of novel applications has been slow and steady (reviewed in Khalil and Collins, 2010). However, in industrial biotechnology such as in the field of protein engineering, directed evolution is still the tool of choice rather than rational design (see Porcar, 2010). Mainly this is because the possible search space is vastly greater in directed evolution than rational design. But it is also partly because the development of foundational tools for making biology easier to engineer is only just starting to gain traction. Synthetic biologists have been described as the Wright Brothers, still "putting pieces of wood and paper together" (Kwok, 2010). A number of new computational tools have recently emerged, including computer aided design tools such as Genocad (Cai et al., 2010), which allow biological constructs to be rapidly designed on a computer. However, until recently the protocols for manipulating DNA had not changed since they were first developed in the 1970's, and many labs are still cutting and pasting DNA with restriction enzymes and ligases. Soon this will changes as a number of new tools for the synthesis of long sequences of DNA have also recently emerged, as discussed next.

Foundational tools for DNA synthesis

A significant bottlekneck in the design and fabrication of biological circuits is that DNA synthesis has remained slow, expensive and error prone (Algire et al., 2010). However

DNA synthesis is also one of the areas that has seen a number of new tools and methods emerge. The biggest recent advance was the whole synthesis and booting up of a bacterial genome (Gibson et al., 2010), which was based on the Gibson assembly (Gibson et al., 2009), a method for stitching together overlapping oligos into lengths of several hundred kilobases. Other tools and methods for DNA synthesis have been addressing the previous bottlenecks of cost and error rate. For example, Kosuri et al. (2010) developed a technique that used microarrays to assemble DNA oligos, they managed to reduce the current cost of synthesis by more than tenfold, to <$0.01 per nucleotide (Algire et al., 2010). Matzas et al. (2010) produced another method to reduce the error rate by verifying the oligos by sequencing them before recovering them from the sequencer.

The cost of DNA synthesis from the development of new methods such as these has been continuing to rapidly fall (Carlson, 2009) and with it comes an unprecedented ability to write and manipulate DNA. These abilities will allow synthetic biologists to test new paradigms for engineering biology that were previously unimaginable due to the cost and time involved in manually cloning the constructs. It will soon become trivial to design, construct and test large genetic systems and to transfer sets of genes and pathways from one organism to another. The biological engineer will then be able to rapidly prototype biological circuits *in vivo*, and design, build and test novel engineered functions.

How could advances in synthetic biology change space exploration?

The whole genome synthesis described above proves that life can be replicated with not much more than a linear digital code. Now let us take the idea of biological *in situ*

resource utilization discussed throughout this thesis one step further, and imagine sending a biological compiler to the moon or Mars. This imaginary compiler would be capable of 'printing' physical DNA from whatever digital signal of A's, C's T's and G's it received. If humanity had this capability of printing and eventually booting up a pre-programmed synthetic cell anywhere that a radio signal could be sent, then this could represent the modern day equivalent of the Cambrian explosion, and see a rapid diversification of life throughout the solar system and beyond.

It could be argued that the political will is what is preventing humanity from settling the solar system. However, at the beginning of this thesis, I argued that the cost of sending things into space is the main problem and that we can solve this problem with biological *in situ* resource utilization. As costs fall, there is less need for governments to be running space programs and more private organizations and individuals will be able to go into space. If synthetic extremophiles could be designed to be productive in space, then synthetic biology would offer a low mass, self-replicating, reprogrammable technology for use there.

In this thesis, I examined life in extreme environments on Earth in order to be able to understand the mechanisms that these organisms use. The future goal is to be able to transfer these mechanisms to other organisms to enable them to live in space. The Earth offers a far milder environment than the rest of the solar system for an organism that depends on carbon biochemistry and uses liquid water as a solvent. It is not yet known what the true limits of life in space are and if it will be possible to engineer life for

survival and productivity there. However, if the surface of the moon or Mars does prove too inhospitable for life, then perhaps a physical shield, such as a system of underground bioreactors should be considered instead. These organisms could then still be used for the production of food, fuels and materials from *in situ* resources whilst being protected from the harsh space environment. This ability to produce resources in space via biological means rather than take them with us from the Earth could be the key to long-term human settlement of the solar system.

My personal vision for the next 50 years of space exploration and settlement is a robotized manufacturing infrastructure for biological *in situ* resource utilization (BISRU) that prepares cached consumables in space for humans to collect in the future. I detailed in Chapter 1 how the moon has the resources for such an activity. The moon is also the logical choice because it is the closest body to Earth and because humans have already been there. The Google Lunar Xprize (googlelunarxprize.org) is a $30M incentive prize to spur non-governmental innovation in robotic space exploration of the moon. I hope that the prize will be successful in fostering a robotic capability for lunar exploration. If this happens, then a new frontier will be opened for resource utilization in space that would allow private individuals and companies to begin to exploit the resources there.

As outlined in this thesis, synthetic biology is a suitable technology for resource utilization in space. The last decade of genomics and the recent advances in whole genome synthesis have demonstrated that life is a digital technology that can be read, saved, modified and rewritten again. I hope to see a robust synthetic biology industry on

Earth that makes bio-based products and replaces chemical manufacturing with biological manufacturing. These organisms and products could then in theory, be sent anywhere in space that a DNA synthesis machine could be sent. This thesis has demonstrated that Earth life would find the lunar radiation and temperature very difficult to be productive in however. If life cannot be engineered to survive the harsh environments of space, then I would shield it from these environments underground, which is also where I predict the first human settlements will be built.

In the words of Carl Sagan:

"Since, in the long run, every planetary civilization will be endangered by impacts from space, every surviving civilization is obliged to become spacefaring - not because of exploratory or romantic zeal, but for the most practical reason imaginable: staying alive... If our long-term survival is at stake, we have a basic responsibility to our species to venture to other worlds."

Pale Blue Dot, 1994.

APPDENDIX: SUPPLEMENTAL DATA

This appendix contains supplemental data that was not included in the main chapters of the thesis. Fig. A.1. shows the results of a FACS analysis of species CCMP 3187 (Samoa). FACS was attempted on this species in order to try and separate individual cells from aggregates of cells. The x-axis is the fluorescence level and the y-axis is the number of events. Three populations of cells can be seen in this diagram, a set of weak fluorescent objects on the left hand side and two sets of brighter objects on the right hand side. The purple line/gate surrounds the two populations of events in the upper right hand corner. It was hypothesized that the left event was single cells, whilst the right event with brighter fluorescence was aggregates of more than one cell. Although this initial FACS analysis showed potential utility, it was not continued due to the slow flow rate of the FACS, the lack of microscopy facilities at the FACS core and the expense ($100 / hour) of operating the equipment.

Another method to try and remove cell aggregates from single cells was via a sucrose gradient as shown in Fig.A.2 and Fig.A.3. Although sucrose gradients were successful for small volumes of cells as shown in Fig.A.2, they were not a practical solution for larger volumes of cells, as when the cells were washed after the gradient, to remove the sucrose, they began to again form cell aggregates.

Fig.A.4 and A.5 both show the controls for the environmental growth chamber used. The temperature was monitored with wireless temperature buttons over time to ensure that the temperature was maintained correctly. The light level was also monitored with a

spectrophotometer to ensure that the light level was even and distributed and to record the particular spectra of the bulbs used.

Fig.A.6 shows a pH assay that changed the pH in the growth medium (BG11). As pH increased, optical density at 730 nm also increased. However, cell aggregates can be seen particularly in higher pH such as at pH 9. Because 171 (Antarctica) survived at a wide range of pH, it was not a variable that was studied further in this thesis. Although pH would be an important area of study in the future in order to understand how different tolerances might be suitable for different space environments.

In Fig.A.7 and A.8, freeze thaw tolerance was investigated in more detail. In Fig.A.7, naked genomic DNA from 1991 (Hawaii) was repeatedly freeze-thawed in liquid nitrogen for a number of repetitions before being run on an agarose gel. No detectable breakages were shown on the gel after increasing rounds of freeze thawing. This assay is not particularly sensitive however and other forms of damage may be occurring to the DNA that cannot be assayed via gel electrophoresis. In Fig.A.8 cells were investigated via microscopy after being frozen in liquid nitrogen and then thawed at room temperature. The assay shows that cell size was much smaller in the cells that had been exposed to liquid nitrogen, although the cells could be plated and grown again after this treatment.

Fig.A.9 and A.10 show binocular microscopy of species growing on agar plates. Fig.A.9 shows an interesting biofilm that formed when cells of CCMP 1991 (Hawaii) were left to

grow on an agar plate for three months. It is hypothesized that this biofilm could form some sort of protective membrane. However, as the assays in the main thesis all were performed on individual cells rather than biofilms, protection from biofilms was not investigated further. Fig.A.10 shows a number of species grown in different shaded light conditions and the pigments they produced. Cell densities were not high enough to perform pigment extractions and when this assay was repeated with a higher density of cells, the same result was not observed.

APPDENDIX: SUPPLEMENTAL FIGURES

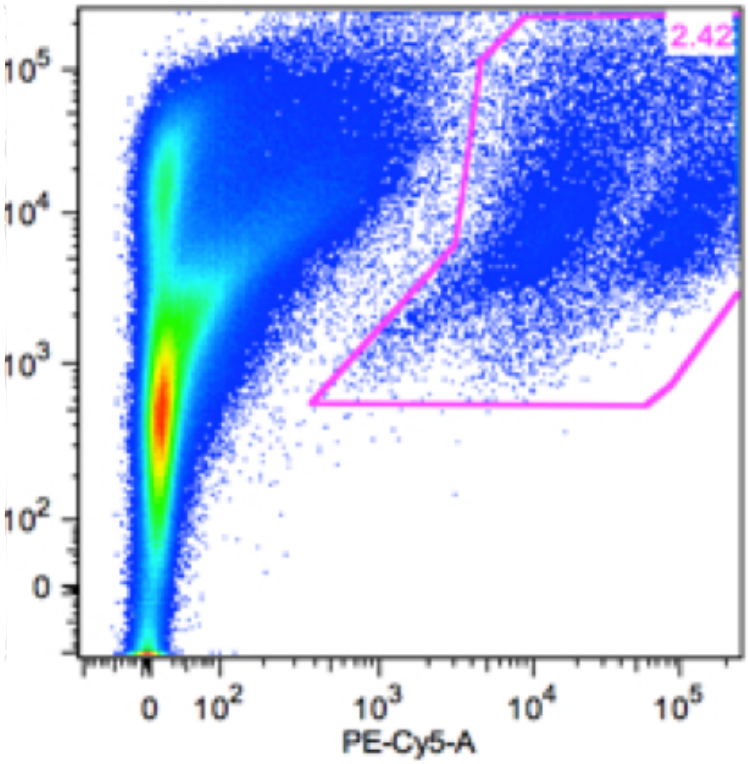


Fig. A.1. CCMP 3187 (Samoa), identification and sorting of single cells via FACS. The purple gate drawn in each example indicates the population of individual cells. The x-axis is the relative phycoerythrin fluorescence and the y-axis is the number of events recorded.

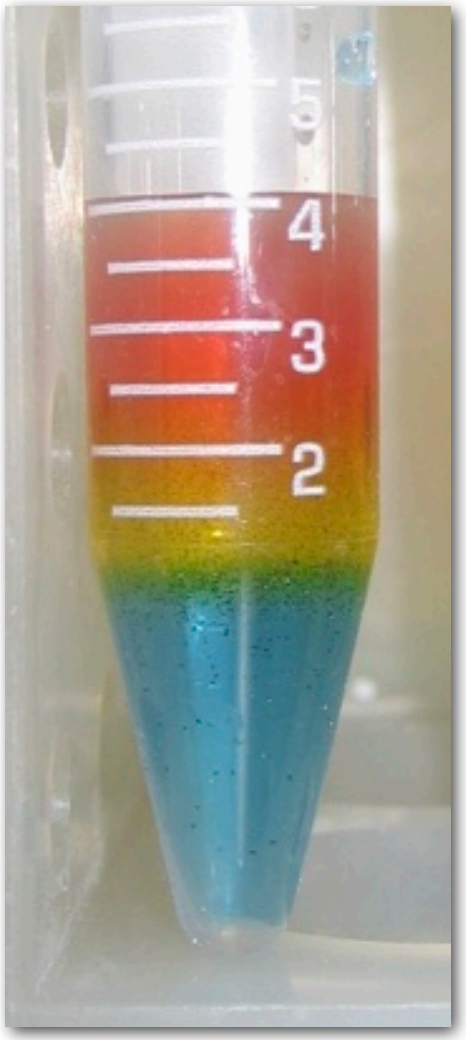


Fig. A.2. Sucrose gradient performed to try and separate clumps of cells from single cells. Food coloring was added to aid visualizing the different densities of sucrose. Red = 15% sucrose, yellow = 20 % sucrose, blue = 25 % sucrose. Clumps of dark cells can be see in the green layer, the boundary between the yellow and blue layers. Cells were left for four hours to settle after loading.

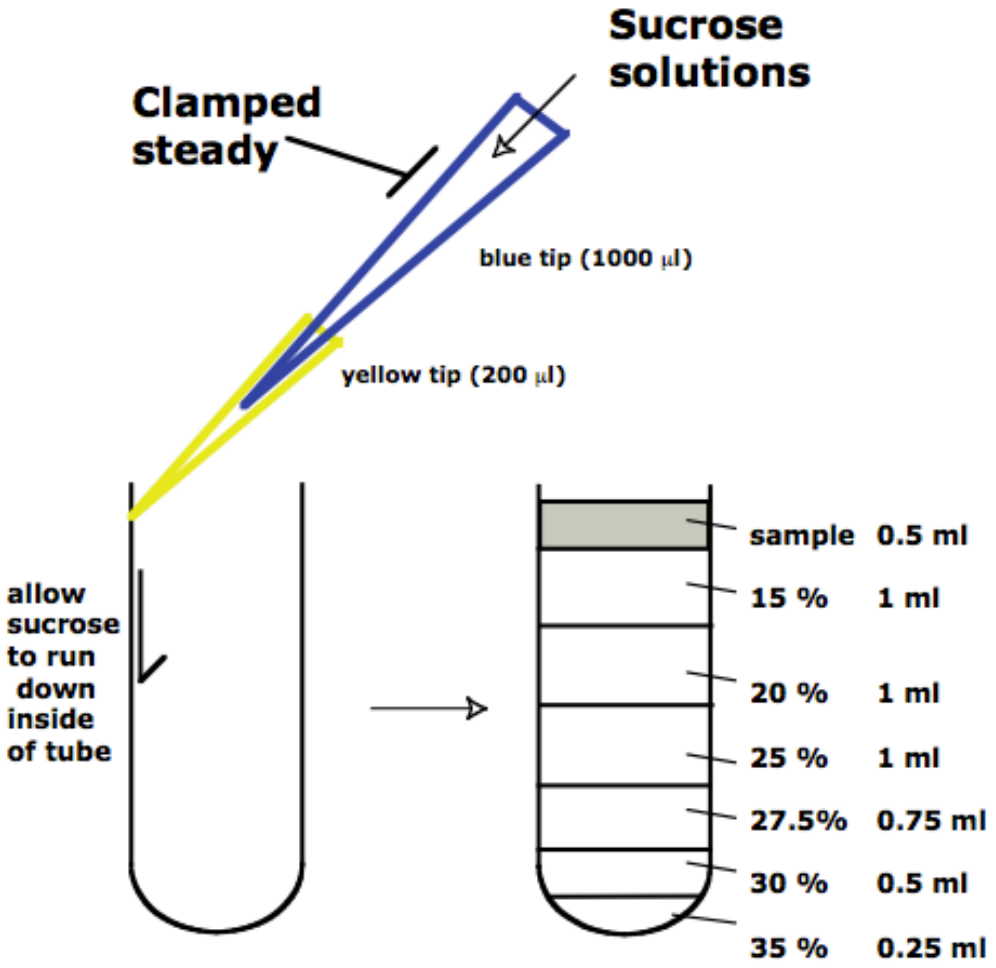


Fig. A.3. For sucrose gradients, the Mitosciences protocol was followed as above. Image: Mitosciences.

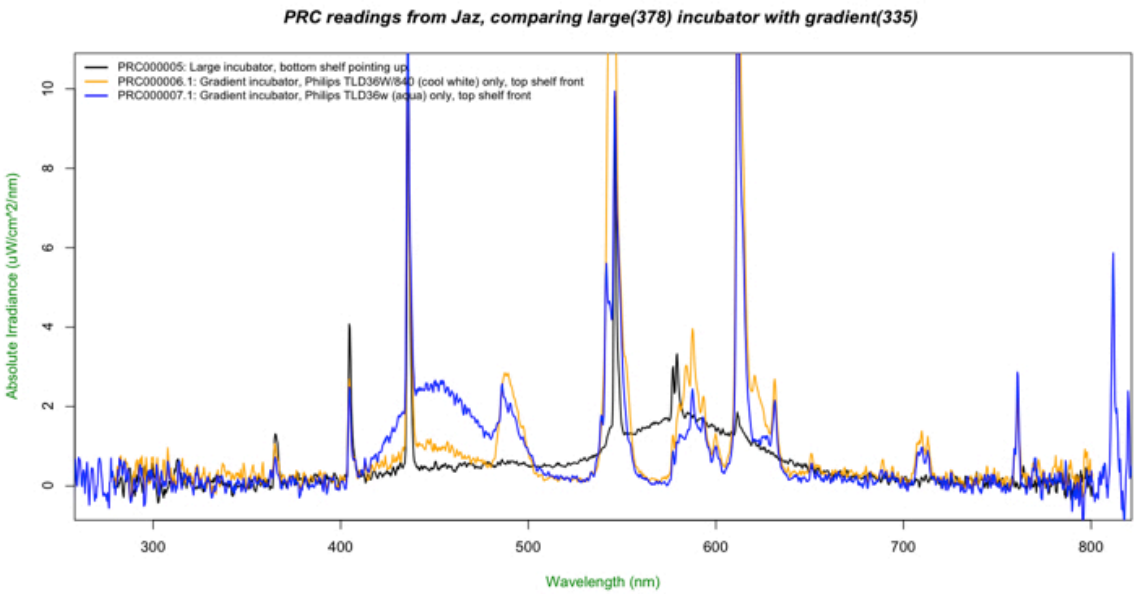


Fig. A.4. The light level was checked in each of the growth incubators used. The black line is a lower shelf, which is shaded by an upper shelf (yellow line). The dark blue line is the spectra of the gradient incubator which contained a mix of Philips TLD36W/840 (cool white) and Philips TLD36w (aqua) fluorescent light at 54±2 µmol photons·m^{-2}·s^{-1} as measured with a PAR detector (PMA2100/PMA2132, Solar Light Company, PA, USA). The spectra were taken with a Jaz Spectrophotometer (Ocean Optics Inc. USA.) The gradient incubator was used for all experiments, whilst the other incubator was used for growing stocks.

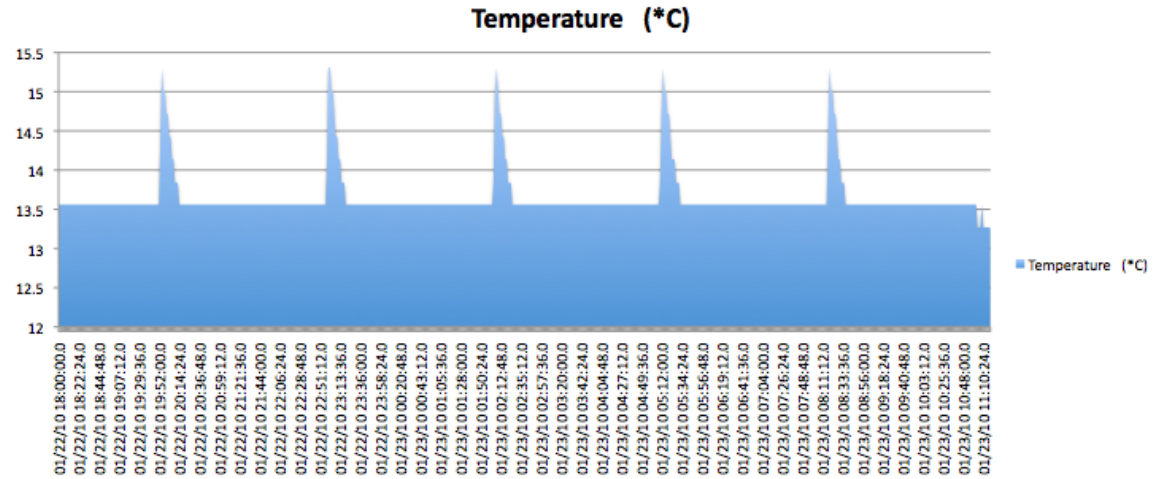


Fig. A.5. Temperature response of the gradient incubator. Temperature was checked with wireless temperature loggers and fluctuations are changes in light dark cycle.

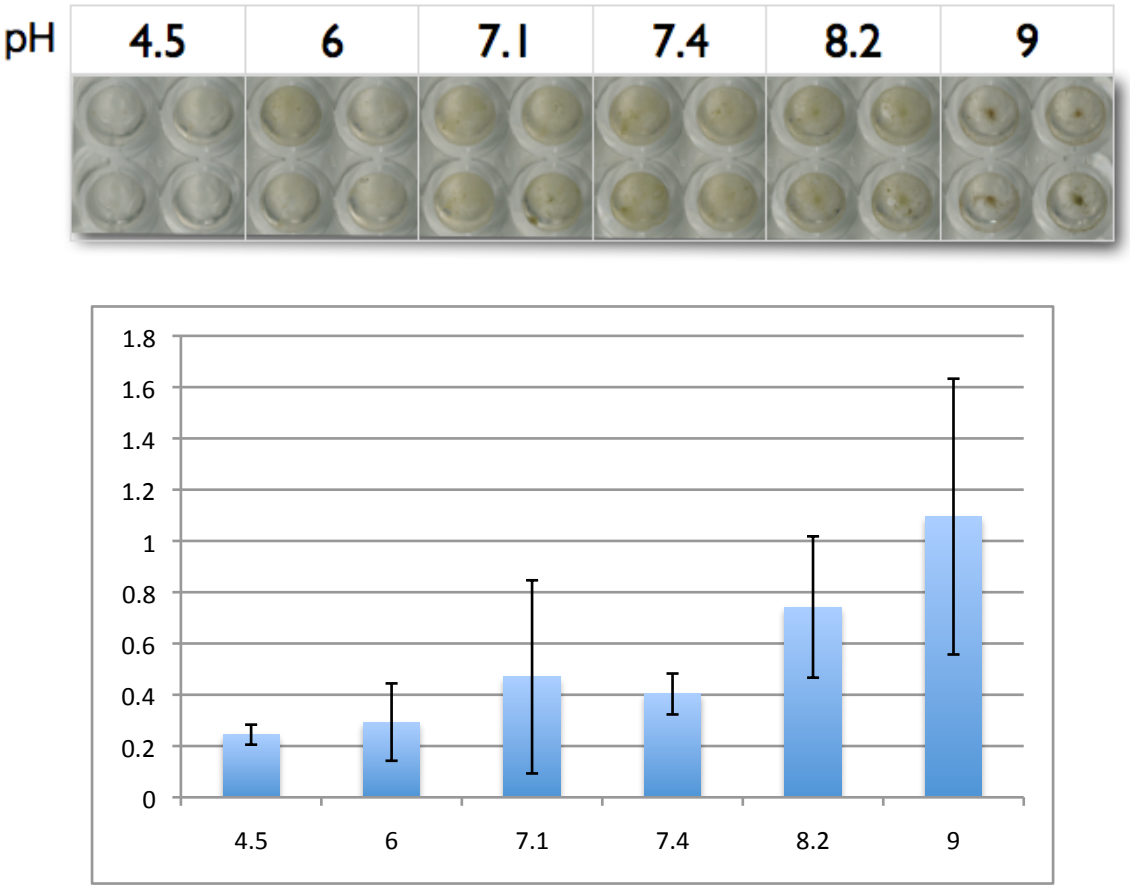


Fig. A.6. Optical density (730 nm) after one month of growth at different pH's (SpectraMax 384, Molecular Devices, Inc. Sunnyvale). Cells of species 171 (Antarctica) were grown in a 96 well plate (as shown in the Figure on top). Error bars represent standard deviations of four replicates. Optical density increases with pH.

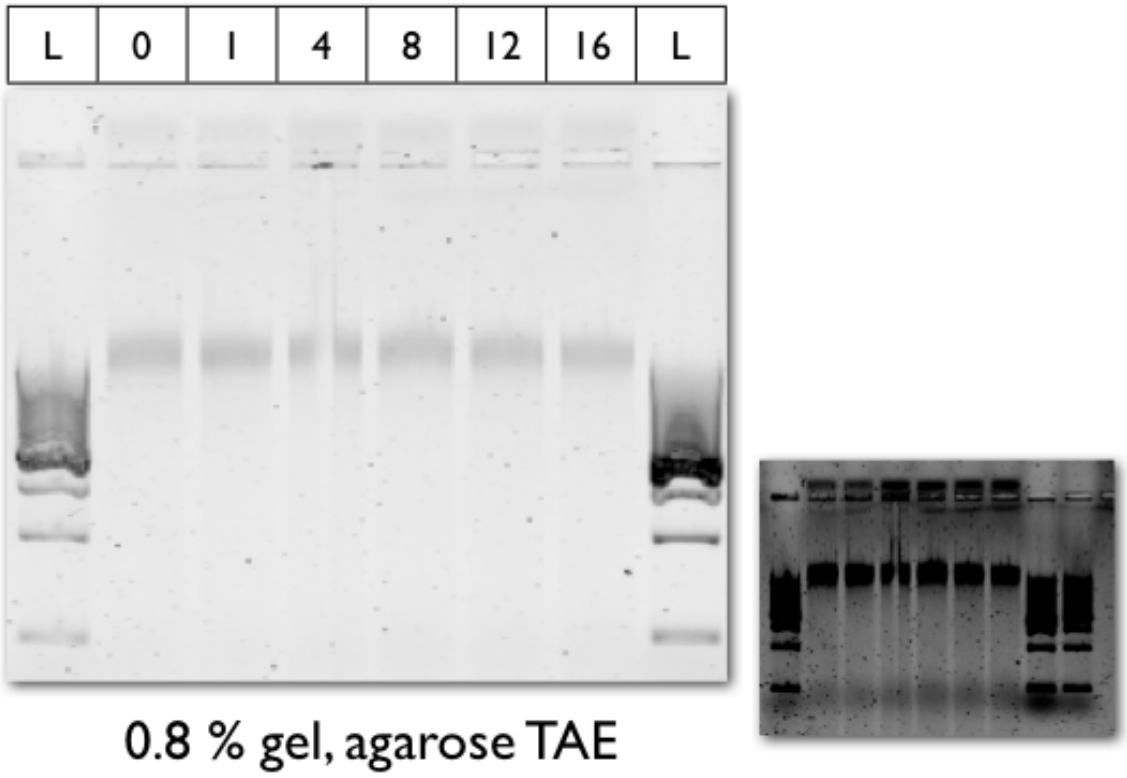


Fig. A.7. Freeze thawing of naked DNA in liquid nitrogen. Genomic DNA was extracted using the Plant DNEasy kit (Qiagen, USA). Aliquots of 3 µL of 100 ng / µL DNA of species CCMP 1991 (Hawaii) was repeatedly frozen for 30 seconds in liquid nitrogen and then thawed at room temperature. All samples were then run on a 1.5 % agarose gel (TAE), the darkest band of column L is a 1 Kb DNA ladder. Numbers along the top represent freeze thaw cycles. The right image shows a darker exposure. Both images show no noticeable change in the integrity of the genomic DNA after 16 freeze thaw cycles.

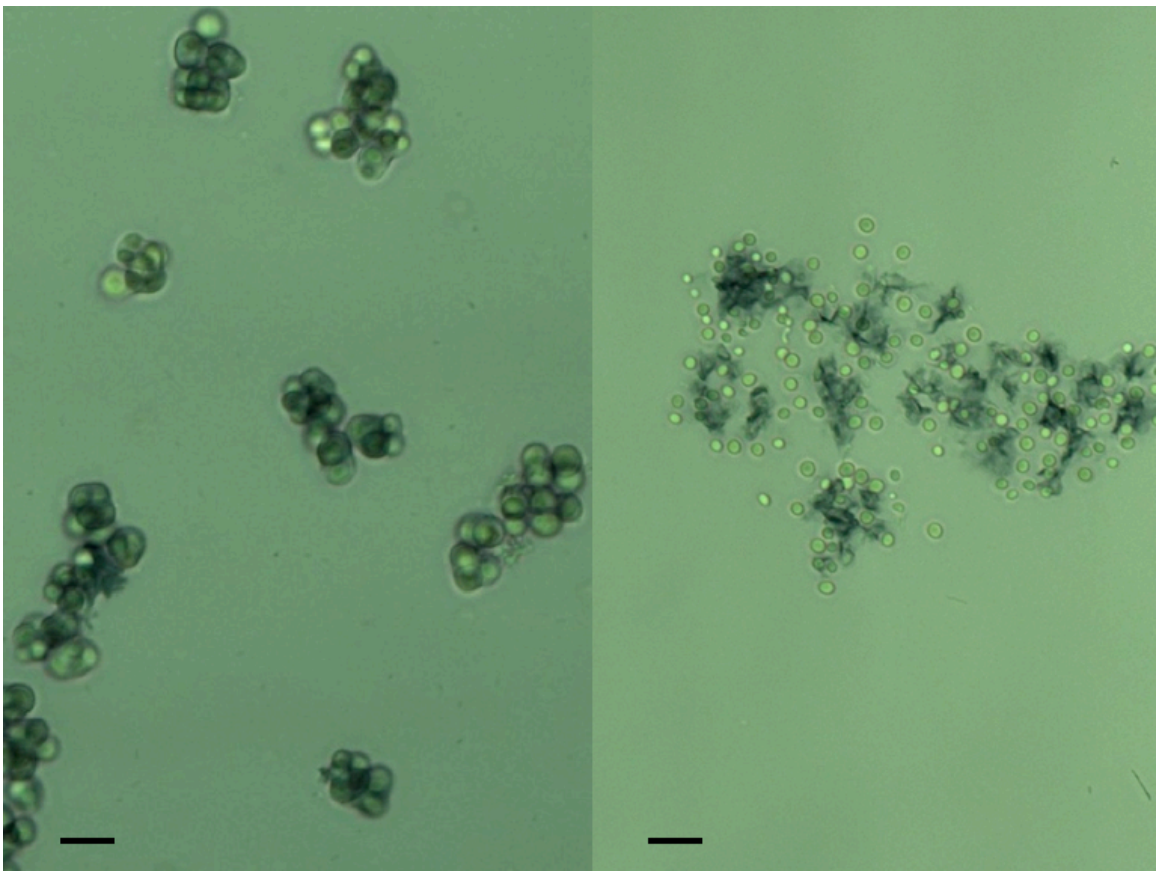


Fig. A.8. Freeze thaw changes to cell morphology, Species CCMP 3187 (Samoa). In this assay 500 uL of cells in exponential phase were added to two 1.5 mL tubes. One tube (right) was frozen in liquid nitrogen for 30 seconds and then thawed for five minutes. The second tube (Left) was not exposed. Scale bar = 17 μM.

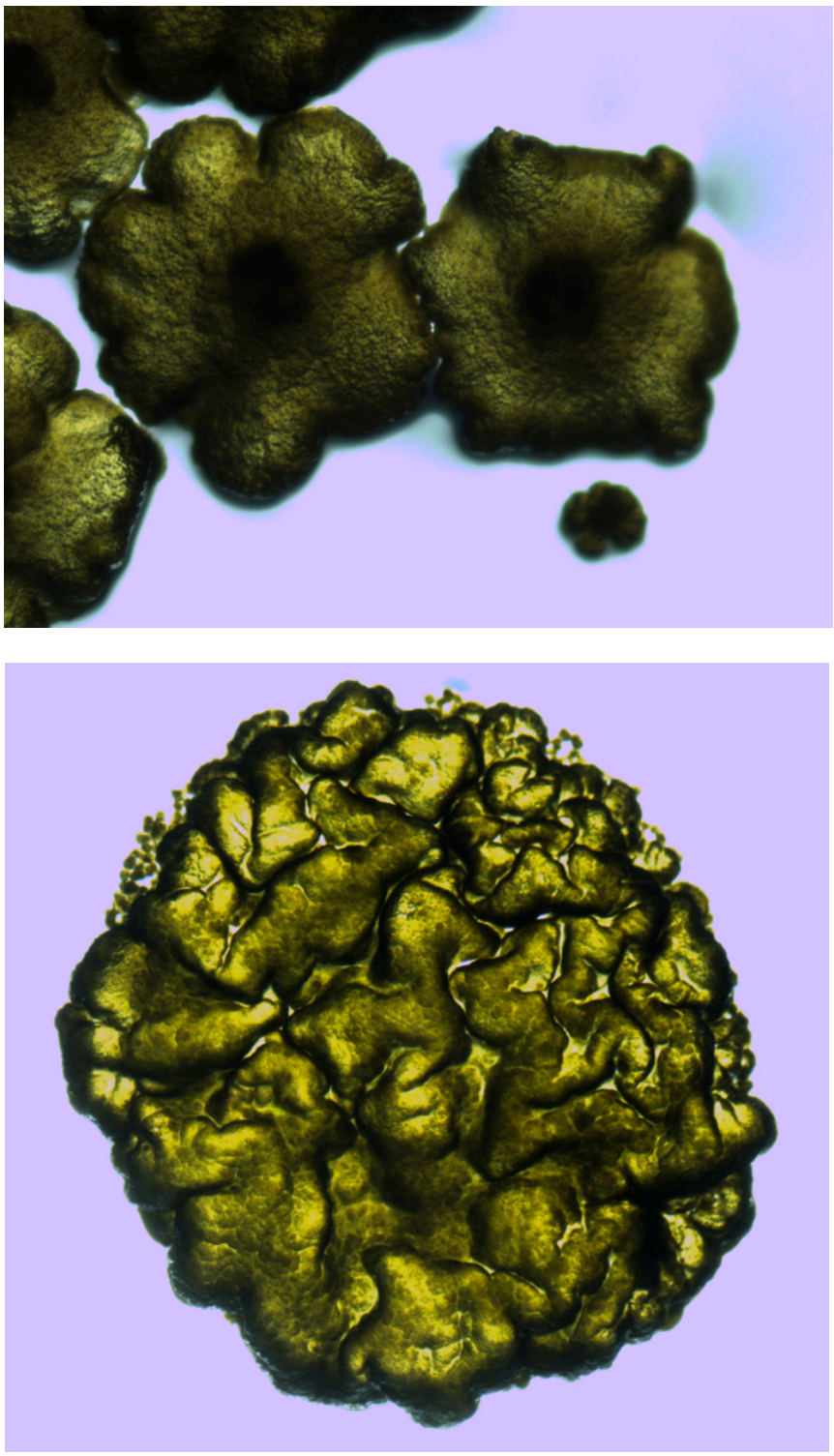


Fig.A.9. Two images of species 1991 (Hawaii) after three months of growth on an agar plate. This interesting phenotype becomes apparent when cells grow over each other resembling a velvet biofilm, which could be a protective envelope. Top image is 0.5 cm across whilst bottom is 1 cm across.

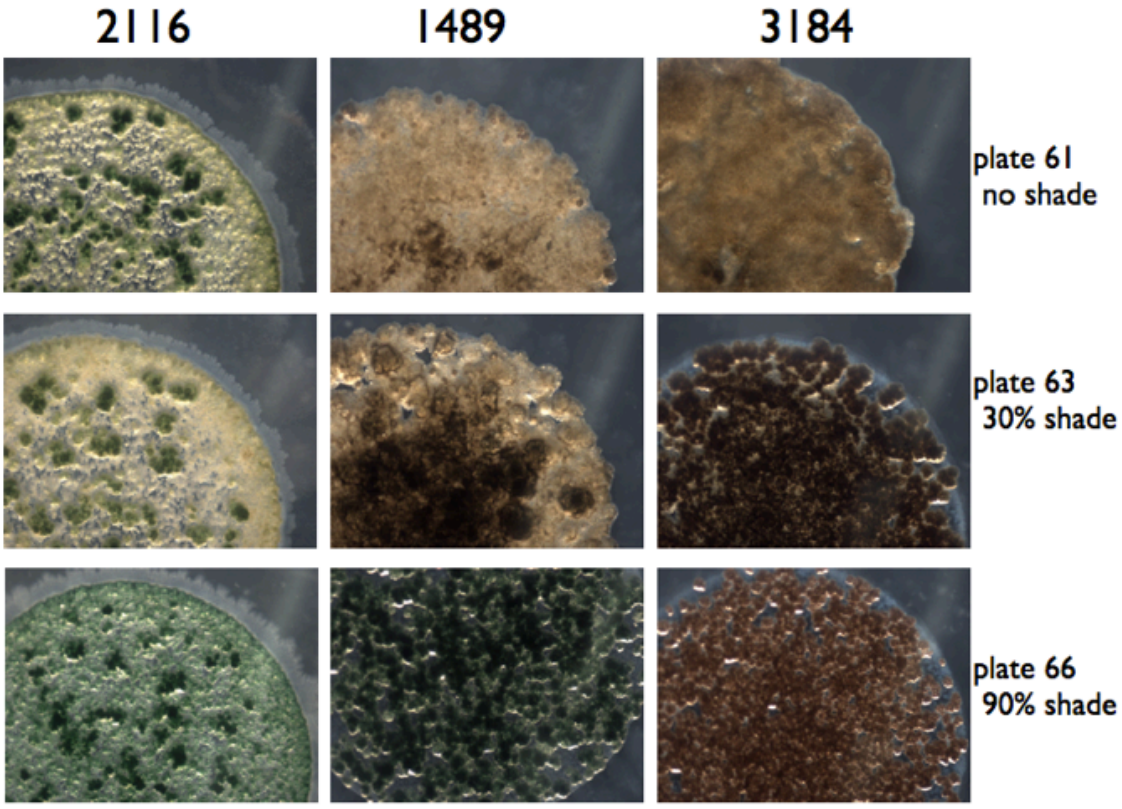


Fig.A.10. Figure of three species at three different levels of light. 2116 (Madagascar), 1489 (Galapagos), 3184 (Samoa). Showing that different pigments are produced under different amounts of light using neutral density filters, photographed after two weeks. Width of each image is 0.75 cm.

APPENDIX

Table A.1: ABBREVIATIONS AND DEFINITIONS

Baeocytes - smaller cells produced initially inside of a mother cell.

BISRU – Biological *in situ* resource utilization, the idea of using biology to extract resources in space, rather than taking them from Earth (Cumbers and Rothschild, 2010)

Chroococcales - Subsection I of the cyanobacteria, unicellular and coccoidal.

Chroococcidiopsis – the genus of cyanobacteria studied in this thesis.

Cryptoendoliths - organisms occupying porous cavities.

Cyanobacteria – a monophyletic clade of prokaroytes capable of oxygenic photosynthesis.

Cyanophage - phage that attach cyanobacteria (Millard et al., 2004; Lindell et al., 2004; Coleman et al., 2006).

DAPI -4′,6-diamidino-2-phenylindole, dihydrochloride, a nucleic acid stain.

desC1 - Δ9 acyl-lipid desaturase, an enzyme that adds double bonds to membranes.

Desiccation tolerance – an organism defined as able to survive drying to 10% water content (0.1g $H_2O \cdot$g dry mass, Alpert, 2005).

Endoliths - organisms that live inside of rocks.

Extremophile – an organism capable of living in an extreme environment.

FACS - Fluorescence activated cell sorter, a technique for counting and analyzing cells.

gltX - glutamyl-tRNA synthetase, the enzyme that charges the glutamyl tRNA.

Gy – a billion years

Gya – Billions of years after

Halophile – an organism capable of living in high salt environments.

Halotolerance – organisms tolerant of salt.

HGT – horizontal gene transfer, the process of genes being transferred from one organism to another.

Hypoliths - organisms that live under rocks.

ISRU – *In situ* resource utilization, the idea of utilizing resources in space rather than taking them from the Earth.

kGy – Kilagray, a unit of radiation dosage.

kJ – Kilajoule, a unit of radiation dosage.

Mesophile – an organism living in moderately temperate environments.

Moderate halophile - organisms that require salt to survive and grow, but cannot live in high salt concentrations.

Monophyletic - a clade containing all the descendants of the closest common ancestor.

My – a million years.

Mya – Millions of years after

Nostocales - Subsection IV, *Stigonematales*, Subsection V of the cyanobacteria, filamentous species that can differentiate into heterocysts, distinct cell types capable of fixing nitrogen (Bryant, 1994), or akinetes, specialized resting cells (Herdman, 1987).

Oscillatoriales - Subsection III, filamentous species that produce vegetative cells.

Pleurocapsale - Subsection II of the cyanobacteria, reproduce by rapid binary fission producing large cells containing many smaller cells called baeocytes.

Pleurocapsales – Subgroup II of the cyanobacteria (Waterbury, 1989).

Polyphyletic - a clade of organisms derived from more than one ancestral type.

Psychrophile – an organisms with a peak growth rate around 15°C that are unable to grow above 20°C.

rbcL - large subunit of RuBisCO (ribulose-1,5-bisphosphate carboxylase oxygenase), the enzyme that catalyzes the first step in carbon fixation.

ROS - reactive oxygen species, chemically-reactive molecules containing oxygen.

SOD - superoxide dismutase, an antioxidant enzyme.

Stigonematales - Subsection V of the cyanobacteria, filamentous species as Nostocales but with a complex branching pattern.

Thermophile – an organism living in thermal environments.

Thymine dimer - two T's on a strand of DNA fused together (usually by UV radiation).

TRMR - "trackable multiplex recombineering" pronounced 'tremor' is a method for genome engineering, (Warner et al., 2010).

UVA - 400-315 nm, ultraviolet radiation not blocked by the ozone layer on Earth

UVB - 315-280 nm, ultraviolet radiation mostly blocked by the ozone layer on Earth.

UVC - 280-100 nm, ultraviolet radiation fully blocked by the ozone layer on Earth.

UVR / UV – Ultraviolet radiation, radiation in the wavelengths between 100 and 400 nm.

Water activity – vapor pressure of a liquid / vapor pressure of pure water at the same temperature; a measure of the amount of water available for biological use.

Xerophile – see desiccation tolerance.

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